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STUDY ON EUGENOL AS A POTENTIAL ANTIBACTERIAL AGENT AGAINST: *Streptococcus mutans and Escherichia coli*

Sheenas N C[1], Dhanyamol Manoharan[2]
[1]*Lecturer, Department of Biochemistry, Annoor Dental College and Hospital, Muvattupuzha, Kerala, Ernakulam, India.*
[2]*Lecturer, Department of Biochemistry, Annoor Dental College and Hospital, Muvattupuzha*

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ABSTRACT
The increased population of antibiotic resistant bacteria limits the successful treatment of infectious diseases. The World Health Organization reports the antibiotic resistance shows a serious risk to public health since the bacteria are becoming resistant to antibiotics for two main reasons. This makes it harder to treat infections and highlights the need for better monitoring solutions. Eugenol a secondary metabolite obtained from clove buds has high potential in the field of biomedicine. Natural products are appealing due to their effectiveness, lower toxicity, and easy availability. Historically, these remedies have been used for treatment since ancient times, before the advent of modern antibiotics. Therefore this study aims to evaluate the antibacterial activity of eugenol as an alternative remedy for antibiotic resistance.Two pathogenic bacteria were tested: ***Streptococcus mutans and Escherichia coli.*** *The inhibitory effect of commercially available eugenol was screened by Kirby Bauer method, Minimum Inhibitory Concentration, Maximum Bactericidal Concentration (MIC and MBC). Confocal Laser Scanning Microscopy(CLSM) are utilized to distinguish between live and dead bacterial cells by using fluorescent dyes on biofilms. In contrast, Scanning Electron Microscopy (SEM) is used to examine the surface structure and material makeup of the cells.The following day after culturing, zones of inhibition were observed and measured in millimeters from the culture plates. Both bacteria showed zones of inhibition, but a larger zone was observed for S. mutans compared to E.coli. Images from Confocal and scanning microscopy indicated a higher proportion of red fluorescence that signifies dead cells, suggesting that cell membrane disruption contributes to cell death. The study reveals that eugenol effectively control the growth of these pathogenic bacteria and can be exploit as a natural antibiotic with lesser side effects.The present study differs by focusing on the bacterial strains that are responsible for many infectious diseases worldwide. By examining these specific pathogens, this research aims to provide new insights into the effectiveness of eugenol against a broader range of infectious agents.*

KEY WORDS: ***Antibiotic Resistance, Eugenol, Streptococcus mutans and Escherichia coli***

2: INTRODUCTION
The advancement of antimicrobial agent has significantly transformed human and animal health by a dramatic change in the war against infectious diseases which resulted in the survivability of both humans and animals. But now there is a growing concern regarding the increasing number of pathogenic bacteria becoming resistant to antimicrobial compounds. In microbes specifically, the bacteria can modify them quickly to resist antibiotics and drugs. The mechanism of resistance is complicated and varied. Though medical research has attained high quality treatment and therapy, still we are taking benefit of ancient anti-microbial principles and resources for the treatment.

Recent findings suggest that the speed at which bacteria acquire resistance far outpaces the development of new or modified antibiotics. The resistance of bacteria against antimicrobial agents is due to the mutation, by plasmid or transposoon acquisition, or by the intrinsic properties present in microorganisms that confirm the resistance. *S.mutans* and *E.coli* are among the bacteria's responsible for most of the infectious diseases worldwide that becomes a major public concern.

It is therefore pertinent to find alternate strategies and compounds which are more effective with high healing potential. Scientists researched natural products rather than those developed chemically; one such product is essential oil. These have been used in folk medicine since ancient times. These are used as powerful tool for the treatment of infectious diseases like respiratory, urinary, and dental

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problems. Herbs and spices are particularly suitable for this purpose, with cloves standing out as a prime example. Cloves have a long history of use in both Ayurvedic and Chinese medicine, with the flower buds being the primary component employed to address various health issues. In the current research we have selected eugenol as an essential compound due to the higher antimicrobial and antioxidant capability. While numerous studies have examined these topics, but the ongoing but still there is a grey area in relation to research the pertaining to the two bacteria

3. MATERIALS AND METHODOLOGY

Eugenol (commercially available), glassware's, BHI media for *S. mutans*, nutrient broth, nutrient agar and the two screened bacteria's in this study were obtained from MTCC (Microbial Type Culture Collection).One Gram- positive and one Gram-negative bacteria were tested.

Table 1: List of bacterial strains used

S.No	BACTERIA	STRAIN TYPE	MTCC NUMBER
1	***E coli*** dh5alpha	Gram negative	MTCC1652
2	***S mutans***	Gram positive	MTCC497

3.1Maintenance of culture

a) Preparation of Liquid Media

Weigh 0.325 gm of nutrient broth and mix with 25ml of distilled water in conical flask.(1.85 gm of BHI was weighed and added to 50ml of distilled water in a beaker for ***S mutans***). Mix well till it dissolve and the pH of the solution was adjusted to 7.4. After proper mixing the media was distributed to different conical flasks and kept in autoclave for sterilization.

b) Preparation of Solid Media

Weigh 3.25gm of nutrient broth (1.85gm of BHI in case of ***S mutans***) and 3.75 gm of bacteriological agar; add both of these to 250ml distilled water in beaker. The solution is mixed well and it was then autoclaved.

c) Preparation of Inoculums

Autoclaved flasks containing the 50 ml nutrient media were taken and then with the help of sterile inoculating loop 2microgram of streptococcus mutans from preserved culture plates /slants were scraped and put in their respective flasks and all the flasks were shaken and kept overnight in an incubator set at 35℃. Turbidity observed the next day indicate the presence of bacterial growth.

d) Preparation of Media Plates

The autoclaved molten agar was poured into the desired number of plates. After 15 minutes the media was solidified and then the media plates were stored in the refrigerator.

The methodology employed in this study is the Kirby- Bauer disk diffusion antibiotic sensitivity testing protocol followed from Jan Hudzicki -2009 American Society for Microbiology.

Table 2: Protocol for Kirby Bauer Method

Eugenol (µl)	1	2	3	4	5	0
DMSO (µL)	14	13	12	11	10	15
Total	15	15	15	15	15	15

3.2. Minimum Bacterial Concentration

The MBCs were determined by observing the growth of bacteria on nutrient agar plates containing increasing concentrations of EO. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5% killing of the original inoculums.

3.3. Minimum Inhibitory Concentration;

This term typically applies to chemicals called antibiotics, drugs that kill bacteria, and thus microorganisms called bacteria. The MIC is typically stated in micrograms/milliliter (MIC in Pharmacology).

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Table 3: Protocol to determine MBC and MIC

Test tube No	Quantity of eugenol (µl)	Quantity of DMSO(µl)	Bacterial media (µl)	Bacteria(µl)
1	2	28	265	5
2	4	26	265	5
3	8	22	265	5
4	16	14	265	5

The eugenol was diluted to 100 times, 1000 times and 10000 times. According to the protocol all were pipetted in a microtitre plate. Incubated for 24 hrs at 37C. The final readings were taken in ELISA reader at 595nm

3.4. Live Dead Cell Assay

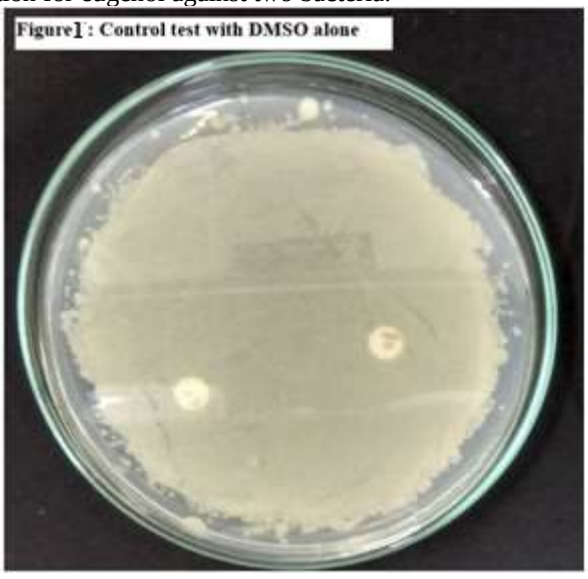
For live dead cell assay freshly grown bacterial cells were treated with eugenol and were washed. Then it is stained with FITC and PI to detect the fluorescence.Record fluorescent and view the image in CLSM. The red spots show the dead cell. The green spot represents the live cell.

3.5. Scanning Electron Microscopy (SEM)

SEM was performed using JOEL-JSM 6510 LV instrument to understand the sample topography and composition. Here ***S. mutans*** was taken as a representative organism. The bacterial cells were treated with EU and incubated for 3-5 hrs. The non-treated bacterial cells were served as a control. All the cells were washed with PBS (pH-7.4) several times followed by fixing the cells with 2.5 % glutaraldehyde at room temperature for 20 min. After fixation step again wash the cells several times with PBS. The obtained pellet was dehydrated with increasing concentration of ethanol after this microscopic examination was performed. Finally sample was coated with gold and analyzed by SEM (University Sophisticated instrumentation center, AMU).

4. RESULTS

The disk diffusion method was used to screen the antimicrobial activity. Next day zone of inhibition were observed and recorded. Below are the images of zone of inhibition for eugenol against two bacteria.



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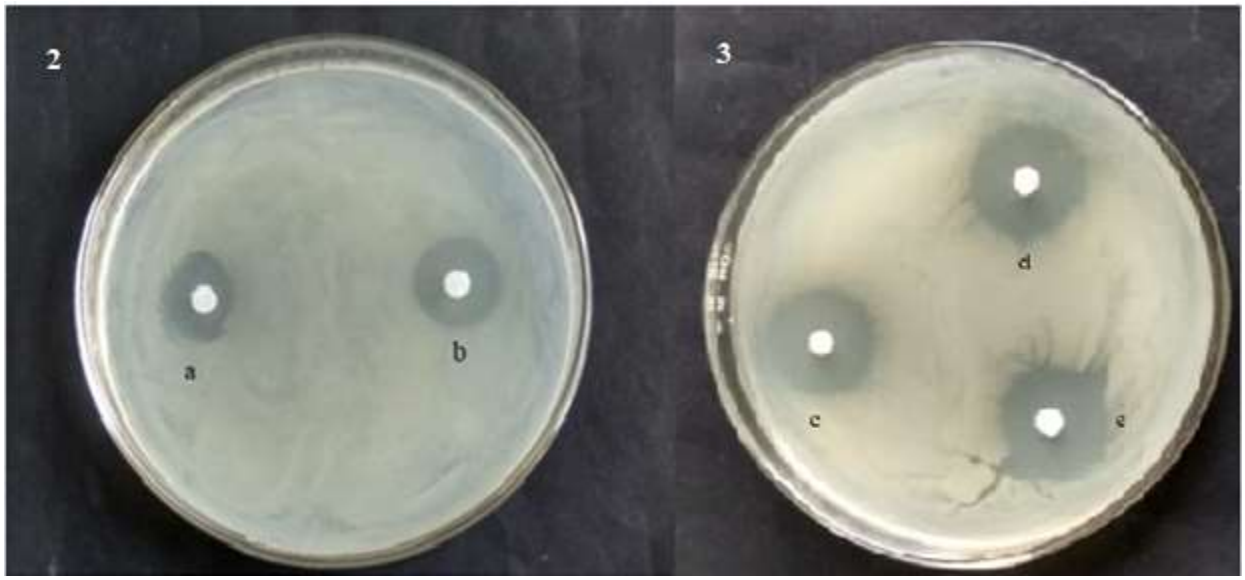

Figure 2&3: Standardized zone of inhibition of eugenol against S *mutans* (a) 1µl of eugenol (b) 2 µl (c) 3µl (d) 4µl and (e) 5 µl of EU. The zone of inhibition was measured in mm.

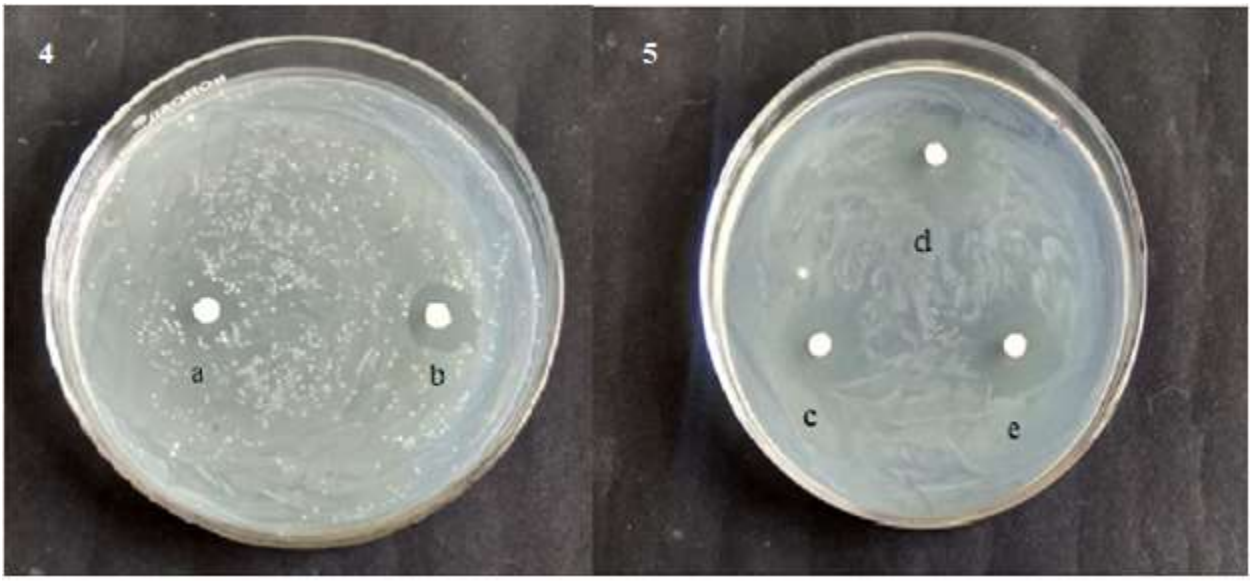

Figure 4& 5: Standardized zone of inhibition of eugenol against E. *coli*(a) 1µl of eugenol (b) 2 µl (c) 3µl (d) 4µl and (e) 5 µl of EU. The zone of inhibition was measured in mm.

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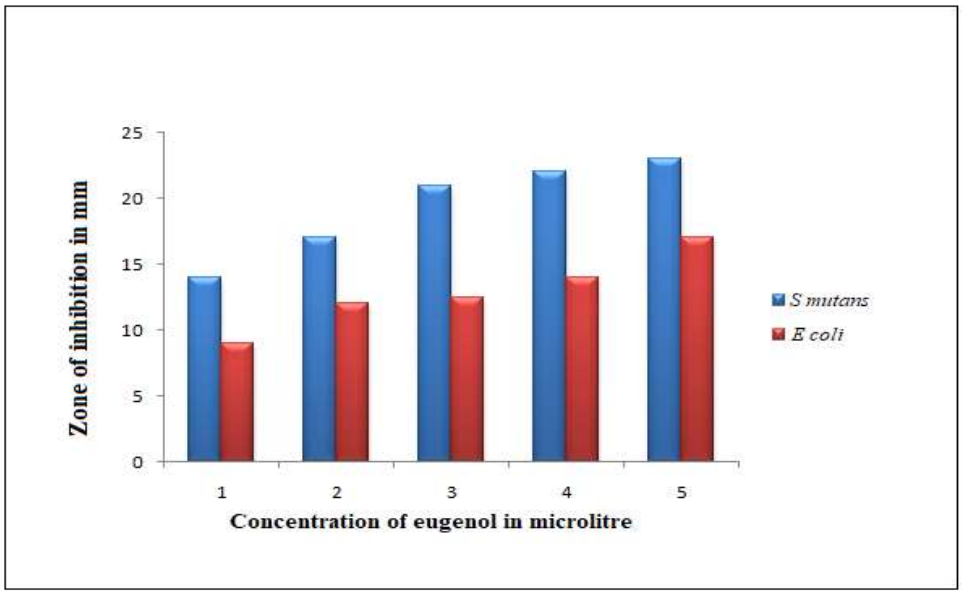

Figure 6: Depicts antibacterial activity of eugenol against the two bacteria which reveals that *S mutans* show more inhibition as compared to *E coli.*

Determination of MBC and MIC

Table 4: Table for MBC and MIC values of two bacteria

	Bacteria	MBC (µg/ml)	MIC (µg/ml)
Eugenol	S *mutans*	50.4 µg/ml	40.32 µg/ml
	E *coli*	403.2 µg/ml	201.6 µg/ml

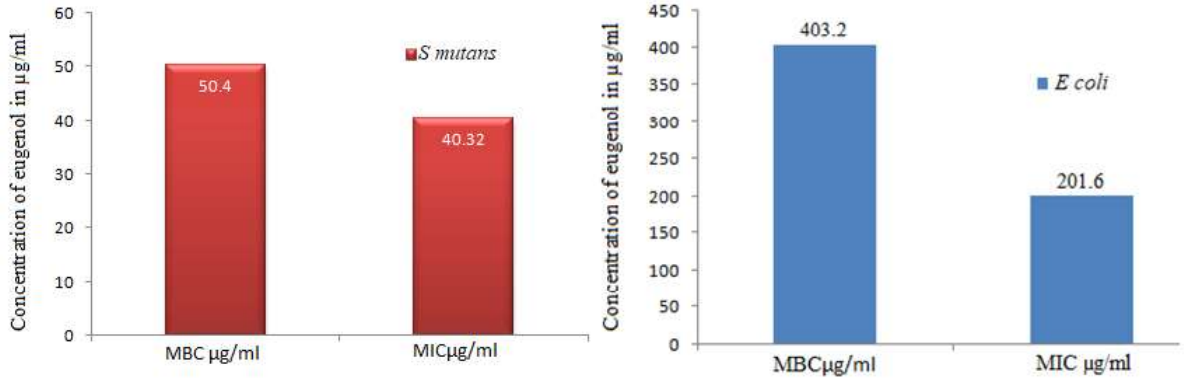

Figure 7: Graphical representation of MBC and MIC values of S *mutans* and E *coli*

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Live Dead Cell Assay

Table 5: Live/dead bacterial cell analyzed by CLSM

	E Coli (Gram Negative)		S Mutans (Gram Positive)	
	Control	Treated	Control	Treated
Fluorescence Color	Only Green	Both Green And Red	Only Green	Both Green And Red
Color intensity	Higher green fluorescence	Higher red fluorescence with moderate green fluorescence	Higher green fluorescence	Higher red fluorescence with moderate green fluorescence
% of Bacterial Cell Killed	-	~80	-	~80

SEM analysis of S *mutans*

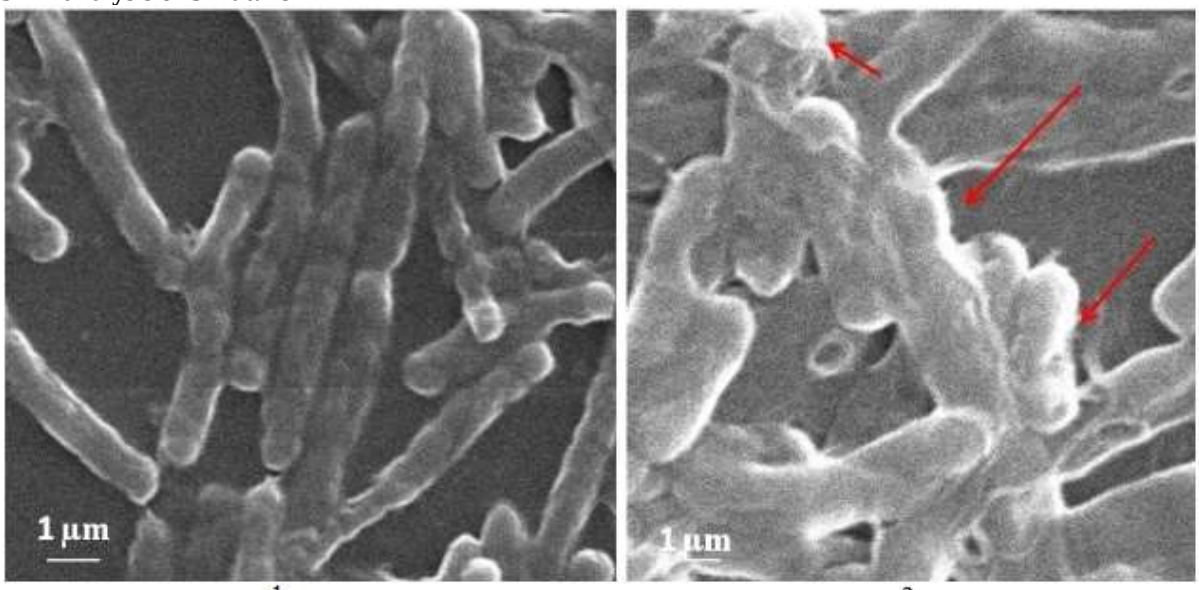

Figure 8: SEM analysis of S *mutans* (1) control of S *mutans* not treated with eugenol. (2) S *mutans* treated with eugenol.

5. DISCUSSION

The addition of eugenol to the broth culture effectively inhibited the growth of both S. *mutans* and E. *coli*, with a more pronounced effect observed on the gram-positive S. *mutans* compared to the gram-negative E. *coli*. Zone of inhibition of S mutans is as follows (14mm, 17mm, 21mm, 22mm, and 23mm), and in E coli is observed as (9mm, 12mm, 12.5mm, 14mm, and 17mm). Some authors have reported that Gram-positive bacteria have higher sensitivity than Gram-negative bacteria. This study also reveals the same. The efficiency of eugenol against these pathogenic bacteria provides a scientific ground for the application of eugenol in the treatment of infectious diseases.

MBC value for S mutans is 50.4µg/ml and MIC obtained is 40.32µg/ml. This suggests that even in small quantities eugenol shows potential activity against S mutans. The MBC value obtained for E *coli* is 403.2µg/ml and MIC is 201.6µg/ml.

In CLSM both the bacteria and non-treated bacterial cells emitted higher intensities of green fluorescence without any red fluorescence indicating all live or healthy cells (table 10). In case of E. coli when the cells were treated at MBC (at mention the values) for about 4-5 hrs there was both visible red along green fluorescence however the fluorescent intensity of the red color was much higher than green indicating 80 % of the dead cells. Similarly in the case of S mutans the fluorescence intensity of red color was observed along with moderate green fluorescence indicating 80% of the dead cells therefore equally bactericidal against both the tested organisms. From above results, it can be speculated that eugenol effectively induces bacterial cell membrane damage making them permeable which ultimately results in the release of intercellular metabolites and consequently cell death.

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SEM images inferred that the eugenol acts on the cell membranes of the bacteria. Disruption of the bacterial cell membrane is the most frequently reported mechanism contributed by essential oils. The most frequently reported mechanisms are disruption of bacterial membranes contributes to the antibacterial properties of most EOs. Damage to membrane proteins (e.g. enzymes), cell content leakage, depletion of the motive proton force, and coagulation of the cytoplasm are also common effects. Although the site of action of individual EO components has been established in many cases, the mechanism itself is often still not completely understood.

The result of the study presents eugenol as a potential alternative antibacterial agent that combats infection-causing bacteria such as S *mutans* and E *coli*.

6. CONCLUSION

- The research indicates that eugenol may serve as an effective antibacterial agent against the pathogenic bacteria examined, with gram-positive bacteria showing greater resistance.
- The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for *Streptococcus mutans* were found to be 50.4 µg/ml and 40.32 µg/ml, respectively.
- In contrast, for *Escherichia coli*, the MIC and MBC values were 403.2 µg/ml and 201.6 µg/ml, suggesting that this gram-negative bacterium also exhibits sensitivity to eugenol.
- Eugenol's antibacterial mechanism involves disrupting the integrity of the bacterial cell wall, as evidenced by scanning electron microscopy (SEM) images.
- Consequently, eugenol has potential as a natural antibacterial agent for treating infections caused by *S. mutans* and *E. coli*, and no adverse side effects have been reported.

LIST OF ABBREVIATIONS

1.	BHI	Brain heart infusion
2.	CLSM	Confocal Laser Scanning Microscopy
3.	E *coli*	*Escherichia coli*
4.	EO	Essential oil
5.	EU	Eugenol
6.	FITC	Fluroscein isothiocyanate
7.	ISO	International Organization for Standardization
8.	MBC	Minimum Bactericidal Concentration
9.	MDR	Multiple drug resistance
10.	MIC	Minimum Inhibitory Concentration
11.	PI	Propidium iodide
12.	S *mutans*	*Streptococcus mutans*
13.	SEM	Scanning Electron Microscopy

AVAILABILITY OF DATA

All data analyzed during this study are included in this published article.

REFERENCES

1. *Ríos JL, Recio MC. Medicinal plants and antimicrobial activity. Journal of Ethnopharmacology [Internet]. 2005 Aug; 100(1-2):80–4. Available from: https://www.sciencedirect.com/science/article/pii/S0378874105003247*
2. *Bassolé IHN, Juliani HR. Essential Oils in Combination and Their Antimicrobial Properties. Molecules. 2012 Apr 2; 17(4):3989–4006. DOI:10.3390/molecules17043989*
3. *Alekshun MN, Levy SB. Molecular Mechanisms of Antibacterial Multidrug Resistance. Cell [Internet]. 2007 Mar; 128(6):1037–50. Available from: https://www.sciencedirect.com/science/article/pii/S009286740700311X.DOI: 10.1016/j.cell.2007.03.004*
4. *Anil Kumar Maurya, Agarwal K, Akhilesh Kumar Gupta, Saxena A, Zulfa Nooreen, Tandon S, et al. Synthesis of eugenol derivatives and its anti-inflammatory activity against skin inflammation. Natural Product Research. 2018 Dec 22;34(2):251–60. DOI: 10.1080/14786419.2018.1528585*
5. *Hammer KA, Carson CF, Riley TV. Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology. 1999 Jun;86(6):985–90.available form https://doi.org/10.1046/j.1365-2672.1999.00780.x*

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EPRA International Journal of Research and Development (IJRD)
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6. *Onyeagba RA, Ugbogu OC, Okeke CU, Iroakasi O. Studies on the antimicrobial effects of garlic (Allium sativum Linn), ginger (Zingiber officinale Roscoe) and lime (Citrus aurantifolia Linn). African Journal of Biotechnology [Internet]. 2004 Oct 31 [cited 2020 Oct 23];3(10):552–4. Available from: https://www.ajol.info/index.php/ajb/article/view/15016/58920*
7. *Chouhan S, Sharma K, Guleria S. Antimicrobial Activity of Some Essential Oils – Present Status and Future Perspectives. Medicines [Internet]. 2017 Aug 8;4(3):58. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5622393/*
8. *Hudzicki, J. Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. American Society for Microbiology; (2009)*
9. *C. D, Pereira V, De Vasconcelos MCBM, Rosa EA, Saavedra MJ. Antibacterial activity evaluation of 15 eucalyptus species essential oils against clinically relevant pathogenic bacteria.American Journal of Microbiology. 2014 Feb 1; 5(2):41–8. DOI:10.3844/ajmsp.2014.41.48*
10. *. Pawley J. Handbook of Biological Confocal Microscopy. Springer Science & Business Media; 2013.*
11. *Bär W, Bäde-Schumann U, Krebs A, Cromme L. Rapid method for detection of minimal bactericidal concentration of antibiotics. Journal of Microbiological Methods. 2009 Apr; 77(1):85–9.*