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RESEARCH ARTICLE

EVALUATING THE ANTI-MICROBIAL EFFECT OF EUGENOL EXTRACTED FROM *OCIMUM SANCTUM***Basak Piyali*², Paul Pritha¹, Kundu Torsha¹, Mallick Priyadarshini²**¹Department of Biotechnology, Heritage Institute of Technology, Kolkata, India-700107²School of Biosciences and Engineering, Jadavpur University, Kolkata, India-700032*Corresponding Author's Email: piyali_basak@yahoo.com

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ABSTRACT

Eugenol is a phytochemical present in herbal and medicinal plants. It possess anti tubercular, anti-inflammatory, anti-mutagenic properties. Commercial or synthesized eugenol is used extensively in the market nowadays. The aim is to evaluate the anti-microbial property of eugenol extracted from both the powder and leaf samples of *Ocimum sanctum* (tulsi) and to have a comparative analysis of the synthetic eugenol and the naturally extracted eugenol from tulsi leaves. The eugenol is extracted from tulsi leaves by steam distillation. For quantitative analysis of the natural eugenol, HPLC and UV Spectroscopy are performed with commercial eugenol as the reference. While Raman Spectroscopy is performed for qualitative analysis of the constituents of tulsi leaves. Membrane casting is done with eugenol as the core ingredient and porosity of the membrane is checked by SEM. Further microbial assay is performed to evaluate the effect of eugenol. From the results it can be concluded that the eugenol extracted from the powdered dry leaves and fresh leaves of tulsi has anti-microbial effect and the membrane composed of eugenol has the capability to retain the eugenol.

Keywords: *Ocimum sanctum*, eugenol, anti-microbial, membrane, anti-microbial.

MATERIALS AND METHODS

Ocimum sanctum L. is a perennial herb and is extensively found in tropical and warm temperate regions with the greatest number of species in Africa.¹ It is commonly known as Tulsi and is an important medicinal plant. The leaves of Tulsi contain essential oil. The composition of the essential oil of Tulsi is eugenol, urosolic acid, carvacrol, linalool, limatrol, caryophyllene, methyl carvicol. Various studies showed that eugenol has many therapeutic properties.^{2,3,4}

Eugenol (C₁₀H₁₂O₂) is a phenyl propene; an allyl chain-substituted guaiacol. It is a pale yellow colored oily liquid. The IUPAC name of eugenol is 4-Allyl-2-methoxyphenol. It has boiling point at 225 degree Celsius and melting point at -9.1 degree Celsius. It is miscible with alcohol, chloroform, ether, soluble in glacial acetic acid, in aqueous mixed alkali hydroxide solutions. Allergic reaction is reported for some patients. Mainly contact dermatitis/ stomatitis; and true allergic reaction have observed. However, there have been rare reports of hypersensitivity reaction to eugenol. It possesses non-mutagenic and non-carcinogenic properties and a very powerful fat-soluble anti-oxidant.^{5,6}

Commercial (synthetic) eugenol is well known for its

anti-bacterial^{7,8,9} and anti-tubercular activity and is extensively used as antiseptic.¹⁰ But limited studies have reported the anti-inflammatory properties of naturally extracted eugenol from tulsi leaves.^{11, 12} Commercial synthetic eugenol may be toxic to human body if more than recommended dose is used. Another problem with commercial eugenol is its high price due to laboratory synthesis process. On the other hand natural elements such as clove, basil, nutmeg are the rich source of natural eugenol. Eugenol extracted from natural source is not as toxic as pure eugenol. Moreover eugenol from natural sources is also cost effective.

Our primary goal is to to compare properties of eugenol present in leaf extract with the commercial and to evaluate the anti-microbial properties of naturally obtained eugenol. So that natural eugenol can substitute the commercial eugenol successfully. Therefore we performed High Performance Liquid Chromatography (HPLC)^{13,14,15} and Raman spectroscopy^{16,17} to compare the composition of leaf extracts from both fresh leaves and powdered dry leaves of tulsi with the commercial eugenol. Subsequently we have checked the anti-microbial properties of the eugenol. For that we have performed microbial assay of the membranes containing eugenol.

MATERIALS AND METHODS

Apparatus: Clevenger apparatus, 1000ml round bottom flask with three necks, Condenser, Blower, Calibrated traps, Weigh machine, Thermometer, Heater, Magnetic stirrer, Collection tubes, Petri plates.

Chemicals: Commercial eugenol from Sigma Aldrich, Litmus paper, Potassium permanganate, Bromine, Polyvinyl alcohol, Gelatin, Glutaraldehyde, Commercial eugenol, Anhydrous Na₂SO₄, Powdered leaves of tulsi, Double distilled water.

Methods:

Steam distillation: For steam distillation first a setup is established using heater, round bottom flask, Clevenger apparatus. 10g of powdered leaves is mixed with 30ml of normal water and the mix is put into the round bottom flask. Then the mix is heated at 70⁰ C to 90⁰ C for about 30 minutes. The liquid is collected after first distillation and the process is repeated again to get double distilled extract. From 40ml mixture we have got approximately 38ml of double distilled final extract.

Chemical Analysis: Reaction with bromine solution, potassium permanganate and litmus test are performed for chemical analysis of the eugenol.

UV Analysis: UV analysis is performed to determine the concentration of eugenol extracted from *Ocimum sanctum* leaves as well as powder by steam distillation. In this UV reading of commercial eugenol was used to get standard curve which is used as reference for finding concentration of eugenol extracted from *Ocimum sanctum* leaves. For the UV Spectrophotometry 0.5ml of eugenol was diluted with 9.5ml of water.

Membrane Casting: We have tried to propose a new idea of using bio-friendly membranes which are made using three types of leaf extracts. In the first method polyvinyl alcohol was mixed with eugenol in the ratio of 3:1 and glutaraldehyde was added to the mixture as a

cross linker in a beaker. The mixture was then placed in the magnetic stirrer for around 30 minutes. The liquefied viscous mixture was poured in the petri dish and is well covered with the aluminium foil so as to avoid contamination. Then the covered petri dish is kept in the blower for around 6 days. In the second method gelatin was mixed with eugenol in 3:1 ratio and glutaraldehyde was added. And the same protocol is followed. In the last method of membrane casting eugenol and glutaraldehyde was added to the beaker containing gelatin in water and gelatin has been solubilised in water through heating. The same procedure is been followed.

High Performance Liquid Chromatography:

HPLC has been used to analyze the amount of Eugenol in the powder extract as well as in fresh leaf extract and to understand whether any of this extracts can be used as a successful substitute for commercial Eugenol. Here commercial eugenol has been kept as a set parameter. Chromatographic analysis is carried out in Zorbax C18 column with a mobile phase of mixture of water and methanol in the volume ratio of 3:2.

Porosity Test of Membranes: The size distribution of pores on the surface of the membrane is quantified through the image analyzer from the images visualized by Scanning Electron Microscope (SEM).

Microbial Assay of Membranes: Eugenol crystals remain entrapped inside membrane. We want to use this membranes as effective mode of drug delivery so it is very important to examine whether the eugenol entrapped in membranes still shows its anti-microbial properties or not. For this purpose we did microbial assay of casted membranes. For this assay we place a small portion of each of three types of membranes on two types of bacterial stains; one plate of *E. coli* colony culture and another of *S. aureus*. The plates are then kept in incubator for 24 hours to allow microbial growth and then observation results are recorded.

RESULTS AND DISCUSSIONS

Table 1: Chemical analysis of Eugenol

Test	Fresh Leaf Extract	Powdered Leaf Extract	Commercial Eugenol
Bromine Test	Pale yellow coloured precipitate	Pale yellow coloured precipitate	Pale yellow Coloured precipitate
Bayer's Test	Muddy brown Precipitate	Muddy brown precipitate	Muddy brown precipitate
Litmus Test	No colour change	Blue colour changes to red	No colour change

In table 1, all the three samples of eugenol have shown positive result in Bayer's Test and Bromine Test indicating presence of alkyne groups in the eugenol. Only in litmus test, the eugenol obtained from plant powder is weakly acidic in nature thus changing the blue litmus colour to red, while no change in coloration is observed in the other two samples.

Quantitative estimation of eugenol by UV-Spectroscopy:

The concentrations of eugenol can be determined by UV-VIS spectrophotometer by putting the absorbance of eugenol obtained from various extracts of *Ocimum sanctum* in the standard calibration curve.

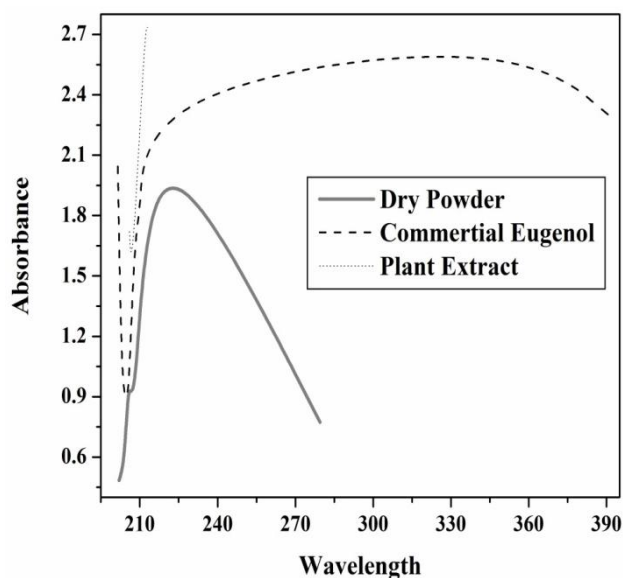


Figure 1: UV analysis of the eugenol extracted from dry powder, plant extract and commercial eugenol

From the graphs in Fig.1 it is quite evident that the eugenol is present both in leaf and powder extract in relatively good amount. Moreover, the eugenol obtained from fresh leaf extract contains more concentration of eugenol since this graph has more overlapping areas with the graph of commercial eugenol compared to the eugenol obtained from powder.

Quantitative estimation of eugenol by HPLC method:

In HPLC test we analysed the presence of eugenol in the two leaf extracts and tried to compare which extract contains eugenol in high concentration. In this study number of peaks was observed which corresponds to the components present in leaf extracts by means of their retention time and absorbance.

Retention time for eugenol is around 14.9 minutes¹³. Peak around that retention time for powdered leaf extract and fresh leaf extract shows the presence of eugenol in both type of extract.

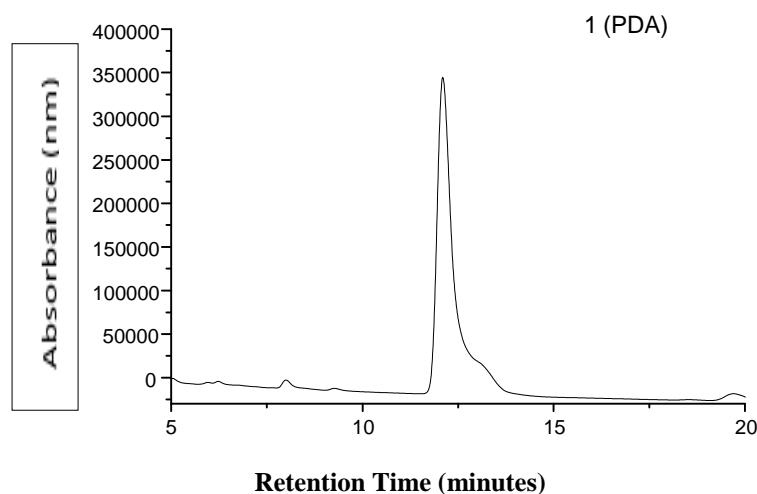


Figure 2a: HPLC of powdered leaf extract

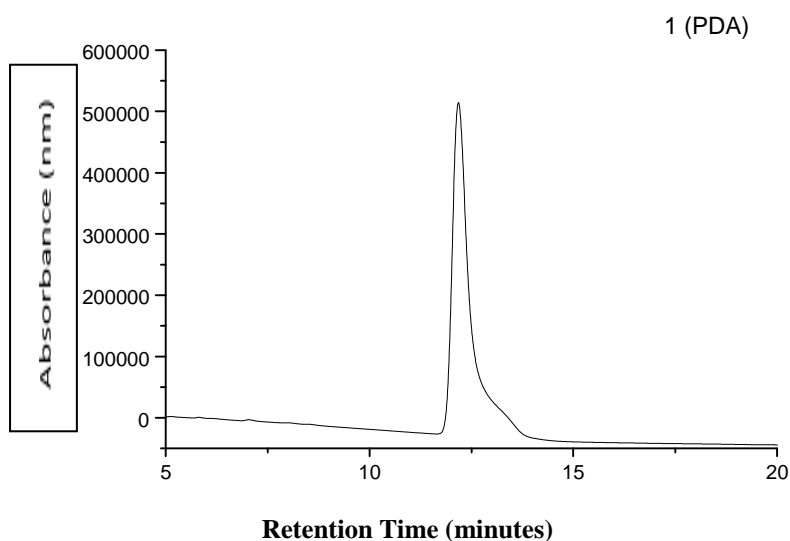


Figure 2b: HPLC of fresh leaf extract

In Fig: 2a the peak intensity of 350000 nm comes for retention time 12.47min. and in fig: 2b the peak intensity of 500000 nm comes for retention time 12.32 min.

Now, 500000 nm > 350000 nm, so it's clear that eugenol is present in both the extracts and the concentration of eugenol in fresh leaf extract is higher than that in powdered leaf extract.

Qualitative Analysis of Raman Spectroscopy:

The peaks which are obtained from Raman Spectroscopy are overlapping as shown in the figure 3 thus indicating that the three samples of eugenol possess the same chemical constituents. There is difference in the intensity between the samples due to varying concentrations of eugenol.

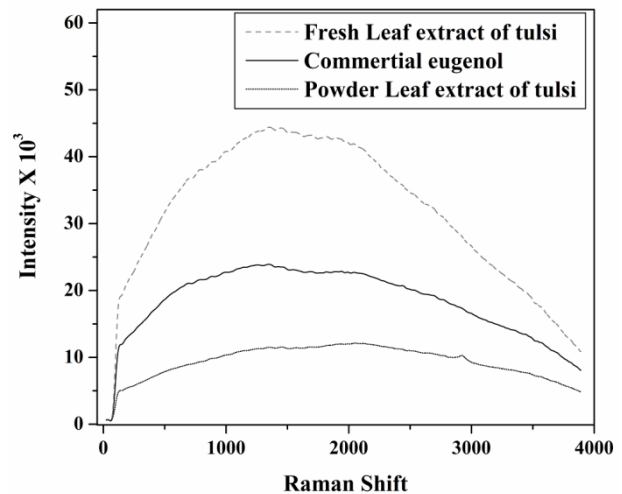


Figure 3: Raman Spectroscopy of Commercial eugenol, fresh and powdered leaf extract of tulsi

Porosity test of the casted membranes by SEM:

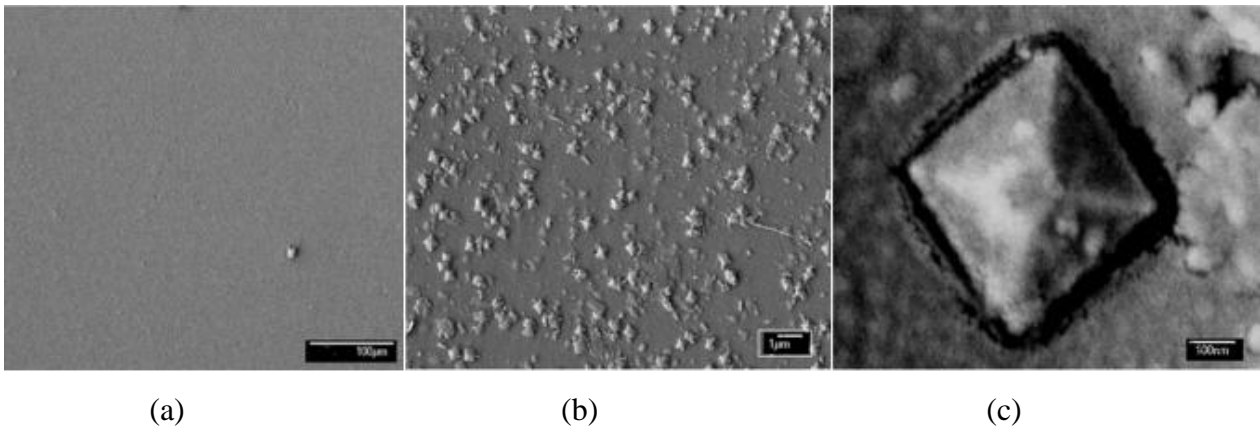


Figure 4: (a) SEM of the membrane at low magnification; (b) SEM of the membrane at high magnification; (c) SEM of a single crystal.

In Fig 4(a) the membrane possesses a uniform surface that indicates uniformity of the membrane composition. In Fig 4(b) the crystals formed are clearly visible. The Eugenol remains trapped inside these crystals. The

crystals are formed with similar shapes and are equally distributed throughout the membrane. These crystals are eugenol embedded within membrane.

Microbial assay of casted membranes:

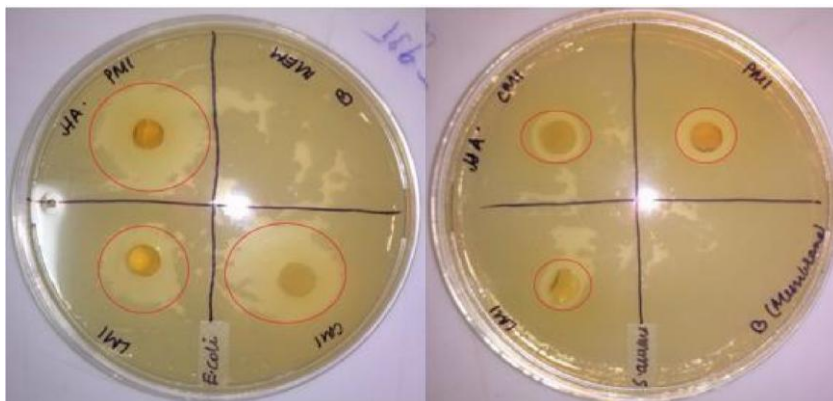


Figure 5a: *E. coli* culture plate

Figure 5b: *S. aureus* culture plate

In *E. coli* culture plate (Fig.5) it can be seen that each of the three types of membrane: LM1 (membrane containing fresh leaf extract), PM1 (membrane containing powdered leaf extract) and CM1 (membrane containing commercial eugenol). The areas around each membrane with in red circles are the areas where growth of microbes has been inhibited by the eugenol present in each membrane. Here if we compare the effect of inhibition then PM1 and CM1 shows maximum inhibition of *E. coli* growth. In *S. aureus* culture plate it can also be seen that the areas around each membrane with in red circles are the areas where growth of microbes has been inhibited by the eugenol present in each membrane. Here if we compare the effect of inhibition then LM1, PM1 and CM1 shows similar amount of inhibition of *S. aureus* growth. From the above figures it is clear that even eugenol entrapped in the membranes do not lose its antimicrobial properties. This proves the anti-microbial nature of eugenol. Microbial inhibition is observed in both the *E. coli* and *S.aureus* strains. Thus it may be said that eugenol is retained within the casted membrane.

CONCLUSION

From the graphs mentioned in the result, it is quite evident that there is presence of huge amount of eugenol in the essential oil obtained from both the leaves and powder extract. Furthermore, the eugenol content is much higher if the source is the basil leaves compared to the leaf powder. From HPLC and Raman spectroscopy it is evident that the chemical constituents of commercial and natural eugenol are same. Moreover from microbial assay it may be concluded that the membrane can retain the eugenol and it is also inhibiting the growth of microbes. This paves a new alternative in the study of anti-microbial properties also of the naturally obtained eugenol instead of the commercial eugenol. This will be much cost effective and also can be used for medicinal purposes. Moreover it does not have toxic effects compared with the commercial eugenol. For further study animal testing must be performed. Furthermore, anti-inflammatory studies may be carried out in animal model.

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