Academic Journal of Sukuna – AJoS, A Peer-reviewed Interdisciplinary Journal
Volume 5 (Issue 1) 2025 July (2082 Ashad), Pp. 160 – 179, ISSN 2594-3138 (Print)
Research Management Cell (RMC – Sukuna), Sundarharaincha, Morang

Extraction of Eugenol from Clove Buds and Testing its Antimicrobial Activity

Doi: https://doi.org/10.3126/ajos.v5i1.81842

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Abstract

Eugenol, the primary component of clove oil, is a natural compound produced by Eugenia caryophyllata for protection against microorganisms and pests. Due to its easy isolation and potential as an antimicrobial medicine, eugenol has gained interest in the medical and food industries. This interest is driven by the negative perception of synthetic drugs and the traditional use of plant-based medicines. As a result, a project was conducted to assess the antimicrobial activity of eugenol against various human pathogenic bacteria and fungus. The test organisms included both Gram-positive bacteria {e.g., Bacillus spp., Streptococcus pyogenes, and methicilline-resistant Staphylococcus aureus (MRSA)} and Gram-negative bacteria (e.g., Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Shigella sonnei). Additionally, Aspergillus genus fungus was used. Eugenol was isolated from hydro-distilled clove oil, and three different concentrations of the test solution were prepared: 1% (v/v), 0.5% (v/v), and 0.25% (v/v). The antimicrobial activity of eugenol was evaluated by applying 100µl of each eugenol concentration to wells made in Muller Hinton agar (MHA) and Potato dextrose agar (PDA) plates, on which the pathogens were swabbed. The results displayed a positive outcome, as evidenced by the presence of a zone of inhibition indicating the inhibition of microbial growth. Overall, the study demonstrated the antimicrobial

effectiveness of eugenol against various microorganisms, including Gram-positive and Gram-negative bacteria, as well as the Aspergillus genus of fungus. These findings highlight the potential of eugenol and similar phytochemicals in the development of antimicrobial medicines and their applications in the food industry for safety and preservation purposes.

Keywords: Eugenols, phytochemicals, anti-biotic resistance, zone of inhibition, anti-microbial activity

Introduction

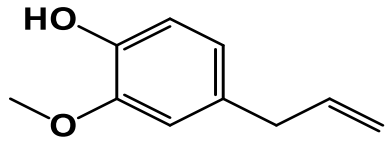
One of the serious issues in the community is public health. It is always under the threat of infectious diseases, which guide the economy of any country. The alarming threat to the economy is partially due to the increasing properties of antimicrobial resistance among fatal pathogenic microorganisms (Jeyakumar & Lawrence, 2021). So, there is a need for research on new fields or classes of antibiotics. Nowadays, it is believed that plant-origin bioactive compounds, i.e., phytochemicals, are alternatives to synthetic and resistant antibiotics. The challenge of the development of this class of antibiotics is due to a lack of sufficient knowledge about the mechanism of drug interaction at the molecular level (Jeyakumar & Lawrence, 2021). So, eugenol (EUG) could be one of the antibiotics derived from the plant. EUG belongs to the phenylpropanoid group of essential oils. Phenylpropanoid is synthesized through the mevalonate and shikimate acid metabolic pathways. Essential oils are volatile, and they are secondary plant metabolites. So, they can be obtained from the essential oils of clove bud (*Eugenia caryophyllata*) by hydro-distillation (Patra, 2012).

Clove essential oil (EO) is one of the cheap sources of EUG, which is a light yellow, transparent liquid with a specific clove aroma and a yield of 12.8% (v/w). Among the 95.8% of the total amount of EO, EUG (76.23%) was found to be a major component of the essential oil, followed by -caryophyllene (11.54%), caryophyllene

(4.29%), and eugenyl acetate (1.76%) (Xu et al., 2016). The structure of eugenol is given below,

Figure 1

Structure of Eugenol



4-allyl-2-methoxyphenol

Some of the properties, Molecular formula and Molecular weight of 4-allyl-2methoxy phenol are given below.

Table 1

Molecular formula and Molecular weight

S.N.	Formula and Weight	Composition
1	Molecular formula	$C_{10}H_{12}O_2$
2	Molecular weight (g/mol)	164.20
3	Solubility	water, alcohol, chloroform, ether
4	Melting point (°C)	− 9.2
5	Boiling point (°C)	254

It is believed that deep research on phytochemicals having antimicrobial properties, the preparation of different derivatives, and testing on them could lead to an alternative to antibiotic resistance. EUG extracted from the essential oil of clove buds (*Eugenia caryophyllata* or other plant species belonging to the corresponding plant family) could be an anti-microbial agent for human pathogenic microorganisms. So, testing the antimicrobial strength of EUG purified from hydro distillation clove buds on different drug-resistant human pathogenic microorganisms is for the search of new drugs (Levy, 2005).

Most of the known antibiotic-resistant bacteria continue to increase in frequency and number globally and are one of the emerging problems that complicate and impede

the treatment of critical infectious diseases (Levy, 2005).*Staphylococcus aureus*vancomyc in resistance, gram-negative pathogens wide spectrum lactamases resistance, and *Escherichia coli* and *Neisseria gonorrhoeae* fluoroquinolone resistance are some of the examples of increased drug tolerance by microorganisms in patients suffering from infectious diseases (Levy, 2005). To identify new lucrative applications for antibiotics for chronic diseases, many major pharmaceutical companies have pulled back from the field of antibiotic discovery based on microbial origin (Levy, 2005).With the alarming decrease of new anti-microbials coming to market and with new threats arising from gram-negative infections at the same time, the number of drug options for us on the market is perilously close to none or only a single effective agent for some life-threatening infections(Levy & Marshall, 2004). So, testing the antimicrobial strength of EUG purified from hydro distilled clove buds on different drug-resistant human pathogenic microorganisms, i.e., gram-positive (*Bacillus spp.*, *Streptocuspyogenes,* and MRSA) and gram-negative (*Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa, Klebsiellapneumoniae, and Shigellasonnei*) bacteria, as well as fungus (*Aspergillus spp.),* is for the search of new drugs. Which should be with public health; parallelly, it should not give rise to drug-resistant microbes (Levy & Marshall, 2004). About twenty-seven species of *Syzygiumaromaticum* are found all over the world, and about twenty-two of them are growing in China these days. Actually, *S. aromaticum* (clove) belongs to the *Syzygiumgaertn* genus of the plant family Myrtaceae, which originated on the Moluccas Islands of Indonesia. The composition of clove oil is found to be completely different due to differences in soil, water, climate conditions, i.e., producing area, and extraction method (Hu et al., 2018). Although the chemical composition of clove oil varies according to producing area and extraction method, the most abundant chemical component is EUG. By using gas chromatography-mass spectrometry (GC-MS) technique to analyze chemical composition, 13 samples of EO

of *Syzygiumaromaticum* from Indonesia, Madagascar, and two provinces of China have been analyzed (Hu et al., 2018). From this chemical composition analysis, it has been found that 21 to 36 kinds of individual chemical species are present in each sample separately. Altogether, 72 different individual chemical species, i.e., kinds of molecules were present, where the proportion of volatile components was 96.16% to 99.91%, where EUG (48.2 to 50.22) %, α-selinene (41.13 to 42.88) %, and cis-α-bisabolene, ocimene, santolinatriene, and humulene are 3.60% to 4.21%. From the above information, we can obtain EUG from clove buds for testing antimicrobial properties in a testable amount at a reasonable cost.

The current top concern for food safety authorities, the food processing sector, and ultimately the general public is microbial foodborne illness. Consumers are simultaneously concerned about food preservatives. The spread of methicillin-resistant bacteria, including one of the antibiotic-resistant pathogens, MRSA, has prompted researchers to resurrect their hunt for antibacterial complexes derived from natural plant sources. Herbs and spices have been added to food systems since antiquity, not just to improve flavor but also as food preservatives and folk remedies (Khalil et al., 2017). Over the past few decades, plant phytogenic chemicals have drawn a lot of attention because of their diverse biological and biochemical roles. Eugenol, a polyphenol found in clove oil, has been shown to have strong antibacterial properties against a variety of strains of both gram-positive (*Enterococcus faecalis, Staphylococcus epidermidis, Streptococcus pyogenes, Staphylococcus aureus, Streptococcus pneumonia, Listeria monocytogenes, Bacillus cereus, and Bacillus subtilis*) and gram-negative (*E. coli, Proteus valgaris, Salmonella choleraesuis, Salmonella typhi)* b acterias. In gram-negative and gram-positive bacteria, eugenol destroys the cell membrane and cell wall, causing cell lysis and the release of intracellular fluid along with the lipid and protein contents. Studies on biofilms conducted in vitro and in vivo show that eugenol has a

potent eradicative and inhibitory effect. In the case of biofilms generated by methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant susceptible *Staphylococcus ureus* (MSSA) strains, 50% inhibition was seen at a concentration of 0.5×MIC (minimum inhibitory concentration (Khalil et al., 2017). Very early on in the creation of magic medication, antibiotic resistance was reported. In an original study published in 1929, Sir Alexander Fleming reported that some bacteria, notably the organism now known as *Escherichia coli,* were immune to the effects of penicillin. Edward Abraham and Ernest Chain first noted the existence of a penicillin-destructive enzyme in *E. coli* in 1940. Several years passed before the medication was frequently utilized to treat patients. Many of the early fungal cultures were infected with bacteria that weakened the antibiotic as it was being generated, making it difficult to initially produce large amounts of penicillin. Bacterial antibiotic resistance has developed into a common and well-researched issue in the following decades (Guilfoile, 2006).

Methods and Materials

Materials

In the process of obtaining Eugenol (EUG), various materials and equipment were utilized. To extract EUG clove buds, dichloromethane, diethylether, dil.HCl and dil.NaOH were used. For the media preparation in microbiological experiments, nutrient agar (NA), Muller Hinton agar (MHA), and potato dextrose agar (PDA) were used. The test organisms were *Escheriachia coli, Salmonella typhi, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Shigella sonnei, Bacillus spp., streptococcus pyogenes, and MRSA,* as well as the fungus *Aspergillus.* Then during this experiment required equipment were a distillation setup, separating funnel, autoclave, electronic weighing machine, glassware, incubator, micropipette, micropipette tips, and a refrigerator. A sample of eugenol was taken only from the distillation method because it was cheap and easy to obtain. Test bacteria were taken from clinically isolated samples in

Bishweshwar Prasad Koirala Institute of Health Science (BPKIHS). These materials and tools collectively facilitate the extraction and testing processes in the laboratory.

Methods

Distillation of clove buds

80 grams of clove buds were ground into powder, and 20 grams of the powder were then subjected to four steam distillations in a 500-ml distillation flask. The oil underwent a four-hour distillation process before collection of the entire oil-water mixture.

Separation of clove oil form distillate

Since clove oil components were soluble in non-polar organic solvents like dichloromethane (DCM), distillate was combined several times with 15 ml of DCM until the DCM became clear. Finally, oil was collected from the separated DCM-oil mixture by evaporating at about 45°C.

Isolation of eugenol from oil

The most common method for making eugenol from natural oil sources involves combining the essential oil with dil. NaOH (3%) solution and stirring the mixture until a phenolic alkali salt is formed. The insoluble non-phenolic fraction is subsequently removed using a solvent DCM. The undissolved component is taken out, the alkali solution is acidified at low temperatures, and the released eugenol is purified by fractional distillation (Kamatou et al., 2012).

So, clove oil was treated with excess dil. NaOH and the remaining components of oil were separated by using the non-polar solvent DCM. Then the separated aqueous solution was neutralized with dilute HCl. Neutralization was tested using litmus paper. Then the neutralized solution was treated with 15 ml of diethylether (DEE) several times. Finally, EUG was obtained by evaporating the DEE-EUG separated mixture at about 45 °C.

Figure 2
Isolation of eugenol from oil

Preparation of eugenol's different concentration

DMSO (dimethyl sulfoxide) is an organosulfur compound with the formula $(CH_3)_2SO$. This colorless liquid is an important polar aprotic solvent that dissolves both polar and non-polar components and is miscible in a wide range of organic solvents as well as water. It has no antimicrobial activity. So, it was one of the best solvents for antimicrobial screening. 20µl of EUG was mixed with 1980µl of DMSO to make a 1% (v/v) solution by volume. By dilution, half of the 1% (v/v) solution was converted into 0.5% (v/v), and similarly, 0.25% (v/v) was obtained. Then, these stoke solutions were ready for antimicrobial screening.

Preparation of standard inoculum of test organism

The anti-bacterial activity of EUG was to be tested against seven bacteria: *Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa, Klebsiella pneumoniae, Shigella sonnei, Bacillus, Streptococcus pyogenes, and* MRSA, along with one fungus, *Aspergillus.* The stock culture of the organism was inoculated on Nutrient Agar plates, and the organism that grew on the nutrients agar (NA) plates was preserved in Nutrient Agar slants. Nutrient broth was inoculated with freshly sub-cultured bacteria and

incubated at 37 °C for 5 hours to match the turbidity to that of 0.5 McFarland standards. Such prepared inoculum was used to spread onto Muller-Hinton agar using a sterile cotton swab to make a lawn of bacteria.

Screening of EUG for Antimicrobial activity (Agar well diffusion Method)

In order to see the anti-microbial activity of EUG agar, the well diffusion assay was performed. In this assay, Muller Hinton Agar (MHA) plates were used for the growth of each bacterial species, and Potato Dextrose Agar (PDA) was used for the growth of fungus species. A total of seven MHA and one PDA plate were prepared in order to test the antibacterial activity of seven different bacterial species and one fungal species. In the wells of 5mm diameter created in the inoculated agar media with sterile cork borer, test solution was loaded into each well and incubated at 37 °C for 24 hours for bacteria and at 25 °C for 3 days for fungus. Then, plates were checked to determine the effect of the EUG on desired bacteria by the appearance of a zone of inhibition around the well.

Testing the value of MBC&MFC in the range of 1% (v/v) to 0.0078% (v/v)

In this testing, 96 well plates were used.

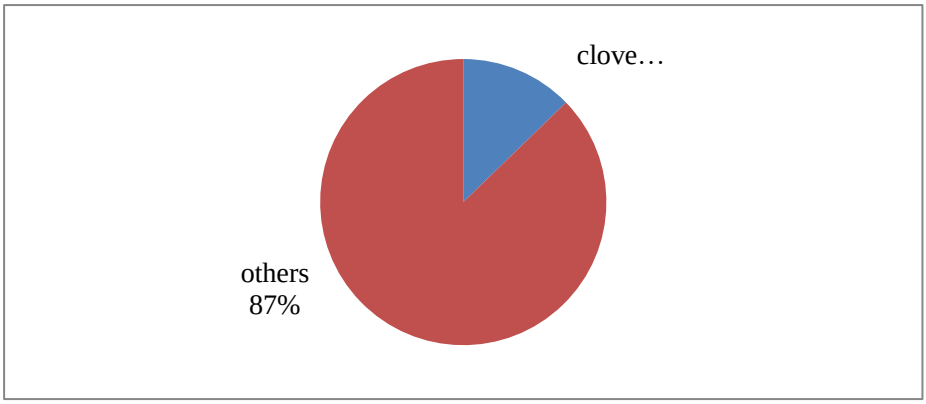
Result and Discussion

Hydro distillated clove oil

Clove EO is one of the cheap sources of EUG, which is a light yellow, transparent liquid with a specific clove aroma and a yield of 12.8% (v/w) (Xu et al., 2016). About 9.5 ml of clove oil was obtained from 80 grams of clove buds, which is about 11.87 % (v/w) of the clove buds, which was a very low amount of yield. For a greater yield, a different method should be applied.

Figure 3

Clove oil yield on hydro distillation of clove buds

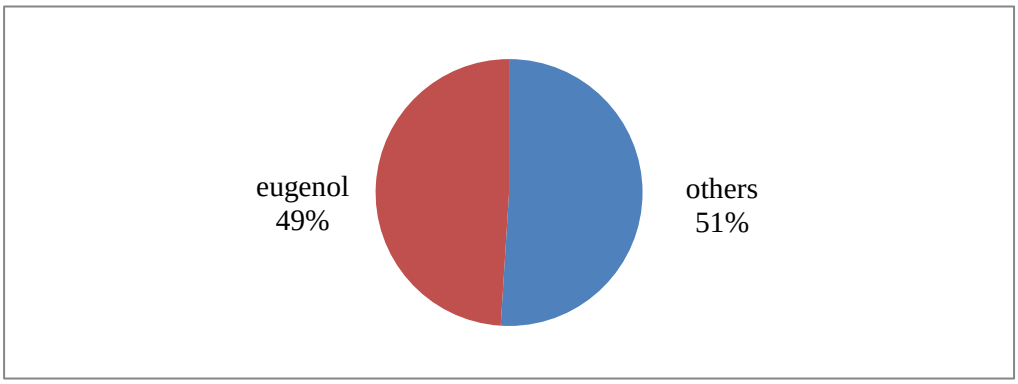


Eugenol in clove oil

On isolation of EUG from clove oil, 5 ml of EUG was obtained, which is about 49% by volume of clove oil. This, somehow, according to the literature, is just at the lower limit of the given range. According to Xu et al. (2016) clove EO is one of the cheaper sources of EUG. Among the 95.8% total amount of EO, the EUG (76.23%) was found to be the major component of the essential oil, followed by -caryophyllene (11.54%), caryophyllene (4.29%), and eugenyl acetate (1.76%).

Figure 4

Clove oil yield on hydro distillation of clove buds

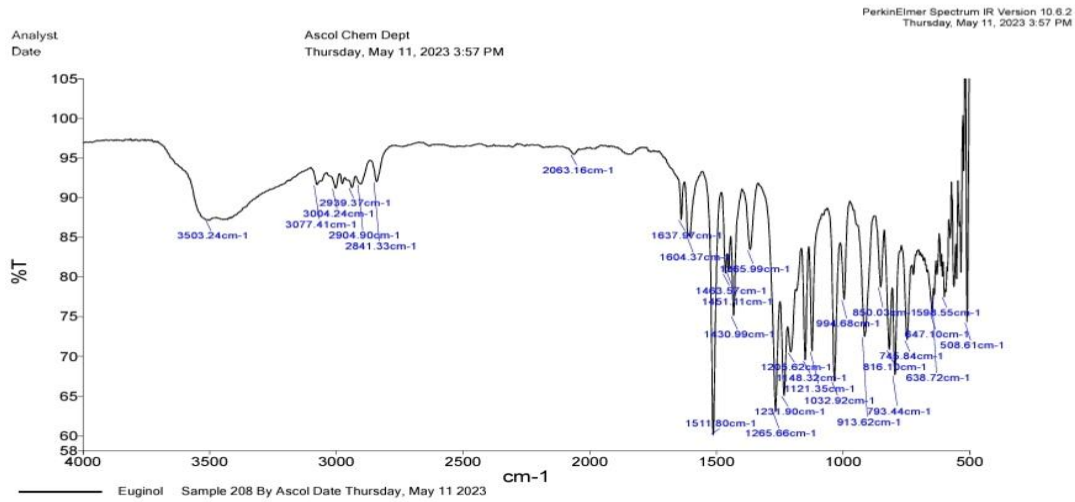


Some relevant conformation of EUG

EUG has phenolic, etheric, and allyl groups on it, so it must show some reactions related to these functional groups. Here, two tests were performed: one on the complex-forming nature of the phenolic group and one on the unsaturation nature of the allyl group. It gives a sky-bluecolor complex with ferric chloride. Unsaturated compounds (alkenes and alkynes) decolorize or discharge the violet color of Baeyer's reagent (alkaline $KMnO_4$ solution) by giving an addition reaction (Khadka et al., *2*018). Benzene rings don't give this test, so it must be given by the allyl group of EUG.

Figure 5

IR diagram of EUG sample



In IR analysis, the broad absorption peak around 3503.24 cm^{-1} regions indicates stretching of both OH and hydrogen attached to sp^3-carbon. The sharp absorption at 1511.8 cm^{-1} indicates that the compound has a benzene ring. Further, the absorption in the range of 1121.35 cm^{-1}–1265.6 cm^{-1} seems to be C-O stretches, which must be ether. So, all the information from IR spectrum implies the conformation of eugenol.

Anti-microbial activity of EUG

From the bar diagrams given below, we can clearly see that the anti-microbial activity of EUG is great. According to Khalil et al. (2017) eugenol, a polyphenol found in clove oil, has been shown to have strong antibacterial properties against a variety of strains of both gram-positive (*Enterococcus faecalis, Staphylococcus epidermidis, Streptococcus pyogenes, Staphylococcus aureus, Streptococcus pneumonia, Listeria monocytogenes, Bacillus cereus, Bacillus, and Bacillus subtilis*) and gram-negative (*E. coli, Proteus valgaris, Salmonella choleraesuis, Salmonella typhi*). In gram-negative and gram-positive bacteria, eugenol destroys the cell membrane and cell wall, causing cell lysis and the release of intracellular fluid along with the lipid and protein contents. Studies on biofilms conducted in vitro and in vivo show that eugenol has a potent eradicative and inhibitory effect. In the case of biofilms generated by methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant susceptible *Staphylococcus aureus* (MSSA) strains, 50% inhibition was seen at a concentration of 0.5× MIC (minimum inhibitory concentration).

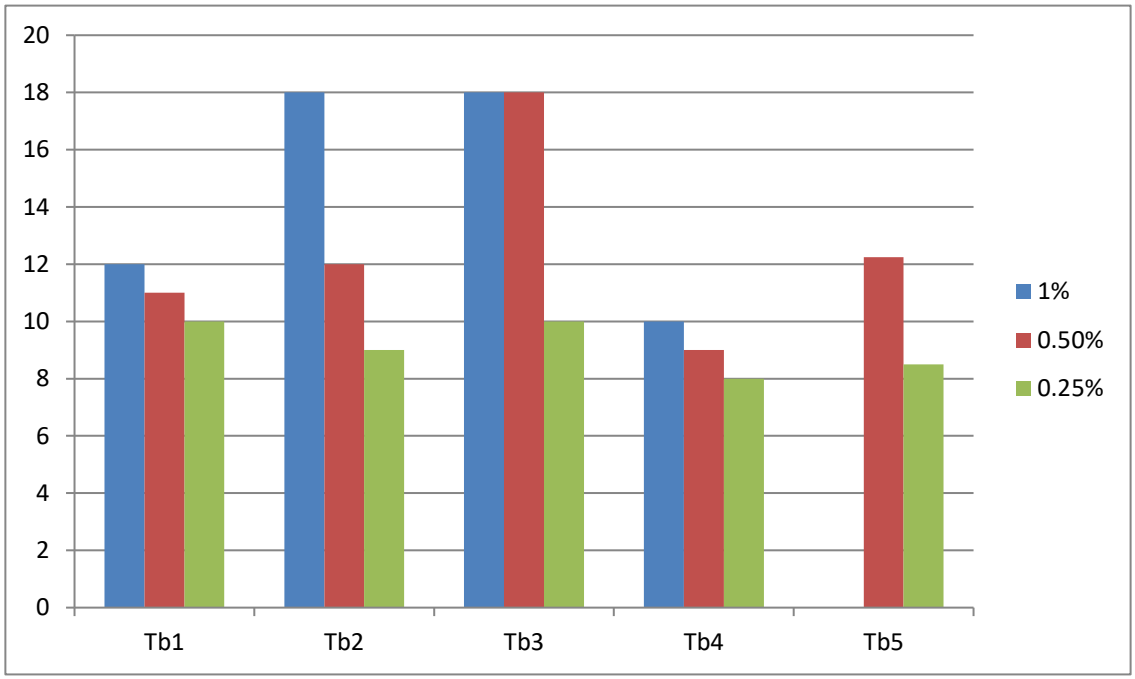
According to Silva et al. (2018) Eugenol was found to have a MIC of 1200 g/ml against S. aureus bacteria, which is consistent with the 1000 g/ml MIC found in the current study. Eugenol exhibits inhibition halos with diameters of 9.25 mm and 7.75 mm, respectively, for strains of *E. coli* and *S. aureus*. To identify the minimum inhibitory concentration (MIC), which prevents the bacteria from growing visibly, microdilution tests were performed on the derivatives previously indicated to have inhibition halos larger than 6mm.

Action of EUG on Gram-negative bacteria

A significant amount of anti-bacterial effect can be seen in the case of gram-negative bacteria in general.

Figure 6

Action of eugenol at three different concentrations on gram-negative bacteria



Where y-axis represents the inhibition zone's diameter in mm and 1%, 0.5% and 0.25% are concentration of EUG in dimethyl sulfoxide (DMSO)

Tb1: *Escherichia coli*, Tb2: *Salmonellatyphi*,

Tb3: *Pseudomonas aeruginosa*

Tb4: *Klebsiellapneumoniae*, Tb5: *Shigellasonnie*

At a concentration of 1000 g/ml, eugenol has been shown to suppress the development of *P. aeruginosa*. At 2000 g/ml, the whole inhibitory effect against these bacteria is visible (Nejad et al., 2017). The actions of EUG on Tb1 at these concentrations don't have a very significant difference, which show 0.25% EUG is equally effective as 1%. But the effectiveness of 1% EUG is much higher in the case of Tb2 than in the other two concentrations. In the case of Tb3, both 1% and 0.5% have

equally high effectiveness compared to 0.25%, where the anti-microbial action of eugenol is on Tb4 among these gram-negative bacteria, but action is significant in all three concentrations. No anti-bacterial action was seen at 0.25% concentration in the case of Tb5, but there was a significant effect at 1% and 0.5% in the same bacteria.

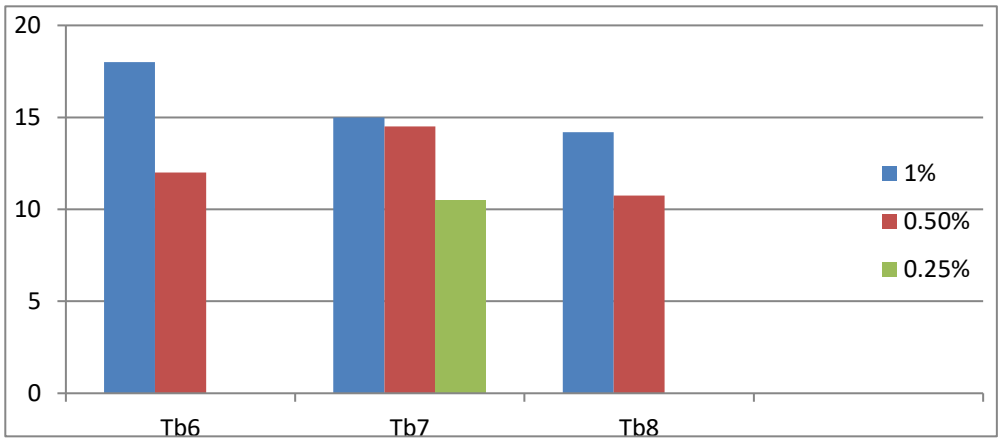
In a comparative study on these five bacteria, the anti-bacterial effect of eugenol can be seen in the case of 1% on Tb2 and both 1% and 0.25% on Tb3.

Action of EUG on Gram-positive bacteria

Significant amount of anti-bacterial effect can be seen in case of Gram-positive bacterial in general.

Figure 7

Action of eugenol at three different concentration on gram-positive bacterias



Where the y-axis represents the inhibition zone's diameter in mm and 1%, 0.5%, and 0.25% are concentrations of EUG in DMSO,

Tb6: *Bacillus*; Tb7: *Streptoccus pyogenes*; Tb8: Methicillin-resistant *Straphylococcusaureus* (MRSA).

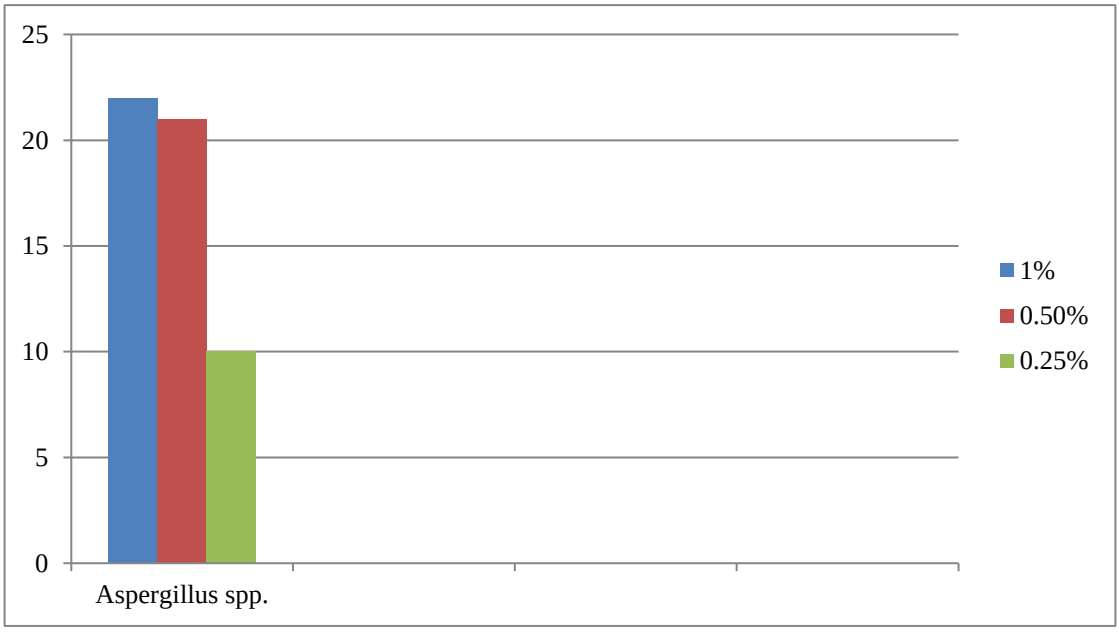
The most significant anti-bacterial effect can be seen at 1% on Tb6, but nil at 0.25%. In the case of Tb7, it is affected by all 1%, 0.5%, and 0.25%, as can be seen. Similarly, Tb8 is affected by both 1% and 0.5% but not by 0.25%.

Action of EUG on fungus

Clove oil has a powerful antifungal effect. Aspergillusflavus and *Aspergillusparasiticus* are foodborne fungi that produce the poisonous secondary metabolite aflatoxin (Nurdjannah & Bermawie, 2012). At 0.6 mg/ml, clove oil exhibited inhibitory activity against *A. parasiticus's* ability to produce aflatoxin. All isolates of the fungi *Eurotium spp., Aspergillus spp.,* and *Penicillium spp*. that frequently contaminate bakery goods showed some antifungal activity when exposed to clove oil. Significant amount of anti-fungal effect can be seen in case of *Aspergillus spp.*

Figure 8

Action of eugenol on fungus



Where the y-axis represents the inhibition zone's diameter in mm and 1%, 0.5%, and 0.25% are concentrations of EUG in DMSO. This result shows that it is also affected by EUG at all three concentrations, where it is badly affected by 1% solution and both 0.5% and 0.25% solutions, so EUG is also effective against this fungal species.

Value of MBC and MFC

From table no. 3, we can say that the MBC values of Tb4 and Tb5 lie in the range of 1% to 0.0078% EUG. We can also say that all others lie outside the test range of EUG concentrations. Eugenol displayed a broad range of antibacterial activity against the test pathogens, with MIC values ranging from 0.0312 to 8 g/ml (Jeyakumar & Lawrence, 2021). The EUG had a 2-4-fold higher matching MBC. The MIC values of *Stapgylococcusaureus* (ATCC 6538) and MRSA were established accordingly at 0.1 mg/ml and 0.1 to 0.15 mg/ml, respectively (Apolonio et al., 2014).

In an in vitro investigation, clove oil and eugenol had MBCs of 40 and 25 g/ml for *Bacillus cereus*, respectively, and also, according to this study, the inhibitory action of eugenol against *E. coli, Salmonella, P. aeruginosa, and L. monocytogenes* (Hu et al., 2018). The MIC value for the fungus *Aspergillusniger* is greater than 2000 g/ml (Filocamo et al., 2015).

Conclusion

From the results of experiment, it is clear thateuginol is an effective anti-microbial phytochemical. This can affect the microbes, i.e., bacteria as well as fungus. It is found that EUG in different concentrations shows inhibitory activity towards different human pathogenic bacteria as well as fungi to a variable extent. The test microbial organisms were both gram-positive and gram-negative bacteria as well as fungi. Gram-negative bacteria were *E. coli, Salmonella typhi, Pseudomonas aeruginosa, Klebsiellapneumoniae, and Shigellasonnie*. Gram-positive bacteria were *Bacillus spp., Streptococcus pyogenes*, and methicillin-resistant *Staphylococcus aureus* (MRSA), and one fungal species, *Aspergillus spp.*, was taken. In this test, Gram-negative bacteria were badly affected by all three concentrations of eugenol, but Gram-positive bacteria Tb6 and Tb8 were affected by only two higher concentrations. From this result, we can say that EUG is more effective against gram-negative bacteria to gram-positive bacteria.

Also, it is an amazing anti-fungal phytochemical; in this experiment, Aspergillus was badly affected.

Finally, it can be concluded that clove bud is a source of eugenol which is costly. The cost of EUG becomes Rs. 50 per ml without adding the cost of extraction and purification. Clove buds contain about 13% clove oils by weight, and from these oils, about 49% eugenol can be recovered. The phytochemical research has opened up a new perspective in pharmaceutical research, and it can be used for the development of potential novel anti-microbial agents for the treatment of bacterial as well as fungal diseases. The incorporation of this eugenol into drugs as well as food formulations is urgent. The biopharmaceutical industry is in need of eco-friendly alternatives as well as antibiotic-resistant alternatives for the treatment of diseases caused by human pathogenic bacteria and fungi. So, it could be a prospective source of alternative antimicrobial agents and may play an important role in the discovery of new drugs.

Acknowledgement

We are thankful to Sukuna Multiple Campus and Central Campus of Technology, Hattisar Dharan for providing laboratory help during experiment. Especially we would like to thanks Mrs. Bijaya Laxmi Maharjan (assistant professor of Central Campus of Technology, Hattisar Dharan) for guiding me during antimicrobial test and providing IR data of sample using Amrit Science Campus chemistry department.

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Appendix

Table 2

Zone of inhibition in mm against test bacteria

Sample concentration(v/v)	**Gram-negative bacterias**					**Gram-positive bacterias**		
%	Tb1	Tb2	Tb3	Tb4	Tb5	Tb6	Tb7	Tb8
1%	12	18	18	10	13	18	15	14.2
0.5%	11	12	18	9	12.25	12	14.66	10.75
0.25%	10	9	10	8	8.5	0	10.5	0

Tb1: *Escherichaicoli*, Tb2: *Salmonellatyphi*,
Tb3: *Pseudomonas aeruginosa*, Tb4: *Klebsiellapneumoniae*,
Tb5: *Shigellasonnie*, Tb6: *Bacillus*,
Tb7: *Streptoccuspyogenes*, Tb8: MRSA

Table 3

Zone of inhibition in mm against test fungus

Sample concentration (v/v) %	**Fungus (Tf_1)**
1%	22
0.5%	21
0.25%	10

Table 4

The value of MBC & MFC in the range of 1% to 0.0078%

Gram-negative Bacteria	Concentrations							
	1 %	0.5 %	0.25 %	0.125 %	0.0625 %	0.0312 %	0.0156 %	0.0078 %
Shigellasonnei								
Salmonella typhi								
Pseudomonas aeruginosa								
Klebsiella pneumoniae								
Escherichia coli								
Gram-positive Bacetria								
Bacillus spp.								
Streptococcus pyogenes								
MRSA								
Fungus								
Aspergillus								
	Yellow colour means no any grown microbes Black colour means fully grown microbes							