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## Studies on antibacterial properties of stingless bee honey fortified with plant extracts

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### Abstract

Being the only available natural sweetener, honey has important role in human nutritional as well as medicinal properties. The present study was conducted to investigate different formulations of stingless bee honey with various plant extracts against five strains of bacteria which include *Escherichia coli*, *Salmonella species*, *Staphylococcus species*, *Proteus species* and *Pseudomonas species*. The plant extract from turmeric, neem and Malabar nut plants were prepared using soxhlet extraction method, mixed with honey and even compared to respective water extracts. Zone of inhibition studies were conducted using bacterial strains grown on Muller Hinton Agar (MHA). Antibacterial activity of the honey was doubled using the fortification with neem extracts. Clear zone of bacterial inhibition ranging from 14.2 to 26.6 mm were observed for all microbial strains except *Pseudomonas species*. The formulation of honey with different plant extracts provides a promising results for the enhancement of antibacterial activity of honey.

**Keywords:** Antibacterial activity; Ayurveda; Stingless bee honey; Soxhlet extraction method

### 1. Introduction

Honey has been used as a food as well as medicinal product since from the early beginning of mankind especially from stone ages. It is natural substances produced from a variety of honey bees including (*Apis mellifera*, *Apis Indica*, *Trigona iridipennis*) and considered as the only natural sweetener [1-3]. Honey has been used in medicinal properties especially for burns, cataracts, ulcers and wound healing [4, 5]. Natural honey is thick, syrupy, translucent, pale yellow or yellowish brown liquid deposited in the honey comb by the bee (Family: Apidae). It has a characteristics odour and a sweet, faintly acidic taste which contains invert sugar (62-83%), sucrose (0-8%), dextrin (0.26-7%) together with water and traces of other nutrients (Figure 1). Honey is levorotatory and is acidic to litmus paper with a unique mixture having fat soluble as well as water soluble vitamins [6-8]. The composition of the honey varies with floral sources, type of bees, seasonal and environmental factors.

Antimicrobial agents play a major role in reducing infectious diseases, but the disease resistance among pathogen is a big challenge. In this global scenario, alternative antimicrobial strategies based on plant and plant based products especially honey have a promising role in this research and clinical challenge [9-11]. Some sort of honey (*Leptospermum scoparium*) inhibits around 60 species of bacteria, while the potential antimicrobial activity of natural honey is also contributed by the hydrogen peroxide as well as the hygroscopic nature [12-14].

Honey has been used in "Ayurveda" (Indian traditional medicine) for at least over 4000 years and well described in Indian traditional medicinal books. Overall, more than 600 remedies with honey as one of the ingredients [15]. The turmeric plant (*Curcuma longa* L.) is an important colouring agent in Indian culinary, also an important medicinal herb used by Ayurvedic medicine practitioners. The turmeric and its essential component (curcumin) are used to treat a number of medical disorders, including digestive disorders, liver problems, and skin diseases. Phytochemicals found in turmeric have been investigated in preliminary research for their potential effects on diseases such as cancer [16-19], Alzheimer's disease [20], arthritis, diabetes [21] and other clinical disorders [22-24].

The Malabar nut tree (*Adhatoda zeylanica*) has been used in "Ayurvedic" system of medicine for the treatment of various ailments of respiratory tract in both children and adults. All the parts of the plant have been used for their therapeutic beneficiary effect from ancient times [25]. The plant is used as an ingredient of numerous popular formulations including cough syrups used in combination with ginger (*Zingiber officinale* Roscoe) and tulsi (*Ocimum tenuiflorum* L.)

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as an expectorant and antispasmodic [26-30]. In India, neem tree (*Azadirachta indica*) is variously known as "Sacred Tree" as well as "Village Pharmacy". Neem tree products are commonly used in India for over two millennia due to their medicinal properties includes anthelmintic, antifungal, antidiabetic, antibacterial, antiviral, contraceptive and sedative. It is considered a major component in "Ayurvedic" and "Unani" (traditional medicines of Muslim community) and particularly prescribed for skin disease [31].

Given lacking qualitative and quantitative data on amendments of honey with plant extracts, our objectives were to (1) enhance the antimicrobial activity of honey and (2) validation of the amendments using zone of inhibition studies on major bacterial strains causing food spoilage and food borne diseases.

## 2. Materials and Methods

### 2.1 Plant samples collection

Fresh plant materials of neem (*Azadirachta indica*), turmeric (*Curcuma longa* L.) and Malabar nut (*Adhatoda zeylanica*) were collected from the herbal garden of Mar Augustinose College. The herbal garden was established on 2006 as a central government scheme form Medicinal Plant Board to educate the students about the local medicinal plants. The garden has a size of 0.1 ha with more than 200 species of medicinal plants (Figure 2) which were mainly maintained by the students. For turmeric, only the roots were collected, while leaves were collected for the other two varieties. The plant materials were thoroughly washed with distilled water and fresh weight were determined. The samples are then oven dried (KOA4, KEMI lab equipments, Ernakulam, India) at 60 °C for 24 h. The dried samples were powdered using a waring blender (Magic V2, Preethi Kitchen Appliances Pvt Ltd, Chennai, India) and stored in air-tight polyethylene bottles until further analysis.

### 2.2 Preparation of extracts

Five gram of plant powdered was loaded into thimble of soxhlet apparatus (Jain Scientific Glass Works, Ambala, India) with 250 ml distilled water as extractant. The solution was concentrated to 10 ml by supplying constant heat at 100 °C using mantox heater (KHM 4, Ambala, India). The final concentrates were transferred to an airtight polyethylene bottle and later for microbial inhibition studies.

### 2.3 Honey collection

The honeys for the experiments were collected locally from farmer keeping stingless bees (*Trigona iridipennis*) of 12 colonies. The extraction of the honey was carried out by filtering and squeezing through muslin cloth (70 µm) and later stored in sterile polyethylene bottles.

### 2.4 Microorganisms

Bacterial strains were obtained from bacterial stock, Department of Biotechnology, Mar Augustinose College, Ramapuram, India which was previously collected from Microbial Type Culture Collection, Chandigarh, India. The selected bacterial strain includes *Escherichia* species, *Staphylococcus* species, *Proteus* species, *Pseudomonas* species and *Salmonella* species. The cultures were maintained at 4 °C on nutrient agar slants.

### 2.5 Agar well diffusion tests

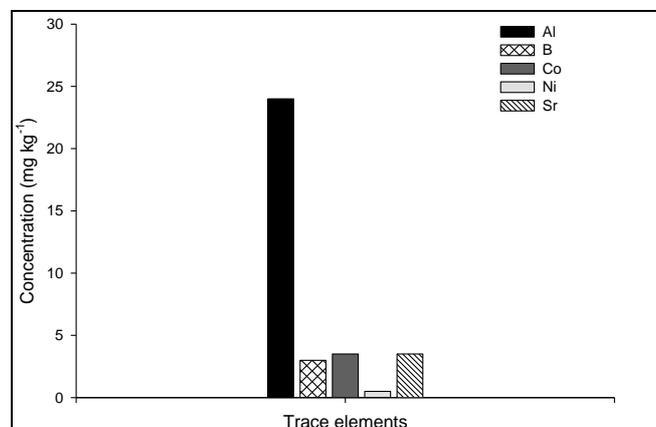
The antibacterial activity of different plant extracts was determined by agar well-diffusion method according to Ahmed *et al.* For this, 150µL of 12-16 hrs incubated cultures of bacterial species were mixed in molten Muller Hinton Agar (MHA) medium (Himedia Laboratories, Mumbai, India) and poured in pre-sterilized petri plates. A well puncher of six mm diameter (Hindustan Syringes & Medical Devices Ltd, New Delhi, India) used to punch wells in solidified medium and later filled with extracts. Three treatments were used including 45 µl of honey with plant extract (50:50), plant extract in water and 25% honey. All the treatments were replicated thrice to avoid possible errors and misinterpretations. The plates were incubated at 37 °C for 24 hrs in incubator and the diameter of the zone of inhibition was measured and recorded. The antibacterial activity was interpreted from the size of the diameter of zone of inhibition measured to the nearest mm as observed from the clear zones surrounding the wells.

### 2.6 Statistical analysis

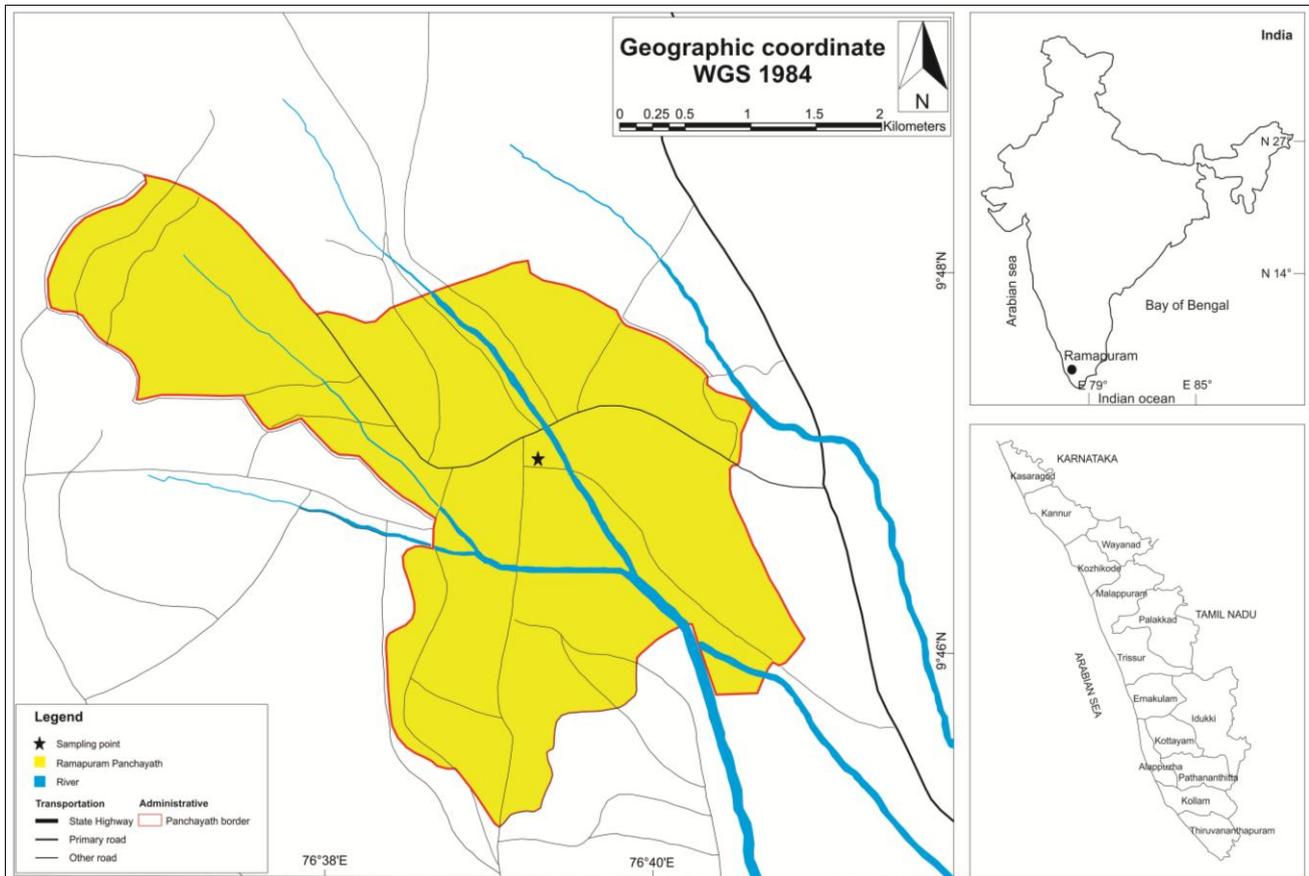
Descriptive statistics using SPSS 12.0 (SPSS Inc., Chicago, IL, USA) were conducted to summarize the data and graphs were generated using Sigma Plot 7 (Systat Software Inc., Chicago, IL, USA).

## 3. Results

From the zone of Inhibition measurements, turmeric extract does not seem to have any good antibacterial activity against all above mentioned the test microorganisms (Figure 3). The test honey sample showed good antibacterial effects on *Proteus sp.* ( $12.3 \pm 0.6$ ), but when turmeric extract was mixed with honey the antibacterial activity is further enhanced against *Proteus sp.* ( $22.6 \pm 0.6$ ). Surprisingly, this combination showed clear zone of inhibition of *Staphylococcus sp.* ( $24.6 \pm 1.5$ ; Table1). Neem extract alone shows antibacterial activity against *E. coli* and *Salmonella sp.* (Figure 4, Table 2). Honey amended with neem extracts showed an increase in zone of inhibition against *Salmonella sp.*, *Proteus sp.*, *Staphylococcus sp.* and *E. coli*. *Adhatoda zeylanica* extract does not show any antibacterial activity against any of the test bacterial strains. However when plant extract was mixed with honey, visible zone of inhibition were obtained against *Staphylococcus sp.* as well as *Pseudomonas sp.* (Figure 5, Table 3).



**Fig 1:** Upper limit of trace elements especially aluminium (Al), boron (B), cobalt (Co), nickel (Ni) and strontium (Sr) found in honey [1].



**Fig 2:** Map of Ramapuram showing the location of the sample collection point.

**Table 1:** Inhibition zone of various treatments (honey+turmeric, turmeric and honey) against various microorganisms using Muller Hington Agar (n=3, values in mm).

Microorganism	Treatments		
	Honey+Turmeric	Turmeric	Honey
<i>E.coli</i>	-	-	-
<i>Staphylococcus sp.</i>	24.6 ± 1.5	-	-
<i>Proteus sp.</i>	22.6 ± 0.6	-	12.3 ± 0.6
<i>Pseudomonas sp.</i>	-	-	-
<i>Salmonella sp.</i>	-	-	-

Numbers represent means ± one standard deviation (SD) of the mean, -: no inhibition

**Table 2:** Inhibition zone of various treatments (honey+neem, neem and honey) against various microorganisms using Muller Hington Agar (n=3, values in mm).

Microorganism	Treatments		
	Honey+Neem	Neem	Honey
<i>E. coli</i>	14.3 ± 1.5	12 ± 1.1	-
<i>Staphylococcus sp.</i>	26.6 ± 1.2	-	-
<i>Proteus sp.</i>	25.0 ± 1.0	-	12.3 ± 0.6
<i>Pseudomonas sp.</i>	-	-	-
<i>Salmonella sp.</i>	15.7 ± 1.2	14 ± 1.3	-

Numbers represent means ± one standard deviation (SD) of the mean, -: no inhibition

**Table 3:** Inhibition zone of various treatments (honey+ Malabar nut, Malabar nut and honey) against various microorganisms using Muller Hington Agar (n=3, values in mm).

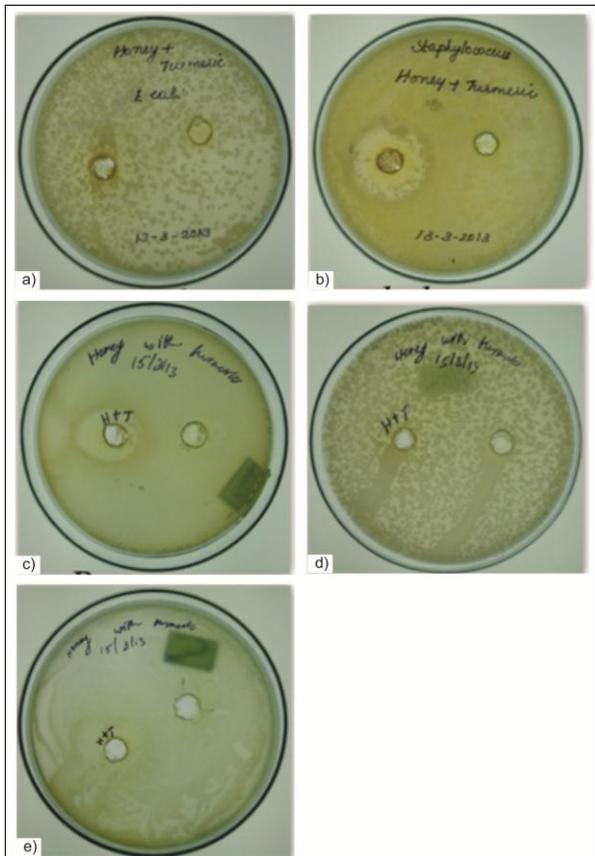
Microorganism	Treatments		
	Honey+ Malabar nut	Malabar nut	Honey
<i>E.coli</i>	-	-	-
<i>Staphylococcus sp.</i>	22.3 ± 0.6	-	-
<i>Proteus sp.</i>	-	-	12.3 ± 0.6
<i>Pseudomonas sp.</i>	14.3 ± 0.6	-	-
<i>Salmonella sp.</i>	-	-	-

Numbers represent means ± one standard deviation (SD) of the mean, -: no inhibition

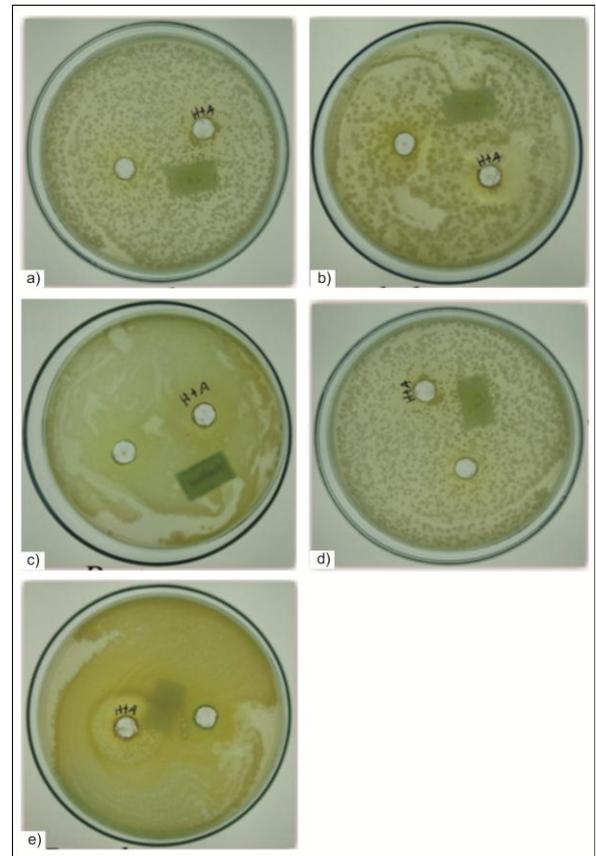
**4. Discussion**

Antibiotic resistance among bacterial is of high concern in clinical areas. The selected bacterial strains are a common cause for food poisoning, food spoilage and many other gastrointestinal troubles and have the possibility to become antibiotic resistant. The present study shows the improvement of antibacterial activity of honey with different plant extracts. The antimicrobial property of honey is well known and mainly due to the presence of peroxide and non-peroxide compounds as well as its high water activity. Neem extract with honey was able to obtain larger zone of inhibition for

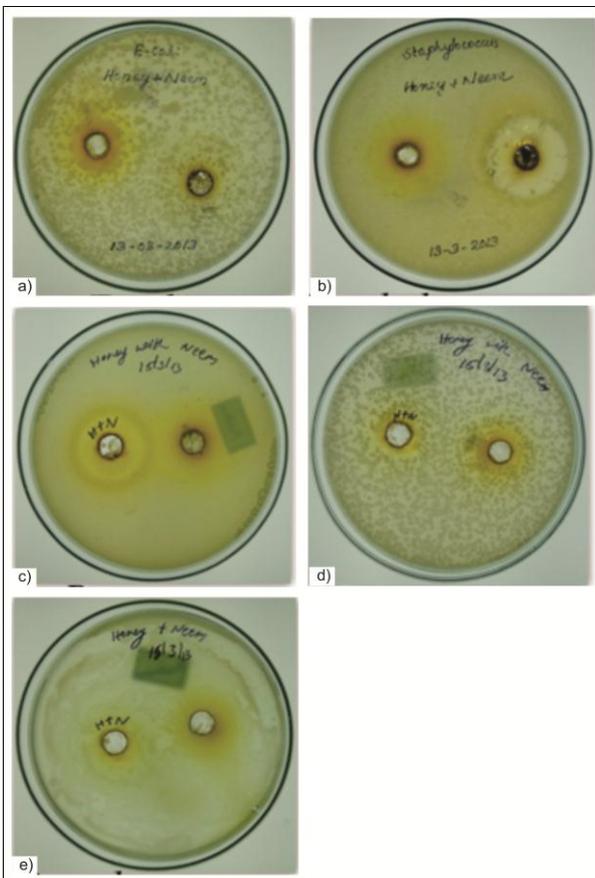
majority of the test bacterial strains except *Pseudomonas sp.* when compared to other plant extracts or their combined formulations with honey. The combination of Malabar nut extracts with honey was able obtain a zone inhibition against *Pseudomonas sp* and *Staphylococcus sp.* while the turmeric formulation with honey was also able to obtain a zone of inhibition against *Staphylococcus sp.* and *Proteus sp.* The above mentioned observations clearly show the possibility to use the different plant extract and honey formulations and creating a market for local medicinal plant.



**Fig 3:** Inhibition zone produced by the combination of turmeric with honey on different bacterial strains, a) *Escherichia species*, b) *Staphylococcus species*, c) *Proteus species*, d) *Pseudomonas species* and e) *Salmonella species*.



**Fig 5:** Inhibition zone produced by the combination of neem with honey on different bacterial strains, a) *Escherichia species*, b) *Staphylococcus species*, c) *Proteus species*, d) *Pseudomonas species* and e) *Salmonella species*.



**Fig 4:** Inhibition zone produced by the combination of neem with honey on different bacterial strains, a) *Escherichia species*, b) *Staphylococcus species*, c) *Proteus species*, d) *Pseudomonas species* and e) *Salmonella species*.

### 5. Conclusions

Amendment of honey with various plant extract seems a promising effect on antibacterial activity which can be used as initiative to reduce antibiotic resistance. It can be also a milestone for new clinical treatments, replacing these antibiotics with phytochemicals. The study proves that honey enhances the antibacterial activity of plant extracts giving them activity against a broad range of bacteria compared to the extract in water. Further study should be also conducted to reduce the research gaps in this area.

### 6. Conflict of interest statement

We declare that we have no conflict of interest.

### 7. Acknowledgements

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