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A Comparative study for the Antimicrobial and Antibiofilm Activities of *Trigona* Honey against Opportunistic Microorganisms

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Abstract— Biofilms formed by *Pseudomonas aeruginosa* and *Streptococcus pyogenes* are associated with devastating infections specifically in immunocompromised patients. The emergence of multidrug resistance in bacteria raised the ultimate need to establish an alternative drug to eradicate biofilm infections. Malaysian stingless bee honey (*Trigona*) has been aroused as a potential food with antibacterial and antibiofilm activities. However, there is limited knowledge about the effect of *Trigona* honey on the opportunistic bacteria during the early stage development of infections. Hence, this study aimed to evaluate the devastating effect of*Trigona* honey on *P. aeruginosa* and *S. pyogenes*growth and biofilm formation. The minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Trigona* honey were determined by the broth dilution method. Biofilm formation assay for *P. aeruginosa* and *S. pyogenes* was conducted using microtiter plates. Degradation assay for established biofilms was determined after the tested bacteria were exposed to 20% of *Trigona* honey. *Trigona* honey showed MIC and MBC values of 20% and 25%, respectively against both bacterial strains. *Trigona* honey successfully degraded 45.67% and 61.94% of *P. aeruginosa* and *S. pyogenes* biofilms, respectively.In conclusion *Trigona* honey can be used as a topical agent to prevent and treat bacterial infections.

Keywords: Trigona honey, Biofilm, Antibacterial, Malaysia.

Introduction

Stingless bee honey is a natural syrup with a complex mixture of monosaccharides, such as glucose and fructose, disaccharides, such as sucrose, amino acids, vitamins, minerals, and phytochemical compounds of phenolic acids, and organic acids (1, 2). There are high similarities between the honey of stingless bees and the honey of *Apis* bees in their compositional properties. Nevertheless, several characteristics outbalance the stingless bee honey over the *Apis* honey. These characteristics include moisture content, acidity, viscosity, sucrose content, and the presence of mineral elements (3, 4). Stingless bees are reared on a variety of tropical flowers distributed around their nests and usually, they utilize the botanical flora of over a hundred plants in various seasons. The phytochemical constituents of honey are extensively relying on several factors in characterizing the nutritional and therapeutic values of honey. These factors include the botanical source of nectar, the environmental and climatic conditions, as well as the species of the stingless bee. Additionally, the physicochemical properties of honey are fundamental for providing information to classify the types of honey and determine their nutritional and therapeutic properties (5).

Many clinical applications have been reported for stingless bee honey, such as antioxidant, antimicrobial, anti-inflammatory, anticancer activities, and wound healing properties (6). In addition, stingless bee honey has beneficial effects in treating neurological disorders, urinary tract diseases, and gastrointestinal tract diseases (7). The biological usefulness of honey and its medicinal activities have gained much attention for a long time. Nowadays, enormous techniques are used to study the major biological factors that contribute to the medical applications of honey (8). Honey contains a mixture of a huge number of bioactive compounds which is crucial for medicinal applications in clinical settings (9). Several studies have focused on the bioactive compounds of stingless bee honey and have explored constituents ofvariable and great biological and clinical importance. For instance, phenolic compounds, flavonoids, and other biochemical organic and nonorganic compounds derived from different nectar botanical origins have beneficial effects on health (10, 11).

Materials and methods

Honey Samples

Malaysian *Trigona* honey of *Trigona itama spp.* was selected for the current study and another two Jordanian honey samples; *Centaureahyalolepis* honey, and *Citrus* honey of *Apis melliferaspp.* were used as reference honey samples in the experiments of phytochemical analysis.Three honey samples of Malaysian *Trigona* honey of *Trigona* itama spp. was selected for the current study and another two Jordanian honey samples; *Centaureahyalolepis* honey, and *Citrus honey* of Apis mellifera spp. were used as reference honey samples.Honey Samples were stored in well-closed amber glass bottles, labeled accordingly, and kept in a dark place at room temperature for further investigations. Honey samples have been freshly prepared for each experiment.

Bacterial Strains

Two bacterial strains were used in this study, Gram-negative bacteria *Pseudomonas aeruginosa*ATCC10145 and Gram-positive bacteria *Streptococcus pyogenes*ATCC19615. The bacterial strains were provided by the microbiology laboratory of the Faculty of Medicine, Universiti Sultan Zainal Abidin, and Malaysia. The bacterial strains were purchased from the American Type Culture Collection (ATCC) organization.

Bacterial Culture

Three morphologically identical colonies from each bacterial strain culture were aseptically picked up and grown aerobically in 20 mL of tryptic soy broth (TSB)(Sigma-Aldrich, Belgium) in a shaker incubator at 150 rpm at 37 ^{o}C for 24 hours for further investigations.

Viable Bacterial Count for *P. aeruginosa* and *S. pyogenes*

To achieve an accurate bacterial count to be utilized in biofilm experiments, an overnight culture of *P. aeruginosa*ATCC10145 and *S. pyogenes*ATCC19615 bacteria were incubated at 37 ^{o}C for 16 hours in nutrient broth (NB) with constant shacking at 150 rpm. Afterward, to count the viable bacterial number in the media and to standardize the absorbance against the bacterial number, the absorbance of the overnight culture was adjusted to around 0.1 using a spectrophotometer at 600 nm wavelength (McFarland standard of 0.5). A Series of dilutions was conducted for the bacterial suspension in a ratio of 10-fold dilution. Following that, 100 µL of the serial dilution was applied on nutrient agar plates and dispensed using an L shape dispenser. Then, the plates were incubated at 37 ^{o}C for 16 ± 2 hours. Following the incubation, the colonies on each plate were counted individually and the optical density at 600 nm (OD_{600}) for the appropriate dilution was adopted for further investigations. The

number of colonies for each bacterial strain was counted on the corresponding countable plate and the colony-forming unit per mL (CFU/mL) was calculated using the following formula:
CFU/mL = (no. of colonies x dilution factor) / volume of the culture plate.
Eventually, the viable bacterial count for each bacterial strain was adjusted to approximately 1.0×10^8 CFU/mL (12).

Antimicrobial Activity of the Honey Types

To determine the antimicrobial activity of the three honey samples against the microbial strains of*P. aeruginosa* ATCC10145 and *S. pyogenes*ATCC19615, the agar well diffusion method was conducted. The standard protocols of the antibacterial susceptibility testing according to the Kirby Bauer disk diffusion susceptibility test were applied with modifications (13, 14).

Initially, bacterial strains were grown at 37 °C for 22 ± 2 hours in nutrient broth. Muller Hinton agar was used for conducting the agar well diffusion test. The bacterial concentration was adjusted according to the turbidity of 0.5 McFarland standard. A sterile swab was used for inoculation by soaking it in the bacterial suspension and streaking it on the Muller-Hinton plates. Pores of 9 mm were bunched on the agar to allow proper filling of the honey samples. As a control for the test, a reference drug ciprofloxacin was used as a positive control for the antibacterial activity. Bacterial plates were incubated at 37 °C, and the growth of the microbial cells was evaluated after 22 ± 2 hours by measuring the zone of inhibition around the wells. The test was performed in triplicate and the average value for each bacterial strain was considered.

Determination of Minimum Inhibitory Concentration of *Trigona* Honey against *P. aeruginosa*and *S. pyogenes*

For the determination of the MIC of *Trigona* honey against two strains of *P. aeruginosa* ATCC10145 and *S. pyogenes*ATCC19615. Standard protocols of antibacterial susceptibility testing according to the Kirby Bauer test were applied with modifications (15, 16).

Overnight culture of the bacterial strains was grown at 37 °C in nutrient broth. Subsequently, the bacterial growth of both *P. aeruginosa* and *S. pyogenes* was adjusted using a spectrophotometer (Eppendorf, USA) to around 0.1 OD_{600} in Muller Hinton broth to be equivalent to 0.5 McFarland standard for the determination assay. Five concentrations of *Trigona* honey including 10%, 20%, 30%, 40%, and 50 % (w/v) were prepared and added to test tubes containing bacterial suspension. Following that, the tubes were incubated at 37 °C and the growth of microbial cells was evaluated after 22 ± 2 hours by naked eyes to examine the turbidity in the tubes. Finally, the tubes were inoculated on Muller Hinton agar and the lowest honey concentration that showed bacterial growth was considered the MIC concentration, while the lowest honey concentration without bacterial growth was considered MBC concentration as it has a bactericidal effect (17).

Biofilm Formation and Quantification for *P. aeruginosa*and *S. pyogenes*

A biofilm formation assay was performed using the crystal violet method in 96well microtiter plates (Fisher Scientific, UK). Bacterial cultures of *P. aeruginosa*ATCC10145 and *S. pyogenes*ATCC19615 were grown separately overnight in a TSB at 37 °C with constant shaking at 150 rpm. The optical density at 600 nm for *P. aeruginosa*and *S. pyogenes*cultures was adjusted to 0.25 OD_{600} and 0.65 OD_{600}, respectively into a fresh TSB supplemented with 1% (w/v) glucose to be relevant to 1×10^8 CFU/mL. For each type of bacteria, 200 µL of the bacterial suspension was pipetted into each well in 96-well microtiter plates, growth medium without bacteria was used as a negative control. The plates were incubated at 37 °C for 24 hours. After incubation, the bacterial cells were discarded by inverting the plates upside down and washed with distilled water to remove any excess unattached cells and allowed to be air-dried. After that, 200 µL of 0.1% crystal violet solution was added to each well of

the microtiter plate and incubated at room temperature for 15 minutes. Following the incubation, the plates were rinsed with phosphate buffer saline (PBS)(Invitrogen, UK) three times to remove the unabsorbed stain, then the plates were tapped gently on a towel paper (18, 19).

For quantification of biofilm formation, 200 µL of 95% ethanol was added to each well to solubilize the stained biofilms. Finally, the absorbance at a wavelength of 570 nm was measured using an Elisa plate reader (Tecan Infinite 200 PRO, Austria).

The experiment was performed in triplicates, and the average value was considered.

The classification of the biofilm formation for each bacterial strain was measured using the following formulas: Non-adherent (NA= $OD \leq OD_C$), Weak adherent (WA= $OD_C < OD \leq (2\times OD_C)$), Moderate adherent (MA= $(2\times OD_C) < OD \leq (4\times OD_C)$) and strong adherent (SA= $(4\times OD_C) < OD$)(20)(18).

Results

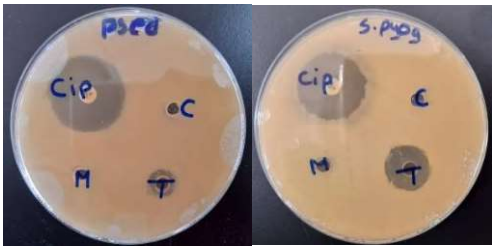
Antimicrobial Activity of the Honey Types

A wide variation in the antimicrobial activity of the analyzed honey types determined by the Kirby Bauer disk diffusion susceptibility test was observed. The antimicrobial activity of *Trigona* honey against *P. aeruginosa*and*S. pyogenes* is demonstrated in Table 1 and Figure 1. *Trigona* honey showed a wide zone of inhibition against the bacterial strains. Meanwhile, *C. hyalolepis* and *Citrus* honey did not show a noticeable antimicrobial activity against both bacterial strains. Both bacterial strains showed susceptibility toward ciprofloxacin (21) which was used as a positive control for bacterial susceptibility.

Table 1 Zone of inhibition diameters for the honey types against *P. aeruginosa*and*S. pyogenes.*

Type of honey	*P. aeruginosa*	*S. pyogenes*
Trigona Honey	11 ± 1.3	15 ± 1.7
C. hyalolepis Honey	-	-
Citrus Honey	-	-
Ciprofloxacin*	28 ± 2.1	25 ± 1.9

*Ciprofloxacin**:** positive control.



T: *Trigona* honey, H: *C.hyalolepis*honey, C: *Citrus* honey, Cip: Ciprofloxacin.

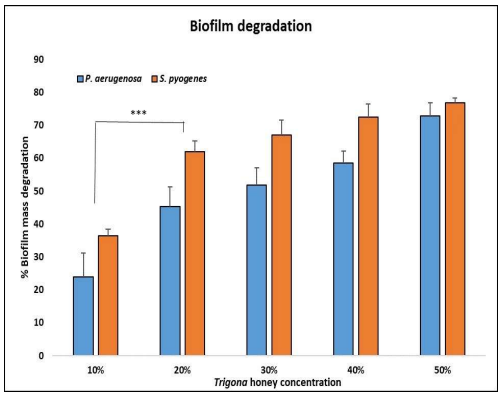
Figure 1: Antimicrobial activity of *Trigona*, *C.hyalolepis,* and *citrus* honey against*P. aeruginosa*and*S. pyogenes.*

Biofilm Degradation Activity of *Trigona* Honey against *P. aeruginosa* and *S. pyogenes*

Trigona honey successfully degraded the established biofilms of both *P. aeruginosa* and *S. pyogenes* using concentrations ranging from 10% to 50% (w/v). *Trigona* honey exhibited a 23.8%, 45.6%, 52%, 58.8%, and 73% reduction in *P. aeruginosa*biofilms following treatment with 10%, 20%, 30%, 40% and 50% (w/v), respectively. Whereas, there was a relevant reduction in *S. pyogenes*biofilms of

36.48%, 61.9%, 67%, 72.5% and 76.8% following treatment with 10%, 20%, 30%, 40% and 50% (w/v), respectively. These findings showed that *Trigona* honey exerted a sequential decomposition of the biofilm biomass for both bacterial strains in relevance to the concentration of honey used in treatment, as shown in Appendix A.1 and A.2.

Figure 2 demonstrates a clear biofilm degradation activity of *Trigona* honey against both bacterial strains of *P. aeruginosa* and *S. pyogenes* at concentrations from 20% to 50% (w/v). Despite the concentration of 10% (w/v) showed a biofilm degradation activity, there was a highly significant difference ($p \leq 0.001$) in the degradation activity between 10% and 20% concentrations. This result supported the usage of the 20% (w/v) concentration of *Trigona* honey which is the MIC against both bacterial strains in the microarray experiment.



*** $p \leq 0.001$

Figure 2:Percentages of biofilm degradation for *P. aeruginosa*and *S. pyogenes*.

Discussion

In the regions of Northern Australia, Asia, and South America stingless bee honey is highly valued and appreciated in the traditional practice(22). These considerations were raised from the usage of honey as an essential alternative medicine in treating many illnesses (10). The importance of honey has been addressed and proven scientifically during the healing process for the treatment of several infections, such as skin bruises, ulcers, wounds, eye, and ear infections (23). Many scientists reported broad-spectrum antimicrobial activity of stingless bee honey against Gram-positive bacteria, such as *S. aureus*, *S. pyogenes,* and *Bacillus cereus*, Gram-negative bacteria, such as *Escherichia coli, Salmonella serotype Typhimurium* and*P. aeruginosa*. In addition to antifungal activity against *Candida albicans*. Therefore, stingless bee honey has been used in treating various infectious diseases caused by different pathogenic microorganisms (24)(7)(25).

Various tribes and cultural communities believe in the superiority of stingless bee honey over other types of honey. The aboriginal people of Australia and Latin American trust that honey produced by bees derived from the *Meliponini* tribe is more valuable and has stronger medicinal and antimicrobial effects rather than honey produced by other bee tribes (26).

Antimicrobial Activity of the Honey Types

Honey is a natural product with a great medicinal grade and valuable nutritional characteristics. One of the main characteristics of honey is the ability to defend against bacteria and fungi species. The antimicrobial activity of honey is attributed to several factors, such as low pH level, containing hydrogen peroxide, high sugar content, and bee defensin-1 secreted by the bee (27). Several studies have revealed that the concentration of the individual constituents in honey is substantially very low

to exert antimicrobial activity. However, the therapeutic characteristic of honey is attributed to the synergistic effect of combining different ingredients of the phytochemical compounds (6)(28).

In the current study, *Trigona* honey showed a wide range of antibacterial activity over the other two kinds of *Apis,* honey. *Trigona* honey showed a high zone of inhibition measurements. Consistently, the phytochemical analysis revealed the highest concentration of polyphenolic compounds, and the lowest pH value of *Trigona* honey. Substantially, these properties confer significant antimicrobial and antibiofilm activities.

Several regimens in alternative medicine recommend the use of specific honey types, such as Sidr honey from Yemen, Manuka honey from Australia, and stingless bee honey from tropical and subtropical regions due to their exceptional therapeutic effects. Meanwhile, several types of honey are used on a commercial scale as food and dietary supplements. Different types of honey vary in their antibacterial activity. A study conducted by Nishio*,* on two types of Brazilian stingless bee honey against Gram-positive and Gram-negative bacterial strains showed cell wall disruption and inhibition of cellular division. *ScaptoTrigonabipunctata* stingless bee honey caused zone of inhibition diameters of 12 mm for *P. aeruginosa*, 14 mm for *S. pyogenes,* and 19 mm for *S. aureus*. Whereas treating the same bacterial strains with*ScaptoTrigonapostica* stingless bee honey results in a zone of inhibition diameters of 8 mm for *P. aeruginosa*, 8 mm for *S. pyogenes,* and 15 mm for *S. aureus* (29). Additionally, Ghramh*,* demonstrated the antimicrobial activity of Sidr honey from Saudi Arabia against *P. aeruginosa* and *E. coli* which caused inhibition of bacterial growth on agar plates with a zone of inhibition diameter of 11.9 mm for *P. aeruginosa* and 13.5 mm for *E. coli*(30).

Determination of MIC of *Trigona* Honey against *P. aeruginosa* and *S. pyogenes*

Several plants or plant-derived products show an ability to suppress the growth of infectious microorganisms. This feature provides value for such products in medical uses over other plants with no therapeutic activities. For instance, some plants show strong antimicrobial activity while other plants suffer from recurrent microbial infections. The therapeutic properties are related to the presence of bioactive compounds with antimicrobial activity. Honey is a well-known plant-derived sugary product with antimicrobial, antioxidant, and wound-healing activities (31).

Malaysian stingless bee honey of *Trigona* species was investigated deeply in this research based on the high phenolic and flavonoid content, as well as, the robust antimicrobial activity over the two Jordanian reference honey of *Apis* species. *Trigona* honey showed a strong MIC value of 20% (w/v). This concentration was sufficient to cause an inhibitory effect against both bacterial strains. These results are congruent with Zainol*,* who reported a MIC value of 20% (v/v) for *Trigona* honey against *P. aeruginosa*and *E. coli*(32). Nweze reported MIC values ranged between 12.50% and 25% (v/v) of *HypoTrigona* honey against isolates of multidrug-resistant *E. coli* and 6.30% to 25% (v/v) of *Melipona* honey against*P. aeruginosa*(24). Moreover, Maddocksshowed distorting the planktonic cells of *S. pyogenes* at MIC of 20% Manuka honey (33).

On the other hand, other types of *Apis* honey showed weak antibacterial activity when compared with *Trigona* honey. As Bhushanam*,* reported that the MIC of *Apis* honey against a set of pathogenic isolates was 25% (v/v)(34). Likewise, E. Brown reported a lower MIC of 16% (v/v) for stingless bee honey against four common pathological strains of *S. aureus*, *E. coli*, *S. pyogenes,* and *Haemophilus influenza* compared with the MIC of 32% (v/v) for *Apis* honey against same strains (35). These findings suggest that the variation in the antimicrobial activity of different honey kinds is attributed to

several factors, such as bee species, geographic origin, and type of multiflora, as well as, other factors like the handling procedure, storage duration between sampling and conducting the experiments. Such factors can exaggerate or reduce the antimicrobial activity of different honey types (36).

Biofilm Formation for *P. aeruginosa*and *S. pyogenes*

Biofilms can be explicitly described as communities of microorganisms that are attached to a surface. They encompass one single kind of microorganism or multiple microorganisms that are attached to a surface (31). Biofilms are dominant in many environments and establish a variety of infections on the surfaces of medical devices and implants. *P. aeruginosa*is one of the most commonly biofilm-forming Gram-negative bacteria. Likewise, the Gram-positive bacteria, *S. pyogenes*can establish strong biofilms on many surfaces (37).

In biofilm development, there are gross morphological changes occur on the level of the individual cells and the lifestyle of microbes (38). There are several methods for the determination of biofilm formation and the extent of the biofilm strength, such as crystal violet staining and observation by confocal microscope (39). In this study, the crystal violet method was used to determine the biofilm mass for the two opportunistic bacteria in the conditions of the presence or absence of *Trigona* honey. Both bacterial strains showed strong biofilms. Similarly, Benedikt*et al.* reported a strong biofilm formation for*S. aureus*, *P. aeruginosa,* and *S. pyogenes*on 17 differentsurfaces(37). Moreover, Kirchhoff *et al.* showed a strong biofilm formation for both *P. aeruginosa*and *S. pyogenes*on cochlear implantsat 24 hours (40).

Several factors affect the formation of biofilm biomass, such as nutrient availability, temperature, surface type, and quorum sensing (41). In this study, the conditions for biofilm formation were adjusted to achieve representative biofilms, the findings were consistent with the results of other research groups. Karami*,* reported a strong biofilm formation of *P. aeruginosa*from clinical isolates in Luria Bertani medium following incubation for 24 hours at 37 ^{o}C(42). Additionally,Matysikreported a strong biofilm formation of *S. pyogenes* incubated for 24 hours in a Todd-Hewitt liquid medium supplemented with0.5% (w/v) glucose (43). The modifications in the initial bacterial inoculum, the type of culture media, and the incubation temperature have drastically affected the biofilm formation. Concomitantly, several studies point out that environmental signals are an essential driving force for microbial development.
Furthermore, environmental cues play a vital role in biofilm formation (41)(44).

Biofilm Degradation Activity of *Trigona* Honey against *P. aeruginosa* and *S. pyogenes*

It is a well-known fact that the formation of bacterial biofilms leads to establish persistent infection in hospitalized patients. Biofilms provide a protective shield for bacterial communities against different antimicrobial agents(45). Biofilms do not only protect bacteria but also supply nutrients. Furthermore, the bacteria grown in biofilms can easily detach from the polysaccharide layer to establish a secondary infection (46).

Currently, an alternative medicine using bee-based products as a treatment for human diseases, known as apitherapy, is being practiced (47)(48). Several research groups have studied the antibacterial and antibiofilm activities of honey against pathogenic microorganisms, such as nosocomial bacteria, as well as food spoilage bacteria (49)(50). In this study, the MIC of 20% (w/v) of *Trigona* honey has successfully degraded the biofilms formed by the two opportunistic bacteria of

P. aeruginosa and *S. pyogenes*. Since MIC is the lowest concentration that provides inhibition of planktonic bacteria, it also showed a capability to cause a biofilm degradation effect. *Trigona* honey affects the integrity of bacterial cell wall through altering the cellular structure and pertaining a lytic effect on*P. aeruginosa* and *S. pyogenes*(20). A study has reported that Manuka honey at a concentration of 12% (w/v) affected the structure and viability of *P. aeruginosa*(51)(52). Additionally, a study conducted using Manuka honey showed that a concentration of 10% (w/v) honey affected the structure of *S. aureus*(53). Moreover, Maddocksdemonstrated the percentages of biofilm degradation for clinical isolates following treatment with Manuka honey. They reported 33% degradation for *P. aeruginosa* biofilms following treatment with MIC of 10% Manuka honey. Whereas, the MIC of 20% Manuka honey degraded the *S. pyogenes* biofilms by 27% and the MIC of 8% Manuka honey degraded the *S. aureus* biofilms by 30% (54). Additionally, the reduction in *S. pyogenes* biofilm established for 24 hours was 77% following two hours of treatment with 20% Manuka honey. Whereas the treatment with 10% Manuka honey resulted in a 72% reduction in *S. pyogenes* biofilm (33).

According to the data generated from the biofilm degradation test, *Trigona* honey has a strong capability in all concentrations to exert biomass degradation of both strains of *P. aeruginosa* and *S. pyogenes*. This degradation was not only limited to the high concentration of *Trigona* honey of 50% (w/v). On the contrary, the concentration of 10% (w/v) honey showed also biodegradation for the bacterial biofilms. The antibacterial and antibiofilm activities of honey are mainly attributed to four main reasons, such as the acidity of honey, the activity of non-hydrogen peroxide compounds, the effect of high osmotic substances, and the presence of phytochemical compounds (55, 56). Combining these factors is beneficial in controlling bacterial colonization and additionally disrupting biofilms. Honey acidity is accounted as one of the major limiting factors for preventing bacterial growth in the environment and inhibiting the bacterial contamination of honey and honey-based products. The low pH value of *Trigona* honey donates an unfavorable condition for bacterial growth as the preferred pH value for bacterial growth is in the range of 7.2 to 7.4. The acidity of honey is related to the reaction of glucose with water and oxygen which give rise to gluconic acid (5). Another limiting factor for bacterial contamination is the high osmotic effect of honey as the high sugar content in honey will produce a hyperosmotic solution of honey which in turn will extract water from any microorganism that will grow in honey (5). In addition to the mentioned factors, the presence of phytochemical components in the honey, such as flavonoids, phenols, and tannins have a robust inhibitory effect on bacterial growth and antibiofilm property. The inhibitory mechanism of these compounds is exerted through the antioxidant effect or through altering the cellular signaling pathways to alter bacterial growth or transform the phenotype from a sessile to a planktonic form (57).

Conflict of interest

The authors declare no conflict of interest

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