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Characterization Of Antioxidants And Antioxidative Properties Of Various Unifloral Honeys Procured From West Bengal, India.

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Abstract : *The present study deals with the investigation of erstwhile undocumented fifty four samples of West Bengal honey from ten different floral origins - sesame (Sesamum indicum), hizal (Barringtonia acutangula), mustard (Brassica spp.), drumstick (Moringa oleifera), litchi (Litchi chinensis), eucalyptus (Eucalyptus spp.), karanza (Pongamia glabra), khalsi (Aegiceras corniculatum), goran (Ceriops decandra) and bain (Avicennia officinalis) for its quantification and comparison of antioxidative capacity by chemometrics. The average total polyphenol and flavonoid content of honeys varied from 9.9 ± 0.6 (bain) to 44.7 ± 2 mg (hizal) gallic acid equivalent and 5.12 ± 0.23 (drumstick) to 19.4 ± 1.38 (bain) mg of quercetin per 100 g respectively. Dark brown hizal honey with ABS_{450} value of 2055.6 mAU exhibited the most potent DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenger (IC_{50} = 23.92 ± 1.12 mg/mL) and a FRAP (ferric reducing ability of plasma) value of 381 ± 1.9µM Fe (II).*

Keywords : *DPPH, FRAP assay, principal component analysis, sundarban honey, total phenolic content*

I. Introduction

Honey is considered as a valuable and unique dietary source of antioxidants. Sugars pre-dominate the composition of honey but its minor constituents contribute to the beneficial therapeutic potentials of honey. 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 [1].

Polyphenols are the most important group of plant secondary metabolites that exhibit antioxidant property through various mechanisms as free radical scavenger, metal ion chelator or hydrogen donor. These bioactive phenolic compounds enter into honey from the plants that are used by the bees to collect nectar and honeydew. Their presence and concentration in honey can vary depending upon the floral source, geographical, climatic and storage conditions.

Studies indicate that consumption of honey alone or in combination with other beverages significantly increases antioxidant capacity of human serum [2]. It is also effective in preventing deteriorative enzymatic browning of fruits and vegetables [3] and lipid oxidation in meat [4].

The Indian state of 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) [5]. Although numerous reports have highlighted the importance of this ecosystem, the natural products from these regions have not been characterized systematically. This leaves a huge void in understanding the prophylactic impact of the natural products procured from these regions. A potent anti-oxidative source of natural products includes honey, which hitherto has not found much interest among the scientific and industrial community. 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. The state of West Bengal contributes a major chunk of the ~ 65000 million tones to the honey produced in India [6] but has not attracted any major research on its nutritional benefits till now. Consequently, the honey samples procured from the geographical locations inside West Bengal merits systematic and holistic research on its health benefits.

The present study entailed to evaluate and compare the antioxidant parameters of honeys of West Bengal (Indian) origin to corroborate the data as authentic by performing multivariate analytical techniques including principal component analysis (PCA) towards classifying different botanical origin honey samples.

II. Materials And Methods

2.1. Chemicals and instruments

DPPH (2, 2-diphenyl-1-picrylhydrazyl) and TPTZ (2, 4, 6-tri (2-pyridyl)-s-triazine) were purchased from E. Merck India Pvt. Ltd., Kolkata, India. Folin–Ciocalteu's phenol reagent and quercetin were obtained

from Sigma Aldrich Chemical Co., Milwaukee, Wis., U.S.A. All absorbance were recorded with the Perkin Elmer (uv/vis) spectrophotometer (L6100013).

2.2. Honey samples

Fifty four honey samples were obtained from various bee-keepers association of different regions of West Bengal. Out of these, eighteen samples were from West Bengal Sundarban mangrove region, donated by West Bengal Forest Department. The honey samples were collected in 2011-2012 and 2012-2013 flowering seasons. The initial verification of the floral origin of each honey sample was provided by the beekeepers 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 [7]. The honey samples were classified into ten groups, namely sesame (*Sesamum indicum*), hizal (*Barringtonia acutangula*), mustard (*Brassica spp.*), drumstick (*Moringa oleifera*), litchi (*Litchi chinensis*), eucalyptus (*Eucalyptus spp.*), karanza (*Pongamia glabra*), khalsi (*Aegiceras corniculatum*), goran (*Ceriops decandra*) and bain (*Avicennia officinalis*). Khalsi, goran and bain were classified as mangrove honeys. All the samples were stored at 0-4ºC and were analyzed within 3 months of storage period. The honey samples were kept at room temperature overnight before the analyses were performed.

2.3. Determination of total polyphenol content

To measure the total polyphenol content of honey samples, Folin–Ciocalteu assay was employed [8]. 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 (0–100 mg/mL) as standard and the result of polyphenol content was represented as mg of gallic acid equivalents (GAE) per 100 g of honey.

2.4. Determination of flavonoid content

Aluminium chloride method [9] 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 (0-50 mg/mL). The results were expressed as mg of quercetin equivalent (QE) per 100 g of honey.

2.5. 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 [1].

2.6. FRAP Assay

FRAP values of honey samples were evaluated by the method of Benzie and Strain [10]. 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.

2.7. DPPH radical scavenging activity

DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity of honey samples was evaluated by the method of Velazquez [11] with minor modifications. Initially, methanolic solution of honey samples were 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 (0.02mg/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.

2.8. Hydroxyl radical scavenging activity

To evaluate the hydroxyl radical scavenging activity of honey samples, the method as described by Singh [12] was used. Different concentrations (50, 100 and 150 µg GAE polyphenol) of aqueous honey solution were taken separately in screw capped tubes. 1 mL of Iron EDTA (0.1% ferrous ammonium sulphate and 0.26% EDTA) was mixed with 0.5 mL of EDTA (0.018%) and 1 mL of dimethyl sulphoxide (0.85% in 0.1 M phosphate buffer with pH 7.4). To commence the reaction 0.5 mL of ascorbic acid (0.22%) was added and held in a water bath at 80 – 90 ºC for 15 min. 1 mL of ice cold TCA was added to stop the reaction. For preparation of Nash reagent 75 g of ammonium acetate was mixed with 3 mL of glacial acetic acid and 2 mL of acetyl acetone and the volume was made upto 1 L with distilled water. 3mL of Nash reagent was finally added and incubated for 15 min at room temperature for yellow color development. Then the absorbance of the reaction mixture was taken at 412 nm against reagent blank. Percentage of hydroxyl radical scavenging activity was calculated as follows: [1 - (sample absorbance \ blank absorbance)] X100.

2.9. Statistical analysis

Analysis of variance according to Duncan's test was done to compare the variables like polyphenol content, flavonoid content, color intensity, FRAP values, DPPH and hydroxyl radical scavenging activity of the different honeys collected from West Bengal. In Table 1 and Table 2, within each column, averages denoted with the same letter were not significantly different by this test ($P < 0.05$). Principal component analysis (PCA) was further used to find out the variables that better differentiate between the honey types. The SPSS software version 16.0 was used for all the statistical calculations.

III. Results And Discussions

3.1. Total polyphenol content

The total phenolic content of honeys, expressed as gallic acid equivalent, was specified in TABLE 1. It varied in the range from 9.9 ± 0.6 mg of GAE per 100 g in bain honey to 44.7 ± 2 mg per 100 g in hizal honey. The total phenolic content of West Bengal honeys followed the order: hizal > litchi > sesame > karanza > eucalyptus > mustard > khalsi > goran > drumstick > bain. In the present study, total amount of polyphenol content were found to be significantly higher than those observed by Berreta *et al.* [1] and Bertoncelj *et al.* [13] in acacia and clover honey. The comparable amount of total phenolic content were reported by the latter authors in case of lime, chestnut, fir, spruce, buckwheat, honeydew, dandelion, chicory and sulla honey, whereas considerably greater values were observed in case of strawberry and few African honeys.

TABLE 1.Total polyphenol and flavonoid content of different floral origin of honeys collected from West Bengal.

Floral origin of honey	Total polyphenol content (mg of Gallic acid equivalent/100g of honey)	Flavonoid content (mg of quercetin equivalent/100g of honey)
Sesame (n=7)	28.9 ± 0.6^{e}	$13.2 \pm 1.8^{c,d}$
Hizal (n=5)	44.7 ± 2^{g}	19.4 ± 1.38^{e}
Mustard (n=6)	20.2 ± 0.79^{c}	5.9 ± 0.83^{a}
Drumstick (n=5)	10.5 ± 0.68^{a}	5.12 ± 0.23^{a}
Litchi (n=5)	35.4 ± 1.8^{f}	15.67 ± 0.83^{b}
Eucalyptus (n=4)	22.5 ± 0.72^{d}	6.475 ± 0.5^{a}
Karanza (n=4)	23.6 ± 0.56^{d}	9.5 ± 0.2^{b}
Khalsi (n=6)	14.9 ± 2.1^{b}	12.3 ± 1.28^{c}
Goran (n=6)	14.4 ± 1.4^{b}	9.4 ± 0.44^{b}
Bain (n=6)	9.9 ± 0.6^{a}	5.65 ± 0.35^{a}

Values are represented as mean ± SD. Values followed by different superscript letter(s) within each column are significantly different at $P < 0.05$ by Duncan's test.

Polyphenol content: Hizal^{g} > Litchi^{f} > Sesame^{e} > Karanza^{d} > Eucalyptus^{d} > Mustard^{c} > Khalsi^{b}> Goran^{b} > Drumstick^{a} > Bain^{a}.

Flavonoid content: Hizal^{e} > Litchi^{d} > Sesame^{c,d} > Khalsi^{c} > Karanza^{b} > Goran^{b} > Eucalyptus^{a} > Mustard^{a} > Bain^{a} > Drumstick^{a}.

3.2. Flavonoid content

The observed level of mean total flavonoid content of West Bengal honeys varied from 5.12 ± 0.23 to 19.4 ± 1.38 mg of quercetin per 100 g honey with the highest and lowest values obtained in hizal and drumstick honeys respectively (TABLE 1). The flavonoid content of West Bengal honey as measured by $AlCl_3$ method exhibited the order: hizal > litchi > sesame > khalsi > karanza > goran > eucalyptus > mustard > bain > drumstick. The observed flavonoid content is considerably higher than multifloral honeys from Burkina Faso [9] and Romanian acacia honeys [14]. However similar values were observed in case of sunflower and lime honeys

of Romania [14]. A high correlation (r = 0.816) was observed between polyphenol and flavonoid content, which was found to be concomitant with the previous study by [14] (TABLE 2).

TABLE 2. Correlation matrix (pearson's correlaion coefficient) between various variables of honey samples.

	Polyphenol	Flavonoid	ABS_{450}	$DPPH(1/IC_{50})$	FRAP
Flavonoid	0.816				
ABS_{450}	0.981	0.797			
$DPPH(1/IC_{50})$	0.803	0.665	0.732		
FRAP	0.469	0.438	0.563	0.029	
Hydroxyl	-0.613	-0.207	-0.546	-0.594	0.089

3.3. Color intensity (ABS_{450})

The color intensity of 50% honey solutions were calculated spectrophotometrically and net absorbance varied from 422 ± 49.7 mAU in pale white drumstick honey to 2055.6 ± 132.9 mAU in dark brown hizal honey (Fig. 1). The net absorbance of different West Bengal honeys were seen to decrease in the order hizal > litchi > karanza > sesame > eucalyptus > mustard > khalsi > goran > bain > drumstick. ABS450 values of Slovenian and Italian honeys were reported to vary in the range of 70 to 495 mAU and 25 to 3413 mAU respectively [1, 13]. The color intensity (ABS_{450}) might be related to the presence of pigments like carotenoids, flavonoids, Maillard reaction products which are known to have antioxidant activity. A high positive correlation observed between color intensity and polyphenol content (r = 0.981), flavonoid content (r = 0.797) and $1/IC_{50}$ value of DPPH (r = 0.732) was indicative of the fact that darker honeys have good radical scavenging properties which may be due to the presence of polyphenols and flavonoids (TABLE 2).

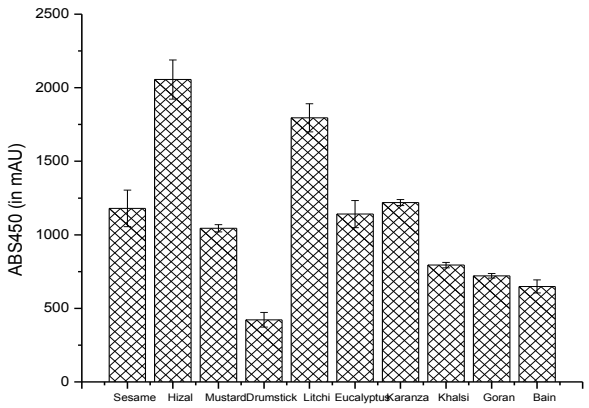

Fig. 1. Color intensity (ABS_{450}) of various floral origin of honeys collected from West Bengal
Values are presented as mean ± SD. Values followed by different superscript letter(s) within each column are significantly different at $P < 0.05$ by Duncan's test.
Color intensity: $hizal^{h} > litchi^{g} > karanza^{e,f} > sesame^{f} > eucalyptus^{e} > mustard^{d} > khalsi^{c} > goran^{b,c} > bain^{b} > drumstick^{a}$.

3.4. FRAP assay

FRAP assay is a reliable test that is widely used for measuring total antioxidant capacity and is based on the capability of the sample to reduce the Fe^{3+} to Fe^{2+} in the presence of TPTZ, forming a blue coloured Ferrous - TPTZ complex with an absorption maxima at 593 nm. The mean FRAP values, expressed as Fe (II) (μM) 10% of honey solution, was found to be highest in case of litchi honey (622.1 ± 3.5) followed by eucalyptus > mustard > goran > khalsi > hizal > sesame > bain > karanza > drumstick (TABLE 3). These results are similar to those obtained by Bertoncelj *et al.* [13], Perna *et al*. [15] for Slovenian and Italian honeys respectively. Mean FRAP value of West Bengal eucalyptus honeys (443.16) were found to be higher than those observed in case of Italian eucalyptus honeys (252.02).

TABLE 3. FRAP values and DPPH radical scavenging activity of different floral origin of honeys collected from West Bengal.

Floral origin of honey	FRAP Values (μM Fe (II) equivalence)	DPPH radical scavenging activity (IC_{50} in mg/ml)
Sesame (n=7)	$340.6 \pm 2.8^{c,d}$	39.5 ± 0.4^{b}
Hizal (n=5)	$381 \pm 1.9^{d,e}$	23.92 ± 1.12^{a}
Mustard (n=6)	400.1 ± 1.8^{e}	60.37 ± 1.57^{d}
Drumstick (n=5)	101.6 ± 1.9^{a}	82.22 ± 1.03^{g}
Litchi (n=5)	622.1 ± 3.5^{g}	72.14 ± 1.28^{f}
Eucalyptus (n=4)	443.16 ± 3.2^{f}	55.42 ± 1.45^{c}
Karanza (n=4)	244.15 ± 2.05^{b}	59.22 ± 1.07^{d}
Khalsi (n=6)	386.8 ± 2.4^{e}	89.88 ± 1.42^{i}
Goran (n=6)	387.9 ± 2.6^{e}	67.15 ± 1.18^{e}
Bain (n=6)	$281.85 \pm 2.3^{b,c}$	84.29 ± 1.22^{h}

Values are presented as mean ± SD. Values followed by different superscript letter(s) within each column are significantly different at $P < 0.05$ by Duncan's test.
FRAP values: litchi^{g} > eucalyptus^{f} > mustard^{e} > goran^{e} > khalsi^{e} > hizal^{d,e} > sesame^{c,d} > bain^{b,c} > karanza^{b} > drumstick^{a}
DPPH radical scavenging activity: hizal^{a} >sesame^{b} > eucalyptus^{c} > karanza^{d} > mustard^{d} > goran^{e} >litchi^{f} > drumstick^{g} > bain^{h} > khalsi^{i}.

3.5. DPPH assay

DPPH is stable nitrogen centered free radical and is extensively used for determining antioxidant activity. DPPH assay measures hydrogen (or electron) donating ability of the samples thereby decolourising DPPH radical from purple to yellow converting it to its reduced form. TABLE 3 shows the scavenging ability of the studied honey samples expressed as IC_{50} value which is inversely proportional to its antiradical potency. Our study indicates that khalsi honey is the least active with an IC_{50} value of 89.88 ± 1.42 mg/mL however hizal honey with an IC_{50} value of 23.92 ± 1.12 mg/mL is the most potent DPPH scavenger among the studied West Bengal honey samples. DPPH radical scavenging ability decreases in the sequence: hizal > sesame > eucalyptus > karanza > mustard > goran > litchi > drumstick > bain > khalsi. As depicted in TABLE 2, strong positive correlation between $1/IC_{50}$ and polyphenol content ($r = 0.803$) lead us to conclude that phenolic compounds in honey might be responsible for its antiradical potency.

3.6. Hydroxyl radical scavenging activity

Hydroxyl radical belongs to the group of extremely reactive oxygen species and is known to cause lipid peroxidation and cellular damage in significant proportions by removing hydrogen atoms from unsaturated fatty acids. Hydroxyl radicals generated by ascorbic acid, iron and EDTA inside *in vitro* system were reduced significantly due to the hydroxyl radical scavenging activity of honey samples. Hydroxyl radicals formed as a consequence of oxidation reacts with DMSO to yield formaldehyde, which were detected by treatment with Nash reagent. Khalsi and goran honey exhibited a significantly higher hydroxyl radical scavenging activity of 80.89 ± 1% and 79.2 ± 2.1% respectively at 150μg honey polyphenol (Fig. 2). This is in agreement with the findings of several other researchers who have reported a dose-dependent activity in other foods such as sesame coat and pomegranate peel [16, 12]. Hydroxyl radical scavenging activity of West Bengal honeys at 150μg honey polyphenol followed the order- khalsi > goran > bain > litchi > drumstick > mustard > karanza > eucalyptus > sesame > hizal. With the increase in polyphenol concentration percentage of hydroxyl radical scavenging activity was found to increase. It can be concluded that inspite of having low polyphenol and flavonoid content, mangrove honeys (khalsi, goran and bain) exhibited potent hydroxyl radical scavenging ability at the same polyphenol concentration. The reduction in rate of chain reaction of reactive oxygen species during lipid peroxidation can thus be attributed to the ability of these honeys to quench hydroxyl radicals which inhibits its subsequent propagation.

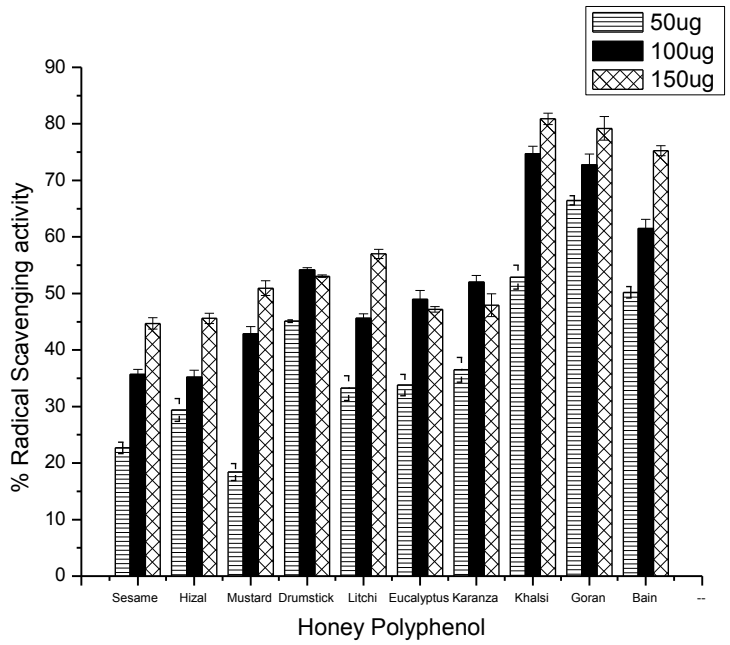

Fig. 2. Hydroxyl radical scavenging activity of different floral origin of honeys collected from West Bengal.

The values are represented as mean ± SD. Different superscript letter(s) are significantly different $P < 0.05$. At150 μg polyphenol level, khalsi^{g} > goran^{g} > bain^{f} > litchi^{e} > drumstick^{d} > mustard^{c} > karanza^{b} > eucalyptus^{b} > sesame^{a} > hizal^{a}.

3.7. Principal Components Analysis

PCA was done to present a data structure study in a reduced dimension, retaining the maximum amount of variance present in the data. The results of PCA based on chosen dimensions were shown in Fig. 3 and Fig. 4. The first three principal components explained 96.14% of the total variation (eigen values: 3.926, 1.275 and 0.568 respectively) (TABLE 4). The third principal component with eigen value less than 1 (0.568) was also included in the PCA study as it could explain another 9.464% of the variance. Also the Scree plot stabilizes after the third component (Fig. 4). According to the rotated component matrix, color intensity, polyphenol content, flavonoid content and DPPH radical scavenging activity ($1/IC_{50}$) were the dominant parameters in the first principal component (PC1) explaining 65.428% of the variance (TABLE 5). The second component (PC2) accounts for 25.251% of variance was dependent on hydroxyl radical scavenging while the third component (PC3) that represents 9.464% of the variance was primarily the function of FRAP values. In terms of the variables hydroxyl radical scavenging activity and FRAP were significantly different from the rest of the analyzed variables, whereas variable reduction can be done from color intensity, polyphenol content, flavonoid content or DPPH radical scavenging activity as they all construct the principal component 1.

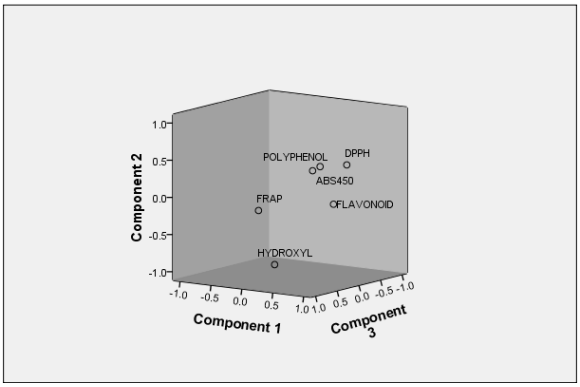

Fig. 3. Component plot in rotated space

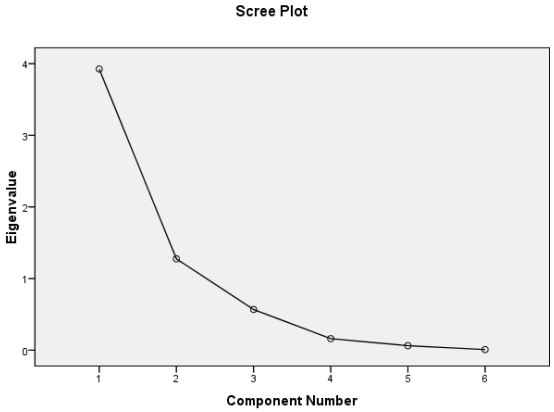

Fig. 4. Scree plot of principal component analysis

TABLE 4. Principal component analysis

Total variance explained			
Principal component	Initial Eigen values	% of variance	Cumulative %
1	3.926	65.428	65.428
2	1.275	21.251	86.679
3	0.568	9.464	96.143
4	0.161	2.677	98.820
5	0.063	1.043	99.863
6	0.008	0.137	100.000

TABLE 5. Rotated component matrix[a]

	Principal components		
	1	2	3
Color intensity	0.717	0.455	0.501
Polyphenol	0.763	0.500	0.394
Flavonoid	0.918	-0.003	0.306
FRAP	0.171	-0.067	0.972
Hydroxyl radical	-0.209	-0.965	0.057
DPPH($1/IC_{50}$)	0.832	0.456	-0.124

Extraction method : Principal component analysis.
Rotation method : Varimax with Kaiser normalization.
[a] Rotation converged in 5 iteratons.

The scatter plots reported in Fig. 5 indicates the antioxidant behavior of different honeys collected from various regions of West Bengal on the basis of the three new variables PC1, PC2 and PC3. When the honey samples were projected on PC1 and PC2 (Fig. 5a), moving along PC1 from left to right in the graph, we found intermediate polyphenol containing honeys (mustard, eucalyptus, karanza, drumstick) separated and grouped in the first quadrant. Hizal honey, which had the highest polyphenol content, was quite far from the others. However, mangrove honeys (khalsi, goran and bain) were sharply separated from the others along the PC2 axis. When samples were plotted on PC2 and PC3 (Fig. 5b), drumstick honey was well separated from litchi honey along the PC3 axis, on account of their differences in FRAP values.

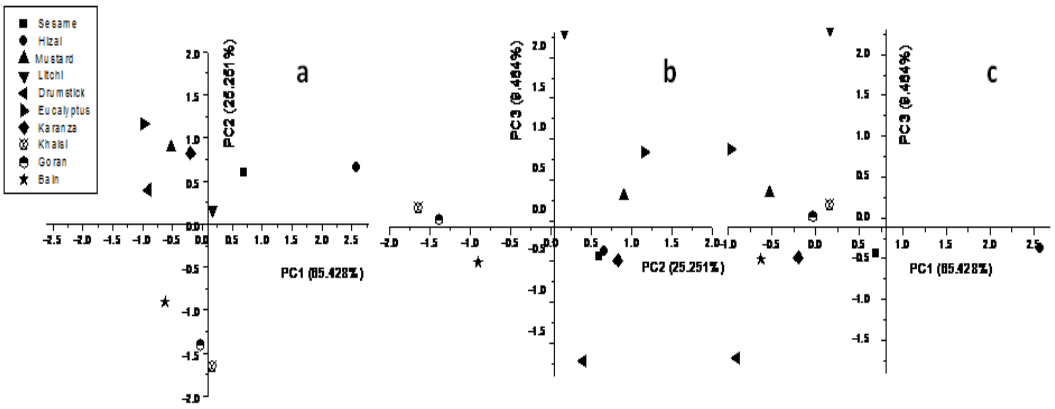

Fig. 5. Scatter plots of principal component analysis for various West Bengal honeys of different floral origins (a) principal components 1 and 2 (b) principal components 2 and 3 (c) principal components 1 and 3

IV. Conclusion

The study on various parameters determining antioxidative properties of honey samples of various regions from West Bengal, India, in a stochastic process of different statistical analysis helped to explore the individual benefits of each cluster of honey sample. The various parameters/constituents contributing to the antioxidative property of honey was also established while working on the honey samples as a consequence of multiple analysis of honey variance. The most reveling feature of the study was that honey samples from mangrove forests of *Sundarban* had significantly better hydroxyl radical scavenging activity than the rest of the sample at the same polyphenol concentration. This study also inspires further research to exploit natural products from *Sundarban* delta region as a source of higher nutritional benefits.

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