Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

J E Z S Journal of Entomology and Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2018; 6(1): 1564-1570

Received: 05-11-2017
Accepted: 06-12-2017

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Physical characterization and antibiotic potential of honey collected from *A. florea* combs in district Khairpur

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Abstract
This work was directed to study the inhibitory effects of honey collected from different geographical regions of District Khairpur against certain pathogenic bacteria. It has been observed that the valuable use of honey in the management of bacterial infection is when it can be applied directly to the bacteria without dilution. There are few published reports on the physicochemical and antibacterial characteristics of honey from *A. florea*, the dwarf honeybee native to Pakistan. Current study explores the variation in physicochemical properties and the level of antimicrobial potential of honey samples collected from wild bee combs of *A. florea* shows potential genetic diversity from District Khairpur. The acacia honey found effective to stop the growth of isolates except *Proteus* and *Shigella*. The antibacterial action of honey was attained in high concentrations of honey both in well diffusion as well as disc diffusion methods.

Keywords: Honey, Khairpur, *A. florea*, physicochemical, genetic diversity

Introduction
The use of honey as a remedy against bacteria is expanding in modern medicine as a result of its antimicrobial activity and wound healing properties. In particular, certain types of honey exhibit broad-spectrum antimicrobial activity and are helpful to treat antibiotic resistant bacteria. Variations in the type and level of antimicrobial activity in honey are directly associated with the flora. In addition, the difference in the level of hydrogen peroxide activity in floral source also causes variation among honeys from within the same floral species. This may be due to the geographical origin of the floral source and the prevailing environment or to different factors related to honey bee [8].

The characterization of honey helps to understand its therapeutic, antibacterial and antioxidant characteristics [1]. Mainly its physicochemical properties can be used to divulge adulteration; for that reason, assessment of certain quality parameters is considered necessary to ensure the purity of honey [23]. In Pakistan, 55 to 60 tons of honey is collected each year that involves approximately 15,000 persons to complete the job [1, 16]. Therefore, apiculture and its products have some significance in the economy of Pakistan that certainly needs expansion. Commercial and economic importance of honey due to its nutritional value signifies the importance of certain quality factors. The quality of honey is generally determined by physico-chemical indicators including moisture content, electrical conductivity, reducing sugars, sucrose content, minerals, free acidity and HMF, as recommended by the International Honey Commission [5].

The variation in the composition of honey constituents is contributed by various physiological factors including climate, soil, flora, etc., besides bee species [15, 44]. *A. florea* has been reported from various countries for its role in crop pollination and production of quality honey, beeswax and honey for certain therapeutic applications. [7, 26, 37].

A. florea honey has been reported as superior in quality and used in traditional medicine probably due to high amount of a glucose oxidase enzyme that shows antibiotic activity [20]. Different flora and geographical locations translate into the variation in color, flavor, aroma, texture, granularity, viscosity, antibacterial properties and pre-biotics composition of honey [31]. The variety of flora, seasonal, environmental and geographical factors such as temperature and humidity on which the antibacterial activity of honey is highly dependent also contribute to the variation in honey quality [32, 42]. Honey is recognized in history as a medicine specially treating the infected wounds for more than two millenniums that was a long time earlier before

the bacteria were considered as a source of infection. The use of honey as a therapeutic agent revived during 1990s and currently being utilized as a remedy for injury care in Australia [19], New Zealand [31] and UK [40]. The antimicrobial potential of honey has been reported against a number of gram-positive and gram negative bacteria [40] including pathogens such as *S. aureus*, *P. aeruginosa*, *E. coli*, *P. mirabilis*, *S. pyogenus*, *S. flexneri* and *S. typhi* [24, 32, 335]. In addition, honey has been reported as conventional medicine for dyspepsia and peptic ulcer [25]. It has been demonstrated previously that honey inhibits the development of wound infecting bacterial strains, including *E. coil*, *S. aureus* and *Salmonella* [7, 46]. The reason for the antibacterial potential of honey is the osmolarity, acidity, presence of hydrogen peroxide and many other unknown substances derive from flora [3, 33]. There are few published reports on the physicochemical and antibacterial characteristics of honey from *A. florea*, the dwarf honeybee native to Pakistan. The aim of this study is to explore the variation in physicochemical properties and the level of antimicrobial activity of honey samples collected from wild bee combs of *A. florea* showing potential genetic diversity from District Khairpur.

Materials and Methods

Collection and Preparation of Honey Samples The honey samples were collected from combs of *A. florea* in different locations of Distict Khairpur in varying geography, topography and flora in vicinity. A total number of 21 honey samples of *Acacia sp.*, *Ziziphus* sp. and *Brassica* sp. were obtained from District Khairpur. The samples were strained through cheesecloth to remove unwanted material such as wax, sticks, and dead bees and comb particles before analysis and stored in dark glass bottles at room temperature.

Honey Color

Colors of 21 honey samples were determined using Honey Color Analyzer C 221 (Hanna Instruments, Rhode Island, and USA) as per manufacturer instructions. Honey color is defined as millimeters on a Pfund scale for optical density with glycerol as reference.

pH of Honey Samples

To determine the pH of honey 10g of honey diluted in 10ml of water to make a solution. The pH of honey was determined using a pH meter (Metler Toledo MP220 pH meter) at 20 ^{0}C.

Electrical Conductivity

Electrical conductivity of the honey samples was determined in mS.cm-1 at 20 ^{0}C using 20% solution of honey in double distilled deionized water on a conductivity meter.

Water Contents

Water contents (% moisture) of honey samples at 20 ^{0}C measured using a refractometer (Erma, Tokyo, Japan).

Ash Contents

Ash contents of honey samples were determined by calcination in a muffle furnace at 550 ^{0}C for two to four hours depending on when mass reaches a constant value.

Total and Reducing and Non Reducing Sugar Contents

Total and reducing sugar contents were determined by titration using a Lane-Eynon method with Fehling's solution. Non-reducing sugar contents were obtained from the difference between total and reducing sugar contents.

Bacterial Isolates

The hospital isolates used in this study were obtained from the Department of Microbiology, University of Karachi. The reference strains were revived, sub cultured on nutrient agar, the bacterial slants were incubated overnight at 37 ^{0}C. The revived cultures were stored in a refrigerator.

Disc Diffusion Assay

Honey solution from Acacia honey, Mustard honey and Ziziphus honey collected from randomly selected areas of District Khairpur was prepared by adding 2g of well mixed honey to 2ml of sterile de-ionized distilled water (ddH2O) in universal bottles and placed at hot plate stirrer for 30 minutes to mix properly by stirring to make a 50% (w/v) solution of each. The bacterial cultures were inoculated in 5ml nutrient broth and incubated overnight in orbital shaker at 37 ^{0}C; the other day inoculums was properly spread on the nutrient agar plates with the help of the cotton swab. The sterilized honey dipped filter paper discs of 6mm diameter were applied and pressed gently with the help of sterilized forceps to ensure complete contact with agar. The sterile absorbent discs were placed in honey for 10 minutes before being placed directly to the culture spread agar plates, control sterile discs of antibiotics were also placed on the same plate for comparison. Plates were placed in incubator at 37 ^{0}C; the readings of zone of inhibition were recorded at 8hrs. The diameter of zones was recorded in mm.

Well Diffusion Assay

The agar well diffusion assay was employed to determine the antimicrobial activity of honey collected from seven different randomly selected areas of District Khairpur. Bacterial cultures were inoculated in 5ml luria broth an incubated overnight in orbital shaker at 37 ^{0}C, the other day the larger end of sterile Pasteur pipette was used to make four wells (6 mm diameter) in Mueller Hinton (HM) agar plates and the inoculum was properly spread on the muller hinton agar plates with the help of cotton swab. Acacia honey, Mustard honey and Ziziphus honey (50% w/v) were deposited into each of the four wells on the Petri plates. Three replicates of each plate were made to test each honey with each of the seven bacteria and methyline blue was used as a control. The plates were incubated aerobically at 37 ^{0}C for 12 hours. The diameter of zones of bacterial growth around the well was measured.

Analysis

The data obtained from three replications of each bacterial strain and honey was analyzed using SPSS statistical software for windows. The mean and standard deviation of observed values were calculated. The Analysis of variance (ANOVA) was performed on the data to compare the significant difference in the zone of inhibition between honey samples at (P<0.05).

Result

Physicochemical Characteristics of Honey

The color of honey samples in this study ranges from light amber to very dark amber. Apparently three color ranges were categorized into three groups, dark amber (honey obtained from the comb of *A. florea* on *Acacia arabica*), light amber (honey obtained from comb of *A. florea* on *Brasicca campestris*) and amber (honey obtained from comb on *Ziziphus acacia spina*) (See Figure 1). The average Pfund scale values were 125.27+2.2, 0.91.11+7.1 and 105.61+3.7 for *A. Arabica*, *B. campestris* and *Z. spina*, respectively (Table 1).

The pH of honey collected from the *A. florea* combs on *Acacia sp.*, *Brassica sp.* And *Ziziphus sp.*, in different localities of District Khairpur was measured and found to be on average 4.8+0.2, 3.7+0.2 and 4.1+0.2 for honey samples obtained from *A. florea* combs on *Acacia sp.*, *Brassica sp.* and *Ziziphuscacia sp.*, respectively. (Table 2)

The electrical conductivity is defined as mS/cm measured at 20 ^{0}C in a 20% solution of honey and turned out to be highest for honey from *Acacia sp.* which is 0.79+0.04. Honey samples from *Brassica sp.* and *Ziziphus sp.* showed electrical conductance of 0.77+0.02 and 0.69+0.03. (Table 2)

Water content (%) was found to be highest for honey from *Acacia sp.* in this study. It was found to be 19.1%, however, the standard deviation for this observation was also as high as +2.3. In *Brassica sp.* and *Ziziphus sp.* honey samples the water contents were 17.3+0.3 and 18.4+0.5, respectively, Ash contents were also highest in *Acacia sp.*, honey sample 0.101+0.01%. In *Brassica sp.* and *Ziziphus sp.* honey samples ash contents were 0.086+0.02 and 0.67+0.02, respectively and sugar contents in the honey samples from *Acacia sp.* were 76.4+3.7%, from *Brassica sp.* were 82.7+3.9% and from *Ziziphus sp.* were 81.2+4.2%. Reducing sugars in the honey samples were found to be 72.8+3.9% for *Acacia sp.*, 77.7+3.6 for *Brassica sp.* and 76.3+3.8% for *Ziziphus sp.* Amount for non-reducing sugar were estimated by finding the difference in total sugar and reducing sugar for each type of honey (Table 2).

Antibacterial Activity of Honey

It has been observed in this study that various samples of honey have inhibited the growth of tested bacterial strains with well diffusion assay more consistently than disc diffusion assay. Each experiment was performed in a triplicated to get an average result. We measured visible clear ring around the disc. Where there was (-) inhibition on the disc diffusion assay, there was no clearly visible ring around the absorbent disc. The average zone of inhibition (30mm) in a disc diffusion method for mustard and ziziphus honey were recorded for *E. coli* where penicillin and carbenecillin were found ineffective. The zone of inhibition (30mm) for acacia, mustard and ziziphus have been recorded for *S. aureus*, the results for carbenecilin and penecilling were found nill to inhibit the growth. The average zone of inhibition (26mm) was found for acacia honey. The honey did not inhibit growth of *P. aeruginosa* and *Streptococcus*. The affect of Carbenecillin, penicillin and chloremphenicol was also found nill at the growth of *P. aeruginosa* and *Streptococcus.* The highest zone of inhibition (29mm) was recorded for *gentamycin.* The highest inhibition zone (33.3±1.1mm) was recorded from acacia honey of Akra for S. aureus, where as the lowest inhibition zone was recorded as (26.3±6.6mm) from Basiro. The highest zone of inhibition for mustard honey of Kotlalu was found to be (26±6.6mm), whereas lowest zone was found to be (19.6±3.2mm) from Karondi respectively. The highest zone of inhibition for ziziphus honey of Akra was found to be (29.3±0.5mm), whereas lowest zone was found to be (23.3±2.3mm) from Pacca. On the contrary for *E.coli* highest zone of inhibition for acacia honey of Pacca was recorded as (21.6±1.5mm), simultaneously the lowest zone was found to be (6±0) in Akra for acacia honey. The highest zone of inhibition for mustard was found to be (23±0mm) in Kotlalu, while the lowest zone was recorded to be (5.6±0.5mm). The highest zone of inhibition for ziziphus honey was recorded as (21.3± 1.52mm) from Kandiari and lowest inhibition zone (8.6±0.5mm) was recorded from Akra.

The highest zone of inhibition for *P. aeruginosa* (23.3±0.5mm) have been recorded for acacia honey from Kotlalu, where as the lowest zone of inhibition (10±1.0mm) have been recorded from Basiro. The highest zones of inhibition for mustard (19±2mm) have been recorded from Kotlalu whereas the lowest zone of inhibition (7.6±1.1mm) was found to be in Akra. The highest zone of inhibition for Ziziphus (22±1.7mm) was recorded from Kotlalu, simultaneously the lowest zone of inhibition (5.6±0.5mm recorded from Akra. The highest zone of inhibition in Klbesella (20±0mm) has been recorded for acacia honey from Pacca, whereas the lowest zone of inhibition was found to be (9.6±1.1mm) from Akri. The highest zone of inhibition for mustard honey was found to be (18.3±2.0mm), whereas, the lowest zone of inhibition (8.3±0.5mm) was recorded from Karondi. The highest zone of inhibition in Klbesella (20.3±0.5mm) have been recorded for Ziziphus honey from Basiro, whereas the lowest zone of inhibition was found to be (9.3±0.5mm) from Akri.

Table 1: Standard color and average Pfund (mm) scale for each of the honey types collected from combs of *A. florea* on Acacia arabica, Brassica campestris and Ziziphus spina in seven different locations in Disrtict Khairpur.

Plant Type	P fund (mm)	Color
Acacia	125.27±2.2	Dark Amber
Brasicca	091.11±7.1	Light Amber
Ziziphus	105.61±3.7	Amber

Table 2: Means and standard deviations of pH, electrical conductivity, water contents, ash contents, total sugars, reducing sugars and non-reducing sugars measured in n=7 for each of *Acacia Arabica*, *Brasicca campestris* and *Ziziphus spina*.

	pH	Electrical Conductivity (mS/cm)	Water Content (%)	Ash Content (%)	Total Sugars (%)	Reducing Sugars (%)	Non-Reducing Sugar* (%)
Acacia sp.	4.8	0.79	19.1	0.101	76.4	72.8	3.6
	±.02	±0.4	±0.5	±0.01	±3.7	±3.9	
Brassica sp.	3.7	0.77	17.3	0.086	82.7	77.7	5.0
	±.02	±0.02	±0.3	±0.02	±3.9	±3.6	
Ziziphus sp.	4.1	0.69	18.4	0.067	81.2	76.3	4.9
	±.02	±0.03	±0.3	±0.02	±4.2	±3.8	

*Amount for non-reducing sugar obtained as the difference between total sugar and reducing sugar percentages.

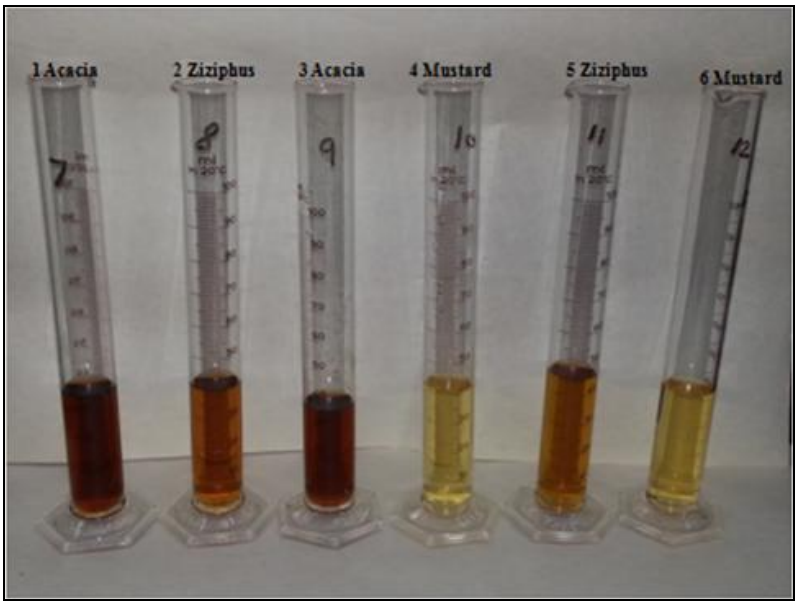

Fig 1: Color of the honey types collected from combs of *A. florea* on *Acacia arabica* first and third from left, *Brassica campestris* fourh and sixth from left and *Ziziphus spina* second and fifth from left.

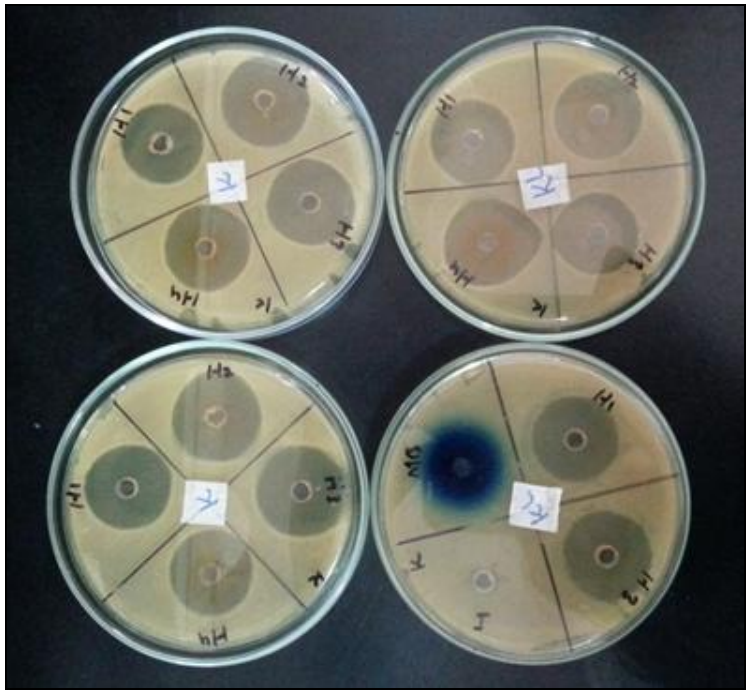

Fig 2: Well diffusion assay results of *Klebsella* measurement of zone of inhibition (mm) using Acacia, Mustard and Ziziphus honey.

Discussion

Honey color is the basic indicator for the classification of honey quality standards. It varies from white or light yellow through to dark amber [29]. In this study, honey samples were studied having only three plant sources, therefore, showed only light amber, amber and very dark amber colors. Honey samples from Acacia sp. had the darkest amber colour. Acacia sp. honey has been reported to show light color and possess low antioxidant activity when compared to honeys that have a dark color [30]. However, in our study a very dark amber color of honey from Acacia sp. found. Other workers have reported both light and very dark amber honey colors from Acacia sp. [30]. In this study the mean Pfund value turned out to be 123.2± 2.2 which is found to be comparable to that of Moniruz-zaman [29]. They found most of the Acacia honey samples dark amber in color at 120+23.46 mm Pfund value. The pH values of the honey samples from District Kairpur from different plant source were observed to be within recommended limits (pH 3.4 to 6.1) for fresh honey. Highest pH (4.8) has been observed from samples of Kandiari for Acacia sp. honey. The pH of Acacia sp. honey has been reported to be 5.4 from Germany [4]. The honey collected from same type of flora but different geographical area has been reported for different chemical composition and antibacterial activity [47]. The pH (4.0) of Acacia sp. honey reported from Romania seems closer to our findings [31]. However, from Pakistan the pH 3.2 for Acacia sp. honey has been reported [11]. The reason behind the difference of pH in the honey of Acacia sp. from Pakistan may be the collection of honey samples from different geographical areas and the type of honeybee and flora. Another prominent difference may be the honey reported from Pakistan has been collected from managed bee that might be *A. mellifera* and *A. cerana* whereas the honey samples reported in this study have been collected from wild bee combs of *A. florea*.

The pH for honey from Brassica sp, found to be 3.7+0.02. Baltrusaityt reported the pH values of Brassica sp. honey from Luthiana ranging from 3.9 to 4.8 [5]. In Pakistan, the highest pH values (4.1) and lowest (3.9) have been reported by [15] for Brassica sp. honey from Pakistan. We found the pH values 4.1+.02 for Ziziphus sp. honey from District Khairpur. Iftekhar reported the pH values (> 6) for Acacia honey from different localities of Pakistan which seems to be much different from our results. pH of honey appears to be affected by different flora, geographical localities, soil type, topography and climate of the sampling area [12, 14].

Our results show highest electrical conductivity in honey from Acacia sp., which is 0.79+0.4 mS/cm. Honey samples from Brassica sp. and Ziziphus sp. have electrical conductivities of 0.77+0.02 and 0.69+0.03 mS/cm, respectively. Our results are in agreement with those reported by [10, 22].

The percentage water contents appear to be on higher side in all samples with 19.1% for Acacia sp. and 18.4% for Ziziphus sp. Brassica sp. honey showed water content as 17.3%. Our results match to those of [41] in the sense that they also found highest moisture contents (18.6%) in Acacia sp. honey. Nevertheless, geographically and climatically the area where that study was conducted is entirely different from Khairpur.

Ash contents 0.1% found to be highest in honey from Accacia sp, although in comparison to some other plant types, it is not high [37], low content of total ash relates to a low level of the specific conductance. Honey originating for Ziziphus sp. was shown to have lowest ash content at 0.067% that compares with its low electrical conductivity [41]. However, found a higher percentage of ash content in Ziziphus sp. than Acacia sp. However, we found it otherwise which corresponds to the electrical conductivity.

The sugar contents were higher in honey from Acacia sp. (82.7+3.9%) and Ziziphus sp. (81.2+4.2%) whereas honey from Brassica sp. only had 76.4+3.7. Higher total sugar in Acacia sp. honey is found to be in agreement to the finding of [37] and [41].

The highest zones of inhibition have been observed on S. aureus (33mm) for acacia honey at 100% concentration, however the zone of inhibition (14.46mm) has been reported for acacia honey of A. dorsata combs on S. aureus from Malaysia [48].

The highest zone of inhibition in *E. coli* was found to be (21.6mm) for acacia honey, our results are in agreement with previous studies from Pakistan, the zone of inhibition for E. coli and Klebsella have been reported to be (19mm) with acacia honey [39].The highest zone of inhibition for *P. aeruginosa* was found to be (23.3mm) for acacia honey. The mustard honey was found to be less effective than acacia, the possible reason was the difference of potency may be origin of honey sample and type of flora, it has been reported previously that the inhibition of certain bacteria depends on the type of honey origin [13].The honey samples were found to have both bacteriostatic and bactericidal properties on both gram-negative and gram-positive bacteria. In particular, pure honey is a very potent inhibitor of growth of bacteria such as clinically isolated *E. coli*, *K. pneumoniae*, *S. aureus*, *Salmonella*, *P. aeruginosa*; however none of the honey expressed effect at the growth of *Proteus*, *Shigella* and *Bacillus.*

It can be seen that Acacia honey has a good antibacterial activity against different isolates, even though Acacia honey shows potential for use as a low-cost mild agent against bacteria its usefulness clinically is not known yet, Clinical trials are necessary to endorse the information. Honey Quantification of inhibition of microbial growth was determined by measurements of the diameters of clear zones around the well in agar using a well diffusion assay as well as measuring zones of inhibition in the disc diffusion assay. The agar diffusion assay has been considered an ideal method for honey bacterial activity. It has been reported to be used for manuka honey batch analysis. The subjective nature of this assay limits the interpretation of results. It is also time consuming and laborious method [43].

Pakistan is considered as a manufacturer of some of the best forms of natural honey in the cosmos, the Acacia honey of Pakistan is considered as the most valuable in the world grabbing the average line of approximately US$ 9Kg, -1 in the year 2000, and total export is 400MT to different nations such as China, Saudi Arabia, United Arab Emirates, Qatar, Kuwait and Yemen. This work was directed to study the inhibitory effects of honey collected from different geographical regions of District Khairpur against certain pathogenic bacteria. It has been observed that the valuable use of honey in the management of bacterial infection is when it can be applied directly to the bacteria without dilution.

The geographical variation plays an important role in the variation of physicochemical properties of honey reported in literature; honey can be classified and characterized on the basis of their geographical origin [23]. The honey samples used in this study show the antimicrobial activity. The acacia honey found effective to stop the growth of isolates except *Proteus* and *Shigella*. The antibacterial action of honey was attained in high concentrations of honey both in well diffusion as well as disc diffusion methods. This indicates that the honey should be applied directly to the bacteria without any dilution. The factors affecting bacterial growth might be due to the Osmosis, low pH and their sensitivity to hydrogen peroxide in our samples. The pH of honey is another potential reason of high antibacterial potential of honey. The honey samples used in this work were obtained from natural honey combs and were not heat processed. As for the antibacterial activity of honey on different bacterial strains it was observed that the S. aureus was the most inhibited bacteria among all. The results revealed by honey samples in relation to S. areas may be significant, particularly in recent decades; there have been problem in treating the skin and underlying tissue infections connected to S. aureus [26]. The development of *P. Aeruginosa* was also inhibited in this study not as much as in the case of *S. aureus*. None of the honey samples showed activity against the Proteus.

The antibacterial activity of honey and its mode of action on bacterial isolates is not completely revealed until now, however its contents, such as Osmolarity, Acidity, Hydrogen peroxide and Photochemical components are considered to play an important role. The honey applications as a medicine for the management of gastrointestinal disorders such as peptic ulcers, gastritis, gastroenteritis has been reported in literature from Eastern Europe and Arab countries [9]. Honey is slightly acidic in nature with pH ranges between 3.2 to 4.5, gluconic acid is brought out in the honey by the secretion of glucose oxidase by honey bees, its catalysis results in the oxidation of glucose to gluconic acid, the low pH itself inhibits the increase of many pathogenic bacteria or could exert inhibitory effects in topical applications

Conclusion

It could be concluded that the honey collected from combs of *A. florea* inhibit the growth of bacterial strains and that honey can be used as complementary antimicrobial agent against selected pathogenic bacteria.

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