PROLONGED HEATING OF HONEY INCREASES ITS ANTIOXIDANT POTENTIAL BUT DECREASES ITS ANTIMICROBIAL ACTIVITY

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Abstract

Background: Honey is renowned for its antioxidant, antimicrobial and medicinal properties. It is typically heated and filtered during processing to avoid crystallization. However, few studies have reported its heating effect and the antioxidant and antimicrobial activities of honey. **Materials and Methods:** In this study, honey samples were collected from eight different honey-producing sites in Bangladesh and heated to 50ºC, 70ºC and 90ºC for 12 hours, while the antioxidant potentials and antimicrobial properties of the heated samples were compared with those of the unheated samples. Antimicrobial properties were investigated against one Gram-positive and eight Gram-negative bacteria. Other physicochemical and biochemical properties were also determined. **Results:** Interestingly, there was a proportionate increase in the amount of antioxidant compounds (phenolics and flavonoids) as well as antioxidant potentials proportional with the amount of heat introduced, whereas the antimicrobial properties of the honey samples were reduced with increasing heat. **Conclusion:** In conclusion, though prolonged heat treatment of honey can increase its antioxidant potential, antimicrobial activities are compromised.

Keywords: Honey, heat, antioxidant, antimicrobial.

List of Abbreviations: DPPH: 1,1-diphenyl-2-picrylhydrazyl; HMF: Hydroxymethylfurfural; TPTZ: 2,4,6-tris(1-pyridyl) -1,3,5-triazine; CEQ: Catechin Equivalents; FRAP: Ferric reducing/antioxidant power; ANOVA: Analysis of Variance; MIC: Minimum Inhibitory Concentration; ZOI: Zone of Inhibition.

Introduction

Honey [a mixture of sugars (38.3% fructose, 30.3% glucose, 7.1% maltose, 1.3% sucrose) and other compounds (water 20%, 0.5% acids, 0.3% proteins, 0.2% minerals, vitamins, phenols, together with more than 180 other substances)] is considered a natural sweet food supplement with medicinal value (National Honey Board, 2002). It also contains trace compounds including flavonoids, phenolic acids, ascorbic acid, enzymes (catalase and peroxidase), carotenoids and products of Maillard reactions, all of which are considered as the prime contributors to its antioxidant capacity (Gheldof, 2002). Free radicals ($O_2^{\bullet-}$, OH•, O•, H_2O_2) are natural by-products of metabolism that are produced within organisms given a complex redox reactions and can cause severe cellular damage. The antioxidants found in honey can bind these dangerous molecules and protect cells against their harmful effects (Jaganathan, 2009).

The crystallization of honey is a natural and spontaneous process by which honey changes from a liquefied to a semi-solid state (Kretavičius, 2010). However, this property is considered undesirable during all stages of honey handling, processing and marketing. Therefore, honey prepared for commercial market is usually heated and filtered to avert crystallization since the heat helps in melting invisible glucose crystals present, thereby keeping the honey in liquid state for many months. Moreover, honey contains high moisture content and osmophilic (sugar-tolerant) yeast in varying amounts (White, 1980). Due to this fact, unprocessed (e.g., unheated and unfiltered) honey is prone to fermentation at ambient temperature within a few days of storage; therefore, honey is typically heat-processed prior to storage. It is believed that the heat applied in processing of honey can eliminate the microorganisms responsible for spoilage and reduce the moisture content to a level that retards the fermentation process (Subramanian, 2007). Nevertheless, thermal treatment can result in some chemical changes in honey. One important chemical change is the condensation of sugars and free amino acids to produce brown pigments as a result of non-enzymatic Maillard reactions, which create Maillard reaction products that are believed to contribute to the antioxidant potential of honey (Manzocco, 2000). Dewanto et al. (2002) established that high temperature (88°C) enhances antioxidant activities in tomatoes, whereas Turkmen et al. (2006) found an increased antioxidant activity in honey after exposure to prolonged continuous heat for 12 days. Similarly, Dustmann (1979) reported that the antimicrobial activity of honey can be altered by exposure to heat, possibly because of lower catalase activity.

Some compounds [phenolics, flavonoids, hydroxymethylfurfural (HMF) and hydrogen peroxide] and properties (low pH and high osmolarity)] of honey have shown antimicrobial activities (Alvarez-Suarez, 2010; Bang, 2003; Snowdon, 1996; White Jr, 1963). Indeed, the growth of a wide range of bacteria, fungi, protozoa and viruses has been reported to be effectively inhibited by honey (Molan, 2006). Previous studies have reported potent antimicrobial activities of honey against *Pseudomonas aeruginosa, Salmonella typhi, Shigella flexneri, Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus* (Andualem, 2013; Ewnetu, 2013; Mandal, 2011; Tan, 2009). In this study, we investigated the effects of heat on the antibacterial properties of honey against nine bacterial species: one Gram-positive (*Staphylococcus aureus)* and eight Gram-negative bacteria *(Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Salmonella paratyphi A, Shigella dysenteriae, Chromobacterium violaceum and Vibrio cholerae).* These organisms are clinical pathogens and cause various types of healthcare-associated infections of skin, respiratory tract, urinary tract and gastrointestinal tract (Nasser, 2003; Tan, 2009). To our knowledge, this was the first study revealing the effects of high temperatures (50°C, 70°C and 90°C) with prolonged (12 hour) heating time on both the antioxidant properties and antibacterial activities of honey samples against nine bacterial species.

Methods
Chemicals and Reagents

The standards catechin, gallic acid, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and 2,4,6-tris (2-pyridyl)-1,3,5-triazine (TPTZ) were purchased from Sigma-Aldrich (St. Louis Missouri, USA). Trichloroacetic acid, tannic acid, L-ascorbic acid, ammonium molybdate, sodium carbonate (Na_2CO_3), aluminum chloride ($AlCl_3$), sodium nitrite ($NaNO_2$), ferrous sulfate hepta-hydrate ($FeSO_4 \cdot 7H_2O$) and sodium hydroxide (NaOH) were purchased from Merck Co. (Darmstadt, Germany). Folin-Ciocalteu's phenol reagent was purchased from LOBA Chemie (Mumbai, India). Mueller Hinton agar was purchased from HIMEDIA (Mumbai, India). All of the chemicals and reagents used in this study were of analytical grade.

Collection of Honey Samples and Honey Treatments

Honey samples (two uni-floral and six multi-floral) were collected from six different parts of Bangladesh (Satkhira, Khulna, Jessore, Sirajgang Faridpur and Sylhet) (Fig. 1), all of which are locally known to produce honey of superior quality. The samples were collected into sterilized plastic containers and transported to the lab on ice at 4ºC. The samples were kept in the same container at 4ºC in the laboratory for a few days prior to the analysis.

The samples were evaluated for physicochemical, antimicrobial and antioxidant properties at ambient temperature (without heat treatment). To investigate the influence of heat on phenolics and flavonoids, antioxidant potentials [Ferric-reducing Antioxidant Power (FRAP) and DPPH] and antimicrobial properties of honey, the samples (30 ml) were individually heated at three different temperatures (50ºC, 70ºC and 90ºC) for 12 hours each. A water bath was used (Memmert, Schwabach, Germany) to maintain uniform heat. All determinations of the physicochemical, biochemical and antioxidant measurements were performed in triplicate.

Microorganisms

All clinical bacterial specimens used in this experiment were collected from Bangladesh Institute for Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM). The antibacterial activity of honey against nine bacterial species was investigated: one Gram-positive (*Staphylococcus aureus)* and eight Gram-negative bacteria *(Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumonia, Salmonella typhi, Salmonella paratyphi A, Shigella dysenteriae, Chromobacterium violaceum* and *Vibrio cholerae)*. The bacterial species were selected on the basis of various organ-targeted diseases (skin, eye, liver, urinary tract, respiratory tract, soft tissue, bone, joints and small intestine), infections (burns, wounds and blood infections).

Physicochemical Analyses
pH

Briefly, 10% (w/v) solutions of each honey samples were prepared using distilled water based on the AOAC method (AOAC, 2000). Then, the pH of the samples were measured using a pH meter (Elico pH analyzer, Elico Pvt Ltd., Mumbai, India).

Moisture Content

The moisture content determination was also based on the method of AOAC (2000). Briefly, the honey samples were transferred to a flat-bottom dish. After measuring the weight, the sample was kept overnight in an oven (100–110°C) and reweighed. The loss in weight is considered as the moisture content, as calculated by the following formula:

$$\text{Moisture (\%)} = \frac{\text{Weight of fresh sample} - \text{Weight of dry sample}}{\text{Weight of fresh sample}} \times 100$$

Color Intensity
Absorbance at 450 nm (ABS_{450})

The mean absorbance of the honey samples was determined by the method of Beretta et al. (2005). Briefly, the honey samples were diluted to 50% (w/v) with warm (45 - 50°C) distilled water and the solution was filtered using a 0.45-μm filter (PVDF, Merck Millipore, Darmstadt, Germany) to eliminate large particles. The absorbance was measured using a spectrophotometer at 450 and 720 nm and the difference in absorbance was expressed as mAU.

Biochemical Analyses
Total Protein

The total protein content was determined by Lowry's method (1951) of protein estimation, which was based on the formation of a copper-protein complex and the reduction of the phosphomolybdate and phosphotungstate present in Folin-Ciocalteau's reagent to hetero-polymolybdenum blue and tungsten blue, respectively. Bovine serum albumin (BSA) (100, 200, 400, 600, 800 and 1000 μg/ml; $r^2 = 0.987$) was used as a standard for preparing the calibration curve. The absorption was measured against the standard solution at 710 nm using a spectrophotometer. The results were expressed as mg/kg honey.

Reducing Sugars

The reducing sugar content was determined based on the Nelson-Somogyi method (1952). Briefly, a 10% (w/v) honey solution (1 ml) was mixed with 0.2% benzoic acid (1 ml) and copper reagent (2 ml). The solution was then boiled for 15 min. After cooling, a color reagent

[arsenomolybdate (2 ml)] was added. A calibration curve was prepared using a standard solution of glucose (20, 40, 60, 80 and 100 μg/mL, r^2 = 0.993). The reducing sugars reduce copper from the cupric to cuprous state when heated with alkaline copper tartrate to form cuprous oxide. When cuprous oxide was treated with arsenomolybdic acid, the reduction of molybdic acid to molybdenum blue occurs. The developed blue color was compared with a set of standards at 620 nm using a colorimeter. The results were reported as g/100 g honey.

Total Phenolic Acid Content

Phenolic acids from honey samples (heated and unheated) were estimated using the modified spectrophotometric Folin–Ciocalteu method by Singleton et al. (1999). Briefly, 200 μL of a 10% (w/v) honey solution was mixed with 1 mL Folin and Ciocalteu's phenol reagent. After 3 min, 1 mL of 10% Na_2CO_3 solution was added to the mixture, which was adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was measured at 725 nm using a spectrophotometer. Gallic acid was used to calibrate the standard curve (20, 40, 60, 80 and 100 μg/mL, r^2 = 0.996). The results were expressed as milligrams of Gallic acid equivalents (GAEs) per kilogram honey.

Total Flavonoids

The total flavonoid content of the honey samples (heated and unheated) was determined according to the colorimetric assay developed by Zhishen et al. (1999). Briefly, a 10% (w/v) honey solution (200 μL) was mixed with 4 mL of distilled water. At baseline, 0.3 mL of $NaNO_2$ (5%, w/v) was added. After 5 min, 0.3 mL of $AlCl_3$ (10% w/v) was added, followed by the addition of 2 mL of NaOH (1 M) 6 min later. Immediately afterward, the volume was increased to 10 mL by the addition of 2.4 mL distilled water. The mixture was vigorously shaken to ensure adequate mixing, and the absorbance was measured at 510 nm. A calibration curve was prepared using a standard solution of catechin (20, 40, 60, 80 and 100 μg/mL, r^2 = 0.993). The results were expressed as mg catechin equivalents (CEQ) per kg of honey.

Ascorbic acid Content

The ascorbic acid content of the honey samples was determined by colorimetric method (Mochnik, 1994). The conversion of the ascorbic acid present in the extract to dehydroascorbic acid was coupled to 2,4-dinitrophenyl hydrazine to form a bis-2,4-dinitro dinitrophenyl hydrazine derivative, which yields a stable brownish-red color. The development of the colored derivative was monitored at 520 nm using a spectrophotometer and was expressed as ascorbate equivalents (mg of AE/kg of honey) by reference to a standard curve of L-ascorbic acid (r^2=0.958).

Analysis of Antioxidant Activity
Ferric-reducing Antioxidant Power (FRAP) Assay

The FRAP assay was performed according to the modified method described by Benzie & Strain (Benzie, 1999). Briefly, 200 μL of properly diluted honey (10% w/v) with distilled water was mixed with 1.5 mL of FRAP reagent. The reaction mixture was then incubated at 37°C for 4 min, and the absorbance was measured at 593 nm against a blank that was prepared using distilled water. The FRAP reagent was pre-warmed at 37°C and freshly prepared. This was performed by mixing 10 volumes of 300 mM/L acetate buffer (pH 3.6) with 1 volume of 10 mmol 2,4,6-tris(1-pyridyl)-1,3,5-triazine (TPTZ) solution in 40 mM/L HCl with 1 volume of 20 mM ferric chloride ($FeCl_3.6H_2O$). A calibration curve was prepared using an aqueous solution of ferrous sulfate ($FeSO_4.7H_2O$) at 100, 200, 400, 600 and 1000 μM/L (r^2=0.993). The FRAP values were expressed as micromoles of ferrous equivalent (μM Fe [II]) per kg of honey. The test was performed for all the heated and unheated honey samples.

DPPH Free Radical-scavenging Activity

The antioxidant properties of both the heated and unheated honey samples were also studied by investigating the free radical-scavenging activity of the DPPH radical. The determination was based on the method proposed by Ferreira et al. (Ferreira, 2009). Briefly, honey extract (1 mL) was mixed with 2.7 mL of methanolic solution containing the DPPH radical (0.024 mg/mL). The mixture was vigorously shaken and left to stand for 15 min in the dark (until the absorbance unchanged). The reduction of the DPPH radical was determined by measuring the absorbance at 517 nm.

% of DPPH inhibition= [(A_{DPPH}- A_S) /A_{DPPH}] × 100

Where,

1) A_S was the absorbance of the solution when the sample extract is added at a particular level

2) A_{DPPH} was the absorbance of the DPPH solution.

The IC_{50} was determined as the concentration of the tested extract samples causing 50% reduction of the initial DPPH concentration, measured from the linear regression concentration curve of the test extract (mg/ml) against percentage of radical scavenging inhibition.

Antibacterial Activity
Test by Agar Well Diffusion Assay

To investigate the antibacterial activities of the honey samples, a simple agar well diffusion method was followed based on the method established by Perez et al. (1990), with some modifications. Briefly, a freshly prepared (24 hour) bacterial culture was suspended in sterile distilled water to produce a solution with a turbidity of 0.5 McFarland units. The final inoculum size was adjusted to 5 X 10^5 CFU/mL. A bacterial lawn was prepared using the experimental bacteria with a spread plate technique on a nutrient agar plate (nutrient broth + 1.8% agar

powder). Wells (8 mm diameter) were created into the agar before the addition of 100 µl of each 50% (w/v) honey samples prepared with distilled water into each. A negative control well containing sterile distilled water and a standard antibiotic solution (100 μg/ml) of streptomycin (positive control) were included on the same plate. The plates were incubated at 37°C for 24 hours. At the end of the incubation period, the antibacterial activities were assessed by measuring the zone of inhibition (ZOI, in millimeters) for each respective honey sample. The relative antibacterial potency of the honey samples was estimated by comparing the diameter of the ZOI with that of the standard antibiotic streptomycin.

Determination of the Minimum Inhibitory Concentration (MIC) of Honey

Six bacteria species (*Salmonella paratyphi A, Escherichia coli, Chromobacterium violaceum, Staphylococcus aureus, Salmonella typhi* and *Vibrio cholerae)* were selected on the basis of growth sensitivity towards honey samples by the agar well diffusion method. Then, minimum inhibitory concentration (MIC) was determined by broth dilution method. Initially, each of the honey samples (50%, w/v) was serially diluted by transferring 5 ml of the sterile honey into 5 ml of sterile nutrient broth to obtain the following dilutions: 25% (w/v), 12.5% (w/v), 6.25% (w/v) and 3.125% (w/v) (Ibekwe, 2001). Other desired concentrations [15, 20, 30, 40 and 50% (w/v)] were also prepared. Thus, four groups (unheated, 50°C, 70°C and 90°C) of honey samples at different concentrations were prepared. Then, each concentration of honey was inoculated with 0.1 ml of a standardized bacterial cell suspension (approximately 10^6 CFU/ml) and incubated at 37°C for 24 hours. The lowest concentration of heated and unheated honey that inhibited the growth of the test organism was taken as the MIC.

Statistical analysis

All analyses were carried out in triplicate, and the results were reported as the mean ± standard deviation (SD). The data were analyzed using MS Excel 2007 and SPSS 16.0. A one-way analysis of variance (ANOVA) followed by a Tukey's honestly significant difference *post hoc* test ($p < 0.01$) was used to compare parameters between the different honey types. The paired sample *t*-test was performed to detect significant differences ($p < 0.01$) among the different antioxidant compounds (phenolic acids and flavonoids) and antioxidant potentials (DPPH and FRAP) between the unheated and heated honey samples.

Results and Discussion
Physicochemical Analysis
pH

All honey samples were found to be acidic, with pH values ranging from 3.7 ± 0.0 to 4.2 ± 0.0 (table 1); this was not surprising because honey was reported to be naturally acidic. As a low pH of honey was important to maintain its shelf life and stability, the acidic pH value of all the honey samples reflects a higher shelf life and stability. These results are similar to those reported for honey samples from India (pH 3.7 - 4.4) (Saxena, 2010).
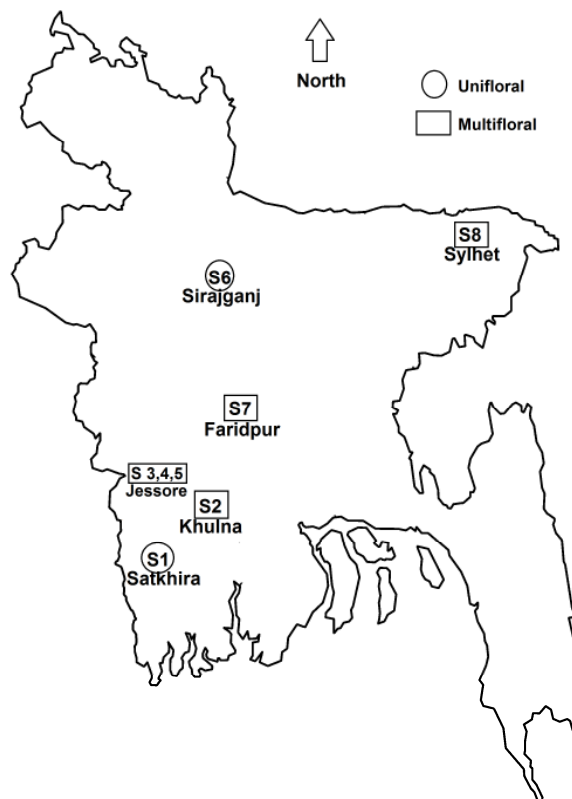

Figure 1: Eight areas of honey sampling in Bangladesh and the honey types.

Moisture Content

Six out of the eight investigated honey samples were within the maximum limit for moisture content per the Codex standard for honey (2001), which was below 20%. However, the mean (19.16 ± 0.1%) was slightly higher than that of honey samples from some Mediterranean countries, such as Portugal (16.65 ± 1.7) (Silva, 2009) and Spain (16.3 ± 1.13) (Terrab, 2004), which may be attributed to the humid tropical climate of Bangladesh.

Color Intensity (ABS$_{450}$)

ABS$_{450}$ was a reliable index for confirming the presence of pigments with antioxidant properties, such as polyphenols and some flavonoids (Antony, 2000). The color intensity of the honey samples ranged from 138.6 ± 0.5 to 408.3 ± 3.7 mAU; which was similar to Slovenian honeys (70 - 495 mAU) (Bertoncelj, 2007) but lower than honeys from India (524 - 1678 mAU) (Saxena, 2010) and Algeria (724 - 1188 mAU) (Khalil, 2012). Overall, the multifloral honeys had higher color intensity when compared to the unifloral honeys, indicating their superior antioxidant properties. As suggested by Frankel et al. (1998), the color intensity (ABS$_{450}$) was strongly related to pigments such as carotenoids, flavonoids and phenolics, which are known antioxidants.

Biophytochemical Analysis
Total Protein

The protein level in honey samples was dependent on the type of flora from which it was derived, which does vary. This variance can be attributed to the presence of enzymes introduced by the bees themselves and other proteins derived from the nectar (Alvarez-Suarez, 2010). The protein contents of the investigated honey samples were between 6078.0 ± 55.0 (S1) and 14508.3 ± 52.0 (S3) mg/kg, which was higher than that of honey samples from Algeria (3007.33 ± 3.54 - 4095.00 ± 3.54 mg/kg) (Khalil, 2012) but is similar to honey samples from South Africa (6200 - 12900 mg/kg) (Serem, 2012).

Reducing Sugars

Reducing sugars (invert sugars), i.e., mainly fructose and glucose, are reported to be the major constituents of honey (Küçük, 2007). The reducing sugar content of the honey samples ranged from 52.3 ± 0.7 g/100 g to 66.5 ± 1.5 g/100 g, which was similar to honey samples from India (42.95 - 60.31 g/100 g) (Saxena, 2010).

Total Phenolic Acid Content

The phenolic acid contents of the honey samples investigated at ambient temperature were between 269.8 ± 4.1 to 826.6 ± 3.7 mg GAE/kg (mean of 506.42 mg GAE/kg). The level was higher than that of honey samples from Algeria (459.8 ± 1.9 mg GAE/kg) (Khalil, 2012) or that of Tualang honey samples from Malaysia (383.7 ± 13.5 mg GAE/kg) (A-Rahaman, 2013), indicating that the collected honey samples from Bangladesh had superior antioxidant properties.

Total Flavonoids

Generally, all of the investigated honey samples had a lower content of flavonoids than polyphenols, at 25.0 ± 1.7 mg CEQ/kg. Sample S7, which was a multi-floral honey, had the highest total flavonoid content (25.0 ± 1.7 mg CEQ/kg). In comparison, sample S1, which was a unifloral honey, had the lowest (10.7 ± 0.0 mg CEQ/kg) flavonoid content. These findings are similar to those reported for honey samples from Cuba, for which the unifloral honey flavonoid content were also low (10.9 ± 0.3 mg - 25.2 ± 0.3 mg CEQ/kg) (Alvarez-Suarez, 2010). It can be postulated that the blending of different varieties of nectars from diverse flower types led to the superior antioxidant properties of the multi-floral honey samples when compared to the unifloral honey samples.

Ascorbic Acid Content

In addition to polyphenols, honey contains a number of compounds known to act as antioxidants, including ascorbic acid (glucose oxidase and catalase) (Alvarez-Suarez, 2010). The ascorbic acid content of the honey samples ranged from 84.4 ± 1.4 mg/kg to 198.1 ± 0.5 mg/kg, with a mean of 141.6 ± 42.1 mg/kg, which was similar to that reported for honey samples from Portugal (143.2 ± 0.4). Although honey samples from Cuba showed a good phenolic content, ascorbic acid was not detected (Alvarez-Suarez, 2010), indicating that the antioxidant potential of honeys from Cuba may be of inferior quality.

The effects of heat treatment on antioxidant compounds and potentials
Total Phenolic Acid Content

Heat treatment appeared to have positive effects on the phenolic content, whereby the content increased with increasing heat (Fig. 2). This finding was similar to that reported by Turkmen at al. (2006), who also found that the antioxidant activity increased linearly with increments (50ºC, 60ºC and 70ºC) of prolonged heat (up to 12 days). Our finding was similar to that reported for Manuka honey from New Zealand (Akhmazillah, 2013): the phenolic content was found to increase by 20.1% and 16.3% following heat treatments at 50ºC and 70ºC, respectively. In our experiment the amount of phenolics may have increased due to the denaturation and exposure of reactive protein sites, degradation of endogenous antioxidants and the production of some non-nutritional antioxidants as a result of Maillard reaction as also suggested by by Kusznierewicz et al. (2008) and Serpen et al. (2012).
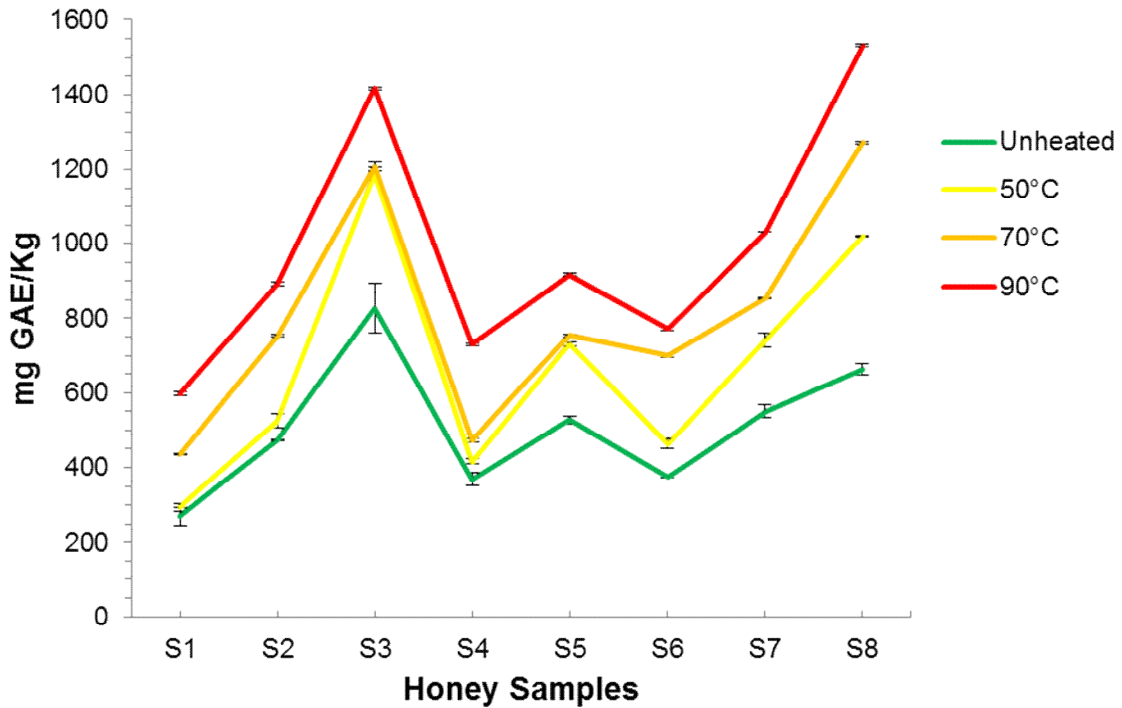
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Figure 2: Gradual increase in the phenolic acid content in honey samples as a result of increasing heat treatment.

Total Flavonoids

Interestingly, as with phenolic acid content, the mean flavonoid content also increased (to 9.6%, 23.0% and 43.8% with increasing temperatures of 50ºC, 70ºC and 90ºC, respectively) in all of the honey samples (Fig. 3). The rise of flavonoid content after heat treatment may be due to the increment of bio-accessibility of certain heat activated flavonoids during thermal treatment, as previously suggested by Dewanto et al. (2002). Moreover, from the suggestion of Šarić et al. (2013), it can be predicted that the increment of flavonoids may be because of the activation of some thermostable compounds which can contribute to the antioxidant activity.
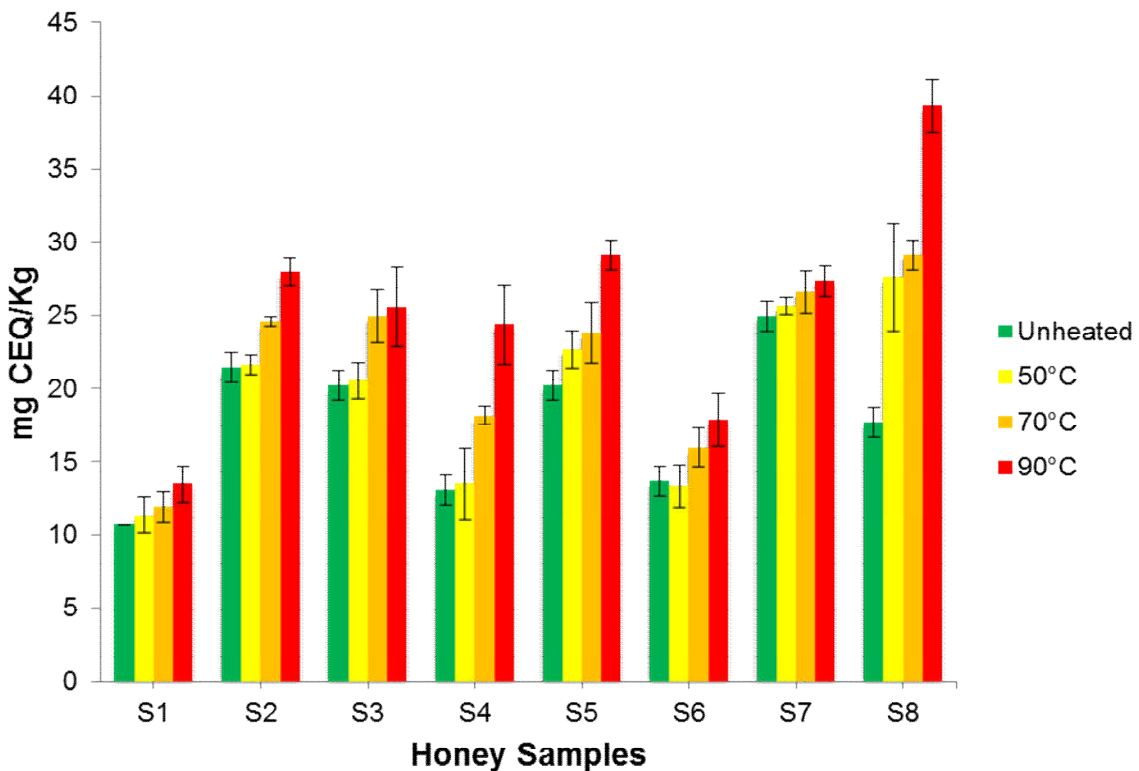

Figure 3: Gradual increase in flavonoid contents in honey samples as a result of increasing heat treatment

Table 1: Physicochemical and phytobiochemical properties of honey samples from Bangladesh

Sample	Name of Honey	Source	pH	Moisture (%)	Color Intensity (mAU)	Protein (mg/kg)	Reducing Sugar (g/100 g)	Ascorbic Acid (mg/kg)
S1	Unifloral	*Litchi chinensis (Apis dorsata)*	3.8 ± 0.0^e	20.2 ± 0.2^b	138.6 ± 0.5^h	6078.0 ± 55.0^h	61.4 ± 0.9^d	93.7 ± 2.4^g
S2	Multifloral	Mixed *(Apis dorsata)*	4.1 ± 0.0^b	18.3 ± 0.1^f	345.3 ± 4.0^c	10300.0 ± 50.0^c	55.5 ± 0.5^f	117.0 ± 1.0^f
S3	Multifloral	Mixed *(Apis dorsata)*	3.9 ± 0.0^d	22.1 ± 0.1^a	334.0 ± 3.6^d	14508.3 ± 52.0^a	57.4 ± 0.3^e	164.1 ± 0.0^d
S4	Multifloral	Mixed *(Apis dorsata)*	4.0 ± 0.0^c	17.7 ± 0.2^g	352.0 ± 2.6^b	7158.3 ± 52.0^g	53.7 ± 1.0^g	84.4 ± 1.4^h
S5	Multifloral	Mixed *(Apis dorsata)*	3.9 ± 0.0^d	17.4 ± 0.1^h	408.3 ± 3.7^a	9058.3 ± 62.9^d	66.5 ± 1.5^a	174.5 ± 4.2^c
S6	Unifloral	*Litchi chinensis (Apis dorsata)*	4.2 ± 0.0^a	19.1 ± 0.2^d	172.6 ± 2.5^g	8308.3 ± 80.3^e	63.2 ± 0.8^c	124.9 ± 4.6^e
S7	Multifloral	Mixed *(Apis dorsata)*	3.7 ± 0.0^f	18.5 ± 0.1^e	307.0 ± 1.6^e	11550.0 ± 66.1^b	65.3 ± 1.3^b	176.5 ± 0.8^b
S8	Multifloral	Mixed *(Apis dorsata)*	4.2 ± 0.0^a	19.6 ± 0.1^c	226.3 ± 1.5^f	7225.0 ± 50.5^f	52.3 ± 0.7^h	198.1 ± 0.5^a

Data are expressed as the mean $\pm$ SD. Significantly different values are represented by different letters (a, b, c, d, e, f, g, h) ($p < 0.01$)

Table 2: FRAP and DPPH-scavenging activities of heated and unheated honey samples

Sample	IC$_{50}$ of DPPH inhibition (mg/mL)				FRAP (μM Fe(II)/kg)			
	#	50ºC	70ºC	90ºC	#	50ºC	70ºC	90ºC
S1	17.6	6.15	5.93	5.90	240.4 ± 5.0	256.6 ± 4.9	475.5 ± 3.1	483.3 ± 1.2
S2	15.5	5.53	5.15	5.03	545.2 ± 12.6	850.0 ± 1.0	1403.3 ± 2.0	1518.8 ± 2.3
S3	14.9	5.17	4.89	4.86	526.4 ± 9.0	1001.1 ± 2.6	1123.3 ± 6.3	1553.3 ± 2.7
S4	14.6	5.31	4.60	5.40	939.3 ± 20.7	1318.8 ± 1.8	1425.5 ± 6.1	1446.6 ± 2.3
S5	15.4	5.31	5.56	4.83	735.0 ± 6.4	795.5 ± 5.3	996.6 ± 0.6	1263.3 ± 2.3
S6	13.4	6.68	5.13	5.06	229.0 ± 26.9	462.2 ± 6.1	597.7 ± 5.4	797.7 ± 2.8
S7	11.3	5.74	5.11	4.99	573.5 ± 7.8	678.8 ± 1.5	1044.4 ± 2.8	1717.7 ± 5.7
S8	12.4	5.50	5.14	4.22	890.2 ± 3.0	1534.4 ± 1.3	1963.3 ± 1.1	2036.6 ± 2.0
Mean	14.3	5.67	5.19	5.04	584.9 ± 11.5	862.2 ± 3.1	1128.8 ± 3.5	1352.2 ± 2.7

FRAP data are expressed as the mean $\pm$ SD.

Unheated

DPPH Free Radical Scavenging Activity

The mean 50% maximum inhibitory concentration (IC_{50}) values of the DPPH-scavenging activities were 14.3 mg/mL (table 2). A decrease in the mean from 14.43 to 5.04 mg/mL was recorded for the IC_{50} values of the honey samples during heating, which further confirms that the total antioxidant activity also increased with increased heat treatment. Our result was similar to the findings of Yang et al. (2013), whereby the content of DPPH decreased significantly ($p < 0.01$) with a gradual increase in temperature (50ºC, 70ºC, 90ºC), representing an increase in antioxidant activity. The antioxidant activity increased possibly due to the production and/or activation of some thermo resistant compounds which activates at high temperature and contributes to increased antioxidant activities (Dewanto, 2002; Kusznierewicz, 2008).

FRAP assay

The FRAP assay was performed to investigate the antioxidant potential of the different honey samples. The mean FRAP value of the unheated honey samples was 584.8 ± 11.4 μmol Fe (II)/kg. Again, the unifloral honey samples S1 (240.4 ± 5.0 μmol Fe (II)/kg) and S6 (229.0 ± 26.9 μmol Fe (II)/kg) tended to show lower FRAP contents than the multi-floral honey samples, with the highest amount observed in sample S4 [939.3 ± 20.7 μmol Fe (II)/kg)] (table 2).Very interestingly, after thermal treatment, the mean FRAP values dramatically increased by 47.4% at 50ºC, 92.9% at 70ºC and 131.8% at 90ºC. At 90ºC, the multi-floral honey (sample S4) again demonstrated the highest FRAP value [2036.7 ± 2.0 μmol Fe (II)/kg)] (table 2). Similarly, Xu et al. (2007), who heated honey samples to 90ºC, 120ºC and 150ºC for 30 min, and Inchuen et al. (2011), who heated honey samples to 60ºC, 75ºC, 90ºC, 105ºC and 120ºC for up to 60 min, also found that the FRAP values increased with increasing temperature. The increment of FRAP value was possibly due to activation of some heat resistant compounds which contributes to increased antioxidant potentials (Dewanto, 2002; Kusznierewicz, 2008).

Antimicrobial Properties

The quantitative assessment of the antibacterial activity of the honey samples and their potencies were determined by measuring ZOI (table 3). All of the honey samples showed good antibacterial activities against six of the investigated microorganisms compared to the standards (except for *Klebsiella pneumoniae*, *Shigella dysenteriae* and *Pseudomonas aeruginosa)*. Among other honey samples, the highest antibacterial activity was displayed by the multi-floral honey (sample S5), again indicating that multi-floral honey tends to have better antibacterial properties. For example, the highest ZOI (32.2 ± 0.2 mm) was found against *Salmonella typhi* by the multi-floral honey sample S8, whereas the smallest ZOI (17.0 ± 0.0 mm) was against *Vibrio cholerae* by the honey sample S4.
Previous studies (Bang, 2003; White Jr, 1963) have indicated that honey samples containing a high sugar but a low water content and acidity tend to have good antibacterial properties. Similar results were found in our study: sample S5, which showed a low pH (3.9 ± 0.0) and the lowest level of moisture (17.4 ± 0.1), had the highest color intensity (408.3 ± 3.7) and reducing sugar content (66.5 ± 1.5). Correspondingly, sample S5 had the best mean antimicrobial activity (26.0 ± 0.3 mm) compared to the other honey samples. Variations in the antimicrobial activities may also be due to different floral sources and geographical regions and/or the presence of variable amounts of hydrogen peroxide, phenolic acids and flavonoids (Molan, 2006; White Jr, 1963).

The effects of heat (50ºC, 70ºC, 90ºC) on the antibacterial properties of honey were compared with unheated samples. The MICs of the investigated honey samples ranged from 6.25% to 20.00% (w/v) (table 4). Notably, the lowest MIC was found only in the case of unheated honey samples (except for the MIC against *Vibrio cholera)*. An increased MIC was observed with increased heat for all of the honey samples, indicating decreases in antibacterial activities (table 4). For example, the unheated honey samples S1, S7 and S8 exhibited the lowest MIC, at 6.25% (w/v), against *Salmonella typhi.* The antibacterial activity decreased even further when the honey samples were heated to 90ºC. At 90ºC, the S6 and S8 honey samples completely lost their antibacterial activities against *Salmonella paratyphi A*. Similarly, samples S2, S6 and S7 failed to inhibit the growth of *Staphylococcus aureus* (table 4), a property that was initially demonstrated prior to the honey being heated.

A high content of glucose oxidase was an important property of honey. In several studies, it has been shown that hydrogen peroxide was the main compound responsible for the antibacterial action of honey (Brudzynski, 2006; Weston, 2000; White Jr, 1963) and was produced mainly during glucose oxidation, which is catalyzed by the bee enzyme glucose oxidase (White Jr, 1963). If a honey contains a high content of this oxidizing compound, bacteria cannot respond normally to proliferative signals, and their growth remains arrested even when the honey is used in diluted forms (Brudzynski, 2011). According to Kretavičius et al. (2010), when honey was liquefied, glucose oxidase was activated and produces its metabolic products. During honey de-crystallization (55 - 70ºC), the stability and activity of glucose oxidase declines. The reduction in the glucose oxidase level may contribute to a higher MIC, as high temperature (50 - 90ºC) may deactivate glucose oxidase. As a result, the production of hydrogen peroxide was also decreased, inhibiting the growth of organisms at a lower honey concentration. For this reason, the lowest MIC values against the investigated pathogenic bacteria were observed for the unheated honey samples, and it is postulated that the MIC values increased with increasing temperature and decreasing hydrogen peroxide. Further investigations to determine the exact mechanism by which the antimicrobial properties of honey are decreased with increasing heat are warranted.

Conclusions

In this investigation, eight honey samples from different areas of Bangladesh were heated to 50ºC, 70ºC and 90ºC for 12 hours and the amount of antioxidant compounds (phenolic acids and flavonoids), antioxidant potentials (FRAP and DPPH) and antibacterial properties of the heated samples were compared with those of unheated samples. The physicochemical and biochemical properties (total protein and reducing sugars) were also determined. In general, multi-floral honeys tend to have higher antioxidant and antibacterial properties than unifloral honeys. Regulated and prolonged heat can improve the antioxidant potential of honey by gradually increasing the antioxidant compounds (phenolic acids and flavonoids) and antioxidant potentials (DPPH and FRAP) with increasing heat. The antimicrobial activity of honey decreased and was even lost with increases in h€eat treatment. Therefore, though prolonged heat treatment was recommended for enhancing the antioxidant potential of honey, the temperature should be limited to preserve honey's antimicrobial activity.

Conflict of Interests: The authors declare that there was no conflict of interests regarding the publication of this paper.

Table 3: Antibacterial activity of honey samples and their zones of inhibition

Test Organisms	Zones of inhibition (mm)								
	S1	S2	S3	S4	S5	S6	S7	S8	Streptomycin (100 µg/ml)
Salmonella paratyphi A	27.6 ± 0.7[a]	27.5 ± 0.5[a]	24.5 ± 0.0[ab]	24.0 ± 0.0[b]	25.2 ± 0.5[ab]	23.5 ± 0.2[b]	24.0 ± 0.0[b]	23.2 ± 0.5[b]	16.33 ± 0.58
Klebsiella spp.	*	*	*	*	*	*	*	*	*
Escherichia coli	25.5 ± 0.5[a]	23.2 ± 0.7[ab]	25.7 ± 0.2[a]	19.2 ± 0.2[c]	19.5 ± 0.5[c]	19.0 ± 0.0[c]	20.2 ± 0.5[bc]	22.2 ± 0.5[bc]	18.16 ± 1.04
Chromobacterium violaceum	23.0 ± 0.5[ab]	21.8 ± 0.6[ab]	21.6 ± 0.7[ab]	20.7 ± 0.5[b]	23.5 ± 0.2[ab]	22.2 ± 0.7[ab]	22.7 ± 0.2[ab]	25.0 ± 0.5[a]	16.67 ± 0.58
Staphylococcus aureus	21.2 ± 0.2[a]	20.0 ± 0.5[ab]	20.0 ± 0.5[ab]	17.5 ± 0.0[b]	18.0 ± 0.0[ab]	17.3 ± 0.1[b]	18.5 ± 0.2[ab]	21.0 ± 0.0[a]	15.83 ± 0.76
Salmonella typhi	31.2 ± 0.7[a]	29.7 ± 0.2[a]	29.7 ± 0.2[a]	31.0 ± 0.5[a]	30.0 ± 0.5[a]	31.0 ± 0.5[a]	31.5 ± 0.2[a]	32.2 ± 0.2[a]	16.20 ± 0.55
Shigella dysenteriae	*	*	*	*	*	*	*	*	*
Pseudomonas aeruginosa	*	*	*	*	*	*	*	*	16.00 ± 0.50
Vibrio cholera	18.0 ± 0.5[bc]	17.2 ± 0.5[c]	19.0 ± 0.0[abc]	17.0 ± 0.0[c]	20.5 ± 0.2[ab]	*	*	21.7 ± 0.2[a]	14.50 ± 0.50
Mean	24.4 ± 0.5	23.2 ± 0.5	23.4 ± 0.2	25.4 ± 0.1	26.0 ± 0.3	22.6 ± 0.1	23.3 ± 0.2	24.2 ± 0.3	

Data are expressed as the mean ± SD. Significantly different values are represented by different letters (a, b, c, d, e, f, g, h) ($p < 0.01$)
*No inhibitory zones observed

Table 4: Comparison of MIC values between unheated and heated honey samples

	MIC % (w/v)																							
	Salmonella paratyphi A				*Escherichia coli*				*Chromobacterium violaceum*				*Staphylococcus aureus*				*Salmonella typhi*				*Vibrio cholera*			
Temperature	#	50º	70°	90º	#	50º	70°	90º	#	50º	70°	90º	#	50º	70°	90º	#	50º	70°	90º	#	50º	70°	90º
S1	12.5[c]	15[d]	20[e]	30[c]	15[b]	15[c]	25[c]	30[c]	15[b]	20[b]	25[c]	30[c]	12.5[c]	20[c]	25[c]	30[b]	6.25[b]	15[c]	25[c]	40[b]	15[a]	20[b]	30[a]	40[b]
S2	12.5[c]	20[c]	25[d]	40[b]	20[a]	25[a]	30[b]	50[a]	15[b]	25[a]	30[b]	50[a]	20[a]	25[b]	30[b]	*	12.5[a]	25[a]	30[b]	40[b]	15[a]	25[a]	30[a]	40[b]
S3	15[b]	12.5[e]	20[e]	30[c]	15[b]	15[c]	20[d]	30[c]	20[a]	20[b]	30[b]	40[b]	20[a]	25[b]	30[b]	50[a]	12.5[a]	20[b]	30[b]	50[a]	12.5[b]	25[a]	30[a]	50[a]
S4	15[b]	20[c]	25[d]	30[c]	20[a]	20[b]	25[c]	30[c]	20[a]	25[a]	30[b]	50[a]	20[a]	25[b]	30[b]	50[a]	12.5[a]	25[a]	30[b]	50[a]	15[a]	15[c]	30[a]	50[a]
S5	15[b]	25[b]	30[c]	50[a]	20[a]	25[a]	40[a]	50[a]	15[b]	25[a]	40[a]	50[a]	20[a]	25[b]	40[a]	50[a]	12.5[a]	25[a]	40[a]	50[a]	12.5[b]	20[b]	30[a]	50[a]
S6	20[a]	30[a]	40[b]	*	20[a]	25[a]	40[a]	50[a]	15[b]	25[a]	40[a]	50[a]	20[a]	25[b]	40[a]	*	12.5[a]	25[a]	30[b]	40[b]	x	x	x	x
S7	15[b]	20[c]	25[d]	40[b]	20[a]	20[b]	25[c]	40[b]	20[a]	20[b]	25[c]	40[b]	12.5[c]	30[a]	40[a]	*	6.25[b]	25[a]	40[a]	50[a]	x	x	x	x
S8	20[a]	30[a]	50[a]	*	20[a]	25[a]	40[a]	50[a]	12.5[c]	25[a]	40[a]	50[a]	15[b]	25[b]	40[a]	50[a]	6.25[b]	15[c]	25[c]	40[b]	12.5[b]	20[b]	30[a]	50[a]

Significantly different values are represented by different letters (a, b, c, d, e, f, g, h) ($p < 0.01$)
*No antibacterial activity observed
x Not performed
Unheated

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