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Original Article

Effect of some plant oils on swarming motility and Biofilm formation in *Proteus, Aeromonas*, and *Pseudomonas*

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ABSTRACT

Uropathogens use many mechanisms for survival in stresses in the bladder such as starvation and immune responses. Through biofilms and undergoing morphological changes, they could persist and cause infections. Numerous uropathogens were encoded in variable ranges of virulence factors/antimicrobial resistance separation. Increased rates for UTIs suggested where antibiotics do not have a therapeutic effect. Therefore, using oils as new alternatives to antibiotics is recommended because of their diverse and nontoxic effect. In this study, antimicrobial and biofilm inhibition and anti-swarming potential of essential oils (cinnamon, clove, eucalyptus, garlic, pomegranate, and tea tree oils) were assessed against four gram-negative uropathogenic bacteria (*Proteus*, *Aeromonas*, and two *Pseudomonas* isolates) in *vitro*. Results showed that among the oils tested, cinnamon, eucalyptus, and tea tree oil demonstrated antibacterial activity, while garlic and pomegranate had little or no effect in inhibiting the bacteria studied. By using two antibiofilm screening assays, tube, and tissue culture plate methods, all oils presented potent antibiofilm activity at different concentrations against all bacteria used in the present work. Cinnamon and eucalyptus oils not only exhibited antibacterial and antibiofilm activity but also clearly showed an anti-swarming effect. Therefore, essential oils might be considered as one of the sources for new antimicrobial agents and could be used for synergy with synthetic antibiotics.

Keywords: Anibiofilm, Antimicrobial, Antiswarming, Plant oils

Introduction

Uropathogens use different means for survival as a reaction to stresses in the bladder. By forming biofilms and undergoing morphological changes, uropathogens can cause recurrent infections.[1] The high incidence of recurrent UTIs implies that antibiotics are not effective treatments for all UTIs. [2] Therefore, plant oils can be used as new alternatives.[3] Plants are possible sources of new antimicrobial drugs because of their diverse and nontoxic effects.

The significant bacterial capability to colonized new conditions is related to biofilm formation. The close attention paid to biofilms is because of the prevalence of this sort of microbial symbiosis both in the natural environment and in industrial systems.[4]

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Biofilms are one of the most common types of bacteria in most natural conditions. [5-7] Biofilms are defined as communities of microbes that grow up in a matrix adhered to inert material or live tissue. These communities may include single or different bacterial species and even different genera. Bacterial biofilms are formed in the tooth, epithelial, bone, blood vessel, and medical devices [8]. Biofilm behavior is related to high tolerance to external stress. Therefore, the treatment of biofilms with antibiotics or other biocides can be ineffective at eradicating them. In medical habitats, biofilms causing infections implicated in more than 80% of microbial cases, releasing toxins, and even harming catheters [9]. It is estimated that more than 65% of microbial infections are caused by microorganisms when they grow in biofilms.[10] Bacteria of clinical importance such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and others proliferate on medical instruments and form biofilms which give them up to 1000 times resistance to antibiotics compared with planktonic forms.[11] An example is demonstrated by the bacillus *P. aeruginosa* which can adapt and survive in harsh environments with minimal nutrition. *P. aeruginosa* is a multidrug-resistant pathogen associated with hospital-acquired infections.[12] Once rigidly established, the biofilm becomes difficult for removing due to the slime matrix produced, which leads to poor

susceptibility to conventional antimicrobial agents and host defense resulting in chronic infections.[13, 14]

Bacterial motility could be sensitive to colonize in the subsequent formation of biofilms. Losing flagella renders bacteria less motile, which may affect the colonization surface. The swarming phenomenon is also associated with invading human cells, expression of virulent genes, and an increase in resistance to antibiotics. This can explain why the inhibition of *P. mirabilis* swarming is of medical importance.

Medicinal plants' extracts have been used for ages due to their therapeutic significance and have become common in health care. The present study aimed to seek new antibacterial and antibiofilm agents that could inhibit biofilm production to counteract virulence rather than bacterial growth in some gram-negative bacteria.

Materials and Methods:

Bacterial Isolates: Four clinical motile gram-negative bacteria were obtained from **the** bacterial bank, Mosul University, Biology Department (two *Pseudomonas aeruginosa* isolates, *Proteus mirabilis*, and Aeromonas).

Essential Oils: The following plant essential oils were assayed in the current study; Cinnamon, Clove, Garlic, Eucalyptus, Tea tree, and Pomegranate oils.

Antibacterial activity assay: The antibacterial activity was assessed using the agar disc diffusion method.[15] An antibiotic sensitivity test was done on Mueller-Hinton agar plates which were inoculated with 0.1 ml broth of an overnight culture of each bacterial isolate and spread with a sterile glass rod spreader. Plates were left for 10 min, saturated with the essential oilsو added to the inoculated agar plate and tested organisms incubated at 37°C. After 24 hours of incubation, diameters of the inhibition zone were measured in millimeters (mm).

Determination of biofilm production:

Tube method: A loopful of colony suspension from an overnight culture was inoculated into 10 ml brain heart infusion broth and incubated for 24 h at 37°C. Then, tubes were inverted to discard the culture and washed by PBS (pH 7.3). Tubes were stained by 0.1% crystal violet, rinsed many times with running tap water to remove excess stains, and biofilm formation was scored as 0-absent, 1-weak, 2-moderate, 3-strong. [16, 17].

Congo red Method: The method was performed by Congo Red Agar (CRA) medium.[18] Biofilm production was indicated through the demonstration of black colonies with a dry crystalline consistency.

Antibiofilm activity of essential oils: Methods described in the literature to detect biofilm production as TCP and tube method (TM), were implemented to determine the antibiofilm activity of six essential oils against the gram-negative bacterial isolates.

Tissue culture plate (TCP): The modified Crystal Violet (CV) assay that is a semi-quantitative biofilm formation analysis was used.[19] The principle of the assay is that CV binds to extracellular molecules that are negatively charged, including polysaccharides in biofilms, and can be used to determine the density of the attached cells. Briefly, 180 µL of brain heart infusion broth and 20 µL of bacterial suspension of each bacterial isolate were added to the microtiter plate. Brain heart infusion was used as a negative control. Essential oils were added in three concentrations (0.5, 1, and 1.5 %) for each oil. Post incubation (37°C; 24 h), the medium was decanted from the microtiter plate by inverting and wells were washed with sterile distilled water. The dye was discarded and the plates were dried for 15 min. Strains were efficient in biofilm formation if absorbance at 630 nm was equal to or greater than 0.15.[20]

Tube method: The tube method (TM) was used for detecting biofilm production.[21] Different concentrations of each essential oil were prepared in polystyrene test tubes containing brain heart infusion broth, inoculated with 0.1 ml of a 24 hours old culture for 24 h at 37°C. Crystal violet-stained test tubes were rinsed twice with PBS to remove the stain. Post drying, the occurrence of visible film lined walls, and the bottom of the tube indicated biofilm production.

Antiswarming activity of Essential oils: Swarming motility activities was determined.[22] One µl of overnight grown cultures were spot inoculated at the center of swarming agar medium consisting of nutrient broth medium with 0.3% and 0.5% agar, respectively. The plates were incubated at 28±2 °C without disturbance for 24 to 48 hours.

Results:

Antibacterial activity: Agar disc-diffusion testing was used to evaluate the antibacterial efficacy of plant essential oils against four gram-negative bacterial isolates figure (1).

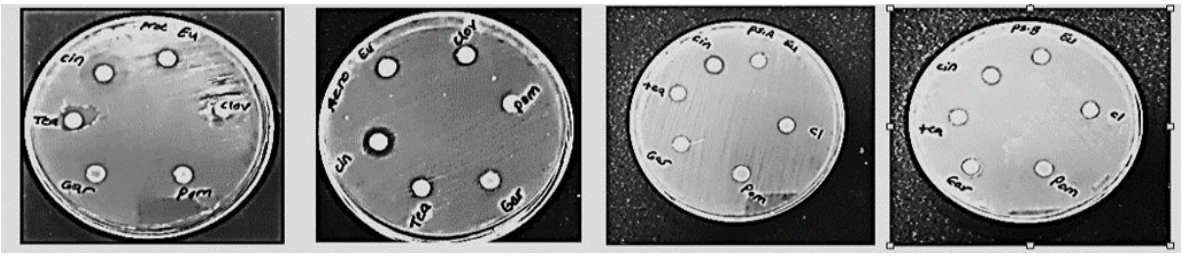


Figure 1: Antibacterial activity of essential plant oils on gram-negative bacteria.

Inhibition zone values measured in mm are listed in Table 1.

Table 1: Antibacterial activity of plant essential oils (inhibition zones measured in mm).

Bacterial isolate	Cinnamon Oil	Tea tree Oil	Garlic Oil	Pomegranate Oil	Clove Oil	Eucalyptus Oil
Aeromonas	14	13	7	Zero	8	15
Proteus	19	14	Zero	8	9	12
Pseudomonas A	10	9	Zero	Zero	7	14
Pseudomonas B	11	9	8	Zero	8	9

Table 2: Standard inhibition zones.

Zones of inhibition	Inferences
<10 mm	May be expressed as inactive
10-13 mm	Partially active
14-19 mm	Active
>19 mm	Very active

According to the standard zones of inhibition, the greatest antimicrobial activity *in vitro* was related to cinnamon oil (Table 2). It was active against *Proteus*, *Aeromonas*, *Pseudomonas* A, and *Pseudomonas* B. The essential oils of cinnamon have been reported to have antibacterial effects on *Salmonella typhi*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*.[23]

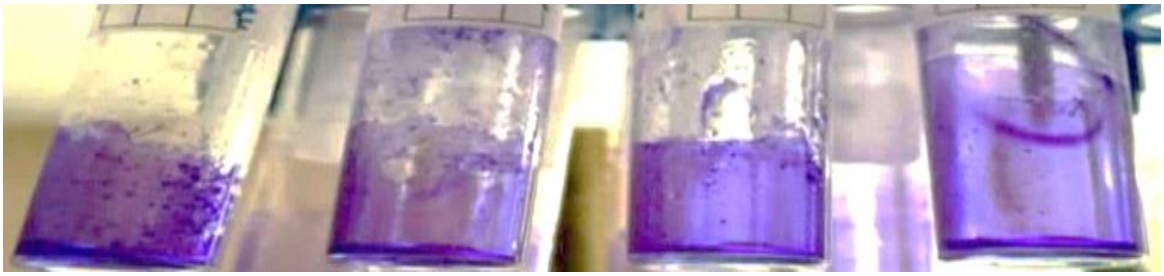
A study on *C. zeylanicum* stalks revealed the presence of 17 essential oil in the extract. The main compounds were monoterpene hydrocarbons, phenolic compounds, and cinnamaldehyde (80.42%).[24] A study by [25] revealed cinnamaldehyde antibacterial activity against gram-positive and gram-negative bacteria. In their study, scanning electron microscopy morphologically revealed the increase of protein and nucleic acids in the bacterial suspension. The cell membrane was damaged and according to the authors, cinnamaldehyde disrupted the bacterial cell membrane. A study by [26] also implied that exposure to cinnamon oil may exert changes in the cell membrane that differ from one bacterial species to another.

Eucalyptus oil also showed antibacterial activity against three out of four gram-negative bacteria used in the present work (*Aeromonas*, *Proteus*, and a *Pseudomonas* isolate). This agrees with recent studies that demonstrated the potential antimicrobial activity of *Eucalyptus globulus* essential oils against pathogenic bacterial species including *Pseudomonas aeruginosa*. [27, 28] Antibacterial activity for eucalyptus was explained by the presence of 1,8-cineol (or eucalyptol that is the main active component of essential oils of *Eucalyptus*).[29]

Tea tree oil had antibacterial activity against *Proteus* and *Aeromonas*. On the other hand, clove, pomegranate, and garlic oils did not inhibit the growth of all tested bacterial isolates.

Determination of biofilm production:

Tube method: Tube adherence assay is a simple and reliable biofilm screening method implemented for the detection of biofilm-producing organisms. A visible film lining the wall and the bottom of the tube demonstrates the biofilm-producing ability of the bacteria used in the present study as shown in Figure 2.



Pseudomonas A (+++) *Proteus* (++) *Aeromonas* (++) *Pseudomonas* B(+++)

Figure 2: Biofilm producing activity of gram-negative isolates.

The disadvantage of the tube test is the difficulty to distinguish between weak and non-biofilm production. However, the tube method is more qualitative and reliable as compared to Congo Red Assay (CRA).[30]

Congo red agar Method: Spot inoculation of the gram-negative bacterial isolates on Congo red agar revealed strong biofilm formation by the two *Pseudomonas* isolates (Figure 3), while *Aeromonas* and *Proteus* demonstrated weak biofilm formation unlike what was demonstrated by the tube method

which agrees with many workers in that this method is less sensitive than the tube and microtiter plate method.[31-33].

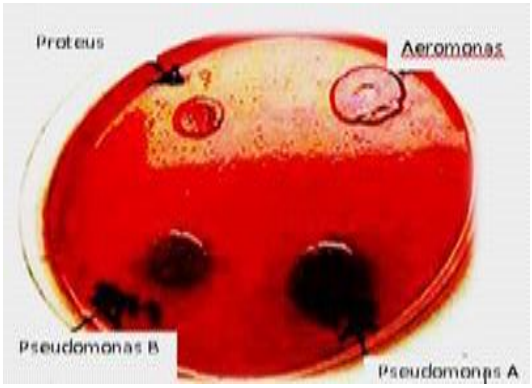


Figure 3: biofilm production on Congo red agar.

Antibiofilm assay methods:

Tube method: The tube method is considered one of the qualitative procedures for antibiofilm detection by observing biofilm lined on the bottom and walls of the tube and it was implemented in the present work. Tubes were photographed to record the amount of biofilm adhered to the glass surface. Eucalyptus oil showed a potent antibiofilm activity against all isolates assayed as there was a clear progression in biofilm formation as the concentration increased. In the case of *Aeromonas* and *Pseudomonas* A, biofilm was almost diminished at 3% concentration (Figure 4)

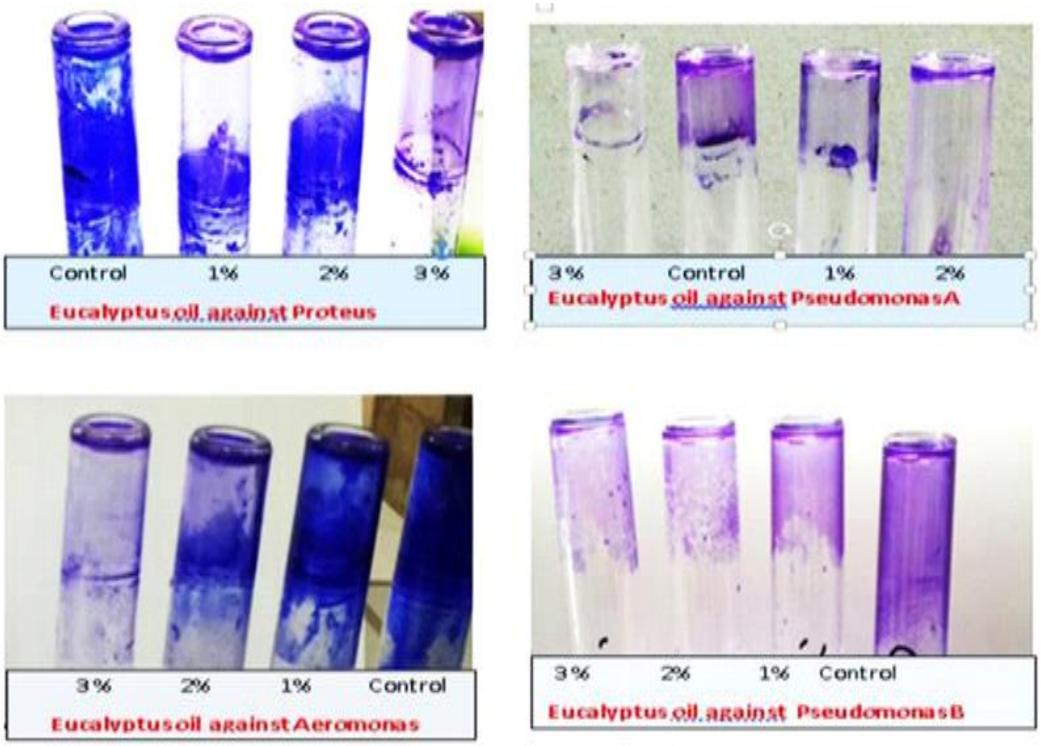


Figure 4: Antibiofilm activity of eucalyptus oil against gram-negative bacteria.

These results correlate with many recent studies [34, 35]. Although the latter concluded that essential oils from dried leaves of eucalyptus inhibit the formation of biofilms but had little or no activity on removing preformed biofilm to most bacteria tested. This could be related to high exopolysaccharide production, which protects metabolically active bacteria within the biofilm community, therefore, any antibiofilm agent used could only abolish the cells near the interface biofilm.[36] The medicinal value of *Eucalyptus* is related to Eucalyptol as *Eucalyptus* oil (EO).[37] Also, eucalyptus has an antimicrobial and antibiofilm effect.[38]

Cinnamon was also an effective antibiofilm agent against the gram-negative bacteria tested except *Proteus* where there was a slight decrease in biofilm progression (Figure 5).

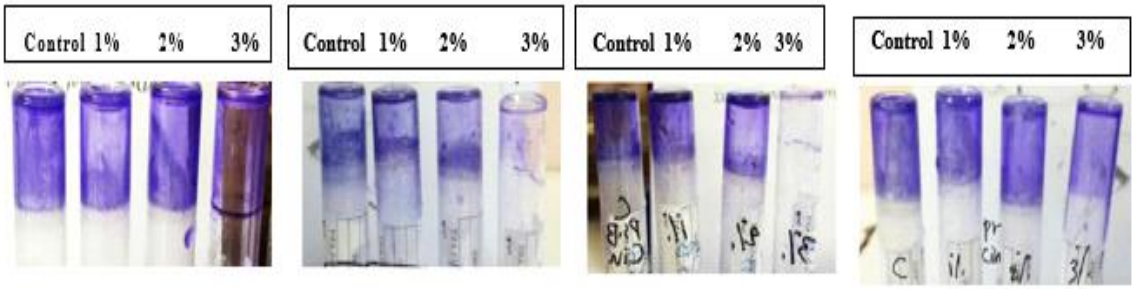


Figure 5: Antibiofilm activity of cinnamon oil against *Pseudomnas* B, *Pseudomonas* A, *Aeromonas,* and *Proteus* (from left to right).

It is assumed that the effect of cinnamon is due to phenolic compounds such as eugenol that can cause a disturbance in energy production. [39] Numerous factors influence the yield and composition of essential oils including the plant, seasonal variations, and maturity. [40] Essential oils and (E)-cinnamaldehyde inhibit Gram-positive and negative bacteria in planktonic form. Also, they inhibit the formation of biofilms, which are directly related to infections. Bioactivity is associated with the presence of high content of (E)-cinnamaldehyde.[41]

Tea tree oil also demonstrated a potent antibiofilm activity against all gram-negative bacteria in the present study especially at the concentration of 3% where there was complete biofilm eradication (Figure 6). Terpinen-4-ol is a major component of TTO and has been considered the main antimicrobial component of the oil.[42]

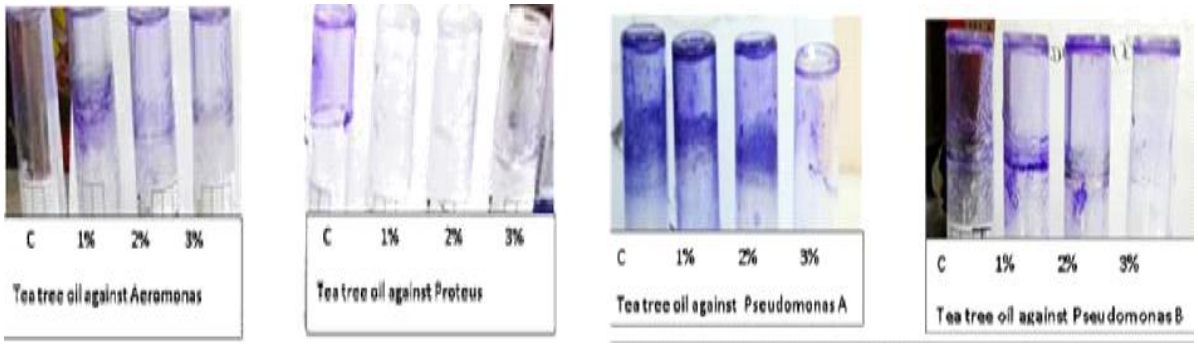


Figure 6: Antibiofilm activity of Tea Tree oil against gram-negative bacteria.

On the other hand, clove oil not only failed to inhibit the biofilm formation but interestingly stimulated biofilm formation by all gram-negative bacteria tested as shown in Figure 7.

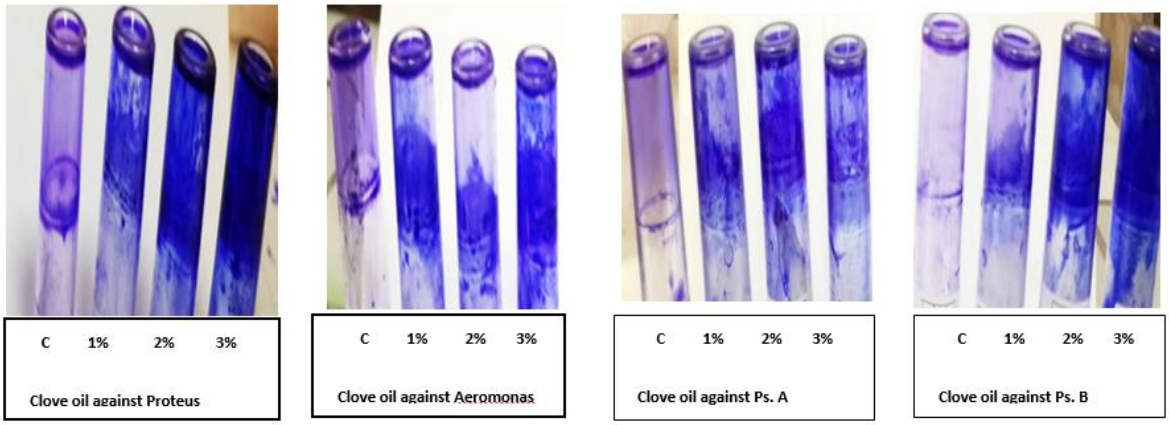


Figure 7: Antibiofilm activity of clove oil against gram-negative bacteria.

Our results are in agreement with the relationship between the stimulation of biofilm formation and the quorum-sensing system of the bacteria.[43] Other reported antimicrobial agents could stimulate biofilm formation by different bacteria as a self-protective reaction.[44, 45]

Tissue Culture Plate (TCP) Method: TCP method detected biofilm formation and antibiofilm activity of essential oils used based on crystal violet staining of polysaccharides or slime**.** The antimicrobial activity of the essential oils included in the present work is shown in Table 3.

Table 3: Antimicrobial activity of essential oils against gram-negative bacteria.

Bacteria	Eucalyptus oil			Tea tree oil			Clove oil			Cinnamon oil		
	0.5%	1%	1.5%	0.5%	1%	1.5%	0.5%	1%	1.5%	0.5%	1%	1.5%
Aeromonas	N	N	N	N	N	M	M	N	N	N	M	N
Proteus	N	N	N	N	N	N	M	N	N	M	N	N
Pseudomonas A	N	N	N	N	N	M	N	N	N	M	M	M
Pseudomonas B	M	N	N	M	M	M	M	M	M	N	M	S

*N= no biofilm; M= Moderate biofilm; S= Strong biofilm

In vitro quantification of biofilm formation was monitored by TCP assay using crystal violet dye and measured spectrophotometrically at a wavelength of 630 nm. Biofilm formation was determined by the formula:
BF = AB – CW where BF= Biofilm formation, AB is the OD at 630 nm of test bacteria, and CW is the OD of control.

OD value greater than 0.1 is considered positive for biofilm formation.[46] Results demonstrate the antibiofilm activity of all essential oils used in one concentration or another. *Aeromonas* and *Proteus* were more sensitive to the antibiofilm action, while *Pseudomonas* B had some degrees of resistance as demonstrated by the formation of moderate adhesion. Results obtained by this method correlated well with the TCP method for producing strains of biofilm. Our results agree with those of [47, 48]. TCP method is considered as a standard test for the detection of biofilm formation.

Antiswarming activity: The ability to swarm was assessed by the distance of swarming from the central inoculation site.[49] Cinnamon and eucalyptus not only demonstrated an antibacterial effect on gram-negative bacteria but also had a potential anti-swarming activity on *Pseudomonas*, *Aeromonas,* and *Proteus* isolates. As can be seen from Figures 8 and 9, the greatest inhibition of swarming motility was exerted by cinnamon and eucalyptus.

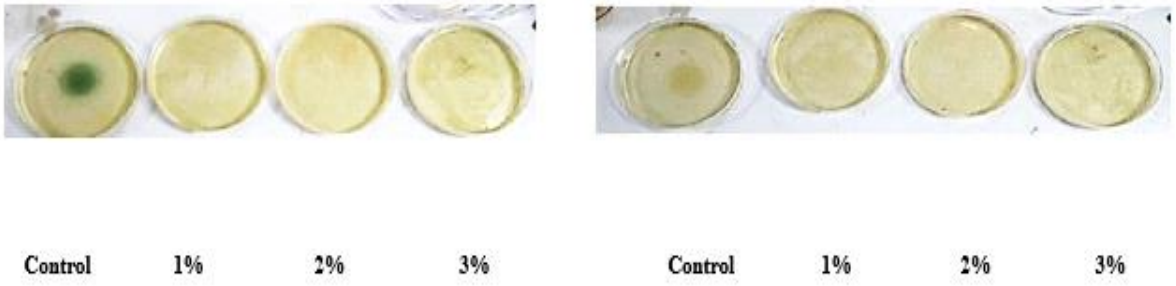


Figure 8: Anti-swarming activity of cinnamon on *Pseudomonas aeruginosa* A. (left) and *Aeromonas* (right).

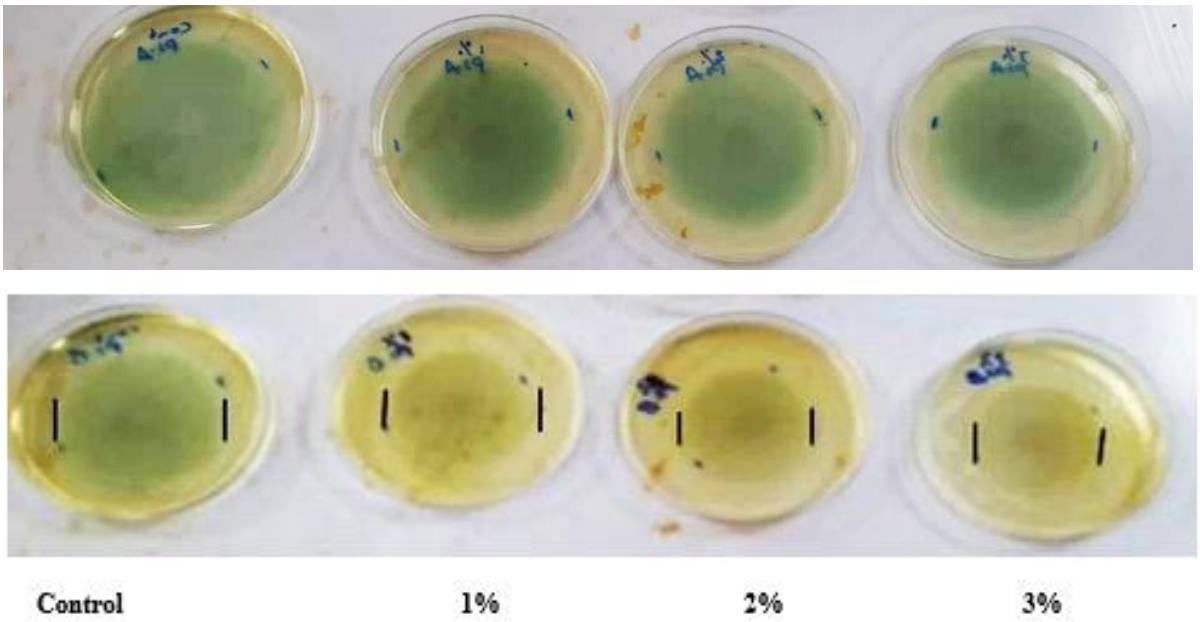


Figure 9: Anti-swarming activity of eucalyptus oil on *Pseudomonas* A (top) and *Pseudomonas* B (bottom).

Swarming motility is one of the major virulence factors that are known to play a role in early colonization, biofilm development, initiation of catheter-associated urinary tract infection, and the subsequent spread of a biofilm over a catheter surface. Therefore, the inhibition of swarming is of medical importance.[50]

Conclusion:

In conclusion, the results of the current study elucidated the antibacterial and antibiofilm activity of cinnamon, eucalyptus, and tea tree oil against gram-negative uropathogens. Clove oil was found to stimulate biofilm production. Also, cinnamon and

eucalyptus oils demonstrated antiswarming activity against gram-negative bacteria used in this study.

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