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Antimicrobial Efficacy and Chemical Profiling of Tribal and Commercial Honey Varieties: A Comparative Study of it's Therapeutic Potential

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ABSTRACT

Background: Honey, a natural product made by honeybees is composed of floral nectar and sweet deposits secreted by non-floral parts of the plant. Natural composition and secondary metabolites present in honey gives it a high therapeutic value because of which it is widely used in the field of allopathy and ayurvedic medicine. The world's oldest medical texts do indeed mention the medicinal value of honey and since ancient period, it has been understood to have antimicrobial and wound-healing properties.

Methods: Characterization and comparative bactericidal activity being the objective, the current study carried out involved bactericidal activity of tribal honey in comparison with processed honey against various bacteria typed by MTCC (Microbial type culture collection). The antagonistic effect of tribal and processed honey in comparison was tested against ten different bacterial strains, namely *Enterobacter aerogenes* (*E. aero.*), *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Bacillus subtilis* (*B. subtilis*), *Bacillus cereus* (*B. cereus*), *Pseudomonas aeruginosa* (*P. ae*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Shigella, Streptococcus* and *Proteus*. Honey samples were subjected to various physical tests such as pH, colour, moisture, turbidity and specific gravity, Chemical characteristics namely protein content and minerals such as sodium, potassium, calcium and phosphates were quantitatively analysed, qualitative assessed for vitamin C and secondary metabolites like alkaloids, terpenoids, flavonoids and phenols. Bactericidal activity was performed using agar well diffusion technique.

Result: The results obtained through the same were indicative that the unprocessed tribal honey showed maximum anti-bacterial activity in comparison with the other labels of processed honey. Tribal honey sample showed antagonistic effect against *Klebsiella pneumoniae, Streptococcus* and *Staphylococcus aureus* colonies whereas the label Dabur, one of the processed honey samples showed maximum effect on the growth of *Escherichia coli* (*E. coli*).

Key words: Agar well diffusion technique, Antagonistic effect, High therapeutic value, Honey, Secondary metabolites, Specific gravity.

INTRODUCTION

Honey is a naturally occurring sweet substance made by honeybees. It is known for its high nutritive value and benefits to health. Its usage as a food and medicinal product dates back to 7000BC. This complex mixture exhibits large variations in composition and characteristics. Its key characteristics are closely related to the source of flowers or the nectar that bees gather.

The major components of honey are directly dependent on the flower source, season, environmental factors and method of processing. It is composed of a range of nutrients. In honey, the major sugars present include, glucose and fructose as the monosaccharides. There are also 25 forms of oligosaccharides, such as sucrose, maltose, *etc*. (Bogdanov *et al*., 2016a). The total amount of fructose (38.5%), glucose (31%), maltose and sucrose (12%) in honey sums up to 81.5% of total carbohydrates (Khan *et al*., 2007).

Proteins and amino acids (proline, glutamic acid, aspartic acid, *etc*.), enzymes (invertase, oxidase, amylase), minerals (potassium, sodium, iron, copper, *etc*.), vitamins and organic acids, such as citric acid are also the primary constituents of honey (Hermosin *et al*., 2003; Jeffry *et al*., 1996; Alaqrni *et al*., 2014). The amino acid and protein content is quite less, *i.e*., about 0.7% in which content of proline, which is the main amino acid, is about 50-85%. The remaining amino acids include glycine, threonine, aspartic acid, histidine, tryptophan, lysine, ornithine, glutamic acid (Engin Gundogdu *et al*., 2019). Honey is also composed of tiny amounts of vitamins, of which the most composition is that of derivatives of vitamin B, including

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thiamine (B1), biotin (B8), riboflavin (B2), pantothenic acid (B5), nicotinic acid (B3), along with minor amounts of vitamin C (El-Bialee *et al*., 2011). Invertase, Amylase and Glucose oxidase are the enzymes which are manufactured in the glands of the worker honeybees called hypopharyngeal glands. Acid phosphatase and Catalase is extracted from plants (Engin Gundogdu *et al*., 2019).

The chemical characteristics affects viscosity, which in turn influences extraction methods, processing, preservation and packaging of honey (Tatjana pavlov *et al*., 2018). Since honey has a long shelf life, it can be kept for several years without being processed or given preservatives (Szweda, 2017). The honey colour ranges from light yellow or pale yellow to a dark reddish-brown colour (Engin Gundogdu *et al*., 2019). Suspended materials like pollen, propolis and bee wax is filtered out by straining the freshly harvested honey after it has been harvested for commercialisation. 5-hydroxy methylfurfural (HMF) is a parameter which determines how fresh the honey is. The concentration of this HMF will increase during processing or aging when compared to the fresh extracts of honey (Eshete and Eshete, 2019; Shapla *et al*., 2018).

Honey is also considered for various medicinal purposes. A number of studies conducted showed that being a natural reservoir of antioxidants, honey has the ability to reduce the risk of many diseases, such as heart diseases, cancer, different inflammatory diseases, *etc*. Components found in small amounts (flavonoids, phenolic acids, carotenoids, *etc*.) and the resultant substances of Maillard reaction exhibits many biological features, such as action against microbes and the ability to fasten the process of healing of injuries. The antagonistic effect of honey on microbes is associated with its high sugar concentration, low pH, as well as the action of phytochemical antibacterial compounds present in honey (Bucekova *et al*., 2019; Bogdanov *et al*., 2008). Since honey has a water activity of about 0.6 and a greater osmotic environment, which inhibits microbial growth, honey is thought to be self-stabilizing for extended periods of time (Raziuddin *et al*., 2021). The sugar molecules in honey use up the water molecules, hence there will be no water molecules available to assist the growth of microorganisms like bacteria, which die due to dehydration. The water content mainly depending upon many factors such as humidity throughtout the hive, weather (Malika *et al*., 2004). The natural acidic nature (pH ranges between 3.2-4.5) will inhibit the growth of many pathogens (Varlová *et al*., 2005). Hydrogen peroxide (H_2O_2) is the product of glucose oxidation by glucose oxidase which is introduced when nectar is being harvested. As H_2O_2 decomposes, it results in the formation of highly reactive free ions which reacts and kills bacteria (Manyi-Loh *et al*., 2011). The measured turbidity of honey from turbidimeter which is an indicator of honey granulation (Paula A. Conforti *et al*., 2006; White *et al*., 1963).

$$\text{Glucose} + H_2O + O_2 \rightarrow \text{Gluconic acid} + H_2O_2$$

(Manyi-loh *et al*., 2011)

The geographic region where the honeybees are raised, nectar of the flower, climatic conditions, *etc*., affect the anti-bacterial strength of the honey (Abd-El Aal *et al*., 2007). Defensin-1 (Def-1) is one of the main components for anti-bacterial activity generally against gram-positive bacteria (Kwakman *et al*., 2011; Tseng *et al*., 2011). Using high performance liquid chromatography (HPLC), several chemical compounds with anti-bacterial action have been found in the ether extract of honey (3,5-dimethoxy-4-hydroxybenzoic acid and methyl-3,5-dimethoxy-4-hydroxyl benzoate) (Selvamohan *et al*., 2016). Cerumen (made of wax) which is sectreted by the glands present in the abdomen region of worker bee and propolis which is derived from the plant's resin used in order to seal the extra space between the nest and cavity. This propolis carries antifungal, antibacterial and antiviral properties (depending upon the resin content of the plants) (Temaru *et al*., 2007).

The current study is done to gauge the bactericidal properties of honey against various bacterial strains, *i.e*., *Enterobacter aerogenes* (*E. aero*.), *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Bacillus subtilis* (*B. subtilis*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Bacillus cereus* (*B. cereus*), *Streptococcus, Pseudomonas aeruginosa* (*P. ae*), *Shigella* and *Proteus*. The above-mentioned strains are typed under microbial type culture collection, research institute in Chandigarh (MTCC). Along with the test for confirmation of anti-bacterial activity, the test for some anti-bacterial compounds and health promoting nutrients was also carried out.

MATERIALS AND METHODS

Collection of honey

To perform this experiment three samples of processed honey from renowned honey brands, namely Dabur(P1), Saffola(P2), Lion(P3) along with one unprocessed honey, our primary test sample were extracted and the origin, source and location of the test sample was recorded. The sample was taken from different botanical sources in northern Karnataka. A tribal group from Haryana who procure honey directly from the bee hives provided this sample, which is also used to feed bears at Bannerghatta bear rescue centre (BBRC) as a refreshment. The experiment was conducted at the microbiology laboratory of Jain (deemed-to-be) University during December 2021. The honey sample was stored in Eppendorf vials at room temperature in the dark.

Tests for biochemical characterization and bactericidal activity

The biochemical tests were conducted with known techniques and the results were recorded. The antimicrobial activity was tested using the agar-well diffusion technique. Agar-well diffusion method-Following the solidification of the media, 10 ml of pure bacterial culture from test tubes were inoculated onto the solidified nutrient agar media using the spread plate technique (Fig 1, Fig 2, Fig 3, Fig 4, Fig 5).

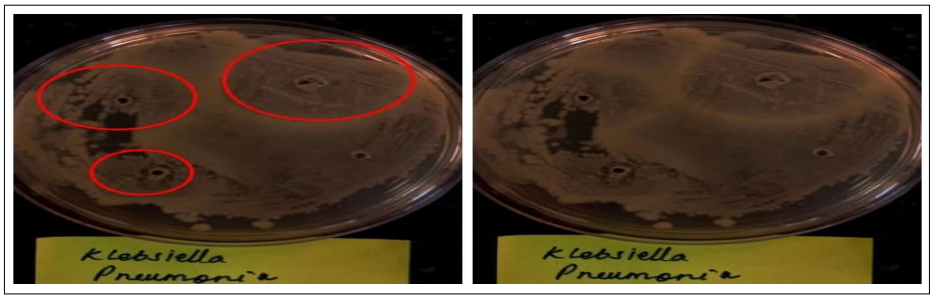

Fig 1: Zone of inhibition *Klebsiella pneumoniae* culture plates.

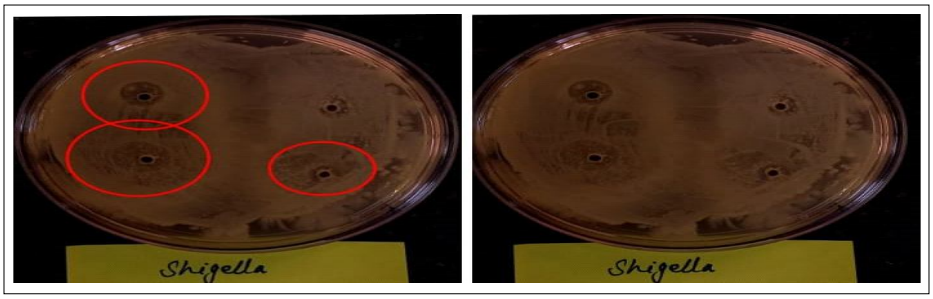

Fig 2: Zone of inhibition *Shigella* culture plates.

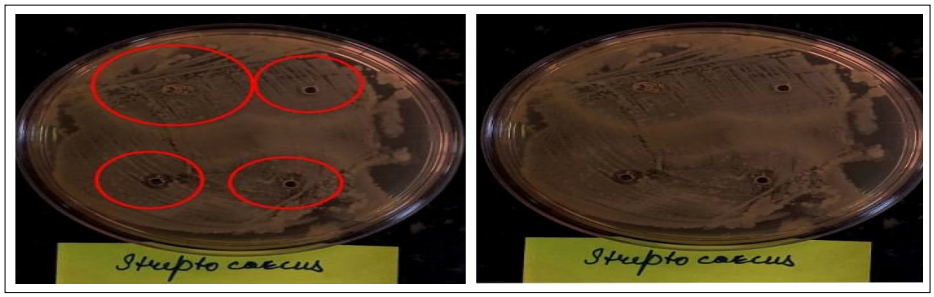

Fig 3: Zone of inhibition *Streptococcus* culture plates.

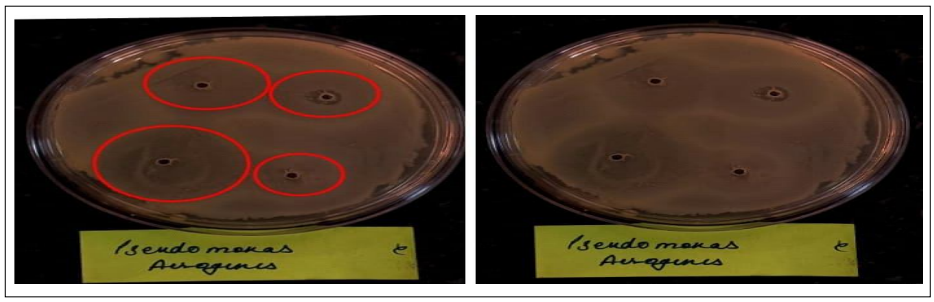

Fig 4: Zone of inhibition in *Pseudomonas aeruginosa* culture plates.

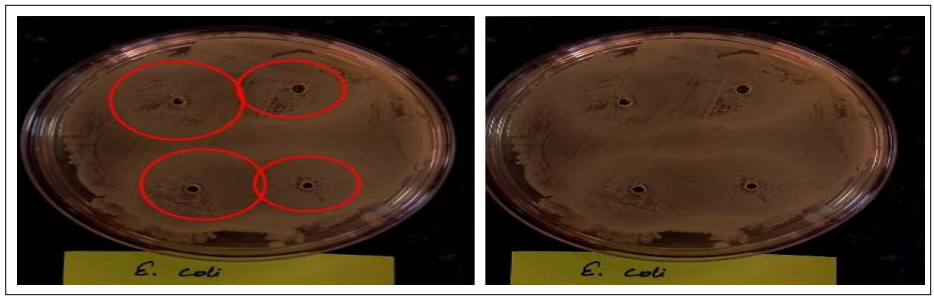

Fig 5: Zone of inhibition in *Escherichia coli* culture plates.

The plates were then incubated for 24 hours. Following a 24-hour incubation period, honey samples were added to the wells that had been made using sterile gel puncture. The plates were then incubated again in the incubator for 24 hours at 37°C. The zone of inhibition/clearance developed around each well following incubation was observed, photographed and the zone's diameter was measured. (Table 4).

RESULTS AND DISCUSSION

Composition of honey

Anti-bacterial effect of honey

Moisture content

The FSSAI prescribed limit for moisture content of the honey is 20% (20 g/100 g). The analysis of the test samples presented that all the honey samples lie within the range of permissible limits except for sample P1. The sample's content was found to range between 11.2% and 25.7% (Table 1), with sample P1 having the highest of 25.7, slightly exceeding the limits.

Protein content

The protein content of the tribal sample (96mg/10g) was comparable to two of the processed samples, Saffola (100 mg/10 g) and Lion (125 mg/10 g). Whereas Dabur (12 mg/10 g) sample showed significantly low amount of protein. The proximate analysis of the four different samples of the processed and unprocessed honey showed that the specific gravity ranged between 1.36-1.38 mg/10 g. The protein content ranged between 12-125 mg/10 g.

Total fat

The fat content in all the samples was found to be nil which is comparable to the standard range 0 g/100 g of the honey. Honey is mainly just sugar; it has almost no or very minute amount of fat, with little amount of fibre and protein. There were no significant differences (P>0.05) in the value obtained for specific gravity of the processed and unprocessed honey samples.

Calcium and potassium contents

A similar observation as protein was made in the calcium and potassium contents of the samples. The levels of calcium and potassium were significantly different in P1 sample as compared to other samples. The calcium content of the P1 sample was lower than the others. The value lied in the range of 12-23 mg/10 g. Whereas, the potassium levels of P1 sample was much higher than the others. The range was observed to be 10-65 mg/10 g. (tribal sample having the lowest of 10 mg).

From the results obtained by this study, the levels of potassium found in tribal honey samples was less compared to the other processed honey samples. As nutritionists mainly recommend the addition of honey to the low-potassium diet of a patient, the main reason behind this is because the potassium content of good-quality honey is low. This diet is mainly recommended for patients who are on haemodialysis. From this study, it was observed that potassium levels for other processed samples of honey were higher, even reaching up to 65. But the value for the tribal honey samples remained consistent at 10. This shows that the use of tribal honey in a low-potassium diet is much more effective rather than using the other commercial samples of honey (Brancati FL *et al*., 1996; Choi *et al*., 2013).

Sodium and phosphate content

Sodium content lies in the range of 3-40 mg/10 g, P3 having the least of 3 mg. The phosphate levels lied in the range of 0.02-0.18 mg/10 g (Table 1).

Colour analysis

The colour of the honey depends on various parameters which includes geographical origin, floral source, method of extraction, *etc*. Generally, the colour of the honey can range from being yellowish to reddish or greenish. The colour of the test sample was observed to be yellowish brown (tribal sample) to darker shades of brown (processed samples). The colour might also depend on the temperature, time of storage and the presence of antioxidant pigments like flavonoids and carotenoids (Kaur *et al*., 2016).

Vitamin C and total phenols

Table 2 represents the tests for phenols, terpenoids, flavonoids, tannins, alkaloids and vitamin C. The results showed the presence of vitamin C and all the polyphenols in

Table 1: Physical and chemical parameters.

Analysis	Dabur	Saffola	Lion	Tribal
Colour	Dark brown	Dark brown	Light brown	Yellowish brown
Specific gravity	1.38	1.36	1.39	1.36
Fat (mg)	0	0	0	0
Protein (mg)	12	100	125	96
Calcium (mg)	12	21	17	23
Potassium (mg)	65	42	32	10
Phosphate (mg)	0.06	0.18	0.02	0.10
Sodium (mg)	20	03	40	12
Moisture (%)	14.94	25.71	11.27	13.80
Turbidity (NTU)	045	051	048	062

Table 2: Secondary metabolites.

1	Terpenoids	+	+	+	+
2	Flavonoids	+	+	+	+
3	Phenol	+	+	+	+
4	Tannins	+	+	+	+
5	Alkaloids	+	+	+	+
6	Vitamin c	+	+	+	+

Table 3: Zone of clearance.

	Tribal (cm)	Dabur (cm)	Saffola (cm)	Lion (cm)
Bacillus subtilis	1.5	1.2	1	1
E. coli	0	1.2	0	0
Streptococcus	1	1.1	0	0
Staphylococcus aureus	0.9	1.9	1	1
Shigella	0.5	1	0.4	1
Klebsiella pneumoniae	1.5	1	0.5	0

Table 4: Zone of clearance.

	Tribal (cm)	Dabur (cm)	Saffola (cm)	Lion (cm)
Enterobacter aerogenes	Nil	Nil	Nil	Nil
Bacillus cereus	Nil	Nil	Nil	Nil
Pseudomonas aeruginosa	Nil	Nil	Nil	Nil
Proteus	Nil	Nil	Nil	Nil
Streptococcus	Nil	Nil	Nil	Nil

the test samples. The antioxidant content in honey is made up of the existing polyphenols and flavonoids. The antioxidant content confers many health benefits to the honey. It keeps in check the ROS reactive oxygen species in the cells, whose accumulation may cause damage. The accumulation of this may also lead to premature aging, type 2 diabetes and heart diseases (Poljsak *et al*., 2013) (Table 2).

Antibacterial activity of the honey samples

The primary screening with the agar-well diffusion assay disclosed the anti-bacterial activity against *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Bacillus subtilis* (*B. subtilis*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Shigella* and *Streptococcus* (Table 3). The antagonistic effect against other strains was not prominent since it did not show distinct zones. Considerable anti-bacterial activity of the tribal honey sample was seen against *Bacillus subtilis*(1.5cm) and *Klebsiella pneumoniae* (1.5 cm) which was marked by eminent zones of inhibition in the agar-well diffusion assay. Whereas, sample P1 was found to be more prominent against other strains of the test bacteria (Table 4).

From the study made on the antibacterial properties of honey, it was found that the tribal samples were the only honey samples that showed effective anti-bacterial activity against two bacterial species, *i.e*., *Bacillus subtilis* and *Klebsiella pneumoniae*. The other honey samples showed anti-bacterial activity against either one of the bacterial species or none. *Bacillus subtilis* is mainly found on the skin and in the intestinal tracts of human beings. Along with certain enzymes, it produces subtilisin, which is a toxin that causes irritation in the lungs. This toxin also causes skin allergies and continuous exposure can also lead to bronchitis (You *et al*., 1996). Hence, the use of the tribal honey sample can effectively produce an anti-bacterial activity against *Bacillus subtilis* and prevent the production of subtilisin. *Klebsiella pneumoniae* is one among the most significant pathogenic bacteria that causes urinary tract infections on entering urinary tract. This bacterium is mainly found in hospital settings and can spread efficiently from the site of wound infection to other parts of the body, even causing septicaemia. *K. pneumoniae* which is foodborne has shown resistance to certain antibiotics, making them more dangerous (Wyres *et al*., 2020). This study has shown that tribal honey samples are capable of producing anti-bacterial activity against *K. pneumonia*e indicating that the consumption of this honey is suitable for fighting off and preventing infections by *K. pneumoniae.*

CONCLUSION

Since ancient times, honey, made by bees from flower nectar, has been prized for its therapeutic and antimicrobial properties. It is well known for its broad-spectrum bactericidal activity. Numerous elements, such as the presence of hydrogen peroxide, the concentration of phenolic and flavonoid acids, the low pH and the osmolarity, all contribute to the antibacterial activity. These elements have

bacteriostatic or bactericidal effects on a variety of bacteria. The ongoing findings on honey as a potent antibacterial agent and their efficacy to reduce the possible emerging MDR (multidrug resistance) in bacteria is very promising.

The antimicrobial potency of the honey can be attributed to the complex composition of the honey including a vast variety of components such as amino acids, vitamins, organic acids, minerals, enzymes, flavonoids and many more. Though the concentration of each of these may differ according to the source, storage conditions, storage duration and the climatic conditions prevailing at that time, their application as a potent antibacterial agent is a product of the effects of either these individual components or a combination of these in right concentrations. Another factor adding to its antimicrobial activity is the high affinity of the molecules towards water, leaving no water for the growth of bacteria.

A survey of these underlying factors can influence the development of a potent medical grade honey which is effective against broad-spectrum of MDR bacteria. Hence the need for precise composition of a natural honey which can be manipulated in laboratories to combat a wide range of bacterial infections is the motto, of the project.

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Conflict of interest

All authors declared that there is no conflict of interest.

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