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Original Research Article

Isolation of pathogenic microorganisms from burn patient and *in vitro* determination of antibacterial activity of honey against antibiotic resistance isolates

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ABSTRACT

Background: Honey is a natural therapeutic agent which manifest antimicrobial activity against a wide range of bacteria. Therefore, the current study was designed to isolate pathogenic bacteria from burn wound and also to determine the anti-bacterial traits of natural and processed honey against infectious agents.
Methods: Wound samples were collected from burn unit of Dhaka Medical College Hospital and conventional cultural methods were applied to identify pathogenic microorganisms. A total of six samples including three each of natural and processed honey were tested for the determination of antimicrobial activity by agar well diffusion method.
Results: Among ten wound samples highest load of total viable bacteria was recorded up to 3.7×10^6 cfu/ml. The maximum load of *Pseudomonas* spp. and *Staphylococcus* spp. were found up to 1.6×10^4 cfu/ml and 8.7×10^4 cfu/ml respectively. Significant *in vitro* antimicrobial activity was found in all the samples. Natural honey showed a little bit more efficacy than processed honey. The samples exhibited antibacterial traits against *Staphylococcus aureus* with a wide zone of inhibition and moderate zone of inhibition against *Pseudomonas* spp. when they are subjected to 100% concentered honey. *E. coli* and *Klebsiella* spp. were remained to be unaffected at 75% and 50% concentrated honey, while *S. aureus* and *Pseudomonas* spp. were found to be sensitive at those concentrations.
Conclusions: The *in vitro* efficacy of different types of honey tested against the bacteria dependent on the type of honey and the concentration at which it was administered. In our study 100% concentred honey was more efficient in inhibiting all the tested isolates.

Keywords: Burn wound, Pathogen, Honey, Antibiotic resistance, Anti-bacterial activity

INTRODUCTION

Burn patient especially with first degree or second degree injuries, are frequently exposed to microbial infection.[1-3] Burn wound infection accelerated by opportunistic pathogen or exogenous infectious agent which acquired through exposure to the hospital environment, hospital personnel or medical devices.[4-9] Any bacterium could be a likely pathogen in burn wounds considering the extent and depth of the injury; however, coagulase-negative *Staphylococci*, *Staphylococcus aureus* and *Enterococcus* spp. have been reported to be the most common gram positive pathogens, and *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and *Acinetobacter* spp. are the most common gram negative microorganisms.[7,10-13] Physical condition of host and virulence factors of microbial flora enhances the risk of disease progression.[14,15] Unfortunately resistant pathogens are continue to develop and spread in the environment and as a result effectiveness of antibiotics is being diminished day by day. Moreover, vigorous bacterial resistance was reported against the latest

generation of antimicrobial which is very alarming to public health.[12,16] Therefore plant and plant-based product have chosen as an alternative antimicrobial agent and among them honey is among the most prominent ones.[17]

Honey is the natural sugary substance collected and stored by honey bees from flower. The honey has been used from ancient times as a method of accelerating wound healing, and the potential of honey to assist with wound healing has been demonstrated repeatedly.[18-22] There are many reports in the clinical literature of honey being used with success in treatment of a wide range of burn wound infection. It inhibits a broad spectrum of bacterial species.[23] Honey is gaining acceptance as an agent for the treatment of ulcers, bed sores and other skin infections resulting from burns and wounds because the antibacterial properties of honey speed up the growth of new tissue to heal the wound.[24] However, one of the most important properties seems to be its antibacterial action. High sugar concentration and low pH of honey is very effective to prevent microbial growth. Besides honey absorbs water out from the environment and as a consequence's bacteria dehydrated. Previous studies revealed that the honey exhibit effectiveness against methicillin-resistant *S. aureus* (MRSA), beta hemolytic *Streptococci* and vancomycin resistant *Enterococci* (VRE).[25]

Multidrug resistance bacterial strains become apparent because of over and non-selective use of antibiotics especially methicillin-resistance *Staphylococcus aureus* which is the major contributor of skin infections.[25,26] To get over this global challenge, like plants and plant-based products such as honey have currently get more attention.[17] Considering all these fact, we designed the study to investigate the antimicrobial traits of honey against the isolate collected from burn wounds.

METHODS

Sampling from burn wound

Taken consent from the patient of burn unit of Dhaka Medical College Hospital (within April 2019 to July 2019) surface swabs were collected from burn wounds after the removal of dressings and topical antimicrobial agents and cleansing of the wound surface with 70% alcohol.[3] Specimen was collected on sterile cotton swab by rotating with sufficient pressure. Samples were homogenized in 4 ml sterile saline.

Microbiological and biochemical analysis

Samples were immediately cultured on NA (nutrient agar), Mannitol salt agar (MSA), MacConkey agar and PA (*Pseudomonas* agar) plates for the isolation of Total viable bacteria, *Staphylococcus* spp., coliform group bacteria and *Pseudomonas* spp. respectively. After inoculation, plates were kept at 37°C for 24-48h.[27,28] A series of several biochemical tests were performed following the standard protocol to identify the bacteria isolated from the wound samples.[3]

Determination of antimicrobial susceptibility

The standard agar disc diffusion method known as the Kirby-Bauer method was applied.[29,30] A suspension of the test organisms were prepared by adjusting the turbidity of the broth in phosphate buffer saline by comparing with McFarland 0.5 solutions. Each bacterial was prepared on Muller Hinton agar plates by sterile cotton swab. Commercially available antimicrobial discs (Oxoid, Hampshire, UK) were applied aseptically (neomycin 10 µg, chloramphenicol 10 µg, polymyxin B 30 µg, ofloxacin 5 µg, amoxicillin 10 µg, ciprofloxacin 5 µg, cefpodoxime 30 µg, nalidixic acid 30 µg, imipenem 10 µg, tetracycline 30 µg) on the surface of the inoculated plates at appropriate spatial arrangement by means of a sterile needle. Susceptibility to the specific antibiotic was interpreted by the presence of clear zone around the disc.[29]

Honey sampling

Three kinds of natural honey (honey from Khalisha tree, Poshur tree and Gewa tree) were collected from beekeepers of the Sundorbon zone and three types of processed honey of different brands were collected from super shop by using purposive sampling technique. Honey was collected in sterile screwed cups/culture bottle.

Preparation of honey solutions

Hundred percent pure honey (100% v/v) was obtained after filtration using sterile gauze. To get 1 ml of 75%, 50% and 25% concentrated honey solution (v/v); 0.75 ml, 0.5 ml and 0.25 ml of honey was diluted in 0.25 ml, 0.5 ml and 0.75 ml distilled water constitutively. For processed honey same methods are followed.[31]

Determination of antimicrobial efficiency of honey

The antimicrobial activity both natural and processed honey samples were was performed by agar well diffusion method.[29,30] At first, the inoculum (with standard turbidity compared to that of the McFarland standard of 0.5) of each of the test bacteria; i.e., *Pseudomonas* spp., *Klebsiella* spp., *Staphylococcus* spp. and *E. coli* was prepared and by using sterile cotton swab uniform lawns were produced on MHA. Wells were then made spanning the MHA by means of sterile cork-borer. 100 µl of honey with the concentration of 75%, 50%, and 25% was added to the wells in the plate. Plates were incubated at 37°C for 12 h. The mean diameters of inhibition zones were measured in mm, and the results were recorded. A positive control well was equally filled with vancomycin 30µg, while sterile distilled water used as negative control.[29,31]

RESULTS

Prevalence of microorganisms in burn wound samples

Out of 10 samples, 8 were found to be hugely populated with bacteria ranging from 10^5-10^7 CFU/ml, among which almost all were found to harbor *Pseudomonas* spp. in the range of (10^3-10^5 CFU/ml) and *S. aureus* (10^3-10^6 CFU/ml). Among the enteric bacteria, *Klebsiella* spp. was found to prevail among 6 samples in the range of (10^3-10^4 CFU/ml) and a comparative lower frequency was observed in case of *E. coli* (in 2 samples) (Table 1).

Drug-resistance traits of the isolates

Out of 10 common antibiotics, amoxicillin, tetracycline and chloramphenicol were found to be effective against *E. coli* isolates. Imipenum, tetracycline and chloramphenicol were found to be effective against *Klebsiella* spp. Imipenum and cefpdoxime were found effective against *Pseudomonas* spp. and ciprofloxacin, tetracycline and ofloxacin were found to be effective against *S. aureus* (Table 2).

Bacteriostatic/bactericidal efficacy of natural honey and processed honey

Table 3 and 4 demonstrate the inhibitory action of three natural honey and three processed honey on the tested bacterial strains. Different types of honey possess different efficacies and mechanisms against the same type of bacteria. 100% concentrated honey samples exhibited best results against almost all isolates. 100% Khalisha flower honey showed its highest antibacterial activity against *S. aureus* (38 mm) and *Pseudomonas* spp. (25 mm).

Table 1: Bacterial load (CFU/ml) in burn wound samples.

Sample	Total viable bacteria (CFU/ml)	*Pseudomonas* spp. (CFU/ml)	*S. aureus* (CFU/ml)	*E. coli* (CFU/ml)	*Klebsiella* spp. (CFU/ml)
01	2.13×10^6	6.8×10^4	1.12×10^5	0	6.7×10^4
02	1.48×10^6	8.9×10^4	1.8×10^4	0	6×10^3
03	2.33×10^6	6.3×10^4	4.5×10^4	0	0
04	3.19×10^6	1.32×10^5	2.7×10^4	0	1.7×10^4
05	1.40×10^6	7.9×10^4	2.8×10^4	4×10^3	0
06	9.8×10^5	2.5×10^3	3.1×10^3	0	0
07	3.65×10^6	7.7×10^4	4.3×10^4	0	6.5×10^4
08	7.6×10^5	1.6×10^4	9×10^3	0	0
09	4.24×10^6	1.43×10^5	8.7×10^4	0	3.4×10^4
10	1.54×10^6	1.21×10^5	3.3×10^5	7×10^3	9×10^3

All the experiments have been performed three times and one reproducible data has given.

Table 2: Antimicrobial susceptibility pattern of different pathogenic isolates in the burn wound sample.

Organisms antibiotics	*E. coli* (n=2)		*Klebsiella* spp. (n=6)		*Pseudomonas* spp. (n=10)		*Staphylococcus* spp. (n=10)	
	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)
CIP (5 µg)	67	33	98	2	70	30	40	60
CPD (30 µg)	80	20	100	0	34	66	ND	ND
AMO (10 µ)	33	67	87	12	80	20	100	1
IPM (30 µg)	90	10	0	100	20	80	ND	ND
N (10 µg)	73	27	60	40	ND	ND	ND	ND
CHL (10 µg)	45	55	24	76	66	34	ND	ND
TE (30 µg)	20	80	18	82	ND	ND	30	70
PB (30 µg)	80	20	ND	ND	ND	ND	ND	ND
NA (30 µg)	80	20	75	25	40	60	ND	ND
OFL (5 µg)	70	30	ND	ND	ND	ND	22	78

S - susceptibility, R - resistance, ND - not done, (CIP - ciprofloxacin, CPD - cefpodoxime, AMO - amoxicillin, IMP -imipenem, N - neomycin, CHL - chloramphenicol, PB - polymyxin B, NA - Nalidixic acids, OFL - ofloxacin, TE -tetracycline.

Table 3: Anti-bacterial activity of natural honey against burn wound isolates.

Raw honey	Zone of inhibition in diameter (mm)			
	E. coli **(n=2)**	***Klebsiella*** **spp. (n=6)**	***Staphylococcus*** **spp. (n=10)**	***Pseudomonas*** **spp. (n=10)**
Khalisha flower honey 100% concentrated	14	15	38	25
Khalisha flower honey 75% concentrated	0	0	22	14
Khalisha flower honey 50% concentrated	0	0	15	0
Poshur flower honey 100% concentrated	16	8	42	23
Poshur flower honey 75% concentrated	0	0	21	12
Poshur flower honey 50% concentrated	0	0	16	8
Gewa flower honey 100% concentrated	15	13	36	18
Gewa flower honey 75% concentrated	0	0	23	16
Gewa flower honey 50% concentrated	0	0	14	0

All the experiments have been performed three times and one reproducible data has given.

Table 4: Anti-bacterial activity of processed honey against burn wound isolates.

Processed honey	Zone of inhibition in diameter (mm)			
	E. coli **(n=2)**	***Klebsiella*** **spp. (n=6)**	***Staphylococcus*** **spp. (n=10)**	***Pseudomonas*** **spp. (n=10)**
Sample 1 100% concentrated	12	15	32	18
Sample 1 75% concentrated	9	0	24	10
Sample 1 50% concentrated	0	0	13	0
Sample 2 100% concentrated	9	8	37	17
Sample 2 75% concentrated	0	0	21	9
Sample 2 50% concentrated	0	0	12	0
Sample 3 100% concentrated	11	10	40	13
Sample 3 75% concentrated	0	0	26	11
Sample 3 50% concentrated	0	0	16	0

All the experiments have been performed three times and one reproducible data has given.

75% and 50% concentrated forms of this honey have no anti-bacterial activity against *E. coli* and *Klebsiella* spp. but a significant zone of inhibition recorded against *S. aureus* in its all three concentration. In case of Poshur flower honey and Gewa flower honey a wide clear zone of inhibition was observed against *S. aureus* (42 mm and 36 mm respectively). Other three isolates also exhibited remarkable zone of inhibition when exposed to 100% concentrated honey. However, 75% and 50% concentrated honey had no antibacterial activity on *E. coli* and *Klebsiella* spp. and limited activity on *Pseudomonas* spp. Moderate zone of inhibition was reported in case of *S. aureus*. On the other hand, all the three processed samples were able to effectively inhibit the growth of *S. aures* and *Pseudomonas* spp. Unlike natural honey, 100% concentrated processed honey reveal a clear zone of inhibition against *S. aures* and

Pseudomonas spp., *E. coli* and *Klebsiella* spp. were remained unaffected at 75% and 50% concentration of honey.

DISCUSSION

Effective drug against wound infections have been a problem in the field of medicine for a long time and nowadays antimicrobial resistance increases which leads, to a continued search for new agents.[32] Broad spectrum antibacterial activity of honey against gram positive and negative bacteria had reported earlier.[33] Floral honey has expressed efficacy against *S. aureus*, *E. coli* and *Klebsiella* spp. which can be vary from more than 100 folds, depending on its geographical, seasonal and botanical source as well as harvesting, processing and storage conditions.[34] Honey contains sugar mainly (glucose, fructose, sucrose) in high concentration up to 82%, H_2O_2, phenolic compounds, phytochemical components such as methylglyoxal and a wide range of minerals those are effective for the treatment of infections, burns, wounds.[34,35] Hydrogen peroxide is the major contributor to the antimicrobial activity of honey, and the different concentrations of this compound in different honeys result in their varying antimicrobial effects.[36] Therefore, it has been shown that the antimicrobial activity of honey may range from concentrations <3% to 50% and higher.[37] Our present study exhibited that 100% concentrated honey showed the higher effectiveness, on the contrary 75% and 50% concentrated honey expressed less activity. Besides H_2O_2, an endogenous enzyme glucose oxidase, produced by honey has also antimicrobial activity.[36] The bactericidal effect of honey is reported to be dependent on concentration of honey used and the nature of the bacteria.[37] The antibacterial property of honey is also derived from the osmotic effect of its high sugar content and low moisture content, along with its acidic properties of gluconic acid.[33] In current study, we observed that compare to the antibiotics, honey has the better antimicrobial activity against pathogenic isolates and some studies proved that honey has a potential role in the decontamination of wound-infecting antibiotic-resistant strains of bacteria like MRSA.[38-40] This evidence supports the existing local traditional practice of using honey to treat wound infections.

CONCLUSION

Currently, the emerging antimicrobial resistance trends are a serious challenge to limiting virulence properties of burn wound bacterial pathogens. Therefore, honey is very promising natural antimicrobial agent. In our current investigation both natural and processed honey proved their efficiency against burn wound infectious agent whereas commercial antibiotics found less functional. From ancient period to till date honey act as a healing agent so it could definitely be listed as a potential therapeutic agent.

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