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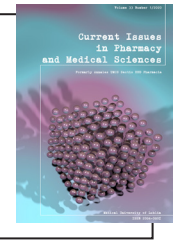

Effect of *Achillea santolina* essential oil on bacterial biofilm and its mode of action

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ABSTRACT

Increased multidrug resistance prompted researchers to search for a new drug that has the ability to overcome antibiotic resistant pathogens. Essential oils have been used in folk medicine for centuries, therefore, they could be employed as an effective alternative to antibiotics without having secondary side effects.

The aim of the present study was to test the antibacterial and antibiofilm activity of the essential oil of *Achillea santolina* and to ascertain its mode of action.

Minimum Biofilm Inhibitory Concentration (MBIC) susceptibility assays were performed using a biofilm inoculator with a 96-well plate with peg led. Minimum Inhibitory Concentration (MIC) was performed in normal microtitre plates using a twofold dilution series.

Achillea santolina essential oil (ASEO) was able to overcome the resistance of all tested bacteria. The MIC values were in the range of 250-1000 μg/ml, while the MBC values were in the range of 500-2000 μg/ml. ASEO increased leakage of potassium ions from the cell membrane and increased release of cellular materials – suggesting that the cell membrane is the target and site of action of ASEO. Moreover, ASEO was able to inhibit initial adherence of methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300) at sub-inhibitory concentrations through alterations to cell membrane.

INTRODUCTION

The emergence of resistant bacteria has become a major health problem worldwide. Antibiotics misuse and overuse are believed to be behind antibiotic resistance. The existence of multidrug-resistant (MDR) bacteria or "superbugs" is a growing threat worldwide. About 70,000 people die each year worldwide from infections caused by antibiotic-resistant pathogens [1].

Methicillin-resistant Staphylococcus aureus (*MRSA*) kills more USA citizens each year than AIDS, Parkinson's disease, emphysema and homicide combined. The global spread of drug resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, *Acinetobacter* and Enterococcus species poses the biggest threat [2].

The organization of bacteria into biofilms increases their ability to resist antibiotics. Bacterial biofilm is a group of microbial cells attached to a surface and enclosed in matrix of extracellular polymeric biomolecules [3]. The matrix which occupies 80-85% of the biofilm's volume is composed of polysaccharides, proteins, lipids and DNA. The matrix provides structural stability and protection to the biofilm. Over 90% of bacteria form biofilm and about 65% of microbial infections are associated with biofilms [4,5]. Biofilms can be formed by single or multiple microbial species. Biofilm can occur on different surfaces, including teeth, heart valves, lungs of cystic fibrosis patients, middle ear, intravenous catheters and stents [6]. Bacterial biofilms show extreme tolerance to antimicrobial agents (as these cause chronic infections) and to hostile environmental conditions like starvation. Microbes in biofilms can be a thousand times more tolerant to antimicrobial agents than planktonic ones [7,8]. Additionally, microbes in biofilms are protected against the host immune system through impaired activation of phagocytes and the complement system [9]. Infections caused by biofilms, including

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endocarditis, cystic fibrosis, and indwelling device mediated infections, are often untreatable and develop into a chronic state [5].

Biofilm resistance can be attributed to many reasons, including restricted antibiotic penetration, higher rate of mutation, decreased growth rates, nutrient and oxygen availability to the bacterial cells and increase in expression of efflux pumps [4,10]. Bacterial biofilm formation begins with reversible attachment to a surface. This is followed by irreversible binding to the surface and the formation of microcolonies and production of polymer matrix around the microcolonies [11]. The biofilm initial attachment step depends on interactions between microbial cell surface and attachment surface, including electrostatic and hydrophobic interactions, steric hindrance and van der Waals forces. It is believed that hydrophobic interaction is the main player in the initial attachment step [12,13].

Essential oils are volatile, natural compounds that are formed by aromatic plants as secondary metabolites. Essential oils are known for their antibacterial, antiviral, antifungal, antioxidant and antiseptic effects, beside sedative, pain relief, aromatherapy and anti-inflammatory attributes [14]. Essential oils are used in fragrance and make-up products, in sanitary products, as food preservers, flavors and additives and as natural medicine [15,16]. Naturally, essential oil protects plants from different pathogens. *Achillea santolina* is a medicinal plant with aromatic smell. In traditional medicine it is used for antidiabetic, antidiuretic, antiinflammatory, hypertension and antimicrobial effects. It is also used against gastrointestinal disorders and to relieve colic and symptoms of common cold.

The increase in microbial resistance to antibiotics, besides the known issues of drug side effects, increases the need for new drugs. The microbial resistance to known antibiotics increases the need for new drugs that are different from that currently in use [17]. Essential oils have been reported to exhibit antimicrobial activity. They are cheap, easily available, do not exhibit side effects and are able to overcome the resistance of many pathogen due to their multiple mechanisms of action [18].

The aim of the present study was to test antibacterial and antibiofilm activity of essential oil of *Achillea santolina*, and to ascertain its mode of action.

MATERIALS AND METHODS

Essential oil of *Achillea santolina*

The chemical *composition* of the *Achillea santolina* was previously published by our research group [19]. In short, fresh aerial parts of *Achillea santolina* was collected, from Mutah Alkarak, south Jordan, then finely chopped and subjected to hydrodistillation using a Clevenger-type apparatus. Chemical analysis of the essential oils was carried out by applying gas chromatography–mass spectrometry (Agilent (Palo Alto, USA) 6890N gas chromatograph). Identification of the oil components was based on computer search using the library of mass spectral data and comparison of calculated Kovats retention index (KRI) with those of available authentic standards and literature data.

Forty-six components accounting for 100% of the oil were identified. The major identified compounds were trans-Sabinene hydrate acetate – 30.09 %, Iso-Ascaridole – 16%, α-Terpinene – 14.31%, p-Cymene – 7.1%, cis-Carvone oxide – 6.08 %, Terpinen-4-ol – 2.75%, cis-Pulegol – 2.58%, cis-Rose oxide – 2.31%,1-Terpineol – 1.93%, Z-β-Ocimene – 1.9%, trans-Verbenol –1.88%, and trans-Piperitol – 1.52%.

The major oil components were oxygenated monoterpene – 70.22%, monoterpene hydrocarbon –26.95%, and sesquiterpene hydrocarbon – 1.04%.

Maintenance and Preparation of Cultures

The effect of *Achillea santolina* essential oil (ASEO) on bacterial biofilm was examined using six bacterial strains: *Staphylococcus epidermidis* (ATCC 35984), Methicillin-susceptible *Staphylococcus aureus* (MSSA) (ATCC 25923), *Methicillin-resistant Staphylococcus aureus* (*MRSA*) (ATCC 43300), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), and the non-biofilm forming strain *Staphylococcus epidermidis* (ATCC12228). Cultures were stored on tryptone soya agar (TSA) (Oxoid, Hampshire, UK) at 2-4°C and subcultured every 3-4 weeks or whenever required.

Minimum Inhibitory Concentration (MIC)

MIC was determined using 96 well broth microdilutions described by Rachid *et al*. [20]. ASEO stock solutions were prepared by dissolving the essential oils in dimethyl sulfoxide (DMSO) (Carlo Erba, France). The stock solutions of ASEO in DMSO were diluted in TSB to give EO concentrations of 16000 µg/ml. Two fold serial dilutions of ASEO in tryptone soya broth (TSB) (Oxoid, Hampshire, UK), were then carried out in microtitre plates to give essential oil concentrations of 8000, 4000, 2000, 1000, 500, 250, and 125 µg/ml.

Bacterial cells were grown overnight to mid-log phase by inoculating TSB (100 mL) and incubating at 37°C until the OD at 600 nm (OD_{600}) reached approximately 0.6, then diluting to 1×10^6 cfu/m and seeding (100 µL) to the wells containing ASEO, mixing and incubating at 37°C for 24 h aerobically. The MIC was taken as minimal concentration of ASEO that inhibited visible growth of the strain. Determination of MIC was carried out in triplicate using three independent experiments.

Minimum bactericidal concentration (MBC)

To determine the MBC values, a volume of 30 µL from each well that did not show an apparent growth as confirmed by MIC determination, was taken and plated on TSA agar. The plates were subsequently incubated at 37°C for 48 h. The MBC was defined as the lowest essential oil concentration able to reduce and kill more than 99.9% of the initial inoculum [21].

Minimum Biofilm Inhibitory Concentration (MBIC) Assay

Biofilm susceptibility assays were performed using MBIC (Minimum Biofilm Inhibitory Concentration) (Innovotech, Inc., Edmonton, AB, Canada), a biofilm inoculator with

a 96-well plate (Figure 1), according to the method reported by Ceri *et al*. [22]. Bacterial strains were cultured overnight in tryptone soya broth (TSB) (Oxoid, Hampshire, UK) and then diluted to give a final concentration of 1×10^6 cfu/mL. After this, 150 µL of inoculums was added to each well of 96-well MBEC biofilm inoculators and the peg lid was then fitted onto the plates. After 24 h incubation at 37°C, biofilms were formed on the pegs. Peg lids were then rinsed three times in phosphate-buffered saline (PBS) (Sigma Aldrich) to remove non-adherent cells, and then the peg lid was transferred to a new 96-well plate containing serially diluted essential oil. The microtiter plate was then incubated at 37°C for 24 h. Following incubation with essential oil, the pegs were rinsed three times with PBS and placed in a fresh 96-well plate containing 100 mL of TSB (recovery plate). The bacteria were removed from the pegs by sonicating the plates for 5 min on high speed with a Decon F51 006 sonicator. The peg lids were then discarded and replaced with standard lids. The OD650 was measured before and after incubation at 37°C for 6 h. Biofilm susceptibility assays were carried out in three independent experiments in triplicate for each strain. OD650 value of 0.05 was regarded as absence of biofilm. The minimum biofilm inhibitory concentration (MBIC) value was read as the concentration of essential oil that inhibited visible growth of bacteria confirmed by no increase in optical density compared with the initial reading. A shift in susceptibility of more than two doubling dilutions in either direction was considered to be a significant change.

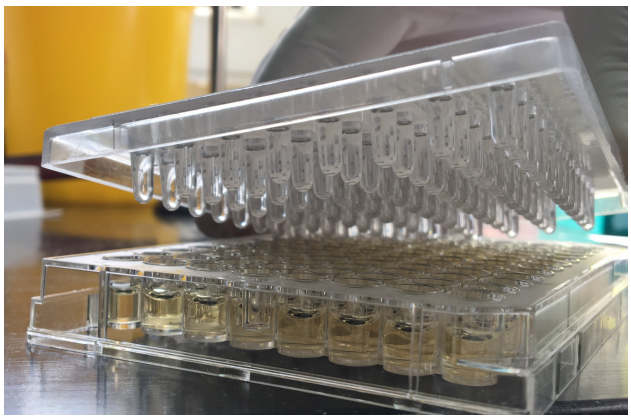

Figure 1. Biofilm inoculator with a 96-well plate with peg lids (Innovotech, Inc., Edmonton, AB, Canada)

Minimum Biofilm Eradication Concentration (MBEC)

The MBEC values was determined by taking a 30 µL volume from each well that did not show an apparent growth as confirmed by MBIC determination, then plating this on TSA agar. The plates were incubated at 37°C for 48 h. The MBEC was defined as the lowest essential oil concentration at which no bacterial growth occurred on the TSA plates.

Leakage of Potassium Ion

The leakage of potassium ion (K^+) into the bacterial suspension was measured using a Kalium/Potassium kit (Quantofix, Macherey-Nagel GmbH & Co. KG, Duren, Germany). *S. epidermidis* (ATCC 35984), *MRSA* (ATCC 43300), *P. aeruginosa* (ATCC 27853), and *E. coli* (ATCC 25922) were exposed to essential oils at MIC value in sterile peptone water (0.1 g/100 mL). The extracellular potassium concentration was measured at 0, 30, 60, 90, 120, and 240 minutes. A culture flask without AEO was used as a control. Results were reported as the amount of free potassium ion (mg/L) in the bacterial suspension at each time interval.

Integrity of the Cell Membrane (Release of cellular material)

The function of the cell membrane is to hold different components of the cell together and protect it from the extracellular environment. Thus, the release of cellular materials, especially DNA, RNA and proteins outside the cell indicates damage within the cell membrane. Essential oils at the MIC concentration were added to 2 mL of *S. epidermidis* (ATCC12228), MSSA (ATCC 25923), *E. coli* (ATCC 25922) (10^7 cfu/mL) in sterilized peptone water (0.1 g/100 mL) and then incubated at 37°C. After 0, 30, 60, 90, 120, 180 and 240 minutes of treatment, cells were collected then centrifuged at 3000 rpm. UV absorbance at 260 nm of the supernatant was measured using a spectrophotometer. A tube without bacteria in sterilized peptone water was used as control [23].

Adherence of Bacterial Cells to Polystyrene

Initial adherence of *MRSA* (ATCC 43300) to polystyrene was determined using a previously reported method [24]. Briefly, bacteria were grown overnight in 10 mL TSB at 37°C and then diluted 1:100 in fresh TSB containing ASEO at the required concentration. A quantity of 5 mL of the bacterial suspensions was then poured into Petri dishes and incubated for 30 min at 37°C. The plates were washed five times using 5 mL PBS, air dried and stained for 1 min with 0.4% crystal violet. The number of the adhered cells was determined microscopically (CETI 60243T UK) by counting the number of bacteria in 20 fields of view. The essential oil concentrations tested were 1/10 of MIC, 1/2 MIC, and the MIC concentration. Adherence was calculated as the total number of cells adhered per square centimetre examined. Each ASEO concentration was assayed in triplicate and the adherence of ASEO treated cells compared with untreated controls. Assays were performed three times on different days and the same result was obtained for each occasion.

Statistical analysis

All experiments were done in triplicate. The obtained results are expressed as mean values with the standard error. The statistical analyses were performed using Student's t-test to compare the controls and treated samples at a significance level of 5%.

RESULTS

MIC and MBC results

Table 1 shows the MIC and MBC results of ASEO. The MIC values were in the range of 250-1000 µg/ml. Regarding MIC, the most susceptible isolates were *S. epidermidis* (ATCC12228) and *S. epidermidis* (ATCC 35984) with MIC value of 250 µg/ml, while the most resistant strain were *MRSA* (ATCC 43300) with MIC value of 1000 µg/ml. The MBC values were in the range of 500-2000 µg/ml.

The most susceptible isolate in MIC and MBC was *S. epidermidis* (ATCC12228) with MBC of 500 µg/ml, while the most resistant MBC was *MRSA* (ATCC 43300) with MBC value of 2000 µg/ml. MBC values are higher than MIC values for all tested strains.

Table 1. MIC and MBC of *Achillea santolina* (µg/ml) for the bacterial isolates

Isolate Number	Isolate name	MIC µg/ml	MBC µg/ml
1	*S. epidermidis* (ATCC 35984)	250	1000
2	MSSA (ATCC 25923)	500	1000
3	MRSA (ATCC 43300)	500	2000
4	*P. aeruginosa* (ATCC 27853)	1000	1000
5	*E. coli* (ATCC 25922)	500	1000
6	*S. epidermidis* (ATCC 12228)	250	500

MBIC and MBEC for the bacterial isolates

The results of MBIC and MBEC of *Achillea santolina* are shown in Table 2. The MBIC test revealed that the values of this test were in the range of 500-2000 µg/ml. The most susceptible isolate was *S. epidermidis* (ATCC 12228), with MBIC value of 500 µg/ml, while the most resistant strains were *MRSA* (ATCC 43300) and *P. aeruginosa* (ATCC 27853) with MBIC values of 2000 µg/ml.

The MBEC test results show that values for this test were in the range of 500-4000 µg/ml. The most susceptible isolate was *S. epidermidis* (ATCC12228) with MBEC of 500 µg/ml while the most resistant were *MRSA* and *P. aeruginosa* (ATCC 27853) with MBEC of 4000 µg/ml. The MBEC values are higher compared to MBIC values for all tested strains.

Table 2. MBIC and MBEC of *Achillea santolina* (µg/ml) for the bacterial isolates

Isolate Number	Isolate name	MBIC µg/ml	MBEC µg/ml
1	*S. epidermidis* (ATCC 35984)	1000	2000
2	*MSSA* (ATCC 25923)	1000	2000
3	*MRSA* (ATCC 43300)	2000	4000
4	*P. aeruginosa* (ATCC 27853)	2000	4000
5	*E. coli* (ATCC 25922)	1000	2000
6	*S. epidermidis* (ATCC12228)	500	500

Leakage of Potassium Ion

Four bacterial strains were used to measure cell membrane permeability based on leakage of potassium ions: *S. epidermidis* (ATCC 35984), *MRSA* (ATCC 43300), *P. aeruginosa* (ATCC 27853), and *E. coli* (ATCC 25922). When bacterial strains were treated with ASEO at the MIC concentration, a sharp increase in potassium ions leakage was observed as shown in Figure 2. All tested bacterial strains treated with ASEO showed increased leakage of K^+ ions with time, and there is a sharp increase in leakage of potassium ions with increase in incubation time as shown in Figure 2. Herein, the extracellular potassium ions concentrations increased from 50 µM to about 300 µM in all tested bacterial strains.

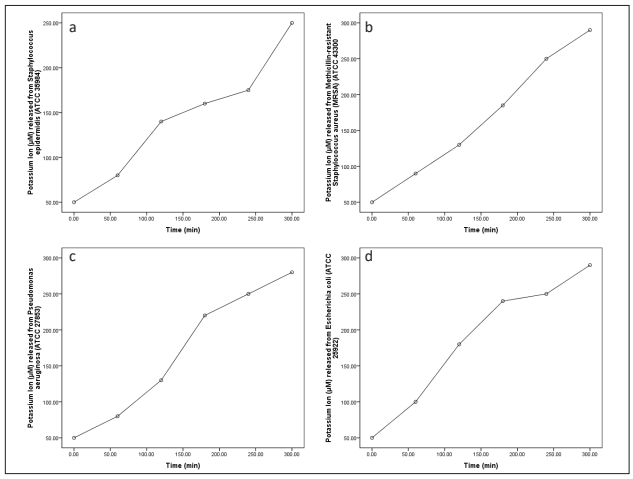

Figure 2. Effect of ASEO on potassium release of: a. *S. epidermidis* (ATCC 35984), b. *MRSA* (ATCC 43300), c. *P. aeruginosa* (ATCC 27853), d. *E. coli* (ATCC 25922)

Integrity of the Cell Membrane (Release of cellular material)

Cellular materials release, especially of DNA and RNA, to outside the cell is another test that indicates damage to the cell membrane. When *S. epidermidis* (ATCC12228), MSSA (ATCC 25923), and *E. coli* (ATCC 25922) were treated at MIC concentration of ASEO, there was a continual increase in 260 nm absorption over the incubation time (Figure 3). There was also elevated release of cell materials according to time of exposure in all tested bacterial strains.

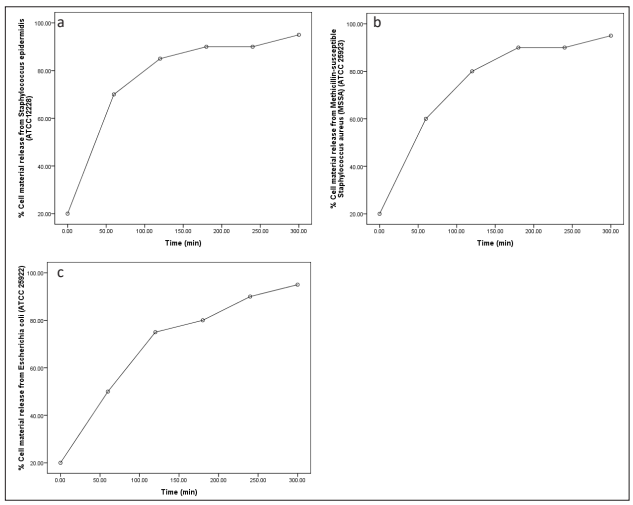

Figure 3. Effect of ASEO on cell material release of: a. *S. epidermidis* (ATCC12228), b. MSSA (ATCC 25923), c. *E. coli* (ATCC 25922)

5. Adherence of Bacterial Cells to Polystyrene

Optical density 600 (OD600) was used to measure the planktonic growth, while OD490 was used to measure biofilm growth. The essential oil concentrations tested were 1/10 of MIC, 1/2 MIC, and the MIC concentration. Adding ASEO to polystyrene Petri dishes containing a suspension culture of the *MRSA* (ATCC 43300) strain reduced the number of individual cells adhering to the polystyrene surface after 30 minutes incubation period (Figure 4). The number of adhered bacteria also decreased with increase in the concentrations of ASEO.

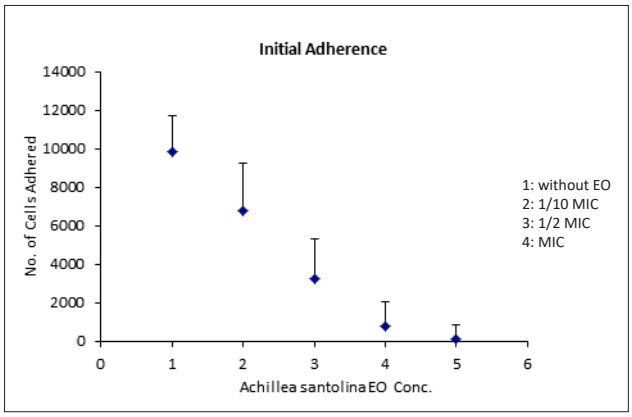

Figure 4. Effect of ASEO on initial adhesion of *MRSA* (ATCC 43300); 1: without EO, 2: 1/10´MIC, 3: 1/2´MIC, 4: MIC

DISCUSSION

Essential oils *are* complex volatile compounds that have been used in traditional medicine for treatment of many different diseases. These oils are known to have antibacterial, antifungal and antiviral activity beside their antioxidant effects.

In the present study, the results of MIC determination showed that essential oil of *Achillea santolina* exhibits potent activities against all tested bacteria, with MIC values ranging from 250 to 1000 µg/ml and MBIC values between 500-2000 µg/ml. The results clearly indicate that essential oil of *Achillea santolina* was able to overcome the resistance shown by all tested microorganisms and their biofilm – especially *MRSA* (ATCC 43300) and *P. aeruginosa* (ATCC 27853). One of the best chemical properties of essential oil is hydrophobicity. Essential oils are hydrophobic which allows them to penetrate into microbial cells – causing disturbance to their structure that result in increased cellular leakage and death.

MRSA poses a significant problem due to its resistance to most known antibiotics. Recently, its resistance to vancomycin has become noted with alarm, as this antibiotic is relied upon for treating MRSA infections [25]. MRSA is a leading cause of endocarditis, bacteraemia, osteomyelitis and skin and soft tissue infections. Infections induced by MRSA causes increased morbidity and mortality, besides longer hospitalizations. The formation of biofilms by MRSA increases its ability to resist antibiotics. MRSA biofilm infections include hospital-acquired infections, artificial heart valves, urinary tract infections and catheter infections [26].

The most distinguishing challenge with *P. aeruginosa* is its ability to rapidly develop resistance during the course of treating an infection. *P. aeruginosa* is responsible for 30% of all pneumonias, 19% of all urinary tract infections, and 10% of all bloodstream infections [27]. The basic bacterial mechanisms of resistance include acquired resistance genes, express efflux systems and modification of drug target site.

The antibacterial activity of ASEO is attributed to its components such as terpinen-4-ol, cis-sabinene hydrate, 1,8-cineole, α-cadinol, α-terpinol, camphor and p-cymene. Terpinen-4-ol has also been found to be one of the major ASEO constituents (10.6%), and Terpinen-4-ol was discovered to be active against clinical skin isolates of MRSA [28]. Terpinen-4-ol is an amphipathic molecule and its activity target is believed to be the cell membrane. Terpinen-4-ol, therefore, has enough hydrophilicity to diffuse through the surrounding water to reach the bacterial cytoplasmic membrane, and enough hydrophobicity to penetrate through the bacterial cell membrane [29].

Cis-sabinene hydrate is another major component of ASEO. This constitutes 10.64% of the total oil. Ramos *et al.* [30] concluded that cis-sabinene hydrate in the essential oil of *Origanum majorana* was the most active ingredient and was the one responsible for inhibition of tested bacterial growth. The terpene 1,8-cineole is a natural monoterpene (terpene oxide), also known as eucalyptol, and is non-toxic to tissue. Clinical trials have found strong evidence that 1,8-cineole has anti-inflammatory activity [31]. Moreover, 1,8-cineole inhibits the leukotrienes (LTB4) and PGE2 that are generated from the pathways of the arachidonic acid metabolism [32]. It is also often used in the treatment of bronchitis, sinusitis and rheumatism [33]. Constituting 4.69% of the total ASEO, α-cadinol has been reported to have antimicrobial and antitumor activities in a variety of cell lines [34,35].

The terpene α-Terpinol makes up 3.97% of the total extracted oil, and has been reported to have a strong antimicrobial activity. It is believed that α-Terpinol disrupts the cytoplasmic membrane, increases its permeability and depolarizes its potential [33].Camphor composes 2.01% of the total ASEO, and has been reported to have antimicrobial, antiviral and anticancer activities [36]. The terpene p-cymene shows a diverse range of activity, including antimicrobial, antioxidant, anti-inflammatory and anticancer effects. It has been reported that *p*-cymene targets several sites in the cell - notably the cell membrane and mitochondria. The mechanism of action of *p*-cymene involve alteration in the lipid of the plasma membrane that results in disrupting the cytoplasmic membrane permeability and fluidity [37].

The mechanism of action of ASEO depends on their chemical composition, and their antimicrobial activity is not attributable to a single mechanism, but is instead a series of reactions involving the entire bacterial cell. Studies have reported the synergistic effect between constituents of essential oils against different pathogens. The primary target for ASEO, as it can be concluded from its constituents, is the cell membrane – resulting in loss of integrity and increased permeability.

The results of this study showed increased leakage of potassium ions and increased leakage of 260 nm absorbing cellular materials. These results indicate that the cell membrane structure was damaged by essential oil application, as compared to the control group. Furthermore, the results indicate that the subinhibitory levels of ASEO markedly reduced the number of *MRSA* (ATCC 43300) cells adhered to polystyrene. It is believed that the subinhibitory level of ASEO caused damage and changes to the cell membrane of *MRSA* (ATCC 43300) and prevented it from adhering to the polystyrene surface.

The activity of essential oil may not be attributed to any particular major constituents [38,39]. Sometimes the minor components of essential oil can be important. The overall

activity of essential oil may be attributed to synergistic effects of several chemical constituents [40-42].

CONCLUSION

ASEO showed high antibacterial and antibiofilm activity and were able to inhibit initial adherence of tested bacteria at sub-inhibitory concentrations.

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