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Physicochemical and Antioxidant Properties of Honey: Effect of Heat and Storage

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Abstract
Seven commercially processed and two unprocessed honey samples of different origins were evaluated for their physicochemical, biochemical and anti-oxidant properties. The initial values of the different parameters varied significantly ($p < 0.05$) and ranged as follows: pH, 3.65 - 4.16; colour intensity, 103 - 1029 mAU; HMF, 1.00 - 58.85 mg/kg; proline, 34.51 - 520.11 mg/kg; phenolic, 32.48 - 86.73 mg GAE/100g; flavonoid, 2.27 - 9.73 mg QE/100g; IC_{50}, 90.00 - 198.95 mg/mL. Except proline, the different parameters tested were found to conform to the values set by the Codex Alimentarius Commission (2002). The impact of thermal treatment was also investigated on the physicochemical and biochemical parameters. The results showed a tremendous increase in the HMF in samples originated from Mauritius (**4)** and India (**8**) as the temperature was increased which may be an indication of adulteration while the proline content showed a decrease for all the samples compared to their initial values. Anti-oxidant properties of the honey samples were found to increase and showed strong correlation with the phenolics, flavonoids and colour intensity. No significant variation was observed in the different parameters of the honey samples stored for 3 months after opening except for HMF value for sample **4**.

***Keywords*:** Honey, Physicochemical parameters, Anti-oxidant, HMF, Proline

1. INTRODUCTION

Honey is a supersaturated solution of sugars containing a complex mixture of enzymes, vitamins, minerals, amino acids, phenolic, organic acids, and flavonoids among others [1]. Its composition depends on the seasonal, geographical and their floral origins [2]. Honey has been traditionally used as alternative medicine and natural therapy [3, 4]**.** It is a functional food and also used in modern medicine for its pharmacological properties which include anti-inflammatory, antidiabetic, antitumour, antibacterial and anti-oxidant [5-7]. The physicochemical properties such as colour intensity, pH, proline and hydroxymethylfurfural (HMF) contents are the main quality indicators of honey [8-10].

Many researchers have reported that the quality or certain nutritional properties of honey can be degraded during processing, thermal treatment and storage [11-13]. During processing honey is heated at temperatures ranging from 30 to 140 °C to reduce crystallisation and water content in order to increase its shelf life [14, 15]. The preferred level of HMF in fresh honey samples is less than 15 mg/kg. According to the standard Codex Alimentarius Commission, 2000, the level of HMF in honey should not exceed 40 and 80 mg/kg for European and tropical countries respectively. 5-Hydroxymethylfurfural (HMF), which is carcinogenic and cytotoxic to human health and is normally not present in fresh honey, however it may be formed after processing and thermal treatment [16]. Proline is the major free amino acid and it is a measure of the level of total amino acids in honey [17, 18]. According to the International Honey Commission, the minimum acceptable limit for proline content in honey is 180 mg/kg [19]. A low proline and high HMF contents is an indication that the honey sample is not fresh and it may contain adulterants [20].

Mauritius imports several brands of honey from different countries, which are consumed by the local population. Honey is taken either directly or indirectly as traditional medicine or for cooking purposes.

The main objectives of this research were to determine the physico-chemical, biochemical and anti-oxidant properties of nine honey samples commonly consumed in Mauritius. The study is also extended to understand the effect of heat treatment and storage on the quality of the honey samples.

2. EXPERIMENTAL

2.1 General

All solvents were of analytical grade purity. Methanol and HPLC-grade of propan-2-ol were supplied by Romil Ltd (Cambridge, England). Ethylene glycol monoethyl ether, L-ascorbic acid, gallic acid, quercetin, 2,2'-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu phenol reagent and HMF standards (Grade: Analytical standard, Assay: ≥ 98 %) were purchased from Sigma-Aldrich while formic acid, ammonia (17.5 % w/v) and ninhydrin were obtained from BDH.

2.2 Samples

Nine honey samples (**1 - 9),** which are commonly consumed by Mauritians and originated from different countries were selected for this study (Table 1). Samples **1** and **2** were fresh honey samples while the remaining seven honey samples were commercial brands available on shelf in supermarkets and all of them were of unknown floral origin and used without pretreatment. All the honey samples were stored at ambient temperature (23 – 28 °C) and kept in the dark until further analysis.

TABLE 1. Brand and Origin of honey samples

Sample code	Sample Brand/Name	Country Origin/ Geographical region	Details
1	NA	Mauritius	Manufacturing date: NA Expiry date: NA; Lot no: NA
2	**Les Verges de Labourdonnais** Pure Honey	Mauritius	Manufacturing date: NA Expiry date: 29/07/19; Lot no: 230/17
3	**Dabur** Pure Honey	India (Bihar and in Madhya Pradesh)	Manufacturing date: 07/16 Expiry date: 06/18; Lot no: BD 2310
4	**Sunny** Pure Honey	Mauritius	Manufacturing date: NA Expiry date: 14/02/19; Lot no: NA
5	**Alshifa** Natural Honey	Saudi Arabia	Manufacturing date: 03/17 Expiry date: 02/22; Lot no: NA
6	**Hosen** Pure Honey	China	Manufacturing date: 12/03/17 Expiry date: 11/03/19; Lot no: NA
7	**Food Lovers** Honey	South Africa	Manufacturing date: NA Expiry date: 16/03/17; Lot no: NA
8	**Tropic** Pure Natural Honey	India	Manufacturing date: 04/16 Expiry date: 03/18; Lot no: TP-005
9	**Wescobee** Pure Honey	Western Australia	Manufacturing date: 13/06/17 Expiry date: 12/06/20 Lot no: W13/06/17

2.3 Thermal treatment

The honey samples (**1** - **9**) were subjected to thermal treatment, involving conventional heating on a hot plate to 40, 60, 80, and 100 °C for 1 min, oven heating for 20 min at 180 °C and microwave heating by irradiation for one min at 60 °C. The samples were also exposed to sunlight for 5 consecutive days.

2.4 Physicochemical analysis

2.4.1 pH

Honey solutions (10% (w/v) on a dry weight basis) were prepared in distilled deionised water and the pH were measured at room temperature using a pH meter (Jenway, 3510 pH Meter).

2.4.2 Colour analysis

The colour of the different honey samples was determined according to the methods described [21]. Honey samples (**1**-**9**) diluted to 50 % (w/v) solution with warm (45 - 50 °C) ultra-purified water and filtered through a 0.45 µm filter paper. The absorbance was read at 450 and 720 nm using a spectrophotometer (Biochrom Libra S22 UV-Vis spectrophotometer). The colour intensity ABS_{450} of the honey samples was obtained by the net difference in the absorbance values and was expressed in mAU units.

The absorbance (*A*) of the honey solutions was also measured at 635 nm and the colour intensity was determined on Pfund scale (mm) according to equation 1 [22].

$$Pfund\ (mm) = -38.70 + 371.39A \qquad \text{Eq 1}$$

2.4.3 Hydroxymethylfurfural (HMF) analysis

HMF was determined using HPLC method [23]. Honey samples (1 ± 0.1 g) were diluted into 10 mL with deionized water; filtered using 0.45 µm PTFE syringe filter and injected into an HPLC system (Dionex Ultimate 3000 UHPLC). The HPLC column was a Luna Phenomenex with phase C18 (2), dimension 150 x 2 mm and 3 µm particle size. The HPLC conditions were: an isocratic mobile phase, ammonium formate buffer and methanol in the ratio 90: 10; flow rate, 0.2 mL/min; column oven (temperature); 30°C. The chromatograms were monitored at 285 nm. The HMF content of the honey samples was calculated by comparing corresponding peak areas of the standard solutions against that of honey samples.

2.5 Determination of proline content

The proline content was determined by the method reported [24]. 5 mL of the honey solution (0.05 g/mL) was diluted with 1 mL of 80 % formic acid and 1 mL of ninhydrin solution containing 3 % ethylene glycol mono ethyl ether and the mixture was shaken for 15 min. The resulting mixture was kept in boiling water for 15 min and at 70 °C for a further 10 min in which propan-2-ol (5 mL, 50 %) was added and the mixture was allowed to cool. The absorbance was read at 510 nm and water was used as blank. 0.032 mg/mL solution of proline was used as standard solution. The proline concentration in the samples was expressed in mg/kg and calculated using the equation 2.

$$[Proline] = \left(\frac{As}{A0}\right) * \left(\frac{m1}{m2}\right) * 80 \qquad \text{Eq 2}$$

where As is the absorbance of the sample solution; Ao is the absorbance of the proline standard solution; m1 is the mass of proline in mg in the standard solution; m2 is the mass of honey sample in grams; 80 is the dilution factor.

2.6 Determination of total phenolic content

The honey sample (0.125 mL, 0.2 g/mL) was mixed with Folin-Ciocalteu reagent (1.25 ml, 0.2 N) and sodium carbonate (0.5 mL, 80 g/L). The mixture was incubated for 2 h at ambient temperature (25 °C) and the absorbance was read at 760 nm against a methanol blank [25]. Gallic acid (0 - 10 µg/mL) was used as standard solution and total phenolic content was reported as the mean ± standard deviation and expressed in mg of gallic acid equivalents (GAE)/100 g of honey.

2.7 Determination of total flavonoid content

0.5 mL of the honey sample (0. 2 mg/mL) was added to a solution of $AlCl_3$ (0.5 mL, 2 %) in methanol and the absorbance of the resulting solution was read at 440 nm [25]**.** Quercetin (0 - 0.05 mg/mL) was used as standard solution. The total flavonoid content was reported as mean ± standard deviation and expressed in mg of quercetin acid equivalents (QE)/100 g of honey.

2.8 DPPH radical-scavenging activity

The honey sample (100 µL, 0.2 g/mL) was serially diluted with methanol in a 96 well microplate. The solution was mixed with DPPH (100 µL, 0.045 mg/mL) and absorbance was read at 492 nm using a Microplate Reader (Perlong DNM-9602). The percentage scavenging activity of the samples was calculated according to equation 3 [25].

$$\%\, Inhibition = [Ao - As)/Ao] * 100 \qquad \text{Eq 3}$$

where A_o is absorbance of control and A_s is absorbance of honey sample.

A graph of % inhibition was plotted against concentration (200 – 6.25 mg/mL). From the graph, the IC_{50}, the concentration of the honey sample required to inhibit 50% of free radical scavenging activity was determined.

2.9 Statistical Analyses

All the tests were performed in triplicate and the results are expressed as mean ± standard deviation. Results were input and analysed by MS Excel and SPSS Version 20. A principal component analysis (PCA) has been performed to determine any association between parameters (HMF, proline, phenolic, flavonoid and IC_{50}) when honey samples were stored for a period of three months away from sunlight.

3. RESULTS AND DISCUSSION

The initial physicochemical and biological properties at room temperature of the nine honey samples (**1**- **9**) are presented in Table 2.

3.1 Physicochemical properties

Colour is a primary characteristic of honey and its intensity depends on its concentration of phenols, minerals and other minor components. Honey is normally classified into seven colour categories [26]. The colour of the honey samples (1-9) in this study varied from white to Amber. The colour was also graded according to the Pfund scale [22] and these values were in agreement with results obtained by other researchers [21, 27]. The significant variation in colour can be explained by the presence of varying number of phytochemicals, which could be due to their different geographical and/or floral origin.

pH is another parameter which influences the texture, stability and shelf life of honey [28]. The pH of all the tested samples was acidic in the range of 3.65 - 4.16 and was within the required standard limit of the Codex Alimentation (pH 3.40 - 6.10).

The amount of HMF in a honey sample depends on its quality, processing and storage time rather than its origin [29]. The initial HMF content of the honey samples (**1**-**2** & **5**-**9**) were found to be in the range of 1.00 - 6.81 mg/kg. The HMF content for samples **3** and **4** (53.31 & 58.85 mg/kg) were higher than the limit set for European countries (40 mg/kg) but lower than that required for tropical countries (80 mg/kg) [23]. This study also shows that natural and untreated honey (**1** and **2**) have lower HMF content compared to processed honey (**4**) even if they are from the same geographical origin. The low concentration of HMF of most of samples tested indicated that the honey consumed in Mauritius is of good quality.

Honey contains several amino acids with proline being the most predominant. The initial proline content of the commercial honey samples (**5**, **7**, **8** & **9**) was above the level while the other samples (**1**, **2**, **3**, **4** and **6**) were lower than the required level of 180 mg/kg. It is known that if the proline content is below the value recommended by the Codex Alimentarius, indicates adulteration in honey [30].

TABLE 2. Physico and biochemical characteristics of honey samples (**1**–**9**) at room temperature

Samples	Origin	Pfund Scale (mm)	Colour	Colour intensity (mAU)	pH	HMF (mg /kg)	Proline (mg/kg)	phenolic (mg GAE/100 g)	Flavonoid (mg QE/100 g)	IC_{50} (mg/ml)
1	Mauritius	46.58	Extra light amber	216.0±8.5	4.16±0.00	5.08± 0.36	34.52 ± 3.35	35.39 ± 2.33	2.55 ± 0.13	159.72
2	Mauritius	60.22	Amber	792.5±16.2	3.75±0.02	5.07± 0.36	135.28 ± 5.68	52.24 ± 2.48	2.78 ± 0.23	166.38
3	India	76.85	Amber	813.0±16.9	3.99±0.01	53.31± 3.81	173.27 ± 4.76	68.61 ± 1.09	6.25 ± 0.33	90.60
4	Mauritius	53.56	Light amber	324.0±4.2	3.71±0.00	58.85± 4.20	80.06 ± 6.18	44.69 ± 1.45	5.17 ± 0.76	160.19
5	Saudi Arabia	84.17	Amber	979.0±22.6	3.80±0.03	2.89± 0.21	501.99 ± 5.94	70.03 ± 1.62	8.93 ± 0.39	95.82
6	China	31.94	White	103.0±9.9	3.75±0.01	2.63± 0.19	66.73 ± 4.34	32.48 ± 0.23	2.27 ± 0.04	198.95
7	South Africa	87.83	Amber	776.0±31.1	3.65±0.00	1.60± 0.11	520.12 ± 11.41	73.29 ± 0.76	8.03 ± 0.23	140.15
8	India	101.80	Amber	998.5±38.8	3.89±0.00	6.81± 0.49	308.42 ± 10.28	86.73 ± 0.85	7.84 ± 0.35	90.00
9	Western Australia	108.46	Amber	1029.5±30.4	3.73±0.00	1.00± 0.07	498.58 ± 8.31	74.73 ± 0.65	9.73 ± 0.68	103.62

3.2 Biochemical characteristics

In general, dark honey samples have a significant higher content of flavonoid and phenolics compounds compared to light coloured honey samples. The total phenolic and flavonoid contents of the honey samples (**1** - **9**) analysed were found to be in the range of 32.48 - 86.73 mg GAE/ 100 and 2.27 - 9.73 mg QE/ 100 g respectively.

The antioxidant properties of honey are related to the presence of enzymatic and non-enzymatic substances [22]. The anti-oxidant activity was determined using the 2, 2'-diphenyl-1-picryhydrazyl (DPPH) assay. The free radical scavenging activity was expressed as IC_{50} which represents the concentration of antioxidant required to inhibit 50 % of free radicals. The samples tested were found to have IC_{50} values ranging from 90.00 - 198.95 mg/mL. The variation in the anti-oxidant properties is highly dependent on the concentration of polyphenols which is mainly influenced by the floral and geographical origins [31, 32]. In addition, many researchers have reported that colour intensity, which is directly related to the phenolic content has a direct impact on the anti-oxidant property [5]. In the present work it was also observed that the white coloured honey (**6**) had lowest activity (IC_{50} 198.95 mg/mL) and the activity was found to increase as the colour intensity of the honey samples increases.

3.3 Correlation analysis

Correlation analysis was performed to identify the relationships between physicochemical and biochemical parameters of the honey samples tested at the initial stage (Table 3). In general, a significantly high correlation is observed between the IC_{50} and phenolic, flavonoid, proline and the colour intensity. Therefore, these parameters are related to the anti-oxidant activity of honey. Specifically, phenolic content is positively correlated with flavonoid and proline. However, it shows a negative correlation with IC_{50}. Hence, as the levels of phenolic, flavonoid and proline increase, IC_{50} decreases significantly. Colour intensity is also highly correlated with these parameters in the sense that with the increase in colour intensity, phenolic, flavonoid and proline increase while IC_{50} decreases.

TABLE 3. Correlation matrix showing the interrelation among the different parameters at room temp

	IC_{50}	Phenolic	Flavonoid	Proline	HMF	Colour intensity
IC_{50}	1					
Phenolic	-.874	1				
	.002					
Flavonoid	-.821	.874	1			
	.007	.002				
Proline	-.621	.789	.904	1		
	.075	.012	.001			
HMF	-.098	-.113	-.082	-.411	1	
	.803	.772	.833	.272		
Colour	-.839	.927	.805	.775	-.184	1
Intensity	.005	.000	.009	.014	.635	
pH	-.173	-.188	-.311	-.444	.102	-.204
	.656	.628	.416	.231	.793	.598

The values in brackets are the p-values

*: significant at 5% level.

**: significant at 1% level.

3.4 Thermal effect

The objective of this study was to investigate the influence of different heat treatments at various temperatures (40, 60, 80 and 100 °C), oven, microwave and sunlight on the different parameters of honey samples (**1-9**). The results are summarised in Table 4.

It was observed that the different thermal treatments had no influence on the acidity of the honey samples (**1-9**) since the pH remain constant. The average colour intensity increased when the samples were heated from 40 to 100 °C. Moreover, oven heating triggered the formation of brown pigment considerably resulting in an increase in colour intensity as compared to microwave and direct sunlight heating.

It is known that the rate of the Maillard reaction increases with increasing temperature where sugars react with amino acid resulting in the formation of a variety of brown pigments and HMF as intermediate product [14]. HMF is also be formed by the acid-catalyzed dehydration of hexoses [33, 34]. Thermal, microwave, oven and sunlight heating resulted in the increase of HMF content except in samples **2**, **6** and **9** (Figure 1a)**.** Samples **3**, **4**, **5** and **8** exceeded the acceptable limit of 80 mg/kg for tropical countries. Sunlight heating did not affect the HMF content of the honey samples significantly. The results regarding the increase of the HMF content with increasing temperature from 40 to 100° C (Figure 1b) were concordant with the results obtained by other researchers [14, 35, 36].

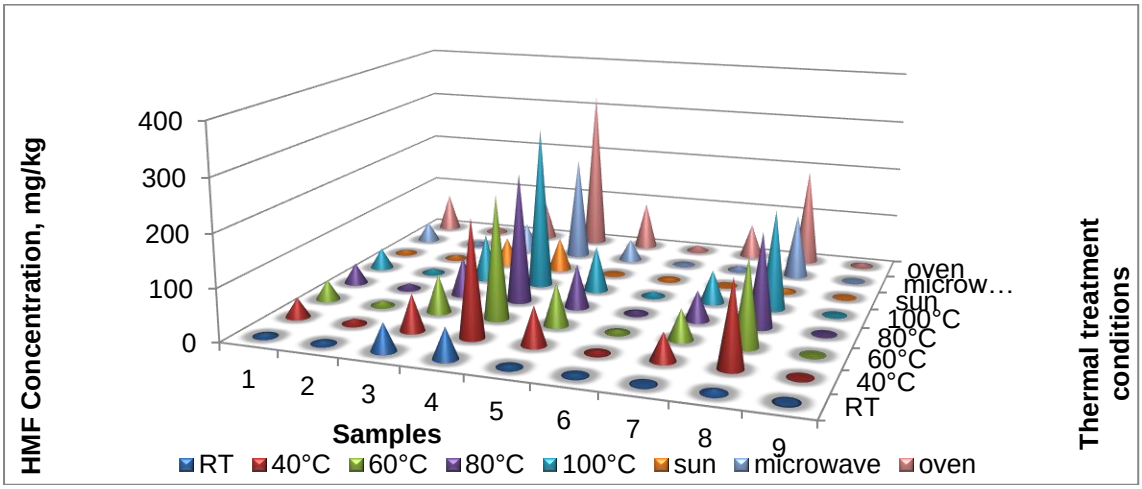

FIGURE 1a. Thermal effect on HMF concentration

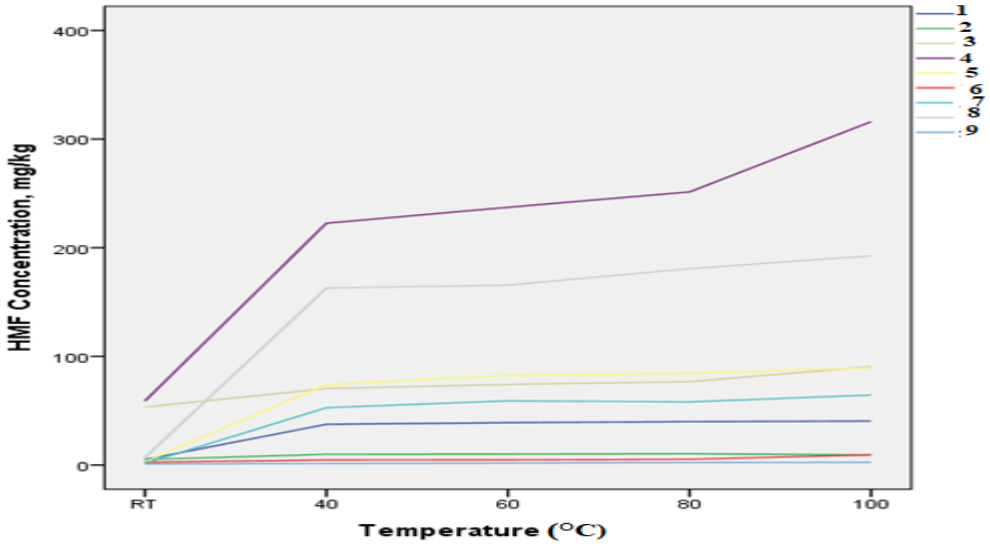

FIGURE 1b. Thermal effect on HMF concentration on heating from 40 to 100 °C

On the other hand, it was observed that different thermal treatments resulted in a decrease in proline content, which is usually associated with denaturation of protein [12]. Compared to the different treatments, oven heating caused a marked decrease in the proline content.

Although many researchers indicated a positive effect for anti-oxidant activity which increases with thermal treatment [15, 37] while some others presented contradictory results. Table 4 shows the changes in the phenolic, flavonoid and anti-oxidant activity after the honey samples were subjected to various heat treatments. As the temperature, increases from 40 to 100 °C both the phenolic and flavonoid contents were observed to increase. Heating at 180 °C in an oven, showed a tremendous increase while when the samples were exposed to direct sunlight no significant change was observed. In this study a constant decline in IC_{50} was observed indicative of an increase in anti-oxidative activity with increase in temperature (Figure 2). For oven and microwave heating a decrease in IC_{50} was observed while sunlight exposure showed no significant effect on the IC_{50} values. The increase in the antioxidant activity may be due to the formation of thermo resistant compounds, which are activated at high temperature and also due to the increase in phenolic and flavonoid contents.

Paired samples T-Test was done to identify whether there were significant differences ($p < 0.05$) between results obtained for different parameters tested (pH, ABS_{450}, HMF, Proline, TPC, TFC and IC_{50}) at room temperature (RT) and other thermal conditions. Significant differences were observed for proline, TPC, TFC and IC_{50} except for TFC under microwave treatment (Table 5).

TABLE 4. Effect of thermal treatment physico and biochemical characteristics of honey samples (**1**–**9**)

	Sample No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
pH	RT	4.16±0.00	3.75±0.02	3.99±0.01	3.71±0.00	3.80±0.03	3.75±0.01	3.65±0.00	3.89±0.00	3.73±0.00
	40	4.16±0.01	3.75±0.00	4.00±0.00	3.71±0.00	3.78±0.01	3.75±0.00	3.65±0.01	3.90±0.01	3.73±0.00
	60	4.16±0.00	3.76±0.01	3.98±0.00	3.71±0.00	3.78±0.00	3.75±0.00	3.66±0.00	3.90±0.01	3.73±0.01
	80	4.15±0.00	3.76±0.00	3.98±0.01	3.72±0.00	3.79±0.01	3.74±0.02	3.64±0.01	3.89±0.01	3.73±0.03
	100	4.16±0.02	3.75±0.01	3.98±0.01	3.71±0.01	3.77±0.02	3.74±0.01	3.64±0.02	3.89±0.01	3.72±0.02
	sun	4.14±0.01	3.75±0.03	3.99±0.02	3.71±0.02	3.80±0.02	3.76±0.01	3.65±0.00	3.89±0.00	3.73±0.02
	microwave	4.15±0.01	3.74±0.00	4.00±0.01	3.71±0.02	3.79±0.03	3.75±0.00	3.64±0.02	3.87±0.02	3.72±0.01
	oven	4.15±0.00	3.74±0.00	3.98±0.01	3.69±0.01	3.76±0.00	3.74±0.03	3.64±0.00	3.89±0.00	3.72±0.02
ABS_{450} (mAU)	RT	216.0±8.5	792.5±16.3	813.0±16.9	324.0±4.2	979.0±22.6	103.0±9.9	776.0±31.	998.5±38.9	1029.5±30.4
	40	217.0±9.9	799.5±13.4	846.0±29.7	335.5±20.5	982.5±26.2	101.5±6.4	779.0±31.1	1001.0±35.4	1052.0±32.5
	60	220.0±6.3	812.0±22.6	858.0±16.9	336.0±12.7	1000.5±32.8	109.0±12.7	778.0±24.1	1073.0±335.3	1080.5±40.3
	80	231.0±14.8	827.5±6.4	879.5±14.8	346.5±14.8	1013.0±24.4	114.0±17.0	814.0±39.6	1088.5±29.0	1117.5±31.8
	100	277.0±9.9	848.0±12.7	914.5±19.1	409.5±9.2	1024.0±18.4	136.0±15.6	850.5±34.6	1133.5±27.6	1197±26.9
	sun	223.0±16.3	797.0±16.9	874.0±12.7	343.5±31.8	976.5±19.1	109.0±12.7	784.0±35.4	992.5±28.9	1029.5±28.9
	microwave	218.0±9.9	792.0±14.8	838.0±14.8	343±24.0	985.0±35.4	202.0±7.1	816.5±26.2	999.5±37.5	1035.5±27.5
	oven	314.5±6.4	838.0±8.5	937.0±16.9	426.5±4.9	1081.0±35.4	264.5±12.0	890.5±23.3	1176.5±41.7	1201±24.04
HMF (mg/kg)	RT	5.08 ± 0.36	5.07±0.36	53.31±3.79	58.85±4.18	2.89±0.21	2.63±0.19	1.60±0.11	6.81±0.48	1.00±0.07
	40°C	37.53 ± 2.66	9.91± 0.70	70.38±5.00	222.6±15.80	74.01±5.25	4.62±0.33	52.78±3.75	162.84±11.56	1.40±0.10
	60°C	39.03 ± 2.77	10.13± 0.72	74.2±5.27	237.23±16.84	82.64±5.87	4.79±0.34	59.11±4.20	165.59±11.76	1.86±0.13
	80°C	39.95 ± 2.84	10.44± 0.74	76.75±5.45	251.38±17.85	84.43±5.99	5.34±0.38	58.12±4.13	180.75±12.83	2.33±0.17
	100°C	40.51 ± 2.88	9.32± 0.66	90.56±6.43	316.05±22.44	89.35±6.34	9.33±0.66	64.41±4.57	192.59±13.67	2.46±0.17
	sun	6.17 ± 0.44	6.37± 0.45	54.9±3.90	65.53±4.65	2.06±0.15	3.68±0.26	0.53±0.04	6.45±0.46	1.40±0.10
	microwave	39.85 ± 2.83	6.46± 0.46	57.2±4.06	206.44±14.66	42.76±3.04	2.85±0.20	8.7±0.62	126.15±8.96	1.12±0.08

	oven	74.72 ± 5.31	12.1± 0.86	79.39±5.64	324.83±23.06	93.91±6.67	11.12±0.79	67.39±4.78	192.15±13.64	5.96±0.42
Proline (mg/kg)	RT	34.52±3.35	135.27±5.68	173.27±4.76	80.06±6.18	501.99±5.94	66.73±4.34	520.12±11.41	308.42±10.28	498.58±8.31
	40°C	33.89±1.63	125.22±2.93	158.12±4.24	52.37±3.03	374.49±11.52	63.83±2.84	379.56±5.80	199.53±3.60	437.79±3.44
	60°C	27.93±1.14	112.55±4.78	123.90±7.58	48.79±1.99	223.69±6.50	43.88±7.36	348.90±2.60	184.82±3.33	379.77±8.23
	80°C	24.13±1.40	76.06±2.89	114.91±3.82	37.41±2.02	198.35±4.79	25.83±1.29	326.97±8.68	182.34±2.22	342.80±9.2
	100°C	19.17±1.71	75.06±2.66	77.72±4.34	34.13±1.47	195.97±3.17	17.41±2.94	254.42±7.75	126.29±1.14	300.04±11.8
	sun	27.11±1.42	98.72±7.83	104.38±8.33	48.64±5.45	291.01±9.55	34.08±4.08	333.03±8.51	179.63±1.74	363.41±2.7
	microwave	23.77±2.05	74.41±3.12	96.44±4.82	43.90±2.57	250.02±8.22	29.01±3.95	291.56±13.5	180.97±3.93	304.97±4.2
	oven	14.87±1.16	28.91±4.30	68.43±4.53	13.33±4.62	185.73±2.68	9.88±2.27	237.17±2.96	104.97±3.85	282.13±2.64
TPC	RT	35.39±1.37	52.24±2.00	68.61±0.62	44.69±0.51	70.03±0.80	32.48±1.30	73.29±2.2	86.73±1.79	74.73±0.74
	40°C	37.72±1.88	55.19±1.68	72.81±0.59	48.64±0.84	71.85±0.28	33.37±1.52	75.01±0.9	89.52±0.48	77.82±1.08
	60°C	39.92±1.38	58.06±3.53	72.84±0.78	49.88±0.42	71.99±1.09	34.92±0.70	76.51±0.9	93.64±1.46	80.23±1.11
	80°C	40.81±2.72	60.84±1.33	73.43±0.18	50.18±2.66	72.93±1.32	35.65±3.16	79.29±1.9	97.61±1.00	81.95±1.41
	100°C	43.71±2.05	62.46±1.88	80.17±1.58	54.49±2.75	75.78±1.74	40.15±1.58	80.16±1.2	98.37±2.07	87.79±0.97
	sun	42.38±2.07	55.52±2.72	74.82±3.22	54.35±0.52	73.23±0.90	36.10±0.57	85.88±1.5	88.00±0.66	78.79±0.70
	microwave	35.92±1.10	59.42±2.77	74.86±1.33	50.13±2.77	75.30±3.06	37.34±1.36	77.19±2.0	92.97±0.34	78.36±1.06
	oven	44.01±1.19	68.18±0.39	81.78±9.08	54.59±1.19	80.94±1.32	52.87±0.61	99.96±0.1	104.66±1.35	95.53±0.65
TFC	RT	2.55±0.32	2.78±0.08	6.25±0.31	5.17±0.12	8.93±0.46	2.27±0.08	8.03±0.20	7.84±0.75	9.73±0.42
	40°C	2.76±0.17	4.13±0.13	7.53±0.31	5.54±0.20	9.15±0.08	2.36±0.07	8.32±0.60	8.21±0.51	10.79±0.83
	60°C	3.01±0.89	4.85±0.10	8.01±1.34	5.80±0.21	9.63±0.28	2.73±0.07	8.57±0.56	8.44±0.25	11.20±1.31
	80°C	3.13±0.10	5.44±0.28	8.10±0.28	6.16±0.16	9.80±0.50	3.01±0.47	8.60±0.40	9.01±0.59	11.49±1.85
	100°C	3.19±0.15	7.31±0.70	8.38±1.26	7.70±0.40	10.9±0.30	3.30±0.70	10.26±0.48	10.53±1.08	13.99±1.19
	sun	2.57±0.20	2.16±0.39	6.28±0.03	4.86±0.55	8.21±0.13	2.35±0.36	8.16±0.63	7.24±0.70	9.39±0.20
	microwave	2.67±0.79	4.22±0.19	7.19±0.14	5.22±0.31	8.99±0.92	2.67±0.17	8.35±1.18	7.31±0.40	9.98±1.13

	oven	3.34±0.17	7.86±0.07	8.87±0.03	7.92±0.22	12.40±1.08	3.29±0.13	14.43±0.80	11.09±0.47	14.42±0.43
IC_{50}	RT	159.72	166.38	90.60	160.19	95.82	198.95	140.15	90.00	103.62
	40°C	153.71	152.94	85.97	152.23	91.19	188.86	133.06	85.31	100.97
	60°C	141.62	146.41	82.287	148.95	85.93	172.07	126.97	81.85	97.74
	80°C	133.25	140.49	75.967	141.20	86.08	164.90	118.05	74.20	91.18
	100°C	126.80	131.04	72.20	133.05	78.14	155.79	115.24	69.41	88.19
	sun	154.47	163.53	87.53	155.86	92.24	191.63	134.24	82.30	101.93
	microwave	150.30	153.70	62.32	120.08	87.05	161.69	124.85	76.77	101.62
	oven	119.59	120.89	62.12	119.67	72.56	136.74	94.37	73.99	81.65

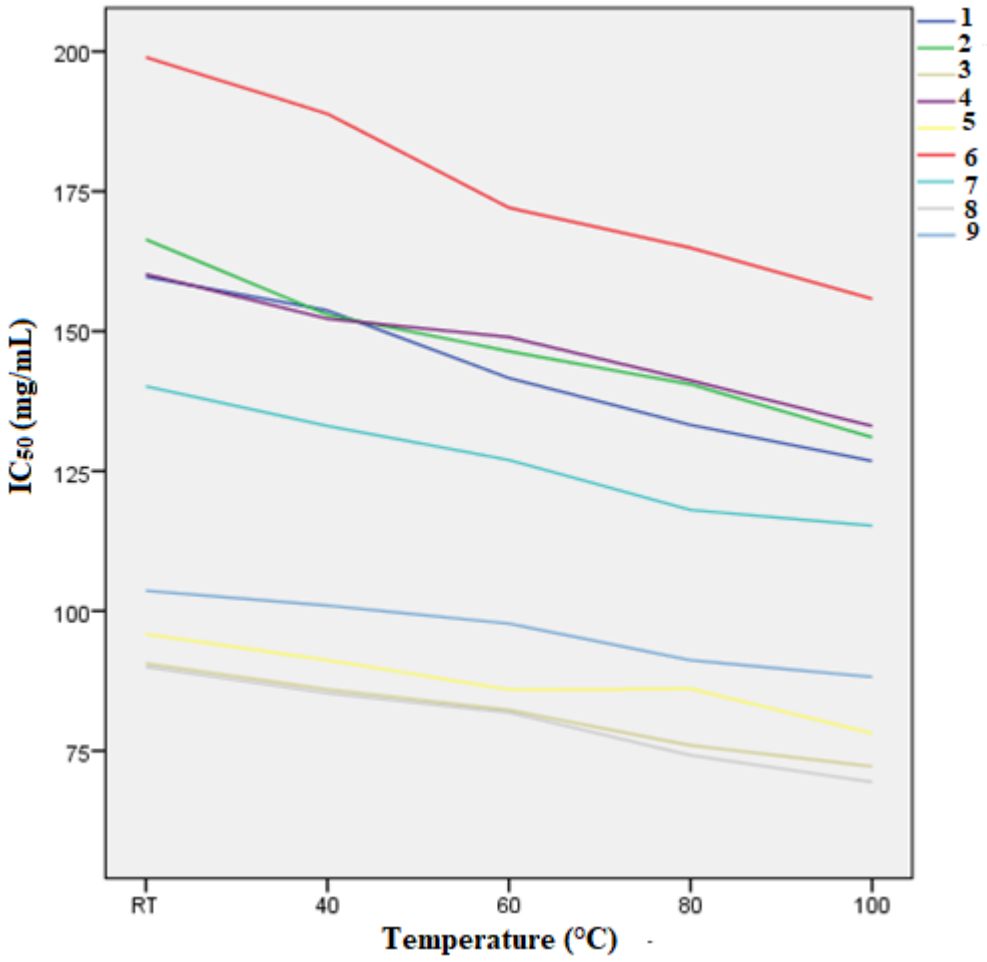

FIGURE 2. Thermal effect on IC_{50} on heating from 40 to 100 °C

TABLE 5. Correlation matrix showing the inter-relation among the different parameters at different conditions

	IC_{50}	Phenolic	Flavonoid	Proline	HMF	Colour intensity
IC_{50}	1					
Phenolic	-.893	1				
	.001					
Flavonoid	-.786	.813	1			
	.012	.008				
Proline	-.640	.769	.879	1		
	.063	.016	.002			
HMF	.186	-.215	-.116	-.437	1	
	.631	.579	.766	.240		
Colour Intensity	-.847	.945	.767	.779	-.375	1
	.004	.000	.016	.013	.320	
pH	-.171	-.183	-.307	-.439	-.168	-.204
	.660	.637	.422	.238	.665	.598

The values in brackets are the p-values

*: significant at 5% level.
**: significant at 1% level.

3.5 Effect of storage

The honey samples are normally not purchased directly from beehivers, but they are purchased from the markets, opened and consumed over a period of time. Keeping this in mind the changes in the anti-oxidant activity, HMF and proline contents were analysed by storing the samples at room temperature (25 - 30°C) away from sunlight for a period of three months after opening the bottles (Table 6).

The HMF content of the honey samples (**1**-**9**) was found to increase significantly after the three months. Honey samples, **3** (60.43 mg/kg), **6** (54.16 mg/kg) and **8** (60.23 mg/kg) were above 40 mg/kg but below the acceptable limit of tropical countries (80 mg/kg). Compared to all the honey samples tested, sample **4** was above the allowed maximum limit of 80 mg/kg for tropical countries, which could be an indication of adulteration with inverted sugars [38]. The HMF content increases with storage time [39], therefore, it can be concluded that honey stored at room temperature (25 – 30 °C) should generally, be consumed within one year.

TABLE 6. Effect of storage on the different parameters of the honey samples

sample	HMF (mg/kg)		Proline (mg/kg)		Phenolic (mgGAE/100g)		flavonoid (mg QE/100g)		IC_{50} (mg/mL)	
	Before storage	After storage	Before storage	After storage	Before storage	After storage	Before storage	After storage	Before storage	After storage
1	5.08	3.43	34.52±3.35	33.48±1.43	35.39±2.33	30.56±1.29	2.55±0.13	2.32±0.09	159.72	162.12
2	5.07	5.25	135.28±5.68	129.14±5.54	52.24±2.48	50.87±0.62	2.78±0.23	2.18±0.79	166.38	168.12
3	53.31	60.43	173.27±4.76	170.68±1.60	68.61±1.09	67.25±1.79	6.25±0.33	5.14±1.59	90.6	93.28
4	58.85	193.67	80.06±6.18	79.45±1.94	44.69±1.45	41.96±2.47	5.17±0.76	4.86±0.56	160.19	164.47
5	2.89	7.36	501.99±5.94	495.24±5.20	70.03±1.62	69.24±2.39	8.93±0.39	7.29±0.60	95.82	97.71
6	2.63	54.16	66.73±4.34	65.69±1.33	32.48±0.23	30.73±2.96	2.27±0.04	1.23±0.79	198.95	204.42
7	1.6	6.67	520.12±11.41	495.02±3.64	73.29±0.76	65.18±3.01	8.03±0.23	6.78±0.60	140.15	142.36
8	6.81	60.23	308.42±10.28	300.63±4.43	86.73±0.85	84.48±0.49	7.84±0.35	6.24±1.02	90.00	94.32
9	1.0	2.12	498.58±8.31	498.50±2.05	74.73±0.65	74.28±1.45	9.73±0.68	9.48±1.28	103.62	104.60

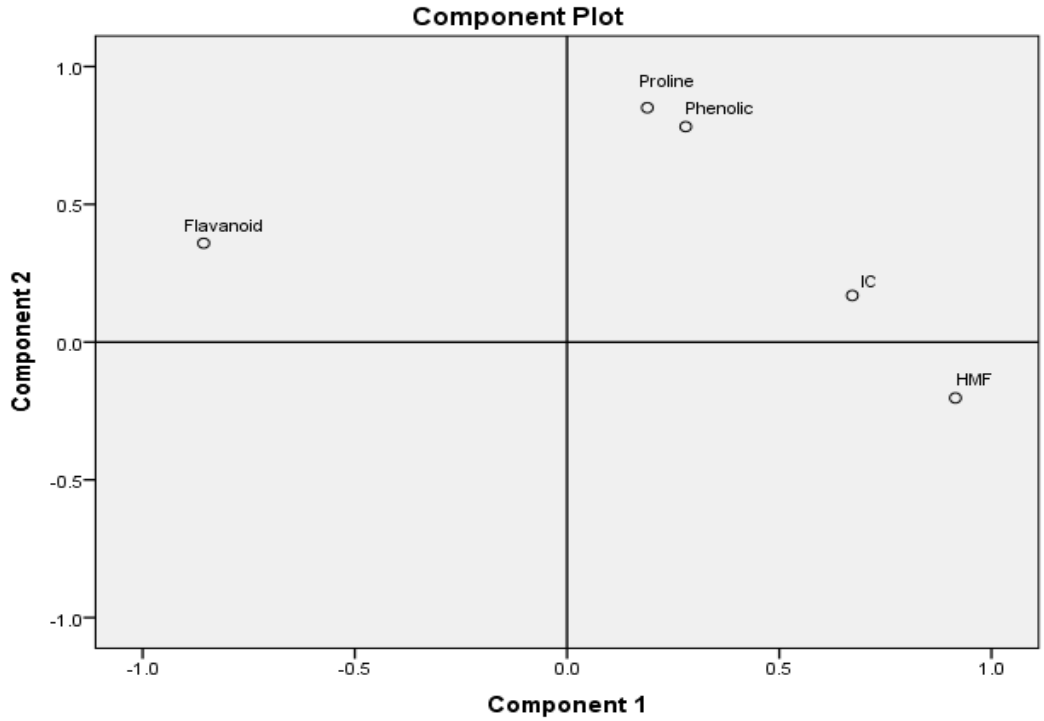

FIGURE 3. % change in concentration due to storage projected on two axes

Some researchers observed no significant change in anti-oxidant activity while others observed a decrease in anti-oxidant activity of commercial honey after storage [40]. In the present study, the total phenolic and flavonoid contents of the samples were found to decrease compare to its initial values after storage. Results obtained for DPPH assay showed an increase in the IC_{50} values after storage in comparison to the initial values indicated that the anti-oxidant activity decreases. Correlation study was performed on the honey samples after storage. The parameters showed similar correlation patterns as observed at the initial stage. Significant differences for IC_{50} (p = 0.000), flavanoid (p = 0.001) and phenolic (p = 0.012) contents while no significant difference of HMF (p = 0.097) and proline (p = 0.062) were observed in the honey samples after opening and storage for three months.

Principal component analysis (PCA) was performed to determine which of these parameters (HMF, proline, phenolic, flavonoid and IC_{50}) showed similar changes in concentration during storage (Table 7). The % change in concentration due to storage of honey samples for three months away from sunlight was calculated for each parameter and two components have been extracted (Figure 3). The % change in phenolic and proline contents was grouped in the same quadrant. This indicated that storage have similar effect on these two parameters across the different samples and a small negative change were observed for both parameters. Flavonoid content, HMF and IC_{50} were not interrelated since they are found in different quadrants. HMF concentration showed major positive change that is the concentration of HMF increases consequently during storage. Moreover, for the IC_{50} a minor positive change while for flavonoid content a high negative change was observed.

TABLE 7. Principal Component Analysis

	% change in concentration and contents				
Samples	HMF (mg/kg)	Proline (mg/kg)	Total phenolic content (mg GAE/100g)	Total flavonoid content (mg QE/100g	IC_{50} (mg/kg)
1	-32.48	-3.01	-13.65	-9.02	1.50
2	3.55	-4.54	-2.62	-21.58	1.05
3	13.36	-1.49	-1.98	-17.76	2.96
4	229.09	-0.76	-6.11	-6.00	2.67
5	154.67	-1.34	-1.13	-18.37	1.97
6	1959.32	-1.56	-5.39	-45.81	2.75
7	316.88	-4.83	-11.07	-15.57	1.58
8	784.43	-2.53	-2.59	-20.41	4.80
9	112.00	-0.02	-0.60	-2.57	0.95

4. CONCLUSION

The quality of different honey samples commonly consumed were assessed in terms of their physiochemical and biochemical properties, A high correlation was found between the antioxidant activity of honey and its total phenolic, flavonoid and proline contents, indicated that the antioxidant activity of honey is mainly due to its phenolic and amino acid components. The tested honey samples may be considered easily accessible natural sources of antioxidants and valuable additions to everyday diet. Elevated concentrations of HMF and low concentrations of proline are indicators of overheating and storage in poor conditions. In this study, conventional heating up to 100 °C and oven heating were found to degrade the quality of the honey samples more than microwave heating since the HMF content was found to increase while that of proline decreases significantly. However, the antioxidant capacity increases by thermal treatment, which has positive effects on human health. Keeping the honey exposed to sunlight for 5 days did not cause significant change in the physico and biochemical properties.

Storing honey samples away from sunlight for three months increased concentration of HMF consequently, however, phenolic and proline content of honey decreases slightly. The factor analysis revealed an association between phenolic and proline content as their concentrations varied similarly during storage.

DECLARATION OF INTEREST

The author(s) declare no potential conflicts of interest with respect to research and publication of this article.

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