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Biochemical, antimicrobial, antioxidant and palynological activities of honey.

Gauthami, Annapoorna, Rama Bhat P*.

PG Department of Biotechnology, Alva's College, Moodbidri, Karnataka, India.

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ABSTRACT

Aims & Objectives: The aim of the study was to examine the biochemical, antimicrobial, antioxidant and palynological studies of different honey samples collected from different locations of Dakshina Kannada, Karnataka, India.

Materials & Methods: Ten types of honey samples were collected from ten locations of Dakshina Kannada. Physical properties and biochemical constituents were determined by following standard protocols. Antimicrobial activity test was done against *Klebsiella pneumoniae* and *Streptococcus mutans* by well diffusion method.

Results: The color of honey samples were differed from light to intense and pH varied from 3.48 to 6.18. The protein content was in the range of 0.13-0.62 mg/g. The honey quality varied from 4.2 to 15 Brix %. Total sugar content was in the range of 4.5-9.8mg/g. The total phenolic content varied considerably with the highest value obtained for Processed society honey, Butterfly Park, Beluvai. The different shapes of pollen grains observed.

Conclusion: The honey samples exhibited different chemical composition and pH. The local natural honeys were abundant in specific plant pollens due to wider biodiversity both cultivated and natural vegetations in the collected areas. So local honey samples can be utilized as an excellent dietary source of antioxidants as well as the antibacterial agents, and hence duly be active for both therapeutic and prophylactic application.

*Author for Correspondence: bhat_pr@rediffmail.com

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INTRODUCTION

Honey is defined by the European Union as "the natural sweet substance produced by *Apis mellifera* bees from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects or the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store, and leave in honeycombs to ripen and mature" [1, 2].

Monofloral honey is arising predominantly from a single botanical origin with above 45% of total pollen content from the same plant species and is named after that plant, such as citrus, manuka and acacia honey [3].

The main composition of honey is carbohydrates or sugars, which represent 95% of honey's dry weight. Honey is a complex mixture of concentrated sugar solution with the main ingredients of fructose and glucose. The average ratio of fructose to glucose is 1.2:1 [4]. Sucrose is present in honey at about 1% of its dry weight. The exact proportion of fructose to glucose in any honey depends largely on the source of the nectar. It also contains bioactive compounds like organic acids, proteins, amino acids, minerals, polyphenols, vitamins, and aroma compounds [5, 6].

The protein content of honey is normally less than 0.5% with a small fraction of enzymes. The overall quality of honey such as taste, color, and other physical properties are contributed by the non-volatile compounds like sugar, amino acids, minerals, and phenolic compounds while the aroma of honey is mainly contributed by the volatile components [7, 8].

Honey has various biological properties including antimicrobial, anti-viral, anti-inflammatory, wound and sunburn healing, antioxidant, anti-parasitic, anti-diabetic, anti-mutagenic, and anti-tumoral activities [9]. Recent pharmacological studies have revealed that natural honey has the potential to reduce the risk of gastric and cardiovascular diseases [3] and has beneficial effects on fertility and ameliorating hormones related to fertility [10].

The pure honey is thick in texture and will settle at bottom of glass or bottles. The honey can be collected from both wild and domesticated bees and the practice is known as apiculture or beekeeping. In domestic beekeeping, human-made hives domesticate the insects to harvest the excess honey. In a hive or wild nest, there are three types of bees are found they are female queen; female worker and male drew bees. Most of the microorganisms do not grow in honey, so sealed honey can be stored for thousands of years. The well-known antimicrobial activity of honey and its recent use in clinical settings has reinvigorated further investigation of bioactive honey i.e., kinds of honey marketed as having therapeutic potential. Some kinds of honey show broad-spectrum activity against antibiotic-resistant bacteria [11], while others are very effective against biofilm-forming clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* [12]. Honey was shown to be effective in alleviating inflammation associated with wound infections and enhancing healing [13]. The honey dressing was effective in decreasing morbidity associated with first and second-degree burns and assisting in reducing the time required for rehabilitation [14].

Honey constitutes 81% sugar, 17% water and 1–2% of other enzymes and compounds [15]. This 2% of the remaining compounds are important contributors to the bactericidal activity of the honey and their composition determines the variability of honey [16]. Honey is a supersaturated solution of sugar, made by bees and it is a sweet viscous food substance. Honey is the golden color produced in the honey sacs of different various bees collected from the nectar of flowers. Bees store honey in wax structures called honeycombs. The commercially desirable honeybees are produced from clover by the domestic honeybee. The uses of honey and production have a long and ancient activity. It is used as food and medicine. Honey is healthy and it contains fiber, proteins, carbohydrates like fructose, glucose, sucrose, maltose, and little amount of trisaccharides and other minerals, vitamins, and enzymes that bring sweetness to the honey. Honeybees are the only insects that produce food that is consumed by humans, animals etc. and it is a social insects. Honey contains compounds that function as antioxidants. The antioxidant compounds in honey are chrysin, pinobanksin, vitamin C, catalase, and pinocembrin [17].

Honey is used as an ingredient in a range of manufactured products. Throughout history, it has been valued as food and as a healing product for cough, sore throat and, swelling and helps in healing wounds because it has antimicrobial activities. It is also used as a beauty product, antioxidant, and antiviral activities too. The objective of the present study was to study the physical, palynological parameters, antimicrobial and antioxidant activities of different honey samples.

MATERIALS AND METHODS

The honey samples from 10 different locations in Dakshina Kannada district, Karnataka were collected, numbered, and brought to the laboratory for the experiment. They were properly labeled, and details were entered during the collection stage itself.

Sample 1: Dadey brand honey, Karkala (raw)

Sample 2: Domesticated honey, Butterfly Park, Beluvai (raw and unprocessed)

Sample 3: Society honey, Butterfly Park, Beluvai (processed)

Sample 4: Natural honey from a tree branch, Sanoor

Sample 5: Reared honey, Muggerkala

Sample 6: Old honey sample, Andar

Sample 7: Reared honey, KoilaBantwal

Sample 8: Processed honey, Sampige, Moodbidri

Sample 9: Reared honey, Sajipa, Bantwal

Sample 10: Natural honey obtained in Arecanut tree, Mittamajal, Bantwal

Bacterial strains used: *Klebsiella pneumoniae* and *Streptococcus mutans*

Color measurement: Actual colors of the 10 honey sample were compared.

pH measurement: The pH meter is used to measure the pH of a solution of honey prepared in milli-Q water (deionised water).

Determination of total protein content (TPC): The amount of protein present in the 10 honey samples were estimated spectrophotometrically.

Determination of Brix content honey by ERMA hand Refractometer: ERMA Hand Refractometer, is an analog instrument to check the quality of honey and also very easy to measure the Brix content in the honey sample. Here a drop of the honey sample was placed between a measuring prism and a small cover plate. Cover the plate so that the honey sample equally spreads throughout the prism. The result was viewed through a magnifying eyepiece. The blue line indicates the Brix level of the honey. The temperature maintained in this instrument is 20°C.

Estimation of total sugar content: The total sugar content of 10 honey samples was estimated by phenol sulphuric acid method [18].

Measurement of Optical density: One gram of honey is dissolved in 9 ml of distilled water and centrifuged for 10 min at 3000 rpm. The absorbance of the filtrate supernatant was measured at 530nm against distilled water as a blank using colorimeter [19].

Phenol estimation of the honey sample was done by Folin ciocalteu reagent method.

Determination of the antimicrobial activity of honey: Bacterial strains and inoculums standardization: *Klebsiella pneumonia* and *Streptococcus mutans*. Before the experiment, the bacterial strains were inoculated onto the surface of nutrient agar media. Four wells punched on the inoculated media using cork borer except for the part where the antibiotic chloramphenicol was placed. Different concentrations of honey samples 25µL and 50µL were introduced into two wells and one well was introduced with distilled water, which was taken as a negative control. Chloramphenicol served as a positive control. The plates were incubated for 24 hours at laboratory temperature and the diameters of the zone of inhibition were measured.

Palynological study of honey carried out using sediment content of honey: Based on the method of Louveaux et al. (1978) [20], two grams of honey were dissolved in 4 mL of warm distilled water (40°C). The solution was centrifuged for 10min at 2500rpm. The solution was poured into the small tube and centrifuged again for 10min at 2500rpm. The entire sediment was put on a slide and spread out over an area of about 20x20mm, after drying by slight heating at 40°C. The sediment was mounted with glycerine gelatin, liquefied by heating in a water bath at 40°C, and observed under a microscope for pollen grains.

RESULTS AND DISCUSSION

All the ten honey samples were compared to note the color and it was seen that sample 10 (honey sample from Arecanut tree, Mittamajal, Bantwal) has got highly intense color whereas sample 2 (Domesticated, raw and unprocessed honey from Butterfly Park, Beluvai) has got least intense color, and this may depend on the source of the nectar. The average pH of honey is 3.9 but can ranged from 3.4-6.1. Honey contains many kinds of acids both organic and amino. However, different types and their amounts vary considerably, depending on the type of honey. These acids may be aromatic or aliphatic (nonaromatic).

It was found that sample 4 (natural honey from a tree branch, Sanoor) is more acidic which is pH 3.48 whereas sample 8(processed honey from Sampige, Moodbidri) was least acidic which is pH 6.18. While rest of the honey samples showed intermediate acidic pH in comparison to samples 4 and sample 8 (Table 1). The Egyptian (4.41±0.09), Yemini (4.460±0.02), and Kashmir honey (4.637±0.03) [21] contain an acidic content compared to the samples of the present study. The acidic and low pH value of Melipona honey was highly contributed to inhibiting the presence and growth of micro-organisms. Melipona honey has a low pH value [22]. Korcha's sample pH is 4.18, Mexi pH is 3.96 Shake's pH is 3.96 and Gobito's sample pH, 4.0 is found [23].

Table 1. pH of the honey samples collected from ten different locations.

Samples	pH
1	5.06
2	4.49
3	4.03
4	3.48
5	4.30
6	4.07
7	4.03
8	6.18
9	3.70
10	4.27

The pH values of the light-colored honey sample (LNH1) and dark-colored honey sample (LNH2) were 5.2 and 5.0, respectively in the natural honey samples from Malda as reported earlier by Roy et al. (2016) [24]. The qualitative analysis of phyto-components revealed the presence of flavonoids, steroids, phenol, terpenoids, and quinone, and the absence of glycosides, in both the honey samples they tested.

The protein content of honey revealed that Sample 5: an old honey sample from Andar contains a high concentration of protein in honey0.62mg/g and Sample 8 and 10: contain the least concentration of

protein in honey 0.13mg/g. the rest of the samples like Sample 2 contains 0.55mg/g, sample 3: 0.21mg/g, sample 4: 0.16mg/g, sample 6: 0.24mg/g, sample 7: 0.14mg/g, sample 9: 0.18mg/g, sample 1: 0.17mg/g of concentration of proteins (Fig. 1). Kashmir honey showed the highest protein content (4.67±0.171mg/g) followed by Yemini (2.64±0.025mg/g) and Saudi (2.42±0.172mg/g) while the lowest value of protein content was registered in Egyptian honey (1.69±0.015mg/g) [21]. The protein results for both Monofloral and Multifloral in the study are similar to results obtained for honey from Bangladesh, Malaysia and Algeria which range from 2-5mg/g [25].

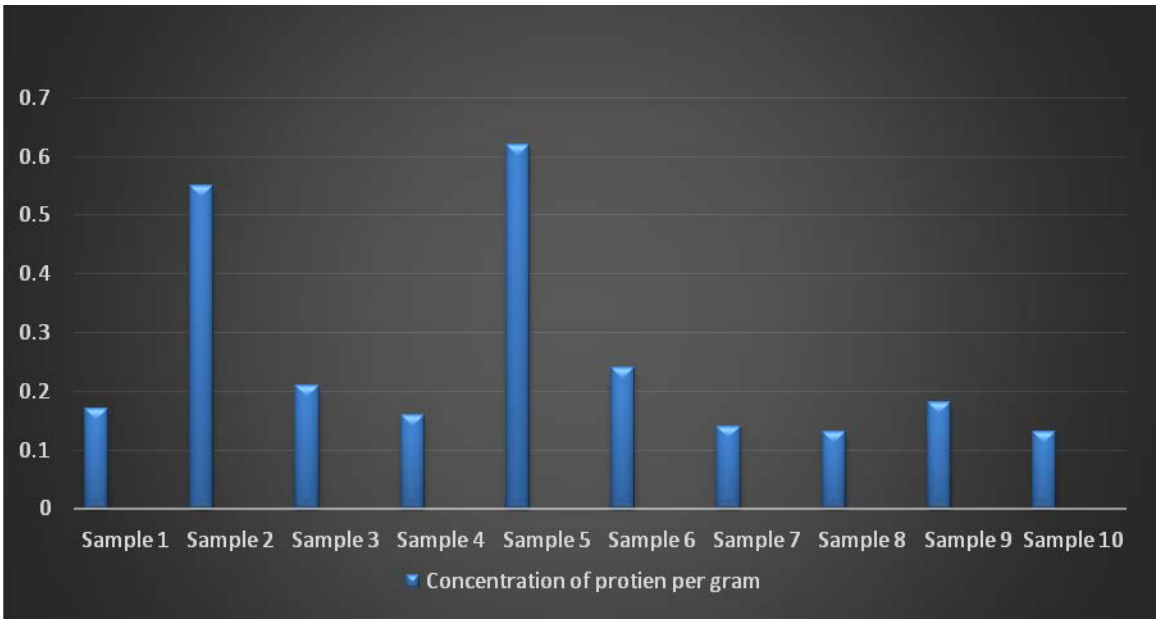

Figure 1. Protein contents in 10 different honey samples as determined by Lowry's method.

The Brix content of the honey can be calibrated using the Instrument. Sample 2 (domesticated, raw, and unprocessed honey from Butterfly Park, Beluvai) showed a high amount of Brix content in the honey sample whereas sample 5 (reared honey from Muggerkala) showed the least amount of Brix content in the honey sample. The rest of the honey samples showed intermediate Brix content in the honey samples (Table 2, Fig. 2).

Table 2. Quality of 10 different Honey samples.

Samples	Brix %
1	13.8
2	15
3	9.2
4	4.3
5	4.2
6	11
7	12
8	14
9	8.6
10	10.5

The total sugar content present in the given honey sample is estimated by using phenol sulphuric acid method as sample was shown in figure 3 indicated varied amount of the sugar content in the honey samples. Sample 2 (unprocessed, raw domesticated honey sample from Butterfly Park Beluvai) contains high concentration of sugar which was 9.8 mg/g and sample 5 (reared honey sample, Muggerkala) contains least concentration of sugar content which is 4.5mg/g whereas the rest of honey samples like Sample 1: 9.2mg/g, Sample 3: 4.9mg/g, Sample 4: 5.7mg/g, Sample 6: 7.3mg/g, Sample 7: 7.7mg/g, Sample 8: 8.2mg/g, Sample 9: 6.5 mg/g, and sample 10: 8.3 mg/g.

The optical density of different honey sample is determined using a colorimeter. The optical density of different honey samples showed wide variation (Table 3). Sample 6 (old honey sample from Andar) showed a high optical density of 0.24 and sample 8 (processed honey from Sampige, Moodbidri) exhibited the least optical density of 0.01. The remaining honey sample showed an intermediate optical density (Fig. 4).

Phenol concentration of all the honey samples was estimated by using the folin ciocalteu reagent method (Fig. 5). From the standard plot of concentration v/s optical density (OD), phenol concentration of honey samples was estimated, Phenolic content in honey samples revealed that Sample 3: processed society honey, Butterfly Park, Beluvai contains highest phenol concentration of 19.6mg/100mg and Sample 8: processed honey Sampige, Moodbidri contains the least amount of phenol concentration of 5.3mg/100mg. Where Sample 1 and 5 contain 11.5mg/100mg, Sample 2 and 4 16mg /100mg, Sample 6: 14.4mg/100mg, Sample 7: 12.3mg/100mg, Sample 9: 14.5mg/100mg, and Sample 10 contains 16.2mg/100mg of phenol content in sample (Fig. 5). However, the honey samples do not show any

significant differences in phenol concentration among them. Honey samples like Polyfloral had high phenolic content compared to Manuka honey had a 0.71 mg GIAE/g sample which was collected from the place Skamnia [26].

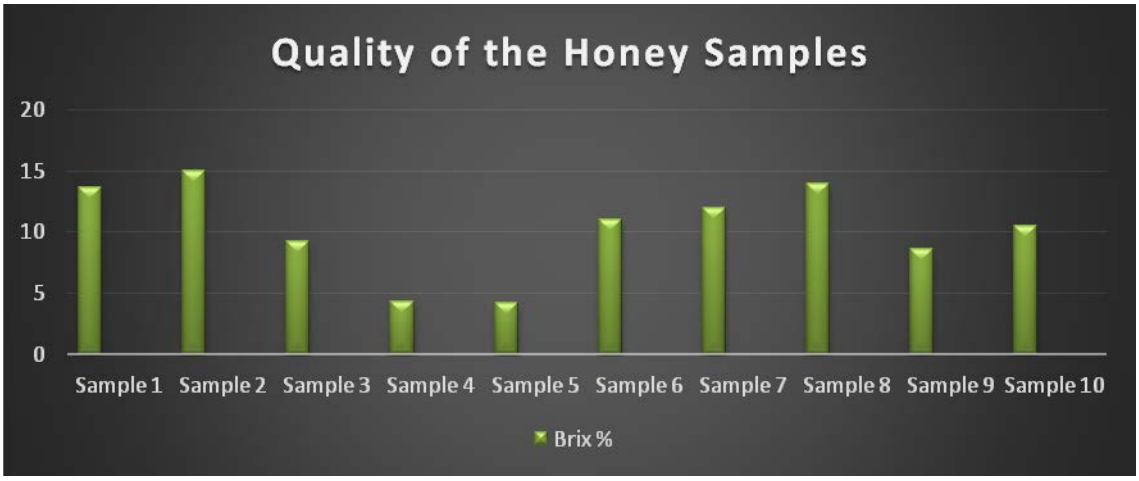

Figure 2. Honey quality of different honey samples.

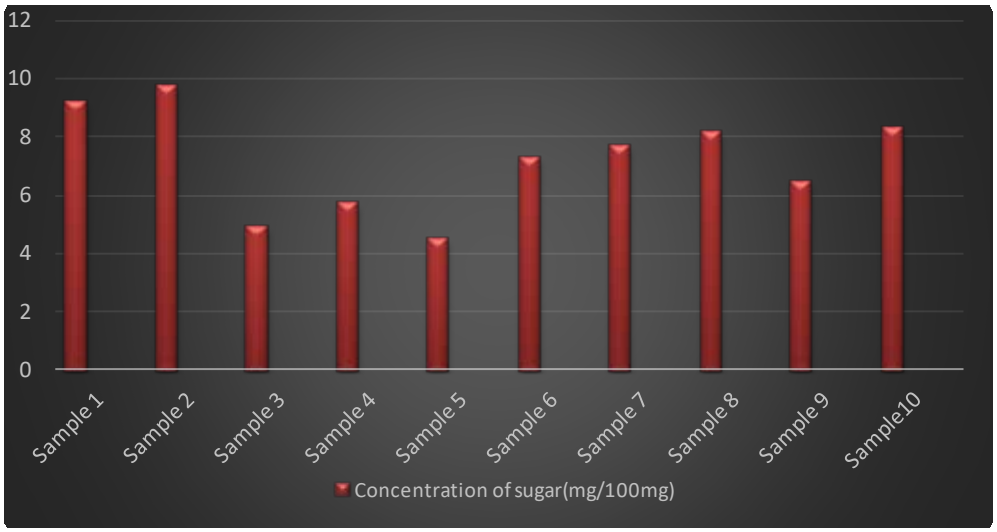

Figure 3. Total sugar contents of honey samples.

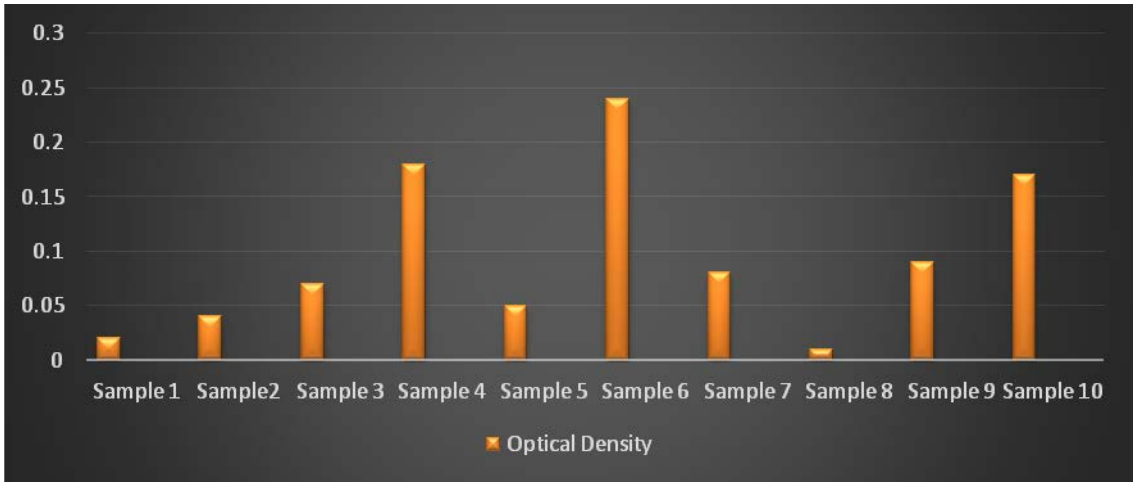

Figure 4. Optical density of different honey samples.

Table 3. Optical desity of 10 different honey samples.

Samples	Optical density
1	0.02
2	0.04
3	0.07
4	0.18
5	0.05
6	0.24
7	0.08
8	0.01
9	0.09
10	0.17

Table 4. *In vitro* antibacterial activities of Honey samples from ten different places against *Streptococcus mutans* and *Klebsiella pneumonia*.

Honey sample	Conc. of sample (mg/ml)	Zones of inhibition (cm)	
		S. mutans	*K. pneumoniae*
01. Dadey brand honey, Karkala (raw)	25	0.12	0.13
	50	0.24	0.22
	Control	0.0	0.0
	Chloramphenicol	0.7	2.0
02. Domesticated honey, Butterfly Park, Beluvai (raw and unprocessed)	25	0.23	0.12
	50	0.36	0.19
	Control	0.0	0.0
	Chloramphenicol	0.9	2.1
03. Society honey, Butterfly Park, Beluvai (processed)	25	0.16	0.18
	50	0.28	0.29
	Control	0.0	0.0
	Chloramphenicol	2.3	2.5
04. Natural honey from tree branch, Sanoor	25	0.10	0.09
	50	0.22	0.10
	Control	0.0	0.0
	Chloramphenicol	0.9	2.2
05. Reared honey, Muggerkala	25	0.13	0.17
	50	0.24	0.27
	Control	0.0	0.0
	Chloramphenicol	3	3.1
06. Old honey, Andar	25	0.12	0.15
	50	0.29	0.31
	Control	0.0	0.0
	Chloramphenicol	2.5	2.4
07. Reared honey, Koila, Bantwal	25	0.08	0.10
	50	0.21	0.24
	Control	0.0	0.0
	Chloramphenicol	2.7	2.5
08. Processed honey, Sampige, Moodbidri	25	0.11	0.13
	50	0.22	0.26
	Control	0.0	0.0
	Chloramphenicol	2.5	2.6
09. Reared honey, Sajipa, Bantwal	25	0.16	0.14
	50	0.28	0.31
	Control	0.0	0.0
	Chloramphenicol	2.4	1.6
10. Natural honey obtained in Arecanut tree, Mittamajal, Bantwal	25	0.12	0.17
	50	0.28	0.31
	Control	0.0	0.0
	Chloramphenicol	2	1.1

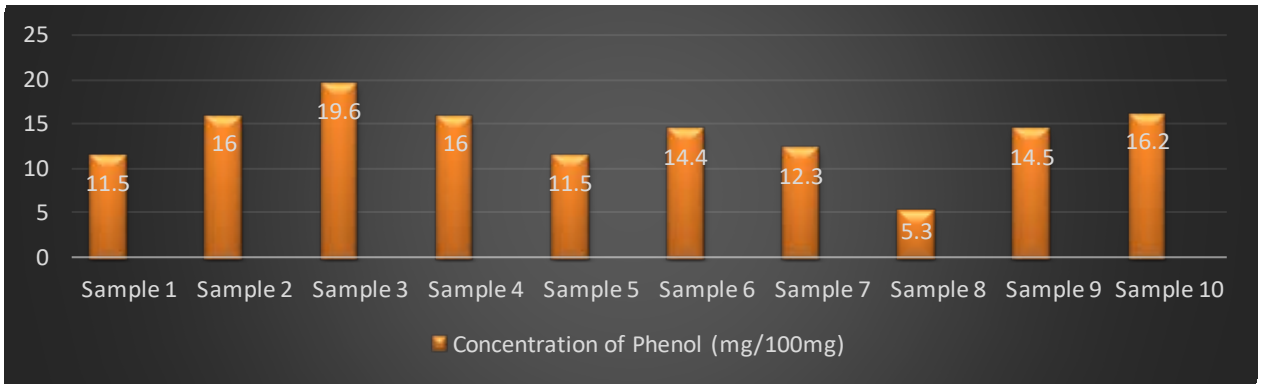

Figure 5. Phenol contents of honey samples.

The TPC of BB samples, as measured by the Folin Ciocalteu method, ranged from 6.49 (sample 12) to 14.64 mg (GAEs)/g sample (sample 18). BB samples (3, 7, 11, and 17) also exhibited high TPC (Table 1). The TFC of the tested samples, as measured by the aluminum chloride colorimetric method, ranged from 2.56 (sample 10) to 5.49 mg (QE)/g sample [27].

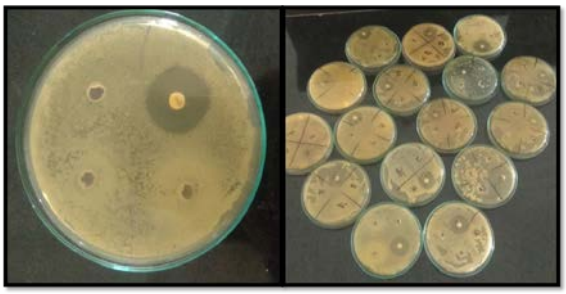

Plate 1. Antimicrobial activity of honey samples with *Streptococcus mutans.*

There was no zone of inhibition for honey samples. It indicates there is no antibacterial activity against Streptococcus mutants (Table 4, Plate 1). Honey samples 1 and 2 showed some antibacterial activity against *Klebsiella pneumoniae* and the rest of the honey samples are not showed any antibacterial activity against *Klebsiella pneumoniae* (Table 4, Plate 2).

Plate 2. Antimicrobial activity of honey samples with *Klebsiella pneumonia.*

Comparatively, the antibacterial activity of Commercial honey was very less when compared to the Natural honey samples. Further, since honey is a cheap, easily available, and also a non-toxic antimicrobial agent due to its properties, it can be very effectively used for medical purposes.

The antibacterial activity of the honey sample LNH1 showed growth inhibitory action against Gram-positive bacteria: *Staph. aureus* (ZDIs: 28-32 mm, for non-autoclaved honey and 27-28 mm, for autoclaved honey), as well as Gram-negative bacteria: *E. coli* ATCC 25922 (ZDIs: 30-35 mm, for non-autoclaved honey and 28-33 mm, for autoclaved honey), *P. aeruginosa* (ZDIs: 25-30 mm, for non-autoclaved honey and 24-28 mm, for autoclaved honey) and *S. enteric serovar. Typhi* (ZDIs: 26-28 mm, for both non-autoclaved and autoclaved kinds of honeys).

The ZDIs for the Gram-negative bacteria *E. coli* ATCC 25922 and *S. enteric serovar*. Typhi ranged from 31 to 34 mm for both non-autoclaved and autoclaved kinds of honey and 18 to 22 mm and 17 to 20 mm, respectively, for non-autoclaved and autoclaved kinds of honey; for *Ps. aeruginosa*, the ZDIs ranged from 19 to 22 mm for non-autoclave (at 48 h incubation). After 48 hours of incubation, the ZDIs against *Staph. aureus* was 15–21 mm due to the activity of honey sample LNH2 [24].

All the tested 21 honey types from Mount Olympus, Greece exhibited antibacterial activity against *S. aureus* and *P. aeruginosa*. In any case, compared to the effects on *P. aeruginosa*, the antibacterial effects of the studied honey types (including Manuka honey) were stronger against *S. aureus*, as shown by bigger inhibition zones. The results of the *S. aureus* and *P. aeruginosa* well diffusion assay were analyzed using Spearman's correlation. This research showed that the antibacterial properties of honey against these two bacterial species were unrelated [26].

In 14 out of 18 samples tested against methicillin-resistant *S. aureus* (MRSA), 16 out of 18 samples tested against *P. aeruginosa*, 10 out of 18 samples tested against *S. typhimurium*, and 10 out of 18 samples

tested against *K. pneumoniae*, the MIC and MBC values of each sample were the same. These findings imply that chemicals in bee bread that harm bacterial cells permanently are probably responsible for the food's antibacterial properties. Bacteriostatic chemicals, however, cannot be completely ruled out. Compared to Gram-negative bacteria, 11 out of 18 samples showed reduced MIC values against *S. aureus*. This observation led to the lowest MIC value against *S. aureus* being recorded (sample 14). The sample with the lowest MIC and MBC against *S. aureus* (3.9 mg/mL), *P. aeruginosa* (15.6 mg/mL), and *S. Typhimurium* (7.8 mg/mL) among all the samples was number 14. In comparison to the other samples, Sample 4 had the lowest MIC and MBC values (9.9 mg/mL) for *K. pneumoniae*. It's interesting to note that certain samples showed lower MIC values for Gram-negative bacteria than for Gram-positive bacteria. In contrast to the equivalent findings against other bacteria (24 mg/mL against *P. aeruginosa*, and 12 mg/mL and 24 mg/mL *against S. typhimurium* and *K. pneumoniae* respectively), sample 3 revealed higher MIC and MBC values against *S. aureus* (48 mg/mL in both cases). Interestingly, sample 5 displayed identical MIC and MBC values (23.5 mg/mL) for all pathogens [27].

The palynological study is nothing but the study of pollen grains. The different shapes of pollen grains can be observed. In samples 4 (natural honey from a tree branch, Sanoor), 5 (reared honey Muggerkala), and 9 (reared honey, Sajipa, Bantwal) more number and different shapes of pollen grains are observed whereas, the sample 8 processed honey, Sampige, Moodbidri) and 2 (raw, unprocessed and domesticated honey from Butterfly Park, Beluvai) least number and only one type of pollen grain were observed. And none of the pollen grains are observed in samples 1 (Dadey brand honey from Karkala) and 3 (processed society honey from Butterfly Park, Beluvai). These results clearly show that the processed honey contains the least number of pollen grains and also the least type of pollen grains compared to the processed one (Table 6).

Table 6. Pollen grain shapes in different honey samples.

Samples	Shape of pollen grains observed
1	-
2	Irregular
3	-
4	Round, ovale, bean, boat, curved
5	Irregular, curved, boat, round, bean
6	Irregular, bean, round
7	Round, curve, boat
8	Irregular
9	Bean, irregular, round, boat
10	Round and bean

Didaras et al. (2021) [27] conducted a palynological study on 18 samples to see whether there is a relationship between the antioxidant, antibacterial properties, and botanical origin. For each sample, they estimated the pollen grain content (percent). We discovered that Sample 18 was monofloral (99.8% *Castanea sativa* from Mount Athos). Additionally, sample 13 might be regarded as monofloral (*Cistus* spp. 78%). In samples 5 (*Hedera helix* 52.4% and sample 11), dominating plant species/genera were found (*Borago* spp. 54.8%). Pollen grains primarily from the Brassicaceae family made up Sample 8.

CONCLUSION

In this study, 10 different honey samples from different places in Dakshina Kannada were analyzed for their antioxidant, antimicrobial activity, and palynological study. All the processed/commercial honey samples clearly showed antioxidant properties and also lesser pollen grains. Natural honey has a high amount of antioxidant properties compared to commercial honey. when the Brix content of the commercial and natural honey samples was analyzed, the commercial honey samples contain a high amount of Brix content compared to the natural honey samples. The phenolic profile of commercial honey also has been compared to natural honey samples. The protein content of the commercial honey is very low compared to the honey samples.

The local natural kinds of honey, citrus honey, and mango honey can be utilized/exploited as an excellent dietary source of antioxidants as well as antibacterial agents, and hence duly be active for both therapeutic and prophylactic application. The creation of broad-spectrum antibacterial medicines using such natural kinds of honey could help stop the spread of bacterial drug resistance. However, more research is necessary to determine the antibacterial and antioxidant effects of honey's bioactive components through chemical analysis.

CONFLICT OF INTEREST

None declared.

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