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ANTIMICROBIAL ACTIVITY OF HONEY AGAINST HUMAN PATHOGENS

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ABSTRACT

Objective: The present study was conducted to evaluate the *in vitro* antimicrobial activity of Indian natural honey at different concentrations (5%v/v, 10%v/v, 20%v/v, 25%v/v, 50%v/v and 100%v/v) against clinical isolates of *Staphylococcus aureus* and *Escherichia coli*. **Methods:** Six multi-drug resistant pathogenic *S. aureus* and *E. coli* strains were selected for this study. Antimicrobial susceptibility of these isolates to different concentrations of honey was determined by minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), cell viability assay and biofilm formation assay. **Results:** Results shows that Natural honey exhibited antibacterial activity, where as commercial honey, a well known non-antimicrobial agent does not reveal so. For multi-drug resistant *S. aureus* and *E. coli* strains, the MIC concentrations of Natural honey were 50%v/v and 25%v/v, respectively, and the MBC concentrations was 50%v/v. Natural honey significantly decreases ($P<0.05$) the cell viability and biofilm formation of multi-drug resistant *S. aureus* and *E. coli* strains by 32.88%, 21.27% and 32.98%, 27.27%, respectively. **Conclusion:** Natural honey may contain compounds with therapeutic potential against our local isolates of *Staphylococcus aureus* and *Escherichia coli*.

KEYWORDS: *Staphylococcus aureus*, *Escherichia coli*, multi-drug resistant bacteria, folk medicine, honey, antimicrobial activity.

1. INTRODUCTION

Honey bees transform nectar into honey by a process of regurgitation and evaporation. They store it as a primary food source in wax honeycombs inside the beehive. Honey gets its sweetness from the monosaccharide fructose and glucose, and has approximately the same relative sweetness as granulated sugar. It has attractive chemical properties for baking and a distinctive flavor that leads some people to prefer it over sugar and other sweeteners. Most microorganisms do not grow in honey because of its low water activity of 0.6. However, honey sometimes contains dormant endospores of the bacterium *Clostridium botulinum*, which can be dangerous to infants, as the endospores can transform into toxin-producing bacteria in infants' immature intestinal tracts, leading to illness and even death.[1]

Honey (*Apis mellifera*) has been used as an eco-friendly medicine throughout the ages and recently regarded for its potential in treatment of burns and peptic ulcer, infected wounds, bacterial gastro-enteritis and eye infection. Honey has a potent broad-spectrum antibacterial activity and studies have demonstrated that manuka honey with a high antibacterial activity is likely to be non-cariogenic.[2] Repeated use of antibiotics increases the percentage of resistant micro-organisms to various antibiotics. Honey increases the sensitivity of micro-organisms to antibiotics and decreases the microbial resistance to antibiotics.[3]

Honey is mostly sugars and contains only trace amounts of vitamins or minerals. Honey also contains tiny amounts of several compounds thought to function as antioxidants, including chrysin, pinobanksin, vitamin C, catalase, and pinocembrin. The specific composition of any batch of honey depends on the flowers available to the bees that produced the honey. Typical honey analysis consists fructose (38.2%), glucose (31.3%), maltose (7.1%), sucrose (1.3%), water (17.2%), higher sugars (1.5%), Ash (0.2%), other/undetermined components (3.2%).[4] Its glycemic index ranges from 31 to 78, depending on the variety. Honey has a density of about 1.36 kg/litre.[5]

The use of traditional medicine to treat infection has been practiced since the origin of mankind, and honey produced by *A. mellifera* is one of the oldest traditional medicines considered to be important in the treatment of several human ailments. Currently, many researchers have reported the antibacterial activity of honey and found that natural unheated honey has some broad-spectrum antibacterial activity when tested against

pathogenic bacteria, oral bacteria as well as food spoilage bacteria.[6]

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.[7-9] Honey is gaining acceptance as an agent for the treatment of ulcers, bed sores and other skin infections resulting from burns and wounds.[10,11] The healing properties of honey can be ascribed to the fact that it offers antibacterial activity, maintains a moist wound environment that promotes healing, and has a high viscosity which helps to provide a protective barrier to prevent infection.[6] There are many reports of honey being very effective as dressing of wounds, burns, skin ulcers and inflammations; the antibacterial properties of honey speed up the growth of new tissue to heal the wound.[12] The medihoney and manuka honey have been shown to have *in vivo* activity and are suitable for the treatment of ulcers, infected wounds and burns.[11,13]

The *Leptospermum scoparium* (*L. scoparium*) honey, the best known of the honeys, has been reported to have an inhibitory effect on around 60 species of bacteria, including aerobes and anaerobes, gram-positives and gram-negatives.[14] Tan et al., reported that Tualang honey has variable but broad-spectrum activities against many different kinds of wound and enteric bacteria.[15] Manuka honey has been reported to exhibit antimicrobial activity against pathogenic bacteria such as *Staphylococcus aureus* and *Helicobacter pylori* (*H. pylori*) making this honey a promising functional food for the treatment of wounds or stomach ulcers.[16] The manuka, jelly bush and pasture honeys are capable of stimulating the monocytes, the precursors of macrophages, to secrete TNF-α.[17,18] On the other hand, glycosylated proteins can induce TNF-α secretion by macrophages, and this cytokine is known to induce the mechanism of wound repairing. Furthermore, the ability of honey to reduce 'reactive intermediates release' may well limit tissue damage by activated macrophages during wound healing.[18]

The present study has been designed to determine the *in vitro* antibacterial activity of honey on isolates of *Staphylococcus aureus* and *Escherichia coli*.

2. MATERIALS AND METHODS

2.1 Culture media and chemicals

Mueller-Hinton broth, nutrient agar, luria broth, tryptic soy broth, agar powder, RPMI-1640, crystal violet, cell culture grade DMSO were purchased from Himedia, India. Sodium chloride, potassium dihydrogen phosphate (KH_2PO_4), di potassium hydrogen phosphate (K_2HPO_4), NaOH, ethanol were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. 3-(4, 5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co., USA. All other the chemicals, reagents, were purchased from Himedia, India; SRL Pvt. Ltd. Mumbai, India and were of the highest grade available.

2.2 Bacterial strains

Six multi drug resistant pathogenic *Staphylococcus aureus* and *Escherichia coli* strains were used for this study. *S. aureus* and *E. coli* strains were clinically isolated from the pus samples by Gram staining and standard biochemical tests.[19,20] Bacterial culture was done at 37ºC throughout the experiment.

2.3 Collection of honey and drug preparation

Natural honey was collected from nearby honey collector and a commercial honey was purchased from market. Several doses of honey (5-100% v/v) were prepared using sterile PBS (pH 7.4). In this study, all these doses were charged against multi-drug resistant pathogenic *S. aureus* and *E. coli* strains.

2.4 Methods

2.4.1 Culture of microorganisms

Staphylococcus aureus and *Escherichia coli* strains were cultured in luria broth media and were shaken in a shaking incubator at 37°C for overnight. Bacterial cultures were grown on Nutrient agar (NA) media. Broth and agar culture media were prepared time to time for this study.

2.4.2 Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of natural honey and commercial honey was determined against multi drug resistant pathogenic *Staphylococcus aureus* and *Escherichia coli* strains by broth dilution method using Mueller-Hinton broth (MHB), as recommended by the National Committee for Clinical Laboratory Standards (NCCLS).[21] About 5 x 10^4 bacterial cells in MHB were treated with different concentrations of natural honey and commercial honey and shaken for 16 h at 37°C. The minimum concentration at which there was no visible turbidity was taken as the MIC.

2.4.3 Determination of Minimum Bactericidal Concentration (MBC)

The Minimum Bactericidal Concentration (MBC) of natural honey and commercial honey was determined against multi drug resistant pathogenic *Staphylococcus aureus* and *Escherichia coli* by the methods of Okore.[22] This is an extension of the MIC Procedure. Natural honey and commercial honey treated bacterial culture showing growth or no growth in the MIC tests were used for this test. Bacterial culture used for the MIC test were inoculated onto the Mueller-Hinton agar and incubated at 37°C for 24 hr. Microbial growth or death were ascertained via no growth on Mueller-Hinton agar plate. The minimal concentration that produced total cell death is the MBC.

2.4.4 Cell Viability Assay

Cell viability of multi drug resistant pathogenic *Staphylococcus aureus* and *Escherichia coli* cells was performed after 12 h of treatment with natural honey and commercial honey by 3-(4, 5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) method according to Mosmann.[23] Drug treated bacterial cultures were centrifuged in 1000×g for 10 min followed by repeated wash for two times with sterile PBS (pH-7.4). Thereafter, the medium was replaced with fresh RPMI (without Phenol Red and FBS) containing 0.5 mg/ml of MTT. After additional 3 h incubation at 37°C, HCl-isopropanolic solution was added and after 15 min of incubation at room temperature, absorbance of solubilized MTT formazan product was measured in Hitachi U2001 UV/Vis spectrophotometer at 570 nm.

2.4.5 Biofilm Formation Assay

Virulence factor in terms of biofilm formation was measured by the method of Lauriano et al.[24] Natural honey and commercial honey treated *Staphylococcus aureus* and *Escherichia coli* cells were grown overnight in LB broth and then normalized to identical densities based on OD_{600}, and 5 µl was inoculated into 500 µl of LB broth in 10 ml borosilicate glass tubes. The tubes were then incubated statically at 30°C for 22 h. The tubes were rinsed with distilled water, incubated with 600 µl of 0.1% crystal violet for 30 min, and rinsed again with distilled water. 1.0 ml of dimethyl sulfoxide was then added, the tube was vortexed and allowed to stand for 10 min, and the optical density was measured in Hitachi U2001 UV/Vis spectrophotometer at a wavelength of 570 nm.

2.5 Statistical analysis

The experiments were performed three times and the data are presented as mean±S.E.M., n=6. Comparisons of the means of control, and experimental groups were made by two-way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA 01060 USA) with multiple comparison t-tests, *P<0.05* as a limit of significance.

3. RESULTS

3.1 Minimum inhibitory concentration (MIC)

Particular concentration of honey was noted where no visible growth appears in broth culture, both in case of natural honey (NH) and commercial honey (CH). In case of natural honey (NH), the MIC value was 50% (v/v) for multi-drug resistant *Staphylococcus aureus* and 25% (v/v) for multi-drug resistant *Escherichia coli* strains (Fig. 1a). On the other hand commercial honey (CH) has no inhibitory effect on both multi-drug resistant *Staphylococcus aureus* and *Escherichia coli* (Fig. 1b).

3.2 Minimum bactericidal concentration (MBC)

Particular concentration was noted where no visible growth appears on agar plate, both in case of natural honey (NH) and commercial honey (CH). In case of natural honey (NH), the MBC value was 50% (v/v) for both multi-drug resistant *Staphylococcus aureus* and *Escherichia coli* strains (Fig. 2a); where as commercial honey (CH) has no bactericidal effect on both multi-drug resistant *Staphylococcus aureus* and *Escherichia coli* (Fig. 2b).

3.3 Cell viability by MTT assay

Natural honey (NH) significantly decreases (*P<0.05*) the cell viability of multi-drug resistant *Staphylococcus aureus* and *Escherichia coli* strains by 32.88% and 21.27%, respectively. On the other hand commercial honey (CH) has no significantly (*P<0.05*) killing activity against both multi-drug resistant *Staphylococcus aureus* and *Escherichia coli* strains (Fig. 3).

3.4 Biofilm formation

Natural honey (NH) significantly decreases (*P<0.05*) the biofilm formation of multi-drug resistant *Staphylococcus aureus* and *Escherichia coli* strains by 32.98% and 27.27%, respectively. On the other hand commercial honey (CH) has no significantly (*P<0.05*) reducing activity of virulency against both multi-drug resistant *Staphylococcus aureus* and *Escherichia coli* strains (Fig. 4).

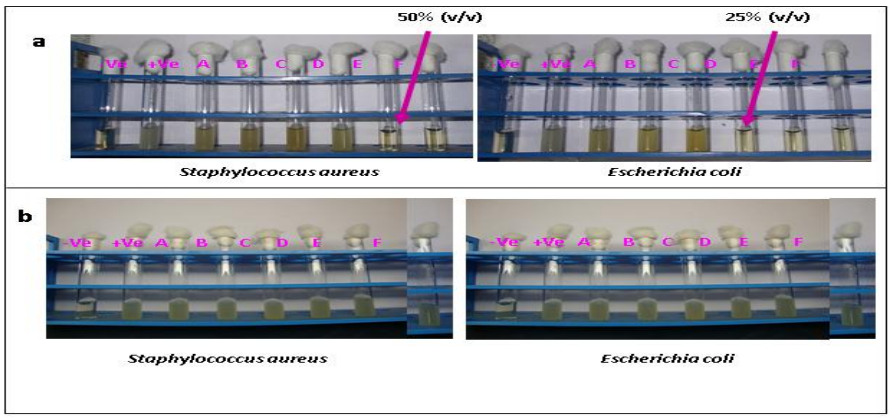

Fig. 1

Fig. 1: Minimum Inhibitory Concentration determination of natural honey (NH) [a] and commercial honey (CH) [b] against multi-drug resistant *Staphylococcus aureus* and *Escherichia coli* strains. Here, –ve = negative control, +ve = positive control, A=5% (v/v), B=10% (v/v), C=20% (v/v), D=25% (v/v), E=50% (v/v), F=100% (v/v).

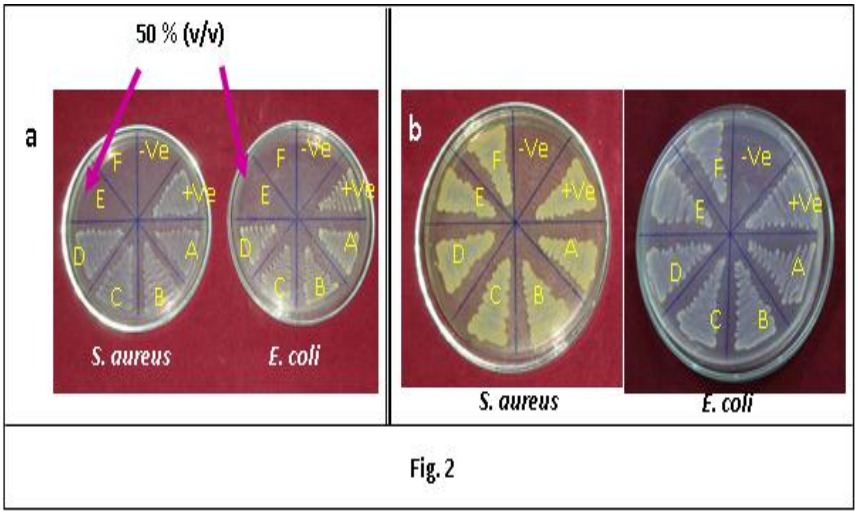

Fig. 2: Minimum Bactericidal Concentration determination of natural honey (NH) [a] and commercial honey (CH) [b] against multi-drug resistant *Staphylococcus aureus* and *Escherichia coli* strains. Here, –ve = negative control, +ve = positive control, A=5% (v/v), B=10% (v/v), C=20% (v/v), D=25% (v/v), E=50% (v/v), F=100% (v/v).

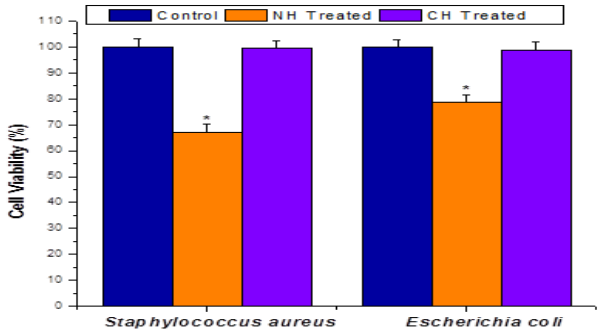

Fig. 3: Cell viability of *Staphylococcus aureus* and *Escherichia coli* strains against natural honey (NH) and commercial honey (CH). All the experimental results are presented as mean±S.E.M., n=6. * indicate statistically significant (*P<0.05*) difference as compared to control group.

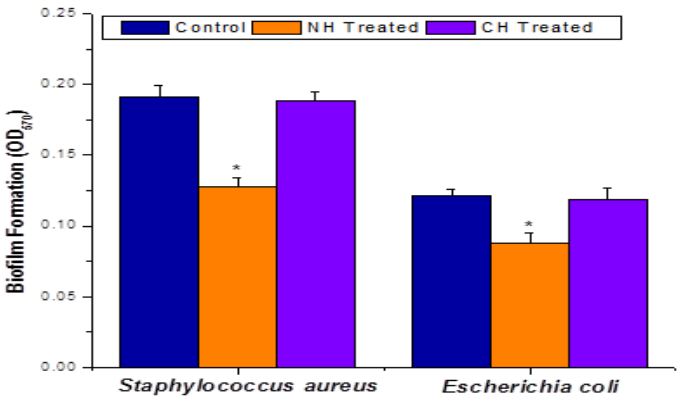

Fig. 4: Biofilm formation of *Staphylococcus aureus* and *Escherichia coli* strains against natural honey (NH) and commercial honey (CH). All the experimental results are presented as mean±S.E.M., n=6. * indicate statistically significant (*P<0.05*) difference as compared to control group.

ABBREVIATIONS

A. mellifera	*Apis mellifera*
CH	Commercial Honey
DMSO	Dimethyl sulphoxide
E. coli	*Escherichia coli*
H. pylori	*Helicobacter pylori*
H_2O_2	Hydrogen peroxide
L. scoparium scoparium	*Leptospermum*
LB	Luria broth
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
NA	Nutrient agar
NCCLS	National Committee for Clinical Laboratory Standards
NH	Natural Honey
PBS	Phosphate buffer saline
S. aureus	*Staphylococcus aureus*

4. DISCUSSION

Despite recent advances in antimicrobial therapy, several types of wound still show resistance to routine wound treatment. The emergence of resistant microbial strains with multiple patterns reduces the efficacy of conventional therapies. Indiscriminate and irrational use of antibiotics is primarily responsible for the emergence and spread of resistance in almost all the microorganisms.[14]

In the present study, all *Staphylococcus aureus* strains were resistant to penicillin G, ampicillin, cephotaxime, gentamycin, streptomycin, tetracycline, erythromycin, chloramphenicol, norfloxacin, methicillin and vancomycin; and all *Escherichia coli* strains were resistant to penicillin G, ampicillin, gentamycin, streptomycin, tetracycline, erythromycin and chloramphenicol. Hence, the relentless emergences of antibiotic resistant strains of pathogens often with multiple antibiotic resistance lead to the need to find alternatives. This has forced the re-evaluation of traditional remedies in the search of appropriate antimicrobial agents.[25]

Manuka, kanuka and pasture honey are known to have antimicrobial activity.[8,26] Not much work has been done on *in vitro* antibacterial activity of various Indian honeys except Subrahmanyam et al.[25] Hence, till date, Indian honey has been used mostly as home remedy. Due to lack of adequate scientific research and documentation, the medicinal properties of Indian honeys still remain mostly in dark.

The present study included one natural honey and one marketed processed commercial honey. Amongst the documented Indian studies regarding antibacterial activity of honey, sterility of honey sample were not considered except by Subrahmanyam et al. who used Jambhul honey which is found sterile. This coincides with present results as Jambhul honey was the only honey found to be sterile among all other honey samples.[25]

In the present study, the natural honey sample showed antimicrobial activity against all *Staphylococcus aureus* and *Escherichia coli* strains; whereas the honey sample procured from local market failed to show any antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* strains. This finding corroborates that of Postmes et al. who reported supermarketed honey had no antimicrobial activity.[27]

In the present study, the MIC values for *Staphylococcus aureus* and *Escherichia coli* ranged from 50-100% (v/v) and 25-100% (v/v), respectively. The MBC values for *Staphylococcus aureus* and *Escherichia coli* ranged from 50-100% (v/v). In the present study, the cell viability and virulency of *S. aureus* and *E. coli* are decreased with treatment of natural honey. Any of these above mentioned changes were not found in commercial honey treatment. *S. aureus* and *E. coli*, are species that has developed resistant to many antibiotics and has become a predominant agent of wound sepsis and urinary tract infection are very susceptible to the antimicrobial activity of honey. This finding is consistent with that reported by Bannur et al., and Subrahmanyam et al. from India.[25,28]

In the present study, the antibacterial activity of honey against *Staphylococcus aureus* and *Escherichia coli* are may be due to the hydrogen peroxide which is produced enzymatically in honey. The *glucose oxidase* enzyme is secreted from the hypopharyngeal gland of the bee into the nectar to assist in the formation of honey from the nectar. The hydrogen peroxide and acidity produced by following two reactions has antimicrobial potential.[2]

$$\text{Glucose} + O_2 \longrightarrow \text{Gluconic acid}$$

$$\text{Gluconic acid} + H_2O \longrightarrow \text{Honey}$$

The beneficial role of honey is attributed to its antibacterial property with regards to its high osmolarity, acidity (low pH) and content of hydrogen peroxide (H_2O_2) and non-peroxide components, i.e., the presence of phytochemical components like methylglyoxal (MGO).[29,30] The antimicrobial agents in honey are predominantly hydrogen peroxide, of which the concentration is determined by relative levels of *glucose oxidase*, synthesized by the bee and catalase originating from flower pollen.[29] Most types of honey generate H_2O_2 when diluted, because of the activation of the enzyme *glucose oxidase* that oxidizes glucose to gluconic acid and H_2O_2, which thus attributes the antimicrobial activity. But, in some cases, the peroxide activity in honey can be destroyed easily by heat or the presence of catalase.[31]

Besides H_2O_2, which is produced in most conventional honeys by the endogenous enzyme *glucose oxidase*, several other non-peroxide factors have been found to be responsible for the unique antibacterial activity of honey.[9] Honey may retain its antimicrobial activity even in the presence of catalase (absence of *glucose oxidase*), and thus this type of honey is regarded as "non-peroxide honey".[14,9] Several components are known to contribute the nonperoxide activity, such as the presence of methyl syringate and methylglyoxal, which have been extensively studied in manuka honey that is derived from the manuka tree (*L. scoparium*).[30,31]

5. CONCLUSION

The present study was undertaken to evaluate the *in vitro* antibacterial activity of honey on isolates of *Staphylococcus aureus* and *Escherichia coli*. Natural honey and commercial honey were charged against multi drug resistant pathogenic *Staphylococcus aureus* and *Escherichia coli* strains and determination of minimum inhibitory concentration, minimum bactericidal concentration, cell viability and virulency factor were assessed. It was observed from the present study that natural honey showed antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*; where as commercial honey has no antimicrobial activity.

In conclusion, it may be concluded that natural honey may be used to discover natural bioactive products that might lead to the development of new drugs. On the basis of present work, a new drug therapy may be developed to fight against microbial infection mediated disorder.

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Declaration of interest

The author reports no conflicts of interest. The author alone is responsible for the content and writing of the paper.

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