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Protection from free radical insult by commercially available green tea extract: an *in vitro* study

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Abstract

Aim of present study is to explore the free radical scavenging and antioxidant property of commercially available Green Tea extract (GTE). The ability of the extract to protect cells from free radical induced cytotoxicity and cellular DNA damage were investigated. Free radical scavenging activity and antioxidant potential was assayed using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay and lipid peroxidation inhibition assay. Cytoprotective property was performed on mouse spleenocytes treated with various concentrations of H_2O_2 and GTE by trypan blue exclusion method. Alkaline single cell gel electrophoresis was performed on mouse spleenocytes treated with H_2O_2 and GTE to assess the extracts potential in protecting cellular DNA. GTE was found to exhibit potent free radical and antioxidant property. The cellular DNA was protected from free radical induced damage as observed from the decreased comet parameters. The extract also protected the cells from H_2O_2 induced cytotoxicity. GTE could cancel out the detrimental effect of oxidative stress and prevent cell death. The possible mechanism could be protection of cellular membrane and DNA from oxidative damage.

Keywords: Green Tea extract, cytotoxicity, DNA damage, Alkaline single cell gel electrophoresis

1. Introduction

Naturally occurring free radicals like reactive oxygen species (ROS) are normally produced in small quantities in the body during normal cellular metabolism. Though they are beneficial in inducing immunological response against infections and in intracellular signal transduction (Lander, 1997; Valko et al., 2007), they elicit deleterious effects when there is an overproduction in the biological system or a deficiency in the systemic antioxidant scavenging capacity, or both. The toxic effect of free radicals includes damage to the genetic material, proteins, membrane lipids, etc (Valko et al., 2004; Slater, 1984). Free radicals also have been attributed as a major reason for ageing (Harman, 1956).

The living cells have inbuilt antioxidant defence mechanism to keep free radicals under control, but when the generation of free radicals is increased due to some pathological or physiological conditions, the intrinsic antioxidant activity will be overwhelmed resulting in various toxic effects. This can be prevented by supplementing cellular antioxidants with extrinsic dietary antioxidant molecules or formulations.

Green tea has attracted attention both in the scientific and consumer communities for its health benefits. The main flavanols found in green tea are (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), and (-)-epicatechin (EC) (Henning et al., 2003). The health benefits of green tea are mainly attributed to its antioxidant properties and the ability of its polyphenolic catechins to scavenge reactive oxygen species (Yang, 1999).

The present work is aimed to investigate free radical scavenging potential of a commercially available

green tea extract, as well as to assess its ability in protecting genomic DNA from free radical induced damage under *in vitro* condition.

2. Materials and Methods

2.1.Extract and Chemicals

Green tea extract was obtained as a gift to CKKN from Parry India, for research studies. All other chemicals and reagents used in this study were of analytical grade.

2.2. Animals

Swiss albino mice of 8-10 weeks old, weighing 22-25 g was obtained from the Small Animal Breeding Section (SABS), Kerala Agricultural University, Mannuthy, Thrissur, Kerala. They were kept under standard conditions of temperature and humidity in the Centre's Animal House Facility. The animals were provided with standard mouse chow (Sai Durga Feeds and Foods, Bangalore, India) and water *ad libitum*. All animal experiments in this study were carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC) and were conducted strictly adhering to the guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of Government of India.

2.3. DPPH radical scavenging activity

The free radical scavenging activity of GTE was determined by the method of Gadov et al (1997) (Gadov et al., 1997) with minor modifications (Gandhi NM and Nair CKK, 2004). Methanolic solution of DPPH (63.4 μM) was incubated at ambient temperature with various concentrations of the GTE (0.062 – 1 mg/ml) and A_{515} was measured using a spectrophotometer. The percent of inhibition of DPPH (decolourization) was calculated according to the formula

$$\% \text{ inhibition} = \frac{A_{control} - A_{GTE} \times 100}{A_{control}}$$

2.4. Effect of GTE in mitigating free radical induced lipid peroxidation

100μl of mouse liver homogenate (10% w/v) prepared in phosphate-buffered saline was treated with various concentrations of H_2O_2 (0.25 mM – 1 mM) and GTE (50 mg/ml, 100 mg/ml). Levels in peroxidation of membrane lipids were done based on the method of Buege and Aust (1987) (Buege and Aust, 1978). The final result was expressed as nanomoles of malonedialdehyde per mg protein.

2.5. Cytoprotecive effect of GTE

Spleen was obtained from Swiss albino mice and made into a single cell suspension of spleenocytes. The cell suspension (10^6 cells/ml) was treated with different concentrations of green tea extracts (0.1 - 10 mg/ml) in the presence of oxidative stress produced by the addition of 0.5 mM H_2O_2. The viability of cells was determined at various time intervals (0 - 3 hr) by trypan blue exclusion method (Guptha and Bhattacharya, 1974). Percentage cytotoxicity or mortality was calculated.

2.6.Effect of GTE on oxidative cellular DNA damage

Liver cells were (10^6 cells/ml) incubated for 5 minutes in presence and absence of 0.5 mM of H_2O_2 and various concentration of green tea extract (5 - 20 mg/ml). DNA damage was analyzed by employing the technique of alkaline single cell gel electrophoresis (comet assay).

2.7. Alkaline single-cell gel electrophoresis (comet assay)

Alkaline single-cell gel electrophoresis was performed using the method given by Singh (2000) (Singh, 2000), with minor modifications (Chandrasekharan et al., 2009). Microscope slides were coated with normal melting point agarose (1% in PBS) and kept at 4°C till the agarose was solidified. On these slides, 200 μl of 0.8% low melting point agarose containing 50 μl of treated cells were added. After solidification of the low melting agarose, the slides were immersed in pre-chilled lysing solution containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, pH 10, 1% DMSO, 1% Triton X and kept for 1 hour at 4°C for lysis of the cells. After lysis, the slides were drained properly and placed in a horizontal electrophoretic apparatus filled with freshly prepared electrophoresis buffer containing 300 mM NaOH, 1 mM EDTA, 0.2% DMSO, pH $\geq$ 13. The slides were equilibrated in buffer for 20 minutes and electrophoresis was carried out for 30 minutes at 20 V. After electrophoresis the slides were washed gently with 0.4 mM Tris-HCl buffer, pH 7.4, to remove alkali. The slides were again washed with distilled water, kept at 37°C for 2 hours, to dry the gel and silver staining was carried out. The comets were visualised under a binocular microscope and the images captured were analysed using the software 'CASP' to find out the extent of DNA damage measured in terms of different comet parameters such as % DNA in tail, tail length, Tail Moment (TM) and Olivetail Moment (OTM) (Konca et al., 2003). The parameter tail moment is the product of tail length and % DNA in tail, and olive tail moment is the product of the distance between the centre of gravity of the head and the centre of gravity of the tail and % DNA in tail. Results are presented as mean ± standard deviation.

2.8. Statistical analysis

The results are presented as mean ± standard deviation of the studied groups. The statistical analyses of the results were performed using ANOVA with Tukey–Kramer multiple comparisons test.

3. Results

3.1. *DPPH radical scavenging activity*

The results showed that GTE could scavenge DPPH radical in a concentration dependent manner. Figure 1 represents the DPPH radical scavenging activity of various concentration of the extract. IC_{50} value for GTE was found to be 0.03 mg/ml.

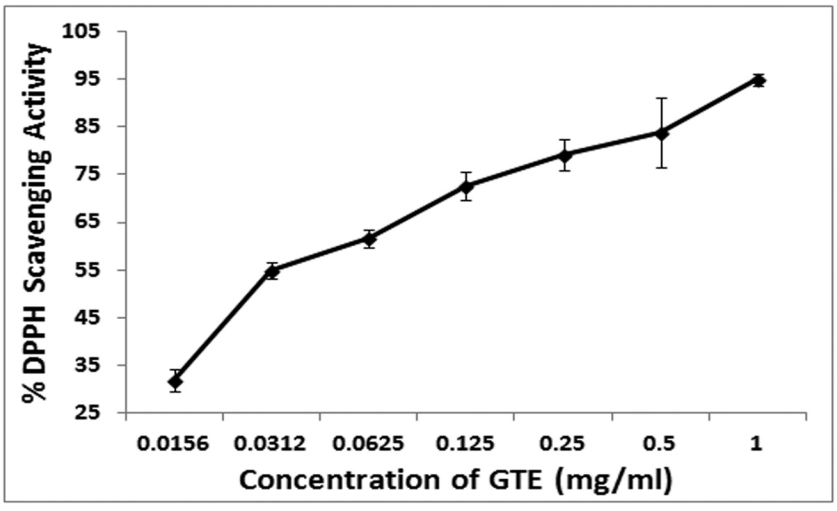

Figure 1: DPPH radical scavenging activity of GTE.

3.2. *Effect of GTE in mitigating free radical induced lipid peroxidation*

The capacity for GTE to prevent lipid peroxidation was assayed using malondialdehyde formation as an index of oxidative breakdown of membrane lipids, following incubation of mouse liver homogenates with various concentrations of oxidant chemical species H_2O_2 (0.25 – 1 mM). From figure 2 it can be observed that, H_2O_2 induced peroxidation of membrane lipids in a dose dependent manner while the presence of GTE during H_2O_2 treatment resulted in a significant lowering of peroxidation.

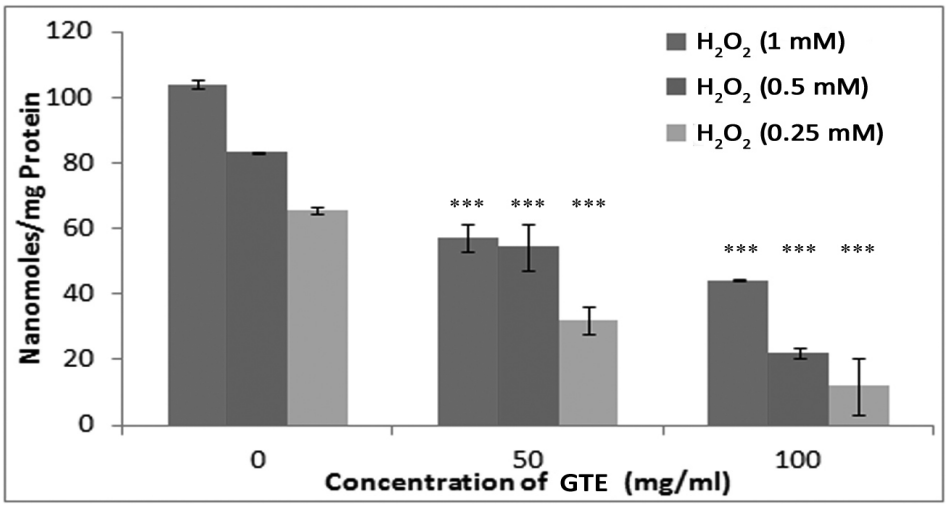

Figure 2: Lipid peroxidation assay of GTE. Values are expressed as mean ± SD (n = 5). * indicates 'P< 0.001' when compared with respective controls.**

3.3.Cytoprotective activity of GTE

The effect of GTE in protecting spleenocytes from free radical induced mortality is illustrated in figure 3. From the figure, it is clear that treating the cells with H_2O_2 induced a high percentage of cytotoxicity (41.97 %) even at the second hour of treatment. Presence of GTE along with H_2O_2 treatment protected the cells from cell death and the highest concentration of GTE (10 mg/ml) maintained 100% viability even after 3 hours of treatment.

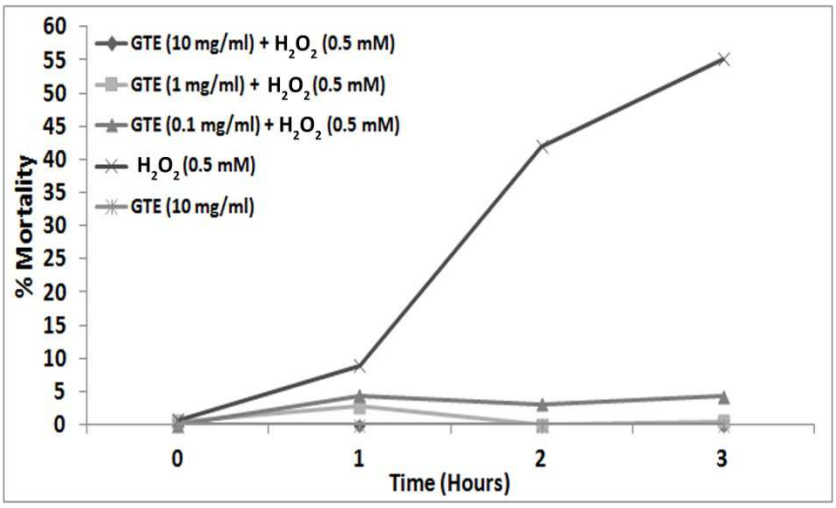

Figure 3: Effect of GTE in protecting spleenocytes from free radical induced cytotoxicity.

3.4. Effect of GTE in preventing oxidative cellular DNA damage

It can be seen from figure 4 that exposure of mouse liver cells to free radicals generated from 0.5 mM H_2O_2 induced damages to cellular DNA as the comet parameters such as tail DNA%, tail length, tail moment and Olive tail moment showed a significant increase. Presence of GTE during free radical insult mitigated the damage to cellular DNA in a concentration dependent manner.

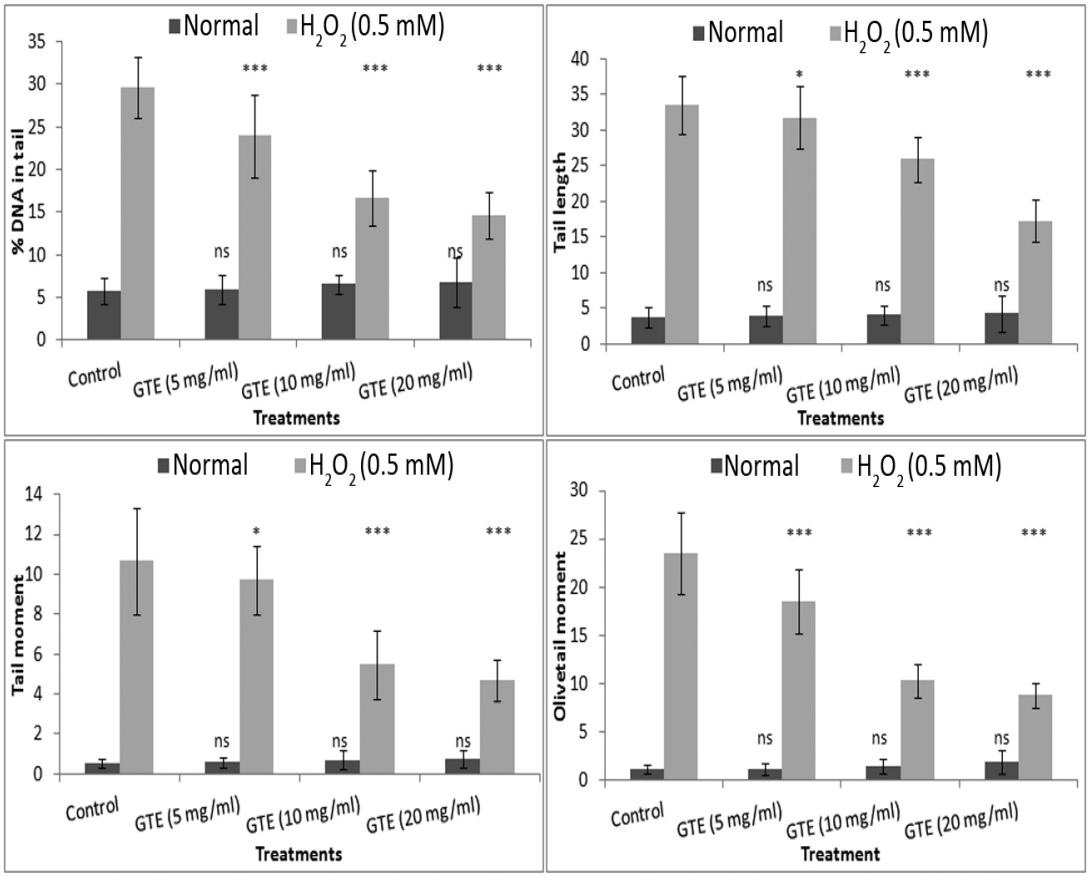

Figure 4: Effect of GTE on H_2O_2 induced cellular DNA damage in mouse liver cells. Comet parameters such as percentage DNA in tail, tail length, tail moment and Olivetail moment are expressed as mean ± S.D. (* indicate $p \leq 0.001$).**

4. Discussion

Oxygen free radicals and other reactive oxygen species are produced as by products through numerous physiological and biochemical processes in living system. Oxygen related free radicals (superoxide and hydroxyl radicals) and reactive species (hydrogen peroxide, nitric oxide, peroxynitrile and hypochlorous acid), are produced in the body, primarily as a result of aerobic metabolism (Halliwell, 1994; Poulson et al., 1998). Free radicals have very important role in origin of life and biological evolution (McCord, 2000). Oxygen radicals are involved in many biochemical activities of cells such as signal transduction, gene transcription and regulation of soluble guanylate cyclase activity. Nitric oxide (NO) is an important signalling molecule that essentially regulates the relaxation and proliferation of vascular smooth muscle cells, leukocytes adhesion, platelets aggregation, angiogenesis, thrombosis, vascular tone and hemodynamics (Zheng, 2000). Though oxidative property of oxygen plays a crucial in diverse biological phenomena, uncontrolled oxidation can aggravate the damage within the cell (Shinde et al., 2006). Living beings are constantly exposed to free radicals created by electromagnetic radiation from the manmade environment such as pollutants and cigarette smoke. Natural resources such as radon, cosmic radiation, as well as cellular

metabolisms (respiratory burst, enzyme reactions) also add to the free radical load. The most common cellular free radicals are hydroxyl (OH·), superoxide (O_2^{-}·) and nitric monoxide (NO·) and some other molecules like hydrogen peroxide (H_2O_2) and peroxynitrite (ONOO–) generate free radicals through various chemical reactions (Gilgun-Sherki, 2001). It has been a common knowledge that overproduction of free radicals results in damaging various vital targets in the cell, including genomic DNA, membrane lipids, proteins etc (Halliwell and Gutteridge, 1999; Marnett, 1999; Slater, 1984) resulting in various conditions including cancer (Dreher and Junod, 1996), pulmonary disorders (Greene, 1995) and Heart diseases (Jialal and Fuller, 1993). All organisms are naturally endowed with indigenous antioxidant defence mechanisms with antioxidants, such as glutathione, arginine, citrulline, taurine, creatine, selenium, zinc, vitamin E, vitamin C, vitamin A and tea polyphenols and antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase which function in removing free radicals (Poulson et al., 1998; Uday et al., 1990; Fang et al., 2002) and minimise the harmful effects of free radicals. Uncontrolled production of free radicals occurs when the body experiences stress from exogenous chemicals, radiation and pathogens (Valko et al., 2006; Maeda and Akaike, 1998; Nair et al., 2001).

In the present work, green tea extract is evaluated for its possible role to counteract the deleterious effects of oxidative stress. H_2O_2 was used to induce oxidative stress in an *in vitro* system and various parameters such as lipid peroxidation, cytotoxicity, cellular DNA damage, etc were used as the endpoints to measure the protective ability of green tea extract. The extract prevented the extent of H_2O_2 induced cell death and protected spleenocytes from oxidative death. The studies on lipid peroxidation and cellular DNA damage analysis by means of alkaline comet assay revealed that the possible mechanism for protection offered by green tea extract may be through prevention of oxidative stress induced lipid peroxidation and cellular DNA damage. The extract was found to possess excellent free radical scavenging potential.

Since the current approach of research on antioxidants is regulation of ROS and oxidation rather than eradication; further *in vivo* studies are needed to be done on the extract to ascertain the usefulness of it as a dietary supplement.

5. Acknowledgment

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A floristic survey of Ilapozhuthu sacred grove - a small sacred grove in Kottayam district

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Abstract

Sacred groves are forest patches conserved by the local people interwined with their socio-cultural and religious practices. These groves harbour rich biodiversity and play a significant role in the conservation of biodiversity. The present paper reports a floristic survey of Ilapozhuthu sacred grove, situated in Kottayam district, Kerala. The study revealed that this sacred grove contains a total of 63 species of angiosperms covering 61 genera under 34 families. While dicots represent 59 species belonging to 57 genera under 31 families, monocots covers 4 species representing 4 genera under 3 families. More over, many pteridophytes, bryophytes and algae are also present in this sacred grove. This sacred grove is a store house of valuable medicinal and other plants having high economic value, and serve as a refuge to threatened species.

Key words: Sacred groves, biodiversity, angiosperms, pteridophytes

1. Introduction

The sacred groves of Kerala are the remnants of evergreen forest patches with a great repository of many endemic, endangered and economically important plant species protected and conserved based on religious beliefs. The study reports revealed that floristic diversity indices of the sacred groves of Kerala are equal or nearly equal to the forests of the Western Ghats (Gadgil and Vartak, 1976). It is also revealed that these isolated patches are self-sustainable ecosystems function as a bioresource centre and closed system for the nutrient and water cycles for the nearby areas (Hughes and Chandran, 1998).

In Kerala, it was a common practice among Hindus to assign a part of their land near the Tharavadu or house as the abode of goddess Durga or Serpent God Naga or Shasta and the place is called Kavu or Sarpakavu. Sacred Grove represents the major effort to recognize and conserve biodiversity (ethnic diversity) traditionally. The age old system of every village having a temple, a tank and associated sacred grove explains the ancient method of water harvesting and sharing and may be considered as the backbone of village economy. People were prohibited from felling trees and even removing a twig was considered as taboo. Some of the trees such as Borassus, Alstonia scholaris, Antiaris toxicaria, Hopea parviflora, Strychnos nux-vomica, Ficus religiosa etc are being worshipped in many sacred groves (Pushpagandan et al., 1998).

Sacred groves act as an abode for many rare, endemic, endangered species and economically important plants of fruit bearing and medicinal properties. Apart from conserving biological diversity, sacred groves that are situated in the middle of the human habitation are responsible for conserving water and soil. This is evident from the perennial nature of ponds, wells and tanks, which are situated near the sacred groves. The fertility of the agro-ecosystems is very high due to the humus and nutrients generated in the sacred groves (Induchoodan and Balasubramanyan, 1991;Gadgil and Vartak, 1975).

In India, sacred groves are scattered all over the country, and do not enjoy protection via federal legislation. Some NGOs work with local villagers to protect such groves. Each grove is associated with a presiding deity, and the grove are referred to by different names in different parts of India.

Ecological significance of sacred groves are:- Fast growth of infra-structural facilities and on-farm activities are the prime causes of deteriorating quality status of the groves. As in the forest, many of the tree species are valuable timber species, they have been largely extracted for timber during the past few decades and thus, subsequently replacing the climax forests (Oak) to early successional pine forests. This has done considerable ecological damage in the region, making the soil more acidic and adversely affecting nutrient cycling and soil fertility. Growth of tourism industry is also deteriorating the faith towards deity and groves. Sacred groves are the victims of this grim tragedy. The groves located near the settlements are disappearing at a faster rate. Groves are disappearing, as the forests are being cleared and utilized for construction and repairing of deity houses. Most of the temple groves are seen disappearing due to inevitable factors like animal grazing and human interference. Other threats to the sacred groves include invasion by invasive species, like the invasive weeds *Chromolaena odorata*, *Lantana camara* and *Prosopis juliflora*.

The present study investigated the floristic diversity of a small sacred grove in the Kottayam District known popularly as "Pathi" or "Ilapozhuthu Devikshethram.

2. Methodology

In the course of investigation for a period of one year (2011-2012), the area was frequently surveyed. Several attempts were made for study in different seasons. Known plants were identified on the spot. In the systematic enumeration of the taxa, the families and the species under them are arranged alphabetically. A general index of angiosperm taxa stating family its number of genus/genera and species indicating habit has been prepared. In addition summarized table showing total number of families, genera and species under dicots and monocots has been prepared.

2.1. Study Area

The present sacred grove known popularly as "Pathi" or "Ilapozhuthu Devikshethram" is located in Kottayam district. It is placed 8 km away from the Pala town of Kottayam. The grove is spread over an area of 1 acre on a land of Nechipuzhoor Village. The diety of the grove is '*Bhadhrakaali*'. This sacred grove is for *Harijans*. There exists a practice of offering worship to the diety of the grove during the first day of every Malayalam month. Since the grove is abode of God, people neither cut any plant for the grove nor foul the serenity of the area, thus strictly adhering to the taboos and ethics. The folk belief goes that worshipping the diety gives well being and prosperity to the villagers.

3. Results and Discussion

On a rough estimate Kerala has about 2000 sacred groves which are distinct and unique in biological diversity. Most of the sacred groves represent the relics of once gregarious and abundant low lying evergreen forests of the Western Ghats. The vegetation in the undisturbed groves is luxuriant and with multi layered trees mixed with shrubs, lianas and herbs. The ground is humus laden and abundant with fungus and ferns. The floristic composition is highly influenced by exposure to anthropogenic pressures, cattle grazing, edaphic and climatic variations.

3.1. Rites and Rituals

There exists a practice of offering worship to the diety of the grove during the first day of every Malayalam month. Here the festival starts on the third week of '*Kumbhamasam*', lasting for four days. Various cultural activities are held on the first day. On the second and third day there will be '*Mudiyattam*' and '*Pethullal*' respectively. During *Pethullal* the *Velichappadu* will tell the fortune of that area. As part of the festival, special rituals like *Kozhivettu, Deshamvittupoka*l etc. are also there. The diety of the grove is '*Bhadhrakaali*'. This sacred grove is for *Harijans.*

3.2. Flora of the Grove

Floristic survey of the Ilapozhuthu Sacred grove revealed a total of 63 species of angiosperms covering 61 genera under 34 families. While dicots represent 59 species belonging to 57 genera under 31 families, monocot covers 4 species representing 4 genera under 3 families (Table 1 and 2). Among the total angiosperms, herbs, shrubs, trees and climbers are 21, 10, 25 and 7 species respectively (Table 3). More over some of the taxa have medicinal values. These are *Achyranthus aspera, Biophytum sensitivum, Centella asiatica, Holostemma anuler, Eclipta alba, Desmodium latifolium, Leucas aspera, Ocimum sanctum, Sida cordifolia, Mimosa pudica, Ixora coccinea, Glycosmis pentaphylla, Scoparia dulcis and Asparagus racemosus.*Many endangered and threatened species are effectively consereved in this sacred grove. In addition to rich flora of angiosperms, the grove harbours many pteridophytes and other lower plants.

Owing to continued protection offered on socio - cultural grounds, the sacred grove provides optimum conditions suitable for the growth of plants. As a result, some of the floristic elements attain maximum dimensions in terms of shape and size. Some trees like *Hydnocarpus wightina*, *Mangifera indica* etc provides safe sanctuary for a good number of birds. Apart from angiosperms the grove also houses few fungi and pteridophytes (*Pteris sp.*).

Sacred groves are a social institution, which permits management of biotic resources through people's participation. A scientific understanding of the sacred groves would be significantly important for designing strategies for rehabilitation of degraded landscapes, involving local people's participation, and training for promotion of traditional and social norms. There is a need of preservation, restoration and proper management of existing groves. Various traditional approaches to conservation of nature require a belief system, which includes a number of prescriptions and proscriptions for restrained resource use. These forestlands need proper conservation and protection by formulating consistent conservation strategies in order to save them from the verge of further degradation. Proper legislative support and specific policies should be provided. Mushrooming infrastructure facilities in the area are deteriorating the proper functioning of social institutions, which reflect that sacred groves are no longer getting the privilege they had in the past. Human interference should be regulated by encoding various indigenous practices along with scientific implications rather than only old religious prescriptions and proscriptions.

4. Conclusion

The sacred grove in essence represents the traditional Indian way of insitu conservation of plant resources. It is also indicator of the rich vegetation that had existed here in the past. Further more, the grove acts as a social space where people not only exchange their cultural identity but also find community solidarity. Therefore, this study calls for the continued protection of the sacred grove.

Table 1: List of Angiosperms of Ilapozhuthu Sacred Grove

Family and species	Local name	Habit
I. Dicots		
Acanthaceae		
(1)*Strobilanthus heynianus*	Karimkurinji	H
Amaranthaceae		
(1)*Achyranthus aspera*	Cherukadaladi	H
(2)*Phyllanthus reticulatus*	Kattukariveppila	H
Anacardiaceae		
(1)*Holigarna arnottiana*	Cheru	T
(2)*Mangifera indica*	Mavu	T
Annonaceae		
(1)*Uvaria narum*	Kuttipanal	S
Apiaceae		
(1)*Biophytum sensitivum*	Mukkutti	H
(2)*Centella asiatica*	Kudakan	C
Apocynaceae		
(1)*Plumeria acutifolia*	Chempakam	S
(2)*Tabernae montana dichotima*	Koonambala	T
(3)*Tabernae montana divaricata*	Nandhyar vattam	S
Asclepiadaceae		
(1)*Calotropis gigantea*	Erikku	S
(2)*Holostemma anuler*	Adapothian	C
(3)*Tylophora indica*	Vallipala	S
Asteraceae		
(1)*Eclipta alba*	Kayyonni	H
(2)*Elephantopus scaber*	Anachuvadi	H
(3)*Emilia sonchifolia*	Muyalcheviyan	H
Caesalpiniaceae		
(1)*Cassia fistula*	Kanikonna	T
(2)*Saraca indica*	Ashokam	T
Combretaceae		
(1)*Terminalia arjuna*	Neermaruthu	T
(2)*Terminalia paniculata*	Maruthu	T
Convolvulaceae		
(1)*Argeria hirsuta*	Veenda	C
(2)*Evolvulus alsinoids*	Vishnukranthi	C
Daliscaceae		
(1)*Tetrameles nudiflora*	Cheeni	T
Depterocarpaceae		
(1)*Vateria indica*	Thelli	T
Euphorbiaceae		
(1)*Aporosa bourdillonii*	Karivetti	T
(2)*Hydnocarpus wightiana*	Marotti	T
(3)*Phyllanthus niruri*	Kizharnelli	H
(4)*Ricinus communis*	Castor	S
(5)*Tragia involucrata*	Kodithoova	H

(Table 1. Conti..)

Fabaceae		
(1)*Clitoria ternatea*	Shanku pushpam	C
(2)*Desmodium latifolium*	Orila	H
(3)*Pterocarpus marsupium*	Venga	T
Lamiaceae		
(1)*Leucas aspera*	Thumba	H
(2)*Ocimum sanctum*	Thulasi	H
(3) *Pogostemon paniculatum*	Poothachadayan	H
Loganaceae		
(1)*Strychnos nux-vomica*	Kangiram	T
Malvaceae		
(1)*Hibiscus rosasinensis*	Chemparathi	S
(2)*Sida cordifolia*	Kurunthotti	H
Mimosae		
(1)*Acasia intscia*	Incha	S
(2)*Mimosa pudica*	Thottavadi	H
Moraceae		
(1)*Artiaris toxicaria*	Arayani	T
Polygalacea		
(1)*Xanthophyllum angustifolium*	Madakka	T
Rubiaceae		
(1)*Anthocephalus cadamba*	Kattukadambu	T
(2)*Chasalia curviflora*	Kattukappi	S
(3)*Ixora coccinea*	Chethi	S
Rutaceae		
(1)*Aegle marmelos*	Koovalam	T
(2)*Glycosmis pentaphylla*	Panal	S
Scrophulariaceae		
(1)*Scoparia dulcis*	Kallurukki	H
(2)*Torenia bicolor*	Kakkapoovu	H
Solanaceae		
(1)*Datura metel*	Ummam	H
(2)*Physalis minima*	Njottanjodiyan	H
Simarobaceae		
(1)*Ailanthus excelsa*	Perumaram	T
Sterculiaceae		
(1)*Sterculia urens*	Thondi	T
Verbenaceae		
(1)*Clerodendron viscosum*	Perungilam	S
(2)*Tectona grandis*	Teak	T
Vitaceae		
(1)*Cissus repens*	Njarala valli	C
II. Monocots		
Araceae		
(1)*Colocasia esculenta*	Chembu	H
Arecaceae		
(1)*Corypha umbraculifera*	Kudappana	T
(2)*Curyota urens*	Choondapana	T
Liliaceae		
(1)*Asparagus racemosus*	Sathavary	C

Table 2: Angiosperms taxa of Ilapozhuthu Sacred Grove

Sl.no	Family	Genus	Species				Total
			Herb	Shrub	Tree	Climber	
I	**Dicots**						
1	Acanthaceae	1	1	0	0	0	1
2	Amaranthaceae	2	2	0	0	0	2
3	Anacardiaceae	2	0	0	2	0	2
4	Annonaceae	1	0	1	0	0	1
5	Apiaceae	2	1	0	0	1	2
6	Apocynaceae	2	0	2	1	0	3
7	Asclepiadaceae	3	0	1	1	1	3
8	Asteraceae	3	3	0	0	0	3
9	Bombacaceae	1	0	0	1	0	1
10	Caesalpiniaceae	2	0	0	2	0	2
11	Combretaceae	1	0	0	2	0	2
12	Convolvulaceae	2	0	0	0	2	2
13	Datiscaceae	1	0	0	1	0	1
14	Depterocarpaceae	1	0	0	1	0	1
15	Euphorbiaceae	5	2	1	2	0	5
16	Fabaceae	3	1	0	1	1	3
17	Lamiaceae	3	3	0	0	0	3
18	Loganaceae	1	0	0	1	0	1
19	Malvaceae	2	1	1	0	0	2
20	Mimosae	2	1	1	0	0	2
21	Moraceae	1	0	0	1	0	1
22	Polygalacea	1	0	0	1	0	1
23	Rubiaceae	3	0	2	1	0	3
24	Rutaceae	2	0	1	1	0	2
25	Sapotaceae	1	0	0	1	0	1
26	Scrophulariaceae	2	2	0	0	0	2
27	Solanaceae	2	2	0	0	0	2
28	Simarobaceae	1	0	0	1	0	1
29	Sterculiaceae	1	0	0	1	0	1
30	Verbenaceae	2	0	1	1	0	2
31	Vitaceae	1	0	0	0	1	1
II	**Monocots**						
32	Araceae	1	1	0	0	0	1
33	Arecaceae	2	0	0	2	0	2
34	Liliaceae	1	0	0	0	1	1

Table 3: Summary of different angiospermic taxa of Ilapozhuthu Sacred Grove

Group	Families	Genera	Species				Total
			Herb	Shrub	Tree	Climber	
Dicots	31	57	19	11	23	6	59
Monocots	3	4	1	0	2	1	4
Total	34	61	20	11	25	7	63

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Studies on the prodigiosin production by *Serratia marcescens* obtained as a lab isolate

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Abstract

Prodigiosins, the red pigment produced by *Serratia marcescens* possess diverse biological activities such as anti-bacterial, anti-malarial, anti-tumor and immunosuppressive activities. The present work compared the prodigiosin biosynthesis by the isolated *Serratia marcescens* spp. by growing it in different media, nutrient broth supplemented with peanut, sesame seeds, coconut, Sesame oil, coconut oil, dextrose or starch. The results showed that Dextrose was found to enhance maximal cell growth and pigmentation while the media containing sesame oil gave maximum production of prodigiosin per cell. The presence of sodium dodecyl sulphate was found to enhance the pigment production and the results confirmed that the pigment was found to be associated with the cell surface hydrophobic characteristics.

Key words: Carbon source, Sodium dodecyl sulphate, prodigiosin, Serratia marcescens

1. Introduction

Serratia marcescens is a rod-shaped, Gram negative, facultative bacterium belonging to the Enterobacteriaceae family characterized by its ability to produce the red pigment prodigiosin (Khanafari *et al* 2006; Gerber 1975). The production of red pigment, prodigiosin, is present only in some strains and most strains that cause infection fail to produce pigment and form colourless colonies which are difficult to distinguish from other coliform organisms (Phillips and King 1977). Pigment production is highly variable among species and is dependent on many factors such as species type, incubation time, pH, carbon and nitrogen sources and inorganic salts (Pandey *et al* 2009; Williams *et al* 1961). The spontaneous occurrence in pigmented Serratia strains of nonpigmented mutants is assumed to be due to the deficient hydrophobic surface characteristics (Hermansson *et al* 1979). This has led investigators to assume that the pigment prodigiosin (Rosenberg *et al* 1983) is responsible for the hydrophobic surface properties of the strains.

Prodigiosins, originally isolated from *Serratia* as a red pigment, are a new class of tripyrrole compounds with diverse biological activities such as anti-bacterial, anti-malarial, anti-tumor and immunosuppressive activities (Moraes *et al* 2009; Pandey *et al* 2007; Stepkowski *et al* 2002; Montaner *et al* 2000, Francisco *et al* 2007, Llagostera *et al* 2005).

Many types of differential and selective media have been used for *Serratia* growth and prodigiosin production. Regular prodigiosin production can be carried out in nutrient broth containing sesame seeds, maltose broth, peptone glycerol broth (Giri *et al* 2004; Wai and Chen 2005), etc. However, it would be desirable to design a new nutritious and economically cheap medium to enhance *S. marcescens* growth and prodigiosin biosynthesis. In this context, we studied the prodigiosin producton potential of a *S. marcescens* strain obtained as lab isolate using various carbon sources. Having an insight on the

composition of already published media, the prodigiosin biosynthesis by the isolated *Serratia marcescens* spp. was compared by growing it in different media. Nutrient broth was supplemented with peanut, sesame seeds, coconut, Sesame oil, coconut oil, dextrose or starch.

2. Materials and methods

2.1. Isolation and characterization of Serratia marcescens

The culture was isolated from an air exposed nutrient agar plate in the microbiology laboratory, St. Mary's college, Thrissur. The culture was characterized as belonging to the *Serratia* by various tests, the major identifier being the gram staining character of the organism, culture morphology and production of cell associated prodigiosin.

2.2. Effect of carbon source on growth of Serratia and Prodigiosin production

To study the effect of different carbon sources on the extent of bacterial growth and prodigiosin production, different media were designed so as to contain 2% crushed peanut, crushed sesame seeds, crushed coconut, Sesame oil, coconut oil, dextrose or starch. Briefly the media contained 0.3% Beef extract, 0.5% Peptone, 0.5% Sodium chloride and 2% of either supplementation (crushed peanut, crushed sesame seeds, crushed coconut, Sesame oil, coconut oil, dextrose or starch). The media were prepared as agar as well as broth, sterilized by autoclaving and were inoculated with the isolated *S. marcescens.* The tubes were incubated at room temperature with shaking for 72 hours. The bacterial biomass from agar plates was scrapped into sterile distilled water. The level of bacterial growth and prodigiosin production were measured as described below (Mekhael and Yousif 2009)

$$\text{Prodigiosin unit/cell} = \frac{(OD_{499} - (1.381 \times OD_{620})) \times 1000}{OD_{620}}$$

OD_{499} – pigment absorbance, OD_{620} – bacterial cell absorbance and 1.381 – constant

2.3. Isolation of Prodigiosin from Serratia marcescens

To obtain the pigment, prodigiosin, the organism grown in liquid media or solid media scrapped into sterile distilled water was centrifuged. The pigment in supernatant was separated by extracting with ethyl acetate. The pigment from the cell pellet was extracted with acetone and the white pellet was discarded after the extraction by centrifuging at 10,000 rpm for 15 minutes. The pigment containing acetone fraction was mixed with ethyl acetate fractions and evaporated to obtain the dried pigment.

2.4. Effect of SDS on Prodigiosin production

Nutrient agar plates were prepared and a lawn culture of *S. marcescens* was prepared and after 20 min, sterilized filter paper disks impregnated with different concentrations of SDS (0.1 to 100 mg/ml) were placed on the surface of the inoculated agar plates. Plates were then incubated for 24 h.

2.5. Hydrophobic surface characteristics of Serratia and Prodigiosin production

A loop full of *Serratia marcescens* colony showing pigment production was mixed well in sterile saline to obtain a suspension. This suspension was kept aside for some time and afterwards the supernatant was

used to inoculate onto different media designed for prodigiosin production. After incubation, the plates were observed for bacterial growth and pigment production.

3. Results and Discussion

3.1. Isolation and characterization of Serratia marcescens

The culture was characterized as belonging to the Serratia by various tests, such as gram staining character of the organism, culture morphology and production of cell associated prodigiosin. The cells were Gram negative rods and produced opaque, circular, convex, mucoid colonies which have entire margins and umbonate elevation. Colonies on nutrient agar are pink, or red (Figure 1a and 1b). The colonies produced the red pigment prodigiosin and formed red colonies in response to incubation at 30^{o} C, but not at 37^{o} C which is an example of temperature-regulated phenotypic expression.

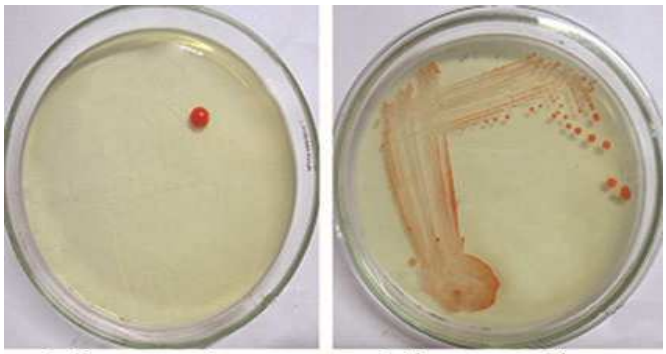

Figure 1. Figure 1a. Plate after air exposure and incubation showing a single colony of *Serratia marcescens*. Figure 1b. Growth of *Serratia marcescens* on nutrient agar plate.

3.2. Effect of carbon source on growth of Serratia and Prodigiosin production

To study the effect of different carbon sources on the extent of bacterial growth and prodigiosin production, different media were designed so as to contain 2% peanut, sesame seeds, coconut, Sesame oil, coconut oil, dextrose or starch. The media were prepared as agar as well as broth, sterilized by autoclaving and were inoculated with the isolated *S. marcescens.*

After incubation at room temperature for 72 hours, the liquid media in the tubes showed pink to red coloration indicating the growth of bacteria and pigmentation (Figure 2). Since the media contained crushed materials and oil, the spectrophotometric analysis of the extent of the pigment production was not done. The tubes were observed manually for the coloration and it was found that the supplementation of nutrient broth with dextrose yielded maximum red pigmentation (Figure 2). The broth culture was used for carrying out the isolation of the pigment.

The growth of *Serratia marcescens* showed varying levels of growth and pigmentation in solid media too (Figure 4). The bacterial biomass from these plates was scrapped into sterile distilled water. The level of

bacterial growth and prodigiosin production were measured as described in materials and method section (Mekhael and Yousif 2009) and are given in Table 1 and Figure 3.

Table 1. The levels of Prodigiosin or pigmentation per cell when the bacteria were grown in different media.

Media	Prodigiosin unit/cell
Control	174.02
Sesame seed	283.61
Sesame oil	576.05
Coconut	459.17
Coconut oil	499.21
Peanut	530.17
Dextrose	520.25
Starch	335.59

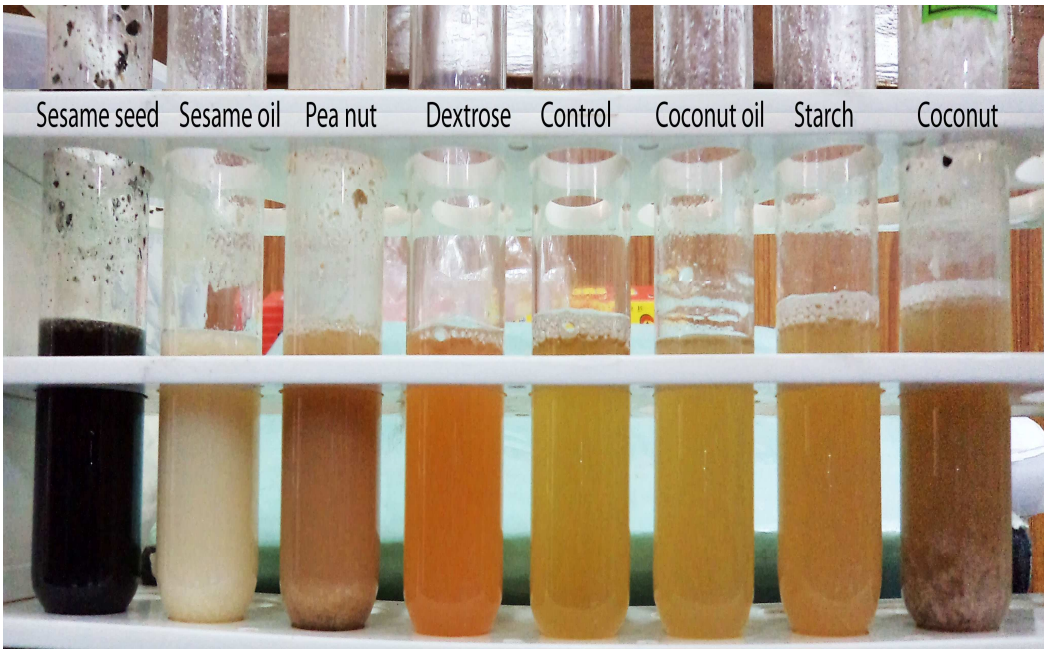

Figure 2. Broth cultures after 72 hours of incubation showing varying extent of pigmentation.

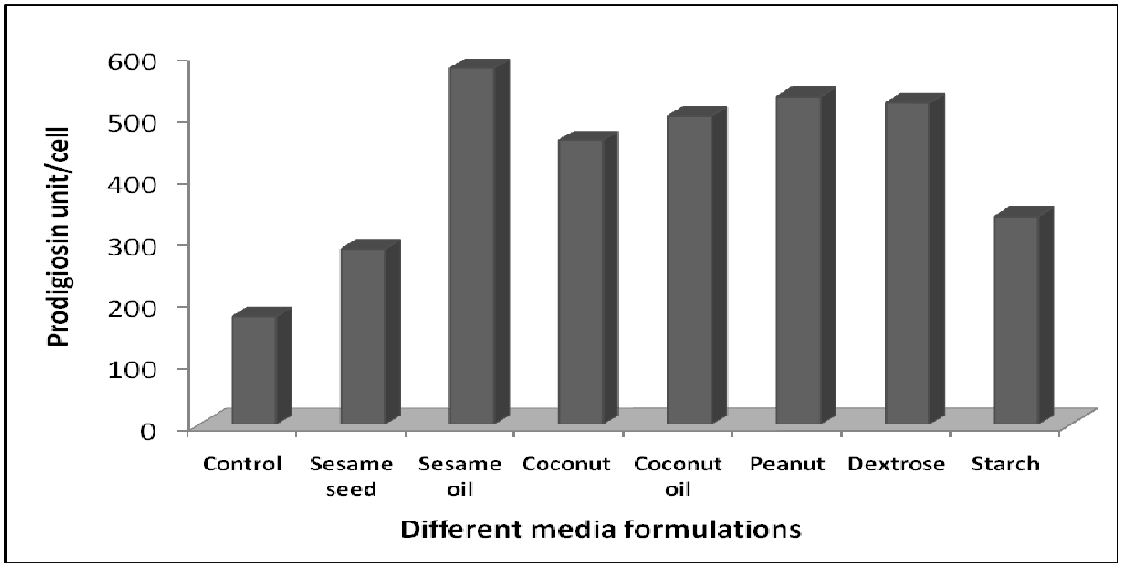

Figure 3. The levels of Prodigiosin or pigmentation per cell when the bacteria were grown in different media.

3.3. Isolation of Prodigiosin from Serratia marcescens

The pigment from the cell pellet (the organism grown in liquid media or solid media scrapped into sterile distilled water and centrifuged) was extracted with acetone and the white pellet was discarded after the extraction by centrifuging at 10,000 rpm for 15 minutes. The pigment in supernatant was separated by extracting with ethyl acetate. The both fractions were mixed and evaporated.

3.4. Effect of SDS on Prodigiosin production

Since it was already reported by Feng *et al* that SDS enhances the production of prodigiosin by Serratia, the effect of SDS on the present isolate was analysed by disc diffusion technique (Feng *et al* 1982).

After incubation time, the bacteria grew well in the nutrient agar plate and the lawn was light orange-red color. When there was an enhancement of pigmentation, the red color around the disk appeared deeper than the adjacent area. This coloration was found maximum near discs impregnated with 10 and 100 mg/ml concentration of sodium dodecyl sulphate. These higher concentrations inhibited the growth of bacteria immediate adjacent to the disc and the coloration of bacteria was found to be higher near this inhibition zone.

3.5. Hydrophobic surface characteristics of Serratia and Prodigiosin production

When the supernatant of a pigmented colony of *Serratia marcescens* suspension prepared in distilled water was used as the inoculum, after incubation, the plates showed bacterial growth with no pigment production. Thus it may be assumed that the organisms that came to the aqueous phase are those lacking

the ability to produce the pigment as it was already speculated that the pigment prodigiosin is responsible for the hydrophobic surface properties of the strains (Rosenberg *et al* 1983.)

4. Conclusions

Serratia marcescens isolated by plate exposure technique was found to produce the pigment prodigiosin and among the various media tested for the production of the pigment, the media containing Dextrose was found to enhance maximal cell growth and pigmentation while the media containing sesame oil gave maximum production of prodigiosin per cell. The presence of sodium dodecyl sulphate was found to enhance the pigment production and the pigment was found to be associated with the hydrophobic characteristics of the bacteria.

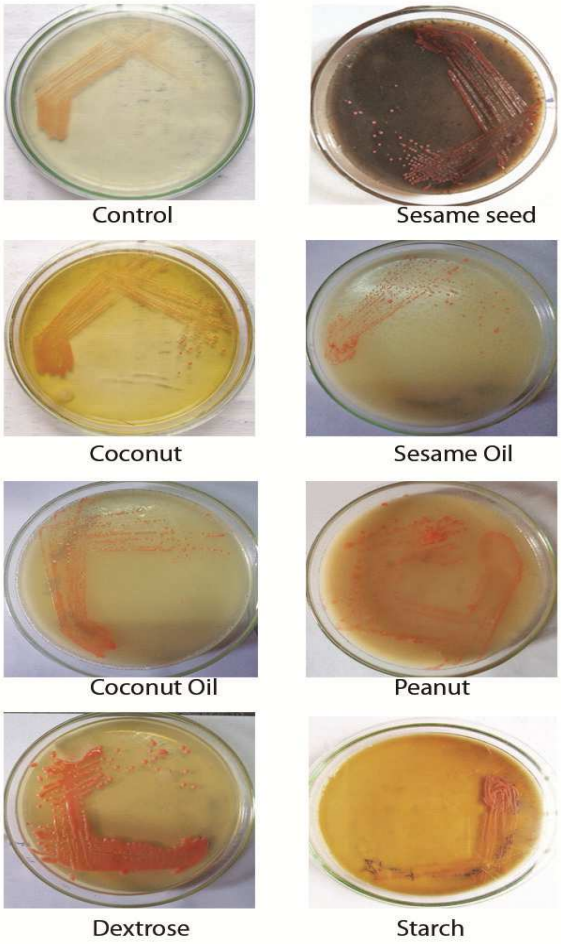

Figure 4. Agar plates after 72 hours of incubation showing varying extent of Growth and pigmentation.

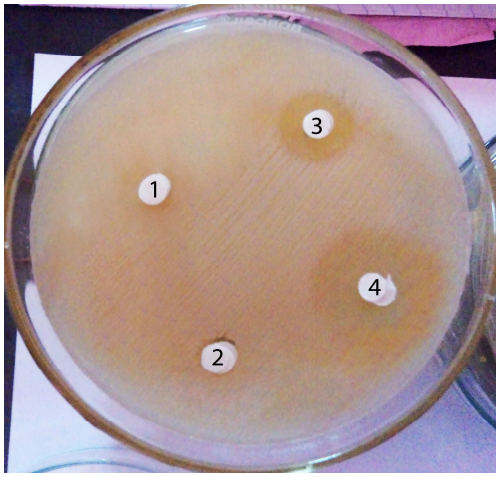

Figure 5. Effect of SDS on prodigiosin production by *Serratia marcescens.* 1- 0.1 mg/ml, 2 – 1mg/ ml, 3 – 10 mg/mll and 4 – 100 mg/ ml.

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Bacteriologic potability analysis of drinking water samples from various sources

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Abstract

The objective of this work is to study the changes in water quality resulting from increasing human intervention on the natural sources. Waste water, agricultural run off, grazing of livestock, drainage from mining areas, runoff from urban areas, domestic and industrial discharges may all lead to deterioration in physical, chemical, or biological/bacteriological water quality within a reservoir. Ground waters are, generally characterized by higher concentrations of dissolved solids, lower levels of colour, higher hardness (as compared with surface water), dissolved gas and freedom from microbial contamination. For this purpose, most probable number (MPN) test was done to detect the coliform in water samples collected from wells and tap water supplied by corporation. The study revealed that the number of coliforms was very high in water samples collected from certain wells. The bacteria were identified as *Escherichia coli.* Bacteriological examination of water samples collected from different sources showed that the water of area was not potable while the municipal tap water was found to be safe for drinking.

Keywords: E.coli, Coliform,Water quality

Abbreviations: TFTC-Too Few To Count,TNTC- Too Numerous To Count

1. Introduction

Water, the elixir of life is considered to be an important component in the life of all living things. It plays a vital role in the development of all living things. The water constitute about major portion of the human body. Therefore water used for human consumption should be free from contamination. These days natural water bodies are contaminated by the microorganisms and pathogens due to the dumping of waste into water bodies and excretions of animals. These waters are therefore unsafe and unhygienic for daily use.

1.1. *Types of water bodies*

Natural water bodies can be grouped into four types: 1) Atmospheric water 2) Surface water 3) Ground water 4) Stored water

Atmospheric water includes rain water and snow. Microorganisms in the form of cells, dormant propagules and dust particles remain suspended in water and snow. Considerable amount of bacteria can

be isolated from rain water. After heavy rain or snowfall, the dust particles and bacteria are washed from the atmosphere. Therefore atmosphere for sometimes remain free from microorganisms.

Surface water is present in earth surface. It is found in the form of several water bodies such as rivers, streams, ocean, lakes, etc. As soon as raindrops or snow touches the earth it become contaminated by the microorganisms present in the soil.

Ground water is the subterranean water that occurs where all the pores in the soil and rocks are saturated. Ground water are generally considered to be free from bacteria because of the filtering action of the earth, when the water passes through it. Not only the microorganisms but also the suspended organic materials in the water get removed by this filtering action. Springs form when the ground water reaches the surface through rock tissue or through porous soil.

Stored water is present in ponds, lakes, etc. During storage in general, the number of microorganisms gets reduced. Thus to some it establishes the purity and stability. However in stored water the microorganisms are affected by several factors such as sedimentation, ultra violet light, temperature, osmotic effects, food effects and activities of other microorganisms in soil.

1.2. *Waterborne diseases*

Waterborne pathogens make their entry into the water bodies through a number of sources. Recycling of treated/inadequately treated wastewater by mixing them with natural water bodies adds microorganism. Waterborne pathogens are transmitted through water.

Waterborne diseases are classified into several categories: Bacterial diseases, Viral Diseases and Protozoan Diseases.

1.2.1. *Bacterial diseases*

1.2.1.1. *Enteric fever/Gastroenteritis*

Enteric fever or typhoid fever and gastroenteritis are among the most common waterborne diseases. The symptoms of typhoid fever include continued fever, inflammation of the intestine, and formation of ulcers and enlargement of the spleen. The causative organism is *Salmonella typhi*

1.2.1.2. *Bacillary dysentery/Shigellosis*

Waterborne bacillary dysentery is common in developing countries. The disease is characterized by inflammation of the walls of large intestine. The causative organisms belong to the genus *Shigella* and include *S.dysentery, S.flexneri, S.boydi and S.sonnei.*

1.2.1.3. *Cholera*

Cholera is a severe gastrointestinal disease of human. The major virulence factor is the cholera endotoxin produced by the causative agent *Vibrio cholera*. Symptoms include severe vomiting, diarrhoea and increased blood activity/acidity.

1.2.2. *Viral diseases*

1.2.2.1. *Infectious Hepatitis*

Hepatitis A virus that causes infectious hepatitis is the only documented waterborne viral pathogen. It is transmitted through drinking water supplies or shell fish beds contaminated by feaces from infected persons. The disease is characterized by a yellow jaundice of the skin caused by an enlarged liver, vomiting and abdominal pain.

1.2.2.2. *Poliomyelitis*

Poliomyelitis is caused by *Polio* virus, which along with echovirus and *Coxsackie* viruses, constitute the *Enteric* virus group. The causative agent is released in feaces. Fecal oral route is the major mode of transmission.

1.2.3. *Protozoan diseases*

1.2.3.1. *Giardiasis*

It is long lasting diarrhoea caused by the flagellated protozoan parasite *Giardia lambia*. It is the most common protozoan parasite associated with the waterborne outbreaks worldwide. Common symptoms include chronic diarrhoea, abdominal cramps, fatigue and weight loss.

1.2.3.2. *Amoebiasis*

Amoebiasis, also called amoebic dysentery, is caused by the protozoan *Entamoeba histolytica*. The pathogen multiplies in the large intestine and adheres to enterocytes. This results in ulceration and watery, bloody diarrhoea. In severe cases fever and chills accompany acute dysentery. Incubation period ranges from two to four weeks.

1.2.3.3. *Cryptosporidiosis*

Cryptosporidium, an intestinal protozoan causes cryptosporidiosis. *C.parvum* has been found to be responsible for extensive outbreaks. Incubation period is about five to ten days. Symptoms include anorexia, vomiting, abdominal pain and diarrhea.

Good health is dependant upon clean, potable water supply. This means drinking water should be free of pathogenic microorganisms dissolved toxins and disagreeable turbidity, colour, odour and taste. To

ensure water is free of infectious agents, its microbial content must be periodically monitored. (Talaro A, Talaro K, 1993) The practice, therefore, is to test for fecal pollution, assuming that it points to the possibility of the presence of enteric pathogens also. By definition coliform is a facultative anaerobe that ferments lactose to produce gas and is a gram negative non sporing rod. *E coli* is a coliform and the best indicator of fecal contamination because they are always present in the human intestine and are excreted in large numbers. They live longer in water than other intestinal pathogens. Their presence in water is easily detected and this alerts health officials to the possible presence of other human or animal intestinal pathogens.

2. Materials and Methods

The primary test employed as an indicator of fecal pollution of water is the presence of coliform bacteria because they are invariably present in the faeces of human beings and other warm blooded animals in large numbers and can be easily detected in water, even in high dilutions. Though coliform bacteria are not exclusively of fecal origin, they serve as presumptive evidence, to be confirmed by the detection of thermo tolerant *E.coli which* provides definite proof of faecal pollution. (Dubey and Maheshwari, 2000).

Other bacteria are also sometimes used as indicators of fecal pollution. These indicates *Fecal Streptococci (*resistant to 45°C, 40% bile, potassium tellurite and sodium azide concentrations inhibitory to coliforms) and *Clostridium perfringens*

Guidelines have been laid down for the collection of water samples for bacteriological tests. Sodium thiosulphate should be added to samples of chlorinated water to inactivate residual chlorine which may lower bacterial counters by continued activity. Samples should be sent to the laboratory and tested without delay.

The following tests are generally done for routine bacteriological analysis of water

2.1. *Standard plate count*

A rapid method for testing the total bacterial levels in water is the standard plate count. This consists of counting the numbers of colonies formed in pour plate cultures of the water samples, on nutrient agar incubated aerobically, in parallel, at 37°C for 1-2 days or at 22°C for 3 days. Those that grow at 37°C are those most likely to be associated with organic material of human or animal origin, whereas those growing at a lower temperature are mainly saprophytes that normally inhabit water or are derived from soil and vegetables. The agar count at 37°C is a more important index of dangerous pollution. A rise in colony count is the usual signal of some defect in filter beds demanding immediate attention.

2.2. *Most probable number of coliform/multiple tube test*

The actual test for detecting the presence of coliforms in water is done by standard multiple tube fermentation technique (MPN). This method involves the three routine standard tests (a) the presumptive test (b) the confirmed test, and(c) the completed test. MPN table was proposed by Hawkins.(Prescott and, Harley J P, Klind A 1999)

(a)*Presumptive Test:* The Presumptive test is specific for detection of coliform bacteria. Measured aliquots of the water to be tested are added to a lactose fermentation broth containing an inverted gas vial. Because these bacteria are capable of using lactose as a carbon source, their detection is facilitated by use lactose broth. Lactose broth also contains a surface tension depressant, bile salt, which is used to suppress the growth of organisms other than coliform bacteria.(Cappuccino, Sherman).Tubes of lactose medium are inoculated with 10-ml, 1-ml,0.1-ml,aliquots of the water sample. The series consists of at least three groups, each composed of three tubes of the specified mediumThe greater the number of tubes per group, the greater the sensitivity of the test. Development of gas in any of the tubes is *presumptive* evidence of the presence of coliform bacteria in the sample. The presumptive test also enables the microbiologist to obtain some idea of the number of coliform organisms present by means of the most probable number test (MPN) (Cappuccino. Sherman).

(b) *Confirmed Test*: If a positive test of gas production is obtained, it does not mean that coliforms are present. The other organisms too also give false positive presumptive test because they are also capable of fermenting lactose with formation of acid and gas. The positive presumptive test is resulted due to synergism, ie.joint action of two microorganisms on a carbohydrate with production of gas which is not formed if both are grown separately. In addition, if yeasts, species of *Clostridium* and *Bacillus* some other microorganisms are present, gas is also produced. Therefore, a confirmed test is performed for the presence of coliforms. All fermentation tubes showing gas within 48 hours at 35°C are used for confirmed test. It is of two types as described below.

The positive presumptive fermentation test tube is gently shaken. A drop of its culture is transferred to brilliant green lactose bile broth fermentation tube. The tubes are incubated for 48 hour at 35°C.The appearance of gas within this period indicates for positive confirmed test. The dye (brilliant green) inhibits the Gram positive bacteria and synergistic reactions of Gram positive and Gram negative bacteria for a common food base.

The second confirmed test is done by Eosin Methylene Blue (EMB) agar or Endo agar method. Eosin-methylene blue contains the dye methylene blue, which inhibits the growth of Gram positive organisms. In the presence of an acid environment, EMB forms a complex that precipitates out onto the coliform colonies, producing dark centers and a green metallic sheen. This reaction is characteristic for *Escherichia coli*, the major indicator of fecal pollution.

(c) *Completed Test:* In the last the completed test is performed to ascertain about the presence of coliforms in water. The purpose of the completed test is to determine whether (a) the colonies growing on EMB or Endo agar are again capable of fermenting lactose and forming the acid and gas, and (b) the organisms transferred to agar slants show the morphological appearance of coliform group. Each colonies form positive confirmed test is transferred to lactose fermentation tube and to nutrient agar slants. The tubes are incubated at 35°C for 48 hours. Production of gas in fermentation tubes and demonstration of Gram negative, nonspore forming rods on the agar slants constitute a positive completed test for coliforms. The absence of gas and the rod production confirms for negative test of coliforms.

3. Results and discussion

Out of 20 samples collected from wells of *Kundvara region*, Thrissur, 1 sample was from Corporation tap of that area. All these samples showed MPN ranging from 3-2400.

After incubation in double strength and single strength Lactose Broth it was presumed that coliforms are present in the water sample. This was then used to determine the MPN of coliforms present in 100 ml of water sample. The confirmed test requires that selective and differential media such as Eosin Methylene Blue (EMB), being streaked from a positive lactose broth tube obtained from presumptive test. The EMB agar contains the dimethylene blue which inhibits the growth of Gram negative organisms. In the presence of acid environment EMB forms a complex that precipitates out onto the coliform colonies producing dark centers and green metallic sheen. This reaction is characteristic for *E coli.* The major indicator for fecal pollution *Enterobacter aerogenes* form large pinkish mucoid colonies without metallic sheen.

Out of 20 samples, 10 samples showed the presence of *E coli* on EMB agar plate and 7 samples showed presence of *Enterobacter species*. Samples 10,11 and 17 showed no growth in EMB agar plate. These samples were collected from disinfected wells, which are treated with bleaching powder. Samples 7 and 16 collected from well, which is not used for drinking purpose showed, a immense load of microbial colonies in SPC.

The completed test is the final analysis water sample and isolated colonies is picked up from the confirmatory test plate and inoculated into a tube of BGLB and streaked on nutrient agar slant to perform a Gram staining. Following inoculation and incubation tubes showing acid and gas production in lactose broth and presence of Gram negative bacilli on microscopic examination are indicative of positive completed test. They provided final evidence for presence of fecal coliforms.

Out of 20 samples collected from various sources of *Kunduvara region*, 1 sample is collected from Corporation tap (drinking source)of that area. All these samples showed MPN ranging from 3->2400.

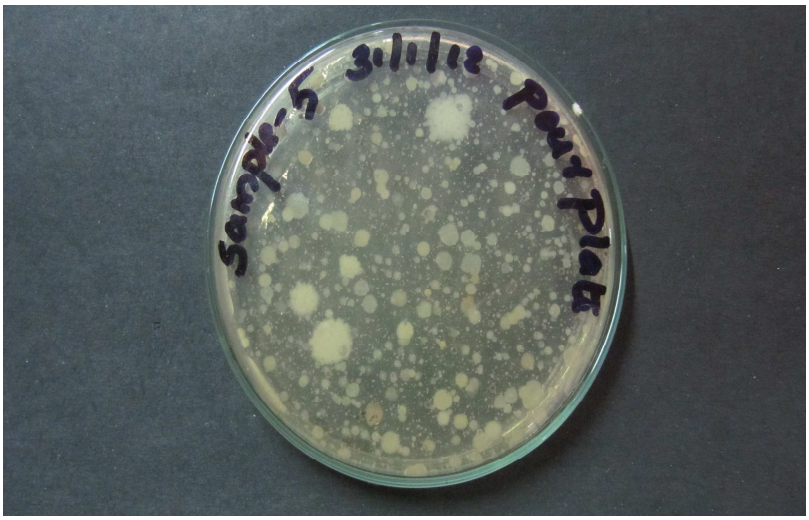

Figure 1. Pour plate of water sample showing bacterial colonies

Table 1. Presumptive test of various water samples

Sl. No	Source	DSLB	SSLB	SSLB	MPN index
1	Well water	3	3	0	240
2	Well water	3	3	0	240
3	Well water	3	0	0	23
4	Well water	3	3	0	240
5	Well water	3	3	0	240
6	Tap water	3	3	0	240
7	Well water	3	3	0	240
8	Well water	3	0	1	39
9	Well water	3	3	0	240
10	Well water	3	0	0	23
11	Well water	0	0	1	3
12	Well water	3	2	1	150
13	Well water	3	3	3	2400
14	Well water	3	0	1	39
15	Well water	2	0	0	9
16	Well water	3	1	0	43
17	Well water	1	0	0	4
18	Well water	3	0	2	64
19	Well water	3	3	2	1100
20	Well water	3	0	2	64

Table 2. Confirmed test of various water samples

Sl.No	EMB agar	Water Samples	*E. coli*	*Enterobactrer aerogenes*
1	Pink colonies	Used bleaching powder	-	+
2	Metallic sheen & pink colonies		+	+
3	Pink colonies		-	+
4	Pink colonies		-	+
5	Pink colonies		-	+
6	Pink colonies	Corporation water	-	+
7	Metallic sheen	Not used	+	-
8	Metallic sheen & pink colonies		+	+
9	Metallic sheen & pink colonies		+	+
10	No growth		-	-
11	No growth	Used bleach 2 months before	-	-
12	Pink colonies	Used bleach twice in a month	-	+
13	Metallic sheen		+	-
14	Metallic sheen & pink colonies		+	+
15	Metallic sheen		+	-
16	Metallic sheen & pink colonies	Not used for drinking	+	+
17	No growth	Used bleach once in month	-	-
18	Metallic sheen & pink colonies		+	+
19	Metallic sheen & pink colonies		+	+
20	Pink colonies		-	+

Table 3. Completed test of various water samples

SI.No	Gas formation	Differential staining(Gram's staining)
1	+	Gram -ve
2	+	Gram -ve
3	+	Gram -ve
4	+	Gram -ve
5	+	Gram -ve
6	+	Gram -ve
7	+	Gram -ve
8	+	Gram -ve
9	+	Gram -ve
10	-	----------
11	-	----------
12	+	Gram -ve
13	+	Gram -ve
14	+	Gram -ve
15	+	Gram -ve
16	+	Gram -ve
17	-	----------
18	+	Gram -ve
19	+	Gram -ve
20	+	Gram -ve

Table 4. Standard plate count of various water samples

SL.NO	STANDARD PLATE COUNT
1	208
2	TNTC
3	100
4	TNTC
5	TNTC
6	176
7	TNTC
8	TLTC
9	TLTC
10	TLTC
11	100
12	55
13	256
14	169
15	233
16	TNTC
17	125
18	123
19	137
20	118

After incubation in double strength and single strength Lactose Broth it was presumed that coliforms are present in the water sample. This was then used to determine the MPN of coliforms present in 100 ml of water sample. The confirmed test requires that selective and differential media such as Eosin Methylene Blue (EMB), being streaked from a positive Lactose Broth tube obtained from presumptive test. Out of 20 samples,10 samples showed the presence of *E coli* on EMB agar plate. Samples 10,11and 17 showed no growth in EMB agar plate. These samples were collected from disinfected wells, which is treated with bleaching powder. Samples 7 and 16 collected from well, which is not used for drinking purpose showed, a immense load of microbial colonies in 1ml of sample.

The completed test is the final analysis water sample and isolated colonies is picked up from the confirmatory test plate and inoculated into a tube of BGLB and streaked on nutrient agar slant to perform a Gram staining. Following inoculation and incubation tubes showing acid and gas production in lactose broth and presence of Gram negative bacilli on microscopic examination are indicative of positive completed test. They provided final evidence for presence of fecal coliforms. Following completed test 10 samples were found to be non potable which showed the presence of *E.coli* and 10 samples were found to be potable which showed absence of *E.coli* on EMB Agar plates.

4 Conclusion

Out of 20 water samples, 10 samples were found to be non potable. Water from disinfected wells as well as corporation tap water were found to be potable. The standard plate count of water samples range from

TLTC-TNTC. This study emphasizes the importance of the standard procedures to be practiced such as boiling, filtering and use of chemical disinfectants. This study also accentuates need of routine maintenance practice and cleaning of natural water bodies.

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Protection of cellular DNA and membrane from deleterious oxidative free radicals by lycopene: A Mechanistic study.

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Abstract

Lycopene, a hydrocarbon carotenoid, was investigated for its protecting activity against oxidative stress induced DNA and membrane damages. The free radical scavenging activity of the compound was studied by viz DPPH (1, 1-diphenyl-2-picryl-hydrazyl) radical scavenging assay. Membrane damage due to oxidative stress was measured as the extent of peroxidation of lipids in terms of Thiobarbituric acid reacting substance (TBARS). Damage to cellular DNA induced by oxidative stress was monitored by alkaline single cell gel electrophoresis in bone marrow cells of mice. Cytoprotective activity of lycopene was also be assayed. Lycopene effectively scavenged the free radicals in a concentration dependent manner. Presence of lycopene prevented peroxidation of membrane lipids in mouse liver homogenate. Lycopene effectively protected DNA from stress induced strand breaks and also has cytoprotective activity. Present study concluded that Lycopene can be used as a natural antioxidant.

Key words: Lycopene . DPPH , Comet Assay , Cytotoxicity ,Antioxidant

1. Introduction

Oxidative stress elicits several oxidative free radicals includes OH, CHO_2, H_2O_2 *etc*, results in the manifestations of various deleterious consequence in living organisms (Moller and Wallin, 1998). Oxidative stress has been recognized as underlying cause of several pathological states such as cardiac arrest, rheumatoid arthritis *etc* (Devasagayam *et al*., 2003). The present study was carried out to examining the compound lycopene can mitigate the oxidative stress of H_2O_2.

Free radicals oxidize proteins, nucleic acids, carbohydrates and lipids such as polyunsaturated fatty acids giving rise to Lipid Peroxidation (LPO) (Chessman and Slater, 1993) causing tissue damage. The products of lipid peroxidation may cause DNA damage (Rajagopalan *et al*., 2002) including strand breaks, base damage, sugar damage, and crosslinks of inter and intrastrand types (Scholes, 1982; Sun *et al*., 1998). These cellular damages when not efficiently repaired by indigenous defence mechanisms can lead to various pathological conditions (Cooke MS *et al*., 2003 ; Wojcik *et al*., 2010).

This relates to the fact that antioxidants can prevent free radicals, primarily highly reactive oxygen and nitrogen species, from damaging human health (Yashin *et al*., 2011).There is much interest in exploiting compounds from natural sources as antioxidants as there is a toxicity concern over the synthetic ones (Rebecca *et al*., 2011).

Lycopene is an unsaturated acyclic carotenoid with 11 linear conjugated and two non-conjugated double bonds (Stahl and Sies, 1996). Lycopene is found in some red vegetables and many red fruits, including tomatoes, watermelon, pink grapefruit, apricots, pink guava and papaya (Srinivasan *et al*., 2007). Various studies indicate that Lycopene rich diets may have a protective effect against chronic diseases, including cancer, heart disease (Rao and Agarwal, 2000; Sesso *et al*., 2004) and diabetics (Ibiebel *et al*., 2005). Lycopene has antioxidant (Yilmaz *et al*., 2006; Wood *et al*., 2008) antitumour and anti-inflammatory properties (Andic *et al*., 2009).

The present study explains the efficacy of lycopene to prevent oxidative damage ,cytotoxicity , and genotoxicity in mammalian cells underlying *invitro* conditions.

2. Materials and methods

2.1. Chemicals

DPPH (2,2-diphenyl 1-picryl hydrazil) , TBA (thio barbituric acid) purchased from Sigma Chemical Company Inc., St Louis, MO, USA. Lycopene was presented to Dr. C. K. K. Nair by Parry India, for research studies. All other chemicals and reagents used in this study were of analytical grade.

2.2. DPPH Radical scavenging Assay

DPPH (1, 1 diphenyl -2 – picryl –hydrazyl) is a stable free radical and has been used as a model free radical compound to evaluate the effectiveness of antioxidants. The free radical scavenging activity of the crude extract was determined by the method of Gadow *et al* (1997) with some modification. Freshly prepared methanolic solution of DPPH (634 μM) was incubated at an ambient temperature with the crude extract of various concentrations and A_{515} was measured using spectrometer. The percent of inhibition of DPPH reduction (decolourization) was calculated according to the formula

$$\% \text{ of inhibition} = \frac{A_0 - A_{20}}{A_0} \times 100$$

2.3. Measurement of lipid peroxidation

Damage to membrane in cells and tissues can be assessed in terms of peroxidation of membrane lipids according to the method of Buege and Aust (1978). 10% mice liver homogenate were prepared in ice cold PBS, pH-7.4. The homogenate were centrifuged at 6,000 x g for 10 minutes at 4^{o}C and the supernatant was exposed to different concentration of H_2O_2 (0.25 – 1mM) and various concentrations of lycopene (50-100mg/ml). The samples were analysed for the presence of thiobarbituric acid reacting substances (TBARS). Briefly the reaction mixture contained 100 μl of liver homogenate (10%), 0.375% thiobarbituric acid, 0.025 N HCl ,15% trichloroacetic acid and 6.0 x 10^{-3}mol dm^{-3} EDTA. The reaction mixture was heated at 90 ^{0}C for 30 minutes, cooled and centrifuged at 10,000 x g for 10 minutes. The amount of TBARS in the supernatant was estimated by measuring the absorption at 532 nm. The lipid peroxidation values are expressed as nano moles of MDA per mg protein. 1,1,3,3,-tetraethoxypropane was used as the standard. Proteins were estimated with Lowry's method (Lowry *et al*., 1951).

2.4. In vitro cytotoxicity assay

Single cell suspensions (1 x 10^6) of spleen was prepared in PBS followed by exposed to 0.5 mM H_2O_2 and different concentrations of lycopene (10–0.1mg/ml) . The percentage mortality of cells was estimated at different time intervals (0–3 hour) by means of Trypan blue dye exclusion method (Patel *et al*., 2009). The percentage mortality of cells was plotted against time.

2.5. Effect of lycopene on cellular DNA damage evaluated through comet assay

Alkaline single-cell gel electrophoresis was performed using the method of *Singh (2000)*with minor modifications.(chandrasekharan *et al*., 2008). 40 μl single cell suspension of bone marrow following H_2O_2 treatment with or without lycopene was mixed with 350 μl of low melting point agarose at 37^{o}C and layered on frosted slides pre-coated with 200 μl high melting point agarose. After solidification of agarose, the cover slips are removed and the slides were kept in pre-chilled lysing solution containing 2.5

M NaCl, 100 mM Na_2–EDTA: pH 10.0, 10 mM Tris HCl, 1% sodium sarcosinate with freshly added 1% TritonX-100 and 1% DMSO at 4 ^{0}C for 1 h. The slides were removed from the lysis solution and placed on a horizontal electrophoresis tank filled with the alkaline buffer (300 mM NaOH, 1 mM Na_2–EDTA, 0.2% DMSO, pH 13.0). The slides were equilibrated in the same buffer for 20 min. Electrophoresis was carried out for 30 min at 25 V (180 mA) using a compact power supply. After electrophoresis, the slides were stained by layering on the top with 50 μl of propidium iodide was visualised in flurosence microscope. The quantitation of the DNA strand breaks of the stored images was done using the imaging software CASP by which the percentage DNA in tail, tail length, tail moment, and olive tail moment can be obtained directly.

2.6. *Statistical Analysis*

The results are presented as mean ± SD of the studied groups. Results were performed using ANOVA with Tukey–Kramer multiple comparisons test.

3. Results

3.1. Free radical scavenging activity of Lycopene

The stable free radical DPPH (1,1- diphenyl-2-picryl-hydrazyl) with characteristic absorption at 515 nm was reduced by lycopene resulting in decrease in the absorption, which is directly related to the electron scavenging capacity of the lycopene. The compound lycopene reduced DPPH radical in a concentration dependent manner and IC_{50} of the reduction of DPPH under the experimental condition was observed at a concentration of 0.30 mg/ml. (Fig. 1).

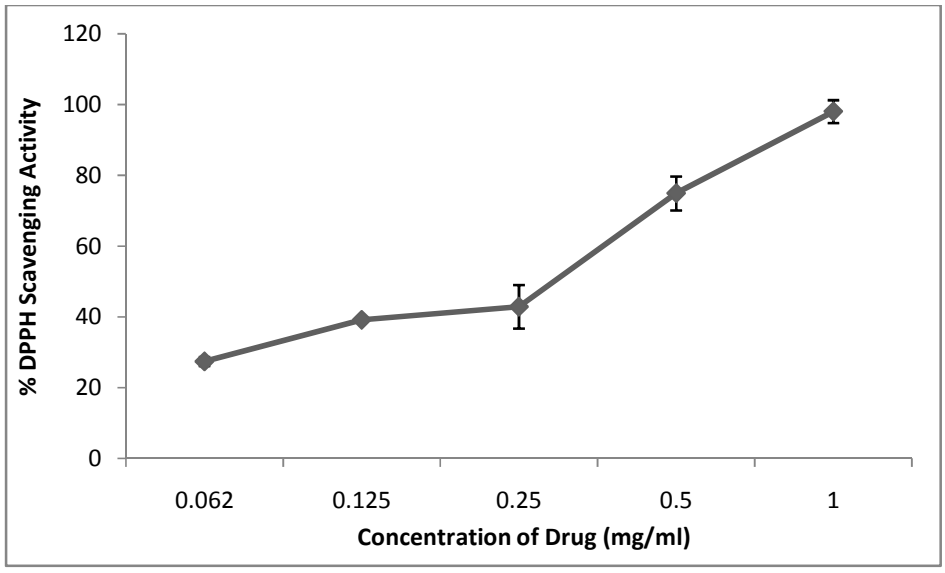

Figure 1. Free radical scavenging activity of Lycopene . Each point represents the mean ± SD.

3.2. Protection against oxidative stress induced lipid peroxidation in liver tissue homogenate by lycopene

Mouse liver homogenate subjected to oxidative stress by exposing to different concentration of H_2O_2 (oxidative stress), showed significant increase in peroxidation of membrane lipids. The presence of

lycopene along with H_2O_2 showed effective protection by inhibiting the lipid peroxide formation, measured as TBA reacting substance as illustrated in Figure 2.

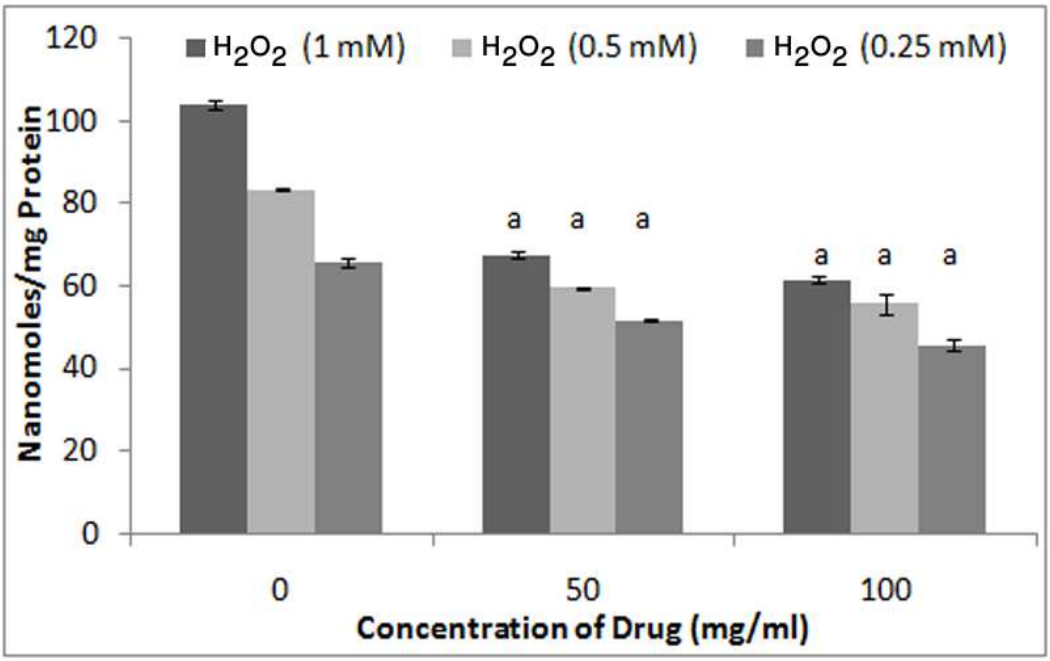

Figure 2. Effect of lycopene extract on oxidative stress induced lipid peroxidation in mouse liver homogenate. The lipid peroxidation values are expressed as nano moles of MDA per mg protein. % Inhibition of peroxidation of lipids is presented above the bars. Each point represents the mean±SD. (a = p < 0.001 when compared with respective control).

3.3. Cytoprotective effects of lycopene against oxidative stress

Exposure of splenocytes to H_2O_2 caused cytotoxicity that ultimately lead to 64.85 % death at 3 hour. In the presence of various concentrations of lycopene (0.1, 1, 10 mg/ml) cytotoxicity was found to be reduced to 22.5 %, 18. 47 %, 15.01 % respectively that was shown in fig 3.

3.4. Effect of Lycopene extract on cellular DNA damage in bone marrow cells evaluated through comet assay

As depicted in fig 4 all the comet parameters (percent DNA in tail, tail length, tail moment and Olive tail moment) increased in bone marrow cells of animals subjected to H_2O_2 respective to their control. In H_2O_2 treated group tail moment increased to 4.667 ± 2.663 from 0.533 ± 0.211 , % DNA in tail increased to 12.672 ± 3.552 from 4.567 ± 1.553 ,tail length increased to 17.567 ± 3.996 from 3.667 ±1.443 and olivetail moment increased to 6.582 ± 4.229 from 1.166 ± 0.443. The presence of lycopene reduce all these parameters, this suggest that lycopene reduce stand breaks induced by H_2O_2.

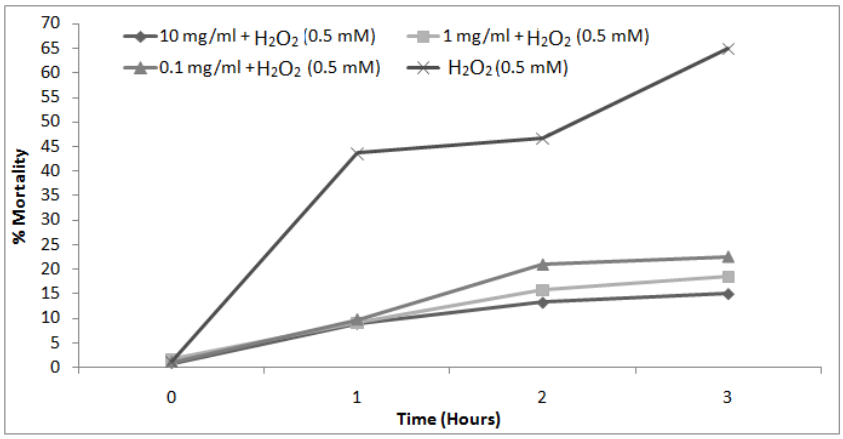

Figure 3. Effect of lycopene on cytotoxicity induced by 0.5 mM H_2O_2. Each point represents the mean±SD.

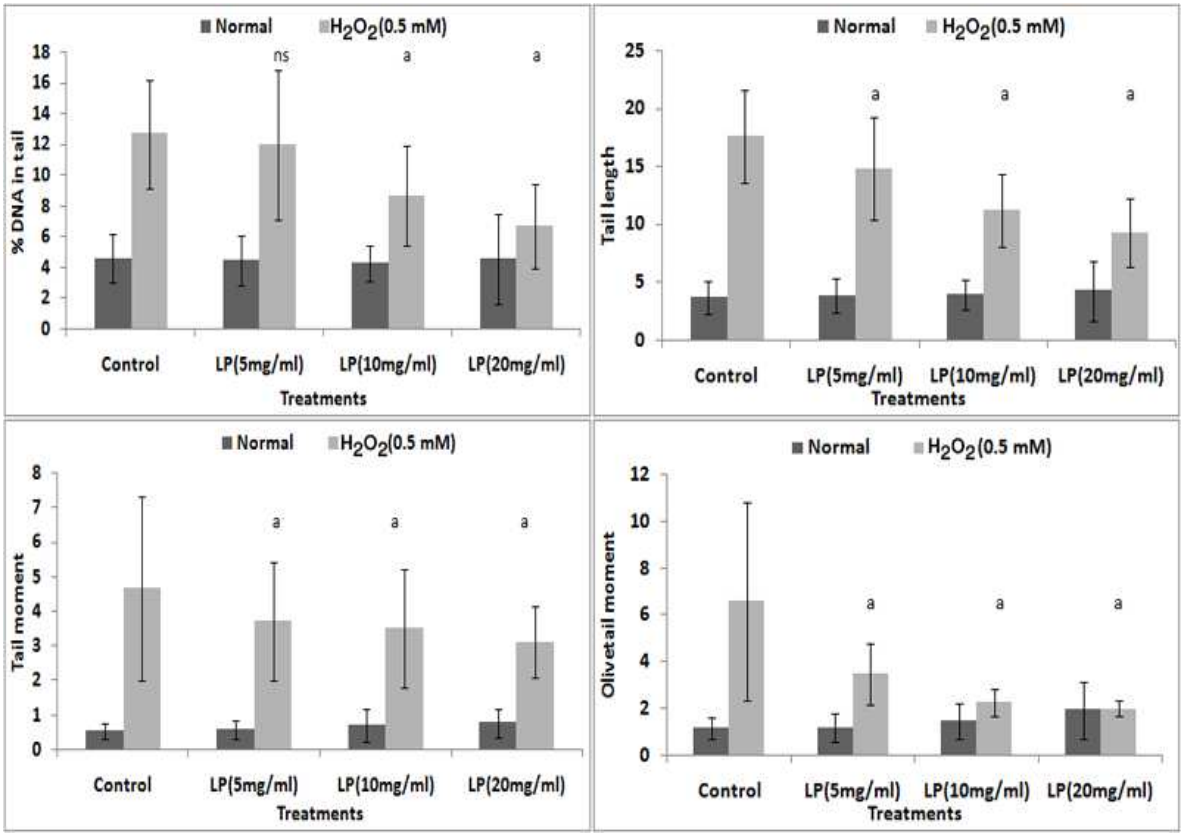

Figure 4. Effect of lycopene on DNA damage in bone marrow cells induced by oxidative stress exposure assayed by Comet . Statistical analysis of the results were performed using ANOVA with Tukey–Kramer multiple comparisons test (a = $p < 0.001$ when compared with respective control, ns = non significant).

4. Discussion

Oxidative stress is one of the major risk factors for chronic diseases. Free radicals or oxidants are potential contributors leading to oxidative stress. Reactive species have extremely short life span, like $OH^{\cdot}$, which is produced in locations where it can cause damage by interacting with its nearby molecules (Sawyer and Valentine, 1981). The high reactivity of radicals and their short life span illustrate the potential toxic effect. To prevent the interaction between radicals and biological targets, the antioxidant should be present at the location where the radicals are being produced in order to scavenging them.

The antioxidant properties of lycopene are commonly evaluated in two ways. One is through examining its protective effect against oxidative damage to biological molecules like DNA, lipids, and protein *in vivo* and *in vitro*. The other way is through measuring its capability to scavenge free radicals directly (Wenli *et al*., 2001).

Lycopene have free radical scavenging activity, because of its high number of conjugated double bonds that acts as singlet oxygen quenching moeity (Mascio *et al*., 1989). Because of this quenching activity, lycopene mitigate the damaging effects of oxidative stress.

Lipid peroxidation is a common product of oxidative stress in biological tissues such as lipoproteins, liposomes, microsomes, and membranes. The ability to reduce lipid peroxidation has become an important factor in examining the biological benefits of antioxidants (Woodall *et al*., 1997). Lycopene also inhibit the formation of TBARS that are mutagenic to cells (Gandhi and Nair, 2005). The mechanism may be interruption of lipid perooxidation by eliminating the lipid peroxyl radicals by donating hydrogen atoms to form lipid peroxide (El-Agamey *et al*., 2004).

Reactive oxygen species interacts with cellular biomolecules, such as DNA, leading to modification and potentially serious consequences for the cell (Cooke *et al*., 2003). In this study lycopene mitigates the effect of oxidative stress induced DNA damage which was analysed by comet assay. Lycopene reduced strand breaks in DNA due to its high free radical scavenging activity (Mascio *et al*., 1989).

5. Conclusions

These results favorably supported the significant role of lycopene rich foods in prevention of oxidative stress , that must be correlated with further study .

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Isolation of microbes from hard surfaces (Key board and mouse, telephone, coins, currencies)

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Abstract

Microorganisms are ubiquitous and transmissible via air, food, water and other interpersonal contacts and in most cases they cause diseases. Isolation of microbes from currency notes, coins, key board and mouse and telephone has been done. Total 20 samples were collected from different sources. All the tested samples were found to be contaminated with various types of microbes mainly normal flora and few of them contaminated with pathogenic microorganism and their presence confirmed by using selective media and biochemical tests. From this study bacterial isolates were associated with oral, nasal, and skin contamination. Implanting measures by providing community education for hygienic standards inorder to prevent transmission and cross contamination of pathogenic microorganism.

Key words : *Streptococcus* sp, *Pseudomonas aeruginosa, Staphylococcus aureus, Plate count*

1. Introduction

Microorganisms are ubiquitous and mixtures of microbes are often transferred to every day objects from the environment and infected individuals. Pathogenic microbes are transmissible via air, food, water and other interpersonal contacts, and in most cases they cause disease and infections. Transmission of these infectious agents typically involves their escape from host and entry in to a new host. These organisms can also be transmitted through the sharing of instruments and materials such as telephones, key board and mice, coins, currencies (Smith S I, and Opere B *et al* 2009). People believe that microbes are only present in research labs or hospitals and clinics and thus they have misleading feeling of security in other places. Lack of knowledge about where germs prowl could be the cause of health problems. In fact 80% of infections are spread through hands or other objects (Reynolds *et al.*2005).

In places where there a lot of people moving in and out, such as offices and internet cafes, there is likely to be a good number of people sick, and through them comes new bacteria that will eventually settle on the key board through air or from physical contact. These formites may be additional reservoirs for transmission of micro organisms and become vectors for cross-transmission. (D.N.A.Tagoe, F.K *et al* 2011). People now use telephones every where such as their homes, offices, schools, hotels, hospitals and it is used by both the healthy and sick. During usage bacterial agents can be transferred from an infected individual or an asymptomatic carrier of a microorganism to the surface of telephones through either direct contact with parts of the body, such as mouth via sneezing or coughing, ear, skin, or indirect contact with aerosols, saliva droplets and infectious particles to other users of the telephone.. The microorganisms well adapted to transmission and are highly resistant to desiccation and drying thus, their easy movement from one host to another. Some of known normal flora sometimes cause diseases when the tissue defenses of host are lowered; eg:-*Staphylococci* can be carried frequently from the nose and moistened areas of skin, where they do not cause disease, but when they land on other parts of the body they may cause diseases or infections especially in immunosuppressed individuals(A.K.Al-Ghamdi *et al* 2011). Paper currency notes and coins are transferred from one individual to other are known to carry bacteria which can spread human diseases. An individual living in unhygienic conditions having

unhygienic habits will contaminate notes with microbes, and these notes will act as a vehicle delivering bacteria to contaminate the hands of the next user. The money makes for easy transfer of bacteria and thus cross contamination occurs. Storage of these notes in humid and dark conditions favors the growth of bacteria on these notes (Sushil Kumar Barolia *et al* 2011).

2. Materials and Methods

2.1. Sample collection from keyboard and mouse and telephone

5 Swabs were collected from keyboard, mouse and telephone from different locations. All swabs collected with aseptically moistened swab sticks using normal saline solution and transported to laboratory. Serial dilution and plating of all the samples were done within 3 hours and it is prepared as 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8} dilutions. This was done by transferring 0.1ml of test sample in to 9.9ml of sterile water and 0.1ml aliquot transferred in to the next tube using micropipette.0.1ml from the 10^{-2},10-^{4},10^{-6},10^{-8} dilutions are dispensed on to a Nutrient agar plate and to spread plate method is applied find the plate count. All plate incubate at 37^0C (Smith S I *et al* ,2009)

2.2. Sample collection from coins and currency

Coins and currency notes collected from bus conductor, greengrocers, butchers, and autodrivers and also from bank. Individuals were asked to put the currency note and coins in sterile polythene bags. Notes were not touched by the researcher using bear hands. With the aid of a pair of sterile forceps, each currency note was transferred aseptically in to a 10ml saline in a boiling tube. The boiling tubes were plugged and shaken vigorously by hand for about 2 minutes to dislodge microorganisms in to the fluid. The resulting fluid served as test sample. The currency note was removed aseptically from the boiling tube with a sterile forceps, rinsed with water and dried to recover the notes. Serial dilutions were prepared from the test sample as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} .This was done by dispensed 1ml of test sample to a 9ml sterile water and then 1ml aliquot to the next tube using a micropipette. 0.1ml from 10^{-1}, 10^{-2},10^{-3},10^{-4} dilutions dispensed on to Nutrient agar and spread plate method applied to find the plate count. Kept uninoculated Nutrient agar plate as control. All plates were incubated at 37^oC.

Test sample is also swabed to different selective media like Mac conkey agar, Mannitol salt agar, Cetrimide agar, chocolate agar and blood agar etc. Control plates were also kept. All the plates were incubated at 37^oC. From coins, samples were collected using swabs and placed in 2ml saline, serial dilutions of test sample is prepared(10^{-2},10^{-4},10^{-6},10^{-8}). 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8} dilutions are plated using spread plate method, kept one uninoculated plate as control and incubated at 37^oC(Patrick feglo[1]*and Micheael Nknsah 2010). Swab is directly streaked on to Mac conkey agar, Mannitol salt agar, cetrimide agar, chocolate agar within 3 hours of the sample collection and control pates were also kept and incubated at 37^oC.

2.3. Identification of organisms

Pure isolated colonies were Gram stained. Biochemically identified using Indole, Methyl red, Vogues prosker and Citrate, Oxidase, Catalase tests, urease tests, Carbohydrate fermentation, Gelatin liquefaction, and Motility tests were also performed.

3. Results and Discussion

A total of 20 samples yield various microorganisms. The microorganisms isolated from keyboard and mouse, coins, currencies, and from telephone. The rates of contamination, appearance in selective media, Gram staining and morphology are shown in table1 to 8. Biochemical tests were performed for the

organism isolated from different selective media for further confirmation (cetrimide agar, Mannitol salt agar).By doing biochemical test *Pseudomonas aeruginosa*, *Staphylococcus aureus* are identified. *Streptococcus* sp was also obtained from blood agar plates. It was noticed from this study that the frequency of isolation of the bacterial agents vary with the location and species. The percentage of different isolates recorded from computer keyboard and mice of internet cafés was significantly higher than personal computers. Also the percentage of isolates obtained from coins and rupee notes from banks are lower than the coins and rupee notes obtained from other sources.

The occurrence of *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the isolated organism. This occurrence may be due to its resistance to drying which favors its transmission and its presence as part of the normal flora of nose, mouth and skin. Its transmission from one host to another susceptible host is known to be responsible for epidemic pyogenic infections in hospitals as well as epidemic diseases. The staphylococcal infections can be transmitted by the holding of telephones contaminated with *staphylococcus aureus*.All tested surfaces were found to be contaminated with mixed growth, Gram positive and Gram negative bacteria were isolated. Keyboards have become reservoirs for pathogens especially in hospitals, internet café and schools. One should also note here that reason for the increased percentage of contamination of computers is the difficulty of cleaning and disinfection. This study demonstrates that microbial contamination of computer keyboards, computer mouse, telephone buttons is prevalent and that commensal skin organism are the commonest contaminating microbes. The present investigation emphasizes the importance of good hand hygiene and adequate decontamination procedures applied to computer keyboards, mice and telephone buttons.

The currencies and coins serve as vehicle for transmission of diseases and represents an often overlooked enteric diseases reservoir. Food vendors use their bare hands which they occasionally wash in a bowl of water. This is not a flowing tap water but a stagnant bowl of water. This is not a flowing tap water but a stagnant bowl of water which is kept for as long as the food remains, until it is sold out to another over looked reservoir.

From this study bacterial isolates were associated with oral, nasal and skin contamination. This is an indication that money contamination is associated to unhygienic practice of people. These practices include indiscriminate sneezing, coughing and defecation with indecent handling of currency notes. It is therefore suggested that individuals should improve upon their personal health consciousness by washing hands after handling of currency notes.Food sellers should be educated to avoid possible cross

Table 1. Microorganisms from telephone

SI. No.	Sample collected from	No of colonies (Cfu/ml)
1	Telephone(bus stand)	TNTC
2	Thrissur town	TNTC
3	Department	$54X10^2$
4	Library	TNTC
5	College phone booth	TNTC

Table 2. Table showing results in selective media and on gram staining

Sample	Selective media				
	Cetrimide agar	**Macconkey agar**	**Mannitol salt agar**	**Blood agar**	**Chocolate agar**
Telephone (bus stand)	White colored, Pin point colonies	Lactose fermenting colonies	No growth	No growth	No growth
Gram staining and morphology	Gram negative, Rod shaped	Gram negative, Rod shaped	_	_	_
Thrissur town	Cream colored colonies	Non lactose fermenting colonies	No growth	No growth	No growth
Gram staining and morphology	Gram negative, Rod shaped	Gram negative, Rod shaped	_	_	_
Telephone department	No growth	Lactose fermenting colonies	Yellow colonies	No growth	No growth
Gram staining and morphology	_	Gram negative, Rod shaped	Gram positive, Cocci	_	_
Library	No growth	Lactose fermenting colonies	No growth	No growth	No growth
Gram staining and morphology	_	Gram negative, Rod shaped	_	_	_
College common phone	_	Non lactose fermenting colonies	No growth	No growth	No growth
Gram staining and morphology	_	Gram negative, Rod shaped	_	_	_

Table 3. Microorganisms from keyboard and mouse

Sample collected from	**No of colonies (Cfu/ml)**
Internet café(near bus stand)	TNTC
College net café	TNTC
BCA computer lab	$2.54x10^4$
Library	$3.03x10^4$
Department	$2.55x10^4$

Table 4. Table showing Results in selective media and on gram staining

Sample	Selective media				
	Cetrimide agar	**Macconkey agar**	**Mannitol salt agar**	**Blood agar**	**Chocolate agar**
Internet café (near bus stand)	No growth	Lactose fermenting colonies	No growth	Alpha hemolytic colonies	No growth
Gram staining and morphology	–	Gram negative, Rod shaped	–	Gram positive, cocci	–
College net café	White colored colonies	Lactose fermenting colonies	No growth	No growth	No growth
Gram staining and morphology	Gram negative, Rod shaped	Gram negative, cocci	–	–	–
BCA computer lab	No growth	Lactose fermenting colonies	No growth	No growth	No growth
Gram staining and morphology	–	Gram negative, Rod shaped	–	–	–
Library	No growth	Lactose fermenting colonies	Yellow colonies	No growth	No growth
Gram staining and morphology	–	Gram negative, Rod shaped	Gram positive,Cocci	–	–
Department	No growth	Lactose fermenting colonies	Yellow colonies	No growth	No growth
Gram staining and morphology	–	Gram negative, Rod shaped	Gram positive,cocci	–	–

Table 5. Microorganisms from coins

SI. No.	Sample collected from	No of colonies
1	Green grocer	TNTC
2	Butcher	$5.075x10^5$
3	Bus conductor	$6x10^5$
4	Bank	$3.04x10^4$
5	Auto driver	$6.225x10^5$

Table 6. Table showing results in selective media and on gram staining

Sample	Selective media				
	Cetrimide agar	**Macconkey agar**	**Mannitol salt agar**	**Blood agar**	**Chocolate agar**
Green grocer	No growth	Lactose fermenting colonies	No growth	No growth	No growth
Gram staining and morphology	–	Gram negative ,Rod shaped	–	–	–
Butcher	No growth	Lactose fermenting colonies	Yellow colonies	No growth	No growth
Gram staining and morphology	–	Gram negative ,Rod shaped	Gram positive,cocci	–	–
Bus conductor	No growth	Lactose fermenting colonies	No growth	No growth	No growth
Gram staining and morphology	–	Gram negative, Rod shaped	–	–	–
Bank	No growth	No growth	No growth	No growth	No growth
Gram staining and morphology	–	–	–	–	–
Auto driver	No growth	No growth	No growth	No growth	No growth
Gram staining and morphology	–	–	–	–	–

Table 7. Microorganisms from rupee notes

SI. No.	**Sample collected from**	**No of colonies**
1	10 rupee note from Green grocer	TNTC
2	10 rupee from bus conductor	$5x10^3$
3	10 rupee from auto driver	$10.9x10^3$
4	500 rupee from bank	$5.7x10^2$
5	5 rupee from general store	$1.01x10^4$

Table 8. Table showing results in selective media and on gram staining

sample	Selective media				
	Cetrimide agar	**Macconkey agar**	**Mannitol salt agar**	**Blood agar**	**Chocolate agar**
10 rupee note from Green grocer	White colored colonies	Non lactose fermenting colonies	No growth	No growth	No growth
Gram staining and morphology	Gram negative, Rod shaped	Gram negative rod shaped	_		_
10 rupee from bus conductor	White colored colonies	Non lactose fermenting colonies	No growth	No growth	No growth
Gram staining and morphology	Gram negative, Rod shaped	Gram negative rod shaped	_	_	_
10 rupee from auto driver	No growth	No growth	No growth	No growth	No growth
Gram staining and morphology	_	_	_	_	_
500 rupee from bank	No growth	Non lactose fermenting	No growth	No growth	No growth
Gram staining and morphology	_	Gram negative, Rod shaped	_	_	_
5 rupee from general store	No growth	Non lactose fermenting colony	No growth	No growth	No growth
Gram staining and morphology	_	Gram negative, Rod shaped	_	_	_

contamination between currency notes and food by avoiding handling currency notes as they sell. These should be public awareness of the fact that currency note could be a source of infection and could be dangerous to health

4. Conclusion

In this work we focused on contamination of currency notes, coins, keyboard, and mouse and telephone buttons. Sample was collected from different sources. Total of 20 samples were collected. All the tested samples were found to be contaminated with various types of microorganisms mainly normal flora and a

few of them contaminated with pathogenic microorganisms. . So appropriate hand hygiene is important in order to prevent the transmission of this pathogenic organism.

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Study on the antibacterial potential of commercially available liquid hand wash using four different bacteria.

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Abstract

The killing or removal of microbes from hands is a critical factor in food safety. The main medical purpose of washing hands is to cleanse the hands to remove pathogens (including bacteria or viruses) and chemicals which can cause personal harm or disease. In response to this concern, a number of novel hand-washing products have appeared on the market and are marketed using claims that they are 'twice as effective as ordinary soap' and 'more effective at removing germs than conventional soaps', and that they 'kill *E. coli* the toilet germ'. This study is performed to find out the efficacy of available liquid hand wash in killing bacteria, using *Staphylococcus*, *Pseudomonas*, *Escherichia coli*, and *Klebsiella* as the model organisms. In this study we found out that most of the liquid hand wash are effective when they are using in their full strength and not in dilutions. Also it is found out that most of the hand wash are effective against both the tested Gram positive and Gram negative organisms but the efficiency varies.

Key words: Hand wash, Triclosan, Nosocomial Infections, E.coli, S.aureus, Gram negative, Gram positive.

Abbreviations:- NI – Nosocomial Infections; MRSA- Methicillin Resistant Staphylococcus aureus; VRE- Vancomycin Resistant Enterococcus ; HIV- Human Immunodeficiency Virus ; RSV-Respiratory Syncytial Virus ; EDTA Ethylene Diamine Tetra Acetic acid-; ICU- Intencive Care Unit.

1. Introduction

The killing or removal of microbes from hands is a critical factor in food safety as many studies have shown that the hands to be both an important source of microbes and powerful agents of cross-contamination in hospital and domestic Situations. The association between hand hygiene and prevention of infection has been well recognized and antimicrobial hand washes are in common use in institutions such as hospitals and the food industry. Hand washing for hand hygiene is the act of cleaning the hands with or without the use of water or another liquid, or with the use of soap, for the purpose of removing soil, dirt, and/or microorganisms. It protects best against diseases transmitted through fecal-oral routes (such as many forms of stomach flu) and direct physical contact (such as impetigo).

Microorganisms carried on the skin of the human body are divided into two distinct populations: resident and transient. Resident microorganisms are considered permanent inhabitants of the skin of most people and are found on the superficial skin surface. Resident microorganisms include *staphylococci*; members of the *Corynebacterium*, *Propionibacterium* and *Acinetobacter* species; and certain members of the *Enterobacteriaceae* family. Some individuals carry *Staphylococcus aureus* on their skin which is the only true pathogenic organisms included in the resident micro flora group of skin. Transient microorganisms are organisms that are found on and within the epidermal layer of skin, as well as other areas of the body.

Almost all disease-producing microorganisms belong to this category. Pathogens that may be present on the skin as transient types include: *Escherichia coli*, *Salmonella* spp, *Shigella* spp., *Clostridium perfringens*, *Giardia lamblia*, Norwalk virus and Hepatitis A virus.

The application of water alone is inefficient for cleaning skin because water is often unable to remove fats, oils, and proteins, which are components of organic soil. Therefore, removal of microorganisms from skin requires the addition of soaps or detergents to water. Solid soap, because of its reusable nature, may hold bacteria acquired from previous uses. A hand sanitizer or hand antiseptic is a non-water-based hand hygiene agent. Hand sanitizers containing a minimum of 60 to 95% alcohol are efficient germ killers. Alcohol rub sanitizers kill bacteria, multi-drug resistant bacteria (MRSA and VRE), tuberculosis, and some viruses (including HIV, herpes, RSV, rhinovirus, vaccinia, influenza, and hepatitis) and fungus. The efficacy of alcohol-free hand sanitizers is heavily dependent on their ingredients and formulation.

The two main ingredients of commercially available hand wash are Triclosan and chlorhexidine gluconate. Other ingredients such as tetra sodium, EDTA, and Triclocarban are also used in liquid hand wash.

1.1. *Triclosan*

Triclosan (Irgasan), an antimicrobial active against *staphylococci* and coliform bacteria, has been formulated for use as a hand washes (Faoaquali et al., 1995). It is a polychloro phenoxy phenol. Some bacterial species can develop low-level resistance to Triclosan at its lower bacteriostatic concentrations which results in a decrease of Triclosan effect (Fan et al., 2002). Some bacteria have innate resistance to Triclosan at low, bacteriostatic levels, such as *Pseudomonas aeruginosa*, which possesses multi-drug efflux pumps that "pump" Triclosan out of the cell (Chuanchuen et al., 2003)

1.2. *Chlorhexidine Gluconate*

Chlorhexidine is a chemical antiseptic. It is effective against both Gram positive and Gram negative bacteria. It has both bactericidal and bacteriostatic mechanisms of action. It is also useful against fungi and enveloped viruses. Chlorhexidine is harmful in high concentrations, but is used safely in low concentrations in many products, such as mouthwash, contact lens solutions and in hand wash (Kuyyakanond *et al* 1991). Studies show that *S. aureus* growth was inhibited by 1.0% and 0.5% chlorhexidine gluconate (Redding *et al* 1991).

1.3. *Tetrasodium EDTA*

Adding Tetrasodium EDTA is also a common way to create an antibacterial soap. This chemical is a chelating agent which sequesters metals that the bacteria need in order to grow.

1.4. *Triclocarban*

Disrupting the bacteria's cell membrane is also a way how antibacterial soaps work. This is done by adding a chemical called Triclocarban. This chemical also inhibits an enzyme that is needed for building cell membranes in bacteria.

Transient hand carriage is associated with the transmission of many viruses, such as rhinovirus (D'Alessio et al., 1976), RSV (Hall C. B, 2000), astrovirus (Esahli et al., 1991), and cytomegalovirus (Demmler et al., 1987). Approximately 2 million nosocomial infections (NIs) occur annually in the United States (Esahli et al., 1991). The most common NIs is urinary tract infections, lower respiratory tract infections, surgical-site infections, and primary septicemia (Soleto et al., 2003). They lead to additional days of

treatment (Piednoir et al., 2003), increase the risk of death (Garcia-Martin et al., 2001), and increase treatment costs.

S. aureus is the most common gram-positive bacterium causing NIs (Mayon-White et al**.,** 1988). The most common type of NI caused by *S. aureus* is the surgical-site infection (Kampf et al., 1996). The transmissibility of transient bacteria depends on the species, the number of bacteria on the hand, their survival on skin, and the dermal water content (Marples and Towers, 1979).

Escherichia coli is the most common gram-negative bacterium, causing mainly urinary tract infections. *Pseudomonas aeruginosa* is also very common, chiefly causing lower respiratory tract infections (Kim et al., 2000). In the majority of cases, both types of infection are device associated (McLaws and Taylor, 2003) and are often found among patients in ICUs (Kampf et al., 1998).

The decision to use products containing antibacterial ingredients to prevent disease transmission rests on whether there are proven benefits in a specific setting (for example, home or institution) and whether there are risks. This study is performed to find out the efficacy of available liquid hand wash in killing bacteria, using *Staphylococcus*, *Pseudomonas*, *Escherichia coli*, and *Klebsiella* as the model organisms.

2. Materials and Methods

2.1. Liquid Hand Wash

Hand washes of different brands were purchased from local super market. Hand washes like Dettol, Santoor , Chandrika, Lux and Palmolive were used. The different varieties of these samples used in this study were 1)Dettol original 2) Dettol sensitive 3) Dettol skin care 4) Dettol fresh 5) Lifebuoy active fresh 6) Santoor hand wash essential oil 7) Santoor hand wash extra moisturizing 8) Lux hand wash - strawberry and cream with mint oil 9) Chandrika hand wash with essential oil 10) Palmolive naturals liquid hand wash.

2.2. *Cultures*

Overnight broth cultures of *Escherichia coli*, *Klebsiella* species, *Staphylococcus aureus* and *Pseudomonas* species were used.

2.3. Antibacterial activity by well diffusion method

Sterile nutrient agar plates containing 10 - 20 ml of medium were prepared. Hand washes of different dilutions 10^{-2}, 10^{-4}, 10^{-6} were prepared by transferring 0.1 ml from each test tube to another using a 100 micro liter micro pipette. The plate were labeled corresponding to each dilutions. Using swab, each cultures were spread on its specific nutrient agar plates and made the lawn cultures. Wells were cut on the lawn culture plate using a well cutter. Hand washes of 0.1 ml in full strength and various dilutions 10^{-2}, 10^{-4} and 10^{-8} were added to each well. The plates were incubated at 37^{0}c for 24-48 hours.

3. Results and discussion

The results of the present study are summarized in Table 1. Dettol original, Dettol sensitive, Dettol skin care and Dettol fresh were found to be effective against both Gram positive and Gram negative organisms in full strength and not in dilutions. Life Buoy active fresh was more effective against both Gram positive organisms than the Gram negative ones in full strength and not in dilutions. Santoor hand wash essential oil was more effective against the Gram negative organisms and also effective against the Gram positive *Staphylococcus* species. This particular hand wash was also effective against *Escherichia coli*, *staphylococcus* sp when diluted and further dilutions reduced the effectiveness of hand wash against all these organisms. Santoor hand wash extra moisturizing was more effective against the Gram negative

organisms like *Escherichia coli* and *Klebsiella* sp, and less effective in case of *pseudomonas* sp, and it was also effective against the Gram positive *Staphylococcus* species. It was found to be not effective when diluted. Lux hand wash strawberry and cream with mint oil was more effective against both Gram

positive and Gram negative organisms in full strength and not in dilutions. Chandrika hand wash with essential oil was more effective against the Gram negative organisms, *Escherichia coli* and *Klebsiella* sp and less effective in case of *Pseudomonas* sp. It was less effective against the Gram positive *Staphylococcus* sp. This particular hand wash was also effective against *Escherichia coli* and *Klebsiella sp* when diluted and further dilutions reduced the effectiveness of hand wash against all these organisms. Palmolive naturals liquid hand wash was more effective against Gram positive and less active against Gram negative organisms. And it was found to be not effective when diluted.

Table 1. The effectiveness of various handwashes in full concentration and their dilutions against four bacterial species. The Zone of inhibition is given in mm.

		Hand washes									
		DO	**DS**	**DSC**	**DF**	**LF**	**SEO**	**SEM**	**LSM**	**CEO**	**PN**
E. coli	**FS**	19	15	15	18	20	30	28	20	42	16
	10^{-2}	_	_	_	_	_	25	_	_	36	_
	10^{-4}	_	_	_	_	_	_	_	_	_	_
	10^{-6}	_	_	_	_	_	_	_	_	_	_
Klebsiella sp	**FS**	20	23	20	20	15	24	25	25	40	18
	10^{-2}	_	_	_	_	_	_	_	_	30	_
	10^{-4}	_	_	_	_	_	_	_	_	_	_
	10^{-6}	_	_	_	_	_	_	_	_	_	_
Pseudomonas	**FS**	15	18	14	15	18	12	15	16	16	21
	10^{-2}	_	_	_	_	_	_	_	_	_	_
	10^{-4}	_	_	_	_	_	_	_	_	_	_
	10^{-6}	_	_	_	_	_	_	_	_	_	_
Staphylococcus	**FS**	23	20	23	20	35	25	30	20	15	35
	10^{-2}	_	_	_	_	_	20	_	_	_	_
	10^{-4}	_	_	_	_	_	_	_	_	_	_
	10^{-6}	_	_	_	_	_	_	_	_	_	_

FS- Full Strength, **DO** - Dettol original, **DS** - Dettol sensitive, **DSC** - Dettol skin care, **DF** - Dettol fresh, **LF** - Lifebouy active fresh, **SEO** - Santoor hand wash essential oil, **SEM** - Santoor hand wash extra moisturizing, **LSM** - Lux hand wash strawberry & cream with mintoil, **CEO** - Chandrika hand wash with essential oil and **PN** - Palmolive naturals liquid hand wash

4. Conclusion

The present study shows that of the different liquid hand washes analyzed, Chandrika liquid hand wash is the most effective one against the four tested bacterial species. It seemed to be very much effective against the coliform bacteria, *Escherichia coli* and *Klebsiella* species. But it was less effective to the Gram positive bacteria, *staphylococcus*. Whereas in the case of Lifebuoy and Palmolive, they were more effective against the Gram positive *staphylococcus* species than to the Gram negative organisms. Dettol Original showed better result than that of its different Dettol varieties. The liquid hand wash varieties of Santoor were effective against the coliform bacteria, *E coli*.

Hand hygiene is indicated after using the restroom in cases of diarrhea and after blowing the nose in cases of an upper respiratory tract infection. The use of antimicrobial soaps in all these situations will probably be less effective in preventing cross-transmission of nosocomial pathogen. Only some of the liquid hand washes were effective against the infectious microorganisms.

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In vitro antimicrobial activity of mace *(Myristica fragrans Houtt.)* against pathogenic bacteria

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Abstract

Spices and their essential oils have varying degrees of antimicrobial activity. In this study the antimicrobial activity of aqueous and methanol extracts of mace (the dried seed covers of *Myrsitica fragrans* Houtt.) against 6 species of pathogenic bacteria were investigated. Methanol extract inhibited the bacterial growth more effectively. Growth of *Klebsiella*, *Salmonella typhi* and *Staphylococcus aureus* were inhibited by the methanol extracts of mace. Growth of *Proteus* and *Salmonella paratyphi* were unaffected by the extracts. Aqueous extract appreciably inhibited the growth of *Escherichia coli*.

Keywords: Myristica fragrans, nutmeg, mace, antimicrobial, disc diffusion, methanol extract, aqueous extract

1. Introduction

Spices are roots, bark, seeds, buds, leaves or fruits of aromatic plants added to foods as flavouring agents. However, it has been known since ancient times that spices and their essential oils have varying degrees of antimicrobial activity. Few studies have focused on the mechanism by which spices or their essential oils inhibit microorganisms. Since it has been concluded that the terpenes in essential oils of spices are the primary antimicrobials, the mechanism most likely involves these compounds. Their mode of action might be related to those of other phenolic compounds, which interferes with functions of the cytoplasmic membrane, including PMF and active transport (Zaika, 1988; Dorman and Deans, 2008; Prabuseenivasan et al., 2006; Palmer et al., 1998).

The nutmeg tree is any of several species of trees in genus *Myristica*. The most important commercial species is *Myristica fragrans* Houtt. Nutmeg grows to the height of twenty or thirty feet and is clothed with numerous oblong, pointed, smooth, shining green leaves. Small bunches of pale yellow flowers grow from the axils of the leaves. The tree blossoms throughout the year, bearing ripe fruit and flowers. The fruit, when at maturity, is as large as a moderate sized pear and of a similar form of a reddish-yellow colour on the outside and nearly white within. The fruits when ripe split in the centre and exposes the arillus or mace with which the nut is enveloped. Mace, the dried seed covers of nutmeg, is the net-like layer, brilliant scarlet red in colour, and glossy in appearance, that surrounds the seeds. Beneath this is a hard shell containing the nutmeg, which when ripe is perfectly smooth, but on drying becomes shrivelled (Ahmed, 2010).

Nutmeg is primarily used as a spice but is also known for its pharmacological effects. It is used for flavouring, hair dye and traditional medicine. Two antimicrobial agents namely Malabaricone B and Malabaricone C have been isolated and characterized from mace (Orabi et al., 1991). These molecules exhibit significant level of antibacterial and antifungal activity which has been associated with the phenolic hydroxyl groups and other structural elements of the molecule. Nutmeg is a warm, aromatic, stimulant tonic and is used for vitiated conditions of kapha and vata, inflammations, cephalagia, helminthiasis, halitosis, dyspepsia, flatulence, colitis, cough, asthma, catarrh, diarrhoea, vomiting, strangury, ulcers, skin diseases, cardiac disorders, fever, insomnia, splenopathy and impotency (Varrier, 2002). It has multiple uses such as sedative, stomachic and stimulant. In India nutmeg has

been used for treatment of intestinal disorders, headaches, nerve fevers, cold fevers and bad breath. It has also been employed as a warmth-producing and stimulating drug aiding in digestion. In this investigation, the antimicrobial activities of aqueous and methanol extracts of mace were tested against six species of pathogenic bacteria.

2. Materials and Methods

2.1. Collection of Sample

Mace (*Myristica fragrans* Houtt.) was collected in a sterile screw capped bottle (100 ml) from Kumily and Ettumanoor, Kerala, India. Attempts were made to study the antimicrobial activity of aqueous and methanol extracts of mace.

2.2. Bacterial Cultures

For antimicrobial assay, six bacterial strains namely *Escherichia coli*, *Proteus* sp., *Klebsiella* sp., *Salmonella typhi*, *Salmonella paratyphi* and *Staphylococcus aureus* were used. Bacterial cultures used in this study were obtained from the culture collections of School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala, India.

2.3. Preparation of Extracts of Mace

Freshly collected sample of mace was oven dried at 60 °C for 2 days. The dried samples were ground well into a fine powder with a sterile mortar and pestle. The powder was stored in air sealed containers at room temperature before extraction. A modified method of Alade and Irobi (1993) was adopted for preparation of mace extracts. A fixed weight (5 g) of powdered material was soaked separately in 10 ml of sterile distilled water or methanol for 48 h. Each mixture was stirred at 8 h interval using a sterile glass rod. After 48 h, contents were transferred into sterile mortar and ground well using a pestle. Contents were transferred into a sterile container and added water or methanol. The sample was extracted by boiling for 15 min. At the end of the extraction, each extract was passed through Whatman No. 1 filter paper. The filtrate was transferred into a screw capped bottle and stored at 4 °C till use.

2.4. Preparation of Bacterial Culture Suspension

Pure isolated colonies of bacterial strains were inoculated into 0.1 % peptone water and inoculated at 37 °C for 48 h. After incubation these culture suspensions were used for lawn culture preparation on Mueller-Hinton Agar (MHA) plates.

2.5. Preparation of Discs

The sensitivity discs of 5 mm diameter were punched off from the Whatman No.1 filter paper and sterilized in a water-proof container by autoclaving at 15 lb pressure for 15 min. The sterile discs were impregnated with the extract by dipping in the extract for 10 min and were allowed to dry. These discs were used for the antimicrobial assay. Discs treated similarly with methanol and sterile water were used as controls.

2.6. Disc Diffusion Assay

The antimicrobial activity was tested against aqueous and methanol extracts of mace. About 15 to 20 ml of Muller-Hinton Agar was poured into sterile Petri dishes and were allowed to solidify. Sterile swabs were charged with 48 h old liquid culture in peptone water and about 0.2 ml of inoculum was spread onto the surface of solidified agar plate. The plates were allowed to dry for 5 min. For agar disc diffusion method, the discs carrying the respective extract were impregnated on the seeded agar

plate (2 discs per plate). The plates were incubated at 37 °C for 48 h. The experiment was performed in duplicates.

2.7. Zone analysis

After incubation the antibacterial activity of the extracts against each bacterial culture was assayed by measuring the diameter of zone of inhibition to the nearest mm. The results were recorded and compared.

3. Results and Discussion

Many spices contain the ability to inhibit the growth of microorganisms due to the presence of essential oils or other bioactive compounds. Several studies have been previously focussed on the antibacterial effects of nutmeg (Zaidi et al., 2009; Braz, 2006; Ceylan and Fung, 2004; Dorman and Deans, 2008; Jaiswar and Singh, 2009; Mahoney et al., 2005; Prabuseenivasan et al., 2006). Very few studies have targeted in querying the potential of mace in inhibition of microbial growth. In this study the antimicrobial activity of aqueous and methanol extracts of mace were assayed against six strains of bacterial pathogens. For these six bacterial strains namely *E. coli*, *Klebsiella* sp., *Proteus* sp. *S. aureus, S. typhi and S. paratyphi* were used.

The extracts of mace exhibited varied antimicrobial activity against the strains of bacteria studied. The antibacterial activity of methanolic extract was more than that of aqueous extract. Maximum antibacterial activity was exhibited by *Klebsiella* sp., followed by *S. typhi* and *S. aureus*, also with similar responses. However, no activity was recorded for *S. paratyphi* and *Proteus* sp. The antimicrobial activity of extracts of mace against test organisms used in the present study is recorded in Tables 1 and 2.

Table 1. Antimicrobial activity of aqueous extract of mace (*Myristica fragrans* Houtt.) against pathogenic bacteria

Spice	Bacterial strains	Diameter of zone of inhibition (mm)
Mace (*Myristica fragrans* Houtt.)	*E. coli*	8
	S. aureus	0
	S. paratyphi	0
	S. typhi	0
	Klebsiella sp.	0
	Proteus sp.	0

The growth of *Klebsiella, S. typhi and S. aureus* were inhibited by the methanol extracts of mace and hence were sensitive to the extract. The diameters of the inhibition zones produced for the organisms were 8 mm, 6 mm and 6 mm, respectively (Table 2). However neither of these strains exhibited growth inhibition around the discs impregnated with the water extract (Table 1). Antimicrobial action of nutmeg extracts against *Salmonella* has been reported by Braz in 2006.

Table 2. Antimicrobial activity of methanol extract of mace (*Myristica fragrans* Houtt.) against pathogenic bacteria

Spice	Bacterial strains	Diameter of zone of inhibition (mm)
Mace (***Myristica fragrans*** **Houtt.)**	*Escherichia coli*	0
	S. aureus	6
	S. paratyphi	0
	S. typhi	6
	Klebsiella sp.	8
	Proteus sp.	0

Neither the water nor the methanol extract produced an appreciable zone of inhibition of growth in the disc diffusion assay in case of *Proteus* sp. and *S. paratyphi* (Table 1 and 2); presumably due to the resistance of these bacterial strains to water soluble and volatile bioactive compounds in mace. Braz (2006) observed that nutmeg extracts exhibited dose dependent activity against *E. coli* and *Salmonella*. Takikawa et al. (2002) also observed antimicrobial activity of nutmeg against *E. coli*. On the contrary, *E. coli* exhibited resistance to water extract alone, in our study, and no response was observed in methanol extract. This shows that water soluble compounds in mace were more effective against *E. coli*, which was not possibly extracted out in methanol.

The *in vitro* effects of bioactive compounds in spices against microbes are well known. Yet very few studies have addressed the effects of these compounds against bacterial pathogens. Orabi et al. (1991) isolated two antimicrobial resorcinols namely Malabaricone B and Malabaricone C from the mace of *Myristica fragrans*. They observed that both the compounds exhibited strong antifungal and antibacterial activities against many microorganisms including *Candida albicans* and *Staphylococcus aureus*. Antimicrobial activity has been reported against *Helicobacter pylori* for water and ethanol extracts of *M. fragrans* (Zaidi et al., 2009).

4. Conclusion

In this paper we present the preliminary report on the sensitivity of six major pathogenic bacteria to mace extracts. This study has provided more evidence into the potential of bioactive compounds in mace in inhibiting the growth of pathogenic bacteria. Extracts of mace of *M. fragrans* were found to inhibit growth of both gram-positive and gram-negative bacteria. The study revealed significant antibacterial activity, particularly anti-*Klebsiella* anti-*E. coli* activity for methanol and aqueous extracts of mace, respectively. Our results confirm that mace can be a good source of antibacterial agents which could be used potentially in combating pathogens and pathogenic diseases. However, complementary tests with more efficient extraction methods and screening of more bacterial cultures would be highly essential in unravelling the exact antimicrobial action effectively.

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Study on hypocholesteremic activity of SPHAG -poly herbal formulation in wistar albino rats

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Abstract

In the present study the hypocholesremic activity in cholesterol induced hypercholesteremic rats were performed. Nutraceuticals are gaining importance in current era therefore diet therapy is an idea which can reduce the dosage of medicines. Research in such plant based diet therapy therefore, has a major role in reducing the risk of atherosclerosis which can lead to development of effective and better management of hyperlipidemia thereby reducing the risk of cardiovascular diseases.
Key words: SPHAG, hypercholesteremia, artherosclerosis; neutraceuticals

1. Introduction

In rural India, 70% of the population is dependent on the traditional system of Ayurvedic medicine, Ayurveda (Bent and KO r, 2004). Hypercholesteremia is the increase in the cholesterol levels in the blood stream above the normal range. Abnormal level of lipids (especially cholesterol) can lead to long – term problems such as atherosclerosis. A high level of LDL (the "bad") cholesterol increases the risk of atherosclerosis and high level of HDL (the "good") cholesterol may decrease risk. The major risk factors for the development of atherosclerosis are hypercholesteremia and elevated low- density lipoprotein cholesterol (LDL-C) concentration. (Ross, 1999).

World ethnobotanical information reported that a number of herbal medicines from plants and vegetables are used for controlling hyperlipidemia and related complications in patient (Dahanukar *et al;* 2002). The effect of amla *(Emblica officinalis*) on hypercholesterolemia in cholesterol-fed rats were studied. The result suggests that amla may be effective for high cholesterol and prevention of atherosclerosis (Kim *et al; 2005*).

2.Materials and Methods

2.1. *Collection of Plants*

The plants namely *Solanum nigrum, Premna corymbosa, Holarrhena pubescens, Alstonia Scholaris and Gymnema sylvestre* were collected from the local market. The species was authenticated by the Pharmacy division of CRIP (Central Research Institute of Panchakarma) and Kerala forest research center, Peachi.

2.2. *Experimental Animals*

Adult Wistar Albino Rats were obtained from the CRIP animal house. Animals weighing between 150-

200g were used for the present study. The animals were housed in polypropylene cages (3 animals per each cage) and maintained under standard laboratory conditions (temperature 25+ 2° c) with dark and light cycles (14 /10 hrs). They were allowed free access to standard dry pellet (Amrut, Bangalore) and water .The rats were acclimatized to laboratory condition for 30 days before commencement of experiment. All procedures described were received and approved by the Institute Animal Ethical Committee (IAEC).

2.3. *Chemicals*

Cholesterol(Nice Chemicals Pvt Limited, Cochin), NaCl (Merck Limited, Mumbai), 40% Formaldehyde (Nice Chemicals Private Limited, Cochin), Serum Glutamate Pyruvate Transaminase (Transasia Lab), Serum Glutamate Oxalo acetate Transaminase (Transasia Lab), Alkaline Phosphatase(Transasia Lab), Cholesterol (Bayer Diagnostics), Triglycerides(Bayer Diagnostics), HDL cholesterol (Bayer Diagnostics).

2.4. Aqueous Extraction of plant materials

The fresh leaves were shade dried, powdered and used for the aqueous extraction. The formulation was having equal proportion of *Solanum nigrum, Premna corymbosa, Holarrhena pubescens, Alstonia scholaris and Gymnema sylvestre.* The aqueous extract or decoction was prepared as per the protocoal of Ayurvedic formulary of India. *i.e.*, one portion of drug with 16 portion of water was boiled continuously till the volume reduced to1/4. The decoction was filtered through fine filter and stored in refrigerator in different aliquots for the usage of present study. This decoction was dark brown, non sticky, weighing 1.2 g.

2.5. *Analysing the hypocholesteremic activity using expirimental animals*

Healthy Wistar albino rats were divided into five groups consisting of six animals each. The first group (I) consisted of normal control rats which received single daily dose of distilled water on all 25 days. The second group (II) was treated with cholesterol (1%) on all 25 days. The third group (III) received single daily dose of plant extract (800 mg/Kg) on all 25 days. The fourth group (IV) was treated with lower dose of plant extract (400 mg/Kg) and cholesterol (1%) on all 25 days. The fifth group (V) was treated with higher dose of plant extract (800 mg/Kg) and cholesterol (1%) on all 25 days.

2.6. *Biochemical Studies*

On twenty-fifth day of the experiment, rats were anaesthetized and the blood was withdrawn through intracardiac puncture to the rats. The animals were fasted 12 hours before the collection of blood. Serum was separated by centrifugation at 2500 rpm for 15 minutes and utilized for the estimation of various biochemical parameters namely Cholesterol, HDL Cholesterol, Triglycerides, Serum Glutamate Pyruvate Transaminase, Serum Glutamate Oxaloacetate Transaminase, and Alkaline Phosphatase.

3. Results and Discussion

3.1. *Changes in Cholesterol, LDL and Triglycerides*

Cholesterol, Triglycerides and LDL levels were found to be significantly decreased in Group IV and Group V animals, when compared with Group II animals (Table 1). Serum Triglyceride values are found elevated in Secondary hyperlipoproteinemia, atherosclerosis, glycogen storage diseases, nephritic syndrome and greatly elevated in diabetes mellitus, chronic hepatitis and alcoholism (Buccolo G and

Davis M, 1973). HDL-Cholesterol determination is a good index of the risk of coronary heart disease (Richmond W, 1973). In the present investigation the cholesterol, LDL and triglycerides were found to be significantly increased in Group II animals. However, the levels of cholesterol, LDL and triglycerides were significantly controlled in Group IV and Group V (Table 1). These observations on cholesterol, LDL and triglycerides strongly support the hypocholesteremic activity of SPHAG.

Table: 1 The Concentration of Cholestrerol , LDL , Triglycerides in Serum of Healthy Control and Experimental Groups

Parameters	**Experimental groups**				
	Healthy control I	**disease control** II	**extract control** III	**lower dose** IV	**higher dose** V
Cholesterol (mg/dl)	69.4±20.26	165.7±28.90^{a*}	59.61±10.50^{b*}	125.50±13.8^{c*}	101.20±21.70^{d*}
LDL (mg/dl)	25.6±7.7	61.2±11.4^{a*}	29.2±4.8^{bNS}	31.0±5.9^{c*}	30.50±5.4^{dNS}
Triglycerides (mg/dl)	130.5±16.30	213.66±43.20^{a**}	133.60±12.55^{b*}	167.40±21.3^{c**}	129.80±29.40^{d*}

Values are expressed as Mean± S.D. n=6 animals in each group.
*p<0.05, **p<0.01 when compared to disease control.
a. Group II compared with Group I d. Group V compared with Group II
b. Group III compared with Group I c. Group IV compared with Group II
* p<0.05 * p<0.01 NS Non Significant

3.2. *Changes in SGOT, SGPT and Alkaline Phosphatase:*

SGOT ,SGPT and Alkaline phosphatase levels were found to be significantly decreased in Group IV and Group V when compared with Group II animal(Table 2).Alkaline phosphatase levels were found to be significantly decreased in experimental groups when compared with control rats. Alkaline phosphatase is present in high concentration in liver, bone, placenta, intestine and certain tumors. Physiologically elevated serum alkaline phosphatase occurs in pregnant women and in growing children. Increased levels of the enzyme occur in liver diseases, bone diseases, congestive hearfailure (Tietz, 1976).

4. Conclusion

In overall the study shows that the *SPHAG* decoction has significant hypocholesterolemic effect. The present study was undertaken to investigate the possible hypocholesterolemic effect of water extract/decoction of formulation *SPHAG* in five groups of Wistar Albino Rats. Tribes use herbs as medicinal medicinal plants but authentic studies were not performed regarding that. Our study focused on

the hypocholesteremic effect of *Solanum nigrum, Premna corymbosa, Holarrhena pubescens, Alstonia scholaris and Gymnema sylvestre* extracts which shed lights on the development of future medicines based on Ayurvedic herbs.

Table: 2 The concentration of SGOT,SGPT and AlKALINE PHOSPHATASE in Serum of Healthy Control and Experimental groups .

Parameters	Experimental groups				
	Healthy control I	**Disease control** II	**Extract control** III	**Lower dose** IV	**Higher dose** V
SGOT(IU/L)	125.7±33.7	148.3±32.6^{a*}	138.7±25.90^{bNS}	128.40±31.60^{c*}	142.30±36.80^{d*}
SGPT(IU/L)	42.2±11.70	74.30±18.30^{a*}	47.80±13.80^{bNS}	40.4±8.4^{c*}	41.3±6.28^{d**}
ALKALINE PHOSPHATASE(IU/L)	76.2±10.80	69.8±5.80.^{aNS}	51.0 ±10.8^{b*}	56.0±15.20^{c*}	60.40±12.20^{d*}

Values are expressed as Mean± S.D. n=6 animals in each group. *p<0.05, **p<0.01 when compared to disease control

a. Group II compared with Group I
b. Group III compared with Group
c. Group IV compared with Group II
d. Group V compared with Group I

* p<0.05 * p<0.01 NS Non Significant

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Sediment Characteristics and Macro benthic Community Structure of the Tropical Mangrove Ecosystems – Cochin Backwaters, South West Coast of India

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Abstract

Macrobenthic infauna and sediment structures were examined seasonally for one year to study the benthic faunal abundance and community composition in relation to sediment characterestics form the two distinct mangrove areas of Cochin backwaters. Macrobenthic densities and biomass varied greatly among sites and seasons. The substratum was dominated by clay and silt at Mangalavanam and the sediment at Puduvypin was sandy except at 2 stations. The organic matter was found to be much higher for the entire study area and showed a good correlation with fine grained sediment. The benthic fauna was dominated by Tanaidaceans and amphipods, followed by polychaetes.

Key words: Macrobenthos, sediment characteristics, organic matter, mangroves, Cochin backwater

1. Introduction

The distribution of mangrove ecosystem on Indian coastlines indicates that the Sundarban mangroves occupy very large area followed by Andaman-Nicobar Islands and Gulf of Kachch in Gujarat. Rests of the mangrove ecosystems are comparatively smaller.

Though the existing mangrove forests of Kerala are highly localized, the species diversity of these mangrove and its associates is comparatively rich.

Mangroves are relatively sheltered areas along tropical estuaries, coastal lagoons and backwaters where the regular ebb and flood tides lead to mixing of fresh water from rain and land drainage with marine, coastal and estuarine waters. The most important components of this ecosystem are water, soil and the biota, which belong to different families of plant and animal kingdom.

This is mainly detritus based system unlike the coastal system which is basically plankton based. The protein rich detritus is mostly consumed by the detritivorous organisms from the riverine or nearshore areas, which come to mangrove swamps for feeding, breeding and utilization as nurseries (Odum and Heald, 1975). Since these detritus rich mangrove areas are used by valued table fishes, prawns, crabs and oysters for their reproduction or growth, such swamps are considered of great economic importance for culture as well as capture fisheries.

Several reports on various aspects are available from the different mangrove ecosystems of west and coast of India(Untawale and Parulekar, 1976; Pillai *et al*, 1979; Gopinathan and Rajagoplan, 1983; Jagtap 1985 and Wafar *et al.* , 1997). Data on primary, secondary and benthic production rates for the Mandovi – Zuari estuarine complex have been summarized by Qasim and Wafar (1990).

Information on the mangrove environments of Cochin backwaters is mainly from the reports of Gopalan *et al.*, (1983), Kurian (1984), Rajagopalan (1985), Rajagopalan *et al.*, (1986) and Purushan (1989).

Studies on the bottom fauna of mangrove area of Cochin backwater are restricted to the work of Sunilkumar (1993, 1995, 1996 and 1999), Sunilkumar and Antony (1994) and Sheeba (2000)

It is recognized that for the sustenance of highly productive nature of the coastal waters, a kin of the benthic system is imperative. An assessment of the health of a particular ecosystem can be achieved only through a careful analysis of the benthic fauna.

Taking into consideration all the viewpoints, the objective of the present investigation was to assess the diversity and spatial/temporal variations in the benthic fauna along with the sediment characteristics.

2. Materials and methods

2.1. Study area (Figure -1).

2.1.1. Mangalavanam, located as a part in the Cochin backwater system (90° 59' N & 76° 11' E). It is a mangrove bitope comprising 3.1 hectares of area which is connected with Cochin barmouth system through a feeder canal. This mangrove area is fed by the tidal waters through this canal. It is having a diversified terrestrial and aquatic flora and fauna including a variety of bats and more than fifty eight species of birds. Mangalavanam is constituted by less than ten species of mangrove vegetation of which species of *Avicennia, Rhizophora, Excoecaria and Acanthus* are some of the dominant halophytes. Oil pollution is a serious threat faced by this mangrove ecosystem. The depth of the water mass is 60cm and this area receives tidal influx daily and it is of mixed semi diurnal type with an average range of 1m.

2.1.2. Pudu Vypin (9°58' to 10°12' N and 76°10' to 76°12' E) a naturally accreted wetland at the southwestern tip of Vypin island, Being located at northwestern bank of Cochin barmouth facing Arabian sea, most of the area is subjected to inundation by semidiurnal type of rhythm in Cochin barmouth. The almost 400 hectares area accreted so far is exclusively marshy with lot of mangrove vegetation. The environment is a shallow salt marsh with a depth of about 1 to 1.5m, a width ranging from 40 to 50m and about a kilometer along the north south axis. The dominant plant species found here are *Bruguiera gymnorhiza, Rhizophora mucronata, Avicennia officinalis, Excoecaria agallocha, Acanthus ilicifolius* and *Derris trifoliata*.

2.2. Sampling and Analytical Methods

Collections were made for sediment and benthic fauna from 5 stations at Pudu Vypin and 2 stations at Mangalavanam mangrove areas. Each station is 50 to 60 m apart. The study area experiences a monsoonal climate, thus seasonal break-up is into monsoon (June-September), post-monsoon (October-January) and pre-monsoon (February-May). Monsoon is characterized by heavy rain fall. Post monsoon is dry and relatively cool and premonsoon is dry, hot and humid.

Duplicate grab samples were collected from all stations using a van Veen grab (0.048m^2) and sediments were sieved through a 500 µ mesh and preserved in 5% neutral formalin mixed with rose bengal stain for subsequent identification (Birkett & McIntyre, 1971). The actual number of organisms counted were converted to No/m^2. The biomass values were expressed as wet weight in g/m^2. Samples for grain size and organic carbon content were treated separately. Grain size and organic carbon were estimated by the methods of Krumbein and Petty John (1938) and El Wakeel and Riley (1957) respectively. Organic matter was calculated by multiplying the organic carbon values by a factor of 1.724 and was converted into percentage (Trask, 1939).

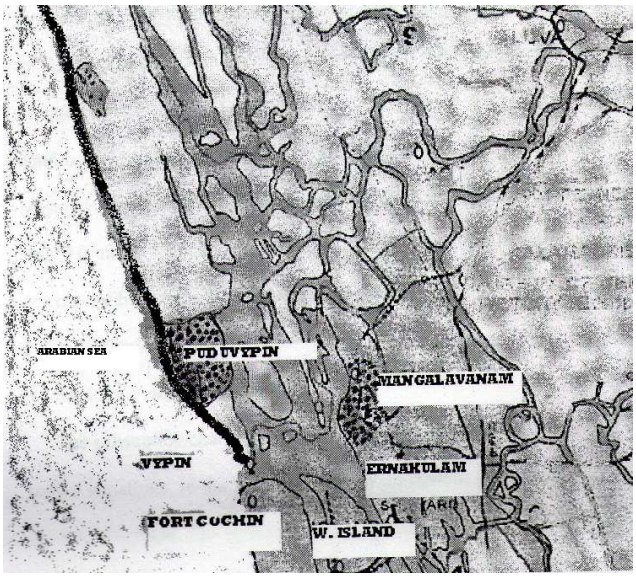


Figure – 1. Study Area

3. Results

3.1. Sediment characteristics

The substratum characteristics of the study area (Figure – 2) showed temporal and spatial variations. In the present study the substratum was dominated by clay and silt at Mangalavanam. The sediment at Puduvypin was found to be more sandy except at station P1 and P2. Clay and silt together can be considered as finer fraction with size approximately 63 microns and less and sand as particles with size greater than 63 microns. The organic matter (Figure – 3) was found to be much higher for the entire study area and showed a good correlation with fine grained sediment.

3.2. Bottom fauna

The seasonal variation in biomass and density are given in Figures 3 & 4. A total of 24 groups/ species were encountered from the mangrove area of Puduvypin. Tanaidaceans were the dominant group represented by 2 species. Amphipods were the second dominant group having 3 species followed by polychaetes with 8 species. Penaeid prawns and juvenile fishes were observed at all stations but with low densities. Among polychaetes the spionid worm, *Prionospio polybranchiata* and the capitellid, *Capitella capitata* that are abundant in highly organic rich area, were recorded here.

A total of 21 groups/species were recorded from the mangrove area of Mangalavanam. Gastropods were the dominat groups followed by tanaidaceans and amphipods. Six species of polychaetes were present. Though 11 groups were encountered from the study area (Table-1), the biomass, number and percentage composition of different groups varied from station to station (Figure 4 & 5 and Table 2).

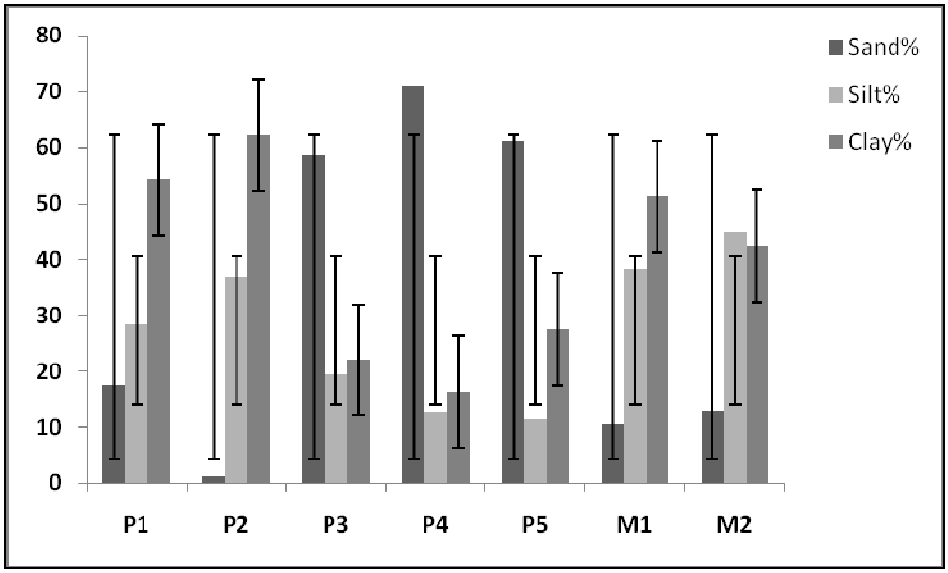


Figure -2. Sediment texture at different stations (Mean ± SD)

Seasonal variation showed higher biomass and density during monsoon followed by post-monsoon and pre-monsoon. The groups namely polychaetes, amphipods, tanaidaceans, decapods and juvenile fishes ere commonly noticed at all stations. Species composition of the different stations in the mangrove area showed that tanaidaceans, the dominant and common group was represented by two species namely *Apseudes gymnophobium* and *A. chilkensis*. The second dominant group amphipod was represented by, *Eriopisa chilkensis, Corophium triaenonyx and Quadrivisio bengalensis*. The third dominant and common group was the polychaete represented by 8 species belonging to 5 families. Among decapods, penaeid prawns were collected from all the stations. The other groups/species present ere the isopod *Asellus* sp, *Mysid* sp., *Littorina littorea*, gastropods, *Dentalium* sp., bivalves, *Cardium* sp., *Cavolina* sp., the insects Chironomid and water beetle, Juvenile fishes and anthozoans.

4. Discussion

Benthic study plays a major role in the strategy of biodiversity conservation. The benthos reflects the effects of pollutants or organic enrichment by responding through detectable changes in population dynamics on a time scale of months to years. Benthic monitoring is also a relatively sensitive, effective

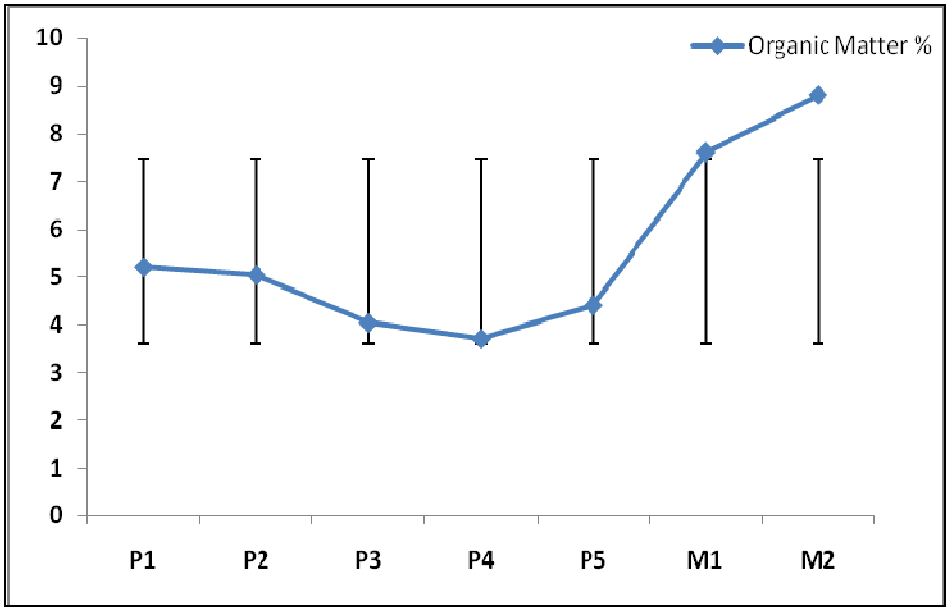
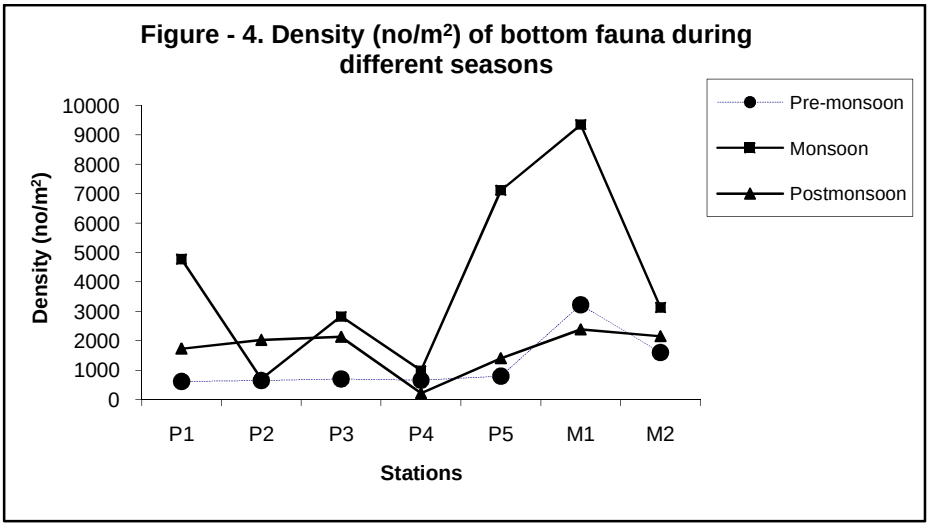


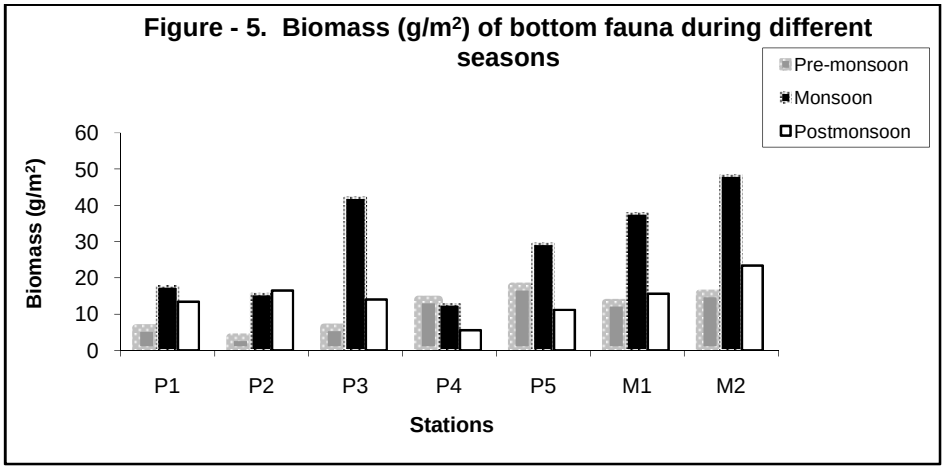
Figure – 3. Organic Matter at different stations (Mean ± SD)

and reliable technique that can detect subtle changes that serve as an early indicator before more drastic environmental changes occur.

Studies on trophic relationships in mangroves generally assume larval, juvenile and adult stages of shrimp, penaeid prawns and fishes to be predators on zooplankton and meio and macrobenthic food sources. Besides food, mangals are also believed to offer shelters and nursery sites to the visiting epibenthos. However, spatial partitioning in the epibenthic distribution in terms of feeding is pronounced. Mangrove forest itself to be invaded for shelter rather than for food during high tides, leaving only the surrounding areas as feeding grounds.

Waters of the mangrove environment are generally turbid because they are shallow regions and subjected to action of tides, waves and currents. The constant wave action and currents disturb surface sediments in the mangrove substratum resulting in high suspended matter in the mangrove ecosystem. The importance of the substratum as an abiotic factor in respect of its physical characters as consistency, water content and grain size etc. and as a biological factor as regards to its content of the organic matter and its microbial turnover was stressed by many workers. Generally the state of preservation depends partly on its texture. In the present study substratum was dominated by clay and silt at Mangalavanam and sandy substratum dominated at Puduvypin. The organic carbon content was found to be much higher for the entire study area (4 - 9%). The preservation of organic matter is exclusively restricted to sediments. The





texture of the sediment plays a very important role in binding the organic matter. Organic matter has a high affinity for fine grained sediment that accumulated in mangroves as it adsorbs onto mineral surfaces. The fine particles may provide increased surface area per unit weight for adsorption of organic matter. So organic matter concentration increase with decreasing grain size. Sandy sediments are relatively poor in organic matter preservation. The organic matter in the mangrove sediment is in excess of the estuarine sediment due to the inherent biological productivity within the mangrove. Decomposition of mangrove foliage and other vegetation remains and their re-suspension also contributes substantially to the organic matter content in mangrove sediment.

It is well established that the distribution of bottom fauna mainly depends on the substratum characteristics as well as organic matter content. The substratum dominated by clay and silt with high organic carbon content favoured the dominance of detritivorous organisms like polychaetes, tanaidaceans and amphipods. Many of the decapod crustaceans depend on detritus for food, in spite of being

Table - 1. List of major groups/ species encountered during the study

Groups/Species	Stations		Groups/Species	Stations	
	Mangalavanam	Pydu-vypin		Mangalavanam	Pydu-vypin
Polychaetes			**Decapods**		
Order Errantia			Penaeid prawn	+	+
Family Nereidae			Crab	+	+
Lycastis indica	+	+	**Isopods**		
Dendronereis aestuarina	+	+	*Asellus* sp.	+	+
Perenereis cavifrons	+	+	**Mysids**		
Family Glyceridae			Mysid	+	+
Subfamily Glycerinae			**Gastropods**		
Glycera longipinnis		+	*Gastropod* sp.	+	-
Order Sedentaria			*Littorina littorea*	+	+
Family Spionidae			*Dentalium* sp.		+
Prionospio pinnata	+	+	**Bivalves**		
Prionospio polybranchiata	+	+	*Bivalve* sp.	+	-
Family Capitellidae			*Cavolina* sp.		+
Capitella capitata	+	+	*Cardium* sp.		+
Family Sternaspidae			**Insects**		
Sternaspis scutata		+	Water beetle	+	+
Amphipods			Chironomid	+	+
Eriopisa chilkensis	+	+	**Fishes**		
Corophium triaenonyx	+	+	Juvenile fish	+	+
Quadrivisio bengalensis		+	**Rare Groups**		
Tanaidaceans			Anthozoa	+	-
Apseudes chilkensis	+	+			
Apseudes gymnophobium	+	+			

Table - 2. Percentage contribution of major groups at different stations

Groups	Stations						
	P1	P2	P3	P4	P5	M1	M2
Polychaetes	17.61	28.30	26.95	20.24	6.55	1.68	16.45
Tanaidaceans	55.65	37.11	45.81	11.55	79.64	20.45	25.34
Amphipods	22.13	28.25	24.76	17.74	12.87	16.95	20.14
Isopods	0.15	-	-	-	-	-	-
Decapods	0.08	0.17	0.40	0.31	0.18	0.16	0.41
Mysids	0.39	0.17	-	0.31	-	-	0.41
Gastropods	3.60	0.52	0.50	20.25	0.35	60.40	30.34
Bivalves	0.08	0.34	0.30	0.31	-	0.12	0.41
Insects	-	-	-	23.96	-	-	-
Fishes	0.23	0.34	0.30	3.12	0.41	0.16	4.20
Others	0.08	4.80	0.98	2.21	-	0.08	2.30

carnivorous. In the energy food web of coastal ecosystem detritus appears to be one of the primary source of carbon and nitrogen (Odum 1971; Benner *et al*, 1986). Not only the sediment disturbances but also the general feeding habits of the dominant species can result in several species being excluded from an environment despite its suitability and hence low diversity is maintained (Rhoads1974 ; Keshavarz 2012).

5. Conclusion

The nature of the substratum showed that the composition of the sediment varied markedly at all the stations investigated. Clay and Silt are the dominant factors at Mangalavanam and sandy substratum was noticed at Puduvpin. Based on the data obtained, the substratum of these mangrove areas can be differentiated into four sediment types - sandy, clayey sand, silty clay and clayey silt. The area is provided with rich organic matter and it has a high affinity with fine grained sediment. This ecosystem supports a number of flora and fauna. From this study it can be inferred that mangrove soil habitat is biologically rich and provides a unique ecological niche to a variety of soil dwelling organisms.

Acknowledgement

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In vitro antibacterial potential of *Premna latifolia* extract and isolation of its active components

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Abstract

In the present study antibacterial and phytochemical screening of *Premna latifolia* extract were evaluated. Turbidimetric method and disc method were used for antibacterial analysis. The strains of bacteria used were *Escherichia coli*, *Salmonella typhi*, *Vibrio parahaemolyticus* and *Bacillus cereus*. In the present study, the ethanol extract of *Premna latifolia* showed antibacterial activity against *Escherichia coli*, *Salmonella typhi* and *Vibrio parahaemolyticus*. There was no inhibitory effect on the growth of *Bacillus cereus*. Maximum zone of inhibition for tetracycline was found to be 24.3mm. In *Premna latifolia* treated culture the zone of inhibition against *Salmonella typhi* was found to be 23.7mm (100 µg/ ml) and 17.3 mm (50 µg/ ml). Phytochemical analysis showed that *Premna latifolia* extract contain alkaloids and phenols. The results showed that the bioactive agents present in the *Premna latifolia* extract may be responsible for its antimicrobial activity.

Keywords: Premna latifolia, phytochemicals, antibacterial activity.

1. Introduction

Nature has been an enormous source of agents of medical importance. Despite of tremendous progress in human medicines, infectious diseases caused by bacteria, fungi, viruses and parasites are still a major threat to public health. Their impact is particularly large in developing countries due to relative unavailability of medicines and the emergence of widespread drug resistance. During the last two decades, the development of drug resistance as well as the appearance of undesirable side effects of certain antibiotics has led to the search of new antimicrobial agents mainly among plant extracts with the goal to discover new chemical structures, which overcome the above disadvantages.

Since time immemorial, different parts of medicinal plants have been used to cure specific ailments in India. Current research on natural molecule and products primarily focuses on plants since they can be sourced more easily and be selected based on their ethno-medicinal uses (Kumar *et al*., 2011). This interest primarily stems from the belief that green medicine is safe and dependable, compared to costly synthetic drugs which are invariably associated with adverse effects.

Plants are used medicinally in different countries and are a source of many potent and powerful drugs (Mahesh *et al*., 2008). A wide range of medicinal plant parts is used for extract as raw drugs and they possess varied medicinal properties. Although hundreds of plants species have been tested for antimicrobial properties, the vast majority of have not been adequately evaluated. Because a large number of plant species still need to be analyzed for their antimicrobial activity against diverse bacteria, it is critical to develop simple systems for rapid antimicrobial screening (Joshi *et al*., 2011). Considering the vast potentiality of plants as sources for antimicrobial drugs with reference to antibacterial agents, a systematic investigation was undertaken to screen the antimicrobial activity of *Premna latifolia*.

2. Materials and methods

2.1. *Plant extract*

Premna latifolia (family-Verbenaceae) was collected locally and 75 % ethanolic extract was made.

2.2. *Tested microorganisms*

Different bacterial strains were collected from Veterinary College, Mannuthy, Kerala. Antimicrobial activity of plant extract was investigated against four bacterial strains. They were *Salmonella typhi*, *Escherichia coli*, *Bacillus cereus* and *Vibrio parahaemolyticus*. The reference strains of bacteria were maintained on Nutrient agar (Hi Media, Mumbai). The cultures were subcultured regularly (every 30 days) and stored at 4^0C.

2.3. *Preparation of culture*

From stock culture aliquot quantity of culture inoculated into 25 ml of nutrient broth and incubated overnight at 37^0C

2.4. *Experimental design*

For antibacterial activity study turbidimetric method and disc method were selected.

2.4.1. *Turbidimetric method*

Varying concentrations of the drug were added into the identical tubes in triplicate. 2.5ml nutrient broth and 0.1 ml culture were also added to the same tubes. Tubes with 0.1 ml culture suspended in 2.5 ml nutrient broth are taken as control. All tubes were incubated at 37^0C and optical density of the control and experimental tubes were measured at different time intervals ranging from 1hr, 5hr and 24hr at 530nm (Brown, 1966).

2.4.2. *Disc method*

15 ml of nutrient agar medium was dispensed into pre-sterilized petridishes to yield a uniform depth for bacterial inoculation. The discs were impregnated with varying concentrations of the extract and dried for 10-15 minutes. The dried discs were placed on the agar surface with flamed forceps and gently pressed down to ensure contact with the agar surface. Tetracyclin (10µg) was used as positive control. The discs were spaced far enough to avoid overlapping rings of inhibition. Finally, the petridishes were incubated for 24 hours at 37^0 C for bacteria. The diameter of zone of inhibition (ZI) (mean of triplicates ± SD) as indicated by clear area which was devoid of growth of microbes was measured (Davis *et al*., 1971). The activity index of the crude plant extract was calculated as

$$\text{Activity index (A.I.)} = \frac{\text{Mean of zone of inhibition of the extract}}{\text{Zone of inhibition obtained for standard antibiotic drug}}$$

2.4.3. *Determination of the minimal inhibitory concentration (MIC)*

The antibacterial activity of the plant extracts was determined using 96-well plates. MIC values, which represent the lowest concentration of extract that completely inhibits the growth of microorganisms, were determined by a micro-well dilution method (Zarai1*et al*., 2011). The inoculum of each bacterium was

prepared and the suspensions were added to each well. Extract was dissolved in 100% ethanol and then dilutions series were prepared in a 96-well plate. Each well of the micro plate included 40 µl of the growth medium, 10 µl of inoculums and 50 µl of the diluted sample extract. Ethanol is used as negative control. The plates were then covered with the sterile plate and incubated at 37°C for 24 h. After that, 40 µl of 3- (4, 5-dimethyl-thiazol-2-yl)- 2,5-diphenyl-tetrazolium chloride (MTT) at a final concentration 0.5 mg/ml freshly prepared in water was added to each well and incubated for 30 min. The change to red colour indicated that the bacteria were biologically active. The MIC was taken to the well, where no change of colour of MTT was observed. The MIC values were done in triplicate.

2.5. *Separation of active compound from Premna latifolia extract by thin layer chromatography (TLC)*

2.5.1. *Identification test for phenols*

TLC plate was loaded with *Premna latifolia* extract and allowed to dry. It was run with the solvent methanol: chloroform in the ratio 1:5. Dried plate sprayed with Folin– Ciocalteu reagent and kept for sometime. Band was visualized under UV inspection box (Chhetri *et al*., 2008).

2.5.2. *Identification test for alkaloid*

TLC plate was loaded with *Premna latifolia* extract. After drying it was run with suitable the solvent methanol: chloroform in the ratio 1:5. It was sprayed with Dragendroff's reagent and kept for drying. Band was visualized under UV inspection box. It imparts orange precipitate when alkaloid is present in the sample (Chhetri *et al*., 2008).

2.6. *Statistical analysis*

The experimental results were repeated thrice and zone of inhibition were determined in millimeter. All the results were statistically expressed as the mean ± SD.

3. Results

In the present study antibacterial and phytochemical screening of *Premna latifolia* extract were evaluated.

3.1. *Effect of Premna latifolia extract against different strains of bacteria using turbidimetric method*

The optical density of the control and experimental tubes were measured at different intervals of time and values were listed in Table 1. The turbidity readings for *Salmonella typhi* was found to be 1.264, while the reading for *Premna latifolia* extract (50 and 100 µg/ ml) against *Salmonella typhi* were 0.905, 0.701 respectively. There was a concentration dependent inhibition of growth of bacteria observed in *Premna latifolia* treated cultures. After 24h treatment, *Escherichia coli* showed optical density 0.604 and the extract treated group showed the optical densities 0.471 and 0.384. Optical density value was found to be lower than the control value indicating that effectiveness of the drug against this bacterium. There was no inhibition of growth of *Bacillus cereus* was observed by the *Premna latifolia* extract. The optical density of *Vibrio parahaemolyticus* and 50 and 100 µg/ ml drug treated groups were 0.714, 0.573, 0. 449 respectively. In this case also a concentration dependent inhibition of the growth of bacteria was observed.

3.2. *Effect of Premna latifolia extract against bacteria using disc method*

Premna latifolia extract showed potential inhibitory action against *Salmonella typhi* 23.7mm (100 µg/ ml) and 17.3 mm (50 µg/ ml). Maximum zone of inhibition for tetracycline was found to be 24.3mm

(Table 2). Activity index of *Premna latifolia* extracts against this bacterium varies from maximum 1 for (100 µg/ ml) to the minimum 0.45 for (10 µg/ ml).

Table 1: Antibacterial activity of *Premna latifolia* extract against different strains of bacteria by turbidimetric method

Strains of Bacteria	Concentration	Optical density at 530nm		
		1h	5h	24h
Salmonella typhi	Control	0.365	0.696	1.264
	Premna latifoilia (50 µg/ ml)	0.363	0.555	0.905
	Premna latifoilia (100 µg/ ml)	0.328	0.415	0.701
Escherichia coli	Control	0.355	0.462	0.604
	Premna latifoilia (50 µg/ ml)	0.348	0.353	0.471
	Premna latifoilia (100 µg/ ml)	0.284	0.324	0.384
Bacillus cereus	Control	0.245	0.609	1.269
	Premna latifoilia (50 µg/ ml)	0.363	0.678	1.275
	Premna latifoilia (100 µg/ ml)	0.328	0.695	1.286
Vibrio parahaemolyticus	Control	0.345	0.649	0.714
	Premna latifoilia (50 µg/ ml)	0.358	0.503	0.573
	Premna latifoilia (100 µg/ ml)	0.393	0.485	0.449

Table 2: Antibacterial activity of *Premna latifolia* extract against *Salmonella typhi* by disc method

Antibiotic/plant extract	Zone of inhibition (mm)	Activity index
Tetracyclin (10 µg / ml)	24.3 ± 1.5	
***Premna latifoilia* (10 µg/ ml)**	11.0 ± 2.6	0.45
***Premna latifoilia* (50 µg/ ml)**	17.3 ± 2.1	0.71
***Premna latifoilia* (100 µg/ ml)**	23.7 ± 3.1	1.0

3.3. *Minimum inhibitory concentration (MIC) of Premna latifolia extract*

The minimum inhibitory concentration (MIC) of the ethanolic extract of *Premna latifolia* for different strains of bacteria ranged from 3.125 -25 µg/ml (Table 3).

Table 3: Minimal inhibitory concentration of *Premna latifolia* extract against different strains of bacteria

Strains of bacteria	Minimal inhibitory concentration(MIC)
Salmonella typhi	3.125 µg
Escherichia coli	6.25 µg
Bacillus cereus	25 µg
Vibrio parahaemolyticus	12.5 µg

3.4. *Preliminary phytochemical analysis of Premna latifolia extract*

Preliminary phytochemical analysis of the ethanol extract of *Premna latifolia* showed the presence of phenol and alkaloid. Bands for phenol and alkaloids were observed under UV inspection box.

4. Discussion

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is *in vitro* antibacterial activity assay. In the present study, the ethanol extract of *Premna latifolia* showed the activity against *Escherichia coli*, *Salmonella typhi* and *Vibrio parahaemolyticus*. The antibacterial activity of this extract was evaluated by the turbidimetric method. When compared to the control, growth of inhibition of *Escherichia coli*, *Salmonella typhi* and *Vibrio parahaemolyticus* was observed. There was no inhibitory effect on the growth of *Bacillus cereus*. The effect of *Premna latifolia* extract was also checked by disc method against *Salmonella typhi*. Here also the growth of inhibition was confirmed by the zone of inhibition of bacteria.

Preliminary phytochemical analysis s of the extract showed that this plant extract contains alkaloids and phenols. These compounds are known to be biologically active and therefore aid the antimicrobial activities. Secondary metabolite is crucial for plant defenses which has enabled plants to survive; e.g. - phenolics, alkaloids, steroids, terpenes, saponins, *etc*. Phenolic is one of the major groups of phytochemical that can be found ubiquitously in certain plants. Phenolic compounds are potent antioxidants and free radical scavenger which can act as hydrogen donors, reducing agents, metal chelators and singlet oxygen quenchers. Purified alkaloids as well as their synthetic derivatives are used as medicinal agents for their various biological effects such as analgesic, antispasmodic and bactericidal (Kaur *et al*., 2009). Alkaloids are basic nitrogenous compounds with definite physiological and pharmacological activity. The active components usually interfere with growth and metabolism of microorganisms in a negative manner (Mohanta *et al*., 2007). These secondary metabolites exert antimicrobial activity through different mechanisms. Several proposed mechanisms include membrane damage, changes in intracellular pH, membrane potential, and ATP synthesis.

5. Conclusion

The results obtained from this work revealed that *Premna latifolia* contained bioactive agents which are connected with its antimicrobial property. From the studies, it is also concluded that the traditional plants may represent new sources of antimicrobials with stable, biologically active components that can establish a scientific base for the use of plants in modern medicine. These local ethno-medical preparations and prescriptions of plant sources should be scientifically evaluated and then disseminated properly and the knowledge about the botanical preparation of traditional sources of medicinal plants can be extended for future investigation into the field of pharmacology, phytochemistry, ethnobotany and other biological actions for drug discovery.

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Amelioration of Oxidative Stress Induced Cellular and Molecular Insults by *Spirulina* Extract

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Abstract

Aim of the study is to evaluate the protective effect of *Spirulina* extract (SPE) against free radicals induced oxidative stress under *in vitro* condition. The radical scavenging activity of SPE was analysed by antioxidant assays (DPPH, GPx and GSH). The cytoprotective activity was studied by trypan blue dye exclusion method. The protective effect of SPE on membrane damages and DNA damages were monitored by lipid peroxidation assay and alkaline single cell gel electrophoresis assay respectively. The protective efficacy of the extract against the oxidative damages induced by H_2O_2 under *in vitro* condition was studied. From the decreased comet parameters in spleenocytes, it is evident that SPE could protect the cellular DNA from the oxidative damage induced by H_2O_2. The increased antioxidant status and cytoprotective activities suggest that the protection could be mediated by strong radical scavenging activity of *Spirulina* extract. Since SPE rendered protection from free radical induced DNA and membrane damages thereby reduced the mortality of cells, further *in vivo* studies are essential to support the use of it as a dietary supplement.

Key Words: Spirulina extract, Cytoprotection, Oxidative stress, antioxidant defence system

1. Introduction

The production of reactive oxygen species (ROS) can induce oxidative damage to vital cellular molecules including DNA, protein and lipids there by damaging the cellular antioxidant defence mechanism (De Groot, 1994; Gracy et al., 1999) in variety of cells. In the living cell, the most important target of free radicals is genomic DNA and the type of damages on DNA includes single and double strand breaks, base damage, sugar damage etc, (Jagetia, 2007). The oxidative stress induced by free radicals is one of the main causes of these DNA damages (Pradhan et al., 1972). Apart from these DNA damages, membrane damage is also considered to be a critical event of free radicals in the living cells (Agarwal and Kale, 2001).

The deleterious effects of ROS are controlled by cellular antioxidant defence system, including non-enzymatic radical scavengers and enzymes that can either directly detoxify ROS or indirectly regulate their levels (Marian Valko et al., 2007). Many natural and synthetic compounds were found to protect against radical induced damage in biological systems (Nair et al., 2001). However, most of them exhibit inherent toxicity and side effects at the protective concentrations. Hence, the interest in search of effective and non-toxic free radical scavenging compounds lead to investigations on naturally occurring antioxidants and phytoceuticals.

Spirulina is a multicellular photosynthetic filamentous cyanobacterium (Blue green Alga) consisting of Blue green filaments of cylindrical cells (1 – 12 μm diameter) (Belov A.P et al., 1997). The genus *Spirulina* has gained importance and international demand for its high value phytonutrients, pigments, which have application in health foods, therapeutics and diagnostics (Becker, 1994; Richmond, 1992). It has been hailed as the 'Food of the future' besides being considered as an ideal food for astronauts by NASA (Cornet et al., 1990 and Basiraath et al.,1992). The biomass of *Spirulina* has been recognized as "wonderful food for health" since it contain high proteins and various bioactive compounds such as essential Fatty Acids (linolenic and γ-Linolenic acid), essential amino acids, B complex vitamins,

Bio pigments (phycocyanin and chlorophyll-a) and Carotenoids (Mani et al., Juarez-Oropeza et al., Chopra et al.,2008) .

Recently, much attention has been focused on the micro algae, particularly Blue green micro alga (Cyanobacteria) as a source of novel biologically active compounds such as phycobiline, phenols, terpenoids, steroids and polysaccharide (Zhang et al., 2006 and Abd El – Baky et al., 2008). One of the components mainly responsible for its antioxidant activity is the biliprotein, phycocyanin (Romay et al., 1998; Gonz´alez et al., 1999). In the present study, the ability of SPE to scavenge free radicals thereby preventing cell mortality was investigated under *in vitro* condition.

2. Materials and Methods

2.1. Chemicals

DPPH (1,1-diphenyl-2-picryl-hydrazyl) and TBA (thiobarbituric acid) were from Sigma Chemical Company Inc., St Louis, MO, USA. Hydrogen peroxide was from Merck Specialities Pvt. Ltd Mumbai. All other reagents were of analytical grade and purchased from reputed Indian manufacturers.

2.2. Spirulina Extract

SPE was presented to Dr.CKK Nair for research studies from Parry, India.

2.3. Free radical scavenging activity of Spirulina extract

The free radical scavenging activity of SPE was determined by the method of Gadov *et al* (1997) (Gadov et al.,1997) with minor modifications (Gandhi and Nair,2004). Methanolic solution of DPPH (63.4 μM) was incubated at ambient temperature with various concentrations of the SPE (0.062–1 mg/ml) and the absorbance was measured at 515 nm using spectrophotometer.

Antioxidants react with DPPH, which is a stable free radical and is reduced to the DPPH-H and as a consequence the absorbance decreased from the DPPH radical to the DPPH-H form. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability (Oktay et al., 2003). Lower the absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH Scavenged (\%)} = [(A_{control} - A_{test})/A_{control}] \times 100$$

where $A_{control}$ is the absorbance of the control reaction and A_{test} is the absorbance of the extract.

2.4. Inhibition of membrane damage induced by H_20_2

Levels of peroxidation of membrane lipid were assayed based on the method of Buege and Aust (1978) (Buege et al., 1978). Hydroxy radicals generated by the treatment with H_20_2 leads to the production of lipid radicals which subsequently react with oxygen to form lipid peroxy radicals after undergoing molecular rearrangement of conjugation in double bonds and eventually a chain reaction is initiated (Pandey and Mishra, 2000). Malondialdehyde (MDA) is one of the products of lipid peroxidation eventually forms adduct with cellular DNA. TBARS assay (Thiobarbituric acid reactive substances assay) is used to quantify this end-product of lipid peroxidation. (Lef'evre., et.al., 1998; Janero., 1990).

100μl of mouse liver homogenate (10% w/v) prepared in phosphate-buffered saline (PBS) was treated with various concentrations of H_2O_2 (0.25 mM – 1 mM) and SPE (50 mg/ml, 100 mg/ml). The final result was expressed as *nanomoles of malonedialdehyde per mg protein.* Protein levels were measured by the method of Lowry (1951) (Lowry et al., 1951).

2.5. Cytoprotective activity assay by trypan blue dye-exclusion method

A single cell suspension (10^6 cells/ml) of spleenocytes of mice was prepared with PBS. The cell suspension was treated with various concentrations (0.1-10*mg/ml*) of SPE in the presence of 0.5mM H_2O_2. The viability of cells were measured at various time intervals (0-3hrs) using trypan blue dye exclusion method (Guptha et al., 1974). Cytotoxic substances make pores on the membrane through which dye enters into the dead cells. Since live cells are excluded from staining it is easy to distinguish viable cells from dead cells.

2.6. Measurement of DNA damage by the use of alkaline single cell gel electrophoresis (Comet assay)

DNA damages in single cell was analysed by using comet assay in mouse intestinal cells. Single cell suspension (10^6 cells/ml) prepared in PBS was exposed to 0.5mM H_2O_2 and treated with various concentrations (5-20mg/ml) of the extract. The cells were embedded along with low melting agarose (0.8%) in normal melting agarose (1%) coated slides. After solidification these slides were kept in pre-cooled lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, pH 10, 1% DMSO and 1% Triton X) to ensure cell lysis for one and half hours and drained accurately. The slides were kept in alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, 0.2% DMSO, pH $\geq$ 13) for 10-20 *minutes* and electrophoresed at 20V for 30 *minutes*. The slides were then washed with distilled water and allowed to dry. The dried slides were stained with fluorescent dye, propidium iodide (50μg/ml) and viewed under fluorescent microscope (Klaude et al., 1996; Anderson et al., 1998). The comet parameters such as tail DNA%, tail length, tail moment and olive tail moment were calculated by using software 'CASP'. The tail moment (TM) is the product of %DNA in tail and tail length. Olive tail moment (OTM) is the product of the distance between the centre of the head and the centre of the tail and % DNA in tail (Cerda et al., 1997).

2.7. Statistical analysis

The results are represented as mean ± SD. Statistical analyses of the results were performed using ANOVA with Tukey-Kramer multiple comparisons test.

3. Results and Discussion

3.1. DPPH radical scavenging activity of Spirulina extract

Figure 1 represented the radical scavenging activity of various concentrations (0.062 - 1*mg/ml*) of SPE. Increase in the concentration of the extract showed increased percentage of the DPPH radical scavenging.

3.2. Inhibition of TBARS formation

Two concentrations of SPE (50 and 100*mg/ml*) were used to analyse its power to protect cells from H_20_2 induced membrane damages in mouse liver cells. The highest drug concentration showed higher inhibition in the formation of MDA, the end-product of lipid peroxidation than its lower concentration as represented in figure 2.

3.3. Cytoprotective activity of Spirulina extracts

Figure3 represented the cytoprotective activity of extract in spleenocytes of mouse. Cells treated with H_2O_2 showed higher mortality rate even at one hour counting of cells. The administration of H_2O_2 along with increase in concentrations of the extract showed decrease in mortality of the cells in a concentration dependent manner. Thus *Spirulina* could protect the cells from free radical induced cell death.

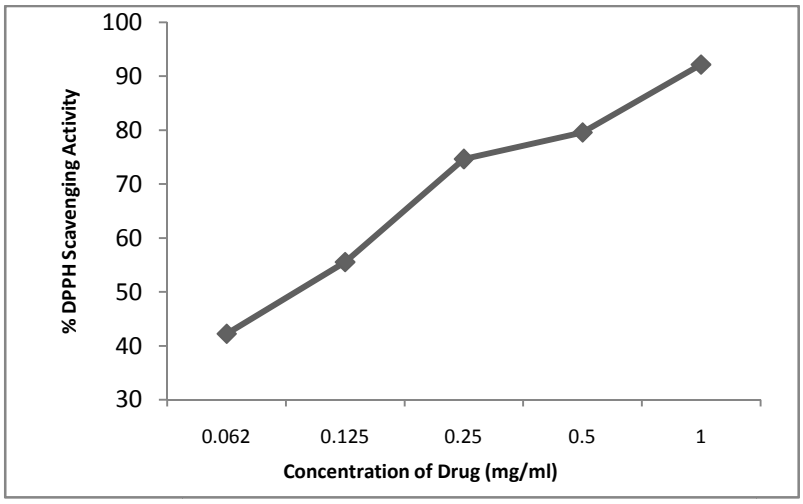

Figure 1. Free radical scavenging activity of *Spirulina* extract by DPPH assay.

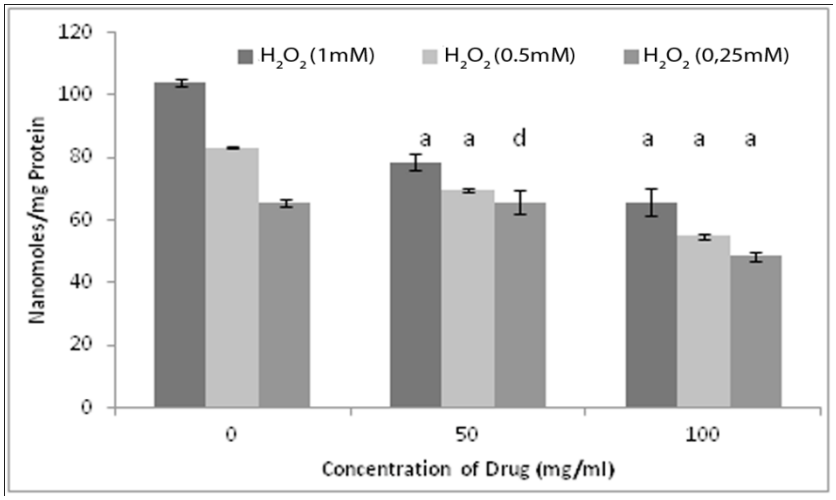

Figure 2. Effect of *Spirulina* extract on membrane damages induced by various concentrations of H_20_2 (a= significant, p<0.001; d=non significant when compared with respective controls)

3.4. Assessment of single cell DNA damages by comet assay

As illustrated in figure 4, exposure of small intestinal cells to H_2O_2 showed increased comet parameters such as percentage DNA in tail, tail length, tail moment and olivetail moment. When cells were exposed to H_2O_2 in the presence of various concentrations of SPE showed decrease in comet parameters in a concentration dependent manner. Cells were treated with highest concentration of SPE did not show any sign of DNA damage indicating the protective effect of it against DNA damages induced by oxidative stress.

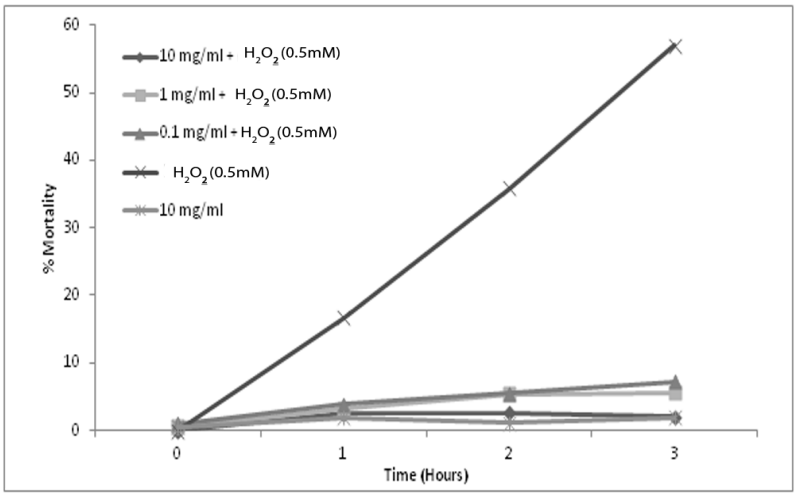

Figure 3. Cytoprotective activity of *Spirulina* extract against oxidative stress.

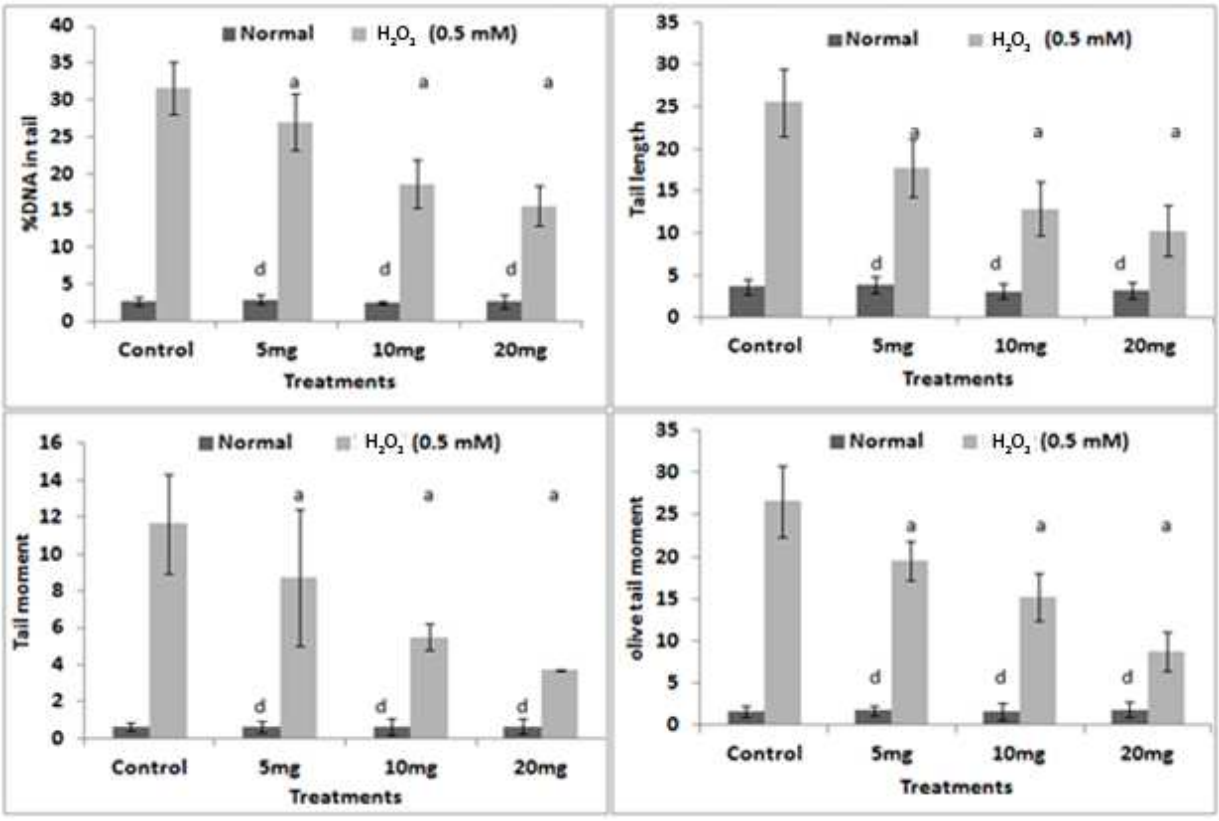

Figure 4. Effect of SPE on H_2O_2 induced cellular DNA damage in mouse spleenocytes. Comet parameters such as percentage DNA in tail, tail length, tail moment and olivetail moment are expressed as mean ± S.D. (a indicate $p \leq 0.001$; d indicate 'non significant' when compared with respective controls).

4. Conclusion

Free radicals are highly reactive molecules since they possess an unpaired electron in the outer shell. These radicals have greater tendency to oxidize intracellular molecules such as lipids, DNA and proteins giving rise to alterations in cell structures. The consequences of these damages affect number of biological reactions, increase the mutation rate and alter the constituents of cellular membranes (Valko et al., 2006). From the cytotoxicity assay it is evident that exposure of cells to H_2O_2 initiated to cause damages to cells. The probable mechanism for its cytotoxic activity is the production of highly reactive free radicals thereby initiating a chain reaction affects the functioning of entire body system.

SPE has great importance as an antioxidant capable to scavenge free radicals. The present study mainly focused to analyse its potent antioxidant activity against oxidative stress induced by H_2O_2 under *in vitro* condition. Dye exclusion assay showed that the mortality of cells decreased by the administration of SPE in a concentration dependent manner. The results from DPPH assay strongly supports potent free radical scavenging activity of SPE that decreases DNA damages and peroxide formation in cells. This may be the reason for the cytoprotective activity of the extract. Moreover the study also revealed that the extract does not induce any DNA damage itself. Further *in vivo* studies to confirm the activity of the extract from *Spirulina* against oxidative stress are worthwhile.

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A search on the inhibitory activities of *Solanum xanthocarpum* Schrad. and Wendl. against selected bacterial pathogens

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Abstract

The development of new infectious diseases, that appear to have been controlled and the increase in bacterial resistance have created the necessity for studies directed towards the development of new antibacterial agents. These led scientists to give great importance to the optimization of screening methods used for the identification of new antibacterial agents from other natural sources. Biologically active compounds from natural sources have always been of great interest to scientists working on infectious diseases. In Ayurveda and traditional medicinal system several plants are used for treatment of manifestations caused by microorganisms. The objective of the present study is to evaluate the *in vitro* antibacterialial activity of methanol and aqueous extracts of *Solanum xanthocarpum* Schrad. and Wendl., a well known ingredient of Dasamoolarishtom with wide spectrum of medicinal properties. The studies were conducted against *Escherichia coli*, *Pseudomonas aeruginosa* gram negative and *Staphylococcus aureus* gram positive bacteria. The results were compared with results obtained using standard antibiotic ampicillin (10mg/disc). The results obtained from the study revealed that both the extracts have significant inhibitory activity against bacterial strains in a dose dependent manner. ($P < 0.01$) when compared with control. Aqueous extract was more effective than the methanol extract. Among the bacterial strains tested, *Escherichia coli* showed more susceptibility in aqueous extract with a diameter of inhibition zone13.7± 0.81 and *Staphylococcus aureus* showed high susceptibility in methanol extract with a diameter of inhibition zone10.6 ± 0.52.

Key words: Solanum xanthocarpum, antibacterial activity, Inhibition zone

1. Introduction

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Many of these isolations were based on the uses of the agents in traditional medicine. Plants have been a valuable source of natural products for maintaining human health, especially in the last decade. More intensive studies are being conducted for devising natural therapies. The use of plant compounds for pharmaceutical purposes has gradually increased all over the world. The search for herbal remedies and natural substances and understanding their mechanisms of action in the body is on the rise.

According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understand their properties, safety and efficacy (Nascimento et al., 2000). About 80% of individuals from developed countries relying traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficiency (Ellof, 1998).

The number of multi-drug resistant microbial strains and the appearance of strains with reduced susceptibility to antibiotics are continuously increasing. This increase has been attributed to indiscriminate use of broad-spectrum antibiotics, immunosuppressive agent, intravenous catheters etc (Rello et al., 2007). In addition, treatments of disease in developing countries using synthetic drugs are not only expensive but are also with adulterations and side effects. Therefore, there is need to search new infection-fighting strategies to control microbial infections (Sieradzki et al., 1999).

The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency (Isso et al., 1995). Even though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased. Bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents (Cohen, 1992). Biologically active compounds from natural sources have always been of great interest to scientists working on infectious diseases and there has been a growing interest to evaluate plants possessing antibacterial activity for various diseases (Clark and Hufford, 1993). Contrary to the synthetic drugs, antimicrobials of plant origin are not associated with many side effects and have an enormous therapeutic potential to heal many infectious diseases (Iwu et al., 1999). Plant derived drugs serve as a prototype to develop more effective and less toxic medicines. (Perumal samy and Ignacimuthu, 1998)

Solanum xanthocarpum Schrad and Wendle (Solanaceae) (syn. Kandakari), is a prickly diffuse bright green perennial herb with white or violet flowers, woody at the base, 2-3 M height found throughout India. The fruits are glabrous, globular berries, green and white striped when young but yellow when matured. The fruits are known for several medicinal uses like anti asthmatic, anti pyretic, laxative, and anti inflammatory activities (Kirtikar and Basu, 1994). The stem flowers and fruits are prescribed for relief in burning sensations on the feet accompanied by vesicular eruptions (Chopra et al., 1956). The fruit paste is applied externally to the affected area for treating pimples and swellings. The roots of this species are used in Ayurvedic medicine as “Dasamoola” for chest pain, cough and asthma. The plant is reported for its anthelmintic activity Gunaselvi et al., (2010), wound healing activity Neeraj kumar et al., (2010), larvicidal efficiency (Bansal et al., 2008 ,Lalit et al., 2007) hypoglycaemic activity Kar et al., (2006) Isolation of steoidal glycosides from *S. xanthocarpum* and studies on their antifungal activity was done by Okram, (2007).

Methanolic extract of the plants was used for the isolation of carpesterol and 4 steroidal glycosides using spectroscopic analysis. Phytochemical investigations of this plant revealed that fruits contain several steroidal alkaloids like Solanacarpine, solasodine, solamargine (Siddqui et al., 1983).With all these wide spectrum of medicinal properties, the present study aims to evaluate the antibacterial activities of aqueous and70% methanolic extract of *Solanum xanthocarpum* against *Escherichia coli*, *Pseudomonas aeruginosa* gram negative and *Staphylococcus aureus* gram positive bacteria, so that we can use this plant for the treatment of manifestations caused by microorganisms.

2. Materials and methods

2.1. Collection of plants

Solanum xanthocarpum was collected from Munnar, Wayanad and Nelliyampathy and authenticated by Dr. N.Sasidharan,Taxonamist, KFRI, Peechi,Thrissur, Kerala, India.

2.2. *Preparation of extract*

Solanum xanthocarpum plants were dried at 45 to 50℃ for one week, powdered and (50g) was defatted with petroleum benzene and successively extracted with methanol using a soxhlet extraction system. The solvent of extraction was concentrated in a rotary evaporator at 45ºC for methanol elimination, and the extracts were kept in sterile bottles under refrigerated conditions until use. The dry weight of the extracts was obtained by allowing the solvent to evaporate and was used to determine concentration in mg/ml. (Betoni et al., 2006*)*.The same methodology was followed for water extract.

2.*3. Test bacteria*

Escherichia coli MTCCB 82, *Pseudomonas aeruginosa MTCCB 741*, *Staphylococcus aureus* MTCCB *737.* The pure cultures were obtained from the standard laboratories. ***Escherichia coli*** are gram negative, straight, rod measuring 1-3x0.4-0.7mm arranged singly or in pairs. It is a parasite living only in the human or animal intestine. Main clinical infections of E-coli are -Urinary infection, Pyogenic infection, Septicemia, Diarrhea. ***Pseudomonas aeruginosa*** is a gram negative aerobic rod shaped bacterium with unipolar motility. It is also an opportunistic pathogen of plants. ***Staphylococcus aureus*** are spherical cocci, approximately 1mm in diameter, characteristically arranged in a grape like clusters. The common diseases caused are-skin and soft tissue infection, wound infection, carbuncle, folliculitis, absces etc.

2.4. Standard disks used

The standard disk -Ampicilin was used. Disk concentration was 10mg/disc.

2.5. Agar disc diffusion method

6 mm filter paper discs (Whatman, no. 3) were impregnated with 20 ml of each of the different dilutions of both extracts. The discs were allowed to remain at room temperature until complete diluents evaporation and kept under refrigeration until ready to be used. Discs loaded with plant extract were placed onto the surface of the agar. Commercial ampicillin discs (10 mg) and paper discs impregnated with 20 ml of diluents used to dilute natural products were used as control. Test microorganism were seeded in to the respective medium by spread plate method 10 µl (10^6 cells/ml) with the 24 hours cultures of bacterial growth in nutrient broth. Discs loaded with plant extract were placed onto the surface of the agar. Discs were placed at well spaced intervals from each other. When the discs were in firm contact with agar medium, the antimicrobial agent diffused in to the surrounding medium and came in contact with the multiplying organism. Then it was incubated at 37º C for 24 hours. After incubation the plates were examined for the presence of inhibition of bacterial growth around the discs. Diameter of zone of inhibition was measured in mm

2.6. Statistical evaluation

Results were expressed as mean value ± standard deviation of the mean of growth inhibition zones diameters obtained with those extracts. Statistical differences between the two variants of diffusion were detected by analysis of variance (ANOVA) followed by Dunnet test. P values lower than 0.05 ($p < 0.05$) were considered as significant.

3. Results and Discussion

The results obtained from the present study revealed that *Solanum xanthocarpum* aqueous and methanol extracts possess potential antibacterial activity against *E.coli, P. aeruginosa* and *S.aureus*. Table.No. 1 represents the comparative antibacterial activity (zone of inhibition) of aqueous and methanol extracts of *S. xanthocarpum.* The values were slightly lesser than that of the standard disc ampicillin, in all the treatments. In the present investigation aqueous extract was found to be more effective than the methanol extract with the diameter of inhibition zone13.7 ± 0.81 for *E.coli,* (Fig. No.1), 10.8 ± 0.75 for *P. aeruginosa,* (Fig. No.2) and 13.3 ± 0.81 for *S.aureus,* (Fig. No.3). In methanol extract inhibition zone was 9.6 ± 0.49 for *E.coli,* (Fig. No.4), 9.6 ± 0.49 for *P. aeruginosa* ,(Fig. No.5) and 10.6 ± 0.52 for *S.aureus,* (Fig,No.6,) at the concentration of 80 mg/disc. Inhibitory activity was found to be increasing in a dose dependent manner.

Plants are rich in a wide variety of secondary metabolites such as tannins terpenoids, alkaloids, flavonoids, etc, which show wide range of *in vitro* antibacterial and antifungal activity. Phytoconstituents such as phenolic compounds, saponins,flavonoids, glycosides and steroids have been reported to inhibit bacterial growth and to be protective to plants against bacterial and fungal infections. (Alam et al 2010). So the development of new antibacterial agents, the most feasible way to combat the problem of microbial resistance and for substitution with ineffective ones. Moreover, it is presumed that the broad spectrum effectiveness of plant species may provide a suitable basis for new antimicrobial therapies (Haque et al., 2011). Phytochemical investigations of *S. xanthocarpum* revealed that this plant contains several steroidal alkaloids like Solanacarpine, solasodine, solamargine and carpesterol (Siddqui et al., 1983).The antibacterial activity of the plant may be due to the presence of these compounds.

Water is a universal solvent and is generally used in traditional settings to prepare the plant decoctions for health remedies (Doughri et al 2008). It has been reported that many natural products including pigments, enzymes and bioactive components are soluble in water, which explaining the highest yield of extract while, some of the solvents especially acetone are selective for tannins (Majorie, 1999). Present investigation also proved that water extract is more effective than methanol extract.

The test organisms used in this study are associated with various forms of human infections. From a clinical point of view, *E. coli* causes septicemias and can infect gall bladder, lungs, meninges, surgical wounds and skin lesions especially in debilitate and immunodeficient patients (Black, 1996). *P. aeruginosa* causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections (Kenneth2009). *S. aureus* causes wound infections, abscesses, osteomyelitis, endocarditis, pneumonia (Charles 2012) that may severely harm or kill the infected person. The demonstration of activity of *S. xanthocarpum* against both gram-negative and gram-positive bacteria is an indication that the plant can be a source of bioactive substances that could be of broad spectrum activity. The *E.coli*, which is already known to be multi-resistant to drugs, was also resistant to the plant extracts. Normally it was susceptible only to benzoic acid and cinnamic acid. Nascimanto et al., (2000).*S. xanthocarpum* showed inhibitory activity to *E.coli.* On the other hand, *P. aeruginosa* (Chandler et al., 1982), which is also resistant to different antibiotics, had its growth inhibited by the extracts. The fact that the plant was active against both clinical and laboratory isolates is also an indication that it can be a source of very potent antibiotic substances that can be used against drug resistant microorganisms' prevailing in hospital environments.

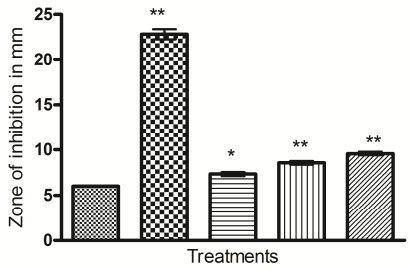

Figure 1. Effect of methanol extract of *S. xanthocarpum* against *Escherichia coli*

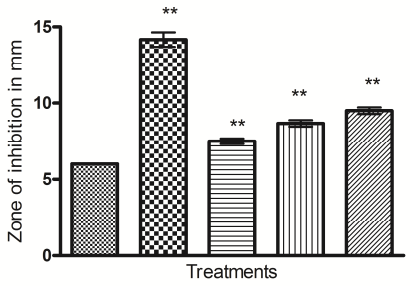

Figure 2. Effect of methanol extract of *S. xanthocarpum* against *P. aeruginosa*

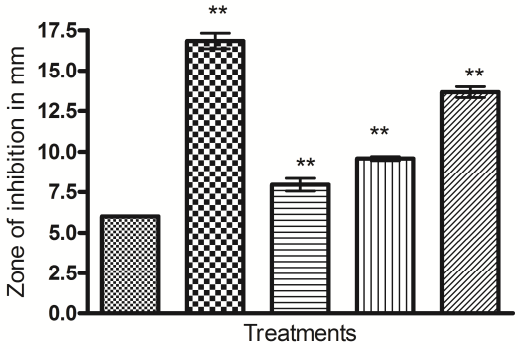

Figure 3.Effect of methanol extract of *S. xanthocarpum* against *Staphylococcus aureus*

Control Ampicillin 10 mg/Disc Methanol Extract 40 mg/Disc

Methanol Extract 60 mg/Disc Methanol Extract 80 mg/Disc

* P < 0.05, **P< 0.01 as compared to control

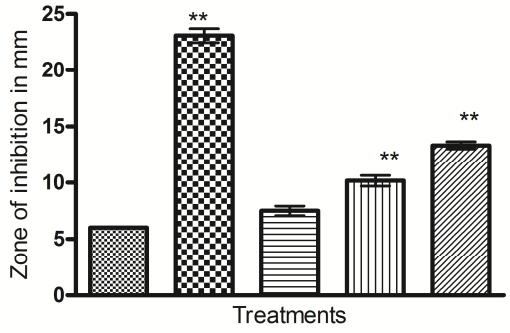

Figure 4.Effect of water extract of *S. xanthocarpum* against *Escherichia coli*

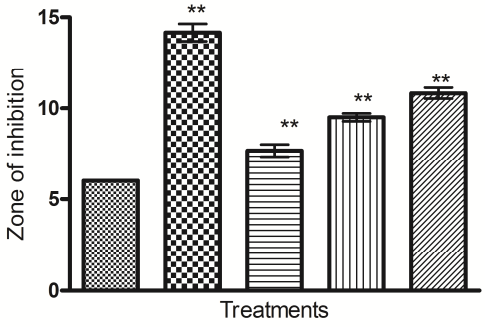

Figure 5.Effect of water extract of *S. xanthocarpum* against *P. aeruginosa*

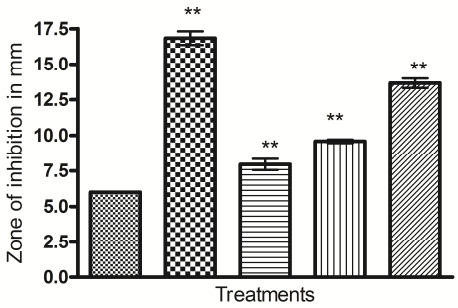

Figure 6.Effect of water extract of *S. xanthocarpum* against *Staphylococcus aureus*

Control Ampicillin 10 mg/Discs Water Extract 40 mg/Disc

Water Extract 60 mg/Disc Water Extract 80 mg/Disc

* P < 0.05, **P< 0.01 as compared to control

Table 1. Antibacterial activity of aqueous and methanol extract of *Solanum xanthocarpum* against bacterial species tested by disc diffusion assay

Bacterial pathogen		*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Control		6 ± 0	6 ± 0	6 ± 0
Ampicillin	**10mg**	23.1 ± 1.49**	17.6 ± 1.03**	17.6 ± 1.03**
Water Extract	**40mg**	7.5 ± 1.05	7.9 ± 0.96**	7.6 ± 0.81**
	60mg	10.2 ± 1.16**	9.5 ± 0.54**	9.6 ± 0.29**
	80 mg	13.7 ± 0.81**	10.8 ± 0.75**	13.3 ± 0.81**
Methanol Extract	**40mg**	7.3 ± 0.52*	7.5 ± 0.44**	7.6 ± 0.51**
	60mg	8.6 ± 0.47**	8.7 ± 0.47**	9.3 0.51**
	80 mg	9.6 ±0.49**	9.6 ± 0.49**	10.6 ± 0.52**

Values are mean inhibition zone in (mm). ± SD of 6 replicates * P < 0.05, **P< 0.01 as compared to control

4. Conclusion

Plants are important source of potentially useful agents for the development of new chemotherapeutic drugs. The result of the present study indicates that *S.xanthocarpum* possess antibacterial activity against both gram-positive and gram-negative bacteria. The broad-spectrum antibacterial activity of the plant extract is possibly due to the identified steroids and glycoalkaloids Bioactive substances from this plant can therefore be employed in the formulation of antimicrobial agents for the treatment of various bacterial infections, These results do not reveal that which chemical compound is responsible for its antibacterial activity. So further clinical studies of this plant is required in order to understand their antibacterial principles which will allow the scientific community to recommend their use as an accessible alternative to synthetic antibiotics.

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Mitigation of oxidative stress by carotenoids

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Abstract

The overall aim of the study was to explore the underlying mechanism of protection offered by carotenoids on murine spleenocytes against oxidative stress induced damages. Oxidative stress was induced in spleenocytes by H_2O_2 treatment, the cellular mortality and lipid peroxidation and DNA damage were studied. Ability of the carotenoids to scavenge stable free radical- 2,2-diphenyl-1-picrylhydrazyl (DPPH)was investigated. The carotenoids exhibited effective free radical scavenging activity as revealed in DPPH assay and protected the spleenocytes from H_2O_2 induced lethality as assessed by trypan blue dye exclusion test. H_2O_2induced DNA damage was mitigated by carotenoids. Carotenoids are highly potent natural antioxidant capable of protecting membrane lipid and cellular DNA from oxidative damage.

1. Introduction

Free radicals are multifaceted and can cause tissue damage by reacting with various biomolecules such as polyunsaturated fatty acids, nucleotides in DNA, and critical sulfhydryl bonds in proteins (Machlin and Bendich, 1987).Free radicals have both endogenous and exogenous origin. Endogenous source are normal metabolic reactions and electron leakage during mitochondrial electron transport (Cadenas et al., 2000) while exogenous source such as air pollutants, components of tobacco smoke and indirectly through the metabolism of certain solvents, drugs, and pesticides aswellas through radiation exposure (Pryor,1997;Balanehruand Nagarajan, 1992). Even though free radicals are essential for fighting against pathogens, to induce various biological processes such as gene expression by stimulating signal transduction components such as Ca(2+)-signaling and protein phosphorylation (Sharma et al.,1995; Suzuki YJ et al., 1997) they cause much tissue/organ injuries by oxidative damages.

Oxidative stress results in many chronic health problems such as emphysema, cardiovascular and inflammatory diseases, cataract, and cancer. The extent of tissue damage is the result of the balance between the free radicals generated and the antioxidant protective defense system. Body defenses against free radical damage include carotenoids, tocopherol (vitamin E), ascorbic acid (vitamin C), glutathione, uric acid, bilirubin, and several metalloenzymes including glutathione peroxidase (selenium), catalase (iron), and superoxide dismutase (copper, zinc, manganese) and proteins such as ceruloplasmin (copper) (Machlin and Bendich, 1987). Several dietary micronutrients contribute greatly to the protective system (Woodall et al,1997).

Carotenes are naturally occurring pigments in plants that are involved in light harvesting reaction in photosynthesis as well as protection of cell organelles from the attack of singlet oxygen(Harry,2004). Dietary carotenoids could act as antioxidants against oxidative stress induced by free radicals (Dutta et al.,2005).Carotenoidsare absorbed into body in reasonable amounts, they have antioxidant properties and immunomodulating effects and may possibly influence gene expression enhancing gap junction communication by which they can inhibit progression of carcinogenic pathway (Gester,1993).

2. Materials and methods

2.1. Chemicals

Carotenoids were a presented to Dr. C. K. K. Nair by Parry India. All other chemicals and reagents used in this study were of analytical grade.

2.2 Animals

Swiss albino mice of 8-10 weeks old, weighing 22-25 g was obtained from the Small Animal Breeding Section (SABS), Kerala Agricultural University, Mannuthy, Thrissur, Kerala. They were kept under standard conditions of temperature and humidity in the Centre's Animal House Facility. The animals were provided with standard mouse chow (SaiDurga Feeds and Foods, Bangalore, India) and water *ad libitum*. All animal experiments in this study were carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC) and were conducted strictly adhering to the guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of Government of India.

2.3 DPPH radical scavenging activity

The free radical scavenging activity of Carotenoids was determined by the method of Gadov*et al.*,(1997) with some modifications (Gandhi and Nair, 2004). Methanolic solution of DPPH (63.4 μM) was incubated at ambient temperature with various concentrations of the Carotenoids (0.062 – 1 mg/ml) and A_{515} was measured using a spectrophotometer. The percent of inhibition of DPPH (decolourization) was calculated according to the formula

$$\% \text{ inhibition} = \frac{A_{control} - A_{GTE}}{A_{control}} \times 100$$

2.4. Effect of Carotenoids in mitigating free radical induced lipid peroxidation

100 μl of mouse liver homogenate (10% w/v) prepared in phosphate-buffered saline was treated with various concentrations of H_2O_2 (1 mM – 0.25 mM) and Carotenoids (100 mg/ml, 50 mg/ml). Levels in peroxidation of membrane lipids were estimated by the method of Buege and Aust (1987). The result is expressed as nanomoles of malonedialdehyde per mg protein.

2.5. Cytoprotecive effect of Carotenoids

Spleens were collected from Swiss albino mice and made into single cell suspension ofspleenocytes. The cell suspension (10^6 cells/ml) was treated with different concentrations of Caroteniods (10 mg/ml, 1 mg/ml, and 0.1 mg/ml) in the presence of 0.5mM, H_2O_2. The viability of cells were determined at various time intervals (0 hr, 1 hr, 2 hr, 3 hr) by trypan blue dye exclusion method (Guptha and Bhattacharya 1974). Percentage cytotoxicity was calculated.

2.6. Effect of Carotenoids on oxidative cellular DNA damage

Spleens were collected from Swiss albino mice and spleenocytes were isolated. The cell suspension (10^6 cells/ml) was incubated for 5 minutes in presence and absence of 0.5 mM of H_2O_2 and various concentrations of Carotenoids (20 mg/ml, 10 mg/ml, 5 mg/ml). DNA damage was analyzed by employing the technique of alkaline single cell gel electrophoresis (comet assay).

2.6.1. Alkaline single-cell gel electrophoresis (comet assay)

Alkaline single-cell gel electrophoresis(Singh, 2000) was performedwith minor modificationsas detailed by Chandrasekharan et al., 2009. Microscope slides were coated with normal melting point agarose (1% in PBS) and kept at 4°C till the agarose was solidified. On these slides, 200 μl of 0.8% low melting point agarose containing 50 μl of treated cells were added. After solidification of the low melting agarose, the slides were immersed in pre-chilled lysing solution containing 2.5 M NaCl, 100 mM EDTA, 10 mMTris-HCl, pH 10, 1% DMSO, 1% Triton X and kept for 1 hour at 4°C for lysis of the cells. After lysis, the slides were drained properly and placed in a horizontal electrophoretic apparatus filled with freshly prepared electrophoresis buffer containing 300 mMNaOH, 1 mM EDTA, 0.2% DMSO, pH $\geq$ 13. The slides were equilibrated in buffer for 20 minutes and electrophoresis was carried out for 30 minutes at 20 V. After electrophoresis the slides were washed gently with 0.4 mMTris-HCl buffer, pH 7.4, to remove alkali. The slides were again washed with distilled water, kept at 37°C for 2 hours, to dry the gel and silver staining was carried out. The comets were visualized under a binocular microscope and the images captured were analysed using the software 'CASP' to find out the extent of DNA damage measured in terms of different comet parameters such as % DNA in tail, tail length, Tail Moment (TM) and Olivetail Moment (OTM) (Konca et al.,2003) The parameter tail moment is the product of tail length and % DNA in tail, and olive tail moment is the product of the distance between the centre of gravity of the head and the center of gravity of the tail and % DNA in tail. Results are presented as mean ± standard deviation.

2.7. Statistical analysis

The results are presented as mean ± standard deviation of the studied groups. The statistical analyses of the results were performed using ANOVA with Tukey–Kramer multiple comparisons test.

3. Results

3.1. DPPH free radical scavenging activity

The samples were tested for their ability to scavenge DPPH free radical at different concentrations ranging from (0.062 to 1 mg/ml) and the readings were observed as decrease in the absorbance indicating the extent of radical scavenging property. Figure 1 represents the percentage of DPPH free radical scavenging activity of carotenoids. The IC_{50} was found to be 0.0325 mg/ml.

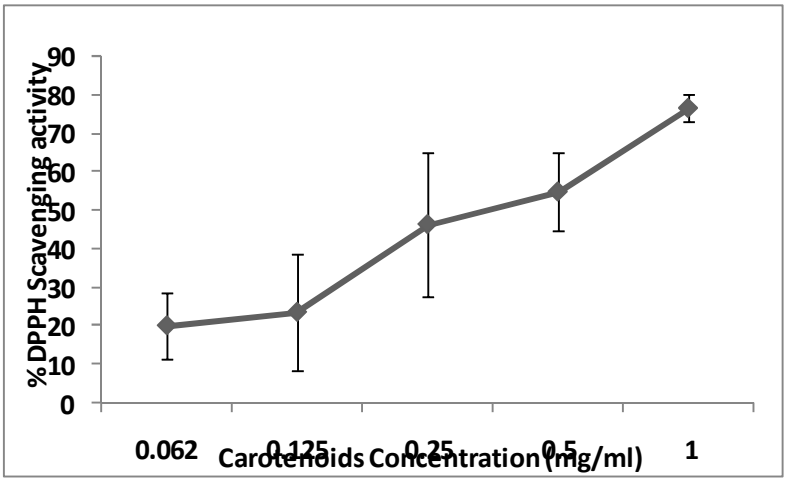

Figure 1. DPPH free radical scavenging activity of carotenoids

3.2. Effect of carotenoids on free radical induced membrane peroxidative damage

The effect of the carotenoids on free radical induced membrane peroxidative damage to liver tissue is presented in figure-2. H_2O_2 induced lipid peroxidation was found to increase in a dose dependent manner (0.25 - 1 mM), while the presence of carotenoids(50 and 100 mg/ml) reduced the preoxidative damage.

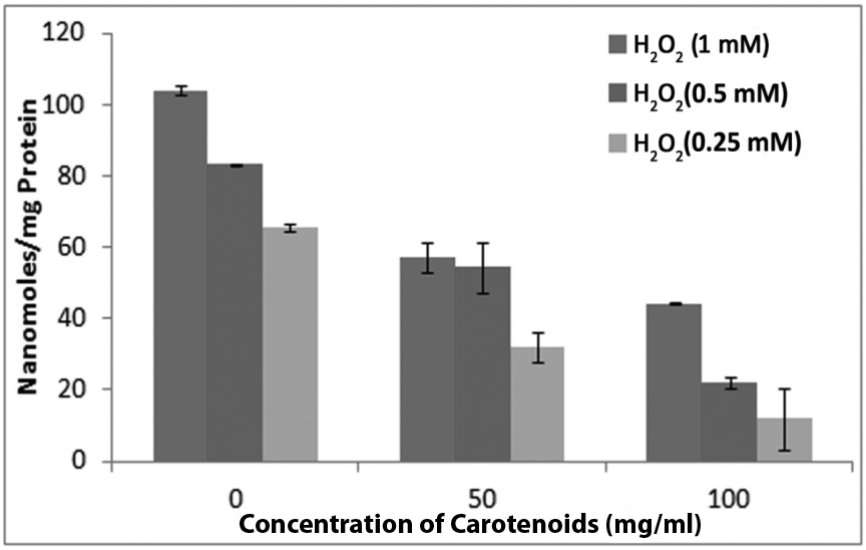

Figure 2. Effect of carotenoids on peroxidative damage on membrane lipids induced by H_2O_2.

3.3. Cytoprotective activity of carotenoids against oxidative stress induced cytotoxicity

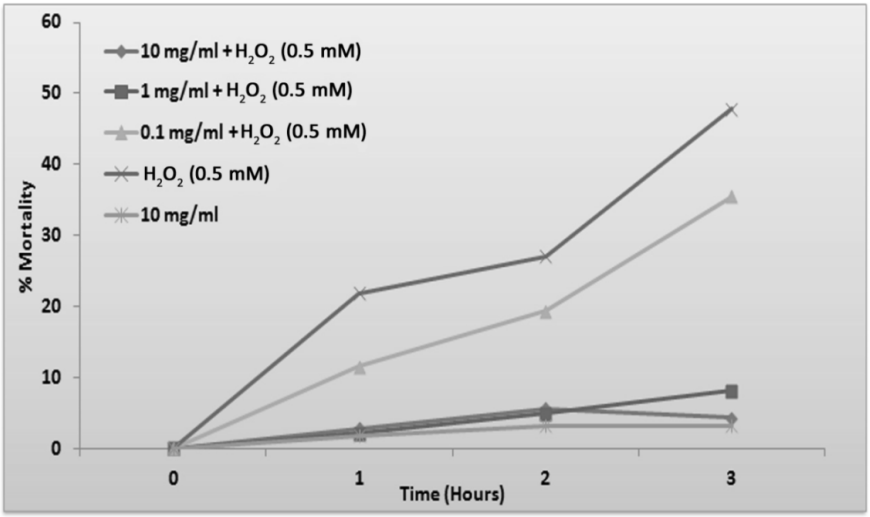

Figure 3. Effect of carotenoids on oxidative stress induced lethality in mouse spleenocytes.

The effect of the carotenoids on H_2O_2 induced mortality in spleenocytes is represented in figure 3. The carotenoids was found to be nontoxic to spleenocytes even at the highest concentration used for the experiment. Exposure of the cells to H_2O_2 (0.5 mM) resulted in 47.84% mortality at 3 hours. When spleenocytes were exposed to 0.5 mM H_2O_2 in the presence ofcarotenoids (10 mg/ml) the percentage of

mortality was found to be reduced to 4.38%. The carotenoids offered cytoprotective property in a concentration dependent manner.

3.4. Protection of cellular DNA from H_2O_2 induced oxidative stress

As evident from figure 4, spleen cells when exposed to H_2O_2 at a concentration of 0.5 mM induced DNA strand breaks, as evident from the comet assay result which showed the production of long tail due to the migration of DNA from the comet head. Spleen cells exposed to H_2O_2 in the presence of various concentrations of carotenoids (5-20 mg/ml) showed a shorter tail, indicating that carotenoids could prevent DNA strand breaks induced by oxidative stress in a concentration dependent manner. Spleen cells incubated in the presence of the highest concentration of carotenoids (20 mg/ml) appeared as homogenous discs without any tail suggesting that carotenoids did not induce any genomic DNA damage

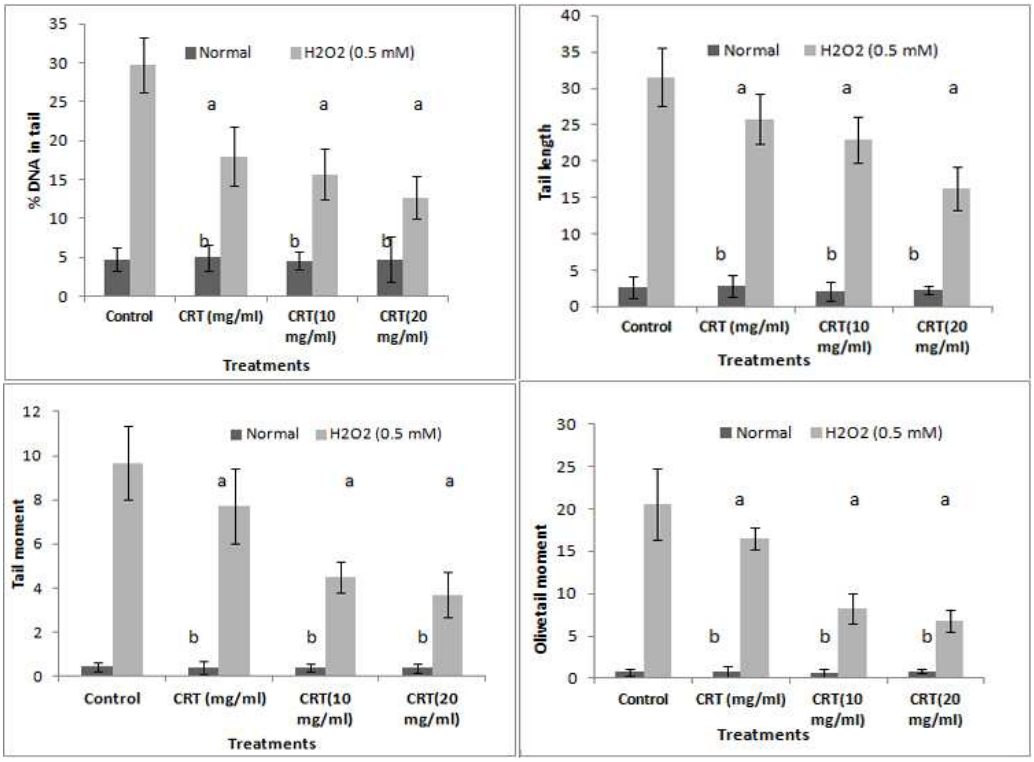

Figure 4. The effect of carotenoids on H_2O_2 induced cellular DNA damage in mouse spleenocytes. Comet parameters such as percentage DNA in tail, tail length, tail moment and olive tail moment are are expressed as mean ± S.D. (a ' indicate P ≤ 0.001(significant)).

4. Discussion

Free radicals cause many diseases like atherosclerosis, autoimmune disorders, neuronal degeneration, cancer etc. Mutation in cycle check point genes leads to cancer(Kaufmann andPaules,1996) which may arise due to oxidative stress.

Carotenoids are capable of inhibiting free-radical-induced lipid peroxidation in liposomes as well as singlet oxygen-induced lipid peroxidation (Krinsky and Deneke,1982). A specific role of carotenoids on immune response was first reported by Bendich and Shapiro, 1986. Certain carotenoids can effectively reduce the toxic effect of reactive oxygen species (ROS), so that it can reduce the risk of cancer,

cardiovascular, neurodegenerative diseases and aging. In recent studies, it has been found that carotenoids have obvious role in gene regulation apoptosis and angiogenesis (Park, 2004).

Many earlier studies on beta-carotene reported a marked stimulatory action of beta -carotene on the growth of the thymus gland and a large increase in the number of thymic small lymphocytes (Seifter et al., 1981). Beta-carotene also have cell invasion reducing property (Huang et al., 2005). Leutin and zeaxanthin are xanthophyll carotenoids found in vegetables and egg yolk, which can prevent stroke and heart disease, reduce the risk of breast cancer and lung cancer (Judy et al., 2004).The carotenoids Lycopene has anti-cancer property on hepatoma and prostate cancer. It also inhibits metastasis by upregulating the expression of metastasis suppressor gene nm23-H1 in highly invasive hepatoma cell line (Huang et al., 2005).

The present work, demonstrated the concentration dependent antioxidant activity of carotenoids against detrimental effect of oxidative stress. The potential oxidant H_2O_2 was used to induce oxidative stress, in vitro. The cytoprotective ability of carotenoids was evaluated using various parameters such as, in vitro cytotoxicity assay, estimation of lipid peroxidation and DNA damage analysis by comet assay. Carotenoids prevented theH_2O_2induced cytotoxicity inspleenocytes. The result on cellular DNA damage analysis and the extent of lipid peroxidation also showed the cytoprotective efficacy of the carotenoids.

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Nutraceuticals and natural compounds as antioxidants

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Abstract

Oxidative stress is involved in the pathogenesis of certain human diseases, including cancer, atherosclerosis, inflammatory diseases and neurodegenerative processes associated with aging. Therefore, inhibition of free radical induced oxidative damage by supplementation of antioxidants has become an attractive therapeutic strategy for reducing the risk of these diseases. In the search for sources of natural antioxidants, in the last years some medicinal plants and fruits have been extensively studied for their antioxidant and radical scavenging activity.

Key words: Free radicals, antioxidants, nutraceuticals, phenolics

1. Introduction

Reactive oxygen species and their intermediates, such as super oxide radical (O_2 -•), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and singlet oxygen (O_2 ^{1}) are produced as by-products of normal cell metabolism, although environmental stresses can cause an increase in their levels. These radicals lead to several diseases, including cancer, cardiovascular diseases, cataracts, atherosclerosis, diabetes, arthritis, immune deficiency diseases and ageing. Of late, the words “free radicals” and “antioxidants” have become well known for the health conscious consumer. An antioxidant is defined as any substance that when present at low concentration comparable to oxidizable substrate delays significantly or prevents substrate oxidation. Antioxidants or antioxidative nutraceuticals can be antioxidative enzymes, hydrogen donating compounds, metal chelators, and singlet oxygen quenchers. The use of synthetic antioxidants is an old practice and their safety could be questioned by the consumer. Recently, there is an increasing interest in finding natural antioxidants from plants because they can protect the human body from the attack of free radicals and retard the progress of many chronic diseases, as well as lipid oxidation.

1.1 Enzymatic antioxidants

The removal of free radicals is achieved through enzymatic and non-enzymatic reactions. The principal defense systems against oxygen free radicals are glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), and antioxidant nutrients (Fang et al., 2002). Antioxidant enzymes convert ROS into nonreactive oxygen molecules. SOD converts superoxide anion into hydrogen peroxide and oxygen. Glutathione peroxidase is the most important hydrogen peroxide removing enzyme existing in the membrane. Catalase is involved in cellular detoxification and can convert hydrogen peroxide into water and oxygen. The antioxidant defense systems in the body can only protect it when the amount of the free radicals is within the normal physiological level. But when this balance is shifted towards more of free radicals, it leads to oxidative stress, which may result in tissue injury and subsequent diseases.

1.2 Synthetic antioxidants

The antioxidant molecules can be classified into synthetic and natural antioxidants. Many synthetic antioxidants such as BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) are very effective and are used for industrial processing, but they may possess some side effects and toxic properties to human health (Anagnostopoulou et al., 2006). For this reason and because of the growing consumer preferences for natural products, there is an increasing interest in the investigation of naturally occurring antioxidants from plants. Therefore, the assessment of antioxidant and radical scavenging properties of traditionally used plants and plant extracts is an important issue in the quest for new sources of natural antioxidants for functional foods and nutraceuticals. This will also provide 'natural' alternatives to synthetic antioxidants in the food industry, since food-preserving compounds are being restricted due to their inherent risk of carcinogenicity.

1.3 Polyphenolic compounds as antioxidants

The β-carotene and carotenoids such as α-carotene, γ-carotene and β-cryptoxanthin are potent antioxidants of plant origins. Phenolic and polyphenolic compounds such as flavonoids and catechin in edible plants exhibit strong antioxidant activities. Epidemiological data indicated that flavonoids are the family of antioxidant compounds with a C6-C3-C6 skeleton structure and exhibit several biological activities in the prevention of a multitude of disease state, including cancer, inflammations, cardiovascular disease and neurodegenerative disorders. The antioxidant effect of plant products is mainly attributed to phenolic compounds, therefore, these substances have been proposed as health-promoting natural products (Capecka et al., 2005).

1.4 Antioxidative nutraceuticals

A whole range of plant derived dietary supplements, phytochemicals and provitamins that assist in maintaining good health and combating disease are now being described as functional foods, nutriceuticals and nutraceuticals. A wealth of information and scientific evidences are rapidly accumulating that shows the beneficial effects of a wide variety of food components on human health. Nutraceuticals or functional foods are any food or food ingredients that may provide beneficial health effects beyond the traditional nutrients they contain (Wildman, 2001). If reactive oxygen species and free radicals are the major causes of aging processes, antioxidative nutraceuticals can reduce the level of reactive oxygen species and free radicals, slow the aging process and increase lifespan. Antioxdative nutraceuticals can be antioxidative enzymes, hydrogen donating compounds, metal chelators, singlet oxygen quenchers, tocopherols and tocotrienols, ascorbic acid, carotenoids, polyphenols and lipoic acids. They can donate hydrogen atoms to free radicals, scavenge free radicals and prevent lipid oxidation. Nutraceuticals are supposed to hold the key to a healthy society in the coming future. Free radical scavengers, which react with peroxyl radicals before the polyunsaturated fatty acids (PUFA) react with peroxyl radicals, can prevent lipid oxidation. Chain breaking antioxidants donate hydrogen atoms to peroxyl radicals and convert them to more stable and non-radical products. Antioxidant radicals formed from hydrogen donating antioxidants can react with alkyl, alkoxyl and peroxyl radicals and generate non-radical stable compounds (Lee et al., 2004).

2. Conclusion

The natural antioxidants could be used to produce a natural dietary antioxidant supplement or added to healthy food products such as cereals, legumes, fruit bars or drinks to prevent chronic diseases where free

radicals are involved. Various biological activities attributed to natural antioxidants increase the potential interest for improving the efficacy of different products as nutraceutical and pharmacological agents.

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Protease Inhibitors: Kunitz and BBI– A review

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Abstract

Protease inhibitors are any compounds which inhibit or antagonize biosynthesis or actions of proteases whereby inhibiting the splitting of proteins into smaller peptide fractions and amino acids by a process known as proteolysis. Among the protease inhibitor families, the Bowman-Birk and Kunitz inhibitor families are most widely distributed, and the most abundant in the seeds of leguminous plants as well as the most intensively studied groups. This review aims at giving a brief description regarding the occurance and physiological importance of these inhibitors which can be used as a therapeutic agent to the mankind.

Key words: protease inhibitors, Bowman-Birk inhibitor, Kunitz inhibitor

Abbreviations: BBI-Bowman-Birk inhibitor, PI- protease inhibitor, BBIC- Bowman-Birk inhibitor concentrate.

1. Introduction

The protein-proteinase inhibitor (PIs) in nature was described as early as 1894 after the discovery of the "anti-trypsin activity" of serum (Fermi and Pernossi 1894). The PIs are specific towards inhibiting proteolytic enzymes such as elastase, thrombin, plasmin, kallikrein, trypsin, chymotrypsin, chymase, tryptase, bacterial enzymes like subtilisin, fungal enzymes, endogenous plant proteinases and insect digestive enzymes (Garcia-Olmedo *et al.*, 1987; Hilder *et al.*, 1990; Richardson, 1991). They are predominant in many plant species. Enzyme inhibitors were isolated first from seeds in 1940s; with the characterization of Kunitz and BBIs from soybean and α-amylase inhibitor from cereal grains. The natural PIs constitute a final regulatory step in the control of protease activities by inhibiting the proteases. During homeostasis there is a biological balance between proteases and their natural inhibitors (Losso, 2008). An imbalance between proteases and their endogenous inhibitors in the body may trigger uncontrolled proteolysis which leads to irreversible tissue destruction such as inflammation processes, rheumatoid arthritis, periodontitis, hypertension, gastric ulcer, muscular dystrophy, pathological angiogenesis, or tumor growth and metastasis (Von der Helm et al., 2000; Noel et al., 1997). PIs are widely classified into five families on the basis of the catalytic mechanisms and conditions of action of the proteases inhibited by them: serine, cysteine, metallo, aspartic, and threonine protease inhibitors. The most extensively studied of the PIs are those that inhibit the serine proteases trypsin and chymotrypsin. The role of serine protease inhibitors as defensive compounds against predators is particularly well established. Because of their ability to inhibit the enzymes involved in the digestive processes of humans and animals, protease inhibitors have been referred to as ''antinutritional compounds'' so far. Eventhough these proteins are antinutritional factors they are beneficial to plants serving as protective agents against insects, fungi or microbial predators (Chilosi et al., 2000). Some of the PIs have now been favourably reconsidered in view of the potential exploitation of their biological behaviour in pharmacological and medical applications. An inhibitor domain is defined as the segment of the amino acid sequence containing a single reactive site after removal of any parts that are not directly involved in the inhibitor activity. Among the protease inhibitor families, the Bowman-Birk and Kunitz inhibitor families are most widely distributed, and the most abundant in the seeds of leguminous plants as well as the most intensively studied groups.

2. Kunitz inhibitor family

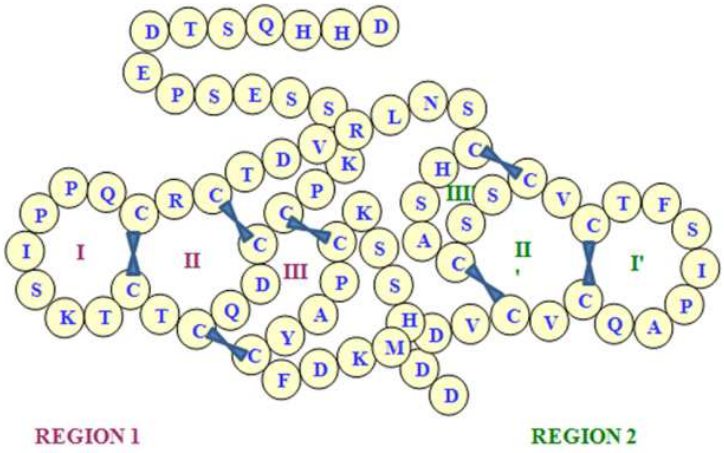
The first plant proteinases inhibitor to be isolated and characterized was Kunitz soybean trypsin inhibitor (KTI) found in the seeds of *Glycine max* and is inhibitory to trypsin from a wide variety of sources. Soy bean seeds generally have more Kunitz than BBIs. Kunitz inhibitors are single chain polypeptides of ~18–24 kDa, with two intra-chain disulphide bridges and form 1:1 complex with the target proteinase. KTIs are proteins with one or two polypeptide chains and low Cys content, generally with four Cys residues connected by two disulfide bridges, each comprising 170-180 amino acids and a single reactive site. The inhibitor is inactivated by heat and gastric juice. The purification, crystallization, kinetics of interaction and complex formation of KTI with trypsin (Kunitz, 1947) is a major landmark in the study of plant proteinases inhibitors. Since KTI inactivates the anionic form of human trypsin, it is believed to be an antinutritional factor. The members of this family are mostly active against serine proteases, but may also inhibit other proteases. Kunitz-type inhibitors are also produced under stress, as has been found in potato tubers (*S. tuberosum*) (Ledoigt et al., 2006). The inhibitors in Kunitz family are widespread in plants and have been described in legumes, cereals and in solanaceous species (Ishikawa et al., 1994; Laskowski and Kato, 1980).

3. Bowman-Birk inhibitor (BBI)

There are several sources of BBI. BBIs are distributed among both dicots and monocots. Among dicots majority of the inhibitors are concentrated in legumes. The BBI is one of the major serine protease inhibitor commonly found in leguminous plants. In Leguminosae, the suborders Caesalpinieae and Mimosaceae were considered to be primitive where as Fabaceae was considered to have evolved. Tropical trees or shrubs belonging to Caesalpinieae and Mimosaceae had the Kunitz family inhibitors but temperate herbs of new tribes contained BBI family. Herbs are thought to be more evolved than trees. The leguminous plants which had only the Kunitz family inhibitors, gradually acquired the BBI family during evolution from trees to herbs, as they adapted to temperate zone. This view is supported by the advanced biochemical nature of BBI family of inhibitors.

Sunflower trypsin inhibitor-1 (SFTI-1)1 is the smallest and only known BBI from compositae and the most potent known peptidic trypsin inhibitor in the BBI class of proteins. This peptide is shorter than other members of the BBI family, exhibits a novel cyclic structure, and has considerably enhanced potency relative to other peptides of similar length. SFTI-1 inhibited β-trypsin and it also inhibited cathepsin G. SFTI-1 has considerable selectivity; it proved to be 74-fold less inhibitory for chymotrypsin, and was found to be 3 orders of magnitude less inhibitory for elastase and thrombin. In contrast, it had no effect on Factor Xa (Luckett et al., 1999). Soybean BBI, isolated by Bowman and characterized by Birk, and was therefore named after the two scientists, serves as a prototype for the BBI family. While the soybean BBI is the classical representative of the BBI family and has been extensively studied, related BBI from other monocotyledonous and dicotyledonous seeds are being gradually identified and characterized. Bowman Birk inhibitors (BBI) are small protease inhibitors found in the seeds of legumes in particular (Laskowski, 1980). Their molecular masses are in the range of 6-9 kDa. They comprise of a binary arrangement of two sub domains with a conserved array of seven disulphide bridges, which play a pivotal role in the stability of the inhibitors. These inhibitors interact simultaneously and independently with two molecules of proteinases.

BBI has been demonstrated to be effective in preventing or suppressing radiation- and chemical carcinogen-induced transformation in a wide variety of in vitro assays (Kennedy, 1998). In vivo, BBI has also been found to inhibit carcinogenesis in the colon, oesophagus, liver, lung and the oral cavity (Losso, 2008). BBIC exhibits very favourable safety profile in pre-clinical studies and in clinical trials in patients with benign prostatic hyperplasia, pre-cancerous conditions, such as oral leukoplakia, and



Schematic representation of the major BBI from horsegram which consists of two domains, each consisting of three peptide chain rings made by disulphide bridges

ulcerative colitis (Lichtenstein et al., 2008). The lack of toxicity and the potent anti-inflammatory effect of BBIC in animals when assessed in an acute colitis model conferred the potential for BBIC to benefit patients with active ulcerative colitis.

In experimental autoimmune encephalomyelitis (EAE), significant disease suppression of clinical and histological EAE has been reported. Therefore BBIC acts as an excellent candidate for oral therapy in multiple sclerosis patients (Gran et al., 2006). BBI has been shown to be an effective inhibitor of several human proteases associated with inflammation-mediating cells, including elastase, cathepsin G and mast cell chymase. BBI had an immunoregulation effect through inhibition of proteases released from inflammation-mediating cells. BBI reduced autoimmune inflammation and attenuated neuronal loss in a mouse model of multiple sclerosis (Touil et al., 2008). Dittmann et al. (2001) reported that pre-incubation of normal human skin fibroblasts with BBI, time-dependently blocked radiation-induced activation of tyrosine kinase and inhibited the activation of epidermal growth factor by enhancing specific tyrosine phosphatases. BBI was found to act as a potent selective normal-tissue radioprotector in vitro and in vivo, apparently without protecting tumors, and thus has the potential to improve clinical radiotherapy. Li et al., (2011) summarised that BBI has the ability to inhibit lipopolysaccharide-mediated macrophage activation, reducing the release of pro-inflammatory cytokines and subsequent neurotoxicity in primary cortical neural cultures. Thus BBI, through its anti-inflammatory properties, protects neurons from neurotoxicity mediated by activated macrophages.

BBIs have attracted much interest due to their anti-carcinogenic activity, although the exact mechanism of this activity has yet to be established. Human populations consuming large quantities of BBIs in their diet exhibit lower rates of colon, breast, prostate, and skin cancers. The purified BBI works as well as BBIC, an anticarcinogenic agent over a range of doses in both *in vitro* transformation systems and *in vivo* carcinogenesis assay systems. Clemente et al., (2005) reported BBIs from recombinant pea (*Pisum sativum* L.) seed protease inhibitors, rTI1B and rTI2B differ in their inhibitory activity, on the growth of human colorectal adenocarcinoma HT29 cells in vitro. A significant and dose-dependent decrease in the growth of HT29 cells was observed using all protease inhibitors, with rTI1B showing the largest decrease. The relative effectiveness of rTI1B and rTI2B correlate to the variant amino acid sequence within their respective chymotrypsin inhibitory domain, in agreement with a chymotrypsin-like protease as a potential target. But the studies with MSTI, a BBI from snail medic seeds (*Medicago scutellata*), (Catalano *et al*., 2003) revealed that

antichymotryptic activity was not a strict requirement for the antitumoral effect wherein the inhibitory activity and antitumoral activity are not correlated.

BBI isolated from the seeds of Faba bean (*Vicia faba* cv. Giza 843) inhibited HIV-1 reverse transcriptase activity with an IC_{50} of about 0.76 µM. Furthermore, this inhibitor showed specific antiproliferative activity toward HepG2 hepatoma cells by inducing chromatin condensation and cell apoptosis (Fang et al., 2011). Dietary supplements with BBIC or the antioxidant formulation significantly reduced the prevalence and severity of the lens opacifications in the mice exposed to iron-ion radiation. Treatment with BBIC or the antioxidant formulation also decreased the severity of the lens opacifications in the mice exposed to proton radiation. These results indicated that BBIC and the antioxidant formulation evaluated could be useful for protecting astronauts against space radiation-induced cataracts during or after long-term manned space missions (Davis et al., 2010).

Owing to the positive aspects, BBI has been incorporated in many commercial soy foods (Blanca et al., 2009), such as soymilk, soy-based infant formula, tofu and bean curd.

4. Physiological role in seeds

BBI is to seeds what α1-antitrypsin is to humans. The physiological significance of BBI-type proteins is associated with their three major roles in plants: regulation of endogenous seed proteinases, storage of sulphur amino acids, and defence against pathogens and insect attack. Many trypsin inhibitors from diverse classes are implicated in the protection of plants against insects and fungi. In the latter case, they appear to have an antidigestive effect, through proteolysis inhibition. During seed germination, the concentration of BBI inhibitors decreases. The result was evaluated by the studies on germination in which protease K1 initiates the degradation of BBI followed by extensive proteolysis by protease B2. Wound-induced leaf expression of BBI was reported as an example of anti-pest function for BBI (Brown et al., 1985).

5. Conclusion

Proteases play key roles in pathogenesis. A large number of human disorders result from an imbalance in proteolytic activity. In this context, PIs are key players in the endogenous defense system, as they help regulate and balance protease activities. Synthetic PIs currently form a part of the combinational therapy and have potential to be used as drugs against many other diseases. Although plant PIs have been isolated and characterized from a large number of sources, and that the natural inhibitors have been made available by gene therapy and through transgenic plants overexpressing specific inhibitors with therapeutic significance, the potential for the natural inhibitors in medicine and agriculture is enormous, awaiting full-scale exploration. BBI is one among the major PIs emerging as an investigatory drug.

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