



Original Research Article

Antibacterial Activity of Some Types of Monofloral Honey Against *Clostridium acetobutylicum* and *Clostridium perfringens*

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A B S T R A C T

The aim of the present study was to evaluate the antibacterial activity of eleven types of monofloral honey as well as to evaluate their synergistic effect with sulphamethoxazole against *Clostridium acetobutylicum* DSM1731 and *Clostridium perfringens* KF383123 using agar well diffusion method. Flavonoid content for all types of tested honey was also measured by folin-ciocalteu reagent. The best antibacterial activity against *C. perfringens* KF383123 was shown by Palm honey with a zone of inhibition of 31.33 ± 0.67 mm and it showed also the highest value of total flavonoid content ($11.68 \mu\text{g}/100\text{g}$ honey). The antibacterial activity of Palm honey was followed by Acacia and Cotton honey with similar zones of inhibition. On the other hand Coriander, Acacia, Cotton, Clover, Commercial Citrus and Commercial Clover honey showed no hindrance activity against *C. acetobutylicum* DSM1731. Seven common antibiotics were used as reference antibacterial agents. All types of tested honey exhibited synergistic effect against both tested clostridia strains when combined with sulphamethoxazole with the highest effect was shown by Sider honey with zones of inhibition of 46.33 ± 0.88 and 26.00 ± 0.58 mm against *C. perfringens* KF383123 and *C. acetobutylicum* DSM1731, respectively. The results indicated that different types of monofloral honey exhibited different antibacterial activity against clostridial strains when used separately and exhibited different synergistic effect when used in combination with sulphamethoxazole. These results suggest the possibility of using honey either separately or in combination with antibiotics to overcome the growing problem of antimicrobial resistance among clostridia strains.

Keywords

Anti-microbial activity,
Honey,
Clostridium acetobutylicum,
Clostridium perfringens;
Well diffusion assay.

Introduction

The genus *Clostridium* consists of over 100 species, many of them are closely related to both human and animal health (Miyakawa et al., 2007, Dong et al. 2010, ESR, 2010). *C. perfringens* (CPE) is an important example

of clostridium species that causes a wide range of enterotoxemic and histotoxic diseases in both humans and animals (McClane, 2007 and ESR, 2010). On the other hand, some species are of

biotechnological importance including *C. acetobutylicum* which is used for solvent production (Jones and Woods, 1986). There are five types of *C. perfringens* based on toxin type (A, B, C, D, and E). Due to the fact that the intestine is an environment that favors CPE to multiply and sporulate leading to clinical manifestation as diarrhea (Cheung et al., 2010). CPE plays an important role in the pathogenesis of both food-borne and non-food-borne human gastrointestinal illnesses (McClane, 2001). Antibiotic resistance of *C. perfringens* strains are becoming a major health concern (Tansuphasiri et al. 2005).

Honey is collected by bees, primarily from floral nectars. Fructose and glucose are the major components and large numbers of other chemical compounds are existed in small quantities as well as low moisture (The British Pharmacopeia, 1993). It contains flavonoids, phenolic acids, ascorbic acid, tocopherol, catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH), and peptides (Hegazi 2012 and Eteraf-Oskouei & Najafi, 2013). Honey has been used in medical practice since ancient times (Ayaad et al., 2009 and Smith et al, 2009). The use of honey as therapeutic substance has been rediscovered due to its ability to inhibit both Gram-positive and Gram-negative bacteria (Hegazi, 2011; Hegazi and Abd Allah, 2012 and Khalil et al., 2013). Honey exhibits a variety of biological activities including antioxidant activity (Frankel et al., 1998 and Hegazi and Abd El-Hady, 2009). It has been also used to treat several conditions including bed sore cure (Tousson et al., 1997), bacterial gastroenteritis in infants (Haffejee and Moosa, 1985) and liver disease (Yoirish, 1977). The antibacterial activity of different types of honey was studied by many authors (Molan et al. 1994; Chute et al., 2010; Kwakman et al., 2010;

Halawani and Shohayeb 2011, Hegazi 2011, Hegazi & Abd Allah, 2012 and Hegazi et al. 2002, and 2014a, b and c). Hammond and Donkor (2013) investigated susceptibility of *C. difficile* to Manuka honey and they concluded that Manuka honey may offer an effective treatment to infections caused by *C. difficile*. The aim of the present investigation was to evaluate the total flavonoid contents and antibacterial activity of eleven types of monofloral honey as well as their synergistic combination with sulphamethoxazole against *C. acetobutylicum* DSM1731 and *C. perfringens*.

Materials and Methods

Honey samples

A total of eleven monofloral types of honey produced by bees collecting nectar from predominantly one plant species were used in this experiment. Ten types were obtained from Egypt and one (Sider honey) was kindly provided by El-Yahia Company, Saudi Arabia (2011, flowering season). Eight out of the ten types collected in Egypt were from apiary farm including Acacia, Coriander, Citrus, Sesame, Eucalyptus, Cotton, clover and Palm. The last two types were Commercial Citrus and Commercial Clover obtained from local market in Egypt. Honey samples were stored at 5°C in dark glass containers to prevent photo degradation until being used (Pimentel et al., 2013).

Preparation of microbial suspensions

Two clostridium reference strains were used in this study including *Clostridium acetobutylicum* DSM1731 and *Clostridium perfringens* KF383123. A suspension of bacterial strain was freshly prepared by inoculating fresh stock culture from the tested reference strain into broth tube

containing 7 ml of Muller Hinton Broth. The inoculated tubes were incubated anaerobically at 37°C for 24 h. Serial dilutions were carried out for each strain, dilution matching with 0.5 Mc-Farland scale standard was selected for screening of antibacterial activities.

Antibacterial activity of pure monofloral honey brands using agar-well diffusion method

The antibacterial activity of honey against the tested bacterial strains was evaluated by using agar-well diffusion method (Katirciolu and Mercan, 2006). A volume of 100 µl of cell culture suspension matching with 0.5 Mc-Farland of target isolate was spread onto the plates. To investigate the antibacterial activity, 50 µl of different honey samples were added in individual wells. Plates were left for 1 h at 25 °C to allow a period of pre-incubation diffusion in order to minimize the effect of variation in time between the applications of different solutions. The plates were re-incubated anaerobically at 37 °C for 24 h to allow bacterial growth. After incubation, plates were observed and the zones of inhibition were measured to evaluate the antimicrobial activity for each of the tested honey samples. The experiment was carried out in triplicates for statistical relevance and the Mean± SE of results was calculated.

Antibiotic sensitivity testing (AST)

Seven common antibiotics were used as reference in this study including Cefotaxime (30 µg/disc) CTX, Ciprofloxacin (5µg/disc) CIP, Erythromycin (15 µg/disc) E, Oxytetracycline (30 µg/disc) OT, Cephalexin (Cephem/Cephalosporin I) (10 µg/disc) CN, Tobramycin (30 µg/disc) TOB and Sulphamethoxazole (100 µg/disc) RL. Antibiotic susceptibility was determined

using disc diffusion method according to the guidelines published by the British Society for Antimicrobial Chemotherapy (BSAC) standardized disc susceptibility testing method (Andrews, 2007), except that Mueller-Hinton agar (MHA; Oxoid, Cambridge, UK) was used in place of iso sensitest agar (Poilane et al., 2007).

Testing for synergistic combinations of honey and Sulphamethoxazole by AST

To evaluate the combined antibacterial activity of different types of honey and Sulphamethoxazole to check if there is any synergistic activity, disc diffusion tests were repeated on MHA. Sulphamethoxazole disk used for sensitivity test was saturated with 50µl of one type of honey at a time. The same procedure as in AST was applied. The experiment was carried out in triplicates for statistical relevance and the Mean± SE of results was calculated. The resulted means were compared with both the means obtained when each type of honey was used alone as well as the means obtained from Sulphamethoxazole discs alone to check the presence of synergism.

Measurement of Total Flavonoid Content Using Folin-Ciocalteu Assay

Total phenolic contents of different types of honey were determined spectrophotometrically according to the Folin-Ciocalteu colorimetric method (Singleton et al., 1999). Total flavonoid content was determined using the method of Meda et al. (2005) with minor modifications. In brief, 0.25 mL of sample (0.1 mg/mL) was added to a tube containing 1 mL of double-distilled water followed by 0.075 mL of 5% NaNO₂, 0.075 mL of 10% AlCl₃ and 0.5 mL of 1 M NaOH at 0, 5 and 6 min, sequentially. Finally, the volume of the reaction solution was adjusted to 2.5 mL

with double-distilled water. The absorbance of the solution was measured at 410 nm wave length using a spectrophotometer. Caffeic acid, a ubiquitous flavonoid was used as a standard to quantify the total flavonoid content of honey and the results were expressed in microgram Catechin Equivalents (CE) $\mu\text{g}/100\text{g}$ honey.

Statistical analysis

The *in vitro* antibacterial activity was conducted in triplicate. The data were then subjected to SPSS Ver. 21 (IBM, New York, US) software for statistical analysis. Duncan Test of Post Hoc Multiple Comparisons in one way ANOVA was applied for comparison between and within the groups. All the data were given mean \pm standard deviation (SD). A probability value $P < 0.05$ was taken as significant (Steel and Torrie, 1980).

Results and Discussion

Eleven honey samples were obtained from Egypt and Saudi Arabia as following: eight monofloral honey types collected from apiary farm in Egypt including Acacia, Coriander, Citrus, Sesame, Eucalyptus, Cotton, Clover, and Palm; Sider honey was kindly provided from Saudi Arabia; and two Commercial samples including Citrus and Clover were obtained from local market in Egypt. The antibacterial activity was evaluated according to the following criteria: zone of inhibition range >18 showed significant activity, 16-18 good activity, 13-15 low activity, 9-12 non-significant activity, and <8 no activity. The antibacterial activity of different types of honey against *C. acetobutylicum* DSM1731 and *C. perfringens* KF383123 is shown in (Chart 1). The results revealed that the antibacterial activity of different types of honey against *C. perfringens* KF383123 was

higher than that against *C. acetobutylicum* DSM1731. The highest antibacterial activities against *C. perfringens* KF383123, was exhibited by Palm with zone of inhibition of 31.33 ± 0.67 mm. This was followed by Acacia, Cotton and Commercial Clover honey with zones of inhibition of 30.33 ± 0.88 mm for both Acacia and Cotton; and 30.00 ± 0.58 mm for Commercial Clover. Lower activities was shown by Coriander and Sider honey with zone of inhibition of 29.67 ± 0.33 mm for both; and Citrus honey with zone of inhibition of 29.67 ± 0.88 mm. The zone of inhibition for Sesame honey was 29.17 ± 0.60 mm while the zone of inhibition for both Clover, and Commercial Citrus was 29.00 ± 0.58 mm. On the other hand Eucalyptus honey showed the least antibacterial activity against *C. perfringens* KF383123 with the least zone of inhibition (9.00 ± 0.58 mm). On the contrary, Eucalyptus honey showed the highest activity against *C. acetobutylicum* DSM1731 with highest zone of inhibition of 25.00 ± 0.58 . This was followed by Sesame and Palm with zones of inhibition of 18.33 ± 0.88 and 15.67 ± 0.33 mm respectively. Meanwhile, Coriander, Acacia, Cotton, Clover, Commercial Citrus and Commercial Clover honey showed no hindrance activity against *C. acetobutylicum* DSM1731 (Chart 1).

Antibiotic sensitivity testing (AST) was carried out to investigate the antibacterial activities of reference antibiotics against the tested reference strains; *C. perfringens* KF383123 and *C. acetobutylicum* DSM1731. The results revealed that reference antibiotics exhibited antibacterial activities with different levels as shown by zones of inhibition. The best antibacterial activity was shown by CIP5 with zone of inhibition of 18.00 ± 0.58 mm for both strains. This was followed by CN10 with zones of inhibition of 17.00 ± 0.58 and 16.00 ± 0.58 mm against both strains respectively. TOB30

showed zones of inhibition of 10.00 ± 0.58 and 11.33 ± 0.88 mm while CTX30 showed zones of inhibition of 8.67 ± 0.33 and 8.33 ± 0.33 mm against tested strains, respectively. On the other hand E15 and OT30 showed no hindrance activities against tested reference strains (Chart 1).

The antibacterial activity of sulphamethoxazole against clostridia reference strains and the synergistic effect of the eleven tested honey samples with sulphamethoxazole were shown in (Chart 2). The results revealed that sulphamethoxazole alone exhibited weak antibacterial activities against tested reference strains with zones of inhibition of 10.33 ± 0.88 mm and 8.00 ± 0.58 mm against *C. perfringens* KF383123 and *C. acetobutylicum* DSM1731, respectively. On the other hand, Sider honey showed great synergistic activity with sulphamethoxazole with zones of inhibition of 46.33 ± 0.88 and 26.00 ± 0.58 mm against *C. perfringens* KF383123 and *C. acetobutylicum* DSM1731, respectively. Lower anticlostridial activities were shown with Commercial Citrus, 35.00 ± 4.04 and 20.33 ± 1.45 mm; Commercial Clover, 34.00 ± 1.15 and 20.33 ± 0.88 mm; Clover, 30.33 ± 0.88 and 23.33 ± 0.88 mm; and Cotton honey, 30.33 ± 0.33 and 20.00 ± 1.15 mm against *C. perfringens* KF383123 and *C. acetobutylicum* DSM1731, respectively.

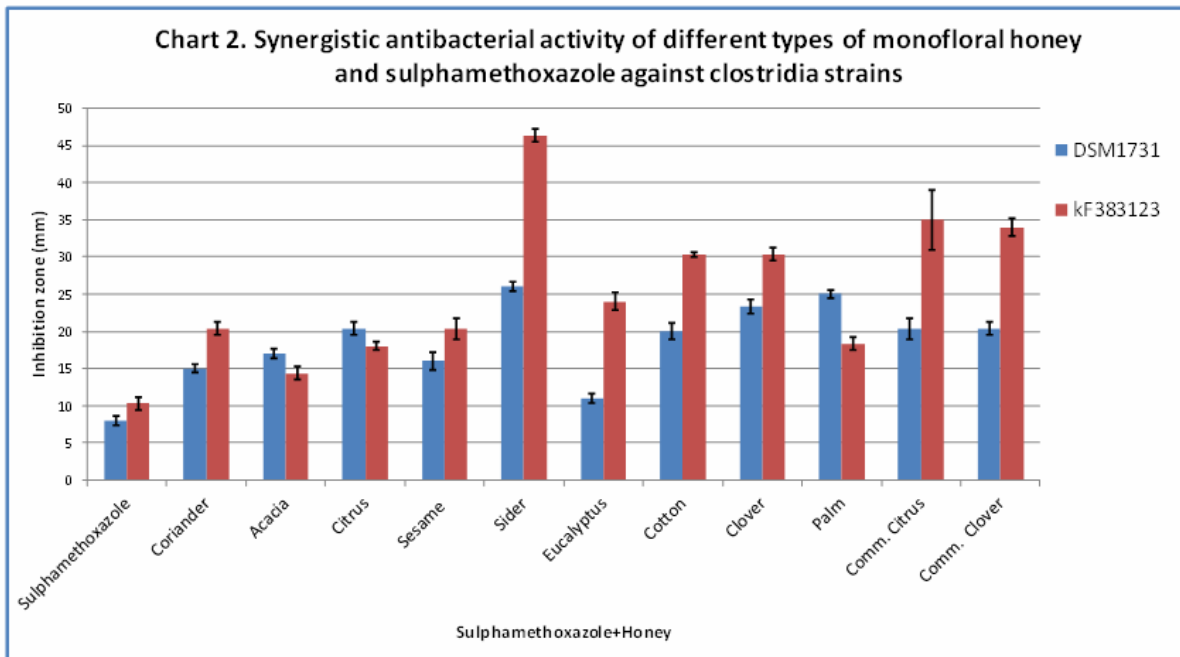
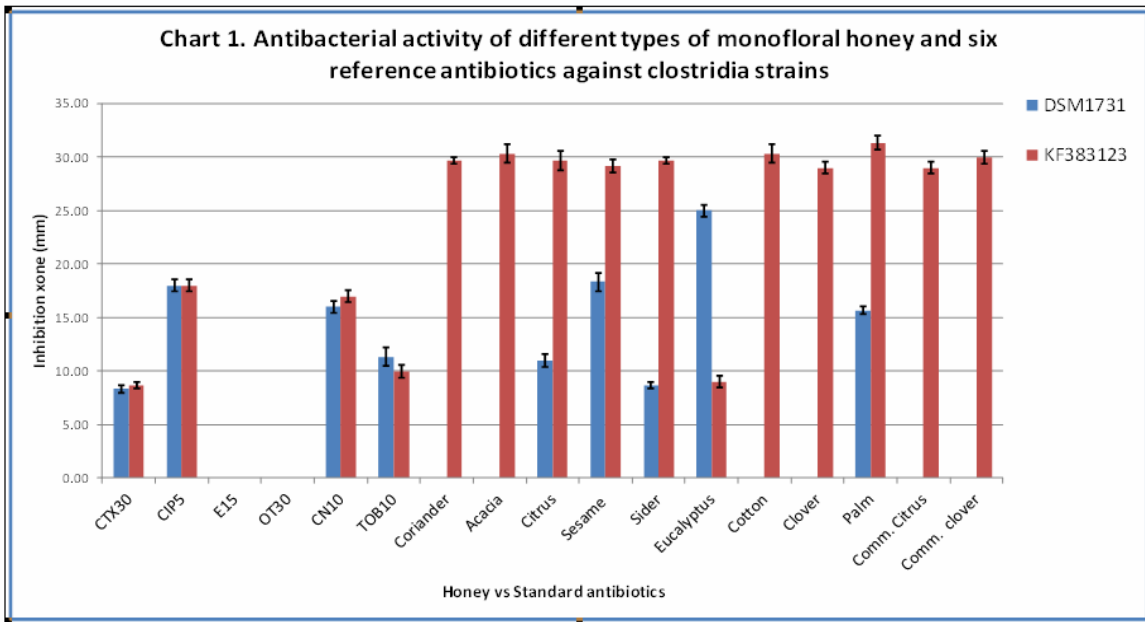
Palm honey showed low synergistic activity as shown by the zone of inhibition against *C. perfringens* KF383123 (18.33 ± 0.88 mm) and it showed better activity against *C. acetobutylicum* DSM1731 with zone of inhibition of 25.00 ± 0.58 mm (Chart 2).

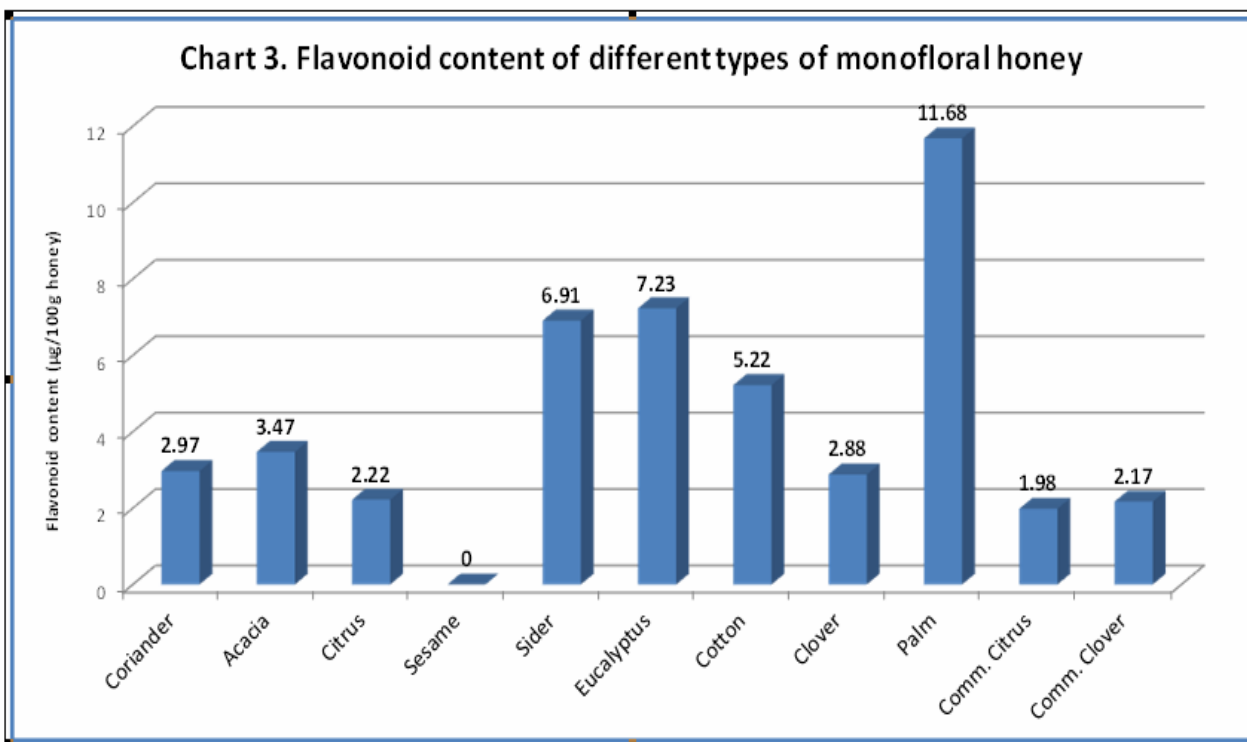
Quantitative determination of the total flavonoid content was done photometrically using Caffeic acid as a standard. The highest value of total flavonoid content (11.68

$\mu\text{g}/100\text{g}$ honey) was obtained in Palm honey (Chart 3). This was followed by Eucalyptus with total flavonoid content of $7.23 \mu\text{g}/100\text{g}$ honey and Sider honey with flavonoid content of $6.91 \mu\text{g}/100\text{g}$ honey. Commercial Citrus and Commercial Clover showed low flavonoid content with values of 2.17 and 1.98 $\mu\text{g}/100\text{g}$ honey respectively. While the total flavonoid content of Sesame was 0 $\mu\text{g}/100\text{g}$ honey.

Antimicrobial agents are necessary for controlling infectious diseases. However, the effectiveness of antimicrobial agents is diminished as a result of developing and spread of many drug resistant pathogens. Pathogens became resistant to all kinds of antibiotics including the major last-resort drugs (Mandal *et al.*, 2009). Antibiotic resistance represents very serious threats to public health, major problems in hospitals and now it is also recognized among various groups in the community, such as pigs and cattle breeders (Fischbach. and Walsh, 2009). Natural products either separately or in combination with antibiotics has been used successfully to overcome the problem of antibiotic resistance in infectious diseases (Hemaiswarya *et al.*, 2008 and Genilloud, 2012). Honey was described by Maddocks and Jenkins, (2013) as the sweet solution to the growing problem of antimicrobial resistance. The antibacterial activity of both monofloral and polyfloral types of honey has been documented earlier against different microbial strains including both Gram-positive and Gram-negative (León-Ruiz *et al.*, 2013 and Zainol *et al.*, 2013).

In earlier study in our lab to investigate the antimicrobial activity of Egyptian cotton flower honey against different microbial strains it was concluded that pure and diluted cotton flower honey can be used beneficially as antimicrobial agent.





Pure honey showed strong bactericidal effect against *S.typhimurium*, *S.typhi*, *Sh. sonnei* followed by *S. aureus*, *Streptococcus* then *E. coli* O157 then *Asperigillus*, *Klebsiella* and *L. monocytogenes*, *E. coli* and *E. fecalis* then *Pseudomonas aeruginosa* followed by *C. albicans* and finally with least hindrance abilities against *Bacillus*, *S. flexneri* and *Citrobacter*.

Diluted cotton flower honey (10%) showed bacteriostatic effect against *Sh. flexneri*, *S. typhimurium*, *E. coli* and *Klebsiella* with zone of bacteriostatic effect equals 40, 35 and 30 mm, respectively, followed by *Pseudomonas aeruginosa*, *Citrobacter* and *E. fecalis* with zone of bacteriostatic 26, 20 and 19 mm, respectively (Abd El-Moez et al., 2013).

Researchers have failed to point out the

active ingredient responsible of the antibacterial activities of honey. Over 100 substances were found to be candidates for such antibacterial activity (Simon et al., 2009). While antibiotics destroy bacteria by attacking the cell wall honey draws moisture out of the environment and dehydrates the bacteria with the aid of its hyperosmolar properties (Khan et al., 2007; Simon et al., 2009; Molan, 2006). Furthermore, honey has a mean pH of 4.4, so the acidification of honey can reduce bacterial colonization (Molan, 1992, Rushton, 2007 and Schneider et al., 2007). It was found that Vegetative *C. perfringens* cells were inactivated below pH 5 (Bates and Bodnaruk, 2003). Other factors that contribute to antimicrobial activity of honey include the high sugar concentration, hydrogen peroxide, methylglyoxal, and the antimicrobial peptide bee defensin-1 (Kwakman and Zaat., 2012). It was found that both

hydrogen peroxide and the non-peroxide components contribute to the bacteriostatic and bactericidal activity of honey. Also, H₂O₂ in honey was involved in oxidative damage causing bacterial growth inhibition and DNA degradation, but these effects were modulated by other honey components (Brudzynski et al., 2011).

The minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of Manuka honey for three *C. difficile* strains were investigated by Hammond and Donkor, (2013). The MIC values of the three *C. difficile* strains were the same (6.25% v/v). Similarly, MBC values of the three *C. difficile* strains were the same (6.25% v/v). Cooper et al., (1999) reported the antibacterial activity of Manuka honey against 58 isolates of *S.aureus*. In another report, Cooper and Molan, (1999) determined the MIC of Manuka honey for 20 strains of *P. aeruginosa*. Furthermore, Cooper et al., (2002) proved medium level of activity of Manuka honey against 17 strains of *P. aeruginosa*. Wilkinson and Cavanagh, (2005) reported that Manuka honey was effective against many organisms including *S. aureus*, *E. coli*, *S. typhimurium* and *P. mirabilis*. In general, earlier studies indicated that antibacterial (Tan et al., 2009, Hegazi, 2011 and Hegazi & Abd Allah, 2012) and anti-fungal (Koc et al., 2011) activities of honey are among several health beneficial effects of honey.

The results showed considerable variation in total flavonoid content with Palm honey contains the highest flavonoid content and showed the highest antibacterial activity. Phenolic compounds in general in their many forms are the main components responsible for the functional properties, such as antioxidant capacity (Kerem et al., 2006; Almaraz et al., 2007 and Hegazi &

Abd el Hady, 2009) and antibacterial capacity (Huang et al., 2006; Theodori et al., 2006 and Hegazi & Abd Allah 2012). Frankel, et al., (1998) studied the antioxidant capacity of 14 types of unifloral honey and they found that honey had significant antioxidant activity . Honey contains flavonoides (such as apigenin, pinocembrin, kaempferol, quercetin, galangin, chrysin and hesperetin), phenolic acids (such as ellagic, caffeic, p- coumaric and ferulic acids), ascorbic acid, tocopherols, catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH), Millard reaction products and peptides. Most of these compounds work together to provide a synergistic antioxidant effect (Hegazi, 2012 and Eteraf-Oskouei & Najafi, 2013).

Honey is a supersaturated solution of sugars of which fructose (38%) and glucose (31%) are the main carbohydrates. A wide range of minor constituents is also present in honey, many of which are known to have antioxidant properties. These include phenolic acids and flavonoids (Moniruzzaman et al., 2014), certain enzymes (glucose oxidase, catalase), ascorbic acid, Mailard reaction products (White, 1975), organic acids (Cherchi et al., 1994) and amino acids (White & Rudyj, 1978). Honey phenolics were found to be different due to the geographical origin. The actual composition of honey in general varies depending on many factors such as the pollen source, climate and environmental conditions (Gheldof et al., 2002; Azeredo et al., 2003). Furthermore, earlier studies indicated that honey contains enzymes such as glucose oxidase, diastase, invertase, catalase and peroxidase (Bogdanov et al., 2008) and these enzymes may play an important role in the antimicrobial activity of honey.

Also, honey contains other bioactive constituents such as organic acids, ascorbic acid, trace elements, vitamins, amino acids, proteins and Maillard reaction products (Bogdanov et al., 2008). Monofloral honey possess highly characteristic aromas indicating the presence of various volatile components some of which are probably derived from the sources of nectar; some are dependent on the physiology of the bee and others arise during processing after harvest. For example the flavonoid glycosides present in nectar are hydrolyzed to give the corresponding aglycons by glycosidases of bee salivary glands (Sabatier et al., 1992) and therefore only the aglycons are detected in honey, as shown in a study on citrus nectar and honey (Ferrerres et al., 1993). The phenolic compounds present in honey in general can originate from flower nectar, propolis (and/or beeswax), and pollen (Meda et al, 2005).

The results also revealed that the combination of honey with sulphamethoxazole as a common antibiotic was beneficial and exhibited a great synergistic effect that suggests the possibility of using honey in combination with antibiotics to overcome the problem of antibiotic resistance in some bacterial strains including clostridia. Synergy between oxacillin and Manuka honey in the inhibition of methicillin-resistant *Staphylococcus aureus* (MRSA) has been reported (Jenkins and Cooper, 2012). Manuka honey, therefore, seems to offer real potential in providing novel synergistic combinations with antibiotics for treating wound infections of multi-drug resistant (MDR) bacteria. In this study a selection of antibiotics which affect a wide variety of cellular target sites was tested for synergistic activity with medical grade Manuka honey in order to identify novel

therapies and five combinations were identified.

Another two research groups have reported synergy between gentamicin and honey (Karayil et al., 1998; Al-Jabri et al., 2005). It is likely that the botanical origin of honey influences its biological activity because different antibacterial components have been identified in different honey samples (Kwakman *et al.*, 2011). This fact confirms the importance of selecting an appropriate honey for specific antibacterial use.

The current study highlights the importance of screening of different types of monofloral honey for their antimicrobial activity, and ranking them based on their effectiveness against specific bacterial strains. This will help to standardize their use as potent antimicrobial agents either separately or in combination with some other antibiotics to overcome the growing problem of antibiotic resistance especially among clostridia strains. Further studies to evaluate the antibacterial activity of honey *in vivo* are of great importance to investigate the indirect effect of honey on the bacteria through the modulation of host immune system during infection.

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