Biological Sciences

Impacts on Modern Civilization, Current and Future Challenges

Anupam Guha

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Dr. Subires Bhattacharyya
M.Sc., Ph.D.
Vice-Chancellor
University of North Bengal

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UNIVERSITY OF NORTH BENGAL
Accredited by NAAC with Grade A
Website : http://www.nbu.ac.in
Email : nbuvc@nbu.ac.in
subires.bhattacharyya@gmail.com

Raja Rammohunpur, P.O. North Bengal University, Dt. Darjeeling, West Bengal, India, PIN – 734013
Phone : (0353) 2776 366 (O), (0353) 2776 308 (R), Fax : (0353) 2699001

FOREWARD

The 21st Century is the age of "Biological Sciences". New technologies, tools, and approaches, often spanning several disciplines, are revolutionising biology and providing unprecedented opportunities to advance the frontiers of bioscience knowledge. Our understanding of the complex and dynamic processes that govern life will be transformed, and with this will come exciting opportunities to apply new knowledge for the benefit of society and the economy of the country, and have the most impact in addressing some of the 21st Century's greatest challenges around ensuring food security, clean growth and healthy ageing.

The power of biological sciences blows all of our lives, from sustainable and resilient agriculture, safe nutritious food, new pharmaceuticals and better health to new low-carbon 'greener' energy, materials and everyday products.

The great challenge and opportunity for biological sciences, as we move into the 21st century is to understand biological systems in all their complexity while preserving and exploiting biological systems in a sustainable fashion. The tools for dealing with this complexity will require the adaptation and application of emerging technologies.

Among the many new tools that are or will be needed, some of those having highest priority are: Bioinformatics, Computational biology, Functional imaging tools using biosensors and biomarkers, Transformation and transient expression technologies, Nanotechnologies are likely to accelerate the development and application of new technologies to the biological sciences.

During the COVID-19 pandemic there has been much more frequent discussion in the media and among educationists, scientists/ researchers, politicians about the emerging science and breakthrough medical technologies that are being used to combat the critical situation in the Globe. We can expect this to result in a broader public understanding of what the Biological sciences ecosystem is capable of creating and the value it can bring to the modern society.

With lots of conflicts, Technology is changing fast and innovations being made to cater to human and social needs to attain our goals of using knowledge of biology and to reap maximum benefits from biotech-products. I am confident that the readers will find in this well edited book "Biological Sciences: Impacts on Modem Civilization, Current and Future Challenges" a comprehensive coverage comprising various aspects of biological sciences and be inspired for future reading.

(Dr. Subires Bhattacharyya)
Vice-Chancellor

Preface

Biological Sciences: Its modern research is in the midst of a revolutionary change due to the integration of powerful technologies along with new concepts and methods derived from inclusion of physical sciences, chemical Sciences, mathematics, computational sciences, and engineering. As never before, advances in biological sciences hold tremendous promise for surmounting many of the major challenges confronting the world. Historically, major advances in science have provided solutions to economic and social challenges. At the same time, those challenges have inspired science to focus its attention on critical needs. Scientific efforts based on meeting societal needs have laid the foundation for countless new products, industries, even entire economic sectors that were unimagined when the work began.

The book contains thirty eight articles covering various phases of Biological Sciences. The topics cover fundamental as well as advanced and modern aspects of biological sciences and its integrated subjects. It is very difficult to keep pace with the recent developments in various fields. Here, an attempt has been taken to present some recent findings with review works in a manner considered suitable for the scientific community.

I am thankful to the contributors for writing authoritative and informative articles for this volume. The opinions and text contained herein are those of the authors and I have tried to honour their ideas in the original shape. While dealing with such a voluminous work, errors are likely to occur despite best efforts. However, the onus of the technical contents rests with the contributors.

This effort will definitely serve not only as an excellent reference material but also as a guide for research communities and students in the field of Biological Sciences. I would very much appreciate receiving suggestions from readers so that shortcomings, if any, are corrected in future editions.

I am gratified to my family members for their fullest co-operation in many invisible ways and for constant encouragement during the preparation of this book. I am thankful to all the faculty members and specially to Dr. Chitra Pal, Principal-in-Charge, Rabindranath Thakur Mahavidyalaya, Bishalgarh, Tripura for their constant support and courage during this effort. I also highly appreciate the all-round co-operation and support of Sri Dipanjan Mukherjee, founder member of New Delhi Publishers for presenting and publishing this work with patience, care and interest.

Bishalgarh **Anupam Guha**

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CHAPTER

Andrographolide: from bench to bedside in colorectal cancer

Aditi Banerjee*, Neha Sharda, Jared Huse, Steven J. Czinn and Thomas G. Blanchard

Department of Pediatrics, University of Maryland School of Medicine, Bressler Research Building, 13-043, 655 W. Baltimore Street, Baltimore, MD 21201, Maryland USA
E-mail: aditi.banerjee@som.umaryland.edu,

Abstract: Colorectal cancer (CRC) has traditionally been associated with older age, However, young onset colon cancer (yo-CRC), defined by diagnosis under the age of 50, has been increasing in prevalence worldwide. It is estimated to increase 90% by2030. In most cases, individuals with yo-CRC remain undiagnosed until the cancer has reached an advanced, metastatic stage.At this stage, the cancer becomes extremely difficult to treat and is therefore associated with a high likelihood of death. There are currently no effective treatments for late stage CRC including yo-CRC. CRC is driven by a subpopulation of cells called cancer stem cells (CSC) that make tumors resilient against treatment and chemotherapy. Self-renewal of CSC is accompanied by the promotion of angiogenesis through the secretion of proangiogenic factors such as Vascular Endothelial Growth Factor (VEGF). Moreover, CRC including yo-CRC is due to genetic and epigenetic alterations, diet,and interaction of genetic and environmental factors. Consumption of alcohol is another factor that contributes to CRC development. Chemotherapy continues to play a pivotal role in the management of many cancers including CRC. CRC can be treated by surgery or through chemotherapy, immunotherapy, or combinational therapy, but unfortunately, conventional therapies work on proliferating and differentiating cells, and not on CSCs. Therefore, a new therapeutic approach that also targets CSC is needed to overcome CRC. Andrographolide (AGP), a plant metabolite labdane, causes apoptotic CRC cell death through the unfolded protein response (UPR)-mediated endoplasmic reticulum (ER) stress. The molecular mechanism is angiogenic inhibition, reactive oxygen species induction, oncogene inhibition and tumor suppressor gene upregulation. AGPalso reverses 5-FU (a commonly used drug) resistance in CRC. Moreover, it inhibits CSC proliferation through the reduction of the stemness properties. Additional studies have demonstrated its clinical impact on CRC.

Keywords: Colon cancer, metastasis, ER stress, angiogenesis, tumor suppressor gene, andrographolide

Colorectal cancer (CRC) is the third most common malignancy diagnosed globally and the fourth leading cause of cancer related death worldwide, with its burden predicted to increase by 60% by 2030[1]. Moreover, young onset colon cancer (yo-CRC), as noted by incidence in individuals under 50 years old, is a severe health problem[2]. The number of yo-CRC cases in people less than 35 years old is estimated to increase 90% by2030[3]. Approximately 65% of CRC cases are sporadic with absence of family history or apparent genetic predisposing[4,5]. Though detection and treatment protocols have improved significantly, late stage cancer patients or metastatic cancer patients often succumb to the disease through relapse[6-10]. In addition, the metastatic stage of CRC (mCRC) is still associated with a poor 5-year overall survival rate of less than 10% [11]. A well accepted cause of CRC relapse is the failure of conventional therapies to eradicate the cancer stem cells (CSC) in the tumor, resulting in recurrence, metastasis and resistance to chemotherapy[12,13]. Unfortunately, most conventional therapies only target proliferating and differentiating cancer cells[14–17]. Additionally, most chemotherapy agents have undesirable side effects including nausea, diarrhea, decreased blood cell counts, fatigue, nerve damage, and pain[18]. Therefore, new therapeutic approaches and strategies are needed to overcome CRCs including CSC.

Colon Cancer and Diet

Colon cancer is associated with the interaction of genetic and environmental factors[19]. Nineteen percent of CRC can be attributed to intake of energy dense foods along with less physical activity. Based on global epidemiological and scientific studies, evidence suggests that the risk of CRC is increased by processed and unprocessed meat consumption but suppressed by fiber, and that food composition affects colonic health and cancer risk via its effects on colonic microbial metabolism[20]. It is a serious concern particularly in young populations. Moreover, animal studies show high fat diet (HFD) consumption is associated with obesity which is it self considered a CRC risk factor [21]. However, the composition of total dietary fat and fatty acid (FA) composition alters the pathway which mediates CRC. Additionally, quantity and frequency of red meat intake and alcohol consumption are directly correlated with CRC progression. Further research found a 12% reduction of CRC associated with fish consumption, a 25% reduction of CRC risk associated with consuming 20g/day fiber, and a 26% reduction of CRC risk with consuming 525ml/day of milk [22]. Therefore, it can be predicted that our daily lifestyle, including diet, may have a role in maintaining our health.

Molecular Mechanisms of Colorectal Cancer

Genetic alteration in CRC

There are several molecular mechanisms that initiate cancer progression, including CRC. One such mechanism that results in the transformation of colon epithelial cells

to malignant cells is the imbalance between cellular on cogene activation and the loss of tumor suppressor gene (TSG) function[23]. Moreover, genetic and epigenetic alterations are common and are driving forces of colon tumorigenesis [24]. In addition, alcohol-mediated CRC is due to the activation of mast cells in response to oncogenic stimuli, suggesting that it could have an impact on intestinal mucosal inflammation, which is supported by several studies [25]. Further studies have demonstrated the key role of an impaired mucosal barrier in the adenoma to carcinoma transition by enabling cancer permissive and inflammatory signaling[26–29]. A growing body of scientific evidence suggests that~85% of sporadic CRCs exhibit chromosomal instability (CIN), with changes in chromosome number and structure [27,30]. Additionally, CIN tumors are recognized by several oncogene mutations and their accumulation, such as KRAS proto-oncogene GTPase (KRAS) and B-raf protooncogene serine/threonine kinase (BRAF), as well asTSG mutations such as RASSF1A, APC, and tumor protein p53 [31–33]. Microsatellite instability (MSI)[34,35], the high overall mutation frequency mediated by the inactivation of DNA mismatch repair (MMR) genes[36], develops due tomutational inactivation of TSGs.

Angiogenesis in CRC

In addition to the genetically controlled development of tumor cells, tumor angiogenesis, formation of new blood vessels from a preexisting vasculature, plays a critical role in tumor development including CRC [37]. Self-renewal of CSC and initiation of the tumor is accompanied by the promotion of angiogenesis through the secretion of proangiogenic factors such as Vascular Endothelial Growth Factor (VEGF) [38]. The size of the tumor depends on the angiogenic vessel formation [36, 39]. Overproduction of pro-angiogenic growth factors such as VEGF turn on the angiogenic switch through the stimulation of hypoxic tumor cells. The upregulation of its receptors (VEGFR1 and VEGFR2) in tumor cells is due to the activation of oncogenes (FoxM1, and PTTG1) and inactivation of TSGs (RASSF1A, PTEN) during neoplastic transformation[40]. Overexpression of VEGF and a high blood vessel density within the tumor tissue are associated with poor prognosis in cancers including CRC [36, 41].

ER Stress and CRC

Alteration of protein folding in the ER due to disturbances in redox, Ca^{2+} levels, glycosylation, or other environmental elements causes accumulation of misfolded proteins[42,43]. Eukaryotic cells activate a series of signal transduction cascades that are collectively termed the unfolded protein response (UPR) [44]. Glucose-regulated protein, 78kDa (GRP78) is a well characterized ER chaperone and a master regulator of ER stress sensors. ER stress signaling is regulated by three major transducers located in the ER membrane:inositol-requiring enzyme-1 (IRE-1), activating transcription

factor 6 (ATF6) and PKR-like ER kinase (PERK)[44–46]. One mechanism of inducing apoptosis (programmed cell death) is through activation of the UPR via ER stress. At present, several studies have demonstrated a correlation in between induction of ER stress and inhibition of colon carcinogenesis [47–50] using chemotherapeutic agents, repositioning drugs, or immunotherapy.

Present treatment strategies for Colon cancer

CRC could be treated with local treatments including colon or rectal surgery and radiation therapy orsystematic treatments. Treatments may further include either chemotherapy, targeted therapy, or immunotherapy. Depending on the stage of the CRC (stage I-IV) and other factors, different types of treatment strategies could be considered. Usually, earlier stages of CRC are treated through surgery alone, whereas advanced stages are treated first with surgery followed by single or combined chemo or immuno therapy depending on the patient's age and health condition. Commonly used chemotherapies for CRCs include FOLFOX (5FU, leucovorin, and oxaliplatin) and CapeOx (capecitabine and Oxaliplatin). Advanced stages of CRC or other cancers could be treated with chemo or targeted therapies. It could be treated using combinations adding either a drug that targets VEGF (bevacizumab [Avastin], ziv-aflibercept [Zaltrap], etc.) or a drug that targets EGFR (cetuximab [Erbitux] or panitumumab [Vectibx]) (https://www.cancer.org/cancer/colon-rectal-cancer/treating/by-stage-colon.html). Unfortunately, drug resistance is a major contributor to poor prognosis; therefore, effective treatments for CRC, including yo-CRC, are urgently needed for drug resistant cancer.

1. Impact of Andrographolide on Colon cancer

Plant metabolites may constitute a vast source of potential new drugs; therefore, many natural food components have been screened for protective or anticancer activities[44,51]. Andrographolide (AGP) is a lactone (bicyclic diterpenoid) derived from *Andrographis paniculata*, anAsian herb, commonly known as Kalmegh or"king of bitter"[52]. This compound is well documented to possess several pharmacological activities due to its anti-pyrogenic[53,54], anti-inflammatory[55], immunomodulator[51,56–58], antioxidant[44,59] and anticarcinogenic activities[44,60–63]. Recently, AGP has been hypothesized to be a molecular target for thewidespread disease of COVID-19, due to its potential antiviral effects as a combinational drug[64]. The molecular mechanism of its anticarcinogenic properties include inhibiting cell cycle progression, matrix metalloproteinase expression, inducing apoptosis, reducing cell migration or invasion[36,65,66] and dysregulation of signaling pathways[44,51]. Recent studies have demonstrated that AGP causes apoptotic CRC cell death through UPR-mediated ER stress[44]. AGP-induced CRC cell cytotoxicity occurs primarily through IRE-1 activity as shown by

the overexpression of IRE-1 as well as depletion of IRE-1 with siRNA[44]. Additional studies have reported that AGP-induced ER stress/UPR leading to apoptosis is dependent on the induction of oxidative stress (Fig.1)[51].

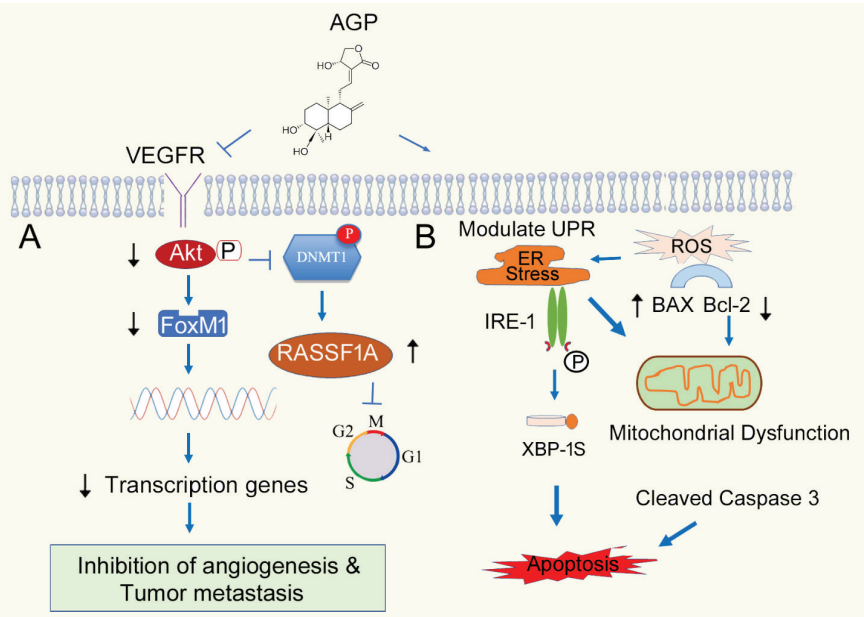

Fig. 1: *Schematic representation of the molecular mechanism of AGP's cytotoxic effects in CRC cells. A.AGP causes angiogenic inhibition through downregulation of Akt and FoxM1 level. Lower levels of activated Akt results in less stable DNMT which then results in reduced methylation of the RASSF1A gene, allowing for increased RASSF1A expression. B. AGP induced apoptosis signaling is dependent on ER stress, ie., upregulation of IRE-1 and its downstream signal XBP-1. Upregulation of proapoptotic molecule BAX and downregulation of anti-apoptotic Bcl-2 are observed in AGP treated cells which ultimately causes apoptotic CRC cell death through the mitochondrial dysfunction and ROS generation.*

It is also documented that in addition to inducing apoptosis via the UPR, AGP blocks the survival molecules, i.e., Akt phosphorylation results in decreased levels of mTOR and suppresses Cyclins D1 and B1 of the cell cycle progression pathway[36]. Further studies have demonstrated that AGP inhibits angiogenic signals through the down regulation of angiogenic receptors, VEGFR1 and VEGFR2, oncogenic protein FoxM1, and PTTG1[36]. Moreover, AGP also upregulates TSG expression (RASSF1A, PTEN, and CDKN2A) in CRC cells, CRC tissues, patient tissue-derived organoids, and mouse CRC tissues. Notably, AGP-activated RASSF1A expression is dependent on ER stress and AGP inhibited angiogenic signaling suppresses Akt activation, a critical event in the upregulation of RASSF1A expression (Fig.1)[67–69]. Additionally, AGP has anti-CSC properties as it diminishes stemness properties in different cancer models[70,71]. Interestingly, an additional study has shown that AGP reverses 5-FU resistance in

CRC and that the mechanism is increased levels of BAX as AGP interacts with BAX preventing its degradation and enhancing mitochondria-mediated apoptosis[70]. There have been several studies to explore translational activities of AGP. Some clinical trials are ongoing for acute bronchitis and tonsillitis (https://clinicaltrials.gov/ct2/show/NCT03132623). Additional clinical studies have been performed (https://clinicaltrials.gov/ct2/show/NCT01993472) to implicate AGP withcolon cancer treatment.

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CHAPTER 2

Vitamin D deficiency is a disease of modern civilization

Dilip K. Deb

Navigator -Nephrohub, Feinberg Cardiovascular and Renal Research Institute
Northwestern University, Chicago
E-mail: dilip.deb@northwestern.edu

Abstract: Vitamin D is synthesized in the skin upon exposure to sunlight, hydroxylated in the liver and kidneys to make metabolically active 1,25-dihydroxyvitamin D [1,25(OH) D]. Melanin inhibits synthesis of vitamin D due to which dark-skinned people have less vitamin D, compared to white skin. The synthesis of vitamin D can decrease due to age, health conditions which prevents absorption of vitamin D, and also due to cholesterol lowering statin drugs. The activated form of vitamin D binds to the vitamin D receptor (VDR) and heterodimerizes with Retinoid X Receptor (RXR), the dimerized complex then binds to the Vitamin D Response Element (VDRE) in the promoter regions of target genes, to regulate expression. Patients with insufficient level of vitamin D are usually supplements given to counteract the deficiency. To avoid a need for a supplement, including a good source of vitamin D containing well-balanced diet, and exposure of body to the sunlight is helpful to avoid a need for a supplement. While taking high doses of vitamin D supplement; patients should be monitored carefully, since high dosage can have adverse effects in human body. Vitamin D's major biological function is to maintain homeostasis of blood's calcium and phosphate levels, including immune modulation and general cellular function in different type of cells. Recently, mortality and severity of COVID-19, patients have been attributed to vitamin D, which will be discussed here.

Keywords: Vitamin D; lifestyle; cytokine storm; mortality; COVID-19

Vitamin D (represents D2, or D3, or both) was discovered in 1920 after a long search to find a cure for diseases which caused weak and soft bones, stunted growth, and, in severe cases, skeletal deformities during childhood [1,2]. These diseases were cured with fortified vitamin D foods within a decade. Vitamin D deficiency occurs due to inadequate sun exposure and is a global health problem, with several adverse consequences. Vitamin D deficiency (<20 ng/mL) and insufficiency (20-30 ng/mL) affect almost 1 billion people worldwide[3]. The vitamin-D deficiency is associated with

osteoporosis, bone fractures, cancer, cardiovascular disease, diabetes, autoimmune diseases, and depression including higher mortality rate in COVID-19 patients [4,5,6].

COVID-19 causes the overproduction of proinflammatory cytokines (tumor necrosis factor [TNF], IL-6, and IL-1β) during infection, which lead to vascular hyperpermeability, multiorgan failure, and eventually death may occur [7]. Vitamin D modulates immune cells to reduce the production of proinflammatory cytokines and increases anti-inflammatory cytokines

This review will discuss mainly the factors that affect synthesis of vitamin D, measurement of vitamin D level, consequences of vitamin D deficiency and connection between COVID -19 mortality rates and vitamin D deficiency [8].

Biosynthesis of Vitamin D

Upon exposure to the sunlight UVB light interacts with 7-Dehydrocholesterol (precursor of cholesterol) protein, converting it into vitamin D3 (Chocalciferol) [9]. People can also get it from the diet but this is biologically inert. At first, hydroxylation of vitamin D3 occurs in the liver and form vitamin 25(OH)D, also known as calcidiol. Then, the in the second step hydroxylation of 25(OH)D occurs in kidneys which results in the formation of the biologically active metabolite 1,25(OH)D, also known as calcitriol [10].

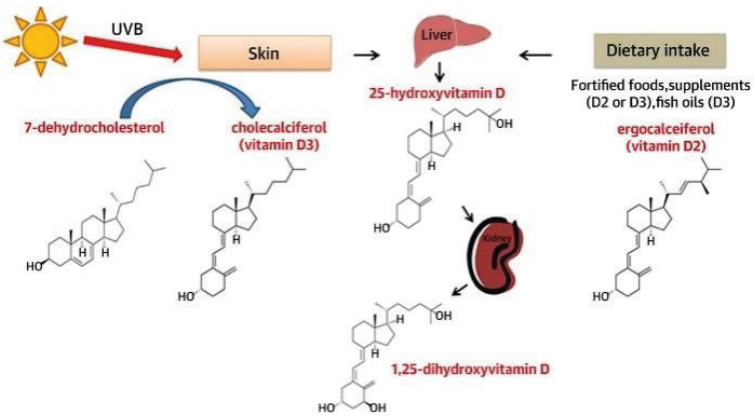

Fig. 1: *Schematic representation of 1,25 hydroxyvitamin D in humans. The figure adapted from Mheid, and Quyyumi JACC 2017;70:89-100* [11]

Factors Affecting Vitamin D Synthesis

Since sunlight is the best source of vitamin D in order to maintain sufficient level of it in blood, people should expose themselves for 20–30 minutes of mid-day sunlight, several days per week. High level of melanin significantly inhibits synthesis of vitamin

D in dark-skinned people. Due to industrial revolution most of the people do not expose themselves to adequate sunlight, since urban and sedentary lifestyles have resulted in less time spent outdoors for work or leisure. People block sun light exposure with heavy clothing, traveling in cars, blocking UVB with glass windows, air pollutions, cultural practice (such as burqa and purdah), application of sunscreen, changing food habits. People are not eating vitamin D fortified foods, although they might be living far from the equator, where there is little sun year-around [12].

There are other risk factors which can also be involved in vitamin D deficiency, such as being elderly, or overweight. There is a decline in the ability of the kidney to synthesize 1,25 (OH)D as people age. People with obesity may have low vitamin D level in blood, since it is stored in fat, and therefore is not always available, when needed [13].

Vitamin D synthesis drops with age in people's skin, and only one-fourth of vitamin D is generated over 65 years of age, compared to when they are in their 20's. People with several health conditions such as inflammatory bowel disease, kidney and liver diseases, or cystic fibrosis, may have trouble absorbing vitamin D, which can lead to deficiencies. Most people don't easily realize vitamin D deficiency, even if they're having a significant negative effect on quality of life.

The sufficient level of 25(OH)D is usually between 40 - 60 ng/mL [14], therefore, measuring serum 25(OH)D is needed to determine the lowest daily Vitamin D3 dosage required for optimal benefit. There are differences among researchers regarding 25(OH)D levels in the blood that defines vitamin D insufficiency and deficiency. However, most experts agree that lower than 20 ng/mL of 25(OH)D is suboptimal for skeletal health.

Vitamin D Foods

There are only very few foods in the nature which contains vitamin D, such as egg yolk, oily or fatty fish (such as salmon, sword fish, mackerel, and tuna), cod liver oil, beef liver, cheese and sun light grown mushroom (vitamin D2). Unfortunately, for vegetarians there is not much choice of vitamin D from foods until they eat fortified vitamin D foods. The cholesterol lowering statin drugs also reduced vitamin D concentrations in 21.4% patients, compared to control group [15], since cholesterol and 7-*dehydrocholesterol* use a common biosynthetic pathway. It is fat-soluble, and is absorbed best in bloodstream when mixed with high-fat foods, so vitamin D supplements should be taken with a meal to enhance absorption in the blood.

Mechanisms of Action

The molecular actions of the 1,25(OH)D are mediated by vitamin D receptor (VDR), which acts as a transcription factor into the target cells. The VDR is expressed in most organs, including the brain, heart, skin, gonads, prostate, and breast. The VDR binds to the physiologically active 1,25(OH)D to mediate biochemical signals in the cells. The VDR protein binds to the active form of vitamin D and heterodimerizes with other nuclear protein, RXR. The dimerized VDR:RXR complex binds to the consensus VDR binding DNA sequences, which is VDRE, in the promoter regions of target genes. As a result, gene expression regulation occurs by repression or induction of gene [16,17]. In a specific cell, Vitamin D's physiological activity is dependent on the available concentrations of 1,25(OH)D.

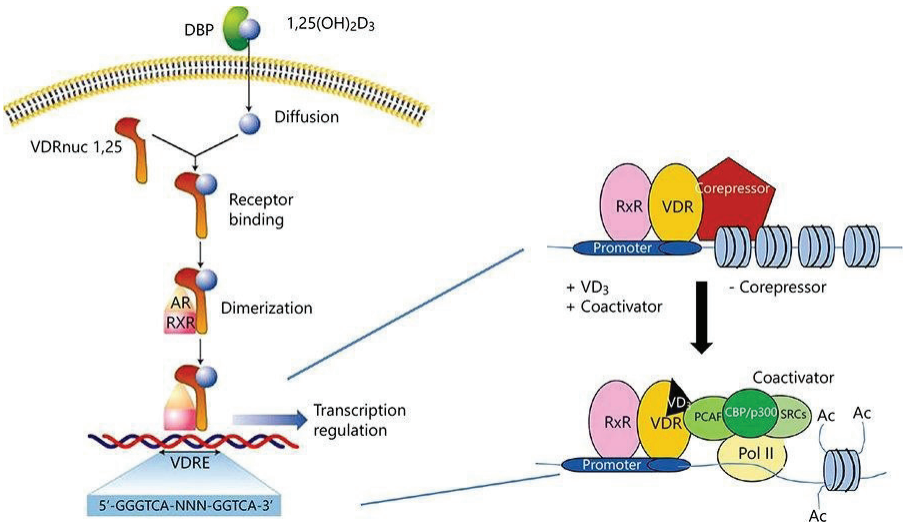

Fig. 2: *Schematic representation of the transcriptional control of gene expression by 1,25(OH)2D via VDR. Adapted from Gil & Mesa, M. (2018) Vitamin D: Classic and Novel Actions. Annals of Nutrition and Metabolism, 72(2) [18].*

Vitamin D Measurement

The best way to determine whether an individual is vitamin D deficient or sufficient is by measuring the circulating level of 25(OH)D. It remains stable in the blood and has circulating half-life of 2-3 weeks [19]. It also gives a good estimate of vitamin D status in an individual since its synthesis is not influenced by any hormone. The 25(OH) D level reflects the amount of vitamin D produced from the skin, also obtained from food, and or as well as from supplement. However, 25(OH)D levels do not reflect the amount of vitamin D stored in body tissues.

If a patient has hypercalcemia or decreased kidney function then 1,25(OH)D is measured, since the hydroxylation of 25 (OH)D occurs in the kidney. Active form of circulating 1,25(OH)D and its concentration is also regulated by parathyroid hormone, calcium, and phosphate [18]. It has a short half-life of 15 hours and hence, is not a good indicator to estimate vitamin D status.

Vitamin D Supplement

The two commonly available forms of vitamin D supplements are: vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). Vitamin D3 is the naturally occurring form of supplement, and is more preferable rather than vitamin D2, since it may raise vitamin D levels more effectively in the blood serum. Regularly eating a good source of vitamin D containing well- balanced diet, and exposing body to the sunlight might avoid the avoid for a supplement. Most people don't need vitamin D supplement more than 600 to 800 IU per day which is sufficient amount [20]. However, some people may need a higher dose of supplement to treat vitamin D deficiency, and doses are given sometimes for a specified time frame, under the observation of a physician. While taking high doses of vitamin D, the patient's level should be carefully monitored by physician. High dosage of vitamin D supplement can be dangerous as body can become hypercalcemic. As a result, nausea and vomiting, weakness, and frequent urination may occur and the toxicity might cause bone pain and also increase the risk to develop stones in kidneys [21].

Vitamin D Function

Vitamin D's major biological function is to maintain homeostasis of blood's calcium and phosphate levels, that are required for bone health, muscle contraction, nerve conduction, immune function and general cellular function in all cells of the body. It is also found to be important for immune function, for inflammation, cell proliferation, and differentiation.

We have demonstrated that combination of high blood pressure medicine losartan and vitamin D analogs are more effective in preventing kidney disease in type1 and type 2 diabetic mouse models [22,23]. The vitamin D deficiencies are body ache, muscle pains, joint paints, depression and anxiety, and even fractures. The lower vitamin D status is linked to multiple acute and chronic disorders, including cancer, obesity, diabetes, cardiovascular diseases, autoimmune diseases such as rheumatoid arthritis, Crohn's disease and multiple sclerosis, aging and respiratory tract infections, including tuberculosis and Covid-19. It has been medically proven that taking vitamin D supplement may treat and prevent such disorders.

Dr. Manson from Harvard University performed a large study with vitamin D and she observed that those volunteers who were given vitamin D supplement for two years, had a 25% lower chance of dying from cancer, compared to the placebo-controlled group. Before the antibiotic era, in order to enhance the immunity in tuberculosis (TB) patients, cod liver oil which is rich in vitamin D was introduced in 1833 by a German physician Dr. Henkel [24]. Vitamin D deficiency is associated with the risk of tuberculosis (TB), and plays a large role in the innate response against tuberculosis infection, activation and progression. Vitamin D also down regulates the production of pro-inflammatory cytokines such as TNF- α, IL-6, IFN-γ. etc., and could protect the host from excessive tissue damage at the site of infection [25].

COVID-19 and Vitamin D

Spain and Italy have higher COVID-19 mortality rates worldwide and also have high rates of vitamin D deficiency. On the other hand, the Nordic countries (Denmark, Finland, Iceland, Norway and Sweden), have lower rates of COVID-19 infection and mortality and they also have higher vitamin D levels [26]. Recently, University of Chicago' physicians have observed that vitamin D deficiency is more common in the elderly population and subsequently they are severely affected by COVID-19 infection[27]. In Chicago, more than half of COVID-19 cases were observed in African-American individuals who were at the greater risk for vitamin D deficiency.

COVID-19 enters the body through the mouth or nose and also may enter through the eyes. After inhaling, spike proteins of the virus bind to Angiotensin-converting enzyme 2 (ACE2) receptors of epithelial cells. ACE2 helps to regulate people's blood pressure, but the COVID- 19 requires it to enter a cell. This can be considered '*devil*', being the 'entry door' for the virus [28]. The ACE2 expression increases of elderly populations, and very low ACE2 expression appear in infants and very young children. Young adults have lower expression of ACE2 compared to adults [28]. The decreasing number of ACE2 expression in children, make them less susceptible to COVID-19, since it is very critical for allowing virus infectivity. Researchers are trying to block prevent COVID-19 entry with Anti-ACE2 antibodies and the recombinant soluble ACE2 to neutralize COVID-19 [30]. The spike proteins of the virus bind to ACE2 and perform endocytosis to enter the cells, which inhibit ACE2's normal function. This can result in and causes pulmonary vasoconstriction to precipitate COVID-19. The expression of ACE2 can be associated with the virus-induced acute lung injury (ALI), as well as it can damage organs when people have other health conditions such as older age, hypertension, diabetes and cardiovascular disease. If people are infected with COVID-19 with these health conditions they may have more severe complications. The ALI or pulmonary injury is caused due to by "cytokine storm", which is a sudden

over expression of different pro- inflammatory cytokines such as IL-6, IL-1, TNF- α, and interferon -γ [8, 31]. Elevated levels of IL-6 is the most reported cytokine linked with COVID-19 mortality rate, which causes damage of vascular barrier, capillary and diffuse alveolar in the lung, multiorgan failure and ultimately death may occur [32].

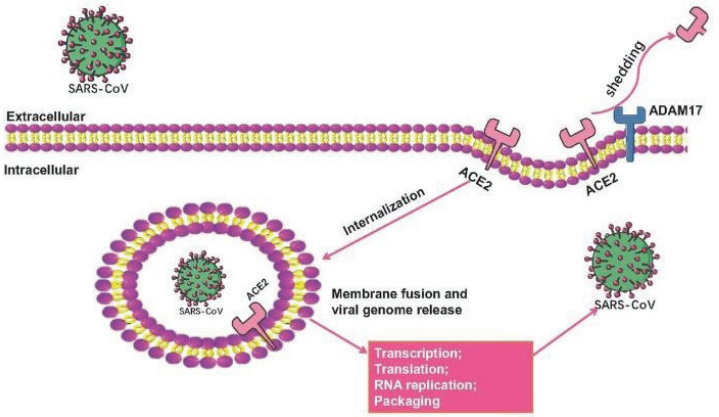

Fig. 3: *Schematic diagram of SARS-CoV infecting cells through ACE2. Adapted from Liu, et.al., (2020). Journal of Translational Internal Medicine, 8(1), 9-19.* [33].

It has been shown that administration of recombinant-ACE2 reduced inflammation and lung damage in several animal models, and also increased oxygenation. Researchers have also demonstrated that vitamin D modulates the expression of ACE2 in lung tissue and protects from infections. We have demonstrated in mouse model that diabetes induced angiotensinogen (Ang) expression can be suppressed by vitamin D analog [34, 35] which is a precursor of angiotensinogen II (Ang II). Ang II expression was significantly elevated in vitamin D knockout mice [36], and its accumulation and proinflammatory activity was reduced by vitamin D supplement.

Vitamin D suppressed the synthesis of pro-inflammatory cytokines during influenza A in lung epithelial cells [37]. To prevent respiratory infection, the 25 (OH) D level should be 40–60 ng/mL which is necessary to have an impact on immune response[15]. Vitamin D deficiency is associated with cytokine storm, and might be a possible molecular mechanism in reducing complications, inflammation and cytokine storm by vitamin D [8,38].Vitamin D supplementation could be especially important for older people as they are at high risk from COVID-19 and of vitamin D deficiency. However, there is no evidence that Vitamin D prevents COVID -19, but high-dose of vitamin D intervention might have potential benefit in decreasing risk of COVID-19 severity and mortality. This might results in a new treatment strategy which will increase ACE2 expression and decrease expression of AngII, this might be a way to fight against the COVID-19 infection.

Conclusion

The pandemic of vitamin D deficiency is a global health problem for the modern civilization, which has severe consequences in human health. There are several factors that can affect the production of vitamin D in the skin such as physical activity, and spending time outdoors, life style factors, living place, age, skin color, weight, what people eat, and certain health conditions. Minimum amount of a vitamin D supplement should be taken if needed to maintain sufficient level of vitamin D in the blood serum. Too much vitamin D can be toxic to the body and can be responsible calcium build up in the blood, which can lead to kidney stones. The vast majority of human population can get adequate Vitamin D levels from diet and sunlight.

There are supportive evidences that shown vitamin D treatment modulate the expression of ACE2 level, which decreases the mortality and severity in COVID-19 patients, by suppressing the production of cytokine storm. COVID-19 leaves us with no choice but to take precautionary and prophylactic measures to stand a better chance to fight this pandemic. It's very important for people to maintain adequate vitamin D levels to prevent getting infected or in case in occurs, to recover without mortality. Adoption to the modern civilization is the main cause of vitamin D deficiency in most people, and as a result infections and civilization of diseases are increasing rapidly.

Conflict of Interest: None

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3
CHAPTER

Lipid based nutraceuticals and nanoformulations: emerging applications in pharmaceuticals and cosmetics industries

Olivia Dhara and Pradosh P. Chakrabarti*

Centre for Lipid Science and Technology
CSIR- Indian Institute of Chemical Technology, Uppal Road, Tarnaka, Hyderabad, India
E-mail: pradosh@iict.res.in

Abstract: Oils and fats are generally regarded as a source of energy. Apart from providing more than twice the energy per unit weight as compared to protein and carbohydrate, they also supply essential fatty acids and oil-soluble vitamins. Oils and fats also contain many nutraceuticals that have proven beneficial effects against some specific diseases. These components of oils and fats have already created a huge impact as their demands in the world market are increasing steadily. Scientists and practitioners of medicines are regularly exploring the use of these nutraceuticals in cancer therapy, gene therapy, in the treatment of brain-related disorders, cardiovascular diseases, age-related macular degenerations and many more. In this review, the applications of lipid-derived nutraceuticals for treatment and cure of some specific diseases will be discussed. In the last few decades, applications of nanoscience and nanotechnology were explored in every area of science, wherever it is possible. Similarly, scientists have worked on various types of nanoformulations and lipid-based drug/ bioactive delivery systems. The biocompatibility of these formulations always gave an edge over other delivery vehicles. The science is still growing, however many formulations are reported to have a significant impact on the treatment of diseases like cancer, sclerosis, Alzheimer's diseases, Parkinson's disease, retinal problems, etc. These particular characteristics of the nanoformulations were also attempted to deliver specific functional properties in different types of cosmetic products. In this review, the latest developments of lipid-based nanoformulations are also discussed.

Keywords: Nutraceuticals, Cancer Therapy, Nanoformulations, Solid Lipid Nanoparticles (SLN), Nanostructured Lipid Carrier (NLC)

Oils and fats are important components in our food and the calorific value of per unit of oils is more than double of that of proteins and carbohydrates. Oils and fats also provide the essential fatty acids required for various physiological functions and are the source for oil-soluble vitamins like A, D, E and K. Some oils and fats contain

higher amounts of nutritionally beneficial components that are having specific benefits against some diseases. On the other hand, Nanoscience or nanotechnology has been a buzz word in many fields of research. This area of research has grown tremendously in the last two to three decades. In fact, nanoscience or nanotechnology is nothing but science or engineering research conducted with nano-scale (specifically 10^{-9} meters) materials. However, what makes this particular field interesting is its widespread added uses and advantages in addition to the regular applications. It is seen that with proper manipulations, properties of nanoparticles can be tuned significantly which can result in novel features in physical, chemical, and biological applications [1]. It is well known that lipids can self assemble into nanofilms, micelles, reverse micelles, etc.[2]. As a result, nanoscale lipid and lipid-soluble materials are being utilized for many typical nanotechnology-based applications. The importance of nanolipids lies in the fact that they can easily overcome the limitation of other colloidal systems like emulsions, liposomes, and polymeric nanoparticles. Nanolipids exhibits good release profile and targeted drug delivery with excellent physical stability[3]. Apart from the excellent biocompatibility compared to polymeric counterparts, lipids nanoparticles exhibit excellent kinetic stability as well as rigid morphology, thereby making them popular over vesicular lipid colloidal systems like liposomes, niosomes, etc.[4,5]. Lipid nanoparticle, thus, has been an emerging field and is being explored for many applications. Although the studies were initially focused on the preparation aspects, later the focus got shifted more to usage and compositions. Lipid nanoparticles can be broadly divided into two categories: nanospheres - having a homogeneous structure across the particle and nanocapsules - having a typical core-shell structure[6]. Solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs), Lipid-drug conjugates(LDCs), and Lipid nanocapsules (LNC) are major forms to be more specific.

Nutraceutical is a term usually given to the nutritionally enriched food products or part of food that has clinically proven medicinal or health benefits and that can be used for treatment of specific diseases [7]. The nutraceutical market has seen very steady growth in Europe and the USA and also in South East Asia and the estimated global market is in the range of 152 billion USD. This market is expected to grow more rapidly in post-COVID -19 scenarios. In this article, the importance of some of these nutraceuticals in the treatment of various types of cancers, cardiovascular diseases, and applications of these natural products in cosmetics and dermatological formulations will be discussed. It is evaluated that the efficacies of these compounds and other antioxidants or essential oil-based nutraceuticals improves many a fold if these are either encapsulated or delivered through some nanoscale formulations using lipid molecules. The details of these nanoformulations and their uses in cancer therapy, treatment of brain diseases, gene therapy, protein and peptide delivery, anti-oxidant and vitamin delivery, and use in cosmetic products will also be discussed.

Liposome to Nanoformulated lipid

Liposomes have been the most potential and practically used carrier system for in-vivo drug delivery especially for lipophilic drugs. Liposomes are simple microscopic aqueous vesicles enclosed by a lipid membrane composed of lipid molecules. This membrane is actually a phospholipid bi-layer arranged in a micelle structure. It is this success of liposome in various therapeutic applications that has inspired the development of various advances in nanolipids [8]. Recent advancements in nanotechnology have motivated the development of various novel liposome-like nanostructures with improved drug delivery performance. In spite of the growing functionality of the conventional liposomes, their (<100 nm size) application was limited by their intrinsic instability. Proper surface modifications of the liposomes were of urgent need in order to restrict their strong interaction with serum proteins, cells, and tissues, resulting in fast immune clearance and undesired off-target toxicity [9, 10].

In order to stabilize the liposomes and achieve stealth ability, in the next stage of development, various polymers like polyethylene glycol (PEG), poloxamers, poloxamines, dextran, sialic acid derivatives, polyacrylic and polyvinyl polymers, etc. have been used to modify liposome surface properties [11,12]. PEG has shown the best results amongst others under various conditions and is often considered as the gold standard for polymeric liposomes. PEG layer provides steric hindrance and prevents liposomes from fusing with one another thereby enhancing the in vivo circulation of the drugs and pharmaceuticals [13]. In recent years super-hydrophilic zwitterionic polymers are also being tried to achieve stronger hydration, lesser interaction with lipid bilayers, and better liposome stabilization [14].

However, these polymer-coated liposomes showed some distinct drawbacks in anti-microbial drug delivery for treating infections. Along with stabilizing the liposomes against fusion with each other, these polymers also prevented the liposomes from fusing with the bacterial membranes, to which the antimicrobial payloads need to be delivered [15]. Thus scientists started looking for structures that will resume their fusion activity once they reach the infection site. This led to the development of an emerging strategy where tiny nanoparticles were attached to the liposome surface [16]. Non-specific adsorption of charged nanoparticles onto phospholipid bilayers has been reported to provide steric and electrostatic repulsion thereby preventing liposomes to form large vesicles [17]. These tailor-made nanostructures (especially gold) infused liposomes have shown a wide range of applications, especially in treating infections of human skin [18, 19]. In spite of having such specific and many-fold advantages liposomes were bound to the limited applications, primarily due to disadvantages like short shelf life, poor stability, low encapsulation efficacy, rapid removal by the reticuloendothelial

system & cell interactions [20]. Demand for advanced control over drug release and drug delivery led to the path for the development of other kind of nanolipids like solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLC) [21].

Types of Nanolipids

Solid lipid nanoparticles (SLN): SLN is the most potent alternative to traditional colloidal systems [4]. Ranging over a size of 50 to 1000 nm, SLNs are physiological lipids dispersed in water or in aqueous surfactant solutions. Lipid includes triglycerides, partial glycerides, fatty acids, steroids, phospholipids and waxes. Surfactants of varying charge and molecular weights have been used for stabilizing the system [22]. Owing to its small size, large surface area, high drug loading, and the interaction of phases at the interface, these particles are widely used for pharmaceutical applications [23]. SLNs can be prepared in various methods which include high-pressure homogenization (both hot and cold), ultrasonication/high-speed homogenization, solvent emulsification-diffusion, spray drying method, double emulsion method, precipitation technique, film-ultrasound dispersion etc.[4,23-28]. SLN offers advantages like targeted drug release, improved stability of pharmaceuticals, enhanced drug content, feasibilities of carrying both lipophilic and hydrophilic drugs, easy scale up etc.[24]. However, it comes with some major disadvantages like unpredictable gelation tendency and unexpected dynamics of polymeric transitions [22, 25].

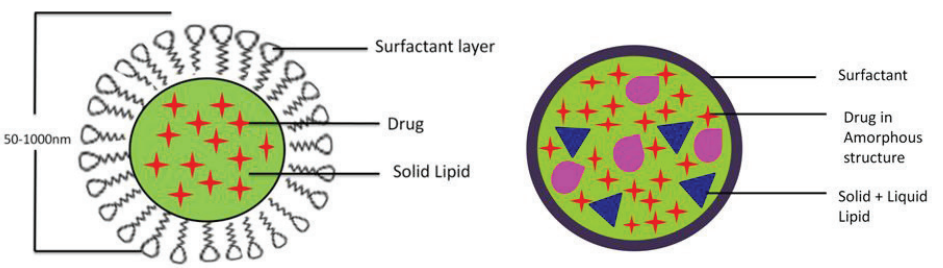


Fig 1: *(a) Solid lipid nanoparticles, (b) Nanostructured lipid carriers*

Nanostructured lipid carriers (NLC): Nanostructured lipid carriers (NLC) were developed as second-generation nanocarriers mainly to overcome the disadvantages of SLN [29]. NLC is similar to SLN. However, the only difference being that in NLC the core matrix has both liquid and solid lipid blend and dispersed in the aqueous phase containing surfactants. Due to the presence of both the phases, the crystal lattice formed is imperfect. It is this imperfect core and possible amorphous matrix that assists better drug loading and lowers drug escape [30]. The preparation of NLCs is very similar to that of SLN, the difference being the use of a liquid lipid. Homogenization, emulsification, ultrasonication, etc. are the widely preferred and practiced methods[31]

for the preparation of NLC. NLCs help in improving the bioavailability of poorly water-soluble drugs targeting various systems like the pulmonary, ocular tissues, brain tissues, cancer tissue, etc.[32-34]. Currently, NLC incorporated cosmetic products and dermal creams are marketed [4]. Oral and topical applications of NLC are also focused on using these in cosmetic and dermal creams [35].

Lipid-drug conjugates (LDC): SLN and NLC both were more suited for lipophilic drugs. Thus to overcome low drug loading capacities of SLN and NLC for hydrophilic drugs LDC was developed. Generally, LDC is prepared using salt or a covalent linkage which is further nanosized using a high-pressure homogenizer using aqueous surfactants [36, 37]. Conjugate drugs are synthesized with fatty acids, steroids, glycerides, phospholipids, etc. LDC exhibits several advantages over conventional nanolipids like improved oral bioavailability, improved targeting to the lymphatic system, enhanced tumor targeting, reduced toxicity, etc. [38].

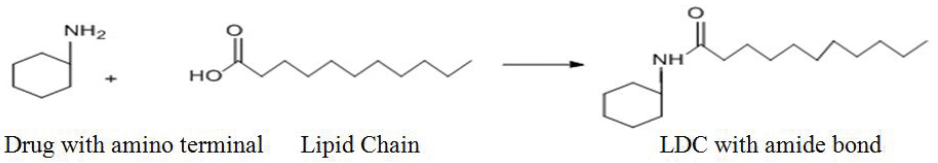


Fig 2: *Lipid-drug conjugate*

Lipid nanocapsules (LNC): Lipid nanocapsules are the latest nanoparticles of lipid origin having a lipoprotein-like structure. Based on a phase-inversion temperature process, LNC has an oily core surrounded by a tensioactive rigid membrane [39]. It has been developed in recent years as a hybrid between polymeric nano-capsules and liposomes [40].

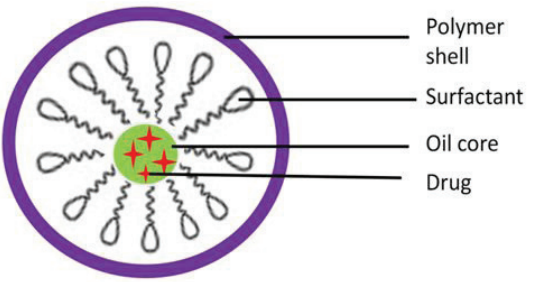


Fig 3: *Lipid nano-capsules*

These were some of the basic lipid nano-particles that are being explored presently for drug and active delivery with several therapeutic, cosmetic, and technological motives. Some of the applications are discussed here.

Medicinal Applications of Lipid-based nutraceuticals

Oils and fats are a chemical entity where three-OH groups of glycerol molecule are esterified with three fatty acids. These fatty acids play an important role in nutritional qualities of oils and fats. Fatty acids having more than one unsaturation are called polyunsaturated fatty acids (PUFA). It is well known that PUFA may have an important role against coronary heart diseases and also against various types of nervous system disorders like Alzheimer's, Schizophrenia, metabolic syndrome, etc. [41-44]. ω-3 and ω-6 fatty acids also have widespread biological applications [45]. Dietary α-linoleic acid has shown to have a significant effect on lowering cholesterol and hypertension [46]. Some oils like evening primrose, hempseed, borage, etc contain gamma-linolenic acid (GLA). This fatty acid is having some role in treating diseases like eczema, psoriasis, and pre-menstrual syndromes [47]. Eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) are two more essential fatty acids present in fish oil which play important role in proper brain functioning, retina health, Alzheimer's and Parkinson's diseases [48,49]. Foetal development and growth in the early years are also influenced by these fatty acids and they are usually used in infant formulations [50]. Conjugated linoleic acid also has an important role in lipid metabolism, prevention of heart diseases and different types of cancer and also improves immune functions [51]. Phytosterols, steryl esters, and phytostanols are present in most of the vegetable oils and these compounds are known for their capabilities of lowering serum cholesterol levels [52-54]. 2g/day dosage of phytosterol or phytostanol reduces the risk of heart diseases by around 25% [55]. Phospholipids are another group of nutraceuticals present in oils like soybean, rice bran, sunflower etc. They show anti-oxidant properties [56] and are used in pharmaceutical formulations and also in drug delivery vehicles [57, 58]. The abnormal metabolism of phospholipid is believed to be associated with cancer [59, 60]. Lysophosphatidyl choline has also been associated with anti-cancer activities [61]. A number of studies also showed that phosphatidylcholine is another type of phospholipid that has uses in anticancer formulations and has a major role in brain damage related issues.

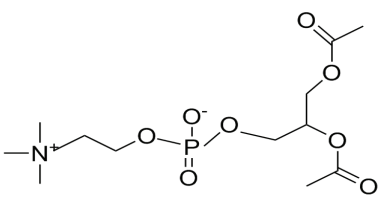
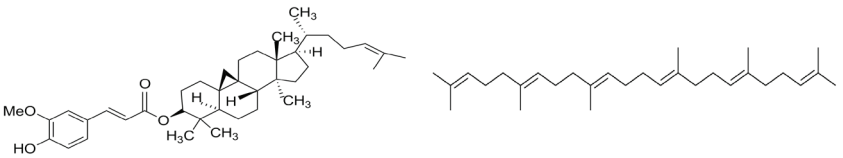


Fig 4: *Phosphatidylcholine*

Rice bran oil contains oryzanol- a unique nutraceutical that has many medicinal applications including treatment of hyperlipidemia, insulin uptake, cirrhosis of the liver, etc.[62-64]. Carotenoids are pigments present in various oils– red palm oil having

the maximum amount of carotenoid. β-carotene is the precursor of vitamin A and play a significant role in maintaining proper health of the eye, the retina in particular[64]. β-carotene also shows anti-obesity, hyperlipidemic activities, and reduces the risk of degenerative diseases [65-67]. α-carotene has shown anti-cancer properties [65]. Sesame oil and Flaxseed oil contain another group of nutraceuticals called lignans [68]. Flaxseed lignans can protect against various cancers like breast, colon, skin, and prostate cancers [69,70]. These nutraceuticals can also reduce atherosclerosis and diabetes [71,72]. Sesamin- a lignan present in sesame oil can reduce hypertension and decrease LDL and increase HDL [73,74]. Sesame lignans are being explored as an alternative to hormone replacement therapy for postmenopausal women [75]. There are many other such nutraceuticals present in oils and fats like isoflavones, medium-chain triglycerides, which has significant medicinal applications.

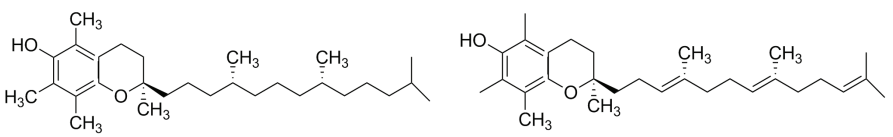


(a) (b)

Fig 5: *(a) Oryzanol, (b) Squalene*

Cosmetic Applications of Lipid-based nutraceuticals

The major lipid based nutraceuticals used in the cosmetic industry is vitamin E or tocopherol and tocotrienols. Most of the oils contain tocopherol in minor quantities whereas oils like palm and rice bran contain tocotrienol. Wheat germ oil is rich in this vitamin E. The use of vitamin E in many skin cream and other dermatological formulations are well-known. They also show anti-aging properties[76]. These nutraceuticals have the capacity to significantly lower the risk of cardiovascular diseases and also can have a role in the treatment of breast cancers [77]. Squalene is another lipid-based nutraceutical present in wheat germ oil, shark liver oil, rice bran oil, etc. which has tremendous scope for use in the cosmetic industry [78]. It is projected to be a hydrating agent and may help to regenerate healthy skin cells [46]. Squalene is also used as an adjuvant in many vaccine formulations [79, 80]. As a result, the importance of squalene has increased considerably in the post-COVID-19 scenario.



(a) (b)

Fig 6: *(a) α- tocopherol, (b) α-tocotrienol*

Lipid based Nanoformulations in Pharmaceutical Applications

As discussed earlier, the next step of development was use of liposomes. These are lipid-based vehicles for the administration of pharmaceuticals and nutraceuticals for specific purposes. These are spherical vesicles having one or more lipid bilayer. This particular vesicle has shown tremendous promises for drug delivery for cancer therapy[81-83], gene delivery [81, 84 and 85] and in the cosmetics industry [86, 87]. However, the liposomes had intrinsic disadvantages. Nanoformulations consisting of nutraceuticals and pharmaceuticals showed better performances in the case of medicinal and cosmetic applications. In the last two decades, many nanoformulations were prepared using solid and liquid lipid molecules as matrices. They show better stability and hence are being explored for various applications. In the following section, a few examples of these applications will be discussed.

Cancer therapy

Due to the enhanced permeability and retention effect, carefully designed lipid-based nanoformulations can easily extravasate into the tumor and are retained there[6]. These can be used for cancer cell-selective cytoxicity[88]. Lipid nanoparticles also help in overcoming multidrug resistance [89]. SLN based controlled delivery system was used for the delivery of paclitaxel [90]. A nanoformulation having cholesteryl butyrate and propionate as a lipid matrix was used for the treatment of tumors and for anti-inflammatory pathologies[91, 92]. PEG-based nanoformulations were prepared including bioactives like castor oil, ethyl oleate, maize, soybean, coconut, olive oil, etc. and these are tried for treatment of osteoarthritis and rheumatoid arthritis and also as anticancer nanomedicine [93, 94]. In another patented process, LNC type nanomaterials were used to deliver p-glycoprotein inhibitor for reducing tumor drug resistance [95]. SLN is also proven to be an important drug carrier. An anticancer drug like Tamoxifen is incorporated in SLN formulations for prolonged-release for breast cancer patients [96]. The anti-cancer properties of a nanoformulation consisting of rosemary essential oil and specific human cell phospholipids were evaluated [97]. Maragheh and coworkers used another nanoformulation containing cherry pit oil and evaluated its anticancer effects on both breast cancer murine model and MCF-7 cell lines [98]. Mitomycin C, ginger, sorbitan monooleate, sorbitan monolaurate, and essential oils encapsulated in a nanoformulation were evaluated for the cytotoxicity and apoptosis in breast and cervical cancer cells [99]. Targeted treatment of lung cancer was tried by developing some nanoscale oil bodies [100] and these NLC formulations showed a huge effect on antitumor activities. It was shown that nanoformulations containing linalool had much better in vitro antiproliferative effects on hepatocarcinoma and lung adenocarcinoma cell lines compared to free linalool [101]. Hybrid polymerized nanoparticles based on *Nigella sativa* L. oil (black cumin) was found to be suitable for controlled release of lipophilic bioactive drugs like 5-fluorouracil, α-tocopherol, curcumin, etc.[102].

There are many such examples that clearly show that lipid-based nanoformulations have added advantages over other drug delivery systems and these nanoformulations may become a much sought after media to fight against cancer.

Brain related problems

Medicinal formulation of drug riluzole was prepared by Bondi and his colleagues using one type of NLC and treatment of multiple sclerosis was attempted [103]. Lipid nanoparticles were prepared as a vehicle that can be used for overcoming the blood-brain barrier [6]. SLN was having a deposit encapsulated in it and this formulation was used to treat brain tumors [104]. Another SLN was used to prepare an injectable with idebenone for the treatment of neural damage after brain trauma [105]. A mixture of curcumin, folic acid, lecithin, and HU-211 loaded in another SLN formulation was used as an anti-depressant [106]. SLN formulations were used to deliver drugs and bioactives like granisetron and carbidopa into brain even through nose and ear [107, 108].

Gene therapy

Gene therapy is one of the most modern technologies of treatment and in recent years nanoformulations based on lipid moieties are gaining more and more importance in delivering the actives or the drugs. Both the SLN and NLC types of nanoformulations were used for delivery of nucleic acid [109], SiRNA [110, 111], p-DNA [112]. There exist tremendous scopes for working in this area.

Delivery of Vitamins and antioxidants

A SLN formulation was prepared with vitamin E derivatives in the lipid moiety and an antibiotic in aqueous phase [112]. Tocotrienol was also delivered using SLN/NLC nanoformulations. Vitamin D_3 and retionoic acid were also delivered using SLN for treatment of diseases like age–related macular degeneration, diabetic retinopathy, cancer, osteoporosis etc.[113].

There are many other examples where lipid based nanoformulations were used for delivering lipid and non-lipid nutraceuticals and drugs or bioactives for treatment of specific diseases.

Use of Lipid-based Nanoformulations in Cosmetics Industry

Apart from the treatment of different diseases, these nanoformulations are also used in cosmetics industries for delivering some functional properties. Rice bran and raspberry seed oil-based nanoformulations are used in skin care products [114]. Solar protection formulations were prepared using TiO_2 [115]. A nanoformulation comprising of glycerol palmitostearate, hydrogenated phosphatidylcholine, monoglyceride, polyoxyethylene sorbitan monooleate and squalene was used in another cosmetic formulation [116]. Polyoxyethylene and soy lecithin based SLN were used to deliver sesamol from sesame

seed oil. This can reduce oxidation and photodegradation. This area of science is also maturing gradually and nanoformulations are having considerable role in designing newer cosmetics products.

Conclusion

It is quite evident from the above discussion that lipid based nutraceuticals play an important role in physiological functions and their applications are being explored in pharmaceuticals industries. They are being prescribed to the patients requiring treatment for cardiovascular diseases, different types of cancer therapies, cholesterol lowering activities and antioxidant properties. However, the emerging trend is to explore different types of nanoformulations prepared by using lipid moieties and having lipid or non-lipid bioactives or pharmaceuticals within it. These formulations could achieve much efficient and safer method for the treatment of various specific diseases including cancer. The same nutraceuticals and nanomaterials showed tremendous scope for applications in formulation of cosmetics. More research in the area of lipid-based nanomaterials as a delivery vehicle would definitely help in developing safer pharmaceuticals and cosmetics products having higher efficiencies.

Acknowledgement

IICT Communication no.: IICT/PUBS/2020/245

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CHAPTER

White blood cell diameter as a biomarker for rapid diagnosis of septicemia and antibiotic sensitivity screening

Shabana Bano[1#], Anirban Mukherjee[2,3#], Manjila Gupta[2], Sourav Ghosh[2], Arindam Roy[1,2], Madhusmita Mishra[4], Debkishore Gupta[5], SujoyPanchadhyayee[6], Shaon Ray Chaudhuri[1$,2$,7*]

[1]Department of Biotechnology, Maulana Abul Kalam Azad University of Technology, West Bengal, BF-142, Sector 1, Salt Lake, Kolkata, India.
[2]Centre of Excellence in Environmental Technology and Management, Maulana Abul Kalam Azad University of Technology, West Bengal, BF-142, Sector 1, Salt Lake, Kolkata, India.
[3]Department of Microbiology, Gurudas College, 1/1 Suren Sarkar Road, Beliaghata, Phoolbagan, Kolkata, India.
[4]Analytical Researcher, Shell Technology India Market Private Limited, Bangalore, India.
[5]Clinical Microbiology and Infection Control, Calcutta Medical Research Institute, 7/2 Diamond Harbour Road, Ekbalpur, Kolkata, West Bengal, India
[6]Department of General Medicine, Burdwan Medical College, Burdwan, West Bengal
[7]Department of Microbiology, Tripura University, Suryamaninagar, West Tripura, India
[#]Equal Contribution
[$]Formerly affiliated to those organizations when the work was conducted in her laboratory.
*E-mail: shaonraychaudhuri@tripurauniv.in

[Shabana Bano was involved in testing the effect of anticoagulants on WBC dimension; Anirban Mukherjee was involved in carrying out the work with Candida sp; Manjila Gupta was involved in the development of the database; Sourav Ghosh was involved in phytochemical extraction that was further used for conducting in vivo experiment; Madhusmita Mishra was involved in the editing of the paper; Debkishore Gupta and Sujoy Panchadhyayee provided clinical samples for the experiments;Debkishore Gupta also provided the corresponding VITEK 2 data; Shaon Ray Chaudhuri had designed the entire study carried out by the students including the in vivo study carried out using outsourcing services; obtained the funding for the work]

Abstract: A considerable morbidity and mortality rate is globally reported due to the presence of the microbial (bacterial and fungal) pathogen in blood circulation. The regular process of detecting blood infection is through blood culture and biomarkers like the CReactive Protein (CRP). The detection is time-consuming, and so is the screening of the antibiotic sensitivity profile. This delay is responsible for the mortality associated with Septicemia. Fast, accurate, and low-cost detection of microbial blood infection and antibiotic sensitivity response is of utmost importance during Septicemia. Methodology: Light microscopic visualization of white blood cells (WBC) dimensions as a surrogate marker was studied for the rapid detection of blood infection. Data was generated for understanding variation in the WBC dimension with gender and age. Variation in WBC diameter was compared between non-infected (control) and microbe seeded blood samples collected from the human population. Thein vitro findings were cross-checked through an in vivo testing in the mouse model. Results: Insignificant (p-value>0.05) variation in the WBC dimension with variation in gender and age was observed (205 sample size). Significant variation (p-value<0.05) in the WBC dimension was observed upon infecting healthy blood withpure bacterial and yeast strains from clinical origin. A similar cell shrinkage was observed duringin vivo mouse model testing, which was reversed on appropriate antibiotic treatment. The microscopic assay developed during this study is highly sensitive, requiring less technical skill and rapid compared to the current techniques available.About 2 hours of the lead timewas required for the setup to result delivery (versus 24–48h of conventional culture). This method can be adopted as a rapid microscopic on-demand testing technique for blood microbial infection detection and antibiotic sensitivity screening without needing a culture-based assay.

Keywords: Septicemia; White Blood Cell; Antibiotic sensitivity; *in vivo* analysis; Bacteria; *Candida sp.*

Sepsis remains one of the significant causes of death worldwide, and the mortality, morbidity rate is disproportionately high in underdeveloped and developing countries[1-5]. According to a recent global survey, about 70% of the 9 million annual neonatal and infant deaths are due to this clinical condition, and half of these cases are reported in Sub African and Asian continent[6-7]. The high occurrence in these demographic areas is attributed to environmental degradation, malnutrition, and high incidence of bacterial, parasitic, and HIV infection[8-9].

Septicemia is a complex clinical manifestation triggered by an infected stimulus leading towards an exaggerated immune response [10-11]. The usual course of the inflammatory response that is evoked against pathogenic and commensal microbes ultimately affects and damages various organs. This disease is more prevalent in hospitalized patients, especially infants and geriatric patients' due to the immunocompromised state. The inefficiency of the immunological effector mechanism causes a devastating state of bacterial infection in the blood (bacteremia), leading to severe sepsis and septic shock [12-14].

Importantly the most causative pathogens among hospitalized patients in the descending order are Gram-negative, Gram-positive, and mixed bacterial pathogens [15].

Besides, fungi have emerged as a significant causative agent of infection, especially in a hospitalized setting, and *Candida* is found to be the fourth common agent identified in the blood culture of sepsis patients [16-18]. The other common etiologic agents are *Staphylococcus aureus* and *Escherichia coli*. Anaerobic bacteremia condition is found to be rare in pediatric patients than in adults. It was also observed that bacteremia infected children have an increased tendency for *Staphylococcus*, *Streptococcus pneumoniae*, and *Meningococcus*[19].

The conventional line of treatment in sepsis involves stepwise patient history taking, assessment of risk factors, the presence of infection, their possible source identification followed by appropriate treatment with antibiotics. The conventional laboratory and radiographic testing facilities are used for locating the infection site and evidence of organ dysfunction. The standard laboratory studies include hematology test (WBC count, hemoglobin, hematocrit, platelet count) and biochemical tests (electrolytes, bicarbonate, glucose, creatine, coagulase, catalase, bile solubility). The standard antimicrobial screening method is testing the potency of the drugs against the bacteria on a Muller Hinton Agar Plate. All these conventional tests are expensive, time-consuming, and are at the discretion of examiner [20]. In successive years of medical science evolution, the successful introduction of antibiotic susceptibility testing of microorganisms took place, which still required 48 to 72 hours for the final result delivery [21]. The first hour after detecting infection is significant in disease management as it directs the appropriate use of antibiotics instead of an application of a broad-spectrum drug, which can later lead to multidrug resistance. There were a very few studies illustrating a unique system of detecting contamination in blood in an in-vitro system within 4 h [22-23].

In 2013, this same research group reported real-time PCR based detection of cell count of both/either Gram-positive and negative organisms in case of urinary tract infection within a short span of 5 h and scaled over conventional enrichment culture technique of 24-48 h [24]. The group's next objective was to explore the hematopoietic system to improvise the detection procedure of microbes in body fluids. Hematic cells are known to have a stipulated life span and are susceptible to internal and external stimuli to undergo a stereotyped sequence of changes, including shrinkage and cell collapse [25]. Further methodical scrutiny, based on the above method, for improvising the detection procedure of microbes in body fluids like blood lead to the study of bacteria-induced WBC size shrinkage which was reported in the year 2013 as a microscopic method for rapid screening of antimicrobial activity of phytochemicals from *Mentha spicata* leaves against MDR *Pseudomonas sp.*[26].
The objective of the current study was as follows:

I. To test the impact of seeded *Candida* and phytochemical administration on WBCdiameter.
II. To test the effect of gender, age, and anticoagulant on WBC diameter.
III. To test the effect of seeded bacterial strains on WBC diameter.
IV. To test bacteria (*Pseudomonas sp*) induced WBC shrinkage (in-vivo system) in the mouse model and its reversal on antibiotic application.

Material and Mothods

Blood fungi infection: In vitro

Fungal strains used in this study, *Candida albicans* (S 08) and *Candida glabrata* (S 01) were isolated from different environmental sources and identified in a previous study [27]. Approximately 20-30 cells (20 µl) of *Candida* sp. were seeded in the blood sample. The whole set was incubated at 37°C in a water bath for up to 4 hours. Samples were collected aseptically at 1-hour interval to check for the shrinkage of WBC.

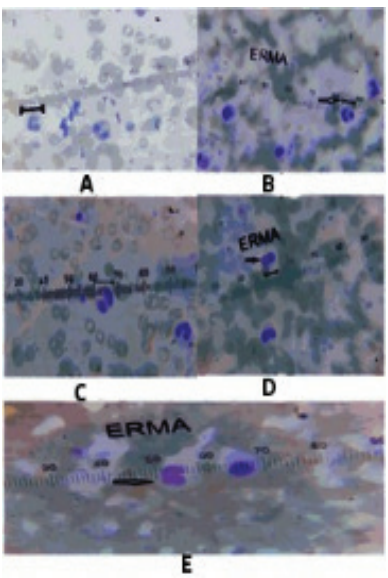


Fig. 1: *Blood cells after Leishman staining under 100X magnification (A) Control WBC (non-seeded) (B) Blood seeded with Candida albicans isolate (S 08) (C) Blood seeded with Candida albicans isolate (S 08) in presence of combination drug (D) Blood seeded with Candida glabrata isolate (S 01) (E) Blood seeded with Candida glabrata isolate (S 01) in presence of combination drug.*

Each set {Control (unseeded); blood seeded with *Candida albicans* (S 08); seeded blood (S 08) with Plumbagin; seeded blood (S 08) with Fluconazole; seeded blood (S 08) with the combination (Plumbagein and Fluconazole); blood seeded with *Candida glabrata* (S 01), seeded blood (S 01) with Plumbagin; seeded blood (S 01)

with Fluconazole as well as seeded blood (S 01) with the combination (Plumbagein and Fluconazole)} of the experiment was performed in a replicate of three for the statistical validation. For each experiment, three slides were prepared, and at least 30 WBC diameters were measured from each slide resulting in a total of at least 90 WBC diameter in each case (Fig. 1).

WBC dimension variation with gender, and age

A total of 205 individuals were included in the study. The data set consists of blood samples collected from different males and females of age group ranging from 5 to 80 years.Blood collection was done with the help of a disposable syringe or by blood lancet from the volunteer. Each individual's three slides were prepared by making thin clean blood smears for studying the WBC dimension. The blood slides were observed under 40 x magnifications in LEICA DM 750 and LEICA DM3000 microscope. For each blood, three slides were prepared, and from each slide, 10 WBCs were identified. Photographs were taken, and with the help of software LAS EZ measured the diameter of each WBC. For each individual, 30 WBC diameters were measured throughout the smear on the slide.

Blood Anticoagulant Interaction

Earlier reports showed a significant decrease in WBC dimension with a bacterial infection in seeded blood [22-23] and the inhibition of this shrinkage in the presence of appropriate antibiotics [26]. The same method could also be used for screening the antimicrobial property of potential drugs as an alternative to the conventional method of plate clearing assay hence saving in terms of the time required for detection (4 hours as compared to 24 to 48 hours in conventional method) [26]. The purpose of this section was to reduce the chances of developing an artifact during the investigation, which could produce false results. The two steps which could result in the development of artifacts were using EDTA as anticoagulant and Leishman staining (involving heating of the smear) for staining WBC.

EDTA (ethylenediaminetetraacetic acid) is the most commonly used anticoagulant in blood collection tubes. It inhibits the clotting process by removing calcium from the blood. This chemical has been used to prevent clotting in blood specimens since the early 1950s and has certain advantages over other anticoagulants. A smear of blood, with and without EDTA (1mg/ml), was drawn on grease-free slides. The smear was observed under the microscope at 40X magnification. The diameter of the WBC was measured, and statistical analysis was carried out.

Assessing the time of incubation with bacteria for WBC shrinkage

To ensure that this assay was not specific for the selected genus of bacteria, similar studies were carried out using well-characterized strains of other bacterial genera, namely *Pseudomonas aureginosa*, *Escherichia coli*, *Bacillus* sp, *Staphylococcus aureus*, *Klebsiella pnuemoniae*, and *Pseudomonas sp*[24] from clinical origin. The bacterial strains were enriched in Luria-Bertani (LB) broth and were inoculated into the blood at a final concentration of 1% (with similar CFU for each strain). The blood cultures were incubated at 37°C for different time intervals (1, 2, 3 hours) and after that visualized under a microscope to investigate the shrinkage kinetics of the WBC. Each set of experiments was performed in a replicate of three for the statistical validation. One-way ANOVA validated the shrinkage kinetics at 95% confidence level.

Correlation of WBC shrinkage with the genotype

If WBC shrinkage is dependent on the genetic composition of the individual, then the next task would be to assess if this assay works in the case of both types of populations (those whose WBC shrinks and those whose WBC do not shrink in response to EDTA). To understand the same, the population mentioned above was divided into two groups: those not showing shrinkage (group 1) and those showing significant shrinkage (group 2). Blood (100µl) from subjects of both these groups in the presence of EDTA was seeded with 5 µl well-characterized *Staphylococcus haemolyticus* from clinical origin. The seeded blood was incubated at 37°C in a water bath for 2 hours while smears were made at different time intervals (0 hours, 1 hour, 2 hours); observed under the microscope at specifications as mentioned earlier and compared with that of control set (non-seeded) treated under identical condition (except the bacterial seeding).

Blood bacterial infection: In vivo

The above findings reveal this method to be a substitute for the conventional method of blood infection detection. It could also be used for antimicrobial susceptibility testing with known pathogens. However, there might be variation in the efficacy of the method *in vivo* compared to the *in vitro* analysis. To test the viability of this method *in vivo,* neutropenic mice were infected with approximately 10^7 CFU/animal of *Pseudomonas aeruginosa*. The experiments were performed, as shown in the tables below.

Table 1: *Details of the infection method of the mice.*

Group	Inoculum strength (*P. aeruginosa*)	Time points for the Blood collection PI (h)	No of animals sampled for smears
1	Approximately 10^7 CFU /animal	0 & 2	5
2		1 & 4	5
3		8 & 24	5

Table 2: *Details of the method used for the treatment of infected mice.*

Group No	Groups	Description	No of Animals
1	Early/Pre-treatment control	Sampled at the beginning of treatment	10
2	Late control	Vehicle	10
3	Reference control	Ciprofloxacin -10 mg/kg, single dose, oral	10
4	Test -1	Phytochemical- 500mg/kg, single dose, oral	10
5	Test -2	Phytochemical- 1000 mg/kg, single dose, oral	10
6	Test -3	Phytochemical- 2000 mg/kg, single dose, oral	10

Neutropenia was induced to the mice by the procedure described subsequently. Four days before the day of infection, the mice were administered with a single intraperitoneal injection of cyclophosphamide derivative having cyclophosphamide (150 mg/kg). The process was repeated on the 4^{th} day again with a dose equivalent to 100 mg/kg of Cyclophosphamide. Before starting the infection process, all animals were divided into different groups as specified in experimental design (Table 1, 2).

On the day of infection, the overnight grown culture was adjusted spectrophotometrically to an optical density (OD) of approximately 1.0 at 600nm wavelength. That corresponded to approximately 10^9 CFU/ml. The culture was diluted 10-fold with sterile 5% (wt/vol) hog gastric mucin in broth to obtain approximately 10^8 CFU/ml. This, in turn, was used (100µl) to infect the animals intraperitoneally using sterile hypodermic syringes (1 ml, 26G) (approximately 10^7 CFU/animal). The precautions adopted were gentle shaking of inoculum between the periods of administration to two animals, placing the inoculum in ice, and completing the infection within 30 minutes to minimize *in vitro* multiplication.

The first set of studies was to analyze the alteration of cell dimensions for a total of 24 to 26 hrs. In the other set of investigation, post two hours infection mice subjects were treated with single oral doses of vehicle, ciprofloxacin (10 mg/kg) and different concentration of phytochemical [5ml/kg (500 mg/kg), 10ml/kg (1000 mg/kg) and 20 ml/kg (2000 mg/kg)]. Blood samples were collected at different time points (0, 2, 8, 24 hours) by the tail prick method, and blood smears were prepared on glass slides. Blood was collected in 2 ml autoclaved centrifuge tube containing sodium heparin, and thin blood smears were prepared on the glass slide for WBC cell size determination. The conventional Leishman staining procedure aided the microscopic visualization of cells. An average of 30 WBCs was analyzed per slide, and micrographs were captured by a Light microscope (Nikon) at 100 X magnification under oil immersion. The diameters of WBCs (neutrophils) were measured using microscope software Mshot® as per manufacturer's instruction. Three measurements were taken per WBC, and thereby the average diameter of the 30 cells was estimated.

For statistical analysis, data were analyzed by using GraphPad prism (v 5.0). Mean (± SEM) diameters of WBCs and Mean (± SEM) Log10CFU/ml at 8 hours post-treatment was analyzed using a one-ANOVA followed by Dunnett's multiple comparison tests (with normal control) at 95 % confidence intervals.

Improvement of the culture-based antibiotic sensitivity detection

In the present study, we described a simple antibiotic susceptibility detection of the microbes directly from body fluids like blood, urine, peri intestinal fluid, ascetic fluid, and throat swab. Luria Bertani (LB) broth (pH 6.8-7.2) was used to cultivate microbes as it was considered a universal growth medium for microbes. 1ml of the media was poured into a 24 well tissue culture plate. Clinical samples like throat swab and clinical body fluid like blood, urine, peri intestinal fluid were collected from Peerless Hospital, Kolkata, India. All the swab samples were first inoculated in LB tubes aseptically. The tubes were put at 37˚C in a shaker incubator for two hours (Lab companion SI 300, Korea) at 250rpm (revolutions per minute). 1% of the grown culture in the LB was inoculated at each well and incubated at 37˚C. Two wells were kept as a negative control with the medium but no inoculum for contamination check during the procedure. Two wells were kept as a positive control with inoculum in the medium to check the extent of growth by the microbes in the body fluid. In the rest of the wells, the inoculated medium also had the selected antibiotic disc. For the rest of the body fluids, 1% of the clinical samples were seeded in the same manner. The occurrence of growth of the seeded microbes was manually checked every hour. After requisite hours of growth (based on the observation of the positive control well), the cultures were used for optical density measurement and plated on LB agar plate for CFU determination. This assay was performed on the mixed culture in the body fluid. The purpose was to determine the antibiotic sensitivity for the microbe in the body fluid. Hence the antibiotic working for the total microbial population would be the one chosen for the therapeutics.

Results and Discussion

Blood fungi infection: In vitro

Candida cells were able to shrink WBC diameter at different concentrations. The minimum amount of yeast cells that were able to shrink WBC diameter significantly was approximately 10^3cell/ml at 37^{o}C within 4 hours of incubation as compared to 22 cells/ml at 37^{o}C within 3 hours of incubation in case of bacteria [26].This is expected due to yeast's long generation time compared to the bacterial isolates used in these two studies. Therefore, further investigation of interaction was carried out according to the above mentioned optimum value of cell concentration and incubation time.

Both Candida species, *Candida albicans* (S 08), and *Candida glabrata* (S 01), we're able to cause shrinkage of the diameter of WBC (Fig. 1B and Fig. 1D). The extent of shrinkage was found to vary between 35% for *Candida glabrata* and 38% in *Candida albicans*. Moreover, combination drug-treated samples most effectively retain the WBC diameter, as depicted in Fig. 1C and Fig. 1E. For each experiment, a total of at least 90 WBC diameter were measured in each case (Fig. 1A). The variation in diameter was significant in each case. The grand average of all 90 WBC diameters of the control sample was calculated as 10.099 ±0.498 µm whereas, in case of *Candida albicans* (CA) and *Candida glabrata* (CG) seeded samples the values were 6.26± 0.906 µm and 6.534± 0.823 µm respectively. To validate statistically, the Z score was computed and found to be -39.11 and -42.34 for *Candida glabrata* and *Candida albicans* treated samples, respectively. We set the cut-off for the rejection of the Null hypothesis as Z-score <1.65 at a 5% level of significance. According to that, both of the calculated Z-score fall within the rejection area and resultantly, the Null hypothesis was rejected. The result was significant at $p < 0.05$, and it can be concluded that the diameter of both of the yeast cell treated samples was less than that of healthy cells.

Next, we focused on the plausible effect of WBC interaction in the presence of Candida and antimicrobial agents. This study helped us understand the effect of antimicrobial agents on blood yeast interaction and provided a sensitivity profile. It is well known that the clinical drug resistance has increased dramatically in recent decades due to irrational use and lack of efficient antimicrobial agents. This problem is now not limited to the bacterial community only where MDR (multidrug resistance) severely narrows down the choice of antibiotics for therapeutic purposes. Antifungal resistance is particularly problematic as an initial diagnosis of systemic fungal infection can be delayed, and there are few antifungal drugs available. Though only a few antifungals are available for the treatment of Candida infections [28], there are various undesirable properties, most importantly, the dose-related toxicity [29]. Bioactive components are significantly crucial in research and development of new drugs either individually or in combination with other synthetic drugs [30]. The synergistic combination of drugs and plant extract could be an active mode of treatment as the new compound generated as a result of combination is less likely for the development of resistance [31]. To overcome drug resistance, the urge for combination research showed significant importance.

The same seeding experiment was carried out with plumbagin, fluconazole, and their combination to understand the impact of combinatorial drug treatment on WBC dimension. It was found that the WBC diameter with plumbagin was 8.91± 0.56 µm and 7±0.51 µm for *Candida albicans* (CA) and *Candida glabrata* (CG) respectively. Whereas, in the case of fluconazole the value was 8.2±0.703 µm and 7.72±0.728 µm for *Candida albicans* (CA) and *Candida glabrata* (CG) respectively.

Surprisingly, when both of these agents were used in combination, the WBC diameter was found to be around 9.28±0.72 µm and 9.5±0.843 µm, respectively. The shrinkage of WBC was reduced by 78.94 to 83.31% with the combination treatment compared to fluconazole (32.34 to 50.7%), and plumbagin (12.5 to 68.94%) alone. Based on the above observation, the data of combination treatment was statistically compared with the control. We calculated the Z-score and accepted the null hypothesis as the Z score of 6.03 and 3.80 were within the non-rejection area in the case of *C.albicans*, and *C. glabrata* treated cells, respectively. The cutoff point is 1.645, and thus Z score greater than 1.645 will be accepted. The result was significant at $p < 0.05$. Hence, it can be concluded that the diameter of healthy and combination drug-treated cells are similar. It emphasizes the higher efficacy of combination treatment.

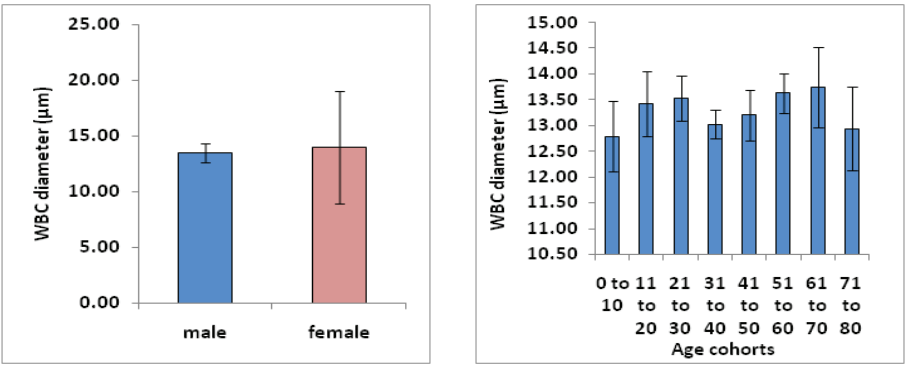
The above finding can be summarized as follows: yeast infection induced by well-characterized *Candida albicans* and *Candida glabrata*, showed a 38% and 35.3% reduction in WBC cell size. Both Candida species were able to cause significant shrinkage of WBC diameters within 2 hours of incubation ($p<0.0001$). In this study, it was confirmed that a combination drug of plumbagin and fluconazole effectively performs against Candida infection in mammalian blood. The entire setup required <4 hours of time frame as compared to the conventional method. Thus, this assay could be used for rapid fungal infection detection in blood and the screening of potential antimicrobials. However, only in vitro analysis is insufficient to suggest the synergistic effect between components from different plant extracts for therapeutic purposes.

WBC dimension variation with gender and age

The dimension of WBC of both healthy females (105) and male donor(100) are depicted in Fig. 2A. The result reflected a reduction in the mean diameter of WBC in the male sample than the female samples. On performing ANOVA at 95% confidence level, the variation was insignificant (p-value 0.29). The dependency of the WBC dimension on age was also checked by plotting the mean dimensions of WBC according to the different age groups in Fig. 2B. A correlation study exhibited a very low correlation (p-value 0.089 at 95% confidence level) between the WBC dimension and the donor's age. Therefore, the WBC dimension does not vary with gender or age.

Blood Anticoagulant Interaction

Of the 20 subjects tested in this experiment, there was a significant change in the WBC dimension (Fig. 3) in the case of 14 subjects (p-value of 0.0045 to 5E-13) (Group 2). The statistical analysis of the data revealed a p-value of 0.115 to 0.738 for the six individuals without significant variation (Group 1). It might be possible that the shrinkage of WBC depends upon the genotypic characteristics of the subject.



A B

Fig. 2: *Comparison of variation in WBC dimension (A) with gender, and (B) with age. In these cases, at least 30 WBC diameter were measured per individual, and the data were statistically analyzed using ANOVA. (A) The figure represents the mean±sd (Standard deviation) of the dimensions of WBC of healthy male and female volunteers. The one-way ANOVA was performed to validate the differences at 95% confidence level. The p-value was 0.29. (B) The figure represents the mean±sd (Standard deviation) of the WBC dimensions of healthy male and female volunteers of different age groups.*

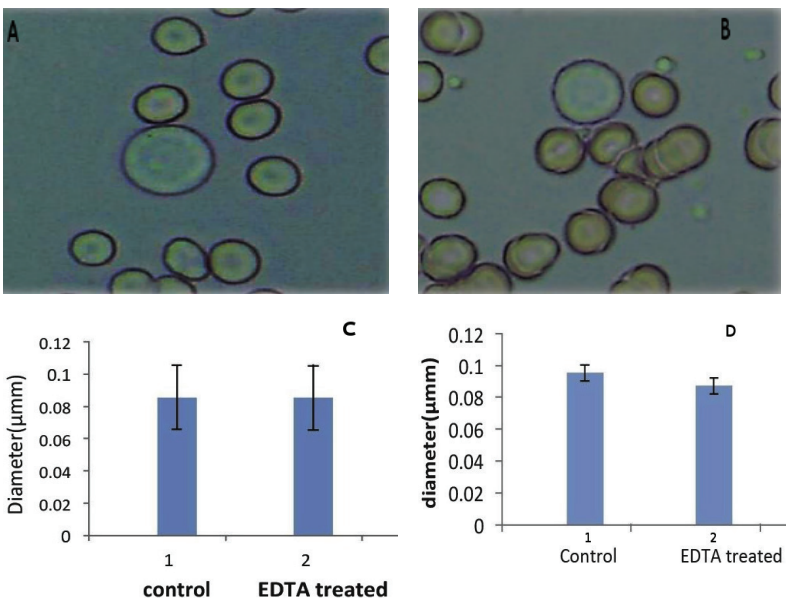
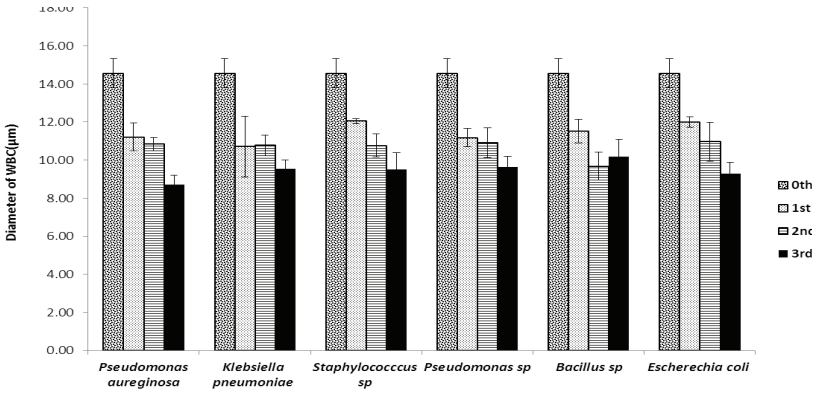


Fig. 3: *Unstained blood smear (A) without (control) and (B) with (test) EDTA showing WBC and RBC at 40X magnification under bright field Leica DM-750 microscope. (C), and (D) are the bar diagram representing the WBC diameter with and without EDTA in two individuals showing two different responses. In these cases, at least 30 WBC diameter were measured per individual, and the data were statistically analyzed.*



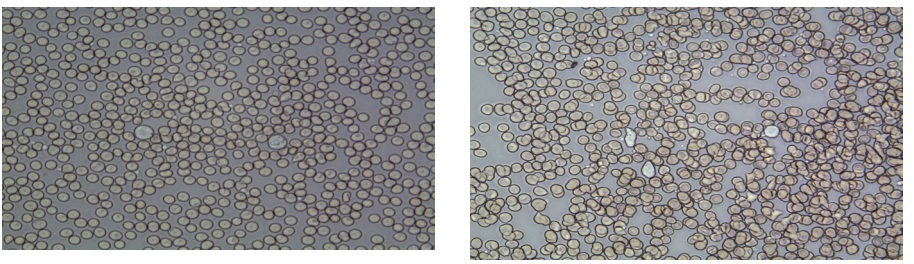


Fig. 4: *A.Comparison of variation in WBC dimensionwith time in response to different bacterial seeding. In these cases, at least 30 WBC diameter were measured per treatment, and one-way ANOVA validated the data of WBC shrinkage at a 95% confidence level. The calculated p-values were 8.97E-08, 4.58E-08, 0.000135, 2.16E-05, 0.018388, 0.004031, for Pseudomonas aureginosa, Escherichia coli, Bacillus sp, Staphylococcus aureus, Klebsiella pnuemoniae, Pseudomonas sp, respectively. B.Microscopic image (Leica DM-750) at 40X magnification of WBCs of control blood (non-seeded) with EDTA use as an anticoagulant; C.microscopic image under a similar condition as mentioned above showing significant shrinkage in the WBC dimension of the blood due to the bacterial seeding.*

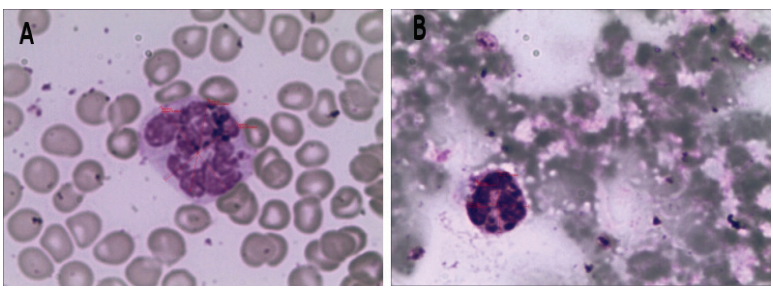


Fig. 5: *Photomicrographs of WBCs stained with Leishman's stain. A) Uninfected mice; B) Mice infected with P. aeruginosa. Magnification 100 X. Cell sizes were determined using Mshot® micrometry- the diameter of each cell was measured in 3 axes indicated as red lines. Mshot® software manual (http://www.m-shot.com)*

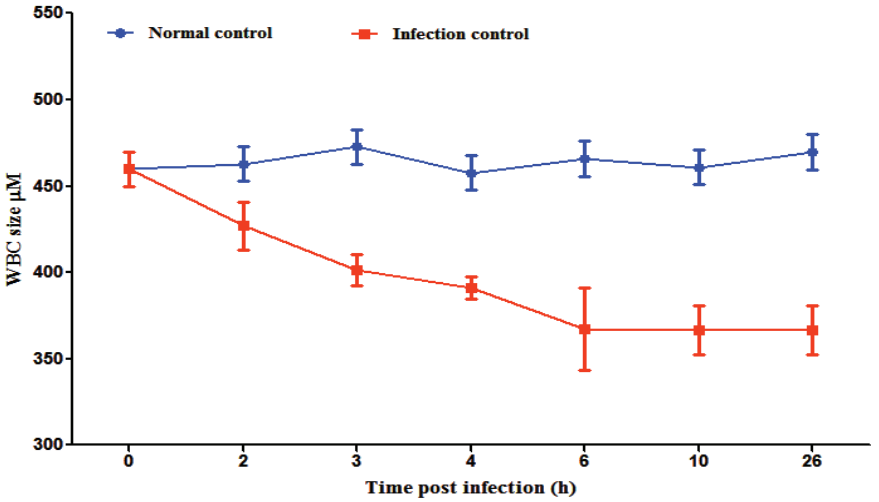


Fig. 6: *Mean WBC size in neutropenic mice blood over time following infection with P. aeruginosa. Error bars indicate the SEM. The values of WBC size in Y-axis are represented as the represented value x10^{-2}µm.*

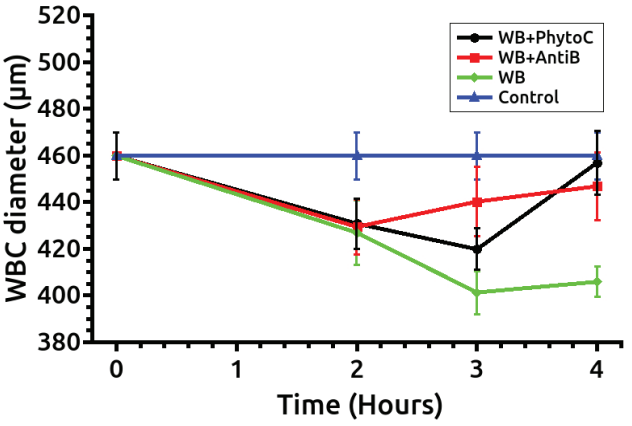


Fig. 7: *Recovery of WBC dimension post-treatment with Antimicrobials in a mouse model. The figure shows the regain of the WBC dimension to the normal size when treated with antibiotics and phytochemicals. Control=No Bacteria, WB= WBC with bacteria, WB+AntiB= WBC with bacteria plus antibiotic (Ciprofloxacin), WB+PhytoC=WBC with bacteria plus phytochemical (20ml/kg).The values of WBC size in Y-axis are represented as the represented value x10^{-2}µm.*

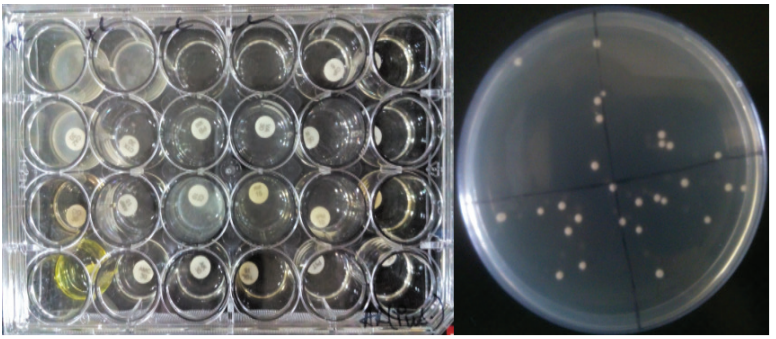


Fig. 8: *A representative image of the experimental setup for antibiotic sensitivity profiling. The first two wells in row one (from top) were positive control (+C), inoculated with body fluid, showing growth. The third and the fourth well in row one were the negative control (-C), which were not inoculated, while the rest of the wells had the antibiotic disc with the inoculated medium. The optical density of the culture was measured. The corresponding CFU was also determined through plating.*

Assessing the optimum time of incubation with bacteria for WBC shrinkage

There was distinct shrinkage of WBC with each strain of bacteria used within one hour of incubation. The calculated p values were 8.97E-08, 4.58E-08, 0.000135, 2.16E-05, 0.018388, and 0.004031, for *Pseudomonas aureginosa*, *Escherichia coli*, *Bacillus* sp, *Staphylococcus aureus*, *Klebsiella pnuemoniae*, and *Pseudomonas sp* respectively (Fig. 4). The above findings indicate that the fastest microscopy-based detection of microbial blood infection is possible within one hour of infection with bacterial pathogens. When the extent of shrinkage among the strains was statistically (one way ANOVA at 95% confidence level) compared at 1st, 2nd and 3rd hour of incubation the p-values were 0.0680, 0.1628, 0.0046 respectively at the three hours indicating nosignificant difference in shrinkage in respect to infection with different strains up to two hours ofinfection. The significant difference with various strains was seen only in the three hours of infection/seeding.

Correlation of WBC shrinkage with the genotype

It was observed that irrespective of the response with EDTA, significant shrinkage was observed with a bacterial infection in both the groups [Group 1: p-value 1.24E-34; Group 2: p-value 0.020659; at 95% confidence level] within 1 hour of incubation, and the shrinkage was more in case of Group 1 as compared to Group 2. However, at 2nd hour of incubation, the extent of shrinkage was similar [Group 1: p-value 4.36 E-36; Group 2: p-value 1.24E-36; at 95% confidence level].

Blood bacterial infection: In vivo

The mean diameter of WBCs in the uninfected neutropenic mice remained approximately similar over a time span of 26 hours study whereas, in the case of the bacterial infection, the mean diameter of WBC (Fig. 5) was found to be reduced by ~ 20% at 24 hours' time point. The cells showed significant shrinkage by 2 hours post-infection, and maximum shrinkage was observed at around 6 hours post-infection (Fig. 6). The result validated the *in vitro* finding of detecting blood infection within 2 hours. Treatment with antibiotics and phytochemical showed recovery of cell size, and hence WBC dimension was restored to standard size (Fig. 7). Though the *in vivo* experiment was conducted with bacterial strain only, it could be assumed from the outcome of *in vitro* analysis that yeast cell infection in the mouse model could also reveal similar results. The above statement has also been validated *in vitro*by Ghosh *et al.* [26]. The present report not only supported the previous hypothesis but also proved its efficacy even *in vivo*. Thus, this could be proposed as a highly efficient means for rapid detection of septicemia and the rapid identification of antimicrobial drugs' efficacy irrespective of the microbes. This method hasbeen filed as an Indian patent [32] in the year 2016.

Improvement of the culture-based antibiotic sensitivity detection:

Since the gold standard for antibiotic sensitivity detection is still culture-dependent, hence attempt was made to make that process faster and compare with the VITEK 2 system-generated data. The culture sensitivity test was done in 24 well tissue culture plates (Fig. 8), and the OD of the growth was measured and compared with the manual observation. Though swab samples required pre-incubation for 2 hours before antibiotic sensitivity testing, the body fluid could directly get into antibiotic sensitivity screening. The results were documented in Table 3 with the corresponding data generated through VITEK 2 system. The method reported here requires less time than the conventional process and can be easily performed in a laboratory. Table 3 summarizes the qualitative estimation of antibiotic sensitivity of the microbes from different body fluids. There was a 94.44% similarity with the VITEK 2 automated data. Thus the adopted process for antibiotic sensitivity was successful in making the process faster. The process was slower than the automated system but faster than the conventional system and much cheaper than the automated process. All the results obtained were within 10 hours of incubation, the fastest being within the 6^{th} hour of incubation.

Table 3: *Representative data of qualitative antibiotic sensitivity of the body fluids with corresponding VITEK 2 data done in 24 well tissue culture plate. Here R represents the resistant nature of microbes for the respective antibiotic, S represents sensitive nature of microbes for the respective antibiotic and ND represents not detected.*

Sample	Sample 1 (Blood)			Sample 2 (Blood)			Sample 3 (Urine)			Sample 4 (Urine)			Sample 5 (Blood)		
Antibiotics	6 H	24H	Vitek 2	6 H	24H	Vitek 2	6 H	24H	Vitek 2	6 H	24H	Vitek 2	6 H	24H	Vitek 2
Nitrofuratoin (NIT300)	R	R	R	S	S	S	S	S	S	R	R	R	S	S	S
Cefepime (CPM30)	S	S	S	S	S	S	R	R	R	R	R	S	R	R	R
Cefuroxime (CXM30)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Cefoperazone (CFS 75/30)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Imipenem (IMP 10)	R	R	R	R	R	S	R	R	R	S	S	S	S	S	R
Vancomycin (VA 30)	R	R	R	S	S	S	S	S	S	R	R	R	S	S	S
Tetracyclin (TE 30)	S	S	S	S	S	ND	R	R	R	S	S	ND	R	R	S
Ampicillin (AMP 10)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Rifampicin (R 30)	S	S	S	S	S	ND	R	R	R	R	R	ND	S	S	S
Gentamycim (G10)	S	S	S	S	S	S	R	R	R	S	S	S	S	S	S
Polymixin B (PB 300)	S	S	ND	S	S	ND	R	R	ND	R	R	ND	S	S	ND
Amikacin (AK 30)	R	R	R	R	R	S	R	R	R	S	S	S	R	R	R
Colistin (CL 10)	S	S	S	S	S	S	R	R	R	S	S	S	R	R	R
Ceftriaxone (CTR 30)	S	S	S	R	R	R	R	R	R	R	R	R	R	R	R
Meropenem (MRP 10)	R	R	R	R	R	S	R	R	R	S	S	S	R	R	R
Trimethoprime (TR 25)	R	R	R	R	R	R	S	R	S	R	R	R	R	R	R
Ciprofloxacin (CIP 5)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Ertapenem (ERT 10)	S	S	S	R	R	S	R	R	R	S	S	S	R	R	R
Amoxyclab (AMC 30)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Piperacillin (PIT 10)	S	S	R	S	S	S	R	R	R	R	R	R	R	R	R
Tigecyclin (TIG 15)	R	R	S	S	S	S	S	S	S	R	R	R	S	S	S

The concentrations of the cells and their generation time play a significant role, as reported earlier [33]. Simple visualization of the microbes' growth (turbidity) through naked eyes might draw wrong conclusions due to manual errors. Thus quantitative estimation of growth in terms of the absorbance of cells gave an unambiguous idea of the cell growth in Table 4. Thus the end-users can also validate the data by the procedure mentioned above.

Table 4: *Representative data of quantitative estimation of microbial growth from different body fluids in presence of different antibiotic. Here R represents the resistant nature of microbes for the respective antibiotic, S represents sensitive nature of microbes for the respective antibiotic and ND represents not detected.*

Antibiotics	Sample 1 (Urine)		Sample 2 (Ascitic fluid)		Sample 3 (Pleural fluid)		Sample 4 (Throat swab)	
	7th hour	Absorbance at 600nm	9th hour	Absorbance at 600nm	6th h	Absorbance at 600nm	8th hour	Absorbance at 600nm
Nitrofuratoin (NIT300)	R	1.398	S	0.029	S	0.064	S	0.036
Cefepime (CPM30)	R	1.911	R	1.364	R	1.054	R	1.398
Cefuroxime (CXM30)	R	1.875	S	0.084	S	0.0546	R	1.881
Cefoperazone (CFS 75/30)	R	1.654	R	1.941	S	0.0213	S	0.054
Imipenem (IMP 10)	S	0.054	R	1.899	R	1.411	S	0.066
Vancomycin (VA 30)	R	1.881	R	2.035	S	0.045	ND	-
Tetracyclin (TE 30)	R	1.695	S	0.054	R	0.9984	S	0.004
Ampicillin (AMP 10)	R	2.112	R	2.016	R	1.094	R	1.992
Rifampicin (R 30)	R	1.724	S	0.034	R	1.894	R	1.784
Gentamycim (G10)	R	1.992	S	0.054	S	0.054	R	1.519
Polymixin B (PB 300)	R	1.784	S	0.041	ND	-	ND	-
Amikacin (AK 30)	R	1.712	R	1.398	S	0.077	R	2.112
Colistin (CL 10)	S	0.036	S	0.048	S	0.038	R	1.695
Ceftriaxone (CTR 30)	S	0.021	R	1.875	ND	-	R	1.881
Meropenem (MRP 10)	S	0.015	R	2.111	S	0.067	S	0.081

Trimethoprime (TR 25)	S	0.019	R	1.364	ND	-	R	1.677
Ciprofloxacin (CIP 5)	R	1.398	R	0.084	-	1.677	ND	-
Ertapenem (ERT 10)	R	1.911	S	0.141	R	1.043	S	0.071
Amoxyclab (AMC 30)	R	1.875	R	1.899	S	0.043	R	1.411
Piperacillin (PIT 10)	S	0.034	R	2.035	S	0.045	R	1.767
Tigecyclin (TIG 15)	ND	-	S	0.091	S	0.021	S	0.058

Conclusion and Future Prospects

The present understanding of infections' identification is based on methods developed in the nineteenth century by Louis Pasteur and Robert Koch. Emerging diseases and the generation of multi-drug-resistant microbes are among the most deadly threats in the present era, shifting the paradigm of earlier diagnosis methods. The prognosis of any disease depends on how quickly the infection is detected and treatment begins. This is even more valid for Septicemia. The rapid and accurate antibiotic therapy would be of utmost importance to the successful diagnostic approach described above. Antibiotic sensitivity assay is an essential assay in clinical microbiology, and researchers practiced it for many years. To date, several antibiotic assays were recommended by WHO. Broth dilution, antimicrobial gradient method, disc diffusion assay were among them. The use of automation in the 20th century has taken an essential place in the above assays. It leaped over the disadvantages of the conventional assays (time consumption, manual handling, and irregular interpretation of results). Vitek 1-2, BD Phoenix, Sensititre ARIS 2X systems with developed software helped to interpret results with accuracy. The most noteworthy point of these systems was very little time consumption for antibiotic assay than conventional methods. The high cost of the automated machines and the complex organization were the points of criticism [33-35].

The appropriate approach for sepsis management is rapid recognition and a protocoled targeted intervention. This demands a prompt and accurate diagnosis of sepsis, which can help early localization along the sepsis spectrum of illness. The reported novel approach for rapid detection of bacterial and fungal infection in blood has been developed, which can confirm the presence of causative agents much faster. This method can identify infection within 1 hour and 2 hours of bacterial and yeast infection, respectively. The conventional methods used currently take one to three days for a culture-based assay of microbes. Some strains can also take up to 10 days to show a positive culture depending on its nature. The current method's efficacy has been

corroborated by *in vivo* tests in mice. The present method of detection of Septicemia will be a new ray of hope towards the proper ailment cure of patients worldwide.

The commercialization of such a technique could lead to faster detection of microbial blood infection and hence facilitate the disease management of sepsis within the first few critical hours of infection. The *in vivo* assay could also be used to detect a patient's response to an antibiotic regime during intravenous administration and minimize the incidence of inducing multidrug resistance due to the administration of an inappropriate drug for an extended period. Hence this could revolutionize the field of antibiotic selection and therapy.

Conflict of Interest

There is no conflict of interest.

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CHAPTER

Genomic characterization of tea garden soil micro-flora from Darjeeling hills

Indrani Sarkar and Arnab Sen*

NBU Bioinformatics Facility, Department of Botany,
University of North Bengal, Siliguri, India
E-mail: arnab.sen@nub.ac.in

Abstract: Actinobacteria is a group of bacteria with high Guanine and Cytosine content. They are mainly important for their niche diversity. Members of actinobacteria belong to both aquatic and terrestrial environment. Moreover, some of them are found in extreme conditions, for example, hydrothermal vent, desert stone etc. They are important source of several anti-bacterial and anti-cancer agents which are produced as secondary metabolites from different Actinobacterial strains especially Streptomyces. This genus has a pivotal significance due to its antibiotic producing capacity. Along with this, they produce several other biosynthetic metabolites with immense medicinal and industrial value. Presently, several genomes of Streptomyces are available in public domain database however; there is hardly any report from Indian subcontinent especially from the Indian Tea gardens. Tea gardens being well exposed to a range of insecticides and pesticides, experience a drastic change in their soil microbiome from its natural microbial ecology as well as microbial behaviour. Hence, a comparative study on the 'dark (microbial) matter' from tea garden soils exposed to chemical and organic fertilisers may reveal some truly interesting facts about the impact of fertilisers on the soil-microbial population. We isolated two Streptomyces genomes from tea garden soil of Northern part of West Bengal, India. Along with this, an extensive high-throughput Next Generation Sequencing (NGS) based comparative study was performed between the soil microbial population of two tea gardens one of which uses chemical fertilizers and the other one was totally organic. The two Streptomyces isolated from tea garden soils showed considerable antibiotic producing capacity along with excess melanin producing ability. This feature can establish them as industrially important strains since melanin is one of the major components used in the commercial sun screens. Moreover, enhanced xenobiotic degradation along with condition-dependent metabolite producing capacity was observed among the microbial populations of both the tea garden soils analyzed through NGS technique.

Keywords: Genomic characterization; tea garden, NGS technique; Dergeeling

The phylum Actinobacteria represents one of the largest taxonomic units among major lineages currently recognized within Bacteria in terms of number and variety of identified species. It comprises gram-positive high G+C content bacteria. The G+C content generally ranges from 51% (some *Corynebacterium*) to more than 70% in *Streptomyces* and *Frankia*[1]. An exception is the genome obligate pathogen *Tropherymawhipplei*, whose G+C content is less than 50%. Actinobacteria exhibit a broad range of morphologies, from rod-coccoid (*Arthrobacter*) or coccoid (*Micrococcus*) to fragmented hyphal forms (*Nocardia* spp.) or highly differentiated branched mycelium (*Streptomyces*spp.)[2]. They also exhibit diverse metabolic and physiological properties, such as the formation of a wide variety of secondary metabolites and production of extracellular enzymes[3]. Some of these secondary metabolites especially produced by *Streptomyces* species have been proved to be potent antibiotics[4]. This trait turned *Streptomyces* into primary antibiotic-producing organisms exploited by the pharmaceutical industry [5]. Moreover, diverse forms of lifestyles are encountered among Actinobacteria. For instance pathogens (*Mycobacterium*, *Nocardia*, *Tropheryma*, *Corynebacterium*, *Propionibacterium*etc.), plant commensals (*Leifsonia*), nitrogen-fixing symbionts (*Frankia*), soil inhabitants (*Streptomyces*), thermal (*Acidothermus*), stone dwellers (*Geodermatophilus*) and gastrointestinal tract (GIT) inhabitants (*Bifidobacterium* spp.) [6].

Abnormal developmental features are also displayed by many actinobacterial genera, for example, the continual non-replicating state exhibited by certain mycobacteria or sporulating aerial mycelium formation in *Streptomyces* species [7]. Actinobacteria are extensively distributed equallyin terrestrial and aquatic (including marine) ecosystems, especially in soil, where they play a vital role in the recycling of unruly biomaterials by decomposition as well as humus formation [8]. Furthermore, some *Bifidobacterium* species are used as active component in a variety of so-called functional foods due to their apparent health-promoting and probiotic properties. They have been reported to take part inbile salt hydrolase activity, protection against pathogens mediated through the process of competitive exclusion, immune modulation along with the ability to stick to mucus or intestinal epithelium [9]. Thus actinobacteriamaybe relevant to human and veterinary medicine, biotechnology, and ecology. Moreover the genomic heterogeneity observed from already sequenced actinobacterial genomes reflects their biodiversity.

Taxonomic reclassification of Actinobacteria

Ray fungi, also called as Strahlenpilze by Lieske or Actinobacteria with a tricky taxonomy were historically considered as ‘intermediary’ between bacteria and fungi[10]. The name itself comes from the colony morphology on agar exhibiting radial growth, a characteristic common to fungi. The mycelial shape and musty smell of

actinobacteria is also similar to fungi. Moreover, the peptide glycan based cell wall of this group is also a property shared to Firmicutes [11]. The very primitive taxonomical treatment of actinobacteria was proposed by Buchanan who proposed an order name Actinomycetales. Some taxonomical works have been based on the perception of evolutionary trend from a simple form to more complicated features like hyphae and sporangia [12]. However most of the taxonomists were not satisfied since morphological features were not consideredwhich may result in faulty positioning, especially in classifications specifically dependentupon a dichotomous scheme [13].

Chemical criteria were initially very few and could not provide a solid base for taxonomy, yet they have gathered a solid set of data compensating for few lost functions. Now-a-days, to explain a species, it is compulsory to afford a measure G+C content of DNA which is a characteristic of the genome; phospholipids analysis whichis an important feature of membrane; the di-amino acids characterising cell wall; or quinones which represent the respiratory chain of organisms [14]. None of these elements taken independently is adequate to recognize a microbe; however, taken as a whole they can give way a solid taxonomic basis in amalgamation with growth characteristics and morphology. These approaches have allowed the classification of several actinobacterial lineages, especially the genera *Streptomyces* and *Mycobacterium*[15].

The arrival of sequencing of 16SrRNA, 16SrRNA gene classification and advanced molecular phylogenetic toolshave advanced the search for a molecular clock that is required to be present in all lineages. These techniques presented Actinobacteria as a consistent subdivision of 'Gram-Positive bacteria' along with the firmicutes (low G+ C), *Bacillus* and *Clostridium*. This confirmation supported Actinobacteria as coherent, with little genetic distances [16-20].

Actinobacteria are generally aerobic and possess filaments (in branching pattern) except Bifidobacteriales. This treatment was reserved in many consecutive editions of Bergey's Manual and the class name was available in 1997 although untenably because no type order was proposed at the time [21]. However, 16SrRNA genes sequences and phylogenetic reconstructions may not be considered as the golden benchmarkin bacterial taxonomy for several reasons. First, there are examples of species with more than one copy of the 16SrRNA gene differing by approximately 6%, as in *Thermomonosporachromogena*, which was taken as support of lateral transfer. Secondly, the 16SrRNA gene may be plasmid-borne as in *Bacillus megaterium*, further following the idea of lateral transfer [22]. Finally, the 16SrRNA gene is a single marker that thus disobeys one basic principle of biology, that any investigation should be considered for reproducibility. This constraint led to multi-locus sequence analysis (MLSA) developed originally for *Neisseria* [23], where five or more preserved genes were sequenced and analysed. This approach is now commonly

used, mainly to distinguish species within a genus. It helped the re-characterization of the genera *Nocardia* and *Streptomyces*, with some noteworthydeviations from the topological pattern obtained by 16SrRNA gene sequences, indicating extensive recombination [24]. The most current taxonomical treatment of Actinobacteria, based fundamentally on the 16SrRNA gene phylogeny, has notice ablytailored all levels of their taxonomy. There are six classes comprising of five basal ones (*Acidimicrobiia, Coriobacteriia, Nitriliruptoria, Rubrobacteria* and *Thermoleophilia*) each withone or two orders. The main class *Actinobacteria* comprises of 15 orders (*Actinomycetales*, '*Actinopolysporales' 'Bifidobacteriales'*, '*Catenulisporales'*, '*Corynebacteriales'*, '*Frankiales'*, '*Glycomycetales'*, '*Jiangellales'*, '*Kineosporales'*, *Micrococcales*, '*Micromonosporales'*, '*Propionibacteriales'*, '*Pseudonocardiales'*, *Streptomycetales* '*Streptosporangiales'*) [25].

Gao and Gupta proposed an approach of concatenating 35 proteins to re-classify actinobacterial phylogeny, with anidea of indels tocorroborate the major lines of that classification. A Study revealed the need of reassessment in the orders '*Frankiales'* and *Micrococcales*. Another study performed a detailed phylogenetic reconstruction exclusively for Actinobacteria using both bootstrapping MLSA and Prunier approach and spitted *Frankiales* into *Frankiales* ord. nov., *Geodermatophilales* ord. nov., *Acidothermales* ord. nov. and *Nakamurellales* ord. nov. Moreover, *Micrococcales*was also divided into several monophyletic orders – *Micrococcales (Micrococcus, Kocuria, Rothia, Arthrobacter, Tropheryma, Microbacterium, Clavibacter* and *Leifsonia); Cellulomonales (Beutenbergia, Cellulomonas, Xylanimonas, Jonesia* and *Sanguibacter); Brachybacteriales (Brachybacterium) and Dermacoccales (Kytococcus, Intrasporangium)*[25].

Actinobacteria as nitrogen fixers

Frankia, a genus with the ability to fix nitrogen, can live both as soil saprophytes as well as endophytic symbionts and are competent to establish mutualistic symbiotic association with non-leguminous plants notably *Alnus*, *Casuarina* and *Elaeaginus* allowing their growth in nitrogen-poor condition[26]. Elucidation of the *Frankia* genomes has revealed novel possibilities in metabolic diversity, stress tolerance and natural product biosynthesis, which aid in establishment of *Frankia*-actinorhizal symbiosis. Depending upon the host specificity, *Frankia* has been clustered in four major groups. Members of lineage I infect plants of the Betulaceae, Myricaceae, and Casuarinaceae families (except Gymnostoma)[27]. Dryadoideae (all actinorhizal Rosaceae), Coriariaceae, Datiscaceae, and the genus Ceanothus (Rhamnaceae) are infected by strains of lineage II. The lineage III stains are most promiscuous infecting Elaeagnaceae, Colletieae (all actinorhizal Rhamnaceae except Ceanothus), Myricaceae, Gynmmostomam (Casuarinaceae), and occasionally Alnus. On the contrary, Frankia

of lineage IV are 'atypical' strains either unable to infect plant or produce i.e. effective root nodules which do not participate in nitrogen fixation.

Several genes have been reported to be involved in nitrogen fixation. Among them, nitrogen fixing (nif) genes are most important. Strains of cluster I, II and III own their nif genes however, in lineage IV this gene is totally absent which probably lead to their inability to fix nitrogen [28]. Eleven- twelve nif genes remain clustered together as an operon on 11. 6-14 kb region and generally one copy of each nif gene is present in that cluster. However, exceptions are there. For instance, nifV gene of lineage III strains has been reported to be present at a distant from the nif cluster and two copies of nifU were reported in lineage II [29]. Interestingly, six non-nif genes (orf1, orf2, hesAorf3, orf4 and fdx) were also found to be located between nif genes. Another important part of frankialN_2 fixation is the presence of specialized thick-walled vesicles. Their formation is controlled by partial pressure of oxygen. Vesicles play a pivotal role in protecting nitrogenase from oxygen inactivation.A natural pentacyclictriterpenoid lipid called hopanoid formed a multilayed envelop surrponding the vesicle structure which act as the oxygen barrier [30-35]. In *Frankia* hopanoid remain as bacterioho-panetetrol and bacterio-hopanotetrolphenylacetate monoester derivatives. Generally, there are two pathways for hopanoid biosynthesis viz. mevalonate pathway and methyl erythritol phosphate (MEP) pathway. However, in *Frankia*, only MEP pathway has been identified. Surprisingly, hopanoid biosynthetic gene cluster have been identified in all *Frankia* strains including non-nitrogen fixer lineage IV which indicates that their functional implication is nitrogen independent. Hence, these genes are not only crucial for nitrogen fixation besides, they are also important as primary cell wall component conferring the membrane integrity [36].

Concurrently, Ni-Fe hydrogenase or uptake hydrogenase (hup) operons are also imperative for Frankia life style. There are two hup operons in lineage I and II strainsAmong them, operon I is expressed under free living condition whereas, operon II is expressed under symbiotic condition. However, all hup genes were found to be clustered in one operon for lineage III and IV strains. Truncated hemoglobin genes, which are broadly classified into three groups - group I (HbN), group II (HbO), and group III (HbP) act either as NO scavengers or protect nitrogenase from oxygen inactivation by buffering the oxygen level [37].

Blastp analysis revealed the complete absence of canonical nodABC gene (NodA-acyl transferase, NodB-chitin deacetylase, NodC-chitin synthase) cluster among *Frankia* strains except *Candidatus* Frankia Datisca Dg1. There prevail two hypotheses behind this finding. First one is, Dg1 follow an identical symbiotic pathway to legume-rhizobium relation and second one is, while sequencing, Dg1 has incorporated genes from other organisms as a part of contamination[38]. Actinorhizal nodules are

well known to contain several bacteria within them hence, genes of other microbes can be accidentally mixed to the genome of Dg1 resulting the exceptional presence of nod genes within the genome. However, Host plant induced *Frankia* have been reported to exert a signal triggering the expression of reinfection genes in host plant as well as Ca^{2}+ level. Their additive effect results in biologically active symbiotic signal [39].Moreover, next-generation proteomic analysis has also provided plethora of information regarding the proteome footprinting of frankial metabolomics altered by root exudates of compatible and non-compatible host plants. This study revealed that, proteins having functionality in the early steps of host recognition and infection were expressed highly in *Frankia* induced by root exudate of compatible host plant [40].

Bioinformatic analysis has also explored a relation between the genome size and niche specificity as well as biogeographic ranges among this genus. For instance, the genome size of narrow-host-range- *Casuarina* strain*Frankia* sp. CcI3 is 5. 4 Mb whereas, the size expands to 8.9 Mb for *Frankia* sp. Ean1pec which is a broad-host-range *Elaeagnus* strain as well as soil cosmopolitan. The infectivity of *F. alni*ACN14a is restricted mainly to *Alnus* and Myricaceae hence, they possess a genome with intermediate size. Thus a hypothesis prevailed that, the genome size of *Frankia* changes with their host range and it held true with all the strains of this genus sequenced so far. Furthermore, study with horizontal gene transfer (HGT) and insertion elements (IS) in *Frankia* has availed us with information regarding their genomic plasticity. *Frankia* sp. Eul1c appeared to be the most stable genome with a very few HGT and IS elements [41]. Thus, our understanding regarding the infection process of *Frankia* could be enhanced through a detailed comparative study.

Actinobacteria as plant protector and plant defence elicitor

Actinomycetes are well recognized for their capacity to produce a broad range of metabolites which can act against plant pathogens. The mechanisms by which actinobacteria inhibit the pathogens include antibiosis, nutrient competition, quorum quenching, nitrous oxide and degradative enzyme production. Some strains also produce siderophores with the ability to chelate iron and thus deprive other microbes from this essential nutrient. Some water soluble chitinolytic enzymes produced from soil actinobacteria are also effective against fungal infection and soil borne pathogens. Broad range of antibiotics produced by this particular microbial population is well recognized plant protectors against pathogenic bacteria, oomocytes, nematodes and fungi [42-46]. Moreover, production of nitrous oxide by some *Streptomyces* strains elicits the plant defense mechanism. Streptomycetes can also degrade a signalling molecule responsible for pathogenicity in *Pectobacteriumcarotovorum*. Thus, actinobacteria can act as a soldier of plant defense mechanism [47].

Actinobacteria help in plant growth

Several nitrogen fixing actinobacteria have been found to be associated with the plant root system. Majority of them aid in the development of plant. For example, *Corynebacterium* sp. AN1, which was isolated from forest phyllosphere can reduce acetylene thus substituting the role of nitrogenous fertilisers and helping in plant growth promotion. *Pseudonocardiadioxanivorans* CB1190 has also been reported to fix dinitrogen [48-52]. Above all, *Streptomyces*, accounting an abundant percentage of soil microflora and an effective plant colonizer is well recognized as source of plant growth promoters and other biocontrol agents. Besides, endophytic (microbes remain within the plant host for a long time without causing any harm to host) streptomycetes can aid in their host plant growth by auxin production [53].

In particular, Actinobacteria produce the majority of the naturally occurring antibiotics. They are important source of several anti-bacterial and anti-cancer agents which are produced as secondary metabolites from different actinobacterial strains especially *Streptomyces*[54-57]. This genus has pivotal significance due to its antibiotic producing capacity. The first antibiotics discovered in Actinobacteria were actinomycin from a culture of *Streptomyces antibioticus* in 1940, streptothricin from *Streptomyces lavendulae* in 1942 and streptomycin from *Streptomyces griseus* in 1944, all of which were discovered by Waksman and colleagues. Streptomycetes have been the major source of clinical antibiotics and are responsible for over 80% of all antibiotics of actinobacterial origin [58]. That actinomycin, streptomycin, and streptothricin were the first to be found is not surprising, as these molecules occur at much higher frequencies than many other antibiotics. For example, streptothricin is found in some 10% of all streptomycetes isolated randomly from soil and streptomycin is found in 1% and actinomycin in 0. 1%, while conversely, erythromycin and vancomycin are found in around 10-5 soil isolates, and daptomycin is found only at a frequency of around 10-7. Major classes of clinical antibiotics produced by actinomycetes are the following: aminoglycosides (neomycin, kanamycin, streptomycin, angucyclines (auricin; also, antitumor agents like landomycin and moromycin, ansamycins (rifamycin, geldanamycin), anthracyclines (primarily antitumor agents, e.g., daunorubicin), lactams (cephamycins) and also the important lactamase inhibitor clavulanic acid, chloramphenicol, glutarimides (cycloheximide), glycopeptides (vancomycin, teichoplanin), lipopeptides (daptomycin), lantibiotics (mersacidin, actagardine), macrolides (clarythromycin, erythromycin, tylosin, clarithromycin), oxazolidinones (cycloserine), streptogramins (streptogramin) and tetracyclines. The producing capacity of individual actinomycetes can also vary enormously [59-63]. Some *Streptomyces* species produce a single antibiotic, while others produce a range of different compounds and compound classes. Besides antibiotics, Actinobacteria also produce a wide variety

of other secondary metabolites with activity as herbicides, antifungals, antitumor or immunosuppressant drugs, and anthelmintic agents [64]. Along with this they produce several other biosynthetic metabolites with immense medicinal and industrial value. Presently, several genomes of *Streptomyces* are available in public domain database however; there is hardly any report from Indian subcontinent especially from Indian Tea Gardens. Tea gardens being well exposed to a range of insecticides and pesticides, experience a drastic change in their soil microbiome from its natural microbial ecology as well as microbial behaviour [65]. Hence a comparative study on the 'dark (microbial) matter' from tea garden soils exposed to chemical and organic fertilizers may reveal some truly interesting facts about the impact of fertilizers on the soil-microbe population. We isolated two *Streptomyces* genomes from tea garden soil of Northern part of West Bengal, India (Fig.1 & 2). Along with this, an extensive high-throughput Next Generation Sequencing (NGS) based comparative study was performed between the soil microbial population of two gardens one of which uses chemical fertilizers and the other one was totally organic [66].

Fig. 1: *Tea garden at North Bengal University Campus*

Fig. 2: *Researchers working in the field*

The two *Streptomyces* isolated from the gardens showed considerable antibiotic producing capacity along with excess melanin producing ability. This feature can establish them as industrially important strains since melanin is one of the major components used in the commercial sun screens. Moreover enhanced xenobiotic degradation along with condition-dependent metabolite producing capacity was observed among the microbial populations of both the tea garden soil analyzed through NGS technique.

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6
CHAPTER

Biodiversity status, threats and conservational measures in Rudrasagar lake, a Ramsar site of Northeast India

Moitree Taran, Prabir Barman, Yapri Jamatia, Sajib Das and Sourabh Deb*

Department of Forestry and Biodiversity, Tripura University
Suryamaninagar, Tripura, India
E-mail: drsourabhdeb@gmail.com

Abstract: Rudrasagar lake, a Ramsar site situated in Tripura, Northeast India offers a range of ecosystem services. The contribution of Rudrasagar lake to the humanity has not been estimated so far. The preliminary study aims to assess the biodiversity status and ecosystem services of Rudrasagar lake. The main provisional services provided by the lake are food (aquatic plants and fishes), fuel wood and timber whereas, the cultural services provided are boat raiding, tourism and recreational activities due to its historical importance. The main intimidations to the wetland are increasing silt loads due to deforestation, expansion of agricultural land and land conversion due to population pressure. To reduce stress on the lake, better monitoring, planning, restoration and management are essential. Different restoration activities like awareness programme, consultation and capacity building activities were conducted in the area. Restoration activities like *Hydrilla* based fish feed was introduced in the waterbody which becomes a good alternate source of food for many edible fishes. The water hyacinth based craft preparation was conducted for improving the livelihood of the common people. Proper conservation by restoration and sustainable management will help to enjoy the various services of the lake in a sustainable way.

Keywords: Biodiversity, Ecosystem services, Threats Management, Restoration.

Rudrasagar is a natural wetland located in Melaghar block, Sonamura subdivision under Sepahijala district of Tripura. Rudrasagar Lake is productive because of its ecological diversity and socio-economic importance [1]. It is designated as a Ramsar site in the year 2005 as the lake complies with the criteria's of the wetland and considered as a national as well as of international importance. Criteria 2, 3 and 8 suggest that the wetland should support endangered, threatened species, animal and plant species which maintains biological diversity and important source of food for fishes respectively [2]. It

is the habitat of endangered Baer's Pochard, near-threatened Ferruginous Pochard and IUCN Red list Three striped roof turtle *Kachuga dongka.*

Rudrasagar is an oval shaped, perennial lake [1] and an inland natural waterlogged wetland [3]. It was known as Rudijala before the Lake got the name as Rudrasagar. Rudrasagar catchments fall under the Sub-basin of Gumti River. The catchment area extends upto 13,909 ha in the north of Gumti River. There are three streams that drain into the wetland. The major stream is Noacherra which is thickly populated as compared to other catchment areas. This is causing in persistent drainage problems around the Rudrasagar lake area. The other two rivulets are Kemtali cherra and Durlavnarayan cherra which drain from northern and north-western side respectively. The combined catchment area of the three inflows to the wetland is about 150 sq.km. The outflow is through Gumti River via a regulatory channel called Kachigang [4]. Kachigang channel is constructed to regulate the water level in the wetland and also to control the backflow of water from Gumti River.

The total area of the wetland is 688 Hectares. According to the Melaghar Revenue Circle, the total water area of the lake is 585.58 acres. Agricultural plot in the lake has not been identified by the revenue department. While as stated by the fisherman society the water area is 364.61 acres, agriculture land 1465.86 acres and homestead land, road and tanks 241.31 acres. The water depth during dry season varies between 1.5 to 2.5 m and 7.5-8.5 m during monsoon [1]. Rudrasagar is a floodplain and it gets inundated periodically when there is high rainfall [4]. The lake is composed of macrophytes and aquatic weeds such as marginal-floating and emergent-submerged weeds. The soil within the lake area is of silt clayey loam to clayey loam [1]. Pisciculture and agriculture are the two main occupation practiced by the local community. Local people are now turning into non-farm activities for work diversification. Diversifying the work has made the local people easier to sustain themselves. The lake is valuable not only for its provisioning services, but also because of its historical heritage (water palace) and endangered waterfowls and migratory birds. In addition to pisciculture and agriculture the members of the Samity also does tourism work.

Materials and Methods

The present study was conducted in Rudrasagar lake, located at Melaghar in Sepahijala district of Tripura, India (Fig. 1). The wetland lies in between 23° 29′ 0′′ N. latitude and 90° 01′ 0′′ E. longitude. To identify the ecosystem services provided by the wetland one questionnaire based survey was carried out in ten villages present near the lake. Biodiversity assessment was done by consulting experts from each filed. Data collection was carried out to determine the potentiality and stress on this wetland. To ensure the ecosystem services provided by lake two invasive species were introduced

in the area namely *Hydrilla verticillata* (to make fish feed), *Eichhornia crassipes* were (to make crafts) by conducting several awareness and training activities.

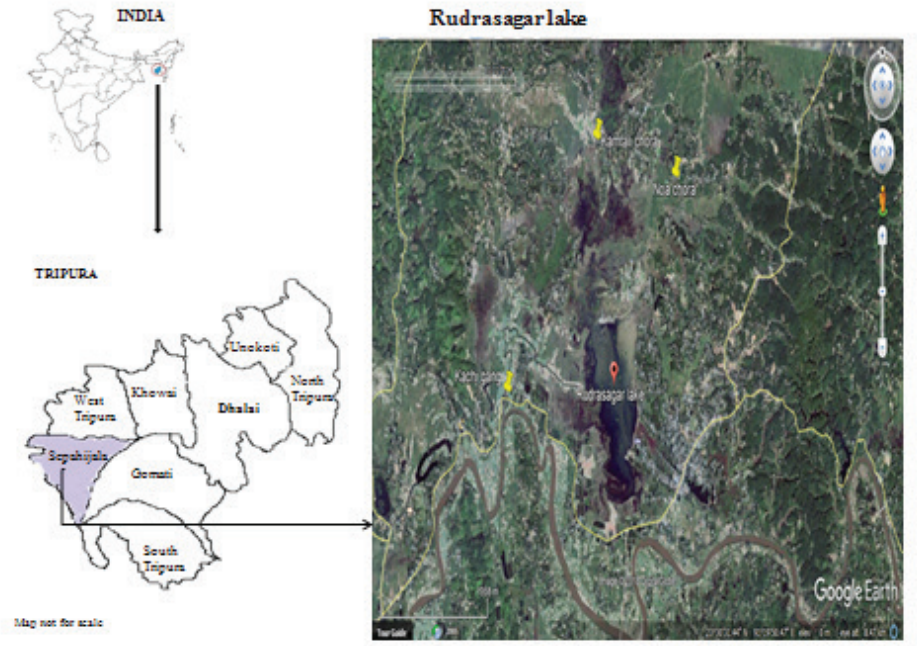

Fig. 1: *Map of study area in Rudrasagar lake.*

Fig. 2: *Restoration activities in Rudrasagar lake*

1. Awareness programme on wetland restoration; 2. PRA Exercise; 3. Resource map; 4. Preparing a hyacinth plant through the stand machine. 5. Training on craft making; 6. Sewing the product; 7. Collection of *Hydrilla* plant from the lake. 8. Distribution of dustbins 9. Cleaning activities in the lake 10. Celebration of World wetland day 11. Plantation in the catchment area12. Bamboo fencing around the plant.

Results and Discussion

Biodiversity and Ecosystem services

In the present study it is observed that the villagers around the Rudrasagar lake are highly dependent on this lake for fishing, irrigation of agricultural land, animal husbandry and for other daily activities. This lake support variety of flora and fauna. Fishing is one of the daily activities of the villagers present in this area. There are a total of 2000 fishermen families present near the wetland area and fully depend on this lake for their income as this lake is a good source of fishes. Tribal community present near the lake preferred molluscs as food but crabs are preferred by both tribal and non-tribal community. There are 31 plankton species identified from the lake (Table 1) and many of them are reported first time from the region. Total 37 aquatic plants belongs to 20 different families were identified from Rudrasagar lake in which some are edible, some have medicinal value and some are used as fencing and fertilizer. The lake supports numerous plant species surrounding it, which are used as timber and fuelwood. It also contributes to the total revenue of the Rudrasagar lake. 14 tree species recorded surrounding the lake and few timbers were used for construction and furniture making. Rudrasagar lake provides provisional services like food (aquatic plants, fishes, crab and molluscs), timber and fuelwood. There are 55 species of fishes

reported from the lake. However only few of them are commercially produced in the lake. During the project period 21 birds species recorded from the area and many other species are expected from the lake.

Table 1: *List of flora and fauna recorded from Rudrasagar lake.*

Category	Scientific name
Planktons	Cymbella sp., Chara sp., Spirogyra indica, Volvox sp., Tetraedron trigonum, Gomphosphaeria sp., Nostoc vaginicola, Euglena acus, Moina micrura, Daphnia magna, Chydorus sphaericus, Alona intermidia Diaphanosoma brachyurum, Cyclops sp.. Mesocyclops edax, Cyclops bicolor, Cyclocypris glodosa, Cyclocypris sharpie, Cyclocypris ample, Cyclochpries nahctta, Trichotria sp., Brachionus quadridentatus, Brachionus patulus. Synechocystis sp., Pediastrum tetras, Aphanocapsa sp., Schroederia sp., Navicula sp., Gomphonema sphaerophorum, Pinularia braunii, Scenedesmus sp.,
Aquatic plants	Alocasia indica, Amischophacelus axillaris, Azolla pinnata, Ceratophyllum demersum, Cyperus haspan, Eichhornia crassipes, Enhydra fluctuans, Euryale ferox, Grangea maderaspatana, Hydrilla verticillata, Hygroryza aristata, Ipomoea fistulosa, Isachne globosa, Ludwigia adscendens, Ludwigia octovalvis, Marsilea minuta, Najas marina, Nymphaea micrantha, Nymphaea pubescens, Nymphaea stellate, Nymphoides indica, Panicum laxum, Pennisetum polystachyon, Pistia stratiotes, Polygonum barbatum, Polygonum hydropiper, Sagittaria guayanensis, Salvinia cucullata, Trapa natans, Utricularia aurea, Vallisneria spiralis. Chara globularis, Cynodon dactylon, Cyperus iria, Hydrocharis morsus-ranae, Polygonum barbatum L, Monochoria hastate,
Trees	Albizia procera, Anogeissus acuminata, Bauhinia variegata, Cassia fistula, Cassia siamea, Dillenia pentagyna, Dysoxylum procerum, Gmelina arborea, Shorea robusta, Sterculia villosa, Tectona grandis, Toona ciliata, Trema orientalis, Trewia nudiflora.
Molluscs	Viviparus spp., Lamellidens spp., Pila spp
Arthropoda (crustaceans)	Macrobrachium rosenbergii, Macrobrachium rude
Fishes	Acanthocobitis botia, Alia coilia, Amblypharyngodon mola, Anabas testudineus, Botia rostrata, Catla catla, Chanda nama, Channa marulius, Channa striata, Channa marulius, Channa striata, Channa punctatus, Chitala chitala, Cirrhinus mrigala, Cirrhinus reba, Clarias batrachus, Clupisoma garua, Colisa sp., Crossocheilus latius, Ctenopharyngodon Idellus, Cyprinus carpio, Danio rerio, Gagata cenia, Glossogobius giuris, Gudusia chapra, Heteropneustes fossilis, Hypothalmichthys molitrix, Labeo calbasu, Labeo gonius, Labeo rohita, Lepidocephalichthys guntea, Macrognathus pancalus, Mastacembalus armatus, Monopterus cuchia, Mystus cavasius, Mystus gulio, Mystus tengara, Mystus vittatus, Nandus nandus, Notopterus notopterus, Ompok bimaculatus, Ompok pabda, Parambassis ranga, Puntius puntius, Puntius conchonius, Puntius ticto, Rasbora daniconius, Rita rita, Salmophasia bacaila, Sperata aor, Sperata seenghala, Tetraodon cutcutia, Trichogaster fasciata, Wallago attu, Xenentodon cancila.

Turtle	Kachuga dhongoka (Reported earlier but not available at present)
Birds	Microcarbo niger, Phalacrocorax fuscicollis, Ardeola grayii, Egretta garzetta, Ardea alba, Leptoptilos dubius, Anas crecca, Anas acuta, Alcedo atthis, Halcyon pileata, Motacilla maderaspatensis, Motacilla cinerea, Ardea alba, Aythya nyroca, Haliastur indus, Pandion haliaetus, Riparia chinensis, Hirundo rustica Linnaeus, Dicrurus macrocercus, Tringa glareola, Metopidius indicus

The Rudrasagar is a place of cultural significance well known by the local people as well as national and international communities. Here the peak season for tourism is October to December. Sagarmahal is a hotel situated near Rudrasagar lake which is a great source of income for government by tourist visit here. Cultural services provided by Rudrasagar lake are *viz.,* boat riding, tourism and recreational value due to its historical importance which signifies the status of the lake. Neermahal the water palace existing in the Rudrasagar Lake is a place of beauty and to reach the palace tourists needs to take boat ride which give them pleasure. However, they also prefer boat riding to enjoy the surrounding beauty of the lake. Every year in September Rudrasagar Jaal Udshav is organised by government which encourage people to visit this lake. Boat racing competition is one of the attraction of this programme.

The major profitable provisional services are aquatic plants, fish, timber, fuelwood, crab and molluscs. According to the study the highest revenue comes from fish followed by timber whereas, lowest revenue comes from crab and molluscs. Some of the common fish of Rudrasagar lake are *Amblypharyngodon mola*, *Catla catla*, *Clarius batrachus, Labeo calbasu, Labeo rohita, Macrobrichium* sp., *Mystus tengra*, *Ompok pabda*, *Puntius puntius* and *Wallago auttu*. Aquatic plants *viz., Euryale ferox* are taken by local tribal people and also used in medicinal purpose whereas, *Trapa natans* by local Bengali community. So both the plants are economically important as every year the Rudrasagar Udbastu Fisherman Samabaya Samity auction these plants and earn good amount from it. The management and conservation of the lake is controlled by the Samity from a long time. This wetland support various plant species surrounding its lake area viz., *Albizia procera* (Roxb.) Benth., *Anogeissus acuminata* Wall., *Gmelina arborea* Roxb., *Microcos paniculata* L. and *Shorea robusta* C.F.Gaertn. These are generally use as fuelwood and timber by villagers present nearby wetland area.

Economic value of cultural services of the lake was measured on the basis of tourism, boat riding and recreational purposes of wetland. Every year in September boat racing event takes place which inspires local people for participation and to earn prize money from Samity. The Neermahal palace of Rudrasagar lake was built by king

Maharaja Bir Bikram Kishore Manikya Bahadur in the year 1939 as a summer resort. For this historical value people are interested to visit Rudrasagar lake.

Threats to Rudrasagar lake

Wetlands are often considered as the kidneys of the landscape but it is also one of the most threatened environments of the world. Due to various anthropogenic activities in and around the wetland, there is continuous degradation of the overall habitat of the wetland. As much of the environmental services of the wetland are open access and are not limited, the degradation of the wetland is also predominant [5]. Major threats of Rudrasagar lake are-

Siltation: It is one of the main issues of the Rudrasagar wetland. Due to siltation the water depth decreased greatly. Encroachment and the reclamation of land are easy when the water depth decreases. The inflow is from three perennial streams known as Noacherra, Durlavnarayan cherra and Kemtali cherra. As a result, silt gets deposited into the lake extensively. The huge catchment area of the wetland is also an important cause for heavy silt loads. The main part of the lake area i.e. Raj ghat has a massive siltation problem. The western part of the Lake is a high silt aspect zone. Expansion of agricultural lands and land conversion due to population pressure also contribute to massive silt load problems. Earlier in the western part of the lake, there were brick kilns which were operated and it must have contributed in the land and soil degradation of the Rudrasagar. Due to siltation the water depth decreased greatly.

Pollution and other anthropogenic activities: the physicochemical parameters of the lake get affected because of the agricultural runoff that is proliferating in the wetland continuously. There is increased use of chemical fertilizers and pesticides which promotes in the massive growth of water hyacinth. Use of chemical fertilisers like Super phosphate, potash, urea, DAP is common in the wetland area. Brick kilns has been shut down but is has immensely polluted the periphery of the lake area. Immersion of idols in the lake has also added to the water quality issues. The anthropogenic activities around the lake could disrupt the aquatic system as well. The unregulated and unplanned tourist activities also impacts negatively on the lake. There is a picnic spot in Raj ghat for the tourists where foods can be cooked and all the wastes are thrown near by the lake. Other anthropogenic activity that is polluting the lake and fishes is the use of plastic that is being thrown within the lake. Ingestion of plastic molecules by the fish is an emerging issue that needs to be addressed. The water is very much polluted that local people can't use it as if it causes lots of skin disease and other diseases. During last fifteen years the famous IUCN Red listed endangered three-striped Roof turtle (*Kachuga dhongka*) are not seen due to over hunting.

Encroachment: The hydrological regime of the wetland changed due to the construction of Kachigang channel. The channel blocked the backflow of water from Gumti to the wetland. It was constructed for agricultural expansion within the lake. During the lean season, periphery and some pockets of the lake area are used for paddy cultivation. Due to gradual expansion of agricultural plot within the lake, the total water area has drastically shrunk especially in the dry season. Insufficient water flow tends to low production of fishes. Rudrasagar serves as a breeding ground with a wide range of indigenous fishes and ornamental fishes. Reduction in water level may tend to the disappearance of variety of fishes and the fishing areas are drastically reduced.

Invasive species: water hyacinth intrusion in the lake over the years is a serious issue of the wetland. Since the growth is not controlled, there is rapid infiltration of the weed throughout the lake depriving sunlight to plants and oxygen to the fish faunas. Furthermore breeding sites will be reduced and fishermen take longer to reach fishing grounds. Transport by ships or boats will be hindered. Also, evapotranspiration is increased as loss of biodiversity in the water body covered by the water hyacinth. These mats like structure are habitats for certain snails and mosquitoes which spread malaria. *Hydrilla* is also another invasive weed that thrives during the monsoon. The rapid growth of Hydrilla is also a troublesome for the fish in the wetland as it could form thick mats that cover the entire surface of the wetland. Invasive species may obstruct the flow of water and boating in the Rajghat area. It also clogs the lake and hampers agricultural activities, fishing. Hydrilla can be used as an aquatic feed as it contains nutrients carbohydrates 20.15%, 32.12% crude fibre, 2.92% fat, 17.82% protein and 28.82% ash.

Restoration activities in Rudrasagar lake

Cooperation and coordination in terms of sustainable mitigation process among various stakeholders are needed for proper and long term rejuvenation of the wetland. As all the threats related to the wetland are interlinked with one another, strategic interdisciplinary approach among the stakeholders with local institution and communities are much needed. At the same time approach needs to be flexible and open at every level so that there are no challenges faced while implementing the management process. The gap in the management process between the local institution and other stakeholders needs to be addressed thoroughly. The first and foremost mitigation process is the identification of all the issues related to the wetland and after that assessing and monitoring the seriousness of each problem. Early warning of the causing factors and demarcation of the vulnerable areas i.e. spatial planning is an important step for effective and efficient management.

Catchment protection

There should be a concrete formulation of the overall catchment management of the wetland in order to avoid the long term deterioration of the wetland. Catchment protection majorly includes Integrated Water Resource Management i.e. there should be coordinated development and management of water, land and related resources without compromising the sustainability of vital ecosystem. The degraded forests in certain part of the catchment area need to be afforested. There should be a thorough check on the water activities and the pattern of using resources by the people living in the upper catchment areas. An activity that takes place in the catchment area eventually affects the water quality draining from the upper catchment to lower catchment. To improve catchment environment, riparian plantation and agricultural runoff should be managed. Riparian plantation can enhance the restoration process of wetlands. It will help in the regeneration of existing grasslands and vegetation as well as in the rehabilitation process of degraded forests. Riparian restoration improves the water quality and in turn will help the fishes and aquatic fauna to thrive more. Monitoring wastewater disposal and drainage pattern in the riparian areas will also improve water quality. Agricultural runoff and soil erosion will be minimized as bank of the lake will be stabilized. Regular and thorough catchment monitoring with the help of local communities should be made. Knowing the current state of the catchment and the activities practiced will improve the quality of the monitoring process. The hydrogeological regime of the wetland has altered gradually especially due to the anthropogenic activities. Reduced lake area due to continuous reclamation of the wetland area has disturbed the ecology of the wetland. Reduced lake area and decreased water depth along with ever increasing silt load is threatening the existence of the lake.

In depth catchment protection and management in succession will help in the rejuvenation of the Rudrasagar wetland. Noacherra is one of the stream that is accelerating the silt load in the Rudrasagar area. Systematic monitoring and treatment of catchment area will contribute in the minimization of siltation. Watershed development activities should be incorporated along with the catchment area treatment for effective results such as forestry, soil and agriculture conservation, aquatic fauna conservation and livelihood. Dredging should be done to increase the storage capacity of the lake. There should be no new construction of embankments and regulators as natural flow of the lake has already been altered due to the Kachigang channel.

Biodiversity protection

Rudrasagar wetland is a biodiverse habitat where aquatic plants, migratory waterfowl, raptors as well as variety waders coexist. The winter migratory waterfowl

population have declined over the years. This signifies the constant deterioration of the lake habit and its decreased water area. Water quality, temperature, aquatic plants, etc. are important factor that has effects on the existence of migratory birds. Excessive siltation is detrimental to aquatic plants. Silts accumulate on bottom where sunlight penetration is suspended and this affect negatively in their production. This is one of the limiting factors for the migratory birds as their aquatic feed habitat is disturbed. Change in the availability of natural resources and loss of habitat is one of the main reasons for the significant decline in the migratory birds in Rudrasagar. Conservation measures should not only focus on their breeding grounds but also on their non-breeding grounds and stopover areas. Knowing the range of migratory birds is vital for the delineation of protected location. Delineation of key protected areas will be effective for migratory bird protection. Delineation will also help in better determination of habitat location for perching and foraging.

Ecotourism

Practice of ecotourism in Rudrasagar will ensure better ecology and sustainability of the lake. Ecotourism plays a significant role in raising awareness about migratory water birds and its protection of breeding grounds. Regulating tourism will bring in constructive habitat improvement and conservation of water birds. Bird watching program could be involved in the tourism management. Green belts should be formed in the periphery of the wetland. This will help in the management of indigenous trees and grasses favourable for bird roosting. Green belts will also help in the development of vegetative cover. Ecotourism will certainly help in the management of water quality. To improve the tourism and to preserve the palace and the overall Lake, animals should not be allowed for grazing inside the Palace premises. Therefore, involving local communities in the understanding of ecotourism will boost the conservation measure. To improve the environment of the Lake overall catchment management is requisite. Enhancing the biodiversity in the catchment area will ultimately improve the condition of the Lake ecosystem.

Wetland zones

Zoning of wetland is also a necessary step for mitigating the wetland threat. Reclamation of wetland areas for agriculture needs to be stopped at the earliest for controlling the risk factor of the wetland. To address this issue zoning of wetland is crucial. Zoning of wetland could be effective by making 3 zones- buffer zone (limited use zone), wise use zone and risk-control zones [6]. Buffer zone or limited use zone will act as a buffer to minimize the anthropogenic activities in wetland. Wise use zone will be the area where agriculture could be practiced along with pisciculture. Conservation of catchment area could be the risk control zone. In accordance to the

Rudrasagar wetland, in wise use zone, fishing could be practiced in sustainable manner in Raj ghat but for agriculture there is a risk attached to it. Therefore, demarcation of waterway and upto how much the lake could be encroached upon should be closely monitored. To address the issue emergent issue of agriculture and pisciculture could be the practice of paddy cum fish culture. Rice and fish is the staple food and practicing paddy cum fish culture could provide the help in the improvement of food security as well as the socio-economic of farmer and fishermen community [7]. Above all, this could be one of the useful methods in curbing the on-going exploitation of wetland areas in Rudrasagar. Rice cum fish cultivation used to be practiced in Tripura for years [8] and the indigenous knowledge of the cultivation should be utilised wisely for the betterment of the environment as well as for livelihood purpose.

Buffer zone could be created along the catchment area of Rudrasagar. To achieve a sustainable buffer zone and for effective function of the buffer system, multidisciplinary approach of management is required. For sustaining healthy wetland, plantation composition is an important factor. Bamboo plantation, horticultural plants and other tree species and grasses could be used for plantation activities. For effective siltation prevention in Rudrasagar, buffer zone could be created along the Noacherra stretch. Noacherra is one of the main streams that drain into the Rudrasagar bringing loads of silt. Bamboo species that could be used in buffer zones are *Melocannabaccifera* (muli), *Bambusapolymorpha* (barak), *Bambusatulda* (mritinga), *Bambusateres* (paora), *Bambusapallida* (makal), *Bambusaaffinis* (kanakkaich), *Dendrocalamuslongispathus* (rupai), *Neohuzeauadullooa* (dolu). Bamboo and other tree species could be planted and buffer width could be 10m. Buffer strips could be created in the Kachigang zone in the same manner.

Wise use of resources

Wise use of wetland is an approach for maintaining the ecosystem services to ensure the biodiversity is preserved for long term [9]. This maintenance will be beneficial for human well-being as well. Continuous encroachment and degradation of wetland leads to the reduction in the delivery of wetland ecosystem services [9]. One of the critical point of the wetland management is to ensure thorough management of the ecosystem services. Wide variety of ecosystem services is provided by the Rudrasagar wetland to the people. Provisional and cultural services are prominent. At the same time regulating services and supporting services are an important determinant. Ecosystem services will be beneficial if the wetland ecosystem is resilient enough to provide for human needs and sustain biodiversity. For the wetland ecosystem to be resilient, integrated water management needs to be incorporated. One of the main challenges is to ensure there are no conflicts of interests among the stakeholders involved in the wetland management.

Management and awareness activities

The main goal in the wetland management should be to address wetland sustainability and to ensure there is no additional stress that will degrade its ecological importance. The management process should be in such a way that the ecosystem of the wetland and livelihood of the communities is not disturbed. For effective management process, local communities should be involved in every level of conservation plan.

Several restoration activities by community involvement have been initiated in Rudrasagar Lake by the Department of Forestry & Biodiversity, Tripura University in collaboration with Rudrasagar Udbastu Samabay Samity, Melaghar with the financial assistance from National Mission on Himalayan Studies (NMHS), Almora. Its main focus is to promote optimum utilization of wetland ecosystem services and its sustainable management and conservation. Many awareness programmes have been organized to increase responsiveness in local people and motivate them towards restoration of this wetland. The assistance from public representatives of that area have also been taken for disseminating the information about the lake to the local people and also to appeal for conservation and restoration of the lake. The PRA exercise was conducted with 141 local people from 18 villages around the lake and resource map of the area was prepared. Most of the participants preferred to start income generation activities based on the lake apart from fishing and agriculture activities.

It was observed that the water hyacinth plants are rapidly growing and spreaded all over the wetlands during the rainy season. So villagers especially females were trained on crafts making from water hyacinth plant to prepare mats, hand bags, round bag, pen stand, etc. Many of the participants are ready to adopt this water hyacinth craft making livelihood option due to low cost of production and availability of raw materials in and around the lake. Beside this many steps have been taken for improving the quality of this wetland ecosystem like all the brick industries nearby the lake are being closed, water level of the lake is now maintained even during winter season. *Hydrilla*, an invasive species is a submerged plant but can grow to the surface and form dense mats. It poses significant ecological damage to the lake system. Therefore, periodic cleaning is necessary. It thrives more during the monsoon. *Hydrilla* can be used as an aquatic feed as it contains nutrients carbohydrates 20.15%, 32.12% crude fibre, 2.92% fat, 17.82% protein and 28.82% ash. Hydrilla was removed from the lake in the month of August with the help of local fishermen. It was then dried in direct sunlight for two days. The weed can be used as fish food after drying and when it is applied in pond it helps in the growth of the fishes because of very high nutrient content. We observed lower pH, Dissolved oxygen, BOD in ponds after using hydrilla based fish food. It was also confirmed that use of hydrilla based fish foods increases the size and taste of the fish specially in grass carps. This might be due to higher protein content in hydrilla

plant. Therefore the above management of two plant species ie., *Hydrilla verticillata* & *Eichhornia crassipes* suggests that despite of its invasiveness and ill effects on lake ecosystem. The former can be used as a fish feed and the later can be of good use in making crafts which will mutually benefit the health of lake while removing them for such purpose and also create a source of income for people living near this lake.

Soil erosion from the shoreline land areas is one of prominent factors responsible for lowering depths of Rudrasagar lake from the last few decades. Moreover nearby villagers are also effected by losing their land near shorelines. So to mitigate such situation plantation activity conducted in catchment areas. The area selected is very much prone to soil erosion as it is surrounded by lake with three sides. Medium sized saplings were used for the plantation activity which comprises of Nageswar, Ashok, Neem, Sajina, Horitoki. Each saplings were well fenced with bamboo sticks so that they can get protection from grazing animals. After plantation the GPS reading of the all the planted locations were recorded. Other activities like lake cleaning, plantation, organic farming, optimal water depth management are also considered for the restoration of the lake.

Conclusion

Overall, the study concludes that the Rudrasagar lake is having great provisional value as it is linked with the livelihood of the local community. It also has high cultural and supporting value for its traditional and historical importance as well as facility to provide habitat. There is a rich diversity of aquatic plants, fishes and other aquatic fauna in the lake. Conservation of certain macrophytes present in the lake can help in restoration of avifaunal species specially the migratory waterfowls as well as wader birds. The lake is also culturally linked with people as every year boat race is organised, which is a very motivating festival for local people. This lake is under high stress and immediate conservation through its restoration is necessary for enjoying its various services sustainably. The project sponsored by National Mission on Himalayan Studies (NMHS), MoEF&CC, Almora for restoration of ecosystem services of Rudrasagar lake is a great initiative for the sustainable development of the lake through community involvement.

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CHAPTER

Morphological analysis of biogenic nanocrystals prepared from banana plant waste material & its application

Sumit Sarkar

Directorate of Biotechnology, Department of Science, Technology & Environment, Agartala, Tripura, India
E-mail: sarkarsumit07phy@gmail.com

Abstract: In subtropical region, banana plant is widely available and a large number of wastes have been produced from different parts of this plant. As it is mostly available in our society, its utilization is very important in an economic point of view. Therefore, a unique experimental technique has been introduced for utilization of banana plant waste product. In this work, X-ray diffraction study confirms the purity and crystalline nature of the prepared materials. Further, the average size is 50-80 nm with morphology of spherical shape of the prepared nanocrystals which has been confirmed from Scanning Electron Microscopic (SEM) study. Considering different concentration of the prepared solution, it has been found that a particular concentration of the material solution is effective for the good wealth of soil. Thus, this approach is a promising and very rapid method for the enhancement of soil pH.

Keywords: Biogenic Nanocrystals; XRD; SEM; Soil pH.

In tropical and subtropical regions of the world, Banana (Musa paradisiaca, family Musaceae) is one of the central fruit crop grown on about 8.8 million hectares[1]. It is possibly the world's oldest cultivated crop[2]. In different countries, about 300 varieties of bananas are grown, of which a vast majority are grown in tropical Asia[3]. Bannana wastes are generated in massive amounts once a year. Due to the massive handiness and composition wealthy in compounds that might be utilized in alternative processes, there's a great interest on the utilization of banana plant extract, each from economical and environmental points of view. The economic facet is predicated on the actual fact that such wastes could also be used as low cost raw materials for the production of different value added compounds. The environmental worry is to reduce the contamination revolt from the waste discharge. The main residual wastes of the banana

crops are leaves and pseudostem. This pseudostem consists of concentric layers of leaf sheath and column of large leaves, both containing high level of lignocelluloses[4]. Currently, immeasurable tones of banana pseudostem are dropping in our country as waste and most of the farmers face vast troubles in disposing the accumulated banana pseudostem.

However, soil pH has been also affected by land use and management. Further, vegetation type impacts soil pH. For example, areas of forestland tend to be more acidic than areas of grassland. Conversion of land from forestland or grassland to cropland may end up in drastic pH scale changes once some years. These changes have been occurred due to the drastically reduce of organic matter, removal of soil minerals once crops are harvested, erosion of the surface layer, and effects of different fertilizers etc. Therefore, the identification of soil pH and nutrient analysis are more important before designing any experimental

Soil acidity has been determined by the number of H+ activity in soil solution and is influenced by edaphic, climatic, and biological factors of natural prevalence. Soils that develop from granite parent materials acidify at a quicker rate than soils derived from chalky parent materials. Sandy soils with comparatively few clay particles acidify sooner because of their smaller reservoir of alkaline cations and better leaching potential. Excessive precipitation influences the speed of soil acidification depending on the rate of percolation of water through the profile. Organic matter can decay to make acid and different weak organic acids. The additive result of those factors is much immeasurable over the course of some years and, therefore, might contribute comparatively very little to total soil acidity [5].

Here, in this work the banana plant waste as raw materials have been prepared after drying the waste of banana plant. The prepared raw materials have been investigated by X-ray diffraction (XRD). Further, the raw materials have been applied in a suitable proportion to enhance the soil pH.

Materials and Methods

Preparation of Raw Materials

The collected banana plant wastes have been dried in sunlight for 7-11 days. Subsequently, the product has been burnt and prepared in powder form which is shown in figure A. Further, to study the soil pH, 5 gm of soil is collected having low pH and diluted accordingly in distilled water to measure the soil pH and after that the prepared materials of 0.30 gm (T_1), 0.55 gm (T_2) and 02 gm (T_3) have been mixed with the above mentioned solution. The prepared solution has been vigorously stirred by rotator shaker for 10 to 20 min.

Fig. A: *Powder form of the prepared banana plant waste product*

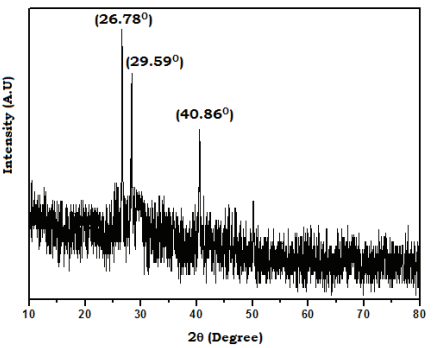

Fig. B: *X-ray diffraction pattern of the prepared sample.*

Characterization

X- ray diffraction (XRD) studies of the prepared banana plant waste powder have been carried out using a D8-Advance Bruker system equipped with Cu Kα radiation (λ= 0.1541nm). UV/vis measurements have been carried out with the Perkins Elmer spectrophotometer Lambda 11, where light source is the deuterium tungsten halogen lamp. Further, Fourier Transform Infra Red (FTIR) spectra of the prepared samples have been recorded on a Perkin Elmer FTIR Spectrophotometer in 800–4000 cm−1 range in the form of KBr pellets. The size and elemental analysis of the prepared samples have been investigated using a Scanning Electron Microscope, Model - Sigma 300, Carl Zeiss.

Results and Discussion

X-ray Diffraction Study

In solid state physics, material science and solid state chemistry, X-ray diffraction is one of the most important characterization techniques used [6-9] for the estimation of the average grain size as well as the confirmation of crystalline nature. XRD measurement has been performed on a D8-Advance Bruker system equipped with Cu Kα radiation (λ= 0.1541nm). Figure: B shows the XRD pattern of the prepared sample and all the detectable peaks (2θ) at 26.78^0, 29.59^0 and 40.86^0 have been clearly indicated the crystallinity of the prepared sample. However, it has been observed that at 27.51^0 is the high intense peak for the prepared material.

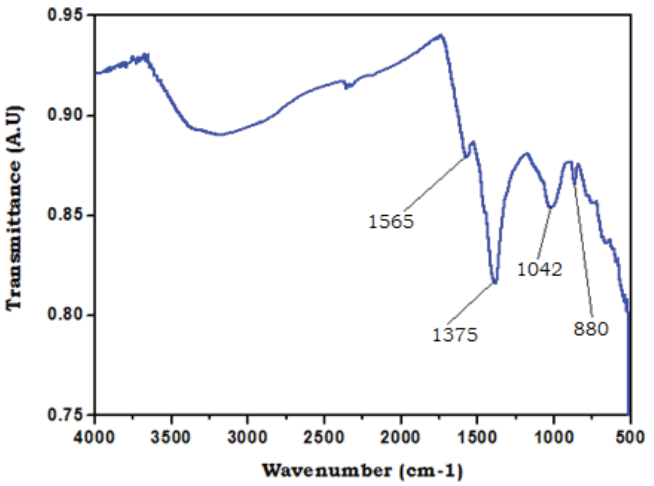

Fig. C: *FTIR spectrum of prepared Biogenic Nanocrystals*

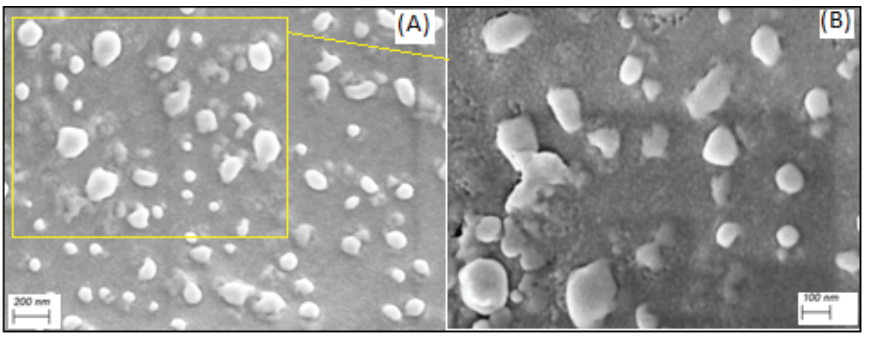

Fig. D: *(A) & (B) SEM images of the prepared Nanocrystals*

Spectroscopic Analysis of Constituents

For the analysis of molecules or compounds, Infrared spectroscopy is is extremely helpful which is basically connected with vibrational energy spectrum of atoms or cluster of atoms in a material. As, IR spectra, in general, show several bands, two different compounds or molecules cannot have the similar infrared spectrum and for this reason, IR spectrum provides the "finger print" of a molecule [10-11]. Here, Fig. C shows the FTIR spectra of the prepared materials. Here, the band observed around 880 cm^{-1} corresponded to β-1, 4 glycosidic linkages while the band around 1042 cm^{-1} corresponded to ring vibrations and C-O-C links. The band in the region around 1415–1325 cm^{-1} has been assigned to C-H in plane deformation of CH_2 groups [12-13]. Besides, C=C stretching bond in the lignin component can be observed from peaks in

the range of 1450–1590 cm^{-1}[12-14]. The FTIR spectra clearly indicate the presence of functional groups such as glycosidic bonds, ether groups, alkynes groups and alkenes in the selected adsorbents.

Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDX) analysis

Here, the SEM images confirm the formation of nanocrystals (Fig: D) and mostly spherical in nature. SEM image clearly shows that a common characteristic of the particles is their spherical in shape. It is also visible that particles are mutually different in size varying between 30 to 100 nm.

Application of Biogenic Nanocrystals for Enhancement of Soil pH

Soil acidity is a major problem in relation to plant growth and therefore, acid soils are called a problem soil. Therefore considering its importance, we are interested to prepare the banana plant waste powder for the enhancement of soil pH. The pH of banana plant waste powder has been measured by a suitable ratio and it has been observed that the pH value of the powder product is 9.08. Therefore, for the enhancement of soil pH, some soil sample has been collected from tea garden and it is found that pH value is 5.03. In this regard, the soil has been treated with the prepared nanocrystals and the following results have been observed which is shown in table: 1.

Table 1: *Influence of powder sample concentration on acceleration of soil pH*

Sl No	Water (ml)	Soil Sample (gm)	Powder Sample (gm)	pH value
01.	150	50	Nil	5.60
02.	150	50	0.15	5.82
03.	150	50	0.25	6.20
04.	150	50	0.30	6.35
05.	150	50	0.35	6.70

Further, the experimental study indicate that suitable dose banana plant waste powder for better wealth of soil is 0.30 gm for 50 gm soil diluted in 150 ml of water. After carefully examining the experiment, it has been observed that at the combination of 150 ml mixed with 50 gm of soil having 0.25 gm of powder sample and 150 ml mixed with 50 gm of soil having 0.30 gm of powder sample are suitable for obtaining the pH value at 6.20 to 6.35. Here, the prepared nanocrystals may consume H+ in soil solution, especially in acidic soils, to produce different ions, which consequently increases the soil pH.

Conclusion

Here, in this work the banana plant waste materials have been prepared in powder form. The materials have been characterized by XRD, SEM and FTIR. X-ray diffraction

study clearly indicates the banana plant wastes material is crystalline in nature, whereas Scanning Electron Microscopy confirms the formation of nanocrystals. Further, FTIR spectra indicate the presence of functional groups in the materials. Here, the materials have been utilized with a different concentration for enhancement of soil pH and it is found that 50 gm of soil diluted in 150 ml water having 0.30 gm of powder sample are suitable for obtaining the pH value at 6.20 to 6.35. Therefore, this study suggests that banana plant waste materials are suitable for enhancement of soil pH.

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8
CHAPTER

Quercetin act as potential anticancer agent against HT-29 human colon cancer cell line

Asish Bhaumik*[1], Samaresh Datta[1] and Tejendra Bhakta[2]

[1]Department of Pharmaceutical Chemistry, Narayan Institute of Gopal Narayan Singh University, Jamuhar, Bihar, India.
[2]Department of Pharmaceutical Chemistry, Regional Institute of Pharmaceutical Science and Technology, Abhoynagar, Agartala, Tripura, India.
*E-mail: bhaumik.asish@gmail.com

Abstract: Colorectal cancer (CRC), also known as bowel cancer, colon cancer, or rectal cancer, is the development of cancer from the colon or rectum (parts of the large intestine). Signs and symptoms may include blood in the stool, a change in bowel movements, weight loss, and feeling tired all the time. Most colorectal cancers are due to old age and lifestyle factors, with only a small number of cases due to underlying genetic disorders. Risk factors include diet, obesity, smoking, and lack of physical activity. Dietary factors that increase the risk include red meat, processed meat, and alcohol. Consuming fruits and vegetables of all kinds have long been associated with a reduced risk of many lifestyle related health conditions. Many studies have suggested that increasing consumption of plant foods like apple decreases the risk of a variety of human disorders, including: prostate cancer, prostatic hyperplasia, colorectal cancer and CVS disorders. Molecular Docking is an important component of computer-assisted drug discovery. It helps in predicting the intermolecular framework formed between a protein and ligand and outputs the appropriate binding between the molecules. The main aim and objective of the present research work was the isolation of bioflavonoid quercetin from methanolic extract of peels of apple (ME-PA) and evaluation of *in vitro* anticancer activity toward HT-29 human colon cancer cell line followed by molecular docking against target protein thymidylate synthase. The extraction was done by reflux condensation method and preliminary phytochemical screening of ME-PA was carried out for the identification of bioactive molecules such as carbohydrates, fats, proteins, sterols, alkaloids and the bioflavanoid present in ME-PA which was confirmed by qualitative confirmatory chemical tests. The bioflavanoid quercetin was characterized by spectral analysis such FTIR, ^{1}H-NMR and ^{13}C-NMR and LC-MS. Molecular docking of isolated compounds was carried out against target protein *thymidylate synthase enzyme* with PDB id 1100 by Auto dock program and the best dock pose was selected based on the interaction study analysis. *In vitro* anticancer activity ME-PA was evaluated by SRB assay against HT-29 human colon cancer cell line and the test was performed at different concentrations. In silico molecular docking studies

the binding energy of isolated compound quercetin was found to be -6.17 which indicated that the compound had high binding affinity towards target protein thymidylate synthase and inhibit the function of this particular enzyme efficiently in comparison with standard drug 5-FU (-3.59). A preliminary anticancer screening displayed that the ME-PA was able to inhibit the proliferation and caused apoptosis of more than 70% of human colon cancer cells. The maximum growth of inhibition by ME-PA was found to be at 400 µg/ml (IC_{50} 4.2 µg/ml) and at this concentration the percentage of cell growth inhibition was found to be 83.9% which was compared with the standard drug 5-FU (IC_{50} 3 µg/ml, 92.1%). The recent findings proved that ME-PA possessed potential anticancer activity against human colon cancer cells which may be mainly due to the present of bioactive molecule quercetin.

Keywords: Colorectal cancer, molecular docking, anticancer activity, bioflavanoid and apoptosis etc.

Phytochemistry is the study of phytochemicals, which are chemicals derived from plants. Specifically, phytochemistry describes the large number of secondary metabolic compounds found in plants. Many of these are known to provide protection against insect attacks and plant diseases. They also exhibit a number of protective functions for human consumers. Phytochemistry can be considered sub-fields of Botany or Chemistry. Activities can be led in botanical gardens or in the wild with the aid of Ethnobotany. The applications of the discipline can be for Pharmacognosy, or the discovery of new drugs, or as an aid for plant. Phytochemicals exist as long as plants exist but we only know about hundred years about their existence. Medicinal plants are traditionally used all over the world. It is likely that the knowledge of traditional medicine developed through trial and error over many centuries [1].

Today, most new pharmaceuticals are not discovered in plants but are new synthetic creations. Recently there is a renewed interest in the discovery of phytochemicals. This renewed interest is our awareness has already developed many chemicals, which still have to be discovered. New modern laboratory techniques have made it easier to discover and identify new phytochemical Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. They are non-essential nutrients, meaning that they are not required by the human body for sustaining life. It is well-known that plant produce these chemicals to protect themselves but recent research demonstrate that they can also protect humans against diseases. There are more than thousand known phytochemicals. Some of the well-known phytochemicals are lycopene in tomatoes, isoflavones in soy and flavanoids in fruits [2]. The development of modern pharmacognosy took place a simultaneous advancement in the area of Organic chemistry, Biochemistry, Medicinal chemistry, Biosynthesis and modern methods and techniques of analysis like TLC, Paper chromatography, HPLC, UV-Visible, IR, NMR and Mass spectroscopy etc. Thus a wide variety of active principles were isolated from different parts of various plants and established to possess a wide

range of pharmacological and antimicrobial activities [3]. Apples might help to prevent a variety of diseases and disorders such as Alzheimer's disease, hypertension, CVS disorders, cancers especially colorectal cancer, asthma, osteoporosis and inflammation. Peels of apples possessed antioxidant, anticancer, anti-inflammatory, antimicrobial activities [4, 5].

Materials and Methods

Experimental Phytochemistry

Chemicals: Chemicals used for the extraction and phytochemical screening was provided by Institutional store.

Extraction methodology: Weigh 5 g of peels of apples paste (can be mashed to prepare a paste) transfer into 250 ml of round bottomed flask. Add a mixture of 50 ml of methanol and 60 ml of dichloromethane. Heat the mixture under reflux for 5 min on water bath with frequent shaking. Filter the mixture under suction and transfer the filtrate to a separating funnel. Wash this mixture containing bioactive compounds with three portions of 150 ml each with sodium chloride solution [6]. Dry the organic layer over anhydrous magnesium sulphate. Filter and evaporate most of the solvent in vacuum without heating and obtained methanolic Extract of Peels of Apple (ME-PA).

Phytochemical screening: Preliminary Phytochemical screening of ME-PA had shown the presence of various bioactive compounds such as carbohydrates, amino acids and peptides, phytosterols, carotenoids, and polyphenols etc [7-9].

Instrumentation: TLC, FTIR, ^{1}H and ^{13}C-NMR and LC-MS are used for the isolation and characterization of bioflavanoid quercetin present in methanolic extract of peels of apple (ME-PA).

Isolation and characterization of bioflavonoid quercetin in ME-PA

Thin layer chromatography (TLC) procedure: The TLC developing was set as twin through chamber were examined in various solvent systems, such as chloroform and methanol in the ratio 7:3, 1:1. The fractions were run on silica gel 60 F254 pre-coated aluminium plate, of 0.2 mm thickness. The optimal solvent for the identification of compound was determined by varying the ratios of solvents for developing the solvent system. Visualization was carried out by dipping the plate in vanillin sulphuric acid reagent and heating till the colour of the spot appears. Retardation factor (R_f) was calculated using the formula: R_f = Distance moved by the solute/Distance moved by the solvent.

Infrared (IR) and Nuclear magnetic resonance (NMR) analysis: IR spectra were recorded on Bruker Alpha TKBR and ATR spectrophotometer. ^{1}H and ^{13}C-NMR

spectra were run on a Bruker AV NMR instrument equipped with 5 mm ^{1}H and ^{13}C operating at 500 MHZ, respectively with tetramethylsilane (TMS) as an internal standard.

Computational Chemistry

Molecular Docking is an important component of computer assisted drug discovery. It helps in predicting the intermolecular framework formed between a protein and ligand and outputs the appropriate binding between the molecules. Molecular docking may be defined as an optimization problem, which would describe the "best-fit" orientation of a ligand that binds to a particular protein of interest[10]. During the course of the docking process, the ligand and the protein adjust their conformation to achieve an overall "best-fit" and this kind of conformational adjustment resulting in the overall binding is referred to as "induced-fit"[11].

Docking Study: Thymidylate Synthase with Quercetin and 5-Fluorouracil.

Protein name: Human Thymidylate Synthase, PDBID= 1I00, Organism= Homo sapiens.

Resolution: 2.50 Å, Sequence Length= 290.

Ligand name: Quercetin, Molecular, **Formula**: $C_{15}H_{10}O_7$. Molecular, **Weight**: 302.23 g/mol.

Ligand name: 5-Fluorouracil. Molecular, **Formula**: $C_4H_3FN_2O_2$. Molecular, **Weight**: 130.08 g/mol.

Methodology: Docking was performed by AutoDock 4.2.6 program, using the implemented empirical free energy function and the Lamarckian Genetic Algorithm (LGA). The grid maps were calculated using Auto Grid. In all dockings, a grid map with 60×60×60 points and a grid-point spacing of 1.000 Å was applied.

Experimental oncology

Method used: Sulforodamine B (SRB) assay.

Principle: Sulforodamine B (SRB) is a bright pink aminoxanthine dye with two sulfonic acid group. Under mild acidic conditions SRB dye binds to basic amino acid residues in trichloro acetic acid (TCA) fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of visible at least two order of magnitude [12].

Reagents: PBS (Phosphate buffer saline), 40-50% TCA, 1% acetic acid solution, Sulforhodamine B (0.4% in 1% TCA) and 10 Mm Tris (P^H = 10.5). The standard drug 5-FU was provided by ACRC.

Cell culture: The cell culture HT-29 human colon cancer cell line was provided by Amala Cancer Research Centre, Thrissur, and Kerala. and were grown in Eagles Minimum Essentia Medium (EMEM) which contained 10% fetal bovine serum (FBS). All cells were maintained at 37°C, 100% relative humidity, 5% CO2, 95% air and the culture medium was changed twice a week.

Procedure: The monolayer cell culture was trypsinized and the cell count was adjusted to 0.5-1.0 x10^5 cells/ml using medium containing 10% new born sheep serum. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately) 10,000 cells was added. After 24 hrs, when a partial monolayer was formed, the supernatant was flicked off, washed once and 100 µg/ml, 200 µg/ml, 300 µg/ml and 400 µg/ml of different concentration ME-PA were prepared and added to the cell in micro titre plate. The plates were incubated at 37 ^{0}c for 72 hrs in 5% CO2 incubator, microscopic examination was carried out and observations were recorded every 24 hrs. After 72 hrs, 25µl of 50% TCA was added to wells gently such that it forms a thin layer over the extract to form overall concentrations 10%. The plates were incubated at 4^{0}c for 1 hr. The plates were flicked and washed five times with tap water to remove traces of medium sample and serum and were then air dried. The air dried plates were stained with 100 µl SRB and kept for 30 mints at room temperature. The unbound dye was removed by rapidly washing four times with 1% acetic acid. The plates were then air dried. 100 µl of 10 mM Tris base was then added to the wells to solubilise the dye. The plates were shaken vigorously for 5 mints [13]. The absorbance was measured using micro plate reader at a 540 nm. The % growth inhibition was calculated by the following formula: % cell growth inhibition = 100-{(At-Ab/Ac-Ab)}x 100.

Where: At = Absorbance value of test compound, Ab = Absorbance value of blank, Ac = Absorbance value of control. Positive control for anticancer activity (Test): cells treated with a cytotoxic drug/sample extract +SRB + solubilizing buffer. Negative control for anticancer activity (control): cells left untreated + SRB + solubilizing buffer. Blank: medium without cells + SRB + Solubilizing buffer.

IC_{50} Determination: IC_{50} is the acronym for "*half maximal inhibitory concentration*". IC_{50} value indicates the concentration needed to inhibit a biological or biochemical function by half (e.g. inhibition of enzymes, affinity to cell receptors). IC_{50} is calculated by the following formula [14]: IC_{50} = (50% - Low Inh%)/(High Inh% - Low Inh%) x (High Conc-Low Conc) + Low Conc. Where, Low Inh%/High Inh%: % inhibition directly below / above 50% inhibition and Low Conc/High Conc: Corresponding concentrations of test compound.

Results and Discussion

Phytochemistry and spectral analysis

Table 1: *Phytochemical present in ME-PA*

Sl. No.	Phytochemical	Present (+) / absent (-)
1.	Carbohydrates	++
2.	Proteins	+
3.	Amino acids	+
4.	Phytosterols	-
5.	Carotenoids	++
6.	Polyphenols (phenolic compounds)	+++
7.	Alkaloids	-

Thin layer chromatography: In the TLC mobile phase solvent ratio of chloroform: methanol (1:1) showed R_f value of 0.46 equal to that of standard quercetin.

Fourier transforms infrared (FT-IR) spectrum analysis: The FT-IR spectrum of isolated compound was shown in fig. 2 and their corresponding characteristic peak positions were listed in table 1. The broad absorption peak at around 3290 cm-1 was assigned to the OH stretching vibration of phenol. C=O aryl ketonic stretching vibrations are observed at 1668 cm-1. The absorption peaks positioned at 1612 cm-1, 1516 cm-1 and 1429 cm-1 are assigned to the C---C, C=O and C=C aromatic stretching vibrations respectively. OH bending vibrations of phenols were observed at 1359 cm-1. The absorption peak at 1315 cm-1 and the peaks at the lower frequencies between 950 cm-1 and 600 cm-1 were assigned to the C-H bending vibrations of aromatic hydrocarbons. C-O stretching vibrations of aryl ether and phenols were observed at 1240 cm-1 and 1210 cm-1 respectively. C-CO-C stretching and bending vibrations of ketones were observed at 1163 cm-1, which confirms that the isolated compound is flavonoid quercetin.

NMR spectrum of isolated compound: NMR studies were carried out to confirm the positions of proton and carbon binding sites. The isolated compound displayed a better resolved ^{1}H-NMR spectrum. The ^{1}H-NMR spectrum of the isolated compound showed aromatic hydrogen groups from 6.18-7.66 ppm and phenolic-OH groups from 9.36-12.48 ppm respectively. The ^{13}C-NMR spectrum showed carbonyl group at 176.2 ppm and aromatic carbon group from 93.8-164.3 ppm. The corresponding ^{1}H NMR and ^{13}C NMR peak positions for isolated compound were shown in table 2.

Table 2: *Peak positions and probable inter-atomic bonds of isolated quercetin*

Peak position	Inter-atomic bond
3290.58	O-H stretching vibration of phenol
1668.24	C=O Aryl ketonic stretch
1612.16	C---C Aromatic ring stretch
1516.26	C=O aromatic stretch
1429.54	C=C aromatic stretch
1359.37	O-H bending of phenols
1315.58	C-H bond in Aromatic hydrocarbon
1240.55	C-O stretch of Aryl ether
1210.97	C-O stretch of phenol
1163.60	C-CO-C stretch and bending in ketone
932.70, 815.46, 705.65, 596.88	C-H bending of aromatic hydrocarbons

Table 3: *^{1}H NMR and ^{13}C NMR data for isolated compound quercetin*

^{1}H NMR spectrum of quercetin	^{13}C NMR spectrum of quercetin
6.19 (d, 1H, J = 7.2 Hz, Ar-H)	93.8, 98.6, 103.4, 115.4,116.0 (Ar-C)
6.41(d, 1H, J = 6.9 Hz, Ar-H)	120.4, 122.4, 136.1, (Ar-C)
6.88(d, 1H, J = 5.1 Hz, Ar-H)	145.5, (Ar-C)
7.54(q, 1H, J = 6.9 Hz, Ar-H)	147.2, (Ar-C)
7.66(d, 1H, J = 7.4 Hz, Ar-H)	148.1, (Ar-C)
9.36 (s, 2H, Ar-OH)	156.6, (Ar-C)
9.65(s, 1H, Ar-OH)	161.1, (Ar-C)
10.87(s, 1H, Ar-OH)	164.3, (Ar-C)
12.48(s, 1H, Ar-OH)	176.2, (Ar-C=O)

Liquid chromatography/tandem mass spectrometry (LC/MS/MS identification): The isolated compound was analyzed by LC-MS-MS. It has been successfully applied for a quick separation and identification of the isolated compound from fenugreek. The chromatogram of the isolated compound and the fragment pattern m/z 302.95 was found in its first order mass spectrum, and it is speculated that they may correspond to the fragment patterns of quercetin. Comparison to the reference substance and a mass spectral library system confirmed that the isolated compound is found to be quercetin. So the proposed structure of isolated quercetin was found to be as followed:

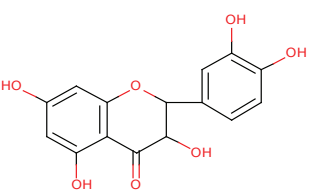


Fig 4: *Structure of isolated quercetin*

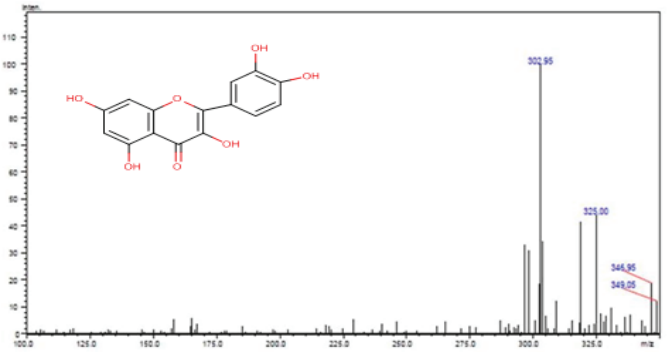


Fig 5: *The total ion chromatogram of the isolated compound*

Molecular docking analysis

Table 5A: *Docking results analysis*

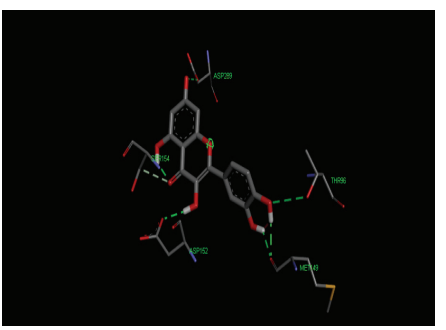
Ligand name	Binding energy (k.cal/ml)	Final IME (kcal/mol)	vdW + Hbond + desolv Energy (kcal/mol)	Electrostatic Energy (kcal/mol)	Torsional Free Energy (kcal/mol)
Quercetin	-6.17	-6.13	-5.86	-0.27	+1.79
5-FU	-3.59	-3.59	-3.44	-0.15	+0.00

Table 5B: *Docking results analysis*

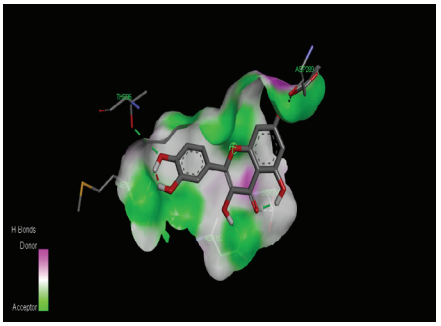
Ligand name	No. of H-bonds	Interacting residues
Quercetin	04: (H1:Distance= 2.61 Å) (H2:Distance= 3.28 Å) (H3:Distance= 2.13 Å) (H4:Distance= 2.25 Å)	ASP289 (H1) THR96 (H2) MET149 (H3) ASP152 (H4) SER154
5-FU	03: (H1:Distance= 1.88 Å) (H2:Distance= 2.95 Å) (H3:Distance= 2.65 Å)	ASN226 (H1) GLN214 (H2) ASP218 (H3)

Quercetin with Thymidylate synthase Model-1

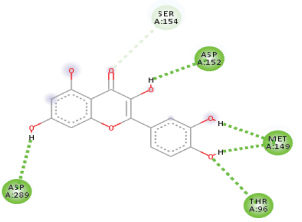
Quercetin with Thymidylate synthase Model-2



Interaction of ligand functional grp with active site of protein Model-1



Interaction of ligand functional grp with active site of protein Model-2



Receptor-ligand complex

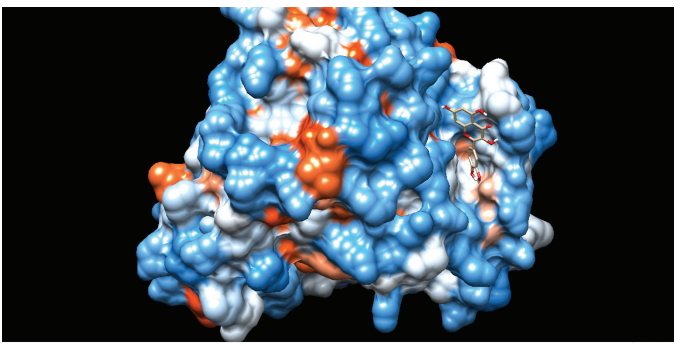
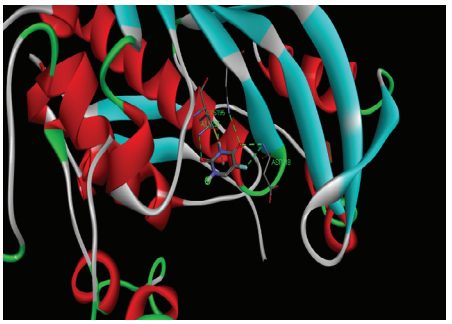


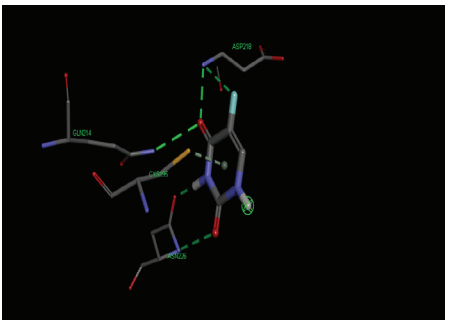
Fig-6-A: *Binding interaction of ligand quercetin with target protein thymidylate synthase*

Most of the scoring functions in molecular docking are physics based molecular mechanics force fields that estimate the energy of the binding pose; a low (negative) energy indicates a stable system and thus a likely binding interaction. Molecular docking is performed to find out the binding affinity or molecular interaction energy (kcal/mol) of docked compounds. Lowest (negative value) energy of docked molecule indicates high binding affinity with the target protein/compound. In silico molecular docking studies the binding energy of isolated compound quercetin was found to be -6.17 which indicated that the compound had high binding affinity towards target protein thymidylate synthase and inhibit the function of this particular enzyme efficiently in comparison with standard drug 5-FU (-3.59). The binding of incoming functional positioned on ligand involved hydrogen bonding and other interaction with active amino acid residues of target protein.

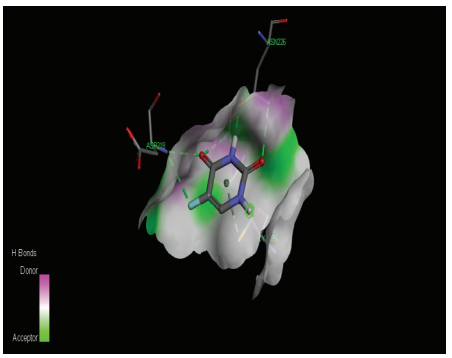
5-FU with Thymidylate synthase Model-1



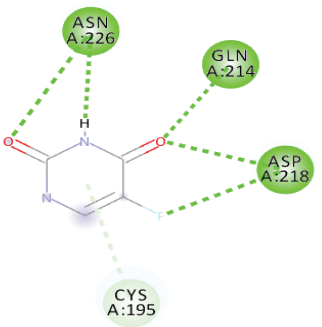
5-FU with Thymidylate synthase Model-2



Interaction of ligand functional grp with active site of protein Model-1



Interaction of ligand functional grp with active site of protein Model-2



Receptor-ligand complex

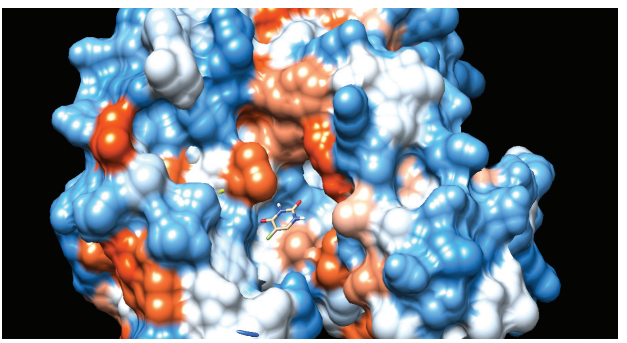


Fig-6-B: *Binding interaction of ligand 5-FU with target protein thymidylate synthase.*

Analysis of in vitro anticancer activity

In vitro anticancer activity ME-PA was evaluated by SRB assay. A preliminary screening displayed that the ME-PA was able to inhibit the proliferation and caused apoptosis of more than 70% of HT-29 human colon cancer cell line.

Table 6: *Percentage Cell Growth Inhibition (%) of by ME-PA*

Sl. No.	Concentration of the Extracts	Absorbance of extracts	PCGI (%)	IC_{50} value (µg/ml)
1	100 µg/ml*	0.083±0.21	69.51±0.24	6.3
2	200 µg/ml*	0.074±0.24	74.13±0.28	5.1
3	300 µg/ml**	0.065±0.41	79.1±0.36	4.9
4	400 µg/ml***	0.069±0.13	83.9±0.14	4.2
7	75 µg/ml (5-FU)***	0.016±0.01	92.15±0.21	3.0
8	Control	0.291±0.08	0	-

P<0.001= ***, highly significant. P<0.01= **, moderate significant P<0.05= *, significant. P>0.05= ns. Here the values were expressed as MEAN ±SD of triplet. The data was statistically analysed by one way ANOVA followed by Tukey Kramer multiple comparison test.

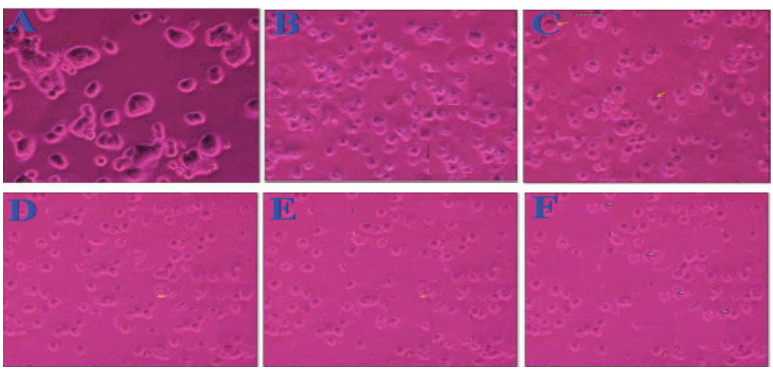


Fig 7: *Morphological changes in HT-29 cell line observed under phase contrast microscope at a magnification of 20X. A: Control (Untreated cells), B: 100 µg/ml ME-PA treated cells, C: 100 µg/ml ME-PA treated cells, D: 100 µg/ml ME-PA treated cells, E: 100 µg/ml ME-PA treated cells and F: 75 µg/ml standard drug 5-FU treated cells.*

In the present study it showed that ME-PA had the potential apoptotic effect on HT-29 human colon cancer cells with different concentrations. The pattern of HT-29 cells growth inhibition and apoptosis by ME-PA was found to be dose dependent. With increasing the concentration of ME-PA the rate of HT-29 cell proliferation decreased gradually at optimal population after 72 hours and lead to apoptosis. The apoptosis of HT-29 human colon cancer cells characterized by cell shrinkage, membrane blebbing and nuclear fragmentation etc. The maximum anticancer activity of ME-PA was found to be at highest concentration i.e. at 400 µg/ml and the standard drug 5-FU was found to cause apoptosis at concentration 75 µg/ml (IC_{50} 3 µg/ml). In mammalian cells, 5-FU is converted to fluorodeoxyuridine monophosphate (FdUMP), which forms a stable complex with thymidylate synthase (TS), and thus inhibits deoxythymidine monophosphate (dTMP) production. dTMP is essential for DNA replication and repair and its depletion therefore causes Cytotoxicity [15].

Conclusion

From the present experimental data, here we concluded that the ME-PA contained various bioactive molecules which were confirmed by their qualitative confirmatory chemical tests and ME-PA displayed the presence of bioactive flavanoid quercetin which was characterized by FTIR, NMR and LC-MS spectrometry. In silico molecular docking studies of quercetin displayed its high binding affinity towards Human Thymidylate Synthase, PDBID=1I00 and inhibit the function thymidylate synthase enzyme efficiently in comparison with standard drug 5-FU having the low binding affinity (binding energy: -3.59 K.cal/mol) than the quercetin (-6.17K. cal/mole). *In vitro* anticancer studies of ME-PA showed 70% apoptosis of human colon HT-29 cells. So the ME-PA displayed potential cytotoxicity against HT-29 human colon cancer cell line may be due to the present of bioactive molecule quercetin which was proved by in silico molecular docking studies.

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CHAPTER

Mapping the molecular basis and preventive strategies of Covid-19: a review towards evoking public awareness

Soumitra Nath*, Aniket Naha and Mehdi Hasan Chowdhury

Gurucharan College, Silchar, Assam, India
*E-mail:nath.soumitra1@gmail.com

Abstract: The current pandemic engendered by the novel SARS-CoV-2 or COVID-19 has invoked worldwide fear and fatality by affecting the upper respiratory tracts of humans. Its originary outbreak was recorded in China during December 2019; however, no specifically effective strategy for neutralizing its virulence is currently existent. Therefore, the creation of public awareness, adoption of safety precautionary measures, and strategic strengthening of immunity are pivotal prophylactic responses. Public awareness, early detection, and diagnosis are vital tools to flatten the curve. Preventive practices include maintenance of personal hygiene, proper exercise, consumption of immunity-boosting foods, and sound sleep. Abiding by the rules and regulations issued for public welfare will aid in bringing a significant downfall in the viral outrage.

Keywords: 2019-nCoV; coronavirus; COVID-19; pandemic; SARS-CoV-2.

These are strange times, when we are healthier than ever but more anxious about our health [1]. The microbial universe and human beings share an intersectional relationship which is frequently defined by conflicts in the form of immunological invasions of the human body. Human history has witnessed several epidemics and pandemics caused by various mutated or novel strains of pathogenic bacteria and virulent viruses. The most destructive history of global pandemics can be traced back from the year 1918-20 (Spanish Flu), 1957-58 (H2N2), 2002-04 (SARS-CoV), 2009 (H1N1), and the most recent being COVID-19 from December 2019. The Coronaviruses (CoVs) belong to the Family of Coronaviridae, constituted by four distinct genera, namely, Alpha Coronavirus (α-CoV), Beta Coronavirus (β-CoV), Gamma Coronavirus (γ-CoV) and Delta Coronavirus (δ-CoV). All of these genera are reported to cause various mild and fatal disease symptoms and predominantly infect the upper respiratory tracts of

humans and various animals along with malfunctioning of the intestine, kidney, liver, and nervous system [2]. The α-CoV and β-CoV originate from mammals, particularly from bats, whereas, γ-CoV and δ-CoV originate from pigs and birds [3]. Among the four genera, α-CoV causes asymptomatic or mildly symptomatic infections, whereas β-CoVs (SARS-CoV-2 or COVID-19) are the most infectious to humans causing severe disease and fatal complications.

In December 2019, an eerie pneumonia of unknown origin was reported in Wuhan, a city in the Hubei Province of China. The infection was observed to be caused by a new coronavirus, named as "2019 novel coronavirus" (2019-nCoV) by the World Health Organization (WHO) on 12th January 2020. Initial studies revealed genetic resemblance with the coronavirus which caused the SARS outbreak in 2002 (SARS-CoV), and therefore, it was renamed as 'Severe Acute Respiratory Syndrome Coronavirus 2' or SARS-CoV-2 by the International Committee on Taxonomy of Viruses. 'COVID-19' is the name given by the WHO on 11th February 2020, for the disease caused by the novel coronavirus SARS-CoV-2. The acronym COVID-19 stands for COronaVIrus Disease of 2019 which has gained the status of a pandemic and wreaked havoc worldwide with paradigm-shifting repercussions. This review paper aims at exploring the epidemiological and pathophysiological conditions, along with the various methods adopted to combat the outrage of the disease and it is imperative for generating public awareness.

SARS-CoV, MERS-CoV and SARS-CoV-2/ COVID-19

SARS-CoV-2 outbreak ranks seventh in the history of corona viral attack on humans. In the past two decades, two large-scale pandemics of Corona viruses have occurred: Severe Acute Respiratory Syndrome (SARS) in 2002 and the Middle East Respiratory Syndrome (MERS) in 2012. The outbreak of SARS originating from Guangdong, Southern China, was reported to be the first viral pandemic to be transmitted by air travel. The characteristic feature of SARS is flu-like symptoms, causing breathing stress in the hosts within 2-10 days after viral exposure. In April 2003, the WHO announced that the pathogen belongs to β-CoV genera, and designated the virus as Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) [4]. Experimental analysis by Hu and his team illustrated high similarity between SARS-CoV strains isolated from Chinese chrysanthemum bats and humans. The study revealed that the Chrysanthemum bat was a natural host and harbors the SARS virus [5]. The Middle East Respiratory Syndrome (MERS), another member of newly discovered β-CoVs (MERS-CoV), was first identified in Saudi Arabia in 2012 and has been referred to as Saudi Arabia's SARS-like virus. In the same year, researchers isolated a virus from the feces of Egyptian tomb bats, which is 100% homologous to human MERS-CoV,

which also caused similar respiratory and pathophysiological infections similar to that of SAS-CoV. The findings concluded that bats might be the true host of MERS-CoV [6].

In the year 2019, SARS-CoV-2 (or COVID-19) finally succeeded in making its transition from animals to humans in the Huanan seafood market in Wuhan, China[7]. However, the exact route of transmission is still under investigation. The average reproduction number (R_0) or transmission rate for SARS-CoV-2 was computed to be 2.2, which implies that one infected patient has the potential of transmitting the infection to two more healthy individuals in the vicinity. The initial clinical indicator of the SARS-CoV-2 was pneumonia and other flu-like symptoms. However, recent reports suggest gastrointestinal symptoms and asymptomatic infections, especially among young children [8].

Configuration of Covid-19: Genes and Proteins

COVID-19 or SARS-CoV-2 is an enveloped RNA virus with positive sensed, non-segmented single-stranded genome spanning around 27-34 kb in size [9,10]. This viral genome was first characterized by Tyrell and Bynoe in 1966, who isolated the viruses from patients with common colds [11]. The virus resembles a solar corona because of the presence of a core-shell with spherical virions and surface projections radiating outwards (Fig. 1). Therefore, they are termed as coronaviruses (Latin: corona = crown). The virus harbors four structural proteins and 16 non-structural proteins (Nsps). The structural proteins include membrane (M), nucleocapsid (N), envelope (E), and spike (S) glycoprotein which are vital for vaccine response and viral sub-typing, while the Nsps play an important role in viral pathogenesis, replication and nine other accessory factors [12].

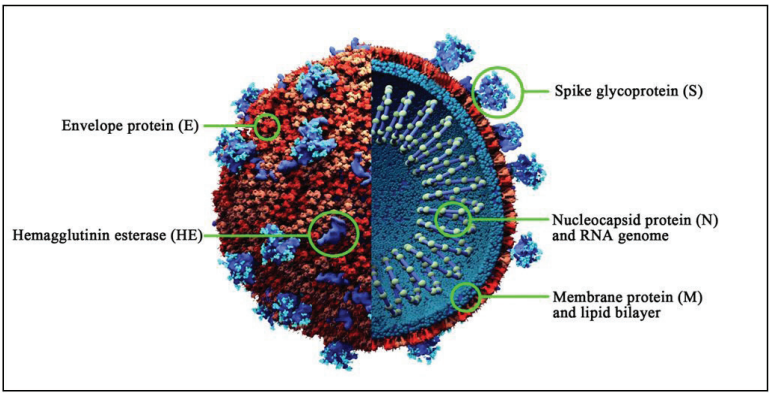

Fig. 1: *Schematic diagram of SARS-COV-2 and its structural proteins*

Single nucleotide polymorphisms (SNPs) in the RNA-dependent-RNA-polymerases facilitate the emergence of two major subtypes commonly designated as L and S. The L subtype was found to be more contagious and displayed more virulence in 70% of the cases reported in Wuhan, China [13]. Further mutations led to the emergence of numerous strains, phenotypes, and clusters among which three clusters were predominantly identified. Clusters A and C were dominant in Europe and America, while cluster B prevailed in East-Asian countries [14]. Phylogenetic analysis revealed that the cluster A had the closest homology to Bat SARS-related coronavirus (Bat-CoV RaTG13). The SARS-CoV-2 genomes display a high sequence similarity of 96.2% with Bat-CoV genomes [15]. Malayan Pangolin (*Manis javanica*) harboring Pangolin-CoV serves as a putative intermediate host for SARS-CoV-2. Pangolin-CoV showed a high percentage of genome similarity of 90.55 % with Bat-CoV RaTG13 and a percentage of 91.02 % with SAS-CoV-2 [12].

Natural or Engineered

Throughout human history, diseases have emerged in one form or another, of which medical and socio-historical discourses have alerted us time and again, and therefore, the immunological invasion by COVID-19 is not surprising. Entailed in the debate on the genesis of COVID-19 are the issues of whether the virus had emerged through natural mutation or laboratory-based genetic engineering. In this regard, a cross-section of views suggests that the outbreak was man-made, while some opine that the virus leaked from a Chinese lab. Others suggest that the virus was engineered to spread among humans as bio-weapons. Regardless of all these claims, the genome sequence data from SARS-CoV-2 reveals that SARS-CoV-2 originated through natural processes [9]. Scientists have analyzed the genetic template for S proteins that protrude from the surface of the virus. The coronavirus uses these spikes to grab the outer walls to invade the host's cells. They specifically looked at the gene sequences responsible for two key features of these S proteins: (i) the receptor-binding domain (RBD) that hooks onto host cells; and (ii) the cleavage site that allows the virus to open and enter those cells. The RBD portion of the SARS-CoV-2 S proteins indicates that SARS-CoV-2 had evolved to effectively target and bind with a high affinity to angiotensin-converting enzyme 2 (ACE2) receptors from humans, ferrets, cats and other species with high receptor homology [16,17]. Andersen *et al.* [9] have proposed two scenarios that can explain the origin of SARS-CoV-2: (i) natural selection in an animal host before zoonotic transfer; and (ii) natural selection in humans following the zoonotic transfer.

Mechanism of Pathogenesis and Immune Responses

The human receptor for SARS-CoV-2 is expressed in the alveolar epithelial cells of type I and II by the ACE2. The viral surface S protein gets activated by transmembrane

protease serine-2, leading to the subsequent disruption of the alveolar cells of the lungs[17].

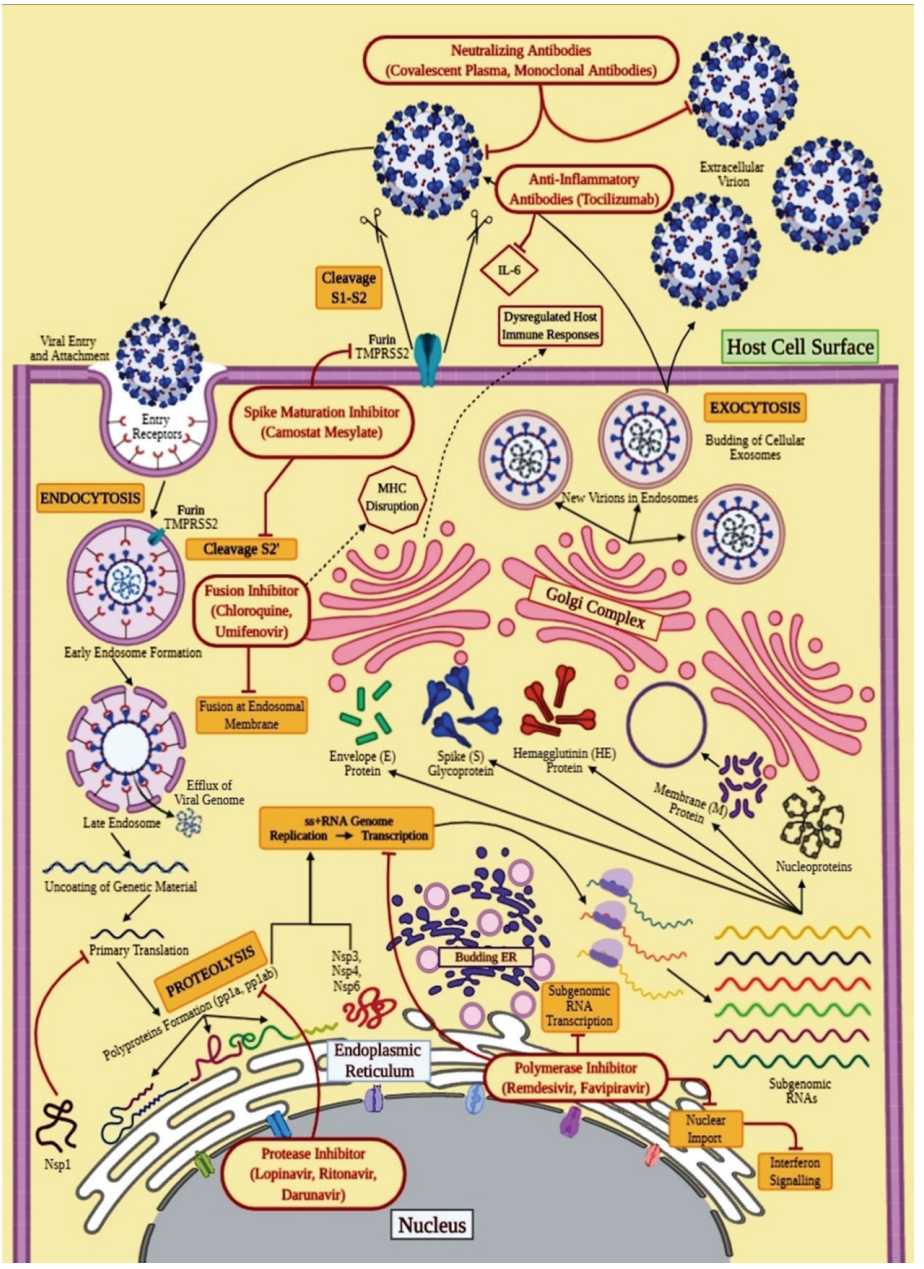

Fig. 2: *Life-cycle and pathogenesis of SARS-CoV-2 along with the inhibitory mechanisms caused by several investigational drugs*

Besides, over expression of ACE2 is seen in vascular endothelium, intestinal epithelium which renders heart failure, kidney dysfunction, hypertension, and atherosclerosis leading to multi-organ failure [16]. The prevalence of higher expression of ACE2 is seen significantly higher in males than in females; and overall, Asians show high expression profiles of ACE2 compared to Americans and Africans. Mortality is mainly caused due to acute respiratory distress syndrome (ARDS) and secondary haemophagocytic lymphohistiocytosis (sHLH) which is characterized by elevated levels of interleukin (IL)-2, IL-7, tumor necrosis factor (TNF)-α, interferon-γ, macrophage inflammatory protein and granulocyte colony-stimulating factor [18]. These factors are extensively diagnosed and examined through several laboratory examinations of bronchoalveolar lavage, nasal secretions, sputum, and blood. Specific genes are studied through molecular approaches like real-time PCR (RT-PCR) or Northern blotting, while viral antigens are traced using immunofluorescent assay (IFA). Serological tests employ ELISA or Western blot analysis to detect COVID-19 proteins [19]. The life-cycle and pathogenesis of SARS-CoV-2 along with the inhibitory mechanisms caused by several investigational drugs are represented in Fig. 2.

Treatment and Recovery for Covid-19

Till date, no effective antiviral treatment or vaccines of any kind have been discovered for treating COVID-19. However, a random multicentre clinical trial is undergoing to assess the effectiveness and safety parameters of patients against COVID-19. Simultaneously, several treatment strategies using antiviral drugs in combination with antibiotics and systemic corticosteroids have been adopted, which mainly curtail the disease symptoms and boost immunity in patients. Popular antiviral drugs such as Remdesivir (nucleoside analog), combinational therapy of Lopinavir/ Ritonavir (anti-HIV) along with Ribavirin (SARS treatment) showed great potential in the treatment strategy for SARS-CoV-2 [20]. Similarly, Chloroquine (antimalarial) and hydroxychloroquine (Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE) treatment) in combination with Azithromycin are administered, which blocks the viral entry by evading the receptor binding mechanism [21]. Antagonists of IL-6, namely Tocilizumab and Sarilumab, showed high success rates in clinical trials which are mainly used in the treatment of rheumatoid arthritis.

Several reports have revealed that attempts are underway to develop monoclonal antibodies and subunit vaccines which can hinder cell invasion and viral replication. Recombinant human ACE2 has shown great potency to reduce lung injury by decreasing levels of IL-6 and angiotensin II [16,22,23]. In India, phase-I trials conducted by Bharat Biotech proclaimed that the inactivated SARS-Cov-2 vaccine (named as Covaxin) is safe for human usage.

Table 1: *Drugs undergoing clinical trials for the treatment of COVID-19*

Sl. No.	Drug	Brand Name	Class	Parent Disease	Route of Administration	Current Status	Country Tested	Mechanism of Action	Refere-nce
1.	Baricitinib	Olumiant	Janus Kinase (JAK) inhibitor	RA	Oral	Phase-III	US	Inhibits JAK1 and JAK2; Reduce inflammatory cytokine storm	[25]
2.	Bemcentinib	BGB324	AXL Kinase Inhibitor	Cancer, Ebola and Zika virus	Oral	Phase-II	UK	AXL leads to immune escape by tumor cells and drug resistance	[26]
3.	Bevacizumab	Avastin	VEGF inhibitor (Monoclonal Antibody)	Acute Lung Injury (ALI), Acute Respiratory Distress Syndrome (ARDS)	Intravenous	--	China	Targets and reduce vascular endothelial growth factor (VEGF)	[27]
4.	Camostat Mesylate	Foipan	Spike maturation inhibitor	Chronic pancreatitis	Oral	Investi-gation	Japan	Inhibitor of TMPRSS2 and related serine proteases	[28]
5.	Chloroquine Phosphate	Avloclor	Anti-malaria drug	Malaria, RA and SLE	Oral	--	China	Increase endosomal pH and interfere glycosylation of receptor	[29]
6.	Darunavir	Daruvir	Protease inhibitor	HIV	Oral	--	China	Inhibits viral replication	[30]
7.	Favipiravir	Faviflu, Favivir	Viral polymerase inhibitor	Ebola and Influenza	Oral	Phase-II	India, US	Inhibits viral replication	[31]

8.	Hydroxy-chloroquine and azithromycin	Zithromax, Plaquenil	Fusion inhibitor	Malaria and Antibacterial	Oral	Phase-I	India, France, China	Activates innate immune system; Inhibits viral replication	[32]
9.	Leronlimab	PRO-140	CCR5 antagonist	HIV	Subcutaneous	Phase-II	New York	Reduction of plasma IL-6; Restoration of CD4: CD8 ratio	[33]
10.	Lopinavir, Ritonavir and Oseltamivir	Kaletra, Tamiflu	Antiviral drug	HIV	Oral	--	Bangkok, Thailand	Antiretroviral drugs, Inhibits cytochrome P450	[20]
11.	Remdesivir	Cipremi, Veklury	Antiviral drug	MERS-CoV, SARS-CoV	Intravenous	Phase-III	India, US, UK, China	Inhibits viral replication	[34]
12.	Tocilizumab	Actemra	IL-6 receptor antagonist	RA	Intravenous	Phase-III	--	Inhibits IL-6 signal transduction pathway	[35]
13.	Umifenovir	Arbidol	Antiviral drug	Influenza	Oral	--	Russia, China	Inhibits viral RNA synthesis and the fusion of the virus to host cell receptor	[36]

Table 2: *Investigational vaccines for the treatment of COVID-19*

Sl. No.	Vaccine	Type	Manufacturer	Laboratory	Country Tested	Phase	Reference
1.	Covaxin™	Inactivated Vaccine	Bharat Biotech	Indian Council of Medical Research (ICMR) The National Institute of Virology (NIV)	India	Phase-III	[24]
2.	Sputnik V	Adenoviral Vector-Based Vaccine	Sistema	Gamalei National Research Centre for Epidemiology and Microbiology	Russia	Phase-III	[37]
3.	mRNA-1273	mRNA vaccine	Moderna, Inc.	National Institute of Allergy and Infectious Diseases (NIAID)	--	Phase-I (18th May 2020) Phase-III (August 2020)	[38]
4.	AZD1222 or ChAdOx1 nCoV-19	--	AstraZeneca	Oxford University's Jenner Institute	Southern England	Phase-I and II (Completed)	[39]
5.	INO-4800	DNA vaccine	Inovio Pharmaceuticals, Inc.	--	--	Phase-I (Completed)	[38]
6.	Ad5-nCoV	Recombinant vaccine	CanSino Biologics Inc.	Beijing Institute of Biotechnology (BIB)	China	Phase-I	[38]
7.	NVX-CoV2373	Adjuvant vaccine (MatrixM™)	Novavax, Inc.	--	Australia	Phase-I / II (Completed)	[40]
8.	CoronaVac	--	Sinovac Biotech Ltd.	--	China	Phase-I and II (China) Phase III (Brazil)	[41]
9.	BNT162 (BNT162b1 and BNT162b2)	mRNA format and target antigen	Pfizer Inc. and BioNTech SE	--	US	Phase-I and II (Completed)	[42]
10.	Ad26.COV2-S	--	Johnson & Johnson	--	--	Phase-I and II	[43]
11.	SCB-2019 (Trimer-Tag©)	Subunit vaccine	Clover Pharmaceuticals	--	--	Phase-I	[44]

The vaccine is developed from a strain of SARS-Cov-2, isolated by the ICMR-National Institute of Virology, Pune. The safety parameters were assessed after two dosages of the vaccine, administered on 375 volunteers from 12 sites in India [24]. Table 1 and 2 accounts the usage of different investigational drugs and vaccines undergoing rigorous clinical trials for the successful treatment for COVID-19 respectively.

Currently, COVID-19, as documented by the WHO, accounts for 21,616,628 confirmed cases with a mortality rate of 768,984 (3.5 %) worldwide. In India, a total of 2,590,501 people have been infected with COVID-19 with the mortality rate of 1.9 % as recorded on 15th August 2020. Out of 21,616,628 confirmed cases, 14,331,736 (66.3 %) patients have completely recovered from the clutches of COVID-19. However, from a total tally of 6,515,908 current active patients, 64,445 (< 1%) patients' condition is considered critically ill [45].

The battle against COVID-19 can be averted with several precautionary measures, proper diet, and regular exercise. Precautionary measures include proper wearing of facial masks, gloves, repeated proper washing of hands — with alcoholic sanitizers, and face with clean water, maintaining safe and adequate distance from infected people as the aerosol transmission is thought as the primary cause for viral contagion. Proper intake of vitamins and proteins, along with consumption of certain medicinal herbs such as Tulsi, Cinnamon, Clove, Ginger, Turmeric, Garlic, Ashwagandha, Brahmi, Giloy, Vasaka, and Nilavembu are considered to have tremendous impacts as immunity boosters as these herbs possess several antiviral, hepatoprotective, cardioprotective, antidiabetic properties which check disease progression in the body [46,47]. Simultaneously, adopting deep breathing exercises, meditation, and yoga along with good sleep, can also enhance blood circulation, which facilitates disease resistance in the human body [48].

Clinical Manifestations

The proportion of individuals infected by SARS-CoV-2 who remain asymptomatic throughout infection has yet not been scientifically well-addressed. However, extensive community transmission has accelerated the asymptomatic cases with people showing mild or no symptoms at all. In symptomatic patients, the clinical manifestations of the disease usually start within a week of viral exposure, consisting of fever, cough, nasal congestion, fatigue, and other signs of upper respiratory tract infections. Loss of smell (anosmia) and taste (ageusia) are also observed in critically ill patients [37]. A comparative clinical manifestation in symptomatic and asymptomatic patients is represented in Figure 3. The infection can progress to severe form with dyspnoea and pneumonia. Prominent signs of viral pneumonia include decreased oxygen saturation, blood gas deviations, changes visible through chest X-rays and other imaging techniques, with

ground glass abnormalities, patchy consolidation, alveolar exudates, and interlobular involvement, eventually indicating deterioration. Lymphopenia appears to be common, and inflammatory markers (C-reactive protein and pro-inflammatory cytokines) are elevated [37]. Older people, and those with underlying medical problems like high blood pressure, heart and lung problems, diabetes, or cancer, are at higher risk of developing a serious illness. Fig. 3 illustrates the various symptoms, diagnoses and several preventive measures to be adopted for symptomatic and asymptomatic cases.

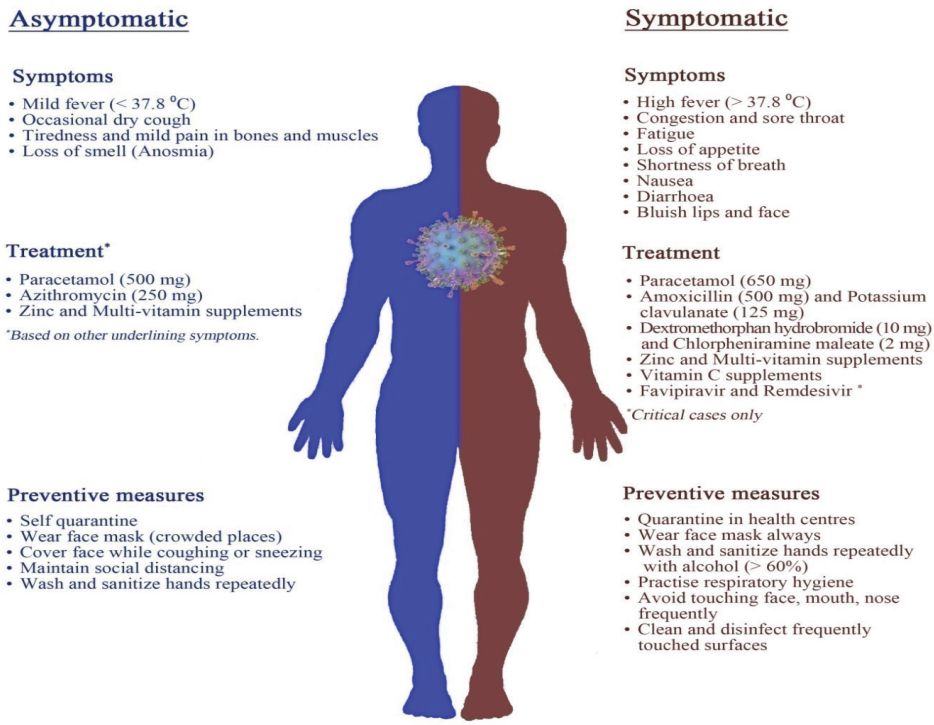

Fig. 3: *Clinical manifestation and treatment options for SARS-CoV-2 symptomatic and asymptomatic patients.*

Policy and Outbreak Control

In order to combat the COVID-19 pandemic, countries across the globe have implemented a range of public health and social measures, including augmentation of healthcare infrastructure, restrictions in mobility, partial or complete closure of schools, businesses and other spaces of public assembly, the establishment of quarantine centers in specific geographic areas and international travel restrictions. In the present scenario, the control strategies are operationalized through a combination of public health measures- such as rapid identification, diagnosis, and management

of the cases, identification and follow up of the contacts, infection prevention and control in healthcare settings, implementation of safe health measures for travelers, awareness-raising among the population and risk communication [45]. The government of India is taking crucial steps to ensure preparedness in facing the challenge and threat posed by the burgeoning pandemic of COVID-19 [49]. National Informatics Centre under the Ministry of Electronics and Information Technology, Govt. of India, has also taken a significant initiative by launching a mobile application called 'Aarogya Setu' which enables health care professionals to track diseased population and provide technological inputs through information about the precautions to be adopted, thereby aiding in the mitigation of contagion [50]. Indeed, the most crucial factor in preventing the local spread of the virus is to enlighten the common citizens with the right information and undertaking strict precautions and surveillance as per the advisories issued by the government or any national advisory body.

Conclusion

The aphoristic epigraph from Roy Porter in the present paper summarises the ironic predicament of human existence amidst pestilential microbes. The immunological invasion by COVID-19 has reiterated the precariousness of human life in the fragile ecosphere with its myriad and competing stakeholders, including infinitesimal yet dreaded microbes and anthropocentrically defined human beings. The present planetary health crisis offers a critical window to visualize and rethink our pandemic preparedness and the vital imperative to invest in and nurture public health infrastructure and research. Notwithstanding the coronavirus's persistency, the contemporaneous burden and dread of the disease will certainly peter out with the spread of public awareness and achievement of scientific breakthrough. Under the present circumstances, early detection, reporting, isolation, diagnosis, and treatment are essential tools to stop or curtail the viral contagion. A decline in the intensity of transmission will encourage countries to gradually re-open workplaces to stimulate economic activity.

Conflict of Interest Statement

The authors declare that there is no conflict of interest.

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New record of coffee locust, Aularches miliaris (Linnaeus, 1758) in Tripura
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CHAPTER

New record of coffee locust, *Aularches miliaris* (Linnaeus, 1758) in Tripura

Dhrubajyoti Paul[1], Rajib Chowhan[2], Navendu Nair[2*], Budhachandra Thangjam[2] and Mithu Rani Debnath[3]

[1]State Agriculture Research Station, Govt. of Tripura, India
[2]College of Agriculture, Tripura, India
[3]Office of the Superintendent of Agriculture, Mohanpur, Govt. of Tripura, India
[*] E-mail:navendunair@gmail.com

Abstract: In the month of June, 2020 the coffee locust, *Aularches miliaris* L. has been recorded for the first time to be appeared in a large number on arecanut and banana plantations in Dhalai district, Tripura. The population was so high that the young plantation of arecanut and banana were severely infested. Observations on behaviour of *A. miliaris* have been taken both in field condition and laboratory. It is evident from the present observations and available literatures that *A. miliaris* is a polyphagous pest and occasionally causes serious damage to many cultivated crops. As this is the new record of this pest species in Tripura on banana and arecanut, regular monitoring of crops is necessary to check further population build up and spread of this pest. Due to sluggishness and congregating nature of this pest mechanical method of controlling the nymphs and adults would be a wise proposition unless use of chemical pesticide is really warranted.

Keywords: Coffee locust; *Aularches miliaris*; Pyrgomorphidae; Banana.

The family Pyrgomorphidae (Orthoptera: Caelifera) contains some of the most colourful grasshoppers in the world, which is why they are also known as gaudy grasshoppers. Currently, there are 487 valid species in 149 genera in this family [1].

Aularches miliaris Linn. (Orthoptera: Pyrgomorphidae) has been called by a variety of names including Coffee locust, Ghost Grasshopper, Northern Spotted Grasshopper, or Foam Grasshopper. It is distributed across many parts of India, Bangladesh, Cambodia, Sri Lanka, the Malay Peninsula, Thailand, Union of Myanmar, Java, Pakistan, Tibet, Nepal and China [2-4]. In India, it has been reported from Assam[5], Chhattisgarh[6], Andhra Pradesh[7], Kashmir[8], Himachal Pradesh[9], Kerala[10-12], Karnataka[13], Odisha[14] and some other states. In the year 2020 outbreaks of *A. miliaris*

have been reported from Kerala, Karnataka, Tamil Nadu, Andhra Pradesh, Meghalaya, Nagaland on many cultivated and wild plants[15].

Green[16] and Hutson[17] claimed that nymphs and adults are highly polyphagous. They remain congregated on the foliages and consume voraciously. Several crops like banana, coffee, coconut, areca nut, teak, *Erythrina lithosperma*, cardamom, cassava, castor, durian, guava, maize, mango, mulberry, oil palm, rice, sugar cane, chillies, cocoa, cotton, custard apple, jute, pigeon pea, rubber, sesame, sorghum, pine, etc. have been reported to be occasionally damaged by this pest [17-19].

Materials and Methods

Surveys were conducted to the infested field in Dhalai district of Tripura in the month of June, 2020. Behaviour of the species in the field itself was studied. Live nymphs were collected and brought to the laboratory of Dept. of Agricultural Entomology, College of Agriculture, Tripura. Three plastic containers were taken and 30 nymphs were kept in each container. Banana leaves were provided to them as food. The open mouth of the containers were covered with plastic net and markin cloth and tied with jute rope. The containers were cleaned every day and fresh leaves were provided. Every day observation was taken on the behaviour of the nymph. After adult emergence the description and behaviour of the adult insects were also noted.

Results and Discussion

In the month of June, 2020 the coffee locust, *Aularches miliaris* L. has been recorded for the first time to be appeared in a large number on arecanut and banana plantations in Dhalai district, Tripura. The population was so high that the young plantation of arecanut and banana were severely infested. However, only nymphs were recorded in the field during the survey and these were brought to the laboratory for further study and identification of species and after adult emergence identity of the pest as *Aularches miliaris* has been confirmed.

This species of grass hopper is a colourful one. Nymphs are blakish with yellow lateral lines. Adult females are slightly larger than the males. Females are 6.2-6.8 cm long whereas the males are 5.3-5.7 cm long. Body shining black in colour with a broad yellow band across the head and sides of the pronotum; pronotum rough and tuberculated, dorsally black with anterior tubercles and lateral lobes yellow; wings large, fore wings long, olive green with many yellow spots of different sizes; hind wing smoky black, abdomen black with reddish orange transverse bands. This is in conformity with Prabakar and Radhakrishnan [12].

Both nymphs and adults remain congregated on the leaves of host plants. They remain off the ground on the foliages of host plants. Banana and young areca nut

plants have been recorded to be severely infested by thousands of nymphs of this pyrgomorphid species. According to Roffey[4] this species is phytophilous, generally remaining off the ground, inhabiting plants from near ground level to the tops of trees.

They possess highly powerful mandibles and feed voraciously on the banana leaves. While feeding a grazing sound is produced. They feed on the banana leaves from the margin inward and even the entire leave including the mid rib of full grown leaves are consumed. Their mandibles are so powerful that they cut open large holes on the plastic net and cloth which were used for covering the open end of raring container in the laboratory. A group of nearly 30, 5^{th} -6^{th} instar nymph has a capacity of consuming a full grown banana leave of a matured plant within one day. According to Ragesh *et al.* [15] in case of banana plants, with its 10 months of growing period, any substantial damage to its stand will surely cause yield reduction. A leaf area reduction of > 50% will invariably lead to reduced bunch weight. Shil *et al.*[20] isolated *Klebsiella* from guts of larvae of *Aularches miliaris*, *Propylea quatuordecimpunctata* and nymphal stage of *Oxya velox*is reported to be a cellulose degrading bacterium. They remain congregated and no cannibalism has been found when sufficient food was given. But in absence of food slight cannibalism has been noticed. The adults can survive several days without food.

They are very sluggish in nature. So they can be easily captured by hands. The adults are also weak flier. However, they possess some defensive mechanism to protect themselves from their natural enemies. When disturbed they jump to short distance from the foliage to the ground, shows clumping behaviour, make a squeaking sound and discharge a slimy frothy material from openings distributed on the thorax. This is in conformity with the findings of Hingston[21], Carpenter[22], McCann[23], Vander Laan[24], Whitman[25], Whitman & Vincent [26]. Thus, inspite of its sluggish nature, this insect is escaped from many vertebrate predators and capable of developing huge population posing threat to cultivated crops. According to Roffey[4] adults are moderate fliers, with long wings, and will swarm and migrate short distances.

It is, thus, evident from the present observations and available literatures that *A. miliaris* is a polyphagous pest and occasionally causes serious damage to many cultivated crops. Though it is not as dangerous as the desert locust, *Schistocerca gregaria* that recently invaded northwestern and central India, causes substantial crop losses sporadically. As this is the new record of this pest species in Tripura on Banana and arecanut, regular monitoring of crops is necessary to check further population development and spread of this pest since a pest causing economic damage to banana is definitely a matter of concern to the farming community of Tripura. Due to sluggishness and congregating nature of this pest mechanical method of controlling the nymphs and adults would be a wise proposition unless use of chemical pesticide

is really warranted. Roffey [4] noted that while the number of species of food plants recorded is large, *Aularches* appears to be only a minor pest of most. This species is listed as lower risk and near-threatened for South India on the International Union for Conservation of Nature and Natural Resources (IUCN) regional conservation status assessment. However, keeping in view the damaging potential of this pest, Ragesh *et al.* [15] opined that periodical assessments should be done based on strong scientific methodologies by a dedicated group of scientists from Agriculture and Conservation science to remove ambiguities about its status, as no subsequent reassessments were done, even after 22 years. Josephrajkumar, *et al.* [27] recommend that in south India *A. miliaris* needs to be conserved at the present time but during localized outbreaks, they recommend that *A. miliaris* be managed via mechanical collection of nymphs and adults, and destruction of egg pods, rather than intervention using insecticides. Ragesh *et al.*, [15] also suggested non chemical methods of management like mechanical, botanical and biological control as eco-friendly strategies against this pest to reduce the economic damage in particular and to help conserve the species in the delicate eco-system in general.

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Impact of edaphic factors in seasonal fluctuation of soil oribatid mite faunal abundance In : *Biological Sciences: Impacts on Modern Civilization, Current and Future Challenges* by *Anupam Guha* © New Delhi Publishers, New Delhi: 2021, 123-128. ISBN: 978-81-947417-9-4, DOI: 10.30954/ndp.bio.2020.11

Impact of edaphic factors in seasonal fluctuation of soil oribatid mite faunal abundance

Sandipan Das[1, 2], Arindom Nath[2], Soumitra Nath[1], Suchismita Das[2] and Shubhadeep Roychoudhury[2]*

[1]Bioinformatics Centre, Gurucharan College, Silchar, Assam, India
[2]Department of Life Science and Bioinformatics, Assam University, Silchar, India
*E-mail: subhadeep1@gmail.com

Abstract: In the present study, an attempt has been made to study the impact of four different edaphic factors (viz. temperature, pH, organic content and soil moisture) on the numerical abundance of orbiatid mite community in the sampling site of Kailashahar, Tripura. The number of mite *Protoribates magnus* show significant relation with all the four factors and are significant at $p<0.05$. But the number of *Scheloribates thermophillus* and *Galumna magnipora* show positive influence with temperature, organic content and soil moisture (at $p<0.05$). On the other hand, *Scheloribates huancayensis* only show significant relation with organic content ($r=0.85$) whereas *Eremobelba hamate* mite failed to demonstrate any such relationship with any one of the edaphic factors.

Keywords: Oribatida, numerical abundance, edaphic factors, soil

The oribatid mites are commonly known as moss mites or beetle mites and explore almost every possible types of habitats. They are considered as a dominant group of mite among the soil-dwelling meso-fauna having higher level of species diversity and species composition [1-3]. Oribatids are key components of decomposer food web, nutrient cycle, energy flow, and play a significant role in enhancing fertility and productivity of soil [4,5]. Besides this, they may use as a natural indicator for various biological and anthropogenic activity [6]. The contribution of oribatids in soil ecology is indeed significant, and their role in the soil also influences human welfare due to our dependency on soil productivity [7]. The diversity and abundance of oribatids greatly influenced by the presence of organic matters due to which their highest abundance is observed in the forest, where deposition of organic matters is highest [8,9]. Oribatids mainly feed on dead organic matter, fungi, nematodes etc., and also contribute in the

degradation of plant bulk materials using enzymes produced by the gut microbes their organically rich excreta also contribute in the soil productivity [1,10] In Tripura, research on oribatids was initiated by the researchers like Subías and Sarkar [11], Sarkar [12,13], Sanyal and Saha [14], Chakrabarti and Bhattacharya [15], Bhattacharya and Chakrabarti [16] and others. Most of the works on oribatids are restricted to the Southern and Western part of the state, but no such extensive work was reported from the Northern part of the state. Recently, Ghosh [17,18] tried to explore mites diversity and abundance, but still, all the areas are not fully covered. In this present investigation, we selected a grassland to analyze the mite abundance and influence of edaphic factors on mite abundance.

Materials and Methods

Study area and sampling plot

The study area lies in the Unakoti district of Tripura, and the sampling sites were located at Kailashahar. The climatic condition of the state is subtropical, warm and humid with moderate rainfall during the monsoon season [17]

Collection of soil sample

Collection of soil was done by selecting a single 4 × 4 m area for sampling and collected in monthly interval for a period of one year (Jan – Dec, 2016). Samples were collected from the surface of the soil and up to a depth of 10 cm with the help of a rectangular steel borer, by following the procedure as described by Dhillon and Gibson [19]. For each sampling site, randomly three cores were drawn from each examining plot and those were blended and treated as a 'sample of soil', so that a comprehensive representation of mite community can be achieve and also to counter the patchy mood of distribution exhibited by some species [17]. Soil samples were loaded in in modified BerleseTullgren funnel in order to collect the mite species [20]. The collected oribatid mites were stored in vials containing a mixture of 90% alcohol and lactic acid (v/v). Further identification was done with the help of expert acarologists of Zoological Survey of India, Kolkata using the identification keys of Balogh [21], Balogh and Balogh [22] and Hammer [23].

Edaphic factors

Soil temperature: Assessment of soil temperature was performed by inserting soil thermometer directly in all sampling plots immediately after collecting the sample from the study site.

Soil pH and moisture: The digital pH meter was used to calculate the soil pH and moisture using Infra-Red Moisture Balance.

Organic content of soil: Rapid titration method of Walkley and Black [24] was followed to calculate the organic matter content of the soils.

Statistical analyses: The obtained data involving four edaphic factors and number of mites extracted from each sample were subjected to statistical analysis. Regression analysis was done in order to establish the relationship between each species of mite and edaphic factors with the help of MS Excel-2010 and $p<0.05$ is considered as statistical significant.

Results and Discussion

The lowest temperature was recorded in the month of January (14.18±0.08), while the highest temperature was observed in the month of July (28.40 ± 0.078). The monthly variation in edaphic factors is represented in table 1. Similarly, highest pH observed in the month of December (5.08±0.02) and lowest pH observed in the month of August (4.12±0.02). The lowest organic content observed in the month of July (3.84±0.08) and highest in the month of February (6.27±0.01). Highest soil moisture observed in July (24.61±0.008) and lowest observed in January (11.4 ± 0.07). An attempt was taken to establish the relation between edaphic factors with individual mite species. Significant influence of the four edaphic factors observed in *Protoribates magnus* numerical abundance and values were significant at $p<0.05$ for all these four factors i.e. temperature ($r=0.87$), pH ($r=0.70$), organic content ($r=0.59$) and soil moisture ($r=0.89$). In case of *Scheloribates thermophillus,* a significant influence is observed by the edaphic factors temperature ($r=0.77$), organic content ($r=0.85$) and moisture ($r=0.65$) and values are significant at $p<0.05$ but not such significance observed for pH. In contrast *Galumna magnipora* species show a strong relation with temperature ($r=0.79$), organic content ($r=0.88$), moisture ($r=0.75$) and values are significant at $p<0.05$, but such relation absent for pH. On the other hand *Scheloribates huancayensis* only show significant relation with organic content ($r=0.85$) whereas *Eremobelba hamate* mite don't show any such kind of relationship with any one of edaphic factors.

A total of 348 adult mites were collected from the study site representing five species. The monthly variation in mite species numerical abundance was represented in Fig. 1. The finding indicates that the edaphic factor such as temperature, pH, organic content and soil moisture may influence the numerical abundance and overall population size of the oribatid mite species. A recent study by Ghosh [17] from North Tripura found that organic content and pH show positive correlation with orbatid mite, while temperature and soil moisture show a negative correlation. Another study reported from South 24-Parganas, West Bengal, by Banerjee and his group found that soil moisture and organic carbon content has a strong positive correlation with oribatids abundance [25].

Table 1: *Monthly variation (year 2016) of edaphic factors (mean ± standard error) in the study site*

Parameters	Month											
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Temperature (°C)	14.18 ± 0.083	20.13± 0.090	22.51± 0.079	24.56± 0.060	27.06± 0.050	26.53± 0.069	28.40± 0.078	26.60± 0.078	23.40 ± 0.070	22.30± 0.078	20.61± 0.064	19.31 ± 0.098
pH	4.93 ± 0.010	4.83 ± 0.008	4.66 ± 0.010	4.26 ± 0.013	4.37 ± 0.007	4.43 ± 0.010	4.32 ± 0.012	4.12 ± 0.025	4.64 ± 0.013	4.53 ± 0.012	4.84 ± 0.010	5.08 ± 0.027
Organic Content (%)	6.00 ± 0.018	6.27 ± 0.010	5.91 ± 0.007	4.66 ± 0.009	4.36 ± 0.012	3.90 ± 0.009	3.84 ± 0.008	3.93 ± 0.011	3.87 ± 0.010	4.22 ± 0.007	4.61 ± 0.008	4.89 ± 0.010
Moisture (%)	12.66 ± 0.010	14.12 ± 0.105	17.58 ± 0.054	20.63 ± 0.038	18.18± 0.083	23.51± 0.079	24.61± 0.008	23.93± 0.010	20.08 ± 0.027	16.27± 0.010	13.36± 0.012	11.40 ± 0.070

However, the present study reports that the individual mite has their specific response toward the four selected edaphic factors, which indicates that temperature, pH, organic content and moisture influences oribatids abundance individually.

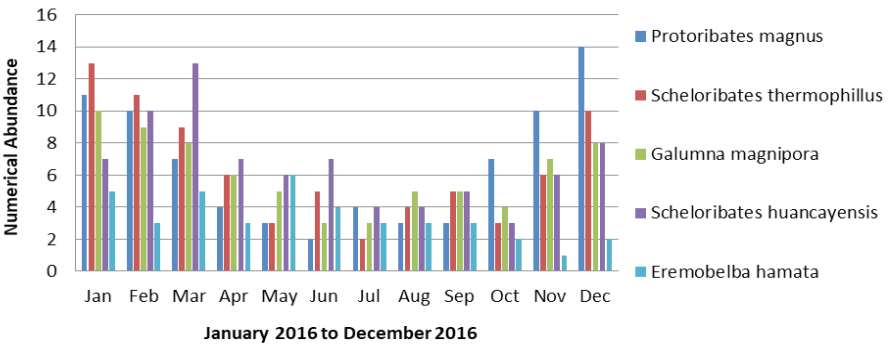

Fig. 1: *Monthly variation of Oribatida numerical abundance.*

Conclusion

The present study revealed that edaphic factors such as temperature, pH, organic content and moisture has significant positive influence in most of the mite species numerical abundance extracted from the study site. However, it is very difficult to conclude the exact reason behind the difference in individual mite species response towards the edaphic factors as we select a small area as our study site. However, to explore the influence of edaphic factors in mite numerical abundance, a large scale study is needed.

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CHAPTER

Bioactive potentials of some foliar endophytic fungi isolated from *Artocarpus heterophyllus* Lam and Citrus reticulata blanco of Tripura

Sanchita Bhattacharya*[1], Sanjit Debnath[2] and Ajay Krishna Saha[2]

[1]Department of Botany, M.B.B. College, Agartala, Tripura, India.
[2]Mycology and Plant Pathology Laboratory, Department of Botany, Tripura University, Suryamaninagar, Tripura, India
*Email: sanchita270873@rediffmail.com

Abstract: Present study deals with the evaluation of bioactivities (antibacterial and antioxidant activitities) of some foliar endophytic fungi isolated from *Artocarpus heterophyllus* and *Citrus reticulata*. Six fungal strains (*Aspergillus fumigatus*, *Colletotrichum gleosporioides*, *Nodulisporium gregarium*, *Penicillium citrinum*, *Pestalotiopsis versicolor* and *Xylaria feejeensis*) were sampled by random sampling method. Fungal metabolites of the mycelial mat and the culture broth were extracted with ethyl acetate and methanol. The antimicrobial activity of extracts from mycelial mat and culture broth were determined by paper disc diffusion method against *Staphylococcus aureus* (MTCC-96) and *Escherichia coli* (MTCC-40). Highest antibacterial activity in terms of inhibition zone was obtained in case of ethyl acetate exrtact treated set of mycelial mat of *Xylaria feejeensis* against *Staphylococcus aureus*. Antioxidant potential was evaluated by DPPH radical scavenging assay. Lowest EC_{50} value was recorded for ethyl acetate extract of mycelial mat of *Penicillium citrinum*. Extraction solvent and culture component part dependent differences were observed in antimicrobial and antioxidant potential of extracts. The different solvent extracts of endophytic fungi on both the pathogenic bacterial strains (*S. aureus* and *E. coli*) significantly differed ($p < 0.05$) among endophytic fungi of various host plant.

Keywords: Endophytic fungi; Antibacterial activity; Antioxidant assay.

Fungal endophytes reside for at least part of their life cycle, inside the tissue of plants without causing visible damage to the host plants [1]. They used to colonize inter and/or intracellular spaces in healthy plant tissues of stem, petiole, roots and leaves [2]. It was reported that variation in chemical profiles of the host plant might affect the differential distribution of endophyte assemblages in different hosts [3]. As bioresource,

endophytic fungi can be considered as an important reservoir to exploit novel bioactive metabolites [4]. Endophytic fungi can produce bioactive secondary metabolites with activities identical or almost same of their respective hosts[5]. Microorganisms associated with host plants rather than the plants produce bioactive metabolites with high therapeutic potential [6]. They are capable of producing novel anti-microbial, anti-cancerous, antioxidant and immunomodulatory metabolites which may be considered as an alternative against increasing levels of drug resistance to human pathogens [7]. Antibiotics from endophytic microbes have the potentiality to inhibit a variety of pathogens as well as they are also a prospective source of novel natural antioxidants[8]. Endophytes were screened for antibacterial and antioxidant compounds[9]. One of the fundamental difficulties in drug discovery from endophytic fungi is the development of efficient strategies to recover bioactive strains. Therefore multiple approaches using different explants tissues, growth conditions and various substrate types might increase bioactivity and secondary metabolite production [10]. *Artocarpus heterophyllus* considered to be a rich source of antioxidants and antibacterial agents[11]. *Citrus reticulate* also reported possessing pharmacological and medicinal activities[12]. Therefore, evaluation of antimicrobial and antioxidant activities of sampled fungal endophytes from the two host plants was carried out to identify prospective fungal strains with bioactive potential.

Materials and Methods

Collection of explants

Green leaves from healthy and disease free host plants of Jackfruit (*Artocarpus heterophyllus* Lam.) and Orange (*Citrus reticulata* Blanco) were selected to minimize the presence of pathogenic and saprobic species. Explants were collected from the sampling sites of different districts of Tripura in sterile bags and processed within 24 hours of collection. Collection of samples was carried out from December 2016 to July 2018.

Isolation of Endophytes

Isolation of fungal endophytes was done according to the standard protocol[13] with slight modification. Sterilization protocol was validated by the standard method[13]. Four to five segments of plant tissues were placed on potato dextrose agar (PDA) and/or Malt Extract Agar (MEA) plate supplemented with streptomycin (100 µg/ml), and incubated in a BOD incubator for 21 days at 25± 2°C. Hyphal tips from fungus growing out from the samples were subsequently transferred onto fresh PDA and or MEA plates to isolate pure colony.

Identification of fungi

Identification was done using macroscopic characteristics of colonies and microscopic characteristics. The standard manuals and literatures were used for the identification of fungi [14, 15].

Cultivation and extraction of fungal metabolites

Selected endophytic fungal isolates were further inoculated into Erlenmeyer flasks containing 100 ml Potato Dextrose Broth and incubated at 21ºC for 21 days under stationary conditions. The broth culture was filtered to separate the mycelia and filtrate. Separated mycelia were dried at 40°C. Dried mycelium was pulverized and extracted with ethyl acetate and methanol separately. The extract was concentrated in Rotary evaporator (Rotavap: PBV-7D) to yield the final extract. Extracts were stored in dark at 4 °C before being used for the antimicrobial assay and for *in vitro* DPPH radical scavenging assay.

Evaluation of Antibacterial activity

The antimicrobial activity of mycelial extracts and culture broth were determined by paper disc diffusion method[16] against *Staphylococcus aureus* (MTCC-96), *Escherichia coli* (MTCC-40). In each petriplates four discs of sterile filter paper (6 mm) were placed and inoculated with 20 µl of extracts. Streptomycin was used as a positive control. The petriplates were incubated at 37°C in B.O.D for 24 h. The antibacterial activity was evaluated by the formation of inhibition zones.

Antioxidant assay: DPPH (1, 1-diphenyl-2-picryl-hydrazyl) radical scavenging activity

Free radical scavenging (FRS) activities of fungal extracts were measured by the slightly modified method of Miliauskas *et al.* [17]. Methanolic stock solutions of extracts were prepared and various concentrations of extracts were obtained by serial dilution. Ascorbic acid was used as a control. EC_{50} value (mg/ml) is the effective concentration at which DPPH radicals were scavenged by 50% and the value was obtained by interpolation from a linear regression analysis.

Statistical analysis

All the assays were performed in triplicate and the results were expressed as Mean ± SE values. The mean values and standard error were calculated using Origin version 7.0. Two-way analysis of variance (ANOVA) was conducted to test the significance among the different solvent extract with endophytic fungal strains isolated from host plants.

Results and Discussion

Among all experimental sets, maximum inhibition zone was obtained in case of ethyl acetate extract treated set of mycelial mat of *Xylaria feejeensis* against *Staphylococcus aureus* (Table 1). But against *Escherischia coli*, maximum inhibition was observed also for ethyl acetate extract treated a set of mycelial mat of *X. feejeensis* (Table 2). Considering only methanolic extract treated sets, mycelial mat extract of *X. feejeensis* against *S. aureus* (Table 1). Among extracts obtained from culture filtrate, maximum inhibition zone was recorded for ethyl acetate treated *Penicillium citrinum* extract against *S. aureus* (Table 1). However, some of the methanolic extracts of the mycelial mat (*Nodulisporium gregarium* and *Penicillium citrinum* against *E. coli*) and culture filtrates (*Nodulisporium gregarium* and *Penicillium citrinum* against *S. aureus*; *Aspergillus fumigatus* and *Colletotrichum gleosporioides* against *E. coli*) were not effective against tested bacterial strains (Table 1 and 2). It was reported that Gram-positive bacterial strains were more sensitive to crude fungal extracts than Gram-negative bacteria which supported the present experimental results [18].

Table 1: *Antimicrobial activity of foliar endophytic fungi against Staphylococcus aureus*

Sl. no.	Name of the fungi /control	Host plant	Inhibition zone (mm)			
			Staphylococcus aureus			
			Mycelial Mat		Culture filtrate	
			Ethyl Acetate	Methanol	Ethyl Acetate	Methanol
1	*Aspergillus fumigatus*	*Artocarpus heterophyllus*	12.78± 1.00	10.667±0.473	11.82±0.41	8.2±0.41
2	*Pestalotiopsis versicolor*	*Artocarpus heterophyllus*	16.08 ± 1.00	13.85±0.67	11.50 ± 0.94	10±0.471
3	*Colletotrichum gleosporioides*	*Citrus reticulata*	13±0.471	11.66±0.471	10.333±0.943	9.66.± 0.473
4	*Nodulisporium gregarium*	*Citrus reticulata*	12.66±0.471	10±0.473	11.33±0.471	0
5	*Penicillium citrinum*	*Artocarpus heterophyllus* and *Citrus reticulata*	14.8 ±0.67	12.33 ±0.41	12.66.± 0.943	0
6	*Xylaria feejeensis*	*Artocarpus heterophyllus* and *Citrus reticulata*	17.80 ± 0.471	15.33.± 0.943	12.05 ± 0.473	8.333±0.471
9	Antibiotic (Streptomycin)	-	22±0.471 .			

Table 2: *Antimicrobial activity of foliar endophytic fungi against Escherischia coli*

Sl. no.	Name of the fungi/control	Host plant	Inhibition zone (mm)			
			Escherischia coli			
			Mycelial Mat		Culture filtrate	
			Ethyl Acetate	Methanol	Ethyl Acetate	Methanol
1	*Aspergillus fumigatus*	*Artocarpus heterophyllus*	11.667±0.471	10.66±0.41	10±0.943	0
2	*Pestalotiopsis versicolor*	*Artocarpus heterophyllus*	14.00 ± 0.61	11.33±0.471	8.333±0.473	7.66±0.943
3	*Colletotrichum gleosporioides*	*Citrus reticulata*	11.667±0.471	9.667±0.473	10.2±.0.47	0
4	*Nodulisporium gregarium*	*Citrus reticulata*	12.05 ± 1.00	0	9.667±0.473	8.66±0.943
5	*Penicillium citrinum*	*Artocarpus heterophyllus* and *Citrus reticulata*	11.50 ±0 .41	0	10±0.473	7.667±0.471
6	*Xylaria feejeensis*	*Artocarpus heterophyllus* and *Citrus reticulata*	15.5 ± 0. 43	12.25±0 .41	11.33 ± 0.52	10.66 ± 0.52
7	Antibiotic (Streptomycin)		19.33±0.943 .			

To find out the significant effect of different solvent extracts of endophytic fungi on pathogenic bacterial strains (*S. aureus* and *E. coli*) and endophytic fungi of the various host plant (Table 1 and 2) were analyzed by ANOVA Two Way. The different solvent extracts of endophytic fungi on both the pathogenic bacterial strains (*S. aureus* and *E. coli*) significantly differed ($p < 0.05$) among endophytic fungi of various host plant. The DPPH, a stable free radical was used to study the radical scavenging effects of the extract. The scavenging effects of the sample were evaluated along with the standard Ascorbic acid. Synthetic antioxidant ascorbic acid, which was used as a standard, had superior EC_{50} (0.072 mg/ml) value in comparison to all the six tested samples. Lowest EC_{50} value signifies better antioxiidant potential of extracts.

Among all experimental sets, the lowest EC_{50} value was recorded for ethyl acetate extract of the mycelial mat of *Penicillium citrinum*. For methanolic extracts of selected fungal strains lowest EC_{50} value was recorded for the methanolic extract of mycelial mat obtained from *Xylaria feejeensis*. Among extracts obtained from culture filtrates lowest EC_{50} value was also observed for ethyl acetate extract of *Penicillium citrinum* (Table 3).

Table 3: *EC_{50} (mg/ml) values of tested endophytic fungal strain for antioxidant activity (DPPH Assay)*

Sl. no.	Name of the fungi/control	Mycelial Mat		Culture filtrate	
		Ethyl Acetate	Methanol	Ethyl Acetate	Methanol
1	*Aspergillus fumigatus*	583.57	672.84	684.17	1012.45
2	*Pestalotiopsis versicolor*	263.33	437.44	583.33	792.34
3	*Colletotrichum gleosporioides*	334.10	487.13	379.08	963.10
4	*Nodulisporium gregarium*	.215	562.12	377.69	852.67
5	*Penicillium citrinum*	162.51	593.02	276.12	683.14
6	*Xylaria feejeensis*	197.25	387.52	457.20	803.55
7	Ascorbic acid (Control)	0.072			

Ethyl acetate was reported as more effective solvent among different extraction solvent to elute the components with antioxidant potential. At the same time, the solubility of bioactive components usually differed depending on the extraction solvent used [19]. This statement corresponded with the present findings.

It was reported that *Aspergillus fumigatus* had antibacterial potential against both gram-positive and gram-negative bacteria [20]. Present experimental results correlated with the above finding as extracts of mycelial mat and culture filtrate (except methanolic extract of culture filtrate against *E. coli*) showed antibacterial activity. Bioactive constituents like alkaloids, carbohydrates, sterols and coumarins were reported to be present in ethyl acetate extract of *Colletotrichum gloeosporioides* [21]. The ethyl acetate extract of fermentation broth of *C. gloeosporioides* exhibited antimicrobial activity against gram-positive and gram-negative bacteria [22]. Above findings correlated with present findings where extracts of *C. gleosporioides*. showed antibacterial and antioxidant activity. Importance of different media and physicochemical parameters in the production of secondary metabolites with antioxidant properties of *Xylaria feejeensis* was evaluated [23]. Endophytic *Xylaria* sp. isolated from the medicinal plant *Ginkgo biloba,* reported to have antioxidant compounds [24]. In the present experiment, extracts obtained using two extraction solvents from *X. feejeensis* also exhibited differential antioxidant activity against DPPH. An earlier report stated that ethyl acetate extract of *Penicillium citrinum* showed significant antioxidant activity in DPPH assay[25] which corresponded with present experimental findings. Moreover, isolation of bioactive metabolites from ethyl acetate extracts of culture broth and mycelia of *P. citrinum* was reported [26]. Citriquinone A was reported from the methanolic extract of *P. citrinum* which exhibited antibacterial activity against *Bacillus* sp [27]. These earlier reports supported the present results where antioxidant and antimicrobial potential were observed in case of extracts of mycelia mat and culture filtrate of *P. citrinum*. Compounds like pestacin and isopestacin were obtained from the endophytic fungus

Pestalotiopsis microspora possess antioxidant activity [28]. The antioxidant activity against DPPH radical exhibited by crude fungal extracts obtained from mycelial mat and culture filtrate of *Pestalotiopsis versicolor* might be correlated with the above findings. In a previous report it was stated that *Nodulisporium* sp. isolated from *Acanthus ilicifolius var. xiamenensis* showed antibacterial activity against *S. aureus*[29]. The statement corresponded with present findings.

According to Kusari and Spiteller [30], production of bioactive compounds by endophytes, similar to their host plants, is important from the molecular, biochemical as well as ecological perspective. The production of such secondary metabolites has its application as alternative and sustainable sources of bioactive compounds. Commercial production of these desirable compounds also has great future perspective. Comprehensive understanding of the host-endophyte relationship at the molecular and genetic levels is essential to stimulate and optimize secondary metabolite production under laboratory conditions using endophytes to produce such metabolites continuously. Thus present study has its relevance in identifying fungal endophytes with bioactive potential from the sampled host plants for isolation and structural elucidations of bioactive compounds along with the development of the suitable strategy of commercial production in near future.

Conclusion

Isolation and screening of endophytic fungi from different host plants and habitat may increase the possibility of identifying fungal strains with the ability to synthesize novel bioactive metabolites. They can also act as biosystem elicitors for their respective host plants. Endophytic fungi also regarded as an important source for pharmacologically active substances with low toxicity level and negligible environmental impact. The fungal endophytes have immense potential as sources of antimicrobials and thus may be useful for the development of new drugs. But the development of suitable cultivation and extraction protocols are required for their marketable exploitation. To obtain the pharmaceutically applicable effective bioproducts such as antimicrobial and antioxidant compounds from fungal endophytes, a comprehensive approach is required.

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CHAPTER

Concomitant administration of selenium and vitamin B12 ameliorates arsenic-induced oxidative stress in male wistar rats

Sudipta Pal*[1] and Ajay Kumar Chatterjee[2]

[1]Department of Human Physiology, Tripura University, Suryamaninagar, West Tripura, India
[2]Ex-Professor and H.O.D, Department of Human Physiology, University of Calcutta, Kolkata, India
*E-mail: sudiptapal@tripurauniv.in, sudiptap12@yahoo.co.in

Abstract: Arsenic toxicity is a serious environmental issue globally. Both chronic and acute exposure cause adverse health effects affecting almost all organ systems. It is a challenge to combat against arsenic toxicity to keep healthy life. Various natural and synthetic compounds have been tried to ameliorate arsenic induced organ toxicity. In the present study protective effect of selenium and vitamin B_{12} co-administration was assessed against arsenic-induced oxidative stress in liver tissue of male Wistar rats. Intraperitoneal administration of sodium arsenite at a dose of 5.55 mg/kg body weight/day (equivalent to 35% of LD_{50}) produced depletion of reduced glutathione (GSH) content of liver, associated with enhanced lipid peroxidation (LPO) level and free hydroxyl radical (·OH) formation. Activities of antioxidant enzymes like glutathione reductase (GR), superoxide dismutase (SOD), catalase were inhibited after arsenic exposure, indicating disturbed pro-oxidant-antioxidant equilibrium in rat liver tissue. Liver NADPH oxidase activity increased significantly following arsenic treatment, and thus enhances superoxide radical production. The same treatment of arsenic also cause liver injury as reflected by the elevated activities of serum γ-glutamyl transpeptidase (γ-GT), glutamate-oxaloacetate transaminase (SGOT), and reduced serum glutamate-pyruvate transaminase (SGPT) activity. Concomitant administration of selenium and vitamin B_{12} with arsenic appreciably restored almost all of these parameters to their control levels. Combination of selenium with vitamin B_{12} restored liver NADPH oxidase and serum GPT activities to their respective control values. In addition, they exhibited better efficacy to restore liver LPO level, SOD and catalase activities, serum γ-GT activity and carbonylated protein content. These results suggest that co-administration of selenium and vitamin B_{12} is capable of reducing arsenic-induced oxidative and degenerative changes in rat liver.

Keywords: Arsenic, oxidative stress, free radical, selenium, vitamin B_{12}, antioxidant

Arsenic, a toxic environmental pollutant, produces adverse effects on human health. Various types of acute symptoms like immediate gastrointestinal symptoms, subacute sequela resulting in polyneuropathy [1-2] and chronic symptoms like degenerative, inflammatory and neoplastic changes of skin, cardiovascular, nervous, endocrine and reproductive systems are the manifestations of arsenic toxicity [3-4]. Several reports have indicated that inorganic arsenicals are capable of generating reactive oxygen species (ROS), especially superoxide and hydrogen peroxide in both cultured cells and experimental animals [5,6]. Arsenic can also alter the tissue metabolic activities through generation of ROS which causes oxidative damage to cellular components including DNA, tissue proteins, fatty acids etc. Arsenic attacks the thiol groups of the tissue proteins and causes oxidative damage leading to various metabolic dysfunctions within the cell [7]. Arsenic-induced lipid peroxidation through generation of ROS may lead to production of various lipid peroxides like conjugated diens, alkenes and aldehyde products [8]. Among several mechanisms, oxidative stress due to production of ROS is thought to be an important causative factor in arsenic-induced toxicity [9].

Some antioxidants like Vitamin E, ascorbic acid, N-acetylcysteine, S-adenosyl methionine and melatonin have some protective role in amelioration of arsenic-induced certain metabolic toxicities [10-12]. It was observed by Chen *et al* [13] that the risk of premalignant skin lesions was consistently greater among participants with blood selenium lower than the average level. The observations establish the hypothesis that selenium intake through diet may minimize the risk of premalignant skin lesions among arsenic exposed population. This may be due to some interaction of selenium and arsenic metabolites. Synergistic effect of folic acid and vitamin B_{12} was found in ameliorating arsenic-induced oxidative damage in pancreatic tissue of rat [14]. Additionally, vitamin B_{12} in combination with folic acid was found to have significant protective effect against arsenic-induced cardio-toxicity [15].

So, the present study is intended to evaluate whether arsenic-induced oxidative stress and generation of free radicals can be scavenged by administration of selenium or vitamin B_{12} alone or in combination to make evaluation of potency of such compound as protective agents against arsenic toxicity.

Materials and Methods

Materials

Sodium arsenite ($NaAsO_2$), S-adenosyl methionine, 5,5'DTNB (Ellman Reagent), NADPH, Na_4, γ-glutamyl p-nitroanilide, Amediol buffer, Fast blue BB salt were purchased from Sigma Chemicals (USA), Dimethylsulfoxide, EDTA, Sodium selenite, Vitamin B_{12} were procured from SRL, India. Other chemicals used in the experiments were of analytical grade.

Animal selection

Twenty four male rats of Wistar strain weighing 130-150 g were chosen for the present experiment. They were housed in laboratory condition for 10 days for adaptation on a 12 hour photoperiod in the animal house facility at 25±1˚C and 50-80% relative humidity. The 18% protein diet was used as it is considered as an adequate (normal) dietary protein level, which was used on earlier occasions [11, 16-17].

Animal treatment

Thirty rats were divided into five groups of equal average body weight and equal number (N=6). Animals were procured from the Chakraborty Enterprise, Kolkata (India), an authorised animal supplier nominated by Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Govt. of India and animal experimentation was performed as per the guideline of CPCSEA. The animals of one of the groups were treated i.p. with sodium arsenite at a dose of 5.55 mg/kg body weight (equivalent to 35% of LD_{50} value) per day for a period of 30 days [12, 18-19] and marked as arsenic-treated group. The animals (N=6) of the control group received the vehicle (0.9% NaCl, i.p.) only.

Selenium supplementation

For evaluating the effects of selenium, some of the animals (N=6) were administered arsenic at the same dose and duration as mentioned before and then selenium was administered at a dose of 6µg of selenium/kg b.w./day orally for the last fourteen days prior to sacrifice and marked as arsenic-treated plus selenium-supplemented group.

Vitamin B_{12} supplementation

To study the effect of vitamin B_{12}, one of the arsenic treated group of animals (N=6) was orally fed with vitamin B_{12} at a dose of 3 µg/kg b.w./day for the last fourteen days of arsenic treatment. At the same time another group of arsenic-treated rats were administered with selenium and vitamin B_{12} conjointly at the aforesaid doses and duration to see whether their combined exposure may have any better response than their single exposure.

Collection of serum and tissues

After the period of treatment was over the animals of each group were kept fasted overnight. The animals were then sacrificed by cervical dislocation following diethyl ether anaesthesia according to the procedure of animal ethics. Blood was collected from hepatic vein and allowed to clot to separate serum. Serum was collected in a micro centrifuge tube and kept at -20^{o}C until analysis. Liver tissue was removed, washed in ice-cold saline, blotted dry and stored at -20^{o}C until analysis.

Preparation of tissue homogenate

A 10% tissue homogenate of liver tissue was prepared in 0.1 M potassium phosphate buffer (pH 7.4) using all glass homogenizer and kept at -20°C until biochemical analysis was performed.

Biochemical analysis

Reduced glutathione (GSH) content of liver tissue

For the determination of liver GSH content [20], the tissue homogenate was treated with 20% TCA containing 1 mM EDTA to allow precipitation of proteins. The centrifugate was then treated with Ellman's reagent and used for the final reaction. The reading was taken in a spectrophotometer at 412 nm. Tissue glutathione level was calculated from the standard curve generated using aliquots of solution having known concentration of GSH.

Liver lipid peroxidation (LPO) level

LPO level in the homogenates of liver was measured as described by Buege and Aust [21] with slight modification. The optimal density was read at 533 nm. The molar extinction co-efficient, of malonaldehyde was used to calculate the malonaldehyde production.

Liver free hydroxyl radical (·OH) production

For free hydroxyl radical estimation, the animals of each group were treated with 30% dimethylsulfoxide at a dose of 0.4 ml per 100g boy weight 2 hours before sacrifice. A 10% tissue homogenate (in distilled water) of liver tissue was prepared and used to determine the free hydroxyl radical formation according to the method of Babbs and Steiner [22]. Fast blue BB salt was used for production of yellow coloured methane sulfinic acid which was read at 425 nm in spectrophotometer.

Glutathione reductase (GR) activity of liver tissue

The enzyme activity was quantitated at 25°C by measuring the disappearance of NADPH at 340 nm in an UV-vis spectrophotometer by the method of Carlberg and Mannervik [23]. The GR activity was calculated in terms of nmoles of NADPH oxidized/ minute/ mg of protein.

Catalase activity of liver tissue

The catalase activity was measured by calculating the rate of degradation of H_2O_2, the substrate of the enzyme [24]. The activity was represented as mmole H_2O_2 used/ minute/ mg of protein.

Superoxide dismutase (SOD) activity of liver tissue

The SOD activity of liver was assayed by the method of Martin *et al.* [25] on the basis of enzyme-mediated decrease in the rate of autooxidation of hematoxylin in aqueous alkaline solution, which yields a chromophore with maximum absorbance at 560 nm. The enzyme activity was expressed as units/ minute/ mg of protein.

NADPH oxidase activity of liver tissue

The 10,000 g supernatant of 10% tissue homogenate (in 0.25 M sucrose, pH 7.4) was employed for estimation of the enzyme activity by the method as employed by Chen *et al.* [26]. The decrease in optical density was noted at 340 nm and the enzyme activity was expressed as nmoles of NADPH oxidized per minute per mg of protein.

Glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase activities of serum

The transaminase activities of serum were measured by the method of Reitman and Frankel [27]. The activities of the enzymes were expressed as mg of pyruvate formed/ minute/ ml serum.

Serum γ-glutamyl transpeptidase (γ-GT) activity

Serum γ-GT activity was estimated as per the protocol of Szasz [28] at 405 nm in a spectrophotometer and expressed in terms of units/ml serum.

Tissue protein content

Proteins were estimated by the method of Lowry *et al.* [29] using bovine serum albumin as the standard protein.

Statistical analysis

The values were expressed as Means ± SEM. The data were statistically analysed using single way ANOVA followed by 'multiple comparison t test' to analyse the data of four different groups. The $p < 0.05$ was considered statistically significant [30].

Results

There was 44.93% decrease ($p^a<0.001$) in liver GSH content following arsenic treatment, which was restored to 75% of the control value after selenium supplementation (Table 1). Exposure of selenium in combination with vitamin B_{12} exhibited 84.6% restoration of depleted GSH content of the control value. Change in LPO level in liver shows that arsenic treatment caused 32.8% increase ($p^a<0.001$) in liver LPO level than the control value. Selenium supplementation alone was found to partially check the increased liver LPO level ($p^b<0.001$) due to arsenic treatment. The counteraction was found to be 8.8% ($p^b>0.05$). Combined treatment of selenium and vitamin B_{12} has much pronounced effect in restoration of liver LPO to the control value. The restoration

was found to be more than 100% of the control. Table 1 further demonstrates that the increased production of free ·OH radical in liver ($p^a<0.001$) in arsenic-exposed animals was completely counteracted by selenium and vitamin B_{12} co-supplementation.

Table 1: *Effects of selenium and combination of selenium and vitamin B_{12} on arsenic-induced changes in tissue glutathione (GSH) contents, LPO levels and free hydroxyl radical productions in liver tissue*

Groups of animals	Liver GSH content (nmoles/100 mg tissue)	Liver LPO level (nmoles of MDA produced/g tissue)	Free ·OH radical formation (µmoles/g tissue)
Pair-fed control (6)	110.45±6.69	56.63±1.92	10.78±0.38
As-treated (6)	60.82±1.77 $p^a<0.001$	75.2±2.68 $p^a<0.001$	19.79±0.62 $p^a<0.001$
As-treated + Selenium-supplemented (6)	82.95±2.03 $p^a<0.05$ $p^b<0.001$	68.58±1.38 $p^a<0.05$ $p^b<0.001$	15.41±0.46 $p^a<0.05$ $p^b<0.05$
As-treated+ Selenium+ Vitamin B_{12}-supplemented (6)	93.47±1.26 $p^b<0.001$ $p^c<0.05$	46.12±2.89 $p^b<0.01$ $p^c<0.001$	11.14±0.2 $p^b<0.001$ $p^c<0.05$

Values are Means±S.E.M. Figures in the parentheses indicate the number of animals.
p^a compared with pair-fed control group.
p^b compared with As-treated group.
p^c compared with As-treated+selenium-supplemented group.

Table 2 shows that arsenic-induced decreased glutathione activity in liver was partially counteracted by selenium alone. The restoration was found to be more than 83.2% of the control value. Hepatic superoxide dismutase activity decreased significantly ($p^a<0.001$) in arsenic-treated group (Table 2). Selenium supplementation appreciably restored the decreased superoxide dismutase activity in liver following arsenic treatment. The restoration was 69.1% of the control value. Change in catalase activity (Table 2) shows that arsenic treatment decreased the catalase activity in liver by 28.54%. Selenium supplementation alone restored the enzyme activity appreciably (88.4% of the control value). Selenium and vitamin B_{12} when administered conjointly exhibited better potentiality in restoration of those enzyme activities as represented in Table 2. The restoration was found to be 97.3% for GR activity and more than 100% for superoxide dismutase and catalase activities.

Table 2: *Effects of selenium and combination of selenium and vitamin B_{12} on arsenic-induced changes in glutathione reductase, SOD and catalase activities in liver tissue*

Groups of animals	GR activity (nmoles of NADPH oxidized/min/mg protein)	SOD activity (units/min/mg protein)	Catalase activity (µmoles of H_2O_2 utilised/min/mg protein)
Pair-fed control (6)	56.83±2.04	3.14±0.09	22.7±0.72
As-treated (6)	39.3±1.35 $p^a<0.001$	1.75±0.07 $p^a<0.001$	16.22±0.87 $p^a<0.001$
As-treated + selenium-supplemented (6)	47.27±1.14 $p^a<0.01$ $p^b<0.01$	2.17±0.1 $p^a<0.05$ $p^b<0.001$	20.08±0.75 $p^a>0.05$ $p^b<0.01$
As-treated+ Selenium+ Vitamin B_{12}-supplemented (6)	55.3±2.39 $p^b<0.001$ $p^c<0.05$	3.58±0.33 $p^b<0.001$ $p^c<0.05$	25.27±0.51 $p^b<0.001$ $p^c<0.05$

Values are Means ± S.E.M. Figures in the parentheses indicate the number of animals.
p^a compared with pair-fed control group.
p^b compared with As-treated group.
p^c compared with As-treated+selenium-supplemented group.

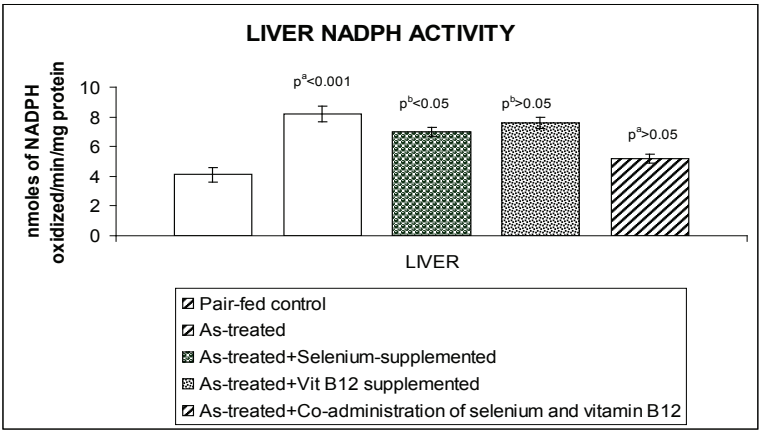

Fig.1: *Changes in liver NADPH oxidase activity following exposure to arsenic with or without selenium or selenium and vitamin B_{12} co-supplementation*

Values are mean ± S.E.M., $p \leq 0.05$ is considered statistically significant
The vertical line on the top of the bar graph indicates ± S.E.M.
p^a compared with pair-fed control group.
p^b compared with As-treated group

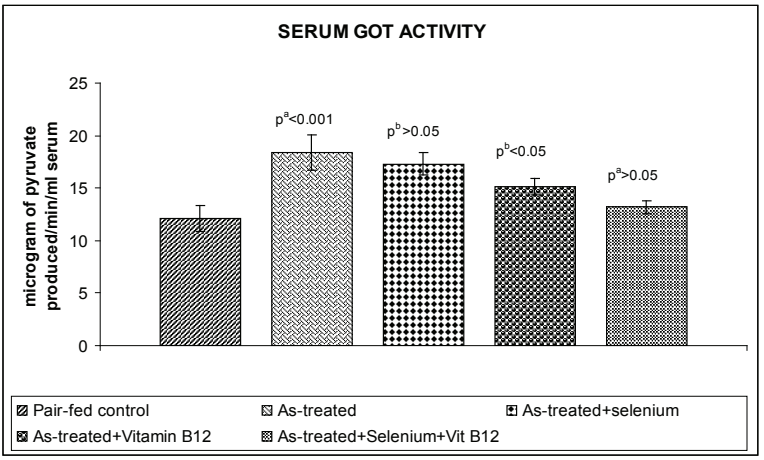

Fig.2: *Changes in serum GOT activity following exposure to arsenic with or without selenium or selenium and vitamin B_{12} co-supplementation.*

Values are mean ± S.E.M., $p \leq 0.05$ is considered statistically significant
The vertical line on the top of the bar graph indicates ± S.E.M.
p^a compared with pair-fed control group.
p^b compared with As-treated group

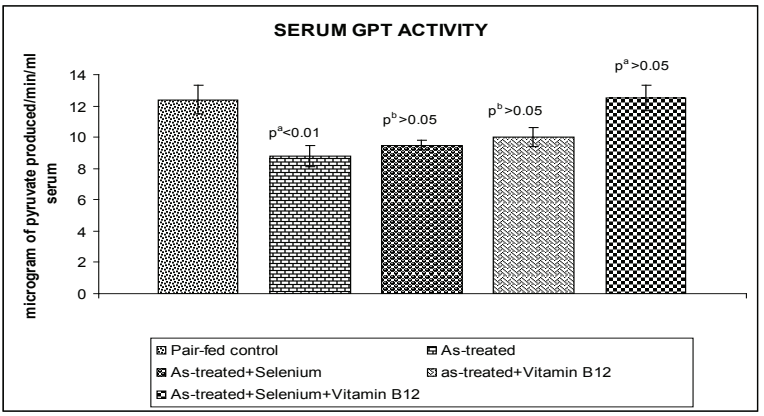

Fig. 3: *Changes in serum GPT activity following exposure to arsenic with or without selenium or selenium and vitamin B_{12} co-supplementation.*

Values are mean ± S.E.M., $p \leq 0.05$ is considered statistically significant
The vertical line on the top of the bar graph indicates ± S.E.M.
p^a compared with pair-fed control group.
p^b compared with As-treated group

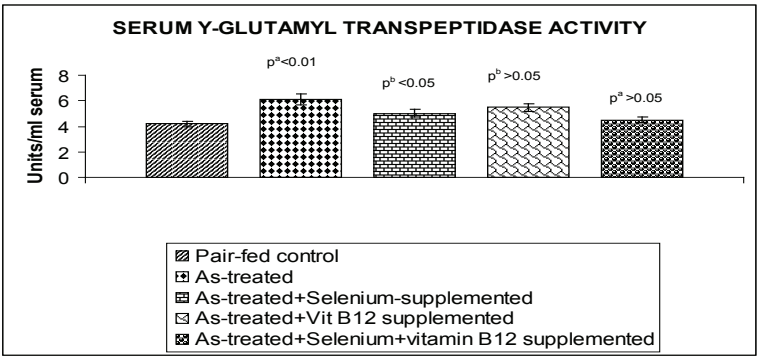

Fig. 4: *Changes in serum γ-glutamyl transpeptidase activity following exposure to arsenic with or without selenium or selenium and vitamin B_{12} co-supplementation.*

Values are mean ± S.E.M., p≤0.05 is considered statistically significant
The vertical line on the top of the bar graph indicates ± S.E.M.
p^a compared with pair-fed control group.
p^b compared with As-treated group

The results presented in Fig. 1 reveal that co-supplementation of selenium and vitamin B_{12} exhibited significant effect in counteracting the increased NADPH oxidase activity in liver following arsenic treatment. Fig. 2 shows that arsenic-induced increased serum glutatmate-oxaloacetate transaminase activity (p^a<0.001) was partially counteracted by selenium and vitamin B_{12} supplementation (p^b<0.05). It is demonstrated by Fig. 3 that decreased serum glutamate-pyruvate transaminase activity (p^a<0.001) in arsenic-treated animals, was counteracted by selenium and vitamin B_{12} co-administration (p^b<0.05). Change in g-glutamyl transpeptidase activity in serum (Fig.4) reveals that arsenic treatment increased the enzyme activity significantly (p^a<0.001). Selenium and vitamin B_{12} supplementation together caused partial counteraction (by 28.94%) of the change in enzyme activity due to arsenic treatment.

Discussion

The present study reveals that arsenic treatment at the present dose and duration causes depletion of reduced glutathione content of liver. Czarnecki *et al.* [31] reported that arsenic is thiol loving and binds with tissue proteins and these interactions have generally been regarded as the basis for the effects of this metalloid on the structure and function of these protein molecules. GSH depletion may result in the accumulation of free radicals that may initiate lipid peroxidation, resulting in oxidative injury [32]. Ito *et al.* [33] demonstrated that arsenic depletes GSH in cells which in turn enhances the expression of several stress proteins by activating the stress protein gene, thereby induces oxidative stress. Perturbation of liver glutathione in arsenic intoxication was also reported earlier [34].

The present study also demonstrates that selenium and vitamin B_{12} supplementation both in arsenic-exposed rats appreciably restored the depleted GSH content in liver. Their combined supplementation exhibited better protection in restoration of depleted GSH level following arsenic treatment. Selenium is a co-factor and required for the activity of several selenoenzymes involved in stress response and maintains the high antioxidant levels in tissues [35]. Moreover, it is a potent antioxidant and nutritionally acts through various selenoproteins to control the level of cellular hydroperoxides and redox tone of the cell that can damage the protein, cell organelle and DNA [36]. It is involved in regulation of body's antioxidant glutathione and glutathione peroxidase system, which plays the major role in control of ROS production [37]. Protective effects of selenium in fluoride induced alterations in free radical scavenging enzymes and metabolic enzymes were found in mice brain [38]. Selenium is known to afford protective effects on fluoride toxicity [39]. Similar protective effect of selenium is also found against arsenic induced oxidative damage in rat hepatocyte, as evidenced by the present findings.

Arsenic is considered as prooxidant and disturbs the antioxidant enzyme activities in liver, kidney, brain and other tissues [40]. In the present study the lipid peroxidation is clearly reflected by the increased malondialdehyde level in the liver in response to arsenic toxicity. Overproduction of lipid peroxides due to arsenic exposure causes destabilization in cellular lipid substances, inducing oxidative damages especially of membrane structures [41]. Our present observations further reveal that The LPO level is reversed partially in selenium treatment with arsenic. The increased production of lipid peroxides in arsenic-treated rats was completely counteracted when selenium was administered in combination with vitamin B_{12}. This observation suggests that the protective effect of selenium is enhanced in presence of vitamin B_{12}. The antioxidant effects of selenium and vitamin B_{12} may involve stabilization of membrane lipids and protein molecules that maintain the functional dynamics of the plasma membrane. It was previously studied by Mukherjee *et al* [14] that concomitant administration of either folic acid or folic acid and vitamin B_{12} with arsenic significantly restored the increase in the levels of nitric oxide, malondialdehyde and hydroxyl radical formation in the pancreatic tissue of arsenic-treated rats. They suggested that vitamin B_{12} along with folic acid is capable of reducing arsenic-induced cellular oxidative and inflammatory toxic changes. Thus, in similar way supplement with vitamin B_{12} and selenium may be predicted as a possible nutritional management strategy against arsenic-induced toxicity.

The increased free ·OH radical production in liver tissue by arsenic treatment was also counteracted by selenium with Vitamin B_{12} co-treatment with better efficacy than selenium supplementation alone. It is suggested that selenium, being a peroxide

radical scavenger, may also scavenge the highly toxic ·OH radical and also detoxify precursor of ·OH radical, H_2O_2 and thereby protects the cells from their toxic effects. Additionally, the present observations also reveal that selenium and vitamin B_{12} co-administration in arsenic-treated animals appreciably counteracted arsenic-induced decreased glutathione reductase activity in liver tissue, and thereby helped in the restoration of GSH content to the control value. Dietary selenium supplementation increases the oxidative stability of tissues by increasing the activities of glutathione reductase and glutathione peroxidase and selenium also interferes with redox status by maintaining the redox state of the tissue [42]. Selenium with vitamin B_{12} at the present dose and duration showed appreciable protective response in the restoration of GR activity in liver, and thus helped in the restoration of GSH content in that tissue to the control level.

Moreover, selenium and vitamin B_{12} co-administration showed additive effects in restoration of decreased superoxide dismutase activity in liver tissue following arsenic treatment. The decrease in this enzyme activity may be attributed to enhanced superoxide radical production during arsenic metabolism [43]. Several earlier reports revealed inhibited superoxide dismutase activity in liver due to arsenic treatment [19, 44-45]. Protective effects of selenium against oxidative stress induced changes in antioxidant enzyme and metabolic enzyme activities were found against fluoride toxicity [38]. The investigators suggested that increase in superoxide dismutase activity after selenium administration might be due to protection from superoxide, hydroxyl radicals, peroxyl and alkyl radicals. Similar explanation may be suggested for protective effect of selenium against arsenic induced alteration of liver superoxide dismutase activity. It was earlier established that vitamin B_{12} and folic acid separately or in combination can give significant protection against alterations in oxidative stress and apoptotic markers and also promotes downstream alterations in mitochondria, especially pro-oxidative (such as TBARS, $OH^-$and NO) and antioxidative defense (CAT, SOD and GSH) markers, mitochondrial swelling, iNOS protein expression, caspase-3 activity, Ca^{2+}-ATPase activity, cytochrome C oxidase activity and Ca^{2+} content [46]. The antioxidative effects of selenium and vitamin B_{12} are additively reflected in counteraction of arsenic-induced change in superoxide dismutase activity in liver, when they are conjointly supplemented.

Co-administration of selenium and vitamin B_{12} also exerted additive beneficial effect in restoration of the decreased catalase activity in liver tissue following arsenic treatment. Inhibited catalase activity in liver tissue due to arsenic treatment could initiate the over accumulation of hydrogen peroxide and free hydroxyl radical, inducing oxidative stress. It was already reported that pre-treatment of cells with non-lethal dose of sodium selenite induces the synthesis of proteins which protect the

cells from killing by hydrogen peroxide[47]. It is suggested that selenium may enhance the synthesis of catalase and thereby helps in quick disposal of H_2O_2 from the cells. Concomitant supplementation of vitamin B_{12} and folic acid enhanced the activity of catalase in liver tissue as reported earlier [46], and thus reducing the toxicity of arsenic. As the mechanisms of actions of both of these supplements are methylation related, a combined application was more effective.

The present study further reveals that the NADPH oxidase activity in liver tissue increased significantly following exposure to arsenic. Chen *et al* [26] reported that arsenite is able to induce cellular apoptosis which is triggered by the generation of hydrogen peroxide through activation of flavoprotein-dependent superoxide producing enzymes, such as NADPH oxidase, and H_2O_2, thus produced, might play a role as a mediator to induce apoptosis through release of cytochrome C to cytosol. Lenartowicz [48] reported that inhibition of a-ketoglutarate dehydrogenease by arsenic causes enhanced oxidation of mitochondrial NADPH, NADH and glutathione, and thus resulting in increased formation of ROS and other oxidants. Barchowsky *et al.* [49] previously reported that low levels of arsenite (5μM) not only stimulate the production of reactive oxygen species which is likely to result from stimulation of NADPH oxidase by arsenite. Concomitant administration of selenium and vitamin B_{12} at the present dose and duration exhibited significant counteractive effect on arsenic-induced elevated NADPH oxidase activity in tissues. Selenium was found to protect skeletal muscle cells damaged by fluoride following a disruption of energy metabolism in mitochondria with improving mitochondrial membrane stability [39]. In selenium and vitamin B_{12} co-supplemented rats, some counteractive effects have been found to normalize the NADPH oxidase activities in liver tissue which may be helpful in reducing the oxidation of mitochondrial NADPH, NADH and GSH, and thus resulting in decreased formation of ROS and other oxidants in presence of selenium and vitamin B_{12}.

Besides oxidative stress, arsenic is also known to cause hepatic tissue injury as reflected by the increase in glutamate oxaloacetate transaminase activity in serum, while the same treatment of arsenic decreased the serum glutamate-pyruvate transaminase activity. Most of the blood enzymes arise from tissues. In an earlier study[50] arsenic exposure was shown to elevate the GOT activity and decrease the GPT activity in kidney. Modi *et al.*[51] showed that sodium arsenite (2 mg/kg, orally) for a period of three weeks produced a significant increase in serum glutamate oxaloacetate transaminase activity. The changes in serum glutamate oxaloacetate transaminase and glutamate pyruvate transaminase activities following arsenic treatment as noted in the present investigation may be a reflection of changes in the transaminase activities of the kidney [50]. The changes in serum transaminase enzyme activities were not significantly

counteracted by selenium or vitamin B_{12} alone. However, selenium and vitamin B_{12} co-supplementation appreciably restored the increased serum glutamate oxaloacetate transaminase activity as well as reduced serum glutamate pyruvate transaminase activity to its control value. This may be ascribed to the additive protective effect of vitamin B_{12} with selenium.

The present study further reveals that the increased serum γ-glutamyl transpeptidase activity following arsenic treatment might be an indicator of plasma membrane damage and glutathione linked oxidative damages in tissues, as suggested earlier. Selenium or vitamin B_{12} supplementation alone showed partial protective effect on serum γ- glutamyl transpeptidase activity, while their co-administration exhibited more protection against arsenic-induced changes in serum γ- glutamyl transpeptidase activity. These observations further suggest some additive protective effects of selenium and vitamin B_{12} in restoration of arsenic-induced cellular metabolic stress.

Conclusion

The present study suggests that short-term arsenic treatment produced oxidative stress in rat liver by disturbing the cellular pro-oxidant-antioxidant equilibrium and altering the serum biochemical variables. Selenium and vitamin B_{12}, when supplemented conjointly, imparts additive beneficial effects in the restoration of some of the parameters such as changes in liver LPO level, SOD, Catalase and NADPH oxidase activities, as well as serum GOT, GPT and g-GT activities. The ameliorative effects of selenium and vitamin B_{12} are due to their role in various physiological functions including as biologically active antioxidants. From these observations, it is thus concluded that selenium in combination with vitamin B_{12} may be helpful to give better protection against arsenic-induced oxidative stress.

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CHAPTER

Indigenous use of medicinal fern and fern allies by the people of Tripura, North East India

Barnali Dey

Department of Botany, Maharaja Bir Bikram College, Agartala, Tripura, India
*Email: dey.barnali29@gmail.com

Abstract: Numerous studies have focused on the medicinal properties of angiosperms; however, only limited amount of studies have explored the medicinal potentials of fern and fern allies (Pteridophytes) which are much neglected group of plant kingdom and have an important role in folklore medicine. The present study focuses on the ethno medicinal values of fern and fern allies that are traditionally used by the tribal people of Tripura through a survey of the area (different parts of South Tripura, Gomati, Sepahijala, West Tripura, Khowai and Dhalai District). Extensive field trips were conducted in order to collect the ethno botanical information of study area. The present article gives a brief account of 31 plants species belonging to 21 families used as herbal remedies by local tribes of different parts of Tripura. Questionnaire was made to gather data for botanical name, family, part used, disease treated and manner of using recipes have been recorded.

Keywords: Ethno medicinal; Indigenous; Pteridophytes ; fern.

India is rich in its tribal population from the ancient time with traditional knowledge system which deals with various important aspects and the health issues of the tribal. The folk people get their treatment with the help of local practitioners and own herbal preparations. The use of herbal medicines by the tribal communities is influenced by distinct socio-cultural practices, beliefs, support of traditional authority and services of traditional medicine men. These people have a close relationship with their ambient environment and basically depend on it for primary healthcare as they live in remote localities far away from modern facilities. Tribal people are the ecosystem people who live in close harmony with the nature and maintain a close relationship between man and environment and indigenous cultures are closely maintained by the tribal and other forest dwellers throughout the world.

Tribal communities are highly dependent on the natural resources obtained from the surrounding forest regions for treatment of various ailments and diseases. The most

common ailments treated are skin problems, burns, wounds, and cuts. Other illnesses alleviated by herbal medicines include respiratory infections, coughs, fevers, colds, gastrointestinal problems, abdominal pains, stomach aches, throat infections, snake bites, and nervous disorders [1].

Though, lot of studies have focused on the medicinal properties of angiosperms, information on the medicinal potentialities of the pteridophytes are limite [2-5]. Recently, Ghosh *et al.,*[6] reported some edible pteridophytes as vegetables and medicines. A comprehensive list of Indian pteridophytes has been prepared by Dixit, [7] and Chandra [8]. The present study has been made to explore the Ethno-medicinal uses of pteridophytes. pteridophytes are used in different medicinal field like Ayurveda and Unani. Some pteridophytes are used in Homeopathic industry. Out of 1,000 species of Pteridophytes occurring in India, 170 species have been found to be used as food, flavour, dye, medicine, bio-fertilizers, oil, fibre and bio-gas production [9]. The medicinal value of pteridophytes against bacteria, fungi, virus, cancer rheumatism, diabetes, inflammation, consultant, fertility, diuretic, pesticides, heptoprotective, and sedative had been reported.

In comparison to higher plants fern and fern allies have found little applications in medicine. Hence, in the present investigation, an attempt has been made to explore indigenous and ethno medicinally important fern and fern allies (pteridophytes) and properly document their useful aspects.

Study Area

Tripura is a landlocked state in North East India, spread over 10,491.69 km^2 (4,050.86 sq mi). Tripura is the third-smallest among 29 states in the country, behind Goa and Sikkim. It extends from 22°56'N to 24°32'N and 91°09'E to 92°20'E with the Tropic of Cancer passing through it. The state has three distinct physiographic zones- i) hill ranges ii) undulating plateau land and iii) low-lying alluvial land. Its maximum extent measures about 178 km (111 mi) from North to South, and 131 km (81 mi) East to West. Tripura is bordered by the country of Bangladesh to the West, North and South; and the Indian States of Assam to the North East; and Mizoram to the East.

The present data is outcome of fieldwork carried out in different parts of South Tripura, Gomati, Sepahijala, West Tripura, Khowai and Dhalai District during the year of 2017 to 2020.

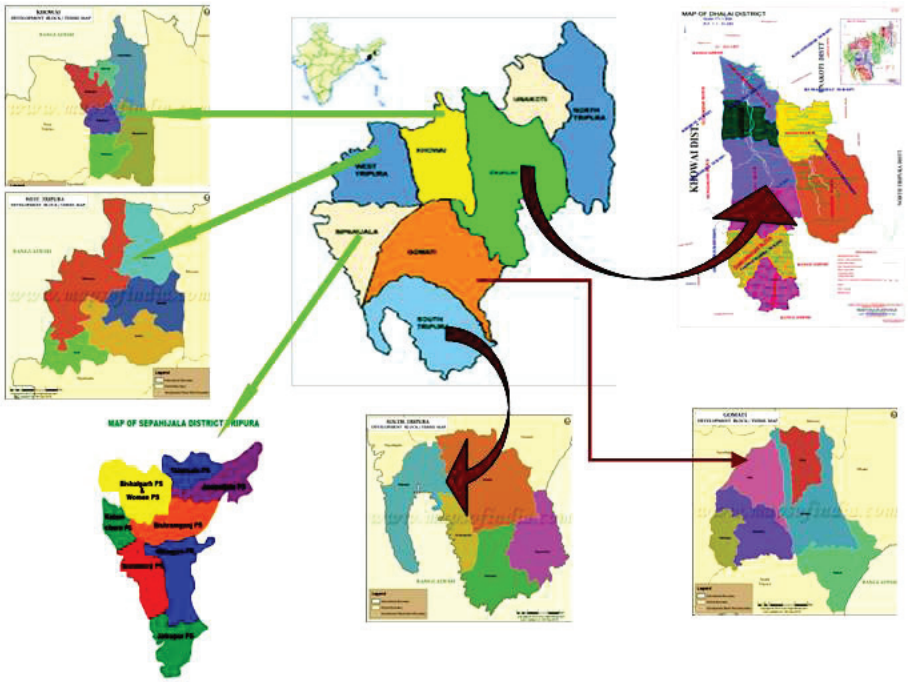

Fig. 1: *Maps of study area (different parts of South Tripura, Gomati, Sepahijala, West Tripura, Khowai and Dhalai District).*

Fig.2: *Author studying ferns in the field*

Materials and methods

Ethno-medicinal survey from tribal inhabitants was undertaken and ethno-medicinal information was gathered from the local inhabitants and intensive interviews were conducted as per the described method [10]. All the specimens were collected from the study area (Fig.1 & 2) and samples of each species were collected to prepare herbarium. About 31 species of pteridophytes with medicinal properties have been collected from the study area. Their botanical name, family, mode of use and parts used are given below. Information about some species, ailments, medicinal uses, methods of preparation of pastes and dosage of cure were also recorded. Interviews with the tribes, herbalists, forest guards, medicine men, witch-men (ojha) and common men were conducted to retrieve information about sustainable utilization of pteridophytes. Knowledge about process of preparation of decoction and application for useful purposes was also gathered. In total 31 species were found to be potentially utilized by the local tribes for different purposes. A detailed account on sustainable utilization and useful aspects of pteridophytes in the livelihood of local tribes are provided below.

Results

A total of 31 species of ferns and fern allies were collected from the different parts of Tripura for this study. All plant parts such as rhizomes, roots, fronds, leaves, stem, and spores are used as medicine. Leaves were the most popular plant part utilized in herbal preparations (37%), rhizome (26%), and whole plant (23%), while fronds (including spores) were used infrequently (14%). The present investigation has brought to highlight the therapeutic value employed to cure skin diseases, burns, wounds and cuts, respiratory infections, coughs, fevers, colds, epilepsy, leprosy, rheumatism, abdominal pains, kidney pains, stomach aches, throat infections, snake bites, nervous disorders, urinary problems, menstrual problem, gastrointestinal problems, and cardiac problems. For the presentation of data, all the medicinally important pteridophytes species are arranged alphabetically followed by family and brief note on medicinal uses (Fig. 3).

1) **Botanical name**: *Adiantum*: *caudatum* L. (**Family**: Adiantaceae)

   Usage in ethno-medicine: It is used in skin disease, diabetes, cough and fever.

2) **Botanical name**: *Angiopteris evecta* (Forest.) Hoffim (**Family**: Angiopteridaceae)

Usage in ethno-medicine: The rhizome paste is applied externally in case of bone fracture along with some other plants. The poultice is applied externally on the broken or fractured part of bone to get cured. This treatment is given to the patients every 3 days of regular interval for a period of 30 days. Apical parts of caudexes is cut into pieces and boiled with water till the contents become half. This extract is applied locally over carbuncle twice a day to get relief from pain, at the same time the abscess dried up within a week.

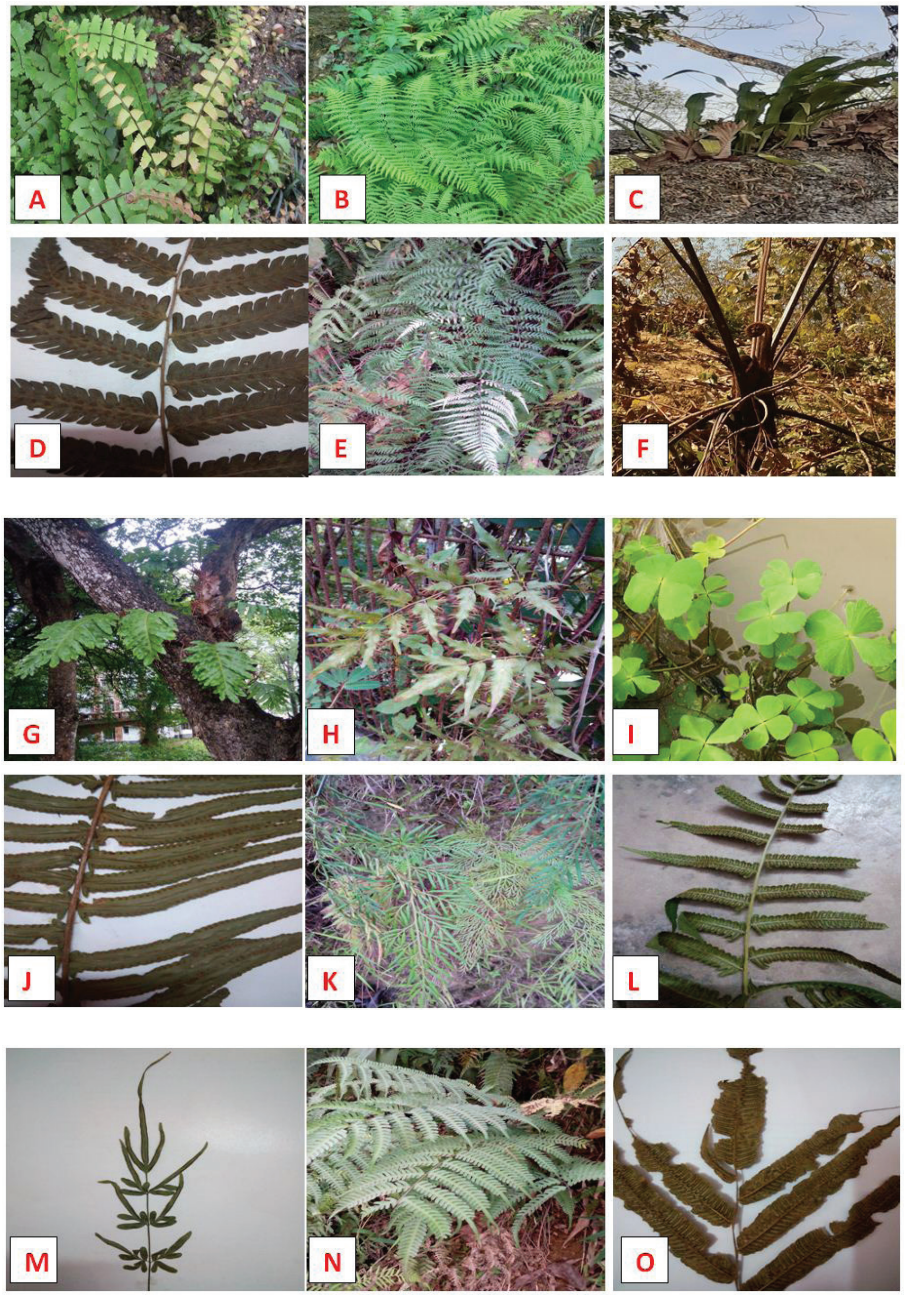

Fig. 3. A. *Adiantum***:** *caudatum B. Angiopteris evecta C. Asplenium nidus D. Christella dentata E. Cleilanthes tenuifolia F. Cyathea gigantea G. Drynaria quercifolia H. Lygodium microphyllum I. Marsilea minuta J. Nephrolepis cordifolia K. Onychium siliculosum J. Osmanda regalis M. Pteris ensiformis N. Pteris wallichiana O. Thelypteris dentata*

3) Botanical name: *Asplenium nidus* Linn. (**Family**: Aspleniaceae).

Usage in ethno-medicine: *Asplenium* has been used as herbal medicine. The rootstock is considered effective against fever, elephantiasis, jaundice and urinary problem. It is used as an emollient, in coughs and diseases of the chest. Leaf is smoked to treat colds. The decoction of *Asplenium* is used for cough and a good hair wash. Species of *Asplenium* is an indicator of nickel. *Asplenium* has aesthetic values for their beautiful habit, graceful shape of the leaves, and beautiful soral arrangement.

4) Botanical name: *Blechnum orientale* L. (**Family**: Blechnaceae).

Usage in ethno-medicine: Fronds showing good antioxidant and antibacterial activities. The hot decoction is used to treat typhoid. Hot decoction of pinnae is applied externally over abscess to liberate pus and also its antiseptic action. Fresh decoction is applied onec a day till abscess dries up.

5) Botanical name: *Botrychum lanuginosum* Wall ex. Hook & Grev. (**Family**: Botrychiaceae).

Usage in ethno-medicine: Plant is anti-dysenteric and antibacterial.

6) Botanical name: *Christella dentata* (Forssk.) (**Family**: Thelypteridaceae)

Usage in ethno-medicine: Fronds The paste obtained from the frond is applied over the swellings over the body.

7) Botanical name: *Cleilanthes tenuifolia* (Blume.f.) sw. (**Family**: Pteridaceae).

Usage in ethno-medicine: Tribal use the extract of rhizome and roots as a general tonic. Roots are prescribed for sickness attributed to witchcraft or the evil eye. Leaves are used as a poultice on swollen limbs. Fronds cut into pieces, made to a paste and applied on abscess in the form of poultice to liberate pus and also used as antiseptic. The poultice is given once a day till the abscess is cured.

8) Botanical name: *Cyathea gigantea* (Wall. ex.Hook.) (**Family**: Cyatheaceae).

Usage in ethno-medicine: Fresh rhizome used to cure for loose motion and anti diabetic. Pith of *Cyathea* is cooked and eaten by tribal in many parts of the country. Apical soft portion of the caudexes cut into pieces and crushed in a mortar and added water to make a paste. The paste is then applied locally on major cuts or wounds for immediate clotting of blood. The same also prevent microbial growth in cut surface so that no abscess could develop. Fresh paste is applied everyday till the wound is healed. Apical portion of the trunk cut into pieces and crushed. The paste so obtained is applied on major cut or wound for immediate arrest of bleeding. The same also prevents microbial growth in the cut portion so that no infection takes place. Fresh paste is applied everyday till the wound is healed.

9) **Botanical name**: *Dicronopteris linearis* (Burm. f.) Undrew (**Family**: Gleicheniaceae).

Usage in ethno-medicine: Decoction of plant is used to treat throat pain. Fronds are used in asthma and antibacterial activity. Freshly extracted fronds juice is slightly heated and the decoction is taken internally during throat pain. Rhizome is anthelmintic; fronds used for asthma.

10) **Botanical name**: *Diplazium esculantum* (Retz.) Sw. (**Family**: Athyriaceae).

Usage in ethno-medicine: Leaves are made into juice and taken orally twice a day to get relief from cold and cough. Fresh uncurled frond of *Diplazium* is eaten in many country. Circinately coiled young and fresh frond is boiled with salt and taken internally for maintaining all round health. Decoction of rhizome along with 2 ml of honey is taken in empty stomach to cure spermatorrhoea (*Rout et al.* 2009, Singh & Khare 2011).

11) **Botanical name**: *Drymoglossum heterophyllum* (Linn) Trimen (**Family**: Polypodiaceae).

Usage in ethno-medicine: Paste obtained by crushing pinnae applied externally in the form of poultice on fractured bones after setting up the bones. Bamboo splints are usefully tried around so as to prevent dislocation of fractured bones.

12) **Botanical name**: *Drynaria quercifolia* (Linn) J. Smith (**Family**: Polypodiaceae).

Usage in ethno-medicine: The rhizome paste mixing with molasses taken internally during cardiac problem. Paste obtained by crushing rhizome applied externally in the form of poultice on fractured bones after setting up the bones. Bamboo splints are usually tried around so as to prevent dislocation of fractured bones. The rhizome juice is inhaled internally cures cardiac and blood coagulation problem. The fronds are useful in poulticing swellings. The rhizome is bitter, it is used as an antibacterial, anodyne, constipating, anti-inflammatory tonic, in the treatment of typhoid fever, phthisis, dyspepsia, cough, arthralgia, cephalalgia, diarrhoea, foul ulcers and other inflammations. It is very specific in the treatment Migraine. The decoction of the plant is used in typhoid fever and is also used as an anthelmintic, pectoral, expectorant, tonic, dyspepsia and astringent. Fronds are useful in poulticing swellings.

13) **Botanical name**: *Dryopteris* cochleata (Ham ex. D. Don)C. Chr. (**Family**: Dryopteridaceae).

Usage in ethno-medicine: The whole plant extract is applied for snake bite. Powdered rhizome is taken with water to cure rheumatism and leprosy. The whole plant is crushed in a bowl and the extract is given (twice a day) orally in case of snake bite, besides, a paste of the plant is also applied on the bite wound to prevent infection.

The rhizome is antibacterial and antiepileptic. The rhizome of the plant is powered and taken with water (twice a day) in rheumatism, epilepsy and leprosy. Juice of roots (about 2 tea spoonful twice a day before meal) is given to treat amoebic dysentery.

14) Botanical name: *Helminthostachys zeylanica* L. (**Family**: Helminthostachtaceae).

Usage in ethno-medicine: The fronds are reported to be a aperients, intoxicant, anodyne, also used in sciatica, as an antiviral, antipyretic, anti inflammatory and intoxicant. The rhizome is used in dysentery, catarrah, sciatica, malaria and also as a tonic and mild aperients. A decoction of the plant is given for curing impotency and the juice of the leaves is used to relieve blisters on the tongue. The decoction of rhizome is used for the treatment of impotency, whooping cough, phthisis. In combination with the roots of *Chlorophytum tuberosum* and roots of *Bombax ceiba* made into a paste when applied for one month to relieve waist pain and used also as a tonic. A paste of rhizome, curd and crushed termite is known to promote strength and vitality. The powder of the rhizome is given for spermatorrhoea and for improving memory power.

15) Botanical name: *Lycopodium cernum* L. Pic.Serm. (**Family**: Lycopodiaceae).

Usage in ethno-medicine: The decoction of the plant is given in beri beri, cough, chest complaints; embrocating of the ashes in vinegar for skin eruptions. The rhizome is used for nervous disorders, rheumatism and also given in fever and dropsy. The decoction of the plant is used for lotion (reducing swelling and itching). The plant also used to cure cough and skin eruption

16) Botanical name: *Lygodium microphyllum* (Cav.) R. Br. (**Family**: Lygodiaceae).

Usage in ethno-medicine: Species of *Lygodium* are used for treatments of stomach ache and diarrhea. Rachis of the plant tied over forehead to reduce headache. The same when tied on head, to be secured from evil spirit. Leaf decoction is given in dysentery. It is used as one of the ingredients in many lotions. Leaves are applied in the form of poultices for skin diseases and swelling. Crushed leaves are used to cure hiccough.

17) Botanical name: *Marsilea minuta* L. (**Family**: Marsileaceae).

Usage in ethno-medicine: The plant leaf is used to cure cough, bronchitis, diarrhea, leprosy and fever. The extract of whole plants is used by the local tribes as aphrodisiac and for increased fertility. The leaves are pounded, cooked with rice and then eaten as a treatment for indigestion the leaf juice is used to stop nose bleeding. The leaves are rolled in a leaf of *Shorea robusta*, the whole is then boiled and then applied to swollen gums in order to reduce the swelling. The leaf extract is also known to reduce cholesterol and triglyceride levels in blood and liver substantially.

18) Botanical name: *Microsorium superficiale* (L.) Copel. (**Family**: Polypodiaceae).

Usage in ethno-medicine: About 20 gm paste obtained by crushing fresh rhizome along with seeds of *Piper nigrum* is taken orally to cure cough and cold. It should be taken thrice a day till the disease is cured. Leaf is ground into juice applied over the affected places twice a day with hot water to heal wounds. It also used for anti-inflammatory and antibacterial activity. Leaf juice is used as a purgative, diuretic and for healing wound.

19) **Botanical name**: *Nephrolepis cordifolia* L. (**Family**: Nephrolepidaceae).

Usage in ethno-medicine: *Nephrolepis* has aesthetic values for their beautiful habit, graceful shape of the leaves, and beautiful soral arrangement. Thus, these characteristics make them horticulturally important plants. The rhizome is reported to be antibacterial and is used in cough, rheumatism, chest congestion, nose blockage and loss of appetities. Pinnae are anti-tussive, styptic, antifungal used in coughs, wounds and for the treatment of jaundice, a decoction of the fresh frond is given as a drink.

20) **Botanical name**: *Onychium siliculosum* (Desv.) C. Chr. (golden fern) (**Family**: Pteridaceae).

Usage in ethno-medicine: Decoction of the fronds are used in dysentery.

21) **Botanical name**: *Ophioglosum peteolatum* Hook. (**Family**: Ophioglossaceae).

Usage in ethno-medicine: *Ophioglossum peteolatum* is an appreciated vegetable, collected sedulously wherever it is common. It is eaten fresh as salad, or cooked, alone or mixed with other vegetables. The leaves should be cooked gently; otherwise they turn completely into slime. In India the leaves are used as a substitute for spinach and it is used as a herbal medicine which is anti-inflammatory and anti-swelling. Leaves boiled in oil are a remedy for wounds. Thick paste of fresh rhizomes and tubers is effective in hair fall.

22) **Botanical name**: *Osmanda regalis* L. (**Family**: Osmundaceae).

Usage in ethno-medicine: The root decoction of *Osmanda regalis* is used for treatment of jaundice. The ointment made from its root is used for application to wound. The extraction of *Osmanda regalis*, commonly known as 'Green oil charity', is used as remedy for wounds. Fronds are used as tonic, styptic and also for rickets, rheumatism and for intestinal gripping. The rhizome is used as aborticacient.

23) **Botanical name**: *Polypodium obliquatum* (**Family**: Polypodiaceae).

Usage in ethno-medicine: The expectorant of *Polypodium* is used as a mild laxative, while the tonic is used for dyspepsia, loss of appetite and hepatic problem.

24) **Botanical name**: *Pteridium aquilinum* (L.) Kuhn. (**Family**: Polypodiaceae).

Usage in ethno-medicine: *Pteridium aquilinum* is used to treat tooth ache and mouth infection. Rhizome and fronds are used in chronic disorders, antihelmintic,

astringent and is useful in diarrohea and for the treatment of inflamation in the gastric and intestinal mucous membranes.

25) Botanical name: *Pronephrium nudatum* (Roxb.) Holttum (**Family**: Thelypteridaceae).

Usage in ethno-medicine: Cold decoction of pinnae is used as mouthwash during acute pyorrhea. 2-3 wash is given a day till it is cured.

26) Botanical name: *Pteris ensiformis* Burm. (**Family**: Pteridaceae).

Usage in ethno-medicine: Leaves of *Pteris sensiformis* are being applied to boils, ulcers and arrow wounds. *Pteris ensiformis* is also used to control menstruation whereas another species of *Pteris* is used internally during child birth. Fronds pounded to paste with water, applied locally twice a day during swelling of joints till it is cured. Decoction of fresh frond is given against dysentery.

27) Botanical name: *Pteris vittata* L. (**Family**: Pteridaceae).

Usage in ethno-medicine: Plant extract is used as demulcent, hypotensive, tonic, antiviral and as antibacterial. Leaves used in worship at the time of illness. Leaf juice reportedly relieved blisters on the tongue.

28) Botanical name: *Pteris wallichiana* J. Agardh (**Family**: Pteridaceae).

Usage in ethno-medicine: Fresh leaves are crushed and applied to stop bleeding and healing of wounds.

29) Botanical name: *Pyrrosia adnascens* (Forest.) Chiny (**Family**: Polypodiaceae).

Usage in ethno-medicine: Cold decoction of rhizome mixed with a little powered seeds of *Piper nigrum* is taken orally during cough and cold twice a day for 7 days. Fronds pounded to paste with water, applied locally twice a day during swelling of joints till it is cured. Fronds pounded to paste with water applied locally around carbuncle for getting it burst and also to reduce pain. Fronds are used medicinally to treat dysentery and burn injuries.

30) Botanical name: *Selaginella involvens* (Sw.) Spring (**Family**: Selaginelliaceae).

Usage in ethno-medicine: Plants is considered to help to rejuvenate life, also used in the prolapsed of rectum, prevents cough, bleeding piles, gravel amenorrhea and as an antibacterial.

31) Botanical name: *Thelypteris dentata* (Forssk.) (**Family**: Thelypteridaceae)

Usage in ethno-medicine: *Thelypteris dentata* has been found to be an herbal medicine, as it has antibacterial properties from the alcoholic and chloroform extracts.

Discussion

In primitive time, pteridophyte possesses an important role in folklore medicine, Ayurvedic, Unani, Homeopathic and other systems. However, now they are neglected due to the migration activity. In the past, several studies in the literature have reported medicinal uses of some fern and fern allies of India. Kirtikar and Basu,[11] have described 27 species of ferns having varied medicinal uses. Chopra *et al.*, [12] have included 44 species and Nadkarni, [13] recorded 11 species of pteridophytes having medicinal importance. Nayar, [14] recorded 29 medicinal ferns. May, 1999 published a detailed review of the various uses of ferns and listed 105 medicinal ferns. Singh, [15] reported 160 species of useful pteridophytes in India on the basis of phytochemical, pharmacological, and ethno-botanical studies. Similar reports on the use of pteridophytes as medicinal purposes are very limited [16]. Contrarily, Sumesh *et al.*, [17] revealed that the leaf extract of *Angiopteris evecta* is used in treatment of dysentery whereas the spores are effective in the treatment of leprosy and other skin diseases [11]. *Actiniopteris radiata* possesses the properties like anthelmintic, astringent, sweet, cooling, acrid, febrifuge, and is used for treating severe conditions of diarrhea, dysentery, helminthiasis, hemopstysis, and fever [18]. Recently, Das and Choudhury, [19] recognized the paste of *Adiantum incisum* is useful in the healing of wounds. In contrast to the general belief that extant *Equisetum arvense* is used in nasal polyps and kidney infections and acidity, *Lycopodium clavatum* decoction used in rheumatism and diseases of lungs and kidneys. The paste of the leaves of *Ophioglossum reticulatum* is used in headache. The fronds of the gleicheniaceous fern *Dicranopteris linearis* Underwood is used for asthma and in woman's sterility, and the rhizomes of *A. evecta* are used for scabies. Singh and Singh, [20] found that 23 species of pteridophytes belonging to 15 families and 18 genera are traditionally used in treating 16 different gynecological and reproductive health-related diseases by the tribal women, about 85% of traditional medicines used for primary healthcare globally derived from plants in India. The fronds of the gleicheniaceous fern *D. linearis* Underwood are used for asthma and in woman's sterility. The rhizomes of *A. evecta* are used for scabies [21]. In this present study, presume that all the fern and fern allies have remarkable effective of medicinal value. In addition, *Hemionitis arifolia* herb mostly available in terrestrial as well as saxiculous nature, the root powder is used to treat hypertension and also good ailment for healing wound. *Dryopteris cochleata* extract is applied for snake bite; powdered rhizome is taken with water to cure rheumatism and leprosy. *Equisetum ramosissimum* paste is externally applied to cure scabies, itches, and allied skin infections. *Lycopodiella cernum* decoction of the plant is used for lotion (reducing swelling and itching) and also used to cure cough and skin eruption.

Conclusion

From this study, it is concluded that ethno-medicinal uses of 31 pteridophytic species used by the tribes of the different parts of Tripura local inhabitants have inherited with rich traditional knowledge on the use of medicinal plants or plant parts for their regular food and medicine. In developing countries, many people still rely on traditional healing practices and medicinal plants for their daily healthcare needs, in spite of the advancement in modern medicine. However, documentation of this indigenous knowledge of healing system still remains at a minimum level. It thus becomes necessary to acquire and preserve this traditional system of medicine by proper documentation and identification of specimens. According to last census 2011, an estimated 65% of Indian population still depend on the traditional medicine, because modern medicine is simply too expensive and treatment is too capital intensive. Therefore, the ethno-medicinal species listed above may be subjected to intensive phytochemical screening and pharmacognosy in view of their immense potential to cure certain vital diseases and ailments.

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Cytotoxicity assessment of diospyros lanceifolia roxb. using Allium cepa test
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CHAPTER

Cytotoxicity assessment of *diospyros lanceifolia* roxb. using *Allium cepa* test

Surochita Basu* and Jain Souh Reang

Department of Botany, Tripura University, Tripura, India
*E-mail:surochitabasu@yahoo.co.in; jainsouh@gmail.com

Abstract: *Diospyros* of family Ebanaceae is better known as ebony for its highly valued timber. Not only timber, all parts of Diospyros are medicinally valued and used in indigenous system of medicine. Several phytochemically active components have been identified from the plants, few used for anticancer and anti-AIDS. The fruits and seeds of the target species *Diospyros lanceifolia* Roxb. is used traditionally as fish poison, plant extract is used to cure skin, teeth disorders, stomach ache and the wood is used for construction and craftsmanship. Notwithstanding its importance, cytotoxicity assessment of the plant extracts conducted to potentiate its use against cancer, however, remains skeptical regarding its toxicity. An attempt to investigate cytotoxicity of aqueous fruit extract through a plant-based bioassay, the '*Allium cepa* test', wherein changes in morphology of treated roots, changes in mitotic index and occurrence of aberrant cells are reliable parameters; indicate significant reduction in cell division rates, appearance of chromosomal aberrations in meristem cells and deformed root morphology beyond specific treatment concentration, duration. The clastogenic effects and low to high dose dependent cytotoxicity of the aqueous fruit extract cautions use of the plant for therapeutic purposes.

Keywords: *Diospyros*; *Allium cepa* test; cytotoxicity; mitotic index, aberrant cells, concentration and duration,

Genus *Diospyros*, the most numerous and important genus of the pan tropical family Ebenaceae with 350 tree and shrub species is represented by about 50 species in India[1], widespread in the evergreen forests of Deccan, Assam, Bengal and a few in North India [2,3,4,5]. Many species of the genus yield economically valuable fruits and highly valued dark timber of excellent durability[6]. About seven species yield edible or medicinally valuable fruits, nine species yield commercial ebony, heartwood of certain species yield textured/colored wood and leaves of two species are used in making candies, bidis etc [7]. *Diospyros* has been well documented for its medicinal properties and almost all parts of the plant have been used in traditional systems of medicine

such as Ayurveda, African folklore and Chinese medicine. In Ayurveda, Unani and Indian traditional medicine, twelve species have been used for [8,9,10,11] treating rheumatism, fever, diarrhea, dysentery, ulcer, menorrhagia, for skin diseases, cuts and wound; as astringent, lithontriptic, haemostat, febrifuge, antimicrobial, abortifacient, carminative laxative etc. Some have been used as fish poison and against scorpion bite. Recently, *Diospyros melanoxylon* Roxb. has been used as anticancer, anti-AIDS and anti-inflammatory. The phytochemically active components of the exploited species include terpenoids, lupanes, ursanes, oleananes, taraxeranes, steroids, napthoquinones, naphthalene-based aromatics, benzopyrones, polyphenols, tannins, hydrocarbons, lipids, aminoacids, carotenoids, sugars and miscellaneous components such as vitamins, free aliphatic, aromatic acids, guanindine derivates, anthraquinones and lignans[7].

Diospyros lanceifolia Roxb. tree grows up to 27m, has simple, oblong elliptical to lanceolate leaves with acuminate apex. The outer brown to black bark has fine cracks, inner bark is bright yellow. The plant flowers during summer, male flowers clustered, very small, salver shaped, sessile; female flowers are solitary, subsessile and urseolate. It fruits during winter, fruit is globose, sub globose, with a small apical beak, hairy with shallow, accrescent spreading calyx [12, 13, 14]. Fruits are rich in ascorbic acid, carotenoids, flavonoids. The barks, leaves and fruits are used as fish poisons in many parts of Southeast Asia [14]. The seeds in Indonesia, both seeds and fruits in Nagaland, Tripura are used as a fish poison [14, 15, 16]. Tribal people of North East Asia use the fruit as piscicide, seeds to expel intestinal worms and to treat viral infections [14]. In North East India it is used as 'ruja' to eradicate wild fish, in Tripura it is used as 'ruthai' by Reang community, both as piscicide as well as for relieving stomach ache. The wood is used for crafts, musical instruments, furniture and timber, also as vegetable tannin source in paper industry and also in breweries [14,16]. Piscicide of plant origin used appropriately [17] is considered environmentally safer and no side effects upon human consumption have been reported [18].

Despite the scientifically validated pharmacokinetics and age old traditional medicinal beliefs, cytotoxicity evaluation of plant extracts, must precede therapeutic applications. Cytotoxicity assessment of several Diospyros species using petroleum ether, ethyl acetate and ethanolic extracts, through Brine Shrimp Lethality Assay and human cancer cell lines- human colon cancer cells HT-29, human pulmonary adenocarcinoma cell line A549 and hepatic carcinoma cell line HepG2, colorectal carcinoma cell line HT29; indicate significant cytotoxicity[19,20,21] and lethality, that potentiate its use as anticancer agent but at the same time contemplate on its potential toxicity.

Here, we attempt to investigate cytotoxic effects, if any, of the aqueous fruit extract of *Diospyros lanceifolia* Roxb. using a convenient, sensitive plant-based bioassay- the Allium cepa Test. Allium cepa test has become the mostly used assay as it is inexpensive, sensitive, fast bioassay, convenient for cytological studies due to good chromosome size and low number, distinct mitotic phases and chromosomal aberrations[22,23,24], as well as, shows good correlation with mammalian system[25]. Changes in root growth form, changes in mitotic index, appearance of chromosomal aberrations are the test criteria for cytoxicity assessment.

Materials and Methods

Fruits, fresh leaves and stem of *Diospyros lanceifolia* Roxb. were collected from Phuldungsai, North Tripura; fresh weight of fruits taken, fruit coat and seeds separated, sun dried, crushed. Crude aqueous extract was prepared by adding different amounts of the powdered fruit coat to adequate amounts of distilled water and kept for 72 hours for slow decoction, thereafter it was filtered, filtrate used for the *Allium cepa* test. Different concentrations 250mg, 500mg, 750mg, 1g, 1.25g, 2.5g, 5g, 7.5g per 100ml of distilled water were used for several treatment durations (6h, 12h, 18h, 24h, 30h, 36h, 42h, 48h). Tap water was used as control. Fresh onion *Allium cepa* L. bulbs with 1-2 cm long slender, healthy roots were arranged in vials so that roots remain immersed in the aqueous extract and control throughout treatment.

For observing changes in root morphology, root size, form, color was noted after each treatment duration. For cytological observations, root tips were excised after treatment, fixed and stained appropriately [26], slides were prepared, cytological observation recorded and photomicrographs obtained. Different mitotic phases and distinct chromosomal aberrations were distinguished and recorded. Micrometric observations recorded include number of cells in different mitotic phases, number of aberrant cells, total number of dividing cells and number of cells in microscopic field (at 40X objective). Mitotic Index (M I) and Frequency of Aberrant cell (FA) calculated by the following formulae:

$$MI = \frac{\text{Total no. of dividing cell / microscopic field}}{\text{Total no. of cell / microscopic field}} \times 100$$

$$FA = \frac{\text{Number of a particular type of aberrant cell}}{\text{Total number. of aberrant cell}} \times 100$$

All treatments were conducted in triplicate and at least 2000 cells were scored for each concentration and duration of treatment along with control. The mean values, standard deviation and standard error of mean were calculated for each attribute. Two-way ANOVA was conducted with Microsoft Excel.

Results and Discussion

The cytotoxic potential of the aqueous fruit extract of *Diospyros lanceifolia* Roxb. was assessed based on certain microscopic parameters - changes in mitotic index (MI) and occurrence, frequency of aberrant cells when compared to negative control and changes in root morphology. Some changes in root morphology of roots immersed in aqueous fruit extract include brown, dark coloration, short, stiff roots and negligible root growth beyond 1g treatment. At higher concentration and duration of treatment root growth gets severely affected. All phases of mitosis and mitotic abnormalities (c-mitosis, chromosome clumping, stickiness, anaphase bridge, disorganized metaphase) were carefully noted; mitotic index and aberrant cell types presented in Table 1-3 and Fig. 1-3.

Table 1: *Mitotic index of root cells at control and after treatment with aqueous extract*

Concentration of extract and control	Treatment duration							
	6 hours	12 hours	18 hours	24 hours	30 hours	36 hours	42 hours	48 hours
Control	7.27±0.57	8.23±0.54	7.01±0.31	8.72±0.61	8.28±0.06	8.44±0.47	8.94±0.45	8.89±0.32
250mg/100ml	16.95±0.80	16.45±0.95	15.09±0.98	14.74±0.52	13.58±0.73	13.55±0.75	11.6±0.78	7.68±0.47
500mg/100ml	15.09±0.98	12.91±0.86	12.74±0.40	11.67±0.52	10.41±0.98	8.93±0.70	8.30±0.78	7.39±096
750mg/100ml	13.15±0.50	9.31±0.54	8.93±0.70	7.65±0.78	6.43±0.36	4.42±0.61	3.86±0.53	3.43±0.57
1g/100ml	7.82±0.63	7.13±0.46	5.69±0.48	5.08±0.34	4.79±0.57	3.63±0.51	3.12±0.38	2.13±0.31
2.5g/100ml	4.64±0.52	4.27±0.64	4.89±0.54	3.51±0.27	2.53±0.45	2.50±0.37	1.10±0.38	0.29±0.14
5g/100ml	4.06±0.34	3.66±0.35	2.01±0.36	1.88±0.28	1.28±0.36	0.64±0.34	0.45±0.21	0.09±0.06
7.5g/100ml	5.57±0.33	4.69±0.48	4.19±0.21	4.04±0.36	3.64±0.21	3.39±0.41	2.66±0.35	1.70±0.17

Table 2: *Occurrence of aberrant cells during treatment with aqueous extract*

Concentration of extract and control	Treatment time							
	6 hours	12 hours	18 hours	24 hours	30 hours	36 hours	42 hours	48 hours
Control	-	-	-	-	-	-	-	-
250mg/100ml	-	-	-	-	-	-	-	-
500mg/100ml	-	-	-	-	-	-	-	-
750mg/100ml	-	-	-	-	-	-	-	-
1g/100ml	-	-	-	-	-	-	-	-
2.5g/100ml	-	-	Disorganized metaphase, 2	-	-		-	Chromosome clumping, 5
5g/100ml	-	-	Chromosome clumping, 3	C-mitosis, 6	Chromosome fragment, 2	Bridge anaphase, 3	-	Sticky anaphase,1 Sticky telophase, 1
7.5g/100ml								

Table 3: *Aberrant cell type and Frequency*

Aberrant Cell type	Number of aberrant cells	Frequency of aberrant cells (%)
C-mitosis	6	26.09
Bridge anaphase	3	13.04
Chromosome fragment	2	8.70
Chromosome clumping	8	34.78
Disorganized metaphase	2	8.70
Sticky Anaphase	1	4.34
Sticky telophase	1	4.34

Average, maximum and minimum MI of control were 8.22, 8.94 at 42 hours, 7.01 at 18hours; average, maximum and minimum MI of treated cells were 6.45, 16.95 at 250mg/100ml 6hours and 0.09 at 5g/100ml, 48 hours of treatment. At lower concentrations (250mg, 500mg, 750 mg) the fruit coat extract has much low cytotoxicity, with short exposure even mitogenic activity (maximum MI of 16.95 observed at 6hour of 250mg extract) noted. Both increase and decrease in MI considered as an indicator of cytotoxicity [23]. Beyond 2.5g/100ml treatment, significant inhibition of mitoses noted, at longer exposure disorganized metaphase, chromosome clumping occurred and with prolonged exposure (>40hrs), cell division almost ceased. At higher concentrations (5g,7.5g) maximum mitotic inhibition noted and all sorts of aberrant cells are observed upon treatment with 5g/100ml concentration of plant extract. Methanolic extract of the plant shows ample antioxidant property at [27], however, petroleum ether, ethyl acetate and ethanolic extract of plants shows significant toxicity[19,21]. Here, water extract evinces considerable cytotoxicity.

Significant reduction in MI at higher concentration and duration of treatment, could be due to several reasons such as delay in cell cycle progression, intervention of cell cycle, spindle disruption and inhibition in DNA, nucleoprotein synthesis [23,28]. Decrease in MI and induction of chromosomal aberrations considered as reliable criteria for genotoxic activity [29]. All sorts of aberrations as c-mitosis, chromosome clumping, chromosome fragments, anaphase bridge, sticky anaphase and telophase observed at higher concentration treatment. C-mitosis is usually due to disturbance in spindle structure and function, chromosome clumping and stickiness indicates severe cell damage and chromosome bridge and fragments indicate structural alterations in chromatin and chromosome. C-mitosis and chromosome fragments indicate weak cytotoxic effect that could be reversible, clumping and stickiness indicate high and irreversible toxicity[22,23]. Statistical analysis shows significant changes in MI at treatment concentration and duration.

Fig. 1: *a- Plant part of Diospyros lanceifolia Roxb., b- seeds, c- Bulbs of Allium cepa L in control and treatment vial, d- control and deformed roots, e- sticky anaphase & disorganised metaphase, f- sticky telophase bridge, g- c-mitosis, h, o- anaphase bridge, i, j, k- chromosome clumping, l-disorganised metaphase, m-chromosome fragment, n-sticky telophase with cell plate formation.*

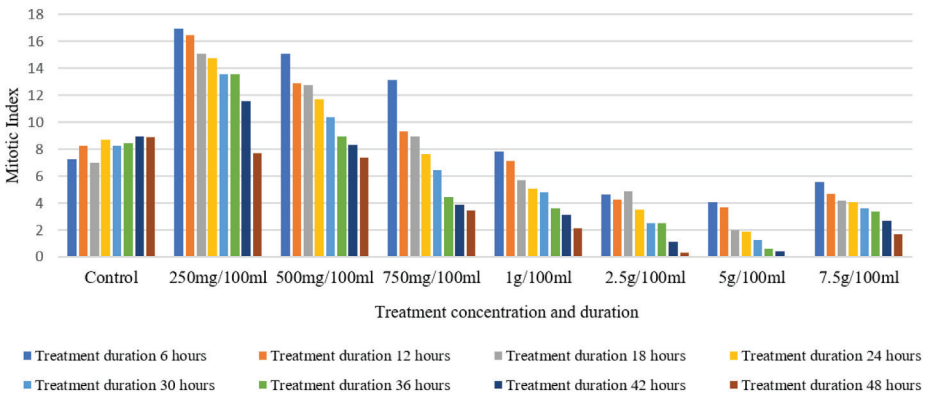

Fig. 2: *Mitotic index of Allium cepa cells at contral and after treatment with aqueous fruit extract of Diospyros lanceifolia*

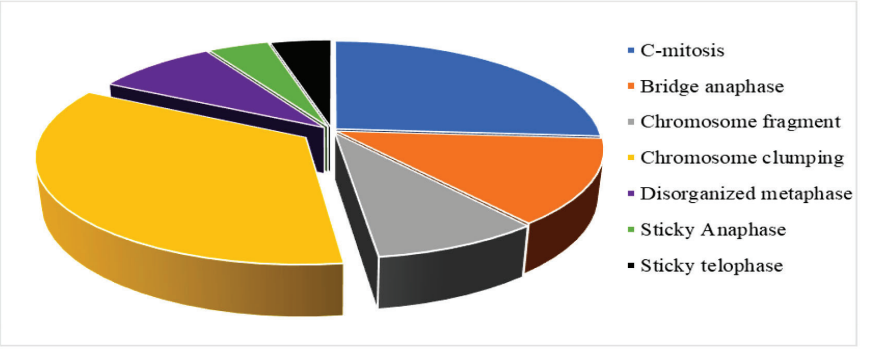

Fig. 3: *Pie diagram respresenting Frequency of Aberrant cell (%)*

Cytotoxicity assessment using *Allium cepa* Test over a two-day period reveal dose dependent low to high cytotoxicity and clastogenic effect of the aqueous fruit extract. Since water extract of fruits of *Diospyros lanceifolia* used as fish poison, with regular use for prolonged duration there is much likelihood of it to cause detrimental effects on the water quality, so the plant extract should be appropriately used intermittently and in low doses for traditional practices.

Conflict of Interest

The authors have no conflict of interest. JSR performed the *Allium cepa* Test and SB analyzed the results, designed and drafted the manuscript.

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CHAPTER

Effect of temperature on the development of *Sclerotium rolfsii*: an isolates of Tripura

Amar Bahadur*, Durga Prashad Awasthi and Prasenjit Debnath

Department of Plant Pathology, College of Agriculture, Tripura, Lembucherra, Agartala, India
*Email: amarpatel44@rediffmail.com / agcollege07@gmail.com

Abstract: *Sclerotium rolfsii* is common soil-born fungus that infecting awide range of cultivated and wild plants species and caused root rot, stem rot, wilt, foot root and rot of fruit in contact with the soil. It is most active during warm, wet weather in tropical and subtropical regions. Suitable climate for the fungus is worm and humid as fast growing mycelia and sclerotia formation within a week. *S. rolfsii* can overwinter as mycelium in infected tissues or plant debris. It usually persists as *sclerotia.* The host range of *S. rolfsii* comprises over 500 plant species. In Tripura last three years observing of *Sclerotium rolfsii* on different host crops *viz,* vegetable, fruits, flower, ornamentals crops in Tripura. Collected fungus mycelia with sclerotia from infected host and isolated in the laboratory on the PDA medium. Isolates of *Sclerotium rolfsii* collected from College of Agriculture, Tripura research farm in infected field of pigeon pea, tomato, sunflower, yam and hibiscus. Five isolates of *Sclerotium rolfsii* collected from different host plants at College of Agriculture, Tripura, Lembucherra research farm during 2017-2019. The isolates cultured in the laboratory and observed cultural characters, vegetative compatibility, colony morphology, mycelial growth, sclerotium formation, sclerotial size, colour and number of sclerotia at different temperatures at 20^0, 25^0 and 30^0C. The isolates varied in colony morphology, mycelial growth rate, sclerotium formation, production of sclerotial size and color.

Keywords: *Sclerotium rolfsii*; hosts; compatibility; sclerotia; temperatures.

Sclerotium rolfsii Sacc. (teleomorph *Athelia rolfsii* (Curzi) Tu & Kimbrough) is an omnivorous, soil-borne fungal pathogen, causes disease more than 500 plant species of agricultural and horticultural crops worldwide and with an extensive host range [1,2]. The genus, *Sclerotium* was characterized by production of small tan to dark brown or black spherical sclerotia with internally differentiated rind, cortex and medulla. The disease caused by *S. rolfsii* in various crops is widely reported and can cause considerable loss to plant stand when soil moisture is high and temperature is warmer

(30°C), which favours sclerotial germination and disease development . The incidence decreases with the age of the crop. The disease causes about 20% yield loss in tropical areas and potential to cause up to 95% losses in chickpea [4]. A study on variability of *S. rolfsii* isolates is important to know changes occurring in the population. Previous studies also reported the variability among the isolates of *S. rolsfii* from various hosts and geographical in terms of morphological and cultural characteristics [5]. The fungus does not produce asexual spores and perpetuates as sclerotia on plant debris and in soil[6]. *Sclerotium rolfsii* Sacc. is a serious disease for a wide range of plants, including vegetables, fruits, ornamental plants, and field crops[7]. Under moist conditions, white mycelial growth which develops on the dead grass and later on sclerotia ranging from white or light to dark brown on the mycelium are observed [8]. S. rolfsii isolates can be diverted into different mycelia compatibility groups (MCGs) based on mycelial interactions among isolates. The role of MCGs is important in defining field populations of fungi and facilitating genetic variation in fungal species, where the sexual reproductive stage (teleomorph stage) of the life cycle has a minimal impact on the disease cycle [9]. The fungus is a destructive pathogen that can infect more than 500 plant species commonly seen in tropical and subtropical regions [8]. A wide range of symptoms are produced by this pathogen on its hostsincluding crown and root rot, stem canker and damping-off and resulting diseases called southern wilt, blight or stem rot [10]. The pathogen is importance when the disease severity is high in the fields and crop loss may between 10-25% in fields [10]. *Sclerotium rolfsii* is the mycelial stage of the basidiomycete *Athelia rolfsii,* a worldwide soil-borne plant pathogenic fungus that lacks a conidial stage. The host range of S. rolfsii comprises over 500 plant species, mostly dicotyledons, but also some monocotyledons [11-13]. The main disease symptoms caused by this pathogen include crown and root rot, stem canker or damping-off. The resulting disease is referred to as southern blight, southern stem rot, or sclerotium root rot [1, 14]. *S. rolfsii* produces abundant coarse, white mycelia on infected host tissues and forms sclerotia [14].The fungus overwinters in soil by means of sclerotia, as well as by mycelia in infected plants or infested plant debris [14].

Sclerotium rolfsii is an important ubiquitous and polyphagous soil borne pathogen of the anamorphic stage, the teleomorph stage is a sexual stage and rarely observed. The teleomorph of fungus is *Athelia rolfsii* (Curzi) C.C. Tu & Kimbr. Belongs to basidiomycete. The fungus was named *Sclerotium rolfsii* by Saccardo [15]. *Sclerotium rolfsii* has an extensive host range; frequently reported to cause root diseases in at list 500 species of dicotyledonous and monocotyledonous plants which represent 100 families [16]. *Sclerotium rolfsii* host reported worldwide and fungus persists in many weed hosts [17,18]. More than 50% yield reduction could occur as a result of Sclerotium infection in some case [19]. Sclerotia serve as the primary inoculums for the initial

infection of host plants. *Sclerotium rolfsii* sacc. is a well known soil inhabiting and most destractive soil born fungus. *S. rolfsii* commonly occurs in the tropics, subtropics, and warm temperate regions. Incidence of collar rot caused by *S. rolfsii* on many dicotyledonous including several monocotyledonous host species have been reported [20]. It perpetuates through sclerotia which are considered to be extremely hardy and relatively resistant survival structures [21], principle means of dispersal [22]. Management of collar rot is difficult since it is soil-borne in nature, has a wide host range and survives for longer periods through the sclerotial bodies *Sclerotium rolfsii* is predominantly distributed throughout the tropical and subtropical regions where the temperature reaches higher level during rainy season. *Sclerotium rolfsii* having sporophytic activity in soil can survive in soil for many years by producing sclerotial bodies. The isolates of *sclerotium rolfsii* are having different modes of survival under adverse climatic conditions, among them the dormant mycelia forms into dark coloured, circular or globe shaped sclerotia. The symptomology of the fungus is varying is according to the host plant they infect, type and age of the plant are major favouring factors for the fungal isolates infection [23]. Disease caused by this pathogen lead to heavy losses in vegetable crop yield especially during the wet season, when weather conditions are favourable for both crop production and for the growth and dissemination of the sclerotia of the pathogen [24].These sclerotia constitute the primary inoculum of the pathogen as well as its principle means of dispersal and the sole organs by which the fungus servives adverse environmental conditions,awaiting germination and infection of susceptible hosts when favourable environments return [25]. *S. rolfsii* attack can make a 25-80% decrease in yield [26].

Seedlings are very susceptible to die once they become infected. Adult plants have woody tissue are girdled by lesions and eventually die. *S. rolfsii* can infect any part of a plant under favorable environmental conditions including stem, roots, fruits, petioles, leaves, and flowers. The first signs of infection dark-brown lesions on the stem at the soil level; the first visible symptoms are progressive yellowing and wilting of the leaves. Fungus produces abundant white, fluffy mycelium on infected tissues and the soil. *Sclerotia* are uniform size produced on the mycelium, roundish and white when immature then becoming dark brown to black. Mature sclerotia resemble mustard seed. Collar rot disease of *Amorphophallus paeoniifolius* is caused by the soil born basidiomeycete fungus *Sclerotium rolfsii* causes rotting of collar region which ultimately topples down and is responsible for heavy reduction in yield and qualitative degradation of the crop. The yield loss varies from 25 to 100% depending upon the nature of cultivars and various predisposing factors [26]. *Sclerotium rolfsii* commonly occurs in the tropics, subtropics, and warm temperate regions. *S. rolfsii* can overwinter as mycelium in infected tissues or plant debris. It usually persists as *sclerotia. Sclerotia*

are disseminated by cultural practices, infested transplant seedlings, water, wind, and possibly on seeds. *S. rolfsii* grows, survives, and attacks plants at or near the soil line. Before the pathogen penetrates host tissue it produces a considerable mass of mycelium on the plant surface, a process which can take 2 to 10 days. Penetration in the host tissue by the pathogen produces an enzyme which deteriorates the hosts' outer cell layer. This results in tissue decay, further production of mycelium and the formation of *sclerotia. S. rolfsii* is able to survive within a wide range of environmental conditions. Growth is possible within a broad pH range, best on acidic soils. Germination is inhibited at a pH above 7.0. Maxiumum mycelial growth occurs between 25 and 35 C. High moisture is required for optimal growth of the fungus. *Sclerotia* fail to germinate when the relative humidity is much below saturation. *S. rolfsii* is able to form new physiological races emergence is a major problem in controlling these pathogens. Each race has a growing ability and a different degree of pathogenicity to the host plants. Present study focused on the growth variation, sclerotia production of *Sclerotium rolfsii* isolated from different hosts on PDA media under different temperature.

Materials and Methods

The most common symptom is a brown to black rot of the stem near the soil line. The stem becomes girdled and the plant wilts suddenly and dies. A coarse, white, cottony fungal growth, containing white, spherical resting bodies (sclerotia) covers the affected area. The sclerotia soon become light brown and resemble mustard seed. As the symptoms showing host plants were collected during the year 2017-19 from the experimental farm of College of Agriculture, Tripura. This research was conducted in the Plant Pathology, College of Agriculture, Tripura. Infected host plants samples were collected from five different crops *viz*., pigeon pea (*Cajanus cajan*), elephant foot yam (*Amorphophallus paeoniifolius*), flowering plants (*Habiscus rosa- sinensis*), tomato (*Lycopersicon esculentum*) and sunflower (*Helianthus annuus*) (Fig.1). On the basis of symptoms and sclerotia formation on coller reign of the host plants with myceia and slerotai and were collected.

Collection of Sclerotium rolfsii isolates

Sclerotial bodies with infected samples as collected from diseased plant were disinfested, washed, inoculated on PDA medium and incubated at 25 ± 2^0C and monitored for the growth of fungal colonies. The isolates were purified by growing single sclerotia from each colony on PDA slants as pure isolates were obtained for further examination. Pure cultures of final isolates were maintained on PDA slants and kept in the refrigerator until required.

Morphological and cultural variability

Growth of *sclerotium rolfsii*. isolated from the five different plants were evaluated on the PDA media for growth variability study. 15 ml of molten potato dextrose agar were dispensed into each of 9 cm sterile petri pates. Plates were incubated in different temperature like 20°C, 25°C, 30°C. Observations of the myceliel growth of *S. rolfsii*. were taken regularly up to 8 days and after that allowed the *sclerotium rolfsii*. to produce scholorotia up to 20 days. The average size of the sclerotia produced by each of the isolates were also determined.

Average diameter of sclerotia in mm 1.3 to 1.5, no of sclerotia per plate was 500 to 1000 at 25^0C. Test weight of 100 nos sclerotia was 05 to .08 gm. Scleortia of the colour is brown to dark brown, mycelia color white to cremish white observed. The plates were incubated at 25± 2^0C with three replicate for morphological characteristics such as radial growth (colony diameter in mm), colony morphology, sclerotial production, size of sclerotia (mm) and colour of sclerotia were studied. Data were taken 7 days after inoculation, while number of sclerotia and colour of sclerotia of each isolate were recorded up to 20 days after inoculation.

Mycelial compatibility

For these studies, mycelial discs (5 mm diameter) taken from the edge of an actively growing colony of each isolate were placed at 40 mm apart on opposite sides of Petri dishes (90 mm d) and incubated at 25 ± 2 ^{0}C. Two isolates were paired in one dish and the test was repeated twice. The pairings were examined macroscopically after 10–15 days for the presence of an antagonistic (barrage or aversion) zone in the region of mycelial contact as described by Punja and Grogan [27].

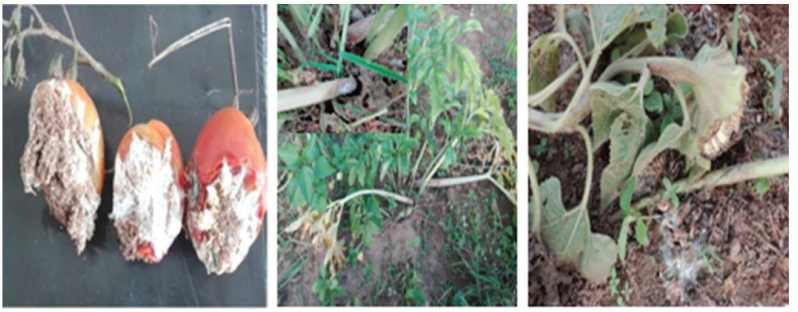

Fig.1: *Mycelia and Sclerotia of Sclerotiium rolfsii on affected crops*

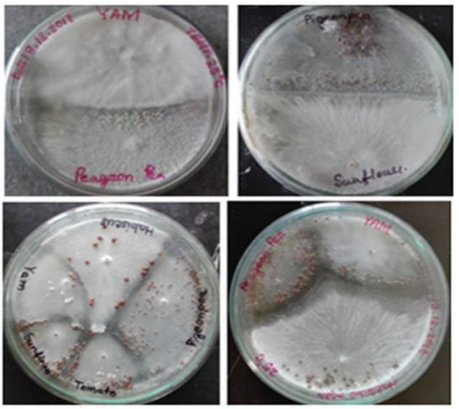

Fig.2: *Incompatibility reaction of Sclerotiun rolfsii*

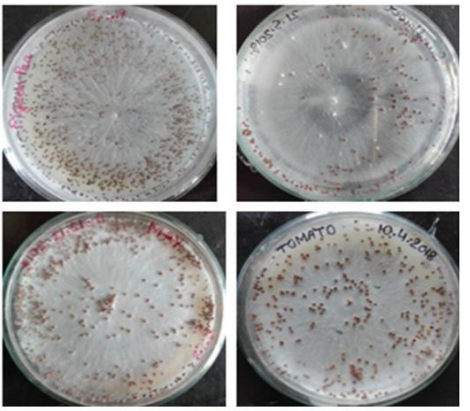

Fig.3: *Mophological variance of Sclerotium rolfsii*

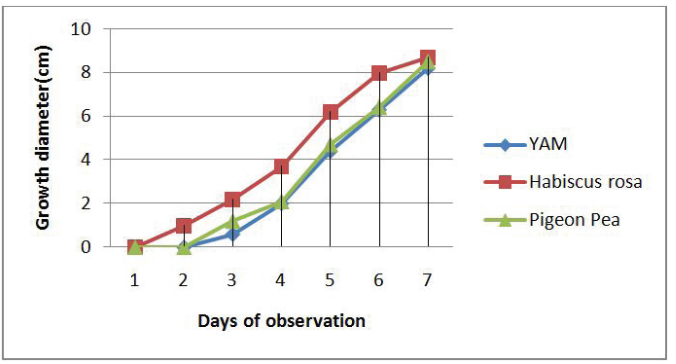

Fig. 4: *Growth curve of Sclerotium rolfsii Sacc. at 20 °C*

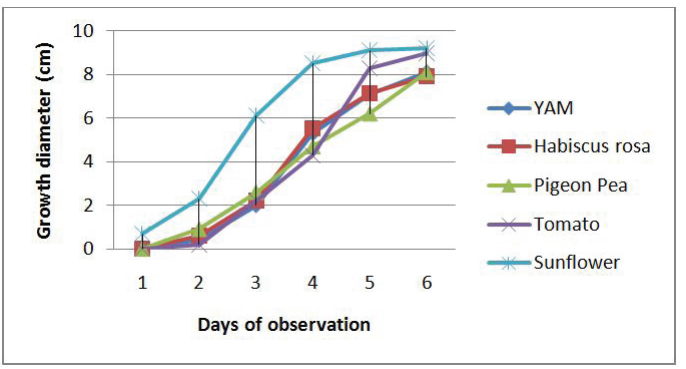

Fig. 5: *Growth curve of Sclerotium rolfsii Sacc. at 25 °C*

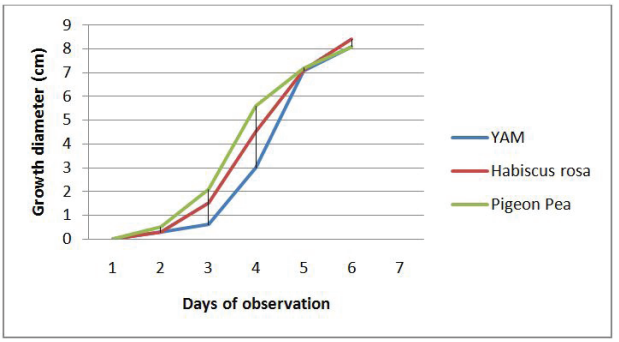

Fig. 6: *Growth curve of Sclerotium rolfsii Sacc. at 30 °C*

Results and Discussion

Isolates of *S. rolfsii* varied in growth parameters *i.e,* colony morphology, mycelial growth, colony colour, sclerotial production, number and size of sclerotia (Fig. 2 & 3). The fungus produces white cottony mycelium. Out of isolates, colonies of 5 were fluffy and wooly. The growth rate of the isolates varied substantially. The variation of growth due to differences in biology, genetic differences and nutrient level in the soil [28]. The fungus is characterized by the production of small spherical sclerotia with differentiated size and colour. The fungus produces sclerotia at the edges of the petri plates from 7 days up to 20 days after inoculation at 25^{0}C when the agar media were completely covered with mycelia. Production of sclerotia varied among isolates. Pigeon pea isolates produced more number of sclerotia (>1000/plate), while majority of the isolates produced fewer nos of sclerotia (300 /plate) in hibiscus isolates. Growth of colony in diameter was higher in sunflower isolates at 25^{0}C (Fig.5) and temperature at 20^{0}C colony growth of diameter was higher in hibiscus isolate (Fig.4), at 30^{0}C the differences was recorded after 2 to 5 day in colony diameter but the trend was same in all of three isolators (Fig 6). The colour of sclerotia of isolates was generally dark to reddish brown at maturity, while sclerotia were light brown in some isolates. Sarma *et al.* [23] reported that isolates of *S. rolfsii* collected from various hosts/soil samples and localities in India varied in colony morphology, mycelia growth rate, sclerotium formation, teleomorph production, sclerotial size and colour. Mycelial compatible reactions of the two isolates intermingled at the zone of interaction a visible border between them (Fig.2). A clear barriage zone of dead mycelia was formed in combinations which showed antagonistic reactions with each other (Fig.2). In all the antagonistic reactions, sclerotia were not formed at the interaction zone. The high rate of antagonistic reactions in the mycelia compatibility test shows the extent of diversity among these isolates of *S. rolfsii* [29]. The death of mycelia at the interaction zone is

attributed to the heterokaryotic condition of the nuclei, but the involvement of toxin cannot be ruled out [30]. The results are in accordance with the findings of who reported morphological variability and mycelial incompatability among twelve *S. rolfsii* isolates collected from various localities of chickpea growing areas of Punjab [29]. Kokub *et al.* [31] observed considerable variation in the growth rate of eight S. rolfsii isolates and mycelial incompatability. Mycelial compatibility groups based on hyphal interactions and barrage zone formation was reported in isolates of *S. rolfsii* from peanut fields [13,32]. Sarma *et al.* [23] observed 8.9% compatibility out of 325 combinations of isolates of *S. rolfsii* collected from various hosts/soil samples from diverse geographical origins of India. Our results are in agreement with the findings of [33-35] who reported cultural variation, mycelial compatability and aggressiveness among *S. rolfsii* on different hosts such as sunflower, mungbean, sugarbeet, lentil, sweet pumpkin, cabbage and cauliflower. The fungus can survive for years as sclerotia in the soil or in host plant debris. Sclerotia spread with soil movement, infested plant material and contaminated equipment. Infection and disease development are favoured by warm, moist conditions. Control of *Sclerotium* diseases is difficult and depends on a combination of cultural, biological and/or chemical methods. In unfavorable environments, *S. rolfsii* is able to form new physiological races in different characters. The emergence of the new physiological races is a major problem in controlling these pathogens. Each race has a growing ability and a different degree of pathogenicity to the host plant. The existence of many races of physiology causes the difficulty of controlling pathogens due to lack of further understanding of the diverse new physiological races.

Control of sclerotium diseases is difficult when soil and weather conditions favour the fungus. Management systems that can reduce the disease severity through the plant residues decomposed before planting, soil solarisation, black plastic mulching (BPM) has been shown to reduce disease incidence and enhanced greater crop yields. buried plant debris at least 10 cm beneath the surface. Non-susceptible crops such as maize in crop rotations to reduce inoculums levels in soil and drench plants with the recommended fungicides. Good cultural practices include roguing, eliminating weed hosts, and avoiding crop injury during cultivation, increasing plant spacings. *S. rolfsii* has a broad host range, crop rotation has less of a chance of being successful as there are few resistant crops. Amendment may increase populations of antagonistic soil microorganisms. *Trichoderma spp., Bacillus subtilis, Penicillium* spp., and *Gliocladium virens* are known mycoparasites of a number of plant pathogens. *T. viride* has been shown to provide good control and *T. harzianum* colonizes *S. rolfsii* hyphae, disrupts mycelial growth and kills the organism. *Gliocladium virens* have been shown to rapidly degrade *S. rolfsii* in soil. Continuous research over the past report, management of the pathogen has remained a chalange. Global worming hit the word

in this connection crop production are a challenge of grower, occurrence of the new pathogen in the nature is very difficult to manipulate them. Chemical pesticides are health hazardous to humane, Environment and beneficial microorganisms.

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CHAPTER

Optogenetics: A technique for neural system

Dhrubjyoti Sharma[1*], Soumitra Nath[1,2] and Bibhas Deb[1,2]

[1,2] Bioinformatics Centre, Gurucharan College, Silchar, Assam, India
[1]Department of Biotechnology, Gurucharan College, Silchar, Assam, India
*Email: dhrubsharma97@gmail.com

Abstract: Optogenetics is a biological technique that involves the use of light to control neuron function that have been genetically modified.Opsin, a photo reactive protein found in the photoreceptor cells of retina is used for this technique. When an opsin is placed in the brain, it functions as a photosensitive molecule and based on the color of illumination, the brain functions accordingly. Various techniques can be used for the transfer of opsin from the microbial cells such as vector method, CRE based technique and use of knock-in mouse. The precision by which the opsin induced by light transfers messages to specific cell type and the speed (milliseconds) it takes makes this technique possibly accurate and most suitable technique for the study of neural network. This approach of using light as a control stick has opened huge area for the study of biology of neural structure and function as well as the study of disease and health.

Keywords: Optogenetics; neuron; opsin; photosensitive.

Light is known for its dual nature i.e. particle and wave character, but because of their short wavelengths, wave characteristics of light generally get undetected [1]. In plants, its effects on growth include phototropism, germination, or elongation of plant parts as well as on differentiation. Photosynthesis, a process by which plants convert light energy into chemical energy which involves movement of stomata, chloroplasts, and leaves is dependent upon the light[2]. In animals, a certain part of the brain is used for visualization, which involves the use of light as a signal[3]. A biological process commonly known as circadian rhythm is a process that involves a cyclical physiological change. It can be found in animals, fungi, plants, and cyanobacteria. This process regulates the sleep-wake cycle of a 24 hours' duration, and for this natural process, it requires the presence of light[4]. The human brain is the central organ of the nervous system which is involved in the functioning of the whole human body. It receives information from the outside world with the help of organs such as ears and eyes, which then transmit the signals via sensory nerves which are further processed at

the brain. The reaction to the message will then be sent via motor neurons to the body[5]. The messages are conveyed via electrical signals with the help of small molecules known as neurotransmitters. The signals are transmitted through neurons based on the voltage difference i.e. uneven distribution of ions or electrically charged particles[6].

Optogenetics is a technology that involves the integration of genetics and optics. It can help in achieving certain events within specific cells or tissue using neurons. A small molecule is inserted into the targeted part of the brain, which helps in the conversion of light radiation into electricity in neurons[7]. Optogenetic approaches have allowed us to modify, to trigger, and to mimic pathological behaviors which can introduce us to innovative therapeutic interventions. Opsins which are required for the processing of the neurons and some devices are required for the study of neurons using lights. In this review, we have shown the basic requirement and methods involved in the optogenetics technique.

Opsins: The Light-Sensitive Protein

All organisms ranging from prokaryotes to eukaryotes express photoreceptor proteins known as rhodopsins which help the organism to sense and respond to light. Opsins are membrane proteins found in animals that act as a light sensor and are related to rhodopsin, a photoreceptive molecule. They have a molecular mass of up to 30-50 kDa. Opsins are classified into two types based on the primary sequence, and mode of action i.e. type I opsin (microbial opsins), found in fungi, archaea, and algae, and type II opsins (animal opsins), found in animals and humans.

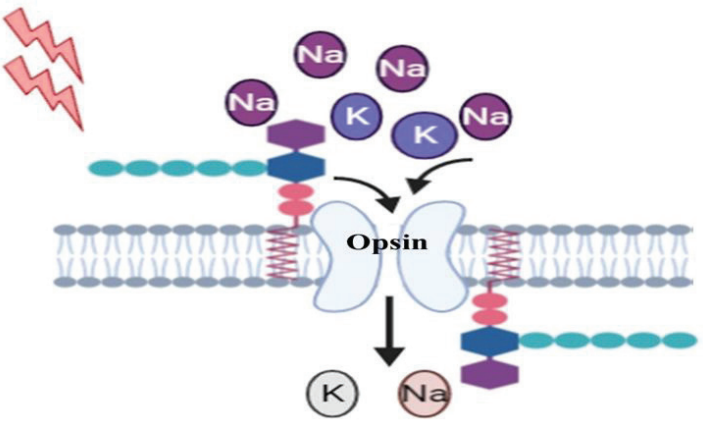

Fig. 1: *Transfer of ions via opsin when stimulated by light.*

Type I opsins include ion channels, light-driven ion pumps, and sensors. When stimulated by light, these opsins mediate transmembrane ionic current and thus cause

specific biological responses such as photophobia and phototaxis (Fig. 1). Microbial opsins are mostly used in research related to optogenetics because of their ease of genetic engineering and faster kinetics. Whereas animal opsins by coupling with G-protein mediated transduction mediate transmembrane ionic potential. They are primarily involved in circadian clock and dim light vision[8].

In humans, there are nine known opsins. Three of which are expressed in cone photoreceptor cells (helps in determining the three colors (i.e. green, blue, and red). Melanopsin helps in the functioning of pupil constriction of the eyes and helps in the circadian regulatory system. Rhodopsin is expressed in rod photoreceptor cells, that helps in functioning under dim light[9]. Encephalopsin is expressed in the brain and retina, it belongs to the light-sensitive transmembrane receptor family[10]. Neuropsin helps in the adaptation of CNS to changes in the external environment. It controls the neurotransmission of GABA (gamma-aminobutyric acid) through NRG-1 (neuregulin-1) and its receptor ErbB4[11].

Table 1: *List of opsins present in humans*

Opsin	Abbreviation	Location (in eye)	Chromosomal location	No of introns
Green opsin	OPN1MW	Cone	Xq28	5
Blue opsin	OPN1SW	Cone	7q32.1	4
Red opsin	OPN1LW	Cone	Xq28	5
Melanopsin	OPN4	ipRGC	10q23.2	9
Rhodopsin	OPN2	Rod	3q22.1	3
Encephalopsin	OPN3	Rod, Cone	1q43	3
Neuropsin	OPN5	Neural retina	6p12.3	6
Retinal-G Protein-coupled Receptor	RGR	RPE cells	10q23.1	6
Peropsin	RRH	RPE cells	4q25	6

RPE: retinal pigment epithelium, ipRGC: intrinsically photosensitive retinal ganglion cells

Neurons Modification and Expression of Opsin Genes

It is necessary to deliver the opsin gene efficiently so that it can be expressed. The delivery of the gene can be done by various methods (Fig.2).

a) The first method is the viral vector-based transfer, which is the most routinely used technique for transgene introduction and expression in the host cell. In this method, an engineered virus containing an opsin gene including promoter is injected into the area of interest in the brain. This method is fast and offers robust expression. The viral vectors generally include adeno-associated virus, lentivirus, rabies virus, herpes simplex virus, and canine adenovirus. Various studies have shown some promising results in model organisms such as rats, mice, zebrafish,

primate models, etc[12]. However, the length of gene material (length of genetic material) these viruses can carry is limited. Thus it limits the size of the promoter it can carry as well as the option of the cell types in which it can transfer its genetic material [13].

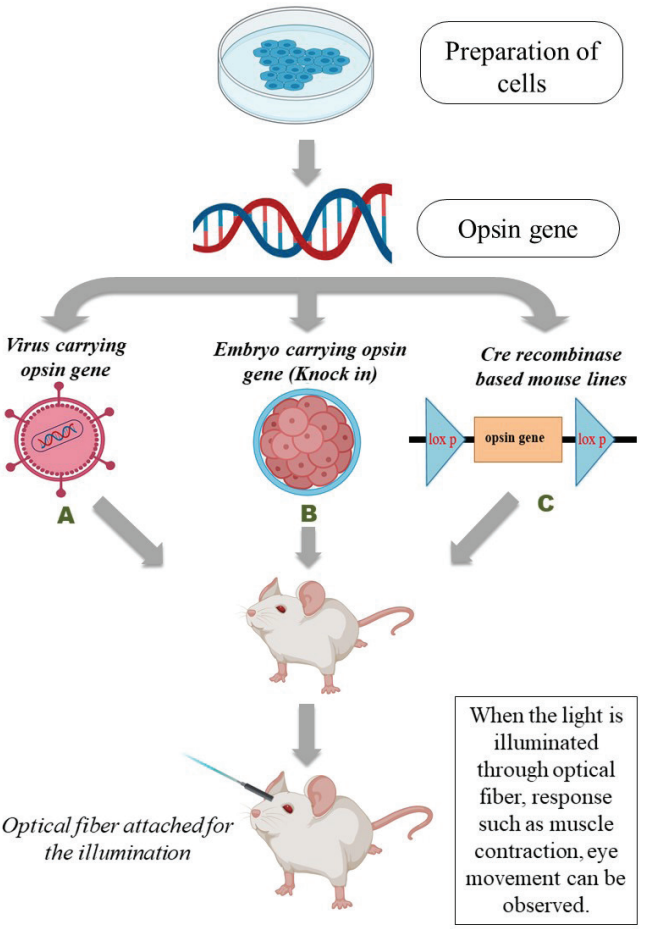

Fig. 2: *Delivery of Opsin gene using (A) Viral vector; (B) Knock-in mouse, and (C) Cre recombinase-based mouse.*

b) The second method is the use of transgenic animals or knock-in animals. In this method, a large promoter fragment can be used for the specific expression of the gene. Using this method, the problem of the limited size of genetic material can be avoided. However, the limitation of generating a stable transgenic line is its high cost. For every desired opsin a new mouse line must be generated which makes this process tedious [14].

c) Third, one of the modern technology used to target genetically defined cell types using Cre recombinase-based mouse lines in combination with viral vectors.This method uses an enzyme Cre recombinase which helps in catalyzing recombination between two loxP sites that flank a gene of interest. The most important advantage of this method is its option for upgradation i.e. an already existing Cre mouse line can be used again for other regions of interest with only change in the use of the viral vector [15].

Response by Opsin in Neurons

The opsins can be switched off or on in the specific regions using illumination. Laser lights are mostly used in optogenetics because they can be coupled with optical fiber efficiently and also because it has the property of narrow bandwidth light [16]. Optical fibers coupled can be used to deliver light to intracranial locations and thus can allow optical control of deep brain structure. Because of its small diameter, it becomes easier to couple it with laser light source efficiently and it causes less tissue damage because of its size [17].

Light-emitting diode (LEDs) can also be used as an illuminator because of their narrow spectral tuning with diverse color options, small size, and low cost. But the low-efficiency coupling of LED optical fiber limits the use of LED light sources for some required wavelengths of light and generating heat during the process can be problematic [17, 18].

Animal Models used for Optogenetics

1. **C. elegans***:* In genetically modified *C.elegans*, neuron activity and muscle wall motor neurons were possible to control by harboring the gene, coding for channelrhodopsin. With the help of ChR2 and NpHR, body wall muscle contraction of *C. elegans*were able to get controlledbidirectionally. Various synaptic protein defects were investigated on a large scale basis because of the positive outcome of light in *C. elegans*[19].
2. **Fly**: Fly was used for the study of appetite odorant learning at the receptor and neuronal basis of the nociceptive response. In fly, it was found that the blue light spectra were difficult to study because of the presence of innate behavioral responses to blue light. This complication can be solved by using the red-shifted optogenetic tools for its excitation and inhibition [19].
3. **Zebrafish:** In zebrafish, the role of somatosensory control of escape behavior was studied. Zebrafish is known for its short generation time and the ease by which a foreign DNA can be inserted [19].

4. **Mouse:** Optogenetic modulation of the mouse was able to control monoaminergic systems. Mice have also been used to study the mechanism of cortical intervention in mouse modelsof depression and amygdala circuits, which are responsible for anxiety and fear. Till now, the mouse has been used the most as a model organism[19].

Therapeutic Applications

Optogenetics is a versatile tool, thus wide ranges of diseases can be diagnosed or cured just by selecting a different neuronal target. Following are some of the diseases which were studied using optogenetics:

1. **Depression**: Optogenetics were applied to improve clinical symptoms of depression. By mimicking burst like patterns of cortical activity delivered via an optical fiber influenced a significant anti-depressant like effect in mice. The simulation was done in the medial prefrontal cortex (mPFC). It was shown that a simulation on mPFC exerted a long-lasting and rapid antidepressant response [20].
2. **Sclerosis**: It is a demyelinating disease, a disease in which immune-mediated demyelination of neuronal fibers impairs impulse conduction and thus causes degeneration of axon in the central nervous system. A repeated long term stimulation of neocortex neurons affected by sclerosis allowed the formation of mature oligodendrocytes. Repeated stimulation of the neurons allowed the remyelination of the damage neurons and also the recovery of the function was observed [21].
3. **Parkinson's Disease**: Halorhodopsin, an inhibitory chloride pump is inserted into human embryonic stem cells by a viral infection, and the cells were then left for differentiation which will finally form into dopamine neuron-like cells. Light activation triggers an influx of chloride ions and therefore reduces neuronal activities. The dopamine neurons were then transplanted, and it was found that transplantation of dopamine neurons helps in restoring motor functions [22].
4. **Controlled Heart Rate**: When rhodopsins are implanted into cardiomyocytes, the cells responsible for the formation of cardiac muscle, heart rate can be controlled directly with the help of optogenetics, though this method is currently in development and is the principle behind optogenetic pacemakers[23].

Conclusion

Optogenetics, a field which changed the way we used to look at neuroscience,which helped us to correct the neural disorders using experiment on a neural network with the help of photosensitive proteins i.e, opsins. It can facilitate in understanding the neural circuit components in normal as well as in dysfunctional behavior such as in the study

of diseases like Parkinson's disease, heart attack, sclerosis, etc. Intercellular responses and neural activity can be stimulated with the help of this technique. Tools required for this technique need to be modified for a better understanding of the neural activity as it is still in the nascent phase. The coming future should see the progress made by this technique in the diagnosis and treatment of diseases and also in the study of neural networks.

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Preliminary report regarding incidence of collar Rot on Durantaerecta L. (Golden Dewdrop) caused by Sclerotium rolfsii Sacc. in Tripura In : *Biological Sciences: Impacts on Modern Civilization, Current and Future Challenges* by *Anupam Guha* © New Delhi Publishers, New Delhi: 2021, 194-197.
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CHAPTER

Preliminary report regarding incidence of collar Rot on *Durantaerecta* L. (*Golden Dewdrop*) caused by *Sclerotium rolfsii* Sacc. in Tripura

Durga Prasad Awasthi*[1], T. Bhattacharjee[2] and N. Paul[3]

[1]Department of Plant Pathology, College of Agriculture, Tripura, India
[2]Department of Horticulture, College of Agriculture, Tripura, India
[3]Department of Soil Science, College of Agriculture, Tripura, India
*E-mail: pathodurga@gmail.com

Abstract: *Durantaerecta* L. (Golden dewdrop) are grown as ornamental plants at road sides, parks and gardens. The plants are often pruned for giving them different desirable shapes. Having high aesthetic value and luxurious growth plants are observed for various diseases. A survey was undertaken in the year 2018-19 and 2019-20to know incidence of significant diseases hampering its growth under agro-climatic condition of Tripura. Plants were observed for different diseases symptoms *in vivo* followed by isolation and further confirmation of causal organism by pathogenicity test *in vitro*. During present study plants were observed for development of symptoms of wilting and the isolated fungi was confirmed to be *Sclerotium rolfsii*Sacc. showing cultural characteristics of white cottony mycelial growthalong with reddish brown sclerotia.

Keywords: Sclerotia; PDA medium; Pathogenicity test.

Durantaerecta (family: *Verbenaceae*) commonly referred to golden dewdrop, pigeon berry, angel whisper, or skyflower is one of the traditional medicinal plants [1].In Tripura, the *Durantaerecta* L.are grown as ornamental plants at road sides, parks and gardens. The plants are often pruned for giving them different desirable shapes. Having high aesthetic value and luxurious growth, plants may suffer from different kinds of diseases. Thus, a survey was undertaken in the year 2018-19 and 2019-20 for knowing incidence of significant diseases hampering its growth under agro-climatic condition of Tripura.

Materials and Methods

Plants were observed *in vivo* condition for development of different diseased symptoms as well as samples were examined and compared *in vitro*for confirmation of disease symptoms with the help of standard disease atlas and recommended text books. Samples from stem portion of the plants near collar regionand roots were collected and brought into the laboratory. Concern pathogen was isolatedaseptically in Potato Dextrose Agar (PDA) slants. For isolation small bits from the diseased plant parts were cut and washed in running tap water followed by washing in distilled water. The washed samples were then dipped in 0.1% $HgCl_2$ (Mercuric Chloride) solution for 30 to 45 seconds for surface sterilization followed by washing in sterile distilled water. The PDA slants were keptfor incubation at 28 ± 1°C in B.O.D. incubator for 14 days. Sub culturing was done by taking 6 mm discs or single sclerotia of fungus. The isolated fungus was inoculated into healthy plants grown in pots for confirmation of Koch postulates.

Results and Discussion

Symptoms observed and pathogenicity test

Among various plants of golden dewdrop some plants show characteristic symptoms of yellow colour discolouration of leaves which later on turns dry, becomes light brown to dark brown, leaves also shows drooping and withering symptoms. When plants are uprooted and collar regions are observed a characteristic white mycelia growth of fungi along with numerous reddish coloured sclerotia are visible. It was also observed that the occurrence of symptoms may appear from young plants to mature plants (Figs. 1 & 2). The pathogen was confirmed to be *Sclerotium rolfsii* Sacc.

1: *Dry leaves*

2: *White Mycelial Growth along with Sclerotia*

Figs. 1 & 2: *Occurrence of symptoms and growth of mycelia*

Cultural characteristics include white light cottony growths of mycelium which on an average completely covers Potato Dextrose Agar (PDA) petri plate of 9.0 cm within 120 hours. Small reddish brown sclerotia were found to be distributed throughout the petri plates.

Results of pathogenicity test further confirm development of above-mentioned symptom after 8 to 10 days of inoculation of inoculums followed by successful re-isolation of the pathogen. In control pots no disease symptoms were developed. Other authors have also conducted similar types of experiments like pathogenicity test of *S. rolfsii* on sunflower was proved by Datar and Bindu[2]. Typical symptoms are produced within a week ofthe inoculation. Maximum mortality of groundnut seedlings was observed in 15 days old plants as reported by Kulkarni *et.al*.[3].

Aycock in the year 1966 reported *S. rolfsii* Sacc. (teleomorph *Atheliarolfsii* (Curzi) Tu & Kimbrough) as a devastating soil-borne plant pathogenic fungus with a wide host range [4]. The fungus forms differentiated sclerotia and sterile mycelia[5]. Although, there are several other sclerotium producing fungi, the fungi characterized by small tan to dark-brown or black spherical sclerotia with internally differentiated rind, cortex, and medulla were placed in the form genus *Sclerotium*. However, the teleomorphic state was discovered later, confirming that the fungus was a basidiomycete. *S. rolfsii* usually causes collar rot, but spotted leaf rot with a single tiny sclerotium in the centre[6].

It is a destructive fungal pathogeninitiating diseases in many mono and dicotyledonous plants encompassing more than 500 host species [7].

Previous studies among different parts of world shows that occurrence of *Clerondendron yellow mosaic virus* on golden dewdrop (*Durantaerecta*) in India [8] and leaf spot disease caused by *Pseudomonas cichorii* and a *Xanthomonas species* in Florida [9].

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Discovery of therapeutic lead molecules from natural resources: A outstanding contribution to modern medicine In : *Biological Sciences: Impacts on Modern Civilization, Current and Future Challenges* by *Anupam Guha* © New Delhi Publishers, New Delhi: 2021, 198-214. ISBN: 978-81-947417-9-4, DOI: 10.30954/ndp.bio.2020.19

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CHAPTER

Discovery of therapeutic lead molecules from natural resources: A outstanding contribution to modern medicine

Sanjoy Das[1*], Biplajit Das[2], Bibek Sinha[3], Alakesh Bharali[4], Taison Jamatia[1], Baishali Deb[1], Kishan Paul[1] and Debashree Bharali[1]

[1]Department of Pharmaceutical Sciences, Dibrugarh University, Dibrugarh, Assam, India
[2]School of Pharmaceutical Sciences, Shoolini University, Solan, Himachal Pradesh, India
[3]Department of Pharmacy, Tripura University, Suryamaninagar, Tripura, India
[4]Girijananda Chowdhury Institute of Pharmaceutical Science, Guwahati, Assam, India
*Email ID: sanjoyeyeconic@gmail.com

Abstract: Over the last few decades, researchers have consecutively focused on natural resources for the discovery of new lead molecules.To develop the scientific evidence of these natural sources, natural derivative especially natural products play a promising role. Natural products have anexclusive chemical innovation ofmulti-dimensional structures, which results in diversity in their biological activities ordrug-like properties and evolve into one of the most valuable resources for developing novelbiologically active compounds and scaffolds.Today, approximately 70% of life-saving drugs like anticancer, antimalarial, antimicrobial, cardiovascular,etc. are synthesized from natural origins or developed from a natural compounds. Natural products will undergo endless use formeeting the demanding need to develop effective drugs and play a vital role in the discovery of newly synthesized drugs for treating chronic human diseases. Therefore, there is great potential for future drug discoveries from natural originor natural products which offer a hugecompetence in novel chemical structures and their new types of activities related to drug development. In this chapter, we highlight the discovery oftherapeutically active lead moleculesderived from natural products, explore the relationship between natural products and modern medicines and also toxicity assessment of natural products in drug discovery and development.

Keywords: Natural products; Drug discovery; Modern medicines; Human diseases; Toxicity assessment.

Since ancient times, humans have utilized natural sources such as plants, animals, marine organisms and microorganisms in various traditional systems of medicine like Indian Ayurveda, Unani and Siddha System of Medicines, Traditional Chinese

Medicines and Traditional Korean Medicines to treat and mitigate various diseases[1,2]. These traditional systems of medicine provide great insight for the use of natural sources especially natural derivatives or products in the form of various herbal or culinary preparations and are of great medicinal importance [3, 4]. Natural products have a wide range of diversity of multidimensional chemical structures, in the meantime, the utility of natural products as biological function modifiers has also gained magnificent attention [5]. With the passing of time, these natural products have undergone interesting and meaningful developments in their innate ability or selectivity to interact with numerous or varied biological targets and some have become the most important therapeutic agents in the health care system. Considering their inimitable chemical diversity and novel mechanisms of action, natural products have continued to play a crucial role in many research programs, drug discovery and development [6-8].

The sources of many new drug molecules and active ingredients of medicines that areavailable in the market today are discovered from natural sources or natural products. The value of natural products in this regard can be assessed by the rate of introduction of new chemical entities of wide structural diversity, the number of diseases cured by these substances and their frequency of use in the disease treatment[9,11]. An analysis of the origins of newly developed drugs from 1981 to 2014 reveals that almost half of the drugs approved are actually being either natural products or directly derived therefrom [12]. There are several examples at hand for new drug development from the natural sources i.e., pharmacologically active compound Morphine was isolated from the opium poppy (*Papaver somniferum*) nearly 200 years ago. Later on, several new drugs have also been developed from natural sources likeantibiotics (Penicillin from Penicillium fungi), antimalarials (Quinine from Cinchona bark) and anticancer (Paclitaxel fromYew tree bark) have undoubtedly revolutionized modern medicine[13,15].

Drug discovery from natural products is a challenging scientific venture to find potent and feasible lead candidates. To overcome this limitation, various screening approaches, like high throughput screening, combinatorial chemistry are being developed to improve the ease with which natural products can be used in the drug discovery process and also virtual screening techniques are being applied to databases of natural products [16,17]. It is expected that the more adequateand effective use of natural products will improve the process of drug discovery.

Methodology for Data Extraction

Considering the significance of this study, a thorough literature survey was conducted through online databases like PubMed, Science Direct, Google Scholar, Research Gate. The title andabstract of articles were searched from previously mentioned databases by using the corresponding Keywords, i.e., drug discovery, natural products, traditional

medicine, modern medicine, biological activity of natural products, structural diversity, toxicity assessment to understand the relationship among natural products, traditional medicines and modern medicine for discovery potential therapeutic lead molecules.

Results and Discussion

Drug Discovery from Natural Sources

Despite the rise of molecular biological techniquesas anintegral part of the drug discovery process, naturalproducts still play a pivotal role as starting raw materialfor drug discovery. These products have become one of the most important reservoirs for developing novel compounds and scaffolds. The drug product have been obtained from various natural sources include plants, animal, marine and microbial sources are discussed below (Fig. 1).

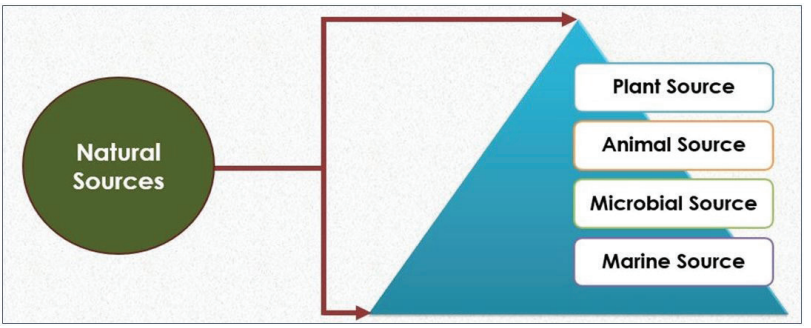

Fig. 1: *Various Natural Sources for Drug Discovery and Drug Development*

1. Plant Sources

Plants have served human kind as sources of medicinal agents sinceits earliest beginnings. The information regarding the use of the plant as a medicine have been recorded in various traditional systems of medicine.These medicines primitivelytaken in theform of crude drugs such as powders,teas, poultices, tinctures and auxiliary herbal preparations [18-20]. In the past two centuries, thechemical investigation and purification of extracts of plants purported to have medicinal properties and those used as toxins and hunting poisons in their native habitats, have yielded numerous purified compounds which have proven to be indispensable in the practice of modern medicine [21]. For example, the use of plants as medicines has involved the isolation of active derivatives beginning with the isolation of Morphine from opium in the early 19th century and are still in use [22]. The discovery of drugs from medicinal plants has evolved toinclude numerous fields of inquiry and various methods ofanalysis. The process typically starts with a botanist, ethnobotanist, plant ecologist or ethnopharmacologist who identify and collects the plant or plant parts. Collection may involve plants with known

biological action for which active derivatives have not been isolated (traditional herbal formulation) or may involve taxa collected randomly for alarge analysis program, just before starting the drug discovery process [23, 24]. After that, phytochemists formulate the extracts from the plantmaterials, subject these extracts to biological screening in pharmacologically relevant assays and commence the processof isolation and characterization of the active compounds [25]. These bioactive compounds in plants can be defined as secondary plant metabolites eliciting pharmacological or toxicological effects in man and animals. The typical bioactive compounds in plants are produced as secondary metabolites [26]. Secondary metabolites are produced within the plants beside the primary biosynthetic and metabolic routes of compounds aimed at plant growth and development, such as carbohydrates, amino acids, proteins and lipids. They can be regarded as products of biochemical 'sidetracks' in the plant cells and not needed for the daily functioning of the plant. These compounds can be categorized into different classes like glycosides, tannins, flavonoids, alkaloids, steroids, etc. and are documented to be used against many devastating diseases [27-29]. Plant products also play an important role in the health care systems of the remaining 20% of the population, mainly residing in developed countries and at least 119 chemical substances, derived from 90 different plant species, can be considered as important drugs currently in use in one or more countries. Out of 119 drugs, 74 % were explored as a result of chemical studies directed at the isolation of the active substances from plants used in traditional medicine[30]. Examples of important drugs obtained from plants include Morphine, Quinine, Digoxin, Cannabinoid, Muscarine, Physostigmine are widely used for treating various types of diseases [31]. However, numerous hurdles are encountered including the procurement of plant materials, the selection and implementation of appropriate molecular bioassays and the scale-up of active compounds [32]. But the searching of therapeutic drugs from natural products has gained much attention day by day, due to huge plant biodiversity and also edible medicinal plants has been grown throughout the world.

2. Animal Sources

Apart from meat and dairy, animals have been extensively used as medicinal resources for the treatment of several human diseases. Although literature on the natural sources focused much on plants and microbes based medications, though interest in animal-based medication is also ever-increasing [33]. The animal body parts, metabolic products like secretions and excreta, and even the non-animal materials like cocoons are used as a source of drug discovery and scientific research. Also, the study of the toxins and venoms from animals has become productive [34, 35]. The achievement of drug discovery from animal sources started with insulin and heparin in the early 1900s. At that time, animal-based drugs were mainly derived from tissue extracts.

With the advancement of purification methods and other technology, different drugs from animal sources are isolated, ranging from Gila monster saliva and snake venom to the most recent genetically engineered goat milk. The commonly used medications from animal sources like toxins, venoms and secretions include Captopril, Lepirudin, Exenatide, Eptifibatide and many more [36-39]. In the last few decades, animal venoms became an invaluable and inexhaustible source of drug discovery. The potent angiotensin-converting enzyme (ACE) inhibitor, Captopril, was derived from the venom of Brazilian pit viper. It was the first FDA approved (1981) ACE inhibitor extensively used today as a first-line antihypertensive drug [40]. Currently, there are six FDA approved venom-derived drugs plus one snake-venom derived serine protease while another two are undergoing clinical trial. Current researches on venom are mainly focused on drug target identification, drug leads, insecticides, treating microbial, parasitic, and viral infections and targeting vectors of disease-causing organisms[41]. Apart from venoms-derived drugs, various drugs like Protamine Sulfate from salmon sperm, Lepirudin from leech saliva, Ursodiol from bear bile, influenza vaccine from chicken eggs, ATryn from genetically engineered goat milk are already being developed [42-44]. Current researches are undergoing on alligator blood, frog skin and Komodo Dragons for antimicrobial/antibacterial properties; scorpions, sea squirts and South American rattlesnake for cancer treatment and many more for the discovery of newer drugs [45, 46]. The above examples clarify the available drugs from animal sources and also demonstrate the possibility for future discoveries. But, like other drug candidates, animal-derived drugs also face a lot of challenges. The number of animal-derived drugs entering clinical trials is increasing but the overall success rate is very low [47]. But still, researches on animal-based medications is ever increasing and this will undoubtedly bring a revolution to medicine.

3. Microbial Sources

The most well-known examples of the natural products obtained from microbial sources are antibiotics [48]. The 'Golden Era of Antibiotics' from the 1950s to the 1970s was sparked by the serendipitous discovery of penicillin by Alexander Fleming in 1928 and its development by Chain and Florey in the 1940s [49]. Since the discovery of penicillin, mostnatural product-derived drugs were obtained from plant sources. The success of penicillin in treating disease led toan expansion in the area of drug discovery from the microbial sources. Microorganisms are a rich sourceof structurally diverse bioactive substances and secondary metabolites that have provided important contributions to the discovery of antibacterial agents (Penicillin), antidiabetic drugs (Acarbose), anticancer drugs (Epirubicin) and many more [50-52]. The example of natural products synthesized from microbial sources namely Lovastatin, the fundamental process for attaining the bioactive compound from the microbial sources

includes isolation and characterization of the microbe, establishing culture condition for the microbe and production of target natural products, isolation, determination of structure and assessment of activity of isolated natural products. Hence, most of these approaches rely on pure culture or a single isolate method. The culture-dependent approaches provides a platform for building the depth and details of microorganisms, and exploration of their microbial physiology and genetics. For long-duration characterization of uncultured bacteria and their biochemical and metabolic potency were ignored [53, 54]. The cultured microorganism cannot substantially represent the microbial world as observed in 'Great Plate Count Anomaly which is the significant discrepancy between the size of the population estimated on dilution plating and microscopic observation [55]. Many microbial flora cannot be cultured in media due to their incapability to grow in common media. On the other hand, it is enormous labor to surge for suitable medium components. In some other cases, the growth requirements for optimal temperature, pH, osmotic pressure, humidity, salinity, etc. hinders the proper propagation in the selected media. Recently, there has been substantial progress in the development of conditions mimicking natural environments in terms of pH, nutrient compositions, culture conditions, etc. But still, these approaches seem inadequate to study the physiology, biochemistry and metabolism of all the microbial resources[56-58].Currently, novel sourceswere expanded beyond soil, which includes marine environments, unusual orextreme habitats (high or low temperature or pressure, highlevel of acidity or alkalinity) and endophytic microorganisms. These new sources have sofar not been exploited to the same extent harbor plentiful microorganisms containing novel bioactive natural productsdue to the lack of modern technology [59, 60]. The advanced technologies now provide opportunities to isolate microorganisms from these extreme environments and lead a resurgence of interest in microorganisms as a source of bioactive compounds for drug development.

4. Marine Sources

Unlike the enduring historical medical application of plants, marine products have a shorter history of utilization in the treatment and prevention of human ailments. In recent years, drug discovery from marine natural products has gained much attention due to theunimaginable level of biodiversity offered by the marine environment [61]. The marine environment is an exceptional storehouse of novel bioactive natural products, with structural and chemical features generally not found in terrestrial natural products. The marine organisms also provide a rich source of nutraceuticals and potential candidates for the treatment of several human diseases. Fish oils are the classic example of marine-derived natural products in use since milestone [62, 63]. Marine natural products are usually secondary metabolites. They are not produced by biological or regular metabolic pathways and have no primary function associated with the growth,

development or multiplication of a species. For that reason, researchers have rapidly persevered the pharmacological potential of bioactive compounds and secondary metabolites from marine organisms [64]. Marine organisms such as sponges, tunicates, fishes, soft corals, nudibranchs, sea hares, echinoderms, bryozoans, prawns, shells, sea slugs, and microorganisms are wealthy sources of novel bioactive compounds [65, 66]. The first biologically active marine natural product Spongosinederived from sponges was formally reported in late 1950[67]. In 1970, it was entrenched that marine plants and animals are biochemically and genetically unique. Around 15,000 such unique natural compounds have been described and out of them 30% products have been isolated from sponges [68]. There are some reports on the evaluation of the antimicrobial potential of marine macro-organisms collected from the Indian coastline have appeared. *Streptomyces* specieshave been the most extensively investigated microbial species from the Indian coastal waters as a source of antibiotics[69, 70]. It has been almost decades since the isolation of Spongothymidine and Spongouridine from the marine sponge *Tethya crypta* that eventually led to the development of Cytarabine (Ara-C, an anticancer agent) and Vidarabine (Ara-A, an antiviral agent), which received US-FDA approval in 1969 and 1976. After the approval of Ara-C and Ara-A as therapeutic agents, that the next marine natural products Ziconotide has been approvedfor the treatment of severe chronic pain [71]. Marine natural products pharmacology has emerged from broad surveys of the marine environment for novel chemical entities regardless of pharmacological activity into what presently is considered the targeted approach to drug discovery [72-74]. After substantially review of marine pharmacology in the past all over the world still there is a need to review the feasibility of the oceans as a source for the development of new drugs, considering the benefits of their abundance in nature and large-scale production.

Stages Involved in the Drug Discovery from Natural Sources

Drug discovery is a process by which new compounds are identified or designed that are useful for treating and curing human diseases. The process of drug discovery began with the identification of the suitable target, lead finding, lead optimization, preclinical phase and clinical phase (Fig. 2). All these stages playing a significant role from initial raw materials i.e., plants, animal parts, microbes, marine organisms, to final therapeutic drug products.

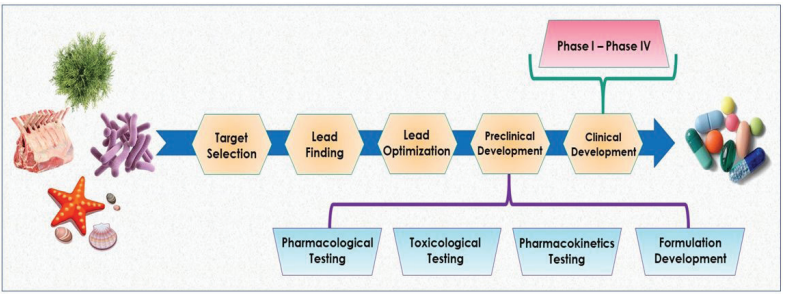

Fig. 2: *Stages Involved in the Drug Discovery Process from Natural Sources*

1. Target Selection

Target selection and validation commence the whole drug discovery process. Naturally existing cellular or modular structures that appear to play a major role in disease progression or pathogenicity are normally targets for therapeutics. A good target needs to be efficient, safe and be usable by the drug molecule or meet the clinical needs of the anticipated patient. After identification of the target, a systematic validation strategy should be attached for the mechanism of action of lead molecule to be evaluated for therapeutic efficacy [75].

2. Lead Finding

Once a diseaseassociated molecular target has been identified andvalidated, the next step is to identify lead molecule that have the desired effect against the identified targets. Initially,screening can be implemented by inquiring for compounds that bind tothe target, but binding is not sufficient for therapeutic activity. Therefore, multiple screening processes must be required to encounter the therapeutic activity of the lead molecules. There are a number of approaches by which lead molecules can be identified, including high-throughput screening, combinatorial chemistry, virtual screening and online databases, etc. [76].

3. Lead Optimization

Lead compounds found by random screening are thebasis for the next stage, lead optimization, where the main aim is to increase the potency of the compound onits target, while upgrading on possible defects of their structures, with a view to producing a preclinical drug candidate. This stage can be used to find out whether the drug metabolizes in the right site of the human body or whether there are currently any adverse effects that are cause for significant concern.For this process, an integrated hybrid approach is recommended i.e., combination of specialists in medical chemistry, computational chemistry, drug metabolism, and other fields that can provide unique insights in the lead optimization process [77].

4. Preclinical Development

The aim of preclinical development is to satisfy all theconditions that have to met before a new moiety or compound is esteemed ready to be the tested for first time in the human model [78].The pre-clinical development includesthe following steps:

- **Pharmacological Testing**: Pharmacological testing to check that the drug doesnot produce any obviously dangerous acute effects like bronchoconstriction, changes of blood pressure and pulse rate, cardiac dysrhythmias and neurodegenerative diseases. This stage is also called safety pharmacology.
- **Toxicological Testing**: Toxicological testing to eliminategenotoxicity, nephrotoxicity, hepatotoxicity and to determine the maximum non-toxic dose of the drug. As well as being checked regularly for weight loss and other grosschanges, the animals so treated are examined hourly and at the end of the study to look for histological and biochemical evidence of tissue damages in animals.
- **Pharmacokinetic Testing**: Pharmacokinetic testing involving the studies on absorption, metabolism, distribution and elimination (ADME studies) in the species of laboratory animals used for toxicological testing. It mainly illustrates about 'What the body does to the drug'.
- **Formulation Development**: Formulation development mainly involves to determine the feasibility of large-scale synthesis and purification, to determine the stability of the compound under different storage conditions and to prepare a formulation convenient for clinical studies.

5. Clinical Development

Clinical development proceeds via four distinct but coinciding phases of clinical trials [79]:

- **Phase I**: Phase I clinical studies test a new biomedical intervention in a small group of people (20 to 100 subjects) for the first time to evaluate safety.
- **Phase II**: Phase II clinical studies investigate the biomedical or behavioral intervention in a larger group of people (100 to 300 subjects) to determine efficacy and to further evaluate its safety.
- **Phase III**: Phase III clinical studies investigate the efficacy of the biomedical or behavioral intervention in large groups of human subjects ((100 to 3000 subjects) by comparing the intervention to other standard or experimental interventions as well as to monitor the adverse side effects.
- **Phase IV**: Phase IV studiesare conducted after the intervention has been marketed. These studies are designed to monitor the effectiveness of the approved

intervention in the general population and to collect information about any adverse effects correlated with extensive use.

Currently Available Marketed Drugs Derived from Natural Sources

During the last few decades,a plentiful drugs derived from natural sources have been launched into the market. The first commercial pure natural product introduced for therapeutic use is morphine marketed in 1826 by Merck [80]. After that many natural products has been marketed as a drug for treating various kind human diseases and all are discussed in below Table 1.

Table 1: *Marketed Drugs Derived from Natural Sources*

Sl. No.	Sources	Isolated From	Drug	Therapeutic Action	Ref (s)
01.	Plant Source	*Papaver somniferum*	Morphine	Analgesic	[81]
		Cinchona officinalis	Quinine	Antimalarial	[82]
		Taxus brevifolia	Paclitaxel	Anticancer	[83]
02.	Animal Source	*Camelus dromedarius*	Insulin	Antidiabetic	[84]
		Camelus dromedarius	Heparin	Anticoagulant	[85]
		Bothrops jararaca	Captopril	Antihypertensive	[86]
03.	Microbial Source	*Penicillium chrysogenum*	Penicillin	Antibiotic	[87]
		Streptomyces hygroscopicus	Sirolimus	Antiproliferative agents	[88]
		Actinoplanes species	Acarbose	Antidiabetic	[89]
04.	Marine Source	*Tethya crypta*	Vidarabine	Antiviral	[90]
		Conus magus	Ziconotide	Analgesic	[91]
		Cryptotheca crypta	Cytarabine	Anticancer	[92]

Toxicity Assessment of Drug Molecules Derived from Natural Sources

Drug molecules derived from natural sources are generally considered less toxic than synthetic drugs. But as said by Paracelsus, the father of toxicology, 'All substances are poisons; there is nothing which is not a poison, only the proper dose makes poison a remedy' [93]. So, the toxicity assessment of drug molecules derived from natural sources is also very important as like other synthetic drugs. This assessment aims to determine the adverse effects and also the limits of exposure level [94]. The toxicity assessment can be classified into two main domains i.e., Pre-clinical toxicity assessment and clinical toxicity assessment.

1. Preclinical Toxicology Testing

Preclinical toxicology testing of pharmaceutical agents is a very important component of the drug development process. This includes a wide range of toxicity tests performed in animals.

- **Acute Toxicity Testing**: It is the first step in the assessment of the toxicity of any drug candidate. Here, the effect of single-dose on a particular test animal is determined. This assessment aims to determine a toxic but sublethal dose. The investigational molecule is administered at the different doses via the oral, dermal, intravenous, intramuscular route in the animal to detect lethal doses [95].
- **Subacute Toxicity Testing**: Subacute toxicity studies are conducted to evaluate anew drug potential adverse effect following a treatment period of 2 to 4weeks durations. This toxicity testing is mainly conducted as range-finding studies in orderto choose dosing limits to be used in subsequent subchronic and chronic toxicity studies [96].
- **Subchronic Toxicity Testing**: Subchronic toxicity testing is also a repeated dose toxicity study that is similar to a subacute toxicity study except that they are conducted for a period of 1 to 3 months (90 days) [96].
- **Chronic Toxicity Testing**: A chronic toxicology study provides inferences about the long-term effect of a test substance in animals and it may be extrapolated to the human safety of the test substance. Chronic toxicity studies are generally performed 6 months to 12 months in duration [96].
- **Carcinogenicity Testing**: Carcinogenicity tests are carried out over the greater portion of an animal's life cycle. During and after liable to test substances, the experimental animals are checked for signs of toxicity. If these are not found, the test may be terminated after 18 months in the case of mice and after 24 months in the case of rats [97].
- **Genotoxicity Testing**: Genotoxicity tests are utilized to recognize gene mutations, alterations in the DNA sequencing and chromosome changes. These tests are generally carried out in diverse species including whole animals, microorganisms and mammalian cells [97].
- **Mutagenicity Testing**: Mutagenicity testsare used to assess submicroscopic variations in the base sequence of DNA, chromosomal abnormalities and structural abnormalities in DNA in addition to duplications, inversions, insertions and translocations [97].
- **Neurotoxicity Testing**: Neurotoxicity testing is performed to determine the effects of a test substance on the central nervous system. Neurotoxic studies may be engaged to investigate the particular histopathological and behavioral neurotoxicity of drug products and are also used to evaluatethe neurotoxic responses such as neuropathological lesions and neurological dysfunctions [97].

2. Clinical Toxicity Assessment

Clinical toxicity study is a discipline within toxicology that is concerned with the impact of drugs and other chemicals on the humans body. Clinical toxicology joins experimental and preventive toxicology with clinical medicine. This area of clinical toxicology has endured serious changes during the last few decades. Traditionally clinical toxicology was involved with the diagnosis and treatment of acute inebriation[98].

Conclusion and Future Perspectives

Contribution of natural sources as a starting point has a mutual promise of transporting the original isolate as a novel drug candidate.The important concerns related to natural products have been the identity of hit rate during different phases of drug discovery. Such identityis expected to be lower in the case of the random pick of candidate species. Hence, there is anurgent need to renew scientific interest toward natural products for drug discovery.An integrative approach by linking the various discovery tools and integrative biology will serve the key for success innatural product drug discovery and development in the near future.

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Nature's call for antimicrobial resistance (AMR): Global public health threat
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CHAPTER

Nature's call for antimicrobial resistance (AMR): Global public health threat

Deepshikha Bhowmik*and Shiela Chetri

Department of Microbiology, Assam University, Silchar, India
*E-mail: krm.deepshikha@gmail.com

Abstract: Antimicrobial resistance (AMR) is one of the biggest public health threats worldwide. Antibiotic resistance occurs when bacteria develops the ability to conquer drugs. Infections due to antibiotic resistance can lead to longer lasting illness eventually leading to death. ESKAPE organism such as *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,and Enterobacter* species are the leading cause of hospital acquired and community acquired infections throughout the world. Multidrug resistance has now become one of the greatest public health challenges in clinical practices which is caused due to the inappropriate use of antimicrobials, substandard pharmaceuticals, and excessive usage of drug. Proper understanding of the resistance mechanism of the bacteria is important for the development of the novel antimicrobial agents to fight these health challenges. However in this review we have summarized the known antimicrobial resistance mechanism of the ESKAPE pathogens.

Keywords: Antibiotic resistance; microorganisms; World Health Organisation; Global Action Plan

The term antimicrobial resistance came after the discovery of penicillin by Alexander Fleming, after 12 years of research in 1940, turned out to be a miracle drug at that period, which can cure many bacterial infections. The discovery of antibiotics has revolutionized healthcare, which leads to the significant advancement in medical science in this 21st century [1]. The post-antibiotic era has lead to the failure of the most effective physician armamentarium viz. antibiotics, anti-tuberculosis, and anti-malarial drugs [2]. However, medicines helped to cure deadly infectious diseases indirectly by assisting the physician by making it feasible to pursue new treatments such as surgery, organ transplantation, and cancer chemotherapy [3].

Microorganisms that develop resistance are generally referred to as super bugs. Antimicrobial resistant-microbes which are found in humans, animals, food, and

the environment (in water, soil and air), can spread the infection between humans and animals, including from food of animal origin and from person to person. Poor infection control, inadequate sanitary conditions, and inappropriate food-handling lead to the spread of antimicrobial resistance. Antibiotic resistance has become a significant problem in recent years due to the slow pace of developing new antibiotics concerning the use of antibiotics. Antibiotic resistance is the problem to bacteria; it also shows its potential to all microbes and may render its drug ineffectiveness [1].

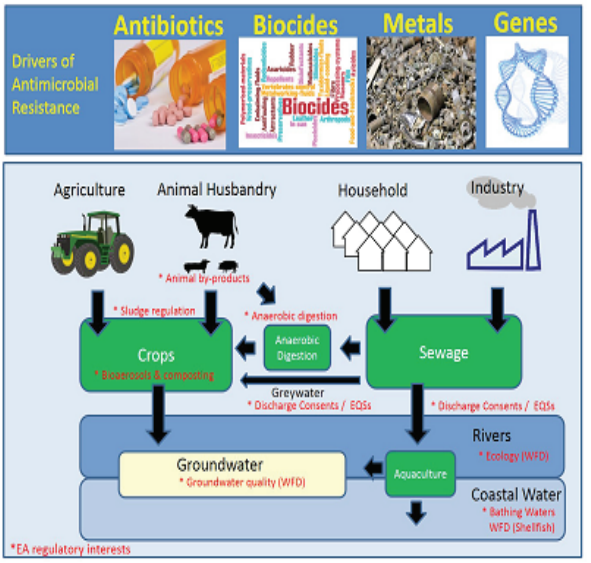
The World Health Organisation (WHO) and its Global Action Plan had announced the following objectives to tackle AMR:

1) Improving awareness and understanding of AMR.
2) Strengthening knowledge through surveillance and research. 3) Reducing the incidence of infection.
4) Optimizing the use of AMR agents and
5) Ensuring sustainable investment in countering AMR. [4]

Antibiotics conferred genes drive resistance in microorganisms, and other environmental factors are also responsible for the prevalence of the resistance gene in the environment. There were well-characterized classes of resistance viz. (a) Antimicrobial (Antibiotics, antiviral, antifungal, antiparasitic); (b) Heavy metals, and (c) Biocides (Fig. 1).

The multidrug resistance has developed due to the frequent and subsequent use of antibiotics. Antibiotic resistance is one of the biggest threats to Global health. The World Health Organization has developed a global priority list of AMR organisms collectively known as ESKAPE to guide the research, design, and development of new antibiotics. Infections due to ESKAPE (*Enterococcus, S. aureus, K. pneumonia, A. baumannii, P. Aeroginosa and E.coli)* organisms are leading cause of healthcare-acquired diseases worldwide. Antibiotic resistance in ESKAPE organisms is associated with significantly higher mortality, morbidity, and socio-economic burden [5].

According to World Health Organisation, there are six WHO regions (African region, region of the Americas, Eastern Mediterranean region, European region, South-East Asia region, and Western Pacific region), of which five areas exceeds 50% of third-generation cephalosporins resistance and fluoroquinolone resistance in *E.coli. While* the six areas had more than 50% resistance to third-generation cephalosporins and two regions had more than 50% resistance to carbapenems for *K. pneumonia*. However, the overall Methicillin-resistant *S. aureus* (MRSA) proportions exceeded 20% in the six WHO regions and even exceeded 80% in three WHO regions, which has become a primary AMR concern worldwide. [6]



Source: Singer *et al.*, 2016

Fig. 1: *Schematics of carriers of Antimicrobial resistance (AMR) and their hotspot*

ESKAPE organism and its Antibiotic Resistance

Enterococcus faecium

Antimicrobial resistance rates of enterococci particularly concern, especially the incidence of vancomycin-resistant *Enterococcus* (VRE), which is mainly associated with *E. faecium*. VRE emerged in North America during the late 1980s, with 61% of *E. faecium* isolates estimated to be vancomycin-resistant by 2002. Six types of Vancomycin-Resistant Enterococci genes (*van*-A–E and *van*-G) known till date, of which *van-A* being the most prevalent one and showing the highest resistance to all glycopeptide antibiotics found both in hospital and in community settings.[7]

Staphylococcus aureus

Staphylococcus aureus, a gram-positive β-lactamase producing bacterium, has now increased to 80% in both community and hospital-associated infections [8,9]. According to the reports, Methicillin Resistance *Staphylococcus aureus* has emerged in the early 1960s and currently accounts for 25% of *S.aureus* isolates [10]. *Staphylococcus aureus*, which is the common cause of severe infections in healthcare facilities and the community, has become resistant to first-line drugs. WHO reported that about 64% of the people with MRSA infections are more prone to death than the non-resistant form of diseases. Vancomycin and teicoplanin are used as first-line antibiotics for the treatment of MRSA infections. In the mid-1990s, VISA first reported in Japan, which

has now emerged in Asian, American, and European countries. VRSA is due to the interspecies exchange of resistance genes from Vancomycin-resistant Enterococci. VRSA may contain both *van* A and *mec* A resistance determinants of VRE and MRSA, resulting in multidrug resistance [11].

Klebsiella pneumonia

Carl Friedlander first discovered *Klebsiella pneumonia* in 1882. *Klebsiella pneumoniae* is a gram-negative, encapsulated, non-motile bacterium, found in the environment and has been associated with pneumonia. Recently, *K. pneumoniae* pneumonia is considered the most common cause of hospital-acquired pneumonia in the United States. The organism accounts for 3% to 8% of all nosocomial bacterial infections. [12] *K. pneumonia* strains can produce a variety of *β*-lactamase enzymes that can destroy *β*-lactam antibiotics such as penicillins, cephalosporins, and carbapenems. Due to the increasing prevalence of carbapenem-resistant *K. pneumoniae* (CRKP), encoded by bla_{KPC} gene [13,14] and the emergence of another super enzyme encoded by blaNDM $_{-1}$ has posed a threat to other antibiotics viz. *β*-lactams, aminoglycosides, and fluoroquinolones [15-16]

Acinetobacter baumanii

Acinetoacter baumannii are lactose-non-fermenting, gram-negative coccobacillus with strong environmental adaptability and drug resistance [17]. Currently, it has been found that the emergence of carbapenemase-producing *A. baumannii* strains to carry imipenem metallo-*β*-lactamases, encoded by *blaIMP*, and oxacillinase serine *β*-lactamases, encoded by *bla*OXA, showed resistance to both colistin and imipenem, and the combination of resistance genes. [18-19]. Polymyxin is considered as the last line of defense against infections caused by multidrug-resistant *A. baumannii*. This multidrug resistant pathogen is the cause of high mortality among ICU patients. [17].

Pseudomonas aeroginosa

Pseudomonas aeroginosa, a gram-negative, rod shaped, facultative anaerobic bacteria, showed a higher carriage rate in hospital inpatients especially in immuno-compromised patients. It is known for its leading cause of morbidity and mortality in cystic fibrosis (CF) patients and of nosocomial infections. *P. aeruginosa* infections are becoming more difficult to treat due to the bacterium›s resistance to many antibiotics and the number of multidrug and pan-drug-resistant strains increasing worldwide. Some strains have been reported to be resistant to almost all class of commonly used antibiotics such as aminoglycosides, cephalosporins, fluoroquinolones, and carbapenems [20]. AmpC production and porin change are the most common mechanism of imipenem resistance in *P.aeroginosa.* These strains produce ESBLs and carry other antibiotic resistance enzymes such as *K. pneumoniae* carbapenemases (KPC),

VIM encoded by bla_{VIM}, and imipenem metallo-β-lactamases. Due to the continuous increase of MDR isolates, complicated situation arises for antimicrobial therapy. However colistin is an effective drug of *P.aeroginosa* [21].

Enterobacter spp.

Enterobacter species, a member of the ESKAPE group, are non fastidious gram-negative rods which can cause opportunistic infections in immuno-compromised patients. These strains contain ESBLs and carbapenemases which includes VIM, OXA, metallo-β-lactamase-1, and KPC. These MDR strains show its resistance to almost all the available antimicrobial drugs, except tigecycline and colistin [18].

Antimicrobial Resistance Mechanisms

Antibiotic-resistant bacteria causing infections are presently estimated to cause ~700,000 deaths each year and the mortality rate is expected to rise to 10 million per year by 2050 [1,19]. Antibiotic resistance has been recognized as one of the most critical challenges to human health by a wide variety of national and international bodies, including the WHO. To solve this crisis, we require new antimicrobials to treat infections caused by resistant pathogens and new approaches to predict and inhibit the spread of resistance in pathogens [22, 23, 24] Antimicrobial resistance (AMR) is rising and poses a significant public health threat. The understanding of antibiotic modes of action (MOA), and bacterial mechanisms of resistance (MOR), is critically important in developing alternative therapies which has been described in Table 1.

The growth in antibiotic resistance in many pathogens has been driven by the spread of a relatively small number of powerful antibiotic-resistant lineages [25-30]. One explanation behind this pattern is that these successful lineages are simply those that, by chance, successfully acquire rare antibiotic resistance genes by mutation or horizontal gene transfer [31, 32]. Alternatively, some strains of bacteria may be more likely to evolve resistance than others, for example because they have an elevated mutation rate [33], or because they carry 'potentiator' genes that open up new genetic paths to evolving resistance [34, 35]. If so, it may be possible to identify strains of bacteria that are at high risk of evolving resistance to antibiotics, and to change antibiotic usage to prevent this outcome and associated treatment failure [36].

Over a decade ago, the formation of reactive oxygen species (ROS) was proposed as a common effector mechanism in bacteria that were challenged with bactericidal antibiotics [37]. Beyond the canonical drug-specific target-corruption MOA, the paradigm shifted toward the system-level disruption of bacteria cellular homeostasis as a common means of antibiotics-induced lethality [38]. Accordingly, several studies reported system-level MOR involving oxidative stress defenses. In 2011, a novel

resistance mechanism mediated by hydrogen sulfide (H2S) was described for several pathogenic bacteria, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Bacillus anthracis* [39]. The model proposed that endogenously produced H2S reduces the cellular formation of ROS, by interfering with the Fenton reaction and by stimulating ROS-scavenging enzymes, thereby contributing to antibiotic tolerance. Genetic and chemical disruption of the H2S biosynthetic pathways resulted in exacerbated antibiotic sensitivity, suggesting that this pathway could be targeted to potentiate antibiotics or even revert resistance. However, recently, exogenous H2S was shown to have a cytotoxic effect on several microbes, including *E. coli* [40,41]. Several approaches to revert the resistance capability of pathogens towards different antibiotics were made in different labs to combat this crucial situation—however, a recent study done by S. Y. Ng in August 2020 claims that by using an H2S-releasing compound to modulate the sulfide content in *A. baumannii*, demonstrated that instead of conferring antibiotic tolerance, exogenous H2S sensitized *A. baumannii* to multiple antibiotic classes, and was able to revert acquired resistance to gentamicin. They also propose that H2S could be used as an antibiotic-potentiator and resistance-reversion agent in bacteria that do not produce it [42].

The emergence and dissemination of antibiotic resistance is now understood as an unavoidable aspect of bacterial evolution following the consumption of antibiotics[43]. This dramatic phenomenon is well illustrated by the relationship existing between the occurrence of resistance and the consumption of antibiotics [44-46]. Mechanistically speaking, the increasing occurrence of antibiotic-resistant bacteria (ARB) has been widely attributed to the selection of resistant variants that pre-exist in susceptible communities [47]. Such resistant bacteria are supposedly outcompeting the rest of the microbial communities in a context where antibiotics are administrated at relatively high levels, which means that local concentrations are well-over the Minimum Inhibitory Concentrations (MICs). Despite the fact that the increasing occurrence of antibiotic resistances among bacteria has been recognized decades ago as resulting from antimicrobial drug consumption, only recently has the seriousness of the situation been considered by international, national and local health organizations/agencies. This awareness led to a series of reports and recommendations intending to educate and improve health professionals and consumers' practices to preserve the effectiveness of our therapeutic potential [48-51]. Considering the correlation between antibiotic consumption and occurrence of resistances in bacteria [45], most recommendations proposed to take action in the public health and veterinary/farming domains by limiting the inappropriate exposure of bacteria to antibiotics to slow down a natural evolution toward resistance and its spread in the downstream environment in a One Health context[52]. Limiting the inappropriate exposure of bacteria to antibiotics means

(i) reducing the need for antibiotics, which can be achieved with infection control measures that would limit the epidemic spread of resistant bacteria, and (ii) a better usage of antibiotics to reduce our overall antibiotic consumption when unnecessary. Even if there is a great disparity between countries regarding the consumption of antibiotics [53], change in practice remains difficult to implement when public health is concerned. In any case, reducing antibiotic resistance requires a coordinated and multi-sectorial approach that combines political commitment, resources, specific governance mechanisms, and practical management, as recently reported by World Health Organization [54]. In its 2018 reports, the ECDC indicated that the overall consumption of antibiotics in the EU did not significantly change in the community and the hospital sectors. A few decreasing and increasing trends were observed for some countries over the 2013–2017 period. Changes in consumption were probably more visible in veterinary medicine. In a report covering the 2011–2016 period on veterinary antibiotics sale, the European Surveillance of Veterinary Antimicrobial Consumption related an overall decrease of 20% aggregated for 25 countries [55]. This was tentatively explained by the implementation of policies and measures aiming at reducing the misuse of antibiotics. Even if the studied period is too short yet to draw a robust conclusion, the first effects of such responsible-use campaigns start to be visible. For instance, in France, an unprecedented national plan to reduce antibiotic consumption in the animal sector has been initiated [56]. This led to a drastic 39% reduction of antibiotic prescription in veterinary medicine in 6 years, all animals considered. The reduction was even stronger for critical antibiotics such as fluoroquinolones (81% reduction) and last generation cephalosporin (75% reduction). According to the French surveillance network of antimicrobial resistance in pathogenic bacteria of animal origin, these measures were followed by a net diminution of pathogenic ARBs [57]. As reported by the French National Public Health Agency [58], using data also presented by the European Food Safety Agency, the proportion of resistant E. coli for C3G went down from 16% to <2% in poultry between 2010 and 2017, which was dramatically increasing before 2010 [58-60]. Although more results are necessary to comfort these results, they tend to demonstrate that better use leading to reduced consumption of antibiotics can rapidly result in a sensible decrease in the relative occurrence of ARBs. If several other reports are rather encouraging to pursue in that direction [61-63], the relationship between the occurrence of resistance and antibiotic consumption does not always follow this trend. Indeed, even if it is not the vast majority of the reported cases, stopping or increasing the consumption of a given antibiotic does not always result in the concomitant decrease or increase of the corresponding resistances, and this may vary according to the studied environment, the public/animal concerned, and the antibiotic and bacteria considered.

Table 1: *Modes of action and resistance mechanisms of commonly used antibiotics*

Antibiotic class	Example(s)	Target	Mode(s) of resistance
β-Lactams	Penicillins (ampicillin), cephalosporins (cephamycin), penems (meropenem), monobactams (aztreonam)	Peptidoglycan biosynthesis	Hydrolysis, efflux, altered target
Aminoglycosides	Gentamicin, streptomycin, spectinomycin	Translation	Phosphorylation, acetylation, nucleotidylation, efflux, altered target
Glycopeptides	Vancomycin, teicoplanin	Peptidoglycan biosynthesis	Reprogramming peptidoglycan biosynthesis
Tetracyclines	Minocycline, tigecycline	Translation	Monooxygenation, efflux, altered target
Macrolides	Erythromycin, azithromicin	Translation	Hydrolysis, glycosylation, phosphorylation, efflux, altered target
Lincosamides	Clindamycin	Translation	Nucleotidylation, efflux, altered target
Streptogramins	Synercid	Translation	C-O lyase (type B streptogramins), acetylation (type A streptogramins), efflux, altered target
Oxazolidinones	Linezolid	Translation	Efflux, altered target
Phenicols	Chloramphenicol	Translation	Acetylation, efflux, altered target
Quinolones	Ciprofloxacin	DNA replication	Acetylation, efflux, altered target
Pyrimidines	Trimethoprim	C1 metabolism	Efflux, altered target
Sulfonamides	Sulfamethoxazole	C1 metabolism	Efflux, altered target
Rifamycins	Rifampin Transcription ADP	ribosylation,	efflux, altered target
Lipopeptides	Daptomycin	Cell membrane	Altered target
Cationic peptides	Colistin	Cell membrane	Altered target, efflux

For instance, Lai *et al.* (2011) reported a negative correlation between a decreasing consumption of cefotaxime and the rate of cefotaxime resistant-Escherichia coli pathogens isolated in a Taiwanese university hospital [64]. Similar trends were reported for the consumption of ceftriaxone and ceftriaxone-resistant E. coli and Klebsiella spp. in a Turkish hospital setting [65]. Negative correlations between antibiotic consumption and the development of resistances can also work the other way around and may depend on the bacterial species considered. In a Korean study covering six

university hospitals, Kim *et al.* (2018) observed contrasted results following increased consumption of fluoroquinolone [66]. The resistance rate for ciprofloxacin in *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, either increased, remained stable or decreased, respectively, over eight years. Surprisingly, the same authors also found negative correlation between decreasing consumption of aminoglycosides and the resistance rate for third-generation cephalosporins and ciprofloxacin, thus disconnecting a given drug consumption from its effect on the corresponding antibiotic resistance, at least for a few documented cases. Further, it is worth noting that carbapenem-resistant *P. aeruginosa* could be isolated from animals that have not been previously treated with carbapenems. In this case, the resistance to carbapenem was attributed to an efflux pump dysregulation (rather than a carbapenemase) resulting from mutations possibly selected by disinfectants and other antibiotics in veterinary practices [67], thus showing that resistant phenotypes can emerge independently from the presence of the corresponding antibiotics. On the other hand, the identification of antibiotic resistance genes in metagenomes from 30,000-years old sediments reminds us that resistance phenotypes and their corresponding genes probably existed before the so-called antibiotic era [68]. These observations indicate that the emergence and the dissemination of antibiotic resistance in bacteria cannot solely be explained by a simple selection process occurring during antibiotics therapy, even if the latter is an important driving parameter in many instances.

Prevention and control

Antibiotic resistance is hastened by the overuse and misuse of antibiotics, as well as with the reduced infection control and prevention. Steps can be taken at all levels of society to reduce the impact and limit the spread of resistance.

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CHAPTER

Plant litter decomposition –An overview

Abhijit Datta*[1] and B.K. Sharma[2]

[1]Department of Botany, Ambedkar College, Fatikroy, Unakoti, India
[2]Department of Microbiology, Tripura University, Suryamaninagar, Agartala, India
*E-mail:abhijitdatta123@gmail.com

Abstract: Decomposition of plant litter refers to the breakdown of dead organic matter to simple inorganic form through the action of soil flora and fauna by a series of events like development of microbial flora on the plant surface followed by colonization of different microbes in a successive manner alongwith the action of soil fauna. The process of decomposition is controlled by various factors like qualitative features of the litter, edaphic and climatological factors etc. among which climate and biomass quality influences the most. Soil enzymes also play major role in decomposition as well as mineralization. The pattern of nutrient dynamics also varies for different litter types. The fungal succession on the decomposing organic matter is a vital aspect of litter decomposition. They explore the nutrients in a sequential mode and perform a key role in litter decomposition. Regardless of the type of soil ecosystem the process of litter decomposition confirms it's growth, development and sustainability.

Keywords: Litter decomposition; Litter quality; Nutrient dynamics; Climatic factors; Fungal succession.

Litter decomposition is described as the biological disintegration of dead bio-organic matter into simple inorganic forms by the process of mineralization of complex organic compounds [1]. It may be any ecosystem; the decomposition of litter has crucial influence upon the growth and development of vegetation. Other than plant remains, fungal and bacterial components and dead residues of various fauna, both micro and macro forms are present along with litter. The functioning of an ecosystem depends upon three distinct subsystems, i.e., products, consumers and decomposers and more importantly transfer of matter and energy among these subsystems to maintain the structural and functional integrity of an ecosystem.

The sequence of events leading to the decomposition can be summed up along these lines: - development of microbes on the phylloplane, microorganisms colonize thereafter, combination and injection by invertebrates result in inclusion of organic

matter into the soil and increase of surface area of the litter. In the tropics the decomposition is high compared to high altitudes which elucidate the influence of climatic and edaphic factors on the decay of litter.

Plant litter quality governs the decomposer community by presenting a diverse range of resources of various microbial decompositibility. The resource quality and accessibility of leaf is high and therefore it is decomposed faster than twigs or other woody materials. Higher rate of turnover is obtained in case of litter rich in mineral nutrients. Irritani and Arnold suggested that the Nitrogen content is to be considered as most significant aspect in determining the pace of decomposition [2]. Vallis and Jones found that polyphenol content of plants determines the rate of decomposition [3]; while Plam and Sanchez proposed the ratio of polyphenol content to N content rather than content of N or polyphenol alone determines the actual rate of decay [4].

Among the litter decomposers, fungi are the most active. They have successive phases of colonization, exploitation and exhaustion of organic substrates during decomposition [1]. Fungi generally colonize the plant litter before it comes in contact with the soil. These fungi are the weak parasites which usually pave the way for the new saprophytes. Later, on the ground, other saprophytes colonize in a gradual manner. In this way the microhabitat of the litter is colonized by other saprophytes in a successive manner, resulting in the biodegradation of litter. Diversity of fungi and microbes is dependent upon the presence of various types of nutrients like Nitogen, Phosphorus, Potasium, Calcium, Magnesium etc.[1] More the nutrients more will be the presence of microbes resulting more microbial diversity and subsequently more mineralization of litter.

Although fungi are the most important group facilitating decomposition, bacteria, actionomycetes, protozoa etc. also are important due to their versatility and large number. Primary fungal colonizer and successive litter attackers are ruled by the soil factors, climate and litter parameters such as moisture, temperature, pH, depth, aeration, organic and inorganic nutrients which are interacting among themselves [5-6]. During winter months low temperature and moisture in the soil is not very conducive for the growth of fungi [7]. Moisture is considered as one of the key factors for the development and fungal activities. After the onset of rainy season, improved moisture content and most favourable temperature supports fungal growth and development, and furthermore these factors are interdependent.

Plant materials are heterogeneous in their composition and a variety of microbes attack them in a successive manner. The general pattern shows the following trend :

Sugar > Amino acids > Hemicellulose > Cellulose > Lignin [8-9].

The role of soil enzymes in litter decomposition and subsequent nutrient cycling is very essential. An immediate enhancement in the quality of nutrient pool, microbial activities and associated enzyme dynamics is a lot dependent on the immediate improvement in the status of organic matter the soil at any time. Some enzymes can only cause breakdown of dead organic matter, while others are involved in nutrient mineralization.

Litter Quality, Climatic factors and Nutrient Dynamics

The process of decomposition is primarily influenced by three factors viz. chemical nature and composition of litter, the array of decomposer organisms and the climatic regimes. Several models of disintegration of plant remainder consider the litter quality[10], under prevailing biotic as well as abiotic aspects [11] and the climatic features[12-13] as factors that manipulate the rate of litter break down. Consequent mobilization of nutrients after litter decay is better correlated to temperature in comparison to soil moisture in the context of litter decomposition [14] or litter residues with comparatively lesser fraction of lignin in addition to C/N ratio which symbolizes the fastest pace of litter weight loss and nutrients release [15]. Among the abiotic factors, OM content and soil fertility are the factors affecting group composition and behaviour of the soil microbes that play a key role in the degradation process revealing the interdependence and interaction of both living and nonliving factors leading to biodegradation and nutrient cycling [16]. Berg & Staaf reported that the higher the mineral content in litter the higher the achieved decomposition rate [17]. Deka & Misra also observed rapid weight loss of bamboo leaf litter which they attributed to the presence of softer cuticle, lower lignin content & higher moisture content and studied higher concentration of labile compounds in the litter from recent jhum fallow than in the old fallow [8]. The labile compounds disappeared before recalcitrants appear. Upadhya showed that the labile fraction of the litter determines the rate of degradation at the early stage [18].

The plant litter comprises a number of key types of organic compounds in various quantities and comparative extent of the said components vary in different plant parts like root stem, and leave as well as between species. Some materials, particularly sugars along-with phenolic substance having lower molecular weight, and some other nutrients, are at first lost from litter in the course of decomposition by dissolution and further leached collectively through the action of opportunistic microorganisms which show rapid growth. Macromolecules, esp. cell wall materials like cellulose, hemicelluloses, lignin etc. reveals slower degradation. During decay, phenolics and lignin-degradation products condenses, alongside nutrients import, bringing about the build up of newly formed substances.

Enumeration of the nutrient flux related to the litter fall is vital for the better understanding of dynamics of the ecosystem. The analysis of quantity, physical and chemical features of the litter and its pace of decay are vastly essential for looking into the process of energy flow, primary production as well as nutrient dynamics in forest and agro-ecosystems.

In the agro ecosystem the leaf litter generally, is of herbaceous nature and decomposes fast due to its soft tissue which are easily degradable. But in a forest ecosystem is comes mainly from large trees and thereby decomposes slowly [19].

Since individual leaf lives are shorter, the species with woody deciduous habit are by and large regarded as faster-return plants with than the woody evergreens [20] and the former decomposes as much as 60% faster than the later. It is valid for evergreen species of gymnosperms as well as angiosperms or for the latter only.

Climate influences and modify the characteristics and pace of decomposition of plant litter on soil surface and thus put forth a vital control upon the nature and ampleness of the organic matter. Moisture content and atmospheric temperature are amongst the most fundamental variables [21-22] for the reason that these aspects influence the plant cover development and the microbial activities which are extremely important soil forming factors. Maximized decomposition of dead organic matter is observed under provision of modest temperature of around 30°C and moisture content of the soil is about 60-80% of upper limit of it's water holding capacity [23]. Linear regressions between microbiotic decomposition of litter and precipitation ($r = 0.80$), decomposition with organic carbon content ($r = 0.75$), and also decomposition with ($r = 0.60$) was observed [24]. The pH result revealed the relationship with rainfall and temperature, enlightening the fact that poor decomposition both at lower pH with lower temperature and higher precipitation and higher pH with higer temperature and lower precipitation, and speedy litter breakdown at intermediary pH levels.

The rate of litter decomposition varies widely. While many factors like temperature, rainfall, soil pH, altitude etc affect the decomposition velocity, it's the varied chemistry of diverse litters that plays a key part in finding out the tempo of litter disintegration and nutrient cycling [25]. The incorporation of tree leaf biomass into the soil helps to retain long term fertility and stability of the soil [26]. The detrital resource is broken down by the collective action of the decomposer community consisting principally of microbes. The decomposed matter is added up to the next bunch litter fall resulting in a build up of forest floor material.

Pattern of decomposition was described as a function of three sets of aspects viz., chemical features of biomass like Nitrogen, Carbon, lignin in addition to polyphenol contents; climatic aspects i.e. temperature, precipitation etc. along-with conditions of

the soil like soil pH, organic carbon, total Nitrogen and Phosphorus etc. Out of these three groups, climatic features and qualitative features of the biomass influences the decomposition rates mostly.

After investigation of the changes in Nitrogen, Phosphorus, Potasium, Calcium and Magnesium in decomposing leaf litter of *Bambusa bambos* it was revealed that the release of nutrient varied element by element and took place after an preliminary phase of accumulation which was in a order of K →Ca→ Mg →N →P [27].

Similar nutrient dynamics study in *Quercus serrata* leaf litter decomposition in Japan revealed that release of elements during the phase of litter decay followed the following order of K→ P →C →Mg →Ca →N [28]. Another pattern of nutrient release was monitored in leaf litter of *Bambusa bambos* as:

i) Increase in concentration of N, Mg and Ca while there is a decrease in litter mass
ii) Decrease in concentration of K and C and also a decreased nutrient mass or
iii) Fluctuation of both concentration of P and Mg and litter mass.

Fungal Succession during Decomposition

Distribution of fungal decomposers and successional changing patterns taking place all through the decomposition route have been comprehensively scrutinized on a number of plant litter varieties and through different isolation methods [29-31]. Fundamental mechanisms of fungal successions in addition to advantages and biases of the diverse techniques were reassessed and discussed by Frankland [32-33].

Microbial succession is an important factor which influences decomposition substantially in various ecosystems. During the process of decomposition various fungi appear in a successional manner and exploration of organic substrates are purely sequential. The greater the species richness of the populations of decomposer groups the greater is the rate of decay. Many workers have worked on fungal succession [34-37].

Soluble components are lost occurs during the foremost phase, and subsequently in the second stage decay of holocellulose takes place and in the third and last stage lignin becomes a leading constituent when mass loss of the organic litter slows down approaches towards humus formation.

During the decomposition of plant litter by soil-organisms, the organic compounds are mineralized to return the elements into the environment [38]. In this way organic N, K, P, C etc. are converted by the soil dwelling microbes to inorganic elements, thus, replenishing the supply of elements in the soil [39]. It also increases the nutrient availability to the vegetation because majority of the mineral elements on which plants depend for growth can only be taken up when they are in their inorganic form [40]. So biogeochemical cycling or recycling of matters in nature is successfully operated by soil microbes among which mycoflora takes a leading role.

In an aerobic environment the fungi play major role in the decomposition process by secreting a large number of exo-enzymes directly in the environment [41]. Under tropical field condition fungal enzyme production is influenced by moisture and temperature [42], chemical composition of litter [43] and altitude as well as vegetation cover[44].

During the process of litter decomposition the group of fungi which appear first are those which utilize the available sugars are basically the phylloplane microbes coming in contact with the leaves through their spores from air. Later true decomposers of the soil appear on the decomposing litter which break down soluble substances and lastly with accumulation of lignin fungi which are having lignolytic capability like basidiomycetes predominates in the litter.

Conclusion

In terrestrial ecosystems, litterfall is considered to be the prime pathway of the returning back of nutrients to the soil. The release of carbon content of plant litter as CO_2 through decomposition mechanism by soil microflora can contribute to about 20% or more outward flow of CO_2 or efflux to surface of the soil, which is frequently termed as soil respiration. During decomposition, nitrogen (N), phosphorus (P), and calcium (Ca) are liberated from the plant litter where these may become accessible for plant and microbial uptake.

The velocity of the process of Litter decomposition are ruled by three core factors viz. temperature, moisture content, and quality of the dead organic matter alongwith microbial as well as micro/macro faunal community structure, which is certainly recognized as another possible key factor. Exponential increase of the activity of the soil microbial community with soil temperature in presence of substrate can be observed, and microorganisms can also be limited by many edaphic aspects like moisture content etc., resulting in, amplification of tempo of fresh litter decay with both rising temperature along with precipitation [45]. This common blueprint of litter degradation can also be manipulated by variable nature of the litter quality. Quality refers to distinctive features of the litter like chemical, physical features etc. that affect the vulnerability of the litter to decay. High percentage of labile components of the detritus like sugars, amino acids etc. leads to rapid decomposition because of readily metabolization of these compounds by soil microflora or leaching. For instance, readily decomposing structural compounds such as cellulose are rapidly broken down into simpler sugar sub-units by a number of extracellular enzymes, which yet again are metabolised by microbial flora. On the contrary, recalcitrant structural components like lignin and chitin are too big to move through plasma membranes, and exozymes work slowly on them.

Many views explain how primary features of the litter manipulate litter decomposition and N release from decomposing litter. Some proposes that litter disintegration and Nitrogen liberation reveals positively co-relation with initial litter quality. In the initial phase the C/N fraction possibly is the best forecaster of mass loss from the plant remain and release of Nitrogen, and amount of lignin becomes gradually more significant later on in the subsequent phases of decomposition. Other views suggests, variations in litter quality in the early stage like the ratio of lignin: Nitrogen and lignin: cellulose modify the pace of decomposition and nutrient release near the beginning stages of decomposition. Since substrate quality of plant litter lessens during the ongoing progression of decomposition, primary litter parameters has a diminishing control on rapidity of decomposition during later stages. At this point, decay rate is under control of weather conditions, soil texture, and exogenic supplies of labile Carbon and nutritive substances instead. Another hypothesis advocates that litter disintegration and paces of N liberation are negatively correlated to Nitrogen based estimations of initial features of the organic matter. A higher Nitrogen composition may perhaps in point of fact hold up decomposition rates afterwards in the degradation process, mainly if lignin concentration is also high. No matter what the fundamental mechanisms are, recurring assessments of litter-fall and subsequent decomposition by means of standard protocols may provide necessary information on Carbon and ecological recycling for all major soil ecosystems.

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CHAPTER

Gold nanoparticles: types, preparation and their application in diagnostics and therapeutics

Bireswar Bhattacharya* and Pinkan Sadhukhan

Department of Pharmaceutical Sciences, Dibrugarh University, Dibrugarh, Assam, India
*Email: bireswar9360@gmail.com

Abstract: Nanotechnology is an emerging inter disciplinary field of science which have amazed the scientists and researchers over the past century. Nanoparticles which are considered to be of less than 100nm in size in at least one of the three dimensions, are in great focus in the recent times due to their unique physical and optical characteristics for which they are being extensively studied, invested upon and researched. Gold nanoparticles also known as colloidal gold are a class of noble metal nanoparticles that are being used from the earliest centuries till the modern time and also in the present COVID-19 pandemic situation. Due to their varied characteristics, they are prepared into different products like nanoshells, nannocages, nanorods etc and are being employed into material science, diagnostics and even therapeutics. In the current review, we aimed at summerizing the overall scenario of Gold nanoparticles in a brief manner, focusing our description mainly upon the diagnostic and therapeutic uses and also the challenges associated with its use in healthcare.

Keywords: Nnaoparticles, gold nanoparticles, nanoshells, diagnostics.

According to Nature research, nanoparticles are defined as particles that exist in nanometer scale (below 100nm), and possesses definite physical properties such as uniformity, conductance and optical characteristics by virtue of which they are widely being studied and extensively being employed into material science, surface chemistry, electrochemistry and more importantly in Pharmaceuticals as nanocarrier dosage forms also known as nano-formulations. [1-2]

Metal nanoparticles are specific type of nanostructures which are synthesized by reducing metal salts in aqueous solutions. They have gained huge attention from the scientific community and are a subject of specific interest in varying fields of science for over a century. Metal nanoparticles have been subjected to various modifications with different chemical groups from time to time that allow them to be conjugated

with ligands, antibodies, and even drugs, thus, diversifying their applications. Gold Nanoparticles also known as colloidal gold is a suspension of nanometer sized gold particles. The first ever person in the modern times to work with colloidal gold was Michael Faraday in 1850s when he made observations which concluded to the fact that colloidal gold solutions have different properties from that of bulk gold. The earliest use of colloidal gold dates back to 4th century AD when it was used as a pigment due to its characteristic red colour to stain glasses and porcelain. In Europe, the procedure for making ruby glass was rediscovered by a German chemist named Johann Kunkell in the 17th century where he added gold nanoparticles precipitated from aqua regia to molten glass. Even the use of aurum potabile (drinkable gold) to cure range of diseases is centuries old, which was prepared by quenching a heated metal into wine. [3-4]

Gold nanoparticles can be easily prepared with a high degree of accuracy and precision, and in a broad spectrum of diameters by altering various parameters in the methodology which are described briefly in this paper for each type of them viz. Nanospheres, nanorods, nanoshells, nanocages and SERS substrates. Once prepared, they remain stable for longer periods of time by virtue of which they are being used in multiple areas of science. The diagnostic and therapeutic applications of gold nanoparticles have been discussed here briefly and also with reference to the recent COVID-19 pandemic. Though gold nanoparticles have found itself in use in different areas, but there lies certain challenges to its use, especially as nanoformulations in Pharmaceuticals which are briefly discussed in the end of the review.[5]

Preparation and Types of Gold Nanoparticles

The gold nanoparticles have varied subtypes depending on their shape, size and physical properties. Among these, the first ever synthesised and studied extensively are the gold nanospheres. The other subtypes along with all of their synthetic procedures have been briefly discussed below:

- **Gold Nanospheres**: The gold nanospheres though not exactly spheres (in most cases), are mostly synthesized by the Turkevich method in which $HAuCl_4$ aqueous solution is reduced using a citrate (Commonly Sodium citrate). One of the biggest advantage of this method is that by varying the citrate/gold ratio, the size of the nanospheres can be varied. This method is relatively easy to reproduce and nanoparticles in the range of 10-30nm can be easily synthesized. But if the size is to be increased more than 30nm, the particles start becoming less spherical and the size distribution broadens. Another method as reported by Giersig and Mulvaney in 1993 in which they electrophoretically deposited citrate and alkanethiol gold colloids onto carbon coated copper grids which formed ordered monolayers. This technique was inspired from the two phase system method used by Faraday in

1857 which was capable of producing stable gold nanospheres. Gold nanospheres in general display a single absorption peak in the visible range of the UV spectrum, between 510 nm and 550 nm. As the particle size increases, the absorption peak shifts to a longer wavelength and the width of the absorption spectra is related to the size distribution range.[6-8]

- **Gold Nanorods**: Gold nanorods have gained wide attention in the field of nanochemistry due to their unique optical characteristics by virtue of their characteristic morphoplogy. They have been vastly employed in the field of bio sensing and diagnostics. The mostly accepted approach for the preparation of gold nanorods is the seed-mediated synthesis reported by Jana el al. In this technique, a strong reducing agent such as $NaBH_4$ is used to chemically reduce a gold salt to form gold seeds, which further serve as the nucleation sites of nanorods. These are then added to a growth solution of gold salth with a weak reducing agent such as Ascorbic acid and hexahexadecyltrimethylammonium bromide. Jana *et al.* also reported separately that quantitative production of gold nano rods can be achieved by the addition of $AgNO_3$. [9-11]
- **Gold Nanoshells**: These are spherical layers of gold with a hollow or filled core. Gold nanoshells got their fame due to the unique property of Surface Plasmon Resonance (SPR), which can be tuned by changing the ratio of core size to surface thickness. The most widely used preparation technique of gold nanoshells is the seed-mediated growth method based on a silica template, in which silica cores are grown using the Stober process, the basic reduction of tetraethyl orthosilicate in ethanol. Small gold nanospheres (2–4 nm in diameter) are attached to the silica core using an amine- terminated silane as a linear molecule which allows the additional gold to be reduced until the seed particles coalesced into a complete nanoshell. Here, the diameter of the silica core template largely dictates the diameter of the gold nanoshell whereas the thickness of the shell depends upon the amount of gold deposited on the surface of the core. This technique is a very complex and a time consuming event. Researchers have also explored the synthesis of gold nanoshells using varying substances as the core template. One such is the in-situ gold nanoparticle formation using thermosensitive microgel as core-shell templates. This technique offers reduced particle aggregation and better thickness control of the gold nanoshells. [12-16]
- **Gold Nanocages:** These are novel class of cubic shaped nanocarriers which are hollow inside supported by porous walls. They are prepared by a technique known as galvanic replacement reaction in which polyol reduction is carried out between metal precusrsor salts and silver nanostructures. The reduced metal deposits over the Ag nanostructures due to the difference in their electrochemical potential. In

case of gold nanocages, $HAuCl_4$ is used as the metal precursor and the Au is deposited in a well defined orientation on the surface of Ag nanocubes adopting the cubic structure of the template. He inside Ag is then oxidised and removed which produces the final hollow gold nanoczages. The dimension and wall thickness of the gold nanocages can be adjusted according to the needs by adjusting the molar ratio of Ag to HAuCl4. [17-18]

- **Gold nanoparticles as SERS substrates**: Surface-enhanced Raman Spectroscopy or Surface-enhanced Raman Scattering (SERS) is a surface sensitive technique in which the Raman scattering of molecules increases which are adsorbed into metal nanostructures also otherwise called as SERS substrates. This technique holds a superior place in analytics and diagnostics due to its high sensitivity even detecting a single molecule and robustness. The two widely accepted theories for the enhancement of Raman spectra are Electromagnetic enhancement (EM) and chemical enhancement. In any of the cases, samples are placed nearby the SERS substrate where the enhancement occurs due to the interaction among the incoming light, the target molecule and the metal nanostructure surface. Gold and silver nanostructures as a SERS substrate are widely used since they are not Raman active. In a pioneering report by Cao et al in 2002, 13nm gold nanospheres were modified with Cy3- labelled, alkylthiol capped oligonucleotide strands which were then used as probes to detect the presence of specific target DNA strands. Similarly, many others have reported the use of gold nanoparticles as SERS substrate by applying variety of modifications. For example in another study 60nm gold nanospheres were encoded with a Raman label and stablisized with a layer of thiolated PEG. [19-25]

Gold Nanoparticles in Diagnostics

Gold nanoparticles have several advantageous physicochemical properties, including their excellent light absorbing and scattering properties. When a matter is exposed to light, either the light gets absorbed or it scatters at the same frequency as of the incoming light. The absorbed light can be re-emitted (i.e. Fluorescence) and the local Electromagnetic field of the incoming light can be boosted. In the case of Gold nanoparticles, all of these events are heightened due to the exceptional interaction of light with the free electrons in the metal. When gold nanoparticles are exposed to light radiation, the electric field of the light causes a collective oscillation of the conduction band electrons and this oscillation of the free metal electrons in resonance with the electromagnetic field is called the surface plasmon resonance (SPR). Compared to fluorescence labeling, Gold nanoparticles are more stable as they do not suffer from photobleaching which is a major limitation for fluorescence-based methods. SPR

scattering of the nanoparticles facilitate the visualization of the cells labeled with the anti-body conjugated gold nanoparticles clearly under monochromatic light illumination using a scanning laser confocal reflectance microscope, this provides effective optical labeling of the cancer biomarkers. [26-27]

The scattering light strength is very sensitive to the size and accumulation state of the particles. The ability to fast and non-invasively image molecular features of cancer using low-priced imaging systems can improve screening for and early detection of carcinoma, and can provide a more effective determination of tumor margins that can be used to study response to treatment in a noninvasive manner. The range of applications for Gold nanoparticles is growing rapidly, including their use in diagnostics to detect biomarkers of heart disease, as a contrast agent for clinical application of coronary angiography [28-29], cancer, and biosensing purpose. Gold nanoparticles can efficiently deliver DNAzymes and substrates into cells and serve as part of the sensor systems, such as signal reporters for light scattering and SERS or quenchers for fluorescence. [30- 32]

Recently, rapid diagnostic assays named COVID-19 Ag Respi-Strip are designed based on a membrae technology with colloidal gold nanoparticles using monoclonal antibodies directed against the SARS-CoV and SARS-CoV-2 highly conserved nucleoprotein antigen. Consequently another technology came into existence called P-FAB: A Fibre-Optic Biosensor Device for Rapid Detection of COVID-19, integrated U-bent fiber probe, where the biosensor matrix needs to be developed by first immobilizing the gold nanoparticles on the probe. Among others, a colorimetric assay is also developed based on gold nanosensors functionalized by antisense oligonucleotides, to target nucleocapsid phosphoprotein of COVID-19 for diagnosis of SARS-CoV-2-infected people using the naked eye. [33-35]

Gold Nanoparticles in Therapeutics

Gold nanoparticles have been used for a long time for the delivery of molecules into cells. For this purpose, the molecules are adsorbed on the surface of the Gold nanoparticles and the whole composite is introduced into the cells. Gold nanoparticles can protect nucleic acids from destruction by nuclease. They can help nucleic acid transfect cells and play a role in targeting, as gene therapy presents an ideal strategy for the treatment of genetic as well as acquired diseases.[36-37] Gold nanoparticles are capable of delivering all kinds of oligonucleotides such as plasmids, double-stranded DNA, single-stranded RNA, single-stranded DNA, and it can be done by Gene Guns, which are mainly used for delivery of DNA into animal cells.[32] They can also be used as nano-carriers for protein-peptide molecule delivery. It is also reported that Gold nanoparticles have been modified by chitosan for the effective delivery of insulin. Prophylactic vaccination is one of the most effective interventions in medicine and is

responsible for substantial decreases in morbidity and mortality by many pathogens worldwide. Gold nanoparticles have shown potential for delivering synthetic carbohydrate-based Streptococcus pneumoniae type 14 conjugate vaccine. Geiser and co-workers showed that gold nanoparticle delivery is an effective strategy for therapeutic targeting of alveolar epithelial cells and macrophages in chronic obstructive pulmonary disease (COPD). Gold nanoparticles have also been effectively employed in HIV/acquired immune deficiency syndrome (AIDS) vaccine development, and have also emerged as a siRNA carrier and target-specific gene silencing against viral and cancerous diseases. These nanoparticles can be easily modified for intranasal delivery and can readily diffuse into lymph nodes thus activating CD8+ (T-killer) cell-mediated immune response.[38-43]

Challenges in Using Gold Nanoparticles

Gold nanoparticles are broadly used in biomedical imaging and diagnostic tests. Based on their established use in the laboratory and chemical stability, gold nanoparticles were expected to be safe. However, recent studies have shown some unusual toxicity of using gold nanoparticles. water-soluble gold nanoparticles stabilized by triphenylphosphine derivatives having size 1.2-1.4nm showed predominantly rapid cell death by necrosis within 12 h of inclusion. Low-level inflammation has been shown after long-term exposure (90 d in a whole-body inhalation chamber) to the gold nanoparticle (4–5 nm) in the pulmonary alveoli in rats. Research suggests that gold nanoparticles with a particle size of 10 nm are potentially nephrotoxic due to their interaction with drugs. So, it can be understood that the smaller particles induce more toxic effects as it is easier to be taken up into the cells and then produce damage. In another study, it was found that the gold nanoparticles can catalyze NO production from endogenous RSNOs in blood serum. The later reaction of the released NO can eventually result in cell apoptosis. Gold nanoparticles that produce more reactive oxygen species(ROS) after entering the cells, lead to further oxidative stress-related cytotoxicity such as DNA damage, and cell death. Chompoosor et al reported the observed effects of Gold nanoparticles on carcinoma cells (A549 cells) in which gold particles were translocated to mitochondria from lysosomes, that resulted in the down regulation of mitochondrial membrane potential, increased oxidative stress and cell death. [44-50]

Conclusion

Gold nanoparticles though being widely employed in various sectors, their use in Pharmaceuticals should be extensively monitored due to some arising issues of toxicity that are being reported by many researchers. Apart from this, Gold nanoparticles have gained at most fame in the field of material science, diagnostics and analytical

chemistry. Their use as SERS substrate has made us capable to detect even a single molecule of any particular type in a given sample. Due to their small size and other characteristic physical properties they were found to be very efficient in working as protein or drug carriers, and hence are being thought to be useful in cancer treatment. With the advancement of technology, day by day researchers are being able to conjugate different functional entities and modify Gold nanoparticles to provide them with improved properties for a better outcome.

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Phytoremediation by aquatic plants: Its importance and applications – A review

Anwesha Deb and Kingsuk Das*

Department of Botany, Serampore College, 9, William Carey Road.
Serampore, Hooghly, West Bengal, India
*E-mail: daskingsukbotany@gmail.com

Abstract: Different water bodies – rivers, ponds, lakes and ditches contain ample aquatic plants. Aquatic plants contain aquatic angiosperms, aquatic ferns and thallophytes. These plants have a great role in maintaining balance in aquatic ecosystem and not only that; they can scavenge water by absorbing different heavy metal pollutants. In the present article, from the experiments performed by different research workers, it is evident that, water bodies can be purified by different aquatic plants. Pollutants, industrial effluents, agro-wastes and heavy metals are the common outcomes of environmental pollution which can be bioaccumulated by different plant bodies at different situations. This phytoremediation is a natural process where environmental stability can be restored without application of any cost effective technologies. From the elaborated works done by different workers, it is prudent that, different works are carried on aquatic flora to establish the phytoremediation potential for different pollutants. However, more works are needed to obtain effective phytoremediating strategy for different plants to make pollution-free environment.

Keywords: Heavy metal; pollutant; water hyacinth; *Marsilea*; lead

Pollution in water bodies through metals and other industrial effluents is a serious environmental problem. It jeopardizes the health of aquatic ecosystem and contaminates drinking water causing serious health hazards in human beings by accumulating along the food chain (bioaccumulation) [1,2]. Arsenic (As), Cadmium (Cd), lead (Pb) and Mercury (Hg) are present in industrial wastes and are well known toxic metals for causing health hazards in both human body and other animals. Such heavy metals are very persistent and can accumulate in different sedimentation layers [3]. Heavy metal can be removed and mobilized through different conventional methods such as sedimentation, adsorption, complexation, reverse osmosis, ion exchange, electrodialysis etc. But most of these are metal specific, energy intensive process and are quite costly [4,5].

Natural floras as well as major crop plants are facing irreparable damages by the toxic metals and other pollutants. Heavy metals are hazardous to soil and water bodies even in very less concentrations [6]. Heavy metal cadmium (Cd) is largely released from battery industry, waste incinerators, power stations, heating systems has a threatening effect on plants and other organism [7]. Multivalent heavy metal chromium (Cr) has toxic effect on plant system [8]. Another metalloid is arsenic (As) which causes major health problems. It is a burning health hazards in West Bengal scenario [9]. Copper (Cu) is required for growth and development of plant in very less concentration but when it exceeds that concentration and it causes deleterious effect on plants [10].

Recently, plants have been used as an alternative for phytoremediation to extract heavy metals from contaminated water bodies [11-13]. Use of plants for removal of heavy metals from waste water is a promising technology [5, 14]. Aquatic macrophytes can accumulate heavy metals from liquid environment to its body [15-19]. If a plant can accumulate or concentrate pollutants in minimum percentage varying from metal to metal such as, >1000 mg/Kg of dry weight for Ni, Cu, Co, Cr, Pb or >10,000 mg/Kg for Zn and Mn it is called a hyperaccumulator [20].

Nowadays different plants have been used as a promising alternative to extract heavy metals from water than the old practices like physical or chemical treatment [11- 13]. Different aquatic plants such as *Myriophyllum aquaticum, Elodea Canadensis, Potamogeton crispus, Eichhornia crassipes, Pistia sp, Lemna minor* have high capability to absorb Cu, Zn, Fe, Hg, Pb, Cd etc from aqueous solutions.

Heavy metal and water pollution

Different industries generated ample amount of heavy metals and lead (Pb), cadmium (Cd), arsenic (As), mercury (Hg), chromium (Cr) and aluminium (Al) are predominant among them [21]. Tannery industry is one of the most important industries which can cause water pollution. The effluents excreted from tannery contains noticeable amount of metals like Cr, Cu, Ni and Pb. Physico-chemical studies of tannery waste water revealed that Cr, Cu, Ni and Pb are present at the concentration of 4.67, 0.26, 0.08 and 0.04 mg/L respectively [22]. Cr in higher concentration in drainage and tannery waste water is due to the use of chromic acid during tanning process makes it toxic and thus it is a matter of concern [23].

Divalent cation Cadmium (Cd) is an effective pollutant available in soil, particularly in industrial zones. After absorption, Cd affects whole cellular metabolism in plants. Cd is fairly soluble in water and a relevant pollutant in water bodies. Hydrophytes are very prone to cadmium toxicity particularly in the industrial zone [24].

Several authors reported that Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) levels of drainage water are 342.89 ± 24.91 mg/L and 1420.06

± 98.61 ml/L. They are much higher than their respective prescribed standard (i.e. 30 and 250 mg/L) for the final discharge of tannery effluent into the inland surface water[25].

Water hyacinth- a potent accumulator of heavy metals (several reports)

Eichornia crassipes well known as water hyacinth is a potent accumulator of heavy metals. It is a free floating perennial plant usually grows on polluted water bodies. It can potentially accumulate Cr in its roots and shoots but concentration is higher in shoots. It can concentrate Copper (Cu) 16.85 µg/g dry weight in its roots [26]. Nickel (Ni) is another heavy metal which can accumulate successfully in its roots and shoots and the concentration is beyond the safer limits for Indian standards which is 1.5µg/g dry weight [27]. Several workers studied that water hyacinth can concentrate nickel (Ni) around 4.23µg/g and 2.89 µg/g of dry weight in root and shoot respectively which reflects that translocation of Ni is very poor from roots to shoots [22]. *Eichornia crassipes* can accumulate relatively high amount of arsenic (As), mercury (Hg) and cadmium (Cd) over a short period of time [28]. Different researchers used water hyacinth as a sample to study of its metal accumulation patterns. After exposure to 9.1 mg/L of cadmium, this plant can accumulate 6.1 µg/g cadmium in the top of the plant in just 24 hrs [29].

Other Aquatic angiosperms and metal accumulation

Aquatic angiosperms such as *Pistia sp, Lemna minor, Hydrilla verticillata, Ipomoea aquatica* are the potent metal accumulators. They are very useful for phytoremediation. *Lemna minor* can accumulate 25623±1949 µg/g of Cu, 58800±4982 µg/g of Zn, 8066±1800 µg/g of Cd and 22533±4935 µg/g of Pb from the treatment containing 10^{-4} M of each salt respectively [30]. *Hydrilla verticillata* grows on submerged water and make a carpet on the bottom of the waterbodies. *Hydrilla* plants accumulate Cu 1.01 µg/g d.wt. in its shoots and Pb concentration is 4.24 µg/g d.wt. in the roots [22]. Several researchers reported that the maximum concentrations of methylmercury, total Hg, Pb, and Cd in *Ipomoea aquatica* were 221, 2,590,530 and123 µg/kg dry weight, respectively from the cultivation area of Greater Bangkok region of Thailand [31]. Water lettuce or *Pistia sp* is a free floating plant. It can be used for the removal of surface waste of water. It is a hyperaccumulator of different metals like Cr, Cu, Fe, Mn, Ni, Pb, and Zn. Usually these metals remains in the roots in higher proportion than in the shoots [32]. Al, Cr, Cu, Ni, and Pb are such metal which *Pistia* roots can absorb. Other metals like Ca, Cd, Co, Fe, Mg, Mn, and Zn are adhered on its external surface [32].

Aquatic ferns and heavy metal accumulation

Different aquatic fern species have varying capacity to accumulate heavy metals. *Azolla, Marsilea, Salvinia* can potentially accumulate heavy metals from

water bodies[24]. Study of some workers indicates that, *Marsilea* is prone to allocate cadmium in its leaves. and they are sensitive to ROS induced lipid peroxidation and MDA content increases according to Cd concentration [33]. *Marsilea* plants are sensitive to oxidative stress at high concentration of Cd (100µM). Atomic absorption spectroscopic data revealed that when the plants were treated with 50 µM and 100 µM $CdCl_2$, the amount of accumulated Cd is different in leaves and roots or rhizomes. The 50 µM treatment plant accumulated 65.3 ±1.31mg/kg. 16.53±1.03mg/kg and 100 µM treatment plant accumulated 310.2 ± 10.75 mg/kg, 43.1 ± 2.01 mg/kg in leaves and rhizomes respectively [33]. Chinese Brake Fern (*Pteris vittata* Linn.) has been reported to be arsenic (As), lead (Pb), nickel (Ni) and selenium (Se) accumulators [34-36]. Maximum accumulation of aluminium in *Salvinia* was recorded in 480 µM which was 4.56 fold over the control plant by correlating (r^2=0.85) it with the amount of tissue loss [37]. Whole *Azolla* plant accumulates Cd 10441±740ppm/dry weight, 1904±126 ppm/d.wt. of Cr, 9224±109 ppm/d.wt. of Cu, 8814±15ppm/d.wt. of Ni and 6408±109 ppm/ d.wt. of Zn [38]. Heavy metal such as Cu, Cr and Ni concentration is much high in roots than the shoots of *Azolla* plant. The difference in the amount of accumulation directly reflects poor translocation rate of heavy metals from root to shoot [38]. Several researches reveals that even dried-dead *Azolla* plants can absorb Cu, Ni and Zn around 3% of its dry weight, Cr around 1.4% of its d.wt. and highest in Cd (4.1% of d.wt.) [38].

Marsilea and Salvinia – potent accumulator of heavy metals

Marsilea and *Salvinia*- these two ferns are reported as potent metal accumulator in several experiments. *Marsilea* is semi-aquatic in nature and generally it grows in the muddy portion of water bodies where land and water meet. On the other hand, *Salvinia* is a free-floating aquatic fern which usually exist on ponds and ditches. Leaves of *Marsilea* are softer and more tender than *Salvinia*.

Experiment shows that *Marsilea* plant is able to accumulate significant amount of cadmium (Cd) (262.1 µg/mg DW maximum) from 200 µM of Cd salt [33]. Moreover, different concentrations of cadmium alter the responses of antioxidative enzymes [39].

Salvinia – another aquatic fern which is capable to accumulate notable quantity of aluminium (Al). Researches on *Salvinia* reveal that it is able to accumulate 260 µg/g DW from 480 µM of Al salt [40]. Additionally, activities of different antioxidative enzymes and reactive oxygen species (ROS) are altered by different concentrations of Cd salt.

Conclusion

Our environment is very much susceptible to different toxic agents. Aquatic environment is significantly affected by pollutants which are the outcome of different anthropogenic

activities. Aquatic angiosperms and aquatic ferns are capable to scavenge different heavy metals and toxicants which are evident from the works of different researchers. From the above discussion, it is prudent that, the aquatic angiosperm – water hyacinth and the aquatic ferns – *Marsilea* and *Salvinia* are potent enough to intake toxic heavy metals. Different workers are carrying on their works on different aquatic plants in search of their phytoremediation potential. Phytoremediation strategy is a sustainable process by which nature can restore its pollution free form. More elaborative researches are in progress to achieve this goal.

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Physicochemical parameters and detritivore faunal diversity as an ecological indicator of soil health status under exotic rubber plantations of South Tripura In : *Biological Sciences: Impacts on Modern Civilization, Current and Future Challenges* by *Anupam Guha* © New Delhi Publishers, New Delhi: 2021, 254-261. ISBN: 978-81-947417-9-4, DOI: 10.30954/ndp.bio.2020.24

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CHAPTER

Physicochemical parameters and detritivore faunal diversity as an ecological indicator of soil health status under exotic rubber plantations of South Tripura

Gourab Roy*[1], Anirban Bhattacharya[2], Bijoy Kumar Jha[3] and Niladri Paul[4]

[1] Dept. of Zoology,M.B.B. College, Agartala, West Tripura, India
[2] Dept. of Botany, Iswar Chandra Vidyasagar College, Belonia, South Tripura, India
[3] Debagram High School, Nanur, Birbhum, West Bengal, India
[4] College of Agriculture, Lembuchara, West Tripura, India
*E-mail: roygourab2002@gmail.com

Abstract: Tripura is second largest rubber cultivator after Kerala and a major cash crop of the state which contributes a considerable part in states GDP. Many literatures suggested that, extensive cultivation of exotic rubber has deleterious impact on the ecological process and biodiversity of the cultivated area especially the rhizosphere of the plantation site. In this paper an effort is made toevaluate therhizosphere region of the plantation site of exotic rubberwith respect to floral and faunal community which could be used as tool of biological indicator for the ecological healthand biodiversity of the area under cultivation. Soil analysis of rubber garden showed the soils are sandy loam type and has very less water holding capacity (WHC as low as 28.6 %)mean pH range 3.8 with low organic carbon (0.68%) and CEC. Analysis of microbial parameters revealed that lower organic matter and low pH may be responsible for lower microbial biomass and reduced soil respiration. The faunal parameters indicated that diversity of many detritivore fauna was also very low but many groups showed dominance in the monoculture plantation sites as they were adapted to the ecological conditions. The overall results suggested that, monoculture plantation of exotic rubber may not be favourable for overall ecological process of the area under cultivation and alternative plantation and management practice is required for maintaining soil fertility and biodiversity.

Keywords: exotic rubber cultivation, monoculture, ecological indicator, rhizosphere, plantation practice.

Commercial cultivation of natural rubber (*Hevea brasiliensis*) was introduced in India by the British, although the experimental efforts to grow rubber on a commercial scale

in India were initiated as early as 1873 at the Botanical Gardens, Calcutta. The first commercial *Hevea* plantations in India were established at Thattekadu which comes under Ernakulam Distric of Kerala in Southern India in 1902 on the Periyar river bank see. [1] The Indian Rubber Board was established on the 19th April 1947 to look after the rubber plantation industry in the country. In Tripura, a north eastern state of India, rubber plantation was introduced in 1963 by the forest department to check soil degradation due to slash and burn agriculture practiced by the local tribal people and till 2015-16 total 74335 ha land is under rubber cultivation (Fig.1) comprising eight districts more or less. Majority of the plantation sites are in South and Dhalai then come West and North Tripura Districts respectively (Fig.2). Rubber is an important cash crop in the economy of Tripura where it is cultivated in more than 74,335 ha area over hill slopes, hillocks and plains [19]. Tripura is the second largest natural rubber (NR) producing state of India after Kerala. As most of the NR plantation in the state is rain dependent, it is observed that fluctuation in monsoon poses a serious threat to plantation growth. The effect of such shift and fluctuation in climate ranges from depletion of surface and ground water level, air quality and soil degradation and loss of biodiversity of local flora and fauna changing the community structure of the ecosystem [17].

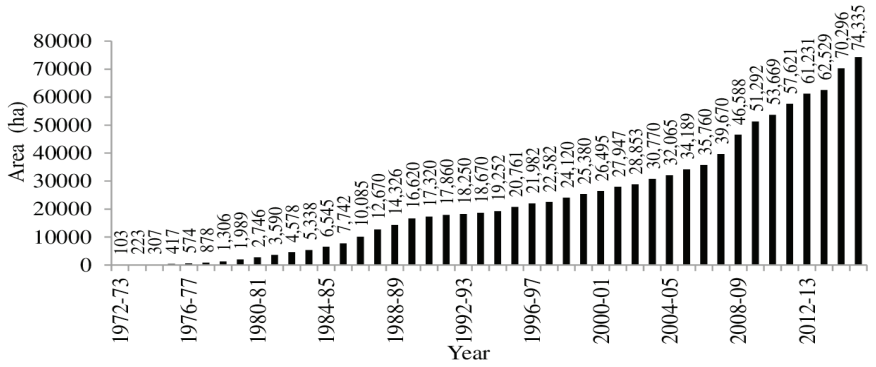

Fig.1: *Total area under rubber cultivation 1972-73 to 2015-16 [20]*

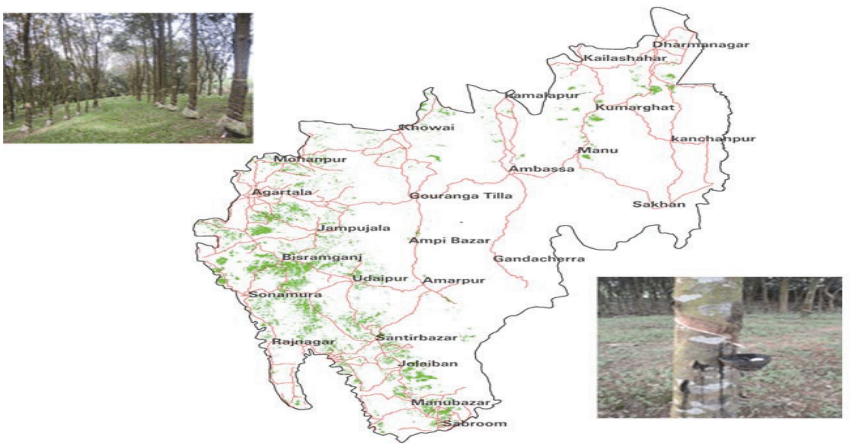

Fig. 2: *District wise distribution and plantation regime of natural rubber in Tripura* [20]

Soil organisms are major components of all soils. Often their biomass is low compared with the mineral or humus fraction, but the organism activity is absolutely crucial for a functioning soil. The soil biota can be regarded as the "biological engine of the earth" and is implicated in most of the key functions soil provides in terms of ecosystem services, by driving many fundamental nutrient cycling processes, soil structural dynamics, degradation of pollutants, and regulation of plant communities [2] and hence their parameters can be considered as an bio indicator for evaluating soil heath and biodiversity status of exotic rubber plantation sites of the state.

Review of Literature

Human-driven land-use changes increasingly threaten tropical forests and biodiversity associated with it where both species diversity and human interventions on natural environments are high. Replacing natural forests with rubber has led to rapid biodiversity loss, depleted water resources (as rubber trees suck up huge amounts of water and lowers the aquifer level) and even regional climate change which is observed recently in many parts of the tropics an example is Xishuangbanna region in Chaina which has become measurably warmer and drier within last decade [8]. Due to the rapid replacement of vast areas of primary forests with monoculture plantations of rubber, oil palm and other crops. As a result, Indonesia has been identified as one of the main global contributors of greenhouse gasses stemming from deforestation, forest degradation [7] suffering associated with biodiversity loss, land degradation, and impairment of ecosystem services, along with resulting negative effects on local livelihoods by creating major economic loss [6]. Agenda 21 calls for the conservation of biodiversity as well as actions to ensure sustainable development in the agricultural

and other sectors. A major feature of global change in the tropics is that of land use associated with agricultural intensification [9]. In addition to plants, soil is the habitat of a diverse array of organisms: archaea, bacteria, fungi, protozoans, algae and invertebrate animals, the activities of which contribute to the maintenance and productivity of agro-ecosystems by their influence on soil fertility [3-5].

This is mediated through four basic activities:

i) Decomposition of organic matte
ii) Nutrient cycling
iii) Bio-turbation and finaly
iv) Suppression of soil-borne diseases and pests.

In thispaper, the impact of monoculture rubber plantations has been evaluated in relation to soil fertility status, loss of biodiversity and productivity of forest cover and crops for the district South Tripura. The study is also important as till date no such work is done in the state and on the basis of the findings a roadmap can be designed for future to save natural mixed forests and biodiversity associated with it which in turn is related to the livelihood of the common tribal community who are socio-economically dependent on rubber cultivation. At the end of this research hopefully a sustainable conclusion can be drawn which will contribute to a new dimension of Joint Forest management (JFM) Practice which may prevent degradation of environment and biodiversity loss.

Materials and Methods

Soil sample was collected from monoculture rubber plantation sites of different locations (09 nos.) of south Tripura district in different seasons like summer, Rainy and winter during March 2015 to February 2016 and to maintain specific locations for multiple sampling during each sampling GPS was used and for batter interpretation all the data is cumulatively converted to mean and represented. Physiochemical analysis of soil samples like soil temperature, texture, moisture, water holding capacity (WHC), pH, organic carbon (OC) was evaluated by taking standard protocols of Jackson, 1967[10]. Microbial and soil respirationwas done following the standard protocols of Nannipieri, 1995[11]. Likewise soil sample collected from natural forest was analysed and compared with monoculture rubber garden soil. To analyse the loss of biodiversity and soil degradation due to excessive monoculture rubber plantation community structure of bioindicator species associated with detritivore cycle like microarthropods (Collembolla) nematodes and oligocheates was extracted from soil samples by “Tullgren extraction chamber” and Pit fall technique following the method given by Dombos M.A.(2002) [12]. Microarthropods were preserved for further

taxonomic grouping and identification was done by following the method of Ruiz and Lavelle, 2008 [13]. Finally statistical analysis of data analysis like mean, SD and graphs were prepared by XLSTAT.

Results and Discussion

In Tripura rubber was generally cultivated in hill locks and degraded waste lands where the bellow ground vegetation was almost negligible and due to lack of vegetation the top soil was eroded due to rain water runoff and so the physical characters of soil revealed that there were very low clay content with very low WHC (28.6± 2.1) and the soil is sandy type and soil temperature is on higher side. On the other hand natural forest had considerable amount of bellow ground vegetation and so the soil texture was clay laterite type due to higher amount of clay matter with moderate soil temperature due to retention of moderate amount of moisture and which was reflected with higher value of WHC (Table-1).

Table-1: *Physical characters of soil under rubber plantation and natural forest.*

Parameters	Rubber Plantations	Natural Forest
Temperature (^{0}C)	22.2 ± 2.3	19.6 ± 1.2
Soil Type	Sandy	Clay laterite
Sand	48.26	40.76
Silt	26.62	25.02
Clay	25.12	34.22
WHC (%)	28.6 ± 2.1	34.2 ± 2.16

± are *sd values with n = 0*

Table-2: *Physicochemical and microbial parameters of soil in Rubber Plantation and Natural forest*

Parameters	Rubber Plantation	Natural forest
pH	3.83 ± 0.63	5.8 ± 0.95
Organic carbon (%)	0.68 ± 0.32	1.2 ± 0.23
CEC cmol (+) kg^{-1}	5.8 ± 0.16	9.2 ± 0.88
Mean count of soil microbial population (CFU g^{-1} soil × 10^6)		
Bacteria	11.5 ± 2.12	19.2 ± 1.66
Fungi	5.4 ± 1.20	6.6 ± 0.82
Actinomycetes	7.6 ± 1.65	8.8 ± 1.26
Soil Respiration (gm/sqm/hr.)	0.34± 0.02	0.57 ± 0.12

± are *sd values with n = 05*

When chemical characters like pH, organic carbon and CEC was analysed it showed that rubber plantation soil had lower pH (3.83), low organic carbon (0.68) and hence the CEC value was also on the lower side as compared to the natural forest. This trend could be due to lesser rate of decomposition of the rubber leaflitter due to lack

of sufficient primary and secondary decomposer fauna. Lower rate of decomposition of rubber litter was also due to low microbial biomass as reflected by lesser number of primary decomposers like bacteria, fungi and actinomycetes (Table-2).

The natural forest soils are reported to harbour higher number of microbial populations and their activity. Being one of the most important components of soil environment, microorganisms exert considerable influence on soil fertility and plant growth [14]. Soil respiration study also supported the previous observations with microbial populations and it was quite clear from the data that due to low pH, organic carbon and moisture decomposition of organic matter was on lower side in rubber plantation as compared to natural forest where the rate of soil respiration was considerably high due to higher microbial load and presence of moderate amount of organic carbon. Other than the factors mentioned seasonal parameters like temperature and moisture also plays vital role as explained by Rout and Gupta [15] while studding the mixed forests concluded that, high rate of soil respiration during rainy season could be attributed to rapid organic matter mineralization by microorganisms due to favourable soil water, litter moisture and moderate soil temperature.

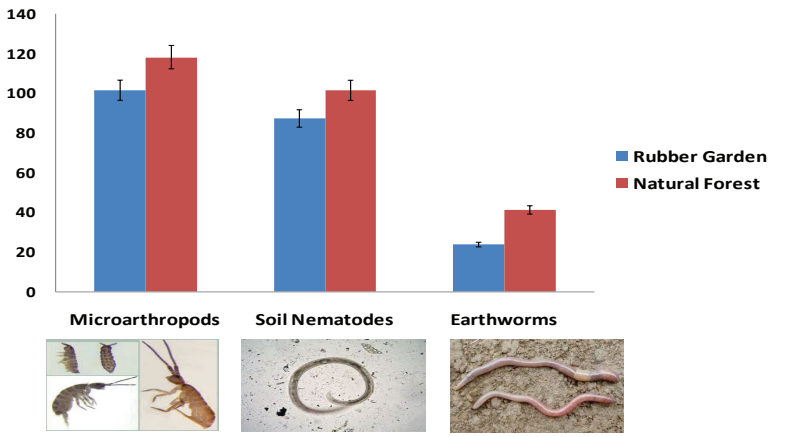

Fig. 3: *Source: reproduced from Roy et al, 2018 [18]*

Detritivore soil fauna like micro-arthropods, nematodes and oligochaetes plays vital role for providing different eco system services in forest ecosystem like decomposition of organic matter, nutrient cycling and soil formation thus maintaining biology and fertility of soil. As disused earlier it was observed that, due to lower pH, organic carbon and less moisture content in rubber plantation soil the biological activity was much low with respect to slow rate of decomposition which was actually due to reduced number of detritivore fauna (Microarthropods, nematodes and oligochaetes)

evident from the graph (Fig. 3) where the mean number of different decomposer faunal groups were considerably low as compared to the natural forest. Another interesting observation was, in most of the rubber plantation sites under study there was not much difference between total number soil fauna m^{-2} but the faunal diversity was much low in monoculture rubber plantation sites where there were very few of the genera of collembolan (*Onychiurus sp. and Isotomurus sp.*) and oligochaetes (*Octochaetona sp. and Gordiodrilus sp.*) were dominant but in natural forest diversity of the faunal groups were very high but dominance of a particular genera was low.

So it is quite clear from the study that, soil fauna could play the vital role of ecological indicators over various spatial and temporal scales to detect incipient change in ecosystem structure, function and composition in response to natural and anthropogenic influences. To date few terrestrial arthropod ecological indicators have been used in monitoring, and the potential for their future application rests on the outcome of the ecological indicator selection process [16].

Conclusion

From this ecologically oriented study it was very clear that the soil of monoculture plantation of rubber was not very fertile to support diverse group of soil fauna as compared to the natural forest. Because of low pH, organic carbon and poor cation exchange capacity and rate of decomposition the bellow ground vegetation was also sparse. It was also noticed that as per number of soil faunal community and diversity of detritivore soil faunal groups natural forest was much richer when compared to monoculture rubber plantation sites but some of the species got adapted to the niche of the monoculture plantation site and showed higher dominance but species diversity was on the lower side. So these observations of the detritivore faunal community could be considered for the bio monitoring practice to identify the biology and fertility status of exotic plantation practices.

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CHAPTER

Endophytic fungi as a new biological tool: A short review

Krishna Talapatra

Department of Botany, Rabindranath Thakur Mahavidyalaya, Bishalgarh, Tripura, India
E-mail: krishna.talapatra@gamil.com

Abstract: Without causing any obvious harm to their hosts endophytes stay in the internal healthy plant tissues during a part or/all of its life cycle. All most all plants are host of one or more endophytic fungi. Endophytic fungi produce different chemical compounds including: alkaloids, steroids, flavonoids, terpenoids, quinones and phenols. Researches on endophytic fungi show that they are new source of biocontrol agents. Also they have affect on the physiological activities of their host plants and enhance their host resistance against abiotic stress, disease, insects and mammalian herbivores. Fungal endophytes can consider as an alternative source of active compounds produced by its host plants also they can be alternative source of novel natural products for exploitation in modern medicine, agriculture and industries. The review represents general idea on endophytic fungi isolation, classification, functions and several classes of their secondary metabolites that they produced.

Keywords: Endophytic fungi; chemical compounds; medicine; agriculture and industries.

For over one hundred years the existence of endophytes has been known. Endophytes are those microorganisms that shows no apparent harm to host and occupy interior of plants particularly leaves, stems and roots [1]. From Greek word endophyte is derived 'endon' meaning within, and 'phyton' meaning plant. In 1866 de Bary first introduced this term. Nearly all classes of vascular plants and grasses that are studied are found to host of endophytic organisms [2]. It has been reported that each plant is the host of one or more endophytes [3,4]. Different groups of organisms such as fungi, bacteria, actinomycetes and mycoplasma are reported as endophytes of plants [5]. Different studies show that they can influence the distribution, ecology, physiology, and biochemistry of the host plants [6]. Nearly 200 years ago, studies of endophytic fungi were initiated in the plant *Sphaeria typhena,* now known as *Epichloe typhina* (Pers.) Tul. [7]. Plants have been associated with endophytes 400-million-year-ago, fossils study indicated that. A petro graphic thin segment of the Rhynie chert plant *Nothia aphylla* shows the presence

of three fungal endophytes in the prostrata axes of this plant [8]. Recent studies shows that with an estimate of 1 million species of endophytic fungi residing in plants [9] and even in lichens [10]. Endophytic fungi live as imperfect fungi most of the time and the relationships between fungal endophytes and their host plants, shows a variety ranging from mutualistic or symbiotic to antagonistic or slightly pathogenic [11,12]. Different studies shows that endophytes can produce different substances that are useful in agriculture, industry and modern medicine such as novel antibiotics, antimycotics, immunosuppressant and anticancer compounds[13]. The works of endophytic fungi and their relationships with host plants provides information on the ecology and evolution of both the endophytes and their hosts[14]. As the natural products are probable personalized to a specific function in nature, so organisms that inhabit novel biotopes should concentrate search for novel secondary metabolites. About 6500 endophytic fungi isolated from herbaceous plants and trees, and screened them for biologically active compounds [15]. Fungal endophytes present within the medicinal plants reports to contain novel metabolites of pharmaceutical importance. [16-18].Various natural products produced by endophytic fungi have exclusive structures and great bioactivities, which offers an enormous potential for exploitation in medicinal, agricultural and industrial uses [19,20], so the aim of this review is to provide recent data about fungal endophytes products and their uses.

Isolation of endophytic fungi from plants

Endophytic fungi reside within intercellular spaces of the tissues and possibly within the plant cells. Structural tools like X ray and electron diffraction are not very helpful, and it remains to be seen whether more specialized analytical methods can be developed that will enable scientists to successfully probe under the outer surface of a plant. Conventionally, staining techniques have been used so that fungal hyphae could be distinguished from the cell tissues. Small plant samples (leaf, stem or roots) are collected from the field and stored in plastic bags (preferably cooled) for transportation to a laboratory. In the laboratory, after soaking the plant sample in 70% ethanol the plant surfaces are sterilized to remove all microbial epiphyte. Next, inside the laminar-flow with a sterilized knife, the outer tissues of the sample are cut so as to expose the interior surface to agar medium on a covered culture plate. After few days of incubation at room temperature, the hyphal tips of fungi growth can be seen exuding from the plant sample. Then tiny cuts of these growths are transferred onto new agar medium plates or onto more nutrient-rich potato dextrose plates and repetitive re-plating of the microbial colonies is continued until a pure culture is obtained. Identification of endophytes can be done in preliminary level by studying differences in morphology, shape and colour.

Identification of isolated endophytic fungi

Endophytic fungal strains morphological identification is based on the morphology of the fungal culture colony or hyphae, the characteristics of the spores, and reproductive structures. Based on these features[21,22], following the procedures[23] and with the help of molecular methods identification of some major groups of fungi were done. The fungal strains were separately inoculated on potato dextrose agar, potato carrot agar, and water agar in petri dishes for inducing sporulation. For measurements of all fungal characters slides were made and the slides were subsequently mounted in lacto phenol and sealed with parafilm and observed under microscope. All experiments and observations were repeated at least twice. Those cultures that failed to sporulate were grouped as sterile mycelia, and divided into different morph species according to their cultural characteristics. The common problem during the identification of endophytes[24, 25] is that some fungal isolates not be identified up to the species level. To solve this problem the new approaching methods to identify endophytic fungi is by using polymerase chain reaction (PCR) and subsequent DNA sequencing[25]. The PCR can be performed on cultured endophytes using primers that amplify DNA encoding ribosomal RNA. By amplification of Internal Transcribed Spacer (ITS) regions identification of fungal endophytes can be accomplished, which are repeating units of DNA encoding ribosomal RNA. These regions evolve rapidly and there is a very large database of sequences on Genbank and the AFTOL (Assembly of the Fungal Tree of Life) project. Sometimes another sequence helpful in identification of fungal species is the 18S RNA. By sequencing the amplified DNA and comparing it with the database help to determine whether the specimen is novel or not; if the sequence is known, then the species can be identified.

Endophytic fungi classification

Based on differences in taxonomy, host range, colonization transmission patterns, tissue specificity and ecological function endophytic fungi divided into two major classes[26]. These are Clavicipitaceous endophytes (C-endophytes) and Non-clavicipitaceous endophytes (NCendophytes). But according to [27] there are four classes of endophytic fungi. Based on phylogeny data and life history traits there are two major endophytic groups (Clavicipitaceous and Nonclavicipitaceous) and on the basis of colonization and transmission, *in planta* biodiversity and fitness benefits conferred to hosts they classified nonclavicipitaceous endophytes into three functional groups.

Beneficial role of endophytic fungi

Endophytic fungi as a source of different secondary metabolites

The metabolites of fungi are diverse including those associated with proteins synthesis and respiration. A number of different secondary metabolites frequently have been isolated and chemically defined. Many of them are waste products while some others are pigments, toxins, and antibiotics that having biological functions. One of them is alkaloids. It is a chemical compounds containing basic nitrogen atoms. In endophytes alkaloids are quite common secondary metabolites, and some of them reported [28] to have anti-microbial activities. Endophyte cultures from a variety of the host plants [29] reported to produce phenols and phenolic acids. These compounds are reported to have significant anti-fungal activity against plant pathogens [30]. Many steroids are produced by endophytes, but most of the isolated compounds showed moderate antimicrobial activities [31, 29]. Terpenoids are also isolated from endophytic fungi; the most important terpenoids isolated from endophytes [29] are Sesquiterpenes, diterpenoids and triterpenoids. Researcher [32] isolated 127 terpenoids from endophytic fungi and they show different biological activity such as anti-microbial, anti-cancer and antiprotozoa [32]. Quinones are also known to isolate from some endophytes and some of them shows growth inhibition activity against some phytopathogens[18]. Peptides produced by endophytes have significant antimicrobial activities, e.g. leucinostatin A produced by *Acremonium* sp. [33]. Endophytic fungus *Periconia* sp. F-31 isolated from the medicinal plant *Annona muricata* [34] known to produce a new polyketide synthase–nonribosomal peptide synthetase hybrid pericoannosin B. Endophytic fungus CR127A isolated from white yemeri tree *Vochysia guatemalensis* in Costa Rica produces a tryptophan–polyketide hybrid Codinaeopsin, that shows activity against *Plasmodium falciparum*, the causative agent of the most lethal form of malaria, with IC50 = 2.3 μg mL-1 or 4.7 μM [35]. Endophytic fungus from *Salvia miltiorrhiza* produces salvianolic acid C like its host plant [36]. *Cryptosporiopsis cf. quercina* an endophytic fungus produces cryptocin in culture. This is a tetramic acid shows antimycotic activity against several plant pathogenic fungal strains including *Pythium ultimum* and *Pyricularia oryzae* [37].

Endophytic fungi as a producer of antibiotics, antifungal and antiviral compounds

Natural products isolated from endophytic fungi are reported to inhibit or kill a wide variety of harmful disease-causing agents including, phyto-pathogens, bacteria, fungi, viruses, and protozoans that generally affect humans and animals. Hydroxylated quinone altersolanol A, isolated from an endophytic fungi *Phoma multirostrata* and it shows activity against bacteria [38]. Endophytic fungi *Phomopsis* sp. produces a secondary metabolite phomopsichalasin that shows antibacterial activity against

Bacillus subtilis, *Salmonella enterica* and *Staphylococcus aureus* in disk diffusion assays [39]. *Colletotrichum gloeosporioides* an endophytic fungi collected from *Artemisia mongolica* produces colletotric acid and this metabolite shows activity against bacteria as well as against the fungus *Helminthsporium sativum* [40]. Endophytes fungi *Cryptosporiopsis* species, *Abies Alba* and *Pleurophomopsis* species have antibiotic activity [41]. Some endophytic fungi like *Alternaria*, *Fusarium*, *Cladosporium*, *Curvularia*, *Trichocladium* and sterile mycelia recognized for antibacterial activity[42]. The imperfect stage of *Pezicula cinnamomea* is *Cryptosporiopsis quercina* it was isolated as an endophyte fungi from a medicinal plant native of Eurasia *Tripterigeum wilfordii* [43]. When it was tested against some important human fungal pathogens viz. *Candida albicans* and *Trichophyton* sp., it shows excellent antifungal activity. Peptide antimycotic termed cryptocandin was isolated and characterized from *C. quercina*[43] and it also shows activity against a number of plant-pathogenic fungi including *Sclerotinia sclerotiorum* and *Botrytis cinerea*. Endophytic fungi *Fusarium* sp. isolated from the plant *Selaginella pallescens*, shows antifungal activity, CR377, a new pentaketide antifungal agent was isolated from the culture broth of this fungus and showed potent activity against *Candida albicans* [44]. Endophytic fungi *Colletotrichum* sp. isolated from *Artemisia annua* have activity against human-pathogenic fungi and bacteria and also show its activity against plant-pathogenic fungi [45]. Antibiotic products isolated from endophytic fungi are reported to have activity against viruses. Such as from the solid state fermentation of the endophytic fungus *Cytonaema* sp., cytonic acids A and B two novel human cytomegalovirus protease inhibitors have been isolated. By mass spectrometry and NMR methods their structural isomers are elucidated as p-tridepside [46]. Some endophytic fungi like *Alternaria*, *Fusarium*, *Monilia, Penicillium*, *Phialocephala*, *Trametes* recognized for the production of podophyllotoxin [47], the potent compound with anticancer, antiviral, antioxidant, antibacterial, immunostimulation and anti-rheumatic activities. In addition, this compound has been used as a precursor for chemical synthesis of the anticancer drugs like etoposide, teniposide and etopophose phosphate [48].

Role of endophytic fungi in agriculture

In the crop field crops are attacked by different insects and cause the damage of crops. Different chemical insecticides are used to prevent this damage. But these insecticides have harmful effects. Different studies proved the ability of endophytes to control disease agents [49]. Between 1981 to1985 it was established the existence of plant protection against herbivore insects given by endophytic microorganisms. An endophytic fungus *Phomopsis oblonga* protected elm trees against the beetle *Physocnemum brevilineum* by reducing the elm Dutch disease causal agent *Ceratocystis ulmi* [50]. Another report [51] shows the control of insects-pests by endophytic fungi

perennial ryegrass *Lolium perenne* against the sod webworm. Studies shows that when the genus *Acremonium*, plants free of endophytic fungi are severely attacked by insect[52,53]. One of the most important pathogen of cacao is *Moniliophtora perniciosa* which caused witches (broom disease) of cacao. Different fungal strains are used to control the disease and among them *Gliocladium catenulatum* decrease the infection of disease in cacao seedling to 70%[54]. Other scientists found that European corn borer (*Ostrinia nubilalis*) can be control by isolated *Beauveria bassiana* from maize (Zea *mays*)[55]. There are many studies on controlling the nematode and insects by endophytic fungi that, which are isolated from plants hosts like soybean, maize and sugarcane[56]. From various abiotic and biotic stresses endophytes increase the resistance in plants. Endophytic fungi released different compounds that accelerate the process of nutrient uptake in the environment effect plant growth directly or indirectly. *Piriformospora indica* an endophytic fungus reported to increase the growth of different hosts and which shows its advantage for promotion for plant growth[57]. Different endophytes isolated from *Eucalyptus* by preventing diseases able to increase growth of seedlings[58]. *G. citricarpa* release large amounts of enzymes like pectinases, endoglucanases and amylases considered in the improvement of citrus black spot, such as pectin-lyases that degrade pectin more effectively in the pathogenic strains.

Conclusion

In plant tissues discovery of endophytic fungi raised new possibilities in the investigation for metabolically active compounds. In nature, endophytic fungi have close interaction with plants. Endophytic fungi gain special attraction and interest by worldwide researchers because of its rich source of novel natural compounds with interesting biological activities, a high level of biodiversity and also they can produce several compounds of pharmaceutical significance. Endophytic fungi secondary metabolism is particularly active because of their metabolic interactions with their hosts. Acquiring more knowledge about endophytic fungi throughout the world can give a good opportunity in the field of agriculture, industry and medicine. Why endophytes are so important discussed below[59]:

1. The endophtyic study offer high taxon diversity; can be finished in the relative comfort of a laboratory with smallest fieldwork, and use a well-known traditional methodology that any enthused student can follow.
2. Easily identification of sporulating isolates [at least to genus] as there are less than 50 characteristic genera.
3. For mycelia sterilia different procedure can be applied to help sporulation; molecular methods can be used to classify these comparatively fast growing morphotypes.

4. Sophisticated statistics can be used to those isolates that "appear" to have been derived from single random units and can be satisfy the demands of any unforgiving non-fungal ecologist.
5. Those endophytes which are relatively fast growing and "highly" diverse are the best tools for screening and discovery of novel compound and can be easily lodged in culture collections.

As these endophytic fungi are brilliant source of bioactive natural metabolites with broad range of functions and structural diversity so it can be used as a bio fertilizer. In conferring resistance to biotic and abiotic stress conditions they also play a vital role. Endophytic fungi are very easy to culturing and they can easily fulfil the future demands in medical, agriculture and pharmaceutical industries.

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Biofeedback monitoring for evaluation of e-learning content using physiological correlates

Jayeeta Banerjee[1] and Moushum Bhattacharyya[*2]

[1] Ananda Mohan College, Kolkata, India
[2] Government Degree College, Dharmanagar, Tripura (North), India
*E-mail: moushum78@gmail.com

Abstract: As the COVID-19 Pandemic runs its course, many governments are implementing measures that limit the number of people congregating in public places. Such measures have disrupted the normal functioning of schools and universities. Because the duration of such measures has been extensive – and is likely to continue in some countries for a certain time until a vaccine becomes available – leaders of public and private education institutions have put in place alternative methods for students and teachers to continue with their lessons when attending school is not possible and are working on methods that will make schools fit for working in a safe environment. Many academic institutions that were earlier reluctant to change their traditional pedagogical approach had no option but to shift entirely to online teaching–learning. However, it has been observed that these e-learning contents are often poorly designed without keeping in mind about the end-users. Evaluation is often one of the weakest areas of any e-Learning program. There may be no standards against which to evaluate. Outcomes may not be defined. The purpose may not be determined, and questions about who benefits (teacher-learner, school, or student) may not be developed. One of the measures that might be used in evaluating the appropriateness of the e-learning contents is by using the technique of biofeedback monitoring. In this technique the user's physiological correlates are estimated in real time while performing the task. Various researchers have attempted to measure brain and other bodily functions associated with attention, focus and presence in a virtual environment. In this article, we attempt to review the various methods for measuring cognitive brain functions and other physiological correlates, which might help us gain an insight in designing for an optimum e-learning content for the users.

Keywords: e-learning; physiological; biofeedback; electroencephalography; eye-tracker.

The COVID-19 has resulted in schools shut all across the world. While countries are at different points in their COVID-19 infection rates, worldwide there are currently

more than 1.2 billion children in 186 countries affected by school closures due to the pandemic. In India, it has impacted over 240 million children of the country who are enrolled in schools. As a result, education has changed dramatically and schools all over the world planned to make e-learning part of their 'new normal', whereby teaching is undertaken remotely and on digital platforms. Now-a-days, digital education is one of the most effective ways to ensure continuity in school education. However, there are some drawbacks of online learning when compared to experiential learning. Experiential learning encompasses sight, smell, touch, hearing, and taste, as well as talking and feeling, which embed learning in the long-term memory portion of the brain. On the contrary, online learning is not the optimum method for deep learning, because it only involves the senses of sight and hearing. In experiential learning, information moves along neural pathways in the brain and takes it from short-term to long-term memory [1]. Knowledge gained from online learning is present only in the working memory and never moves to long-term memory [2]. Additionally, increased screen time has detrimental health and social consequences too. Continuous viewing causes eye strain and headaches. The physical, social, and emotional needs of the learner go undetected since there is no visual contact between the teacher and the student [3]. The primary concern of online/e-learning is helping students to pay attention. It is extremely challenging for educators who are delivering online teaching to be successful when they lack a basic understanding of how the attention system works.

Attention is the behavioral and cognitive process of selectively concentrating on a discrete aspect of information, whether considered subjective or objective, while ignoring other perceivable information. It is a state of arousal. In 1890, William James wrote that "Attention is the taking possession by the mind, in clear and vivid form, of one out of what seem several simultaneously possible objects or trains of thought. Focalization, concentration, of consciousness are of its essence [4]. Although the human brain is highly adept at processing information, it has a limited capacity meaning it is unable to attend to everything received from stimuli and memories at once. Attention is the capability of the brain to choose one aspect on which to concentrate while ignoring everything else in the environment. It lies in three areas of the brain:

(i) The prefrontal cortex, located behind the forehead and spanning to the left and right sides of the brain, handles willful concentration. It helps an individual to focus attention on a goal;

(ii) The parietal cortex, behind the ear, is for sudden events that require action; and

(iii) The Reticular Activating System (RAS), which includes a number of nerve fibers such as the thalamus, hypothalamus, brain stem, and cerebral cortex.

The RAS accounts for shifts in levels of involvement in surroundings, for instance, when it is operating fully, the person is awake, alert, and attentive, but when it is less active, the person feels tired and inattentive. The RAS is responsible for sorting sensory stimuli at the spinal cord and sending the only most relevant information to appropriate destinations in the conscious brain. Information that gets this far is carried to the amygdale, the emotional filter, which determines what passes to the prefrontal cortex, the place where the highest cognition and emotional reflection takes place. The brain's goal is to choose the stimulus that is the most immediately relevant and valuable, so it is easiest to pay attention when information is interesting [5]. For effective learning to take place, students must focus their attention on the learning activity. Paying attention during an on-line class is a task educators take for granted; they rarely stop to think about the complex neurocognitive processes involved. However, it is an important topic for e-learning educators who are often concerned about the superficial elements of their courses and neglect to learn how the brain works. After all, paying attention is the first step in the learning process, so ensuring learners pay attention is fundamental. However, voluntarily keeping attention is a challenging task. In on-line classes, the less engaging the class, the more difficult it is for students to hold their attention. In order to create interesting classes, e-learning educators must include meaningful learning situations and opportunities throughout the class to maintain attention and retain information in the long-term working memory.

There is often an underlying assumption that the main goal for designing virtual environments like e-learning/on-line classes generated by a computer that simulate some characteristic of reality is to promote a sense of presence of the user. Cummings and Bailenson in 2016, noted that a heightened sense of presence enhances the user's attention, focus and capacity for interaction with the simulation [6]. The users we are talking about here are students who are attending virtual on-line classes. Several studies have explored attention, focus and the sense of presence and its possible physiological correlates in a virtual simulated environment, but there is a lack of an overview and critical analysis of the various methods. One of the attempts used to measure attention, focus and presence in virtual settings is by estimation of various human physiological correlates while performing the task. This type of monitoring is known as the biofeedback monitoring of physiological functions while performing a particular task, like on-line learning, during the course of a virtual classroom [7]. Biofeedback can be used as a monitoring tool to identify students who are facing difficulty to focus during an on-line session. It has been used by schools all over the world to improve the focus and attention, reduce impulsiveness and to generally improve on-line classroom management for a better outcome. In view of the above, in this article, we intend to review the scope of engaging the technology of biofeedback

monitoring by use of physiological correlates to understand how the cognitive brain functions and other bodily functions respond in individuals who are involved in the on-line mode of education as the only means of learning during this extended COVID-19 pandemic.

Different types of Biofeedback monitoring techniques for e-learning

There are different types of physiological correlates that have been used in biofeedback monitoring of e-learning. There have been numerous attempts to use these human physiological measures as indices for attention, focus and presence [8], in virtual simulated environments. These physiological indices can be coarsely divided into two families: brain-related and nonbrain-related (Fig. 1).

I. Brain-realted biofeedback (neurofeedback) monitoring

Electroencephalography (EEG) - Among the brain-related biofeedback monitoring measures, electroencephalography (EEG) is one of the most commonly used within the field of cognitive science and has found extensive use in relation to the sense of presence, attention and focussing [9]. EEG measures the electrical activity of the human brain in a passive and non-invasive way: many neurons disposed perpendicularly to the scalp and firing at the same time produce an electrical potential that is possible to measure from outside the scalp. EEG signals can be analyzed in several different ways. Continuous EEG signals can be divided into frequency bands (usually delta, theta, alpha, beta, and gamma), and those oscillatory neural activities can be interpreted, for example, in connection with human behavior and cognitive processes [10]. Attention has been often investigated using the EEG-based technique of event-related potentials[9]. ERPs represent brain activity generated as a response to an event (a stimulation that can be, e.g., visual or auditory). This activity is generally averaged across many samples (trials), in order to reduce signal noise and obtain a reliable estimate for the brain activity related to the response to the stimulation [11]. The ERP methodology takes advantage of the good time resolution (milliseconds) of the EEG recording and it is widely used in cognitive research (attention, perception, consciousness, etc.). However, EEG has somewhat imprecise signal localization (low spatial resolution). EEG is sometimes also utilized to identify the physical sources of brain signals and connectivity among brain areas and this methodology has also been implemented for the spatial individuation of the physiological correlates of attention [12]. EEG offers several advantages. It is relatively affordable, even for unspecialized laboratories. The latest developments of portable consumer-grade equipment have made EEG equipment cost-effective and easy to operate. However, the setup of the equipment for the experimental phase is rather lengthy compared with other methods, and data analysis requires specialized expertise. Furthermore, the recording

is somewhat sensitive to movements, and data quality could be impaired in virtual environments (VEs) where movements are essential. For the latter reason, EEG is not adaptable to all kinds of VEs.

Functional Magnetic Resonance Imaging (fMRI) - A further brain-related physiological measure that is used for the study of attention, focus and presence is functional magnetic resonance imaging [13]. fMRI is a brain imaging technique that allows for spatially precise (millimeters) identification of activity in the human brain. The fMRI scanner can identify the flow of oxygenated blood in the brain in a relatively short amount of time (seconds). This identification occurs due to blood oxygenation-dependent imaging (BOLD), which highlights the activated brain areas. However, the spatial precision of fMRI comes at the cost of temporal resolution, which is much lower than that of EEG [14]. Furthermore, fMRI is very expensive and is generally used for clinical purposes, sometimes only available within hospital infrastructures. The machinery is complex to operate, and specialized medical staff are often required for its correct operation and to limit possible usage risks, further increasing its operational costs. Moreover, there are some non-negligible risks and restrictions for the participants of fMRI studies. For example, a participant cannot participate in fMRI studies if he/she has permanent metal prostheses on his/her body (or, e.g., a pacemaker), as the magnetic field of the scanner may interact with the metal. Eventual presentation of the stimuli (e.g., via VR goggles) needs to be mediated by MRI-scan compatible equipment, i.e., not interacting with magnetic fields. Additionally, the experiment is performed in an unnatural lying-down position, and the head of the subject needs to be immobilized, often causing discomfort. These limitations make this methodology challenging to apply in most, if not all, on-line simulated VEs.

II. Nonbrain-realted biofeedback monitoring

Generally, brain-related physiological measures are more expensive and less adaptable compared to non-brain physiological measures.

Galvanic Skin Response (GSR)/ Electro-Dermal Activity (EDA)/ Skin Conductance (SC) - Several studies have explored the use of galvanic skin response (GSR), also known as electrodermal activity (EDA) or skin conductance (SC), which measures how the electrical variations in the skin trigger eccrine sweat glands, a phenomenon that allows SC measurement. The use of SC is well documented in the literature on human emotion and cognition [15]. SC is associated, for example, with stress, excitement, engagement, and frustration, and arousal, among other factors. Furthermore, stimuli that promote attentional processes and attention-demanding tasks relate to several characteristics of the SC signal. SC data can be analyzed in several ways (e.g., decomposing phasic-transient and tonic activity), even though researchers are

still in the process of understanding the exact meanings of those different components of SC[16]. SC is easy to set up and minimally invasive, while SC sensors are affordable. However, SC data are sensitive to movements (especially of the part of the body where the sensors are attached) and to all those activities that may modulate the activity of the eccrine sweat glands. Furthermore, SC may not be an optimal proxy for the measure of attention, focus and presence, as SC modulation is highly dependent on the content of the immersive experience. SC can also be significantly modulated by external (often non-controlled) factors, like room temperature or environmental humidity [17].

Heart Rate (HR) and Hear Rate Variability (HRV) - Heart rate (HR) has also been investigated as a possible correlate of attention. In one of our previous study we have seen that heart rate (HR) and heart-rate variability-HRV are the most reliable methods in psychophysiological research [18]. HR is the calculation/estimation of the average heartbeats per timeframe (generally 1min). HRV, instead, is the measure (in milliseconds) of the changes (i.e., the variability) between successive heartbeats. This time period is called the R-R interval. The experimental methodology generally preferred for HR studies is electrocardiography (ECG or EKG). ECG records changes in electrical potential associated with the heartbeat. Due to its affordability compared to ECG, PPG (photoplethysmography) was also used to measure heart rhythm. The PPG can detect blood volume changes in the microvascular tissue, and it is often measured using a pulse oximeter, which illuminates the skin and can detect changes in the light absorption and, with that, indirectly measures the heart rhythm. Heart rate sensors are cheap, especially PPG sensors. HR is generally less affected by movement than EEG and SC. On the other hand, PPG provides fewer analysis possibilities compared to ECG, giving only the number of heartbeats per time but without giving information on the beat components. However, ECG is more expensive, more challenging to operate, and more invasive than PPG.

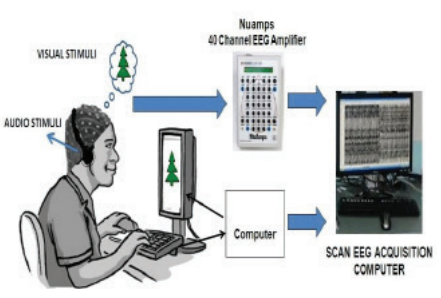
Skin temperature (ST) - is a rather simple experimental methodology in which a sensor records the temperature of the participant. Even though the use of skin temperature in psychological research date back many years [19] and has attracted attention in cognitive science research due to its cost-effectiveness and simplicity of use [20], this method has not found as many applications as the other methodologies listed above.

Electromyography (EMG) - is a technique for recording the electrical activity produced by skeletal muscles. Its setup is easy and inexpensive. However, it finds limited application within the field of cognitive psychology. Due to its connection to subject behavior (muscle movements), it has generally been less used to study cognitive phenomena except the quite widely used technique of facial electromyography [21]. Its

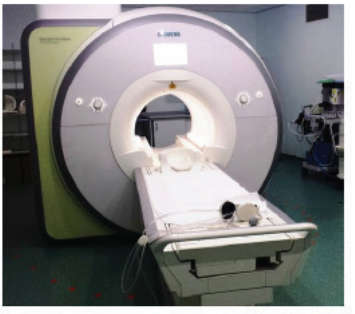
robust connection with human behavioral responses makes this technique difficult to use in many VR contexts.

Eye Tracking – Eye tracking can be used to improve the functionalities of an e-learning system. It can dynamically capture users' behaviors in such a way that determines what they are doing, how much attention they are giving to each topic, where they are stuck, at and in what order they are reading content. Eye movement parameters are considered as important physiological measures for assessment of cognitive workload [22]. Eye tracking is nothing more than using a device to track the movement of the eyes to understand where the subject is looking and for how long. There are a few different types of eye-tracking devices. Head-mounted devices are by far the most suitable for studying reading behavior. On the other hand, devices that are integrated into TFT monitors or standalone eye-tracking units are more typically used for market research and usability. These devices operate by measurements of electric and photoelectric signals, tracking a number of visual features in the image of the eye, measuring relative reflection of infra-red (IR) light, and using either mechanical or optical levers or a magnetic field. The eye movement measurements that correlate well with mental workload and cognition are blink rate, pupil diameter, mean fixation duration and total gaze duration. Rates of blinking provide insight into the cognitive state of an individual. Blink rates increase with anxiety levels or fatigue and decrease when people are especially alert. Blink rate varies with cognitive workload. When there is an increase in cognitive workload blink rate decreases. During fatigue blink rate increases than normal resting level. Pupil diameter changes due to light reflex and near reflex. It is found to correlate to the number of items held in recent memory and is sensitive to various workload components, such as perception/visual load, mental processing and response. Pupil size is also an indication of the person's cognitive state. When a greater degree of mental effort is required to do a task, the size of the pupil increases. The pupil size also is an indication of the amount of interest a person shows in a particular subject [23]. The two other important eye movement parameters i.e. mean fixation duration and total gaze duration are also important indicator of cognitive workload. The first well-known applied uses of eye trackers in the study of human visual attention were those conducted by Rayner *et. al* in 1989 [24]. The primary goal of early eye trackers was to support research in human visual data acquisition. But as instrumentation technology continued to evolve, it eventually led to applications in a variety of settings where understanding of human perception, attention, search, tracking and decision making are of great importance. Real time knowledge of the eye, pupil diameter, eye movement and eye scanning patterns are invaluable indicators of thought and mental processing involved during visual information extraction. Studies conducted by us on young adults revealed that data collected from eye-tracking devices

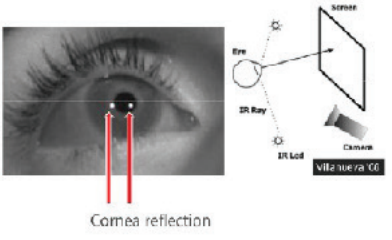
indicates the person's interest level and focus of attention [25, 26]. From eye position tracking and indirect measures, such as fixation numbers and duration, gaze position, and blink rate, it is possible to draw information about the user's level of attention, stress, relaxation, problem solving, successfulness in learning, tiredness, and more. Even emotions can be tracked, and based on the data; the eye-tracking system can provide more personalized learning [27].

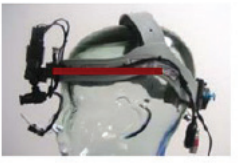


Electroencephalogram (EEG) technique



Functional Magnetic Resonance Imaging (fMRI) technique





Eye-tracker technique

Fig.1: *Different types of physiological monitoring techniques used in biofeedback.*

While physiological measures could be more objective indices of the level of attention, focus and presence experienced by a subject, there is no consensus on which measure is the best to use. Furthermore, many of these measures have methodological limitations (e.g., requiring the subject to be still, requiring a long preparation time, and being costly) and therefore cannot be applied for all situations and in all virtual environments.

Studies on physiological measures of attention, focus and presence in virtual environments

Numerous studies aimed to find physiological measures that correlate with attention, focus and presence. EEG was commonly analyzed using both spectral signal decomposition and ERPs. The ERP paradigms commonly assessed attention indirectly, specifically by studying the attentional processes allocated toward the simulated vs. the "external" real environment. In order to do so, the researchers used a traditional auditory dual-task oddball paradigm. In this paradigm, a series of frequent, repeated auditory tones are followed by less frequent tones of a different pitch, a phenomenon that generates a cognitive mismatch in the listener. This experimental design is often used in the study of subjective awareness, in combination with EEG [28]. Clemente *et al.* [29] used EEG to measure presence for navigation in virtual environments, using consumer-grade EEG equipment (Emotive EPOC EEG).

Among the non-brain physiological indices, attention was found to positively correlate with SC activity [30]. One of the report suggested that HR is a better correlate for presence [31]. Another study [32] found no association between attention and SC (using the sum of skin responses for each of the three conditions employed in their experiment). HR was found to be one of the most reliable indices of presence among the physiological indices, and it can be compared directly with other physiological measures. Meehan *et al.* in 2002, investigated ST as a possible index of presence[31]; even though the authors expected skin temperature to be an index for enhanced attention in their more highly arousing experimental condition, there was no connection between the phenomena. Unfortunately, the use of ST as a possible index of presence was not found in any articles. More studies are needed to gain a better understanding of ST as an index for attention. One study also combined EMG with SC to assess the sense of presence [33]. The results of the EMG confirmed those from SC. However, in the context of the study of Poels *et al.* in 2012 [33], EMG was used as a measure of arousal and not of presence. Unfortunately, such a finding was not replicated nor attempted by other studies, and therefore it is challenging to give an interpretation to it. The prevalence of the psycho physiological indices reported in the present review may be characteristic of the sample of studies retrieved and may be difficult to generalize when also considering studies that have been excluded from the current review.

Discussion

A wide range of physiological measures was used, attempting to identify physiological correlates of the sense of presence and attention. The most prevalent measure in the retrieved literature was EEG. The attempts to use SC and HR or to exploit other cognitive phenomena during EEG recordings to assess the sense of presence have

revealed mixed results. Furthermore, findings from the physiological responses were obtained using a variety of methodologies and diverse phenomena within the same methodology (e.g., brain wave oscillation analysis vs. event-related potentials; peak amplitude analysis vs. overall skin conductance). There are several benefits with psycho physiological measures, in case reliable ways to measure attention with only physiological measures are identified. These benefits include the possibility of continuous data collection and, therefore, the ability to study the association of physiological activity with the effect of a contemporary stimulus presentation in experimental paradigms. Thus, this modality will provide a more accurate analysis of the physiological state of a person compared to bare behavior. Consumer-oriented, cost-effective, small, and easy to handle EEG systems are now available and have already been used for the study of the sense of presence [12]. EEG is widely implemented in the development of brain-computer interface (BCI), and therefore it is expected that in the next decades the use of this methodology will grow exponentially in the tech sector and possibly in the study of brain activity during immersive experiences. The fMRI studies agree on the involvement of the dorsolateral prefrontal cortex in the experience of the sense of presence and on the role of the insula. However, Clemente *et al.* [34] reported the inactivation of the dorsolateral prefrontal cortex in a different, inferior location. Related brain areas were also shown to activate in EEG studies [12]. However, the source localization attempt carried out in the EEG study of Clemente *et al.* [12] was conducted with a sub-optimal configuration. Further studies are needed for a more precise spatial localization of the phenomenon and to confirm it. The activation of the insula is related to cognition and behavior, as for emotion, regulation of the body's homeostasis, perception, motor control of hands and eyes, self-awareness, cognitive functions, and interpersonal experience [12]. Self-awareness, sense of agency, and sense of body ownership are essential in this context, as directly linked with the sense of presence. Attentional and behavioral components are crucial for the development of the sense of presence, such as increasing the ability to understand the dynamics, predict, and interact with the virtual environment (VE). Clemente *et al.*[12] acknowledged that their interpretation of the data was speculative and that the brain areas activated during their VE navigation task are possibly not directly related to the sense of presence per se. The reviewed literature showed that little effort had been made to replicate the physiological indices proposed for the feeling of presence. SC and HR studies generally showed more consistent findings, with a higher level of immersion correlating with greater SC or increased HR. While fMRI and EEG are more likely, in the future, to pinpoint specific brain-related activity patterns or areas directly involved (or highly correlated) with attention, SC and HR are more likely to identify secondary effects of presence, as well as experiences modulated by or together with presence, such as, for example, arousal, emotion, and stress [15]. An approach to indirectly measure attention

via, for instance, non-invasive and reliable physiological measurements, would be ideal for understanding the ongoing presence level of the users. Furthermore, several problems are present in the use of physiological measures. Recording equipment is very sensitive to motion (EEG, SC, and HR), and, in fMRI, motion by the subject is not allowed at all due to technical constraints. Therefore, not all experimental scenarios will be suitable for those methods. Moreover, VE content can affect the recorded data and thus constitute an uncontrollable variable when comparing among different experimental settings. It may be challenging to isolate the phenomena of attention, as many other cognitive or perceptive factors (e.g., emotional charge of the environment, arousal of the subject, and image quality) may profoundly influence the physiological data. Additionally, understanding and measuring attention has become necessary due to the various applications of virtual reality in different fields. Furthermore, several studies demonstrated that attention could be a crucial factor to consider when using VE outside the entertainment context, for example, as a training tool in work environments or to increase the performance of users.

In online learning, there is a need to create more effective interaction between e-learning content and learners. In particular, increasing the learners' motivation by stimulating their interests is very important. However, for any e-learning system to be effective, the knowledge transfer must occur in a usable, accessible, and functional manner. Evaluation of online content for learning by biofeedback monitoring using physiological measures like those discussed in this article should be encouraged by educators for a better outcome of the efforts undertaken in this pandemic situation.

Future Perspectives

Considering that in the coming years, online technology users will probably increase in number, there is a need for research on standard practices and standardization in the area to understand the effect of those technologies on the end-user, as well as to help in the development of better ones in the future. At the current state of research, no physiological measure has collected enough evidence to be considered "good enough" to be reliably used alone. As such, more studies should be performed based on current technology and devices. Furthermore, some studies should aim to replicate old findings using new devices. This line of research can help with the development of a more comprehensive theoretical framework for measuring attention and related constructs during online learning. Future research should also focus on the role of presence in shaping human performance. More empirical research is needed to understand better how attention (and related factors, e.g., emotional involvement) may represent a positive or a negative factor for human performance.

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Effect of salicylic acid and microbial antagonist Cryptococcus Laurentii on crown rot disease of banana caused by Fusarium Semitectum, leading to increased shelf life In : *Biological Sciences: Impacts on Modern Civilization, Current and Future Challenges* by *Anupam Guha* © New Delhi Publishers, New Delhi: 2021, 285-292. ISBN: 978-81-947417-9-4, DOI: 10.30954/ndp.bio.2020.27

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CHAPTER

Effect of salicylic acid and microbial antagonist *Cryptococcus Laurentii* on crown rot disease of banana caused by *Fusarium Semitectum*, leading to increased shelf life

Anirban Bhattacharya[1*], Probal Kumar Chowdhury[2] and Gourab Roy[3]

[1] Dept. of Botany, Iswar Chandra Vidyasagar College, Belonia, South Tripura, India
[2] Department of Botany, Govt. Degree College, Dharmanagar, North Tripura, India
[3] Dept. of Zoology, M.B.B. College, Agartala, India
*Email: b_anirban17@rediffmail.com

Abstract: Crown rot is a major post-harvest disease of banana caused by *Fusarium semitectum*. Defence elicitor salicylic acid (SA) was able to control the colony growth and reduce the spore production of *F. semitectum* under in vitro condition. Treatment with SA increased the shelf life of banana by delaying the onset of disease. It also reduced the percentage of infected banana fruits as well as their complete decaying. Pre-inoculation of banana with microbial antagonist *Cryptococcus laurentii* provided protection against the Crown rot disease. *C. laurentii* successfully delayed the expression of disease symptoms on banana and also reduced the percentage of infected fruits.

Keywords: Salicylic acid; microbial antagonist; *Fusarium semitectum*

Fruits supply vital growth factors such as sugars, minerals and vitamins to human nutrition and thus form important components of human daily diet. Relatively short shelf-life period is one of the limiting factors that reduce the economic value of fruits. This is caused by pathogen attack during harvesting and post-harvest handling. About 20-25% of the harvested fruits are decayed by pathogens during post-harvest handling even in developed countries [1, 2]. In Tripura banana is one of the most important fruits locally produced by the farmers. Post harvest diseases greatly reduce the shelf life of banana leading to severe economic loss. Correct characterization of such diseases and proper identification of the pathogens are central to the selection of an appropriate disease control strategy.

Presently the scientific community is in search for safer and more eco-friendly alternatives than chemical control for plant diseases. The use of antagonistic microorganisms is one such approach which is gaining popularity all over the world. Different microbial antagonists like *Cryptococcus laurentii*, *Bacillus subtilis* and *Trichoderma harzianum* are in use globally for controlling postharvest diseases [3]. Microbial antagonists are applied either before or after harvest, but post-harvest applications are more effective than pre-harvest applications [3]. Use of biocontrol agents such as salicylic acid (SA), methyl jasmonate and chitosan has also given good results in controlling post-harvest diseases [4-6]. Plant defence system against pathogen infection can be constitutive or induced. Induced plant defence against pathogen can be stimulated by exogenous defence elicitors [7]. Salicylic acid, methyl jasmonate, and chitosan (CHN) are important defence elicitors used for manipulating defence pathways in plants [8].

Aim of the present investigation is to isolate and identify the causal organism of Crown rot of banana from South Tripura district and increase the shelf life of banana by controlling the severity of the disease by the application of defence elicitor salicylic acid and microbial antagonist *Cryptococcus laurentii*.

Materials and Methods

Isolation and identification of the pathogen

Infected banana fruits showing symptoms of Crown rot (Fig. 1a) were collected from local markets of South Tripura district. For isolation of the pathogen, small tissue segments from the diseased fruits were scooped off and transferred to Petri plates containing solidified Rosebengal Chloramphenicol Agar (RCA) medium (Himedia) to allow selective growth of fungus only. Operation was carried in front of Laminar Air Flow. Plates were sealed with parafilm and incubated under room temperature in diffused light. After 3 days, whitish fungal colonies were observed on RCA around the inoculated pieces. Mycelial tips from the radiating fungal colonies were scooped off and inoculated on sterile Potato Dextrose Agar (PDA) medium (Himedia) in Petri plates, which were sealed and allowed to incubate. Tip culture technique was repeated three times, allowing each time 3 days for incubation, to get the pure culture of the pathogen. Pure culture was maintained by repeated sub-culturing in PDA and stored at 4°C (Fig.1b).

Morphological study of the fungal pathogen was done using fully grown colonies on PDA medium. For microscopic observations, a very small piece of the sporulating was mounted on slide in a drop of glycerol with a tinch of lactophenol cotton blue and observed under microscope. Spore morphology including shape, number of cells

etc. was recorded. Spores were measured using stage and ocular micrometer and measurement was recorded. Identification of the pathogen was done on the basis of colony characters on PDA including appearance, growth nature, coloration of the mycelia and spore morphology [9]. Identification was confirmed by ARI, Pune. Koch's postulates were studied to confirm the pathogenicity of the isolated fungus.

Fig. 1a: *Affected banana fruits showing symptoms of Crown rot, 1b. Isolated Fusarium semitectum in pure culture on PDA medium*

Effect of defence elicitor salicylic acid (SA) on in vitro growth and sporulation of Fusarium semitectum

Effect on colony growth

Effects of SA on colony growth of *F. semitectum* were studied on PDA plates [10] amended with three different concentrations of the SA (0.05%, 0.10% and 0.20%). Unamended PDA was used as control (C). Three plates per elicitor concentration were inoculated with small (about 2mm in diameter) mycelial clumps from periphery of 14 days old colony from stock PDA plates. Control plates were inoculated in similar way. Plates were incubated at room temperature under diffused light. Colony diameter was measured (in replicates of ten / treatment) after 96 hours and percentage of growth inhibition (PGI) in all the treatments was calculated in relation to the average colony diameter in PDA control (C) according to the formula PGI= [(dc-dt) / dc] x 100 [11], where dc = mean colony diameter in control plate (here C1) and dt = colony diameter in test plates.

Effect on spore count

To determine the effects of SA on spore count of *F. semitectum,* fungal colony was raised on PDA plates amended with three different concentrations of the elicitor (0.05%, 0.10%, and 0.20%). Unamended PDA plate was used as control. Ten plates per SA concentration as well as control were inoculated at the centre with a single

mycelial clump and plates were incubated at room temperature under diffused light. Spores were harvested from 14 days old colonies [12]. 5ml distilled water was added to the petri dish with sporulating colonies and the surface was rubbed with sterile glass rod. Suspensions from ten plates were accumulated together, filtered to remove mycelia and diluted with sterile distilled water to 100ml. Conidial number /ml spore suspension was determined (ten replicates/treatment) by haemocytometer.

Effect of salicylic acid on occurrence of Crown rot disease of banana

Fresh and healthy and properly ripen banana fruits were collected from local markets. Fruits were surface sterilised by 70% alcohol. Next they were soaked in SA solution (0.20%) for one hour. Control fruits were soaked in sterile distilled water. Next both treated and control fruits were inoculated with small mycelial clumps of *F. semitectum* at the stalk portion and incubated at room temperature in sterile moist plastic zipper bags. Fruits were regularly observed for the appearance of crown rot symptoms. Three replicates of ten fruits each were used.

Effect of pre-inoculation with microbial antagonist [Cryptococcus laurentii (MTCC No: 3954)] on occurrence of Crown rot disease of banana

Fresh and healthy and properly ripen banana fruits were collected from local markets. Fruits were surface sterilised by 70% alcohol. *Cryptococcus laurentii* culture was procured from Microbial Type Culture Collection and Gene Bank, Chandigarh 160036, INDIA. Banana fruits were treated with microbial antagonist by dipping them in liquid culture containing *C. laurentii* cells (3×10^5 cfu ml^{-1}). Cell free liquid culture treated fruits were used as control. Two days later both treated and control fruits were inoculated with small mycelial clumps of *F. semitectum* at the stalk portion and incubated at room temperature in sterile moist plastic zipper bags. Fruits were regularly observed for the appearance of crown rot symptoms. Three replicates of ten fruits each were used.

Results

1. **Identification of the pathogen**: In the present study *Fusarium semitectum* was detected as the pathogen responsible for Crown rot of banana.
2. **Effect of SA on colony growth of F. semitectum**: SA inhibited the colony growth of *F. semitectum* after 96 hrs. of incubation (Table-1). With the increasing concentrations of SA, percent growth inhibition (PGI) of *F. semitectum* colony increased as compared to the control (C).
3. **Effect of SA on spore count of F. semitectum**: SA treatments reduced the spore formation of *F. semitectum,* as compared to the control (C). With increasing

concentrations of SA, number of spores / ml of culture filtrate gradually decreased than the control (Table-1).

Tabl. 1: *Effect of salicylic acid (SA) on colony growth (after 96 hours of incubation) and spore count of Fusarium semitectum:*

Growth medium	Mean colony diameter (mm)	Percent growth inhibition (PGI) [Mean ± SD]	Number of spores (conidia) / ml spore suspension (Mean)
PDA control (C)	22.00	-	156 x 10^4
SA 0.05% in PDA	18.42	12.00 ± 2.02	48 x 10^4
SA 0.10% in PDA	11.23	47.00 ± 3.41	23 x 10^4
SA 0.20% in PDA	6.48	73.00 ± 2.63	12 x 10^4

4. **Effect of salicylic acid on occurrence of Crown rot disease of banana**: Treatment with SA was able to increase the shelf life of banana against the Crown rot disease caused by *Fusarium semitectum.* SA treatment delayed the onset of disease as well as reduced the percentage of infected banana fruits as compared to the control. Also complete decaying of the fruits was reduced by SA treatment (Table-2).

Table. 2: *Effects of salicylic acid on occurrence of Crown rot disease of banana:*

Type of treatment	First appearance of symptom		Complete decaying of fruits	
	Days after inoculation	% of fruits affected (Percent disease index)	Days after inoculation	% of fruits affected
Control	03 - 04	80 - 90	10 - 12	100
Treated	08 - 10	30 - 40	10 - 12	35 - 45

5. **Effect of pre-inoculation with microbial antagonist Cryptococcus laurentii on occurrence of Crown rot disease of banana**: Pre-inoculation with the microbial antagonist *Cryptococcus laurentii* was able to provide protection against the Crown rot disease of banana (Table-3). As compared to the control, microbial pre-inoculation with *Cryptococcus laurentii* was able to delay the expression of disease symptoms on banana fruits and reduced the percentage of infected fruits as well as their complete decay.

Table. 3: *Effects of pre-inoculation with microbial antagonist Cryptococcus laurentii on occurrence of Crown rot disease of banana:*

Type of treatment	First appearance of symptom		Complete decaying of fruits	
	Days after inoculation	% of fruits affected (Percent disease index)	Days after inoculation	% of fruits affected
Control	04 - 05	85 - 90	10 - 12	100
Treated	09 - 10	30 - 40	10 - 12	35 - 40

Discussion

Crown rot is one of the main postharvest diseases of banana in banana-producing countries [13, 14]. In the present study Crown rot was found to be the most important post-harvest disease of banana from the region under study (South Tripura district) and *Fusarium semitectum* was identified as the pathogen responsible the disease. Several other workers also find the involvement of *Fusarium spp.* in banana crown rot [15]. *Fusarium semitectum* was isolated at high frequency from collected banana cultivar showing crown rot symptoms from commercial markets in Egypt [16].

Salicylic acid (SA) showed direct antifungal activities against *F. semitectum*. SA controlled the colony growth and spore production of the pathogen in a concentration dependent manner. Direct antifungal activity of SA has been recorded by several workers. There is evidence of antifungal activity of SA against *Penicillium expansum* where SA inhibited the germination of *P. expansum* and the blue mould incidence in apples [17]. SA also inhibited mycelial growth of *Eutypa lata* [18]. Treatment with SA was able to increase the shelf life of banana against the Crown rot disease caused by *F. semitectum.* SA increases plant's response to stress conditions (biotic and abiotic) by increasing the resistance of the plant [19]. SA is a defense-related plant hormone and exogenous SA application induces local and systemic acquired resistance in different plant species against various types of pathogens, including *Fusarium sp*, *Alternaria sp*, *Magnaporthe sp*, *Colletotrichum sp*, *Xanthomonas* spp., different kinds of viruses and etc [20]. Increased protection of banana fruits against Crown rot pathogen *F. semitectum* could be due to a combination of direct antifungal activity of SA and its resistance inducing ability.

Different mode of actions of biocontrol-active microorganisms in controlling fungal plant diseases include hyperparasitism, predation, antibiosis, cross-protection, competition for site and nutrient and induced resistance [21]. Similar mechanisms were also suggested by other workers [22]. In the present experiment pre-inoculation with the microbial antagonist *Cryptococcus laurentii* was able to provide protection against the Crown rot disease of banana. This resulted probably from any of the above mentioned mechanisms or a combination of several such mechanisms.

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CHAPTER

Medicinal and aromatic plants of Bihar and their potential application: A review

Shyama Roy and Meenu Gupta*[1]

Department of Botany, J.D.Women's College, Patna, India
*Email: meenugupta.999@gmail.com

Abstract: Revival of interest in Aayurveda has caused increased demand and consumption of medicinal plants and crude drugs. Medicinal and aromatic plants are very important for mankind as they serve as scaffold for the production of medicines, cosmetics, flavors and perfumes. Bihar is rich in small, dense forests where medicinal plants have natural protection. Approximately 5000 plant species have been studied worldwide as possible sources of new drugs for human use. Present paper is based on medicinal and aromatic plants of Bihar and their medicinal and ethno botanical significance.

Keywords: Medicinal and Aromatic Plants; Aayurveda; Ethno botanical significance.

Bihar is 13th largest state of India which covers around 94,163 sq km area and is situated between 24°20'10"-27°31' 15"N latitude and 83°19'50" - 88°17'40" E longitude. It has been divided into 9 divisions and 37 districts. Bihar has been divided in to three topographic regions ie northern lower Himalayan mountain range (foothills) tarai region of marshy, swampy habitat, the southern undulating table land of Kaimur and Chotanagpur plateau and Lower Gangetic plains covers the entire plain area between south of tarai and north of plateau regions [1]. The Climate of Bihar ranges from subtropical to tropical with average rainfall 100 cm (west central part) to ≥ 150 cm (North part) and relative humidity varies from 38% (April-May) to 94% (August-September). Flora of Bihar consists of about 2963 species of 1151 genera of 186 families. Out of which 857 species in 276 genera and 38 families are of Monocots and 2106 species, 875 genera and 148 families are of Dicots [2]. Generally the vegetation of Bihar is tropophilous with patches of true forest considered as protected areas. Forests of Bihar has been grouped in to, tropical dry deciduous forest, tropical moist deciduous forest and grassland [3, 7].

Methodology

All the description and details of the medicinal and aromatic plants given in the present paper and their application is truly based upon literature and herbarium study. Medicinal uses of flora is based upon traditional systems [4 - 7] and bioactivity on the information given in the literature [8 - 27].

Results and Discussion

Ethnobotany is a branch of science which connects humans with nature. Search of new sources of food, drugs and other commercial products has compelled man to go back to the nature. National inventories of medicinal plants are essential for rational use and exploitation. Inventories should cover the climatic and geographical distribution of medicinal and aromatic plants, their source (wild or cultivated or commercial plantation) and the status of their relative abundance or scarcity. For each plant, the utilization (folk medicine, traditional, pharmaceutical), commerce (local use, trade, export) and the description of its constituents, pharmacological properties and therapeutic indications should be known. These all information (utilization, commerce) will be helpful in taking conservation measure and their potent use as drugs. A large number of medicinal plants are used in traditional forms of medicine in Bihar. Biological screening, pharmacological and clinical studies are useful to assess their safety and therapeutic efficacy. Therefore, in the present paper, bioactivities of traditionally useful plants of the state are based on detailed description, pharmacological properties clinical studies and therapeutic efficacy [7]. All plants are enumerated alphabetically with latest botanical name followed by family, habit and bioactivities given in Table 1.

Table 1: *Medicinal plants of Bihar and their bioactivity is given in the table, based on detailed description, pharmacological properties clinical studies and therapeutic efficacy.*

Botanical Name of Plant	Family	Habit	Potential Application
Abelmoschus esculentus	Malvaceae	Under shrub	Anticancer
Abelmoschus esculentus	Malvaceae	Shrub	Antitumor, fungitoxic
Abrus precatorius	Fabaceae	Twinning shrub	Anti fertility , Anti estrogenic, cytotoxic and CVS active,
Abutilon indicum	Malvaceae	Shrub	Analgesic, antifungal, antipyretic, CNS active, febrifuge, hypoglycemic, hypothermic
Acacia auriculaeformis	Mimosaceae	Medium sized tree	Anti filarial, CNS & CVS active
Acacia catechu	Mimosaceae	Medium sized tree	Anti fertility, anti-inflammatory, antiviral, hepatoprotective, hypoglycemic, hypotensive, spasmolytic

Acacia nilotica	Mimosaceae	Small tree	Analgesic, Anti fertility, antifungal, antiviral, anti-inflammatory, antiprotozoal, hypoglycemic ,hypotensive
Acalypha indica	Euphorbiaceae	Annual herb	Antibacterial
Achyranthes aspera	Amaranthaceae	Under shrub	Abortifacient, antibiotic, antifungal, Anti inflammatory, hypoglycemic, uterine stimulant
Acorus calamus	Araceae	Herb	Analgesic, anticonvulsant, antipyretic, insecticidal, piscicidal, spasmolytic
Adansonia digitata	Bombacaceae	Tree	Antioxidant
Aegle marmelos	Rutaceae	Medium sized tree	Anti helminthic, antifungal, antibacterial, antitumor, antiviral, CVS active, cytotoxic, hypoglycemic, hypotensive, spasmolytic
Aerva lanata	Amaranthaceae)	Herb	Ant diabetic, antimicrobial, cytotoxic, inotropic
Ageratum conyzoides	Asteraceae	Small herb	Antibacterial, antifungal, antiulcer, CVS depressant, haemostatic
Ailanthus excelsa	Simaraubaceae	Large tree	Anticancer, antitumor, cytotoxic, hypotensive, spasmolytic
Alangium salviifolium	Alangiaceae	Small tree	Anticancer, antiprotozoal, hypoglycemic, hypotensive, spasmolytic
Allamanda cathartica	Apocynaceae	Shrub	Antileukaemia, hypotensive
Allium sativum	Liliaceae	herb	Antibacterial, antifungal, antiinflammatory antitumor, hypo-cholesterolemic, hypoglycemic, hypotensive
Alpinia galangal Wild	Zingiberaceae	Under shrub	Antioxidant, antiulcer, CNS & CVS active,diuretic, febrifuge, antipyretic, nematicidal
Alstonia scholaris	Apocynaceae	Evergreen tree	Anticancer, antifungal, hypotensive
Alternanthera paronychioides	Amaranthaceae	Prostrate herb	CNS depressant
Alternanthera sessilis	Amaranthaceae	Herb	Antibacterial, anticancer, antiulcer, Hypothermic ,antipyretic
Alysicarpus vaginalis	Fabaceae	Herb	Anticancer
Amaranthus viridis	Amaranthaceae	Small herb	Juvenomimetic
Abroma augusta	Sterculiaceae	Shrub or small tree	Abortifacient, anti-implantational, galactoprotective
Ammannia baccifera	Lythraceae	Herb	Analgesic, CNS active, diuretic
Anagallis arvensis	Primulaceae	Small herb	Anti fertility, antiviral, diuretic, hypotensive, spasmolytic
Andrographis paniculata	Acanthaceae	Erect herb	Antibacterial, anti diabetic, antifungal, antileukaemia, antimalarial, antioxidant, antityphoid, hepatoprotective. hypotensive
Anethum graveolens	Apiaceae	Aromatic herb	Fungicidal

Anisomeles indic	Lamiaceae	Aromatic Under shrub	Anti arthritic, antibacterial, anti-inflammatory, antipyretic
Annona squamosa	Annonaceae	Small tree	Anticancer, Anti fertility, antihistamine, antiimplantational, antispasmodic, antitumor, bronchial asthma, diuretic, cytotoxic, hypotensive
Anthocephalus chinensis	Rubiaceae	Medium sized tree	Antihelmintic, antimalarial, hypoglycemic
Antirrhinum majus	Scrophulariaceae	Annual herb	Anticancer, antiulcer
Aphanamixis polystachya	Meliaceae	Evergreen tree	Anticancer, antifeedant, CVS active, immunosuppressive
Ardisia solanacea	Myrsinaceae	Shrub	CVS active
Argemone mexicana	Papaveraceae	Annual under shrub	Acetylcholine, antihistamine, antiviral, CVS active
Argyreianervosa	Convolvulacea	Twiner	CVS active, spasmolytic
Aristolochia indica	Aristolochiaceae	Twining Under shrub	Anticancer, Anti fertility, antiimplantational, diuretic
Artabotrys hexapetalus	Annonaceae	Evergreen shrub	Antifungal, CVS & CNS active, hypotensive, spasmolytic, uterine stimulant,
Arundo donax	Poaceae	Grass	Acetylcholine, antifeedant, antihistamine, curarimimetic, hypotensive, uterine stimulant
Asclepias curassavica	Asclepiadaceae	Under shrub	Anticancer, cardiotonic, hypotensive, spasmogenic
Asparagus racemosus Willd	Liliaceae	Twining shrub	Antiallergic, antibacterial, anticancer, antifungal, spasmolytic
Atalantia monophylla	Rutaceae	Small tree	Antibacterial, antifungal, insecticidal, antitubercular
Azadirachta indica	Meliaceae	Tree	Anti-inflammatory, antimalarial, antitumor, CVS active, cytotoxic, diuretic, hypoglycemic, hypotensive, spasmolytic
Bacopa monnieri	Scrophulariaceae	Prostrate herb	Anticancer, antiepileptic
Baliospermum montanum	Euphorbiaceae	Under shrub	Anticancer, CVS active, effect on respiration
Barleria cristata	Acanthaceae	Under shrub	Hypoglycemic, spasmolytic, oxytoxic, CNS depressant, hypothermic, antiarrhythmic
Barringtonia acutangula	Lecythidaceae	Small tree	Antiprotozoal, CNS active, hypoglycemic, hypothermic
Bauhinia variegata	Caesalpiniaceae	Medium sized tree	Antibacterial, hypothermic
Benincasa hispida	Cucurbitaceae	Climbing shrub	Anti helminthic, antiviral,CNS depressant
Berberis asiatica	Berberidaceae	Perennial shrub	Anticancer, CVS active, spasmolytic
Bischofia javanica	Euphorbiaceae	Evergreen tree	Antiulcer
Bixa orellana	Bixaceae	Shrub	Hypoglycemic
Blumea lacera	Asteraceae	herb	Anti arrhythmic, anti-inflammatory, antifungal, antimicrobial

Blumea oxyodonta	Asteraceae	herb	CNS depressant, diuretic
Boerhavia diffusa	Nyctaginaceae	Perennial herb	Analgesic, antiarrhythmic, anticonvulsant antiviral, hepato protective, spasmolytic
Bombax ceiba	Bombacaceae	Tall tree	Antiviral, cardiac stimulant, hypoglycemic oxytoxic, musculotropic
Boswellia serrata	Burseraceae	Tree	Analgesic, anticancer, anti-inflammatory, CNS active, hypoglycemic, sedative
Breea arvensis	Asteraceae	Annual under shrub	CVS active, spasmolytic
Bryonopsis laciniosa	Cucurbitaceae	Annual climber	Spasmolytic
Buchanania lanzan	Anacardiaceae	Tree	Anticancer, CVS active
Butea monosperma	Fabaceae	Tree	Anti helminthic, antiestrogenic, Anti fertility, antimicrobial, hepatoprotective
Caesalpinia bonduc	Caesalpiniaceae	Shrub	Antidiarrhoeal, Anti fertility, antiviral, hypoglycemic
Cajanus cajan	Fabaceae	Biennial shrub	Hypoglycemic
Calendula officinalis	Asteraceae	Annual herb	Antibacterial, anticancer, antifungal, antimicrobial, antiprotozoal, antiviral, cytotoxic, spasmolytic
Calophyllum inophyllum	Clusiaceae	Tree	CVS & CNS active, hypothermic, spasmolytic
Calotropis gigantea	Asclepiadaceae	Tall shrub	Antiarrhythmic, anticancer, antispasmodic, hypotensive, spasmolytic
Calotropis procera	Asclepiadaceae	Shrub	CVS active, Antibacterial, anticancer, antifungal, anti-inflammatory
Campsis grandiflora	Bignoniaceae	Twining shrub	Analgesic, anti-inflammatory, bronchial dilatational effects
Canna indica	Cannaceae	Perennial Herb	Hypotensive, enterokinase activity
Cannabis sativa	Canriabaceae	Under shrub	Analgesic, CVS & CNS active, spasmolytic
Capsicum annuum	Solanaceae	Under shrubs	Hypocholesterolemic
Carica papaya	Caricaceae	Tree	Anti helminthic, anticoagulant, Anti fertility cardiostimulent, hypotensive
Carissa carandas	Apocynaceae	Tall shrub	Cytotoxic, hypotensive
Carthamus tinctorius	Asteraceae	Annual under shrub	Analgesic, anti-inflammatory, sedative, spasmolytic
Cassia auriculata	Caesalpiniaceae	Shrub	Antiviral, spasmolytic
Cassia occidentalis	Caesalpiniaceae	Herb or under Shrub	Antibacterial, anticancer, diuretic, hepatoprotective
Cassia sophera	Caesalpiniaceae	Shrub	Intestinal & bronchial muscle relaxant, spasmolytic
Casuarina equisetifolia	Casuarinaceae	Tall tree	Hypoglycemic
Catharanthus roseus	Apocynaceae	Erect herb Or under shrub	Anticancer, antihypertensive, CNS depressant, diuretic, hypoglycemic
Cayratia trifolia	Vitaceae	Climbing shrub	CNS active, hypothermic
Ceiba pentandra	Bombacaceae	Tree	CNS active, diuretic

Celastrus paniculatus	Celastraceae	Twining shrub	Anti fertility, antimalarial, antispermatogenic, antiviral, hypotensive, spasmolytic
Centella asiatica	Apiaceae	Creeping herb	Antiprotozoal, sedative, spasmolytic
Cestrum diurnum	Solanaceae	Shrub	Antibacterial, antitumor, CNS depressant, hypothermic, spasmolytic
Chenopodium album	Chenopodiaceae	Annual herb	CNS active, hypothermic
Chrysanthemum coronarium	Asteraceae	Annual herb	Insecticidal
Chrysanthemum indicum	Asteraceae	Herb	Anti-inflammatory, antitumor
Cicer arietinum	Fabaceae	Herb	Antihyperlipidemic, antipyretic, antistress, Hypocholesterolemic
Cinnamomum camphora	Lauraceae	Evergreen tree	Antibacterial, CVS & CNS active,
Citrus reticulata	Rutaceae	Small tree	Antibacterial, anticancer, antioxidant
Cleome viscosa	Cleomaceae	Annual herb	Anticancer, antifungal, anti-inflammatory, Hepatoprotective
Clerodendrum inerme	Verbenaceae	Shrub	Analgesic, antifeedant, antimicrobial
Clerodendrum multiflorum	Verbenaceae	Shrub	Anti helminthic, hepatoprotective, hypoglycemic
Clitoria ternata	Fabaceae	Twiner	Hypothermic, insecticidal, sedative
Coccinia grandis	Cucurbitaceae	Climbing shrub	Antiprotozoal, hypoglycemic
Cocculus hirsutus	Menispermaceae	Twining shrub	Diuretic
Codiaeum variegatum	Euphorbiaceae	Evergreen shrub	Anticancer
Coix lacryma	Poaceae	Annual grass	Anticancer, spasmolytic, antimicrobial, Hypoglycemic
Coleus amboinicus	Lamiaceae	Perennial herb	Antifungal, antimicrobial
Coleus barbatus	Lamiaceae	Perennial herb	CNS active, diuretic, hypotensive, hypothermic, spasmolytic
Convolvulus prostratus	Convolvulaceae	Prostrate herb	Antifeedant, antifungal, hypotensive, sedative
Corchorus aestuans	Tiliaceae	Small herb	Anticancer, anti-inflammatory, antiviral, CVS active
Cordia dichotoma	Ehretiaceae	Medium sized tree	Antimicrobial, anti-inflammatory, diuretic
Coriandrum sativum	Apiaceae	Annual herb	Antiviral
Costus speciosus	Costaceae	Perennial	Anti fertility, anti-inflammatory, Antiviral, CNS depressant, hypotensive, spasmolytic
Crotalaria medicaginea	Fabaceae	Annual herb	CNS depressant, spasmolytic
Croton bonplandianus	Euphorbiaceae	Erect herb	Hypotensive, spasmolytic
Cuminum cyminum	Apiaceae	Annual herb	Abortifacient, Anti fertility, Immunostimulatory
Curculigo orchiofdes	Hypoxidaceae	Perennial herb	Anticancer, hypoglycemic, spasmolytic
Curcuma amada	Zingiberaceae	Perennial herb	Antifungal, anti inflammatory, antimicrobial, CNS active, hypothermic

Curcuma longa	Zingiberaceae	Perennial herb	Antiarthritic, antibacterial, anti-inflammatory, antiparasitic, antiprotozoal, antispasmodic, CNS active, spasmolytic
Cuscuta reflexa	Convolvulaceae	Parasitic, twining herb	Anti fertility, antifungal, antiviral, hypotensive, nematicidal
Cyamopsis tetragonoloba	Fabaceae	Erect herb	Hypoglycemic, hypolipidaemic
Cymbopogon martinii	Poaceae	Perennial Aromatic herb	Antibacterial, antifungal, larvicidal, nematicidal acivities
Cynodon dactylon	Poaceae	Perennial herb	Antifungal, antimicrobial, antiviral, Hypoglycemic
Cyperus rotundus	Cyperaceae	Small sedge	Diuretic, anti-inflammatory, antipyretic, antimalarial, liver-protective
Dalbergia lanceolaria	Fabaceae	Deciduous tree	Antiarthritic
Dalbergia sissoo	Fabaceae	Deciduous tree	Spasmolytic
Datura metel	Solanaceae	Erect shrub	anticholinergic, anti helminthic, CVS active, spasmogenic, spasmolytic, Anticancer
Datura stramonium	Solanaceae	Under shrub	Antifungal, antifeedant, insecticidal
Daucus carota	Apiaceae	Herb	Antiesterogenic & antiprogestational activity, hepatoprotective
Delonix regia	Caesalpiniaceae	Medium- sized tree	CVS active
Dendrophthoe falcata	Loranthaceae	Parasites on trees	Antiviral, hypotensive
Desmodium gangeticum	Fabaceae	Erect herb	Analgesic, Anti fertility, anti-inflammatory
Desmostachya bipinnata	Poaceae	Perennial grass	Diuretic
Dianthus barbatus	Caryophyllaceae	Small herb	Analgesic, anti-inflammatory
Digera muricata	Amaranthaceae	Annual herb	Diuretic
Dillenia indica	Dilleniaceae	Tree	Antimicrobial, CNS active and CNS depressant hypothermic
Dioscorea bulbifera	Dioscoreaceae	Rhizomatous shrub	Diuretic
Diospyros malabarica	Ebenaceae	Small tree	Antiprotozoal, anti-stress, anti-ulcer, antiviral, hypoglycemic
Dodonaea viscosa	Sapindaceae	Evergreen shrub	Antibacterial, hypoglycemic
Drypetes roxburghii	Euphorbiaceae	Evergreen tree	Fungicidal
Echinochloa crusgalli	Poaceae	Annual grass	Anticancer
Edipta prostrata	Asteraceae	Erect or prostrate herb	Anticancer, antihepatic, antiviral, CVS active, hepatoprotective, spasmolytic
Embelia basaal	Myrsinaceae	Deciduous shrub	Anti helminthic, antibacterial, ascaricidal, contraceptive, diuretic, oxytocic
Emblica phyllanthus	Euphorbiaceae	Small tree	Antibacterial, anti diabetic, antifungal, antispasmodic, antiviral, CNS depressant, CVS active, spasmolytic

Eriobotrya japonica	Rosaceae	Evergreen tree	Antifungal, anti-inflammatory, antirheumatic, hepatoprotective, hypoglycemic
Eryngium foetidum	Apiaceae	Annual herb	Antistrychnine ,CVS & CNS active, diuretic, hypothermic
Erythrina variegata	Fabaceae	Tree	Anticancer, anticonvulsant, CNS depressant, diuretic, spasmolytic
Eschscholzia califomica	Papaveraceae	Annual herb	Antifungal, anti-inflammatory, sedative, Spasmolytic
Euphorbia hirta	Euphorbiaceae	Annual herb	Antibacterial, anticancer, antidiarrhoeal, antifungal, antiprotozoal, CVS active, hypoglycemic, spasmolytic, sedative
Euphorbia pulcherrima	Euphorbiaceae	Shrub	Anticancer
Euphorbia thymifolia	Euphorbiaceae	Prostrate herb	Antifungal, antimicrobial, antispasmodic, Bronchodilator
Evolvuius nummularius	Convolvulaceae	Herb	Anti helminthic, antibacterial, anticonvulsant, antioxidant
Ficus benghalensis	Moraceae	Tree	Hypoglycemic
Ficus elastica	Moraceae	Tree	Diuretic
Ficus religiosa	Moraceae	Large Tree	Anti helminthic, antibacterial, antiprotozoal, antitumor, antiviral
Flacourtia indica	Flacourtiaceae	Small spinous tree	Anti strychnine, CVS & CNS active, diuretic, hypothermic, spasmolytic
Foeniculum vulgare	Apiaceae	Annual herb	Abortifacient, antibacterial, antifungal, antioxidant, oxytocic
Fumaria indica	Fumariaceae	Annual herb	CNS depressant
Gardenia gummifera	Rubiaceae	Small tree	Anti helminthic, antispasmodic
Gloriosa superba	Liliaceae	Perennial herb	CNS depressant, hypothermic, oxytocic, spasmolytic, uterine stimulant
Gmelina arborea	Verbenaceae	Deciduous tree	Antiviral, anti-inflammatory, hypoglycemic
Grevillea robusta	Proteaceae	Tall tree	CNS depressant, diuretic, spasmolytic
Gymnema sylvestre	Asclepiadaceae	Twining shrub	Anti diabetic, antiviral, insulinotropic
Hedychium coronarium	Zingiberaceae	Perennial herb	Anti helminthic, anti-inflammatory, cytotoxic
Helianthus tuberosus	Asteraceae	Herb	Antimicrobial
Helicteres isora	Sterculiaceae	Shrub	Cytotoxic, spasmolytic, uterine stimulant
Heliotropium indicum	Boraginaceae	Annual herb	Anticancer, antifungal, antineoplastic
Hemidesmus indicus	Periplocaceae	Twining shrub	Antibacterial, anti-inflammatory, antiviral, hypotensive
Hibiscus rosa-sinensis	Malvaceae	Evergreen shrub	Abortifacient, analgesic, antifungal, antipyretic, CNS depressant, hypotensive
Hibiscus sabdariffa	Malvaceae	Shrub	Antibacterial, antifungal
Holarrhena pubescens	Apocynaceae	Deciduous tree	Antifungal, antiprotozoal, CVS active, hypoglycemic
Holmskioldia sanguinea	Verbenaceae	Straggling shrub	Oestrogenic
Hybanthus enneaspermus	Violaceae	Herb	Antigonorrhoeic, diuretic

Hydrocotyle sibthorpioides	Apiaceae	Prostrate herb	Antitumor
Hyptis suaveolens	Lamiaceae	Under shrub	Anticancer, antimicrobial, hypoglycemic
Iberis amara	Brassicaceae	Small herb	Antifeedant, anti-inflammatory. cytotoxic
Ichnocarpus frutescens	Apocynaceae	Evergreen shrub	Antiviral
Impatiens balsamina	Oxalidaceae	Annual herb	Anticancer, antifungal
Indigofera linifolia	Fabaceae	Herb	Antimicrobial
Indigofera tinctoria	Fabaceae	Herb	CNS depressant, hypoglycemic
Ipomoea aquatica	Convolvulaceae	Aquatic herb	Spasmolytic
Ipomoea nil	Convolvulaceae	Annual twiner	Anticancer
Ixora coccinea	Rubiaceae	Shrub	CNS depressant, hypothermic
Jacarandamimosaefolia	Bignoniaceae	Tree	Antimicrobial, cytotoxic
Jasminum grandiflorum	Oleaceae	Twining shrub	Diuretic, CNS depressant
Jasminum sambac	Oleaceae	Shrub	CVS & CNS active
Jatropha curcas	Euphorbiaceae	Shrub	Antimicrobial ,CNS depressant, diuretic
Justicia gendarussa	Acanthaceae	Erect Under shrub	Hypotensive
Kaempferia galanga	Zingiberaceae	Perennial Rhizomatous herb	Cytotoxic, insecticidal
Kalanchoe pinnata	Crassulaceae	Succulent herb	Analgesic antibacterial, anti-inflammatory
Kigelia africana	Bignoniaceae	Medium sized tree	Antimalarial, diuretic
Lactuca sativa	Asteraceae	Annual herb	CNS active
Lagenaria siceraria	Cucurbitaceae	Climbing shrub	Anti helminthic, insect-repellent
Lagerstroemia reginae	Lythraceae	Deciduous tree	Antiviral, anti-inflammatory, CNS active, Cardio stimulant, hypoglycemic, spasmogenic
Lantana camara	Verbenaceae	Shrub	Antibacterial, antifungal, hepatoprotective, nematicidal
Launaea procumbens	Asteraceae	Small herb	Antioxidant, hypoglycemic, spasmolytic
Lawsonia inermis	Lythraceae	Erect shrub	Antibacterial, anti-inflammatory
Lepidium sativum	Brassicaceae	Annual herb	Antiviral, diuretic, hypotensive, spasmolytic
Leutaena latisiliqua	Mimosaceae	Small tree	CNS active, diuretic, spasmolytic
Leucas aspera	Lamiaceae	Aromatic herb	Antibacterial, antifungal, CNS active, diuretic, hypothermic
Limonia acidissima	Rutaceae	Medium sized tree	Antibacterial, antifungal, antimicrobial, spasmolytic
Litsea glutinosa	Lauraceae	Small tree	Anti helminthic, antibacterial, antifungal, antispasmodic
Macrotyloma uniflorum	Fabaceae	Twining or trailing herb	Diuretic
Madhuca iongifoiia	Sapotaceae	Medium sized tree	Antibacterial, hypotensive

Magnolia grandiflora	Magnoliaceae	Evergreen tree	Antibacterial, antifungal, antileukaemic
Mailotus philippensis	Euphorbiaceae	Evergreen tree	Antibacterial, anticancer, hypoglycemic, Spasmolytic
Malvastrum	Malvaceae	Sub erect herb	CVS active
Mangifera indica	Anacardiaceae	Deciduous tree	Antiviral
Manilkara zapota	Sapotaceae	Evergreen tree	Anti-allergic
Martynia annual	Martyniaceae	Annual under shrub	CVS active
Melia azedarach	Meliaceae	Medium sized tree	Antibacterial, anti helminthic, antiviral, CNS depressant, spasmolytic
Melothria maderaspatana	Cucurbitaceae	Climber herb	Antihepatotoxic
Mentha piperita	Lamiaceae	Perennial aromatic herb	Antibacterial, antifungal
Mesua ferrea	Clusiaceae	Evergreen tree	Anti asthmatic, antibacterial, antifungal, antimicrobial, diuretic, spasmolytic
Michelia champaca	Magnoliaceae	Small tree	CNS active, hypoglycemic
Millettia peguensis	Fabaceae	Medium sized tree	Biocidal, fungicidal
Millingtonia hortensis	Bignoniaceae	Tree	Diuretic
Mimosa pudica	Mimosaceae	Under shrub	Antiviral, diuretic, spasmolytic
Mimusops elengi	Sapptaceae	Medium sized tree	Antimicrobial, diuretic, spermicidal, Spasmolytic
Mirabilis jalapa	Nyctaginaceae	Herb	Abortifacient, antifungal, antiviral, spasmolytic
Momordica charantia	Cucurbitaceae	Climbing or trailing herb	Antifeedant, cytotoxic, hypoglycemic, Insecticidal
Moringa oleifera	Moringaceae	Small tree	Antibacterial, anticancer, antifungal, Anti fertility, hypoglycemic, hypotensive, Spasmolytic
Morus alba	Moraceae	Medium sized tree	Antimicrobial, anti-inflammatory, Hypoglycemic
Mucunapruriens	Fabaceae	Twining shrub	CNS active, hypoglycemic, spasmolytic
Murraya koenigii	Rutaceae	Aromatic shrub	Antiprotozoal, CVS active, hypoglycemic, spasmolytic
Murraya paniculata	Rutaceae	Evergreen shrub	Antibacterial, antifungal,cytotoxic
Myrtus communis	Myrtaceae	Erect shrub	Anti-inflammatory, antioxidant, hypoglycemic
Nerium indicum	Apocynaceae	Shrub	CNS active, spasmolytic
Nicotiana plumbaginifolia	Solanaceae	Annual herb	Anticancer, antiviral, hypotensive, spasmolytic
Nyctanthes arbor-tristis	Nyctanthaceae	Small tree	Antimalarial, CNS depressant, hypothermic
Ocimum basilicum	Lamiaceae	Aromatic herb	Antibacterial, antifungal
Ocimum tenuiflorum	Lamiaceae	Under shrub	Anti asthmatic, antibacterial, antifungal, Anti inflammatory, hypoglycemic, nematicidal, spasmolytic
Opercuiina turpethum	Convolvulaceae	Perennial shrub	Anti-inflammatory, laxative

Opuntia stricta	Cactaceae	Shrub	CNS active, diuretic
Oroxylum indicum	Bignoniaceae	Small tree	Diuretic, spasmolytic
Paederia scandens	Rubiaceae	Twining shrub	Anti helminthic, anticancer, CNS active, hypothermic, spasmolytic
Pandanus odoratissimus	Pandanaceae	Bushy shrub	Antispasmolytic, antirheumatic
Pentanema indica	Asteraceae	Annual herb	Anti-inflammatory, antipyretic
Papaver somniferum	Papaveraceae	Annual herb	Analgesic, anticancer, antiprotozoal, CVS active, hypoglycemic, spasmolytic
Parthenium hysterophorus	Asteraceae	Herb	Antibacterial, antifungal, insecticidal
Pedilanthus tithymaloides	Euphorbiaceae	Succulent shrub	Anti-inflammatory, CNS active, hypothermic
Phyllanthus amarus	Euphorbiaceae	Herb	Antibacterial, antihepatotoxic, antiulcer, ' antiviral, hypoglycemic, hepatoprotective
Physalis minima	Solanaceae	Annual herb	Abortifacient, anti-inflammatory, antimalarial, diuretic
Piper longum	Piperaceae	Creeping herb	Antibacterial, Anti fertility, anti-inflammatory, antimicrobial, antipyretic, CNS stimulant, hypotensive, hypoglycemic, spasmogenic
Pitheceliobium dulce	Mimosaceae	Medium sized tree	Haemolytic, spasmolytic, spermicidal
Plantago ovata	Plantaginaceae	Annual herb	Hypocholesterolemic, nematicidal
Pluchea lanceolata	Asteraceae	Erect annual herb	Anti-implantation, anti-inflammatory
Plumbago zeylanica	Plumbaginaceae	Under shrub	Abortifacient, antibacterial, anticoagulant, antifungal, antiimplantation, cytotoxic, hypothermic
Plumeria rubra	Apocynaceae	Deciduous tree	Anesthetic, antibacterial, antibiotic, antiviral
Polyalthia longifolia	Annonaceae	Evergreen tree	Antifeedant, cytotoxic, fungitoxic, nemacidal
Polygonum glabrum	Polygonaceae	Erect herb	Antibacterial, antiviral, CVS active, Hypothermic
Polygonum hydropiper	Polygonaceae	Erect herb	Antibiotic, antifeedant, Anti fertility, antiovulatory, antioxidant, cytotoxic
Portulaca oleracea	Portuiacaceae		Annual herb Antibacterial, Anti fertility
Pongamia pinnata	Fabaceae	Medium sized tree	Antibacterial, antifungal, antiplasmodic, Antipyretic
Prosopis cineraria	Mimosaceae	Medium sized tree	Anti-inflammatory
Psidium guajava	Myrtaceae	Small tree	Antimicrobial, anti-mutagenic, spasmolytic
Psoralea corylifolia	Fabaceae	Erect herb	Antibacterial, anti helminthic, antifungal, antioxidant, cytotoxic, nematicidal
Pterocarpus marsupium	Fabaceae	Medium sized tree	Antifungal, antimicrobial, CVS active, hypoglycemic, hypotensive
Pterospermum acerifolium	Sterculiaceae	Evergreen tree	CNS active, hypothermic
Punica granatum	Punicaceae	Shrub or small tree	Antibacterial, Anti fertility, antifungal, CNS depressant, diuretic, hypoglycemic
Quisqualis indica	Combretaceae	Twining shrub	Anti helminthic

Ranunculus sceleratus	Ranunculaceae	Annual herb	Antibacterial, antifungal, antiviral
Raphanus sativus	Brassicaceae	Biennial herb	Hypotensive
Rauvolfia serpentina	Apocynaceae	Under shrub	Hypotensive, CVS & CNS active
Ricinus communis	Euphorbiaceae	Shrub or small tree	Anticancer, antiprotozoal, antiviral, diuretic, hepatoprotective, hypoglycemic
Rumex vesicarius	Polygonaceae	Annual herb	Diuretic
Ruta chalepensis	Rutaceae	Erect herb	Antibacterial, antipyretic, spasmolytic
Saccharutn spontaneum	Poaceae	Herb	Diuretic
Santalum album	Santalaceae	Semi parasite tree	Antibacterial, antifungal, anticancer, Anti inflammatory, antiviral, CVS active
Sapindus emarginatus	Sapindaceae	Medium sized tree	Antifungal, piscicidal
Saraca asoca	Caesalpiniaceae	Small tree	Anticarcinogenic, antifungal, antitumor, CNS active, hypothermic, spasmolytic
Scoparia dulcis	Scrophulariaceae	Herb	Anti-inflammatory, hepatoprotective, spasmolytic
Semecarpus anacardium	Anacardiaceae	Tree	Anti helminthic, anticancer, antimicrobial antirheumatic, CVS active, hypoglycemic
Sesamum indicum	Pedaliaceae	Herb / under shrub	Antioxidant
Sesbania sesban	Fabaceae	Shrub or small tree	Antibacterial, Anti fertility, antiviral, CVS active, cardiac depressant, hypoglycemic
Sida acuta	Malvaceae	Under shrub	Anti arrhythmic, antibacterial, antimicrobla hypotensive
Sida rhombifolia	Malvaceae	Erect Under shrub	Anti helminthic, antibacterial, antifungal, antimalarial, sedative, spasmolytic
Soianum nigrum	Solanaceae	Erect herb	Hepatoprotective,hypothermic, hypotnsive, spasmolytic
Soianum virginianum	Solanaceae	Under shrub	Antiarrhythmic, anticancer, antifungal, antiviral, CVS active, spasmolytic, spermicidal
Sorghum halepense	Poaceae	Herb	Diuretic
Spathodea campanulata	Bignoniaceae	Medium sized tree	Antimalarial, hypoglycemic
Spilanthes oleracea	Asteraceae	Herb	Anti-inflammatory, antimicrobial
Spirodela polyrhiza	Lemnaceae	Aq. floating herb	CNS depressant, diuretic
Spondias pinnata	Anacardiaceae	Medium sized tree	CVS & CNS active, diuretic, hypothermic
Stellaria media	Caryophyllaceae	Annual herb	Anti-inflammatory, antioxidant
Sterculia foetida	Sterculiaceae	Tree	Carcinogenic
Stereospermum chelonoides	Bignoniaceae	Medium sized tree	Anticancer, antiviral, hypoglycemic
Streblus asper	Moraceae	Evergreen small tree	Anticancer, antifilarial, antitumor, antiviral, insecticidal
Strychnos nux-vomica.	Stiychnaceae	Medium sized tree	Analgesic, anti-inflammatory, cardiotonic, spasmolytic

Swietenia macrophylla	Meliaceae	Evergreen tree	Antimalarial
Swietenia mahagoni	Meliaceae	Evergreen tree	CVS active, diuretic
Syzygium cumini	Myrtaceae	Medium sized tree	Analgesic, antibacterial, Anti fertility, hypoglycemic, hypotensive
Syzygium jambos	Myrtaceae	Medium sized tree	Anti-inflammatory, Anti fertility, diuretic
Trewia nudiflora	Euphorbiaceae	Deciduous tree	Antileukaemic, cytotoxic
Trianthema portulacastrum	Aizoaceae	Prostrate herb	Anti-inflammatory, CNS depressant, CVS active, hepatoprotective, spasmolytic
Tribulus terrestris	Zygophyllaceae	Prostrate herb	CVS active, cardiotonic, hepatoprotective, nematicidal, spasmolytic
Tridax procumbens	Asteraceae	Small herb	Diuretic, hepatoprotective, insect-repellent activity
Trigonella foenum-graecum	Fabaceae	Small herb	CNS depressant, cardiotonic, diuretic, hypoglycemic, hypotensive
Tropaeoium majus	Tropaeolaceae	Annual herb	Antibacterial, antifungal
Tylophora indica	Asclepiadaceae	Twining shrub	Antiallergic, antiasthmatic, anticancer, Anti inflammatory, antileukaemic
Typhonium trilobatum	Araceae	Small herb	Nemacidal
Uraria picta	Fabaceae	Erect herb or Under shrub	Antiarrhythmic, hypothermic
Urena lobata	Malvaceae	Under shrub	Analgesic,CNS depressant, hypotensive, hypothermic
Vallaris solanacea	Apocynaceae	Straggling shrub	CNS depressant, hypothermic
Verbascum chinense	Scrophulariaceae	Annual herb	Anticancer, antiviral, hypothermic
Vernonia cinerea	Asteraceae	Herb	Anticancer, antimalarial, antiviral, spasmolytic
Vetiveria zizanioides	Poaceae	Perennial herb	Antibacterial, antifungal, CNS active
Viola tricolor	Violaceae	Annual herb	Antocoagulent, anti-inflammatory
Vitex negundo	Verbenaceae	Large shrub or small tree	Analgesic, anti-inflammatory, antiimplantation, CNS depressant
Wedelia chinensis	Asteraceae	Perennial herb	Nematicidal
Withania somnifera	Solanaceae	Under shrub	Antibacterial, anti-inflammatory, antispasmodic, antitumor, antiviral, hypotensive, sedative
Woodfordia firuticosa	Lythraceae	Shrub	Abortifacient, antitumor
Wrightia arborea	Apocynaceae	Small tree	Antileukaemic
Xanthium indicum	Asteraceae	Under shrub	Anti-inflammatory, antimicrobial, antitumor
Zea mays	Poaceae	Herb	Antitumor, antiviral, cytotoxic
Zingiber officinale	Zingiberaceae	Rhizomatous herb	Antifungal, antioxidant, antiulcer
Zinnia elegans	Asteraceae	Annual herb	Haemolysis
Ziziphus mauritiana	Rhamnaceae	Deciduous tree	Anticholinergic activity, hypotensive
Ziziphus nummularia	Rhamnaceae	Thorny shrub	Antibacterial, antifungal, anti-inflammatory

These results show biological active medicinal plants resources of the state. These plant resources should bring under cultivation to maintain regular supply of raw materials to different pharmaceutical companies. It can be also concluded that several plants mentioned in table 3.1 are used to validate their application. Furthermore, the conservation of all these plants should not be neglected. As, many of the plants has become endangered in the wild because of human interference, conservation efforts are urgently needed to save these plants from going extinct [28].

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CHAPTER

Socio economical status of *Musa acuminate* colla in Tripura state

Saswati Chakraborti

Department of Botany, Ramthakur College, Agartala, Tripura, India
E-mail: chakraborti_saswati @yahoo.in

Abstract: Present paper deals with the economical aspect of *Musa acuminata* in Tripura along with its traditional value. The species is commonly known as Ram kala to Bengali community people and Thailik Bolong to Tripuri community people of the state. Inflorescence and innermost soft pseudo stem of the plant is gaining its importance as a wild vegetable in terms of its palatability to the local people. Market demand of the vegetable is also worked out through market survey and is found highly relevant with income generation of economically poor tribal and other communities of this region. Traditional food items and the variety of recipe preparation using the vegetable are recorded along with nutritional value assessment in terms of biochemical parameters.

Keywords: *Musa acuminata*; wild vegetable; market status; traditional recipes

Diversified food habit is very common and distinctive feature among the peoples residing in this land locked hillock state Tripura. The state is inhabited by heterogeneous population of Bengali, different tribal communities and other minor communities. This ethological diversity has not only reflected the sociological and cultural aspect but also revealed unique traditional food habit [1]. Different food habit with less known non-conventional plants has been recorded as distinctive feature through ages by tribals[2]. In Tripura 19 scheduled tribes are found, most of the tribal economies have been engaged in agriculture, jhum, piggery, fishery and hunting. Tribal peoples have traditional knowledge about the utility of many plants species as their food by making different delicious recipes, which are unknown to modern society. Wild vegetables are an important source of food, not only in the rural parts of Tripura but in the whole world. Wild edible plants are those that are harvested or collected from their wild natural habitats and used as food or for other purposes for human consumption [3]. They provide staple food for indigenous people, serves as supplementary food for non-indigenous people and are one of the primary sources of income for poor communities[4-6]. The

traditional use of wild edible plants used by different communities along with the economic importance of the species in the livelihood of such communities needs to be investigated properly. It has been observed that non-conventional forest resources which are used by hill peoples of Tripura have not yet exploited economically and botanically in spite of having their immense potentialities as useful vegetable with good nutritive value [7].

Musa acuminata Colla belonging to the family Musaceae, is a non-conventional wild vegetable used by different communities in Tripura. The hill ranges of Baramura, Atharamura and Jampui are the common places where this plant can be found growing wild [7]. The species is commonly known as wild banana and in Bengali - Ram kala, in Tripuri - Thailik Bolong, in Manipuri – Ching and in Reang - Thailik. Mainly the inflorescence of the plant is used as popular vegetable among the local people of the stateafter making different delicious dishes.

Although scattered information is available on the use of wild banana as vegetable no attempt has been taken so far to evaluate the consumption pattern of this vegetable and its production potential in Tripura. In view of the above, an attempt has been taken to record the market potentiality of the vegetable in West District of Tripura. Present work also highlights some traditional wild banana consumption pattern for recipe development along with nutritional value assessment in terms of biochemical estimates.

Materials and Methods

Knowledge based information on use and utility of *M. acuminata* as food vegetable are generated through visit and interaction with different communities residing in different parts of the state. The details of indigenous food preparation methods used in different recipes are recorded and described. Biochemical analysis of the fresh samples of inflorescence was carried out. Biochemical characters like protein, soluble sugar, total phenolics and ascorbic acid as vitamin C was estimated following standard biochemical methods respectively [8-11]. The market status and potentialities of the *M. acuminata* inflorescence as food vegetable was recorded through market survey. Survey was conducted through frequent visit and interaction with seller in different markets in West district of Tripura.

Results and Discussion

During the course of investigation, frequent visit was made in different localities and it was found that *M. acuminata* grows wild in hilly areas in different parts of the state without any organized cultivation. The inflorescence (Fig. 1) of the species is

mainly used as vegetable and known as Muikhon in Kokborok, in Bengali – Mocha, in Manipuri – Laphutharo and in Reang – Muikhan.

At the time of flowering, the true stem or growing point of the plant emerges from the center of the tightly rolled bunch of leaves. This odd-looking "flower cluster" is actually an elongated, plump, purple to green "bud", which at first displays large female flowers. As the "bud" elongates, it exposes semicircular layers of female flowers, then neutral flowers, and finally small, generally non-functional (with no viable pollen) male flowers. Each group of flowers is arranged radially on the stem in nodal clusters (Fig. 2). Each flower cluster (Fig. 3) is borne on a prominence on the stem bearing the fruit (peduncle) and covered by a bract. About 12–20 flowers are produced per cluster. Dissection of individual bisexual flower shows perianth, labellum, androecium with staminode and gynoecium (Fig. 4). Collectively, the flowering parts and fruit are referred to as the bunch. Individual clusters of fruits are known as hands, and individual fruits are known as fingers.

Table 1: *Some of the recipes using M. acuminata collected and documented from Tripura*

Name of the recipe	Popular among the community	Ingredients	Mode of preparation
Mui awandru	Tripuri	Inflorescence (muikhon)& tender core stem (bugili) of *Musa*, rice, dry fish, chopped onion, green chili and salt	Rice is soaked in water for few hours. Then this rice is grinded into fine paste. Washed muikhon and bugili are cut into small pieces and boiled for 15 – 20 minutes. After that chopped onion, washed dry fish, rice paste, green chili and salt are added to this. Cooked well after proper mixing. Serve with rice.
Gudak	Tripuri	Muikhon, dry fish, potato, bean, green chili and salt	After washing muikhon, peeled potato and beans are cut into small pieces. These vegetables are boiled along with green chili, after boiling dry fish and salt are added to this, 5 minutes before removing from flame. Excess water is removed in a container and kept aside. These boiled vegetables are mixed well using as pestle. After mixing the excess water which was kept aside is again added to this and mixed properly. Then it is cooked for some time. After cooking the dish is ready to serve.

Mwiborok	Tripuri	*Diplazium esculenta* twigs, *Enhydra fluctuans*twigs, *Eryngium foetidum* leaves, *Monochoriahastata, Mentha spicata, Musa acuminata* inflorescence,*Lecuas aspera*twigs, green chili,onion, garlic and salt.	In case of preparing Mwiborok*Diplazium esculenta, Enhydra fluctuans, Eryngium foetidum, Monochoriahastata, Mentha spicata, Musa acuminata* and *Lecuas aspera* are cut into small pieces, then washed thoroughly and boiled along with dry fish, onion, green chili, turmeric powder, garlic and salt. Cooked well and served hot
Laphu-tharoeromba	Manipuri	*Musa* inflorescence (Laputharo), peeled potato, chili, dry fish, *Houttuynia cordata* and *Eryngium foetidum*leaves, salt to taste	Inflorescence of *Musa* is cut into small pieces and boiled in water. Excess water is removed. Boiled potato pieces, chopped green chili, washed and roasted dry fish and salt are added to the boiled lapotharo. Mixed properly and fine pieces of *Houttuynia* and *Eryngium* leaves are added to this. After mixing cooked well and serve hot with rice
Pungre-eeromba	Manipuri	Tender innermost stem of *Musa* (pungree) dry fish, chopped leaves of *Houttuynia cordata* and *Eryngium foetidum,* green chili and salt	Preparation method is same as laphutharoeromba. Only exception is here no potato is needed.
Gudak	Tripuri & Mog	Banana inflorescence, *Cajanus cajan*,green chili, dry fish, onion and salt to taste	At first all the spathes were taken out from the inflorescence. Now from each small yellow blossom inner stamen and translucent white labellum at the base were removed. All the flowers along with *Cajanus cajan*podswere boiled in water with salt and green chili. After proper boiling, these vegetables are kept in a bowl, then dry fish (*Puntius ticto*) was roasted and all scales were removed, now other ingredients like salt, few pieces of onion, roasted dry fish were added with boiled banana flower and mixed properly. Now tasty goudak of banana flower is ready to serve.
Boglinong-gudak	Mog	Innermost portion of the banana pseudo-stem; green chili, salt.	Preparation is same as Gudak

Laifungmui	Tripuri	Banana stem, green chili, dry fish, potato, salt, turmeric powder, mustard oil, onion, garlic.	First banana stem was washed properly with water. Then banana stem cut into small pieces. Now mustard oil was heated in a pan. Paste of onion and garlic were added in oil. Now sauté banana stem with onion, garlic, and turmeric powder and salt. After sauté some amount of water and was added and cover the pan with a lid until it cooked properly. This is an easy healthy and delicious banana stem curry.

After peeling the outer layer of stem of the young plant the inner immature soft portion are also used as vegetable among different communities in the state and known as Laifung in Kokborok and Pungree in Manipuri. After harvesting the fruit, the innermost soft pseudo-stem (Fig. 5) is also used as vegetable and known as, Bugili in Kokbork, Chubui in Bengali. The fruit/green banana (Thailikkwthung- in Kokbork) are also sometimes eaten as vegetable by the tribals of this state. Different recipes (Fig. 6) recorded and documented are presented in Table -1. The recipes used by the tribals and other communities are unique and this know-how information so generated and documented could be of great significance in exploring such knowledge for the common people.

Table 2: *Biochemical estimations of fresh samples of inflorescence of Musa acuminata*

Sample	**Biochemical parameters *Mean ± SD (expressed in mg/g.fr.wt.)**			
	Protein	Phenolics	Soluble sugar	Ascorbic acid
Fresh	6.71 ± 0.16	0.56 ± 0.18	0.98 ± 0.13	0.22 ± 0.01

* Mean of 5 replications

Biochemical characters of fresh *Musa acuminata* inflorescence used as a vegetable by the common people of the state was analyzed (Table – 2).Higher protein value and the total soluble sugar content with lower phenolics and ascorbic acid was recorded in the present sample and clearly indicates the nutritional value of this wild vegetable.

Market potential of the *M. acuminata* inflorescence was studied (Fig. 7). A total of 5 markets were visited during the present investigation in West district of the state. Market survey in all the markets clearly indicated that the vegetable was available thorough out the weekand the price of each inflorescence varies from Rs. 15 to 20 depending upon the size of the inflorescence. Even the price varies according the season of availability. One retail seller can sell as many as 15 to 20 inflorescence per day. Total selling price was found to vary from Rs. 300 to Rs. 350 per day. Retail

seller collects such vegetable from villages in bulk amount in comparatively at a lower price and sell in the local markets. In some markets tribal people directly collects the vegetable from the natural habitat and sold it in the markets. Due to its high palatability the vegetable has very good demand in such markets and also gaining its acceptance among other communities like Bengali in the state. Such preliminary data so collected through present investigation needs further detailed analysis in terms of economic status of the vegetable.

The plant is also medicinally important as traditionally the green banana and flowers are used in combating diarrhea and dysentery, cooked flowers and unripe fruits are useful in diabetes, the ash of the leaves, stem and whole plant is considered as antihelmithentic, juice of stem and root are useful in blood disorders, the ripe fruits are considered good in gout, hypertension, constipation and cardiac diseases.

During the present study it was also observed that there was no organized cultivation of this vegetable in the state in spite of having socio-economic value. Therefore, the economic status along with documentation of recipe and nutritional value estimation of this vegetable so generated through presentinvestigation could be helpful to explore economic potentiality of *M. acuminata* as an alternative source of income generation to the rural common people of the state.

Wild edible plants play an important role in ensuring food security and improve nutrition in the diets of many people in developing countries [3]. The tradition of using wild food is at risk of disappearing throughout the world. Northeast India including Tripura is very rich in plants especially herbs because of plenty of rainfall and availability of dense forest. Tribal people are the eco-system people who live in harmony with nature and maintain a close link between man and environment. Phillips and Gentry [12] reported that wild edible plant knowledge is gained early in life and increases with age. Wild vegetables and medicinal plants are the gift of nature for the ethnic communities because the poor hilly peoples can collect the vegetable from the forest and it is free of cost which they couldn't otherwise afford to get from the market due to their socio-economic condition. The vegetables help to upgrade the nutritional need of the ethnic communities [13].

At present, about 90 % of global food production comes from less than 30 species and more than 85–90% of the total caloric intake is obtained from 12 domesticated species [14]. In future, this can be a reason of unbalanced pressure on agricultural food sources. The majority of the wild edible plants were neglected that grow naturally in the wild and does not have to be tended prior to producing edible parts [15]. Wild vegetables and spices were an important source of food for mankind before the dawn of civilization.

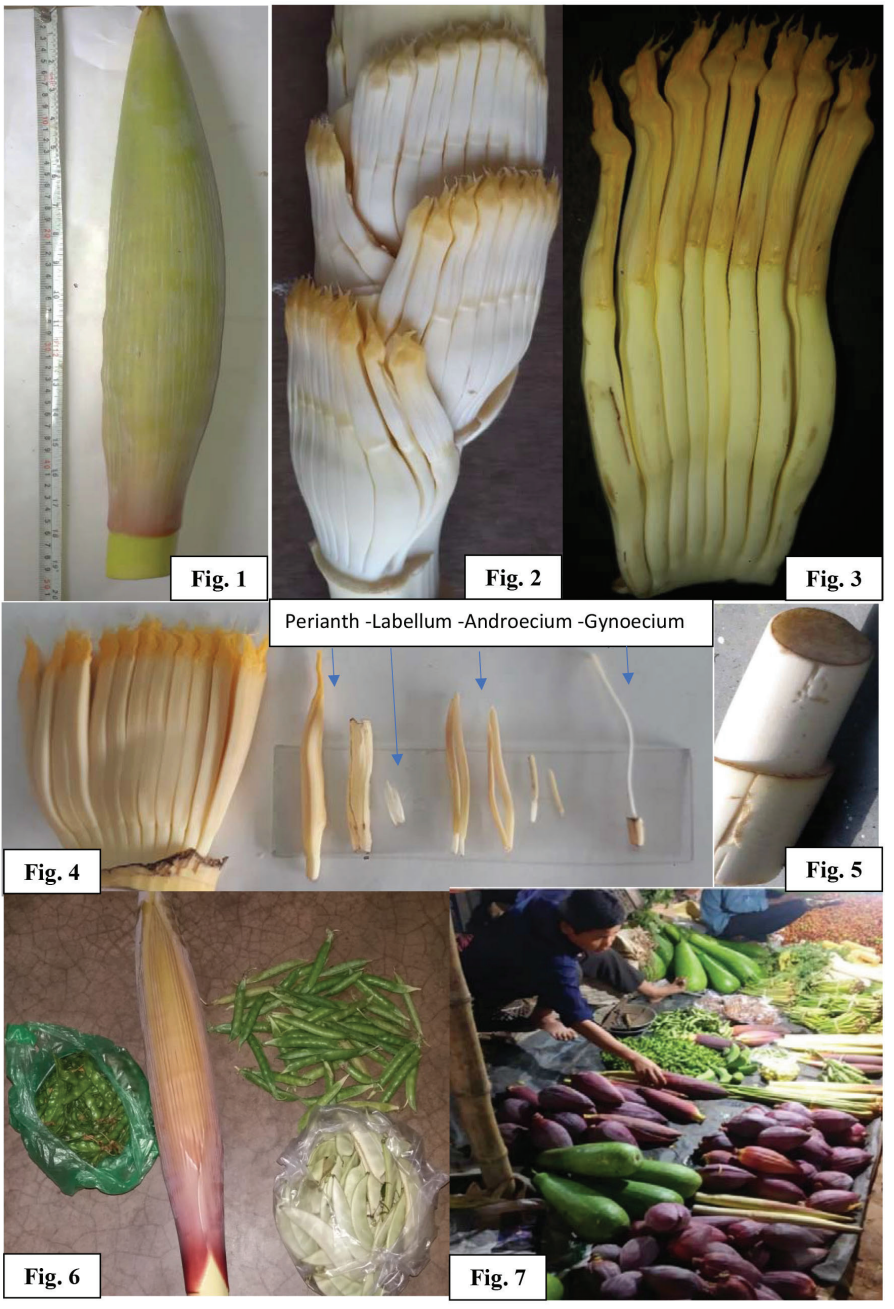

Fig. 1-7: *1. Inflorescence of Musa acuminata; 2. Arrangement of flowers; 3. Individual flower cluster; 4. Different parts of dissected flowers with young flower cluster; 5. Pseudo-stem; 6. Ingredients of one traditional recipe; 7. Inflorescence sold in one local market*

The Utilization of wild plant resources and its popularity in the day-to-day life of people in urban areas has been declining in recent years due to loss of cultural practices. It is seen that due to rapid urbanization, people from present generation have migrated from their native place to urban areas. Many people of the ethnic groups from present generation have started having food belonging to other communities which have led to their change in taste and preferences. The urgent need to maintain and popularize this important source of non-conventional food is needed so as to keep the traditional knowledge intact.

Through present investigation, the traditional significance of *Musa acuminata* is documented as one of the useful nutritional diet to the rural and tribal people of this region. However, it is extremely important to establish nutritional quality parameters along with economic potentiality of the vegetable by undertaking extensive and intensive study on this species in the light of modern scientific knowledge using latest analytical tool. The work directed to this angel will give the platform of general acceptability of the vegetable to the common people.

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Vermicompost a cost effective and eco-friendly component of organic farming
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CHAPTER

Vermicompost a cost effective and eco-friendly component of organic farming

Poulami Saha

Seven Sister Development Assistance, SeSTA (www.sesta.org), Tripura, India
E-mail:gpsahapoulami@gmail.com

Abstract: Vermicompost production and use is an 'environment friendly, protective and restorative' process as it diverts waste from ending up in landfills and also reduces emission of greenhouse gases (GHG) due to very small amount of energy used in its production process Vermicompost is produced from a 'cheap raw material' (community wastes including farm wastes) which is in plenty all over the world and is growing in quantity with the growing human population while the chemical fertilizers are obtained from 'petroleum products' which are not only very 'costly raw materials' but also a 'vanishing resource' on earth. Vermicompost can be produced 'on farms' by all farmers, but the chemical fertilizers has to be produced in 'factories' at a high economic and environmental cost so vermicompost can be afforded by all farmers. The earthworms itself after producing the vermicompost can be sold to fishery, poultry, dairy and pharmaceutical industries for economic benefits.

Keywords: Vermicompost, fertilizers, raw materials, earthworms, economic

The word vermicomposting is derived from the Latin word 'Vermis' which mean 'worms' [1]. Vermicomposting can also be defined as a waste management technology that involves decomposition of organic fraction of solid waste in an eco-friendly way to a level that can be easily stored, handled, and applied to agricultural fields without any adverse effects [2]. Role of vermicompost in nourishing agricultural crops has attracted the attention of researchers throughout the globe in past few decades. Vermicompost is the method of using earthworms to transform organic waste into nutrient rich compost. The earthworms' casting is nutritive organic manure rich in humus, NPK, micronutrients, beneficial microbes, antibiotics, enzymes, growth hormones, etc. There are many reports in literature showing beneficial effects of imbedding vermicompost in soil. Application of vermicompost as organic manure in soil built-up organic carbon, improve nutrient status, microbial activities, microbial biomass carbon and enzymatic activities. The earthworms' castings also have pest repellent

attributes. Beside that vermicompost also improves soil structure, soil aggregation and improve water retention capacity. Vermicompost is a good quality manure that contain several essential nutrients needed by the crops such as nitrogen, phosphorus potassium, calcium, magnesium and micronutrients viz. iron, zinc, copper, manganese in sufficient quantity that increase the productivity and quality of crops. Application of vermicompost in soil improves physical, chemical and biological properties of the soil. It improves soil structure due to which soil become porous and permeable to soil and water. Vermicompost contain sufficient amount of vitamins, amino acids, antibiotics, enzymes and hormones that are helpful in growth and development of plants. Vermicompost provide complete nutrition to plants and create resistance in plants against insect–pest and diseases. Vermicompost increase water holding capacity of the soil and save irrigation. Further, it also reduces the expenditure on costly chemical fertilizer inputs thus, reducing overall cost of cultivation. Vermiculture is basically the science of breeding and raising earthworms. It defines the thrilling potential for waste reduction, fertilizer production, as well as an assortment of possible uses for the future[3]. Vermicomposting is the process of producing organic fertilizer or the vermicompost from bio-degradable materials with earthworms. Composting with worms avoids the needless disposal of vegetative food wastes and enjoys the benefits of high quality compost.

Materials and Methods

Study Area

This study, carried out at the five numbers of cluster villages of Jirania block, west Tripura district among 35 No's of Self help Group (SHGs) members. Under the piloting project related to social mobilization and livelihood promotion supported by government as well as private donors like Indigo for promotion of sustainable livelihood with women farmers enhancing their income generation.

Phase 1: field experiment

Construction of the vermicompost Unit

A vermicompost unit of poly bag size $10 \times 8 \times 3$ m^3 ($l \times w \times h$) was set up in a shaded area, following the standard method of vermicomposting unit. The vermicomposting units were set up at the farmer's field area using the vermitech pattern reported by Ismail (2005) [4] The roof of the unit was made of zinc/ tin sheets with underneath isolation paper to ensure a cool environment and concreted the wall or the sides with bamboo structure.

Preparation of culture bed

1st layer: The basal layer of vermibed comprising broken bricks, then a layer of sand to the thickness of 6–7.5 cm was set up to ensure proper drainage.

2nd layer: Loamy soil up to the height of 15 cm, which was moistened.

3rd layer: The soil was then covered with dry grass clippings/ rice straw up to 10 cm thickness.

4th layer: Lumps of fresh/dry cattle dung with banana stem or leaves were scattered over the soil even the kitchen waste such as leaves or peels of vegetables, fruits, roughages of animals by chopping them into small pieces can use in the unit.

5th layer: Again, spread about 1-1.5 feet layer of cow dung uniformly and sprinkler sufficient quantity of water. Spread about one kg vermiculture (contain about 800 – 1000 earthworms) over the layer of cow dung and at the final layer the earthworms, Eisenia foetida, were inoculated then again spread 2-3 inch layer of green leaves viz neem (*Azadirachta indica*) leafs or moringa leafs (*Moringa oleifera*) or ipomoea (sweet potato) leafs on availavlity basis of the raw materials uniformly over the layer of FYM and then sprinkle water that it should kept moist by sprinkling of water twice a week and turned once a week, up to the harvest of the vermicompost. The entire unit was covered with banana leaves to protect the earthworms from sunlight and birds. And finally the vermicompost bed would cover with the help of jute/gunny bags. For maintaining optimum moisture and temperature conditions in the vermicompost bed, regularly sprinkler the water over the gunny bags. There should be about 35-40% moisture and 15-30^{o}C temperature in the bed. Hence, regularly sprinkle water to maintain optimum conditions for earthworm growth and functioning.

By following above the steps, vermicompost is ready in about 8–10 weeks time. The vermicompost appear dark brown in colour on maturity and is very porous, granulated and free of any foul smell (Fig. 1-4).

Fig. 1: *The above figure depicts worms (Eisenia fetida.)*

Fig. 2: *The above figure depicts inoculation of E.fetida*

Fig. 3: *The above figure depicts preparation of vermibed which contains sandy soil, rice hull, rice straw*

Fig 4: *The vermibed constructed with improvised roofing to protect the worms from direct sunlight and excessive rain.*

Results and Discussion

Effect of Vermicompost on plant growth:

Vermicompost is an organic fertilizer obtained from the earthworms by passing out the organic wastes through the digestive systems, vermicompost has also found to have positive effect on early as well as later stages of plant life cycle [5] reported that seedling emergence of petunias seeds grown in mixture at (produced from cattle manure, food waste and paper waste).Vermicompost is found to have positive influence on crop productivity and quality in wide range of crops [6] such as tomato, eggplant, okra, lettuce, cabbage, coriander, cucumber etc it greatly enhances crop productivity than inorganic fertilizers (Table 1 & 2). The leaf number, stem circumference were found maximum in vermicompost amended soil rather than chemical amended soil.

An example of successful farmer like Smt Jharna Debbarma who adopted vermicompost production, enhanced the livelihood status, improved soil health and conserved beneficial soil micro-organisms. she argued herself by the quality product which is demanded by nearby farmers, NGO's and government organizations of Jirania block, Tripura west. The success of any production system basically depend on need, availability of inputs and marketing channels by which one can get the remunerative price by using locally available resources.[7] The key to the success of organic farming system is the production of all inputs like, manures, plant protection etc, and on-farm utilizing the local resources wherein animal husbandry plays a catalytic role. It is essential to clearly define a national policy on organic farming by supporting private sector groups, NGOs or associations, and encouraging farmers to produce their own fertilizer in respective countries.[8]

Table.1: *Nutrient concentration in the vermicompost*

Nutrient	Content (%)
Organic carbon	9.15 - 17.98 %
Total nitrogen	1.5 - 2.10 %
Total phosphorus	1.0 - 1.50 %
Total potassium	0.60 %
Ca and Mg	22.67 - 47.60 meq/100g
Available S	128 - 548 ppm
Copper	2 - 9.5 ppm
Iron	2 – 9.30 ppm
Zinc	5.70 – 11.5 ppm

Source: Online: http://agritech.tnau.ac.in/org_farm/orgfarm_vermicompost.html; http://www.hillagric.ac.in/edu/ coa/agronomy/lect/agron-3610/ Lecture-10-BINM-Vermicompost.pdf

Table. 2: *Comparison between nutritive value of vermicompost and farmyard manure*

Element	Vermicompost	Farmyard manure
C:N Ratio	15.5	31.3
N (%)	1.6	0.5
P (%)	0.7	0.2
K (%)	0.8	0.5
Ca (%)	0.5	0.9
Mg (%)	0.2	0.2
Fe (mg kg-1)	175	146.5
Cu (mg kg-1)	5.0	2.8
Zn (mg kg-1)	24.5	14.5
Mn (mg kg-1)	96.5	69.0

Source: Online: http://agri.and.nic.in/vermi_culture.htm

Conclusion

The vermicomposting of dry grass clippings, rice straw and cow manure (1:1 ratio) using *Eisenia foetida* was a successful model. The produced vermicompost had a dark color, a mull-like soil odor and was homogeneous. It had all the essential macro- and micro-plant nutrients like N, P, K, Ca, Mg, Mn, Cu, Zn and Fe, indicating the achievement of getting an environment friendly nutrient-rich fertilizer for the agriculture sector. Vermicompost is a potential input for sustainable agriculture. Planning for global organic farming and sustainable agriculture can truly bring in 'economic prosperity' for the farmers, 'ecological security' for the farms and 'food security' for the people. This practice will ensure economic viability and environmental sustainability. The vermin-composting technology helps in organic waste management. The method is in accordance with the principles of sustainable development.

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Attitude of parents and teachers towards adolescent and reproductive health in females in North and Unakoti district of Tripura, India: A study In : *Biological Sciences: Impacts on Modern Civilization, Current and Future Challenges* by *Anupam Guha* © New Delhi Publishers, New Delhi: 2021, 323-332.
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CHAPTER

Attitude of parents and teachers towards adolescent and reproductive health in females in North and Unakoti district of Tripura, India: A study

Rajib Biswas [1], Rithi Majumdar[2] and Soumitra Nath*[3]

[1] Dept of Zoology, Govt. Degree College, Dharmanagar, India
[2] Dept of Zoology, Rabindranath Thakur Mahavidyala, Bishalgarh, Spheiajala, India
[3] Dept of Biotechnology, Gururcharan College, Silchar, Assam, India
*E-mail: nath.soumitra1@gmail.com

Abstract: Adolescence is a phase of tremendous physical and psychological changes, along with development in social perceptions and expectations. The public health challenges for adolescents encompasses pregnancy, risk of maternal and infant mortality, STI, RTIs and the rapidly rising incidence of HIV in this age group. Thus it is important to influence the health-seeking behavior of adolescents as their situation will be central in determining India's health, mortality and morbidity; and the populace growth consequence. For the same proper understanding of adolescence, reproductive health is of utmost significance to teachers' and parents as they are the guiding poles of adolescent's and reproductive aged females. The study intends to find out the attitude of parents and teachers towards reproductive health and how adolescents perceive their phenomenal changes. We surveyed 1000 females of adolescent age group and reproductive age group. The study also stresses on parents occupation, education, accessibility and schools environment because all these factors have direct implications for the population's reproductive health, with potential consequences leading to disability, infertility, sexual violence and abuse, and even death. Nutritional status, access to educational opportunities, access to reproductive health services and commodities, and some life style behavior's may also impact on the future reproductive health of the current and the next generations. The findings were very dishoarding as it was found that maximum girls are not able to open up their issues with parents or teachers as they are not heard upon. They suppress their menstrual and health issues which led to infections and other health issues. The challenges in providing services for adolescent health is not providing newer technologies or interventions as they would not bring the adolescents in purview of health care but to make the 'adolescent friendly health services' more accessible, equitable, acceptable, appropriate, comprehensive, effective and efficient. All this is possible only when the guardians of today's society accepts openly the issues related to reproductive health.

Keywords: Adolescence; Reproductive health; Tripura.

Today's children's are the future citizens.An individual of reproductive ages faces tremendous number of developmental tasks. We tend to think primarily of the biological changes that the adolescents are experiencing, such as puberty and social changes. To fully understand adolescence, it is critical to understand the diversity of changes that adolescents experience beyond the biological and social. These changes include emerging sexuality, acquiring interpersonal skills for dealing with members of the opposite sex, and intimate relationships [1]. Many adults think that children do not have any problems. Today's families are very complex in structure and day-to-day schedules of families are complicated and lead to stress in family members. Adults' family stress impacts on children too. Children express stress in their behavior [2]. Adolescents face various issues at family, school and society, such as poverty, adjustment problem, broken family, frequent fights at home, death of the parents, single child, no place to play, homework issues, difficulty in understanding the lesson, and other developmental issues. If suitable help is not provided to them in time, it affects their future as an adult. The formal academic system may not permit a teacher to handle children with psycho- social issues. A teacher's role is limited to classroom teaching. A good quality of life of the child includes good housing, health services, financial stability, family environment, social network; practical coping skills, etc. Ninety percent of the children in India have a poor quality life. Child and adolescent mental health have given less importance. The overall development of any country is dependent on the mental development of its children. Alcohol and drug abuse in children has increased tenfold. Poverty, malnutrition, illiteracy, poor health and hygiene are adversely affecting millions of children in India [3]. Children living in families characterized by unemployment, substance abuse, inadequate healthcare, poor quality child care, and high level of child abuse and neglect can lead to higher level of impairment in the children's social, behavioural, and academic functioning. Similarly, poverty also leads to serious social and personal issues [4]. Communication within the family is particularly important during adolescent years, especially concerning reproductive health issues. Family communication affects the adolescent's identity formation and role-taking ability. Adolescents who experience the support of their families feel free to explore identity issues. Discussions between parents and children significantly facilitate the development of higher levels of moral reasoning in adolescents [5].

Females are more prone to RTI (includes HIV) due to larger mucosal surface exposed during sexual intercourse. Young reproductive females are even more risk zoned because of the immature lining of cervix. Quite a many, young women do not even realize they have developed RTI as they have no symptoms and unaware of them. Undiagnosed and untreated, the infection continues to plague them into adult life and

it may lead to an ectopic pregnancy and eventually infertility. Attitudes for preventing such reproductive disorder's must be based on sound understanding of young people's and societal beliefs and behavior's keeping in view the context of cultures they dwell in. Understanding of reproductive health and adolescence by parents and teachers will help in better understanding and in long terms benefit reproductive health of adolescents [6].

A major domain of concern is reproductive and sexual health as the adolescents do not have adequate awareness and knowledge about these. The occurrence of STIs, teenage pregnancy and unsafe abortions are significantly high among adolescents [7].

The role of school and parents is not just confined to imparting education while and not only for feeding and financing but also expands their horizon to making them capable of all round survival [8-10]. Apart from assigning the regular school assignments and tasks it's important to organize them and impart adolescence education to them for better social, mental and physiological acceptance of their own-self and their fellow mates. At home parents are to be friendly with children to know their feelings and guide them at their time of disparity [11-13]. This is the age of springs and storms and hence the adolescents are too sensible and needs to be treated with utmost care and love in accordance to appropriate measures for discipline for their overall up-bringing in all spheres of life [15-18]. More research is being performed regularly in this field for standardizing the norms for adolescence care as this forms the basis of the upbringing of nation's future generation [19]. Objectives of the present study include assessment on the source approached by them for the solution. To find out family background to address the psycho-social issues of the child and to determine their knowledge about reproductive health and natural phenomena's and finally determine their approachability to parents, teachers' and friends during problems.

Methodology

During this we surveyed literature regarding adolescence and issues regarding to it and made a detailed questionnaire for our survey by discussion among the team members. We selected following schools for our survey keeping in mind the various societal norms, education structure, location and status of schools. We divided the group into two parts and surveyed respondents. The survey was conducted by discussion method and face to face interview. Since the schools are closed due to lockdown, interview was done individually at respondents respective homes and over telephone in some cases. After the completion of the survey the data obtained were checked for complete surveyed forms and collected sequentially. The data entry was performed for tabulation and further analysis. The sample comprised of female students of reproductive age from 50different schools of Dharmanagar and Unakoti district. 20 female students

from each school were selected for the survey based on availability of approach. Total respondents were 1000 female students.

Results and Discussion

The family education data reveals that about 1/4th of the respondents parents were primary school attendee while about ½ of the respondents parents were high school/ M.E. level attendee while only 1/10th were higher secondary pass out and graduate (Fig.1). Insight into parent's profession revealed that maximum students belonged from poor families. 1/5th of their parents were farmer and 1/5th are small businessman, on the other side 3/10th were laborers and 1/10th were drivers on hire. Very few were govt. employed amounting to only 1/10th and about 1/20th were religious personal (Fig. 2). Most of the adolescents are highly attached with the family (83 percent) while 12 percents are moderately attached with their family. Adolescents are emotionally misbalanced in this period, the effects of that 5 percent students are less attached with their family (Fig. 3). Students in this adolescence period not very comfortable with their parents and teachers but adolescents among parents and teachers comfortable with their problem sharing with their mother (42 Percent). They are less comfortable with their teacher. Higher percent students are not at all comfortable with their father and teachers in case of problem sharing (Fig. 4). The data reveals that the 34 percent students want their teacher should treat and behave with them friendly. After that 32 percent student wants teachers pay lot of attention towards them. 19 percent and 15 percent students want their teacher strict and passive (Fig. 5). The collected information reveals that 79 percent students are not aware about the sex education and they don't have any knowledge about this topic. Only 21 percent students are more or less aware about this topic it may students are now a day's most connected with the internet world (Fig. 6). Insight into the data which we collected through survey 88 percent students don't have any knowledge about counseling and very less 12 percent students have idea about counseling (Fig. 7). Highest percentage (46 percent) of students is watching TV for 1 to 2 hours and then 38 percent students are watching for 3 to 4 hours. Around 16 percent are watching TV above 4 hours (Fig. 8). During the Adolescence period the children's think differently, 32 percent students are watching romantic TV shows, 30 percent prefer violence, 18 percent Sport shows, 8 percent students cartoon shows and 12 percent students prefer all types of TV shows (Fig. 9). When questioned indirectly regarding dressing sense of girl child 15 percent of the students responded with negative implications while 18 percent had a positive approach towards the necessity of dressing as per circumstance. Among the remaining population 45 percents were suggestive regarding the sensational issues while 22 percent had forceful implications to the concerned matter (Fig. 10). Regarding idea about sexual harassment by their relatives or close associates of elderly age 22 percent student had no idea while 57

percent reported affirmatively on the other hand 16 percent of them were suggestive and 7 percent were forceful (Fig. 11). When questioned regarding idea of family planning the response was missed. 24 percent had no idea regarding family planning, 62 percent had some idea and 14 percent didn't know anything of the issue (Fig. 12). When tactfully enquired about consequence of family planning 22 percent retorted that it would get them distracted and disturb from their studies, while 28 percent had the view that this would make them too aware of the opposite sex, contrastingly 16 percent had the opinion that it would make them responsible. While 12 opted that it would make them self-conscious and 22 percent had the view that this would encourage them for future experimenting (Fig. 13). Regarding conceptual knowledge of sexual activities during adolescence 16 percent reported affirmatively, 57 percent had no idea and 17 percent didn't know anything in the perspective (Fig. 14). Regarding beating of child by parents 17 percent believed that it's a form of violence and 40 percent believed that it's necessary for keeping them in control. Strangely 23 percent believed that it's a mode of expression of love and 20perccent expressed objection to it (Fig. 15). Finally on enquiring about tendency towards physical attraction during adolescence 54 percent had positive outlook, treating it as a normal behavior while 13 percent had negative approach toads this natural phenomenon. 29 percent students believed that it's an universal process and 4 percent had no idea on this issue (Fig. 16). Regarding menstruation and conceptual norms regarding societal orders or customary laws related to menstrual cycle 36 percent retorted it as a normal phenomenon,18 percent still had no proper knowledge about the issue, 14 percent still believed in age log superstitions and 19 percent had wrong dietary conceptions while 12 percent believed in isolation practice (Fig. 17).

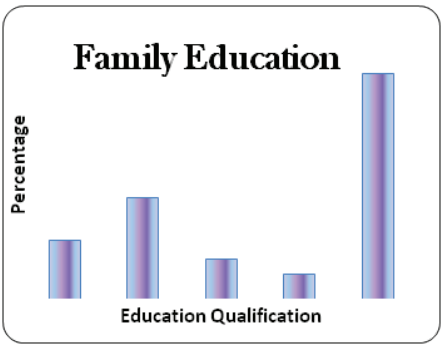

Fig 1: *Graphical representation of Family Education*

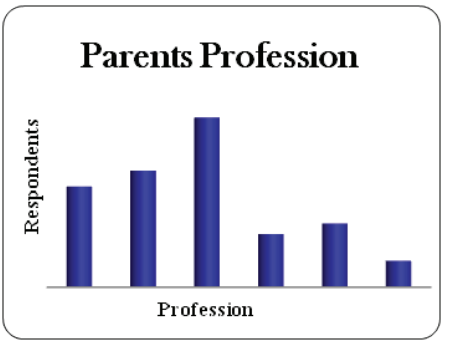

Fig 2: *Graphical representation of Parents Profession*

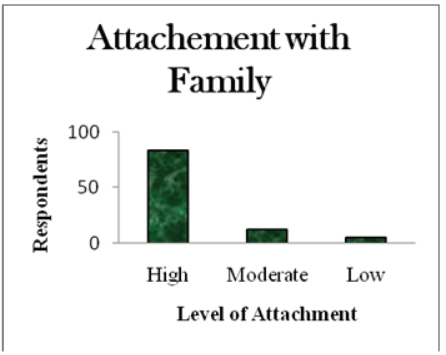

Fig 3: *Graphical representation of family attachment*

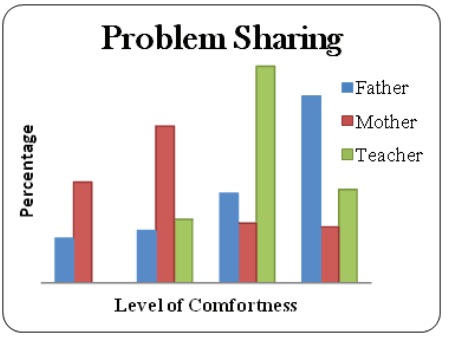

Fig 4: *Graphical representation of problem sharing*

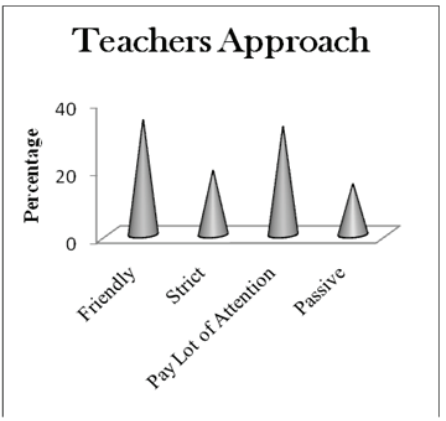

Fig 5: *Graphical representation of expected Teachers, approach*

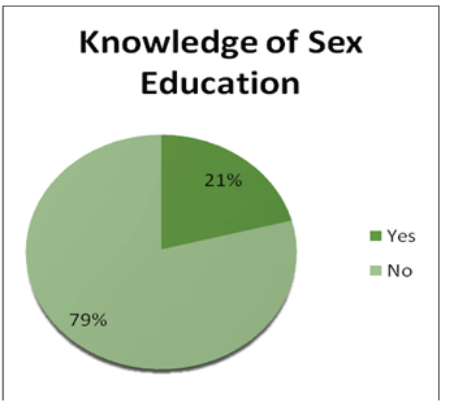

Fig 6: *Graphical representation of knowledge of sex education*

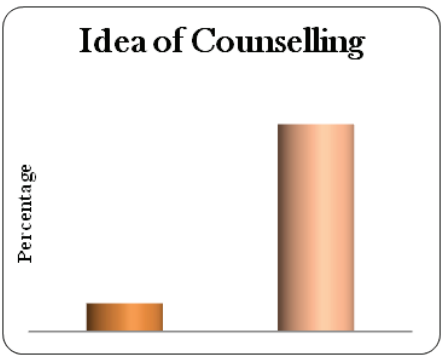

Fig 7: *Graphical representation of family attachment*

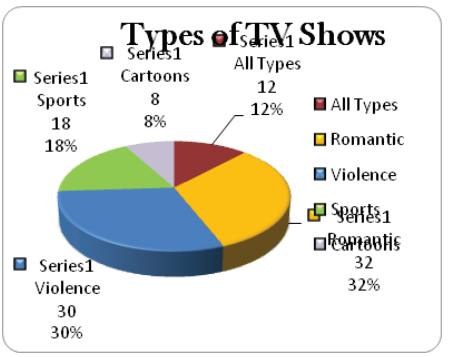

Fig 8: *Graphical representation of Duration of watching TV*

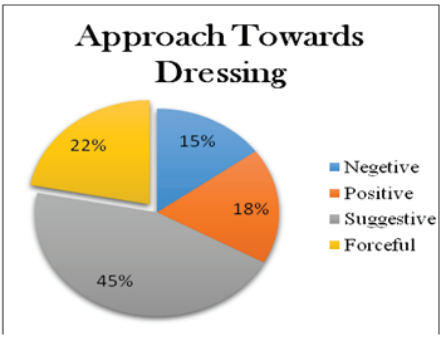

Fig 9: *Graphical representation types of TV shows*

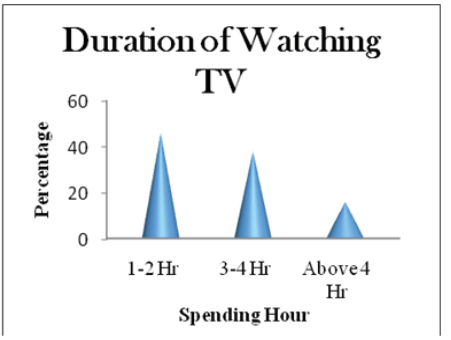

Fig 10: *Graphical representation of approach towards dressing*

Fig 11: *Graphical representation of ideas of sexual harassment*

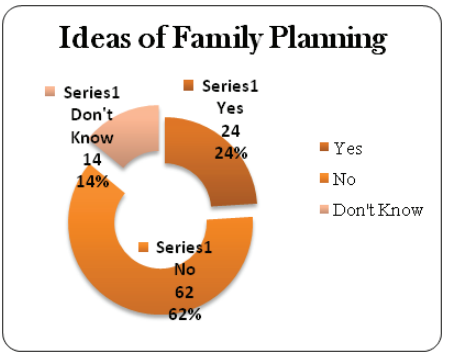

Fig 12: *Graphical representation consequence of family Planning*

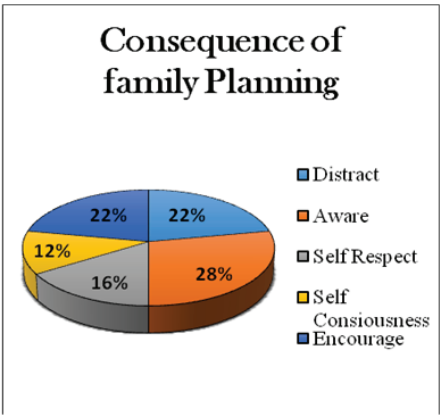

Fig 13: *Graphical representation of consequences of family planning*

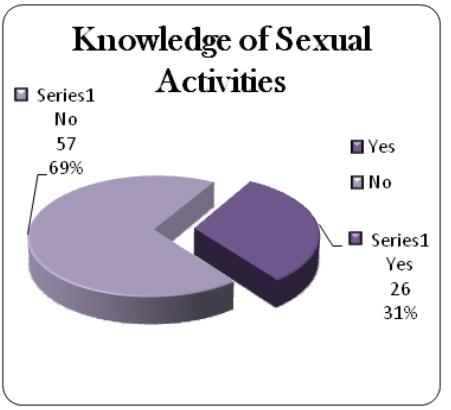

Fig 14: *Graphical representation of knowledge of sexual activity*

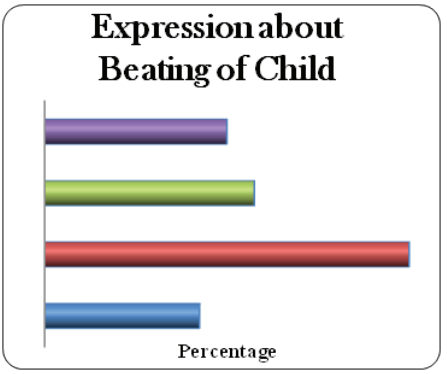

Fig 15: *Graphical representation of expression about beating of child*

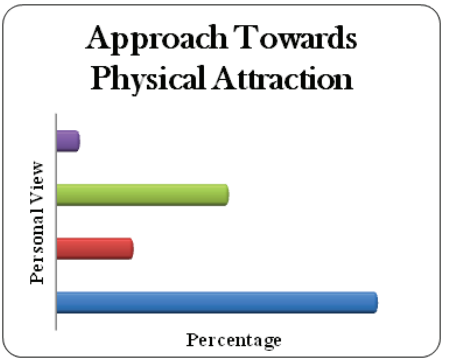

Fig 16: *Graphical representation of expression about beating of child*

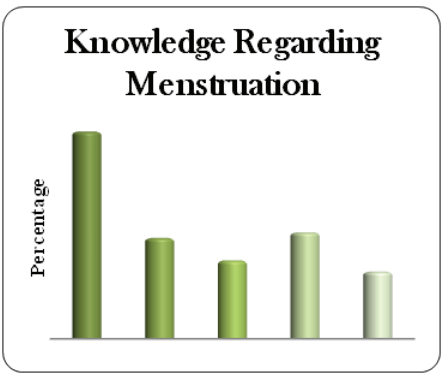

Fig 17: *Graphical representation of Knowledge regarding Menstruation*

A= It is a normal process for adolescent girls and women of child bearing age

B= During menstrual periods girl/woman can continue with sports activities.

C= during menstrual periods a girl/woman should not visit sacred places

D= during menstrual periods a girl/woman should not eat pickles

E= during menstrual periods a girl/woman should be isolated.

Conclusion

Thus from the above discussion and results obtained we can clearly see that there is a sheer lacking of proper knowledge of reproductive health and reproductive health education among the students. Parents and teachers are unaware about the health issues and reproductive care for adolescents. Parents and teachers are unaware of their menstrual health and proper care is absent in almost all cases. The female students are left with no options for sharing their problems and develops depression and low self esteem. The introduction of reproductive health and the biological aspects of adolescence should be made known to students, parents and teachers for the better upbringing and stable adolescent females. Only this can prevent the occurrence of female unhygienic diseases and build a better society for them where they can feel free to discuss their traumatic issues and live a better life.

The dares in providing amenities for adolescent health is not as long as newer technologies or involvements as they would not bring the adolescents in purview of health care but to make the 'adolescent friendly health services' more accessible, equitable, acceptable, appropriate, comprehensive, effective and efficient. Moreover, strong political will and commitment is necessary to boost up the adolescent health.

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A report on the occurrence of fungal pathogen Saprolegnia parasitica on a freshwater fish Puntius ticto (Ham.) in a pond ecosystem of Tripura, India In : *Biological Sciences: Impacts on Modern Civilization, Current and Future Challenges* by *Anupam Guha* © New Delhi Publishers, New Delhi: 2021, 333-341. ISBN: 978-81-947417-9-4, DOI: 10.30954/ndp.bio.2020.32

A report on the occurrence of fungal pathogen *Saprolegnia parasitica* on a freshwater fish *Puntius ticto* (Ham.) in a pond ecosystem of Tripura, India

Saumen Chakrabarti

Department of Zoology, Women's College, Agartala, Tripura, India
*E-mail: drsaumen@gmail.com

Abstract: The present observation makes a report on the occurrence of an aquatic fungal species *Saprolegnia parasitica* on the epidermis of a freshwater fish *Puntius ticto*(Ham,). The fungus infected fish was collected from a pond ecosystem of Tripura, India during a period from September 2018 to February 2019. The appearance of *Saprolegnia parasitica* was cotton-fibre like tufts of mycelium and observed on the muscle epidermis, fins and caudal region of the fish. A deep ulceration was observed on the base of the pectoral fin of the infected *Puntius ticto* .Important biotic and abiotic(water quality) features of the studied pond were also noted during the occurrence of this fungal species on the body of fish. During the occurrence of this fish pathogen, pond water showed lower temperature (9-16^{0}C), alkaline pH (7.6-8.4 ppm) and moderate levels of dissolved oxygen (6.2-7.8 ppm).The prevailing biotic as well as suboptimal water quality characteristics indicate the development of stressed condition in the pond ecosystem which might be helpful for the occurrence of *Saprolegnia parasitica* over the epidermis of *Puntius ticto.*

Keywords: *Saprolegnia parasitica* ; *Puntius ticto*; water quality; freshwater pond; Tripura.

Diseases in freshwater fishes are a great threat to achieve optimum production and become a limiting factor to economic success of aquaculture [1]. In aquaculture, large scale fish mortalities occur due to infectious microbial and parasitic diseases caused due to high dense culture or by pollution mediated environmental stress[2, 3] . The open water capture fish has been suffering such as epizootic ulcerative syndrome(EUS), septicaemia, tail and fin rot disease, gill rot disease, viral disease, bacterial disease and fungal disease[4, 5] . Fungal infection of fish by oomycetes commonly called water moulds are widespread in freshwater and represent the most important fungal group

affecting wild and cultured fish[6] . Dominant fungal pathogens reported in aquaculture are oomycetes including the genera *Achlya*, *Aphanomyces* and *Saprolegnia*[7,8]. *Saprolegnia* may occur anywhere on the body of fish but normally appears as a conspicuous, circular or crescent-shaped, white cotton-like mycelium, particularly around the head, caudal and anal fins [9, 10]. It may spread over the body by radial extensions until adjacent lesions merge [11] . In *Saprolegnia* species longer lived secondary zoospores emerge from cysts produced by primary zoospores. This pattern of re-emergence called polyplanetism, may be repeated several times [12] and most likely functions to allow several opportunities to contact a new host [13] . Saprolegniasis is more prevalent in lower water temperature and most of the *Saprolegnia* associated mortalities are confined to late autumn,winter and early spring seasons in intensive culture ponds[1, 5]. *Saprolegnia* infections usually result from a wound on fish skin,while eggs are another main infection target on which it may spread and cover more than 80% of body surface[14]. The encysted zoospores of *Saprolegnia parasitica* are decorated by long hooked hairs that are thought to aid in attachment to the fish host [15, 16]. Research reports have shown that susceptibility of farmed fish to fungal or fungal-like microbes depends on several factors including rapid drops in ambient temperatures [6,17], low water levels, failure to remove dead fish or eggs, primary infection by other organisms[34] and pollution[18, 19], all of which can reduce ecosystem function[12] and lead to an increase in emerging infectious diseases (EIDs)[20, 21]. A look into the existing literature explicitly reveals that a number of research works on fish pathogen were carried out in India and Abroad but in Tripura no in-depth knowledge on the occurrence of fish pathogen is available till-date. Hence, the present observation makes a report on the occurrence of a fungal parasite *Saprolegnia parasitica* on a freshwater fish *Puntius ticto* (Ham.) in a pond ecosystem of Tripura.

Materials and Methods

The present study was carried out in a freshwater pond located at Melaghar(Latitude $23^0 50' 15''$N and Longitude $91^0 15' 45''$E), Sonamura Sub-division, Sepahijala District of Tripura, India during a period from September 2018 to February 2019. The pond is perennial and rectangular shaped, the surface area is about 0.62 ha. The depth of the water column varies from 1.0 to 2.5m. The littoral zone of the studied pond harbous some species of macrophytes such as *Eichhornia crassipes, Trapa bispinosa, Lemna* minor. Fungal infected fish *Puntius ticto*(Ham.) was collected from the studied pond under observation brought immediately in the laboratory for isolation of fungi. Isolation of fungi was carried out by taking small pieces from muscles about 2mm in diameter from different portions of the body of the infected fish and then washed thoroughly with distilled water. These tissues were then incubated over

plates containing PDA (Potato Dextrose Agar) media. The isolates were incubated at 18^0C-22^0C for 3-4 days until fungal mat developed. Isolates were then used to make pure cultures. Pure cultures were prepared by taking small piece of media with fungal growth and transferred to the plates containing baits like hempseeds, sesame seeds, mustard seeds and broken pulses with sterile tap water. These cultures were incubated at18^0C-22^0C for growth of colony on bait and development of sexual characters for identification of the fungal species. Isolated fungal species was identified following keys and monographs of Cocker [11] and Khulbe[22].The Water quality parameters of pond water as water temperature, transparency, pH, dissolved oxygen, free carbon dioxide, bicarbonate, dissolved organic matter, silicate, phosphate-phosphours, nitrate-nitrogen were analyzed following the methodology of APHA [2].

Results and Discussion

Fig. 1: *Growth of Saprolegnia parasitica on Puntius ticto*

The present study infers that a fungal species *Saprolegnia parasitica* was observed on the outer body part (epidermis) of three freshwater fishes *Puntius ticto* (Ham.) The appearance of *Saprolegnia parasitica* was cotton-fibre like tufts of mycelium (Fig. 1) and observed on the fins, fin rays, muscle epidermis and caudal region of fish. Fin rays become brittle. A deep ulceration was marked on the base of pectoral fin of the infected fish.

The well grown *Saprolegnia parasitica in the* PDA medium looks like cotton wool and it grows radially and spreads mycelia (Plate1 and 2).

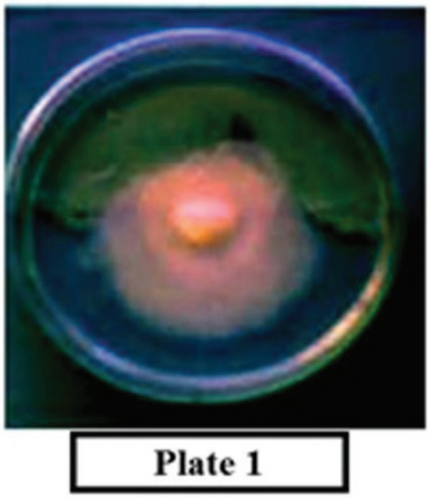

Plate1: *Saprolegnia parasitica in a in a control PDA disc method culture*

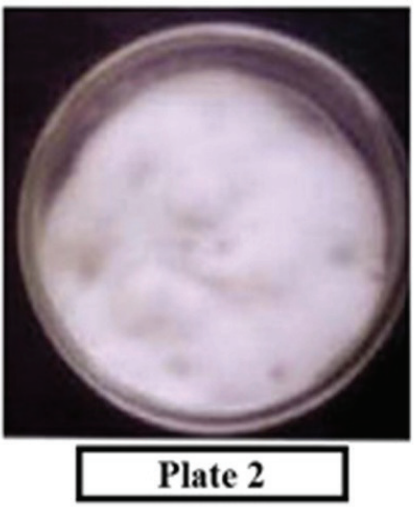

Plate 2: *Fully grown Saprolegnia parasitica in a control PDA disc method culture*

The growing young mycelia on potato dextrose agar (PDA) media from serial cultured plate was teased and observed under microscope (Plate 3). The vegetative thallus is tubular, multinucleate and variably branched with transparent envacuolated hyphae (Plate 4).

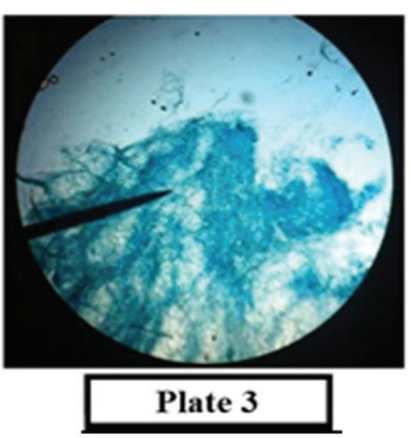

Plate3: *Cluster of mycelia under X40 magnification*

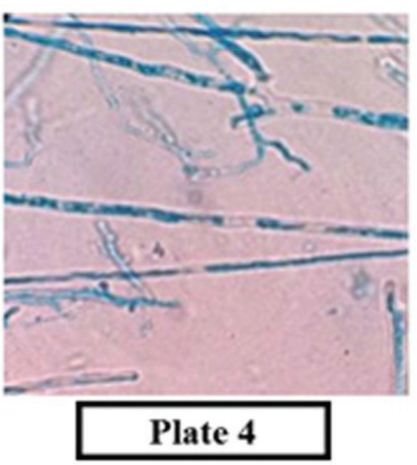

Plate 4: *Single hyphae under X100 magnification*

During the abundance of this fungal species, notable characteristics of water quality were found to be observed (Table 1).

Table 1: *Water quality parameters of the studied pond*

Physico-chemical factors	Range	Mean
Water temperature (^{0}C)	9-16	12.2
Transparency (cm)	4-12	5.8
pH	7.6-8.4	7.8
Dissolved oxygen (ppm)	6.2-7.8	6.4
Free carbondioxide (ppm)	1.6-3.0	2.6
Bicarbonate aklalinity(ppm)	48-74	56
Dissolved organic matter (ppm)	9-19.6	14.4
Silicate (ppm)	7-12.8	9.6
Phosphate phosphorus (ppm)	0.5-0.8	0.6
Nitrate nitrogen (ppm)	0.6-09	0.7

The distribution and seasonal occurrence of aquatic fungi in relation to water quality parameters have been investigated by several noteworthy researchers[23,24,25]. Water temperature, pH and oxygen availability are the important water quality parameters regulating the growth of aquatic fungi [24]. Low water temperature, alkaline pH, moderate values of dissolved oxygen and low concentration of chloride found to be significant for the growth of fungi in winter season [10] and these findings are in agreement with the present observation. Micro-organisms (fungi, bacteria and protozoa) are constant component of the host's environment and appear to follow a disturbance what might be conceived as a dynamic equilibrium between pathogen and host[16]. The most common fungal parasite affecting fish is *Saprolegnia parasitica* and this fungal parasite has been associated with a wide range of infections such as ulceration, exfoliation of the skin followed by haemorrhage, blindness, congestion at the base of pectoral and anal fins in a number of freshwater fishes[23]. It is a common inhabitant of the aquatic environment and has been isolated from both unpolluted and polluted freshwater[26-32]. . Under stress conditions, when there is some injury on the fish body, the fungal parasite *Saprolegnia parasitica* grown on the dead tissue at the site of the injury and form a thick layer of fungal hyphae which imparts a cotton-wool like appearance[3] Injured fishes are generally attacked by *Saprolegnia* sp. in ponds with abundant decaying organic matter which could be seen as tufts of whitish thread outgrowth[29] (Fig. 1). The present observation shows moderately high range of dissolved organic matter (9-19.6 ppm) in the studied pond water. Experimental studies of several researchers [6, 33-42] also suggest that *Saprolegnia parasitica* infection on fish in fresh water ecosystem occur due to certain stress condition in the physico-chemical parameters of water. It is further recorded that the availability of this fungus sp. is very much specific in regard to the nature of peripheral vegetation as well as phytoplanktonic abundance [23] . The availability of this fungus sp. is species specific

in regard to the selection of fish species as their suitable host [17,43-48]. The present study redescribed *Saprolegnia parasitica*, for which the most remarkable features are put together after comparing with own observations.

Conclusion

The abiotic and biotic observations reveal that the occurrence of *Saprolegnia parasitica* in the fresh water ecosystem might be due to suboptimal condition in the physico-chemical parameters of water. Although the fungal species *Saprolegia parasitica* was reported earlier by many researcher from some other states of India[16,17,24,27,28,29,40] and aboard[6,8,9,11,13,35,41,42,44], the present report also confirms its nature of cosmopolitan distribution.

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Conflict of Interest: The author declares that there is no conflict of interest.

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Pollen fertility, viability and in vitro pollen germination studies in Mucuna Bracteata DC. Ex Kurz from Tripura In : *Biological Sciences: Impacts on Modern Civilization, Current and Future Challenges* by *Anupam Guha* © New Delhi Publishers, New Delhi: 2021, 342-349. ISBN: 978-81-947417-9-4, DOI: 10.30954/ndp.bio.2020.33

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CHAPTER

Pollen fertility, viability and *in vitro* pollen germination studies in *Mucuna Bracteata* DC. Ex Kurz from Tripura

Somnath Kar*[1], Debasree Lodh[1], Dipanwita Chaudhuri Sil[1] and Badal Kumar Datta[2]

[1] Department of Botany, Holy Cross College, Jubatara, Lembucherra, Tripura, India
[2] Plant Taxonomy and Biodiversity Lab, Department of Botany, Tripura University, Suryamaninagar, Tripura, India
*E-mail: somnathagt21@gmail.com

Abstract: The present work aimed to determinepollen fertility and viability and *in vitro* pollen germination of pollens of *Mucuna bracteata*. The freshly opened flowerswere collected in the morning and evaluated for pollen fertility, viability and *in vitro* pollen germination. For pollen viability studies five different stains were used. *In vitro* pollen germination was estimated using different pollen germination solutions.Out of the pollen fertility and viability stains, Fluorescein Diacetate stain differentiate viable and non-viable pollen more accurately than other stains. Among the *in vitro* pollen germination media, 25% sugar + 300ppm boric acid showed maximum percent of pollen germination and in 25% sugar+ 100ppm boric acid pollen tube growth.

Keywords: *Mucuna bracteata*, pollen fertility and viability, *In vitro* pollen germination

Mucuna bracteata DC. Ex Kurz is a leguminous herbaceous and an important cover crop which grows as an annual or perennial plant across the tropics. The seeds of *Mucuna* contain high amount of L-DOPA (L-3, 4-dihydroxyphenylalanine), which is used in the treatment of Parkinson's disease and mental disorder. *Mucuna* seeds have high content of alkaloids, glycosides, terpenoids, reducing sugars, tannins, saponins etc. [1]. Traditionally the plant has been used against neuropathy, oedema, fever, delirium, amenorrhea, elephantiasis, constipation, ulcers etc. The seeds used as nervine tonic, astringent, aphrodisiac; the hairs of pods are vermifuge; the leaves used in treatment of ulcers, inflammation, helminthiasis and cephalagia; the roots are bitter, emollient, stimulant, thermogenic, purgative and diuretic. The bark powder mixture with dry ginger is applied over painful rheumatic joints etc. [2]

Successful pollination is a prerequisite for fertilization and seed set for most of the plants. A successful fertilization depends on the viability, germination capacity and quality of pollen grains [3-4]. The quality and amount of pollen viability and germination by a flower play an important role in plant breeding. Therefore, pollen qualityassessment is a necessary and it is determined by pollen viability and vigour, i.e., the proportion of pollen grains that are viable. Pollen viability and fertility are very important for fertilization programme, because the pollen germination percentage affects the seed formation of any plant [5]. *In vitro* germination involves germination of pollen grains on artificial media and determining germinability and pollen tube growth[6]. To study the *in vitro* pollen viability and the germination of pollen grains different culture media are available and various methods are used for estimation of pollen viability and germinability [7]. This paper deals with pollen viability, pollen fertility, *in vitro* pollen germination in *Mucuna bracteata* DC. Ex Kurz.

Materials and Methods

Flowers were collected from two populations growing in their natural habitat *i.e.* Bhallukiatila (23°52'07.88"N, 91°17'45.67"E) and Tripura University Campus (23°45'38.70"N, 91°15'52.87"E) and observations were made on a day-to-day basis.

Pollen morphology

For pollen morphology, Acetolysis methodology proposed by Erdtman [8] was carried out. The sizes of the pollen grains were measured by mounting the pollen grains in glycerine jelly [9] using a standard ocular micrometer. Pollen grain size was measured randomly from different flowers. The morphology and pollen grains size were studied. The terminology used is in accordance with [8,10-12].

Pollen fertility and viability

The pollen fertility was studied using Muntzing's mixture technique [13], 2% Acetocarmine staining [14] and Lacto-phenol cotton blue technique [15].

The stained pollen grains were treated as fertile while unstained pollen grains were counted as sterile. The pollen viability was assessed using 1% TTC [16] solution and FDA [17].

In vitro Pollen germination

These studies were worked out in various concentration of sucrose (2%, 5%, 10%, 15%, 20%, 25%, 30%, 35% and 40%) alone and in combination with boric acid and calcium carbonate respectively. The fresh pollen samples were collected before anthesis and then spread on the grooved slides having the solution of sucrose alone as well as in combination with boric acid at different concentrations. Slides were placed into petri-plates having moist filter paper. Slides were observed at different time

intervals following the method of [18]. Pollen grains, which had produced pollen tubes double in size of the pollen in the respective medium, were recorded as germinated pollen [19]. Length of pollen tubes in different concentrations was recorded after 4 hours of the commencement of germination in humid chamber and percentage of pollen germination was calculated and the average lengths of pollen tubes were measured.

Results and Discussion

Pollen Morphology

Walker and Doyle [11] opined that palynology is the study of pollen and spores and it can provide incredible amount of information from a small material in a little time.

In present study pollens were found as tricolporoidate type. While Agostini *et al.* [20] observed tricolpate type of pollens in *M. Japira*and *M. urens.*

Table1: *Palynological information of Mucuna bracteata*

Pollen Parameters	Measurements
Pollen type	Tricolporoidate, Zonoaperturate
Pollen axis (μm) (Mean ± S.E.)	(40.81-) 43.38±0.71 (- 48.32)
Equatorial diameter (μm) (Mean ± S.E.)	(38.05-) 40.48±0.55 (- 43.52)
P/E ratio (Mean ± S.E.)	(1.04-) 1.07±0.007 (-1.11)
Exine size (μm) (Mean ± S.E.)	(2.81-) 3.27±0.11 (- 3.85)

Pollen fertility and pollen viability

Different tests were carried out for determining pollen fertility and viability of the selected species showed in the Fig. 2. Pollens of this species showed good colour to differentiate between fertile/ sterile pollens and viable/ non-viable pollens. Pollens of studied species showed fertility percentage of 95.4%, 94.32%, 94.51% in three fertility tests *i.e.,* Muntzing Mixture test, 2% Aceto-carmine test and Lacto phenol cotton blue test respectively. In two pollen viability tests *i.e.,* TTC test and FDA test, viability expressed by pollens of studied species are 87.23% and 82.02%.

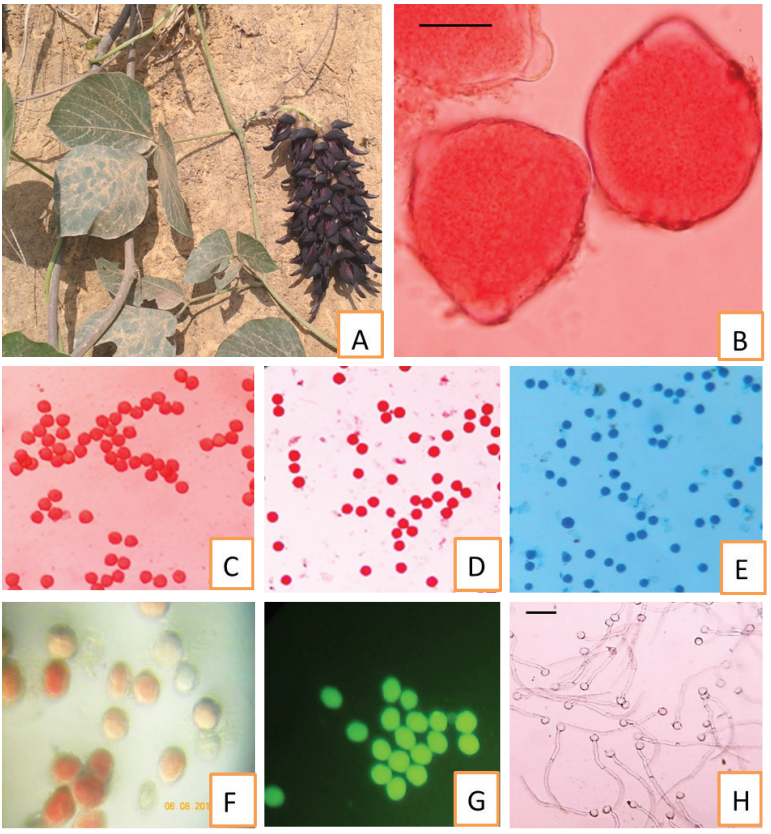

Fig. 1: *Mucuna bracteataDC. Ex Kurz (A) Flower bearing twigs; (B) Pollens; (C-H) Pollen germinations in ---(C) Muntzing's mixture technique, (D)2% Acetocarmine staining; (E) Lacto phenol cotton blue technique; (F) 1% TTC(2, 3, 5- Triphenyl tetrazolium chloride); (G)Fluorescein Diacetate;(H) Pollen germination25% sugar + 300ppm boric acid.*

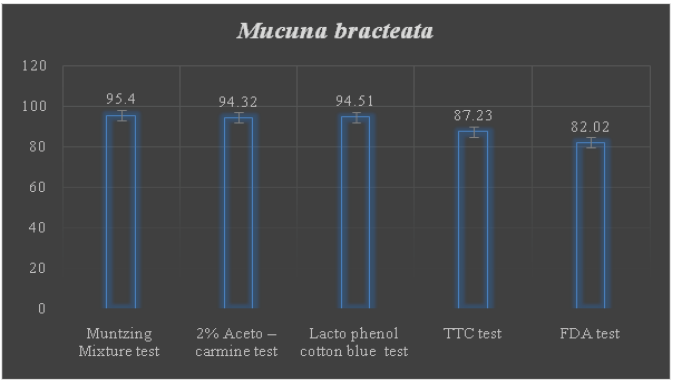

Fig. 2: *Pollen fertility and viability of Mucuna bracteata*

Pollen is like any living organism; its behaviour and survival are influenced by both environment and genotype. Therefore, pollen quality must be measured under defined conditions, and thought about experimental and plant growth conditions is always required. Pollen production and quality vary from hour to hour, day to day and season to season. Many species have strong diurnal rhythms- in the summer anther dehiscence may occur at 05:00am and pollen collected 12h later may be in viable[21-22]. The nutrition of the parent plant can also have large effects on pollen viability may affect results from the *in vitro* germination tests discussed below, more than growth on the stigma. Some pollen shows no viability in tests unless it is correctly preconditioned by, for example, leaving in a humid atmosphere before testing. For most species, the requirements for such preconditioning or post-maturation are not established, so cytological methods to estimate viability may give an unrealistic estimate of quality.

Most pollen samples have a few such non-viable grains, but the presence of many aborted grains indicates substantial infertility. The causes may include genetic sterility of hybrids, or severe environmental stress.

Stains are specific to pollen components can also be used to test for viability [23] However, immature or non-viable pollen sometimes contains enough cytoplasm and cellulose that cause staining, and viable pollen of some species does not stain well [24]. Therefore, stains provide (at best) only rough estimates of viability.

Pollen viability is taken to be an essential parameter of pollen quality [25]. In present observation, pollens of *Mucuna bracteata* flowers possess higher percentage in all pollen fertility and viability tests.

In vitro pollen germination

In vitro pollen germination of the selected taxa was carried out. The study revealed that *Mucuna bracteata* showed highest pollen germination in 25% sugar + 300ppm boric acid *i.e.,*77.92% ± 1.14 (Fig.3).

Highest pollen tube length in *Mucunabracteata* in 25% sugar+100ppm boric acid 405.37µm ± 4.96 (Fig.4)

In accordance with Pfahler *et al.*[26], the lack of fertility can be explained by the *in vitro* pollen germination and pollen tube growth. Studied species exhibit high pollen germination and pollen tube growth in sugar in combination with boric acid as well as sugar in combination with $CaCO_3$. This is attributed to the fact that sucrose is necessary for proper pollen nutrition, osmotic control and in combination with boric acid promoted pollen germination [27]. Boron may boost up the sucrose uptake and also trigger the germinating ability [28]. Gauch and Duggar [29] stated that boron associates with sugar to form a sugar-borate complex which is translocated with better

proficiency rather than non-borate sugars. It is also observed that the adding of $CaCO_3$ with sucrose solution outcomes in the rise of percentage of pollen germination as well as pollen tube enlargement. [30] established that in pollen ion channels regulated by Calcium-dependent kinases.

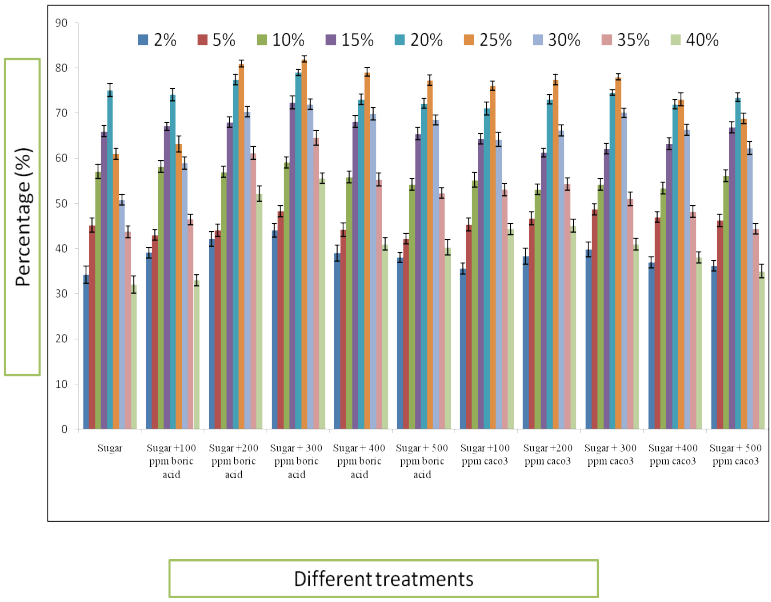

Fig. 3: *Germination percentage of pollens of Mucunabracteata in different pollen germination treatments(in vitro).*

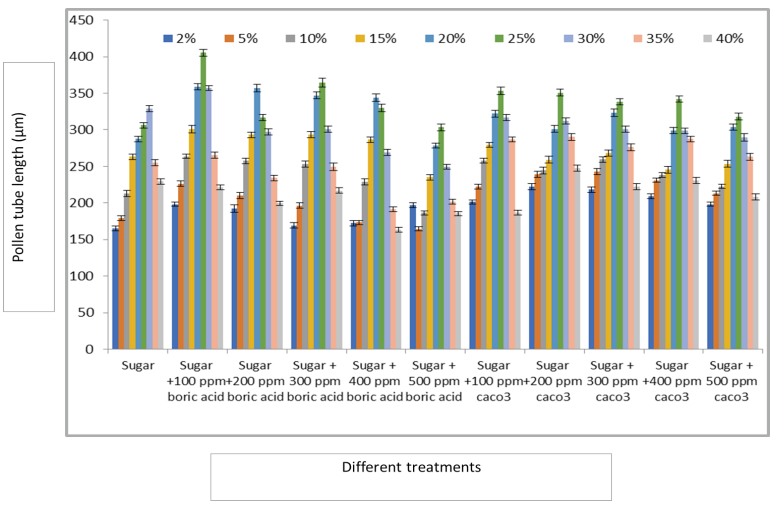

Fig. 4: *Pollen tube length of pollens of Mucunabracteata in different pollen germination treatments (in vitro).*

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CHAPTER

Study on anti-mitotic and genotoxic effects of herbicide pretilachlor using *Allium cepa* assay

Probal Kumar Chowdhury*[1] and Anirban Bhattacharya[2]

[1] Department of Botany, Govt. Degree College, Dharmanagar, North Tripura, India
[2] Dept. of Botany, Iswar Chandra Vidyasagar College, Belonia, South Tripura, India
*E-mail: probalkc@gmail.com

Abstract: Anti-mitotic and genotoxic activities of herbicide pretilachlor was studied using *Allium cepa* assay.Pretilachlor showed inhibitory effects on the root tip mitosis of *A. cepa* irrespective of concentrations and treatment durations which are indicated by the mitotic indices (MI). The herbicide also induced abnormality in the mitotic cells. Different types of abnormalities were clumping of metaphase chromosomes, formation of anaphasic bridge, early separation, laggard formation and formation of micronuclei. In case of control frequency of different stages of mitosis showed closeness, but in treatments frequency of prophase was visibly higher than other stages irrespective of concentration of the herbicide and treatment durations. This indicated the presence of some sort of prophase arrest in the cell cycle. Overall, pretilachlor showed anti-mitotic and genotoxic effects in the root tip cells of *A. cepa.*

Keywords: *Allium cepa*; pretilachlor; mitotic index;genotoxicity.

Use of synthetic pesticides including herbicides, insecticides and fungicides is a common agricultural practice carried out in order to increase the crop yield. Being usually non-degradable, these synthetic chemicals accumulate in the ecosystem leading to serious environmental problems. Besides, these chemicals often cause harmful effects on non-target species. Pretilachlor [2-chloro-N-(2,6-diethylphenyl)-N-(2-propoxyethyl) acetanilide] is a chloroacetanilide herbicide[1] which is sold under several brand names such as Rifit, Omit, Race, Craze, Pretigan, etc. This is used as pre-emergence and early post-emergence herbicide for the control of annual grasses and some broad-leaved weeds such as *Echinocloa crusgalli* and *Ischaemum rogosum* in the rice fields [1]. In recent years several workers have found harmful effects of different herbicides on non target species [2-4] including that of pretilachlor [1].

Higher plants are now frequently used for the assessment of genotoxicity and cytotoxicity induced by environmental pollutants and *Allium cepa* is a standard material for such studies because the chromosomes of *A. cepa* are large enough and its karyotype is also well known. Also protocol for the preparation of mitotic chromosomes of *A. cepa* is well established [5]. Aim of the present study was to find out the effects of pretilachlor on root tip mitosis of *A. cepa* and to determine probable genotoxic potential of the herbicide.

Materials and Methods

Preparation of pretilachlor solution

Pretigan (pretilachlor 50% EC) was purchased from local market and used as 100% stock solution of pretilachlor. From this 1% stock solution was prepared initially in distilled water. Later 1% stock was diluted to three different concentrations of pretilachlor (0.05%, 0.10%, and 0.20%) with distilled water.

Treatment of root tips and staining

Healthy Allium cepa bulbs of approximately equal size and weight were rooted in moist sand beds. Fresh root tips were collected and dipped into solutions of different concentrations of the herbicide for 2, 4 and 6 hours each. Distilled water was used as control. Treated root tips were fixed in 3:1 acetic ethanol for overnight [5]. Next day root tips were stained using standard aceto orcein staining method, squashed on 45% acetic acid and observed under microscope (10X eyepiece, 40X objective). Photographs were takes as required.

Recording of data

Total number of cells per microscopic field was counted. Also the number cells under different stages of mitosis (prophase, metaphase, anaphase and telophase) were recorded. From this total number of dividing cells was determined. Number of abnormal mitotic stages and their types were also recorded. Ten (10) observations were recorded for control as well as each treatment. Data were presented as mean ± SD. Mitotic index (MI) was determined according to the formula: MI= (No. of cell in mitotic stages/ total no. of cells counted) X 100 [5] and percentage of abnormal cells was determined according to the formula:% of abnormal cell= (No. of abnormal cell / total no. of dividing cells counted) X 100.

Statistical analysis

All statistical analyses were performed using Microsoft Office Excel 2007. Quantitative changes in different parameters were analyzed by analysis of variance (ANOVA).

Results

Effect on MI: Pretilachlor (PC) was found to have inhibitory effects on the root tip mitosis of *A. cepa*. Irrespective of the concentration of the herbicide and duration of treatment, the mitotic index values (MI) of *A. cepa* root tips were significantly lower than that of the control. (Table 1).

Table 1: *Effect of pretilachlor on mean mitotic index (MI) of root tip of A. cepa:*

Treatment	Duration of treatment		
	2h	4h	6h
Control (dH_2O)	33.36±1.84	32.21±1.86	32.84±2.04
Pretilachlor 0.05%	10.72±1.67[a]	7.12±1.68[a]	5.35±1.70[a]
Pretilachlor 0.10%	10.21±1.42[a]	6.59±2.19[a]	5.22±2.01[a]
Pretilachlor 0.20%	8.15±1.50[a]	6.03±1.67[a]	4.93±1.45[a]

*Each value represents mean ± SD ** values followed by same letter differ significantly from control

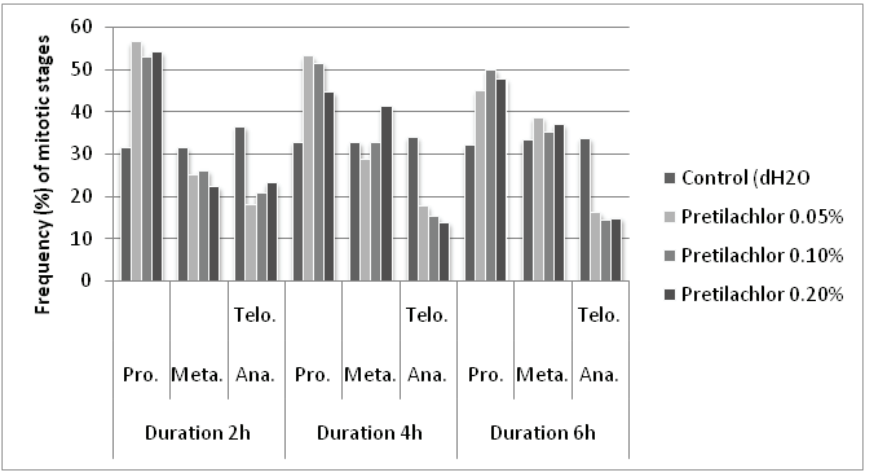

Fig. 1: *Graphical representation of frequency (%) of different stages of mitosis in root tip of A. cepa in respect to total dividing cells under the treatment of pretilachlor:*

Effect on frequency of abnormal mitotic stages

As compared to the control, pretilachlor induced significantly higher levels of abnormality in the mitotic cells of *A. cepa* root tips. (Table 2). Different types of abnormalities that were observed include clumped metaphase, anaphasic bridge, early separation, laggard formation and micronuclei formation (Fig. 2).

Table 2: *Effect of Pretilachlor on mean percentage of abnormal mitotic cells in root tip of A. cepa:*

Treatment	Duration of treatment		
	2h	4h	6h
Control (dH_2O)	6.46±0.25	7.66±0.22	7.51±0.17
Pretilachlor 0.05%	12.29±0.07[a]	13.11±0.48[a]	13.73±0.38[a]
Pretilachlor 0.10%	14.74±0.11[a]	15.41±0.08[a]	16.00±0.53[a]
Pretilachlor 0.20%	17.12±0.06[a]	19.33±0.04[a]	21.62±0.17[a]

*Each value represents mean ± SD ** values followed by same letter differ significantly from control

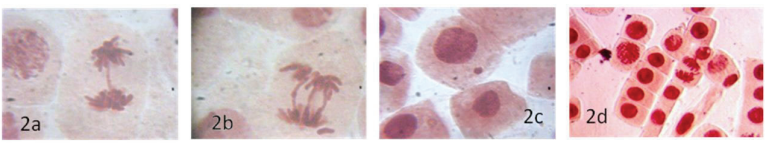

Fig 2: *Abnormality in mitotic cells of A. cepa – (2a) single anaphasic bridge formation, (2b) multiple anaphasic bridge formation with early separation, (2c) micronucleus formation, (2d) anaphase with laggard chromosome.*

Effect on frequency of different stages of mitosis

Fig. 1 shows the frequency (%) of different stages of mitosis under the treatment of different concentrations of pretilachlor for three different time durations. In case of control frequency of different stages of mitosis are very close to each other, indicating a synchronized pattern of cell division. In case of treatments frequency of prophase is visibly higher than other stages.

Discussion

Present study shows the anti-mitotic activity of pretilachlor in *A. cepa.* Mitotic index is a measure for the proliferation status of a cell population. It is define as the ratio between the numbers of cells in mitosis and the total number of cells. The mitotic index is a well known parameter that reflects the frequency of the cell division and thus offers an indirect assessment of growth of cells or tissues [5]. Reduced mitotic activity under the influence of herbicides has been noticed by several workers in *A. Cepa* [3,4,6] and other non target species [2]. Non target physiological effects of pretilachlor have been studied previously [1]. But present work probably records non-target cytogenetical effects of pretilachlor for the first time. In pretilachlor treated *A. cepa* cells frequency of prophase is visibly higher than other stages. This indicates the probable prophase block caused by the herbicide. Similar type of effect has been recorded earlier [3].

Different types of mitotic abnormalities caused by the herbicideare clumping of metaphase chromosomes, formation of anaphasic bridge, early separation, laggard formation and formation of micronuclei. This type of effect under the influence of herbicide treatment is supported by earlier reports [1, 3, 4, 6]. Micronuclei result from

chromosomal fragments or whole chromosome lagging during cell division and micronuclei assay is an effective technique to assess the genotoxic damage occurring by environmental pollutant and toxic substances, such as pesticides, heavy metals, in both plant cells and animal cells [7]. The presence of dicentric chromosomes and unequally exchanged chromatids undergoing translocation has been reported to be responsible for chromosomal bridges at anaphase [8]. Stickiness leading to clumped metaphase is due to the inhibition of spindle formation[9]. Improper folding of chromosome fibres that makes the chromatids connected by sub-chromatid bridges lead to form sticky chromosomes[10]. Lagging chromosomes fail to reach the pole before the chromosomes relax to uncoil to form daughter nuclei and the either disintegrate or form micronuclei.

In conclusion this may be stated that exposure to herbicide pretilachlor may be harmful for the non-target species. Such exposure may result in slow or reduced growth due to anti-mitotic activity of the chemical and also may cause abnormal chromosomal behaviour leading to chromosomal mutations. This study further demonstrates the usefulness of *A. cepa* assay in assessing the anti-mitotic activity and genotoxicity of harmful chemicals such as synthetic herbicides.

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Cd(II) and Hg(II) metal complexes biological activity: A future perspective

Priyatosh Nath[1], Moushum Bhattacharyya[2], Abhijit Datta[3] and Suman Adhikari*[4]

[1] School of Health Sciences, The Assam Kaziranga University, Jorhat, Assam, India.
[2] Department of Human Physiology, Govt. Degree College, Dharmanagar, Tripura, India.
[3] Department of Botany, Ambedkar College, Fatikroy, Unakoti, Tripura, India
[4] Department of Chemistry, Govt. Degree College, Dharmanagar, Tripura(N), India.
**E-mail : sumanadhi@gmail.com*

Abstract: Therapeutic function of metal based complexes is still an untouched area of study and may be helpful in the development of novel drugs. These complexes find application as potential drugs in neurodegenerative diseases, as well as chemotherapeutic, antimicrobial, antifungal and anticancer agents. In the field of medicinal chemistry, interaction between metal drugs and metalloproteins as drug targets is a new area of interest. The design and synthesis of novel metal-based drugs is still a challenge of the new millennia. The novel metal based drugs, may lead to future generations of medicines that can overcome few shortcomings related to current drug therapies, together with lessening of side-effects, broad spectrum of action, and resistance. The toxic effects of cadmium and mercury to the human body are well known. However, complexes of these two metals with other molecules or groups were found to acts as effective antibacterial and antifungal agents against many micro-organisms. These two metal complexes also show promise as potential anti-oxidants and provide evidence to protect the cell from oxidative stress damage. Design and application of chelating agents to eradicate the toxicity of these two metals, are key challenges of bioinorganic and medicinal chemistry. In this review we will discuss about the recent advancements of primary bioinorganic phenomena associated with metallo-organic interactions which will help in the future enormously and also provide an insight towards development of novel cadmium and mercury complexes that would help in identifying compounds with zero toxicity successful therapeutic efficacy in the future.

Keywords: Metal-complexes; medicinal chemistry; cadmium and mercury

The metal-based drugs were used since the early days of civilization [1]. Metal-based compounds have been found to have wide use in ancient Indian medicine. In Egypt, during ancient times copper was utilized for sterilization of water. There is also

evidence that the famous Greek physician Hippocrates used mercury as early as in 400 BC. The onset of the 20th century noticed an impact of metals on present day medicines through discovery of an organo-metallic drug Salvarsan by Erlich which is based on Arsenic for syphilis treatment. Further metal-based treatments meant for contagious ailments using Antimony compounds to treat parasitic disease like lesihmaniasis as well as gold cyanide for tuberculosis were also discovered. The present review was attempted to discuss about the different types of metal complexes formulated till date as therapeutics by various researchers with special reference to their structural, chemical and biological peculiarities. We also tried to review the efficacy of two toxic metals, cadmium and mercury, as potential antibacterial, antifungal and antioxidant agent and discuss about the formulations adopted by researchers towards development of novel cadmium and mercury complexes that could potentially exert a lesser amount of toxicity along with superior therapeutic efficacy.

Metal-based compounds have historically been extensively utilized in the treatment of disease disorders, but the main problem was a comprehensible difference between medicinal and harmful doses. But when Barnett Rosenberg discovered cisplatin (Fig. 1) in 1960, a breakthrough was observed in the development of metal-based compounds used to treat cancers.

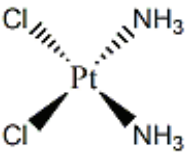

Fig. 1: *Cis Platin*

Types of metallic complexes in medicine

Macrocyclic metal compounds, transition metal complexes with Schiff-base ligand, metal terpyridine complexes, hydrazone derivatives, benzimidazole compounds have found wide applications in biology and medicine as antitumor, anticancer, antibacterial and antifungal agents. Macrocyclic natural products evolved to accomplish several biochemical roles. Moreover their intense pharmacological characteristics have guided to their advancement as pharmaceutical substances. Additionally, the variety of biological tasks performed by natural counterparts can be primarily concluded by the nature of the metal ions along with their detention in the enclosed pocket of natural macrocycles.

The Schiff bases have appeared as antimicrobial substance due to their wide-ranging in vitro as well as in vivo chemotherapeutic potential. Besides their broad spectrum applications in catalytic, industrial as well as analytical practices, they show

complexation with nitrogen and oxygen donor atoms and are employed as models for studying biomolecules as well as biological processes [2] in addition to their interactions with DNA [3]. Transition metal complexes with Schiff-base ligand are vital category of compounds in therapeutic as well as pharmaceutical advancement owing to their extensive variety of applications, that include, antibacterial [4] antifungal [5] and anticancer [6] properties. The interest in Schiff bases along with their complexes having O and N donor atoms is attributable to their noteworthy antifungal action[7,8] Amino-acid (L-glycine) reduced Schiff base complexes with cobalt, copper, and cadmium demonstrate oxidative DNA cleavage alone or in presence of H_2O_2, or sodium ascorbate [9]. Schiff base metal compounds were described as effective radical scavengers or antioxidants with their activity depending on the types of the Schiff base [10-12]. In clinical applications as well as biochemistry, functionalized terpyridines have found a ample variety of possible utilizations that ranges from colorimetric metal determination to DNA binding agents [13]. Metal terpyridine complexes because of the binding to nucleic acids possess the capacity to act like antiparasitic, antibacterial, and anticancer preparations [14, 15]. The specific mechanisms are unknown in a number of cases and may engage protein or membrane binding. It has been discovered and reported that the ligands with oxygen and nitrogen donor active sites can hamper production of enzyme[16]. The enzymes that allow such groups to function show increased susceptibility to deactivation via the central metal ions after coordination. Metal ion coordination has been reported to decrease polarity of metal ions also as Tweedy chelation theory [16,17] of the central metal ions as a result of donating of its positive charge through the donor atoms. The reaction of coordination helps to boost the lipophilic character of the metal ions that help its infiltration through the lipid layers of the cell membrane. This increase in hydrophobic behaviour along with liposolubility of the complexes bring about passing through the cellular membrane of the organism leading to cell respiratory activity disruption and restricts further organism growth [18]. The hydrazine derivatives demonstrated a broad spectrum of biological activities such as anticancer, antifungal, antitubercular anti-inflammatory, analgesic, antiplatelet, antimalarial, antiviral, antimicrobial, anticonvulsant, antidepressant, and cardio protective [19].

Cadmium complexes

The interaction between Cd(II) ion and biomolecules is one of the most researched areas in coordination chemistry. Although Cadmium is a toxic metal, it is extensively utilized in several industrial practices [20, 21]. Main organ of our body where toxicity of cadmium is noticeable is the kidney. The S1 section of the proximal convoluted tubule is the chief target of Cd deposition, with medically evident defects in the reabsorption of important nutrients and salts (Fanconi syndrome) which is the resultant of Cd-

induced oxidative injury to transport proteins along with mitochondria that possibly stimulate apoptosis of tubular cells [22-25]. Cadmium may be connected with impairment of metabolism of Vitamin D in the kidney as well [26], and subsequent injurious effect on bones. Cadmium(II) compounds are familiar human carcinogens [20] and may result in lower toxicity through their complexation.

Despite of the above mentioned toxic effects, cadmium complexes have shown some convincing therapeutic applications. Some schiff base cadmium compounds were reported to be active against *Candida albicans* or *Aspergillus niger* [27, 28]. Terpyridinecadmium (II) compound has been established to have strong antibacterial activity due to chelation and existence of I−anion in its structure [29]. Cadmium (II) complexes of N-dependent macrocyclic ligands have strong antibacterial activity [30]. The augmented activity in complex is not caused by any ionized Cd^{2+} moiety because the complexes reveal elevated stability. The amplified activity of the compounds in some cases compared to ligands can be elucidated by Chelation theory [31]. The possible mechanism is the disturbance in the respiration process of the cell and blocking of the synthesis of protein by the metal complex, which restricts further growth of the organism [32]. The schiff base Cd (II) complex has been reported to have strong antibacterial activities owing due to their increased lipophilic character [33]. The schiff base Cd (II) complex reported to have antioxidant and radical scavenging activities more in comparison to that of only ligands is attributed to the chelation of ligand with the central metal atom [33, 34].

The bovine serum albumin (BSA) and human serum albumin (HAS) protein binding activity of the Cd(II) compounds has been accomplished through absorption and fluorescence spectroscopic study. The schiff base Cd (II) complex binds with BSA and HSA proportionately with increasing concentration pointing toward the formation of a ground state complex [35]. The Cadmium (Cd) complex of Pyrimethamine-Ibuprofen was reported to have antibacterial and antimalarial activities [36]. Malarial animal model of mice induced with the organism Plasmodium berghei and subsequently treated with Cadmium (Cd) complex of Pyrimethamine-Ibuprofen showed percentage reduction in parasitaemia. The ligands and the complexes were compared which indicated that the complexes were additionally effective than their parent ligands. It could be a result of the coordination of the ligands to the metal ions which improve the effectiveness of the drug.

The Cd(II) complexes with 2-phenylamino-N-(thiophene-2-yl) methylene) acetohydrazide ligand also shows strong antibacterial and antioxidant activities[37]. Increased lipophilicity and chelation are responsible for most of the biological activities. The azomethine linkage present in these compounds possibly are the basis for their significant antibacterial actions [37]. A key factor in developing a complex

with distinct biological as well as pharmaceutical attributes is its capacity to interact with DNA. Cd(II) picrate complex with Bis (N-benzylbenzimidazol-2-ylmethyl) aniline (Bebba) demonstrated binding with DNA in an intercalation mode [38]. In view of above evidences it may be emphasized the cadmium complexes shows considerable promise as potential antibacterial, antimalarial, antifungal and antioxidant therapeutic agents. However the dangers of cadmium toxicity in these complex formulation needs careful investigation and remains a matter of future speculation. Singh and colleagues reported a series of binary ligand based Cd(II) and Zn (II) complexes with potential antimicrobial activities [39]. They employed flexible polyamines and 1, 1-dicyanoethylene-2, 2-dithiolate ligands for metal ion coordination and explored the antimicrobial action of the synthesized complexes. The structural features of the complexes were well illustrated by X-ray crystallographic analysis.

Mercury complexes

Mercury as well as its compounds are a type of persistently deadly pollutant of global concern and reveal bio-accumulation because of manmade and natural emissions. It imposes elevated level of hazard to human as well as environmental wellbeing [40, 41]. Mercury is known to accumulate in human organs and has toxic effects on placenta, brain, intestine, kidney, liver, intestine and kidney [42].

In spite of its known deleterious effects on the human body, mercury complexes have shown promise in several medicinal applications The Hg(II) complexes acetohydrazide has been reported to have strong antibacterial activity [43]. Similarly schiff base Hg (II) complex found to have strong antimicrobial and antioxidant activity[44]. Thioether and thiosemicarbazone can bind to metals as neutral molecules or as anionic ligands after deprotonation, and can take up a number of different mode of coordination. Studies have reported strong antibacterial and antifungal activities with the mercury complexes from a pentadentatethioether ligand [45], mercury complexes of 2-Formylpyridine thiosemicarbazone [46] and hydrazinecarbothioamide complex[47]. These activities are due to increase in the lipophilic nature of the metal complex that supports its penetration via the lipid layer of the cell membrane of bacteria. Additional factors which increase the activity are namely solubility, conductivity along with bond length among metal and the ligand as well. In some cases metal complexes forms hydrogen bonds with the active sites of the cell constituents, that interferes with the the regular cellular functions[48]. N-heterocyclic carbenes (NHCs) interacts with DNA and binds in a sequence specific manner makes them suitable for use as spectroscopic probes, diagnostic as well as site-specific nucleic acid cleaving agents along with in chemotherapy [49, 50]. Rosenani *et al.*, also reported nuclease and DNA binding capabilities of Hg(II) N heterocyclic carbene complexes [51]. The above mentioned studies provide evidence on the advancement in development of new

mercury complexes and a significant progress in the synthesis of these complexes as therapeutic agents in the field of medicinal chemistry. Recently Adhikari and colleagues reported antimicrobial activity of neutral, non-polymeric bimetallic and trimetallic supramolecular architecture of Cd(II) and Hg(II) with polyamines and 1, 1-dicyanoethylene-2, 2-dithiolate dipotassium salt [52]. The synthesized complexes revealed wide spectrum with comparatively strain-specific antibacterial action in comparison to streptomycin.

Future Perspective

Since metals have been found to be toxic to the human body in general, hence, in order to make them more applicable as drug or medicine, we should primarily aim towards reducing their toxicity. By application of recent advanced scientific research and techniques based on bioinorganic chemistry in solving the challenges of toxicities associated with metal based agents, they can be used for excellent fungicidal agent in agriculture and poultry industry without any risk of bioaccumulation and food chain toxicity. Based on their diverse range of biological and pharmacological activities designing metal complex for selective targets will help us delivering drug particularly to the defective or diseased structure or part of the body without harming the healthy portion. Drugs targeting particular sugars, steroids, folates, or peptides which are over-expressed on the surface of cancerous or infected cells will help in finding novel drugs for various ailments. As most of the metals and their complex exhibits toxicity, future research should be more focused in alteration of structural features of the metal-based complexes to boost selectivity in addition to reduction of noxious consequences on normal cellular features as well as other activities.

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The science of taxonomy in India: Present challenges and future prospects

Kizhakke Veettil Radhakrishnan*[1], Rithi Majumdar[2] and Tanmoy Acharjee[2]

[1] Dept. of Fisheries Resource Management, College of Fisheries, Central Agricultural University (Imphal), Lembucherra, Agartala, Tripura, India
[2] Dept. of Zoology, Rabindranath Thakur Mahavidyalaya, Bishalgarh, Sepahijala, Tripura, India
*E mail: radhakrishnan.frm.cof@cau.ac.in

Abstract: It is estimated that only less than 10% of the the biological diversity of earth is so far been described. At the current pace, nearly 1000 more years will still need to name all the living beings; unfortunately, several hundreds of species may become extinct before we discover them with the growing human interventions with nature. While it is being increasingly recognized that taxonomy is the only tool for identification and evaluation of living organisms, there is a growing apprehension that interest in this important branch of science is rapidly declining worldwide. India is a mega-biodiversity country harbouring about a million species of living organisms, but fewer than 100,000 of these have been formally described. Guided by the United Nations' Millennium Development Goals, India agreed to quantify and protect the existing biodiversity. High quality taxonomic research is vital for poverty reduction through sustainable agriculture, forestry, fisheries, combating pests and human diseases and for sustainable national and international trade in biological products in our country. However, taxonomy has many confronting issues here, the subject itself is being considered as outdated and hence presently there is a serious dearth of scientists/researchers in this field. Students seldom show enthusiasm in taking up taxonomy as a career oriented subject. There are shortage of taxonomic centers, sophisticated repositories and libraries, lack of in-depth trainings, lack of field guides and authentic publications, insufficient funding, inter- and intra-discipline cooperation, and international collaborations. Contradicting results between different taxonomical techniques, struggle in publishing taxonomic work, and impoverished rules curtailing exchange of specimens between countries are other hurdles. Nevertheless, there is still a way forward. One of the best ways to popularize the science of taxonomy in India is, incorporating the relevant topics of the subject in the school curricula itself. Equally important is the need to develop awareness of this subject among people, particularly the younger generation. Train and support more taxonomic experts and establish more taxonomy centers, repositories/museums and libraries. Integrating different taxonomic methods to reach a consensus on results, publication of user-friendly taxonomic keys, field

guides and manuals, sharing information and consolidating taxonomic research among institutions in the country, promoting collaborations with international universities and other research bodies, co-operation with local Govt. bodies and NGOs for fast finishing the inventory works, regular monitoring and updating of bio-resources, amendments of acts and rules which limit the free scientific research, and sophistication of taxonomic techniques are suggested as solutions to boost taxonomic research in India. It is imperative on the part of the governments to approach the issues faced by this important scientific field in proper perspectives and vision with policy formulations, financial and infrastructural support.

Keywords: Taxonomy; challenges; future prospects; India

Taxonomy is the language of biology. The multitude of species occurring in nature are studied, classified, and named so that they can be identified in the future [1]. Taxonomy provides a working, conceptual framework enabling us to make biological comparisons and predictions on the properties of various taxa. Before starting any kind of studies, one need to know the correct scientific name of the organism and that name is a functional label using which various pieces of information concerning that organism, including all the past work done on it, can be retrieved and stored ensuring easy reference [2, 3]. Taxonomic research, therefore, has applications in other branches of biology as well as in the economy and well being of mankind [1].

The biological diversity of earth is estimated to harbour as many as 30 million species of which only 1.75 million have been described [2-5]. This includes a million species of animals and 0.5 million species of plants. We spent almost 230 years to find out this merely 10% of the existing biodiversity and it may take several thousand years still to identify and describe the remaining species. However, the mass destruction of habitats, especially forests in tropical countries are causing large scale depletions in populations and eventual extinction of many numbers of species. In order to know which species is endangered or threatened, we must know what they are and what we have to conserve. Here lies the importance of taxonomy. If the number of bona fide taxonomists is not increased from the present state and if the taxonomic research is not get accelerated, several hundreds of species may become extinct before we discover them [2]. The famous spokesman of conservation of world's biodiversity, Edward Wilson cautioned that "*if nothing more is done, one-fifth of all the plant and animal species now on earth could be gone or on the road to extinction by 2030*" [2].

Materials and Methods

The materials for this review are collected from various published literature on taxonomical research and its relevance in India. The knowledge and experiences gathered during taxonomic research of the authors for the past many years has greatly contributed in bringing out the results and discussion.

Results and Discussion

The conclusions and opinions derived from a synthesis of the published literatures are detailed and discussed below.

Status of Taxonomy in India

India is considered to be one of the mega-biodiversity centers of the world containing diverse ecosystems with many novel organisms [6]. The country holds only 2% of the World's total land surface but harbours over a million species of living species including 7.43% of the world's total of animals. Having said that, fewer than 100,000 of these species have been formally described and classified. As highlighted by Heywood [7], this precious natural bio-richness is vulnerable; most species are yet to be described, and the majority of the current extinctions are going unrecorded. Guided by the United Nations' Millennium Development Goals, India agreed to quantify and protect the existing biodiversity. High quality taxonomic research is vital for poverty reduction through sustainable agriculture, forestry, fisheries, combating pests and human diseases and for sustainable national and international trade in biological products without endangering indigenous plant and animal species in our country [8].

Present challenges for Taxonomy in India

While it is being increasingly recognized that taxonomy is only the tool for identification and evaluation of living organisms, there is also a growing apprehension that interest in this important branch of science is rapidly declining worldwide and so is in India, despite its mega-diversity status. The greatest threat is that taxonomy is considered as an outdated science that doesn't need the best of minds. Another is that taxonomy doesn't need hard work [9]. Field sampling for fresh specimens is essential for correctly identifying, but there are seldom researchers and students interested in that. On one hand, there is mounting pressure imposed through steep rates of species extinction due to disturbance and degradation of ecosystems and on the other hand, there is a serious deficiency of trained taxonomists, in the country [5]. It is important to reprioritize the focus on issues like targeting the neglected taxonomic groups, networking of people and conservation agencies, modern approaches in systematics and bioprospecting programmes for a better conservation strategy through a participatory manner. These activities will provide a better future for biosystematics in India [2, 6, 9].

The Convention on Biological Diversity (CBD) has recognized the existence of what is termed the "taxonomic impediment", a situation where there is scarcity of taxonomic information, constraints within taxonomic institutions and gradual decline in taxonomic expertise across all biological groups, which is limiting the implementation of the Convention [2]. CBD has formed the Global Taxonomic Initiative (GTI) as a means for overcoming this "impediment" and facilitate good biodiversity

management [2]. The various issues and problems faced by taxonomy as a science in India, in concurrence with the "taxonomic impediment" depicted by CBD are briefly dealt with below.

1. Dearth of bona fide taxonomists

As years pass by, we find that a generation of Indian taxonomists, who contributed immensely in the post-independent India, had retired and there are no suitable replacements [2]. These established stalwarts are leaving numerous 'orphan' taxa behind and with few students entering into the field of taxonomy [10, 11]. Seberg [12] stated that for describing all the existing taxa on earth, traditional taxonomy at the current rate will require nearly 1000 years. At no time has there been a greater need for taxonomists than now, when the biodiversity is in grave crisis. Unless serious steps are taken at the national level to reverse the situation, soon we will reach a stage where we will be compelled to approach foreign taxonomists to identify indigenous/common species of our backyard.

2. Shortage of taxonomic centers, sophisticated repositories and libraries

Taxonomic research in India now mainly confined to the specific institutions such as Botanical Survey of India (BSI), Zoological Survey of India (ZSI), etc. and only a very few authentic and large scale research in universities and colleges [6,13]. Unfortunately, most of our museums/repositories and libraries where rich and diverse fauna and flora kept and sometimes await discovery, lack adequate infrastructure facilities. For studying taxonomy of any group of animals or plants, all relevant literature, both old and new has to be secured. Even papers published 100 years ago are also important in taxonomic research. Taxonomic descriptions of tropical organisms published western journals are often not accessible and the back volumes of many journals such as zoological records, etc. are not available in most libraries.

3. Lack of in-depth Trainings

Short as well as long term courses should be offered to students and researchers for getting in-depth knowledge on the subject. For example, undergraduate and postgraduate students can be given different levels of assignments/projects on taxonomy under the guidance of teachers who have competence in taxonomy. Apart from that, short duration (preferable 1-3 months) courses on taxonomy can be organized regularly. Taxonomy should be introduced as a compulsory subject in the syllabi and curriculum of graduate and postgraduate students of life sciences. Besides, taxonomy can also be offered as a special subject at master's level as suggested by the University Grants Commission of India, 1990 [2]. A Field-oriented taxonomy project under the guidance of an expert taxonomist is one of the most effective ways to train budding taxonomists [14].

4. Lack of authentic publications, field guides and open access resources

Field guides are prerequisites for preliminary identification of flora and fauna and without authentic descriptions, monographs and revisions, it is practically impossible to confirm an identity. Interestingly, the rate of taxonomic publication has been increasing only slightly in the past few years [13]. It is important to increase the accessibility of resources needed by taxonomists to do high quality work (e.g. electronic libraries, hardware, etc.). Electronic mediums/platforms help taxonomists to deliver the product quickly and efficiently. The Biodiversity Heritage Library [15] is a wonderful example of how accessibility can move taxonomy ahead. Universities and research institutes should ensure free and open access to electronic tools and services for taxonomists to increase their efficiency.

5. Reluctance among the students

Taxonomy is a science of 'expertise by experience' and somebody has to undergo systematic practical identification trainings with equal wisdom from theoretical knowledge, to master in the technique. However, taxonomy has not been given the value and credit it deserves, by many scientists. The quantitative and qualitative deterioration in taxonomic training in our teaching and scientific institutions might have been due to many reasons. Manilal [16] is of the opinion that the current curricula is good enough but the taxonomy is taught by teachers who themselves are not taxonomists. Consequently their teaching would hardly inspire any student to develop a liking of the subject of taxonomy and the students, in general, would have no special liking to the subject of taxonomy. Taxonomy demands persistent hard work mostly in the form of difficult field work in inhospitable areas and the student will naturally opt a subject that he can study in comfort and also more fashionable in the modern society. Above all, a student will also think of the job opportunities that he would have after completing his studies. Being a subject that is considered as having no 'applied value', Taxonomy would not figure in his list of priorities [16].

6. Insufficient funding

Taxonomic research is relatively hectic and costly. It starts from rigorous sampling surveys over a considerably large geographical area. Usually such dedicated research works are carried out by a team, whose travelling and stay at remote places for longer days incur huge money. Unfortunately, majority of the scientific research projects are sanctioned by the authorities by making a comparison between practical application and the budget outlay. Though no research work can be started without correct species identification, taxonomy is still treated as a 'basic science' having no any practically utility directly to the end users, typically, the farmers. It is rare now to find national and state level bodies fund a pure taxonomic research work. It is important that the

funding agencies and universities earmark enough funds for taxonomic research. Students should be given scholarships/fellowships for taking up research in taxonomy. The financial load of maintaining taxonomic collections/specimens is another issue and average universities in India can seldom bear this. These collections are seats of permanent information storage and unique scientific records of biological diversity of organisms. Government should support universities in maintaining taxonomic collections [17]. Steps may also be taken to allocate enough funds for the national libraries to procure old research papers so as to reprint and republish them.

7. Inter- and intra-disciplinary cooperation

This is an emerging issue, not only concerned with taxonomy, but also many other science fields. It is important to encourage capacity-building and support access to and generation of taxonomic information, and strengthening of networks for cooperation between institutions who work on a broad objective. For instance, if the objective is to develop a database on flora and fauna of a particular geographical region, and different institutes are given task of bringing up results on specific taxa, the data could be shared between the institutes. In spite of many efforts and initiatives that contribute to institutional co-operation and collaborations, it is well-established that the results are still not encouraging. A study in these lines [18] found that the researchers involved in inter-departmental collaborations tend to be drastically more productive (by all considered productivity measures), collaborative (measured by the number of co-authorship relations) and institutionally important (in terms of the betweenness centrality in the co-authorship network) compared to those who collaborate only with colleagues from their own research departments.

8. International collaborations

Quality taxonomic research requires extensive collaboration and cooperation among specialists and institutions across continents, as the type specimens (original reference specimens) of even closely related species may be held in museums in different continents [19]. It is important to encourage national and international collaboration in taxonomic research and free exchange of specimens for basic, non-commercial research and promote fundamental research. Unfortunately, many a times we restrict ourselves to a small geographic area while describing a new fish species.

9. Barriers in publishing taxonomic work

At present, there are very few journals in the world and especially in the third world countries for exclusively publishing lengthy taxonomic papers such as monograph, revision or reviews. Valdecasas *et al.* [20] argued that the Science Citation Index (SCI) could have a detrimental effect on taxonomic studies, as science policy makers and bureaucrats routinely base their evaluation of particular scientist on citations.

Taxonomists will generally have a low SCI because they tend to publish in either smaller museum journals. The core journals that feature highly in citation indices accept taxonomic papers only under exceptional circumstances. Given the restriction over where one can publish a taxonomic paper, the SCI does not help in assessing how good a journal, taxonomic paper or taxonomist may be, as such indices do not signify the level of taxonomic or nomenclatural quality [13].

10. Impoverished rules

The Biological Diversity Act, 2002 seriously curtails the scientific freedom of individual taxonomists by putting impoverished measures and regulations on the free exchange of specimens for taxonomic research [19]. Too much stringent restrictions preventing collection and movement of specimens and type materials can have damaging implications on taxonomic research. Bona fide taxonomists must be permitted to collect and freely exchange specimens for taxonomic research, and every step should be taken to ensure that national and international legislation does not impede these activities of the taxonomists. The information housed in taxonomic collections all over the world should be made available to the countries of the origin of the specimens [19]. Hence it is very important to amend and include appropriate provisions in such rules for the growth of taxonomy in the country.

11. Contradictions between morphological and molecular taxonomy

Taxonomic research in our country remains mostly morphology-based. However, many researchers also pointed out the serious drawbacks of morphological tools and warned for over-depending on morphological methods. With the advent of modern science, there are innovative tools to do our work faster and better than ever before, including molecular methods [5]. However, there are many examples were morphological and molecular methods were not analogous to each other and this still remains as a puzzle.

The Way forward

One of the best ways to popularize the science of taxonomy in India is, incorporating the relevant topics of the subject in the school curricula. There is a need to present/package taxonomy as a potential career option and, even otherwise, popularize it for the benefit of general public, particularly the young generation, and furtherance of the science and its understanding. It also should be mentioned that, very recently, there are some new awareness has developed among many of the developed countries to intensify taxonomic research and take the subject forward [1]. Hopefully, the waves of the same would also be reaching developing tropical countries like ours, in the coming days. Nevertheless, it is important to reset our priorities and make an earnest effort to give due importance to research in Taxonomy [2]. The scientific community of the country

has already submitted a set of recommendations that needs immediate consideration by the concerned, to successfully achieve the targets of international treaties such as the Convention on Biological Diversity in India [8]. Some of the significant steps which could be taken to bring back the grace of the science of taxonomic in the country are listed below.

1. Establishing more taxonomy centers and repositories

It is very important to establish more national and regional centers of taxonomic research and development. Such centers should incorporate conventional and modern molecular, digital, and computational tools and approaches to taxonomy [2, 14]. They would facilitate the completion of inventories of the country's flora and fauna and compilation of people's biodiversity registers, train systematic biologists in national and international institutions of repute, offer basic systematic biology courses in the curriculum for undergraduate biology students, facilitate the use of information technology to organize and disseminate taxonomic data, and support biodiversity portals to engage civil society in collating highly dispersed but immense biodiversity information, and recognizing the need for specialist training courses for students and teachers interested in taxonomy the two organizations need to establish a series of training/diploma courses [14]. It also became a necessity to increase the number of repositories in the country for preserving innumerable type specimens and other valuable biological material for posterity at suitable locations.

2. Integrating taxonomic methods

There should be a shift from complete dependence of one method for taxonomic identification to an integrated approach wherein a consensus between different methods is made for taxonomic confirmation. Such integration should also incorporate a large number of characters derived through the advanced aspects of biology in our biosystematic research. Information regarding evolutionary aspects of the species under consideration should also be taken into account while selecting the methods. Above all, applications of several methods provide insights into the processes that make them separate species like divergent selection on some traits, behavior and consequent adaptation. Thus integrative taxonomy improves rigour contributing to efficient biodiversity inventorisation [21].

3. Publication of user-friendly taxonomic keys, field guides and manuals

The outcome of a taxonomic research must be published with user-friendly keys and people- oriented manuals in both print and electronic versions. Each taxonomic description should be accompanied with photographs of the species (preferably in live condition) and labeled diagrams of diagnostic features. The conventional checklists with lot of technical jargons and poor quality line drawings must be avoided. In the

Indian bio-systematic literature the nomenclatural aspects were considered weak which also needs to be strengthened. Incorporate data on biogeography, ecological status, environment induced phenotypic changes, traditional knowledge of the people, chemical data (wherever it is applicable), wild relative status, other usefulness including its economic and ecological potentials to strengthen species information. Encourage publication of field guides to identify our flora and fauna to draw students and young people to the study of taxonomy, and to create awareness on the importance of biodiversity [6].

4. Consolidating taxonomic research in a collaborative mode

In India, the Botanical Survey of India (BSI) and Zoological Survey of India (ZSI) are the two main departments responsible for the survey, collections, maintenance/ storage and systematic/taxonomic research [6]. These departments also provide advisory service to the government and the public. There are several, R & D centers, national and state institutions of higher learning seriously carrying out biodiversity related studies and contributing to our knowledge on the rich bio-wealth of our country. A better cooperation and coordination among researchers at a local, regional and national level is very much needed for exchange of specimens and other vital information such as specific issues concerning the identification and systematic position of the taxa, etc. [6]. There is an urgent need for a consolidation of efforts on the taxonomic front, prioritizing the activities in the short and long-term levels and setting targets to achieve in the next five years.

5. Collaborations with international Universities and other research bodies

Encourage national and international collaboration in taxonomic research and free exchange of specimens for basic, non-commercial research and promote fundamental research in biology by suitably amending the Biological Diversity Act, 2002 [8]. For accurate generic and species determinations, it is essential to study specimens from across political boundaries and continents. Unless and until the type specimens are studied, the identity of the taxa concerned remains questionable. The guidelines being formulated by the Ministry of Environment and Forests and the National Biodiversity Authority should be viewed in this backdrop [19].

6. Co-operation with local Govt. bodies and NGOs

Many local bodies, both governmental and non-governmental, can assist in conducting sampling surveys, cataloguing and building up an authentic database on biodiversity of a particular region. One of the best examples is the State Forest Departments (SFDs). They are the custodians of the 'biodiversity-rich landscapes' of our country, having an established infrastructure both in the field and cities. Hence, the involvement of SFDs is highly essential in inventorying our biological wealth. The

SFD can serve as a nodal point where it can bring the specialists and interested people (or the associations or societies) together to carry out this task [6].

7. Seek alternatives and broaden areas of research for publication

Taxonomy, like many other fields, becoming is increasingly multidisciplinary and taxonomists need to keep up with the evolution of the discipline, gaining competence in molecular methods, interactive databasing and identification keys, dissemination of data over the Internet, etc. The budding taxonomy researchers are encouraged to consider alternative strategies for publishing their work and consider broadening their research scope to improve their competitiveness. Large monographic treatments are idealistic, but for training graduate students, several smaller publications, and publications in higher ranking journals, will usually be preferable when the time comes to apply for a job. Integrating descriptive taxonomy with other biological fields, such as phylogenetics, biodiversity conservation, molecular biology, ecology, and biogeography, can only improve the taxonomy-based products, gain access to high-impact publication venues, and improve the trainees' chances of employment and scientific perceptions [22].

8. Regular monitoring and updating of biological resources:

Producing a taxonomic checklist of a region is not the end point in conservation and environmental management. In fact a a taxonomist has much more bigger jobs and the biodiversity inventories must lead us to a sound bio-resource management practice for a meaningful conservation strategy, through participatory approach, by different key stakeholders, including the poor and neglected sections of the community living in and around the biodiversity-rich locations [6]. Bio-prospecting of natural resources is a viable answer to many issues related to biodiversity conservation. It is important on the part of Govt. agencies to ensure necessary funding and facilities for members of the local bodies/societies/conservationists to continue regular field studies and monitoring to update the status of bio-resources in and around their area [6].

9. Amendments of acts and rules

The guidelines being formulated by the Ministry of Environment and Forests and the National Biodiversity Authority should be reviewed. It is essential to study specimens from across political boundaries and continents. Bona fide scientists/ researchers should be allowed to exchange and examine specimens across the globe for taxonomic confirmation, keeping all the bio-ethical and intellectual right formalities existing in the country. Unless and until the type specimens are studied, the identity of the taxa concerned will only remain questionable.

10. Sophistication of taxonomic techniques:

Under the present circumstances, it is very essential that the traditional taxonomic community in India should incorporate modern tools to speed up the process of describing and identifying species [5]. In this way only the taxonomy in India can survive and flourish in the twenty-first century. Modern techniques such as molecular systematics should be used more extensively. Over the past decade several renowned taxonomists and a number of organizations at both national and international levels entrusted with cataloguing biodiversity (e.g. BioNET and INBIO) have added their voices to the rising demand for computer aided taxonomy (CAT), and now the biological community is gifted to use tools like DAISY, SPIDA [23, 24] and ABIS [25] for the identification of various taxa [5]. This is the era of internet and the taxonomic information is also heading toward a Web-based system. A virtual system for accessing morphological, audio and video data would be a fundamental step, because text-based descriptions alone will not deal with either the taxonomic hurdles or identification problems successfully. New identification tools can allow anybody to make identifications, and numerous well-illustrated interactive online keys are now in use for a variety of taxa and the software for such practices is being rapidly enhanced and easily accessible to all [5]. Automated identification of objects is a concept wherein a taxon is identified automatically, taking less time and effort [26]. In this age of information technology, taxonomic institutions need to capitalize on the capacities of the electronic medium to disseminate and widen the base of taxonomic knowledge. Development of modules for cyber-taxonomy is work/man intensive task and would need coordinated efforts on the national front [14].

Conclusion

The Senegalese conservationist Baba Dioum once said "*in the end we will conserve only what we love, we will love only what we understand, we will understand only what we are taught*". Just the wisdom of taxonomy can only help us in learning the biodiversity what we have and take decision on which species is endangered and which have to be conserved. At a time when human-induced species extinctions far outnumber the natural processes, it would be a sorry state of affairs if species were to disappear even before they are discovered, named and classified. In the present-day scenario where students from higher education Institutions has no enthusiasm in taxonomy and concomitantly these systems being able to produce far fewer schools of taxonomists, the task of reviving interest in taxonomy seems indeed very hard. There is an urgent need to train and support more taxonomic experts in order to discover and understand world's biodiversity. It is imperative on the part of the governments to investigate these issues in proper perspectives and vision and formulate appropriate policies for bringing back the science of taxonomy in India in its glorious stature, again.

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Multifaceted role of Arbuscular Mycorrhiza: A potential mutualistic rhizospheric mycosymbiont In : *Biological Sciences: Impacts on Modern Civilization, Current and Future Challenges* by *Anupam Guha* © New Delhi Publishers, New Delhi: 2021, 378-394. ISBN: 978-81-947417-9-4, DOI: 10.30954/ndp.bio.2020.37

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CHAPTER

Multifaceted role of *Arbuscular Mycorrhiza*: A potential mutualistic rhizospheric mycosymbiont

Kripamoy Chakraborty *, Dimpi Das and Panna Das

Microbiology Laboratory, Department of Botany, Tripura University, Suryamaninagar, Tripura, India
*E-mail:kripachakraborty@gmail.com

Abstract: Plant roots are colonized by the diverse group of microbes residing at plant rhizosphere and these microbes are considered as a critical component of terrestrial ecosystem. Mycorrhizal symbiosis may be considered as the most primeval known symbiosis on the earth where both the participating partners are benefited resulting in the formation of mutualistic approaches. In this type of symbiotic association, myco partner facilitates phyto partners to absorb the requisite minerals from the nutrient deficient sites and in returns plant furnishes the photosynthates to the fungus. In addition to nutritional support mycorrhizal association also ensures some other aspects such as resistance to phytopathogens and pest, alleviate drought and salinity stress, increase reproductive capability, improvement of stomatal conductance and water retention capacity, enhancement of foliar biomolecules and plant productivity and diversity, thus can be considered as the decisive components for ecosystem function and nutrient cycling.

Keywords: Mycorrhiza; Phytopathogens; Ecosystem; Plant productivity; Nutrient cycling

Mycorrhiza is a Greek word, where 'myco' means "fungus" and 'rhiza' means "root"[1]. Mycorrhizal symbiosis is widespread in natural ecosystem hence it is the norm rather than exception [2]. This symbiosis is the integral part of the natural ecosystem[3] and approximately 95% of all vascular plants have a mycorrhizal association[4]. Mycorrhizal habit has a long evolutionary history [5], possibly associated with the evolution of land plants [6]. Lewis [7] endorsed an evolution towards biotrophic nutrition has occurred in all the fungi capable of forming mycorrhizal associations. The mycorrhization process can be divided into distinctive steps, consisting of germinating spores, hyphae differentiation, appressorium formation, penetration of the host root, intraradical hyphae formation, intercellular growth along with developed external

mycelium (extraradical hyphae), and arbuscule formation, subsequently exchanging nutrients and carbohydrates between the host and fungus [8]. Arbuscular mycorrhizal fungi (AM fungi) protect the host roots from certain root pathogens [9] and improve water relations[10] especially under nutrient limitation and also metal toxicity condition have been reported [11]. AM fungi are associated with improved growth of host plant species due to increased nutrient uptake, production of growth promoting substances, tolerance to drought, salinity and synergistic interactions with other beneficial microorganisms [12]. The variability of effects of different AM fungi on host species and their assemblages have already been demonstrated [13]. AM fungi produce hyphae, vesicles, and arbuscules inside the root cortex, while the spores and sporocarps along with hyphae are produced outside the root tissues [14], arbuscules, which are considered the functional site of nutrient exchange [15]. AM morphology is distinguished into *Arum* type, *Paris* type, and Intermediate type [16]. *Arum* type of AM fungal morphology has been reported to be abundant in agricultural crops, whereas the *Paris* type is frequent in plants inhabiting natural ecosystems [17-20]. AM fungi, accounting for somewhere between 5 and 50% of the biomass of soil microbes [21]. These obligate mutualistic symbionts colonize the roots of the vast majority of plants, including most crop plants[22]. The AM association has been observed in 200 families of plants representing 1,000 genera and about 300,000 plant species [23] including most crop and pasture plants, in particular legumes and cereals[24], as well as in many tree crops [25-27].

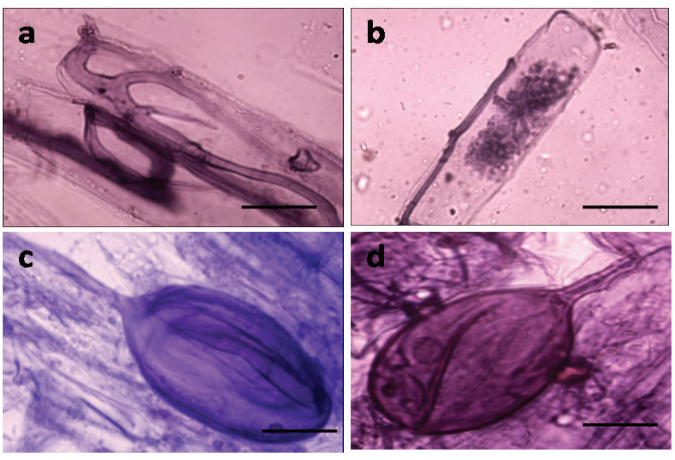

Fig. 1: *Light microscopic images of Arbuscular mycorrhizal colonization. (a) Root segments showing intra cellular hyphal coils in root portion, (b) Root segments showing Arum type of AM fungal morphology,(c) Root segments showing vesicles,(d) Root segments showing AM fungal spores. Scale bars (a-d=150µm)*

AM fungi are ubiquitous plant root symbionts that have been considered as 'keystone mutualists' in terrestrial ecosystems as they form a link between biotic and

abiotic components of ecosystems via carbon and nutrient fluxes that pass between plants and fungi in the soil [28]. According to the fossil record and molecular data, the origin of the AM symbiosis goes back at least to the Ordovician, 450–500 million years ago [29-30]. The taxonomy of AM fungi is based primarily on the morphology of their spores, which is now often supplemented with molecular biological data, the study of AM fungal communities and of their diversity is also based primarily on the retrieval of spores [31-34]. Traditional methods to identify AM fungi have for a long time been based on spores captured from soil, either by direct extraction or via so-called 'trap-culturing' with plants [35]. Bever *et al.* [36] proclaimed that a total of 37 taxa were detected by extensive trap-culturing of spores over many years from an old field. AM fungal spores are able to germinate in the absence of the host, but are unable to produce extensive mycelia and to complete their life cycle without establishing a functional symbiosis with a host plant. The lack of host-regulated spore germination did not represent a selective disadvantage, since AM fungi coevolved with heir host plants for more than 360 and 400 million years [29-30]. AM fungi being obligate biotrophs are unculturable in the absence of their host and depend on the plant for their viability and in particular for carbohydrates [37]. AM fungi are not host-specific [38], although there may be ecological specificity or selectiveness among symbionts [13].

AM fungi in plant productivity and diversity

AM fungal symbiosis is important for maintaining and promoting the productivity of crop lands and may be decisive to the maintenance of biodiversity [38-39] and such symbiosis is considered to be the primary determinant of plant health and soil fertility in terrestrial ecosystems [40]. AM fungi can play significant roles in the growth of plants in metal contaminated soils and in salt marshes [41]. AM fungal inoculation of soybean, corn, millet, trifoliate orange, rice and seventeen tropical legumes has demonstrated an improved economic impact on agriculture and horticulture [42-45]. The application of AM fungi facilitates the growth of plants by enhancing seedling growth and rooting of cuttings, reducing phosphate and nitrate requirements, increasing survival rate and development of plantlets, increasing resistance to abiotic stresses, increasing flowering and fruiting, and by increasing crop uniformity [46]. AM fungi may be considered as indispensible biotechnological tools with regards to augmentation of plant productivity and diversity.

AM fungi in nutrient acquisition and improvement of soil fertility

The health of soil is crucial for sustainable development although its health is deteriorated with various anthropogenic disturbances resulting in the decrease of agricultural soils fertility [47]. AM fungi, the obligate symbiont depend on plant roots and prevent the plants from the micro-organisms attack especially where there is

limiting supply of phosphorus (P) [48]. Glomalin deposited in the neighbouring walls of AM fungal extraradical hyphae and soil particles is the best-known soil adhering component[49]. The consequence of this adherement is the development of a sticky-string like bags that play a climacteric role in the entangling the soil particles resulting in the formation of a unique structure known as "macroaggregates", the basic element of soil structure formation [50]. These aggregations are the influential elements of soil carbon production of [51]. There is a progressive activity of AM fungi at the site of soil toxicity [52-53] and restoration of soil [54]. AM fungi may be used as a cursor of changing soil pattern and soil quality [55-56]. AM fungi intensify the plant survival rates in polluted areas by means of availing proper resistance, improving bioremediation and depleting cesium uptake in plants [57]. AM fungi scale down the environmental risk factors by attenuation of detrimental soil conditions [58]. AM fungi result in improvements in plant fitness and nutrition [59]. The level of AM fungi in soils and their efficacy decreases with soil degradation, pollution or over fertilization and high soil P fertility and/or fertilization, the use of systemic fungicides, and the use of nonmycotrophic or low mycotrophic crops in rotation have an adverse effect on mycorrhizal symbiosis [60]. AM fungi can respond to localized sources of soil nutrients by hyphal proliferation[61] and it is often assumed that AM fungal hyphae take up nutrients along their entire length[62]. AM fungi are able to deliver P which would otherwise not be directly accessible for the plant [63]. The preferred form of phosphorus taken up by plants is ortho-phosphate[64]. The hyphae may have a much more efficient biochemical pathway of incorporating inorganic ortho-phosphate followed by transfer into the vacuole and transport towards the root, thereby avoiding a negative feedback of increased internal P status on P uptake[65]. AM fungi successfully and effectively transport orthophosphate besides nitrogen, zinc, and other nutrients [66]. Nitrogen is synthesized and stored in the extraradical mycelium in the form of arginine and is transported to the intraradical mycelium in host cells following its generation from arginine breakdown [67]. AM fungi enhance the uptake of phosphorus, but they also contribute to the absorption of other immobile ions, such as zinc and copper [68]. AM fungal applications could increase plant soluble sugars contents [69] that do not only act on the metabolic activities of the host but also as a signal regulators in processes related plant growth and development[70]. AM fungal association can significantly reduce the uptake of Al^{3+} by roots and its translocation within plants [71]. AM fungi may enhance the reabsorption of nutrients lost through root exudation [72]. Zinc (Zn) is considered as an essential micronutrient associated with growth and yield of the plant [73]. AM fungi have an immense role in Zn uptake and reduction of Zn toxicity resulting from high accumulation [74]. The AM fungal hyphal network takes active participation in Zn transportation to the host plant. Hence there is a great influence of AM fungi in uptaking zinc present in the soil. The necessity of Molybdenum (Mo) in the plant was first piercing out by [75] from

Tomato where Mo deficiency of lead to molting lesions in leaves and alteration of leaf morphology such as lamellae became involuted and a peculiar phenotype occurs known as "whiptail". AM fungi also delivered the Mo co-factor present as a transitional micronutrient through a mechanism mediated by vacuoles that are hypothesized to serve as the carrier of this complex [76].

AM fungi in alleviating drought stress

Plant-symbiosis with AM can improve overall plant growth by improving root length, leaf area, plant biomass, and nutrient uptake under drought condition [77-78]. AM symbiosis resulted in a greater leaf water potential, improved gas exchange, increased stomatal conductance and transpiration and photosynthetic rates in mycorrhizal plants under drought [79-80]. Regarding abiotic stress, several studies for years have demonstrated that AM symbiosis confers tolerance to drought [81]. In Junggar Basin, the majority of desert ephemerals form mutualisms with AM fungi [82-83], and mycorrhizal colonization increased plant growth, nutrition uptake, productivity and community restoration [84-85]. The possible reason is AM fungi can help host plants to uptake enough water for their growth even under drought condition because AM fungi can increase the absorption area of hosts [86]. AM fungi improve physiological processes and general metabolic activities of the plant and help in the mitigation of physiological drought, which is often imposed under saline conditions [87].

AM fungi in improvement of salt tolerance

AM fungi, are a normal part of the root system in most natural and agro-systems including stressed soils [88]. They can be found even under severe saline conditions in nature, both in saline inlands and coasts [89] and in salt marshes [90]. AM fungi have been found to improve salt tolerance in different plant species [91-92]. In addition to improved mineral nutrition, the involvement of AM fungi in improving plant photosynthetic capacity, stomatal conductance, root hydraulic conductivity, water use efficiency, accumulation of enzymatic and nonenzymatic antioxidants, compatible organic solutes (help in detoxification of damaging reactive oxygen species), and osmotic adjustment (protect integrity of cell membrane and organelle and stabilize proteins) has been evidenced in AM plants growing under salinity stress[93-95]. Improved nutrient acquisition, particularly P by AM fungi, could ascribe to be the primary mechanism by which mycorrhizal fungi mitigate the adverse effects of salinity stress on plant growth [96]. Increased photosynthetic rates and stomatal conductance in AM fungal compared to non-AM fungal plants under salinity stress [97]. The increased rate of photosynthesis in AM fungi-colonized plants under salinity stress has been correlated with the lower intercellular CO_2 concentration in mycorrhizal plants, since the higher photosynthetic capacity increases water use efficiency for the assimilation of more carbon per unit water transpiration [98].

AM fungi as proficient bioprotectant

AM fungi and their associated interactions with plants reduce the damage caused by plant pathogens [99]. AM fungi are a major component of the rhizosphere of plants and may affect the incidence and severity of root diseases [100]. The AM inoculated plants possess a strong vascular system, which imparts greater mechanical strength to diminish the effects of pathogens [101]. Phenolic compounds have been shown to be formed after mycorrhizal colonization [102] and are thought to play a role in disease resistance [103]. Improvement of phosphorus nutrition following AM colonization of phosphorus-deficient roots results in a decrease in membrane permeability and reduction in root exudation [104]. AM inoculation increased the quantities of sugars and amino acids in plant tissue which may be responsible for the reduction of nematode infestation [105].

Reduction of soil erosion and nutrient leaching by AM fungi

The rate of soil erosion has been accelerated through various human activities at a global scale [106] that imparted negative effects including loss of topsoil, decrease in soil organic matter and pollution of surface water [107]. Soil erosion is related to the susceptibility of soil to both detachment and transport of soil particles[108]. Vegetation biomass has been acknowledged to play a pivotal role in decreasing soil erosion [109]. It is postulated that soil biota indirectly decrease soil erosion through the formation and stabilization of soil aggregates[110]. The root inhabiting AM fungi are known for their rule in increasing the soil aggregation[111]. AM fungi promote soil aggregation processes by various mechanisms on different hierarchical levels [110]. Two key mechanisms are the physical stabilisation through entanglement of soil particles by fungal hyphae and chemical stabilisation by glue-like fungal exudates [112]. The AM fungal hyphal network endorsed plant growth and root system development [113] that protects the soil from erosion by wind and water. AM fungi also increase the surface roughness lead to decrease in near-ground wind velocities [114] which in turns reduce soil erosion. Glomalin, a soil glycoprotein produced by AM fungi was identified as an additional important agent in the stabilization of soil aggregates [115]. It is known for its extractability and immuno-reactive properties[116]. Existing literature survey revealed the fact that inoculation of mycorrhizal propagules associated with the improvement of soil physical, chemical and biological properties resulting in the enhancement of the establishment of vegetation in degraded environments[117-118]. Studies under experimental condition have shown that mycorrhizal fungi improve the soil resistance to water erosion and the fraction of water stable aggregates [119]. Surface soil erosion can be decreased through microtopog- raphy (surface roughness) and soil cohesion due to a dense root mat[120]. A considerable amount of N and P fertilizers is lost from agro-ecosystems via leaching, causing serious groundwater pollution and eutrophication[121]

This losses can be regained by the application of AM fungi as they have decisive role in nutrient cycling[122]. Works from different groups of workers suggest that AM fungi are capable of reducing P losses via leaching[123-124]. In addition AM fungi have the ability to assimilate N in different forms [125] and reduce the losses of ammonium (NH_4^+) and nitrate (NO_3^-) from the ecosystem [126] although their impact on decreasing mineral N losses is variable[126]. The ability of AM fungi to decrease N losses has been pointed out by several workers [127-128] and it was proclaimed that AM fungi can retain nutrients much higher in systems with high N losses [128]. AM fungi enhance the nutrient interception ability of soils and decrease the nutrient leaching risk by enhancing nutrient uptake and immobilizing nutrients [129]. AM fungal hyphae improve soil structure by stabilizing soil micro- and macro-aggregates [50] and this is another way that can affect soil water relations and leaching volume. The effect of AM fungi on P losses is especially relevant for sandy soils where P loss can be substantial [130-131]. In other soils, P leaching losses are often very low and P loss through surface run-off is much more important [132]. Auge [133] showed that AM fungi improve soil moisture retention in a sandy soil. Thus, AM fungi have a direct and indirect impact on the retention of the amount of nutrients lost from the soil.

AM fungi enhance foliar biomolecules and plant growth regulator content

AM fungi residing at soil rhizosphere are the most promising soil mycobiota associated with the betterment of plant growth and yield. Reports are available on their impact on the improvement of secondary plant metabolites along with the medicinally important compounds. Many studies have shown that application of commercial AM fungal inoculum benefits crops under agricultural conditions [134]. Numerous studies have shown that AM fungi can increase plant health and yield [135-136]. AM fungi interfere with the phytohormone balance of host plants, thereby influencing plant development (bioregulators) and inducing tolerance to soil and environmental stresses (bioprotector[135]. One important aspect of this is the promotion of root system development[137].

Significant increase in the biomolecules contents in AM fungi inoculated plants may be corroborates with the vegetative response to the fungal colonization [138], which may ameliorate the various metabolic processes mediated by better P and N absorption promoted by the symbiosis [138]. AM fungi also stimulate the photosynthetic activity through the enhancement of photosynthetic pigment levels and by the drain of carbon resulting in a higher export of triose phosphate to the cytoplasm; there is a larger activation of the Calvin cycle which results in a higher production of primary metabolites, which are precursors of secondary metabolism pathways [139]. Increase in photosynthetic rates is coupled with the storage and export of photosynthates at the same period [140]. The higher content of foliar carbohydrate in AM fungal inoculated plants may be due to

the increase in quantity and size of chloroplasts as reported[141-142]. The increased leaf protein content in the AM fungi inoculated plants may be due to the increased nitrogen and phosphorus content[143-144] which is attributable with the potentiality of AM fungal hyphae for supplying N to mycorrhizal plants through atmospheric N fixation[143] and increasing in the percentages of N, P in plant, organic acids added to soils increased the plant uptake of P from a water soluble P [145].The enhanced protein content may also be due to the presence of fungal proteins or post infectional stimulation of protein synthesis by AM fungi [146]. The plants with higher extent of mycorrhizal colonization have the higher phenolic contents which may be due to the reaction of the plant against mycorrhizal colonization[147]. Accumulation of phenolic compounds is associated with plant defense response against microorganisms[148]. Carotenoids originated from primary carbon metabolism[149], include lycopene and βcarotene found in the species that tend to be the most effective naturally existing radical sequesters for oxygen[150]. The AM fungi inhabiting in the roots stimulate carotenoid metabolism[151]. The enhancement in the carotenoid pigments content in AM fungi inoculated plants is associated with improvement of photosynthetic performance through enhancement of water and soil nutrient uptake [152].

Conclusion

The use of synthetic chemical fertilizers and unscientific anthropological practices over a longer period of time lead to the destruction of native soil microbiota leading to reduction of soil fertility which in turns trim down plant productivity and diversity. To tide over with such difficulties, plant and soil scientists are in search of potential alternative and soil microbiota especially AM fungi can serve as indispensable bio resources capable of increasing the plant growth and yield. AM fungi belonging to monophyletic group Glomeromycota possess potent activity on the improvement of soil nutrient status and thus assist in the nutrient acquisition of the native plant communities in available form from nutrient deficient sites through mutualistic symbiotic association. AM fungi also help in various physiological processes of associated plant growth and yields and also act as a potential bio-control agent by improving the plant resistant capability against certain phtytopathogens and pests.AM fungi are also capable of mitigating certain abiotic stresses such as drought and salinity stress. Thus, AM fungi may be considered as admirable biotechnological tools for the promotion of plant productivity and diversity.

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CHAPTER

Decoding the role of inflammation in lung cancer development

Debasish Maiti* and Debabrata Majumder

Department of Human Physiology, Tripura University (A Central University)
Suryamaninagar, India
*E-mail: debasish.maiti@tripurauniv.in

Abstract: Lung cancer is the leading cause behind cancer related deaths and is responsible for one-quarter of all cancer deaths. The pulmonary diseases that are associated with the greatest risk for lung cancer are characterized by abundant and deregulated inflammation. Pulmonary disorders such as COPD are characterized by profound abnormalities in inflammatory pathways. Inflammation predisposes all that required to transform the cells in airways and alveoli into cancer cells. Inflammation is required to promote the survival of cancer cells in tumor microenvironment and apart from that it also helps the tumor cells to evade the immune response and also reduces their response towards chemotherapeutic drugs. Two important transcription factors viz. NF-κB and Nrf2 are involved in inflammation induced lung cancer development. Carcinogen activated NF-κB in lung tissue is reported to induce the expression of various inflammatory cytokines as well as matrix metalloproteinases and finally NF-κB facilitates lung carcinogenesis positively. Inflammation also required for epithelial–mesenchymal transition (EMT) of lung tissue cells. But less information is available about the molecular mechanism involved in this. So in this review the connection between inflammation and lung cancer development will be highlighted.

Keywords: Lung cancer, Inflammation, Cyclooxygenase, NF-κB, Oxidative stress.

Cancer is the uncontrolled proliferation of transformed cells which are capable of infiltrating into normal tissues to destroy their function [1]. After cardiovascular diseases, cancer is the second cause of deaths worldwide. Among the different forms of cancers lung cancer remains the front runner of all cancer related deaths [2]. Lung cancer can be characterized into two major groups namely small-cell lung cancer (SCLC) and non-small-cell cancer (NSCLC). NSCLC is reported to spread slower than SCLC and if a patient of NSCLC is diagnosed at an earlier stage, is potentially curable [3].

According to Colotta *et al.*, one of the important hallmarks of cancer is persistent inflammation [4]. Chronic inflammation in tumor microenvironment is responsible for the increased survival of cancer cells. Inflammation not only promotes the survival of cancer cells in tumor microenvironment, it also helps the tumor cells to evade the immune response and also reduces their response towards chemotherapeutic drugs [5].

The major cause of lung cancer development is cigarette smoking and consumption of tobacco products [6]. The tobacco smoking up-regulates the recruitment of inflammatory cells in the lung tissue which further increase the production of reactive oxygen or nitrogen species (ROS and RNS) in the lung tissue. The increased ROS and RNS may bind to DNA and thus lead to genomic alterations which is the main requirement for cancer initiation. Apart from this, inflammation increases the level of different cytokines and growth factors in lung tissue that are required to support the initiation and promotion of lung cancer [7].

Two important transcription factors viz. NF-κB and Nrf2 are involved in inflammation induced lung cancer development [8]. Carcinogen activated NF-κB in lung tissue is reported to induce the expression of various inflammatory cytokines as well as matrix metalloproteinases and finally NF-κB facilitates lung carcinogenesis positively. NF-κB is also reported to induce the survivality and proliferation of mutant cells in cancer microenvironment [9]. On the other hand, Nrf2 is known to regulate the expression of various cytoprotective genes in response to xenobiotic stress [10]. Smoking is reported to suppress the expression of Nrf2. During reduced expression of Nrf2, benzo(a)pyrene (BaP), a carcinogen, increases ROS level in lung tissue to exaggerate the pathogenesis of lung carcinogenesis [11]. Some popular anticancer drugs like paclitaxel, vinblastine etc. are reported to induce NF-κB which contributes resistance to anticancer therapy in lung cancer treatment. From the available data it can be concluded that smoking's non-genotoxic mode of action associated with lung carcinogenesis is mainly dependent of activation of NF-κB and inhibition of Nrf2. So identification of phytochemical which can inhibit NF-κB and also up-regulate the Nrf2 will be a breakthrough in lung cancer treatment.

Epidemiology and Etiology of Lung Cancer

According to World health organisation (WHO) lung cancer is the second most common cancer in men and women around the world. Almost as many people die of lung cancer every year than die of prostate, breast and colon cancer combined (https://www.who.int/news-room/fact-sheets/detail/cancer). A relative increase is observed in the numbers of cases of lung cancer in developing countries. Approximately half (49.9%) of the cases now occur in developing countries whereas in 1980, 69% of cases were in developed countries. The estimated numbers of lung cancer cases worldwide has

increased by 51% since 1985 (a 44% increase in men and a 76% increase in women). The rate of lung cancer incidence varies around the world, reflecting geographical alterations in tobacco consumption and air quality [12]. The rate of lung cancer incidence in female is increasing Worldwide [13]. For instance, female lung cancer incidence in Europe has been rising for most of the 21st century and in 2017 exceeded breast cancer mortality rates for the first time, 14.6 lung cancer deaths per 100,000 compared with 14 per 100,000 for breast cancer. Indoor air pollution and occupational exposures play a greater role in female lung cancer in some regions, particularly Asia [14]. Similar to the US, there is significant geographical and ethnic variation in lung cancer incidence and mortality within regions.

The extent of the effect of cigarette smoking is greater than all other factors leading to lung cancer [15]. An increase in cigarette smoking as well as consumption of tobacco products has been closely linked to the rise in the incidence of lung cancer. According to the WHO, each year near about 3,000,000 people die worldwide as a result of smoking. The risks of dying of lung cancer are 22 times higher for male smokers and 12 times higher for female smokers than for people who have never smoked [16]. As the duration of smoking and the number of cigarettes smoked per day increase, the risk for lung cancer increased accordingly.

However, Smokers are not the only individuals who are susceptible to lung cancer development. Passive smoking from environmental tobacco smoke also increases the risk of lung cancer deaths. As per the reports of Environmental Protection Agency, each year about 3,000 non-smoking adults die of lung cancer as a result of breathing the smoke of others' cigarettes [17].

Older people have a greater probability of developing lung cancer incidence. Independent of sex, race, and histology the percentage of patients with advanced-stage disease increases with age. As other health related problems increase with age, this fact heightens the risks associated with treatment [17].

Persistent Inflammation in Lung and Cancer Induction

Lung cancer is the result of mutational events taken place in the epithelia cells lining the respiratory tracts and alveoli [18]. However, the molecular pathways related to the lung cancer development are still unclear. Till date various studies linked the persistent inflammation in lung taken place due to infection and smoking with lung carcinogenesis[19]. Inflammatory cells along with stromal cells in the lung create a micro environment in lung which facilitates lung carcinogenesis (Fig. 1). The pulmonary diseases which are associated with abundant and deregulated inflammation are known to facilitate lung cancer [20-22]. Pulmonary disorders such as Chronic

obstructive pulmonary disease (COPD) are characterized by profound abnormalities in inflammatory pathways [23, 24].

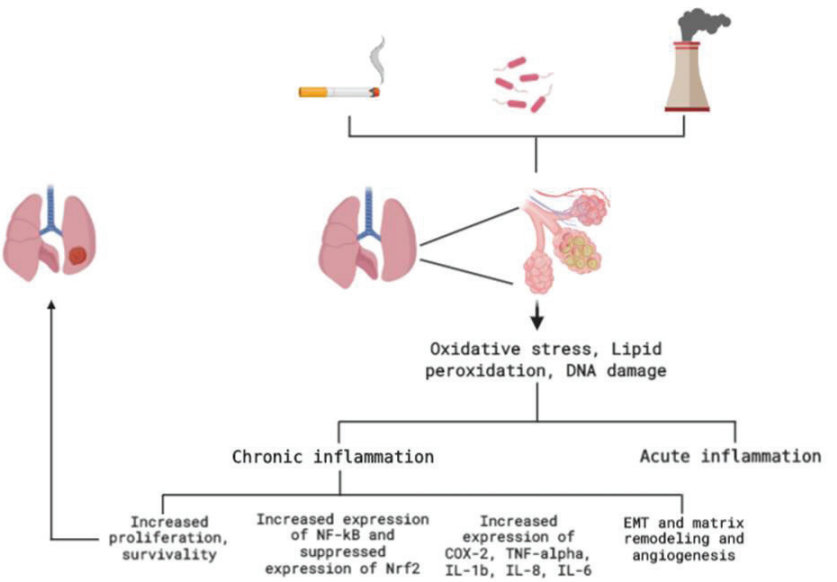

Fig. 1: *Inflammation induced changes in lung which facilitates various stages of lung cancer.*

Interleukin (IL)-1b, prostaglandin (PG) E2, and transforming growth factor (TGF)-b are among the cytokines, growth factors, and mediators released in these lung diseases and the developing tumor microenvironment which have been found to have damaging properties that concurrently pave the way for EMT and destruction of specific host cell–mediated immune responses against tumor antigens [25, 26]. The harmonies in smoking, COPD, and lung cancer begin with the profound changes induced by cigarette smoke, which comprises of known carcinogens as well as high levels of reactive oxygen species (ROS). The ready induction of ROS after tobacco smoke exposure leads to impairment of epithelial and endothelial cell function as well as inflammation [27]. When COPD progresses, the percentage of the airways that contain macrophages, neutrophils, T cells, B cells, and lymphoid aggregates containing follicles increases [27].

Inflammation in Lung Introduces Epithelial–Mesenchymal Transition (EMT)

The EMT ensues during typical embryonic development, tissue renewal, organ fibrosis, and wound curing. It is a highly vigorous process, by which epithelial cells can alter into a mesenchymal phenotype. However, it is also involved in tumor

advancement with metastatic growth, and the generation of tumor cells with stem cell assets that play a major role in insensitivity to cancer treatment [28, 29]. Whereas in cancer development and progression, EMT is unregulated with selective features of the course augmented while other aspects are circumvented [25]. The EMT process consists of the disruption of cell–cell adhesion and cellular polarity, remodeling of the cytoskeleton, and changes in cell–matrix adhesion. It is associated with advance in migratory and invasive properties [30]. Thus, EMT is operative in a variety of malignancies [31], including lung cancer. Recently the connection between inflammation and EMT progression in lung cancer development and resistance to therapy has been emphasized. Interleukin-1 beta (IL-1β), a proinflammatory cytokine, correlates with tumor progression in non-small cell lung cancer (NSCLC) patients in multiple studies. IL-1β exposure immediately deregulated many signaling pathways, including the AP-1, NF-κB, JNK, and AKT pathways, as well as cellular programs, such as cell migration and differentiation. Pro-inflammatory cytokines like IL-1β also has the capacity to up-regulate the zinc-finger E-box–binding transcriptional repressors of E-cadherin, including Zeb1, Snail, and Slug, thus leading to EMT progression [25]. Recently a direct link between EMT and gain of epithelial stem cell properties is reported [32]. Thus, inflammation may impact a diminution of E-cadherin, an induction of vimentin and other mesenchymal markers (FSP1, N-cadherin and MMP-9), an expression of EMT-TFs, the acquisition of enhanced migratory and invasive properties in the pathogenesis of lung cancer. The stimulation of EMT by TNF-α, particularly in synergy with TGF-β or other inflammatory factors, has been described. As per recent studies TNF-α and TGF-β induce EMT-like changes via a NLRP3/Snail1 axis-dependent way (NLRP3 stands for 'NOD-like receptor family, pyrin domain containing 3') in lung cancer. Recently researchers found that benzo[a]pyrene induced EMT-related genes in lung cancer cells; while fibronectin and Twist were induced, E-cadherin expression was deceased [33]. Tobacco-specific carcinogen 4-(nmethyl-n-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) has also been found to promote EMT via induction of E-cadherin transcriptional repressors in human bronchial epithelial cells [25].

The Molecular Players Involved in Inflammation Mediated Lung Cancer Induction

Smoking or pathogen induced epithelial abnormalities can serve both as targets for uncharacteristic inflammatory responses and as initiators of derestricted inflammation. Cytokines, chemokines, and growth factors released by alveolar macrophages, lymphocytes, neutrophils, endothelial cells, and fibroblasts may act to promote epithelial dysfunction and malignant progression [34]. For example, a group of scientists used KrasLA1 mice, which develop lung adenocarcinoma due to somatic stimulation of the KRAS oncogene, to evaluate the significance of ligands

for chemokine receptor CXCR2 in the pathogenesis of lung cancer [35]. Vascular endothelial cells and neutrophils with high expression of CXCR2 ligands and CXCR2 were found in premalignant alveolar lesions of KrasLA1 mice. Importantly, CXCR2 inhibition blocked the expansion of early alveolar neoplastic lesions. These studies are consistent with other recent findings indicating that the CXCR2 ligand CXCL8 plays a critical role in Kras-induced tumorigenesis [36]. By implicating CXCL8, these findings highlight another common pathway in the pathogenesis of COPD and lung cancer.

Epithelial cells can also serve as a site of deregulated inflammatory responses in pulmonary tumorigenesis. For example, chronic exposure to tobacco compounds can lead to loss of p53 and Kras mutation. These in turn can lead to deregulated inflammation and angiogenesis. Komarova found that p53, by acting to suppress NF-kB activity, could serve as a ‘‘buffer’’ for inflammatory responses. The induction of oxidative stress-mediated inflammatory signal is regulated by NF-κB and NF-κB is known as a connecting link between inflammation and lung cancer [37]. This is unfailing with the p53 tumor suppressor functions. As noted above, Kras mutations can serve as a driving force for the generation of the proangiogenic CXC chemokines such as CXCL8. On the other hand the products of COX-2 mediated oxygenation of arachidonic acid are related to various forms of immune modulation associated with lung carcinogenesis. Several studies have documented high constitutive expression of COX-2 in precursor lesions as well as established human lung cancer. Estimated 80% of lung cancers are attributed to cigarette smoking and smokers tended to have more COX-2 expression than non-smokers. Cigarette smoke exposure (tobacco carcinogens as nicotine and NNK) can induce COX-2 expression and lead to PGE2 release from alveolar macrophages and lung dendritic cells. Additionally, carcinogen products stimulating the production of PGE2 may facilitate the pro-inflammatory environment, a suitable scenario for the development of lung tumours. Over expression of COX-2 is related to cell proliferation, down regulation of apoptosis, angiogenesis, metastasis and resistance to anti-cancer drugs. As per recent reports, elevated COX-2 expression has been observed with greater staining in lymph node metastases than in the primary tumor, and tumor COX-2 expression has been found to be a poor prognostic indicator of lung cancer [38]. These findings, along with studies documenting increased COX-2 expression in precursor lesions [39], an association between a common polymorphism in the COX-2 gene and increased risk of lung cancer [40], and epidemiological studies indicating a decreased incidence of lung cancer in individuals who regularly use aspirin, support involvement of COX-2 and its enzymatic products in the pathogenesis of lung cancer. COX-2 induction or overexpression is associated with an increased production of PGE2, one of the foremost products of COX-2 which is known to modify cell

proliferation, cell death, and tumor invasion [41]. On-going chemoprevention studies in patients at risk for lung cancer are now assessing blockade of the eicosanoid pathway.

Whereas the COX enzymes are expressed at low constitutive levels in the normal lung, a variety of factors may contribute to up-regulation of COX-2 in the developing lung cancer environment. COX-2 contributes to immune circumvention and resistance to cancer immunotherapy. The activity of COX-2 -PGE2-EPs signal pathway can subdue Dendritic cells (DCs), natural killer (NK), T cells, type-1 immunity, but promote type-2 immunity, which uphold tumor immune evasion. Various inflammatory markers like IL-1b and TGF-b are required for the over expression of COX-2 in lung cancer tissue [42]. Once COX-2 is up-regulated in lung cancer cells, its promotion may be upheld by aberrations in signaling pathways required to down-regulate COX-2. Two such abnormalities are loss of IL-10 receptor expression and constitutive nuclear localization of STAT-6 [43].

Conclusion

Overall this review suggests some potential links between chronic inflammation and lung cancer development. Moreover, no unvarying outline exists to evaluate and enumerate lymphoreticular infiltrates, leading to difficulties in comparisons between studies and the generalization of results. Lungs having a large surface area are being exposed to various kinds of insults throughout the whole day. This insults results in a persistent or chronic inflammation in lung. Chronic inflammation has been related to various stages involved in tumorigenesis, including cellular alteration, promotion, survival, proliferation, invasion, angiogenesis, and metastasis. Furthermore, epidemiologic studies and meta-analysis have revealed that sustained use of non-steroid anti-inflammatory (NSAID) drugs decreases the risk of lung cancer. Strong lines of evidence suggest that the chemopreventive properties of chronic NSAID administration are based on their COX-inhibitory activity. Nonetheless, unrelenting refinements in our indulgent learning of the complex interaction between different components inflammation associated with lung cancer are vital to identify potential new treatment approaches for this devastating disease.

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About the Book

The book inspires the scientific spirit to promote biological science research in various arenas. The pace at which the globe is losing biological resources very rapidly makes it imperative to take stock of the situation from time to time and develop suitable strategy for conserving natural resources through genes and gene pools. The content of the book encompasses major areas of research like medical science, nanotechnology, plant systematic, diversity, ethno botanical knowledge, cytogenetics, nutrient stock estimation, bio-pesticides, below ground biodiversity etc. The contents of the book will be useful to post graduate students, researchers, teachers and the scientific community working in related fields. I believe that the book will add value to the wide range of curriculum like Microbiology, Natural Resource Management, Cell Biology, Plant Pathology, Biotechnology, Nanotechnology and Bioinformatics.

Key Features

- Biomedical Science
- Cytogrnetics and Biotechnology
- Plant systematic and diversity
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- Microbiology and Bio-informatics

About the Editor

Anupam Guha, M. Sc, Ph. D
Associate Professor in Botany

Dr. Guha did his schooling at Melaghar Class XII School, Tripura. He completed his college education from Maharaja Bir Bikram College, Agartala, Tripura. After obtaining his Master's Degree from Tripura University (A Central University), he also obtained his Ph. D degree in the field of Plant Cytogenetics and Tissue Culture from the same University. Presently, he is working as an Associate Professor in Botany under Department of Higher Education, Government of Tripura at Rabindranath Thakur Mahavidyalaya, Bishalgarh, Tripura. He has been teaching for nearly 17 years and actively associated with research. At present, he is involved in the field of plant biodiversity, specially, in relation to ethnomediacinally important plants of North Eastern Regions of India. He has attended many national and international seminars/conferences and published 26 scientific papers in national and international journals. Dr. Guha had organised a UGC sponsored National Seminar: *"Recent Trends in Biodiversity Conservation and Bioresource Utilization*" in the year 2017 and edited a book (www.Amazon.com) published by New Delhi Publishers (www.ndpublisher.in). He has contributed seven book chapters. He is also life members of several professional societies.

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