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Remedial Power of Honeyfrom Different Floral Sources on Some Bacterial trains Isolated from Infected Wounds

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Remedial effect of different Saudi honey types on some Gram positive bacteria(Staphylococcus aureus, Bacillus subtilis) and Gram negative bacteria (Escherichia coli, Pseudomonas aeruginosaandKlebsiella pneumonia) was investigated. The honey types used in this investigation were different in their floral sources, N. Sativa and Ziziphus- Spinachristilocally known asSamra and Sidir, respectively. Well agar diffusion assay for the antibacterial effect of honey was employed. Zones of complete and partialgrowth inhibition of all bacterial species tested weredetected.Tested activity of thehoney types was compared to that of phenol. 50% (w/v) of each of those honey types was equivalent to 3.3 and 4.5% phenol (w/v). Comparing the antibacterial activity ofSamra and Sidir honey types with some commercial antibiotics at 30 g/ml revealed that, some of the antibiotics used were not effective especially against P.aeruginosa. This indicated that honey could be used effectively in some cases where some antibiotics are not effective.Further investigation on the nature of the antibacterial substance in honey through high pressure liquid chromatography (HPLC) was attempted. Cinnamic acid, methyl-4-hydroxy-3, 5-dimethoxy benzoate and 4-hydroxy-3, 5-dimethoxy benzoic were the standard references available during this study. No correlation was found between their quantity and the antibacterial activity of the honey types. The present study clearly demonstrated thatsome Saudi honey types possess antibacterial activity against some bacterial species causing wound infections

Key words: Honey, Antibacterial, HPLC, phenol, Saudi Arabia.

Usage of honey as an effective remedy has been documented in many cultures a long time ago1,2,3besides its antibacterial activity4,5,6.Three factors (osmolarity, acidity and hydrogen peroxide) have been accepted as playing a major role contributing to the antibacterial activity of honey7 in addition to the presence of antibacterial phytochemical components8,9,10. Furthermre, defensin-1, has recently been found to contribute in the antibacterial activity of honey11.Some components with antibacterial activity have been identified in the ether extract of manuka honey by preparative thin-layer chromatography12,13. 3,5 - dimethoxy-4-hydroxybenzoic acid (syringic acid), methyl 3,5 - dimethoxy-4-hydroxybenzoate (methyl syringate), 3,4,5,-trimethoxybenzoic acid and 2-hydroxy-3-phenylpropionic acid.Comparison of the antibacterial action of honey in different dilutions with the antibacterial effect of commonly used antibiotics on nine types of pathogenic organisms, isolated from urine samples of 149 patients with urinary tract infection was documented by14. Results indicated that 30-50% dilutions of honey were superior to all tested antibiotics including gentamicin. Some honey types of low antibacterial

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activity were not inhibiting to S. aureus up to a concentration of 50% in an agar diffusion assay, other active honey types inhibited the same organisms when diluted to a concentration of 1%15.

Honey has special endorsement by the Saudi consumers as a source of food, primarily due to the fact that the Holy Koran as well as the Hadith have clearly referred to the efficacy of honey as a healer of disease16. Chemical characteristics of imported and locally-produced honey samples was performed17. Results obtained indicated that imported honey samples tested contained significantly higher amounts of moisture, water insoluble solids, total acidity and HMF. Saudi honey types contained significantly higher amounts of calcium and magnesium.The healing potential of Saudi bee honey has been investigated also in limited studies. Natural honey was reported to be inhibitory in vitroto the growth of Helicobacter pylori at 20% concentration18. Natural honey was shown to prevent indomethacin and ethanol-induced gastric lesions in rats19. Natural honey also accelerated the healing of indomethacin-induced antral ulcers in rats20. The antimicrobial activity of Saudi honey was addressed in many previous studies21,22,23,24. Generally, not all honey types created an equal antimicrobial activity because of differences in floral source. Hereby, this study aimed to investigate the curing effect of selected Saudi bee honey types on bacterial species causing wound infections in relation to floral-type utilized by the bee besides some honey components.

MATERIALS AND METHODS

Honey samples

Honey samples were obtained from commercial apiarists throughout the Kingdom of Saudi Arabia. Care was taken to select honey samples differing in their floral sources. Honey samples were supplied and stored in air-tight glass containers at 4 C under dark conditions.The honey samples assayed were unpasteurized; they were tested before and after each assay for possible microbial contamination. Description of the honey samples included in this study isshown in table 1.

Properties of the honey samples

Some physical and chemical tests were performed on the honey samples through the Honey Quality Laboratory (Riyadh, Saudi Arabia) to identify some of their properties as indicated in table 2.

Standard test organisms

The standard test organisms used in this study were obtained from (Difco Laboratories, Mi-U.S.A.). They were selected as representative of Gram positive and Gram negative pathogenic bacteria causing primarily wound infections, except for B.subtilis (Table 3).

Clinical isolates

Six clinical isolates of S. aureus and P.aeruginosa were obtained from six different patients in Riyadh Armed Forces Hospital, Saudi Arabia (Table 4).

High Performance Liquid Chromatography (HPLC)

Waters company series for HPLC fitted with an automated gradient controller model 680, pump model 45, injector wisp model B712, refractive index detector model R401 and recorder model 730.

Standardization of inoculums

The inoculum size of each bacterial tested organism used in the conducted experiments was standardized using Macfarland tube no. 1 to give a concentration of 1x10 organisms per ml. The turbidity standard was prepared according to Lorian (1980) by adding 0.5 ml of 0.048 MBacl2 (1.175% w/v Bacl2 2H20) to 99.5 ml of 0.35N H2S04 (1% v/v) and agitated on a vortex mixer just prior to use.

Assay of the antibacterial activity of honey

The antibacterial activity of honey samples was assayed by agar well diffusion method. Sterile nutrient agar seeded with the organism under test and distributed aseptically into the petri-dishes and allowed to set. The plates were stored for onehour before use. Wells were cut in the agar using a cooled flamed 6 mmcork borer, and the cut discs of agar were removed. Honey samples were tested in four replicates by adding 100µl of the honey sample to each well. All honey samples were prepared aseptically, and handled away from direct bright light. A blank of 100µl sterile distilled water was used per plate. Plates were incubated for 18 hours at 37ºC. The diameter of the clear zone was measured in mm.

Comparison of the antibacterial activity of honey to that of known antimicrobial agents

The antimicrobial activity of the honey typeswas compared to antimicrobial agents of

known antibacterial activitynamely, phenol and antibiotics. Only the diameter of zone of complete inhibition (C.I) was measured.

Comparison of the antibacterial activity of selected honey types to that of phenol

For construction of the standard curve,different dilutions of the honey types were prepared. A 50% (w/v) solution of each honey sample was diluted with sterile distilled water to give a series of 10 concentrations in the range 5-50% (w/v). Solutions of 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10% (w/v) phenol in sterile distilled water were prepared to be used as a standard. All above samples were assayed for their antibacterial activity in agar well diffusion plates seeded with S. aureus.

Antibacterial activity of selected honey types against clinical isolates of *Pseudomonas sp*. and *Staphylococcus sp*.

Clinical isolates of Pseudomonas sp.and Stapylocuccus sp. were obtained from Riyadh Armed Forces Hospital, Saudi Arabia. Honey types were tested at 100%, 50%, 25% (w/v) concentrations. The effect of four commercial antibiotics, Velosef, Chloromycetin, Pentrexyl and Tetracycline at final concentration of 30 g/ml were also assayed for their antibacterial activity against the same clinical isolates.

Determination of the inhibine number

The inhibine number of the honey types was determined21. Five nutrient agar plates which contain graded amounts of diluted honey(20% - 4%) were prepared.All plates were inoculated with a fresh culture of S. aureusand incubated for 24 hours then examined. The inhibine number was taken as the number of the plate with the lowest concentration of honey that does not permit any visible growth of the bacteria.The minimum inhibitory concentration (MIC) of the honey types which inhibited the growth of S. aureus in solid media was also determined by incorporating honey in different concentrations into the growth media and examining plates for determining the lowest concentration of honey that did not allow any visible growth of the bacteria under test.

Statistical analysis

Data were statistically treated with the statistical programme JMP 5.1 Start Statistics, third edition (SAS Institute, Inc., Cary, North Carolina, USA). The variations among the different treatments were tested using One-Way-ANOVA. The results presented are means (4 replicates ± SE).

RESULTS AND DISCUSSION

Detection of the antibacterial effect

In the present work it was aimed to establish the ability of different Saudi honey types from different floral sources to exert an inhibitory effect on the growth of different microorganisms causing wound infections. In Saudi Arabia, there is a wide diversity of ecological and geographical floral sources and bee honey is known to be influenced in quality by the type of flowers and season of its production[26, 27]. Dark honey from conifer forests of the mountainous regions of

Table 1. Description of honey types collected from different floral sources in the kingdom of Saudi Arabia

Honey Type	Location of Production	Season of Production	Floral Source	
			Species	Family
Samra	South	Summer	*Nigella sativa*	Ranunculaceae
Sidir	South	Summer	*Ziziphusspina- christi*	Rhamnaceae

Table 2. Chemical characteristics of the honey samples

Honey Sample	Chemical Tests							
	Moisture %	Invert Sugars			Sucrose Enzyme	Diastase	Acidity	Ash %
		GLU	FRUC	F/G				
Samra	17	31	36	1 .2	-	8.21	35	0.54
Sidir	14	27	5.6	0 .2	2.9	10.34	18	0.065

Central Europe and honey from sweet chestnut (*Castanea saliva*) have been found to have particularly high activity[4,28]. Furthermore, clover (*Trifolium spp*.) honey has been found to haveconsistently low activity[29].Very large differences have been found between honeys from different floral sources in thermal stability of their glucose oxidase content[25]. These information might support our finding regarding different responses of microorganism to honey from different floral sources *Nigella sativa* (Ranunculaceae) *and Z. spina-christi* (Rhamnaceae). By the use of seeded agar well diffusion assay, two kinds of antibacterial effects

Table 3. Standard tested bacterial strains, source, classification and the infectious diseases they cause

Bacterial species	Source	Classification	Infection
Staphylococcus aureus	ATCC 35923	Gram positive	Abscesses, wound infections, carbuncles, impetigo, boils
Escherichia coli	ATCC 35922	Gram negative	Diarhoea, wound infections, septicaemia, urinary infections
Pseudomonas aeruginosa	ATCC 27853	Gram negative	Urinary infections, wound infections
Bacillus subtilis	ATCC 6633	Gram positive	Non pathogenic
Klebsiella pneumoniae	ATCC 13883	Gram negative	Pneumonia, urinary infections

Table 4. Clinical bacterial isolates of S*taphylococcus aureus* and *Pseudomonas aeruginosa* information

Clinical Isolate	Isolate No.	Patient's Sex; Age (Years)	Isolation Site
P. aeruginosa	1	M;31	Wound/diabetic foot
	2	M;65	Pus/leg
	3	M;65	Wound/right leg
S. aureus	6	M;42	Blood
	7	M;10	Wound/unspecified site
	8	M;61	Urine

Table 5. Effect of dilution of Samra honey on the growth inhibition of the standard microorganism

Bacterial species	100%	75%	50%	25%	10%	5%
Staphylococcus aureus	35.5 ± 1.5	31.3±1.2	30.7±0.6	24.3±1.6	15±1.7	7.3±0.6
Escherichia coli	34.3 ± 3.2	29.3±1.5	28±2	24.3±2	20±1	13.7±1.5
Ps. Aeruginosa	15.7 ± 2.1	10±0	8.7±0.9	8±1.7	7.3±0.6	-
B. Subtilis	15.6 ± 0.6	12±1	11.3±1.15	8±1	6.3±1.5	-
K. Pneumoniae	36 .0± 1.0	34.3±2.1	33.3±2.9	23±3	22±1	17.3±2.1

Diameter of growth inhibition zone (mm) is an average of 4 replicates ± SE)

Table 6. Effect of dilution of Sidir honey on the growth inhibition of the standard microorganism

Bacterial species	100%	75%	50%	25%	10%	5%
Staphylococcus aureus	35.0 ± 1.0	31.7±1.5	29.7±0.6	23.7±1.5	16.7±1.32	12.3±2.5
Escherichia coli	35.6 ± 0.6	31±3	30±2	22.3±1.5	21±1.7	13±1.6
Ps. Aeruginosa	17.0 ± 1.7	15.3±5.1	12.7±2.1	10.7±1.2	9.3±1.1	-
B. Subtilis	14.3 ± 2.5	13.7±1.5	11.3±1.1	11.7±0.6	8±1.7	-
K. Pneumoniae	33 .0± 1.5	29.7±0.6	26±1.7	23.7±3	23.3±3.9	15.3±1.1

Diameter of growth inhibition zone (mm) is an average of 4 replicates ± SE)

of honey were detected. As shown in Figure 1,all tested honey samples caused zone of partial inhibition of growth (P.I.) as result of a bacteriostatic action of honey on the tested organism. However, another clear zone of no growth which was much smaller than the (P.I.) was referred to the zone of complete inhibition of growth (C.I.) as result of a bactericidal action of honey on the tested organisms. Complete and partial inhibitory effects were seen persistent for both Gram positive and Gram negative test organisms. The range of the zone of partial inhibition diameter and the zones of complete inhibition as a result of various honey samples on the test organisms wereranged between 13.3 to 37.7mm and between 9.5 to 21.0 mm, respectively.

Many reports confirmed the formation of partial or complete inhibitory effects of honey on growth of different tested organisms[30,31]. However, the inhibitory effect of honey varied among different bacterial species. In case of Gram positive bacteria,*S. aureus* was found to have the most sensitivityto honey types showing higher degrees of antibacterial activity.Previous studies confirmed that S.*aureus*was very sensitive to honey[21,22,32]. *E. Coli* has been considered one of the bacterial species generally sensitive to many of the honey types tested[21,33,34]. Another study indicated that only 100% of a different honey type was required to reach complete inhibition of growth of *K.pneumoniae*[34].Same observations for *P. aeruginosa* and *B. subtilis* were also detected[33,35] when different Saudi honey types were applied. Differences in bacterial responses might be attributed to the osmotic effect and the sensitivity of these organisms to hydrogen peroxide which are unsuitable for bacterial growth[33,36]. Our present results indicated that the diameter of the zone of partial inhibition decreased with increasing the dilution of honey (Tables 5,6) as reported earlier[27,35]. The decrease in the diameter of the zone of partial inhibition could be regarded as due to a decrease in the osmotic effect of honey on the test organism[36,37].

Comparison of the antibacterial activity of selected honey types to that of phenol

Comparingthe antibacterial activity of the honey types to that of phenol has indicated as 50% honeySamra and Sidir are equivalent to 3.3 and 4.5% phenol (w/v), respectively. In a similar

Table 7: Diameter of growth inhibition zone (mm) for six clinical isolates treated with different dilution of the honey types samra and Sidir beside their sensitivity to different antibiotics

Clinical	Isolate	Samra			Sidir			Antibiotics			
Isolate	No.	100%	50%	25%	100%	50%	25%	AMP	TET	CEPH	CHL
P. aeruginosa	1	17	14	0	18	10	0	15	12	13	0
	2	14	13	10	17	13	8	16	17	12	16
	3	12	10	0	14	11	0	0	0	0	0
S. aureus	6	12	11	0	13	8	0	0	0	12	13
	7	12	10	0	11	9	0	0	15	0	15
	8	22	18	9	18	15	10	0	0	0	0

Diameter of growth inhibition zone in mm (average of 4 replicates)

Table 8. Concentration (mg/g) of cinnamic acid, 4-hydroxy-3, 5-dimethoxy benzoic and methyl-4- hydroxy-3, 5-dimethoxy benzoate in Saudi honey samples

Honey Sample	Cinnamic Acid	4-hydroxy-3, 5-dimethoxy benzoic	Methyl 4-hydroxy-3, 5 dimethoxy benzoate
Samra (S-S)	0.300	3.034 (F)	0.002
Sidir (S-S)	0.007	0.207	-

(F) Mixture of more than one component.
Results are the mean of duplicate assays using HPLC system (A).

study on several types of New Zealand honeys found that their antibacterial activities were found

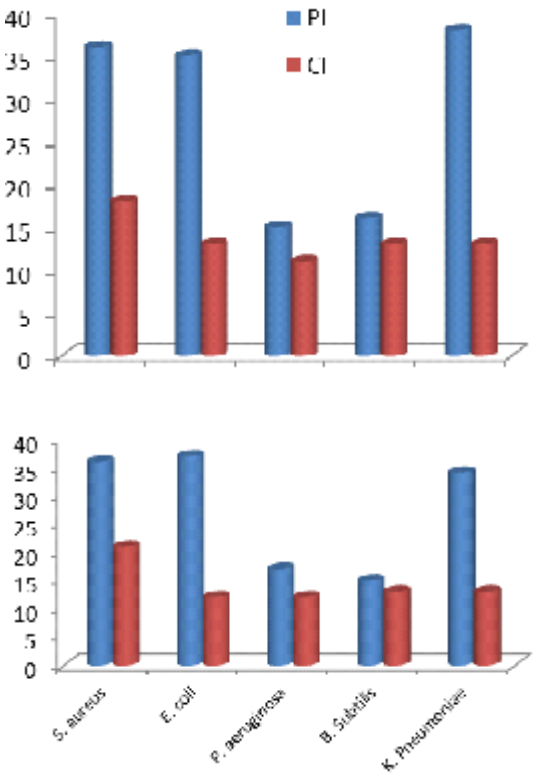

Fig. 1. Comparison between complete inhibition (C.I.) and Partial inhibition (P.I.) for the growth of standard tested organisms with Samra (A) and Sidir (B) in mm

to be equivalent to 14.1 and 13.6% (w/v) phenol, respectively[37]. A recent study on 24 honey types showed activityequal to (5.5-8.4%) w/vto that of phenol[24].

Antibacterial activity of selected honey types against clinical isolates of *Pseudomonas* and *Staphylococcus*

The six clinical isolates of *Pseudomonas sp.* and *Staphylococcus sp.* exhibited a high degree of sensitivity to the honey types tested Samra and Sidir as shown in table 7.*Pseudomonas* isolates were sensitive to the honey types at 100% and 50% concentrations. The complete growthinhibitionzones at those concentrations were (10-17mm) and (11-19mm) for samra and sidir,respectively. At 25% honey however, only one *Pseudomonas* isolates was affected. On the other hand, *Staphylococcus* isolates showed diameter of the zone of complete inhibition of growth for 100% and 50% concentration ranged from (10-22mm) and (8-18mm) for samra and sidir, respectively. At 25% honey however, only one *Staphylococcus* isolates was affected. The four commercial antibiotics tested exerted a selective inhibitory effect on the isolates tested. Similar observations were also detected by[37] and the dilution of honey to 50% inhibited the growth of *S. aureus.*

Determination of the inhibine number of selected honey types

The inhibine number for honey types Samra and Sidirfor the bacterial species *S. aureus* was found to be 4, and 5, respectively. The minimum concentration of each of the honey types which totally inhibited the growth of *S. aureus* when incorporated into the nutrient agar medium were found to be 5% (w/v) for Samra and 5% (w/v) for Sidir, same range of observations was also detected when *S. aureus* was treated with honey types from Yemen[34].

On the other hand, cinnamic acid, Methyl 4-hydroxy-3, 5 dimethoxy benzoate and 4-hydroxy-3, 5-dimethoxy benzoic were detected in the studied samples (Table 8). The three substances are among the components of honey and in the meantime are extensively used as preservatives in pharmaceutical preparations as well as food and cosmetics. They possess antibacterial and antifungal properties to honey[9,10,40]. Our results indicated that there is not much correlation between the presence of these compounds and the higher level of antibacterial activity of honey

CONCLUSION

Results from this investigation revealed that the daily application of bee honey on septic wounds, might give favorable results. Thus, honey could be regarded as a successful alternative to conventional antibiotics normally used as well as the significant cost efficiency in using honey, its application and removal as a dressing could be easier.

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