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ARTICLES

Diversity, nutritional composition and medicinal potential of Indian mushrooms: A review

Hrudayanath Thatoi and Sameer Kumar Singdevsachan

Nanosilver: Potent antimicrobial agent and its biosynthesis

Vikas Sarsar, Krishan K. Selwal and Manjit K. Selwal

Genetic polymorphism of milk protein variants and their association studies with milk yield in Sahiwal cattle

Shahlla N Mir, Obaid Ullah and Riazuddin Sheikh

Selection and optimization of extracellular lipase production using agro-industrial waste

Pabline Rafaella Mello Bueno, Tatianne Ferreira de Oliveira, Márcio Caliar, Gabriel Luis Castiglioni and Manoel Soares Soares Júnior

New fruit and seed disorders in Papaya (*Carica papaya* L.) in India

P. L. Saran, R. Choudhary, I. S. Solanki and P. R. Kumar

Development of specific primers for the detection of HVA1 from barley in transgenic durum wheat by polymerase chain reaction (PCR) technology

Marouane Melloul, Driss Iraqi, Sripada M Udupa, My Abdelaziz El Alaoui, Sanaa Amine Alaoui, Mohammed Ibriz and Elmostafa Elfahime

Age-dependent prevalence of *Loa loa* microfilaremia and microfilaremia status as defined by two markers: microfilaria and specific IgG4

Djikeussi, E. D., and Akue, J. P.

Phytochemical and antimicrobial study on the leaf extracts of *Erythrophleum africanum* (Caesalpinaceae)

M. Mohammed, A. M. Musa, M. A. Garba, A. A. Adeiza and U. A. Hanwa

Table of Contents: Volume 13 Number 4, 22 January, 2014

Seasonal variation in phytochemicals and antioxidant activities in different tissues of various *Broccoli* cultivars

Shiva Ram Bhandari and Jung-Ho Kwak

Antibacterial activity of honey and medicinal plant extracts against Gram negative microorganisms

Ramalivhana JN, Obi CL, Samie A, Iweriebor BC, Uaboi-Egbenni P, Idiaghe J.E. and Momba M. N. B

Establishment of a heterologous immunoassay for detecting enrofloxacin residues in poultry muscles

Jin-qing Jiang

Review

Diversity, nutritional composition and medicinal potential of Indian mushrooms: A review

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Mushrooms are the higher fungi which have long been used for food and medicinal purposes. They have rich nutritional value with high protein content (up to 44.93%), vitamins, minerals, fibers, trace elements and low calories and lack cholesterol. There are 14,000 known species of mushrooms of which 2,000 are safe for human consumption and about 650 of these possess medicinal properties. Among the total known mushrooms, approximately 850 species are recorded from India. Many of them have been used in food and folk medicine for thousands of years. Mushrooms are also sources of bioactive substances including antibacterial, antifungal, antiviral, antioxidant, antiinflammatory, anticancer, antitumour, anti-HIV and antidiabetic activities. Nutraceuticals and medicinal mushrooms have been used in human health development in India as food, medicine, minerals among others. The present review aims to update the current status of mushrooms diversity in India with their nutritional and medicinal potential as well as ethnomedicinal uses for different future prospects in pharmaceutical application.

Key words: Mushroom diversity, nutritional value, therapeutic potential, bioactive compound.

INTRODUCTION

Mushroom is a general term used mainly for the fruiting body of macrofungi (Ascomycota and Basidiomycota) and represents only a short reproductive stage in their life cycle (Das, 2010). Mushroom can be epigeous or hypogeous, large enough to be seen with the naked eyes and can be picked by hand (Chang and Miles, 1992). From the taxonomic point of view, mainly basidiomycetes but also some species of ascomycetes are mushroom forming fungi. Total mushrooms on the earth are estimated to be 140,000 species in which 10% (14,000 approximately) are known. Assuming that the proportion of useful mushrooms among the undiscovered and

unexamined mushrooms will be only 5%, implies that there are 7,000 yet undiscovered species, which if discovered will be provided with the possible benefit to mankind (Hawksworth, 2001).

Mushrooms have a long association with humankind and provide profound biological and economical impact. From ancient times, wild mushrooms have been consumed by man with delicacy probably, for their taste and pleasing flavor (Das, 2010). They have rich nutritional value with high content of proteins, vitamins, minerals, fibers, trace elements and low/no calories and cholesterol (Agahar-Murugkar and Subbulakshmi, 2005; Wani et al., 2010).

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Abbreviations: NEH, North-eastern hills; FRAP, ferric reducing antioxidant power; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2-azobis-3-ethylbenzthiazoline-6-sulphonilic acid; RPA, reducing power ability; FRS, free radical scavenging activity; NOS, nitric oxide synthase; EAC, Ehrlich's ascites carcinoma; DLA, Dalton's Lymphoma Ascites.

Many of them have been used in folk medicine for thousands of years. Some of them are nutraceuticals (natural food having potential value in maintaining good health and boosting immune system of the human body) while others can produce potent nutraceuticals (compounds that have medicinal and nutritional attributes and are consumed as medicines in the form of capsules or tablets but not as food) (Elmastas et al., 2007; Ribeiro et al., 2007). Mushrooms are known to be rich sources of various bioactive substances like antibacterial, antifungal, antiviral, antiparasitic, antioxidant, antiinflammatory, antiproliferative, anticancer, antitumour, cytotoxic, anti-HIV, hypocholesterolemic, antidiabetic, anticoagulant, hepatoprotective compounds, among others (Wasser and Weis, 1999; Lindequist et al., 2005; Ajith and Janardhanan, 2007). Out of approximately 14,000 known species, 2,000 are safe for human consumption and about 650 of these possess medicinal properties (Rai et al., 2005). In developing countries like India with rich biodiversity, mushrooms are a boon for progress in the field of food, medicine and unemployment because of several nutraceuticals and medicinal mushrooms that have been found to be useful towards human health development as food, medicine, minerals and drugs among others. (Rai et al., 2005; Sheena et al., 2005; Wani et al., 2010). The present review provides information on mushroom diversity in India and their nutritional and therapeutic importance of various Indian mushrooms towards human health and benefits such as food, medicine, minerals, drugs among others.

MUSHROOM DIVERSITY IN INDIA

In India the total recorded mushrooms are approximately 850 species (Deshmukh, 2004). There are references to the use of mushrooms as food and medicine in India in the ancient medical treatise, Charaka Samhita (3000±500 BC). However, the scientific study of mushrooms in India started with the identification and description of *Podaxis pistillaris* (L.: Pers.) by Linnaeus in the 18th century which was collected and sent by Koenig from Tamil Nadu State. Later, Sir J.D. Hooker made extensive collection mostly from Assam, Darjeeling, Sikkim and Khasi hills which led to the publication of a series of papers by an English mycologist, Revd M.J. Berkeley between 1850 and 1882 (Natarajan, 1995).

Collection and scientific study of mushrooms in India really began during the 19th century and continued till date (Kaul, 2002). The period can be divided into three phases. The first phase lasted from 1825 to 1899 and in addition to Berkeley and Montagne, recorders during this phase included Fries, Léveillé, Currey, Cooke, Masee, Watt and Lloyd (Sathe, 1979; Natarajan, 1995). The second phase (1900-1969) started with Paul Henning's significant contributions which have described another 32 genera and 68 species from India (Natarajan, 1995). A

significant feature of the second phase was the involvement, besides European and American workers, of a number of Indian workers in research on higher fungi (Sathe, 1979). Special mention should be made of the work on Indian fungi by E.J. Butler at Pusa (Bihar) in the post of Imperial Mycologist who has produced the first authoritative list, *Fungi of India*, in collaboration with G.R. Bisby (Butler and Bisby, 1931). This publication was updated until the latest edition by Sarbhoy et al. (1996). Notable Indian workers of this period were Professor S.R. Bose (Calcutta, West Bengal) and Professor K.S. Thind (Punjab University, Chandigarh). The third phase of the work is said to have started in the early 1970s and continuing till date provided much needed impetus with the development of an edible mushroom industry in India. After that, several researchers are continuing their study all over India on mushroom diversity and their uses as food and medicine (Patil et al., 1995; Swapna et al., 2008; Das, 2010; Sachan et al., 2013). Details of region wise studies undertaken on mushroom diversity in India have been discussed below.

North India

Several reports on higher fungi and mushroom have been conducted from northern India which includes North Western region, Eastern Himalaya proper and North-Eastern hilly areas. North western region of India includes Punjab, Haryana, Chandigarh and Gujarat while the Eastern Himalaya proper includes the northern parts of Assam, the whole of Arunachal Pradesh and Sikkim, and North-Eastern covers the hilly states of Nagaland, Meghalaya, Manipur, Mizoram and Tripura (Khoshoo, 1992). Berkeley in 1876 was probably the first to report higher fungi from the Kashmir valley. Later, T. N. Kaul and his group provided fragmentary records on higher fungi in the late 1960s working at the Regional Research Laboratory, Srinagar and Kashmir. Due to sustained work carried out by Kaul, Kapoor and Abraham from Northern India, 262 higher fungi have been recorded from Kashmir valley, among which 226 taxa were agarics (Abraham, 1991). They described a number of species of *Coprinus*, *Morchella*, *Pleurotus*, *Lycoperdon*, *Calvatia* and *Helvella*. A significant contribution to their study was made by Professor Watling from Edinburgh, UK, who, besides providing constant guidance to these workers, published a list of 119 species of higher fungi from the Kashmir valley, based on his personal collection (Watling and Gregory, 1980).

North-Western Himalaya has been the centre of intensive research on higher fungi since the 1950s. An edible species of *Agaricus*, namely *Kbasianulosus* was first reported from Punjab of North-West Himalaya by Paracer and Chahal (1962). Now there are two active centers (Department of Botany, Punjab University,

Chandigarh and Punjabi University, Patiala) of research on macrofungi in this region and collections have mostly been made from North-West Himalaya. Professor K. S. Thind (Punjab University, Chandigarh) has published a series of papers on operculate discomycetes, particularly *Pezizales*. A total of 226 operculate discomycetes have been recorded from India so far; the major contribution from Thind and his associates. Thind and his associates have also worked on clavarioid homobasidiomycetes in the Himalaya, recording 181 taxa in 20 genera from Indo-Himalaya (Thind, 1961; Kaul, 1992). Thind and his colleagues have also published a series of papers on the *Polyporaceae* of India, and later Rattan (1977) recorded 198 species of resupinate aphylophoroid taxa from North-West Himalaya.

A number of reports on mushroom from North Western Himalaya have been provided by Atri and Saini since 1988 to till date from Department of Botany, Punjab University, Patiala (second center). Atri and Saini (1989) reviewed work on the *Russulaceae* worldwide including the Indian components. They have described many species of mushrooms which include *Russula*, *Lactarius* (Saini et al. 1988, Atri et al. 1991a), *Agaricus campestris* (Atri et al. 1991b), *Termitomyces* (Atri et al., 1995), *Agaricales* and *Gasteromycetes* (Saini and Atri, 1995), *Lepiota* (Atri et al., 1996). Atri et al. (1997) also studied the taxonomy, distribution, ecology and edibility of 30 taxa of genus *Russula* which are new records from India. To date only 81 taxa (55 of *Russula* and 26 of *Lactarius*) have been recorded from India.

Some fleshy fungi from Himachal Pradesh were described by Sohi et al. (1964). They prepared a list of 15 fleshy fungi of which 3 (viz; *Macrolepiota procera*, *Cantharellus minor* and *Cantharellus cibarius*) were noted as edible. Sohi et al. (1965) also described 10 species in which four belonging to *Morchella* (viz; *M. hybrida*, *M. angusticeps*, *M. conica*, *M. esculenta*) and two of *Helvella* (viz; *Emitra*, and *E. crispa*) are edible. Three important centers of work on macrofungi in the state of Himachal Pradesh are the Biosciences Department, University of Himachal Pradesh, Shimla; the Agricultural and Horticultural University, Solan, and the National Research Centre on Mushrooms (ICAR), Solan. Professor Lakhanpal, working at the University of Himachal Pradesh, Shimla, has made a major contribution with a list of 190 species of *Agaricales* occurring over the entire North-west Himalayan region (Lakhanpal, 1995). Agarwal et al. (1984) made additions to fleshy fungi of India by recording *Collybia albijorida*, *Calvaria flava*, *Amanita phalloides*, *Lysurus borealis* and *Calyatia* species from Palampur district of Himachal Pradesh. A review of Himalayan *Agaricales* was also conducted by Lakhanpal (1993) and listed all genera by family and the number of species present in India and their distribution in both north-western and eastern Himalaya. An attempt has been made by Pande et al. (2004) to give an assessment of the species diversity of

epigeous ectomycorrhizal fungi of the temperate forests of Western Himalaya, based on studies carried out in this region. They have reported several major genera in terms of species of *Amanita* (15 sp.), *Russula* (13 sp.), *Boletus* (12 sp.), *Lactarius* (9 sp.), *Hygrophorus* (4 sp.) and *Cortinarius* (4 sp.).

A new record of twenty eight species of the macrofungi distributed in eighteen genera belonging to ten families of the order *Agaricales* have been reported by Upadhyay et al. (2007) from North Western Himalaya, India (Table 1). Futher, Vishwakarma et al. (2011) have reported some medicinal mushrooms (*Ganoderma lucidum*, *A. campestris*, *Hydnum repandum*, *Coprinus comatus*, *M. esculenta* and *Cantharellus cibarius*) from Garhwal Himalaya, Uttarakhand, India. Later, macrofungal diversity in moist temperate forests of Garhwal Himalaya has reported by M.P. Vishwakarma and his group (Vishwakarma et al., 2012). As a result of their study, total 40 taxa belonging to 11 families were identified (Table 1).

Sharma and Sidhu (1991) reported the occurrence and distribution of *Geoglossaceae* in the Eastern Himalayan ranges of India. They maintained that the Himalaya in general and Eastern Himalaya and adjoining hills in particular are relatively rich in *Geoglossaceae*. They surveyed localities in and around West Bengal, Meghalaya, Assam and Arunachal Pradesh states and recorded 12 species distributed among nine genera with ecological notes (Table 1). In India as a whole, the family is represented by 48 species within nine genera. Verma et al. (1987) described fleshy fungal flora of the north-eastern hills (NEH) India from Manipur and Meghalaya belonging to the family *Auriculariaceae*, *Clavariaceae*, *Cantharellaceae*, *Tricholomataceae*, *Pluteaceae*, *Paxillaceae*, *Cortinariaceae*, *Cycoperdaceae*, and *Sclerodermataceae* of Basidiomycotina and *Halvellaceae* of Ascomycotina. Again, Verma et al. (1995) recorded the results of a macrofungal survey of the NEH and confirmed ninety five species of higher fungi. Among these, 85 species were new records from the NEH region and others were from different locations of India.

Three new species of *Lactarius* (*L. sanjappae*, *L. mukteswaricus* and *L. verbekeneae*) in different regions of Kumaon Himalaya were extensively studied and described by Das et al. (2004). Total 126 wild mushrooms from Barsey Rhododendron Sanctuary of the state Sikkim were also recently reported by Das (2010) which were enlisted with their scientific names, common names, distribution, growing period and status of edibility. Medicinally important 46 mushrooms were also highlighted with their medicinal properties. Acharya et al. (2010) have reported 151 species of *Agaricales* belonging to 42 genera from the Darjeeling and Sikkim hilly areas of Sikkim Himalaya. The number of representative species under each of the 42 genera varied with 13 genera having a single species each and the genera *Mycena* and *Collybia*, having 20 and 16 species, respectively. A total of 11 edible macrofungi

Table 1. Diversity of Indian mushrooms and their location.

Mushrooms diversity¹	Location in India	References
<i>Agaricus compestris</i>	West Bengal	Bose and Bose (1941)
<i>Cantharellus aurantiacus</i>		
<i>Cantharellus cibarius</i>		
<i>Coprinus comatus</i>		
<i>Lentinus subnudus</i>		
<i>Termitomyces microsporus</i>		
<i>Talbutinosa</i>		
<i>Volvariella terastius</i>		
Truffles and <i>Boletus</i> sp.		
<i>Volvariella diplasia</i>	Baroda State	Moses (1948)
<i>Pleurotus ostreatus</i>		
<i>Boletus crocatus</i>		
<i>Agaricus arvensis</i> and some puff-balls		
15 fleshy fungi of <i>Macrolepiota procera</i> , <i>Cantharellus minor</i> and <i>C. cibarius</i>	Himachal Pradesh	Sohi <i>et al.</i> (1964)
10 species of which four belonging to <i>Morchella</i> (viz; <i>M. hybrida</i> , <i>M. angusticeps</i> , <i>M. conica</i> , <i>M. esculenta</i>) and two of <i>Helvella</i> (viz; <i>Emitra</i> , and <i>E. crispa</i>)	Himachal Pradesh	Sohi <i>et al.</i> (1965)
<i>Calocybe indica</i> and <i>Termitomyces eurhizus</i>	West Bengal	Purkayastha and Chandra (1974; 1975)
3 species belong to <i>Calvatia</i> , 2 to <i>Lycoperdon</i> and one each to <i>Geastrum</i> and <i>Bovista</i>	West Bengal	Gupta <i>et al.</i> (1974)
58 species of <i>Agaricus</i> , <i>Amanita</i> , <i>Chlorophyllum</i> , <i>Coprinus</i> , <i>Macrolepiota</i> , <i>Pleurotus</i> , <i>Termitomyces</i> and <i>Volvariella</i>	Lucknow, Uttar Pradesh	Pathak and Gupta (1979)
<i>Collybia albijorida</i> , <i>Calvaria flava</i> , <i>Amanita phalloides</i> , <i>Lysurus borealis</i> and <i>Calyatia</i> sp.	Palampur, Himachal Pradesh	Agarwal <i>et al.</i> (1984)
<i>Agaricus argyropotamicus</i> , <i>Agaricus solidipes</i> and <i>Strophariapokhraensis</i>	Garhwal district, Uttar Pradesh	Dancholia and Bahukhandi, (1988)
12 species distributed in nine genera: <i>Cudonia</i> , <i>Leotia</i> , <i>Maasoglossum</i> , <i>Microglossum</i> , <i>Mitrula</i> , <i>Thuemenidium</i> , <i>Spathularia</i> , <i>Trichoglossum</i> and <i>Geoglossum</i> .	West Bengal, Meghalaya, Assam and Arunachal Pradesh	Sharma and Sidhu (1991)
<i>Auricularia delicata</i> , <i>A. auricula- Judea</i> , <i>A. mesentrica</i> , <i>Phellorina inquinans</i> , <i>Boletus spp.</i> , <i>Termitomyces microcarpus</i> , <i>Tstriatus</i> , <i>Pleurotus pulmonarius</i> , <i>R. ostreatus</i> , <i>P. sapidus</i> , <i>P. sajor-caju</i> , <i>Agaricus compestris</i> , <i>Volvariella bombycina</i> , <i>V. speciosa</i> and <i>Lepiota</i> spp.	Rajasthan, India	Sharma <i>et al.</i> (1992)

Table 1. Contd.

Mushrooms diversity ¹	Location in India	References
<i>Termitomyces mammiformis</i> and <i>T. tyleranus</i>	Punjab, India	Atri <i>et al.</i> (1995)
<i>Cantharellus luteocomus</i> , <i>Lentinus giganteus</i> , <i>Marasmins caryotea</i> , and <i>Pholiota ealaensis</i>	South India	Joseph <i>et al.</i> (1995)
<i>Volvariella nigrodisca</i> , <i>V. taylori</i> , <i>Vapalotricha</i> and <i>V. glandiformis</i>	Kerala, India	Pradeep <i>et al.</i> (1998)
<i>Lentinus sanjappae</i> , <i>L. mukteswaricus</i> and <i>L. verbekena</i>	Kumaon Himalaya	Das <i>et al.</i> (2004)
<i>Morchella esculenta</i> , <i>M. conica</i> (Pers.) Fr., <i>M. deliciosa</i> (Fr.) Jct., <i>M. angusticeps</i> Peck, <i>M. crassipes</i> (Vent.) Pers. and <i>M. semilibera</i> (DC.) Fr.	Himachal Pradesh, Punjab, Jammu and Kashmir and Uttaranchal	Negi (2006)
Eighteen genera belonging to ten families of the order <i>Agaricales</i> are <i>Agaricaceae</i> (<i>Agaricus</i>), <i>Amanitaceae</i> (<i>Amanita</i>), <i>Bolbitiaceae</i> (<i>Agrocybe</i>), <i>Coprinaceae</i> (<i>Lacrymaria</i>), <i>Cortinariaceae</i> (<i>Cortinarius</i> , <i>Gymnopilus</i> , <i>Phaeocollybia</i>), <i>Entolomataceae</i> (<i>Entoloma</i>), <i>Hygrophoraceae</i> (<i>Hygrotrama</i>), <i>Pluteaceae</i> (<i>Pluteus</i>), <i>Strophariaceae</i> (<i>Phollota</i>) and <i>Tricholomataceae</i> (<i>Collybia</i> , <i>Laccaria</i> , <i>Lepista</i> , <i>Calocybe</i> , <i>Clitocybe</i> , <i>Melanoleuca</i> , <i>Tricholomopsis</i>)	North Western Himalaya	Upadhyay <i>et al.</i> (2007)
<i>Ganoderma lucidum</i> , <i>Agaricus campestris</i> , <i>Hydnum repandum</i> , <i>Coprinus comatus</i> , <i>Morchella esculenta</i> , <i>Cantharellus cibarius</i>	Garhwal Himalaya, Uttarakhand, India	Vishwakarma <i>et al.</i> (2011)
<i>Xyrocomus chrysenteron</i> <i>Suilleus spraguei</i> <i>Russula aquosq</i> <i>Russula solaris</i> <i>Russula violacea</i> <i>Nictalis asterophora</i> <i>Agaricus campestris</i> <i>Macrolapiota procera</i> <i>Amanita veginata</i> <i>Amanita pantherina</i> <i>Amanita caesarea</i> <i>Termytomyces microcarpus</i> <i>Termytomyces hemi</i> <i>Ganoderma lucidum</i> <i>Thelephora caryophyllea</i> (Schaeff.) Pers., <i>Coltricia</i> <i>cinnamomea</i> (Pers.) Murr., and <i>Guepinia</i> <i>helvelloides</i>	Amarkantak Biosphere Reserve, Madhya Pradesh, India	Dwivedi <i>et al.</i> (2012)

Table 1. Contd.

Mushrooms diversity ¹	Location in India	References
Total 40 taxa belonging to 11 families. Two families and 2 taxa belonged to class- Ascomycetes viz: Family- Helvellaceae and Morchellaceae and nine families and 38 taxa to class-Basidiomycetes viz: Family-Agaricaceae, Amanitaceae, Boletaceae, Cantharellaceae, Coprinaceae, Ganodermataceae, Hydnagiaceae, Lycoperdaceae and Russulaceae.	Garhwal Himalaya, Uttarakhand, India	Vishwakarma et al. (2012)
<i>Agaricus bisporus</i> Quell <i>Agaricus campestris</i> L. <i>Auricularia auricular</i> (Hook.) <i>Cantharellus cibarius</i> Fr. <i>Ganoderma lucidum</i> (Leys ex. Fr.) karsten <i>Lenzites betulina</i> Fries <i>Lycoperdon perlatum</i> Pers. <i>Termitomyces mummiformis</i> (Heim.) <i>Tricholoma luscinum</i> Fr. <i>Tricholoma colossus</i> Fr. (Quell.) <i>Tricholoma strictipes</i> Fr	Dhemaji District, Assam, India	Gogoi and Sarma (2012)
<i>Scleroderma citrinum</i> , <i>Psilocybe subtropicalis</i> , <i>Ganoderma applanatum</i> , <i>Cyptotrama asprata</i> and <i>Entoloma serrulatum</i>	Rajouri district, Jammu & Kashmir, India	Anand and Chowdhry (2013)
<i>Gomphus floccosus</i> (Schw.) Singer <i>Tricholoma viridiolivaceum</i> stev. <i>Craterellus odoratus</i> (Schwein.) Fr. <i>Lactarius volemus</i> (Fr.) Fr. <i>Cantharellus cibarius</i> <i>Tricholoma saponaceum</i> (Fr.) P. Kumm. <i>Tricholoma</i> sp. <i>Laccaria lateritia</i> Malencon <i>Albatrellus</i> sp. <i>Ramaria</i> sp. and <i>Clavulina</i> sp. <i>Russula sharmae</i> , <i>R. dubdiana</i> and <i>R. sikkimensis</i>	Khasi hills of Meghalaya, India	Khaund and Joshi (2013)
<i>Russula emetica</i> <i>Russula delica</i> <i>Termitomyces eurrhizus</i> <i>Termitomyces</i> sp. <i>Agaricus silvaticus</i> <i>Agaricus</i> sp. <i>Volvorella volvacea</i> <i>Volvorella</i> sp. <i>Lentinus sajor-caju</i> <i>Lentinus</i> sp. <i>Pleurotus ostreatus</i> <i>Pleurotus</i> sp.	West district of Sikkim, India	Das et al. (2013)
	Similipal Biosphere Reserve, Odisha, India	Sachan et al. (2013)

Table 1. Contd.

Mushrooms diversity ¹	Location in India	References
<i>Lycoperdon</i> sp.		
<i>Calvatia gigantea</i>		

¹Mushroom diversity listed in chronological order from the year 1941 to 2013.

species (Table 1) in Dhemaji district, Assam were explored by Gogoi and Sarma (2012). Recently, Khaund and Joshi (2013) have reported diversity of wild edible mushrooms from Khasi hills of Meghalaya, India. During their study period, a total of 11 different species were identified based on morphology that belongs to 9 genera and 8 families (Table 1). Further, three new species of *Russula* (*Russula sharmae*, *R. dubdiana* and *R. sikkimensis*) from Sikkim (India) have been reported by Das et al. (2013).

Another centre of work in the northern India is the National Botanical Research Institute, Lucknow, Uttar Pradesh. Pathak and Gupta (1979) reported 58 species of agaricus from Lucknow area distributed among 25 genera. Prominent genera were *Agaricus*, *Amanita*, *Chlorophyllum*, *Coprinus*, *Macrolepiota*, *Pleurotus*, *Termitomyces* and *Volvariella*. Danchola and Bahukhandi (1988) discovered *Agaricus argyropotamicus*, *Agaricus solidipes* and *Stropharia pokhraensis* as new *Agaricus* from Garhwal district of Uttar Pradesh.

One of the important areas of northern India is Rajasthan. An intensive survey of wild mushrooms was conducted throughout the state by A. Doshi working at the Department of Plant Pathology, Rajasthan College of Agriculture for 8 years (1989-1996). Sharma et al. (1992) reported fifteen species of fungi from Rajasthan and many of these are first record of genus *Auricularia*; (*A. delicata*, *A. auricula-judea*, *A. mesentrica*), *Phellorina inquinans*, *Boletus* sp., *Termitomyces microcarpus*, *Termitomyces striatus*, *Pleurotus pulmonarius*, *Pleurotus ostreatus*, *Pleurotus sapidus*, *Pleurotus sajor-caju*, *Agaricus compestris*, *Volvariella bombycina*, *Volvariella speciosa* and *Lepiota* spp. Doshi and Sharma (1997) provided a detailed list of macrofungi occurring in the region with mycoecological notes. A total of 173 species belonging to 95 genera were recorded from this area. Most genera (18) were gasteromycetes or aphylloroid taxa (17). Special mention should be made of two edible gasteromycetes, *Phellorinia inquinans* Berk and *Podaxis pistillaris*, tonnes of which can be collected from desert areas. *P. inquinans* is associated with sand dunes in the area (Singh, 1994).

Morchella has a wide distribution in India and is very common in the temperate zones of forests in Jammu and Kashmir, Punjab, Himachal Pradesh and Uttaranchal (Negi, 2006). Six species of *Morchella* have been identified by Negi (2006), which include *M. esculenta*, *M. conica*, *Morchella deliciosa*, *M. angusticeps*, *Morchella crassipes* and *Morchella semilibera* (Table 1). Besides

that, recently Anand and Chowdhry (2013) reported five wild mushrooms (*Scleroderma citrinum*, *Psilocybe subtropicalis*, *Ganoderma applanatum*, *Cyptotrampa asprata* and *Entoloma serrulatum*) from Rajouri district of Jammu and Kashmir (JandK), India.

Central India

Mushroom research of central part of India covers Maharashtra, Madhya Pradesh, West Bengal and Odisha states. Moses (1948) identified the edible mushrooms of Baroda state including *Volvovella diplasia*, *P. ostreatus*, *Boletus crocatus*, *Agaricus arvensis* and some puff-balls. Mushroom recording in Maharashtra was neglected for a long time and only 21 species of agarics had been recorded from the state by year 1967 (Kamat et al., 1971). Intensive work in the region began only after 1974 when A.V. Sathe and his group, working at the Maharashtra Association for Cultivation of Sciences, published a series of papers mainly on *Agaricales* (Sathe, 1979; Sathe and Kulkarni, 1987). Later a comprehensive list of 231 mushrooms recorded from all regions of Maharashtra state was published by Patil et al. (1995). Recently Bhosle et al. (2010) reported 15 species and 3 varieties of *Ganoderma lucidum* (of which one variety remains unidentified) from the Western parts of Maharashtra (India) and in their study, only 9 valid *Ganoderma* species have been reported from India. Karwa and Rai (2010) also surveyed six different zones of Melghat forest of Amravati District, Maharashtra State from July 2005 to December 2008 for the availability of wild edible and medicinal mushrooms. In their study, out of total 153 species, ten species of *Agaricus* were recorded from different localities. Of these, seven species namely *Agaricus bitorquis*, *A. subrufescens*, *A. augustus*, *A. placomyces*, *A. essettei*, *A. basioanolosus* and *Agaricus* sp. nov (a new species) are being reported for the first time from this region (Table 1). Biodiversity of mushrooms of Amarkantak Biosphere Reserve, Madhya Pradesh have been reported by Dwivedi et al, (2012). They have collected 52 mushroom samples from Amarkantak region forests which were belonging to different genera out of which only 14 mushroom samples were identified up to species level (Table 1). Rests were identified only up to the genus level.

Bose (1921) reported a few edible species from undivided

Bengal. In 1940, Bose and Bose prepared a list of about 28 varieties of edible species including *A. campestris*, *Cantharellus aurantiacus*, *C. cibarius*, *C. comatus*, *Lentinus subnudus*, *T. microsporus*, *Termitomyces albuminosa*, *Volvariella terastia*, *Truffles* and *Boletus* sp. Two edible species, namely *Calocybe indica* and *Termitomyces eurhizus* have been reported by Purkayastha and Chandra (1974) from West Bengal. The former was a new species while the later was first recorded from India. Ten species of *Calvatia* and *Lycoperdon* have been incorporated in the list of edible fungi by Gupta et al. (1974). Out of the 10 species described, 7 were edible in their immature stage. Among seven edible mushroom, 3 species belong to *Calvatia*, 2 to *Lycoperdon* and one each to *Geastrum* and *Bovista*. Purkayastha devoted attention to wild edible mushrooms of West Bengal and succeeded in cultivating one of them, *C. indica* Purkayastha and Chandra. Purkayastha and Chandra (1985) compiled lists of Indian edible mushrooms which included 283 species of higher fungi. Recently a total of 14 species of fleshy mushrooms belonging to 8 genera and 6 families (Table 1) were reported by authors from Similipal Biosphere Reserve, Odisha, India (Sachan et al., 2013).

South India

Study on mushrooms in South India such as Tamil Nadu, Kerala, Karnataka and Andhra Pradesh was neglected as regards to studies on agarics until 1975 (Natarajan, 1995). The genus of *Volvariella* from Kerala, India was first time investigated by Pradeep et al. (1998). Out of ten species of *Volvariella* treated, *Volvariella nigrodisca*, *Volvariella taylori*, *Volvariella apalotricha* and *Volvariella gandiformis* are described and illustrated for the first time from Kerala by Pradeep et al. (1998). Natarajan listed 115 species of mushroom from Kerala (Kaul, 1992) and a macrofungal survey of Kerala was carried out at the Plant Pathology Department of Kerala Agricultural University at Vellayani, Thiruvananthapuram, by Ms Bhavani Devi from 1985 to 1988. The collections were made from 12 agroclimatic zones in four monsoon seasons and revealed the presence of 134 species of mushrooms (including 14 gasteromycete species) belonging to 45 genera (Bhavani Devi, 1995). Edible fruiting bodies included species of *Termitomyces*, *Volvariella*, *Pleurotus*, *Macrolepiota*, *Boletus* and *Calvatia*. *Tuber magnatum* Vitt. (Ascomycotina), a highly prized truffle, is regularly collected and consumed by tribal people in the forest area of the southern part of this state.

Staff of the Botany Department of Calicut University and the Tropical Botanic Garden and research Institute at Thiruvananthapuram were also surveyed macrofungi, but it was at only a preliminary stage. Natarajan started work at the Centre of Advanced Studies in Botany, University

of Madras in 1975 and his group had collected mushrooms from the entire southern and south-western region. They started a series entitled 'South Indian Agaricales', publishing over two dozen papers. Natarajan (1995) presented a list of 230 agaric and bolete species distributed among 67 genera from southern Indian states excluding Kerala.

A survey of macrofungi diversity has been conducted in semi-evergreen and in moist deciduous forest of Shimoga District-Karnataka, India during 2005 to 2007 by Swapna et al. (2008). In their survey, a total of 778 species of macrofungi belonging to 43 families, 101 genera were enumerated of which 242 species were identified to genus level and 73 were identified to species level. Further, Pushpa and Purushothama (2012) have studied the biodiversity of Mushrooms in and around Bangalore (Karnataka), India and recorded 90 species in 48 genera belonging to 19 families in 05 orders. Among them, 28 species were found to be recorded for the first time in India.

NUTRITIONAL POTENTIAL OF MUSHROOMS

Man has been hunting for the wild mushrooms since ancient time (Cooke, 1977). Thousands of years ago, the fruiting body of higher fungi has been used as a source of food (Mattila et al., 2001) due to their chemical composition which is attractive from the nutrition point of view. During the early days of civilization, mushrooms were consumed mainly for their palatability and unique flavors. Present use of mushrooms is totally different from traditional because, lot of research has been done on the chemical composition of mushrooms, which revealed that mushrooms can be used as a diet to combat diseases. The early history regarding the use of mushrooms in different countries has been reviewed by number of workers (Rolfe and Rolfe, 1925; Bano and Rajarathnam, 1982; Wani et al., 2010). Rolfe and Rolfe (1925) mentioned that mushrooms like *A. campestris*, *M. esculenta*, *Helvella crispa*, *Hydnum coralloides*, *Hypoxylon vernicosum* and *Polyporus mylittae* were used much earlier in India. Lintzel (1941) recommended that 100 to 200 g of mushrooms (dry weight) is required to maintain an optimal nutritional balance in a man weighing 70 kg. Several researchers have determined the nutritional value of different mushrooms. Among them, Bano et al. (1963) determined the nutritive value of *Pleurotus flabellatus* as 0.974% ash, 1.084% crude fibre, 0.105% fat, 90.95% moisture, 0.14% non-protein nitrogen and 2.75% protein. Bano (1976) suggested that food value of mushrooms lies between meat and vegetables. Gruen and Wong (1982) indicated that edible mushrooms were highly nutritional and compared favorably with meat, egg and milk food sources. Crisan and Sands (1978) observed that mushrooms in general contain 90% water,

10% dry matter with the protein content varying between 27 and 48% and carbohydrates are less than 60% and lipids are between 2 to 8%. Orgundana and Fagade (1981) indicated that an average mushroom is about 16.5% dry matter out of which 7.4% is crude fiber, 14.6% is crude protein and 4.48% is fat and oil. In case of Indian mushrooms, several reports on nutritional status of different mushrooms have been published in different manner which is discussed below.

PROXIMATE COMPOSITION

Protein and amino acids

Protein is an important constituent of mushrooms (Agrahar-Murugkar and Subbulakshmi, 2005; Wani et al., 2010). Protein content of mushrooms depends on the composition of the substratum, size of pileus, harvest time and species of mushrooms (Bano and Rajarathnam, 1982). Protein content in *Pleurotus* sp. has been documented to range between 8.9 and 38.7% on dry weight basis (Bano and Rajarathnam, 1982). Rai and Sohi (1988) also reported protein content of *Agaricus bisporus* to be 29.3% on dry weight basis. Purkayastha and Chandra (1985) found 14 to 27% crude protein on dry weight basis in *A. bisporus*, *L. subnudus*, *C. indica* and *Volvariella volvacea*. Samajipati (1978) found 30.16, 28.16, 34.7 and 29.16% protein in dried mycelium of *A. campestris*, *A. arvensis*, *M. esculenta* and *M. deliciosa*, respectively. Sharma et al. (1988) reported 14.71 to 17.37% and 15.20 to 18.87% protein in the fruiting bodies of *Lactarius deliciosus* and *Lactarius sanguifusus*, respectively. Nutritional analysis of two edible wild mushrooms (*Schizophyllum commune* and *Lentinula edodes*) from northeast India have been studied by Longvah and Deosthale (1998) and reported that protein content of *L. edodes* (26%) is much higher than the *S. commune* (16%). Nutritional values of seven wild edible mushrooms were analyzed by Agrahar-murugkar and Subbulakshmi (2005) which are commonly consumed in the Khasi hills of Meghalaya and reported that 27.3, 27.5, 21.1, 24.1, 21.1, 21.2, 19.0% protein content present in *Calvatia gigantea*, *Clavulina cinerea*, *C. cibarius*, *Ramaria brevispora*, *Russula integra*, *Gomphus floccosus* and *Lactarius quieticolor*, respectively.

Pushpa and Purushothama (2010) have analyzed the nutrition of five mushroom species and found 21.60, 41.06, 27.83, 26.25, 18.31% protein in *C. indica*, *A. bisporus*, *P. florida*, *Russula delica* and *Lyophyllum decastes*, respectively. Jagadeesh et al. (2010) analyzed the proximate composition of *V. bombycina* and found 25.5% crude protein in mycelia and 28.3% in fruit body (Table 2). Nutrient composition of *Lentinus tuberregium* in both wild and cultivated type were analyzed by Manjunathan and Kaviyarasan (2011) and found that the cultivated variety had higher concentration of protein (25%) than the

wild one (18.07%). The nutritional values of 10 edible mushrooms from Western Ghats of Kanyakumari district have been analyzed by Johnsy et al. (2011) and reported that edible mushrooms are highly valued as a good source of protein ranged from 28.93 to 39.1% of dry weight (Table 2). Manjunathan et al. (2011) reported the proximate composition of four wild mushrooms from Tamil Nadu, India in which *A. polytricha* had the highest concentration of protein (37%) and *Clitocybe* sp. had the least (24.8%). Recently, nutrient content of 15 selected mushrooms of Nagaland, India have been studied by Kumar et al. (2013) and found 22.50 to 37.80% protein (Table 2). Further, Singdevsachan et al. (2013) reported the nutrient values of two wild mushrooms (*Lentinus sajor-caju* and *Lentinus torulosus*) from Similipal Biosphere Reserve, Odisha, India where highest protein content (28.36%) was found in *L. sajor-caju* and lowest (27.31%) in *Lentinus torulosus*. However, protein contents of mushrooms were reported to vary according various factors such as mushroom strain/type, composition of growth media, time of harvest, management techniques, handling conditions, and the preparation of the substrates (Manzi et al., 2001).

In terms of the amount of crude protein, mushrooms rank below animal meats but well above most other foods including milk (Chang, 1980). Mushrooms in general have higher protein content than most other vegetables (Bano and Rajarathnam, 1988). On a dry weight basis, mushrooms normally contain 19 to 35% proteins as compared to 7.3% in rice, 12.7% in wheat, 38.1% in soybean and 9.4% in corn (Crisan and Sands, 1978; Bano and Rajarathnam, 1988). Verma et al. (1987) reported that mushrooms are very useful for vegetarians because they contain some essential amino acids which are found in animal proteins. Mushrooms contain all the essential amino acids required by an adult (Hayes and Haddad, 1976). Gupta and Sing (1991) reported 41.4% essential amino acids in *P. pistillaris*. Longvah and Deosthale (1998) also analyzed the amino acid content of two edible wild mushrooms (*Schizophyllum commune* and *L. edodes*) from northeast India and reported that 34% and 39% essential amino acids are present in *S. commune* and *L. edodes* respectively. Agrahar-murugkar and Subbulakshmi (2005) also analyzed the essential amino acid of seven wild edible mushrooms from the Khasi hills of Meghalaya and found average ranges between 16.3 (lysine) and 45.8% (methionine). The digestibility of *Pleurotus* mushrooms proteins is as that of plants (90%) whereas that of meat is 99% (Bano and Rajarathnam, 1988). The protein conversion efficiency of edible mushrooms per unit of land and per unit time is far more superior compared to animal sources of protein (Bano and Rajarathnam, 1988).

Carbohydrate

The carbohydrate content of mushrooms represents the

Table 2. Proximate composition of some Indian mushrooms shown in percentage.

Species	Protein	Carbohydrate	Lipids/fats	Ash	Fiber	References
<i>Agaricus arvensis</i>	32.87	32.91	-	0.18	0.14	Kumar et al. (2013)
<i>Agaricus bisporus</i>	41.06	28.38	2.12	7.01	18.23	Pushpa and Purushothama (2010)
<i>Agaricus bisporus</i>	33.48	46.17	3.10	5.70	20.90	Manikandan (2011)
<i>Agaricus heterocystis</i>	32.23	48.55	2.90	11.42	19.7	Manimozhi and Kaviyaran (2013)
<i>Agaricus langei</i>	35.14	34.83	-	14.10	3.28	Kumar et al. (2013)
<i>Auricularia auricula</i>	4.20	82.80	8.30	4.70	19.80	Manikandan (2011)
<i>Auricularia auricula</i>	36.3	33.23	1.63	7.07	8.4	Johnsy et al. (2011)
<i>Auricularia auricula-judae</i>	36.30	33.23	-	7.07	2.81	Kumar et al. (2013)
<i>Auricularia polytricha</i>	37.0	38.48	0.74	6.87	21.97	Manjunathan et al. (2011)
<i>Boletus aestivalis</i>	32.76	52.07	-	14.97	12.13	Kumar et al. (2013)
<i>Calocybe indica</i>	17.69	64.26	4.10	7.43	3.40	Manikandan (2011)
<i>Calocybe indica</i>	21.60	49.20	4.96	12.80	13.20	Pushpa and Purushothama (2010)
<i>Calvatia gigantea</i>	27.3	-	1.0	6.3	22.0	Agrahar-murugkar and Subbulakshmi (2005)
<i>Cantharellus cibarius</i>	21.1	-	1.6	13.2	12.8	Agrahar-murugkar and Subbulakshmi (2005)
<i>Cantharellus cibarius</i>	34.17	47.00	-	7.78	1.40	Kumar et al. (2013)
<i>Clavulina cinerea</i>	27.5	-	2.5	13.9	8.4	Agrahar-murugkar and Subbulakshmi (2005)
<i>Clitocybe sp.</i>	24.8	42.0	1.24	15.73	13.04	Manjunathan et al. (2011)
<i>Cookeina sulcipes</i>	28.93	50.20	-	6.55	0.16	Kumar et al. (2013)
<i>Flammulina velutipes</i>	17.60	73.10	1.90	7.40	3.70	Manikandan (2011)
<i>Gomphus floccosus</i>	21.2	-	5.3	8.0	9.2	Agrahar-murugkar and Subbulakshmi (2005)
<i>Grifola frondosa</i>	31.47	40.77	1.49	5.13	7.0	Johnsy et al. (2011)
<i>Hypsizygus tessulatus</i>	37.80	51.20	-	9.09	12.90	Kumar et al. (2013)
<i>Lactarius hygrophoroides</i>	44.93	42.00	-	2.00	10.58	Kumar et al. (2013)
<i>Lactarius quieticolor</i>	19.0	-	2.6	6.6	14.4	Agrahar-murugkar and Subbulakshmi (2005)
<i>Lentinus edodes</i>	32.93	47.60	3.73	5.20	28.80	Manikandan (2011)
<i>Lentinus edodes</i>	22.8	64.4	2.1	6.0	-	Longvah and Deosthale (1998)
<i>Lentinus sajor-caju</i>	28.36	68.24	02.42	04.88	-	Singdevsachan et al. (2013)
<i>Lentinus squarrosulus</i>	37.13	47.83	2.58	8.33	11.33	Johnsy et al. (2011)
<i>Lentinus tigrinus</i>	18.07	60.0	2.25	5.14	14.69	Manjunathan et al. (2011)
<i>Lentinus torulosus</i>	27.31	64.95	1.36	13.16	-	Singdevsachan et al. (2013)
<i>Lentinus tuber-regium</i>	28.93	50.2	2.17	6.56	12.17	Johnsy et al. (2011)
<i>Lepiota lilacea</i>	28.12	49.33	-	8.09	11.98	Kumar et al. (2013)
<i>Lepiota magnispora</i>	27.55	35.00	-	3.05	5.20	Kumar et al. (2013)
<i>Lepista irina</i>	26.12	50.20	-	3.16	6.08	Kumar et al. (2013)
<i>Lyophyllum decastes</i>	18.31	34.36	2.14	14.20	29.02	Pushpa and Purushothama (2010)
<i>Macrolepiota rhacodes</i>	34.31	48.0	2.25	11.80	4.78	Manjunathan et al. (2011)
<i>Melanoleuca grammopodia</i>	36.27	33.04	-	4.13	8.12	Kumar et al. (2013)
<i>Panus fulvus</i>	27.06	33.04	-	3.11	6.08	Kumar et al. (2013)
<i>Pleurotus florida</i>	27.83	32.08	1.54	9.41	23.18	Pushpa and Purushothama (2010)
<i>Pleurotus ostreatus</i>	30.40	57.60	2.20	9.80	8.70	Manikandan (2011)
<i>Pleurotus ostreatus</i>	37.63	43.4	2.47	10.17	4.2	Johnsy et al. (2011)
<i>Pleurotus pulmonarius</i>	37.63	43.40	-	10.17	4.12	Kumar et al. (2013)
<i>Pleurotus roseus</i>	30.27	42.97	2.02	5.57	4.2	Johnsy et al. (2011)
<i>Pleurotus sajor-caju</i>	39.1	38.57	1.17	5.73	4.9	Johnsy et al. (2011)
<i>Pleurotus sajor-caju</i>	19.23	63.40	2.70	6.32	48.60	Manikandan (2011)

Table 2. Continued.

Species	Protein	Carbohydrate	Lipids/fats	Ash	Fiber	References
<i>Ramaria brevispora</i>	24.1	-	1.3	10.9	8.8	Agrahar-murugkar and Subbulakshmi (2005)
<i>Russula delica</i>	26.25	34.88	5.38	17.92	15.42	Pushpa and Purushothama (2010)
<i>Russula integra</i>	21.1	-	4.5	11.5	6.4	Agrahar-murugkar and Subbulakshmi (2005)
<i>Schizophyllum commune</i>	15.9	68.0	2.0	8.0	-	Longvah and Deosthale (1998)
<i>Schizophyllum commune</i>	22.50	32.43	-	10.10	6.50	Kumar et al. (2013)
<i>Termitomyces heimii</i>	34.2	39.03	2.11	16.8	9.73	Johnsy et al. (2011)
<i>Termitomyces microcarpus</i>	29.4	46.53	2.33	11.2	11.5	Johnsy et al. (2011)
<i>Volvariella bombycina</i> (Fruit body)	28.30	38.90	2.72	10.90	24.60	Jagadeesh et al. (2010)
<i>Volvariella bombycina</i> (Mycellia)	25.50	34.75	1.15	9.03	31.80	Jagadeesh et al. (2010)
<i>Volvariella volvacea</i>	37.50	54.80	2.60	1.10	5.50	Manikandan (2011)
<i>Volvariella volvacea</i>	30.57	43.53	2.04	10.37	9.67	Johnsy et al. (2011)

-: No results.

bulk of fruiting bodies accounting for 50 to 65% on dry weight basis. Free sugars amounts to about 11%. Nutritional analysis of two edible wild mushrooms (*S. commune* and *L. edodes*) from northeast India have been studied by Longvah and Deosthale (1998) and reported that 64.4% carbohydrate content present in *L. edodes* and 68% in *S. commune* (16%). Jagadeesh et al. (2010) reported that 34.75 and 38.9% of carbohydrate content present in mycelia and fruit body of *V. bombycina*. Pushpa and Purushothama (2010) have analyzed the nutrition of five mushroom species and found 49.20, 28.38, 32.08, 34.88, 34.36% carbohydrate content in *C. indica*, *A. bisporus*, *P. florida*, *R. delica*, and *L. decastes*, respectively. Nutrient composition of *L. tuberregium* in both wild and cultivated type were analyzed by Manjunathan and Kaviyarasan (2011) and found 58.05 and 55.8% carbohydrate in cultivated variety and in wild variety respectively. Manikandan (2011) reported that total carbohydrate content varies from 26-82% on dry weight basis in different mushrooms (Table 2). Nutritional values of wild mushrooms have been studied by Johnsy et al. (2011) and found good source of carbohydrates ranged from 33.23% in *A. auricula* to 50.2% in *L. tuberregium* (Table 2). Proximate composition of four wild mushrooms have been revealed by Manjunathan et al. (2011) and found highest carbohydrate (48%) in *M. rhodocus* in comparison to other studied mushrooms. Kumar et al (2013) reported the carbohydrate contents of 15 selected mushrooms from Nagaland, India ranged from 32.43% in *S. commune* to 52.07% in *Boletus aestivalis* (Table 2). Recently total carbohydrate contents of two wild mushrooms was studied by Singdevsachan et al. (2013) and found highest in *L. sajor-caju* (68.24%) and

lowest in *L. torulosus* (64.95%).

Lipid/fat

In mushrooms, the fat content is very low as compared to proteins and carbohydrates. The fats present in mushroom fruiting bodies are dominated by unsaturated fatty acids. Singer (1961) determined the fat content of some mushrooms as 2.04% in *Suillus granulatus*, 3.66% in *Suillus luteus* and 2.32% in *A. campestris*. Crude fat content in the range of 1.08 to 9.4% with an average of 2.85% has been reported in *Pleurotus* species (Bano and Rajarathnam, 1982). On fresh weight basis, the fat content of 0.10 to 0.19% in *Pleurotus* species has been reported by Rai et al. (1988). Fat content of fresh *A. bisporus* (Lange) Sing and *P. ostreatus* (Jacq: Fr.) Kumm was analyzed by Manzi et al. (2001) and found to be 0.3 and 0.4 g/100 g, respectively. Longvah and Deosthale (1998) has reported that crude fat content (2%) were similar in two edible wild mushrooms (*S. commune* and *L. edodes*) from northeast India. Agrahar-murugkar and Subbulakshmi (2005) also reported the fat content (ranged from 1.0% in *C. gigantean* to 5.3% in *G. floccocus*) of seven different wild mushrooms collected from the Khasi hills of Meghalaya (Table 2). Kavishree et al. (2008) have analyzed twenty-three species of naturally grown and collected mushroom fruiting bodies from different geographic locations of India for their total fat and fatty acid contents and mushroom species were found to contain 0.6-4.7% total fat. These mushroom species were also high in unsaturated fatty acids (52-87%), compared to saturated fatty acids. Jagadeesh et al. (2010) also

reported that 1.15 and 2.72% lipid contents were present in mycelia and fruit body of *V. bombycina*, respectively. Pushpa and Purushothama (2010) have also analyzed the fat content of five mushrooms which were 4.96, 2.12, 1.54, 5.38, 2.14% in *C. indica*, *A. bisporus*, *P. florida*, *R. delica*, and *L. decastes*, respectively. Manjunathan and Kaviyarasan (2011) reported that the fat content in the cultivated variety (1.54%) of *L. tuberregium* was lower than that in the wild one (1.6%). Johnsy et al. (2011) have studied the nutritional values of wild mushrooms from Western Ghats of Kanyakumari district and revealed very less amounts of fats ranged from 1.17% to 2.58% (Table 2). According to proximate composition of four wild mushrooms studied by Manjunathan et al. (2011), the fat contents was very less ranged from 0.74% to 2.25% (Table 2). Further fat contents of two wild mushrooms was determined by Singdevsachan et al. (2013) and found lowest amount of fats (2.42 and 1.36%) in both studied mushrooms (*L. sajor-caju* and *L. torulosus*, respectively).

Vitamins

Mushrooms are one of the best sources of vitamins especially vitamin B (Mattila et al., 1994, 2000). Manning (1985) gave a comprehensive data of vitamin content of mushrooms and some vegetables which are present in the world. But in India, the information on vitamin content of mushrooms has been lacking. Agrahar-murugkar and Subbulakshmi (2005) determined the vitamin C content (mg/g) in seven wild edible mushrooms commonly consumed in the Khasi hills of Meghalaya, India and found that 14.9, 41.8, 41.9, 28.0, 19.6, 25.8, 18.1 vitamin C present in *C. gigantea*, *C. cinerea*, *C. cibarius*, *R. brevispora*, *R. integra*, *G. floccosus* and *L. quieticolor*, respectively. Recently, vitamin content such as thiamine, riboflavin and ascorbic acid were analyzed by Singdevsachan et al. (2013) in wild mushrooms (*L. sajor-caju* and *L. torulosus*) from Similipal Biosphere Reserve, Odisha, India. The highest thiamine content was found in *L. torulosus* (0.19 mg/g) and lowest in *L. sajor-caju* (0.13 mg/g). Both the studied wild mushrooms were showed good quantities of ascorbic acid (17.75 mg/g in *L. sajor-caju* and 52.91 mg/g in *L. torulosus*) whereas riboflavin was not detected (Singdevsachan et al., 2013). Unfortunately, information on the bioavailability of vitamins from mushrooms has been lacking.

Mineral constituents

Ash content of different mushrooms is usually 0.18-15.73% of dry matter (Table 2). The fruiting bodies of mushrooms are characterized by a high level of well assimilated mineral elements. Major mineral constituents

in mushrooms are Na, K, Ca, Mg, P, S and elements like As, Cd, Cr, Co, Cu, Fe, Mo, Mn, Ni, Pb, Se, Zn among others form minor constituents (Bano and Rajarathanam, 1982; Bano et al., 1981). The mineral content of wild edible mushrooms has been found to be higher than cultivated ones (Mattilla et al., 2001). Kaul (1978) has reported that *M. esculenta* contains Ca (0.57 mg/g), P (3.31 mg/g), Fe (1.21 mg/g) and K (3.83 mg/g). Bano et al. (1981) and Bisaria et al. (1987) have also assessed the minerals and heavy metals content in *Pleurotus* sp. which are given in Table 3. Longvah and Deosthale (1998) analyzed the two species of mushrooms (*S. commune* and *L. edodes*) from northeast India and found that both mushrooms appear to be rich in minerals (Table 3).

Micronutrient profile of seven wild edible mushrooms were also analyzed by Agrahar-murugkar and Subbulakshmi (2005) which are commonly consumed in the Khasi hills of Meghalaya (Table 3) and reported that the calcium (g%) content ranged from 0.42 in *C. cibarius* to 1.91 in *C. cineria*. Phosphorus (g%) levels were the highest in *C. cibarius* (0.58), followed by *R. brevispora* (0.51) whereas *R. integra* had the lowest levels with 0.24. *C. cinerea* had a very high content of iron (mg%) at 75.2. The rest fell in the range 7.17 (*R. brevispora*) to 56.2 (*R. integra*). Manganese (mg%) levels ranged between 4.41 in *C. gigantea* to 11.4 in *R. brevispora*. The copper (mg%) of the mushrooms studied was between 1.39 (*C. gigantea*) and 23.9 (*C. cinerea*). Zinc (mg%) levels varied between 6.76 in *R. brevispora* and 39.4 in *L. quieticolor*. Sodium (mg%) ranged from 0.14 in *G. floccosus* to 0.56 in *R. integra*. Potassium (mg%) levels varied between 17.0 (*L. quieticolor*) and 52.1 (*C. cinerea*). Magnesium (mg%) content was between 25.3 in *L. quieticolor* to 327 in *R. virescens*. The content of selenium ($\mu\text{g}/\text{kg}$), ranged from negligible levels in *G. floccosus* to very high levels in *L. quieticolor* (975) and *C. cibarius* (295). Mineral composition of *L. tuberregium* in both wild and cultivated type were also analyzed by Manjunathan and Kaviyarasan (2011) and found that the potassium concentration in the cultivated mushroom (90.8%) was higher than in the wild (7.53%). Zinc was distributed such that the cultivated variety had a higher concentration (4.9%) than the wild one (0.41).

Proximate composition of four wild mushrooms has been studied by Manjunathan et al. (2011) with their macro- and micromineral contents. Macro mineral such as calcium content was 208 mg/g for *Clitocybe* sp., and 195 mg/g for *M. rhodocus*. The highest sodium and potassium content (858.4 and 1369.1 mg/g respectively) found in *Clitocybe* sp. whereas *M. rhodocus* had the highest magnesium content (250 mg/g) (Table 3). Further, micro-mineral such as Iron content varied from *A. polytricha* with 16.3 mg/g to *M. rhodocus* with 85.6 mg/g. Copper content ranged from *A. polytricha* (0.3 mg/g) to *M. rhodocus* 9.0 mg/g. Manganese content in *M. rhodocus*, *Clitocybe* sp., *A. polytricha*, and *L. tigrinus* were 3.4, 2.7,

Table 3. Mineral nutrients of some Indian mushrooms.

Species	Ca	P	Fe	Mn	Cu	Zn	Na	K	Mg	Se	Cr	Pb	References
<i>Agaricus Heterocystis</i> ³	81.0	-	39.0	39.0	3.72	1.9	39.0	422.0	39.0	-	-	-	Manimozhi and Kaviyarasan (2013)
<i>Auricularia polytricha</i> ³	607	-	16.3	1.3	0.3	1.0	858.4	588.4	136	-	-	-	Manjunathan et al. (2011)
<i>Calvatia gigantea</i> ¹²	630	330	10.7	4.41	1.39	10.3	0.18	22.3	150	91.2	-	-	Agrahar-murugkar and Subbulakshmi (2005)
<i>Cantharellus cibarius</i> ¹²	420	580	53.5	7.68	4.36	6.83	0.29	47.9	46.2	295	-	-	Agrahar-murugkar and Subbulakshmi (2005)
<i>Clitocybe sp.</i> ³	208	-	61.4	2.7	9.0	6.2	858.4	1369.1	120	-	-	-	Manjunathan et al. (2011)
<i>Coprinopsis cinerea</i> ¹²	1910	420	75.2	6.79	23.9	11.1	0.33	52.1	43.8	0.17	-	-	Agrahar-murugkar and Subbulakshmi (2005)
<i>Gomphus floccosus</i> ¹²	1370	340	22.3	7.04	3.48	13.0	0.14	18.7	136	X	-	-	Agrahar-murugkar and Subbulakshmi (2005)
<i>Lactarius quieticolor</i> ¹²	1460	420	19.4	5.32	1.41	39.4	0.21	17.0	25.31	975	-	-	Agrahar-murugkar and Subbulakshmi (2005)
<i>Lentinus edodes</i> ³	127	493	20.1	-	0.9	4.3	-	-	200	-	0.140	-	Longvah and Deosthale (1998)
<i>Lentinus sajor-caju</i> ⁴	-	0.10	2.37	0.12	-	-	-	0.14	-	-	-	-	Singdevsachan et al. (2013)
<i>Lentinus tigrinus</i> ³	248	-	36.2	0.6	1.2	4.9	37.3	90.8	14	-	-	-	Manjunathan et al. (2011)
<i>Lentinus torulosus</i> ⁴	-	0.24	2.94	0.05	-	-	-	0.85	-	-	-	-	Singdevsachan et al. (2013)
<i>Lentinus tuberregium</i> (Wild type) ¹	2.66	-	0.53	0.08	0.11	0.41	1.2	7.53	2.45	-	-	-	Manjunathan and Kaviyarasan (2011)
<i>Lentinus tuberregium</i> (Cultivated type) ¹	87	-	6.5	1.7	1.0	4.9	37.3	90.8	30.4	-	-	-	Manjunathan and Kaviyarasan (2011)
<i>Macrolepiota rhacodes</i> ³	195	-	85.6	3.4	9.0	3.8	274.4	294.3	250	-	-	-	Manjunathan et al. (2011)
<i>Pleurotus eous</i> ³	23	1410	9.0	-	17.8	82.7	78	4570	242	-	-	1.5	Bano et al. (1981); Bisaria et al. (1987), Rai (1994)
<i>Pleurotus flabellatus</i> ³	24	1550	12.4	-	21.9	58.6	75	3760	292	-	-	1.5	Bano et al. (1981); Bisaria et al. (1987), Rai (1994)
<i>Pleurotus florida</i> ³	24	1850	18.4	-	15.8	11.5	62	4660	192	-	-	1.5	Bano et al. (1981); Bisaria et al. (1987), Rai (1994)
<i>Pleurotus sajor-caju</i> ³	20	760	12.4	-	12.2	29	60	3260	221	-	-	3.2	Bano et al. (1981); Bisaria et al. (1987), Rai (1994)
<i>Ramaria brevispora</i> ¹²	530	510	7.17	11.4	16.7	6.76	0.31	35.5	217.2	5.28	-	-	Agrahar-murugkar and Subbulakshmi (2005)
<i>Russula integra</i> ¹²	1270	240	56.2	7.28	3.33	10.5	0.56	41.0	327	26.9	-	-	Agrahar-murugkar and Subbulakshmi (2005)
<i>Schizophyllum commune</i> ³	188	408	12.3	-	0.9	5.7	-	-	227	-	0.133	-	Longvah and Deosthale (1998)

¹Ca, P, Fe, Mn, Cu, Zn, Na, K and Mg contents in mg%; ²Se content in µg/kg; ³All mineral contents in mg/100g; ⁴P and K in g/100 g and rest of the metals in mg/kg; X: negligible quantities; -: No results.

1.3 and 0.6 mg/g, respectively (Manjunathan et al., 2011). Recently, Singdevsachan et al. (2013) have reported the mineral contents of two wild mushrooms (*L. sajor-caju* and *L. torulosus*) from Similipal Biosphere Reserve, Odisha, India. *L. torulosus* showed the highest iron (2.94 mg/kg), potassium (0.85 mg/kg) and phosphorus (0.24 mg/kg) contents whereas *L. sajor-caju* showed the highest manganese (0.12 mg/kg) and nickel (0.05 mg/kg) contents (Table 3). However, both mushrooms did not show the presence of cobalt and cadmium content (Singdevsachan et al. 2013). The mineral proportions vary according to the species, age and the diameter of the fruiting body. It also depends upon the type of the substratum (Demirbas, 2001).

MEDICINAL POTENTIAL OF MUSHROOMS

Medical mycology is as old as traditional uses of mushrooms. They have been used in medicine since the Neolithic and Paleolithic eras (Samorini, 2001). Although mushrooms as medicine have been used in China since 100 A.D. (Gunde-Cimmerman, 1999), but it was only in 1960 that scientists investigated the basic active principles of mushrooms which are health promoting. Mushrooms have been used in health care for treating simple and age old common diseases like skin diseases to present day complex and pandemic disease like acquired immunodeficiency syndrome (AIDS). Mushrooms in the twentieth century are well known to people all over Asian countries as an important bio-source of novel secondary metabolites. In India, particularly the alternative systems of medicine, utilize the curative properties of mushrooms. The secondary metabolites of these mushrooms are chemically diverse and possess a wide spectrum of biological activities, which are explored in traditional medicines (Rai et al., 2005). In India, several mushrooms have been reported as medicinal mushrooms which have antioxidant, antimicrobial, anti-inflammatory activity with antitumor and other properties.

Antioxidant properties

Several researchers investigated that a number of medicinal mushrooms occurring in India possessed promising antioxidant properties. Extracts from fruiting bodies and mycelia of *Ganoderma lucidum*, *Phellinus rimosus* and several *Pleurotus* sp. occurring in South India were found to possess antioxidant activity with high free radical scavenging activity (Jones and Janardhanan, 2000; Ajith and Janardhanan, 2001; Lakshmi et al., 2003). Ethyl acetate, methanol and aqueous extract of *G. lucidum* has been reported to effectively scavenge the O_2^- and $\cdot OH$ radicals, however the aqueous extract was not effective to inhibit the ferrous ion induced lipid

peroxidation (Jones and Janardhanan, 2000) whereas ethanol extracts of the mycelium of *G. lucidum* showed high antiperoxidative activity (Lakshmi et al., 2003). The methanol extract of fruiting bodies of *P. florida* was found to possess $\cdot OH$ radical scavenging and lipid peroxidation inhibiting activities (Jose and Janardhanan, 2000). The extract also showed significant reducing power and radical scavenging property as evident from ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals scavenging assay. Ethyl acetate, methanol and aqueous extracts of the *Phellinus rimosus* were effective to scavenge O_2^- generated from the photoillumination of riboflavin, $\cdot OH$ generated from Fenton's reaction, nitric oxide radical released from aqueous solution of sodium nitroprusside in a dose dependent manner (Ajith and Janardhanan, 2001). All these three extracts of *P. rimosus* also inhibited dose dependently ferrous ion induced lipid peroxidation in the rat whole liver homogenate.

Sheena et al. (2005) also studied the therapeutic potential of *G. lucidum* and reported that the sample of *G. lucidum* from South Indian tropics has greater antioxidant activity through suppression of formation capacity of the free radicals and also possesses high 2,2-azobis-3-ethylbenzthiazoline-6-sulphonilic acid (ABTS) and DPPH scavenging activity. The antioxidant potential from water and methanolic extracts of fruiting bodies of 23 species of mushrooms naturally grown in different geographic locations of India (forest area of Himachal Pradesh and Kerala) was measured by Puttaraju et al. (2006). The antioxidant ability of each species was analyzed for the total antioxidative status, employing multimechanistic antioxidative assays such as inhibition of lipid peroxidation, determination of reducing power, and free radical scavenging ability with determination of total phenolics, because the phenolics are known to contribute largely to antioxidant potential. *Termitomyces heimii* was identified as the best variety, which showed 37 mg of phenolics/g of sample, 418 units of reducing power ability (RPA)/g, and an IC_{50} of ~ 1.1 mg (dry weight)/mL, free radical scavenging activity (FRS) in the water extract followed by 11.2 mg of phenolics/g, 275 units of RPA/g, and an IC_{50} of ~ 2.7 mg (dry weight)/mL of FRS in the methanolic extract. Antioxidant and nitric oxide synthase (NOS) activation properties of *Armillaria mellea* Quel was evaluated by Rai et al. (2009). *In vitro* evaluation of antioxidant activities of *A. mellea* Quel showed significant inhibition of lipid peroxidation, potent hydroxyl and DPPH radical scavenging activity with IC_{50} values of crude, boiled and ethanolic extracts ranging from 36.3 to 388.92 $\mu g/ml$. Among three kinds of extracts, the ethanolic extract was the most effective in relation to antioxidant activity and NOS activation property. Antioxidant activity and bioactive compounds from six wild mushrooms (*Lycoperdon perlatum*, *C. cibarius*, *Clavaria vermiculris*, *Ramaria formosa*, *Marasmius oreades*, *P. pulmonarius*)

of Western Ghats of Karnataka, India were studied by Ramesh and Pattar (2010). All the mushrooms showed high phenol and flavonoid content with promising antioxidant properties (Ramesh and Pattar, 2010). Antioxidant and phytochemical properties of ethanolic extracts from the wild edible mushroom *Termitomyces reticulatus* and their individual parts (Cap and Stipe) were evaluated by Loganathan et al. (2010) through the reducing power, β -carotene bleaching, ABTS and DPPH radicals scavenging activity methods. Antioxidant components like total phenol, flavonoid, β -carotene and lycopene were also determined and the amount of phenol was correlated with the antioxidant property. All the extracts showed potent antioxidant activities, in which the entire mushroom extract showed more antioxidant property when compared with the other two extracts (Cap and Stipe). Kumari et al. (2011) have investigated the antioxidant activity of *Cantharellus friessi*, *Cantharellus subcibarius*, *Cantharellus cinerius* and *P. florida* collected from North-Western Himalayan region of India including their bioactive compounds such as phenol, flavonoid, ascorbic acid and β -carotene. Among them *C. friessi* showed significantly higher antioxidant activity through β -carotene bleaching method and with the high phenol content (16.80 mg/g) than the other mushroom species. Methanolic extracts of cap and stipe of commercially obtained mushrooms *A. bisporus*, *Hypsizyguis ulmarius*, and *C. indica* were analysed by Babu and Rao (2011) for their antioxidant activity in different chemical systems including reducing power, free radical scavenging, ferric reducing antioxidant power (FRAP), superoxide scavenging, peroxide scavenging, and metal chelating activities. All the three commercially grown mushrooms exhibited moderate to high antioxidant activities. All the activities increased steadily with increase in the concentration. *Hypsizyguis ulmarius* cap showed excellent DPPH radical scavenging, peroxide scavenging, FRAP and reducing power abilities which may be attributed to its highest total phenol content whereas the excellent ferrous ion chelation and superoxide scavenging abilities exhibited by *A. bisporus* cap may be attributed to its highest flavonoid content (Babu and Rao, 2011). It has been reported that free radical-scavenging activity is greatly influenced by the phenolic composition of samples (Cheung et al., 2003) and the reducing power of mushrooms might be due to their hydrogen-donating ability (Shimada et al., 1992). Accordingly, above studied mushrooms might contain higher amounts of reductone, which could react with free radicals to stabilise and block radical chain reactions. Beside that, the activity difference among the results obtained from antioxidant studies with the mushroom extracts might be related to the different conditions of measurement and the sensitivity of the assays. Therefore several methods should be used in parallel to elucidate the complex field of antioxidants and oxidation (Ciz et al., 2010).

Antimicrobial properties

The petroleum ether, chloroform, acetone and water extracts of the mushroom *Osmoporus odoratus* have been tested for their antibacterial activity against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*; the water extract alone showed antibacterial activity against the tested organisms and the results were comparable with that of ampicillin rather than chloramphenicol (Sivakumar et al., 2006). Two edible mushrooms (*A. bisporus* and *P. sajor-caju*) were assayed *in vitro* for their antimicrobial activities by Tambekar et al. (2006) using aqueous and organic solvent extracts. In their study, *E. coli* 390, *E. coli* 739, *Enterobacter aerogenes*, *P. aeruginosa* and *Klebsiella pneumonia* were most sensitive to aqueous, ethanol, methanol and xylene extracts of these mushrooms. *Pleurotus* species had a narrow antibacterial spectrum against Gram-negative bacteria and strongly inhibited the growth of the Gram-positive bacteria tested, including *B. subtilis*, and *M. luteus* (Loganathan et al., 2008). The antimicrobial activity of various solvent extracts (methanol, ethanol, acetone and aqueous extract) of *G. lucidum* was tested by Quereshi et al. (2010) against six species of bacteria: *E. coli*, *S. aureus*, *K. pneumoniae*, *B. subtilis*, *S. typhi* and *P. aeruginosa*. Acetone extract exhibited maximum antibacterial activity, while the most susceptible bacterium observed was *K. pneumoniae*. Methanolic extracts of six wild mushrooms (*L. perlatum*, *C. cibarius*, *C. vermiculris*, *R. formosa*, *M. oreades*, *P. pulmonarius*) of Western Ghats of Karnataka, India showed significant antimicrobial activity against *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa* and *Candida albicans* (Ramesh and Pattar, 2010).

Manjunathan and Kaviyarasan (2010) studied the solvent based effectiveness of antibacterial activity of edible mushroom *L. tuberregium* (Fr.). *In vitro* antimicrobial properties of *L. tuberregium* culture filtrate extracted using four different solvent systems (Hexane, dichloromethane, chloroform and ethyl acetate) were the most active to inhibit the growth of *S. aureus*, *Micrococcus luteus*, *E. coli*, *Salmonella typhi* and *Shigella flexneri*. The antibacterial activity of *V. bombycina* extracts (hexane, chloroform, ethyl acetate and methanol) were evaluated by Jagadeesh et al. (2010) against the clinically important bacterial strains *B. subtilis*, *S. aureus*, *E. coli*, *K. pneumoni* and *P. aeruginosa* and reported that the *V. bombycina* extracts possess compounds which have the antibacterial properties.

The antibacterial and antifungal activity of methanol and aqueous extract of fruit bodies from *Phellinus* was tested by Balakumar et al. (2011) against five bacterial pathogens such as *E. coli*, *P. aeruginosa*, *S. typhi*, *S. aureus* and *Streptococcus mutans* and five fungal strains *Penicillium* sps., *Aspergillus fumigatus*, *Aspergillus*

niger, *Aspergillus flavus* and *Mucor indicus*. The fruit body of *Phellinus* showed potential antibacterial activities against the selected strains whereas aqueous extract showed maximum inhibition zone (42 mm) against *P. aeruginosa* and the methanolic extract showed the maximum antifungal activity against *A. flavus* (35 mm). Ethyl acetate extract of four different edible mushrooms (*P. sajor-caju*, *V. volvaceae*, *A. bisporus* and *P. ostreatus*) were investigated for their antimicrobial activity against *B. subtilis*, *S. aureus*, *E. coli*, *K. pneumoniae* and *Proteus vulgaris* (Surekha et al., 2011). Among them, only *A. bisporus* and *P. ostreatus* showed effective inhibition zone against all pathogenic strains.

Anti-inflammatory properties

Ethanol extract of cultured mycelium of *M. esculenta* were investigated by Nitha et al. (2006) for their anti-inflammatory activity. The extract showed significant dose-dependent inhibition of both acute and chronic inflammation in mice model which was comparable to that of the standard reference drug, Diclofenac. In yet another report, the acute and chronic anti-inflammatory activities of ethyl acetate and methanolic extracts from *G. lucidum* were determined by Sheena et al. (2005) through carrageenan induced acute and formalin induced chronic inflammatory models in mice. Both the extracts showed significant effect on carrageenan induced acute and formalin induced chronic inflammation in mice which was comparable with standard drug, diclofenac. However chloroform extract of *G. lucidum* also exhibited significant anti-inflammatory activity (Joseph et al., 2009).

Antitumor properties

The methanolic and aqueous extracts of *G. lucidum* were tested by Jones and Janardhanan (2000) for antitumor activity which effectively inhibited Ehrlich's ascites carcinoma (EAC) cell line induced solid tumor in mice when administered orally (Jones and Janardhanan, 2000). Methanol extract of the fruiting bodies of *P. florida* and *P. pulmonarius* occurring in South India also showed profound antitumor activity against the EAC cell line induced solid tumor model in mice (Jose and Janardhanan, 2000; Jose et al., 2002). The three different extracts (ethyl acetate, methanol and aqueous) of *P. rimosus* were found to inhibit the Dalton's Lymphoma Ascites (DLA) cell line induced solid tumor and EAC cell line induced ascites tumor in mice whereas the antitumor effect was high in ethyl acetate extract than the other extracts (Ajith and Janardhanan, 2003). Antitumor activity of *G. lucidum* was again confirmed by Sheena et al. (2005) through the EAC cell line induced solid tumor model in mice where both the extracts (methanol and aqueous) showed significant antitumor properties by inhibiting the tumor development. Polysaccharides, extracted from mycelium

and fruiting bodies of *L. tuberregium* effectively inhibited solid tumour proliferation in mice (Manjunathan and Kaviyarasan, 2010). Antitumor activity of the ethanolic extract from cultured mycelium of morel mushroom, *M. esculenta* was determined by Nitha et al. (2006) using both DLA cell line-induced solid tumor and EAC cell line-induced ascites tumor models in mice. The extract exhibited significant antitumor activity against both ascites and solid tumours (Nitha et al., 2006).

Immunomodulatory properties

In India, only a few studies have been carried out with mushrooms for immunomodulatory properties. Structural aspects of mushroom polysaccharides from *P. osreatus*, *Astraeus hygrometricus*, *P. sajor-caju* and *P. florida* has been studied at Vidyasagar University, West Bengal, India (Chakraborty, 2004; Rout et al., 2004; 2005) with their immunomodulatory effects. A water soluble glucan (Figure 1) was isolated from *P. florida* fruit bodies and investigated for its structural characterization and immunomodulatory effects by Rout et al. (2004) which exhibited significant macrophage activity through the release of nitric oxide whereas Chakraborty et al. (2004) investigated another water-soluble glucan (Figure 2) from an edible mushroom, *A. hygrometricus* and found that glucan is a strong immune-enhancing material showing strong splenocyte activation. A glucan (Figure 3) that was soluble in aqueous sodium chloride was isolated from the aqueous extract of the fruiting bodies of *P. florida* which stimulated the phagocytic activity of macrophages (Rout et al., 2005). Roy et al. (2009) studied an immune-enhancing water-soluble glucan (Figure 4) isolated from hot water extract of an edible mushroom, *P. florida*, cultivar Assam Florida and found that the glucan stimulates macrophages, splenocytes, and thymocytes. A heteroglycan (Figure 5) isolated from an aqueous extract of an edible mushroom, *L. squarrosulus* and analyzed for its structural characterization with immune-enhancing activity by Bhunia et al. (2010) which showed macrophage as well as splenocyte and thymocyte activation. Dey et al. (2010) have also analyzed an immunoenhancing water-soluble polysaccharide (Figure 6) of an edible mushroom, *P. florida* blue variant and they have also found that the molecule activated macrophages, splenocytes, and thymocytes. A structural and biological study of a heteropolysaccharide (Figure 7) from aqueous extract of an edible mushroom, *P. ostreatus* has also been conducted by Maity et al. (2011) and revealed that the heteroglycan stimulates macrophages, splenocytes, and thymocytes (Table 4).

ETHNOMYCOLOGICAL KNOWLEDGE OF ETHNIC-TRIBES IN INDIA

The traditional uses of the mushroom are known to the



Figure 1. Repeating unit of glucan isolated by Rout et al. (2004).

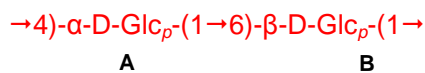


Figure 2. Repeating unit of glucan isolated by Chakraborty et al. (2004).

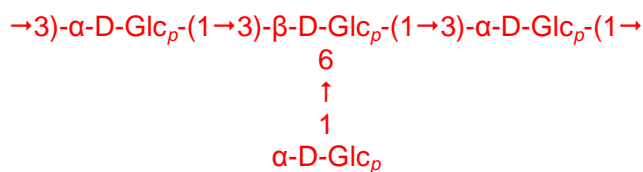


Figure 3. Repeating unit of glucan isolated by Rout et al. (2005).

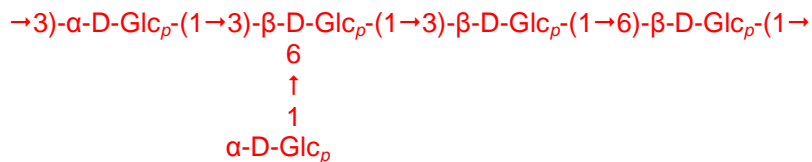


Figure 4. Repeating unit of glucan isolated by Roy et al. (2009).

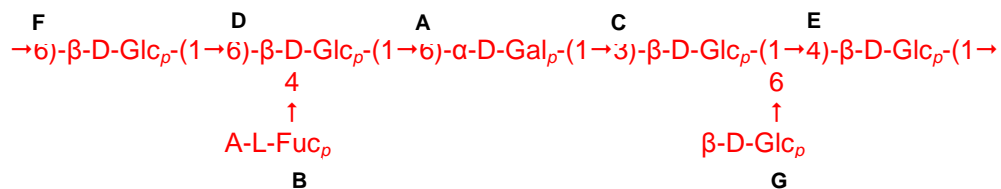


Figure 5. Repeating unit of heteroglycan isolated by Bhunia et al. (2010).

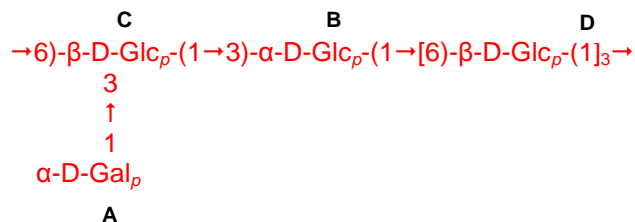


Figure 6. Repeating unit of polysaccharide isolated by Dey et al. (2010).

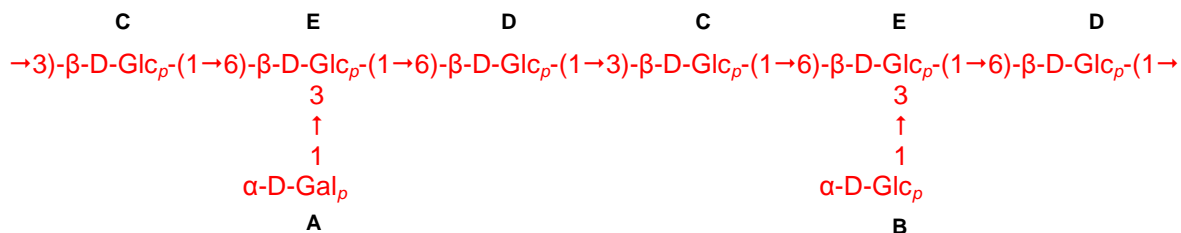


Figure 7. Repeating unit of heteropolysaccharide isolated by Maity et al. (2011).

Table 4. Medicinal properties of some Indian mushrooms.

Biological source	Medicinal properties	References
<i>Agaricus bisporus</i>	Antioxidant activity, antimicrobial activity, antiproliferative activity	Jagdish et al. (2009)
<i>Astraeus hygrometricus</i>	Immunoanancing activity	Chakraborty et al. (2004)
<i>Cantharellus cibarius</i>	Antioxidant activity, antimicrobial activity	Ramesh and Pattar (2010)
<i>Clavaria vermicularis</i>	Antioxidant activity, antimicrobial activity	Ramesh and Pattar (2010)
<i>Ganoderma lucidum</i>	Antioxidant activity, antimutagenic activity	Jones and Janardhanan (2000); Lakshmi et al. (2004)
<i>Lentinus squarrosulus</i>	Immunoanancing activity	Bhunja et al. (2010)
<i>Lentinus tuberregium</i>	Antibacterial activity	Manjunathan and Kaviyaranan (2010)
<i>Lycoperdon perlatum</i>	Antioxidant activity, antimicrobial activity	Ramesh and Pattar (2010)
<i>Marasmius oreades</i>	Antioxidant activity, antimicrobial activity	Ramesh and Pattar (2010)
<i>Phellinus rimosus</i>	Antioxidant activity and antitumor activity	Ajith and Janardhanan (2002; 2006)
<i>Pleurotus florida</i>	Antioxidant activity, antiinflammatory, immunoanancing activity	Jose and Janardhanan (2000); Jose et al. (2002); Roy et al. (2009); Dey et al. (2010)
<i>Pleurotus ostreatus</i>	Immunoanancing activity	Maity et al. (2011)
<i>Pleurotus pulmonarius</i>	Antioxidant activity, antimicrobial activity	Ramesh and Pattar (2010)
<i>Pleurotus pulmonarius</i>	Antitumor activity	Jose et al. (2002)
<i>Pleurotus sajor-caju</i>	Antibacterial activity	Tambekar et al. (2006)
<i>Ramaria formosa</i>	Antioxidant activity, antimicrobial activity	Ramesh and Pattar (2010)
<i>Volvariella bombycina</i>	Antibacterial activity	Jagadeesh et al. (2010)

aboriginals of Africa, India, Brazil and other countries. Wild mushrooms are a valuable non-timber forest resource used by mycophilic societies and their use has been documented in many countries around the world (Chang and Lee, 2004, Roberto G.O et al., 2005; Sarma et al., 2010). They are sold in traditional markets (Roberto G.O et al., 2005) or commercially exploited as food (Bhaben et al., 2011) or medicines (Sachan et al., 2013). In Nigeria, Puff balls (*Lycoperdon pusillum* and *C. gigantea*) are used to cure sores, abrasion or bruises, deep cut, haemorrhages and urinary infections (Buswell and Chang, 1993). Traditional mycological knowledge of most Indian ethnic groups has proven to be extensive and profound, consuming nearly 283 species of wild mushrooms out of 2000 species recorded world over (Purkayastha and Chandra, 1985).

Ethnomycological aspects were also dealt with by few workers in different parts of India and world over (Harsh et al. 1993; Bulakh, 2001). Some of the wild edible mushrooms have also been reported from Manipur and Arunachal Pradesh of North East India (Sing and Sing, 1993; Sing et al., 2002) whereas, from Assam, Baruah et al. (1971) reported few Basidiomycetous fungus of Sibsagar District. In Central India *G. lucidum* is used as herbal medicine by the Baiga tribes to cure asthma and *Agaricus* sp. is used in goiter and *L. pusillum* in wound healing and also for controlling bleeding (Rai et al., 2005). Sarma et al. (2010) reported that some Ethnic Tribes of Western Assam use the wild edible mushroom as food source. Ethnic tribes such as Garos, Adivashis, Bodos and Rajbangshis of Western Assam are consuming at least seven species of mushrooms as

vegetables. But the potentialities of such species are yet to be studied in detail (Sarma et al., 2010). According to Bhaben et al. (2011), the ethnic tribes of Nagaland, India were also using wild edible mushrooms for food purposes. Their investigation revealed that more than 12 ethnic groups of Nagaland were found to be mycophilic and to have traditional mycological knowledge (complete data not available). A total of 13 species of fleshy fungi under 9 genera and 6 families were identified which are being used by tribals of Nagaland. Srivastava et al. (2011) conducted an ethnobotanical survey for distribution and utilization of *Termitomyces* species in Gorakhpur forest division of Uttar Pradesh, India and reported that tribal people and forest dwellers are using *Termitomyces* species as food and for medicinal purposes (not clearly known but used in malnutrition, weakness and their nutritional disorders). Traditional uses and medicinal potential of *Cordyceps sinensis* has been studied by Panda and Swain (2011) in Sikkim, India and found that most local folk healers/traditional healers use cordyceps in their herbal medicine for the treatment of 21 ailments including cancer, bronchial asthma, bronchitis, TB, diabetes, cough and cold, erectile dysfunction, BHP, jaundice, alcoholic hepatitis, among others. Beside that, seven numbers of wild mushrooms such as *P. sajor-caju*, *T. heimii*, *T. microcarpus*, *V. volvaceae*, *A. auriculata*, *L. fusipes* and *L. tuberegium* are also consumed by the Kaani tribes of Kanyakumari district in their different recipe (Sargunam et al., 2012). Recently, a survey has been conducted by Sachan et al. (2013) on indigenous knowledge of ethnic tribes from Similipal Biosphere Reserve, Odisha for utilization of wild mushrooms as food and medicine. All these studied mushrooms (Table 1) are used by several tribals (Santal, Kolha, Munda, Khadia, Bhumija, Bhuyan, Bathudi, Kudumi, Ho, Mankdias) living in the Similipal forest for their food as well as herbal medicinal purposes to cure malnutrition, weakness, other nutritional disorder like diarrhea, high blood pressure, fever, asthma among others. (Sachan et al., 2013).

CONCLUSION

India with diverse habitats with varied ecological conditions, harbors wide varieties of mushrooms potentially rich with nutritional and medicinal values. Several mushrooms are known to be the sources of various bioactive substances like antibacterial, antifungal, antiviral, antiparasitic, antioxidant, antiinflammatory, antiproliferative, anticancer, antitumor, cytotoxic, anti-HIV, hypocholesterolemic, anti-diabetic, anticoagulant, hepatoprotective among others. These mushrooms have been used as ethnomedicines by tribals for treatment of various diseases. Many mushrooms still remain unreported and their nutritional as well as health benefits are unknown to us. If discovered, some of them may have high nutritional value and serve as valuable sources of bioactive compounds with many

pharmaceutical applications. Further, several products such as antidiabetic mushroom powder, weight gain powder, sex enhancement medicine, mushroom chyawanprash, mushroom pickles among others. have been developed from mushrooms in India and other medicines such as anticancer, anti-tumor, anti-inflammatory among others. are under trials.

REFERENCES

- Abraham SP (1991). Kashmir fungal flora-an overview. In: Nair MC (ed). Indian Mushrooms. Kerala Agricultural University, Vellanikkara, India. pp. 13-24.
- Acharya K, Rai M, Pradhan P (2010). Agaricales of Sikkim Himalaya: A Review. Researcher. 2(5):29-38.
- Agrahar-Murugkar D, Subbulakshmi, G (2005). Nutritional value of edible wild mushrooms collected from the Khasi hills Meghalaya. Food Chem. 89:599-603.
- Ajith TA, Janardhanan KK (2001). Antioxidant and antiinflammatory activities of methanol extract of *Phellinus rimosus*. Indian J. Exp. Biol. 39:1166-1169.
- Ajith TA, Janardhanan KK (2003). Cytotoxic and antitumor activities of a polypore macrofungus, *Phellinus rimosus*. J. Ethnopharmacol. 84:157-162.
- Ajith TA, Janardhanan KK (2007). Indian medicinal mushrooms as a source of antioxidant and antitumor agents. J. Clin. Biochem. Nutr. 40:157-162.
- Anand N, Chowdhry PN (2013). First report on five hitherto unreported macro fungi from Rajouri district of Jammu and Kashmir (JandK), India. Ann. Biol. Res. 4(5):62-70.
- Atri NS, Saini SS (1989). Family *Russulaceae* Roze-a review. In: Trivedi ML, Gill GS, Saini SS (eds). Plant Science Research in India. Today and Tomorrow's Printers and Publishers, New Delhi, India. pp. 115-125.
- Atri NS, Saini SS, Gupta AK (1991b). Systematic studies on *Agaricus campestris* (L.) Fr. Geobios New Report. 10:32-37.
- Atri NS, Saini SS, Kaul G (1995). Taxonomic studies on North Indian Agarics-The genus *Termitomyces*. Mushroom Res. 4:7-10.
- Atri NS, Saini SS, Kaur G (1996). Three species of agarics from Patiala. Mushroom Res. 5:77-80.
- Atri NS, Saini SS, Mann DK (1991a). Further studies on north-west Indian Agaricaceae-Systematics of *Lactarius deliciosus* (Fr.) S.F. Gray. Geobios New Report 10:106-111.
- Atri NS, Saini SS, Saini MK (1997). Studies on genus *Russula* Pers. from North Western Himalaya. Mushroom Res. 6(1):1-6.
- Babu DR, Rao GN (2013). Antioxidant properties and electrochemical behavior of cultivated commercial Indian edible mushrooms. J. Food Sci. Technol. 50(2):301-308.
- Balakumar R, Sivaprakasam E, Kavitha D, Sridhar D, Kumar JS (2011). Antibacterial and antifungal activity of fruit bodies of *Phellinus* mushroom extract. Int. J. Biosci. 1(3):72-77.
- Bano Z (1976). Nutritive value of Indian mushrooms and medicinal practices. Eco. Bot. 31:367-371.
- Bano Z, Bhagya S, Srinivasan KS (1981). Essential amino acid composition and proximate analysis of Mushroom, *Pleurotus florida*. Mushrooms News Lett. Trop. 1:6-10.
- Bano Z, Rajarathanam S (1982). *Pleurotus* mushrooms as a nutritious food. In: Tropical mushrooms -Biological nature and cultivation methods. Chang ST, Quimio TH (eds). The Chinese University press, Hongkong. pp. 363-382.
- Bano Z, Rajarathanam S (1988). *Pleurotus* mushroom part II. Chemical composition, nutritional value, post harvest physiology, preservation and role as human food. Crit. Rev. Food Sci. Nutr. 27:87-158.
- Bano Z, Srinivasan KS, Srivastava HC (1963). Amino acid composition of the protein from a mushroom (*Pleurotus flabellatus*). Appl. Microbiol. 11:184-187.
- Baruah HK, Sing DK, Islam, M (1971). On the distribution of higher Basidiomycetes in the Sibsagar district, Assam. Bull. Bot. Surv. India.

- 13(3and4):285-289.
- Bhaben T, Gurung L, Sarma GC (2011). Wild edible fungal resources used by ethnic tribes of Nagaland, India. *Indian J. Tradit. Know.* 10(3):512-515.
- Bhavani Devi S (1995). Mushroom flora of Kerala. In: Chadha KL, Sharma SR (eds). *Advances in Horticulture 13, Mushroom*. Malhotra Publishing House, New Delhi, India. pp. 277-316.
- Bhosle S, Ranadive K, Bapat G, Garad S, Deshpande G, Vaidya J (2010). Taxonomy and diversity of *Ganoderma* from the western parts of Maharashtra (India). *Mycosphere*. 1(3):249-262.
- Bhunia SK, Dey B, Maity KK, Patra S, Mandal S, Maiti S, Maiti TK, Sikdar SR, Islam SS (2010). Structural characterization of an immunoenhancing heteroglycan isolated from an aqueous extract of an edible mushroom, *Lentinus squarrosulus* (Mont.) Singer. *Carbohydrate Res.* 345:2542-2549.
- Bilgrami KS, Jamaluddin, Rizvi AM (1991). *The Fungi of India Part III (List and References)*. Today and Tomorrow's Printers and Publishers, New Delhi.
- Bisaria R, Madan M, Bisaria VS (1987). Biological efficiency and nutritive value of *P. sajor-caju* cultivated on different agro-wastes. *Biological Wastes*. 19:239-255.
- Bose SR (1921). Possibilities of mushrooms industry in India by cultivation. *Agr. J. India*. 16:643-45.
- Bose SR, Bose AB (1940). An account of edible mushrooms of India. *Sci. and Culture*. 6:141-149.
- Bulakh EM (2001). Medicinal Mushrooms of the Russian far east in nature. *Int. J. Med. Mushr.* 3:125.
- Buswell JA, Chang ST (1993). Edible Mushrooms. Attributes and Applications. In: Chang ST, Buswell JA, Miles, PG (eds). *Genetics and Breeding of Edible Mushrooms*. Gordon and Breach, Philadelphia. pp. 297-394.
- Butler EJ, Bisby GR (1931). *The Fungi of India*. Calcutta, India: Imperial Council of Agriculture Research, India. Scientific Monograph 1. pp. 237.
- Chakraborty I, Mondal S, Pramanik M, Rout D, Islam SS (2004). Structural investigation of a water-soluble glucan from an edible mushroom, *Astraeus hygrometricus*. *Carbohydrate Res.* 339:2249-2254.
- Chang ST (1980). Mushroom as human food. *Bio. Sci.* 30:339-401.
- Chang ST (1999). Global impact edible and medicinal mushrooms on human welfare in the 21st century: non green evolution. *Int. J. Med. Mushr.* 1:1-7.
- Chang ST, Miles PG (1992). Mushroom biology-A new discipline. *Mycologist*. 6:64-65.
- Chang YS, Lee SS (2004). Utilization of macrofungi species in Malaysia. *Fungal Divers.* 15:15-22.
- Cheung LM, Cheung PCK, Ooi VEC (2003). Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chem.* 81:249-255.
- Ciz M, Cizova H, Denev P, Kratchanova M, Slavov A, Lojek A (2010). Different methods for control and comparison of the antioxidant properties of vegetables. *Food Contr.* 21:518-523.
- Cooke RC (1977). *Fungi, man and his environment*. Largman, London, New York. pp. 144.
- Crisan EW, Sands (1978). A nutritional value. In: Chang ST, Hayes WA (eds). *The biology and cultivation of edible mushrooms*. Academic press, New York. pp. 172-189.
- Das K (2010). Diversity and conservation of wild mushrooms in Sikkim with special reference to Barsey Rhododendron Sanctuary. *NeBIO*. 1(2):1-13.
- Das K, Atri NS, Buyck B (2013). Three new species of *Russula* (Russulales) from India. *Mycosphere*. 4(4):722-732.
- Das K, Sharma JR, Montoya L (2004). *Lactarius* (Russulaceae) in Kumaon Himalaya. 1. New species of subgenus *Russularia*. *Fungal Divers.* 16:23-33.
- Davidson SS, Johnsy G, Selvin Samuel A, Kaviyaran V (2012). Mushrooms in food culture of the Kaani tribe of Kanyakumari district. *Indian J. Tradit. Know. Knowledge*. 11(1):150-153.
- Demirbas A (2001). Concentrations of 21 metals in 18 species of mushrooms growing in the east Black Sea region. *Food Chem.* 75:453-457.
- Deshmukh SK (2004). Biodiversity of tropical basidiomycetes as sources of novel secondary metabolites. In: Jain PC (ed). *Microbiology and Biotechnology for Sustainable Development*. CBS Publishers and Distributors, New Delhi, India. pp. 121-140.
- Dey B, Bhunia SK, Maity KK, Patra S, Mandal S, Maiti S, Maiti TK, Sikdar SR, Islam SS (2010). Chemical analysis of an immunoenhancing water-soluble polysaccharide of an edible mushroom, *Pleurotus florida* blue variant. *Carbohydrate Res.* 345:2736-2741.
- Dhancholia S, Bahukhandi D (1988). New records of agarics from India. *Indian J. Mushroom.* 14:51-56.
- Doshi A, Sharma SS (1997). Wild mushrooms of Rajasthan. In: Rai RD, Dhar BL, Verm RN (eds). *Advances in Mushroom Biology and Production*. Mushroom Society of India, National Research Centre for Mushroom, Solan, Chambaghat (HP), India. pp. 105-127.
- Dwivedi S, Tiwari MK, Chauhan UK, Pandey AK (2012). Biodiversity of mushrooms of Amarkantak Biosphere Reserve forest of Central India. *Int. J. Pharm. Life Sci.* 3(1):1363-1367.
- Elmastas M, Isildak O, Turkecul I, Temur N (2007). Determination of antioxidant activity and antioxidant compounds in wild edible mushrooms. *J. Food Comp. Anal.* 20: 337-345.
- Gogoi Y, Sarma TC (2012). An ethnomycological survey in some areas of Dhemaji District (Assam). *The Ecoscan*. 1:403-407.
- Gruen VEC, Wong HX (1982). Immunomodulatory and antitumour activities of a polysaccharide-peptide complex from a mycelial culture of *Trichoderma* sp. *Sciences* 57: 269-281.
- Gunde-Cimmerman N (1999). Medicinal value of the genus *Pleurotus* (fr). P Karst (*Agaricales* s.l. *Basidiomycetes*). *Int. J. Med. Mushr.* 1: 69-80.
- Gupta KK, Agarwala RK, Kumar S, Seth PK (1974). Gasteromycetes of Himachal Pradesh. *Indian Phytopath.* 27: 45-48.
- Gupta S, Sing SP (1991). Nutritive value of mushroom *Podaxis pistillaris*. *Indian J. Mycol. Plant Pathol.* 21:275-276.
- Harsh NSK, Rai BK, Ayachi SS (1993). Forest Fungi and Tribal Economy-A Case Study in Baiga tribe of Madhya Pradesh (India). *J. Trop. Forestry*. 9: 270-279.
- Hawksworth DL (2001). Mushrooms: the extent of the unexplored potential. *Int. J. Med. Mushr.* 3: 333-7.
- Hayes WA, Haddad N (1976). The food value of the cultivated mushrooms and its importance in industry. *Mushroom J.* 40: 104-110.
- Jagadeesh R, Raaman N, Periyasamy K, Hariprasath L, Thangaraj R, Srikumar R, Ayyappan, SR (2010). Proximate analysis and antibacterial activity of edible mushroom *Volvariella bombycina*. *Int. J. Microbiol. Res.* 1(3): 110-113.
- Jagadish LK, Krishnan VV, Shenbhagaraman R, Kaviyaran V (2009). Comparative study on the antioxidant, anticancer and antimicrobial property of *Agaricus bisporus* (J. E. Lange) Imbach before and after boiling. *Afr. J. Biotechnol.* 8: 654-661.
- Johnsy G, Davidson Sargunam S, Dinesh MG, Kaviyaran V (2011). Nutritive Value of Edible Wild Mushrooms Collected from the Western Ghats of Kanyakumari District. *Bot. Res. Int.* 4(4):69-74.
- Jonathan SG, Fasidi IO (2003). Antimicrobial activities of two Nigerian edible macro-fungi *Lycoperdon pusillum* (Bat. Ex) and *Lycoperdon gigantum* (Pers). *Afr. J. Biomed. Res.* 6: 85-90.
- Jones S, Janardhanan KK (2000). Antioxidant and antitumor activity of *Ganoderma lucidum* (cult ex Fr.). P. Karst-Reshi (Aphyllophoromycetieae) from south India. *Int. J. Med. Mushr.* 2: 195-200.
- Jose N, Ajith TA, Janardhanan KK (2002). Antioxidant, anti-inflammatory and antitumor activities of culinarymedicinal mushroom *Pleurotus pulmonarius* (Fr.) Quel. (Agaricomycetideae). *Int. J. Med. Mushr.* 4: 329-335.
- Jose N, Janardhanan KK (2000). Antioxidant and antitumor activity of *Pleurotus florida*. *Curr. Sci.* 79: 941-943.
- Joseph S, Sabulal B, George V, Smina TP, Janardhanan KK (2009). Antioxidative and antiinflammatory activities of the chloroform extract of *Ganoderma lucidum* found in South India. *Sci. Pharm.* 77: 111-121.
- Kamat MN, Patwardhan PG, Rao VG, Sathe AV (1971). Fungi of Maharashtra, Bulletin 1. Mahatma Phule Krishi Vidyapeeth, Rahuri (M.S.), India.

- Karwa A, Rai MK (2010). Hitherto unreported *Agaricus* species of Central India. *Nusantara Biosci.* 2(3): 141-145.
- Kaul TN (1978). Nutritive value of some edible *Morchellaceae*. *Ind. J. Mushroom.* 4: 26-34.
- Kaul TN (1992). Conservation of Mushroom Resources in India: Introduction and Action Plan. World Wide Fund for Nature-India, New Delhi.
- Kaul TN (2002). Conservation of Mycodiversity in India: an Appraisal. In: Watling R, Frankland JC, Ainsworth AM, Isaac S, Robinson CH (eds). *Tropical Mycology*. CABI Publishing. 1: 131-147.
- Kaushal SC (1991). Systematics of N.W. Himalayan species of *Helvella* (operculate discomycete). In: Khullar SP, Sharma MP (eds). *Himalayan Botanical Researches*. Ashish Publishing House, New Delhi, India. pp. 61-75.
- Kavishree S, Hemavathy J, Lokesh BR, Shashirekha MN, Rajarathnam S (2008). Fat and fatty acids in Indian edible mushrooms. *Food Chem.* 106: 597-602.
- Khaund P, Joshi SR (2013). Wild edible macrofungal species consumed by the Kashi tribe of Meghalaya, India. *Indian J. Nat. Prod. Resour.* 4(2):197-204.
- Khosho TN (1991). Conservation of biodiversity in biosphere. In: Khosho TN, Sharma M (eds). *Indian Geosphere Biosphere Programme: Some Aspects*. Har-Anand Publications, Vikas Publishing House Private, New Delhi, India. pp. 183-233.
- Khosho TN (1992). Plant diversity in the Himalaya: Conservation and utilization. Govind Ballabh Pant Institute of Himalayan Environment and Development, Kosi, Almora, India.
- Kumar R, Tapwal A, Pandey S, Borah RK, Borah D, Borgohain J (2013). Macro-fungal diversity and nutrient content of some edible mushrooms of Nagaland, India. *Nusantara Biosci.* 5(1):1-7.
- Kumari D, Reddy MS, Upadhyay RC (2011). Antioxidant activity of three species of wild mushroom genus *Cantharellus* collected from North-Western Himalaya, India. *Int. J. Agric. Biol.* 13: 415-418.
- Laganathan KJ, Ramalingam S, Venkatasubbu V, Venketesan K (2008). Studies on the phytochemical, antioxidant and antimicrobial properties of three indigenous *Pleurotus* species. *J. Mol. Biol. Biotech.* 1: 20-29.
- Lakhanpal TN (1993). The Himalayan *Agaricales*-status of systematics. *Mushroom Res.* 2: 1-10.
- Lakhanpal TN (1995). Mushroom flora of North-west Himalaya. In: Chadha KL, Sharma SR (eds). *Advances in Horticulture 13: Mushrooms*. Malhotra Publishing House, New Delhi, India. pp. 351-373.
- Lakshmi B, Ajith TA, Sheena M, Nidhi G, Janardhanan KK (2003). Antiperoxidative, anti-inflammatory and antimutagenic activities of ethanol extract of the mycelium of *Ganoderma lucidum* occurring in South India. *Teratogen Carcinogen Mutagen.* 22: 1-13.
- Lindequist U, Niedermeyer THJ, Julich WD (2005). The pharmacological potential of mushrooms. *eCAM.* 2(3): 285-299.
- Lintzel W (1941). The nutritional value of edible mushroom proteins. *Biochem. Acta.* 308: 413-419.
- Loganathan JK, Gunasundari D, Hemalatha M, Shenbhagaraman R, Kaviyaran V (2010). Antioxidant and phytochemical potential of wild edible mushroom *Termitomyces reticulatus*: individual cap and stipe collected from south eastern part of India. *Int. J. Pharma. Sci. Res.* 1(7): 62-72.
- Longvah T, Deoshthale YG (1988). Compositional and nutritional studies on edible wild mushrooms from northeast India. *Food chem.* 64(3): 331-334.
- Maity KK, Patra S, Dey B, Bhunia SK, Mandal S, Das D, Majumdar DK, Maiti S, Maiti TK, Islam SS (2011). A heteropolysaccharide from aqueous extract of an edible mushroom, *Pleurotus ostreatus* cultivar: structural and biological studies. *Carbohydrate Res.* 346: 366-372.
- Manikandan K (2011). Nutritional and medicinal values of mushrooms. In: Singh M, Vijay B, Kamal S, Wakchaure GC (eds). *Mushrooms Cultivation, Marketing and Consumption*. Director of Mushroom Research, Solan, India. pp. 11-14.
- Manimozhi M, Kaviyaran V (2013). Nutritional composition and antibacterial activity of indigenous edible mushroom *Agaricus heterocystis*. *Int. J. Adv. Biotechnol. Res.* 4(1):78-84.
- Manjula B (1983). A revised list of the agaricoid and boletoid basidiomycetes from India and Nepal. *Proceedings of the Indian Academy of Sciences (Plant Science).* 92: 81-213.
- Manjunathan J, Kaviyaran V (2010). Solvent based effectiveness of antimicrobial activity of edible mushroom *Lentinus tuberregium* (Fr). *Int. J. Pharmatech. Res.* 2(3): 1910-1912.
- Manjunathan J, Kaviyaran V (2011). Nutrient composition in wild and cultivated edible mushroom, *Lentinus tuberregium* (Fr.) Tamil Nadu, India. *Int. Food Res. J.* 18: 59-61.
- Manjunathan J, Subbulakshmi N, Shanmugapriya R, Kaviyaran V (2011). Proximate and mineral composition of four edible mushroom species from South India. *Int. J. Biodivers. Conserv.* 3(8):386-388.
- Manning K (1985). Food value and chemical composition. In: Flegg PB, Spencer DM, Wood DA (eds). *The biology and technology of the cultivated Mushroom*. John Willey and sons, New York. pp. 221-230.
- Manoharachary C, Sridhar K, Singh R, Adholeya A, Suryanarayanan TS, Rawat S, Johri BN (2005). Fungal biodiversity: Distribution, conservation and prospecting of fungi from India. *Curr. Sci.* 89(1): 58-71.
- Manzi PA, Agguzzi A, Pizzoferrato L (2001). Nutritional mushrooms widely consumed in Italy. *Food Chem.* 73: 321-325.
- Mattila P, Konko K, Eurola M, Pihlawa JM, Astola J, Vahteristo Lietaniemi V, Kumpulainen J, Valtonen M, Piironen V (2001). Contents of vitamins, mineral elements, and some phenolic compounds in cultivated mushrooms. *J. Agric. Food Chem.* 49: 2343-2348.
- Mattila PH, Piironen VI, Uusi R, Koivistoinen, PE (1994). Vitamin D contents in edible mush. *J. Agr. Food Chem.* 42: 2449-2453.
- Mattila PK, Konko M, Eurola J, Pihlava J, Astola L, Vahteristo V, Hietaniemi J, Kumpulainen N, Valtonen V, Piironen V (2000). Contents of vitamins, mineral elements and some phenolic compounds in the cultivated mushrooms. *J. Agric. Food Chem.* 49: 2343-2348.
- Moses ST (1948). A preliminary report on the mushrooms of Baroda. *Dept. of Fisheries, Baroda State, Baroda, Bulletin No. XIV, 1-3.*
- Natarajan K (1995). Mushroom flora of South India (except Kerala). In: Chadha KL, Sharma SR (eds). *Advances in Horticulture 13 Mushroom*. Malhotra Publishing House, New Delhi, India. pp. 381-397.
- Negi CS (2006). Morels (*Morchella* sp.) in Kumayun Himalaya. *Nat. Prod. Rad.* 5(4): 306-310.
- Nitha B, Meera CR, Janardhanan KK (2007). Anti-inflammatory and antitumour activities of cultured mycelium of morel mushroom, *Morchella esculenta*. *Curr. Sci.* 92(2): 235-239.
- Orgundana SK, Fagade O (1981). The nutritive value of some Nigerian edible mushrooms. In: *Mushroom Science XI, Proceedings of the Eleventh International Scientific Congress on the Cultivation of Edible Fungi, Australia.* pp. 123-131.
- Panda AK, Swain KC (2011). Traditional uses and medicinal potential of *Cordyceps sinensis* of Sikkim. *J. Ayurveda Integr. Med.* 2(1):9-13.
- Pande V, Palni UT, Singh SP (2004). Species diversity of ectomycorrhizal fungi associated with temperate forest of Western Himalaya: a preliminary assessment. *Curr. Sci.* 86(12): 1619-1623.
- Paracer CS, Chahal DS (1962). A new edible species of genus *Agaricus* in the Punjab (India). *Mycopathologia.* 18(4): 267-270.
- Pathak NC, Gupta S (1979). *Agaric flora of Lucknow*. In: Chattopadhyay SB, Smajpati N (eds). *Advances in Mycology and Pathology*. Oxford and IBH Publishing Co., New Delhi, India. pp. 76-80.
- Patil BD, Jadhav SW, Sathe AV (1995). Mushroom flora of Maharashtra. In: Chadha KL, Sharma SR (eds). *Advances in Horticulture 13, Mushroom*. Malhotra Publishing House, New Delhi, India. pp. 317-328.
- Pradeep CK, Vrinda KB, Mathew S, Abraham TK (1998). The genus *Volvariella* in Kerala state, India. *Mushroom Res.* 7(2): 53-62.
- Purkayastha RP, Chandra A (1974). A new species of edible mushroom from India. *Trans Brit. Mycol. Soc.* 62: 415-418.
- Purkayastha RP, Chandra A (1985). *Manual of Indian Edible Mushrooms*. Today and Tomorrow's Printers and Publishers, New Delhi, India.
- Pushpa H, Purushothama KB (2012). Biodiversity of Mushrooms in and Around Bangalore (Karnataka), India. *American-Eurasian J. Agric. Environ. Sci.* 12(6):750-759.

- Pushpa H, Purushothoma KB (2010). Nutritional analysis of wild and cultivated edible medicinal mushrooms. *World J. Dairy Food Sci.* 5(2): 140-144.
- Puttaraju NG, Venkateshaiah SU, Dharmesh SM, Urs SM, Somasundaram R (2006). Antioxidant Activity of Indigenous Edible mushrooms. *J. Agric. Food Chem.* 54: 9764-9772.
- Quereshi S, Pandey AK, Sandhu SS (2010). Evaluation of antibacterial activity of different *Ganoderma lucidum* extracts. *People's J. Scientific Res.* 3(1): 9-13.
- Rai M, Biswas G, Chatterjee S, Mandal SC, Acharya K (2009). Evaluation of antioxidant and nitric oxide synthase activation properties of *Armillaria mellea* Quel. e *J. Biological Sci.* 1(1):39-45.
- Rai M, Tidke G, Wasser SP (2005). Therapeutic potential of mushrooms. *Nat. Prod. Rad.* 4(4): 246-257.
- Rai RD, Saxena S, Upadhyay RC, Sohi HS (1988). Comparative nutritional value of various *Pleurotus* species grown under identical conditions. *Mushroom. J. Tropics.* 8: 93-98.
- Rai RD, Sohi HS (1988). How protein rich are mushrooms. *Indian Horticulture.* 33: 2-3.
- Ramesh C, Pattar MG (2010). Antimicrobial properties, antioxidant activity and bioactive compounds from six wild edible mushrooms of Western Ghats of Karnataka, India. *Pharmacognosy Res.* 2(2): 107-112.
- Rattan SS (1977). The Resupinate Aphylophorales of the North-western Himalaya. *J. Cramer, Vaduz, Liechtenstein.*
- Ribeiro B, Valentao P, Baptista P, Seabra RM, Andrade PB (2007). Phenolic compounds, organic acids profiles and antioxidative properties of beefsteak fungus (*Fistulina hepatica*). *Food Chem. Toxicol.* 45: 1805-1813.
- Roberto Garibay-Orijel J, Cifuentes, Estrada-Torres A, Caballero J (2005). Fungal Biodiversity, People using macro-fungal diversity in Oaxaca, Mexico.
- Rolfe RT, Rolfe FW (1925). *The Romance of the fungus world.* Chapman and Hall Ltd., London. pp. 309.
- Rout D, Mondal S, Chakraborty I, Pramanik M, Islam SS (2004). Structural characterization of an immunomodulating polysaccharide isolated from aqueous extract of *Pleurotus florida* fruit-bodies. *Med. Chem. Res.* 13: 509-517.
- Rout D, Mondal S, Chakraborty I, Pramanik M, Islam SS (2005). Chemical analysis of a new (1→3), (1→6)-branched glucan from an edible mushroom, *Pleurotus florida*. *Carbohydrate Res.* 340: 2533-2539.
- Roy SK, Das D, Mondal S, Maiti D, Bhunia B, Maiti TK, Islam SS (2009). Structural studies of an immunoenhancing water-soluble glucan isolated from hot water extract of an edible mushroom, *Pleurotus florida*, cultivar Assam Florida. *Carbohydrate Res.* 344: 2596-2601.
- Sachan SKS, Patra JK, Thatoi HN (2013). Indigenous knowledge of ethnic tribes for utilization of wild mushrooms as food and medicine in simlipal biosphere reserve, Odisha, India. *J. Agric. Technol.* 9(2):335-348.
- Saini SS, Atri NS (1995). Mushroom flora of Punjab. In: Chadha KL, Sharma SR (eds). *Advances in Horticulture 13, Mushroom.* Malhotra Publishing House, New Delhi, India. pp. 374-386.
- Saini SS, Atri NS, Bhupal MS (1988). North Indian Agaricales-V. *Indian Phytopathol.* 41: 622-625.
- Saini SS, Atri NS, Gupta AK (1997). Studies on the Genus *Agaricus* L: Fr. The subgenus *Agaricus* section *Sanguinolenti* Schaeff. et Moller from North West India. *Mushroom Res.* 6(2): 53-58.
- Samajipati N (1978). Nutritive value of Indian edible mushrooms. *Mushroom Sci.* 10: 695-703.
- Samorini G (2001). *Fungi Hallucinogeni. Studi etnomicologici.* Teleserion. Dozza, Bologna, Italy. pp. 250.
- Sarbhoy AK, Aggarwal DK, Varshney JL (1996). *Fungi of India.* CBS Publishers and Distributors, Daryaganj, New Delhi, India.
- Sarma TC, Sarma I, Patiri BN (2010). Wild edible mushrooms used by some ethnic tribes of western Assam. *The Bioscan.* 3: 613-625.
- Sathe AV, Kulkarni SM (1987). A check list of wild edible mushrooms from South-West India. In: Kaul TN, Kapur BM (eds). *Indian Mushroom Science II. Regional Research Laboratory, Jammu, India.* pp. 411-413.
- Sathe AV. 1979. Agaricology in India-a review of work on Indian Agaricales. *Biovigyanam.* 5: 125-130.
- Sharma PM, Sidhu D (1991). Notes on Himalayan *Geoglossaceae*. In: Khullar SP, Sharma MP (eds). *Himalayan Botanical Researches.* Ashish Publishing House, New Delhi, India. pp. 13-29.
- Sharma RP, Kaisth KR, Lakhanpal TN (1988). Protein and mineral content of two edible *Lactarius species*. *Indian J. Mushrooms.* 14: 44-47.
- Sharma SS, Doshi A, Trivedi A (1992). Records and Reports. *Mush Res.* 1: 41.
- Sheena N, Lakshmi B, Janardhanan KK (2005). Therapeutic potential of *Ganoderma lucidum* (Fr.) P. Karst. *Nat. Prod. Rad.* 4(5): 382-386.
- Shimada K, Fujikawa K, Yahara K, Nakamura T (1992). Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *J. Agric. Food Chem.* 40:945-948.
- Sing NI, Sing SM (1993). Edible fleshy fungal flora of Manipur. *Bioveel.* 4(2): 153-158.
- Sing NI, Sing SM, Th C (2002). Fleshy fungi of Manipur. In: Vij SP, Kondo K, Sharma ML, Gupta A (eds). *Plant Genetic Diversity: Exploitation, Evaluation, Conservation.* Affiliated East West Press Pvt. Ltd., New Delhi, India. pp. 9-13.
- Singdevsachan SKS, Patra JK, Thatoi HN (2013). Nutritional and Bioactive Potential of Two Wild Edible Mushrooms (*Lentinus sajor-caju* and *Lentinus torulosus*) from Similipal Biosphere Reserve, India. *Food Sci. Biotechnol.* 22(1):137-145.
- Singer R (1961). *Mushrooms and Truffles,* Leonard Hill Books Ltd. pp. 272.
- Singh RD (1994). Edible mushrooms of arid zones of Rajasthan. Abstracts of Papers Presented at the National Symposium on Mushrooms. Mushroom Society of India, Solan, India. pp. 8.
- Sivakumar R, Vetrichelvan T, Rajendran NN, Indira Devi M, Sundaramoorthi K, Shankar ASK, Shanmugam S (2006). Antibacterial activity of mushroom *Osmoporus odoratus*. *Indian J. Bacteria. Sci.* 68(4): 523-524.
- Sohi HS, Kumar S, Seth PK (1964). Some interesting fleshy fungi from Himanchal Pradesh. *Indian Phytopath.* 17: 317-322.
- Sohi HS, Kumar S, Seth PK (1965). Some interesting fleshy fungi from Himachal Pradesh. *J. Indian Bot. Soc.* 54: 69-73.
- Srivastava B, Dwivedi AK, Pandey VN (2011). Ethnobotanical survey, distribution and utilization of *Termitomyces* species in Gorakhpur forest division. *Plant Sciences Feed.* 1(3): 28-33.
- Surekha Ch, Kaladhar DSVGK, Raju Srikakarlupudi JR, Haseena (2011). Evaluation of antioxidant and antimicrobial potentiality of some edible mushrooms. *Int. J. Adv. Biotech. Res.* 2(1): 130-134.
- Swapna S, Abrar S, Krishnappa M (2008). Diversity of macrofungi in semi-evergreen and moist deciduous forest of Shimoga District-Karnataka, India. *J. Mycol. Plant Pathol.* 38(1): 21-26
- Tambaker DH, Sonar TP, Khodke MV, Khante BS (2006). The novel antimicrobials from two edible mushrooms: *Agaricus bisporus* and *Pleurotus sajor caju*. *Int. J. Pharmacol.* 2(5): 584-587.
- Thind KS (1961). *The Clavariaceae of India.* Indian Council of Agricultural Research, New Delhi, India.
- Upadhyay RC, Kaur A, Semwal KC (2007). New records of fleshy fungi from North Western Himalaya. In: Rai RD, Singh SK, Yadav MC, Tewari RP (eds). *Mushroom Biology and Biotechnology.* Mushroom Society of India, Solan, India. pp. 13.
- Verma RN, Singh GB, Bilgrami KS (1987). Fleshy fungal flora of N.E.H. India- I. Manipur and Meghalaya. *Indian Mush. Sci.* 2: 414- 421.
- Verma RN, Singh GB, Mukta Singh S (1995). Mushroom flora of north-eastern hills. In: Chadha KL, Sharma SR (eds). *Advances in Horticulture 13 Mushroom.* Malhotra Publishing House, New Delhi, India. pp. 329-349.
- Vishwakarma MP, Bhatt RP, Gairola S (2011). Some medicinal mushrooms of Garhwal Himalaya, Uttarakhand, India. *Int. J. Med. Arom. Plants.* 1(1):33-40.
- Vishwakarma MP, Bhatt RP, Joshi S (2012). Macrofungi diversity in moist temperate forests of Garhwal Himalaya. *Indian J. Sci. Technol.* 5(1):1928-1932.
- Wani BA, Bodha RH, Wani AH (2010). Nutritional and medicinal importance of mushrooms. *J. Med. Plants Res.* 4(24): 2598-2604.
- Wasser SP, Weis AL (1999). Medicinal properties of substances

occurring in higher basidiomycetes mushrooms: current perspectives (review). *Int. J. Med. Mushrooms*. 1:31-62.

Watling R, Gregory NM (1980). Higher fungi from Kashmir. *Nova Hedwigia*. 32:493-564.

Review

Nanosilver: Potent antimicrobial agent and its biosynthesis

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Silver ions and its salts are well known for their potent antimicrobial agent. These days, the nanosilver are widely used in a growing number of applications ranging from home disinfectants and medical devices to water purifier due to properties of silver at the nano level. Nanosilver has a large ratio of surface which dramatically increases the potential for silver ions to be accessed anywhere in body where larger silver particles cannot. The synthesis of silver nanoparticles has been reported using chemical and physical methods. This review describes a cost effective and ecofriendly approach for the synthesis of silver nanoparticles. Thus, in this review we focus on the role of microorganisms and plants in the synthesis of nanosilver and their potent application as antimicrobial agent.

Key word: Nanosilver, antimicrobial, disinfectant, nanoparticles.

INTRODUCTION

Nanoscale science is the discipline that examine the unique behaviors and properties of materials that emerge at the size range of 1 to 100 nanometers (a billionth of a meter). Nanobiotechnology is a sub-discipline of nanoscience that has arisen more recently. It manipulates unique behaviors and properties at the nanoscale to manipulate materials for various applications in biology. Nanobiotechnology already impact in medicine as therapeutic agent.

Nanobiotechnology promises to be critical in advances in other related fields. Silver has been valued for many of its properties that are useful for mankind. It is used in precious goods such as currencies, ornaments, jewelry, electrical contacts and photography, among others. The one of the most potent uses of silver as antimicrobial agent that is toxic to bacteria, fungi, and viruses. Due to their size, nanosilver have a very large surface area which typically results in greater biological activity, chemical reactivity and catalytic behavior compared to larger particles of the same chemical composition (Garnett and Kallinteri, 2006; Limbach et al., 2007). Nanotechnology is being utilized in offering many new developments in the

fields of biosensors, biomedicine, bio nanotechnology diagnosis, therapeutic drug delivery and the development of treatments for many diseases and disorders. Nanosilver have novel or enhanced properties based on specific characteristics such as size, distribution and morphology. Nanosilver show potential of applications in various fields such as catalysts in chemical reactions, electrical batteries and in selective coatings for absorption of solar energy, as optical elements, pharmaceutical components and in chemical sensing and biosensing (Kamat, 2002), plasmonics (Maier et al., 2001), optoelectronics (Gracias et al., 2002), biological sensor (Mirkin et al., 1996; Han et al., 2001) and pharmaceutical applications like their potential in drug delivery formulations and routes.

The synthesis of nanosilver has been remarkable developments in the field of nanotechnology from the decade. A large numbers of methods have been reported for the synthesis of nanosilver of particular shape and size depending on specific requirements (Figure 1). Nanosilver was produced by physical and chemical methods. Some of the commonly used physical and chemical

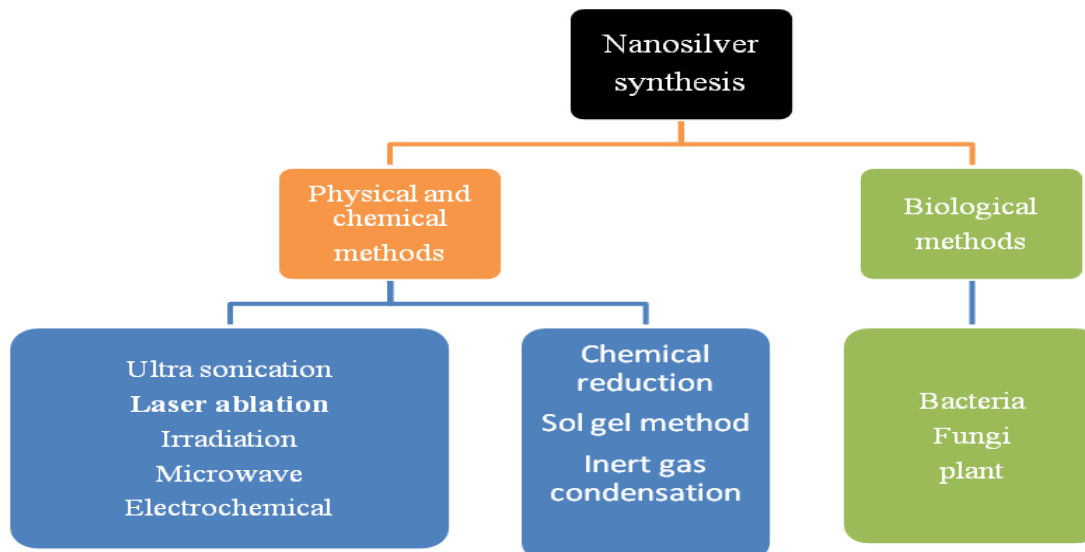


Figure 1. Different methods for nanosilver synthesis.

methods are ion sputtering, thermal synthesis, Inert gas condensation, reduction and sol gel technique.

Unfortunately, nanosilver synthesis involves the use of hazardous chemicals, low material conversions, high energy requirements, difficult and wasteful purifications. Further, the synthesis of nanosilver including physical and chemical processes is costly. Therefore, there is a growing need to develop environmentally friendly processes for nanoparticle synthesis without using toxic chemicals. Biosynthetic methods employing either microorganisms or plant extracts have emerged as a simple and viable alternative to chemical synthetic procedures and physical methods.

The development of cheaper reliable, eco-friendly processes for the synthesis of nanosilver is an important aspect of nanotechnology today. Many biological synthesis protocols have been reported using bacteria (Kalimuthu et al., 2008; Fu et al., 1999) fungi (Bhainsa and D'Souza, 2006; Vigneshwaran et al., 2006; Basavaraja et al., 2008; Kathiresan et al., 2009) and plants (Selwal et al., 2013; Singh et al., 2010; Dubey et al., 2009). In this review, we focus on the biological synthesis of silver nanoparticles, potential and the possible mechanism of antimicrobial actions.

NANOSILVER SYNTHESIS- AN OVERVIEW

Nano silver are one of the promising products in the nanotechnology industry. The development of consistent processes for the synthesis of silver nanoparticles is an important aspect of current nanotechnology research. One of such promising process is green synthesis. Silver nanoparticles can be synthesized by several physical, chemical and biological methods.

However for the past few years, various rapid chemical methods have been replaced by greens synthesis because of avoiding toxicity of the process and increased quality.

Approaches for nanosilver synthesis

Basically there are two approaches for nanoparticle synthesis namely the Bottom up approach and the Top down approach (Figure 2).

Top down approach

In the Top down approach involve production of nanosilver involve mechanical grinding of a bulk piece of the material.

Bottom up approach

The Bottom up approach involves chemical and biological methods to make nanostructures and nanoparticles. These processes involve controlled condensation of solute molecules that are formed during a chemical reaction. The restriction of the condensation or growth leads to the formation of particles of desired size and shape.

Biosynthesis of nanoparticles is a kind of scale down or bottom up approach where the main reaction occurring is reduction/oxidation. The microbial enzymes or the phytochemicals with anti oxidant or reducing properties are usually responsible for reduction of metal compounds into their respective nanoparticles.

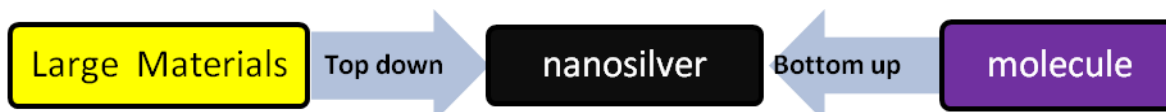


Figure 2. The top-down approach versus the bottom-up approach.

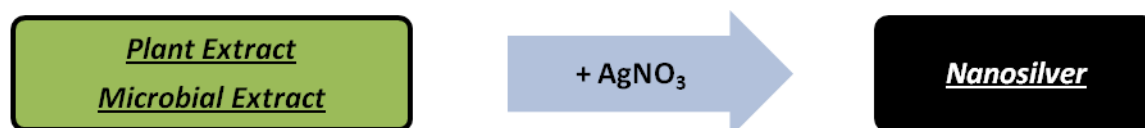


Figure 3. Plant and microbial mediated synthesis of nanosilver.

Physical and chemical methods of nanosilver synthesis

Some of the very successful physical methods for the synthesis of nanoparticles include radiolysis, ultrasonication, laser ablation, microwave and electrochemical methods. However, physical methods have had limited success and therefore chemical methods for the synthesis of inorganic nanoparticles are widely accepted and commonly practiced. Nanosilver can be synthesized chemically by reduction or oxidation of metal ions, inert gas condensation, or by the sol gel methods.

Disadvantages of using physical and chemical methods

The synthesis of nanoparticles including physical and chemical processes is costly. The use of these synthesis methods requires both strong and weak chemical reducing agents and protective agents like sodium borohydride, sodium citrate and alcohols. These agents are mostly toxic, flammable, cannot be easily disposed off due to environmental issues and also show a low production rate.

There is need to search of cheaper path-ways for nanoparticle synthesis, so used microorganisms and plant extracts for synthesis. The biosynthesis of nano-scale nanosilver may contribute to the development of relatively new and largely unexplored area of research.

Biological synthesis of nanosilver

Green plant and microorganisms have a remarkable ability to form exquisite nanosilver. Biological synthesis using plant and microorganisms is eco-friendly and cheaper. Nowadays a large number of microorganisms and plant extracts are used for nanosilver synthesis (Figure 3).

Bacterial mediated synthesis of nanosilver

The probability of the bacterial mediated synthesis of silver nanoparticles has been successfully reported (Table 1). Bacterial cells are used to produce silver nanoparticles. These particles possess interesting physical and chemical properties (Figure 4).

Fungus mediated synthesis of nanosilver

The probability of the fungal system for the synthesis of silver nanoparticles has been successfully demonstrated (Table 2). The fungal system shows the capability of both intracellular and extracellular synthesis of silver nanoparticles. In the recent past, research work using the fungal system has been carried out using both aspects of intracellular and extracellular methods for synthesis of nanosilver.

Plant mediated synthesis of nanosilver

The probability of the plants extract for the synthesis of silver nanoparticles has been successfully reported (Table 3). Plants extracts can be used in the synthesis of silver nanoparticles. However, the mechanism for the synthesis of silver nanoparticle from plants is not yet fully understood. Phytochemicals are assumed to be responsible for the formation of silver nanoparticles.

APPLICATIONS OF NANOSILVER

Nanosilver as powerful antimicrobial

The potent application of nanosilver is use as antimicrobial agent. Nanosilver shows strong antimicrobial activity is a major reason for the development of nano-

Table 1. List of bacteria synthesizing silver nanoparticles.

Bacteria	Size of the particle (nm)	Reference
<i>Bacillus licheniformis</i>	50	Kalimuthu et al., 2008
<i>Bacillus megaterium</i>	46.9	Fu et al., 2009
<i>Pseudomonas stutzeri</i>	200	Tanja et al., 1999
<i>Corynebacterium sp.</i>	10-15	Zhang et al., 2005
<i>Bacillus licheniformis</i>	50	Kalishwaralal et al., 2008
<i>Geobacter sulfurreducens</i>	200	Law et al., 2008
<i>Morganella sp.</i>	20-25	Parikh et al., 2008
<i>Bacillus subtilis</i>	5-60	Saifuddin et al., 2009
<i>Escherichia coli</i>	1-100	Gurunathan et al., 2009b
<i>Klebsiella pneumonia</i>	50	Ahmad et al., 2007
<i>Proteus mirabilis</i>	10-20	Samadi et al., 2009
<i>Bacillus sp.</i>	5-15	Pugazhenthiran et al., 2009
<i>Staphylococcus aureus</i>	1-100	Nanda and Saravanan, 2009
Lactic acid bacteria	11.2	Sintubin et al., 2009
<i>Brevibacterium casei</i>	50	Kalishwaralal et al., 2010
<i>Cryphonectria sp.</i>	30-70 nm	Dar et al., 2013

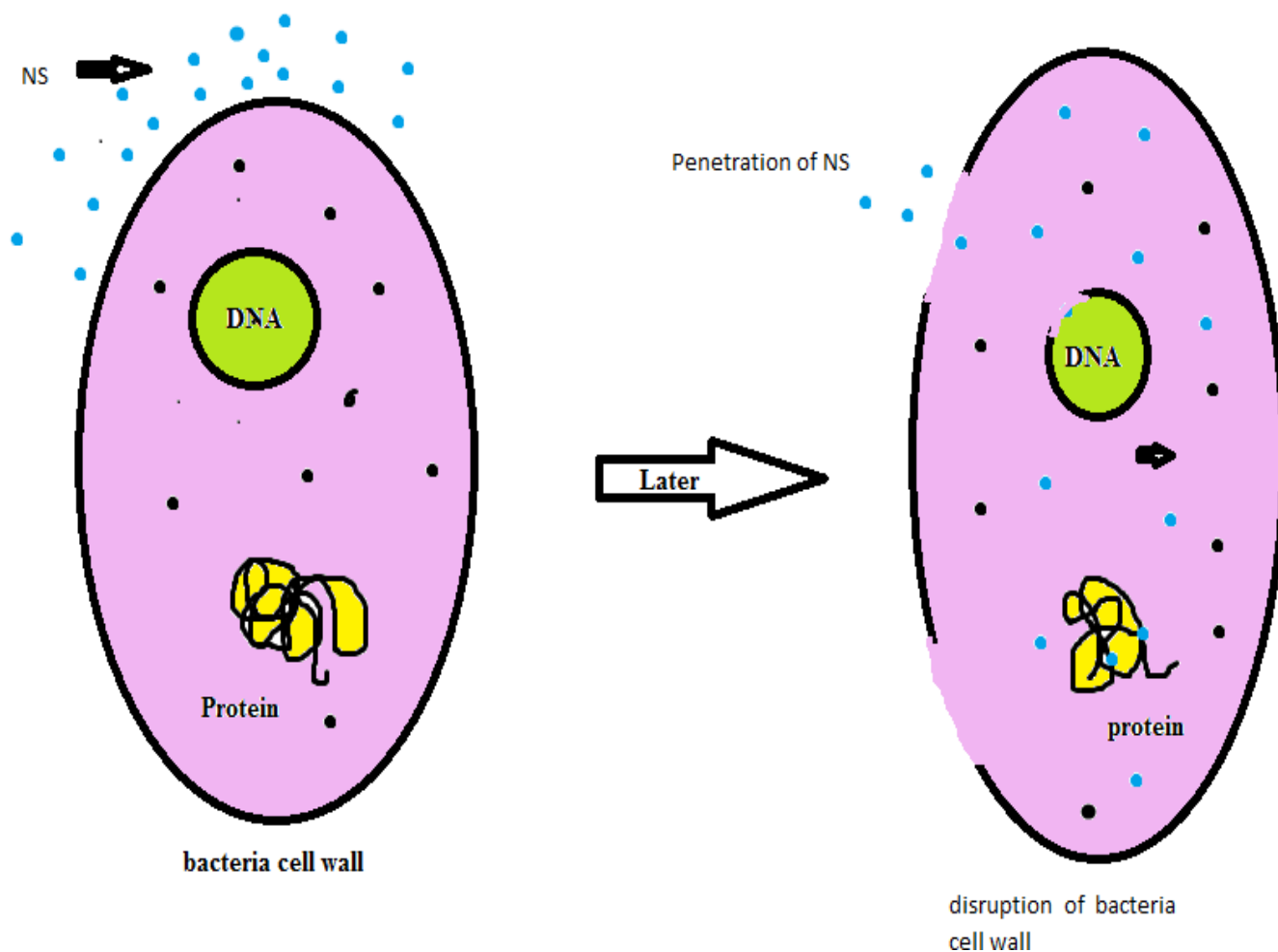
**Figure 4.** Attachment and penetration of nanosilver with bacterial cell membrane.

Table 2. List of Fungi synthesizing silver nanoparticles.

Fungi	Size of the particle (nm)	Reference
<i>Aspergillus fumigates</i>	5-25	Bhainsa et al., 2006
<i>Phaenerochaete chrysosporium</i>	100	Vigenshwaran et al., 2006
<i>Fusarium semitectum</i>	10-60	Basavaraja et al. 2008
<i>Penicillium fellutanum</i>	1-100	Kathiresan et al, 2009
<i>Fusarium oxysporum</i>	5-50	Ahmad et al., 2003
<i>Trichoderma viride</i>	5-40	Fayaz et al., 2010
<i>Fusarium oxysporum</i>	5-40	Kumar et al., 2007
<i>Fusarium semitectum</i>	13-18	Basavaraja et al., 2007
<i>Aspergillus niger</i>	20	Gade et al., 2008
<i>Fusarium acuminatum</i>	5-40	Ingle et al., 2008a
<i>Trichoderma asperellum</i>	13-18	Mukherjee et al., 2008
<i>Penicillium</i>	10-100	Sadowski et al., 2008
<i>Phoma glomerata</i>	60-80	Birla et al., 2009
<i>Fusarium solani</i>	5-35	Ingle et al., 2008b
<i>Coriolus versicolor</i>	67.6-74.52	Sanghi and Verma, 2009
<i>Cladosporium cladosporioides</i>	10-100	Balaji et al., 2009

Table 3. List of plant synthesizing silver nanoparticles.

Plant	Size of the particle (nm)	Reference
<i>Mangifera indica</i>	-	Selwal et al., 2013
<i>Argemone mexicana</i>	10-50	Singh et al., 2009
<i>Eucalyptus hybrid</i> (Safeda)	50-150	Dubey et al., 2009
<i>Brassica juncea</i>	15-25	Lamb et al., 2001
<i>Azadirachta indica</i>	5-100	Shankar et al., 2004
<i>Emblica officinalis</i>	10-20	Ankamwar et al., 2005
<i>Aloe vera</i>	15±4.2	Chandran et al., 2006
<i>Capsicum annum</i>	-	Li et al., 2007
<i>Helianthus annuus</i>	-	Leela and Vivekanandan, 2008
<i>Gliricidia sepium</i>	10-50	Raut et al., 2009
<i>Jatropha curcas</i>	10-20	Bar et al., 2009
<i>Carica papaya</i>	60-80	Mude et al., 2009
<i>Coriandrum sativum</i>	26	Sathyavathi et al., 2010
<i>Artemisia nilagirica</i>	70-90	Vijayakumar et al., 2013
<i>Rhinacanthus nasutus</i>	11.5- 22	Pasupuleti et al., 2013

silver containing products. Nano-silver have used for thousands of years without ill effects. Nanosilver is an effective killing for bacteria, fungus, and viruses.

Nanosilver as antibacterial

Nanosilver is an effective killing agent against a broad spectrum of Gram negative and Gram- positive bacteria (Burrell et al., 1999; Wijnhoven et al., 2009; Yin et al., 1999) including antibiotic-resistant strains (Percival et al.,

2007; Wright et al., 1998). Silver nanoparticles show potential antimicrobial effects against infectious organisms including *Escherichia coli*, *Bacillus subtilis*, *Vibrio cholera*, *Pseudomonas aeruginosa*, *S.typhus*, and *Staphylococcus aureus*. It is important that silver is a natural element that is commonly used throughout the world at low concentrations with no evidence to support a concern for widespread bacterial resistance. Nanosilver has a large surface which increases the potential for silver ions to interact with the bacteria and reduction in size of silver at nanoscale make enable it to be released

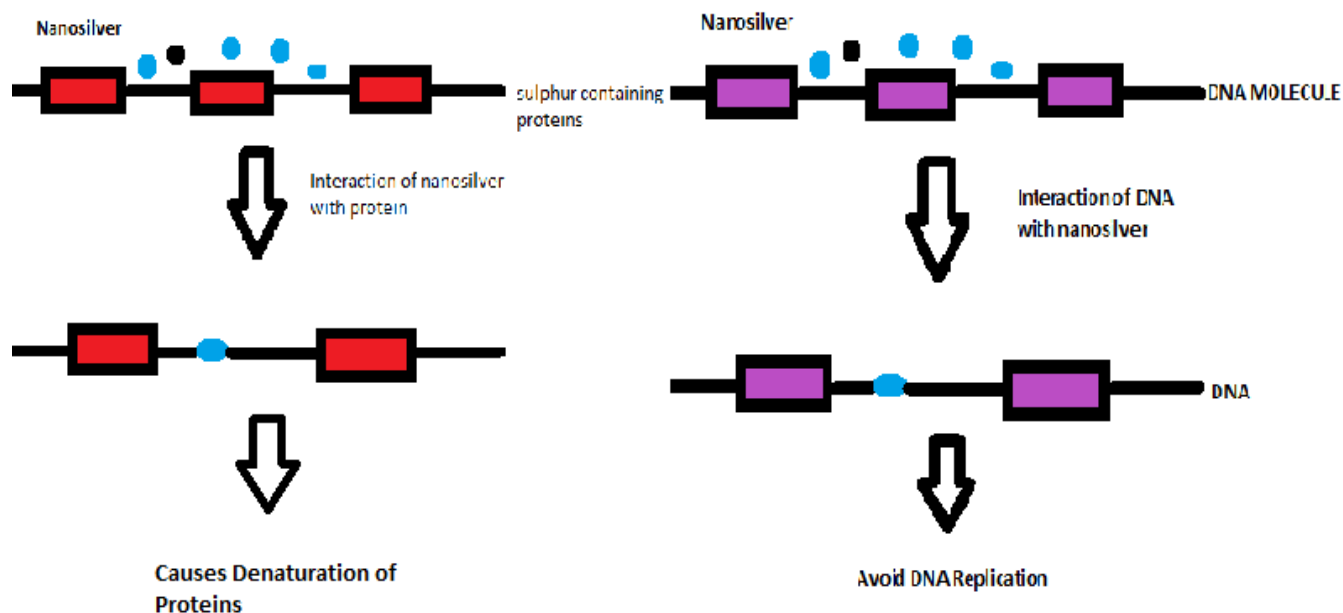


Figure 5. Interaction of nanosilver with proteins and DNA.

anywhere in body where larger silver particles cannot. The exact mechanism of silver nanoparticles is not scientifically justified and is a debated topic. There are however various theories are put forwarded on the possible mechanism for the antimicrobial action of nano silver. The possible mechanisms of action are:

1. Nanosilver as nanometer scale silver provides an extremely large surface area for better contact with bacteria. These silver nanoparticles get attached to the cell membrane and easily penetrate inside the bacteria (Rai et al., 2009).
2. Nanosilver attack the respiratory chain in microbial mitochondria which lead in to the death (Sondi and Salopek-Sondi, 2009).
3. Nanosilver interacts with sulphur containing proteins on microbial cell membrane causing disruption (Liau et al., 1997) (Figures 4 and 5).
4. The surface of microbes having phosphorus containing compound like DNA, nanosilver inhibit their functions (Matsumura et al., 2003).
5. Nanosilver release Ag^+ ion inside the microbial cell which may create free radicals and induce oxidative stress, thus further enhancing their bactericidal activity (Kim et al., 2007).

Nanosilver as antifungal

Nanosilver is an effective, fast-acting fungicide against a broad spectrum of fungi including genera such as *Aspergillus*, *Candida*, and *Saccharomyces*. The mecha-

nisms similar to that of the antibacterial actions have been proposed for fungi (Wright et al., 1999).

Nanosilver as antiviral

Nanosilver (average diameter ~10 nm) inhibit HIV-1 virus replication (Sun et al., 2005). Size- dependent antiviral activity of silver nanoparticles has been shown with HIV-1 virus (Elechiguerra et al., 2005). It is evident that nanosilver due to its biological and physiochemical properties is potent as antimicrobials and therapeutic agents. They can be used for many challenges in the field of nanomedicine.

Nanosilver in medicine

Nanosilver shows potent antibacterial activities against various pathogens and immensely useful in the medical field (Guzman et al., 2012). Earlier studies have suggested that nanosilver can inhibit the gamma interferon and alpha tumor necrosis factor which are involved in inflammation (Shin et al., 2007). These anti-inflammatory responses induced by nanosilver make it an anti-inflammatory agents. Polymethyl methacrylate along with nanosilver is being considered as bone cement as the nanosilver can encourage antimicrobial activity (Bechert et al., 2004). The antimicrobial property of nanosilver shows enormous potential to be used in disinfectants (Brady et al., 2003).

The plasmonic properties of nanosilver can effectively

biosense that makes it ideal biosensors for a large number of proteins that normal biosensors find more difficulty in detections. This unique advantage that nanosilver has, can be utilized for detecting various abnormalities and diseases in the human body including cancer (Moore and Goettmann, 2006; Zhang et al., 2009; Ghodselahi et al., 2011). Due to potent application as antimicrobial agent nanosilver used for development of novel chitin/nanosilver composite scaffolds for wound dressing (Madhumathi et al., 2010), nanosilver are currently being used in many household products such as cloths, washers, water purification systems, tooth paste, shampoo, fabrics, deodorants, filters, paints, kitchen utensils, and toys to impart antimicrobial properties (Baker et al., 2005).

CONCLUSION AND FUTURE PROSPECTS

Silver nanoparticles are conveniently prepared through leaves extract. The biological synthesis of silver nanoparticles is fast, ecofriendly and cheaper. The biological method for synthesis of silver nanoparticles with high antimicrobial activity has been developed. Nanosilver is an excellent antimicrobial and has been used for many years. Chemical and physical methods of nanosilver synthesis have been used for decades, but they are found to be expensive and the use of various toxic chemicals for their synthesis therefore, the biological synthesis is the most preferred option. Plant, bacterial, and fungal extract can be used for nanosilver synthesis, the easy availability, the nontoxic nature, the various options available, and the advantage of quicker synthesis make plant extracts the best and an excellent choice for nanosilver synthesis.

Rapid synthesis and excellent reproducibility of silver nanoparticles from the plant extract is a time saving and advantage over microbial synthesis because laborious and lengthy procedures are involve in mantainace of microbial cultures. Nanosilver contributes into potential application as antimicrobial agent. This has been utilized in various processes in the medical field and has hence been exploited well.

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REFERENCES

Ahmad A, Mukherjee P, Senapati S, Mandal D, Khan MI, Kumar R (2003). Extracellular biosynthesis of silver nanoparticles using the fungus *Fusarium oxysporum*. Coll. Surf. B. 28:313-318
 Ahmad RS, Sara M, Hamid RS, Hossein J, Ashraf-Asadat N (2007). Rapid synthesis of silver nanoparticles using culture supernatants of

Enterobacteria: a novel biological approach. Process Biochem. 42:919-923
 Ankamwar B, Damle C, Ahmad A, Sastry M (2005). Biosynthesis of gold and silver nanoparticles using *Emblica officinalis* fruit extract, their phase transfer and transmetalation in an organic solution. J. Nanosci. Nanotechnol. 5:1665-1671.
 Baker C, Pradhan A, Pakstis L, Pochan DJ, Shah SI (2005). Synthesis and antibacterial properties of silver nanoparticles. J. Nanosci. Nanotechnol. 5:244-249
 Balaji DS, Basavaraja S, Bedre Mahesh D, Prabhakar BK, Venkataraman A (2009). Extracellular biosynthesis of functionalized silver nanoparticles by strains of *Cladosporium cladosporioides*. Coll. Surf. B. 68:88-92.
 Bar H, Bhui DK, Gobinda PS, Priyanka S, Santanu P, Ajay M (2009). Green synthesis of silver nanoparticles using seed extract of *Jatropha curcas*. Colloids Surf A Physico Chem. Eng. Asp. 348:212-216.
 Basavaraja S, Balaji SD, Lagashetty A, Rajasab AH, Venkataraman A (2008). Extracellular biosynthesis of silver nanoparticles using the fungus *Fusarium semitectum*, J. Mater. Res. Bull. 43:1164- 1170.
 Basavaraja S, Balaji SD, Lagashetty A, Rajasab AH, Venkataraman A (2008). Extracellular biosynthesis of silver nanoparticles using the fungus *Fusarium semitectum*. Mater. Res. Bull. 43(5):1164-1170.
 Bechert T, Steinrücke P, Wagener M, Seidel P, Dingeldein E, Scheddin D, Domann E, Schnettler R (2004). Nanoparticulate silver. A new antimicrobial substance for bone cement. Orthopade. 33:885-892.
 Bhainsa KC, D'Souza SF (2006). Extracellular synthesis using the fungus *Aspergillus fumigates*, Coll. Surf. B. 47:152-157.
 Birla SS, Tiwari VV, Gade AK, Ingle AP, Yadav AP, Rai MK (2009). Fabrication of silver nanoparticles by *Phoma glomerata* and its combined effect against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Lett. Appl. Microbiol. 48:173-179.
 Brady MJ, Lissay CM, Yurkovetskiy AV, Sarwan SP (2003). Persistent silver disinfectant for environmental control of pathogenic bacteria. Am. J. Infect. Control. 31:208-214
 Burrell RE, Heggors JP, Davis GJ, Wright JB (1999). Efficacy of silver-coated dressings as bacterial barriers in a rodent burn sepsis model. Wounds. 11:64- 71.
 Chandran SP, Chaudhary M, Pasricha R, Ahmad A, Sastry M (2006). Nanotriangles and silver nanoparticles using *Aloe vera* plant extract. Biotechnol. Progr. 22:577-583
 Dar MA, Ingle A, Rai M (2013). Enhanced antimicrobial activity of silver nanoparticles synthesized by *Cryphonectria sp.* evaluated singly and in combination with antibiotics. Nanomed. Nanotechnol. Biol. Med. 9:105-110.
 Dubey M, Bhadauria S, Kushwah BS (2009). Green synthesis of nanosilver particles from extract of *Eucalyptus hybrid* (Safeda) leaf Dig. J. Nanomater. Biostruct. 4:537-543.
 Elechiguerra JL, Burt JL, Morones JR, Camacho-Bragado A, Gao X, Lara HH, Yacaman MJ (2005). Interaction of silver nanoparticles with HIV-1. J. Nanobiotechnol., June 29:3-6.
 Fayaz M, Tiwary CS, Kalaichelvan PT, Venkatesan R (2010). Blue orange light emission from biogenic synthesized silver nanoparticles using *Trichoderma viride*. Coll. Surf. B. 75(1):175-178.
 Fu JK, Zhang WD, Liu YY, Lin ZY, Yao BX, Weng SZ, (1999). Characterization of adsorption and reduction of noble metal ions by bacteria. Chem. J. Chin. Univ. 20:1452-4.
 Gade AK, Bonde P, Ingle AP, Marcato PD, Dura'n N, Rai MK (2008). Exploitation of *Aspergillus niger* for synthesis of silver nanoparticles. J. Biobased Mater Bioenergy. 3:123-129.
 Garnett MC, Kallinteri P (2006). Nanomedicines and nanotoxicology: some physiological principles. Occ. Med. 56:307-311.
 Ghodselahi T, Nejad TN, Vesaghil MA Salimi KZ, Mobasheri H (2011). Synthesis of Silver Nanoparticles Array and Application of Their Localized Surface Plasmon Resonance in Biosensor Design IPCBEE vol.2
 Gracias DH, Tien J, Breen T, Hsu C, Whitesides GM (2002). Forming electrical networks in three dimensions by self assembly, Sci. 289:1170-1172.
 Gurunathan S, Kalishwaralal K, Vaidyanathan R, Venkataraman D, Pandian SRK, Muniyandi J, Hariharan N, Eom SH (2009b). Biosynthesis, purification and characterization of silver nanoparticles

- using *Escherichia coli*. Colloids Surf B. 74(1):328-335
- Guzman M, Dille J, Godet S (2012). Synthesis and antibacterial activity of silver nanoparticles against gram-positive and gram-negative bacteria. Nanomed. Nanotechnol. Biol. Med. 8:35-45.
- Han M, Gao X, Su JZ, Nie S (2001). Quantum-dotted microbeads for multiplexed optical coding of biomolecules, Nature Biotechnol. 19:631-635.
- Ingle A, Rai M, Gade A, Bawaskar M (2008b). *Fusarium solani*: a novel biological agent for the extracellular synthesis of silver nanoparticles. Journal of Nanoparticle Research. 10: in press.
- Ingle AP, Gade AK, Pierrat S, Sønnichsen C, Rai MK (2008a). Mycosynthesis of silver nanoparticles using the fungus *Fusarium acuminatum* and its activity against some human pathogenic bacteria. Curr Nanosci. 4:141-144.
- Kalimuthu K, Babu RS, Venkataraman D, Bilal M, Gurunathan S (2008). Biosynthesis of silver nanocrystals by *Bacillus licheniformis*, Coll. Surf. B. 65:150-153.
- Kalishwaralal K, Deepak V, Pandian SRK, Kottaisamy M, BarathManiKanth S, Kartikeyan B, Gurunathan S (2010). Biosynthesis of silver and gold nanoparticles using *Brevibacterium casei*. Coll. Surf. B. 77(2):257-262
- Kalishwaralal K, Deepak V, RamkumarPandian S, Nellaiah H, Sangiliyandi G (2008). Extracellular biosynthesis of silver nanoparticles by the culture supernatant of *Bacillus licheniformis*. Mater Lett. 62:4411-3
- Kamat PV (2002). Photophysical, photochemical and photocatalytic aspects of metal nanoparticles, J. Phy. Chem. B. 106:7729-7744.
- Kathiresan K, Manivannan S, Nabeal MA, Dhivya B (2009). Studies on silver nanoparticles synthesized by a marine fungus, *Penicillium fellutanum* isolated from coastal mangrove sediment, Coll. Surf. B. 71:133-137.
- Kim JS, Kuk E, Yu KN, Kim JH (2007). Antimicrobial effects of silver nanoparticles, Nanomed.:Nanotechnol., Biol. Med. 3:95-101
- Kumar SA, Ayoobul AA, Absar A, Khan MI, (2007). Extracellular biosynthesis of CdSe quantum dots by the fungus, *Fusarium Oxysporum*. Journal of Biomedical Nanotechnology. 3:190-194.
- Lamb AE, Anderson WN, Haverkamp RG (2001). The induced accumulation of gold in the plants *Brassica juncea*, *Berkheya coddii* and *chicory*. Chem. New Zealand. 9:34-36.
- Law N, Ansari S, Livens FR, Renshaw JC, Lloyd JR (2008). The formation of nano-scale elemental silver particles via enzymatic reduction by *Geobacter sulfurreducens*. Appl. Environ. Microbiol. 74:7090-7093
- Leela A, Vivekanandan M (2008). Tapping the unexploited plant resources for the synthesis of silver nanoparticles. Afr. J. Biotechnol. 7:3162-3165.
- Li S, Qui L, Shen Y, Xie A, Yu X, Zhang L, Zhang Q (2007). Green synthesis of silver nanoparticles using *Capsicum annum* L. extract. Green Chem. 9:852-858.
- Liau SY, Read DC, Pugh WJ, Furr JR, Russell AD (1997). Interaction of silver nitrate with readily identifiable groups: relationship to the antibacterial action of silver ions. Lett. Appl. Microbiol. 25:279-283
- Limbach LK, Wich P, Manser P, Grass RN, Bruinink A, Stark WJ (2007). Exposure of engineered nanoparticles to human lung epithelial cells: influence of chemical composition and catalytic activity on oxidative stress. Environ. Sci. Technol. 41:4158-4163.
- Madhumathi K, Kumar PT, Abhilash S, Sreeja V, Tamura H, Manzoor K, Nair SV, Jayakumar R (2010). Development of novel chitin/nanosilver composite scaffolds for wound dressing applications. J. Mater. Sci. Mater Med. 21:807-813.
- Maier SA, Brongersma ML, Kik PG, Meltzer S, Requicha AAG, Atwater HA (2001). Plasmonics- A Route to Nanoscale Optical Devices, Adv. Mater. 19:1501-1505.
- Matsumura Y, Yoshikata K, Kunisaki SI, Tsuchido T (2003). Appl. Mode of Bactericidal Action of Silver Zeolite and Its Comparison with That of Silver Nitrate. Environ. Microbiol. 69:4278-4281
- Mirkin CA, Letsinger RL, Mucic RC, Strohof JJ (1996). A DNA-based method for rationally assembling nanoparticles into macroscopic materials. Nature 382:607-609.
- Moores A, Goettmann F (2006). The plasmon band in noble metal nanoparticles: an introduction to theory and applications. New J. Chem. 30:1121-1132
- Mude N, Ingle A, Gade A, Rai M (2009). Synthesis of silver nanoparticles using callus extract of *Carica papaya* - a first report. J. Plant Biochem. Biotechnol. 18(1):83-86
- Mukherjee P, Ahmad A, Mandal D, Senapati S, Sainkar SR, Khan MI, Parishcha R, Ajaykumar PV, Alam M, Kumar R, SastryM (2001). Fungus-mediated synthesis of silver nanoparticles and their immobilization in the mycelial matrix: a novel biological approach to nanoparticles synthesis. Nano Lett. 1:515-519
- Nanda A, Saravanan M (2009). Biosynthesis of silver nanoparticles from *Staphylococcus aureus* and its antimicrobial activity against MRSA and MRSE. Nanomedicine 5(4):452-456.
- Parikh RY, Singh S, Prasad BL, Patole MS, Sastry M, Shouche YS (2008). Extracellular synthesis of crystalline silver nanoparticles and molecular evidence of silver resistance from *Morganella*. sp.: towards understanding biochemical synthesis mechanism. Chem. Biochem. 9:1415-1422
- Pasupuleti VR, Prasad TNKV, Shiekh RA, Balam SK, Narasimhulu G, Reddy CS, Rahman IA, Gan SH (2013). Biogenic silver nanoparticles using *Rhinacanthus nasutus* leaf extract: synthesis, spectral analysis, and antimicrobial studies. Int. J. Nanomed. 8:3355-3364.
- Percival SL, Bowler PG, Dolman J (2007). Antimicrobial activity of silver-containing dressings on wound microorganisms using an in vitro biofilm model. Int. Wound. J. 4:186-191
- Pugazhenthiran N, Anandan S, Kathiravan G, Prakash NKU, Crawford S, Ashokkumar M (2009). Microbial synthesis of silver nanoparticles by *Bacillus sp.* J. Nanopart. Res. 11(7):1811-1815
- Rai M, Yadav A, Gade A (2009). Silver nanoparticles as a new generation of antimicrobials. Biotechnol. Adv. 27:76-83
- Raut R, Jaya SL, Niranjana DK, Vijay BM, Kashid S (2009). Photosynthesis of silver nanoparticles using *Gliricidia sepium* (Jacq.). Curr. Nanosci. 5(1):117-122
- Sadowski IH, Maliszewska B, Grochowalska I, Polowczyk T, Koźlecki M (2008). Synthesis of silver nanoparticles using microorganisms, Mater. Sci. Poland 26:2.
- Saifuddin N, Wong CW, AA Nur yasumira (2009) Rapid biosynthesis of silver nanoparticles using culture supernatant of bacteria with microwave irradiation. Eur. J. Chem. 6:61-70
- Samadi N, Golkaran D, Eslamifar A, Jamalifar H, Fazeli MR, Mohseni FA (2009). Intra/extracellular biosynthesis of silver nanoparticles by an autochthonous strain of *Proteus mirabilis* isolated from photographic waste. J. Biomed. Nanotechnol. 5(3):247-253
- Sanghi R, Verma P (2009). Biomimetic synthesis and characterisation of protein capped silver nanoparticles. Bioresour. Technol. 100:501-504.
- Sathyavathi R, Krishna MB, Rao SV, Saritha R, Rao DN (2010). Biosynthesis of silver nanoparticles using *Coriandrum sativum* leaf extract and their application in nonlinear optics. Adv. Sci. Lett. 3(2):138-143
- Selwal KK, Sarsar V, Selwal MK (2013). Green synthesis of silver nanoparticles using leaf extract of *Mangifera indica* and evaluation of their antimicrobial activity J. Microbiol. Biotech. Res. 3 (5):27-32
- Shankar SS, Rai A, Ahmad A, SastryM (2004). Rapid synthesis of Au, Ag and bimetallic Au core-Ag shell nanoparticles using Neem (*Azadirachta indica*) leaf broth. J. Coll. Int. Sci. 275:496-502
- Shin SH, Ye MK, Kim HS, Kang HS (2007). The effects of nano-silver on the proliferation and cytokine expression by peripheral blood mononuclear cells. Int. Immunopharmacol. 7:1813-1818
- Singh A, Jain D, Upadhyay M, Khandelwal N (2010). Green synthesis of silver nanoparticles using *Argemone mexicana* leaf extract and evaluation of their antimicrobial activities. J. Nanomater. 5:483-489.
- Sintubin L, De Windt W, Dick J, Mast J, Ha DV, Verstraete W, Boon N (2009). Lactic acid bacteria as reducing and capping agent for the fast and efficient production of silver nanoparticles. Appl. Microbiol. Biotechnol. 84(4):741-749
- Sondi I, Salopek-Sondi B (2009). Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria. J. Colloid Interface Sci. 275:177-82.
- Sun RW, Chen R, Chung NP, Ho CM, Lin CL, Che CM (2005). Silver nanoparticles fabricated in HEPES buffer exhibit cytoprotective activities toward HIV-1 infected cells. Chem. Commun. (Camb.):5059-5061.
- Tanja K, Ralph J, Eva O, Claes-Gé (1999). Silver-based crystalline

- nanoparticles, microbially fabricated. *Proc Natl. Acad. Sci.* 96:13611-13614
- Vigenshwaran N, Kathe A, Nacahe PV, Balasubramanya RH (2006). Biomimetics of silver nanoparticles by white rot fungus, *Phanerochaete chrysosporium*, *Coll. Surf. B.* 53:55-59.
- Vijayakumar M, Priya K, Nancy FT, Noorlidah A, Ahmed ABA (2013). Biosynthesis, characterization and anti-bacterial effect of plant-mediated silver nanoparticles using *Artemisia nilagirica*. *Ind. Crop. Prod.* 41:235-240.
- Wijnhoven SWP, Peijnenburg WJGM, Herberts CA, Hagens WI, Oomen AG, Heugens EHW, Roszek B, Bisschops J, Gosens I, Van de Meent D, Dekkers S, De Jong WH, Zijverden M, Sips AJAM, Geertsma RE (2009). Nanosilver - a review of available data and knowledge gaps in human and environmental risk assessment. *Nanotoxicology.* 3(2):109-138.
- Wright JB, Lam K, Burrell RE (1998). Wound management in an era of increasing bacterial antibiotic resistance: A role for topical silver treatment. *Am. J. Infect. Control.* 26:572-577.
- Wright JB, Lam K, Hansen D, Burrell RE (1999). Efficacy of topical silver against fungal burn wound pathogens. *Am. J. Infect. Control* 27:344-350.
- Yin HQ, Langford R, Burrell RE (1999). Comparative evaluation of the antimicrobial activity of Acticoat antimicrobial barrier dressing. *J. Burn Care Rehabil.* 20:195-200.
- Zhang H, Li Q, Lu Y, Sun D, Lin X, Deng X (2005). Biosorption and bioreduction of diamine silver complex by *Corynebacterium*. *J. Chem. Technol. Biotechnol.* 80:285-290.
- Zhang Y, Zhang K, Haiyan Ma (2009). Electrochemical DNA biosensor based on silver nanoparticles/poly(3-(3-pyridyl) acrylic acid)/carbon nanotubes modified electrode. *Anal. Biochem.* 387:13-19

Full Length Research Paper

Genetic polymorphism of milk protein variants and their association studies with milk yield in Sahiwal cattle

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The objective of this study was to determine the allele and genotype frequencies of genetic variants in five milk protein genes and estimate the effect of these variants on milk yield in Sahiwal cattle. Genotypes of five milk protein genes (*alpha s1 casein*, *beta casein*, *kappa casein*, *alpha lactalbumin* and *beta lactoglobulin*) were detected using SNaPshot genotyping method. All the five milk protein genes studied exhibited polymorphism with high allele frequencies of 0.51 for *alpha s1casein C*, 0.93 for *beta casein A2*, 0.92 for *kappa casein A*, 0.93 for *alpha lactalbumin B* and 0.91 for *beta lactoglobulin B*. Statistically significant differences ($p > 0.05$) were observed in *kappa casein* genotypes AA (AA) and AB (AC) that is, genotype AB had more milk yield in 1st lactation (422 kg) and 2nd lactation (612 kg), respectively. In conclusion, the AB genotype identified in *kappa casein* gene is associated with higher milk production therefore incorporation of AB and BB genotypes for *kappa casein* may help to improve the milk yield in Sahiwal cattle population of Pakistan. To the best of our knowledge, this is the first detailed study involving frequency distribution of genetic variants and their effects on milk yield in *Bos indicus* Sahiwal cattle of Pakistan.

Key words: Genetic variant, milk protein genes, Sahiwal cattle.

INTRODUCTION

For centuries, animal breeders have greatly and effectively manipulated the genomes of livestock species and enhanced production traits in their herds by selecting superior individuals as predecessors for the next generations. This indirect use of hereditary information without involving molecular knowledge may give useful results in terms of production but may ignore some reproductive traits of animal. Hence, there is need to use selection methods that are based on genomic studies (André, 2012). Genes that are correlated with performance parameters can improve the estimation of breeding value and hence can work as a suitable supplement to conventional breeding

procedures. Genetic polymorphism related to the differences in animal performance can be taken into account in the selection process.

Milk is an important source of essential nutrients for lactating calves and a key raw material for human food preparations (Reinhardt et al., 2012). All over the world people fulfill approximately 13% of their protein requirement from milk and milk products. Bovine milk proteins are generally classified as caseins, which make up about 80% of the milk proteins, consisting of four proteins: Alpha S1 (CSN1S1, 39-46% of total caseins), alpha S2 (CSN1S2, 8-11%), beta (CSN2, 25-35%), and kappa (CSN3, 8-

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Abbreviations: NCBI, National Centre of Biotechnology Information; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; MFGM, milk fat globule membrane; RFLP, restriction fragment length polymorphism; SSCP, single strand conformation polymorphism.

15%) (Eigel et al., 1984). Whey proteins make about 16% of the total milk protein and contain two major proteins *alpha lactalbumin* and *beta lactoglobulin*. Other minor part is made by peptones/low molecular weight peptides (3%) and milk fat globule membrane (MFGM) proteins (1%) (D'Alessandro et al., 2011).

Ruminant's milk proteins are coded by highly polymorphic genes, containing an unusually large number of polymorphisms (Nilsen et al., 2009). Since the pioneer work done by Aschaffenburg and Drewry in 1955 who discovered alleles A and B of β lactoglobulin in cattle the researchers worldwide became interested in genetic polymorphisms of major milk proteins. This chain of studies continued and according to a recent review by Caroli et al. (2009) of milk protein variants, 9 α s1 - CN (A, B, C, D, E, F, G, H, I), 4 α s2 -CN (A, B, C, D), 12 β -CN (A1, A2, A3, B, C, D, E, F, G, H1, H2, I), 14 κ -CN (A, A1, B, B2, C, D, E, F1, F2, G1, G2, H, I, J), 11 β -LG (A, B, C, D, E, F, G, H, I, J, W), and 3 α -LA (A, B, C), modified from Farrell et al. (2004) have been reported.

These allelic forms are controlled by codominant autosomal genes according to the Mendelian mode of inheritance. The different genetic variants of milk proteins differ from each other by only a few amino acid substitutions or deletions within the polypeptide chain (Eigel et al., 1984). Several studies have been carried out to determine the frequencies of genetic variants of milk proteins in different cattle breeds (Erhardt, 1996; Baranyi et al., 1996; Lien et al., 1999; Jeichitra et al., 2003; Caroli et al., 2004) and possible relationships between milk protein polymorphism and economically important production traits, milk composition, and quality have been widely studied (Yasemin and Cengiz, 2006) due to the potential use of milk protein types as an aid to genetic selection. However no comprehensive and detailed studies are available in Sahiwal cattle breed. The study of various allelic forms is important in terms of their effect and frequencies in this breed.

The Sahiwal is breed of *Bos indicus*, known to have the greatest potential for milk production, growth and reproductive efficiency in tropical environments compared with other *B. indicus* breeds (Mwandotto, 1994; Dahlin et al., 1998; Khan et al., 1999; Muhuyi et al., 1999). In Pakistan, the major breeding tracts of Sahiwal lie in Montgomery (Sahiwal) district Rachagani et al. (2006). Its ability to endure and produce in harsh environments coupled with its dual purpose role has widened the distribution of this breed in tropical and subtropical countries. Among *B. indicus* breeds, the Sahiwal is the most frequently used in dairy crossbreeding in the tropics (Muhuyi, 1997; Kahi et al., 2000).

Milk protein genetic variability in cattle has been extensively studied at both the DNA and protein levels for evolutionary and biodiversity analyses (Caroli et al., 2004). Typing milk proteins at the DNA level does not require the gene product, which renders feasible the genotyping of males and non-lactating females and even embryos.

Recent advances in single nucleotide polymorphism (SNP) detection allow different genotyping ways of detecting polymorphisms. One of these techniques, SNaPshot genotyping has been optimized to genotype SNPs in the bovine α S1-casein, β -casein, κ -casein, α -lactalbumin and β -lactoglobulin genes. SNaPshot genotyping is a newly optimized rapid and efficient screening procedure that could provide more accurate predictions of breeding values of animals to be selected, and thus improve response to selection.

This study was designed to identify variants in five milk protein genes, three caseins (α S1, β and κ) and two whey proteins (α -lactalbumin and β -lactoglobulin) and to determine allele and genotype frequencies of milk protein genetic variants in Sahiwal cattle of Pakistan. We also investigated the association between observed milk protein genetic variants and milk yield in this group of animals. Moreover, we report the effects of identified milk protein genetic variants on milk yield. Although polymorphism was observed in genes of all five studied proteins, only *Kappa casein* polymorphism had statistically significant ($p > 0.05$) association with milk yield in Sahiwal cattle. Genotype AB of *Kappa casein* had more milk yield in 1st lactation (422 kg) and 2nd lactation (612 kg), respectively. Hence genotype information at an early age can help the farmers to improve milk yield by replacing tradition selection methods currently being used in Pakistan.

MATERIALS AND METHODS

Phenotypic data and genomic DNA samples

Phenotypic data of the selected animals was collected which include animal's age, milking record, calving record, sire and dam names. Blood samples of 120 Sahiwal cattle breed were collected from Research Centre for Conservation of Sahiwal cattle (RCCSC, Khanewal) and Livestock Production Research Institute (LPRI, Okara). DNA was extracted using a standard protocol (Sambrook et al., 2001).

Primers design

Primers were designed using the bovine α s1casein, β casein, *kappa casein*, α -lactalbumin and β -lactoglobulin gene sequences available at the National Centre of Biotechnology Information (NCBI) website. Promoter regions of α -s1 casein and α -lactalbumin were amplified by polymerase chain reaction (PCR) using primers AS1CN and PLAL while exon VII of *beta casein*, exon IV of *kappa casein* and exon IV of *beta lactoglobulin* were amplified by PCR using primers X7.1Bcn, KCN and X4Blg, respectively (Table 1). Primers for SNaPshot SNP genotyping were designed using the website <http://www.basic.northwestern.edu/biotools/oligocalc.html>. The primers were tailored to immediately end 5' site before the target SNP (Table 1).

Genomic DNA amplification

PCR reaction (25 μ l total volume) included 100 ng of bovine genomic DNA, 0.16 pmol of amplification primer (Table 1), 2.5 U *Taq* DNA polymerase, 2 mM $MgCl_2$ and 100 μ M dNTPs. Thermal profile included initial denaturation for 3 min at 94°C, followed by 35

Table 1. Primer sequences.

Marker	Primer sequences (5'-3')	T _m (°C)	Amplicon size
AS1CN (a)	F-TGCATGTTCTCATAATAACC R-GAAGAAGCAGCAAGCTGG	52	310
7.1BCN (b)	F-GATTTGTTTTCTTCTTTCCAGGAT R-GTTGGAGGAAGAGGCTGGTG	58	357
KCN (c)	F-ATCATTATGGCCATTCCACCAAAG R-GCCCATTTGCCTTCTCTGTAACAGA	60	350
PLAL (d)	F-AGATTCTGGGGAGGAAAGGA R-GGGTGGCATGGAATAGGAT	58	250
X4BLG (e)	F-TGGGGGCTGACCAGAAAC R-CTCCTCCTCCTGGGAATCAA	60	282
AS1CN (f)	Forward 1st round TGCATGTTCTCATAATAACC	56	310
	Reverse 1st round GAAGAAGCAGCAAGCTGG		
	Extension Primer sequence GAGAGTTTACAACAAAGAAG		21
BCN (g)	Forward 1st round AACATCCCTCCTCTTACTCAAACCCCT	66	338
	Reverse 1st round ATATCTCTCTGGGGATAGGGCACTGCT		20
	Extension Primer sequence GTTGAGCCCTTACTGAAA		
KCN (h)	Forward 1st round AACATCCCTCCTCTTACTCAAACCCCT	60	350
	Reverse 1st round ATATCTCTCTGGGGATAGGGCACTGCT		20
	Extension Primer sequence GTTGAGCCCTTACTGAAA		
ALAL (i)	Forward 1st round AGATTCTGGGGAGGAAAGGA	58	250
	Reverse 1st round GGGTGGCATGGAATAGGAT		
	Extension Primer sequence GGGGTCACCAAAATGAT		19
BLG (j)	Forward 1st round Primer TGGGGGCTGACCAGAAAC	60	282
	Reverse 1st round Primer CTCCTCCTCCTGGGAATCAA		
	Extension Primer sequence AGCCCGAGCAAAGCCTGG		19

a, Sequencing primers for promoter region of *alpha s1 casein* gene; b, sequencing primers for exon 7 of *beta casein* gene; c, sequencing primers for exon 4 of *kappa casein* gene; d, sequencing primers for promoter region of *alpha lactalbumin* gene; e, sequencing primers for exon 4 of *beta lactalbumin* gene; f, SNaPshot primer set for *alpha s1 casein* gene; g, SNaPshot primer set for *beta casein* gene; h, SNaPshot primer set for *kappa casein*; i, SNaPshot primer set for *alpha lactalbumin* gene; j, SNaPshot primer set for *beta lactoglobulin*, bp: base pair; T_m, melting temperature.

cycles of 45 s at 94°C, 45 s at 56°C, 45 s at 72°C and final extension for 10 min at 72°C using a GeneAmp PCR System 9700 (Applied Biosystems, CA). Annealing temperatures for studied milk proteins are also mentioned in Table 1.

PCR amplification for sequencing

The PCR products from 120 cattle DNA samples were purified by Microcon (Millipore Corporation, MA) and sequenced using the Big Dye Terminator Cycle Sequencing v3.1 Ready Reaction kit on an ABI PRISM 3730 automated sequencer (Applied Biosystems, CA).

PCR amplification for SNaPshot genotyping

The SNaPshot® Multiplex Kit a single-tube was used to interrogate SNPs at known locations using recommended protocol from Applied Biosystems, CA.

Genotyping by SNaPshot protocol

The minisequencing reaction produced one (homozygote) or two (heterozygote) peaks depending on the genotype at this *locus*. Three genotypes each for *alpha s1 casein*, *kappa casein* and *beta lactoglobulin* were studied in 120 animals, while *beta casein* and

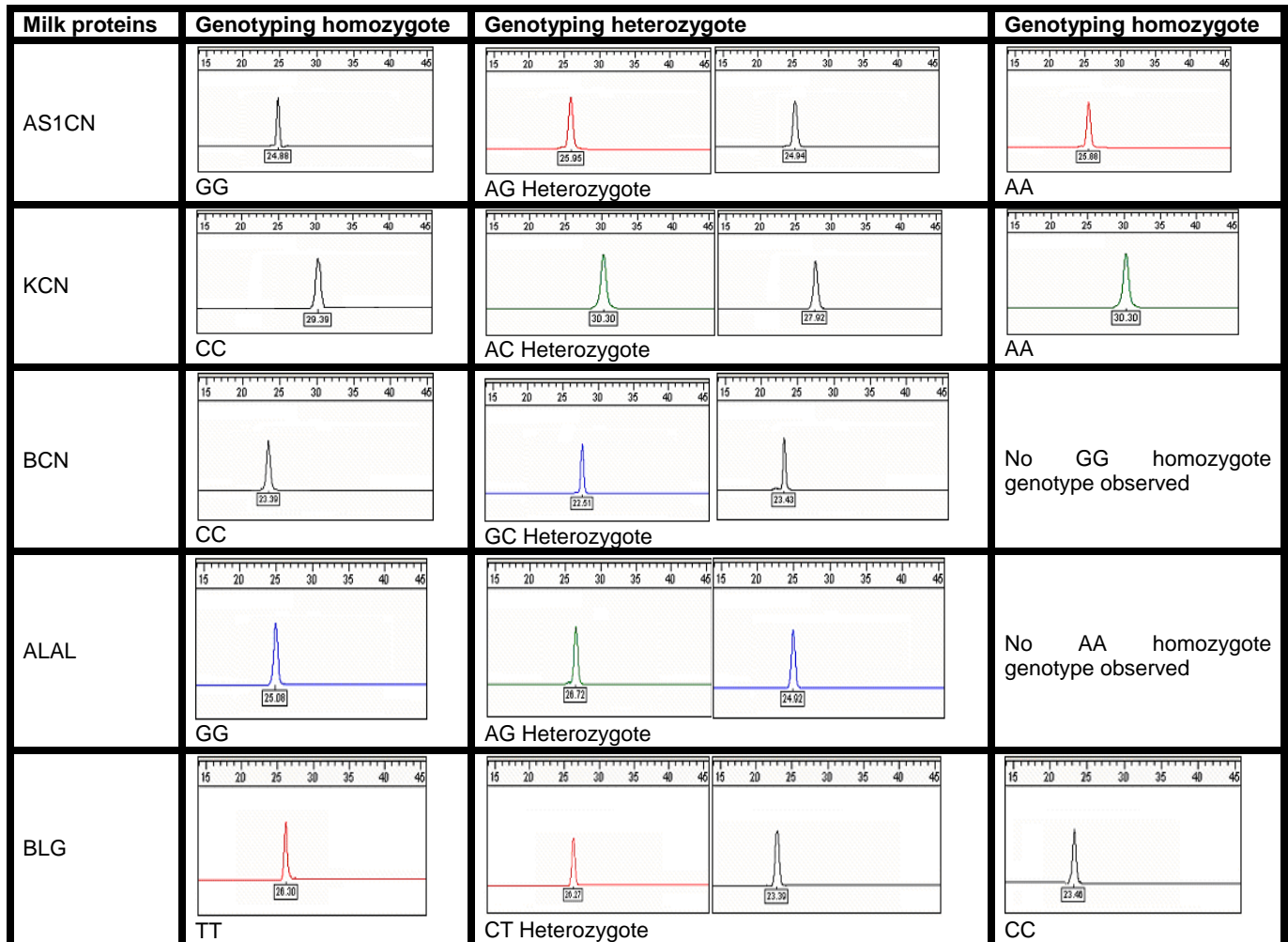


Figure 1. SNaPshot genotyping. AS1CN, *alpha s1 casein*; KCN, *Kappa casein*; BCN, *beta casein*; ALAL, *alpha lactalbumin*, BLG, *beta lactoglobulin*.

alpha lactalbumin showed only two genotypes. For the mini-sequencing technique, black color is assigned to ddCTP and blue color is assigned to ddGTP which is opposite to sequencing where black color depicts ddGTP and blue stands for ddCTP. Forward extension primers were used for *beta casein*, *kappa casein*, *alpha lactalbumin* and *beta lactoglobulin* whereas reverse extension primer was used to genotype *alpha s1 casein* reported variant. As a result, red color peak for A allele (B variant) and black color peak for G allele (C variant) were observed (Figure 1).

Statistical data analysis

Gene counting method (Chang, 1995) was used to estimate allele and genotypic frequencies of the five milk proteins. The chi-square test was used to find association of different milk protein genotypes with milk production. Influences of milk protein loci on milk yield were analyzed by linear model without interaction as follows:

$$Y_{ijkl} = \mu + P_i + G_i + e_{ijkl}$$

Where, Y_{ijkl} , is the Observed value of milk yield; μ , is the population

mean; P_i , is the fixed effect of parity; G_i , is the fixed effect of milk protein genotypes ($i = 1, 2, \dots$); and e_{ijkl} , is the random residual effect.

All data were input by Excel and Statistical Program for Social Sciences (SPSS Version 15.0) was used to adjust 1st lactation and 2nd lactation milk records obtained from dairy farms, those greater than 150 and less than 305 days were fixed to 305 days (simple linear regression model) and one way Analysis of Variance for statistical significance of genotypes.

RESULTS AND DISCUSSION

Allele and genotype frequency distribution

The SNaPshot protocol was successfully used to genotype all the alleles of five milk protein genes. Genotyping one hundred and twenty animals revealed ten DNA polymorphisms (alleles) in five milk protein genes and their allele frequencies were also calculated. Figures 2 and 3 summarize the estimated genotype and allele frequencies of the *alpha*

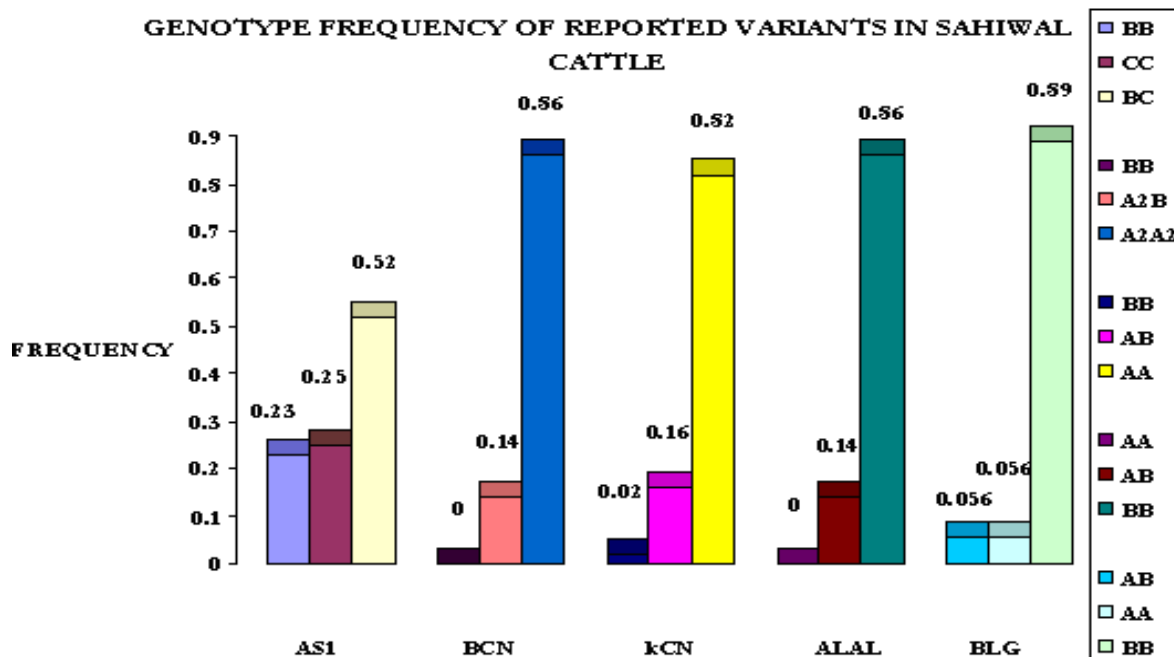


Figure 2. Genotype frequencies of reported variants. AS1: *alpha s1 casein*, BCN: *beta casein*, KCN: *kappa casein*, ALAL: *alpha lactalbumin* and BLG: *beta lactoglobulin*.

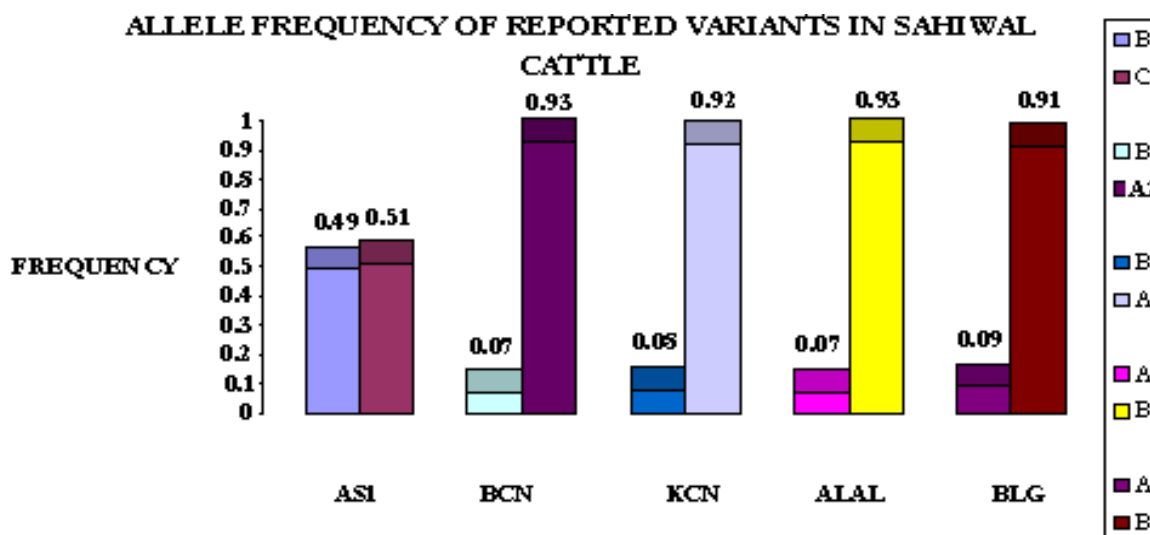


Figure 3. Allele frequencies of reported variants. AS1, *Alpha s1 casein*; BCN, *beta casein*; KCN, *kappa casein*; ALAL, *alpha lactalbumin*; BLG, *beta lactoglobulin*.

s1 casein, *beta casein*, *kappa casein*, *alpha lactalbumin* and *beta lactoglobulin* in Sahiwal cattle breed, respectively.

In *alpha s1 casein*, the genotype frequencies of Sahiwal animals were 0.23, 0.25 and 0.52 for BB, CC and BC genotypes, respectively. Alpha s1casein locus showed an approximately even distribution of alpha s1casein B (0.49) and C (0.51) alleles in Sahiwal cattle.

Most common allelic forms observed in different breeds are B and C. The present data agree with previous reports (Aschaffenburg, 1968; Aschaffenburg et al., 1968) that predominance of the *alpha s1 casein* C allele in the Sahiwal cattle (*B. indicus*) contrasts with the high frequency of the *alpha s1 casein* B allele (90 to 95%) in *Bos taurus* breeds. A frequency close to 0.9 was

reported for the C variant in *B. indicus* (Ivana and Marco, 1997) and *Bos grunniens* (Eigel et al., 1984) while in *B. taurus* its frequency ranges from 0.2 to 0.4 (Baker and Manwell, 1980; Baranyi, 1992; Golijow et al., 1999; Yasemin and Cengiz, 2006). This asymmetric distribution in *B. indicus* breeds and European cattle breed has been explained by the different processes of domestication to which these animals were submitted (Grosclaude et al., 1974). Ivana and Marco (1997) studied frequency distribution at *alpha s1 casein* locus in Brazilian Zebu cattle (Gyr, Guzerat, Sindi, Nelore) and reported predominance of allele B (0.000 - 0.136) over C allele (0.864 - 1.000). Caroli et al. (2008) also reported dominance of B allele (0.8) over C allele (0.08) in Carora cattle. Creangă et al. (2010) reported allele B is more frequently encountered with a higher frequency than 0.7 and allele C had the frequency of 0.2 for the animals of Romanian Grey Steppe Breed.

At β -CN locus, A_2A_2 and A_2B genotypes had frequencies of 0.14 and 0.86, respectively (Figure 2). B allele was less frequent (0.07) and the A_2 allele more frequent (0.93) within the *beta casein* locus (Figure 3). There was no A1 genotype in animals under study. The present data agree with previous reports (Ng-Kwai-Hang et al., 1984; Ivana and Marco, 1997; Malik et al., 2000; Jann et al., 2002) that indicate predominance of the β -CN A_2 allele in the Sahiwal cattle (*B. indicus*). Mishra et al. (2009) also reported absence of A1 allele in most of Indian cattle breeds which is predominant in most of *B. taurus* breeds (Kaminski et al., 2007). The frequency of A1 allele in different breeds varies between 0.01-0.06 (Guernsey), 0.09-0.22 (Jersey), 0.31-0.66 (Holstein), 0.43-0.72 (Ayrshire) and 0.71 (Danish Red) as reviewed by Kaminski et al. (2007). Frequency of B allele in Sahiwal cattle (0.07) is close to Polish Red and Red Danish dairy cattle (0.06) as reported by Erhardt et al. (1998).

Recently Ganguly et al. (2013) reported frequency distribution at *beta casein* locus in Frieswal (HF X Sahiwal Crossbred) hence allele A1 is hypothesized to be of *B. taurus* origin rather than *B. indicus*. The frequency of A2 and A1 allele was 0.65 and 0.35, respectively and this high frequency of A1 in Frieswal is due to crosses between Holstein (*B. taurus*) and Sahiwal (*B. indicus*). Olenski et al. (2010) have reported the frequencies of A1 (0.35) and A2 (0.65) alleles from Polish Holstein-Friesian bulls which are found to be very similar to Frieswal population.

Kappa casein (CSN3) is the most extensively studied milk protein in cattle which showed two predominant DNA genotypes AB and AA in present study with 0.16 and 0.82 frequencies respectively (Figure 2), two out of 120 animals studied were with CC genotype (frequency = 0.02). Allele A was more frequent in Sahiwal cattle than allele B, that is, 0.92 and 0.08, respectively (Figure 3) corroborating several findings (Ng-Kwai-Hang et al., 1984; Pinder et al., 1994; Kemenes et al., 1999; Golijow et al., 1999; Lara et al., 2002; Yasemin and Cengiz, 2006;

Alipanah et al., 2008) that observed this in *B. indicus* and *B. taurus*. The genotyping results are also similar to earlier studies reported for Korean native cattle, Japanese brown, Angus, Hereford, Charolais and Holstein cows by Hung et al. (1995, 1998). Malik et al. (2000) reported similar observations in fourth-generation crossbred cattle (50% Holstein, 25% Jersey, 25% Zebu, that is, Haryana and Sahiwal cattle). Rachagani and Gupta (2008) studied the dominance of A allele in their study performed on Sahiwal and Tharparker breeds of India. High frequency of A allele was also observed in Brazilian zebu Gyr, Guzerat and Nelore cattle (Azevedo et al., 2008). Bonvillani et al. (2010) and Ren et al. (2011) observed a higher frequency of allele A and lower frequency of allele B in Holstein cows. Contrary to these findings, Ceriotti et al. (2004) reported that allele B has higher frequency than allele A in *B. taurus* breeds as compared to *B. indicus* breeds. Moin and Supriyanto (2012) also compared allele frequencies at *kappa casein* locus in the breeds of *B. taurus* and reported higher frequency of B alleles than the breeds of *B. indicus*. Lukac et al. (2013) studied genotype distribution of *kappa casein* in Serbian Holstein-Friesian and found that this locus is at Hardy-Weinberg equilibrium ($P > 0.05$), similar to that found by Hanusová et al. (2010) in Holstein cattle of Slovakia.

Alpha lactalbumin gene had AB and BB genotypes with frequencies of 0.14 and 0.86 while genotype AA was absent in this study (Figure 2). Alleles A and B of *alpha lactalbumin* were observed in this study with frequency of 0.07 for allele A and 0.93 for allele B (Figure 3). The observed higher frequency of the *alpha lactalbumin* B allele was consistent with results reported previously that *B. indicus* had high frequency for B allele than A allele compared to *B. taurus* breeds (Blumberg and Tombs, 1958; Bhattacharya et al., 1963; Bleck et al., 1993b; Ivana and Marco, 1997). Several workers have reported two genetic variants A and B at this locus in *B. indicus* and the droughtmaser (*B. indicus* x *B. taurus*) (Blumberg and Tombs, 1958; Bhattacharya et al., 1963; Bell et al., 1970; Osterhoff and Pretorius, 1966; Chianese et al., 1988), whereas a third variant, namely, C was reported in Bali cattle (*B. javanicus*) by Bell et al. (1981).

AB, AA and BB genotypes of *beta lactoglobulin* occurred at frequencies 0.056 for AB and AA each while BB genotype had highest frequency (0.89) in this study (Figure 2). The frequency of allele A and allele B in Sahiwal cattle were 0.09 and 0.91, respectively (Figure 3). These findings are consistent with studies of other authors (Aschaffenburg, 1964; Ivana and Marco, 1997; Litwinczuk and Krol, 2002; Celik, 2003; Yasmin and Cengiz, 2006; Daniela et al., 2008) who observed that allele A has less frequency than B allele in *B. taurus* as well as in *B. indicus* breeds. Karimi et al. (2009) showed that allele frequency of B allele (0.9125) was higher than that of the A allele (0.0875). In contrast to these findings Heidari et al. (2009) reported allele (frequencies of A and

Table 2. 1st lactation record and observed milk protein genotypes.

Protein	Genotype	Mean	Standard deviation	Significance
α -S1 Casein	BB	1952.8	610.93244	0.555 NS
	BC	1715.0	724.01986	
	CC	1636.8	671.68244	
β -Casein	A2A2	1727.7	602.71792	0.188 NS
	A2B	2027.7	802.18974	
κ -Casein	AA	1935.7	465.12855	0.041 S
	AB	2357.5	619.92349	
α -Lactalbumin	BB	1638.2	287.13450	0.068 NS
	AB	1879.4	396.66632	
β -Lactoglobulin	BB	1728.7	683.86772	0.872 NS
	AB	1936.4	166.05696	
	AA	1906.7	1402.89278	

NS: Non-Significant, S: Significant.

Table 3. 2nd lactation record and observed milk protein genotypes.

Protein	Genotype	Mean	Standard Deviation	Significance
α -S1 Casein	BB	2780.6	825.22309	0.823 NS
	BC	2746.6	937.67345	
	CC	2975.0	1049.21113	
β -Casein	A2A2	2529.6	964.81080	0.671 NS
	A2B	2379.2	635.35901	
κ -Casein	AA	2523.0	811.96566	0.047 S
	AB	3111.8	950.83972	
α -Lactalbumin	BB	2542.1	1000.78859	0.073 NS
	AB	3313.4	2015.84518	
β -Lactoglobulin	BB	2623.6	891.98183	0.910 NS
	AB	2343.0	1319.46125	
	AA	2543.5	1191.47493	

NS, Non-significant; S, significant.

B in Holstein cattle as 0.53 and 0.47, respectively. Lucak et al. (2013) reported similar findings for the Serbian Holstein Friesian cattle that *beta lactoglobulin* locus fitted with Hardy-Weinberg equilibrium ($P < 0.05$), and was similar to that demonstrated by Gouda et al. (2011) in Egyptian Holstein cattle, and Ren et al. (2011) in Chinese Holstein and Jersey cows.

Genotype effects

Data published in research reports regarding correlations between the *alpha s1 casein* gene polymorphism and the

milk traits is controversial that may partly be due to the differences in parameters used and/or variety in cattle breeds. In our study on Sahiwal cattle at *alpha s1 casein* locus all three variants studied (BB, BC and CC) had no effect ($p > 0.05$) on milk 1st lactation, 2nd lactation, (Tables 2 and 3). According to other publications (Ng-Kwai-Hang et al., 1984; Aleandri et al., 1990) *alpha s1 casein* BB genotype correlated with higher milk production than those with either AB or BC genotype. Results of Hristov et al. (2013) also agree with the dominance of the B allele over the C allele relative to the milk production. On the other hand Havliček (1996) and Micinski et al. (2007)

reported superiority of the heterozygous BC genotype with reference to milk yield.

In this study, *beta casein* reported variant had no effect ($p > 0.05$) on 1st lactation, 2nd lactation milk yield (Tables 2 and 3). There are similar findings (Aleandri et al., 1990; Mao et al., 1992; Famula and Medrano, 1994) which agree with our data. But according to Ng-Kwai-Hang et al. (1984), Bovenhuis et al. (1992), Ortner et al. (1995) and Cardak (2005), the *beta casein* genotypes had an effect on milk. Morris et al. (2005) showed superiority of A2 genotype with reference to milk yield in Holstein cattle. Tolenkhomba and Yadav (2012) also studied *beta casein* genotypes in Indian Sahiwal cattle and showed that cows with AB genotype produced more milk (11.81 ± 2.10) than those with AA genotype whose values were 6.48 ± 0.41 . However genetic characterization of *beta casein* is important because A2 allele that is predominant in Sahiwal cattle is beneficial with reference to human health (Chatchatte et al., 2001).

Kappa casein has significant effect ($p < 0.05$) on 1st and 2nd lactation in this study (Tables 2 and 3) which agrees with studies of other authors (Ng-Kwai-Hang et al., 1986; Mao et al., 1992; Cardak, 2005) who suggested that the *kappa casein* is associated with high production for milk during the first lactation, but there is difference in genotypes effecting milk production and even some studies indicated no effect at all (Ng-Kwai-Hang et al., 1990; Lundén et al., 1997). In our study, the animals with genotype AB had a higher 1st and 2nd lactation milk yield than those with genotypes AA and BB. A similar effect had been noticed by Ng-Kwai-Hang et al. (1986) Bovenhuis et al. (1992) and Hirstov et al. (2013) who showed that animals with *kappa casein* AB genotype were better milk producers than either of the animals homozygous for this gene in Ayrshire, Holstein, Jersey, brown Swiss, Canadienne and Guernsey and Bulgarian Rhodopean cattle breeds. Contrary to these findings, Gonyon et al. (1987), Curi et al. (2005), Cardak (2005), Sitkowska et al. (2008) and Ahmadi et al. (2008) reported AA genotype was associated with higher milk production than BB. In contrast, Lin et al. (1986), Van Eenennaam and Medrano (1991), Mao et al. (1992) and Rachagani (2008) reported BB genotype to be significantly affecting milk production than those with genotypes AA and AB. However, Ikonen et al. (1999) showed that the *kappa casein* genotypes had no distinct effect on milk production, which was in agreement with previous studies (Ng-Kwai-Hang et al., 1986; Gonyon et al., 1987; Aleandri et al., 1990; Bovenhuis et al., 1992; Bovenhuis et al., 1994; Famula and Medrano, 1994). This is indicative of variations among species, environments and management practices adopted at different farms.

In Sahiwal cattle, *alpha lactalbumin* did not have significant effect in 1st lactation ($p = 0.068$) and 2nd lactation ($p = 0.073$) on milk production, however, the p values were very close to significance level (Tables 2 and 3). Our results revealed that animals having AB

genotypes had a higher average milk yield than the animals with BB genotypes (Tables 2 and 3). Hence our results are consistent with findings of Bleck and Bermel (1993b) who reported that *alpha lactalbumin* (+15) A variant was associated with greater milk yield. Sashikanth and Yadev (2011) also reported that BB genotype in zebu cattles (Sahiwal, Hariana, Tharparkar) showed higher milk yield. Zhang et al. (2007) screened Chinese Holstein cattle for *alpha lactalbumin* locus and found no significant association between the two genotypes found and milk production traits in these cattle. Recently Zhou et al. (2013) identified single nucleotide substitution C→T (α -LA2516) at position 2516 of the α -LA gene in Chinese Holstein cattle with frequencies of T and C as 0.67 and 0.32, respectively. There was no significant association between genotypes resulting from this SNP and production traits in cattle. In a future study, increase in number of samples may give significant findings in Sahiwal cattle with reference to *alpha lactalbumin*.

There was no significant effect ($p > 0.05$) of *beta lactoglobulin* genotype on the 1st lactation, 2nd lactation in this study (Tables 2 and 3). Our data agree with the results of Lunden et al. (1997) and Ojala et al. (1997) who reported no significant associations of different *beta lactoglobulin* genotypes on milk production. Nevertheless, there are also reports for the positive influence on the milk quantity of all the genotypes, for example, Pupkova, (1980) and Cardak, (2005) reported that cows having AB genotype produce more milk than cows of AA and BB genotypes, however, Aleandri et al. (1990), Bovenhuis et al. (1992) and Ikonen et al. (1999) observed the rare *beta lactoglobulin* genotype AA was associated with the highest milk production. Similar results describing effects of the *beta lactoglobulin* genotypes on milk production traits that were observed (Ikonen et al., 1999) and have been frequently reported (Ng-Kwai-Hang et al., 1984; 1992; Mao et al., 1992).

Heidri et al. (2009) reported that cows with the AA genotype produced more milk than animals with the BB genotype ($P < 0.006$). Contrary to these findings, Jairam and Nair (1983) and Hirstov et al. (2013) showed that the BB genotype determines higher milk production. Ahmadi et al. (2008) reported strong association between BB genotype and protein percentage while there was no association between *beta lactoglobulin* genotypes and milk yield or milk fat percent. Chi square tests showed significant association between genotypes and milk yield in this study (Table 4).

Genotyping technique

The most commonly used techniques used for DNA typing milk protein genes are restriction fragment length polymorphism (RFLP) and single strand conformation polymorphism (SSCP). These techniques either require high quality and quantity of DNA or the low detection sensitivity when amplicon sizes exceed 200 bp. To milk

Table 4. Chi square test for association of genotypes with milk yield.

Protein	Value	df	p-Value
α -S1 Casein	1.947E5	119	0.000
β -Casein	1.525E5	119	0.000
κ -Casein	1.412E5	119	0.000
α -Lactalbumin	1.597E5	119	0.000
β -Lactoglobulin	2.093E5	119	0.000

df, Degree of freedom; p-value, 0.05.

overcome these limitations SNaPshot genotyping was optimized to type genetic variants in all five milk proteins under study. In a future study, all five variants of major proteins will be multiplexed to make this technique cost effective and hence more efficient for genotyping in Sahiwal cattle.

Conclusion

In the present study, we designed a strategy to avoid long and costly traditional selection methods for dairy purposes in Sahiwal cattle. We worked on reported variants of *alpha s1 casein*, *beta casein*, *kappa casein*, *alpha lactalbumin* and *beta lactoglobulin* in Sahiwal cattle. Statistically significant differences were observed in *kappa casein* genotypes AA (AA) and AB (AC), that is, genotype AB had more milk yield than genotype AA in 1st lactation (422 kg) and 2nd lactation (612 kg), respectively. Results of our studies observe that A allele of *kappa casein* is near fixation limit in Sahiwal cattle so it would be difficult to increase frequency of B allele but we assume that selection of bulls with AB and BB genotype for *kappa casein* may help to increase the frequency of B allele in the Sahiwal cattle population of Pakistan. The favorable allele B of *alpha lactalbumin* in Sahiwal cattle breed is already found to be near fixation limit, hence we do not need to alter frequency. However, large population size of Sahiwal cattle must be studied to observe the statistically significant effects of AB genotypes.

Indirect milk tests are limited to mature lactating females and indirect genotyping of sires. These tests require extremely long times to obtain results which makes them impractical for establishing breeding development programs aimed at increasing the frequency of desired milk protein alleles in progeny. Breeding cows are selected on few performances and even before they start their first lactation, which is costly and does not always, result in precise identification of the animal breeds that produce higher milk yield. In dairy cattle, selection of bulls is mostly based on progeny-tests which involve recording the performances of large groups of female off springs of these bulls. A SNP genotyping method that was optimized in this study is simple and efficient which can be used not only for the selection of sires for artificial insemination but also for

selection of immature cows and for pre-implantation embryos for embryo transfer. To the best of our knowledge, this is the first comprehensive study involving milk proteins in the *B. indicus* Sahiwal cattle breed of Pakistan.

REFERENCES

- Ahmadi M, Muhammadi Y, Damani Kuhi H, Osfoori R, Qanbari R (2008). Association between milk protein genotypes and production traits and somatic cell count in Holstein cows. *J. Biol. Sci.* 8(7):1231-1235.
- Aleandri R, Buttazzoni LG, Schneider JC, Caroli A, Davoli R (1990). The effects of milk protein polymorphisms on milk components and cheese-producing ability. *J. Dairy Sci.* 73:241-255.
- Alipanah M, Alexandrovna KL, Veladimirovich RG (2008). *Kappa casein* and PRL-RSAI genotypic frequencies in two Russian cattle breeds. *Arch. Zootec.* 57(218):131-138.
- André E (2012). The development and application of genomic selection as a new breeding paradigm. *Anim. Front.* 2(1):10-15.
- Aschaffenburg R, Drewry J (1955). Occurrence of different beta-lactoglobulins in cow's milk. *Nature.* 176:218.
- Aschaffenburg R (1964). Protein phenotyping by direct polyacrylamide gel electrophoresis of whole milk. *Biochem. Biophys. Acta.* 82(1):188-191.
- Aschaffenburg R (1968). Genetic variants of milk proteins: Their breed distribution. *J. Dairy Res.* 35: 447.
- Aschaffenburg R, Sen A, Thompson MP (1968). Genetic variants of casein in Indian and African zebu cattle. *Comp. Biochem. Physiol.* 25:177.
- Azevedo ALS, Nascimento CS, Steinberg RS, Carvalho MRS, Peixoto MGCD, Teodoro RL, Verneque RS, Guimarães SEF, Machado MA (2008). Genetic polymorphism of the kappa-casein gene in Brazilian cattle. *Genet. Mol. Res.* 7(3):623-630.
- Baker CMA, Manwell C (1980). Chemical classification of cattle. 1. Breed groups. *Anim. Blood Groups Biochem. Genet.* 11(2): 127-150.
- Baranyi M, Bosze ZS (1992). Genetic Polymorphism of Milk Proteins in Hungarian Spotted and Hungarian Grey Cattle: A Possible New Genetic Variant of β -Lactoglobulin. *J. Dairy Sci.* 7(2):630-636.
- Bell K, Hopper KE, Mckenzie HA, Murphy WH, Shaw DC (1970). A comparison of bovine α -lactalbumin A and B of drought-master. *Biochem Biophys Acta.* 214:437.
- Bell K, Hopper KE, McKenzie HA (1981). Bovine alpha-lactalbumin C and alpha S1, beta and kappa-caseins of Bali (Banteng) cattle, *bos* (Bibos) javanicus. *Aust. J. Biol. Sci.* 34:149-159.
- Bhattacharya SD, Roychoudhury AK, Sinha NK, Sen A (1963). Inherited α -lactalbumin and β -lactoglobulin polymorphism in Indian zebu cattle. Comparison of zebu and buffalo α -lactalbumins. *Nature.* 197:797.
- Bleck GT, Bremel RD, Wheeler MB (1993b). Identification of the α -lactalbumin (+15) genetic marker and correlation to milk production and milk composition in Holstein cattle. *J. Dairy Sci.* 76:2292-2298
- Blumberg BS, Tombs MP (1958). Possible polymorphism of bovine alpha-lactalbumin. *Nature.* 181:683.
- Bonvillani AG, Di Renzo MA, Tiranti IN (2010). Genetic polymorphism of

- milk protein loci in Argentinian Holstein cattle. *Genet. Mol. Biol.* 23(4):819-823.
- Bovenhuis H, Van Arendonk JA, Korver S (1992). Associations between milk protein polymorphisms and milk production traits. *J. Dairy Sci.* 75:2549-2559.
- Bovenhuis H, Weller JI (1994). Mapping and analysis of dairy cattle quantitative trait loci by maximum likelihood methodology using milk protein genes as genetic markers. *Genetics.* 137:267-280.
- Çardak AD (2005). Effects of genetic variants in milk protein on yield and composition of milk from Holstein-Friesian and Simmentaler cows. *S.Afr. J. Anim. Sci.* 35 (1):41-47.
- Caroli A, Chessa S, Chiatti F, Rignanese D, Meléndez B, Rizzi G (2008). Carora cattle show high variability in alpha s1-casein. *J. Dairy Sci.* 91(1):354-359.
- Caroli AM, Chessa S, Erhardt GJ (2009). Invited review: milk protein polymorphisms in cattle: effect on animal breeding and human nutrition. *J. Dairy Sci.* 92:5335-5352.
- Celik S (2003). Beta-lactoglobulin genetic variants in Brown Swiss breed and its association with compositional properties and rennet clotting time of milk. *Intl. Dairy J.* 13(9):727-731.
- Cerriotti G, Marletta D, Caroli A, Erhardt G (2004). Milk protein polymorphism in taurine (*Bos taurus*) and zebu (*Bos indicus*) populations bred in hot climate. *J. Anim. Breed. Genet.* 121:404-415.
- Chang H (1995). *Essentials of Animal Genetic Resources Science*. Agricultural Publishing House of China (1st Ed), Beijing.
- Chatchatte P, Jarvinen KM, Bardina KL, Beyer K, Sampson HA (2001). Identification of IgE and IgG binding epitopes on *beta casein* and *kappa casein* milk proteins in cow's milk allergic patients. *Clin. Exp. Allergy.* 31:1256-1262.
- Chianese, L, DiLuccia A, Maurielle R, Ferrara L, Zehender G, Addeo F (1988). Biochemical polymorphism of proteins milk in podolica cows. *Nutri. Zoo. Anim.* 14:189-197.
- Creangă St, Maciuc V, Bălțeanu AV, Chelmu SS (2010). Genetic polymorphism of main lactoproteins of Romanian grey steppe breed in Preservation. *W. Acad. Sci. Eng. Technol.* 41:240-244.
- Curi RA, De Oliveira HN, Gimenes MS, Silveira AC, Lopes CR (2005). Effect of CSN3 and LGB gene polymorphism on production traits in beef cattle. *Genet. Mol. Biol.* 28:262-266.
- Dahlin A, Khan UN, Zafar AH, Saleem M, Chaundhry MA, Philipsson J (1998). Genetic and environmental causes of variation in milk production traits of Sahiwal cattle in Pakistan. *Anim. Sci.* 66:307-318.
- D'Alessandro A, Zolla L, Scaloni A (2011). The bovine milk proteome: nourishing, nourishing and fostering molecular complexity. An interactomics and functional overview. *Mol. Biosyst.* 7:579-597.
- Daniela I, Aurelia S, Magdin A, Claudia S, Vintila I (2008). Genetic polymorphism at the *β-lactoglobulin* Locus in a dairy herd of Romanian spotted and Brown of maramures breeds. *lucrări științifice zootehnie și biotehologii.* 41(1):104-107.
- Eigel WN, Butler JE, Ernstrom CA, Farrell HM, Halwarkar VR, Jenness R, Whitney RM (1984). Nomenclature of proteins of cow's milk: fifth revision. *J. Dairy Sci.* 67:1599-1631.
- Erhardt G (1996). Detection of a new kappa-casein variant in milk of Pinzgauer cattle. *Anim. Genet.* 27:105-107.
- Erhardt G, Juszczak J, Panicke L, Krick-Saleck H (1998). Genetic polymorphism of milk proteins in Polish Red Cattle: a new genetic variant of *β-lactoglobulin*. *J. Anim. Breed. Genet.* 115:63-71.
- Famula TR, Medrano JF (1994). Estimation of genotype effects for milk proteins with animal and sire transmitting ability models. *J. Dairy Sci.* 77:3153-3162.
- Farrell HM, Jr Jimenez-Flores R, Bleck GT, Brown EM, Butler JE, Creamer LK (2004). Nomenclature of the proteins of cows' milk-sixth revision. *J. Dairy. Sci.* 87: 1641-1674.
- Ganguly I, Kumar S, Gaur GK, Singh U, Kumar A (2013). Status of *β-casein* (CSN2) Polymorphism in Frieswal (HF X Sahiwal Crossbred) Cattle. *Intl. J. Biotechnol. Bioeng. Res.* 4(3):249-256.
- Golijow CD, Giovambattista G, Ripoli MV, Dulout FN, Lojo MM (1999). Genetic variability and population structure in loci related to Milk production traits in native argentine creole and commercial argentine Holstein cattle. *Genet. Mol. Biol.* 22(3):395-398.
- Gonyon DS, Mather RE, Hines HC, Haenlein GFW, Arave CW, Gaunt SN (1987). Associations of bovine blood and milk polymorphisms with lactation traits: Holsteins. *J. Dairy Sci.* 70:2585-2598.
- Gouda EM, Galal MK, Wasfy MA, Abdelaziz SA (2011): Phenotypes, Genotypes and Allele Frequencies of *β-lactoglobulin* in Egyptian Cattle and Buffalo. *J. Agric. Sci.* 3:203-210.
- Grosclaude F, Mahe MF, Mercier JC (1974). Comparison of the genetic polymorphism of the lactoproteins zebu and cattle. *Ann. Genet. Sel. Anim.* 6:305-329.
- Hanusová E, Hub J, Oravcová M, Polák P, Vrtková I (2010). Genetic variants of beta-casein in Holstein dairy cattle in Slovakia. *Slovak. J. Anim. Sci.* 43:63-66.
- Heidari M, Azari MA, Hasani S, Khan ahmadi A, Zerehdaran S (2009). Association of genetic variants of *β-lactoglobulin* gene with milk production in a herd and a superior family of Holstein cattle. *Iran. J. Biotechnol.* 7(4):254-257.
- Hristov P, Teofanova D, Mehandzhiyski I, Zagorchev L, Radoslavov G (2013). Significance of Milk protein genes polymorphism for Bulgarian Rhodopean cattle: comparative studies. *Biotechnol. and Biotechnol. eq.* 27(2):3659-3664.
- Ikonen T, Ojala M, Ruottinen O (1999). Associations between milk protein polymorphism and first lactation milk production traits in Finnish Ayrshire cows. *J. Dairy Sci.* 82:1026-1033.
- Ivana Tramontina da S, Marco Antonio DL (1997). Milk protein polymorphisms in Brazilian zebu cattle. *Braz. J. Genet.* 20(4).
- Jairam BT, Nair PG (1983). Genetic polymorphism of milk proteins and economic characters in dairy animals. *Indian J. dairy. Sci.* 53(1):1-8.
- Jann O, Ceriotti G, Caroli A, Erhardt G (2002). A new variant in exon VII of bovine b-casein gene (CSN2) and its distribution among European cattle breeds. *J. Anim. Breed. Genet.* 119:65-68.
- Kahi AK, Thorpe W, Nitter G, Baker RL (2000). Crossbreeding for dairy production in the Lowland tropics of Kenya In Estimation of individual crossbreeding effects on milk production and reproductive traits and cow live weight. *Livestock Prod. Sci.* 63:39-54.
- Kaminski S, Cieoelinska A, Kostyra E. (2007). Polymorphism of bovine *β-casein* and its potential effect on human health. *J Appl. Genet.* 48(3):189-198.
- Karimi K, Beigi Nassiri MT, Mirzadeh KH, Ashayerizadeh A, Roushanfekr H, Fayyazi J (2009). Polymorphism of the *β-lactoglobulin* gene and its association with milk production traits in Iranian Najdi cattle. *Int. J. Biotechnol.* 7(2):82-85.
- Kemenes PA, Reginato LCA, Rosa AJM, Parker IU, Razook GA, Figueiredo LA, Silva NA, Etchegaray MAL, Coutinho LL (1999). *Kappa casein*, *beta lactoglobulin* and growth hormone allele frequencies and genetic distances in Nelore, Gyr and Guzera, Caracu, Charolais, Canchin and Santa Gertrudis cattle. *Genet. Mol. Biol.* 22:539-541.
- Khan UN, Dahlin A, Zafar AH, Saleem M, Chaundhry MA, Philipsson J (1999). Sahiwal cattle in Pakistan: genetic and environmental causes of variation in body weight and reproduction and their relationship to milk production. *Anim. Sci.* 68:97-108.
- Lara MAC, Gama LT, Bufarah G, Sereno JRB, Celegato EML, de Abreu UP (2002). Genetic polymorphisms at the k-casein locus in pantaneiro cattle. *Arch. Zootec.* 51:99-105.
- Lin CY, McAllister AJ, Ng-Kwai-Hang KF, Hayes JF (1986). Effects of milk protein loci on first lactation production in dairy cattle. *J. Dairy Sci.* 69:704-712.
- Litwinczuk Z, Krol J (2002). Polymorphism of main milk proteins in beef cattle maintained in east central Poland. *Anim. Sci. papers reports.* 20(1):33-40.
- Lukac D, Vidović V, Nemeš Ž, Stupar M, Popović-Vranješ A (2013). Genotypic frequencies of the *β-lactoglobulin*, *κ-casein* and transferrin in Serbian Holstein-Friesian dairy cattle. *Mljekarstvo.* 63 (4): 203-210.
- Lunden A, Nilsson M, Janson L (1997). Marked effect of *β-lactoglobulin* polymorphism on the ratio of casein to total protein in milk. *J. Dairy Sci.* 80:2996-3005.
- Malik S, Kumar S, Rani R (2000). Kappa-casein and Beta-casein alleles in crossbred and Zebu cattle from India using polymerase chain reaction and sequence-specific oligonucleotide probes. *J. Dairy Res.* 67:295-300.
- Mao IL, Buttazzoni LG and Aleandri R (1992). Effects of polymorphic milk protein genes on milk yield and composition traits in Holstein cattle. *Acta Agric. Scand.* 42:1-7.
- Medjugorac I, Kustermann W, Lazar P, Russ I, Pirchner F (1994). Marker derived phylogeny of European cattle supports demic

- expansion of agriculture. *Anim. Genet.* 25:19-27.
- Miciński J, Klupczyński J, Mordas W, Zablotna R (2007). Yield and composition of milk from Jersey cows as dependent on the genetic variants of milk proteins. *Pol. J. Food Nutr. Sci.* 57(3):95-99.
- Mishra BP, Mukesh M, Prakash B, Sodhi M, Kapila R, Kishore A (2009). Status of milk protein, β -casein variants among Indian milch animals. *Ind. J. anim. sci.* 79(7):722-725.
- Morris CA, Hickey SM, Cullen NG, Prosser CG, Anderson RM, Tate ML (2005). Associations between β -casein genotype and milk yield and composition in grazing dairy cows. *NZ. J. Agric. Res.* 48(4):441-450
- Muhuyi WB (1997). A comparison of the productivity of Kenya Sahiwal and their crosses in large scale dairy dual purpose and beef production systems. Ph.D. thesis, University of Nairobi, Kenya.
- Muhuyi WB, Lokwaleput I, Sinkeet SN (1999). Conservation and utilization of the Sahiwal cattle in Kenya. *Anim. Genet. Res. Info.* 26:35-44.
- Mu'in MA, Supriyanto A (2012). Allelic Frequency of Kappa-Casein Locus (Asp148/Ala) in F1: Simmental (Bos Taurus) x Ongole Grade (Bos indicus). *Media Peternakan.* 35(3):157-162.
- Mwandotto BAJ (1994). Phenotypic, genetic and environmental parameter estimates of weight growth and maturing patterns in Sahiwal females. *Bull. Anim. Prod.* 42:61-67.
- Ng-Kwai-Hang KF, Hayes JF, Moxley JE, Monardes HG (1984). Association of Genetic Variants of Casein and Milk Serum Proteins with Milk, Fat, and Protein Production by Dairy Cattle. *J. Dairy Sci.* 67:835-840.
- Ng-Kwai-Hang K F, Hayes JF, Moxley JE, Monardes HG (1986). Relationships between milk protein polymorphisms and major milk constituents in Holstein-Friesian cows. *J. Dairy Sci.* 69:22-26.
- Ng-Kwai-Hang K.F, Monardes HG Hayes JF (1990). Association between genetic polymorphism of milk proteins and production traits during three lactations. *J. Dairy Sci.* 73:3414-3420.
- Nilsen H, Olsen HG, Hayes B, Sehested E, Svendsen M, Nome T, Meuwissen T, Lien S (2009). Casein haplotypes and their association with milk production traits in Norwegian Red cattle. *Genet. Sel. Evol.* 41:24.
- Ojala M, Famula TR, Medrano JF (1997). Effects of milk production genotypes on the variation of milk production traits of Holstein and Jersey cows in California. *J. Dairy Sci.* 80:1776-1785.
- Oleński K, Kamiński S, Szyda J, Cieslińska A (2010). Polymorphism of the beta-casein gene and its associations with breeding value for production traits of Holstein-Friesian bulls. *Livest. Sci.* 131:137-140.
- Osterhoff DR, Pretorius AMG (1966). Inherited biochemical polymorphism in milk proteins. *Proc. South African Soc. Anim. Prod.* 5:166.
- Ortner M, Essl A, Sölkner J (1995). For breeding significance of various milk protein genotypes in cattle. *Züchtungskunde.* 67:353-367.
- Pinder SJ, Perry BN, Skidmore CJ, Savva D (1994). Analysis of polymorphism in the bovine casein genes by use of the polymerase chain reaction. *Anim Genet.* 22:11-20.
- Pupkova GV (1980). Milk protein polymorphism and milk production of Estonian Black Pied cows. *Dairy Sci.* 45:6620.
- Rachagani S, Gupta ID, Gupta N, Gupta SC (2006). Genotyping of β -lactoglobulin gene by PCR-RFLP in Sahiwal and Tharparkar cattle breeds. *BMC Genet.* 7:31.
- Rachagani S, Gupta ID (2008). Bovine k-casein gene polymorphism and its association with milk production traits. *Genet. Mol. Biol.* 31(4):893-897.
- Reinhardt TA, Lippolis JD, Nonnecke BJ, Sacco RE (2012). Bovine milk exosome proteome. *J Proteom.* 75(5):1486-92.
- Ren DX, Miao SY, Chen YL, Zou CX, Liang XW, Liu JX (2011). Genotyping of the κ -casein and β -lactoglobulin genes in Chinese Holstein, Jersey and Water buffalo by PCR-RFLP. *J. Genet.* 90: 1-5.
- Sambrook J, Russell DW (2001). *Molecular Cloning: A Laboratory Manual*. 3rd Edition. Cold Spring Harbor Laboratory Press, New York.
- Sashikanth MA, Yadav BR (2011). *Alpha lactalbumin* polymorphism in three breeds of Indian zebu cattle. *J. Anim. Breed. Genet.* 115:403-405.
- Sitkowska B, Neja W, Wiśniewska E (2008). Relations between kappa-casein polymorphism (csn3) and milk performance traits in heifer cows. *J. Cent. Eur. Agric.* 9(4):641-644.
- Tolenkhomba TC, Yadav BR (2012). Effect of β -casein genotypes on milk production traits in Sahiwal cattle. *Indian J. Dairy Sci.* 65(3):225-228.
- Van Eenennaam AL, Medrano JF (1991). Differences in allelic protein expression in the milk of heterozygous κ -casein cows. *J. Dairy Sci.* 74:1491-1496.
- Yasemin O, Cengiz E (2006). Milk protein polymorphisms in Holstein cattle. *Intl. J. Dairy Technol.* 5(3).
- Zhang J, Sun D, Womack JE, Zhang Y, Wang Y and Zhang Y (2007). Polymorphism Identification, RH Mapping and Association of α -Lactalbumin Gene with Milk Performance Traits in Chinese Holstein. *Asian-Aust. J. Anim. Sci.* 20(9):1327-1333.
- Zhou JP, Dong CH (2013). Association between a polymorphism of the α -lactalbumin gene and milk production traits in Chinese Holstein cows. *Genet. Mol. Res.* 12(3):3375-3382.

Full Length Research Paper

Selection and optimization of extracellular lipase production using agro-industrial waste

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The aim of this study was to isolate and select lipase-producing microorganisms originated from different substrates, as well as to optimize the production of microbial lipase by submerged fermentation under different nutrient conditions. Of the 40 microorganisms isolated, 39 showed a halo around the colonies and 4 were selected (*Burkholderia*, *Bacillus* sp., *Penicillium lanosum* and *Corynebacterium glutamyl*), where strains showing lipolytic halo Radius (R) / colony radius (r) ratio greater than 2.0 were selected. The results of submerged fermentation expressed as enzymatic activity revealed that the genera of microorganisms significantly influenced the enzymatic reaction, and lipase obtained from *Burkholderia cepacia* was the most promising, with activity of 0.0058 U.mL⁻¹. It was also observed in the optimization step of lipase production that the sodium nitrate content (NaNO₃) had a positive effect on enzyme production, and its increase was indicative of higher enzymatic activity. The addition of sources of organic nitrogen (corn steep liquor, p = 0.2398), carbon (soybean oil, p = 0.3379), magnesium MgSO₄·7H₂O (p = 0.4189) and potassium KH₂PO₄ (p = 0.8562) had no significant effects on the lipase production and could result in decreased production of extracellular lipases.

Key words: *Burkholderia cepacia*, hydrolytic enzymes, residue, submerged fermentation.

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC3.1.1.3) are water-soluble enzymes that catalyze the hydrolysis of ester bonds of insoluble triacylglycerols, releasing free fatty acids, mono-or diacylglycerol in the oil-water interface (Linko et al., 1998; Treichel et al., 2010). Lipases may be of animal, microbial or plant origin, with variations in their catalytic properties, and can be obtained by solid-state fermentation or by submerged fermentation (Annibale et al., 2006; Rigo et al., 2010; Papagora et al., 2013). These enzymes catalyze a wide range of reactions such as hydrolysis, esterification, trans-esterification, alcoholysis, acidolysis and aminolysis (Joseph et al., 2008). Lipases represent an important group of enzymes with various applications in the food, chemical and

pharmaceutical industry (Salihu et al., 2012), besides having great interest for the treatment of effluents with high lipid content (Dharmstithi and Kuhasuntisuk, 1998; Jung et al., 2002; Jeganathan et al., 2007; Ertugrul et al., 2007). However, the high cost of commercial enzymes makes treatment costly in the production of enzymes and in the search for new microorganisms. In this context, the use of agroindustrial waste contributes in the reduction of production costs (Leal et al., 2006; Salihu et al., 2012). In the production of lipases, studying the optimization of fermentation conditions, relating the carbon and nitrogen sources to temperature and pH conditions is of fundamental importance (Silva et al., 2005). Some studies (Nascimento et al., 2007; Kona et al., 2001) reported the

use of some low-cost substrates in the production of enzymes such as corn steep liquor and soybean or olive oil, food industry by-products, as an alternative to reduce production costs, thus contributing to agro-industrial sustainability.

Corn steep liquor is a by-product rich in carbohydrates, amino acids, peptides, minerals, vitamins and phosphates and is thus considered as an excellent culture medium for enzyme production (Lee et al., 2003; Rivas et al., 2004).

Some studies have also shown that producing lipase by bacterial strains is more common because they offer higher enzymatic activity compared to fungi, tend to have optimal pH at neutrality or alkalinity, they are often thermostable and are mostly extracellular, facilitating their extraction from the fermented medium (Hasan et al., 2006; Li et al., 2010).

Thus, the aim of this study was to isolate and select lipase-producing microorganisms originated from different substrates (yeast extract, peptone and soybean oil), as well as to optimize the production of microbial lipase by submerged fermentation under different nutrient conditions of substrate (corn steep liquor, yeast extract, peptone, soybean oil), sources of sodium (Na_2HPO_4), magnesium ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), potassium (KH_2PO_4) and nitrate (NaNO_3).

MATERIALS AND METHODS

Collection of effluent and microorganisms

Microorganisms were isolated from effluent collected in the "Cicopa Ltda" Potato chip industry, Senador Canedo, GO, and in the Sewage Treatment Plant (STP) in Goiânia-GO, Brazil. *Burkholderia cepacia* strain ATCC 25416 (BC25416) was obtained from the "Fundação Tropical André Tosello", Campinas-SP, Brazil, originated from the collection of tropical crops. Other organisms such as *Candida tropicalis* CCT 5846 (UCT), *Candida* sp. (UCS), *Zymomonas mobilis* CCT 4494 (UZM), *Escherichia coli* (UEC), *Kluyveromyces marxianus* (UKM), *Kocuria rhizophila* (UKR), *Bacillus subtilis* NRRL 14819 (UBS14), *Corynebacterium glutamyl* (UCG), *Bacillus* sp. NRRL 41094 (UBS41) and *Penicillium lanosum* NRRL 3442 (UPL) were obtained from strains provided by the Laboratory of Wastewater Treatment and Fermentation Processes (LARPF), Faculty of Food Engineering, State University of Campinas-SP, Brazil.

Isolation of microorganisms

The effluents collected were inoculated in Petri dishes in the following media: potato agar (PA), violet red bile lactose agar (VRBA) and standard count agar (PCA), according to methodology of the American Public Health Association (APHA, 2001). After incubation, they were analyzed for the development of microorganisms. The colonies were selected by visual analysis. For isolation, the selected colonies were inoculated in Petri dishes containing nutrient agar using the exhaustion technique and incubated at 30°C for 48 h in a bacteriological incubator. The microorganisms were maintained in test tubes containing nutrient agar pH 7.0 at 4°C.

Selection of lipase-producing microorganisms

The pre-selection of lipase-producing strains was performed by

analyzing the formation of transparent halo around colonies on medium containing tributyrin, according to the methodology proposed by Freire (1997). A small fraction of each strain was inoculated in Petri dishes containing 0.5% peptone, 0.3% yeast extract, 2% agar and 0.1% tributyrin, pH 6.0 and incubated at 30°C for 48 h. Strains showing lipolytic halo radius (R)/colony radius (r) ratio greater than 2.0 were selected according to the study of Freire (1997) and Colen et al. (2005) choosing only the strains lipolytic that showed higher halos for further studies and submitted to submerged fermentation. The cultivation on solid medium was performed in a completely randomized design (CRD) with forty microorganisms and three replications, and the means were compared by the Tukey test at 5% probability.

Lipase production via submerged fermentation

The microorganisms previously selected were used in the liquid medium cultivation. In the preparation of inoculum, microorganisms were scraped with platinum loop and transferred to 125 ml Erlenmeyer flasks containing 50 ml medium culture solution of (g L^{-1} of distilled water): yeast extract (5.0), peptone (5.0), and 1%_{w/w} soybean oil in the proportion of 1:20 inoculum in relation to the culture medium, incubated at 30°C for 48 h under stirring in a water bath (Tecnal TE-184). After the incubation period, 10 ml of the inoculum was transferred to each reactor to carry out fermentations. Fermentations were carried out in Erlenmeyer flasks with capacity of 125 ml and incubated at 30°C for 72 h under stirring (100 rpm) in water bath. Fifty milliliter (50 ml) of medium culture solution of (g L^{-1} of distilled water): yeast extract (5.0), peptone (5.0) and soybean oil (1%_{w/w}) were part of the culture medium. After fermentation, extracellular enzymes were extracted, where an aliquot of 5 ml of the reaction medium was centrifuged (TG16-WS Tabletop High Speed Centrifuge) at 2000 xg for 15 min. The supernatants were used as enzyme extract for analysis of the enzymatic activity.

Lipase activity determination

The enzymatic activity was determined by spectrophotometric method (Spectrophotometer BEL photonics), according to the methodology described by Parul et al. (2005). The Elisa plate well was added of 100 μL of solution containing sodium phosphate buffer pH 7 (0.1 M), 0.9% Triton X-100 and 0.27 M sodium chloride, as well as 100 μL of sample and 20 μL p-nitrophenyl phosphate substrate (pNPP). The mixture was incubated for 30 min in water bath at 37°C and readings were made in a spectrophotometer at 405 nm. The enzymatic activity unit was defined as 1 $\mu\text{mol} \cdot \text{min}^{-1}$ of p-nitrophenol released from the substrate.

Optimization of the production of lipases

To optimize the production of lipases from microorganism with the highest lipolytic potential, a 2⁶⁻² fractional factorial exploratory design was initially performed, which evaluated the effect of the corn steep liquor (5 to 30 g L^{-1}), soybean oil (4 to 12%) sources of sodium (0 to 4 g L^{-1} Na_2HPO_4), magnesium (0.1 to 0.3 g L^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), potassium (2 to 4 g L^{-1} KH_2PO_4) and nitrate (0 and 6 g L^{-1} NaNO_3). Peptone and yeast extract were used at concentration of 1 g L^{-1} . Each effect varied in three levels (-1, 0, +1), with duplicates of the central point, totaling 18 assays. Fermentations were performed at 30°C for 72 h under stirring in water bath. The two nutrients that showed the greatest effect on the enzyme activity were chosen and evaluated by means of a central composite design. Each effect varied in five levels (-1.42, -1, 0, +1, +1.42) with triplicate of the central point, totaling 11 assays. The model validation was performed in triplicate.

RESULTS AND DISCUSSION

Isolation and selection of microorganisms

Overall, 29 microorganisms were isolated, 24 from the Potato chip industry and 5 from the sewage treatment plant (STP). The coding for microorganisms isolated from industrial effluents was: IEA1, IEA2, IEB1, IEB2, IEB3, IEB4, IEV1, IEV2, ITEA1, ITEA2, ITEB1, ITEB2, ITEB3, ITEB4, ITEV1, ITEV2, ITA1, ITA2, ITB1, ITB2, ITB3, ITB4, TV1, TV2, and for isolates from the STP: ETDB1, ETDB2, ETDB3, ETDB4 and ETDB5. Of the 40 microorganisms used for the lipase activity evaluation (29 isolates from the effluents and 11 from research institutes), 39 showed a halo around the colonies, indicating the presence of degradation reaction of tributyrin by extracellular enzymes, inducing lipase production. Of these 39 microorganisms, 4 showed lipolytic halo radius (R) / colony radius (r) ratio greater than 2.0 (Table 1), among them are *B. cepacia* ATCC 25416, *Bacillus* sp. NRRL 41094, *P. lanosum* NRRL 3442 and *C. glutamil*. These results showed that there was a variation of 23.1% in the hydrolysis halos, where strain BC25416 was the one showing the highest R/r ratio, and this difference in intensity between halos is due to the amount of extracellular lipase secreted by microorganism (Cardenas et al., 2001). Similar results were found by Griebeler et al. (2009), who studied the production capacity of lipolytic enzymes from different microorganisms on solid medium containing tributyrin, and obtained for strain *Penicillium* sp. and *B. cepacia*, the greatest hydrolysis halo (radius) of 9.35 and 9.1 mm, respectively, suggesting a probable lipolytic potential of these microorganisms.

Table 1 shows that although none of the organisms isolated from effluents have been pre-selected for submerged fermentation, this confirmed that lipase-producing microorganisms can be found in various habitats, especially those containing lipids (Sharma et al., 2001).

Submerged fermentation

The results of the submerged fermentation expressed in enzymatic activity of the 4 previously selected microorganisms are shown in Table 2. The results obtained reveal that the pre-selected microorganisms significantly influenced ($p \leq 0.05$) the enzymatic degradation reaction of p-nitrophenyl palmitate. Among the microorganisms that differ from one another, the lowest activity was 0.0022 U.mL⁻¹, referring to lipase from *C. glutamil* (UCG), which showed 62% less activity than lipase from *B. cepacia* ATCC 25416 (BC25416), with activity of 0.0058 U.mL⁻¹. The enzymatic activities of microorganisms *Bacillus* sp. NRRL 41094 (UBS41) of 0.0032 U.mL⁻¹ and *P. lanosum* NRRL 3442 (UPL) of 0.0027 U.mL⁻¹ were 45 and 53% less than the maximum activity found in the present study. These genera of microorganisms *Burkholderia*, *Bacillus* and *Penicillium* have been extensively studied for the production of extracellular lipases in order to optimize the

production of enzymes for various applications, where there is great variation between the results, probably due to the different cultivation media, methods for determining the enzymatic activity and microorganisms are used.

Wolski et al. (2009) compared the production of lipase from *Penicillium* sp. in solid and submerged fermentation and found greater lipase production and activity at 42°C of 15.17 U.mL⁻¹. Annamalai et al. (2011) studied the production of thermostable lipase from *Bacillus licheniformis* isolated from the marine environment, using peanut oil as substrate and reported hydrolytic activity of 730 U.mL⁻¹ after 48 h of incubation at 55°C.

Optimization of the lipase production

Strain *B. cepacia* ATCC 25416 was selected to study the optimization of the production of extracellular lipase, where the effects of different nutrients on the lipase production were assessed. The results are shown in Table 3, where the enzymatic activity values ranged from 0 to 0.0181 U.mL⁻¹. Table 4 shows the results of the effects of variables on the enzymatic activity response. It was observed that the sodium nitrate concentration (NaNO₃) had a positive effect on the enzymatic activity, and the increase in the concentration of this salt in the reaction medium from 0 to 6 gL⁻¹ resulted in increased response, indicating increased production of extracellular lipase from *B. cepacia* ATCC 25416, and consequently, higher enzyme activity. Generally, microorganisms provide high lipase yields when organic nitrogen sources are used (Supakdamrongkul et al., 2010); however, in this experiment, the high yield was due to inorganic sources. Na⁺ ion has positive and significant effect only when sodium nitrate was used. For sodium phosphate monobasic (Na₂HPO₄), a positive effect was found, although not significant ($p = 0.2352$), and only sodium nitrate was statistically significant ($p = 0.0463$). The addition of sources of organic nitrogen (corn steep liquor, $p = 0.2398$), carbon (soybean oil, $p = 0.3379$), magnesium MgSO₄.7H₂O ($p = 0.4189$) and potassium KH₂PO₄ ($p = 0.8562$) had no significant effects on the lipase production. However, these effects had a negative sign, indicating that increases in the concentration of these nutrients showed little tendency to lower the enzymatic activity and could result in decreased production of extra-cellular lipases.

Some studies have revealed that vegetable oils such as soybean, corn, sunflower, olive, palm and cotton are referred to as inducers of lipase production, being used by microorganisms as carbon source in the reaction medium (Paques and Macedo, 2006). However, this phenomenon was not observed in this study with results obtained by varying the soybean oil concentration, being not statistically significant in the production of extracellular lipases from *B. cepacia* ATCC 25416. The induction of lipase production appears to be related not only to the length of the carbon chains of fatty acids in triacylglycerols and to the number of insaturations, but also to the concentration of the substrate used (Lima et al., 2003).

Table 1. Measurements of the radius of colonies (r), lipolysis halos (R) and R / r ratio in solid culture medium.

Code	Radius (cm)		
	Radius of colonie (r)	Lipolysis halo (R)	R/r
UCT	0.25 ± 0.10	0.29 ± 0.04	1.15
UCS	0.29 ± 0.09	0.34 ± 0.04	1.17
UZM	1.06 ± 0.05	0.18 ± 0.02	0.16
UEC	0.50 ± 0.07	0.09 ± 0.04	0.18
UKM	0.25 ± 0.03	0.29 ± 0.04	1.15
UKR	0.25 ± 0.06	0.35 ± 0.14	1.40
UBS14	0.38 ± 0.12	0.65 ± 0.06	1.70
UCG	0.33 ± 0.08	0.70 ± 0.13	2.10
UBS41	0.33 ± 0.05	0.76 ± 0.08	2.30
UPL	0.34 ± 0.05	0.72 ± 0.07	2.15
EA1	0.30 ± 0.08	0.41 ± 0.02	1.33
EA2	0.45 ± 0.03	0.10 ± 0.04	0.22
EB1	0.27 ± 0.06	0.50 ± 0.11	1.87
EB2	0.25 ± 0.02	0.47 ± 0.04	1.86
EB3	0.44 ± 0.04	0.48 ± 0.07	1.09
EB4	0.38 ± 0.05	0.11 ± 0.04	0.30
TEB1	0.29 ± 0.07	0.29 ± 0.04	1.00
TEB2	0.35 ± 0.11	0.41 ± 0.09	1.18
TEB3	0.25 ± 0.00	0.47 ± 0.06	1.86
TEB4	0.30 ± 0.07	0.31 ± 0.04	1.04
TEV1	0.30 ± 0.07	0.47 ± 0.08	1.53
TEV2	0.43 ± 0.16	0.46 ± 0.04	1.09
BC25416	0.24 ± 0.05	0.91 ± 0.05	3.76
TA1	0.28 ± 0.03	0.02 ± 0.01	0.08
TA2	0.30 ± 0.13	0.41 ± 0.04	1.38
TDB1	0.24 ± 0.05	0.10 ± 0.02	0.42
TDB2	0.40 ± 0.03	0.46 ± 0.04	1.16
TDB3	0.41 ± 0.18	0.19 ± 0.04	0.45
TDB4	0.38 ± 0.06	0.19 ± 0.07	0.50
TDB5	0.23 ± 0.07	0.34 ± 0.04	1.50
TV1	0.34 ± 0.04	0.41 ± 0.04	1.22
TV2	0.26 ± 0.11	0.36 ± 0.04	1.38
EV1	0.29 ± 0.04	0.28 ± 0.07	0.96
EV2	0.25 ± 0.04	0.31 ± 0.07	1.25
TB1	0.25 ± 0.06	0.35 ± 0.07	1.40
TB2	0.35 ± 0.11	0.68 ± 0.10	1.95
TB3	0.60 ± 0.08	0.23 ± 0.07	0.38
TB4	0.34 ± 0.12	0.29 ± 0.04	0.85
TEA1	0.26 ± 0.14	0.26 ± 0.04	1.00
TEA2	0.55 ± 0.00	0.00 ± 0.00	0.00

UCT: *Candida tropicalis* CCT 5846; UCS: *Candida* sp.; UZM: *Zymomonas mobilis* CCT 4494; UEC: *Escherichia coli*; UKM: *Kluyveromyces marxianus*; UKR: *Kocuria rhizophila*; UBS14: *Bacillus subtilis* NRRL 14819; UCG: *Corynebacterium glutamyl*; UBS41: *Bacillus* sp NRRL 41094; UPL: *Penicillium lanosum* NRRL 3442; BC 25416: *Burkholderia cepacia* ATCC 25416; Industrial effluent: IEA1, IEA2, IEB1, IEB2, IEB3, IEB4, IEV1, IEV2, ITEA1, ITEA2, ITEB1, ITEB2, ITEB3, ITEB4, ITEV1, ITEV2, ITA1, ITA2, ITB1, ITB2, ITB3 e ITB4, TV1 e TV2; sewage treatment plant: ETDB1, ETDB2, ETDB3, ETDB4 e ETDB5.

These results show that the reduced lipase production at higher olive oil concentrations may be explained by the lower oxygen transfer to the medium, since lower oxygen supply could decrease the metabolism of microorganisms

and consequently the lipase production (Elibol and Özer, 2000). From the results previously obtained and all the substances tested, only sodium nitrate was significant for lipase production; however, another variable was selec-

Table 2. Enzymatic activity of pre-selected microorganisms after submerged fermentation.

Microorganism	Enzymatic activity (U mL ⁻¹)
<i>Corynebacterium glutamicum</i>	0.0022 ^d
<i>Penicillium lanosum</i> NRRL 3442	0.0027 ^c
<i>Bacillus</i> sp. NRRL 41094	0.0032 ^b
<i>Burkholderia cepacia</i> ATCC 25416	0.0058 ^a

Same letters indicate no significant difference ($p > 0.05$) in the production of lipase from microorganisms under study.

Table 3. Matrix of the 2⁶⁻² fractional factorial exploratory design used to determine the enzymatic activity of lipases from *Burkholderia cepacia* ATCC 25416 with different nutrients.

Experiment	CST (g L ⁻¹)	Na ₂ HPO ₄ (g L ⁻¹)	OS (g L ⁻¹)	MgSO ₄ ·7H ₂ O (g L ⁻¹)	KH ₂ PO ₄ (g L ⁻¹)	NaNO ₃ (g L ⁻¹)	Enzymatic activity (U·mL ⁻¹)
1	5	0	4	0.1	2	0	0.0029
2	30	0	4	0.1	4	0	0.0023
3	5	4	4	0.1	4	6	0.0181
4	30	4	4	0.1	2	6	0.0090
5	5	0	12	0.1	4	6	0.0089
6	30	0	12	0.1	2	6	0.0022
7	5	4	12	0.1	2	0	0.0000
8	30	4	12	0.1	4	0	0.0080
9	5	0	4	0.3	2	6	0.0113
10	30	0	4	0.3	4	6	0.0017
11	5	4	4	0.3	4	0	0.0012
12	30	4	4	0.3	2	0	0.0063
13	5	0	12	0.3	4	0	0.0011
14	30	0	12	0.3	2	0	0.0025
15	5	4	12	0.3	2	6	0.0114
16	30	4	12	0.3	4	6	0.0011
17	15	2	8	0.2	3	3	0.0036
18	15	2	8	0.2	3	3	0.0034

CST, Corn Steep Liquor; Na₂HPO₄, sodium phosphate monobasic; OS, soybean oil; MgSO₄·7H₂O, magnesium sulfate heptahydrate; KH₂PO₄, potassium phosphate monobasic; NaNO₃, sodium nitrate.

Table 4. Estimate of the effect of each nutrient added to the culture medium on the enzymatic activity of lipase from *Burkholderia cepacia* ATCC 25416.

Sources of variation	Effect	Standard deviation	t (11)	p-value	Conf. Lim. -95%	Conf. Lim. +95%
CST	-0.00273	0.002	-1.243	0.23975	-0.00756	0.00210
Na ₂ HPO ₄	0.00276	0.002	1.256	0.23517	-0.00207	0.00758
OS	-0.00220	0.002	-1.002	0.33794	-0.00703	0.00263
MgSO ₄	-0.00184	0.002	-0.840	0.41890	-0.00667	0.00299
KH ₂ PO ₄	-0.0004	0.002	-0.186	0.85619	-0.00523	0.00442
NaNO ₃	0.00493	0.002	2.245	0.04632*	0.00010	0.00975

* Significant at 5% probability. CST, corn steep liquor; Na₂HPO₄, sodium phosphate monobasic; OS, soybean.

ted, corn steep liquor, because it is a by-product from the food industry rich in nutrients such as carbohydrates, amino acids, peptides, vitamins and minerals, reducing

the culture medium costs. The results obtained in the optimization stage are shown in Table 5, which are expressed as enzymatic activity response as a function of

Table 5. Matrix of the central composite design used to determine the enzymatic activity of lipase from *Burkholderia cepacia* ATCC 25416 due to the enrichment of the medium with corn steep liquor (CST) and sodium nitrate (NaNO_3).

Experiment	CST (g L^{-1})	NaNO_3 (g L^{-1})	Enzymatic activity (U.mL^{-1})
1	10	1	0.0068
2	30	1	0.0063
3	10	5	0.0025
4	30	5	0.0034
5	20	3	0.0110
6	20	3	0.0103
7	20	3	0.0089
8	5.858	3	0.0056
9	20	5.828	0.0044
10	34.142	3	0.0024
11	20	0.172	0.0039

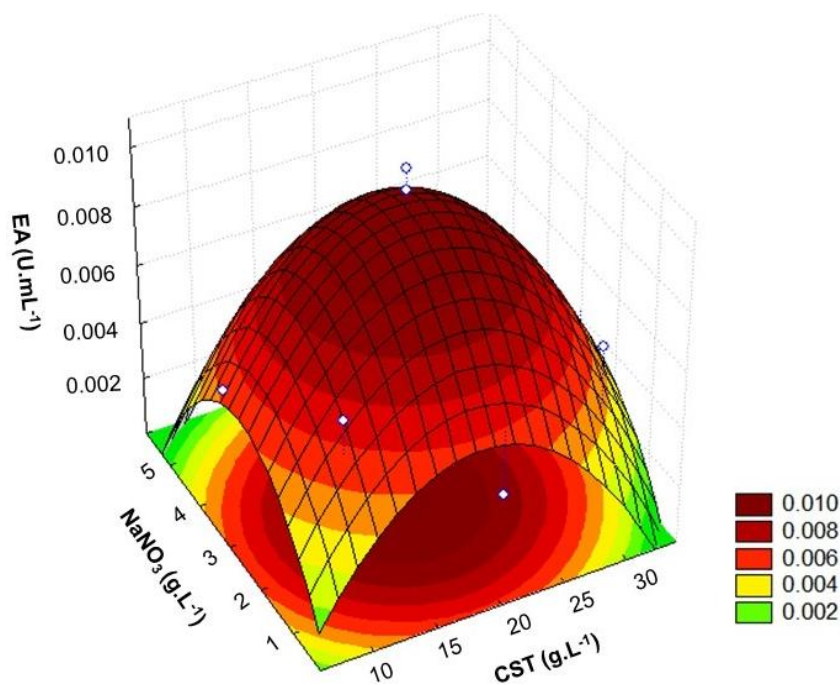


Figure 1. Response surface for the activity of lipase from *Burkholderia cepacia* ATCC 25416 under different corn steep liquor (AMM) and sodium nitrate concentrations.

the corn steep liquor and sodium nitrate contents, which ranged from 0.0024 to 0.0110 U.mL^{-1} . These results also show that both nutrients produced a negative effect on the enzymatic activity response, that is, the increase in the sodium nitrate and corn steep liquor concentration decreased the production of extracellular enzymes by *B. cepacia* ATCC 25416. This significant effect of the concentration of these nutrients was quadratic, indicating that there was an increase in the production of enzymes up to a maximum value, declining from this point on.

From the data obtained for the effect of each nutrient, a

second-order mathematical model was built. Equation 1 represents the model representing the enzymatic activity (EA) versus STP (X_1) and NaNO_3 concentration (X_2). Words in bold refer to statistically significant variables ($p \leq 0.05$). According to these results and Figure 1, it was found that the optimization of the production process was achieved, and the optimum production was achieved for concentrations from 13.8 to 26.2 g L^{-1} of corn steep liquor and from 1.5 to 3.9 g L^{-1} of sodium nitrate, with maximum response for the enzymatic activity of 0.0100 U.mL^{-1} , which allows reduction of the culture medium costs in this process.

$$EA = 0.001007 - 0.002864X_1^2 - 0.000826X_2 - 0.002806X_2^2 \quad (1)$$

These results also showed that the lowest activity found (0.0040 U.mL⁻¹) by the model had response 60% lower than the maximum activity obtained, found when sodium nitrate concentrations higher than 5 g L⁻¹ and lower than 0.17 g L⁻¹ were used. Nitrogen is an essential nutrient for the production of enzymes, since these are composed of amino acids, which have nitrogen in their structure (Rodeva et al., 2010). Thus, sodium nitrate corn steep liquor both supplied nitrogen to microorganisms to produce lipases. These results also show that the fact that intermediate concentrations of these compounds provided high lipase production was probably a result of the balance between carbon and nitrogen concentrations in the medium, and usually, the carbon concentration must be greater than that of nitrogen in order to favor the physiological needs of microorganisms for lipase biosynthesis (Freire et al., 1997). Complex nitrogen sources have been traditionally used in the production of lipases (Lima et al., 2003). Roveda et al. (2010) evaluated the production of lipases from microorganisms isolated from dairy effluents using sodium nitrate as nitrogen source at fixed concentration of 0.1% and obtained satisfactory lipase activity for fungus of the genus *Aspergillus*. The validation of the model was performed by testing the enzymatic activity of the lipase enzyme extract of *B. cepacia* ATCC 25416 in culture medium containing 20 g L⁻¹ corn steep liquor and 3 g L⁻¹ sodium nitrate.

The results were a response estimated by the model of 0.0100 U.mL⁻¹, and the average response experimentally obtained (EA) was 0.0110 U.mL⁻¹, indicating good fit of the experimental points. This enzymatic activity is equivalent with almost 6.2% of the enzymatic commercial enzyme lipase calculated under the same experimental conditions.

Conclusion

According to the experimental results, in the step of pre-selection of microorganisms with hydrolytic capacity performed with 40 microorganisms, 39 showed halo around the colonies, indicating degradation reaction of tributyrin by extracellular enzymes, inducing the production of lipases. It was possible to isolate microorganisms from agro-industrial waste. In the submerged fermentation stage, the results expressed as enzymatic activity showed that the highest activity observed was for strain *B. cepacia* ATCC 25416. The optimization step for the production of extracellular lipase allowed establishing appropriate conditions for the production of lipases when using corn steep liquor and sodium nitrate in the culture medium.

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REFERENCES

- American Public Health Association (2001). 4nd ed., Compendium of Methods for microbiological examination of foods. Technical Committee on Microbiological Methods for Foods, Washington, USA.
- Annamalai N, Elayaraja S, Vijayalakshmi S, Balasubramania T (2011). Thermostable, alkaline tolerant lipase from *Bacillus licheniformis* using peanut oil cake as a substrate. Afr. J. Biochem. Res. 5(6): 176-181.
- Annibale A, Sermani GG, Federici F, Petruccioli M (2006). Olive-mill wastewater : a promising substrate for microbial lipase production. Bioresour. Technol. 97(15): 1828-1833.
- Cardenas FE, Alvarez MS, Castro-Alvarez JM, Sanchez-Montero M, Valmaseda SW, Elson JV (2001). Screening and catalytic activity in organic synthesis of novel fungal and yeast lipases. J. Mo. Catal. B: Enzymatic. 14 (4-6): 111-123.
- Colen G, Junqueira RG, Moraes-Santos T (2005). Isolation and screening of alkaline lipase-production fungi from Brazil savanna soil. World J. Microb. Biot. 22 (8): 881-885.
- Dharmstithi S, Kuhasuntisuk B (1998). Lipase from *Pseudomonas aeruginosa* LP602: biochemical properties and application for wastewater treatment. J. Ind. Microbiol. Biot. 21(1-2): 75-80.
- Elibol M, Özer D (2000). Lipase production by immobilized *Rhizopus arrhizus*. Process. Biochem. 36: 219-223.
- Ertugrul S, Donmez G, Takac S (2007). Isolation of lipase producing *Bacillus* sp. from olive mill wastewater and improvising its enzyme activity. J. Hazard. Mater. 149:720-724.
- Freire DM, Teles EMF, Bom EPS, Sant'Anna JrGL (1997). Lipase production by *Penicillium restrictum* in a bench-scale fermenter: effect of carbon and nitrogen nutrition, agitation, and aeration. Appl. Biochem. Biotechnol. 63-65: 409-421.
- Griebeler N, Polloni A, Remonato D, Arber F, Vardanega R, Cechet JM, Oliveira D, Treichel H, Cansian R, Rigo E, Ninow J (2009). Isolation and screening of lipase-producing fungi with hydrolytic activity. Food Bioprocess Tech. 4 (4): 578-586.
- Hasan F, Shah AA, Hameed A (2006). Industrial applications of microbial lipases. Enzyme Microb. Tech. 39 (2): 235-251.
- Jeganathan J, Nakhla G, Bassi A (2007). Hydrolytic pretreatment of oily wastewater by immobilized lipase. J. Hazard. Mater. 145 (1-2): 127-135.
- Joseph B, Ramteke PW, Thomas G (2008). Cold active microbial lipases: some hot issues and recent developments. Biotech. Ad. 26 (5): 457-470.
- Jung F, Cammarota MC, Freire DMG (2002). Impact of enzymatic pre-hydrolysis on batch activated sludge systems dealing with oily wastewaters. Biotechnol. Lett. 24: 1797-1802.
- Kona RP, Qureshi N, Pai JS (2001). Production of glucose oxidase using *Aspergillus niger* and corn steep liquor. Bioresource Technol. 78: 123-126.
- Leal MC, Freire DMG, Cammarota MC, Sant'Anna JRGL (2006). Effect of enzymatic hydrolysis on anaerobic treatment of dairy wastewater. Process Biochem. 41 (5): 1173-1178.
- Lee PC, Lee SY, Hong SH, Chang HN (2003). Batch and continuous cultures of *Mannhettmiasucciniciproducens* MBEL55E for the production of succinic acid from whey and corn steep liquor. Bioprocess Biosyst. Eng. 26 (1): 63-67.
- Li N, Zong M (2010). Review: lipases from the genus *Penicillium*: production, purification, characterization and applications. J. Mol. Catal. B: Enzymatic. 66: 43-54.
- Lima VMG, Krieger MI M, Sarquis DA, Mitchell LP, Ramos JD (2003). Effect of the nitrogen and carbon sources on lipase production by *Penicillium aurantiogriseum*. Food Tech. Biotech. 41: 105-110.
- Linko YY, Lamsa M, Uosukainen E, Seppalla J, Linko P (1998). Biodegradable products by lipase biocatalysis. J. Biotech. 66 (1): 41-50.

- Nascimento WCA, Silva CR, Carvalho RV, Martins MLL (2007). Optimization of a culture medium for protease production by *Bacillus SP. thermophilic*. *Food Sci. Technol.* 27 (2): 417-421.
- Papagora C, Roukas T, Kotzekidou P (2013). Optimization of extracellular lipase production by *Debaryomyces hansenii* isolates from dry-salted olives using response surface methodology. *Food Bioprod. Process.* 91(4): 413-420.
- Paques FW, Macedo GA (2006). Lipases of plants latex: properties and industrial applications. *Química Nova.* 29: 93-99.
- Parul J, Sulakshana J, Gupta MN (2005). A microwave-assisted micro-assay for lipases. *Anal. Bioanal. Chem.* 381(7): 1480-1482.
- Rigo E, Ninow JL, Luccio MD, Oliveira JV, Polloni AE, Remonato D, Arbert F, Vardanega R, Oliveira D, Treichel H (2010). Lipase production by solid fermentation of soybean meal with different supplements. *Food Sci. Technol.* 43 (7): 1132-1137.
- Rivas B, Moldes AB, Dominguez JM, Marajó JC (2004). Development of culture media containing spent yeast cells of *Debaryomyces hansenii* and corn steep liquor for lactic acid production with *Lactobacillus rhamnosus*. *Int. J. Food Microbiol.* 97 (1): 93-98.
- Roveda M, Hemkemeier M, Colla LM (2010). Evaluation of lipase production using different strains of microorganisms isolated from dairy effluents through submerged fermentation. *Food Sci. Technol.* 30 (1): 126-131.
- Salihi A, Alam MZ, Abdulkarim MI, Salleh HM (2012). Lipase production: as insight in the utilization of renewable agricultural residues. *Resour. Conserv. Recy.* 58: 36-44.
- Sharma RY, Chisti UC, Banerjee UC (2001). Research review paper: production, purification, characterization, and applications of lipases. *Biotech. Adv.* 19: 627-662.
- Silva WOB, Mitidieri S, Schrank A, Vainstein MH (2005). Production and extraction of an extracellular lipase from the entomopathogenic fungus *Metarhiziumanisopliae*. *Process Biochem.* 40 (1): 321-326.
- Supakdamrongkul P, Bhumiratana A, Wiwat C (2010). Characterization of an extracellular lipase from the biocontrol fungus, *Nomuraearileyi* MJ, and its toxicity toward *Spodopteralitura*. *J. Invertebr. Pathol.* 105 (3): 228-235.
- Treichel H, Oliveira D, Mazutti MA, Luccio M, Oliveira JV (2010). A review on microbial lipase production. *Food Bioprocess Tech.* 3 (2): 182-196.
- Wolski E, Menusi E, Remonato D, Vardanega R, Arbert F, Rigo E, Ninow J, Mazutti MA, Luccio M, Oliveira D, Treichel H (2009). Partial characterization of lipases produced by a newly isolated *Penicillium* sp. in solid state and submerged fermentation: A comparative study. *Food Sci. Technol.* 42 (9): 1557-1560.

Full Length Research Paper

New fruit and seed disorders in Papaya (*Carica papaya* L.) in India

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Systematic field screening was conducted in 14 important papaya germplasm lines to observe the severity of bumpy fruit incidence. The disease severity, fruit yield and economic losses due to physiological disorders were observed. Pune Selection-3 was most sensitive (64.6%) for the disorder while Pusa Selection Red was most tolerant (4.2%). The maximum yield was obtained in Pune Selection-3 (62 kg/plant) while lowest yield in Mokama Local (20 kg/plant). The lowest market acceptability and highest economic loss were also observed in Pune Selection-3 (Rs. 3.3/kg and 11, 21,580/ha, respectively) due to highest incidence of bumpy fruit disorder as compared to other germplasm. The highest frequency of deformed seeds/fruit (83.6%) and economic loss (Rs. 84,400/ha) under fruit production for market was noticed during February, while the lowest deformed seeds/fruit (2.7%) were observed during December. The maximum incidence of vivipary and white seed disorders were observed during May (13.4 and 75.7%, respectively) while least incidence was observed in February and March (10 and 5%, respectively). The increased incidence of vivipary was observed in harvested fruits from February to May with increasing average monthly temperature. The low and high temperatures during seed maturation period aggravate deformed and vivipary seeds, respectively.

Key words: *Carica papaya*, physiological disorders, bumpy, vivipary, white seeds.

INTRODUCTION

Papaya (*Carica papaya* L.) belongs to the family Caricaceae, one of the most important fruits cultivated throughout the tropical and subtropical regions of the world (Anonymous, 2000). The natural habitat of papaya lies in tropical, central and South America. Papaya flourishes in the frost-free and humid areas of the tropics and subtropics. It is regarded as an excellent source of ascorbic acid, a good source of carotene, riboflavin and a fair source of iron, calcium, thiamin, niacin, pantothenic acid, vitamin B-6 and vitamin K. Each and every part of papaya plant from root to shoot is used for medicament purposes. Seeds are also rich source of amino acids; scented oil was extracted, used in treatment of sickle cell disease and poisoning related disorders (Saran and Choudhary, 2013). The challenges that Indian Agriculture

faces in the coming years remain enormous. Ensuring food and nutritional security and eliminating hunger, including hidden hunger, remain a high national priority. In order to effectively counter this demand, this crop will play an even more critical role in determining the productivity and food security. Papaya has gained more importance owing to its high palatability, early fruiting and highest productivity per unit area and multifarious uses like food, medicine and industrial input. It is cultivated in the world in an area of 3.83 lakh ha with a production of 8.05 million tones. In India, it is cultivated in 73,000 ha with a production of 23.17 lakh tones (Singh et al., 2010). The seed production in papaya with 100% genetic purity is difficult because of dioecious nature of the plant. Therefore, the seed should be produced either strictly

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under controlled condition or in an isolated area.

Under diverse agro-climatic conditions of North Bihar, the autumn sown crop is best suited for seed production (Ram and Ray, 1992; Singh et al., 2010). Quality seed production, market value and export of papaya fruit suffer from several limiting factors including physiological disorders. Different nutrient deficiencies of boron, zinc and sulphur and environmental stresses cause several physiological disorders such as bumpy fruit which may lead to deformed/discolored seeds. Bumpy fruit, deformed / discolored seeds, vivipary seeds (Saha, 2007; Saran et al., 2013a) and white seeds (disturbed sarcotesta) were identified as new physiological disorders of papaya under diverse agro-climatic conditions of India. Deformity first starts in young fruits, but symptoms become more severe over fruits close to physiological ripening or of older age. The bumpiness begins in areas on the fruit epidermis and is due to boron deficiency, stopping the fruit growth (Wang and Ko, 1975; Chen and Raveendranathan, 1984). In addition to this, infected tissue continues increasing in size and ends forming a protuberance or “bump”, similar to a ball. High incidence before maturity has also been a serious concern in papaya production.

During fruit and seed development period, proper temperature also plays important role. Keeping aforementioned facts in mind, the present study was conducted on the varietal screening for bumpiness; morphology and economics of bumpy fruits, deformed/discolored seeds, vivipary and white seeds (disturbed sarcotesta) and relationship between temperature and disorders (deformed seed and vivipary).

MATERIALS AND METHODS

The investigations were carried out at Indian Agricultural Research Institute Regional Station Pusa, Samastipur, Bihar (India) (elevation 52 m; latitude 25.98°N and longitude 85.67°E). The details of different experiments conducted are given as follows:

Screening of papaya germplasm

Screening of ten papaya germplasm lines was carried out during 2011 and 2012 for different physiological disorders. The plants selected were of uniform age (14 months). Ten plants of each germplasm line were randomly chosen for observations on bumpy fruit at fruit maturity. Each tree represented a replication and ten times replicated. Four germplasm lines, namely, Pune Selection-3, Pusa Dwarf, Pusa Nanha and Pusa Selection Red were studied for bumpy fruit incidence. Each tree of different selected germplasm was observed carefully and numbers of damaged fruits were recorded at weekly intervals. The values of different observations obtained from these trees were averaged to get the mean value. The bumpy fruit severity was calculated by own formular as given as follows:

$$\text{Disease severity (\%)} = \frac{\text{Disease category scale} \times \text{Number of plants} + \text{Disease category scale} \times \text{Number of plants}}{\text{Higher category scale} \times \text{Total number of plants}} \times 100$$

The observations on fruit yield were recorded to compare the yield of affected and non-affected trees of each germplasm in selected

orchard of uniform age (first crop). The yield of ten normal plants was recorded for estimating average fruit yield (kg/tree). A survey was carried out during the year; 2008 to 2013. The data on rates of fruits/kg was collected for normal and bumpy fruits to work out the market acceptability. The rates of bumpy and normal fruits of papaya were collected from the fruit sellers. The economic loss was calculated by working out the difference between gross return from normal and bumpy fruits as rupees per hectare during study years. An economic loss was estimated by calculating the difference between normal and diseased (bumpy) plants yield per unit area and it was multiplied by the market rate of fruits. The data presented are the pooled mean of two years. The statistical analysis of the data was carried out using standard statistical procedures.

Studies on morphological and quantitative traits

The observations were recorded from December, 2008 to May, 2013. The average monthly temperature was recorded from IARI RS Pusa observatory. Morphological observations were recorded for fruit surface, seed attachment, seed color, seed surface, sarcotesta, removal of sarcotesta and vivipary in both varieties namely, Pusa Dwarf and Pune Selection-3. The fruits of Pusa Dwarf were observed with vivipary and morphological observation for fruit surface, seed attachment, seed color, seed surface, seed size, sarcotesta and removal of sarcotesta during fruit harvesting (initiation of colour turning stage) and seed extraction. The data were also recorded on the total number of seeds/fruit, number of normal seeds/fruit, number of deformed seeds/fruit, white seeds/fruit, vivipary seeds/fruit, deformed seeds (%), seed harvest (%) and economic losses (Rs/ha) in Pusa Dwarf at seed crop harvesting (December to May) due to deformed seed disorder in autumn crop. Each fruit was cut into two equal halves for observing the incidence. The deformed/discolored seeds were recorded by counting the seeds in different categories. An economic loss was estimated by calculating the seed yield per unit area in kilograms.

Loss was estimated by observing seed harvest (normal and deformed percent) in particular months and multiplied by our sale price (at Rs. 40,000/kg). Relationship between average monthly temperatures, time of fruit harvest and seed disorders, namely, deformed seed and vivipary seeds from December to May were shown during the study years.

RESULTS

Field screening of papaya cultivars

The field screening results had divulged that among all the selected germplasm, the bumpy fruit was significantly higher in Pune Selection-3 (64.6%), Pune Selection-2 (45.6%) and Pune Selection-1 (41.7%) followed by Co-7 (35.2%) and Pusa Dwarf (25%) while Pusa Selection Red (4.2%) was least affected. The maximum yield was obtained in Pune Selection-3 (62 kg/plant) followed by Pusa Selection Red (57.8 kg/plant), while lowest in Mokama Local (20 kg/plant) and Co-2 (22 kg/plant). The lowest market acceptability and highest economic losses were also observed in Pune Selection-3 (Rs 3.3/kg and Rs11, 21,580/ha), respectively due to highest incidence of bumpy fruit disorder as compare to other germplasm (Table 1).

Table 1. Screening of different papaya germplasm lines.

Papaya germplasm	Bumpy fruit disease severity (%)	Fruit yield/plant (kg/tree)	Market acceptability (Rs/kg)		Economic loss (Rs/h)
			Normal	Abnormal	
Pusa Dwarf	25.0	41.0	8.3	5.1	3,54,240
Pusa Nanha	05.5	34.0	8.1	7.9	13,770
Pusa Delectious	11.0	44.0	8.1	6.2	2,25,720
Pusa Majesty	14.5	36.2	8.8	7.3	1,46,550
Pune Selection -1	41.7	54.0	10.0	7.1	4,22,820
Pune Selection -2	45.6	56.0	9.2	5.0	6,35,040
Pune Selection -3	64.6	62.0	10.0	3.3	1,121,580
Co-2	18.2	22.0	7.9	6.8	65,340
Co-6	25.0	40.0	8.7	7.2	1,62,000
Co-7	35.2	25.0	7.0	5.2	1,21,500
Pusa Selection Red	04.2	57.8	8.7	8.1	93,660
Pusa Papaya 2-8	09.1	43.0	10.0	7.5	2,90,250
Pusa Papaya Selection 1-11	07.4	41.0	9.7	9.0	77,490
Mokama Local	11.1	20.0	8.9	6.9	1,08,000
C.D. (P = 0.05)	1.9	8.4	1.6	1.3	8,988

Table 2. Morphological traits of normal and bumpy fruits in both 'Pune Selection-3' and 'Pusa Dwarf'.

Trait	Normal fruit	Bumpy fruit
Fruit surface	Smooth	Rough
Seed attachment	Loose	Tight
Seed color	Black	Brownish
Seed surface	Smooth	Rough
Sarcotesta	Shining	Disturbed
Removal of sarcotesta	Easily-removable	Non-removable
Deformed seeds	None	Almost

Studies on morphological traits

There were visual differences in normal and bumpy fruits and seeds of Pune Selection-3 and Pusa Dwarf. Morphological observations of fruit and seed, namely, fruit surface, seed attachment, seed color, seed surface, sarcotesta, removal of sarcotesta and vivipary in normal fruit were smooth, loose, black, smooth, shining, easily removable and none while rough, tight, brownish, rough, disturbed, non-removable and almost in bumpy fruits, respectively, were observed in both cultivars (Table 2, Figures 1 and 2). The highest frequency of deformed seeds/fruit (83.6%) and economic loss (Rs. 84,400/ha) were recorded and noticed during February, whereas the lowest frequency of deformed seeds/fruit (2.7%) were observed during December. The lowest economic loss (Rs. 5,200/ha) was noticed in April (Table 3). A positive relationship between average monthly minimum temperature and deformed seeds (%) was observed from December to May during the years from 2008 to 2009 to 2013. The

increased incidence of deformed seeds was observed from December to February and decreased onward months harvested fruits with decreased and increase average monthly minimum temperature, respectively during study period. The maximum incidence was observed during February due to lower temperature during January which is seed maturity period for February harvest (Figure 3).

The vivipary fruits were also rough surfaced, while the normal fruits were smooth surfaced. The visual observations for vivipary were tight seed attachment, white seed color, rough seed surface, smaller size, disturbed sarcotesta and no need of removal of sarcotesta for seed germination, while just reverse of it was observed in case of normal seeds (Table 4 and Figure 4). Vivipary and white seeds (disturbed sarcotesta) are a serious economic problem only in Pusa Dwarf and the severity of damage varies with the harvesting/fruit maturity months. For vivipary and white seed disorders, the highest incidence were observed during May (13.4 and 75.7%, respectively), followed by April (8.1 and 66.3%, respectively), March (1.1 and 34.6%, respectively), and the lowest incidence was observed in February (0.0 and 10.0%, respectively). The maximum economic loss (Rs. 53,600/ha) was observed in April, while least during February (Rs. 16,800/ha) in autumn sown crop of Pusa Dwarf (Table 5). The higher incidence of these seed disorders during May harvested fruits might have got aggravated due to high temperature during seed maturation period. Positive relationship between average monthly maximum temperature and vivipary (%) was observed from December, 2008 to May, 2013. The incidence of vivipary was observed increased from February to May harvested fruits with increased average monthly maximum temperature during



Figure 1. Bumpy fruits of papaya.



Figure 2. Deformed and normal seeds in papaya cultivar, 'Pune Selection-3'.

Table 3. Average incidence and economic loss due to deformed seed disorder in autumn sown papaya cultivar, Pusa Dwarf during 2011 and 2012.

Month of harvest	Total number of seeds/fruit	Number of healthy seeds/fruit	Number of deformed seeds/fruit	Deformed seeds/fruit (%)	Economic loss (Rs/ha)
December	318.8	312.2	8.6	2.7	58,400.00
January	658.6	374.1	284.5	43.2	62,400.00
February	638.4	103.2	535.2	83.6	84,400.00
March	549.2	419.8	129.4	22.0	16,000.00
April	514	456.6	58.8	12.1	5,200.00
C.D. (P = 0.05)	31.653	4.646	25.369	2.7	1,466.18

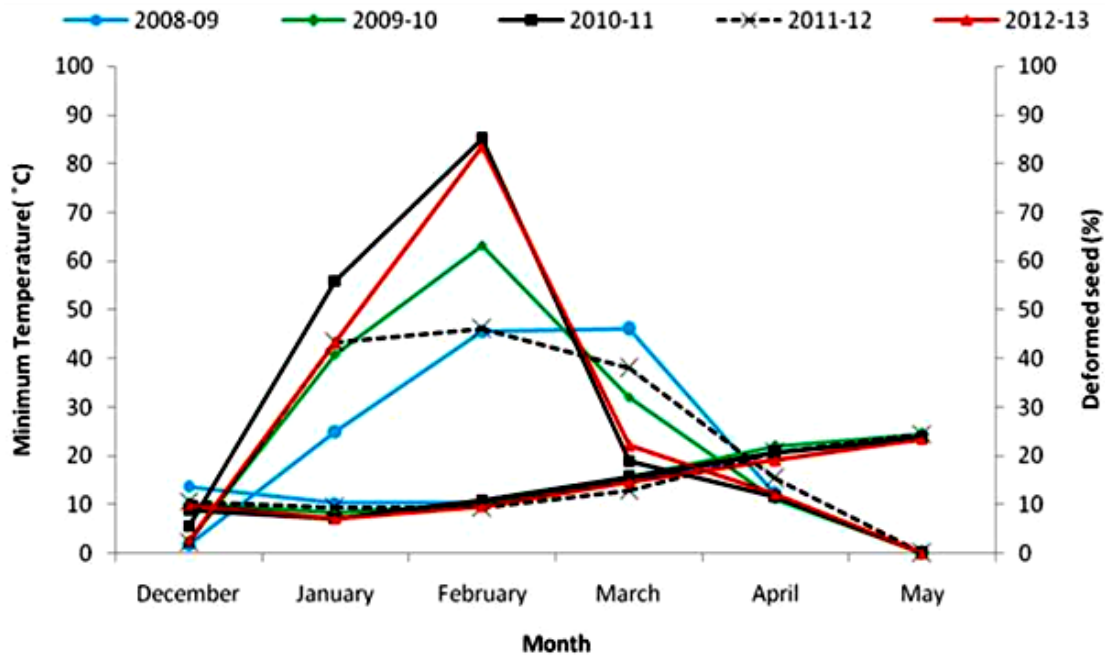


Figure 3. Relationship between average monthly minimum temperature and deformed seed (%).

Table 4. Morphological description of vivipary fruits of papaya cultivar, 'Pusa Dwarf'.

Observation	Normal fruit	Vivipary fruit
Fruit surface	Smooth	Rough
Seed attachment	Loose	Tight
Seed color	Black	White
Seed surface	Smooth	Rough
Seed size	Large	Small
Sarcotesta	Shining	Disturbed
Removal of sarcotesta	Easily-removable	Broken sarcotesta



Figure 4. Normal, white and vivipary seeds in papaya cultivar, 'Pusa Dwarf'.

Table 5. Average incidence and economic loss due to vivipary and white seeds disorder (disturbed sarcotesta) in autumn sown papaya cultivar, 'Pusa Dwarf' during 2011 and 2012.

Month of harvest	Normal seeds (%)	Vivipary seeds (%)	White seeds (%)	Economic loss (Rs./ha)
February	90.0	0.0	10.0	16,800.00
March	64.4	1.1	34.6	42,800.00
April	25.7	8.1	66.3	53,600.00
May	11.0	13.4	75.7	21,600.00
C.D. (P = 0.05)	9.3	1.4	4.5	481.00

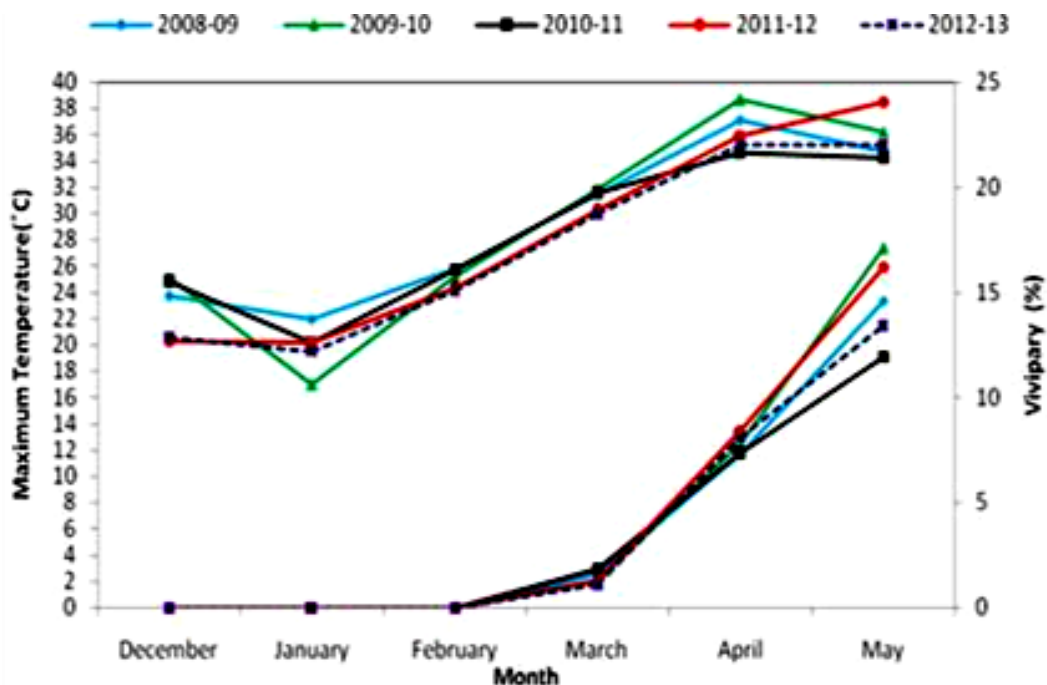


Figure 5. Relationship between average monthly maximum temperature and vivipary (%).

study period (Figure 5).

DISCUSSION

Ram and Ray (1992) reported fruit yield in different papaya cultivars under agroclimatic conditions of North Bihar in autumn sown crop. Screening of different papaya varieties have been carried out against expression of boron deficiency symptoms by different workers (Wang and Ko, 1975; Chen and Raveendranathan, 1984) and fruit deformity has been found associated with boron deficiency. Visual differences in normal and bumpy fruits and seeds of Pune Selection-3 and Pusa Dwarf were observed. Similarly, the bumpy surface on the fruit epidermis at fruit maturity stage was also reported (Chen and Raveendranathan, 1984). The stress associated with bumpy fruits may aggravate deformed seed incidence in Pune Selection 3. Fruit and seed disorders in papaya cause high economic losses in seed production during

autumn-sown crop (Saran et al., 2013a). The economics of Pusa Dwarf seed production and seed yield has already been reported (Ram and Majumder, 1990; Sing et al., 2010). Similarly, the economic loss was maximum in April and least in February in autumn-sown crop of 'Pusa Dwarf' under seed production at North Eastern Plains Zone of India (Saran et al., 2013b). Under normal conditions, the seed yields are 60 to 75 kg/ha in dioecious varieties. The high incidence of deformed seeds in Pusa Dwarf may be due to lower temperature during seed maturation period (January) in autumn sown crop. The stress associated with bumpy fruits may aggravate the incidence in Pusa Dwarf. The natural occurrence of precocious germination has been reported in papaya (Balakrishnan et al., 1986). These observations have revealed that the temperature during seed maturation period play an important role.

The low and high temperatures during seed maturation period aggravate deformed and vivipary seeds, respectively.

Conclusions

Fruit and seed disorders in papaya cause great economic losses in seed production during autumn sown crop. We observed that Pune Selection-3 and Pusa Dwarf were the most susceptible germplasm lines for bumpy fruit and deformed seed disorders, while vivipary and white seeds (disturbed sarcotesta) are a serious economic problem only in Pusa Dwarf. The incidence of deformed seeds was increased from December to February and decreased onward months harvested fruits and incidence of vivipary was increased from February to May harvested fruits with maximum temperature.

REFERENCES

- Anonymous (2000). Organic farming in the tropics and subtropics (exemplary description of 20 crops). Naturlande.V– 1st edition.
- Balakrishnan K, Balamohan TN, Veerannah L, Kulasekaran M, Shanmughavelu KG (1986). Seed development and maturation in papaya. *Prog. Hortic.* 18: 68-70.
- Chen YK, Raveendranathan P (1984). Differential sensitivity of papaya varieties in expression of boron deficiency symptoms. *Mardi Res. Bull.* 12(3): 281-286.
- Ram M, Majumder PK (1990). Cost of seed production in gynodioecious and dioecious papaya under controlled pollination. *Seed Res.* 18: 17-20.
- Ram M, Ray PK (1992). Influence of fruiting season on seed production of papaya in north Bihar. Abstracts - VIII. All India Seed Seminar on Recent Advances and Future Strategies in Seed Science and Technology, June 9-11, 1992, Nauni, Solan (India). p. 27.
- Saha M (2007). *In vitro* propagation of precociously germinated seedlings of *Carica papaya* L. variety Madhubindu. *Bionano Front.* 1(1): 55-59.
- Saran PL, Choudhary R (2013). Drug bioavailability and traditional medicaments of commercially available papaya– a review. *Afr. J. Agric. Res.* 8(25): 3216-3223.
- Saran PL, Choudhary R, Solanki IS, Singh K (2013a). New bottlenecks in seed production of papaya under North Eastern Plains Zone. *New Initiatives. ICAR NEWS, A Sci. Tech. News Lett.* 19(3): 4.
- Saran PL, Kumar PR, Choudhary R, Devi G, Solanki IS (2013b). New physiological disorders in papaya (*Carica papaya* L.) fruit or seed production, market acceptability and economic losses in North-East Plain Zone. *Agric. Econ. Res. Rev. (Abstract)*, 26: 207.
- Singh K, Ram M, Kumar A (2010). Forty years of papaya research at Pusa, Bihar, India. *Acta Hort.* 851: 81-88.
- Wang Der-Nan, Ko WH (1975). Relationship between deformed-fruit disease of papaya and boron deficiency. *Phytopathology* 65: 445-447.

Full Length Research Paper

Development of specific primers for the detection of HVA1 from barley in transgenic durum wheat by polymerase chain reaction (PCR) technology

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Genetic transformation is a widely employed tool in both basic research and commercial plant breeding programs. Its application requires that transgenes be stably integrated and expressed in the plant genome. When transgenic plants are developed, it is essential to determine which plants contain the transgene. Detection methods are usually based on amplification of the target transgene. This paper describes a development of detection method based on conventional and real time polymerase chain reaction (PCR) for simultaneous detection of barley *HVA1* transgene and its transcript in transformed durum wheat. Since there exist a high homology between the barley *HVA1* gene and the wheat gene, development of a specific sets of primers is needed for PCR-based characterizations, and the study of the transgene. Based on the alignment of the two genes sequences obtained from public databases, several primers were designed to detect and distinguish between the transformed and non-transformed plants. Real time PCR has been employed because of its inherent sensitivity and quantitative nature. It has been possible to design the following primers pairs *F2/MMR*, *F2/R10* and *F14/R10* as highly specific and suitable for the detection of *HVA1* DNA by conventional and real-time PCR. Nonetheless, the primers used were allowed to reach high efficiencies and did not show any cross-reactivity with DNAs extracted from various plants. The sensitivity achieved was 6.4 pg. The primer pair *F2/R10* was considered as highly specific for the detection of both DNA and mRNA of the *HVA1* by real-time PCR. The assays proved to be accurate, specific, sensitive and sufficiently reproducible for further application in high-throughput molecular characterization of transgenic lines.

Key words: HVA1, durum wheat, transgenic plant, real time polymerase chain reaction (PCR), drought.

INTRODUCTION

Abiotic stresses, such as drought, salinity, extreme temperatures, chemical toxicity and oxidative stress are serious threats to agriculture and result in the deterior-

ation of the environment (Boyer, 1982). Abiotic stress leads to a series of morphological, physiological, biochemical and molecular changes that adversely affect

plant growth and productivity (Wang et al., 2001). Among the abiotic stresses, the availability of water is the most important factor that limits the productive potential of higher plants (Rodriguez et al., 2006). Drought is one of the most severe environmental stresses that affect almost all plant function (Yamaguchi Shinozaki et al., 2002) and is also considered as the primary limitation to wheat production worldwide (Mardeh et al., 2006).

Durum wheat (*Triticum durum* L.) is the most important cereal crop in the Mediterranean basin. In Morocco, durum wheat is grown over an area ranging from 1 to 1.2 million hectares annually, and ranks the third after bread wheat and barley, with respect to production (MAPM, 2011). The country's wheat productivity has been affected by various biotic and abiotic stresses (Karrou, 2003). To increase yields or reduce yield losses under such adverse conditions, it is necessary to improve tolerance to environmental stress (Shinozaki and Yamaguchi-Shinozaki, 2007). Developing a line of drought tolerant crops would alleviate the economical strain of losing crop yield due to insufficient water availability. Traditional breeding for increasing drought tolerance is difficult because of limited genetic diversity and the complex biochemical response plants have to drought stress (Bacsó et al., 2008). It is important, therefore, to look for alternative strategies to develop stress tolerant crops. They will provide an attractive and complementary option for improving a plant's performance under stress conditions.

Genetic engineering has undoubtedly opened a new avenue to overcome crop losses due to various abiotic stresses prevalent in the agricultural ecosystems (Bajaj et al., 1999). It provides novel opportunities for the transfer of agronomically useful genes in an elite cultivar without disturbing its genetic background (Rohila et al., 2002). A number of stress related genes have been isolated which can potentially improve the stress tolerance in plants (Bajaj et al., 1999). Late embryogenesis abundant (LEA) proteins are among the best known of water stress-induced proteins. These proteins accumulate during the late stage of seed formation and in vegetative tissues under drought, heat, cold and salt stress conditions or with abscisic acid (ABA) application (Sivamani et al., 2000). A LEA gene *HVA1* (which encodes a group 3 LEA protein) from barley was engineered in rice (Chandra et al., 2004; Rohila et al., 2002; Xu et al., 1996), the transformed lines produced showed enhanced tolerance to drought stress. It has been introduced also into wheat (Debasis and Paramjit, 2003; Sivamani et al., 2000), and the transgenic plants showed improved biomass productivity and water-use efficiency under water-deficit conditions. *HVA1* has been used successfully to confer stable tolerance to osmotic stress in oat (Maqbool et al.,

2002; Oraby et al., 2005) and mulberry (Lal et al., 2008). To produce transgenic plants, selection systems are used that lead to the selective growth of transformed cells. In many plant transformation systems, the availability of selectable markers is essential to recover transgenic plants. Genes conferring resistance to selective chemical agents, such as antibiotics and herbicides, are used routinely (Darbani et al., 2007). They have been found to be particularly effective for selection and provide a means for rapidly identifying transformed cells, tissues, and regenerated shoots (Goodwin et al., 2005). In many cases it has been regarded as unnecessary to carry out molecular analysis using vector DNA other than the gene of interest and the selectable marker (Smith et al., 2001). To confirm definitively the presence of a transgene, a sample with a positive signal in screening should be further analyzed using a construct-specific or event-specific method (Griffiths et al., 2002).

Expression of a transferred gene can be initially silenced or inactivated over time or generations by either transcriptional gene silencing (TGS) or post-transcriptional gene silencing (PTGS) triggered by diverse host defense responses (Matzke et al., 2000). Success at developing improved cultivars through genetic engineering depends on stable and predictable expression of the inserted gene. Thus it is widely considered necessary to confirm the expression of the transgenes before proceeding to downstream functional characterization. Polymerase chain reaction (PCR) is one of the most sensitive techniques for detecting the integrated gene in the transgenic plant genome (Wong and Medrano, 2005). Especially, real time PCR has been regarded as the most powerful tool for the detection and quantification of GMO despite its high expense (Tripathi, 2005). Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a rapid, sensitive, and reliable method for the detection of messenger RNAs (mRNAs) (Bustin et al., 2005).

The objective of the study was to design specific primers sequences of barley *HVA1* gene in transgenic wheat for conventional PCR and real time PCR analysis. Designing highly specific primers to amplify the transgene will help either in the detection of the gene of interest (*HVA1*) in transformed plants and, in the measurement of its mRNA abundance and changes. The barley *HVA1* and the wheat genes are highly similar at both nucleotide level and predicted amino acid level (Curry et al., 1991), this sequence homology between the two genes makes the detection of the *HVA1* gene and mRNA in wheat very difficult by PCR or real time PCR. To our knowledge, this is the first report describing the development of specific primers able to detect DNA and mRNA of *HVA1* in transgenic durum wheat by conventional PCR and

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Abbreviation: LEA, Late embryogenesis abundant; ABA, abscisic acid; TGS, transcriptional gene silencing; PTGS, post-transcriptional gene silencing; PCR, polymerase chain reaction; CTAB, cetyltrimethyl ammoniac bromide; CT, cycle threshold.

quantitative real time PCR, respectively.

MATERIALS AND METHODS

Leaves from untransformed, transformed durum wheat line 1804, developed at National Institute of Agronomical Research, Laboratory of Biotechnology (Rabat -Morocco), kindly provided, were used in this study. For the specificity assessment, we used three durum wheat varieties, three bread wheat varieties, two maize, rice and oat.

DNA extraction

DNA was extracted from transformed, untransformed and other plants from frozen leaves. The cetyltrimethyl ammoniac bromide (CTAB) protocol was used for DNA extraction as described by Murray and Thompson (1980). The concentration of genomic DNA was determined by Nanodrop 8000 spectrophotometer (Thermo Scientific). Genomic DNA was measured by absorption at 260 nm, while DNA purity was evaluated using the ratio at 260/280 nm.

RNA isolation and cDNA synthesis

Total RNA was extracted from leaves by the Spectrum Plant Total Kit (Sigma Aldrich, St louis, US) in accordance with the manufacturer's instructions. To avoid possible DNA contamination, a digestion by Ambion Turbo DNase (life technologies, US) was carried out on the RNA samples. RNA concentrations were measured at 260 nm by Nanodrop 8000. The quality and purity of the preparations were determined by measuring the OD260: OD280 nm absorption ratio (1.8-2.0), and the integrity of the preparations was ascertained by denaturated agarose gel electrophoresis (2%). DNase-treated RNA samples were reverse-transcribed using The SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo (dT)20 (Invitrogen) on 1 µg total RNA following the manufacturer's recommendations (final volume of 20 µl). The cDNAs were then stored at -20°C until used in real-time PCR amplification.

Primers design

As *HVA1* barley gene show high sequence similarity with the endogenous gene in wheat, the primers were designed manually using an alignment performed by ClustalW program of software Geneious (Drummond et al., 2011), of the *HVA1* sequence in the plasmid PBY520 with its homologous in durum wheat on the basis of sequences present in the GenBank database (AJ613581, AJ716526, BE428267, BE428883) (Figure 1). Different primers sets were designed to target the *HVA1* gene, two forward primers and five reverse primers. The plasmid *PBY520* contained the linked selectable marker/herbicide resistance bar (phosphinothricin acetyl transferase) gene (driven by cauliflower mosaic virus 35S promoter and the nopaline synthase nos terminator). 35S and NOS primers were used, for the screening of the transformed plants (Pietsch et al., 1997). To verify the amplification of extracted DNAs, a pair of primers CDC(a).F/CDC(a).R targeting the cell division control gene was used (Gimenez et al., 2011). The primers were synthesized using Polyplex machine (Gene machine, USA) at functional genomic platform - UATRS-CNRST. Table 1 lists all the primers used in this study.

PCR conditions

Reactions were performed on genomic DNA (100 ng) extracted

from transformed lines, on plasmid as positive control and on non-transformed lines as negative control for each designed primer. PCR's were performed with 1 U platinum Taq DNA polymerase (Invitrogen), 1x PCR Buffer, 2.5 mM MgCl₂, 0.2 mM dNTP mix, and 400 nM of each primer in a 25 µl reaction volume under the following conditions: preheating at 96°C for 3 min, then 35 cycles of denaturation at 96°C for 30s, annealing at 50-60°C for 30 s and extension at 72°C for 60 s, followed by final extension of 10 min employing a veriti PCR thermal cycler (Applied Biosystems, USA). The following primers combinations were used: *F2/R1*, *F2/R4*, *F2/R5*, *F2/MMR*, *F2/R10* and *F14/R10*. The amplified fragments were electrophoresed on 2% agarose gels and detected using ethidium bromide along with molecular weight markers. Different primers pairs have been tested for the *HVA1* gene amplification by qualitative PCR and those giving a specific product were also tested by the more sensitive real time PCR to confirm their specificity.

Real time PCR: SYBR green detection

DNA

Reactions for real-time PCR using SYBR green detection consisted of 12.5 µl of SYBR green JumpStart Taq Ready Mix (Sigma-Aldrich, US), 400 nM forward and reverse primers, 100 ng of DNA template in 25 µl reaction volume. The PCR conditions were as follow, first denaturation at 94°C for 2 min, 45 cycles of denaturation at 94°C for 15 s, annealing and extension at 60°C for 60 s followed by a melting curve analysis with a temperature gradient of 0.1°C/s from 70 to 95°C.

Sensitivity, efficiency and reproducibility

Genomic DNA was serially diluted five-fold and conducted in triplicates to establish the standard curves. The extracted DNA was serially diluted to a final concentration of 20, 4, 0.8, 0.16, 0.032, 0.0064 and 0.00128 ng/µl. The amount of DNA per reaction is 20, 4, 0.8, 0.16, 0.032, 0.0064 and 0.00128 ng. Cycle threshold (CT), that is, the number of PCR cycles necessary to reach the threshold fluorescence level, was automatically determined by the Rotor gene software (Corbett Research, Sydney, Australia) based on the second derivative maximum method. Individual measurements for replicate real-time PCR reactions were plotted separately to obtain correlation coefficient (R^2) that reflect the variation of the data set. The PCR efficiency was calculated using the slope of the standard curve as follows: PCR efficiency = $10^{-1/\text{slope}}$ whereby an efficiency of 1 corresponded to 100% PCR efficiency, when all target molecules double in one PCR cycle and an efficiency of 0 indicates no amplification (Cankar et al., 2006). Each dilution was tested, using real-time PCR amplification, as previously described.

RNA

First strand cDNA was used as template for real time PCR amplifications with gene specific primers (confirmed specific by conventional PCR and real time PCR). KAPA SYBR fast qPCR Kit (Kapa Biosystems, cape town, SA) was used for the detection of *HVA1* transcript. The PCR consisted of 10 µl SYBR green fast Ready Mix, 300 nM forward and reverse primers, 200 ng of cDNA and made up to 20 µl with water. The PCR's mixtures was denatured at 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30s and extension at 72°C for 1 s. The amplification period was followed by a melting curve analysis as mentioned above to exclude amplification of unspecific products. The real time PCRs for DNA and RNA were carried out on a RotorGene 6000 (Corbett Research, Sydney, Australia). The

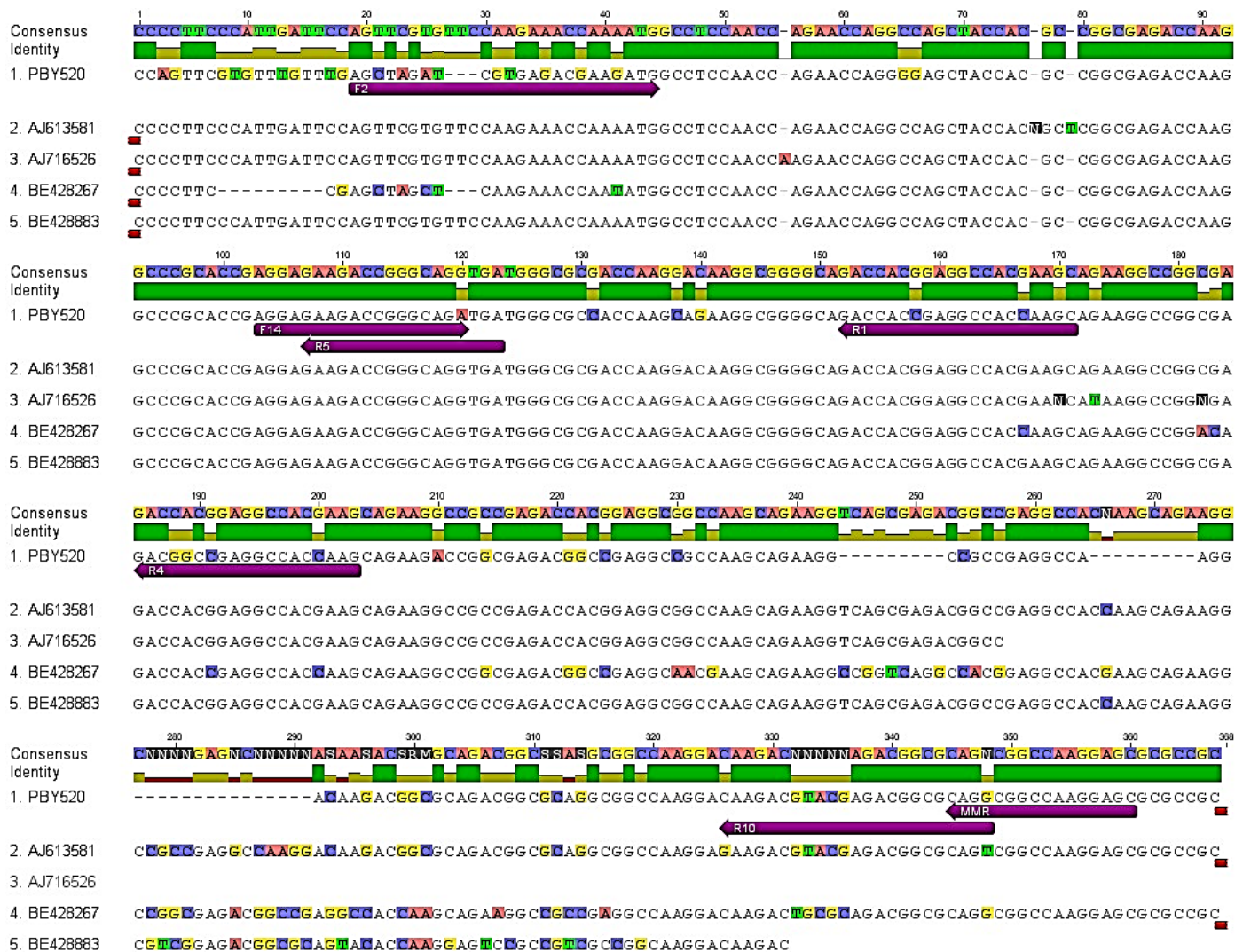


Figure 1. Sequence alignment of barley *HVA1* cDNA with its homologous in durum wheat on the basis of 4 sequences present in the GenBank database (AJ613581, AJ716526, BE428267, and BE428883). Positions of the primers are indicated by arrows.

Table 1. Primer sequences and length of PCR amplification products.

Primer	Target	Orientation	Sequence (5'-3')	Product length (bp)	Reference
CDC(a)_F	CDC	Sense	CAGCTGCTGACTGAGATGGA	77	Gimenez et al. 2011
CDC(a)_R		Antisense	ATGTCTGGCCTGTTGGTAGC		
35S_F	35S	Sense	GCTCCTACAAATGCCATCA	195	Pietsch et al. 1997
35S_R		Antisense	GATAGTGGGATTGTGCGTCA		
Nos_F	NOS	Sense	GAATCCTGTTGCCGGTCTTG	189	Pietsch et al. 1997
Nos_R		Antisense	TTATCCTAGTTTGCGCGCTA		
F2	<i>HVA1</i>	Sense	AGCTAGATCGTGAGACGAAGATG		This work
F14		Sense	AGGAGAAGACCGGGCAGA		This work
R1		Antisense	GCTTGGTGGCCTCGGTGGTC		This work
R4		Antisense	CTTGGTGGCCTCGGCCGTC		This work
R5		Antisense	TCATCTGCCCGTCTTC		This work
R10		Antisense	CCTGCGCCGTCTCGTACGTCTTGG		This work
MMR		Antisense	CTCCTTGGCCGCTG		This work

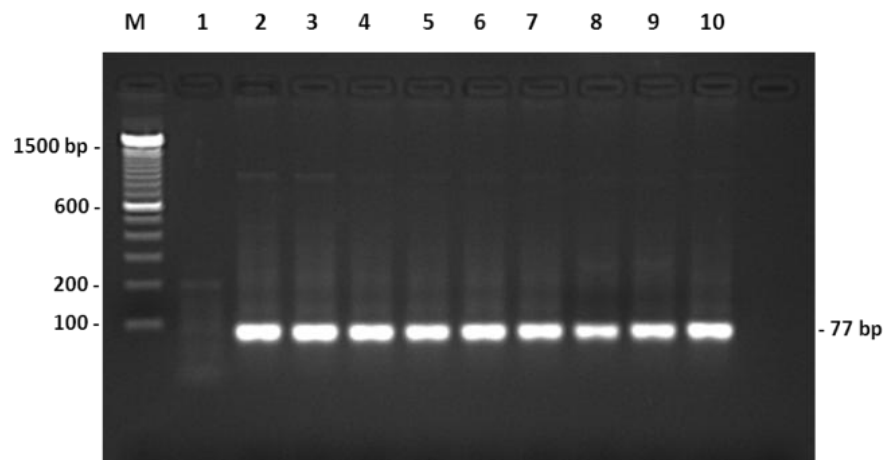


Figure 2. 2% Agarose gel electrophoresis of cell division control (CDC) PCR products amplified from wheat genomic DNA. M, 100 bp DNA ladder; lane 1, negative control; lanes 2, 3, and 4, non-transformed wheat; lanes 5 to 10, transformed wheat.

SYBR green PCR product was analyzed on 2% agarose gel with ethidium staining.

Sequencing analysis

The products that were positive after conventional PCR analysis of DNA and real time PCR for RNA were subjected to sequencing, after purification using USB ExoSAP-IT reagent (Affymetrix). The sequences of each strand were determined by means of automated fluorescence sequencing on an ABI PRISM 3130xl sequencer (Applied Biosystems, USA) using BigDye Terminator v3.1 cycle sequencing Kit (Applied Biosystems) with the same primers used in PCR amplification. Sequence alignments were performed using the software MEGA 5.0 (Tamura et al., 2011).

RESULT

Qualitative polymerase chain reaction (PCR)

Amplification of DNA is generally influenced by the presence of PCR inhibitors, the extent of DNA damage and the length of the extracted DNA fragment. The endogenous gene CDC was used to confirm the presence of amplifiable wheat DNA, using the plant-specific primer pair CDC(a)_F/CDC(a)_R. Fragments of the expected target size (77 bp) were amplified in all tested samples, untransformed and transformed (Figure 2). Screening based on the 35S-PCR and Nos-PCR systems was performed using primer pairs previously described by Pietsch (Pietsch et al., 1997). The desirable PCR products of 195 bp fragment of CaMV 35S promoter and 180 bp fragments of NOS terminator (Figure 3) were amplified only in transgenic plants, while no such products were detected in non-genetically modified plants. After assessing the initial quality of the DNA extracted from the samples, and their screening using the

selectable markers, we proceed to the detection of *HVA1* with the aim of obtaining specific amplicons, using the following primers pair *F2/MMR* (300bp), *F2/R1* (147bp), *F2/R4* (212 bp), *F2/R5* (100 bp), *F2/R10* (290 bp) and *F14/R10* (212 bp). Except the primer pair *F2/R1* (presence of two bands), clear and intense bands, with the expected size for all the primers pair were obtained in all transformed plants, including the positive control (Figure 4); however primers pair *F2/R1*, *F2/R4* and *F2/R5* failed to yield a specific assay for *HVA1*. A weak 147 bp band corresponding to primers pair *F2/R1* was observed in the 3 untransformed samples. A smear was observed in the non-transformed samples using the primer pair *F2/R4*. The primer pair *F2/R5* was unable to differentiate the transgenic from non-transgenic plant, a weak intensity band with expected amplifications products were present in non-transformed plants. These primers could not distinguish between transgenic and non-transgenic wheat, whereas the primers pair *F2/MMR*, *F2/R10* and *F14/R10* were successful to yield appropriately sized fragments in only transgenic wheat. No amplification of non-transgenic wheat was observed. These primers pairs were specific to transgenic wheat, and no amplification was found in non-genetically modified wheat. They were chosen to perform real time PCR to check their specificity.

Real time PCR

The specificity and sensitivity of primer pairs *F2/MMR*, *F2/R10* and *F14/R10* has been tested with real time PCR using SYBR green detection. This intercalating molecule fluoresces upon binding to dsDNA, allowing the detection of any products accumulated during amplification, including non-specific reaction products, such as primer-

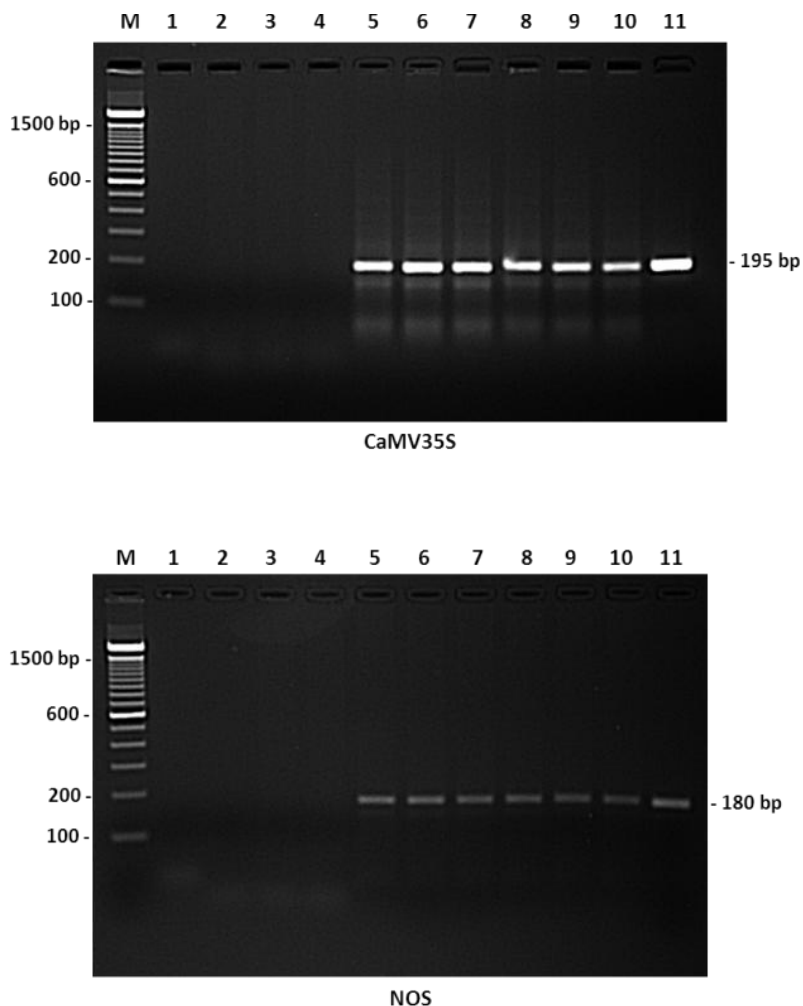


Figure 3. Detection of GMO target DNA (CaMV35S and NOS fragments) by 2% agarose gel electrophoresis. M: 100 bp DNA markers; 1: Negative control; 2, 3, and 4: non-transformed wheat; 5 to 10: transformed wheat; 11: Positive control (Plasmid *PBY520*).

dimers. The real time PCR is specific and sensitive enough to distinguish between transformed and non-transformed plants. The evaluation of the dissociation curves makes it however possible to identify specific amplicons from other products, by assessing their own melting temperatures.

In order to assess the specificity of the selected primer pairs, amplifications have been carried out using DNAs extracted from various plants. The results indicate that no amplification was observed from the untransformed plants and from any of the species tested other than the target (Data not shown). With the primer pair *F2/MMR*, the melting curve analysis (Figure 5A) showed one product corresponding to transformed plants and positive control. No amplification was observed in non-transformed plants, whereas a signal was observed in non-genetically modified plants when using the primer pair *F14/R10* with lower size (Figure 5E), this amplification

was also observed in qualitative PCR. With primers pairs *F2/R10* fluorescent signals were detected only for transformed samples; no amplification was detected for non-transformed samples and the NTC (no template control). The dissociation analysis (Figure 5C) showed that the PCR reactions only produced one specific product; the curves were shaped and clear with no trace of nonspecific products or of primer dimers. These indicated that the primers *F2/R10* were highly specific for the detection of *HVA1*. The potentiality of the real time PCR system to detect *HVA1* has been demonstrated. The SYBR green product of the three primers pair was analyzed on 2% electrophoresis gel, a clear bands corresponding to the expected size were detected in all transformed plants and the positive control, whereas no amplification products were detected in non-transformed plants (Figure 5B, 5D, and 5F).

To assess the sensitivity of these real-time PCR

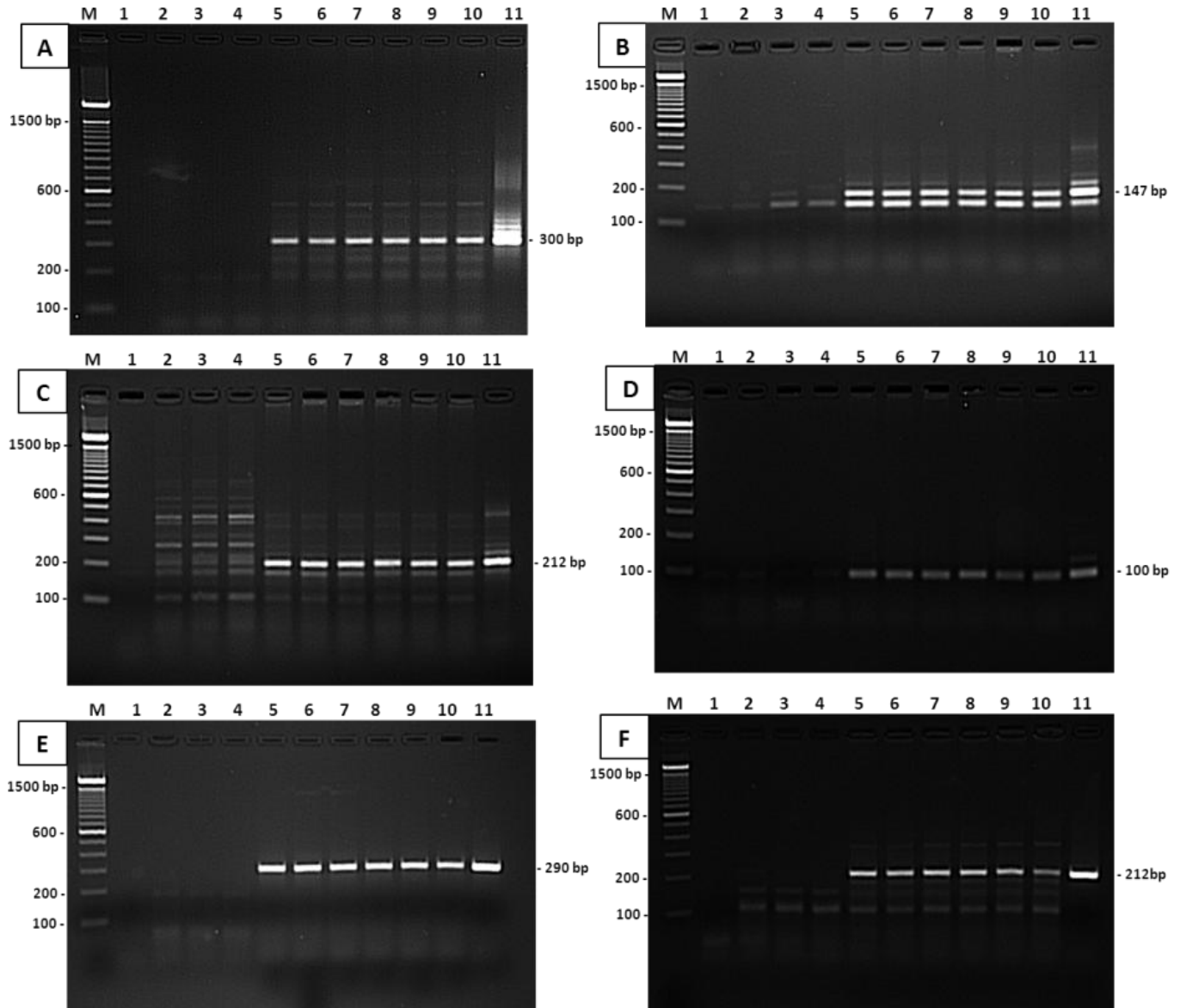


Figure 4. PCR amplification of the *HVA1* DNA after gene transfer into wheat using qualitative PCR. M, 100 bp marker; 1, negative control; 2 to 4, non-transformed wheat; 5 to 10, transformed wheat; 11, positive control (Plasmid *PBY520*). Forward primer F2 is combined with different reverse primers as follow: (A) *F2/MMR*; (B) *F2/R1*; (C) *F2/R4*; (D) *F2/R5*; (E) *F2/R10*. (F) Corresponds to primers combination *F14/R10*.

assays, standard curves were generated with genomic DNA using the primer pairs *F2/MMR*, *F2/R10* and *F14/R10* (Data not shown). The fluorescent signal could be detected when the amount of the DNA template was lowered to 6.4 pg. A very efficient amplification was obtained as indicated by the slope of the linear regression analysis (-3) with a good correlation coefficient (0.99) in all standard curves (Table 2). To further demonstrate the reproducibility of the quantitative system, we ran the real time PCR in triplicate with six dilutions of durum wheat genomic DNA using the 3 primers pair (*F2/MMR*, *F2/R10* and *F14/R14*). The coefficients of variation (CV%) values

and the standard deviation (SD) values for the 3 primers pair are indicated in Table 3. These data showed that the CV values and the SD values derived from these tests were relatively small and that this quantitative PCR detection system worked stably and reliably.

For the detection of *HVA1* transcript we used the primer pair *F2/R10* in all tested samples. Robust amplification of the *HVA1* mRNA was observed in transgenic samples (Figure 6). The post-amplification melting curve (Figure 6A) showed a sole, symmetric and sharp curve, indicating that only one product was accumulated. The real time PCR product was analyzed on electrophoresis

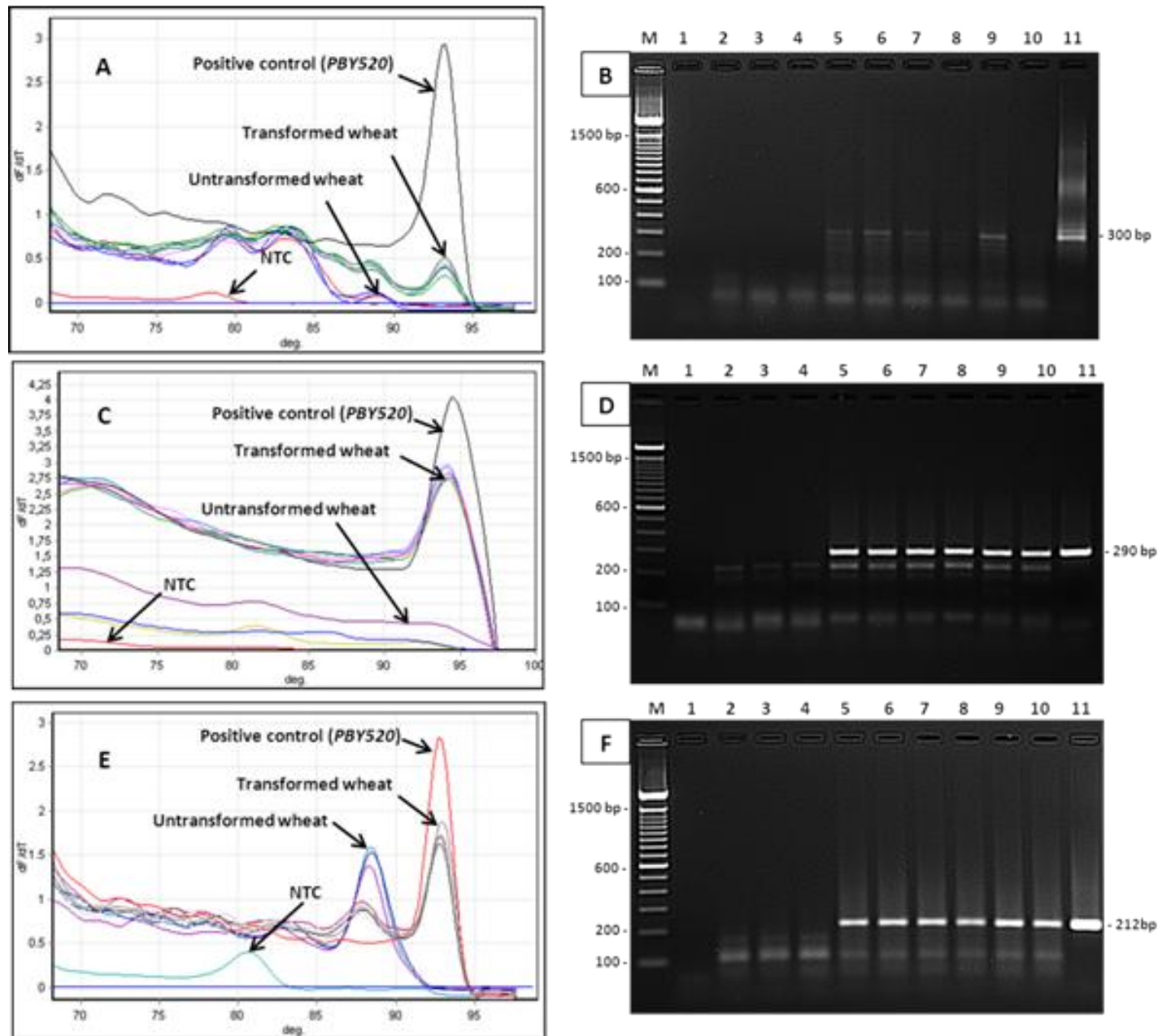


Figure 5. Melting curves analysis, showing the specific amplification of the target gene *HVA1*, and confirmation by agarose gel electrophoresis analysis. (A, B): Primers F2/MMR; (C, D): primers F2/R10; (E, F) primers F14/R10. NTC (no template control), transformed, non-transformed wheat and positive control are shown by arrows on the amplification plots. Lane M: DNA marker 100 bp; lane 1: water control; lanes 2 to 4: non-transformed wheat; lanes 5 to 10: transformed wheat; lane 11: positive control (Plasmid *PBY520*).

gel 2%, (Figure 6B); the reliability of the specific primers was confirmed by the unique fragment with expected size (290 bp). In controls with cDNAs from non-transformed plants, or with samples not treated with reverse transcriptase, no amplification products were detected. This result indicates that genomic DNA was completely removed from the RNA samples used for cDNA synthesis, thus, the *HVA1* gene is transcribed in transgenic samples. The

primer pair F2/R10 was considered highly specific for the detection of *HVA1* mRNA.

Sequencing

The identity of the PCR product has been checked by nucleic acid sequencing to confirm the specific amplifica-

Table 2. Real time PCR amplification efficiencies of HVA1 using genomic DNA obtained with three pairs of primers.

Primer pair	Slope (gDNA)	Interception (gDNA)	Correlation coefficients (gDNA)	PCR efficiencies (gDNA)
F2/MMR	-3.114	29.91	0.9986	109
F2/R10	-3.120	26.65	0.9980	109
F14/R10	-3.218	30.64	0.9941	105

Table 3. Reproducibility of the Ct measurements by real-time PCR for six levels of concentration of genomic DNA from transformed durum wheat using the 3 pair of primers (F2/MMR, F2/R10 and F14/R14).

Starting concentration (ng/μl)	Ct Value			Average	SD ^a	CV ^b
	1	2	3			
Primer pair F2/MMR						
0.0064	36.34	34.62	34.36	35.11	1.08	3.076
0.032	33.76	32.44	33.43	33.21	0.69	2.077
0.16	30.28	30.43	30.39	30.37	0.08	0.263
0.8	28	28	28.08	28.03	0.04	0.142
4	25.64	25.72	25.7	25.69	0.04	0.155
20	23.77	23.83	23.75	23.78	0.04	0.168
Primer pair F2/R10						
0.0064	29	31.59	29.05	29.88	1.48	4.953
0.032	28.04	26.7	27.53	27.43	0.68	2.479
0.16	24.71	24.88	24.71	24.77	0.1	0.403
0.8	22.6	22.84	22.82	22.75	0.13	0.571
4	20.13	20.16	20.09	20.12	0.04	0.198
20	18.24	18.31	18.42	18.32	0.09	0.491
Primer pair F14/R10						
0.0064	32.08	31.34	32.05	31.82	0.42	1.319
0.032	30.42	29	29.37	29.6	0.74	2.5
0.16	27.71	27.34	27.71	27.59	0.21	0.761
0.8	25.21	25.09	25.03	25.11	0.09	0.358
4	22.74	22.7	22.51	22.65	0.12	0.529
20	20.76	20.76	20.74	20.75	0.01	0.048

^aStandard deviation. ^bCoefficient of variation.

tion of the expected region on the templates. The conventional PCR products amplified with the primers pair *F2/MMR*, *F2/R10* and *F14/R10* and the RT-real time PCR product amplified with the primers *F2/R10* were sequenced. Using MEGA software, we aligned the sequences with the Reference sequence (Plasmid PB520), the alignment showed a complete homology with the sequence on which they were designed (data not shown). The alignment analysis indicated that the isolated fragment came from the transgenic construct.

DISCUSSION

Genetic engineering makes it feasible to transform drought resistance genes into plants; however a series of experiments are needed to confirm whether the genes

are successfully transformed and the target genes are expressed. The detection of transgenic plants in most crop species requires the use of selectable marker genes and selective agent. Selectable marker genes are introduced into plant genome to express a protein generally with an enzymatic activity, which allows distinguishing transformed from non-transformed cells, however, the detection of the transgene of interest and the study of mRNA transcribed from the introduced DNA provides far more information than a screening method. A transgene incorporated into the plant genome is integrated randomly and in unpredictable copy numbers, often in the form of repeats, which can influence its expression. The integration site also has a profound effect on the expression of the transgene, which is affected by intrinsic and extrinsic factors that can trigger methylation and reduce the stability of the expression

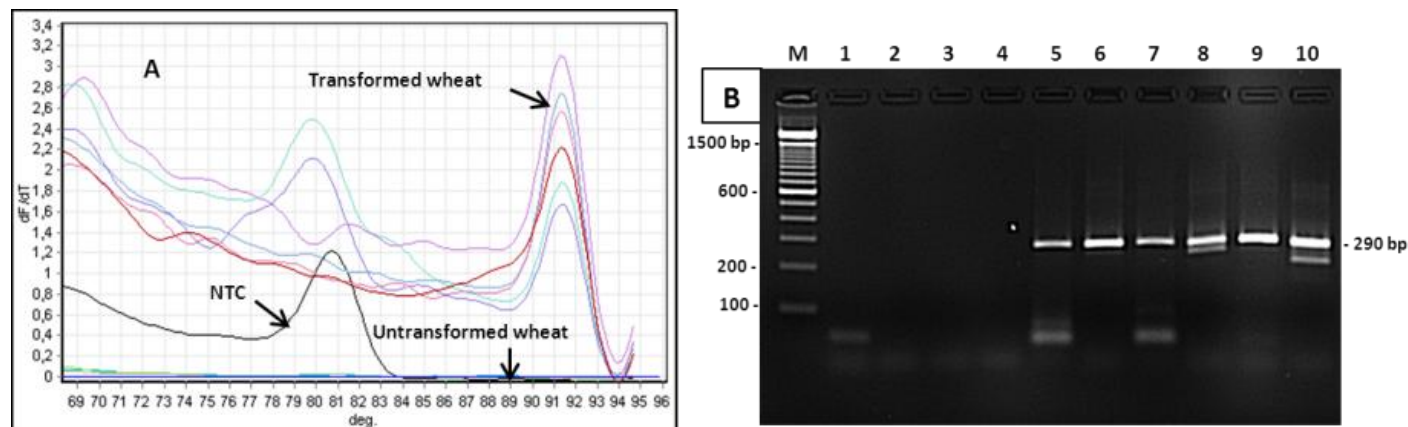


Figure 6. Real time RT-PCR to detect *HVA1* mRNA transcript. (A) Post-amplification melting curves of total *HVA1* RNA extraction from transgenic wheat obtained by real-time PCR using primer pair *F2/R10* and (B) confirmation of amplification by electrophoretic analysis of the real-time PCR products. Arrows show specific amplification at 92 °C. NTC: no template control; Lane M: DNA marker 100 bp; lane 1: water control; lanes 2 to 4: non-transformed wheat; lanes 5 to 10: transformed wheat.

(Kooter et al., 1999). Silencing of introduced genes is routinely observed in polyploid plant species such as wheat. Such transgene silencing has been observed in transgenic wheat plants generated by microprojectile bombardment (Alvarez et al., 2000). There have been many reports about loss of transgene expression in subsequent generations (Brandle et al., 1995) and under certain environmental conditions (Meyer et al., 1992). Thus initial laboratory testing supplemented by extensive analyses of gene expression of the transgene are important. In some cases, the detection and the study of gene expression of a transgene is difficult due to sequence homology between the transgene and the endogenous genes. Because the wheat genome contains homologues of the barley *HVA1* gene (Curry et al., 1991), the detection method of *HVA1* transgene in the previous works, was only based on southern blot analysis using a probe that target a promoter (Sivamani et al., 2000), or a marker gene probe (Debasis and Paramjit, 2003).

The aim of this work has been to develop PCR primers to detect the introduced DNA from a durum wheat line transformed to express a barley gene (*HVA1*). This work was designed to investigate also the ability to detect the mRNA of the transgene in transformed plant samples by real time PCR. The polymerase chain reaction is a highly specific and sensitive method for the detection of small amounts of target DNA. In the past few years, several PCR methods have been developed to detect GMOs for screening purposes including the detection of specific genes of interest (Zimmermann et al., 1998). Primers for PCR were designed to avoid the amplification of genomic DNA with 90% identity in sequence to the corresponding cDNA sequence of the transgene. Information available was used to set up a PCR based detection system. We used several primer combinations in this study to detect the *HVA1* transgene. Primer pairs *F2/R1*, *F2/R4* and *F2/R5* were not able to distinguish the transgene from the

endogenous gene, whereas primer pairs *F2/MMR*, *F2/R10* and *F14/R10* were successful to amplify a 300, 290 and 212 bp fragments respectively from transgenic DNA, and no fragment was amplified from non-transgenic samples. The primers were specific to amplify the expected band sizes from DNA template. Identification of assay conditions that yield a single *HVA1* transgene fragment without any amplification of non-transformed samples yields the potential of adaptation of our assay to real time quantification. Real-time quantitative PCR is considered to be an easy-to-use, accurate, specific, quantitative method. We amplify the *HVA1* gene by the primers pair *F2/MMR*, *F2/R10* and *F14/R10*. The specificity of the primers was confirmed by the absence of any fluorescence signal with the untransformed plants, and did not show any cross-reactivity with DNAs extracted from various plants. Analysis of the dissociation curves in real time PCR showed a specific amplification of target gene for all the primers. The assessment of the primers sensitivity, efficiency and reproducibility of the tests was determined by generating a standard curves. The analysis showed an acceptable efficiency. Each assay was 100% specific, reproducible and consistently detected DNA concentrations ≤ 7 pg.

Among the three primers, the primer pair *F2/R10* clearly was the highly specific primers as no fluorescence corresponding to non-transformed samples or primer dimers was detected. This specificity is basically attributable to specific primer pair designed to amplify the *HVA1* gene. We have concluded that the primer pair *F2/R10* can reliably be defined highly specific for amplification of *HVA1* transgene in wheat. Since the *HVA1* transgene is under the control of the Actin promoter, the transgene is expected to be constitutively expressed. We tested the primer pair *F2/R10*, for the amplification of *HVA1* mRNA in transgenic samples by real time PCR. Real-time RT-PCR is, at present, the most sensitive method for the

detection of low abundance mRNAs (Bustin, 2000). A major challenge was to detect *HVA1* transcript. After several experiments, we successfully amplified the mRNA of *HVA1* in transgenic samples, no fluorescence was observed in any of non-transgenic plants, the electrophoresis gel of the real time PCR product showed a clear bands corresponding to the expected size. The sequencing of PCR and real time RT-PCR products confirmed that the sequences corresponded to the target gene.

We described here an assay based on PCR and real time PCR assays for specific and sensitive detection of *HVA1* in transgenic durum wheat. The methods are reproducible since all experiments were repeated at least twice. We demonstrate in this study that *HVA1* transgene can be specifically amplified even in a complex genetic background containing homologous gene, facilitating the identification and study of transformed plants. Development of specific primers capable of distinguishing the transgene transcript from other homologous transcript is essential for the study of transgene transcriptional regulation. The successful detection of *HVA1* transcript will allow for the assessment of transgene transcription throughout plant development.

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REFERENCES

- Alvarez ML, Guelman S, Halford NG, Lustig S, Reggiardo MI, Ryabushkina N, Shewry P, Stein J, Vallejos RH (2000). Silencing of HMW glutenins in transgenic wheat expressing extra HMW subunits. *Theor. Appl. Genet.* 100(2):319-327.
- Bacsó R, Janda T, Galiba G, Papp I (2008). Restricted transpiration may not result in improved drought tolerance in a competitive environment for water. *Plant Sci.* 174(2):200-204.
- Bajaj S, Targolli J, Liu L-F, Ho T-H, Wu R (1999). Transgenic approaches to increase dehydration-stress tolerance in plants. *Mol. Breed.* 5(6):493-503.
- Boyer JS (1982). Plant productivity and environment. *Science (New York, N.Y.)*. 218(4571):443-448.
- Brandle JE, Mchugh SG, James L, Labbe H, Miki BL (1995). Instability of transgene expression in-field grown tobacco carrying the *csrl-1* gene for sulfonylurea herbicide resistance. *Bio-Technology* 13(9):994-998.
- Bustin SA (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* 25(2):169-193.
- Bustin SA, Benes V, Nolan T, Pfaffl MW (2005). Quantitative real-time RT-PCR--a perspective. *J. Mol. Endocrinol.* 34(3):597-601.
- Cankar K, Stebih D, Dreo T, Zel J, Gruden K (2006). Critical points of DNA quantification by real-time PCR-effects of DNA extraction method and sample matrix on quantification of genetically modified organisms. *BMC Biotechnol.* 6:37.
- Chandra Babu R, Zhang J, Blum A, David Ho TH, Wu R, Nguyen HT (2004). *HVA1*, a LEA gene from barley confers dehydration tolerance in transgenic rice (*Oryza sativa* L.) via cell membrane protection. *Plant Sci.* 166(4):855-862.
- Curry J, Morris CF, Walkersimmons MK (1991). Sequence-Analysis of a Cdna-Encoding a Group-3 Lea Messenger-Rna Inducible by Aba or Dehydration Stress in Wheat. *Plant Mol. Biol.* 16(6):1073-1076.
- Darbani B, Eimanifar A, Stewart CN Jr., Camargo WN (2007). Methods to produce marker-free transgenic plants. *Biotechnol. J.* 2(1):83-90.
- Debasis P, Paramjit K (2003). Genetic transformation of Indian bread (*T. aestivum*) and pasta (*T. durum*) wheat by particle bombardment of mature embryo-derived calli. *BMC Plant Biol.* 3(1):5-5.
- Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Duran C, Field M, Heled J, Kearse M, Markowitz S, Moir R, Stones-Havas S, Sturrock S, Thierer TAW (2011). Geneious v5.4, Available from <http://www.geneious.com/>.
- Gimenez MJ, Piston F, Atienza SG (2011). Identification of suitable reference genes for normalization of qPCR data in comparative transcriptomics analyses in the Triticeae. *Planta* 233(1):163-173.
- Goodwin JL, Pastori GM, Davey MR, Jones HD (2005). Selectable markers: antibiotic and herbicide resistance. *Methods Mol. Boil. (Clifton, N.J.)*. 286:191-202.
- Griffiths K, Partis L, Croan D, Wang MN, Emslie DKR (2002). Review of technologies for detecting Genetically modified materials in commodities and food. Department of Agriculture, Fisheries and Forestry - Australia. 1-126.
- Karrou M (2003). Conduite du blé au Maroc (behavior of wheat in Morocco). National Institute of Agricultural Research (INRA Editions), Rabat, Maroc. 57.
- Kooter JM, Matzke MA, Meyer P (1999). Listening to the silent genes: transgene silencing, gene regulation and pathogen control. *Trends Plant Sci.* 4(9):340-347.
- Lal S, Gulyani V, Khurana P (2008). Overexpression of *HVA1* gene from barley generates tolerance to salinity and water stress in transgenic mulberry (*Morus indica*). *Transgenic Res.* 17(4):651-663.
- MAPM (2011). Situation de l'Agriculture Marocaine (Agriculture situation in Morocco), Ministry of Agriculture and Fisheries, Rabat, Morocco. 204.
- Maqbool B, Zhong H, El-Maghraby Y, Ahmad A, Chai B, Wang W, Sabzikar R, Sticklen B (2002). Competence of oat (*Avena sativa* L.) shoot apical meristems for integrative transformation, inherited expression, and osmotic tolerance of transgenic lines containing *hva1*. *TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik* 105(2-3):201-208.
- Mardeh ASS, Ahmadi A, Poustini K, Mohammadi V (2006). Evaluation of drought resistance indices under various environmental conditions. *Field Crop Res.* 98(2-3):222-229.
- Matzke MA, Mette MF, Matzke AJ (2000). Transgene silencing by the host genome defense: implications for the evolution of epigenetic control mechanisms in plants and vertebrates. *Plant Mol. Biol.* 43(2-3):401-415.
- Meyer P, Linn F, Heidmann I, Meyer H, Niedenhof I, Saedler H (1992). Endogenous and Environmental-Factors Influence 35s Promoter Methylation of a Maize A1 Gene Construct in Transgenic Petunia and Its Color Phenotype. *Mol. Gen. Genet.* 231(3):345-352.
- Murray MG, Thompson WF (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 8(19):4321-4325.
- Oraby HF, Ransom CB, Kravchenko AN, Sticklen MB (2005). Barley Gene Confers Salt Tolerance in R3 Transgenic Oat. *Crop Sci.* 45(6):2218.
- Pietsch K, Wailblinger HU, Brodmann P, Wurz A (1997). Screeningverfahren zur Identifizierung gentechnisch veränderter pflanzlicher Lebensmittel. 93:2.
- Rodriguez M, Canales E, Borroto CJ, Carmona E, Lopez J, Pujol M, Borrás-Hidalgo O (2006). Identification of genes induced upon water-deficit stress in a drought-tolerant rice cultivar. *J. Plant Physiol.* 163(5):577-584.
- Rohila JS, Jain RK, Wu R (2002). Genetic improvement of Basmati rice for salt and drought tolerance by regulated expression of a barley *Hva1* cDNA. *Plant Sci.* 163(3):525-532.
- Shinozaki K, Yamaguchi-Shinozaki K (2007). Gene networks involved in drought stress response and tolerance. *J. Exp. Bot.* 58(2):221-227.

- Sivamani E, Bahieldin A, Wraith JM, Al-Niemi T, Dyer WE, Ho TD, Qu R (2000). Improved biomass productivity and water use efficiency under water deficit conditions in transgenic wheat constitutively expressing the barley HVA1 gene. *Plant Sci.* 155(1):1-9.
- Smith N, Kilpatrick JB, Whitelam GC (2001). Superfluous Transgene Integration in Plants. *Crit. Rev. Plant Sci.* 20(3):215-249.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28(10):2731-2739.
- Tripathi L (2005). Techniques for detecting genetically modified crops and products. *Afr. J. Biotechnol.* 4(13):1472-1479.
- Wang WX, Vinocur B, Shoseyov O, Altman (2001). Biotechnology of plant osmotic stress tolerance: physiological and molecular considerations. *Acta Hortic.* 560:285-292.
- Wong ML, Medrano JF (2005). Real-time PCR for mRNA quantitation. *BioTechniques* 39(1):75-85.
- Xu D, Duan X, Wang B, Hong B, Ho T, Wu R (1996). Expression of a Late Embryogenesis Abundant Protein Gene, HVA1, from Barley Confers Tolerance to Water Deficit and Salt Stress in Transgenic Rice. *Plant Physiol.* 110(1):249-257.
- Yamaguchi Shinozaki K, Kasuga M, Liu Q, Nakashima K, Sakuma Y, Abe H (2002). Biological mechanisms of drought stress response. *JIRCAS Working Rep.* 23(1341-710X):1-8.
- Zimmermann A, Hemmer W, Liniger M, uuml, thy J, Pauli U (1998). A Sensitive Detection Method For Genetically Modified MaisGard™ Corn Using a Nested PCR-system. *Lebensmittel-Wissenschaft und-Technologie* 31(7/8):664-667.

Full Length Research Paper

Age-dependent prevalence of *Loa loa* amicrofilaremia and microfilaremia status as defined by two markers: microfilaria and specific IgG4

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Loiasis infection is characterised by long term stability in infection status. The bases of such stability are not well known. As preliminary step toward verification of possible genetic involvement in this stability, a survey in a homogeneous population (n = 106) of a village from an endemic zone of Gabon was undertaken. The distribution of *Loa loa* microfilaremia according to age revealed a significant relationship between age and the presence of microfilariae in the blood (p = 0.0059). The proportion of microfilaremic individuals increased with age until 45 years old, and did not exceed 34% as its maximum. The other marker (specific IgG4) increased also significantly with age (p = 0.0038), but in contrast to microfilaremia, the prevalence of specific IgG4 in the group from 45 years onward reached 100%. These observations show the importance of age for the definition of the amicrofilaremic or microfilaremic individual status in an endemic area and are in agreement with the hypothesis suggesting the existence of genetic factors controlling the outcome of the parasitological status in *L. loa* infection.

Key words: *Loa loa*, prevalence, age, IgG4, genetic.

INTRODUCTION

Loiasis is caused by the human filarial *Loa loa* which is endemic in the west and Central African forest block. An estimated 10 millions individuals are thought to harbour the parasite (Sasa, 1976; Akue et al., 2011; Zouré et al., 2011) and in some regions, the loiasis is the second cause of hospital consultation after malaria (Boulestix and Carme, 1986). Several clinical symptoms varying from mild to severe have been documented in *L. loa* infection (Nutman et al., 1988; Klion et al., 1991; Akue, 2011). The treatment available (diethylcarbamazine

citrate and Ivermectine) are mostly active on circulating microfilariae (Richard-Lenoble et al., 1988). Vector control is an unrealistic approach.

Therefore, a better understanding of this infection in human population from endemic area is required in order to elaborate new strategy for its control. Epidemiological studies have shown the diversity of infection status and the microfilaraemic status was used for long time to define loiasis (Richard-Lenoble, 1980). However, it is admitted today that some are infected without any

microfilariae detectable in their blood by microscopic technique even after blood concentration exist (Dupont et al., 1988). These individuals are designated as amicrofilaraemic or occult infected. A good characterisation of these occult infected is principal for the understanding of mechanisms which control the infection. Besides the microscopic technique and the ocular passage of adult worms, new methods such as detection of *Loa loa* specific IgG4 (Akue et al., 1994) are now available. Furthermore, by using some of these methods, it appears clearly that occult infected individuals are more prevalent than microfilaraemic ones (Akue et al., 1996). Interestingly, different studies have shown the stability within the space concerning the prevalence of microfilariae carriers which never exceed one third of the Bantu population in any endemic country (Pinder et al., 1988). In contrast, Pampiglione et al. (1979) have shown up to 88% of microfilariae carriers in Bambutis Pygmy of Noth Zaire. There is not only stability in time for infection status but also for the density of parasite in the blood (Garcia et al., 1995; Akue et al., 1996). The existence of different parasitological status temporally stable suggests that some individuals once infected are capable of controlling durably their microfilaraemia (occult infected); in contrast to the others (microfilaraemic), remain susceptible for life span.

The phenomenon can be under control of some genetic factors as it was noticed in other parasitic infections (Wakelin and Blackwell, 1988; Bouchery et al., 2012). This study was carried out in the light of new tools for diagnosis to understand whether the absence of microfilaria is just time dependent or a genetic character for some people.

MATERIALS AND METHODS

Study population

The survey was carried out in the village of Dienga. A field station of Centre International de Recherches Medicales de Franceville (CIRMF) to study infectious diseases. Dienga is situated in the south-east of Gabon, Ogooue Lolo province, near the Congolese border. The climate is of equatorial type with the vegetation of savannah. Habitations are grouped along the main road. The population was recently evaluated at 1050 individuals. A proportional random sample was obtained within the population aged over five years which was divided into "stratums" according to age and sex. This random sampling method ensures the representativity of the results, and the structure of the sample to reflect the one in the general population from which the sample was taken. It also allows having sample from individuals of all age, and all level of exposure to infective bites of chrysops vector. Stratums were designed as follow: 1) Less than 6 years, very protected in general. 2) From 6 to 15 years old, spending most of their time in the villages and most often are school children. 3) From 16 to 45 years old, active population, farmers in general. 4) More than 45 years, less active, but sometimes participating during harvesting time.

Each of those individuals had 5 ml of blood taken once, after their agreement, and treated if found positive. Blood was taken in tube containing EDTA and kept at 4°C for a maximum of 72 h at Dienga before it was taken to CIRMF laboratory where 1.1 ml was used

for microscopic examination for the presence of microfilariae. The remaining blood samples were centrifuged and the plasma kept at -20°C.

Examination for the presence of microfilariae in the blood

A wet preparation of 10 µl of uncoagulated blood plus a drop of saponine was systematically examined under microscope. In addition, a concentration technique was performed according to Akue et al. (1996). Briefly, in a conical 15 ml tube, the following mix was made: 1 ml of blood, 200 µl of saponine and 9 ml of phosphate buffered saline (PBS). This mixture was incubated at room temperature for 5 min, then centrifuge for 10 min at 500 g. The whole pellet was examined under microscope. Microfilariae (mf) were counted and the total obtained was expressed as mf/ml. The identification of microfilariae species was based on their size, mobility, the presence or the absence of sheath.

Diagnosis of *L. loa* infection by specific IgG4 detection

This was based on an enzyme linked immunosorbent assay (ELISA) using adult worm *L. loa* antigen as describe elsewhere (Akue et al., 1994). Briefly, adult worms removed from eye of patient during their ocular migration were washed and homogenised in Tris-HCl 10 mM buffer plus protease inhibitors and antigen extracted with 1% sodium deoxycholate. The ELISA was performed with 10 µg/ml of antigen and ant-human IgG4 (HP 6011) diluted at 1/30000 as described. Individuals with a mean optical density (OD) higher than the mean OD of African outside endemic zone of *L. loa* (Gambie, Mali) plus one standard deviation of this mean were considered positive.

Statistical analysis

The analysis were performed using Epi info 6 program, Chi square test was used for the comparison between group and Yates correction was performed when necessary. Fischer exact test was used for comparison of prevalence of microfilariae between age group, and for seroprevalence result in men and women. $P \leq 0.05$ was considered significant.

RESULTS

Characteristic of the study population

The individuals are Bantu from Nzebi ethnic group, farmers in general with women mostly involved in agriculture. Most of the children attended a school which is situated in the centre of the village. Only people who agreed were enrolled. Finally, the study population was formed by 106 persons from both sexes with age varying from 6 to more than 45 years old. Sex ratio (women/men = 1.25) with mean age of 26 is shown in Table 1.

Prevalence of microfilaraemia

Among the 106 individuals examined, 13 had microfilariae of *L. loa* (12.26%), 95% CI (confidence interval): 6.18-18.36. The microfilaraemia vary from 3 to 7475 mf/ml. *Mansonella perstans* was also present but less

Table 1. Study population.

Age group (years)	Sex		Total
	Male (n)	Female (n)	
6-15	24	19	43
16-45	13	21	34
>45	9	20	29
Total	46	60	106

Table 2. Prevalence of microfilaraemia in different age groups.

Age group	Microfilaraemic individuals (n)	Number (N)	Percentage (%)
6-15	1	43	2.35
16-45	4	34	11.43
>45	8	29	26.66
Total	13	06	12.26

Table 3. Prevalence of specific IgG4 positive/negative individuals in different age group.

Age group	Specific IgG4+ n (%)	Specific IgG4- n (%)
6-15	24 (55.81)	19 (44.19)
16-45	27 (79.41)	7 (20.59)
>45	26 (100)	0 (0)
Total	77 (74)	29 (26)

prevalent. Only three individuals had microfilariae of *M. perstans*. The level of *M. perstans* microfilariae vary from 1 to 56 mf/ml. Only one individual had a double infection: *L. loa* /*M. perstans*. The prevalence of the *L. loa* microfilariae carriers according to age group (Table 2) revealed a significant difference among the three groups ($p = 0.0037$). However, when males and females were taken separately the age did not affect the risk of being microfilaraemic in females ($p = 0.235$) in contrast to males where age affect this risk ($p = 0.0015$). No significant difference was seen between male and female for the risk of being microfilaria carrier ($p = 0.134$). When study population was split into ten years group, it was shown that the proportion of microfilariae carriers increase until 45 years (Figure 1, micro-prevalence curve); at this age, the prevalence reached a maximum of 33.33%, then plateau and decreased at 65 years.

Prevalence of infection as detected by specific IgG4 test

Samples ($n = 103$) were analysed for the prevalence of specific IgG4 by ELISA. As shown in Table 3, it appears that 74.75% (95% CI: 65.55 - 82.47) of individuals were positive using this test. Again, there was a significant relationship between age and seroprevalence ($p = 0.0038$). The risk of being IgG4 positive increase with increasing age ($p = 0.00118$). When males and females

were analysed separately, a significant difference was seen among female ($p = 0.0052$) and male ($p = 0.042$). The sex was not a risk factor for loiasis infection according to the serological test ($p = 0.54$). Following the dynamic of specific IgG4 (Figure 1, sero prevalence curve), the seroprevalence was already high between 6 and 15 years age (55.26%), but continued to increase to reach 100% of individuals at 45 years and above. Specific IgG4 remained in plateau at that level until 85 years olds.

DISCUSSION

Although, previous studies did not agreement with this (Garcia et al., 1995; Noireau et al., 1989; Pampiglione et al., 1979), it is shown that age affects the prevalence of microfilaraemic in such a way that the percentage of microfilariae carriers increases and reaches a plateau at about 45 years with a maximum of 33.33% in this study. It seems likely that individuals potentially "susceptible microfilariae carriers" are recruited gradually until they all become infected.

The low proportion of microfilaraemics in the age group of 6-15 years is in agreement with this hypothesis. The latter is supported by the fact that: Firstly, exposition time of this young people has not been sufficient to facilitate biting by infected chrysops. Another explanation may be related to the prepatent period during which larvae are

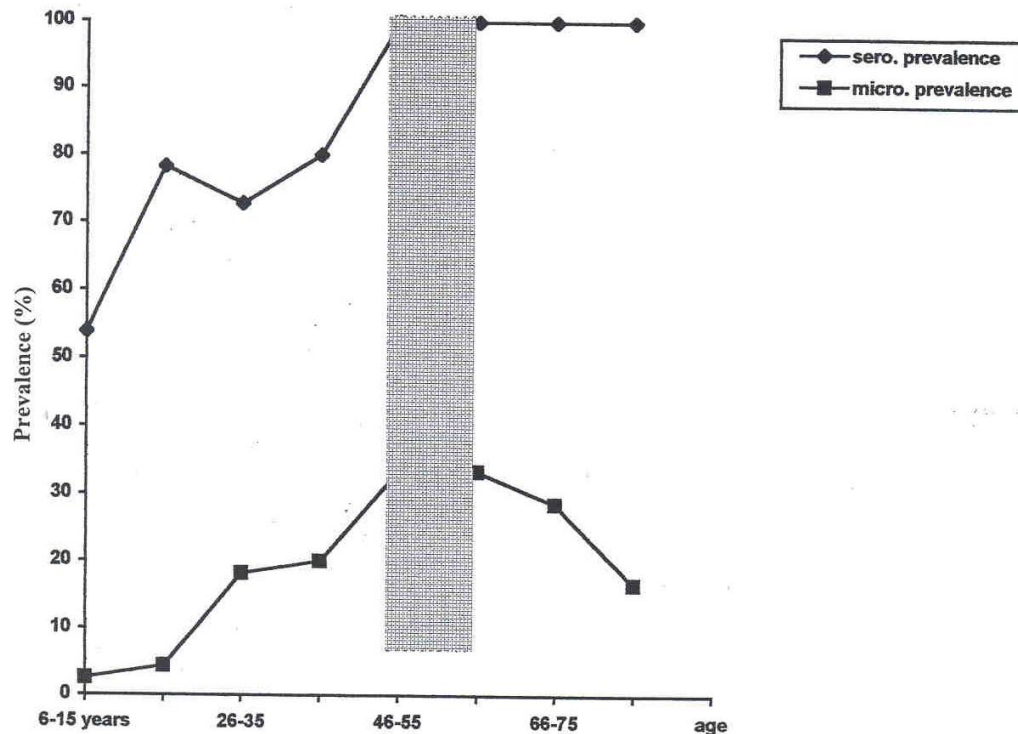


Figure 1. Definition of infection status according to age/microfilaremia/serology. Both microfilaria and specific IgG4 prevalence were plotted against age. The grey band show the probable age for acquisition of a definitive infection status in Dienga.

still immature to produce microfilariae. An alternative is the possible existence of single sex infection (Eveland et al., 1975). Interestingly, evolution of microfilariae carriers prevalence every ten years showed clear increase, then plateau followed by decrease at age 65. This plateau will not be surprising if we accepted the possible involvement of concomitant immunity which acts against establishment of new infection while the first infection remains (Rajakumar et al., 2006; Specht et al., 2011).

At 65 years, the decline of microfilariae carriers may be concomitant to the natural disappearance of infection acquired in younger age and to the low level of exposure as majority of the individuals over 45 years old age group stay indoor. This is substantiated by the evolution of the mean level of microfilariae density which is low in the younger age class (400 mf/ml) then increases for individuals aged between 16 and 45 (3006 mf/ml) and decreases for individuals over 45 years (627 mf/ml). Although no statistical significant difference was found when comparing means level of microfilariae density between age groups, similar trend was found in south-Cameroon (Garcia et al., 1995). When the prevalence of microfilaraemics is examined according to sex, it appears that relationship between sex and existence of microfilaraemia was significant only in males and not in females. Ripert et al. (1977) also mentioned this fact. Although certain studies have found a link between prevalence of

microfilariae carriers and sex (Van Hoegarden et al., 1987; Noireau et al., 1989), our survey did not completely support these findings, but the small size of the sample in the group with defined status (45 years and over) as well as the level of exposure to infective larvae and a previous chemotherapy may affect the interpretation of this result.

Age is therefore a non-negligible factor in the definition of infection status. Thus, only individuals who have been in contact with infective larvae and without circulating microfilariae will be considered as amicrofilaremic. However, evidence to show that an individual has been in contact with L3 larvae is not clearly defined in natural conditions. It becomes necessary to define a "cut-off age" beyond which the infection status of an individual will be finally determined.

When loiasis infection was defined using specific IgG4 serology, sex did not affect the parasitological status. The curve of distribution of specific IgG4 test increases gradually and reaches a plateau at about 45 years. This plateau remains stable. Interestingly, the age of the beginning of this plateau is the same as the one determined by microfilaremia (Figure 1, grey vertical area). Thus individuals in the group of "good responders" that is, individuals capable of controlling their microfilaraemia will not be identified in another way other than taking into account the notion of "cut-off age".

These results suggest the fact that in a given popula-

tion, the minority have a predisposition of being microfilaric. These individuals will become micro-filariae positive in continual conditions of transmission before a certain age. This age is probably under influence of transmission intensity and the degree of exposure to infective bite. In the region of Chaillu mountains of Congo, for example, the beginning of pla-teau in the curve showing the prevalence of microfilariae according to age, has been found at 20 years old (Noireau et al., 1989).

In South-Cameroon, it is about 40-50 years old (Garcia et al., 1995). The majority of individuals seem to behave as "good responders". Meaning that their defence system, probably immunological, has capability of controlling microfilariae level. These individuals may also be infected before the "cut of age" In the case of Dienga, it is likely that 33% of the population may be predisposed to the microfilaraemic status but this will be apparent only after a long experience of 45 years. Therefore, during the follow up of a group, about 33% of individuals will probably be microfilariae carriers at 45 years old and the whole population infected before this age if there is transmission remains identical through out the follow-up time.

It has been shown that transmission intensity does not affect the prevalence of microfilaremia and the density of microfilaria (Akue et al., 2002). Similarly, the prevalence of occult infection or amicrofilaremic does not depend on transmission intensity. This suggests that it is only after a sufficient length of exposure that the real status (micro-filaric or amicrofilaremic) of an individual will be revealed. It is likely that the transmission will only either accelerate (High transmission) or decelerate (low transmission) the outcome. This need to be taken into account while the objective of the study is to search for genetic or protective mechanisms involved in the control of *L. loa* microfilaria in a given population. However, the fact that microfilaremia did not exceed 34% while the serological test shows that 100% of individuals have been infected, suggests the existence of genetic factor which limit microfilaria appearance in only some individuals (Bouchery et al., 2012).

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REFERENCES

- Akue JP, Devaney E, Wahl G, Moukana H (2002). Expression of filarial-specific IgG subclass under different transmission intensities in region endemic for loiasis. *Am. J. Trop. Med. Hyg.* 66:245-250.
- Akue JP, Egwang T G, Devaney E (1994). High levels of parasite-specific IgG4 in the absence of microfilaremia in *Loa loa* infection. *Trop. Med. Parasitol.* 45:246-248.
- Akue JP, Hommel, M. Devaney E (1996). Markers of *Loa loa* infection in permanent residents of loiasis endemic area of Gabon. *Trans. R. Soc. Trop. Med. Hyg.* 90:115-118.
- Akue JP, Nkoghe Dieudonné, Padilla cindy, Moussavou Ghislain, Moukana Hubert, Mbou Moutsimbi Roger Antoine, Ollomo Benjamin, Leroy Eric Maurice (2011). Epidemiology of concomitant infection due to *Loa loa* and *Mansonella perstans* in Gabon. *PLoS Negl Trop Dis* 5(10): e1329 doi: 10. 1371/ journal.pntd. 0001329
- Bouchery T., Gaelle Dénécé, Tarik Attout, Katharina Ehrhardt, Nathaly Lhermitte- Vallarino, Muriel Hachet-Haas, Jean Luc Galzi, Emilie Brotin, Françoise Bachelerie, Laurent Gavotte, Catherine Moulia, Odile Bain, Coralie Martin (2012). The chemokine CXCL12 is essential for the clearance of the filarial *Litomosoides sigmodontis* in resistant mice. *PLoS ONE* 7(4): e 34971. doi: 10.1371/ journal.pone.0034971
- Boulestix G, Carme B, (1986). Encephalite au cours du traitement de la filariose par la diethylcarbazine. A propos de 6 observations. *Bull. Soc. Pathol. Exotique* 79:649-654.
- Dupont A, Zué-Ndong J, Pinder M (1988). Common occurrence of amicrofilaremic *Loa loa* filariasis within the endemic region. *Trans. Roy. Soc. Trop. Med. Hyg.* 82: 730.
- Eveland L K, Yermakov V, Kenney M (1975). *Loa loa* infections without microfilaremia. *Trans. Roy. Soc. Trop. Med. Hyg.* 69: 354-355.
- Garcia A, Abel L, Cot M, Stephan Ranque, Philippe Richard, Michel Boussinesq, Jean Philippe Chippaux (1995). Longitudinal survey of *Loa loa* filariasis in Southern Cameroon: Long term stability and factors influencing individual microfilarial status. *Am. J. Trop. Med. Hyg.* 4: 370-375.
- Klion CL, Massougbodji A, Sadeler B C, Ottesen E A, Nutman TB (1991). Loiasis in endemic and non endemic populations: Immunologically mediated differences in clinical presentation. *J. Infect. Dis.* 163:1318-1328.
- Noireau F, Carme B, Ampembet JD, Gouteux J P (1989). *Loa loa* and *Mansonella perstans* filariasis in the Chaillu mountains, Congo : Parasitological prevalence. *Trans. Roy. Soc. Trop. Med. Hyg.* 83:529-534.
- Nutman TB, Reese W, Poindexter RW, Ottesen EA (1988). Immunologic correlates to the hyperresponsive syndrome of loiasis. *J. Infect. Dis.* 157:144-550.
- Pampiglione S, Najera E, Ricciadi M L, Junginger L (1979). Parasitological survey on pygmies in Central Africa III. Bambuti group (Zaire). *Rivista di Parasitologia.* 40: 187-234..
- Pinder M (1988). *Loa loa* a neglected filarial. *Parasitology Today.* 4 : 279-284.
- Rajakumar Sethu, Wilfrid Bleiss, Susanne Hartmann, Peter Schierack, Anorte Marko, Richard Lucius (2006). Concomitant immunity in a rodent model of filariasis: the infection of *Meriones unguiculatus* with *Acanthocheilonema vitae* J. *Parasitol.* 92(1): 41- 45
- Richard-Lenoble D, Kombila M Y, Rupp E A, Pappayliou E S, Gaxotte P, Nguri C, Aziz M H (1988). Ivermectin in loiasis and concomitant *O. volvulus* and *M. perstans* infections. *Am. J. Trop. Med. Hyg.* 39: 480-483.
- Richard-Lenoble D, Kombila M, Carme B., Gilles J C, Delattre PY (1980). Prevalence des filarioses Humaines sanguinolées au Gabon. *Bull. Soc. Pathol. Exotique.* 73 :192-199.
- Rippert C, Ambroise-Thomas P, Riedel D, Rousselle-Sauer C, Zimflou A, Ibrahim H (1977). Epidemiologie des filarioses à *Loa loa* et *D. perstans* dans sept villages de la province du Centre- sud du Cameroun. *Bull. Soc. Pathol. Exotique.* 7: 619-626.
- Sasa M (1976). Human filariasis. University Park Press, 122-152.
- Specht S, Frank JK, Alferink J, Dubben B, Layland LE, Denece G, Bain Odile, Först I, Kirschning CJ, Martin C, Hoerauf A (2011). CCL17 controls mast cells for the defense against filarial larval entry. *J. Immunol.* 186(8):4845-4852
- Wakelin D M, Blackwell J M (1988). Genetics of resistance to bacterial and parasite infections. Taylor and Francis. London.
- Zouré HG, Wanji S, Noma M, Amazigo UV, Diggle PJ, Tekle AH, Remme JH (2011). The Geographic Distribution of *Loa loa* in Africa: Results of Large-Scale Implementation of the Rapid Assessment Procedure for Loiasis (RAPLOA). *PLoS Negl. Trop. Dis* 5(6): e1210. doi:10.1371/ journal. Pntd.0001210.

Full Length Research Paper

Phytochemical and antimicrobial study on the leaf extracts of *Erythrophleum africanum* (Caesalpinaceae)

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The leaf of *Erythrophleum africanum* was exhaustively extracted with ethanol using cold maceration techniques. This was subsequently partitioned with petroleum ether, chloroform, ethylacetate and n-butanol. The agar diffusion method was used to determine the antimicrobial activity against the following micro-organisms *ethicillin resistant Staphylococcus*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Proteus vulgaris*, *Candida albicans*, *Candida krusei* and *Candida tropicalis*. Minimum Inhibitory Concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined. The *in vitro* antimicrobial screening revealed that the extracts exhibited diverse activities against different microbes with zones of inhibition ranging from 12 to 36 mm, MIC ranging from 3.25 to 60 mg/ml and MBC/MFC of 3.25 to 60 mg/ml for sensitive organisms at the tested concentrations. The activities observed could be attributed to the presence of terpenoids, saponins, flavonoids, alkaloids and tannins. The results justify the ethnomedicinal use of this plant in the treatment of sores, boils, wounds, dysentery, diarrhea and sexually transmitted infections.

Key word: *Erythrophleum africanum*, phytochemistry, antimicrobial activity.

INTRODUCTION

Despite the tremendous progress in human medicine, infectious diseases caused by bacteria, fungi, virus and parasites are still a major threat to public health. Their impact is particularly large in developing countries due to relative unavailability of medicine and emergence of drug resistance (Zampini et al., 2009). During the last two decades, the development of drug resistance as well as the appearance of undesirable side effects of certain antibiotics (Okemo et al., 2003), has led to the search for new biologically active compounds from natural sources as new antimicrobial agent with the view to discover new chemical structures, which could overcome the above disadvantages (Bouamama et al., 2006; Meenakshi et al.,

2001). The development of resistance to current antibiotics by disease causing microbes has also reinforced research for discovery of new ones. Current trends in drug development process are focused on natural sources, especially of plant origin due to some proven correlation between the folkloric medicinal uses of some of these plants to biological activity. Therefore, the use of plant materials to prevent and treat infectious diseases successfully over the years has continued to attract the attention of scientists worldwide (Osawa et al., 1990; Kunle and Egharevba, 2009; Sophon et al., 2002; Begum et al., 2002).

Erythrophleum africanum (African black wood), is an

endangered African medicinal plant. Studies have revealed that, it is extremely toxic to livestock all over the world especially to goats, sheep and cows. Several species which include *E. quineense*, *E. invoreense*, *E. lasicanthum* and *E. chlorostachys* are known to be poisonous (Watt and Breyer Brandwijk, 1963; Dalziel et al., 1959; Griffin et al., 1971). This plant is reputed for its uses as an ordeal poison for executing capital punishment for witches and also to kill or scare away stubborn pest from cultivated farm land (Dalma, 1970; Mattocks, 1987; Loder et al., 1974).

The leaf of *E. africanum* is used in the treatment of various ailments which include emetics, as an anti-inflammatory agent, as an analgesic and also in sore and wound dressing. It is also used to treat chicken pox and gangrenous sores. The leaf decoction of this plant is well known by the traditional healers in Congo, (Democratic Republic of Congo), Zaire, Eastern province of Cameroon and India who used it empirically for several ailments including cardiovascular disease, various inflammation, diabetes, simple goiter, dysentery, diarrhea and as an astringent (Dalziel, 1999). This plant is reported to contain flavonoids and anthocyanidins and as such was used as a tooth pick for oral hygiene (Nwude and Chineme, 1981; Watt and Bayer Bradwyle, 1962; Burkill, 1995). Some alkaloids (pyrolizine alkaloids, PAS) from the leaf of this plant have been implicated to be gastrointestinal tract irritants, cholinesterase inhibitors and also affect the nervous system by causing drowsiness, salivation, labored breathing, trembling, loss of consciousness, coma and death due to paralysis (Roberts and Wink, 1998; Ahmad et al., 1994).

In the northern part of Nigeria (Africa), Gwaska as it is called by the Hausas, the leaf decoction with natron is taken for the treatment of sexually transmitted disease, as an abortifacient agent and is also used in the treatment of leprosy (Jinju, 1990). The aqueous leaf extract of this plant is also used to cure cancer of the blood and mentally related sickness (Watt and Breyer Brandwijk, 1963; Dalziel, 1999; Jinju, 1990). Consequently, we decided to screen the leaf part of this plant with a view to validate the folkloric claim.

MATERIALS AND METHODS

Plant materials

Fresh plant material *E. africanum* was obtained from Nimbia forest in Sanga Local Government Area of Kaduna State, Nigeria. The plant was identified at the Herbarium Biological Science Department, Ahmadu Bello University Zaria, Nigeria. Voucher specimen was kept with No. 1047.

Extraction

The dried powdered leaf (500 g) of *E. africanum* was macerated with 96% ethanol four times for 6 h each at room temperature. The alcoholic extract was concentrated *in vacuo* to afford (47.8 g). This

was suspended in water and then partitioned successively with petroleum ether (3 × 2 L), chloroform (4 × 2 L), ethylacetate (2 × 1.5 L) and n-butanol (5 × 2 L). The partitioned portion of the extracts were concentrated using rotary evaporator to afford petroleum ether (2.542 g), chloroform soluble (4.834 g), ethylacetate soluble (2.113 g), n-butanol soluble (6.811 g) and aqueous residue (4.201 g). These extracts were subjected to phytochemical screening (Table 1) using standard protocols (Sofowora, 2008; Trease and Evans, 2002; Brain and Tuner, 1975). All the extracts were stored at room temperature until required.

Test microorganisms

The test microorganisms were isolates obtained from the Medical Microbiology Department, Ahmadu Bello University Teaching Hospital, Zaria. These include *Mithicillin R. staph.*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Proteus vulgaris*, *Candida albicans*, *Candida krusei* and *Candida tropicalis*.

Antimicrobial assay

The minimum inhibitory concentration of the various leaf extracts against the test microorganisms were determined using broth dilution techniques (Vollokova et al., 2001; Sidney et al., 1978). Mueller-Hinton agar broth was prepared according to manufacturer's instruction using 10 ml of the broth to be dispensed into test tubes. The broth was sterilized at 121°C for 15 min after which the broth was allowed to cool. Test microbes were inoculated and incubated at 37°C for 6 h.

Mcfarland's turbidity scale number 0.5 was prepared to give a turbid suspension of the microorganisms. Dilution of the test microbes was done in normal saline until the turbidity matches with that of the Mcfarland's scale by visual comparison (concentration of about 1.5×10^8 cfu/ml). Two fold serial dilutions of the extract was made in the sterile broth to obtain the concentrations of 60, 30, 15, 7.5 and 3.25 mg/ml, respectively. The initial concentration was obtained by dissolving 0.6 g of the extract in the sterile broth. 0.1 ml of each test microbe in the normal saline was then inoculated into the different concentrations. After incubation at 37°C for 24 h, each test tube was observed for turbidity. The lowest concentration of the extract for which no turbidity was recorded was the minimum inhibition concentration (Osadebe and Okueze, 2004; Cowan, 1991).

MBC/MFC

The minimum bactericidal and fungicidal concentration of the extracts was determined (Table 4). Mueller-Hinton agar was prepared and sterilized at 121°C for 15 min poured into sterile petri dishes and allowed to cool and solidify. The content of the MIC in the serial dilution was then sub-cultured onto the medium. Incubation was made at 37°C for 24 h, after which the plates were observed for colony growth. The lowest concentrations of the extracts for which no microbial growth was observed were registered as the minimum bactericidal or minimum fungicidal concentration (Ogbulie et al., 2007).

RESULTS AND DISCUSSION

The sensitivity test or solvent used for the reconstitution of different extracts was carried out. It was observed that

Table 1. Phytochemical screening of the ethanolic leaf extract of *Erythrophleum africanum*.

Constituent	Test	Observation	Inference
Carbohydrate			
General test	Molisch	Red colour	+
Monosaccharide	Barfoed's	Red. ppt	+
Reducing sugar	Fehlings	Red. ppt	+
Tannins	Lead ethanoate	White ppt	+
	Methanol's	Red. ppt	+
	Iron (III) chloride	Blue-black	+
Saponins	Frothing	Persist frothing	+
Sterols	Liebermann, Burchad	Blue-green	+
	Salkowski	Red ring at interphase	+
Terpenoids	Liebermann, Burchard	Brown ring with brown interphase	+
Alkaloids	Dragendoff's	-	+
	Mayer's	-	-
	Wagner's	-	+
Flavonoids	Shinoda	-	+
	Ferric chloride	-	+
	Sulphuric acid	-	+
Cardiac glycoside	Keller-Kilanis	Reddish brown	+
	Legal's	Deep red colour	+

different concentrations of the extracts had different responses against the tested isolates. Increased concentrations of the extracts tend to increase the zone of growth inhibition of the sensitive micro-organisms (Table 3). The extract was found not to inhibit the growth of *S. feacalis*, *S. typhi* and *C. krusei*, respectively. The highest zone of growth inhibition of 36 mm diameter was exhibited by *S. aureus* while the lowest zone of growth inhibition was observed for *P. vulgaris* (12 mm diameter).

Results obtained in this study indicated that the various extracts of *E. africanum* inhibited the growth of some of the micro-organism except *S. feacalis*, *S. typhi* and *C. krusei*. This therefore shows that the extract contains substance(s) that can inhibit the growth of some micro-organisms. Other workers have also shown that the extracts of plants inhibited the growth of various micro-organisms at different concentrations (Akujobi et al., 2004; Nweze et al., 2004; Ntiejumokwu and Alemika, 1991; 2004; Osadebe and Ukwueze, 2004). The observed antimicrobial effects on the microorganism could be attributed to the presence of saponins, glycosides, flavonoids, tannins, alkaloids and terpenoids which have been shown to possess antimicrobial activities (Draughon, 2004; Cowan, 1999). The presence

of these metabolites suggests great potential for the use of the studied plant as a source of phytomedicines. The presence of alkaloids in plants is known for decreasing blood pressure and balancing the nervous system in case of mental illness. The presence of tannins could also show that it is an effective astringent, that is, helps in wound healing and as anti-parasitic. The presence of terpenes suggest its possible use as an anti-tumor and anti-viral agent as some terpenes are known to be cytotoxic to tumor cells, while the saponins in plants are believed to have antioxidant, anti cancer, anti-inflammatory and anti-viral agent (Ronan et al., 2009; Yoshida et al., 1990; Balas and Agarwal, 1980).

The large zone of inhibition exhibited by the plant extract against *Methicillin R. staph aureus*, *S. aureus*, *P. aeruginosa*, *E. coli* and *P. vulgaris* justified its use by the traditional medical practitioners in the treatment of sores, boils, open wounds, sexually transmitted diseases and in the treatment of dysentery (Table 2). *S. aureus* and *P. aeruginosa* have been implicated in cases of boils, sores and wounds (Braude et al., 1982). The MIC exhibited by the plant extract against *S. aureus* is of great significance in the health care delivery system, since it could be used as an alternative to antibiotics in the treatment of

Table 2. Zone of inhibition obtained from the microorganisms.

Test organism	n- hex (mm)	CHCl ₃ (mm)	EtoAc (mm)	n- BuOH (mm)	Aq (mm)
<i>Mithicilin R. Staph. aureus</i>	17	27	25	18	20
<i>Staphylococcus aureus</i>	18	25	24	36	21
<i>Streptococcus feacalis</i>	-	-	-	-	-
<i>Escherichia coli</i>	19	20	21	28	22
<i>Pseudomonas aeruginosa</i>	15	24	22	29	19
<i>Salmonella typhi</i>	-	-	-	-	-
<i>Proteus vulgaris</i>	12	25	22	25	18
<i>Candida albicans</i>	17	24	22	25	20
<i>Candida krusei</i>	-	-	-	-	-

n-hex, n-hexane; CHCl₃, chloroform; EtoAc, ethylacetate; n-BuOH, n-Butanol; Aq, aqueous.

Table 3. Minimum inhibitory concentration of the extracts against test micro organism.

Test-organism	n-Hexane (mg/ml)					CHCl ₃ (mg/ml)					EtoAc (mg/ml)					n-BuoH (mg/ml)					Aq (mg/ml)				
	60	30	15	7.5	3.25	60	30	15	7.5	3.25	60	30	15	7.5	3.25	60	30	15	7.5	3.25	60	30	15	7.5	3.25
<i>Mithicilin R. staph. aureus</i>	-	-	OX	+	++	-	-	-	OX	+	-	-	-	OX	+	-	-	OX	+	++	-	-	-	OX	+
<i>S. aureus</i>	-	-	OX	+	++	-	-	-	OX	+	-	-	-	OX	+	-	-	OX	+	++	-	OX	+	-	++
<i>S. feacalis</i>			Nil					Nil					Nil					Nil					Nil		
<i>P. aeruginosa</i>	-	-	OX	+	++	-	-	-	OX	+	-	-	-	OX	+	-	-	-	-	OX	-	-	-	OX	+
(-) <i>E. coli</i>	-	OX	+	++	+++	-	OX	+	++	+++	OX	-	+	++	+++	-	-	OX	+	++	-	OX	+	++	+++
<i>S. typhi</i>			Nil					Nil					Nil					Nil					Nil		
<i>P. vulgaris</i>	-	-	OX	+	++	-	-	-	OX	+	-	-	-	OX	+	-	-	OX	+	++	-	-	OX	+	++
<i>C. albicans</i>	-	-	OX	+	++	-	-	-	OX	+	OX	-	+	++	+++	-	-	OX	+	++	-	OX	+	++	+++
<i>C. krusei</i>			Nil					Nil					Nil					Nil					Nil		
<i>C. tropicalis</i>	-	-	OX	+	++	-	-	OX	+	++	-	-	OX	+	++	-	-	-	OX	+	-	OX	+	++	+++

n-hex, n-hexane; CHCl₃, chloroform; EtoAc, ethylacetate; n - BuOH, n-Butanol; Aq, aqueous; ox, MIC.

infections caused by this microbe, especially as it frequently develops resistance to known antibiotics (Singleton, 1999). Their use will also reduce the cost of obtaining health care delivery. The inability of the extract to inhibit *S. typhi* may be

due to a bacterial mechanism for detoxifying the active principle contained in the extract. Some bacteria are known to possess mechanisms by which they convert substances that inhibit their growth to non-toxic compounds (Singleton, 1999;

Srinivasan et al., 2001). Therefore, the observed antimicrobial properties of this plant *E. africanum* corroborate its use in folkloric medicine.

Traditionally, extract from this plant is used in sore and wound dressing, treatment of boils,

Table 4. Minimum bactericidal/fungicidal concentration of the extracts against test micro organisms.

Test-organism	n-Hexane (mg/ml)					CHCl ₃ (mg/ml)					EtoAc (mg/ml)					n-BuoH (mg/ml)					Aq (mg/ml)				
	60	30	15	7.5	3.25	60	30	15	7.5	3.25	60	30	15	7.5	3.25	60	30	15	7.5	3.25	60	30	15	7.5	3.25
<i>M. R. staph. aureus</i>	Ox	+	++	+++	++++	-	-	Ox	+	++	Ox	+	++	+++	++++	Ox	+	++	+++	+++	Ox	+	++	+++	++++
<i>S. aureus</i>	Ox	+	++	+++	++++	-	Ox	+			-	Ox	+	++	+++	Ox	+	++	+++	+++	-	Ox	+	++	+++
<i>S. faecalis</i>			NIL					Nil					Nil					Nil					Nil		
<i>P. aeruginosa</i>	Ox	+	++	+++	++++	Ox	+	++	+++	++++	Ox	+	++	+++	++++	-	Ox	+	++	+++	-	Ox	+	++	+++
(-) <i>E. coli</i>	Ox	+	++	+++	++++	Ox	+	++	+++	++++	Ox	+	++	+++	++++	Ox	+	++	+++	++++					
<i>S. typhi</i>			Nil					Nil					Nil					Nil					Nil		
<i>P. vulgaris</i>	Ox	+	++	+++	++++	-	Ox	+	++	+++	Ox	+	++	+++	++++	Ox	+	++	+++	++++	Ox	+	++	+++	++++
<i>C. albicans</i>	Ox	+	++	+++	++++	-	Ox	+	++	+++	Ox	+	++	+++	++++	Ox	+	++	+++	++++	Ox	+	++	+++	++++
<i>C. krusei</i>			Nil					Nil					Nil					Nil					Nil		
<i>C. tropicalis</i>	Ox	+	++	+++	++++	-	Ox	+	++	+++	Ox	+	++	+++	++++	Ox	+	++	+++	++++	Ox	+	++	+++	++++

n-hex, n-Hexane; CHCl₃, chloroform; EtoAc, ethylacetate; n - BuOH, n-Butanol; Aq, aqueous; Nil, absent; ox, MIC; -, no turbidity (no growth); +, turbid (light growth); ++, moderate turbidity, +++, high turbidity; +++++, extremely high turbidity.

sexually transmitted diseases, dysentery and also in the treatment of cancer.

Conclusion

The leaf extract of *E. africanum* provides a promising solution in the ethno medicine practice of disease control. This can hence be used to replace the synthetic antibiotics used in the treatment of related ailments as phytochemicals from natural resources are generally considered safer, available and affordable compared to the synthetic drugs in the treatments of infectious diseases.

REFERENCES

- Ahmad W, Ahmad Z, Najumul S, Kazim H, Malik A (1994). Pyrrolizidine alkaloid content of the genus *Senecio*. J. Chem. Society Pak. 16:64-81.
- Akujobi C, Anyawu BN, Onyeze C, Ibekwe VI (2004). Antibacterial activities and preliminary phytochemical screening of four medical plants. J. Appl. 7(3):4328-4338.
- Balas RK, Agarwal R (1980). Chemical Examination of *E. hirta*. In Book of abstracts II Micher, E. Reinhard E. (Eds). International Research Congress on National Products as Medicinal Agents Strasbourg, France. Pp. 25
- Begum S, Hassan SI, Siddiqui BS, Shaheen F, Ghaayur AH (2002). Triterpenoid from the leaves of *Psidium guajava*. Phytochemistry 61:399-403.
- Bouamama H, Noel T, Villard J, Benharref A, Jana M (2006). Antimicrobial activities of the leaf of the leaf extract two Moroccan *Citrus* L. species. J. Ethnopharmacol. 104: 104-107.
- Brain KR, Tuner (1975). The practical evaluation of phytochemistry pharmaceuticals Wright Science Chila Bright pp. 120-140.
- Braude AI (1982). W.B. Microbiology. Saunders Company, London.
- Burkill HM (1995). The useful plants of West Tropical Africa. Royal botanic garden Kew (UK), 3.
- Cowan MM (1999). Plants products as an antimicrobial agent's Clin. Microbiol. Rev. 12 (4) 564-582.
- Dalma G (1970). The erythrophleum alkaloids. In "The Alkaloids" Ed. Manske, H. F. Academic Press, New York. 4:265-273
- Dalziel JM (1959). The useful plants of West Tropical Africa: An Appendix to the Flora of West Tropical African. Crown Agents for Overseas Governments and Administrative, London. pp. 192-194.
- Dalziel JM (1999). Useful plants of tropical West Africa. Crown Agents London. pp. 432-434.
- Griffin WJ, Phippard JH, Culvenor CCJ, Loder JW, Neam R (1971). Alkaloids of the leaves of *Erythrophleum chlorostachys*. Phytochemistry 10:2793-2797.
- Jinju MH (1990). African Traditional Medicine. A case study of Hausa Medicinal Plants and Therapy. Gaskiya Corp. Ltd. Zaria. Nigeria Pp 43-50.
- Kunle OF, Egharevba HO (2009). Preliminary studies on *Vernonia ambigua*: Phytochemistry and Antimicrobial screening of the whole plant. Ethnobotanical leaflet 13:1216-1221
- Loder NJ, Culvenor CCJ, Neam RH, Russel GB, Stanton FW (1974). Tumor inhibitory plants New Alkaloids from the bark of *Erythrophleum chlorostachys* (Leguminosae). Aust. J. Chem. 27:179-185.
- Mattocks AR, Jukes R (1987). Improved field tests for toxic pyrrolizidine alkaloid. J. Nat. Prod. 50:161-166.
- Meenakshi S, Jain DC, Darokar MP, Sharma RP (2001). Antibacterial activity of *Ailanthus excels* (Roxb). Phytother. Res. 15:165-166.
- Ntiejumokwu S, Alemika TOE (1991). Antimicrobial and phytochemical investigation of the stem bark of *Boswellia dalzielii*. W. Afr. J. pharmacol. Drug Res. 10:100-104.
- Nweze, EI, Okofor JI, Njoku O (2004). Antimicrobial activities of methanolic extracts of *Trema guineensis* (Schum and Thom) and *Morinda lucida* Benth use in Nigerian Herbal Medicinal Practice. J. Bro. Res. Biotechnol. 2(1):39-46.
- Nwude N, Chineme CN (1981) Toxic effects of the leaves of *E. africanum* (Harms) in Sheep. Bull. Anim. Hlth. Prod. Afr. 29:349-354.

- Ogbulie JN, Ogueke CC, Okoli IO, Anyanwu BN (2007). Antibacterial activities and toxicological potential of crude ethanolic extracts of *Euphorbia hirta* J. Afr. J. Biotechnol. 6(13):1544-1548.
- Okemo PO, Bais HP, Vivanco JM (2003). In vitro activities of *Moesa lanceolata* extracts against fungal plant pathogens. *Fitoterapia*, 74:312-316.
- Osadebe PO, Ukwueze SE (2004). A comparative study of the phytochemical and Antimicrobial properties of the Eastern Nigerian species of Africa Mistletoe (*Loranthus micranthus*) Sourced from different host trees. *J. Beol. Res. Biotechnol.* 2(1):18-23.
- Osawa K, Matsumoto T, Maruyama T, Tagikuchi T, Okuda K, Takazoe I (1990). Studies of the antimicrobial activity of plant extracts and their constituents against perioontopathic bacteria. *Bul Tokyo Dent. Coll.* 31:17-21.
- Roberts MF, Winks M (1998) Alkaloids: Biochemistry, Ecology and Medical Applications: Edited Roberts and Wink, Plenum Press, New York. pp. 397-424.
- Ronan B, Ademir JSJ, Alaide BO (2009). Plant- derived Antimicrobial agents: New leads and Efficient Phytomedicine. Part 11. Non alkaloid Natural products- A Review. *Molecules* 14, 3037-3072.
- Sidney MF, William JM, Elvyn GS (1978). Baily and Scotts Microbiology. C. V. Mosby: st. Louis. pp 385-403.
- Singleton P (1999). Bacteria in Biology, Biotechnology and Medicine. 4th edn. John Willey and son Ltd, New York.
- Sofowora A (2008). Medicinal plants and Traditional Medicine in Africa, Spectrum Books Limited Nigeria. P0 10-7.
- Sophon R, Kasan S, Nongmy J, Narongsak C, Amoro P (2002). Crystal structure of Ipolamiide Monohydrate from *Stachytarpheta indica*. *Anal Sci.* 18(9):1063-1064.
- Srinivasan D, Nathan S, Suresh T, Perumalsam PZ (2001). Antimicrobial activities of certain Indian medicinal plants used in folkloric medicine. *J. Trop. Biosci.* 3:17-20.
- Trease GC, Evans WC (2002). Trease and Evans pharmacognosy .W.B Sanders Edinborough.
- Vollokova AD, Kostalova, Sochorova (2001). Isoquinoline alkaloid from mahonia Aquifolium stem bark is active against malseizia species. *Fol. Microbiol* 46:107-111.
- Watt JM, Breyer - Brandwijk MG (1963). Medicinal and Poisonous plants of Southern and Eastern Africa. E. S Living Stone Edinburgle publishers. pp. 1046-1048.
- Yoshida T, Namba O, Chen L, Okuda T (1990). Euphorbin E., a hydrolysable tannin dimmer of highly oxidized structure from *Euphorbia hirta*. *Chem . Pharm. Bull.* 38(4):1113-1115.
- Zampini IC, Cuello S, Alberto MR, Ordonez RM, Solorzano E, Isla MI (2009). Antimicrobial activity of seleted plant species from the the Argentine puna against sensitive and multiresistantbacteria. *J. Ethnopharmacol.* 124:499-505.

Full Length Research Paper

Seasonal variation in phytochemicals and antioxidant activities in different tissues of various *Broccoli* cultivars

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Florets, leaves, and stems of twelve commercial broccoli cultivars grown in the spring and fall seasons at the National Institute of Horticultural and Herbal Science (NIHHS), Rural Development Administration (RDA), Suwon, South Korea were evaluated for glucosinolates, vitamin C, total phenol, and total flavonoid contents and antioxidant activity. The levels of all phytochemicals and antioxidant activity were significantly influenced by cultivar (C), plant part (P), and growing season (S). Among the glucosinolates, glucoraphanin and glucobrassicin were the major constituents. The highest total glucosinolate content was found in the florets of plants grown in both seasons. Phenols and flavonoids were highest levels in leaves, while vitamin C was highest in stems, suggesting that broccoli leaves and stems may be good sources of such phytochemicals. The levels of all phytochemicals were generally higher in florets in the spring than in the fall, but were higher in leaves and stems during the fall than the spring. Furthermore, higher cultivar-dependent and tissue-dependent variation was observed in the spring than in the fall. Total phenol content exhibited a strong positive correlation ($r = 0.674^{**}$) with antioxidant activity, followed by total flavonoid content ($r = 0.497^{**}$), indicating their significant contribution to total antioxidant activity.

Key words: Antioxidant activity, broccoli, glucosinolate, seasonal variation, total phenol, vitamin C.

INTRODUCTION

Broccoli is one of the most commonly consumed green vegetables. Like other species of the *Brassica* family, broccoli is a source of health-promoting phytochemicals. Broccoli is known mainly for its wide range of bioactive compounds and is rich in both nutritional and non-nutritional antioxidants, including vitamin C, vitamin E, and phenolic compounds including flavonoids, carotenoids, and glucosinolates (Lin and Chang, 2005) which possess both antioxidant and anticancer activities (Williamson et al., 1998; Cohen et al., 2000; Chu et al., 2002; Gundgaard

et al., 2003; Podsedek, 2007). Glucosinolates constitute a major group of natural plant compounds in the family Brassicaceae. They are responsible for the hot and pungent flavor of crucifers and exhibit anti-cancer activity (Fahey et al., 2001). Glucosinolates can be used as an alternative to synthetic pesticides for pest and disease control (Kirkegaard and Sarwar, 1998). Vitamin C is a health-promoting antioxidant compound that protects against cell death, directly scavenges superoxide radicals, hydrogen peroxide singlet oxygen, and hydroxyl radicals

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Abbreviations: DEAE, Diethyl aminoethyl; DPPH, 2,2-diphenyl-1-picrylhydrazyl; HPLC, high performance liquid chromatography; UPLC, ultra-performance liquid chromatography; PDA, photodiode array; C, cultivar; P, plant part; S, season.

(Gliszczynska-Swiglo et al., 2006) and cooperates with vitamin E to regenerate membrane-bound oxidized α -tocopherol, creating an 'antioxidant network' (Valko et al., 2006). Phenolic compounds are secondary metabolites that can neutralize or quench free radicals (Picchi et al., 2012). Flavonoids and their derivatives are the largest group of plant polyphenols (Hounsome et al., 2009). They possess strong antioxidant activity due to their ability to scavenge reactive oxygen species and inhibit oxidative stress (Rice-Evans et al., 1995; Pourcel et al., 2007). Variation in the amounts of these phytochemicals depends upon factors such as cultivar genotype, developmental stage, growing conditions, season, soil properties, and post-harvest storage conditions (Kurlich et al., 1999; Vallejo et al., 2002; Jeffery et al., 2003; Singh et al., 2007; Nath et al., 2011; Samec et al., 2011; Samec et al., 2013).

Although genotypic differences in the contents of glucosinolates, vitamin C, phenolics, and total flavonoids and antioxidant activity in broccoli florets have been reported (Zhang and Hamazu, 2004; Singh et al., 2007; Koh et al., 2009; Balouchi et al., 2011; Naguib et al., 2012), information regarding the content of such phytochemicals specifically in leaves and stems is limited. Characterization of such phytochemicals to establish their distribution patterns in leaves and stems would also be useful. Seasonal variation in glucosinolate content in broccoli cultivars has been reported (Rosa and Rodrigues, 2001; Vallejo et al., 2003); however, most of the research focused only on variations in glucosinolate profiles. This study was conducted to evaluate glucosinolate, vitamin C, total phenol, and total flavonoid contents and to measure antioxidant activity in commercially cultivated broccoli cultivars in South Korea and to evaluate the cultivar- and season-dependent variation in such compounds in florets, leaves, and stems of different broccoli cultivars.

MATERIALS AND METHODS

Authentic standards and chemicals

Nine glucosinolate standards, glucoiberin, progoitrin, glucoraphanin, sinigrin, gluconapin, glucobrassicinapin, glucoerucin, glucobrassicin, and gluconasturtiin, were purchased from Cfm Oskar Co. (Germany). Authentic standards for diethyl aminoethyl (DEAE) Sephadex-A25, aryl sulfatase from *Helix pomatia*, vitamin C, glucose, sucrose, fructose, gallic acid, and catechin hydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chemicals, including sodium hydroxide, sodium carbonate, sodium nitrite, aluminum chloride, Folin-Ciocalteu reagent, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals including acetonitrile (high performance liquid chromatography (HPLC)) grade, methanol (HPLC grade), and formic acid (ACS reagent) were purchased from J.T. Baker (Phillipsburg, NJ, USA).

Plant materials and growing conditions

Twelve commercial broccoli cultivars, 05-C3, AMaGi, BaeRiDom, CheongJae, Diamond, Grace, Grandeur, JikNok No. 28, NokJae, NokYeom No. 1, TS-2319, and YuDoRI No. 1 were used in this study

study. The cultivars were grown in the field at the National Institute of Horticultural and Herbal Science (NIHHS), Rural Development Administration (RDA), Suwon, South Korea in the spring and fall growing seasons of 2011. Sowing dates were 5 March 2011 for the spring season and 25 July 2011 for the fall season. For both seasons, seedlings were transplanted to the cultivation field 33 days after sowing. Seedlings were planted in rows with 50 cm between plants and 60 cm between rows. The plants were harvested 35 and 30 days after planting in the spring and fall seasons, respectively. During the field experiments, water, fertilizers, and pesticides were applied according to standard cultural practices at the NIHHS, RDA. After harvest, the plants were separated into different parts (florets, stems, and leaves), cut into small pieces, and freeze-dried. The samples were ground into a fine powder and stored at -80°C for subsequent analyses of glucosinolates, vitamin C, total phenol, and total flavonoid content and antioxidant activity.

Glucosinolate analysis

For glucosinolates analysis, freeze-dried broccoli powder (0.1 g) was extracted with 1 mL of boiling methanol (70%) for 20 min and centrifuged at 12,000 rpm for 10 min at 4°C . The pellet was re-extracted once following the same procedure, and the supernatants were combined. Desulfoglucosinolates were then prepared and quantitatively determined by ultra-performance liquid chromatography (UPLC) using purified sulphatase isolated from *H. pomatia* according to Lee et al. (2013). Briefly, the extract was loaded onto a Mini Biospin chromatography column (Bio-Rad) containing 0.5 mL of DEAE-Sephadex A 25 which was preactivated with 0.1 M sodium acetate (pH 4.0). Then, desulfation was carried out by the addition of 200 μL of purified aryl sulphatase (EC 3.1.6.1, type H-1 from *H. pomatia*). The column was capped and left for 24 h at room temperature. The desulphoglucosinolates were eluted with 1.5 mL distilled water, filtered through a 0.2 μm syringe filter, and 10 μL samples were injected into a UPLC system (H-Class, Waters Co., USA) equipped with a sample manager flow-through-needle auto injector, quaternary solvent manager, and a photodiode array (PDA) eA detector set at 229 nm. Separation was performed using a BEH-C18 column (2.1 \times 100 mm, 1.7 μm , Waters Co., USA) at 25°C with a gradient elution of solvent A (100% distilled water) and solvent B (20% acetonitrile) with a flow rate of 0.2 mL min^{-1} . The gradient program used was as follows: a linear step from 1 to 99% of solvent B within 6 min, a constant step for 10 min, followed by a rapid dropdown to 1% solvent B at 12 min, and isocratic conditions with 1% solvent B for 3 min. Authentic glucosinolate standards were used for the identification and quantification of the peaks.

Vitamin C analysis

Vitamin C content was determined according to a method described by Spinola et al. (2012) with modifications. Dried and powdered broccoli samples (0.5 g) were extracted in 5% metaphosphoric acid solution. After centrifugation and filtration (with a 0.20 μm syringe filter), the sample was analyzed using a UPLC system (Waters, USA), an Acquity UPLC[®] HSS T3 (2.1 \times 100 mm, 1.8 μm) column, and a PDA detector (Waters, USA) set at a wavelength of 254 nm. The mobile phase used was 99% methanol and 1% distilled water with 0.1% formic acid solution at a flow rate of 0.3 mL min^{-1} . An authentic ascorbic acid standard was used for identification and quantification of the peak.

Determination of total phenol content

Total phenolic content was estimated using the Folin-Ciocalteu colorimetric method based on the procedure of Singleton and Rossi (1965) using gallic acid as a standard phenolic compound. Freeze-dried powdered samples (1 g) were extracted in 80% methanol for

15 h at room temperature on an orbital shaker. The extracts were centrifuged and filtered through 0.45 μm syringe filters and 1 mL of each supernatant was mixed with 3.0 mL distilled water in 15 mL Falcon tubes. After adding 1 mL Folin reagent, the solutions were incubated in a water bath at 27°C for 5 min followed by addition of 1 mL of saturated sodium carbonate. After 1 h, absorbance of the extracts at 640 nm was measured using a micro plate reader (EON-C) (BioTek, USA) using 80% methanol as a blank. Gallic acid standards of various concentrations (5.0, 10.0, 25.0, 50.0, 75.0, and 100.0 ppm) were used for calibration and total phenol content was expressed as milligrams of gallic acid equivalent per gram (mg GAE g^{-1}) dry weight.

Determination of total flavonoid content

The broccoli extracts obtained for total phenol analysis were also subjected to total flavonoid analysis using a colorimetric method described by Zhishen et al. (1999). One milliliter of methanol extract (80%) was added to a 15 mL Falcon tube, mixed with 4 mL distilled water, and 0.3 mL 5 % sodium nitrite added. After 5 min, 10% AlCl_3 was added to the solution. At the sixth minute, 2 mL 1 M NaOH was added and the solution was brought to a final volume of 10 mL with distilled water. The solution was mixed thoroughly and absorbance was measured at 510 nm in a micro plate reader (EON-C) (BioTek, USA) using 80% methanol as a blank. Catechin hydrates of different concentrations (5.0, 10.0, 25.0, 50.0, 75.0, and 100.0 ppm) were used as standards and total flavonoid was expressed as milligrams of catechin hydrate equivalent per gram (mg CE g^{-1}) dry weight.

Determination of antioxidant activity

Antioxidant activity in extracts of different broccoli tissues was determined using the DPPH radical-scavenging method according to Koleva et al. (2002) with modifications. The extracts obtained for total phenol analysis were also used for the measurement of antioxidant activity. A 400 μM DPPH solution in 80% methanol was prepared. Then, 100 μL of the DPPH solution were mixed with 100 μL of various concentrations (0.5, 1.0, 2.5, 5.0, 7.5, and 10.0 mg mL^{-1}) of the extracts in 96-well plates. After 30 min in darkness at room temperature, absorbance at 517 nm was measured in a micro plate reader (EON-C) (BioTek, USA) using 80% methanol without DPPH as a blank. Similarly, absorbance of samples was also measured after mixing 100- μL samples with 100 μL of 80% methanol. Free-radical-scavenging activity (%) was calculated using the following equation:

$$\% \text{ DPPH radical-scavenging activity} = (B - A) \times 100/B$$

Where, A is the absorbance of [(Sample + DPPH) – (Sample + Methanol)] and B is the absorbance of [(Methanol + DPPH) – (Methanol)]. The IC_{50} value, which is the concentration required to obtain 50% antioxidant capacity, was calculated and was used to compare the antioxidant activities of sample extracts.

Statistical analysis

Means of at least two independent sample replications were used for all statistical analyses. Data were analyzed using the SAS (version 9.2) software. The statistical significance of differences among cultivars, growing seasons, and plant tissues was assessed using a fixed-factor analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Glucosinolates content

Variations in glucosinolate profiles in florets, leaves, and

stems of different broccoli cultivars during different growing seasons are presented in Tables 1, 2, and 3, respectively. Glucoraphanin was the most abundant glucosinolate in all three tissue types in both cultivars in both growing seasons. Glucobrassicin was the second most abundant glucosinolate in florets and leaves. Glucoerucin was present only in stems and was the second most abundant glucosinolate after glucoraphanin. Progoitrin, sinigrin, and gluconapin were found in only some cultivars, which showed different cultivar-dependent distribution patterns according to tissue type and growing season. Similar variations in glucosinolate distributions in different growing seasons were reported by Vallejo et al. (2003). In florets, the highest content of total glucosinolates was measured in the TS-2319 cultivar (17.81 $\mu\text{mol g}^{-1}$) in the spring season, while the JikNok No. 28 cultivar (13.80 $\mu\text{mol g}^{-1}$) had the highest glucosinolate levels in the fall season (Table 1). In both seasons, we observed lower glucosinolate levels than reported by Lee et al. (2012), who found total glucosinolate levels of 15.90–59.30 $\mu\text{mol g}^{-1}$ from analysis of 95 broccoli accessions. This difference in values might be due to differences in genotypes and other environmental factors. Florets showed higher total glucosinolate levels (7.45 $\mu\text{mol g}^{-1}$) in the spring than in the fall (6.96 $\mu\text{mol g}^{-1}$); however, no specific trends were found among the cultivars. This result was somewhat similar to those of Charron and Sams (2004) and Justen et al. (2012) who also reported higher total glucosinolate amounts in spring growing conditions. These changes in glucosinolate concentration might be due to the interactions of several factors (Charron and Sams, 2004; Fabek et al., 2012). Two major glucosinolates, glucoraphanin and glucobrassicin, were present in all of the cultivars in both seasons, but other glucosinolates showed cultivar-dependent distribution patterns. The average total glucosinolate content was lower (4.20 $\mu\text{mol g}^{-1}$) in the spring than in the fall (5.35 $\mu\text{mol g}^{-1}$). Among the 12 cultivars, the highest total glucosinolate contents in the spring and fall seasons were found in NokYeom No. 1 (5.76 $\mu\text{mol g}^{-1}$) and AMaGi (6.73 $\mu\text{mol g}^{-1}$), respectively, suggesting their superiority in terms of leaf glucosinolate content.

Stems contained one additional glucosinolate, glucoerucin, not found in florets or leaves. Glucoerucin was one of the three major glucosinolates in stems in all cultivars and constituted approximately 25 and 10% of total glucosinolates in the spring and fall seasons, respectively (Table 3). Other major glucosinolates included glucoraphanin (spring, 2.19 $\mu\text{mol g}^{-1}$; fall, 3.60 $\mu\text{mol g}^{-1}$) and glucobrassicin (spring, 0.27 $\mu\text{mol g}^{-1}$; fall, 0.29 $\mu\text{mol g}^{-1}$). The average total glucosinolate content in stems showed a pattern similar to that in leaves with higher total glucosinolate levels in fall (4.96 $\mu\text{mol g}^{-1}$) than in spring (4.18 $\mu\text{mol g}^{-1}$). Among the 12 cultivars, only 2, BaeRiDom and TS-2319, contained all 6 glucosinolates analyzed in this study in their florets, leaves, and stems in both seasons. Among the three tissue types, florets exhibited the highest total glucosinolates, followed by leaves and stems, in both seasons, sug-

Table 1. Seasonal variation in glucosinolate contents ($\mu\text{mol g}^{-1}$, dry weight) in florets of broccoli cultivars.

Cultivar	Progoitrin		Glucoraphanin		Sinigrin		Gluconapin		Glucobrassicin		Total glucosinolates	
	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall
05-C3	0.00±0.00 ^z	0.17±0.00	2.30±0.15	4.03±0.04	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.58±0.10	4.31±0.10	3.88±0.25	8.51±0.07
AMaGi	2.25±0.08	1.83±0.01	2.61±0.11	1.41±0.01	0.00±0.00	0.00±0.00	0.57±0.02	0.60±0.01	1.54±0.09	2.57±0.11	6.95±0.28	6.40±0.12
BaeRiDom	1.28±0.00	1.15±0.02	1.81±0.05	1.37±0.06	0.21±0.00	0.19±0.00	0.32±0.01	0.34±0.02	0.68±0.03	1.56±0.13	4.29±0.07	4.60±0.24
CheongJae	0.19±0.01	0.56±0.04	1.67±0.00	2.62±0.20	0.00±0.00	0.10±0.00	0.06±0.00	0.18±0.02	0.34±0.00	2.35±0.13	2.26±0.01	5.79±0.01
Diamond	0.00±0.00	0.00±0.00	4.34±0.02	2.05±0.14	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	3.51±0.06	1.63±0.06	7.85±0.08	3.68±0.20
Grace	0.00±0.00	0.00±0.00	5.83±0.33	1.75±0.10	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.46±0.10	1.42±0.08	7.29±0.23	3.17±0.18
Grandeur	0.00±0.00	0.00±0.00	4.28±0.22	2.37±0.07	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	4.21±0.03	4.09±0.04	8.48±0.01	6.46±0.02
JikNok No. 28	2.58±0.25	3.72±0.14	3.38±0.26	5.12±0.52	0.00±0.00	0.00±0.00	0.83±0.01	0.97±0.02	1.98±0.21	4.00±0.04	8.76±0.73	13.8±0.32
NokJae	0.00±0.00	0.00±0.00	3.74±0.40	3.54±0.31	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.88±0.07	2.49±0.07	5.62±0.33	6.03±0.38
NokYeom No. 1	2.64±0.27	2.55±0.01	2.16±0.06	3.32±0.12	0.00±0.00	0.00±0.00	0.56±0.06	0.82±0.01	2.94±0.18	2.80±0.10	8.30±0.20	9.49±0.21
TS-2319	4.48±0.11	2.11±0.19	7.64±0.07	1.92±0.09	0.54±0.00	0.26±0.01	0.49±0.01	0.69±0.05	4.68±0.28	1.27±0.11	17.81±0.31	6.24±0.46
YuDoRi No. 1	1.86±0.07	2.36±0.19	3.31±0.09	3.49±0.09	0.30±0.03	0.00±0.00	0.44±0.01	0.61±0.04	2.09±0.18	3.00±0.20	7.99±0.18	9.45±0.53
Average	2.18	1.80	3.59	2.75	0.35	0.18	0.47	0.48	2.24	2.62	7.46	6.96

^z Each value is the mean ± SD of two independent replications.**Table 2.** Seasonal variation in glucosinolate contents ($\mu\text{mol g}^{-1}$, dry weight) in leaves of broccoli cultivars.

Cultivars	Progoitrin		Glucoraphanin		Sinigrin		Gluconapin		Glucobrassicin		Total glucosinolates	
	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall
05-C3	0.00±0.00 ^z	0.00±0.00	3.18±0.23	4.70±0.29	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	2.10±0.01	1.55±0.08	5.28±0.24	6.24±0.21
AMaGi	0.34±0.02	0.60±0.02	2.05±0.16	3.38±0.06	0.00±0.00	0.00±0.00	0.20±0.00	0.25±0.01	2.31±0.01	2.51±0.01	4.90±0.13	6.73±0.03
BaeRiDom	0.26±0.02	0.22±0.01	2.00±0.09	1.36±0.11	0.07±0.00	0.06±0.01	0.18±0.01	0.10±0.01	1.16±0.01	0.68±0.01	3.66±0.05	2.41±0.12
CheongJae	0.00±0.00	0.19±0.01	1.66±0.11	5.05±0.06	0.00±0.00	0.06±0.00	0.00±0.00	0.08±0.00	0.21±0.00	1.74±0.11	1.87±0.11	7.11±0.18
Diamond	0.00±0.00	0.00±0.00	1.27±0.09	4.13±0.31	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	2.02±0.02	2.16±0.02	3.29±0.10	6.28±0.33
Grace	0.00±0.00	0.00±0.00	3.56±0.03	4.11±0.05	0.00±0.00	0.00±0.00	0.12±0.01	0.00±0.00	0.75±0.03	1.02±0.02	4.42±0.05	5.13±0.07
Grandeur	0.00±0.00	0.00±0.00	2.40±0.07	3.70±0.31	0.00±0.00	0.00±0.00	0.10±0.00	0.00±0.00	2.22±0.06	3.14±0.17	4.72±0.01	6.84±0.14
JikNok No. 28	0.48±0.00	0.34±0.02	2.66±0.13	1.61±0.06	0.00±0.00	0.00±0.00	0.24±0.00	0.12±0.00	1.55±0.10	1.34±0.08	4.91±0.03	3.40±0.04
NokJae	0.00±0.00	0.00±0.00	3.39±0.11	7.45±0.33	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.84±0.08	1.27±0.06	5.23±0.03	8.72±0.39
NokYeom No. 1	0.54±0.00	0.45±0.04	1.92±0.00	2.07±0.11	0.00±0.00	0.00±0.00	0.20±0.00	0.12±0.00	3.10±0.00	1.68±0.08	5.76±0.00	4.31±0.23
TS-2319	0.10±0.01	0.38±0.01	1.56±0.04	2.82±0.07	0.05±0.00	0.08±0.00	0.11±0.00	0.15±0.01	2.01±0.03	0.77±0.03	3.82±0.01	4.19±0.05
YuDoRi No. 1	0.21±0.01	0.27±0.01	1.09±0.06	1.41±0.10	0.00±0.00	0.06±0.00	0.17±0.00	0.11±0.00	1.08±0.00	0.96±0.01	2.54±0.09	2.80±0.12
Average	0.32	0.35	2.23	3.48	0.06	0.06	0.16	0.13	1.70	1.57	4.20	5.35

^z Each value is the mean ± SD of two independent replications.

Table 3. Seasonal variation in glucosinolate contents ($\mu\text{mol g}^{-1}$, dry weight) in stems of broccoli cultivars.

Cultivar	Progoitrin		Glucoraphanin		Sinigrin		Gluconapin		Glucoerucin		Glucobrassicin		Total glucosinolate	
	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall
05-C3	0.00±0.00 ^z	0.00±0.00	2.95±0.10	6.42±0.40	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.89±0.01	1.17±0.06	0.52±0.00	0.33±0.02	4.35±0.11	7.92±0.35
AMaGi	1.16±0.04	1.01±0.00	2.39±0.14	3.22±0.06	0.00±0.00	0.00±0.00	0.33±0.00	0.32±0.03	1.56±0.01	0.28±0.01	0.30±0.01	0.54±0.00	5.74±0.07	5.37±0.09
BaeRiDom	0.88±0.01	0.84±0.05	1.7±0.06	2.05±0.19	0.13±0.01	0.12±0.02	0.15±0.01	0.13±0.00	1.59±0.10	0.27±0.02	0.21±0.01	0.15±0.00	4.64±0.08	3.55±0.12
CheongJae	0.00±0.00	0.31±0.00	1.00±0.04	3.07±0.24	0.00±0.00	0.05±0.00	0.00±0.00	0.11±0.01	0.00±0.00	0.15±0.00	0.04±0.00	0.40±0.00	1.04±0.04	4.08±0.25
Diamond	0.00±0.00	0.00±0.00	2.65±0.12	3.98±0.12	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.36±0.01	0.48±0.03	0.39±0.00	0.24±0.01	3.39±0.13	4.70±0.09
Grace	0.00±0.00	0.00±0.00	2.98±0.25	3.79±0.06	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	2.41±0.07	0.54±0.00	0.29±0.03	0.29±0.01	5.68±0.21	4.61±0.04
Grandeur	0.00±0.00	0.00±0.00	2.77±0.24	5.82±0.18	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.48±0.00	0.49±0.01	0.53±0.04	0.56±0.01	3.77±0.20	6.86±0.18
JikNok No. 28	1.22±0.01	1.29±0.06	1.55±0.00	1.40±0.03	0.00±0.00	0.00±0.00	0.29±0.01	0.32±0.00	1.20±0.02	0.18±0.01	0.17±0.00	0.23±0.00	4.41±0.02	3.41±0.10
NokJae	0.00±0.00	0.00±0.00	4.18±0.33	7.22±0.08	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.09±0.07	0.37±0.01	0.26±0.01	0.22±0.00	5.53±0.27	7.81±0.07
NokYeom No. 1	1.11±0.02	1.33±0.10	1.16±0.07	2.46±0.13	0.00±0.00	0.00±0.00	0.97±0.04	0.20±0.00	1.29±0.04	0.30±0.01	0.23±0.03	0.24±0.00	4.76±0.13	4.52±0.02
TS-2319	0.62±0.00	0.82±0.01	1.65±0.07	2.15±0.20	0.11±0.00	0.09±0.01	0.16±0.00	0.14±0.00	0.78±0.03	0.53±0.00	0.15±0.00	0.11±0.00	3.46±0.04	3.84±0.36
YuDoRi No. 1	0.78±0.06	0.74±0.06	1.31±0.12	1.59±0.02	0.10±0.00	0.08±0.01	0.12±0.01	0.13±0.01	0.95±0.08	0.23±0.02	0.23±0.01	0.15±0.00	3.47±0.29	2.91±0.08
Average	0.96	0.90	2.19	3.60	0.11	0.09	0.33	0.19	1.14	0.42	0.27	0.29	4.19	4.96

^z Each value is the mean \pm SD of two independent replications.

gesting that florets are a good source of glucosinolates. Variation in glucosinolate amounts, measured as the coefficient of variation (CV %), was highest in florets in both the spring (CV 51.9%) and fall (CV 42.4%) seasons. Two major glucosinolates (glucobrassicin and glucoraphanin) as well as total glucosinolates exhibited higher variation in florets in the spring than in the fall, but the opposite was the case in leaves and stems. In our study, amounts of total as well as individual glucosinolates were significantly dependent on cultivar (C), plant part (P), and season (S) (Table 4) in most cases. Most of the interactions were significant, except for C x S and C x S x P for gluconapin and S x P for glucoerucin (Table 4). This result is in agreement with Rosa and Rodrigues (2001) and Vallejo et al. (2003) who also reported cultivar- and season-dependent variation in total

and individual glucosinolate amounts. Such variation in total as well as individual glucosinolate contents in different plant tissues may be due to differences in the control mechanisms of the glucosinolate biosynthetic pathway, alteration of substrate availability, and degradation and mobilization of glucosinolates (Sang et al., 1984; Chen and Andreasson, 2001).

Vitamin C content

Vitamin C contents in different tissue types of broccoli cultivars cultivated in the spring and fall seasons are presented in Figure 1. Vitamin C amounts varied according to cultivar, tissue type, and season. In florets, vitamin C amounts ranged from 2.42 (JikNok No. 28) to 5.53 mg g⁻¹ (Grandeur) with an average of 3.94 mg g⁻¹ in the

spring, and from 1.22 (NokJae) to 5.27 mg g⁻¹ (BaeRiDom) with a lower average value of 3.05 mg g⁻¹ in the fall. Variation in vitamin C amounts was higher in the fall (CV 41.7%) than in the spring (CV 23.0%). In leaves, the average vitamin C content (4.28 mg g⁻¹) was similar in both seasons, but variation was higher in the fall (CV 33.1%) than in the spring (CV 15.4%). In contrast, stems showed higher vitamin C levels (5.54 mg g⁻¹) in most of the cultivars and higher cultivar-dependent variation (CV 18.0%) in the spring than in the fall (CV 15.0%). The presence of higher vitamin C levels in the spring growing season might be due to higher temperature and radiation, as was reported previously for broccoli and other plants species (Merzlyak and Solovchenko, 2002; Hakala et al., 2003; Yao et al., 2005; Nilsson et al., 2006; Aires et al., 2011). However, no cultivar has

Table 4. Results of analyses of variance for antioxidant amounts and activities in broccoli cultivars.

Source of variance	Progoitrin	Glucoraphanin	Sinigrin	Gluconapin	Glucorucin	Glucobrassicin	Total glucosinolate	Vitamin C	Total phenol	Total flavonoid	Antioxidant activity
Cultivar (C)	***	***	***	***	***	***	***	***	***	***	***
Season (S)	NS	***	***	NS	***	*	***	***	**	***	***
Plant Parts (P)	***	**	***	***	***	***	***	***	***	***	***
C X S	***	***	***	NS	***	***	***	***	***	***	***
C X P	***	***	***	**	***	***	***	***	***	***	***
S X P	*	***	***	*	NS	***	***	***	***	***	***
C X S X P	***	***	***	NS	***	***	***	***	***	***	***

NS, *, **, ***: Non-significant or significant at $p < 0.05$, 0.01 , and 0.001 , respectively.

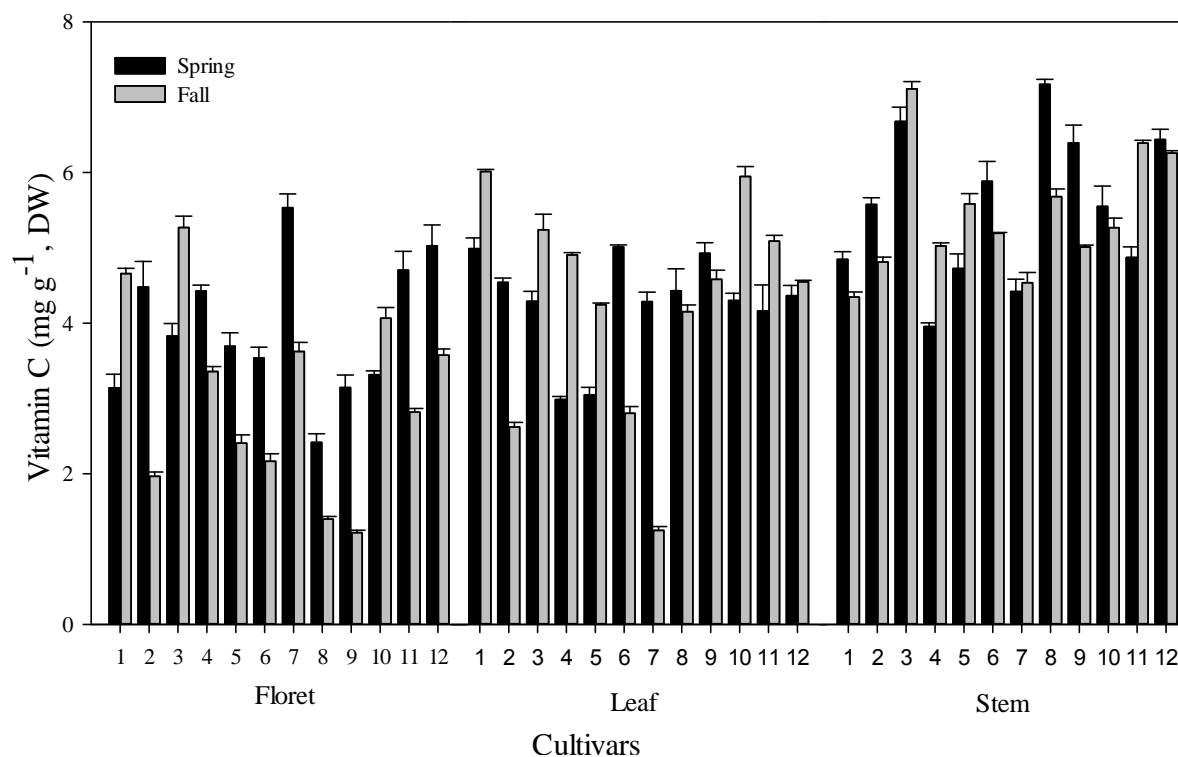


Figure 1. Seasonal variation in the vitamin C contents of florets, leaves, and stems of broccoli cultivars. Each bar represents the mean \pm SD of three independent replications. 1, 05-C3; 2, AMaGi; 3, BaeRiDom; 4, CheongJae; 5, Diamond; 6, Grace; 7, Grandeur; 8, JikNok No. 28; 9, NokJae; 10, NokYeom No. 1; 11, TS-2319; and 12, YuDoRi No. 1.

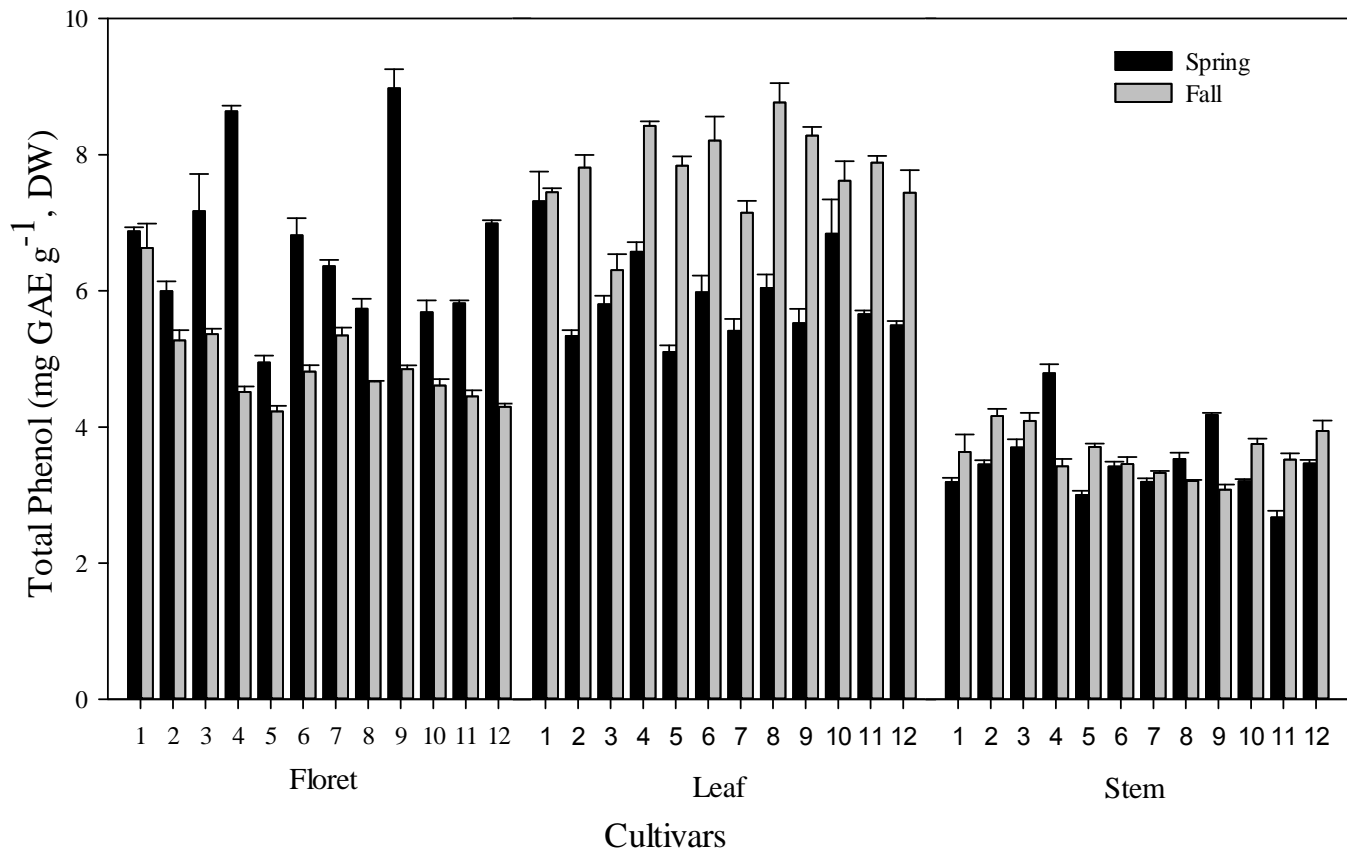


Figure 2. Seasonal variation in the total phenol contents of florets, leaves, and stems of broccoli cultivars. Each bar represents the mean \pm SD of three independent replications. 1, 05-C3; 2, AMAgi; 3, BaeRiDom; 4, CheongJae; 5, Diamond; 6, Grace; 7, Grandeur; 8, JikNok No. 28; 9, NokJae; 10, NokYeom No. 1; 11, TS-2319; and 12, YuDoRi No. 1.

higher vitamin C contents in both seasons as well as their respective parts (florets, leaves and stems), which suggests that genotype is one of the most important factors and that the effects of tissue types and environmental factors associated with growing seasons on vitamin C content are dependent on the genotype.

The average vitamin C content was highest in stems (spring, 5.54 mg g^{-1} ; fall, 5.44 mg g^{-1}), followed by leaves, and was lowest in florets, indicating that stems are a good source of vitamin C in broccoli. In contrast, cultivar-dependent variation was highest in florets in both growing seasons. The data revealed that vitamin C levels were significantly ($p < 0.001$) affected by C, P and S (Table 4). Similarly, all interactions (C \times S, C \times P, S \times P, and C \times S \times P) were statistically significant. Kurilich et al. (1999) also reported significant differences in vitamin C content among various cultivars in different growing seasons. Our study is the first report, to our knowledge, of changes in vitamin C content in different plant tissues and in different growing seasons in broccoli. Although our results suggest that the vitamin C content in broccoli plants is higher in stems than in florets or leaves and higher in the spring than in the fall, further careful investigation of vitamin C content using fresh samples is required.

Total phenol content

Phenolic compounds, which are important secondary metabolites, possess various biological activities, the most important of which is antioxidant activity associated with reduced cancer risk (Manach et al., 2005; Picchi et al., 2012). The seasonal variation in total phenol content in different tissues of various broccoli cultivars is presented in Figure 2. In florets, the total phenol content in the spring growing season ranged from $4.95 \text{ mg GAE g}^{-1}$ (Diamond) to $8.98 \text{ mg GAE g}^{-1}$ (NokJae), with an average of $6.67 \text{ mg GAE g}^{-1}$; this was higher than the average phenol content in the fall season ($4.92 \text{ mg GAE g}^{-1}$). The variation in total phenol content in florets was higher in the spring (CV 17.9%) than in the fall (CV 13.5%). In contrast, leaves and stems exhibited higher total phenol contents in the fall ($7.76 \text{ mg GAE g}^{-1}$ and $3.61 \text{ mg GAE g}^{-1}$, respectively) than in the spring ($5.92 \text{ mg GAE g}^{-1}$ and $3.48 \text{ mg GAE g}^{-1}$, respectively). Similar to florets, variation was higher in leaves (CV 11.3%) and stems (CV 15.9%) in the spring than in the fall. Similar to the results of Howard et al. (2002), we found higher total phenol content in florets ($6.67 \text{ mg GAE g}^{-1}$) than in leaves or stems in the spring growing season, possibly due to the

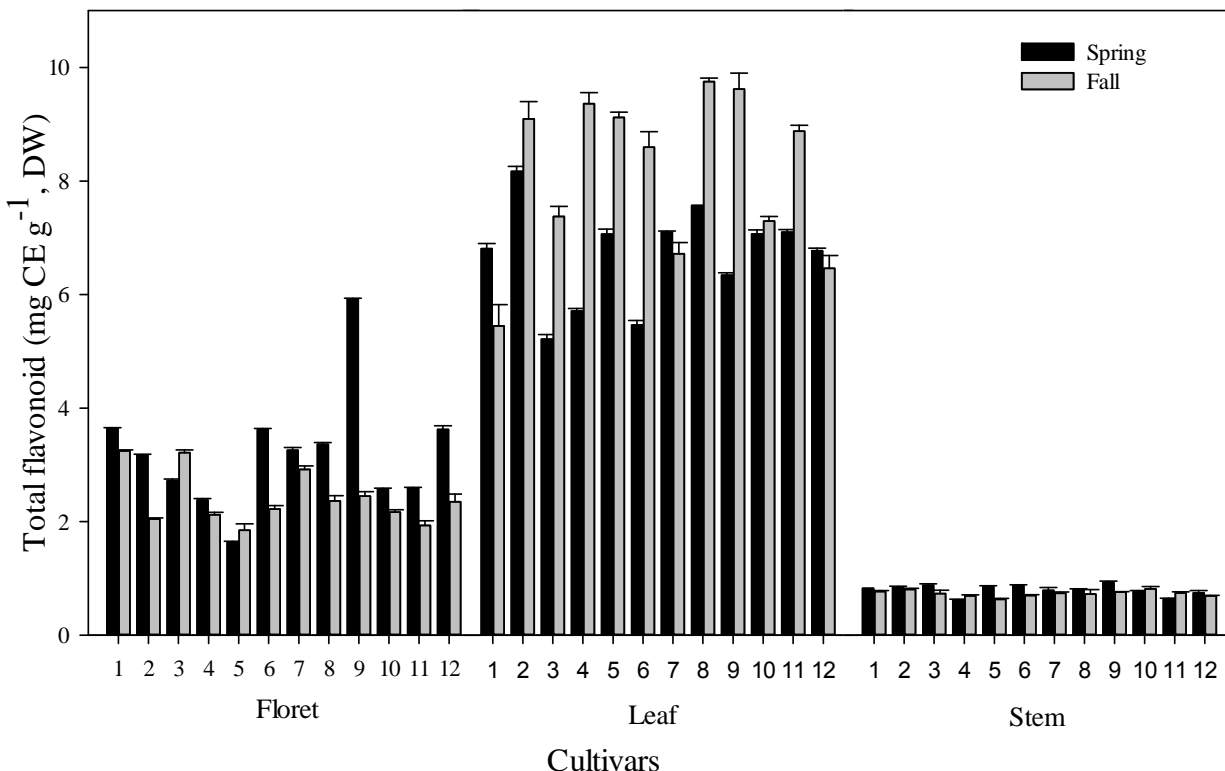


Figure 3. Seasonal variation in the total flavonoid contents of florets, leaves, and stems of broccoli cultivars. Each bar represents the mean \pm SD of three independent replications. 1, 05-C3; 2, AMaGi; 3, BaeRiDom; 4, CheongJae; 5, Diamond; 6, Grace; 7, Grandeur; 8, JikNok No. 28; 9, NokJae; 10, NokYeom No. 1; 11, TS-2319; and 12, YuDoRi No. 1.

influence of biosynthesis of phenolic compounds; however, average total phenol contents were higher in leaves ($7.76 \text{ mg GAE g}^{-1}$) and stems ($3.60 \text{ mg GAE g}^{-1}$) in the fall season. Among the three tissue types, cultivar-dependent variation was highest in florets in both seasons (spring season, 17.9%; fall season, 13.5%). We found that the total phenol contents of broccoli were significantly affected by C, P, S and interactions between these factors (Table 4). However, seasonal variation was lower than cultivar-dependent and tissue-dependent variation in this case. Similar cultivar- and season-dependent variation was also reported by Eberhardt, et al. (2005), Singh et al. (2007) and Balouchi et al. (2011). However, this study describes the variation in total phenol among various tissue types of several broccoli cultivars.

Total flavonoid content

Flavonoids are important secondary plant metabolites (Koh et al., 2009) that possess strong antioxidant activity due to their ability to scavenge reactive oxygen species and inhibit oxidative stress (Hounsoume et al., 2009). The seasonal variation in total flavonoid content in florets, leaves, and stems of broccoli cultivars is presented in Figure 3. Total flavonoid content in florets ranged in the spring from $1.63 \text{ mg CE g}^{-1}$ in Diamond to $5.92 \text{ mg CE g}^{-1}$

in NokJae, with an average of $3.21 \text{ mg CE g}^{-1}$; this was higher than the average flavonoid content in the fall season ($2.41 \text{ mg CE g}^{-1}$). Total flavonoid content was also higher in stems in the spring ($0.80 \text{ mg CE g}^{-1}$) than in the fall ($0.74 \text{ mg CE g}^{-1}$). However, leaves had higher average total flavonoid contents in the fall ($8.14 \text{ mg CE g}^{-1}$) than in the spring ($6.70 \text{ mg CE g}^{-1}$). Flavonoid contents ranged from 5.45 to $9.75 \text{ mg CE g}^{-1}$ in the 05-03 and JikNok No. 28 cultivars, respectively. Among the three tissue types, leaves had a significantly higher average total flavonoid content ca. threefold higher than in florets and eightfold higher than in stems in both seasons, indicating that leaves are a good source of flavonoids. Florets showed the highest cultivar-dependent variation in both the spring (CV 32.7%) and fall (CV 19.7%), while the variation was lowest in stems (spring, CV 11.8%; fall, CV 7.0%). No cultivar exhibited higher flavonoid contents in all tissue types and in both growing seasons. We found that total flavonoid content in broccoli was significantly ($p > 0.001$) affected by C, P, S and their interactions (Table 4), suggesting that total flavonoid content in broccoli cultivars is markedly influenced by genotype, growing season, and tissue type. Similar cultivar- and season-dependent variation was also reported by Koh et al. (2009) and Balouchi et al. (2011). However, this study addresses the variation in the total flavonoid contents in various tissue types of several broccoli cultivars.

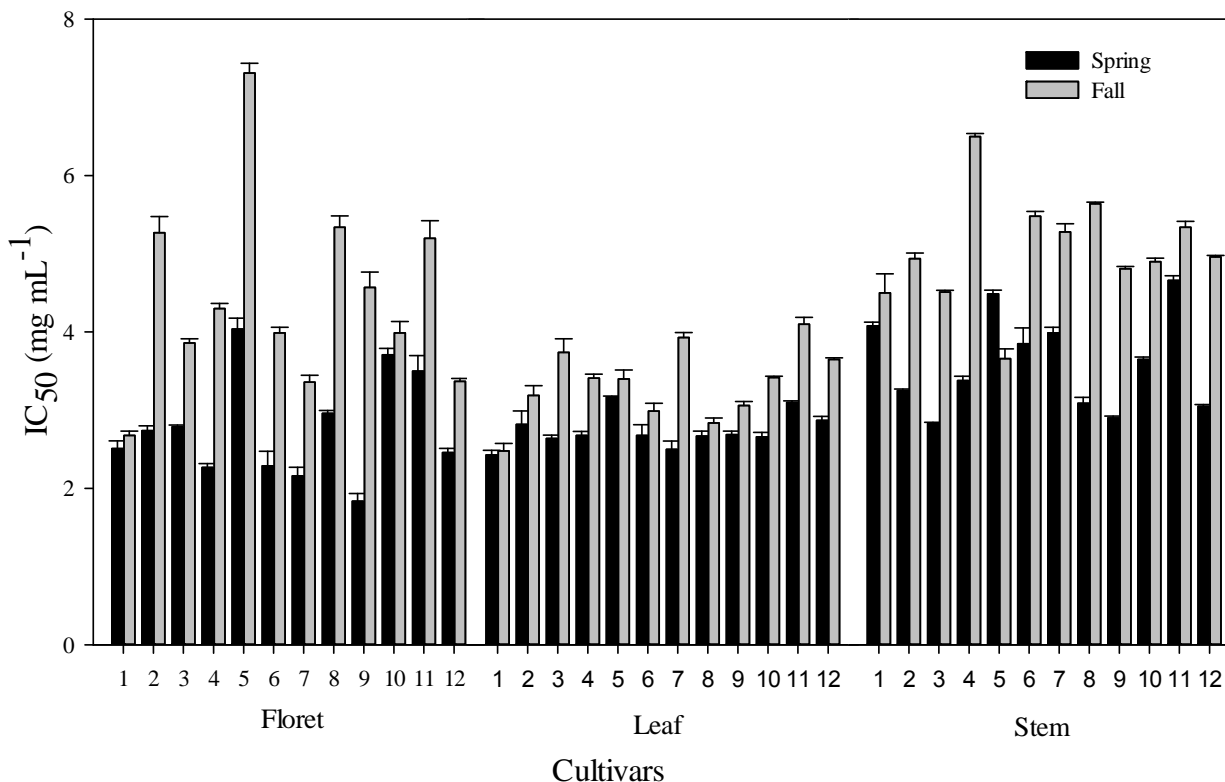


Figure 4. Seasonal variation in the antioxidant activities in florets, leaves, and stems of broccoli cultivars. Each bar represents the mean \pm SD of three independent replications. 1, 05-C3; 2, AMaGi; 3, BaeRiDom; 4, CheongJae; 5, Diamond; 6, Grace; 7, Grandeur; 8, JikNok No. 28; 9, NokJae; 10, NokYeom No. 1; 11, TS-2319; and 12, YuDoRi No. 1.

Antioxidant activity

Antioxidant activities of broccoli samples were evaluated by measuring the DPPH radical scavenging activity of different concentrations of methanol extracts. IC₅₀ (50% of inhibition) values were calculated after linear regression analysis of the observed inhibition percentages vs. concentration (Figure 4), where lower IC₅₀ values indicate higher antioxidant activity. Measurement of DPPH radical scavenging activity is a technically simple and rapid method for the evaluation of antioxidant activity (Fukumoto and Mazza, 2000). In the spring season, the highest antioxidant activities were exhibited by NokJae (IC₅₀ 1.84 mg mL⁻¹), 05-C3 (IC₅₀ 2.43 mg mL⁻¹), and BaeRiDom (IC₅₀ 2.83 mg mL⁻¹) in florets, leaves, and stems, respectively. In the fall, the antioxidant activities in florets, leaves, and stems were highest in 05-C3 (IC₅₀ 2.68 mg mL⁻¹), 05-C3 (IC₅₀ 2.48 mg mL⁻¹), and Diamond (IC₅₀ 3.66 mg mL⁻¹), respectively (Figure 4). Almost all of the cultivars showed higher antioxidant activity in the spring than in the fall. The average antioxidant activities in the spring season in florets, leaves, and stems were 2.77, 2.74, and 3.60 mg mL⁻¹, respectively. This might be due to the higher temperature in the spring because temperature has an important influence on antioxidant activity (Aldrich et al., 2011). Among the three tissue types, leaves exhibited the highest antioxidant activity,

followed by florets and stems. Cultivar-dependent variation was higher in florets in both the spring (CV 24.2%) and fall (CV 27.6%) than in leaves or stems. Similar to vitamin C, total phenol, and total flavonoid contents, antioxidant activity was also significantly influenced by C, P S and their interactions (Table 4).

Corelationships among phytonutrients

Several studies have evaluated the relationship between antioxidant activity and several antioxidants, such as vitamin C, phenolics, and flavonoids (Robards et al., 1999; Zhou and Yu, 2006; Sun et al., 2007; Aires et al., 2011; Naguib et al., 2012). To clarify the contribution of antioxidants to antioxidant activity and among phytonutrients (glucosinolates, vitamin C, total phenol and total flavonoid), we evaluated the correlations between vitamin C, phenolics, and flavonoids and antioxidant activity (Table 5). In this study, regardless of P or S, all of the glucosinolates showed significantly positive correlations with total glucosinolate contents, with the exception of glucoerucin, which was present only in stems and exhibited a non-significant negative correlation with total glucosinolates. Similarly, among the antioxidants, the total phenol content exhibited a significant positive correlation with total flavonoid content (r 0.808**), but a negative correlation with vitamin C content (r - 0.282**), and a non-

Table 5. Correlation coefficients among antioxidants and antioxidant activities in broccoli cultivars.

	Glucoraphanin	Sinigrin	Gluconapin	Glucorucin	Glucobrassicin	Total glucosinolate	Vitamin C	Total phenol	Total flavonoid	Antioxidant activity
Progoitrin	0.562**	0.983**	0.689**	-0.100	0.503**	0.837**	-0.367**	-0.205	-0.385**	-0.228*
Glucoraphanin		0.638**	0.234*	-0.098	0.269**	0.712**	-0.077	0.12	0.039	0.003
Sinigrin			0.799**	0.356	0.706**	0.881**	-0.188	0.091	-0.230	0.049
Gluconapin				-0.055	0.402**	0.594**	-0.439**	-0.204	-0.307**	-0.179
Glucorucin					-0.277**	-0.037	0.354**	-0.184	-0.169	0.247
Glucobrassicin						0.666**	-0.479**	0.378**	0.309**	0.286**
Total glucosinolate							-0.218**	0.086	-0.037	0.027
Vitamin C								-0.282**	-0.231**	-0.028
Total phenol									0.808**	0.674**
Total flavonoid										0.497**

*, **, Correlation is significant at $p < 0.05$ and 0.01 , respectively.

significant positive correlation with total glucosinolate content ($r = 0.086^{NS}$). Similarly, antioxidant activity exhibited a significantly positive correlation with total phenol content ($r = 0.674^{**}$) and total flavonoid content ($r = 0.497^{**}$). In contrast, non-significant positive and negative correlations were observed between antioxidant activity and total glucosinolate ($r = 0.027^{NS}$) and vitamin C ($r = -0.028^{NS}$) contents, respectively. The stronger correlations between antioxidant activity and total phenol content in this study are in agreement with Tavarini et al. (2008), Olajire and Azeez (2011), and Naguib et al. (2012), possibly due to the contribution of the high concentration of phenolics to antioxidant activity.

Conclusion

In this study, we identified changes in the amounts of various phytochemicals and in antioxidant activity in various broccoli tissues in different growing seasons. In most cases, the amounts of phytochemicals and antioxidant activities were

significantly affected by C, P, S and their interactions. The average contents of vitamin C, glucosinolate, total phenol, and total flavonoid and antioxidant activities were significantly higher in florets in the spring than in the fall. Furthermore, florets exhibited the highest cultivar-dependent and season-dependent variation of all of the phytochemicals relative to leaves and stems. Similarly, leaves exhibited higher contents of phytochemicals in the fall. Stems exhibited higher levels of some of the phytochemicals in both seasons, indicating that seasonal variation in the phytochemical content of broccoli is dependent not only on genotype but is also affected by tissue type and the particular phytochemical compound. Similarly, leaves and stems had higher contents of vitamin C, total phenol, and total flavonoids than florets, indicating that leaves and stems are good sources of these phytochemicals.

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REFERENCES

- Aires A, Fernandes C, Carvalho R, Bennett RN, Saavedra MJ, Rosa EAS (2011). Seasonal effects on bioactive compounds and antioxidant capacity of six economically important *Brassica* vegetables. *Molecules* 16: 6816–6832.
- Aldrich HT, Kendall P, Bunning M, Stonaker F, Kulen O, Stushnoff C (2011). Environmental temperatures influence antioxidant properties and mineral content in broccoli cultivars grown organically and conventionally. *J. Agron. Crop Sci.* 2: 1–10.
- Balouchi Z, Peyvast G-A, Ghasemnezhad M, Saadatian M (2011). Changes of antioxidant compounds of broccoli (*Brassica oleracea* L. var. *Italica*) during storage at low and high temperatures. *South-west. J. Hortic. Biol. Environ.* 2: 193–212.
- Charron CS, Sams CE (2004). Glucosinolate content and myrosinase activity in rapid-cycling *Brassica oleracea* grown in a controlled environment. *J. Am. Soc. Hortic. Sci.* 129: 321–330.
- Chen S, Andreasson E (2001). Update on glucosinolate metabolism and transport. *Plant Physiol. Biochem.* 39: 743–758.

- Chu Y-F, Sun J, Wu X, Liu RH (2002). Antioxidant and antiproliferative activities of common vegetables. *J. Agric. Food Chem.* 50: 6910–6916.
- Cohen JH, Kristal AR, Stanford JL (2000). Fruit and vegetable intakes and prostate cancer risk. *J. Natl. Cancer Inst.* 92: 61–68.
- Eberhardt MV, Kobira K, Keck AS, Juvik JA, Jeffery EH (2005). Correlation analyses of phytochemical composition, chemical, and cellular measures of antioxidant activity of broccoli (*Brassica oleracea* L. Var. *italica*). *J. Agric. Food Chem.* 53: 7421–7431.
- Fabek S, Toth N, Redovnikovic IR, Custic MH, Benko B, Zutic I (2012). The effects of nitrogen fertilization on nitrate accumulation, and the content of minerals and glucosinolates in broccoli cultivars. *Food Technol. Biotechnol.* 50: 183–191.
- Fahey JW, Zalcmann AT, Talalay P (2001). The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 56: 5–51.
- Fukumoto LR, Mazza G (2000). Assessing antioxidant and prooxidant activities of phenolic compounds. *J. Agric. Food Chem.* 48: 3597–3604.
- Gliszczynska-Swiglo A, Ciska E, Pawlak-Lemanska K, Chmielewski J, Borkowski T, Tyrakowska B (2006). Changes in the content of health-promoting compounds and antioxidant activity of broccoli after domestic processing. *Food Addit. Contam.* 23: 1088–1098.
- Gundgaard J, Nielsen JN, Olsen J, Sorensen J (2003). Increased intake of fruit and vegetables: estimation of impact in terms of life expectancy and healthcare costs. *Public Health Nutr.* 6: 25–30.
- Hakala M, Lapvetelainen A, Huopalahti R, Kallio H, Tahvonen R (2003). Effects of varieties and cultivation conditions on the composition of straw berries. *J. Food Comp. Anal.* 16: 67–80.
- Hounsom N, Hounsom B, Tomos D, Edwards-Jones G (2009). Changes in antioxidant compounds in white cabbage during winter storage. *Postharvest Biol. Technol.* 52: 173–179.
- Howard LR, Pandjaitan N, Morelock T, Gil MI (2002). Antioxidant capacity and phenolic content of spinach as affected by genetics and growing season. *J. Agric. Food Chem.* 50: 5891–5896.
- Jeffery EH, Brown AF, Kurilich AC, Keck AS, Matusheski N, Klein BP, Juvik JA (2003). Variation in content of bioactive components in broccoli. *J. Food Comp. Anal.* 16: 323–330.
- Justen VL, Fritz VA, Cohen JD (2012). Seasonal variation in glucosinolate accumulation in turnips grown under photosensitive nettings. *Hortic. Environ. Biotechnol.* 53: 108–115.
- Kirkegaard JA, Sarwar M (1998). Biofumigation potential of brassicas. *Plant Soil* 201: 71–89.
- Koh E, Wimalasiri KMS, Chassy AW, Mitchell AE (2009). Content of ascorbic acid, quercetin, kaempferol and total phenolics in commercial broccoli. *J. Food Comp. Anal.* 22: 637–643.
- Koleva II, van Beek TA, Linssen JPH, de Groot A, Evtatieva LN (2002). Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. *Phytochem. Anal.* 13 (1): 8–17.
- Kurilich AC, Tsau GJ, Brown A, Howard L, Klein BP, Jeffery EH, Kushad M, Wallig MA, Juvik JA (1999). Carotene, tocopherol, and ascorbate contents in subspecies of *Brassica oleracea*. *J. Agric. Food Chem.* 47: 1576–1581.
- Lee JG, Bonnema G, Zhang N, Kwak JH, de Vos RC, Beekwilder J (2013). Evaluation of glucosinolate variation in a collection of turnip (*Brassica rapa*) germplasm by the analysis of intact and desulfo glucosinolates. *J. Agric. Food Chem.* 61: 3984–3993.
- Lee JG, Kwak JH, Um YC, Lee SG, Jang Y-A, Choi CS (2012). Variation of glucosinolate contents among domestic broccoli (*Brassica oleracea* L. var. *italica*) accessions. *Korean J. Hortic. Sci. Technol.* 30: 743–750.
- Lin CH, Chang CY (2005). Textural change and antioxidant properties of broccoli under different cooking treatments. *Food Chem.* 90: 9–15.
- Manach C, Mazur A, Scalbert A (2005). Polyphenols and prevention of cardiovascular diseases. *Curr. Opin. Lipidol.* 16: 77–84.
- Merzlyak MN, Solovchenko AE (2002). Patterns of pigment changes in apple fruits during adaptation to high sunlight and sunscald development. *Plant Physiol. Biochem.* 40: 679–684.
- Naguib AEMM, El-Baz FK, Salama ZA, Hanaa HAEB, Ali HF, Gaafar AA (2012). Enhancement of phenolics, flavonoids and glucosinolates of broccoli (*Brassica oleracea*, var. *italica*) as antioxidants in response to organic and bio-organic fertilizers. *J. Saudi Soc. Agric. Sci.* 11: 135–142.
- Nath A, Bagchi B, Misra LK, Deka BC (2011). Changes in post-harvest phytochemical qualities of broccoli florets during ambient and refrigerated storage. *Food Chem.* 127: 1510–1514.
- Nilsson J, Olsson K, Engqvist G, Ekvall J, Olsson M, Nyman M, Akesson B (2006). Variation in the content of glucosinolates, hydroxycinnamic acids, carotenoids, total antioxidant capacity and low-molecular-weight carbohydrates in *Brassica* vegetables. *J. Sci. Food agric.* 86: 528–538.
- Olajire AA, Azeez L (2011). Total antioxidant activity, phenolic, flavonoid and ascorbic acid contents of Nigerian vegetables. *Afr. J. Food Sci. Technol.* 2: 22–29.
- Picchi V, Migliori C, Lo Scalzo R, Campanelli G, Ferrari V, Di Cesare LF (2012). Phytochemical content in organic and conventionally grown Italian cauliflower. *Food Chem.* 130: 501–509.
- Podsedek A (2007). Natural antioxidants and antioxidant capacity of *Brassica* vegetables: a review. *LWT- Food Sci. Technol.* 40: 1–11.
- Pourcel L, Routabou JM, Cheyrier V, Lepiniec L, Debeaujon L (2007). Flavonoid oxidation in plants: from biochemical properties to physiological functions. *Trends Plant Sci.* 12: 29–36.
- Rice-Evans CA, Miller NJ, Bolwell PG, Bramley PM, Pridham JB (1995). The relative antioxidant activity of plant-derived polyphenolic flavonoids. *Free Radic. Res.* 22: 375–383.
- Robards K, Prenzler PD, Tucker G, Swatsitang P, Glover W (1999). Phenolic compounds and their role in oxidative processes in fruits. *Food Chem.* 66: 401–436.
- Rosa EAS, Rodrigues AS (2001). Total and individual glucosinolate content in 11 broccoli cultivars grown in early and late seasons. *HortScience* 36: 56–59.
- Samec D, Bogovic M, Vincek D, Martincic J, Salopek-Sondi B (2013). Assessing the authenticity of the white cabbage (*Brassica oleracea* var. *capitata* f. *alba*) cv. 'Varaždinski' by molecular and phytochemical markers. *Food Res. Int.* <http://dx.doi.org/10.1016/j.foodres.2013.07.015>.
- Samec D, Piljac-Zegarac J, Bogovic M, Habjanic K, Gruz J (2011). Antioxidant potency of white (*Brassica oleracea* L. var. *capitata*) and Chinese (*Brassica rapa* L. var. *pekinensis* (Lour.)) cabbage: The influence of development stage, cultivar choice and seed selection. *Sci. Hortic.* 128: 78–83.
- Sang JP, Minchinton IR, Johnstone PK, Truscott RJ (1984). Glucosinolate profiles in the seed, root and leaf tissue of cabbage, mustard, rapeseed, radish and swede. *Can. J. Plant Sci.* 64: 77–93.
- Singh J, Upadhyay AK, Prasad K, Bahadur A, Rai M (2007). Variability of carotenes, vitamin C, E and phenolics in *Brassica* vegetables. *J. Food Comp. Anal.* 20: 106–112.
- Singleton VL, Rossi Jr JA (1965). Colorimetric of total phenols with phosphomolybdenic-phosphotungstic acid reagents. *Am. J. Enol. Viticult.* 16: 144–158.
- Spinola V, Mendes B, Camara JS, Castilho PC (2012). An improved and fast UHPLC-PDA 388 methodology for determination of L-ascorbic and dehydroascorbic acids in fruits and vegetables. 389 Evaluation of degradation rate during storage. *Anal. Bioanal. Chem.* 403: 1049–1058.
- Sun T, Powers JR, Tang J (2007). Evaluation of the antioxidant activity of asparagus, broccoli and their juices. *Food Chem.* 105: 101–106.
- Tavarini S, Degl'Innocenti E, Remorini D, Massai R, Guidi L (2008). Antioxidant capacity, ascorbic acid, total phenols and carotenoids changes during harvest and after storage of Hayward kiwifruit. *Food Chem.* 107: 282–288.
- Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem. Biol. Interact.* 160: 1–40.
- Vallejo F, Tomas-Barberan FA, Garcia-Viguera C (2002). Potential bioactive compounds in health promotion from broccoli cultivars grown in Spain. *J. Sci. Food Agric.* 82: 1293–1297.
- Vallejo F, Tomas-Barberan FA, Garcia-Viguera C (2003). Effect of climatic and sulphur fertilization conditions, on phenolic compounds and vitamin C, in the inflorescences of eight broccoli cultivars. *Eur. Food Res. Technol.* 216: 395–401.
- Williamson G, Faulkner K, Plumb GW (1998). Glucosinolates and phenolics as antioxidants from plant foods. *Eur. J. Cancer Prev.* 7: 17–21.

- Yao L, Caffin N, D'arcy B, Jiang Y, Shi J, Singanusong R, Liu X, Datta N, Kakuda Y, Xu Y (2005). Seasonal variations of phenolic compounds in Australia-grown tea (*Camellia sinensis*). *J. Agric. Food Chem.* 53: 6477–6483.
- Zhang D, Hamauzu Y (2004). Phenolics, ascorbic acid, carotenoids and antioxidant activity of broccoli and their changes during conventional and microwave cooking. *Food Chem.* 88: 503–509.
- Zhishen J, Mengcheng T, Jianming W (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem.* 64: 555–559.
- Zhou K, Yu L (2006). Total phenolic contents and antioxidant properties of commonly consumed vegetables grown in Colorado. *LWT- Food Sci. Technol.* 39: 1155–1162.

Full Length Research Paper

Antibacterial activity of honey and medicinal plant extracts against Gram negative microorganisms

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There has been a steady rise in antibiotic resistance of bacteria and this urgently calls for the discovery of alternative therapeutic agents. Honey possesses therapeutic potentials which includes antimicrobial activity. Although the antimicrobial activity of honey has been effectively established against an extensive spectrum of microorganisms, it differs depending on the type of honey. To date, not much extensive studies of the antibacterial properties of South African honeys on enteric microorganisms have been conducted. The objective of this study was to compare the antibacterial activity of extracts of six different honeys with those of medical plants commonly used in South Africa. Using a broth dilution method, the antibacterial activity extracts of six South African honeys and medicinal plants against six enteric microorganisms viz- *Enterobacter cloacae*, *Escheriachia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii* isolated from geophagia samples and *Aeromonas hydrophila* and *Plesiomonas shigelloides* isolated both from stool and water samples using agar well diffusion method was done. Different concentrations of honey and plant extracts were tested against each type of microorganism. Briefly, two-fold dilutions of honey solutions were tested to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against each type of microorganism. Extracts from both South African honeys and medicinal plants showed zones of inhibition that ranged from 6.94 to 37.94 mm. The most susceptible bacteria were *Escheriachia coli*, *Aeromonas hydrophila* and *Plesiomonas shigelloides*. MIC and MBC values of extracts were found in the range of 0.625 to 5.000 mg/ml. Extracts of honey showed good antibacterial activity against most organisms than the standard antibiotics such as Ampicillin and Gentamycin. Honey extracts showed antibacterial activity against most microorganisms which were showing some degree of resistance to commercial antibiotics. Extracts from South African honeys and medicinal plants exhibited variable activities against different microorganisms. This result suggests that the honeys could potentially be used as an alternative therapeutic agent against certain microorganisms.

Key words: Agar well diffusion assay, honey, minimum inhibitory concentration, minimum bactericidal concentration.

INTRODUCTION

Traditional medical practise has assumed exalted status in various communities around the world (Mathabe et al., 2006). People living in rural areas prefer using traditional

medicines for the treatment of various diseases and disorders to orthodox medicines (ref). According to the World Health Organization (1999), an estimated 80% of

people living in developing countries rely on harvested wild plants for their primary health care. Several reports on the antibacterial activities of medicinal plants against pathogenic organisms abound in literatures (Obi et al., 2003; Samie et al., 2009, 2010; Eloff et al., 2005; Kaushik et al., 2009; Yasutan et al., 2009). Furthermore, plant extracts and other natural substances have been in use as per the Indian system of medicine (Ayurveda) for the treatment of diseases requiring antimicrobial drugs. One of the popular natural antimicrobial substances described in Ayurveda as a potent medicine for several uses was honey.

Honey has been used for its medicinal properties to treat a wide variety of ailments since ancient times. In particular, it has been used in wound dressings (Molan and Cooper, 2000; Kingsley, 2001). Honey in general has high sugar content but a low water content and acidity, which prevent microbial growth (Farouk et al., 1988; Tan et al., 2009). Most types of honey generate hydrogen peroxide when diluted due to the activation of the enzyme glucose oxidase, which oxidizes glucose to gluconic acid and hydrogen peroxide (Bogdanov, 1984; Bang et al., 2003). Hydrogen peroxide is the major contributor to the antimicrobial activity of honey, and the different concentrations of this compound in different honeys result in their varying antimicrobial effects (Molan, 1992).

The bactericidal action could also be ascribed to the normal acidity of honey, its high sugar content, nitrogenous or other compounds (Radwan et al., 1974; Adeleke, 2006; Basualdo et al., 2007; Namias, 2003). Honey can inhibit the growth of a wide range of bacteria, fungi, protozoa and viruses (Molan, 1992; Blair et al., 2005). Microorganisms such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* frequently are isolated from skin wounds (Tan et al., 2009). There are many reports of honey being very effective as an adjunct in the treatment of wounds, burns, skin ulcers and as an anti-inflammatory agent (Lusby, 2002). Honey also contains various constituents such as water, carbohydrates, proteins, vitamins, amino acid, energy and minerals (Abhishek, 2010). It is also known to cure anaemia and improves calcium fixation in infants (Heerng, 1998) and also reduces and cures eye cataracts and conjunctivitis (Ilechie et al., 2012). The bactericidal effects of honey are reportedly dependent on concentrations of honey used and the nature of the bacteria (Adeleke, 2006). The aim of this study therefore was to ascertain the antibacterial activities of honey in comparison to commercial antibiotics and known medicinal plants extracts.

MATERIALS AND METHODS

Collection of honey samples

Raw honey samples were collected from rural areas in the Limpopo

Province of South Africa.

Bacterial isolates

The following bacterial isolates: *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae* and *Citrobacter freundii* were isolated from geophagic samples while *Aeromonas hydrophila* and *Plesiomonas shigelloides* were isolated from water and stool samples. The isolates were identified using a range of biochemical and morphological techniques, and the Microscan walk away automated bacterial identification instrument (McDonnell Douglas Health System Company). The isolates were stored on Protect Bacterial Preserver Beads (LabSupply Pierce) at -70°C .

Plant materials used in the study

The following medicinal plants: *Carissa edulis*, *Erythrina lysistemon*, *Momordica balsamina*, *Psidium guajava* and *Ficus syscomorus* were collected based on the information received from herbalists and their consorts on the basis of their effectiveness against microbial diseases. Plant parts used were leaves, bark, roots, ripe fruits, unripe fruits and twig tips. The plants were collected from their natural environment and stored in the dark at room temperature until they were dry.

Extraction of honey

Extraction of honey was performed by using methanol; 10 g of honey was placed in a centrifuge with 25 ml of solvent and then mixed well by vortexing and shaking with hands for about 30 min. This was centrifuged at 3000 rpm for 20 min at 25°C . Supernatant was collected from each centrifuged tube in a round bottom flask by filtration. The resulting supernatant was dried under nitrogen gas at a temperature of 50°C . All extracts were put in DMSO at a concentration of 100 mg/ml as the extract of honey. All the extracts dissolved in DMSO were collected in sterilized glass tubes and used within 24 h for the evaluation of bacteriostatic and bactericidal activity.

Extraction of medicinal plants

Collected plant materials were ground into fine powder using a local traditional grinding system (Musi and Mutuli, Tshivenda). Extractions were done as previously reported by Samie et al. (2010). About, 50 g of the ground materials of each plant was extracted in 500 ml methanol under continuous shaking for 24 h. The extract was filtered through a $22\ \mu\text{m}$ paper filter. The filtrate was evaporated to dryness using a rotatory evaporator at 40°C . The residues in the form of powder materials were preserved in sterile glass bottles at room temperature until further use.

Antibacterial activity test

Bacterial suspensions were done as described by Ramalivhana and Obi (2010). Agar diffusion and micro-dilution methods were used to determine the antibacterial activity of the medicinal plant extracts against bacterial isolates. Brain heart infusion broth (BHIB) was used for the preparation of bacterial cultures. The determination of the minimum inhibitory concentrations (MICs) was done as recommended by the manufacturer's. Brain heart infusion agar

(BHIA) was used to determine the activity of the plant extracts against bacterial organisms, this was prepared according to the manufacturer's instruction.

Agar diffusion assay

Bacterial isolates were prepared to match 0.5 McFarland standards. Using the micropipette, 100 µl of organisms (BHIB or SDB) was spread over the surface of an agar plate. This procedure was the same for all test organisms. Using a sterile glass pipette, five holes were punched in each of the culture plates. One of the holes was punched in the center of the plate where 10 µl of Gentamicin was added as positive control; 10 µl of DMSO was added as a negative control in the other hole; 10 and 15 µl of the plant extracts were put in the remaining two holes. The culture plates were then incubated at 37°C for 24 h. The clear zone of inhibition around the plant extract was measured in mm. The experiments were done in triplicate.

Microdilution assay

The microdilution method was used to determine the minimum inhibitory concentrations (MICs) of the plant extracts using 96 well microtitration plates as previously described by Samie et al. (2005). One hundred and eighty-five microliter (185 µl) of the broth was added into each well in the first row of microtitration plate and 100 µl to the rest of the wells from the second row downwards. Fifteen microliter (15 µl) of the plant extracts was then added into each well on the first row (row A), starting with the positive control (Gentamicin for bacteria and Floconazole for yeast, all the antibiotics were from MAST), followed by the negative control (the 20% DMSO used to dissolve the plant extracts) and the plant extracts in the rest of the wells on that row. A twofold serial dilution was done by mixing the contents in each well of the first row and transferring 100 µl to the second well of the same column and the same was done up to the last well of the same column and the last 100 µl from the last well was discarded. Then 100 µl of yeast suspensions was added.

The results were observed after 24 h incubation at 37°C, followed by the addition of 40 µl of a 0.2% Iodo Nitro Tetrazolium (INT) solution after a further incubation of 4 h at 37°C.

Determination of minimum inhibitory concentration (MIC)

Prior to testing, each isolate was cultured from preserver beads by inoculating two beads into 9 mL of TSB and incubating for ~16 h at 37°C. Cultures obtained were diluted with TSB to obtain 2 to 3 × 10⁷ cfu/mL, the minimum to produce confluent growth at inoculation positions. The minimum inhibitory concentration (MIC) of active extract was evaluated by tube dilution method. The MICs of all the extracts were determined by dilution of the extract to various concentrations (5.000 to 0.150 mg/mL). Decreasing concentrations of methanol extracts were prepared in serial twofold dilutions using Mueller Hinton Broth (MHM). Controls were included. After an overnight incubation at 37°C, the tubes were examined for turbidity indicating the growth of the microorganisms. The lowest solution of the extract that inhibited the growth of the microorganism as detected by the lack of visual turbidity (matching the negative growth control) was designated the minimum inhibitory concentration.

Determination of minimum bactericidal concentration (MBC)

The bactericidal activities of the extracts (both honey and plant

extracts) were tested as follows: the number of the bacteria in the initial microorganism suspension was counted by the surface plate method. After ascertaining the MIC, the number of bacteria was counted in each of the tubes of broth that showed no visible turbidity after overnight incubation, and was compared with the number of bacteria in the initial microorganism suspension. According to NCCLS (1997), the lowest concentration of the extract solution that allowed less than 0.1% of the original inoculum to survive was taken to be the minimum bactericidal concentration.

Antibiotic susceptibility testing

The susceptibility of isolates to antimicrobial agents was examined by an agar diffusion method using paper disks containing the following antibiotic concentrations: Amikacin (30 µg), Ampicillin (10 µg), Gentamicin (10 µg), Cefotaxime (30 µg) and Ciprofloxacin (30 µg). Disks were purchased from Oxoid. Antimicrobial activities were interpreted according to the National Committee for Clinical Laboratory Standards (NCCLS, 1997). *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control strains.

RESULTS

In this study, we compared the MIC values of six honey extracts in comparison with the plants extracts and some commonly used antibiotics. The MBC values of honey extracts also were compared. Under visual inspection, the zones of inhibition for honey extracts ranged from 7.0 to 28.0 mm, while those for plants ranged between 7.0 mm to 29.5 mm and antibiotics from 14.5 mm to 36.0 mm (Table 1). Honey B had the highest *in vitro* activity of 28.0 mm when compared with other honey extracts against *A. hydrophila*. This was followed by extracts of honey E and F against *C. frundii* with 19.0 mm and 19.5 mm zones of inhibition respectively. All the test isolates showed some degree of sensitivity to the different honey extracts as the zones of inhibition ranged from 7.0 mm in honey D extract against *E. coli* to 28.0 mm of honey B extract against *H. hydrophila*.

On the action of honey B extract against the test isolates, the highest antibacterial activity was observed against *A. hydrophila* with 18.5 mm zone of inhibition and lowest activity of 8.5 mm against *E. cloacae* (Figure 1). Similarly, Honey B extract demonstrated the highest antimicrobial activity against *A. hydrophila* with 28.0 mm zone of inhibition and lowest activity of 8.0 mm zone of inhibition against *E. Cloacae* (Figure 2). Honey C extract showed the least activity as the highest zone of inhibition of 10.0 mm was observed against three of the test isolates; *K. pneumonia*, *A. hydrophila* and *P. shigelloides* and the lowest activity of 8.5 mm was against *E. coli* (Figure 3). The lowest activity of 7.0 mm was demonstrated by honey D extract against *E. coli* while the highest activity of this extract was observed on *A. hydrophila* with a zone of inhibition of 15.5 mm (Figure 4). Both extracts from honey E and F had the highest antibacterial activities of 19.0 and 19.5 mm zones of inhibition respectively against *C. frundii* while the lowest activity of Honey E was observed against *E. cloacae* and

Table 1. *In vitro* antibacterial activities of honey, antibiotics and medicinal plants against Gram negative bacteria.

Parameter	Zone of inhibition (mm diameter)					
	<i>Enterobacter cloacae</i>	<i>Escherichia coli</i>	<i>Klebsiella pneumonia</i>	<i>Citrobacter freundii</i>	<i>Aeromonas hydrophila</i>	<i>Plesiomonas shigelloides</i>
Honey A	8.5	10.0	9.0	15.5	18.5	10.0
Honey B	8.0	11.0	8.5	12.0	28.0	15.5
Honey C	9.0	8.5	10.0	9.0	10.0	10.0
Honey D	8.5	7.0	11.5	8.5	15.5	8.5
Honey E	9.0	9.5	12.0	19.0	10.0	9.0
Honey F	9.5	9.0	10.0	19.5	9.5	9.5
Antibiotics						
Amikacin (30 µg)	26.0	22.0	16.5	33.0	35.0	36.0
Ampicillin (10 µg)	15.5	8.5	28.0	15.5	28.0	27.0
Gentamicin (10 µg)	25.0	28.0	22.0	33.0	15.5	35.0
Cefotaxime (30µg)	15.0	33.0	19.5	22.0	15.5	19.0
Ciprofloxacin (5µg)	14.5	19.5	17.0	22.5	19.0	15.0
Medicinal plants						
<i>Carissa edulis</i>	19.0	19.5	12.0	19.0	20.0	19.0
<i>Erythrina lysistemon</i>	18.5	10.0	19.0	15.5	18.5	20.0
<i>Momordica balsamina</i>	15.0	21.0	15.5	22.0	23.0	15.5
<i>Psidium guajava</i>	19.5	25.0	20.0	29.5	19.5	20.5
<i>Ficus syscomorus</i>	8.5	7.0	11.5	8.5	15.5	8.5
DMSO	0	0	0	0	0	0

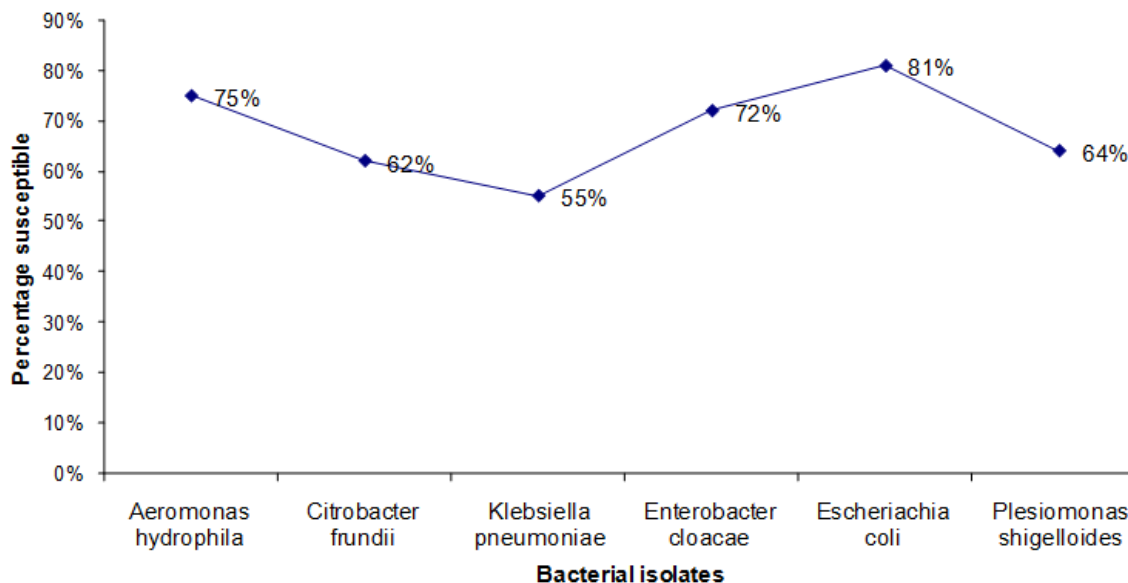


Figure 1. Antibacterial activity of Honey A against Gram negative bacteria. The highest activity was against *E. coli* and moderate activity against *K. pneumonia*.

P. shigelloides with the lowest activity of honey F extract was against *E. coli* (Figures 5 and 6). Comparing the MICs of the honey extracts to those of the plants against the bacterial isolates, the MIC of all the honey extracts against *Aeromonas hydrophila* was 1.25 mg/ml while for

the plant extracts, 0.625 mg/ml was observed for *Dodonea angeostifola* and *E. lysistemon* respectively on the same isolate. For *C. frundii*, 0.625 mg/ml of extracts of honey A and E was the lowest MIC while on the same isolate the lowest MIC of 0.3125 was noted on extracts

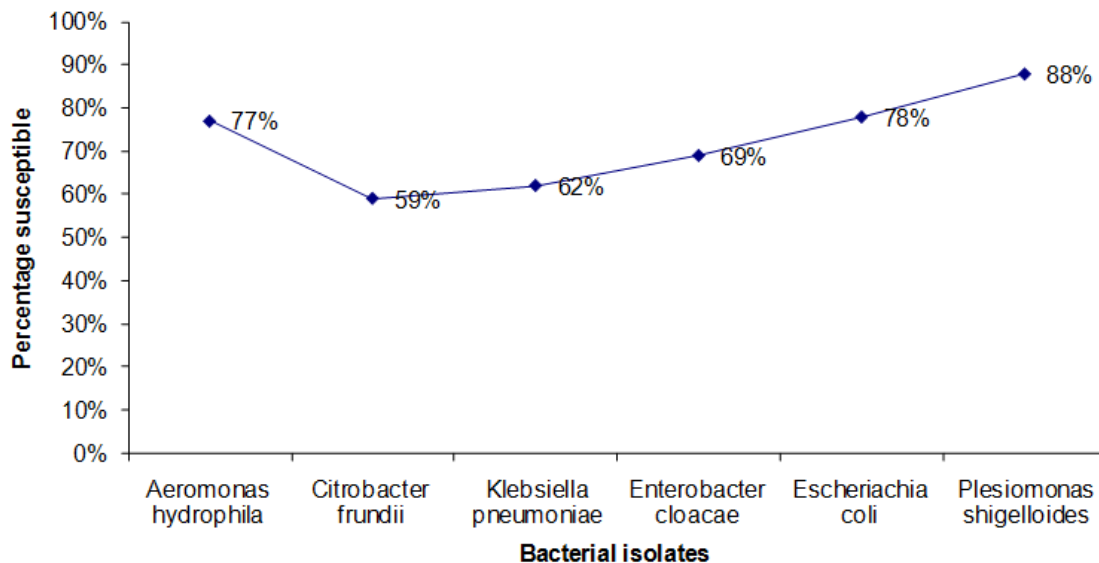


Figure 2. Antibacterial activity of Honey B against Gram negative bacteria. Activity was greatest on *P. shigelloides* and lowest in *C. frundii*.

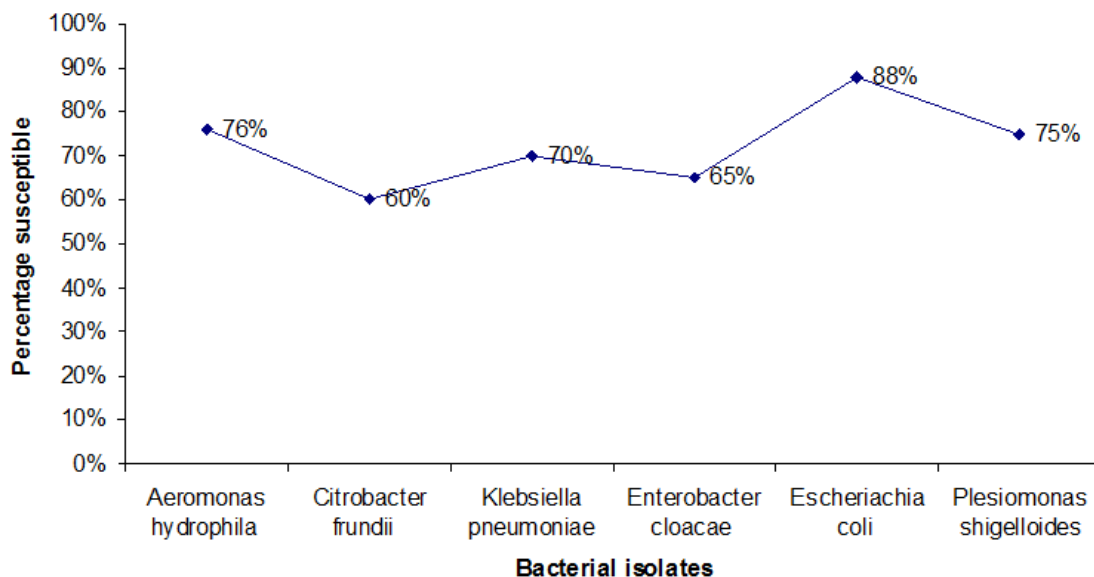


Figure 3. Antibacterial activity of Honey C against Gram negative bacteria. *E. coli* exhibited the greatest sensitivity while *C. frundii* showed moderate sensitivity.

from *D. angeostifolia* and *F. sycomorus* respectively. The MIC of 0.625 mg/ml of honey C was the most effective against *K. pneumonia* while the MIC of 0.3125 mg/ml of *P. guajava* was the lowest of all the plant extracts. The lowest MIC of 0.625 mg/ml of the honey extracts against *E. cloacae* was that from honey C while those of the plant extracts of 0.625 were those of *P. guajava* and *M. balsamina* respectively on the same bacteria isolate. For *E. coli*, the lowest MIC 0.625 was from honey E while for

the plant extracts, 0.3125 mg/ml *P. guajava* and *E. lysistemon* were noticed. Similarly, the lowest MIC of 0.625 mg/ml of honey B was noticed against *P. shigelloides* with a similar MIC for extracts of *P. guajava* and *E. lysistemon* as shown in Table 2.

Comparison of the minimum bactericidal concentrations of the both the honey and plant extracts against the test isolates showed that the bactericidal activities of the honey C and E along with those of *Dodonea angeostifolia*

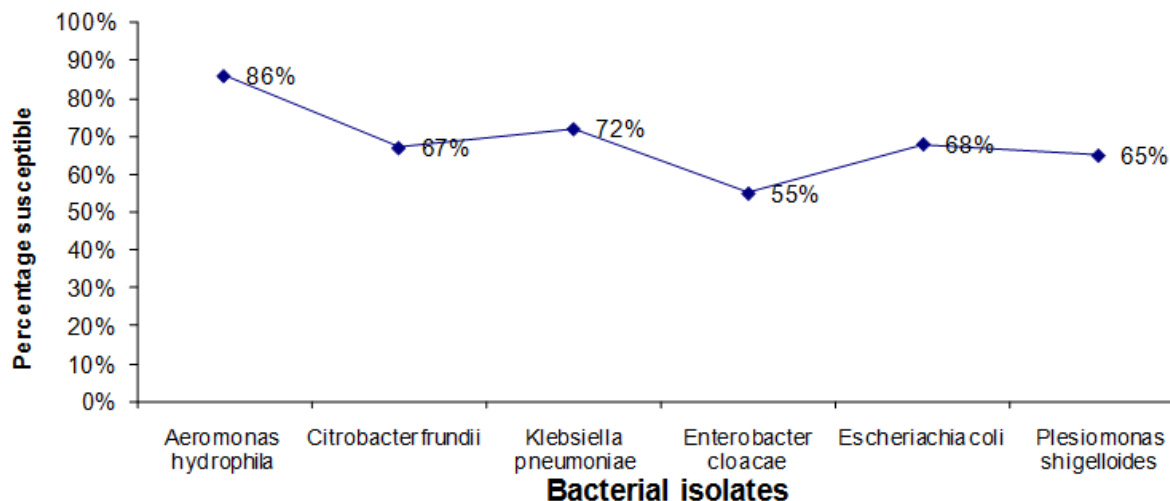


Figure 4. Antibacterial activity of honey D against gram negative bacteria. Honey D extract demonstrated a very high activity of 86% susceptibility against *A. hydrophila* with moderate effect of 55% on *E. Cloacae*.

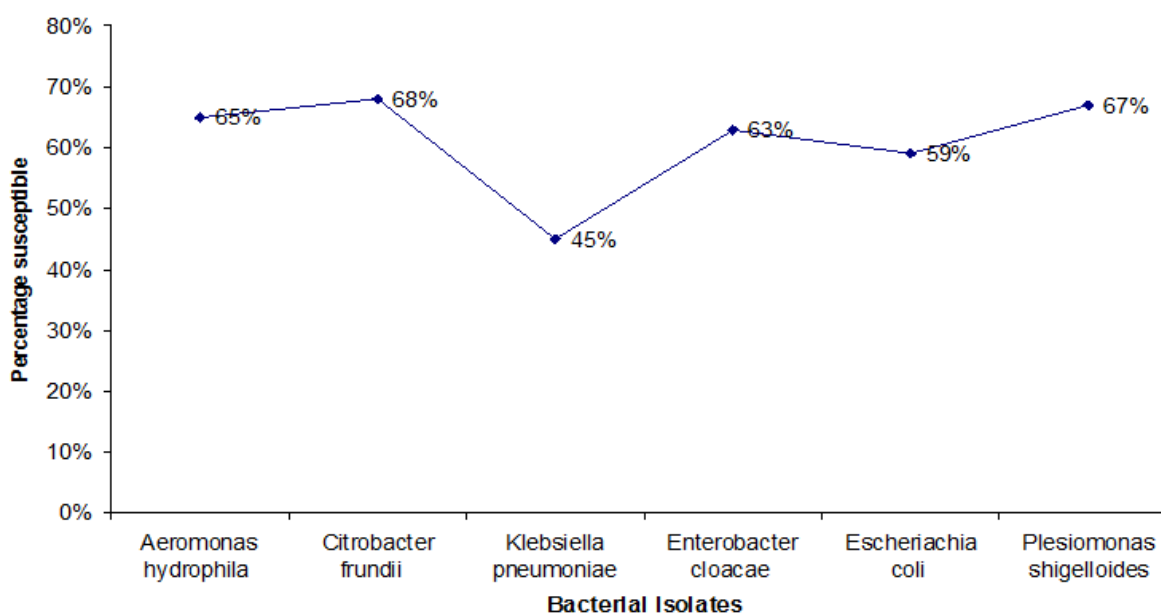


Figure 5. Antibacterial activity of Honey E against Gram negative bacteria. The highest antibacterial effect was on *C. freundii* and with the lowest activity of 45% was on *K. Pneumonia*.

and *Erythrina lysistemon* had the lowest concentration of 0.625, respectively against *Aeromonas hydrophila* while honey C and D had the lowest MBC of 0.3125 each on *Citrobacter freundii* and *Psidium guajava* and *E. lysistemon* demonstrated the lowest MBC of 0.625 on the same organism (Figure 7). For *K. pneumonia*, honey A and F extracts showed the lowest MBC of 0.3125 while *P. guajava* and *M. balsamina* demonstrated the lowest MBC of 0.625 respectively against the isolate. Honey A, B and plant extracts of *P. guajava* and *M. balsamina* each

exhibited the lowest MBC of 0.625 against *E. cloacae*. The MBC of extracts of honey A, *P. guajava*, *M. balsamina* and *E. lysistemon* against *E. coli* were 0.625 each while on *P. shigelloides*, extracts of honey C, E, *D. angeostifolia* and *E. lysistemon* demonstrated the lowest MBC of 0.625 as shown in Table 3.

The lowest MIC value of 0.312 mg/ml was observed against *K. pneumoniae*, *E. coli* and *C. freundii* using *P. guajava*, *F. sycomorus*, *D. angeostifolia* and *E. lysistemon* extracts respectively.

Table 2. MIC values of different extracts of Honey and medicinal plants.

Zone diameter of inhibition (in mm) including the diameter of well (6 mm) Isolates	Honey A	Honey B	Honey C	Honey D	Honey E	Honey F	<i>Psidium guajava</i>	<i>Momordica balsamina</i>	<i>Dodonea angeostifolia</i>	<i>Ficus sycomorus</i>	<i>Erythrina lysistemon</i>
	MIC(mg/ml)										
<i>Aeromonas hydrophila</i> (n= 200)	1.25	1.25	1.25	1.25	1.25	1.25	2.5	1.25	0.625	1.25	0.625
<i>Citrobacter freundii</i> (n=49)	0.625	1.25	1.25	1.25	0.625	1.25	0.625	0.625	0.3125	0.3125	2.5
<i>Klebsiella pneumoniae</i> (n=55)	1.25	0.625	1.25	1.25	1.25	1.25	0.3125	2.5	2.5	5	1.25
<i>Enterobacter cloacae</i> (n=46)	1.25	1.25	0.625	1.25	1.25	1.25	0.625	0.625	2.5	2.5	1.25
<i>Escheriachia coli</i> (n=88)	1.25	1.25	1.25	1.25	0.625	1.25	0.3125	1.25	1.25	1.25	0.3125
<i>Plesiomonas shigelloides</i> (n=89)	1.25	0.625	1.25	1.25	1.25	1.25	0.625	2.5	1.25	2.5	0.625

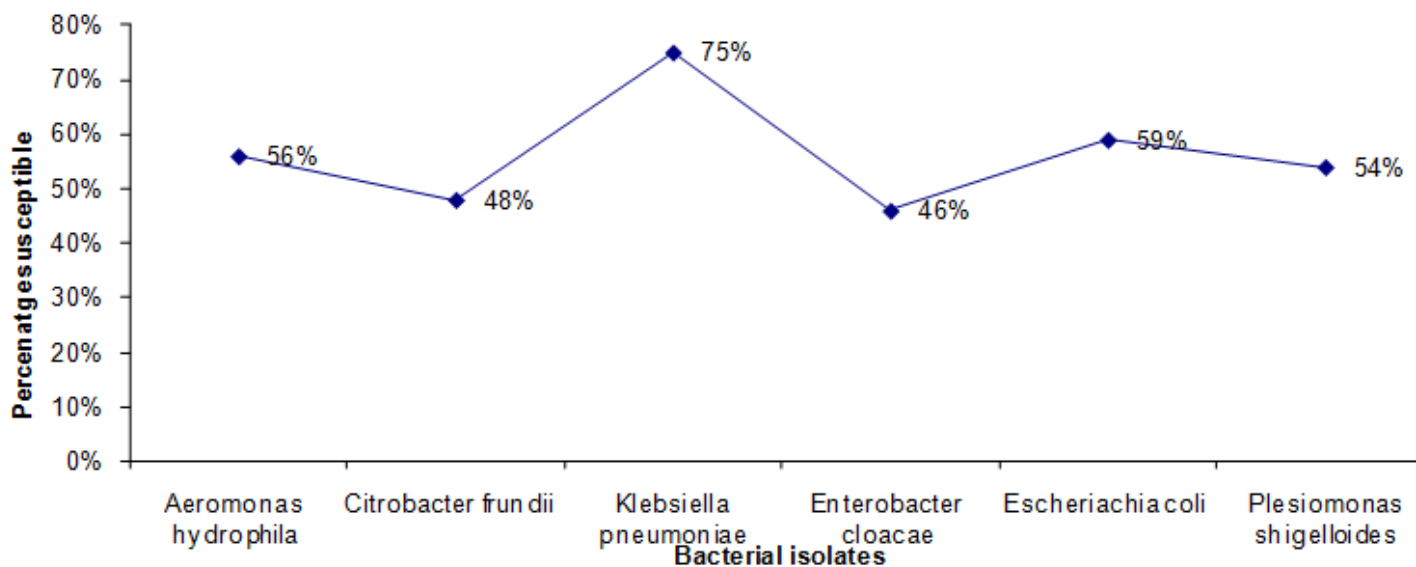


Figure 6. Antibacterial activity of Honey F against Gram negative bacteria. Honey F extract had the highest antibacterial effect on *Klebsiella pneumonia* while against the other test isolates it was moderate.

DISCUSSION

The antibacterial activities of six South African honeys and medicinal plants were investigated on

some common entero pathogens isolated from geophagist and water samples. In this study, we found that extracts of different honeys and medicinal plants commonly used in South Africa

has variable but broad-spectrum activities against many different species of enteric bacteria (Figure 8). Lusby et al. (2005) reported that honeys other than the commercially available honeys can have

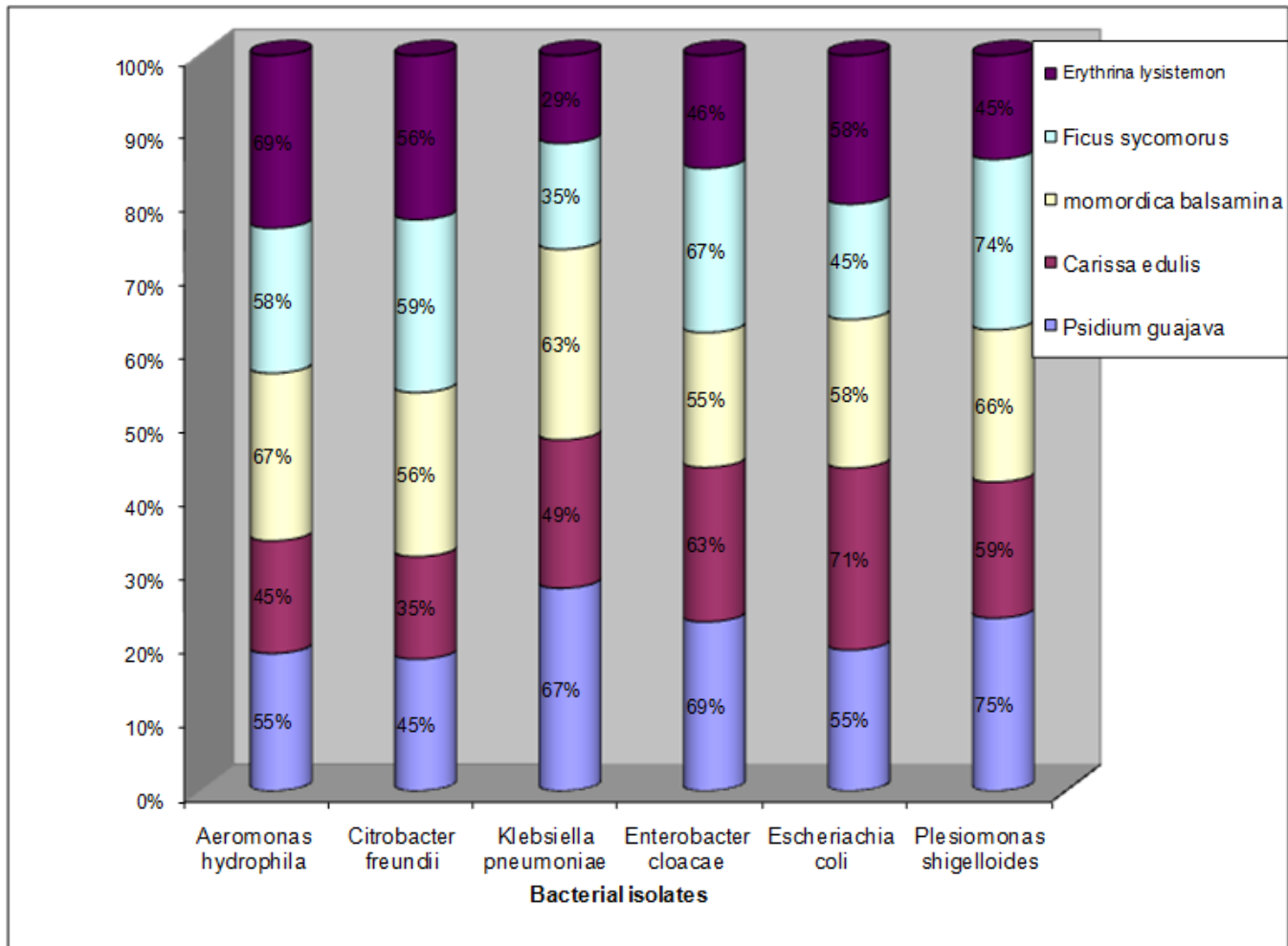


Figure 7. Antimicrobial activity of medicinal plants against Gram negative bacteria. The graph depicts the percentage activities of the different medicinal plants extracts against the test bacterial isolates. The highest antimicrobial activity of 89% was on *A. hydrophila* by the extract of *E. lysistemon* followed by 75% of *P. guajava* extract on *P. shigelloides* and 71% of *C. edulis* on *E. coli*.

equivalent antibacterial activity against some bacteria, whereas Basson and Grobler (2008) found no exceptionally high antimicrobial activity of honeys from indigenous wild flowers from South Africa. Ali et al., (1991), reported the inhibitory effects of natural honey on *Helicobacter pylori*. Analysis of the inhibition of bacterial growth caused by different honey extracts revealed similarities in the pattern of inhibition exhibited by the 6 bacterial isolates tested in this study.

Most bacteria showed similar growth inhibition patterns for all the six honeys tested, but some variations were detected. The observed differences might reflect how each type of bacteria reacts to honey treatment. That honey is effective in treating bacterial gastroenteritis in infants has been reported by Haffejee and Moosa (1985). Honey was reported to be effective when used as a substitute for glucose in oral rehydration and its antibacterial activity shortened the duration of bacterial diarrhoea (Tan et al., 2009). In our study, the growth of

bacterial species that cause gastric infections, such as *C. frundii*, *P. shigelloides* and *E. coli*, were inhibited by honey extracts.

Results obtained reveal the varying levels of the antibacterial activities of honey against bacterial isolates studied. The observations are consistent with the reports of Ibrahim (1985) on the bactericidal activity of aqueous solution of honey on *Salmonella spp.* and *Shigella spp.* and other enteropathogenens such as *E. coli*, *Vibrio cholera*, other Gram-negative and Gram-positive bacteria. Similarly, Allen et al. (2000) reported the antibacterial properties of honey against two laboratory isolates of *P. aeruginosa* and *E. coli*.

Our study is also in agreement with the study done by Samie et al. (2007) who reported on the activities of medicinal plants against 14 Gram negative microorganisms. Similarly, Obi et al. (2003) reported on the inhibitory properties of medicinal plants against a total number of fifty isolates of *E. coli* from various pathologic sources.

Table 3. MBC values of different extracts of honey and medicinal plants.

Isolate	Honey A	Honey B	Honey C	Honey D	Honey E	Honey F	<i>Psidium guajava</i>	<i>Momordica balsamina</i>	<i>Dodonea angeostifolia</i>	<i>Ficus sycomorus</i>	<i>Erythrina lysistemon</i>
	MBC(mg/ml)										
<i>Aeromonas hydrophila</i> (n= 200)	2.5	1.25	0.625	1.25	0.625	2.5	1.25	1.25	0.625	1.25	0.625
<i>Citrobacter freundii</i> (n=49)	0.625	0.625	0.3125	0.3125	2.5	0.625	0.625	2.5	1.25	1.25	0.625
<i>Klebsiella pneumoniae</i> (n=55)	0.3125	2.5	2.5	5	1.25	0.3125	0.625	0.625	2.5	2.5	1.25
<i>Enterobacter cloacae</i> (n=46)	0.625	0.625	2.5	2.5	1.25	0.625	0.625	0.625	2.5	2.5	1.25
<i>Escheriachia coli</i> (n=88)	0.3125	1.25	1.25	1.25	0.625	0.625	0.3125	0.3125	2.5	1.25	0.3125
<i>Plesiomonas shigelloides</i> (n=89)	2.5	1.25	0.625	1.25	0.625	2.5	1.25	1.25	0.625	1.25	0.625

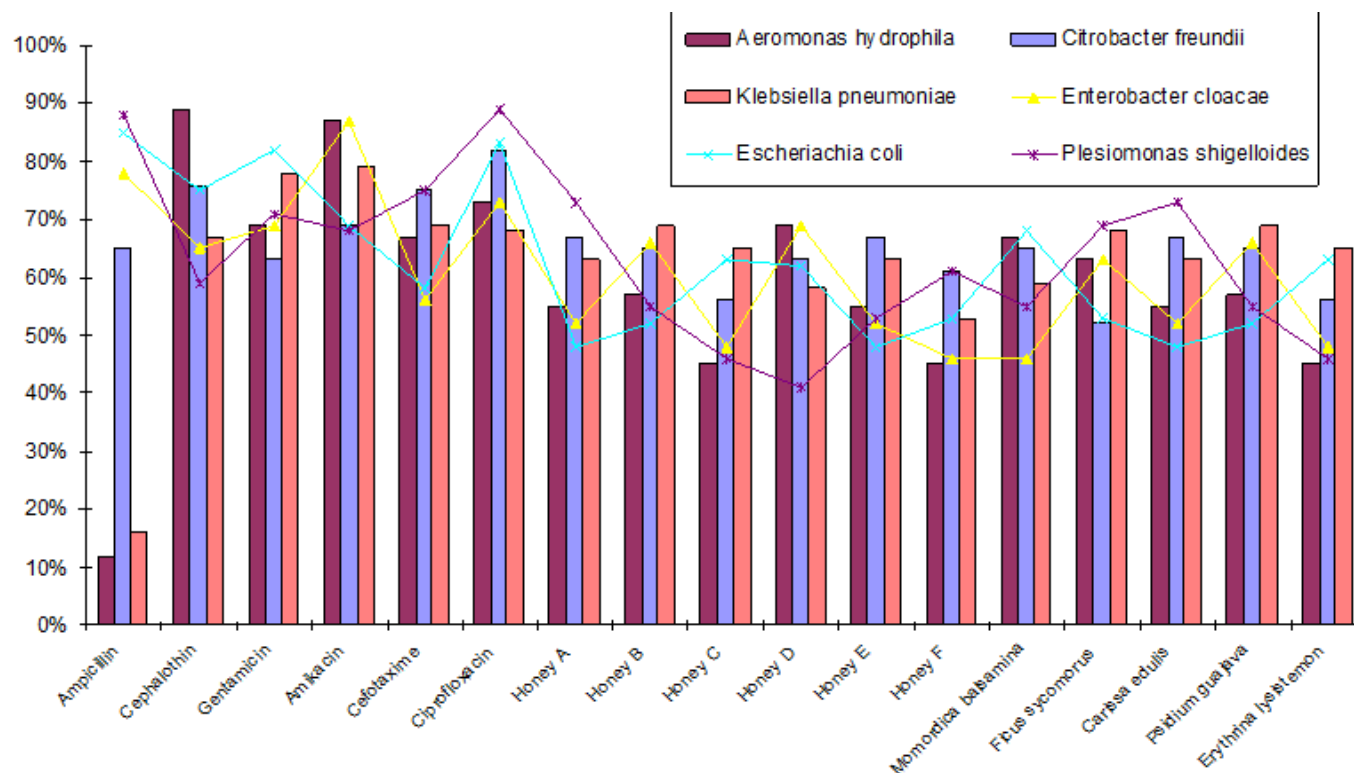


Figure 8. Summary of antimicrobial activities of antibiotics, medicinal plants and honey on bacterial isolates.

Conclusion

The six honeys and plant extracts exhibited variable activities against many different microorganisms and in some cases they showed equivalent or better activities than some antibiotics. The potency of these honeys and plants against certain microorganisms suggests their potential to be used as an alternative therapeutic agent in the face of antibiotic resistance. It will also be of great advantage if they are administered together as they could have synergistic action.

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REFERENCES

- Adeleke OE, Olaitan JO, Okepeke EI (2006). Comparative antibacterial activity of honey and Gentamicin against *Escherichia coli* and *P. aeruginosa*. Ann. Burn, fire disasters 19: 4.
- Ali AT, Chowdhury MN, Al-Humayyd MS (1991). Inhibitory effects of natural honey on *Helicobacter pylori*. Trop. Gastroenterol. 12: 139-143.
- Allen KI, Radwan S, Reid GM (2000). Antimicrobial activity of honey on some microbial isolates. J. Medic. Pharmaceut.Sci,2:75-79.
- Bang LM, Bunting C, Molan PC (2003). The effect of dilution on the rate of hydrogen peroxide production in honey and its implications for wound healing. J. Alter. Compl. Med. 9(2):267-273.
- Basson NJ, Grobler SR (2008). Antimicrobial activity of two South African honeys produced from indigenous *Leucospermum cordifolium* and *Erica* species on selected micro-organisms. BMC Compl. Alter. Med. 8:41.
- Basualdo C, Sgroy V, Finola MS, Juam M, et al. (2007). Comparison of the antibacterial activity of honey from different provenance against bacteria usually isolated from skin wounds. Vet. Microbiol. 124(3-4): 375-381.
- Blair SE, Carter DA (2005). The potential for honey in the management of wounds and infections. J. Austr. Infect. Control 10(1):24-31.
- Bogdanov S (1984). Characterization of antibacterial substance in honey. Lebensm Wiss Technol, 17(2): 74-76.
- Farouk HF, Kash SA, Khalid M, Mahasin W et al (1988). Studies on sudanese bee honey. Int. J. Crude Drug Res, 26: 161-168.
- Haffejee IE, Moosa A (1985). Honey in the treatment of infantile gastroenteritis. Br. Med. J. 290:1866-1867.
- Heerng W (1998). Immunochemical screening for antimicrobial drug residue in commercial honey, 123(12): 2759-2762.
- Ibrahim AS (1985). Antibacterial action of honey. Bull. Islam Med, 1: 363-365.
- Ilechie AA, Kwapong PK, Mate-Kole E, Kyei S, Darko-Takyi C (2012). The efficacy of stingless bee honey for the treatment of bacteria-induced conjunctivitis in guinea pigs. J. Expt. Pharm. 4: 63-68.
- Kaushik P, Goyal P, Chauhan A, Chauhan G (2009). *In vitro* evaluation of antibacterial potential of dry fruit extracts of *Elettaria cardamomum* Maton (Chhoti Elaichi). Iran. J. Pharm. Res. 9(3).
- Kingsley A (2001). Supplements. The use of Honey in treatment of infected wounds. Case studies. J. Nurs. 10(22):Sup 13-Sup 20.
- Lusby PE, Coombes A, Wilkinson JM (2002) Honey: A potent agent for wound healing? J. Wound Ostomy Continence Nurs. 29:295-300.
- Lusby PE, Coombes AL, Wilkinson JM (2005). Bactericidal activity of different honeys against pathogenic bacteria. Arch. Med. Res. 36(5):464-467.
- Molan PC (1992). The antibacterial activity of honey. Bee world 73(1):5-28.
- Molan PC, Cooper RA (2000). Why honey and sugar as dressing for wound and ulcer. Trop. Doc. 4:249-258.
- Namias S (2003) Honey in the management of infections. Surg. Infect (Larchmt), 4:219-226.
- NCCLS (1997). National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grows aerobically. Approved Standards, M7-A4, Wayne, Pa.
- Nzeako BC, Hamdi J (2000). Antimicrobial potential of Honey. Med. Sci. 2:75-79.
- Radwan SA, El-Essawy Sarhan MM et al (1984). Experimental evidence for the occurrence in honey of specific substances active against microorganisms. Zentral Mikrobiol, 39:249-255.
- Shears P (2000) Antimicrobial resistance in the tropics. *Tropical Doctor*, 30(2):114-116.
- Tan HT, Rahman RA, Gan SH, Halim AS, Hassan SA, Sulaiman SA, Kirnpal-Kaur BS (2009). The antibacterial properties of Malaysian tualang honey against wound and enteric microorganisms in comparison to manuka honey. BMC Complementary and Alternative Medicine 9:34 doi:10.1186/1472-6882-9-24.
- World Health Organization (1999). Drug information. WHO, Geneva, 13(4):230-233.

Full Length Research Paper

Establishment of a heterologous immunoassay for detecting enrofloxacin residues in poultry muscles

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A heterologous indirect competitive enzyme linked immuno sorbent assay (icELISA) has been developed for the determination of enrofloxacin (ENR) residues in poultry. For this purpose, carbodiimide active ester method was employed to synthesize the artificial antigen of ENR- bovine serum albumin (BSA) while mixed-anhydride technique was used to synthesize the coating antigen of ENR- ovalbumin (OVA), to pursue the heterologous sensitivity. By square matrix titration, an icELISA method was developed, and the linear range was from 0.02 to 86.3 ng/mL, with limit of detection (LOD) and IC_{50} value of 0.8 and 0.01 ng/mL, respectively. After optimization, 5% of NaOH was used in the assay buffer and this ELISA system can tolerate methanol not higher than 30%. The correlation coefficients (R^2) between concentration spiked and concentration determined were 0.9975 in chicken muscle and 0.9959 in duck muscle, respectively. Therefore, this assay has the potential to be incorporated into a quantitative monitoring program for the rapid screening of ENR residue in poultry muscles.

Key words: Enrofloxacin, artificial antigen, polyclonal antibody, indirect competitive enzyme linked immuno sorbent assay (ELISA), heterologous, poultry.

INTRODUCTION

Enrofloxacin (ENR) is the first specific fluoroquinolone developed for veterinary application, which belongs to the second generation of quinolone antibiotics fluorinated in position 6 and bearing a piperazinyl moiety in position 7. Similar to other fluoroquinolones, enrofloxacin is used in the treatment of systemic infections including urinary tract, respiratory, gastro-intestinal and skin infections (Tong et al., 2010). Due to the very broad spectrum of activity against both gram-negative and gram-positive bacteria and lower side effects, ENR has also been widely used for the treatment of some infectious diseases in pets and livestock. However, ENR residues may

persist in animal body and may result in the development of drug-resistant bacterial strains or allergies. In the present years, awareness of residual antibiotics in animal-derived food is growing as their application increases in both human and veterinary medicine (Yan et al., 2011).

In order to monitor enrofloxacin residue levels in livestock and poultry products, simple and rapid analytical methods are required. Various analytical methods, such as high performance liquid chromatography (HPLC) (Christodoulou et al., 2008), liquid chromatography-mass spectrometry (LC-MS) (Delepine et al., 1998; San Martin

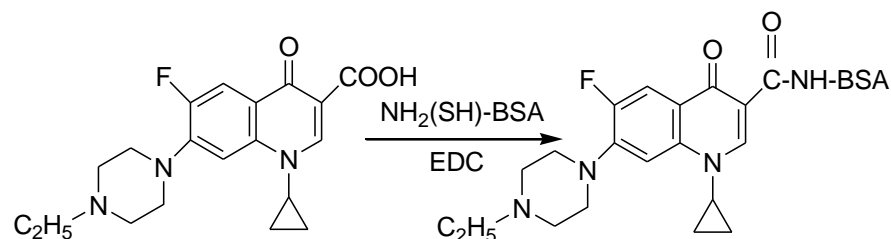


Figure 1. The synthesis procedure for ENR immunogen through EDC method.

et al., 2007), and LC-MS/MS (Dufresne et al., 2007; Hermo et al., 2008) have been reported. However, many of the instrumental methods used to monitor the residues are time-consuming, solvent intensive, and costly; therefore, chromatographic and mass spectrometry methods are not practical for screening large numbers of food samples for ENR. The enzyme-linked immunosorbent assay (ELISA) was widely employed because of its sensitivity of detection and ease of use. Several papers have reported the development of an immunoassay for the determination of ENR residue (Watanabe et al., 2002; Wang et al., 2007; Zhang et al., 2011). However, there have not yet any reports of preparation a rabbit polyclonal antibody (pAb) for ENR and development of a heterologous immunoassay. In this study, we aimed to prepare the artificial antigen of ENR and produce anti-ENR pAb. We have also developed an indirect competitive heterologous immunoassay for monitoring enrofloxacin residues in poultry muscles.

MATERIALS AND METHODS

Materials and equipment

Enrofloxacin, ciprofloxacin and other fluoroquinolones (FQs) were provided by Sigma (St. Louis, MO). 1-ethyl-3-(3-dimethylaminopropyl) (EDC), Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) were obtained from Pierce while N-hydroxysuccinimide (NHS) was from Japan; MSDS available. O-(Carboxymethyl) hydroxylamine hemihydrochloride, succinic anhydride, bovine serum albumin (BSA) and ovalbumin (OVA) were supplied by Sigma while Dialysis bag (8000-14000 Da) was from Solarbio company. The peroxidase-conjugated rabbit anti-mouse IgG (GaRlgG-HRP) was purchased from Sino-American Biotechnology Company (Shanghai, China). 3,3',5,5'-tetramethylbenzidine (TMB), phenacetin, urea peroxide were obtained from Sigma Company. All other solvents and reagents were of analytical grade or higher, unless otherwise stated. A spectrophotometric microtitre reader, Multiskan MK3 (Thermo company, USA), provided with a 450 nm filter, was used for absorbance measurements. Fresh meat samples of poultry (chicken tissue and duck) were purchased in retail outlets in Xinxiang, China. Female New Zealand white rabbits weighing 2–2.5 kg were obtained from the Laboratory Animal Center, Beijing Medical University, China, and raised under strictly controlled conditions in our laboratory chamber.

Synthesis of immunogen and coating antigen

The immunogen of ENR-BSA was prepared by carbodiimide active ester method. Briefly, a total of 4.18 mg of ENR, 20.64 mg of EDC, and 5.76 mg of NHS were added to 2 mL dimethylformamide (DMF) respectively. The mixture solution was incubated for 24 h at room temperature in dark. Then 2.5 mL of phosphate buffer saline (PBS) (0.01 mol/L, pH 7.4) with 26.4 mg BSA was added slowly to the mixture solution with stirring, followed by 4 h incubation at room temperature. The synthesis procedure is shown in Figure 1. Finally, the reaction mixture was dialyzed under stirring against PBS for 6 days with repeated changes of the PBS solution to remove the unconjugated hapten. The solution was stored at -20°C. An ENR-OVA conjugate was prepared as a modified previous paper (Huang et al., 2010) and the mixed-anhydride technique route was as shown in Figure 2.

Polyclonal antibody generation

Two female New Zealand white rabbits were subcutaneously immunized at multiple sites in the back with ENR-BSA conjugate, and under control of the local Ethical Committee for Research. The initial immunization was injected with 500 µg of conjugate in 0.5 mL of PBS and 0.5 mL of FCA. Subsequent 4 booster injections (0.5 mg of conjugate in 0.5 mL of PBS plus 0.5 mL of FIA) were performed three weeks later and then at 15 days intervals. Ten days after the last boost, all rabbits were exsanguinated by heart puncture under general anesthetic. The antiserum was prepared by allowing the blood to clot overnight at 4°C, followed by centrifugation at 10000 r/min for 20 min to remove particulate materials. The crude serum was purified by saturated ammonium sulfate (SAS) precipitation method (purified three times using SAS). The purified serum was then aliquotted and stored at -70°C.

Development of a heterologous icELISA

The checkerboard procedure was used to optimize the coating antigen and the primary antibody concentrations, resulting in the following optimized protocol. To each well of a 96 well plate, 100 µL of selected coating antigen was added and incubated for 2 h at 37°C. The plate was washed three times with PBST (PBS containing 0.05% Tween-20) and blocked with 250 µL/well of blocking buffer, followed by incubation for 1 h at 37°C. After another washing procedure, varying concentrations of ENR or competitive fluoroquinolones (50 µL/well) was added, followed by equal volume of ENR antiserum previously diluted 10,000 folds in PBS according to the optimal data. The following steps were similar to the indirect ELISA (Jiang et al., 2011). Absorbances were corrected by blank reading and the results were expressed in percent inhibition rate.

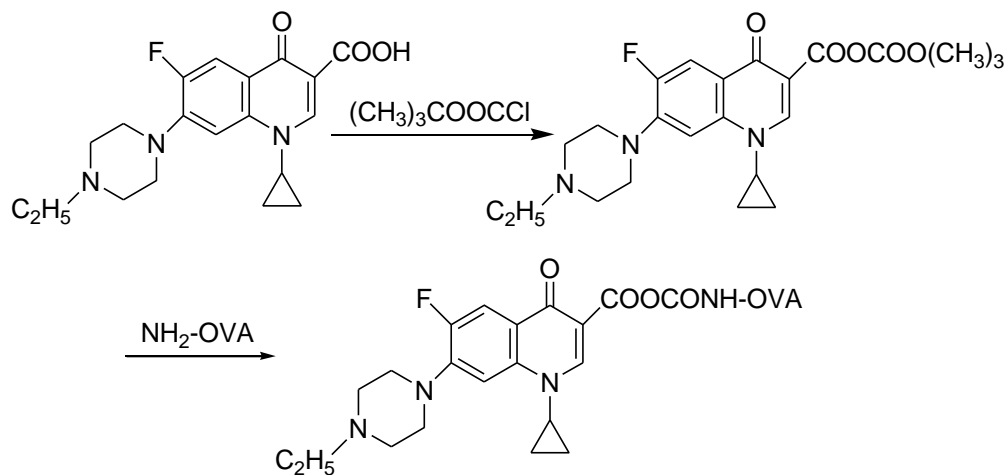


Figure 2. The synthesis procedure for ENR-OVA coating antigen through mixed-anhydride technique.

Sensitivity was evaluated according to the inhibition rate, and the data were calculated using the IC_{50} values, which represented the concentration of ENR that produced 50% inhibition of antiserum binding to the hapten conjugate. The limit of detection (LOD) was defined as the lowest concentration that exhibits a signal of 15% inhibition (Jiang and Wang, 2011). The dynamic range for the icELISA was calculated as the concentration of the analyte providing a 20–80% inhibition rate (IC_{20} – IC_{80} values) of the maximum signal. Specificity was defined as the ability of structurally related chemicals to bind to the specific antibody. The cross-reactivity was calculated as: $(\text{IC}_{50} \text{ of ENR}) / (\text{IC}_{50} \text{ of competitors}) \times 100$. The lower the CR, the higher the specificity of ENR pAb (Lei et al., 2010).

Chemical effects on assay performance

It is commonly acknowledged that immunoassay performance is often affected by chemical parameters such as ionic strength, pH values, organic solvent concentration, and other substances in the sample matrix. The effects of these parameters were estimated by the maximum absorbance (A_{max} , the absorbance value at zero concentration of ENR) and half-maximum inhibition concentration (IC_{50} , the value represents the concentration of ENR that produce 50% inhibition of antibody binding to the hapten), and the maximal $A_{\text{max}}/\text{IC}_{50}$ ratio was chosen (Hao et al., 2009). In our study, the concentrations of NaOH and methanol contents in the assay buffer have determinant effects on the standard inhibition curve, which was checked for the optimal data.

Spiking tests in poultry samples

Prior to running the assay, poultry samples (chicken and duck muscles) were homogenized, then 1 g of each meat sample was weighed into a 10 mL polypropylene centrifuge tube. Next 2 mL of 0.1 mol/L sodium hydroxide-acetonitrile (1:10) was added, and the mixture was agitated on a shaker for 10 min. The samples were centrifuged at 5000 r/min for 10 min, and 1 mL of supernatants was diluted with 9 mL of PBS (0.01 mol/L). 50 μL of this buffer solution was used in the ELISA. For spiking tests, a standard stock of ENR

was prepared by diluting the initial solution prepared in HCl (0.03 mol/L) to give a final stock solution at 1 mg/mL. The stock solution was serially diluted with PBS to give the working standard solutions. The recoveries were calculated by interpolation of the mean absorbance values on a standard curve constructed by icELISA in PBS.

RESULTS AND DISCUSSION

Hapten conjugation

UV-vis spectrum for ENR-BSA, ENR, and BSA are presented in Figure 3. The absorbance for ENR-BSA (279 and 321 nm) gave a significant shifted peak at 279 nm compared with the 269 nm peak for ENR (269, 321, and 333 nm), which indicated the ENR was successfully conjugated with BSA. The coating antigen of ENR-OVA gave a UV pattern similar to that of immunogen. Calculated from the formula, molar ratio of 18:1 for ENR-BSA conjugates was obtained.

Heterologous icELISA Standard curve

Checkerboard titrations were performed, taking into account the optimal dilutions. The optimal reagent concentrations were determined when the maximum absorbances (A_{max}) were between 1.5 and 2.0, and the dose-response curve of inhibition ratio versus the ENR concentration pursued the lowest IC_{50} values. From the checkerboard assays, a representative standard inhibition curve was obtained (Figure 4). As can be seen, the optimum concentration of coating antigen was 1.0 $\mu\text{g}/\text{mL}$ and pAb was 1:10,000 dilutions. This assay allowed the detection of ENR (20–80% inhibition of color development) from 0.02 to 86.3 ng/mL, with an IC_{50} value

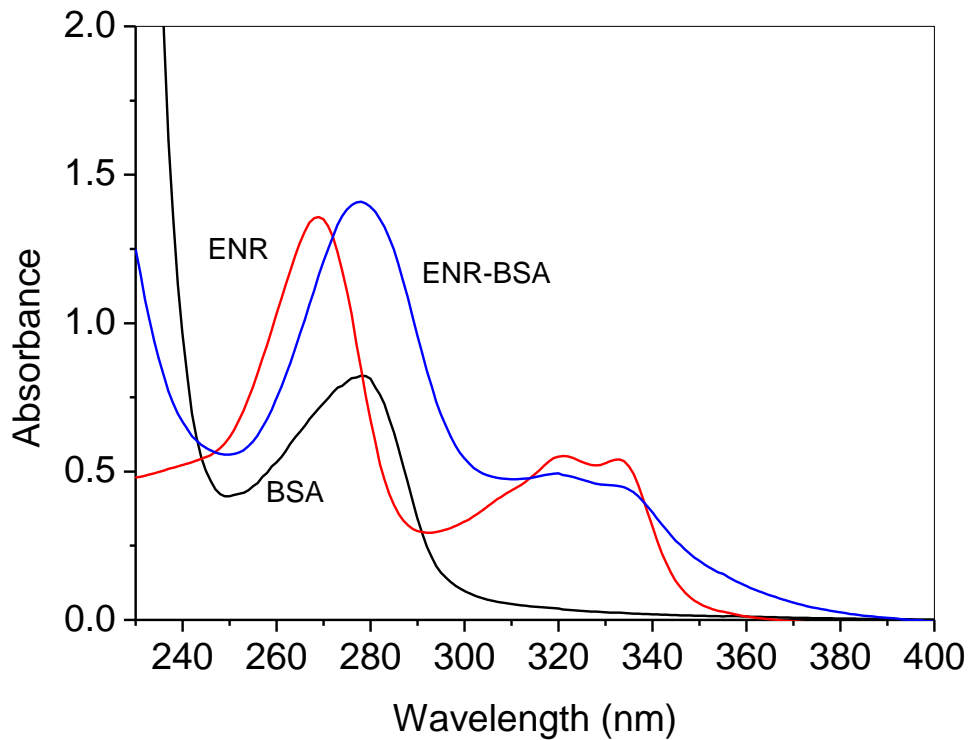


Figure 3. UV-vis spectrum for artificial antigen of ENR-BSA, BSA and ENR.

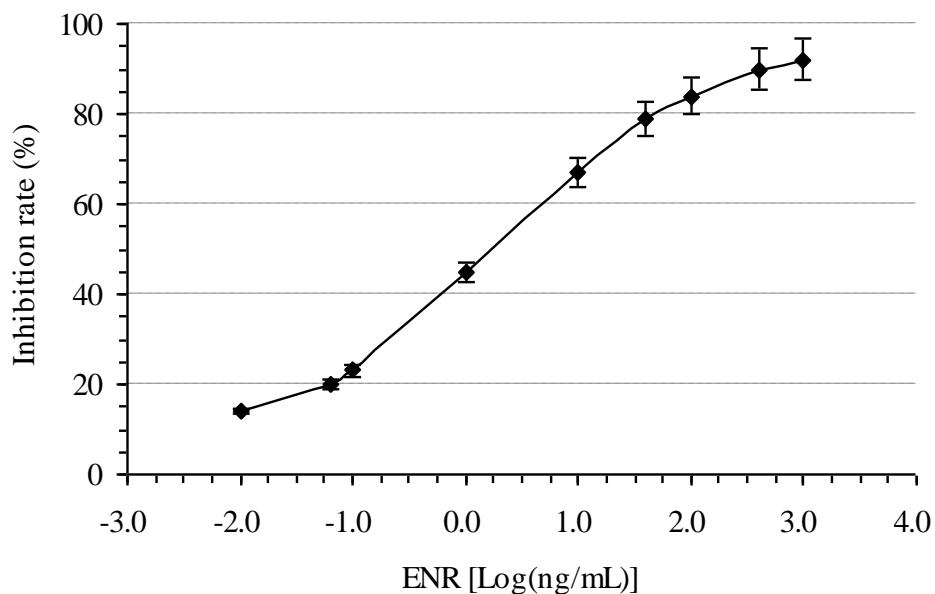


Figure 4. Optimized standard heterologous icELISA inhibition curve for ENR. Data were obtained by averaging three independent curves, each run in triplicate. ENR-OVA (1 $\mu\text{g}/\text{mL}$) as coating antigen was prepared in CBS (pH 9.6), purified anti-serum produced by ENR-BSA as immunogen was diluted 1:10 000 in PBS (pH 7.4), ENR was prepared in PBS, containing 10% methanol; GaR1gG-HRP was diluted 1:1000 in incubation buffer.

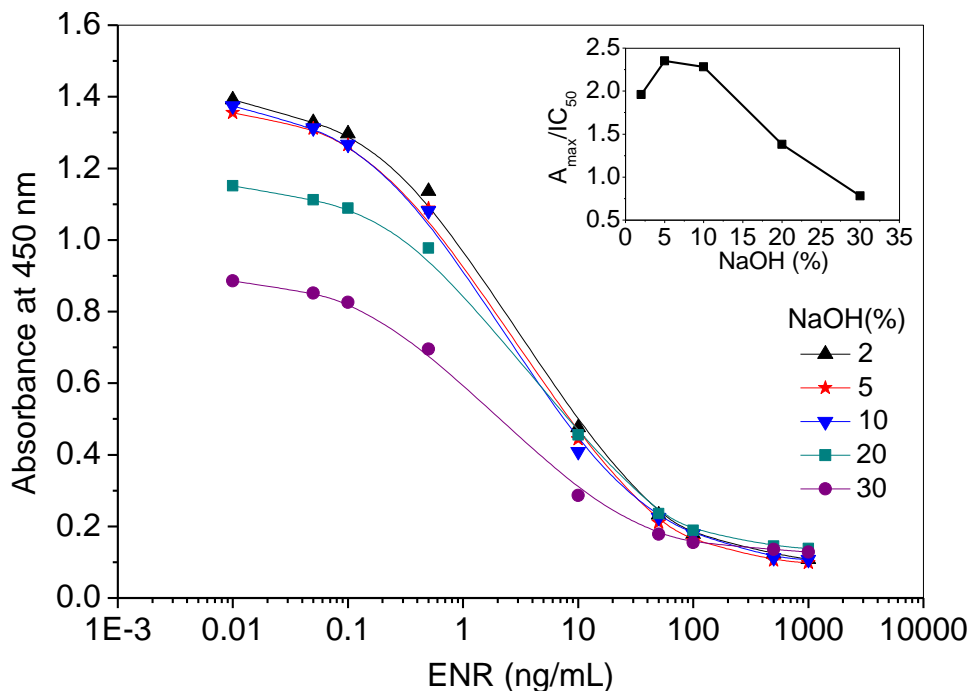


Figure 5. Effects of NaOH on the icELISA inhibition curve. Insets indicate the fluctuations of A_{\max}/IC_{50} (Y-axis) as a function of NaOH concentration (X-axis). Each value represents the mean of three replicates.

of 0.8 ng/mL. The LOD of the assay was 0.01 ng/mL.

Chemical effects

To study the influence of NaOH on the assay characteristics, competitive curves were prepared using standards in PBS which contained 2, 5, 10, 20, and 30% of NaOH. Figure 5 presents the effects of NaOH in assay solution on the ELISA. This result indicates that higher NaOH concentrations may change the ionization state of the antibody binding site, disrupt the ionic interactions, and resist the entrance of the standards. Accordingly, 5% NaOH in assay buffer provides the best conditions for the binding of antibody and coating antigen that was selected for the immunoassays.

Methanol that may interfere with antigen-antibody binding and increase the solubility of analytes, was tested for its effects on the ELISA. Figure 6 shows the normalized dose-response curves at various solvent concentrations. The results show that the IC_{50} values of the immunoassay were varying obviously when increasing the amounts of methanol. It indicated that a reproducible inhibition curve could be observed, only when the concentration of the solvent was not higher than 30%. Therefore, to accurately determine the

concentration of ENR, methanol contents in the assay buffer should be minimized.

Specificity

Specificity is a phenomenon inherent to all immunoassays, which was evaluated by determination of the cross-reactivity based on the IC_{50} values of individual chemicals. In this work, the study was undertaken by adding various competitors of functional related analogues. The cross-reactivity rate for each compound is presented in Table 1. Of all the cross-reacting analogues, this assay exhibited a high cross-reactivity to ciprofloxacin (56%), but negligible cross-reactivity to other chemicals.

Correlations between concentrations spiked and determined in poultry samples

The accuracy of the analysis was studied by comparative detection of fortified ENR in poultry muscle samples, and the measurement correlations between the fortified and analyzed concentrations are shown in Figure 7. We can find that the data spots were nearly distributed on both

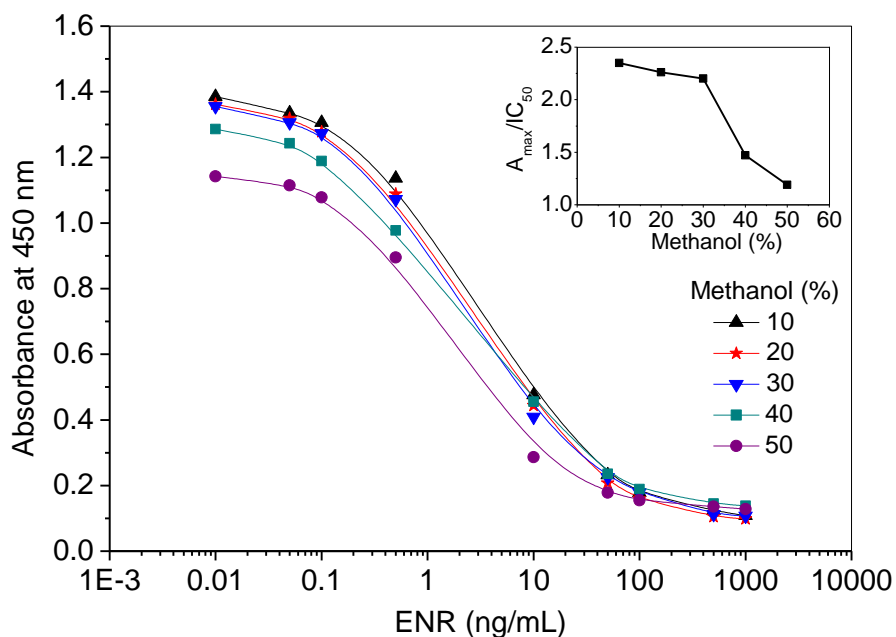


Figure 6. Effects of methanol on the icELISA inhibition curve.

Table 1. Cross-reactivities of FQs analogues in the ENR immunoassay.

Analogue	IC ₅₀ (ng/mL)	CR (%)
Enrofloxacin	0.8	100
Ciprofloxacin	1.43	56
Pefloxacin	>1600	<0.05
Danofloxacin	>1600	<0.05
Sarafloxacin	>1600	<0.05
Norfloxacin	>1600	<0.05
Lomefloxacin	>8000	<0.01
Ofloxacin	>8000	<0.01
Enoxacin	>8000	<0.01
Flumequin	>8000	<0.01

sides of the trendline. This indicates that an excellent correlation between concentration spiked and concentration determined was found, and the results also suggest the veracity of the icELISA method for detecting ENR residue in poultry tissues.

Conclusions

We have prepared a high-quality polyclonal antibody with

high specificity for ENR. The feasibility to apply this antibody in a competitive ELISA has been explored, and the results show that this heterologous icELISA has been shown to be capable of detecting ENR residue in muscle, and it can also be potentially applied in other matrices.

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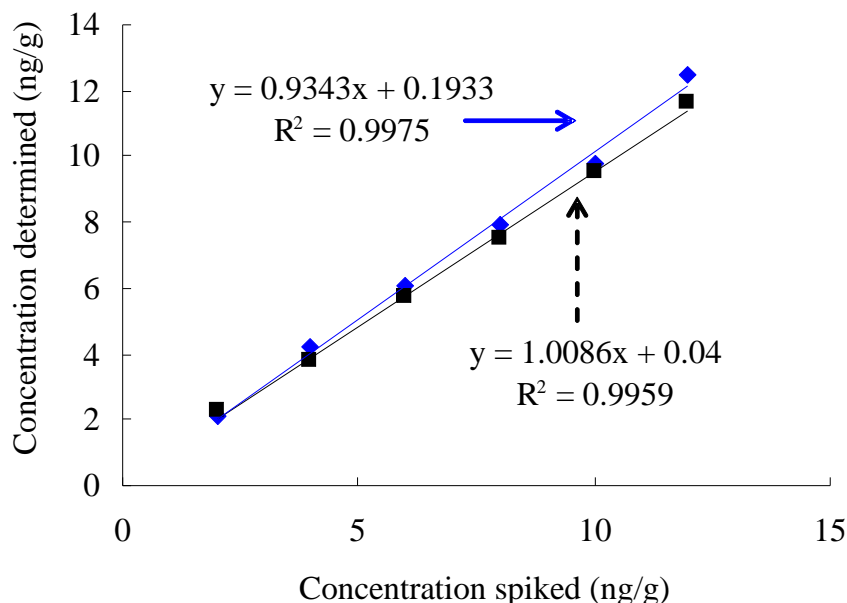


Figure 7. Correlations between concentration spiked and concentration determined in poultry muscle samples fortified with ENR. ◆, Chicken muscle; ■, duck muscle.

Abbreviations: ENR, Enrofloxacin; icELISA, indirect competitive enzyme linked immuno sorbent assay; pAb, polyclonal antibody; BSA, bovine serum albumin; OVA, ovalbumin; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; GaRiGg-HRP, the peroxidase-conjugated rabbit anti-mouse IgG; A_{max} , maximum absorbance; IC_{50} , half-maximum inhibition concentration; LOD, limit of detection; CR, cross-reactivity; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; NHS, N-hydroxysuccinimide; FQs, fluoroquinolones; PBS, phosphate buffer saline; TMB, 3,3',5,5'-tetramethylbenzidine; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; pAb, polyclonal antibody.

REFERENCES

- Christodoulou EA, Samanidou FV, Papadoyannis IN (2008). Development of an HPLC multi-residue method for the determination of ten quinolones in bovine liver and porcine kidney according to the European Union Decision 2002/657/EC. *J. Sep. Sci.* 31(1):119-127.
- Delepine B, Hurtaud-Pessel D, Sanders P (1998). Simultaneous determination of six quinolones in pig muscle by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. *Analyst.* 123(12): 2743-2747.
- Dufresne G, Fouquet A, Forsyth D, Tittlemier SA (2007). Multiresidue determination of quinolone and fluoroquinolone antibiotics in fish and shrimp by liquid chromatography/tandem mass spectrometry. *J. AOAC. Int.* 90(2):604-612.
- Hao XL, Kuang H, Li YL, Yuan Y, Peng CF, Chen W, Wang LB, Xu CL (2009). Development of an enzyme-linked immunosorbent assay for the alpha-cyano pyrethroids multiresidue in Tai lake water. *J. Agric. Food Chem.* 57(8):3033-3039.
- Hermo MP, Nemutlu E, Kir S, Barrón D, Barbosa J (2008). Improved determination of quinolones in milk at their MRL levels using LC-UV, LC-FD, LC-MS and LC-MS/MS and validation in line with regulation 2002/657/EC. *Anal. Chim. Acta.* 613(1): 98-107.
- Huang B, Yin Y, Lu L, Ding H, Wang L, Yu T, Zhu JJ, Zheng XD, Zhang YZ (2010). Preparation of high-affinity rabbit monoclonal antibodies for ciprofloxacin and development of an indirect competitive ELISA for residues in milk. *J. Zhejiang Uni. Sci. B.* 11(10): 812-818.
- Jiang Y, Huang X, Hu K, Yu W, Yang X, Lv L (2011). Production and characterization of monoclonal antibodies against small haptenciprofloxacin. *Afr. J. Biotechnol.* 10: 14342-14347.
- Jiang J, Wang Z, Zhang H, Zhang X, Liu X, Wang S (2011). Monoclonal antibody-based ELISA and colloidal gold immunoassay for detecting NT residue in animal tissues. *J. Agric. Food Chem.* 59(18): 9763-9769.
- Lei H, He Z, Yuan H, Wu J, Wen L, Li R, Zhang M, Yuan L, Yuan Z (2010). Generation and characterization of a monoclonal antibody to penicillic acid from *Penicillium cyclopium*. *Afr. J. Biotechnol.* 9: 3026-3031.
- San Martín B, Cornejo J, Iragüen D, Hidalgo H, Anadón A (2007). Depletion study of enrofloxacin and its metabolite ciprofloxacin in edible tissues and feathers of white leghorn hens by liquid chromatography coupled with tandem mass spectrometry. *J. Food Prot.* 70(8): 1952-1957.
- Tong C, Zhuo X, Liu W, Wu J (2010). Synchronous fluorescence measurement of enrofloxacin in the pharmaceutical formulation and its residue in milks based on the yttrium (III)-perturbed luminescence. *Talanta.* 82(5): 1858-1863.
- Wang Z, Zhu Y, Ding S, He F, Beier RC, Li J, Jiang H, Feng C, Wan Y, Zhang S, Kai, Z, Yang X, Shen J (2007). Development of a monoclonal antibody-based broad-specificity ELISA for fluoroquinolone antibiotics in foods and molecular modeling studies of cross-reactive compounds. *Anal. Chem.* 79(12): 4471-4483.

- Watanabe H, Satake A, Kido Y, Tsuji A (2002). Monoclonal-based ELISA and immunochromatographic assay for enrofloxacin in Biological matrices. *Analyst*. 127(1): 98-103.
- Yan H, Wang H, Qin X, Liu B, Du J (2011). Ultrasound-assisted dispersive liquid-liquid microextraction for determination of fluoroquinolones in pharmaceutical wastewater. *J. Pharm. Biomed. Anal.* 54(1): 53-57.
- Zhang L, Jiang JQ, Li GL, Wang ZL, Yang XF, Ma JY, Zhang HT, Huang HG, Liu XY (2011). Development and optimization of a monoclonal antibody-based indirect competitive ELISA for detecting fluoroquinolone residue in milk. *J. Food Agric. Environ.* 9(2): 113-120.

UPCOMING CONFERENCES

3rd Biotechnology World Congress, Dubai, UAE, 10 Feb 2014



3rd Biotechnology World Congress
February 10th - 12th, 2014, Dubai, UAE

7th Annual World Congress of Industrial Biotechnology (IBIO-2014), Dalian, China, 25 Apr 2014

ibio-2014 BIT's 7th Annual Congress of **Industrial Biotechnology**

Theme: Greener Manufacturing in Synbio Era

Date: April 25-28, 2014 Place: Dalian, China

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