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Research Article

Physicochemical and antibacterial activities of *Apis* honey types derived from Coorg, Karnataka, India

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Abstract
Natural honey has various ingredients in it that contribute to its incredible properties. The aim of this investigation was to evaluate the physicochemical and antibacterial activity of various *Apis* honey from Coorg, Karnataka. Four samples of *Apis* honey viz., *A. florea, A. mellifera, A. cerana* and *A. dorsata* were collected from various regions of Coorg, Karnataka. The honey samples' physicochemical properties and antibacterial activities against *Streptococcus* sp., *Staphylococcus aureus*, *Bacillus subtilis* and *Enterococcus* sp were determined in vitro. The moisture and ash content varied from 13.6 - 17.2% and 0.32 – 0.49%, respectively. Hydroxy methyl furfurals) content of *A. dorsata* honey samples was highest with 9.2±0.5 mg/Kg and least was recorded with 6.8±0.4 mg/Kg for *A. florae* honey. The reducing sugar content of *A. florea* honey sample was highest with 87.5±3.2 (%) and the peroxide levels were in the range of 10.2 – 14.9 µg/g/h at 20°C. The antibacterial assay revealed that *S. aureus*, *Enterococcus* sp and *Streptococcus* sp were most susceptible against the honey varieties tested and minimum inhibitory concentration (MIC) values between 25-6.5 (%v/v) were determined. In conclusion, honey varieties from Coorg could be used in specific antibacterial prophylaxis as the activity depends on the honey bee species, their metabolism and floral sources in specific geographical regions.

Keywords: *Apis* honey, Coorg, Antibacterial, Peroxide, Physicochemical

INTRODUCTION

Honey is an easily digestible foodstuff containing a wide range of nutritiously important complementary elements. Besides the high content of a range of saccharides, there are also organic acids, amino acids, mineral matter, colouring-aromatic substances, and a trace of fats (Bogdanov *et al.,* 1999). Honey also contains very valuable but unstable compounds, such as enzymes, substances of hormonal character, vitamins, and few minor compounds (Qui *et al.,* 1999). The medicinal use of honey in wound treatment is derived from diverse ancient civilizations. A wide range of microbial species has shown to be inhibited by honey (Cooper *et al.,* 2002). Antimicrobial use of honey has been reported since ancient times but modern dressings and antibiotic therapy superseded its use as an anti-infective agent. However, the emergence of bacterial pathogens and the potential of region specific honey variety have confounded the relook of honey and its antibacterial activity. Antibacterial resistance to honey is unlikely and has not been reported in the literature (Hussain, 2018) due to its synergistic antibacterial components (Sanz *et al.,* 2005). In contrast to antibiotics, when consumed orally, beneficial gut flora is not disrupted by honey (Hussain *et al.,* 2015) and also it enhances the growth of normal flora in the gastrointestinal tract (Mohan *et al.,* 2017). Honey exhibits a unique, multifac-

eted antibacterial activity against pathogenic bacteria as revealed by molecular and cellular studies (Blair *et al*., 2009; Kwakman *et al.,* 2010). The antimicrobial properties of honey, along with activation of the immune system and healing process, are one of the main reasons for its medicinal use. Given the importance of a study that honeys might exert antimicrobial activity against pathogenic bacteria in a relationship with the honey bee varieties and floral sources, this investigation aimed to evaluate the physico-chemical and antimicrobial activity of various *Apis* honey from Coorg, Karnataka.

MATERIALS AND METHODS

Study area

Honey samples of *A. florea, A. mellifera, A. cerana* and *A. dorsata* were collected from Galibeedu region of Coorg, (12.3375° N, 75.8069° E) Karnataka. Coorg is a diversified forest with multifloral region. The physico-chemical properties of the honey samples were determined by following the standard methods.

Determination of physicochemical parameters

pH

The honey was diluted to 10% using distilled water and the pH was determined in a pH meter (Bogdanov *et al.,* 2004).

Moisture and ash content

Moisture content in the honey was calculated by measuring the refractive index at 40ºC. Ash content of the honey was determined by heating the honey at 600°C in a muffle furnace for 2 hours followed by cooling (Bogdanov *et al.,* 2004).

Estimation of HMF (5-hydroxymethylfurfuraldehyde)

Five grams of honey were dissolved in 25 ml of water, transferred quantitatively into a 50 ml volumetric flask. To this, added 0.5 ml of Carrez solution I and 0.5 ml of Carrez II and the volume was made upto the mark with distilled water. The solution was filtered through paper, rejecting the first 10 ml of the filtrate and 5ml of aliquots were put in two test tubes. To one tube was added 5 ml of distilled water (sample solution); to the second was added 5 ml of sodium bisulphite solution 0.2% (reference solution). The absorbance of the solutions at 284 and 336 nm was determined using a UV-Vis spectrophotometer (White, 1979).

Estimation of reducing sugars

Reducing sugars present in the honey were estimated by dinitrosalicylic acid (DNS) method. A volume (0.1 ml) of the honey sample previously dispersed in DMSO mixed with 0.4 ml of distilled water was reacted on a boiling water bath for 8 minutes with 1 ml of DNS reagent. After cooling in an ice bath for 3 min, the absorbance was read at 546 nm on the spectrophotometer. The reducing sugar concentrations were calculated from the calibration curve using fructose as the standard.

Determination of sucrose content

Sucrose content of the honey was determined by the method described by White (1977). In brief, 1 g of honey was diluted in 15 ml of water, boiled for 30 seconds, cooled and the volume was made up to 100 ml. From this solution, 5 ml was taken and diluted to 50 ml, followed by 5 ml of glucose oxidase-catalase reagent. The mixture was placed in 40°C water bath for 1 hour, cooled and 2 ml was transferred to a fresh tube. To this, 2 ml of invertase was added and the tube was held at room temperature for 30 minutes. The absorbance was read at 520 nm using glucose as standard.

Peroxide content

Screening for peroxide accumulation was carried out by dissolving 10 g of honey in 40 ml of water. After 1 hour, peroxide test strip is dipped into the honey solution and the blue colour obtained is read after 15 seconds and compared with the colour scale. The obtained value, multiplied by five, gives the amount of hydrogen peroxide accumulation in micrograms per gram honey per hour at 20°C (Kerkvliet, 1996).

Antimicrobial activity of honey

Agar well diffusion assay

The antibacterial efficacy of the Coorg honey samples was performed on the basis of the Clinical and Laboratory Standards Institute (CLSI) guidelines (Anthimidou and Mossialos, 2013). Briefly, overnight bacterial cultures (*Streptococcus* sp. (BU202031), *Staphylococcus aureus* (BU202016), *Bacillus subtilis* (BU201907) and *Enterococcus* sp (BU202055) procured from Department of Microbiology, Bangalore University were grown in Mueller-Hinton broth were adjusted to 0.5 McFarland turbidity standard (~1.5×10^8 CFU/ml).Mueller-Hinton agar plates were inoculated with 10^6 CFUs of bacterial cultures over the entire surface of the plate. Wells of 6 mm in diameter were cut into the surface of the agar and 100 µl (50% v/v in phosphate-buffered saline) of the tested honey samples were added separately to each well. Standard antibiotic discs of chloramphenicol (c^{30}), tetracycline (TE^{30}) and Ciprofloxacin (CIP^5) were used as positive control in the antibacterial assay. The plates were incubated at 37°C for 16-18 h. The diameter of the inhibition zones, including the diameter of the well, was recorded. Each assay was carried out in triplicate.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of the hon-

ey types was determined in 96-well microtiter plates using a spectrophotometric bioassay, as previously described (Patton *et al.,* 2006). Approximately, 5×10^4 CFUs of bacterial cultures in 10 µl Mueller-Hinton broth were added to 190 µl of 2-fold diluted test honey (honey concentration ranged from 100 to 1% v/v) in Mueller-Hinton broth. The optical density was determined at 630 nm using a micro-plate reader.MIC was defined as the lowest concentration of honey that completely inhibits bacterial growth.

Statistical analysis

The MIC results were expressed as mode (the value that appears most often) and the comparison of the antibacterial activity of the samples was evaluated by applying *t*-test. $p \leq 0.05$ values were considered to indicate statistically significant differences.

RESULTS AND DISCUSSION

The physicochemical characteristics of *Apis* honey samples collected from Coorg are presented in table-1. The pH of *A. dorsata* honey samples was highest with 5.3 and of *A. florea* was least with 3.2. The moisture content of honey samples varied from 13.6 to 17.2% and the highest was recorded in *A. dorsata,* whereas the least was found in *A. florae* and the difference in the moisture might be due to low rate of honey fermentation.The total ash content of *A. mellifera* honey was highest (0.49%) and of *A. cerana* was least (0.32%).The HMF (Hydroxy methyl furfurals) content of *A. dorsata* honey samples was highest with 9.2±0.5 mg/Kg and least was recorded with 6.8±0.4 mg/Kg for *A. florae* honey. The reducing sugar content of *A. florea* honey sample was highest with 87.5±3.2 (%) and was least recorded with 53.4±1.5 (%) for honey of *A. dorsata*. The sucrose content of *A. dorsata* honey was highest with 6.5±0.7 (%) and least with 4.8±0.4 percent for *A. florea* and *A. cerena* honey. The peroxide content of *A. florae* honey was the highest with 14.9±0.1 µg/g/h at 20°C and of *A. dorsata* was least with 10.2±0.5 µg/g/h at 20°C. The probable reasons for the variations of physicochemical characteristicsis due to the honey bee species and their floral sources.

All the honey samples showed different levels of growth inhibition on all the bacteria tested. The results of antibacterial assay revealed that *S. aureus*, *Enterococcus* sp and *Streptococcus* sp were most susceptible against the honey varieties tested (Fig.1). *A. florea* honey had highest antibacterial activity against *S. aureus* with a zone of inhibition of 4.5 mm whereas *Enterococcus* sp was the most susceptible organism (4.1 mm) for *A. dorsata* followed by *Streptococcu*s sp (3.9 mm). *A. mellifera* was effective against *S. aureus* while *A. cerena* was equally effective against both *S. aureus* and *Streptococcus* sp. Varying antibacterial activity of honey samples might be influenced by the bee's source of nector, floral type and phytochemicals present in the honey. This findings is in accordance with previous studies reported that different honey types possess different efficacies and mechanisms against the same bacteria (Cebrero *et al*., 2020; Al-Masaudi, 2020, Al-Masaudi *et al*., 2017&2020; Lu *et al*., 2014; Carnwath *et al*., 2014)

The results of minimum inhibitory concentration values for the honey samples against the different bacteria tested are shown in Fig.2. The highest inhibition zone was recorded against *S. aureus* (3.9±0.7mm) while it was 3.1±0.1mm against *Enterococcus sp.* The data was significant at $p<0.01$ for *A. florea* and *A. cerana* honey samples and $p<0.05$ for *A. mellifera* and *A. dorsata* honey samples. Cebrero *et al*. (2020) reported that the minor constituents such as phenolic compounds, antioxidant enzymes present in the honey contribute substantially to the antibacterial activities of honey. As this study used four different honey varieties, variations in the antibacterial activity is justified based on the nature and composition of honey samples.

The use of honey for treating microbial infections is an ancient process (Molan, 1992) and one of the predominant antimicrobial agents in honey is hydrogen peroxide generated when the honey is peroxide levels were in the range of diluted (Weston, 2000). In this study the peroxide levels were found to be in the range of 10.2-14.9 µg/g/h.Both Gram-positive and Gram-negative pathogenicbacteria are susceptible to honey and the antibacterial activity is attributed to its osmolarity, H_2O_2content, low pH, phenolic compounds and flavonoids(Jenkins *et al.,* 2014; Lusby *et al*., 2005; Manyi-Loh *et al.,* 2006). Similarly, Nayaka *et al*., (2020) re-

Table 1. Physicochemical characteristics of Apis honey samples from Coorg, Karnataka.

Honey Types	pH	Moisture Content (%)	Ash (%)	HMF (mg/kg)	Reducing Sugars (%)	Sucrose (%)	Peroxide µg/g/h at 20°C
A. florea	3.2±0.6	13.6±2.0	0.41±0.2	6.8±0.4	87.5±3.2	4.8±0.4	14.9±0.1
A. mellifera	4.7±0.1	14.5±0.0	0.49±0.5	8.0±1.8	77.4±0.2	3.6±0.5	11.4±0.7
A. cerana	4.2±0.2	15.8±2.1	0.32±0.9	8.5±0.2	66.2±0.7	4.8±0.7	10.9±0.8
A. dorsata	5.3±0.4	17.2±0.0	0.41±0.3	9.2±0.5	53.4±1.5	6.5±0.7	10.2±0.5

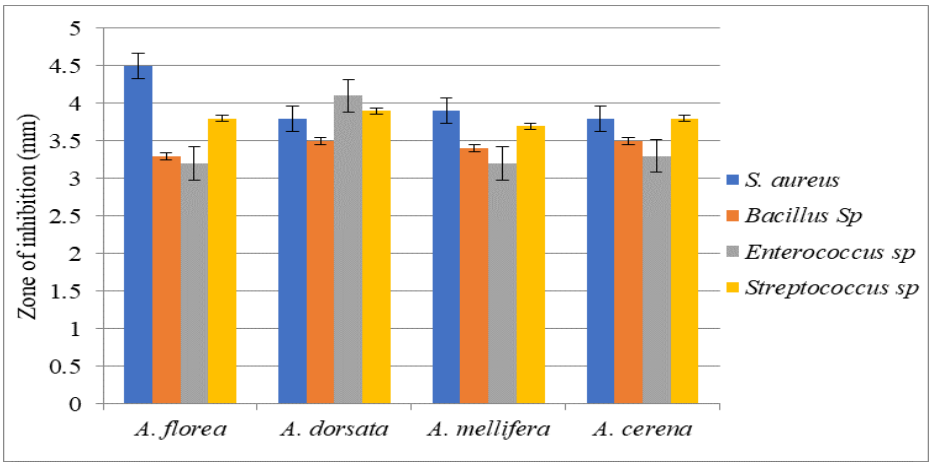

Fig. 1. *Zone of inhibition of Apis honey samples from Coorg, Karnataka.*

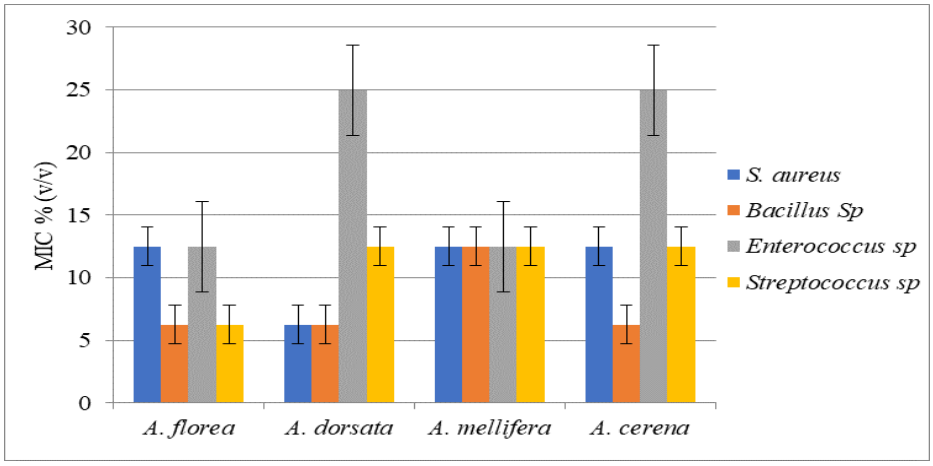

Fig. 2. *MIC % (v/v) values of Apis honey samples from Coorg, Karnataka.*

ported that bioactivities of honey are influenced by geographical regions and phytochemical profiles of the honey. The other components with antimicrobial potential of honey are catalase, glucose oxidase, non-peroxide components and methylgloxal (Simon *et al.,* 2009). It is known that acidic pH is inhibitory to many bacterial pathogens and the natural acidic nature of honey is good enough for its antibacterial properties (Haniyeh *et al.,* 2010). The four honey samples derived from Coorg were with pH between 3.2 and 5.3 and that might have attributed to the antibacterial activity of the honey samples tested. The pH values were in accordance with the results of Bogdanov (1997) and Jyothi (2006). Floral sources is the another factor influences the varying antibacterial nature of the honey samples tested. It has also been reported that physical property, geographical distribution may play important role in the antimicrobial activity of honey (Nayaka *et al*., 2020).

The higher the concentration of honey the greater its antibacterial activity (Badawy *et al.,* 2004) and 50% v/v in phosphate-buffered saline was used for antimicrobial assay in this present study. Further, honey concentration ranged from 100 to 1% v/v was used in MIC assay to determine the usefulness of honey varieties in controlling bacterial growth. Earlier studies by Albaridi, (2019), Anand *et al*., (2019), Matzen *et al*. (2018) and Adeleke *et al*. (2006) mentioned the use of diluted honey in controlling the bacterial growth and the dilutions could be confirmed through *in vivo* and clinical studies.

Conclusion

Physicochemical properties of the honey play a substantial role in its antibacterial activity. Variations in the antibacterial activity could be attributed by the honey bee species, floral varieties even it is collected from the same geographical region hence identification of appropriate honey type to control the specific bacterial growth is required. Further deciphering of phytochemicals in the effective honey variety is important in order to use the honey against specific pathogens.

Conflict of interest

The authors declare that they have no conflict of interest.

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