Anti-Oxidative and Antagonistic Activity of Honey Samples Collected from Different Geographical Areas of Tamilnadu

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Abstract: Honey has abundant salutary properties which depend mainly on the flower- sources. The study was intended to personify the antioxidative and antibacterial properties of seven types of honey samples collected from different areas of southern India. The total phenolic content was determined by the modified Folin - Ciocalteu method, the total protein content by Lowry’s method and proline content. The antioxidant property of the honey samples was assessed by 2, 2-diphenyl-1-picryl-hydrazy (DPPH) for antiradical activity and Ferric reducing/ antioxidant power (FRAP) assay. The antimicrobial activity was studied by the agar disc diffusion method using four bacterial strains. Among the seven honey samples analyzed, sample SBH1 showed the highest antioxidative property and antimicrobial activity against Eschiershea coli at 75% dilution. Hence the honey sample SBH1 was subjected to LC/MS (Liquid chromatography Mass Spectroscopy) analysis to determine its composition and the analysis revealed that it contained six flavonoids and two alkaloids. The above obtained results of the honey samples studied proved to be a good source of antioxidative and antimicrobial activity, thus it would enhance the human health.

Key words: Honey samples • Antioxidative activity • Antimicrobial activity and flavonoids

INTRODUCTION

Honey is an imperative, primary product of beekeeping that is quantitative and a profitable product. Honey is a natural substance formed by honeybees as a food source from the nectar of blossoms and from the secretions of the living parts of the plants. The chemical composition of honey is quite complex; it contains about 181 substances with sugars, proteins, moisture, vitamins, minerals, enzymes, flavonoids, phenolic acids, compounds, etc.

From ancient days honey has been used in the treatment and prevention of many diseases [1-3]. It has also revealed that honey has been served as natural antioxidants, which has reduced the risks of heart disease cancer, decline in the immune system etc [4]. The chemical composition of the honey mainly depends upon the flowers foraged by the honeybees, also the environmental, processing and the storage conditions [5, 6]. Traditionally honey has been used for different purposes and it has been served as a very good natural food antioxidant [7]. Honey contains various enzymes which serve as good antioxidants by promoting the removal of the oxygen [8]. Consuming of the honey has been effective in increasing the total plasma antioxidant and reducing capacity in humans [9]. Among the five honey sample collected from different parts of India. Honey sample AC3 showed the good antioxidative property and antimicrobial agent [10].

The healing capacity of the honey is mainly because of its physical property of osmosis and also the antibacterial properties of the hydrogen peroxide [11]. According to the literature hydrogen peroxide is the antibacterial substance present in honey [12]. The phenolics present in honey are mainly from the nectar as glycosides which are hydrolysed and transferred to the honey [13]. There are also various other acids and enzymes that contribute to the antibacterial capacity of
the honey, but they are all small compared to that of the hydrogen peroxide [14]. The antibacterial activity of honey has been collectively researched methodically and subjugated medicinally for the therapeutic consequence of honey, which is due to the substantial property of osmosis and the antibacterial properties of hydrogen peroxide [13].

Although various studies on the antioxidative and antimicrobial activity of Indian honey samples have been done, but there is a lack of information on the honey samples collected from Tamilnadu. Therefore the main objective of this study was to identify the anti-oxidative, antiradical, total phenolic content and antagonistic activity of honeys collected from different geographical areas of Tamilnadu.

MATERIALS AND METHODS

Chemicals Used: The chemicals used for the present study were ascorbic acid, Folin-Ciocalteu phenol reagent, gallic acid, bovine serum albumin (0-100µg/ml), Sodium carbonate (0.2g/ml), potassium ferric cyanide, trichloroacetic acid (TCA), Ferric chloride, DPPH solution and Kanamycin.

Honey Samples Collected from Tamilnadu: For the present study, seven honey samples were procured from diverse ecological localities of Tamilnadu. These samples were collected directly from the apiary as well as from conventional honey seekers and it was packed, sealed in the containers and stored at 4°C. These seven samples were examined in order to assess its total phenolic, antiradical, antioxidative and antibacterial effects.

Biochemical Analysis of Honey Samples

Total Protein Content: The total protein content was determined by Lowry’s method for protein estimation which is based on the formation of a copper protein complex [15]. The absorbance was measured at 660 nm. Bovine serum albumin was used as the standard for preparing the calibration curve. For all the honey samples three trials were done and the mean values were calculated and expressed in mg/Lt.

Proline Content: Proline content of the honey samples was determined as per the modified methodology [16]. 0.5 ml of solution of every honey sample (0.05 g/ml) was taken and 1 ml of formic acid (80%) and 1 ml of Ninhydrin solution (0.2 g in 100 ml of rectified spirit) were added into the test tubes and incubated at 70°C on a water bath for 15 minutes. 5 ml solution of 50% 2-propanol was used as supplements and the mixture was left to cool at room temperature. After 45 minutes the absorbance was read at 510 nm. 0.032 mg/ml solution of proline was taken as a standard and the proline content was repeated for three trials and the mean value was calculated and expressed in mg/Kg.

Total Phenolic Content: The total phenolic content was determined by the Folin-Ciocalteu method [17]. The absorbance was measured at 765nm using a spectrophotometer. Gallic acid was used as standard to produce the calibration curve. Each sample was repeated for three trials and the mean was expressed in milligram Gallic acid equivalent (mg GAE) /100g of honey.

Analysis of the Antioxidative Property of Honey Samples

DPPH Radical Scavenging Activity: The antiradical scavenging activity of honey was determined by the DPPH (2, 2-diphenyl-1-picrylhydrazyl) [18]. Honey sample was dissolved in ethanol and mixed with a fixed volume of DPPH solution in ethanol, the mixtures were left for 15 minutes at room temperature and then the absorbance was measured at 517nm. Blank sample contained ethanol. The antiradical scavenging activity was calculated using the formula.

\[ \text{Inhibition\%} = \left[1-\frac{(T1-B2)}{B1}\right] \times 100 \]

where, T1, B1 and B2 are the observance of the samples, sample blank and DPPH blank, respectively. The mean values of the three trials were calculated and the values were expressed in mg/ml.

Ferric Reducing/Antioxidant Power (FRAP) Assay: The reducing power of the honey samples was determined according to the methodology [19]. The absorbance was measured at 700nm. Ascorbic acid (0-125mg/ml) was used as a reference standard. The mean value of the triplicates was calculated and the value was expressed in mg/ml.

Antibacterial Efficacy of Honey Samples: The antibacterial efficacy of different concentrations of the honey samples (25%, 50%, 75% and 100%) was studied against four microorganisms of which one gram-positive bacteria (Staphylococcus aureus) and three gram-negative bacteria (Escherichia coli, Pseudomonas aeruginosa and Bacillus subtilis). The nutrient agar was prepared and poured in sterile Petri plates, after solidification, one milliliter of bacterial culture was spread
on Petri plates. Then five wells were cut with the help of sterile cork borer in the inoculated agar and then the wells were filled with honey sample. The Petri plates were incubated for 24 hours at room temperature. At the end of the incubation period the zones of inhibition on the medium and measured in millimeter (mm).

**Liquid Chromatography/Mass Spectrometry (LC/MS) analysis of honey sample SBH1:** The LC/MS analysis of the honey sample SBH1 was performed using a 150 mm - 3.9 mm i.d. 5-im XTerra RP18 instrument (Waters, MA) with diode array detection (DAD) at 285 and 340 nm. Major peaks were further isolated and their identities were confirmed by LC/MS. Positive-ion-mode electro spray ionization (ESI)-MS spectra were recorded on a Waters Alliance 2690 LC/MS system (Waters, MA) using the following conditions: source temperature 150°C, desolvation temperature 275 °C, capillary voltage 3.53 eV and cone voltage 16 eV. Spectra were scanned over a mass range of m/z 120-650. Chromatographic separation was done on a 150 mm - 2.1 mm i.d, 5µm Discovery C18 (Supelco, PA) column with UV detection set at 285nm and 340 nm.

**Statistical Analysis:** The result was investigated statistically, the entire assay was carried out in triplicate and the data was represented as mean and standard deviations. One way analysis of Variance (ANOVA) followed by least difference (LSD) was compared with the data. Differences between means at the 95% (p ≤ 0.05) level were considered statistically significant.

**RESULTS**

**Biochemical Analysis of Honey Samples**

**Total Phenolic Content:** The total phenolic content of the seven honey samples was in the range of 0.62 (mg/Lt) in AH1 to 1.13 (mg/Lt) in SBH1 (Table 1 and graph 1). The Gallic acid standard calibration curve was obtained by plotting different concentrations of Gallic acid against observance that was read at 765 NM. For the honey samples collected from India were 1.1 mg/Lt [10]. The polyphenolic content present in honey mainly depends upon the floral sources in honey [20].

**Protein and Proline Content:** For the present study the total protein content of the honey present in the honey samples ranged from 3881 to 4655 µg/ml (Table 1 and Graph 3), which was determined by using BSA (Bovine Serum Albumin) as standard and it was relatively high in sample AH5. This result was compared with the Algerian honeys which were relatively higher, ranging from 3700 to 9400 lg/g [21]. The normal range of protein content in honey is less than 5 mg/g [22]. The protein content mainly depends on the presence of the enzymes, which may be introduced by the honeybees themselves and sometimes it may be derived from the nectar of the flora and thus it is variable. The predominant amino acid present in honey is proline and the content of proline ranged from 2.17 to 6.52 mg/Kg.

**Analysis of the Antioxidative Property of Honey Samples**

**DPPH Radical Scavenging Activity:** The free radical scavenging activity of various samples is done by the DPPH which is a stable nitrogen centered radical. According to the literature higher the DPPH scavenging activity, the higher is the antioxidant capacity of the sample. The DPPH scavenging activity percentage out of the seven honey samples ranged from 62 to 191 and its percentage was higher in the honey sample SBH1 (Table 1 and Graph 2). The scavenging activity of the seven honey samples was greater than 50%. The DPPH scavenging activity percentage of the Indian honeys were from 44 to 71 [7].

![Graph 1: Total Phenolic content and FRAP assay of the honey samples](image-url)
Table 1: Antioxidative and biochemical properties of honey samples

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Sample no</th>
<th>TPC (mg/lt)</th>
<th>FRAP (mg/ml)</th>
<th>DPPH (g/ml)</th>
<th>Protein (µg/ml)</th>
<th>Proline (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AH1</td>
<td>0.62±0.02</td>
<td>1.80±0.10</td>
<td>62±0.17</td>
<td>4357±2.59</td>
<td>3.26</td>
</tr>
<tr>
<td>2</td>
<td>AH2</td>
<td>0.82±0.02</td>
<td>2.41±0.11</td>
<td>82±0.32</td>
<td>4486±5.19</td>
<td>2.61</td>
</tr>
<tr>
<td>3</td>
<td>AH3</td>
<td>0.91±0.01</td>
<td>2.65±0.08</td>
<td>91±0.31</td>
<td>4633±2.81</td>
<td>2.17</td>
</tr>
<tr>
<td>4</td>
<td>AH5</td>
<td>0.91±0.01</td>
<td>2.10±0.09</td>
<td>84±0.34</td>
<td>4655±5.07</td>
<td>2.61</td>
</tr>
<tr>
<td>5</td>
<td>SBH1</td>
<td>1.13±0.03</td>
<td>2.81±0.12</td>
<td>191±0.41</td>
<td>4649±4.81</td>
<td>4.35</td>
</tr>
<tr>
<td>6</td>
<td>SBH2</td>
<td>1.02±0.02</td>
<td>2.56±0.09</td>
<td>113±0.34</td>
<td>3881±1.39</td>
<td>5.40</td>
</tr>
<tr>
<td>7</td>
<td>SBH3</td>
<td>1.10±0.04</td>
<td>2.48±0.12</td>
<td>110±0.34</td>
<td>4458±0.57</td>
<td>6.52</td>
</tr>
</tbody>
</table>

Ferric Reducing/Antioxidant Power (FRAP) Assay:
The Ferric reducing antioxidant power (FRAP) assay of the five honey samples ranged from 1.80 to 2.81 mg/ml (Table 1 and Graph 1). Honey samples that showed higher reducing power had a higher absorbance value at 700nm. If the absorbance value is higher than then there is more reduction of ferric ions to ferrous ions. In Turkey honeys also the same results were observed [20]. In Indian honey samples the absorbance values of FRAP assay varied from 0.38 to 0.59 [7].

Antibacterial Activity of Honey Samples: Honey sample SBH1 showed a good antibacterial activity, SBH1 was effective against bacterial strains (Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Bacillus subtilis). Bacillus subtilis and Pseudomonas aeruginosa, showed relatively high inhibitory zones in 100% dilution. Staphylococcus aureus in the inhibitory zones were also higher 100% dilution. Escherichia coli showed the highest zone of inhibition at 75% dilution compared to other species. The activity seen with dilute solutions of honey at different concentrations clearly indicates that there is a high sugar content of honey involved in its antibacterial action. This supplementary antibacterial activity is due to hydrogen peroxide formed by enzymatic activity in the honey. From the above results it is evident that that honey sample SBH1 showed a good sign of antibacterial effect in Escherichia coli (Table 2 and Graph 4).

LC MS Analysis of the Honey Sample SBH1: The LC MS analysis of the specific honey sample was done and the composition of honey was found to be as follows
Table 3: LC-MS Compound details (Major MS Values)

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Name</th>
<th>Formula</th>
<th>Exact mass</th>
<th>Values obtained</th>
<th>Flavonoid/Alkaloid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2H,6H-Benz[1,2-b:5,4-b']dipyran-6-one, 7-[4-(acetyloxy)phenyl]-5-hydroxy-2,2-dimethyl-</td>
<td>C_{22}H_{19}O_{5}</td>
<td>378.4</td>
<td>377.4</td>
<td>Flavonoid</td>
</tr>
<tr>
<td>2</td>
<td>(S)-2,3,9,10-Tetrahydro-5-hydroxy-2-(4-hydroxyphenyl)-8,8-dimethyl-4H,8H-Benz[1,2-b:3,4-b']dipyran-4-one</td>
<td>C_{22}H_{19}O_{5}</td>
<td>340.4</td>
<td>339.6</td>
<td>Flavonoid</td>
</tr>
<tr>
<td>3</td>
<td>8-Demethyleucalyptin</td>
<td>C_{18}H_{17}O_{5}</td>
<td>312.3</td>
<td>311.4</td>
<td>Flavonoid</td>
</tr>
<tr>
<td>4</td>
<td>Amentoflavone</td>
<td>C_{18}H_{17}O_{5}</td>
<td>538.5</td>
<td>537.6</td>
<td>Flavonoid</td>
</tr>
<tr>
<td>5</td>
<td>Eucalyptin</td>
<td>C_{18}H_{17}O_{5}</td>
<td>326.4</td>
<td>325.5</td>
<td>Flavonoid</td>
</tr>
<tr>
<td>6</td>
<td>Naringenin triacetate</td>
<td>C_{18}H_{17}O_{5}</td>
<td>398.4</td>
<td>397.7</td>
<td>Flavonoid</td>
</tr>
<tr>
<td>7</td>
<td>11-Methoxyuncarine C</td>
<td>C_{18}H_{17}O_{5}</td>
<td>398.5</td>
<td>397.7</td>
<td>Alkaloid</td>
</tr>
<tr>
<td>8</td>
<td>Humantenmine</td>
<td>C_{18}H_{17}O_{5}</td>
<td>326.4</td>
<td>325.5</td>
<td>Alkaloid</td>
</tr>
</tbody>
</table>

Graph 5: The peaks obtained from the LC/MS analysis of the honey sample SBH1.

Discussion

In this study, the total phenolic content (mg of GAE/100gm of honey) varied from 0.62 to 1.13mg GAE/kg using Gallic acid as standard calibration curve (R^2 = 0.9988). It was high in the sample SBH1. The total phenolic content of Australian Unifloral honey samples ranged from 14 to 195.96 mg GAE/kg [23]. Honeydew honeys had higher levels of phenolic content compared to that of the other honey sample [24]. Honeydew honey contains high total polyphenol content and honey is a rich source of antioxidants [25]. The phenolic content of Algerian and Slovenian honeys ranged from 64 to 1304 and 448 to 2414 mg GAE/100 g, respectively [5, 21]. The total phenolic content of Indian honeys was found in the range of 47 to 98, which was determined using Gallic acid as standard (R^2 = 0.9988) [7]. The antiradical scavenging activity determined by DPPH was higher in the honey sample SBH1, higher the DPPH scavenging activity higher is the antioxidant capacity. The Ferric reducing antioxidant power (FRAP) of the honey sample SBH1 was high i.e., 2.81mg/ml. The total protein content and proline content was also in the honey sample SBH1.

From the above seven honey samples, SBH1 was chosen to find out the antimicrobial efficacy against four bacterial strains (Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Bacillus subtilis). The antimicrobial efficacy of the sample SBH1 was recorded high against Escherichia coli. Hence it is
evident that the honey sample SBH1 had higher levels of phenolic, antiradical, Ferric reducing antioxidant power, protein and proline content.

Further the honey sample SBH1 was subjected to LC/MS analysis and the major peaks of the mass spectrometry revealed that the composition of the sample had six flavonoids and two alkaloids. These are the contents that have made the sample SBH1 a good source for antioxidative and hence it can act as an effective antimicrobial agent, effective wound healing property and also food preservative which is rich in nutrition and would enhance the human health.

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