Screening, Isolation and Identification of Antimicrobial and Antioxidant Substances from Some Common Lichens of Darjeeling Hills

Thesis submitted to the
University of North Bengal
For the Award of Doctor of Philosophy in Botany

BY
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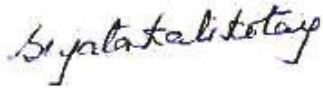
January 2017

Declaration

I declare that the thesis entitled "**Screening, Isolation and Identification of Antimicrobial and Antioxidant Substances from Some Common Lichens of Darjeeling Hills"**, has been prepared by me under the guidance of Dr.Binod Chandra Sharma, Associate Professor, A.B.N Seal, College, Coochbehar. No part of this thesis has been formed the basis for the award of degree or fellowship previously.



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Date: 03.01.2017

CERTIFICATE

I certify that Miss Sujata Kalikotay has prepared the thesis **"Screening, Isolation and Identification of Antimicrobial and Antioxidant Substances from Some Common Lichens of Darjeeling Hills"**, for the award of Ph.D. degree of the University of North Bengal, under my guidance. She has carried out the work at P.G Department of Botany, Darjeeling Government College, University of North Bengal. No part of this thesis has been formed the basis for the award of degree or fellowship previously

Miss Kalikotay has followed the new rules and regulations (Course work) as laid down by the University of North Bengal for the fulfillment for requirement for the award of the degree of Doctor of Philosophy in Science (Botany).

16.11.2016

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Abstract

This study pertains to the assessment of **"Screening, Isolation and Identification of Antimicrobial and Antioxidant Substances from Some Common Lichens of Darjeeling Hills".**

A short introduction to the above study is given which is followed by review of literature based on the antimicrobial and antioxidant properties of lower plant groups to higher. It also comprises reports on work done by earlier scientists on synergistic effect of plants with antibiotics or with plants together. A glance on identification of various lichen compounds and its various therapeutic uses are presented. This review has been done in an elaborated manner and divided into different groups. Lichen and its forms; Industrial and Medicinal significance of lichens; Antimicrobial activity of higher vascular plants; Antimicrobial activity of lower plant groups; Antimicrobial activity of Algae; Antimicrobial activity of Bryophytes; Antimicrobial activity of Fungi; Antibacterial and antifungal activity of lichens; Antiviral and anti tumor activity of lichens; Antioxidant activity of higher vascular plants; Antioxidant activity of lichens; Synergistic activity; Spectral analysis of lichens and other plants; Methodology for determination of antimicrobial and antioxidant activity of lichen extracts

The study was carried out with following objectives:

- To study the antimicrobial property of some high altitude lichen members of Darjeeling Hills.
- To study the antioxidant property of some high altitude lichen members of Darjeeling Hills.
- To study the synergistic antimicrobial and antioxidant activity of such lichens in combination with selected local medicinal plants.
- To identify active principle compounds from the lichens showing potential antimicrobial and antioxidant activity.

The methodology adopted for carrying out the above objectives has been described in the materials and method chapter.

Lichen samples were collected from the barks of trees like *Alnus*, *Erythrina, Macaranga, Citrus*, *Betula*, *Prunus* as well as rocks from different places in Darjeeling district. The taxonomic identity of lichen samples was confirmed from the Lichenology Laboratory, National Botanical Research Institute, Lucknow and Uttar Pradesh, India. The ethanolic and methanolic extracts of studied lichen specimens were screened for antimicrobial activity against eight test microorganisms (*Bacillus subtilis* MTCC 3972, *Bacillus megaterium* MTCC 7192, *Alcaligenes faecalis* MTCC 9780, *Staphylococcus aureus* MTCC 7443, *Pseudomonas aeruginosa* MTCC 424, *Escherichia coli* MTCC 6365, *Enterobacter aerogenes* MTCC 111 and *Candida albicans* MTCC 4748). Test microorganisms were obtained from Institute of Microbial Technology, Chandigarh, India. Broad spectrum antimicrobial activity was shown by extracts of *Everniastrum* sp*, P. reticulatum, R. hossei* and *U. baileyi* during preliminary screening which was evaluated on the basis of inhibition zone produced around the filter paper discs. In few instances zones of inhibitions produced by the extracts of *U. baileyi*, *Everniastrum* sp, *P. reticulatum* and *R. hossei* was greater than the standard antibiotic taken. Further, lowest Minimum inhibitory concentration of lichen extracts (methanolic extract of *U. baileyi* and *Everniastrum* sp, ethanolic extract of *P. reticulatum, S. pomiferum, Everniastrum* sp, ethanolic and methanolic extract of *R. hossei)* was 500µg/ml.

Besides the antimicrobial screening of four medicinal plants (*Berginia ciliata, Panax pseudoginseng, Urtica dioica* and *Sapindus mukrossi*) in their ethanolic and methanolic extract, MIC was estimated in the similar manner as employed to lichens. *P. pseudoginseng* exhibited a good degree of antimicrobial activity*, U. dioica* was least active, *S. mukrossi* and *B. ciliata* was moderately active. It was also noted that the MIC value of medicinal plants ranged from 100µg/ml (*S. mukrossi*) to 500µg/ml. The combined extracts of lichen and medicinal plants were screened for the presence of synergistic effect. This combined effect was manifested by ethanolic extracts of (*P. pseudoginseng* + *Everniastrum* sp), methanolic extract of (*P. pseudoginseng* + *Everniastrum* sp, ethanolic extract of (*S. pomiferum* + *U. dioica)* with relatively strong antimicrobial activity evidenced by inhibition zones greater than the control antibiotic. Besides this ethanolic extract of (*U. baileyi* + *U. dioica)*, methanolic extracts of (*U. baileyi* + *U. dioica)* and (*R. hossei* + *U. dioica)* also possessed synergistic effect. The activity of ethanolic

and methanolic extract of *S. mukrossi* was enhanced by its combination with ethanolic and methanolic extracts of *Everniastrum* sp and *P. reticulatum.*

Various parameters namely DPPH radical scavenging activity, total antioxidant activity, reducing power ability, total phenolic content and total flavonoid content have been used to monitor and estimate the antioxidant activity lichen extracts. These analysis required special equipment and technical skills. Synergistic effect was observed on the basis two parameters namely DPPH radical scavenging activity and total antioxidant activity. Strong antioxidant activity (by Phosphomolybdenum method) was shown by ethanolic extracts of *R. hossei* and *U. baileyi* among the lichen extracts and ethanolic and methanolic extract *B. ciliata* among medicinal plants. The best synergistic effect was shown by ethanolic extracts of (*U. baileyi* + *P. pseudoginseng)* and (*U. baileyi* + *B. ciliata)*. α-tocopherol was taken as standard compound for comparing total antioxidant property.

A rapid and simple method to measure antioxidant capacity of lichens involves the use of the free radical, 1, 1-Diphenyl-2-picrylhydrazyl (DPPH). DPPH widely used to test the ability of compounds. The lichen and medicinal plant samples subjected to this method changed its colour from purple to yellow according to the molar absorptivity of the DPPH radical. Ethanolic extract of *Everniastrum* sp proved to be a potent radical scavenger, followed by ethanolic extract of *U. baileyi*, methanolic extract of *P. reticulatum, Everniastrum* sp, ethanolic extract of *R. hossei* and *P. reticulatum* which showed quite appreciable amount of radical scavenging activity. A low radical scavenging activity was shown by ethanolic and methanolic extract of *S. pomiferum*. Ethanolic extract of *U. dioica* and *B. ciliata* were good radical scavengers, which was quite near to the standard compound. Ethanolic extracts of *B. ciliata* and *U. dioica* and (*P. reticulatum* + *B. ciliata)*, methanolic extract of (*U. baileyi* + *B. ciliata)*, ethanolic extract of (*S. pomiferum* + *U. dioica)* were good in combination.

The reducing power ability was monitored by of Perl's Prussian blue. Methanolic extract of *R. hossei* and ethanolic extract of *Everniastrum* sp possessed high reducing ability, other extracts also showed moderate reducing capacity.

Several studies had been conducted to evaluate the correlation between phenolic compounds and antioxidant activity; hence estimation of total phenolics in lichen extracts was determined using modified Folin-

Ciocalteu reagent using tannic acid as standard. Higher phenol content was shown by mainly ethanolic and methanolic extract of *P. reticulatum* and *U. baileyi*. Consequently low flavonoid content was noted from ethanolic and methanolic lichen samples under study. Descriptive statistics to determine the mean and standard deviation was performed; One way ANOVA was performed to see significant variation in the five parameters for determining antioxidant activity of lichens. Pearson's correlations test was performed to determine correlation between the total antioxidant activity, reducing power ability and DPPH radical scavenging activity.

Effect of lichen extracts on antioxidant enzymatic activities: like catalase and peroxidase was estimated. *Everniastrum* sp as 1.68 enz/min/gm tissue exhibited high catalase activity and high peroxidase activity was noted in case of *Ramalina hossei* (2.4 enz/min/gm tissues).

Effect of lichen extracts on metabolism of microorganisms like growth and total cellular protein content of culture filtrate of test organisms was performed. The growth of microorganisms were interrupted by the lichen extract, the duration of log phase was decreased. This could be clearly observed from the prepared growth curves.

The culture filtrate were subjected to different concentration of lichen extracts, the present protein concentration decreased with the increasing concentration of all test lichen extracts (from 0.1 to 0.5µg/ml). This may have occurred due to inhibition of protein synthesis by the inoculation of extracts which may be one of the factors responsible for the antimicrobial activity described previously.

Thin Layer Chromatography was followed to determine the compounds present in each of the lichens under study. Bioautographic assay of lichen substances was made following the method of Santiago *et. al.*, (2010).

The TLC plates showed different coloured spots which revealed the presence of different bioactive acids from lichens. A good number of spots separated during TLC shows that the lichen under study contains diverse group of phytochemicals which are responsible for antimicrobial as well as antioxidant properties as described in earlier part of this work.

Bioautographic assay of lichen substances was followed taking Gram positive and Gram negative bacteria. Spots of lichen compounds from *S. pomiferum* and *Everniastrum* sp also inhibited the growth of *S. aureus*.

Similarly spots of *R. hossei* from (solvent system B) and *R.hossei, P. reticulatum* (solvent C) inhibited the growth of *E.coli.*

LCMS peaks obtained from SAIF, CDRI, Lucknow yielded the presence of different classes of lichen substances such as β-Orcinol Depsidones, Xanthones, Aliphatic acids, Usnic acid derivatives, Terpenoids, Naphthaquinones, Pulvinic acid derivatives and others which are responsible for making lichens a capable antimicrobial and antioxidant agent.

The findings of present study have been discussed in detail and compared with the results of other prominent works.

It could be concluded that the significant broad spectrum antimicrobial activity of the lichen compounds from Darjeeling Hills could serve either as novel antimicrobial agent and could furnish derivatives with better activity and less or no side effect.

The combination of lichens with medicinal plants extract could be significantly helpful to the progress of new approach in providing barrier against microbial agents because the use of combined extracts showed reduced microbial growth. The extracts mixture contain mixture of bioactive compounds which makes the survival of microorganisms difficult in comparison to the single plant extracts and also minimize the chance of side effects. Use of only combined plant extracts without combining antibiotics may also decrease risk of side effects caused by the antibiotics.

The identified compounds from four lichen species (*Everniastrum* sp, *Ramalina hossei, Stereocaulon pomiferum* and *Usnea baileyi)* call attention to future research in this direction. The results obtained from this present study could serve as a base line data for future research works. More information like safety studies and clinical trials would be needed to determine the viability of the isolated components for development into drugs for treatment of microbial infections caused by diverse groups of pathogenic microbes. Thus the effort made in this investigation provided scientific evidence for their antimicrobial and antioxidant activity thereby validating the rationale behind traditional uses of lichens from Darjeeling Hills in folk medicines.

(SUJATA KALIKOTAY)

Preface

Lichens are symbiotic organisms formed by associations of a fungus and green algae or cyanobacteria. Lichens have a new anatomical, morphological and physiological properties which unlike organisms that they constitute. Lichens are used as food, dyes, perfume as well as in pharmaceutical industries since time immemorial.

Works on antimicrobial and antioxidant properties lichen of have been performed by various workers worldwide. Hence our investigation is based on assessing antimicrobial and antioxidant efficiency five common lichens Everniastrum sp, Ramalina hossei, Stereocaulon pomiferum, Parmotrema reticulatum and Usnea baileyi from Darjeeling hills against seven bacteria and a fungus. Preliminary antimicrobial screening was preceded by determination of Minimum inhibitory concentration in nutrient agar (NA) medium. Four medicinal plants namely (Bergenia ciliata, Panax pseudoginseng, Urtica dioica and Sapindus mukrossi) were combined and its synergistic antimicrobial and antioxidant efficacy was estimated. It may be assumed that the prevalence of antimicrobial and antioxidant activity of lichens from Darjeeling Hills may be due to the presence of different lichen compounds. Further studies on isolation of metabolites and their bioactivities are suggested in future line of investigation.

With regard to this research work I would like to express my deep and sincere thanks of gratitude to my supervisor Dr. Binod Chandra Sharma Associate Professor of Botany, ABN Seal College, Coochbehar (formerly Postgraduate Department of Botany, Darjeeling Government College), who gave me this opportunity to do the research work and his invaluable inputs which provided insight and expertise into my work from the beginning until the end. His dynamic supervision, constructive criticism, unceasing motivation and enthusiasm have been the constant source of inspiration to carry out this research work successfully.

I am immensely grateful to Professor Lalita Rai Ahmed, former Principal of Darjeeling Govt. College and Dr. Projjwal Chandra Lama, Officer-in-Charge, Darjeeling Govt. College for providing infrastructure facilities for the work.

My sincere thanks goes to authorities of The University of North Bengal for granting me permission for research works which enabled me to complete my study within stipulated time. I would also like to extend my sincere words of admiration and appreciation to Professor A. Saha (HOD, Department of Botany, University of North Bengal), Professor A. P. Das, Professor P. K. Sarkar, Professor B. N. Chakraborty, Professor U. Chakraborty, Professor A. Sen, Dr. S. C. Roy, Dr. P. Mandal and Dr. M. Choudhury for their presence in the University of North Bengal as my moral support.

I thank to the Dr. Arun Chettri, Assistant Professor, Sikkim University, Dr. Dhiraj Brahman and Miss Nishika Jaishee as per my need. I would also like to acknowledge my humble gratitude to the members of IMTECH, Chandigarh, India for supplying bacterial and fungal cultures for my research work and SAIF, CDRI, Lucknow. I also extend my sincere words of thanks, Dr. Himanshu Rai and Miss Roshni Khare (NBRI, Lucknow) for identification of lichens.

My sincere thanks must also go to my beloved sisters Ms. Bimala Rai, Ms Sulaxana Baraily, and dear friend Ms Deewa Basnett for their moral support throughout my research. My heartfelt thanks to Dr. Smriti Gurung, Dr. Rashi Subba, Ms Sachina Yonzone, Dr. Ranju Tamang, Dr. Sabina Pradhan, Miss Deepa Ghising, Mr. Jayant Chowdary and all the teaching and non-teaching staff of P.G Department of Botany, Darjeeling Govt. College, who supported me directly or indirectly throughout my investigation.

I offer profoundest gratitude to Mr. P. P. Kalikotay (my father) and Mrs. R. Kalikotay (my mother), Mr. Bhasker Kalikotay (brother), Mrs.G. Sunam and Mrs.S. Barua (aunties), Mr. Pranay Baraily (brother in law) and for their love, prayers, sacrifices and unwavering support.

I convey my heartfelt thanks to husband Mr. Basant Baraily and notorious son Pukul for being in my life and supporting me during my lows.

Finally, I thank Almighty God for showering the blessings throughout my research work.

(Sujata Kalikotay)

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

ABBREVIATIONS and ACRONYMS

($^1O_2$)	Singlet oxygen
°C	Degree Celsius
µg	Microgram
µg/ml	Microgram per milliliter
µl	Micro liter
$0H^-$	Hydroxy radicals
AA/g	Acetic acid per gram
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
$AlNO_3$	Aluminum nitrate
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BHA	Butylated Hydroxyl Anisole
BHT	Butylated Hydroxyl Toluene
BSA	Bovine Serum Albumin
CAT	Catalase
CDRI	Central Drug Research Institute
CFU	Colony forming unit
conc.	Concentration
d. H_20	Distilled water
DMSO	Dimethyl Sulphoxide
DPPH	2, 2 –Diphenyl -1- picrylhydrazyl
enz	Enzyme
FIC	Fractional Inhibitory Concentration
FRAP	Ferric Reducing Power Assay
g/tissue	Gram per tissue
g^{-1}	Gram per litre
gm	Gram
GSHPX	Glutathionine peroxidase

H_20_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HEC	Human endometrial carcinoma
HIV	Human Immunodeficiency Virus
IMTECH	Institute of Microbial Technology
KH_2PO_4	Potassium Dihydrogen Phosphate
LAB	Lactic Acid Bacteria
LC/MS	Liquid Chromatography/ Mass Spectroscopy
M	Molar
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
mg/gm	Milligram Per Gram
MIC	Minimum Inhibitory Concentration
min	Minute
ml	Milliliter
mM	Milimolar
mm	Millimeter
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSSA	Methicillin Sensitive *Staphylococcus aureus*
MTCC	Microbial Type Culture Collection
N	Normal
N.A.	Nutrient Agar
N.B.	Nutrient Broth
$Na_2HPO_4.2H_2O$	Di sodium Hydrogen Phosphate
$NaH_2PO_4.2H_2O$	Sodium Dihydrogen Phosphate
NBRI	National Botanical Research Institute
nm	Nanometer

O.D	Optical Density
$P0_4$	Phosphate
PG	Propylgallate
pH	Potential Hydrogen
Rf	Retention Factor
RF	Representative Fraction
RO	Reverse osmosis
ROO	Peroxyl
ROS	Reactive Oxygen Species
RPM	Rotations Per Minute
SAIF	Sophisticated Analytical Instrument Facility
SD	Standard Deviation
SOD'S	Super Oxide Dismutase
TBHQ	Tertabutyl Hydroquinone
TEAC	Trolox Equivalent Antioxidant Capacity
$TiSO_4$	Titanium Sulphate
TLC	Thin Layer Chromatography
UV	Ultraviolet
v/v	Volume by volume
w/v	Weight by volume

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources; many of these isolations were based on the uses of the agents in traditional medicine. This plant-based, traditional medicine system continues to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care (Grabley and Thiericke, 1999; Owolabi *et. al.*, 2007).

Historically, a large portion of the world's medicine has been derived from plants. Natural products chemistry actually began with the work of Serturner, who first isolated morphine from Opium. This, in turn, was obtained from opium poppy (*Papaver somniferum*) by processes that have been used for over 5000 years. Many such similar developments followed. Quinine from Cinchona tree had its origin in the royal households of the South American Incas. Before the first European explorers arrived, the native people of the Americas had developed complex medical systems replete with diagnosis and treatment of physical as well as spiritual illnesses (Patwardhan *et. al.*, 2004). Salicin from *Salix alba* which on oxidation was converted to salicylic acid is the active ingredient in aspirin (Piria, 1938; Jeffreys, 2005) which has been used for millennia as an effective pain reliever and fever reducer. Drugs such as aspirin, vincristine, vinblastine, cocaine, digitoxin and morphine are also isolated from plants (Newman *et. al.*, 2000; Butler, 2004; Gilani and Rahman, 2005).

In the recent years, research on medicinal plants has attracted a lot of attentions globally. Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, etc. which have been found to have antimicrobial properties *in vitro* (Dahanukar *et. al.,* 2000). The so-called secondary metabolites (Evans *et. al.*, 1986) can be classified as chemotherapeutic and antimicrobial (Purohit and Mathur, 1999). The use of plant extracts and phytochemical both with known antimicrobial properties is of great significance, in the past few years a number of investigations have been conducted worldwide to prove antimicrobial activities from medicinal

plants (Nascimento *et. al.*, 1990; Alonso *et. al.*, 1995; Islam *et. al.*, 2008; Manjulata *et. al.*, 2012).

'Doctrine of signatures' written in 15th century stated "A plant could treat a disease it most looked like", This formed the basis of phyto-therapeutics in traditional system of medicines like Traditional Indian Medicine (TIM) or Ayurveda, Traditional Chinese Medicine (TCM) and Western Medical Herbalism (Bown,2001). The ancient Egyptians were familiar with many medicinal herbs and were aware of their usefulness in the treatment of various diseases (Abu-Shanab *et. al.,* 2004).

According to a report issued by the World Health Organization (WHO), plant species that were used for medicinal purposes are about 20,000 (Vartia, 1973). Current estimates indicate that about 80% of people in developing countries still rely on traditional medicine based largely on various species of plants and animals for their primary healthcare (Goodwin *et. al.*, 1997).

For example, the use of bearberry (*Arctostaphylos uvaursi*) and cranberry juice (*Vaccinum macrocarpon*) to treat urinary tract infections is reported in different manuals of phytotherapy, while species such as lemon balm (*Melissa officinalis*), garlic (*Allium sativum*) and tea tree (*Melaleuca alternifolia*) are redescribed as broad-spectrum antimicrobial agents (Rios and Recio, 2005).

Since the discovery of penicillin (1929) and its use in chemotherapy in 1941 as a response to the great fatalities in the Second World War, a great number of important antibiotics have been found (El- Bana, 2007). The success story of chemotherapy lies in the continuous search for new drugs to counter the challenge posed by resistant strains of microorganisms. The investigation of certain indigenous plants for their antimicrobial properties may yield useful results. Many studies indicate that in some plants there are many substances such as peptides, unsaturated long chain aldehydes, alkaloidal constituents, some essential oils, phenols, which are water, ethanol, chloroform, methanol and butanol soluble compounds. These plants then emerged as compounds with potentially significant therapeutic application against human pathogens, including bacteria, fungi or virus (Elmastal *et. al.,* 2005).

Plants are an important source of potentially useful structures for the development of new chemotherapeutic agents (Gomathi *et. al.*, 2011). The search for novel bioactive compounds from natural resources to improve pharmaceutical, cosmetic and agriculture applications is an ancient practice and currently it is regaining more rapid importance. Most citrus and dried fruits, cruciferous vegetables, garlic, onions, carrots, tomatoes, sweet potatoes, sesame and olive oil are rich sources of antioxidants. Antioxidants are molecules that can delay or prevent an oxidative reaction (Velioglu *et. al.*, 1998) catalyzed by free radicals.

Indian medicinal plants represent a rich source of antibacterial agents (Mahesh and Satish, 2008). To the botanist the Himalayas are the treasure-house for not only the study of the plants growing there but also for plants new to science and particularly those plants which are of great medicinal and economic value for the welfare of mankind.

Lichens are another type of organisms that may hold the potential for medical exploration. The word lichen is derived from Greek word "Leprous" and refers to use of lichens in treating skin diseases due to peeling–skin appearance. Lichens comprise a unique group that consists of two unrelated organism, a fungus and an alga, growing together in a symbiosis. Lichens with blue green algae symbionts, contribute significantly for forest nitrogen fixation. They are distributed universally and are occurring in varied climatic conditions ranging from the poles to the tropics. They may look like crust, spreading rapidly over the surface (crustose) or leafy and loosely attached to the surface (foliose) and branched and shrubby, hanging from tree twigs or branches, with a single attachment (fruticose). Besides many other uses, lichens are also used as pollution monitors (Nash and Wirth, 1988; Richardson, 1992; Stolte *et. al.*, 1993; Slack, 1998; Garty, *et. al.*, 2000; Nash and Gries, 2002; Kumar, 2009).

In the folklore of many European countries, lichens were used as a remedy for pulmonary tuberculosis and in treatment of wounds and disorders. These medicinal uses to some extent been confirmed by studies which showed that many lichen metabolites such as depsides, depsidones and usnic acid are active against mycobacteria and gram positive bacteria (Vartia,1973).

Lichens like *Lobaria pulmonaria* (Stictaceae) and *Parmelia sulcata* (Parmeliaceae) have been used in the treatment of pulmonary and cranial diseases, respectively. Similarly, *Xanthoria parietina* (Lobariaceae), being yellow, was used to cure jaundice (Bown, 2001).

Various biological activities of some lichens are known, such as: antimicrobial, antiviral, anti-tumor, anti-inflammatory, analgesic, antipyretic, antiproliferative and antiprotozoal (Lawrey, 1986; Huneck, 1999;Halama and Van, 2004). The lichen compounds are not an exception in this field. Currently the interest on the lichen secondary compound is increasing because of ineffectiveness of some known previously reliable drugs (Huneck, 1999).

A lot of attention has been paid to lichens as a source of natural antioxidants (Behera *et. al.*, 2006; Gulluce *et al.*, 2006; Kinoshita *et. al.,* 2010; Rankovic *et. al.*, 2010; Dzomba *et. al.*, 2012). Antioxidant activity of some other lichen was studied by other researchers. For example, Gulcin *et al.*, (2002) reported that the aqueous extracts of *Cetraria islandica* had a strong antioxidant activity. Similar results found by Behera *et. al.*, (2005) for different extracts from the lichen *Usnea ghattensis*. Antioxidant activity for the extracts of the lichen *Parmotrema pseudotinctorum* and *Ramalina hossei* was also documented (Kekuda *et. al.*, 2009).

India is a rich center of lichen diversity contributing of about 15% of the 13,500 species of lichens so far recorded in the world (Negi, 2000). In India parmeloid lichens are extensively used in traditional medicine to treat several diseases and disorders e.g., headache, skin diseases, urinary trouble, boils, vomiting, diarrhoea, dysentery, heart trouble, cough, fever, leprosy and as blood purifier (Chandra and Singh, 1971).

Lichens of Eastern Himalayan regions, particularly of Darjeeling District have been studied extensively by Chopra (1934) and Awasthi and Agarwal (1970) from taxonomic point of view, but the antimicrobial and antioxidant properties of these lichens have not yet been explored to that extent. Gupta and Paul (1995) reported antimicrobial property of *Usnea floria, Physcia* sp*, Usnea pendulata, Cladonia cristatella, Parmelia perforata* and *Ramalina calicaris* collected from Darjeeling Hills against *Bacillus megaterium, Staphylococcus aureus.* In another study from Darjeeling hills

Ray *et. al.*, (2003) screened the extract of *Usnea articulate, Ramalina jamesii* and *Parmelia tinctorum* against both Gram positive and negative bacteria and antimicrobial activity was reported to most of the tested micro organisms. The extracts were also found to be inhibitor of protein synthesis, energy metabolism and growth of selected bacteria.

In view of the limited information on *in vitro* activity of lichen members and the abundant distribution of lichens in the varied locations of Darjeeling Hills, the present study includes determination of potential pharmaceutically relevant antimicrobial and antioxidant activities of selected lichen species from profusely grown locations of Darjeeling Hills.

Objectives

The study was carried out with following objectives:

- ✓ To study the antimicrobial property of some high altitude lichen members of Darjeeling Hills.

- ✓ To study the antioxidant property of some high altitude lichen members of Darjeeling Hills.

- ✓ To study the synergistic antimicrobial and antioxidant activity of such lichens in combination with selected local medicinal plants.

- ✓ To identify active principle compounds from the lichens showing potential antimicrobial and antioxidant activity.

REVIEW OF LITERATURE

2.1a. Lichens and its forms

Lichens are "composite organisms" made up of two, or may be three, completely different kinds of organisms with its part fungus and usually the other species as photosynthesizing alga, but sometimes it can be a photosynthesizing bacterium known as a cyanobacteria. On the basis of external morphology or growth forms, lichen thalli are distinguished into four categories namely Crustose *(Lecanora, Lecidia*, *Lepraria, Vezaea* etc.), Squamulose (*Cladonia, Endocarpon, Psora, Tonina* and some species of *Parmeliella* and *Pannaria),* Foliose and Fruticose (Alexopoulos and Mims,1979). Lichens reproduce in two main ways i.e., by production of spores and fragmentation

2.1b.Diversity of photobiont and mycobiont in lichens

Out of 43 genera of photobionts associated as a primary or secondary constituent of a lichen taxa 14 genera of Cyanobacteria, 27 genera of Chlorophyceae (green algae), one genus of Xanthophyceae (yellow green algae) and Phaeophyceae (brown algae) have been confirmed (Ahmadjian,1958). The Cyanobacterial genera *Gloeocapsa, Nostoc* and *Scytonema* and the green algal genera *Myrmecia, Protococcus, Pseudotrebouxia, Trebouxia* and *Trentepholia* are associated as photobiont in majority of lichen taxa.

Lichen forming fungi are referred to as lichen mycobionts like plant or animal pathogens or mycorrhizal fungi. They differ from non-lichenized taxa by their manifold adaptations to symbiosis with a population of minute photobiont cells. Lichenization is regarded as a successful nutritional strategy, almost 20% of all fungal species being lichenized. About 14000 species of lichen-forming fungi are so far described, approximately 99% of them being ascomycetes classified (Kirk *et. al.,* 2001). Less than 50 species of homobasidomycetes are distinctly lichenized; these belong to the Agaricales (*Lichenomphalia*, *Semiomphalia*), Atheliales (*Dictyonema*), Cantharellales (*Multiclavula*) and Polyporales (*Lepidostroma*), as summarized by Nelsen *et. al.*, (2007).

2.2 Industrial and medicinal significance of lichen

In India many species lichens are extensively used in traditional systems of medicine (Chevallier, 1996; Kumar and Upreti, 2001; Bown, 2001). Use of lichens in commercial, ethno botany and Ayurvedic and Unani systems of medicine in India is well documented (Upreti, 1996). Naturally, the most important application of lichens is the one in traditional medicine for treatment of animals and human diseases. Sodium usnate have been successfully used for the control of various plant diseases in green house (Ark *et. al.*, 1960). *Ramalina thrausta* is used in Finland for treatment of wounds, athlete's foot or other skin diseases and taken to relieve sore throat and toothache (Vartia, 1973). The desert species *Lecanora esculenta* is considered as "biblical manna" (Trease & Evans, 1978). Two lichen species *Parmelia caperata* and *Umbilicaria* sp. are reported in study of Chilean traditional medicine (Munoz *et. al.*, 1981). Several lichen species have been used in folk medicine for treatment of stomach diseases, diabetes, whooping cough, pulmonary tuberculosis, cancer treatment and skin diseases (Richardson, 1991; Baytop, 1999; Huneck, 1999).

Usnea species in Asia, Africa and Europe used for pain relief and fever control (Okuyama *et. al.,* 1995) and in New Zealand Maori traditionally for nappies and sanitary pads (Perry *et. al.*, 1999). *Usnea densirostra*, known as "barba de la piedra" served for a curing for various disorders in Argentina (Correch *et. a*l., 1998). *Usnea* has been used as antibiotics (Sharnoff, 1997). *Usnea longissima* was used as a dermatological aid for dressing wounds in the Pacific North West. Ethno botanical uses of *Usnea* include its use as aromatic in health recipes (Rai *et. al.,* 1998) in Darjeeling and Sikkim Himalayas. *Cetraria islandica* is ancient cough remedy known as "tonicum amarum" accepted as a mucilage drug (Muller, 2001). It was observed by Behera *et. al.,* (2005), metabolic products that have antibiotic activity may have function of protecting the organisms from attack by other fungi. The intestinal worms are treated by *Flavoparmelia caperata* and dried powder of the thallus can be applied on burns (Haq *et. al.*, 2012).

Litmus, a blue coloring matter from lichen fermentation, was used as dye for textile and beverages (Beecken *et. al.,* 1961). Extracts of some species of lichens, like *Evernia prunastri*, are contents of perfumes (Trease & Evans, 1978). Lichens are used in nutrition of many animals and humans during

famine throughout the ages and getting colours, perfumes, alcohol and in the medicine industry (Richardson, 1988; Romagni and Dayan, 2002; Kirmizigul *et. al.,* 2003; Karagoz *et. al.,* 2009)

Paper strips impregnated with litmus, a water extracted dye from *Roccella* sp., are used as pH indicators in laboratories from ancient times till present. Lichen species are used as deodorants, and herbal colouring, dyes and decorative materials and for production of clothes, and perfumes (Sharnoff, 1997 and Brodo *et. al.,* 2001) and also as bioindicator for determining atmospheric pollution (Richardson, 1988; Romagni and Dayan, 2002; Kirmizigul *et, al.,* 2003; Freitas, 2011). Lichen dyes had considerable economic importance in 18th century in some parts of the world as in the Canary Islands. It is also known that Romans dyed their togas with orchil, a purple pigment from *Roccella* sp. and crottal, brown pigment from *Parmelia*, *Ochrolechia* and *Evernia* sp (Muggia *et. al.*, 2009).

In various system of traditional medicine worldwide (Richardson, 1991) including the Indian system of medicine, lichen species are said to be effective in curing of dyspepsia, bleeding piles, bronchitis, scabies, stomach disorders and many disorders of blood and heart (Saklani and Upreti, 1992; Negi and Kareem,1996)

2.3 Antimicrobial activity of higher vascular plants

The microbiologist to be interested in antimicrobial plant extracts are as it is very likely that these phytochemical will help into the production of antimicrobial drugs. The scientists realize that the effective life span of any antibiotic is limited, so new sources especially plant sources are also being investigated. The people are becoming increasingly aware of the problems with the over prescription and misuse of traditional antibiotics. Interest in plants with antimicrobial properties has revived as a result of current problems because of increasing antibiotic resistance of microorganisms associated with the use of antibiotics (Abu Shanab *et. al.,* 2004).

In an extensive work by various scientists for searching antibacterial activities of plants in India alone 880 plant species have been worked out (Bhakuni *et. al.,* 1969).

The water and methanol extracts of some medicinal plants displayed antifungal activity against *Fusarium oxysporum,* which causes yellows of

Japanese radish strongly inhibited the growth of *Streptomyces scabies,* which causes common scab of potato (Fujii *et. al.*, 1991).

Studies on the antimicrobial activity was performed on *Rhus glahra* (Anacardiaceae), further its bioassays leading to the isolation of three antibacterial compounds, the methyl ester of 3, 4, 5 trihydroxy benzoic acid (methyl gallate), 4-methoxy and 3, 5dihydroxybenzoic acid and gallic acid (Saxena *et. al.,* 1994).

Among different solvent extracts of aerial parts of *Drymaria cordata,* observed that the methanol extract was most effective against *S. aureus*, *E. coli, Bacillus subtilis, B. pumilis* and *P. aeruginosa* (Mukherjee *et. al.,* 1997).

Additional investigations on plants as a source of human disease management as well as various phytochemical constituents has been made in by various scientists (Gomathi *et. al., 2*011).

Juniperus oxycedrus is widely used as traditional folk medicine in Turkey for treatment of different infectious diseases. It had inhibitory effects on the growth of 57 strains of 24 bacterial species of the genus *Acinetobacter, Bacillus, Brevundimonas, Brucella, Enterobacter, Microccous, Pseudomonas, Staphylococcus* sp and *Xanthomonas* (Karaman *et. al.*, 2003). Further action of medicinal plants against microbes have also been studied by various authors (Das *et. al.,* 2008)

T. bellirica is used as an antioxidant, antimicrobial, antidiarrheal, anticancer, antidiabetic, antihypertensive and hepatoprotective agent (Elizabeth, 2005).

Many reports also cite the inhibitory activity of *Lawsonia inermis* (Henna) against gram negative and gram positive organisms (Muhammad and Muhammad, 2005).

Hibiscus sabdariffa possessed antibacterial effect against *S. aureus, Bacillus stereothermophilus, Micrococcus luteus, Serratia mascences, Clostridium sporogenes, E. coli, K. pneumoniae, Bacillus cereus* and *Pseudomonas fluorescence* (Tolulope, 2007).

The antimicrobial substances from *Geranium glaberrimum, Geranium stepporum* and *Geranium psilostemon* can be used as biological control agents as they possessed antimicrobial and free radical scavenging activity (Didem *et. al.*, 2007).

Plants from Western Mediterranean regions of Egypt namely *Mesembryanthemum crystallinum*, *Blackiella aellen*, *Arthrocnemon glaucum*,

Atriplex halimus, *Thymelaea hirsute*, *Carduus getulus*, *Nicotiana glauca*, *Alhagi maurorum*, *Atractylis carduus* and *Echinops spinosissimus* have been found to possess antimicrobial properties that can be used to cure infectious diseases (Rahman *et. al.*, 2011a).

The leaf extract of *Hallea ledermannii* (Rubiaceae) was screened for antibacterial activities against pathogenic organisms like *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Klebseilla pneumoniae* and its antioxidant activity was also determined (Adsegun *et. al.,* 2012).

In later years extracts of *Semecarpus anacardium* was evaluated against medicinally important bacteria *Staphylococcus epidermidis* (ATCC 12228), *Micrococcus luteus* (ATCC10240), Methicillin-resistant *Staphylococcus aureus* (Hospital-isolate), *Propionibacterium acnes* (MTCC 1951) and yeast, *Malaassezia furfur* (MTCC 1374) using the MIC and MBC/MFC analysis. Leaves of *S. anacardium* could be used as a source for the development and formulation of drugs, thus also in herbal medicines to treat a variety of infectious conditions caused by test microorganisms (Parag and Raman, 2013).

2.4 Antimicrobial activity of lower plant groups

2.4.1 Antimicrobial activity of Algae

The lower groups of plants like algae, bryophytes and fungi have been screened earlier for antimicrobial activity. Many photobiont floras of lichens have antimicrobial properties as observed before. The use of algae for therapeutic purposes has a long history and the systematic examination of algae for biologically active substances especially antibiotics began in the year 1950. Various strains of cyanobacteria are known to produce intracellular and extracellular metabolites with, diverse biological activities such as antibacterial, antifungal and antiviral activity (Noaman *et. al.*, 2004). The aqueous and solvent extracts from algae were tested against gram positive and gram negative bacteria (Prashanthkumar *et. al.*, 2006)

Microalgae are sources of biologically active compounds such as phycobilins, phenols, terpenoids, steroids and polysaccharides have recently found immense application in human and animal medicine (Qi *et. al.*, 2006).

Chlorophycean members such as *Chlorella* sp., *Scenedesmus* sp. have been reported as a few among the main groups of microalgae to produce

antimicrobial substances (Ghasemi *et. al.,* 2007). Subramaniyan *et. al.*, (2011) found that Cyanophycean member such as *Chrooccocus minor* also possess antimicrobial properties

Table 2.1 Antimicrobial activity of algae

Algae	Activity against	References
Cyanobacteria	*E. coli, Bacillus subtilis* and *Pseudomonas aeruginosa*	(De Mule *et. al.,* 1991)
Oscillatoria, Phormidium and *Lyngbya*	Gram positive and Gram negative organisms	(Vijaya and Prabu, 2010)
Freshwater cyanobacteria like *Oscillatoria latevirns, Phormidium corium, Lyngbya martensiana, Chrooccocus minor* and *Microcystis aeruginosa*	*Bacillus subtilis, Staphylococcus aureus, Streptococcus mutans, Escherichia coli, Micrococcus mutans, Klebsiella pnuemoniae, Saccharomyces cerevisiae* and *Candida albicans*	(Subramaniyan *et. al.*, 2011)
Desmococcus, *Chlorella* and *Scenedesmus*	Gram-positive and Gram-negative	(Ordog *et. al.,* 2004)
Gloeocapsa, Synechocystis sp.	*Bacillus cereus*, *Escherichia coli* and *Candida albicans*	(Najdenski *et. al.*, 2013).
Spirogyra sp	*Pseudomonas solanacearum* and *Escherichia coli*	(Naik *et. al.,* 2012)

2.4.2 Antimicrobial activity of Bryophytes

Bryophytes are traditionally used in Chinese, European, North American and Indian medicine, to treat illness of cardiovascular system, tonsilitis, bronchitis, tympanitis, in skin diseases and burns. They also possess anticancer and antimicrobial activity due to their unique chemical constituents (Banerjee and Sen, 1979).

Many bryophytes have been investigated for their antimicrobial activity and have stated that bryophytes are one of the important sources of antibiotics and biologically active, naturally occurring compounds (Singh *et. al.,* 2007; Dulger *et. al., 2*009).

Marchantia palmata were tested against the growth of four human pathogenic gram negative bacteria namely *Escherichia coli*, *Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae* and two gram positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* (Khanam *et. al.,* 2011).

2.4.3 Antimicrobial activity of Fungi

Fungi have long been used as a valuable food source and as traditional medicines around the world since ancient times, especially in Japan and China. A number of medicinal mushrooms, such as *Aleurodiscus, Coprinus, Clitocybe, Daedalea, Marasmius, Merulius, Pleurotus, Polyporus, Poria, Psathyrella,* and *Tricholoma* spp are rich sources of ß-glucan, proteoglucan, lectin, phenolic compounds, flavonoids, polysaccharides, triterpenoids, diatery fibre, lentinan, schizophyllan, lovastatin, pleuran, steroids, glycopeptides, terpenes, saponins, xanthones, coumarins, alkaloid, purin, purimidin, kinon, fenil propanoid, kalvasin, volvotoksin, flammutoksin, porisin, AHCC, maitake D-fraction, ribonuclease, eryngeolysin and also have been used extensively in traditional medicine for curing various types of diseases such as antimicrobial, antiviral, anticancer, antitumor, anti-inflammatory, cardiovascular diseases, immunomodulating activities etc. (Benedict and Brady, 1972; Conchran, 1978; Chihara, 1993; Gunde-Cimerman, 1999; Bobek and Galbavy 2001; Jose *et. al.,* 2002, Wasser 2002; Periasamy 2005; Carbonero *et. al.,* 2006)

Table 2.2. List of some common fungus showing antimicrobial activity

Fungus	Activity against	References
Pleurotus eryngii var. *ferulae*	*Bacillus megaterium* DSM 32, *Staphylococcus aureus*, , *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* FMC 5, *Candida albicans* FMC 17, *Candida glabrata* ATCC 66032, *Trichophyton* spp., and *Epidermophyton* spp.	Mehmet and Sevda, 2009
Camptotheca acuminate	*Rhizoctonia solani* and *Fusarium oxysporum* f. sp. *vasinfectu*	Ding *et. al.,* 2010

2.5 Antibacterial and antifungal activity of lichens

After the discovery of penicillin from a fungus, numbers of lichens were screened for antibacterial activity in the 1940s and 1950s (Varita, 1973; Lawery, 1986). Studies by Burkholder *et. al.,* (1944*)* on 100 species of American lichens showed that 52% of lichens were active only against Gram positive bacteria. Owing to pronounced antimicrobial activity of some of their

secondary metabolites, lichens (together with algae, micro fungi and higher plants) attracted much attention among researchers as significant new source of bioactive substance (Ingolfsdottir, 1997; Hostettmann, 1997).

Lichen metabolites exert a wide variety of biological actions including antibiotic (Lawery *et. al.,* 1986), anticancer (Willams *et. al.,*1998*),* antiHiv (Huneck and Yoshimura*,* 1996), antimycobacterial (Ingolfsdottir *et. al.,* 1998; Gupta *et. al., 2*007), antiviral, anti-inflammatory, analgesic, antipyretic (Muller 2001), antiproliferative and cytotoxic effects (Perry *et. al.,*1999; Ingolfsdottir 2002). Even though these manifold activities of lichen metabolites have now been recognized, their therapeutic potential has not yet been explored and thus remains pharmaceutically unexploited (Muller, 2002).

Turkey lichens, *Evernia prunastri, Pseudeovernia furfuracea a*nd *Alectoria capillaris* were active against Gram-positive bacteria and the *Candida albicans* (Rowe *et. al.,* 1989).

Lichens produce a wide range of organic compounds that can be grouped as primary metabolites and secondary metabolites (Elix, 1996). Primary metabolites such as proteins, lipids, carbohydrates, and some other organic compounds are needed for the lichen's metabolism and structure. Secondary metabolites are produced by the fungus alone and secreted onto the surface of lichen's hyphae either in amorphous forms or as crystals. About 350 secondary lichen metabolites have been found and the chemical structures of approximately 200 of them have been established (Galun, 1988). Huneck (1999) the listed the antifungal and antibacterial activities of lichen compounds and lichens against bacteria and fungi.

Usnic acid (Dobrescu,1993) evernic acid, and vulpinic acid inhibited the growth of gram positive bacteria *Staphylococcus aureus*, *Bacillus megaterium*, but the acids had no affect on gram negative bacteria *Escherichia coli* or *Pseudomonas aeruginosa* (Lawery,1986). Lauterwein *et. al.*, (1995) determined *in vitro* activities of (+)usnic acid, (-)usnic acid and vulpinic acid against aerobic and anaerobic microorganisms. It was again reported that both forms of usnic acid inhibited the growth of *Mycobacterium tuberculosis* and *Mycobacterium tufu in vitro* at a relatively low concentration (Krishna and Venkataramana, 1992). But later Ingolfsdottir (2002) listed the antimicrobial activity of (+)- and (+)-usnic acid in a table against Gram-positive, Gram-negative, anaerobic bacteria, mycobacterium, and

yeast/fungi with the relevant references. Usnic acid being one of the most common and investigated lichen compounds, its antimicrobial, antiprotozoal, antiviral, antiproliferative, anti-inflammatory, analgesic, antipyretic and antitumor activities as well as some other properties such as UV protection, allergen, and toxicity has been summarized in two reviews (Cocchietto *et. al.*, 2002; Ingolfsdottir, 2002).

Usnic acid is extensively distributed in species of *Cladonia, Usnea, Lecanora, Ramalina, Evernia, Parmelia* and other lichen genera (Ingolfsdottir, 2002) and *Alectoria* species yielding of up to 6% of usnic acid have been reported (Proska *et. al.,*1996). In addition Behera *et. al.,* (2005) determined the antimicrobial and antioxidant activities of *Usnea ghattensis* and found that *U. ghattensis* was active against *S. aureus, B. licheniformis, B. subtilis* and *B. megaterium* (Behera *et. al., 2*005). Another species *Usnea barbata* showed significant activity against the Gram-positive bacteria like *Bacillus subtilis, Enterococcus faecalis, Micrococcus viradans* and *Staphylococcus aureus* (Madamombe and Afolayan, 2003; Weckesser *et. al., 2*007).

However, Kharel *et. al.,* (2000) and Behera *et. al.*, (2008) isolated compounds like dehydrocollatolic acid from *P. nilgherrense* and salazinic acid from *Parmotrema tinctorum* respectively, and conducted antibacterial activity against some animal pathogenic bacteria. Their study supported their finding, as the crude extracts possess all these active compounds, which are responsible for the inhibition of bacterial growth (Louwhoff and Crisp, 2000), and also have recovered malanoprotocetraric acid from *P. conformatum,* but its antibacterial activity was not tested then.

Hypogymnia apinnata (atranorin), *Letharia columbiana* (vulpinic acid), *Lobaria pulmonaria* (Stictic acid, constictic acid and norstictic acid) and *Usnea filipendula* (usnic acid and salazinic acid) have been reported to have significant antibiotic activity against *Micrococcus luteus, Staphylococcus aureus, Salmonella gallinarum* and *Serratia marcescens,* respectively (Crockett, 2003). Parietin, anthraquinone isolated from methanol extract of *Caloplaca cerina* (Teloscistaceae) has been reported to have significant antifungal activity (Manojlovic, 2005). Extracts of Andean lichens *Protousnea poeppigii* (Parmeliaceae) and *Usnea florida var. rigida* demonstrated antimicrobial activity against the pathogenic fungi *Microsporum gypseum, Trichophyton mentagrophytes* and *T. rubrum* isodivaricactic acid, 5-

propylresorcinol, divaricatinic acid and usnic acid were identified as antifungal agents (Schmeda, 2008).

Antibacterial activity of acetone, chloroform, diethyl ether, methanol and petroleum ether extracts of *Parmelia sulcata* displayed its antimicrobial activity against *Aeromonas hydrophila, Bacillus cereus, Bacillus subtilis*, *Listeria monocytogenes, Proteus vulgaris, Yersinia enterocolitica, Staphylococcus aureus, Streptococcus faecalis, Candida albicans, Candida glabrata, Aspergillus niger, Aspergillus fumigatus* and *Penicillium notatum* (Candan *et. al.,* 2007) and also its Salazinic acid constituent was active against the tested bacteria and fungi except against *Listeria monocytogenes, Proteus vulgaris, Yersinia enterocolitica* and *Staphylococcus aureus.*

Ramalina farinaceae (Esimone and Adikwu, 1999; Turk *et. al.,* 2004) and *Ramalina pacifica* (Hoskeri *et. al.*, 2010) were active against pathogenic strains of bacteria and fungi namely *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Candida albicans*, *Aspergillus niger*, *Trichophyton rubrum* and *Trichophyton mentagrophytes.* (Turk *et. al.,* 2004) concluded *Ramalina farinacea* and its (+)-usnic acid constituent showed antimicrobial activity against *Bacillus subtilis*, *Listeria monocytogenes*, *Proteus vulgaris*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Yersinia enterocolitica*, *Candida albicans*, and *Candida glabrata.* Protolichesterinic acid from lichen *Cetraria aculeata* inhibited the growth of *Escherichia coli*, *Staphylococcus aureus*, *Aeromonas hydrophila*, *Proteus vulgaris*, *Streptococcus faecalis*, *Bacillus cereus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Listeria monocytogenes* (Turk *et. al.,* 2003).

Cladonia foliacea were found active against 9 bacteria and fungi namely *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis*, *Proteus vulgaris, Aeromonas hydrophila, Streptococcus faecalis* and *Listeria monocytogenes,* the yeasts *Candida albicans* and *Candida glabrata* whose growth were inhibited by the extracts (Yilmaz *et. al.,* 2004; Turk *et. al.*, 2004). Antibacterial screening of the light petroleum extracts of *Thamnolia subuliformis* showed it is active *in vitro* against Gram positive organisms as well as against *Escherichia coli* and *Candida albicans* (Eugene *et. al.,* 2004).

3-hydroxy physodic acid isolated from *Hypogymnia tubulosa* (Parmeliaceae) showed antimicrobial activity against *Aeromonas hydrophila, Bacillus cereus, Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae,*

Listeria monocytogenes, Proteus vulgaris, Salmonella typhimurium, Staphylococcus aureus, Streptococcus faecalis and *Candida albicans* (Yilam, 2005).

A year later, Candan (2006) reported extracts of *Xanthoparmelia pokornyi* (Parmeliaceae) and its constituents (gyrophoric and stenosporic acid) have been reported to be potential antimicrobials. The antimicrobial activity of *Rocella montagnei* were assayed against six human pathogenic microorganisms namely *Staphylococcous aureus*, *Salmonella para- typhi*-B, *Proteus vulgaris, Klebsiella pneumonia* and fungal culture of *Candida albicans,* which proved to be a better antimicrobial agent screened against human pathogenic microorganisms (Balaji *et. al* ., 2006).

Acetone, choroform, diethyl ether, methanol and petroleum ether extracts of *Parmelia sulcata* and its constituent (salazinic acid) demonstrated antibacterial activity against *Aeromonas hydrophila, Bacillus cereus, Bacillus subtilis, Listeria monocytogenes, Proteus vulgaris, Yersinia enterocolitica, Staphylococcus aureus, Streptococcus faecalis, Candida albicans, Candida glabrata, Aspergillus niger, Aspergillus fumigates* and *Penicillium notatum* (Candan, 2007) . In the same year Rankovic *et. al.,* (2007) screened the antimicrobial properties lichens *Lasallia pustulata, Parmelia sulcata, Umbilicaria crustulosa, Umbilicaria cylindrica* and all of them manifested antibacterial and antifungal activity.

Lichens *Lecanora frustulosa* and *Parmeliopsis hyperopta* and their divaricatic acid and zeorin constituents was screened *in vitro* against the following species of microorganisms: *Bacillus mycoides*, *Bacillus subtilis*, *Staphylococcus aureus*, *Enterobacter cloaceae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Botrytis cinerea*, *Candida albicans*, *Fusarium oxysporum*, *Mucor mucedo*, *Paecilomyces variotii*, *Penicillium purpurescens*, *Penicillium verrucosum*, *Trichoderma harsianu* (Rancovic *et. al.*, 2007), these lichen extracts and lichen compounds demonstrated a strong antimicrobial effect against the tested microorganisms.

In a screening made by Chand *et. al.,* (2009) on antimicrobial activity of 15 lichen species from Indian Himalayan flora, lichen *Everniastrum* was found active to both Gram- positive and Gram- negative bacteria. Later bactericidal activity of crude extracts from lichen *Ramalina pacifica* were screened against 20 clinical pathogenic strain isolated from different

infectious sources which belong to *Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhi, Salmonella paratyphi, Escherichia coli* and *Staphylococcus aureus* (Hoskeri *et. al.*, 2010).

Antimicrobial activity of the acetone, methanol and aqueous extracts of the lichens *Lecanora atra, L. muralis, Parmelia saxatilis, P. sulcata* and *Parmeliopsis ambigua* of which strongest antimicrobial activity was found in the acetone extract of the lichen *Parmelia sulcata* where the least measured MIC value was 0.78 mg/ml. Bacteria *Bacillus mycoides* and the fungi *Botrytis cinerea* and *Candida albicans* were the most sensitive species. *Escherichia coli* was resistant to all extracts of lichens tested (Rankovic and Kosanic, 2010).

Usnea baileyi, Ramalina dendriscoides, *Stereocaulon massartianum* and *Cladonia gracilis* were inhibitory against Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus)* and Gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa), R. dendriscoides* were observed to be the most active. Selected lichen extracts also showed activities against *S. aureus.* Barbatic acid, stictic acid, diffractaic acid, galbinic acid, norstictic acid, salazinic acid, and usnic acid proved to be the bioactive lichen acids (Santiago *et. al.,* 2010).

In-vitro antifungal activity of *Parmotrema tinctorum* (Despr. Ex. Nyl.) Hale. was investigated against ten plant pathogenic fungi viz. *Aspergillus niger*, *Aspergillus flavus, Aspergillus fumigatus, Alternaria alternata, Fusarium oxysporum, Fusarium solani, Fusarium roseum, Ustilago* spp., *Albugo candida* and *Penicillium citrinum* was performed by Tiwari *et. al.*, (2011), it was observed that extracts of *Parmotrema tinctorum* were more effective against five broad spectrum plant pathogenic fungi (*Aspergillus fumigatus, Fusarium solani, Fusarium roseum, Penicillium citrinum* and *Ustilago* spp).

Generally the extracts of *Usnea longifolia*, *Cetraria* spp, *Parmotrema reticulatum* and *Evernastrium nepalense* demonstrated antimicrobial effect which suggested a possibility of their use in treatment of various diseases caused by these and similar microorganisms. The extracts obtained showed the presence of volatile oil, saponins, coumarins and quinines, flavonic glycosides and carotenoids (Baral *et. al.,* 2011).

Cladonia furcata was the most active antimicrobial agent with minimum inhibitory concentration values ranging from 0.78 to 25 mg/mL.

All extracts were found to show strong anticancer activity toward both cell lines (Rankovic *et. al.*, 2011).

Antibacterial activity of *Cladonia digitata* was observed against *Clostridium perfringens* and *Staphylococcus aureus* at the concentration of 100% and 50%, respectively (Dzomba *et. al., 2*012).

Several studies have been made on antimicrobial activity of *Flavoparmelia caperata* against *K. pneumoniae* (Seaman *et. al.*, 2007), *Mycobacterium tuberculosis* H37Rv and *M. tuberculosis* H37Ra (Gupta *et. al.,* 2007), against *E. faecalis* ATCC 29212, *B. cereus* (clinical strain), *S. aureus* subsp. *aureus* ATCC 25923, *E. coli* ATCC 25922, *P. mirabilis* (clinical strain) and *S. enterica* serovar *typhimureum* (clinical strain). Acetone extract of *F. caperata* didn't show antimicrobial activity against *E. coli*, *E. faecalis*, *P. mirabilis* and *P. vulgaris* (Duman, 2009; Aydin and Kinaliodu, 2013).

Ethanolic and methanolic extracts of *Flavoparmelia caperata* and *Roccella phycopsis* were tested against seven gram positive and nine gram negative bacteria, their antibacterial activities compared with penicillin, tetracycline and gentamicin. It was observed from the studies that the most resistant bacteria were *Bacillus megaterium* and the most sensitive bacteria was *Proteus vulgaris.* It was concluded that methanol and ethanol extracts of *Flavoparmelia caperata* and *Roccella phycopsis* extracts have antibacterial activity on both gram negative and gram positive bacteria and could be an alternative of the antibiotic to cure the diseases (Aydin and Kinaliodu, 2013).

Usnea ghattensis G. Awasthi (Usneaceae) endemic fruticose lichen found growing luxuriantly in Northern Western Ghats of India; it also contains Usnic acid as a major chemical and tested against some human pathogenic bacteria. Ethanol extract was most effective against *Bacillus cereus* and *Pseudomonas aeruginosa* whereas acetone and methanol extract demonstrated almost similar activity against *Staphylococcus aureus*, only methanol extract was showing activity against *Streptococcus faecalis* (Srivastava *et. al.,* 2013).

Thirty four North American lichens screened against four pathogenic bacteria. *Staphylococcus aureus, Pseudomonas aeruginosa* and methicillin-resistant *S. aureus* (MRSA). In addition, extracts from three species, *Letharia columbiana* (Nutt.) J. W. Thomson (Parmeliaceae), *Letharia vulpina* (L.) Hue

(Parmeliaceae), and *Vulpicida canadensis* (Rasanem) J.-E. Mattsson & M. J. Lai (Parmeliaceae) was also effective against *Escherichia coli.* Generally, viewing acetone extracts were found to be more effective than methanol extracts (Shrestra *et. al.*, 2014).

Parmotrema reticulatum (Taylor) M. Choisy, was screened *in vitro* for its antifungal activity against *Aspergillus niger*, *Aspergillus flavus, Fusarium oxysporum* and *Fusarium solani.* Maximum inhibition was shown by acetone extract against *Fusarium solani* (17.0±0.0 mm) which was higher than the standard control Ketoconazole (15.3±0.2 mm) (Preeti, *et. al.,* 2014).

Parmotrema perlatum exhibited inhibitory activity against human pathogens *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Bacillus subtilis*, *Streptococcus pneumoniae*, *Streptococcus faecalis*, and *Staphylococcus aureus* which recommended the use of this lichen in healing numerous diseases caused by these pathogenic microorganisms and related organisms (Revathy *et. al.,* 2015). In this lichen compounds similar to usnic acid, 3-ketooleanane, tridecyl myristate, icosan-1-ol, azolitmin, erythrolein, orcin, spaniolitmint, atranorin and parmelanostene permelabdone were also present (Caccamese *et. al.*, 1985; Sharma *et. al.*, 2012; Thippeswamy *et. al.*, 2012).

2.6 Antiviral and antitumor activity of lichens

Kupchan and Koppennan (1975) studied and found *Evernia, Acrosyphus* and *Alectoria sp.* (Takeda *et. al.,* 1972) have antitumor activity. Anthraquinones, especially the polyphenolic and polysulphonate substituted types commonly found in lichens have been shown to exhibit potent antiviral properties (Sydiskis *et. al.,*1991; Schinazi *et. al.*, 1990).

Table 2.3 Antiviral and antitumor activity of lichens

Sl No.	Name of lichen	Compound	Activity/used in	References
1	*Evernia* sp., *Acrosyphus* sp.,	#	Antitumor	Kupchan and Koppennan,1975
2	*Ramalina* sp.	#	liver diseases and stomach cramp	Takeda *et. al.,* 1972
3	*Usnea* sp	usnic acid	Callus formation and hormone regulation	Nishitoba *et. al.,* 1987
4	*Usnea longissima*	+(-)usnic acid	Epstein –Barr virus	Yamamoto *et. al.,* 1995
5	Lichens	Lichenan	T.M.V	Stubler and Buchenaver, 1996
6	*Cetraria islandica*	Protolichesterinic	Breast cancer cell lines and mitogen stimulated lymphocytes	Ogmundsdottir *et. al.,* 1998
7	*Umbilicaria esculenta*	#	HIV virus	Brodo *et. al.,* 2001
8	*Teloschistes chrysopthalmus*	Parietin	Virucidal for Junin and Tacaribe arena virus	Fazio *et. al., 2*007
9	*Parmelia perlata*	Crude polysaccharide fraction	Yellow fever virus	Esimone *et. al., 2*007
10	*Thamnolia vermicularis*	#	Anticancer	Manojlovic *et. al.*, 2010
11	*Xanthoria parietina*	#	Anticancer	Trigiani *et. al., 2*009

name of compound not mentioned

Ingolfsdottir *et. al.*, (1997) revealed that protolichesterinic acid showed to exhibit antitumor activity against solid-type Ehrlich carcinoma in mice, point *in vitro* inhibiting activity of against the DNA polymerase activity of human immunodeficiency virus type 1 reverse transcriptase, and inhibitory effects on arachidonate 5-lipoxygenase from porcine leukocytes. Antiviral properties have been attributed to specific lichen secondary metabolites, Antitumor activities of lichens are of major importance, Usnic acid exhibited an antiproliferative effect on human leukemia cells (K562) and endometrial carcinoma (HEC-50) cells (Carderelli *et. al.,* 1997; Ingolfsdottir, 2002; Krismundsdottir *et. al.,* 2002).

Another lichen compound depsidone pannarin inhibited cell growth and induced apoptosis in human prostate carcinoma DU-145 and human melanoma M14 cells (Russo *et. al.*, 2006, 2008).

2.7 Antioxidant activity of plants

Oxygen is an essential element for life to perform biological functions such as catabolism of fats, proteins and carbohydrates in order to generate energy for growth and other activities. However, oxygen may be a toxic agent for living tissues. The biological combustion produces harmful intermediates called free-radicals. A free-radical is simply defined as any species capable of independent existence that contains one or more unpaired electrons, an unpaired electron being one that is alone in an orbital. It may be superoxide (O_2^-), thiyl (RS.) or nitric oxide (NO.) in which the unpaired electron delocalized between both atoms. Reactive oxygen species (ROS), which include superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and singlet oxygen ($^1O_2$), are different forms of activated oxygen (Sies, 1993, Halliwell,1995; Squadriato and Pelor, 1998; Gulçin *et. al.,* 2003) are continuously produced *in vivo*. Oxygen, may not be harmful by itself, it is involved in the generation of various kinds of "reactive oxygen species" (ROS). ROS, primarily play an important role in the host defense mechanism by acting against microorganisms, but the increased production of ROS react with macro-molecules of crucial biological significance (DNA, lipids, protein) and destroy their structure and function what accelerates ageing and might lead to degenerative diseases, including cancer (Wiseman and Halliwell, 1996; Kehrer,1993), inflammations (Sco *et. al.,* 1995), neuro-degeneration (Leboritz *et. al.*, 1996), Parkinson's disease (Jenner, 1994), artherosclerosis (Witztum, 1994) and per-mature aging (Orr and Sohal, 1994).

Peroxidation (auto-oxidation) of lipids exposed to oxygen is responsible not only for deterioration of foods (rancidity) but also damage to tissue *in vivo* (Rice-Evans and Miller, 1997). The dangerous effects are considered to be caused by free radicals (ROO, RO, OH^-) produced during peroxide formation from fatty acids containing methylene-interrupted double bonds i.e., those found in the naturally occurring polyunsaturated fatty acid. Lipid peroxidation is a chain reaction producing a continuous supply of free radical that initiates further peroxidation. Since the molecular precursor for

the initiation process is generally the hydroperoxide product ROOH, lipid peroxidation is a chain reaction with potentially devastating effects. To control and reduce lipid peroxidation, both humans in their activities and nature invoke the use of antioxidants.

Certain portion of reactive oxygen species (ROS) is generated in normal human metabolism and the production rate is precisely controlled by specialized system of antioxidant defense (Ignatowicz, 1994). This well-balanced ROS synthesis is impaired by inflammatory events, where activated macrophages and neutrophils upon contact with proinflammatory stimuli; release substantial amounts of aggressive oxygen and nitrogen-centered

Radicals (Halliwell, 1999).

Natural antioxidant defense system involves enzymes (superoxide dismutase, catalase, glutathione peroxidase), other proteins (albumin, ferritin, ceruloplasmin) and numerous smaller molecules (eg. reduced glutathione, α-tocophrol, α-carotene, bilirubin, uric acid) of various modes of action. Antioxidant molecules counteract ROS and diminish their deleterious effects (Halliwell, 1990; Yu 1994). This protective barrier can be enhanced by the use of antioxidant micronutrient (vitamins C, E, α –carotene) and non-nutrient ingredients of edible plants, like polyphenols. Polyphenol subgroup of chemicals, flavonoids, is the extensively examined group of antioxidants (Cotelle, 2001).

ROS damage membrane proteins by causing lipid peroxidation in membranes by attacking to unsaturated fatty acids (Ames *et. al.*, 1993). The damage to membrane proteins decreases the membrane permeability, activities of enzymes and receptors and activation of cells. When free radicals attack DNA, cancer-causing mutations may occur. Therefore, antioxidant defense systems including antioxidant enzymes, food and drugs are important in the prevention of many diseases (Pietta *et. al.,* 1998; Yen and Hsieh, 1998).

Several studies have shown that plant derived antioxidant neutraceuticals scavenge free radicals and modulate oxidative stress-related degenerative effects (Joshep *et. al.,* 1999). Free radicals contribute to more than one hundred disorders in humans including atherosclerosis, arthritis,

ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer, AIDS, diabetes, neurodegenerative disorders and aging (Yu, 1994; Cook and Samman, 1996; Kumpulainen and Salonen, 1999).

Previously it was suggested that higher intake of antioxidant rich food is associated with decreased risk of degenerative diseases particularly cardiovascular diseases and cancer (Thatte *et. al.,* 2000). Because of this, there is need for the development and use of natural antioxidants for their capacity to protect organisms without any negative effects from damage induced by oxidative stress (Gulcin *et. al.*, 2004; Naveena *et. al.*, 2008).

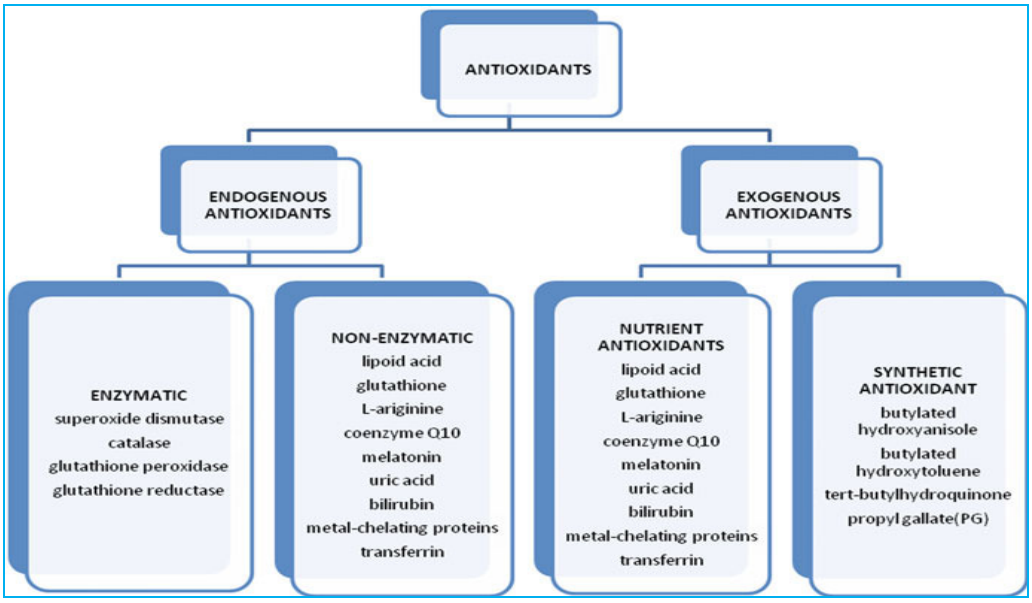


Fig. 2.1c Endogenous antioxidants and exogenous antioxidants for protecting the body from damage caused by oxidative stress

(source Kosanic and Rankovic, 2015)

Antioxidants, both synthetic and natural, are substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress (Souri *et. al.*, 2008).

The antioxidant activity of putative antioxidants have been attributed to various mechanisms, like prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Diplock, 1997). Synthetic antioxidants like BHA, BHT, gallic acid esters etc., have been suspected to cause or prompt negative health effects hence strong

restrictions have been placed on their application (Branen, 1975; Barlow, 1990). It was also suspected that at the present time, synthetic antioxidants such as butylated hydroxyl anisole (BHA), butylated hydroxytoluene (BHT), tertbutylhydroquinone (TBHQ) and propyl gallate (PG) have toxic and carcinogenic effects (Grice, 1986; Zhang *et. al.*, 2009).

In recent years much attention has been devoted to natural antioxidant and their association with health benefits (Ali *et. al.,* 2008). Studies on medicinal plants, fruits and vegetables owing to the presence of low molecular weight phenolic compounds, strongly supported this idea that plant, constituents with antioxidant activity are capable of exerting protective effects against oxidative stress in biological systems and which are known to be potent natural antioxidants (Block and Patterson, 1992; Cao *et. al.,* 1996; Ness and Powles, 1997; Eastwood 1999; Wang *et. al.*, 1999). Antioxidant properties of numerous crude extracts, primary and secondary metabolites of many plants have been widely reported previously (Hidalgo *et. al.,* 1994; Pietta *et. al.*, 1998). Extracts of 12 medicinal and aromatic plants were investigated by Miliauskas *et. al.,* (2004) for their radical scavenging activity using DPPH and ABTS assays. The plants *G. macrorrhizum* and *P. fruticosa* extracts possessed very high radical scavenging activity (RSA), higher than that of *S. officinalis* extract.

The anti-inflammatory effects of sesquiterpenes isolated from *Curcuma zedoaria* have been studied (Yoshioka *et. al.*, 1998 and Makabe *et. al.*, 2006). The antioxidant activity of Du-Zhong (*Eucomnia ulmoides*) (Yen and Hsieh, 1998), ear mushrooms (Chao, 2001) and anise (*Pimpenella anisum* L.) seed (Gulcin *et. al.,* 2003) were found to correlate with the phenolic compounds.

Phenolic components are potential antioxidants, free radical terminators (Kaushik *et. al.*, 2010). These compounds are the main agents that can donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first initiation step. They also scavenge radicals such as singlet oxygen, superoxide and hydroxyl radicals may be explained by their phenolic hydroxyl groups (Sawa *et. al.*, 1999)]. Flavonoids are also the

most important natural phenolics and they possess a broad spectrum of chemical and biological activities including radical scavenging properties (Mohammed *et. al.,* 2010). Numerous researches found a high correlations

between antioxidative activities and phenolic content (Rankovic *et. al.,* 2010; Odabasoglu *et. al.,* 2004).

Table 2.4 Antioxidant activity of higher plants

Sl. No	Plants	Parts used	Activity	References
1	*Daphyniphyllum calycinum*	leaves	Antioxidant	Gamez *et. al.,* 1998
2	*Mentha spicata*	Whole plant	Antioxidant and radical scavenging	Elmastas *et. al., 2*005*;* Kiselova *et. al.*, 2005; Kanatt *et. al., 2*007
3	*Curcuma longa*	rhizome	Antioxidant and radical	Mau *et. al.*, 2003
4	*Anthurium versicolor*	leaves	Radical scavenging	Aquino *et. al.,* 2001
5	*Artemisia apiaceae*	Whole plant	Radical scavenging	Kim *et. al.*, (2003)
6	*Chionanthus virginicus*	Root bark	antioxidant	Gulcin *et. al.,* 2007
7	*Cosmos caudatus, Polygonum minus,*	Whole plant	antioxidant	Faujan *et. al.,* 2009
8	*Cucurma longa, Coffea arabica, Tribulus terrestris,*	Whole plant	antioxidant	Satisha *et. al.,* 2011
9	*Celtis africana*	leaves	antioxidant	Adedapo *et. al.,* 2009
11	*Biebresteinia multifida* and *Polypodium vulgare*	plant	antioxidant	Souri *et. al.,* 2008
12	*Salix* sp and *Allium hirtifolium*	plant	radical scavenging	Souri *et. al.,* 2008
13	*Curcuma domestica, Piper betel, ;Pandanus odorus, Garnicia atroviridis, Morinda citrifolia, Centella asiatica, Zingiber officinale, Manihot asculenta,*	Whole plant	antioxidant	Huda-Faujan *et. al.*, 2007; Jayamalar and Suhaila, 1998; Noriham *et. al., 2*004; Zainol *et. al.,* 2003)

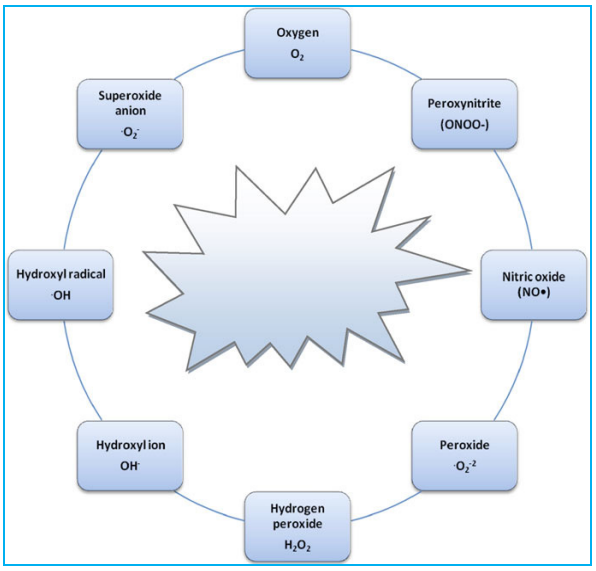


Figure 2.1a Free radicals (reactive oxygen species and reactive nitrogen species. (source Kosanic and Rankovic, 2015)

2.8 Antioxidant activity of lichens

Thamnolia vermicularis has commonly been used as a tea with the local name of "snow tea" in traditional Chinese medicine for hundreds or thousands of years, this lichen can be also used as a novel source of natural antioxidant (Luo *et. al.,* 2006).

Gulcin *et. al.*, (2002) reported that the aqueous extracts of *Cetraria islandica* had a strong antioxidant activity. Similar results found (Behera *et. al.*, 2005) for different extracts from the lichen *Usnea ghattensis*. (Kekuda *et. al.*, (2009) found an antioxidant activity for the extracts of the lichen *Parmotrema pseudotinctorum* and *Ramalina hossei*.

In vitro antioxidant activities of the methanol extracts of *Cladonia foliacea*, *Dermatocarpon miniatum*, *Everinia divaricata*, *Evernia prunastri*, and *Neofuscella pulla* was performed in which only *D. miniatum* provided 50% inhibition at 396.1 µg/ml concentration in the former and gave 49% inhibition in the latter. Total phenolic constituents of extracts from lichen species tested (*C. foliacea.*, *D. miniatum.*, *E. divaricata.*, *E. prunastri*, and *N. pulla.*) were 1.7% (w/w), 2.9% (w/w), 3.0% (w/w), 2.6% (w/w), and 1.5% (w/w), respectively (Aslan *et. al.,* 2006).

Extracts of *Usnea articulata, Ramalina jamesii* and *P. tinctorum* inhibited the growth of *Staphylococcus aureus, Sar. lutea* and *Ser.*

marcescens by reducing protein synthesis, inhibiting energy metabolism (Ray *et. al.*, 2003).

A yellow and new dark red pigments were isolated from *Lethariella sernanderi, L. cashmeriana, and L. sinensis* as antioxidant components from which yellow pigment was identified as canarione (Kinoshita *et. al.,* 2004) and the others were determined to be 1,2-quinone derivatives and rubrocashmeriquinone (Kinoshita *et. al., 2*005) by analysis of their spectroscopic data.

The antioxidant activity of some species of lichens, such as *Bryoria fuscescens, Cetraria islandica, Dermatocarpon intestiniformis, Parmelia saxatilis, Peltigera rufescens, Platismatia glauca, Ramalina pollinaria, R. polymorph, Umbilicaria nylanderiana , Usnea ghattenis,* and *U. longissima* were tested and some of them have very good antioxidant activity (Behera *et. al.*, 2006; Gulluce *et. al.*, 2006).

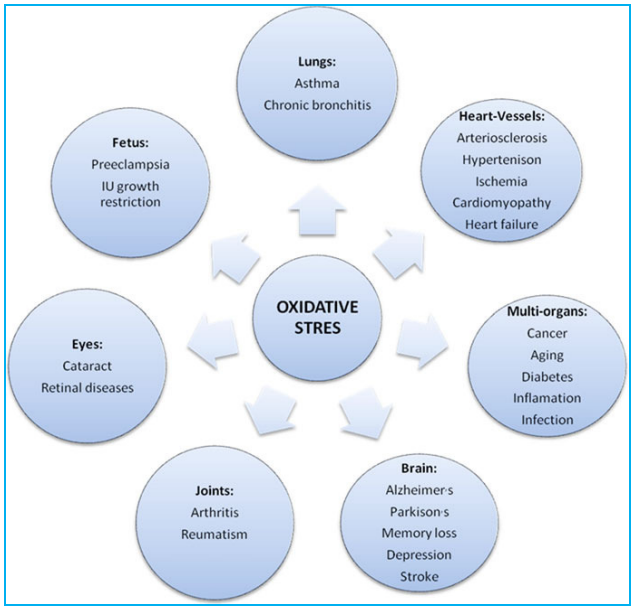


Fig 2.1b Diseases induced by oxidative stress

(source Kosanic and Rankovic, 2015)

Rankovic *et. al.,* (2011) tested lichens *Cladonia furcata, Lecanora atra* and *Lecanora muralis* for antioxidant activity. Of the lichens tested, *Lecanora atra* had largest free radical scavenging activity (94.7% inhibition), which was greater than the standard antioxidants.

Acetone, methanol and aqueous extracts of the lichen *Cetraria islandica, Lecanora atra, Parmelia pertusa, Pseudoevernia furfuraceae* and

Umbilicaria cylindrica exhibited strong antioxidant activity (Kosanic and Rankovic, 2011). A marked antioxidant activity of *Everniastrum cirrhatum* (Parmeliaceae) may be attributed due to its phenol content (Kekuda *et. al.,* 2011). It can be assumed that antioxidative nature of the extracts might depend on their phenolics. Phenolic components are potential antioxidants, free radical terminators and antibacterials (Shahidi and Wanasundara, 1992; Kaushik *et. al.*, 2010; Pereira *et. al.,* 2007; Oliveira *et. al.*, 2008; Gursoy *et. al.,* 2009; Turkoglu *et. al.,* 2010; Roman *et. al.*, 2010; Gulumser *et. al.*, 2010). These compounds are the main agents that can donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first initiation step. This high potential of phenolic compounds to scavenge radicals such as singlet oxygen, superoxide and hydroxyl radicals may be explained by their phenolic hydroxyl groups (Sawa *et. al.,* 1999). Flavonoids are also the most important natural phenolics and they possess a broad spectrum of chemical and biological activities including radical scavenging properties as observed by Mohammed (2010). Previous workers like Rankovic (2010), Odabasoglu *et. al.,* (2004), Mukherjee *et. al.*, (2011) found a high correlations between antioxidative activities and phenolic content. Interestingly, Odabasoglu *et. al.*, 2004 reported that in some lichens extracts no correlation was found between the total phenol and the antioxidant activity, suggesting that the antioxidant activity of different lichens may also depend on other, non-phenol components. Gulcin *et. al.*, (2002) reported that the aqueous extracts of *Cetraria islandica* had a strong antioxidant activity. Similar results were reported by Behera *et. al.,* (2005) for different extracts from the lichen *Usnea ghattensis*.

Katalinic *et. al.,* (2006) analyzed the total phenolic content (TPC) and antioxidant capacity of 70 medicinal plant infusions by FRAP assay. The best results were obtained for *Melissae folium* infusions.

Hexane extract of *Ramalina roesleri* was highly active against *Staphylococcus aureus* and *Streptococcus mutans*. The 1, 1-diphenyl-2-picryl-hydrazil (DPPH) radical scavenging activity of extracts ranged from 29.42% to 87.90%. Atranorin, protolichesterinic acid, usnic acid, 2-hydroxy-4-methoxy-6-propyl benzoic acid, homosekikaic acid, sekikaic acid, benzoic acid, 2, 4-dihydroxy-6-propyl and 2, 4-dihydroxy-3,6-dimethyl benzoate were isolated from the hexane extract. Maximum DPPH radical scavenging activity

was exhibited by sekikaic acid followed by homosekikaic acid (Sisodia *et. al.*, 2013).

Antioxidant potential and anticancer activity against breast carcinoma of 70% methanolic extract of lichen, *Parmotrema reticulatum* was performed by Ghate *et. al.*,(2013), phytochemical analysis of methanolic extract confirmed the presence of various phytoconstituents like alkaloids, carbohydrates, flavonoids, glycosides, phenols, saponins, tannins, anthraquinones and ascorbic acid; among which alkaloids, phenols and flavonoids were found in abundant amount. High performance liquid chromatography (HPLC) analysis of the lichens methanolic extract revealed the presence of catechin inferred that methanolic extract of *P. reticulatum* possessed antioxidant property which may be further developed into an anticancer agent., purpurin, tannic acid and reserpine. It was hence

In vitro antioxidant activity, phenolic content of different extracts from four lichens species *Cladonia ragiferina*, *Cladonia sylvatica* , *Evernia prunastri* and *Usnea barbata* collected in the Republic of Mari El in Russia: was evaluated. Studies of lichen's antioxidant, radical scavenging activity in relation to responsible compounds were essential to identify natural compounds that could be used to reduce oxidative stress associated with many human diseases (Koptina *et. al.*, 2013).

2.9 Synergistic activity

In addition to determining the antimicrobial and antioxidant activities of plant extracts, studies have also been made wide over the world on synergistic activities of plants together with other plants or antibiotics. Synergism, this is when combined action is significantly greater than the sum of the both effects (Eugene *et. al.*, 2004). There have been many studies about the beneficial role of bioactive plant extracts and pure isolated compounds in increasing the *in vitro* efficacy of commonly used antibiotics against variety of microorganisms, these studies have reported the use of plant extracts in combination with antibiotics, with significant reduction in the minimum inhibitory concentrations of the antibiotics (Hu *et. al.,* 2002; Nagoshi *et. al.*, 2006; Betoni *et. al.*, 2006; Purushotam *et. al.,* 2010)

The combined plant extracts of black thyme (*Thymbra spicata* L), fennel (*Foeniculum vulgare* Mill), sage (*Salvia pilifera*), wild tea (*Stachys pumilia*) and wild mint (*Micromeria fruticosa*. L) had strong antibacterial

activity against pathogenic bacteria and lactic acid bacteria (Yasar *et. al.*, 2004). The synergistic antimicrobial activity of tea and various antibiotics against enteropathogens was reported by Tiwari *et. al.*, (2005). Tea extract showed synergistic activity with chloramphenicol and other antibiotics like gentamycin, methicillin and nalidixic acid against test strains

The combination of ethanolic extracts of the plants *Mentha longifolia, Melissa officinalis* and *Rosa damascena* showed synergistic antibacterial activity against MRSA (methicillin resistant *Staphylococcus aureus*) strains (Bassam *et. al.,* 2006).

Combination of *Vangueria spinosa* with doxycycline and ofloxacin against gram positive and gram negative bacteria was also tested (Chatterjee *et. al.*, 2009). Synergism between 13 antimicrobial drugs and plant extracts namely "guaco" (*Mikania glomerata*), guava *(Psidium guajava*), clove (*Syzygium aromaticum*), garlic (*Allium sativum*), lemongrass (*Cymbopogon citratus*), ginger (*Zingiber officinale*), "carqueja" (*Baccharis trimera*) and mint (*Mentha piperata*) against *Staphylococcus aureus* strains reported (Betoni *et. al.,* 2006*).* Esimone *et. al.*, (2006) investigated the herb-drug interaction between tea extract and penicillin G against *Staphylococcus aureus* and showed additive interactions. Nostro *et. al.*, (2006) demonstrated that combinations of propolis extract with clarithromycin and *Zingiber officinale* with clarithromycin could control *Helicobacter pylori* associated with gastroduodenal disease.

Sibanda and Okoh (2008) showed potentials of synergy between acetone extracts of *Garcinia kola* seeds and amoxicillin, ciprofloxacin, tetracycline and chloramphenicol against pathogenic microorganisms. *Balanites aegyptiaca* (L) Del. (Balanitaceae), *Hyptis sauveolens* Poit (Lamiaceae), *Lawsonia inermis* L. (Lathyraceae), *Leucas aspera* L.(Lamiaceae), *Nicotianae folia* Roth.ex. Roem and Schult (Lobileaceae) and *Phyllanthus madraspatana* (Euphorbiaceae) treated individually and in combination for their antimicrobial activity against five different diarrhaegenic bacteria and proved that there is a scope to develop antimicrobial agent by combinations of plants and antibiotics (Karmegam *et. al.,* 2003).

Water extracts of *Psidium guajava*, *Rosmarinus officinalis*, *Salvia fruticosa*, *Majorana syriaca*, *Ocimum basilucum, Syzygium aromaticum*, *Laurus nobilis* and *Rosa damascene* was screened alone and then in

synergy testing of these extracts with known antimicrobial agents of different mechanisms (protein synthesis inhibition: oxytetracycline HCl and gentamicin sulfate; cell wall synthesis inhibition: penicillin G and cephalexin; folic acid synthesis inhibition: Sulfadimethoxine as sodium; and nucleic acid synthesis inhibition: enrofloxacin) using both well-diffusion and microdilution method against five *S. aureus* isolates; one is Methicillin-resistant *Staphylococcus aureus* (MRSA) and 4 Methicillin-sensitive *Staphylococcus aureus* (MSSA). As observed by many researchers synergistic effect between antimicrobial agent and plant extract occurred in both sensitive and resistant strains but the magnitude of minimum fold inhibition in resistant strains especially MRSA strain was higher than the sensitive strains. Shangmugam *et. al.,* (2008) and Matias *et. al.*, (2011) concluded that the use of plant extracts with antibiotics shows a low risk of increasing bacterial resistance to the action of antibiotics modifying agents, as the extracts contain mixtures of different bioactive compounds, which make microbial adaptability very difficult comparing to single-constituent antibiotics and using plant products with antibiotics could decrease undesirable side effects of antibiotics, so the combinations of antibiotics with plant extracts could be a significant basis for development of new approach in resistance.

A study was made, to formulate new cost effective antimicrobial agent for multi drug resistant organisms, based on the synergistic activity of tetracycline with methanolic extract of *Tectona grandis* (Purushotam *et .al.,* 2010).

Salvadora persica contains a number of medicinally beneficial properties including abrasives, antiseptics, astringent, detergents, enzyme inhibitors and fluoride Almas *et. al.*, (2004). Eight years later Ahmed *et. al.*, (2010) investigated *Salvadora persica*, the same medicinal plant of repute for its antimicrobial activities with two antibiotics viz., penicillin and tetracycline against *Staphylococcus aureus* separately and in combination (synergistic), showed their synergistic effect was much more effective. The highest inhibition was noticed (31.5 mm) when *S. aureus* was exposed to tetracycline plus *Salvadora* stem extract.

Chatterjee *et. al.*, (2009) also studied the combined use of ethanolic leaf extracts of *Vangueria spinosa* Roxb. (Rubiaceae) and antibiotics (doxycycline and ofloxacin) against Gram-positive bacterium (*Staphylococcus*

aureus) and three Gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and observed synergistic actions in all the cases except against *P. aeruginosa.*

Varthemia iphionoides exhibited MIC value of 0.03mg/ml against ATCC strains of *Staphylococcus aureus*, *Bacillis subtilis*, *S. epidermidis* and 4.0mg/ml against *E. coli, V. iphionoides* and Cefotaxime was synergistically tested and it was seen that FIC (Fractional inhibitory concentration against *B. subtilis* (ATCC 6633) and *S. aureus* was 0.75 to .0875mg/ml but FIC against *E. coli* and *S. epiderdimis* 2.5 to 16.4mg/ml (Awni *et. al., 2*009).

Parmotrema pseudotinctorum (des. Abb.) Hale and *Ramalina hossei* H. Magn & G. Awasthi has been screened individually and in combination with honey for its antimicrobial and antioxidant activity but the combination was not as effective as scavenging potential of individual lichen extracts. The bacteria namely *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were screened for their sensitivity towards the lichen extracts, honey and their combination by agar well diffusion method (Tepe *et. al.,* 2004). Even in antibacterial assay extracts of *Parmotrema pseudotinctorum* and *Ramalina hossei* exhibited marked antibacterial activity individually than in combination with honey. Thus, combination of lichen extracts and honey was not found to exert any synergistic action (Kekuda *et. al.*, 2009).

Antimicrobial and antifungal activity of *Lawsonia inermis*, *Punica granatum* and *Hibiscus sabdariffa,* again their synergistic effect by mixing plant extracts with antibiotic was performed. The methanolic extract of *Punica granatum* showed the highest antibacterial activity especially against *Staphylococcus aureus,* while*, Klebsiella pneumoniae* and *Escherichia coli* showed the least sensitivity to it, combining antibiotics to plant extract showed synergistic antibacterial activity especially with Ciprofloxacin and Erythromycin on *Pseudomonas aeruginosa,* and *Staphylococcus aureu*s, respectively. Mixture of Ketoconazole and Fluconazole drugs with aqueous extract of *Hibiscus sabdariffa* was highly effective. Nystatin, an antifungal showed higher activity when mixed with plant extract (Abdelraouf *et. al.*, 2011).

Methanol extract of *R. farinaceae* and ampicillin showed synergism and penetrated and prevented the growth of certain Gram negative bacteria which proved that methanol extract of *R. farinaceae* and ampicillin could be used as an antibiotic (Agboke and Esimone, 2011).

The combinations of acetone and ethyl acetate extract of *Cichorium intybus* and antibiotics (amoxicillin and chloramphenicol) resulted in additive and indifferent effects against tested bacteria (Stefanovi *et. al.*, 2012).

In a recent study (Jonshon *et. al.,* 2015) it was suggested that the combination any two plants extracts may not always lead to synergism but in some cases their individual efficacies may be inhibited in combination and which may lead to antagonism. Methanolic extract of leaves of *Kigelia africana*, *Alafia bateri*, *Anthocleista djalonesis* and the stem bark of *Harungana madagascarensis* were screened for phytochemicals, free radical scavenging activity and total phenolic content. The antioxidant activity of combination of these plants resulted in antagonism.

2.10 Spectral analysis of lichens and other plants

The identification of active principle compounds in various plant samples is carried out using methods like TLC, LCMS and GCMS analysis.

Previously Manojlovic (2010), developed HPLC method for characterization of xanthones and anthraquinones in extracts of lichen *Laurera benguelensis*. Lichexanthone, secalonic acid D, norlichexanthon, paretin, emodin, telochistin, and citreorosein were thus detected by relative retention time and spectral data.

Kinoshita *et. al.*, (2010), worked out a yellow and new dark red pigments isolated from *Lethariella sernanderi*, *L. cashmeriana* and *L. sinensis* as antioxidant components. The yellow pigment was identified as canarione (1), and the others were determined to be 1,2-quinone derivatives, rubrocashmeriquinone (2) and 7-chlororubrocashmeriquinone (3), and 7-chlorocanarione (4) by analysis of their spectroscopic data.

Nagarajan *et. al.*, (2011), characterized the bioactive principle compound, a fatty acid derivative namely methyl 6, 7-dithia stearate from *Wrightia tomentosa* through mass spectrum $WTEF_{24}$ analysis.

Bangajaualli and Ramasuhramanian (2015), investigated and determined possible 16 bioactive components from ethanol bark and 24 compounds from leaf of *Aglaia elaeagnoidea* using GCMS analysis. The identified compounds possessed antioxidant, antitumour, analgesic, antiinflamatory and antimicrobial properties. Two important bioactive compounds namely squalene and phytol thus found to possess

chemopreventive activity against colon carcinogenesis and effective against different different stages of arthritis respectively.

2.11 Methodology for determination of antimicrobial and antioxidant activity of lichen extracts

The lichen specimens can be identified with the help of guidelines described in the communication of British Lichen Society. Each specimen are observed for their morphology, anatomy, colour reaction, thin layer chromatography, etc. For identification of genus and species, the key to macro lichens (Awasthi, 1988) and key to micro lichens (Awasthi, 1991) are generally consulted. Extraction of lichen compounds are usually done following the procedure of (Turk *et. al.*, 2003).

The Kirby and Bauer disk diffusion method (National Committee for Clinical Laboratory Standards, 1993) is generally used to determine the antimicrobial activity of lichen extracts against test bacteria and fungi. MIC determinations of the extracts can be carried out using above mentioned method. It is usually calculated for the test bacteria only that show antimicrobial activity following agar diffusion method (Perez *et. al.*, 1990).

Minimum inhibition concentrations (MICs) are determined by checking the inhibition zones formed. Bioautographic assay of lichen substances is usually done by the methods described by Turk *et. al.,* (2003). Sometimes bioautographic assay is done after the separation of compounds in thin layer chromatography (Culberson and Amman, 1979; Schumm, 2002). MICs, are determined a certain volume of extract of lichens is spotted on silica gel thin layer chromatography (TLC) plates and then the TLC plates are developed in three solvent systems usually employed in the TLC of lichen substances. Solvent system A contained a mixture of toluene/dioxane/ glacial acetic acid (36:9:1 v/v), the solvent system B contain hexane/diethyl ether/formic acid (24:18:4 v/v), the solvent system C contain toluene/ glacial acetic (20:3 v/v) TLC. It is characterized by checking its Rf values in different solvent systems with the ones given in the literature and its melting point (Schumm, 2002).

Numerous methods and modifications have been proposed to evaluate antioxidant activity and to explain how antioxidants function, of these total antioxidant activity, reducing power, DPPH assay, metal

chelating, ROS quenching assays are commonly used for evaluation of antioxidant activities of extracts (Duh *et. al.*,1999)

The lichen extracts can be precisely investigated for antioxidant activity by five different methods: DPPH radical scavenging, reducing power, determination of total phenolic compounds and determination of total flavonoid content. Different antioxidant activities of the test extracts are studied in comparison to known antioxidants such as ascorbic acid, butylated hydroxyl anisole (BHA) or butylated hydroxyl toluene (BHT) ,and α-tocopherol. These methods have been employed since many years with slight modification. The free radical scavenging activity of the fractions are measured *in vitro* by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (Gadow *et al.*, 1997; Dorman *et. al., 2*004; Liyana et.al., 2005).

However in general Ascorbic acid and BHT are used as references. The ability to scavenge DPPH radical is calculated by the following equation: DPPH radical scavenging activity (%) = [(Abs control – Abs sample)]

(Abs control)] x 100

where Abs control is the absorbance of DPPH radical + methanol; Abs sample is the absorbance of DPPH radical + sample extract /standard.

The method of (Oyaizu, 1986) was used to determine the reducing power of extracts. The determination of the total antioxidant activity (FRAP assay) in the extract is done with a modified method by (Benzie and Strain 1996). The determination of total phenolics content employs Folin and Ciocalteu's phenol reagent which response depending on the chemical structure of phenolics (i.e. the higher the number of functional –OH group the higher the total phenolics content). For determination of total soluble phenolic compounds in the lichen extracts Folin- Ciocalteu reagent are used according to the method of Slinkard and Slingleton (1997). Total phenolic content are expressed as mg/g tannic acid equivalent using the following equation based on the calibration curve: $y = mx+c$, and finding R^2 ,where x was the absorbance and y was the tannic acid equivalent (mg/g). Estimation of the total flavonoids in the plant extracts was carried out using the method of Meda *et. al.,* (2005); Ordon Ez *et. al.,* (2006). Total flavonoid content was calculated as quercetin equivalent/mg using the following equation based on the calibration curve: $y = mx+c$, and finding R^2 where x is the absorbance and was the quercetin equivalent/mg. The total antioxidant activity by phosphomolybdenum method as in total antioxidant capacity of turmeric oil,

and its different fractions are evaluated by the method of (Prieto *et. al.,* 1999). Evaluation of antioxidant capacity is also performed by the colorimetric assay as Trolox equivalent antioxidant capacity (TEAC) (Nedeljko *et. al.,* 2010).

MATERIALS AND METHODS

3.1. Media used *(g/l unless otherwise stated)*

(pH of the media was adjusted before autoclaving unless otherwise stated).

3.1.1 Nutrient Broth

Beef Extract	3.0
Peptone	5.0
NaCl	5.0
pH	6.8±2

3.1.2 Nutrient Agar

Beef Extract	3.0
Peptone	5.0
NaCl	5.0
Agar	20.0
pH	7.0±2

3.1.3 King's medium B

K_2PO_4	3.0
Na_2HPO_4	1.0
NH_4Cl	1.0
$MgSO_4.7H_2O$	0.4
Glycerol	15ml
Agar	15.0

3.2. Reagents used

3.2.1 Protein estimation

a. *Biuret reagent*

0.2N NaoH Solution:

Dissolved 8gm of Sodium hydroxide in 1000ml of dH_2O. Dissolved 3gm $CuSo_4.5H_2O$ and 9 gm of sodium potassium tartarate in 500ml of 0.2N of NaOH solution. Potassium iodide 5gm was added and volume was made to 1 litre by adding 0.2N NaOH solution.

b. BSA stock solution

Powdered 1200mg of BSA was weighed out into 100ml volumetric flask and was dissolved gradually in 10ml distilled water with careful shaking; a few drops of 0.2 (N) NaOH solution was added to facilitate the solution and volume was made up to 100ml.

3.2.2. Determination of DPPH Radical scavenging activity

a. 0.3mM of DPPH

Dissolved 1mg of DPPH in 25ml ethanol (100%)

3.2.3 Determination of total antioxidant activity

a 0.6M H_2SO_4

5.8ml of H_2SO_4 was added to dH_2O and volume was made up to 100ml.

b. 28 mM Sodium phosphate

Dissolved 1.064gm NaH_2PO_4 in 100ml dH_2O.

c. 4 mM Ammonium molybdate

Dissolved 494 mg of $NH_4.MoO_4$

in 100ml dH_2O.

3.2.4 Determination of Reducing power ability

a. Phosphate buffer (pH 6.6)

Dissolved 2M Monobasic sodium phosphate 276g/l (stock solution A) and 2M Dibasic sodium phosphate 284g/l (stock solution B).62.5 (A) +37.5(B), and volume was made up to 200ml by adding water.

b. Potassium ferricyanide

Dissolved $K_3Fe(CN)_6$ 1.64 gm in

100ml of dH_2O

c. Trichloroacetic acid (10%)

Dissolved 1.63gm of Trichloroacetic acid in dH_20 and volume made up to 100ml.

d. Ferric chloride (1%)

One gm of $FeCl_3.6H_2O$ in dH_2O
and volume made up to 100ml

3.2.5 Determination of Total phenolic content

*a. Folin ciocalteu reagent (*1:10 v/v with water)

To 10 ml of reagent 100 ml of dH_2O was added

b. Sodium carbonate (75g/lit)

Dissolved 75 gm of sodium carbonate and volume was made up to 1000 ml by adding of dH_2O

3.2.6 *Determination of Total Flavonoid content*

a. Aluminium nitrate (10%) in 80% ethanol

Dissolved 2 gm of aluminum nitrate in 20 ml ethanol (80%)

b. Potassium acetate (1M)

Dissolved 1.96gm of *Potassium acetate* in 20ml ethanol (80%).

3.2.7 *Determination of Catalase activity*

a. 0.05M phosphate buffer pH 6.5

Dissolved 1.56gm of $NaH_2PO_4.2H_2O$ in 50 ml of d H_2O (A) + 1.78gm of $Na_2HPO_4.2H_2O$ in 50 ml of dH_2O (B)

34.25 ml of reagent (A) + 15.75 ml of reagent (B), pH was adjusted to 6.5 and volume was made up to 100 ml.

b. *0.0025M H_2O_2*

H_2O_2 (0.028 ml) 30% mixed to 100 ml of d. H_2O

c.*0.1% Titanium sulphate*

$TiSO_4$ (15%) 0.1ml added to 14.9 ml of 25%

H_2SO_4.

c.*25% H_2SO_4*

(Conc. H_2SO_4) 25.5ml was added to 74.5 ml of dH_2O

3.2.8 *Determination of Peroxidase activity*

*a.300µM phosphate buffer (*pH -6.8*)*

Dissolved 2.34gm of $NaH_2PO_4.2H_2O$ in 50 ml dH_2O(A).Dissolved 2.66gm of $Na_2HPO_4.2H_2O$ in 50 ml dH_2O(B)

25.5 ml of (A) + 24.5 ml (B) was mixed and volume was made up to 50 ml. By adding d.H_2O

b. *Pyragallol*

Dissolved 189mg of Pyragallol in 100ml of dH_2O.

c. 5% H_2SO_4

Added 2ml of 25% H_2SO_4 to 8ml of distilled water.

3.3. Instruments used

1. Laminar air flow cabinet (MSW-161, Macro Scientific Works, New Delhi, India).

2.Orbital shaking incubator (MSW-232, Macro Scientific Works, New Delhi).

3. Digital Balance (Sartorius, Germany)

4. Hot air oven (MSW-211, Macro Scientific Works, New Delhi)

5. Spectrophotometer (Shimadzu UV-1700, Japan)

6. Sonicator (Labsonic M, 100 W, Sartorius, Japan)

7. Cooling centrifuge (REMI, C-30 BL, India)

8. Rotary evaporator (Ricon, India)

9. UV-Transilluminator (Bangalore Genei)

10. Vortex mixer (REMI, India)

11.Digital camera (SONY, Japan)

3.4. Methodology

3.4.1. Area of Study

Darjeeling District (27° 13" N to 26° 27" N and 88° 53" E to 87° 59" E), is the northernmost District of West Bengal. The area under Darjeeling District is flanked by North Dinajpur District from South, Bangladesh from South-East, Bihar from South-West, Nepal from West, Sikkim from North and Bhutan from North-East.

The District has four Administrative Sub-divisions namely, Darjeeling, Kalimpong, Kurseong and Siliguri. The area of Darjeeling District falls under the Northern Hill Zone and Teesta is the most important river of this zone. It's principle tributaries are the Rangphu and Relli on the left bank; Rangeet, Reang and Sevoke on the right bank. In the west of Teesta, the Mahanadi, Balasan and Mechi merge into Ganges.

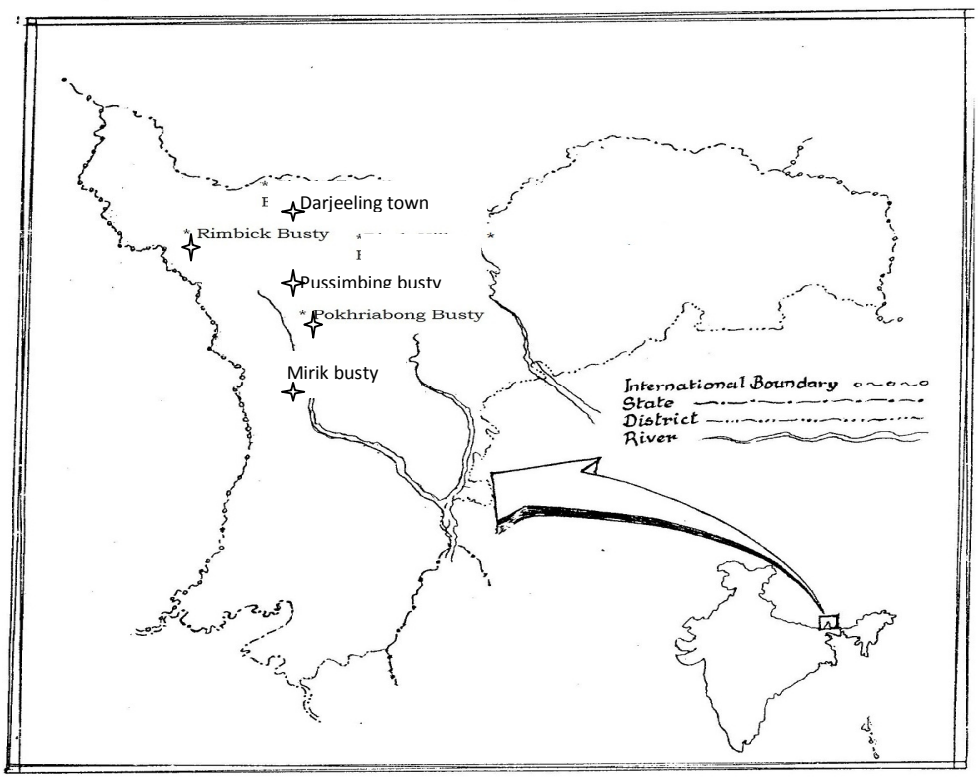


Figure 3.1 Area of study showing sampling sites

The geographical area of hill areas of Darjeeling District is 247800 hectare. About 18% of the soil is highly acidic (pH below 4.9), 60% is

moderately acidic (pH 5.0 to 5.9) and the rest 22% is normal (above 6.0). The rainfall varies from 2941mm to 1391mm of which 80% is received during June to September. The average maximum and minimum temperature round the year records are 20˚C and 2˚C respectively. Details of climatic conditions are given in Table 3.1 and 3.2 respectively.

Table 3.1 Climatic conditions of Darjeeling District- Average monthly rainfall for last five years

Rainfall (mm)					
Month	2011	2012	2013	2014	2015
January	0	0	0	0	0
February	39	0	28.2	3.4	0
March	14	0	19.2	33.6	0
April	48	204	54	47	98.7
May	123	81	294.9	159	87
June	629	339	261.6	331.8	244
July	961	529	310.5	452.6	356.6
August	667	284	319.2	543.5	278.1
September	438	505	199.7	305.8	326.6
October	14	18	159.7	4.8	0
November	8	0	0	0	0
December	0	0	0	0	0
Total	2941	1960	1647	1881.5	1391

Source: Deputy Director of Agriculture (Administrative), Darjeeling)

Table 3.2.Climatic conditions of Darjeeling District- Average monthly temperature for last five years

Month	Temperature (°C)									
	2011		2012		2013		2014		2015	
	Mean maxi	Mean Mini	Mean Maxi	Mean Mini	Mean Maxi	Mean Mini	Mean Maxi	Mean Mini	Mean Maxi	Mean Mini
January	9.8	1.3	9.5	2.0	8.1	1.5	9.5	1.7	10.2	1.7
February	15.1	4.3	10.5	4.0	9.1	1.9	12.4	3.3	12.6	3.8
March	19.1	7.6	10.5	6.0	13.5	5.6	20.2	8.1	18.4	5.7
April	19.7	8.0	22.3	9.0	16.9	9.2	19.5	9.1	20.5	8.3
May	20.0	12.3	21.5	8.8	17.8	11.2	19.8	11.3	22.2	9.8
June	19.4	14.5	20.0	16.0	18.6	13.4	20.3	13.5	24.1	15.1
July	18.5	15.0	20.0	15.5	19.1	14.3	20.8	14.0	21.5	15.0
August	19.9	14.8	22.0	15.6	18.8	14.1	21.5	15.8	22.2	16.0
September	20.2	14.2	21.5	15.0	18.3	13.2	24.2	13.5	23.4	15.2
October	18.8	12.7	20.1	12.0	16.3	9.9	19.5	11.1	18.6	13.0
November	12.0	6.8	17.0	6.5	12.8	5.9	15.8	6.2	14.8	6.5
December	12.5	4.5	14.0	5.5	9.6	2.4	14.6	4.4	12.9	6.2

Source: Deputy Director of Agriculture (Administrative), Darjeeling

3.4.2. Sampling sites: The sampling sites were selected on the basis of elevation, vegetation and population status (Table 3.3.). Two sampling sites located at sub-tropical region are Mirik, Pokhriabong Busty and three other are located at temperate region [Darjeeling town, Pussimbing Busty and Rimbick busty].

Table 3.3 Elevation and habitat of sampling sites

Sl No.	Sampling sites	Altitude (msl)	Habitat
1.	Darjeeling town	2130	*Alnus* trees
2.	Pussimbing Busty	2000	*Erythrina* trees, rock
3.	Mirik Busty	1300	*Citrus* trees
4.	Pokhriabong Busty	1550	*Macaranga*, *Alnus* trees
5.	Rimbick Busty	2600	*Prunus*, *Betula*, *Alnus* trees

3.4.3. a. Collection of lichen samples:

Lichen samples were collected in paper polypacks from different sampling sites described in Table 3.3. Samples were collected from the barks of trees like *Alnus*, *Erythrina, Macaranga, Citrus*, *Betula*, *Prunus* as well as rocks and brought to the laboratory. Each specimen was preliminarily identified with the help of available literature, Key to Macrolichens (Awasthi, 1988). The taxonomic identity of lichen samples was confirmed from the Lichenology Laboratory, National Botanical Research Institute, Lucknow, Uttar Pradesh, India and the voucher specimens were deposited in the Herbarium of the Postgraduate Department of Botany, Darjeeling Government College, Darjeeling, India.

3.4.3. b. Collection of medicinal plant samples:

The medicinal plants under study were collected from profusely grown places of Darjeeling and surrounding areas. The lichen and medicinal plant samples are deposited in P.G Department of Botany, Darjeeling Government College, Darjeeling, West Bengal, India

3.4.4. Extraction of samples

Each lichen and medicinal plant sample was washed to remove debris, dried and ground to powder and was stored in sterile glass bottle in the refrigerator. The 10g portions of sieved powder were added to 100 ml of solvents (ethanol and methanol), sonicated for 30 min and left overnight at room temperature. The crude extract was prepared by decanting, followed by filtration through muslin cloth and further filtered with Whatman No. 1 filter paper to obtain a clear filtrate. Fifty ml of the filtrate was evaporated to obtain 10 ml of concentrated extract and sterilized by membrane filtration using 450 nm bacteriological filters. Such sterilized filtrate was stored in screw capped airtight containers in the refrigerator and used for antimicrobial screening. The remaining (50 ml) filtrates were concentrated to paste in reduced pressure at 40°C using a rotary evaporator and were used for the determination of minimal inhibitory concentration (MIC) value, protein content and growth of the test microorganisms and antioxidant assays.

Table 3.4 List of lichens and medicinal plant samples

Sl No.	Name of lichens and medicinal plants	Extraction solvent	Extract code
1	*Everniastrum* sp	ethanol	EVRE
		methanol	EVRM
2	*Parmotrema reticulatum*	ethanol	PARE
		methanol	PARM
3	*Ramalina hossei*	ethanol	RARE
		methanol	RARM/CLRM
4	*Sterecaulon pomiferum*	ethanol	STRE
		methanol	STRM
5	*Usnea baileyi*	ethanol	USRE
		methanol	USRM
6	*Berginia ciliata*	ethanol	BERE
		methanol	BERM
7	*Panax pseudoginseng*	ethanol	PNXE
		methanol	PNXM
8	*Sapindus mukrossi*	ethanol	SAPE
		ethanol	SAPM
9	*Urtica dioica*	methanol	URRE
		methanol	URRM

3.4.5. Extract yield (%) of extracts

Extract yields of dried extracts were calculated the following equation: % Extract yield = (W_1x100) / W_2. W_1 shows the remaining solid lichen extract weight after evaporation of the solvent used in extraction; W_2 shows the weight of lichen powder form used in extraction (Aydin and Kinadiglu, 2013).

3.4.6. Test microorganisms

Test microorganisms (seven bacteria and one fungus) were obtained from Institute of Microbial Technology, Chandigarh, India (table.3.5).the bacterial culture was preserved in N.A medium and fungal culture in King's medium B.

Table 3.5. List of test microorganism

Sl No.	Test Microorganisms	Gram nature	MTCC Code
1	*Alcaligenes faecalis*	Gram negative	MTCC9780
2	*Bacillus megaterium*	Gram positive	MTCC 7192
3	*Bacillus subtilis*	Gram positive	MTCC 3972
4	*Candida albicans*	-	MTCC 4748
5	*Escherichia coli*	Gram negative	MTCC 6365
6	*Enterobacter aerogenes*	Gram negative	MTCC 111
7	*Staphylococcus aureus*	Gram positive	MTCC 7443
8	*Pseudomonas aeruginosa*	Gram negative	MTCC 424

3.4.7. Screening of antimicrobial activity

This procedure is based on disc diffusion method of Bauer *et. al.,* (1966). Overnight grown bacterial cultures of approximately (0.1ml) were spread plated on nutrient agar plates to achieve semi confluent growth. Sterile filter paper discs were soaked in concentrated extracts, allowed to dry between the applications and placed on plates which were then incubated at 37°C for 24 hrs. Streptomycin (10µg/ml) and sterile distilled water were taken as positive control and negative control respectively. Growth was evaluated and inhibition zone were measured. All the experiments were repeated thrice and data presented are average of three independent readings.

3.4.8. Determination of minimal inhibitory concentration of extracts against test microorganisms

The filtrate was evaporated to dryness under a rotary evaporator and the residues were dissolved in Dimethlyl sulfoxide (DMSO) and further diluted with sterile distilled water to obtain the concentrations (250 µg/ml, 500 µg/ml, 1000 µg/ml, 5000 µg/ml and 10000 µg/ml), (Javeria *et. al.*, 2013).

All of the test microorganisms in this study were used and the bioactivity was based on agar-diffusion assay (Dubey and Maheshwari, 2002). Overnight grown cultures (0.1 ml) were spread plated on nutrient agar plates. Cylindrical holes were made with the help of sterile cork borers on the petriplates containing test microorganisms. Different extract concentrations were filled to the holes and after overnight incubation at 37°C; the plates were screened for the production of inhibition zone. Minimum extract concentration which yielded inhibition zone was considered as their respective MIC value. The MIC values of lichen sample were compared to antibiotic, Streptomycin.

Plate 3.1 Lichen samples under study

Plate 3.2 Medicinal plants under study

3.4.9. DPPH radical scavenging assay

The free radical scavenging activity of the extracts was measured *in vitro* by 1, 1-diphenyl-2- Picryl-hydrazyl (DPPH) assay (Nagarajan *et. al.*, 2008). Solution having strength of 0.3 mM DPPH in ethanol and methanol was prepared and 1 ml of this solution was added to 3 ml of the extract residue dissolved in ethanol/ methanol at different concentrations (25-200 µg/ml). This mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm. Ascorbic acid was taken as reference. The ability to scavenge DPPH radical was calculated by the following equation (Adedapo *et. al.*, 2009):

$$\text{DPPH radical scavenging activity (\%)} = \frac{[(Abs_{control} - Abs_{sample})]}{(Abs_{control})]} \times 100$$

Where $Abs_{control}$ is the absorbance of DPPH radical + solvent; Abs_{sample} is the absorbance of DPPH radical + sample extract /standard.

For the study of combined activities respective solvents extracts from different samples were mixed in equal proportion and used.

3.4.10. Reducing power ability

The reducing power was investigated by the Fe^{3+}-Fe^{2+} transformation in the presence of the extracts as described (Nagarajan *et. al.*, 2008). The Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. One ml of the extract (25-200 µg/ml), 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide were incubated at 50°C for 30 min. 2.5 ml of 10% trichloroacetic acid was added to the mixture and centrifuged for 10 min at 3000 rpm. Supernatant (2.5 ml) was diluted with 2.5 ml of water and was shaken with 0.5 ml of freshly prepared 0.1% ferric chloride. The absorbance was measured at 700 nm using spectrophotometer. Butylated hydroxy toluene, BHT (25-200 µg/ml) was used as the standard. All tests were performed in triplicate and the graph was plotted with the average of the three determinations. For the study of combined activities respective solvents extracts from different samples were mixed in proportion and used. The more change in colour of extracts to blue indicates the increased reduction of Fe^{3+} to Fe^{2+}.

3.4.11. Determination of total antioxidant capacity

The total antioxidant capacity of the extracts was determined with phosphomolybdate method using α-tocopherol as the standard (Nagarajan *et. al.*, 2008). An aliquot of 0.5 ml of the extracts solution was combined with 5 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95°C for 90 min. The extracts concentration (25-200 µg/ml) was prepared by dissolving the extract residue in respective solvents (ethanol and methanol). After the samples had cooled to room temperature, the absorbance was measured at 695 nm against the blank using an UV spectrophotometer. The blank solution contained 1.0 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as rest of the sample. All tests were performed in triplicate, the total antioxidant capacity was expressed as µg equivalents of α-tocopherol by using the standard α-tocopherol graph ($Y=0.141x-0.039$; $R^2 = 0.941$).

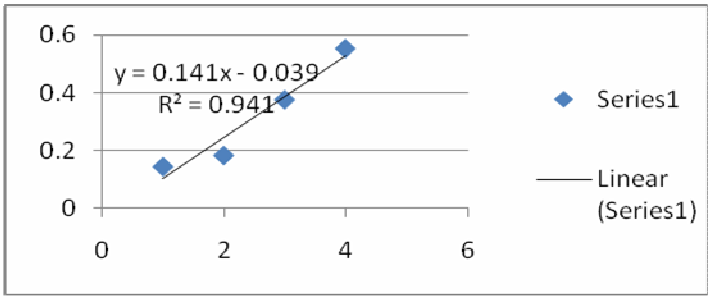


Fig 3.2 Standard α-tocopherol graph

3.4.12. Estimation of total flavonoid content

Total soluble flavonoid content of the extracts was determined with aluminium nitrate using quercetin as the standard (Nagarajan *et. al.*, 2008). One mg of the extract was added to 1ml of 80 % ethanol. An aliquot of 0.5 ml was added to test tubes containing 0.1 ml of 10 % aluminium nitrate, 0.1 ml of 1M potassium acetate and 4.3 ml of 80 % ethanol. The absorbance of the supernatant was measured at 415 nm in UV spectrophotometer after incubation at room temperature for 40 min. The lichen extracts concentrations (25-200 µg/ml) were made by dissolving the residue extract in respective solvents (ethanol/methanol). The total flavonoid content in the

extracts was determined as µg quercetin equivalent by using the standard quercetin graph using following equation based on calibration curve:- y=356x -0.461; R^2 = 0.697.

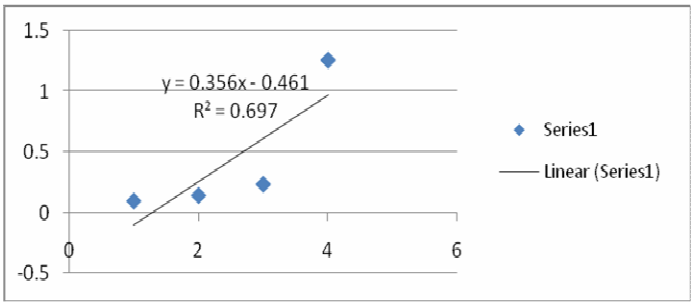


Fig.3.3 Standard quercetin graph

3.4.13 Determination of total phenolics

Total soluble phenol contents in the extracts were determined using Folin-Ciocalteu reagent using tannic acid as standard with some modifications (Adedapo *et. al.*, 2009). Five ml Folin-Ciocalteu reagent (previously diluted with water at 1:10 v/v) was added to 4 ml (75 g/l) of sodium carbonate and 0.1mg/ml extract. The mixtures were vortexed for 15 sec and allowed to stand for 30 min at 40°C for colour development. Absorbance was then measured at 765 nm using the UV spectrophotometer. The lichen extract (25-200µg/ml) were made by dissolving the residue extract in respective solvents i.e., ethanol and methanol separately. Total phenolic content were expressed as mg/g tannic acid equivalent using the following equation based on the calibration curve: - y=0.007x-0.186; R^2= 0.938.

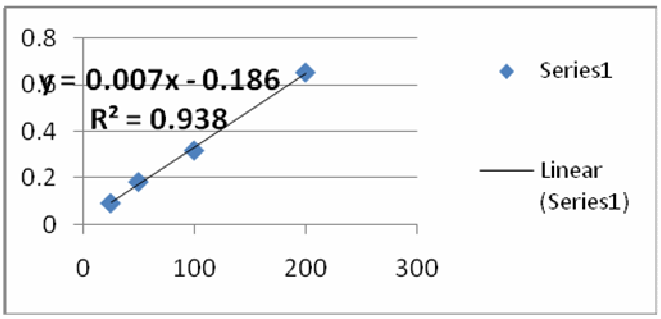


Fig. 3.4 Standard tannic acid curve

3.4.14. Catalase activity

One gm of freshly collected lichen thallus was homogenized with 10 ml of 0.05 M cold phosphate buffer (pH-6.5). The homogenate was then centrifuged at 5000 rpm for 10 minutes and supernatant was taken as enzyme extracts. One ml of 0.0025M H_2O_2 added to 1ml of crude enzyme extract, the mixture was then incubated at 37°C for 5 minutes. The reaction was stopped by adding 2 ml of 0.5 % $TiSO_4$. The content was further centrifuged for further 10 minutes and O.D of the golden yellow supernatant was measured using the UV spectrophotometer at 420nm. For control set, $TiSO_4$ added to the enzyme extract before the addition of H_2O_2 was taken and same procedure as for reaction mixture was followed. The enzyme activity was calculated by the formula of Luck (1974).

$$\text{Enzyme activity} = \frac{\Delta A \times TV}{t \times V}$$

[Where Δ A= O.D. difference; TV = total volume of enzyme extract; t = time of incubation taken; V= volume of enzyme extract taken for the reaction].

Peroxidase activity

One gram of freshly collected lichen thallus was homogenized with 10 ml of 300µM cold phosphate buffer (pH-6.8). The homogenate was then centrifuged at 5000 rpm for 10 min and supernatant was taken as enzyme extract. For reaction mixture 1ml of phosphate buffer and 1ml of pyragallol added to 1ml of enzyme extract which was incubated at 25° C for 5 minutes. The reaction was stopped by adding 5% H_2SO_4 and mixture was further centrifuged at 5000 rpm for 10 minutes. The O.D of the yellow coloured supernatant was measured using the UV spectrophotometer at 430 nm. For contol set 1ml of 5% H_2SO_4 added to the mixture of enzyme extract, pyragallol and H_2O_2 before the addition of buffer. The enzyme activity was calculated by the formula of Luck (1974):

$$\text{Enzyme activity} = \frac{\Delta A \times TV}{t \times V}$$

(Where Δ A=O.D. difference; TV = total volume of enzyme extract; t = time of incubation; V= volume of enzyme extract taken for the reaction).

3.4.16. Effect of lichen extracts on total protein content of the test microorganisms

Effect of lichen extracts on total protein content of the microorganisms grown culture filtrate was performed following the method of Ray *et. al.*, (2003) with some modification. Different concentration of extracts (100-500µg/ml) was prepared filtered and sterilized. Sterile nutrient broth each of 5 ml was inoculated with 24 hours old broth cultures of different microorganisms (0.1ml) each, to which was added 0.5ml of extract of different concentration. The whole content was incubated at 37ºC for 24 hours. The content was sonicated and filtered through Whatman No.1filter paper and was centrifuged at 10,000 rpm for 5 minutes.

The clear supernatant was taken and Biuret method was employed for protein estimation. Three ml of Biuret reagent was added to 3 ml of the supernatant incubated at 37°C for 10 minutes and was allowed to stand at room temperature for 30 minutes. Then absorbance of the mixture was measured at 540nm. The OD_{540} values were recorded and total protein content of the supernatant was estimated using BSA as standard.

3.4.17. Effect of lichen extracts on the growth of the test microorganisms

Effect of lichen extracts on growth of microorganisms was performed following the method of Ray *et. al.*, (2003) with some modifications. Nutrient broth (50 ml) were inoculated with different volume (0.2ml, 0.4ml, 0.6ml, 0.8ml and 1ml) of lichen extracts (ethanol and methanol) from the stock (1mg/ml) mixed well, inoculated with 0.5ml of overnight grown different test microorganisms and incubated in shaking incubator (150 rpm) at 37ºC. Broth without any lichen extract was taken as control. OD_{620} value of the content was taken at initial i.e., 0 hour and at intervals of one hour repeatedly until stationary phase of the test cultures was obtained. Incubation time taken by the microorganisms to reach stationary growth

phase was determined using a plot with O. D. values on Y axis and time interval on the X axis.

3.4.18. Preliminary separation of lichen compounds by TLC

Extraction and preliminary identification of lichen compounds was done following the method of Santiago *et. al.*, (2010). One gram, air-dried thalli of lichen specimens were initially ground to powder and soaked overnight in 10 ml acetone. Acetone was used for the microscale extraction of the lichen acid, as most lichen substances are soluble in this solvent (Huneck and Yoshimura, 1996). After 24 hours, the extracts were filtered, concentrated by air-drying for 4 – 5 days or until the extracts crystallized, and the weight/yield of the crude extracts were determined. To identify the lichen acids present, crude extracts were dissolved in acetone to a final concentration of 10 mg/ml. The crude extracts were then spotted on silica gel thin layer chromatography (TLC) plates (silica gel 60 F_{254} aluminum plates, Merck) and run in three different solvent systems: (1) Solvent System A- 36:9:1 toluene/dioxane/glacial acetic acid, (2) Solvent System B- 24:18:4 hexane/diethyl ether/formic acid, and (3) Solvent C- 20:3 toluene/glacial acetic acid (Culberson *et. al.*, 1972). Each TLC plate was then sprayed with 0.5 ml glacial acetic acid and 1 ml 97 % sulfuric acid and heated at 105°C for 5 minutes to visualize the lichen acids (Santos and Mondragon, 1969). The RF values for each spot were determined using the formula:

$$RF = \frac{\text{distance travelled by the solute}}{\text{distance travelled by the solvent}}$$

3.4.19. Determination of the bioactive lichen compounds using TLC bioautography

To determine the bioactive lichen acids (Santiago *et. al.*, 2010), lichen extracts from representative lichen species were initially spotted on TLC plates. The TLC plates were then run in Solvent System A (36:9:1 toluene/dioxane/glacial acetic acid) 2) Solvent System B: 24:18:4 hexane/diethyl ether/formic acid, and (3) Solvent C: 20:3 toluene/glacial acetic acid (Culberson *et. a*l., 1972) and the spots were visualized under ultraviolet (UV) light (254 nm). Prior to the TLC bioautography, the TLC plates were allowed to air-dry for at least 24 hours to remove any traces of

the solvent system. A bacterial suspension was prepared from a 24-hour old *S. aureus* and *E. coli* culture. About 100 µl of the bacterial suspension were mixed with 100 ml cooled, melted NA. The seeded NA was poured on top of the base medium (approximately 15 ml solidified NA) and allowed to solidify.

The TLC plates were placed on top of the seeded layer and stored for two hours inside a refrigerator. This was to allow the metabolites to diffuse directly into the seeded layer without allowing the growth of the test organisms. After two hours, the TLC plates were carefully removed and the culture plates were then incubated at 37°C for 18-24 hours. After incubation, the spots having zones of inhibition were noted.

3.4.20. Identification of active principle in lichen extract

Four lichen samples was air dried at room temperature (26°C) for until complete drying and then it was ground to powder. Powdered lichen material (10g) was added to 100ml methanol, sonicated and shaken for 7 days in shaking incubator at room temperature. The extract was filtered through whatman filter paper no 42 and was concentrated using a rotary evaporator the obtained extracts were sent to SAIF, CDRI, Lucknow for LCMS analysis.

The mass spectrum as LCMS chromatogram of EVRM, USRM, STRM and CLRM obtained from SAIF was studied following the literature - A catalogue of standardized chromatographic data of synthetic relationship for lichen substances (Elix, 2014) and lichen substances were determined.

3.4.21Statistical analysis:

Statistical analysis were calculated using Excel software (Microsoft 2007) and SPSS version 21.0 for Windows 2007. Statistical significance was determined by One way ANOVA using Duncan's post hoc test. Correlation coefficients (r) were determined by using Pearson's bivariate correlation test. All the results are shown as mean ± standard deviation (SD) of three parallel measurements.

RESULTS AND DISCUSSION

4.1. Lichen samples

Lichen samples were collected in paper polypacks from different sampling sites. Samples were collected from the profusely grown habitats such as bark of trees like *Alnus, Macaranga, Citrus, Betula, Prunus* and rock surface and brought to the laboratory. Each specimen was preliminarily designated with sample codes and later identified from the Lichenology Laboratory, National Botanical Research Institute, Lucknow, Uttar Pradesh, India which are presented with LWG code provided by the institute (Table 4.1)

Table 4.1. List of lichen samples collected, identified and used for the study

Sl no.	Sampling sites	Altitude (msl)	Habitat	Sample code	Lichen species	LWG code
1.	Darjeeling town	2130	*Phoenix* trees	USR	*Usnea baileyi*	08-0017193
2.	Pussimbing Busty	2000	Rock surface	STR	*Stereocaulon pomiferum*	Not alloted
3.	Mirik Busty	1300	*Citrus* trees	EVR	*Everniastrum* sp	09-0017195
4.	Pokhriabong	1550	*Macaranga*, *Alnus* trees	PAR	*Parmotrema reticulatum*	09-0017196
5.	Rimbick Busty	2600	*Prunus*, *Betula*, *Alnus* tree	RAR	*Ramalina hossei*	09-0017194

Five lichen species were collected from their natural habitats keeping in mind not to scrap off the whole population. *Usnea baileyi* and *Everniastrum* sp was collected from the bark of *Phoenix* trees and *Citrus* trees respectively. *Stereocaulon pomiferum* was collected from the rock surface; *Parmotrema reticulatum* from the barks of *Macaranga* and *Alnus* trees; *Ramalina hossei* from the barks of *Prunus*, *Betula* and *Alnus* trees.

Discussion

In all the sampling sites from Darjeeling Hills wide diversity of occurrence of lichen species both habitat wise as well as elevation wise was

observed which is due to negligible air pollution in the area. One lichen species from each sampling site was choosen based on their population density.

4.2. Morphological properties of lichen samples under study

The lichen samples were morphologically characterized taking different parameters like growth form, length and colour of the thallus, type of branches and reproductive structure present.

Table 4.2. Morphological properties of lichen samples

Lichen samples	Growth forms	Length/ diameter of the thallus (cm)	Description of the branches	Colour and texture of thallus	Presence and absence of reproductive structure
Usnea baileyi	fruticose	5.5-6	sympodial	Greyish green	Apothecia present
Stereocaulon pomiferum	fruticose	2-3	sympodial	Dark grey	Cephalodia present
Everniastrum sp	foliose	4-5	dicharial	Green	Isidium present
Parmotrema reticulatum	foliose	4-5	sympodial	Light green	Isidium present
Ramalina hossei	fruticose	3-3.5	sympodial	Light green	Apothecia present

The three lichen species under study namely *Usnea baileyi* (greyish green), *Stereocaulon pomiferum* (dark grey) and *Ramalina hossei* (light green) were fruticose in form with sympodial branching. *Everniastrum* sp (green) with dicharial branching and *Parmotrema reticulatum* (light green) with sympodial branching were foliose in form.

4.3. Extraction of lichen samples and percentage yield of extract residue

Lichen samples were extracted in ethanol and methanol as described in materials and method section (3.3.4) which were then completely evaporated and extract residue percentage were determined (Table 4.3).

Table 4.3.a Percentage yield of lichen extract residue

Sl. No.	Lichen samples	Solvent	Yield %	Colour	Consistency
1	*Usnea baileyi*	Ethanol	7.41	Green	paste
		Methanol	5.94	Green	paste
2	*Stereocaulon pomiferum*	Ethanol	9.95	Dark green	paste
		Methanol	8.5	Dark green	Paste
3	*Everniastrum* sp	Ethanol	13.7	Greenish brown	Paste
		Methanol	11.3	Greenish brown	Paste
4	*Parmotrema reticulatum*	Ethanol	13.4	Green	Paste
		Methanol	8.4	Green	Paste
5	*Ramalina hossei*	Ethanol	10.36	Green	Paste
		Methanol	9.55	Green	Paste

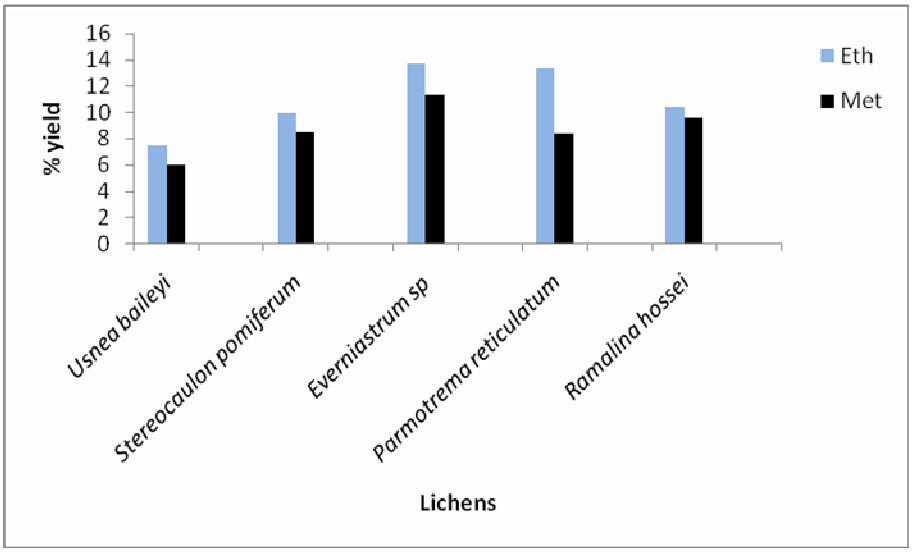


Figure 4.1. Percentage yield of ethanolic and methanolic lichen extract residue

Table 4.3.b. Percentage yield of medicinal plant extract residue

Sl. No.	Plant samples	Solvent	Yield %	Colour	Consistency
1	*Berginia ciliata*	ethanol	7.99	green	Paste
		methanol	7.55		
2	*Panax pseudoginseng*	ethanol	14.27	green	Paste
		methanol	15.67		
3	*Urtica dioica*	ethanol	10.63	green	Paste
		methanol	7.72		
4	*Sapindus mukrossi*	ethanol	6.78	Reddish brown	Paste
		methanol	15.59		

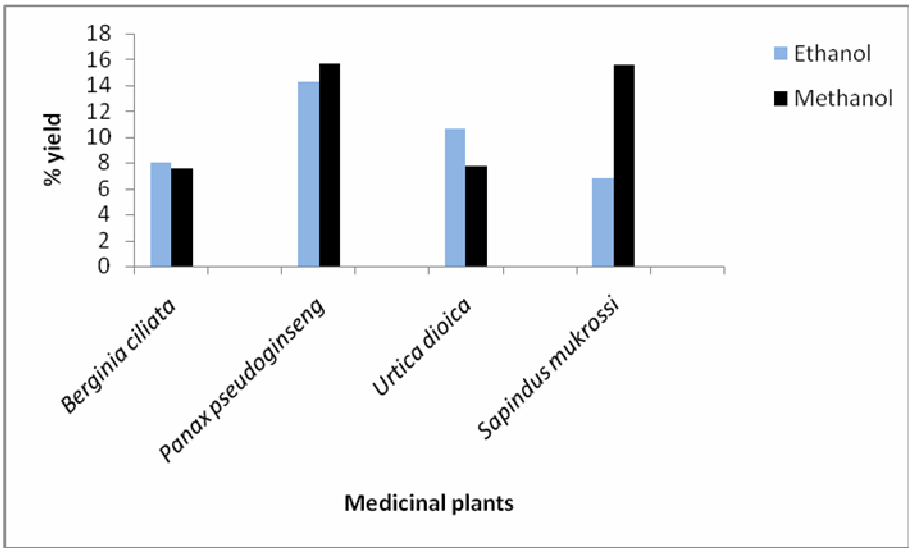


Figure 4.2. Percentage yield of ethanolic and methanolic medicinal plant extract residue

Usnea baileyi yielded 7.41% residue in ethanol and 5.94% in methanol, both of them were green in colour. *Stereocaulon pomiferum* residue with dark green colour showed little higher yield i.e., 9.95% in ethanol and 8.5% in methanol. *Everniastrum* sp yielded highest residue (greenish brown colour) among the lichen samples, ethanol (13.7%) and methanol (11.3%). Ethanolic extract residue yield of *Parmotrema reticulatum* (13.4%) was very close to that of *Everniastrum* sp whereas methanolic extract residue (8.4%) was very close to *Stereocaulon pomiferum. Ramalina hossei* residue yield was also higher with 10.36% (ethanol) and 9.55% (methanol*).* *Parmotrema reticulatum* and *Ramalina hossei* yielded green coloured residue. All the lichen species under study yielded the extract residue with paste consistency.

The medicinal plant *Panax pseudoginseng* yielded different residue in its ethanolic and methanolic extract. Methanolic extract of *Sapindus mukrossi* also yielded high percentage of 15.59%. Comparitively low residue was obtained from *Berginia ciliata*.

Discussion

All the lichen species yielded residue with green base and paste consistency. The residue amount is remarkable and little lower than previously reported that of *Roccella phycopsis* (ethanol -16.28% and methanol-19.38%) and also *Flavoparmelia caperata* (ethanol -12.34% and methanol-14.65%), Aydin and Kinalioglu (2013). The difference in percentage yield of studied lichen and medicinal plant extracts varied as the samples were collected from different places under different growing conditions. The habitat of occurrence lichens also differed and the laboratory techniques adopted for extraction of samples in the present study must have been different.

4.4. Screening of antimicrobial activity of lichen extracts

Both ethanolic and methanolic extracts of all the lichen samples under study were subjected to screening for antimicrobial activity. Streptomycin was taken as positive control and sterile distilled water as negative control in all the screening experiments.

4.4.1. Screening of antimicrobial activity of *Usnea baileyi*

Table 4.4. Antimicrobial activity of extracts of *Usnea baileyi* by disc diffusion method

Sl No.	Test organisms	Inhibition zone (mm)			
		SDW	USRE	USRM	Streptomycin
1	*A. faecalis*	0	6	7	16
2	*B. subtilis*	0	0	15	15
3	*B. megaterium*	0	0	18	14
4	*C. albicans*	0	0	12	9
5	*E. aerogenes*	0	17	11	16
6	*E. coli*	0	10	15	10
7	*P. aeruginosa*	0	15	12	12
8	*S. aureus*	0	6	13	16

SDW = sterile distilled water

The antimicrobial activity of ethanol and methanol extracts of lichen samples against the microorganisms was estimated on the basis of the

presence or absence of inhibitory zones. Ethanolic extract of *U. baileyi* inhibited the growth of Gram negative bacteria namely *A. faecalis*(7mm), *E. aerogenes* (17mm), *P. aeruginosa* (15 mm), *E. coli* (10mm) and one gram positive bacteria *S. aureus* (6mm).The ethanolic extract of *U. baileyi* could not inhibit the growth of *B. subtilis, B. megaterium* and *C. albicans* (fungus). The methanolic extracts of *U. baileyi* was active against all the microorganisms tested producing larger inhibition zones measuring 18mm and 15mm against *B. megaterium* and *B. subtilis* respectively. Methanolic extract of *U. baileyi* produced larger inhibition zone than streptomycin against *C. albicans* and *E. coli* (Plates 3.3, 3.4, 3.5, 3.6, 3.8 and 3.9).

Discussion

Many reports cite the inhibitory activity of *Usnea* sp against gram positive and gram negative organisms. The ethanolic and methanolic extracts of *U. baileyi* were active against both gram positive and gram negative bacteria. In some cases (i.e., against *B. megaterium, C. albicans, E. coli)* inhibition zones produced by lichen extracts were even greater than the control antibiotic. From the results it can be seen that methanolic extract of *U. baileyi* was more active than ethanolic extract. The results were consistent with previous works (Santiago *et. al.,* 2010) where acetone extract of *U. baileyi* was partially active against *E. coli* (10-12 mm). *U. ghattensis* was active against *S. aureus, B. licheniformis, B. subtilis* and *B. megaterium* (Behera *et. al.,* 2005). *Usnea subflorida* was active against *E. coli, P. mirabilis, B. subtilis* and *B. megaterium.* (Cansarana *et. al.,* 2006), similarly it was observed that ethanolic and methanolic extract of *U. baileyi* inhibited the growth of *E. coli* and *P. aeruginosa* whereas its methanolic extract inhibited the growth of *B. subtilis* and *B. megaterium* (Table 4.4).

The demonstration of activity by *Usnea baileyi* against both Gram negative and Gram positive bacteria indicated that the lichen could be a source of bioactive substances that and possessed a broad spectrum activity

4.4.2. Screening of antimicrobial activity of *Stereocaulon pomiferum*

Table 4.5. Antimicrobial activity of extracts of *Stereocaulon pomiferum* by disc diffusion method

Sl No.	Test organisms	Diameter of inhibition zone (mm)			
		SDW	STRE	STRM	Streptomycin
1	*A. faecalis*	0	11	0	16
2	*B. subtilis*	0	0	0	15
3	*B. megaterium*	0	11	9	14
4	*C. albicans*	0	7	0	9
5	*E. aerogenes*	0	9	12	16
6	*E. coli*	0	0	0	10
7	*P. aeruginosa*	0	0	9	12
8	*S. aureus*	0	0	0	16

SDW = sterile distilled water

Disc diffusion assay was followed to determine the antimicrobial activity of *S. pomiferum*. It was observed that ethanolic extract of *S. pomiferum* had inhibitory effect against *A. faecalis, B. megaterium, E. aerogenes* and *C. albicans,* while no zone of inhibition was observed against *B. subtilis, E. coli, P. aeruginosa* and *S. aureus*. The methanolic extract of *S. pomiferum* were active against *B. megaterium* and *E. aerogenes* (Table 4.5), (Plates 3.7, 3.9 and 3.10).

Discussion

In our study it was evident that ethanolic and methanolic extract of *S. pomiferum* was inactive towards *E. coli* similarly Duman (2009), worked out that lichen *Flavoparmelia caperata* did not show any activity against *E. coli, P. mirabilis* and *P. vulgaris*.

It was evident that ethanolic and methanolic extract of *S. pomiferum* was partially active against Gram positive bacteria, Gram negative bacteria and a fungus. The results obtained were also similar to that of Santiago *et. al.*, (2010) in which the extracts of *S. massartianum* was inactive against *S. aureus***.** Compounds like (methyl β-orcinol carboxylate and atranol) isolated from *Stereocaulon vesuvianum* (Caccamese *et. al.*, 1986) and *S. alpinum* (Paudel *et. al.,* 2008) exhibited antibacterial activity.

Similarly another fruticose lichen *Cladonia floiaceae* were found active against nine microorganisms (*Bacillus subtilis, Staphylococcus aureus, Bacillus cereus, Proteus vulgaris, Aeromonas hydrophila, Streptococcus*

faecalis and *Listeria monocytogenes, Candida albicans* and *Candida glabrata* (Yilmaz *et. al.*, 2004).

4.4.3. Screening of antimicrobial activity of *Parmotrema reticulatum*

Table 4.6. Antimicrobial activity of extracts of *Parmotrema reticulatum* by disc diffusion method

Sl No.	Test organisms	Inhibition zone(mm)			
		SDW	PARE	PARM	Streptomycin
1	*A. faecalis*	0	17	11	16
2	*B. subtilis*	0	10	12	15
3	*B. megaterium*	0	12	7	14
4	*C. albicans*	0	8	10	9
5	*E. aerogenes*	0	11	11	16
6	*E. coli*	0	0	9	10
7	*P. aeruginosa*	0	10	10	12
8	*S. aureus*	0	16	10	16

SDW = sterile distilled water

From the results (Table 4.6) it was evident that the methanolic and ethanolic extract of *P. reticulatum* was active against all the microorganisms tested. The ethanolic extract of *P. reticulatum* showed largest inhibition zone (17mm) against *A. faecalis* and (16mm) against *S. aureus*. The ethanolic extracts was active against *B. subtilis, B. megaterium, C. albicans*, *E. aerogenes* and *P. aeruginosa* but was inactive against *E. coli*. The methanolic extract of *P. reticulatum* moderately inhibited the growth of all the test microorganisms with inhibition zone of 7-12mm (Table 4.6), (Plates 3.3, 3.4, 3.5, 3.6, 3.8 and 3.9).

Discussion

Generally it was observed in table 4.6 that ethanolic and methanolic extract of *Parmotrema reticulatum* exhibited potent antimicrobial activity. Acetone, methanol, ethyl acetate and benzene extract of *Parmotrema nilgherrense* exhibited antibacterial activity against *Pseudomonas aeruginosa, Pseudomonas fluroscens, Proteus vulgaris, Shegilla flexneri, Klebsiella pneumonia* and *Salmonella typhi* (Javeria *et. al.*, 2013).

Another work by Kekuda *et. al.*, (2010) showed that methanolic extract of *Parmotrema pseudotinctorum* showed promising antibacterial activity against pathogenic bacteria *Salmonella typhi, Clostridium perfringens*, *E. coli* and *P. aeruginosa* causing hospital infections, food poisoning, gastroenteritis, burn infections etc, similarly from present result

it was concluded that methanolic extract of *Parmotrema reticulatum* inhibited the growth of *S. aureus*, *E. coli* and *P. aeruginosa* which are clinical pathogens.

In vitro antifungal activity of *Parmotrema tinctorum* (Despr.ex Nyl.) Hale, Tiwari *et. al.,* (2011) suggested that acetone, methanol and chloroform extract of *P. tinctorum* was most effective against the tested fungus which is also supported by present finding on the antifungal activity of *P. reticulatum* against *C. albicans*. The methanolic extract of *P. paresorediosum* inhibited the growth of human pathogenic bacteria *Proteus mirabilis* and *Salmonella typhi* (Balaji and Hariharan, 2007).

4.4.4. Screening of antimicrobial activity of *Ramalina hossei*

Table 4.7. Antimicrobial activity of extracts of *Ramalina hossei* by disc diffusion method

Sl No.	Test organisms	Inhibition zone(mm)			
		SDW	RARE	RARM	Streptomycin
1	*A. faecalis*	0	10	12	16
2	*B. subtilis*	0	9	14	15
3	*B. megaterium*	0	17	18	14
4	*C. albicans*	0	16	17	9
5	*E. aerogenes*	0	16	12	16
6	*E. coli*	0	15	7	10
7	*P. aeruginosa*	0	12	0	12
8	*S. aureus*	0	15	11	16

SDW = sterile distilled water

Ramalina hossei proved to be a potent antibacterial agent from this study as the ethanolic and the methanolic extract inhibited the growth of four gram positive bacteria, three gram negative and a fungus. Both ethanolic and methanolic extract of *R. hossei* produced largest zones of inhibition measuring 17 mm and 18 mm respectively against *B. megatarium*. The extracts were also active against *C. albicans* with zones measuring 16 mm and17 mm. Both extracts were moderately active against *A. faecalis*, *B. subtilis* and *E. aerogenes*. Ethanolic extract of *R. hossei* showed a higher antibacterial activity against *S. aureus* (15 mm) and *E. coli* (15 mm),(Plates 3.3, 3.4, 3.5, 3.6, 3.8 and 3.9).

Discussion

Study on phytochemical constituents, antibacterial, antifungal and cytotoxic properties of lichen member *R. farinaceae* was conducted earlier by (Esimone and Adikwu, 1999)where acetone extract of *R. farinaceae* and its (+)- usnic acid showed antimicrobial activity against *B. subtilis, Listeria monocytogenes, Proteus vulgaris, Staphylococcus aureus, Streptococcus faecalis , Yersima enterocolitica*, *Candida albicans* and *C. glabrata*. Present study also revealed that ethanolic and methanolic extract of *R. hossei* inhibited the growth of all test bacteria and a fungus.

The ethanolic extract of *R. hossei* was highly active against *E. coli* (15 mm) whereas the methanolic extract was weakly active against *E. coli* (7 mm) which is consistent with the work of Santiago *et. al.*, (2010) in which *R. dendriscoides* were found partially active against *E. coli* (10-12 mm). Another related species of *R. pacifica* were found inhibitory against *P. aeruginosa, Klebsiella pneumoniae, Salmonella typhi, Salmonella paratyphi, E. coli* and *Staphylococcus aureus* (Hoskeri *et. al.*, 2010).

The ethanolic extract of *R. hossei* (Table 4.7) and *R. pacifica* (Hoskeri *et. al.*, 2010) produced similar inhibition zones measuring 15 mm and 16.5 mm respectively against *S. aureus*. The ethanolic extract of *R. hossei* (Table 4.7) and *R. pacifica* (Hoskeri *et. al.*, 2010) inhibited the growth of *E. coli* an opportunistic pathogenic. Behera *et. al.,* (2005) observed metabolic products that have antibiotic activity which may have the function of protecting the organisms from attack by other fungi, hence extracts of *R. hossei* was also able to inhibit the growth of *C. albicans.*

4.4.5. Screening of antimicrobial activity of *Everniastrum* sp

Table 4.8. Antimicrobial activity of extracts of *Everniastrum sp* by disc diffusion method

Sl No.	Test organisms	Inhibition zone(mm)			
		SDW	EVRE	EVRM	Streptomycin
1	*A. faecalis*	0	8	10	16
2	*B. subtilis*	0	9	0	15
3	*B. megaterium*	0	15	13	14
4	*C. albicans*	0	19	14	9
5	*E. aerogenes*	0	8	13	16
6	*E. coli*	0	14	0	10
7	*P. aeruginosa*	0	12	0	12
8	*S. aureus*	0	11	10	16

SDW = sterile distilled water

Ethanolic extract of *Everniastrum* sp. were very active against all the microorganisms tested, whereas the methanolic extract was active against *A. faecalis* (10mm), *B. megaterium* (13mm), (Plates 3.7and 3.10), *C. albicans* (14mm), *E. aerogenes* and *S. aureus* (10mm). *C. albicans* and *B. megaterium* was highly sensitive to ethanolic extract of *Everniastrum* sp. Methanolic extract of *Everniastrum* sp was unable to inhibit the growth of *B. subtilis*, *E. coli* and *P. aeruginosa.*

Discussion

Generally the ethanolic extract of *Everniastrum* sp were active against all the test microorganisms. Turkey lichens *Evernia prunastri*, *Pseudoevernia furfuraceae* and *Alectoria capillaries* (Rowe *et. al.*, 1989) were active against Gram positive bacteria and *C. albicans*. Present study also showed that the ethanolic and methanolic extract of *Everniastrum* sp were mostly active against gram positive bacteria and *C. albicans* (Table 4.8).

All these studies indicate that the lichens inhibit mostly Gram-positive bacteria. Even though most of the lichens have been reported to be active against Gram-positive bacteria, the actual factors that affect the selective antibiotic activity have not been identified. However, this may be attributed to the biochemical and physiological variations between Gram-positive and Gram-negative bacteria. If so, it is of great interest to note that *Everniastrum* sp inhibited the growth of both Gram positive and Gram negative bacteria.

Studies on 100 species of American lichens (Burkholder *et. al.*, 1944) showed that 52% of the lichens in America were active against Gram positive bacteria.

Lichen *Rocella belangeriana* had antimicrobial activity against both Gram positive and Gram negative bacteria (Dahake *et. al.,* 2010)**,** similarly ethanolic extract of *Everniastrum* sp also manifested antibacterial activity against these two gram negative and gram positive bacteria. As reported earlier most of the lichens have been active against Gram positive bacteria, but the extracts of *Everniastrum nepalense* and *Usnea longifolia* equally inhibited the growth of both Gram negative and positive bacteria (Baral *et. al.*, 2011).

4.4.6. Screening of antimicrobial activity of common medicinal plants

4.4.6.1. Screening of antimicrobial activity of *Urtica dioica*

Table 4.9. Antimicrobial activity of extracts of *Urtica dioica* by disc diffusion method

Sl No.	Test organisms	Inhibition zone (mm)			
		SDW	URRE	URRM	Streptomycin
1	*A. faecalis*	0	13	0	16
2	*B. subtilis*	0	0	0	15
3	*B. megaterium*	0	10	11	14
4	*C. albicans*	0	0	0	9
5	*E. aerogenes*	0	12	0	16
6	*E. coli*	0	0	0	10
7	*P. aeruginosa*	0	0	0	12
8	*S. aureus*	0	0	0	16

SDW = sterile distilled water

The results showed that ethanolic and methanolic extracts of *Urtica dioica* possessed a weak antimicrobial property. The ethanolic extract of *U. dioica* produced inhibition zones measuring 13mm, 10mm, 12mm against *A. faecalis, B. megaterium* and *E. aerogenes* respectively, whereas the methanolic extract could inhibit the growth of *B. megaterium* only. No gram negative bacteria and fungus was sensitive to methanolic extract of *U. dioica* (Plates 3.7and 3.10).

Discussion

Urtica dioica herbs are used to treat stomachache (Yesilada *et. al.*, 2001), rheumatic pain, for cold and cough (Sezik *et. al.*, 1997) and liver diseases (Yesilada *et. al.*, 1993). As reported by Tolulope *et. al.*, (2007) our data also showed that *U. dioica* did not have any effect against *C. albicans*.

When zone of inhibiton is greater than 6mm a plant extract is considered active against both bacteria and fungi Muhammad and Muahmmad (2005)**,** but here it may be observed that no zone of inhibition is produced against most of the microorganisms. In another study conducted by Gulcin *et. al.*,(2004) water extracts of *U. dioica* inhibited the growth of *E. coli* (8mm), *P. mirabilis* (8mm), *Citrobacter koseri* (9mm), *S. aureus* (8mm), *Streptococcus pneumoniae* (9mm), *Micrococcus luteus* (13mm), *Staphylococcus epidermis* (11mm) and *C. albicans* (8mm). In the present study both the ethanolic and methanolic extract was not active against *C. albicans*, *E. coli*

and *S. aureus*. This may be due to difference in the type of solvent taken for extraction as the compounds of *U. dioica* can be mostly extracted in water than in ethanol and methanol. The solvents used in extraction in their increasing order of polarity play an important role in selecting the best solvent for extraction of active principles (Vries *et. al.,* 2005; Das *et. al.,* 2010). Gram negative test microorganisms were found to be more resistant as compared to gram positive microorganisms by methanolic extract of *U. dioica*. The higher resistance of gram negative bacteria than gram positive bacteria towards plant derived products was also reported by Yagi *et. al.,* (2012).

4.4.6.2. Screening of antimicrobial activity of *Berginia ciliata*

Table 4.10. Antimicrobial activity of extracts of *Berginia ciliata* by disc diffusion method

Sl No.	Test organisms	Inhibition zone(mm)			
		SDW	BERE	BERM	Streptomycin
1	*A. faecalis*	0	12	12	16
2	*B. subtilis*	0	7	6	15
3	*B. megaterium*	0	0	0	14
4	*C. albicans*	0	10	0	9
5	*E. aerogenes*	0	0	9	16
6	*E. coli*	0	12	11	10
7	*P. aeruginosa*	0	10	0	12
8	*S. aureus*	0	9	11	16

SDW-sterile distilled water

B. ciliata is a perennial herb belonging to the family Saxifragaceae. The hot water extract of whole dried plant of *B. ciliata* is orally taken by human for renal and urinary calculi (Mukherjee *et. al.*, 1984).

The results showed that mostly Gram negative bacteria were sensitive towards the ethanolic extracts. The methanolic extract of *B. ciliata* was moderately active against all test microorganisms. *B. megaterium* and *E. aerogenes* could survive against the ethanolic extract of *B. ciliata* whereas *B. megaterium*, *C. albicans* and *P. aeruginosa* survive against the methanolic extract. Both the extracts were partially active against *A. faecalis*, *E. coli* and *S. aureus* (Plates 3.3, 3.4, 3.5 and 3.6).

Discussion

Different species of *Berginia ciliata* harbour chemical compounds like polyphenols arbutin, hydroquinine, methyl arbutin, hydroquinone and

methylether (Furmanowa and Rapezewska, 1993; Fuji *et. al.*, 1996b) which may be suspected for its antibacterial activity.

The result obtained in the study (Table 4.10) was same as that of Islam *et. al.*, (2002) in which the ethanolic extract of root of *B. ciliata* produced inhibition zone of 10mm against *C. albicans.*

Reports are available which evidenced weak antifungal activity by root extracts of *B. schnnidtii* against *Aphanonnysees euteiches* (Kakwaro, 1976).

4.4.6.3. Screening of antimicrobial activity of *Sapindus mukrossi*

Table 4.11. Antimicrobial activity of extracts of *Sapindus mukrossi* by disc diffusion method

Sl No.	Test organisms	Inhibition zone(mm)			
		SDW	SARE	SARM	Streptomycin
1	*A. faecalis*	0	0	0	18
2	*B. subtilis*	0	0	0	19
3	*B. megaterium*	0	0	0	19
4	*C. albicans*	0	18	0	9
5	*E. aerogenes*	0	0	0	19
6	*E. coli*	0	11	10	18
7	*P. aeruginosa*	0	0	12	19
8	*S. aureus*	0	0	0	19

SDW-sterile distilled water

It was observed (Table 4.11) that ethanolic and methanolic extract of *S. mukrossi* have weak antimicrobial activity against most of the bacteria and test fungi. Ethanolic extract of *S. mukrossi* was active against *C. albicans* and *E. coli* with inhibition zone measuring 18mm and 11mm respectively.

Growth of *A. faecalis, B. subtilis, B. megaterium, C. albicans, E. aerogenes* and *S. aureus* were not inhibited on exposure to methanolic extract of *S. mukrossi*.

Discussion

Thota *et. al.,* (2012), examined that methanol, acetone and 1, 4 dioxan extract of *S. saponaria* showed good inhibitory activity against *S. aureus, P. vulgaris, P. aerogenes* and *Microccous albus*.

The methanolic leaf extract of *S. saponaria* produced inhibition zone measuring 11mm approx against *E. coli* (Table 4.11). Inhibiton zone measuring 12mm was produced by methanolic extract of *S. mukrossi* against *P. aeruginosa. S. emarginatus* (soapnut tree) belongs to the family

Sapindaceae found to content of saponins and sugar in the pericarp, which may be one of the factors for growth inhibition (Gupta and Ahmed, 1990).

S. emarginatus commonly known as soapberry or ritta. It showed antimicrobial against *C. albicans, Trichophyton rubrum* and *Epidophyton flocossum* (Manjulata *et. al.*, 2012). Our report is very much consistent with this result in which the ethanolic extract if *S. mukrossi* is highly active against *C. albicans* with inhibition zone measuring 18mm.

4.4.6.4. Screening of antimicrobial activity of *Panax pseudoginseng*

Table 4.12. Antimicrobial activity of extracts of *Panax pseudoginseng* by disc diffusion method

Sl No.	Test organisms	Inhibition zone(mm)			
		SDW	PNXE	PNXM	Streptomycin
1	*A. faecalis*	0	0	8	18
2	*B. subtilis*	0	17	19	19
3	*B. megaterium*	0	17	19	19
4	*C. albicans*	0	11	0	9
5	*E. aerogenes*	0	11	13	19
6	*E. coli*	0	0	9	18
7	*P. aeruginosa*	0	14	0	19
8	*S. aureus*	0	13	10	19

SDW-sterile distilled water

It could be observed that the methanolic extract of *P. pseudoginseng* was better than the ethanolic extract (Table 4.12). Both ethanolic and methanolic extract was highly active against gram positive bacteria. However the lichen extracts were completely unable or weakly able to restrict the growth of Gram negative bacteria.

Discussion

Antimicrobial activity may be due to numerous free hydroxyl ions that have the capability to combine with the carbohydrates and proteins in the cell wall of bacteria and may attach to the enzyme site making the microorganisms inactive**.** Similarly the antimicrobial activity as observed may be due to the presence of such free hydroxyl ions in *P. pseudoginseng. P. pseudoginseng* showed no antibacterial activity against *Chromobacterium violaceum* CV026 and *P. aeruginosa* PAO1 (Siew *et. al.*, 2012).

The results proved that bacteria are more sensitive to the antimicrobial compound than fungi (Hugo *et. al.,* 1983). The reason for different sensitivity between the fungi and bacteria may be because of

different transparency of the cell wall (Yang *et. al.,* 1999). The cell wall of the Gram-positive bacteria consists of peptidoglycans (mureins) and teichoic acids, the cell wall of the gram-negative cells consists of lipo polysaccharides, and lipopoliproteins (Hugenholtz, 2002), whereas the cell wall of fungi consists of polysaccharides such as chitin and glucan (Griffin, 1994).

4.4.7. Antimicrobial activity of combined extracts of lichens and medicinal plants

Synergistic antimicrobial assay was performed by combining equal proportions of lichen and medicinal plant extract. Zone of inhibition thus produced was measured.

4.4.7.1. Screening of antimicrobial activity of *Usnea baileyi* and *Urtica dioica*

Table 4.13. Screening of antimicrobial activity of *Usnea baileyi* and *Urtica dioica* by disc diffusion method

Inhibition zone (mm)			
Sl No.	Test organisms	USNE+URRE	USRM+URRM
1	*A. faecalis*	16	12
2	*B. subtilis*	0	0
3	*B. megaterium*	19	19
4	*C. albicans*	17	12
5	*E. aerogenes*	0	0
6	*E. coli*	17	15
7	*P. aeruginosa*	13	13
8	*S. aureus*	15	0

The ethanolic and methanolic extract of *U. baileyi* and *U. dioica* exhibited a good antibacterial and antifungal activity against the test organisms. However ethanolic extract of *U. dioica* (Table 4.9) could not inhibit the growth of *E. coli* but when it was combined with *U. baileyi* growth of *E. coli* was inhibited (Table 4.13). Good antimicrobial activity was shown by combined ethanolic extract of *Usnea baileyi* and *Urtica dioica* which greatly inhibited the growth of *B. megaterium*

Methanolic extract of *U. baileyi* with *U. dioica* inhibited the growth of gram positive and gram negative bacteria under study. Combined antimicrobial effect of ethanolic extract of *U. baileyi* and *U. dioica* was observed better against *A. faecalis* and *S. aureus* (Table 4.13).

Discussion

The ethanol extract of *U. baileyi* and *U. dioica* (Table 4.9) did not individually inhibit the growth of *C. albicans* but combined extracts could produce large inhibition zone. This synergistic effect may be useful in the treatment of fungal infection.

Two different cases can be observed as the combined ethanolic extract *U. baileyi* and *U. dioica* was antagonistic to each other for its activity against *E. aerogenes* and *P. aeruginosa* (Table 4.13).

4.4.7.2. Screening of antimicrobial activity of *Usnea baileyi* and *Berginia ciliata*

Table 4.14. Antimicrobial activity of combined extracts of *Usnea baileyi* and *Berginia ciliata* by disc diffusion method

Inhibition zone (mm)			
Sl No.	Test organisms	USNE+BERE	USRM+BERM
1	*A. faecalis*	9	0
2	*B. subtilis*	6	7
3	*B. megaterium*	8	15
4	*C. albicans*	14	9
5	*E. aerogenes*	7	9
6	*E. coli*	11	12
7	*P. aeruginosa*	7	0
8	*S. aureus*	10	6

Overall results show that methanolic and ethanolic extract of *B. ciliata* and *U. baileyi* proved to be potent antimicrobial agent (Table 4.14).

The activity of combined ethanolic extract of *B. ciliata* against *A. faecalis* (9mm), *B. subtilis* (6mm) and *B. megaterium* (8mm) was quite weak (Table 4.14) than methanolic extract. The fungus *C. albicans* was greatly inhibited by combined extract. *B. megaterium* also inhibited by the combined extracts as a zone of 14mm was produced.

The methanolic extract of the mixture did not inhibit the growth of *A. faecalis* and weakly restricted the growth of *B. subtilis* (7mm) and *S. aureus* (6mm).

Almost all Gram positive and Gram negative bacteria and a fungus tested was inhibited by combined ethanolic and methanolic extract.

Discussion

A moderate synergistic activity was observed when *U. baileyi* and *B. cilata* was combined. Synergistic effects resulting from the combination of

antibiotic with plant extract have been documented earlier (Muroi and Kubo, 1996).

But results of such effect on combination of two plants are very scanty. An antagonistic effect of combined methanolic extract was observed against *S. aureus* and *A. faecalis*.

Methanolic extract of *B. ciliata* did not alone resist the growth of *B. megaterium*, *C. albicans* and *P. aeruginosa*. But in combination with *U. baileyi* could inhibit growth producing synergistic effect.

An antagonistic effect was noted as of ethanolic extract of *U. baileyi* could strongly restrict the growth of *E. aerogenes* and *P. aeruginosa* individually but combined ethanolic extract did not inhibit the growth of these gram negative organisms.

Antagonistic effect of combination of plant extract was mainly observed against gram negative bacteria, this may be due to the biochemical and structural uniqueness in gram negative bacteria which may have prevented the entry active molecules through the cell boundary.

4.4.7.3. Screening of antimicrobial activity of *Parmotrema reticulatum* and *Urtica dioica*

Table 4.15 Antimicrobial activity of combined extracts of *Parmotrema reticulatum* and *Urtica dioica*

Inhibition zone (mm)			
Sl No.	Test organisms	PARE+URRE	PARM+URRM
1	*A. faecalis*	10	7
2	*B. subtilis*	0	0
3	*B. megaterium*	0	0
4	*C. albicans*	8	7
5	*E. aerogenes*	0	0
6	*E. coli*	10	9
7	*P. aeruginosa*	10	11
8	*S. aureus*	9	7

The antimicrobial effect of ethanolic and methanolic extract of *P. reticulatum* and *U. dioica* was quite weak. It was noted that both the combined extract (ethanolic and methanolic) did not at all inhibit the growth of *B. megaterium, B. subtilis* and *E. aerogenes*.

It was observed that *A. faecalis, C. albicans, E. coli, P. aeruginosa* and *S. aureus* were weakly inhibited by the plant and lichen combined extract (Table 4.15).

Out of eight microorganisms under test the mixed methanolic extract of *P. reticulatum* and *U. dioica* could show a considerable synergistic effect

against only five microorganisms (*A. faecalis*, *C. albicans, E. coli, P. aeruginosa* and *S. aureus*) whereas no effect was observed against *B. megaterium, B. subtilis* and *E. aerogenes.*

Discussion

Additive effect of the combined ethanolic extract of lichen and *U. dioica* was observed against *E. coli, P. aeruginosa* and *S. aureus*, the microorganisms whose growth was not restricted by single ethanolic extract of *U. dioica* alone.

This additive effect may be due to the presence of different compounds (phenols, flavonoids, tannins, coumarins, alkaloids and terpenoids) which effect growth and metabolism of microorganisms.

Ethanolic extract *U. dioica* inhibited the growth of only *B. megaterium* but when combined with *P. reticulatum* growth of gram positive bacteria, gram negative bacteria and a fungus was inhibited. Hence there existed a synergism between the two plant extracts.

4.4.7.4. Screening of antimicrobial activity of *Parmotrema reticulatum* and *Berginia ciliata*

Table 4.16. Antimicrobial activity of combined extracts of *Parmotrema reticulatum* and *Berginia ciliata* with by disc diffusion method

Inhibition zone (mm)			
Sl No.	Test organisms	PARE+BRRE	PARM+BRRM
1	*A. faecalis*	9	7
2	*B. subtilis*	13	7
3	*B. megaterium*	9	9
4	*C. albicans*	10	7
5	*E. aerogenes*	11	10
6	*E. coli*	11	12
7	*P. aeruginosa*	11	7
8	*S. aureus*	12	0

Extracts of *P. reticulatum* combined with *B. ciliata* showed moderate antimicrobial activity. Growth of gram negative bacteria *A. faecalis* (9mm), *E. aerogenes* (11mm), *P. aeruginosa* (11mm) was inhibited by the combined extract of *P. reticulatum* and *B. ciliata*. Methanolic extract of *P. reticulatum* and *B. ciliata* in combination not at all inhibited the growth of *S. aureus* (Table 4.16).

Very small inhibition zones were observed against *A. faecalis*, *B. subtilis*, *B. megaterium, C. albicans, E. aerogenes, E. coli* and *P. aeruginosa*.

The inhibitory effect of combined ethanolic extract of *P. reticulatum* and *B. ciliata* was quite high than methanolic extract. Growth of all microorganisms tested was inhibited by ethanolic extract whereas methanolic extract could not inhibit the growth of *S. aureus*.

Discussion

Few studies on synergism is reported which have been done using Kirby and Bauer (Betoni *et. al.,* 2006) method.

An antagonistic effect of combined plant extract was revealed as ethanolic and methanolic extract of *P. reticulatum* and *B. ciliata* was higher individually than in combination against *A. faecalis*. The antibacterial activity of one plant was lowered by other plant.

The combined ethanolic extracts of *B. ciliata* with *P. reticulatum* is more active towards the test microorganisms than the methanolic extracts, this may be due to differences in solubility of active principles in ethanol and methanol.

A distinct synergistic effect was noted as both ethanolic and methanolic extract as *B. ciliata* could not inhibit the growth of *B. megaterium* but the combination extract with *P. reticulatum* could make it possible. It may be inferred that the antimicrobial activity of *B. ciliat*a was enhanced by the presence of *P. reticulatum.*

4.4.7.5. Screening of antimicrobial activity of *Ramalina hossei* with *Urtica dioica*

Table 4.17. Antimicrobial activity of combined extracts of *Ramalina hossei* and *Urtica dioica* by disc diffusion method

		Inhibition zone (mm)	
Sl No.	Test organisms	RARE+URRE	RARM+URRM
1	*A. faecalis*	0	10
2	*B. subtilis*	0	0
3	*B. megaterium*	0	14
4	*C. albicans*	0	16
5	*E. aerogenes*	0	0
6	*E. coli*	11	0
7	*P. aeruginosa*	9	17
8	*S. aureus*	12	17

Ethanolic extract of *Ramalina hossei* together with *U. dioica* did not at all inhibit the growth of *A. faecalis*, *B. subtilis*, *B. megaterium*, *C. albicans*, and *E. aerogenes* (Table 4.17). Combined methanolic extract of *R. hossei* and *U. dioica* greatly inhibited the growth of *B. megaterium* (14mm), *C. albicans* (16mm*), P. aeruginosa* and *S. aureus* (17mm*).*

Discussion

The influence of combined plant extract was antagonistic as the growth of both gram positive and gram negative bacteria was not restricted by combined plant extract in some cases. This may be because the activity of ethanol extract of *R. hossei* is lowered by the ethanolic extract of *U. dioica*.

In another case the effectiveness of methanolic extract of *U. dioica* was increased by *R. hossei* as the combination could restrict the growth of *A. faecalis, C. albicans, P. aeruginosa* and *S. aureus.*

4.4.7.6. Screening of antimicrobial activity of *Ramalina hossei* and *Berginia ciliata*

Table 4.18 Antimicrobial activity of combined extracts of *Ramalina hossei* with *Berginia ciliata* by disc diffusion method

		Inhibition zone (mm)	
Sl No.	Test organisms	RARE+BERE	RARM+BERM
1	*A. faecalis*	11	0
2	*B. subtilis*	11	14
3	*B. megaterium*	10	11
4	*C. albicans*	12	9
5	*E. aerogenes*	12	11
6	*E. coli*	7	9
7	*P. aeruginosa*	7	13
8	*S. aureus*	0	11

. Ethanolic and methanolic extract of *R. hossei* and *B. ciliata* depicted moderate antimicrobial activity when combined. The combination of extracts inhibited the growth of gram positive and gram negative bacteria with moderate inhibition zones (Table 4.18). The inhibition zones produced were small as compared to standard antibiotic.

The combined ethanolic extract could not inhibit the growth of *S. aureus* and combined methanolic extract these plants also could not inhibit the growth of *A. faecalis* (Table 4.18).

Discussion

The activity of *B. ciliata* was enhanced by *R. hossei* as its mixed ethanolic extract could restrict the growth of *B. megaterium* and *E. aerogenes*, so there existed a synergism between the two extract.

The only tested fungus was not inhibited by methanolic extract of *B. ciliata* but combined plant extract combined with *R. hossei* prohibited its growth.

The antagonistic influence was seen in case of *S. aureus*, in which combined ethanolic extract could not inhibit its growth which may be due to the reason that the activity of one extract was not enhanced instead suppressed by the other extract.

4.4.7.7. Screening of antimicrobial activity *Stereocaulon pomiferum* with *Urtica dioica*

Table 4.19. Antimicrobial activity of combined extracts of *Stereocaulon pomiferum* with *Urtica dioica by* disc diffusion method

Inhibition zone (mm)			
Sl No.	Test organisms	STRE+URRE	STRM+URRM
1	*A. faecalis*	6	7
2	*B. subtilis*	0	0
3	*B. megaterium*	20	0
4	*C. albicans*	7	0
5	*E. aerogenes*	0	0
6	*E. coli*	0	0
7	*P. aeruginosa*	0	0
8	*S. aureus*	0	10

A highest antimicrobial activity was shown by combined ethanolic extract of *S. pomiferum* and *U. dioica* in which it produced inhibition zones measuring 20mm against *B. megaterium* but could not at all or weakly inhibit the growth of other microorganisms tested. Combined methanolic extract of *S. pomiferum* and *U. dioca* showed very weak antimicrobial activity as it inhibited the growth of only two microbes *A. faecalis* and *S. aureus.*

Discussion

An example of best synergism was noted where the mixed ethanolic extract of plant greatly inhibited the growth of *B. megaterium*. The activity of either *S. pomiferum* or *U. dioica* was increased by the other (Table 4.5 and Table 4.9)

Only three of the test microorganism (*A. faecalis, B. megaterium* and *C. albicans*) tested was sensitive to combined plant extract it may be due to the fact that lichen extract was well as the plant extract could not individually penetrate the cell membrane of gram positive and gram negative bacteria

According to some authors, both active compounds from lichens and medicinal plants directly or indirectly attach to the same site on bacterial cell still mechanism of synergy could be still insufficiently researched (Sibanda, 2007; Horiuchi, 2007). Similarly the synergy between the plant and lichen extract in inhibiting the bacterial growth could be further analysed.

4.4.7.8. Screening of antimicrobial activity of *Stereocaulon pomiferum* and *Berginia ciliata*

Table 4.20 Antimicrobial activity of combined extracts of *Stereocaulon pomiferum* with *Berginia ciliata* by disc diffusion method

Inhibition zone (mm)			
Sl No.	Test organisms	STRE+BERE	STRM+BERM
1	*A. faecalis*	10	0
2	*B. subtilis*	0	0
3	*B. megaterium*	6	7
4	*C. albicans*	6	6
5	*E. aerogenes*	7	6
6	*E. coli*	9	0
7	*P. aeruginosa*	6	6
8	*S. aureus*	10	6

A weak antimicrobial activity was shown by ethanolic and methanolic extract of *S. pomiferum* and *B. ciliata* very small inhibition zone was observed against *A. faecalis, B. megaterium, C. albicans*, *E. aerogenes*, *E. coli*, *P. aeruginosa* and *S. aureus*. The methanolic combined extract was weak to inhibit the growth of *A. faecalis*, *B. subtilis* and *E. coli* (Table 4.20).

Out of eight test microorganisms *B. subtilis* was totally insensitive to combined ethanolic and methanolic extract.

Discussion

The combined extract of *S. pomiferum* and *B. ciliata* had not much synergistic or additive effect. In many cases the activity of *S. pomiferum* was lowered by extracts of *B. ciliata*. Hence it was evident that the plant extracts had antagonistic effect with each other.

4.4.7.9. Screening of antimicrobial activity of combined extracts of *Everniastrum* sp and *Urtica dioica*

Table 4.21Antimicrobial activity of combined extracts of *Everniastrum* sp and *Urtica dioica* by disc diffusion method

Inhibition zone (mm)			
Sl No.	Test organisms	EVRE+URRE	EVRM+URRM
1	*A. faecalis*	0	10
2	*B. subtilis*	0	0
3	*B. megaterium*	0	18
4	*C. albicans*	14	15
5	*E. aerogenes*	0	0
6	*E. coli*	0	0
7	*P. aeruginosa*	15	16
8	*S. aureus*	0	19

Combined ethanolic extract of *Everniastrum* sp and *U. dioica* was strong as it inhibited the growth of *C. albicans* (14mm) and *P. aeruginosa* (15mm) respectively but had no effect against *A. faecalis*, *B. subtilis*, *B. megaterium, E. aerogenes, E. coli* and *S. aureus.*

Comparitively the combined methanolic extract of *Everniastrum* sp and *U. dioica* showed positive results (Table 4.21) as *A. faecalis* (10mm), *B. megaterium* (18mm), *C. albicans* (15mm), *P. aeruginosa* (16mm) and *S. aureus* (19mm) were sensitive to the mixture of extracts.

The combined effect of *U. dioica* and *Everniastrum* sp. was close to the effect of antibiotic as noted in *B. megaterium* (18mm) and *S. aureus* (19mm), (Table 4.21).

Discussion

The influence of synergistic effect of plant extract could be clearly seen as combined methanolic extract of plants inhibited the growth of gram positive bacteria, gram negative bacteria and a fungus.

At the same time antagonism could be observed in activity of combined ethanolic extract of plants against *A. faecalis*, *B. subtilis*, *B. megaterium*, *E. aerogenenes*, *E. coli*, and *S. aureus*. In this context the antibacterial activity of one plant extract is lowered by the other plant. Methanolic extract were more active towards test fungi (*C. albicans*) indication the presence of methanol soluble active constituents.

4.4.7.10. Screening of antimicrobial activity of *Everniastrum* sp with *Berginia ciliata*

Table 4.22. Antimicrobial activity of combined extracts of *Everniastrum* sp with *Berginia ciliata* by disc diffusion method

Inhibition zone (mm)			
Sl No.	Test organisms	EVRE+BERE	EVRM+BERM
1	*A. faecalis*	9	0
2	*B. subtilis*	10	10
3	*B. megaterium*	11	11
4	*C. albicans*	11	9
5	*E. aerogenes*	10	10
6	*E. coli*	17	12
7	*P. aeruginosa*	11	10
8	*S. aureus*	10	10

B. ciliata and *Everniastrum* sp (ethanolic extract) in combination moderately inhibited the growth of all the test microorganisms. *E. coli* was very sensitive to the combined extracts as inhibition zone measuring (17mm) was clearly observed in table 4.22. Growth all the tested microorganisms except *A. faecalis*, was inhibited by the combined methanolic extract of *B. ciliata* and *Everniastrum* sp (Table 4.22).

Both the combined ethanolic and methanolic extract of lichen and medicinal, plants showed equal antimicrobial property.

Discussion

As discussed earlier (Tab 4.10) ethanolic extract of *B. ciliata* couldnot inhibit the growth of *B. megaterium* and *E. aerogenes* but alone this was possible by combination of ethanolic extract of *B. ciliata* and *Everniastrum* sp which showed synergistic effect.

An antagonistic effect was observed where the individual activity of the extract of *Everniastrum* sp. against *C. albicans* and *B. megaterium* was lowered in combined extract.

4.4.7.11. Screening of antimicrobial activity of *Everniastrum* sp and *Panax pseudoginseng*

Table 4.23. Antimicrobial activity of combined extracts of *Panax pseudoginseng* and *Everniastrum* sp by disc diffusion method

Inhibition zone (mm)			
Sl No.	Test organisms	PNXE+EVRE	PNXM+EVRM
1	*A. faecalis*	0	10
2	*B. subtilis*	12	20
3	*B. megaterium*	14	11
4	*C. albicans*	20.7	17.1
5	*E. aerogenes*	9	14
6	*E. coli*	18	18.9
7	*P. aeruginosa*	17.1	18.9
8	*S. aureus*	22.05	10.8

Combined ethanolic extract of *P. pseudoginseng* and *Everniastrum*, was active against all the microorganisms except (*A. faecalis*)(Plate 3.15) where highest inhibition zone was observed against *S. aureus* (22.05mm) and lowest against *E. aerogenes* (9mm).

Methanolic extract inhibited all the test organisms. Mixture of ethanolic extract of *P. reticulatum* and *Everniastrum* sp was observed to produce even larger inhibition zone against *S. aureus* (22.05mm) than that of Streptomycin (19mm)(Plates 3.11,3.12,3.13 and 3.14).

Discussion

A good synergism was observed as both ethanolic and methanolic extract of *P. pseudoginseng* and *Everniastrum* sp greatly inhibited the growth

of test microorganisms. This combination could be exploited as potent antimicrobial agent.

4.4.7.12. Screening of antimicrobial activity of *Usnea baileyi* and *Panax pseudoginseng*

Table 4.24. Antimicrobial activity of combined extracts of *Panax pseudoginseng* with *Usnea baileyi* by disc diffusion method

Inhibition zone (mm)			
Sl No.	Test organisms	PNXE+USRE	PNXM+USRM
1	*A. faecalis*	0	8
2	*B. subtilis*	14	9
3	*B. megaterium*	14	11
4	*C. albicans*	11.7	13.5
5	*E. aerogenes*	9	14
6	*E. coli*	16.2	9
7	*P. aeruginosa*	15.3	6.3
8	*S. aureus*	11.25	13.05

Combined ethanolic extract of *P. pseudoginseng* and *U. baileyi* acted against *B. subtilis* (14mm), *B. megaterium* (14mm), *E. coli* (16.2mm), *P. aeruginosa* (15.3 mm) and *S. aureus* (11.25).

Methanolic combined extract was not as effective as ethanolic extract as it inhibited the growth of *C. albicans*(13.5mm), *E. aerogenes*(14mm) and *S. aureus*(13.05mm),(Table 4.24) whereas other microorganisms were weakly inhibited.

Only *A. faecalis*, Gram negative bacteria was not sensitive to combined plant extract, out of eight microorganisms studied (Plates 3.11.3.12, 3.13, 3.14 and 3.15).

Discussion

The mixed ethanolic extract of plants against *P. aeruginosa* and methanolic extract against *A. faecalis* and *S. aureus* was indifferent to each other as their combined effect was equal to the individual effect of plant extract.

Synergistic influence was observed in case of combined ethanolic extract of plants against *C. albicans*. The antimicrobial property of one extract was enhanced by the other.

4.4.7.13. Screening of antimicrobial activity of *Sapindus mukrossi* with *Usnea baileyi*

Table 4.25 Antimicrobial activity of combined extracts of *Sapindus mukrossi* with *Usnea baileyi* by disc diffusion method

Inhibition zone (mm)		
Sl No.	Test organisms	SAPE+USRE
1	*A. faecalis*	12
2	*B. subtilis*	9
3	*B. megaterium*	0
4	*C. albicans*	9.9
5	*E. aerogenes*	0
6	*E. coli*	13.5
7	*P. aeruginosa*	9
8	*S. aureus*	11.25

S. mukrossi and *U. baileyi* when combined together was moderately controlled the growth of *A. faecalis, B. subtilis, C. albicans, E. coli, P. aeruginosa* and *S. aureus*. Its methanolic extract in combination could not restrict the growth of microorganisms under test (Table 4.25)(Plates 3.11.3.12,3.13,3.14, 3.15).

The combined ethanolic extract proved to be better than methanolic extract.

Discussion

A better synergism was observed as in this case as ethanolic extract of *S. mukrossi* and *U. baileyi* individually could not inhibit the growth of *A. faecalis*, *B. subtilis* and *S. aureus* whereas its combined extract restricted their growth.

A distinct antagonistic effect was seen as two gram negative bacteria *E. coli* and *P. aeruginosa* was inhibited by methanolic extract of *S. mukrossi* and *U. baileyi* individually but its mixed extract could not prohibit the growth of all the microorganisms under study.

4.4.7.14. Screening of antimicrobial activity of *Parmotrema reticulatum* and *Panax pseudoginseng*

Table 4.26. Antimicrobial activity of combined extracts of *Parmotrema reticulatum* with *Panax pseudoginseng* by disc diffusion method

Inhibition zone (mm)			
Sl No.	Test organisms	PARE+PNXE	PARM+PNXM
1	*A. faecalis*	6	0
2	*B. subtilis*	10	8
3	*B. megaterium*	0	0
4	*C. albicans*	9	10
5	*E. aerogenes*	14	9
6	*E. coli*	13	8
7	*P. aeruginosa*	17	8
8	*S. aureus*	13	9

In the study 40% of microorganisms under test (*E. coli, E. aerogenes, P. aeruginosa* and *S. aureus*) was less sensitive towards combined ethanolic extracts of *P. reticulatum* and *P. pseudoginseng*. Methanolic extract of *P. reticulatum* and *P. pseudoginseng* weakly prohibited the growth of test microorganisms (Table 4.26)(Plates 3.16,3.17,3.18,3.19, 3.20)

Among the combined extracts the activity of ethanolic extract of was stronger than methanolic against the test microorganisms.

Discussion

As observed earlier ethanolic extract of *P. reticulatum* (Table 4.6) and *P. ginseng* (Table 4.12) individually did not inhibit the growth of *E. coli* but their combination was able to restrict the growth as may be antibacterial activity of mixed extract was stronger.

In contrary to the above result the combined extract of lichen and *P. pseudoginseng* could not restrict the growth of *B. megaterium* which was made possible by the individual extract this may have been occurred due to loss in antibacterial activity of each other when combined together. The growth of only fungus under test *C. albicans*, was prohibited by the combined extract by showing their synergistic effect.

4.4.7.15. Screening of antimicrobial activity of *Everniastrum* sp and *Sapindus mukrossi*

Table 4.27 Antimicrobial activity of combined extracts of *Sapindus mukrossi* with *Everniastrum sp* by disc diffusion method

Inhibition zone (mm)			
Sl No.	Test organisms	SAPE+EVRE	SAPM+EVRM
1	*A. faecalis*	17	11
2	*B. subtilis*	12	12
3	*B. megaterium*	0	0
4	*C. albicans*	16	11
5	*E. aerogenes*	12	7
6	*E. coli*	14	9
7	*P. aeruginosa*	16	6
8	*S. aureus*	15	13

It was evident that combined methanolic extract of *S. mukrossi* and *Everniastrum* sp was strongly active towards 90% microorganisms under test except *B. megaterium* (Table 4.27). The combined methanolic extract was moderately active against the bacteria and fungi under test.

Out of four gram negative bacteria three gram positive bacteria and a fungus under test growth of *A. faecalis*(17.2mm), *C. albicans*(16.2mm), *E. coli* (14.72mm), *P. aeruginosa* (16.74mm) and *S. aureus* (15.84mm)was greatly inhibited (Plates 3.16,3.17,3.18,3.19 and 3.20).

Discussion

Ethanolic extract of *S.mukrossi* was weak as it could inhibit growth of *C. albicans* and *E. coli* (Table 4.11), but when combined with *Everniastrum* sp it inhibited the growth of both gram positive (*B. subtilis* and *S. aureus*) and gram negative bacteria. (*A. faecalis*, *E. aerogenes*, *E. coli* and *P. aeruginosa*) as the weak antibacterial activity of *S. mukrossi* was increased by presence of *Everniastrum* sp.

Similarly methanolic extract of *S. mukrossi* in combination with *Everniastrum* inhibited the growth of 90% of microorganism under test except *B. megaterium*.

4.4.7.15. Screening of antimicrobial activity of *Parmotrema reticulatum* and *Sapindus mukrossi*

Table 4.28. Antimicrobial activity of combined extracts of *Parmotrema reticulatum* with *Sapindus mukrossi* by disc diffusion method.

		Inhibition zone (mm)	
Sl No.	Test organisms	PARE+SARE	PARM+SARM
1	*A. faecalis*	6	7
2	*B. subtilis*	10	0
3	*B. megaterium*	0	0
4	*C. albicans*	6	10
5	*E. aerogenes*	10	7
6	*E. coli*	11	10
7	*P. aeruginosa*	15	10
8	*S. aureus*	11	12

All the eight microorganisms under test except *B. megaterium* were sensitive towards combined ethanolic extract of *P. reticulatum* and *S. mukrossi*. *A. faecalis* and *C. albicans* were not very sensitive towards the combined extracts (Table 4.28).

In another case combined methanolic extract of *P. reticulatum* and *S. mukrossi* was moderately active towards *C. albicans* (10 mm) and *S. aureus* (12 mm), while growth of *B. subtilis* and *B. megaterium* was not at all inhibited (Table 4.28).

Only *B. megaterium* was not sensitive to the combined extract. Among all the test microorganisms, it was revealed that mostly Gram negative bacteria were more sensitive to the combined extracts (Plates 3.16, 3.17, 3.18, 3.19 and 3.20).

Discussion

An antagonistic effect was observed as antimicrobial activity of ethanolic extract of *P. reticulatum* was lowered against *A. faecalis* (6.24mm), *C. albicans* (6mm), *B. megaterium* (0), *E. aerogenes* (10mm) and *S. aureus* (11.22mm) in combination with *S. mukrossi*

But the activity of methanolic extract of *S. mukrossi* was enhanced by presence of methanolic extract of *P. reticulatum* (Table 4.28).

According to wide screening of antimicrobial activity of lichen extracts combined with medicinal plants it seems that their inhibitory activity vary with growth condition of samples, solvent system etc. Synergism between lichen and medicinal plants open the door for further investigation related to the development of treatment system for combatment of diseases caused by antibiotic resistant microorganisms.

4.5. Determination of Minimum Inhibitory Concentration (MIC) of lichen extracts against the test organisms

MIC of the sample wise result is tabulated followed by result and discussion.

4.5.1. Minimum Inhibitory Concentration (MIC) of extracts of *Usnea baileyi* against the test organisms

Table 4.29. Minimum Inhibitory Concentration (MIC) of extracts of *Usnea baileyi* against the test organisms

Sl No.	Microorganisms used	MIC (µg/ml)		
		USRE	USRM	Streptomycin
1	*A. faecalis*	5000	500	10
2	*B. subtilis*	500	500	10
3	*B. megaterium*	>10000	500	10
4	*C. albicans*	500	500	10
5	*E. aerogenes*	>10000	500	10
6	*E. coli*	500	500	5
7	*P. aeruginosa*	>10000	>10000	5
8	*S. aureus*	>10000	>10000	10

The methanolic extract of *U. baileyi* showed MIC of 500µg/ml against *A. faecalis B. subtilis*, *B. megaterium, C. albicans, E. aerogenes* and *E. coli.* The MIC of ethanolic extract of *U. baileyi* was 500µg/ml against *B. subtilis*, *E. coli*, *P. aeruginosa* and *C. albicans* (Plates 3.21, 3.22, 3.23, 3.31, 3.32, 3.33, 3.34).

Discussion

Extract of *U. baileyi* contained potent antimicrobial activity. Ethanolic extract of *U. baileyi* showed lower MIC value 500µg/ml against Gram

negative bacteria *E. coli,* similarly such low MIC value was exhibited by another lichen *Usnea barbata* (Madamombe and Alfolayan, 2003), against *Enterococcus faecalis, B. subtilis, Micrococcus viradans* and *S. aureus*. The MIC of its methanolic extract was also appreciable as its low concentration inhibited the growth of gram positive bacteria, gram negative bacteria and a fungus.

4.5.2. Minimum Inhibitory Concentration (MIC) of extracts of *Parmotrema reticulatum* against the test organisms

Table 4.30. Minimum Inhibitory Concentration (MIC) of extracts of *Parmotrema reticulatum* against the test organisms

Sl No.	Microorganisms used	MIC (µg/ml)		
		PARE	PARM	Streptomycin
1	*A.faecalis*	1000	>10000	10
2	*B. subtilis*	500	5000	10
3	*B. megaterium*	10000	5000	10
4	*C. albicans*	1000	>10000	10
5	*E. aerogenes*	500	10000	10
6	*E. coli*	>10000	>10000	5
7	*P. aeruginosa*	5000	>10000	5
8	*S. aureus*	500	500	10

The MIC of ethanolic extract of *P. reticulatum* against Gram positive and Gram negative bacteria *B. subtilis*, *E. aerogenes* and *S. aureus* was 500µg/ml. The methanolic extract of *P. reticulatum* showed MIC of 5000 µg/ml against *B. subtilis* and *B. megaterium* and 500µg/ml against *S. aureus* (Plates 3.24,3.25,3.26,3.27, 3.28, 3.29 and 3.38).

Discussion

It was observed that methanolic and ethanolic extract of *P. reticulatum* exhibited similar inhibitory activity against the tested microorganisms. This agrees with previous work where *P. pseudotinctorium* showed MIC values of 500 µg/ml, 350µg/ml and 400µg/ml against *S aureus*, *P. aeruginosa* and *E. coli* (Kekuda *et. al.*, 2010).

4.5.3. Minimum Inhibitory Concentration (MIC) of extracts of *Stereocaulon pomiferum* against the test organisms

Table 4.31. Minimum Inhibitory Concentration (MIC) of extracts of *Stereocaulon pomiferum* against the test organisms

Sl No.	Microorganisms used	MIC (µg/ml)		
		STRE	STRM	Streptomycin
1	*A. faecalis*	5000	>10000	10
2	*B. subtilis*	>10000	>10000	10
3	*B. megaterium*	>10000	>10000	10
4	*C. albicans*	>10000	>10000	10
5	*E. aerogenes*	>10000	10000	10
6	*E. coli*	>10000	>10000	5
7	*P. aeruginosa*	>10000	10000	5
8	*S. aureus*	>10000	>10000	10

S. pomiferum did not possess a potent antimicrobial activity as all its ethanolic extract exhibited MIC greater than 10000µg/ml against all the test microorganisms (Table 4.31) except against *A. faecalis* it is 5000 µg/ml. Methanolic extract of *S. pomiferum* had MIC greater than 10000µg/ml against *A. faecalis, B. megaterium, C. albicans, E. coli*, and *S. aureus*. Only two gram negative bacteria *E. aerogenes* and *P. aeruginosa* were inhibited by 10000µg/ml of methanolic extract.

Discussion

Both methanolic and ethanolic extract of *S. pomiferum* were found less effective against test microorganisms because the growth of microorganisms could not be inhibited by even the highest concentration of extracts (10000 µg/ml) used in the experiment.

Compounds like atanorin, a depside isolated from *Stereocaulon alpinum*, showed MIC value of 250µg/ml against *Mycobacterium aurum (*Ingolfsdottir *et. al.*, 1998*).* Kumar *et. al.*, (2010a) and Ullah *et. al.,* (2009) reported the antifungal activity of lichens and Schmeda *et. al.*, (2008) identified antifungal agents like Isodivaricatic acid, 5-propylresorcinol, divaricatinic acid and usnic acid. Divaricatic acid and other compounds were identified from LCMS Chromatogram of *S. pomiferum.* Highest MIC value may be due to antagonism between phytochemicals.

4.5.4. Minimum Inhibitory Concentration (MIC) of extracts of *Everniastrum* sp against test organisms

Table 4.32. Minimum Inhibitory Concentration (MIC) of extracts of *Everniastrum* sp against test organisms

Sl No.	Microorganisms used	MIC (µg/ml)		
		EVRE	EVRM	Streptomycin
1	*A. faecalis*	500	>10000	10
2	*B. subtilis*	>10000	>10000	10
3	*B. megaterium*	>10000	500	10
4	*C. albicans*	500	500	10
5	*E. aerogenes*	>10000	1000	10
6	*E. coli*	500	>10000	5
7	*P. aeruginosa*	500	>10000	5
8	*S. aureus*	>10000	>10000	10

The highest concentration 10000µg/ml of ethanolic extract of *Everniastrum* used for MIC determination could not inhibit four out of eight microorganisms (Table 4.32) but the same extract showed MIC value of 500µg/ml against three gram negative bacteria (*A. faecalis*, *P. aeruginosa*, *E. coli* and one fungi).

Methanolic extract of *Everniastrum* sp. was found unable to inhibit the growth of five test bacteria even at 10000µg/ml. The MIC value against *E. aerogenes* was resulted as 1000µg/ml and against *B. megaterium* and *C. albicans* was 500µg/ml (Plates 3.40 and 3.41).

Discussion

The ethanolic extract of *Everniastrum* sp was much active against Gram negative bacteria and fungus than against the other microorganisms.

Similarly the methanolic extract of *Everniastrum* sp also gave a low MIC of 500µg/ml against *C. albicans* and *B. megaterium* as compared to that against other microorganisms under study. The MIC value of these extract were much higher than the standard antibiotic streptomycin. The study done by Marijiana *et. al.*, (2010) reported the fungi to be more resistant towards lichen extracts than bacteria. But the present study is consistent to the result of (Baral *et. al.*, 2011) showing strong antifungal activity by lichens, who reported that *Cetraria* spp and *P. nilgherensis* exhibited the

specific antifungal activity while least or ineffective toward bacterial pathogens.

4.5.5. Minimum Inhibitory Concentration (MIC) of extracts of *Ramalina hossei* against the test organisms

Table 4.33. Minimum Inhibitory Concentration (MIC) of extracts of *Ramalina hossei* against the test organisms

Sl No.	Microorganisms used	MIC (µg/ml)		
		RARE	RARM	Streptomycin
1	*A. faecalis*	500	5000	10
2	*B. subtilis*	500	500	10
3	*B. megaterium*	1000	500	10
4	*C. albicans*	500	1000	10
5	*E. aerogenes*	500	500	10
6	*E. coli*	500	5000	5
7	*P. aeruginosa*	500	>10000	5
8	*S. aureus*	500	1000	10

It was evident from (Table 4.33) the MIC of ethanolic extract of *R. hossei* against all the test microorganisms was 500µg/ml except for *B. megaterium* where it was 1000µg/ml. The MIC of methanolic extract of *R. hossei* was different with different organisms tested. MIC of 500µg/ml was observed against *B. subtilis*, *B. megaterium* and *E. aerogenes*, *A. faecalis* and *E. coli*. MIC of 1000 µg/ml against *C. albicans* and *S. aureus* was shown by its methanolic extract. A higher MIC greater than 10000 µg/ml against *P. aeruginosa*.

Discussion

These results revealed that ethanolic and methanolic extract of *R. hossei* exhibited a varying MIC value against test microorganisms. Turk *et. al.*, (2004) reported that the MIC value of lichen *R. farinaceae* was 6.6 µg/25 µl against *B. subtilis* 3.3 µg /25µl against *S. aureus* and 3.3 µg /25µl against *C. albicans*.

Another study conducted by Hoskeri *et. al.*, (2010) on *Ramalina pacifica* showed MIC value of 1µg/ml against clinical pathogenic strains isolated from different infectious sources (*Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Salmonella typhi*, *S. paratyphi*, *E. coli* and *S. aureus*).

Bioactive column fractions from *Ramalina farinaceae* were active to clinical isolates of *Staphylococcus aureus* (Esimone and Adikwu, 1999).

4.6. Determination of Minimum Inhibitory Concentration (MIC) of medicinal plant extracts.

4.6.1 Minimum Inhibitory Concentration (MIC) of extracts of *Urtica dioica* against test organisms

Table 4.34. Minimum Inhibitory Concentration (MIC) of extracts of *Urtica dioica* against test organisms

Sl No.	Microorganisms used	MIC (µg/ml)		
		URRE	URRM	Streptomycin
1	*A. faecalis*	500	5000	10
2	*B. subtilis*	>10000	>10000	10
3	*B. megaterium*	>10000	>10000	10
4	*C. albicans*	>10000	>10000	10
5	*E. aerogenes*	>10000	>10000	10
6	*E. coli*	>10000	>10000	5
7	*P. aeruginosa*	>10000	>10000	5
8	*S. aureus*	>10000	>10000	10

MIC value against all the tested microorganisms except *A. faecalis* (500µg/ml) for ethanolic extract of *U. dioica* was noted whereas other MIC values were greater than 10000µg/ml.

The MIC of methanolic extract of *U. dioica* was greater than 10000µg/ml against three gram negative bacteria; three gram positive bacteria and a fungus under test (Plates 3.35 and 3.53).

Discussion

The MIC of both ethanolic and methanolic extract of *U. dioica* was higher than10000µg/ml in most of the cases, which indicated that *U. dioica* was weak for its antimicrobial activity.

In a study carried out by Chahardehi *et. al.*, (2012), MIC of *U. dioica* against pathogenic bacteria *B. cereus* and MRSA using butanol extraction method was 8.33 and 16.33 mg/mL respectively, while using ethyl acetate against *Vibrio parahaemolyticus* was 0.13 mg/mL.

MIC of *U. dioica* leaves essence was 1.8 µg/ml against *B. cereus* 3.75 µg/ml against *S. aureus*, *P. aeruginosa* and *K. pneumoniae*; 7.5 µg/ml against *E. faecalis* and *E. coli* (Ramtin *et. al.*, 2014) which was dissimilar to the results presently obtained (Table 4.34) in which MIC value was greater than 10000µg/ml against *S. aureus, P. aeruginosa* and *E. coli.*

4.6.2. Minimum Inhibitory Concentration (MIC) of extracts of *Berginia ciliata* against the test organisms

Table 4.35. Minimum Inhibitory Concentration (MIC) of extracts of *Berginia ciliata* against the test organisms

Sl No.	Microorganisms used	MIC (µg/ml)		
		BERE	BERM	Streptomycin
1	*A. faecalis*	>10000	>10000	10
2	*B. subtilis*	5000	>10000	10
3	*B. megaterium*	>10000	>10000	10
4	*C. albicans*	5000	>10000	10
5	*E. aerogenes*	10000	>10000	10
6	*E. coli*	>10000	5000	5
7	*P. aeruginosa*	5000	10000	5
8	*S. aureus*	>10000	>10000	10

Ethanolic extract of *B. ciliata* had MIC greater than 10000µg/ml against *A. faecalis, B. megaterium*, *E. coli* and *S. aureus*. A MIC of 5000µg/ml was observed against *B. subtilis, P. aeruginosa* (Plate 3.30) and *E. aerogenes*.

Mostly MIC greater than 10000µg/ml was exhibited by methanolic extract of *B. ciliata* against all the microorganisms under study except for *E. coli* where it was 5000 µg/ml.

Discussion

The MIC value of ethanolic and methanolic extract of *B. ciliata* was higher against test bacteria. A quite low MIC of 5000µg/ml was observed against the fungus *C. albicans* in its ethanolic extract.

4.6.3. Minimum Inhibitory Concentration (MIC) of extracts of *Sapindus mukrossi* against the test organisms

Table 4.36. Minimum Inhibitory Concentration (MIC) of extracts of *Sapindus mukrossi* against the test organisms

Sl No.	Microorganisms used	MIC (µg/ml)		
		SARE	SARM	Streptomycin
1	*A. faecalis*	5000	10000	10
2	*B. subtilis*	100	5000	10
3	*B. megaterium*	>10000	100	10
4	*C. albicans*	>10000	5000	10
5	*E. aerogenes*	>10000	>10000	10
6	*E. coli*	>10000	>10000	5
7	*P. aeruginosa*	>10000	>10000	5
8	*S. aureus*	>10000	>10000	10

A low MIC value of 100µg/ml of *S. mukrossi* ethanolic extract was observed against *B. subtilis* but MIC value greater than 10000µg/ml was observed against *B. megaterium*, *C. albicans*, *E. aerogenes, E. coli*, *P. aeruginosa* and *S. aureus*. In gram negative bacteria (*A. faecalis*) MIC of 5000µg/ml was obtained.

Considering methanolic extract of *S. mukrossi* a high MIC value greater than 10000µg/ml was noted against *E. aerogenes*, *E. coli, P. aeruginosa* and *S. aureus*. In contrary MIC of only 100µg/ml was observed against *B. megaterium* (Plates 3.51 and 3.55).

Discussion

A high MIC value greater than 10000µg/ml, was observed for ethanolic and methanolic extract of *S. mukrossi* against most of microorganisms under study.

Manjulata *et. al.*, (2012) also tested the antibacterial activity of *S. emarginatus* against *E. coli*, *S. aureus*, *Staphylococcus pyogenes* and *Klebsiella pneumoniae* in different solvents with MIC values 500µg/ml in response to butanol fraction of pericarp and butanol fraction of seed, and 1000µg/ml against methanol fraction of seed.

As observed in the present study the MIC value of *S. mukrossi* against *C. albicans* was 10000µg/ml taking ethanolic extract and 5000µg/ml taking methanolic extract. This result is nearer to that of study conducted by Manjulata *et. al.*, (2012) extracts of *S. emarginatus* in which 7.8mg/ml MIC was observed against yeast *C. albicans* 15.6 mg/ml against dermatophyte *Trichophyton rubrum*, 62.5 µg/ml against, *Epidermophyt*on *floccosum* in which the MIC value against *E. coli* and *S. aureus* was greater than 1000µg/ml. The MIC of extracts of *S. emarginatus* was obtained ranging from 500µg/ml to 2000 µg/ml against *E. coli*, *S. aureus* and *Streptococcus pyogenes* (Manjulata *et. al.*, 2012).

4.7. Determination of MIC of combined extract of lichen and medicinal plants against the test organisms

4.7.1. Minimum Inhibitory Concentration (MIC) of extracts of *Panax pseudopseudoginseng* against the test organisms

Table 4.37. Minimum Inhibitory Concentration (MIC) of extracts of *Panax pseudoginseng* against the test organisms

Sl No.	Microorganisms used	MIC (µg/ml)		
		PNXE	PNXM	Streptomycin
1	*A. faecalis*	10000	1000	10
2	*B. subtilis*	1000	>10000	10
3	*B. megaterium*	>10000	>10000	10
4	*B. albicans*	10000	10000	10
5	*E. aerogenes*	>10000	>10000	10
6	*E. coli*	>10000	>10000	5
7	*P. aeruginosa*	>10000	>10000	5
8	*S. aureus*	500	>10000	10

The ethanolic extract of *P. pseudoginseng* exhibited quite high MIC greater than 10000µg/ml against *B. megaterium*, *E. aerogenes*, *E. coli*, *P. aeruginosa* and 500µg/ml for *S. aureus*. The methanolic extract of *P. pseudoginseng* was also quite weak in its antimicrobial activity as the entire MIC observed was greater than equal to 10000µg/ml(Plates 3.52 and 3.54).

Discussion

The value of ethanolic and methanolic extract *P. pseudoginseng* was higher than 10000µg/ml against gram positive bacteria, gram negative bacteria and fungus tested except for *S. aureus.*

The extracts of *P. pseudoginseng* could not easily inhibit the growth of microorganisms may be the particles of extracts of *P. pseudoginseng* could not enter the cells of gram positive and gram negative bacteria tested. A quite high concentration 10000µg/ml could restrict growth of *C. albicans.*

4.7.2. Minimum Inhibitory Concentration (MIC) of combined extracts of *Everniastrum* sp and *Panax pseudopseudoginseng* against the test organisms

Table 4.38. Minimum Inhibitory Concentration (MIC) of combined extracts of *Everniastrum* sp and *Panax pseudopseudoginseng* against the test organisms

Sl No.	Microorganisms used	MIC (µg/ml)		
		EVRE+PNXE	EVRM+PNXM	Streptomycin
1	*A. faecalis*	1000	>10000	10
2	*B. subtilis*	500	>10000	10
3	*B. megaterium*	1000	10000	10
4	*C. albicans*	500	>10000	10
5	*E. aerogenes*	>10000	>10000	10
6	*E. coli*	1000	1000	5
7	*P. aeruginosa*	5000	>10000	5
8	*S. aureus*	1000	500	10

The combined ethanolic extract of *Everniastrum* sp and *P. pseudoginseng* had significant MIC. MIC values of 1000µg/ml and greater than that were observed against *A. faecalis* and *B. megaterium, E. aerogenes, E. coli, P. aeruginosa* and *S. aureus.* Low MIC values of 500µg/ml were noted against *B. subtilis* and *C. albicans.* MIC value of 500µg/ml and 1000 µg/ml was shown by methanolic extract of combined extract (Plates 3.42- 3.50).

Discussion

It can be concluded from the result that combination of *Everniastrum* sp with *P. pseudoginseng* showed moderate antimicrobial property.

It could be traced out from above results that the combined extracts *Everniastrum* sp and *P. pseudoginseng* was able to inhibit the growth of mostly Gram positive bacteria and fungus at low concentration.

4.7.3. Minimum Inhibitory Concentration (MIC) of combined extracts of *Everniastrum* sp and *Berginia ciliata against* test organism

Table 4.39. Minimum Inhibitory Concentration (MIC) of combined extracts of *Everniastrum* sp and *Berginia ciliata* against test organisms

Sl No.	Microorganisms used	MIC (µg/ml)		
		EVRE+BERE	EVRM+BERM	Streptomycin
1	*A. faecalis*	>10000	>10000	10
2	*B. subtilis*	>10000	>10000	10
3	*B. megaterium*	>10000	>10000	10
4	*C. albicans*	>10000	>10000	10
5	*E. aerogenes*	>10000	>10000	10
6	*E. coli*	5000	>10000	5
7	*P. aeruginosa*	>10000	500	5
8	*S. aureus*	5000	>10000	10

MIC of combined ethanolic extract of *Everniastrum* sp and *B. ciliata* was greater than 10000µg/ml in all cases except for *E. coli* (5000µg/ml) and *S. aureus* (500µg/ml).

The MIC of combined methanolic extract of *Everniastrum* sp and *B. ciliata* was greater than 10000µg/ml except for *P. aeruginosa* where it was 500µg/ml.

Discussion

The mixture of ethanolic extracts of *Everniastrum* sp and *B. ciliata* exhibited high MIC value greater than 10000µg/ml against majority of test microorganisms.

A distinct synergistic effect of combined extract of these two plants was observed against *P. aeruginosa* and *E. coli* where the MIC was reduced to 500 µg/ml and 5000µg/ml respectively. In other cases the extracts of lichen and *B. ciliata* was mostly indifferent to each other.

4.7.4. Minimum Inhibitory Concentration (MIC) of combined extracts of *Stereocaulon pomiferum* and *Berginia ciliata* against the test organisms

Table 4.40. Minimum Inhibitory Concentration (MIC) of combined extracts of *Stereocaulon pomiferum* and *Berginia ciliata* against the test organisms

Sl No.	Microorganisms used	MIC (µg/ml)		
		STRE+BERE	STRM+BERM	Streptomycin
1	*A. faecalis*	>10000	>10000	10
2	*B. subtilis*	>10000	>10000	10
3	*B. megaterium*	>10000	>10000	10
4	*C. albicans*	>10000	>10000	10
5	*E. aerogenes*	>10000	>10000	10
6	*E. coli*	>10000	>10000	5
7	*P. aeruginosa*	>10000	>10000	5
8	*S. aureus*	5000	>10000	10

The MIC of combined ethanolic extract of *S. pomiferum* and *B. ciliata* was greater than 10000µg/ml against all the microorganisms under test except *S. aureus* where it was 5000µg/ml.

The MIC value of ethanolic extract of *S. pomiferum* and *B. ciliata* was very high as it was greater than 10000µg/ml against the four gram negative, three gram positive and a fungus tested.

Discussion

The mixture of ethanolic extract of *S. pomiferum* and *B. ciliata* did not show much synergistic effect.

An antagonistic effect was observed as MIC *of B. ciliata* against *B. subtilis* (5000µg/ml), *C. albicans* (5000 µg/ml) and *E. coli* (5000µg/ml) (Table 4.35) individually but when mixed with *S. pomiferum* its MIC was higher than 10000µg/ml against these bacteria and fungi.

4.7.5 Minimum Inhibitory Concentration (MIC) of combined extracts of *Usnea baileyi* and *Berginia ciliata* against the test organisms

Table 4.41. Minimum Inhibitory Concentration (MIC) of combined extracts of *Usnea baileyi* and *Berginia ciliata* against the test organisms

Sl No.	Microorganisms used	MIC (µg/ml)		
		USRE+BERE	USRM+BERM	Streptomycin
1	*A. faecalis*	>10000	500	10
2	*B. subtilis*	>10000	>10000	10
3	*B. megaterium*	>10000	5000	10
4	*C. albicans*	>10000	>10000	10
5	*E. aerogenes*	>10000	>10000	10
6	*E. coli*	>10000	>10000	5
7	*P. aeruginosa*	>10000	>10000	5
8	*S. aureus*	>10000	5000	10

The MIC value of ethanolic extract of mixture of *U. baileyi* and *B. ciliata* was higher than 10000µg/ml against all the microorganisms tested including fungi.

The MIC of value of combined ethanolic extract of *U. baileyi* and *B. ciliata* was greater than 10000 µg/ml against gram negative organisms (*B. subtilis, E. aerogenes, E. coli* and *P. aeruginosa*), fungus (*C. albicans*) and 5000 µg/ml against gram positive organisms (*S. aureus* and *B. megaterium*). MIC value of combined methanolic plant extracts was lowest (500 µg/ml) against *A. faecalis*

Discussion

An antagonism was observed as MIC of ethanolic extract of *U. baileyi* alone was 500µg/ml (against *B. subtilis C. albicans* and *E. coli*) but in its combined extract it was raised to 10000µg/ml against them.

Similarly the MIC of methanolic extract of *U. baileyi* was 500µg/ml against *B. subtilis*, *C. albicans*, *E. coli* and *E. aerogenes* but when combined with *B. ciliata,* it was enhanced to 10000µg/ml. The antimicrobial activity of one plant extract was lowered by the other.

The results indicate that either of the lichen or medicinal plant possesses antagonistic principle which has resulted in increasing the MIC values of combined extracts than individual extract.

4.7.6. Minimum Inhibitory Concentration (MIC) of combined extracts of *Ramalina hossei* and *Berginia ciliata* against the test organisms

Table 4.42. Minimum Inhibitory Concentration (MIC) of combined extracts of *Ramalina hossei* and *Berginia ciliata* against the test organisms

Sl No.	Microorganisms used	MIC (µg/ml)		
		RARE+BERE	RARM+BERM	Streptomycin
1	*A. faecalis*	>10000	>10000	10
2	*B. subtilis*	>10000	5000	10
3	*B. megaterium*	>10000	>10000	10
4	*C. albicans*	500	>10000	10
5	*E. aerogenes*	5000	>10000	10
6	*E. coli*	>10000	>10000	5
7	*P. aeruginosa*	>10000	500	5
8	*S. aureus*	>10000	>10000	10

The combination of ethanolic extract of *R. hossei* and *B. ciliata* yielded MIC value greater than 10000µg/ml against both gram positive and gram negative bacteria tested. MIC against a fungus was 500µg/ml. In addition the MIC against Gram negative *E. aerogenes* was 5000µg/ml.

The mixture of methanolic extract of *R. hossei* and *B. ciliata* was higher than 1000µg/ml against *A. faecalis*, *B. megaterium*, *C. albicans*, *E. aerogenes*, *E. coli*, and *S. aureus*. The MIC value 5000µg/ml and 500µg/ml was observed against a gram positive (*B. subtilis*) and a gram negative (*P. aeruginosa*) bacteria respectively.

Discussion

The MIC of *B. ciliata* for its ethanolic and methanolic extract against the test organisms ranged from 1000µg/ml to 10000µg/ml.

An evident antagonistic effect was noted because low MIC of 500µg/ml was observed against ethanolic extract of *R. hossei* (Table 4.33) against all the test microorganisms except *B. megaterium* (10000µg/ml) and *C. albicans* (500µg/ml).

Synergism was observed in two cases as lower MIC against ethanolic and methanolic combination *C. albicans* (500µg/ml) and *P. aeruginosa* (500µg/ml) was observed.

The combination of two plant extract could significantly lead to the development of new approach in providing barrier against resistance microorganisms because the use of extracts shows low risk of increasing bacterial resistance to their action. As the extracts mixture contains mixture of bioactive compounds which makes the survivality of microorganisms difficult as compared to the single plant extracts.

Use of only combined plant extracts without combining antibiotics may also decrease risk of side effects caused by the antibiotics (Matias, 2011; Shanmugam, 2008).

4.7.7. Minimum Inhibitory Concentration (MIC) of combined extracts of *Everniastum sp* and *Urtica dioica* against the test organisms

Table 4.43. Minimum Inhibitory Concentration (MIC) of combined extracts of *Everniastum sp* and *Urtica dioica* against the test organisms

Sl No.	Microorganisms used	MIC (µg/ml)		
		EVRE+URRE	EVRM+URRM	Streptomycin
1	*A. faecalis*	>10000	>10000	10
2	*B. subtilis*	>10000	500	10
3	*B. megaterium*	>10000	>10000	10
4	*C. albicans*	500	>10000	10
5	*E. aerogenes*	>10000	500	10
6	*E. coli*	>10000	>10000	5
7	*P. aeruginosa*	>10000	>10000	5
8	*S. aureus*	>10000	5000	10

The MIC of combined ethanolic extract of *Everniastrum* sp and *U. dioica* was higher than 10000µg/ml against all the bacteria tested except for a fungus where the MIC value was 500µg/ml.

The MIC of mixture of methanolic extract of *R. hossei* with *U. dioica* was varying against different microorganisms under study. MIC was higher than 10000µg/ml against *A. faecalis, B. megaterium, C. albicans, E. coli* and *P. aeruginosa*, 5000µg/ml against *S. aureus* and 500µg/ml against *B. subtilis* and *E. aerogenes*.

Discussion

U. dioica proved to be a weak antimicrobial agent than *Everniastrum* sp when tested separately (Table 4.34). It could be concluded that synergism was observed while determining the MIC of test microorganisms by the combined extracts.

Association of ethanolic lichen and medicinal plant showed MIC value 500µg/ml against *C. albicans*, *B. subtilis* and *E. aerogenes* in methanolic extract. Therefore the ethanolic extract of lichen *Everniastrum* sp in combination with extract of traditionally known medicinal plant, *U. dioica* may be used as potent antifungal agent and it may be assumed that the high MIC of *U. dioica* was lowered by *Everniastrum* sp.

4.7.8. Minimum Inhibitory Concentration (MIC) of combined extracts of *Ramalina hossei* and *Urtica dioica* against the test organisms

Table 4.44. Minimum Inhibitory Concentration (MIC) of combined extracts of *Ramalina hossei* and *Urtica dioica* against the test organisms

Sl No.	Microorganisms used	MIC (µg/ml)		
		RARE+URRE	RARM+URRM	Streptomycin
1	*A. faecalis*	>10000	>10000	10
2	*B. subtilis*	>10000	500	10
3	*B. megaterium*	500	>10000	10
4	*C. albicans*	500	500	10
5	*E. aerogenes*	>10000	500	10
6	*E. coli*	>10000	>10000	5
7	*P. aeruginosa*	>10000	1000	5
8	*S. aureus*	>10000	500	10

The mixture of ethanolic extract of *R. hossei* and *U. dioica* was showed MIC of 500µg/ml against *B. megaterium* and *C. albicans***.** The extract mixture showed MIC greater than 10000µg/ml against remaining six bacteria.

MIC value of 500µg/ml was observed for combined methanolic extract of *R. hossei* and *U. dioica* against *B. subtilis*, *C. albicans* and *S. aureus*. MIC higher than 10000µg/ml was observed against *A. faecalis, B. megaterium* and *E. coli.*

Discussion

Combination of ethanolic and methanolic extract of *U. dioica* and *R. hossei* exhibited synergism against *B. megaterium*, *C. albicans*, *E. aerogenes* and *S. aureus* with MIC values of lower than the individual value of *U. dioica* against the respective test organisms. In a study conducted by Agboke and Esimone *et. al.*, (2011) methanol extract of *R. farinacea* was combined with antibiotic ampicillin; the lichen extract enhanced the potency of ampicillin against *S. aureus*. It was also observed that combination ratio of lichen: ampcillin was (9:1, 8:2, 6:4, 5:5, 4:6 and 3:7) the interactions were synergistic and at (7:3, 2:8 and 1:9) interactions was additive.

4.7.9. Minimum Inhibitory Concentration (MIC) of combined extracts of *Parmotrema reticulatum* and *Berginia ciliata* against test microorganisms

Table 4.45. Minimum Inhibitory Concentration (MIC) of combined extracts of *Parmotrema reticulatum* and *Berginia ciliata* against test microorganisms

Sl No.	Microorganisms used	MIC (µg/ml)		
		PARE+BERE	PARM+BERM	Streptomycin
1	*A. faecalis*	>10000	>10000	10
2	*B. subtilis*	500	>10000	10
3	*B. megaterium*	>10000	>10000	10
4	*C. albicans*	>10000	>10000	10
5	*E. aerogenes*	>10000	>10000	10
6	*E. coli*	>10000	500	5
7	*P. aeruginosa*	>10000	>10000	5
8	*S. aureus*	1000	>10000	10

The combined ethanolic extract of *P. reticulatum* and *B. ciliat*a revealed MIC greater than 10000 against *A. faecalis, B. megaterium, C. albicans, E. aerogenes, E. coli* and *P. aeruginosa.* A lower MIC 500 µg/ml and 1000 µg/ml were observed against *B. subtilis* and *S. aureus* respectively.

A combined methanolic extract of *P. reticulatum* and *B. ciliata* exhibited MIC greater than 10000µg/ml against all microorganisms tested except *E. coli* where MIC value was 500µg/ml.

Discussion

Synergism was observed for combined methanolic extract against *E. coli* with MIC of 500µg/ml because as seen earlier MIC was 5000µg/ml (for methanolic extract of *B. ciliata*) and greater than 10000 µg/ml for methanolic extract of *P. reticulatum*.

4.7.10. Minimum Inhibitory Concentration (MIC) of combined extracts of *Usnea baileyi* and *Urtica dioica* against the test organisms

Table 4.46. Minimum Inhibitory Concentration (MIC) of combined extracts of *Usnea baileyi* and *Urtica dioica* against the test organisms

Sl No.	Microorganisms used	MIC (µg/ml)		
		USRE+URRE	USRM+URRM	Streptomycin
1	*A. faecalis*	500	>10000	10
2	*B. subtilis*	>10000	>10000	10
3	*B. megaterium*	>10000	>10000	10
4	*C. albicans*	>10000	500	10
5	*E. aerogenes*	>10000	500	10
6	*E. coli*	>10000	500	5
7	*P. aeruginosa*	>10000	>10000	5
8	*S. aureus*	500	>10000	10

The mixture of ethanolic extract of *U. baileyi* and *U. dioica* exhibited MIC value greater than 10000µg/ml against *B. subtilis, B. megaterium, C. albicans, E. aerogenes E. coli* and *P. aeruginosa.* MIC of 500µg/ml was observed against *A. faecalis* and *S. aureus*.

Similarly the MIC value of mixture of methanolic extract of *U. baileyi* and *U. dioica* was higher than 10000µg/ml against all the microorganisms under study except for *C. albicans* (500 µg/ml), *E. coli* (500 µg/ml) and *E. aerogenes* (500µg/ml).

Discussion

A distinct synergistic effect was observed as for mixture of ethanolic extract of *U. baileyi* (Table 4.29) and *U. dioica* (Table 4.34) against *A. faecalis* with low MIC (500 µg/ml) as their individual they showed MIC value of 5000 µg/ml against test organisms.

MIC of mixture of methanolic extract of *U. baileyi* with *U. dioica* showed additive effect against *C. albicans*, *E. aerogenes* and *E.coli* out of eight test microorganisms.

It seemed that active compounds from both the plants directly or indirectly attach to the same site on bacterial cell. Synergistic effect may have been insufficiently occurred. It may be that phytocompounds disturb cell wall or increase permeability of the cytoplasmic membrane and thereby facilitate the influx of antibiotics, produce efflux pump inhibitiors (Sibanda and Okoh, 2007; Horiuchi *et. al.*, 2007). Similar activity may be shown by lichen as well as plant extract.

4.8. Estimation of DPPH radical scavenging activity of lichens and medicinal plants

The free radical scavenging activity of the extracts was measured *in vitro* by 1, 1, diphenyl pycryl hydrazyl (DPPH) assay (Nagarajan *et. al.*, 2008). DPPH is usually used as a substrate to evaluate the antioxidative activity of antioxidants (Luo *et. al.*, 2006). It is model of stable lipophilic radical which reacts with antioxidants either by addition of an electron or hydrogen atom, which reduces the number of free radicals. The absorption which is measured at 517nm is proportional to the amount residual DPPH and is observed by discolouration from purple to yellow. Free radicals are also implicated in the pathology of diseases such as coronary heart diseases and cancer (Dzomba *et. al.*, 2012).

4.8.1. DPPH radical scavenging activity of Ascorbic acid

Table 4.47. DPPH radical scavenging activity of Ascorbic acid

ASCR (µg/ml)	DPPH Radical Scavenging activity (%)
25	18.87±0.06
50	28.37±0.06
100	38.78±0.06
200	70.58±0.06

4.8.2. DPPH radical scavenging activity of *Usnea baileyi*

Table 4.48. DPPH radical scavenging activity of *Usnea baileyi*

USR (µg/ml)	Extract	DPPH Radical Scavenging activity (%)
25	Ethanolic	12.77±0.15
	Methanolic	16.50±0.07
50	Ethanolic	24.27±0.06
	Methanolic	19.40±0.35
100	Ethanolic	37.07±0.06
	Methanolic	24.37±0.6
200	Ethanolic	49.67±0.06
	Methanolic	31.49±0.09

Free radical scavenging ability of the ethanolic and methanolic extract of *U. baileyi* was compared with that of ascorbic acid. The results showed that absorbance decreased with increasing concentration of extract (25-200 µg/ml) but the percentage of scavenging activity increased. Hence the percentage radical scavenging activity of extracts was directly proportional to concentration of extracts.

Discussion

Significant correlation was found between the free radical scavenging activity and the concentration of lichen extract and ascorbic acid used as positive control. The ethanolic extract of *U. baileyi* exhibited a highest radical scavenging activity of 49% at concentration 200µg/ml.The scavenging activity of ethanolic and methanolic extract of *U. baileyi* was lower than that of ascorbic acid.

Earlier work by Luo *et. al.,* (2006) indicated that removal of free radical increased by 36-72% in accordance with the increase in concentration of the extract from 0.2 to 2 mg/ml in methanol extract of *Thamnolia vermicularis*, similarly the scavenging ability of *U. baileyi* increased from 12.77% to 49% for ethanolic extract and from 16% to 31.49% for methanolic extract. The obtained results reveal that significant quantities of antioxidant substances are present in the lichen *U. baileyi.*

4.8.3. DPPH radical scavenging activity of *Everniastrum* sp

Table 4.49. Free radical scavenging activity of *Everniastrum* sp

EVR (µg/ml)	Extract	DPPH Radical Scavenging activity (%)
25	Ethanolic	20.70±1.07
	Methanolic	28.18±0.97
50	Ethanolic	30.86±0.97
	Methanolic	30.34±1.02
100	Ethanolic	33.07±0.35
	Methanolic	32.22±0.80
200	Ethanolic	53.59±0.94
	Methanolic	36.59±2.05

Ethanolic and methanolic extracts of tested lichen showed a good scavenging effect on DPPH radical. The scavenging activity of ethanolic

extract of *Everniastrum* sp ranged from 20.7% to 53.59% in the concentration dependent manner (i.e., from 25-200µg/ml). The highest radical scavenging activity was shown by ethanolic extract as 53.59% at concentration 200µg/ml. At concentration (25 and 50µg/ml) free radical scavenging activity of methanolic extract of *Everniastrum* sp (28% and 30%) was even greater than the standard compound ascorbic acid (18.87% and 28.37%) respectively.

The percentage of inhibition on DPPH radical by ethanolic extract of *Everniastrum* sp (30.86%) was greater than ascorbic acid (28%) at concentration 50µg/ml.

Discussion

It was observed that the scavenging activity of ethanolic extract of *Everniastrum* sp was better than that of methanolic extract. The effect of extracts on DPPH is thought to be due to their hydrogen donating ability. DPPH scavenging activity of the ethanol extract of *Everniastrum* sp was greater than ascorbic acid at some concentrations showed that the extracts have the proton donating ability and could serve as free radical inhibitors or scavengers as primary antioxidants.

Our results are consistent with that of previous result obtained by Kosanic and Rankovic *et. al.*, (2011) in which the methanolic extract of *Pseudoevernia furfuraceae* exhibited a free radical scavenging activity of 57.88% similarly the tested lichen *Everniastrum* sp possessed this activity as 53.59% using ethanol for extraction. The capacity of selected lichens like *Everniastrum* sp to scavenge DPPH radical even more than ascorbic acid opens the door for further investigation and use of *Everniastrum* sp as antioxidants.

4.8.4. DPPH radical scavenging activity of *Parmotrema reticulatum*

Table 4.50. DPPH radical scavenging activity of *Parmotrema reticulatum*

PAR	Extract	DPPH Radical Scavenging
25	Ethanolic	24.76±0.70
	Methanolic	9.65±2.24
50	Ethanolic	29.55±0.95
	Methanolic	16.20±1.13
100	Ethanolic	33.17±1.39
	Methanolic	34.48±0.66
200	Ethanolic	36.40±0.21
	Methanolic	40.79±0.86

The scavenging of DPPH radicals by the studied lichen *P. reticulatum* was moderate and relatively less than the standard ascorbic acid.

The scavenging activity of the ethanolic extract of *P. reticulatum* ranged in from 24.76% to 36.40% using extract concentration from 25µg/ml to 200µg/ml. The methanolic extract exhibited scavenging activity of 9.65% at concentration 25µg/ml and 16.20% at 50µg/ml but reached maximum to 40.79% at concentration 200µg/ml.

Discussion

It was observed that scavenging activity of methanolic extract of *P. reticulatum* was better than ethanolic extract. The antioxidant activity of the plant extract may be due to the presence of terpenes, tannins and flavonoids (El-Massy *et. al.*, 2009; Maestri *et. al.,* 2006).

Hence it can be clearly observed that methanolic and ethanolic extract of *P. reticulatum* potent as free radical scavengers.

4.8.5. DPPH radical scavenging activity of *Ramalina hossei*

Table 4.51. DPPH radical scavenging activity of *Ramalina hossei*

Extract conc. (µg/ml)	Extract	DPPH Radical Scavenging activity (%)
25	Ethanolic	4.99±0.52
	Methanolic	14.25±0.91
50	Ethanolic	13.40±1.6
	Methanolic	24.93±0.58
100	Ethanolic	24.78±1.79
	Methanolic	25.62±1.02
200	Ethanolic	36.59±0.82
	Methanolic	30.63±0.90

The ethanolic extracts of *R. hossei* possessed moderate scavenging ability in this dose dependent study. Moreover the activity of methanolic extract of *R. hossei* is better than the ethanolic extract.

The scavenging ability of extracts of *R. hossei* is quite lower than the standard ascorbic acid. The maximum activity of the extract was observed as 36.59% at concentration 200µg/ml.

The scavenging activity of methanolic extract of *R. hossei* was close to ascorbic acid at concentration 50µg/ml. Overall it could be observed that ethanolic extract of *R. hossei* exhibited a good radical scavenging potency.

Discussion

From the above results it was observed that the scavenging activity of ethanolic and methanolic extract of *R. hossei* which was evaluated by bleaching of stable DPPH radical was moderate. The bleaching power of extract increased from lower to higher in a dose dependent manner *i.e.,* from 25 -200µg/ml. The results show that the lichen *Ramalina hossei* may possess a significant amount of antioxidant molecules.

4.8.6. DPPH radical scavenging activity of *Stereocaulon pomiferum*

Table 4.52.a DPPH radical scavenging activity of *Stereocaulon pomiferum*

Extract. conc. (µg/ml)	Extract	DPPH Radical Scavenging activity (%)
25	Ethanolic	19.03±0.2
	Methanolic	6.13±2.01
50	Ethanolic	20.27±0.42
	Methanolic	13.36±1.39
100	Ethanolic	25.80±0.35
	Methanolic	20.14±1
200	Ethanolic	27.40±0.53
	Methanolic	30.2±2.03

The studied fruticose lichen *S. pomiferum* possessed a lower radical scavenging ability. The percentage of inhibition of DPPH radical for ethanol and methanol extracts of this lichen were 19%, 20%, 25%, 27% and 6.1%,13%,20% and 30.2% respectively. The scavenging ability of the extracts was lower than that of ascorbic acid.

Discussion

In the present study the rapid change in colour of purple DPPH suggested that ethanolic and methanolic extract of *S. pomiferum* might contain antiradical activity. In another conducted by Bhattarai *et. al.*, (2008), the strength of antioxidant activity in terms of discoloration of

DPPH was shown to be stronger by the constituents of *S. alpinum*, *Cladonia regalis* and *Cladonia* sp.

Table 4.52b. Statistical analysis of DPPH radical scavenging activity of lichens at 200(µg/ml)

Samples	(µg/ml)
Ascorbic acid	70.58±0.06$^{(h)}$
EVRE	53.59±0.94$^{(g)}$
EVRM	36.59±2.05$^{(d)}$
PARE	36.40±0.21$^{(d)}$
PARM	40.79±0.86$^{(e)}$
RARE	36.59±0.82$^{(d)}$
RARM	30.63±0.90$^{(c)}$
STRE	27.40±0.53$^{(b)}$
STRM	30.2±2.03$^{(a)}$
USNE	49.67±0.06 $^{(f)}$
USNM	31.49±0.09$^{(c)}$

Duncan's post hoc test indicates the values in the column with similar superscripts inside bracket are not significantly different and with different superscript are significantly different (P<0.05),(Appendix I)

The results above (Table 4.52b) signify that the radical scavenging activity of lichen extracts and ascorbic acid differ significantly (p<0.05).

Pearson's bivariate test for correlation exhibit that the DPPH radical scavenging activity showed positive and significant correlation with Total antioxidant activity and Reducing power Ability in this study (where r=0.647 and r=0.528) at significant level P<0.01(Appendix L)

4.8.7. DPPH radical scavenging activity of medicinal plants

Table 4.53. DPPH radical scavenging activity of medicinal plants

DPPH Radical Scavenging activity (%)					
Concentration (µg/ml)	Ascorbic acid (Control)	URRE	URRM	BERE	BERM
25	18.87±0.06	11.2±0.02	21.6±0.35	17.6±0.17	15.2±0.51
50	28.37±0.06	22.09±0.16	38.6±0.55	21.8±0.41	28.0±0.01
100	38.78±0.06	30.9±0.2	53.3±0.34	37.9±0.20	49.9±0.48
200	70.58±0.06$^{(d)}$	38.7±0.11$^{(a)}$	56.9±0.91$^{(c)}$	49.4±0.26$^{(b)}$	56.9±0.50$^{(c)}$

Duncan's post hoc test indicates values in the row with similar superscripts inside bracket are not significantly different and with different superscript are significantly different ($P<0.05$),(Appendix M)

Some specialized plant metabolic compounds that can act as antioxidants are phytochemicals (Otkay *et. al.*, 2003; Wangesteen *et. al.*, 2004). The phytochemicals participate in redox systems and act as electron donors hydrogen donors and singlet oxygen quenchers (Kahkonen *et. al.*, 1999).

Two medical plants were screened for its radical scavenging activity. The ethanolic and methanolic extracts overall possessed a moderate radical scavenging activity. But it could be traced out from table 4.53 that URRE exhibited a highest radical scavenging activity at concentration 200µg/ml as 38.7% and URRM a relatively high inhibition percentage of 56.9% (Table 4.53).

The ethanolic and methanolic extract of *B. ciliata* also possessed an appreciable radical scavenging activity. The percentage of activity increased with increase in concentration of extract. A high value of 56.9% activity was observed at concentration 200µg/ml by BERM.

Discussion

Urtica dioica which is a member of Urticaceae class, its Latin name is Nettle it has been used traditionally in treatment of many diseases. There are many reports which show this plant is very effective in the treatment of blood pressure, diabetes and prostate hyperplasia, rheumatoid arthritis and allergic rhinitis (Fathi *et. al.*, 2005). DPPH which can be used as indicators for radical scavenging abilities of biological samples are widely used (Wang and Zhang, 2003).

Previous reports of antioxidant activities and traditional uses of the plant also support the findings of present studies. The methanolic extract of *U. dioica* had greater DPPH radical scavenging activity than of the ethanolic extract and standard ascorbic acid at concentrations 25, 50 and 100µg/ml respectively. Similarly in a screening conducted by Gulcin *et. al.*, (2004), the scavenging effect of water extract of *U. dioica* was 32% at a concentration of 60 µg/ml and that of standards quercetin and BHA on the DPPH radical were 93% and37% respectively.

B. ciliata is considered to be one important medicinal plant. Its rhizome extracts is proved to have anti-bacterial and anti-tussive properties. It is reported to be helpful in dissolving kidney stones. *B. ciliata* is used in traditional ayurvedic medicine for the treatment of several diseases in Nepal, India, Pakistan, Bhutan and some other countries. Methanolic extract to be more active radical scavenger than aqueous extract. Similar findings are also reported by Rajkumar *et. al.*, (2010).

The obtained results signify that the radical scavenging ability of medicinal plants vary significantly with ascorbic acid ($p<0.05$).

4.9. Estimation of DPPH radical scavenging activity of lichen extracts in combination with medicinal plants

Table 4.54. DPPH radical scavenging activity of lichen extracts in combination with medicinal plants

DPPH Radical Scavenging activity (%)								
Conc. (µg/ml)	Ascorbic acid	USRE+URRE	STRE+URRE	STRM+URRM	USRM+BERM	PARM+BERM	EVRM+BERM	PARE+BERE
25	18.87±0.06	5.45±0.43	27.63±3.10	25.73±1.76	34.20±3.16	24.43±1.71	9.94±0.97	30.38±4.22
50	28.37±0.06	10.93±0,42	32.44±0.66	34.23±2.02	40.34±0.76	47.13±4.04	18.84±2	40.72±0.99
100	38.78±0.06	14.88±1.57	39.97±0.65	42.05±1.48	49.55±0.74	54.97±0.76	21.96±2.45	42.72±2.85
200	70.58±0.06	26.41±0.60	47.43±1.86	45.12±1.07	52.01±1.92	72.12±1.83	32.78±1.72	52.20±0.62

After screening the individual scavenging power of lichens and medicinal plants their combined effect was determined. It was clear from the results that the combined antioxidant effect of *Usnea baileyi* (Table 4.48) with *Urtica dioica* (Table 4.53) was less than their individual effect (Table 4.54).

The activity of combined ethanolic extract of *U. dioica* and *S. pomiferum* was quite appreciable. At concentration 25µg/ml, 50µg/ml, 100µg/ml and 200µg/ml the percentage of radical scavenging activity was greater than the activity of individual extracts

The table 4.54 shows that the radical scavenging percentage of the combined methanolic extracts of the plants were less than the individual activity of one of them i.e., *U. dioica*. But it was also observed that at all

concentrations the activity of combined extracts was greater than that of *S. pomiferum* alone.

The combined methanolic extract of *U. baileyi* and *B. ciliata* showed greater activity than the individual plant extracts at concentration 25 µg/ml, 50µg/ml and 100µg/ml. But at concentration 200µg/ml there was a decrease in percentage of antioxidant activity than the individual extracts of *U. baileyi* and *B. ciliata*.

The activity of combined methanolic extract of *P. reticulatum* and *B. ciliata* was very high about 72.12% at 200µg/ml. This value was higher than that of the individual extracts and ascorbic acid.

At high concentration 200µg/ml the combined radical scavenging activity was 32.78%, which was a low value as the individual methanolic extract of *Everniastrum* sp exhibited activity of 53% and that by *B. ciliata* was 56% respectively.

Combined ethanolic extract of *P. reticulatum* and *B. ciliata* exhibited a good scavenging activity which was 52% at concentration 200µg/ml. Even at lower concentration of extracts at 25µg/ml the percentage of activity was 30% which was appreciable value.

Discussion

The ethanolic extracts of *U. baileyi* and *U. dioica* was antagonistic to each other. Similar case was observed by (Johnson *et. al.,* 2015), methanolic extract of leaves of *Kigelia africana*, *Alafia bateri*, *Anthocleista djalonesis* and the stem bark of *Harungana madagascarensis* were screened for antioxidant activity in combination, which resulted in antagonism. It may be hence indicated that the mixture of two cannot be always effectively used to scavenge free radicals.

It may be assumed from table 4.54 that the radical scavenging activity of lichen *S. pomiferum* was enhanced by the presence of the medicinal plant *U. dioica*. But the combination was not synergistic may be due to the reason the proportion of *U. dioica* would have to be increased to achieve synergistic effect.

Comparitively synergism was also clearly observed in table 4.54 it may be concluded that the radical scavenging activity of one plant increased the activity of the other extract, at 200µg/ml the combined

activity of BERM and USRM was little greater than individual lichen extract (USRM).

A good synergistic activity was seen between the methanolic extract of *B. ciliata* and lichen *P. reticulatum.* The combined plant extracts had percentage of activity directly proportional to the concentration.

As observed in table 4.54 the methanolic extract of *Everniastrum* sp and *B. ciliata* was antagonistic to each other. Because some individual plants in a multicomponent preparation may have negative effect on overall potential of the multicomponent herbal formulation due to masking and other chemical or physical interaction, which in turn may result in antagonism (Johnson *et. al.*, 2015).

Synergistic activity exhibited by the combined ethanolic extract of *P. reticulatum* and *B. ciliata*, than the individual plant extract and even higher than standard compound which may be attributed to the presence of diverse natural phytochemicals in the lichen and plant samples.

4.10. Estimation of total antioxidant activity of lichens and medicinal plants under study

The total antioxidant capacity of extract was determined with phosphomolybdenum method using α-tocopherol as the standard expressed as µg equivalent of α-tocopherol by using the standard tocopherol graph (Nagarajan *et. al.*, 2008).

In this assay the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) complex at acidic pH occur. The total antioxidant capacity is expressed as α- tocopherol equivalent following the phosphomolybdate method which is quantitative. The total antioxidant capacity was observed from the calibration curve y= 0.141x-0.039: R^2= 0.941.

4.10.1. Total antioxidant activity of lichen extracts under study

Table 4.55. Total antioxidant activity of lichen extracts at conc. 200 µg/ml

Samples	µg/ml
α-tocopherol	4.19±.005[k]
EVRE	1.39±0.01[d]
EVRM	1.54±0.01[e]
PARE	0.81±0.01[b]
PARM	1.63±0.3[g]
RARE	3.19 ±0.01[j]
RARM	1.59 ± 0.02[f]
STRE	1.88± 0.01[h]
STRM	1.24± 0.01[c]
USNE	2.12±0.02[i]
USNM	0.71±0.01[a]

Duncan's post hoc test indicates values in the column with similar superscripts inside bracket are not significantly different and with different superscript are significantly different (P<0.05), (Appendix G)

α- tocopherol equivalent/ mg equivalent as total antioxidant activity are in given (table 5.55). The highest antioxidant activity was measured as 3.19±0.01µg α-tocopherol equivalent of ethanolic extract of *R. hossei* which is followed by ethanolic extract of *U. baileyi* 2.12 µg α- tocopherol equivalent/mg.

Comparatively very low total antioxidant capacity was shown by methanolic extract of *U. baileyi* (0.71±0.01) and ethanolic extract of *P. reticulatum* (0.81±0.01).

Similar range of antioxidant capacity was observed in ethanolic extract of *Everniastrum* sp (1.3±0.01), methanolic extract of *Everniastrum* sp (1.54±0.01), methanolic extract of *P. reticulatum* (1.63±0.03), methanolic extract of *R. hossei* (1.59±0.02), ethanolic extract of *S. pomiferum* (1.88±0.01) and its methanolic extract (1.24±0.011).

Discussion

The result from table 4.54 depicted that methanolic extracts of lichen samples possessed a greater total antioxidant value than the ethanolic extracts.

Pramoda *et. al.*, (2014) where the total antioxidant activity measured by phosphomolybdenum method and the value shown by water extract of lichen *Punctelia subrudecta* was 6.49±1.4µg AA/g followed by *Cladonia fimbriata*, *Evernia mesomorpha* and *Permiolopsis ambigua.*

Studies made by previous workers showed that environmental factors play an important role in antioxidant activity of lichens. For example as high light, air pollution, desiccation, rehydration and high temperature affecting decreased antioxidant activity and reducing synthesis of antioxidants by lichens (Bartak *et. al.*, 2004; Weissman *et. al.*, 2005, 2006), hence even in our study different lichens exhibited different levels antioxidant activity.

The obtained results show that there is significant difference between the total antioxidant activity of lichen extracts and the standard compound (α-tocopherol) $p<0.05$ (Appendix G).

Pearson's bivariate test for correlation reveal that total antioxidant activity showed positive and significant correlation with Reducing power ability and DPPH radical scavenging activity in this study (where r=0.680 and r=0.647) at significant level $P<0.01$ (Appendix L)

4.10.2. Total antioxidant activity of medicinal plants under study

Table 4.56. Total antioxidant activity of medicinal plants extracts in at conc. 200 µg/ ml

Medicinal plants	µg α- tocopherol equivalent/ mg
α-tocopherol	4.19±.005[(b)]
URRE	2.7±0.012[(a)]
URRM	2.45±0.005[(a)]
BERE	5.43±0.005[(b)]
BERM	7.03±0.007([c)]
SAPM	3.4±0.018
PNXE	3.8±0.009

Duncan's post hoc test indicates values in the column with similar superscripts inside bracket are not significantly different and with different superscript are significantly different ($P<0.05$), (Appendix N)

The total antioxidant value of ethanolic and methanolic extract of *Urtica dioica* are 2.7 and 2.45 µg α-tocopherol equivalent/mg respectively. On the other hand the antioxidative value of ethanolic and methanolic extract of *Berginia ciliata* is high as 5.43 and 7.03 µg α-tocopherol equivalent /mg respectively. A moderate antioxidant value was observed in case of methanolic extract of *Sapindus mukrossi* as 3.4 µg α-tocopherol

equivalent /mg and that of *Panax pseudoginseng* as 3.8 µg α-tocopherol equivalent/mg.

Discussion

In a study conducted by Gulcin *et. al.*, (2003) water extract of nettle *Urtica dioica* (WEN), was subjected to for antioxidant, antimicrobial, antiulcer and analgesic properties including antimicrobial activity against nine microorganisms and antioxidant activity. The total antioxidant activity of WEN increased concentration dependently. WEN (50, 100 and 250 µg) showed higher antioxidant activities than that of α-tocopherol 100 µg. But the antioxidant activity of ethanolic and methanolic extract of *U. dioica* was less than that of the standard α-tocopherol. Therefore among the medicinal plants under study *Berginia ciliata* found to possess highest total antioxidant activity.

In our findings the antioxidant value of ethanolic and methanolic extract of *Berginia ciliata* was higher than the standard α-tocopherol at concentration 200µg/ml. Lastly methanolic extract of *Sapindus mukrossi* (Table 3.4) and ethanolic extract of *Panax pseudoginseng* (Table 3.8) exhibited a lower antioxidant activity than α-tocopherol.

The obtained results show that there is significant difference between the total antioxidant activity of medicinal plants extracts and the standard compound (α-tocopherol), however ethanolic extract of *B. ciliata* had no significant difference with α-tocopherol($p<0.05$).

4.11. Total antioxidant activity of lichen and medicinal plant extracts in combination

Table 4.56. Total antioxidant activity of lichen and medicinal plant extracts in combination at conc. 200 µg/ ml

Combined extracts of lichens with medicinal plants	∞-tocopherol equivalent /mg (Mean±S.D)
PARM + SAPM	4.22±0.032
USNE + BERE	6.36±0.23
USNE + URRE	4.98±0.33
PARM + URRM	3.9±0.73
RARM + URRM	2.9±0.40
RARM + BERM	4.09±0.03
EVME+ URRE	4.27±0.13
EVRM + BERM	3.62±0.22
STRM + URRM	3.00±0.09
STRM +BERM	2.68±0.27
USNE + PNXE	6.3±0.24
EVRM + SAPM	5.3±0.24
USRM + SAPM	4.3±0.45

A synergistic effect could be observed in case of combined methanolic extract of *P. reticulatum* (1.63) and *S. mukrossi* (3.4) as its antioxidant value is higher i.e., 4.22µg α-tocopherol equivalent/mg than its individual extracts. Another synergistic effect could be observed in combined ethanolic extract of *Usnea baileyi* with ethanolic extract of *Berginia ciliata* and *Urtica dioica* as 6.36 and 4.98 respectively.

Heights of synergism was also observed in case of combined ethanolic extract of *U. baileyi* and *P. pseudoginseng* as 6.3 α-tocopherol equivalent/mg. A very moderate synergistic effect between methanolic extract *Stereocaulon pomiferum* and *Urtica dioica* was clearly seen in (Table 4.56) with a value of 3µg α-tocopherol equivalent/mg.

The antioxidant activity of methanolic extract of *U. baileyi* was increased to 4.33µg α- tocopherol equivalent/mg when combined with methanolic extract of *S. mukrossi.*

No synergism was observed in case of combined methanolic extract of *Stereocaulon pomiferum* and *Berginia ciliata.*

Discussion

As synergism is observed between most of the combined extracts of plants this may be due to the fact that potential of one plant improved the performance of the other.

Phenolics are the largest group of phytochemicals and have been said to account for most of the antioxidant activity of plant extracts (Thabrew *et. al.*, 1998). Lichens like *Everniastrum* sp contained phenol (Kekuda *et. al.*, 2011) which may also attribute to its antioxidant activity and increase the antioxidant activity of the other plant.

In some cases no synergistic activity could be observed as the activity of one plant extract lowered the activity of the other in combined methanolic extract of *Stereocaulon pomiferum* and *Berginia ciliata*.

Again phenolic compounds are considered to contribute to the antioxidant activities of the plant extracts (Velioglu *et. al.*, 1998). It is reported that the methanolic extract of *B. ciliata* are rich in phenolic content (Rajkumar *et. al.*, 2010) which may have enhanced the antioxidant capability of *U. baileyi* raising its value to 6.36µg α-tocopherol equivalent/mg.

In general it is observed that use of lichens and medicinal plants together is suitable for antioxidant activity.

4.12. Reducing power ability of lichen extracts under study

The reducing power of the lichen extract was determined by the Fe^{3+}-Fe^{2+} transformation (Nagarajan *et. al.*, 2008). The reducing features are mainly related to the reductones (Kosanic and Rancovic *et. al.*, 2011).The reductones destroy the free radicals chain by donating hydrogen atom. The Fe^{2+} can be monitored by formation of Perl's Prussian blue at 700nm (Nagarajan *et. al.*, 2008).

4.12.1. Reducing power ability of lichen extracts and BHT as control

Table 4.57. Reducing power ability of lichen extracts and BHT as control

Duncan's post hoc test indicates the values in the column with similar superscripts inside bracket are not significantly different and with different superscript are significantly different (P<0.05), (Appendix H).

Conc. (µg/ml)	Extraction solvent	*U. baileyi*	*Everniastrum* sp	*P.reticulatum*	*S.pomiferum*	*R. hossei*	BHT(control)
50	Eth	0.41±0.0006	0.48±0.0021	0.20±0.0006	0.35±0.0050	0.45±0.0011	
	Meth	0.39±0.0006	0.37±0.0042	0.36±0.0006	0.39±0.0067	0.57±0.0050	0.56±0.001
100	Eth	0.42±0.0006	0.54±0.0031	0.20±0.0006	0.38±0.0059	0.53±0.0068	
	Meth	0.41±0.0006	0.39±0.0059	0.39±0.0006	0.42±0.0042	0.62±0.0070	0.75±0.0006
200	Eth	0.42±0.0006	0.57±0.0032	0.34±0.0012	0.42±0.0059	0.55±0.0061	
	Meth	0.43±0.0012	0.44±0.0040	0.41±0.0006	0.49±0.0064	0.67±0.0067	0.81±0.0015
300	Eth	0.43±0.0012$^{(b)}$	0.61±0.0040$^{(g)}$	0.38±0.0010$^{(a)}$	0.52±0.0046$^{(d)}$	0.58±0.0044$^{(f)}$	
	Meth	0.50±0.0012$^{(d)}$	0.49±0.0068$^{(c)}$	0.49±0.0006$^{(c)}$	0.54±0.0075$^{(e)}$	0.72±0.007$^{(h)}$	0.89±0.0010(i)

The absorbance of ethanolic extract *U. baileyi* ranged from 0.41±0.0006 to 0.43±0.0012 and that of methanolic extract it ranged from0.39±0.0006 to 0.50±0.0012. At high concentration of 300µg/ml the methanolic extract of *U. baileyi* possessed greater reducing capacity than the ethanol extract (Table 4.57).

The results of reducing power assay of methanolic and ethanolic extracts of *S. pomiferum* are presented in table 4.57 high absorbance of

extract means high reducing power. The reducing power of compounds can serve as indicator of potent antioxidant properties (Ailyu *et. al.*, 2008).

A highest reducing power was that of methanolic extract of *S. pomiferum* at concentration 200 µg/ml as 0.54±0.007. It was also observed that the reducing power of the extract increased with the increase in concentration 50 -300µg/ml. The absorbance of ethanolic extract of *S. pomiferum* was appreciable with high value at concentration 200 µg/ml as 0.52±0.0046.

The absorbance varied from 0.35±0.0050 to 0.52±0.0046 for ethanolic extract and from 0.39±0.0067 to 0.54± 0.0075 for methanolic extract of all tested lichens. The different concentrations of lichen extract showed reducing power as total antioxidant capacity in a dose dependent manner.

The ethanolic and methanolic extract of *U. baileyi* exhibited a good reducing power. The absorbance increased from lower concentration to higher concentration i.e., from 50µg/ml to 300µg/ml.

The absorbance of ethanolic extract *P. reticulatum* was low at 50 µg/ml(0.20±0.0006) but gradually increased to 0.38±0.0010 at concentration 300 µg/ml. A better reducing power ability was exhibited by methanolic extract of *P. reticulatum* with a high value of 0.49±0.0006 at concentration 300 µg/ml.

As observed in other extracts the reducing power value of ethanolic and methanolic extract of *P. reticulatum* increased with respect to the concentration (Table 4.57). It is clearly evident that the ethanolic extract of *Everniastrum* sp exhibited a higher reducing power than the methanolic extract, its absorbance increased from 0.48±0.0021 to 0.61±0.0040. Similarly the absorbance of methanolic extract also increased from 0.37±0.0042 to 0.49±0.0068 in accordance with the concentration from 50µg/ml to 300µg/ml. The reducing power ability of the lichen extract was very close to the reducing power of BHT at lower concentration.

Reducing power of both the extracts was compared with that of BHT. High absorbance of the extract samples indicates a potent reducing power with increased concentration of extracts. Measured value of absorbance varied from 0.45±0.0011 to 0.72±0.0075 in the lichen extract.

The reducing power of the lichen extract was very close to that of the standard BHT. It was interesting to note that the absorbance of methanolic

extract of *R. hossei* 0.57±0.0050 was greater than the standard BHT (0.56±0.001) at concentration 50µg/ml.

Discussion

The results present table 4.57 shows that the ferric reducing power of the extract which may be due to the presence of polyphenols react with free radicals to turn them into more stable product and destroy free radical chain reaction (Sashi kumar *et. al* ., 2010).

The values are about half of the chemical compound BHT which indicates the presence of lichen compounds with reducing power ability.

Our result indicated that all samples increased their reducing ability when the concentration was increased. Our result is in consistent with that as reported by Gulcin *et. al.,* (2003), Noriham *et. al*, (2004) and Faujan *et. al.*, (2009) who worked in reducing power of various plant extracts.

The reducing power ability of ethanolic and methanolic extract of *U. baileyi* was not as equal to standard BHT but its activity was appreciable. The reducing ability of BHT at concentration 50µg/ml was 0.56±0.001 and that for methanolic extract of *U. baileyi* was 0.51±0.0012 at concentration 300µg/ml.

The ability to reduce $Fe^{3+}$to Fe^{2+} may be attributed from hydrogen donation from phenolic compounds (Shimanda *et. al.*, 1992) which is also related to presence of reductant agent (Duh, 1998).

From the results obtained it can be estimated that the extracts may act as an electron donor, could neutralize free radicals (Aliyu *et. al.*, 2008) and can be widely used as source of antioxidant in the prevention of diseases.

It was observed that the reducing power lichen *Everniastrum* sp increased with increased concentration.

Works performed on reducing power of lichens *Cladonia fimbriata, Permilopsis ambigua, Punctelia subrudecta, Evernia mesomorpha* by Pramoda *et. al.*, (2014) also indicated that the absorbance of the lichen samples increased with increasing concentration of the extracts.

The standard lichen *R. hossei* exhibited a high reducing power hence it possessed a high antioxidant ability. Our current study is consistent with that of previous study (Krishnaraju *et. al.,* 2009) where the methanol extract of samples exhibited reducing ability greater than the standard

vitamin C similarly methanolic extract of this present lichen possessed a high absorbance than standard BHT at concentration 50µg/ml.

The results reveal that the crude extracts of lichens (in some cases) are even superior in antioxidant activity than the purified standard compounds. Numerous identified as well as unidentified compounds present in the crude extract contributed to higher antioxidant activity.

The obtained results reveals that there is significant difference between the reducing power ability of lichen extracts and BHT ($p<0.05$), (Appendix H).

The more ferric reducing power ability of lichen extracts the more antioxidant property. Previously it has been stated that extreme conditions in lichens increase oxidative stress as a result lichens contain large amount of antioxidant substances leading to higher antioxidant activity (Paz. *et. al.*, 2010).

Similarly, Pearson's bivariate test for correlation reveals that the reducing power ability of the lichen extract at other concentration was close to that of standard BHT. The Reducing Power ability showed positive and significant correlation with Total antioxidant activity and DPPH radical scavenging activity in this study (where r=0.680 and r=0.528) at significant level $P<0.01$, (Appendix L).

4.13. Estimation of Total phenolic content of lichen extracts

Table 4.59. Estimation of total phenolic content of lichens eth- ethanol, met- methanol

Extract Conc.(	Solvent	USR	EVR	PAR	STR	RAR	Tannic acid
25	Eth	46.81±0.46	42.63±0.80	40.21±0.10	37.4±0.26	38.73±0.29	39.20±0.17
	met	53.89±0.71	62.13±1.36	64.31±0.77	41.16±0.32	39.91±0.46	
50	Eth	54.08±0.50	52.66±1.49	66.62±0.14	39.16±-.75	40.±0.49	51.31±0.09
	met	66.6±0.36	82.06±0.41	68.74±0.39	42.53±0.41	40.63±1.57	
100	Eth	67.5±0.17	71.18±1.24	99.15±0.28	42.1±0.1	41.29±0.40	69.43±0.06
	met	79.60±0.32	84.8±0.36	118±3.40	46.66±0.57	42.91±1.62	
200	Eth	106±0.51$^{(g)}$	71±1.3$^{(e)}$	108.4±1.50$^{(h)}$	52.69±1.97$^{(d)}$	42±0.43$^{(a)}$	97.40±1.53$^{(f)}$
	met	141.2±0.34$^{(i)}$	96.86±0.37$^{(f)}$	143.23±1.30$^{(j)}$	49.47±0.12$^{(c)}$	44.85±0.23$^{(b)}$	

Duncan's post hoc test indicates the values in the column with similar superscripts inside bracket are not significantly different and with different superscript are significantly different ($P<0.05$), (Appendix J)

The amount of total phenolics in the lichen extracts was determined by tannic acid equivalent using Folin ciocalteu reagent obtained from the regression equation of calibration curve of tannic acid y=0.007x-0.186; R^2=0.938.

The total phenolic content of ethanolic and methanolic extract of *P. reticulatum* was very appreciable. It was observed that the phenolic content ethanolic extract of *P. reticulatum* was 108.4±1.5 tannic acid equivalent/mg even greater than the phenolic content of standard compound Tannic acid (97.40±1.53) (Table 4.53). Similarly the methanolic extract of this lichen possessed a high phenolic content of (118±3.40) and (143.23 ±1.3) tannic acid equivalent/mg at concentrations 100 and 200 (µg/ml) respectively.

It was also observed that the phenolic content of ethanolic and methanolic extract of *U. baileyi* was quite high. For ethanolic extract the total phenolic content was 106±0.51 and for methanolic extract it was 141.2±0.34 tannic acid equivalent/mg at concentration 200µg/ml.

The ethanolic extract of *Everniastrum* sp possessed a high phenolic content of 96.86±0.37 than the methanolic extract with a little less phenolic content of 71±1.3 tannic acid.

The phenolic content of ethanolic and methanolic extract of *S. pomiferum* was 52.69±1.97 and 49.47±0.12 tannic acid equivalent /mg, which is very moderate value.

The phenolic content of ethanolic and methanolic extract of *Ramalina hossei* was 42±0.43 and 44.85±0.23 tannic acid equivalent /mg respectively.

Discussion

Several studies have found high correlations between antioxidative activities and phenolic content (Odabasoglu *et. al.*, 2004; Rankovic *et. al.*, 2010). According to Adedapo *et. al.*, (2009), both edible and non edible plants have been reported to have phenolic content exhibiting multiple biological effects including antioxidant activity. This antioxidant property of plants phenolics may be due to their redox potential, which allows these to

act as reducing agent, hydrogen donators and singlet oxygen quenchers (Rice and Evans *et. al.*, 1995).

Studies on total phenolic content of various plants has been performed by previous workers like Yen and Hsieh (1998), Siddhuraju and Becker, (2003), Noriham *et. al.*, (2004), Jerez *et. al.*, (2007), Kosanic *et. al.*, 2013 and Johnson *et. al.*, (2015). The antioxidative properties of some plants are partly due to the low molecular weight phenolic compounds, which are known to be potent as antioxidants (Wang *et. al.*, 1999).

Previous studies by Kosanic *et. al.,* (2014b) revealed that lichens *Lecanora muralis, Parmelia saxatilis, Umbilicaria polyphylla* contained high phenolics as high as 50.93±1.91, 53.08±1.269, 52.67±1.211, 55.03±1.096 and 90.09±1.176 respectively.

Our results are also consistent with this report as content of phenolics is high ranging from (42±0.43) to (143.23±1.3) tannic acid equivalent/mg for ethanolic and methanolic extracts of lichens studied. On the basis of this result the lichen appears to be a good and safe natural antioxidant.

The antioxidative nature of the tested lichen extract might or might not depend upon the total phenolic content. Some researchers found that the antioxidant activity of the lichen did not always correlate with phenolic content but its antioxidant activity may be due to presence other non phenolic component (Odabasoglu *et. al.,* 2004).

Although the phenolic content of ethanolic and methanolic extract of *U. baileyi* was very high (Table 4.59) but its total antioxidant activity was moderate.

This findings on phenolic content of lichen extracts (Table 4.59) was higher than that of the lichens studied earlier as *Parmeliopsis ambigua*, *Parmelia pertusa* and *Hypogymnia physoides* exhibited phenolic content of 45.86±1.91, 30.00±1.264 and 38.22±1.211respectively (Kosanic *et. al.*, 2013).

In another study conducted by Kekuda *et.al.*, (2011) on lichen *Everniastrum cirrhatum* the total phenolic content was found to be 101.20±1.86µg/g tannic acid equivalent dry weight of extract, which was in margin with the present result studied (Table 4.59). A highly positive relationship is found between the phenols and antioxidant activity in many

plants (Oktay *et. al.*, 2003; Velioglu *et. al.,* 1998; Vinson *et.al.*, 1998). Hence, the lichen could be used as antioxidant.

As studied in most lichens phenols are important antioxidants because they have the ability to donate hydrogen to free radicals and stop the chain reaction of lipid oxidation at initial stage (Rankovic *et. al.*, 2010). The lichen *S. pomiferum* possess good amount of phenolic content, many of these phytochemicals possess significant antioxidant capacities (Anderson *et. al.*, 2001; Djeridane *et. al.*, 2006).

Marijana *et. al.*, (2010), identified highest phenolic compounds in methanol extract of *Hypogymnia physodes* at 86.76 µg of pyrocatechol equivalent while aqueous extracts of *Cladonia furcata* showed the lowest content at 5.81 µg of pyrocatechol equivalent. High phenolic contents were also found in acetone, methanol and aqueous extract of *Lasallia pustulata* with 84.33, 49.62 and 23.90 µg of pyrocatechol equivalent, respectively.

In our findings the methanolic extract of *R. hossei* exhibited a varying value of phenolics up to 44.85 tannic acid equivalent/mg, similarly, phenolic content in the Antarctic lichen extracts was in the range of 17–47 mg/g, supporting the antioxidant data of TLC analysis (Bhattarai *et. al.*, 2008).

Other than lichens, in a recent study by Johnson *et. al.,* (2015) methanolic extract of leaves of *Kigelia africana*, *Alafia bateri*, *Anthocleista djalonesis* and the stem bark of *Harungana madagascarensis* possessed phenolic content 46.24, 43.82, 33.42, 84.8 respectively which is much similar to our results. The phenolic content of these extracts contributed to its antioxidant activity.

The obtained results indicate that there is significant difference in the phenolic content of lichen extracts and Tannic acid ($p<0.05$), (Appendix J).

4.14. Estimation of total flavonoid content of lichen extracts

Table 4.58. Total flavonoid content of lichen extracts

Lichen extracts	Quercetin equivalent/ mg(Mean ±S.D)
Quercetin	4.9±0.003$^{(d)}$
USRE	1.58±0.01$^{(bc)}$
USRM	1.62±0.03$^{(bc)}$
PARE	1.50±0.02$^{(ab)}$
PARM	1.41±0.01$^{(a)}$
EVRE	1.52±0.01$^{(ab)}$
EVRM	1.66±0.01$^{(bc)}$
STRE	1.53±0.01$^{(ab)}$
STRM	1.55±0.01$^{(ab)}$
RARE	1.69±0.01$^{(c)}$
RARM	1.55±0.01$^{(bc)}$

Duncan's post hoc test reveals that the values in the column with similar superscripts inside bracket are not significantly different and with different superscript are significantly different ($P<0.05$), (Appendix K)

The total flavonoid content of the lichen extract was carried out using method of Nagarajan *et. al.,* (2008) and was determined as microgram quercetin equivalent. Value from graph was calculated following the standard quercetin equation y= 0.356x-0.461:R^2=0.697.

Flavonoids are natural compounds and are most important natural phenolics. These compounds have a large number of biological and chemical activities including radical scavenging properties (Ghafar *et. al.*, 2010).

The total flavonoid content of ethanolic extract of *U. baileyi* was 1.58±0.01µg quercetin equivalent/mg and that of methanol extract was 1.62±0.03µg quercetin equivalent/mg.

In lichen *P. reticulatum* the flavonoid content measured was 1.50±0.02 and 1.41±0.01 in its ethanolic and methanolic extract respectively.

The flavonoid content of the remaining three ethanolic lichen extract exhibited 1.52±0.01, 1.53±0.01 and 1.69±0.01µg equivalent/mg for *Everniastrum* sp, *S. pomiferum* and *R. hossei* respectively.

The total flavonoid content calculated for methanolic extract of lichen was 1.66±0.01 (*Everniastrum* sp), 1.55±0.01 (*Stereocaulon pomiferum*) and 1.55±0.01 (*Ramalina hossei*) µg quercetin equivalent/mg.

Discussion

Flavonoids are a group of phytochemicals found in varying amounts in foods and medicinal plants which have been shown to exert potent antioxidant activity against the superoxide radical. Flavonoids are 15-carbon compounds generally distributed throughout the plant kingdom which are known to be synthesized by plants in response to microbial infection and have been found *in vitro* to be effective against a wide array of microorganisms (Soctanand and Aiyelaagbe *et. al.*, 2009).

In a study conducted by Arunachalam (2011), the total flavonoid content was very high as 122.3±9.9 (mg RE/g extract) of *Gymnostachyum febrifugum*, a medicinal plant endemic to Western Ghat of India.

The results thus obtained show that the flavonoid content of quercetin and lichen extracts are significantly different but there exist no significant difference in the flavonoid content of lichen extracts ($p<0.05$) (Table.4.58), (Appendix K).

The reason for antimicrobial activity of lichens may be probably due to the existence of flavonoids although present in low amount their respective extracts.

4.15. Estimation of Catalase and Peroxidase activity of studied lichens

Table 4.60. Estimation of Catalase and Peroxidase activity of studied lichens

		(enzyme/minute/gramtissue)	
Sl No.	Name of lichens	Catalase activity	Peroxidase activity
1.	*Everniastrum* sp	1.68	1.57
2.	*Stereocaulon pomiferum*	0.651	2.26
3.	*Ramalina hossei*	0.149	2.4
4.	*Usnea baileyi*	0.915	2.058

The catalase and peroxidase activity of the enzymes showed typical pattern in lichens. The catalase activity of lichen was highest in case of

Everniastrum sp as 1.68 enz/min/gm tissue and its peroxidase activity was it was 1.57enz/min/gm tissue.

The activity of catalase was moderate in case of *Usnea baileyi* as 0.915 but in its activity decreased significantly as 0.651enz/min/gm tissue and 0.149 enz/min/gm tissues in case of *Stereocaulon pomiferum* and *Ramalina hossei* respectively. The antioxidative peroxidase activity noted in lichens was comparatively higher than catalase activity.

High peroxidase activity was noted in case of *Ramalina hossei, Usnea baileyi* and *Stereocaulon pomiferum* as, 2.4, 2.058 and 2.26 enz/min/gm of tissue respectively. Investigations revealed a low peroxidase value in lichens 1.59 enz/min/gm of tissue.

Discussion

Peroxidase are widely distributed in nature and are found in plants, microorganisms and animals, where they catalyze the reduction of hydrogen peroxide (H_2O_2) to water, rendering it harmless. At high altitudes accumulation of chemically active molecules and free radicals in plant cell changes the direction of metabolic process (Asada, 1999), but plants possess strong antioxidant system which inhibits free radicals process (Keniya, *et. al.*, 1993; Zenkov and Menshikova, 1993).

H_2O_2 is a common end product of oxidative metabolism, and being a strong oxidizing agent, could prove toxic if allowed to accumulate. Thus, peroxidases serve torid plant cells of excess H_2O_2 under normal and stress conditions (Laloue *et. al.*, 1997). Peroxidases are versatile biocatalyst with an ever increasing number of applications (Colona *et. al.*, 1999).

Lichens are abundantly found at high altitude which increases with respect to temperature and light (Longton, 1988). Lichens have the ability to assimilate significant amounts of carbon during autumn and winter compared to higher plants hence they experience less oxidative stress during high light periods of winter (Lange, 2003).

During the growth of plant they are subject to different type of stress such as heat drought, ultraviolet light air pollution and pathogen attack. The protective mechanisms are developed by plants to control these damage (Syvacy and Sokmen, 2006). The major primary intracellular endogenous antioxidant defenses are the enzyme system. This antioxidant enzymatic system includes superoxide dismutases (SOD's), catalase (CAT),

and glutathione peroxidase (GSHPx) (Yang *et. al.*, 1999; Halliwell and Guttredige, 1990). Hence the provided data on catalase and peroxidase activity of lichens can be regarded as significant factors contributing to its antioxidant property.

ROS damage membrane proteins by causing lipid peroxidation in membranes by attacking to unsaturated fatty acids (Ames *et. al.*, 1993). The damage to membrane proteins decreases the membrane permeability, activities of enzymes and receptors, and activation of cells. When free radicals attack DNA, cancer-causing mutations may occur. Therefore, antioxidant defense systems including antioxidant enzymes, food and drugs are important in the prevention of many diseases (Pietta *et. al.,* 1998; Yen and Hsieh, 1998). Previously it was suggested that higher intake of antioxidant rich food is associated with decreased risk of degenerative diseases particularly cardiovascular diseases and cancer (Thatte *et. al.,* 2000).

4.16. Determination of Total protein content of culture filtrate containing lichen extract

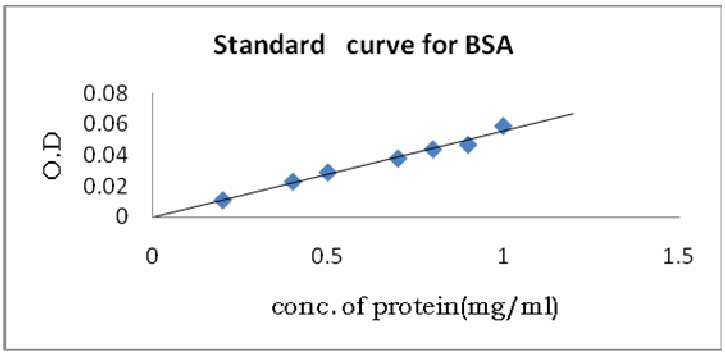


Fig 4.3 Standard curve of protein (BSA)

4.16.1 Total protein content of culture filtrate containing ethanolic and methanolic extracts of *Usnea baileyi*

Test microorganisms	Total protein of culture filtrate(µg/ml)					
	Extract concentration(µg/ml)					
	Control	100	200	300	400	500
A. faecalis	980	870	780	570	490	380
B. megaterium	1110	910	880	830	790	620
C. albicans	1010	910	870	680	670	560
P. aeruginosa	800	780	720	630	590	500
E. coli	760	700	500	450	310	240
S. aureus	900	700	630	550	480	430
E. aerogenes	910	630	560	530	520	430

Table 4.61. Total protein content of culture filtrate containing ethanolic extract of *Usnea baileyi*

Table 4.62. Total protein content of culture filtrate containing methanolic extract of *Usnea baileyi*

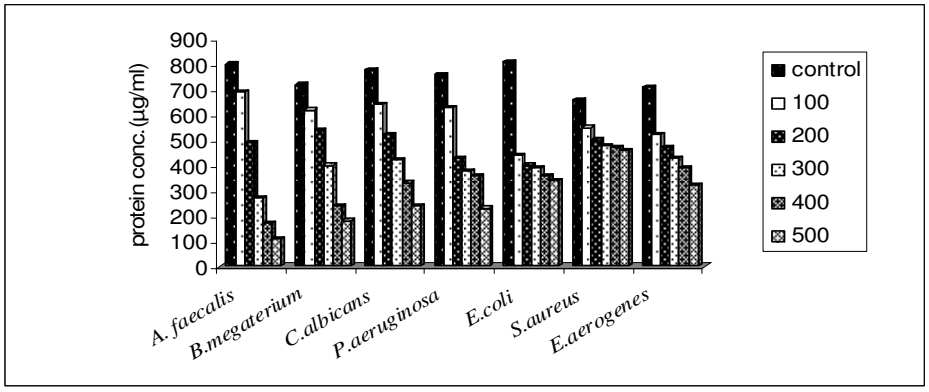


Fig 4.4. Total protein content of culture filtrate containing ethanolic extract of *Usnea baileyi*

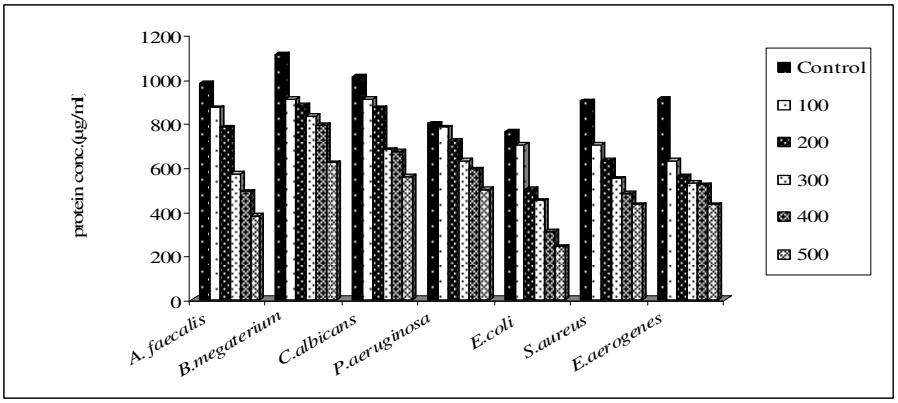


Fig 4.5.Total protein content of culture filtrate containing methanolic extract of *Usnea baileyi*

The protein content of culture filtrate was maximum in the control sets containing *Usnea baileyi* extracts. When the concentration of the extracts USRM and USRE was increased then there was a decrease in the protein content of the culture filtrate.

Highest protein content i.e., 810µg/ml was recorded in the control set inoculated with Gram negative bacteria like *E. coli*. But when the concentration of the USRE was increased up to 500µg/ml the protein content of the microorganism was lowered to 340µg/ml. Similarly the protein content of *B. megaterium* was observed as 1110µg/ml in another control set but after addition of USRM the protein concentration decreased to 620µg/ml (Table 4.62).

4.16.2. Total protein content of culture filtrate containing ethanolic and methanolic extract of *Everniastrum* sp

Table 4.63. Total protein content of culture filtrate containing ethanolic extract of *Everniastrum* sp

Test microorganisms	Total protein of culture filtrate(µg/ml)					
	Extract concentration(µg/ml)					
	Control	100	200	300	400	500
A. faecalis	540	470	450	430	300	230
B. megaterium	300	290	260	250	240	200
C. albicans	400	310	300	290	280	270
P. aeruginosa	800	380	270	230	190	150
E. coli	380	350	290	280	270	170
S. aureus	430	410	340	360	290	210
E. aerogenes	690	380	370	360	320	130

Table 4.64. Total protein content of culture filtrate containing methanolic extract of *Everniastrum* sp

Test microorganisms	Total protein of culture filtrate (µg/ml)					
	Extract concentration(µg/ml)					
	Control	100	200	300	400	500
A. faecalis	610	560	480	450	340	280
B. megaterium	580	510	450	430	360	340
C. albicans	610	570	520	470	430	330
P. aeruginosa	800	780	720	630	590	500
E. coli	550	290	250	210	170	130
S. aureus	540	350	300	260	170	130
E. aerogenes	770	620	480	300	280	270

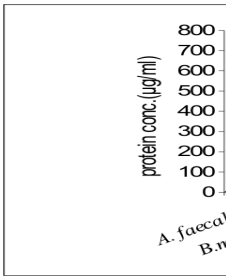


Fig 4.6 Total protein content of culture filtrate containing ethanolic extract of *Everniastrum* sp

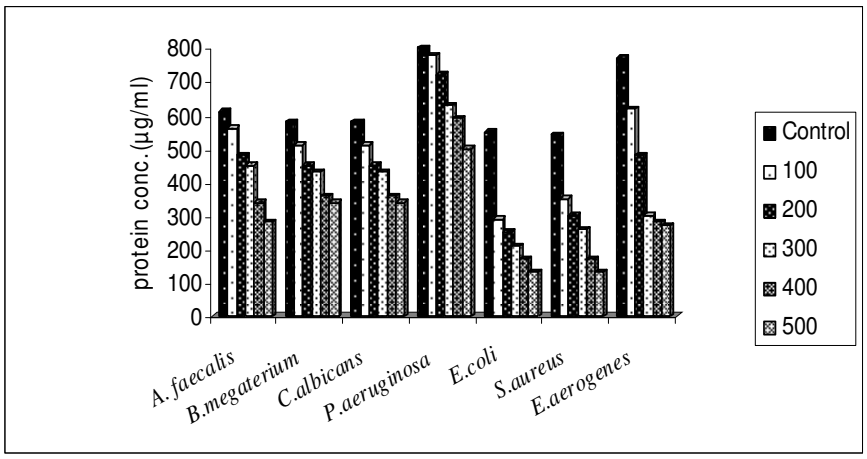


Fig 4.7. Total protein content of culture filtrate containing methanolic extract of *Everniastrum* sp

The protein content of microorganisms is maximum at the control sets, but when the concentration EVRE and EVRM was increased then there was a decrease in the rate of protein content of the microorganisms.

In Gram negative bacteria *Pseudomonas aeruginosa* the control set had highest protein content i.e., 800µg/ml but when the concentration of EVRE was increased i.e., in concentration 500 µg/ml the protein content of the culture filtrate was lowered to 150µg/ml.

Similarly EVRE also could decrease the concentration of *E. aerogenes* grown culture filtrate from 690µg/ml to a significantly lower value 130µg/ml. Even by addition of EVRM the protein content of *P. aeruginosa* decreased from 800µg/ml to 500µg/ml from 770µg/ml to 270µg/ml in case of *E. aerogenes*.

4.16.3.Total protein content of culture filtrate containing ethanolic and methanolic extract of *Parmotrema reticulatum*

Table 4.65. Total protein content of culture filtrate containing ethanolic extract of *Parmotrema reticulatum*

Test microorganisms	Total protein of culture filtrate(µg/ml)					
	Extract concentration (µg/ml)					
	Control	100	200	300	400	500
A. faecalis	810	790	700	610	550	500
B. megaterium	740	700	670	600	530	490
C. albicans	800	750	700	650	600	530
P. aeruginosa	830	770	710	670	580	510
E. coli	790	770	660	600	570	500
S. aureus	820	740	660	610	520	500
E. aerogenes	810	630	580	560	500	480

Table 4.66. Total protein content of culture filtrate containing methanolic extract of *Parmotrema reticulatum*

Test microorganisms	Total protein of culture filtrate(µg/ml)					
	Extract concentration (µg/ml)					
	Control	100	200	300	400	500
A. faecalis	740	710	670	630	580	510
B. megaterium	800	700	650	600	560	510
C. albicans	740	690	650	610	570	530
P. aeruginosa	700	680	620	560	530	480
E. coli	740	700	690	630	570	500
S. aureus	710	660	600	560	510	470
E. aerogenes	760	730	680	600	560	510

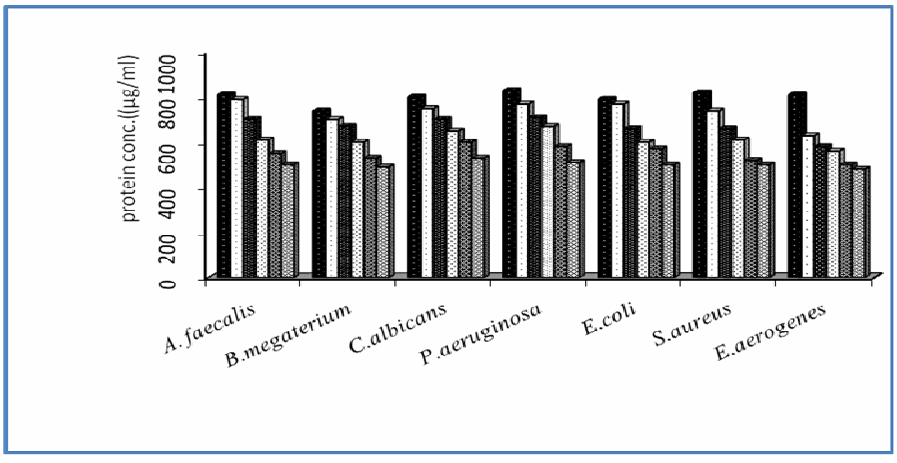


Fig 4.8. Total protein content of culture filtrate containing ethanolic extract of *P. reticulatum*

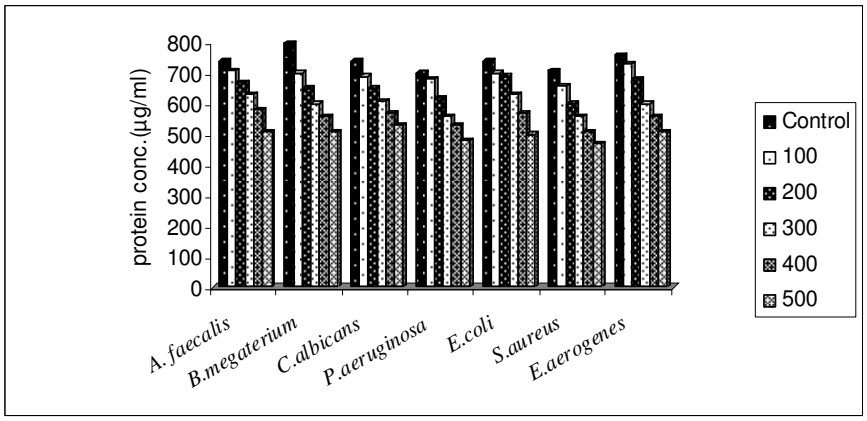


Fig 4.9.Total protein content of culture filtrate containing methanolic extract of *P. reticulatum*

The amount of protein in the culture filtrate was highest in case of *C. albicans* was 800µg/ml which was consequently lowered to 530µg/ml by addition of PARE. In the flltrate previously inoculated into Gram positive bacteria *S. aureus* the value of protein lowered from 820µg/ml to 500µg/ml with increasing concentration of PARE.

With the addition of PARM the concentration of protein of all studied microorganisms inoculated culture filtrate decreased to an extent. The concentration of protein in *B. megaterium* grown culture filtrate decreased from a high 800µg/ml to 510µg/ml and that of *E. coli* from 740µg/ml to 500µg/ml.

4.16.4. Total protein content of culture filtrate containing ethanolic and methanolic extract of *Ramalina hossei*

Table 4.67. Total protein content of culture filtrate containing ethanolic extract of *Ramalina hossei*

Test microorganisms	Total protein of culture filtrate(µg/ml)					
	Extract concentration(µg/ml)					
	Control	100	200	300	400	500
A. faecalis	800	690	490	270	170	110
B. megaterium	720	620	540	400	240	180
C. albicans	780	640	520	420	330	240
P. aeruginosa	760	630	430	380	360	230
E. coli	810	440	400	390	360	340
S. aureus	660	550	500	480	470	460
E. aerogenes	710	520	510	500	490	470

Table 4.68. Total protein content of culture filtrate containing methanolic extract of *Ramalina hossei*

Test microorganisms	Total protein of culture filtrate(µg/ml)					
	Extract concentration(µg/ml)					
	Control	100	200	300	400	500
A. faecalis	850	610	510	450	330	280
B. megaterium	810	620	490	410	390	200
C. albicans	780	590	560	550	540	460
P. aeruginosa	830	640	630	310	290	240
E. coli	780	650	540	500	480	450
S. aureus	640	590	560	540	510	430
E. aerogenes	830	650	540	520	500	470

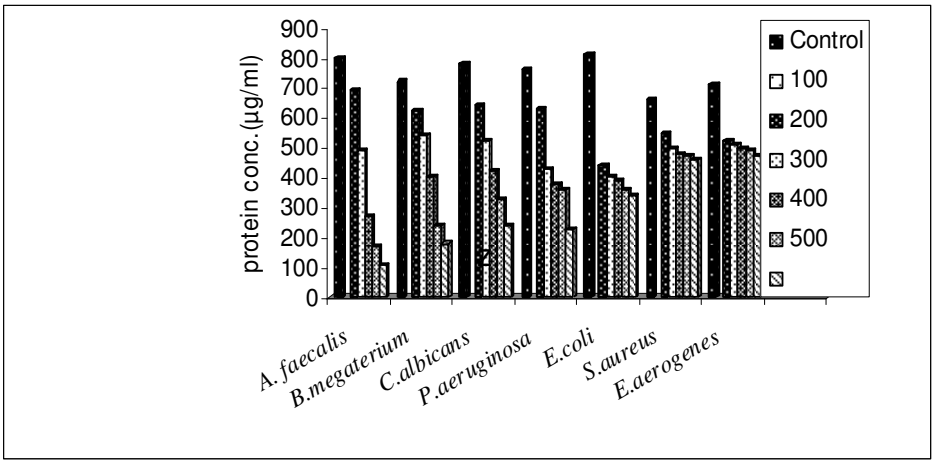


Fig 4.10.Total protein content of culture filtrate containing ethanolic extract of *R.hossei*

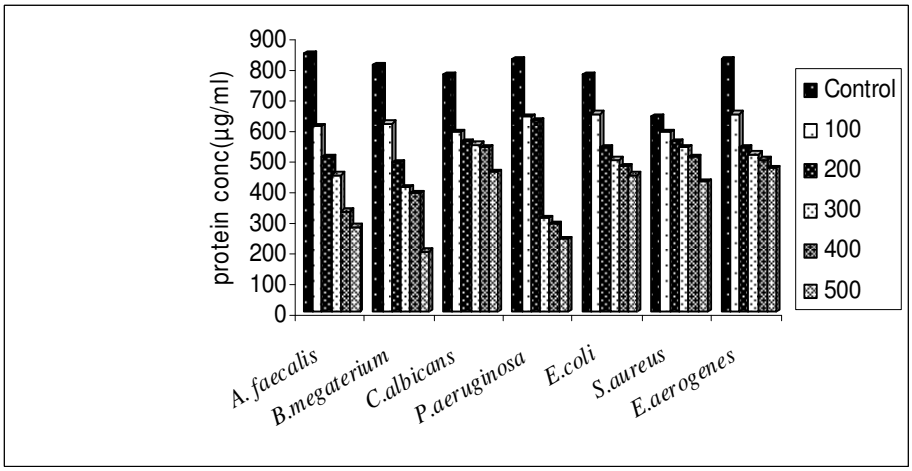


Fig 4.11Total protein content of culture filtrate containing methanolic extract of *R. hossei*

In the culture containing *A. faecalis* as test organism the protein content decreased from a very high value of 800µg/ml to 110µg/ml by adding with different concentration of RARE. The value of protein of *E. coli* also was reduced to 340µg/ml from 810µg/ml.

Table 4.68 showed that concentration of protein from *P. aeruginosa* grown culture filtrate decreased from 830µg/ml to 240µg/ml by the effect of RARM. RARM also affected the protein in *A. faecalis* by lowering it from 850µg/ml to 280µg/ml.

4.16.5. Total protein content of culture filtrate containing ethanolic and methanolic extract of *Stereocaulon pomiferum*

Table 4.69.Total protein content of culture filtrate containing ethanolic extract of *Stereocaulon reticulatum*

Test microorganisms	Total protein of culture filtrate(µg/ml)					
	Extracts concentration(µg/ml)					
	Control	100	200	300	400	500
A.faecalis	980	870	780	570	490	380
B. megaterium	1110	910	880	830	790	620
C. albicans	1010	910	870	680	670	560
P. aeruginosa	800	780	720	630	590	500
E. coli	760	700	500	450	310	240
S. aureus	900	700	630	550	480	430
E. aerogenes	910	630	560	530	520	430

Table 4.70.Total protein content of culture filtrate containing methanolic extract of *Stereocaulon reticulatum*

Test microorganisms	Total protein of culture filtrate (µg/ml)					
	Extracts concentration (µg/ml)					
	Control	100	200	300	400	500
A. faecalis	800	690	490	270	170	110
B. megaterium	720	620	540	400	240	180
C. albicans	780	640	520	420	330	240
P. aeruginosa	760	630	430	380	360	230
E. coli	810	440	400	390	360	340
S. aureus	660	550	500	480	470	460
E. aerogenes	710	520	470	430	390	320

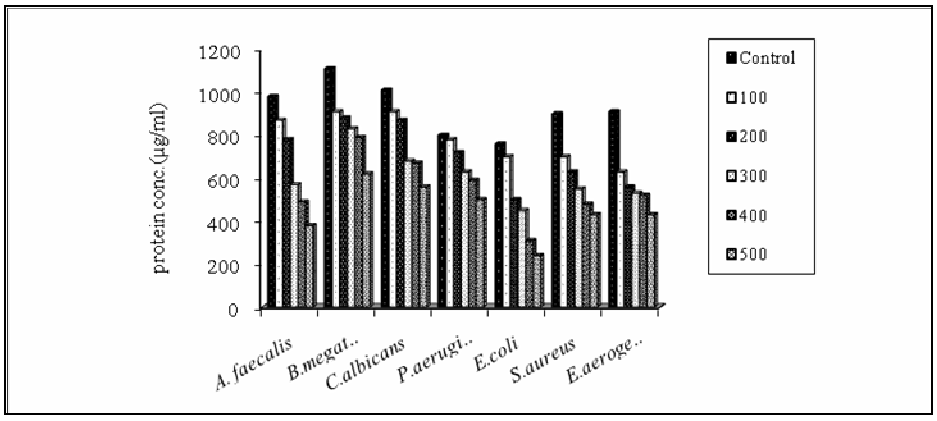


Fig 4.12.Total protein content of culture filtrate containing ethanolic extract of *Stereocaulon pomiferum*

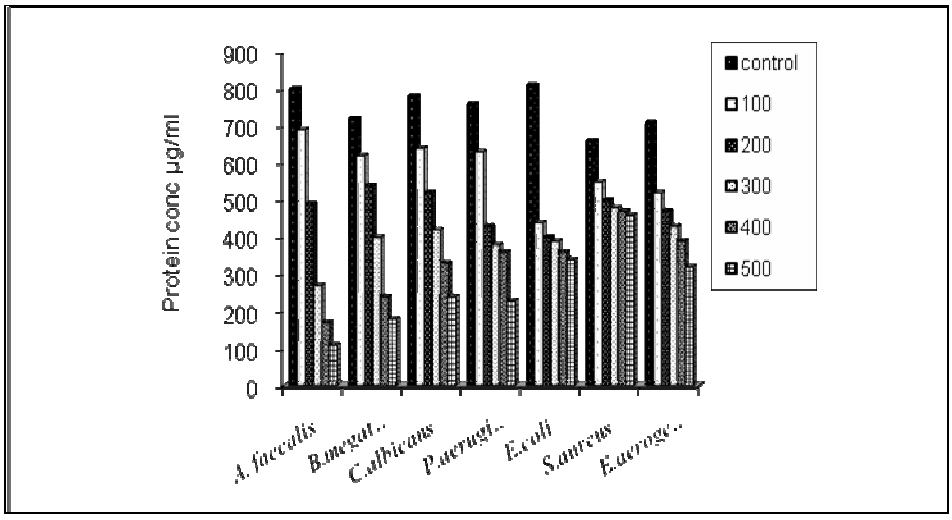


Fig 4.13.Total protein content of culture filtrate containing methanolic extract of *Stereocaulon pomiferum*

The results obtained from table 4.69 and 4.70 indicates that the protein concentration of Gram negative *A. faecalis, E. coli, P. aeruginosa* and *E. aerogenes* grown culture decreased significantly. The protein content in gram positive bacteria and fungus also showed a significant decrease in its concentration. A remarkably feature that was noticed from (table 4.69)is that with the input of STRE the protein content of both gram positive and negative bacteria namely *A. faecalis* and *B. megaterium* decreased from 1110µg/ml and 1010µg/ml to 620 µg/ml and 560 µg/ml respectively

The addition of STRM decreased the protein content from 780µg/ml to 240µg/ml of *C. albicans* and that of *B. megaterium* and *E. coli* it was decreased to 180µg/ml and 340µg/ml respectively.

Discussion

The study of possibility of the plant extract mediated changes in the bacterial protein pattern has been previously made by Akhand *et. al.*, (2008). It could be clearly observed from table 4.61 and table 4.62 that both the USRE and USRM exhibited a strong ability to reduce the concentration of protein with its increasing concentration.

The author (Akhand *et. al.*, 2008) analyzed the expression pattern in proteins of different bacterial strains by treating it with extracts of *Azadirachta indica* and *Terminalia arjuna* followed by Gel electrophorisis (SDS-PAGE) of proteins. They observed the aggregation of protein at the upper portion of stacking and separating gel in extract treated samples and concluded that the appearance of such bands might be due to the extract mediated crosslinking of cellular proteins.

Hence, it could be pointed out that the decrease in the protein content of all tested microorganisms by USRE and USRM might be due to its cross linking of proteins.

The lowering of protein value with the addition of EVRE and EVRM could signify cross linking of proteins. Crosslinking of proteins was also reported by the use of different chemicals and pollutants (Nakashima *et. al.*, 1993; Akhand *et. al.*, 1999; Akhand *et. al.*, 2008).

It was earlier studied by (Akhand *et. al.*, 1999) that the carbonyl compounds present in the plant extracts crosslink cellular proteins. Hence the various compounds present in the lichen extracts may be responsible for lowering the amount of bacterial protein.

After studying the potential antimicrobial and antioxidant activity of PARE and PARM its effect on protein content of microorganisms grown culture filtrate was observed. Table (4.65 and 4.66) depicted the lowering concentration of protein which might have occurred due to presence of carbonyl compounds. These carbonyl compounds in PARE and PARM might reacted with amino group of proteins to make a Schiff-base for protein cross linking thereby decreasing its content in microorganisms (Akhand *et. al.*, 1999; Akhand *et. al.*, 2008).

Carbonyl compounds in RARE and RARM lowers the protein content of microorganisms which may be due to its crosslinking and later on it may cause death of these microorganisms. Gyrophoric acid, Calycin, 20, 24-Epoxydammarane, Coronatoquinone, Pulvinic dilactone [Pulvinic acid] lactone, (-)-Dihydropertusaric acid, 2-Chlorolichexanthone, Methyl haematommate 3β, 12β, 25-triol [Pyxinol] are some of the compounds which were identified from RARM which may be responsible for aggregation of proteins.

As observed from previous section STRE and STRM possess antimicrobial activity. An antibiotic trimethoprim aggregated bacterial protein (Laskowska *et. al.*, 2003) similarly it may be assumed antimicrobial property of STRE and STRM may be one of the important factors for causing the decrease in concentration of bacterial protein by aggregating and crosslinking it.

Ability of all lichen samples under study to inhibit protein synthesis of microorganisms justify their antimicrobial activity as described in previous section.

4.17. Determination of effect of lichen extract on growth of microorganisms

4.17.1. Effect on growth of microorganisms by ethanolic extract of *Everniastrum* sp

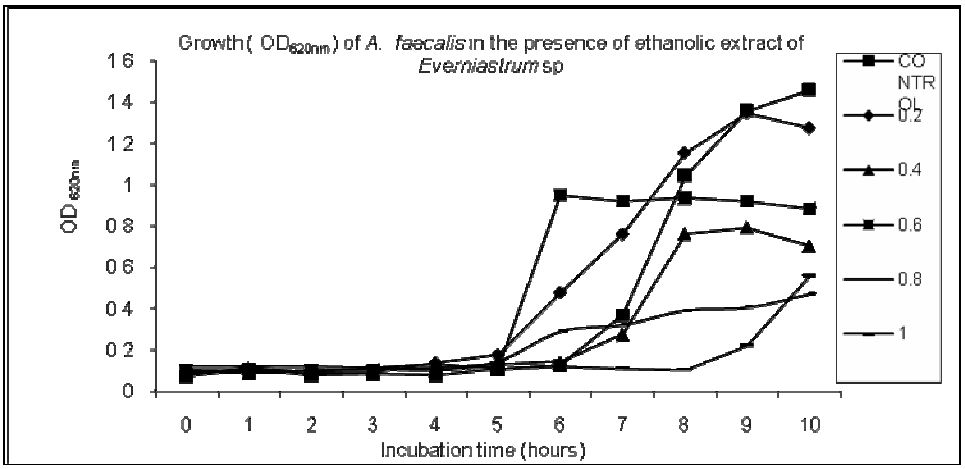


Fig 4.14 Growth curve of *A. faecalis* in the presence of different conc. (µg/ml) of EVRE

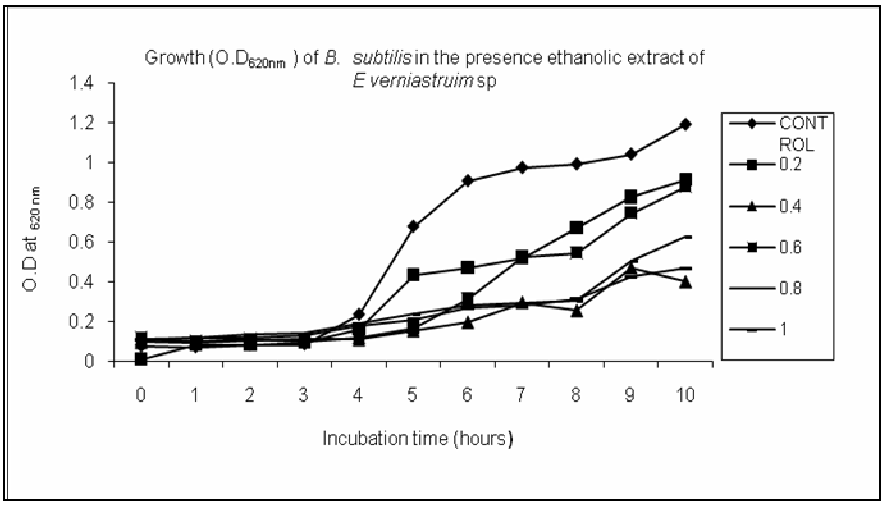


Fig 4.15 Growth curve of *B. subtilis* in the presence of different conc. (µg/ml) of EVRE

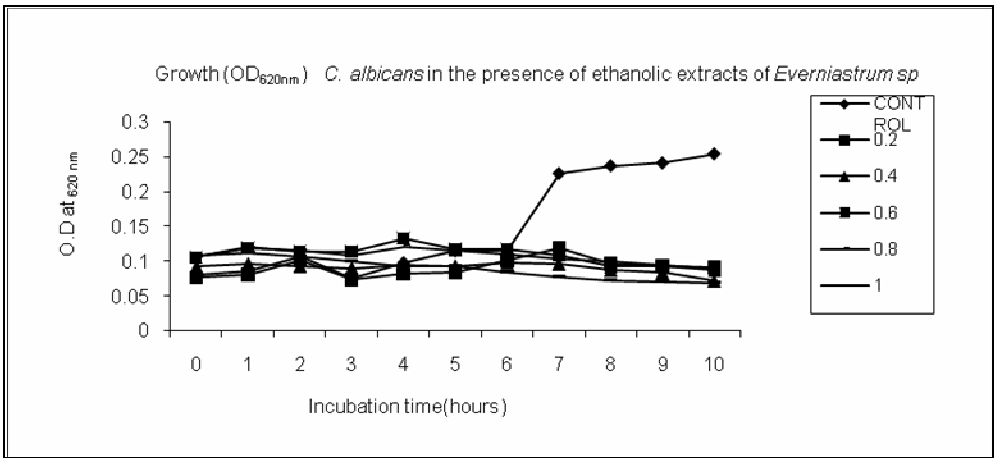


Fig 4.16 Growth curve of *C. albicans* in the presence of different conc. (µg/ml) of EVRE

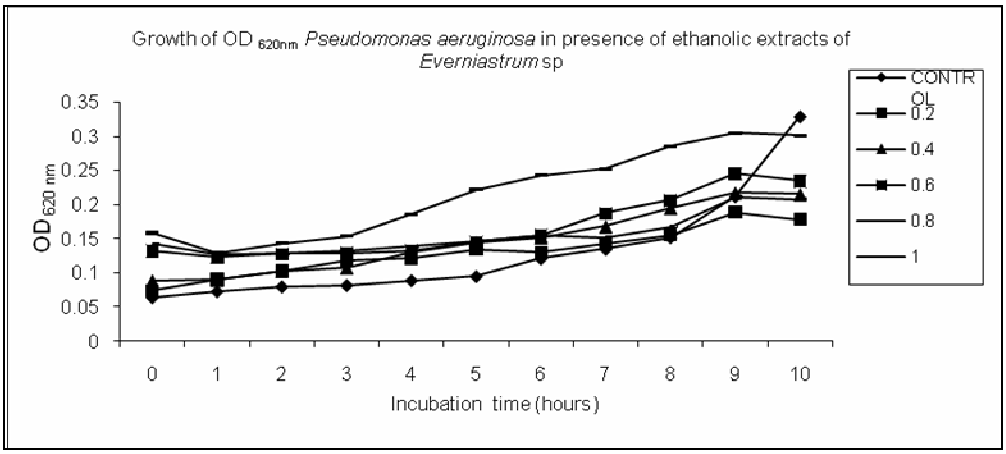


Fig 4.17 Growth curve of *P. aeruginosa* in the presence of different conc. (µg/ml) of EVRE

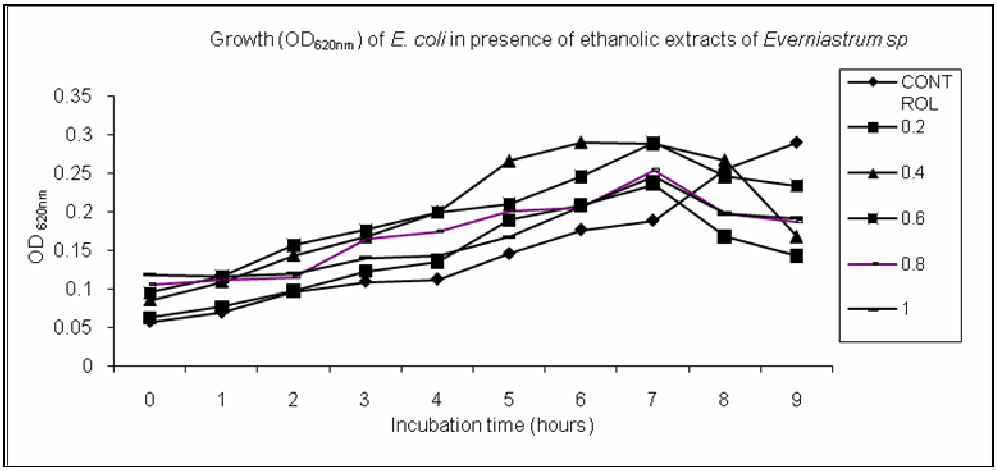


Fig 4.18 Growth curve of *E. coli* in the presence of different conc. (µg/ml) of EVRE

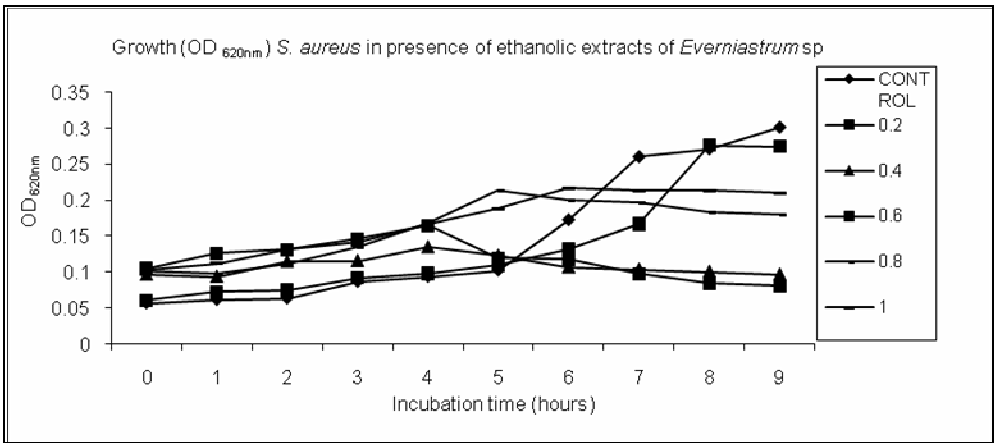


Fig 4.19 Growth curve of *S. aureus* in the presence of different conc. (µg/ml) of EVRE

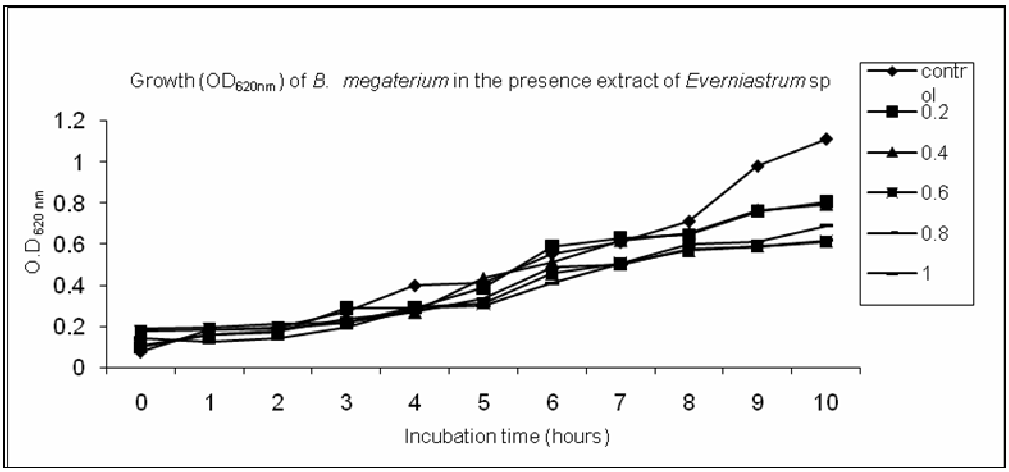


Fig 4.20 Growth curve of *B. megaterium* in the presence of different conc. (µg/ml) of EVRE

The effect of lichen extract on growth of microorganisms was estimated by adding different concentration of extracts in culture broth inoculated with test microorganisms and recording O.D. at 620nm at intervals.

It could be observed (except for few cases) that the lag phase of bacteria persisted for approximately 4-5 hours. The growth of microorganisms was inhibited in the presence of extracts. In other words it was evident that the growth of microorganisms was inversely proportional to the concentration of extracts.

In the (Fig 4.14) it was observed that the lag phase of *A. faecalis* persisted for 5 hours, then followed by exponential phase about 2-3

hours. The stationary phase was very momentary about one hour approx then the bacterial culture soon attended death phase. In the control set (i.e., the culture without any lichen extract) the exponential phase continued even after 10 hours of observation.

The lag phase of Gram positive bacteria *B. subtilis* (Fig 4.15) persisted for 4 hours then the culture attended log phase which persisted for about more than 4 hours of observation. Almost all the microorganisms in the control set did not attend death during 10 hours of observation.

The growth of culture filtrate of *C. albicans* (Fig 4.16) was not that transparent. But it could be observed that the fungus culture soon attended autolytic phase within 10 hours of observation.

In the fig 4.17 it could be observed that the lag phase in *P. aeruginosa* lasted for about 3 hours as culture required much time to adjust to the medium. The log phase here was of greater period of time approximately 6 hours, much bacterial growth occurred during this hour. Soon after the ninth hour of observation the microorganisms either attended stationary phase or death phase directly.

A very short lag phase of not even one hour duration followed by exponential phase of 4-5 hours was seen in the culture filtrate of *E. coli* .Then the culture soon attended death phase in the eighth hour of observation in ethanolic extract of *Everniastrum* sp (Fig 4.18).

Similar to that of culture filtrate of *E. coli*, the culture of *S. aureus* (Fig 4.19) followed a very negligible period of lag phase. Then followed by exponential phase, a very short stationary phase and death phase after seven hours of observation.

In the above graph it was seen that the lag phase of *B. megaterium* (Fig 4.20) was only for 1 to 2 hours. Then the bacterial cells soon started multiplying rapidly in log phase which lasted for 5 to 6 hours, soon after the culture entered the stationary phase. But as usual the microorganisms in the control grew rapidly even after 10 hours of observation.

Discussion

It could be evident from fig 4.14 that the ethanolic extract of *Everniastrum sp* inhibited the growth of *A. faecalis* at different concentration but its growth continued in absence of extract.

Growth in bacteria and other microorganisms usually refers to the change in the total population rather than increase in size and mass of the individual organism the studied graph growth was estimated as increase in population of culture.

The ethanolic extract of *Everniastrum* sp proved to be very weak to inhibit the growth of *B. subtilis* whereas the growth of fungus culture was almost ceased by the extract of *Everniastrum* sp within the observed duration of time.

It was observed from the above graph that the ethanolic extract of *Everniastrum* sp was weak enough to inhibit the growth of *B. subtilis*.

In the fig 4.17 it could be seen that the growth of *Pseudomonas* occurred maximum between 3-9 hours of observation. But soon after the ninth hour death phase of the culture was observed. In the control set the culture continued its growth even till 10 hours of observation; no stationary phase was reached by the control set culture during its period of observation.

The gradual decline in the in the growth rate of bacterial culture with the ethanolic extract of *Everniastrum* sp may be due to its effect in metabolism. The cells during this phase die faster than the new ones are produced.

It could be traced out from the above results that ethanolic extract of *Everniastrum* sp was able to restrict the growth of both gram positive bacteria *S. aureus* and Gram negative bacteria *E. coli.*

The ethanolic extract of *Everniastrum* sp was able to slow down the rate bacterial growth to an extent. The culture with higher concentration of the extract (i.e., 0.8ml) attended stationary phase earlier than the culture with lower concentration of extracts (0.6ml, 0,4ml, 0.2ml).

Lowering the rate of microbial growth in presence of ethanolic extract of *Everniastrum* sp. may be due to the aggregation enzyme proteins (Akhand *et. al.*, 2008) and also its interference to other metabolic reactions. Plant extracts contain a great number of different compounds (phenols, flavonoids, tannins, coumarins, alkaloids, and terpenoids) which have an impact on growth and metabolism of microorganisms (Cowman, 1999).

4.17.2 Effect on growth of microorganisms by methanolic extract of *Everniastrum* sp

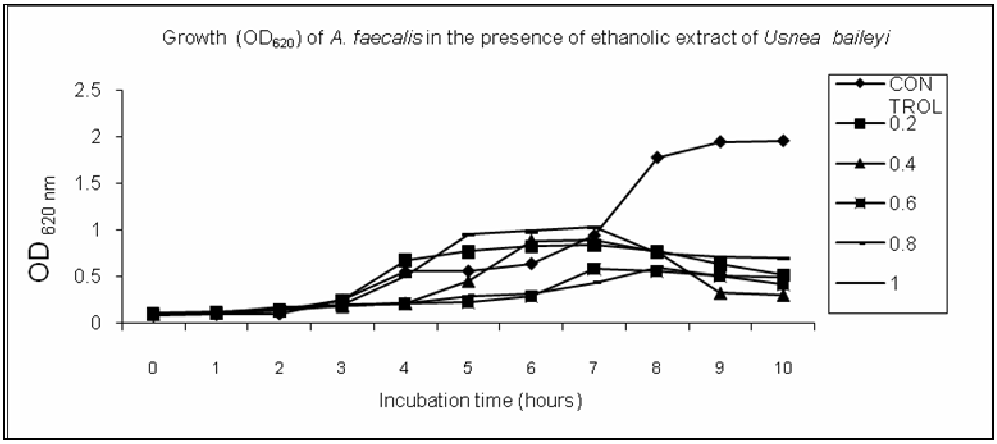


Fig 4.21 Growth curve of *A. faecalis* in the presence of different conc. (µg/ml) of EVRM

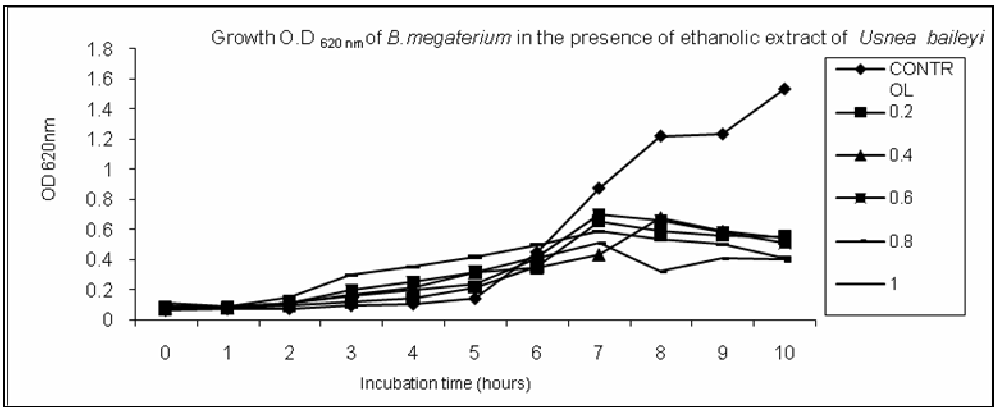


Fig 4.22 Growth curve of *B. megaterium* in the presence of different conc. (µg/ml) of EVRM

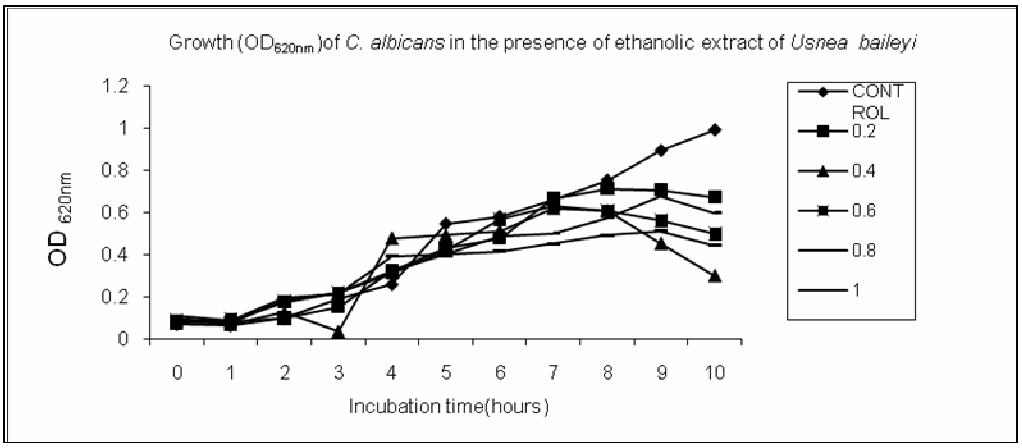


Fig 4.23 Growth curve of *C. albicans* in the presence of different conc. (µg/ml) of EVRM

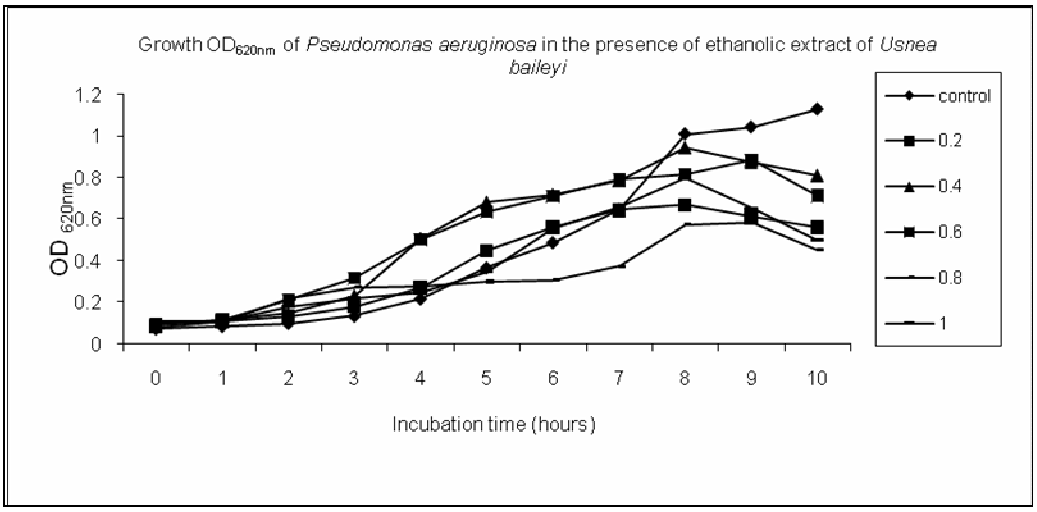


Fig 4.24 Growth curve of *P. aeruginosa* in the presence of different conc. (µg/ml) of EVRM

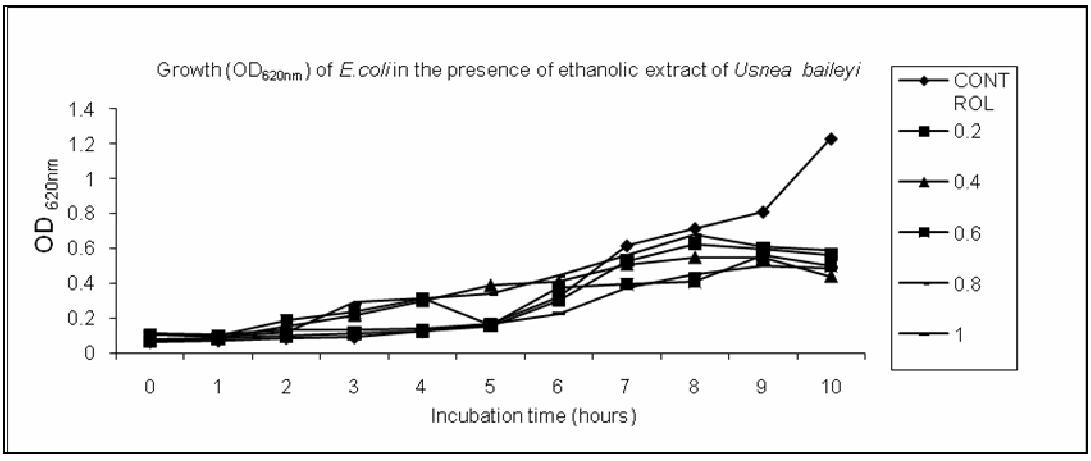


Fig 4.25 Growth curve of *E. coli* in the presence of different conc. (µg/ml) of EVRM

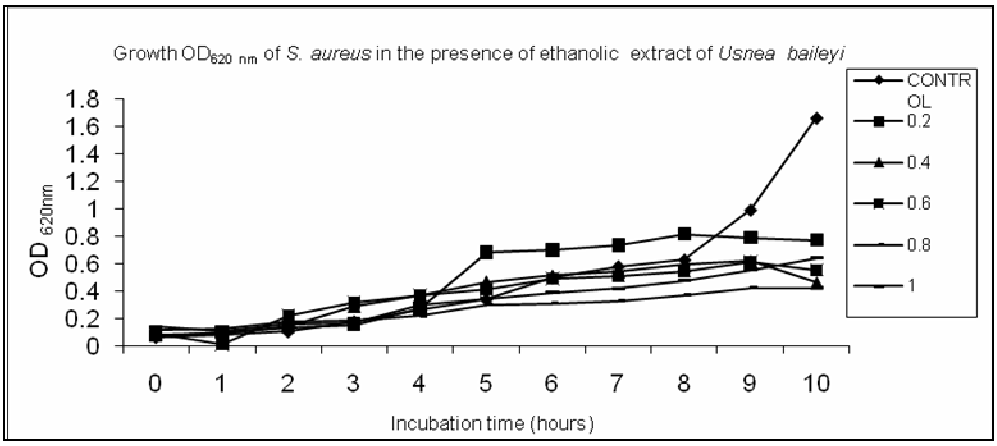


Fig 4.26 Growth curve of *S. aureus* in the presence of different conc. (µg/ml) of EVRM

The culture of *A. faecalis* (Fig 4.21) continued to multiply even after the stipulated observation period. Similarly the culture of *B. megaterium* (Fig 4.22) after attending a short lag phase entered exponential phase and the culture continued to grow without attending any stationary phase during 10 hours of observation.

The growth of only fungus namely *C. albicans* (Fig 4.23) tested was moderately affected by methanolic extract of *Everniastrum* sp. A lag phase of about one hour was followed by log phase. Linear phase persisted for longer period i.e., for seven hours.

It could be observed from the above graph that methanolic extract of *Everniastrum* sp interfered with the growth of *P. aeruginosa* (Fig 4.23). Even after 10 hours of observation growth of microorganisms in all the cultures with different amount of extract continued. No stationary or death phase was attended by microorganisms.

It was observed from the graph the growth of *E. coli* was effected by methanolic extract *Everniastrum* sp. After ninth hour of observation the cultures with different amount of extract started to attend death phase.

No prominent lag phase was noticed in the above graphs in case cultures of *E. coli* and *S. aureus*. A linear log phase was observed, which

persisted during entire period of observation which indicates that cellular metabolism was insensitive to the presence of extracts in the medium

Discussion

The methanolic extract of *Everniastrum* sp was weak to control the growth of *A. faecalis, B. megaterium* and *C. albicans* as no death phase was attended by the test microorganisms as observed from the (Fig 4.21, 4.22 and Fig 4.23).

The methanolic extract of *Everniastrum* sp was not able to control the growth of *P. aeruginosa* as the culture continued its exponential phase even after 10 hours of observation.

The growth of culture of *E. coli* was however slowed down by the lichen extract after 9 hours of observation but the Gram positive bacteria *S. aureus* continued to grow even after observed time.

Everniastrum sp. was found weak to interfere the growth of test microorganisms. The possible reason maybe active principles are sparingly soluble in the solvents used or their content in lichen is very low.

4.17.3 Effect on growth of microorganisms by ethanolic extract of *Parmotrema reticulatum*

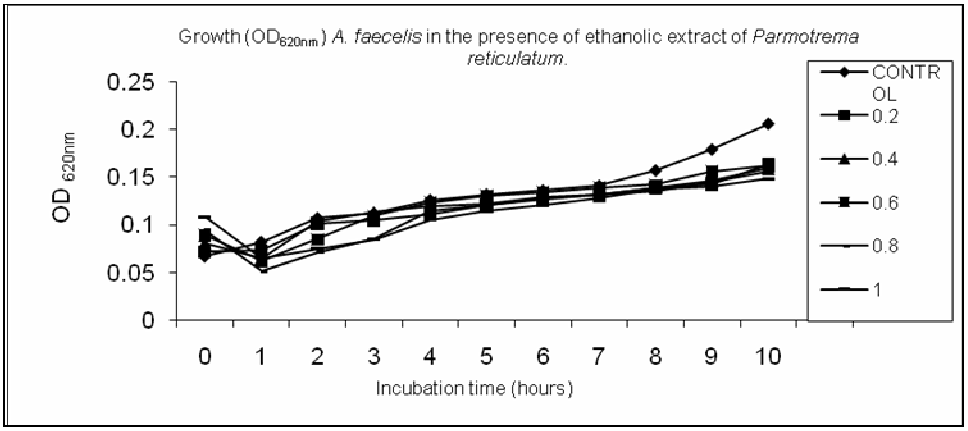


Fig 4.27 Growth curve of *A. faecalis* in the presence of different conc. (µg/ml) of PARE

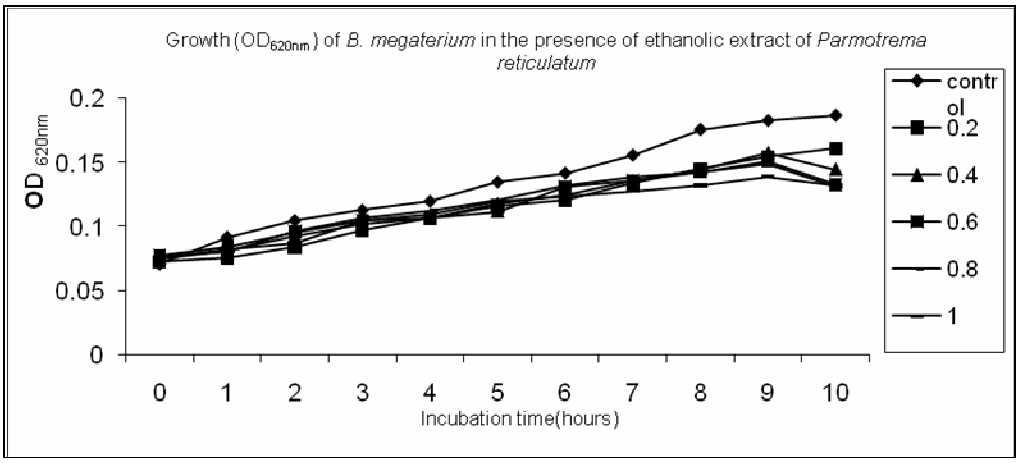


Fig 4.28 Growth curve of *B. megaterium* in the presence of different conc. (µg/ml) of PARE

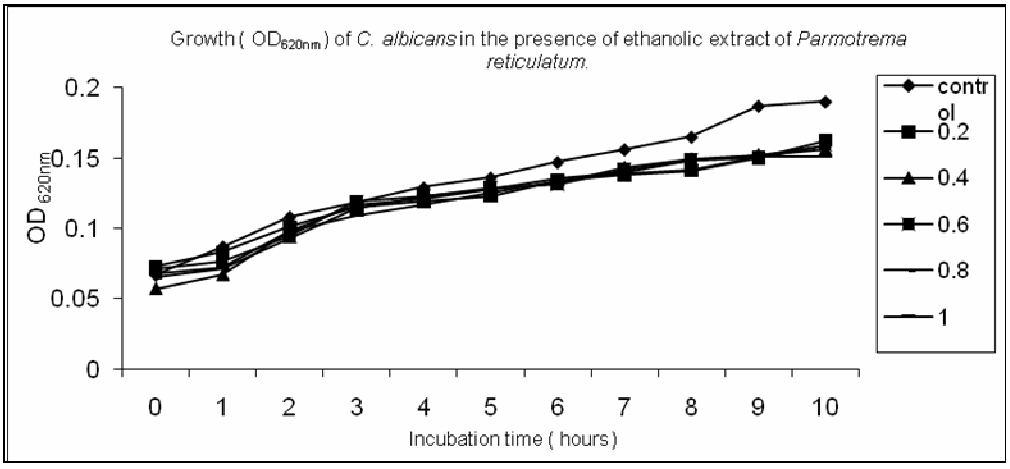


Fig 4.29 Growth curve of *C. albicans* in the presence of different conc. (µg/ml) of PARE

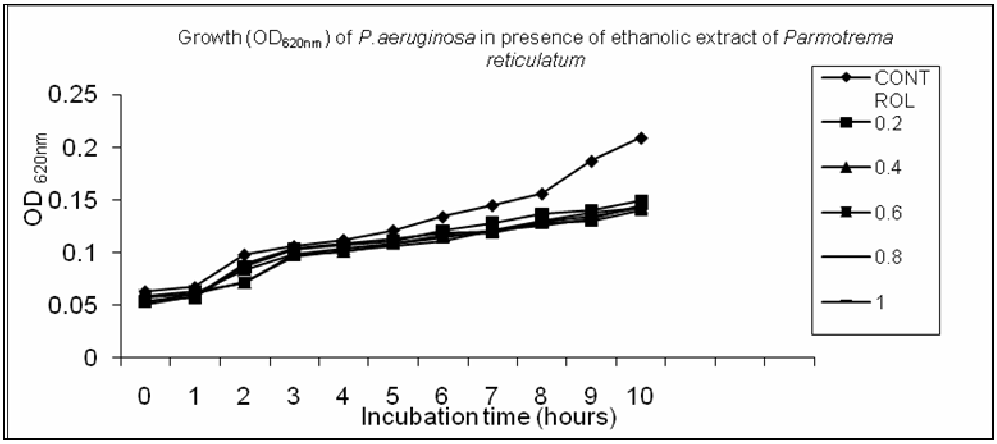


Fig 4.30 Growth curve of *P. aeruginosa* in the presence of different conc. (µg/ml) of PARE

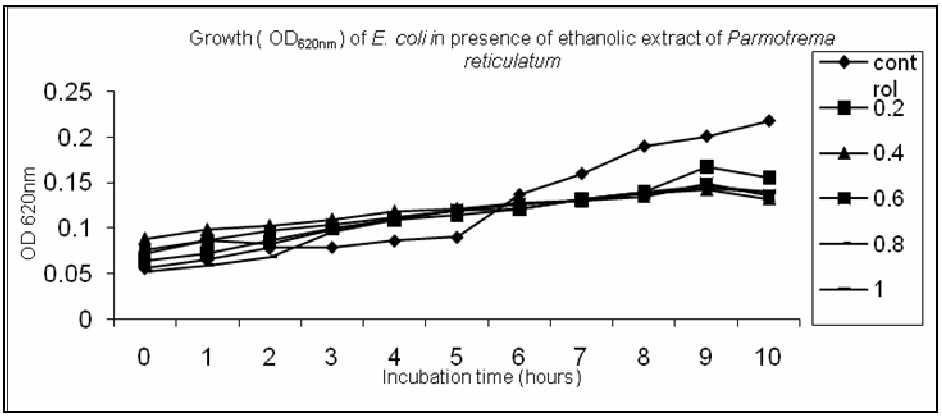


Fig 4.31 Growth curve of *E. coli* in the presence of different conc. (µg/ml) of PARE

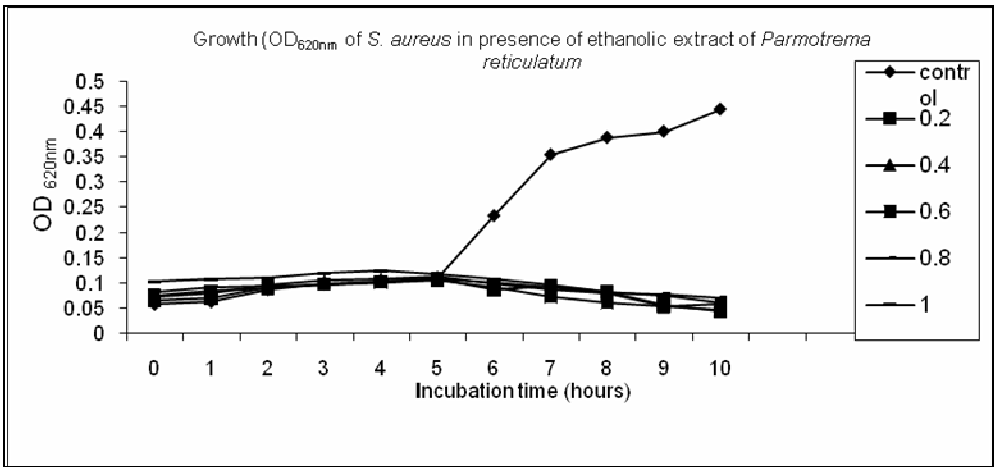


Fig 4.32 Growth curve of *S. aureus* in the presence of different conc. (µg/ml) of PARE

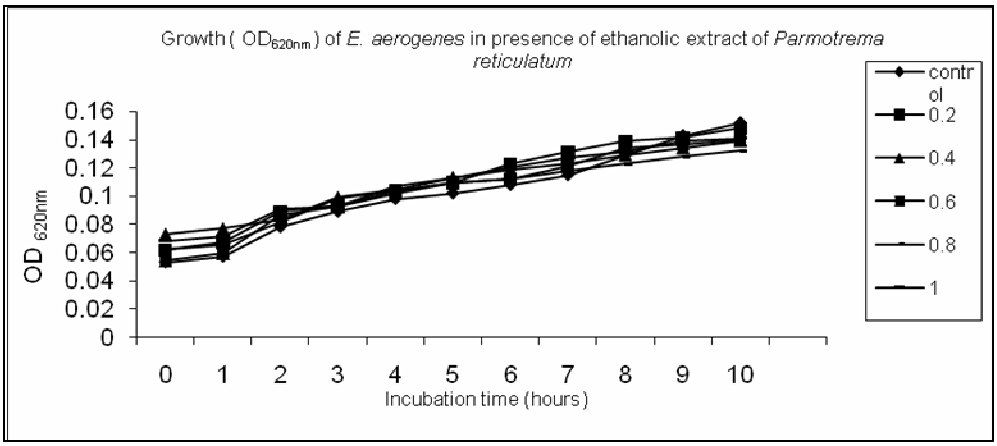


Fig 4.33 Growth curve of *E. aerogenes* in the presence of different conc. (µg/ml) of PARE

The growth of *A. faecalis* in the presence of ethanolic extract *P. reticulatum* could be observed in the (Fig 4.27). The cultures with extracts of different concentration grew continuously without attending stationary or death phase during 10 hours of observation.

The ethanolic extract of *P. reticulatum* was effective in controlling the growth of *B. megaterium* (Fig 4.28) moderately. The cultures with more amount of extract (0.4ml, 0.6ml, 0.8ml and 1ml) soon attended death phase while the cultures with minimum amount of extracts (0.2ml) continued to grow. The bacterial cultures grew up to 9 hours of observation

A lag phase of about one hour and exponential phase of about seven to nine hours was observed in the culture filtrate of *C. albicans* (Fig 4.29). The growth of *P. aeruginosa* with different concentration of *P. reticulatum* extracts grew exponentially without attending stationary or death phase. The culture in control set (i.e., without extract) multiplied more vigorously than other sets.

The fig 4.30 illustrates that a very short lag phase of one hour and exponential phase remained for 8 to 9 hours of growth. The growth rate of the control set of *P. aeruginosa* was quite higher than that of other sets treated with lichen extracts.

A short lag phase of about one hour was observed followed by log or exponential phase which persisted for about 3 to 4 hours in the other sets except for control set. Then the culture of *S. aureus* soon entered stationary and finally death phase. The culture in the control set attended highest phase of growth**.**

Discussion

It could be seen that the growth curve of *A. faecalis* increased with increase in time. It may be assumed the bacterial culture adapted well to the surrounding environment supplied with extracts of different concentration. The cultures with no extract continued to grow at a highest rate.

Fig 4.29 shows very short lag phase of less than half an hour and log phase of same duration by *C. albicans*. Then a linear phase of very long duration i.e., about seven hours was observed. It could be clearly concluded that the ethanolic extract of *P. reticulatum* was not able to

control the growth of fungus. The fungus culture with different concentration of extracts grew indifferently on the on the culture plate.

No slowing down to stationary and autolytic phase could be noticed. It may be concluded that bioactive molecules of lichens are targeted to prokaryotic (test bacteria) structural organization instead of eukaryotes.

As observed in most of the cases the Gram negative bacteria is quite difficult to be inhibited by various lichen extracts. Though the rate of growth was not very rapid but a uniform multiplication of the culture took place. It is assumed that plant extract was unable penetrate the thick outer membrane of bacterial cell wall. An exception could be traced out as ethanolic extract of *P. reticulatum* could restrict the growth of *E. coli*. It may be assumed that ethanolic extract is strong antimicrobial agent.

S. aureus being a Gram positive bacteria its growth was strictly affected by ethanolic extract of *P. reticulatum*. This could be clearly seen in the (Fig 4.28). It may be assumed that the metabolism of bacteria was affected by lichen extract and then the culture attended autolytic stage.

4.17.4 Effect on growth of microorganisms by methanolic extract of *Parmotrema reticulatum*

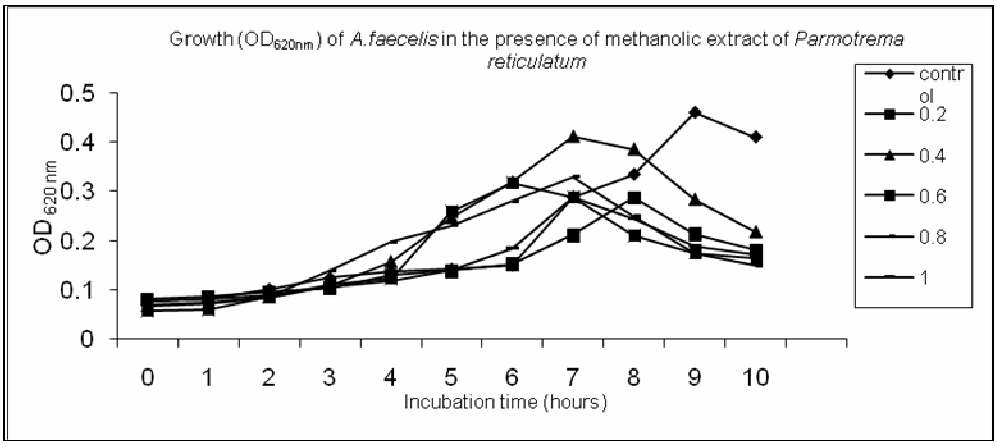


Fig 4.34 Growth curve of *A. faecalis* in the presence of different conc. (µg/ml) of PARM

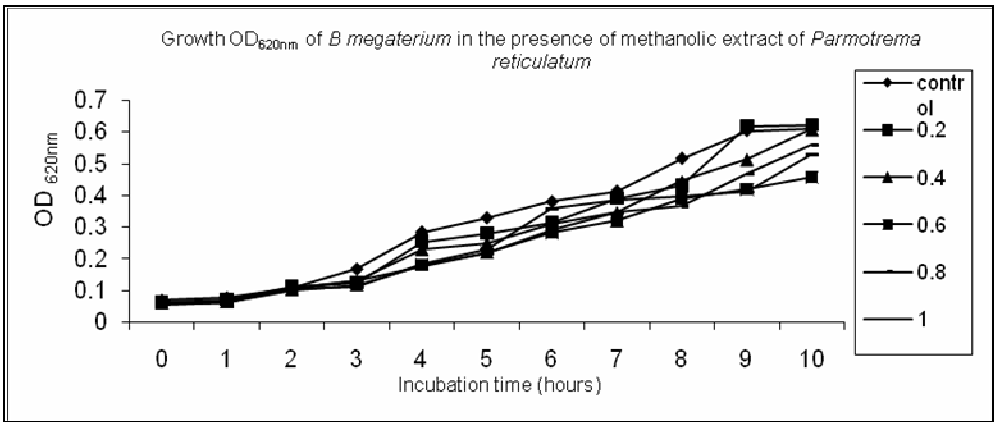


Fig 4.35 Growth curve of *B. megaterium* in the presence of different conc. of (µg/ml) PARM

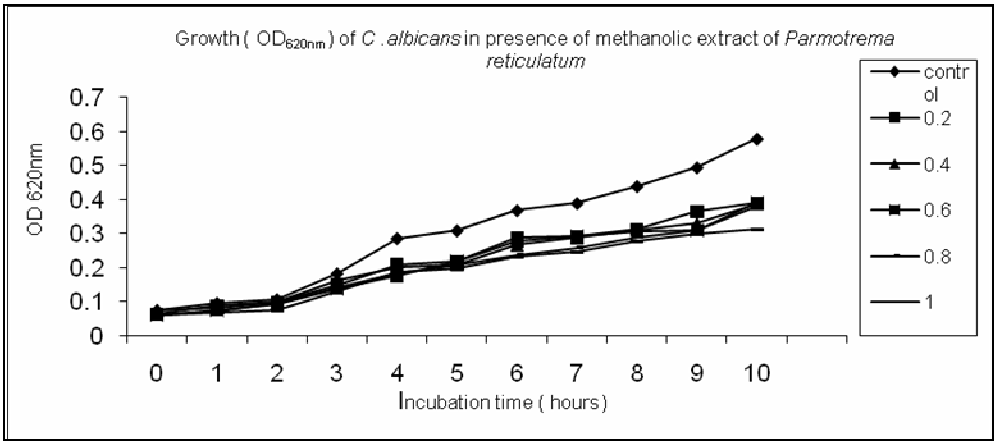


Fig 4.36 Growth curve of *C. albicans* in the presence of different conc. (µg/ml) of PARM

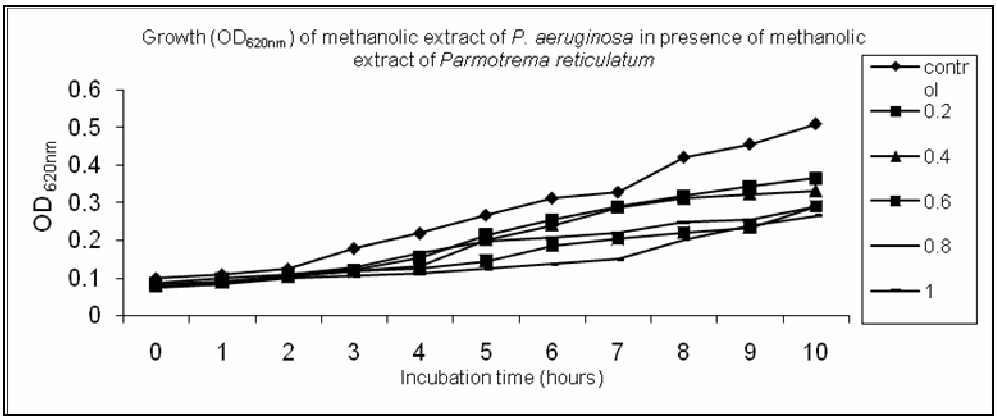


Fig 4.37 Growth curve of *P. aeruginosa* in the presence of different conc. (µg/ml) of PARM

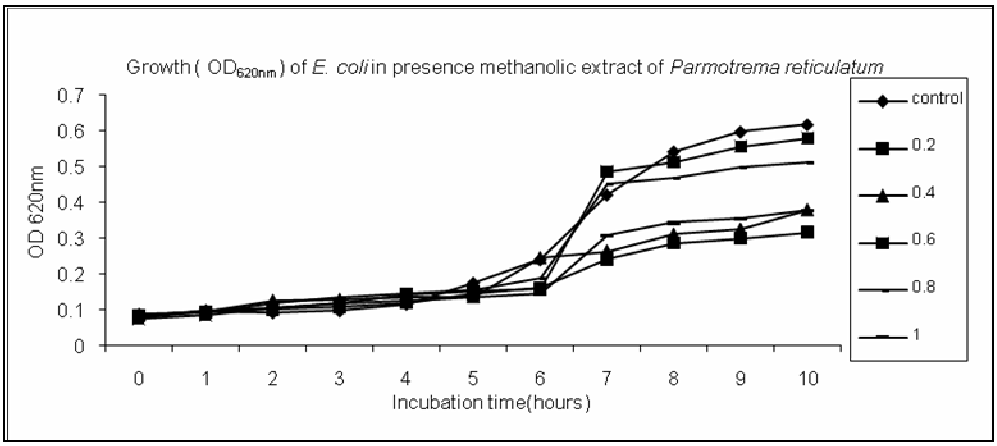


Fig 4.37 Growth curve of *E. coli* in the presence of different conc. (µg/ml) of PARM

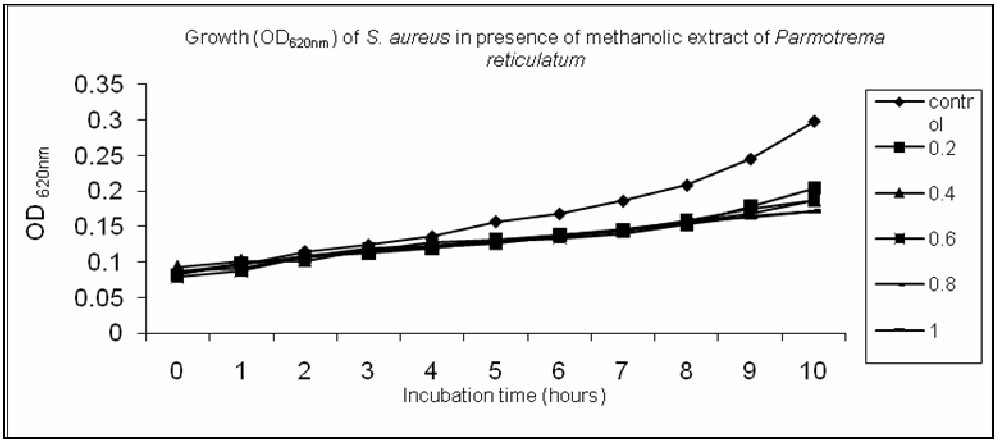


Fig 4.38 Growth curve of *S. aureus* in the presence of different conc. (µg/ml) of PARM

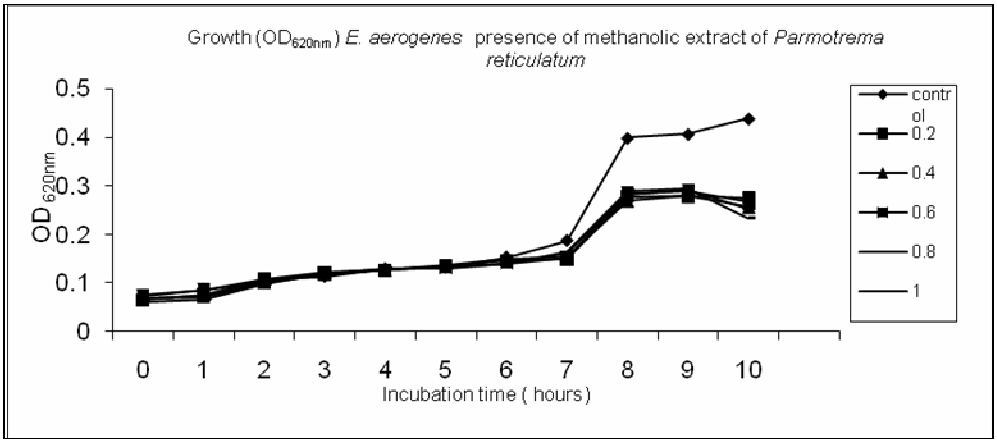


Fig 4.39 Growth curve of *E. aerogenes* in the presence of different conc. (µg/ml) of PARM

The cultures of *A. faecalis* (Fig 4.34)after attending very short lag phase entered log phase abruptly (i.e., for cultures with 0.6ml and 0.4ml of extract) whereas a lengthy lag phase of about 5-6 hours was attended by cultures with 0.2 and 1 ml of extract. Highest growth rate was observed for control set. Second to that was culture with (0.4 ml of extract). But the cultures of *A. faecalis* then attended death phase after completing the exponential and stationary phase.

Culture of *C. albicans* (Fig 4.36) exhibited very short lag phase of one hour duration followed by log phase of an hour again was observed. Exponential phase was of very long duration i.e., seven hours was noticed. No stationary or autolytic phase was attended by cultures with extracts of different concentration. The culture with control set showed elevated population than others.

The gram negative bacteria *P. aeruginosa* (Fig 4.37) attended a lag phase of very short time about 1 to 2 hours and then exponentially grew up to 10 hours of observation. In this case no stationary phase was reached by the methanolic extract of *P. reticulatum* at different concentration. The control set culture grew continuously and at the highest rate. A long duration lag phase was observed in case of concentration 0.8ml of extract

A unique feature was observed in case culture of *E. coli* as lag phase here persisted for about 1 to 6 hours and then the microorganisms entered exponential phase. The bacterial culture with 0.6ml of extract multiplied at a highest rate than compared to the other cultures. No stationary or autolytic phase was noted after stipulated period of observation.

An initial stage a short lag phase was attended and then the cultures of *S. aureus* entered into log phase. Nearly all the cultures grew up simultaneously.

Discussion

The methanolic extract of *P. reticulatum* proved to be quite powerful in inhibiting the growth of gram negative bacteria *A. faecalis*. After attending the log phase the bacteria soon entered a short stationary

and then death phase, The bacteria in the control set also entered autolytic stage as may be depletion of nutrients might have occurred or changes in the nutrient medium might have taken place .

The methanolic extract of *P. reticulatum* proved to be weak antibacterial agent. The growth of *B. megaterium* was resistant to the different concentration of extract.

The methanolic extract of *P. reticulatum* was very weak in its antimicrobial activity. The growth of fungus (*C. albicans*) population rose in accordance with time.

As expected the growth rate of *E. coli* was not affected by methanolic extract of *P. reticulatum* cultures with different amount of extract attended different growth rates.

Extracts of *Parmotrema reticulatum* was more active against Gram negative organisms than Gram positive ones under study. Proper evaluation of this lichen is needed by this lichen for its use against Gram negative bacteria.

4.17.5. Effect on growth of microorganisms by ethanolic extract of *Usnea baileyi*

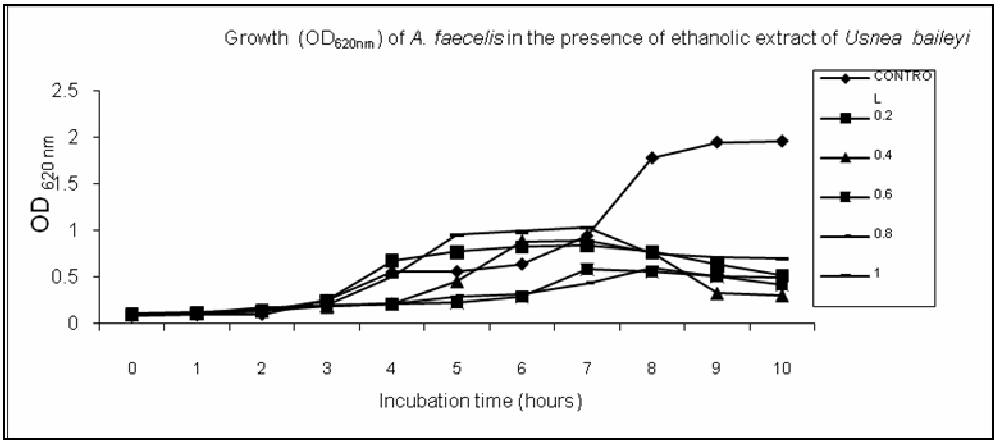


Fig 4.40 Growth curve of *A. faecalis* in the presence of different conc. (µg/ml) of USRE

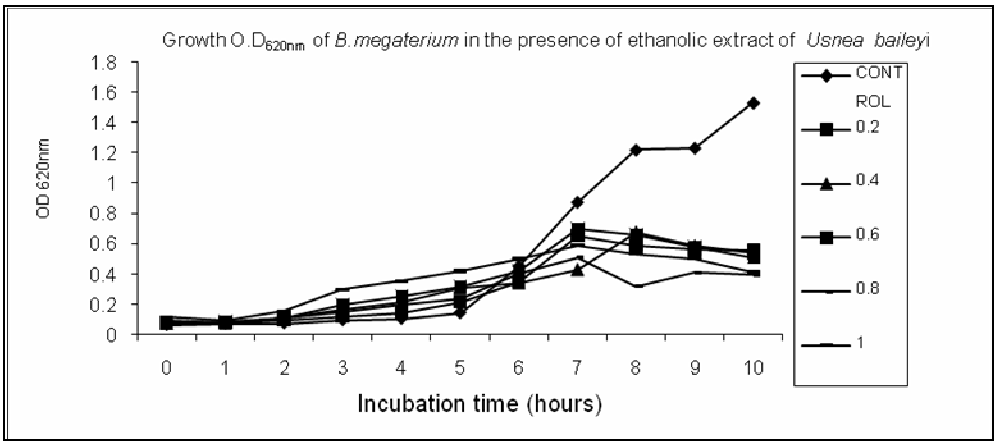


Fig4.41 Growth curve of *B. megaterium* in the presence of different conc. (µg/ml) of USRE

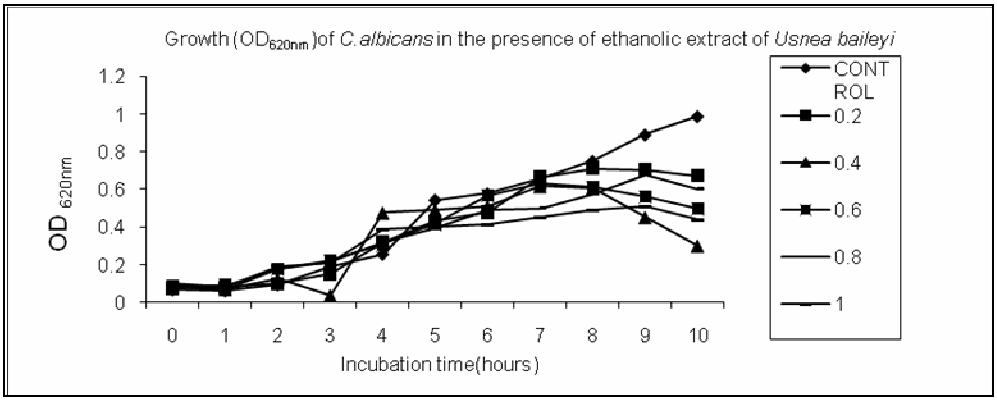


Fig4.42 Growth curve of *C. albicans* in the presence of different conc. (µg/ml) of USRE

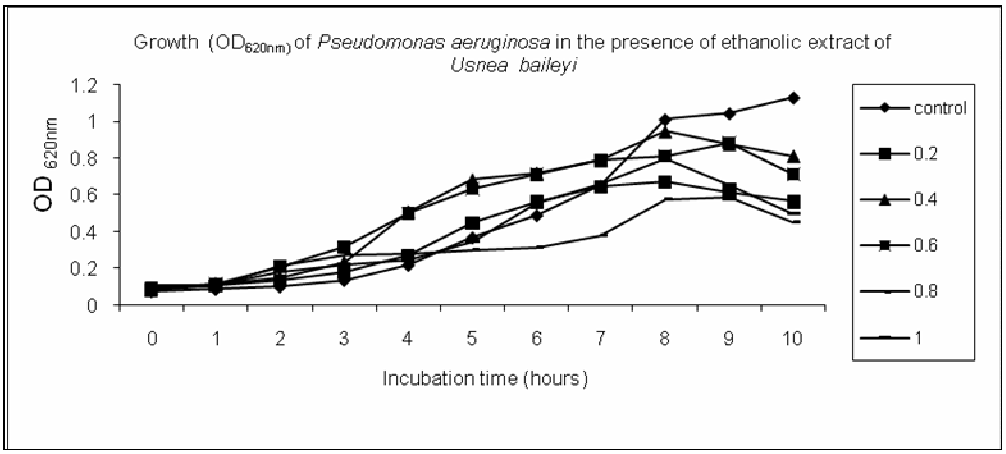


Fig4.43 Growth curve of *P. aeruginosa* in the presence of different conc. (µg/ml) of USRE

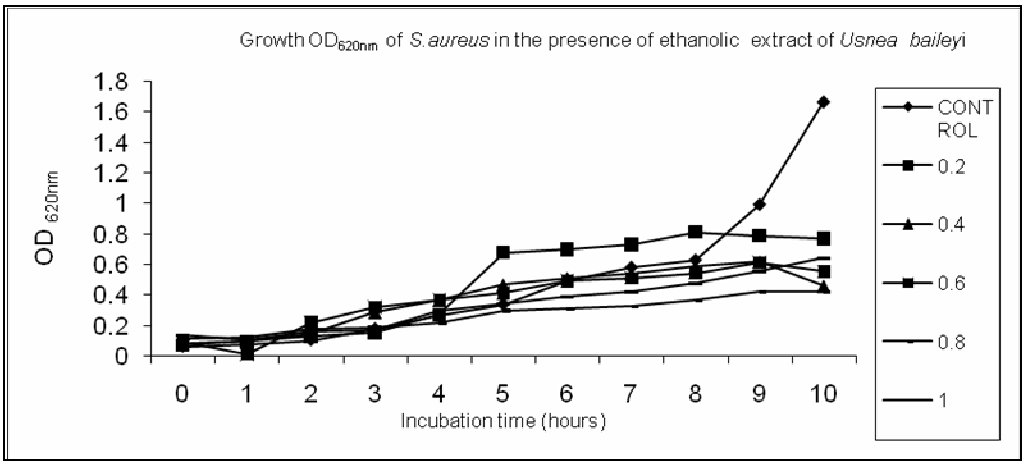


Fig 4.44 Growth curve of *S. aureus* in the presence of different conc. (µg/ml) of *U. baileyi*

The lag phase i.e., the time required by the microorganisms to adjust to the present media was about 1 to 3 hours for *A. faecalis* (Fig. 4.40) Then the culture entered into exponential phase where rapid multiplication of microorganisms occurred. The log or exponential phase of this microorganism was very momentary i.e., about 2 to 3 hours and soon the microorganisms entered stationary phase followed by death phase.

A lag phase of very short duration was shown by culture of *B. megaterium* (Fig 4.41) with 0.4ml, 0.2ml and 0.6ml of extract as compared to long duration lag phase shown by cultures with 0.8ml and 1 ml of extract. It was seen that the bacterial cultures are still growing as different concentration of the cultures have not attended stationary phase.

The lag phase of fungus *C. albicans* (Fig. 4.42) was maximum about 4 hours then it attended exponential phase. The log phase i.e., the growth and multiplication of fungi continued even after 10 hours, no stationary was observed

A short duration lag phase about 1 hour and exponential phase about 6 hours was observed in culture of *P. aeruginosa* (Fig 4.43). Then the microorganisms finally attended stationary phase. An evident death phase could not be observed. But the culture without any extract of *U. baileyi* continued growing even after the estimated incubation period.

The culture of *S. aureus* (Fig 4.44) attended a very short lag phase about 1 hour or even less and then quickly initiated the exponential phase which lasted for 6hours.

Discussion

An unusual case was observed by the presence of ethanolic extract of *U. baileyi*. The microorganisms after multiplying rapidly soon entered stationary phase which may be caused due to interference in cell activity, changes in pH the medium or other factors. The effect of lichen extract on the culture of *A. faecalis* was so effective that soon after stationary phase in eighth to ninth hour of observation the culture attended death phase. There was a gradual decline in the population of microorganisms.

The culture of *B. megaterium* not so much affected by the ethanolic extract *U. baileyi* of as the culture did not attend stationary phase even after 10 hours of observation. The fungus (*C. albicans*) culture with low concentration of extract attended death phase while the control set continued to grow.

The extract was effective in controlling the growth of culture *P. aeruginosa* as the culture with different concentration of extracts attended death phase. The culture of *S. aureus* attended stationary phase, but no distinct death phase was observed which proved that ethanolic extract of *U. baileyi* could not control the growth of *S. aureus*.

4.17.6. Effect on growth of microorganisms by methanolic extract of *Usnea baileyi*

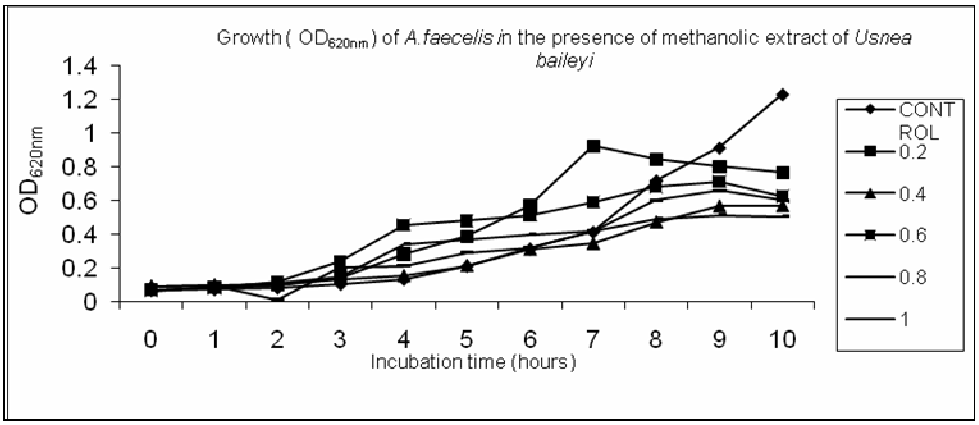


Fig 4.45 Growth curve of *A. faecalis* in the presence of different conc. (µg/ml) of USRM

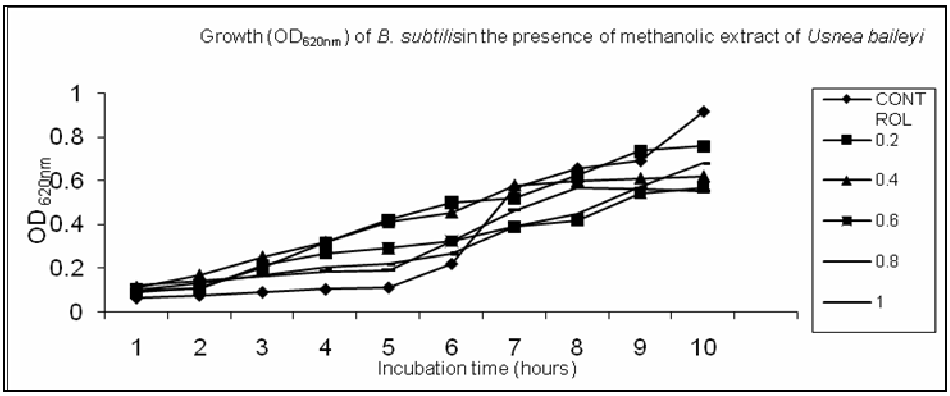


Fig 4.46 Growth curve of *B. subtilis* in the presence of different conc.(µg/ml) of USRM

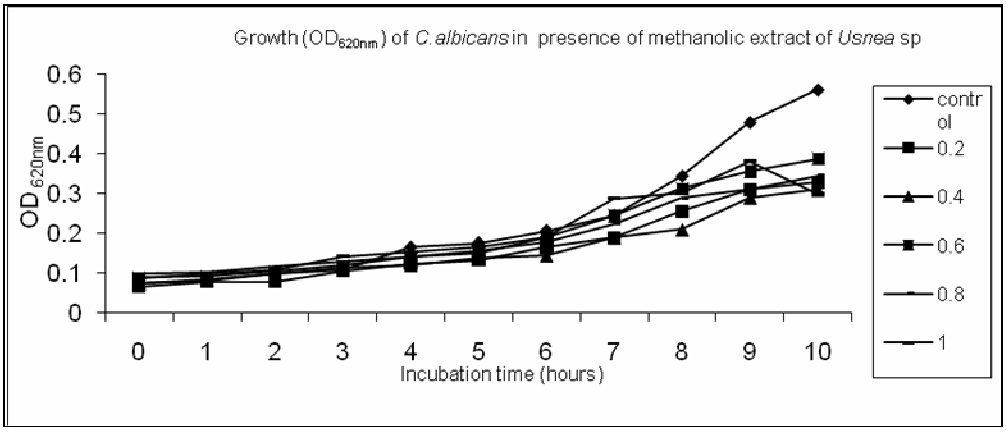


Fig 4.47 Growth curve of *C. albicans* in the presence of different conc. (µg/ml) of USRM

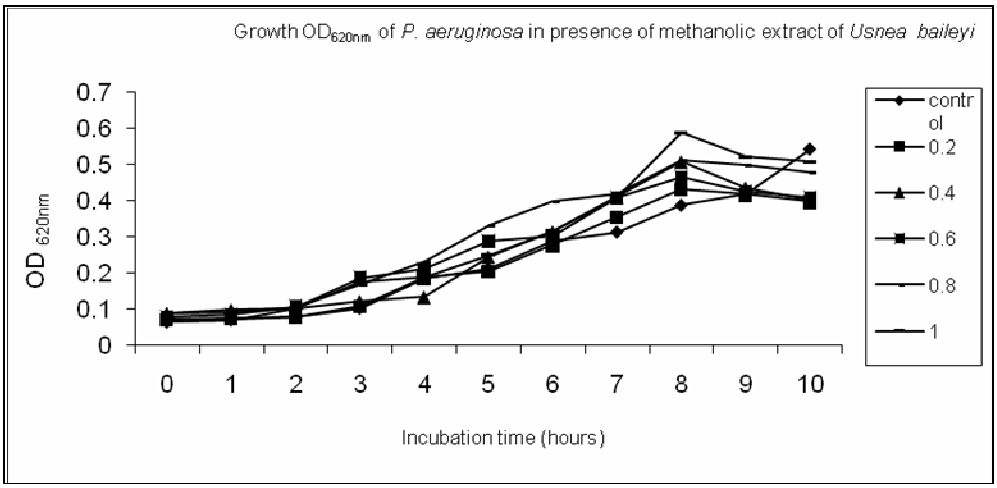


Fig 4.48 Growth curve of *P. aeruginosa* in the presence of different conc. (µg/ml) of USRM

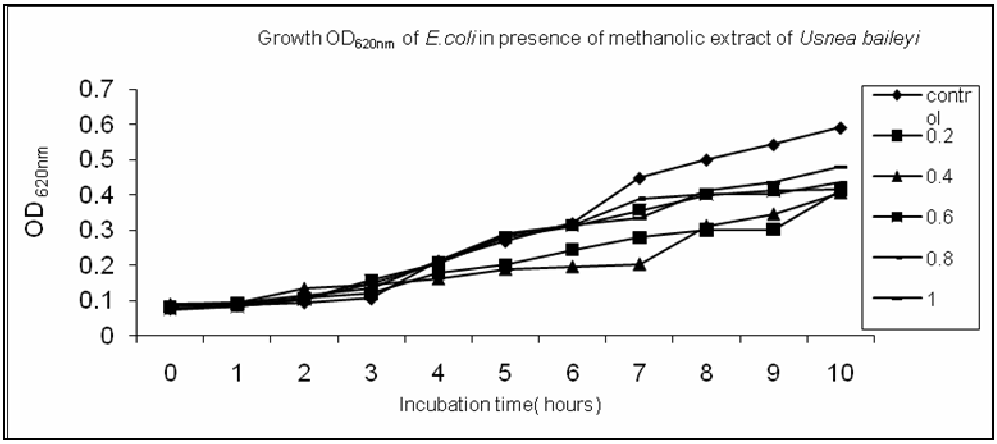


Fig 4.49 Growth curve of *E. coli* in the presence of different conc. of (µg/ml) USRM

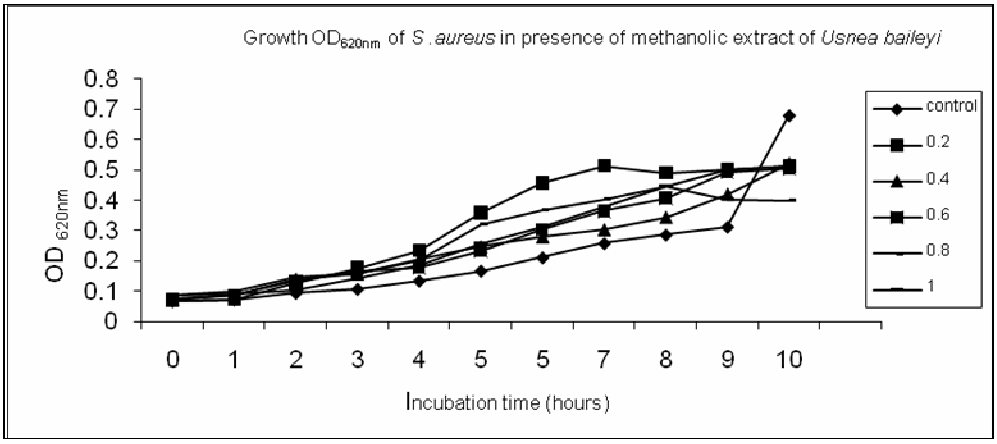


Fig 4.50 Growth curve of *S. aureus* in the presence of different conc. (µg/ml) of USRM

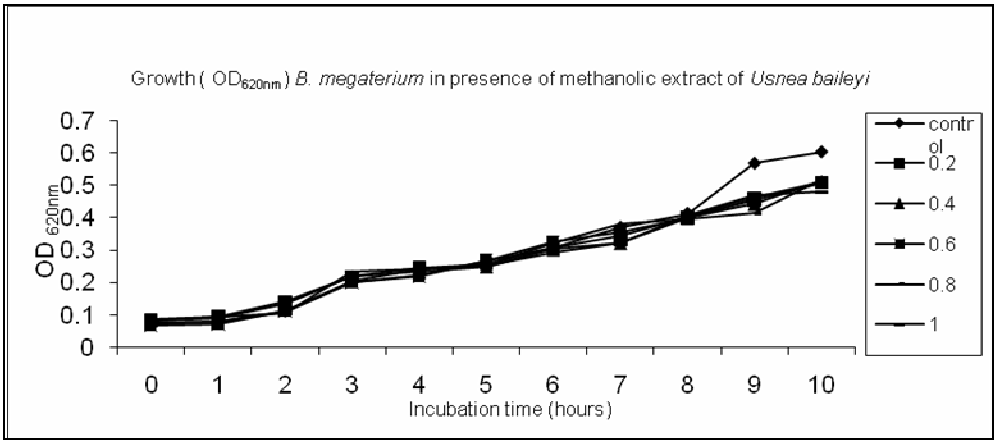


Fig 4.51 Growth curve of *B. megaterium* in the presence of different conc. (µg/ml)

It could be observed that highest rate of growth was attended by the culture of *A. faecalis* (Fig 4.45) with lower concentration of extract and the growth cultures decreased with increase in amount of extract. The bacterial cultures filtrates with highest amount of extract i.e., 1ml attended the stationary phase earlier. The culture with 0.6 ml of extract attended stationary phases within 8-9 hours of observation.

Fig 4.46 shows the effect of methanolic extract of *U. baileyi* on *B. megaterium.* The growth of the control set was continuous even after 10 hours observation. The maximum rate of growth or multiplication of bacteria was observed in the set in which minimum amount lichen extract was present.

The bacterial culture is which high concentration of lichen extract was present attended death phase after 7-9 hours of observation.

Generally observing the fig 4.49 it could be concluded that the growth culture of *E. coli* was not so much affected by the lichen extract. The cultures with all the concentration of extracts (i.e., 0.2ml, 0.4ml, 0.6ml, 0.8ml and 1ml) continued to grow in 10 hours of observation. None of the cultures reached the death phase. The culture of *P. aeruginosa* attended stationary phase in eighth hour of observation.

The methanolic extract of *U. baileyi* was much active against *S. aureus*. From the graph it was seen that a short lag phase of duration 1 to 2 hours was attended by *S. aureus* and then the bacteria started growing with the available nutrients in all the extracts.

Discussion

As reported by (Ray *et. al.*, 2003), extracts of *Usnea articulate, Ramalina jamesii* and *Parmelia tinctorum,* were also found to be inhibitor of protein synthesis, energy metabolism and growth of selected bacteria similarly in our work the growth of microorganisms were affected by the ethanolic and methanolic extract of studied lichen species.

The methanolic extract of *U. baileyi* was able to control the growth of *A. faecalis* as the culture with the extracts attended stationary phase within the observation time.

The methanolic extract of *U. baileyi* may be a weakly active against *B. megaterium* as the culture is still multiplying even ten hours of observation. The lichen extract may not be able to affect the metabolic activity of microorganisms and retard its growth.

The methanolic extract of *U. baileyi* proved to be very weak in its activity against *C. albicans* as the fungi grew continuously without attending any stationary phase or death phase. The metabolic activity of the fungus was not affected by methanolic extract of *U. baileyi*.

From the graph it was seen soon after the culture of *E. coli* was introduced in to the medium with lichen extract the microorganisms started growing after attending short lag phase. No clear stationary phase was observed as the culture of *E. coli* grew and multiplied continuously. The control set was not affected by the lichen extract. It could be observed that the methanolic extract of *U. baileyi* could not inhibit the growth of gram negative *E. coli*. The culture of *E. coli* continued to multiply even after observation of 10 hours. The culture of *S. aureus* was weakly controlled by the methanolic extract of *U. baileyi.*

According to the graphs obtained from EVRE, EVRM, PARE, PARM, USNE and USRM against microorganisms could be generally concluded that the growth of test cultures of microorganisms were either inhibited or slowed down by the addition of lichen extracts. The active components present in lichen samples may have interfered with growth of microorganisms.

4.18 Preliminary separation of lichen compounds by TLC

Preliminary separation of lichen compounds was done taking three solvent systems: Solvent System A- toluene/dioxane/glacial acetic acid (36:9:1), Solvent System B- hexane/diethyl ether/formic acid (24:18:4) and Solvent C- toluene/glacial acetic acid (20:3). Rf values of lichen compounds were calculated and presented in Table (4.71).

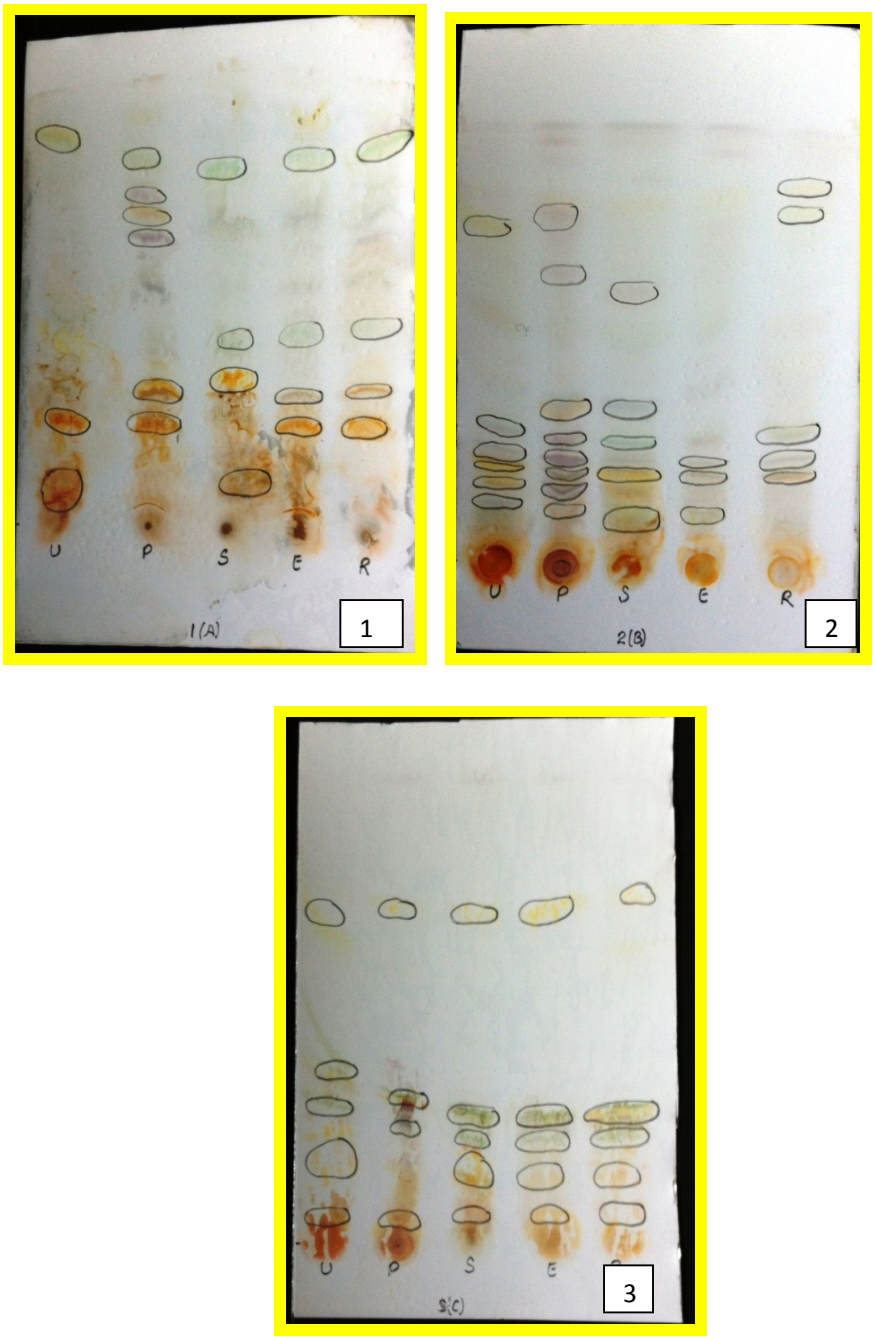


Plate 3.56 (1, 2 and 3) TLC plates of the lichen extracts run in Solvent Systems A, B and C, [U-*U.baileyi*; P-*P. reticulatum,* S-*S. pomiferum; E-Everniastrum* sp*; R-R. hossei*

Table 4.71 Rf value of lichen compounds obtained from TLC plates

Lichen samples	Rf value		
	Solvent A	Solvent B	Solvent C
U. baileyi	0.09	0.09	0.037
	0.22	0.28	0.28
	0.9	0.23	0.33
		0.33	0.66
		0.19	0.71
		0.76	
S. pomiferum	0.09	0.11	0.037
	0.31	0.19	0.2
	0.43	0.38	0.21
	0.81	0.4	0.23
		0.66	0.60
P. reticulatum	0.7	0.17	0.003
	0.72	0.19	0.29
	0.74	0.23	0.66
	0.81	0.26	0.85
		0.28	
		0.36	
		0.77	
		0.8	
Everniastrum sp	0.22	0.09	0.037
	0.27	0.23	0.14
	0.45	0.23	0.22
	0.86		0.29
			0.62
R. hossei	0.22	0.23	0.037
	0.31	0.26	0.13
	0.45	0.28	0.18
	0.86	0.76	0.25
		0.85	0.66

In *U. baileyi* three spots were detected with Rf values ranging from 0.09 to 0.9 in solvent system A, six compounds from value ranging from 0.09 to 0.76 in solvent system B and five compounds from with values from 0.037 to 0.71 in solvent system C.

S. pomiferum showed four spots with Rf value ranging from 0.09 to 0.81 in solvent system A, five spots with Rf value ranging from 0.11 to 0.66 in solvent system B and five spots with values ranging from 0.037-0.66 in solvent system C

TLC plates of *P. reticulatum* showed the four spots in solvent system A (Rf: 0.7-0.81), eight compounds in solvent system B (Rf: 0.17-0.8) and four compounds (Rf: 0.03 to 0.085).

Everniastrum sp. depicted the presence of four compounds (0.22-0.86 in solvent system A), three compounds from solvent system B (Rf: 0.09-0.23) and five compounds from solvent system C (Rf: 0.037-0.62).

TLC plates from *R. hossei* showed the presence of four and five compounds from solvent system A, B and C respectively. The values ranged from 0.22-0.86 in solvent system A, 0.23-0.85 in solvent system B and 0.037-0.66 in solvent

Discussion

The TLC plates showed different coloured spots which revealed the presence of different bioactive acids from lichens. Generally it could be observed that maximum numbers of spots were present in lichens in solvent system B especially in *U. baileyi* and *P. reticulatum*. Highest numbers (16) of spots were marked on TLC plates from *P. reticulatum.* It could be seen that maximum number were observed taking solvent system B, which signified that most of the lichen compounds are soluble in solvent system B followed by C.

A good number of spots separated during TLC shows that the lichen under study contains diverse group of phytochemicals which are responsible for antimicrobial as well as antioxidant properties as described in earlier part of this work.

4.19 Determination of the bioactive lichen compounds using TLC bioautography

The lichen extracts were run on TLC plates in different solvent systems (A, B and C). The different spots on plates were marked and Rf values were calculated (Table 4.71).This was followed by Bioautographic assay taking two bacteria (*E. coli* and *S. aureus*).

The marked spots of *R. hossei* from (solvent system B)(Plate 3.58) and *R. hossei, P. reticulatum* (solvent C) restricted the growth of *E.coli* (Plate 3.59).

Spots of lichen compounds from *S. pomiferum* and *Everniastrum* sp also inhibited the growth of *S. aureus* (Plate.3.60)

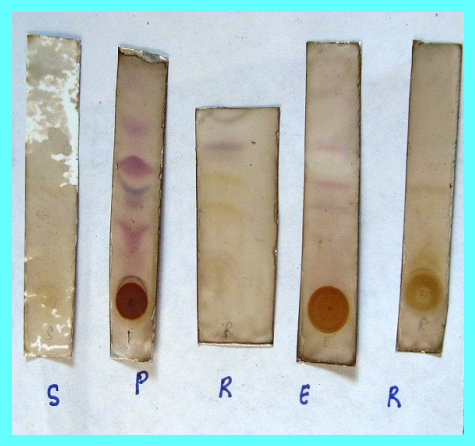


Plate 3.57

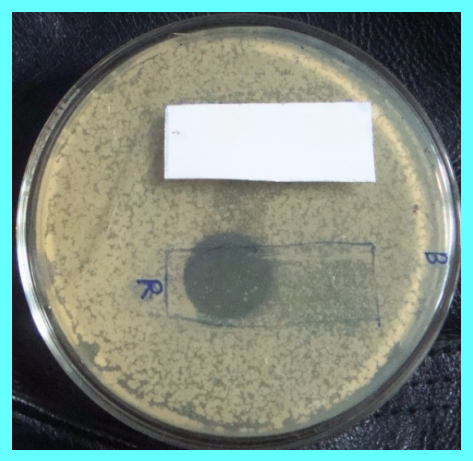


Plate 3.58

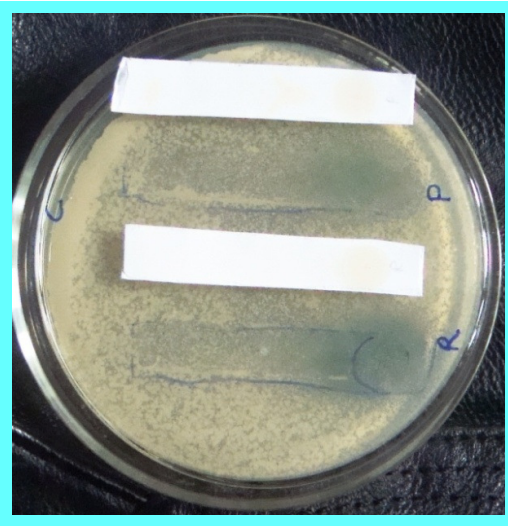


Plate 3.59

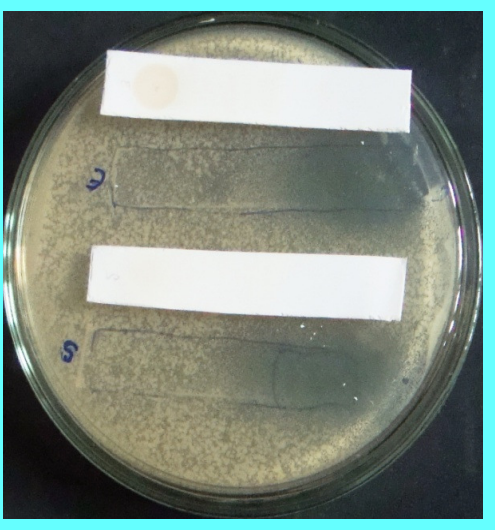


Plate 3.60

Plate 3.57 TLC spots of different lichens separated and used for Bioautographic assay [S- *S. pomiferum*; P- *P. reticulatum;* E-*Everniastrum* sp; R- *Ramalina hossei*

Plate3.58 Inhibition zone produced by marked TLC spots of *R. hossei* against *E. coli in solvent system B*

Plate 3.59 Inhibition zone produced by marked TLC spots of *R. hossei* and *P. reticulatum* against *E. coli* in solvent system *C*

Plate 3.60 Inhibition zone produced by marked TLC spots of *Everniastrum* sp and *S. pomiferum* against *S. aureus* in solvent system *C*

Discussion

The presence of lichen compounds represented by the spots in the TLC plates with respect to P. *reticulatum*, *R. hossei*, *S. pomiferum* and *Everniastrum* sp may be responsible for inhibiting the growth of *S. aureus* and *E. coli.* The spots which are responsible for the production of clear zones on the bacterial lawn may be one or few of the phytochemicals revealed from the LCMS chromatogram of the lichen samples described in the later part of this work.

4.20. Different lichen compounds identified on the basis of LCMS chromatogram

The LCMS chromatogram of the lichen samples unveils the presence of different classes of compound which are enlisted below from four different lichens from Darjeeling hills.

Table 4.72. List of names, classes, mass spectrum and occurrence of lichen substances identified from LCMS Chromatogram of methanolic extract of *Everniastrum* sp (SAIF 7904)

Sl.	Compound	Class	Mass spectrum	Also occurs in	References
1.	Hypostictic acid	â-Orcinol Depsidones	372,354,328 327	*Xanthoparmelia quintaria*	Keogh, 1978
2.	Salazinic acid	â-Orcinol Depsidones	388,370,354,179	*Parmelia sulcata*	Culberson,1969, Chapel Hill 161
3.	Norstictic acid	â-Orcinol Depsidones	372,354,179,177	*Xanthoparmelia substrigosa*	Culberson, 1969,Chapel Hill 156
4.	Thiomelin	Xanthones	342,340,327,325	*Rinodina thiomela*	Elix, *et. al.,* 1987
5.	Chloroatanorin	β-Orcinol Depsides	408,215,213,196		ulberson,1969,Chapel Hill 146
6.	Caperatic acid	Aliphatic acids	402	*Flavoparmelia caperata, haysomii*	Culberson,1969 Chapel Hill: 101
7.	Conloxodin	Orcinol Depsidones	428,396,384,370	*Xanthoparmelia xanthofarinosa*	Begg, *et. al.,* 1979
8.	Usnic acid	Usnic acid derivatives	344,260,233,217	*Usnea* sp	Culberson1969, Chapel Hill: 170
9.	Alectoronic acid áAlectoronic	Orcinol Depsidones	494,468,450,370	*Parmotrema rigidum*	Elix, *et. al.,*1974
10	3-Hydroxycolensoic acid	Orcinol Depsidones	458,440,414,236	*Hypotrachyna osseoalba*	Djura, *et. al.,*1977
11	Erioderimin	β–Orcinol Depsidones	384,382,367,347	*Erioderma sorediatum*	Connolly, *et. al.,*1984
12	Erythrin	Orcinol Depsides		*Roccella physcopsis*	Culberson, 1969: Chapel Hill: 116
13	Decarboxyperlatolic acid	Orcinol Depsides	400, 222, 221,180	*Xanthoparmelia depsidella*	Elix and Wardlaw, 1997
14	Nopannarin	â-Orcinol Depsidones	350,348,215, 213	*Erioderma chilense*	Elix, *et. al.,* 1986
15	Chloroatranorin	β-OrcinolDepsides	408,215,213,196		Culberson, 1969, Chapel Hill: 146
16	2'Omethylnobarbatic acid	â-Orcinol Depsides	360, 197, 196,180	*Pseudocyphellaria norvegica*	Elix, *et. al.,*1990
17.	Placodiolic acid	Usnic acid derivatives	376, 361, 235,233	*Rhizoplaca chrysoleuca*	Huneck, S 1972
18.	Lobaric acid	Orcinol Depsidones	456, 438, 412,235	*Protoparmelia badia*	Culberson, 1969, Chapel Hill: 137
19	Fern-9(11)-ene-3,12-dione	Terpenoids	438, 423, 395,273	Xanthoria resendei	Gonzales, *et.al* ,1974
20	Decarboxyhypothamnolic acid	Orcinol Depsides	362, 209, 192,191	*Pertusaria* sp	Elix, *et. al.,* 1999
21	Ursolic acid	Terpenoids	456, 438, 249,248	in many lichens	Culberson, 1969 Chapel Hill: 205
22	Alectoronic acid Alectoronic acid)	Orcinol Depsidones	494,468,450,370	*Parmotrema rigidum*	Elix *et. al.,* 1974
23	Lanosterol	Terpenoids	468, 454, 453,394	*Evernia prunastri*	Nicollier, *et. al.,* 1979
24	Gyrophoric acid	Orcinol Tridepsides	1, 318, 168, 150	*Punctelia borreri*	Culberson, 1969 Chapel Hill: 114
25	Protocetraric acid	â-Orcinol Depsidones	1, 358, 314, 312	*Flavoparmelia caperata*	Culberson, 1969 Chapel Hill: 159

Table4.73. List of names, classes, mass spectrum and occurrence of lichen substances obtained from LCMS of methanolic extract of *Stereocaulon pomiferum* (SAIF 7904)

Sl.No	Compound	Class	Mass spectrum	Also occurs in	References
1	Haemoventosin	Naphthaquinones	304, 302, 260	*Ophioparma ventosa*	ckand Yoshimura,1996:*NewYork*: 166
2.	Phlebic acid B	Terpenoids	458, 440, 415, 387	*Peltigera aphthosa*	Takahashi, *et. al.,* 1970
3	Methylpseudosalazinate	β-Orcinol Depsidones	402, 384, 369	*Pertusaria* sp.	Elix*, et. al.,* 1997
4.	Stictic acid	â-Orcinol Depsidones	386, 368, 193, 191	*Xanthoparmelia conspersa*	Culberson, 1969 *Chapel Hill*: 163
5.	Loxodin(Methylnorlobariate)	Orcinol Depsidones	456, 424	*Xanthoparmelia flavescentireagens*	Komiya and Kurokawa, S 1970
6.	Divaricatic acid	Orcinol Depsides	1,370, 193, 179	*Canoparmelia texana, Evernia divaricata*	Culberson, C F 1969 *Chapel Hill*: 115
7.	Fulgoicin	Orcinol â-Orcinol Depsidones	370, 368, 333, 325	*Fulgensia fulgida*	Mahandru and Tajbakhsh, 1983
8.	Oxyskyrin	Anthraquinones	596	*Trypetheliopsis*	Santesson, J 1970
9.	Lobaric acid	Orcinol Depsidones	456, 438, 412, 235	*Protoparmelia badia*	Culberson, 1969, Chapel Hill: 137
10	Fragilin	Anthraquinones	318, 284, 277, 275	*Nephroma laevigatum*	Culberson, 1969 Chapel Hill: 184
11.	Xanthorin(1,5,8-trihydroxy-6-methoxy-3-methylanthraquinone)	Anthraquinones	300, 282, 272, 260	*Xanthoria elegans*	Culberson, 1969, *Chapel Hill*: 191
12.	Norsolorinic acid	Anthraquinones	370, 352, 327, 299	*Solorina crocea*	Steglich and Jedtke, 1976
13.	2-*O*-Methylsekikaic acid	Orcinol Depsides	-1, 227, 224, 208	*Ramalina asahinae*	Chester, and Elix, 1978
14.	4-*O*-Methylconhypoprotocetraric acid	β-Orcinol Depsidones	-1, 278, 223, 205	*Xanthoparmelia competita*	Elix and Wardlaw, 2000
15.	Crustinic acid	Orcinol Tridepsides	-1, 301, 151	*Umbilicaria crustulosa*	Huneck *et. al.,*1993
16.	4-*O*-Methylolivetoric acid	Orcinol Depsides	1, 280, 262, 224	*Xanthoparmelia brattii*	Culberson, and Esslinger, 1976
17.	4-*O*-Demethylstenosporic acid	Orcinol Depsides	-1, 224, 206, 196	*Xanthoparmelia*	Culberson *et. al.,*1977

Table 4.74. List of names, classes, mass spectrum and occurrence of lichen substances obtained from LCMS Chromatogram of methanolic extract of *Usnea baileyi* 7904(SAIF)

SlNo	Compound	Class	Mass spectrum (nm)	Also occurs in	References
1.	Bonnic acid	Orcinol Depsides	416, 236, 224, 207	*Ramalina boninensis*	Culberson, 1969, *Chapel Hill*: 129
2.	Methyl pseudosalazinate	â-Orcinol Depsidones	402, 384, 369	*Pertusaria* sp.	Elix, *et. al.,* 1997
3.	6-*O*-Methylaverantin	Anthraquinones	368, 339, 325, 311	*Solorina crocea*	Steglich and Jedtke, 1976
4.	Usnic acid	Usnic acid derivatives	344, 260, 233, 217	*Usnea* sp.	Culberson, 1969 *Chapel Hill*: 170
5.	Stictic acid	â-Orcinol Depsidones	386, 368, 193, 191	Xanthoparmelia conspersa	Culberson, 1969 *Chapel Hill*: 163
6.	Constictic acid	â-Orcinol Depsidones	402, 384, 356, 193	*Xanthoparmelia conspersa*	Yosioka, *et. al.,* 1970.
7.	Eumitrin A1	Ergochromes	680, 621, 561, 501	Usnea baileyi	Yang,*et. al.,*1973

Table4.75. List of names, classes, mass spectrum and occurrence of lichen substances obtained from LCMS Chromatogram of methanolic extract of *Ramalina hossei* 7904(SAIF)

Sl. No	Compound	Class	Mass spectrum (nm)	Also occurs in	References
1.	Gyrophoric acid	Orcinol Tridepsides	-1,318, 168, 150	*Punctelia borreri*	Culberson, 1969 Chapel Hill: 114
2.	Calycin	Pulvinic acid derivatives	306, 250, 161, 153	*Candelariella* spp. and *Pseudocyphellaria aurata*	Culberson, Chapel Hill: 210
3.	2-Chlorolichexanthon	Xanthones	322, 321, 320, 319	*Pertusaria cicatricosa*	Elix, *et. al.,* 1978
4.	Coronatoquinone	Naphthaquinone	320, 318, 303, 302	*Pseudocyphellaria coronata*	Ernst *et. al,* 2000
5.	Pulvinic dilactone [Pulvinic acid lactone]	Pulvinic acid derivatives	290, 261, 234, 178	*Pseudocyphellaria crocata*	Culberson, 1969 Chapel Hill: 214
6.	(-)-Dihydropertusaric acid	Aliphatic acids	368, 353, 326, 293	*Pertusaria albescens*	Huneck, *et. al* 1986
7.	20,24-Epoxydammarane-3â,12â,25-triol	Terpenoids	-1, 463, 417, 400, 381	*Pyxine endochrysina*	Yosioka, *et. al.,* 1972
8.	Methyl haematommate	Monocyclic aromatic derivatives	210, 179, 178	*Stereocaulon ramulosum*	Hickey *et. al.,* 1990

Lichen substances are secondary metabolites of lichens, like Dibenzofurans, Depsidones, Xanthones and Terpene derivatives (Kosanic *et. al.*, 2013). The LCMS chromatogram of studied lichen *Everniastrum* sp revealed the presence of mainly Orcinol Depsidones and Depside compounds with some Aliphatic acids, Xanthones and Usnic acid derivatives. The compounds present are namely Hypostitic acid, Salazinic acid, Norstictic acid, Eriodermin, Nopannarin, Lobaric acid, Alectoronic acid, Protocetraric acid, Conloxodin, Chloroatanorin, Erythrin, Decarboxyperlatolic acid, 2'-O-Methylnobarbatic acid, Caperatic acid, Fern-9(11)-ene-3,12-dione, Ursolic acid, Lanosterol, Thiomelin, Usnic acid, Placodiolic acid and Usnic acid Gyrophoric acid.

The methanolic extract of *S. pomiferum* revealed the presence of mainly β-Orcinol Depsidones and Orcinol depsides classes of compounds some Terpenoids, Anthraquinones, Orcinol Tripepsides and Napthaquinones. The name of compounds identified are as follows Methylpseudosalazinate, Lobaric acid, Fulgoicin, Loxodin, 4-O-Methylconhypoprotocetraric acid, Constictic acid, Stictic acid, Eumitrin A1, Phlebic acid B, Haemoventosin, Oxyskyrin, Fragilin, Norsolorinic acid, Xanthorin, Norsolorinic acid, Divaricatic acid, 2-O-Methylsekikaic acid, 4-O-Methylolivetoric acid, 4-O-Demethylstenosporic acid, Crustinic acid and Haemoventosin.

The presence of Stictic acid, Constictic acid, Methyl pseudosalazinate, Eumitrin A1, Usnic acid, 6-O-Methylaverantin and Bonnic acid could be observed from LCMS peaks obtained from methanolic extract of *U. baileyi.*

Different classes of compounds namely Terpenoids, Pyxinol, Pulvinic acid derivatives Xanthones, Naphthaquinone, Pulvinic acid derivatives, Aliphatic acids, Orcinol Tridepsides could be identified from chromatograms of methanolic extract of *R. hossei.* The compounds identified are namely Gyrophoric acid, 24-Epoxydammarane-3β, 12β, 25-triol[Pyxinol], Coronatoquinone, 2-Chlorolichexanthone, Calycin, 2-Chlorolichexanthone, Coronatoquinone, ,(-)-Dihydropertusaric acid, (-)-Dihydropertusaric acid and Methyl haematommate

Discussion

The lichen substances comprise amino acid derivatives, sugar alcohols, aliphatic acids, macrocyclic lactones, mono-cyclic aromatic compounds, quinones, chromones, xanthones, dibenzofuranes, depsides, depsidones, depsones, terpenoids, steroids, carotenoids and diphenyl ethers

(Clix *et. al.*, 1984). Further lichen substances like aliphatic, cycloaliphatic, aromatic and terpenic components with relatively low molecular weight crystallized on the hyphal cell walls (Chand *et. al.,* 2009). Lichens and their metabolites have many biological activities: antiviral (Esimone *et. al.,* 2007), antibiotic (Rankovic *et. al.,* 2010), antitumor (Manojlovic *et. al.,* 2010), antiherbivore (Lawrey, 1983), ecological roles (Richardson, 1992) and enzyme inhibitory (Romagni *et. al.,* 2000). The methanolic extract of all lichen specimens was sent for identification of active principle components by LCMS analysis, as among different solvent extracts the methanolic extract are usually most effective against microorganisms (Mukherjee *et. al.,* 1997; Otzurk *et. al.,* 1999)

Numerous lichen substances have been recovered from the methanolic extracts of lichen studied, its major role are its UV screening for protection of photobiont cells (especially cortical pigments), it also provides protection from predation by arthropods, snails and other animals. The lichen substances cause membrane permeability effects to facilitate release of carbohydrates from photobiont cells and antimicrobial effects to protect thallus from decomposition.

From our study it could be seen that methanolic extract of *Everniastrum* sp and *Ramalina hossei* showed the presence of xanthones. Xanthones occur in many species namely *Lecanora, Pertusaria, Melanaria, Lecidea* and *Buellia*, lichen xanthones contain larger amount of chlorinated substituent suggesting the availability of chloride in environment may affect the production of these compounds.

Secondly lichen compounds occurring as phenolics with carbonyl as functional groups play an important role in withering of rocks due to complex metal ions which in turn leads to soil formation.

Thirdly the various biological activities of lichen compounds also help in colonization of terrestrial areas as these compounds have been used by man during ancient Chinese and Egyptian evolution (Karunaratne *et. al.*, 2005). Our study has made an attempt to show various secondary metabolites present in lichens. Secondary metabolites are products of polyketide pathway, mainly monocyclic and or bicyclic phenols joined by an ester bond (depsides), both ester and ether bonds (depsidones) and fur

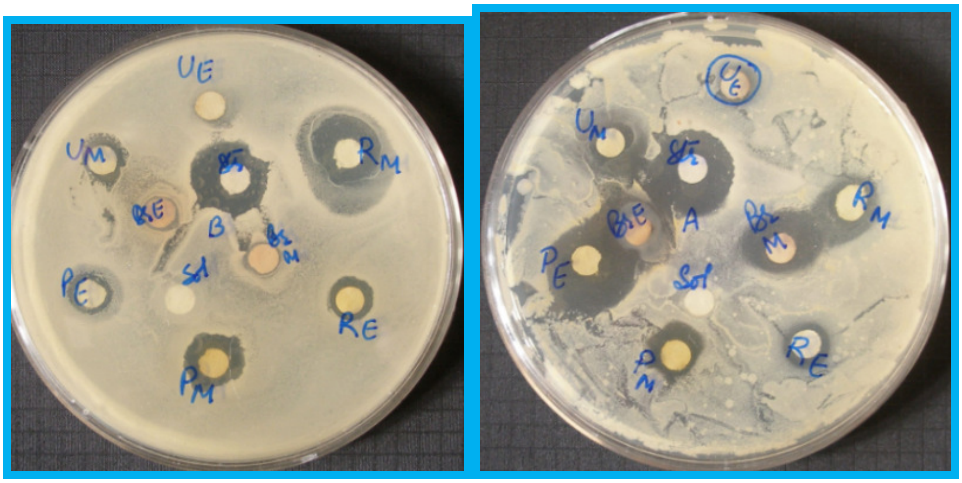


Plate 3.3 Plate 3.4

Plate 3.5 Plate 3.6

Plate 3.3. Inhibition zone produced by USRE (UE), USRM (UM), RARE (RE), RARM(RM), BERE (BRE), BERM (BRM), PARE(PE), PARM (PM), STREPTOMYCIN (STR),Sterile distilled water (Sol) against *B. subtilis*

Plate 3.4. Inhibition zone produced by USRE (UE), USRM (UM), RARE (RE), RARM(RM), BERE (BRE), BERM (BRM), PARE(PE), PARM (PM), STREPTOMYCIN (STR), Sterile distilled water (Sol) against *A. faecalis*

Plate 3.5. Inhibition zone produced by USRE (UE), USRM (UM), RARE(RE), RARM(RM), BERE (BRE), BERM (BRM), PARE(PE), PARM (PM), STREPTOMYCIN (STR), Sterile distilled water (Sol) against *S. aureus*

Plate 3.6. Inhibition zone produced by USRE (UE), USRM (UM), RARE(RE), RARM(RM), BERE (BRE), BERM (BRM), PARE(PE), PARM (PM), STREPTOMYCIN (STR), Sterile distilled water (Sol), against *P. aeruginosa*

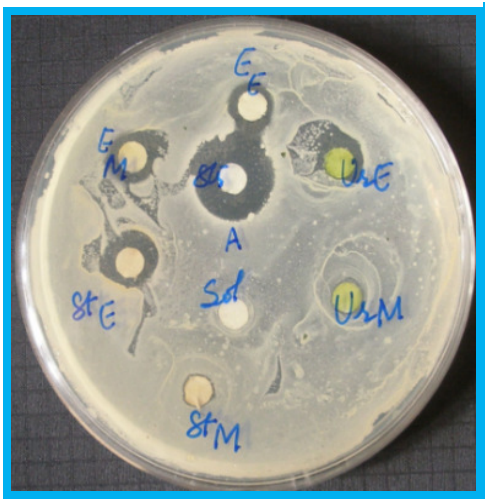


Plate 3.7

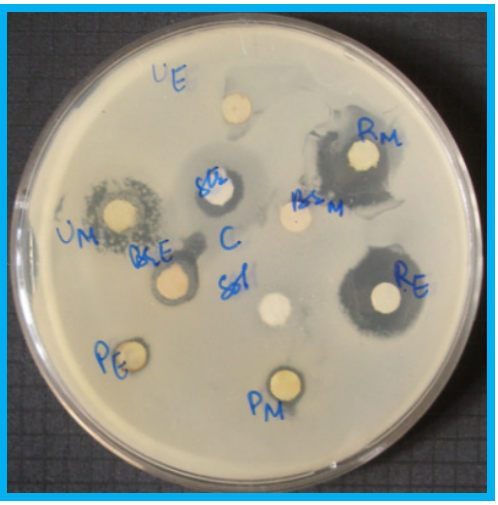


Plate 3.8

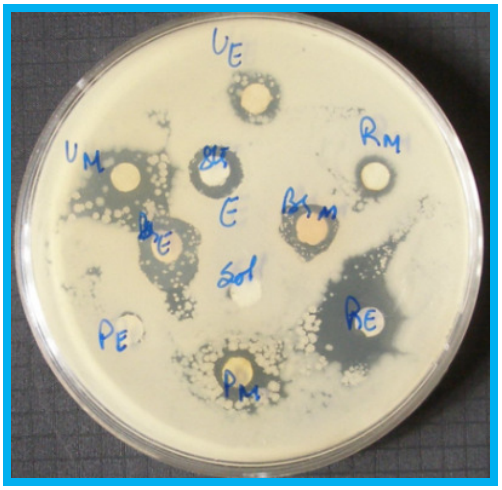


Plate 3.9

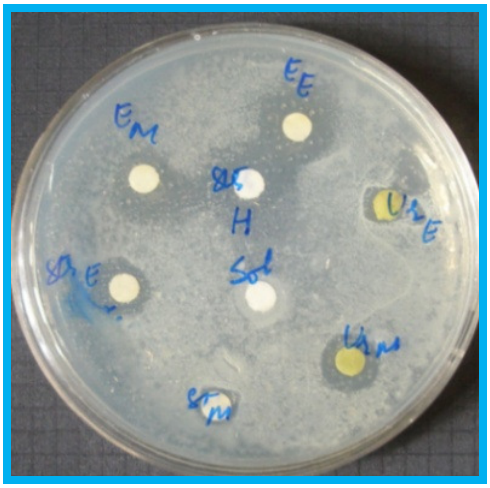


Plate 3.10

Plate 3.7. Inhibition zone produced by EVRE(EE), EVRM(EM),URRE(URRE), URRM(UM), STRE(STE), STRM(STM), STREPTOMYCIN(STR), Sterile distilled water (Sol) against *A. faecalis*

Plate 3.8. Inhibition zone produced by USRE (UE), USRM (UM), RARE (RE), RARM(RM), BERE (BRE), BERM (BRM), PARE(PE), PARM (PM), STREPTOMYCIN (STR) Sterile distilled water (Sol) against *C. albicans.*

Plate 3.9. Inhibition zone produced by USRE (UE), USRM (UM), RARE(RE), RARM(RM), BERE (BRE), BERM (BRM), PARE(PE), PARM (PM), STREPTOMYCIN (STR,) Sterile distilled water (Sol), against *E. coli*

Plate 3.10. Inhibition zone produced by EVRE(EE), EVRM(EM),URRE(URRE), URRM(UM), STRE(STE), STRM(STM), STREPTOMYCIN (STR) Sterile distilled water (Sol), against *B. megaterium*

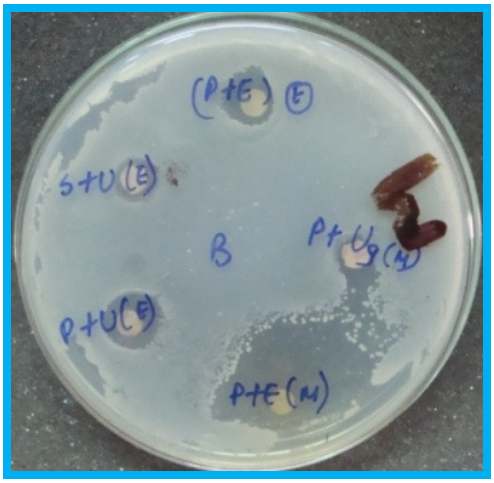


Plate 3.11

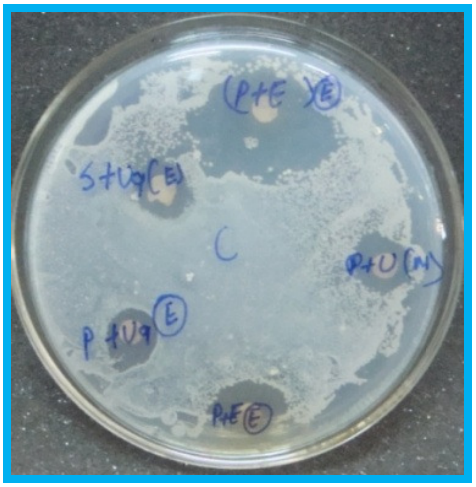


Plate 3.12

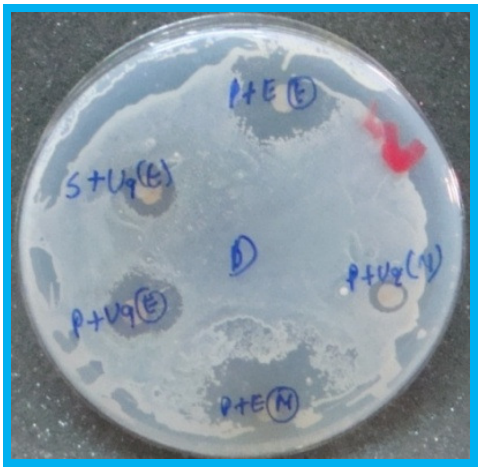


Plate3.13

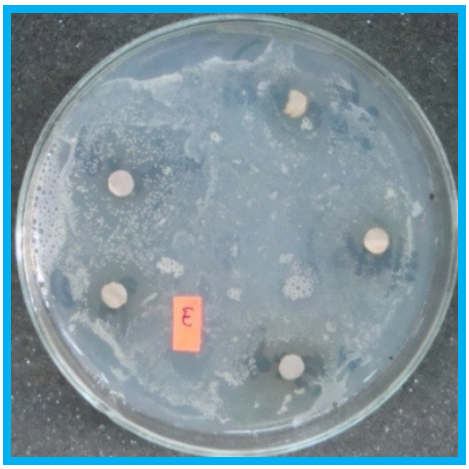


Plate 3.14

Plate 3.11. Nutrient agar spread plates of *B. subtilis* showing Inhibition zone with respect to combined extract of *Panax pseudoginseng* and *Everniastrum* sp (P+E), P. *pseudoginseng* and *U. baileyi* (P+U), *S. mukrossi* and *U. baileyi* (S+U) extracts .[E= Ethanolic ,M= methanolic].

Plate 3.12. Nutrient agar spread plates of *C. albicans showing* Inhibition zone with respect to combined extract of *Panax pseudoginseng* and *Everniastrum* sp (P+E), P. *pseudoginseng* and *U. baileyi* (P+U), *S. mukrossi* and *U. baileyi* (S+U) extracts .[E= Ethanolic ,M= methanolic].

Plate 3.13. Nutrient agar spread plates of *P. aeruginosa* showing Inhibition zones with respect to combined extract of *Panax pseudoginseng* and *Everniastrum* sp (P+E), *P. pseudoginseng* and *U. baileyi* (P+U), *S. mukrossi* and *U. baileyi* (S+U) extracts [E= Ethanolic, M= methanolic]

Plate 3.14. Nutrient agar spread plates of *E. coli* showing Inhibition zones with respect to combined extract of *Panax pseudoginseng* and *Everniastrum* sp (P+E), *P. pseudoginseng* and *U. baileyi* (P+U), *S. mukrossi* and *U. baileyi* (S+U) extracts [E= Ethanolic, M= methanolic]

Plate 3.15

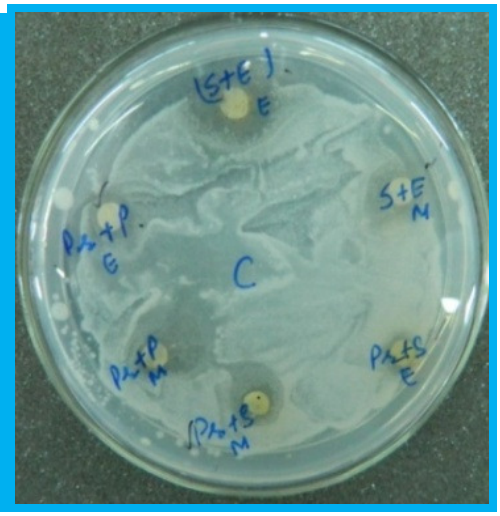


Plate 3.16

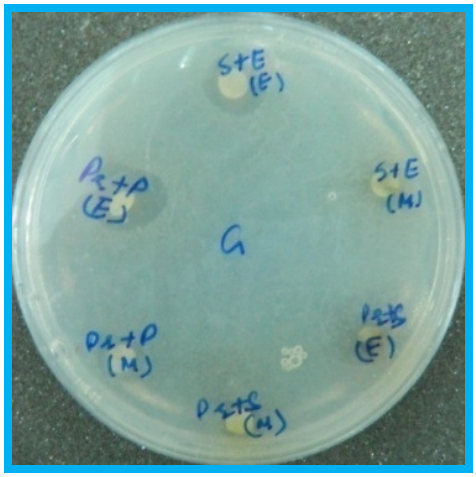


Plate 3.17

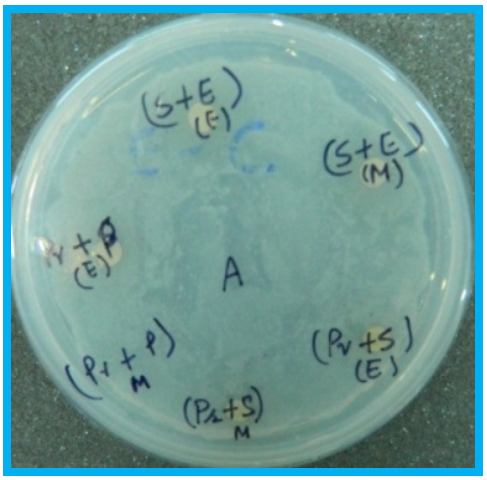


Plate 3.18

Plate 3.15. Nutrient agar spread plates of *A. faecalis* showing Inhibition zones with respect to combined extract of *Panax pseudoginseng* and *Everniastrum* sp (P+E), *P. pseudoginseng* and *U. baileyi* (P+U), *S. mukrossi* and *U. baileyi* (S+U) extracts [E= Ethanolic, M= methanolic]

Plate 3.16. Nutrient agar spread plates of *C. albicans* showing Inhibition zones with respect to combined extract of *S. mukrossi* and *Everniastrum* sp (S+E), *P. reticulatum* and *S. mukrossi*(Pr+S), *P. reticulatum and P. pseudoginseng* (Pr+P) extracts [E= Ethanolic, M= methanolic

Plate 3.17. Nutrient agar spread plates of *B. megaterium* showing Inhibition zones with respect to combined extract of *S. mukrossi* and *Everniastrum* sp (S+E), *P. reticulatum* and *S. mukrossi*(Pr+S), *P. reticulatum and P. pseudoginseng* (Pr+P) extracts [E= Ethanolic, M= methanolic]

Plate 3.18. Nutrient agar spread plates of *A. faecalis* showing Inhibition zones with respect to combined extract of *S. mukrossi* and *Everniastrum* sp (S+E), *P. reticulatum* and *S. mukrossi*(Pr+S), *P. reticulatum and P. pseudoginseng* (Pr+P) extracts [E= Ethanolic, M= methanolic]

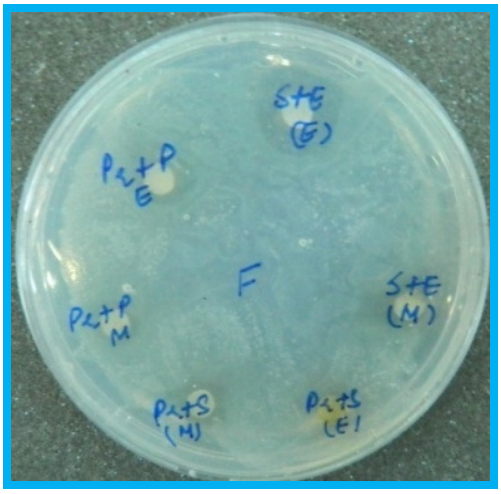


Plate 3.19

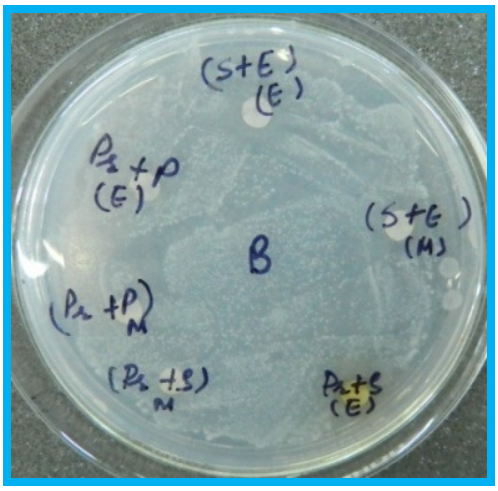


Plate 3.20

Plate 3.19. Nutrient agar spread plates of *S. aureus* showing Inhibition zones with respect to combined extract of *S. mukrossi* and *Everniastrum* sp (S+E), *P. reticulatum* and *S. mukrossi*(Pr+S), *P. reticulatum and P. pseudoginseng* (Pr+P) extracts [E= Ethanolic, M= methanolict]

Plate 3.20 Nutrient agar spread plates of *B. subtilis* showing Inhibition zones with respect to combined extract of *S. mukrossi* and *Everniastrum* sp (S+E), *P. reticulatum* and *S. mukrossi*(Pr+S), *P. reticulatum and P. pseudoginseng* (Pr+P) extracts [E= Ethanolic, M= methanolic]

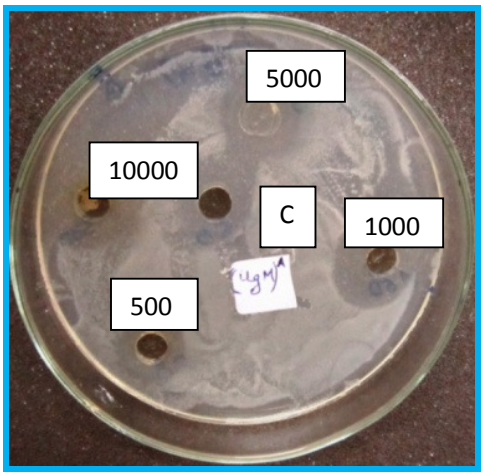


Plate 3.21

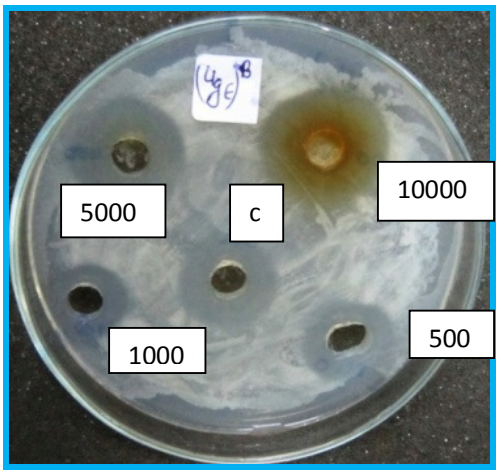


Plate 3.22

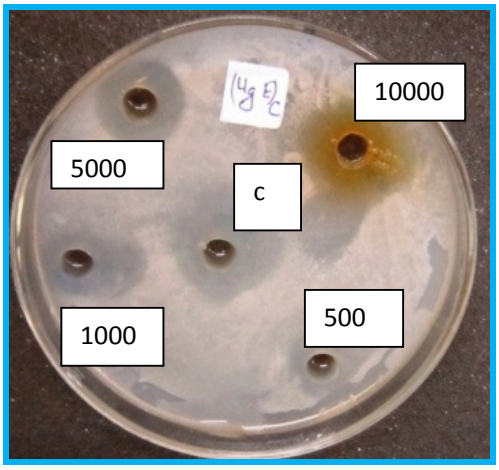


Plate 3.23

* The values given by the side of inhibition zone corresponds to the extracts concentration(µg/ml)

Plate 3.21. Nutrient agar spread plates of *A. faecalis* showing inhibition zones with respect to methanolic extract *U. baileyi* during MIC value determination

Plate 3.22.Nutrient agar spread plates of *B. subtilis* showing inhibition zones with respect to ethanolic extract *U. baileyi* during MIC value determination

Plate 3.23.Nutrient agar spread plates of *C. albicans* showing inhibition zones with respect to ethanolic extract *U. baileyi* during MIC value determination

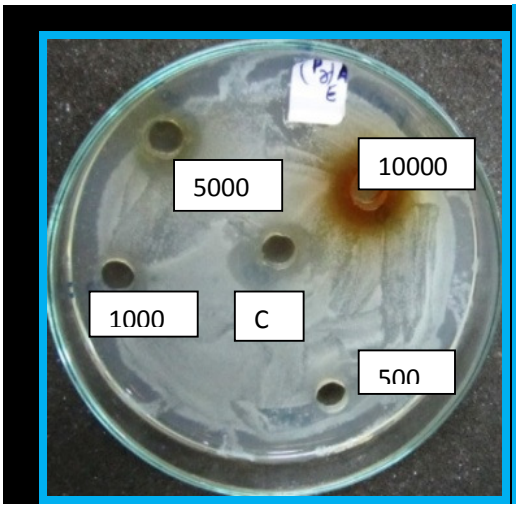


Plate 3.24

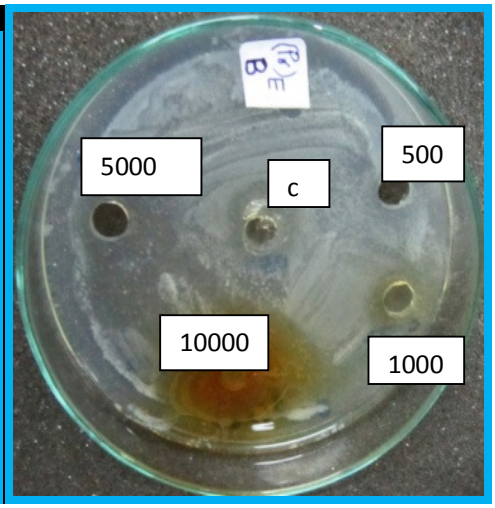


Plate 3.25

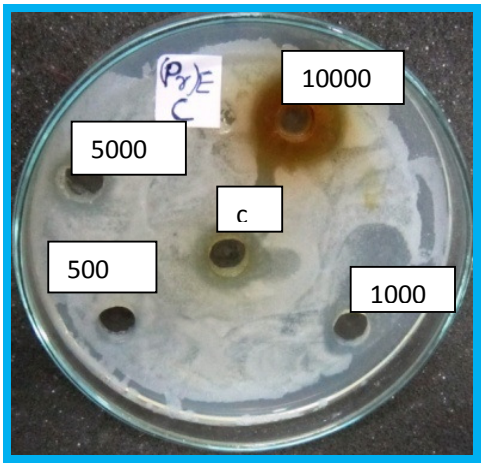


Plate 3.26

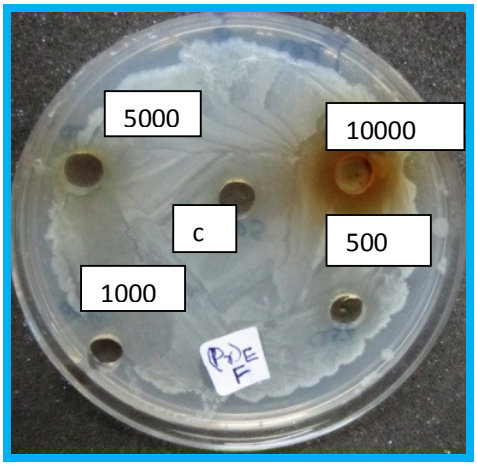


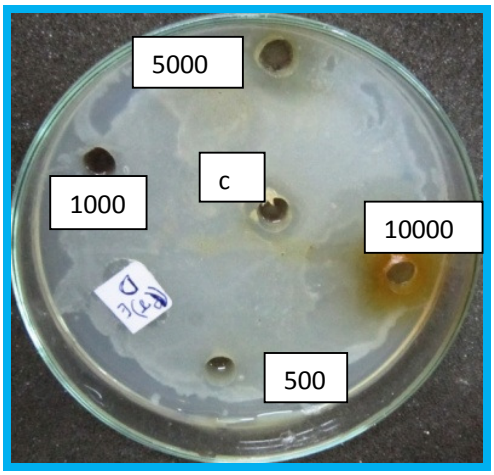
Plate 3.27

Plate 3.24.Nutrient agar spread plates of *A. faecalis* showing inhibition zones with respect to ethanolic extract *P. reticulatum* during MIC value determination

Plate 3.25.Nutrient agar spread plates of *B. subtilis* showing inhibition zones with respect to ethanolic extract *P. reticulatum* during MIC value determination

Plate 3.26.Nutrient agar spread plates of *C. albicans* showing inhibition zones with respect to ethanolic extract *P. reticulatum* during MIC value determination

Plate 3.27.Nutrient agar spread plates of *S. aureus* showing inhibition zones with respect to ethanolic extract *P. reticulatum* during MIC value determination



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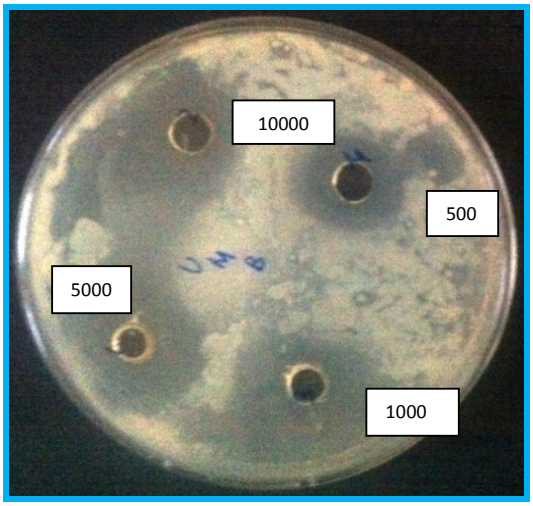


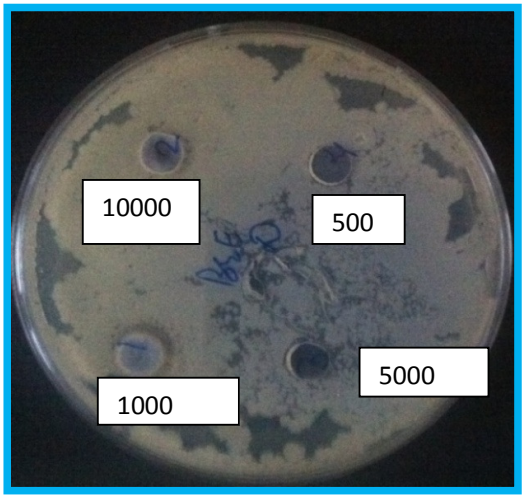


Plate 3.29

Plate 3.30

Plate 3. 28.Nutrient agar spread plates of *P. aeruginosa* showing inhibition zones with respect to ethanolic extract *P. reticulatum* during MIC value determination

Plate 3.29. Nutrient agar spread plates of *B. subtilis* showing inhibition zones with respect to methanolic extract *U. baileyi* during MIC value determination

Plate 3.30. Nutrient agar spread plates of *P. aeruginosa* showing inhibition zones with respect to ethanolic extract *B. ciliata* during MIC value determination

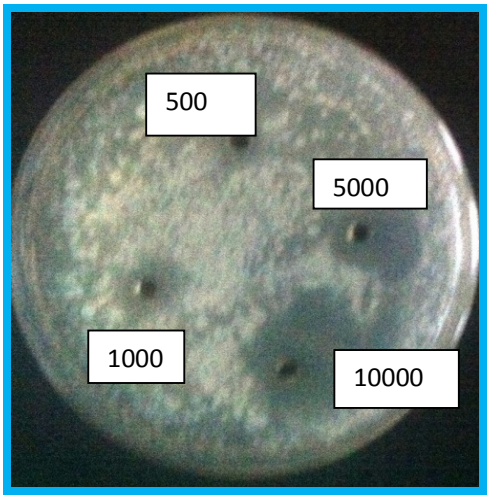


Plate 3.31

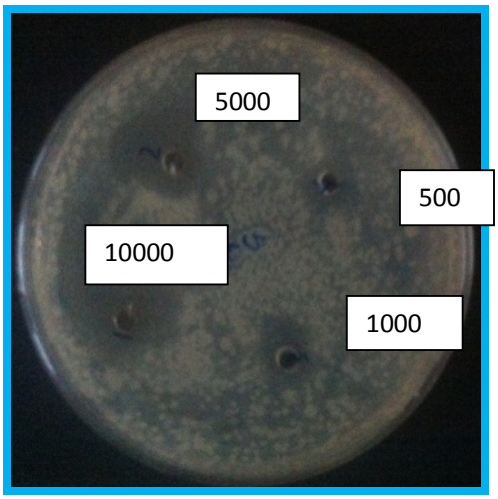


Plate 3.32

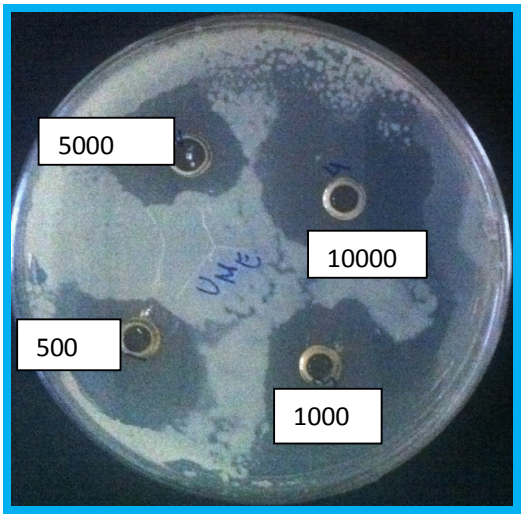


Plate 3.33

Plate 3.31.Nutrient agar spread plates of *E. aerogenes* showing inhibition zones with respect to methanolic extract *U. baileyi* during MIC value determination

Plate 3.32.Nutrient agar spread plates of *B. megaterium* showing Inhibition zones with respect to methanolic extract *U. baileyi* during MIC value determination

Plate 3.33.Nutrient agar spread plates of *E. coli* showing inhibition zones with respect to methanolic extract *U. baileyi* during MIC value determination

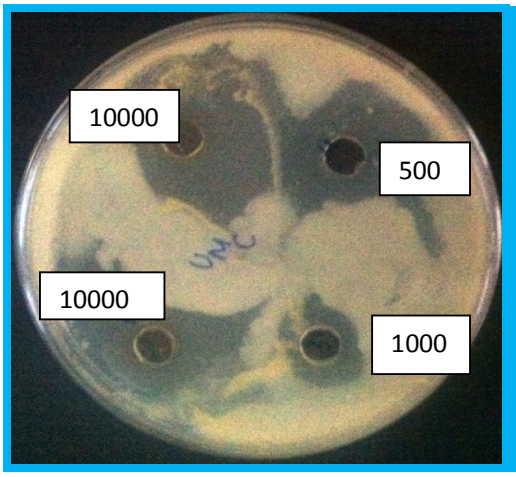


Plate 3.34

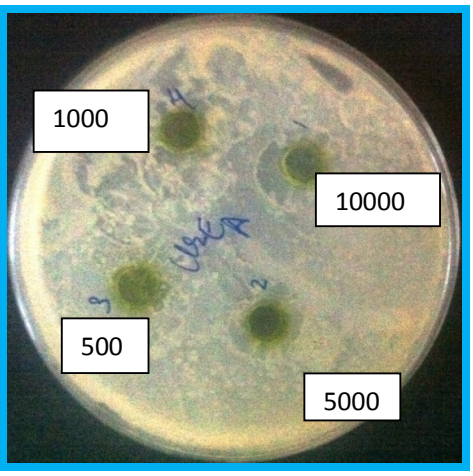


Plate 3.35

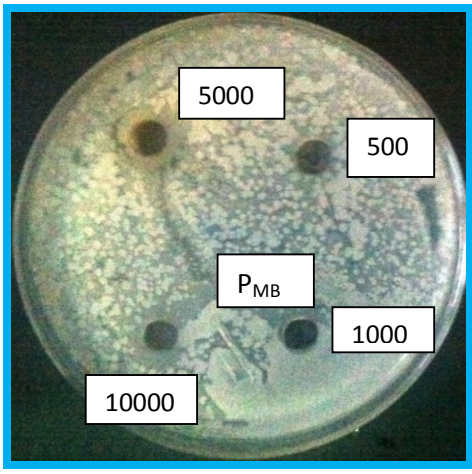


Plate 3.36

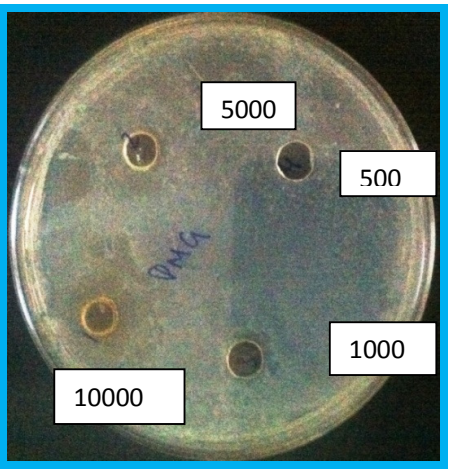


Plate 3.37

Plate 3.34.Nutrient agar spread plates of *C. albicans* showing inhibition zones with respect to methanolic extract *U. baileyi* during MIC value determination

Plate 3.35.Nutrient agar spread plates of *A. faecalis* showing inhibition zones with respect to ethanolic extract *U. dioica* during MIC value determination

Plate 3.36. Nutrient agar spread plates of *B. subtilis* showing inhibition zones with respect to methanolic extract *P. reticulatum* during MIC value determination

Plate 3.37. Nutrient agar spread plates of *B. megaterium* showing inhibition zones with respect to ethanolic extract *P. reticulatum* during MIC value determination

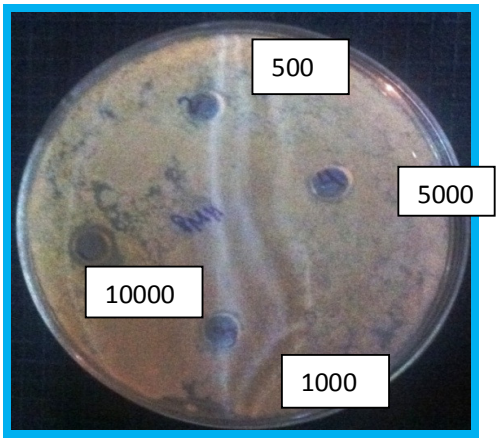


Plate 3.38

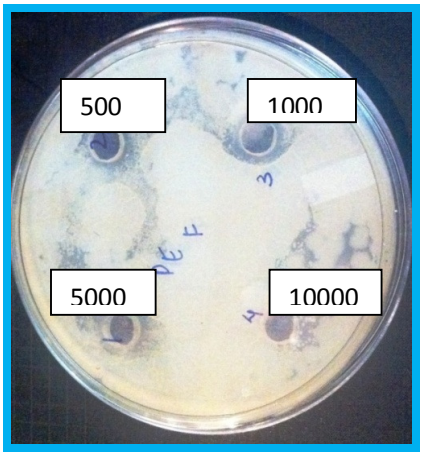


Plate 3.39

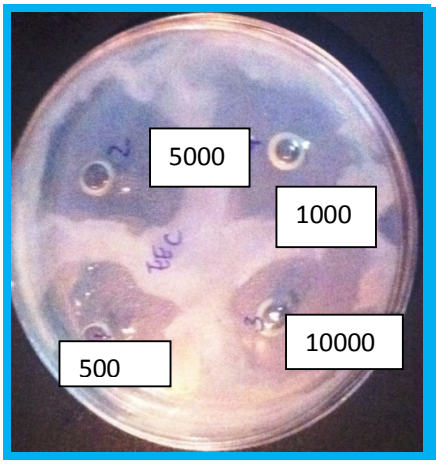


Plate 3.40

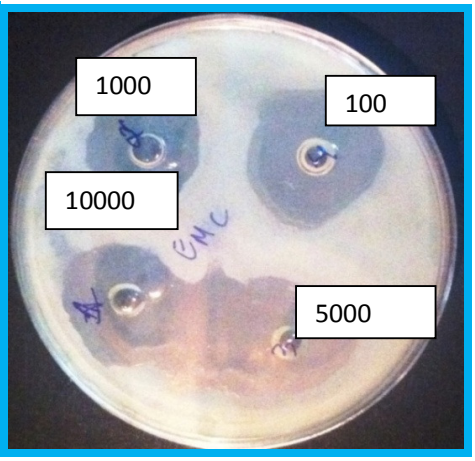


Plate 3.41

Plate 3.38 Nutrient agar spread plates of *E. aerogenes* showing inhibition zones with respect to methanolic extract *P. reticulatum* during MIC value determination

Plate 3.39 Nutrient agar spread plates of *S.aureus* showing inhibition zones with respect to ethanolic extract *P. reticulatum* during MIC value determination

Plate 3.40 Nutrient agar spread plates of *C. albicans* showing inhibition zones with respect to ethanolic extract *Everniastrum* sp during MIC value determination

Plate 3.41 Nutrient agar spread plates of *C. albicans* showing inhibition zones with respect to methanolic extract *Everniastrum* sp during MIC value determination

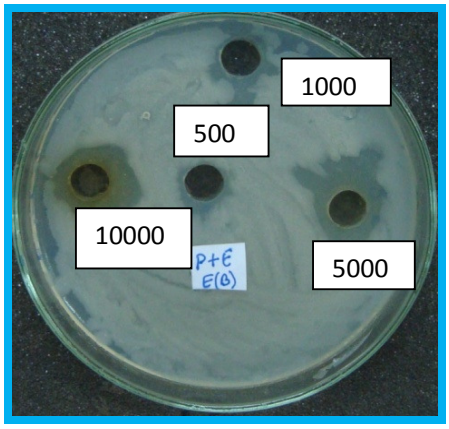


Plate 3.42

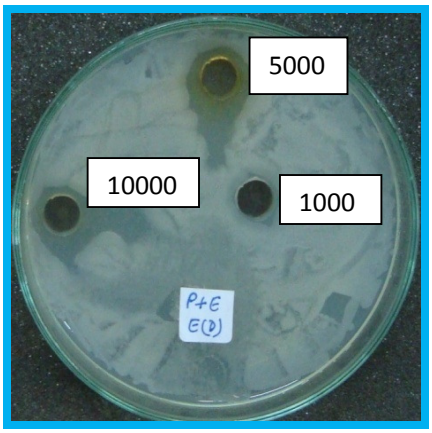


Plate 3.43

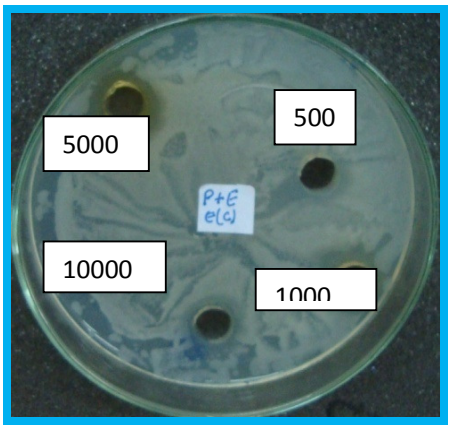


Plate 3.44

Plate 3.42.Nutrient agar spread plates of *B. subtilis* showing inhibition zones with respect to ethanolic extract *P. pseudoginseng* and *Everniastrum* sp during MIC value determination

Plate 3.43. Nutrient agar spread plates of *P. aeruginosa* showing inhibition zones with respect to ethanolic extract *P. pseudoginseng* and *Everniastrum* sp during MIC value determination

Plate 3.44. Nutrient agar spread plates of *C. albicans* showing inhibition zones with respect to ethanolic extract *P. pseudoginseng* and *Everniastrum* sp during MIC value determination

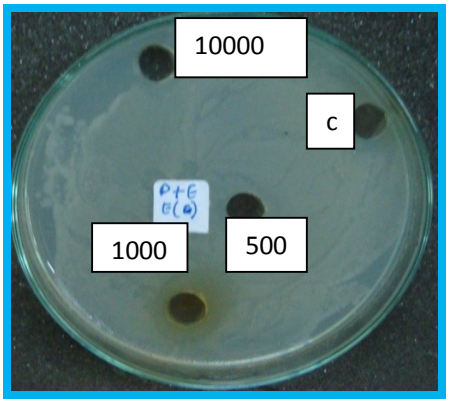


Plate 3.45

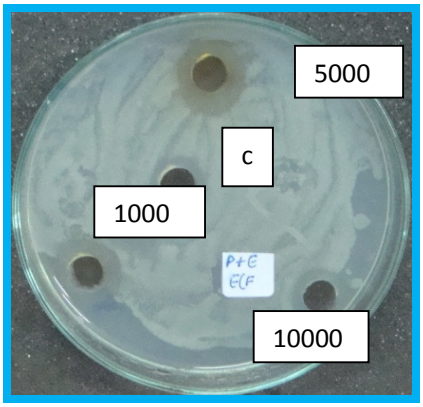


Plate 3.46

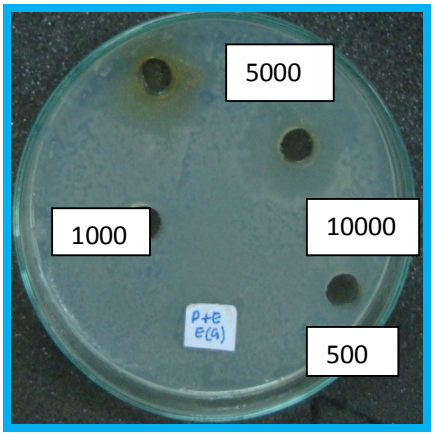


Plate 3.47

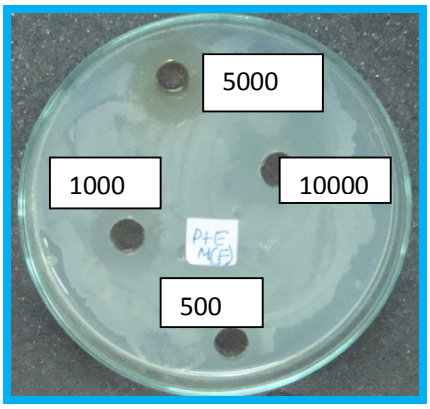


Plate 3.48

Plate 3.45. Nutrient agar spread plates of *B. megaterium* showing inhibition zones with respect to ethanolic extract *P. pseudoginseng* and *Everniastrum* sp during MIC value determination

Plate 3.46. Nutrient agar spread plates of *S. aureus* showing inhibition zones with respect to ethanolic extract *P. pseudoginseng* and *Everniastrum* sp during MIC value determination

Plate 3.47. Nutrient agar spread plates of *B. megaterium* showing inhibition zones with respect to methanolic extract *P. pseudoginseng* and *Everniastrum* sp against during MIC value determination

Plate 3.48.Nutrient agar spread plates of *S. aureus* showing inhibition zones with respect to methanolic extract *P. pseudoginseng* and *Everniastrum* sp during MIC value determination

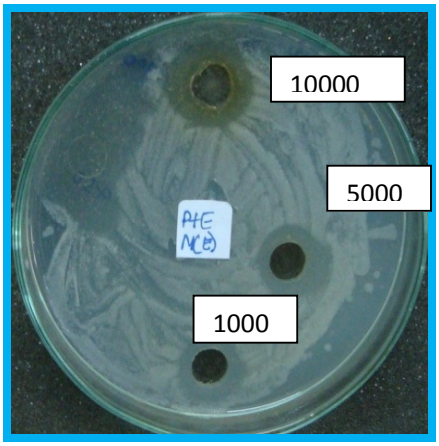


Plate 3.49

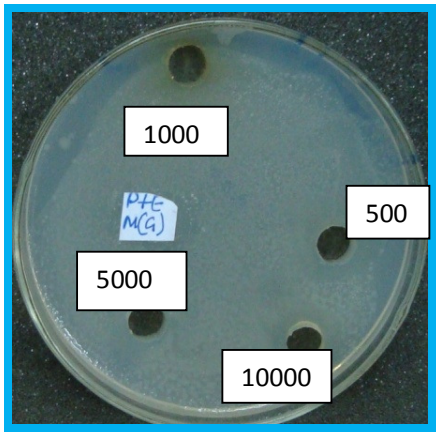


Plate 3.50

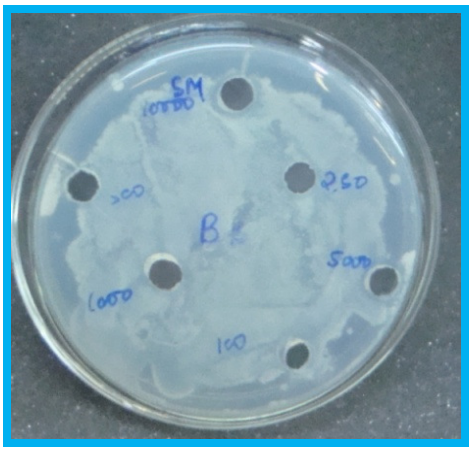


Plate 3.51

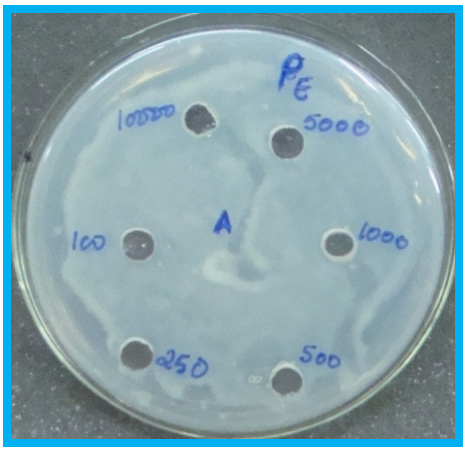


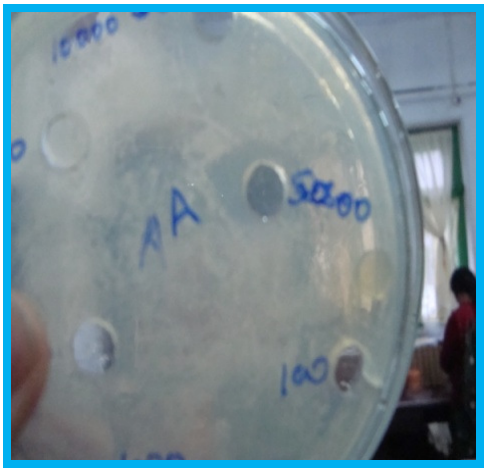
Plate 3.52

Plate 3.49.Nutrient agar spread plates of *E. coli* showing inhibition zones with respect to methanolic extract *P. pseudoginseng* and *Everniastrum* sp during MIC value determination

Plate 3.50.Nutrient agar spread plates of *B. megaterium* showing Inhibition zones with respect to methanolic extract *P. pseudoginseng* and *Everniastrum* sp during MIC value determination

Plate 3.51. Nutrient agar spread plates of *B. subtilis* showing inhibition zones with respect to methanolic extract *S. mukrossi* during MIC value determination

Plate 3.52.Nutrient agar spread plates of *A. faecalis* showing inhibition zones with respect to ethanolic extract *P. pseudoginseng* during MIC value determination



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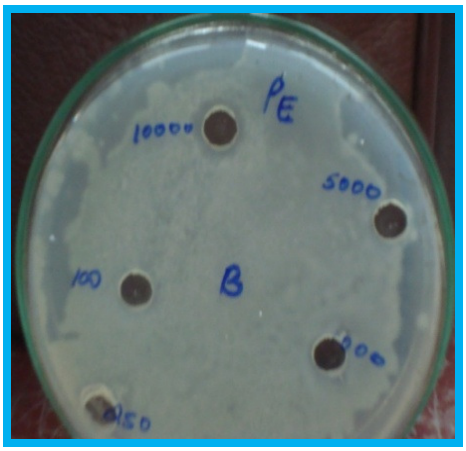


Plate 3.54

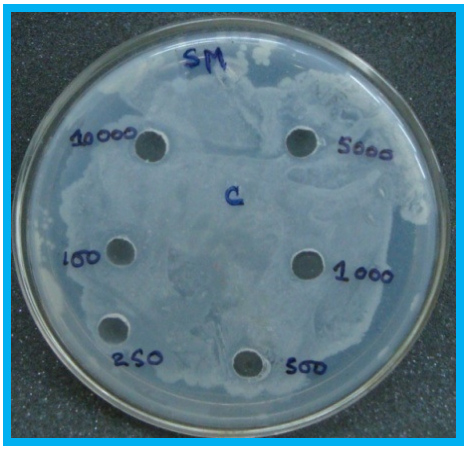


Plate 3.55

Plate 3.53.Nutrient agar spread plates of *A. faecalis* showing inhibition zones with respect to methanolic extract *U. dioica* during MIC value determination

Plate 3.54.Nutrient agar spread plates of *B. subtilis* showing inhibition zones with respect to ethanolic extract *P. pseudoginseng* during MIC value determination

Plate 3.55. Nutrient agar spread plates of *C. albicans* showing inhibition zones with respect to methanolic extract *S. mukrossi* during MIC value determination

heterocycle (dibenzofurans). Numerous depsidones has been identified from our lichens under study. The depsides and depsidones are also said to be reported to have defensive function in lichens. Large amounts of phenolic, fungal melanins are synthesized and accumulated in the thallus in order to absorb UVB light and shelter the photobiont from excessive radiation (Gauslaa and Solhaug, 2001). These metabolites commonly referred to as photoprotecters have great antioxidant capacity (Hidalgo *et. al.*, 1994, Fernandez *et. al.*, 1996) and can be used as preservatives in cosmetic products (Muller, 2001). Some of lichen metabolites are involved in maintaining of the symbiotic equilibrium (Huneck, 1999), while others dissolved rocks for better attachment of lichens (Seaward, 1997).

Studied lichen *Everniastrum* sp, *Stereocaulon pomiferum*, *Usnea baileyi* revealed the presence of salazinic acid, stictic acid and usnic acid respectively such compounds as reported by Paz *et. al.,* (2010) protected human astrocytes from hydrogen peroxide induce damage. Such compound could also act as antioxidant agents in the neurodegenerative disorders associated with oxidative damage (e.g. Alzheimer's disease and Parkinson's disease).

It has been studied earlier that the salazinic acid constituent and *Parmelia sulcata* showed antimicrobial property against food borne bacteria and fungi (Candan *et. al.*, 2007), hence salazinic acid identified from methanolic extract of *Everniastrum* sp may also possess such antimicrobial ability. Similarly usnic acid was obtained from methanolic extract of *Everniastrum* sp and methanolic extract of *Usnea baileyi* which is known to possess antiprotozoal, antiviral, antiproliferative, anti-inflammatory, analgesic, antipyretic and antitumour activities (Cocchietto *et. al.,* 2002; Ingolfsdpttir, 2002).

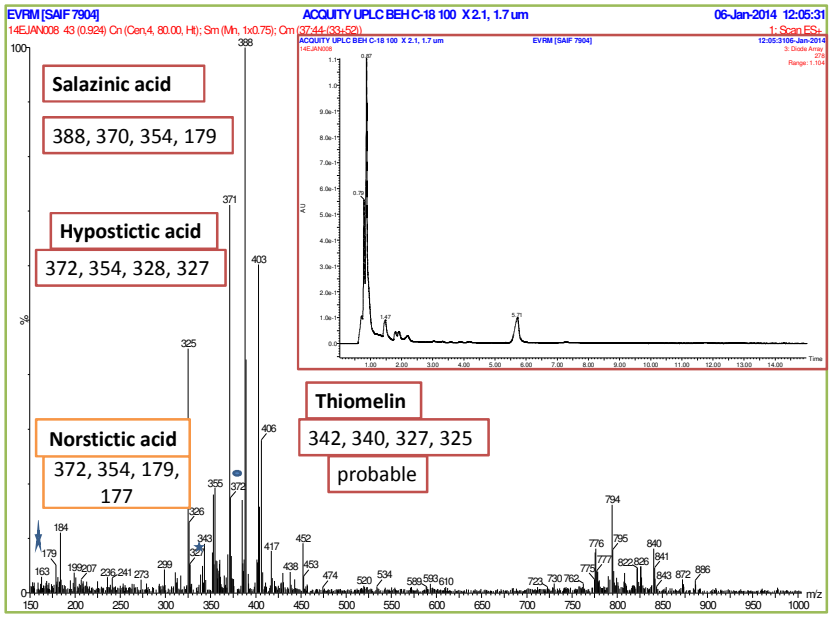
Norstictic acid from *Lobaria pulmonaria* and usnic acid, salazinic acid obtained from *Usnea filipendula* have been reported to possess antibiotic property (Crockett, 2003), these acids were also obtained in this present assay from methanolic extract of *Everniastrum* sp.

Gyrophoric acid from *Xanthoparmelia pokornyi* proved to be potent antimicrobials (Candan, 2006), this acid was also obtained in our LC-MS profile of methanolic extract of *Everniastrum* sp and methanolic extract of *Ramalina hossei* which might prove to be good antimicrobials.

Anthraquinones such as Oxyskyrin, Fragilin, Xanthorin, Norsolorinic acid was obtained from lichen *Stereocaulon pomiferum*, and also studied earlier (Schnazi *et. al.,* 1990 and Sydiskis *et. al.,* 1991) anthraquinones possessed antiviral properties. The above mentioned anthraquinones has also been identified in this work from methanolic extract of *Stereocaulon pomiferum* which may also exhibit such property.

Stictic acid isolated from *Lobaria pulmonaria* resulted moderate anticancer activity, and this compound could used as a lead compound for desgining of novel human colon adenocarcinoma drugs (Pejin *et. al.*, 2013), existence of stictic acid was also identified in methanolic extract of *Usnea baileyi.*

Occurrence of different group of phytochemicals in lichen samples may be exploited for the development of widely acceptable agents to combat disorders without any side effects.



LCMS spectral peaks of EVRM

Fig. 4.52a LCMS spectral peaks and respective compounds of methanolic extract of *Everniastrum* sp

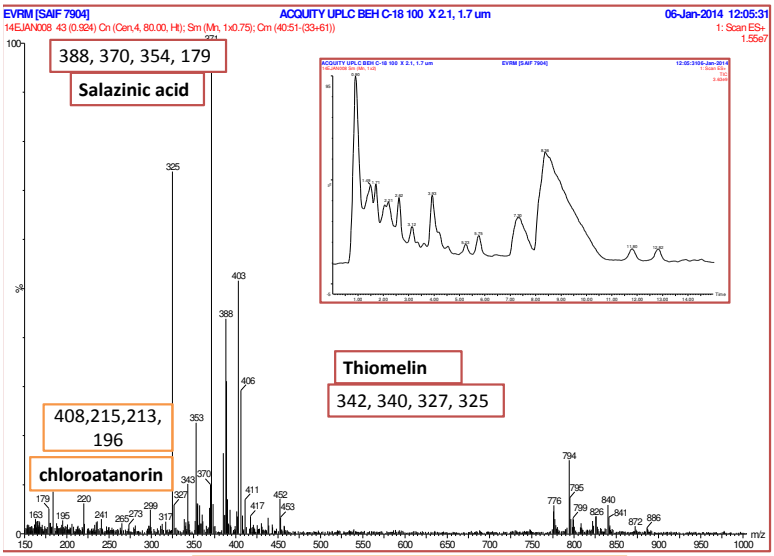


Fig . 4.52b LCMS spectral peaks and respective compounds of methanolic extract *Everniastrum* sp

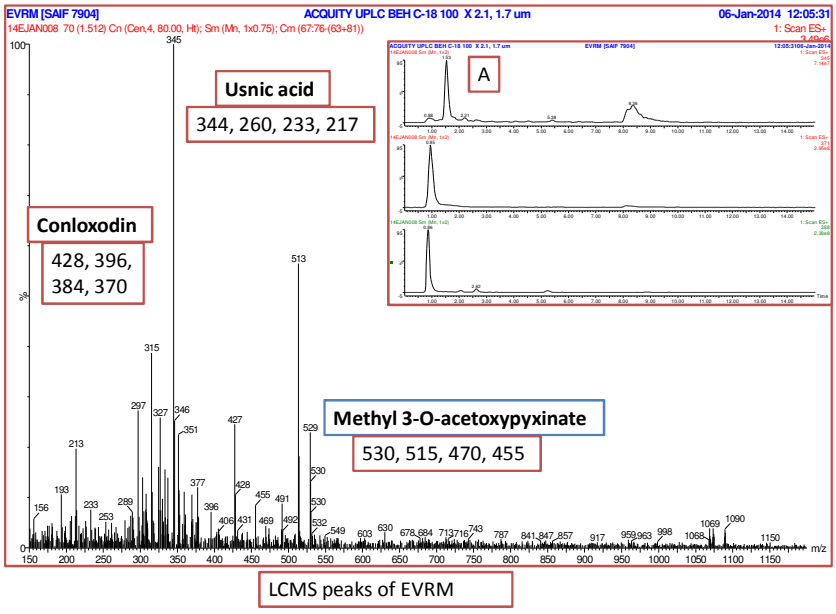
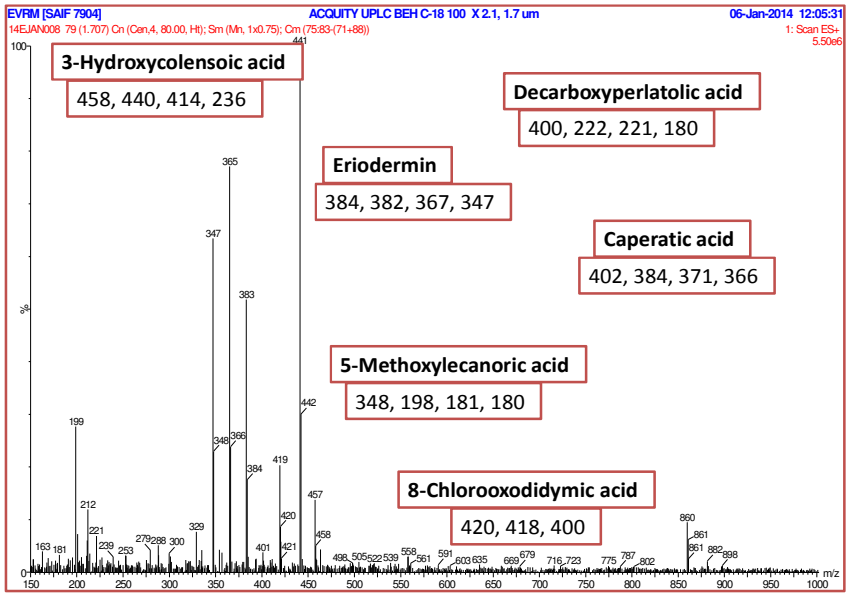
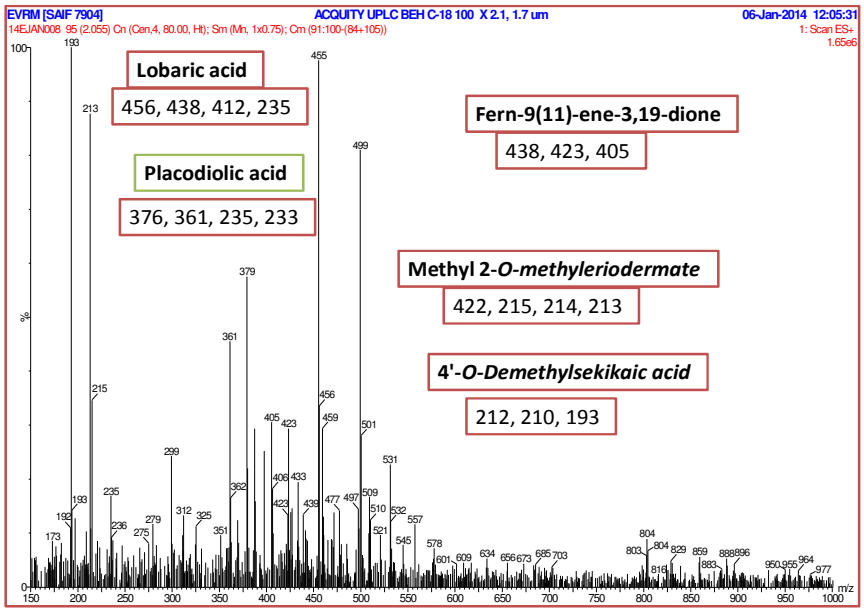


Fig. 4.52c LCMS spectral peaks and respective compounds of methanolic extract *Everniastrum* sp



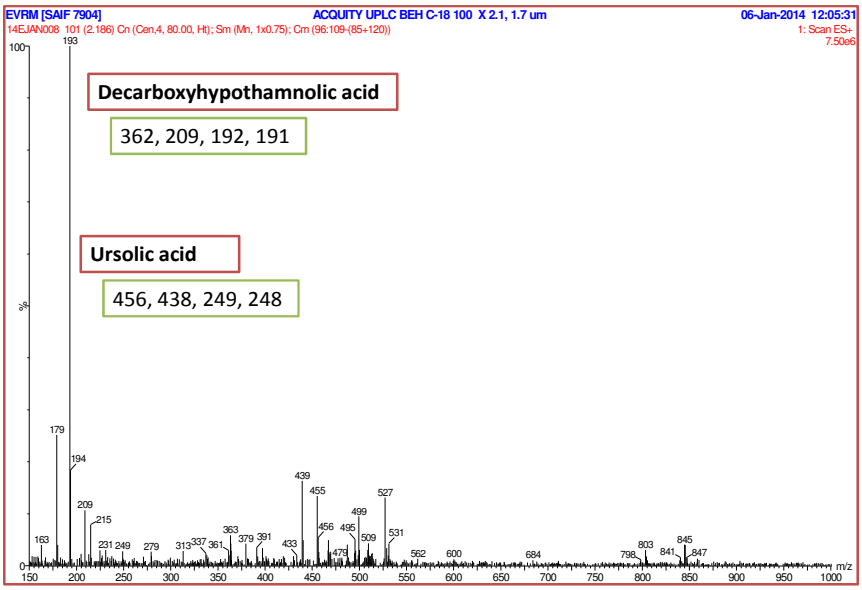
LCMS peaks of EVRM

Fig 4.52d LCMS spectral peaks and respective compounds of methanolic extract *Everniastrum* sp



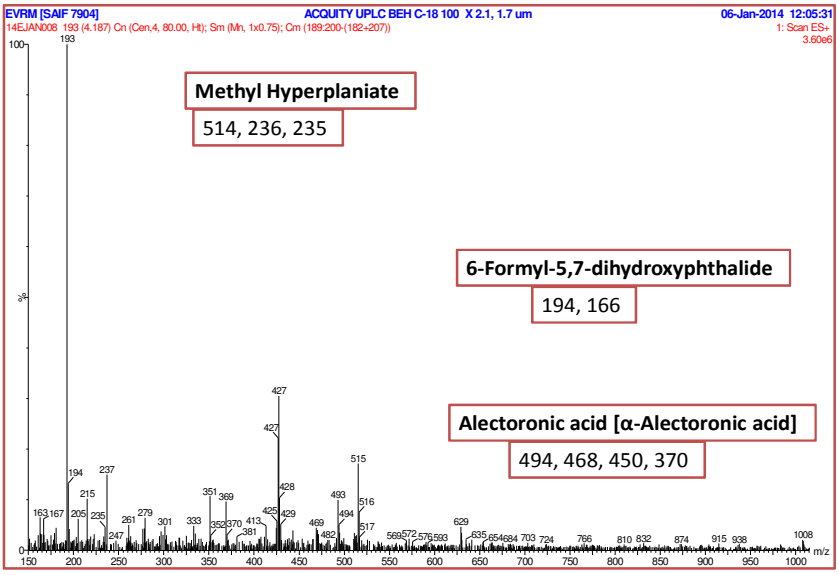
LSMS peaks of EVRM

Fig 4.52e LCMS spectral peaks and respective compounds of methanolic extract *Everniastrum* sp



LCMS peak of EVRM

Fig 4.52f LCMS spectral peaks and respective compounds of methanolic extract *Everniastrum* sp



LCMS peaks of EVRM

Fig 4.52g LCMS spectral peaks and respective compounds of methanolic extract *Everniastrum* sp

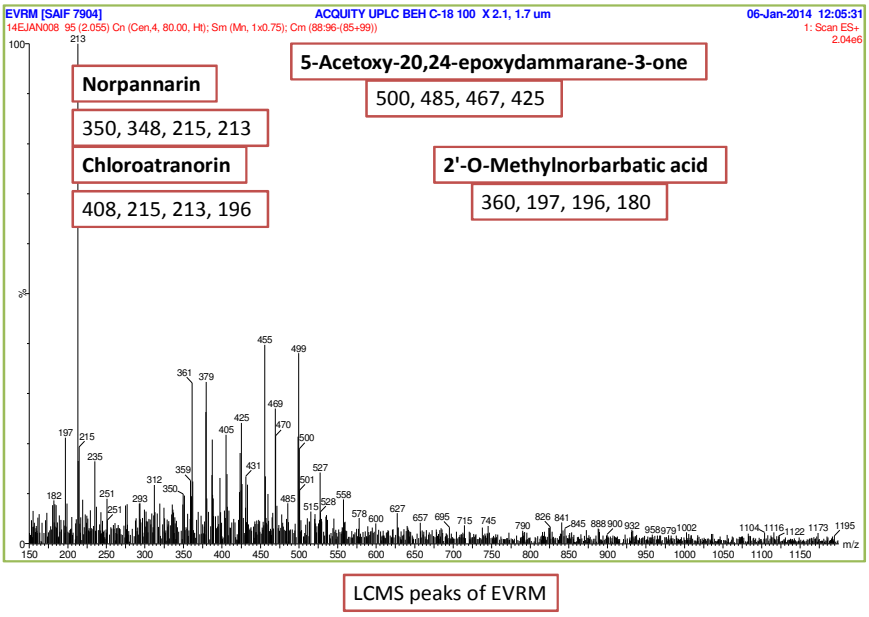


Fig 4.52h.LCMS spectral peaks and respective compounds of methanolic extract *Everniastrum* sp

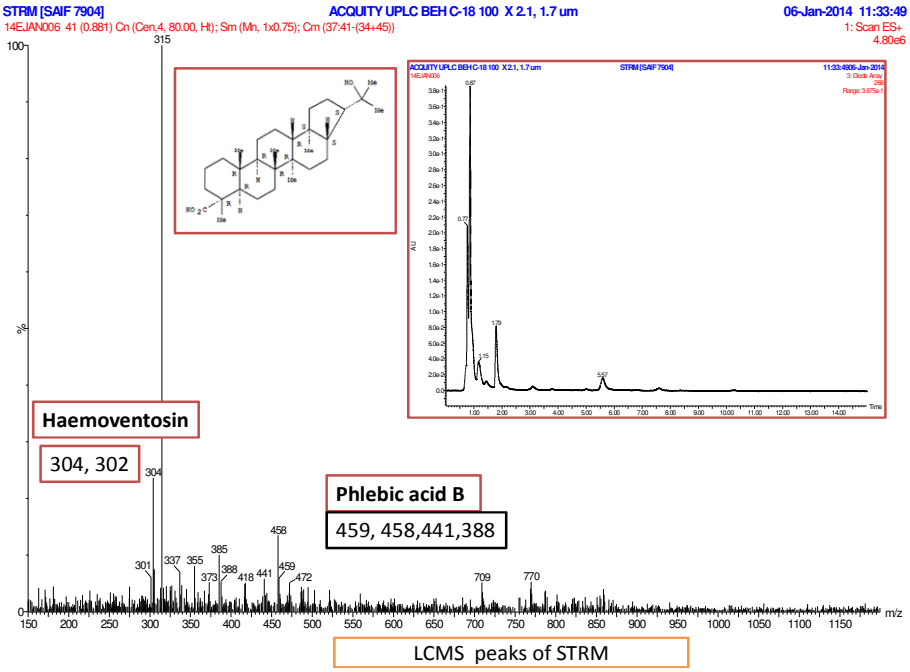


Fig 4.53a LCMS spectral peaks and respective compounds of methanolic extract *Stereocaulon pomiferum*

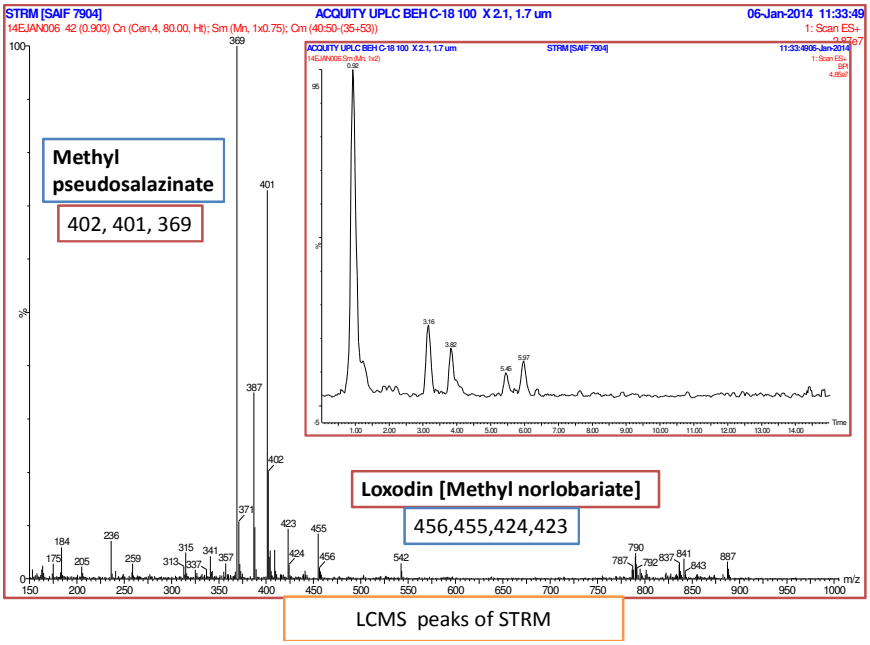


Fig 4.53b LCMS spectral peaks and respective compounds of methanolic extract *Stereocaulon pomiferum*

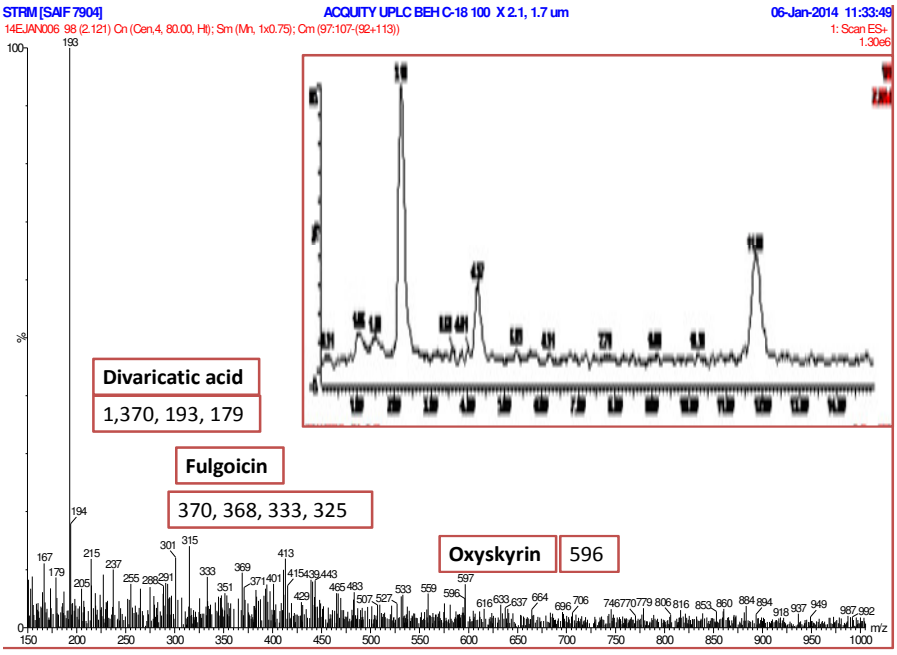


Fig 4.53c LCMS spectral peaks and respective compounds of methanolic extract *Stereocaulon pomiferum*

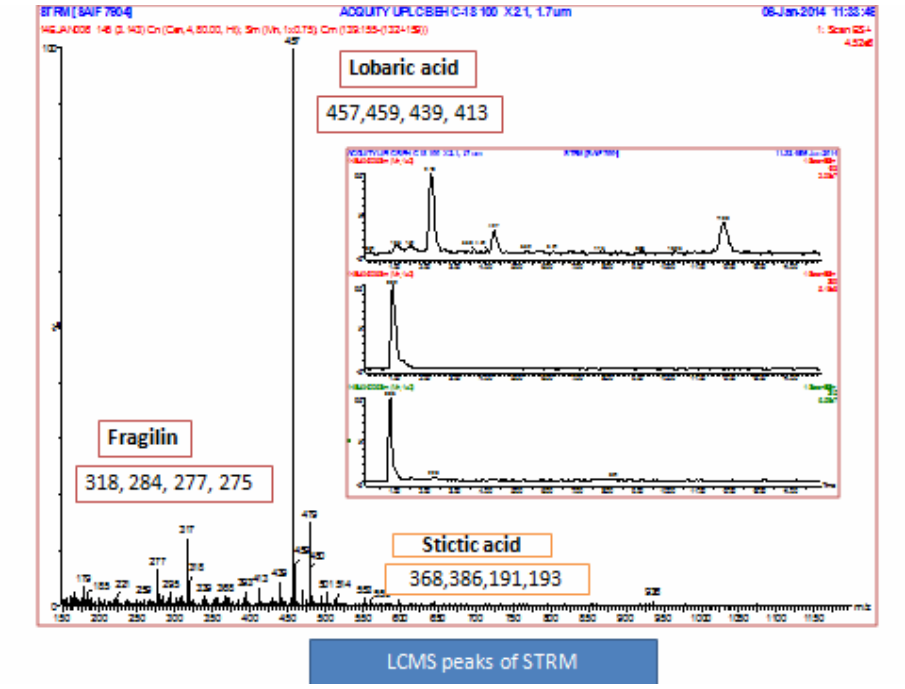


Fig.4.53d LCMS spectral peaks and respective compounds of methanolic extract *Stereocaulon pomiferum*

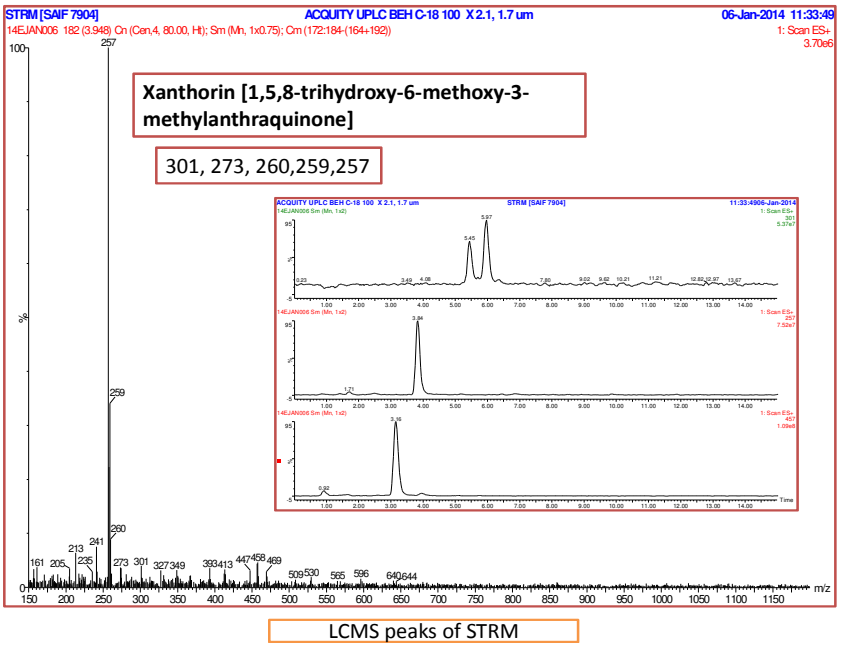


Fig.4.53e LCMS spectral peaks and respective compounds of methanolic extract *Stereocaulon pomiferum*

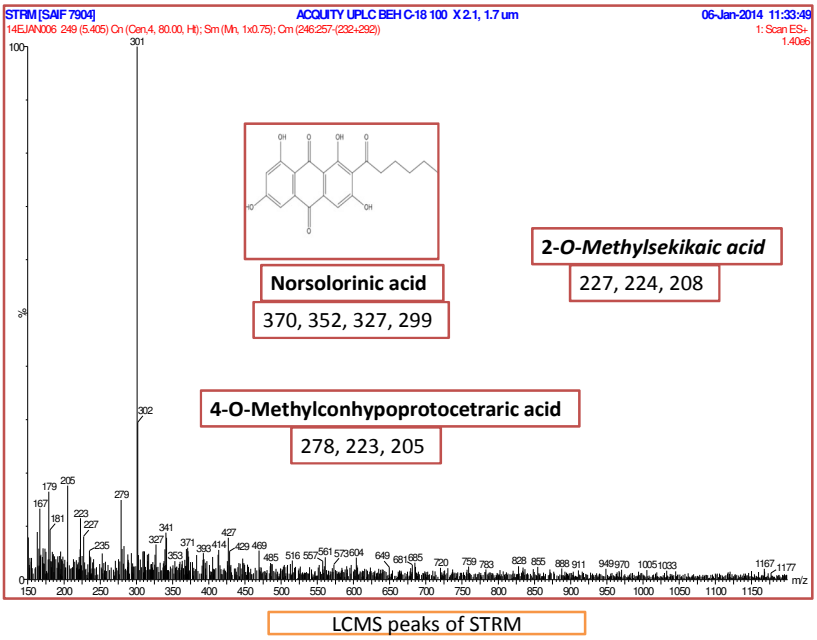


Fig.4.53f LCMS spectral peaks and respective compounds of methanolic extract *Stereocaulon pomiferum*

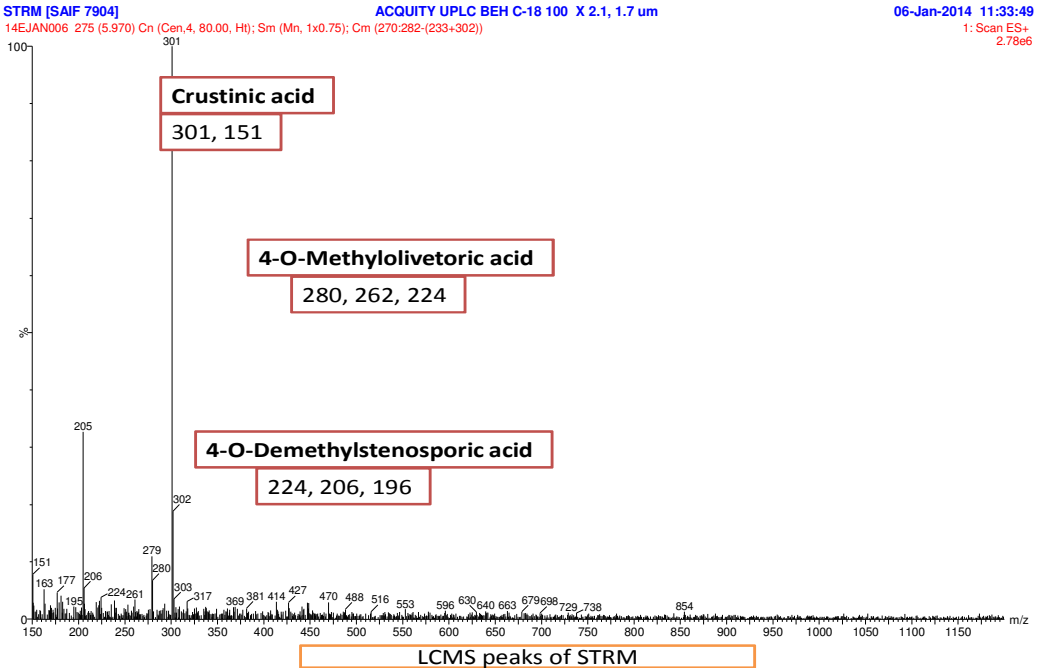


Fig.4.53g LCMS spectral peaks and respective compounds of methanolic extract *Stereocaulon pomiferum*

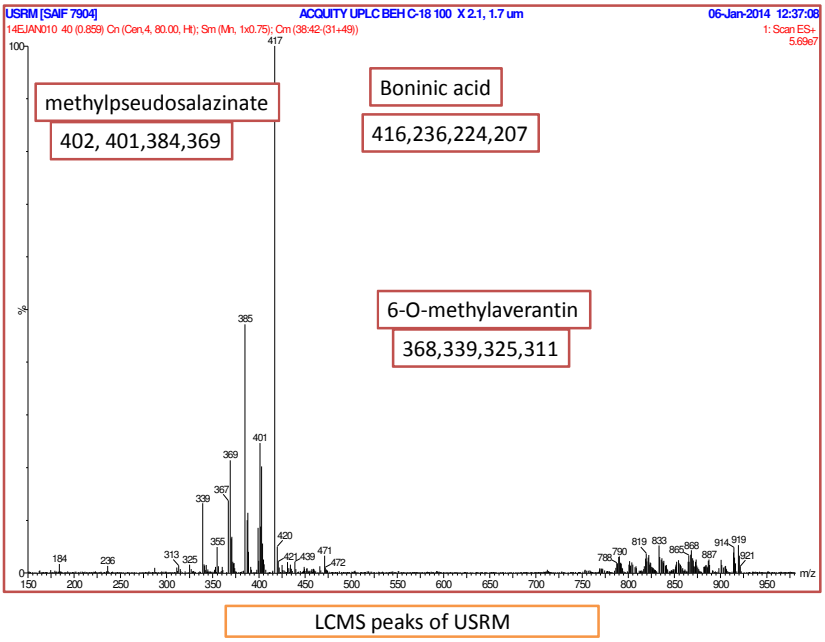


Fig.4.53h LCMS spectral peaks and respective compounds of methanolic extract *Stereocaulon pomiferum*

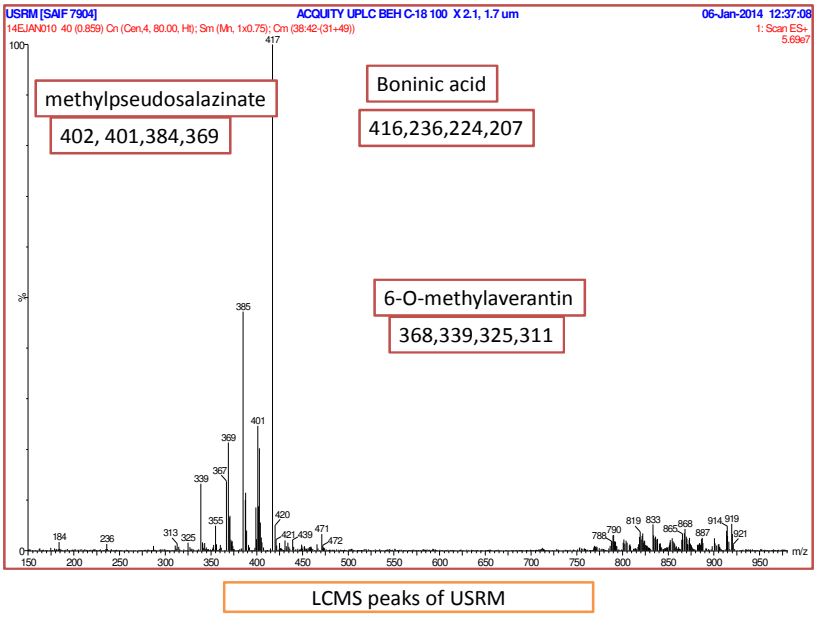


Fig 4.54a LCMS spectral peaks and respective compounds of methanolic extract *Usnea baileyi*

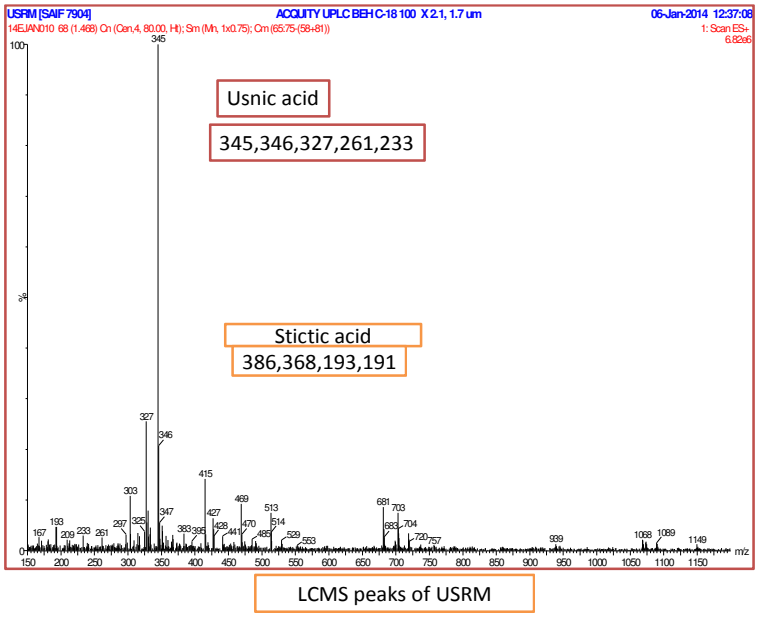


Fig 4.54b LCMS spectral peaks and respective compounds of methanolic extract *Usnea baileyi*

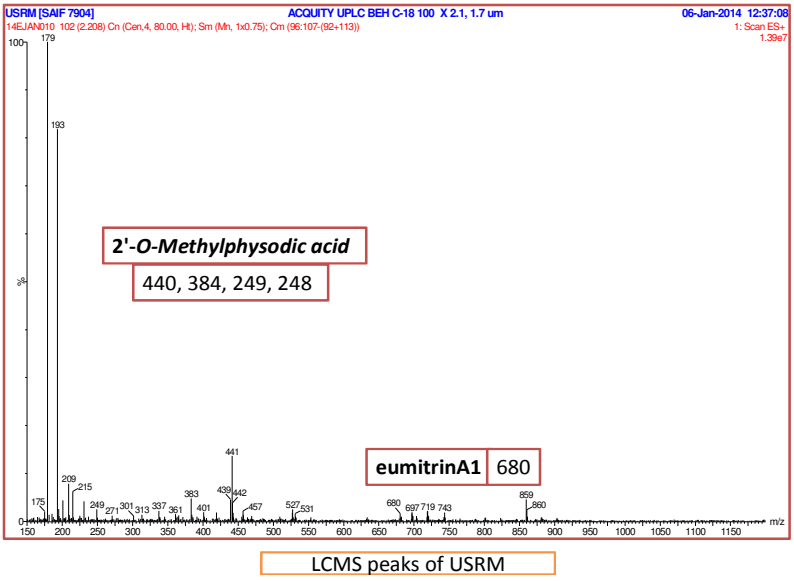
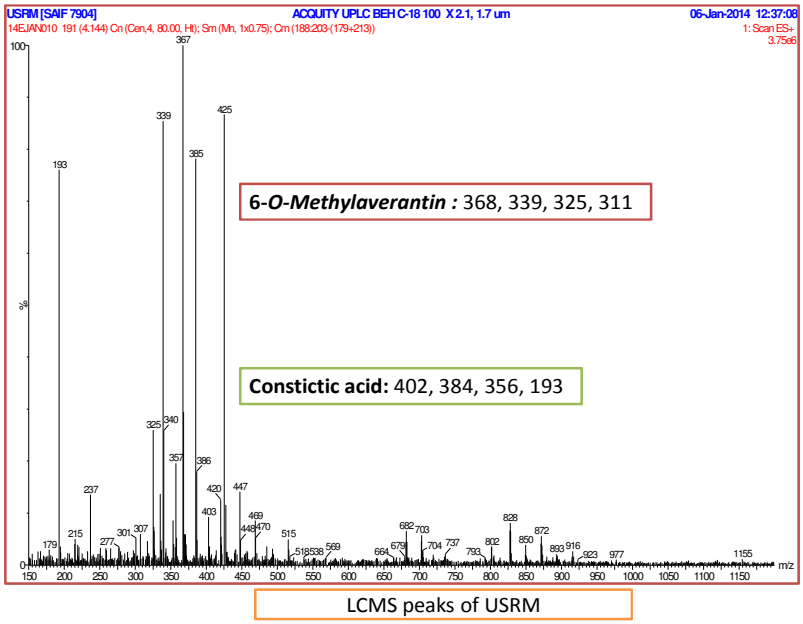


Fig.4.54c LCMS spectral peaks and respective compounds of methanolic extract *Usnea baileyi*



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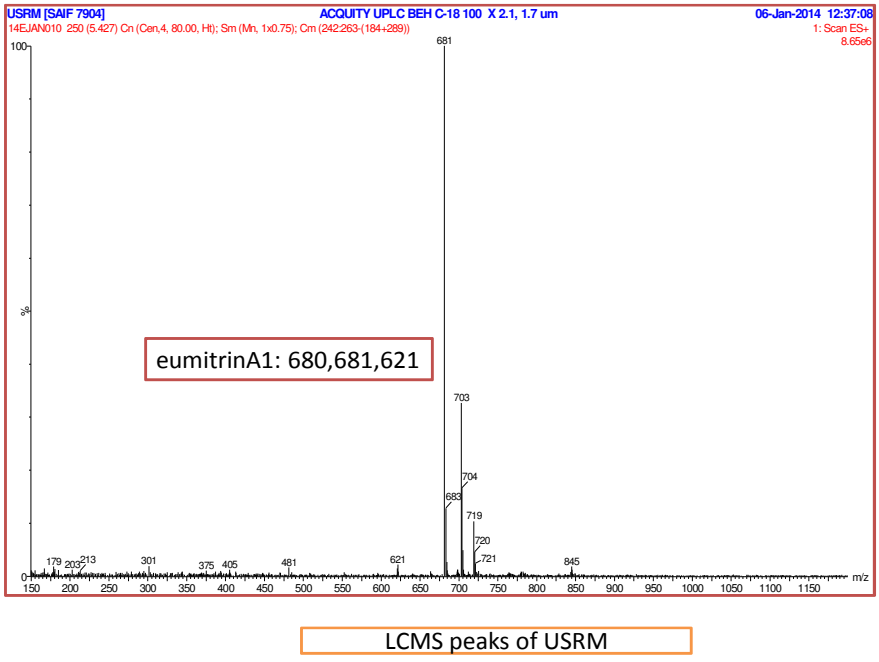

Fig.4.54e LCMS spectral peaks and respective compounds of methanolic extract *Usnea baileyi*

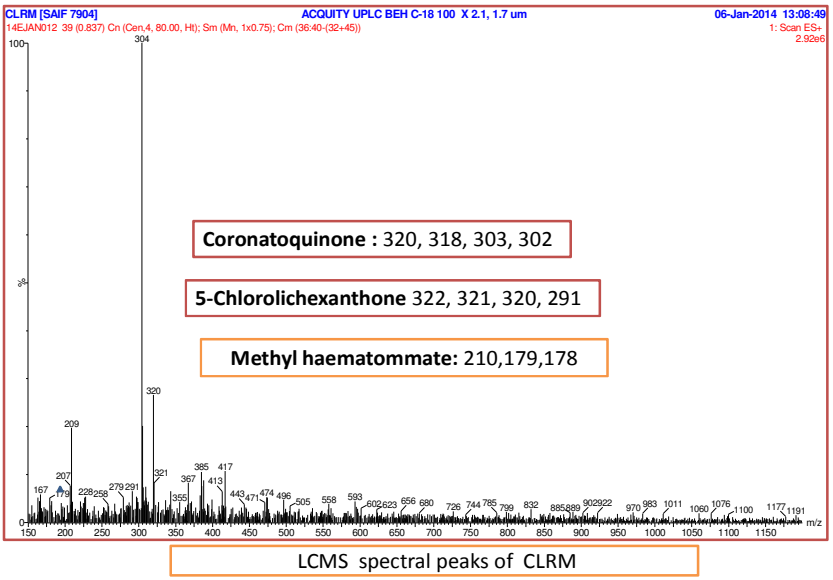

Fig .4.55a LCMS spectral peaks and respective compounds of methanolic extract *Ramalina hossei*

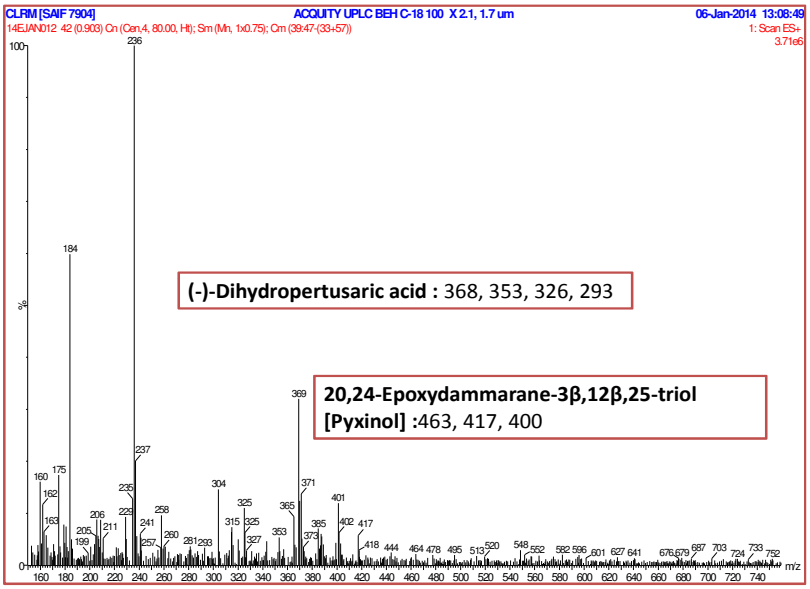

Fig .4.55b LCMS spectral peaks and respective compounds of methanolic extract *Ramalina hossei*

SUMMARY AND CONCLUSION

The present thesis entitled **"Screening, Isolation and Identification of Antimicrobial and Antioxidant Substances from Some Common Lichens of Darjeeling Hills"** was conducted.

The study revealed the following results-

During antimicrobial screening broad spectrum antibacterial activity shown by ethanolic and methanolic extract of *U. baileyi*, *P. reticulatum, R. hossei* and *Everniastrum* sp. Out of the four medicinal plants which underwent antimicrobial screening ethanolic extract of *P. pseudoginseng* exhibited a good degree of antimicrobial activity*, U. dioica* was least active, *S. mukrossi* and *B. ciliata* was moderately active. All the lichens and medicinal plants used in this study exhibited some degree of antibacterial as well as antifungal activity against clinical and phytopathogens.

- Although some lichens and medicinal plants did not show any antimicrobial properties individually, but they exhibited antimicrobial property when their extracts were combined. This combined effect was manifested by ethanolic extracts of *P. pseudoginseng* + *Everniastrum* sp and methanolic extracts of *P. pseudoginseng* + *Everniastrum* sp with relatively strong antimicrobial activity evidenced by inhibition zones greater than the control antibiotic. Besides this ethanolic extract of (*U. baileyi* + *U. dioica)* and methanolic extract of (*U. baileyi* + *U. dioica)* also possessed synergistic effect. The activity of ethanolic and methanolic extract of *S. mukrossi* was enhanced by its combination with ethanolic extract of *Everniastrum* sp, methanolic extract of *Everniastrum* sp, and ethanolic and methanolic extract of *P. reticulatum.*

- A weak combination of ethanolic extract of *S. mukrossi* + *U. baileyi* was observed. This study gives possibility of use of these lichens which are available throughout the growing season.

- The MIC of the different extracts was within the range of 500-10000µg/ml. Among the tested lichen extracts methanolic extract of *U. baileyi*, ethanolic extracts of *P. reticulatum, S. pomiferum,* ethanolic and

methanolic extract of *Everniastrum* sp, ethanolic and methanolic extract of *R. hossei* exhibited MIC of 500µg/ml against test microorganisms.

- It was noted that the MIC value of medicinal plants ranged from 100 µg/ml (*S.mukrossi*) to 500µg/ml. In combination low MIC (500µg/ml) was exhibited by methanolic extract of *R. hossei* + *B. ciliata*, ethanolic extract of *R. hossei* + *B. ciliata*, methanolic extract of *Everniastrum* sp + *B. ciliata*, methanolic extract of (*Everniastrum* sp + *U. dioica)* and ethanolic extract of (*Everniastrum* sp + *U. dioica).*

- In our study higher total antioxidant activity (Phosphomolybdenum method) was shown by ethanolic extracts of *R. hossei* 3.19µg/ml ($p<0.05$) and *U. baileyi* 2.12 µg/ml ($p<0.05$) α-tocopherol equivalent/mg. Among the medicinal plants under study ethanolic and methanolic extract of *B. ciliata* exhibited antioxidant activity 5.43 µg equivalent/mg of plant extract and 7.03($p<0.05$) µg/α-tocopherol equivalent/mg. The best synergistic total antioxidant activity was shown by ethanolic extracts of (*U. baileyi* + *P. pseudoginseng*) and (*U. baileyi* + *B. ciliata*).

- Ethanolic extract of *Everniastrum* sp proved to be a best free radical scavenger ($p<0.05$) followed by ethanolic extract of *U. baileyi*, methanolic extract of *P. reticulatum*, ethanolic extracts of *Everniastrum* sp, *R. hossei* and *P. reticulatum* which showed quite appreciable amount of radical scavenging activity. A low radical scavenging activity was shown by ethanolic and methanolic extract of *S. pomiferum* compared to the other lichen extracts.

- Methanolic extract of *R. hossei* demonstrated strongest reducing power, 0.72±0.007 ($p<0.05$) followed by ethanolic extract of *Everniastrum* sp 0.61 ±0.0040 which was nearer to the reducing power of standard BHT (0.89±0.0010).

- Among the medicinal plant extracts methanolic extract of *U. dioica* and *B. ciliata* possessed a good radical scavenging activity ($p<0.05$), which was quite near to the standard compound. Ethanolic extract of *B. ciliata* and *U. dioica* were also potent radical scavengers. Methanolic extract of *P. reticulatum* + *B. ciliata* in combination possessed a DPPH radical scavenging value of 72.12±1.83 **%** which was greater than the standard

compound Ascorbic acid. Ethanolic extract of *P. reticulatum* + *B. ciliata* and methanolic extract of *U. baileyi* +*B. ciliata,* ethanolic extract of *S. pomiferum* +*U. dioica* and methanolic extract of *S. pomiferum* +*U. dioica* were also able to quench the free radicals.

- An appreciable amount of phenol content was found in all test lichens. Methanolic and ethanolic extracts *U. baileyi* (141.2±0.34 and 106±0.51) and *P. reticulatum* (143.23±1.30 and 108.4±1.50) ($p<0.05$), were recorded as highest. Phenolic content of methanolic extract of *Everniastrum* sp was 96.86±0.37µg tannic acid equivalents which are greater than or nearly equal to the standard compound tannic acid (97.40±1.53).

- The value of flavonoid content ranged from methanolic extract of *P. reticulatum* 1.41±0.01 to ethanolic extract of *R. hossei* 1.66±0.01 which was less than the standard (quercetin).

- The catalase activity of lichen was highest in case of *Everniastrum* sp as 1.68 enz/min/gm tissue and for *Parmotrema reticulatum* it was 1.57enz/min/gm tissue. High peroxidase activity was noted in case of *Usnea baileyi*, *Ramalina hossei* and *Stereocaulon pomiferum* as 2.058, 2.4 and 2.26 enz/min/gm of tissue respectively.

- The total protein content decreased with the increasing concentration of all test lichen extracts (from 0.1 to 0.5ml) extract during determination of total protein content of culture filtrate. The effect of lichen extracts on growth of microorganisms showed that the duration of log phase decreased with increasing concentration of different lichen extracts.

- It appeared that all the five lichen extracts with antimicrobial activity also possessed appreciable antioxidant activity with some altered results.

- The LCMS chromatograms of different lichens (methanolic extracts) have been found to contain a variety of secondary lichen substances which are responsible for antimicrobial and antioxidant activity of lichens. Twenty five such active compounds were isolated from methanolic extract of *Everniastrum* sp alone which includes mainly orcinol, depsidones and depside compounds with some aliphatic acids, xanthones and Usnic acid derivatives. Methanolic extract of *S. pomiferum* mainly yielded orcinol depsidones and depside compounds with some aliphatic acids, xanthones and usnic acid derivatives. Secondary metabolites such as orcinol

depside, β-orcinol depsidones, anthraquinones, usnic acid derivatives, and ergochromes were isolated from methanolic extract of *U. baileyi.*

- The chromatogram of methanolic extract of *R. hossei* revealed the existence of xanthones, pulvinic acid derivatives, orcinol tridepsides, xanthones, naphthaquinone, monocyclic aromatic derivatives, aliphatic acids, terpenoids, and pulvinic acid derivatives in the crude extract.

- In those cases where combined extracts of lichen and medicinal plant were used there is also a possibility of two or more compounds working in consortium to give antimicrobial activity. Combinations of antimicrobials that demonstrate an *in vitro* synergistic effect against microorganisms are more likely to result in successful therapeutic application. The results obtained of these studies may be helpful in developing the plant based natural antimicrobial agents, fungicides and insecticides for preventing and curing the common diseases of humans and to reduce the pathogen population.

- The presence of compounds indicated after LCMS data seem to be responsible constituent or one of active principles of the lichens for its antimicrobial as well as antioxidant properties.

Recommendations

The present investigation dealt with only five lichen specimens of Darjeeling Hills, but other lichen samples are also abundantly present. Hence it is recommended to conduct such studies on other lichen species also.

- Altogether eight microorganisms including Gram positive bacteria, Gram negative bacteria and fungus was taken for determination of antimicrobial activity but other microorganisms can be also taken for antimicrobial assay against lichen extract.

- Four medicinal plants were taken for the determination of synergistic activity with lichens. However other locally available medicinal plants can be explored for the antimicrobial potentiality.

- Only few parameters are taken for determination of antioxidant activity other parameters like lipid peroxidation assay, nitric oxide scavenging assay, hydroxyl radical scavenging assay, hydroxyl radical scavenging assay can be also further performed.

- Lastly, the results obtained in the present study could be used as a database for further use of lichens for medicinal purpose.

Consequently, the antimicrobial effect of lichens and plants tested can be further authenticated with new studies by taking other clinical pathogens and conducting the pharmacological tests. The findings of this study is the database for further research for search and isolation of the lichen metabolites, greater detail investigation in the action of lichen substances for their application is essential. Further clinical trials are warranted beyond this thesis.

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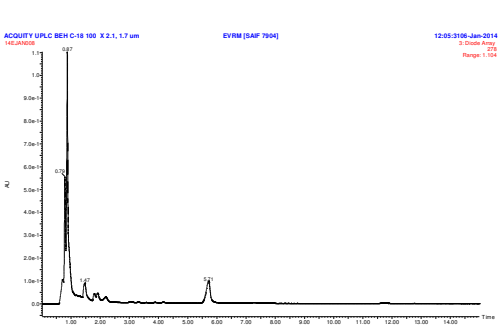
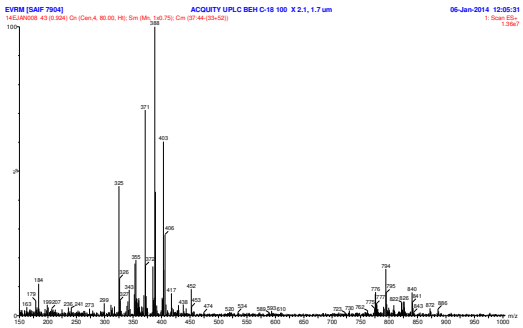
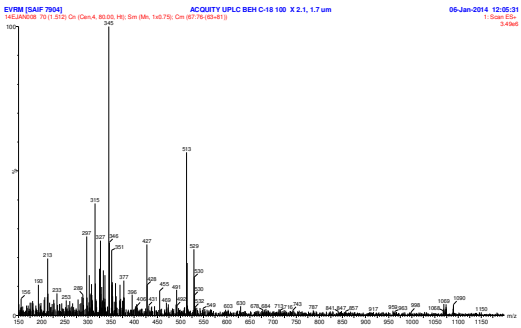
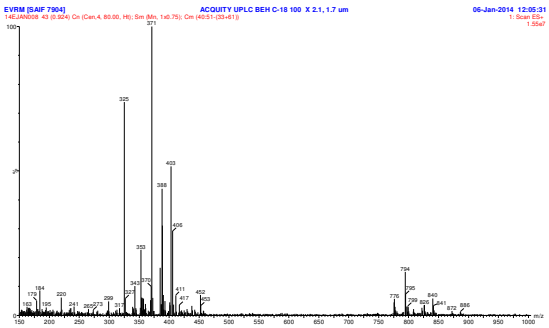
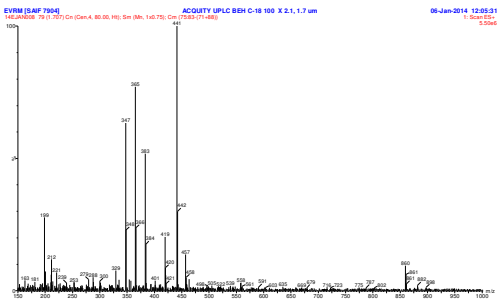
LCMS peaks of *Everniastrum* sp obtained from Lucknow [SAIF7904]

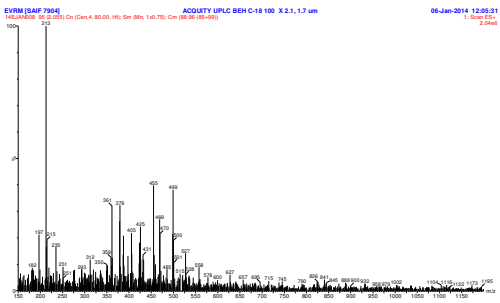




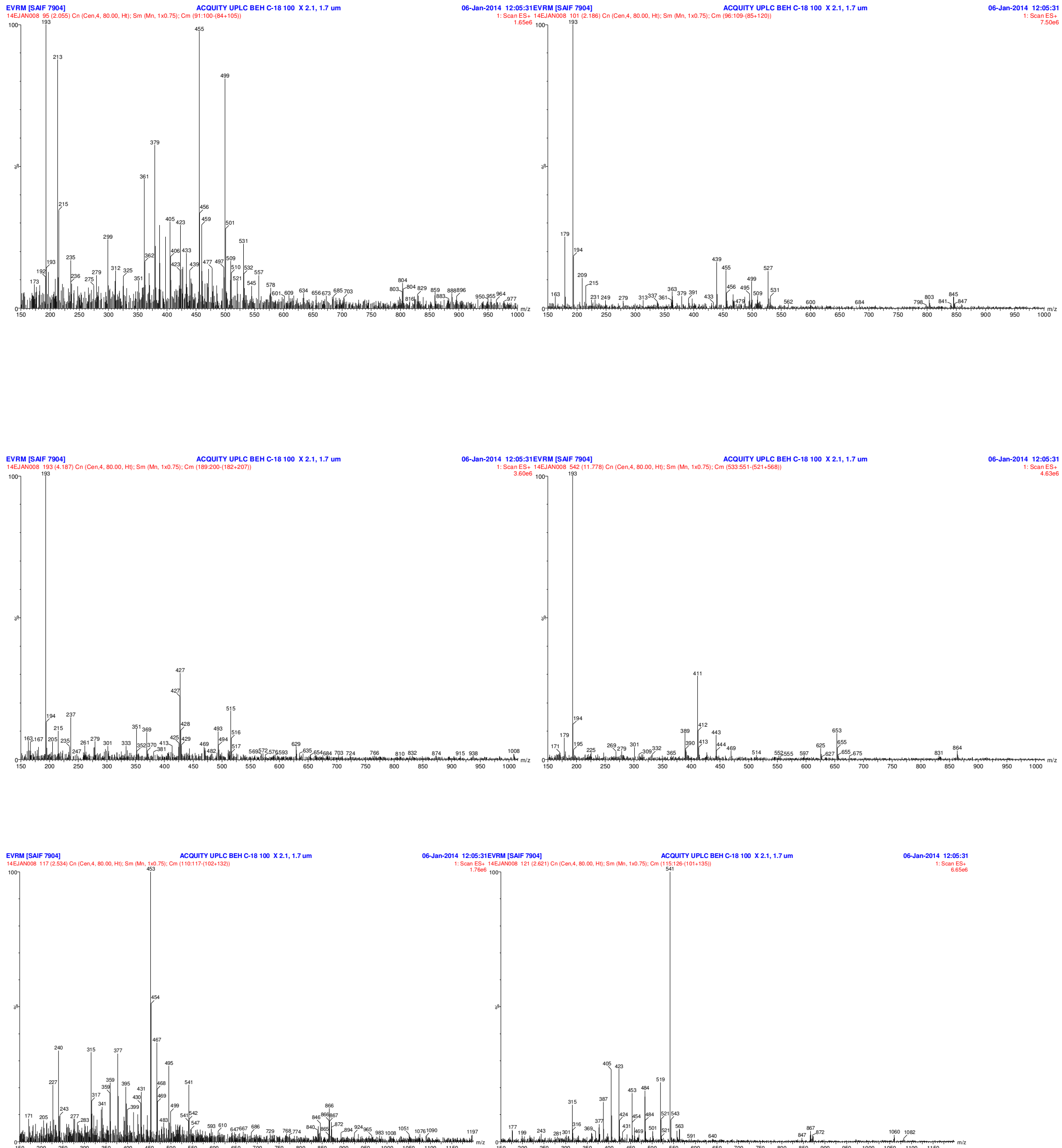






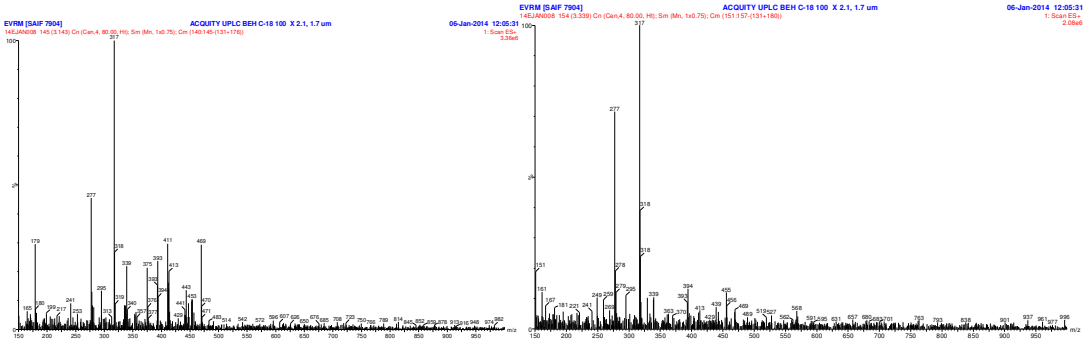


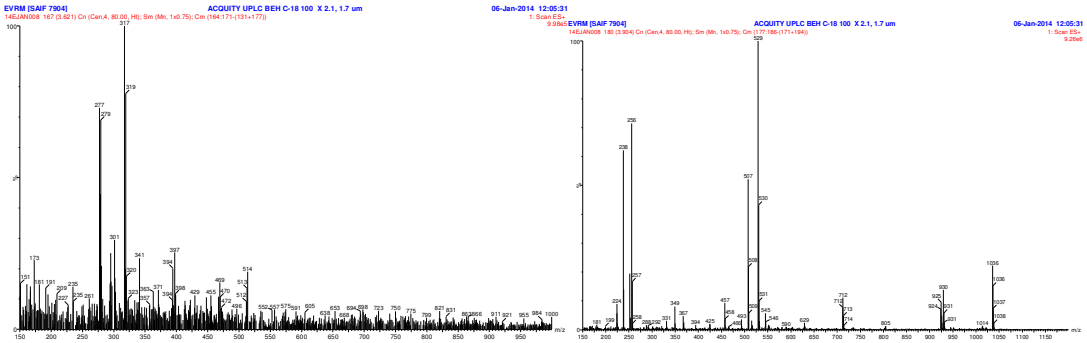
APPENDIX A contd.

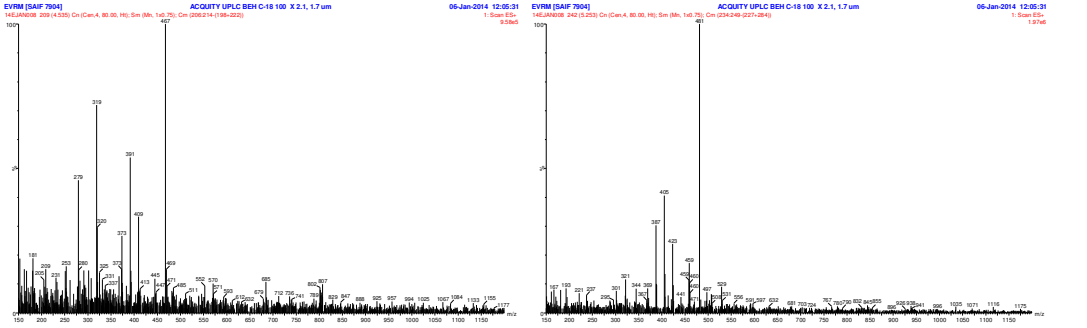


APPENDIX A contd.

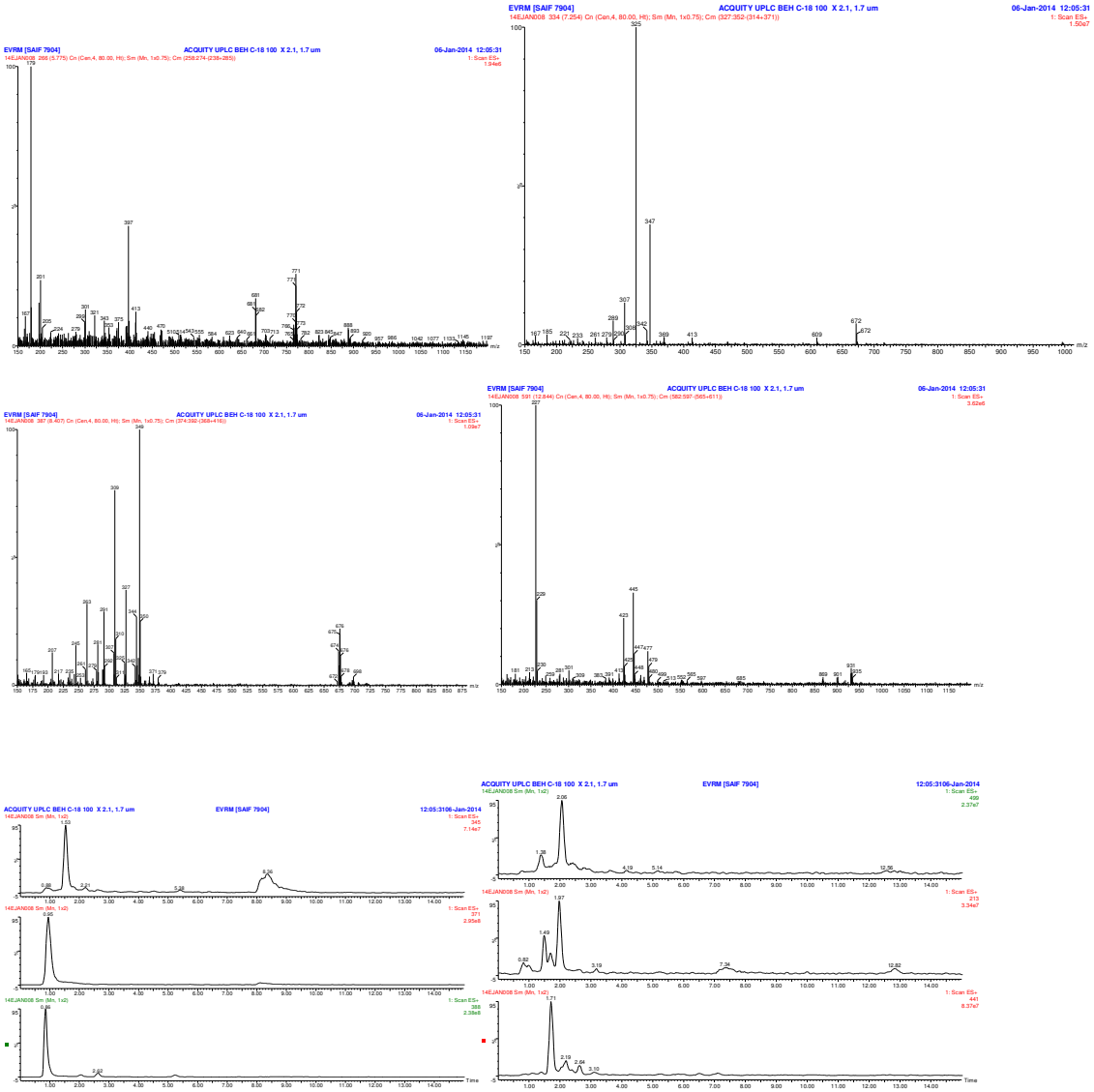
b



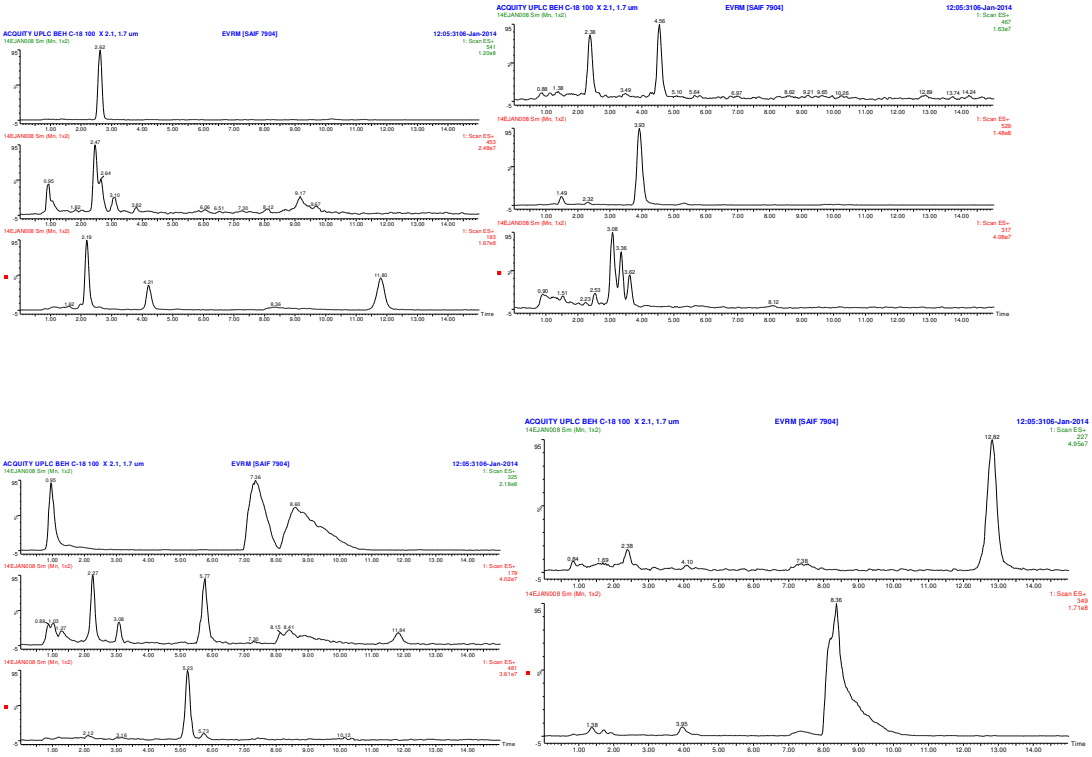




APPENDIX A contd.

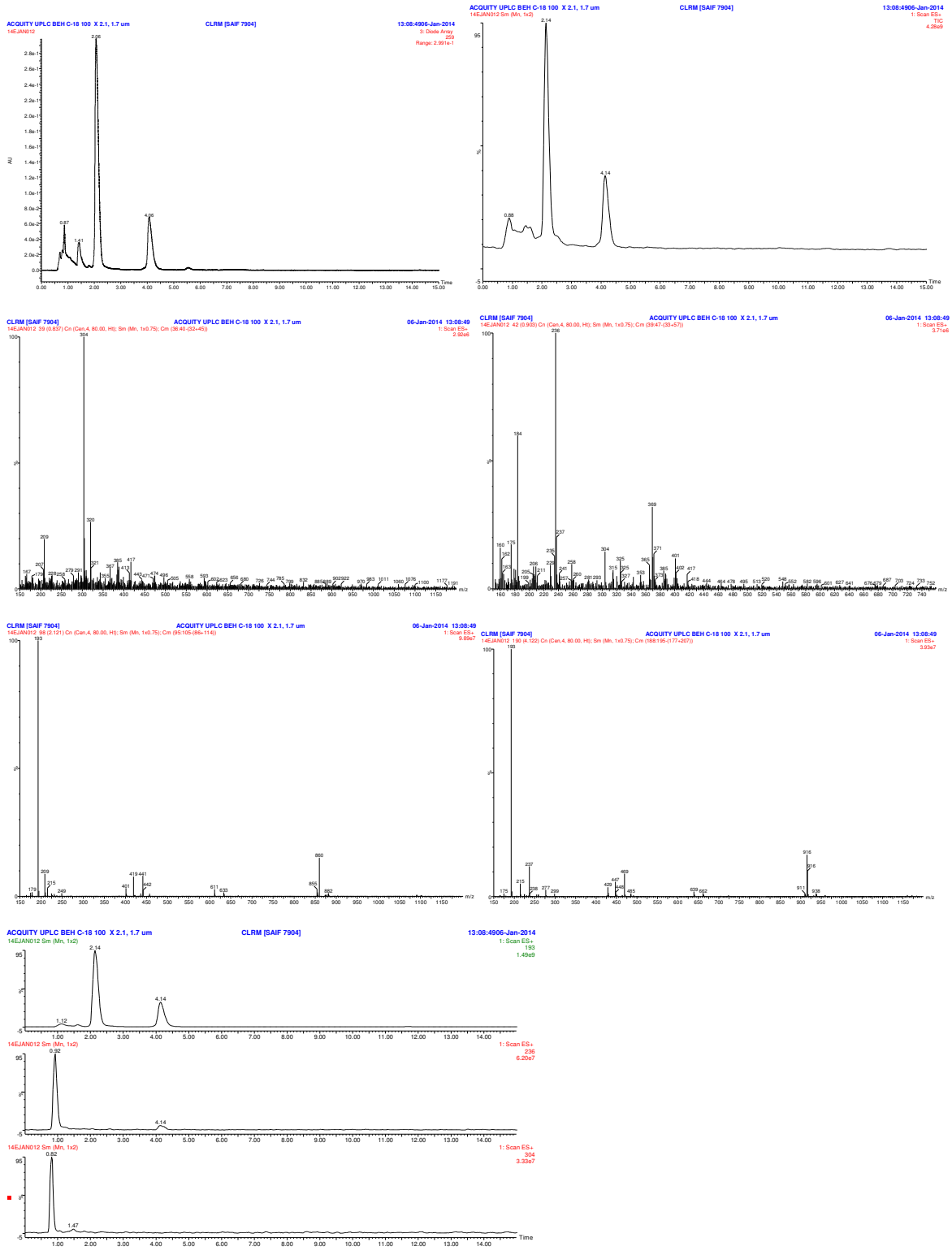


APPENDIX A contd.



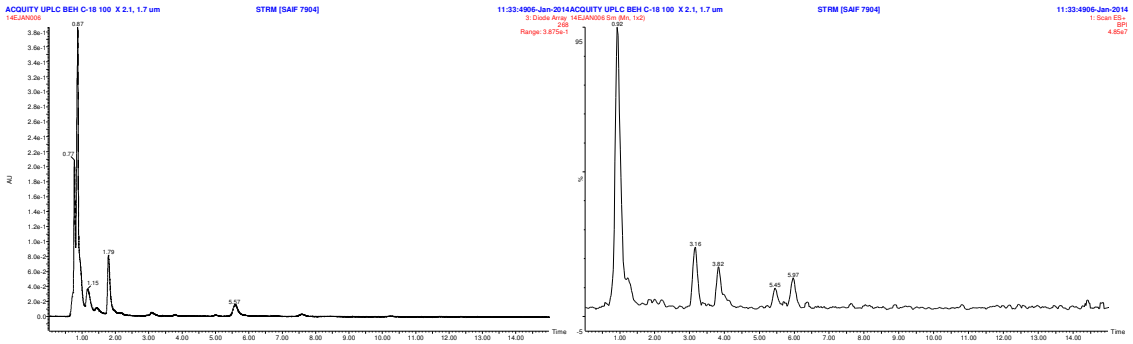
LCMS peaks of *Everniastrum* sp obtained from Lucknow [SAIF7904]

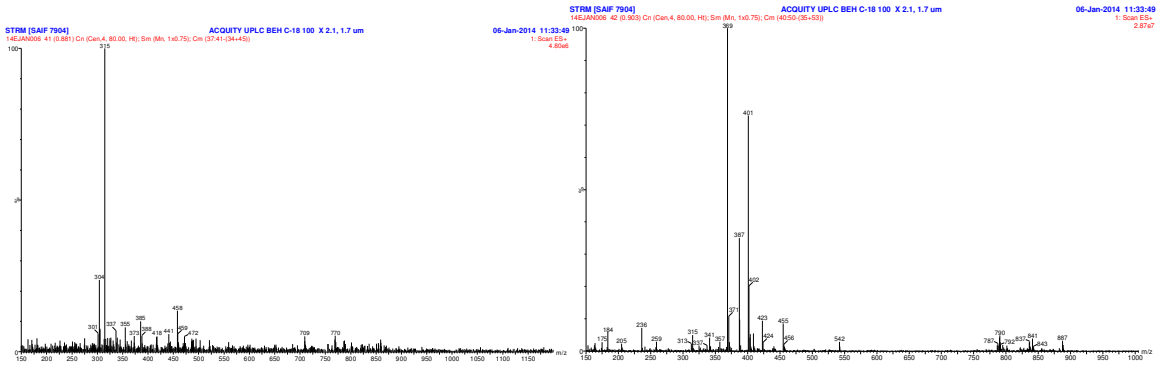
LCMS peaks of *Ramalina hossei* obtained from Lucknow APPENDIX B

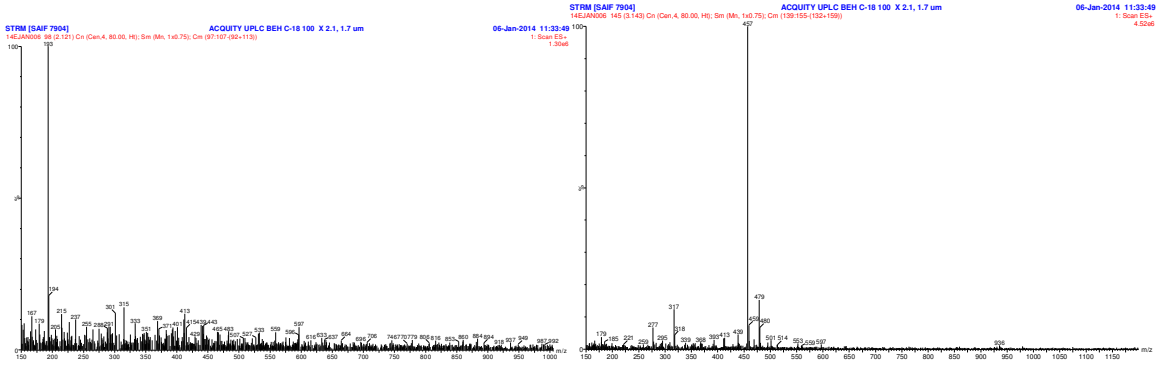


LCMS peaks of *Stereocaulon pomiferum* obtained from Lucknow

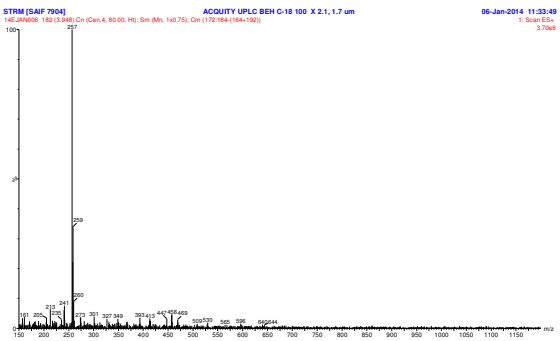
APPENDIX C







APPENDIX C contd.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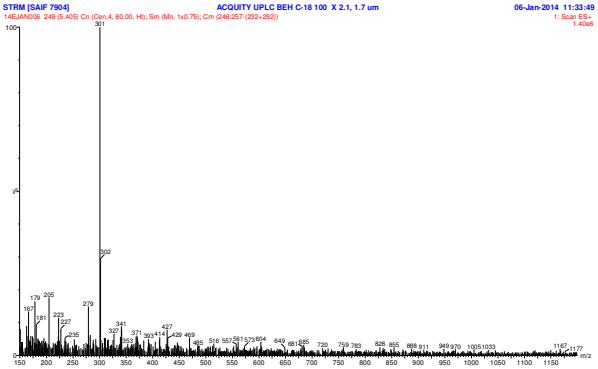
3.70e6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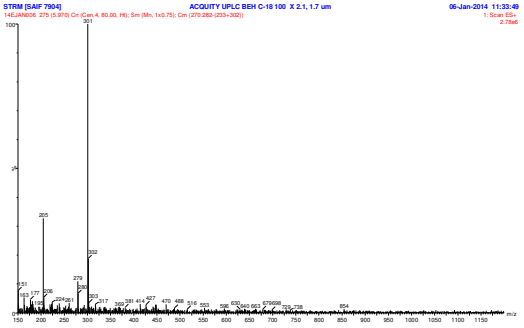
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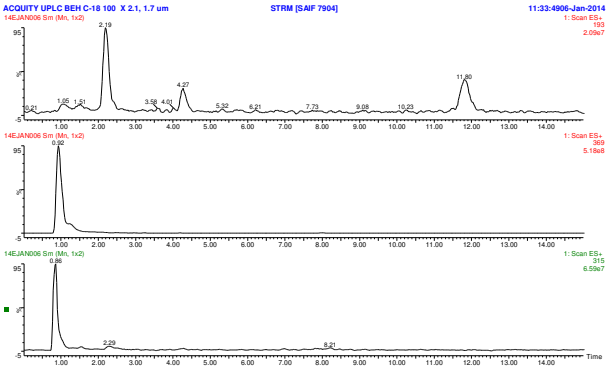
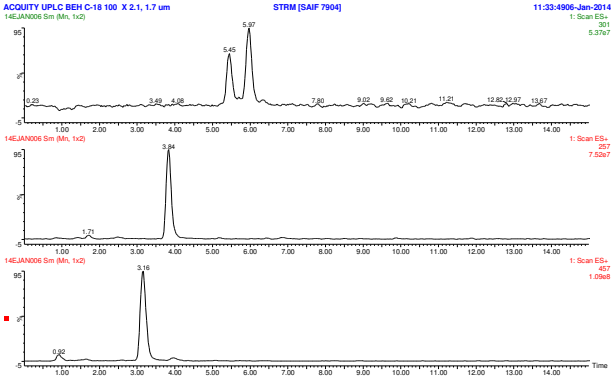
2.78e6


ACQUITY UPLC BEH C-18 100 X 2.1, 1.7 um
STRM [SAIF 7904]
11:33:4906-Jan-2014
14EJAN006 Sm (Mn, 1x2)
1: Scan ES+
193
2.09e7
14EJAN006 Sm (Mn, 1x2)
1: Scan ES+
369
5.18e8
14EJAN006 Sm (Mn, 1x2)
1: Scan ES+
315
6.59e7
Time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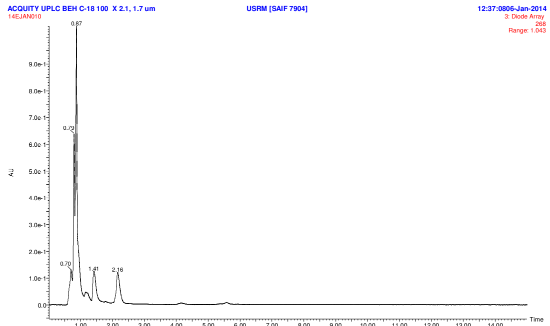
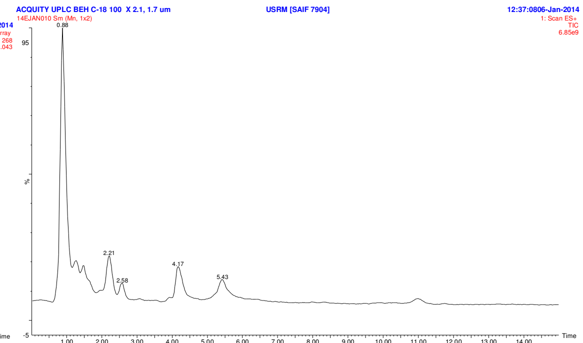
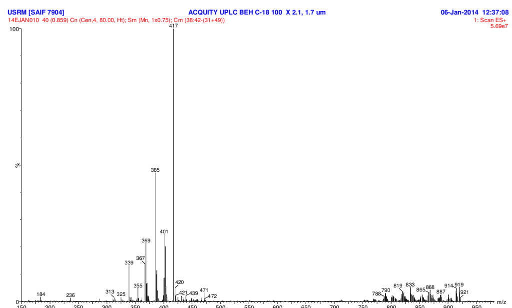
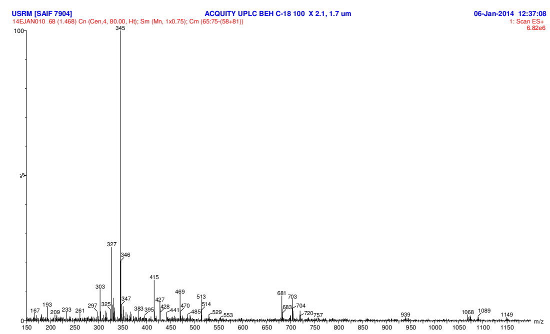
14EJAN006 Sm (Mn, 1x2)
1: Scan ES+
301
5.37e7
14EJAN006 Sm (Mn, 1x2)
1: Scan ES+
257
7.52e7
14EJAN006 Sm (Mn, 1x2)
1: Scan ES+
457
1.09e8
Time

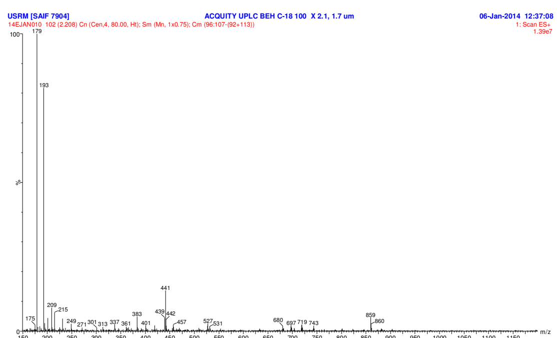
LCMS peaks of *Usnea baileyi* obtained from SAIF, CDRI, Lucknow APPENDIX D

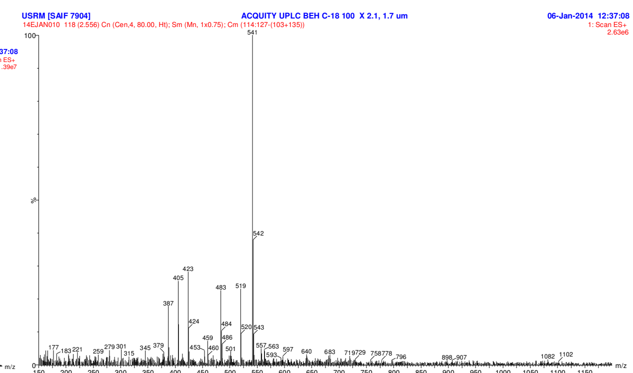


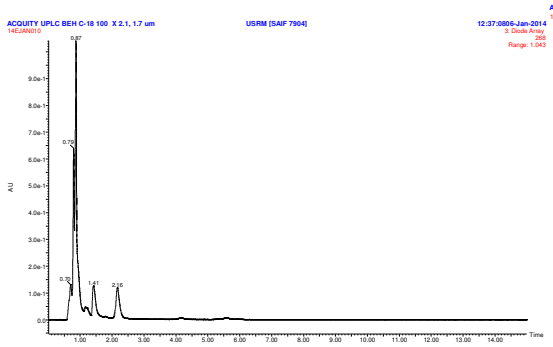








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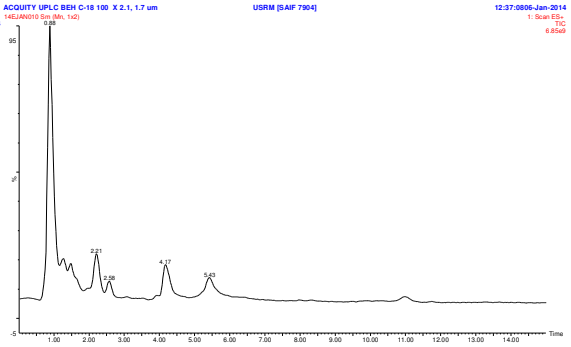
USRM [SAIF 7904]
14EJAN010
12:37:0806-Jan-2014
3: Diode Array
255
Range: 1.043
AU
0.87
0.72
0.37
1.41
2.16
Time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14EJAN010 Sm (Mn, 1x2)
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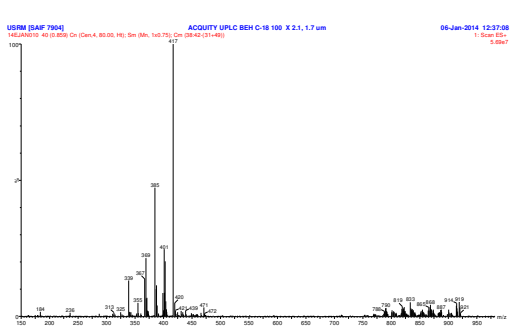
TIC
6.85e9
0.88
2.21
2.58
4.17
5.43
Time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06-Jan-2014 12:37:08
1: Scan ES+
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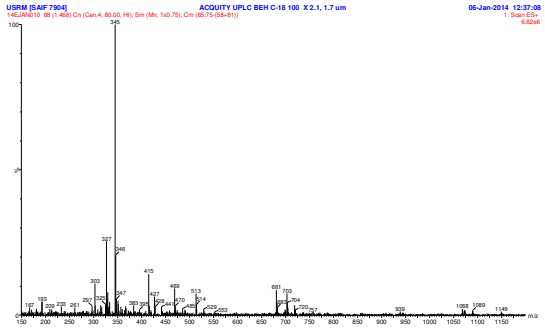
m/z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6.82e6
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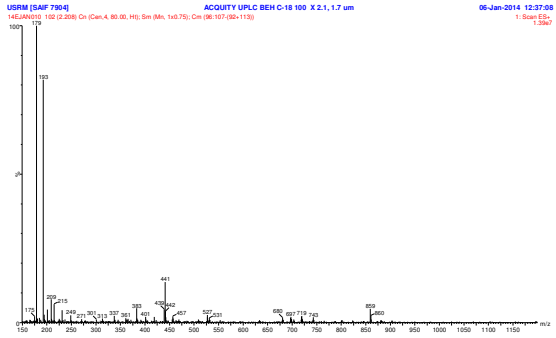
m/z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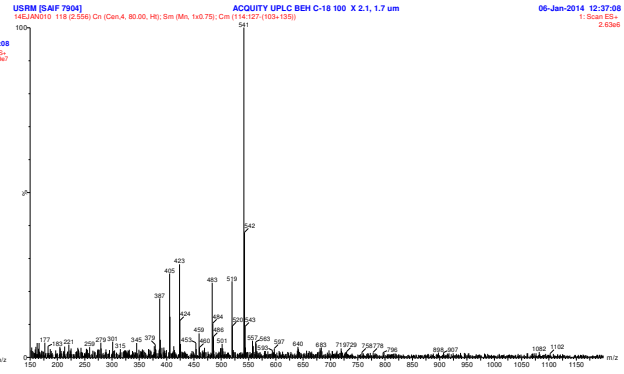
m/z


USRM [SAIF 7904]
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06-Jan-2014 12:37:08
1: Scan ES+
2.63e6
14EJAN010 118 (2.556) Cn (Cen,4, 80.00, Ht); Sm (Mn, 1x0.75); Cm (114:127-(103+135))
m/z

APPENDIX G

AOA

Duncan[a]

Post Hoc test for the Total antioxidant activity of lichen extracts

SAMPLE	N	Subset for alpha = 0.05										
		1	2	3	4	5	6	7	8	9	10	11
2	3	.71333										
3	3		.81000									
8	3			1.24333								
5	3				1.39000							
6	3					1.54333						
10	3						1.59000					
4	3							1.63667				
7	3								1.88000			
1	3									2.12667		
9	3										3.19000	
0	3											4.19333
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

5	3							.61400		
10	3								.72067	
0	3									.89300
Sig.		1.000	1.000	.114	.718	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

[illegible]	[illegible]	.37600								
1	3		.43333							
6	3			.48533						
4	3			.49133						
2	3				.51467					
7	3				.51600					
8	3					.54700				
9	3						.58400			

APPENDIX I

DPPH

Duncan[a]

APPENDIX –I

Post Hoc test for the DPPH radical scavenging activity of lichen extracts

SAMPLE	N	Subset for alpha = 0.05							
		1	2	3	4	5	6	7	8
8	3	25.40000							
7	3		27.40000						
10	3			30.63333					
2	3			31.49000					
6	3				36.23667				
3	3				36.39667				
9	3				36.59333				
4	3					40.79333			
1	3						49.66667		
5	3							53.56667	
0	3								70.56667
Sig.		1.000	1.000	.318	.693	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

APPENDIX J

PHENOLS

Duncan[a] APPENDIX-J

SAMPLE	N	Subset for alpha = 0.05									
		1	2	3	4	5	6	7	8	9	10
9	3	42.00000									
10	3		44.85333								
8	3			49.47667							
7	3				52.69667						
5	3					71.00000					
6	3						96.90000				
0	3						97.43333				
1	3							106.00000			
3	3								108.40000		
2	3									141.20000	
4	3										143.23333
Sig.		1.000	1.000	1.000	1.000	1.000	.512	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

FLAVONOIDS

Duncan[a] APPENDIX- K

Post Hoc test for the Flavonoids activity of lichen extracts

SAMPLE	N	Subset for alpha = 0.05			
		1	2	3	4
4	3	1.41333			
3	3	1.49667	1.49667		
8	3	1.52667	1.52667		
5	3	1.53000	1.53000		
7	3	1.53000	1.53000		
6	3		1.55333	1.55333	
10	3		1.55333	1.55333	
1	3		1.57667	1.57667	
2	3		1.62333	1.62333	
9	3			1.66000	
0	3				4.91000
Sig.		.062	.051	.087	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

AOA

Duncan[a] APPENDIX –N

Post hoc test for Total antioxidant activity of medicinal plants

SAMPLE	N	Subset for alpha = 0.05		
		1	2	3
2	3	2.45000		
1	3	2.70000		
0	3		4.19333	
3	3		5.43000	
4	3			7.03667
Sig.		.708	.086	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Correlations **APPENDIX-L**

Pearson's bivariate test for Correlation

		AOA	RPA	DPPH	PHENOLS	FLAVONOIDS
AOA	Pearson Correlation	1	.680**	.647**	-.235	.[b]
	Sig. (2-tailed)		.000	.000	.188	.
	N	33	33	33	33	0
RPA	Pearson Correlation	.680**	1	.528**	-.313	.[b]
	Sig. (2-tailed)	.000		.002	.076	.
	N	33	33	33	33	0
DPPH	Pearson Correlation	.647**	.528**	1	.258	.[b]
	Sig. (2-tailed)	.000	.002		.147	.
	N	33	33	33	33	0
PHENOLS	Pearson Correlation	-.235	-.313	.258	1	.[b]
	Sig. (2-tailed)	.188	.076	.147		.
	N	33	33	33	33	0
FLAVONOIDS	Pearson Correlation	.[b]	.[b]	.[b]	.[b]	.[b]
	Sig. (2-tailed)	.	.	.	.	
	N	0	0	0	0	0

DPPH

Duncan[a] APPENDIX –M

Post hoc test for DPPHradical scavenging activity of medicinal plants

SAMPLE	N	Subset for alpha = 0.05			
		1	2	3	4
1	3	38.76667			
3	3		49.40667		
2	3			56.90000	
4	3			56.93333	
0	3				70.56667
Sig.		1.000	1.000	.936	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Indian Journal of Fundamental and Applied Life Sciences ISSN: 2231-6345 (Online)
An Online International Journal Available at http://www.cibtech.org/jls.htm
2012 Vol. 2 (1) January- March, pp. 120-126/Sharma et al.
Research Article

ASSESSMENT OF ANTIMICROBIAL ACTIVITY OF EXTRACTS OF FEW COMMON LICHENS OF DARJEELING HILLS

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ABSTRACT
Five common lichens (*Cladonia* sp., *Everniastrum* sp., *Parmelia* sp., *Stereocaulon* sp. *and Usnea* sp.) of Darjeeling hills were extracted from different solvents like ethanol, methanol, petroleum ether, chloroform and aqueous extracts and tested against four Gram positive and four Gram negative bacterial strains. Ethanol extracts exerted stronger inhibitory action followed by methanol extracts. Aqueous extracts manifested less activity to the tested microorganisms. Previous reports on the antimicrobial properties of lichens showed the resistance of Gram negative bacteria but in our investigation such group of bacteria found sensitive to four of the five lichens tested (except *Everniastrum*).

Key Words: *Antimicrobial Activity, Lichens, Different Solvent Extracts, Darjeeling*

INTRODUCTION
Lichens has been used in the folk medicines for centuries; their biological properties explored by Native Americans, Indians and Europeans, who used in their traditional medicines to treat a variety of animals. Lichen synthesize numerous metabolites called lichen substances including aliphatic, cycloaliphatic, aromatic and terpenic components. These metabolites exert a wide variety of biological actions including antibiotic, antimycobacterial, immunomodulatory, antioxidant, cytotoxic, antiherbivore, and antitumour effects (Chand *et. al.*, 2009).

Lichen forming fungi produce antimicrobial secondary metabolites that protect many animals from pathogenic microorganisms. The first study of antibiotic properties of lichens was carried out by Burkholder (1944). Vartia (1973) reported antimicrobial properties of several lichens and other researchers have since then studied the antimicrobial activity of several lichens against gram-positive, gram- negative bacteria as well as several fungi.

The search for novel natural bioactive compounds as a foundation to new drug discovery is receiving attention as previously reliable standard drugs become less effective against the emerging new strains of multiple drug resistant pathogens (Muller, 2001).

India is a rich centre of biodiversity contributing nearly 15 % of the 13500 species of lichens (Negi, 2000). Many lichen species of the Himalayan region are said to effectively cure dyspepsia, bleeding piles, bronchitis, scabies, stomach disorders and many disorders of blood and heart (Saklani and Upreti,1992; Negi and Kareem, 1996; Sochting, 1999). Even though manifold activities of lichen metabolites have now been recognized their therapeutic potential has yet not been fully explored and thus remains pharmaceutically unexploited (Turk *et. al.*, 2003).

Darjeeling Himalaya is situated between 87°59' - 88°53' E and 28°31'-27°13' N in the Eastern Himalayan region of India. The altitudinal range of this hilly region varies from 130 to 3660 m., due to this a wide array of climatic zones are available, which favour the luxuriant growth of diversified and rich vegetation. This region is also the abode of many endemic elements and a number of species which have become rare, threatened and endangered (Das, 1995). It is known that microorganisms have developed resistance to many antibiotics. This creates enormous problems in the treatment of infectious disease, and investigators therefore seek new antimicrobial substances from different sources such as higher plants and lichens (Mitscher *et.al.*, 1987; Crittenden and Porter, 1991; Karaman *et.al.*, 2003). Locally ethnobotanical uses of *Usnea* include its use as aromatic in health recipes (Rai *et. al.*, 1998). Secondary metabolites of

lichens contain many active components; the purpose of the present study is to investigate their antimicrobial activity. A few reports are available on the studies on antimicrobial properties of lichens from Darjeeling hills. Gupta and Paul (1995) studied antimicrobial properties of five lichens and *Usnea floria* was found to be promising against *Bacillus megaterium* and *Staphylococcus aureus*. Ray *et. al.*, (2003) reported the antimicrobial activity of the extracts of *Usnea articulata, Ramalina jamisii and Parmelia tinctorum* against both Gram-positive and Gram-negative bacteria. The extracts were also found to be the inhibitor of protein synthesis, energy metabolism and growth of studied bacteria.

In this present work we have investigated the antibacterial potential of five common lichens i.e., *Cladonia* sp., *Everniastrum* sp., *Parmelia* sp., *Stereocaulon* sp. *and Usnea* sp. of Darjeeling Hills.

MATERIALS AND METHODS

Lichen materials

Five lichen materials were collected from profusely grown sites of Darjeeling Hills. The specimens were provisionally identified as *Cladonia* sp., *Everniastrum* sp., *Parmelia* sp., *Stereocaulon* sp. *and Usnea* sp. following the relevant key and monographs (Sochting, 1999). Voucher specimens of the samples are stored at the Herbarium of Department of Botany, Darjeeling Govt. College.

Microorganisms

Eight bacteria listed below were obtained from the stock culture of Microbiology Research Laboratory, Postgraduate Department of Botany, Darjeeling Government College.

List of bacteria:Alcaligens faecalis, Bacillus subtilis, Bacillus megaterium, Escherichia coli, Enterobacter aerogenes, Pseudomonas aeruginosa, Streptococcus mutans and Staphylococcus aureus.

Preparation of lichen extracts

Each lichen sample was washed to remove debris, dried and ground to powder and was stored in a sterile glass bottle in the refrigerator. The 5 g portions of sieved powder was added to 50 ml of solvents (ethanol, methanol, chloroform and petroleum ether), sonicated for 30 min and left overnight at room temperature. The crude extract was prepared by decanting, followed by filtration through muslin cloth, and further filtered with Whatman No. 1 filter paper to obtain a clear filtrate. The filtrate was evaporated to obtain 10 ml of concentrated extract and sterilized by membrane filtration using 450 nm bacteriological filters. Such sterilized filtrate was stored in screwcapped airtight containers in the refrigerator.

Screening of antibacterial activity

This procedure is based on disc diffusion method of Baur *et. al.*,(1966). Overnight grown bacterial cultures (1.5×10^8 CFU/ml) were spreadplated on nutrient agar plates to achieve semiconfluent growth. Sterile filter paper discs were soaked in extracts, allowed to dry between the applications and placed on plates which were then incubated at 37°C for 24 hrs. Streptomycin (10µg/ml) and sterile distilled water were taken as positive and negative control respectively. Growth was evaluated and inhibition zone were measured. All the experiments were repeated twice and data presented are average of three independent readings.

RESULTS AND DISCUSSION

Cladonia sp. extracts inhibited all the tested bacteria. Aqueous extract was inhibitory only against *Alcaligens faecalis* whereas ethanolic extracts could inhibit all the tested bacteria except *B. subtilis* (Table 1). *Everniastrum* sp. was found to be inhibitory only against two of the Gram positive bacteria i.e., *B. subtilis* and *S. aureus* (Table 2). Aqueous extracts of *Parmelia* sp. inhibited only gram positive bacteria (*B. megaterium* and *S.aureus*, rest of the extracts were inhibitory against both Gram-positive and Gram-negative bacteria (Table 3). Ethanolic fraction of *Stereocaulon* sp. inhibited all the tested bacteria except *S. mutans* (Table 4) whereas *B. subtilis* was sensitive to all the solvent fractions. Aqueous fractions of *Usnea* sp. inhibited only *E. coli* and ethanolic extract could inhibit seven of the tested bacteria and *S. mutans* was not inhibited (Table 5).

Table 1: Antibacterial activity of *Cladonia* sp. against test organisms

Test organisms	Diameter of inhibition zone (mm)						
	* SDW	STR	AQE	ETE	MTE	PEE	CHE
E.coli	-	18	-	13	-	10	11
E. aerogenes	-	19	-	14	20	12	10
P. aeruginosa	-	19	-	10	16	12	15
A. faecalis	-	18	9	8	-	-	-
B. subtilis	-	19	-	15	10	8	12
B. megaterium	-	19	-	-	10	9	-
S. mutans	-	18	-	9	-	-	7
S. aureus	-	19	-	11	17	8	10

* SDW (Sterile distilled water), STR (Streptomycin -10µg/ml), AQE (Aqueous extract), ETE (Ethanolic extract), MTE (Methanolic extract), PEE (Petroleum ether extract), CHE (Chloroform extract)

Table 2: Antibacterial activity of *Everniastrum* sp. against test organisms

Test organisms	Diameter of inhibition zone (mm)						
	* SDW	STR	AQE	ETE	MTE	PEE	CHE
E.coli	-	18	-	-	-	-	-
E. aerogenes	-	19	-	-	-	-	-
P. aeruginosa	-	19	-	-	-	-	-
A. faecalis	-	18	-	-	-	-	-
B. subtilis	-	19	-	-	-	-	-
B. megaterium	-	19	-	10	10	-	15
S. mutans	-	18	-	-	-	-	-
S. aureus	-	19	12	12	10	8	8

* SDW (Sterile distilled water), STR (Streptomycin -10µg/ml), AQE (Aqueous extract), ETE (Ethanolic extract), MTE (Methanolic extract), PEE (Petroleum ether extract), CHE (Chloroform extract)

Table3: Antibacterial activity of *Parmelia* sp. against test organisms

Test organisms	Diameter of inhibition zone (mm)						
	* SDW	STR	AQE	ETE	MTE	PEE	CHE
E.coli	-	18	-	10	8	19	-
E. aerogenes	-	19	-	8	-	16	-
P. aeruginosa	-	19	-	11	20	9	8
A. faecalis	-	18	-	-	8	-	-
B. subtilis	-	19	-	-	8	-	8
B. megaterium	-	19	8	10	9	8	-
S. mutans	-	18	-	9	-	-	8
S. aureus	-	19	8	8	20	8	-

* SDW (Sterile distilled water), STR (Streptomycin -10µg/ml), AQE (Aqueous extract), ETE (Ethanolic extract), MTE (Methanolic extract), PEE (Petroleum ether extract), CHE (Chloroform extract)

Table 4: Antibacterial activity of *Stereocaulon* sp. against test organisms

Test organisms	Diameter of inhibition zone (mm)						
	* SDW	STR	AQE	ETE	MTE	PEE	CHE
E.coli	-	18	10	8	-	-	-
E. aerogenes	-	19	-	8	-	-	-
P. aeruginosa	-	19	-	8	9	-	-
A. faecalis	-	18	-	10	-	-	-
B. subtilis	-	19	17	9	9	12	14
B. megaterium	-	19	8	10	-	8	-
S. mutans	-	18	-	-	-	-	-
S. aureus	-	19	-	10	8	8	-

* SDW (Sterile distilled water), STR (Streptomycin -10µg/ml), AQE (Aqueous extract), ETE (Ethanolic extract), MTE (Methanolic extract), PEE (Petroleum ether extract), CHE (Chloroform extract)

Table 5: Antibacterial activity of *Usnea* sp. against test organisms

Test organisms	Diameter of inhibition zone (mm)						
	* SDW	STR	AQE	ETE	MTE	PEE	CHE
E.coli	-	18	8	8	12	17	15
E. aerogenes	-	19	-	10	-	13	-
P. aeruginosa	-	19	-	12	12	15	10
A. faecalis	-	18	-	14	10	23	17
B. subtilis	-	19	-	12	10	19	14
B. megaterium	-	19	-	10	15	19	14
S. mutans	-	18	-	-	-	-	-
S. aureus	-	19	-	11	17	-	8

* SDW (Sterile distilled water), STR (Streptomycin - 10µg/ml), AQE (Aqueous extract), ETE (Ethanolic extract), MTE (Methanolic extract), PEE (Petroleum ether extract), CHE (Chloroform extract)

Methanolic extract of *Cladonia* sp. was found to be more potent than Streptomycin against *E. aerogenes* (Table 1) and similarly methanolic extract of *Parmelia* sp. also showed larger inhibition zone than standard drug Streptomycin against *Pseudomonas* and *Staphylococcus aureus* (Table 4).

Aqueous extracts of all investigated lichens were less inhibitory to the test bacteria. Earlier studies did not find any antibacterial properties of lichens extracts in water (Tay *et. al.*, 2004). *S. mutans* showed the greatest resistance to the investigated lichen extracts.

Extracts of all investigated lichens showed antibacterial activity. The petroleum ether extract of *Usnea* sp showed strongest antibacterial activity (inhibition zone dia 23 mm) against *A. faecalis*. *Cladonia* sp. and *Parmelia* sp. inhibited all the tested bacteria. The weakest activity was shown by extracts of *Everniastrum* sp., which inhibited only two of the tested bacteria *i. e., B. megaterium* and *S. aureus*. All gram negative bacteria were resistant to the extracts of this lichen.

Ethanol extracts exerted stronger inhibitory action followed by methanol extracts. In general, Gram-negative bacteria were more resistant than Gram-positive bacteria. Aqueous extracts manifested very little activity to the tested microorganisms but such extracts of *Stereocaulon* sp. and *Cladonia* sp. showed significant activity to *B. subtilis* and *S. aureus* respectively. The probable reason for this is that majority of active substances present in the lichen thalli are either insoluble or poorly soluble in water (Karthikaidevi *et. al.*, 2009).

There are reports of inactiveness of purified active components of *Cladonia* sp. against gram negative organisms in literature (Yilmaz *et.al.*, 2004; Lauterwein *et al.*, 1995; Ingolfsdottir, 2002)) but our experiments showed inhibitory action against all such organisms tested.

These similarities and differences in the antimicrobial activity of extracts of different lichen species probably are a consequence of the presence of different components with antimicrobial activity. The results presented here indicate that the investigated extracts manifest strong but varying antimicrobial activity, which suggests that extracted components from various lichens may prove useful in treating many diseases caused by microorganisms.

This broad variation of antimicrobial activity may be attributable to the differently soluble wide variety of bioactive compounds, such as phenolics, flavones, carotenoids and tannins, present in the selected lichen specimens. Moreover, significant differences in antibacterial activity can be attributable to extraction methods, time of collecting samples, environment, and genetic differences between tested samples (Shan

et al., 2005). This difference in sensitivity can be ascribed to morphological differences between the microorganisms, and above all to differences in permeability of the cell wall (Nostro *et. al.*, 2000).

Previous reports on the antimicrobial properties of lichens showed the resistance of Gram negative bacteria but in our investigation such group of bacteria found sensitive to four of the five lichens tested (except *Everniastrum* sp.). As gram negative bacteria are the major pathogens of gastrointestinal diseases, a further study is needed to improve the efficacy of lichen extracts against the microbes tested.

The reason why few extracts did not show antimicrobial activity in the screening may be their low quantities, probably lower than their MICs. Hence, detailed studies on the role of individual phytochemicals involved in the antibacterial activity of specific lichens are required for their use in the pharmaceutical industry.

From the results obtained in this study further research needs to be carried out to determine the exact phytochemicals (and their nature) involved in the antibacterial activity of the lichens. When these facts are harnessed, the studied lichens will surely be useful in the development of some new drugs with broad spectrum of antimicrobial activity.

ACKNOWLEDGEMENTS

The authors are grateful to University Grants Commission for providing scholarship to Miss S. Kalikotay as senior research fellow under Rajiv Gandhi National Fellowship Scheme.

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IOSR Journal of Pharmacy
e-ISSN: 2250-3013, p-ISSN: 2319-4219, www.iosrphr.org
Volume 2 Issue 6 II Nov-Dec. 2012 II PP.54-60

Screening of antioxidant activity of lichens *Parmotrema reticulatum* and *Usnea* sp. from Darjeeling hills, India

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Abstract—Till date many plants have been screened for their antioxidant activity. Oxidative stress can lead to the production of free radicals which may cause many degenerative diseases. These free radicals can be eliminated with the help of antioxidants which may be of a natural origin. The aim of this study was to examine the antioxidant activity of two common lichens namely Parmotrema reticulatum and Usnea sp from Darjeeling hills. The antioxidant assay of different concentration of ethanolic and methanolic extracts of lichens was determined with respect to five parameters i. e., DPPH radical scavenging activity, total antioxidant activity, reducing power ability, flavonoid and phenolic content. The DPPH radical scavenging ranged from 10% to 31.5% for methanol extracts of Parmotrema reticulatum and Usnea sp respectively and for reducing power measured values of absorbance varied from 0.376 to 0.514. In addition, total phenolic content of the extracts were high and total flavonoids content was moderate. Tested lichen species were found to possess considerable antioxidant activities and could be evaluated as good natural sources of antioxidants.

Keywords—Antioxidants, Parmotrema reticulatum, Usnea sp., Darjeeling.

I. INTRODUCTION

Free radicals which have one or more unpaired electrons are produced in normal or pathological cell metabolism. Reactive oxygen species (ROS) react easily with free radicals to become radicals themselves. ROS are various forms of activated oxygen, which include free radicals such as superoxide anion radicals (O^{-2}) and hydroxyl radicals (OH^{-1}), as well as non-free radical species (H_2O_2) and the singlet oxygen ($^1O_2$)[1, 2, 3, 4]. Also, excessive generation of ROS, induced by various stimuli and which exceed the antioxidant capacity of the organism, leads to a variety of pathophysiological processes such as inflammation, diabetes, genotoxicity, and cancer [5, 6, 7]. Exogenous sources of free radicals include tobacco smoke, ionizing radiation, certain pollutants, organic solvents, and pesticides [3, 8, 9, 10]. The most effective way to eliminate free radicals which cause the oxidative stress is with the help of antioxidants.

Antioxidants, both exogenous and endogenous, whether synthetic or natural, can be effective in preventing free radical formation by scavenging and suppressing such disorders [11, 12-14]. Currently, there is a growing interest toward natural antioxidants of herbal resources [10-12]. Epidemiological and in vitro studies on medicinal plants and vegetables strongly supported this idea that plant constituents with antioxidant activity are capable of exerting protective effects against oxidative stress in biological systems [13-16].

Lichens are the symbiotic organisms including a fungal partner and an algal partner and are known to have therapeutic effects on various diseases in folk medicine of many countries. Recently, much attention has been paid to lichens as resources of natural antioxidants. Scientist already investigated the antioxidant activity of some species of lichens, such as *Bryoria fuscescens, Cetraria islandica, Dermatocarpon intestiniformis, Parmelia saxatilis, Peltigera rufescens, Platismatia glauca, Ramalina pollinaria, R. polymorph, Umbilicaria nylanderiana, Usnea ghattenis,* and *U. longissima* and some of them have very good antioxidant activity [17, 18, 19, 20].

India is a rich centre of lichens diversity, contributing nearly 15% of the 13500 species of lichens so far recorded [21]. Darjeeling hills, one of the picturesque hill stations of Eastern Himalayas is situated at coordinates 27°13'N to 26° 27'N and 88°53'E to 87°58'E. It has an area of 3,149 sq km. Its annual mean maximum temperature is 14.9°C and annual mean minimum temperature is 8.9°C and average annual rainfall is 3092mm.

Lichens occur in abundance in Darjeeling Hills of West Bengal, India. Hence this work was set out in order to screen the antioxidant activity of two common lichen species namely *Parmotrema reticulatum* and *Usnea* sp of Darjeeling hills.

II. MATERIALS AND METHODS

2.1 Collection and identification of lichen materials:

The lichen specimens was collected from the trees growing around Darjeeling town and characterised with the help of their morphology, anatomy, colour reaction, thin layer chromatography and identified from National Botanical Research Institute, Lucknow as *Parmotrema reticulatum* 08-0017193 (LWG) and *Usnea* sp 09-0017196 (LWG). Specimen samples namely SK1 and SK2 are preserved in the Herbarium of Darjeeling Government College, Darjeeling.

2.2 Extract preparation:

Lichen specimens were air dried at room temperature to constant weights. The dried plant materials were ground separately to powder. Ten grams of each powdered lichen material were extracted in methanol and ethanol (50 ml each) by shaking for 48 hours in shaking incubator (Orbital Shaking Incubator MSW232, Macro Scientific Works, New Delhi, INDIA). Each extract was filtered with Tarsons Nylon Membrane Filter 66, Riviera, Kolkata of pore size 450nm. Each filtrate was evaporated and residue obtained stored in refrigerator.

2.3 Chemicals used

1,1-Diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, aluminum nitrate, potassium acetate, quercetin, Folin-Ciocalteus's phenol reagent, sodium carbonate, tannic acid, potassium ferricyanide, phosphate buffer, trichloroacetic acid, ferric chloride, butylated hydroxytoluene (BHT), sodium phosphate, ammonium molybdate, α-tocopherol were obtained HiMedia Laboratories, Mumbai, INDIA. All the chemicals used including the solvents were of analytical grade.

2.4 DPPH radical scavenging assay

The effect of the extracts on DPPH radical was estimated using the method of Liyana- Pathiranan and Shahidi [26]. A solution of 0.135 mM DPPH in methanol was prepared and 1.0 ml of this solution was mixed with 1.0 ml of extract in methanol containing 0.02–0.1 mg of the extract. The reaction mixture left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid was used as reference. The ability to scavenge DPPH radical was calculated by the following equation: DPPH radical scavenging activity (%) = [(Abs control – Abs sample)]/ (Abs control) x 100 where Abs control is the absorbance of DPPH radical + methanol or ethanol; Abs sample is the absorbance of DPPH radical + sample extract /standard.

2.5 Determination of ferric reducing antioxidant power (FRAP)

Reducing power of both extracts of the lichen specimens were measured by method of Oyaizu [28] with slight modifications. According to this method the reduction of Fe^{3-} to Fe^{2-} was determined by measuring absorbance of the Perl's Prussian blue complex. This method is based on the reduction of (Fe^{3-}) ferricyanide in stoichiometric excess relative to the antioxidants. For this purpose, different concentrations (25,50,100 and 200 µg/mL) of lichen extracts in ethanol and methanol, and standard antioxidants (BHT) was added to the each tube, volumes were adjusted with distilled water to 0.75 mL, separately. Then, they were mixed with 1 ml of 200 mM sodium phosphate buffer (pH 6.6) and 1 ml (1%) of potassium ferricyanide [$K_3Fe(CN)_6$]. After 20 min of incubation at 50°C, the reaction mixtures were acidified with 1 ml of trichloroacetic acid (10%). Finally, 0.25 ml of $FeCl_3$ (0.1%) was added to this solution. Distilled water was used as control. Absorbance of these mixtures was measured at 700 nm using spectrophotometer [29]. Decreased absorbance indicates ferric reducing power capability of sample [30].

2.6 Determination of total phenolics

Using modified Folin-Ciocalteu method [24], total phenolic contents of the extracts were determined. An aliquot of the extract was mixed with 5 ml Folin-Ciocalteu reagent (previously diluted with water at 1:10 v/v) and 4 ml (75 g/l) of sodium carbonate. The tubes were vortexed for 15 sec and allowed to stand for 30 min at 40°C for color development. Absorbance was then measured at 765nm in a spectrophotometer (UV-1700 PharmaSpec UV-VS Spectrophotometer, Shimadzu, Japan). Samples of extracts were evaluated at a final concentration of 0.1 mg/ml. Total phenolic content were expressed as mg/g tannic acid equivalent using the equation based on the calibration curve: y = .007x-0.186, R2= 0.938, where x was the absorbance and y was the tannic acid equivalent (mg/g).

2.7 Determination of total flavonoids

Estimation of the total flavonoids in the plant extracts was carried out using the method of Ordon Ez et al [25]. To 0.5 ml of sample, 0.5 ml of 2% $AlCl_3$ ethanol solution was added. After one hour at room temperature, the absorbance was measured at 420 nm. A yellow colour indicated the presence of flavonoid. Extract samples were evaluated at a final concentration of 0.1 mg/ml. Total flavonoid content was calculated as quercetin (mg/g) using the following equation based on the calibration curve: y = 0.356x-0.461, R2 = 0.697, where x was the absorbance and was the quercetin equivalent (mg/g).

2.8 Determination of total antioxidant capacity

The total antioxidant capacity of lichen extracts, and its different fractions was evaluated by the method of Prieto et al. [27]. An aliquot of 0.1 ml of sample (100 µg) solution was combined with 1 ml of reagent (0.6 M

sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95° C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank in a spectrophotometer (UV-1700 PharmaSpec UV-VS spectrophotometer, Shimadzu, Japan). A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as rest of the sample. For samples of unknown composition, antioxidant capacity was expressed as equivalents of α-tocopherol (mg/g).

2.9 Statistical analysis

All analysis was done in triplicates. Data were analyzed in Microsoft EXCEL-2007 by taking triplicates and thus mean and Standard Deviation (SD) obtained.

III. RESULTS AND DISCUSSIONS

3.1 DPPH Radical Scavenging Activity

Free radicals contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS [26, 29]. The synthetic antioxidants like BHA, BHT, gallic acid esters etc., have been suspected to cause or prompt negative health effects. Strong restrictions have been placed on their application [30, 31]. In recent years much attention has been devoted to natural antioxidant and their association with health benefits [32]. The antioxidant activity of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging [33]. Numerous antioxidant methods and modifications have been proposed to evaluate antioxidant activity and to explain how antioxidants function. Of these, total antioxidant activity, reducing power, DPPH assay, metal chelating, ROS quenching assays are commonly used for evaluation of antioxidant activities of extracts [34, 35].

Table 1. DPPH radical scavenging of the ethanol and methanol extracts of the lichen *Parmotrema reticulatum*, *Usnea* sp and ascorbic acid.

	control	0.750±0.0057	-
	25	0.638±0.0057	16
	50	0.562±0.0057	26
	100	0.481±0.0057	36.7
	200	0.231±0.0057	69.6
***Usnea* sp (M)**	control	0.76±.0.057	-
	25	0.63±0.0057	17
	50	0.61±0.0057	19.4
	100	0.57±0.0057	24.4
	200	0.52±0.0057	31.5
***Usnea* sp (E)**	control	0.82±0.0057	-
	25	0.71±0.001	12.8
	50	0.62±0.0057	24.3
	100	0.51±0.00057	37.1
	200	0.41±0.0057	49.7
***Parmotrema reticulatum* (E)**	control	.43±.00057	
	25	0.363±0.00057	16.5
	50	0.359±0.00015	17
	100	0.356±0.002	18.1
	200	0.351±0.002	19.3
***Parmotrema reticulatum* (M)**	control	0.796±.0005	
	25	.716±.0005	10.1
	50	.667±.0005	15.5
	100	.619±0.321	21.6
	200	.611±.0011	22.6

Data represented as mean±SD of three independent readings

E- Ethanolic extract; M- Methanolic extract

The change in absorbance produced by reduced DPPH was used to evaluate the ability of test compounds to act as free radical scavengers. It was observed (Table:1) that methanolic extracts of *Parmotrema reticulatum* and *Usnea* sp showed potent radical scavenging activity with IC50 value of 4.39 µg/ml and 3.21 µg/ml respectively, the IC50 value of ethanolic extracts of Usnea sp and Parmotrema reticulatum are 4.023µg/ml and 2.95µg/ml respectively. Removal of free radical increased by 10.1 to 49.7 % in accordance with the increase of the concentrations of the extract from 25µg/ml to 200µg/ml, compared to the negative control and moreover, the scavenging ability of the extract was as moderately less as that of ascorbic acid at all the concentrations tested. Significant correlation was found between the free radical scavenging activity and the concentration of lichen extract or the compound used as positive control. The DPPH assay is a widely used method to evaluate antioxidant activities in a relatively short time compared to other methods [36]. The reduction capability on the DPPH radical is determined by the decrease in its absorbance at 517 nm. The decrease in absorbance of DPPH radical caused by antioxidants is due to the reaction between antioxidant molecules and radical, which results in the scavenging of the radical by hydrogen donation. This is visualized as a discoloration from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate antioxidant activity [37, 38]. In this study, a significant decrease in the concentration of DPPH radical due to the scavenging ability of the extract was observed. Though the DPPH radical scavenging abilities of the lichen extracts was less than that of standard, the study showed that the extracts has proton-donating ability and could serve as a natural antioxidant.

3.2 Reducing power assays

Reduction capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Antioxidant compounds are able to donate electrons to reactive radicals, reducing them into more stable and unreactive species [18, 19].The reducing power of the extracts was compared with standard Butylatedhydroxytoluene (BHT) and it increased with increasing concentration of the extracts. The methanolic extracts of *Parmotrema reticulatum* showed high reducing ability with absorbance of 0.491 at concentration of 300µg/ml and absorbance of methanolic extracts of *Usnea* sp was highest as 0 .514 at 300µg/ml. Antioxidant compounds cause the reduction of ferric (Fe^{3+}) form to the ferrous (Fe^{2+}) form because of their reductive capabilities. Prussian blue coloured complex formed by adding $FeCl_3$ to the ferrous (Fe^{2+}) form. Therefore, reduction can be determined by measuring the formation of Perl's Prussian blue at 700 nm [20, 30]. In this assay, yellow colour of the test solution changes to green or blue colour depending on the reducing power of antioxidant samples. A higher absorbance indicates a higher ferric reducing power.

Table 2: Reducing power of extracts of lichen *Parmotrema reticulatum*, *Usnea* sp and BHT.

	Concentration in µg/ml	O.D value
BHT	50	.555±.002
	100	.751±.0005
	200	.815±.001
	300	.893±.004
***Usnea* sp (E)**	50	.413±.001
	100	.421±.001
	200	.425±.002
	300	.434±.002
***Usnea* sp (M)**	50	.402±.001
	100	.411±.001
	200	.502±.003
	300	.514±.003
***Parmotrema reticulatum* (E)**	50	.195±.003
	100	.205±.003
	200	.342±.003
	300	.376±.002
***Parmotrema reticulatum*(M)**	50	.363±.001
	100	.394±.002
	200	.406±.001
	300	.491±.002

Data represented as mean±SD of three independent readings
E- Ethanolic extract; M- Methanolic extract

3.3 Total phenolic and flavonoids contents

Table 3: Total phenolic and flavonoid content of lichen Parmotrema reticulatum and Usnea sp

Parmotrema reticulatum(E)	113 ±1	1.42±0.01
Parmotrema reticulatum(M)	151 ±0.577	1.38±0.0057
Usnea sp(E)	110 ±0.577	1.498±0.001
Usnea sp(M)	148 ±0.577	1.543±0.0057

Data represented as mean±SD of three independent readings
E- Ethanolic extract; M- Methanolic extract

Results obtained in the present study revealed that the level of these phenolic compounds in the ethanol extracts of the *Parmotrema reticulatum* and *Usnea* sp were considerable. The phenolic content of methanolic extracts of *Parmotrema reticulatum* and *Usnea* sp are 151µg and 148µg tannic acid equivalent. Polyphenolic compounds are known to have antioxidant activity and it is likely that the activity of the extracts is due to these compounds [31, 32]. This activity is believed to be mainly due to their redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [33]. In fact, many medicinal plants contain large amounts of antioxidants such as polyphenols. Many of these phytochemicals possess significant antioxidant capacities that are associated with lower occurrence rates of several human diseases [34, 35]. Phenols are very important constituents because of their scavenging ability due to the presence of hydroxyl groups [39].

Flavonoids are wide group of natural compounds and also the most important natural phenolics. These compounds have a large number of biological and chemical activities including radical scavenging properties [40]. The amount of flavonoids in the lichen extracts were very less such as 1.54µg and 1.49µg for ethanolic and methanolic extracts of *Usnea* sp respectively

3.4 Total antioxidant activity

The phosphomolybdenum method has been used to investigate the total antioxidant capacity of the extracts. This method is quantitative, since the total antioxidant capacity is expressed as α- tocopherol equivalents. The ethanolic and methanolic extracts of *Parmotrema reticulatum* contained 0.781 and 1.58 µg vitamin E equivalent /mg and that of *Usnea* sp was 2.025µg and 0.690µg vitamin E equivalent /mg respectively. In fact, many medicinal plants contain large amounts of antioxidants such as polyphenols. Many of these phytochemicals possess significant antioxidant capacities that are associated with lower occurrence rates of several human diseases [34,35].

Table 4. Total antioxidant activity of lichen *Parmotrema reticulatum* and *Usnea* sp.

Parmotrema reticulatum (E)	0.781±.00057
Parmotrema reticulatum (M)	1.58 ±.00577
Usnea sp(E)	2.025 ±.001
Usnea sp(M)	0 .690 ±.001

Data represented as mean±SD of three independent readings
E- Ethanolic extract; M- Methanolic extract

IV. CONCLUSION

The investigation for bioactive compounds from natural resources to improve pharmaceutical, cosmetic and agriculture applications is extremely advancing till today. The lichen compounds are also being investigated for its phytochemical properties. Peroxidation (auto-oxidation) of lipids exposed to oxygen is responsible not only for deterioration of foods (rancidity) but also damage to tissue in vivo, where it may be a cause of cancer, inflammatory disease, ageing etc. Thus there is an increase in demand for drugs of natural origin for healing several diseases. On the basis of the results, it can be concluded that tested lichen extracts show a strong antioxidant activity in vitro. The intensity of antioxidant activity depended on the tested lichen species and the solvent that used for extraction. Different antioxidant activities of different solvents depend on their different capabilities to extract bioactive substances [41]. Ethanolic and methanolic extracts of *Parmotrema reticulatum* and *Usnea* sp possessed potent antioxidant activity and DPPH radical scavenging activity. Presence of an appreciable amount of phenol and flavonoid content could suggest for the use of these extracts as natural source of antioxidants. Further, the bioactive substances from these samples are under investigation.

ACKNOWLEDGEMENTS

We are grateful to the University Grant Commission for providing financial assistance to Miss Sujata Kalikotay under Rajiv Gandhi National Fellowship Scheme. We acknowledge Department of Science and Technology, Govt. of India for providing instrumentation facility to the department under DST-FIST scheme.

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