IJPSR (2021), Volume 12, Issue 1 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 01 January 2020; received in revised form, 08 April 2020; accepted, 10 April 2020; published 01 January 2021

ANTIMICROBIAL ACTIVITY OF EUGENOL AGAINST HUMAN PATHOGENIC BACTERIA BY MINIMAL INHIBITORY CONCENTRATION, MINIMAL BACTERICIDAL CONCENTRATION AND DISC-DIFFUSION METHODS

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Keywords:

Essential oil, Eugenol, MBC, MIC, Disc-diffusion, Bacteria

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ABSTRACT: Essential oils from plants have been reported to have antibacterial activity. Essential oil is a mixture of many chemicals, and one or more chemicals in essential oil may have antibacterial activity. In our laboratory, essential oil from the leaves of *Ocimum sanctum* L. was found to have antibacterial activity against 18 human bacteria. GC-MS analysis of the *Ocimum sanctum* essential oil revealed the presence of 19 chemicals, and one of them was eugenol. In the present study, eugenol was found to have an antibacterial effect against 4 Gram-negative and 2 Gram-positive human pathogenic bacteria by minimal bactericidal concentration, minimal inhibitory concentration and disc-diffusion methods. The minimal bactericidal concentrations of eugenol were 0.96 mg/ml, 4.17 mg/ml, 16.6 mg/ml, 16.6 mg/ml, 33.3 mg /ml and 33.3 mg /ml against *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Serratia marcescens* and MRSA, respectively. The minimal bactericidal concentrations and minimal inhibitory concentrations in Gram-negative bacteria *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Serratia marcescens* were similar. The minimal inhibitory concentrations are more than those of minimal bactericidal concentrations of Gram-positive bacteria, MRSA, and *Staphy-lococcus aureus*. The diameters of growth inhibition by eugenol were 7, 10, 10, 14, 15, and 23 millimeters for *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Staphylococcus aureus*, MRSA, and *Acinetobacter baumannii*, respectively.

INTRODUCTION: Infectious diseases are one of the most important causes of human death world-wide. Due to the widespread use of antimicrobial agents, many organisms have developed drug resistance to many available antimicrobials. These multidrug-resistant organisms are killed hardly by one or two antimicrobials available today, and soon it is possible that resistance may develop to these few anti-microbials also.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.12(1).330-35

The article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.12(1).330-35

Therefore detection or development of newer antimicrobial agents is the need of the hour. For centuries medicinal plants have been used to treat human diseases. During the last 20 to 30 years, advances in photochemistry and identification of plant components have shown that plant compo-nents can be used as effective antimicrobial agents.

Studies on oils from aromatic and medicinal plants are growing because they are known to have many biological activities such as antibacterial, anti-fungal, antioxidant, and anticancer [1]. The chemical composition and antibacterial effects of Osmium species essential oils have been reported from different parts of the world [2-8]. In our laboratory, the essential oil from *Ocimum sanctum* L. was extracted by Clevenger apparatus and was found

that the essential oil had an antibacterial effect against 18 human bacteria by minimal bactericidal concentration, minimal inhibitory concentration, and gaseous contact exposure methods in our laboratory [2].

The GC-MS analysis of the essential oil from *Ocimum sanctum* L. revealed the presence of 19 chemical constituents, including eugenol and caryophyllene oxide [2]. Among these chemicals, one or more than one may have an antibacterial effect. Therefore evaluation of the antibacterial effect of an individual chemical component in the essential oil is needed so that instead of treating the patient with essential oil (containing all the chemicals in the essential oil), the particular chemical/s in the essential oil with antibacterial activity alone can be used. This approach may reduce the cost and side effects due to other chemicals in the essential oil.

The present work evaluates the antibacterial effect of eugenol (which was found in the essential oil of plants, including *Ocimum sanctum* L) against six human pathogenic bacteria by minimal inhibitory concentration, minimal bactericidal concentration, and disc-diffusion methods.

MATERIALS AND METHODS: American type culture collection (ATCC) strains of bacteria [*Klebsiella pneumonia* (ATCC 1700603), *Acinetobacter baumannii* (ATCC 19606), *Serratia marcescens* (ATCC 14041) and *Pseudomonas aeruginosa* (ATCC 10145)] were purchased from HIMEDIA Pvt. Ltd, Bombay, India (KWIKSTIC). Clinical isolates of bacteria [*Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* (MRSA)] were obtained from the Microbiology department of Mahatma Gandhi Medical College and Research Institute, Puducherry, India. Imipenam (10 mcg /disc) vancomycin (30 mcg / disc), Mueller Hinton broth (MHB) base, Dimethyl sulphoxide (DMSO), sterile susceptibility test discs and McFarland standard were purchased from HIMEDIA Pvt. Ltd., Mumbai, India. Mueller Hinton agar (MHA) was purchased from micro express, Goa, India, and Microtiter plates were purchased from TARSONS, Kolkata, India. Eugenol (PESTANAL, analytical standard) was purchased from SIGMA ALDRICH, USA. (1 vial of eugenol contains 250 mg of eugenol in 234 µl; 1.068 mg of eugenol in 1 µl; product number 35995; a colorless to yellow liquid; purity ≥ 98%; CAS number 97-53-0;

Formula $4\text{-}(H_2C{=}CHCH_2)C_6H_3\text{-}2\text{-}(OCH_3)OH$; Formula weight 164.20;)

Minimal Bactericidal Concentration (MBC) and Minimal Inhibitory Concentration (MIC) Determination by Micro Tube Dilution Method: Minimal inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation in a tube dilution method. Minimal bactericidal concentration (MBC) is the lowest concentration of an antimicrobial that will prevent the growth of an organism in a tube dilution method after subculture on an antibiotic-free medium [9].

Two-fold dilutions of eugenol were carried out in the 96 well microtiter plate [10]. Briefly, 50 µl of MHB was added to well numbers 1 to 9 and 11 to 12 in a 96- well sterile U bottomed microtiter plate. 50 µl of eugenol was added to the 1st well. The content in 1st well was mixed and 50 µl transferred to the 2nd well. Likewise, a serial double dilution was carried out up to the 9th well, and 50 µl was discarded from the 9th well. 100 µl of MHB was added to 10th well (medium control), and 50 µl of eugenol was added to the 11th well (test drug control).

The final concentration of eugenol in the 1st well was 25 µl (equal volumes of neat eugenol and bacterial suspension). The final eugenol concentrations in the 2nd, 3rd, 4th, 5th, 6th, 7th, 8th, and 9th wells were 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19, and 0.09 µl, respectively.

The bacteria to be tested were inoculated on an MHA plate and incubated overnight at 37 ºC. Few colonies of the growth were picked up and mixed with 1 ml of MHB and incubated at 37 ºC for 2 hours. From this suspension, 100 µl was transferred to another tube containing 1 ml of MHB, and the density was adjusted to 0.5 McFarland with MHB to get 1×10^8 CFU (colony forming units)/ml suspension. 100 µl of this suspension was transferred to another tube containing 10,000 µl of MHB to get 1×10^6 CFU/ml suspension [11]. (When 50 µl of this bacterial suspension was mixed with 50 µl of eugenol dilutions in the microtiter plate

wells, the final mixture in the wells had 5×10^5 CFU/ml of bacteria). 50 µl of bacterial suspension was added to all the wells except 10th (MHB media control) and 11th (tested drug control) wells. For each bacterium, the experiment was carried out in triplicate.

The plates were incubated for 24 h at 37 ºC. The wells 1 to 9 were visually observed, and the concentration of eugenol in the well just before the well from which the turbidity appeared was noted as the MIC of the 3 experiments for each bacterium, the highest MIC value is taken as the MIC for that bacterium **Table 1**.

From each well 10 µl suspension was aspirated and inoculated on MHA plates. The plates were incubated at 37 ºC for 24 h, and growth or no growth of bacteria for each well was observed and recorded. There should not be growth in 10 and 11th wells (MHB medium control and test drug control wells, respectively), but the 12th well should have bacterial growth (bacterial growth control). Among wells 1 to 9, the well with the least eugenol concentration up to which there was no growth was taken as the MBC of the 3 experiments for each bacterium; the highest MBC is taken as the MBC for that particular bacterium **Table 2**.

TABLE 1: MINIMAL INHIBITORY CONCENTRATION OF EUGENOL AGAINST BACTERIA

Bacteria	MIC of eugenol - µl / ml		MIC of eugenol- mg / ml	
	Highest MIC out of 3 experiments µl / ml	Mean ± Standard deviation of MIC of 3 experiments µl / ml	Highest MIC out of 3 experiments mg / ml	Mean ± Standard deviation of MIC of 3 experiments mg / ml
1. *Klebsiella pneumonia* (ATCC)	3.9	3.9 ± 0	4.17	4.17 ± 0
2. *Serratia marcescens* (ATCC)	31.2	31.2 ± 0	33.32	33.32 ± *0*
3. *Pseudomonas aeruginosa* (ATCC)	15.6	15.6 ± 0	16.6	16.6 ± 0
4. *Acinetobacter baumannii* (ATCC)	0.9	0.9 ± 0	0.96	0.96 ± 0
5. MRSA	62.4	26 ± 31.5	66.64	27.74 ± 33.68
6. *Staphylococcus aureus*	31.2	20.8 ± 9	33.32	22.21 ± 9.61

1 µl of eugenol = 1.068 mg

TABLE 2: MINIMAL BACTERICIDAL CONCENTRATION OF EUGENOL AGAINST BACTERIA

Bacteria	MBC of eugenol -- µl / ml		MBC of eugenol -- mg /ml	
	Highest MBC out of 3 experiments µl / ml	Mean ± Standard deviation of MBC of 3 experiments µl / ml	Highest MBC out of 3 experiments mg / ml	Mean ± Standard deviation of MBC of 3 experiments mg / ml
1. *Klebsiella Pneumonia* (ATCC)	3.9	3.9 ± 0	4.17	4.17 ± 0
2. *Serratia Marcescens* (ATCC)	31.2	31.2 ± 0	33.3	33.3 ± 0
3. *Pseudomonas Aeruginosa* (ATCC)	15.6	15.6 ± 0	16.6	16.6 ± 0
4. *Acinetobacter Baumannii* (ATCC)	0.9	0.9 ± 0	0.96	0.96 ± 0
5. MRSA	31.2	13 ± 15.8	33.3	13.88 ± 16.82
6. *Staphylococcus aureus*	15.6	10.4 ± 4.5	16.6	11.1 ± 4.8

1 µl of eugenol = 1.068 mg

Disc Diffusion Method to find the Antibacterial Effect of Eugenol: A sterile swab was dipped into the bacterial suspension adjusted to 0.5 McFarland density (as explained above), and the swab was pressed along the sides of the tube to remove excess fluid. The swab was streaked in three directions on MHA plate to get a lawn of bacterial growth [12]. The agar plate was left at room temperature for 15 min. Sterile 6 mm susceptibility test discs were placed on the agar surface. 15 µl each of undiluted (neat) eugenol, 50% eugenol in DMSO (equal volumes of eugenol and DMSO), and 50% DMSO in MHB (diluent control) were dropped on different discs on the agar plate.

Vancomycin disc (for Gram-positive bacteria) or imipenam disc (for Gram-negative bacteria) were placed on the agar plate as bacterial growth inhibitor controls. The plate was incubated for 24 h at 37 ºC, and the diameters of inhibition of growth around discs were measured with a scale and recorded. Of the 3 experiments for each bacterium, the least diameter of growth inhibition was taken as the diameter of growth inhibition by eugenol for that particular bacterium **Table 3**.

TABLE 3: DISC-DIFFUSION METHOD--DIAMETER OF BACTERIAL GROWTH INHIBITION BY EUGENOL

Bacteria	Neat (Undiluted eugenol) Diameter of growth inhibition-mm		50% eugenol in DMSO. Diameter of growth inhibition-mm	
	Least diameter of 3 experiments-	Mean ± Standard deviation of 3 experiments	Least diameter of 3 experiments	Mean ± Standard deviation of 3 experiments
1. *Klebsiella pneumonia* (ATCC)	10	10.33 ± 0.58	8	8 ± 0
2. *Serratia marcescens* (ATCC)	10	10.67 ± 0.58	8	9.33 ± 1.55
3. *Pseudomonas aeruginosa*(ATCC)	7	7 ± 0	-	
4. *Acinetobacter baumannii* (ATCC)	23	23.67 ± 0.58	19	20.33 ± 1.53
5. MRSA	15	15.67 ± 0.58	12	13.33 ± 1.5
6. *Staphylococcus aureus*	14	15.33 ± 1.15	11	13.67 ± 2.31

- No bacterial growth inhibition DMSO-Dimethyl sulfoxide

RESULTS: Of the six bacteria tested, *Acinetobacter baumannii* had the least MIC of 0.96 mg/ml, while MRSA had the highest MIC of 66.64 mg/ ml **Table 1**. *Acinetobacter baumannii* showed the least MBC of 0.96 mg/ml, whereas Serratia marcescens and MRSA showed a MBC of 33.3 mg/ml. *Pseudomonas aeruginosa* and *Staphylococcus aureus* had a MBC of 16.6 mg/ml. *Klebsiella pneumoniae* had a MBC of 4.17 mg /ml.

The MBC and MIC of four bacteria (*Klebsiella pneumoniae*, *Serratia marcescens*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*) were similar. But MICs of MRSA and *Staphylococcus aureus* were more than those of the MBCs of respective bacteria **Table 2**.

Disc–diffusion method **Table 3** showed higher diameter of bacterial growth inhibition by neat (undiluted) eugenol than that of 50% diluted eugenol in DMSO for all the bacteria tested. *Acinetobacter baumannii* had the largest diameter of growth inhibition (23 mm), while *Klebsiella pneumoniae* and *Serratia marcescens* had a growth inhibition diameter of 10 mm. MRSA and *Staphylococcus aureus* had growth inhibition diameters of 15 mm and 14 mm respectively. Undiluted eugeno inhibited the growth of *Pseudomonas aeruginosa* to 7 mm diameter, while 50% diluted eugenol in DMSO did not inhibit the growth of *Pseudomonas aeruginosa* **Table 3**.

DISCUSSION: Essential oils are complex compounds with different types of aldehydes, phenolics, and terpenes. The antimicrobial effects of essential oils and the chemical components (such as eugenol) in the essential oils have been reported by many researchers [2-8].Pathirana *et al.*, (2019) have reported that eugenol had antibacterial activity against pathogenic fish bacteria isolated from cultured olive flounder [13]. They have also reported that eugenol can denature protein and react with phospholipids in the cell membrane of bacteria.

They have also suggested that eugenol affected the transport of ions and ATP and changed the fatty acid profile of different bacteria. Jiangwei *et al.*, (2017) have tested eugenol by broth microdilution method against *Legionella pneumophila* and have reported that eugenol had significant anti-legionella pneumophila activity.

They have shown that eugenol acted on the bacterial envelope of *L. pneumophila*, leading to cell membrane damage, cytoplasm leakage, and bacterial death [14]. Lena Dhara and Anusri Tripathi (2013) have reported that eugenol showed antibacterial activity against *Escherichia coli* and *Klebsiella pneumonia* [15]. They have also stated that microbiological assays and molecular docking experiments indicated antibacterial activity and significant molecular interactions of eugenol with ESBL enzymes of pathogenic bacteria.

Lucy owen and Katie Laird (2018) have reviewed the literature concerning the antibacterial activity of essential oils and their interactions with antibiotics as a potential solution against antibiotic-resistant organisms [16]. Synergistic interactions between Essential oils and their components with antibiotics have been reported, including several instances of antibiotic resensitization in resistant isolates. Wendy *et al.,* (2014) have suggested that anti-biotics with essential oils containing carvacrol, cinnamaldehyde, cinnamic acid, eugenol, and thymol can have a synergistic effect against bacteria as they may act against multiple targets; consequently, the usage of antimicrobials can be reduced [17]. Jadhav *et al.,* (2004) have reported that eugenol is used in perfumes, flavorings, and as a local antiseptic and anesthetic [18].

In our laboratory, the essential oil of *Ocimum sanctum* L. was found to have antibacterial effect on 18 bacteria (*Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Proteus mirabilis*, *Shigella boydii*, *Serratia marcescens*, *Salmonella typhimurium*, *Burkholderia cepacia*, *Enterobacter aerogenes*, *Haemophilus influenzae*, *Salmonella typhi*, *Vibrio cholerae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Methicillin resistant, Staphylococcus aureus* (MRSA), Coagulase-negative Staphylococcus, *Enterococcus faecalis* and *Corynebacterium diphtheriae*). GC-MS analysis of the essential oil of Ocimum sanctum revealed the presence of 19 chemicals (Eugenol, Copane, Caryophyllene oxide, Isoaramadendrene epoxide, Spathulenol, Phytol and others) [2].

In the present study, we investigated the antibacterial effect of one of the chemicals (eugenol) present in the essential oil. Eugenol was found to have antibacterial activity against 6 human pathogenic bacteria by using minimal inhibitory concentration, minimal bactericidal concentration, and disc-diffusion *in-vitro* methods. Eugenol showed antibacterial activity against both Gram-negative and Gram-positive bacteria tested in this study.

The MBC and MIC were the same for four bacteria (*Klebsiella pneumoniae*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*), whereas two bacteria (MRSA and *Staphylococcus aureus*) had higher MICs than MBCs. Essential oil is a mixture of many chemical components in varying amounts in different plants. The antibacterial activity may reside in one or more than one chemical in any essential oil. If we can test each component in the essential oil and find one or more than one chemical is antibacterial, then appropriate *in-vivo* animal experiments can be designed to detect the antibacterial effect of the individual chemical. The present study and earlier studies have shown that eugenol alone can kill bacteria in *in-vitro* experiments. Studies to evaluate the use of each essential oil component (such as eugenol) are required to ascertain their use in human diseases. Further experiments in animal models and subsequent human trials are needed to reach the final goal of identifying the individual chemical/s in essential oil for treating human or animal diseases.

CONCLUSION: The present study reveals that eugenol, a chemical constituent present in plant essential oils, including *Ocimum sanctum* L. has antibacterial activity against human pathogenic bacteria, both Gram-negative and Gram-positive.

ACKNOWLEDGEMENT: Nil

CONFLICTS OF INTEREST: Nil

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How to cite this article:
Jayapal V: Anti-microbial activity of eugenol against human pathogenic bacteria by minimal inhibitory concentration, minimal bactericidal concentration and disc-diffusion methods. Int J Pharm Sci & Res 2021; 12(1): 330-35. doi: 10.13040/IJPSR.0975-8232. 12(1).330-35.