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Phlomis brachydon Essential Oil Against Bacterial Biofilm

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Abstract

Bacteria in biofilms show high resistance to antimicrobial and they cause many persistent and chronic bacterial infections. The failure of antibiotics in eradicating biofilm drives the need for novel approaches to effectively kill bacterial biofilms. Plant essential oils have been used for hundreds of years in traditional medicine to treat infections due to bacteria, fungi, and virus. The aim of the present study is to determine the effects of essential oil of *Phlomis* grown in north Jordan on biofilm-forming bacteria. Six bacterial clinical isolates were used in this study. The Minimum Inhibitory Concentration (MIC) and Biofilm Inhibitory Concentration (BIC) assays were performed in microtitre plates using a twofold dilution series. *Phlomis* essential oil MIC for planktonic bacteria ranged between 0.125 and 2 mg/mL. The most susceptible strains were MRSA and *S. epidermidis.* For bacteria grown in biofilm, the BIC ranged between 0.25 and 4 mg/mL. The most sensitive to *Phlomis* essential oil was *S. epidermidis* while the most resistant were *P. aeruginosa* and *E. coli*. *Phlomis* essential oil was able to inhibit initial adherence in the most tolerant isolate (*E. coli*) at sub-inhibitory concentrations. *Phlomis* essential oil showed a significant activity against all isolates in both planktonic and biofilm growth. It was able to inhibit initial adherence in the most tolerant isolate at sub-inhibitory concentrations.

Keywords: MIC, BIC, biofilm, resistant bacteria, essential oil, Pholims.

1. Introduction

Biofilm is a community of *microorganisms* adhered to surfaces and embedded in a self produced slimy, glue-like matrix. The matrix, which is made up of polysaccharide, protein and DNA, acts as a shield that prevents the access of antimicrobials to biofilm microbes (Veerachamy *et al*., 2014; Stewart and Costerton, 2001). Bacteria in biofilms display a coordinated activity and are able to communicate with each other and exchange signals. Through signalling and sensing, bacteria in a biofilm regulate cell density, formation of channels and pillar like structure for nutrient delivery (Sauer *et al*., 2002; Miller and Bassler, 2001). This is why bacteria in a biofilm are different from planktonic bacteria and they are also found to be different in gene expression (Mikkelsen *et al*., 2007).

Bacterial biofilms show high tolerance to antibiotics and disinfectant hemicals and resist body's defence system. The mechanisms associated with biofilm resistance include: restricted diffusion of antimicrobial agents due to the extracellular matrix, formation of persister cells, adaptive stress responses, slow-growing bacteria (due to depletion of nutrient and oxygen within biofilm) which is less susceptible to antimicrobial agents and expression of biofilm-specific antimicrobial resistance genes (Høiby *et al*., 2010; Patel, 2005).

Biofilm associated *diseases* include cystic fibrosis pneumonia, native valve endocarditis, otitis, bacterial prostatitis, dental plaques, osteomyelitis, musculoskeletal infections, periodontitis and medically associated such as intravascular catheters, urinary catheters and prosthetic implants (Pamp *et al*., 2009; Aparna and Yadav, 2008).

The failure of antibiotics to treat biofilm associated infections increases the need for alternatives. Essential Oils (EO) have been used for centuries in traditional medicine for treating various diseases. Essential oils are very complex mixtures of volatile components produced by aromatic plants as secondary metabolites. They were shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties (Nazzaro *et al*., 2013; Burt, 2004).

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Predictions about the mode of action of crude essential oils require thorough investigations of their constituents' target site, their mode of action, and their interactions with the surrounding environment (Burt, 2004). As crude essential oils contain many constituents, it is very difficult to predict their mode of action. The strongest antibacterial properties against pathogens come from phenolic compounds such as carvacrol, eugenol and thymol. Essential oils are hydrophobic in nature, which enables them to disturb the bacterial cytoplasmic membrane and mitochondria causing leakage of ions and other cell contents (Hyldgaard *et al*., 2012).

Phlomis is a plant used in herbal medicine for respiratory tract diseases, local treatment of wounds, treatment of ulcers and hemorrhoids. In Jordan traditional medicine *Phlomis brachydon* (Boiss.) Zohary is used to treat stomach and intestine pain (Darwish and Aburjai, 2010). In addition, *Phlomis* have shown activities as anti-inflammatory, immuno-suppressive, antimutagenic, anti-nociceptive, antifibriel, free radical scavenging, anti-malarial, and anti-microbial responses (Sarkhail *et al*., 2006).

There are a few reports about the essential oil constituents of *Phlomis brachydon* (Boiss.) Zohary. To the best of our knowledge, the activity of *Phlomis brachydon* against bacterial biofilm has never been reported worldwide.

The aim of the present study is to determine the effects of essential oil of wild *Phlomis brachydon* (Boiss.) Zohary grown in Jordan on clinical isolates of biofilm-forming bacteria. .

2. Materials and Methods

2.1. Essential oil of Phlomis brachydon

Fresh *Phlomis brachydon* was collected from mountains of Qumeim, Irbid, north Jordan, before the flowering period. The plant materials were taxonomically identified and authenticated by the Botanical Survey of Yarmouk University.

The composition of the essential oil from *Phlomis brachydon* was determined using Gas Chromatography-Mass Spectrometry (GC-MS) (Al-Shuneigat *et al*., 2015). 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 (Al-Shuneigat *et al*., 2015).

2.2. Cultures and Media

The effect of *Phlomis brachydon* essential oil on bacterial biofilm was examined using six clinical isolates including: Gram positive bacteria: Methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis*, and *Bacillus subtilis*, and Gram negative bacteria: *Escherichia coli*, *Enterobacter aerogenes*, and *Pseudomonas aeruginosa*. These clinical isolates were isolated from human patients. Cultures were stored on tryptone soya agar (TSA) (Oxoid, Hampshire, UK) at 2-4^0 C 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. Screening for Biofilm Formation

Biofilm formation was quantified in microtitre plates using the method described by Rachid et al (Rachid *et al*., 2000). Bacteria were grown overnight to mid-log phase by inoculating 10 mL tryptone soya broth (TSB) and incubating at 37ºC until the OD at 600 nm (OD_{600}) reached approximately 0.6. Strains were then diluted in fresh TSB supplemented with 0.5% glucose to give cell density of approximately 10^6 cfu/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 37^0 C. 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 (Al-Shuneigat *et al*., 2005).

2.4. MIC Assay

MIC was determined using 96 well microtitre plates as described by Rachid *et al*. (2000). Serial two fold dilutions of *Phlomis brachydon* essential oil in TSB were carried out in microtitre plates, 100 µL of bacterial cells with density of approximately 10^6 cfu/mL were added to the wells, mixed and then incubated at 37^{0}C for 24 h aerobically. The MIC was taken as minimal concentration of *Phlomis brachydon* 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 for MRSA, *Staphylococcus epidermidis*, and *Bacillus subtilis* was vancomycin, chloramphenicol was used for *E. coli* and *Enterobacter aerogenes* and ceftazidime for *P. aeruginosa*. 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 (Rachid *et al*., 2000). Serial two fold dilutions of *Phlomis brachydon* essential oil in TSB were carried out in microtitre plates, 100 µL of the diluted bacterial cells were added to the wells, mixed and then incubated at 37^{0}C 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 0.1% (w/v) crystal violet. The wells were then washed three times with PBS and allowed to air-dry for 60 min. The crystal violet-stained biofilm was solubilized using 95% (v/v) ethanol and the absorbance read 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 *Phlomis brachydon* 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 strain and the same result was obtained for each occasion.

2.6. Adherence of Bacterial Cells to Polystyrene

Initial adherence of bacterial cells to polystyrene was determined using a previously reported method (Heilmann *et al*., 1996). Briefly, bacteria were grown overnight in 10 ml TSB at 37^0 C and then diluted 1 : 100 in fresh TSB containing *Phlomis brachydon* essential oil 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 37^0 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 *Phlomis brachydon* essential oil concentration was assayed in triplicate and the adherence of *Phlomis brachydon* 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

3. Results

3.1. MIC and BIC

Table 1 shows the MIC and BIC values of *Phlomis brachydon* essential oil (mg/mL) for the bacterial isolates used in this study. In general and as expected, the planktonic growth of nearly all bacteria strains tested were more sensitive to *Phlomis brachydon* essential oil than biofilm growth.

The MIC values for planktonic growth were between 0.125 and 2 mg/mL. The most susceptible strains were MRSA and *S. epidermidis.*

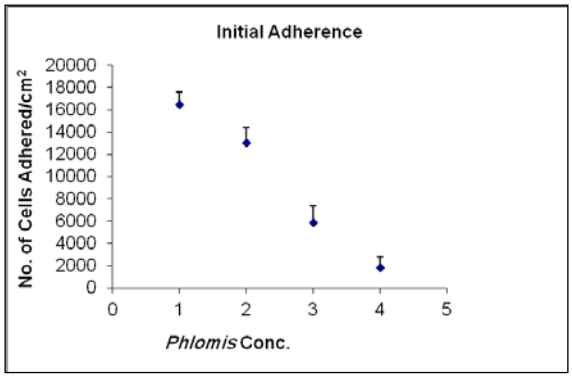
For biofilm, the BIC values were between 0.25 and 4 mg/mL. The most sensitive to *Phlomis* essential oil was *S. epidermidis* while the most resistant were *P. aeruginosa* and *E. coli*. planktonic *E. coli* was very sensitive to *Phlomis* essential oil and the most resistant to *Phlomis* essential oil grown as biofilm

Table 1. MIC and BIC of *Phlomis* (mg/mL) for the bacterial isolates used for this study.

Isolate Number	Isolate name	MIC_{plank}	$BIC_{biofilm}$
1	MRSA	0.125	2
2	*P. aeruginosa*	1	4
3	*E. aerogenes*	2	2
4	*E. coli*	0.25	4
5	*B. subtilis*	2	2
6	*S. epidermidis*	0.125	0.25

3.2. Inhibition of E. coli Adherence to Polystyrene by Phlomis at sub-MIC Levels

As stated above, *E. coli* was very sensitive to *Phlomis brachydon* essential oil in planktonic growth and became very resistant in biofilm growth. Because of the great variations between MIC and BIC values and because BIC value for *E. coli* was one of the highest, *E. coli* was chosen to test the effect of sub-inhibitory concentrations (sub-MIC_{plank}) on its initial adherence to polystyrene.



The results show that adding sub-inhibitory concentrations (sub-MIC_{plank}) of *Phlomis* essential oil to polystyrene Petri dishes containing a suspension culture of the *E. coli* strain were able to reduce the number of individual cells adhering to the polystyrene surface after 30 minutes incubation period (Fig. 1).

Figure 1. Effect of *Phlomis* on initial adhesion of *E. coli*; 1: without EO, 2: 1/10×MIC 3: 1/2×MIC, 4: MIC

4. Discussion

Biofilm is a complex structure that causes chronic infections. Biofilm infections are extremely resistant to antibiotics and conventional antimicrobial agents, and are able to evade host defenses. Nowadays, the known antibiotics became of limited effectiveness in treating biofilms infection. Biofilms are able to withstand 100 to 1000 times the concentrations of antibiotics that can inhibit planktonic cells (Wolcott and Ehrlich, 2008). This means that there is an urgent need for the development of alternatives to antibiotics. A possible alternative is plants derived essential oils. Essential oils have been shown to possess broad-range of antibacterial properties (Hyldgaard *et al*., 2012; Oussalah *et al*., 2007).

Essential oils have been used in folk medicine since ancient times for the treatment of various diseases. The

main cellular targets of essential oil are cell membrane and cytoplasm (Nazzaro *et al*., 2013).

The aim of the present study is to test the effect of essential oil of wild *Phlomis brachydon* on biofilm-forming bacterial clinical isolates. The results show that the MIC values for planktonic growth were between 0.125 and 2 mg/mL while the BIC values were higher for nearly all strains.

The essential oil of *Phlomis brachydon* has been able to overcome the resistance mechanisms of the plankonic growth of two of the most prominent antibiotics resistant pathogens MRSA with MIC of only 0.125 mg/mL and *P. aeruginosa* with MIC of 1 mg/mL.

The results show that in general that the MIC_{plank} values are lower than that of $BIC_{biofilm}$. This is not unexpected as pathogens become more resistant in biofilm than in planktonic form. The essential oil of *Phlomis* was able to overcome the resistance of *S. epidermidis* at very low concentrations in both planktonic and biofilm form. *S. epidermidis* is a very important opportunistic pathogen and the most common source of infections on indwelling medical devices (Otto, 2009). Each year, billions of dollars are spent to replace the infected devices such as intravascular catheters, mechanical heart valves, pacemakers, prosthetic joints, contact lenses, urinary catheters and prosthetic implants (Chen *et al*., 2013).

Sub-inhibitory concentrations (sub-MIC_{plank}) of *Phlomis brachydon* essential oil to polystyrene Petri dishes containing a suspension culture of the *E. coli* strain were able to reduce the number of individual cells adhering to the polystyrene surface. In general, bacterial biofilm formation occurs in two stages (Chen *et al*., 2013; Mack, 1999). The initial phase coincides with adhesion of bacteria to the biomaterial surface and this phase is reversible. It is then followed by an irreversible second phase of cell to cell accumulation as multilayered cell clusters and biofilm form. The reduced bacterial adherence to polystyrene caused by PT can be explained by either the alteration of adherence factors present on the bacterial cell surface or by the modification of the polystyrene surface. Such effects could be unrelated to the mechanism by which the essential oil inhibits growth and may only be produced by relatively high levels of the essential oil.

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