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# Study on the physicochemical parameters, phenolic profile and antioxidant properties of Indian honey samples from extrafloral sources and multi floral sources

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**Abstract**

Physicochemical characteristics, antioxidant activities and phenolic profile of honey obtained from extrafloral nectar (EFH) and the honey obtained from a mixed floral source (wild honey) were compared in this study. The antioxidant capacity of honey samples was examined using *in vitro* assays. The honey samples vary widely in their physicochemical as well as antioxidant properties. EFH samples had Total Phenolic Content (TPC) ranging from 207.4 ± 12.6 mg gallic acid/kg to 230.3 ± 22 mg gallic acid/kg but did not show potent DPPH radical scavenging activity. One of the EFH samples has shown 50% inhibition at the tested concentration (100 mg/100 µL) for hydroxyl radical scavenging activity. HPLC chromatograms showed the presence of gallic acid and chlorogenic acid in the wild and EFH sample. The samples used for HPLC profiling had higher TPC content (680.95 ± 4.47 mg gallic acid/kg for wild honey and 568.73 ± 3.61 mg gallic acid/kg for EFH) than the 24 samples studied.



## Introduction

Honey, the golden yellow liquid processed by the honey bees is valued from ancient time for its medicinal properties. The role of honey as a preservative and as a natural sweetener is well appreciated. In the recent years, there has been an increasing interest in the determination of the antioxidant activity of honey. The antioxidants in honey include both enzymatic and non-enzymatic substances. There are also reports on more than 200 phenolic compounds including flavonoids, flavanols, phenolic acids, catechins and cinnamic acid derivatives (Gheldof *et al*., 2002; Ferreira *et al*., 2009).

Researchers have also reported that honey with high levels of the phenolic antioxidants increases antioxidant activity of the plasma (Schramm *et al*., 2003). Studies indicated that the antioxidant activity of honey varies widely, depending on the floral source. The botanical origin of honey influences its antioxidant activity, while processing, handling, and storage affects honey antioxidant activity only to a minor degree. Several studies have shown that antioxidant activity is strongly correlated with the content of total phenolics (Al-Mamary *et al.*, 2002; Gheldof *et al*., 2002; Gheldof and Engeseth, 2002; Aljadi and Kamaruddin, 2004; Beretta *et al*., 2005; Blasa *et al*., 2006). Beside this, a high correlation was found between antioxidant activity and the color of honey. Many researchers found that honey with dark color have a higher TPC and consequently a higher antioxidant capacity (Beretta *et al*., 2005; Blasa *et al*., 2006). Extrafloral nectaries of rubber tree (Hevea brasiliensis) are a prolific source of honey and the nectar flow period is between January and March. In some regions in India, there is a practice of keeping the Newton hives in rubber plantations aiming at exploiting the extrafloral nectar formed at the axil of new leaves. As the extrafloral nectar is abundantly available from January to March, the honey in circulation throughout the year mainly comes from this source. Apis cerana indica is an indigenous variety and is the most common species domesticated in the rubber plantation that yields around 19 kg/ hive/year.

As studies clearly indicate the variation in honey quality with varying floral source, this study was undertaken to compare the physicochemical characteristics, antioxidant activities and phenolic profile of honey obtained from extrafloral nectar and the honey obtained from the mixed floral source.

## Materials and Methods

### *Samples*

The present study was carried out using four EFH

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samples (collected from Newton hives placed in rubber plantation) and 20 commercial samples purchased from the local market, Thiruvananthapuram, India. Samples are numbered from 1 to 24 of which 14, 15, 17, 18 are EFH samples. The tests were performed within two months following collection. The phenolic profiling of two samples of honey: 1 wild honey and 1 EFH were also performed using HPLC -DAD.

*Chemicals*

Folin–Ciocalteu's phenol reagent, gallic acid, chlorogenic acid and 1,1-diphenyl-2-picrylhydrazyl (DPPH), were purchased from Sigma Chemicals (St. Louis, MO, USA). All other chemicals were purchased from Ranbaxy (New Delhi, India).

*Physicochemical analysis*

Absorbance of honey samples were measured at 660 nm using a Shimadzu model 2450 UV-VIS spectrophotometer by loading into a 1 cm path-length cuvette. The color of the honey was determined by using Tintometer (Lovibond PFX995) in the visible range. The color was measured as Y+5R. Ash, free acidity and moisture were measured according to the AOAC method (AOAC, 1995). The pH of honey samples was assessed in a 10% (w/v) solution of honey in distilled water by a digital mode pH meter. Specific rotation of clear filtered aqueous solution was determined by using polarimeter (Rudolph Research Analytical, USA). Specific rotation was calculated from angular rotation, ray circuit length and grams of sample taken. The temperature of the instrument was 31.7°C and measurement was carried out at 589 nm. Specific gravity of honey samples were determined by recording the weights of empty specific gravity bottle, bottle with distilled water and bottle with honey separately and was calculated using the formula.

Specific gravity at 27°C = (C-A)/(B-A), C = Mass of the bottle with the honey sample

A = Mass of the empty bottle, B = Mass of the bottle with water

*Screening of antioxidant activity--Total phenolic content (TPC)*

The TPC in honey was determined by a modification of the Folin-Ciocalteu method and the results expressed as mg gallic acid/kg honey (Vinson *et al*., 2001). Samples were treated with warm distilled water (500 mg/5 mL water) and sonicated for 5 min. Then 100 µL of the solution, corresponding to 10 mg of fresh honey, were added to 1 mL of Folin-Ciocalteu reagent previously diluted 1:10 with distilled water. The mixture was vortexed for 2 min, and the content transferred into a 1.5 mL cuvette (1 cm path); absorbance was determined after 20 min at 750 nm against the sugar analogue. Determinations were performed in triplicate. As we were working under acidic conditions, there was no interference from the sugar analogue, and no precipitate formed during the analysis. The linearity was checked using gallic acid in the concentration ranging from 10 to 250 µg/mL (dissolved in methanol/water 1:1).

*DPPH scavenging activity*

The scavenging activity (H/e- transferring activity) against DPPH radical was evaluated according to the method of Brandwilliams *et al.* (1995) with minor modifications (Beretta *et al*., 2005).The assay mixture contained 1.9 mL of 130 µM DPPH (final concentration 83.3 µM) dissolved in absolute ethanol, 1 mL of acetate buffer solution (100 mM, pH 5.5) and 0.1 mL of the sample of honey solution containing 600 mg to 1 g/mL native honey, the final volume was 3 mL. The mixture was vortexed and then incubated for 90 min at 25°C in a water bath in the dark, after which the absorbance of the remaining DPPH was determined at 517 nm against a blank. Blank was honey at the same concentration described above containing all reagents except DPPH. The scavenging activity was expressed as $IC_{50}$ (mg/mL). All analysis were performed in triplicate.

*Hydroxyl radical scavenging activity*

2-Deoxyribose is oxidized by OH• that is produced by the Fenton reaction and degraded to malondialdehyde. The reaction mixture contained 0.45 mL of 0.2 M sodium phosphate buffer (pH 7.0), 0.15 mL of 10 mM 2-deoxyribose, 0.15 mL of 10 mM $FeSO_4$ EDTA, 0.15 mL of 10 mM $H_2O_2$, 0.525 mL of water, and 0.1 mL of sample solution in an eppendorf tube. After incubation at 37ºC for four hours, the reaction was stopped by adding 0.75 mL of 2.8% (w/v) trichloroacetic acid and 0.75 mL of 1.0% (w/v) of TBA in 50 mM NaOH, the solution was boiled for 10 min, and then cooled in water. The absorbance of the solution was measured at 520 nm. Hydroxyl radical scavenging ability was evaluated as the inhibition rate of 2-deoxyribose oxidation by hydroxyl radicals. Catechin (0-2 mg/mL) was used as the standard (Nagai *et al*., 2006). Honey at the same concentration described above was used instead of deoxyribose as sample control to eliminate the sugar interference of honey.

*HPLC analysis of phenolic compounds in wild honey and EFH using HPLC-DAD and determination of TPC and TFC*

The phenolic content of 1 wild honey sample and 1 EFH sample were checked using HPLC-DAD. The phenolic compounds were extracted from extrafloral and mixed floral honey according to the method described by Kermasha *et al.* 1995 with 50 mL ethyl acetate 6 times; the extracts were then pooled, filtered and concentrated in a rotary evaporator (Heidolph, Switzerland) and then vacuum dried. The dried extract was redissolved in methanol prior to injection to HPLC. The composition of phenolic acids were analyzed using HPLC (model LC-10A, Shimadzu Corp.) on a reverse phase Phenomenex C18 (2) column (4.6 x 250 mm, 5 micron), using a diode array ultraviolet detector (operating at 280 nm). A binary gradient linear system consisting of acetonitrile (A) and 0.8% formic acid in water (B) was used. Gradient method was generated by starting with 80% B; then decreasing to 60% B in 10 min, to 40% B in 20 min, to 20% B in 30 min, to 10% B in 40 min, to 0% B in 45 min; at a flow rate of 1 mL/min. Quantification of separated peaks was performed by calibration with standard gallic acid and chlorogenic acid. The peak identified at a retention time (RT) of 3.8 min was confirmed as gallic acid and the peak at an RT of 4.4 min was identified as the chlorogenic acid in comparison with standard gallic acid and chlorogenic acid. TPC of the samples were determined according to the method detailed before. The amount of total flavonoids was estimated by the aluminum chloride method (Chang *et al*., 2002). Catechin, which is having a moderate absorbance, was used as the standard. Catechin (10 mg) was dissolved in methanol and then diluted to 10-50 µg/mL. The diluted standard solutions (100-500 µL) were mixed with 0.3 mL of 10% aluminum chloride, 0.3 mL of 5% sodium nitrite and 2 mL of NaOH and the volume was made to 10 mL with water. The absorbance of the reaction mixture was measured at 510 nm with a Shimadzu UV-2450 PC; Shimadzu, Kyoto, Japan. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. Similarly, 100-500 µL of extracts was reacted with aluminum chloride for determination of flavonoid content as described above.

*Statistical analysis*

Statistical analysis was performed using the stat comp software. All the analysis was performed in triplicate and the results are expressed as mean ± SD. Correlations among data obtained were calculated using Pearson's correlation coefficient(r).

## Results and Discussion

*Physicochemical parameters*

Different parameters like absorbance, ash, color, free acidity, moisture, optical rotation, specific rotation, pH, refractive index and specific gravity of the honey samples (24 numbers) were determined, and the results are presented in Table 1.

The absorbance of 24 honey samples were measured at 660 nm and the absorbance of samples varied from 0.14 (sample 14) to 2.4 (sample 15). The absorbance of samples depends on color, granulation, and turbidity. Out of the 4 EFH samples tested, one sample (sample 14) showed lowest absorbance and the other showed the highest value (sample 15). Absorbance showed by samples can be a total effect of color, turbidity and granularity. Samples 1, 15 and 19 with higher absorbance (> 1) showed turbidity, granularity, and foaming. The turbidity of honey increases with granulation and an intensity increase in the absorbance at 660 nm are considered a valid measure of determining the granulation extent (Lupano, 1997). Correlation matrix showed that a negative correlation exists between absorbance and color.

Ash content ranged from 0.04% (sample 11- 0.04 ± 0.009 and sample 20- 0.04 ± 0.002) to 0.2 ± 0.02% (sample 7). Ash content of EFH varied from 0.07 ± 0.001% to 0.18 ± 0.014%. Light-colored honey usually has low ash contents, below 0.1%, while dark-colored honey has higher ash contents. The color was measured in the Lovibond range. For the samples analyzed, Y value ranged from 8.7Y to 73.3Y, and R value ranged from 4R to 33R. Most of the samples had a high Y value except for sample 2 (4.1R+28Y), sample 16 (9R+19Y), sample 19 (33R+8.7Y) and sample 23 (4R+16Y). Sample 19 was dark in color and had high R-value. All EFH samples showed high Y value. The color of natural honey is often an indication of their origin. Natural honey varies greatly in color, ranging from nearly colorless to very dark. The color is also affected by the harvesting method, storage time, and exposure to elevated temperature. The color of honey is closely related to its chemical composition, primarily to the presence of pigments such as chlorophylls, carotenoids, flavonoids and derivatives of tannins and polyphenols. In honeydew honey, the dark, strong color also results from the higher mineral contents and the presence of algae and green algae forming part of the flora of forest trees. Honey color depends on various factors, their mineral content being an important one. It was observed that samples with high Y value showed a lower absorbance and sample with increased R -

Table 1. Physicochemical parameters of commercial honey samples

| Samples | Absorbance at 660 nm | Ash (%) | Color (Lovibond 1"cell) | Free acidity (meq/kg) | Moisture (%) | Optical rotation | pH | Specific gravity | Specific rotation | Refractive index |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1.00 | 0.16±0.09 | 11.6R+68.4Y | 45.75±5.38 | 23.6±2.4 | 0.96±0.00 | 3.4 | 1.413±0.011 | -9.5±0.00 | 1.477±0.012 |
| 2 | 0.65 | 0.06±0.01 | 4.1R+28.0Y | 21.75±4.6 | 20±3.5 | 1.01±0.12 | 3.8 | 1.418±0.021 | -10.1±0.21 | 1.486±0.014 |
| 3 | 0.26 | 0.18±0.02 | 10.6R+72.9Y | 37.25±6.8 | 22.4±1.9 | 0.73±0.01 | 4.1 | 1.403±0.032 | -7.3±0.37 | 1.480±0.005 |
| 4 | 0.34 | 0.05±0.00 | 18.5R+70.0Y | 47.75±9.4 | 22±2.7 | 0.86±0.03 | 4.1 | 1.402±0.034 | -8.4±0.25 | 1.481±0.016 |
| 5 | 0.93 | 0.06±0.00 | 10.0R+72.7Y | 17.75±7.5 | 20.4±1.9 | 0.68±0.01 | 4.4 | 1.418±0027 | -6.6±0.35 | 1.485±0.019 |
| 6 | 0.17 | 0.15±0.03 | 7.0R+70Y | 34.25±4.9 | 18.4±2.2 | 0.74±0.02 | 3.9 | 1.434±0.019 | -7.3±0.24 | 1.496±0.010 |
| 7 | 0.56 | 0.20±0.02 | 10.9R+70.0Y | 27.75±5.3 | 19.2±1.9 | 0.32±0.00 | 3.9 | 1.414±0.005 | -6.2±0.089 | 1.488±0.004 |
| 8 | 0.31 | 0.06±0.01 | 14.6R+72.7Y | 18.25±3.5 | 19.2±2.1 | 0.19±0.01 | 4.3 | 1.413±0.004 | -3.4±0.089 | 1.485±0.016 |
| 9 | 0.27 | 0.10±0.02 | 12.4R+72.9Y | 19.25±4.3 | 21.6±1.8 | 0.19±0.02 | 4.2 | 1.414±0.001 | 1.9±0.130 | 1.482±0.009 |
| 10 | 0.24 | 0.05±0.01 | 11.4R+72.7Y | 19.75±3.9 | 18.8±1.1 | 0.38±0.03 | 4.4 | 1.402±0.014 | -3.3±0.464 | 1.489±0.016 |
| 11 | 0.20 | 0.04±0.01 | 10.5R+71.4Y | 19.25±4.1 | 18±1.6 | 0.35±0.01 | 4.6 | 1.420±0.015 | -3.5±0.14 | 1.491±0.012 |
| 12 | 0.29 | 0.15±0.03 | 16.5R+70.0Y | 25.75±5.0 | 18±1.7 | 0.32±0.01 | 4.4 | 1.411±0.010 | -6.4±0.2 | 1.491±0.015 |
| 13 | 0.26 | 0.05±0.00 | 12.2R+73.3Y | 24.25±2.5 | 20.8±1.2 | 1.78±0.14 | 4.2 | 1.410±0.00 | 18.00±0.18 | 1.484±0.016 |
| 14 | 0.14 | 0.07±0.00 | 8.2R+52.5Y | 39.25±5.9 | 20.4±1.6 | 0.23±0.00 | 3.9 | 1.404±0.010 | 2.50±0.06 | 1.485±0.020 |
| 15 | 2.4 | 0.18±0.01 | 9.3R+59.9Y | 37.25±8.5 | 20.8±1.4 | 0.06±0.09 | 4.0 | 1.402±0.012 | 3.6±1.081 | 1.484±0.011 |
| 16 | 0.70 | 0.17±0.02 | 3.9R+19.0Y | 29.75±4.3 | 18.4±1.5 | 0.46±0.01 | 4.1 | 1.426±0.014 | -4.40±0.96 | 1.490±0.021 |

value (33R + 8.7Y) showed a higher absorbance.

The free acidity values of the honey analyzed ranged from 16.25 ± 2.4 to 49.25 ± 5.6 meq/kg. EFH samples showed free acidity ranging from 37.25 ± to 40.25 ± 6.3 meq/kg. None of the samples exceeded the limit allowed by international regulations (50 meq/kg), indicating the absence of unwanted fermentation (Finola *et al*., 2007). The acidity of honey is due to the presence of organic acids, particularly gluconic acid, pyruvic acid, malic acid and citric acid, in equilibrium with lactones or esters and inorganic ions, such as phosphate and chloride. The variation in acidity among honey samples may be attributed to the plants' floral types (Anklam, 1998).

Moisture in the analyzed honey ranged from 18 ± 1.6% to 23.6 ± 2.4%. EFH sample had the moisture content ranging from 20.4% ± 1.6 to 21.6 ± 2.1%. Abu-Tarboush *et al.* (1993) have reported that moisture content is related to the floral source. The moisture content of honey is an important factor, contributing to its stability against fermentation and granulation during storage. The water content of honey depends on various factors, for example, the harvesting season, the degree of maturity reached in the hive and climatic factors. The maximum amount of water contained in honey is regulated for safety against fermentation (Finola *et al.*, 2007). The refractive index of honey samples ranged between 1.477 ± 0.012 to 1.496 ± 0.010. The refractive index of honey is said to be a rapid, accurate and simple measure of its moisture content. The honey samples varied in the specific rotation. Samples 9, 13, 14, 15, 17, 18, and 23 showed positive values for specific rotation and optical rotation. Among these samples, 14, 15, 17, 18 are EFH samples. Specific rotation is closely correlated with saccharide composition, having a negative value when the dominant sugar is fructose and a positive value in the case of glucose or sucrose. It has been also reported that natural honey containing small amounts of sucrose showed negative values for specific rotation (Nanda *et al.*, 2003).

All studied honey samples were acidic in nature, and the pH values varied in between 3.29 to 4.56. EFH had pH ranging from 3.8 to 4.0. These values fall within the range usually observed for natural honey (Anupama *et al.*, 2003) which may even reach pH 5.0. It is important to note that the pH of honey does not directly reflect the total organic acid, but rather reflects the buffering action of the inorganic cation constituents of the acids present. This parameter is of great importance during the extraction and storage of honey as it influences the texture, stability and shelf life of honey (Terrab *et al.*, 2002). Specific gravity values ranged from 1.402 to 1.434.

*Antioxidant screening*

There are many different antioxidant components of natural origin, and it is relatively difficult to measure each separately. Several methods have been employed to determine antioxidant activity of biological samples, and the results were compared with those of reference antioxidant standards (Rice-Evans *et al*., 1997). The antioxidant capacity of honey samples was examined by comparing to that of the known antioxidants: gallic acid and catechin by employing the following three complementary in vitro assays viz. TPC, DPPH radical scavenging activity (Beretta *et al*., 2005) and hydroxyl radical

Table 2. Total Phenol content and DPPH scavenging activities of commercial honey samples

| Sample | TPC (mg gallic acid/kg) of honey | DPPH (mg/mL, $IC_{50}$ Values) |
|---|---|---|
| 1 | 232.9±20.0 | 28.78 |
| 2 | 202.0±12.0 | 38.76 |
| 3 | 229.5±11.90 | 26.00 |
| 4 | 307.8±25.0 | 18.24 |
| 5 | 226.2±23.0 | 26.85 |
| 6 | 240.5±19.0 | 25.11 |
| 7 | 250.9±14.0 | 25.63 |
| 8 | 264.2±27.0 | 20.00 |
| 9 | 277.1±18.0 | 25.65 |
| 10 | 241.9±17.0 | 20.40 |
| 11 | 282.3±9.0 | 20.22 |
| 12 | 289.5±12.8 | 22.08 |
| 13 | 214.6±33.0 | 32.69 |
| 14 | 209.7±5.9 | 36.37 |
| 15 | 219±7.9 | 37.12 |
| 16 | 210.1±11.0 | 35.08 |
| 17 | 230.3±22.0 | 44.10 |
| 18 | 207.4±12.6 | 46.53 |
| 19 | 245.2±4.9 | 4.80 |
| 20 | 272.9±17.0 | 13.18 |
| 21 | 229.4±14.0 | 4.50 |
| 22 | 258.5±12.0 | 5.80 |
| 23 | 221.1±9.7 | 66.05 |
| 24 | 288.0±8.9 | 17.80 |

scavenging assay (Nagai *et al*., 2006).

*TPC*

The TPC (mg gallic acid/kg) of the different kinds of honey were investigated using the modified Folin-Ciocalteu assay that is sensitive to phenol and polyphenols entities and other electron-donating antioxidants (ascorbic acid, vitamin E). As reported in Table 2, the TPC of 24 samples ranged between 202 ±12 mg gallic acid/kg (sample 2) to 307.8 ± 25 mg gallic acid/kg (sample 4). EFH samples had TPC ranging from 207.4 ± 12.6 mg gallic acid/kg to 230.3 ± 22 mg gallic acid/kg. Out of the 24 samples examined two samples were very light in color (2 and 23), and the sample 19 appeared to be reddish brown, the color values of these three samples were 4.1R + 28Y, 4R + 16Y, 33R + 8.7Y, respectively. Earlier researchers observed that lighter the honey lower the TPC (Beretta *et al*., 2005). The correlation matrix (Table 3) showed a positive correlation (0.61) between TPC and color which is in quite an agreement with the earlier reports. Only sample 13 with high color value (12.2R + 73.3Y) showed a low TPC (214.6 mg gallic acid/kg). Most plants contain an extensive number of polyphenols and flavonoids, and each plant tends to have a distinctive profile. The concentration and type of polyphenolic substances depend on the floral origin of honey and are major factors responsible for biological activities, including antioxidant, antimicrobial, antiviral and anticancer activities.

*DPPH radical scavenging activity*

The DPPH radical scavenging test is one of the shortest available to investigate the overall hydrogen/ electron donating activity of single antioxidants and health promoting dietary antioxidant supplements. The odd electron in the DPPH free radical gives an absorption maximum at 517 nm and is purple in color. The odd electron of DPPH becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolourization was stoichiometric with respect to number of electrons captured. The sample size that can lower the initial absorbance of DPPH solution by 50% has been chosen as the endpoint for measuring the antioxidant activity. Table 2 shows the scavenging ability expressed as $IC_{50}$. The $IC_{50}$ values of the honey samples ranged between 4.5 to 66.05

Table 3. Correlation matrix

| | TPC | DPPH:$IC_{50}$ | Hydroxyl radical scavenging activity | Absorbance | Color |
|---|---|---|---|---|---|
| TPC | -- | -0.55 | -0.46 | -0.24 | 0.61 |
| DPPH:$IC_{50}$ | -0.55 | -- | -0.46 | -0.01 | -0.55 |
| Hydroxyl radical scavenging activity | -0.46 | 0.31 | -- | -0.03 | -0.33 |
| Absorbance | -0.24 | -0.01 | -0.03 | -- | -0.17 |
| Color | 0.61 | -0.55 | -0.33 | -0.17 | -- |

mg/mL. Again the least active were sample 2, 13, 14, 15, 16, 17, 18, and 23 of which 14, 15, 17, and 18 are EFH samples. The most active samples were sample 19, 21, 22 (4.8, 4.5 and 5.8 mg/mL, respectively), the least active was sample 23. Correlation matrix showed a negative relation (-0.55) between $IC_{50}$ and TPC, which showed that higher the TPC value lower the $IC_{50}$ value (Blasa *et al*., 2006). Also, a negative correlation was seen between color and DPPH ($IC_{50}$) indicating that darker the sample lower the $IC_{50}$ value (Table 3). This result is in agreement with the earlier studies reported (Bertoncelj *et al*., 2007). Another finding from the results is that some samples (sample 19, 21, 22) which where darker in color did not have exceptional TPC values but had lower $IC_{50}$ value. This point to the fact that compounds other than phenols (products of Maillard reaction and caramelization) show radical scavenging activity. The fact was checked using jaggery and caramelized sugar, which showed radical scavenging activity (data not shown). Low $IC_{50}$ value discussed in the above samples thus can indicate the chance of adulteration of these samples with jaggery or caramelized sugar.

*Hydroxyl radical scavenging activity*

Radiolysis of water produces a substantial yield of hydroxyl radicals (OH) in free solution. Any molecules present in radiolysed water will react with OH radicals at rates predictable from their established second-order rate constants. Some of these molecules react with OH in aqueous solution rapidly at almost diffusion - controlled rates, and are popularly known as 'hydroxyl radical scavengers. These include simple substances such as mannitol, thiourea, glucose, histidine, butan-1-ol, propan-2-ol, formate, benzoate and many others.

In biological systems, not subjected to high-energy radiation, the majority of OH radicals or oxidants with similar activity such as ferryl ions ($FeO^{2+}$) are probably produced when certain transition-metal ions react with $H_2O_2$ in a Fenton-type reaction. Under the standard conditions of substrate (detector molecule), iron salt EDTA and different concentrations of OH scavengers were added to determine the % inhibition. Hydroxyl radical scavenger will quench the hydroxyl radical generated through Fenton's reaction and thus prevent it from fragmenting deoxyribose and further color development. The scavenging activities of honey samples against hydroxyl radical inhibition, reviewed using Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- +$ OH), is shown in figure 1. Each honey sample showed varied hydroxyl radical scavenging activity (20.23% - sample 4 to 80.19% -sample 13). Sample 5, 10, 13, 14, 16, 19, and 23 showed 50% inhibition at the tested concentration (100 mg/100 µL) among them 14 was EFH sample. DPPH and hydroxyl radical scavenging activity showed a positive correlation (0.309) where as a negative relation was seen between hydroxyl and TPC; and hydroxyl and color: (- 0.46) and (- 0.33), respectively (Table 3). Few reports are available on hydroxyl radical scavenging activity except that reported by Nagai *et al.* (2006), in which they observed that hydroxyl radical scavenging activity was very high in all honeys (over 77% inhibition). Hydroxyl radicals are shown to be capable of abstracting hydrogen atoms from membranes and bring about peroxide reactions of lipids. It is therefore thought that honey species demonstrate antioxidant effects against lipid peroxidation on meat or muscle membrane by scavenging the hydroxyl radicals and superoxide anions at the stage of initiation and termination of peroxy radicals.

*HPLC analysis of phenolic compounds in wild honey and EFH using HPLC-DAD and determination of TPC and TFC*

The phenolic compounds were extracted using ethyl acetate and the extracts were concentrated and dried. Wild honey showed a higher yield than EFH (data not shown). The extracts were redissolved in methanol and injected into HPLC. Figure 2a,b

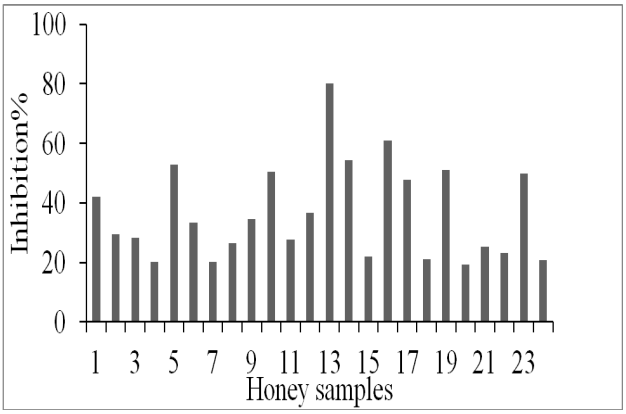


Figure 1. Hydroxyl radical scavenging activity of honey samples

represents HPLC chromatograms of EFH and wild honey. Phenolic acids, gallic acid (RT - 3.8 min) and chlorogenic acid (RT - 4.4 min) were identified in both the sample. The phenolic acids were quantified by plotting a standard curve with the respective standards. There was another relevant peak in wild honey (RT - 19.6 min) that could not be identified. TPC of wild honey was 680.95 ± 4.47 (mg gallic acid/kg) and of EFH was 568.73 ± 3.61 (mg gallic acid/kg) and their TFC content was 100.24 ± 0.71 (mg quercetin/kg) and 37.95 ± 0.81 (mg quercetin/kg), respectively. Among the two phenolic acids identified chlorogenic acid was the most dominant in EFH accounting to 152 mg/kg of honey whereas gallic acid was only 2.2 mg/kg of honey. There was also a small unidentified peak at RT - 19.6 min. Gallic acid and chlorogenic acid content of wild honey samples were 5 mg/kg honey and 9 mg/kg honey, respectively. Though gallic acid content was high for wild honey than EFH, the unidentified peak at RT - 19.6 min may account for its high yield and TPC.

## Conclusions

Physicochemical characteristics, antioxidant activities and phenolic profile of honey obtained from extrafloral nectar and the honey obtained from the mixed floral source were compared in this study. Different parameters like absorbance, ash, color, free acidity, moisture, optical rotation, specific rotation, pH, refractive index and specific gravity of the honey samples were determined. The antioxidant capacity of honey samples were examined by comparing to that of the known antioxidants: gallic acid and catechin by employing the three complementary in vitro assays TPC, DPPH scavenging activity, hydroxyl radical scavenging assay. The honey samples vary widely in their physicochemical as well as antioxidant properties. Among the 24 samples studied, TPC ranged from 202 ± 12 to 307.8 ± 25

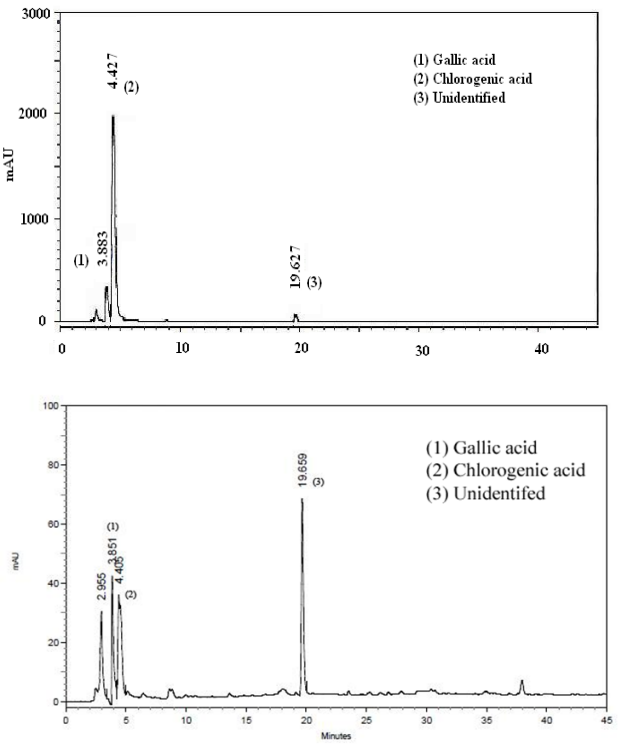


Figure 2. (a) HPLC chromatogram of extrafloral honey; (b) HPLC chromatogram of wild honey

mg gallic acid/kg of honey. EFH samples had TPC ranging from 207.4 ± 12.6 mg gallic acid/kg to 230.3 ± 22 mg gallic acid/kg but did not show potent DPPH radical scavenging activity. One of the EFH samples showed 50% inhibition at the tested concentration (100 mg/100 µL) for hydroxyl radical scavenging activity. The correlation matrix showed a positive correlation (0.61) between TPC and color which is in quite an agreement with the earlier reports. Correlation matrix showed a negative relation (-0.55) between $IC_{50}$ and TPC, which showed that higher the TPC value lower the IC50 value. Also, a negative correlation was seen between color and DPPH ($IC_{50}$) indicating that darker the sample lower the $IC_{50}$ value. DPPH and hydroxyl radical scavenging activity showed a positive correlation (0.31) where as a negative relation was seen between hydroxyl and TPC; and hydroxyl and color: (-0.46) and (-0.33), respectively. HPLC chromatograms showed the presence of gallic acid and chlorogenic acid in wild and EFH. Among the two phenolic acids identified chlorogenic acid was the most dominant in EFH. Though gallic acid content was high for wild honey than EFH, the unidentified peak at RT - 19.6 min may account for its high yield and TPC. This study thus shows that honey can be consumed as a source of natural antioxidants, but the fact about variation in quality of honey on foraging source of bee has to be

considered.

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