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Preliminary study on synergistic combinations of raw honey with gentamicin against Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* of veterinary origin

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ABSTRACT

Objective: To search for further synergistic combinations of gentamicin and raw honey that might have potential in treating wounds.
Methods: The antibacterial activity and synergistic interaction of raw honey and gentamicin was assessed by using agar well diffusion method. Two Gram-negative (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 2154) bacteria were selected for antibacterial activity assay. The cultures of bacteria were maintained in their appropriate agar slants at 4 ˚C throughout the study and used as stock cultures.
Results: Raw honey and gentamicin interacted synergistically to inhibit *Escherichia coli* and *Pseudomonas aeruginosa*.
Conclusions: These results suggest that combinations of raw honey and gentamicin have therapeutic benefits in prophylaxis of infections caused by multidrug-resistant Gram-negative bacilli.

1. Introduction

The infections caused by multidrug-resistant Gram-negative bacteria, particularly *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Escherichia coli* (*E. coli*) are increasing worldwide. However, the prevalence of bacterial resistance to conventional antibiotics has prompted an intensive search for new therapeutic agents including various antimicrobial agents of animal origin[1]. Numerous antipseudomonal antibiotics are used currently for the treatment of bronchial infections, including ceftazidime, tobramycin, ciprofloxacin, imipenem, cilastatin and gentamicin. However, resistance to these agents is becoming more prevalent[2,3]. Combination therapy can be used to expand the antibacterial spectrum, to prevent the emergence of resistant mutants, to minimize toxicity, and to obtain synergistic antibacterial activity. Honey has potent activity against both antibiotic-sensitive and -resistant bacteria, and is an interesting agent for topical antibacterial application to chronic wound infections not responding to antibiotic therapy[4]. In Gram-positive bacteria, a synergistic interaction between honey and antibiotics has been suggested[5]. Although two research groups have reported synergy between gentamicin and honey, this was not replicated here with raw honey[6,7]. This could be due to differences in composition of honey. The objective of this work was to investigate antibacterial activity of six multifloral honey samples obtained from different regions of Algeria against *E. coli* and *P. aeruginosa*, and to explore synergistic activity between gentamicin and raw honey.

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2. Materials and methods

2.1. Honey samples

Six honey samples, produced in different regions of Algeria, were taken directly from the containers that the beekeepers use for the storage of honey. All samples were collected in their original packages and were transferred to the laboratory and kept at 4-5 ˚C until analysis. Each sample of honey was double diluted in series of 0%, 50%, 25%, 12.5%, 6.25% and 3.12% of its original concentration with sterile distilled water.

2.2. Bacterial stains

Two Gram-negative (*E. coli* ATCC 25922 and *P. aeruginosa* ATCC 2154) bacteria were selected for antibacterial activity assay. The cultures of bacteria were maintained in their appropriate agar slants at 4 ˚C throughout the study and used as stock cultures. Gentamicin was used as positive reference standard having a concentration of 5 μg/mL.

2.3. Preparation of standard inoculums

Fresh microbial cultures were prepared by streaking loopful of bacterial suspension into organism specific selective media (Merck, Germany) and incubated at optimal temperature in order to maintain approximately uniform growth rate of each organism. The bacterial cultures from fresh media were compared with 0.5 McFarland turbidity standards, which is equivalent to approximately 1×10^8 bacterial cell counts per mL and it was maintained throughout the experimentation.

2.4. Agar well-diffusion assay

The assay was performed as described by Ahmed *et al*[8]. Briefly, agar plates (90 mm) contained 20 mL of nutrient agar medium for all the bacteria.

An 8 mm diameter well was cut into the agar and 100 μL of 100%, 50%, 25%, 12.5%, 6.25% and 3.12% honey solution (w/v,) prepared in sterile distilled water was aliquoted into the well. The controls were set up with equivalent quantities of water as controls. The plates were incubated at 37 ˚C for 24 h. Zones of inhibition were measured using a vernier caliper (Draper). The antibacterial potential of test compound was determined on the basis of mean diameter of zone of inhibition around the wells in millimeters. Each assay was performed in duplicate and repeated twice. The diameter of the inhibition zones were considered as < 5.5 mm, inactive; 5.5-9.0 mm, very low activity; 9-12 mm, low activity; 12-15 mm, average activity; and > 15 mm, high activity.

2.5. Antibacterial synergism of raw honey and gentamicin

After determination of zones of inhibition of raw honey and gentamicin, various concentrations of raw honey and gentamicin below their zones of inhibition were prepared. Mixtures of gentamicin and raw honey were prepared by mixing various concentrations of gentamicin (100%, 50%, 25%, 12.5%, 6.25% and 3.12%) with various concentrations of raw honey (100%, 50%, 25%, 12.5%, 6.25% and 3.12%). These mixtures were tested against two bacerial described above to identify whether there was synergism between gentamicin and raw honey. Synergism was identified when the zones of inhibition of raw honey and gentamicin in combination was higher than the zones of inhibition of raw honey alone.

3. Results

The present study was undertaken to study the efficacy of raw honey alone and in combination with gentamicin. All six different types of raw honey samples used in this study were effective against *E. coli* and *P. aeruginosa* (Tables 1 and 2). It was found that samples of undiluted honey were found to be more effective against *E. coli* whereas 50 % dilution was most effective against *P. aeruginosa*. However, 3.12% dilution was not effective against two Gram-negative bacteria.

Table 1

The mean zone of inhibition obtained using different concentration of raw honey against *E. coli* ATCC 25922.

Samples	Diameter of inhibition zone (mm)					
	100%	50%	25%	12.5%	6.25%	3.12%
RH1	22.5	36.0	25.0	19.0	10.0	ND
RH2	30.0	12.0	31.0	29.0	4.0	ND
RH3	20.0	18.0	11.0	9.0	6.0	ND
RH4	30.0	34.0	24.0	17.0	0.0	ND
RH5	40.0	23.0	26.0	17.0	6.0	ND
RH6	30.0	29.0	24.0	10.0	0.0	ND

RH: Raw honey; ND: Not detected.

Table 2

The mean zone of inhibition obtained using different concentration of raw honey against *P. aeruginosa* 2154.

Samples	Diameter of inhibition zone (mm)					
	100%	50%	25%	12.5%	6.25%	3.12%
RH1	12	22	19	12	9	ND
RH2	ND	30	20	18	9	ND
RH3	ND	30	22	19	12	ND
RH4	12	29	17	14	12	ND
RH5	15	31	25	20	15	ND
RH6	20	30	25	20	ND	ND

RH: Raw honey; ND: Not detected.

The antibacterial assay indicated that the diameter of the zone of

inhibition ranged from 0 to 40 mm and 0 to 31 mm against *E. coli* and *P. aeruginosa* respectively was varied with the concentrations. The highest zone of inhibition was observed in sample of RH5 (40 mm) (Tables 1 and 2).

The combination effects of raw honey and gentamicin were summarized in Tables 3 and 4. The differences in inhibition were observed for six types of raw honey with gentamicin: for *E. coli,* the sample (RH1 + GNT) has the largest inhibition with an average diameter of 26 mm, followed by the sample (RH2 + GNT) in (21 mm), RH3 + GNT (14 mm), RH6 + GNT (7 mm), RH4 + GNT (6 mm) and finally the RH2 + GNT (5 mm). While, *P. aeruginosa*, the zones of inhibition obtained against undiluted honey and gentamicin in combination was 20 and 34 (mm) respectively. The percentage increase was noticed with each variety and it ranged between 13.46% to 100% and 31.03% to 100%. (Figures 1 and 2).

Table 3

The mean zone of inhibition obtained using different concentration of raw honey and gentamicin against *E.coli* ATCC 25922.

Samples	Diameter of inhibition zone (mm)					
	100%	50%	25%	12.5%	6.25%	3.12%
RH1 + GNT	26 (S)	22 (I)	21 (I)	10	6 (I)	ND
RH2 + GNT	21 (I)	21 (S)	19 (I)	9 (I)	5 (S)	ND
RH3 + GNT	17 (I)	14 (I)	14 (S)	8 (I)	4 (I)	ND
RH4 + GNT	13 (I)	12 (I)	11 (I)	7 (I)	6 (AD)	ND
RH5 + GNT	17 (I)	13 (I)	12 (I)	9 (I)	6 (I)	ND
RH6 + GNT	20 (I)	16 (I)	15 (I)	10 (I)	7 (AD)	ND

RH: Raw honey; GNT: Gentamicin; S: Synergistic; AD: Additivity; I: Indifferent; ND: Not detected.

Table 4

The mean zone of inhibition obtained using different concentration of raw honey and gentamicin against *P. aeruginosa* 2154.

Samples	Diameter of inhibition zone (mm)					
	100%	50%	25%	12.5%	6.25%	3.12%
RH1 + GNT	20 (S)	15 (I)	12 (I)	ND (I)	ND	ND
RH2 + GNT	34 (AD)	22 (I)	17 (I)	11 (I)	4 (I)	ND
RH3 + GNT	23 (AD)	23 (I)	20 (I)	10 (I)	8 (I)	ND
RH4 + GNT	30 (S)	21 (I)	20 (I)	11 (I)	9 (I)	ND
RH5 + GNT	26 (S)	25 (I)	19 (I)	10 (I)	7 (I)	ND
RH6 + GNT	29 (S)	15 (I)	14 (I)	9 (I)	ND	ND

RH: Raw honey; GNT: Gentamicin; S: Synergistic; AD: Additivity; I: Indifferent; ND: Not detected.

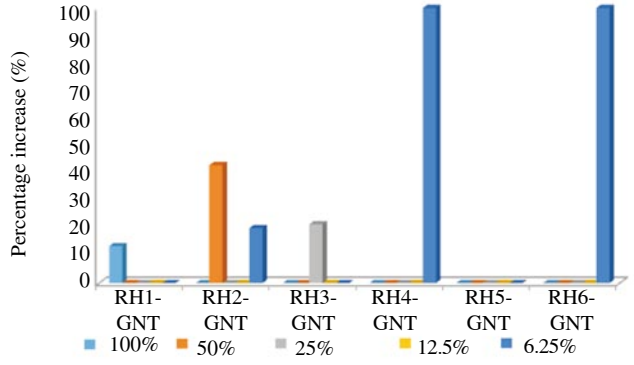

Figure 1. The percentage increase of inhibition zone obtained using different concentration of raw honey and gentamicin against *E.coli* ATCC 25922.
RH: Raw honey; GNT: Gentamicin.

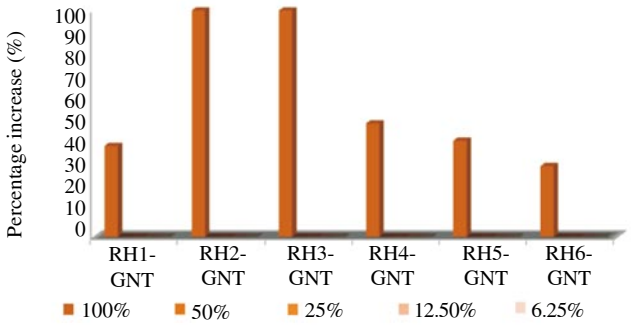

Figure 2. The percentage increase of inhibition zone obtained using different concentration of raw honey and gentamicin against *P. aeruginosa* 2154.
RH: Raw honey; GNT: Gentamicin.

4. Discussion

Many conventional drugs have arisen from bee products, including honey bee. Moreover, few antimicrobial agents have come from this source, with the vast majority in clinical use derived from bee products naturally produced by microorganisms[9-12]. This is the first study to report the synergistic effects of the combination of Algerian honey with gentamicin against *E. coli* and *P. aeruginosa*. Many researchers are studying bee products that could be used as antibiotics against Gram-negative bacteria, and are employing novel dosing regimens and antimicrobials that would be advantageous for combating the therapeutic problems associated with *E. coli* and *P. aeruginosa*. Many studies have found instances of improved efficacies of bee products when they are combined with antibiotics and there is a clinical interest in the use of combinations of bee products and antimicrobial agents to improve the spectrum of drug activity[13-19].

Synergism is a positive interaction created when two agents combined and exert an inhibitory effect (on the targeted organisms) that is greater than the sum of their individual effects. Antagonism results if the combination provides an effect less than the effect of either agent alone or less than the sum of the effects of the individual agents[20]. Recently, synergistic action between honey and curcuma starch was reported for *E. coli* and *P. aeruginosa*[21]; and additivity among of rifampicin, tetracycline and colistin and manuka honey for *P. aeruginosa*[5]. Al-Jabri *et al*. related that Omani honey had a marked synergistic effect on the antibacterial activity of gentamicin towards *Staphylococcus aureus*[7]. Also, Jenkins and Cooper observed a synergistic effect between manuka honey and oxacillin against methicillin-resistant *Staphylococcus aureus*[6].

The antibacterial nature of honey depends on different factors acting singularly or synergistically, the most salient of which

are phenolic compounds, hydrogen peroxide, methylglyoxal and bee defensin-1. Another reason suggested that the antibacterial property of honey was also derived from the osmotic effect of its high sugar content. Combination of bee product like honey with another antibacterial drug gentamicin may, in all probability, turn out to be highly active against the virulent threats caused by a large number of extremely virulent Gram- negative pathogens as is evident from the present study.

In conclusion, our results demonstrate a synergism between raw honey and gentamicin against Gram-negative bacteria (*P. aeruginosa* and *E. coli*). Further study is also needed to determine the underlying mechanism of the synergistic action.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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References

[1] Masco L, Van Hoorde K, De Brandt E, Swings J, Huys G. Antimicrobial susceptibility of *Bifidobacterium* strains from humans, animals and probiotic products. *J Antimicrob Chemother* 2006; **58**; 85-94.

[2] Banerjee D, Stableforth D. The treatment of respiratory pseudomonas infection in cystic fibrosis: what drug and which way? *Drugs* 2000; **60**: 1053–64.

[3] Waterer GW, Wunderink RG. Increasing threat of Gram-negative bacteria. *Crit Care Med* 2001; **29**: N75-81.

[4] Kwakman PHS, de Boer L, Ruyter-Spira CP, Creemers-Molenaar T, Helsper JP, Vandenbroucke-Grauls CM, et al. Medical-grade honey enriched with antimicrobial peptides has enhanced activity against antibiotic-resistant pathogens. *Eur J Clin Microbiol Infect Dis* 2011; **30**(2): 251-7.

[5] Karayil S, Deshpande SD, Koppikar GV. Effect of honey on multidrug resistant organisms and its synergistic action with three common antibiotics. *J Postgrad Med* 1998; **44**: 93-6.

[6] Jenkins RE, Cooper R. Synergy between oxacillin and manuka honey sensitizes methicillin-resistant *Staphylococcus aureus* to oxacillin. *J Antimicrob Chemother* 2012; **67**(6): 1405-7.

[7] Al-Jabri AA, Al-Hosni SA, Nzeako BC, Al-Mahrooqi ZH, Nsanze H. Antibacterial activity of Omani honey alone and in combination with gentamicin. *Saudi Med J* 2005; **26**: 767-71.

[8] Ahmed M, Djebli N, Aissat S, Zerrouki K, Bourabeh A. *In vitro* synergistic antibacterial activity of natural honey combined with curcuma starch and their correlation with diastase number, flavonoid and polyphenol content. *J Plant Pathol Microb* 2013; **4**: 152.

[9] Ayaad TH, Shaker GH, Almuhnaa AM. Isolation of antimicrobial peptides from *Apis florae* and *Apis carnica* in Saudi Arabia and investigation of the antimicrobial properties of natural honey samples. *J King Saud Univ Sci* 2012; **24**: 193-200.

[10] Al-Waili NS, Saloom K, Butler G, Al Ghamdi AA. Honey and microbial infections: a review supporting the use of honey for microbial control. *J Med Food* 2011; **14**: 1079-96.

[11] Ansari AA, Alexander C. Effect of natural honey (produced by *African sculata* in Guyana) against bacteria (*Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus*) and fungus (*Candida albicans*). *World J Dairy Food Sci* 2009; **4**(1): 73-7.

[12] Wu CS, Kim HK, Wezel GPV, Choi YH. Metabolomics in the natural products field-a gateway to novel antibiotics. *Drug Discov Today Technol* 2015; doi: 10.1016/j.ddtec.2015.01.004.

[13] AL-Ani I, Zimmermann S, Reichling J, Wink M. Pharmacological synergism of bee venom and melittin with antibiotics and plant secondary metabolites against multi-drug resistant microbial pathogens. *Phytomedicine* 2015; **22**: 245–55.

[14] Al-Waili NS. Mixture of honey, beeswax and olive oil inhibits growth of *Staphylococcus aureus* and *Candida albicans*. *Arch Med Res* 2005; **36**(1): 10-3.

[15] Muller P, Alber DG, Turnbull L, Schlothauer RC, Carter DA, Whitchurch CB, et al. Synergism between medihoney and rifampicin against methicillin-resistant *Staphylococcus aureus* (MRSA). *PLoS One* 2013; **8**(2): e57679.

[16] Tajik H, Jalali FSS. *In vitro* evaluation of antimicrobial efficacy of natural honey in comparison with sulfonamide derivatives. *J Anim Vet Adv* 2009; **8**: 23-5.

[17] Campeau MEM, Patel R. Antibiofilm activity of Manuka honey in combination with antibiotics. *Int J Bacteriol* 2014; doi: 10.1155/2014/795281.

[18] Fernandes Júnior A, Balestrin EC, Betoni JE, Orsi Rde O, da Cunha Mde L, Montelli AC. Propolis: anti-*Staphylococcus aureus* activity and synergism with antimicrobial drugs. *Mem Inst Oswaldo Cruz* 2005; **100**(5): 563-6.

[19] AL-safi SM. Synergistic effect of propolis and antibiotics on the *Salmonella typhi* TY21. *Kufa J Vet Med Sci* 2013; **4**(1): 118-27.

[20] Kumar SN, Siji JV, Nambisan B, Mohandas C. Activity and synergistic antimicrobial activity between diketopiperazines against bacteria *in vitro*. *Appl Biochem Biotechnol* 2012; **168**(8): 2285-96.

[21] Moussa A, Noureddine D, Saad A, Abdelmalek M, Salima B. The influence of botanical origin and physico-chemical parameters on the antifungal activity of Algerian honey. *J Plant Pathol Microb* 2012; **3**: 132.