



Evaluation of *in vitro* antimicrobial activity of Indian honey on burn wound isolates

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ABSTRACT

This study was aimed at determining the susceptibility of organisms namely *Staphylococcus sp.*, *Pseudomonas sp.*, *Klebsiella sp.*, *Escherichia coli* and *Candida* associated with burn wound infection to some Indian honey. Five swabs from patients with burn wound were cultured. The effect of honey on these pathogens was tested by performing Agar Well Diffusion assay, MIC and MBC. All the honey samples tested showed strong definite antibacterial activity against these organisms but the activity pattern was different for different honeys. The isolates were also sensitive to the antibiotics ciprofloxacin at 1 mg/ml concentration. A comparative study was made between the processed honey and the raw honey. It was seen that processed honey did not show any inhibitory effect on the pathogens under the study supporting the view that processing procedures affect the therapeutic properties. The Honey samples were treated with catalase to group them into either peroxide-based or non-peroxide based honeys. The outcome of this assay was that Acacia and Forest honey samples lost their antimicrobial activity completely whereas Neem honey showed a decreased inhibitory effect. Phytochemical analysis was also carried out which included qualitative and quantitative analysis of the honey samples for Tannins, Alkaloids, Total phenols, antioxidant potential and H_2O_2 levels.

Keywords: Honey, MIC, MBC, Hydrogen peroxide, catalase, phytochemicals

INTRODUCTION

Honey has been used as a medicine in many cultures for centuries [1]. The antibacterial activity of honey was first recognized in 1892, by van Ketel [2], following which extensive research was conducted to further substantiate this claim and to demonstrate factors that contribute to the activity [3]. It has been rediscovered by the medical professionals and is gaining acceptance as an antibacterial treatment of topical infections resulting from burns and wounds [4]. Honey has been reported to have an inhibitory effect on around 60 species of bacteria including aerobes and anaerobes, gram-positives and gram-negatives [3], antifungal action against some yeasts and species of *Aspergillus* and *Penicillium* [1], as well as all the common dermatophytes [5]. Honey based wound care products have been registered with medical regulatory authorities as wound care agents in Australia, Canada, the European Union, Hong Kong, New Zealand and the USA. In most instances these products use Manuka honey from New Zealand or the equivalent honey produced from other *Leptospermum* species in Australia [6].

Honey has several properties that contribute to its antimicrobial activity such as low pH and high osmolarity combined with the enzymatic production of hydrogen peroxide [7,8]. The high osmotic pressure or low water activity of honey is inhibitory to the growth of majority of bacteria and some yeasts and moulds. When applied topically to wounds, osmosis results in the drawing off of water from the wound into the honey, helping to dry the

infected tissue and reduce bacterial growth. The low pH (between 3.2 and 4.5) in honey, arising due to the oxidation of glucose to gluconic acid by the enzyme glucose oxidase, is inhibitory to many pathogenic bacteria [9]. Even low concentrations of H₂O₂, the end product of the glucose oxidase system plays an important role in the wound healing mechanism [10] and in stimulation and proliferation of peripheral blood lymphocytic and phagocytic activity [11]. Other factors, such as low protein content, high C/N ratio, low redox potential due to the high content of reducing sugars, viscosity and other phytochemicals are also likely to play some role in defining antibacterial activity of honey [12]. These phytochemical components of floral origin can confer antimicrobial activity that is stable in the presence of catalase, an enzyme that destroys H₂O₂ [13]. The composition of honey and hence its antimicrobial activity varies widely depending up on the botanical origin of the nectar and also on the geographical location. Variation in activity is also seen within the same floral species due to prevailing environmental conditions that affect the physiology of the plant [5,13,14]; or to bee-related factors such as age or colony health, which may affect the production or activity of glucose oxidase [15,16]. In a clinical setting where honey is used as a topical antimicrobial and wound dressing, non-peroxide activity may be advantageous as it is not destroyed by catalase present in body fluids, and is unaffected by gamma irradiation used for sterilization prior to medicinal use [17].

India is home to diverse and unique floral resources that are exploited by the beekeeping industry. No published data exist on the antimicrobial activity of most Indian honey, and the benefits of this knowledge to both the apiary industry and the health care sector are clear. Therefore, the aim of this study was to survey some of the Indian honey sourced from different native flora for antimicrobial activity on microorganisms routinely isolated from burn wounds such as *Staphylococcus*, *E. coli*, *Klebsiella spp.*, *Pseudomonas* and *Candida albicans*.

EXPERIMENTAL SECTION

Collection of Honey samples

Raw unprocessed honey samples were obtained from local apiarists and stored in the dark at room temperature. The identification was performed by the bee hunters based on their geographical hunting area and floral availability at the location of bee hives (foraging radius). The three types of honey used were: (i) Acacia (RA); a unifloral honey derived from the plant *Acacia mangium* belonging to the Family Fabaceae widely, (ii) Forest (RF); a multifloral honey derived from the Himalayan forest from Coniferaceae plants, (iii) Neem (RN); a unifloral honey harvested from the Neem tree (Indian Lilac) belonging to the Family Meliaceae. The three samples were dispensed into sterile cork-screwed containers. In the laboratory, all the three honeys were extracted by hand pressing and then filtered with a sterile mesh to remove debris. Their purity and confirmation of non-adulteration through physical examination of color, viscosity and non-infected with insects was performed. Processed (pasteurized) honey (PA, PF, PN) from the same source was used for comparison of anti-microbial activity.

Preparation of Artificial Honey

Artificial honey (AF) was prepared by dissolving 76.8 g of fructose and 60.6 g of glucose separately in 100 ml of sterile, deionized water, and by mixing these two solutions in a 1:1 ratio.

Phytochemical Analysis of Honey Samples

Qualitative Studies

Tannins: To 2 ml of diluted honey a few drops lead acetate was added. Yellow precipitate indicates a positive result.

Alkaloids (Wagner's test): To 1 ml of diluted honey 2-3 drops of Wagner's reagent was added. A reddish brown precipitate indicates a positive result.

Quantitative Analysis

Total Phenolic content: Total phenolic contents were estimated according to the spectrophotometric method [18]. Based on the measured absorbance, the concentration of phenolics was calculated from the calibration line; the content of phenolics in extracts was expressed in terms of gallic acid equivalent (mg of GAE/g of tissue).

Estimation of Antioxidants Using DPPH Assay: Procedure of Braca et al., [19] was used for determination of DPPH scavenging capacity of various fractions. A stock solution of ascorbic acid (1000 µg/ml) was diluted ranging from 10-100 µg/ml. 0.1 ml solution from different dilutions was pipetted out in respective tubes. The volume in each tube was made up to 3 ml with DPPH (20 µg/ml). The test tubes were incubated for 10 min at room temperature. The contents of each tube were mixed well and the absorbance was measured at 517 nm against a blank. A control (3.1 ml DPPH) was prepared. The percentage inhibition of DPPH by the samples was calculated as follows:

Scavenging effect (%) = [(OD of control-OD of sample) / (OD of control)] × 100

Collection and Cultivation of Isolates from Clinical Specimens

Collection of clinical specimens were done from five burn patients who were suffering from burn injuries that were less than 1 month old and were being treated at Bangalore Baptist Hospital, Bengaluru. The swabs were obtained from these wounds and cultivated for identification by streaking on Eosin Methylene Blue agar (EMB), Nutrient Agar (NA), MacConkey agar (MAC) and Muller Hinton Agar (MHA) and then inoculated for 24 h at 37 °C. Pure cultures were obtained by sub-culturing each distinctive colony on agar slants. Each isolate was given a number before its identification was done and these numbers were maintained till its identity was confirmed by further biochemical investigations.

Biochemical tests for identification of isolates

The colonies obtained were further used to identify the organisms by performing various tests namely indole, coagulase, oxidase, motility, urease, citrate utilization, triple sugar iron, MR-VP and Gram staining [20].

Well Diffusion Assay

Each of the honey samples at original concentration (100%) were used for this purpose. All the tested honey samples (PA, PF, PN, RA, RF, RN and AF) were adjusted to 40 °C in a water bath in order to aid pipetting. Each of the isolated organisms was picked up with an inoculating loop and suspended in 3-4 ml of nutrient broth and incubated for 2-3 h at 37 °C, then diluted with sterile distilled water to a turbidity that matches 0.5 McFarland standard as a suspension. Each of the test pathogens that was standardized were surface spread on Muller Hinton agar in petri dish. A single well was bored using a 6 mm sterile cork borer into each dried plate. About 0.2 ml of each honey type was aseptically introduced into the well of each plate using a micropipette. Ciprofloxacin (1 mg/ml) were used as the control in order to compare the activity between the honey samples and control. The plates were allowed to dry on the laboratory bench as well as allowing the honey to diffuse for about 2- 3 h and then incubated at 30 °C for 24-48 h. The antibacterial activity was expressed as the diameter of the zone of inhibition (ZOI) calculated as the difference in diameter of the observed zone and the diameter of the well or disc.

Minimum Inhibitory Concentrations (MIC)

The antibacterial activity of processed, raw and artificial honeys was determined using a broth microdilution assay using a 96-well microplate format. Row A-F contained the different honey type with the most effective dilutions obtained by well diffusion assay. Each well of these rows was prepared by pipetting 50 µl of the inoculum which was a 24 h old broth culture (10^6 cfu/ml final concentrations for each microorganism) and 50 µl of the diluted honey sample from row A to row F of a microplate. Row G contained only inoculum and served as a positive control and row H contained sterile MH broth and served as a blank. After overnight incubation of plates at 37 °C in a shaking water-bath, bacterial growth was measured at $A_{595\text{ nm}}$ using the Synergy HT multi-detection microplate reader (Synergy HT, Bio-Tek Instruments). The contribution of color of honeys to the absorption was corrected by subtracting the absorbance values before (zero time) incubation from the values obtained after overnight incubation. The absorbance readings obtained from the dose-response curves were used to construct growth inhibition profiles (GIPs). The minimal inhibitory concentrations (MIC) were determined from the GIPs and represented the lowest concentration of honey that inhibited the bacterial growth. The MIC end point in our experiments was honey concentration at which 90% bacterial growth reduction was observed as measured by the absorbance at $A_{600\text{ nm}}$.

Minimum Bactericidal Concentrations (MBC)

For each set of the test tubes in MIC determination, a loopful of broth was collected from those which did not show any growth and inoculated on sterile nutrient agar plates. The inoculation was carried out using streak method. The plates were then incubated at 37 °C for 24 h. All the plates examined for the presence of growth. The concentration at which no visible growth was observed after incubation period was noted as minimum bactericidal concentration (MBC).

Endogenous H₂O₂ Content

Hydrogen peroxide content in various honey samples was determined [21]. 1 ml of 10 % honey in 50 mM phosphate buffer (pH 7.0) was centrifuged at 10,000 g for 15 min and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. The absorbance of the supernatant was measured at 390 nm.

$$C_{UK} = (A_{UK} \times C_s) / A_s$$

Catalase-treatment of Honey Samples

Honey were treated with catalase (3500 U/mg solid; Sigma-Aldrich) at ratio of 1500 U/ml of 50% honey solution in sterile water for 2 h at room temperature. Overnight cultures of *Staphylococcus* (1.5 ml, adjusted to 10^7 cfu/ml in MH broth) were treated with 50% honey solution in a 1:1 ratio (v/v). After overnight incubation at 37 °C with continuous shaking, the tubes were streaked out on Nutrient agar, incubated at 37 °C for 24 h and checked for growth (Snow and Manley-Harris, 2004); triplicates were used throughout.

RESULTS AND DISCUSSION

Phytochemical analysis of Honey

The phytoconstituents are known to play an important role in the bioactivity of honey. Presence of tannins and alkaloids were observed in all the three types of raw honey (Table 1) suggesting the presence of a number of chemical ingredients, which might be responsible for the various pharmacological actions like antibacterial, antiulcer, anticancer, and chemo protective activities of honey; although their specific roles remain to be investigated. Phenolic compounds have been reported to have multiple biological effects, including antioxidant activity [22]. The total phenolic contents were found to be highest in raw Neem (373.2 ± 4.1 mg GAE/g) followed by raw Forest (297.5 ± 3.1 mg GAE/g) and of raw Acacia honey (205.6 ± 5.8 mg GAE/g) (Table 1). Evidences suggest that honey rich in polyphenolic compounds play a significant role in reducing oxidative stress at the burn wound site [9]

Total antioxidant capacity of the extracts was calculated using % inhibition against ascorbic acid. The results of the present investigation demonstrate that the honey samples can significantly decrease *in vitro* DPPH*concentration, thus suggesting the presence of secondary metabolites with strong antioxidant activity. The antioxidant properties of natural and synthetic antioxidants are believed to be responsible for their beneficial effects during treatment of inflammation disorders. There is a positive relationship between total phenols and antioxidant activity of honey samples, because of the scavenging ability of their hydroxyl groups. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing super oxide anion ($O_2^{\cdot-}$), hydroxyl radical or peroxy radicals, quenching singlet and triplet oxygen or decomposing peroxides [23]. Polyphenolic contents appear to function as good electron and hydrogen atom donors, and therefore, be able to terminate radical chain reaction involved in lipid peroxidation by converting free radicals and reactive oxygen species to more stable products.

Table 1: Qualitative and quantitative analysis of the raw honey samples

Honey	Tannins	Alkaloids	Total Phenols (mg GAE/g)	DPPH (mg QE/g)
Acacia	++	++	205.6 ± 5.882	26.283 ± 4.415
Neem	++	++	373.2 ± 4.166	57.633 ± 4.210
Forest	++	++	297.5 ± 3.174	33.419 ± 2.351

Effect of dilution on H₂O₂ levels in Honey

Formation of H₂O₂ depends on the honey dilution since glucose oxidase is inactive in undiluted honey [24]. It is seen that the dilution of honey increases the H₂O₂ levels for Acacia and Forest honey wherein the maximum levels were observed for 50% dilution (Fig 1). Such correlation was not observed for Neem honey which exhibited higher H₂O₂ levels in the undiluted sample.

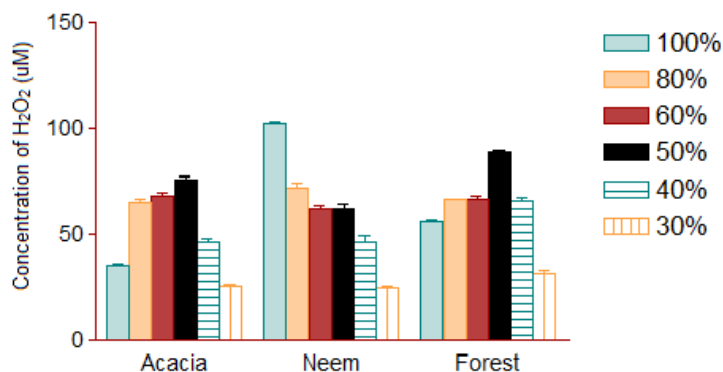


Fig 1: Effect of dilution of Honey on its H₂O₂ levels

H₂O₂ in honey is produced mainly during glucose oxidation catalyzed by the bee enzyme, glucose oxidase (FAD-oxidoreductase, EC 1.1.3.4) (White et al., 1963). The levels of H₂O₂ in honey are determined by the difference between the rate of its production and its destruction by catalases. Glucose oxidase is introduced to honey during nectar harvesting by bees. This enzyme is found in all honeys but its concentration may differ from honey to honey depending on the age and health status of the foraging bees [25] as well as the richness and diversity of the foraged diet [26]. Catalase often of pollen origin efficiently hydrolyzes H₂O₂ to oxygen and water due to its high turnover numbers. The total concentration of catalase depends on the amount of pollen grains in honey [27], and consequently, the H₂O₂ levels in different honeys may vary considerably [24]. A substantial correlation has been found between the level of endogenous H₂O₂ and the extent of inhibition of bacterial growth by honey [8,24]. It is observed that in honeys with a high content of this oxidizing compound, bacteria cannot respond normally to proliferative signals and their growth remains arrested even at high honey dilutions.

Identification of Isolated Organisms

Exposed subcutaneous tissue provides a favorable substratum for a wide variety of microorganisms to contaminate and colonize, and if the involved tissue is devitalized (Ex., ischemic, hypoxic, or necrotic) and the host immune response is compromised, the conditions become optimal for microbial growth. Whereas a minor, healing wound may allow sufficient time for only relatively small number of skin contaminants to take residence, the continued exposure of devitalized tissue associated with a slowly healing chronic wound is likely to facilitate the colonization and establishment of a wide variety of endogenous microorganisms. Based on the colony characteristics (Table 2), Gram character and biochemical tests, the organism corresponding to isolates 1 to 5 (Table 3).

Table 2: Colony characteristics of the organisms isolated from the burn wounds

Isolate no.	Shape	Elevation	Margin	Color
1	Circular	Raised	Undulate	Green
2	Circular	Convex	Entire	Greenish metallic sheen
4	Circular	Convex	Entire	Yellow
5	Circular	Raised	Entire	white

Table 3: Gram staining, morphology and biochemical characteristics of isolates obtained from burn wound infections

Isolate	Morphology	GR	Motility	Cat	Ox	Ind	MR	VP	Cit	Organism
1	Rods	Negative	Motile	+	+	-	-	-	+	<i>Pseudomonas</i>
2	Rods	Negative	Motile	+	-	+	+	-	-	<i>E. coli</i>
3	Fungal appearance	Positive	Non-motile							<i>Candida</i>
4	Cocci in clusters	Positive	Non-motile	+	-	-	+	+	+	<i>Staphylococcus</i>
5	Rods	Negative	Non-motile	+	-	-	-	+	+	<i>Klebsiella</i>

**GR- Gram reaction; Cat-catalase; Ox-oxidase; Ind-indole; MR-Methyl red; VP-Voges-Proskauer; Cit-Citrate; n/c-not carried out.

Well Diffusion Assay

All of the processed honey samples (PA, PN and PF) failed to show any antibacterial activity. Among the raw honey samples (RA, RF and RN), Acacia honey showed the maximum inhibitory effect on all the 5 microorganisms at 50% dilution (Table 4). Neem honey inhibited growth of *Pseudomonas* at all dilutions with maximum inhibition

with undiluted honey. *Candida* was also seen to be inhibited by undiluted honey, whereas 50% diluted honey was found to be effective on both *E. coli* and *Klebsiella*. Forest honey did not show any inhibitory effect on *Staphylococcus* and *E. coli*. *Pseudomonas* growth was inhibited by all the dilutions under consideration with maximum inhibition at 50%. Undiluted honey was found to be effective in the case of *Candida*. The zone of inhibition obtained is given in Table 4.

Table 4: The effect of different concentrations of Raw Acacia (RA), Forest (RF) and Neem (RN) Honey against the pathogens determined by well agar diffusion

The values are means of triplicates expressed in mm.

Test organisms	Antibacterial activity (Zone of Inhibition in mm)												Cipro (1mg/ml)
	Acacia Honey				Forest Honey				Neem Honey				
	Dilution percentage (%)												
	30	40	50	100	30	40	50	100	30	40	50	100	
<i>Pseudomonas</i>	1.6	4.9	28.9	48	0.36	1	0.64	1.69	0.36	0.49	1.56	0.36	55
<i>Staphylococcus</i>	25.6	28.9	32.4	21	-	-	-	-	-	-	-	-	48
<i>E. coli</i>	25.6	19.6	28.9	45	-	-	1.4	0.4	-	-	-	-	21
<i>Klebsiella</i>	0.9	4.0	10	39	-	-	0.25	0.09	0.09	0.16	0.49	0.16	45
<i>Candida</i>	10	22.5	25.6	14.4	-	-	-	4	-	-	-	1.8	39

Table 4 show the results of *in vitro* susceptibility of the extracts of raw honey having varying degree of antibacterial activity against Gram-positive as well as Gram-negative bacteria. These might be due to the osmotic effect, the effect of pH, and the sensitivity of these organisms to H₂O₂ (Fig 1) which are unsuitable for bacterial growth, represented as an “inhibition” factor in honey [13]. Major variations seen in overall antibacterial activity were due to changes in the level of H₂O₂ achieved and in some cases to the level of non-peroxide factors. The content of non-peroxide factors was obviously related to the floral source and sometimes accounted for the major part of the antibacterial activity in honey [28]. However, H₂O₂ concentration produced in honey was typically around 1 mmol/L [3], about 1000 times less than 3% solution commonly used as an antiseptic. The harmful effects of H₂O₂ were further reduced as honey sequesters and inactivates free iron which catalyzes the formation of oxygen free radicals produced by H₂O₂ [28], and its antioxidant components help to mop up oxygen free radicals.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Following recognition of their marked antibacterial activities, the three raw honeys were chosen to determine their MIC and MBC. The MIC value indicates the inhibitory concentration at which honey showed no visible growth of any test organisms. MIC for the active extract was determined by macrodilution method. Results in Table 5 showed that the MIC values for the bacterial pathogens were 50 µg/ml for all types of honey treatment except for *P. aeruginosa* which exhibited 100 µg/ml when treated with neem. The MBC value of all honey samples was in the range 25–45 µg/ml. The MIC values of various honey samples on selected wound pathogens is as shown in Fig 2.

Table 5: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of raw honey samples

Organism	Acacia Honey		Forest Honey		Neem Honey	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
<i>Pseudomonas</i>	50	30	50	25	100	35
<i>E. coli</i>	50	30	-	-	50	20
<i>Candida</i>	100	40	100	45	100	45
<i>Staphylococcus</i>	50	25	-	-	-	-
<i>Klebsiella</i>	50	30	50	30	50	35

Honey has been reported to be effective in the healing of infected post-operative wounds [29]). The *in vitro* antimicrobial activity of honey was reported by Radwan et al., [30] who observed that honey stopped the growth of *Salmonella* and *Escherichia coli*. Honey has a potent antibacterial activity and is very effective in clearing infection in wounds and protecting them from becoming infected. Honey has been useful in the treatment of infected surgical wounds, burn wounds, and decubitus ulcers (bedsores). It maintains a moist wound environment that promotes healing, and its high viscosity helps to provide a protective barrier to prevent infection. Low concentrations of this known antiseptic are effective against infectious bacteria and can play a role in the wound healing mechanism [3] and in stimulation and proliferation of peripheral blood lymphocytic and phagocytic activity. In addition, the mild acidity and low-level H₂O₂ release assists both tissue repair and contributes to the antibacterial activity [31].

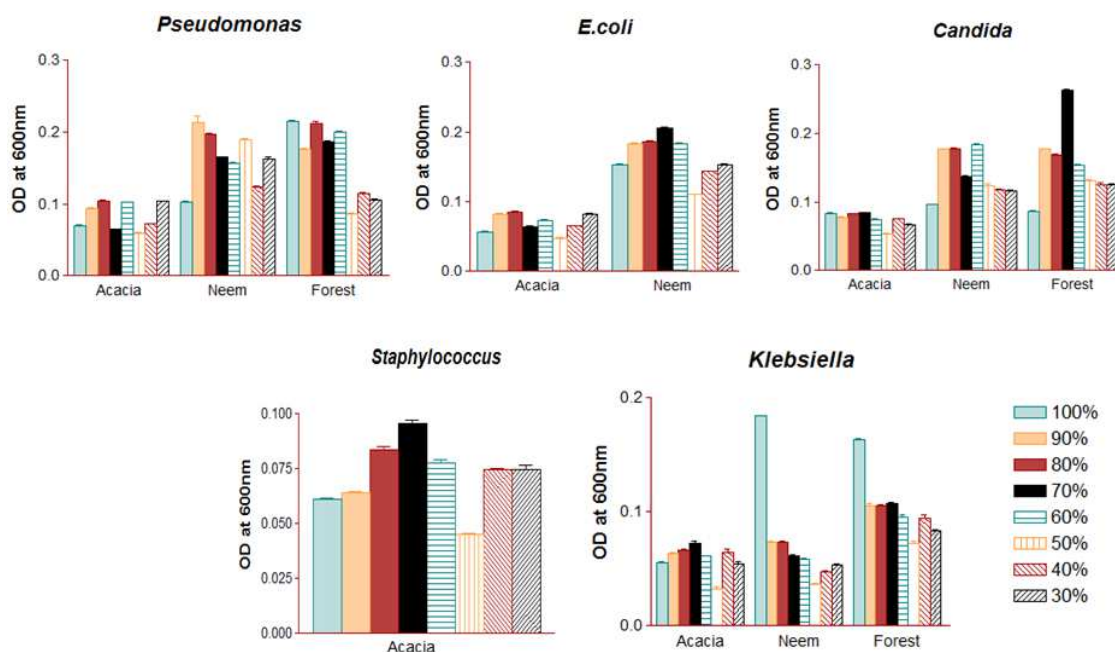


Fig 2: MIC values of various honey samples on selected wound pathogens

Catalase treatment of Honey

In order to determine the degree of involvement of H_2O_2 in the antibacterial effects of the honeys, samples were treated with Catalase solution to breakdown the endogenous H_2O_2 . Pre-treatment of honey with catalase restored, to a certain extent, the bacterial growth, thus suggesting that endogenous H_2O_2 was implicated in the growth inhibition. All the plates inoculated with cultures incubated with catalase-treated Acacia and Forest honey grew well on media without any zone of inhibition but Neem honey showed a very small ZOI compared to the disc diffusion assay did earlier. These results show that the honeys contain complex antibacterial agents, in addition to a small amount H_2O_2 and are also a strong indication that Acacia and Forest honey samples lost their antibacterial activity as the H_2O_2 was broken down by Catalase showing their peroxide dependent role whereas, Neem honey still displayed a reduced inhibitory activity suggesting a peroxide as well as non-peroxide defendant role. The non-peroxide dependent role can be attributed to the polyphenol content in neem.

CONCLUSION

As microbial resistance to honey has never been reported, it is a very promising topical antimicrobial agent against the infection of antibiotic-resistant bacteria and in the treatment of chronic wound infections that do not respond to antibiotic therapy. Thus, honey with effective antimicrobial properties against antibiotic-resistant organisms such as MRSA and MDR *P. aeruginosa*, which have been associated with infections of burn wounds and in nosocomial infections, is much anticipated.

The present study concluded that honey has both bacteriostatic as well as bactericidal activity against many pathogens. The study also demonstrated that honey, akin to antibiotics, provides an alternative therapy against certain bacteria. Aqueous extracts of Honey samples resulted in a broad spectrum of antibacterial activity due to activation of glucose oxidase resulting in the production of H_2O_2 . Neem honey when treated with catalase still possessed antibacterial activity, proving the presence of non-peroxide factors. Therefore, there is need to characterize the non-peroxide active components of honey extracts. At present a number of honeys are sold with standardized levels of antibacterial activity, of which the best known is manuka (*Leptospermum*) honey as well as Tualang (*Koompassia excelsa*) honey. This study demonstrates the fact that locally produced honeys too possess excellent antibacterial activity comparable to the commercial honeys.

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