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Antimicrobial properties of *Dalbergia, Brassica* and *Trifolium* honey against burn Microorganisms

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ABSTRACT

This research aimed at assessing the antimicrobial properties of *Dalbergia*, *Brassica* and *Trifolium* honey samples against microorganisms isolated from infected burned skin of patients in children hospital, Lahore, Pakistan. The isolated microorganisms were identified as *P. aeruginosa, E.coli, K. pneumoniae* and *S. aureus*. The original bacterial inoculum was serially diluted (adjusted to 1.5 X 10^6 CFU) and spread on the nutrient agar plates. Whattmann filter paper discs were soaked in three different concentrations (50%, 70% & 90%) of each of the three unifloral honey samples for 48 hrs. The filter discs were placed on the agar plates seeded with the individual bacteria. Solitary effect of antibiotic discs (Ciprofloxacin, Imipenem, Ceftriaxone, Amikacin, and Vancomycin) and their synergistic effect were also studied. No bacterial growth showed resistance to honey at any concentration when used individually or in combination with antibiotic although bacteria showed resistance to Ciprofloxacin and Ceftriaxone. *Brassica* honey 90% was the most effective at all concentrations with a maximum of inhibition zone 11.13 mm against *P. aeruginosa* followed by 90% *Trifolium* honey with the maximum inhibition zone 10.75 mm against *P. aeruginosa*. The *Dalbergia* honey (50%) was least effective against *P. aeruginosa*, however it inhibited *S. aureus* producing inhibition zone 10.25 mm. Honey-antibiotic combination produced inhibition zone 51 mm that was much larger than the inhibition zone produced by antibiotic or honey when applied individually. Honey, whether used individually or in combination with an antibiotic, was effective against all the bacterial isolates used in this study.

Keywords: *Dalbergia, Brassica,* honey, *Trifolium,* antibiotic discs

INTRODUCTION

Ever since the introduction of antibiotics, the concern of antibacterial resistance has only doubled with about 70% bacterial species resistant to known antibiotics. Gram-positive species of *Staphylococcus*, *Streptococcus*, *Enterococcus* and Gram-negative species of *Klebsiella*, *E. coli*, *Proteus*, *Serratia*, *Pseudomonas*, *Acinetobacter*, *Candida*, Herpes simplex virus, *Varicella zoster* virus are the most commonly isolated, multidrug-resistant microbes of burned. Furthermore, burn infections are characterized by slow healing and scamming. The resistant bacteria causing the burn associated infections further complicate the treatment protocols. Therefore, alternate natural therapies such as honey may offer better treatment avenues compared with the modern antibiotics (Bowler, 2002).

The antimicrobial efficiency of honey against microbial infections has been known since the ancient times. The flower nectar is collected by the honeybees of genera *Apis* and *Meliponinae* and is converted into honey through enzymatic processes involving bee salivary enzymes (Iglesias *et al.,* 2006). Honey is known to contain more

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than180substances including fructose (38%), glucose (31%), carotenoids, vitamins, minerals, aromatic substances, organic acids, proteins and amino acids (Szweda, 2017; Khan *et al*., 2012; Poonkothai *et al*., 2013). Of all the amino acids present in the honey, proline is the most abundant amino acidthat is derived from bee salivary secretions during the conversion of nectar to honey. In addition, gluconic acid is the predominant organic acid produced by glucose oxidase (Karabagias *et al*., 2014). However the honey composition is variable and depends on the type of flower bee visits for collection of nectar.

Anoveruse or misuse of medications including anti-infective drugs results in an antibiotic resistance (Bowler, 2002). Hence, there is a dire need to explore new antibiotics that could be used against resistant untreatable infections. This is where honey offers a unique potential to contain microflora resistant to conventional antibiotics. Thus, honey has been found to be effective against more than 70 microbial species. The probable mechanism of action is through oxidation of glucose to gluconic acid by glucose oxidase, generating hydrogen peroxide (H_2O_2) as a byproduct. H_2O_2 is a known antimicrobial agent and it is only produced when honey is diluted with water activating glucose oxidase. H_2O_2 is a powerful oxidizing agent that inhibits bacterial growth and multiplication. In addition, the gluconic acid, produced in the oxidation process, lowers the honey pH 3.2-4.5 making it unsuitable for bacterial growth (Koochak *et al*., 2010). The low pH range is a significant antibacterial factor for diluted honey and inhibits growth of many microbes such as *E. coli* that grows at a minimum pH 4.3, *Salmonella spp*. (4.0), *P. aeruginosa* (4.4), *S. pyogenes* (4.5) etc (O'Grady *et al*., 1997).

The high osmolarity of honey also offers microbial growth inhibition since honey sugar molecules can 'tie up' water making it unavailable for the microbial growth. Bee defensin-1, also called royalysin, is produced by the hypopharangeal glands of honey bee and it exhibits antibacterial activity against Gram-positive bacteria such as *Bacillus subtilis*, *Staphylococcus aureus*. The concentration of antibacterial, defensin-1 varies with the type of honey. The normal flora composed of approximately 40 lactic acid bacterial (LAB) strains with 13 taxonomically well-defined *Lactobacillus* (9 spp.) and *Bifidobacterium* (4 spp.) species play an important role in the antibacterial activity of honey.

This symbiotic flora produces several bioactive compounds such as organic acids, hydrogen peroxide, antimicrobial peptides, antibiotics and bacteriocins. Bacteriocins are protein complexes that are highly inhibitory towards both Gram positive and Gram negative bacteria (Szweda, 2017).

The present study aims to evaluate the antimicrobial properties of the unifloral honey collected from *Dalbergia, Brassica* and *Trifolium* species. The antimicrobial activity was tested against burn microorganisms, and the degree to which these properties persist when honey is diluted. An intra-comparison of the antimicrobial characteristics of these honey samples as well as with standard antibiotics was investigated.

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MATERIALS AND METHODS

Sixty patients suffering from burn infections were selected and the microbial community was taken from deep of the wound, using a sterile cotton swab. These swabs were then streaked several times, across nutrient agar plates to obtained distinctly unconnected microbial colonies. For identification of the microbes, colony morphology and biochemical tests were performed. The *Dalbergia, Brassica* and *Trifolium* honey samples were diluted with sterile water to produce 50%, 70% and 90% concentrations (wt/wt) respectively. Sterile Whattman filter paper discs (0.6 mm) were left in each of these diluted honey samples for 48 hrs under sterile conditions. Antibiotic sterile discs of ciprofloxacin (CIP), amikacin (AK), imipenem (IMP), ceftriaxone (CRO) and vancomycin (VAN) were applied according to their target action against Gram-positive or Gram-negative or both types of bacteria. These antibiotic discs were also left in each of honey concentrations (50%, 70% and 90%) of the three honeys for 48 hrs and applied to the bacterial culture spread over the nutrient agar plates. These plates were incubated at 37^{o}C for 2 hours and the zone of inhibition was measured and recorded the next day.

RESULTS

Isolation of Microorganisms

Colonies of *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus* and *Klebsiella pneumoniae* were isolated and their morphology was examined under microscope and were identified on the basis of results of biochemical tests given in table I.

Table I: Summary of the results obtained with the biochemical tests

Biochemical Test	*Pseudomonas aeruginosa*	*Escherichia coli*	*Klebsiellapneumoniae*	*Staphylococcus aureus*
Gram Staining	-ve	-ve	-ve	+ve
Motility test	+ve	+ve	-ve	-ve
Catalase test	+ve	+ve	+ve	+ve
Oxidase test	+ve	-ve	-ve	-ve
Indole test	-ve	+ve	-ve	-ve
Methyl red test	-ve	+ve	-ve	+ve
Citrate test	+ve	-ve	+ve	+ve
Urease test	-ve	-ve	+ve	+ve

Results with disc diffusion method

Effect of *Dalbergia* honey

The largest zone of inhibition at 50% concentration of *Dalbergia* honey was produced against *K. pneumoniae* with an average value of 8.00 mm while the least average inhibition zone 6.13 mm was recorded for *P. aeruginosa.* At 70% concentration, the highest inhibition zone, 8.85 mm was recorded for *S. Aureus* while the minimum inhibition zone 6.88 mm was recorded for *E. coli.* At 90% concentration of *Dalbergia* honey, the inhibition zone 10.25 mm was recorded for *S. Aureus* while *E. coli* showed a minimum inhibition zone of 7.00 mm (Table II).

Table II: Average recorded values of inhibition zone (mm) produced by *Dalbergia* honey against microorganisms used in the study.

Zone of Inhibition (mm)			
Microorganism	**50%**	**70%**	**90%**
Pseudomonas aeruginosa	6.13± 0.1	7.13± 0.1	7.50± 0.11
Escherichia coli	6.88± 0.12	6.88± 0.1	7.00± 0.1
Klebsiella pneumoniae	8.00± 0.16	7.80± 0.1	7.75± 0.14
Staphylococcus aureus	7.60± 0.11	8.85± 0.12	10.25± 0.16

Effect of *Brassica* honey

Brassica honey at 50% concentration, showed the largest inhibition zone, 8.50 mm for *K. pneumonia*. This showed the superior sensitivity towards *Brassica* honey compared with other three microorganisms. The least sensitivity was shown by *S. aureus* with an inhibition zone of 7.00 mm. *P. aeruginosa s*howed the largest, 9.75 mm inhibition zone at 70% concentration of *Barssica* honey and the smallest inhibition zone, 8.5 mm for *S. aureus* appeared with 70% honey concentration. At 90% concentration, the highest average inhibition zone 10.75 mm, was obtained for *K. pneumonia* and *E. coli* showed an inhibition zone 10.25 mm. The smallest, 8.63 mm average inhibition zone appeared for *S. Aureus* (Table III).

Table III: Average recorded values of inhibition zone (mm) produced by *Brassica* honey against microorganisms used in the study

Zone of Inhibition (mm)			
Microorganism	**50%**	**70%**	**90%**
Pseudomonas aeruginosa	8.10± 0.1	9.75± 0.13	11.13± 0.25
Escherichia coli	7.25± 0.1	9.00± 0.1	10.25± 0.1
Klebsiella pneumoniae	8.50± 0.14	9.25± 0.11	10.75± 0.1
Staphylococcus aureus	7.00± 0.21	8.50± 0.1	8.63± 0.2

Effect of *Trifolium* honey

Trifolium honey (50%) concentration was the most effective against *K. pneumoniae* with an average inhibition zone 7.75 mm, followed by *S. aureus* with an inhibition zone, 7.30 mm. *P. aeruginosa* produced the smallest inhibition zone, 6.05 mm, at 50% concentration of the honey. At 70%, the largest inhibition zone, 8.67 mm was produced against *S. aureus* and the least sensitivity or the smallest inhibition zone, 7.00 mm, appeared against *E. coli* was 7.65 mm. At 90% honey concentration, the inhibition zone, 10.75 was the largest against *P. aeruginosa* and the smallest inhibition zone 8.03 mm at this concentration was produced against *E. coli* (Table IV).

Table IV: Average recorded values of inhibition zone (mm) produced by *Trifolium* honey against microorganisms used in the study.

Zone of Inhibition (mm)			
Microorganism	**50%**	**70%**	**90%**
Pseudomonas aeruginosa	6.05± 0.1	8.25± 0.13	10.75± 0.23
Escherichia coli	6.60± 0.2	7.65± 0.16	8.03± 0.11
Klebsiella pneumoniae	7.75± 0.13	8.50± 0.19	9.40± 0.2
Staphylococcus aureus	7.30± 0.25	8.67± 0.23	9.65± 0.2

Microorganism inhibition with standard antibiotics

P. aeruginosa was resistant to all three antibiotics but *P. aeruginosa* was sensitive to amikacin. *E*. *coli* showed resistance to ciprofloxacin and ceftriaxone but it had the greatest sensitivity towards amikacin. *K*. *pneumonia* was not resistant to any of the antibiotics applied. It was least sensitive towards amikacin and highly sensitive towards ceftriaxone. Of the two antibiotics applied to *S. aureus*, a greater sensitivity was obtained towards imipenem than with vancomycin (Table V).

Synergistic activity of honey with antibiotics

Of all the antibiotic and honey combinations used against *P. aeruginosa,* the most effective was the combination of antibiotic amikacin with 90% *Trifolium* honey. AK+ 90% *Trifolium* produced a zone of 44 mm while the smallest zone against *P. aeruginosa* was produced by the combination of imipenem with 50% *Trifolium* honey. IMP+ 50% *Trifolium* honey produced a zone of 12.5 mm.

Against *E. coli*, CRO+ 90% *Brassica* produced the largest zone of inhibition of 36.5 mm while the smallest zone of 19 mm was produced by the combination of ciprofloxacin with 50% *Brassica* honey. Against *K. pneumoniae*, AK+ 90% *Trifolium* produced the largest zone of inhibition of 42 mm while the smallest zone of 11 mm was produced by the combination of ciprofloxacin with 70% *Dalbergia* honey. Against *S. aureus*, IMP+ 90% *Trifolium* produced the largest zone of inhibition of 51 mm.

Comparison of results

A comparative study of the three honey sample showed that *Brassica* honey was the most effective at all the three concentrations (50%, 70% and 90%), against all the three microorganisms. *Trifolium* honey was the second best effective honey at all of its three concentrations. *Dalbergia* honey used either singly or in combination with the standard antibiotics, was found to be the least effective against all the three microorganisms. The antibiotics ciprofloxacin and ceftriaxone, when used individually showed no inhibition of the bacterial isolates. However, when these antibiotics were combined with honey they produced a much larger inhibition zone. For example, *E. coli* showed resistance to ceftriaxone and highest sensitivity towards amikacin. However, the combination of CRO+90% *Brassica* honey produced 36.5 mm inhibition zone which was the largest of all the zones produced by antibiotic and honey combination against *E. coli*. In addition, this zone was also much larger than 10.25 mm zone produced by 90% *Brassica* when applied individually.

Table V: Zone of Inhibition (in mm) produced by antibiotic discs against the microorganisms used in the study

Antibiotic inhibition zones (mm) against tested microorganisms					
Microorganism	**Ciprofloxa-cin**	**Imipenem**	**Ceftriaxone**	**Amikacin**	**Vancomycin**
Pseudomonas aeruginosa	0.00	21.50	N/A	27.00	N/A
Escherichia coli	0.00	31.00	0.00	32.00	N/A
Klebsiella pneumoniae	29.50	26.60	35.00	24.60	N/A
Staphylococcus aureus	N/A	34.40	N/A	N/A	18.50

Table VI: Inhibitions zones produced against tested bacteria by honey, antibiotic and combination of antibiotic and honey

Microorganism	Honey	Antibiotic	Antibiotic + Honey
Pseudomonas aeruginosa	90% *Dalbergia* (11.13 mm)	Amikacin (27 mm)	AK+90% *Trifolium* (44.0 mm)
Escherichia coli	90% *Dalbergia* (10. 25 mm)	Amikacin (32 mm)	CRO+90% *Dalbergia* (36.5 mm)
Klebsiella pneumoniae	90% *Dalbergia* (10. 75 mm)	Ceftriaxone (35 mm)	AK+90% *Trifolium* (42 mm)
Staphylococcus aureus	90% *Dalbergia* (10.25 mm)	Imipenem (34.40mm)	IMP+90% *Trifolium* (51 mm)

DISCUSSION

In the present study, *Dalbergia*, *Brassica* and *Trifolium* honey samples were investigated for their antibacterial activities andthe order of antimicrobial activities was established as *Brassica>Trifolium>Dalbergia* honey.

The four bacterial isolates used in the study, *P. aeruginosa, E. coli, K. pneumonia* and *S. aureus*, were multidrug resistant tested by inhibition zone. For example, *P. aeruginosa* and *E. coli* were resistant to ciprofloxacin and ceftriaxone respectively (all of the bacterial isolates were sensitive to imipenem and amikacin). Abdallah (2016) carried out an antibacterial susceptibility test on Gram-negative bacteria (*Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli*) and Gram-positive bacteria (*Staphylococcus aureus*). Amikacin generally works through inhibition of bacterial protein synthesis by binding to 30s ribosome leading to misreading of mRNA (Ahmed *et al*., 2015). However, Kibret and Abera (2011), specifically experimented antimicrobial susceptibility test using *E. coli*, and showed that it was sensitive to ciprofloxacin. However, recent studies have shown that Ciprofloxacin's sensitivity against *E. coli* declines with higher resistance patterns. The formation of these 'super bacteria' is happening because they are rapidly becoming resistant to one antibiotic after another in a very small time span. Resistance to honey has not yet been reported which makes it a very promising topical antimicrobial agent against infection of antibiotic-resistant bacteria (Dixon, 2003; Carter *et al*., 2016). This is likely due to the complex composition of honey, which causes the individual components to act either individually or in synergy to prevent resistance (Cooper *et al*., 2010).

P. aeruginosa is a common pathogen of infected burn wounds. Abd*et al.* (2007) carried out a comparative study of honey and ciprofloxacin against *P. aeruginosa* and showed that the mean inhibition zone of Sider, Acacia and Eucalyptus honey were (12.1, 11.2, 11.05 mm), respectively. These inhibition zones are comparable to 11.13 mm zone produced by 90% *Brassica* honey but *Dalbergia* and *Trifolium* produced smaller zone of 7.50 mm and 10.75 mm, respectively, against *P. aeruginosa*. Nevertheless, except for *Dalbergia* honey, the 10.75 mm zone by *Trifolium* and 11.13 mm zone by *Brassica* are significantly higher than 8 mm against *P. aeruginosa* as reported by Osman

et al. (2003). All this work and the present study, thus, show that *P. aeruginosa* is resistant to Ciprofloxacin but not to a vast majority of honey samples (Kwakman *et al*., 2011). The difference in their antimicrobial activities is according to floral, botanical and geographical origins, and also to bee-origin metabolic products (Almasaudi *et al*., 2016).

A combination of the antimicrobial properties of clinically approved antibiotics and the antibacterial activity of honey could lead to a new spectrum of antimicrobials providing broad-spectrum coverage and consequently improving therapeutic efficiency. Alkhyat *et al*. 2014 blended local Yemeni honey brands and antibiotics to compare the effectiveness of this combination with the individual honey/antibiotic against standard bacterial isolates. All diluted honey samples (25%, 50% and 75%) inhibited growth of the standard bacteria. Mixture of Gentamicin and honey samples showed maximum inhibition zones with *S. abony and S. aureus, P. aeruginosa* as 32, 30 and 16 mm, respectively. From these results, they concluded that honey could effectively complement standard antibiotics, especially in cases of pathogenic infections in general and in burn wounds in particular. Similar results were obtained in the present study using *Dalbergia*, *Brassica* and *Trifolium* honey. *E. coli* is resistant to Ciprofloxacin but when it was blended with *Dalbergia*, *Brassica* and *Trifolium* honey, inhibition zones enlarged upto 27.0 mm, which is 3-folds larger than the inhibition zones produced when honey and antibiotic were used individually. In addition, combined Imipenem and Trifolium honey (90%) produced 6-folds larger inhibition zone (51 mm) against *K. pneumonia.* This was much larger than inhibition zone 9.40 mm produced by *Trifolium* and 26.60 mm produced by imipenem individually. This result aguments that combination of honey and antibiotic can be more efficient especially when bacteria become resistant to antibiotics.

CONCLUSION

The results of this study conclude that *Brassica* honey at 50%, 70% and 90% concentrations was the most effective against the microorganisms (*P. aeruginosa, E. coli, K. pneumoniae* and *S. aureus*) followed by *Trifolium* and *Dalbergia* honey. The results also revealed that when resistant bacterial isolates were exposed to a combination of different honey samples and antibiotics, a much larger inhibition zone was exhibited. Thus, imipenem combined with *Trifolium* at 90% produced an inhibition zone 51 mm against *S. aureus* and 34.40 mm produced by imipenem, applied individually. This shows that once microbes become resistant, they can be constrained by using a combination of honey and an antibiotic.

Conflict of Interest Statement

All authors declare that there is no conflict of interest regarding this manuscript.

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