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Full Length Research Paper

Assessment of physico-chemical and antimicrobial of honey of *Apis dorsata* from different locations of Pakistan

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This study was designed to determine the physicochemical composition and antimicrobial activity against different food pathogens obtained from different honey samples of *Apis dorsata* of different locations of Pakistan, that is, Changamanga (Central Punjab), Multan (Southern Punjab), Mansehra (Upper Khyber Pakhtunkhwa; I) and Islamabad (Federal Area). These samples were analysed for their moisture, ash, nitrogen, hydroxymethylfurfural (HMF), diastase number (DN), reducing sugars, total sugars, sucrose contents, acidity and pH contents. It was observed that there was a significant difference among these tested parameters for these honey samples. The analysis showed moisture contents in the range of 22.87-26.70%, ash contents in the range of 0.03-0.1 g/100 g, nitrogen contents in the range of 0.27-0.39%, sucrose in the range of 2.5-4.57%, reducing sugars in the range of 69.62-73.93%, HMF contents in the range of 37.14-46.60%, acidity in the range of 23.67-43 meq/kg, pH in the range of 3.09-3.61, diastase number (DN) in the range of 18.33-29% and total sugars in the range of 73.67-77.53. Highest antimicrobial activity was recorded for honey collected from Mansehra which gave zone of diameter as 23.3, 26.67 and 22 mm against *Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus* respectively.

Key words: *Apis dorsata*, honey, antimicrobial, hydroxymethylfurfural, physicochemical.

INTRODUCTION

Honey is a super saturated solution of different sugars such as glucose fructose, sucrose and some other found in minor amount (Aurongzeb and Azim, 2011). It is also defined as exudates from plants by combining with specific substance of their own, store in honey comb to ripen and mature (Codex Alimentarius, 2001; Al-Jabri, 2005; National Honey Board, 2008). The main compositional components of honey are sugars and water. Sugars are mainly composed of glucose and fructose and accounts for about 95-99% of total dry weight of honey (Alvarez-Saurez et al., 2009). Similarly, second most important component is water that affects the storage stability of honey. Moisture in honey depends upon several factors such as level of moisture during honey production and temperature in hive and extraction technique (Molan, 2002). Acid found in honey is gluconic acid (2, 3, 4, 5, 6-pentahydroxyhexanoic acid) which produces from enzymatic breakdown of glucose by glucose oxidase present naturally in honey (Bogdanov et al., 2008).

Level of minerals is also very less with potassium as the most abundant mineral. Other commonly found minerals are copper, iron, manganese, calcium and phosphorous (Bogdanov et al., 2007). Vitamin B and C complexes like riboflavin, nicotinic acid and pantothenic acid are also found in honey of different types (Ciulu et al., 2011). Honey contains free amino acids which are 18 in number and an adequate number of proteins (Molan, 2002; Won et al., 2008; Amin et al., 2010). Acid components of honey are very less and round about 0.57% in most honey types (Wang et al., 2011). Hydroxy methyl furfural (HMF) is also found to be produced in honey which is not stored at proper temperature. So the HMF contents of honey may also depict the keeping quality of honey. The more the HMF contents of the

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honey, the lower will be its quality. Since HMF is produced by acid hydrolysis of sucrose, the presence of high level of this compound in honey will determine and indicate its contamination with higher level of invert sugars (Aurongzeb and Azim, 2011).

Honey has shown significant antibacterial activity against many pathogenic bacteria (Oyeleke et al., 2010). Due to its unique properties, it is largely been used as a wound healing agent for infections. Wound healing was probably the first mentioned medical implication of honey. In modern pharmaceutical and therapeutics, there is increasing trend in administration of honey for wound care. Its medicinal properties have been reported both from *in vitro* and i*n vivo* studies and evidences obtained from different diversified researches support its importance (Davis, 2005). Chemical analysis of honey is very important for honey production, packaging and marketing industry in the sense that these chemical parameters are used to test the quality of honey, its storage stability and its effect on flavor, texture and other sensory parameters.

Keeping in view the importance of the subject there is a dire need to access the potential of honey collected from different ecological regions of Pakistan as there is limited information available.

MATERIALS AND METHODS

Sample collection

Fresh honey samples were collected from different areas of Pakistan during the 2012-2013 season (that is, Changamanga, Multan, Mansehra and Islamabad). All these locations were climatically and geographically different from each other. Unwanted material such as wax, dead bees, sticks and comb particles were removed with the help of cheesecloth. All samples were stored in 500 ml food grade plastic bottles in unprocessed form at refrigerated temperature.

Physico-chemical analysis

Moisture content of these samples was determined by using the refractometer Extech RF-15 with moisture range from 0-32% and the final moisture content was equal to the reading corresponding to refractometer value at 20°C in the procedure given in the AOAC: Association of Analytical Chemists (1990). Similarly, the ashes of these samples were measured by weighing 10 g of a sample in ignited petri dishes. It was incinerated in a muffle furnace (Hexatech) at 600°C for 24 h until a constant weight was attained. The difference in weight was expressed as ash (g) /100 g of honey. Nitrogen and sucrose contents were determined according to the method of AOAC (1990), while reducing sugars were determined by using the Fehling's solution A and B. Hydroxymethylfurfural contents were measured by AOAC (1990) method using the spectrophotometer. Acidity of all the samples was determined using the method of AOAC (1990). pH was determined by using a standard pH meter HI-110, which was calibrated by using standard buffer of pH 4 and 7. However, diastase contents were determined according to the method devised by AOAC (1990).

Antimicrobial activity

Antimicrobial activity of these honey samples was measured using different concentrations (20, 25 and 30%) against different pathogenic bacteria namely: *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* by using well diffusion followed by minimum inhibition concentration. Bacterial cultures were serially diluted in 0.1% peptone water to obtain 10^5cfu/ml, and 100 µl of this solution was spread on Mueller Hinton (MH) agar in Petri plates. An aliquot of 10 µl of each honey sample was poured in each well. A well with control (ampicillin) was also placed into the Petri plate. These plates were then inverted and incubated at 37°C for 24 h. Microbial inhibition was determined by measuring the diameter of clear zone of inhibition (ZOI) around each well and recorded as diameter of ZOI in millimeters. All assays were performed in triplicates. For minimum inhibitory concentration (MIC) determination, different concentrations of honey (8-20%) were prepared. MIC was determined as the minimum amount of honey concentration that proved effective to control any visible growth of bacteria.

Statistical analysis

Results obtained were analyzed statistically. Comparison between means of parameters were made using LSD (Least Significant Difference).

RESULTS AND DISCUSSION

The mean and statistical results obtained from honey samples of *A. dorsata* obtained from different regions of Pakistan, that is, Changamanga (Central Punjab), Multan (Southern Punjab), Mansehra (Upper Khyber Pakhtunkhwa, KPK) and Islamabad (Federal Area) are summarized in Table 1.

Moisture

Honey collected from Changamanga from mulberry tree and honey collected from Multan from citrus tree showed higher water content (26.70% ± 1.12 and 25.93% ± 0.12) and are significantly different than the honey collected from Mansehra and Islamabad respectively. Higher moisture contents may be attributed to the higher level of moisture during the honey production time. Water

Table 1. Physicochemical profile of honey samples collected from different areas of Pakistan.

Physicochemical content	Honey sample			
	Changamanga	Multan	Mansehra	Islamabad
Moisture (%)	26.70 ± 1.12^{A}	25.93 ± 0.12^{A}	24.5 ± 0.0^{B}	22.87 ± 0.15^{C}
Ash contents (%)	0.05 ± 0.01^{BC}	0.07 ± 0.012^{B}	0.03 ± 0.01^{C}	0.10 ± 0.012^{A}
Sucrose (%)	3.07 ± 0.15^{C}	2.50 ± 0.26^{D}	3.60 ± 0.30^{B}	4.57 ± 0.31^{A}
Reducing sugars (%)	70.39 ± 0.71^{B}	72.50 ± 1.21^{A}	73.93 ± 0.55^{A}	69.62 ± 0.63^{B}
HMF contents (mg/kg)	37.14 ± 0.58^{C}	39.60 ± 0.57^{BC}	41.03 ± 1.98^{B}	46.60 ± 1.28^{A}
Acidity (meq/k)	43.0 ± 3.0^{A}	23.67 ± 2.0^{C}	34.67 ± 2.0^{B}	31.33 ± 2.52^{B}
pH	3.09 ± 0.03^{C}	3.61 ± 0.03^{A}	3.28 ± 0.03^{B}	3.33 ± 0.29^{B}
Diastase content (DN)	29.0 ± 1.0^{A}	25.67 ± 1.5^{B}	22.33 ± 1.5^{C}	18.33 ± 1.5^{D}
Total sugars (%)	73.67 ± 0.5^{C}	75.0 ± 0.95^{B}	77.53 ± 0.25^{A}	74.37 ± 0.55^{BC}

- All the values are result of triplicate analysis.
- Determination at alpha (α) is equal to 0.05.
- Those means corresponding to same alphabet are statistically non-significant at α=0.05.

contents also depend upon several factors such as temperature in hives and extraction techniques (Molan, 2002). Iftikhar et al. (2011) also found these results that compared different honey types and found average moisture contents of *A. dorsata* honey as 22.06%.

Ash content

Ash contents give floral origin of the honey. Ash content of honey collected from Islamabad is significantly higher (0.10±0.012 g/100g) at level of significance; α≤0.05; than all the other honey samples collected from other regions. This higher level might be due to the higher pollen count in this region. High ash contents may also depend upon the floral origin of honey and the material collected by bees during foraging (Kamal et al., 2002). Ash content actually reflects the mineral analysis of honey. Mineral contents of honey depend upon plant type from which we are collecting honey and soil type. Results of this study are in line with the findings by Kamal et al. (2002) and Aasima et al. (2008), who showed the ash contents of honey from different flora as 0.04 to 0.7 g/100g and 0.05 g/100 g to 0.27 g/100 g respectively.

Sucrose content

Highest level of sucrose was found in honey collected from Islamabad (4.57 % ± 0.31). Highest sucrose contents in honey collected from Islamabad might be due to the composition of nectar which honey collected during foraging. There has been found a relation between the moisture contents and sucrose percent in honey samples. These results showed that moisture level increase gradually with decrease in sucrose contents with very slight variation. Honey collected from Islamabad showed lowest moisture contents, that is, 22.87% ± 0.15 and highest sucrose contents, that is, 4.57% ± 0.31. Sucrose contents in this study are close to those of Kamal et al. (2002), who concluded that the sucrose contents are 1.1 and 3.4% in honey from different flora.

Reducing sugars

Reducing sugars in honey are glucose and fructose. Results obtained from analysis gave highest level of reducing sugars in honey collected from Mansehra region (73.93%±0.55) which differ non-significantly from honey collected from Multan region (72.50%±1.21). Highest level of reducing sugars in honey collected from Mansehra might be due to the botanical origin of honey or the plants from which bee collect nectar for honey manufacturing. Results in this study are very close to the findings of Shahnawaz et al. (2013). They reported that reducing sugar level of different honey samples collected from Gilgit, Pakistan ranged between 68 and 75%. Honey type having highest level of total sugar also possess highest level of reducing sugars with slight variation where sucrose contents played some part as well.

Hydroxy methyl furfural (HMF) contents

HMF contents in honey directly relates to the keeping quality of honey. HMF contents are more in the honey where storage conditions are not up to the standards as HMF contents increases with deteriorated storage conditions. High temperature or heat treatments can also elevate HMF contents in *Acacia* honey (Kamal et al., 2002). Highest level of HMF is found in honey collected from Islamabad (46.60 mg/kg ± 1.28) which indicates the ageing of this honey, but this range is still within the acceptable range for HMF contents as prescribed by Codex Alimentarious Commission (2002). Lowest level of HMF has been found in honey collected from Changamanga (37.14 mg/kg ± 0.58) that accounts for its

good quality as compared to other honey types. HMF is produced by acid hydrolysis of sugars, therefore the presence of high level of this compound will show with higher level of invert sugars which is ultimately subjected towards contamination and its level is inversely proportional to the quality of honey (Aurongzeb and Azim, 2011). Results of this study are found close to the results of Kamal et al. (2002), who found HMF contents of different honey types between 31 and 43 mg/kg.

Acidity

A wide range of different components are present in honey that accounts for its acidity. Gluconic acid (2, 3, 4, 5, 6-pentahydroxyhexanoic acid) is the most abundant acid found in honey which is produced from enzymatic breakdown of glucose by glucose oxidase (Oddo and Piro, 2004). Glucose oxidase is an enzyme found naturally in honey. Acidity in the honey samples collected from Changamanga forest (43.0 mg/kg ± 3.0) is highest and almost double the acidity found in honey collected from Multan region (23.67 mg/kg ± 2). Acidity in honey collected from Islamabad (31.33 meq/kg ± 2.52) is close to that of honey collected from Mansehra (34.67 meq/kg ± 2). This varied range is due to difference in level of glucose oxidase and level of gluconic acid in different honey samples. Results obtained from acidity analysis are found very closer to the findings of Gulfraz et al. (2010), who determined acidity from different flora of Pakistan between 14 and 45 meq/kg.

pH

The acidic pH of honey is basically due to the presence of some acids, mainly gluconic acid, which is formed as a result of glucose degradation by glucose oxidase (Oddo and Piro, 2004). Lowest pH was associated with honey collected from Changamanga region (3.09±0.03) where acidity was highest (43±3.0 meq/kg) because there is a reverse relationship between acidity and pH. Highest pH was observed in honey sample collected from Multan (3.09±0.03) where acidity was lowest (23.67 meq/kg). The above findings have been found very close to the results of Shahnawaz et al. (2013), who determined pH of different types of honey collected from Gilgit, Pakistan between 3.2 and 3.5.

Diastase number

Diastase is one of the major enzymes found in honey. Quantity of diastase (Diastase number, D.N) is more in the fresh honey and less in the old honey. As enzymes is protein in nature so they deteriorate with time due to fluctuating storage conditions and their amount decreases with time making it a significant indicator for the quality of honey. Results of this analysis showed that the highest level of diastase is found in honey collected from Changamanga, that is, 29 D.N ± 1.0 while the lowest level of diastase is found in honey collected from Islamabad (18.33 D.N ± 1.5). Diastase number of Multan and Changamanga honey (25.67 D.N ± 1.5 and 29 D.N ± 1.0 respectively) is closer to each other which might be due to similar climatic conditions in these areas. Likewise diastase number of Islamabad and Mansehra honey (18.33 D.N ± 1.5 and 22.3 D.N ± 1.5) is closer to each other due to some sort of similarity in climate. The above results are in line with the findings of Samina et al. (2008), who found diastase number of different honey types of *A. dorsata* between 5.53 D.N and 29.35 D.N for honey collected from forests of Nepal.

Total sugars

Sugars are important components of honey which not only determine its nutritional property but also plays important role in dictating the physical properties such as crystallization, hygroscopicity and viscosity. Results showed that highest level of total sugars is found in honey collected from Mansehra region (77.53% ± 0.25). This may be attributed to the rich flora of that region from which the bee collected the nectar. Moisture condition during honey production may also be the important factor. Total sugars in honey collected from Mansehra is significantly higher from all other locations of honey collection. Total sugars in honey mainly comprise glucose and fructose (almost above 70% in all types of honey). Very small amount of other sugars are also present such as disaccharides maltose, isomaltose and sucrose. Lowest level of total sugars is found in honey collected from Changamanga (73.67% ± 0.51).

ASSESSMENT OF ANTIMICROBIAL ACTIVITY

Inhibitory action of different concentrations of four types of honey was observed on different bacterial pathogens. Honey has been shown to produce marked antibacterial activity against health hazardous microorganisms such as *E. coli, S. aureus* and *P. aeruginosa.* According to the results, the highest inhibition was observed in the case of *E. coli* where ZOI is highest as compared to the other two bacteria (21, 21, 26.67 and 23.667 mm against honey collected from Changamanga, Multan, Mansehra and Islamabad respectively). Honey collected from Mansehra showed the highest ZOI, that is, 23.3 mm for *E. coli*, 26.7 mm for *S. aureus* and 22 mm for *P. aeruginosa* respectively. Lowest inhibition zone was observed in the case of honey collected from Changamanga where ZOI for *E. coli* was 18.33 mm, that for *S. aureus* was 21 mm and that for *P. aeruginosa* was 19.33 mm respectively. This inhibition may be the combined result of pH, hydrogen peroxide, volatile compounds and antimicrobial peptides (Gulfraz et al., 2010). Honey has shown antimicrobial activity even

Table 2. Antibacterial activity of the four honey samples against foodborne bacterial pathogens (in terms of zone of inhibition; mm).

Bacterial strain	Changamanga	Multan	Mansehra	Islamabad
P. aeruginosa	18.33^{C}	20.33^{B}	23.3^{A}	18.67^{C}
E. coli	21^{C}	21^{C}	26.67^{A}	23.667^{B}
S. aureus	19.33^{C}	20.667^{B}	22^{A}	17.33^{D}

Table 3. Minimum inhibitory concentration (MIC) % of different honey samples against foodborne bacterial pathogens.

Bacterial strain	Changamanga MIC (%)	Multan MIC (%)	Mansehra MIC (%)	Islamabad MIC (%)
P. aeruginosa	20^{A}	16^{C}	14^{D}	18^{B}
E. coli	14^{A}	10^{C}	8^{D}	12^{B}
S. aureus	16^{A}	13^{B}	10^{C}	14^{B}

more than control antibiotics so the use of honey may be recommended in traditional antibiotics which can then be used for tropical treatments. Table 2 shows the ZOI given by four honey samples.

Minimum inhibitory concentration

Honey is a supersaturated solution with low water activity which means that there is no sufficient quantity of moisture in it to support the growth of bacteria. Different concentrations of all types of honey against different bacteria showed encouraging results with respect to inhibition. Every type of honey at a specific concentration showed remarkable antimicrobial activity even against those bacteria which become resistant to antibiotics. Minimum inhibitory concentration for *E. coli* has been lowest as compared to other bacteria, that is, 14, 8, 10 and 12% for Changamanga, Multan, Mansehra and honey collected from Islamabad respectively. Honey with highest MIC value is honey collected from Changamanga with 20, 14 and 16% against *P. aeruginosa, E. coli* and *S. aureus* respectively. Table 3 shows the MIC of different honey samples against pathogenic bacteria.

MIC may be used as a confirmatory test for the validity of the process of antimicrobial activity measured through zones of inhibition. It can be noted that honey type giving largest ZOI will also show lowest value for minimum inhibitory concentration. Results of ZOI and MIC were similar and they showed that honey collected from Mansehra gave the best results against pathogenic bacteria.

Conclusion

This study was conducted to analyze the chemical characteristics of honey collected from different areas of Pakistan. In conclusion, all the physicochemical parameters are within the quality criteria set by Codex Alimentarious (2001) with very negligible differences. This study gave useful information about the chemical composition of honey collected from *A. dorsata* bee from Pakistan on which to our knowledge little information is available. Antimicrobial activity of honey has been well elaborated by antimicrobial assay that gave clear zones of inhibitions for all honey types with comparison of the standard used (ampicillin). Honey is miracle food that has many health benefits especially against potential food pathogens and intensive research on it may solve the problem of emergence of antibiotic resistance among these bacteria.

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