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Activity of Egyptian Cotton Flower Honey as Antimicrobial Agent Against Pathogens of Animal Origin

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Abstract: Two experiments were conducted to evaluate theantimicrobial propertiesof cotton flower honey. In the Experiment 1:10% dilution of honey was evaluated against 6 Gram negative bacteria including *E. coli*, *S.*Typhimurium, *Sh. flexneri*, *Klebsiella*, *Pseudomonas aeruginosa*and Citrobacterand a Gram positive bacteria *E.fecalis* and two fungal strain including mould*(C. albicans)*, yeast (*Aspergillus*). In Experiment 2, pure honey was assessed against five Gram positive bacteria; Bacillus,*Strept. pneumonia, S. aureus*, *E. fecalis* and*Listeria monocytogenes* as well asnine Gram negative bacteria*Citrobacter, E. coli, E.coli* O157, *Salmonella* Typhimurium, *Sh.flexneri, Sh.sonnei*, *Pseudomonas aeruginosa*, *Klebsiella* andCitrobacterandmould*(C. albicans)* and yeast (*Asprigillus*) was evaluated using Agar Well Diffusion Method (AWDM) and Minimum Inhibitory Concentration (MIC). Results indicated that 10% dilution of honey has a 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. Pure honeyshowed strong bactericidal effect against *S.typhimurium*, *S.typhi*, *Sh. sonnei* followed by *S. aureus*, *Streptococcus* then *E. coli* O157 then *Asperigllus*, *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.* In conclusion, pure cotton flower honeycan be used beneficially as antimicrobial agent.

Key words: Cotton flower honey · Bacteriostatic effect · Bactericidal effect · Antifungal properties

INTRODUCTION

Antimicrobial agents are necessary for controlling infectious diseases. However, the effectiveness of the 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 [1]. These antibiotic resistantpathogensrepresenta very serious threats to public health, a major problem in hospitals and now it recognized among various groups in the community, such as pigs and cattle breeders [2]. Also, there is an increasing resistance of *mycotic* spp. to antifungal agents withrisingthe mortality associated with infections by *Candida* spp [3].

Honey has been used since ancient times for the treatment of some diseases and for the healing of wounds, however, its antibacterial activity was first reported by scientists in 1892. Recently, numerous studies have been published on the antimicrobial activities of honey showing its biological activities [4, 5], and as antimicrobial agentagainst antibiotic-resistant bacteria [6, 7]. Antibiotic-susceptible and -resistant isolates of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter cloacae* and *Klebsiellaoxytoca* were killed within 24 h by 10%-40% (vol/vol) honey [8]. Honey has been used to treat adult and neonatal postoperative infection [9,10], burns [11], necrotizing fasciitis [12], infected and non-healing wounds and ulcers [13],

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pilonidal sinus [14], venous ulcers [15] and diabetic foot ulcers [16]. When ingested, honey also promotes healing and shows antibacterial action by decreasing prostaglandin levels, elevating nitric oxide levels and exerting probiotic effects.

Under the current situation there is an urgent need to discover an alternative antimicrobial agents against the antibiotic and antimycotic resistant pathogens. Therefore, the present study was conducted to investigate the*in vitro* antimicrobial effects of diluted (10%) and pure Egyptian cotton flower honey against highly pathogenic bacterial and mycotic isolates of animal origin which have high public health hazards and compare its activity with reference antibiotic and antimycotic drugs.

MATERIALS AND METHODS

Honey Samples: One sample of cotton flowerhoney was collected from El FayoumGovernorate, Egypt in2012.

Preparation of Microbial Suspensions: Highly pathogenic strains including five Gram positive bacteria; *Bacillus,* Streptococcus*, S. aureus*, *E. fecalis*, *L. monocytogenes* and nine Gram negative bacteria; *E. coli*, *E. coli* O157, *Salmonella*Typhi*, S.* Typhimurium, *Sh. flexneri, Sh. sonnei*, *Pseudomonas aeruginosa*, Citrobacter and *Klebsiella* and two mycotic strain; mould (*C.albicans*) and yeast (*Asprigillus*) isolated from animal origin. Agar well diffusion (AWD) (qualitative method) and Minimum Inhibitory concentration (MIC) (quantitative method) were used in this study. Wherein suspensions of bacterial and mycotic strains were freshly prepared by inoculating fresh stock culture from each strain into separate broth tubes, each containing 7 ml of Muller Hinton Broth for bacterial strains and Sabaroud Dextrose broth for fungal strain. The inoculated tubes were incubated at 37°C and 28 °C for 24 h, respectively. Serial dilutions were carried out for each strain, dilution matching with 0.5 Mc-Farland scale standard was selected for screening of antimicrobial activities. Ciprofloxacin 100 µg/ml and fluconazole 100 µg/ml were used as reference drugs (Oxoid).

Experimental Design

Experiment 1: ***Antimicrobial Activity of 10% Diluted Cotton Flower Honey***

Preparation of Microbial Suspensions: Antimicrobial activities of 10% diluted cotton flower (10% honey in distilled water) were evaluated against pathogenic isolates of animal origin and are accused of causing food poisoning in human consuming contaminated animal byproducts including; five Gram positive bacteria; *Bacillus,* Streptococcus*, S. aureus*, *E. fecalis* and *L. monocytogenes* and nine Gram negative bacteria; *E. coli*, *E. coli* O157, *Salmonella* Typhi*, S*. Typhimurium*,Sh. flexneri, Sh. sonnei*, *Pseudomonas aeruginosa*, Citrobacter and *Klebsiella* and two mycoticstrain; mould (*C. albicans*) and yeast (*Aspergillus*).

Isolates were isolated from Broiler carcasses including; *E. coli*, *E. coli* O157, *Salmonella* Typhi*, S*. Typhimurium*, Sh. flexneri, Sh. Sonnei* [17, 18] other isolates were isolated from mastitic cow milk including; *Pseudomonas aeruginosa*, Citrobacter, *Klebsiella, Bacillus,* Streptococcus*, S. aureus*, *E. fecalis* and *L. monocytogenes* [19] as well as mycotic isolates; mould (*C. albicans*) and yeast (*Aspergillus*) [3].

Agar well diffusion (AWD) (qualitative method) and Minimum Inhibitory concentration (MIC) (quantitative method) were used for evaluation.

Experiment 2: ***Antimicrobial Activity of Pure Cotton Flower Honey:*** Antimicrobial activities of pure cotton flower honey were conducted against highly pathogenic strains including five Gram positive bacteria; *Bacillus,* Streptococcus*, S. aureus*, *E. fecalis* and *L. monocytogenes*, nine Gram negative bacteria; *E. coli*, *E. coli* O157, *Salmonella*Typhi*, S.* Typhimurium, *Sh. flexneri, Sh. sonnei*, *Pseudomonas aeruginosa*, Citrobacter and *Klebsiella* and two mycotic strain; mould *(C. albicans)* and yeast (*Aspergillus*) isolated from animal origin. Agar well diffusion (AWD) (qualitative method) and Minimum Inhibitory concentration (MIC) (quantitative method) were used in this study.

Agar Well Diffusion Method: The antimicrobial activity of honey against bacterial and mycotic isolates was evaluated by using agar-well diffusion method [20]. 100 µl of cell culture suspension matching with 0.5 McFarland of target isolate was spread onto the Muller Hinton agar plates. For the investigation of the antibacterial and antimycotic activity, 50µl of honey, 50µl ciprofloxacin (100µg/ml) and fluconazole (100µg/ml) as control positive and DMSO as control negative were added into wells of agar plates directly. Plates were left for 1 h at 25 °C to allow a period of pre-incubation diffusion in order to minimize the effects of variation in time between the applications of different solutions. The plates were re-incubated at 37 °C and 28 °C for 24 h for bacterial and mycotic isolates, respectively. After incubation, plates

were observed for antimicrobial activities by determining the diameters of the zones of inhibition for each of the samples. For an accurate analysis, tests were run in triplicate for each isolate to avoid any error.

Minimum Inhibitory Concentration (MIC) Method: Microtiter dilution plate quantitative method, i.e. the MIC [21] was used for evaluation of the antimicrobial activity of Egyptian honey against tested organisms. Determination of MIC of extract against tested strains was achieved using 96-well sterile micro plates containing Muller Hinton broth. Initial concentration 100 %, then two fold serial dilutions of the Egyptian honey, reference drugs (ciprofloxacin and fluconazole) and DMSO as control negative, Then wells were inoculated with 100µl of tested strains (0.5 Mc-Farland, about 1×10^8cfu/ml) and incubated at 37°C and 28°C for 24 h for bacterial and fungal strains, respectively. After incubation, plates were examined visually for bacterial or fungal growth. The experiment was repeated three times. The lowest concentration that showed complete hindrance of growth was taken as MIC.

Statistical Analysis: Data were statistically computed using SPSS 15 for Windows[22], using Chi Square analysis.

RESULTS

Experiment 1: Results revealed the bacteriostatic effect of the diluted honey against the tested bacterial isolates. On the other hand, the antimycotic activities of diluted honey (10%) did not showed any fungistatic effect on the examined mycotic isolates. The best bacteriostatic effect was shown against *Sh. flexneri*, *S.*Typhimurium, *E. coli* and*Klebsiella*with zone of bacteriostatic effect equals 40mm, 35mm, 30 mm and 30mm, respectively. Followed by *Pseudomonas aeruginosa*, Citrobacter and *E. fecalis*with zone of inhibition of 26, 20 and 19mm, respectively. Results were compared with reference drugs; ciprofloxacin and fluconazole as shown in Table 1.

Table 1: Agar well diffusion method showing antimicrobial activities of 10% honey against bacterial and fungal isolates compared with reference drugs, results given in (mm).

	Mean zone of inhibition (mm)		
Microbes	Honey 10%	Ciprofloxacin 100 µg/ml	Fluconazole 100 µg/ml
Gram positive bacteria			
E. fecalis	19	43	-
Gram Negative Bacteria			
E. coli	30	50	-
S. Typhimurium	35	39	-
Sh. Flexneri	40	32	-
Klebsiella	30	40	-
Pseudomonas aeruginosa	26	45	-
Citrobacter	20	30	-
Fungi			
C. albicans	-ve	-	45
Asperigllus	-ve	-	45

Amount of tested sample or reference drug added in each well =50 µl/well.

Table 2: Agar well diffusion method showing antimicrobial activities conc. honey against bacterial and fungal isolates compared with reference drugs.

	Mean zone of inhibition (mm)		
Sample	Honey 100%	Ciprofloxacin 100 µg/ml	Fluconazole 100µg/ml
Gram Positive Bacteria			
Bacillus	13	36	-
Streptococcus	28	36	-
S. aureus	29	43	-
E. fecalis	18	43	-
L. monocytogenes	18	45	-
Gram Negative Bacteria			
E. coli O157	26	50	-
E. coli	18	50	-
Salmonella Typhi	30	45	-
Salmonella Typhimurium	30	39	-
Sh. Flexneri	12	32	-
Sh. Sonnei	30	46	-
Klebsiella	20	40	-
Pseudomonas aeruginosa	16	45	-
Citrobacter	12	30	-
Fungi			
C.albicans	15	-	45
Asperigllus	20	-	45

Amount of tested sample or reference drug added in each well =50 µl/well.

Experiment 2: The antimicrobial effect of pure cotton flower honey was presented in Tables 2 &3. Results revealed that concentrated honey has antibacterial activities against Gram negative bacteria; *S.* Typhi, *Sh. sonnei* and *S.* Typhimurium with zone of inhibition 30 mm and MIC 1.56, 1.56 and 6.25µg/ml as well as Gram positive bacteria as *S. aureus* with zone of inhibition 29 mm and MIC 6.25 µg/ml. Streptococcus and *E. coli* O157 showed 28 and 26mm zone of inhibition; and MIC 6.25 and 3.125 µg/ml, respectively. The hindrance activity of pure cotton flower honey decreases against Klebsiella, *E. coli, E. fecalis* and *L. monocytogenes*showing zone of inhibition 20 and 18 mm, respectively and MIC 12.5 µg/ml. The lowest zone of inhibition was detected against *Pseudomonas aeruginosa* (16 mm), *B. cereus* (13 mm) and12 mm *for Sh. flexneri* andCitrobacter and MIC

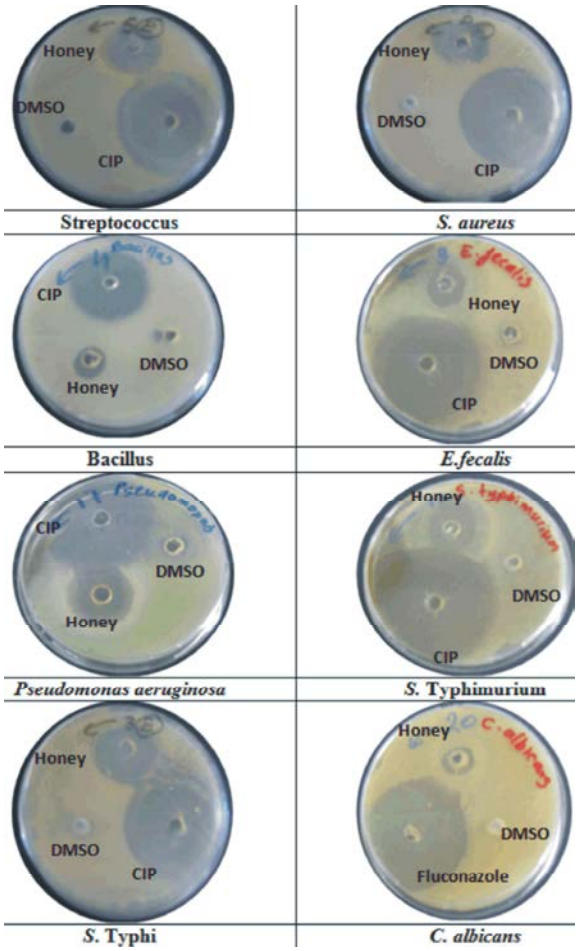

Plate 1: Showing zone of inhibition of honey, antimicrobial reference drugs and DMSO against different microorganisms

25µg/ml for *Pseudomonas aeruginosa* and 100 µg/ml for *B. cereus, Sh. flexneri* andCitrobacter. Pure cotton flower honey expressed a moderate antimycotic effect against*Aspregillus*and *C.albican*s with zones of inhibition of 20 and 15 mm, respectively and MIC 12.5 and 25µg/ml, respectively.

Results of hindrance abilities of honey were compared to highly effective reference drugs including 100µg/ml ciprofloxacin as broad spectrum antibiotic and 100 µg/ml fluconazole as antimycotic (Tables 2& 3). The reference drugs showed great hindrance abilities against tested isolates including Gram negative bacteria; *S.* Typhi, *Sh. sonnei* and *S.* Typhimurium with zone of inhibition 45, 46 and 39 mm and MIC 0.097 µg/ml as well as Gram positive bacteria as *S. aureus* with zone of inhibition 43 mm and MIC 0.39 µg/ml. zone of inhibition was 36 mm against Streptococcus, 50mm against *E. coli* O157 and *E. coli* and 45mm against *L. monocytogenes*. MIC was 3.125

Table 3: Minimum Inhibitory of pure cotton flower honey (100%) against bacterial and fungal isolates compared with reference drugs.

	MIC (%)		
Sample	Honey 100%	Ciprofloxacin 100 µg/ml	Fluconazole 100 µg/ml
Gram Positive Bacteria			
Bacillus cereus	100	0.78	-
Streptococcus	6.25	3.125	-
S. aureus	6.25	0.39	-
E. fecalis	12.5	0.19	-
L.monocytogenes	12.5	0.097	-
Gram Negative Bacteria			
E. coli O157	3.125	0.097	-
E. coli	12.5	0.097	-
*S.*Typhi	1.56	0.097	-
*S.*Typhimurium	6.25	0.097	-
Sh. Flexneri	100	1.56	-
Sh. Sonnei	1.56	0.097	-
Klebsiella	12.5	0.19	-
Pseudomonas aeruginosa	25	0.097	-
Citrobacter	100	1.56	-
Fungi			
C. albicans	25	-	3.125
Asperigllus	12.5	-	0.097

µg/ml against Streptococcus and 0.097 µg/ml against *E. coli* O157, *E. coli* and *L. monocytogenes*.. The hindrance abilities of ciprofloxacin decreased against *Pseudomonas aeruginosa, E. fecalis* and *Klebsiella*, giving zone of inhibition 45, 43 and 40 mm, respectively, while MIC was 0.097, 0.19 and 0.19 µg/ml, respectively. On the other hand, the least hindrance ability was against *Sh. flexneri*, Citrobacter, *B. cereus* with zone of inhibition 32, 30 and 36 mm and MIC 1.56, 1.56 and 0.78 µg/ml. Hindrance activity of fluconazole against mycotic infection with*Aspergillus*and *C. albicans* showed zone of inhibition 45 mm and MIC 0.097 and 3.125 µg/ml, respectively.

DISCUSSION

There is a tremendous need for novel antibacterial agents to treat infections caused by antibiotic-resistant bacteria. Honey, with its long history of usage as an antibacterial agent in traditional and folk medicine [23], has recently brought renewed attention of researchers working in the area of drug discovery and development.

In the present study, cotton flower honey at low concentration (10%) possessed bacteriostatic effect against the tested bacterial isolates. The best bacteriostatic effects of honey were shown against *Sh. flexneri*, *S.* Typhimurium, *E. coli* and*Klebsiella*with zone of b acteriostatic effect equals 40, 35, 30 and 30mm,

respectively. Followed by *Pseudomonas aeruginosa*, Citrobacter and *E. fecalis*with zone of bacteriostatic activity 26, 20 and 19mm, respectively.Similar results on the bacteriostatic effects of honey were previously reported [24].The bactericidal effect of honey was dependent on the concentration of honey used and the nature of the bacteria [25, 28]. Also, Badawy*et al*., 2004 [27] found that the concentration of honey has an impact on antibacterial activity and added that the higher the concentration of honey the greater its usefulness as an antibacterial agent. The factors contributing to antimicrobial activity of honeys identified to date are the high sugar concentration, hydrogen peroxide, methyl glyoxal, the antimicrobial peptide bee defensin-1 and the low pH [28]. Bacteriostatic effect of honeys on methicillin-resistant *Staphylococcus aureus*(MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE) is dose-dependently related to generation of OH from honey H_2O_2 [24]. Furthermore, it was found that both hydrogen peroxide and the non-peroxide components contribute to the bacteriostatic and bactericidal activity of the honey. The honey H_2O_2 was involved in oxidative damage causing bacterial growth inhibition and DNA degradation, but these effects were modulated by other honey components [29]. On the other hand, tualang honey is not effective against Gram positive bacteria [30]. This discrepancy could be due to the type of honey or due to difference in its chemical composition. However, honey at 10% concentration did not show any antifungal effects against the tested mycotic isolates (*C. albicans* and *Aspergillus)*.

Moreover, the present work revealed that concentrated pure cotton flower honey has a strong antibacterial activities against Gram negative bacteria *S.*Typhi, *Sh. sonnei* and *S.* Typhimuriumwith zone of inhibition 30mm and MIC 1.56, 1.56 and 6.25µg/ml, respectively.Also, cotton flower honey has antibacterial effect against Gram positive bacteria; *S. aureus* with zone of inhibition 29mm and MIC 6.25 µg/ml, while, Streptococcus and *E. coli* O157 showed lower zone of inhibition; 28 and 26mm and lower MIC 6.25 and 3.125 µg/ml, respectively. Also, the present study revealed that the hindrance abilities of honey decreases against Klebsiella, *E. coli, E. fecalis* and *L. monocytogenes*with zones of inhibition 20, 18, 18 and 18 mm, respectively and MIC 12.5 µg/ml. The lowest hindrance ability of cotton honey was detected against *Sh. flexneri*, Citrobacter, *B. cereus* with a zone of inhibition 12, 12 and 13mm, respectively and MIC 100µg/ml. Similar results were previously reported [31-33]. Similarly,Agbagwa and Frank-Peterside (2010) [34] examined the antimicrobial activity of 100% Southern Nigerian honey and compared their abilities to inhibit the growth of *S. aureus, P. aeruginosa, E. coli* and *Proteus mirabilis,* an average diameter of zone of inhibition was 15.40±0.15, 3.30± 0.03, 14.80± 0.60 and 7.30±0.07 mm, respectively for the examined microbes. Also, Chauhan*et al.*2010 [35] reported that the extracts of raw and processed honey showed ZDI (6.94-37.94 mm), against Gram-positive bacteria viz., *S. aureus, Bacillus subtilis, Bacillus cereus*, as well as Gram negative bacteria like *E. coli, P. aeruginosa*and *S. entericaserovarTyphi*. Also results agree with Badawy*et al.* 2004 [27] who indicated that the zone diameter of inhibition of different honey samples (5-20%) has been determined against *E. coli* O157: H7 (12 -24 mm) and *S.* Typhimurium (0-20 mm).Rajeswari*et al.* 2010 [33] indicated that the zone diameter of inhibition of Nilgiris honeys are (20-21 mm), (15-16 mm) and (13-14 mm) for *S. aureus, Ps. aeruginosa*and *E. coli*, respectively.Honey is a complex substance and the antibacterial activity is multi-factorial [37].The antibacterial activity of honey can be related to the amount of hydrogen peroxide and the presence of additional antibacterial components derived from the nectar source [28]. More recently, methylglyoxal and the antimicrobial peptide bee defensin-1 were identified as important antibacterial compounds in honey [7].

In conclusion, 10%Egyptian cotton flower honey possessed a bacteriostatic effect against the examined bacterial isolates and raw honey produced a stronger antibacterial and antifungal effect against the tested isolates.

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