



Antibacterial and antibiofilm activity of essential oil of *Achillea biebersteinii* and its mode of action

[Actividad antibacteriana y anti-biopelícula del aceite esencial de *Achillea biebersteinii* y su modo de acción]

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Abstract

Context: Microbial biofilms and antibiotic-resistant bacterial strains pose a challenge in clinical world as they fail to respond to conventional treatments. This failure of antibiotic treatment led researchers to look for alternatives. A possible alternative is plants derived essential oils. Many studies have reported that certain essential oils succeed where antibiotics fail.

Aims: To test antibacterial and antibiofilm activities of essential oil of *Achillea biebersteinii* and its mode of action.

Methods: 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 determined in normal microtitre plates using a twofold dilution series.

Results: *Achillea biebersteinii* essential oil showed good activity against all tested bacteria. The MIC values were in the range of 0.125 - 1 mg/mL while MBIC values were in the range of 0.125 - 4 mg/mL. The mechanism of action of *Achillea biebersteinii* essential oil is related to a strong increase in membrane permeability of 260 nm absorbing materials and potassium ions from the cell membrane. *Achillea biebersteinii* essential oil was able to inhibit initial adherence of methicillin-resistant *Staphylococcus aureus* (ATCC 43300) at sub-inhibitory concentrations.

Conclusions: *Achillea biebersteinii* essential oil has the potential for use as an effective antibacterial and antibiofilm agent that functions by impairing cell membrane permeability resulting in cellular death.

Keywords: *Achillea biebersteinii*; antibacterial; antibiofilm; mode of action.

Resumen

Contexto: Las biopelículas microbianas y las cepas bacterianas resistentes a los antibióticos representan un desafío en el mundo clínico, ya que no responden a los tratamientos convencionales. Este fracaso del tratamiento con antibióticos llevó a los investigadores a buscar alternativas. Una posible alternativa son los aceites esenciales derivados de plantas. Muchos estudios han informado que ciertos aceites esenciales tienen éxito donde los antibióticos fallan.

Objetivos: Evaluar las actividades antibacteriana y anti-biopelícula del aceite esencial de *Achillea biebersteinii* y su modo de acción.

Métodos: Los ensayos de susceptibilidad de concentración mínima inhibitoria de biopelícula (MBIC) se realizaron utilizando un inoculador de biopelícula con una placa de 96 pocillos con clavija led. La concentración inhibitoria mínima (MIC) se determinó en placas de microtitulación normales usando una serie de dilución doble.

Resultados: El aceite esencial de *Achillea biebersteinii* mostró buena actividad contra todas las bacterias probadas. Los valores de MIC estuvieron en el rango de 0.125 - 1 mg/mL mientras que los valores de MBIC estuvieron en el rango de 0.125 - 4 mg/mL. El mecanismo de acción del aceite esencial *Achillea biebersteinii* está relacionado con un fuerte aumento en la permeabilidad de la membrana de los materiales absorbentes de 260 nm y los iones de potasio de la membrana celular. El aceite esencial de *Achillea biebersteinii* fue capaz de inhibir la adherencia inicial de *Staphylococcus aureus* resistente a metilicina (ATCC 43300) en concentraciones subinhibitorias.

Conclusiones: El aceite esencial de *Achillea biebersteinii* tiene el potencial de usarse como un agente antibacteriano y anti-biopelícula eficaz que funciona al afectar la permeabilidad de la membrana celular y provocar la muerte celular.

Palabras Clave: *Achillea biebersteinii*; antibacteriano; anti-biopelícula; modo de acción.

ARTICLE INFO

Received: October 3, 2019.

Received in revised form: November 24, 2019.

Accepted: November 25, 2019.

Available Online: November 26, 2019.

Declaration of interests: The authors declare no conflict of interest.

Funding: This research was supported by the Deanship of Scientific Research, Mutah University, Mutah, Jordan [grant numbers 120/14/63, 16/02/2015].



INTRODUCTION

Bacteria resistant to commonly used antibiotics pose one of the biggest threats to global health (Wernli et al., 2017). Certain bacteria termed as multidrug resistant (MDR) have developed resistance to nearly all currently used antibiotics. Causes of the antibiotic resistance include overuse and abuses of antibiotics, inappropriate and incorrectly prescribing, and extensive use in agricultural (Ventola, 2015).

The emergence of resistant infections caused by multidrug-resistant bacteria has led to high mortality and morbidity needing urgent solutions. Methicillin-resistant *Staphylococcus aureus* (MRSA) kills more people in USA each year than AIDS, emphysema, Parkinson's disease, and homicide crimes combined. Some Gram-negative pathogens are becoming resistant to nearly all known antibiotics (Golkar et al., 2014). In the United States of America, alone 23,000 people die each year as a result of antibiotic-resistant infections. It is estimated that antibiotic resistance cost about \$20 billion a year to U.S. economy (Li and Webster, 2018).

Biofilm is a community of bacterial cells attached to a surface and embedded in a protective extracellular polymeric matrix (Chakraborty and Kumar, 2019). It is believed that more than 90% of bacteria live in biofilms. Biofilm has increased antibiotic resistance. It is believed that 65% of microbial infections are associated with biofilms and cells in biofilm are up to 1,000 times more resistant to antibacterial agents than planktonic bacterial cells (Costerton et al., 1999; Mah and O'Toole, 2001).

With the rise of antibiotics resistance to high levels, there is a need for antibiotics alternatives. One option is essential oil that may provide natural, efficient and cheap alternatives to antibiotics. Essential oils have been reported to possess antimicrobial activity and ability to inhibit biofilm formation (Al-Shuneigat et al., 2014; 2015). Essential oils possess antimicrobial, anti-inflammatory and antioxidative properties and they are relatively safe (Sitarek et al., 2017).

The genus *Achillea* comprises of about 100 species mostly distributed in Europe and Middle East (Rahimmalek et al., 2009). *Achillea biebersteinii* is 20–60 cm high a perennial herb. In traditional medicine *Achillea biebersteinii* is mostly used for wound healing abdominal pain, and diarrhea. *Achillea* species have anti-inflammatory, antispasmodic, diaphoretic, diuretic and was used to treat hemorrhage, pneumonia, stomachache and wounds healing (Saeidnia et al., 2011). Different medicinal properties of these plants such as spasmolytic, choleric, anti-inflammatory and wound healing are well documented (Saeidnia et al., 2011). The antibacterial activities of *Achillea biebersteinii* essential oil were previously reported (Salarbashi et al., 2014). However, its activity against biofilm and its mode of action has never been reported.

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

MATERIAL AND METHODS

Essential oil of *Achillea biebersteinii*

The chemical composition of the *Achillea biebersteinii* was previously published by our research group (Al-Shuneigat et al., 2019). 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-verbenaol 1.88%, trans-piperitol 1.52%. Oxygenated monoterpene were the major oil components (70.22%), monoterpene hydrocarbon 26.95%, and sesquiterpene hydrocarbon 1.04%.

Maintenance and preparation of cultures

The effect of *Achillea biebersteinii* essential oil 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), *Pseudomo-*

nas aeruginosa (ATCC 27853), *Escherichia coli* (ATCC 25922), and the non-biofilm forming strain *Staphylococcus epidermidis* (ATCC 12228). 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. (2000). Briefly, serial two fold dilutions of *Achillea biebersteinii* essential oil in tryptone soya broth (TSB) (Oxoid, Hampshire, UK), were carried out to give essential oil concentrations of 0.125, 0.250, 0.5, 1, 2, 4, 8, and 16 mg/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 (OD₆₀₀) reached approximately 0.6 then diluted to 1×10^6 cfu/mL and seeded (100 µL) to the wells containing *Achillea biebersteinii* essential oil, mixed and incubated at 37°C for 24 h aerobically. The MIC was taken as minimal concentration of *Achillea biebersteinii* essential oil that inhibited visible growth of the strain. Determination of MIC was carried out in triplicate using three independent experiments. The positive control used was gentamicin while the negative control was

only media and essential oil without bacteria. Gentamicin was chosen because it is a potent broad-spectrum antibiotic used for the treatment of Gram-positive and Gram-negative bacterial infections.

Minimum bactericidal concentration (MBC)

To determine the MBC values, a volume of 30 µL from each well, which did not show an apparent growth as confirmed by MIC determination, was taken and plated on TSA agar. The concentrations of essential oil used were 0.125, 0.250, 0.5, 1, 2, 4, 8, and 16 mg/mL. The plates were 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 (Jardak et al., 2017). The positive control used was gentamicin while the negative control was only media and essential oil without bacteria.

Minimum biofilm inhibitory concentration (MBIC) assay

Biofilm susceptibility assays were performed using MBIC (Innovotech, Inc., Edmonton, AB, Canada) a biofilm inoculator with a 96-well plate (Fig. 1) according to method reported by Ceri et al. (2001).



Figure 1. Biofilm inoculator with a 96-well plate with peg lids (Innovotech, Inc., Edmonton, AB, Canada). The MBEC biofilm inoculator has a lid with 96 pegs and a base with 96 individual wells. Biofilm grow and established on the peg lid.

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. Then 150 μ L of inoculums was added to each well of 96-well MBEC biofilm inoculators and the peg lid was then fitted on plates. After 24 h incubations at 37°C biofilms were formed on 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 concentrations of essential oil used were 0.125, 0.250, 0.5, 1, 2, 4, 8, and 16 mg/mL. The microtiter plate was then incubated at 37°C for a 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 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 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. The positive control used was gentamicin while the negative control was only media and essential oil without bacteria.

Minimum biofilm eradication concentration (MBEC)

The MBEC values was determine by taking a 30 μ L volume of each well, which did not show an apparent growth as confirmed by MBIC determination then plated 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.

The positive control used was gentamicin while the negative control was only media and essential oil without bacteria.

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). *Staphylococcus epidermidis* (ATCC 35984), *Methicillin-resistant Staphylococcus aureus* (MRSA) (ATCC 43300), *Pseudomonas aeruginosa* (ATCC 27853), and *Escherichia 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. The positive control used was gentamicin while the negative control was only media and essential oil without bacteria.

Integrity of the cell membrane (release of cellular material)

The function of cell membrane is to hold different components of the cell together and protect it from extracellular environment. Thus, the release of cellular materials especially DNA, RNA and proteins to outside cell indicates damage in cell membrane. Essential oils at the MIC concentration was added to 2 mL of the methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300), *Pseudomonas aeruginosa* (ATCC 27853), and *Staphylococcus epidermidis* (ATCC 35984) (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 (Yang et al., 2015). The positive control used was gentamicin while the negative control was only media and essential oil without bacteria.

Adherence of bacterial cells to polystyrene

Initial adherence of methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300) to polystyrene was determined using a previously reported method (Heilmann et al., 1996). Briefly, bacteria were grown overnight in 10 mL TSB at 37°C and then diluted 1: 100 in fresh TSB containing *Achillea biebersteinii* essential oil at the required concentration. The used concentrations were 1/10 of MIC, 1/2 of MIC, the MIC concentration, and finally five times the MIC. The positive control used was gentamicin while the negative control was only media and bacteria but without essential oil.

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 centimeter examined. Each *Achillea biebersteinii* essential oil concentration was assayed in triplicate and the adherence of *Achillea biebersteinii* essential oil 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

Statistical analyzes were performed using SPSS package for Windows (version 15, Chicago, IL, USA). 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

The MIC and MBC results of *Achillea biebersteinii* essential oil are shown in Table 1. As expected, the MBC values are higher compared to MIC values for all tested strains. The MIC values were in the range of 0.125 mg/mL. While the MBC values were in the range of 0.50-4 mg/mL. The most susceptible isolates in MIC and MBC were *Staphylococcus epidermidis* (ATCC 12228) with planktonic MIC of 0.125/mL and MBC of 0.5 mg/mL while the most resistant MIC was *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922) with MIC of 1 mg/mL while the most resistant MBC was *Pseudomonas aeruginosa* (ATCC 27853) with MBC value of 4 mg/mL. The MIC value for positive control, gentamicin, against methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300) was 1 mg/mL and the MBC 2 mg/mL while for *Pseudomonas aeruginosa* (ATCC 27853) the MIC was 0.125 mg/mL and the MBC was 0.5 mg/mL.

MBIC and MBEC for the bacterial isolates

The MBIC and MBEC results of *Achillea biebersteinii* for bacterial strains are shown in Table 2. The MBEC values are higher compared to MBIC values for all tested strains. The MBIC values were in the range of 0.125- 4 mg/mL. While the MBEC values were in the range of 0.50-8 mg/mL. The most susceptible isolate were *Staphylococcus epidermidis* (ATCC 12228) for both MBIC and MBEC with MBIC of 0.125 mg/mL and MBEC of 0.5 mg/mL while the most resistant for both MBIC and MBEC was *Pseudomonas aeruginosa* (ATCC 27853) with MBIC of 4 mg/mL and MBEC of 8 mg/mL. Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300) show resistance with MBIC of 1 mg/mL and MBEC of 4 mg/mL. The MBIC and MBEC values for the positive control, gen-

tamicin, against methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300) were 1 mg/mL and 2 mg/mL and for *Pseudomonas aeruginosa* (ATCC 27853) were 0.50 mg/mL and 1.0 mg/mL.

Leakage of potassium ion

The permeability of the cell membrane was measured based on leakage of potassium ions from four bacterial strains: *Staphylococcus epidermidis* (ATCC 35984), methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300), *Pseudomonas aeruginosa* (ATCC 27853), and *Escherichia coli*

(ATCC 25922). Increase in membrane permeability of potassium ions into the extracellular space is an index of cytotoxicity of the *Achillea biebersteinii* essential oil that disrupts the structure of membranes and causes damages to cell membrane. *Achillea biebersteinii* essential oil was able to increase extracellular potassium at MIC.

When the four selected bacterial strains were treated with essential oil at the MIC, increase leakage of potassium ions was observed as shown in Fig. 2.

Table 1. MIC and MBC of *Achillea biebersteinii* essential oil (mg/mL) for the bacterial isolates.

| Isolate Number | Isolate name | MIC (mg/mL) | MBC (mg/mL) |
|----------------|--|---------------|-------------|
| 1 | <i>Staphylococcus epidermidis</i> (ATCC 35984) | 0.250 ± 0.072 | 1.0 ± 0.12 |
| 2 | Methicillin-susceptible <i>Staphylococcus aureus</i> (MSSA) (ATCC 25923) | 0.500 ± 0.11 | 2.0 ± 0.25 |
| 3 | Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA) (ATCC 43300) | 0.250 ± 0.072 | 2.0 ± 0.12 |
| 4 | <i>Pseudomonas aeruginosa</i> (ATCC 27853) | 1.000 ± 0.12 | 4.0 ± 0.50 |
| 5 | <i>Escherichia coli</i> (ATCC 25922) | 1.000 ± 0.20 | 2.0 ± 0.12 |
| 6 | <i>Staphylococcus epidermidis</i> (ATCC 12228) | 0.125 ± 0.07 | 0.5 ± 0.02 |
| | Gentamicin against MRSA (ATCC 43300) | 0.500 ± 0.05 | 2.0 ± 0.05 |
| | Gentamicin against <i>P. aeruginosa</i> (ATCC 27853) | 0.125 ± 0.01 | 0.5 ± 0.05 |

Data represented as mean ± SD of three independent readings. MIC: Minimum inhibitory concentration mg/ mL and MBC: Minimum bactericidal concentration mg/ mL. Gentamicin was used as the reference compound against Gram-positive Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300) and Gram-negative *Pseudomonas aeruginosa* (ATCC 27853).

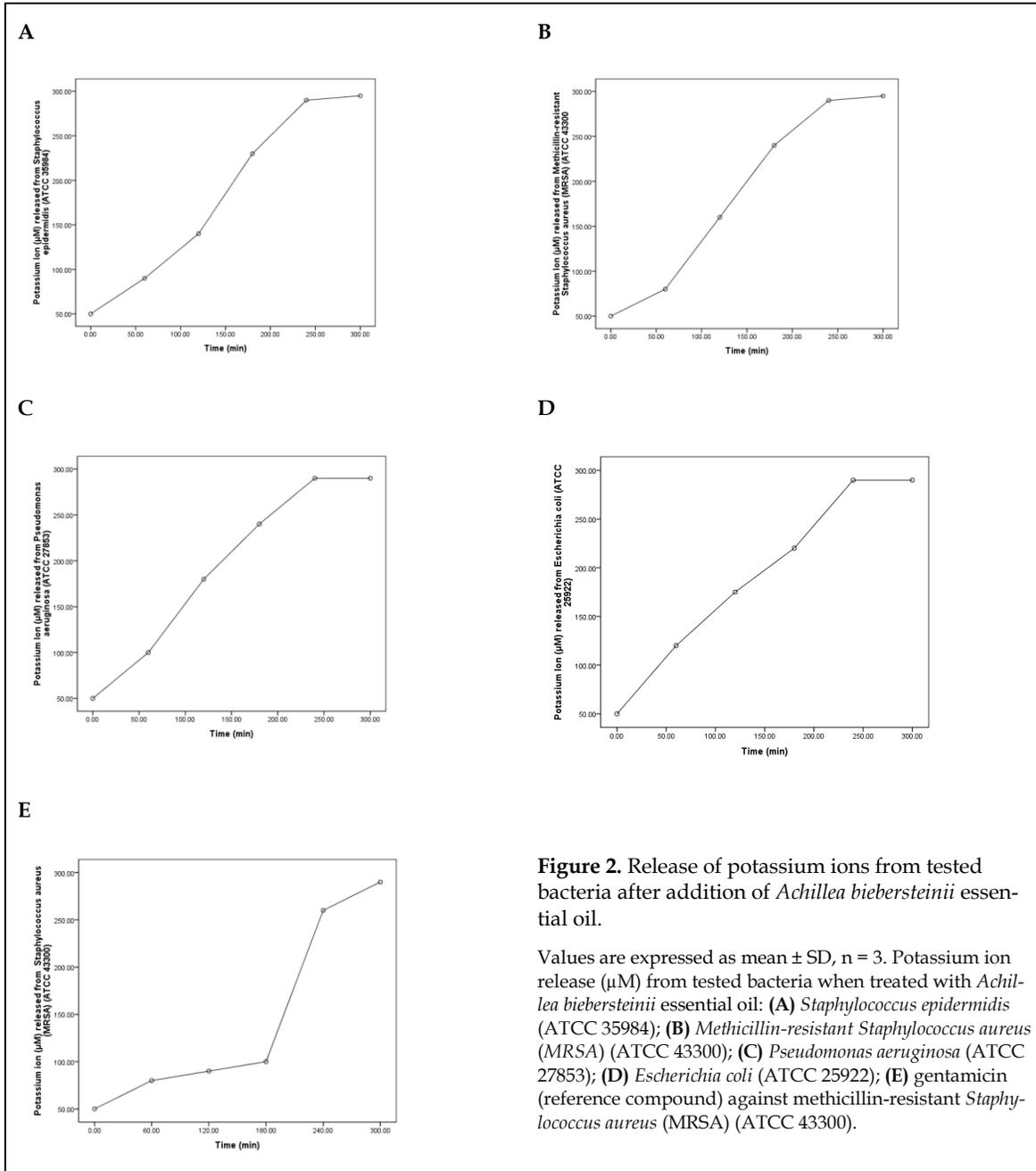
Table 2. MBIC and MBEC of *Achillea biebersteinii* essential oil (mg/mL) for the bacterial isolates.

| Isolate Number | Isolate name | MBIC (mg/mL) | MBEC (mg/mL) |
|----------------|--|---------------|--------------|
| 1 | <i>Staphylococcus epidermidis</i> (ATCC 35984) | 1.00 ± 0.12 | 2.0 ± 0.02 |
| 2 | Methicillin-susceptible <i>Staphylococcus aureus</i> (MSSA) (ATCC 25923) | 1.00 ± 0.15 | 2.0 ± 0.25 |
| 3 | Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA) (ATCC 43300) | 1.00 ± 0.02 | 4.0 ± 0.15 |
| 4 | <i>Pseudomonas aeruginosa</i> (ATCC 27853) | 4.00 ± 0.20 | 8.0 ± 0.50 |
| 5 | <i>Escherichia coli</i> (ATCC 25922) | 1.00 ± 0.12 | 2.0 ± 0.025 |
| 6 | <i>Staphylococcus epidermidis</i> (ATCC 12228) | 0.125 ± 0.01 | 0.5 ± 0.05 |
| | Gentamicin against MRSA (ATCC 43300) | 1.00 ± 0.10 | 2.0 ± 0.12 |
| | Gentamicin against <i>P. aeruginosa</i> (ATCC 27853) | 0.500 ± 0.025 | 1.0 ± 0.025 |

Data represented as mean ± SD of three independent readings. Gentamicin has been used as a reference compound against Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300) and *Pseudomonas aeruginosa* (ATCC 27853). MBIC: Minimum biofilm inhibitory concentration in mg/mL, MBEC: Minimum biofilm eradication concentration in mg/mL.

Release of potassium ions from tested bacteria occurred immediately after addition of essential oil. All tested bacterial strains treated with *Achillea biebersteinii* essential oil showed increased leakage of K⁺ ions with time. There is a sharp increase in

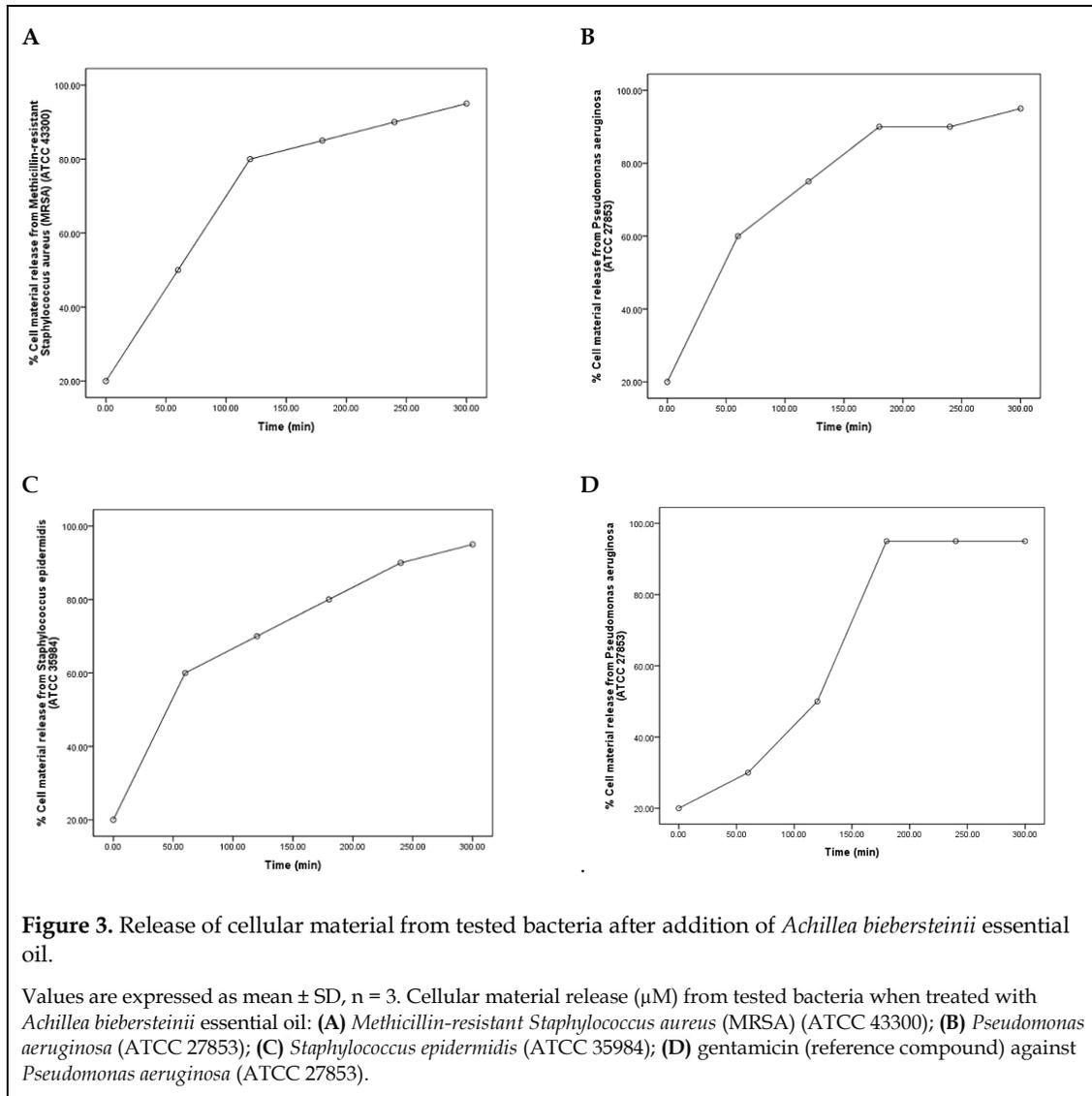
leakage of potassium ions with increase of incubation time as shown in Fig. 2. The positive control used was gentamicin against *Staphylococcus aureus* (MRSA) (ATCC 43300) and results are shown in Fig. 2.



Integrity of the cell membrane (release of cellular material)

In addition to leakage of potassium ion, release of cellular materials especially DNA and RNA to outside cell is another parameter that indicates damage in cell membrane. When tested bacterial strains, methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300), *Pseudomonas aeruginosa*

(ATCC 27853), and *Staphylococcus epidermidis* (ATCC 35984), were treated at MIC concentration of *Achillea biebersteinii* essential oils, there was a continual increase in the concentration of 260 nm absorbing materials over 300 min of incubation (Fig. 3). The positive control used was gentamicin used against *Pseudomonas aeruginosa* (ATCC 27853) and results are shown in Fig. 3.



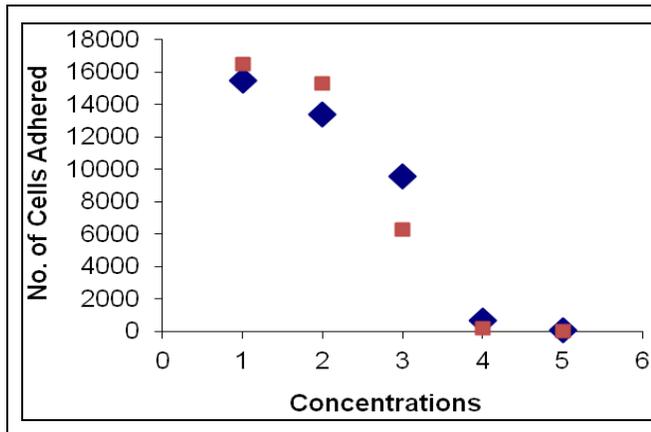


Figure 4. Effect of *Achillea biebersteinii* essential oils on initial adhesion of *Staphylococcus epidermidis* (ATCC 35984).

Concentrations: (1) without essential oil; (2) 1/10 of MIC; (3) 1/2 of MIC; (4) MIC; 5) five times the MIC. ♦ *Achillea biebersteinii* essential oils; ■ gentamicin.

Adherence of bacterial cells to polystyrene

The optical density 600 (OD₆₀₀) was used to measure the planktonic growth (data not shown) while OD₄₉₀ was used to measure biofilm growth. Adding sub-inhibitory concentrations of *Achillea biebersteinii* to polystyrene Petri dishes containing a suspension culture of the *Staphylococcus epidermidis* (ATCC 35984) strain reduced the number of individual cells adhering to the polystyrene surface after 30 minutes incubation period (Fig. 4). The positive control used was gentamicin against *Staphylococcus epidermidis* (ATCC 35984) and results are shown in Fig. 4.

DISCUSSION

Essential oils derived from aromatic plants have been used for centuries in traditional medicine for treatment of various diseases as they exhibit antimicrobial activity on different pathogens. Essential oils are cheap, easily available; and do not exhibit side effects (Burt, 2004).

In the present study, MIC results revealed that essential oil of *Achillea biebersteinii* exhibits potent activities against all tested bacteria, with MIC values ranging from 0.125 mg/mL to 1 mg/mL.

Achillea biebersteinii essential oil was more active against Gram-positive bacteria than Gram negative bacteria. *Pseudomonas aeruginosa* and *E. coli* were more resistant to essential oil than Gram-positive bacteria, which could be attributed to differences in the structures of the cell walls between

Gram-positive and Gram-negative bacteria (Trombetta et al., 2005). Gram-positive bacteria cell wall is made up of peptidoglycan. Essential oils were able to penetrate the cell wall of Gram-positive bacteria and destroy bacteria cell (Kavanaugh and Ribbeck, 2012). In Gram-negative bacteria, there is an outer membrane that covers peptidoglycan layer. This outer membrane is hydrophilic in nature it is impermeable to hydrophobic essential oil (Nazzaro et al., 2013). The presence of this outer membrane is what makes Gram-negative bacteria mainly more resistant to essential oil than Gram-positive bacteria (Nikaido, 2003).

Our results showed that MBC values were higher than MIC and this agreed with published data. MIC is the lowest concentration of antimicrobial agent that inhibits visible growth while MBC is the lowest concentration required to kill 99.9% microorganism. Thus, killing microorganisms normally require higher antimicrobial concentration and the same goes for MBIC and MBEC.

Bacterial biofilm is highly resistant to antimicrobial agent. Infections caused by biofilm including, cystic fibrosis, endocarditis, and intravenous catheters and stents infections are often untreatable and develop into a chronic state (Khattoon et al., 2018).

In the present study, MBIC results showed that essential oil of *Achillea biebersteinii* showed a good activity against all tested bacteria with MBIC values ranging from 0.125 to 4 mg/mL. The most sus-

ceptible tested strain was *Staphylococcus epidermidis* (ATCC 12228) while the most resistant was *Pseudomonas aeruginosa* (ATCC 27853). As stated before, the antibacterial activities of *Achillea biebersteinii* essential oil against *Staphylococcus aureus* (MRSA) (ATCC 43300) were previously reported (Salarbashi et al., 2014). The reported MIC was 1.2 mg/mL while in this study the MIC was 0.25 mg/mL. The differences in the MIC may be attributed to differences in the chemical composition of essential oil. The differences of chemical composition of essential oils of a plant may be attributed to growth stage of plant at time of collection, climate, geographic conditions and the extraction method (Zouari et al., 2012).

Staphylococcus epidermidis (ATCC 12228) is non-biofilm forming strain and this is why it is the least resistant microorganism and its MIC and MBIC values are equal. *Pseudomonas aeruginosa* is an opportunistic pathogen that resists many of the currently available antibiotics and causes high morbidity and mortality in cystic fibrosis patients and immunocompromised patients (Lister et al., 2009). *Pseudomonas aeruginosa* has been listed by World Health Organization for an urgent need for the development of new antibiotics to treat its infections (Pang et al., 2019). Essential oils, unlike antibiotics that only have a single target site, are complex mixtures of a wide diversity of components so that they got different mechanisms of action enabling them to overcome microbial resistance (Yap et al., 2014). *Achillea biebersteinii* essential oil was able to overcome the resistance mechanisms of these pathogens. The hydrophobic nature of essential oils allows them to penetrate into microbial cells and cause alterations in its structure and cell death. The mechanisms of action of the essential oils include the degradation of the cell wall, damage of the cytoplasmic membrane, cytoplasm coagulation, damage to membrane proteins, increased permeability leading to leakage of the cell contents and death (Nazzaro et al., 2013; Bajpai et al., 2014).

Achillea biebersteinii essential oil was able to prevent the formation of biofilm by preventing the first step in biofilm formation, reversible attachment step. 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 production of polymer matrix around the microcolonies (Marshall, 1992). It is believed that the subinhibitory level of *Achillea biebersteinii* essential oil did not cause killing of the microorganism but caused damage and changes to test microorganism's cell membrane and prevented it from adhering to the polystyrene surface. Thus, another possible approach to fight biofilm is through preventing biofilm formation. For example, coating indwelling devices and catheters with anti-adhesion coatings like *Achillea biebersteinii* essential oil could inhibit the formation of biofilm.

The mode of action of *Achillea biebersteinii* essential oil against test bacteria was revealed by two assays: leakage of potassium ion and integrity of the cell membrane (release of cellular material). The primary target of essential oils is pathogens' cell membrane (Stammati et al., 1999). The results indicate that *Achillea biebersteinii* essential oils acted on the cytoplasmic membrane, resulting in loss of integrity and increased membrane permeability. Increased leakage of potassium ions and the increase in optical density at 260 indicates that the cell membrane structure was damaged by essential oil causing the release of intercellular materials such as nucleic acids to the outer solution as compared to the control group. As stated, before essential oils are hydrophobic, which allow them to penetrate into microbial cells and accumulate inside the cells, causing disturbance to their structure that results in increased cellular leakage and death.

CONCLUSIONS

Achillea biebersteinii essential oil has the potential for use as an effective antibacterial and antibiofilm agent that functions by impairing cell membrane permeability resulting in cellular death.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by the Deanship of Scientific Research, Mutah University, Mutah, Jordan [grant numbers 120/14/63, 16/02/2015].

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| Contribution | Al-Shuneigat JM | Al- Sarayreh SA | Al-Qudah MA | Al-Saraireh YM |
|------------------------------------|-----------------|-----------------|-------------|----------------|
| Concepts or ideas | x | x | | |
| Design | x | x | x | x |
| Definition of intellectual content | x | | | |
| Literature search | x | x | | |
| Experimental studies | x | | x | |
| Data acquisition | x | | x | x |
| Data analysis | | | | x |
| Statistical analysis | | | x | x |
| Manuscript preparation | x | x | | |
| Manuscript editing | x | x | | |
| Manuscript review | x | x | x | x |

Citation Format: Al-Shuneigat JM, Al- Sarayreh SA, Al-Qudah MA, Al-Saraireh YM (2020) Antibacterial and antibiofilm activity of essential oil of *Achillea biebersteinii* and its mode of action. *J Pharm Pharmacogn Res* 8(2): 155–166.