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The Inhibition Effects of Eugenol and Pulegone on *Stenotrophomonas maltophilia*: an Opportunistic Pathogen

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Summary: Aerobic, non-fermentative and Gram-negative *Stenotrophomonas maltophilia* is a multidrug-resistant bacilli that is known to be pathogen for human and animals. *S. maltophilia* has been isolated from different animal species and also found in a variety of environments including soil, water, and plants. *S. maltophilia*, which has the ability to form biofilms on surfaces that cause environmental problems, is resistant to many antibiotic classes such as cephalosporins, carbapenems, and aminoglycosides. Here in this study, its aimed to determine the inhibition activities of natural phenolic compounds eugenol and pulegone against *S. maltophilia* MU69. Antibacterial activities of eugenol and pulegone were initially determined by disc diffusion method. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were also determined by tube dilution assay. Antibiofilm activities of the compounds were investigated by crystal violet staining and also monitorized using Scanning Electron Microscopy (SEM). Zone of inhibition measurements were found to be 39 mm and 28 mm for eugenol and pulegone, respectively. MIC and MBC values were found to be 3.12 mg/mL for eugenol while these were 2.5 mg/mL and 5 mg/mL for pulegone, respectively. The highest antibiofilm activity was found to be 56.30±0.45% for MIC of eugenol. To our knowledge, this is the first report of the antibacterial and antibiofilm activity of eugenol and pulegone against *S. maltophilia*. According to the biological activity results, it can be concluded that these natural agents may be potentially used for veterinary sciences, food industry or pharmaceutical applications that aim to manage *S. maltophilia* biofilm.
Key words: Antibacterial, antibiofilm, eugenol, pulegone, *S. maltophilia*.

Eugenol ve Pulegonun Fırsatçı Patojen Bir Bakteri Olan *Stenotrophomonas maltophilia*'ya Karşı İnhibisyon Etkinliğinin Belirlenmesi

Özet: Aerobik, non-fermentatif ve gram-negatif *Stenotrophomonas maltophilia*, insan ve hayvanlar için patojen olarak bilinen çoklu ilaç direncine sahip bir basildir. Farklı hayvan türlerinden izole edilmiş *S. maltophilia* suşu toprak, su ve bitkiler gibi çeşitli çevresel etmenlerden de izole edilebilmektedir. Katı yüzeyler üzerinde biyofilm oluşturabilme yeteneği sayesinde *S. maltophilia* suşu çevre sorunlarına yol açmakla birlikte aminoglikozitler, karbapenemler ve sefalosporinler gibi çeşitli antibiyotik sınıflarına karşı direnç göstermektedir. Bu çalışmada doğal fenolik bileşikler olan eugenol ve pulegonun *S. maltophila* MU69 suşu üzerindeki inhibisyon aktivitelerinin belirlenmesi amaçlanmıştır. Eugenol ve pulegonun antibakteriyel aktivitesi disk difüzyon yöntemi ile araştırılmıştır. Minimum İnhibisyon Konsatrasyonu (MİK) ve Minimum Bakterisidal Konsantrasyon (MBK) ise tüp dilüsyon yöntemi ile belirlenmiştir. Antibiyofilm aktivitenin belirlenmesinde kristal viyole boyama yöntemi uygulanmış ve ayrıca Taramalı Elektron Mikroskobi (SEM) ile görüntülenmiştir. Eugenol ve pulegon için inhibisyon zonu sırasıyla 39 mm ve 28 mm olarak ölçülmüştür. MİK ve MBK değerleri eugenol için 3.12 mg/mL olarak belirlenirken, pulegon için sırasıyla 2.5 mg/mL ve 5 mg/mL olarak tespit edilmiştir. En yüksek antibiyofilm aktivite oranı % 56.30±0.45 ile eugenole ait MİK değeri için gözlenmiştir. Bu çalışma eugenol ve pulegon bileşiklerinin *S. maltophilia* suşuna karşı antibakteriyel ve antibiyofilm aktivitelerinin belirlendiği ilk çalışma olarak sunulmaktadır. Biyolojik aktivite çalışmalarının sonuçlarına göre bu iki doğal bileşik veteriner bilimleri, gıda endüstrisi ve farmasötik uygulamalarda *S. maltophilia*'ya karşı geliştirilen sistemlerde potansiyel kullanım kapasitesine sahiptir.
Anahtar kelimeler: Antibakteriyel, antibiyofilm, eugenol, pulegon, *S. maltophilia*.

Introduction

There is a growing interest in the use of herbal products as an alternative to standart medical therapies. Plants contain various type of bioactive compunds and secondary metabolites that play an important role for using throughout alternative pharmaceutical approaches. Some of these compounds are found in essential oils isolated from plants. Essential oils have distinctive fragrances and/or flavours and are used in cosmetic as well as medical applications (38). Essential oils (EOs) are aromatic and volatile liquids ex-

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tracted from plant materials, such as flowers, roots, bark, leaves, seeds, peel, fruits, wood, and whole plant (19). Essential oils (EOs) can be isolated from by several techniques such as water or steam distillation, solvent extraction, expression under pressure, supercritical fluid extraction, subcritical water extraction, ultra-sound assisted extraction, microwave assisted extraction (7). The main constituents of EOs are mono- and sesquiterpenes, along with carbohydrates, phenols, alcohols, ethers, aldehydes, and ketones, which are responsible for the biological activity of aromatic and medicinal plants as well as for their fragrance (37). Eugenol (2-allyl-4-methoxyphenol) is a phenolic compound that can be obtained from a wide range of plant sources including clove oil, nutmeg oil, cinnamon extract and many other plants (28). The pharmaceutical functions of essential oils isolated from various plants have also thought to be due to the presence of eugenol (36). Eugenol is known to be effective against a number of lifestyle related threats including nervous disorders, digestive complications, reproductive derangements, blood cholesterol irregularity, hyper-tension, elevated blood glucose level, microbial infections, inflammatory actions and carcinogenesis (45). Antioxidant (25), antimicrobial (2), anti-inflammatory (29), anticancer (43), anti-diabetic (39), neuroprotective (35), and antihypercholesterolemic (42) activities of eugenol have been reported. Pulegone [Cyclohexanone; 5-methyl-2-(1-methylethylidene)-] is a monoterpene ketone present in the leaves and flowering tops of several members of the mint family *Lamiaceae*. Pulegone is reported to be the major constituent of, *Mentha pulegium* (22), *M. longifolia* (34), and *Micromeria cilicica* (18). As a pharmacologically active compund, pulegone found to have antimicrobial activity against *Candida albicans* and *Salmonella typhimurium* (18). Gram-negative bacterial pathogens, as *Stenotrophomonas maltophilia*, are often multiple-drug-resistant organisms (MDROs) due to multidrug resistance pumps, plasmids harboring antibiotic resistance genes, and various gene transfer mechanisms involved in the acquisition of antimicrobial resistance (9). *S. maltophilia* which is generally associated with respiratory infections in humans is considered as a nosocomial bacterium (1), but there have been reports of *S. maltophilia* associated with community acquired infections (9). *S. maltophilia* was considered to be the cause of fleece rot in sheep (31), septicemia in crocodiles (23) and the snubnose dart (*Trachinotus ovatus*) (47), ulcerative stomatitis in captive snakes (17), lymphadenitis in goats (26), infectious intussusception syndrome (IIS) in channel catfish (21), gill disease in sea bream (27), and lower airway disease in horses and dogs (1). *S. maltophilia* has the ability to adhere to solid surfaces such as plastics and form bacterial films (biofilms). The drug resistance mechanisms of the *S. maltophilia* are acquired by the horizontal transfer of antibiotic resistance through plasmids (5), transposons (6), integrons (24), efflux pumps, melanin-like pigment and biofilm formation (30).

The published sequence of the *S. maltophilia* genome shows numerous resistance genes, such as genes encoding for multidrug-efflux pumps, β-lactamases, and aminoglycoside-modifying enzymes (13). Discovery of the new strategies to treat the *S. maltophilia* infections has been gaining importance for a long time. In this study, its aimed to determine the antimicrobial and antibiofilm activities of eugenol and pulegone against *S. maltophilia* MU69 as a natural alternative to the use of antibiotics.

Material and Methods

Materials

Eugenol and pulegone were purchased from Sigma-Aldrich. Mueller Hinton Agar (MHA), Tryptic Soya Broth (TSB) and D-Glucose were purchased from Merck. *S. maltophilia* MU69 strain was provided from Mugla Sitki Kocman University Culture Collection and was incubated at 37±0.1°C for 24 h. Inocula was prepared adjusting the turbidity of the medium to match the 0.5 McFarland standard dilutions. The strain was maintained in its appropriate agar slants at 4°C throughout the study and used as stock culture. Eugenol and pulegone concentrations were prepared with 10 % DMSO as two-fold serial dilutions.

Disc Diffusion Assay of Eugenol and Pulegone

The antimicrobial activity was measured based on the disc diffusion method using pour-plating technique (12). 20 mL of MHA (Merck) sterilized and cooled to 45-50°C. After injecting 1000 µL microorganism cultures to sterile plates, media was distributed ans mixed homogenously. Sterile 6 mm paper discs (Schleicher and Schuell) were impregnated with 20 µL of pure eugenol and pulegone and were then placed on the inoculated agar. The plates were incubated at appropriate temperature for performing the bacteria, as mentioned above. At the end of the incubation period, diameters of growth zones around the discs were measured. The experiments were performed in triplicate.

Tube Dilution Method

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of eugenol and pulegone were determined by tube dilution method as recommended by the Clinical and Laboratory Standards Institute (11). *S. maltophilia* was grown in Mueller-Hinton Broth (MHB) at 37° C overnight and diluted to $5x10^5$ colony forming unit (CFU)/mL. Eugenol/ Pulegone solutions (200 µL)+microorganism (20 µL)+culture media (1800 µL) were inoculated into a glass tubes and incubated for 24 h. 10% DMSO is

used as negative control. The MIC was defined as the lowest concentration of eugenol/pulegone showing no turbidity (no visible growth of microorganism). 100 µL cell suspensions from the tubes with no turbidity were poured onto Mueller-Hinton Agar (MHA) plates and incubated overnight and the MBC was defined as the lowest concentration of eugenol/ pulegone at which bacteria did not grow on agar media. The experiments were performed in triplicate.

Effect on biofilm formation

The effect of eugenol and pulegone on biofilm-forming ability of *S. maltophilia* were tested by a microplate biofilm assay (33). Bacterial strain was prepared as above, but Trypticase Soy Broth (TSB) supplemented with 5% D-glucose was used as culture media. Cultures diluted to 1:100 in fresh TSB with 5% D-glucose. Totally 200 µL bacteria suspension and MIC and MIC/2 of eugenol and pulegone were incubated in sterile microplate at 37ºC for 48 h. After incubation, the wells were washed with distilled water twice to remove the planktonic bacteria. The remaining bacteria was subsequently stained with 0.1% crystal violet solution for 10 minutes. Wells were washed again to remove the excess crystal violet solution. After air-drying, 200 µl of ethanol were added to each well and incubated at room temperature for 10 m. 125 µl solution from each well transferred to another sterile tube and the final volume was adjusted to 1 mL with distilled water. Optical density of the solutions was measured at 550 nm (Multiskan GO UV/Vis Microplate Spectrophotometer, Thermo-Fisher Scientific, USA). The effect of eugenol and pulegone on biofilm formation of *S. maltophilia* was calculated with the following equation:

$$\% \text{ Antibiofilm Effect} = [(\text{Control}_{OD} - \text{Sample}_{OD}) / \text{Control}_{OD}] * 100$$

where control is the cell suspensions of bacteria containing 10% DMSO without eugenol/pulegone. The experiments were performed in triplicate.

Scanning Electron Microscopy (SEM)

To monitorize the antibiofilm activity, the highest biofilm inhibiton was also observed by SEM using glass coverslips and compared with the control group (8). Sterile circle glass coverslips (20×20 mm) were placed in biofilm assay tubes which were prepared as mentioned above. Control group was incubated without eugenol or pulegone addition. After incubation period at 37 ºC for 48 h, the coverslips were gently rinsed with PBS (pH 7.4) and fixed with 2.5% glutaraldehyde at 4 ºC for 2 h. Following the glutaraldehyde fixation, the coverslips were washed again with PBS for 1 h and dehydrated by increasing concentrations of ethanol. Specimens were air-dried and coated by gold (Emmitech K550, UK) before examining with a SEM (JEOL, JSM-7600F; JEOL Ltd., Tokyo, Japan).

Results

Zone of inhibition measurements were found to be 39 mm and 28 mm for eugenol and pulegone, respectively. MIC and MBC values were found to be 3.12 mg/mL (both are the same) for eugenol while these were 2.5 mg/mL and 5 mg/mL for pulegone, respectively.

The MIC value of eugenol obtained in this study was found to be same as the MBC value, which suggests that eugenol is bacteriostatic and bactericidal at 3.12 mg/mL concentration. On the other hand, pulegone was found to be bacteriostatic at lower concentration (2.5 mg/mL) than its bactericidal concentration (5 mg/mL) (Table 1).

Table 1. MIC and MBC values of eugenol and pulegone against *S. maltophilia*

	Eugenol (mg/mL)	**Pulegone** (mg/mL)
MIC	3.12	2.50
MBC	3.12	5.00

*mg/mL: miligram/mililiters

Within the results of this study, the highest antibiofilm activity was found to be 56.30±0.45% for MIC of eugenol (Figure 1). Inhibition effect of pulegone was slightly lower than eugenol (39.31±0.79%). Antibiofilm activity of subMIC concentrations were similar for

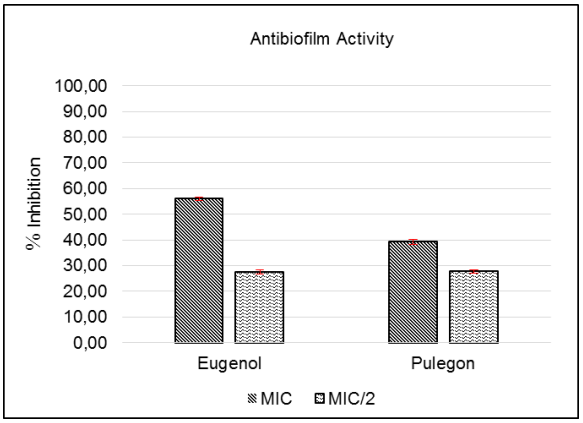

Figure 1. Antibiofilm activity of eugenol and pulegone against *S. maltophilia*. Error bars represent the standard error of the means from three biological replicates

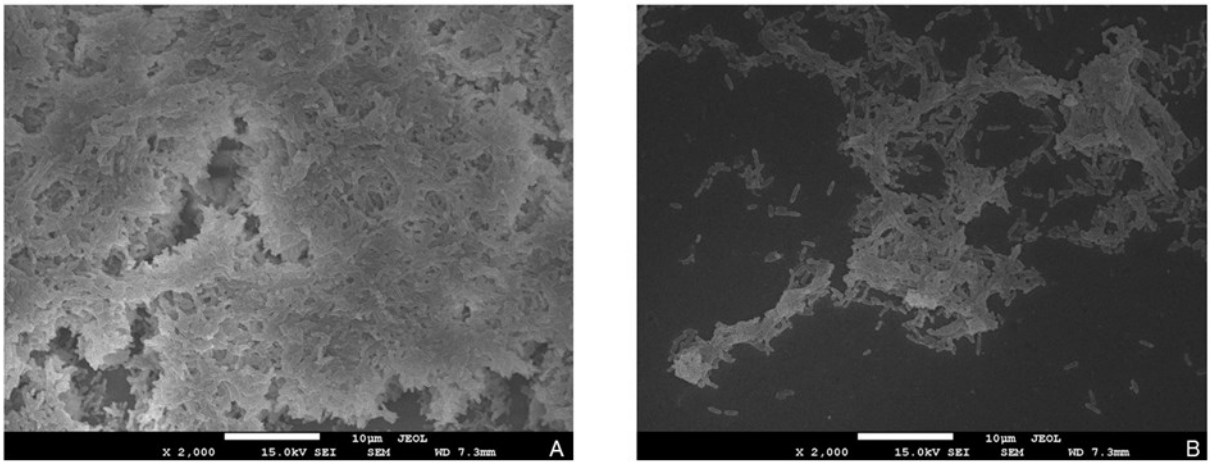

Figure 2. SEM images of *S. maltophilia* biofilm inhibition of eugenol at MIC concentration All images are taken at x2000 magnification. A: Control, B: Eugenol treatment

both eugenol and pulegone.

As the highest biofilm inhibition rate was observed for MIC of eugenol (56.30±0.45%) *S. maltophilia* biofilm layer incubated with MIC of eugenol was also observed by SEM (Figure 2). The biofilm formation on the control group glass coverslips which did not contain eugenol (Figure 2A) were higly intense and it was clear that bacteria cells colonized and accumulated into the biofilm matrix. For the eugenol-incorporated group, the microbial adhesion onto the glass coverslips were found to be decreased (Figure 2B). These findings are the observable proof of the antimicrobial and antibiofilm activity of eugenol against *S. maltophilia*. As the biofilm inhibition rate was found to be lower, *S. maltophilia* biofilm formation with pulegone incorporation was not monitorized using SEM.

Discussion and Conclusion

Within the results of this study, eugenol found to be more effective against *S. maltophilia* than pulegone. Similar studies revealed out that eugenol exhibits an excellent bactericidal activity against a wide range of organisms like *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* (44), *Listeria monocytogenes* (20), and *Salmonella typhi* (16). Recent reports about te antimicrobial susceptibility of *S. maltophilia* strains revelaed that trimethoprim-sulfamethoxazole (TMP-SXT), chloramphenicol, minocycline and levofloxacin are active antibiotics for the treatment of the infections caused by *S. maltophilia* (10,14). According to the literature data, there has been limited study on the antibacterial activity of eugenol and pulegone against a MDRO like *S. maltophilia*, so, the present study is an important data. MIC is the lowest concentration of the drug that allows no visible growth and MBC is defined as the lowest concentration of the drug that kills nearly 99.9% of the original inoculum (32). The MIC and MBC experiments revealed the least concentrations at which eugenol and pulegone act as bacteriostatic and bactericidal agent against *S. maltophilia*, respectively. *S. maltophilia* isolates often display high-level multidrug resistance (41). Recent evidence indicates that antibiotic efflux may be a contributing factor to the intrinsic and acquired multidrug resistance of *S. maltophilia* (3). It is supposed that an effective antibiotic therapy of *S. maltophilia* infections may require the targeting of efflux mechanisms, in order to render the organism more susceptible to available antimicrobial agent (46). Bacterial biofilms are formed when planktonic organisms come together to form a community which attaches to a solid surface and encased in an exopolysaccharide matrix (32). There are a few studies about the antibiofilm activity of eugenol and pulegone for evaluating their clinical usage. Al-Shabib et al. (4) reported eugenol as a broad-spectrum anti-quorum sensing and antibiofilm agent against toxin producing biofilm forming methicillin-resistant *Staphylococcus aureus* (MRSA). In a study of Upadhyay *et al.* (40), it is suggested that eugenol could potentially be used to control *Listeria monocytogenes* biofilms in food processing environments. Biofilm formation by *S. maltophilia* isolates from device-associated nosocomial infections has been investigated by researchers. De Oliveira-Garcia *et al.* (15) suggested that flagella and fimbriae 1 (SMF-1) produced by *S. maltophilia* can be involved in biofilm formation of the bacteria. Recently, natural plant-derived extracts are investigated for their activity on eradication of the biofilm formation of microorganisms. As far as we know, this is the first study about the antibiofilm activity of eugenol and/or pulegone against *S. maltophilia* strain. According to Brooke (9), there is ongoing debate about the use of monotherapy versus combination therapy to treat infections of *S. maltophilia*, so new treatment strategies have included the use of select antibiotics in synergy. Euge-

nol or pulegone may be suggested as an alternative synergistic compunds to enhance the mechanism of antibiotics.

In conclusion, antibiotics play an important role for medical treatment. However, there is growing crisis due to the antimicrobial resistance of microorganisms against the antibiotic drugs used in the pharmaceutical applications. Researchers now focus on the investigations of naturally occurring molecules with antimicrobial and antibiofilm activity. This study results figured out that both eugenol and pulegone are highly active against *S. maltophilia* strain which has been known as multidrug-resistant bacteria. In respect to the higher inhibition zone and antibiofilm activity results, eugenol may be a clinically important compound to be tested with further analysis such as toxicity, pharmacokinetics, pharmacodynamics and drug interactions.

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