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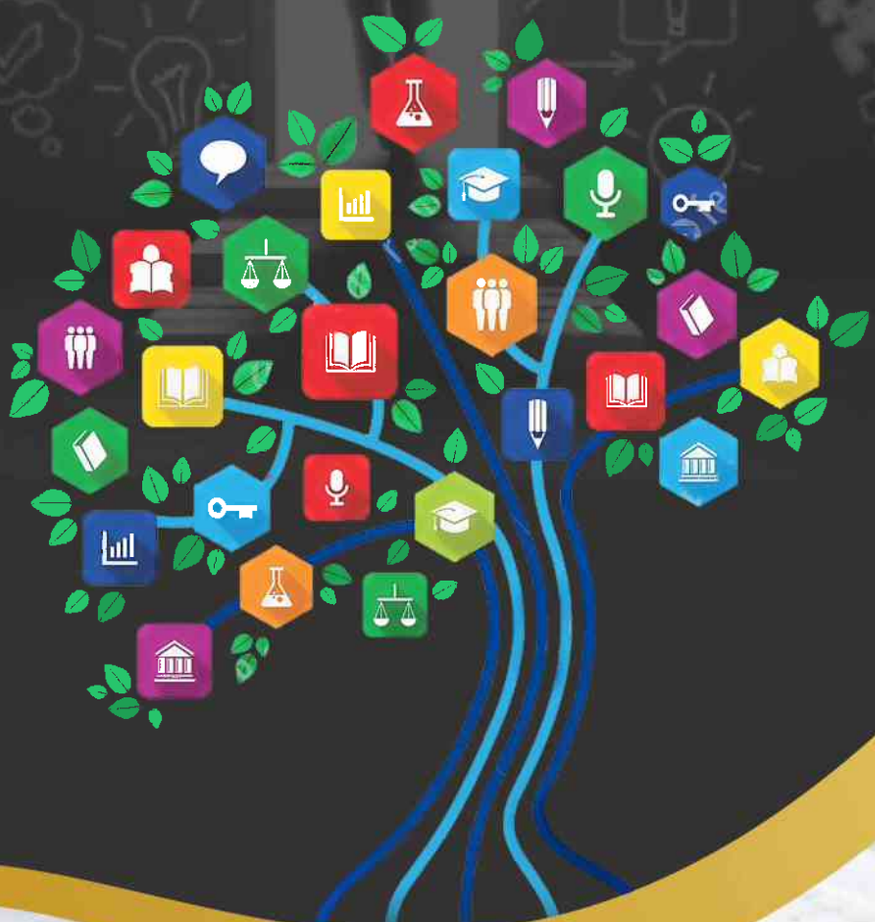

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Asst. Prof. Vinay Shankarrao Hatole
M.Sc (Maths), M.B.A. (Mktg.), M.B.A. (H.R.),
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Cell No. : 9579260877, 9822620877 Ph. No. : (0240) 2400877

E-mail : ajanta5050@gmail.com, www.ajantaprakashan.com

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CONTENTS OF PART - III

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1. Isolation of Microorganism from Raw Milk

Mrs. Sonali Rahul Joshi
Assistant Professor, Microbiology ID, ZSCT's Thakur College of Science,
Kandivali East, Mumbai.
Aditi Tiwari
Student, Microbiology ID, ZSCT's Thakur College of Science, Kandivali East, Mumbai.
Aditya Dhobale
Student, Microbiology ID, ZSCT's Thakur College of Science, Kandivali East, Mumbai.

1. Abstrac

Milk is an important Component of our diet which gives us the strength to build our body and health as it is packed with Calcium, Phosphorus, B vitamins, potassium and vitamin D, it is also filled with protein which helps us to build our bones and structure, even it prevent bone fractures and helps you to maintain a Healthy weight and by preventing osteoporosis, But sometimes it can be contaminated and may be a place for breeding of Microbes like *E.coli, Staphylococcus Aureus, L.acidophilus, Propinobacter, Mycobacterium Tuberculosis* and many more such Harmful microbes. So it is important for us to see which microbes are present in our milk that we consume and that is what motivated us to choose this as our research topic. So three samples were collected from 3 different locations, Then they were diluted using dilution technique and were spread using Spread plate technique on Different agar plates, out of which sample number 2 showed growth on Mannitol Agar plate, which was gram stained and further analysed using biochemical technique and it was found that the microbe we obtained was *Staphlyococcus aureus*, so this paper concludes that there was presence of *S.aureus* in the sample, which can be eliminated by heating and proper pasteurization of milk.

Keywords: Phosphorous, *Staphylococcus aureus*, Pasteurization and *Propinobacter.*

2. Introduction

Raw Milk has been consumed for ages and is still an important entity in terms of our diet, milk is one of the major component consumed in human diet all over the world, but it also serves as an excellent medium of the growth of many microorganisms, especially pathogenic bacteria. Thus, the quality control of milk is considered essential to the health and welfare of a community. (MENNANE *et al.* 2007). Microbes may enter into milk from many sources like

from experiencing clinical or sub clinical mastitis (Shanmugavel Lingathurai. 2010), from farm, any water source and even any utensil or any particular unmaintained storage vessel. (GARIMA GARG *et.al,* AASTHA AGRAWAL et.al, ARPITA MUKHERJEE *et.al.* 2018)

Many Microorganisms are found in raw milk like *Pseudomonas*, *Enterobacteriaceae* and *Bacillus cereus* as spoilage microbes. *Brucella, Staphylococcus aureus, Mybocaterium tuberculosis* and *S. agalactiae* stated as pathogenic microbes with *S. thermophiles*, *S. lactis, S. cremoris and Leuconostoc lactis* grouped as acid fermentating microbes and *L. lactis, L. bulgaricus* and L. acidophilus employed in acid production. (Cristiano CORTES *et.al.* 2021)

3. Composition of Milk

So basically milk contains water with a range of 85.5 percent to 89.5 percentage, with total solid of 10.5 to 14.5 percent, fat with a range of 2.5 to 6.0 percent, proteins with a range of 2.9 to 5.0 percent, lactose with being 3.6 to 5.5 percent and other minerals being in range of 0.6 to 0.9 percent. (Erasmus, 2019)

Milk contains further water than any other element, around 87 for dairy cows. The other rudiments are dissolved, colloidally dispersed, and emulsified in water. The amounts of the main milk ingredients can vary vastly depending on the individual beast, its strain, stage of lactation, age and health status. Herd operation practices and environmental conditions also impact milk composition. (Milk Facts, 2022)

4. Milk Contamination in India

Survey conducted by Consumer Guidance society of India (CGSI), in case of Milk showed us how much there is difference in raw milk and one from branded. So they collected over 413 samples from all over Maharashtra from January to December in the year 2019, and after testing it was found that over 79 percent of all samples were adulterated, out of which 73 were found to be from branded companies and rest of remaining 340 from unbranded sources, and percentage of adulteration in unbranded sources was 78 percent.

With seeing a rise in milk of branded companies seeing a rise of 5 percent increase in adulteration despite of strict regulations being implanted. With being 85 percent of samples being found adulterated and tainted (FOOD navigator-asia.com, 2020)(The Hindu, 2020).

Also our country's food regulator, Food Safety and Standard Authority of India (FSSAI) has discovered and found that nearly about 93 percent of milk samples collected and tested under National Milk and Safety Quality Survey found that contamination in milk can be worrying,

where Out of the total 6,432 samples of milk tested, about 456 were found to failed to land on parameter. Even out of 456 samples, 12 were found to be deliberately adulterated with elements like hydrogen peroxide, urea and detergent(Times of India, 2019).

5. How Milk can get Contaminated

- Animal feces and waste directly coming in contact of milk.
- Mastitis can cause bacterial infection in udder and milk.
- Cow disease like bovine tuberculosis.
- Bacteria that resides on the skin of the animal.
- Other Environmental factors like dust, utensils and equipment's used in processing.
- Lack of sanitation in milk processing plant.
- Roaming and presence of unwanted rodents and insects. (Center for Disease control and prevention, CDC, 2017)

6. Diseases Caused By Contaminated Milk

Food Poisoning

Mainly caused by Salmonella which is the main causative agent of poisoning. According to WHO, World Health Organization they expect it to be around 16 million infections worldwide each year. Basically it can be eliminated by pasteurization. (WHO, 2020)

Diarrhoea

This is a fatal disease caused by *E.coli* which produces Shigatoxin which upon ingestion causes diarrhoea and stomach pain it can be eliminated by simple pasteurization, which if not treated it can cause complications like haemolytic uremic syndrome. (Mayoclinic, 2021)

Urinary Tract Infection

This is caused by *Pseudomonas Aeruginosa*, these are opportunistic pathogens. The species *Pseudomonas fluorescens* is among the most common spoilage agents in milk and mostly found only in milk. The enzymes produced by pseudomonads are heat-stable and they can survive pasteurization to a certain extent. (EHA Consulting group, 2022)

Gastrointestinal Disorders

The bacterium *Bacillus cereus* causes corruption of cream, rubbish and milk and makes them taste rancid, sour or bitter. In addition, it produces poisons which may beget serious gastrointestinal diseases. In February this time, a discounter in Germany had to recall long- life whole milk due to Bacillus cereus impurity. Strict attachment to the surface of the cold chain is

essential to help the bacteria from multiplying. Spores of Bacillus cereus can even survive against heat treatment. (Per Einar Granum *et.al*, 1997)

Botulism

Botulism is poisoning that's due to *Clostridium botulinum* poison and that affects the supplemental jitters. Botulism may do without infection if poison is ingested, fitted, or gobbled. Symptoms are symmetric cranial whim-whams paralysis accompanied by a symmetric descending weakness and limp palsy without sensitive poverties. (Larry M. Bush *et.al*, 2021)

7. How to Get Rid of the Contamination

Pasteurization is that the process of hotting milk to a high enough temperature for an extended enough time to kill illness-causing origins. milk is milk that has skilled this process. (CDC, 2017)

Pasteurization of Milk at Home

Pour the raw milk into the pristine sword pot. However, that will work indeed better to keep the milk from parboiling, if you have a double boiler. However, you can put one pristine sword pot inside a larger pot with many elevation of water at the bottom, if you don't have a double boiler. However, also you'll just need to be careful to heat the milk gently, If you can't achieve this setup.

1. Then slowly heat the milk to 145 degree Fahrenheit, and stir continuously, if you are not using double slider, stir it frequently.
2. Hold the temperature around 145 degree Fahrenheit for 30 minutes. You may even need to increase and decrease temperature to keep temperature constant.
3. Then remove the utensil from heat and place it in a sink or vessel filled with ice. Stir constantly until the temperature is 40 degree Fahrenheit.
4. Store pasteurized milk in the refrigerator. (MotherEarth News, 2022)

8. Methodology

8. a Collection of Samples

- 3 samples were collected from 3 different locations,
- Sample 1 was collected from a local shop,
- Sample 2 was collected from a local dairy farm,
- Sample 3 was collected from home.

8. b Isolation and Enrichment of Samples

Then the samples were diluted and streaked on various sterile nutrient plates and incubated at 37 °Cfor 24 hours.

Growth was observed on sample no. 2 on Mannitol Salt Agar plate which was taken from a local dairy farm.

8.c Identification of Microorganisms by Gram Nature and Biochemical Technique

Morphological and Cultural Identification

Strain of interest was studied and gram stained for its colonies developed on the Mannitol agar were characterised by observing various parameters viz, shape, size, colour, elevation, margin, surface etc.

Biochemical Characterization

Several test were carried out like Oxidase test, Catalase test, H.S production test, Nitrate reduction test, Gelatine Hydrolysis test and Urease test were carried out.

- **Oxidase Test** - Dissolve all the ingredients except the indicator and sugar solution in water. Adjust the ph. Add the indicator and steam sterilize the medium flask at 121^0 C for 30 min. The sugar solution is sterilized separately by filtration and then added as given in the composition. The medium is then distributed in the sterile tubes to depth of about 4 cm in aseptic conditions. . (Microbiologyinfo, 2018)
- **Catalase Test -** Transfer growth from centre of colony on the surface of glass slide. Add on drop 3% H_2O_2 and observe bubble formation. (Student Health Centre Manuals, 2016)
- **H2S production Test -** Firstly inoculate the organism into labeled tube by means of stab inoculation in SIM medium then incubate the inoculated tubes at 37°C for around 24-48 hours then properly observe for the formation of black precipitate on the medium carefully. (Microbionotes, 2018)
- **Nitrate Reduction Test -** At Start Inoculate test organism in nitrate broth then incubate at 37°C for 24 hours then add 1ml of ά -naphthylamine and 1 ml of sulfanilic acid. (Microbiology info.com, 2021)
- **Gelatine Hydrolysis Test -** First inoculate known Gelatinase positive organisms eg. Staphyloccus aureus, Pseudomonas sp. then incubate at 37°C for 24-48 hrs then chill

the tubes at 4°C for 20 minutes and if the medium remain in liquid state, it is a positive test. (Microbesnotes.com, 2020)

- **In IMViC Test -** Indole test, Methyl- red test, Voges-Proskauer Test and Citrate Utilization test were carried out.
- **Indole Test -** First inoculate tryptophan broth with test culture and incubate it at 37°C for around 24 hours then add around 15 drops of Kovac's reagent along the side of the test tube very carefully after that there should be formation of red ring on the top of medium. (Microbiology info.com. 2021)
- **Methyl Red Test** - Before the inoculation, allow medium to equilibrate to room temperature, then using organisms taken from an 18-24 hour culture, slightly inoculate the medium very carefully then incubate it at aerobically at 37 °C for around 24 hours.
- After 24 hours of incubation, aliquot 1ml of the broth to a clean test tube carefully. Then reincubate the remaining broth for more additional 24 hours. Then carefully add 1 to 3 drops of methyl red indicator to aliquot thencarefullyobservefor red color immediately. (Microbeonline.com,. 2021
- **Voges-Proskauer Test** - At first inoculate test organisms in St. Glucose phosphate broth then inoculate at 37°C for 24 hours then, add 0.5 ml of 5% a-naphthol and 0.2 ml 40% KOH, shake gently and carefully. (Onlinebiology.notes, 2018)
- **Citrate Utilization Test -** First prepare Simmon's Citrate agar and pour it in test tube and keep it at a slant position then inoculate test culture on surface of summons's citrate slant by streaking and let it incubate at 37°C. (Microbenotes.com, 2020)
- **Sugar Fermentation Test** - Test for Glucose, Maltose, Mannitol, Lactose, Xylose and Sucrose were carried out. At first saline water was taken in a 500 ml flask and 10 ml of peptone was added and mixed properly and poured into 6 flasks with each flask holding a quantity of 50ml. Then in each flask certain required grams of Glucose, Sucrose, Maltose, Mannitol Lactose and Xylose was added. Then 10 ml was taken from each flask and poured into 6 different test tubes, this was done until we had 3 test tube filled of each sugar. Then Durham's tube was inserted properly and in a way that avoided the formation of bubble in the Durham's tube. Then one drop of Andrade indicator was added in every test tube with the help of micropipettes and pH was checked with the help of pH paper and if it is found to be acidic then NaOH is added

and if it is found to be basic then HCl is added. All the test tubes were sterilized for 121℃ for 20 mins.After sterilization cooled in and inoculate loop full of suspension of the microorganism in test tubes, this is to be done in interest of the microorganism we founded so that it can be identified.

9. Result and Discussion

9.a Collection of Samples

Sample and its location from where it is collected is shown in Table no. 1

Table No. 1

Sr. No	Location
1	Local shop
2	Local dairy farm
3	Home

9.b Isolation and Enrichment of water Sample

Out of 3 samples, Sample 2 taken from a local dairy farm showed significant growth on Mannitol Nutrient Plate and was taken in for the further process of Gram staining and Identification.

9.c Identification of Microbe

Gram of the Microbe has been shown in table no. 2

Table no. 2

Colony Characteristics	Colony 1
Size	1 mm
Colour	Yellow
Shape	Circular
Margin	Smooth
Opacity	Opaque
Consistency	Sticky
Elevation	Low convex
Gram nature	Gram Positive
Morphology	Spherical

Biochemical Testing of Bacteria Shown in Table No.3

Table no.3

Characteristics	Result	Characteristics	Result
	Morphological Characteristics		
Shape	Cocci	Gram Staining	Gram Positive
Size	0.5 – 1.0 µm	Motility test	Non motile
Colour	Yellow or white colonies	Spore	Non Sporulating
	Decomposition/ Enzymatic studies		
Oxidase Test	Negative		
Catalase test	Positive	H_2S production	Negative
Nitrate reduction test	Positive		
		Gelatine Hydrolysis test	Positive
		Urease test	Positive
	IMViC set		
Indole test	Negative	Methyl - red test	Positive
Voges -Proskauer test	Positive	Citrate utilization test	Positive
	Sugar Fermentation Test		
Glucose	Positive	Lactose	Positive
Maltose	Positive	Mannitol	Positive
Sucrose	Variable	Xylose	Positive

From above results and tables we can conclude that the microorganism present in the sample is none other than ***Staphylococcus aureus.***

Discussion

Similar experiments were carried out by Braz J Microbiol *et.al,* . In the region of Sao Paulo, Brazil. With over 208 samples were taken into consideration and out of which 18 samples showed the presence of *S.aureus* (Braz J Microbiol., *et.al,* 2010). Similarly in Brazil, Rall *et al.* collected 162 raw and pasteurized milk samples and found around 20.4% of the pasteurized milk samples were testing positive for *S. aureus* (Rall *et al.*2008)) and in 2006 Guodogan *et.al* analyzed 180 raw milk, pasteurized milk, and ice cream samples of which 56.6% of the pasteurized milk samples were found contaminated with *S.aureus* (Gündoğan *et al.* 2006).

Acknowledgment

We Aditi Tiwari and Aditya Dhobale would like to express our gratitude to our Professor Mrs. Sonali Joshi for helping us and guiding us throughout the process, even all the vendors for voluntarily giving us samples and also would like to express our gratitude towards our college ZSCT's Thakur Shyamnarayan Degree College for letting us a stage to present our research papers.

Conclusion

- **According to present result, the *Streptococcus aureus* is found in the sample taken from lab floor.**

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2. To Study the Antibacterial Properties of Different Types of Liquid Hand Wash

Mrs. Sonali Rahul Joshi
Assistant Professor, Microbiology ID, ZSCT's Thakur College of Science,
Kandivali East, Mumbai.
Kartik Salian
Student, Microbiology ID, ZSCT's Thakur College of Science, Kandivali East, Mumbai.

Abstract

Hand washing with hand wash is one of the most common practice practiced throughout every household in a daily bases. Exposure to different surfaces and environments makes our hands susceptible to different organisms. These organisms may be pathogenic and dangerous to our health if it enters our body through physical actions like eating food using our hands or touching our faces. Effects of the hand wash on selected common potentially pathogenic bacteria *E.coli* (gram negative) & *Klebshella spp* (gram positive) were evaluated using disc diffusion method.

So these 2 species of bacteria were used to test the anti-microbial property of 2 very common household hand wash I.e. Dettol & Santoor. Serial dilutions of each hand wash were used to check their anti-microbial strength at different concentrations. Fresh samples of *E.coli* and *Klebsiella sp.* were swabbed on 2 MIC plates each. Then discs with various dilutions of both the hand washes were transferred to the these plates and the zone of inhibition was observed after 24 hours in incubation of temperature of 37°C. The results obtained from this test give us credence to antimicrobial properties and effectiveness of the hand washes.

Keywords

- Hand wash
- Handwashing
- *E.coli*
- *Klebsiella sp.*
- Antimicrobial property
- Effectiveness

- Dettol
- Santoor

Introduction

Hand perform many functions in our daily life & are exposed to variety of substances like dust, different bodily fluids, contaminated materials, raw substances and various stuff from environment and during personal hygiene. Washing our hands with liquid hand washes stops the spread of microbes or loose transient flora thus preventing infections. But after washing our hands there remains a layer on our skin which protects our normal flora of hands ensuing low rate of different nosocomial infections. In early years people used to have soap to wash their hands. But these soap bars have residues of past usage and dirt on them. So now liquid hand washes are used instead of bar soap to reduce the cross contamination. Most of hand microbes can be killed using hand sanitizers but soiled or tainted hands with muck or organic material must always be washed with liquid hand wash.

Studies have revealed that liquid soaps are more effective than plain soaps as they contain antimicrobial active ingredients which remove more bacteria away. Studies suggest that using plain soap increases the spread of pathogenic bacteria to food by 3 times than using liquid antimicrobial hand wash. Comparing hand washes with alcohol-based solutions and washing with antimicrobial for a median time of 30 seconds, each one showed that the alcohol-based hand washes reduced bacterial contamination 26% more than the antibacterial. The results of a clinical trial with HIV patients found that 100% of itching symptoms are decreased by using liquid hand wash. So all the Indian branded liquid hand wash like Dettol, Lux, Johnson & Johnson, Lifebuoy & Santoor have the ability to remove 65% to 85% of microbial population that are present or get settled on human skin.

Materials & Method

Liquid hand wash

- For this research 2 household liquid hand washs were utilised.
- They were Dettol liquid hand wash & Santoor liquid hand wash

A. Preparation of Serial Dilution

About 10 ml of undiluted sample in taken of dettol liquid hand wash. Then 1 ml of this undiluted solution is then transferred to another test tub containing 9 ml of distilled water forming a 10 ml solution of diluted liquid hand wash with hand wash to water ratio of 1:9

respectively. It creates 10^{-1} dilution of that hand wash. Then repeat the same process again & again to get 10^{-2}, 10^{-3} & 10^{-4} dilutions. Repeat the same process with Santoor liquid hand wash.

B. Bacterial Samples

Fresh samples of *Escherichia coli (E.coli)* & *Klebsiella sp.* were used in this research which was obtained from the laboratory of the Microbiology department.

C. Media

Sterile Mueller-Hilton Agar

Plates of sterile Mueller-Hilton agar were used for the detection of antibiotic susceptibility by MIC & MBC. Due to its low sulfoamide concentration most pathogenic organisms grow perfectly in this medium.

Components	Amount
Beef Extract	2.00gm
Starch	1.50gm
Acid Hydrolysate of Casein	17.50gm
Agar	17.00gm
Distilled Water	1000ml

D. Miscellaneous

1. Test tubes (x8)
2. Sterile Petri Plates (x2)
3. Sterile Cotton Swabs (x2)
4. Sterile Antimicrobial Discs
5. Sterile 10ml pipettes (x2)

E. Antimicrobial Activity of Liquid Hand Wash

Firstly, take 2 test tubes and add 10ml of liquid Dettol hand wash in one and 10ml of Santoor hand wash in other. By using water as a diluent a series of dilutions is made ranging from 10^{-1} to 10^{-3} of each liquid hand wash. Sterile Mueller-Hinton agar was made in the lab and aseptically transferred into 4 sterile petri plates. These plates are later allowed to cool & solidify at room temperature.

After solidifying, the Mueller–Hinton agar plates were swabbed with strains *Escherichia coli* and other 2 with *Klebsiella sp.* respectively. After that, aseptically dip different sterile discs in different test tubes containing different dilutions of dettol hand wash respectively and transfer

the disc in different quadrants of the petri plate containing *E-coli* in one and *Klebsiella sp.* Do the same with santoor hand wash. The plates are then transfered to an incubator and place it in and incubate it at 37^0C for 24 hours.

Results

The main motive of this research on these household handwash was to determine the antibacterial activity in Dettol and Santoor hand washes. Larger zones of inhibitions were noticed at higher concentration and it keeps on decreasing as the concentration decreases.

The undiluted sample of dettol showed 12mm zone in *Escherichia coli* & 10mm zone in *Klebsiella* whereas Santoor showed 16mm zone & 10nm zone on *Escherichia coli & Klebsiella sp.* respectively.

10^{-1}, 10^{-2} & 10^{-3} dilutions of Dettol got 10mm, 4mm & 2mm zones in *E.coli* whereas it got 9mm, 4mm & 3mm zones in Klebsiella respectively. In Santoor, 14mm, 11mm & 7mm zones in E.coli and 5mm, 3mm & 2mm zones in Klebsiella are found for 10^{-1},10^{-2} & 10^{-3} dilutions respectively.

	Samples	Zone of inhibition in diameters	
Dettol	Dilutions	Escherichia coli	Klebsiella spp
Hand	U.D	12nm	10nm
Wash	10^{-1}	10nm	9nm
	10^{-2}	4nm	4nm
	10^{-3}	2nm	3nm

Table 1 : Antimicrobial Activity of Dettol.Hand Wash

	Samples	Zone of inhibition in diameters	
Santoor	Dilutions	Escherichia coli	Klebsiella spp
Hand	U.D	16nm	12nm
Wash	10^{-1}	14nm	5nm
	10^{-2}	11nm	3nm
	10^{-3}	7nm	2nm

Table 2 : Antimicrobial Activity Of Santoor Hand Wash

Discussion

The main objective of this study is to analyze and confirm the reliability and to know the antimicrobial properties of the liquid hand washes that are commonly used by many individuals at different places.

As these hand washes contain disinfectants such as isopropyl alcohol which has bactericidal properties in it. Most of the hand washing can be usual done by hand sanitizers but it is found out that utilization of hand wash on murky and soiled hands is far more effective than sanitizers.

Conclusion

The hand washes has shown antimicrobial properties against tested organisms. Santoor hand wash showed more antimicrobial activity than the Dettol ones. So use of liquid hand wash should be mandatory in every household and is very important in hospitals and healthcare fields to reduce the amount of cross contamination. This paper shows that common house hold hand washes are enough to exterminate the common pathogenic bacteria on our hands & shows that the amount of mocroorganisms on our hand after hand wash is reduced with the microbial eradication rate of 70%. So it's safe to say that hand washing with liquid hand wash is safe. As it contains low amount of alcohol which does not have any side effects, it can be also utilized by or on children's hands to kill the bacteria on them. Further study and research is required to increase the efficiency and usability of hand washes to get better alternatives.

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3. Phytosynthesis of Silver and Gold Nanoparticles in Peel and Bark of Plant *Punica Granatum* (Pomegranate)

Udaybhan Yadav
Coordinator, Department of Microbiology (Interdisciplinary)

Abstract

Nanoparticles are extensively used in biological and medical research due to their unique properties. Use of such nanoparticles in biological & medicinal field gives rise to the concept of biomedical nanotechnology, bio nanotechnology & nanomedicines. Phytosynthesis of nanoparticles is immerging area in plant science research. Different plants are used for this purpose being it is most ecofriendly and convenient method of synthesizing nano scale particles of different salts. The plants are their potent sources of many valuable bioactive constituents and these constituents contributes reduction of salt in the system. In present work, fruit peel and plant bark of *Punica granatum* plant was taken as an experimental system for Phytosynthesis of silver and gold nanoparticles from silver nitrates and gold chloride salt. *Punica granatum* is rich source in secondary metabolites especially polyphenols such as alkaloids, tannins, flavonoids and also steroids, triterpenes etc. which has lots of medicinal importance. The extract reaction mechanism of the nanoparticles synthesis by using biomaterials is yet to elucidate in detail; the work done proposes the involvement of redox enzymes in the reduction of silver and gold ions.

Key Words - Nanoparticles, Phytosynthesis, Pomegranate, Silver Nanoparticles, Gold Nanoparticles, NTA, TEM, UV-Vis.

Introduction

A nanometer is one-billionth of a meter. Norio Taniguichi coined the term "nano-technology" in 1974. Nobel Laureate Dr. Horst Stormer said that, “the nanoscale is more interesting than the atomic scale because the nanoscale is the first point where we can assemble something- it’s not until we start putting atoms together that we can make anything useful”. Biologists, chemist, physicists and engineers are all evolved in the study of substances at the nanoscale. Dr. Stormer hopes that the different disciplines develop a common language and

communicate with one another without a solid background in multiple sciences one cannot understand the world of nanotechnology. Nano biotechnology is the merger of two distant fields of nanotechnology and biotechnology. Nanoparticles are extensively used in biological and medical research communities for various applications. Use of such nanoparticles in biological and medical field gives rise to the concept of biomedical nanotechnology, bionanotechnology and nanomedicine. The integration of nanomaterials with biology has led to the development of diagnostic devices, contrast agents, analytical tools, physical therapy applications, and drug delivery vehicles. The Biological systems specially algal materials, fungal forms and Angiospermic plants of medicinal importance are considered as a most ecofriendly and nontoxic systems with superiority of applications over physical and chemical methods of nanoparticle synthesis.

In present work, *Punica granatum* plant was taken as an experimental system for Phytosynthesis of AgNP's and AuNP's. Bottom up and 'Top down Approach are considered as two important modes of nanoparticle synthesis. In the bottom up approach, materials and devices are built from molecular components which assemble themselves chemically by principles of molecular recognition. While in the top down approach, nano sized particles are constructed from large entities without atomic level control (Maccuspie et.al. 2011). Nanostructures come in various size and shapes and are synthesized by using different metals and other chemicals.

Nanocages, Nanofibers, Nanotubes and Nanodots are the specific types of nanostructures. Nanocages are hollow porous gold nanoparticles ranging in size from 10nm-150nm. These are the product of reaction of silver nanoparticles with chloroauric acid ($HAuCl_4$) in boiling water. Nanocages show different properties than their buildind molecules. E.g. Gold Nanoparticles show absorbance in the visible spectrum of light while gold Nanocages absorbs light in near infrared region. Nanofibers are the specially designed fibers with diameter less than 100 nanometers. They can be produced by interfacial polymerization and electro spinning. Carbon Nanofibers are graphitized fibers and are shaped by catalytic synthesis. Napkins with Nanofibers contain antibodies against numerous biohazards and chemical that signal by changing color. These napkins are chiefly used in identifying the bacteria in kitchens. Nanotubes are nanoscale tube like structures whose diameter ranges from 0.1 to 100 nm and length is much greater. Such nanotubes which exhibit extraordinary strength and unique electrical properties are efficient

conductors of heat. Nanodots or Nanoparticles that consist of homogenous materials, especially those that are almost spherical or cubical in shape.

The size of nanomaterials is similar to that of most biological molecules and structures; therefore, nanomaterial's can be useful for both in vivo and in vitro biomedical research and applications. Thus far, the integration of nanomaterials with biology has led to the development of diagnostic devices, contrast agents, analytical tools, physical therapy applications, and drug delivery vehicle. Several proteins make nanomaterials suitable for bio tagging or labeling. In order to interact with biological target, a biological or molecular coating or layer acting as bioinorganic interface should be attached to the nanoparticles. These biological coatings include antibodies, biopolymers like collagen, or monolayers of small molecules making the nanoparticles biocompatible. This is the reason why the biological metal nanoparticle synthetic methods are favored over physical and chemical methods. As the biosynthetic methods utilize natural solvents for the production of metal nanoparticles they are biocompatible and can be utilized in the medicine. Also, nanoparticles have a further advantage over larger macromolecules as they are better suitable for intravenous delivery.

The shape of the nanoparticles is more often spherical but cylindrical, plate like and other shapes are possible. The size and size distribution might be important in some cases, for example if penetration through a pore structure of a cellular membrane is required. The size and size distribution are becoming extremely critical when quantum-sized effects are used to control material properties. A tight control of an average particle size and a narrow distribution of sizes allow creating very efficient fluorescent probes that emit narrow light in a very wide range of wavelengths. This helps with creating biomarkers with many and well distinguished colors. The core itself might have several layers and multifunctional. For example, combining magnetic and luminescent layers one can both detect and manipulate the particles.

Gold play an important role in pharmaceutical, cosmetic and food industries. Dentists use gold for crowns, and certain medicines, such as sodium aurichloride for rheumatoid arthritis which also contain gold. Small amount of gold sometimes brightens foods such as jelly or liquors, like Goldschlager gold can be catalyze, or speed up, certain chemical reactions more efficiently than other toxic catalysts. It plays an important role in reducing pollution. For example, scientists have recently discovered that gold particles energized by the sun can destroy volatile organic chemicals. Recently, gold nanoparticles were used to detect breast cancer. The

procedure works by identifying the proteins found on the exteriors of the cancer cells. Different types of cancer have different proteins on their surfaces that serve as unique markers. Nanorods, gold nanoparticles shaped like rods, use specialized antibodies to latch onto the protein markers for breast cancer, or for another cancer type. After the nanorods bind to proteins in a blood sample, scientists examine how they scatter light. Each protein-nanorod combination scatters light in a unique way, allowing for precise diagnose.

In medicine, nanoparticles first found use in the diagnosis of tumors in the spleen and liver using magnetic resonance tomography. In cancer therapy a major difficulty is to destroy tumor cells without harming the normal tissues. Radiotherapy attempts to focus irradiation on the tumor but never the less damages healthy tissues which cannot always be protected in the desired way. Magnetic drug targeting employing nanoparticles as the carrier is a promising cancer treatment avoiding side effects of conventional chemotherapy (Akerman et.al.2006). There is also a very significant role in of hyperthermia in cancer drug delivery. There is increasing evidence that hyperthermia at 40-43^0 Celsius enhances the uptake of therapeutic agents in to cancer cells and provides an opportunity for improved targeted drug delivery (Kocbek et.al.2001). Using nanoparticles for drug delivery of anticancer agents has significant advantages such as the ability to target specific locations in the body, the reduction of the overall quantity of drug used, and the potential to reduce the concentration of the drug at non target sites resulting in fewer unpleasant side effects (Joenathan et.al.2006). The use of nanoparticles as drug delivery vehicles for anticancer therapeutics has great potential to revolutionize (Faroji and wipf et.al.2009) the future of cancer therapy. As tumor architecture causes nanoparticles to preferentially accumulate at the tumor site, their use as drug delivery vectors results in the localization of a greater amount of the drug load at the tumor site; thus improving cancer therapy and reducing the harmful nano specific side effects of chemotherapeutics.in addition, formulation of these nanoparticles with imaging contrast agents provides a very efficient system for cancer diagnostics.

Silver derives its broad-spectrum antimicrobial activity from the ability of silver ions to bind irreversibly to a nucleophilic group commonly available in cells of bacteria, viruses, yeast, fungi and protozoa. Binding to cellular components disrupts the normal reproduction and growth cycle resulting in death of the cell. Capitalizing on its potent activity, silver and its compounds have been incorporated over the past several decades in a variety of wound care products such as

dressings, hydrogels, hydrocolloids, creams, gels, lotions, sutures, and bandages. The preferred form of silver in antimicrobial products has been its compounds or salts as the metallic form of the element itself lacks therapeutically effective oligodynamic action. The compounds or salts upon contact with an aqueous medium ionize to yield silver ions that become available for antimicrobial action. The majority of silver compounds is also photosensitive or heat sensitive making their utilization in stable commercial products challenging. It is well known that the proteins and enzymes present in the plant extract are responsible for the reduction of metal ions. However, the shape, size and the stability of metal nanoparticles is due to the phyto-constituents present in the extract. It is also known that the composition of the enzymes and phyto-constituents varies from plant to plants and thus has the impact on the ability to reduce metal ions as well as the shape, size and stability of the metal nanoparticles.

Pomegranate fruit, technically a berry, are globose and up to 6 inches wide. Inside they contain up to six hundred seeds surrounded by transparent sacs that are red when ripe. The seeds are divided into clumps by yellow membranes. Pomegranate aril juice provides about 16% of an adult's daily vitamin C requirement per 100 ml serving, and is a good source of vitamin B5 (pantothenic acid), potassium and polyphenols, such as tannins and flavonoids. Pomegranates are listed as high-fiber in some charts of nutritional value. That fiber, however, is entirely contained in the edible seeds which also supply unsaturated oils.

The most abundant polyphenols in pomegranate juice are the hydrolysable tannins called ellagitannins formed when ellagic acid binds with a carbohydrate. Punicalagins are tannins with free-radical scavenging properties in laboratory experiments and with potential human effects. Punicalagins are absorbed into the human body and may have dietary value as antioxidants, but conclusive proof of efficacy in humans has not yet been shown. During intestinal metabolism by bacteria, ellagitannins and punicalagins are converted to urolithins which have unknown biological activity in vivo. Other phytochemicals include polyphenolic catechins, gallocatechins, and anthocyanins, such as prodelphinidins, delphinidin, cyanidin, and pelargonidin. The ORAC (antioxidant capacity) of pomegranate juice was measured at 2,860 units per 100 grams.

Materials and Methods

i. Collections of Plant Materials

The bark and fruit peel of *Punica granatum* (pomegranate) tree were collected from fruit market and the dust particles were removed. The plant materials are kept in oven for 24 hours at

40^0C. After drying plant materials are converted in to a fine powder with the help of mortar and pestle.

ii. Chemicals

1. Silver nitrate ($AgNo_3$)
2. Chloroauric acid ($HAuCl_4$)
3. Deionized water

iii. Preparation of Bark and Fruit Peel Extract

The 15 gm. of bark powder and 15gm. of peel powder was mixed with sterile D/W in 500ml Erlenmeyer flask. This mixture was then boiled for 25min on heating plate. After boiling mixture were filtered with Whatman filter paper separately. The supernatant was used as a plant extract for the experiment.

iv. Preparation of 1mM aqueous AgNo3 and HAuCl4 solution

76.441mg standard AgNo3 powder and 177.223mg standard HAuCl4 powder was separately diluted with 450ml de-ionized d/w. 1mM aqueous AgNo3 solution and HAuCl4 solution was used for the treatment of the plant extract.

v. Synthesis of Silver Nanoparticles in Pomegranate bark and Peel Extract

Accurately measured bark and peel extract of 25ml and 50ml was separately added to the 75ml and 50ml in 1mM aqueous AgNo3 solution respectively in a jar. The jar was agitated for few minutes and then incubated at room temperature.

vi. Synthesis of Gold Nanoparticles in Pomegranate Bark and Peel Extract

Accurately measured as prepared bark extract and peel extract of 25ml and 50ml was separately added to the 75ml and 50ml in 1mM aqueous $HAuCl_4$ solution respectively in a jar. The jar was agitated for few minutes and then incubated at room temperature.

vii. UV-Vis Spectra Analysis

The bioreduction of Au^{3+} to Au^0 and Ag^{++} to Ag^0 in the aqueous solution was monitored by periodic sampling (0min, 15min, 30min…120min, 24hr) of aliquots of the suspension, if required then by diluting the samples with distilled water and subsequently measuring the UV-Vis Spectra (190nm to 1100nm) of the resulting diluents on the spectrophotometer (Model-Shimazdu UV 1800). Similarly, the spectra of the ions were recorded and compared. Deionized water was used for the base line correction.

viii. Transmission Electron Microscopy (TEM)

TEM samples of the silver, gold nanoparticles synthesized by the peel and bark extract of *Punica granatum* were prepared by first sonicating the samples in sonicator (Vibronics VS80) for 15mins. A drop of the nanoparticle's solution was put on carbon coated copper grids of 3mm diameter, blot to remove excess of solution and later was allowed to dry under Infrared light for 40mins. TEM measurements were then performed on instrument operated at an accelerating voltage at 200 Kv (PHILIPS MODEL CM 200).

Observations

The data obtain on analysis of characters of Phytosynthesized nano scale particles in Peel and Bark extract of *Punica granatum* is tabulated in this chapter.

Table 1: - Observation table for average particle size, shape and distribution frequency of Phytosynthesized **silver nanoparticles** in *Punica granatum* bark & peel extract.

Sr. No.	Salt Used	Plant Materials	NTA analysis		TEM analysis	UV Spectra (nm)
			Mean size (nm)	Particles per frame	Shape of the particles	
1	Silver Nitrate	Peel extract	59	26.99	Oval, Spherical	463
2	Silver Nitrate	Bark extract	61	37.66	Circular	400

Sr. No.	Salt Used	Plant materials	NTA analysis		TEM analysis	UV Spectra (nm)
			Mean size (nm)	Particles per frame	Shape of the particles	
1	Gold Chloride	Peel extract	54	25.51	Spherical	548
2	Gold Chloride	Bark extract	58	10.91	Circular	520

Table 2: - Observation table for average particle size, shape and distribution frequency of Phytosynthesized **gold nanoparticles** in *Punica granatum* bark & peel extract.

Result and Discussion

The present work deals with the aspect of Phytosynthesis of Silver nanoparticles (AgNPs) and Gold nanoparticles (AuNPs) in peel and bark extract of *Punica granatum*. The finely grinded powder of pomegranate peels and bark **(Photoplate-1)** was used to prepare the extract.

Au and Ag NPs have a wide range of application in areas such as catalysis, medical diagnostics, and biological imaging. Various physiochemical method of metal nanoparticles synthesis has been reported, all having their inherent limitations. Development of easy, reliable

and ecofriendly biological methods helps in endorsing extra interest in the synthesis and application of nanoparticles which are good for mankind (Bhattacharya and Gupta, 2005). In this context the utilization of biological systems for nanoparticles synthesis provide move towards this multifaceted approach. Biological systems have shown ability to interact with metal ions and reduce them to form metallic nanoparticles (Beveridge et.al.1997).

Phytosynthesized Silver NP's in Peel and Bark Extract of *Punica Granatum*

UV-Vis Studies

A UV-Vis measurement gives the precise report of the absorbance of the light on the basis of shape of the nanoparticles. Absorption spectra show the production of SNP's within an hour on the reduction of Ag^{++} ions. The UV-Vis spectra of reaction mixture of 1mM aqueous $AgNo_3$ solution and 25% peel and 25% bark extract of plant *Punica granatum* is shown in the **Fig.-1**. The spectra clearly shows the absorption band at around 463nm in the peel extract and at around 406nm in the bark extract of silver nanoparticles. There is a close relationship between the UV-Vis and light absorbance characteristics and size and shape of the absorbate (Ankavmar et.al, 2005). The absorption band is a characteristic feature of anisotropic nanoparticle and occurs in structures such as triangular, spherical and hexagonal particles (Ankavmar et.al, 2005).

PHOTO PLATE 1

Peel powder of *Punica granatum*	Bark powder of *Punica granatum*
	

Fig. 1: UV-Vis Spectra recorded as a function of time for the solutions prepared using silver nitrate (1mM), peel and bark extract of *Punica granatum*.

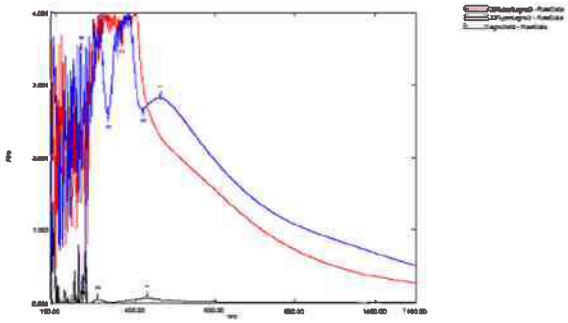
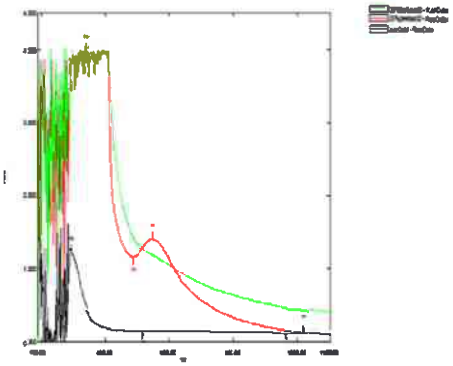


Fig. 2: UV-Vis spectra recorded as a function of time for the solutions prepared using gold chloride (1mM), peel and bark extract of *Punica granatum.*



Nanoparticle Tracking Analysis (NTA)

NTA analysis for silver nanoparticles was done using Nano sight Ver.2.1 Instrument in Institute of Science. NTA studies indicated the average size of SNP's synthesized in the peel extract as 59 nm with the frequency of 26.99 particles per frame **(Fig.-3)**. The nanoparticles synthesized using bark extract showed the average particle size of 61nm with the average frequency of 37.66 particles per frame **(Fig.-4)**. These results indicated the high frequency of SNP' synthesis in bark extracts compare to peel extract. It is to be noted that the average size of Phytosynthesized SNP's was also more in bark extract (61nm) against peel extract (59nm).

TEM Analysis for Silver Nanoparticles

The shape of the Phytosynthesized nanoparticles can be conspicuously observed by TEM analysis. TEM samples of the aqueous suspension of silver nanoparticles after sonication for 15 minutes were produced by placing a drop of the suspension on Carbon coated copper grids and allowing water to evaporate in vacuum. TEM observations were performs on Philips Electron Microscope operated at an accelerating voltage of 200Kv with the resolution of 0.22. The shape of the AgNP's in pomegranate peel extract was spherical and Oval **(Photoplate-2)**. This morphology of silver ions is obtained by reduction of Ag++ to Ag^0. A large density of silver NP's was observed under low magnification. Thus, silver nanoparticles are quite polydispersed and ranged in size from 9-70nm. The SNP's in bark extract showed spherical shape **(Photoplate-3)**. The Silver nanoparticles are quite polydispersed and ranged in the size from 9-22nm. These observations indicated the variations in the shape of nanoscale particles in peel and bark extract.

Fig. 3- NTA analyses of 25% Peel Extract of Silver Nanoparticles

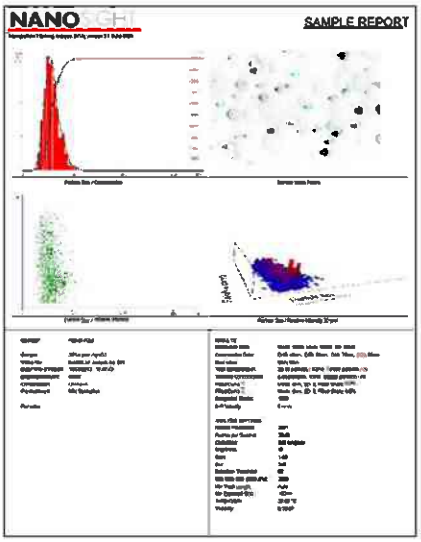


Fig. 4: NTA analyses of 25% bark extract of silver nanoparticles

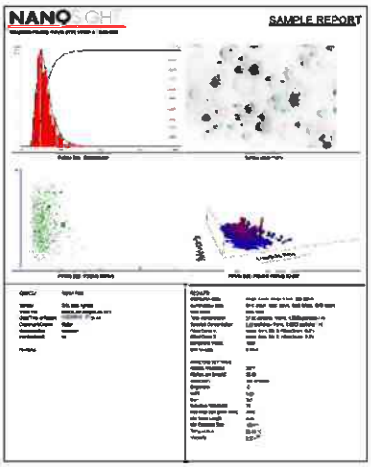
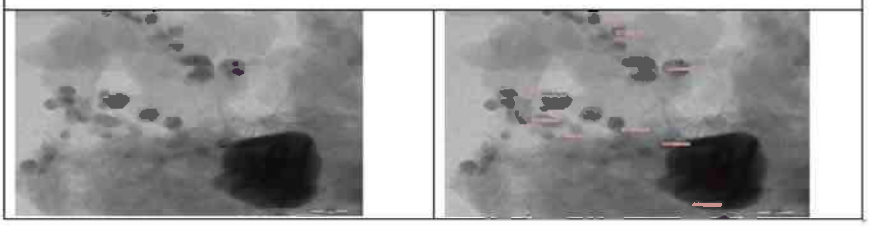


Photo plate 2
Photo plate 2: a, b and c-TEM images of Ag Nanoparticles showing spherical and oval shape nanoparticles in *Punica granatum* peel extract

Photo plate 3
Photo plate 3: a and b- TEM images of Ag Nanoparticles showing circular shape nanoparticles in *Punica granatum* bark extract


Phytosynthesized Gold NP's in Peel and Bark Extract of *Punica Granatum*

UV-Vis Studies

The absorption spectra show the production of NP's within an hour on the reduction of Au^{+++} ions in to Au^{0}. The UV-Vis Spectra of reaction mixture of 1mM aqueous AuCl4 solution and 25% peel and 25% bark extract of plant *Punica granatum* is shown in the Fig.**-2.** The spectra clearly show the absorption band at around 548 nm in the peel extract and at around 520 nm in the bark extract of gold nanoparticles.

Nanoparticle Tracking Analysis (NTA)

NTA analysis for gold nanoparticles indicated the average size of gold NP's synthesized in the peel extract as 54 nm with the frequency of 25.51 particles per frame **(Fig.-5)**. The nanoparticles synthesized using bark extract showed the average particle size of 58 nm with the average frequency of 10.91 particles per frame **(Fig.-6)**. These results indicated the high frequency (25.51) of gold NP's synthesis in peel extracts compare to bark extract (10.91). It is to be noted that the average size of Phytosynthesized gold NP's was also more in bark extract (58 nm) against peel extract (54 nm).

TEM Analysis for Gold Nanoparticles

TEM samples of the aqueous suspension of gold nanoparticles after sonication for 15 minutes were produced by placing a drop of the suspension on Carbon coated copper grids and allowing water to evaporate in vacuum. TEM observations were performs on Philips Electron Microscope operated at an accelerating voltage of 200Kv with the resolution of 0.22. The shape of the Au NP's in pomegranate peel extract was spherical **(Photoplate-4)**. This morphology of gold ions is obtained by reduction of Au+++ to Au^{0}. In TEM analysis, the particles range observed was 8-20nm. The Au NP's in bark extract showed circular shape **(Photoplate-5)**. These

observations indicated the variations in the shape of nanoscale particles i.e. spherical in peel and circular bark extract.

Fig. 5: NTA analyses of 25% Peel extract of Gold nanoparticles

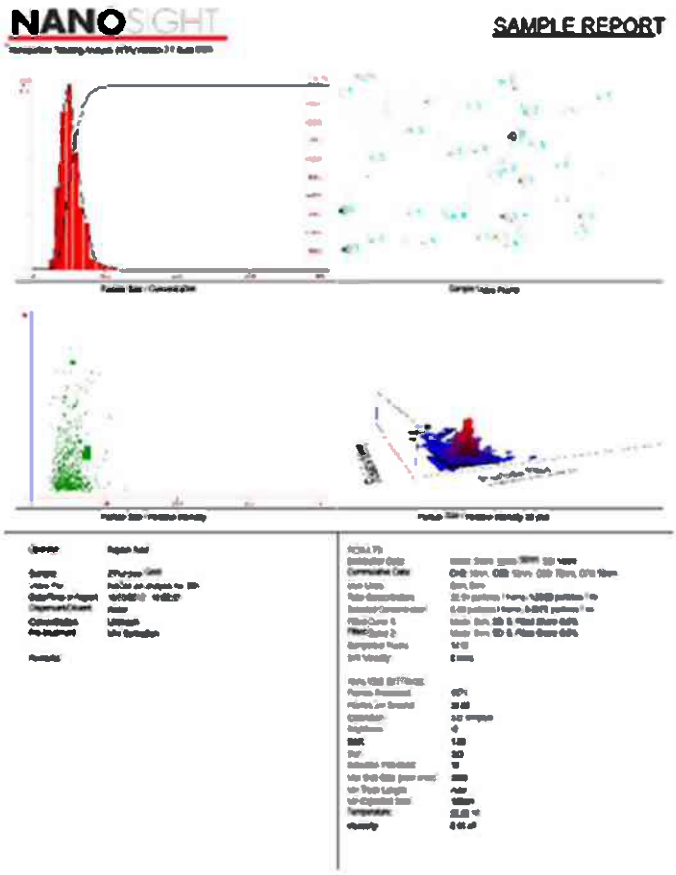


Fig. 6: NTA analyses of 25% bark extract of Gold nanoparticles

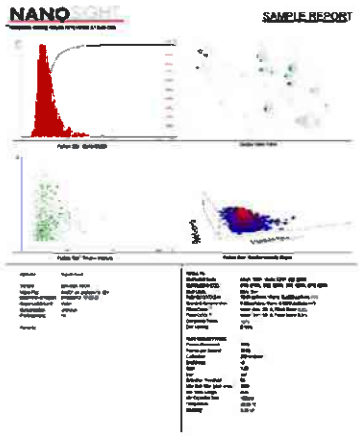


Photo plate 4

Photo plate 4: a, b and c- TEM images of Au Nanoparticles showing spherical shape nanoparticles in *Punica granatum* peel extract

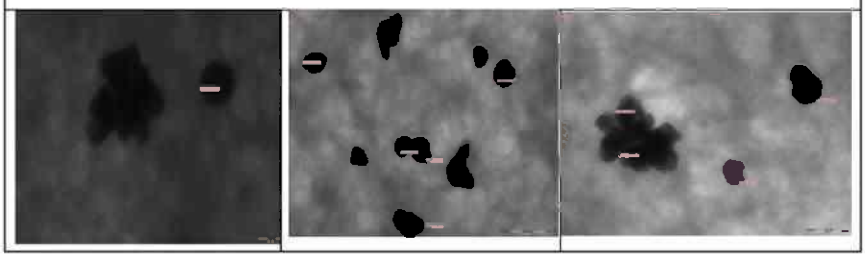
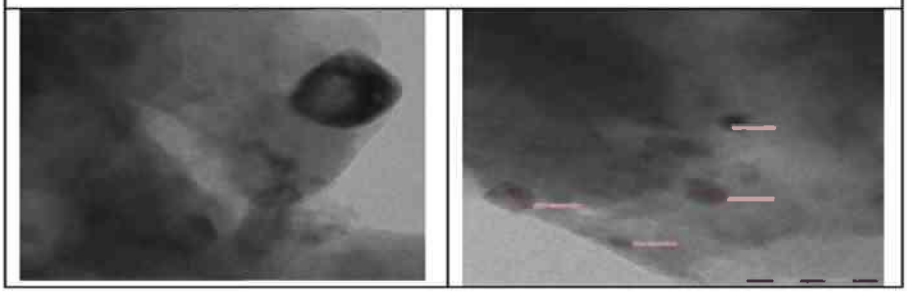


Photo plate 5

Photo plate 5: a and b- TEM images of Au Nanoparticles showing circular shape nanoparticles in *Punica granatum* bark extract



Green synthesis of nanoparticles is a coming up experimental exercise where the biological agents like Bacteria, Fungi, and Higher plants are used (Mukherjee *et. al.,* 2001a, Singaravelu *et. al.,* 2007) have reported the use of plant materials for the synthesis of nanoparticles. The use of inactivated biomass to recover metal ions from solution has been studied extensively. The possibility of using live bacteria for the remediation of metal-contaminated waters has shown the bacterial production of silver-carbon composite materials. Many reports on synthesis of metal and semiconductor nanoparticles using fungi or bacteria have appeared. In our quest for new eco-friendly "green" methods for the synthesis of noble metal nanoparticles, many workers have identified fungi (Mukherjee *et. al.*, 2001), Actinomycetes (Ahmad *et. al.*, 2003), and plant extracts (Shivshankar*et. al.*, 2003), for the synthesis of silver and gold nanoparticles (Shivshankar*et. al.*, 2003).

The synthesis of stable silver, gold and bi-metallic Ag/Au core shell nanoparticles using 20 g of leaf biomass have been reported by Shivshankar*et.al,* (2004) using *Azadiracta indica.* In

vivo synthesis of nanoparticles of gold-silver-copper alloy has been reported by R.G. Haverkamp*et.* al.2007. Most of the above research involves the synthesis of colloidal silver or gold nanoparticles employing plant broths resulting from boiling fresh or dried plant leaves (Shivshankar*et. al.*, 2003, 2004, 2005 and 2006).

In the present work, the attempt was made to synthesize Silver and Gold nanoparticles employing the fruit peel and bark extracts of *Punica granatum* Linn. *Punica granatum* is rich source in secondary metabolites especially polyphenols such as alkaloids, tannins, flavonoids and also steroids, triterpenes etc. which has lots of medicinal importance. The extract reaction mechanism of the nanoparticles synthesis by using biomaterials is yet to elucidate in detail; the work done proposes the involvement of redox enzymes in the reduction of silver and gold ions. Different chemical components present in the plant contribute the stability, shape and size of the phytonanoparticles.

Conclusion

The present work was conducted in the bark and peel aqueous extract of Punica granatum to synthesized silver and gold nanoparticles by treating with 1mM concentration of silver nitrate and gold chloride. The characterization of Phytosynthesized nanoparticles in pomegranate peel and barks was done with the help of UV-Vis spectrometry, nanoparticle tracking analysis and transmission electron microscopy. The results obtained in the present work are concluded as follows

- In both the experimental samples (peel and bark extract of *Punica granatum*, it is concluded that the biomass used yields good response for the Phytosynthesis of silver and gold nanoscale particles.
- The UV-Vis Spectrophotometry analysis revealed the spectrum status for both the samples in accordance with nature of salt used.
- Nano tracking analysis for silver nanoparticles indicates high frequency of nano sized particles in bark extract compare to peel extract of the experimented plant.
- The average size of the silver nanoparticle was more i.e. 61nm for a bark extract when compare to peel extract sample.
- The gold nanoparticles appeared with high frequency in peel extract however the size of nanoparticle was more (58 nm) in the bark extract sample compare to peel extract.
- The TEM analysis indicates circular, spherical, and oval shaped phytonanoparticles.

- Silver nanoparticles in peel extract were spherical as well as oval in shape where as in bark extract only spherical nano sized particles were observed.
- The gold nanoparticles were circular in shape in bark extract where as in peel extract sample the shape of nano scale particles was spherical.

The work conducted was useful to understand the process of nanoparticle synthesis using plant material. The work also given an inside to understand the characters of phyto nanoparticles using analytical techniques. This mode of synthesis is a part of nano biotechnology using green synthesis approach. These aspects generate more interest in plant science researchers because the phytonanoparticles are capped with biological molecules which are either enzymes or the phytoconstituent's derivatives of different bioactive groups. Therefore, the use of these nano size particles in modern medicine and drug delivery technology deserves high importance and generate more scope for research in future.

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4. Effect of Hormones on Diosgenin Production by Callus Cultures of *Trigonella Foenum Graecum L.*

Udaybhan Yadav
Coordinator, Department of Microbiology (Interdisciplinary), Thakur Shyamnarayan Degree College, Kandivali East Mumbai.
Udaybhan Yadav
Coordinator, Department of Microbiology (Interdisciplinary), Thakur Shyamnarayan Degree College, Kandivali East Mumbai.

Abstract

Trigonella foenum-graecum, commonly known as fenugreek, is an annual herbaceous plant that has been a staple of traditional herbal medicine in many countries. Fenugreek seeds have a healthy nutritional profile, containing a good amount of fiber and minerals, including iron and magnesium. Since traditional to modern time fenugreek is used as alternative medicine and has been studied about its efficacy and biologic mechanisms of action. Diosgenin, a glycoside, and the fiber component of the plant are the most intensively act as a bioactive constituent which is present in fenugreek. This compound has been shown many beneficial effects like glucose tolerance, inflammation, insulin action, functioning of Liver, cardiovascular and other metabolic activity. In this article author has been tried to increase the production of diosgenin by means of Callus culture in MS medium with hormones like BAP and 2,4-D under controlled condition.

Key Words: Fenugreek, Diosgenin, Hormones, Callus culture, MS medium.

Introduction

Fenugreek is an annual forage legume crop. It is believed to be native to the Mediterranean region (Petropopulas, 2002) but now is grown as a spice in most part of the world. It is reported as a cultivated crop in part of Europe, northern Africa, west and south Asia, Argentina, Canada, USA and Australia (AAFRD at al 1998). India is the leading fenugreek producing country in the world (Edison et al 1995). Fenugreek, an erect, branched herb finds its value in medicinal due to its phytoconstituent's.

Phytochemical constituents of Trigonella foenum-graecum: The leaves contain at least 7 saponins, known as graecunins. These compounds are glycosides of diosgenin. Seeds contain

0.1% to 0.9% diosgenin and are extracted on a commercial basis. The seeds also contain the saponin fenugrin B. Several coumarin compounds have been identified in fenugreek seeds as well as a number of alkaloids (e.g., trigonelline, gentianine, carpaine). A large proportion of the trigonelline is degraded to nicotinic acid and related pyridines during roasting. These degradation products are, in part, responsible for the flavor of the seed. The seeds also yield as much as 8% of a fixed, foul-smelling oil. The C-glycoside flavones vitexin, vitexin glycoside, and the arabinoside is orientin have been isolated from the plant. Three minors steroidal sapogenins also have been found in the seeds: smilagenin, sarsapogenin, and yuccagenin. The mucilage of the seeds of several plants, including fenugreek, have been determined and their hydrolysates analyzed. Fenugreek gel consists chiefly of galactomannans characterized by their high water-holding capacity. These galactomannans have a unique structure and may be responsible for some of the characteristic therapeutic properties attributed to fenugreek. Plant tissue cultures from seeds grown under optimal conditions have been found to produce as much as 2% diosgenin with smaller amounts of gitongenin and trigogenin.

Traditional uses of *Trigonella foenum-graecu*m: The fresh leaves can be cooked, steamed or in a curry with potatoes in a similar way to spinach, their flavor is quite mild. Leaves can also be dried and there has a more pronounced bitter taste used to flavor dishes. In Egypt the seeds are sweetened and used as a tea and in many middle Eastern cultures, they are used in cakes and confectionary. The seeds are also reputed to have many medicinal properties inducing relieving joint pain, lowering blood sugar level and even restoring hair growth. Fenugreek can also be sprouted as a salad and has a distinctive spicy taste. Fenugreek seeds contain hormone precursors that increase milk supply. Scientists do not know for sure how this happens. some believe it is possible because breasts are modified sweet glands, and fenugreek stimulates sweat production. It has been found that fenugreek can increase a nursing mother's milk supply with in 24 to 72 hours after first taking the herb.

Pharmakon uses of Diosgenin: *Trigonella foenum* – graecum produces diosgenin as asteroid saponin, belonging to triterpene group and has great significance for pharmaceutical industry due to its oestrogenic effect on the mammary gland (Oncina et al .2000). It also plays an important role in the control of cholesterol metabolism variation in the lipoxygenase activity of human erythroleukemia cells. Diosgenin has performed mainly as basic material for partial synthesis of oral contraceptives, sex hormones and the other steroids (Gorez et al 2004). Methyl

protodiosein potent agent with anti tumor properties, has been synthesized from diosgenin (charg et al 2003).

Tissue Culture: Tissue culture is an experimental technique trough which a mass of cells (callus) is produced from an ex-plant tissue or more precisely it is the technique of growing plant cells tissue and organs in an artificial prepared nutrient medium static or liquid under controlled condition. Clones generated through tissue culture are identical in terms of size, developmental stage and rate of metabolic activities. The clones are capable of performing the transformative activity which involves biotransformation to produce primary and secondary metabolites in the tissue culture medium.

Callus culture: For raising the callus tissue, a tissue culturist must have clear understanding of some basic principals. A cell from any part of a plant like shoots apex, bud, leaf, mesophyll cells epidermis, cambium, anthers, pollen, fruit etc; when inoculated in a suitable medium under aseptic laboratory conditions can be able to differentiate and multiply. This results in the formation of an amorphous mass of cells known as callus, which can be induced to re-differentiate an appropriate medium to develop into the plantlets, eventually giving rise to a whole viable plant.

Multiple Shoots: Aerial parts of the plant such as shoot apices, hypocotyls, epicotyls, mesocotyl, cotyledon, node, leaf and stem, segments, have been known to posses the potential for multiple shoot induction in aseptic condition (Bajaj and Gosal, 1981; Mroginski and Kartha,1984).

The persistence of the meristems, a key factor in the plant kingdom offers scope for the experimental interventions in the production of more shoot meristems either by the development of adventitious meristems (multiple shoot apices) by auxiliary meristems (lateral buds) or by somatic embryogenesis (from cells of the plant body).

Tissue culture and Secondary Metabolites: Research in the area of plant tissue culture technology has resulted in the production of many pharmaceutical substances for new therapeutics. For the production of wide variety of pharmaceuticals like alkaloids, terpenoids, steroids, phenolics, flavanoids and amino acids. Successful attempts have been done to produce some of these valuable pharmaceuticals relatively in large quantities by cell culture some of these are illustrated here.

The advantage of this method is that it can ultimately provide a continuous, reliable source of natural products. Large scale plant tissue culture is found to be attractive alternative approach to traditional methods of plantations as it offers a controlled supply of biochemicals independent of plant availability (saje et al. 2000). Current developments in tissue culture technology indicate that transcription factor is efficient. New molecular tools for plant metabolic engineering to increase the production of valuable compounds (Gantet and Memelink, 2002). The production of secondary products is a particularly application of the plant tissue culture and personnel from oil and food producing companies and prominent among student of tissue culture

Materials and Methods

1. Media Preparation

I. Preparation of Plant Growth Hormones Solutions

Auxins- Auxin stock were usually prepared by dissolving in ethanol, not more than 0.3ml/10mg of Auxin to dissolve crystals, rapidly adding 10ml distilled water. Though the Auxin are thermo-stable, IAA is destroyed by low pH, light, oxygen and peroxidase. The NAA and 2,4-D are most stable forms.

Cytokinin's- The cytokinin stock were prepared in few drops of 1N NaOH to dissolve crystals. 10mg /10ml distilled water is used. It is thermostable and store for several month.

ii. Preparation of Murashige and Skoog (MS) Stock

- **Stock-1**
- M1 – Ammonium Nitrate (NH_4NO_3) 16.5 gm in 500 ml DW.
- M2- Magnesium Sulphate heptahydrate ($MgSO_47H_2O$) 3.7 gm in 500 ml DW.
- M3- Calcium Chloride ($CaCl_2$) 4.4 gm in 500 ml DW.
- M4-Potassium nitrate (KNO_3) 19 gm in 500 ml DW.
- M5- Potassium dihydrogen ortho-Phosphate (KH_2PO_4) 17 gm in 500 ml DW.
- **Stock - 2**
- $MnSO_4.4H_2O$– 2.23 gm
- $ZnSO_4$. $7H_2O$– 0.86 gm
- $CUSO_4$. $5H_2O$ – 0.0024 gm
- KI – 0.0830 gm
- $COCl_3$. $6H_2O$– 0.0024 gm
- H_3BO_3 – 0.62 gm

- Na_2MoO_4 – 0.024 gm

All chemicals are dissolved in 500 ml distilled water.

- **Stock – 3**

Dissolve salt of EDTA 3.71 gm in 500 ml of boiling double distilled water, and the gradually add 2.78 gm of $FeSO_4$ $7H_20$ in 500 ml. Keep on magnetic stirrer for at least one hour in hot condition. Solution turns golden yellow. Finally make the volume to 100 ml with distilled water.

iii. Vitamins

- Thymine – 10 mg/l
- Inositol – 100 mg/l
- Pyridoxine - 10 mg/l
- Glycine – 40 mg/l
- Sucrose – 30 mg/l

iv. Preparation of Culture Media

- Measure about 50 ml of stock solution 1 &add 50 ml distilled water & stir continuously in 1000 ml beaker.
- Add about 5 ml of stock solution 2 & add 5ml of stock 3.
- Add stock solution 4.
- Add 30 gm sugar for 1-liter media.
- Make the final volume to 1000 ml by using distill water.
- Adjust the pH to 5.8 using 1N NaOH/HCl.
- Add 7gm of agar to the above mixture & melt it by heating in microwave oven.
- Then it was cooled to 60°C *and medium was distributed in conical* flasks.
- Plug the flasks with cotton and then cover it with news paper and tie with thread.
- Autoclave the media at 15 lbs pressure at 121^0C for about 15 minutes.
- The culture was stored in incubator or culture room, allow the media to solidify prior to use.

v. Hormonal Combination

SR NO	HORMONAL COMBINATION	CONCENTRATION IN mg/l
1	BAP	0.1 0.5 1.0
2	2,4-D	0.1 0.5 1.0
3	BAP:2,4-D	0.1 : 0.1 0.1 : 0.5 0.1 : 1.0 0.5 : 0.1 0.5 : 0.5 0.5 : 1.0 1.0 : 0.1 1.0 : 0.5 1.0 : 1.0

2. Callus Induction

i. Propagation Fenugreek in MS Medium

- The seed were bought from the market and were surface sterilized and the were inoculated in MS medium.
- Surface sterilization for performed as follow.
- The seeds were surface sterilized before they were used for inoculation in media.
- Sterilization was done to avoid any contamination that can arose due to fungal or bacterial spores present on the surface of ex-plant part used.
- Healthy seeds were selected & it first washed with detergent.
- Detergent was removed completely by washing it 5-4 times in distilled water 0.1 % $Hgcl_2$ was used to sterilize the seeds for 2 minutes.
- The seeds were washed with 7-8 times with sterile distilled water under the hood of laminar flow.
- The seeds were blotted on sterile tissue paper before inoculation in conical flask to remove excess of moisture.
- Cultures were maintained at 25°C with continuous light three to four weeks after germination different plant organs were separated and placed saperately in MS culture media.

ii. Explant Inoculants and Subculture

- The plant organs (leaves) were cut into the appropriate sizes of 0.5 – 1.0 cm to inoculate in conical flasks containing MS media & also the different concentration of hormones.
- Once the callus was proliferated it was sub cultured once in 2 weeks.
- The calluses were grown and maintained at 25°C with a 16 h light photoperiod provided by fluorescent tubes.
- Three-time subculture calli were used for making the extract.
- Calli were harvested after six weeks of inoculation.

3. Callus Extracts & HPTLC

- **i. Extraction of calli:** 3gms of fresh callus was crushed in 8 ml methanol and Sonicated for 15 mint. The extract was centrifuge at 2000 rpm for 2 mints. The supernatant was used for analysis of Diosgenin.
- **ii. HPTLC analysis:** Solvent System used: - Chloroform: Methanol: Water (9.4: 0.5: 0.1) 10 µl of callus extract was loaded on pre-coated silica gel TLC plate of uniform thickness of 0.2 mm. Develop the plate in the solvent system till the solvent reached to the $3/4^{th}$ of silica gel plate. The plate was sprayed with 25% methanol sulphuric acid reagent and heated to 100^0c till the coloured bands appeared. Plates were visualized under 254 nm.

Result and Discussions

i. Studies on callus induction

Callus induction from leaf explants was studied in BAP, 2, 4-D at concentrations of 0.1 ,0.5 and 1.0 mg/l. Hormonal combination used were 0.1, 0.5, 1.0 mg/l of BAP each with 0.1, 0.5 ,1.0 mg/l of 2,4-D. The result obtained are shame in table 1 and plates (3 -11).

In MS media supplemented with BAP no callus developed up to 3^{rd} week at 0.1 mg/l concentration, and up to 7 days at 0.5 and 1.0 mg/l concentration. At the end 6^{th} week callus quantity was fair in 0.1 mg/l and good in 0.5 & 1.0 mg/l. More or less similar result were obtained with 0.1 mg/l concentration of 2, 4-D, hormones at 0.5 and 0.1 mg/l of 2,4-D, callus initiation began in the first week itself and good amount of callus were obtained in the 3^{rd} week.

The calli obtained in the MS – media supplemented with BAP and 2,4-D were healthy, initially they were light green in colour, the colour then changed to greenish yellow and finally became light brown.

In comparison to BAP, 2,4-D, proved to be better for callus initiation when incorporated as the sole hormones in MS media. Favorable effect of 2,4-D at a concentration of 1mg/l on *T. foenum geaecum* has also been reported by Shararch Rezaeian (2011) and for other plant by slesak et. al., 2005 (*Brassica napus*) and shoji et al, 2010 (*Tanacetam balsamita*).

Among various hormonal combination used lower concentration of BAP (0.1 mg/l) with various concentration (0.1, 0.5, 1.0 mg/l) of 2,4-D proved unfavorable for callus initiation. In these condition callus did not develop even after 6^{th} week of inoculation similar results were obtained with BAP: 2, 4D concentration of 0.5:0.5 mg/l, 1.0:0.1 mg/l and 1.0: mg Hormonal combination of1.0:0.5 mg/l and 0.5:1.0 mg/l gave best results which callus initiation was seen in the very first week which continued to increase with incubation time up to 6^{th} week reaching maximum in the 5^{th} week for 1.0:0.5 mg/l combination.

Result obtained with 0.5:1.0 mg/l combination were more similar to those obtained with mg/l combination except that the initiation of callus was seen in the 2^{nd} week instead of first.

The calli obtained in the MS media supplemented with the combination of hormones BAP and 2,4-D were healthy and spongy and produced in clump initially they were light green in colour then the colour stains changed to greenish yellow.

Callus growth follows a typical logarithmic pattern involving slow initial cell division induction period requiring Auxin, a rapid cell division phase involving active synthesis of DNA, RNA and protein followed by a gradual cessation of cell division along with differentiation of callus mass (Heartmann et. al., 1999). Among different hormones used for callus induction and proliferation, Auxins are reported to be most effective. Among different Auxins used, 2,4-D is reported to have better result for callus induction and proliferation. The hormone mediated callus induction and subsequent growth is dependent on certain factors which may trigger the complete chain of events that influence the ability of cultured cells to grow in an organized fashion. Plan tissues, therefore, must have receptors for hormones. Mockeviciute and Anisimoviene (1999) reported before that the hormonal levels are increased by specific receptor either on the cell membrane or within the cytoplasm.

ii. Quantification of Diosgenin

Quantification of Diosgenin was done by HPTLC method using the solvent system-Chloroform: Methanol: water (9.4:0.5:0.1). 25% Methanolic sulphuric acid was used to develop

the plates when Diosgenin appeared as dirty green bands. Results quantification studies are depicted in table 2, plate 12 and 13 and fig. 1 and 2.

Calli obtained in MS media incorporated with BAP showed maximum quantity of diosgenin (1.593 mg/l) in the 0.5 m/l concentration followed by 1.0 mg/l (0.649 mg/g) and 0.1 mg/l (0.507 mg/g) quantity of Diosgenin was less than control (0.730 mg/g) in 0.1 and 1.0 mg/l concentration.

Similar trend was seen in calli obtained on MS media incorporated with various concentration of 2,4-D. However maximum diosgenin quantity (0.851 mg/g) obtained at 0.5mg/l concentration of 2,4-D was almost half the quantity of diosgenin obtained with BAP at the same concentration.

Callus on MS media containing hormonal combination of 0.5:1.0 mg/l of BAP: 2,4-D gave maximum diosgenin production (1.680 mg/g), followed by 1.0: 0.5 mg/l and 0.5:0.1 mg/l concentration.

Even through the growth of calli was better in MS media containing hormonal combination of 1.0:0.5 mg/l and 0.5:0.1 mg/l, when compared to the one containing 0.5 mg/l of 2,4-D and 0.5 mg/l of BAP, quantity of diosgenin produced was far less than the later.

Enhancement of diosgenin production over control (0.730 mg/g) was maximum in hormonal combination of 0.5:1.0 mg/l concentration (1.680 mg/g) followed by BAP at 0.5 mg/l (1.593 mg/g) and last being 2,4-D at o.5 mg/l (0.851 mg/g).

These results suggest that leaf calli are the suitable starting materials for diosgenin production in vitro and are in keeping with the result obtained by Sharareh Rezaeian (2011), who reported maximum quantity of diosgenin in 2,4-D (0.5 mg/l), after 45 days in leaf explants of *T. foenum graecum.*

Table 1-Callus Induction in Leaf Explants of *Trigonella Foenum* in MS Media Supplemented With the Different Concentration of Hormones

Callus Induction							
Hormone/ Hormonal combination	**Concentration In mg/L**	**1st week**	**2nd week**	**3rd week**	**4th week**	**5th week**	**6th Week**
BAP	0.1	#	#	#	+	++	++
	0.5	#	+	++	+++	+++	+++
	1.0	#	+	++	+++	+++	+++
2,4-D	0.1	#	#	#	+	++	++
	0.5	+	++	+++	+++	+++	+++
	1.0	+	++	+++	+++	+++	+++

BAP:2,4-D	0.1 : 0.1	#	#	#	#	#	#
	0.1 : 0.5	#	#	#	#	#	#
	0.1 : 1.0	#	#	#	#	#	#
	0.5 : 0.1	#	+	++	+++	++++	++++
	0.5 : 0.5	#	#	#	#	#	#
	0.5 : 1.0	+	++	++	++++	++++	++++
	1.0 : 0.1	#	#	#	#	#	#
	1.0 : 0.5	+	++	+++	+++	++++	++++
	1.0 : 1.0	#	#	#	#	#	#

Sign.	Meaning
#	No Callus development
+	Minimum callus development
++	Fair callus development
+++	Good callus development
++++	Maximum callus development

Table 2: Effect of hormones on Diosgenin production in callus cultures of *Trigonella foenum-graecum*:

Leaf Extract for Control: 0.730

Fig. 1- Effect of

Hormone/ Hormonal combination	Concentration In mg/L	Quantity of Diosgenin (mg/g)
BAP	0.1	0.507
	0.5	1.593
	1.0	0.649
2, 4-D	0.1	0.436
	0.5	0.851
	1.0	0.622
BAP:2,4-D	1.0:0.5	0.681
	0.5:0.1	0.595
	0.5:1.0	1.680

hormones BAP & 2,4-D on Diosgenin production in by callus cultures of *Trigonella foenum-graecum*

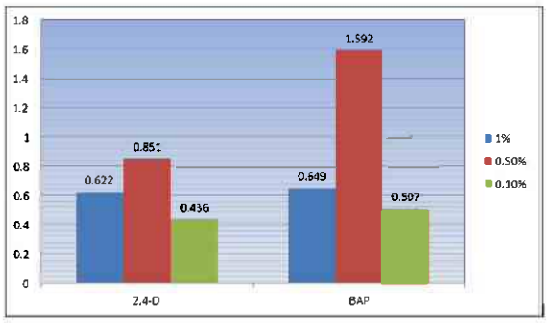


Fig. 2- Effect 0f hormonal combination (BAP & 2,4-D) on Diosgenin production in by callus cultures of *Trigonella foenum-graecum*

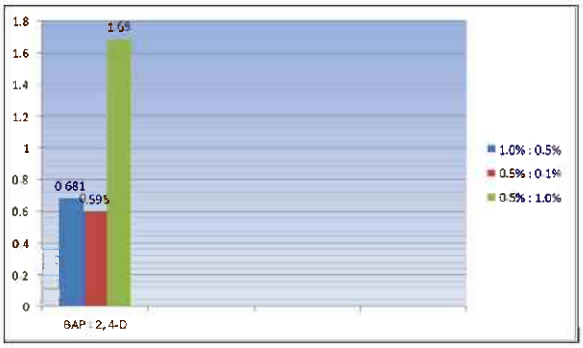


Plate 1: *Trigonella foenum-graecum*: In vitro	Plate 2: Leaves of Trigonella *foenum-graecum*: Control
	

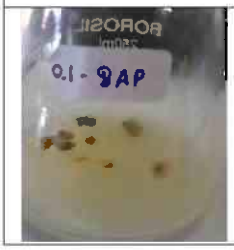
Plate: Callus initiated from BAP		
Plate 3: Callus of leaves elicit with 0.1% BAP	Plate 4: Callus of leaves elicit with 0.5% BAP.	Plate 5: Callus of leaves elicit with 1.0% BAP
		

Plate 6: Callus of leaves elicit with 0.1% 2,4-D	Plate 7: Callus of leaves elicit with 0.5% 2,4-D	Plate 8: Callus of leaves elicit with 1% 2,4-D
		

Plate: Callus initiated from Hormonal Combinations		
Plate 9: Callus of leaves elicit with 0.5% BAP: 0.1% 2,4-D	Plate 10: Callus of leaves elicit with 1.0% BAP: 0.5% 2,4-D	Plate 11: Callus of leaves elicit with 0.5% BAP: 1.0%2,4-D
		

Plate 12: HPTLC of Sample Obtained from Callus: (Before Devt.@254nm)

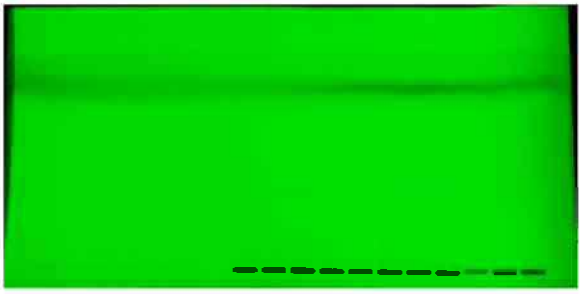


Plate 13: HPTLC of Sample Obtained from Callus: (After Devt.@254nm)

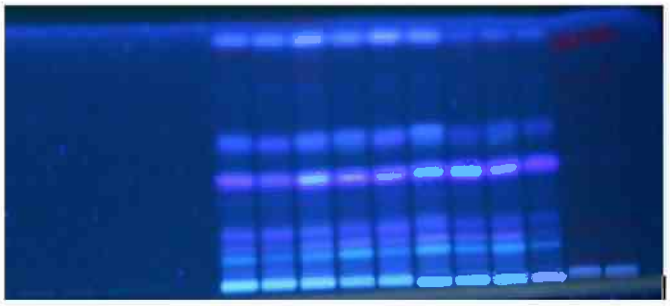


Plate 14: HPTLC of Sample Obtained from Callus: (After Devt.@White Light)

Summary

1. Surface sterilized seeds of fenugreek were germinated in MS medium.

2. Leaf explants of these in – Vitro germinated seeds were used to induce callus in MS medium supplemented with various concentration of BAP, 2,4-D & various combinations of these two hormones.
3. Maximum callus development occurred in BAP: 2,4-D combination at 0.5:1.0 mg/l concentration.
4. Quantification of Diosgenin in calli was done by HPTLC method.
5. Increase in the quantity of Diosgenin over mother plant was observed in 2, 4-D & BAP at 0.5 mg/l and hormonal combination of 0.5: 1.0 mg/l of BAP & 2,4-D.

CONCLUSION

1. Tissue culture is an alternate way of producing secondary metabolites of interest in a short period of time.
2. Production of medicinally valued Diosgenin can be enhanced by manipulation of hormones and their combinations in MS medium.
3. The present work can provide a standard protocol for callus induction, enhancement of Diosgenin production, extraction and quantification of steroidal compounds.

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5. *In- Vitro* Antifungal Activity of *Vitex Leucoxylon* Fresh Extract Against Two Fungal Species *Aspergillus Niger and Fusarium Oxysporum*

Udaybhan Yadav
Coordinator, Department of Microbiology (Interdisciplinary), Thakur Shyamnarayan Degree College, Kandivali East Mumbai.
Kunal Thakur
Assistant Prof. Department of Microbiology ID, Thakur Shyamnarayan Degree College, Kandivali East Mumbai.

Abstract

Plants square measure wealthy supply of medication antifungal and antiviral properties. These properties square measure employed by plants to safeguard themselves from foreign particles or pathogens. With the advancement of techniques currently we tend to were extracting the chemicals and victimisation to cure plant and animal infection. The in vitro antifungal activity of binary compound extract from *Vitex leucoxylon* plants utilized in ancient drugs for the treatment of varied diseases. Extract effectivity was evaluated victimisation the agar well diffusion assay against 2 fungi i.e. fungus genus Niger and Fusarium Exosporium. Zone of inhibition against fungi studied. the importance of those ends up in reference to ethnobotanical information is mentioned.

Keywords: Antifungal activity, healthful plants, *Vitex leucoxylon*, agar well diffusion assay.

Introduction

A variety of microorganisms as well as fungi, bacteria, viruses, harbour the soil. Out of those microorganisms, few area units useful for the soil, some don't damage the soil in any respect neither have the benefit of it, however a number of them have the potential to cause major injury to plant growth moreover because the quality of soil. They will result in reduction in plant growth that takes place by obstruction the nutrients that the plant should absorb, or they could cause physical damage that result in uncommon look of the plant successively reducing the market price. Soil borne illness will be a serious limitation for plants. The infective agent

might stay dormant within the soil and may become morbific once more as shortly because the host is out there. Preventive measures taken for the soil to avoid contamination and healthy environmental conditions will be effective for healthy plant growth. Fungi area unit present and infection caused thanks to them has become common. Morbific fungi area unit to blame for infecting flora diseases of the plants. They cause alterations throughout biological process stages as well as post-harvest. There's a good variety of flora genera that causes issues within the quality of fruit, vegetable or crop that area unit associated with aspects like organic process worth, organoleptic characteristics and restricted period of time. In some cases, fungi area unit indirectly to blame for allergic or cytotoxic disorders among shoppers thanks to the assembly of mycotoxins or allergens.

Generally, for the management of phytopathogenic fungi use of artificial fungicides is finished. These chemical fungicides have tremendous facet effects on the plant moreover as on the animals feeding on them the human intense the plant merchandise because it may contain harmful chemicals. Hence, there has been an increasing demand to create use of natural merchandise which will function antifungal agents inflicting less injury to the surroundings and living organisms. Biologically active compounds found in plants area unit a lot of safer than the artificial fungicides. Hence, extracts and oils of medicative plants has been used since it contains a great deal of secondary metabolites as compared to the other plants.

The effectivity of Murraya koenigii leaf extract on some microorganism and a plant life strain by disc diffusion technique (Mohar Singh 2011). In the gift study Murraya koenigii unremarkably known as "curry leaf" leaves extracts subjected to a screening study to find potential antimicrobial activity against Strains of Escherichia coli, eubacteria genus Cereus, Coccus aureus, Bacillus, causes respiratory illness, coccus epidermidis, bacteria genus aeruginosa, coccus faecalis, antibiotics resistant enterococcus and candida. The antibacterial drug activity of the merchandise was evaluated exploitation colonies growing in solid medium, establishing the zone of inhibition in vitro growth (ZOI). Plant (leaf) extract was conjointly used for the phytochemical tests for compounds that embrace Glycosides, Steroids, Tannins, Alkaloids, Flavonoids, Saponins, Quinone, macromolecule and Sugar in accordance with the strategies. The results showed that almost all of the microorganism strains (except E. coli, B. genus Cereus and S. faecalis) had intermediate impact at low concentration leaf extract (10

and 15%) of Murraya koenigii however the effectivity of the leaf extract might be increased by increasing the concentration of the extract.

Therapeutic Uses and pharmacologic Properties of *Vitex*, and their biologically active compounds (Peyman Mikaili, 2013) is documented in Iran and its leaves, flowers, and cloves are employed in in ancient medication for a protracted time. Analysis in recent decades has shown widespread pharmacologic effects of A. sativum and its organosulfur compounds particularly *Active constituents. Active constituents* represent one amongst most studied among these present compounds.

Materials and Method

Materials

A. Fungi Used

1. *Aspergillus Niger*

Isolation - *Aspergillus* culture was obtained by doing agar plate method and the culture was observed under microscope and sub-culturing was done to obtain pure cultures of *Aspergillus niger*.

Seven days old culture of fungi was used. The culture of *Aspergillus niger* was maintained on PDA medium throughout the project.

Taxonomic Position [According to the classification system of Alexopoulos and Mims (1979)]:

Kingdom	:	Mycetae
Division	:	Amastigomycota
Sub-division	:	Deuteromycotina
Form-class	:	Deuteromycetes
Form-sub-class	:	Hyphomycetidae
Form-order	:	Moniliales
Form-family	:	Moniliaceae
Form-genus	:	*Apergillus*
Form-species	:	*niger*

Figure I: *Aspergillus niger* pure culture

- **Macroscopic Characters** - The colony is black in colour and the reverse surface is white to light yellow in colour.
- **Microscopic Characters** - Hypha is septate and hyaline. Conidiophore is present and arises from the basal foot of the. Its conidiophores are smooth-walled, hyaline or turning dark towards the vesicle. Conidial heads are biseriate with the phialides borne

on brown, often septate metulae. Conidia are globose to sub globose (3.5-5.0 um in diameter), dark brown to black and rough-walled. It is known to create increased amount of pathogenicity in various species of plant.

2. *Fusarium Oxysporum*

Isolation - *Fusarium* culture was obtained by placing banana peel on agar which gave pinkish white colonies surrounding the peel. The colony was first identified under microscope and then sub-cultured and *Fusarium* oxysporum pure cultures were obtained.

Seven days old culture of fungi was used. The culture of *Fusarium oxysporum* was maintained on PDA medium throughout the project.

Taxonomic position [According to the classification system of Alexopoulos and Mims (1979)]

Kingdom	:	Mycetae
Division	:	Amastigomycota
Sub-division	:	Deuteromycotina
Form-class	:	Hyphomycetidae
Form-order	:	Moniliales
Form-family	:	Tuberculariaceae

- **Macroscopic Characters** - Colonies are initially white in colour and turn pinkish or purplish in colour at maturity. The reverse of the plate shows purplish colour.
- **Microscopic Characters** - Hyphae are septate and hyaline. Conidiophores are short and simple (mostly not branched). Conidia maybe ellipsoidal, slightly curved in shape.

B. Plants Used

Vitex leucoxylon - (white wood chaste tree, Family: Lamiaceae). Dried ground leaves of 50 grams were extracted soxlet sequentially in 300ml of Petroleum Ether, Dichloromet hane, Methanol, Ethanol, and Aqueous. The process was run for 48 hours after which the sample was concentrated using rotatory evaporator and freeze dried to powdered form. The dried extracts were weighed and kept in labelled sterile specimen bottles. For centuries it was used as a traditional remedy for most health-related disorders. Vitex is used for many conditions related to the heart and blood system. These conditions include high blood pressure, low blood pressure, high cholesterol, inherited high cholesterol, coronary heart disease, heart attack, reduced blood flow due to narrowed arteries, and "hardening of the arteries" (atherosclerosis).

C. Culture Medium Used

1. Liquid Culture Medium

a. Richard's Broth

Potassium Nitrate	: 10 g
Potassium Monobasic Phosphate	: 5 g
Magnesium Sulphate	: 0.25 g
Ferric Chloride	: 0.02 g
Sucrose	: 50 g
Distilled water	: 1000 ml

All above constituents was added in conical flask. The flask was plugged and autoclaved at the pressure of 15 lbs./sq. inch. at 121°C for 20 minutes. Streptomycin was added before using the broth.

b. Potato Dextrose Broth, (Bilgrami, 1978)

Potato	: 200 g
Dextrose	: 20 g
Distilled water	: 1000 ml

Peeled and chopped potatoes were boiled in distilled water, till the water became starchy. Solution was filtered through muslin cloth and the volume was raised to 1000 ml by adding distilled water. Filtrate was then transferred to a conical flask and dextrose was added. The flask was plugged and autoclaved at the pressure of 15 lbs./sq. inch at 121°C for 20 minutes. The pH of the media was checked (5.6 ±0.2). Streptomycin was added before pouring the plates.

2. Solid Culture Medium

a. Potato Dextrose Agar (PDA), (Bilgrami, 1978)

Potato	: 200 g
Dextrose	: 20 g
Agar	: 20 g
Distilled water	: 1000 ml

Peeled and chopped potatoes were boiled in distilled water, till the water became starchy. Solution was filtered through muslin cloth and the volume was raised to 1000 ml by adding distilled water. Filtrate was then transferred to a conical flask and dextrose and agar were added.

The flask was plugged and autoclaved at the pressure of 15 lbs./sq. inch at 121°C for 20 minutes. The pH of the media was checked (5.6 ±0.2). Streptomycin was added before pouring the plates.

b. Richard's Agar

Potassium Nitrate	: 10 g
Potassium Monobasic Phosphate	: 5 g
Magnesium Sulphate	: 0.25 g
Ferric Chloride	: 0.02 g
Sucrose	: 50 g
Agar	: 18 g
Distilled water	: 1000 ml

All above constituents was added in conical flask. The flask was plugged and autoclaved at the pressure of 15 lbs./sq. inch. at 121°C for 20 minutes. Streptomycin was added before using the agar.

3. Chemicals Used

- **Ethanol -** Used as a solvent for extraction of secondary metabolites from *Vitex leucoxylon.*
- **Dimethyl sulfoxide (DMSO)** -Used to dissolved ethanol extract of *Vitex leucoxylon*

4. Other Requirements

- Petri plates, Conical flasks, Micropipettes, cork borer, forceps, nichrome loop, Laminar Air Flow, etc.
- The entire experiment must be carried out in Aseptic conditions with sterilized glasswares.

Method

A. Aqueous Extract Preparation

Extract Preparation Of *Vitex Leucoxylon*

Vitex leaf were crushed and soaked in Distilled water and kept on shaker overnight (150 rpm for 24 hours). Next day the mixture was filtered using muslin cloth. The extract was stored at 4°C.

Extract of Different Concentration

Concentration (%)	Dried Powder (gm)	Distilled water (ml)
10%	10	100
20%	20	100
30%	30	100
40%	40	100
50%	50	100

B. Ethanolic Extract Preparation

Extract Preparation Of *Vitex Leucoxylon*:

Plant material (leaf) were carefully washed and oven-dried (120°C for 2 hours) and put in shade and aerated place later for drying completely. The dried *leaf* are ground into a fine powder and the powder is then soaked in Ethanol and kept on shaker overnight (150 rpm for 24 hours). Next day the mixture was filtered using muslin cloth then Whatmann filter paper and the concentrations were made using another organic solvent i.e. DMSO (Dimethyl sulfoxide). The concentrations made and used were 5%, 10%, 20%, 30%, 40%.

C. Testing Of Plant Extract (Aqueous/Ethanolic) Of *Vitex Leucoxylon*

1. In Liquid Culture Medium

49 ml of Richard's Broth was added to each conical flask and during the time of experiment 1 ml of plant extract (*Vitex leucoxylon*) was added to it. The total volume should sum up to be 50 ml in each conical flask. A control flask was maintained to compare the growth and efficacy. The flasks were plugged and autoclaved for sterilization and homogenization of Richard's broth and extract.

Preparation of the Concentrations of Aqueous Extract of *Vitex Leucoxylon*

Concentrations g/ml (wt./vol.)	Volume of extract (ml)	Volume of Richard's Broth (ml)
Control	0	50
5%	1	49
10%	1	49
20%	1	49
30%	1	49
40%	1	49
50%	1	49

After 7 days of incubation, the fungal growth was observed visually and biomass was measured for different concentrations.

Biomass Estimation - The flasks filtrate was separated by filtration with Whatmann no. 1 filter paper. The weight of filter paper was taken prior. After complete filtration, the mat left on the filter paper was dried completely in oven/incubator (180°C for 2 hours). The dry weight was taken and biomass was calculated.

Observations are recorded and given below.

2. On Solid Culture Medium

The antifungal activity on Solid medium was studied using Agar well diffusion method. In Agar well diffusion method, sterilized PDA is poured into the petri-plates aseptically. Then

the media was allowed to cool. About 5 mm diameter well (reservoir) was made in the centre of the petri-plate using a sterilized cork borer. The plates were then inoculated with fungal discs at equidistant radii. About 100 µl of the plant extract of respective concentration was filled in the well by using sterilized micropipettes. A control petri-plate was maintained without extract for comparison purpose.

Petri-plates were incubated for 7 days to observe the zone of inhibition. The observations and pictures are given below.

Observations and Results

A. Observations for Aqueous Extracts

1. In Liquid Culture Media

Excellent	+++++
Very Good	++++
Good	++
Fair	++
Poor Growth	+
No Growth	-

Table no 1.a.: For *Vitex leucoxylon* on *Aspergillus Niger*

Concentration of Extract	Weight of Whatmann filter paper (g)	Weight of paper + Biomass (After drying)	Weight of Biomass (g)	Visual Fungal growth in PD Broth
Control	0.830	1.612	0.782	+++++
10%	0.820	1.517	0.697	+++
20%	0.822	1.484	0.662	+++
30%	0.831	1.478	0.647	++
40%	0.821	1.440	0.619	++
50%	0.823	0.874	0.051	-

Figure 1.a.: *Aspergillus niger* grown on PD Broth + *Vitex leucoxylon* aqueous extract

Table No 1.B.: For *Vitex Leucoxylon* on *Fusarium Oxysporum*

Concentration of Extract	Weight of Whatmann filter paper (g)	Weight of paper + Biomass (After drying)	Weight of Biomass (g)	Visual Fungal growth in PD Broth
Control	0.831	1.809	0.978	+++++
10%	0.882	1.158	0.276	+++
20%	0.825	1.078	0.253	+++
30%	0.812	0.988	0.176	++
40%	0.822	0.895	0.073	++
50%	0.841	0.871	0.030	-

Figure 1.b.: *Fusarium oxysporum* grown on PD Broth + *Vitex leucoxylon* aqueous extract

2. On Solid Culture Media

Table no 2-For *Vitex Leucoxylon* on *Fusarium Oxysporum*

Concentration of Extract	Inhibition zone observed on the 8th day (cm)		
	I	II	Average
Control	0.2	0.6	0.3
10%	0.2	0.8	0.5
20%	0.1	0.1	0.1
30%	0.1	0.6	0.35
40%	0.1	0.2	0.15
50%	0.3	0.4	0.35

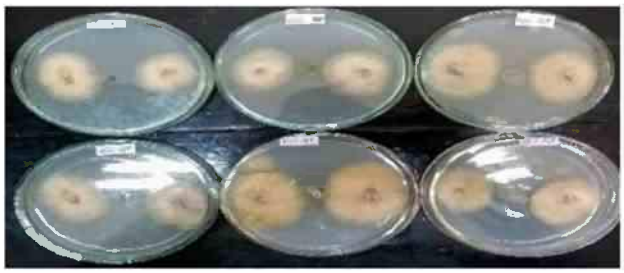

Figure 2: *Fusarium Oxysporum* Inoculated On PDA Plate with *Vitex Leucoxylon* Aqueous Extract

B. Observations for Ethanolic Extracts

1. In Liquid Culture Media

Table no 3.a.: For *Vitex Leucoxylon* on *Aspergillus Niger*

Concentration of Extract	Weight of Whatmann filter paper (g)	Weight of paper + Biomass (After drying)	Weight of Biomass (g)	Visual Fungal growth in Richard's Broth
Control	0.730	1.789	1.059	+++++
5%	0.737	1.728	0.991	++++
10%	0.731	1.650	0.919	+++
20%	0.722	1.642	0.920	+++
30%	0.718	1.548	0.830	++
40%	0.716	1.083	0.367	+

Figure 3.a.: *Aspergillus Niger* grown on Richard's Broth + *Vitex leucoxylon* Ethanolic Extract

Table no 3.b.: For *Vitex Leucoxylon* on *Fusarium Oxysporum*

Concentration of Extract	Weight of Whatmann filter paper (g)	Weight of paper + Biomass (After drying)	Weight of Biomass (g)	Visual Fungal growth in Richard's Broth
Control	0.896	1.199	0.303	++++
5%	0.886	1.102	0.216	+++
10%	0.866	0.982	0.116	++
20%	0.889	0.967	0.078	+
30%	0.857	0.925	0.068	+
40%	0.861	0.898	0.037	-

Figure 3.b.: *Fusarium oxysporum* grown on Richard's Broth+ *Vitex leucoxylon* ethanolic extract

2. On Solid Culture Media

Table no 4: For *Vitex leucoxylon* on *Fusarium Oxysporum*

Concentration of Extract	Inhibition zone observed on the 8th day (cm)		
	I	II	Average
Control	0.5	0.7	0.6
5%	0.2	0.2	0.2
10%	0.1	0.2	0.15
20%	0.2	0.1	0.15
30%	0.2	0.1	0.15
40%	0.2	0.4	0.3

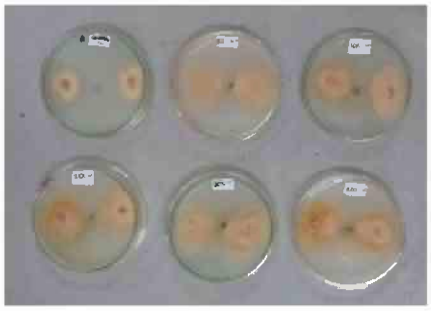

Figure 4: *Fusarium Oxysporum* Inoculated on Richard's Agar plate with *Vitex Leucoxylon* Ethanolic extract

Results

The results indicated that *Vitex leucoxylon* was effective as an antifungal extract because it showed considerable amount of Zone of Inhibition. Although, *Vitex leucoxylon* was resistant, still it did show a fair amount of reduction in biomass at higher concentrations of plant extracts (both aqueous and ethanolic). *Fusarium oxysporum* on the other hand was very sensitive to plant extracts and hence was very slow growing too.

A. On Liquid Culture Medium

For Aqueous Extracts of *Vitex Leucoxylon*

- The growth of *Aspergillus niger* was completely inhibited at 50% concentration of aqueous *Vitex* extract. Other concentrations (10%, 20%, 30%, 40%) showed fair amount of growth.
- The growth was very slow for *Fusarium oxysporum* on *Vitex* aqueous extract on 10%, 20% and 30%. 40% showed a great amount of inhibition whereas, 50% didn't show growth at all. Hence, minimum inhibitory concentration was found in 50%.

For Ethanolic Extract of *Vitex Leucoxylon*

- *Aspergillus* showed a considerable reduction in mycelial growth on 40% ethanolic extract of *Vitex*. It also showed colour changes (brown pigmentation) as compared to the *Aspergillus* control (on Richard's media).
- The growth of *Fusarium* was very slow in ethanolic extract of *Vitex* and it also showed complete inhibition at 40%. The mycelial mat quantity varied considerably in 20%, 30% of ethanolic extract as compared to the Control used.

B. On Solid Culture Medium

Aspergillus niger is very resistant to the plant extracts (Aqueous & Ethanolic). When inoculated on equidistant radii it tends to overgrow on the reservoir in between too. Hence, agar well diffusion method was not an effective method to test the inhibition for *A. niger*.

The agar well diffusion method showed a clear inhibition zone on higher concentrations of both Aqueous and Ethanolic extracts for *Fusarium oxysporum* only.

1. **For Aqueous Extracts of *Vitex Leucoxylon* (*Vitex*)** - As compared to the control plate and other concentrations (10%, 20%, 40%), *Fusarium oxysporum* showed very clear inhibition in 30% and 50% concentrations
2. **For Ethanolic Extract of *Vitex Leucoxylon* (*Vitex*)** - 5% shows overgrowth of *Fusarium oxysporum* on the well. 10% onwards there is a major inhibition seen. 40% show maximum inhibitory zone as compared to rest of the concentrations.

Discussion

Plants are susceptible to fungal attacks which causes major loss of yield and causes damage to the quantity and quality of crops and its product. In order to overcome this problem synthetic fungicides are brought to use to lessen the effects of fungi. The usage of chemical/ synthetic fungicides results in accumulation of toxic chemicals in the plant which may lead to undesirable effects. To avoid these problems and promote healthy growth of plants simultaneously eradicating the fungi from the plant, there is a growing need to create natural fungicides which has minimal or no side effects on the plant.

Both fungi (*Aspergillus niger* and *Fusarium oxysporum*) which fall under the sub division Deuteromycotina, against which antifungal extracts were tested are pathogenic fungi which caused disease of the plants and are most common contaminants of crops worldwide.

The active components present in *Vitex leucoxylon* were extracted using water (aqueous) and ethanol (organic solvent).

According to a research study, *Vitex leucoxylon* consists of organo-sulphur compounds, out of which Active constituents is the main sulphur containing compound. Active constituents is known to show inhibition mostly in ethanolic extracts as compared to aqueous extract. *Active constituents*in *Vitex* extract showed inhibitory activity against murine pulmonary aspergillosis and against *Candida albicans*. High zones of inhibition were noticed against dermatophytic fungi in ethanolic extracts. (Peyman Mikaili, Surush Maadirad, Milad Moloudizargari, Shahin Aghanjanshakeri and Shadi Sarahroodi, 2013).

For testing the efficacy of extracts, (aqueous and ethanolic) of *Vitex leucoxylon* on pathogenic fungi *A. niger* and *Fusarium oxysporum*. 2 types of methods were performed - 1. Liquid culture media method (Broth method) and 2. Solid culture media method (Agar well-diffusion method).

On Liquid culture media for aqueous *Vitex* extract showed complete inhibitory effect on both *A. niger* as well as *F. oxysporum* on 50% concentrations and major reduction in mat at 40% concentration. Minimum Inhibitory Concentration here is 50%.

On solid culture media maximum inhibitory zones were observed at 50% concentration in *Fusarium oxysporum* for *Vitex* aqueous plant extracts. *Aspergillus niger* does not show a zone and also grew on the reservoir.

Ethanolic *Vitex* extract showed reduction at 40% in *A. niger* and complete inhibition in *F. oxysporum* at 40%. On solid media however, *Vitex* showed maximum inhibition zone at 40%.

Conclusion

The above investigation has brought us to the conclusion that Ethanolic and Aqueous plant extract of *Vitex leucoxylon* are effective against the both pathogenic fungi i.e. *Aspergillus niger* (which causes mold disease) and *Fusarium oxysporum* (which is a wilt causing fungi). In this particular study Aqueous extracts showed better inhibitory results as compared to Ethanolic solvents. *Aspergillus niger* showed more resistance against *Vitex* plant extracts at most concentrations. *Fusarium* being sensitive to the antifungal compounds present in *Vitex* extracts has shown considerable decrease in biomass and the plant extracts (aqueous and ethanolic) showed complete inhibition at the highest concentrations used (50% for Aqueous extract and 40% for Ethanolic extract).

The variations in the effects of plant extracts on inhibiting the pathogenic fungi maybe due to the variable concentrations of plant extract we used. Also, the type of solvent used for plant extract preparation may have a profound effect on inhibition.

Studies indicate that the higher the concentration of plant extract (both aqueous and ethanolic) the higher is effect of inhibition. Higher concentrations of *Vitex* extract for both types show tremendous reduction in *Aspergillus niger* growth and complete inhibition at a certain percentage for *Fusarium oxysporum. Aspergillus niger* does not show inhibition zones even higher concentrations whereas, *Fusarium oxysporum.* does.

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6. Use of Essential Oils as Natural Preservative for Cosmetic Products Like Cream & Gel

Udaybhan Yadav
Coordinator, Department of Microbiology (Interdisciplinary)
Kunal Thakur
Assistant Prof. Department of Microbiology ID

Abstract

Skin care is the main part of a person's overall appearance. Our daily routine we use cosmetics which most of them are synthetic product. Many herbal product industries use synthetic material as preservatives like methyl paraben, propyl paraben, butyl paraben which are preserve cosmetic product long life. But some of them carcinogenic. The plant is having antibacterial, antifungal, antioxidant, anthelminthic, carminative, aromatic, stimulant, expectorant, anti-inflammatory properties. Many plant extracts, spices, essential oils having property to kill the micro-organism or to stop their growth and preserve cosmetic product long life. In this study main aim is to estimate moisture content and stability at different temperature. Physicochemical test for herbal cosmetics and analysis of Microbial assays. Preservatives are commonly used to prevent microbial growth and spoiling of the cosmetic products. Plant selected for natural preservatives were dose not change in their pH, moisture content, texture, stability of herbal cosmetic products at 15 days of interval.

Key Words: Skin care, Cosmetics, Microbial Assay, natural Preservatives

Introduction

Natural skin care is the care of the skin using naturally-derived ingredients (such as herbs, roots, essential oils and flowers) combined with naturally occurring carrier agent, preservatives, surfactants, humectants and emulsifiers (everything from natural soap to oils to pure water). The classic definition of natural skin care is based on using botanically sourced ingredients currently existing in or formed by nature, without the use of synthetic chemicals, and manufactured in such a way to preserve the integrity of the ingredients. As a result of this definition, many people who use natural skin care products generally make their own products at home from naturally occurring ingredients. Many people use natural skin care recipes to make

remedies to care for their skin at home. Many spas and skin care salons now focus on using more naturally-derived skin care products.

The term "Cosmetics" is derived from Greek word Kosmin meaning to decorate. These are the natural or synthetic substances applied to a person's body to cleanse, promote attractiveness or alter the appearance. The ancient Egyptians applied perfumes and anointing oils to the body as early as 4000 B.C. Since Vedic period, India has rich and varied flora of herbal and medicinal plants.

The Aryan period witnessed the use of turmeric, saffron, indigo, raktachandan etc., for beautification. Using Mehndi for dying and conditioning hair was also practiced in the older times. Thus, the concept of beauty and herbal cosmetics is as old as mankind and civilization. Natural skin care has its roots in the 4th millennium BC in China and the Middle East. In the modern age many people with unique skin types and needs (sensitive skin, dry skin, and oily skin) have turned to natural skin care solutions.

Some examples of natural skin care ingredients include Jojoba, Safflower oil Some examples of natural skin care ingredients include Jojoba, Sunflower oil, Rose hip seed oil, shea butter, beeswax, witch hazel, aloe Vera, tea tree oil, and chamomile. Many of these natural ingredient combinations can be tailored specifically to the individual's skin type or skin condition.

The term natural has considerable market value in promoting skin care cosmetic products to consumers. preservatives are commonly used to preserve the safety and efficacy in these products.

Why we Use preservatives?

Especially as a cream in jars, cosmetic product come in frequent contact with the non-sterile human skin, there by coming easily contaminated by microbes containing water, oils, peptides and carbohydrates cosmetics are a very good medium for growth of microbes. All these factors contribute to the fact that cosmetic products need very good preservative to prevent microbial growth and spoiling of the cosmetic product and also infection of the skin. Generally, shampoos and other rinse products need less preservative than leave on product as creams and decorative cosmetic.

Preservatives can maintain the shelf life of product for about 2 to 3 years. With ingredients such as essential oils and antioxidants that act as preservative natural skin care

products can be produced with a limited shelf life. Essential oils are natural substances that are powerful preservative but are not extensively used to preserve cosmetic products. They are derived from flowers, leaves, grasses and woody plants.

What Makes a Good Preservative?

To overcome the broad spectrum of microbes, and at the same time, not to be harmful to the skin and deleterious to other ingredients in a cosmetic product it is critical to use the right preservative. The optimal preservative should have the following attributes.

i. Broad spectrum activity (bacteria and fungi)
ii. Be effective over the anticipated shelf life
iii. Be preferably liquid and water soluble
iv. Be effective over a wide pH range
v. Not be deactivated by other ingredients
vi. Be odorless, colorless and safe

Selected Natural Oil and Extract as Preservatives

1. Clove oil *(Syzygium aromaticum L.)*
2. Turmeric oil *(Curcuma longa)*
3. Rosemary oil *(Rosmarinus officinalis)*
4. Lavender oil *(Lavandula angustifolia)*
5. Tea tree oil *(Melaleuca alternifolia)*
6. Cinnamon oil *(Cinnamomum zeylanicum)*
7. Eucalyptus oil *(Eucalyptus globulus)*

Why Select These Oils as Preservatives?

The use of Essential oils in the production of cosmetics and related products may have several advantages. Essential oils in cosmetic formulations at relatively high concentrations are likely to provide skin benefit. Essential oils have been shown to possess antibacterial, antifungal, antiviral insecticidal and antioxidant properties. Some oils have been used in cancer treatment. Some other oils have been used in aromatherapy and fragrance industries. Essential oils are a rich source of biologically active compounds. There has been an increased interest in looking at antimicrobial properties of extracts from aromatic plants particularly essential oils.

Essential oils such as Cinnamon, Clove, Eucalyptus, Geranium, Lavender, Rosemary, and Turmeric have been traditionally used by people for various purposes in different parts of the

world. Cinnamon, Clove and Rosemary oils had shown antibacterial and antifungal activity; Cinnamon oil also possesses antidiabetic property. Cinnamon oil showed promising inhibitory activity even at low concentration. Rosemary oils possess antioxidant property. Lavender oil has shown antibacterial and antifungal activity; it was also found to be effective to treat burns and insect bites.

Materials and Methods

The Experimental Material: - Gel and Cream, Essential oils use as Preservatives (Collected from Gayatri Herbal PVT. LTD.)

Gel Formation

Compos ition: Base Aloe Gel (For ~100ml), 88ml Spring Water, 1g/2ml tsp Xanthan Gum, 10ml Aloe Vera extract, 12 drops(0.6ml/g) Preservative.Method: Measure the water in a jug & pour into a bowl. Weigh 1gm or measure a level 2ml measuring spoon with Xanthan Gum powder.Sprinkle the Xanthan Gum powder over the water little by little, whisking vigorously. If Gel gets lumpy, blend until smooth with a stick blender. When there are no more lumps, stop whisking or blending immediately. Add all of the pre-measured ingredients and mix in to the Gel. All the methods will keep for 1.5-2years.

Cream Formation

Composition

Fat Stage (75-800C)- 6ml Vegetable oil, 2g Bees wax, 2g acetyl Alcohol, 3g VE Emulsifier

Water Stage (75-800C)- 4f MF Emulsifier, 75ml Boiling Spring Water, 4ml/g Glycerin, 12 drops(0.6ml/g) preservative Third Stage (40-350C)- 2ml NFF Moisturizer, 1ml/g Vitamin E oil (undiluted) Fourth Stage (35-250C)- 20 drops Essential oil,

Method

i. Heat the fat stage ingredients in a double boiler until all of the ingredients have melted and the temperature has risen to 75-800C. There is no use a whisk at this stage.

ii. After boiling the Spring Water in a Kettle, measure it according to the recipe and pour it over the MF Emulsifier and the Glycerin, Sorbitol and preservative, which have put into a separate double boiler.

iii. Whisk the water stage ingredients well together, making sure that the MF Emulsifier powder is fully dissolved in the water and that don't have any lump. Then allow the mixture to heat to 75-800C.

iv. When both fat and water stages are over 750C, remove both double boilers from the hob, keeping the water stage mixture hot by leaving it on the top half of the double boiler.

v. Now pour the melted fat stage into the water stage in a thin, steady stream, while continuously whisking the mixture from side to side for 5 minutes. If necessary, use a spatula to scrape the mixture from the sides of the bowl.

vi. Allow the mixture to cool, stirring all the time. Speed up by the cooling process by replacing the hot water in the double boiler with very cold water. In the process of cooling down, the mixture becomes a cream and will reach it thickest consistency when it is has cooled down to room temperature.

vii. Stir in the Third stage ingredients when the mixture has cooled to under 400C. Continue stirring until the mixture has cooled under 300C then thoroughly mix in the Essential oils.

viii. Pour the cream into jar and label.

Concentration of Essential Oil Used as Preservatives

- Clove oil (0.1%, 0.2%, 0.3%, 0.4%, 0.5%)
- Tea tree oil (0.1%, 0.2%, 0.3%, 0.4%, 0.5%)
- Cinnamon oil (0.1%, 0.2%, 0.3%, 0.4%, 0.5%)
- Eucalyptus oil (0.1%, 0.2%, 0.3%, 0.4%, 0.5%)

- Combination of oil (0.1%, 0.2%, 0.3%, 0.4%, 0.5%)

Method

Sample	Microbial assays (Various conc.)	Qualitative test (Various conc.)
Cream	15 day's analysis	15 day's analysis
	30 day's analysis	30 day's analysis
	45 day's analysis	45 day's analysis
	60 day's analysis	60 day's analysis
Gel	15 day's analysis	15 day's analysis
	30 day's analysis	30 day's analysis
	45 day's analysis	45 day's analysis
	60 day's analysis	60 day's analysis

Preparation of Sample

i. Cinnamon oil, Eucalyptus oil, Tea tree oil, Clove oil and the combination of these oils are added in Cream and gel as preservatives separately.

ii. The preservatives are added in cream and gel at different concentration such as 0.1%, 0.2%, 0.3%, 0.4% and 0.5% separately.

Various Microbial Assay & Qualitative Changes was Estimated by Following Methods

i. Microbial Assays
   - Bacterial contamination
   - Fungal contamination

ii. Qualitative Study
   - pH test
   - Visual appearance
   - Stability at 450C and 540C
   - Solubility
   - Skin irritation test

Observation and Result

1. Test for Qualitative Study

Table. 1.1: Preliminary Test (Qualitative Assay) for Gel & Cream

Test	Preliminary Test for Gel	Preliminary Test for Cream
pH	6	6.5
Visual appearance	Soft	Smooth, soften
Stability at 45^0C	Dried	Dried
stability at 54^0C	Dried	Dried, oil layer separated
Skin irritation test after 24 hours	No skin irritation	No skin irritation
Disperse in water	Colorless solution obtained	Turbid solution
Moisture content	98%	80%

Table: 1.2: Preliminary Test for Cream at Various Concentrations

Oil use as Preservative	Conc.	pH	Visual appearance	Stability 45^0 C	Stability 54^0C	Irritation test
Mix oil	0.1%	6.5	Smooth	Dried	oil layer separated	No skin irritation
	0.2%	6.5	Smooth	Dried	oil layer separated	No skin irritation
	0.3%	6.5	Soft	No changes	oil layer separated	No skin irritation
	0.4%	6.5	Soft	No changes	oil layer separated	No skin irritation
	0.5%	6.5	Soft	No changes	oil layer separated	No skin irritation
Cinnamon oil	0.1%	6.5	Smooth	Dried	oil layer separated	No skin irritation
	0.2%	6.5	Smooth	Dried	oil layer separated	No skin irritation
	0.3%	6.5	Soft	Dried	oil layer separated	No skin irritation
	0.4%	6.5	Soft	No changes	oil layer separated	No skin irritation
	0.5%	6.5	Soft	No changes	oil layer separated	No skin irritation
Eucalyptus oil	0.1%	6.5	Soft	Dried	oil layer separated	No skin irritation
	0.2%	6.5	Soft	Dried	oil layer separated	No skin irritation
	0.3%	6.5	Soft	Dried	oil layer separated	No skin irritation

	0.4%	6.5	Soft	No changes	oil layer separated	No skin irritation
	0.5%	6.5	Soft	No changes	oil layer separated	No skin irritation
Tea tree oil	0.1%	6.5	Smooth	Dried	oil layer separated	No skin irritation
	0.2%	6.5	Smooth	Dried	oil layer separated	No skin irritation
	0.3%	6.5	Soft	No changes	oil layer separated	No skin irritation
	0.4%	6.5	Soft	No changes	oil layer separated	No skin irritation
	0.5%	6.5	Soft	No changes	oil layer separated	No skin irritation
Clove oil	0.1%	6.5	Smooth	Dried	oil layer separated	No skin irritation
	0.2%	6.5	Smooth	Dried	oil layer separated	No skin irritation
	0.3%	6.5	Smooth	Dried	oil layer separated	No skin irritation
	0.4%	6.5	Smooth	No changes	oil layer separated	No skin irritation
	0.5%	6.5	Smooth	No changes	oil layer separated	No skin irritation

Table: 1.3: Preliminary Test for Gel at Various Concentrations

Oil use as Preservative	**Conc.**	**pH**	**Visual appearance**	**Stability 45^0 C**	**Stability 54^0C**	**Irritation test**
Mix oil	0.1%	6	Soft	No changes	Dried	No skin irritation
	0.2%	6	Soft	No changes	Dried	No skin irritation
	0.3%	6	Soft	No changes	Dried	No skin irritation
	0.4%	6	Soft	No changes	Dried	No skin irritation
	0.5%	6	Soft	No changes	Dried	No skin irritation
Cinnamon oil	0.1%	6	Soft	No changes	Dried	No skin

						irritation
	0.2%	6	Soft	No changes	Dried	No skin irritation
	0.3%	6	Soft	No changes	Dried	No skin irritation
	0.4%	6	Soft	No changes	Dried	No skin irritation
	0.5%	6	Soft	No changes	Dried	No skin irritation
Eucalyptus oil	0.1%	6	Soft	No changes	Dried	No skin irritation
	0.2%	6	Soft	No changes	Dried	No skin irritation
	0.3%	6	Soft	No changes	Dried	No skin irritation
	0.4%	6	Soft	No changes	Dried	No skin irritation
	0.5%	6	Soft	No changes	Dried	No skin irritation
Tea tree oil	0.1%	6	Soft	No changes	Dried	No skin irritation
	0.2%	6	Soft	No changes	Dried	No skin irritation
	0.3%	6	Soft	No changes	Dried	No skin irritation
	0.4%	6	Soft	No changes	Dried	No skin irritation
	0.5%	6	Soft	No changes	Dried	No skin irritation
Clove oil	0.1%	6	Soft	No changes	Dried	No skin irritation
	0.2%	6	Soft	No changes	Dried	No skin irritation

	0.3%	6	Soft	No changes	Dried	No skin irritation
	0.4%	6	Soft	No changes	Dried	No skin irritation
	0.5%	6	Soft	No changes	Dried	No skin irritation

2. Analysis Of Bacterial Contaminations (In Tvc)

A. Eucalyptus oil use as preservative

Table.2.1: Bacterial contamination observed in Gel (No. of colonies) – (Photo plate 5.A)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
15th day	10	4	3	3	2	1
30th day	14	5	3	2	2	1
45th day	29	5	3	3	2	1
60th day	33	5	5	4	3	1

Table.2.2: Bacterial contamination observed in Cream (No. of colonies) – (Photo plate 5.B)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
15th day	4	4	3	3	2	1
30th day	10	3	3	3	2	1
45th day	20	4	2	3	1	1
60th day	22	5	4	3	2	2

B. Clove Oil as Preservative

Table.2.3: Bacterial contamination observed in Gel (No. of colonies) – (Photo plate 5.A)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
15th day	10	5	4	2	1	1
30th day	14	6	5	3	2	1
45th day	29	7	4	3	2	2
60th day	33	10	7	3	3	2

Table.2.4: Bacterial contamination observed in Cream (No. of colonies) – (Photo plate 5.B)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
15th day	4	4	4	2	1	1
30th day	10	5	4	3	1	1
45th day	20	5	4	3	2	1
60th day	22	7	5	3	2	1

C. Cinnamon Oil as Preservative

Table.2.5: Bacterial Contamination Observed in Gel (No. Of Colonies) – (Photo Plate 5.A)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
15th day	10	5	4	2	2	1
30th day	14	6	4	2	1	1
45th day	29	6	5	4	2	1
60th day	33	8	7	4	1	0

Table.2.6: Bacterial Contamination Observed In Cream (No. Of Colonies)

(Photo Plate 5.B)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
15th day	4	4	3	3	1	1
30th day	10	4	3	2	1	1
45th day	20	6	2	3	2	1
60th day	22	7	5	3	3	0

D. Tea Tree Oil as Preservative

Table.2.7: Bacterial Contamination Observed in Gel (No. Of Colonies) – (Photo Plate 5.A)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
15th day	10	6	4	2	2	1
30th day	14	7	4	2	2	1

45th day	29	8	4	2	1	1
60th day	33	9	6	3	1	1

Table.2.8: Bacterial Contamination Observed in Cream (No. Of Colonies)

(Photo Plate 5.B)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
15th day	4	3	2	2	1	1
30th day	10	4	3	1	1	1
45th day	20	3	3	2	1	0
60th day	22	6	4	3	0	0

E. Combination of All Oil as Preservative

Table.2.9: Bacterial Contamination Observed in Gel (No. Of Colonies) – (Photo Plate 5.A)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
15th day	10	3	3	2	1	1
30th day	14	3	3	4	2	1
45th day	29	4	3	3	1	0
60th day	33	4	2	3	1	0

Table.2.10: Bacterial Contamination Observed in Cream (No. Of Colonies)

(Photo Plate 5.B)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
15th day	4	3	2	2	1	1
30th day	10	3	2	1	1	1
45th day	20	3	3	2	1	0
60th day	22	4	3	2	0	0

3. Analysis of Fungal Contamination (In Tvc)

A. Eucalyptus Oil As Preservative

Table.3.1: Fungal Contamination Observed in Gel (No. Of Colonies) – (Photo Plate 6.A)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
15th day	3	2	2	2	1	0
30th day	5	3	2	2	1	1
45th day	9	4	2	2	1	1
60th day	13	4	3	3	1	1

Table.3.2: Fungal Contamination Observed In Cream (No. Of Colonies) – (Photo Plate 6.B)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
15th day	3	2	2	1	1	0
30th day	4	2	2	1	1	1
45th day	7	2	3	2	1	1
60th day	11	4	3	2	1	1

B. Clove Oil as Preservative

Table.3.3: Fungal Contamination Observed in Gel (No. Of Colonies) – (Photo Plate 6.A)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
15th day	3	3	2	2	1	1
30th day	5	3	2	2	1	1
45th day	9	4	3	2	2	1
60th day	13	6	2	4	2	2

Table.3.4: Fungal contamination observed in Cream (No. of colonies)

(Photo plate 6.B)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
15th day	3	3	2	1	1	0
30th day	4	3	3	1	1	1
45th day	7	3	2	2	1	1
60th day	11	5	4	2	2	1

C. Cinnamon Oil as Preservative

Table.3.5: Fungal Contamination Observed in Gel (No. Of Colonies) – (Photo Plate 6.A)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
15th day	3	3	2	1	1	0
30th day	4	3	3	1	1	1
45th day	7	3	2	2	1	1
60th day	11	5	4	2	2	1

Table.3.6: Fungal Contamination Observed in Cream (No. Of Colonies) – (Photo Plate 6.B)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
15th day	3	2	1	1	0	0
30th day	4	2	1	1	1	1
45th day	7	2	2	1	1	1
60th day	11	3	2	2	1	1

D. Tea Tree Oil as Preservative

Table.3.7: Fungal Contamination Observed in Gel (No. Of Colonies) – (Photo Plate 6.A)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
15th day	3	3	2	1	1	0
30th day	5	3	2	2	1	1
45th day	9	3	3	2	1	1
60th day	13	5	3	2	2	1

Table.3.8: Fungal Contamination Observed in Cream (No. Of Colonies) – (Photo Plate 6.B)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
15th day	3	2	2	1	1	0
30th day	4	2	2	1	1	1
45th day	7	3	2	1	1	1
60th day	11	3	2	2	1	1

E. Combination of all Oil as Preservative

Table.3.9: Fungal Contamination Observed in Gel (No. Of Colonies) – (Photo Plate 6.A)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
15th day	3	2	2	1	0	0
30th day	5	3	2	2	1	0
45th day	9	3	2	2	1	1
60th day	13	3	2	2	2	1

Table.3.10: Fungal Contamination Observed in Cream (No. Of Colonies)

(Photo Plate 6.B)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
15th day	3	1	1	1	0	0
30th day	4	2	1	1	1	0
45th day	7	2	2	1	1	1
60th day	11	2	2	2	1	1

Photo Plates

Moisture content of Gel	Moisture content of Cream
Photo plate 1	Photo plate 2
	

Stability of Cream at 45°C and 54°C respectively		Stability of Gel at 45°C and at 54°C respectively	
Photo plate 3.a	Photo plate 3.b	Photo plate 4.a	Photo plate 4.b
			

Photo Plate 5.A: Analysis for Bacterial Contamination in Gel

Bacterial contamination observed in Gel due to Clove oil, Cinnamon oil, Eucalyptus oil, Tea tree oil, combination of all oil as preservatives.

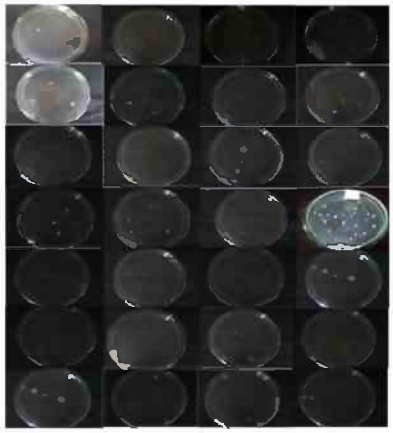


Photo plate 5.B: Analysis for Bacterial contamination in Cream

Bacterial contamination observed in Cream due to use of Eucalyptus oil, Cinnamon oil, Clove oil, Tea tree oil and Combination of all oil as preservatives.

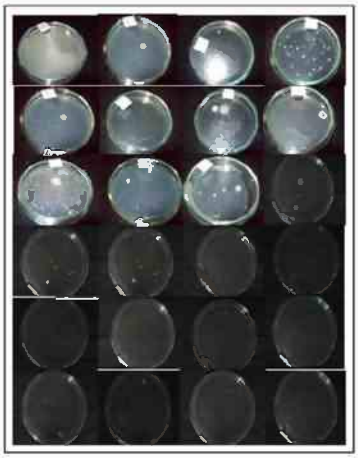


Photo plate 6.A: Analysis for Fungal contamination in Gel

Fungal contamination observed in Gel due to use of Eucalyptus oil, Tea tree oil, Clove oil, Cinnamon oil, combination of all oil as preservatives.

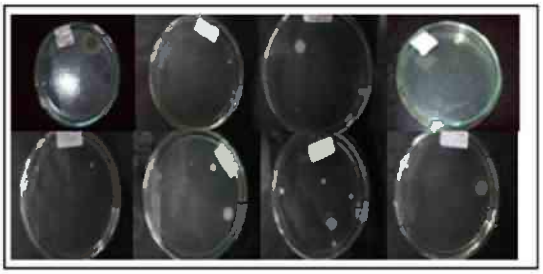
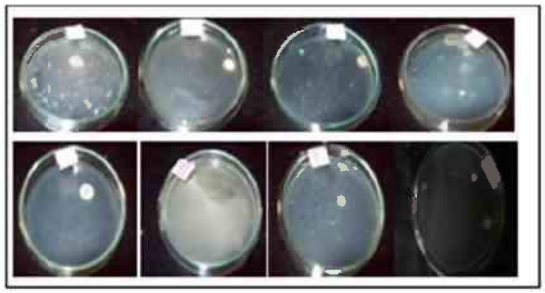


Photo plate 6.B: Analysis for Fungal contamination in Cream

Fungal contamination observed in cream due to use of Eucalyptus oil, Cinnamon oil, Clove oil, Tea tree oil and Combination of all oil as preservatives.



Results and Discussion

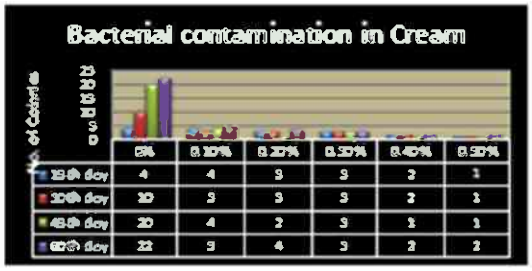
Moisture Content

- Moisture content of cream was 80%. (photo plate 1)
- Moisture content of gel was 98%. (photo plate 2)

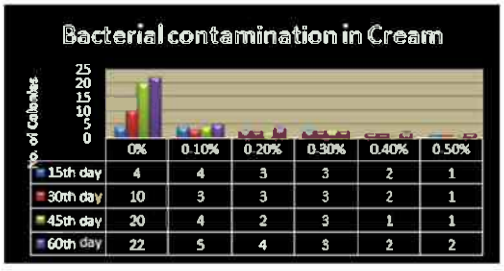
Qualitative Test

- According to Qualitative tests both Cream and Gel shows no difference in pH, visual appearance, stability at 45oC and 54oC, Skin irritation test of 15-day interval. (Table 1.3 and 1.4)

Analysis of Bacterial Contamination for Cream

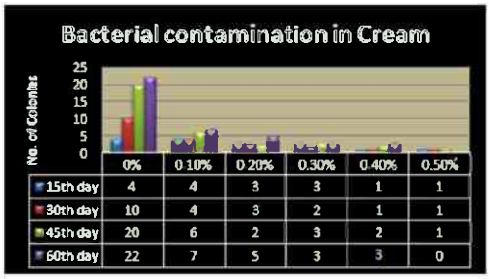


Graph 1.1: Eucalyptus oil added as preservative in cream gave minimum bacterial contamination in 0.4%, 0.5% conc. maximum in 0.1%, 0.2% conc.

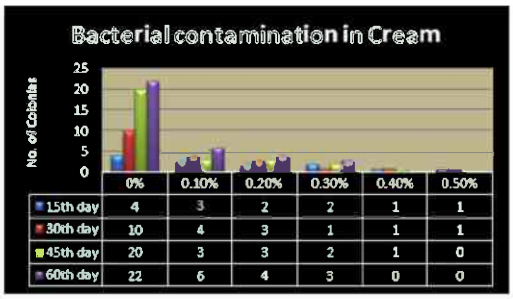


Graph 1.2: Clove oil added as preservative in cream gave minimum bacterial

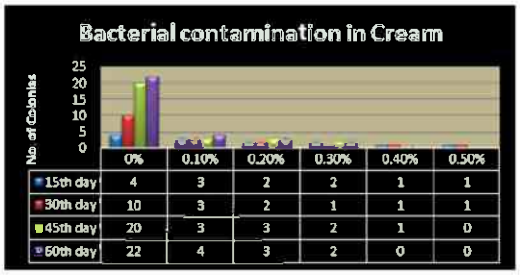
contamination in 0.5% and maximum in 0.1%, 0.2% conc.



Graph 1.3: Cinnamon oil used as preservative in cream gave minimum bacterial contamination in 0.4%, 0.5% conc. and maximum in 0.1% conc.

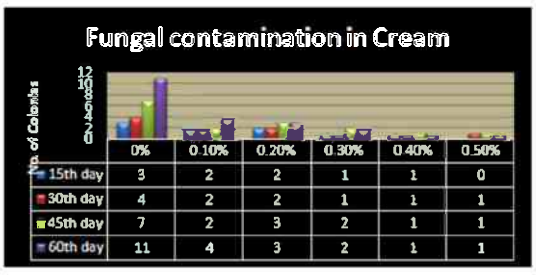


Graph 1.4: Tea tree oil added as preservative in cream gave minimum bacterial contamination in 0.3%, 0.4%, 0.5% conc. and maximum 0.1% conc.

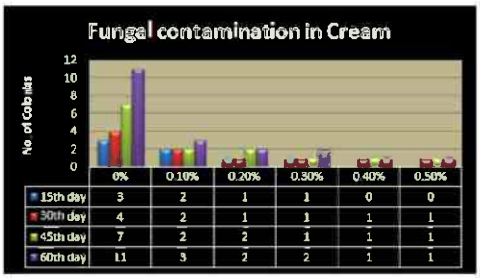


Graph 1.5: Combination of all essential oil used as preservative added to cream gave minimum bacterial contamination in 0.3%, 0.4%, 0.5% as compare to 0.1%, 0.2%, conc.

ANALYSIS OF FUNGAL CONTAMINATION FOR CREAM

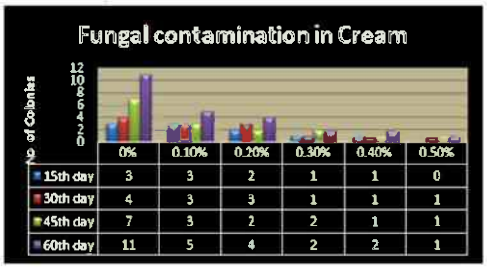


Graph 1.6: Eucalyptus oil added as preservative in cream gave minimum fungal contamination in 0.5% conc. and maximum in 0.1% conc.



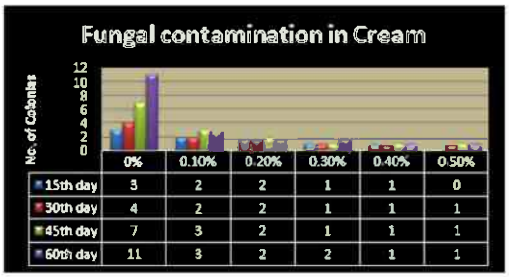
	0%	0.10%	0.20%	0.30%	0.40%	0.50%
15th day	3	2	1	1	0	0
30th day	4	2	1	1	1	1
45th day	7	2	2	1	1	1
60th day	11	3	2	2	1	1

Graph 1.7: Cinnamon oil used as preservative in cream gave minimum fungal contamination in 0.4%, 0.5% conc. and maximum 0.1% conc.



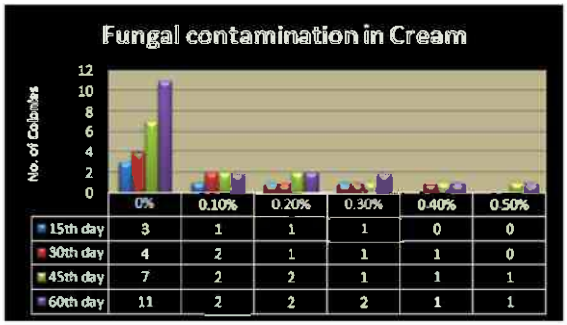
	0%	0.10%	0.20%	0.30%	0.40%	0.50%
15th day	3	3	2	1	1	0
30th day	4	3	3	1	1	1
45th day	7	3	2	2	1	1
60th day	11	5	4	2	2	1

Graph 1.8: Clove oil added as preservative in cream gave minimum fungal contamination in 0.5%, 0.3% and maximum in 0.1%, 0.2% conc.



	0%	0.10%	0.20%	0.30%	0.40%	0.50%
15th day	3	2	2	1	1	0
30th day	4	2	2	1	1	1
45th day	7	3	2	1	1	1
60th day	11	3	2	2	1	1

Graph 1.9: Tea tree oil added as preservative in cream gave minimum fungal contamination in 0.3%, 0.4%, 0.5% and maximum 0.1% conc.



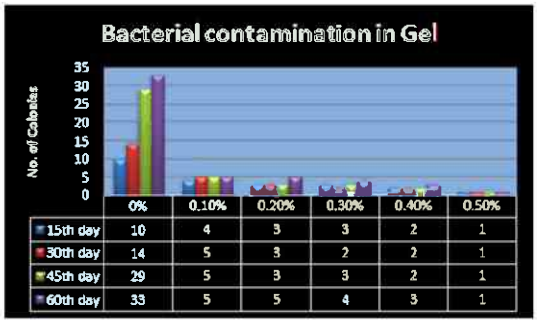
	0%	0.10%	0.20%	0.30%	0.40%	0.50%
15th day	3	1	1	1	0	0
30th day	4	2	1	1	1	0
45th day	7	2	2	1	1	1
60th day	11	2	2	2	1	1

Graph 1.10: Combination of all oil as preservative added in cream gave minimum

fungal contamination in 0.3%, 0.4%, 0.5% conc.

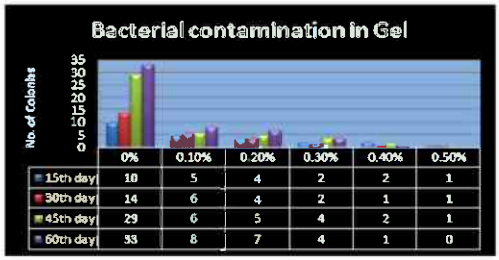
Cinnamon oil, Tea tree oil and Combination of all oil were shown more effective results as a natural preservative, compare to Clove oil and Eucalyptus oil in cream.

ANALYSIS OF BACTERIAL CONTAMINATION FOR GEL

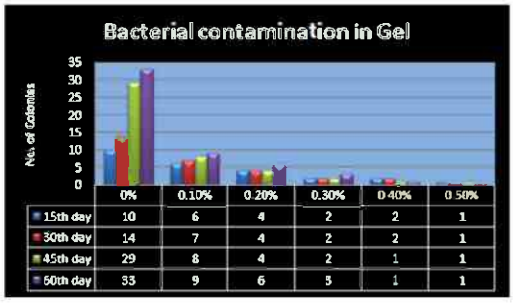


Graph 2.1: Eucalyptus oil added as preservative in gel gave minimum bacterial contamination in 0.5%, 0.4% conc. and maximum in 0.2% conc.

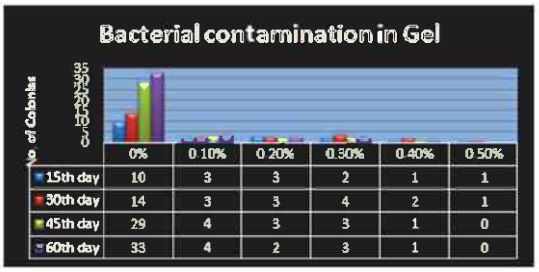
Graph 2.2: Clove oil added as preservative in gel gave minimum bacterial contamination in 0.5%, 0.3% and maximum in 0.1%, 0.2% conc.



Graph 2.3: Cinnamon oil used as preservative in gel gave minimum bacterial contamination in 0.3%, 0.4%, 0.5% as compare to 0.1% and 0.2% conc.



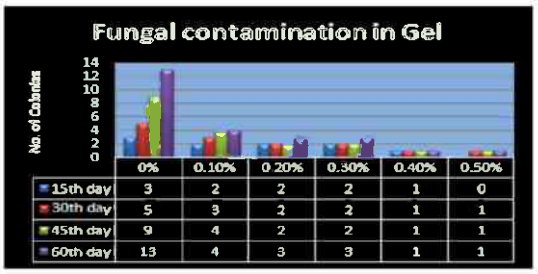
Graph 2.4: Tea tree oil added as preservative in gel gave minimum bacterial contamination in 0.5%, 0.4%, 0.3% as compare to 0.2% conc.



	0%	0.10%	0.20%	0.30%	0.40%	0.50%
15th day	10	3	3	2	1	1
30th day	14	3	3	4	2	1
45th day	29	4	3	3	1	0
60th day	33	4	2	3	1	0

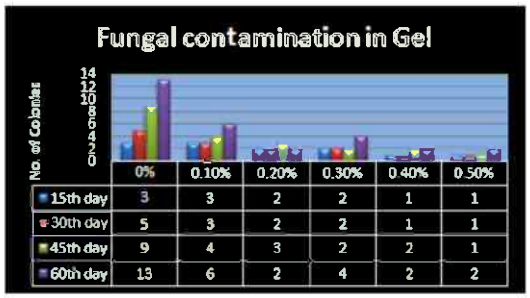
Graph 2.5: Combination of Essential oil as preservative added to gel gave minimum bacterial contamination in 0.3%, 0.4%,0.5% conc.

ANALYSIS OF FUNGAL CONTAMINATION FOR GEL



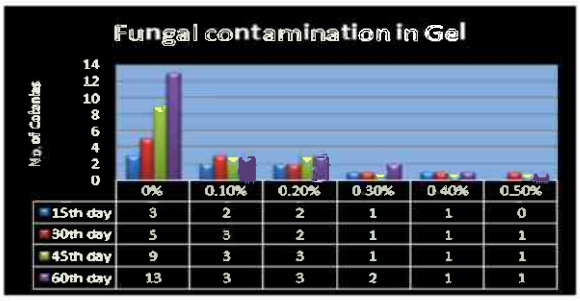
	0%	0.10%	0.20%	0.30%	0.40%	0.50%
15th day	3	2	2	2	1	0
30th day	5	3	2	2	1	1
45th day	9	4	2	2	1	1
60th day	13	4	3	3	1	1

Graph 2.6: Eucalyptus oil used as preservative in gel gave minimum fungal contamination in 0.5% conc.



	0%	0.10%	0.20%	0.30%	0.40%	0.50%
15th day	3	3	2	2	1	1
30th day	5	3	2	2	1	1
45th day	9	4	3	2	2	1
60th day	13	6	2	4	2	2

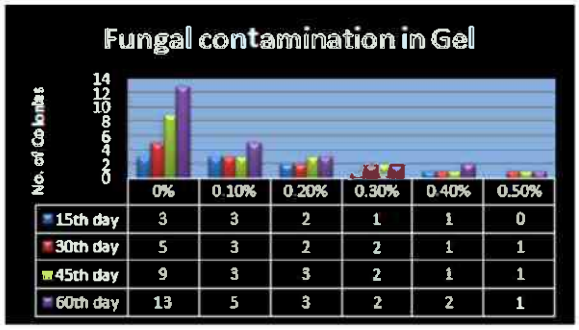
Graph 2.7: Clove oil used as preservative in gel gave minimum fungal contamination in 0.4% and 0.5% conc. and maximum in 0.2%, 0.1% conc.



	0%	0.10%	0.20%	0.30%	0.40%	0.50%
15th day	3	2	2	1	1	0
30th day	5	3	2	1	1	1
45th day	9	3	3	1	1	1
60th day	13	3	3	2	1	1

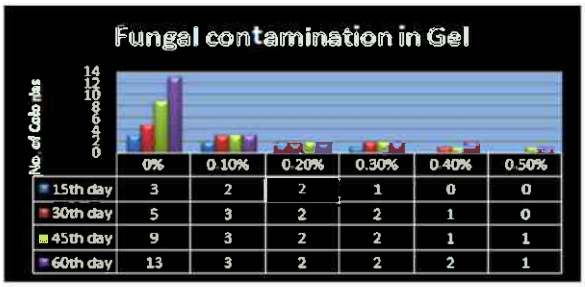
Graph 2.8: Cinnamon oil used as preservative in gel gave minimum fungal

contamination in 0.4%, 0.5% conc. and maximum in 0.1% conc.



	0%	0.10%	0.20%	0.30%	0.40%	0.50%
15th day	3	3	2	1	1	0
30th day	5	3	2	2	1	1
45th day	9	3	3	2	1	1
60th day	13	5	3	2	2	1

Graph 2.9: Tea tree oil used as preservative in gel gave minimum fungal contamination in 0.5% conc. As compare to 0.1%, 0.2%, 0.3% conc.



	0%	0.10%	0.20%	0.30%	0.40%	0.50%
15th day	3	2	2	1	0	0
30th day	5	3	2	2	1	0
45th day	9	3	2	2	1	1
60th day	13	3	2	2	2	1

Graph 2.10: Combination of essential oil used as preservative in gel gave minimum fungal contamination in 0.4%, 0.5% conc.

Cinnamon Oil, Tea Tree Oil, Clove Oil and Combination of all Oil were Shown More Effective Results as a Natural Preservative, Compare to Eucalyptus Oil in Gel.

Discussion

Lavandula officinalis, and Rosmarinus officinalis, to be used as natural cosmetic preservatives in an aqueous cream formulation for antimicrobial activities against bacteria and fungi. All the test microorganisms used in this study were generally more susceptible to the oils during the challenge test in aqueous cream compared to the antimicrobial test performed on agar. The 0.5% (vol/wt) essential oil of Rosemary was completely inhibitory (Dr. Reyhan Irkin et al., 2011). Clove essential oils were the most inhibitory against bacteria and yeasts. Tea tree oil inhibited the yeasts actively. (Reyhan Irkin and Mihriban K. 2009). In this study, I observed that the Tea tree oil was shown most inhibitory against bacteria and yeast as compare to Clove oil.

Eucalyptus, cinnamon exhibited the greatest antibacterial activities. The Gram-positive bacteria were more susceptible to the volatile oils than the Gram-negative. combinations of oils were found to exhibit greater antibacterial activity as compared with each oil used separately.

Maruzzella J.C. and Henry P. A. (2006). In this study, I found that the combination of all oils as well as Cinnamon oil exhibit greater antibacterial activity as compared with each oil used separately.

Conclusion

In this study, the herbal cosmetic products were tested for different test parameters. The two herbal cosmetics selected at regular interval microbial contaminants, were also studied to proven efficacy of essential oil as Natural Preservative and exhibit excellent anti-bacterial, anti-fungal properties. These natural preservatives were dose not change in their pH, moisture content, texture, stability of herbal cosmetic products.

Cinnamon oil, Tea tree oil, Clove oil and Combination of all oil were shown more effective results as a natural preservative, compare to Eucalyptus oil in Gel.

Cinnamon oil, Tea tree oil and Combination of all oil were shown more effective results as a natural preservative, compare to Clove oil and Eucalyptus oil in cream.

Summary

Herbal cosmetic products are made from plant material or plant extract. Herbal or any organic cosmetic product come in frequent contact with the non-sterile human skin, there by coming easily contaminated by microbes containing water, oils, peptides and carbohydrates cosmetics are a very good medium for growth of microbes. To avoid or overcame this situation preservatives are added. Synthetic preservatives if added might have side effects on the consumer's organ on which it is applied. So Natural preservative were taken to study its effect on physicochemical properties and shelf life of cosmetic products.

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7. To determine the effect of *Rubia cordifolia* (manjistha) and *Glycyrrhiza glabra* (Liquorice/ mulethi) on the Herbal Gel as natural Preservatives

Udaybhan Yadav
Assistant Prof. and Coordinator Department of Microbiology ID, ZSCT's Thakur Shyamanarayan Degree College, Kandivali East, Mumbai.
Kunal Thakur
Assistant Prof. Department of Microbiology ID
Bhanupratap Vishwakarma
Department of Microbiology ID
Abhishek Pankaj Jha
Department of Microbiology ID

Abstract

Licorice/ mulethi is a common herb which has been used in medicine for centuries. More than 20 triterpenoids and nearly 300 flavonoids have been isolated from licorice. It possesses many properties Such as antiviral, antimicrobial, anti-inflammatory, antitumor and other activities. The purpose of this study was to evaluate the antimicrobial effect of Rubia cordifolia root on Herbal gel using microbial assays method and find the effects of these natural substances on the herbal gel .we also performed some phytochemical analysis for the Rubia cordifolia (manjistha) and Liquorice (mulethi) to determine the various chemical present in its and to study it's properties and then we performed the microbial assays test for the herbal gel to study and examine the effects of Rubia cordifolia (manjistha) and Liquorice (mulethi) on the herbal gel and using the natural substances as preservatives. We found that they inhibited the growth of the bacteria in the herbal gel this happened due to the presence of the phytochemical present in Rubia cordifolia (manjistha) and Liquorice (mulethi). This shows that we can use the natural substances as preservatives to give them longer life and we can use them alternative of the chemical preservatives.

Keywords - Manjistha, Antimicrobial activity, Essential Oils, Herbal extracts, methylparaben.

Introduction

Licorice is a very well-known herb in traditional Asian medicine. In China, it is called "gancao" (meaning "sweet grass") and has been recorded in the Shennong's Classic of Materia Medica around 2100 BC. In this book, licorice was supposed to have life-enhancing properties. During the following thousands of years licorice has been present in most of Chinese traditional prescriptions. It was believed to have the functions of nourishing qi, alleviating pain, tonifying spleen and stomach, eliminating phlegm, and relieving coughing1. Have always anchored to the mother earth long before man can Set his feet on earth. Mankind has been gifted with Sources for existence much earlier than arrival of life on Earth.

The World Health Organization (WHO) estimates that about 80% of the population still depends upon Herbal medicines for the treatment of various diseases Due to easy availability, economic reasons and lesser side effect.

Availability of medicinal plants is not A problem especially in developing countries like India, which is having rich agro climatic, cultural and ethnic Biodiversity. India is the largest producer of medicinal Herbs and is a appropriately called the botanical garden of world. In the various products there are different types of chemical preservatives which are harmful and also carcinogenic Similarly, The Essential Oils represent an alternative way to chemical preservatives in the food industry against spoilage bacteria, yeast, and mold. The addition of essential oils can be used as a substitute to chemical preservatives, e.g., potassium sorbate that's widely used as a preservative in food industry to increase food shelf life, or by the combination of natural Preservatives and chemical preservatives leading to better results. The objective of this study is therefore to add different essential oils to the various Herbal products to increase their storage life and as well as to reduce the harmful effects of the chemical preservatives. Plant essential oils simply abbreviated as Eos, are aromatic oily liquids obtained from plant materials. Steam distillation is the most commonly used method for commercial production of Eos. Some Eos have antimicrobial properties. In various industries plant Eos are gaining a wide interest for their potential as decontaminating agents, and as they are generally recognized as safe (GRAS). The active components are commonly found in the EO fractions, and it is well established that most of them have a wide spectrum of antimicrobial activity against food-borne pathogens and

spoilage bacteria. The antimicrobial activity of plants essential oils is due to their chemical structure, in particular to the groups of phenolic components and/or lipophilicity of some EO components Usually, the compounds with phenolic groups. Recently a large demand has risen for preservative-free cosmetics and antimicrobial herbal extracts, aimed at reducing the risk of allergies connected to synthetic preservatives such as methylparaben. So, this research is carried out to know the effects of Essential plants as natural Preservatives on Herbal gel.

Taxonomical Classification

Rubia cordifolia (manjistha)	***Glycyrrhiza glabra* (mulethi)**
Kingdom: Plantae	Kingdom: Plantae
Division: Tracheophyta	Division: Tracheophyta
Class: Angiosperms	Class: Angiosperms
Order: Gentianales	Order: Fabales
Family: Rubiaceae	Family: Fabaceae
Genus: Rubia	Genus: *Glycyrrhiza*
Species: cordifolia	species: *glabra*

Materials and Methods

1. Qualitative Analysis on Phytochemical Constituent in Essential Oils

I. Test for tannins: 0.1% FeCl3 Is added to the Essential Oils samples and We observed the brownish green coloration, which showed us the presence of tannins.

II. Test for flavonoids: A few drops of 1% NH3 solution was added to the Aqueous extract of Essential Oils in a test tube. A Yellow coloration confirmed us the presence of flavonoid Compounds.

III. Test for Alkaloids: 2ml Essential Oils + 1% HCl + steam, 1ml filtrate + 6 drops of Dra-Gendroff reagent, orange precipitate indicated the presence of respective alkaloids.

IV. Test for Phenol: To 2-3 ml of oil extract and few drops of 5% FeCl3 solution was added in the test tube. Formation of deep blue-Black colour indicated us the presence of phenols in the essential oils.

V. Test for Cineol: 1,8-cineole and other monoterpenes starting from geranyl diphosphate (GPP), using different terpene synthases. The (4S)-α-terpinyl cation is a key

intermediate; several other terpenes result from it, such as α-thujene, α-pinene, sabinene and limonene.

VI. Test for Cymene: p-Cymene [1-methyl-4-(1-methylethyl) benzene or 4-isopropyltoluene] is formed from the OH + γ-terpinene reaction after H-atom abstraction from the two ring CH2 groups, and its molar formation yield has been measured.

2. Determination of Microbial Content

Methods

1 gm of the sample and add to a sterile tube containing sterile inactivating diluent or sterile diluent broth containing the appropriate neutralizer to give a 1:10 and/or a 1:100 dilution. tubes and mix thoroughly using a vortex mixer. Prepare a 1:100 dilution by adding 1ml of the 1:10 dilution to 9 ml of sterile diluent. 1ml to each of the broth suspension from the 1:10 dilution into two sterile petri dishes for the 1:100 dilution. 15-20ml of sterilized molten plate count agar (held at 44-48^{0}C in a water bath) into the four-petri dished. Transfer 1ml each of the broth suspension from the 1:10 and 1:100 dilutions into two sterile petri dishes each as above. Pour sterilized molten potato dextrose agar (held at 44-480C in a water bath) into the four petri dishes. Rotate the plates gently on a flat surface to ensure thorough mixing and Allow the agar in the plates to solidify at room temperature for about 30-45 Min in dishes. Invert the plates and incubate at 33 ± 20C for 48 hours in the case of count agar for bacterial enumeration and 28±20C for 3-5 days in case of potato dextrose agar for enumeration of yeast and molds. Count the number of colonies on the duplicate plates, obtain an average and multiply by the dilution factor to obtain the colony forming units per gram or per ml (CFU/g or CFU/ml) in the sample. Addition of total bacterial count and yeast and mold counts will give total microbial (viable) count (TVC).

- Total microbial count = Total bacterial count + Yeast and mold count

Observation and Result

Table:1 Phytochemical Analysis Of *Rubia Cordifolia* (Manjistha)

PHYTOCHEMICAL TEST	INFERENCE
TARPINEOL	PRESENT
CINEOL	PRESENT
CYMENE	PRESENT
FLAVONOIDS	PRESENT
NITRATE	PRESENT

TABLE: 2 PHYTOCHEMICALS CONSTITUENTS OF *Glycyrrhiza Glabra* (MULETHI)

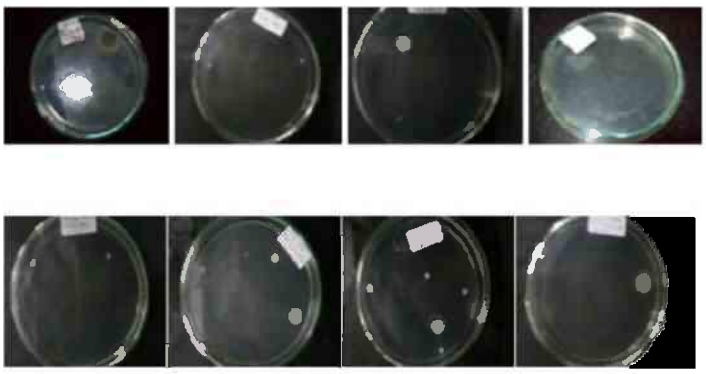
PHYTOCHEMICAL TEST	INFERENCE
FLAVONOIDS	PRESENT
SAPON	PRESENT
PHENOLS	PRESENT
TANNINS	PRESENT
TARPINEOL	PRESENT

The above table 1 and 2 shows that the presence of several phytochemicals which will inhibit the growth of the microorganisms.

Table.3: Observation Table for *Rubia cordifolia* (manjistha) as preservatives of gel against various organisms- (Bacterial colonies observed in gel)

Days	0%	0.1%	0.2 %	0.3%	0.4%	0.5%
05 Days	10	6	4	2	2	1
10 Days	16	7	4	2	2	1
15 Days	30	9	5	2	1	1
20 Days	35	9	6	3	3	1

Photoplate.1: Bacterial Colonies Observed in the Gel

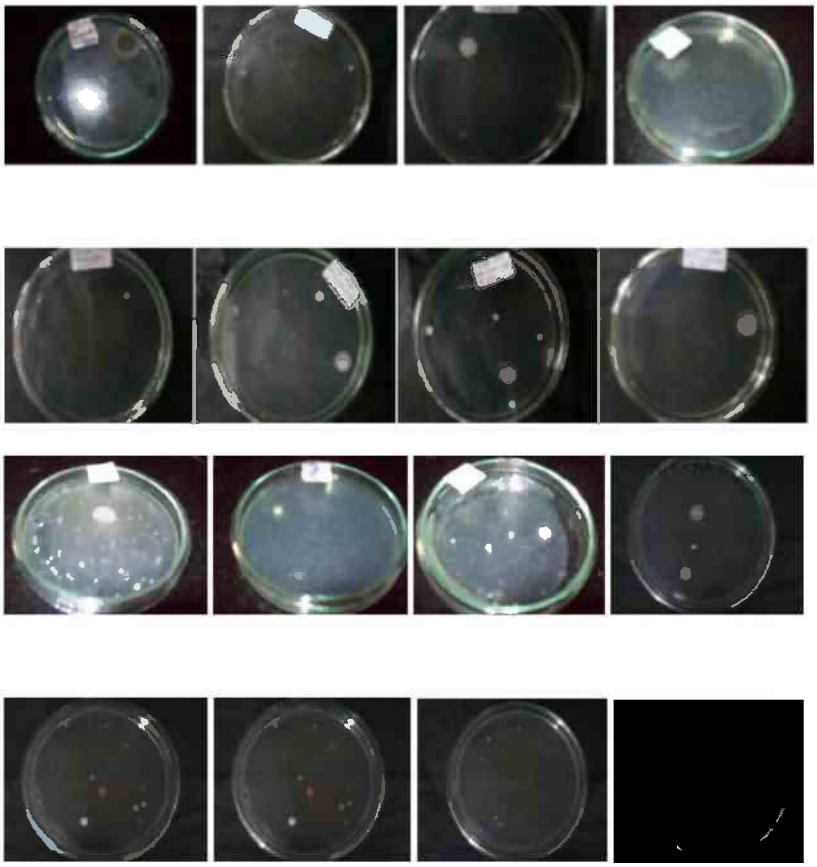


Less Bacterial contamination is seen due to use of the oil as preservatives in the Gel.

Table.3: Observation Table for *Glycyrrhiza glabra* (Mulethi) As Preservatives of gel against various organisms and pathogens. (Bacterial colonies observed in gel)

Days	0%	0.1%	0.2%	0.3%	0.4%	0.5%
05 Days	**10**	**5**	**4**	**2**	**1**	**1**
10 Days	**14**	**6**	**5**	**3**	**1**	**1**
15 Days	**29**	**7**	**4**	**3**	**2**	**2**
20 Days	**33**	**10**	**7**	**3**	**3**	**2**

Photoplate.2: Bacterial Colonies Observed in the Gel



Less Bacterial contamination is seen due to use of the Mulethi as preservatives in the Gel

Here we can clearly see that the inhibited the growth of the micro organisms s due to the presence of phytochemical and other compounds which are present in the essential oil. When we increase concentration of the essential oils there is decrease in the growth of the colony from here we have founded that they possess the Antimicrobial, Antifungal properties. Which can be helpful for us to use these essential oils as preservatives.

Discussion

Rubia cordifolia (manjistha) added as preservative in gel gave minimum bacterial Contamination in 0.5%, 0.4%, 0.3% as compare to 0.2% conc.

Rubia cordifolia (manjistha) oil used as preservative in gel gave minimum fungal contamination in 0.5% conc. As compare to 0.1%, 0.2%, 0.3% conc.

Liquorice (mulethi) added as preservative in gel gave minimum bacterial contamination in 0.5%, 0.3% and maximum in 0.1%, 0.2% conc.

Liquorice (mulethi) used as preservative in gel gave minimum fungal contamination in 0.3% and 0.5% conc. and maximum in 0.2%, 0.1% conc.

All the test microorganisms used in this study were generally more susceptible to the substances during the challenge test in aqueous Gel compared to the antimicrobial test performed on agar.

Rubia cordifolia (manjistha) inhibited the yeasts actively. In this study, I observed that the Rubia cordifolia (manjistha) oil was shown most inhibitory against bacteria and yeast as compare to Liquorice (mulethi).

Conclusion

The herbal cosmetic selected at regular interval microbial contaminants, were also studied to proven efficacy of essential oil as Natural Preservative and exhibit excellent anti-bacterial, anti-fungal properties. These natural preservatives were dose not change in their pH, moisture content, texture, stability of herbal cosmetic products.

Rubia cordifolia (manjistha), Liquorice (mulethi) were shown more effective results as a natural preservative,

The properties of these compounds were shown due to the presence of the various phytochemical compounds in the which were more effective on the gel as Natural Preservatives.

These natural substances have more scope in future for the treatment of the many disease which can't be cure by chemical. These substances show the many other properties which are useful for us.

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8. To Study the Effects of *Bacopa Monnieri and Momordica Charantia* as Natural Preservatives on Herbal Cream

Udaybhan Yadav
Assistant Prof. and Coordinator Department of Microbiology ID, ZSCT's Thakur Shyamanarayan Degree College, Kandivali East, Mumbai.
Kunal Thakur
Assistant Prof. Department of Microbiology ID
Abhishek Pankaj Jha
Department of Microbiology ID
Bhanupratap Vishwakarma
Department of Microbiology ID

Abstract

There is teeming healthful flora that is covert through analysis. The study was conducted to assess the results of Essential Oils on the various cream. The cosmetic business adapts to the wants of shoppers seeking to limit the employment of preservatives and develop of preservative-free or self-preserving cosmetics, wherever preservatives are replaced by raw materials of plant origin.The aim of study was to grasp the results of Essential Oils on the seasonal cosmetics. Essential oils showed higher repressive activity against tested against the cosmetics. This shows that tested extracts and essential oils may replace use of chemical preservatives, at an equivalent time giving a guarantee of microbiological purity of the cosmetic below its use and storage. the employment of Essential oils within the production of cosmetics and connected merchandise might have many benefits. Essential oils in cosmetic formulations at comparatively high concentrations are seemingly to produce skin profit. Essential oils are shown to possess medication, antifungal, antiviral insecticidal and inhibitor properties. Some oils are utilized in cancer treatment, other oils are utilized in aromatherapy and fragrance industries. Essential oils are an upscale supply of biologically active compounds. There has been associate accrued interest in observing antimicrobial properties of extracts from aromatic plants significantly essential oils. Then the varied phytochemical determined by the varied take a look at and also the we've got done the microorganism assay technique to see the overall growth

of the bacterium and fungi on the cream by running it into the agar plate then they were determined for many days and that we supported that the essential oils are often used as natural Preservatives.

Keywords – Antimicrobial activity, Essential Oils, Herbal extracts, methylparaben.

Introduction

Plants Have always anchored to the mother earth long before man can Set his feet on earth. Mankind has been gifted with Sources for existence much earlier than arrival of life on Earth.

The World Health Organization (WHO) estimates that about 80% of the population still depends upon Herbal medicines for the treatment of various diseases Due to easy availability, economic reasons and lesser side effect. Availability of medicinal plants is not a problem especially in developing countries like India, which is having rich agro climatic, cultural and ethnic Biodiversity. India is the largest producer of medicinal Herbs and is an appropriately called the botanical garden of world.

In the various products there are different types of chemical preservatives which are harmful and also carcinogenic Similarly, The Essential Oils represent an alternative way to chemical preservatives in the food industry against spoilage bacteria, yeast, and mold. The addition of essential oils can be used as a substitute to chemical preservatives, e.g., potassium sorbate that's widely used as a preservative in food industry to increase food shelf life, or by the combination of natural Preservatives and chemical preservatives leading to better results. The objective of this study is therefore to add different essential oils to the various Herbal products to increase their storage life and as well as to reduce the harmful effects of

the chemical preservatives. Plant essential oils simply abbreviated as Eos, are aromatic oily liquids obtained from plant materials. Steam distillation is the most commonly used method for commercial production of Eos. Some Eos have antimicrobial properties. In various industries plant Eos are gaining a wide interest for their potential as decontaminating agents, and as they are generally recognized as safe (GRAS). The active components are commonly found in the EO fractions, and it is well established that most of them have a wide spectrum of antimicrobial activity against food-borne pathogens and spoilage bacteria. The antimicrobial activity of plants essential oils is due to their chemical structure, in particular to the groups of phenolic components and/or lipophilicity of some EO components Usually, the compounds with phenolic

groups. Recently a large demand has risen for preservative-free cosmetics and antimicrobial herbal extracts, aimed at reducing the risk of allergies connected to synthetic preservatives such as methylparaben. So, this research is carried out to know the effects of Essential Oils such as *Bacopa monnieri* and *Momordica charantia* natural Preservatives on Herbal cream.

Taxonomical Classification

BACOPA MONNIERI	***MOMORDICA CHARANTI***
Kingdom: Plantae	Kingdom: Plantae
Subkingdom: Viridiplantae	Subkingdom: Viridiplantae
Infrakingdom: Streptophyta	Infrakingdom: Streptophyta
Super division: Embryophyta	Super division: Embryophyta
Division: Tracheophyta	Division: Tracheophyta
Subdivision: Spermatophyte	Subdivision: Spermatophytina
Class: Magnoliopsida	Class: Magnoliopsida
Superorder: Asteranae	Superorder: Rosanae
Order: Lamiales	Order: Cucurbitales
Family: Plantaginaceae	Family: Cucurbitaceae
Genus: Bacopa	Genus: Momordica L.
Species: monnieri (L.)	Species: charantia L.

Materials and Method

1. Procurement of plant material- Bacopa monnieri, Momordica and Herbal cream were purchased from the market
2. Different types of test were also performed to know the constituents present in the extract of Essential Oils they are as follow: -
3. Qualitative analysis on phytochemical constituent in Essential Oils.
4. Determination of Microbial Content
5. Microbial Assays Method

These tests consisted of planting a known amount of the sample on selected culture media specifically suitable for the growth of bacteria and fungi and incubating them for a specified period to permit the development of visual colonies for counting. The number of colonies seen is taken as a measure of the number of micro-organisms present in the sample. Firstly, we have prepared the two different types of media for culturing the microorganisms

For total Bacterial count	**For Yeast and Mold counts**
Plate Count Agar	**Potato Dextrose Agar**
Composition	**Composition**
Tryptone: 5.0 g	Infusion from potatoes: 200.0 g
Yeast extracts: 2.5 g	Dextrose: 20.0 g

Dextrose: 1.0 g Agar: 15.0 g Distilled water: 1000 ml Final pH: 7.0 ± 0.2	Agar: 15.0g Distilled water:1000 ml Final pH: 5.6 ± 0.2

Methods

1 gm of the sample and add to a sterile tube containing sterile inactivating diluent or sterile diluent broth containing the appropriate neutralizer to give a 1:10 and/or a 1:100 dilution. tubes and mix thoroughly using a vortex mixer. Prepare a 1:100 dilution by adding 1ml of the 1:10 dilution to 9 ml of sterile diluent. 1ml to each of the broth suspension from the 1:10 dilution into two sterile petri dishes for the 1:100 dilution. 15-20ml of sterilized molten plate count agar (held at 44-48^{0}C in a water bath) into the four-petri dished. Transfer 1ml each of the broth suspension from the 1:10 and 1:100 dilutions into two sterile petri dishes each as above. Pour sterilized molten potato dextrose agar (held at 44-480C in a water bath) into the four petri dishes. Rotate the plates gently on a flat surface to ensure thorough mixing and Allow the agar in the plates to solidify at room temperature for about 30-45 Min in dishes. Invert the plates and incubate at 33 ± 20C for 48 hours in the case of count agar for bacterial enumeration and 28±20C for 3-5 days in case of potato dextrose agar for enumeration of yeast and molds. Count the number of colonies on the duplicate plates, obtain an average and multiply by the dilution factor to obtain the colony forming units per gram or per ml (CFU/g or CFU/ml) in the sample. Addition of total bacterial count and yeast and mold counts will give total microbial (viable) count (TVC).

Total microbial count = Total bacterial count + Yeast and mold count

Observation and Result

Table:1 Phytochemicals Constituents of *Bacopa Monnieri*

PHYTOCHEMICAL TEST	Inference
ALKALOIDS	PRESENT
TANNINS	PRESENT
FLAVONOIDS	PRESENT
PHENOLS	PRESENT

Table: 2 Phytochemicals Constituents Of *Momordica Charantia*

PHYTOCHEMICAL TEST	Inference
ALKALOIDS	PRESENT
TANNINS	PRESENT
FLAVONOIDS	PRESENT
PHENOLS	PRESENT

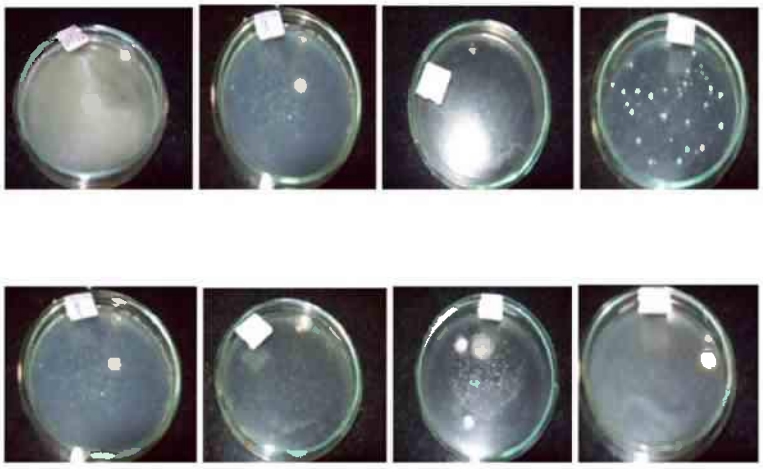
The above table no. 1 &2 shows that the presence of several phytochemicals which will inhibit the growth of the microorganisms.

Table.3: For Bacopa Monnieri as Preservatives

(Bacterial Colonies Observed in the Cream)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
05 Days	5	4	3	3	2	1
10 Days	12	4	2	2	1	1
15 Days	20	8	3	1	2	1
20 Days	30	10	5	1	1	0

Photoplate.1: Bacterial colonies observed in the cream



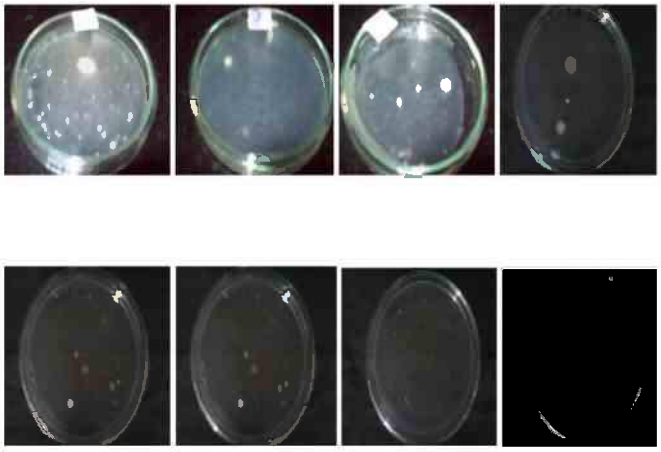
Photoplate.1 Shows the Less Bacterial Contamination Observed in Cream when Bacopa Monnieri is Used as a Preservative

Table.4: For *Momordica Charantia* as Preservatives

(Bacterial Colonies Observed in on Various Days)

	0%	0.1%	0.2%	0.3%	0.4%	0.5%
05 Days	5	4	3	3	2	1
10 Days	12	6	3	3	2	1
15 Days	18	8	2	3	3	1
20 Days	24	12	4	4	4	2

Photoplate.2: Bacterial colonies observed in the cream on various day



Photoplate.2- Shows theless Contamination Observed in Cream is Due *Momordica Charantia* to as Preservatives.

Here, we can clearly see that the essential oil has inhibited the growth of the microorganisms. When we When we increase concentration of the essential oils there is decrease in the growth of the colony from here we have funded that they possess the Antimicrobial, Antifungal properties.

Discussion

There are Many herbal remedies Have been tried on in vitro and in vivo against different pathogens and have been found to be effective. *Bacopa monnieri* and *Momordica charantia* going to be cosmetic preservatives in an aqueous cream formulation for antimicrobial activities against bacteria and fungi. All the test microorganisms used in this study were generally more susceptible to the oils during the challenge test in aqueous cream compared to the antimicrobial test performed on agar. Bacopa monnieri and Momordica charantia exhibited the antibacterial activities. The Gram-positive bacteria were more susceptible to the volatile oils than the Gram-negative. Combinations of oils were found to exhibit greater antibacterial activity as compared with each oil used separately. In this study, We found that the combination of all oils as well as *Momordica charantia* exhibit greater antibacterial activity as compared with Bacopa monnieri used separately.

Conclusion

In this study, the herbal cosmetic products were tested for different test parameters. The herbal cosmetic(cream) selected at regular interval microbial contaminants, were also studied to proven effectiveness of essential oil as Natural Preservative and exhibit excellent anti-bacterial, anti-fungal properties. These natural preservatives were does not change in their pH, moisture

content, texture, stability of herbal cosmetic products. *Momordica charantia* were shown more effective results as a natural preservative, compare to *Bacopa monnieri.*

So, it means that there are many possibilities that we can use these essential oil as as natural Preservatives in cosmetics instead of the chemical preservatives which are harmful as well as carcinogenic.

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9. Estimation of Biological Oxygen Demand (BOD) From Three Different Industrial Sewage Samples of Daman, Navapur & Murbe from Maharashtra

Udaybhan Yadav
Assistant Prof. and Coordinator Department of Microbiology ID, ZSCT's Thakur Shyamanarayan Degree College, Kandivali East Mumbai.
Kunal Thakur
Assistant Professor, Microbiology ID, ZSCT's Thakur Shyamnarayan Degree College, Kandivali, Mumbai.
Meenu Baljeet Jaiswal
Research Student, Microbiology ID, ZSCT's, Thakur Shyamnarayan Degree College, Kandivali, Mumbai.

Abstract

The Biological Oxygen Demand (BOD) is one of the most widely used criteria for water quality assessment. It provides information about the readily biodegradable fraction of the organic load in water. However, this analytical method is time-consuming (generally 5 days, i.e. BOD5), and the results may vary according to the laboratory (20%), primarily due to fluctuations in the microbial diversity of the inoculums used. Work performed during the two last decades has resulted in several technologies that are less time-consuming and more reliable. This review is devoted to the analysis of the technical features of the principal methods described in the literature in order to compare their performances and to identify the pros and cons of each method.

Aim

The aim of this experiment is to measure the BOD and DO of water.

Principle

BOD is expressed as weight of oxygen consumed per unit volume of water during 5 days at 20°C; BOD is related to the amount of biodegradable organic matter in water sample; during oxidative degradation of organic matter, aerobic micro-organisms which perform it, consume oxygen present in water as dissolved gas.

Water sample is diluted by a dilution solution (sometime containing bacteria seed); this sample is incubated during 5 days at 293°k and the consumed amount of oxygen is measured. It is necessary to prepare many solutions corresponding to different dilutions in order to choose the one which presents oxygen consumption equals to 40 to 60% of the initial concentration of oxygen.

Key Words: Bio-chemical Oxygen Demand (BOD), Sewage Treatment Plant (STP), Wetlands.

Introduction

Biochemical oxygen demand (BOD) represents the amount of oxygen consumed by bacteria and other microorganisms while they decompose organic matter under aerobic (oxygen is present) conditions at a specified temperature.

When you look at water in a lake the one thing you don't see is oxygen. In a way, we think that water is the opposite of air, but the common lake or stream does contain small amounts of oxygen, in the form of dissolved oxygen. Although the amount of dissolved oxygen is small, up to about ten molecules of oxygen per million of water, it is a crucial component of natural water bodies; the presence of a sufficient concentration of dissolved oxygen is critical to maintaining the aquatic life and aesthetic quality of streams and lakes.

The presence of a sufficient concentration of dissolved oxygen is critical to maintaining the aquatic life and aesthetic quality of streams and lakes. Determining how organic matter affects the concentration of dissolved oxygen (DO) in a stream or lake is integral to water-quality management. The decay of organic matter in water is measured as biochemical or chemical oxygen demand. Oxygen demand is a measure of the amount of oxidizable substances in a water sample that can lower DO concentrations.

Certain environmental stresses (hot summer temperatures) and other human-induced factors (introduction of excess **fertilizers** to a water body) can lessen the amount of dissolved oxygen in a water body, resulting in stresses on the local aquatic life. One water analysis that is utilized in order to better understand the effect of bacteria and other microorganisms on the amount of oxygen they consume as they decompose organic matter under aerobic (oxygen is present) is the measure of biochemical oxygen demand (BOD).

I took 3 different water samples from different places:

1. **Daman**

2. Navapur

3. Murbe

1. Daman

As we all know that Daman is a Union Territory State there are various kind of big industries. Most of the industries have their own ETP (Environmental Treatment Plant). From some of the industries have waste drainage water mixes to the river, lakes, and ponds etc. Here are some images from Daman as below.


Daman Ganga: Restoration of river choked by Toxin.

2. Navapur

Navapur is a village in Boisar, District Palghar state of Maharashtra. Navapur comes under M.I.D.C (Maharashtra Industrial Development Corporation).


Many of the Fishes Die in the Navapur Area, Due to Wastewater from the Company's image as Above.

3. Murbe

Murbe is a village in Boisar, District Palghar state of Maharashtra. Murbe is close to M.I.D.C (Maharashtra Industrial Development Corporation), Tarapur.

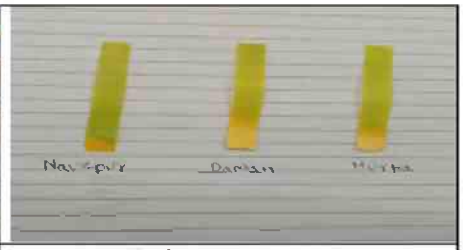

Here The Beaches are Contaminated by all the Industries Situated Near Murbe Image as Above.

Materials and Methods

1. Neutralization of Sample

Ph of the Diluted Sample Should be Adjusted 7.0 ± 0.2 before the Incubation for 5 Days for Proper Result

Collecting samples from the influent raw wastewater stream for BOD measurements at a wastewater treatment plant.



pH of the water sample

- Take 50ml of water sample in a 100ml of beaker.
- Measure the pH of the solution by using the calibrated pH meter.
- Add the 1N Sulfuric acid to adjust the pH if it is higher than 7.00 and if pH is lower than 7.00 then add 1N of Sodium Hydroxide.
- Note down the volume of Sulfuric acid or Sodium Hydroxide used to adjust the pH of 50ml sample to 7.00 ± 0.2.
- Calculate the volume of Sulfuric acid or Sodium Hydroxide required to neutralize the 1000ml sample.
- Add the calculated volume of Sulfuric acid or Sodium Hydroxide to the sample to neutralize. For example, if 2.1ml of 1N Sulfuric acid or Sodium Hydroxide are used to neutralize 50ml of sample to ph 7.00 ± 0.2. Calculate the volume of 1Nsulfuric acid or Sodium Hydroxide to be added to neutralize the 1000ml sample as follows:
- 1N Sulfuric acid or Sodium Hydroxide required = (2.1ml × 1000ml)/ 50ml= 2100/50= 42ml

2. Removal of Chlorine Content

Hydrochlorine acid or any other acid containing chlorine should not be used to neutralize the sample because chlorine interferes the results of wastewater BOD.

Chlorine is the strong oxidizing agent and it can inhibit the Microbial Growth during wastewater BOD analysis. Chlorine can be removed by adding Sodium Sulfite to the sample in the following manner.

- Take 50ml of water sample to be tested in a conical flask.
- Add 2.5ml of acetic acid diluted to 50% of with water.
- Add 2.5ml of 10% w/v solution of Potassium Iodide (KI).
- Add 1ml of starch indicator and titrate with 0.025N Sodium Sulfite solution.
- Note down the volume and calculated to add in 1000ml of the sample as described above in neutralization of sample section.
- Add the calculated volume of Sodium Sulfite solution to the sample and mix well to neutralize the chlorine.

3. Preparation of Phosphate Buffer Solution

Dissolve accurate weighed 8.5gm of Potassium Dihydrogen Phosphate (KH_2PO_4), 21.75gm of Dipotassium Hydrogen Phosphate (K_2HPO_4), 33.4gm of Disodium Hydrogen Phosphate ($Na_2HPO_4.7H_2O$) and 1.7gm of Ammonium Chloride (NH_4Cl) in 500ml dW. Dilute the solution up to 1000ml.

4. Preparation of Alkali-Iodide-Azide-Reagent

Dissolve 500gm of Sodium Hydroxide (NaOH) and 135gm of Sodium Iodide (NaI) in distilled water. Make up the solution to 1000ml of dW. Now dissolve 10gm of Sodium Azide ($Na_2S_2O_3$) in this solution.

5. Preparation of Dilution Water

The dilution water for wastewater BOD analysis must be free from organic content. Dilution water can be prepared by following method:

- Take 5*l* of double dW in a glass container.
- Aerate the water with the clean compressed air for not less than 12hrs.
- Allow to stable for at least 6hrs at 20°C.
- Add 5ml of 27.5% w/v solution of Calcium Carbonate (CaCO).
- Add 5ml 22.5% w/v solution of Magnesium Sulfate (Mg).
- Add 5ml 0.15% w/v solution of Ferric Chloride.
- Add 5ml of Phosphate Buffer solution.
- Mix well and allow to stand for 2hrs.

6. Procedure to Determine the Biological Oxygen Demand of Water

- Take 4 300ml BOD bottles and add 10ml of samples to 2 bottles and filled the remaining volume with dilution water.
- Fill remaining 2 BOD bottles only with dilution water for blank.
- Immediately close the bottles when filled and there should not be any air bubble in the bottle.
- Marked the Bottles as blank and sample.
- Incubate one sample and one blank bottle at 20°C for 5 days.
- Analyze immediately remaining one blank and one sample bottle of dissolved oxygen (DO).
- Analyze incubated bottles for DO after 5 days.

7. Test for Dissolve Oxygen (DO)

- Add 2ml of 36.4% of Manganous Sulfate ($MnSO_4.H_2O$) solution inserting the tip of pipette tip into the sample because the drops of solution can allow inserting the oxygen into the solution.
- Add 2ml of the Alkali-Iodide-Azide-Reagent by above method.
- Allow reacting the solution with the oxygen present in the sample.
- When precipitates are settled down at the bottom add 2ml of Conc. Sulfuric acid by placing the pipette tip very near to sample surface.
- Mix well to dissolve the precipitates.
- Take 203ml of sample from BOD bottle into a Erlenmeyer flask.
- Titrate immediately with 0.025N Sodium Thiosulfate solution using Starch Indicator until blue color disappears and note down the burette readings.
- Determine the Burette Reading for blank in same manner.

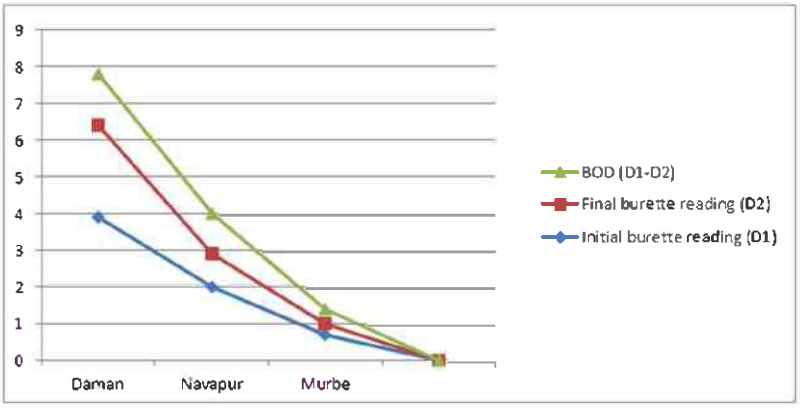
After adding Conc. Sulfuric acid, we can see there is cloud formation

After adding Starch Indicator

Automatically color has been changed after 5 days incubation at 20°C

Observation Table

Bottle number	Initial DO reading (D_1)	Date/time of reading	Final DO reading (D_2)	Date/time of reading	BOD (D_1-D_2)
Daman	3.9	17/12/2021 02:49 pm	2.5	22/12/2021 01:57 pm	1.4
Navapur	2.0	17/12/2021 02:52 pm	0.9	22/12/2021 02:05pm	1.1
Murbe	0.7	17/12/2021 02:58 pm	0.3	22/12/2021 02:14 pm	0.4



Above Graph indicates the BOD of the Water Samples

Result and Discussion

The BOD level for D/W was found to be 0.0 mg/L, which means that this water sample is not polluted and water quality is extremely good. This value is to be expected because D/W is purified water.

The BOD level for waste water was found to be 3.7762 mg/L, which means that the water quality was fair. The level for this sample was slightly on the higher side because waste water contains a lot of pollutants.

Conclusion

As a conclusion, the water source near DAMAN INDUSTRY Lake has been polluted due to the incorrect handling of human activities. The recommendation to solve the problem is to apply water treatment to the water source and to prevent incorrect handling of human activities at the golf field. Then our hypothesis for this experiment is accepted.

BOD Level in mg/liter	Water Quality
1 - 2	**Very Good:** There will not be much organic matter present in the water supply.
3 - 5	**Fair:** Moderately Clean
6 - 9	**Poor:** Somewhat Polluted - Usually indicates that organic matter present and microorganisms are decomposing that waste.
100 or more	**Very Poor:** Very Polluted - Contains organic matter.

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10. To Study the Antimicrobial Properties of *Mangifera Indica* (Mango) Leaves- A Century Old Interrelationship between the Rituals and Science

Udaybhan Yadav
Coordinator, Department of Microbiology (Interdisciplinary), Thakur Shyamnarayan Degree College, Kandivali East Mumbai.
Kunal Thakur
Assistant Prof. Department of Microbiology ID, Thakur Shyamnarayan Degree College, Kandivali East Mumbai.
Kewat Modi
Department of Microbiology ID, TSDC
Shraddha Singh
Department of Microbiology ID, TSDC

Abstract

Many individuals are familiar with the sweet, tropical fruit that comes from the mango tree, but did you know that leaves of mango trees are consumable as well? Young mango leaves are very tender, so they're cooked and eaten in some cultures because the leaves are considered very nutritious food. Mango leaves can be infused into tea. Although the stem, bark, leaves, roots, and fruits are likewise used in traditional medicine, the leaves particularly are believed to help treat like disease like diabetes and other health condition. The leaves of *Mangifera indica*, a certain species of mango, have been used in healing practices like Ayurveda and ancient Chinese medicine for thousands of years. *Mangifera indica L.* comes under the family of Anacardiaceae and is a dominant fruit from South and Southeast Asia. Leaves of the mango plant are being studied for their health benefits, which are accredited to a plethora of phytochemicals like mangiferin, followed by phenolic acids, benzophenones, and other antioxidants such as flavonoids, ascorbic acid and tocopherols. The extracts from mango leaves have been studied for their biological activities, including anti-cancer, anti-diabetic, anti-oxidant, anti-microbial, lipid-lowering, hepatoprotection, and anti-diarrheal. Here, in present study we have taken in to consideration two points; first, to study aeromicroflora from a closed chamber with mango leaves and second, study of Zone of Inhibition with mango leaf extract.

Keywords -*Mangifera indica,* rituals, scientific awareness, Ayurveda, zone of Inhibition, aeromicroflora, MDR.

Introduction

Natural plant as herbal remedies are being used to prevent and cure several illnesses vary in different aspect. These herbal plants are largely raw source are used for the production of modern antibiotics. For a long time, medicine have been explored solely from leaves, flowers and barks of plants; only recently the synthetic drugs used to treat different infections have same chemical components are identified in plants. As reported by WHO, a medicinal plant could be any plant that contains constituents which can be produced from its different parts and can be applied for beneficial purposes or can be predecessor for the production of useful drugs.

Continuous spread of infectious diseases is a significant problem for health organizations, pharmaceuticals think tanks all over the world. Failure of treatment, particularly with the current escalating trends of multi-drug resistance (MDR) to the available modern drugs or antibiotics among emerging and re-appearing bacterial pathogens leads to serious risks. Typhoid fever is a significant health problem due to poor personal hygiene, improper sewage drains contaminated food and unsafe potable water in Pakistan. *Mangifera indica L*, commonly known as mango, is a plant belonging to the family Anacardiaeceae which consists of about 60 genera and 600 species. It is one of the most admired tropical fruit bearing trees in the world. *Mangifera indica* also known as Mango or Aam. Many people used mango leaf as an ayurvedic medicine. In Indian houses they make toran (arch) made of mango leaves and hang in front of the door because it keeps air fresh, specifically when there are many people there. Reason why Santana Dharma encourages to tie mango leaves to our doors.

The leaves of mango are very rich in vitamin B, C and A. Mango leaves are very effective antioxidant, and for that reason it is widely used for curing various diseases. Mango leaves contain compound like polyphenols and terpenoids. Polyphenols have antioxidant and anti-inflammatory properties and it also helps to prevent conditions like obesity, diabetes, heart disease and cancer. Mango leaf helps to manage diabetes due to effects on fat metabolism. Elevated triglycerides levels are associated with insulin resistance and type 2 diabetes. Some research works show that mangiferin in mango leaves may have anticancer potential, it fights oxidative stress and inflammation and treats stomach ulcers and other digestive conditions. Mango leaves extract may reduce signs of skin aging due its antioxidant content and

it also promote hair growth and mango leaf extract may be used in some hair product. Terpenoids also have antioxidants which protect cells from harmful molecules called free radicals. It is also important for optimal vision and immune health. Mango leaf extract helps to manage metabolic syndrome by interfering with fat metabolism.

Aeromicrobiology is the study of all living microbes (bacteria, fungi, viruses, yeasts and protozoans) suspended in the air. Gregory termed these microbes, as "air spora" The percentage of microorganisms present in open environment is less as compared to the number in soil and oceans. Nevertheless, their number is enough to have an impact on the environment. The type and the number of microorganisms depend on the kind of environment, sampling time, and type of culture medium for measuring the fungal and bacteria species present at the location. Most commonly found bacteria in air are Staphylococcus aureus, Candida albicans and Clostridium difficile. Similarly, frequently found fungi in air are Alternaria, Cladosporium, Penicillium and Aspergillus. These airborne microbes cause various diseases including respiratory ailments (allergies and asthma). Exposure to bio-aerosols (air borne particles with biological origin), result in respiratory disorders, hypersensitivity pneumonitis toxic reactions and infections (Yassin and Almuqati, 2010). Wind is the major way of transport of bioaerosols. Bioaerosols are most commonly analysed by 'gravity slide' and 'settle-plate' techniques. In gravity slide technique, a glass slide containing glycerine jelly/petroleum jelly/silicone grease is kept facing upward in air, so that the spores of microbes get trapped in it. These trapped microbes are later analysed under microscope. In settle-plate technique, petri dish containing solid media is horizontally exposed in air for 5 min, followed by incubation at a particular temperature. The number of colonies grown is counted. Both these techniques have been widely used to study fungi and bacteria present in the air.

Materials and Methods

1. Aeromicroflora from a Closed Chamber with Mango Leaves

Indoor air of a closed container was analyzed in order to investigate the level of antimicrobial properties of mango leaves, to prevent the microbial growth in closed container.

Collection of plant Materials: Fresh leaves of *Mangifera indica* L. were collected from the college campus garden.

Preparation of Plant Materials: The freshly collected leaves were dipped into two different test tubes one of ethanol and other of distilled water. It was kept for 18 to 24 hours in room temperature for the extraction purpose.

Media, Chemicals and Solvents used Media used include: Nutrient Agar and Nutrient broth. The media were prepared in accordance with manufacturer's instructions. The media used was sterilized at 121^0C for 15minutes using autoclave and the glass wares were dried at 60^0C using hot air- oven. Pour autoclaved and cooled nutrient agar media in a petri dish (sterile) under sterile conditions and label the plates. Leave the plates undisturbed to solidify. Expose the petri dish for 5 min at different study sites for e.g. inside laboratory.

Preparation of closed environment: A plastic container was used as a closed environment. The container was sterile with alcohol to prevent from any other contamination.

Susceptibility test of microorganisms with and without mango leaves (procedure):

a. Without mango leaves: In the sterile plastic container, petri plate was placed in the center containing sterile nutrient agar. The container was immediately closed with a lid to prevent any other contamination. The container was then under observation for 18 – 24 hours.
b. With mango leaves: In the sterile plastic container, petri plate was placed in the center containing sterile nutrient agar, but this time with presence of mango leaves washed with ethanol were kept inside the container to check their antimicrobial properties. The container was immediately closed with a lid to prevent any other contamination. The container was then under observation for 24 hours.

2. Zone of Inhibition with Mango Leaf Extract

Media, Chemicals and Solvents used Media used include: Nutrient Agar and Nutrient broth. The media were prepared in accordance with manufacturer's instructions. The media used was sterilized at 121^0C for 15minutes using autoclave and the glass wares were dried at 60^0C using hot air- oven.

Chemicals and Solvent used include: Ethanol, Distilled water.

Collection of plant Materials: Fresh leaves of *Mangifera indica* L. were collected from the college campus garden.

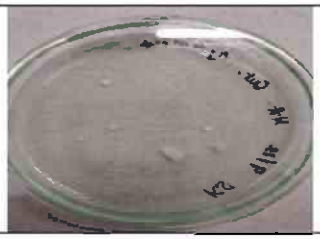
Preparation of Plant Materials: The freshly collected leaves were dipped into two different test tubes one of ethanol and other of distilled water. It was kept for 18 to 24 hours in room temperature for the extraction purpose.

Preparation of Test Organisms: The test organisms used were pure culture of *Escherichia coli, Staphylococcus typhi, Aspergillus niger*. These organisms were obtained from the Laboratory Unit of Microbiology. The said test organisms were inoculated onto nutrient broth for a period of 24 hours and at growth temperature of 37 °C in order to ascertain their viability.

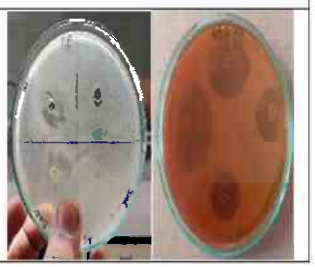
Susceptibility Test of organisms: - The plates were inoculated with the test organisms in duplicates by spread plate method. A cork borer of width 6 mm in diameter was flamed using a Bunsen burner and was used to bore wells on the inoculated plates. The wells were then filled with the extracts at different concentrations. The extracts were allowed for 30 minutes to diffuse into the agar plates and then incubated at 37 °C for 24 hours.

After incubation, the plates are examined and the diameter of the zones of inhibitions measured to the nearest whole millimeter by use of sliding calipers, a ruler, or a template prepared for this purpose. When supplemented medium is used, the measuring device is held on the back of the petri plate.

Observations

Photoplate.1.: Mango Leaves are kept in closed chamber for 24 Hours to study aeromicroflora	**Photoplate.2: Petri plate kept in Closed chamber with mango leaves**	**Photoplate.3: Petri plate kept in open environment without mango leaves**
		

It has been observed that the Petri plate with closed chamber shows very less no of microbial contaminants on the nutrient media where as the petri plate kept in open space for aeromicroflora study shows more no of bacterial and fungal contamination after 48 hours of incubation period.

Photoplate.3: Mango Leaves are kept in alcohol for extract preparation	Photoplate.4: Extract where placed in Petri plate to study the zine of inhibition	Photoplate.5: Zone of Inhibition showed antimicrobial property.
		

It has been observed that the results of the antibacterial assay of the ethanol leave extracts of *Mangifera indica* indicated that the plant exhibited antibacterial activity against the tested microorganisms at different concentration. The potential sensitivity of the extract was obtained against all the microorganisms tested and the zone of inhibition.

Result and Discussion

A Zone of Inhibition Test, also called a Kirby-Bauer Test, is a qualitative method used clinically to measure antibiotic resistance and industrially to test the ability of solids and textiles to inhibit microbial growth. Researchers who develop antimicrobial textiles, surfaces, and liquids use this test as a quick and easy way to measure and compare levels of inhibitory activity.

With this method, approximately one million cells from a single strain are spread over an agar plate using a sterile swab, then incubated in the presence of the antimicrobial object.

If the bacterial or fungal strain is susceptible to the antimicrobial agent, then a zone of inhibition appears on the agar plate.

Advantages of Zone of Inhibition Testing

1. Zone of inhibition testing is fast and inexpensive relative to other laboratory tests for antimicrobial activity.
2. Zone of inhibition testing is especially well suited for determining (albeit qualitatively) the ability of water-soluble antimicrobials to inhibit the growth of microorganisms.
3. A number of samples can be screened for antimicrobial properties quickly using this test method.

4. A variety of antimicrobial product types can be tested using this method. Liquids, coated antimicrobial surfaces, and antimicrobial-impregnated solid products can all be tested for their ability to produce a zone of inhibition.

Disadvantages of Zone of Inhibition Testing:

1. Antimicrobial agents that leach out of the object and into the aqueous agar matrix, such as silver ions, usually show better results than antimicrobials that stay affixed to the object or textile or that are not water-soluble.
2. Zone of Inhibition tests do not necessarily indicate that microorganisms have been killed by an antimicrobial product - just that they have been prevented from growing.
3. Microbial growth agars themselves may interfere with the function of some antimicrobial agents.
4. The method cannot be used to test the activity of antimicrobial agents against viruses, since viruses on agar plates like bacteria.
5. The method has some natural variability, and zones of microbial inhibition do not always have clear or regular boundaries.
6. The method is not classically quantitative (though sometimes the diameter of the zone of inhibition are measured and recorded).

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11. Singh, SatyavratPratap Singh, JitendraKumar Yadav, SandeepYadav, Vibha Kumar, Rajesh Kumar Joshi, Rajesh Antibiogram Pattern of Common Plant Leaves against E. coli, Staphylococcus aureus and Whole Milk Culture

11. Analysis of Sewage and Fresh Pond Water Samples from Thakur College Campus and Their Treatment Using Fungal Organisms

Udaybhan Yadav
Coordinator, Department of Microbiology (Interdisciplinary), Thakur Shyamnarayan Degree College, Kandivali East Mumbai.
Kunal Thakur
Assistant Prof. Department of Microbiology ID, Thakur Shyamnarayan Degree College, Kandivali East Mumbai.

Abstract

Aflatoxin biosynthesis was induced by compounds in filtrates (EF) obtained from cultures consisting of ground maize kernels colonized by *Aspergillus flavus*. Natural poisons in food are as much a part of our life as the oxygen we breathe. It is more important to be able to neutralize these poisons naturally from our diet. This is only possible if we are exposed to very minute and non–toxic amounts which our bodies can handle with ease. It would not only be unrealistic, but impossible to totally eradicate natural poisons from our food. Some mycotoxins directly inhibit the growth of microorganisms. The term Mycotoxin literally means poison from fungi. Mycotoxins are secondary metabolites produced by micro fungi that are capable of causing disease and death in humans and other animals. Because of their pharmacological activity, some mycotoxins or mycotoxin derivatives have found use as antibiotics, growth promotants, and other kinds of still others have been implicated as chemical warfare agents.

Key Words: *Aspergillus flavus,* Mycotoxins, Aflatoxins, Secondary Metabolites.

Introduction

Mycotoxins: The most important mycotoxins are the following: aflatoxins (AFB1,B2, G1, G2), ochratoxin A (OTA), tricothecenes, zearalenoneand fumonisins (B1, B2). Of the tricothecenes, attentionis paid mainly to deoxynivalenol (DON or vomitoxin), nivalenol and T2-toxin. Some laboratories also focused onHT2 toxin, 3-Ac-DON, DAS, Fusarenon-X. One laboratory also included MAS, Neosolaniol and 15-Ac-DON in the range of analyses. Other mycotoxins were also mentioned but only by a few members; these were patulin,

sterigmatocystin and cytochalasin E. This review focuses on the most important ones associated with human and veterinary diseases, including aflatoxin, citrinin, ergot akaloids, fumonisins, ochratoxin A, patulin, trichothecenes, and zearalenone. They are usually named on the basis of the fungus that produces them. For instance, "Aflatoxin" uses the A for Asperigillus and Fla for the species flavus along with the word toxin. There are three major genera of fungi that produce mycotoxins: Aspergilius, Fusarium and Penicilium. Mycotoxin-producing fungi grow on a wide spectrum of feeds that include cereal grains, groundnuts, beans and peas.

Aflatoxins: Aflatoxins are toxic metabolites produced by certain fungi in/on foods and feeds. They are probably the best known and most intensively researched mycotoxins in the world.

The occurrence of aflatoxins is influenced by certain environmental factors; hence the extent of contamination will vary with geographic location, agricultural and agronomic practices, and the susceptibility of commodities to fungal invasion during preharvest, storage, and/or processing periods. Aflatoxins are produced by different species of *Aspergillus niger,* flaves particularly and *parasiticus*, as well as members of the Genera *Penicillium* and *Rhizopus*. Aflatoxins can contaminate corn, cereals, sorghum, peanuts, and other oil-seed crops. *Aspergillus*, which produces aflatoxin, is among the most common grain mold fungi. Nevertheless, aflatoxin's reputation has a potent poison may explain why it has been adopted in bioterrorism The aflatoxins were isolated and characterized after the death of more than 100,000 turkey poults (turkey X disease) was traced to the consumption of a mold-contaminated peanut meal (four major aflatoxins are called B1, B2, G1, and G2 based on their fluorescence under UV light or green) and relative chromatographic mobility during thin-layer chromatography.

Aflatoxins have been associated with various diseases , such as aflatoxicosis in livestock , domestic animals and humans throughout the world Aflatoxins have received greater attention than any other mycotoxins because of their demonstrated potent carcinogenic effect in susceptible laboratory animals and their acute toxicological effects in humans . Aflatoxins are acutely toxic, immunosuppressive, mutagenic, teratogenic and carcinogenic compounds. People look at the direct life threat from culprits, but the job hazards are much more than that. Aflatoxins, like other natural poisons in food, cannot be completely eradicated from food. Aflatoxin poisoning is reported from all parts of world in almost all domestic and non domestic animals and other non human primates. In addition, inhalation of aflatoxin is associated with

disease and injury in both animals and human identification of genetic resistance to the production of aflatoxin in more than five major crops. Aflatoxins are recognized as serious food safety hazards by most countries of the world and more than 50 countries have established or proposed regulations for controlling them in food and feed. There is to our knowledge very little evidence of the occurrence of aflatoxins in European cereals and in some other countries37 .In the U.S. corn, cottonseed, peanuts, and other crops are routinely tested and those containing more than 20 parts per million of aflatoxins cannot be used in human food or fed to dairy cows. It is estimated that crop loss due to aflatoxin contamination costs U.S. producers more than $100 million per year on average. As it the is realized that absolute safety is never achieved , many countries have attempted to limit exposure to aflatoxins by imposing regulatory limits on commodities intended for use as food and feed.

Contents

A careful survey of the early outbreaks showed that they were all associated with feeds, namely Brazilian peanut meal. An intensive investigation of the suspect peanut meal was undertaken and it was quickly found that this peanut meal was highly toxic to poultry and ducklings with symptoms typical of Turkey X disease. Speculations made during 1960 regarding the nature of the toxin suggested that it might be of fungal origin. In fact, the toxin-producing fungus was identified as *Aspergillus flavus* (1961). This discovery has led to a growing awareness of the potential hazards of these substances as contaminants of food and feed causing illness and even death in humans and other mammals. Studies that are summarized in the following sections revealed that aflatoxins are produced primarily by some strains of A. Flavus and by A. niger .most , if not all , strains of A. parasiticus , plus related species, A. nomius Moreover , these studies also revealed that there are four major aflatoxins : **B1** , **B2** , **G1** , **G2** plus two additional metabolic products , **M1** and **M2** , that are of significance as direct contaminants of foods and feeds . The aflatoxins M1 and M2 were first isolated from milk of lactating animals fed aflatoxin preparations; hence, the M designation. Whereas the B designation of aflatoxins B1 and B2 resulted from the exhibition of blue fluorescence under UV-light, while the G designation refers to the yellow-green fluorescence of the relevant structures under UV-light. These toxins have closely similar structures and form a unique group of highly oxygenated, naturally occurring heterocyclic compounds. Their molecular formulas as established from elementary analyses and mass spectrometric determinations are:

- B1 : C17 H12 O6
- B2 : C17 H14 O6
- G1 : C17 H12 O7
- G2 : C17 H14 O7

Aflatoxins B2 and G2 were established as the dihydroxy derivatives of B1 and G1 , respectively . Whereas, aflatoxin M1 is 4-hydroxy aflatoxin B1 and aflatoxin M2 is 4-dihydroxy aflatoxin .Consumption of low concentrations by animals sensitive to aflatoxins can lead to death in 72 hours. In general, at nonfatal levels, the health and productivity of animals fed contaminated feed are seriousimpaired. As a result, the Food and Drug Administration (FDA) has set an action level for aflatoxins in corn at 20 parts per billion (ppb). Corn containing aflatoxin levels of 20 ppbor more cannot be sold in interstate commerce, and, in general, should not be fed to young poultry , swine, and livestock, or to lactating animals, and must not be milled.

Aspergillus Niger is a fungus and one of the most common species of the genus *Aspergillus*. It causes a disease called black mold on certain fruits and vegetables such as grapes, onions, and peanuts, and is a common contaminant of food. It is ubiquitous in soil and is commonly reported from indoor environments, where its black colonies can be confused with those of *Stachybotrys* (species of which have also been called "black mold").Some strains of *A. niger* have been reported to produce potent mycotoxins called ochratoxins, aflatoxin.

Taxonomy: A. niger is included in Aspergillus subgenus Circumdati, section Nigri. The section Nigri includes 15 related black-spored species that may be confused with A. niger, including A. tubingensis, A. foetidus, A. carbonarius, and A. awamori.

Pathogenicity: 1. Plant disease*: A. niger causes black mold of onions. Infection of onion seedlings by A. niger can become systemic, manifesting only when conditions are conducive. A. niger causes a common postharvest disease of onions, in which the black conidia can be observed between the scales of the bulb. The fungus also causes disease in peanuts and in grapes. The spore comes to common trees such as maple.*

2. ***Human and animal disease: A.*** *niger is less likely to cause human disease than some other Aspergillus species, but if large amounts of spores are inhaled, a serious lung disease, aspergillosis can occur. Aspergillosis is particularly frequent among horticultural workers who inhale peat dust, which can be rich in Aspergillus spores. Less commonly, it has been found on*

the walls of ancient Egyptian tombs and can be inhaled when the area is disturbed. A. niger is one of the most common causes of otomycosis (fungal ear infections), which can cause pain, temporary hearing loss and, in severe cases, damage to the ear canal and tympanic membrane.

Aspergillus flavus is a mold fungus. It is a pathogen, associated with aspergillosis of the lungs and sometimes believed to cause corneal, otomycotic, and nasoorbital infections. It is believed to be allergenic and sometimes causes losses in silkworm hatcheries. It is particularly common on corn and peanuts, as well as water damaged carpets, and is one of several species of mold known to produce aflatoxin which is a carcinogenic substance.

Industrial uses: *A. niger* is cultured for the industrial production of many substances. Various strains of *A. niger* are used in the industrial preparation of citric acid (E330) and gluconic acid (E574) and have been assessed as acceptable for daily intake by the World Health Organization. Many useful enzymes are produced using industrial fermentation of *A. niger*. For example, *A. niger* glucoamylase is used in the production of high fructose corn syrup, and pectinases are used in cider and wine clarification. α-galactosidase, an enzyme that breaks down certain complex sugars, is a component of Beano® and other medications which the manufacturers claim can decrease flatulence. Another use for *A. niger* within the biotechnology industry is in the production of magnetic isotope-containing variants of

Material and Methods

1. Preparation of PDA Medium
2. Preparation of PDA plates
3. Streak Plate Method from Mixed Culture to Pure Culture

Sterilization of Maize Seeds

Take the seeds in a conical flask. Wash it in a tap water properly and then with a dilute Savlon or Dettol. Again wash 2 times in a tap water. Wash with 70% alcohol for half min. Transfer the seeds to sterile conical flask containing 0.1 % HgCL2 solution. Plug the flask and shake gently for 5-7 min. Decent the HgCL2 solution from the flask. Rinse the seeds 3-4 times with sterile DW properly. Transfer the seeds in a Petri dish. Seeds germinated in a dark, Calculate percentage of germination. Root formed kept in a light for further growth.

Sterilization of Soil

The garden soil is been taken and placed in a vessel. The vessel had been placed in a cooker so the micro-organisms or any of the contamination will not be there because of high temperature.

A.Extraction of aflatoxins from culture media

Aflatoxin produced by fungi in culture media can be easily extracted in a chloroform and their toxicity can be assayed using bioassays.

Material

- Aspergillus.niger or A.flavus grown for 10 days on Czapek's broth supplemented with casein to give 0.5g nitrogen / L at pH 4.5 as stationary culture.
- Bucher funnel
- Whatman No . 1 filter paper
- Chloroform

For Czapek (Dox) Broth (CDB)

- Sodium Nitrate : 1gm
- Potassium dihydrogen phosphate : 0.5gm
- Magnesium Sulphate : 0.25gm
- Ferrous Sulphate : pinch
- Sucrose : 15.0 gm
- Distilled water : 500ml

Method

For preparation of Czapek Broth mix all the ingredients required to prepare it, adjust Ph to 5.6 and sterilize for 15 min in an autoclave at 1.05 kg f/cm2

Filter the medium through Buchner funnel using Whatman No.1 filter paper. Extract 100ml of the culture filtrate thrice with equal volumes of chloroform. Cool the chloroform extracts and concentrate to dryness in a rotary evaporator. Dissolve the residue in minimum quantity of distilled water. If required for direct bioassay or in chloroform for chromatographic separation its toxicity on plant growth may be assayed without further purification

Bioassay of Aflatoxins

The toxicity of the aflatoxin extract obtained form culture filtrates on plant growth may be assayed using seed germination and seedling growth tests.

Materials

- Aflatoxin extract
- Maize seeds
- Test tubes
- Pipettes, 10 ml
- Petri dishes
- Filter dishes
- Scale.

Methods

Inhibition of Seed Germination

Soak the surface sterilized seeds in 20 ml aliquots of culture filtrate on appropriate dilutions of the toxin extract for 24 hr in test tubes. Spread 100 of the treated seeds on moist filter paper placed in Petri dish. lncubate at room temperature. Maintain suitable control with seeds soaked in dist water .Count the germinated seeds after 7 days. Calculate per cent of germination.

Reduction in Seedling Vigour

Germinate the seed soaked in water in dishes. Select 12 germinated seeds with uniform shoot length measuring about 5mm and place them in dished lined with filter paper moistened with 5ml of the culture filtrate or toxin extract. Maintain control with distilled water. After 6 days, measure the total shoot growth of 10 seedlings avoiding both shortest and longest ones and measure elongation during 5 days incubation period.

When the aflatoxin extract is applied on the maize seed and the growth of specific fungus has been seen. The 40% seed's were germinated out of which only 15% showed development of seedlings . The growth of the maize plant was shunt and slow as compaired to the standard seedlings.

Observation and Result

Calculation

Percentage of germination of

$$\text{Percentage of germination} = \frac{\text{no. of germinated seeds}}{\text{Total no of seeds}} \times 100$$

$$\text{Percentage of germination of the standerd seed of maize is} = \frac{70}{100} \times 100 = 0.70$$

Percentage of germination of the infected seed of maize by Aspergillus. niger

$$= \frac{25}{100} \times 100 = 0.22$$

Result

- Percentage of germination of the standerd seed of maize is = 0.70
- Percentage of germination of the infected seed of maize by Aspergillus . niger = 0.22
- Percentage of germination of the infected seed of maize by Aspergillus .flavus = 0.45

B. Thin Layer Chromatography

The separation and identification of organic compounds is a routine work in many service laboratories. This layer chromatography (tlc) is an easy technique to adopt for the said purpose. It is highly useful in research laboratories to separate, identify and characterize unknown compounds. A variety of small molecules like amino acids, sugar, organic acids, organic acids, lipids etc are separated by tlc technique. The greater advantage of tlc is the spread at which separation is achieved. When volatile solvents are used the time required to effect separation is only about 30 min and with nonvolatile solvents it is seldom longer than 90 min.

Principle

The general priniciple involved in tlc is similar to that of colume chromatography i.e, adsorption chromatography. In the adsorption process, the solute competes with the solvent for the surface sites of the adsorbent. Depending on the distribution coefficients, the compounds are distributed on the surface of the adorbent. Of course, in tlc the partition effect in the separation is also not ruled out. The adsorbent normally used contains a binding agent such as calcium sulphate which facilitates the holding of the adsorbent to the glass plate.

Materials

- Thin Layer Chromatography (tlc) Kit
- Ultra – Violet (UV) Chamber
- Mechanical Shaker
- Toluene
- Ethyl Acetate
- Formic Acid
- Chloroform
- Silica Gel G (tlc Grare)

Procedure

Extraction of Toxins

1. Weigh exactly 50g of ground sample material and transfer it into a 250ml conical flask.
2. Moist the material uniformally by adding 10 -15ml of distilled water and add about 200ml chloroform, stopper the mouth with a cotton plug in aluminium foil.
3. Shake the flask for one hour mechanically. (It is important that the oil – containing materials are defatted prior to extraction.)
4. Filter the slurry through a Buchner funnel under mild suction. Equal amount of a filtering aid such as celite may be mixed before filtering in order to ease filtration. Wash the flask and the slurry thoroughly with additional chloroform (25 ml) and collect the filtrate.
5. Transfer the filtrate quantitatively to a separatory funnel and shake with water one-half volume of chloroform.After the phases separate,drain the bottom (chloroform) phase into a flask containing about 10g sodium sulphate (anhydrous) to absorb any water.
6. Concentrate the clear, chloroform extract over a warm water bath. Make up the concentrate to a known volume with chloroform and store in amber – coloured vials under refrigeration until analysis

Preparation of the Plates

1. Place 30g silica gel G in a stoppered flask, share vigorously with 60-65ml distilled water for about one minute, transfer to the applicator and spread uniformally on glass plates (20 × 20 cm). The exact quantity of chloroform to get a good slurry . The thickness of layer should usually be 0.25 mm.
2. Allow the plates to dry for 1 hr in dust free conditions.
3. Divide the gel into a number of lanes by drawing lines on the gel with a sharp needle.
4. Spot different volumes of the sample extract in various lanes carefully with a capillary on a imaginary line 2.5cm away from one end of the plate.
5. Develop the plate in a solvent system of toluene: ethyl acetate: formic acid (6: 3: 1) in a chromatographic tank for about 50min. By then, the solvent front might have moved up to 20mm below the top end of the plate.
6. Dry the plate at room temperature to remove the solvent. Visualize the fluorescing spot of toxin under UV light; otherwise, eye sight will be affected.

7. Identify each fluorescing spot of the sample extract .Determine the Rf value of each spot.

Calculation

$$Rf = \frac{\text{Distance (cm) moved by the solute from the origin}}{\text{Distance (cm) moved by the solvent from the origin}}$$

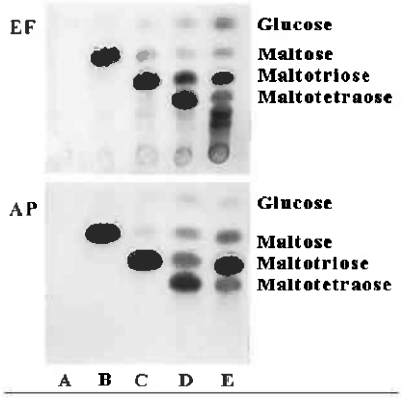
For a fluoresce green (G) G1 $ G2

Rf of Aspergillus niger $= \frac{14.6}{20} = 0.73$

Rf of Aspergillus flavus $= \frac{15.3}{20} = 0.77$

Result

- Rf of Aspergillus niger = 0.73
- Rf of Aspergillus flavus = 0.77



Photoplate.1: Thin-layer chromatographic (TLC) analysis of malto-oligosaccharides digested by maize EF and 2% amylopectin (AP) culture filtrates. Culture filtrates were incubated for 8 h at 40°C with **A,** no substrate; **B,** 0.6% maltose; **C,** 0.6% maltotriose; **D,** 0.6% maltotetraose; or **E,** 0.6% maltoheptaose. After chromatographic development, products were detected by spraying the TLC plates with 30% sulfuric acid solution and charring at 100°C.

C. Extraction and Detection of Nuleic Acids

The nucleic acid consist of Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA).

A. EXTRACTION AND DETECTION OF DNA FROM INFECTED SEEDS

Requirement

- *Racicals of germinating seeds*
- 15 % TCA (Trichloro acetic acid)
- 10 % NaCl (Sodium chloride)
- Chilled etanol
- EDTA (Etylene diamine tetra acetic acid) powder
- Grinder (motor and pestle)
- Centrifuge
- Test tubes.

Extraction of DNA

Weigh 1gm radicals of germinated seeds and cut into small pieces. To this add a pinch of EDTA powder and grind it in a grinder or motor pestle. Then add 5ml of TCA (removes fats and carbohydrates). Take the contents in a centrifuge tube and centrifuge the solution at 5000rpm. Discard the supernatant and to the ppt and 10ml of 10%NaCl (to dissolve DNA solution). Boil the content in a water bath for 30 mins. Cool the solution and again centrifuge at 5000rpm for 5mins.Take the supernatant and chill it. To this add equal quantity of chilled ethanol. DNA appears at the junction of two liquids. Shake the tube and keep it in ice-box for 30mins. After 30mins centrifuge the content to separate the DNA ppt. Discard the supernatant and collect the ppt.

Put a drop of Acetocarmine stain. The DNA ring becomes purplish pink in colour.

Chemical Preparation

i. 15%TCA :1.5gm of TCA in 30ml of distilled water.

ii. 10%NaCl : 10gms of NaCl in 100ml of distilled water.

Detection of Nucleic Acid

The nucleic acids absorb strongly in the ultra violet region of the spectrum (due to conjugate double bond system). They show characteristic maxima at 260 nm and minimum at 230nm but such a low range is not possible so minimum OD at

Result of OD

- Standard material: 0.56
- Aspergillus. flavus : 0.66

- *Aspergillus .niger : 0.45*

***B.* Extraction and Detection of RNA from Plant Material**

Requirement

Maize seeds, saline EDTA (Ethylene diamine tetra acetic acid), Phenol 80%, chilled ethyl alcohol, centrifuge, pestle and mortar.

Procedure

Weigh 1gm of maize and cut it into small pieces. Crush the maize seeds grained in 5ml of saline EDTA or distilled water using pestle and mortar. To this add 10ml 0f 80% Phenol. Shake the mixture and centrifuge it. Take the supernatant and 2 volumes of chilled ethanol. Keep it in refrigerator overnight. Centrifuge the next day and collect the RNA ppt.

Detection of RNA

Dissolve the RNA crystals from the previous experiment in 1ml of water. Also dissolve 0.5gmsof RNA crystals. Use these two materials for descending chromatography on Whatman No .1 PAPER. After running the chromatogram dry it in dry air for 4 hours. And locate the bases under ultra violet light. Both will be same.

Preparation

80% Phenol. Take 80ml of Phenol and 20ml of distilled water

Calculation

$$Rf = \frac{\text{Distance (cm) moved by the solute from the origin}}{\text{Distance (cm) moved by the solvent from the origin}}$$

$$\text{Rf of Pure maize} = \frac{8.1}{8.9} = 0.91$$

$$\text{Rf of Aspergillus niger} = \frac{7.2}{8.4} = 0.84$$

$$\text{Rf of Aspergillus flavus} = \frac{7.3}{8.2} = 0.89$$

Result

- Rf of Pure maize = 0.91
- Rf of Aspergillus niger = 0.84
- Rf of Aspergillus flavus = 0.89

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12. Study of Fungal Mycotoxins I.E. Aflatoxins from Infected Maize Grains by Using *Aspergillus* Spp. *I.E. A. Niger* and *A. Flavus*

Udaybhan Yadav
Coordinator, Department of Microbiology (Interdisciplinary), Thakur Shyamnarayan Degree College, Kandivali East Mumbai.
Kunal Thakur
Assistant Prof. Department of Microbiology ID, Thakur Shyamnarayan Degree College, Kandivali East Mumbai.

Abstract

Natural poisons in food is a major part of our life as the oxygen we breathe. It is very important to neutralize these poisons naturally from our diet to consume the food safely. This is only possible if we are exposed to very minute and non–toxic amounts which our bodies can handle with ease. It would not only be unrealistic, but impossible to totally eradicate natural poisons from our food. Fungi and bacteria are main cause of food poisoning. It takes about 3 days for the symptoms of diarrhea, stomach cramps and fever to develop. Fungi releases out mycotoxins; also called as secondary metabolites for fungi. Aflatoxins are one of the class of mycotoxins. Aflatoxin biosynthesis was induced by compounds in filtrates (EF) obtained from cultures consisting of ground maize kernels colonized by *Aspergillus flavus*.

Key Words - Natural Poison, Mycotoxins, Aspergillus, Aflatoxins, Maize.

Introduction

Mycotoxins are secondary metabolites produced by micro fungi that are capable of causing diseases and death in humans and other animals. Because of their pharmacological activity, some mycotoxins or mycotoxin derivatives have found use as antibiotics, growth promotants, and others have been implicated as chemical warfare agents. Some mycotoxins directly inhibit the growth of microorganisms. This review focuses on the most important ones associated with human and veterinary diseases, including aflatoxin, citrinin, ergot akaloids, fumonisins, ochratoxin A, patulin, trichothecenes, and zearalenone etc.

The most important mycotoxins are aflatoxins (AFB1, B2, G1, G2), ochratoxin A (OTA), tricothecenes, zearalenone and fumonisins (B1, B2). Tricothecenes, mainly include deoxynivalenol (DON or vomitoxin), nivalenol and T2-toxin. Some other examples are HT2 toxin, 3-Ac-DON, DAS, Fusarenon-X. MAS, Neosolaniol and 15-Ac-DON are also included in the in the range of analyses. The other mycotoxins mentioned are patulin, sterigmatocystin and cytochalasin E.

They are usually named on the basis of the fungus that produces them. For instance, "Aflatoxin" uses the A for *Aspergillus* and fla for the species flavus along with the word toxin. There are three major genera of fungi that produce mycotoxins: *Aspergillus, Fusarium* and *Penicillium*. Mycotoxin-producing fungi grow on a wide spectrum of feeds that include cereal grains, groundnuts, beans and peas.

Aflatoxins

Aflatoxins are toxic metabolites produced by certain fungi in / on foods and feeds. They are probably the best known and most intensively researched mycotoxins in the world. The occurrence of aflatoxins is influenced by certain environmental factors; hence the extent of contamination will vary with geographic location, agricultural and agronomic practices, and the susceptibility of commodities to fungal invasion during preharvest, storage, and / or processing periods .

Aflatoxins are produced by different species of *Aspergillus niger, flavus* particularly and *parasiticus*, as well as members of the Genera *Penicillium* and *Rhizopus*. Aflatoxins can contaminate corn, cereals, sorghum, peanuts, and other oil-seed crops. *Aspergillus*, which produces aflatoxin, is among the most common grain mold fungi.

Nevertheless, aflatoxin's reputation has a potent poison may explain why it has been adopted in bioterrorism. The aflatoxins were isolated and characterized after the death of more than 100,000 turkey poults (turkey X disease) was traced to the consumption of a mold-contaminated peanut meal (four major aflatoxins are called B1, B2, G1, and G2 based on their fluorescence under UV light or green) and relative chromatographic mobility during thin-layer chromatography.

Aflatoxins have been associated with various diseases , such as aflatoxicosis, in livestock , domestic animals and humans throughout the world Aflatoxins have received greater attention than any other mycotoxins because of their demonstrated potent carcinogenic effect in

susceptible laboratory animals and their acute toxicological effects in humans . Aflatoxins are acutely toxic, immunosuppressive, mutagenic, teratogenic and carcinogenic compounds. People look at the direct life threat from culprits, but the job hazards are much more than that. Aflatoxins, like other natural poisons in food, cannot be completely eradicated from food. Aflatoxin poisoning is reported from all parts of world in almost all domestic and non - domestic animals and other non - human primates. In addition, inhalation of aflatoxin is associated with disease and injury in both animals and human identification of genetic resistance to the production of aflatoxin in more than five major crops. Aflatoxins are recognized as serious food safety hazards by most countries of the world and more than 50 countries have established or proposed regulations for controlling them in food and feed. There is to our knowledge very little evidence of the occurrence of aflatoxins in European cereals and in some other countries37. In the U.S. corn, cottonseed, peanuts, and other crops are routinely tested and those containing more than 20 parts per million of aflatoxins cannot be used in human food or fed to dairy cows. It is estimated that crop loss due to aflatoxin contamination costs U.S. producers more than $100 million per year on average. As it the is realized that absolute safety is never achieved , many countries have attempted to limit exposure to aflatoxins by imposing regulatory limits on commodities intended for use as food and feed.

Materials and Methods

Part I: Preparation of Potato Dextrose Agar Medium

a. Requirements

- Potato tubers 100g
- Dextrose 10g
- Agar 7.5g
- Peptone 1g
- Distilled water 500ml
- HCl 1N
- NaOH 1N
- Knife
- Muslin cloth
- Heater
- Beaker (500ml capacity)

- Borosil flask (500ml capacity)

b. Procedure

i. Take potato tubers, peel off and weigh 100g.

ii. Chop the tubers into small pieces with the help of a knife.

iii. Transfer the chopped potatoes into a beaker containing about 200ml of distilled water.

iv. Boil the contents with the help of a heater for about 20 minutes.

v. Decant supernatant, filter with four fold of the muslin cloth and collect the filtrate into a beaker. This filtrate is called potato extract.

vi. Transfer dextrose (10g), agar (7.5g) and peptone (1g) into the extract and gently heat and shake to dissolve the ingredients.

vii. Finally transfer this medium into a measuring cylinder of 500ml capacity and make the volume to 500ml by adding more distilled water.

viii. Measure the pH of the medium and adjust to 5.6 by using 1N HCl or NaOH.

ix. Pour the medium into Borosil flask, put cotton plug, and cover the plug with aluminium foil / paper, and autoclave at 121^0 C for 20 minutes.

x. When temperature cools down take out the flasks and use if required or store at room temperature.

Part II: Preparation of PDA Plates

a. Requirements

- PDA medium
- Culture tubes (smooth mouth)
- Test tube stand
- Heater
- Aluminium foil or paper

b. Procedure

i. Before starting autoclaving place few Petri plates into an oven and sterilize them at 200℃ for about half an hour.

ii. When temperature cools down, transfer them into the cabin of the laminar air flow or inoculation chamber built for inoculation work .However, these must be sterilized by UV light 30 minutes before start of the work.

iii. Bring the flask containing PDA medium and pour about 15 – 20 ml medium aseptically into the bottom half of the Petri dishes when temperature remains to about 40°C (care should be taken not to pour too hot medium otherwise it will cause condensation of vapour into water droplets and help contamination).

iv. Place the plates in tiers and wait for about 20-30 minutes to solidify the medium.

v. These plates containing solidified medium are called PDA plates.

vi. Use the plates immediately foe cultivation of fungi or store for future use.

Part III: Streak Plate Method to Get Pure Culture

The colonies on mixed plates are separated by spreading on a plate with good spacing each other using streak method.

a. Requirements

- Tripod and wire gauze
- Burner
- Beaker of water
- Wire loop
- PDA pour plates
- Mixed culture of fungi

b. Procedure

i. Streak the plates following quadrant radiant or T-streak or continuous streak as shown in the Fig.

ii. Keep the streaked plates in inverted position at 25°C for 24 – 48 hours.

iii. Place the Petri dishes upside down to solve the problem of water condensation because if it drops down on the colonies, the organisms of one colony can spread on the other colony.

c. Results

The isolated colony of desired fungi (at the site of last streak) on the plate will be Observed.

Part IV: Preparation of Czapek's Dox Broth

Aflatoxin produced by fungi in culture media can be easily extracted in a chloroform and their toxicity can be assayed using bioassays.

Material

Aspergillus.niger or A.flavus grown for 10 days on Czapek's broth supplemented with casein to give 0.5g nitrogen / L at pH 4.5 as stationary culture.

- Bucher funnel
- Whatman No . 1 filter paper
- Chloroform

For Czapek (Dox) Broth (CDB)

- Sodium Nitrate : 1gm
- Potassium dihydrogen phosphate : 0.5gm
- Magnesium Sulphate : 0.25gm
- Ferrous Sulphate : pinch
- Sucrose : 15.0 gm
- Distilled water : 500ml

Method

For preparation of Czapek Broth mix all the ingredients required to prepare it, adjust Ph to 5.6 and sterilize for 15 min in an autoclave at 1.05 kg f/cm2

Filter the medium through Buchner funnel using Whatman No.1 filter paper. Extract 100ml of the culture filtrate thrice with equal volumes of chloroform. Cool the chloroform extracts and concentrate to dryness in a rotary evaporator. Dissolve the residue in minimum quantity of distilled water. If required for direct bioassay or in chloroform for chromatographic separation its toxicity on plant growth may be assayed without further purification

Part V: Bioassay of Aflatoxins

i. **Extraction of Toxins**
ii. **Preparation of TLC Plates**
iii. **Qualitative Analysis of Aflatoxins**
iv. **Seed Germination**
v. **Detection of Nucleic Acids (DNA and RNA)**

Results and Discussion

Sterilization of Maize Seeds: Take the seeds in a conical flask. Wash it in a tap water properly and then with a dilute Savlon or Dettol. Again wash 2 times in a tap water. Wash with 70% alcohol for half min. Transfer the seeds to sterile conical flask contains 0.1 % HgCL2 solution. Plug the flask and shake gently for 5-7 min. Decent the HgCL2 solution from the flask.

Rinse the seeds 3-4 times with sterile DW properly. Transfer the seeds in a Petri dish. Seeds germinated in a dark, Calculate percentage of germination. Root formed kept in a light for further growth.

Sterilization of Soil: The garden soil is been taken and placed in a vessel. The vessel had been placed in a cooker so the micro-organisms or any of the contamination will not be there because of high temperature.

1. Extraction of Aflatoxins from Culture Media: Aflatoxin produced by fungi in culture media can be easily extracted in a chloroform and their toxicity can be assayed using bioassays.

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2. Bioassay of Aflatoxins

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- Aflatoxin extract
- Maize seeds
- Test tubes
- Pipettes, 10 ml
- Petri dishes

Methods

Inhibition of seed Germination: Soak the surface sterilized seeds in 20 ml aliquots of culture filtrate on appropriate dilutions of the toxin extract for 24 hr. in test tubes. Spread 100 of the treated seeds on moist filter paper placed in Petri dish. incubate at room temperature. Maintain suitable control with seeds soaked in dist water. Count the germinated seeds after 7 days. Calculate per cent of germination.

Reduction in Seedling Vigour: Germinate the seed soaked in water in dishes. Select 12 germinated seeds with uniform shoot length measuring about 5mm and place them in dished lined with filter paper moistened with 5ml of the culture filtrate or toxin extract. Maintain control with distilled water. After 6 days, measure the total shoot growth of 10 seedlings avoiding both shortest and longest ones and measure elongation during 5 days incubation period. When the aflatoxin extract is applied on the maize seed and the growth of specific fungus has been seen. The 40% seeds were germinated out of which only 15% showed development of seedlings. The growth of the maize plant was shut and slow as compared to the standard seedlings.

Calculation

Percentage of germination of

$$\text{Percentage of germination} = \frac{\text{no. of germinated seeds} \times 100}{\text{Total no of seeds}}$$

$$\text{Percentage of germination of the standard seed of maize is} = \frac{70}{100} \times 100 = 0.70$$

Percentage of germination of the infected seed of maize by *Aspergillus niger*

$$= \frac{25}{100} \times 100 = 0.22$$

Percentage of germination of the infected seed of maize by *Aspergillus flavus* =

$$= \frac{45}{100} \times 100 = 0.45$$

Result

- Percentage of germination of the standard seed of maize is = 0.70
- Percentage of germination of the infected seed of maize by Aspergillus niger = 0.22
- Percentage of germination of the infected seed of maize by Aspergillus flavus = 0.45

3. Thin Layer Chromatography

The separation and identification of organic compounds is a routine work in many service laboratories. This layer chromatography (tlc) is an easy technique to adopt for the said purpose. It is highly useful in research laboratories to separate, identify and characterize unknown compounds. A variety of small molecules like amino acids, sugar, organic acids, organic acids, lipids etc. are separated by tlc technique. The greater advantage of tlc is the spread at which separation is achieved. When volatile solvents are used the time required to effect separation is only about 30 min and with nonvolatile solvents it is seldom longer than 90 min.

Principle

The general priniciple involved in tlc is similar to that of column chromatography i.e., adsorption chromatography. In the adsorption process, the solute competes with the solvent for the surface sites of the adsorbent. Depending on the distribution coefficients, the compounds are distributed on the surface of the adsorbent. Of course, in tlc the partition effect in the separation is also not ruled out. The adsorbent normally used contains a binding agent such as calcium sulphate which facilitates the holding of the adsorbent to the glass plate.

Materials

- Thin Layer Chromatography (tlc) Kit
- Ultra – Violet (UV) Chamber
- Mechanical Shaker
- Toluene
- Ethyl Acetate
- Formic Acid
- Chloroform
- Silica Gel G (tlc Grade)

Procedure

Extraction of Toxins

1. Weigh exactly 50g of ground sample material and transfer it into a 250ml conical flask.
2. Moist the material uniformly by adding 10 -15ml of distilled water and add about 200ml chloroform, stopper the mouth with a cotton plug in aluminum foil.
3. Shake the flask for one hour mechanically. (It is important that the oil – containing materials are defatted prior to extraction.)
4. Filter the slurry through a Buchner funnel under mild suction. Equal amount of a filtering aid such as ciliate may be mixed before filtering in order to ease filtration. Wash the flask and the slurry thoroughly with additional chloroform (25 ml) and collect the filtrate.
5. Transfer the filtrate quantitatively to a separatory funnel and shake with water one-half volume of chloroform. After the phases separate, drain the bottom (chloroform) phase into a flask containing about 10g sodium sulphate (anhydrous) to absorb any water.
6. Concentrate the clear, chloroform extract over a warm water bath. Make up the concentrate to a known volume with chloroform and store in amber – coloured vials under refrigeration until analysis

Preparation of the Plates

1. Place 30g silica gel G in a stoppered flask, share vigorously with 60-65ml distilled water for about one minute, transfer to the applicator and spread uniformly on glass plates (20 × 20 cm). The exact quantity of chloroform to get a good slurry. The thickness of layer should usually be 0.25 mm.
2. Allow the plates to dry for 1 hr. in dust free conditions.
3. Divide the gel into a number of lanes by drawing lines on the gel with a sharp needle.
4. Spot different volumes of the sample extract in various lanes carefully with a capillary on a imaginary line 2.5cm away from one end of the plate.
5. Develop the plate in a solvent system of toluene: ethyl acetate: formic acid (6: 3:1) in a chromatographic tank for about 50min. By then, the solvent front might have moved up to 20mm below the top end of the plate.
6. Dry the plate at room temperature to remove the solvent. Visualize the fluorescing spot of toxin under UV light; otherwise, eye sight will be affected.

7. Identify each fluorescing spot of the sample extract. Determine the Rf value of each spot.

Calculation

$$Rf = \frac{\text{Distance (cm) moved by the solute from the origin}}{\text{Distance (cm) moved by the solvent from the origin}}$$

For a fluoresce green (G) G1 & G2

$$\text{Rf of Aspergillus niger} = \frac{14.6}{20} = 0.73$$

$$\text{Rf of Aspergillus flavus} = \frac{15.3}{20} = 0.77$$

Result

- Rf of Aspergillus niger = 0.73
- Rf of Aspergillus flavus = 0.77

4. Extraction and Detection Of Nuleic Acids

The nucleic acid consists of Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA).

A. EXTRACTION AND DETECTION OF DNA FROM INFECTED SEEDS

Requirements

- Radicals of germinating seeds
- 15 % TCA (Trichloro acetic acid)
- 10 % NaCl (Sodium chloride)
- Chilled ethanol
- EDTA (Ethylene diamine tetra acetic acid) powder
- Grinder (motor and pestle)
- Centrifuge
- Test tubes.

Extraction of DNA

Weigh 1gm radicals of germinated seeds and cut into small pieces. To this add a pinch of EDTA powder and grind it in a grinder or motor pestle. Then add 5ml of TCA (removes fats and

carbohydrates). Take the contents in a centrifuge tube and centrifuge the solution at 5000rpm. Discard the supernatant and to the ppt and 10ml of 10%NaCl (to dissolve DNA solution). Boil the content in a water bath for 30 mins. Cool the solution and again centrifuge at 5000rpm for 5mins.Take the supernatant and chill it. To this add equal quantity of chilled ethanol. DNA appears at the junction of two liquids. Shake the tube and keep it in ice-box for 30mins. After 30mins centrifuge the content to separate the DNA ppt. Discard the supernatant and collect the ppt. Put a drop of Acetocarmine stain. The DNA ring becomes purplish pink in colour.

Chemical Preparation

i. *15%TCA: 1.5gm of TCA in 30ml of distilled water.*

ii. 10%NaCl: 10gms of NaCl in 100ml of distilled water.

Detection of Nucleic Acid

The nucleic acids absorb strongly in the ultra violet region of the spectrum (due to conjugate double bond system). They show characteristic maxima at 260 nm and minimum at 230nm.

Result of OD

- Standard material: 0.56
- *Aspergillus flavus*: 0.66
- Aspergillus niger*: 0.45*

B. Extraction and Detection of RNA from Plant Material

Requirement

Maize seeds, saline EDTA (Ethylene diamine tetra acetic acid), Phenol 80%, chilled ethyl alcohol, centrifuge, pestle and mortar.

Procedure

Weigh 1gm of maize and cut it into small pieces. Crush the maize seeds grind in 5ml of saline EDTA or distilled water using pestle and mortar. To this add 10ml 0f 80% Phenol. Shake the mixture and centrifuge it. Take the supernatant and 2 volumes of chilled ethanol. Keep it in refrigerator overnight. Centrifuge the next day and collect the RNA ppt.

Detection of RNA

Dissolve the RNA crystals from the previous experiment in 1ml of water. Also dissolve 0.5gmsof RNA crystals. Use these two materials for descending chromatography on Whatman

No .1 PAPER. After running the chromatogram dry it in dry air for 4 hours. And locate the bases under ultra violet light. Both will be same.

Preparation: 80% Phenol. Take 80ml of Phenol and 20ml of distilled water.

Calculation

$$Rf = \frac{\text{Distance (cm) moved by the solute from the origin}}{\text{Distance (cm) moved by the solvent from the origin}}$$

$$\text{Rf of Pure maize} = \frac{8.1}{8.9} = 0.91$$

$$\text{Rf of Aspergillus niger} = \frac{7.2}{8.4} = 0.84$$

$$\text{Rf of Aspergillus flavus} = \frac{7.3}{8.2} = 0.89$$

Result

- Rf of Pure maize = 0.91
- Rf of *Aspergillus niger* = 0.84
- Rf of *Aspergillus flavus* = 0.89

Discussion

The toxicity of the aflatoxin extract from culture filtrates used on seed germination and percentage germination result for Standard maize seed which is free from fungal infection is 0.70% whereas & growth for A. niger and A. flavus is 0.22 & 0.45 respectively. The Rf value for A. niger and A. flavus is 0.73 & 0.77 respectively. Fungal contamination also shows impacts on Nucleic acid w.r.t to amount of DNA and RNA composition. Here, we find that DNA composition for Standard maize seed which is free from fungal infection is 0.56mg whereas & DNA for A. niger and A. flavus is 0.66 & 0.45mg respectively. RNA composition for Standard maize seed which is free from fungal infection is 0.91mg whereas & growth for A. niger and A. flavus is 0.84 & 0.89mg respectively.

Conclusion

If a seed is infected fungal pathogens the germination percentage will goes down. Apart from the rate of germination, the nucleic acid composition in terms of DNA & RNA will

synthesize in less amount which affect overall growth and development of the plant. Hence, to sowing the seeds it must be taken care of that if is free from any kind of infection. To take precaution one can use sterilization of seeds either with Mercuric chloride or any fungicides.

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CONTACT FOR SUBSCRIPTION

AJANTA

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Vinay S. Hatole

Jaisingpura, Near University Gate, Aurangabad (M.S) 431 004,

Cell : 9579260877, 9822620877 Ph: 0240 - 2400877

E-mail : ajanta5050@gmail.com Website : www.ajantaprakashan.com