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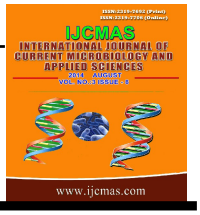

Original Research Article

Isolation and partial purification of antibacterial activity protein from Indian honey *Apis cerana indica*

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A B S T R A C T

The present work has been carried out to identify the antimicrobial compound in Indian honey and the range of organisms susceptible to the antimicrobial action. Antibacterial potential of the precipitated protein, from honey was used to elucidate the antimicrobial activity. In order to purify the sample the protein was subjected to 10kDa molecular weight cut off centrifugation and the positive phase was taken for further purification by thin layer chromatography. The maximum inhibition as produced was observed against *E.coli* (16 to 23 mm), Klebsiella pneumonia (14 to 19 mm), Pseudomonas sp. (15 to 21 mm) and Proteus sp. (15 to 16 mm). The present findings revealed that local Indian honey of *Apis cerana indica* had higher antibacterial activity against the bacterial strains. Active fraction of the gel filtration elutes showing higher concentration of proteins with Rf valaue 1.6 and autobiography assay conforming the zone of inhibition of TLC separated protein.

Keywords

Honey,
Apis cerana indica
India,
Antibacterial,
Protein,

Introduction

Indian hive bee *Apis cerana indica* are the domesticated species, which construct multiple parallel combs with an average honey yield about 6-8 kg per colony per year. They are native of India/Asia. Approximately 10 mm long, smaller, less hairy and even yellow and black stripes on the abdomen. *Apis cerana indica* is found in plain areas and foothills of the region (Verma, 1996). *Apis cerana indica* is a subspecies of honeybee *Apis cerana* and one of the important pollination agents for coconut palms and it's belong to the family Apidae. They usually build multiple combs nest in some tree hollows and some man-made structure.

A great deal of work has been done in an attempt to identify the antimicrobial agents in honey and the range of organisms susceptible to this antimicrobial action. Several studies have been conducting to authenticate this 'forklore' on medicinal properties of honey and there has been a

renaissance in the use of honey as medicine in more recent times (Muli *et al*., 2008). Its antimicrobial properties have been extensively reviewed (Molan, 1992). Honey has a potent antibacterial activity and is very effective in clearing infection in wounds and protecting them from becoming infected. Since ancient times, honey has been used for treatment and prevention of wound infections. Honey are bee products that have been used for centuries in medicine the antimicrobial activity of honey is mainly credited to its acidity, osmolarity and enzymatic generation of hydrogen peroxide via glucose oxidase (Molan ,1992 a). Additional honey components such as aromatic acids or phenolic components may also contribute to the overall antimicriboial activity. Honey consists of various constituents such as water, carbohydrates, proteins, vitamins, amino acid, energy and minerals (Abshishek Chauhan and Vimlendu Pandey et al., 2010). The high sugar concentration, hydrogen peroxide, high osmolarity and the low pH are well-known antibacterial factors in honey and more recently, methylglyoxal and the antimicrobial peptide bee defensin-1 were identified as important antibacterial compounds in honey (Thomes, 1990; Marshall *et al;* 2005). Honey also contains antioxidants and flavonoids that may function as antibacterial agents.

The multi drug resistant gram-positive and gram-negative bacteria were used as test organisms. To analyze the antimicrobial activity of honey the concentrations of honey were increased using different solvents in order to determine the minimal inhibitory concentration for microbial growth. In the present study, the effort has been made to identify and purify the active antibacterial component in Indian honey against *Pseudomonous* sp.

The minimum inhibitory concentration of various types of honeys for various pathogenic bacterial strains have been determined by many authors; in this article for oral bacterial strains and bacterial strains causing wound infections. Lusby et al., 2005 reported that honeys other than the commercially available antibacterial honeys can have equivalent antibacterial activity against bacterial pathogens.

Materials and Methods

Indian honey was collected from beehives of *Apis cerana indica* from remote regions of Mettupalayam, Tamil Nadu, India. They were collected in sterile glass bottles and stored in airtight in the laboratory for further use. Collected samples were subjected to protein precipitation by solvents, acetone and ethanol for total protein precipitation. One volume of honey was taken in a centrifuge tube, mixed with 2.5 volume of ice-cold solvents acetone gently mixed and incubated in -20℃ deep freezer for two hours. The tubes were centrifuged at 10,000 rpm for 10 minutes. The pellet obtained was air dried and suspended in 0.05M Tris HCL buffer (generally 200µL of buffer is used to dissolve pellet obtained from 1mL of sample). Antibacterial potential of the precipitated protein, from honey was used to elucidate the activity.

The antibacterial activity was checked against four different well known human pathogens. The pathogens used were collected from bacterial culture collection, microbial technology Laboratory, Cochin University of Science and Technology, Cochin. The pathogens used were Escherichia coli (NCIM No. 2574), Proteus sp (NCIM No 2501), Klebsiella pneumoniae (NCIM No. 2079) and Psudomonas sp (NCIM No. 2079). The antibacterial efficacy of the precipitated proteins was elucidated by agar well diffusion method. The 6 hour grown bacterial pathogen was swabbed on nutrient agar plates.

Wells of 0.5cm diameter was punctured on the plate using a sterile well cutter and 50µl of precipitated proteins were aseptically loaded onto the wells. The plates were incubated at 37℃ overnight and the size of the zone of inhibition was measured. In order to purify the sample the protein was subjected to 10kDa molecular weight cut off centrifugation tubes and the positive phase was taken for further purification by Gel filtration chromatography. The positive sample showing antibacterial activity after MWCO centrifugation was taken for Gel filtration chromatography on a Sephadex G50 column. 50cm height and 1cm diameter column was taken and resins were filled upto 30cm column mark. Fractions showing antibacterial activity were pooled together lyophilized and suspended in 2mL of 0.05M Tris HCl buffer (pH8.0) and kept for further use.

Silica gel and water were mixed in the ratio 1:2 respectively and were mixed thoroughly using a glass road to make slurry. The slurry was poured on a grease free glass slide to obtain a thin layer of silica gel. The plates were dried and kept in a hot air oven at 60ºC for 10minutes for the activation of thin layer. 10 µl of the sample (Sephadex G50 purified) was spotted onto the TLC plate about 1.0 cm above the lower edge so that the solvent does not touch the sample-loaded region. The solvent mixture Butanol: glacial aceticacid: distilled water in the ratio 8:2:2 respectively were used as the mobile phase. The container was kept closed so that the whole container becomes saturated with the solvent. The sample spotted TLC plate was dipped into the solvent so that the mobile phase travels in the TLC due to capillary action. The solvent moves towards the top of the TLC plate where it also mobilizes the samples and separation occurs according to the migration potential of the compound present in the sample. The plates were taken out of the solvent dried were developed by spraying nin-hydrin solution (0.1% nin-hydrin add 95ml of methanol to mix well) for detecting the bands. The R_f value was then calculated using the formula R_f value = Migration of spot/Migration of solvent

The dried TLC plate was kept on a LB medium and 15ml of half strength agar nutrient medium mixed with 500µl of bacterial pathogen was overlayed on to the plate under aseptic condition in a laminar airflow chamber. The plates were incubated at 37˚C at 8 hours and the zone of inhibition was examined by spraying the plates with 0.5% of tetrazolium salt solution.

Results and Discussion

In the present study, the honey sample was found to be acidic with pH range 3.75. The antibacterial activity of the sample was checked against gram positive and gram-negative bacteria and it was found that it had a vast range of activity against those human pathogens (Figure1). The Zone of inhibition against different human pathogens was listed in table 1. Acetone precipitated protein showed activity against all the pathogens used and so the acetone-precipitated protein was taken for further study. It also showed a maximum range of activity against the human pathogen *Pseudomonas sp.*

The proteins were subjected for purification. Only partial purification was attained and samples were confirmed for its purity. Precipitated proteins were subjected to molecular weight cut off centrifugation. Upon centrifugation two fractions were obtained. The upper phase containing > 30 kDa proteins and the lower phase contains < 30 kDa fraction. Both the fractions were subjected to check the antibacterial activity and it was found to be that the lower fraction contained the higher activity and thus it was confirmed that the protein having the

antibacterial activity is less than 30 kDa. There are also chances for this fraction to contain peptide molecules.

The lower fraction was applied to gel filtration chromatography. Sephadex G50 resins were used and it was found that the active compound was eluted in the elute fractions 15-21. These positive activity elutes were checked for protein concentration at Optical Density (OD) 280 nm and showed the presence of excess proteins when comparing to other fractions. All the active fractions were pooled together and lyophilized. The lyophilized sample was re-suspended in 2mL of buffer and a sub stock used kept which was used for further analysis.

Thin layer chromatography was performed and their R_f value was calculated and the results are tabulated in Tabe2. The separations of the protein were clearly visualized on the silica gel as a thick band (Figure 3). These solvents then subjected TLC plates were taken for autobiography assay

The autobiography assay performed on the TLC plate subjected to solvent for separation. The culture mixed with *Pseudomonas sp*. was poured and after growth upon spraying of tetrazolium salt showed red colorations which indicates the bacterial growth. Exactly at on the plate corresponding to the R_f value of the developed plate a zone of clearance was noted (Figure 3) which shows that the separated protein contains the antibacterial property.

The antimicrobial activity of Indian honey *Apis cerana indica* has been researched here as first time. However, the quality and floral origin of Indian honey have yet to be determined and standardized. When determine the antibacterial activity of inhibition compounds, selection and use of suitable methods are important. The choice of the methods range from agar diffusion, disc diffusion, broth diffusion, or varies of this methods. In addition using this methods, microdilution, the MIC values determined were lower (indicating higher activity) than those obtained using agar well diffusion methods, as diffusion rates of active constituent in agar may be slower than those in broth (Tan et al 2009). Significant antibiotic resistance and multi drug resistance have been identified for *Pseudomonas* strains. These local Indian honeys from *Apis cerana indica* may have potential properties to be used as effective antibacterial treatments for antibiotic-refractory purposes.

Pseudomonas sp is a major nosocomial pathogen, particularly dangerous to cystic fibrosis patients and populations having weak immune system. In wounds, it is having emerged as a multi drug-resistant organism that gives rise to persistent infection in burns patients and chronic venous leg ulcers (Branski LK and Al-Mousawi A et al.,2009; Robinson Bj et al.,2010; Fazli M and Bjarnsholt et al., 2009). Novel antimicrobial interventions are needed. Natural medicinal products have been used for millennia to treat multiple products have been used for millennia to treat multiple ailments. The antibacterial activity of local honey *Apis cerana indica* based on the zone diameter inhibition produced for clinical isolates. The activity of honey against gram-positive, gram-negative bacteria, in order to establish the potential efficacy of such local honey (not studied before) and it was collected from village of the Mettupalayam, Tamil Nadu State, India. The honey samples exhibited varying level of antibacterial activity against all the selected strains as indicating by the zone of

inhibition of growth. In case of raw honey, the maximum inhibition as produced was observed against *E.coli (16mm to 23mm zone size), Klebsiella pneumonia (14mm to 19mm zone size), Pseudomonas sp. (15mm to 21mm zone size) and Proteus sp. (15mm to 16mm size).* However, the acetone and ethanol extract of honey also tested against the above said organisms and it was found to be less activity as minimum inhibition zone observed. However, residue obtained after extraction from acetone was found active against all the tested strains, the maximum activity was observed against *Pseudomonas sp*. The methanol and chloroform extract was completely inactive and no zone of inhibition was recorded, however acetone extract was inhibitory only against gram-negative *Pseudomonas* sp in the zone diameter range of about 15 mm to 21 mm.

Evidently Ibrahim A.S. (1985) and Jeddar et al (1985) reported that the bactericidal activity of aqueous solution of honey on *Salmonella* spp., *Shigella* spp and also entero-pathogenes such as *E.coli, Vibrio cholera* and other gram-negative and gram-positive bacteria. Similarly, Allen et al (2000) also reported that the antibacterial properties of honey against two bacterial isolates namely *P. aeruginosa* and *E.coli*. Adeleke et al. (2006) has also been reported that the strong inhibition properties of honey against a total number of fifty isolates of *P. aeruginosa* and *E.coli* from various pathologic sources. The extracts obtained with zone of inhibition more than 15mm were subjects to macro dilution assay to determine the MIC. To date, there is no published scientific study on the antimicrobial activity against *Pseudomonas sp*. Hence, the antibacterial activity of Indian honey of *Apis cerana indica* towards this bacterium reported in this study could not be compared to any previous published scientific research.

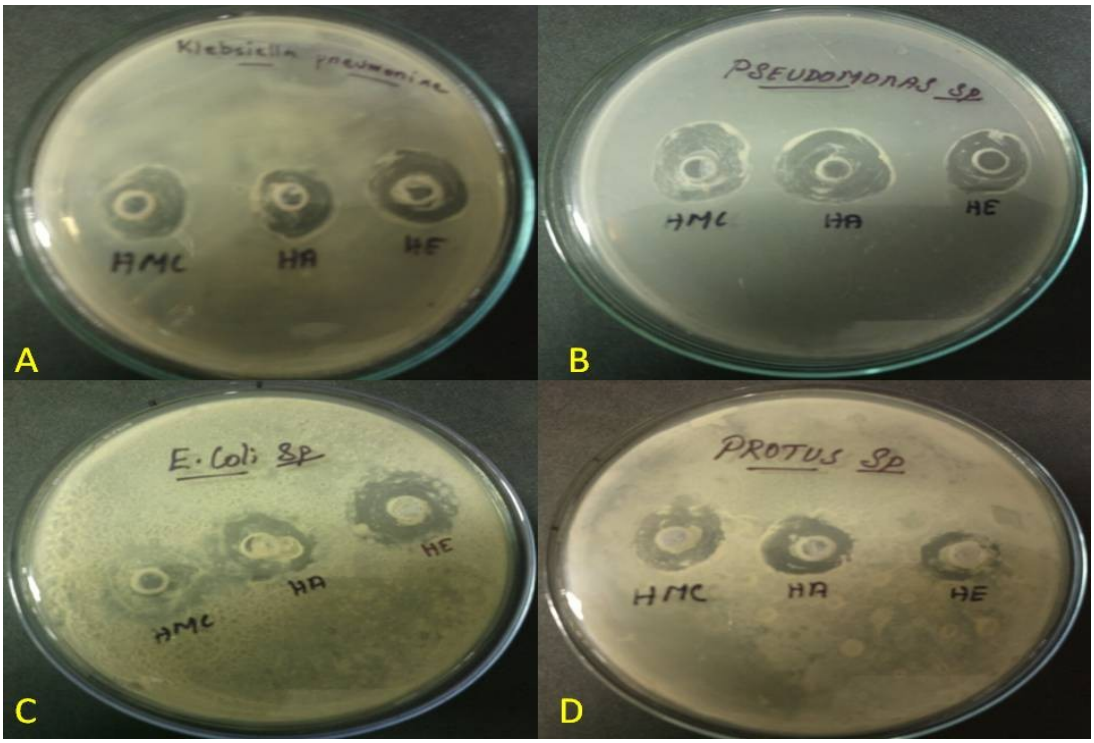
The high antimicrobial activity found against *pseudomonas sp* other bacterial strains. The beneficial role of honey is attributed to its antibacterial property with regards to its high osmolarity, acidity (low pH) and content of hydrogen peroxide (H2O2) and non peroxide components and the presence of phyto-chemical components like methy-glyoxal (MGO) (Manish and Shyamapada., 2011). Evidently, report of Chauhan et al 2010 described that one of the popular natural substances ayurveda as a potent medicine for several uses is honey. The bacterial effect of honey is reported to be depending on concentration of honey used and the nature of the bacteria. In the present study, we use the raw honey of *Apis cerana indica* and its effectiveness against both gram positive and gram-negative bacteria. The antibacterial activities of honey mostly against gram positive than the gram-negative bacteria, both bacteriostatic and bactericidal effects have been reported against *pseudomonos sp*.

Table.1 Antibacterial activity of local Indian honey (*Apis cerena*)

Culture	H* + E* Mm	H*+ AC* Mm	H*+CL,M* Mm
E.coli	22 ± 0.19	16±0.19	0
Klebsiella pneumonia	14±0.15	19±0.14	14±0.13
Pseudomonas sp	0	21±0.17	0
Proteus sp	17±0.12	16±0.15	14±0.14

*H-Honey; E-Ethanol; Ac-Acetone; Cl-Chloroform; M-Methanol.

Figure.1 Antibacterial activity of protein precipitated from Indian honey



1A antimicrobial activity of Indian honey against *Klebsiella pneumonia*
1B antimicrobial activity of Indian honey against *Pseudomonas sp*
1 C antimicrobial activity of Indian honey against *E.coli*
1 D antimicrobial activity of Indian honey against *Protus sp*

Figure.2 Active fraction of the gel filtration elutes showing higher concentration of proteins

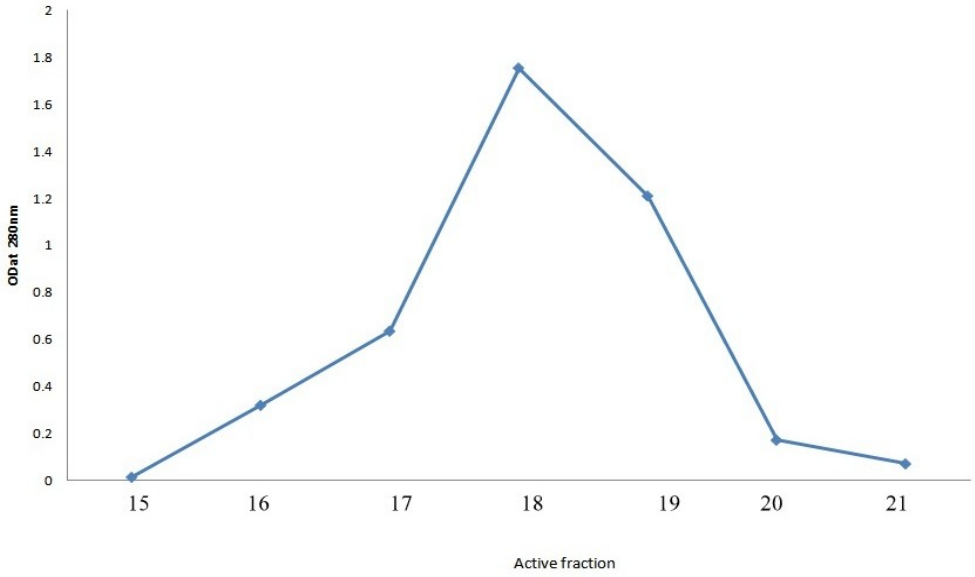
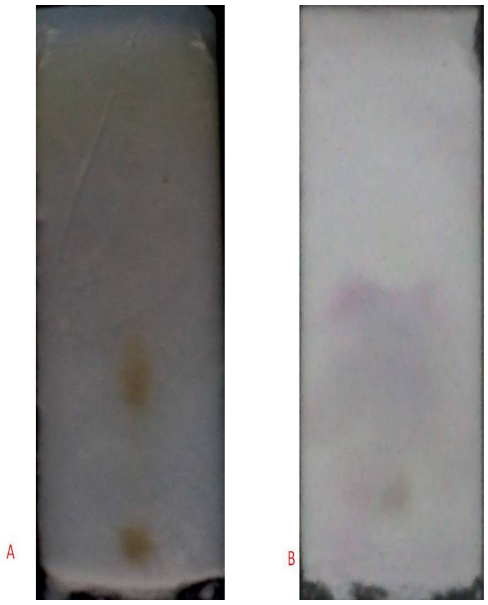


Fig.3 Thin layer chromatography of acetone-precipitated protein showing clear bands (arrow indicates)

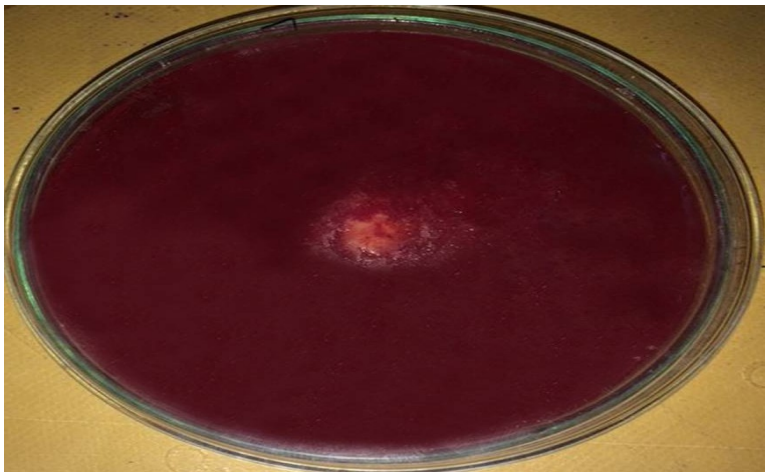


2A – Plate before development
2B - Plate development after applied with nin-hydrin

Table.2 R_f Value of the separated protein

Sample	Distance travelled by (solute) sample	Distance travelled by solvent	Rf value
Acetone precipitated protein	2.7	4.2	1.6

Fig.3 Autobiography assay showing zone of inhibition of TLC separated protein (arrow)



Based on the experimental results and discussion, we can conclude that the well diffusion method is very use full tool to evaluate effect of natural product of honey. Collectively, present findings revealed that local Indian honey of *Apis cerana indica* had higher antibacterial activity against *pseudomonas sp* compared with other bacteria strains.

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