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A Facile Spectrophotometric Determination of Osmium(VIII) in very low concentrations using DL-Pencillamine as Chromogenic Reagent

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ABSTRACT

A simple spectrophotometric technique of high sensitivity and selectivity has been developed for the determination of trace amount of osmium(VIII) using DL-Pencillamine(DL- Pen) as a chromogenic reagent. The method is based on the formation of a pale pink complex at a fast pace at room temperature by the reaction of osmium(VIII) with DL-Pen in strongly acidic solution of pH 1 which absorb at 540 nm. The decrease of absorbance is directly proportional to osmium concentration and obeys the Beer's law in the range of 0.15-1.50 µg/ml. The optimum reaction condition and other analytical requirements were evaluated. The molar absorptivity(5.05 x 10^4 $LMol^{-1}cm^{-1}$), Sandell's sensitivity(3.8 x 10^{-3} μgcm^{-2}), detection limit(0.066 μgml^{-1}) and quantitation limit(0.20 μgml^{-1}) of the method were computed.

Key words

Osmium, Spectrophotometry, DL-Pencillamine , Chromogenic reagent.

1.Introduction

Osmium($Z=76, 5d^6 6s^2$) is a bluish white metal, the heaviest among the platinum metals, exhibits high specific density(22.61g cm-3), high melting point(3050^0C)and hardness(7.0Mhos scale) , occurs in ten different oxidation states from Os(II) to Os(VIII). The metal and its alloys are used for the production of materials of extreme hardness.

The volatile osmium tetroxide, OsO_4, is formed by the oxidation of osmium, is one of the most important compounds of the element and is easily identified by its characteristic unpleasant odour. Osmium tetroxide is a powerful toxic agent, harmful to eyes and respiratory tracts. So special precautions are needed in sample collection, storage and other procedures due to its strong tendency of oxidation to volatile tetroxide and possible losses of the element. The volatility of OsO_4 makes the basis of separation of osmium from its mixtures with other noble metals by distillation. The reduction of OsO_4 to the black osmium dioxide, OsO_2, by biological substances made the basis of its use as a staining reagent for the microscopic examination of tissues.

Compared to the various modern optical methods of analysis including inductively coupled plasma atomic emission spectrometry, electron microprobe analysis, total reflection X-ray fluorescence spectrometry, atomic absorption spectrometry and neutron activation analysis, the visible spectrophotometric methods seems to be the most appropriate analytical approach for the determination of toxic metals like osmium, as it provides sensitive, precise and accurate measurements of suitable analytes and offers practical and economical advantages over other methods. Most of these instruments are highly expensive and their day to day maintenance cost is high and are also not free from various types of inherent interferences (Somasiri LLWet al., 1991,Honegger K .,et al 1987 Ramesh et al., 1992).Besides,visible spectrophotometric detection is much more viable as a useful technique to develop a portable on-line or at line system.

Most of the existing well established spectrphotometric methods using the sulfur containing molecules and ligands like thiourea, thiocynate and 1,5-diphenylcarbazide lack adequate sensitivity and selectivity and necessitate the separation of osmium by extraction or distillation before the determination.

Many organic reagents have been proposed for spectrophotometric determination of osmium, including Perphenazine (Gowda et al., 1984)

In the flotation methods, the molar

absorptivities are 2.2 x 10^5 at 655 nm ,2.0 x 10^5 (with crystal violet and malachite green) (Balcerzak M. 1991) . Larger amounts of osmium can be determined as osmate, OsO_4^{2-}(ε = 2.75 x 10^3 at 340 nm) (Ensafi et al 1991) .Other than direct spectrophotometric methods of determination, certain reagents (Jamshid et al 2000) are employed for the spectrophotometric determination of osmium based on its catalytic activity. Derivative spectrophotometric methods are also used for the determination of osmium under different experimental conditions (Balcerzak M e t al., 1996, Balcerzak M et al.,1997, Kosiorek A et al., 2006) .

In the present work, a reaction of Os(VIII) with DL-pencillamine have been examined at various experimental conditions and the most suitable analytical requirements have been established for the quantitative determination the element in pure aliquots and in the presence of various other metal ions. The proposed method for the element offers many advantages over the conventional spectrophotometric methods.

2.Experimental

Reagents and chemicals:Standard osmium solution (1.5 mg/ml) : carefully broken an accurately weighed glass ampoule containing ~1 g of OsO_4 (J.M. Chemicals Ltd., London)in a beaker containing ~100 ml of water acidified with 3 ml of H_2SO_4 (1+1) wash, dry and weigh the glass fragments of the ampoule and calculate the weight of OsO_4 by difference. Dilute the osmium solution with double distilled water till 1 ml contains precisely 1.5 mg of osmium. Perform all these operations in a fume cupboard, and keep the osmium solution in a bottle with precision ground stopper on account of the toxic properties and offensive odour of the tetroxide(Marczenko Z 1986).

Pencillamine solution (1%): Dissolve 1g of reagent (HIMEDIA) in 100 ml of double distilled water.

Apparatus: The absorbance was measured and absorption spectra were recorded using a systronics double beam uv-vis spectrophotometer 2201with Quartz Cells (1cm). Digital pH meter (M.C. Dalal and Co. Madras) was used for pH measurements.

Procedure:Aliquots of the stock solution containing 0.15-1.5 µg of osmium(VIII) were pipetted out into a series of 10 ml standard flask and made up to the mark using dil. H_2SO_4 (to maintain pH1). 3ml each of these solutions were taken and mixed with 0.1ml of 1% DL-pencillamine solution and shaken well. A pink colour is developed in the solution and measure the absorbance at 540 nm against the reagent blank. The calibration graphs were constructed by plotting the absorbance against the amount of metal ions.

3. Results and Discussion

DL-pen reacts with osmium at room temperature to form a pink coloured complex species having maximum absorbance at 540 nm. Subsequent studies have been therefore carried out at this wavelength. The influence of pH on the absorbance of Os-DLpen chelate, effect of concentration of DL- pen and effect of time has been investigated.

3.1.Effect of pH

The variation of absorbance of known concentration of osmium with pH of the medium was studied. A series of buffer solutions differing by pH 0.5 was prepared, and using each of these buffers the experiment was carried out. The maximum absorbance was found at pH 1. Hence, this pH was maintained throughout the investigation.

3.2. Effect of the concentration of DL-pencillamine

Varying amounts of DL- pen were added to a fixed amount of osmium and the absorbance was measured according to the standard procedure. A volume of 0.1ml 1% solution of DL- pen was found to be optimum for the determination of 0.75µg of osmium. It was noticed that an excess of the reagent does not have any adverse effect on the sensitivity and stability of the complex.

3.3 Effect of time on absorbance

The absorbance of the complex was found to be stabilised within ten minutes and remained unaltered at least for half an hour at room temperature, indicates that the coloured complex species is having substantial stability.

3.4.Analytical Data

The Beer's law was obeyed in the range of 0.15 - 1.5 µg/ml with relative standard deviation in the range of 1-8%(Table 1). The molar absorptivity and sandell's sensitivity for the coloured system was found to be 5.5 x 10^4 L mol^{-1} cm^{-1} and 3.8 x 10^{-3} µgcm^{-2} respectively. The detection limit (D_L =3.3 σ/S) and quantitation limit (Q_L = 10σ/S) (Where σ is the standard deviation of 1m reagent blank (n=5) and S is the slope of the calibration curve) for the osmium determination were found to be 0.066 µg/ml and 0.20 µg/ml respectively.

Table.1 Determination of Os(VIII) in pure aliquots

Osmium taken (μg)	Osmium found (μg)	S.D (μg)	R.S.D. (%)	Relative error
0.15	0.14	0.03	7.14	-0.06
0.45	0.43	0.02	4.65	-0.04
0.75	0.74	0.01	1.35	-0.01
1.20	1.22	0.02	2.45	0.01
1.50	1.54	0.02	1.94	0.02

* Average of five determinations.

3.5.Interference Studies

The effect of mg amounts of several ions on the determination of 0.75 μg of osmium was studied. No interference was noticed in the presence of NO_3^-,NO_2^- , Br^-,SCN^-, SO_3^{2-} ,$AsSO_3^{3-}$,AsO_4^{3-} ,Li^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Cd^{2+}, Hg^{2+}, Al^{3+}, Tl^+, Pb^{2+}, Sb^{3+}, Bi^{3+}, Zn^{2+}, Ce^{4+}, UO_2^{2+}, Se^{4+}, Cr^{3+}, Mn^{2+}, Fe^{2+} , Rb^{3+}, Ni^{2+}, Pt^{4+}, MoO_4^{2-} and WO_4^{2}. The presence of Fe^{3+}, Ru^{3+}, VO_4^{3-}, CrO_4^{2-}, Pd^{2+}, Cu^{2+}, and Co^{2+}gave high recoveries while Ir^{4+}, Zr^{4+}, Ti^{4+}, La^{3+} and Be^{2+} caused low recoveries.

The application of suitable masking agents can sometimes effectively avoid the interference from different cations and anions. The interference due to Fe^{3+}, Zr^{4+}, Ti^{4+}, Be^{2+} and Ir^{4+} was overcome by the addition of a 2% solution of sodium fluoride and the interference due to La^{3+}, Co^{2+}, Cu^{2+}, Pd^{2+}, VO_4^{3-} and Ru^{3+} was eliminated using0.1% solution of EDTA.

4. Conclusion

DL-Pencillamine provide a simple, rapid, sensitive and selective method for the spectrophotometric determination of osmium. The major advantage of the proposed method is its rapidity, as the colour development is instantaneous at room temperature. It does not require heating, cooling or long standing to record constant absorbance. The reagent is available in pure form and hence completely free from multistage synthesis, as is the case with most of the reported methods .The proposed method does not require extraction with a solvent or addition of a surfactant to intensify the colour of the system which is an inevitable part of quite a number of reported methods.The metal salt and the chromogenic reagent are highly soluble in water and hence the entire experiment can be performed without using any other solvent system.

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Evaluation of insecticidal and larvicidal activity and the detection of lipase from *Eupatorium odoratum* leaf extracts.

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ABSTRACT

Eupatorium odoratum belongs to asteraceae family and is used by many people in the field of agriculture as a natural pest repellent. The preliminary phytochemical screening revealed the chemical composition of *Eupatorium odoratum* leaf extract containing tannins, phenols, phytosterols, flavonoids, saponins, coumarins, quinones, cardiac glycosides, terpenoids, anthraquinones, phytosteroids and steroids. This study aims at exploring the plant in various aspects like insecticidal property and larvicidal property against mosquito larvae. The larvicidal potential of *Eupatorium odoratum* leaf extract was tested against third instar mosquito larvae. The insecticidal activity was carried out using white mealy bugs and the mortality was observed at every 30 minutes. The present study also identified the presence of lipase enzyme from this plant. This enzyme has a variety of applications in industry. There are no reports of lipase enzyme from *Eupatorium odoratum.* The current investigation shows the *Eupatorium odoratum* leaf exhibited lipase activity.

Key words:

Lipase, larvicidal activity, insecticidal activity, *Eupatorium odoratum.*

1. Introduction

Eupatorium odoratum is a fast-growing perennial and invasive weed native to South and Central America. It has been introduced into the tropical regions of Asia, Africa and other parts of the world. It is a species of flowering shrub in the sunflower family, Asteraceae. It is also known as *Chromolaena odorata*. It is sometimes grown as a medicinal and ornamental plant. Common names of *Eupatorium odoratum* includes Siam weed, Christmas Bush and common Floss flower.

Owolabi et al 2010 revealed that oil from *Eupatorium odoratum* showed antibacterial activity against *Bacillus cereus* and antifungal activity against *Aspergillus niger*. Ranjini and Nambiar 2015, studied that *Eupatorium odoratum caused* disruptive changes tolarvae of *Orthaga exvinacea* Hampson. *Eupatorium odoratum* is used as a traditional medicine in Indonesia. The young leaves are crushed and the resulting liquid can be used to treat skin wounds. (Panyaphu k., 2011). In traditional medicine, a decoction of the leaf is used as a cough remedy and as an ingredient with lemon grass and guava leaves for the treatment of malaria. 17 major and 26 minor compounds were identified in methanol and aqueous extracts of *Eupatorium odoratum* by GC-MS analysis showing significant antibacterial, antioxidant and other prophylactic activities (Raman et al, 2012).

2. Materials And Methods

2.1 Plant material

The plant leaves were collected from various locations in Thrissur, Kerala. The leaves were washed thoroughly with tap water and shade dried at room temperature. The dried plant leaves were finely powdered using an electric grinder and used for aqueous and organic solvent extraction.

2.2 *Mosquito larvae*

Third instar mosquito larvae were collected and used in the present study'

2.3 Pests

White mealy bugs were collected from the infected plants from Thrissur area and is used for the study.

2.4 Larvicidal Activity

Aqueous extract of *Eupatorium odoratum* leaves were used to analyze the larvicidal activity. 10gms of leaf powder was mixed in 100ml distilled water and kept for few hours. Filtered the mixture using a muslin cloth and centrifuged at 10000rpm for 10minutes. The supernatant was collected. Third instar larvae of mosquitos were used for this study. Five mosquito larvaes were placed in a petridish containing 10ml of leaf extract and a control plate containing 10 ml water was also kept. The larval mortality was monitored in every 10 minutes.

2.5 Insecticidal Activity

The aqueous extract of *Eupatorium odoratum* was used to check the insecticidal activity. To evaluate the insecticidal activity we used white mealy bug. 5-7 mealy bugs were placed in two petriplates. 10ml aqueous extract was poured in one plate. One plate was kept as control. Water was added to the control plate. The insect mortality was monitored in every 30 minutes.

2.6 *Lipase Activity Of Eupatorium Odoratum Leaf Extract:*

Lipase activity in *Eupatorium odoratum* leaf extract was analyzed using a simple method (Rajini Singh, 2011)**.** Simple and few constituents are used for this test (Table).

Table1. Composition of Lipase assay medium:

Components	**Quantity**
Phenol red	0.01%
Substrate	1%
Calcium chloride	10mM
pH	7.3-7.4

Substrate: Gingily oil, Palm oil, Coconut oil, Tween 20.

Two main methods were used for the detection of lipase activity.

1. Plate method:

0.01% Phenol red, 10mM calcium chloride and 2% agar mixed in 100ml distilled water. pH was adjusted with sodium hydroxide (7.3-7.4). Media was equally distributed to three beakers and melted by heating. 250µl substrates (Gingily oil, coconut oil, Palm oil, Tween 20) were added to respective beakers and mixed thoroughly/vortexed and poured to the respective plates and plates were solidified. After solidification, 4 wells were punched on each plate and numbered as 1.DMSO (control), 2. Methanol Extract, 3. Chloroform extract, 4.Aqueous extract. Each extracts were poured to the respective wells and incubated the plates at 37 ˚C overnight.

2. Tube method- modified:

Media composition was same as in the plate method without 2% agar. After the substrate were added to the respective tubes, mixed thoroughly/ vortexed. pH was adjusted with sodium hydroxide. One tube considered as blank in which no substrate was added. In case of control tubes, only media was used, extract not added. All tubes were incubated at 50˚C for 10 minutes. After incubation, the color difference was monitored with respect to control and test tubes

3. Results And Discussion

3.1 Larvicidal Activity Of Eupatorium Odoratum

Eupatorium odoratum was analysed for larvicidal activity against mosquito larvae. The mortality of larvae was gradually increased after each time period. The graph shows mortality of larvae in every 10 minutes.

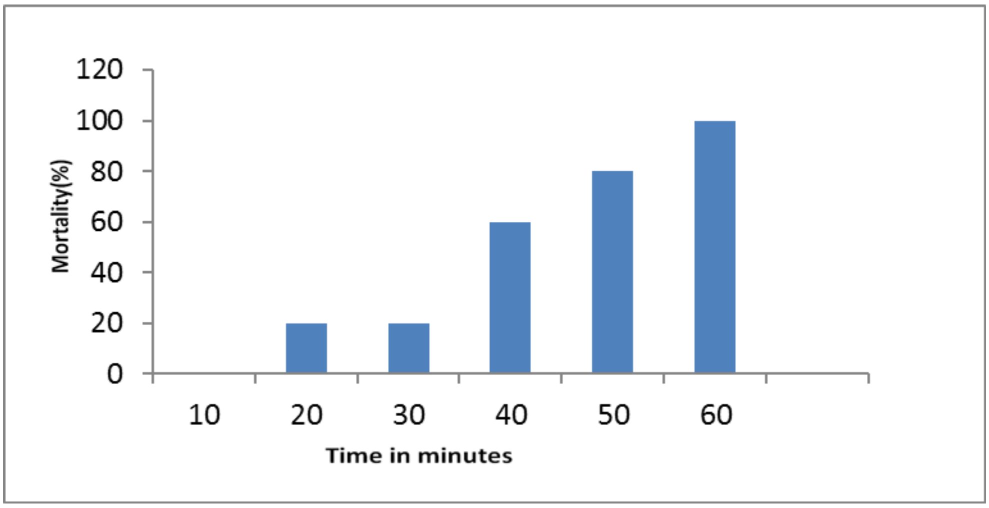

Fig: 1. Larvicidal activity of Eupatorium odoratum leaf extract.

The above data shows the larvicidal activity of *Eupatorium odoratum* leaf extract. After 60 minutes all mosquito larvae were dead.

Ranjini and Nambiar 2015, studied the histomorphological changes due to the effect of leaf extracts of *Clerodendrum infortunatum* and *Eupatorium odoratum* on the midgut tissue of sixth instar larvae of *Orthaga exvinacea*. The studies revealed the effect of extracts on the morphometric and histological changes in the midgut tissue and showed that they can cause degenerative effects in the midgut epithelium. Due to these disruptive changes they can be employed for the management of this pest.

3.2 Insecticidal Activity Of Eupatorium Odoratum

Eupatorium odoratum has insecticidal activity also. We used white mealy bug to check the insecticidal activity of the extract. The graph given below shows the mortality (%) of the insect at an interval of 30 minutes.

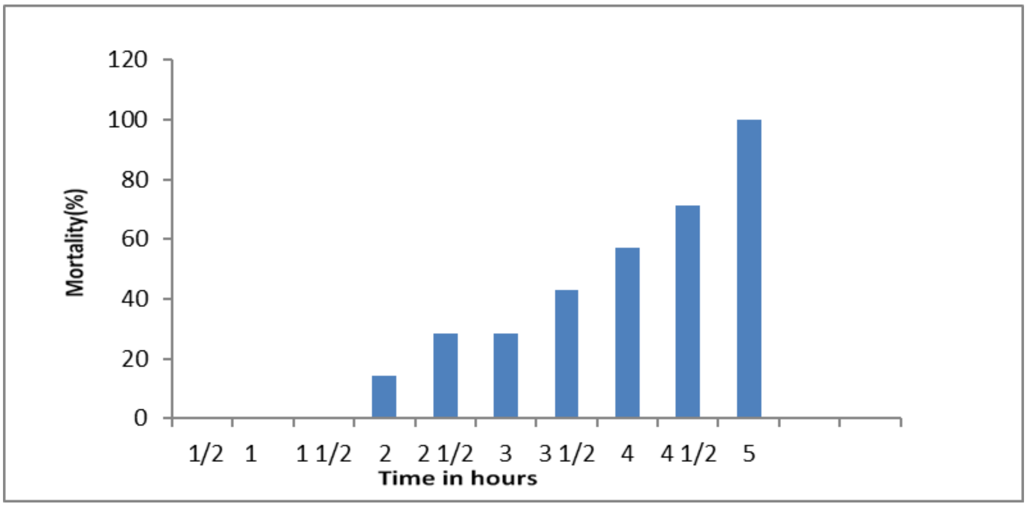

Fig: 2. Insecticidal activity of *Eupatorium odoratum*

From the above data we got a clear idea about the insecticidal activity of *Eupatorium odoratum* leaf extract. All the insects were dead after 5 hours.

Plants are rich source of alternative agents for control of mosquitoes, because they possess bioactive chemicals, which act against limited number of species including specific target-insects and are eco-friendly (Sukumar et al., 1991). Traditionally plant based products have been used in human communities for many centuries for managing insects. Several secondary metabolites present in plants serve as a defense mechanism against insect attacks. These bioactive chemicals may act as insecticides, anti-feedants, moulting hormones, oviposition deterrents, repellents, juvenile hormone mimics, growth inhibitors, antimoulting hormones as well as attractants. Plant based pesticides are less toxic, delay the development of resistance because of its new structure and easily biodegradable (Ignacimuthu, 2000).

Plant based products does not have any hazardous effect on ecosystem. Recent research has proved that effectiveness of plant derived compounds, such as saponine (Wiseman and Chapagain, 2008), steroids (Ghosh et al., 2008), isoflavonoids (Joseph et al., 2004), essential oils (Cavalcanti et al., 2004), alkaloids and tannins (Khanna V G and Kannabirank, 2007) are potential mosquito larvicides. Plant secondary metabolites and their synthetic derivatives provide alternative source in the control of mosquitoes.

3.3 Lipase Activity Of Eupatorium Odoratum Leaf Extract

1. Plate method:

After the lipase assay, color changes were produced around the wells. Color changed from pink to yellow. All extracts shows the changes. This denoted that the leaf extract have oil degrading activity or lipase activity. This is the first report for the presence of lipase from this plant.

2. Tube method:

The results obtained colorimetrically showed the degradation of oils by lipase enzyme present in the leaf extract of *Eupatorium odoratum.* The degrading activity shows more effectively in coconut oil. Here we used different substrates to check the lipase activity. This method is a simple and cost effective method to check for the presence of lipase. The presence of the protein can be analyzed in a short period of time. Only drawback of the method is the inability to quantitize the amount of lipase present. Since the absorption maximums of phenol red at both colors (red and yellow) are at different wavelengths we are unable to quantify the amount of lipase by this method. But the method is very effective in mass screening for the presence of lipses.

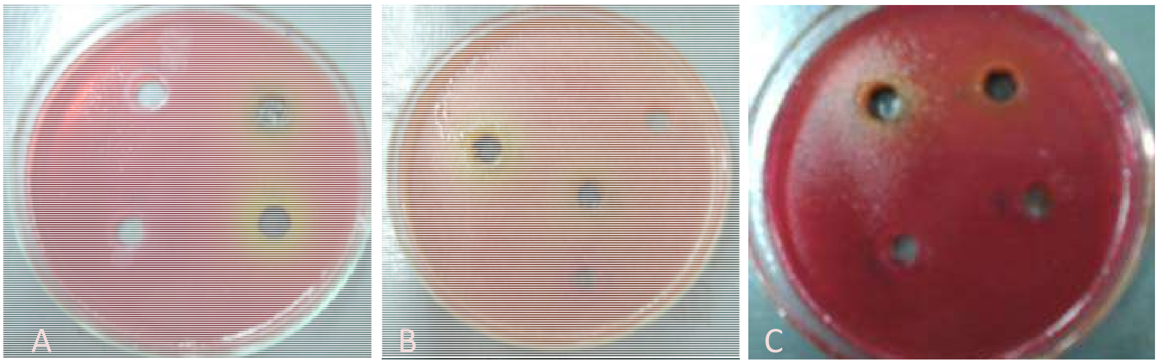

Fig: 3. Lipase activity of *Eupatorium odoratum* leaf extract with different substrates. (A) Coconut oil (B) gingili oil (C) tween20

A B

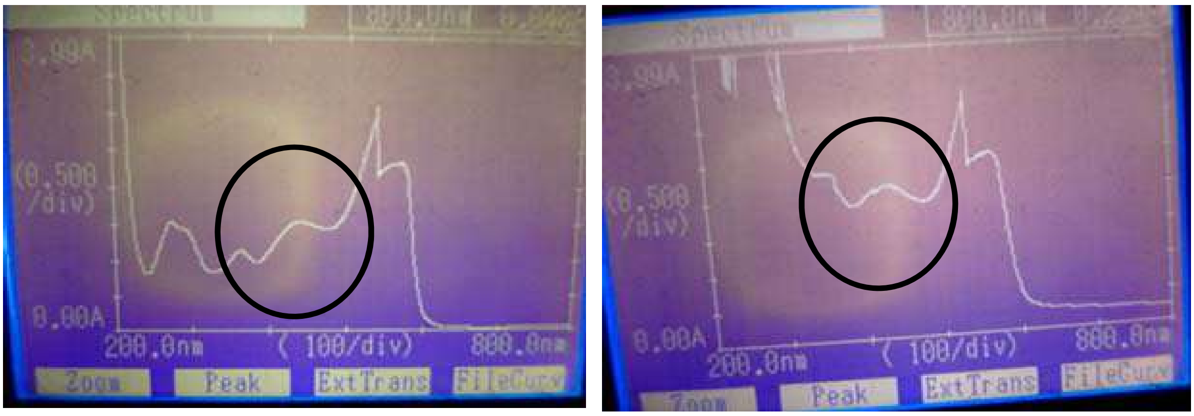

Fig: 4. Spectrum of *Eupatorium odoratum* leaf extract showing lipase activity.

A) Control tube without leaf extract. B) Leaf extract showing lipase activity.

Phenol red shows absorption maximum at 550 nm at alkaline pH. In the figure B, the shift in peak near yellow-orange range is visible due to lipase activity.

Lipase enzyme gains attention due to their wide variety of industrial applications. This enzyme is wide spread in occurrence, i.e., it is seen in unicellular life forms like microbes to the highly evolved plant species, both dicots and monocots and also seen in animals including primates like humans. Lipases can be used in cosmetics, detergents, biodiesel preparation and variety of pharmaceutical applications. Application of lipases in detergents marked a new era in detergent industry. Traditional fossil fuels like petroleum products becoming a limited source made us to think in different way to create new fuels. This led to the development of bio-fuels which employs the potential of lipase in the field. Employing this enzyme, bio-fuels can be developed even from things we considered as waste. Thus this enzyme paves an efficient way in waste management which is the current major issue of the era.

4. Conclusion

Eupatorium odoratum (Asteraceae) is a perennial scandent or semi-woody shrub. It has been distributed into the tropical regions of Asia, Africa and other parts of the world. The present study reveals the significant larvicidal and insecticidal activity of *Eupatorium odoratum* leaf. According to our results, we can suggest that *Eupatorium odoratum* have potent insecticidal and larvicidal activity. Due to its lipase activity and bio enhancer activity of leaf extract, it can provide newer leads and clues for modern drug design and for industrial applications. In fact, waste degradation a main problem in environment, *Eupatorium odoratum* ensure its great role in nature and in advanced activity.

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Biosynthesis of Gold Nanoparticles using Bacteria and Fungi

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Abstract

Nanotechnology is one of the most active areas of research in modern material sciences. This field is developing day by day, making an impact in all spheres of human life making vast implications in life sciences especially biomedical devices and biotechnology since it is expected to open new avenues to fight and prevent disease using atomic scale tailoring of materials. Gold nanoparticles are traditionally being synthesized by chemical and physical methods which needs severe reaction conditions, for example, aggressive agents like sodium borohydride, hydrazinium hydroxide, cetyl triethyl ammnonium bromide, harmful solvent system to environment and ecology, higher temperature and higher pressure etc. On the other hand, in green synthesis approach, both prokaryotic and eukaryotic organisms including bacteria, fungi and yeasts could synthesize nanoparticles by means of bioreduction. This biological route involving micro-organisms provides great advantages over traditional methods, as it has the potential to be cost-effective, simple and environmentally friendly and allow size and shape controlled nanoparticle synthesis.

Key words:

Nanoparticles, Nanotechnology, Green synthesis, Biosynthesis

Introduction

Metal nanoparticles have been gaining importance in the past years because of their unique properties. Substantial research has been directed towards their reliable synthesis to explore their potential applications. Amongst the noble metals (silver, gold, and platinum), gold nanoparticles (GNPs) are of increasing interest due to their use in divergent fields of science. GNPs are being extensively used for colorimetric detection of DNA and proteins, in form of biosensors, bioimaging, diagnostics, and therapeutic agents.

Biosynthesis or green synthesis can be successfully used for the production of smaller particles in large scale. Biological systems like bacteria, fungi, actinomycetes and plants have the ability to produce NPs. Use of microorganisms as potential biofactories for synthesis of AuNPs is a relatively new area of research and is environmentally acceptable, economic, time saving and can be easily scaled up for large scale synthesis.

Among the reported microorganisms, fungus offers the advantage of easy handling and fast downstream processing and allows feasible large scale synthesis of nanoparticles due to the high secretion of enzymes and proteins. Cytosolic extracts of the fungi *Candida albicans*, *Aspergillus niger*, *Penicillium* sp., and *Aspergillus clavatus* have been successfully used for GNP synthesis.

Biosynthesis of nanoparticles by bacteria

Bacteria are well known to produce inorganic materials either intracellularly or extracellularly. Microorganisms are concluded as a potential biofactory for the synthesis of nanoparticles like gold, silver and cadmium sulphide. There are several types of bacteria used to synthesize gold nanoparticles from aqueous $HAuCl_4$ solution either intra or extracellularly.

For the first time microbial synthesis of AuNPs was reported in *Bacillus subtilis* 168 which revealed the presence of 5–25 nm octahedral NPs inside the cell wall. In *Rhodopseudomonas capsulata*, spherical AuNPs with 10–20 nm range have been observed [He et al., 2007] at lower concentration and nanowires with network at higher concentration.

Nakajima [2003] screened 30 species of various microorganisms for gold accumulation. An extremely high ability to accumulate gold from a solution containing Au_{+3} was found in bacterial strains such as *E. coli* and *Pseudomonas maltophila*. The accumulation was very rapid and was affected by the pH of the solution.

The precipitation of gold by magnetotactic cocci bacteria occurred in the Phosphorus–Sulfur–Iron (PSFe) granules which suggest the association of gold with these elements. The reduction and precipitation of gold by dissimilatory iron (III) reducing bacteria were observed. Deplanche and Macaskie [2007] investigated the hydrogenase enzyme on the role of gold precipitation by *Escherichia coli* and *Desulfovibrio desulfuricans* ATCC 29577 and suggested that the periplasmic hydrogenases were involved in the gold precipitation.

Feng et al [2008] discovered that *Rhodobacter capsulatus* were able to precipitate gold nanoparticles within and outside the bacterial cells, forming spherical and irregular gold with average size ranging from 10 to 48 nm. *Bacillus licheniformis* precipitated gold nanocubes (10–100 nm) within 2 days at 25^{0}C [Kalishwaralal et al., 2009]. Monodispersed gold nanoparticles capped with dodecanethiol were biosynthesized extracellularly by *Bacillus megatherium* D01 at 26^{0}C. Nangia et al [2009] reported the biosynthesis of gold nanoparticles by *Stenotrophomonas maltophilia* forming ~40 nm spherical and irregular shapes at 25^{0}C.

Growth conditions play an important role during the production of nanoparticles. When gold ions were incubated with the *Trichothecium* sp. Biomass, under stationary conditions it led to the formation of extracellular gold nanoparticles. While under shaking conditions, this has resulted in the formation of intracellular gold nanoparticles. Venkatesan et al [2011] studied the synthesis and optimization of gold nanoparticles from *Pseudomonas* sp. isolated from soil sample. They found the production of gold nanoparticles with controlled size, shape and composition by optimizing various parameters like pH, temperature and concentration of aurium chloride.

Satyanarayana et al [2010] reported that the NPs were stabilized by the surface-active molecules, that is, surfactin or other biomolecules released into the solution by *B. subtilis*. *Bacillus megaterium* D01 have shown the strong potential of Au_{3+} adsorption. When *B. megaterium* D01 biomass was exposed to the aqueous solution of $HAuCl_4$, monodispersed spherical gold NPs capped with self-assembled monolayer (SAM) of thiol have been synthesized, extracellularly. The gold NPs were stable without any aggregation over a period of several weeks.

Lactobacillus strains, when exposed to gold ions, resulted in formation of gold NPs within the bacterial cells [Nair and Pradeep, 2002]. *Escherichia coli* DH5α can be used in synthesizing gold NPs. In another study, biorecovery of gold from jewellery wastes was obtained using *E. coli* MC4100 (nonpathogenic strain) and *Desulfovibrio desulfuricans* ATCC 29577 [Deplanche and Macaskie, 2007]. *Rhodopseudomonas capsulata* showed the ability to produce gold NPs in different sizes, and the shape of gold NPs was controlled by Ph [He et al., 2007]. *R. capsulate* was capable of producing gold NPs extracellularly and the gold NPs were quite stable in the solution and formation of gold NPs might be due to NADH dependent enzymes secreted by *R. capsulate*.

Southam and Beveridge have demonstrated that gold particles of nanoscale dimensions may readily be precipitated within bacterial cells by incubation of the cells with Au3+ ions [Southam and Beveridge, 1996]. Monodisperse gold nanoparticles have been synthesized by using alkalotolerant Rhodococcus sp. under extreme biological conditions like alkaline and slightly elevated temperature conditions [Ahmad et al., 2003]. Lengke [2006] claimed the synthesis of gold nanostructures in different shapes (spherical, cubic, and octahedral) by filamentous cyanobacteria from Au(I)-thiosulfate and Au(III)-chloride complexes and analyzed their formation mechanisms

[Lengke et al.,2006]. Nair and Pradeep reported the growth of nanocrystals and nanoalloys using Lactobacillus [Nair and T. Pradeep, 2002].

Another example of gold deposition in prokaryotes is the demonstration by Kashefi et al [2001]. They screened a range of anaerobic Fe(III)-reducing bacteria (mesophiles and thermophiles) for their ability to reduce Au_{+3} and observed a complete reduction of Au_{+3} after 25 min using the *Pyrobaculum islandicum*. The use of various strains of *Pseudomonas aeruginosa* for extracellular biosynthesis of gold nanoparticles was described. In different microorganisms, various enzymes are believed to take part in the bioreduction process involving the transport of electrons from certain electron donors to metal electron acceptors. Some studies of nonenzymatic reduction mechanism suggested that some organic functional groups of microbial cell walls could be responsible for the bioreduction process [Crookes-Goodson et al., 2008].

Biosynthesis of nanoparticles by fungi

Fungi appear to be more promising for large scale production of NPs as they are simpler to grow both in the laboratory and at an industrial scale as well as secrete large amount of proteins. Fungi are regarded as more advantageous for GNPs biosynthesis because of the following features;

- Fungal mycelial mesh can withstand flow pressure, agitation, and other conditions in bioreactors compared to bacteria,
- They are fastidious to grow and easy to handle
- They produce more extracellular secretions of reductive proteins and can easily undergo downstream processing [Musarrat et al., 2010].

AuNP formation can occur either in the intracellular or extracellular space. Extracellular AuNP formation is commonly reported for fungi when Au_{3+} ions are trapped and reduced by proteins in the cell wall. Previous work with the fungus Verticillium sp. ruled out the possibility that reduced sugars in the cell wall are responsible for the reduction of Au_{3+} ions and suggested adsorption of AuCl4- ions on the cell-wall enzymes by electrostatic interaction with positively charged groups (e.g. lysine) [Duran et al., 2005]. In the case of intracellular AuNP formation, Au_{3+} ions diffuse through the cell membrane and are reduced by cystolic redox mediators [Das et al., 2010].

Another recent study reports the production of intracellular AuNPs in metal-tolerant *Aspergillus fumigatus* and *Aspergillus flavus*. The intracellular AuNPs have an average diameter of 22 ± 2 nm, slightly larger than those observed in the extracellular space. The enzymatic reduction mechanism of Au_{3+} is essentially the same for intracellular and extracellular AuNPs [Gupta and Bector, 2013]. In viable cells, Au ions are reduced by nicotinamide adenine dinucleotide(NADH)/nicotinamide adenine dinucleotide phosphate (NADPH) oxidoreductases either in the cell surface or in the cytoplasm.

Other mechanisms for fungal AuNP biosynthesis have been proposed. The fungal pathogen *Candida albicans* is capable of synthesizing phytochelatins, which are made of chain links of glucose, cysteine and glycine ((c-GluCys) n-Gly) by the transpeptidation reaction of c-Glu-Cys dipeptide from a succession of glutathione molecules. In the presence of glutathione, metal ions, including Au, trigger phytochelatin synthesis, in which Au^{3+} ions get reduced to AuNPs, which are then capped by glutathione [Chauhan et al., 2011]. AuNP synthesis has been detected by UV-visible spectrophotometry in heat-denatured cell-free filtrate of *Sclerotium rolfsii* in the presence of co-enzyme NADPH and 1 mM of Au^{3+} . This indicates the involvement of thermostable NADPH-dependent enzymes in the AuNP biosynthesis process. Cyclic voltammetry analysis showed that NADH produced from the fermentation of lactate by *Hansenula anomala* reduces Au^{3+} to AuNPs [Kumar et al., 2011]. The involvement of biosynthetic redox mediators in the fungal biosynthesis of AuNPs has been observed in *Yarrowia lipolytica*. These fungi secrete melanin, which interestingly appears to reduce Au^{3+} to AuNPs.

The extracellular synthesis of gold nanoparticles was reported by fungus *Fusarium oxysporum* and *Verticillium* . Shankar et al [2004] demonstrated that gold nanoplates can be synthesized by using fungal extracts. The results obtained from the Gericke and Pinches [2006] study demonstrated the possibility to manipulate the size and shape of gold nanoparticles synthesized by *Verticillium luteoalbum*. Ahmad et al [2003] screened a number of species belonging to different genera of molds and observed the extracellular synthesis of silver and gold nanoparticles by the treatment of the biomass of *F. oxysporum* with silver or gold ions.

An endophytic fungus *Colletotrichum* sp. growing in geranium leaves [Shankar et al., 2003] and alkalotorerant fungus *Tricothecium* sp., when exposed to aqueous chloroaurate ions, synthesized gold nanoparticles. Xie et al [2007] showed that *Aspergillus niger* may also be used for the synthesis of gold nanoparticles. The exposure of the mycelia-free spent medium or extract of *A. niger* to gold ions resulted in the growth of gold nanoparticles of various shapes and sizes.

The rate of particle formation and the size of the nanoparticles could be manipulated by controlling parameters such as pH, temperature, gold concentration and exposure time. The extracellular secretion of the microorganisms offers the advantage of obtaining large quantities in a relatively pure state, free from other cellular proteins associated with the organism with relatively simpler downstream processing.

Gold nanoparticles are one of the most attractive nanomaterials for various applications like antimicrobial, electronic, catalytic, and various biomedical applications. The present review summarises literature on synthesis of gold nanoparticles using bacteria and fungi. This green synthesis is useful since it can be used to produce large quantities of nanoparticles have advantage over the other physical methods as it is safe, eco-friendly and simple to use for the production of gold nanoparticles of desired shape and size.

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Comparative Study on Antibacterial profiles of Kanakasava

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ABSTRACT

Ayurveda is a traditional Indian medicinal system being practiced thousands of years. Considerable research on pharmacognosy, chemistry, pharmacology and clinical therapeutics has been carried out on ayurvedic medicinal plants. The present study was designed to observe antimicrobial activities of the ayurvedic medicine namely Kanakasava against four different common bacterial pathogens. The current study was structured for screening of in-vitro antimicrobial activities of collected samples by agar well diffusion method. The main objective of this study is to know how far the four bacterial species namely *Streptococcus, Staphylococcus, Pseudomonas and Escherichia coli* are sensitive to Kanakasava during different stages of production and also to know which among these will produce highest zone of clearance.

Keywords:

Ayurvedic medicines, Medicinal plants, Antimicrobial study

1.Introduction

There are many traditional systems of medicine in the world, each with different associated philosophies and cultural origins. Ayurveda is the most widely practised of the Indian traditional medicine systems, but there are others such as Siddha and Unani which are also used in the Indian subcontinent (Gogtay et al., 2002). Although there is limited data on the use of Ayurvedic medicines specifically in other areas of the world, the use of traditional medicines has increased significantly in recent years in North America, Europe. Ayurvedic medicines are of various type, so as to meet the diverse requirements in the treatment of illness. They are herbal teas, infusions, decoctions, tinctures, capsules and powders, infused oils, ointments, creams, lotion (Kumar et al., 2010). Arishta and asava are considered as unique and valuable therapeutics in Ayurveda. They are self generated herbal fermentation of traditional ayuredic system. They are alcoholic medicaments prepared by allowing the herbal juices or their decoctions to undergo fermentation with the addition of sugars. Arishtas are made with decoctions of herbs in boiling water while asavas are prepared by directly using fresh herbal juices. The method of preparation of arishta and asava is known as Sandhana kalpana (S.Sekar et al.,2006). Kanakasava is an ayurvedic medicine practiced now-a-days for mainly menstrual irregularities.

Kanakasava is a liquid ayurvedic medicine, widely used in treating asthma, cough, fever etc. It contains 5 – 10 % self generated alcohol in it. This self generated alcohol and the water present in the product act as a media to deliver water and alcohol soluble the active herbal components to the body. It is widely used in the treatment of respiratory conditions such as asthma, cough, fever and bleeding diseases. It is a natural mucolytic and bronchodilator. It is also used in treatment of bleeding diseases, injuries and chronic fever. It helps to relieve chest mucous congestion. A very high dosage of Kanakasava may cause stomach irritation and loose stool. The main ingredients of Kanakasava are: Kanaka (purified and processed Datura metel), Vrushamoola, Yashtimadhu, pippali(long pepper), Vyahgri, Keshara, Vishvabheshaja(shunti-ginger), Bharngi, Talisapatra, Dhataki, Draksha(raisins), water, Sharkara(sugar), Kshaudra (honey).

2. Methodology

2.1 Sample Collection

Kanakasava was prepared according to Sahasrayogha. Samples for analysis were collected aseptically during 30 day fermentation . The in process and quality control for the preparation was strictly controlled and monitored.

2.2 Determination of antibacterial activity

The antibacterial activity of Kankasava can be checked by measuring the zone of clearance on different bacterial culture plates. Agarwell diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts. The nutrient agar plate surface was inoculated by spreading a volume of the microbial inoculum over the entire agar surface. Then, a hole with a diameter of 6 to 8 mm was punched aseptically with a tip, and a volume 50µl of Kanakasava was introduced into the well. Then,agar plates were incubated at 37^0C for 24 hours and checked for the zone of inhibition. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested.

3. Result

Ayurvedic medicine Kanakasava was tested (agar well diffusion method) for their antimicrobial activity against *Staphylococcus species*, *Streptococcus species*, *Pseudomonas species* and *Escherichia coli*. In this antibacterial screening of test product were used at a concentration of 50µl/well. All the tested samples showed antibacterial activity . 12 day,15 day, 18 day fermented samples showed highest zone of inhibition (25mm) against *Streptococcus species*.

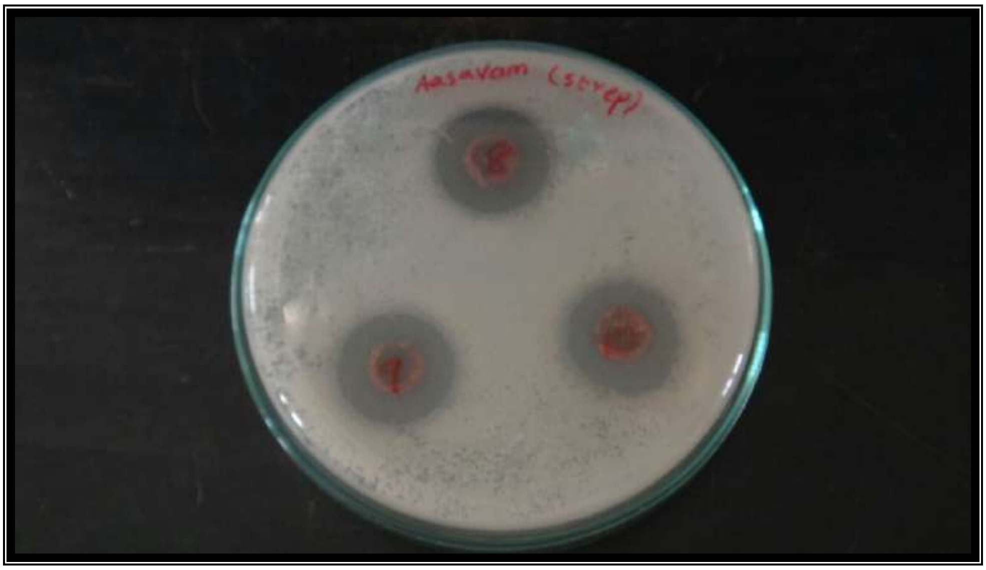

Figure 1: Anbacterial activity of Kanakasava against *Streptococcus species*

4. Discussion and Conclusion

Kanakasava is a liquid medicine widely used in treating asthma, cough, fever, etc. Datura is the main ingredient in Kanakasava that has both antimicrobial and antioxidant activities. Kanakasava is a natural mucolytic and bronchodilator. It is also used in the treatment of bleeding diseases, injuries and chronic fever. It helps to relieve chest mucous congestion. In this present study, Kanakasava showed effective antimicrobial activity against all the test organisms. Samples of Kanakasava showed the highest zone of inhibition against *Streptococcus species*. This study shows the most effective antibacterial activity of Kanakasava against four different bacterial species during different fermentation periods (days).

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Antibacterial effect of citrus fruit juice against enteric and non enteric pathogenic bacteria

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ABSTRACT

The present study was carried out to determine the antimicrobial effect of three different citrus fruit juices against four enteric and two non enteric pathogens named *Escherichia coli* , *Salmonella* , *Proteus, Klebsiella ,Vibrio,* and *Staphylococcus species.* The study was done by well diffusion and disc diffusion method. The use of different concentrations (100%, 75%, 50**%,** 25%) of citrus juice extracts had an effective antibacterial activity. Lemon juice was the most effective against the test organisms in both undiluted and diluted concentration. Pomelo juice showed antimicrobial activity only against *Vibrio* and *Salmonella*. Orange juice showed antimicrobial activity only against *Staphylococcus* and *Escherichia coli* .

Key Words :

citrus fruit, well diffusion , disc diffusion , antimicrobial effect

1. Introduction

Bacterial infections are among the important infectious diseases. Hence, over 50 years of extensive researches have been launched for achieving new antimicrobial medicines isolated from different sources. Despite progress in development of antibacterial agents, there are still special needs to find new antibacterial agents due to development of multidrug resistant bacteria (R.Wise,T. Hart, O.Cars et al. ,1998)

The emergence of resistant organisms presents a major challenge for the antimicrobial therapy of infectious diseases and increases the incidence of mortality and morbidity.The increase in antibiotic resistance bacteria is largely due to the widespread use of antibiotics in medicine in animal care and agriculture (Bansode et al 2012).)Citrus fruits have been of interest for extraction of antimicrobial metabolites by large number of researchers (Kumar et al., 2011; Kumar et al., 2010; Amandeep et al., 2009) but the peels have been less studied. Lemon juice has been used in the treatment of oral thrush in HIV/AID patients (Wright et al., 2009). The antioxidant activities of citrus flavonoids and phenolic compounds exhibited a potent antibacterial activity which is probably due to their ability to complex with bacterial cell walls and disrupt microbial membrane . Studies have shown that concentrated or freshly squeezed lemon juice has antibacterial activity against Vibrio species (Tomotake et al., 2006), Antibacterial properties of plant extract have been a hot topic for the researchers. Besides plants, fruits also have been studied by the researchers for the presence of bioac-tive compounds close related to herbs, commonly referred as phytochemicals such as tannins, carotenoids, polyphe-nols and anthocyanins (Khushwaha et al 2012).

The current research focuses on the extraction and assay of antibacterial component from citrus fruit which are easily available at very low cost.

2. Materials And Methods

2.1 Bacterial cultures - *Escherichia coli* , *Salmonella* , *Proteus, Klebsiella* ,*Vibrio,* and *Staphylococcus species.* were kindly provided from Poly Clinic Pvt Ltd, Thrissur , Kerala , India.

2.2 Citrus fruits – Fresh lemon ,Orange , Pomelo were obtained from local market of Thrissur.

2.3 Taking of juice from fruits

Surface of the fruit was disinfected using ethanol. Fruit was pierced and juice was aspirated and collected in beaker in sterile condition.

2.4 Preparation of different concen-trations of fruit juice

Different concentrations were made by adding sterile physiological saline(0.85%) into the juice. To prepare 1ml of 25% concentration of fruit juice,0.25 ml juice was added to 0.75 ml of saline, 1ml of 50% concentraton,0.5 ml juice was added to 0.5ml saline, for 1ml of 75% concentration ,0.75 ml of juice was added to 0.25ml of saline,and for 1 ml of 100% concentration,1ml juice was used.The physiological saline was used as control.

2.5 Anti-bacterial activity testing

- Well diffusion method

Antibacterial test were carried out by the well diffusion method . Overnight bacterial cultures were diluted in the nutrient broth to obtain a bacterial suspension of 10^8 CFU/ml. Petriplates containing 20 ml of Muller-Hinton Agar media were inoculated with diluted cultures with a sterile cotton swab is dipped into standardized bacterial test suspension and used to evenly inoculate the entire surface of the Muller-Hinton Agar plates. After the agar surface has dried for about 5 minutes, five wells, all are have equal diameter were made in the plate at equal distance. Citrus fruit juice of different concentrations (25,50,75,100µl)and a control (0.85%) saline were poured on each well by using micropipette. Plates were incubated at 37^{0}C for 24h .The antibacterial activity was determined by measuring the inhibition zone.

- Agar disc diffusion assay

The antibacterial activity of the citrus fruits extracts was determined by the disc diffusion method.Briefly ,overnight bacterial culture were diluted in the Muller-Hinton broth to obtain a bacterial suspension of 10^8 CFU/ml. Petriplates containing 20ml of Muller-Hinton Agar media.A sterile cotton swab is dipped into a standardized bacterial test suspension and used to evenly inoculate the entire surface of the Muller-Hinton Agar plate.After the agar surface has dried for about 5 minutes , Citrus fruit juice of different concentrations (25,50,75,100µl) were loaded on to the filter paper discs (whatman No.1 ,6mm diameter) were placed on the inoculated agar surface were allowed to dry completely. Standard saline (0.85%) was placed as control. Plates were incubated at for 37^{0}C 24h. The antibacterial activity was determined by measuring the inhibition zone. (S.Sundar and koilpillai, 2015)

3. Result And Discussion

From three citrus fruits used in the study for evaluation of their antimicrobial activity against four enteric bacteria and two non enteric bacteria initially determined by Muller Hinton Agar well diffusion method. All of the isolated species showed activity against citrus fruit juices. Juice of *Citrus limon* showed highest inhibitory effect against *Vibrio* species with largest DIZ (Diameter of Inhibition Zone) value followed by *Staphylococcus* species ,*Salmonella* species and *Proteus* species. Juice of *Citrus maxima* showed highest inhibitory effect against *Vibrio* species followed by *Salmonella* species. *de Castillo etal* (2000) also reported that freshly squeezed lemon juice inhibited the growth of *V. Cholerae* . Juices of *Citrus sinesis* showed highest inhibitory effect against *Staphylococcus* species followed by *E.coli.*

The antibacterial activity of these pathogens are further determined by disc diffusion method. All isolated bacteria showed susceptibility against citrus fruit juices. Juice of *Citrus limon* showed highest inhibitory effect against *Vibrio* species with largest DIZ followed by *Staphylococcus* species ,*salmonella* species and *Proteus* species. Juice of *Citrus maxima* showed highest inhibitory effect against *Vibrio* species followed by *Salmonella* species. *Citrus sinesis* showed highest inhibitory effect against *Staphylococcus* species followed by *E.coli* . *Citrus limon* showed highest inhibitory activity against *Vibrio* species while lowest activity against *Proteus* species. The results showed in Table 1 and Table 2. (Kumar et al., 2011; Kumar et al., 2010; Amandeep et al., 2009; Nurmahani et al., 2012) have also reported similar results for the various extracts from citrus fruits.On comparison of antimicrobial activity of different citrus fruits the results showed that *Citrus limon>Citrus maxima>citrus sinensis*.

The results of Table 1 and Table 2 revealed that *Citrus limon* juice showed highest activity against almost all enteric pathogens and non enteric pathogens. From the results obtained, the highest inhibitory effect showed by 100µl concentration and least effect by 25µl.

<u>The Table 1. Showing Antibacterial Activity By Well Diffusion Method</u>

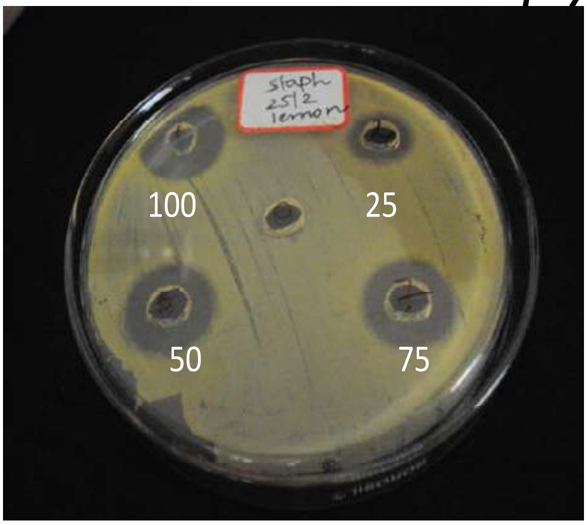
Bacteria	Diameter Of Inhibition Zone(DIZ) in mm.												
	Citrus limon				*Citrus maxima*				*Citrus sinensis*				0.85 % Saline as control
	100 µl	75 µl	50 µl	25 µl	100 µl	75 µl	50 µl	25 µl	100 µl	75 µl	50 µl	25 µl	
Enterobacteriacea													
E.coli	15	10	11	7	------	-----	-----	-----	7	4	2	-----	------
Salmonella	21	19	17	14	8	14	10	-----	------	-----	-----	-----	------
Proteus	19	17	15	11	------	-----	-----	-----	------	-----	-----	-----	------
Klebsiella	13	11	10	3	------	-----	-----	-----	------	-----	-----	-----	------
Non-Enterobacteriacea													
Vibrio	23	22	18	16	21	18	3	-----	-----	-----	-----	----	------
Staphylococcus	22	19	17	14	------	-----	-----	-----	2	10	8	7	------

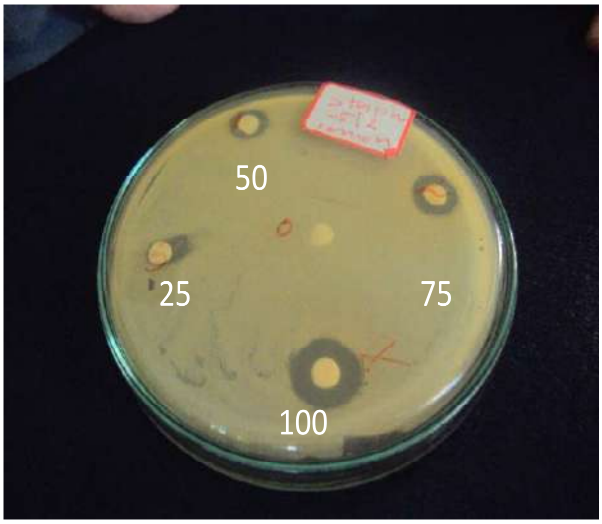
The Table2. Showing Antibacterial Activity By Disc Diffusion Method

Bacteria	**Diameter Of Inhibition Zone(DIZ)** in mm												
	Citrus limon				***Citrus maxima***				***Citrus sinensis***				**0.85 % Saline as control**
	100 µl	**75 µl**	**50 µl**	**25 µl**	**100 µl**	**75 µl**	**50 µl**	**25 µl**	**100 µl**	**75 µl**	**50 µl**	**25 µl**	
Enterobacteriacea													
E.coli	10	7	4	3	-----	----	----	----	4	2	----	----	------
Salmonella	13	12	10	7	10	8	6	----	----	----	----	----	------
Proteus	12	10	8	6	-----	----	----	----	-----	----	----	----	------
Klebsiella	10	9	3	1	-----	----	----	----	-----	----	----	----	------
Non-Enterobacteriacea													
Vibrio	18	14	12	10	18	10	8	5	-----	----	----	----	------
Staphylococcus	15	12	10	7	-----	----	----	----	10	7	6	2	------

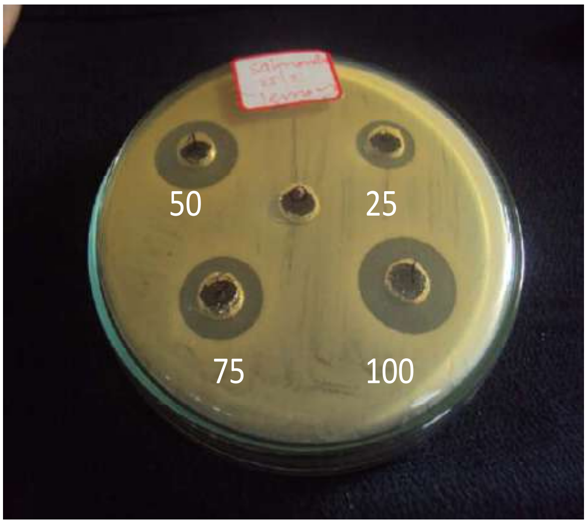
<u>Antibacterial Activity Of Fruit Juice Of *Citrus Limon* By Well Diffusion And Disc Diffusion Methods</u>

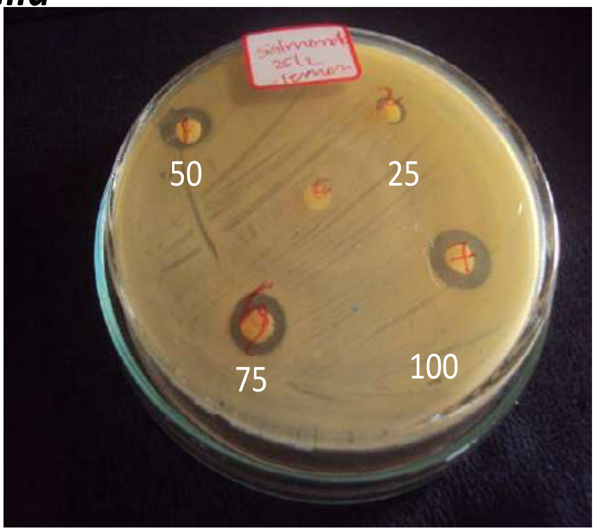
Staphylococcus





Salmonella





4. Conclusion

The study summarised here demonstrate that the microbial load of human body constitute enteric and non-enteric pathogens.The bacterial isolates identified in this study are common human enteric and non-enteric pathogens.This study emphasizes the need of usage of citrus fruits in our diet.Because the results showed that the citrus fruit juice have high antibacterial activity against almost all enteric and non-enteric pathogens .The well diffusion and disc diffusion methods using citrus fruit juice showed antibacterial activity against *Vibrio, Salmonella*, *Staphylococcus, E.coli, Proteus* and *Klebsiella*..The study points out that the infections with enteric and non-enteric pathogens can be successfully eliminated by the use of easily available citrus fruit juices. This approach however go a long way in combining the rising tide of antibacterial resistance.

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Hydrogen Peroxide Scavenging Ability of *Scoparia Dulci*&*Piper Longum Linn*

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ABSTRACT

In this study the *Hydrogen Peroxide Radical Scavenging (H_2O_2) Assay* of various extractants of *Scoparia Dulcis* and *Piper Longum Linn* were studied. Based on phytochemical analysis aqueous methanol extract was found to be the most efficient extract, to evaluate radical scavenging property. Both *Scoparia Dulcis* and *Piper Longum Linn* show antioxidant property. In the case of *Scoparia Dulcis*, its whole plant shows more anti-oxidizing property, than its roots, stem seed and leaves. But in the case of *Piper Longum Linn*, its stem and leaf have no antioxidant properties, while its root shows higher antioxidising property than the whole plant.

Key Words :

phytochemistry, *Scoparia Dulcis* and *Piper Longum Linn*, Aqueous methanol extractant, chloroform, ethanol and water.

1.0 Introduction

1.1,Free radicals are chemically active atoms or molecular fragments that have a charge due to an excess or deficient number of electrons. Free radicals containing oxygen known as reactive oxygen species (ROS) are the most biologically significant free radicals. ROS include the radicals-superoxide and hydroxyl radical, plus derivatives of oxygen that do not contain unpaired electrons, such as hydrogen peroxide, singlet oxygen and hypochlorous acid. Because they have one or more unpaired electrons, free radicals are highly unstable. They scavenge our body to grab or donate electrons, there by damaging cells, proteins and DNA. It is impossible for us to avoid damage by free radicals. Free radicals arise from the sources both inside (endogenous) and outside (exogenous) our bodies. Anti-oxidants are substances like nutrients (vitamins and minerals) as well as enzymes that are capable of counteracting the damaging, but normal, effects of the physiological process of oxidation in animal tissue. They are believed to play a role in preventing the development of chronic diseases such as cancer, heart diseases, stroke, Alzheimer's disease, rheumatoid arthritis and cataracts.

Plants are one of the largest sources of antioxidants. Studies by Harshay Meena *et al*.(2012), Latha B, *et al*. (2013), PiseNavnath, *et al*. (2010), Arulmozhi, S.*et al*.(2010), Rahmat Ali Khan, *et al*.(2012), Naima Saeed, *et al*.(2010), have all concluded that the plant extracts show antioxidant property. Some plants like Daucus crinitus Desf., had antioxidant potential comparable to those of the standard compounds such as gallic acid(Bendiabdellah, A *et al*., 2012).

1.2Hydrogen peroxide radical scavenging (H_2O_2) assay

Hydrogen peroxide occurs naturally at low concentration levels in the air, water, human body, plants, microorganisms, food and beverages. It is widely used as a bleaching agent in the textile, paper and pulp industries. Human beings exposed to H_2O_2 indirectly via the environment are estimated as 0.28 mg/kg/day with intake from leaf crops contributing most to this exposure. Hydrogen peroxide enters the human body through inhalation of vapour or mist and through eye or skin contact. In the body, H_2O_2 is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals ($OH^{\cdot}$) that can initiate lipid peroxidation and cause DNA damage. Hence Hydrogen peroxide radical scavenging (H_2O_2) assay is an important tool in assessing the antioxidant property of plant (Chand S & Dave R 2009).

2.0 Materials& Methods

2.1Collection and processing of plant samples

Scoparia dulcis and *Piper longum linn* were collected from Thrissur district. Plants were identified in the Botany Dept. of St. Mary's College Thrissur. The plants were washed with water to remove all unwanted materials; the plant parts were separated and dried in the dark. These were then grounded to a fine powder and stored in an airtight container.

2.2 Preparation of extracts

The aqueous methanol extract of the plants were prepared by shaking 5g of powdered samples in 50ml of (50:50) methanol and double distilled water for 1 hr using a shaker. The extract was then filtered using double filter paper and stored in an airtight container. The chloroform, ethanol and aqueous extract of the plants were prepared by shaking 5g of powdered samples in 50ml of chloroform, ethanol & water respectively for 1 hr using shaker. The extract was then filtered using double filter paper and stored in an airtight container.

2.3 Phytochemical Screening Methods

The phytochemical tests to detect the presence of alkaloids, carbohydrates, saponins, tannins, flavonoids, phenols and glycosides were performed according to the method described by Harborne JB (1998). Alkaloids were detected by Hager's test Carbohydrates by Molisch's test, glycosides by Salkowski's test, tannins by ferric test, saponins by frothing, Phenols by $FeCl_3$ test and Flavonoids by Lead acetate solution Test.

2.4 Hydrogen Peroxide Radical Scavenging (H_2O_2) Assay

The ability of plant extracts to scavenge hydrogen peroxide is determined according to the method of Ruch *et al.* (1989). A solution of hydrogen peroxide (40 mM) is prepared in phosphate buffer (50mMpH 7.4). The concentration of hydrogen peroxide is determined by absorption at 230 nm using a spectrophotometer (UV-1800 SHIMADZU; UV Spectrophotometer). 1 ml of plant extract is added to2 ml hydrogen peroxide and absorbance at 230 nm is determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging is calculated as follows:

$$\% \text{ Scavenged } (H_2O_2) = (A_0 - A_1 / A_0) \text{ X } 100$$

Where A_0 is the absorbance of control and A_1 is the absorbance of test.

3.0 Results& Conclusion

3.1 Qualitative Analysis

Preliminary phytochemical screening revealed the presence of tannins, phenols, glycosides, carbohydrates, saponins, flavonoids and alkaloids in different extracts of *Scoparia dulcis* and *Piper longum linn.* The results show that these plants contain a number of chemical ingredients, which may be responsible for the various pharmacological actions although their specific roles remain to be investigated. In the phytochemical qualitative analysis of *Scoparia Dulcis*, it has been seen that alkaloids, carbohydrates, glycosides and tannins are present in ethanolic extract, while saponin, phenol and flavonoid are absent. In the case of aqueous methanol extract, alkaloid, carbohydrate, glycosides, flavanoid and tannin are present whereas saponin and phenol are absent. The chloroform extract shows only the presence of carbohydrate, phenol and tannin and others are absent. But in the case aqueous extract, only tannin, phenol and flavonoid are absent and all others are present(Table 1). In the phytochemical qualitative analysis of *Piper Longum Linn*, carbohydrate, phenol, flavonoid and tannin are present in ethanolic extract, while alkaloid, glycosides and saponin are absent. The aqueous methanol extract shows only the absence of saponin all others are present. But in the case of chloroform extract, only carbohydrate, glycosides, and tannin are present, all the rest are absent. In aqueous extract, only tannin and phenol are absent and all others are present(Table 2).

In the case of *Scoparia dulcis*, ethanol extract shows the presence of four constituents, aqueous extract shows the presence of four constituents, chloroform shows only the presence of three constituents and aqueous methanol extract shows the presence of five constituent. In the case of *Piper longum linn*, ethanol extract shows the presence of four constituents, aqueous extract shows the presence of five constituents, chloroform shows only the presence of three constituents and aqueous methanol extract shows the presence of six constituent. From the phytochemical screening, it is understood that aqueous methanol extract is the good extract. Hence the Hydrogen peroxide radical scavenging (H_2O_2) assay was conducted using the aqueous methanol extract.

3.2 Hydrogen Peroxide Scavenging Effects on Scoparia Dulcis & Piper Longum Linn

In the case of *Scoparia Dulcis*, highest anti-oxidizing property was shown by whole plant followed by root, then stem and seed. Its leaf possessed least anti-oxidizing property (Table 3). In the case of *Piper Longum Linn*, root shows higher anti-oxidizing property than whole plant. Its stem and leaves have no anti-oxidizing properties (Table 4).

3.3. Conclusion

Aqueous methanol extract was found to be the most efficient extract, for radical scavenging property. Both *Scoparia Dulcis* and *Piper Longum Linn* show antioxidant property. In the case of Scoparia Dulcis, its whole plant shows more anti-oxidizing property, than its roots, stem seed and leaves. But in the case of *Piper Longum Linn*, its stem and leaf have no antioxidant properties, while its root shows higher antioxidising property than the whole plant.

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Table 1. Phytochemical qualitative analysis of *ScopariaDulcis*

Sl. No.	Phytochemical test for	Ethanol	Aqueous methanol	Chloroform	Water
1	Alkaloid	√	√	×	√
2	Carbohydrate	√	√	√	√
3	Glycosides	√	√	×	√
4	Tannin	√	√	√	×
5	Saponin	×	×	×	√
6	Phenol	×	×	√	×
7	Flavonoid	×	√	×	×

"√"indicates presence and "×"- indicates absence

Table 2. Phytochemical qualitative analysis of *Piper Longum Linn*.

Sl. No.	Phytochemical test for	Ethanol	Aqueous methanol	Chloroform	Water
1	Alkaloid	×	√	×	√
2	Carbohydrate	√	√	√	√
3	Glycosides	×	√	√	√
4	Tannin	√	√	√	×
5	Saponin	×	×	×	√
6	Phenol	√	√	×	×
7	Flavonoid	√	√	×	√

"√"indicates presence and "×"- indicates absence

Table 3. Hydrogen Peroxide Scavenging Effects on *Scoparia Dulcis*

Plant part/ control	UV Asorption value	% of H_2O_2 Scavenging Activity
Control	2.9	
Root	2.2	24.1
Stem	2.4	17.2
Leaf	2.8	3.4
Seed	2.4	17.2
Whole plant	2.0	31.0

Table 4. Hydrogen Peroxide Scavenging Effects on *Piper Longum Linn*

Plant part/ control	UV Asorption value	% of H_2O_2 Scavenging Activity
Control	2.9	
Root	2.2	24.1
Leaf	2.9	0
Stem	2.9	0
Whole plant	2.6	10.3

Effect of plant growth promoting *Proteus* spp.on fenugreek

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ABSTRACT

Rhizospheric microorganisms support the growth of host plants by production of phytohormones, N_2 fixation, synthesis of ACC deaminase, phosphate solubilization and also by means of production of antimicrobial metabolites or siderophores. In the present study, effect of rhizospheric *Proteus* spp. was analysed on the growth enhancement of Fenugreek (*Trigonella foenum-graecum* L.), a well known spice with medicinal properties. A bacterial strain *Proteus* spp.was selected as plant growth promoting rhizobacteria on the basis of preliminary screening of plant growth promotion.The bacterial strain *Proteus* spp. was Gram negative, motile, and positive for catalase and oxidase. Strain was positive for IAA, Ammonia, ACC deamine, siderophore, can solubilize phosphate and fix nitrogen. The plantlets formed from the treatment were statistically analysed for leaf number, shoot length, root length, wet and dry weights. Application of *Proteus* spp. in Fenugreek seedlings significantly increased shoot length (3.2 fold), root length (2.6 fold) dry weights (1.19 fold) and wet weights (1.5 fold), when compared with the control. The obtained results are of great significance as it widens the exploration of rhizosperic bacteria on enhanced growth of medicinal plants.

Keywords

Plant growth promotion (PGP), Fenugreek, Rhizosphere, Indole 3 Acetic Acid (IAA).

1. Introduction

Bacterial strains isolated from the rhizosphere hold great promise as seed inoculants in new agricultural systems to promote plant growth and yield. These bacteria are termed as plant growth promoting rhizobacteria (PGPR), to accentuate their intimate association with roots. Studies with PGPR indicate that the root microflora can profitably be manipulated through use of bacteria which live as epiphytes on roots and beneficially modulate the ecological niche. The direct application of microorganisms to seed or other plant parts give them a competitive advantage over pathogens that must compete for nutrients and sites for attachment prior to infection. Routine use of biological systems in controlling plant diseases and high yield have become more attractive due to the added benefits of enhanced plant growth. Keeping this in view an experiment was conducted to study the effect of (PGPR) on growth and yield of fenugreek. Rhizospheric bacteria have been reported to be equipped with the potential for nutrient cycling, production of antiphytopathogenic chemicals and plant growth regulators (Khalid et al. 2004). In other words they have direct effect on plants through the production of phytohormones, N_2 fixation, synthesis of ACC (1-aminocyclopropane-1-carboxylate) deaminase, phosphate solubilization and indirect effects by means of production of antimicrobial metabolites or siderophores. The extent to which these bacteria can be exploited for the growth modulation of medicinal plants is least explored.

Fenugreek (*Trigonella foenum-graecum*) is a self pollinated crop belongs to family *fabaceae*. The major fenugreek growing states are Rajasthan, Gujarat, Madhya Pradesh, Tamil Nadu and Uttar Pradesh. More than 80 per cent area and production of India is contributed by Rajasthan state alone as fenugreek is fairly tolerant to salinity which makes it suitable for cultivation in major parts of the state. The crop has immense medicinal value and is a good source of vitamins, protein and essential oils. It is mainly used for culinary and medicinal purposes and continues to be important winter season legume spice mainly cultivated in India. Being an important *rabi* spice crop, farmers largely include it in their cropping plan. The seeds contain an alkaloid "Trigonellin (0.12 to 0.38%) is thought to reduce glycosuria in diabetes. Fenugreek seed helps not only reducing blood sugar levels with its high concentration of phytochemicals, but also reduced low density cholesterols and triacylglycerols. The productivity of the crop is below potential due to variety of reasons including environmental factors. Fenugreek (*Trigonella foenum-graecum* L.) is grown as a spice in most parts of the world and is an important source of steroidal sapogenins such as diosgenin which are used extensively by both pharmaceutical and nutraceutical industries. Diosgenin is often used as a raw precursor for the production of steroidal drugs and hormones such as testosterone, glucocorticoids and progesterone. Natural diosgenin is mainly isolated from the tubers of certain wild species of Mexican yam (*Dioscorea* species). However this process is both time consuming and costly, requiring several years for the yam tubers to grow to a size to possess significant concentration of diosgenin to be used for commercial and pharmaceutical purposes (McAnuff et al. 2005; Obour et al. 2015). Hence fenugreek may be a viable alternative for production of diosgenin because of its shorter growing cycle and lower production costs. So enhanced growth of *T. foenum-graecum* can have important applications.

Proteus species with rhizospheric origin can thus offer highly promising agronomical application as plant probiotics and their long term survival in the host is of great significance to be exploited. Due to their remarkable impact on plant physiology, this organism is well expected to have modulatory effect on plant growth. Hence rhizospheric *Proteus* species isolated from our previous study were investigated for the enhanced growth of *T. foenum-graecum*.

2. Materials and methods

Bacterial strains, culture conditions, media and treatment

Proteus species already isolated from Western Ghats forest, Konni, Pathanamthitta having many plant growth promoting properties were used in this study.

50 ml nutrient broth supplemented with 0.2% tryptophan was prepared in a conical flask. *Proteus* spp. was inoculated into it and incubated at room temperature with continuous shaking. One flask with sterile media was taken as control. After 10 days of incubation the culture supernatant of *Proteus* spp. was taken by centrifugation.10 Fenugreek seeds were surface sterilized with 2% sodium hypochlorite for 15 minute and 70% ethanol for 10 minute(Jasim et al. 2014). Then 6 times washed with sterile distilled water and soaked in sterile distilled water for 24 hours.

50 ml of 2% agar was prepared in a wide mouth flask (250 ml) and sterilized. 10 sprouting seedlings were placed on the flask. Then 200 µl of culture supernatant of *Proteus* spp. was applied to seedlings in triplicate to each seedlings in flask. Uninoculated media was taken as control and was used for treating the seeds in control flask. After 4 days the seedlings were transferred to another set of wide mouth flask containing 100g sterilized soil. 200µl of *Proteus* spp. supernatant was added to each seed. Control was treated with 200µl uninoculated media. These flasks were kept for 5 days. After each day, 200µl sterile distilled water was added to each seedling. After 5 days of growth, the plantlets were removed from flask and their shoot length, root length, leaf number, and fresh weight were measured for statistical analysis. Then the plantlets were subjected to drying in hot air oven at 65^0C for 2 hours. Dry weight of plantlets was measured.

Statistical analysis

The results were analyzed by statistical program IBM SPSS Statistics 20. One way ANOVA performed for comparison among groups. By using Post hoc multiple comparison test the significant difference among groups were determined.

3. Results and Discussion

Effect of isolates on growth promotion

Proteus spp. showed significant effect in increasing the biomass of fenugreek plant. This isolate can produce

IAA, Siderophore, ACC Deaminase, Ammonia and can solubilize phosphate and fix nitrogen. The study was carried out on the basis of comparison of different parameters like leaf number, shoot length, root length, wet weight and dry weight of the seedlings after treatment(Figure 1). Shoot length, root length and wet weight showed significant difference when compared with control(Figure 2 & 3).

The highest mean shoot length, 3.239±1.95 cm, was showed by the plants treated with *Proteus* spp. The root length is higher in the case of *Proteus* spp. with a mean root length of 1.0527±0.695 cm. The higher mean wet weight was also showed by *Proteus* spp. treated plants. 0.0093±0.0017g was the highest mean dry weight, which was showed by the plants treated with *Proteus* spp(Table1).

The yield and plant growth enhancement effects of bacteria used in this study on *T. foenum-graecum* could be explained with N2-fixing capacity, IAA production, phosphate solubilization, ACC deaminase, siderophore and ammonia. The positive effects of PGPR on the yield and growth of crops such as apricot, tomatoes, sugar beet, and barley were explained by N2 fixation ability and antimicrobial substance production (Esitken et al. 2006; Esitken et al. 2010; Ghorbani et al. 2008; Karlidag et al. 2007; Şahin et al. 2004) and by IAA production in apricot. In the present study, we have also found that the inoculation of *Proteus* spp. increased yield and growth in fenugreek.

Proposed roles for bacterial IAA synthesis include the determination of rooting capacity(Foga�a and Fett-Neto 2005) , the stimulation of the release of plant metabolites (Lambrecht et al. 2000), and the promotion of root elongation and shoot growth of inoculated plants (Gadagi et al. 2004). Application of IAA to P-deficient plants increased the root surface, carbohydrate release and acid-phosphatase activity (Wittenmayer and Merbach 2005). Also, IAA secreted by a bacterium may promote root growth directly by stimulating plant cell elongation or cell division or indirectly by influencing bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Patten and Glick 2002), which is the immediate precursor of the phytohormone ethylene, and thereby prevents the reduction of plant growth-inhibiting levels of ethylene (Penrose et al. 2001). Also, IAA production contributes to the colonization efficiency and to the growth and survival of bacteria on its host plants (Vandeputte et al. 2005). The presence of high number of bacteria in the rhizosphere is undoubtedly also important, since they may convert organic and inorganic substances into available plant nutrients. IAA dependent growth promotion mechanisms have previously been described in many cases (Jimtha et al. 2014). In the current study, Tryptophan based IAA production analysis showed the isolate *Proteus* spp. observed to influence the enhancement of growth of fenugreek seedlings. In addition to IAA concentration and its synergistic effect with other plant growth promoting factors might have involved in growth promotion of fenugreek seedlings.

PGPRs are getting more attraction in the development of methods for sustainable agriculture. Increased use of biofertilizers with PGPR demand identification of more potent organisms with adaptive mechanisms for various agro-ecological niches and with diverse plant growth promoting and plant protective properties. From this point of view, biodiversity richness of Western Ghat is highly promising as it is very reasonable to consider the evolution of plant beneficial microorganisms along with or before evolution of biodiversity. Hence the potential of microorganisms from such sources will be highly demanding. So the current study was directed towards the microbiome of Western Ghat. The comparatively higher percentage of plant growth promoting properties as identified in the study is highly supportive to the presence and distribution of various plant growth enhancement mechanisms in this organism.

The interactions between plants and rhizobacteria are naturally based on exchanges of information between the plants and the bacteria; it has become one of the most demanding research topics during the last decade. It is well known that some auxins exert positive effects on fruit set and development in various plant species, as strawberry, fig, Citrus and olive (Lange 2005). Thus, producing IAA by *Proteus* spp. may have positive effects on fenugreek seedlings. Our study demonstrates that *Proteus* spp. can promote the growth of fenugreek, specifically, it increases root length, shoot length, dry weight and fresh weight with the bacterial inoculation against the control plants. The results of the present study suggested *Proteus* spp. has a great potential to increase the yield, growth of fenugreek seedlings. The production of plant growth hormones has been suggested as one of the mechanisms by which PGPRs stimulate fenugreek seedlings growth. The growth-promoting effect appears to be direct, with possible involvement of the plant growth regulators indole-3-acetic acid. In view of environmental pollution due to excessive use of fertilizers and high costs of the production of fertilizers, PGPR strains tested in our study have potential to be used for the sustainable and environmentally benign horticultural production.

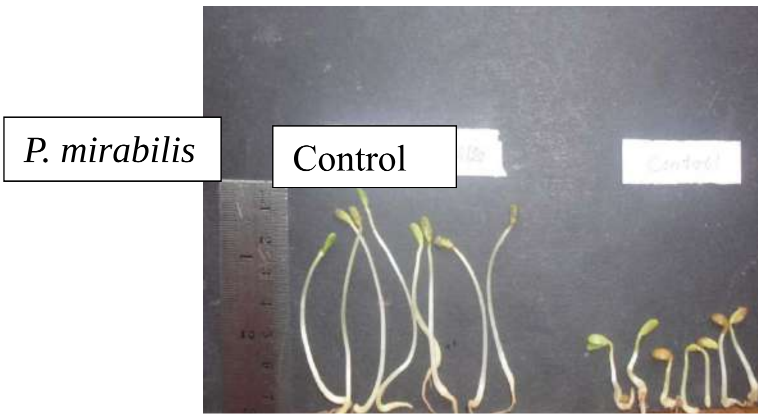

Fig. 1- Plantlets showing plant growth enhancement property when treated with the supernatant of *Proteus* spp. along with control.

	Leaf Number	Shoot length (cm)	Root length (cm)	Wet Weight (g)	Dry Weight (g)
Control	0.833± 0.543a	1.0247±0.678a	0.4130±0.178a	0.0636±0.0161a	0.0078±0.0004a
Proteus mirabilis	0.767±0.274a	3.239±1.95	1.0527±0.695	0.0977±0.036	0.0093±0.0017

Table. 1 Comparative analysis of variation in shoot length, root length, wet and dry mass of fenugreek seedlings under the influence of *Proteus* spp. along with control. Data represented as mean ± SE. n = 10. Mean values followed by the same alphabet are not significantly different by Duncan's multiple range test at *P≤0.05.

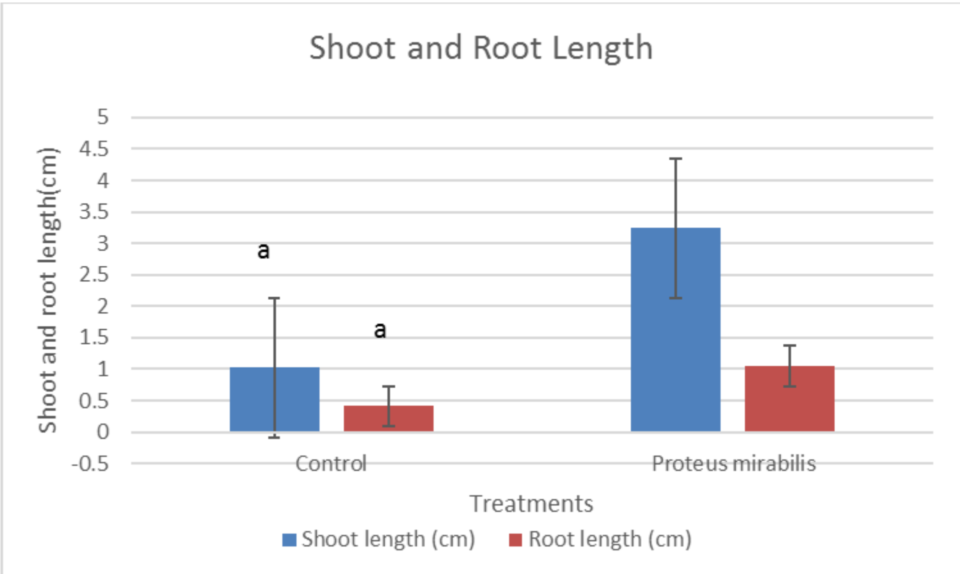

Fig. 2- Graph showing the effect of *Proteus* spp. on shoot and root length of plantlets used for plant growth promotion. Data represented as mean ± SE. n = 10. Mean values followed by the same alphabet are not significantly different by Duncan's multiple range test at *P≤0.05.

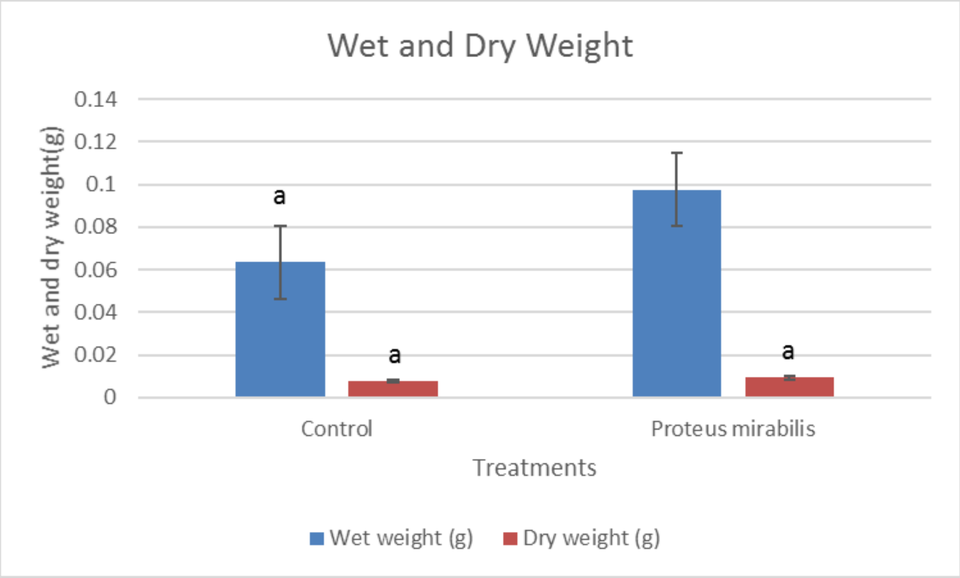

Fig. 3- Graph showing the effect of *Proteus* spp. on wet and dry weight of plantlets used for plant growth promotion. Data represented as mean ± SE. n = 10. Mean values followed by the same alphabet are not significantly different by Duncan's multiple range test at *P≤0.05.

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A study on the antibiotic sensitivity test of selected isolates from facial microflora and their susceptibility to commercial face washes

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ABSTRACT

The skin is a milieu for controlled bacterial growth and it supports the growth of commensal bacteria, with specific genera populating various body regions during particular periods in an individual's life. The resident micro biota on the skin play a major role in the delicate balance which if disrupted can make the skin susceptible to pathogens. The present study was on isolation of facial micro flora followed by their susceptibility testing to some common antibiotics and face washes available in market. About 23 different strains were isolated from facial microflora and the major isolates were tentatively identified to be Staphylococci, Bacillus, Pseudomonas, *E. coli* and Micrococci. Antibiotic sensitivity tests showed that almost all the isolates were susceptible to the common antibiotics with a few exceptions. Antibacterial activity of five face washes *viz*., GARNIER, EVERYOUTH, HIMALAYA, PEARS and VIVEL against the isolated microflora showed that the VIVEL brand was the most potent among the five and could inhibit all the isolates at the dilution tested.

Keywords:

Facial microflora, Antibiotic sensitivity, Antibacterial activity, disc diffusion method

1. Introduction

Skin provides good example of various microenvironments with varying amounts of moisture, body temperatures, and greater concentrations of surface lipids. The skin is a milieu for controlled bacterial growth and it supports the growth of commensal bacteria, which protect the host from pathogenic bacteria. The abundance and types of microbes on the skin (Roth and James, 1989; Fredricks *et al.*, 2001; Hadaway, 2003), is attributed to the skin being the primary external coating of the human body which is exposed to the environment. Human skin has a variety of mechanisms for interacting with microorganisms, which promote the propagation of certain organisms while attacking others. The relationship between microorganisms and human skin is complex with some microorganisms being friendly residents, while others are harmful pathogens. The number of bacteria on an individual's skin remains relatively constant; bacterial survival and the extent of colonization probably depend partly on the exposure of skin to a particular environment and partly on the innate and species-specific bactericidal activity in skin. Also, a high degree of specificity is involved in the adherence of bacteria to epithelial surfaces.

Skin being the primary external coating of the human body is exposed to the environment and is inhabited by a number of bacteria. The majority of the skin microbes are found in the first few layers of the epidermis which is the outermost layer of skin and in the upper regions of the hair follicles. The bacteria found here are mostly commensals whose association is beneficial for the microbe and not harmful to the human. Some of the best-studied long-term and transient bacterial residents isolated from the skin include those from the genera Staphylococcus, Corynebacterium, Propio - nibacterium, Streptococcus and Pseudomonas (Cogen *et al.*, 2008). They are part of the natural environment of the skin and as such are generally benign. The number of bacteria identified from human skin has expanded significantly, and will probably continue to increase as genotyping techniques advance (Gao *et al.*, 2007; Dekio *et al.*, 2005).

Many commensal microbes inhabit our facial skin. The skin microflora acts as a protective mechanism by colonizing the skin and restricting the colonization by other, hostile microorganisms by competitive exclusion. The environment of the skin also predisposes the skin to selective colonization. Glands of the skin secrete compounds called fatty acids. Many organisms will not tolerate these fatty acids. But, the normal microflora of the skin is able to tolerate and grow in the presence of the fatty acids. Also, sweat contains a natural antibiotic known as dermicidin. The normal flora appears to be more tolerant to dermicidin than the invading microbes. Thus, the presence of a normal population of microorganisms on the skin is encouraged by the normal physiological conditions of the body.

In contrast to the protection they bestow, skin microorganisms can cause infections if they gain entry to other parts of the body, such as through cut or during a surgical procedure, or because of a malfunctioning immune system. Bacteria and other microbes that are normal residents of the skin cause some six to ten percent of common hospital-acquired infections.

Fortunately, these problematic bacteria can be easily removed by washing with ordinary soap. However, washing using harsh soaps, or very frequent washing can increase the acidity of the skin, which can counteract some of the protective fatty acid secretions. Also the physical act of washing will shed skin cells. If washing is excessive, the protective microflora will be removed, leaving the newly exposed skin susceptible to colonization by another, potentially harmful microorganism. Health care workers, who scrub their hands frequently, are prone to skin infections and damage. Many hand and face washes are now available with antimicrobial properties capable of acting against such infections.

High quality cleansing products are designed to remove impurities, infective bacteria, make-up and sloughing skin cells without irritating or stripping the skin. Soaps are the combination of fats and oils (of animal or vegetable origin) and salt. Although fats and oils are general ingredient of soaps but some detergents are added to enhance the antibacterial activities of soaps (Friedman and Wolf, 1996). Soaps play an important role in removing and killing bacteria and antibacterial soaps can remove 65 to 85% bacteria from human skin (Osborne and Grube, 1982). Transient bacteria deposited on the skin surface from environmental sources and which cause skin infections including *Pseudomonas aeruginosa* (Fluit *et al*., 2001), *Staphylococcus aureus* (Higaki *et al*., 2000), have been shown to be more susceptible to soaps containing antimicrobial active ingredients as compared to plain soap (Lucet *et al*., 2002). With the use of antibacterial soaps, removal as well as killing of bacteria is effected and antibacterial soaps are thus considered to be more effective than beauty (plain) soaps and deodorant (Toshima *et al*., 2001).

In the present study, an attempt was made to isolate and characterize a few common members of facial microflora. Identification of bacteria was done by biochemical tests followed by examining their susceptibility towards a few selected antibiotics. The study also attempted to investigate the antibacterial efficiency of different brands of face washes obtained in the market.

2. Materials and Methods

2.1 Isolation of micro-organisms from face

Sterile swab sticks damped with sterile saline were used to collect samples from face, which were inoculated into nutrient agar plates and incubated at 37^0C for 24 hours. The most prominent isolates were selected, Gram stained, and then inoculated onto slants containing nutrient agar. The colony characters were recorded and further identification of the organisms was carried out by biochemical characterization.

2.2 Identification of isolated organisms

Identification of bacteria was done by using different biochemical tests based on the gram stain reaction of bacterial strains. Different biochemical tests such as Indole Test, Methyl Red Test, Voges Proskauer Test, Citrate Utilization Test, Urease Test, Catalase Test etc. were performed to identify the isolated microorganisms. Special media like Cetrimide agar and Mannitol Salt agar were employed for the identification of specific organisms.

2.3 Antibiotic sensitivity test of selected isolates

Antibiotic sensitivity of the isolated facial micro flora against five common antibiotics namely, penicillin, erythromycin, tetracycline, chloramphenicol and bacitracin was performed using disc diffusion method. 0.1ml of selected bacterial culture was uniformly spread on nutrient agar plates to prepare lawn culture. Sterile antibiotic discs were placed on the surface of nutrient agar plates at a distance of 2 cm using a sterile forceps. The plates were incubated at 37^0C for 24 hrs and after incubation, zone diameter was measured.

2.4 Antibacterial activity of Face Wash

Face washes of different brands like Garnier, Vivel, Ever youth, Himalaya and Pears were purchased from local super market and used to compare the antibacterial activities according to the standard method. The single dilution was prepared by dissolving 1 ml of face wash in 10 ml sterile distilled water and used for the test. Nutrient agar plates were prepared and 0.1ml of selected bacterial culture was uniformly spread on nutrient agar plates to prepare lawn culture. After solidification, wells were made and each well incorporated with 20 to 30 µl of diluted face washes followed by incubation at 37^oC for 24 hours. The zone of inhibition was determined by measuring the diameter in millimetres of zone to which the face washes inhibited the growth of the organism. After incubation the plates were observed and the results were recorded.

3. Results and Discussion

3.1 Isolation and identification of micro-organisms from face

About 23 different strains were isolated from facial microflora of different volunteers. They were cultured onto nutrient agar plates and after overnight incubation, the colony characters were noted followed by gram staining reaction (Table 3.1.1).

Isolate	Colony characters	Gram staining reaction
1	pale, dry, flat	Gram negative rod
2	mucoid, circular, raised	Gram positive cocci
3	Yellow, flat	Gram positive cocci
4	pale, dry, flat	Gram positive rod
5	yellow, flat	Gram positive rod
6	medium, raised	Gram positive rod
7	yellow, flat	Gram positive rod
8	raised, opaque	Gram negative rod
9	transparent, flat	Gram positive rod
10	opaque, irregular, raised	Gram positive rod
11	opaque, irregular, flat	Gram negative rod
12	transparent, dry, flat	Gram positive rod
13	opaque, pin point, raised	Gram positive cocci
14	Irregular, opaque, flat	Gram negative rod
15	yellow, opaque, mucoid, raised	Gram positive cocci
16	round, mucoid, opaque, raised	Gram positive cocci

17	round, mucoid, yellow, raised	Gram positive cocci
18	round, pin point, opaque, raised	Gram positive cocci
19	large, irregular, opaque, dry, flat	Gram negative rod
20	small, round, raised, opaque, dry	Gram negative rod
21	small, round, white, opaque, dry	Gram positive cocci
22	yellow, raised, small, mucoid	Gram positive cocci
23	irregular, raised, opaque, dry	Gram negative rod

Table 3.1 Colony characteristics of isolated facial microflora

The different strains were further characterized by specific biochemical tests and were tentatively identified as shown in Table 3.2.

Isolate			BIOCHEMICAL TEST						
	Gram stain	**Motility**	**Indole**	**MR**	**VP**	**Citrate**	**Catalase**	**Urease**	**Tentative identification**
1	Gram negative rod	motile	-	+	-	+	+	-	*Pseudomonas
2	Gram positive cocci	non motile	-	-	+	-	+	+	*Micrococci
3	Gram positive cocci	non motile	-	+	-	-	+	-	*Staphylococci
4	Gram positive rod	motile	-	+	-	+	+	-	*Bacillus
5	Gram positive rod	motile	-	-	-	+	+	+	*Bacillus
6	Gram positive rod	motile	-	-	-	+	-	+	*Bacillus
7	Gram positive rod	motile	-	+	+	-	+	+	*Bacillus
8	Gram negative rod	motile	+	+	-	-	+	-	*E. coli*
9	Gram positive rod	motile	-	+	+	-	+	-	*Bacillus
10	Gram positive rod	motile	-	-	+	-	+	-	*Bacillus

11	Gram negative rod	motile	-	+	_	+	+	-	*Pseudomonas
12	Gram positive rod	motile	-	-	-	+	+	+	*Bacillus
13	Gram positive cocci	non motile	-	+	-	-	+	-	*Staphylococci
14	Gram negative rod	motile	+	+	-	-	+	-	*E. coli*
15	Gram positive cocci	non motile	-	-	+	-	+	+	*Micrococci
16	Gram positive cocci	non motile	-	-	-	+	+	-	*Staphylococci
17	Gram positive cocci	non motile	-	-	-	+	+	-	*Staphylococci
18	Gram positive cocci	non motile	-	-	-	+	+	-	*Staphylococci
19	Gram negative rod	motile	-	+	-	+	+	-	*Pseudomonas
20	Gram negative rod	motile	-	+	-	+	+	-	*Pseudomonas
21	Gram positive cocci	non motile	-	+	-	-	+	-	*Staphylococci
22	Gram positive cocci	non motile	-	+	-	-	+	-	*Staphylococci
23	Gram negative rod	motile	-	+	-	+	+	-	*Pseudomonas

Table 3.2 Biochemical characteristics of isolated facial microflora

Special media like Cetrimide agar and Mannitol Salt agar were employed for the identification of Pseudomonas and Staphylococcus species respectively. Cetrimide agar contains the selective agent, cetrimide, which inhibits the growth of other bacteria and enhances the production of Pseudomonas pigments such as pyocyanin and so is used for the selective isolation of *Pseudomonas aeruginosa*. However there was no characteristic blue-green or yellow-green colour which rules out the isolate being *Pseudomonas aeruginosa*.

Mannitol salt agar or MSA contains a high concentration(~7.5-10%) of salt (NaCl), as well as mannitol with phenyl red as indicator, thus being employed as selective media for gram positive *Staphylococci* which can survive at the high salt concentration unlike most other bacteria. Mannitol fermentation ability of Coagulase-positive *Staphylococci* resulted in yellow colonies with yellow zones, whereas non-mannitol fermenting and coagulase-negative *Staphylococci* produced small pink or red colonies with no color change to the medium. Among the isolates, it could be identified that facial microflora comprised of both mannitol fermenting *Staphylococcus aureus* and non-mannitol fermenting *Staphylococcus epidermidis.*

3.2 Antibiotic sensitivity test of selected isolates

The major isolates from facial microflora tentatively identified by biochemical tests as well as use of selective media were Staphylococci, Bacillus, Pseudomonas, *E. coli* and Micrococci. These were subjected to antibiotic sensitivity test against five common antibiotics namely, penicillin, erythromycin, tetracycline, chloramphenicol and bacitracin using disc diffusion method (Table 3.3).

Isolate	**ANTIBIOTIC DISC**				
	Erythromycin	**Tetracycline**	**Chloramphenicol**	**Bacitracin**	**Penicillin**
Pseudomonas	1.2	1.8	2.3	1.7	1.5
Micrococci	0.6	1.6	2.9	**R**	**R**
Staphylococci	2.6	2.8	3	1.4	1.5
Bacillus	2.3	1.5	2.5	0.8	**R**
Bacillus	1	2	2.2	1.6	0.9
Bacillus	2	1.9	2.4	1	**R**
Bacillus	1.2	1.8	2.2	1	1
E. coli	2.9	1.8	2.2	1.5	**R**
Bacillus	1.1	2.6	1.5	1.2	1.2
Bacillus	1.1	1.8	2.3	1	**R**
Pseudomonas	0.7	1.8	2.2	**R**	1
Bacillus	1	1.8	2.1	1.8	**R**
Staphylococci	1	2	2.2	1.2	1
E. coli	2	1.1	2.7	1.2	**R**
Micrococci	1	1.3	2.1	**R**	**R**
Staphylococci	**R**	2	1.8	1	**R**
Staphylococci	2.8	2.4	2.9	1.9	4.5

Staphylococci	2.4	2.5	2.9	1.7	2.2
Pseudomonas	1.7	2.3	2	1.8	1.9
Pseudomonas	3.1	2.5	3	1.3	3.8
Staphylococci	3.1	2.5	4	1.2	3
Staphylococci	3	1.5	3.3	1.2	1.2
Pseudomonas	0.8	2.1	2.4	1	0.9

Table 3.3 Antibiotic sensitivity test of isolated facial microflora

Antibiotic sensitivity tests showed that almost all the isolates were susceptible to the common antibiotics with a few exceptions. These included Micrococci which were resistant to Bacitracin and Penicillin, Bacillus and *E. coli* which were resistant to Penicillin, Pseudomonas which was resistant to Bacitracin and Staphylococci which was resistant to Erythromycin and Penicillin. The facial micro flora being an easy target for becoming vulnerable opportunistic pathogens, antibiotic resistance is a factor that needs special consideration while studying them.

3.3 Antibacterial activity of Face Wash against isolated facial microflora

The facial microflora isolates tentatively identified by biochemical tests as well as use of selective media comprising Staphylococci, Bacillus, Pseudomonas, *E. coli* and Micrococci were subjected to antibacterial activity of Face Washes and the results tabulated (Table 3.3.1).

Isolate	**FACEWASH**				
	GARNIER	**VIVEL**	**EVERYOUTH**	**HIMALAYA**	**PEARS**
Pseudomonas	1.2	2.5	1.5	1.3	1.8
Micrococci	**R**	2.3	**R**	**R**	1.9
Staphylococci	1.1	2.3	1.1	1.2	1.5
Bacillus	1.2	2.8	2	2.5	1.8
Bacillus	1.2	2.5	1.5	1.3	1.8
Bacillus	**R**	2.3	**R**	**R**	1.5
Bacillus	1.5	1.4	1.5	1.5	1.5
E. coli	1.5	2.6	2	2.3	2.5
Bacillus	1.4	2.5	2	2.5	3
Bacillus	1.1	1.8	2.3	1	**R**
Pseudomonas	0.7	1.8	2.2	**R**	1
Bacillus	1	1.8	2.1	1.8	**R**
Staphylococci	1	2	2.2	1.2	1
E. coli	2	1.1	2.7	1.2	**R**
Micrococci	1	1.3	2.1	**R**	**R**
Staphylococci	1	2.1	1.3	1.5	1.9
Staphylococci	1.2	2.1	1.3	1.9	2
Staphylococci	**R**	1.7	**R**	1.5	1.2
Pseudomonas	**R**	1.6	**R**	1.5	1.8
Pseudomonas	1.5	2.5	1.6	2.2	2.2

Staphylococci	2.9	2.8	2.9	2.9	3.6
Staphylococci	2.5	2.1	2.5	2.7	2.7
Pseudomonas	1.2	2.6	1.3	1.1	1.6

Table 3.4 Antibacterial activity of Face Wash against isolated facial microflora

The five antibacterial face washes *viz*., GARNIER, EVERYOUTH, HIMALAYA, PEARS and VIVEL which were tested, had varying activities against the isolated microflora. The single dilution prepared and added into the wells cut in the nutrient agar plates was found in most cases, to be potent against bacterial growth. In few instances, the isolates showed resistance which were as follows:

- Micrococci - Resistant against GARNIER, EVERYOUTH, HIMALAYA, PEARS
- Bacillus - Resistant against GARNIER, EVERYOUTH, HIMALAYA, PEARS
- Pseudomonas- Resistant against HIMALAYA, GARNIER, EVERYOUTH
- *E. coli* - Resistant against PEARS
- Staphylococci - Resistant against GARNIER EVERYOUTH

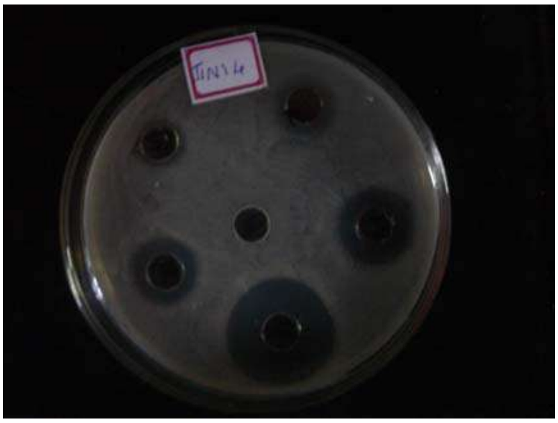

Fig 3. 1 Antibacterial activity of Face Wash against isolated facial microflora

Plate 1 – complete inhibition as against water as control

Plate 2 – 2 isolates resistant and 3 sensitive as against water as control

The VIVEL brand was found to be inhibiting all the isolates at the dilution tested and could be selected as the most potent among the five. This could be an interesting observation which has to be studied in detail with regard to its MIC, MBC etc. So also, similar to the results in antibiotic sensitivity tests, Micrococci were found to be resistant to all the four brands tested except VIVEL and this could also be a potentially interesting observation for further studies.

4. Conclusions

The complex host– microbe and microbe–microbe interactions that exist on the surface of human skin illustrate that the microbiota have a beneficial role in resisting infections by pathogenic microbes. The present studies on isolation of facial micro flora followed by their susceptibility testing to some common antibiotics and face washes available in market, is of considerable merit since an overuse of antimicrobial agents may disrupt the delicate balance of the cutaneous microflora leaving the skin susceptible to pathogens kept in control by the resident micro biota.

The major findings of the study included,

- About 23 different strains were isolated from facial microflora of different volunteers and the major isolates were tentatively identified to be Staphylococci, Bacillus, Pseudomonas, *E. coli* and Micrococci.
- Antibiotic sensitivity tests showed that almost all the isolates were susceptible to the common antibiotics with a few exceptions. These were Micrococci which were resistant to bacitracin and penicillin, Bacillus and *E. coli* which were resistant to penicillin, Pseudomonas which was resistant to Bacitracin and Staphylococci which was resistant to erythromycin and penicillin.
- Antibacterial activity of five face washes *viz*., GARNIER, EVERYOUTH, HIMALAYA, PEARS and VIVEL showed that the VIVEL brand was the most potent among the five and could inhibit all the isolates at the dilution tested.
- Some isolates showed resistance to different face washes and they were Micrococci - Resistant against GARNIER, EVERYOUTH, HIMALAYA, PEARS, Bacillus- Resistant against GARNIER, EVERYOUTH, HIMALAYA, PEARS, Pseudomonas- Resistant against HIMALAYA, GARNIER, EVERYOUTH, *E. coli* - Resistant against PEARS and Staphylococci - Resistant against GARNIER EVERYOUTH.
- However, the majority of isolates could be found to be inhibited by one face wash or the other and the antibacterial activity of these products could be termed satisfactory.

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Bioactive peptides derived from milk: a review

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ABSTRACT

Milk is an excellent source of well-balanced nutrients and also exhibits a range of biological activities that influence digestion, metabolic responses to absorbed nutrients, growth and development of specific organs, and resistance to disease. These biological activities are mainly due to the peptides and proteins in milk. Bioactive peptides are produced during digestion of milk in the gastrointestinal tract, fermentation and food processing. The beneficial health effects may be classified as antimicrobial, antioxidative, antihypertensive and immunomodulary. The present review summarizes isolation of bioactive milk-derived peptides along with their physiological functions.

Key words:

Bioative peptide, antimicrobial, antioxidative, antihypertensive and immunomodulary

Introduction

Milk and derived dairy products are considered as an important constituent of a balanced diet. Moreover, it is a source of many bioactive components, such as high-quality proteins, lipids, carbohydrates, lactose, vitamins, minerals, enzymes, hormones, immunoglobulins, and growth factors, These components help to meet human nutritional requirements and also play a relevant role in preventing various disorders such as hypertension and cardiovascular diseases, obesity, osteoporosis, dental caries, poor gastrointestinal health, colorectal cancer, ageing, and others (Chia-Chein*et al*., 2015).

Milk is a rich source of protein. Bioactive peptides derived from milk proteins, especially caseins and whey proteins are an important source of these compounds. Caseins comprises about 80 percent of the total protein content in bovine milk and are divided into α-, ß- and κ-caseins(Nagpal*et al*., 2011). Whey protein is composed of *β*-lactoglobulin, α-lactalbumin, immunoglobulins, and minor proteins such as lactoperoxidase, lysozyme and lactoferrin.

Milk proteins can be degraded into numerous peptide fragments by enzymatic proteolysis and serve as source of bioactive peptides, may affect the major body systems—namely, the cardiovascular, digestive, immune and nervous systems. During recent years, major whey protein components, *α*-lactalbumin and *β*-lactoglobulin, were also shown to contain bioactive sequences (Srikantsharma*et al.,* 2011).

2. Isolation of bioactive peptide from milk

Bioactive peptides are defined as specific protein fragments that have a positive impact on the functioning or conditions of living beings, thereby improving their health (Nagpal *et al*., 2011). These are derived from milk proteins by different methods like enzymatic breakdown by digestive enzymes or by the proteinase enzymes produced by lactobacilli during the fermentation of milk. Milk-derived bioactive peptides are usually comprised of 2–20 amino acids and become active after release from the precursor protein where they are encrypted either by digestion proteolysis both in vivo or in vitro (Mohanthy *et al*., 2016). Bioactive peptides may be released *in vivo* during gastrointestinal digestion as a result of the degradation of casein with severalproteases such as pepsin; trypsin or chymotrypsin Regulatory peptides can be released by enzymatic proteolysis of food proteins and may act as potential physiological modulators of metabolism during the intestinal digestion of the diet. The possible regulatory effects of peptides relate to nutrient uptake, immune defense, opioid and antihypertensive activities (Kay 2012).

2. Physiological effects of bioactive peptide

2.1. *Effects on cardiovascular system*

Acute and chronic cardiovascular events may result from alterations in the activity of the renin-angiotensin aldosterone system and activation of the coagulation cascade and of platelets. Medications that inhibit angiotensin converting enzyme

(ACE) are widely prescribed in the treatment and prevention of cardiovascular disease. ACE inhibitor peptides are of particular interest due to the presence of encrypted inhibitory peptide sequences. In particular, Ile-Pro-Pro and Val-Pro-Pro are fore runners in ACE inhibition, and have been incorporated into commercial products (Fissesha T Welderufael *et al*., 2012).

2.2. *Bioactive peptide act as anti-hypertensive*

ACE is a multifunctional enzyme that is located in many tissues and plays an important role in blood pressure regulation and in turn hypertension. Therefore, ACE inhibition mainly results in a hypotensive effect. Recent research has shown that enzymatic digestion of casein and whey proteins generate bioactive peptides that have the ability to inhibit ACE. The best known ACE-inhibitory peptides, Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) with IC 50 values (concentration of peptides mediating 50% inhibition of ACE activity) of 9 and 5 µMoles respectively have been identified from a Japanese sour milk drink(Prasad patel et al., 2015).

2.3. *Bioactive peptide act as anti-inflammatory*

Bioactive peptides with known anti-inflammatory effects are potential candidates for improving endothelial dysfunction. Studies were carried out to check the effects of milk-derived bioactive peptides on the expression of the inflammatory phenotype of human endothelial cells and their effects on monocyte adherence to endothelial cells. These results suggested that milk-derived bioactive peptides work as anti-atherogenic agents through the inhibition of endothelial-dependent adhesive interactions with monocytes by inhibiting the NF-κB pathway through a PPAR-γ dependent mechanism.

Peptide-rich milk protein hydrolysates have shown anti-inflammatory effects in cultured endothelial cells and appear to inhibit leukocyte-endothelial interaction which may explain some of their beneficial vascular/endothelial roles in addition to ACE inhibitor effects. While some bioactive peptides such as Ser-Ser-Ser, Glu-Glu-Glu and Val-Pro-Leu have all been shown to attenuate leukocyte-endothelial interactions (SepidehJabbari *et al* 2012).

2.4. *Bioactive peptide act as anti-microbial*

Biologically active peptides produced from milk and milk products have very healthy impact on human physiology and give protection against different type of pathogens. The cell-free supernatants and peptide of *lactobacillus* KSBT46 exhibited antibacterial activity. Lactic acid bacteria have an essential role in most food and beverage fermentation processes. The bacterial isolate have positive impact on their use as starter cultures for traditional fermented foods, with a view to improve the hygiene and safety (Mohanthy *et al*., 2014).

2.5. *Bioactive peptide acts as anti-oxidant.*

Bioactive peptides possess the potential antioxidant property to be used in physiologically functional foods or pharmaceutical industries. Bovine casein hydrolysates were prepared by enzymatic hydrolysis using trypsin alone and with a combination of trypsin and pepsin at different time intervals. Trypsin hydrolysate showed the highest antioxidant activity (13.7 %). Crude casein hydrolysate (CH) was further fractionated into 2 main types by using ultrafiltration. The combined effect of trypsin and pepsin did not prove to be effective in radical scavenging. However, fractionation by ultrafiltration proved to be effective in radical scavenging (SanlidereAloglu *et al.*, 2011)

Goat milk proteins have gained increasing attention especially the bioactive peptides released from the parent proteins by digestive enzymes. Proteins of goat milk were fractionated into caseins (GCP) and whey proteins (GWP), hydrolyzed by pepsin and the generated peptides were examined for radical scavenging activities. The hydrolysates of whey (P-GWP) and casein (P-GCP) proteins exhibited potent superoxide anion ($O_2^{\cdot -}$) scavenging activity in a dose-dependent manner, as investigated using the natural xanthine/xanthine oxidase (X/XOD) system. MALDI-TOF-MS allowed the identification of several antioxidant peptides derived from both caseins and whey proteins, with β-casein and β-lactoglobulin being the major contributors, respectively (Ahemad*et al.,* 2015).

3. Conclusion

Recent research has shown that bioactive peptide have a positive impact on the functioning or conditions of living beings, with antihypertensive, immunostimulating, antimicrobial and antioxidative activity in the human body. Bioactive peptides are encrypted in milk proteins and are only released by enzymatic hydrolysis *in vivo* during gastrointestinal digestion, food processing or by microbial enzymes in fermented products. At present significant research is being undertaken on the health effects of Milk derived peptides. So these Bioactive peptides have the potential to be used in the formulation of health-enhancing nutraceuticals, and as potent drugs with well-defined pharmacological effects.

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