Quality characterization and effect of sonication time on bioactive properties of honey from North East India

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Abstract The North-Eastern region of India is known for its rich flora and has great potential for honey production. Honey samples collected from local markets of different regions of North-East India viz. Shillong, Sohra, Mawsynram, Jorhat and Tezpur were analyzed for physicochemical properties, bioactivity and mineral content. Effect of sonication time on the bioactive properties of honey was determined. All the honey samples had good bioactive properties and high content of potassium, sodium, calcium and iron. The Jorhat sample had the highest phenolic content (296.68 ± 2.16 mg GAE/100 g) and flavonoid content (155.26 ± 2.90 µg quercetin/100 g) whereas highest DPPH radical scavenging activity with an IC₅₀ value of 29.8 ± 0.20 g and a FRAP value of 7291.60 ± 584.6 µM Fe(II)/100 g were noted in Shillong sample. Sonication exhibited various effects on the bioactive properties of the selected honey samples based on their source and treatment time. Honey from Jorhat and Sohra exhibited good quality standards with HMF content less than 80 mg/kg.

Keywords North-East India · Honey · Bioactive properties · Sonication · Minerals · Hydroxymethylfurfural

Introduction

The definition of honey as given by Codex Alimentarius Commission (2001) is “the sweet substance produced by honeybees from the nectar of blossoms or from secretions on living plants, which the bees collect, transform and store in honey combs”. Honey finds its use as a therapeutic agent and its therapeutic benefits have been mentioned in the works of different ancient civilizations of the world including India, Egypt and Greece. Honey is also widely used in traditional medicine. Honey is rich in fructose and glucose. Honey is considered to be important for its composition of vitamins, amino acids, organic acids, minerals and phenolics (Suárez-Luque et al. 2002; Pohl et al. 2012; Can et al. 2015). Honey also exhibits both antibacterial and antifungal properties (El-Haskoury et al. 2018). The phenolic acids, flavonoids, certain enzymes like glucose oxidase and catalase, ascorbic acid, protein and carotenoids that are present in honey impart antioxidant properties (Froschle et al. 2018; Saxena et al. 2010).

The composition and quality of honey is variable and depends upon various factors like botanical origin, geographical region, seasonal variation, processing techniques and storage conditions (Turhan et al. 2008). Although raw honey is the best, the heating of honey is industrially used to delay crystallization. The important quality parameter for heat processed honey is hydroxymethylfurfural (HMF) content. HMF in honey from tropical region should be in the range from 40 to 80 mg/kg as per European Union Guidelines (Codex Alimentarius Commission 2001). Ultrasound has been applied to honey processing mostly to

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reduce crystallization of honey (Kabbani et al. 2011; Janghu et al. 2017) besides the other advantages like slower granulation, microbial inactivation and adulteration detection. Ultrasonication has no effect on its physical properties (Majid et al. 2015). However, limited reports on the effect of sonication on the bioactive properties in honey are available (Lira et al. 2017).

India is one of the significant honey exporters in the world. Even though about half of the total number of floral species known in India is present in the North-Eastern region, this region is not considered among the major producers like Punjab, Himachal Pradesh, Bihar and West Bengal. The North-Eastern states account for over 17 million hectares which is roughly one-fourth of the forest area of the country and is one of the biodiversity hotspots of India. Thus, North-East can become a potential honey producing region and contribute appreciably to the Indian honey production and consumption levels. However, there are no reports of the physicochemical and antioxidant properties of honey produced in this region.

The current work attempted to determine the physicochemical composition and antioxidant properties of honey collected from two of the North-Eastern states of India namely, Meghalaya and Assam. The selected locations were far from densely populated areas as well as far from industrial areas so as to minimize the effect of pollutants from such places which are supposed to affect honey quality (Aghamirlou et al. 2015). The effect of sonication on the bioactive properties of honey was also evaluated.

Materials and methods

Chemicals

The chemicals viz. ethanol, concentrated sulphuric acid, bovine serum albumin, glucose, fructose, resorcinol, bovine serum albumin (BSA), Folin Ciocalteu reagent (FCR), aluminum trichloride, hydrogen peroxide, and ferric chloride were of analytical grade from Merck, India while methanol, glacial acetic acid, and quercetin were of HPLC grade from Merck. Safranin, coomassie brilliant blue dye, 2,4,6-tripyridyl-S-triazine (TPTZ) for ferric reducing antioxidant potential assay (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), phenazine methosulfate (PMS), nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium (NBT) were obtained from HiMedia, India. Hydroxymethyl furfural was obtained from SRL, India. Mineral standards stock solutions (Ca, K, Na, Mg, Fe, Cu, Ni, Zn, Mg and Co), phenolic and flavonoid standards (catechin, caffeic acid, coumaric acid, syringic acid, chlorogenic acid, ferulic acid and rutin) and organic acids (gluconic acid, succinic acid, lactic acid, acetic acid, citric

acid and tartaric acid) and XAD-2 resin were all obtained from Sigma, India.

Sample collection

A total of five honey samples were collected: one each from Shillong, Mawsynram and Sohra from the State of Meghalaya; and one each Jorhat and Tezpur from the State of Assam. All samples were collected during November 2016 to January 2017.

Pollen study

Pollens from honey were extracted using the acetolysis protocol (Erdtman 1960). Honey (5 g) was diluted with 70% alcohol and centrifuged (Hettich, EBA 21, Germany) at $1006\times g$ for 10 min. The supernatant was discarded, 5 mL glacial acetic acid was added to the sediment, allowed to stand for 10 min, centrifuged and the supernatant was discarded. Then 5 mL of acetolysis mixture (concentrated H_2SO_4 and glacial acetic acid, 9:1) was added and heated for 30 min at 80 °C. The mixture was centrifuged; the sediment was rinsed with distilled water and centrifuged again. The pollens were stained with safranin stain and studied under microscope to determine the number of different pollens present. The botanical origin of honey was distinguished as unifloral or multifloral depending on whether or not the dominant pollen percentage was greater than 45% (Louveaux et al. 1970).

Moisture, total acidity, pH, ash content, and fructose content

Moisture content of the honeys was estimated by taking refractive index using an Abbe refractometer (Atago DR-A1, Japan) at 20 °C as given in AOAC (2005). The total acidity of honey was measured according to AOAC (2005). The pH was measured using a pH meter (Eutech pH 700, Singapore) after dissolving 4 g of honey in 20 mL of water. Ash content of honeys was determined after igniting in a muffle furnace at 550 °C to a constant weight (AOAC 2005). Fructose content was determined using resorcinol method (AOAC 2005).

Soluble protein, glucose content, and color

Soluble protein was estimated following the method given by Bradford (1976). Briefly, 0.1 mL of solution (1 g of honey dissolved in 1 mL of water) was added to 5 mL of coomassie brilliant blue dye and vortexed. After 2 min incubation time, the change in absorbance was measured at 595 nm and the protein content was estimated from BSA standard curve.

The glucose content was determined using a D-glucose oxidase/oxidase (GOD/POD) assay kit (Coral, Tulip group, India). Briefly, 10 μ L of the sample or standard was properly mixed with 2.0 mL of the enzyme mix and incubated for 10 min at 37 °C. The absorbance of the sample (A_{sample}) and standard (A_{standard}) was read against a reagent blank at 505 nm. The glucose content was measured from glucose standard curve. CIE Lab measurements for honey samples was carried out using Varian Cary 50 Scan UV–Visible spectrophotometer (Agilent Technologies, Inc, Australia) integrated with color software.

Mineral analysis of honey

For mineral analysis, 1 g of honey sample was taken in a Kjeldahl flask and to it concentrated nitric acid and 30% hydrogen peroxide in the ratio of 2:1 were added. The mixture was digested at 300 °C for 3 h. The digest was cooled and the volume was made up to 100 mL with deionised water. Mineral estimation was done using atomic absorption spectrophotometer (iCE 300 series, Thermo Fisher Scientific, USA). Estimation of various elements was done using standard calibration curves made from Na, K, Ca, Mg, Fe, Cu, Ni, Zn, Mn and Co standards stock solution.

HMF, phenolic acid and organic acid profiles using RP-HPLC

The RP-HPLC protocols were run on a Waters system with a Symmetry 300TM C18 (5 μ m, 4.6 $\times$ 250 mm) column with a binary pump (Waters, 1525) and a UV–Vis detector (Waters, 2489). All the samples were filtered through 0.22 μ m nylon membrane filter prior to sample injection and the sample injection volume was 20 μ L. For HMF content, a mixture of 90% ultrapure water and 10% methanol acidified with 1% glacial acetic acid was used at isocratic condition at the flow rate of 0.7 mL/min and the chromatograms were obtained at 285 nm (Zappalà et al. 2005). The HMF content was estimated from the calibration curve of standard HMF.

For phenolic profiling, the honey samples were extracted using liquid–liquid extraction. Briefly, XAD-2 resin was soaked in equal volumes of water and methanol and packed into a column of 2 $\times$ 25 cm dimension and rinsed with 1 L of deionised water. Honey (50 g of honey dissolved in 250 mL of acidified water at pH 2) was filtered through glass wool and added slowly to the column. The column was rinsed with 200 mL of acidified water (pH 2) followed with 300 mL deionised water and 300 mL methanol. The methanol extract was collected and concentrated to 1 mL in a rotary vacuum evaporator at 40 °C. The methanol extract was used for phenolic profiling. Gradient elution

using two mobile phases was done. Mobile phase A was acidified (0.1% acetic acid, pH 3.2) ultrapure water and mobile phase B was methanol. The gradient conditions were as follows: 80% A (0–8 min), 65% A (9–12 min), 45% A (13–16 min), 30% A (17–20 min), 20% A (21–30 min), 10% of A (31–34 min) and then washing of the column with 65% A (35–39 min) and lastly, with 80% A (40–45 min) at a flow rate of 0.8 mL/min (Saikia et al. 2015). The chromatograms for samples and standards of catechin, caffeic acid, coumaric acid, syringic acid, chlorogenic acid, rutin, quercetin, and ferulic acid were obtained at 254 nm.

The organic acid profiling was carried out using isocratic elution of the diluted honey samples with solvent comprising of 95 mL of 10 mM phosphate buffer (pH 2.8) and 5 mL of methanol at a flow rate of 0.8 mL/min (Ding et al. 2006). The standard calibration curves were made for gluconic acid, succinic acid, lactic acid, acetic acid, citric acid and tartaric acid and the chromatograms were obtained at 220 nm.

Sonication of the honey

Sonication of honey (10g) was carried out at 50 °C in an ultrasonic water bath (230 V, 30 $\pm$ 3 kHz, JSGW, India) which had an ultrasonic power of 100 W for time intervals ranging from 0, 30, 60, 90 and 120 min.

Effect of sonication on the bioactive properties of the selected honey samples

Total phenolic content (TPC)

The method of Saikia and Mahanta (2013) was followed. Briefly, 1 g of honey was made up to 10 mL with distilled water and filtered. Then, 100 μ L of extract was added with 7.9 mL of deionised water and 0.5 mL of 1 N Folin–Ciocalteu and mixed well in a test tube. Within 8 min of mixing, 1.5 mL of 20% sodium carbonate was added and the mixture was vortexed. The tube was then incubated in dark for 30 min at 40 °C. The absorbance was taken thereafter at 765 nm in a spectrophotometer (Cecil Aquarius 7400, England) and the results were expressed as mg GAE/100 g from standard curve of gallic acid ranging from 50 to 300 mg/mL.

Total flavonoid content (TFC)

The protocol for flavonoid content was as described in the works of Chang et al. (2002). The honey extract was obtained as stated in Sect. 2.5. The data were reported in terms of μ g quercetin/100 g.

Ferric reducing antioxidant potential (FRAP)

A modified method of Saikia and Mahanta (2013) was used for FRAP. Here, 0.1 mL of the extract (as stated in Sect. 2.5) was added to 3.9 mL of FRAP reagent. FRAP values were expressed as $\mu\text{M Fe(II)}/100\text{ g}$ from standard curve in the range of 25–250 mM.

Determination of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) activity

Honey in varying concentrations (5–90%) was extracted in methanol and filtered. For studying the effect of sonication on honey, 90% methanolic extract was used for calculation. Precisely, 4 mL of 10^{-4} M DPPH solution in methanol was added to 0.1 mL of honey extract and absorbance was taken at 517 nm in a spectrophotometer after incubation in dark for 30 min (Saikia and Mahanta 2013). Blank contained 0.1 mL methanol and 4 mL of 10^{-4} M DPPH. The scavenging activity was calculated using the equation.

$$\text{Radical scavenging activity (\%)} = [(A_o - A_s)/A_o] \times 100$$

where A_o is the absorbance of blank and A_s is the absorbance of sample. The IC_{50} is the concentration at which the sample showed 50% radical scavenging activity which was calculated from the linear equation generated from the plot for DPPH radical scavenging activity against increasing concentration of honey.

Superoxide radical scavenging activity (SO)

The honey samples were mixed with 0.1 M phosphate buffer of pH 7.4 to obtain solutions varying in concentration from 10–90% and filtered. Concentration of 50% was taken to study the effect of sonication. The superoxide radical scavenging activity was measured using PMS/NADH system. Precisely, 2 mL of extract in a test tube was added with 1.25 mL of 468 μM NADH solution, 1.25 mL of 150 μM NBT solution and 1.25 mL of 60 μM PMS solution and incubated at room temperature for 5 min. The absorbance was read at 560 nm against blank containing 2 mL of phosphate buffer and the other reagents. The activity was calculated similar to DPPH activity (Sect. 2.8). Linear graph of concentration versus percentage inhibition of scavenging activity was prepared and IC_{50} value was calculated.

Total antioxidant capacity (TAC)

The total antioxidant capacity in extract of 1% honey in methanol was estimated according to the protocol

described in the works of Silici et al. (2010). The antioxidant activity was calculated as ascorbic acid equivalents (mg AAE/ g honey).

Statistical analysis

All experiments were carried out at least in triplicates and reported as mean $\pm$ standard deviation of mean. The data were subjected to Duncan's new multiple range test ($p < 0.05$) and Pearson's correlations between sonication time and the bioactive properties were observed to find relations using IBM SPSS Statistic version 20 software.

Results and discussion

Melissopalynological analysis

Of the samples under study, only honey samples from Sohra and Jorhat were unifloral and rest were multifloral. The sample from Sohra had dominant pollens (83%) of *Citrus* sp and pollens belonging to the families of cucurbitaceae, malvaceae and fabaceae were present in lesser numbers. The honey from Jorhat had dominant pollens of *Brassica campestris* which contributed to 96% of the total pollens. On the other hand, honey from Shillong had pollens from polygonaceae, sapindaceae, brassicaceae, poaceae, myrtaceae, lamiaceae and rutaceae families. Honey from Mawsynram consisted of pollen grains from poaceae, malvaceae, brassicaceae and rutaceae families. The sample from Tezpur contained pollens belonging to plants from families of lamiaceae, poaceae, brassicaceae, malvaceae and moraceae. No dominant pollen grains were identified from samples from Shillong, Mawsynram and Tezpur.

Physicochemical parameters

Table 1 represents the physicochemical properties of honey from North East India. The moisture content of the collected honeys ranged between 15.4 and 22.8%. The moisture content in honey collected from Mawsynram (20.1%) and Jorhat (22.8%) was higher than the maximum permissible limit of 20% for honey (Codex Alimentarius Commission 2001). High moisture ($> 21\%$) honey indicates a premature extraction or extraction under high humidity conditions (Ajlouni and Sujirapinyokul 2010). Quality of honeys with higher moisture content deteriorates due to fermentation and subsequent rise in acidity and sour taste (Singh and Bath 1997; Ajlouni and Sujirapinyokul 2010). Various factors like varietal differences, the harvesting time, climatic factors, geographical factors and level of maturity of honey in the hive cause variation in moisture content (Kayacier and Karaman 2008). The pH of

Table 1 Physicochemical properties of honey from North East India

Honey samples	Refractive index at 20 °C	Moisture content (%)	pH	Ash content (g/100 g)	Honey acidity (meq/kg)			Total soluble protein content (mg/100 g)	Glucose content (g/100 g)	Fructose content (g/100 g)	Fructose to glucose ratio
					FA	LA	TA				
Shillong	1.4885 ± 0.0005 ^a	19.20 ± 0.20 ^a	4.00 ± 0.02 ^a	0.14 ± 0.02 ^a	16.06 ± 0.26 ^a	15.22 ± 0.32 ^a	31.28 ± 0.29 ^a	129.11 ± 2.03 ^a	28.93 ± 1.56 ^a	36.91 ± 2.34 ^a	1.27
Sohra	1.4982 ± 0.0003 ^b	15.40 ± 0.40 ^b	3.90 ± 0.01 ^a	0.08 ± 0.01 ^{ab}	10.52 ± 0.56 ^b	12.61 ± 0.43 ^b	23.14 ± 0.99 ^b	177.63 ± 6.42 ^b	29.25 ± 0.96 ^a	41.19 ± 3.10 ^{bc}	1.41
Mawsynram	1.4862 ± 0.0002 ^c	20.10 ± 0.30 ^{c,a}	3.37 ± 0.10 ^b	0.07 ± 0.02 ^{ab}	54.41 ± 1.45 ^c	52.74 ± 2.3 ^c	107.15 ± 1.87 ^c	34.96 ± 2.82 ^c	39.51 ± 2.43 ^b	35.62 ± 2.32 ^a	0.90
Jorhat	1.4795 ± 0.0006 ^d	22.80 ± 0.30 ^d	3.84 ± 0.2 ^a	0.17 ± 0.08 ^c	25.39 ± 1.21 ^d	24.46 ± 0.67 ^d	49.75 ± 0.94 ^d	253.54 ± 4.02 ^d	27.74 ± 1.14 ^a	42.94 ± 4.05 ^{bc}	1.54
Tezpur	1.4895 ± 0.0001 ^e	18.80 ± 0.10 ^{e,a}	3.93 ± 0.04 ^a	0.04 ± 0.03 ^b	17.63 ± 0.87 ^a	17.07 ± 0.43 ^a	34.75 ± 0.65 ^a	58.36 ± 1.39 ^e	37.70 ± 1.80 ^b	30.66 ± 1.87 ^d	0.81

Data reported as Mean ± standard deviation

No. of observations n = 3

The different letters (a,b, c...) in the superscript suggests significant difference between the samples based on Duncan multiple range test at $p < 0.05$

FA, LA and TA indicates free acidity, lactone acidity and total acidity respectively. TA = FA + LA

the honey samples ranged from 3.37 to 4.00 indicating the acidic character of honey. The pH values were found to be similar to Indian honeys reported by Saxena et al. (2010) and also comparable to Australian honeys (Ajlouni and Sujirapinyokul 2010), Tunisian honeys (Boussaid et al. 2014) and Turkish honeys (Kayacier and Karaman 2008). The pH values are important, since acidity can influence the texture, stability, and shelf life of honey (Froschle et al., 2018).

Honey samples from Shillong, Sohra, Jorhat and Tezpur had total acidity (sum of free acidity and lactone acidity) ranging from 23.14 to 49.75 meq/kg, which was in agreement with 33.5–53.5 meq/kg reported in Australian honeys (Ajlouni and Sujirapinyokul 2010), but Mawsynram recorded an exceptionally high total acidity of 107.15 meq/kg probably due to excessive fermentation of the honey (Ajlouni and Sujirapinyokul 2010). Organic acids, particularly gluconic acid and its corresponding lactones, and inorganic ions, such as phosphates, sulfates and chlorides make the honeys acidic (Nanda et al. 2003). The wide variation in honey acidity is attributed to the floral sources of nectar and variations due to harvest season (Perezarquillue et al. 1994; Ajlouni and Sujirapinyokul 2010). Free acidity of all the honey samples except for Mawsynram (54.41 meq/kg) was within the permitted range of no more than 50 meq/kg specified by Codex Alimentarius (2001). Initially, glucose and fructose are converted into carbon dioxide and alcohol and thereafter alcohol is hydrolysed and converted to acetic acid in the presence of oxygen, and the generated acetic acid raises the free acidity in honey. In the studied honey samples, the mean free acidity values in Shillong and Tezpur honey were not significantly different ($p < 0.05$). Lactone content values, it was observed, were almost similar with free acidity values implying that free acidity and lactones contributed to acidity in honey, with Mawsynram having the highest value of 52.74 meq/kg. Organic acids are in a state of variable equilibrium with their corresponding lactones (Froschle et al. 2018).

Ash content in the present study ranged from 0.045 to 0.14% which is comparable with Indian honeys (Saxena et al. 2010). Ash content can be taken as a parameter to determine the botanical origin of honey and there is a difference in ash content between floral, mixed and honeydew honeys; in general floral honeys have the lowest ash contents (Froschle et al. 2018). The total soluble protein in the samples ranged from 34.96 to 253.54 mg/100 g of honey and is related to the botanical origin of honey and the quality of nectars (Anklam 1998). Enzymes like alpha-amylase, invertase, catalase, glucose oxidase, and phosphatase are also included in the protein content of honey. The samples had fructose concentration ranging from 30.66 to 41.19% and glucose content ranging from 28.93 to 39.51%. Fructose/glucose (F/G) ratio is an indicator for

honey granulation because glucose is less water soluble than fructose. Moreover, honey crystallization is slower when F/G ratio exceeds 1.3, and it is faster when the ratio is below 1.0 (Dobre et al. 2012). Accordingly, Mawsynram and Tezpur honeys will crystallize faster than the others.

Color characteristics of the selected honey

Color parameter of honey is studied to judge consumer preference. The CIE Lab readings for honey samples are presented here: Shillong ($L^* = 15.21 \pm 1.12$, $a^* = 29.83 \pm 2.10$, $b^* = 26.59 \pm 0.23$); Sohra ($L^* = 42.75 \pm 2.01$, $a^* = 12.92 \pm 0.22$, $b^* = 58.05 \pm 1.78$); Mawsynram ($L^* = 0.007 \pm 0.00$, $a^* = 3.14 \pm 0.01$, $b^* = 0.70 \pm 0.04$); Jorhat ($L^* = 6.94 \pm 0.12$, $a^* = 30.18 \pm 3.06$, $b^* = 12.28 \pm 1.09$); and Tezpur ($L^* = 59.84 \pm 2.33$, $a^* = 19.70 \pm 1.24$, $b^* = 80.28 \pm 3.11$). It is obvious that the honey samples differed widely in their L^* , a^* and b^* values. The L^* , a^* and b^* values suggest that sample from Mawsynram was the darkest of all the samples and the honey from Tezpur was more on the yellow side and Jorhat honey was more reddish than the rest. Honey color is influenced by the content of minerals, pollen, phenols, and flavonoids and storage conditions (El-Haskoury et al. 2018). Color of honey also darkens on heating due to formation of Maillard reaction products and on storage at all temperatures (Singh and Singh 2018); this implied that Mawsynram was subjected to strong heating conditions. Heating of honey is done to prevent fermentation by sugar-tolerant yeasts as well as crystallization.

Mineral analysis

The variation in mineral composition (Table 2) is due to factors like geographic region of the collected honey, different bee keeping practices, contamination due to processing or pollutants and urbanization of the geographical region from which the honey comes (Pohl et al. 2012). Küçük et al. (2007) reported that honey has high quantity of K, Na and Ca content and is also a good source for Fe and Zn; which is similar to the our results. Also, the mineral content of the studied honeys from North-East India was found to be comparable with the mineral content of North Indian honey for Cu, Zn, Ca and Fe (Nanda et al. 2003). However, the Fe content in the studied samples was on the higher side which could be attributed to higher Fe content in the soil of North-East India that is mostly lateritic and alluvial.

HMF, phenolic profile and organic acid profile

Naturally occurring levels of HMF are about 10 mg/kg (Crane 1990). Fructose and acids present in honey facilitate

Table 2 Mineral content of the honey from North East India

Honey Samples	Calcium	Sodium	Potassium	Iron	Magnesium	Copper	Manganese	Nickel	Zinc
Shillong	392.38 ± 4.65^a	179.14 ± 0.78^a	372.38 ± 8.42^a	31.89 ± 0.86^a	113.65 ± 1.23^a	0.23 ± 0.02^a	5.83 ± 0.31^a	1.91 ± 0.12^a	81.44 ± 4.05^a
Sohra	208.52 ± 3.12^b	125.04 ± 0.63^b	287.25 ± 10.24^b	18.04 ± 0.42^b	35.27 ± 0.05^b	0.40 ± 0.10^b	5.64 ± 0.16^a	6.57 ± 0.84^b	70.27 ± 3.02^b
Mawsynram	152.50 ± 1.76^c	198.26 ± 1.02^c	296.42 ± 15.03^b	57.48 ± 0.28^c	12.58 ± 0.18^c	2.87 ± 0.34^c	3.53 ± 0.22^b	3.97 ± 0.32^c	46.66 ± 1.16^c
Jorhat	339.28 ± 5.68^d	231.27 ± 2.01^d	902.27 ± 10.28^c	90.31 ± 1.09^d	85.99 ± 0.28^d	19.28 ± 1.03^d	4.46 ± 0.53^c	5.44 ± 0.18^d	136.45 ± 2.09^d
Tezpur	31.08 ± 1.03^e	91.39 ± 0.43^e	96.86 ± 0.51^d	57.26 ± 0.98^c	41.05 ± 0.47^e	14.91 ± 0.92^e	3.16 ± 0.12^b	9.89 ± 0.10^e	36.73 ± 1.27^e

Data reported as mean $\pm$ standard deviation

No. of observations $n = 3$

The different letters (a, b, c...) in the superscript suggests significant difference between the samples based on Duncan multiple range test at $p < 0.05$

the formation of HMF on heating and storage. Temperature, duration of heating during processing, storage time and heat stress during storage increases the HMF in honey (Ajilouni and Sujirapinyokul 2010). HPLC study detected HMF in all the honey samples and the retention time for HMF in all the honey samples was 8.7 min. The HMF content of the honey samples varied from 49.87 to 297.93 mg/kg (Table 3). HMF content should be below 40 mg/kg after processing or blending of honey with an exception that tropical honey should have HMF below 80 mg/kg. Among all the samples, only sample from Sohra and Jorhat were found to fulfill the norm set by Codex Alimentarius Commission (2001) as quality standard for HMF. Thus, it can be inferred that the samples from Shillong, Mawsynram and Tezpur were subjected to excessive heat treatment with resultant high HMF content, an indicator of quality deterioration. There was an inverse relationship between HMF value and fructose to glucose ratio (Table 1), implying that fructose was used up for HMF formation.

The phenolic profile of honey samples are as shown in Fig. 1a. Phenolic acids in honey are responsible for the health beneficial properties of honey. The compounds which were detected and quantified are presented in Table 3. The phenolic profiles varied among the samples. Phenolic compounds present in Sohra, Jorhat, Shillong and Tezpur were not detected in Mawsynram even though phenolic compounds that could not be identified were present in larger concentration. Probably high heat treatment during honey processing as indicated by high HMF content might have caused the complete loss of catechin, ferulic acid, rutin and quercetin in Mawsynram. Moreover, some peaks could not be identified due to non availability of reference standards.

The organic acid profiles are shown in the Fig. 1b–f. Table 3 gives the quantification of the identified organic acids. In the honey samples, gluconic acid, acetic acid, citric acid, succinic acid, tartaric and lactic acids were identified. The presence of these acids has also been reported by Suárez-Luque et al. (2002). Gluconic acid was the major organic acid observed in honey samples.

Bioactive properties of the untreated and sonicated honey samples

Total phenolic content (TPC)

The total phenolic content of honey ranged from 74.42 to 290.68 mg GAE/100 g (Table 4). The range was found to be higher than the values reported for various Tunisian honeys (Boussaid et al. 2014), monofloral and multiflora Turkish honeys (Can et al. 2015), commercial Indian honey (Saxena et al. 2010) and Madagascan *Jatropha* honeys

Table 3 RP-HPLC quantification of the phenolics, organic acids and hydroxymethylfurfural (HMF) content in honey from North East India

Samples	Phenolic acids (ppm)					Organic acids (ppm)					HMF content (mg/kg)		
	Catechin	Ferulic acid	Rutin	Quercetin	Gluconic acid	Acetic acid	Citric acid	Succinic acid	Tartaric acid	Lactic acid			
Retention time (min)	11.9	16.5	17.3	19.9	3.8	5.2	6.2	6.9	4.1	4.9	8.7		
Shillong	8.83 ± 0.01 ^a	N.D.	17.45 ± 1.12 ^a	N.D.	331.28 ± 11.23 ^a	15.23 ± 2.10 ^a	3.77 ± 0.49 ^a	19.21 ± 2.69 ^a	N.D.	N.D.	94.41 ± 5.31 ^a		
Sohra	4.64 ± 0.02 ^b	38.89 ± 2.04	9.13 ± 0.50 ^b	N.D.	96.11 ± 6.10 ^b	8.70 ± 1.98 ^b	N.D.	6.40 ± 1.11 ^b	2.41 ± 1.05	0.24 ± 0.02	49.87 ± 1.47 ^b		
Mawsynram	N.D.	N.D.	N.D.	N.D.	102.24 ± 8.34 ^c	8.70 ± 1.56 ^b	N.D.	6.40 ± 1.28 ^b	N.D.	0.99 ± 0.04	297.93 ± 12.45 ^c		
Jorhat	12.80 ± 0.10 ^c	N.D.	20.48 ± 0.31 ^c	5.92 ± 0.28 ^a	143.14 ± 7.46 ^d	N.D.	N.D.	28.8 ± 4.28 ^c	N.D.	N.D.	58.88 ± 3.46 ^d		
Tezpur	N.D.	N.D.	15.14 ± 0.12 ^d	6.99 ± 0.43 ^b	61.35 ± 2.98 ^e	4.35 ± 1.24 ^c	3.77 ± 0.49 ^a	6.60 ± 1.00 ^b	N.D.	N.D.	134.87 ± 7.81 ^e		

Data reported as Mean ± standard deviation

No. of observations n = 3

The different letters (a, b, c...) in the superscript suggests significant difference between the samples based on Duncan multiple range test at $p < 0.05$

N.D. indicates not detected

Fig. 1 a RP-HPLC showing phenolic profiles of honey from North East India b–f RP-HPLC profile for organic acids in the samples from Shillong, Sohra, Mawsynram, Jorhat and Tezpur respectively

Table 4 The bioactive properties of honey from North East India

Honey samples	Total phenolic content (mg GAE/100 g)	Total flavonoid content (μ g quercetin/100 g)	Ferric reducing antioxidant potential (μ M Fe(II)/100 g)	Total antioxidant capacity (mg AAE/g honey)	DPPH radical scavenging activity (IC ₅₀) (g)	Superoxide radical scavenging activity (IC ₅₀) (g)
Shillong	140.83 $\pm$ 3.16 ^a	144.84 $\pm$ 3.78 ^a	7291.60 $\pm$ 584.6 ^a	25.72 $\pm$ 0.49 ^a	29.8 $\pm$ 0.20 ^a	21.18 $\pm$ 0.1 ^a
Sohra	83.85 $\pm$ 1.74 ^b	69.53 $\pm$ 2.34 ^b	2272.21 $\pm$ 67.01 ^b	24.36 $\pm$ 0.31 ^a	73.9 $\pm$ 0.23 ^b	48.44 $\pm$ 0.21 ^b
Mawsynram	126.07 $\pm$ 2.21 ^c	72.49 $\pm$ 2.24 ^b	3213.77 $\pm$ 84.83 ^c	21.84 $\pm$ 1.16 ^b	54.3 $\pm$ 0.21 ^c	N.E
Jorhat	296.68 $\pm$ 2.16 ^d	155.26 $\pm$ 2.90 ^c	5622.44 $\pm$ 115.9 ^d	24.55 $\pm$ 1.33 ^a	35.5 $\pm$ 0.54 ^d	66.22 $\pm$ 0.76 ^c
Tezpur	74.42 $\pm$ 2.63 ^e	25.49 $\pm$ 3.98 ^d	1435.18 $\pm$ 40.09 ^e	22.75 $\pm$ 0.54 ^b	100 $\pm$ 1.20 ^e	N.E

Data reported as Mean $\pm$ standard deviation

No. of observations n = 3

The different letters (a, b, c...) in the superscript suggests significant difference between the samples based on Duncan multiple range test at $p < 0.05$

N.E indicates non estimation as the activity was too low

(Froschle et al. 2018). The variation in the phenolic content is attributed to different floral origin of honey (Froschle et al. 2018; Küçük et al. 2007).

Sonication of the honey samples had mild effect on phenolic content and only a slight decrease in phenolic content at the end of 120 min was observed as compared to non-sonicated samples (Fig. 2a). Altemimi et al. (2016) observed lower extraction of phenolics at temperature higher than 42 °C. Decrease in phenolic content may be due to structural destruction or decomposition of phenolics (Carrera et al. 2012).

Total flavonoid content (TFC)

Flavonoids are an important sub-branch of the polyphenol family (Can et al. 2015). The flavonoid content of honeys under study ranged from 25.45 to 155.26 μ g quercetin/100 g of honey (Table 4). The samples from Jorhat and Shillong showed the highest flavonoid content. It was observed that samples with higher phenolic content also had higher flavonoid content (Table 2). In comparison, total flavonoids were present in the range of 0.65 and 8.10 mg quercetin/100 g in monofloral and polyfloral honeys from Turkey (Can et al. 2015) and 9.58–22.45 mg quercetin/1000 g in Tunisian honeys (Boussaid et al. 2014). A study on Polish honeys suggested that flavonoid content represents approximately 5% of total phenolic content (Jasicka-Misiak et al. 2012), thus higher phenolic content can be related to higher flavonoid content.

Sonication time of 30 min caused an increase in flavonoid content in all the samples except for Jorhat and Sohra and with further increase in sonication time the flavonoid content decreased (Fig. 2b). The initial increase can be attributed to better extraction of flavonoids from the matrix (Annegowda et al. 2010). Prolonged sonication for 120 min decreased the flavonoid content in all honeys except Mawsynram honey which showed an increase of

10.2%. A very high loss of 44.7% of the initial content was noticed in Tezpur honey after 120 min of sonication. The loss of flavonoid content on prolonged sonication may be attributed to sonochemical effects like oxidation (Robak et al. 1991) or glycosylation of flavonoids (Biesaga and Pyrzynska 2013). Also, generation of reactive hydroxyl radicals during ultrasound treatment induces degradation of certain flavonoids (Paniwnyk et al. 2001). Sonication usually inactivates PPO activity and enhances the release of phytochemicals such as phenolics and flavonoids due to breakdown of the cellular matrix because of cavitation (Nadeem et al. 2018). Changes in phenolic compounds are influenced mainly by their composition. Flavonoids are a subclass of phenolics and their increase can sometimes be independent of phenolics. In addition, upon processing, there is a tendency of the phenolic compounds to undergo structural rearrangement which could lead to either an increase or a decrease in their content (Naczka and Shahidi 2004). Individual phenolic acids and flavonoids can have varying degree of susceptibility towards sonication. As reported by Saikia et al. (2016), sonication of pineapple juice resulted in a decrease in gallic acid, chlorogenic acid and quercetin but showed an increase in catechin, syringic acid and kaempferol.

Ferric reducing antioxidant potential (FRAP)

The FRAP for all the honey samples was found to be quite high and ranged from 1435.18 to 7291.60 μ M Fe(II)/100 g (Table 4). Various authors have reported a correlation between honey color and antioxidant capacity, where amber and more crystallized honeys possess stronger antioxidant activity than lighter and transparent (Taormina et al. 2001) and this may be one of the reasons for increased FRAP values. The FRAP values obtained are high and is in congruence with the results in other Indian honeys (Saxena et al. 2010). It is also reported that FRAP

Fig. 2 Changes in **a** total phenolic content, **b** total flavonoid content, **c** FRAP activity, **d** total antioxidant capacity, **e** DPPH activity, and **f** superoxide radical scavenging activity after sonication with varying time from 0 to 120 min. Data reported as Mean $\pm$ standard deviation. No. of observations $n = 3$, The different letters (a, b, c...) in the superscript suggests significant difference between the samples based on Duncan multiple range test at $p < 0.05$

values are directly dependent upon concentration (Küçük et al. 2007).

Again, sonication did not show any distinct effect on FRAP power (Fig. 2c). Among the samples, Mawsynram honey showed an increase in FRAP activity which can be attributed to the increase observed in its flavonoid content during sonication. On the other hand, Tezpur honey that recorded a decrease in flavonoid content on sonication also showed decrease in FRAP value (Fig. 2b).

Total antioxidant capacity (TAC)

The antioxidant activity of the samples measured by phoshomolybdenum method ranged from 21.84 to 25.72 mg AAE/g honey (Table 4). The range was found to be similar to the average ranges of antioxidant power of certain *Rhododendron* honeys (Silici et al. 2010) although variations between samples were observed probably because of floral source and environmental variations. Maillard reaction products formed during heating of honey have antioxidant properties (Turkmen et al. 2006). This explains the antioxidant properties of Mawsynram honey that has very few phenolic acids present (Fig. 1) but high HMF (Table 3).

A significant reduction was seen in the antioxidant power for all the samples after 120 min of sonication (Fig. 2d) as compared to the initial content without sonication. The probable reasons can be the degradation of individual phenolics, organic acid, peptides and enzymes (Elhamirad and Zamanipoor 2012; Majid et al. 2015; Janghu et al. 2017) that are supposed to contribute towards antioxidant capacity (Silici et al. 2010) and also the degradation of ascorbic acid on sonication (Tiwari et al. 2008).

DPPH radical scavenging activity (DPPH)

The DPPH radical scavenging activity of the different honeys varied. The IC_{50} values of the different samples are given in Table 4 and it is to be noted that lower IC_{50} value means better antioxidant property. The honey collected from Tezpur had the least DPPH activity and its IC_{50} value was around 100 g while the sample collected from Shillong showed the highest antioxidant activity followed by Jorhat, Mawsynram and Sohra. Some of the honeys under study showed better scavenging activity than that of

Eucalyptus and Horehound honey collected from Tunisia (Boussaid et al. 2014). Also, the antioxidant activity of honeys from Shillong, Mawsynram and Jorhat were better than other commercial Indian honey samples (Saxena et al. 2010).

DPPH activity showed that sonication affected the antioxidant activity of honey (Fig. 2e). It was observed that after an initial decline in the DPPH activity after 30 min of sonication, the activity was more or less stable in all the samples till 120 min of sonication. The lowering can be due to the effect of reduced level of flavonoids and phenolics on sonication, as they are positively correlated (Bertoncelj et al. 2007). Elhamirad and Zamanipoor (2012) observed that sonication and heating caused degradation of individual phenolics like gallic acid. Zhao et al. (2014) reported that formation of dimers between flavonoid molecules on sonication caused higher loss in antioxidant activity suggesting that a small decrease in phenolics or flavonoids might cause significant losses in antioxidant activity.

Superoxide radical scavenging activity (SO)

IC_{50} of Mawsynram and Tezpur honeys could not be calculated as their superoxide radical scavenging activity was very low. The data are presented in Table 4. The presence of such activity in honey helps in establishing that honey can quench free radicals and can reduce oxidative damage. Honey from Shillong showed the highest scavenging activity with an IC_{50} value of 21.18 g. The superoxide radical scavenging activity is suggested to be related to the phenolic and flavonoid content (Esmaeili et al. 2015).

It was also observed that on sonication of the honey the superoxide radical scavenging activity was depleted and the honey sample from Mawsynram lost its activity completely while in rest of the samples there was loss of activity with time (Fig. 2f).

Statistical correlation between the bioactive properties

The correlation tables S1, S2, S3, S4 and S5 respectively for honey samples from Shillong, Mawsynram, Sohra, Jorhat and Tezpur respectively are supplied as supplementary file. The sonication time was found to be negatively correlated with TAC, SO, DPPH and TFC in the honey sample from Shillong. However, TAC positively correlated to SO and DPPH and SO correlated with DPPH at both significance levels of 0.05 and 0.01. Similarly, in Sohra sample time had a negative effect on TAC, SO, DPPH and TFC. TPC had a positive correlation with TAC, FRAP and TFC and it did not affect SO and DPPH. TAC was positively correlated with SO, FRAP, DPPH and TFC. The SO values showed a positive correlation with that of

DPPH and TPC values. In the Mawsynram honey sample, time of sonication negatively correlated with SO and DPPH but rest of the properties showed no significant changes due to time. TPC was positively correlated with FRAP and TFC. TAC was positively correlated with SO and DPPH. SO negatively correlated with FRAP and TPC but positively correlated with DPPH. Likewise, in the Jorhat sample sonication time had a negative correlation with TPC, TAC, SO, DPPH and TAC but showed no significant effect on FRAP. TPC positively correlated with TAC, SO, FRAP and TFC. TAC positively correlated with SO and TPC. SO was positively correlated with DPPH and TPC. FRAP was positively correlated with DPPH. However, in the honey sample from Tezpur, sonication exhibited a negative correlation with FRAP while other properties were not affected significantly. Tezpur sample did not show SO activity on sonication so no relation with other properties could be determined. But, TAC was positively correlated with FRAP and DPPH. FRAP and DPPH were also correlated positively. The variation in correlation among the samples with the bioactive properties might be due to compositional variation, the effect of the matrix as well as the viscosity differences which might have interfered with the effect of sonication.

Conclusion

All the honeys showed acidic character with low moisture contents. The honeys had good mineral composition, TPC, TFC, and FRAP values, and DPPH activity. However, there were variations among the samples. Mawsynram showed acidity levels and HMF content that were above the standards for honey. The quality of Mawsynram indicates that there is a need for creating awareness among the honey processors of the region to use the modern techniques of honey production. Sonication exhibited varied effect on TPC, TFC and FRAP values of the honey samples based on their source and treatment time. DPPH, total antioxidant capacity and superoxide radical scavenging activity was reduced on sonication. As all the honey samples showed good phenolic content, flavonoid content and antioxidant activities these honeys can be used to enrich foods and also as an additive in the food processing, cosmetics and pharmaceutical industries.

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Compliance with ethical standards

Conflict of interest We declare that there is no conflict of interest.

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