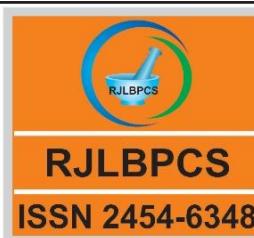


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EVALUATION OF ANTIBACTERIAL ACTIVITY OF STINGLESS BEE HONEY FROM DAKSHINA KANNADA DISTRICT OF KARNATAKA

Anil G B^{1,2*}, Mahesh Pattabhiramaiah², Shankar Reddy M²

1. Department of Zoology, MES Degree College, Malleshwaram, Bangalore, Karnataka, India.

2. Department of Zoology, Bangalore University, Bangalore, Karnataka, India.

ABSTRACT: Stingless bees which belong to the family Apidae and sub-family Meliponinae, are the smallest of the honey-producing bees. They are highly social insects like honey bees living in perennial colonies and nesting in concealed places. Besides, honey production Stingless bees are important and effective pollinators of many crop species. Honey has been identified as a potential alternative to the widespread use of antibiotics, which are of significant concern considering the emergence of resistant bacteria. In this context, this study aimed to evaluate the antimicrobial activity of honey samples produced by a stingless bee species (*Tetragonula iridipennis*) against pathogenic bacteria. The aim of the present research work to investigate the antibacterial activity of 15 honey samples collected from different regions of the Dakshina Kannada district of Karnataka. The inhibitory action of crude honey was evaluated against eight bacterial strains, Gram-positive bacteria viz., *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus*, *Lactobacillus acidophilus* and Gram-negative bacteria viz., *Staphylococcus aureus*, *Proteus mirabilis*, *Streptococcus mutans*, and *Salmonella typhi* by agar well diffusion method. The honey showed the zone of inhibition ranged from 6.5 mm to 14 mm for crude honey. *Streptococcus mutans* showed a maximum zone of inhibition of 14 mm in S13 honey and *Bacillus cereus*, *Staphylococcus aureus* showed a minimum zone of inhibition in S8 and S6 honey. Differences in the antibacterial activity of the different honey samples were observed. This study demonstrated that the natural honey possess in vitro antimicrobial activity against Gram-positive and Gram-negative bacteria.

Keywords: Stingless bees, *Tetragonula iridipennis*, Stingless bee honey, Antibacterial activity, Agar Well diffusion.

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Corresponding Author: Anil G B.

1. Department of Zoology, MES Degree College, Malleshwaram, Bangalore, Karnataka, India.

2. Department of Zoology, Bangalore University, Bangalore, Karnataka, India.

E-mail Address: anil.gb2@gmail.com

1. INTRODUCTION

Stingless bees (Apidae, Meliponini) are native to tropical and subtropical parts of the planet like Central and South America, Africa, Asia, and northern Australia [1]. All stingless bees build elaborate nests with structures that are often characteristic for the species or for higher taxa [2, 3]. Meliponini is the tribe, little studied, with two genera: *Lisotrigona* and *Trigona* found to occur in Asia [4]. The Indian stingless bee or dammar bee, *Tetragonula iridipennis* is predominantly found in India. Various studies have been conducted on *T. iridipennis* colonies located in Karnataka, Kerala, and Tamil Nadu states [5]. The tropical savanna climate, varying physiographic environment, higher altitudes, and luxuriant flora offers an abode for the rich and wide distribution of stingless bee (*T. iridipennis*). Like *Apis* honeybees, most species of eusocial stingless bees, produce honey. Stingless bee honey is potentially valued as a food source. Stingless bee honey has also been utilized as traditional medicine in Central, South America, and Africa [6], suggesting that stingless bee honey may have therapeutic properties that are almost like currently used medicinal honey such as manuka honey from New Zealand [7, 8, 9]. Currently, around 20,000 honeybee species inhabit the most diverse ecosystems around the world. Bees from the Meliponina sub tribe are known as native stingless honeybees, and the *Melipona Illiger* genus, 1806, has a wide number of species scattered across the Neotropical region, with the greatest diversity in the Amazon [10]. The honey that these bees produce has a specific property with a characteristic flavor and aspect. For this reason, it has become a product with high market demand, achieving higher prices than the honey produced by bees of the *Apis* genus. Honey is the natural sweet substance obtained from nectar or blossoms which honey bees collect, transform, and combine with specific substances of their own to ripen and mature [11]. It is stored as honey in the honey cells by worker honeybees. Honey consists of various constituents such as water, carbohydrates, proteins, vitamins, amino acids, energy, and minerals. The composition of honey varies from one honey to a different counting on several factors. A critical factor maybe a floral region is attributable to the nectar collected from various plants which contains the most sugars and trace elements in different compositions. These compositions are influenced by climate, the environment surrounding the plant, and therefore the soil type [12]. In addition to the main components, several minor components of honey may also play a crucial role in the determination of honey's anti-microbial behavior. Honey's antimicrobial activity has been documented in the past only with the use of an aqueous honey solution. The bactericidal effect of

honey is reported to be linked with the concentration of honey used and therefore the nature of the bacteria [10, 13]. Honey is one of the oldest traditional medicines considered as a standard remedy for microbial infections. It's also recognized as an efficacious topical antimicrobial agent within the treatment of burns and wounds [14]. It is widely used in traditional medicine throughout the world. However, it has limited use in modern medicine due to a lack of scientific support [15]. In Ayurveda, Indian system of health care treats as food for health while recommending it as an ancient medicine for some conditions using it externally as well as orally. It is known to cure anemia and improves calcium fixation in infants. Honey also reduces and cures eye cataracts and conjunctivitis and applied honey directly to the eye cures various diseases of the cornea [16]. There are many reports of honey being very effective as dressing of wounds, burns, skin ulcers, and inflammations used in ayurvedic treatments. The antibacterial properties of honey prove that the growth of new tissue to heal the wound at a faster rate [17]. The bactericidal effect of honey is reported to be dependent on the concentration of honey used and the nature of the bacteria [18, 19]. Despite the use of honey as an antibacterial in folklore medicine, this practice has been replaced by synthetic and semisynthetic antimicrobials [20, 21]. However, the indiscriminate use of antibiotics exerts selective pressure on microorganisms, progressively selecting the most resistant [22]. Given this problem, the search for new antimicrobial compounds derived from different natural products, such as honey, to replace conventional antibiotic therapy is of high importance [23, 24, 25]. Indeed, apitherapy has gained attention as a form of folklore and preventive medicine for treating diseases and promoting overall health [26, 27]. The healing effect of honey could be attributed to various physical and chemical properties [28]. The floral source of honey plays a crucial role in its biological properties [29]. Honey is widely used in hospitals, especially for the clinical treatment of ulcers, bedsores, burns, injuries, and surgical wounds. The antibacterial properties of honey could also be particularly useful against bacteria that have developed resistance to several antibiotics, e.g. *Staphylococcus aureus*, which may be a major explanation for wound sepsis in hospitals [30]. Honey is thus a perfect topical wound dressing agent in surgical infections, burns, and wound infections [31]. The utilization of honey as medicine has continued into present-day medicine. Many researchers and clinical laboratories have demonstrated the antimicrobial activity of honey against a broad range of microorganisms, including multi-antibiotic resistant strains. The main objective of this study was to evaluate the antimicrobial activity of stingless bee honey collected from different regions of Dakshina Kannada district using Agar well diffusion method.

2. MATERIALS AND METHODS

Collection and Maintenance of Honey

Fifteen stingless bee honey samples (*Tetragonula iridipennis*) (S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, S13, S14 & S15) were collected during the month of October 2019 to January 2020 from different regions of the Dakshina Kannada district, Karnataka. Crude honey samples were

collected from domesticated stingless bees hive from the beekeepers. All the honey samples were collected in sterilized polythene bottles from the place of honey extraction and it was stored at room temperature (25 – 35°C).

Collection and Maintenance of Test Organisms

Eight different species of bacteria was used in this study to explore the effectiveness of stingless bee honey on the growth inhibition. The bacteria selected for this study are Gram-positive species viz., *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus*, *Lactobacillus acidophilus* and the Gram-negative species viz., *Staphylococcus aureus*, *Proteus mirabilis*, *Streptococcus mutans* and *Salmonella typhi*. Eight Universal bottles containing 10 ml each of nutrient broth were inoculated separately with *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus*, *Lactobacillus acidophilus*, *Staphylococcus aureus*, *Proteus mirabilis*, *Streptococcus mutans* and *Salmonella typhi* using an inoculum loop and then incubated at 37°C for up to 48 hours. The cell suspensions of all the cultures were adjusted to 1-2x 10⁶cells/ml. These bacteria cultures were then stored at 4°C until use (Table 1).

Table 1: List of micro organism

Sl.no	Organism	Gram +/-
1	<i>Pseudomonas aeruginosa</i>	+
2	<i>Escherichia coli</i>	+
3	<i>Bacillus cereus</i>	+
4	<i>Lactobacillus acidophilus</i>	+
5	<i>Staphylococcus aureus</i>	-
6	<i>Proteus mirabilis</i>	-
7	<i>Streptococcus mutans</i>	-
8	<i>Salmonella typhi</i> .	-

Antibiotics

Ciprofloxacin, Mfg. By-Nicolas Piramal India Ltd. was obtained from local pharmacy store. The antibiotics were used in 2.5 µg/ml concentration against every bacterial strain.

Agar well diffusion assay (Zone of Inhibition Evaluation)

Antibacterial activity of all the samples was evaluated by the zone of inhibition using the agar well diffusion assay [32]. 25 ml of Soya bean Casein Digested agar solution was filled in the sterilized petri plates (90 mm). Then, Bacteria were inoculated on petri plates. These were allowed to solidify and then individual plates were marked for the organism inoculated. Each plate was punched to make 8 wells of 5mm diameter with the help of a sterile cork borer at different sites of the plates. 2.5µg/ml of respective samples, 2.5 µg/ml of standard Ciprofloxacin, and solvents (Distilled water

used as control) were pipetted into the wells in assay plates. Plates were incubated at 37°C for 24 hours. The plates were observed for the zone of inhibition around the wells, the diameter of which measured by using a Vernier caliper.

3. RESULTS AND DISCUSSION

As an initial screen to evaluate the antibacterial activity of honey samples against bacterial strains, the AWD assay was applied, and inhibition zones were measured (Table 2). A total of fifteen honey samples from different locations were evaluated for their antibacterial activity against the Gram-positive species such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus*, *Lactobacillus acidophilus* and the Gram-negative species such as *Staphylococcus aureus*, *Proteus mirabilis*, *Streptococcus mutans* and *Salmonella typhi*. In 100% honey concentration, among the fifteen honey samples S 12 honey sample show maximum zone of inhibition against E. coli (13 mm) and S 8 honey sample show minimum antibacterial activity against Bacillus cereus (6.5 mm) (Table 2, Figure 1). S 13 honey sample show maximum antibacterial activity against *Streptococcus mutans* (14 mm) and S 6 honey sample show minimum antibacterial activity against *Staphylococcus aureus* (6.5 mm) (Table 2, Figure 2). The antibiotic ciprofloxacin showed maximum antibacterial activity against *Streptococcus mutans* (30 mm) and minimum antibacterial activity against *Proteus mirabilis* (22 mm) (Table 2, Figure 3). Except S 1 and S 12 honey sample, remaining sample does not show any activity against *Salmonella typhi*. Zone of inhibition were not formed in control.

Table 2: Zone of Inhibition of different 100 % honey samples (mm)

Honey samples	gram positive bacteria				gram negative bacteria			
	<i>P. aeruginosa</i>	<i>E. coli</i>	<i>B. cereus</i>	<i>L. acidophilus</i>	<i>S. aureus</i>	<i>S. mutans</i>	<i>P. mirabilis</i>	<i>S. typhi</i>
S 1	9	8	7	8	7	11	8	9
S 2	-	11	-	7	7.5	9	10	-
S 3	-	10	-	7	-	9	7	-
S 4	-	8	0	7	-	9	9	-
S 5	10	10	8	10	-	10	11	-
S 6	-	8	9	8	6.5	9	11	-
S 7	11	-	7	7	7	10	-	-
S 8	7	-	6.5	7	7	-	8	-
S 9	10	8	10	8	7	-	9	-
S 10	-	-	9	-	-	11	9	-
S 11	10	10	9	9	-	-	10	-
S 12	11	13	11	12	8	11	7	9
S 13	-	9	0	11	-	14	10	-

S 14	-	10	0	12	-	9	-	-
S 15	8	8	0	12	7	-	-	-
CIPROFLAXIN	27	27	26	23	24	30	22	25
CONTROL	-	-	-	-	-	-	-	-

*Note: Zone of inhibition (in mm diameter) including the diameter of well (5 mm), (-) - No zone of inhibition,

Ciprofloxacin 2.5 μ g/ml. control – distilled water.

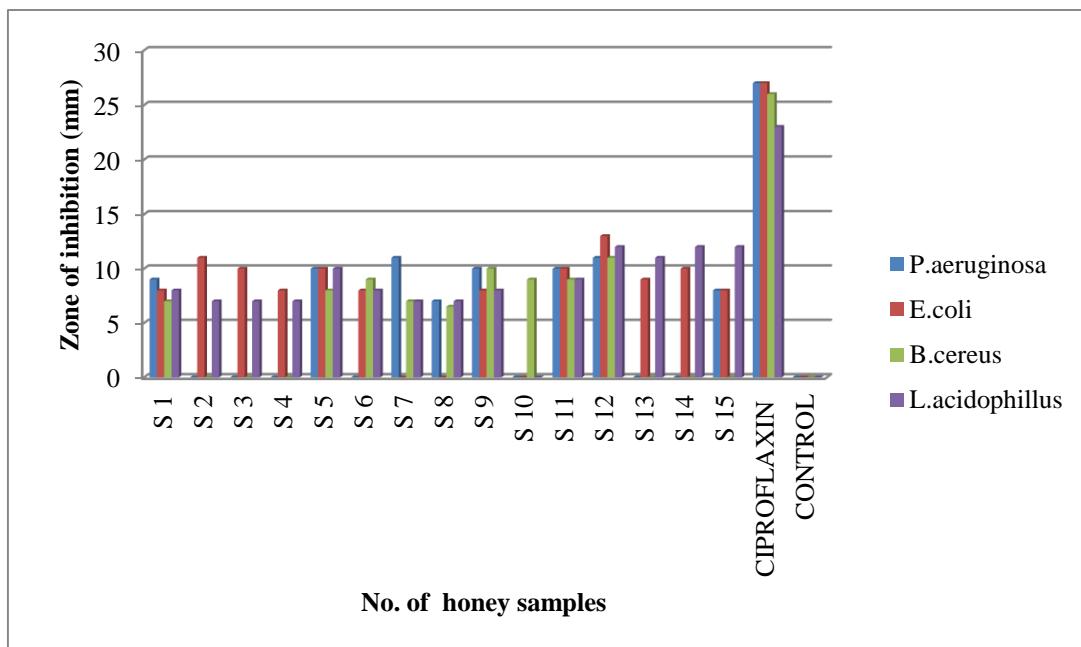


Figure 1: Antibacterial activity of stingless bee honey sample against Gram +ve bacteria

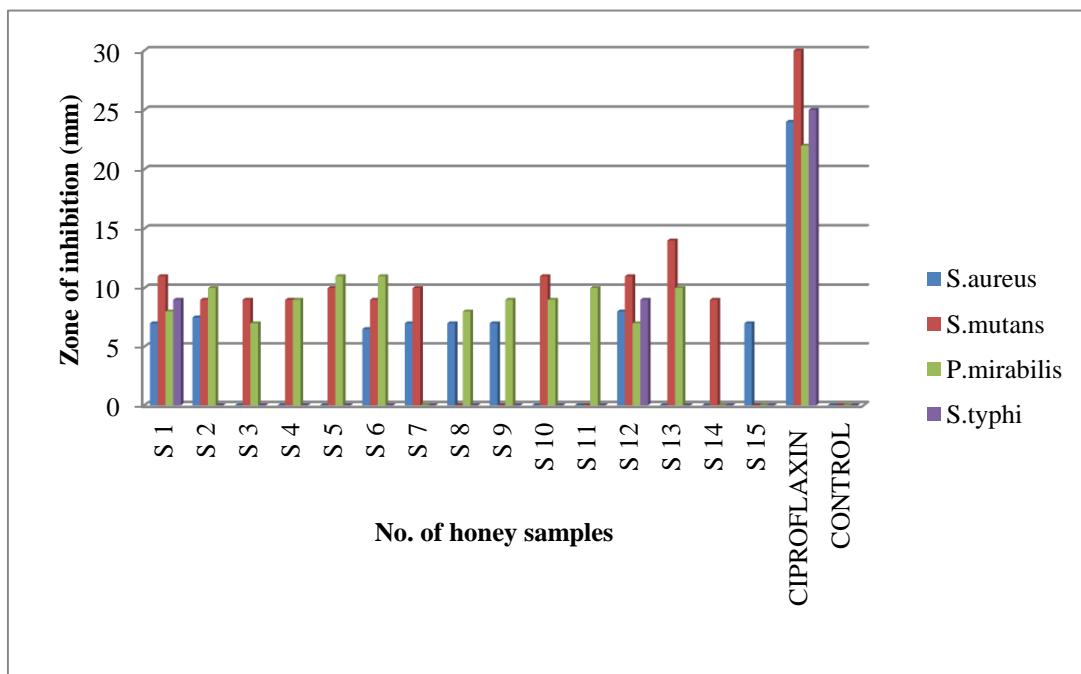


Figure 2: Antibacterial activity of stingless bee honey sample against Gram -ve bacteria

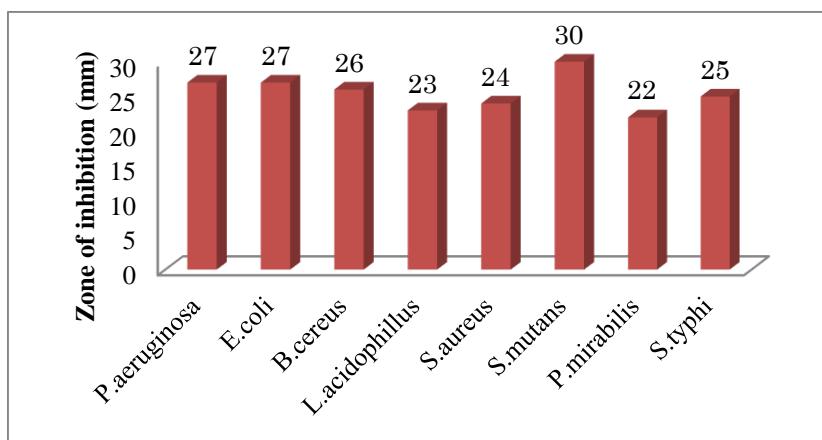


Figure 3: Antibacterial activity of an antibiotic Ciprofloxacin against bacteria

This study demonstrates that stingless bee honey has broad-spectrum antibacterial activity. Significant differences in antibacterial activity were found amongst the 15 stingless bee honey samples, and individual test organisms were also found to differ in susceptibility. A total of 98% of honey samples did not exhibit any antibacterial activity against *S. typhi*. Out of 15, only two honey samples showed antibacterial activity against *S. typhi* (S1, S12). Similarly, some of the honey samples failed to exhibit any antibacterial activity against different bacteria. By comparing with the two results (Graph 1 and Graph 2), gram-negative bacteria *S. mutans* showed the maximum zone of inhibition against gram-positive bacteria. The antibacterial properties of honey can be attributed to several factors like high osmotic pressure, low pH. Natural honey has antibacterial activity against certain bacteria, viruses, and fungi [33, 34]. This result showed that honey had a similar inhibitory effect on *S. mutans*. The process of the antibacterial effect of honey remains speculative at present. Possible explanations are the presence of hydrogen peroxide [35], flavonoids [36], and hypertonic sugar concentration [37]. This shows carbohydrate content of honey has not a significant inhibitory effect on *S. mutans* [38]. Nowadays, it is recognized that most types of honey have antibacterial activity and that this activity is dependent on physical and chemical factors. The viscosity of honey is sufficiently high to create a physical barrier that inhibits the contamination of the wound by infectious agents present in the air. Honey eliminates most bacteria by osmosis due to its high sugar concentration. The antibacterial activity can also partially give results based on the acidity of honey, the presence of phytochemical components such as flavonoids and phthalic acids and, most importantly, the action of oxygen peroxide, produced in honey due to the presence of the glucose oxidase enzyme secreted by the hypopharyngeal glands of honeybees [39]. Osmosis and hydrogen peroxide has long been considered as the main factors responsible for the antibacterial activity of honey [40]. The non-peroxide antibacterial activity in diluted honey shows low concentrations has brought attention to the presence of other antibacterial agents in verification [41]. In most of the studies, chemical components in honey which could be responsible for an antibacterial activity like flavonoids and phenolic acids are the most studied. One reason for such interest is that these molecules present in numerous types of biological activity,

including antibacterial properties [42]. Several researchers have verified the antibacterial activity of flavonoids isolated from honey and prominent results have been reported for stingless bees from Dakshina Kannada district. This activity is probably due to the ability of flavonoids to form complexes with soluble proteins and with the bacteria cell wall [42]. In the past few years, an increase in the number of research groups dedicated to studying the antibacterial activity of honey can be noted, which has promoted the publication of several papers regarding this activity and verifying its efficiency. These findings have also promoted the interest of companies dedicated to the commercialization of the high level of antibacterial activity of honey, which has provided financial support for research in this area, especially concerning clinic assays. The determination of the antimicrobial potential of the honey from stingless bees could identify this honey as an attractive low-cost alternative for treating bacterial infections, along with the possibility of promoting for these native bee products. In this context, the aim of this study was to investigate the antibacterial activity and flavonoid profile of honey produced by the native stingless bees.

4. CONCLUSION

The present study reveals that among the fifteen honey samples tested against the pathogenic bacteria, all the bacteria show antibacterial activity more or less except in *Salmonella typhi*. The results showed that honey has an antibacterial effect on *Streptococcus mutans* after a definite time interval. Results can be due to the production of hydrogen peroxide, inhibition of glucosyl transferase activity, or presence of polyphenols in honey. All these factors are responsible for the antibacterial effect of honey on *Streptococcus mutans*. *Salmonella typhi* shows high resistance to the honey sample. It is clear from this study that different kinds of honey act differently on the same microorganism. This is to be expected since the composition of each honey is different. The composition of each honey will be different according to the different floral sources and the species of bee.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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