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Original Article

Antibacterial Activity and Physicochemical Properties of Florally Diversified Bangladeshi Honeys

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Seven florally diversified Bangladeshi honey samples were assessed for their physicochemical properties as well as their antibiogram profile on different human pathogenic bacterial strains. The average density, total protein content, ascorbic acid content, total phenolics content and the total antioxidant capacity of these honeys were determined as 1.50±0.09 g/ml, 5.63±1.56 mg/g, 91.87±22.16 mg/g, 571.04±289.02 mg gallic acid equivalent/gram, and 320.74±55.06 mg ascorbic acid equivalent/gram of samples, respectively. A significant correlation between the phenolics content and the total antioxidant capacity was observed in commercially available honey samples. However, the natural honey samples showed the best antibacterial activity against different pathogenic multidrug-resistant bacterial strains. Significant antibacterial activities were observed against *Klebsiella pneumoniae*, *Salmonella typhi*, *Micrococcus luteus*, *Pseudomonas* spp. and *Enterobacter* spp. and moderate antibacterial activities was observed on *Shigella boydii*, *Bacillus cereus*, *Bacillus megaterium*, and *Bacillus subtilis*. The antibacterial activity was correlated with the total antioxidant capacity. This study suggested that the Bangladeshi multifloral honeys have clinical potential against multidrug-resistant pathogenic bacterial strains.

Keywords: Honey, antioxidant capacity, multidrug-resistance, antibacterial activity.

Introduction

In the long human tradition, honey has been used not only as a nutrient but also as medicine and preservative[1,2]. The properties of honeys depend largely on their composition and the actual composition of honey varies in association with many factors such as the honey bees and angiosperm species, climate, and the processing it undergoes[3]. At least 181 components, including simple sugars, proteins and free amino acids, vitamins, polyphenols, flavonoids, carotenoids, minerals, and ascorbic acid have been identified in honey[4]. Honey has many biological effects, such as antibacterial, antioxidant, anti-inflammatory, anti-allergenic activities etc. and also shows various metabolic activities in our body[5]. Honey exerts antibacterial properties due to the presence of defensins as well as its consistent amount of hydrogen peroxide and non-peroxide factors, such as flavonoids and polyphenols content, low pH level, osmotic effect (due to high sugar content) etc.[6,7]. Honey has been identified as a potential alternative to the widespread use of antibiotics, which are of significant concern considering the emergence of several multidrug-resistant bacterial strains[1]. Several research works have been done on the antibacterial activity of honey. Previous investigations indicated the antibacterial activity of honey against *Escherichia coli, Campylobacter jejuni, Salmonella enterica, Shigella dysenteriae*, *Mycobacterium tuberculosis*, vancomycin–resistant *Enterococcus faecalis*, and other common gastrointestinal pathogenic bacteria[8,9]. Honey samples collected from Northern Ireland and France showed a significant ability to inhibit the growth of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA)[10]. Honey also inhibits the development of bacterial biofilms formed by *Streptococcus pyogenes* and *Pseudomonas aeruginosa*[11,12]. Nonetheless, the antibacterial and antioxidant activities of a honey sample depend on its physical and chemical factors. Hence, the comparative analyses on physicochemical properties and biological activities of different honeys from various regions of the world have been extensively conducted. Honey is produced and consumed on a large scale in Bangladesh, and some investigations have already been done with the physicochemical, antibacterial and antioxidant properties of Bangladeshi honeys. These include the heavy metal content, antioxidant properties, antibacterial activity and seasonal variation in antibacterial activity etc. of Bangladeshi honey samples[13,14]. However, an extensive study on the antibacterial activity of physicochemically defined diverse categories of

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Bangladeshi honey samples is still absent. This study investigated the antibacterial potentials and physicochemical properties of seven different varieties of honey samples of Bangladesh against pathogenic bacterial strains. Our observation suggested a positive correlation between the antibacterial activity and the total antioxidant capacity of these samples.

Materials and Methods

Sample collection and preparation

Seven different amber honey samples were collected from different regions of Bangladesh. Natural honeys were collected from the comb and commercially available honeys were bought from the market. All samples were stored at room temperature (20-25 °C) before analysis and were treated similarly (Table 1). Individual samples were properly mixed with cold milli-Q water as 1:1 (v/v), and then were filtered through 0.45 ìm syringe filter to remove particles.

Table 1. *Details of seven different honey samples.*

Sample	Source	Type
BDH 1	The Sundarban	Multifloral (Natural)
BDH 2	University of Dhaka	Multifloral (Natural)
BDH 3	Jessore	Multifloral (Natural)
BDH 4	Local Market	Multifloral (Commercial)
BDH 5	Local Market	Multifloral (Commercial)
BDH 6	Local Market	*Nigella sativa* (Commercial)
BDH 7	Local Market	*Brassica campestris* (Commercial)

Basic physicochemical analysis

The densities of the undiluted honey samples were determined using an electronic balance (KERN & Sohn GmbH; Type: ABS 220-4). The total protein content of the diluted honey samples was determined by Lowry's method of protein estimation using bovine serum albumin (Sigma, USA) as standard[15]. The ascorbic acid content was determined by Bessel's titrimetric method using 2,6-dichlorophenol indophenol (Sigma, USA) as the dye and standard ascorbic acid[16]. Total phenolics content of the honey samples were measured as mg gallic acid equivalence using a previously described modified spectrophotometric Folin-Ciocalteu method[17]. Briefly, 200 µl of 1:1 diluted honey sample was mixed well with 1.8 ml distilled H_2O and 200 µl Folin-Ciocalteaue phenol reagent (Scharlau, Spain). The solution was incubated for 5 minutes at room temperature and then was mixed well with 2 ml of 7% Na_2CO_3 (Sigma, USA) and 800 ml distilled water. This solution was incubated in dark for 90 minutes at room temperature. Absorbance of the solution was taken at 750 nm using a spectrophotometer. The total antioxidant capacity was measured as mg ascorbic acid equivalence using the phosphomolybdenum method[17]. For this, a 0.1 ml aliquot of the diluted honey sample was shaken with 1 ml of phosphomolybdenum reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate in distilled water). The test tubes were covered and incubated in a water bath at 95 °C for 90 minutes. After the samples were cooled, the absorbance of the mixture was measured at 765 nm. 0-100 mg of ascorbic acid was used as the standard positive control and Milli-Q water was used as negative control.

Antibacterial activities of honey samples

Antibacterial activities of the honey samples were assessed with the test bacterial strains using agar well diffusion method[18]. For this, 16 test organisms, including 9 Gram-negative and 7 Gram-positive bacterial strains, were collected from the Department of Genetic Engineering and Biotechnology, University of Dhaka repository. Antibiogram of these strains was determined by Kirby-Bauer disk diffusion method[19]. The antibiotics tested were: ampicillin (10 µg), azithromycin (15 µg), ciprofloxacin (5 µg), ceftriaxone (30 µg), methicillin (5 µg), nalidixic acid (30 µg), tetracycline (30 µg), and vancomycin (15 µg) (Oxoid, UK). Briefly, 100 ml of 0.5 McFarland standard[20] inoculum in nutrient broth (Oxoid, UK) was spread in Muller-Hinton agar (Oxoid, UK) plate. The antibiotic disks were placed on top of the agar media or the wells were made on the agar media using sterile tips. 20 µl of diluted honey samples were applied in each well. Plates were then incubated overnight at 37 °C. The diameter of the clear zone of inhibition was measured in millimeter (mm) unit. Milli-Q water was used as a negative control. Susceptibility to a certain antibiotic was justified using the Clinical and Laboratory Standards Institute (CLSI) standard chart.

Statistical Analysis

All the tests were carried out in triplicate. The results were expressed as mean values, the standard deviation (SD), probability (p-value), and the correlation co-efficient (r). The data were analyzed using Microsoft Excel 2007.

Results

Physicochemical properties

Density of the collected honey samples ranged from 1.36 g/ml – 1.61 g/ml with an average 1.50±0.09 g/ml, indicating that the density of commercial honey fluctuates more compared to the natural one. The lowest density was observed with monofloral (*Nigella sativa*) honey sample (BDH 6) (Table 2). Average protein content of these samples was 5.63±1.56 mg/g. The protein content of commercially available honey samples were higher compared to non-commercial one ($p<0.03$). The protein content was the highest in sample BDH 6 and the lowest in sample BDH 1. The highest ascorbic acid content was observed in sample BDH 4 followed by sample BDH 6. On an average, 91.87±22.16 mg ascorbic acid was detected per gram of honey samples. The ascorbic acid contents of natural honeys were closer to the average. The mean phenolics content of these honeys was 571.04±289.02 mg gallic acid equivalent per gram of sample (Table 2). The BDH 6 sample contained the highest phenolics, whereas the sample BDH 3

contained the lowest level of phenolics. The sample BDH 2 possessed the highest antioxidant capacity (368.4±0.3 mg ascorbic acid equivalent per gram) and the sample BDH 1 possessed the lowest (215.8±0.8 mg ascorbic acid equivalent per gram), indicating that the antioxidant activity of the natural honey may fluctuate. The average antioxidant capacity was 320.74±55.06 mg ascorbic acid equivalent per gram of samples (Table 2). There was no significant difference in the antioxidant capacity of natural honeys and commercial honey samples. Interestingly, the antioxidant capacity of the commercial honey samples was strongly correlated with the total phenolics content ($r^2 = 0.93$). Such findings indicated that the total phenolics might have contributed to their antioxidant potentials.

Antibiogram of the strains

Antibiogram of the pathogenic bacterial strains suggested that all of these strains were resistant to methicillin and mostly to vancomycin. The *Shigella dysenteriae* strain was resistant to all tested antibiotics (Table 3). 50% of the tested strains were resistant to ampicillin, whereas 37.5% of them were resistant to ceftriaxone. However, 50% of these strains were sensitive to ciprofloxacin, and 43.75% were sensitive to tetracycline. These data indicated that these strains were mostly multidrug-resistant.

Antibacterial activity of the honey samples

Table 4 summarizes the antibacterial activity of the honey samples against the multidrug-resistant bacterial strains. It was observed that sample BDH 2 showed antibacterial activity against all the strains except *Shigella dysenteriae*. Only the sample BDH 3 and BDH 7 showed excellent antibacterial activity against this strain. The multidrug-resistant strains of *Pseudomonas* spp. and *Enterobacter* spp., and also the *Klebsiella pneumoniae*, *Salmonella typhi*, and *Micrococcus luteus* were highly susceptible to all honey samples. The least antibacterial activity was observed against *Staphyllococcus aureus*, *Staphyllococcus saprophyticus*, *Escherichia coli*, and *Vibrio cholerae* strains (Table 4). Overall, these honey samples could inhibit the growth of mostly the Gram negative than the Gram positive strains. The antibacterial activity was negatively correlated with the density and the phenolics content (r = -0.32 and r = -0.24 respectively).

Table 2. *Summary of the physicochemical properties of the honey samples.*

Sample	Density (g/ml)	Total Protein (mg/g)	Ascorbic Acid (µg/g)	Total Phenolics (µg gallic acid/g)	Antioxidant Capacity (µg ascorbate/g)
BDH 1	1.46±0.0	3.6±0.01	96.88±0.01	709.18±0.002	215.8±0.8
BDH 2	1.42±0.01	4.6±0.006	73.32±0.01	302.84±0.0	368.4±0.3
BDH 3	1.57±0.01	4.6±0.0	89.87±0.004	276.55±0.0	348.4±0.4
BDH 4	1.57±0.006	7.3±0.01	129.53±0.007	852.92±0.01	360.8±0.5
BDH 5	1.61±0.01	5.2±0.01	72.12±0.0	426.85±0.002	292.7±0.4
BDH 6	1.36±0.01	7.9±0.007	110.22±0.0	1016.2±0.002	357.8±0.4
BDH 7	1.53±0.005	6.2±0.01	71.17±0.004	412.77±0.002	301.3±0.5

Table 3. *Antibiotic resistance pattern of the bacterial strains. A=Ampicillin, AZ=Azithromycin, C=Ciprofloxacin, CT=Ceftriaxone, M=Methicillin, NA=Nalidixic acid, T=Tetracyclin, and V=Vancomycin.*

	Bacterial strain	Resistant	Intermediate	Sensitive
Gram Negative	*Escherichia coli*	M, V	A, NA, T	AZ, C, CT
	Klebsiella pneumoniae	A, M, V	NA, T	AZ, C, CT
	Salmonella typhi	M, V	NA, T	AZ, A, C, CT
	Salmonella paratyphi	M, V	A, AZ, CT	C, NA, T
	Shigella boydii	A, M, NA	AZ, V	C, CT, T
	Shigella dysenteriae	A, AZ, C, CT, M, NA, T, V	-	-
	Vibrio cholerae	M, NA, T	C, CT, V	A, AZ
	Enterobacter spp.	A, C, CT, M, NA, V	AZ	T
	Pseudomonas spp.	A, AZ, C, CT, M, NA, V	T	-
Gram Positive	*Bacillus cereus*	A, CT, M, V	AZ, C, T	NA
	Bacillus megaterium	A, M, V	AZ, C, CT	NA, T
	Bacillus subtilis	A, CT, M, V	AZ, T	C, NA
	Staphylococcus aureus	NA, M	CT, V	A, AZ, C, T
	Staphylococcus saprophyticus	CT, M, NA, V	A, AZ, C	T
	Micrococcus luteus	M, V	A, C, NA	AZ, T, CT
	Streptococcus spp.	M, V	AZ, CT, NA,	A, C, T

Table 4. *Antibacterial activity of the honey samples against the test bacterial strains. Data are expressed as mean ± SD, where n=3.*

	Bacterial strain	Diameter of the Zone of Inhibition (mm)						
		BDH 1	BDH 2	BDH 3	BDH 4	BDH 5	BDH 6	BDH 7
Gram Negative	*Escherichia coli*	7±1	9±1	0±0	0±0	0±0	0±0	6±1
	Klebsiella pneumoniae	15±1	19±1	19±1	22±1	19±1	18±1	18±1
	Salmonella typhi	20±1	29±1	10±1	19±1	24±1	20±1	21±1
	Salmonella paratyphi	0±0	10±0	0±0	0±0	6±1	10±0	0±0
	Shigella boydii	9±1	9±1	0±0	0±0	7±1	9±1	0±0
	Shigella dysenteriae	0±0	0±0	24±1	0±0	0±0	0±0	22±1
	Vibrio cholerae	0±0	8±1	0±0	0±0	0±0	8±1	9±1
	Enterobacter spp.	24±1	22±1	20±1	23±1	21±1	25±1	24±1
	Pseudomonas spp.	20±1	20±1	20±1	22±1	23±1	22±1	20±1
Gram Positive	*Bacillus cereus*	7±1	9±1	0±0	7±1	6±1	0±0	0±0
	Bacillus megaterium	6±0	7±1	6±0	8±1	0±0	0±0	7±1
	Bacillus subtilis	6±1	10±1	0±0	10±1	9±1	9±1	7±1
	Staphylococcus aureus	0±0	9±1	0±0	0±0	0±0	0±0	0±0
	Staphylococcus saprophyticus	0±0	9±1	0±0	0±0	7±1	0±0	0±0
	Micrococcus luteus	13±1	14±1	10±1	8±1	7±1	15±1	16±1
	Streptococcus spp.	0±0	8±1	0±0	0±0	0±0	10±0	10±1

However, a positive correlation between the antibacterial activity and total antioxidant capacity was observed (r = 0.25).

Discussion

In this study, we have demonstrated the antibacterial activity of different honey samples and its correlation with the floral sources as well as the basic physicochemical composition. The density, total protein content, ascorbic acid content, total phenolics, and total antioxidant capacity of the collected honeys (Table 2) were moderately higher than previous reports with Bangladeshi honey as well as from the standard manuka honey[13,14,21]. A positive correlation between total antioxidant capacity and antibacterial activity indicated the presence of phytochemical components, like methylglyoxal, and/or short peptides, like defensins[1]. *Salmonella typhi* strain was the most susceptible strain towards these honey samples, which was also observed before[22]. The Gram negative bacterial strains were more susceptible to these honey samples compared to the Gram positive strains. Such observation indicates that osmolarity might also have a contributing factor to inhibit the bacterial growth, although we have diluted our honey samples to decrease the osmolarity. Our data showed that these honeys were mostly active against pathogenic bacterial strains except the *Shigella* strains, and were poorly active against the non-pathogenic strains. Poor susceptibility of *Escherichia coli*, *Bacillus cereus*, and *Bacillus subtilis* to these samples suggested that these honey samples are least likely to affect the normal gastrointestinal microbial flora. As the resistance to antibiotics continues to rise and few new therapies are on the horizon, it is very important to find out potential antibacterial alternative to combat these bugs. We have observed that the methicillin-resistant bacterial strains are mostly susceptible to our honey samples. Most importantly, these honey samples showed antibacterial activity against *Enterobacter* spp. and *Pseudomonas* spp., these strains were resistant to almost all the antibiotics tested in this study (Table 3 and Table 4). In a previous study, *in vitro* antibacterial activity of natural and commercially available honey of Bangladesh was tested against *Staphylococcus aureus* by Shahedur *et al*., where natural honey showed more inhibitory activity against *Staphylococcus aureus* than commercially available honey, which also supports our finding[23]. The antibacterial activities of five different brands of unifloral honey from the northern region of Bangladesh were investigated by Ibrahim *et al*.[24]. These honey showed a significant antibacterial activity against *Shigella dysenteriae*. However, only one unifloral honey samples (*Brassica campestris*) showed excellent antibacterial activity to *Shigella dysenteriae* in our study. Molan and Cooper reported that the difference in antimicrobial potency among the different honeys can be more than 100-fold, depending on its geographical, seasonal and botanical source[25]. Thereby, the variation in antibacterial activity could possibly due to the floral variation. Overall, our study indicates that Bangladeshi honey samples can be utilized as an alternative to antibiotics.

Conclusion

The development of antibiotic resistance imposes a great challenge to public health by limiting the choice of antibiotic treatment in the case of multidrug-resistant pathogenic bacterial strain. The present study showed that some multidrug-resistant bacteria were sensitive to Bangladeshi honeys. Therefore, these honeys could be used as potential alternative therapy against those bacteria. Further studies into the composition and stability of the active constituents of these honeys are warranted.

Conflict of interest

The authors declare no conflict of interest.

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