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Propolis-Sahara honeys preparation exhibits antibacterial and anti-biofilm activity against bacterial biofilms formed on urinary catheters

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ABSTRACT

Objective: To evaluate the antibacterial effect of Sahara honeys (SHs) against bacterial biofilms formed on urinary catheters in combination with propolis-Sahara honeys (P-SHs).**Methods:** Three clinical isolates were subjected to biofilm detection methods. The antibacterial and anti-biofilm activity for SHs and P-SHs were determined using agar well diffusion and the percentage of biofilm inhibition (PBI) methods.**Results:** The PBI for Gram-positive bacteria [*Staphylococcus aureus* (*S. aureus*)] was in the range of 0%–20%, while PBI for Gram-negative bacteria [*Pseudomonas aeruginosa* and *Escherichia coli* (*E. coli*)] were in range of 17%–57% and 16%–65%, respectively. The highest PBI (65%) was produced by SH2 only on *E. coli*. In agar well diffusion assay, zones of inhibition ranged from 11–20 mm (*S. aureus*), 9–19 mm (*Pseudomonas aeruginosa*) and 11–19 mm (*E. coli*). The highest inhibition (20 mm) was produced by SH1 only on *S. aureus*. In addition, the treatment of SHs and P-SHs catheters with a polymicrobial biofilms reduced biofilm formation after 48 h exposure period.**Conclusions:** SHs and P-SHs applied as a natural agent can be used as a prophylactic agent to prevent the formation of *in vitro* biofilm.

1. Introduction

A number of previous studies have shown the urinary tract colonization and infection caused by *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Escherichia coli* (*E. coli*) in patients with indwelling urinary catheters[1,2]. Al-Mathkhury *et al.*[3] demonstrated the Gram-negative opportunistic *P. aeruginosa* common colonization of urinary catheters and biofilm development on them. Several factors may contribute for the pathogenicity of bacterial biofilm formation, including the production of extracellular compounds (*E. coli*: flagellum; *S.*

aureus: lipopolysaccharides, exopolysaccharide; *P. aeruginosa*: flagella and pili), production of resistant “persister cells”, surface adherence and biofilm formation[4-6]. The adhesion of bacteria to a surface depends on various factors (nutrient levels, pH changes, desiccation, ultraviolet radiation and osmotic stress)[7,8]. More recently, some substances showing antibacterial properties, such as nitrous oxide chlorhexidine, nitrofurazone and gentian violet, have been used to modify the surface of urinary catheters[9]. But the biofilms are notoriously difficult to eradicate. In addition to the difficulty of treating biofilms with conventional antibiotics, recently alternative treatments are playing their role in the treatment of biofilms.

The antimicrobial activities of bee products, such as honey and propolis, have been researched over recent years as alternatives for new therapeutic agents for the treatment of bacterial biofilm infections[10,11]. Algerian honey [Sahara honey (SH)] was reported to inhibit the growth of *S. aureus*, *P. aeruginosa* and *E. coli*[12,13].

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Today, no information is available about the effects of SHs on biofilms. Therefore, this study was performed to investigate the role of SHs at different concentrations alone or in combination with propolis-Sahara honeys (P-SHs) on biofilms. We also investigated the effects of P-SHs on biofilms for the first time.

2. Materials and methods

2.1. Honey and propolis samples

The present study was carried out on raw SH of different floral origins, namely, *Euphorbe* (SH1) (*Euphorbia* spp.) and Sidr honey (SH2). The propolis used in this study was obtained from Southern Algeria.

2.2. Preparation of propolis solutions

The propolis was cold-macerated to make an extract with olive oil (20 g of brute propolis/2 mL of olive oil). The mixture was heated at 50 °C for 15 min before microbiological testing.

2.3. Preparation of honey with olive oil – propolis

The mixture was stirred gently with a spatula until homogeneous gel was formed. The mixture was heated at 50 °C for 15 min. For a microbiological test of a mixture of honey, 20 g of propolis was made, where the honey was added in a concentration of 25%, 50% or 100%.

2.4. Bacterial isolates and growth media

The catheters were removed from patients and then cut under aseptic conditions using a sterile scalpel. The catheter was carefully and aseptically cut. Three discs were placed on the surface of Chapman, MacConkey and King A agar plates. Colony formation was monitored by examining plates after 48 h of incubation.

2.5. Antibacterial susceptibility testing

In this study, two different assays were performed to evaluate the antibacterial potential of the honey samples: agar-well diffusion (AWD) and percentage of biofilm inhibition (PBI).

2.5.1. AWD

Antibacterial studies have been evaluated by the method of AWD by Moussa *et al.*[13]. Briefly, agar plates (90 mm) were containing 20 mL of nutrient agar at 37 °C for 24 h and adjusted by diluting fresh cultures to a turbidity equivalent to 0.5 McFarland

scale (approximately 2×10^8 colony-forming unit/mL). An 8 mm diameter well was cut into the agar and 100 μ L of undiluted, and 25% and 50% honey solution (w/v) prepared in sterile distilled water was aliquoted into the well. The controls were set up with equivalent quantities of water. After incubation, the diameters of the inhibition zones were measured.

2.5.2. PBI

The method adopted was described by Akujobi and Njoku with little modification[14]. Briefly, 0.2 mL of 0.5 McFarland standardised culture was added to 4 mL of the test (SHs and P-SHs). Concentration in a test tube while inoculation of 4 mL of nutrient broth with 0.2 mL of the cell suspension was served as the control. The optical density (OD) was determined in a spectrophotometer at 620 nm prior to incubation (T0) and recorded after the cultures were incubated for 24 h in the dark at 37 °C. The OD was determined at T0 and again after 24 h of incubation at 620 nm. The OD for each replicate at T0 was subtracted from the OD for each replicate after 24 h of incubation. The PBI was calculated using the following formula:

$$\text{PBI}\% = [(\text{OD control} - \text{OD experimental})/\text{OD control}] \times 100$$

OD = absorbance at 620 nm.

2.5.3. Biofilm response to SHs and P-SHs

The bacterial anti-adhesive activity of the SHs and P-SHs against bacterial biofilms was qualitatively evaluated by the following method (Table 1).

Table 1

Exposure of SHs and P-SHs treatment on bacterial biofilm.

| Tube | Experiment I | Treatment after 24 h | Experiment II | Incubation |
|--------|---------------------------|---------------------------|--|------------|
| Tube 1 | Nutrient broth + catheter | Negative control | Nutrient broth + catheter bacterial (single and mixed) | 48 h |
| Tube 2 | Nutrient broth + catheter | SHs (25%, 50% and 100%) | Nutrient broth + catheter bacterial (single and mixed) | 48 h |
| Tube 3 | Nutrient broth + catheter | Propolis | Nutrient broth + catheter bacterial (single and mixed) | 48 h |
| Tube 4 | Nutrient broth + catheter | P-SHs (25%, 50% and 100%) | Nutrient broth + catheter bacterial (single and mixed) | 48 h |

Tube 1: Sterile catheter segments were immersed in 5 mL sterile culture tubes nutrient broth and incubated at 37 °C for 24 h (Experiment I), and after 24 h, the tubes were inoculated with 100 μ L of bacterial inoculum (2×10^8 cells/mL) and incubated at 37 °C for 48 h (Experiment II); Tube 2: Sterile catheter segments were immersed in 5 mL sterile culture tubes nutrient broth + SHs (25%, 50% and 100%) and incubated at 37 °C for 24 h (Experiment I), and after 24 h, the tubes were inoculated with 100 μ L of bacterial inoculum (2×10^8 cells/mL) and incubated at 37 °C for 48 h (Experiment II); Tube 3: Sterile catheter segments were immersed in 5 mL sterile culture tubes nutrient broth + propolis and incubated at 37 °C for 24 h (Experiment I), and after 24 h, the tubes were inoculated with 100 μ L of bacterial inoculum (2×10^8 cells/mL) and incubated at 37 °C for 48 h (Experiment II); Tube 4: Sterile catheter segments were immersed in 5 mL sterile culture tubes nutrient broth + P-SHs at (25%, 50% and 100%) and incubated at 37 °C for 24 h (Experiment I), and after 24 h, the tubes were inoculated with 100 μ L of bacterial inoculum (2×10^8 cells/mL) and incubated at 37 °C for 48 h (Experiment II).

3. Results

3.1. Antibacterial activity

Figures 1–3 show the PBI data for the bee product tested against Gram-negative and Gram-positive bacteria. PBI for Gram-positive bacteria (*S. aureus*) were in the range of 0%–20%, while they were 17%–57% and 16%–65% for Gram-negative bacteria *P. aeruginosa* and *E. coli*, respectively. The highest PBI (65%) was produced by SH2 only on *E. coli*.

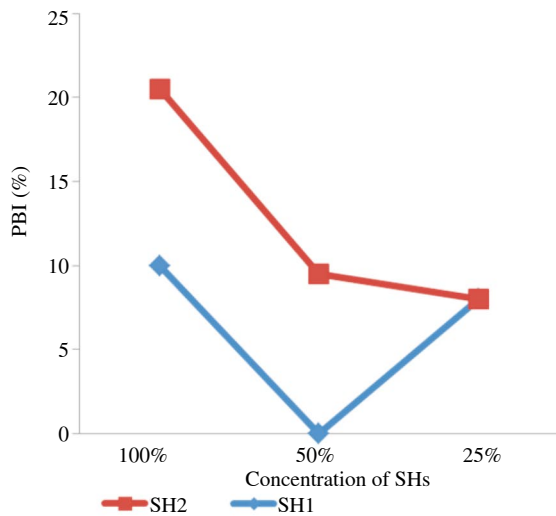


Figure 1. Growth inhibitory activity of the SHs against *S. aureus*.

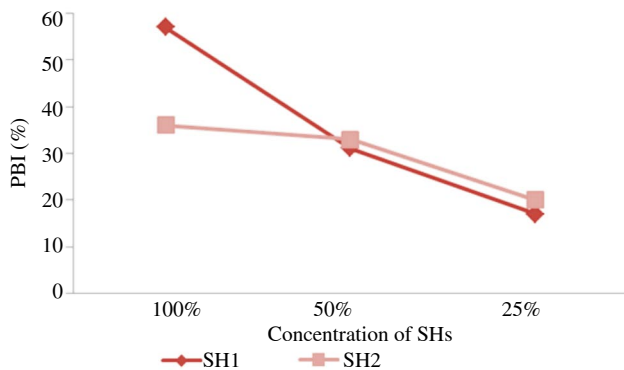


Figure 2. Growth inhibitory activity of the SHs against *P. aeruginosa*.

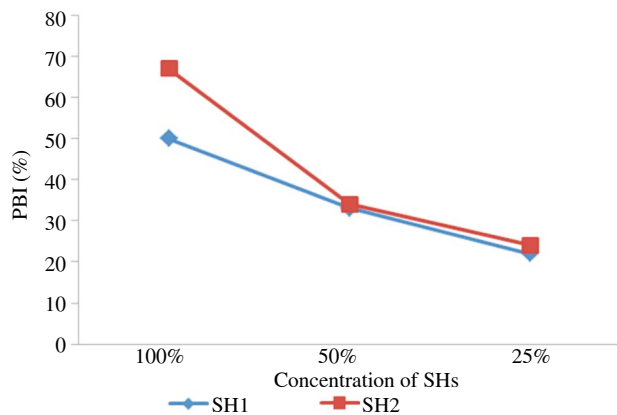


Figure 3. Growth inhibitory activity of the SHs against *E. coli*.

In AWD assays, zones of inhibition ranged from 11–20 mm *S. aureus*, 12–16 mm *P. aeruginosa* and 11–19 mm *E. coli*. The highest inhibition (20 mm) was produced by SH1 only on *S. aureus* (Table 2).

The results of the synergistic effect between SHs and P-SHs are given in Figures 4–6, respectively. In combination with P-SHs, the PBI ranged from 16% to 79% against *S. aureus*, 31% to 87% against *P. aeruginosa* and 22% to 67% against *E. coli*, respectively. The highest PBI (79%) was produced by SH2 in combination with propolis on *S. aureus* (Figure 4).

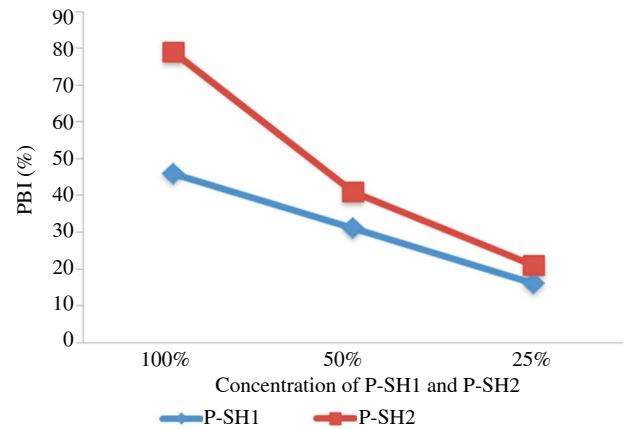


Figure 4. Growth inhibitory activity of the P-SHs against *S. aureus*.

In AWD assays, zones of inhibition ranged from 11 to 20 mm against *S. aureus*, 9–19 mm and 11–19 mm against *P. aeruginosa* and *E. coli*, respectively. The highest concentration required was 100% to simultaneously inhibit all bacteria tested (Table 2).

Table 2

Mean zones of inhibition values of the honey samples against bacterial tested determined by AWD.

| Concentrations | <i>S. aureus</i> | | | <i>P. aeruginosa</i> | | | <i>E. coli</i> | | |
|------------------|------------------|-----|-----|----------------------|-----|-----|----------------|-----|-----|
| | 100% | 50% | 25% | 100% | 50% | 25% | 100% | 50% | 25% |
| SH1 | 20 | 15 | 13 | 16 | 14 | 13 | 17 | 12 | 11 |
| SH2 | 18 | 13 | 11 | 15 | 14 | 12 | 19 | 13 | 11 |
| P-SH1 | 17 | 12 | 11 | 17 | 11 | 9 | 16 | 13 | 11 |
| P-SH2 | 18 | 13 | 11 | 19 | 15 | 12 | 18 | 12 | 11 |
| Negative control | - | - | - | - | - | - | - | - | - |

3.2. Effect of SHs and P-SHs on biofilm-forming bacterial growth

In a second series of experiments, the disruption of preformed biofilms (48-h growth in the absence of SHs and P-SHs) by addition of SHs and P-SHs at various concentrations for 48 h. The SHs and P-SHs inhibited biofilm formation on catheters (Table 3). This suggested that SHs, propolis and P-SHs have a better ability to prevent biofilm formation.

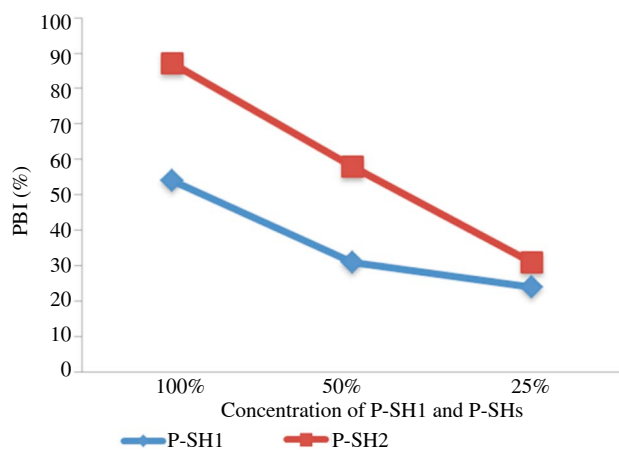


Figure 5. Growth inhibitory activity of the P-SHs against *P. aeruginosa*.

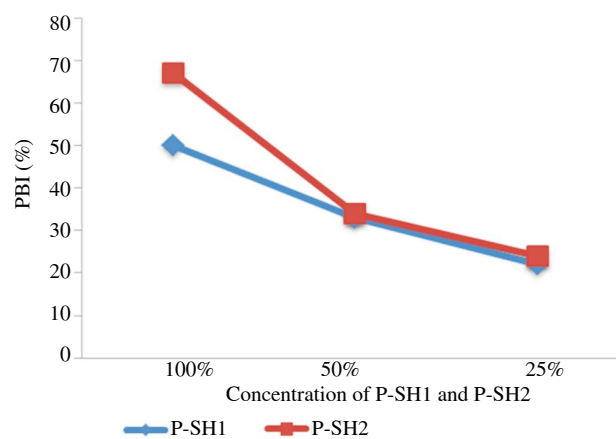


Figure 6. Growth inhibitory activity of the P-SHs against *E. coli*.

Table 3

Effects of SHs and propolis on single and mixed microbial growth and biofilm formation after 48 h of incubation.

| Type of isolates | <i>S. aureus</i> single | <i>P. aeruginosa</i> single | <i>E. coli</i> single | Microbial cultures mixed |
|--|-------------------------|-----------------------------|-----------------------|--------------------------|
| Nutrient broth + catheter (negative control) | BF (++) | BF (++) | BF (++) | BF (++) |
| Nutrient broth + catheter + SHs | BF (--) | BF (--) | BF (--) | BF (--) |
| Nutrient broth + catheter + propolis | BF (--) | BF (--) | BF (--) | BF (--) |
| Nutrient broth + catheter+ SHs + propolis | BF (--) | BF (--) | BF (--) | BF (--) |

BF: Biofilm formation; ++: Adhesion; --: No adhesion.

4. Discussion

Biofilm-producing bacteria are intrinsically resistant to antimicrobial agents, which is a main cause of the pathogenesis of catheter infection[15,16], and the susceptibility of bacteria biofilms to the current therapeutic agents remains low. Currently, researches are focused on the development of anti-biofilm agents that are nontoxic, as it is believed that such molecules will not lead to future drug resistance[17]. Researches aiming at new anti-biofilm originating mainly from bee products have long been revered for their healing. The anti-biofilm properties of bee products as a natural antibiotic agent have been extensively studied. Strong antibacterial activities of propolis and honey against both Gram-positive and Gram-negative bacteria have been reported[11,18]. In addition, several investigators examined the effects of honey and propolis on biofilms. Campeau and Patel[10] reported that Manuka honey has a synergistic interaction with vancomycin against *S. aureus* biofilms and an additive interaction with gentamicin against *P. aeruginosa* biofilms. In addition, Jenkins and Cooper[19] reported that Manuka honey-exposed planktonic *S. aureus* cells were enlarged and had more septa. Cooper *et al.*[20] reported that Manuka honey at concentrations below 10% (w/v) promoted the growth of established biofilms of *S. aureus*. Also, Alandejani *et al.*[21] studied *S. aureus* and *P. aeruginosa* biofilms but only evaluated 50% Manuka and Sidr honey. The therapeutic effects of the propolis and honey are well known. Several aspects of this use indicate that they also have functions such as antibacterial and anti-biofilm properties. Biological activities of honey and propolis are mainly attributed

to the phenolic compounds such as flavonoids[22]. It has recently been reported that several flavonoids reduced biofilm formation in *Vibrio harveyi* and *E. coli* O157:H7[23]. Also, various honeys were investigated for the presence of nitrite/nitrate, the stable nitric oxide metabolites[24]. Barraud *et al.*[25] observed a decrease in biofilm biomass and an increase in planktonic biomass at low concentrations of nitric oxide donors. In addition, honey is a saturated or supersaturated solution of carbohydrates of glucose and fructose[26]. Previous work by Dusane *et al.*[27] has reported that the lauroyl glucose after 48 h of incubation with *P. aeruginosa* and *Pseudomonas aureofaciens* resulted in 51% and 57% of the disruption of preformed biofilms, respectively. Carvacrol (thymol isomer) is present in the essential oil of Algerian propolis (4.47%)[28]. Antibacterial effect of carvacrol and its isomer thymol against *E. coli*, *P. aeruginosa* and *Enterococcus faecalis* have been proved[29]. Several studies have examined the effect of various types of antimicrobial treatment in controlling biofilm formation on these devices [central venous catheters, mechanical heart valves and urinary (Foley) catheters][30]. To our knowledge, this is the first time these novel anti-biofilm agents (SHs and P-SHs) are reported on the tested organisms. SHs, propolis and P-SHs exhibited excellent antibacterial and anti-adhesive properties against *S. aureus*, *P. aeruginosa* and *E. coli*, which was evidenced in our adhesion based assays.

Conflict of interest statement

We declare that we have no conflict of interest.

References

- [1] Hooton TM, Bradley SF, Cardenas DD, Colgan R, Geerlings SE, Rice JC, et al. Diagnosis, prevention, and treatment of catheter-associated urinary tract infection in adults: 2009 International Clinical Practice Guidelines from the Infectious Diseases Society of America. *Clin Infect Dis* 2010; **50**: 625-63.
- [2] Murugan K, Selvanayagi K, Al-Sohaibani S. Urinary catheter indwelling clinical pathogen biofilm formation, exopolysaccharide characterization and their growth influencing parameters. *Saudi J Biol Sci* 2016; **23**(1): 150-9.
- [3] Al-Mathkhury HJ, Ali AS, Ghafil JA. Antagonistic effect of bacteriocin against urinary catheter associated *Pseudomonas aeruginosa* biofilm. *N Am J Med Sci* 2011; **3**: 367-70.
- [4] Hogardt M, Heesemann J. Adaptation of *Pseudomonas aeruginosa* during persistence in the cystic fibrosis lung. *Int J Med Microbiol* 2010; **300**: 557-62.
- [5] Schembri MA, Christiansen G, Klemm P. FimH-mediated autoaggregation of *Escherichia coli*. *Mol Microbiol* 2001; **41**: 1419-30.
- [6] Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. *Annu Rev Microbiol* 1995; **49**: 711-45.
- [7] Flemming HC. Biofilms and environmental protection. *Water Sci Technol* 1993; **27**: 1-10.
- [8] Gilbert P, Das J, Foley I. Biofilm susceptibility to antimicrobials. *Adv Dent Res* 1997; **11**: 160-7.
- [9] Soto SM. Importance of biofilms in urinary tract infections: new therapeutic approaches. *Adv Biol* 2014; doi: 10.1155/2014/543974.
- [10] Campeau ME, Patel R. Antibiofilm activity of Manuka honey in combination with antibiotics. *Int J Bacteriol* 2014; **2014**: 795281.
- [11] Veloz JJ, Saavedra N, Lillo A, Alvear M, Barrientos L, Salazar LA. Antibiofilm activity of Chilean propolis on *Streptococcus mutans* is influenced by the year of collection. *Biomed Res Int* 2015; **2015**: 291351.
- [12] Moussa A, Noureddine D, Mohamed HS, Abdelmelek M, Saad A. Antibacterial activity of various honey types of Algeria against *Staphylococcus aureus* and *Streptococcus pyogenes*. *Asian Pac J Trop Med* 2012; **5**: 773-6.
- [13] Moussa A, Noureddine D, Abdelmelek M, Saad A. Antibacterial activity of various honey types of Algeria against pathogenic Gram-negative bacilli: *Escherichia coli* and *Pseudomonas aeruginosa*. *Asian Pac J Trop Dis* 2012; **2**: 211-4.
- [14] Akujobi CO, Njoku HO. Bioassay for the determination of microbial sensitivity to Nigerian honey. *Global J Pharmacol* 2010; **4**(1): 36-40.
- [15] Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 2002; **15**(2): 167-93.
- [16] Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. *Lancet* 2001; **358**(9276): 135-8.
- [17] Rabin N, Zheng Y, Opoku-Temeng C, Du Y, Bonsu E, Sintim HO. Agents that inhibit bacterial biofilm formation. *Future Med Chem* 2015; **7**(5): 647-71.
- [18] Lu J, Turnbull L, Burke CM, Liu M, Carter DA, Schlothauer RC, et al. Manuka-type honeys can eradicate biofilms produced by *Staphylococcus aureus* strains with different biofilm-forming abilities. *PeerJ* 2014; **2**: e326.
- [19] Jenkins R, Cooper R. Improving antibiotic activity against wound pathogens with Manuka honey *in vitro*. *PLoS One* 2012; **7**: e45600.
- [20] Cooper RA, Jenkins L, Henriques AF, Duggan RS, Burton NF. Absence of bacterial resistance to medical-grade Manuka honey. *Eur J Clin Microbiol Infect Dis* 2010; **29**(10): 1237-41.
- [21] Alandejani T, Marsan J, Ferris W, Slinger R, Chan F. Effectiveness of honey on *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms. *Otolaryngol Head Neck Surg* 2009; **141**: 114-8.
- [22] Viuda-Martos M, Ruiz-Navajas Y, Fernández-López J, Pérez-Alvarez JA. Functional properties of honey, propolis, and royal jelly. *J Food Sci* 2008; **73**(9): R117-24.
- [23] Vikram A, Jayaprakasha GK, Jesudhasan PR, Pillai SD, Patil BS. Suppression of bacterial cell-cell signalling, biofilm formation and type III secretion system by citrus flavonoids. *J Appl Microbiol* 2010; **109**: 515-27.
- [24] Al-Waili NS. Identification of nitric oxide metabolites in various honeys: effects of intravenous honey on plasma and urinary nitric oxide metabolites concentrations. *J Med Food* 2003; **6**(4): 359-64.
- [25] Barraud N, Hassett DJ, Hwang SH, Rice SA, Kjelleberg S, Webb JS. Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. *J Bacteriol* 2006; **188**(21): 7344-53.
- [26] Ansari AA, Alexander C. Effect of natural honey (produced by African scutella in Guyana) against bacteria (*Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus*) and fungus (*Candida albicans*). *World J Dairy Food Sci* 2009; **4**: 73-7.
- [27] Dusane DH, Rajput JK, Kumar AR, Nancharaiah YV, Venugopalan VP, Zinjarde SS. Disruption of fungal and bacterial biofilms by lauroyl glucose. *Lett Appl Microbiol* 2008; **47**(5): 374-9.
- [28] Segueni N, Khadraoui F, Moussaoui F, Zellagui A, Gherraf N, Lahouel M, et al. Volatile constituents of Algerian propolis. *Ann Biol Res* 2010; **1**(2): 103-7.
- [29] Sinha DJ, Sinha AA. Natural medicaments in dentistry. *Ayu* 2014; **35**(2): 113-8.
- [30] Donlan RM. Biofilms and device-associated infections. *Emerg Infect Dis* 2001; **7**(2): 277-81.