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Effects of Wild *Artemisia herba-alba* Essential Oil on Biofilm-Forming Bacteria

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Authors' contributions

This work was carried out in collaboration between all authors. Author JAS designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors SAS, YAS and EA helped in data collection and managed the analyses of the study. Authors MAQ and IAT performed the statistical analysis, literature searches and chemical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of the present study was to determine the effects of essential oil of wild *Artemisia herba-alba* grown in south Jordan on biofilm-forming bacteria.
Study Design: Seven bacterial clinical isolates were used in this study. Biofilm formation was first quantified in microtitre plates. The minimum inhibitory concentration (MIC) and biofilm inhibitory concentration (BIC) assays were performed in microtitre plates using a two fold dilution series. The most tolerant isolate were then used to test the effectiveness of *Artemisia herba-alba* essential oil on initial adherence to polystyrene surface.
Place and Duration of Study: Faculty of Medicine, Mutah University, Mutah, Jordan; Faculty of Science, Department of Chemistry Yarmouk University, Irbid, Jordan; and Faculty of Science, Department of Chemistry, Albalqa' Applied university, Albalqa, Al-Salt, Jordan. Between August 2012 and June 2014.
Methodology: Using a microtitre plate assay we measured inhibitory adherence effect for *Artemisia herba-alba* essential oil against seven biofilm-forming bacterial clinical isolates.
Results: *Artemisia herba-alba* essential oil produced inhibitory effects against all isolates and susceptibility varied considerably. The MIC values were found to be in the range of 0.5-4% v/v. In addition *Artemisia herba-alba* essential oil was able to inhibit initial adherence in the most tolerant isolate (*Pseudomonas aeruginosa*) at sub-inhibitory concentrations.
Conclusions: *Artemisia herba-alba* essential oil showed a significant activity against all isolates. It was able to inhibit initial adherence in the most tolerant isolate at sub-inhibitory concentrations.

Keywords: Antimicrobial efficacy; Artemisia herba-alba; essential oil; biofilm; resistant bacteria.

1. INTRODUCTION

Biofilm is a community of microbial cells attached to a surface and embedded in matrix of extracellular polymeric biomolecules [1]. The matrix contains polysaccharides, proteins and DNA. The matrix provides structural stability and protection to the biofilm. Nearly all microorganisms including over 90% of all bacteria can form biofilm. Biofilms can be formed by both single and multiple species. While biofilms formed by multiple species predominate in the environment, those formed by single species usually exist in infections and on medical implants [2]. Natural surfaces on which biofilm growth can occur include teeth, heart valves (endocarditis), lungs of cystic fibrosis patients (causing chronic bronchopneumonia), middle ear of patients with persistent otitis, prosthetic joints, intravenous catheters and stents [3]. Bacterial biofilm causes chronic infections characterized by persistent inflammation and tissue damage because the contained bacteria show increased tolerance to antibiotics and resist phagocytosis and other components of the body's defense system.

The first step in biofilm formation is reversible attachment to a surface when the bacteria are still susceptible to antibiotics. This is followed by irreversible binding to the surface and multiplication of the bacteria that results in the formation of microcolonies and production of polymer matrix around the microcolonies [4]. Finally the biofilm grows in thickness to produce mature biofilm that shows maximum antibiotic tolerance. The initial attachment depends upon the forces between microbial cell surface and attachment surface. These include electrostatic and hydrophobic interactions, steric hindrance and van der Waals

forces. Probably hydrophobic interaction plays the dominant role in primary adhesion [5] whereas molecular binding between specific adhesins and the surfaces is believed to be responsible for the irreversible attachment [6].

A hallmark of biofilms is their ability to tolerate antimicrobial agents. For example, bacteria living in biofilms can be up to 1,000 times more tolerant to antibacterial compounds than their planktonic counterparts [7,8]. While biofilm resistance against antimicrobial agents commences at the attachment phase, it increases greatly as the biofilm ages. It should be noted that biofilm antibiotic tolerance is not to be confused with antibiotic resistance because, although bacteria within a biofilm tend to survive antibiotic treatment, they usually become susceptible to the treatment when the biofilm is disrupted [3,9]. The increase in microbial resistance to antibiotics beside drugs side effects increases the need for new drugs. Essential oils (EO) have been used for centuries in traditional medicine for treatment against various diseases. EO's are composed of a mixture of secondary metabolites that often possess antimicrobial properties and thus can play an important role for the plant defense. De la Croix was the first to evaluate the antibacterial properties of essential oils using vapors in 1881 [10]. The genus *Artemisia* comprises more than 300 species. *Artemisia herba-alba* has been used in traditional medicine in Jordan for treatment against constipation, hypercholesterolemia, jaundice, abdominal pains, diabetes, parasitic worms, flatulence, inflammations, common cold, kidney sand and stones [11].

The aim of the present study was to determine the effects of essential oil of wild *A. herba-alba* grown in south Jordan on the growth of biofilm-forming bacterial clinical isolates.

2. MATERIALS AND METHODS

2.1 Essential oil of *A. herba-alba*

Fresh amount of the *Artemisia herba-alba* was collected from Mutah, Alkarak, south Jordan, during the flowering period and the vegetative phase. The plant materials were taxonomically identified and authenticated by the Botanical Survey of Yarmouk University.

The composition of the essential oil from *A. herba-alba* was determined using gas chromatography-mass spectrometry (GC-MS) [12]. Fifty eight components accounting for 98.8% of the oil were identified, with oxygenated monoterpenes accounting for about 75% of the total oil content. Major identified compounds were *cis*-chrysanthenol (13.83%), 1,8-cineole (12.84%), *cis*-limonene (12.57%), α-terpinenol (6.97%), and γ-muurolene (4.50%). The concentration of the oil was 0.0032 (wt/wt) and its density was 0.912 g/ml at room temperature.

2.2 Cultures and Media

The effect of *A. herba-alba* essential oil on bacterial biofilm formation was examined using seven bacterial clinical isolates including: Methicillin-resistant *Staphylococcus aureus* (MRSA), Methicillin-sensitive *Staphylococcus aureus* (MSSA), *Staphylococcus epidermidis*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Proteus mirabilis*. These clinical isolates were isolated from human patients. Cultures were stored on tryptone soya agar (TSA) (Oxoid, Hampshire, UK) at 2-4ºC and subcultured every 2 months or whenever required. Isolates were purified on specific nutrient agar plates and characterized

by standard microbiological and biochemical methods like Gram stain, catalase test, coagulase test and an API system (bioMerieux, France).

2.3 Biofilm Formation and Broth Microdilution Assays

Biofilm formation was quantified in microtitre plates using the method described by Rachid et al. [13]. Bacteria were grown overnight in 10 ml tryptone soya broth (TSB) at 37ºC and then diluted 1: 100 in fresh TSB supplemented with 0.5% glucose to give cell density of approximately 10^6 cells/mL. For each test strain 200 µl of inoculum was added to 72 wells of a 96-well plate. A quantity of 200 µl TSB was added to the remaining 24 wells and the plate incubated for 24 h at 37ºC. Following this the optical density at 600 nm (OD_{600}) was measured as an indication of bacterial growth, the plate contents emptied out and washed three times with phosphate-buffered saline (PBS) (Sigma Aldrich). The plates were air-dried and the cells that remained adhered to microwells stained with 0.4% crystal violet (Sigma Aldrich). Optical density at 490 nm (OD_{490}) nm was measured to quantify the amount of crystal violet-stained biofilm. Each strain was assayed in triplicate.

2.4 MIC Assay

MIC was determined using 96 well microtitre plates as described by Rachid et al. [13]. Serial two fold dilutions of *A. herba-alba* essential oil in TSB were carried out in microtitre plates, 100µl of the diluted bacterial cells were added to the wells, mixed and then incubated at 37^0C for 24 h aerobically. MIC was determined as the minimum concentration that caused 30% decrease in optical density. The positive control used for MRSA, MSSA, and *S. epidermidis* was vancomycin, for *E. coli* and *K. pneumonia* was chloramphenicol for *P. aeruginosa* was ceftazidime and finally for *P. mirabilis* was ampicillin. The absorbance was measured at 600 nm as an indication of bacterial growth.

2.5 Biofilm Inhibitory Concentration (BIC) Assay

BIC was determined using 96 well microtitre plates as described by Rachid et al. [13]. Serial two fold dilutions of *A. herba-alba* essential oil in TSB were carried out in microtitre plates, 100µl of the diluted bacterial cells were added to the wells, mixed and then incubated at 37^0C for 24 h aerobically. The wells were washed three times with PBS. The plates were dried using air, and the remaining surface-adsorbed cells of the individual well were stained with 0.4% crystal violet. Absorbance was measured at 490 nm. A well, with no cells and sterile TSB was used as blank (negative control), and a well with cells and TSB but without *A. herba-alba* essential oil was used as a control. The positive control used for MRSA, MSSA, and *S. epidermidis* was vancomycin, for *E. coli* and *K. pneumonia* was chloramphenicol for *P. aeruginosa* was ceftazidime and finally for *P. mirabilis* was ampicillin. BIC was determined as the minimum concentration that caused 30% decrease in optical density. Assays were performed three times on different days for each individual strains and the same result was obtained on each occasion.

2.6 Adherence of Bacterial Cells to Polystyrene

Initial adherence of bacterial cells to polystyrene was determined using a previously reported method [14]. Briefly, bacteria were grown overnight in 10 ml TSB at 37ºC and then diluted 1: 100 in fresh TSB containing *A. herba-alba* essential oil at the required concentration. A quantity of 5 ml of the bacterial suspensions were then poured into Petri dishes and

incubated for 30 min at 37ºC. 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 adhered cells was determined microscopically (CETI 60243T UK) by counting the number of bacteria in 20 fields of view. Adherence was calculated as the total number of cells adhered per square centimetre examined. Each *A. herba-alba* essential oil concentration was assayed in triplicate and the adherence of *A. herba-alba* essential oil treated cells compared with untreated controls.

3. RESULTS

3.1 Quantification of Biofilm Formation

All bacteria tested in this study were able to produce a stable biofilm that adhered to polystyrene microwells. Strains number 6 and 7 had an optical density (OD_{490}) of 0.762 and 0.854 respectively and were quantified as strong biofilm producers. Strains 1 and 5 had an OD_{490} of more than 0.4 and were classified as moderate biofilm producers. Strains 2, 3 and 4 had an OD_{490} less than 0.4 and were classified as poor biofilm producers (Table 1).

Table 1. Isolate name, initial adherence, MIC and BIC of *A. herba-alba* for the bacterial isolates used for this study

Isolate Number	Isolate name	Biofilm (OD490)	MIC_{plank} %v/v	$BIC_{biofilm}$ %v/v
1	MRSA	0.416±0.078	1	1
2	MSSA	0.206±0.072	0.5	1
3	*S. epidermidis*	0.245±0.044	1	1
4	*E. coli*	0.102±0.02	1	2
5	*K. pneumonia*	0.463±0.038	0.5	0.5
6	*P. aeruginosa*	0.762±0.097	2	4
7	*P. mirabilis*	0.854±0.089	0.5	1

3.2 MIC and BIC

A. herba-alba essential oil produced inhibitory effects against all isolates and susceptibility varied considerably (Table 1). The MIC and BIC values were in the range of 0.5-4% v/v. The most susceptible were *K. pneumonia*, *P. mirabilis* and MSSA while the most resistant was *P. aeruginosa* with planktonic MIC of 2 and BIC of 4% v/v. Isolate 6 *P. aeruginosa* was the most tolerant isolate tested.

3.3 Inhibition of *P. aeruginosa* Adherence to Polystyrene by *A. herba-alba* at Sub-MIC Levels

The optical density 600 (OD_{600}) was used to measure the planktonic growth (data not shown) while OD_{490} was used to measure biofilm growth. Adding sub-inhibitory concentrations (sub-MIC_{plank}) of *A. herba-alba* to polystyrene Petri dishes containing a suspension culture of the *P. aeruginosa* strain reduced the number of individual cells adhering to the polystyrene surface after 30 minutes incubation period (Figs. 1 and 2).

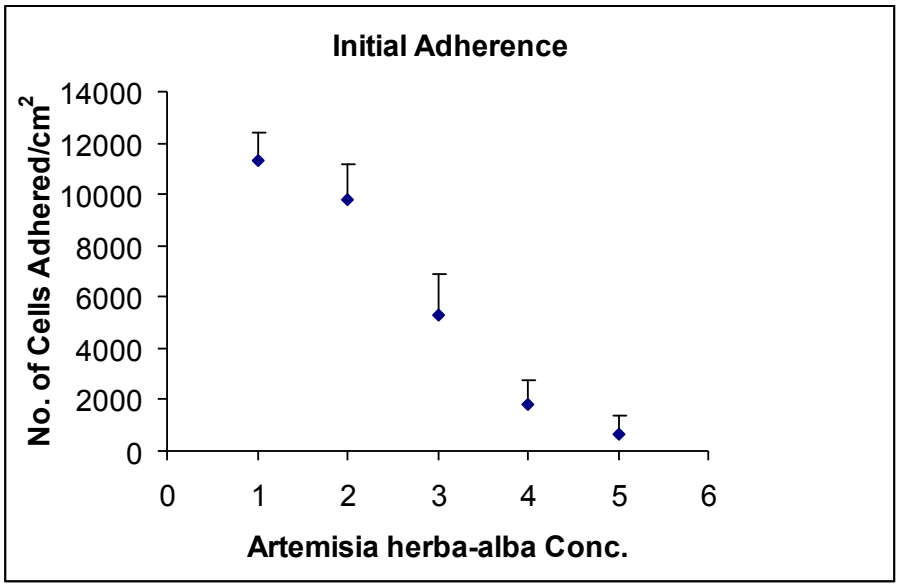

Fig. 1. Effect of *A. herba-alba* on initial adhesion; 1: without EO, 2: 1/10×MIC 3: 1/2×MIC, 4: MIC, 5: 5×MIC

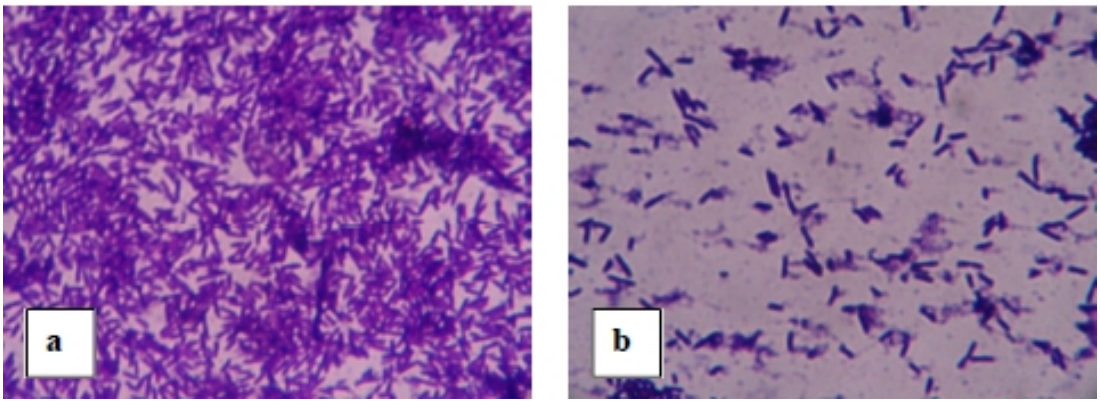

Fig. 2. Microscopic images of *P. aeruginosa* adherence to polystyrene a. without *A. herba-alba* essential oil b. with *A. herba-alba* essential oil (1/2 ×MIC)

4. DISCUSSION

The formation of biofilms is an important strategy used by microbes for survival. Biofilms are extremely resistant to antibiotics as standard antimicrobial treatments typically fail to eradicate biofilms, which can result in chronic infection. Essential oils derived from medicinal plants have been reported to exhibit a good antimicrobial effect against bacteria, yeasts, and viruses. The spread of drug resistant pathogens is one of the most serious threats for successful treatment of microbial diseases. This has prompted researchers into the identification of new antimicrobial with broad activity. Essential oils are potential sources of novel antimicrobial compounds.

The MIC and BIC values were found to be in the range of 0.5-4% v/v for all isolates. Essential oils (EOs) comprise a large number of components therefore their mode of action involves several targets in the bacterial cell and cytoplasm. The hydrophobicity of essential oils enables them to partition the lipids of the bacterial cell membrane and mitochondria, leaving them permeable due to an inability to separate the essential oils from the bacterial cell membrane leading to leakage of cell contents and death [15]. The mechanisms of action of the essential oils include the degradation of the cell wall, damaging the cytoplasmic membrane, cytoplasm coagulation, damaging the membrane proteins, increased permeability leading to leakage of the cell contents, reduction in the intracellular ATP pool via decreased ATP synthesis. Thus, the hydrophobic nature of EOs allows them to penetrate microbial cells and cause alterations in its structure and functionality [16,17].

Isolate 6 *P. aeruginosa* was chosen to test the effectiveness of *A. herba-alba* on initial adherence of this bacterium because it was found to be the most resistant isolate beside the big variation between MIC and BIC values. *A. herba-alba* essential oil was able to inhibit *Pseudomonas aeruginosa* adherence to polystyrene surface at subinhibitory levels (Fig. 2). The reduction in *P. aeruginosa* adherence to polystyrene can be explained in terms of damage to cell membrane and alterations of adherence factors present on the bacterial cell surface. As stated earlier, the mechanisms of action of the essential oils include degradation and damage of cell wall and cytoplasmic membrane. It is believed that at the subinhibitory level *A. herba-alba* essential oil was able to cause alterations to cell wall and to cytoplasmic membrane that prevented the initial adherence of *P. aeruginosa* to polystyrene surface.

5. CONCLUSION

In conclusion, we have demonstrated the antimicrobial efficacy of *A. herba-alba* essential oil against seven bacterial clinical isolates *in vitro*. *A. herba-alba* essential oil showed a good antimicrobial activity against all tested clinical isolates. In addition *A. herba-alba* essential oil was able to inhibit initial adherence in the most tolerant isolate (*P. aeruginosa*) at sub-inhibitory concentrations.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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