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Analysing Physico-chemical Changes Due to Storage in Crude Honey Samples Collected from Sunderban Mangrove Forest

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Abstract—*To study the effect of storage on in vitro antioxidative capacities, raw mangrove honeys, Aegiceras corniculatum, Ceriops decandra and Avicennia officinalis samples were analysed for color, melanoidin content, UV absorbing compounds and antioxidative capacities and were stored at room temperature in dark and dry place for one year and were analysed again. Honey samples were collected from Sundarban mangrove forest of West Bengal in different seasons. DPPH radical scavenging assay, FRAP assay and Nitric oxide assay of peripheral blood mononuclear cells were carried after storage of honey samples for one year. The study suggested that non-enzymatic browning or Maillard reaction may be chiefly responsible for the colour of raw, unheated honey during storage, and as a result appears to be a major contributor to the antioxidant activity of honey.*

1. INTRODUCTION

According to European Union Council Directive, 2002, honey is defined 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 on 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'.

Although studies on the basic composition of honeys started few hundred years ago, the interest in honey phenolics or more specifically honey flavonoids is relatively recent which contribute to its therapeutic health benefits especially its antioxidant behaviour. Although the total antioxidant capacities of honey is the combination of wide range of bioactive components both enzymatic and non enzymatic, including catalase, phenolic acids, flavonoids, amino acids, ascorbic acid, proteins, carotenoids, organic acids and Maillard reaction products, the content of phenolic compounds can significantly reflect its total antioxidant activity to some extent.

Phenolic compounds in honey are mainly flavonoid, aglycones and phenolic acid derivatives like benzoic and cinnamics acids and their respective ester. Phenolic compounds are the phytochemicals, and thus the phenolic composition of honey varies depending on the vegetation of the area visited by the bee. With this in mind, phenolic compounds have been proposed as potential chemical markers for authenticating the geographical and botanical origin of honey. Flavonoids are the most common phenolics in floral honeys, and characteristic profiles could be expected in unifloral honeys depending on the corresponding plant source. A strong correlation has been observed between the antioxidant activity of honeys and their phenolic composition and especially the total phenolic content. Thus, characterization of phenolics and other components in honey that might have antioxidant properties is essential to improve our knowledge about honey as a source of nutraceuticals and would also be an important tool to contribute to their authentication.

The comparative physicochemical characterization of different honeys from various regions of the world has been carried out extensively. Although in India, honey is produced and consumed on a large scale, there is a lack of systemic information on the comparative biochemical properties of Indian honeys, especially the honeys collected from the state of West Bengal. However, the state of West Bengal contributes a major chunk of the ~ 65000 million tones to the honey produced in India (Kumar 2010).West Bengal harbors a natural and unique ecosystem and consequently is a hub of natural products. Prominent features of this ecosystem includes a part of the world's largest ecosystem i.e. the Sundarban mangrove forests (~ 40% of the forest area is present in West Bengal). Although numerous reports have highlighted the importance of this ecosystem and the natural products from these regions this leaves a huge void in understanding the prophylactic impact of the natural products especially honeys procured from these regions. This can be

attributed to the fact that the honey samples from this geographical area and similar ecosystem across West Bengal have not been documented and characterized for their prophylactic and associated impact on health.

Therefore, the objective of the present study was to investigate the changes caused by storage on the biochemical and antioxidative properties of Sundarban mangrove honeys. The novelty of the study lies in the fact that raw mangrove honeys from Sundarban which serves as a major chunk of the Indian honey have been evaluated for antioxidant property.

2. MATERIALS AND METHODS

HONEY SAMPLES

Eighteen honey samples were obtained from West Bengal Sundarban mangrove region, donated by West Bengal Forest Department. The honey samples were collected in 2010-2011, 2011-2012 and 2012-2013 flowering seasons. The initial verification of the floral origin of each honey sample was providedby the West Bengal Forest Department on the basis of corresponding hive location, season and available floral sources. Confirmatory results of the floral origin was further obtained by means of pollen analysis (45% and above), which was calculated as the ratio of frequency of each pollen type in honey with respect to the total number of identified pollens (Louveaux *et al.* 1978).The honey samples were classified into three groups, namely khalsi (*Aegiceras corniculatum*), goran (*Ceriops decandra*) and bain (*Avicennia officinalis*) as depicted in **Table 1**. Khalsi, goran and bain were classified as mangrove honeys. All the samples were stored at 0-4ºC. The honey samples were kept at room temperature overnight before the analyses were performed.

Table 1: Sample information of honeys collected from Sundarban Mangrove forest of India

Sample labeling	Local name	Botanical origin	Geographical origin
K1,K2,K3,K4,K5,K6 (n=6)	Khalsi	*Aegiceras corniculatum*	Sundarban forest
G1,G2,G3,G4,G5,G6 (n=6)	Goran	*Ceriopsdecandra*	Sundarban forest
B1,B2,B3,B4,B5,B6 (n=6)	Bain	*Avicennia officinalis*	Sundarban forest

Determination of *in vitro* antioxidative potential of honeys

Total polyphenol content

To measure the total polyphenol content of honey samples, Folin–Ciocalteu assay was employed (Dhar *et al.* 2011). 0.5 mL of aqueous honey solution (20%) was added with 1 mL of Folin–Ciocalteu's phenol reagent (10 fold diluted). 0.8 mL of 2% sodium carbonate and 60% methanol were added successively. Then the reaction mixture was incubated at room temperature for 30 min and were spectrophotometrically analysed at 740 nm. The calibration curve was plotted using gallic acid (20–100 µg) as standard and the result of polyphenol content was represented as mg of gallic acid equivalents (GAE) per 100 g of honey.

Flavonoid content

Aluminium chloride method (Meda *et al.*2005) was used to quantify total flavonoid content of honey samples. Equal volume of honey solution (10%) was combined with 2% aluminium trichloride ($AlCl_3$) dissolved in methanol. After 10 min of incubation at room temperature, absorbance was measured at 415 nm using a standard curve of quercetin (20-100 µg). The results were expressed as mg of quercetin equivalent (QE) per 100 g of honey.

Color intensity (ABS_{450})

To find out the Color intensity, honey samples were dissolved in warm (45-50 ºC) double distilled water at 50% concentration and were filtered through a Whatman no.1 paper. The OD reading of the honey solutions were taken at 450 and 720 nm respectively. ABS_{450} values were expressed as the difference between the two absorbances (Beretta *et al.*2005).

FRAP Assay

FRAP values of honey samples were evaluated by the method of Benzie and Strain (1996). To prepare working FRAP reagent, 50 mL of 300 mM acetate buffer (pH-3.6) was mixed with 5 mL of 40 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) dissolved in 40 mM HCl and 5 mL of 20 mM ferric chloride. 400 µL of aqueous honey solution (10%) was added to 3 mL of freshly prepared working FRAP reagent. The absorbance at 593 nm was spectrophotometrically measured immediately and after 4 min of incubation at 37 ºC. The change in absorbance was recorded as the final absorbance. For plotting calibration curve, ferrous sulphate ($FeSO_4.7H_2O$) was used as standard at various concentration (100-500 µM/L). The ferric reducing ability of honey sample was expressed as FRAP value (µM of Fe^{II}) of 10% honey solution.

DPPH radical scavenging activity

DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity of honey samples was evaluated by the method of Velazquez *et al.* (2003) with minor modifications. Initially, methanolic solution of honey samples was prepared at different concentration (25-100 mg/mL). 0.75 mL of sample solution was added to 1.5 mL of DPPH dissolved in methanol (0.02 mg/mL). After 15 min of incubation at room temperature, absorbance of the reaction mixture was recorded at 517 nm against methanol blank. The percentage of inhibition DPPH radical was calculated as [(Absorbance of Blank – Absorbance of sample)/ Absorbance of Blank] X 100. IC_{50} value of each honey sample was determined from the graph between sample concentration and the percentage of DPPH radical inhibition.

Difference in the content of UV-absorbing compounds in stored honey

The quantitative data on the levels of UV absorbing compounds were obtained from the absorbance spectrum profiles of honey scanned at wavelengths 200–400 nm. The concentration of UV-absorbing compounds was determined from the area-under the curve (AUC). The concentrations were expressed in arbitrary units as the AUC units.

Determination of Maillard reaction content

The melanoidin content was assessed spectrophotometrically as net absorbance at (A_{450}-A_{720}) (Ramonaityte *et al.*2009).The melanoidin content was in absorption units (AU).

Estimation of the effect of Khalsi honey on nitric oxide release from oxidatively stressed PBMC (peripheral blood mononuclear cell)

Isolation of PBMC from human blood

PBMC were isolated from whole blood obtained from healthy donors with their mutual consent. Separation of blood cells was performed using density gradient centrifugation (with 4 mL aHiSep® and 5.5 mL diluted blood), at 1500 rpm for 30 min. The buffy coat containing PBMC was removed carefully following centrifugation and washed twice in phosphate buffer saline (PBS). Cells were then counted and assessed for viability using trypan blue with the help of haemocytometer (Winkler *et al*. 2006).

Nitric oxide assay

Nitric Oxide (NO) generation during cell culture and by NO chemical donors was measured as accumulated supernatant nitrite (a stable breakdown product of NO) by the Griess reaction in the presence or absence of LPS (1 µg/mL) according to (Green *et al.* 1982).After 24 h of incubation, nitrite production in the culture supernatant was assayed using the Griess reaction by the measurement of the total amount of inorganic nitric oxides. Then, 100 µL of Griess reagent (1% sulfanilamide and 0.1% *N*-1-naphthyl ethylene-diamine dihydrochloride in 2.5% H_3PO_4) were added to each well, and the samples were incubated for 10 min at room temperature. Optical densities at 540 nm were read on an ELISA reader. Nitrite concentration was calculated with reference to a standard curve obtained using $NaNO_2$.

STATISTICAL ANALYSIS

Analysis of variance according to Duncan's test was done to compare the variables of different honeys collected from Sundarban mangrove forest. Correlation coefficient test was applied to test the association between the polyphenol content and the antioxidant activity of the honeys. The SPSS software version 16.0 was used for all the statistical calculations.

3. RESULT AND DISCUSSION

Changes in stored honey colour

As shown in **Table 2**, a significant increase in honey colour, measured as net absorbance (A_{560}-A_{720}) was observed after one year of storage ($p < 0.0025$). Relatively fewer differences in colour change were observed in individual unifloral honey.

The color of honey is one of the important factor determining its price and acceptability by the consumers. The rate of darkening has been related to the composition of honey, initial color of the honey and of the storage temperature (White, 1978). Lynn et al., (1936) indicated that the main causes of darkening in honey could be: (a) reaction aminoacid- aldol (Maillard reaction); (b) combination of tannates and other oxydated polyphenols with ferrum salts; (c) instability of fructose (caramelization reaction). However, there is still controversy over the relative influence of these factors on the darkening of honey.

TABLE 2: List of Mangrove honeys, their floral origin and visual colour.

Floral origin of honey	*Aegiceras corniculatum*		*Ceriops decandra*		*Avicennia officinalis*	
Color intensity	Fresh	Stored	Fresh	Stored	Fresh	Stored
	0.0918± 0.01	0.2509± 0.04	0.1811± 0.02	0.2934± 0.01	0.1973± 0.04	0.1319± 0.04
Melanoidin content	0.2682 ±0.02	0.8061 ±0.03	0.4343± 0.03	0.6621± 0.01	0.5005± 0.03	0.5220± 0.01

Changes in contents of UV absorbing compounds

The honey UV profile results from a summation of spectra from many different compounds, in which each of constituents contributes to the final UV absorbance profile. The total concentration of UV absorbing compounds was determined from the Area-Under-the Curve AUC (200–400 nm) and arbitrarily expressed in AUC units. Comparison of AUCs of fresh and stored honeys showed an extremely significant increase in the content of UV absorbing compounds in the stored honeys independent of their botanical source after one year of storage (the two-tailed ANOVA, $p < 0.0001$) (**Fig. 1**). This process(s) apparently generated novel compounds of the high UV absorbance from the pool of existing substrates.

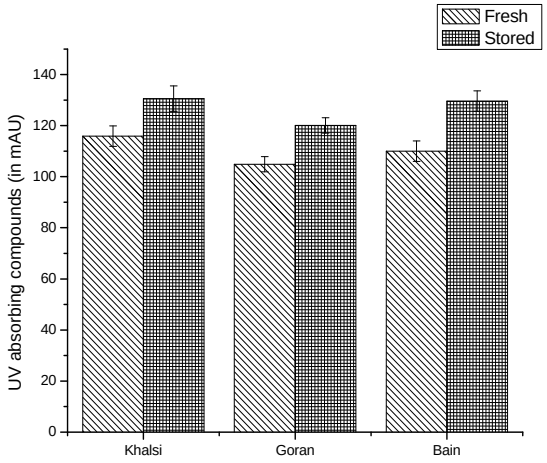

Fig. 1: Changes in the content of UV absorbing compounds in fresh honeys and after one year of storage. Values are expressed as mean ± SD n=6

Melanoidins content in stored honeys

There is vast evidence in literature to demonstrate that the Maillard reaction and subsequent formation of melanoidins is a major cause of non-enzymatic browning in thermally processed and stored foods. Browning of honey upon storage is a well knownphenomenon that adversely affect honey quality and consumeracceptance. We therefore examined stored honeys for the presenceof melanoidins. As shown in **Table 2**, all stored honeys exhibited an increase in thecontent of melanoidins after one year of storage.

Thus the present data indicated that during storage, honey undergone marked chemical changes including a change to darker colours, increased production of UV absorbing compounds and appearance of melanoidins.

Changes in polyphenol and flavonoid content of honey after storage

As reported in **Table 3** total polyphenol and flavonoid content was higher in case of darker honey i.e, *Aegiceras corniculatum* (khalsi) and low in paler *Avicennia officinalis* (bain) honey. After one year of storage, there has a significant increase in both polyphenol and flavonoid content **(Table 3)**.

Table 3: Changes in the content of polyphenol and flavonoid, in fresh honeys and after one-year storage period.

Floral origin of honey		Total Polyphenol (mg GAE/100g)	Total Flavonoid (mg QE/100 g)
Aegiceras corniculatum	Fresh	13.1 ± 1.2	13.7 ± 0.9
	Stored	17.18 ± 0.8	16.3 ± 0.5
Ceriops decandra	Fresh	12.8 ± 0.7	9.97 ± 0.3
	Stored	16.1 ± 0.6	12.4 ± 0.9
Avicennia officinalis	Fresh	9.7 ± 1.1	5.82 ± 0.9
	Stored	15.35 ± 0.6	10.6 ± 0.8

Values are expressed in mean ± SD.

Table 4: Changes in Nitric Oxide release from PBMC before and after storage of Khalsi Honey

	Without LPS treatment. Conc. Of Nitrite(μM)	***LPS treated*** Conc. Of Nitrite(μM)	LPS treatment +Khalsi Honey (30%). Conc. Of Nitrite(μM)	LPS treatment +Khalsi Honey(50%). Conc. Of Nitrite(μM)
Fresh	**8.7± 0.43[a]**	**20.78± 0.73[b]**	**10.68± 0.32[c]**	**9.67± 0.66[d]**
Stored			**13.91± 0.76[e]**	**11.54± 0.56[f]**

Values are expressed as mean ± SD n=6 $^{b>c>d>a}$ where ^{c} vs ^{d} are NS $^{e>c}$ and $^{f>d}$ ***where p<0.05***

Changes in antioxidant activity of honey after storage

The DPPH assay measures hydrogen atom (or one electron) donating activity and hence provides a measure of antioxidant activity. **Fig. 2** showed the DPPH radical scavenging ability expressed as IC_{50} on the DPPH radical, the amount of antioxidant necessary to decrease the initial concentration of DPPH by 50%. The results of DPPH radical scavenging activity revealed that khalsi (*Aegiceras corniculatum)*was the most efficient Sunderban honey while the least effective was bain (*Avicennia officinalis)*. Coming to the effect of storage on DPPH radical scavenging activity, it was found that irrespective of botanical origin of honey there had been a significant decrease in IC_{50} value which was equivalent to increase in DPPH radical scavenging activity.

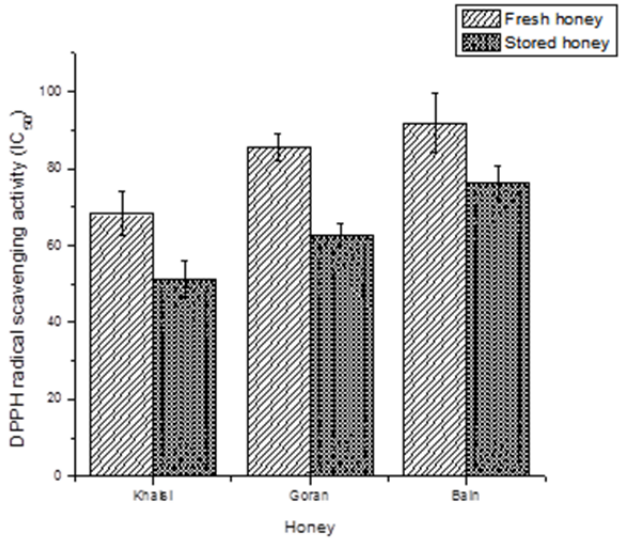

Fig. 2: Changes in the DPPH radical scavenging activity in fresh mangrove raw honeys and after one year of storage. Values are expressed as mean ± SD n=6

To gain further insight on the antioxidant activity of honey, FRAP value of Sundarban honey samples were analysed. FRAP assay is a simple direct test that is widely used for antioxidant activity and is based on the ability of the analyte to reduce the Fe^{3+}/Fe^{2+} couple. Mean FRAP value of Sundarban mangrove honeys were found to concomitant with the results obtained in case of DPPH IC_{50} value. As depicted in **Fig. 3,** FRAP values of analysed samples has showed better results after one year of storage.

Beekeepers are well aware of the fact that honeys of light color often became darker upon storage which decreases its consumer acceptability. Moreover, the change in color is also notable during honey processing and pasteurization, which is known to facilitate the Maillard reaction and non-enzymatic browning (Turkmen *et al.* 2006). This study suggests that non-enzymatic browning or Maillard reaction may be chieflyresponsible for the colour of raw, unheated honey during storage, and as a result appears to be a major contributor to the antioxidant activity of honey.

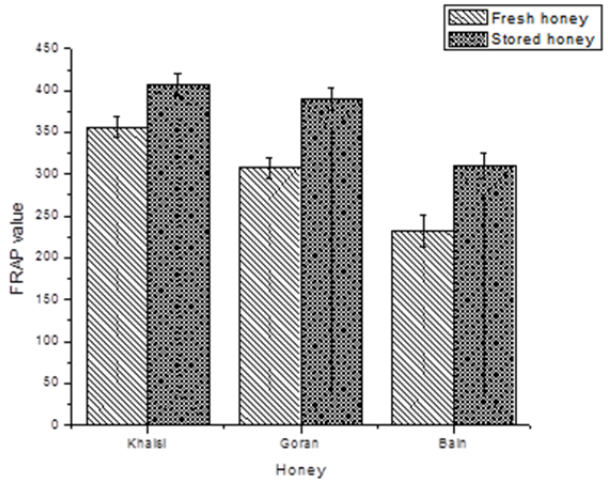

Fig. 3: Changes in the FRAP values of fresh mangrove raw honeys after one year of storage. Values are expressed as mean ± SD n=6

Changes in the Nitric oxide assay due to storage

Nitric oxide (NO) release potential from oxidatively stressed PBMC were investigated to determine the therapeutic potential of Khalsi honey. Nitrite is detected and analyzed by formation of a red pink colour upon treatment of a NO_2^--containing sample with the Griess reagent described as Griess reaction. When sulphanilic acid is added the nitrites form a diazonium salt. When the azo dye agent (N-alpha-naphthyl-ethylenediamine) is added a pink colour develops. This diamine is used in place of the simpler and cheaper alpha-naphthylamine because the latter is a potent carcinogen and moreover the diamine forms a more polar and hence a much more soluble dye in acidic aqueous medium.

Six Khalsi honey samples were used for this analysis. <u>Group 1</u>, represents the control group which was not treated with LPS hence nitric oxide production was low and varied from 7.5-10 uM.Group 2 cells were treated with LPS and NO released near about 20 uM. 30% Khalsi honey with LPS was used in Group 3 and 13-14 uM NO production was observed. Group 4, 50% Khalsi honey was used with LPS and NO production was slightly lower than the previous group, nearing about 10 uM. Therefore, Nitric Oxide production was lowered by the honey samples in presence of LPS. Both 30% and 50% concentration of honey samples reduced Nitric oxide production. After storage for one year the therapeutic potential decreases.

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