Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/kr6h4v86

ACCESSING PAKISTANI COMMERCIAL HONEY FOR ITS COMPOSITIONAL, QUALITY, SAFETY, ANTIBACTERIAL AND PHYTOCHEMICAL ANALYSIS: A PRE-CLINICAL SCREENING

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Abstract
Honey usage as medicine is as old as 4000-years. The application of honey as therapeutic is increasing, the pre-clinical screening is crucial to evaluate its effectiveness to ensure its medical grade. The first ever therapeutic use of honey was for wound healing but is now recognized in many other health ailments owing to its antioxidant, anti-microbial, anti-inflammatory, anti-proliferative, anti-cancer, anti-metastatic, anti-fungal and anti-viral properties. The adulteration with different substances, pesticides, microbes, and heavy metals can prove to be toxic for human health effecting organs. This study aims to access the different Pakistani commercial honey to check its authenticity via different methods including compositional, quality, safety and anti-bacterial analysis. To evaluate its therapeutic potential in-vitro analysis includes total phenolic content (TPC), total flavonoid content (TFC), radical scavenging activity via DPPH and FRAP were carried out. All honey samples fall under the permissible limits set by the international and national legislative bodies. The safety analysis total plate, fungi and mold were also under the permissible limits. No pesticides were detected. The heavy metals such as lead, cadmium and arsenic were found in range of 0.0-2.22, 0.0-0.18 and 0.0-0.2mg/kg. The total phenolic (21.9mgGAE/100g) and flavonoid content (2.76-5.57mgCAT/100g) were found to be highly variable similar their respective antioxidant activity DPPH (18-45%) and FRAP (954-1445µMFeII/100g). Forest Sidr honey showed highest anti-bacterial activity against all 3 bacterial isolates *i.e., Staphylococcus aureus, Escherichia coli* and *Klebsiella pneumoniae*, then any other honey sample. Great variability in composition, and its safety concerns demand rigorous testing before clinical applications.

Keywords: Honey, Adulteration, Pesticides residue, Safety, *In-vitro* anti-bacterial activity.

1. Introduction
The history of adulteration in honey is as old as honey production itself. People used to blend natural honey since ancient times with maple syrup, birch and sorghum. In 1889, Dr. Harvey W. Wiley, the first chief chemist of U.S. Department of Agriculture (USDA) testified honey as widely adulterated throughout the country[1]. Ever since there has been a continuous effort to combat adulteration and to evaluate its authenticity, quality and safety for human consumption through different methods such

as sensory, biological and physico chemical. These methods are used to test the parameters set by different international and national bodies such as Codex Alimentarius, International Honey Commission (IHC). The important physicochemical properties of honey include moisture content, total soluble solids, total proteins, proline, ash, electrical conductivity (EC), pH, Hydroxy methyl-furfuraldehyde (HMF), free acidity, sugar profile, metal, bioactive components of honey such as phenolics and flavonoids. Honey across the world has been classified and authenticated for its quality by using these physicochemical parameters[2,3].

Honey composition mainly consist of fructose (38%), glucose (31%) and sugars (8%) as disaccharides and water (17%)[4]. Apart from sugars, it consists of more than 300 compounds like certain enzymes, minerals, vitamins and bioactive component such as phenols and flavonoids (4%). The vitamins, mineral, phenol and flavonoid content can vary greatly based on honey type. Multi-floral honeys can be much better in bioactive profile than mono-floral ones[5]. The therapeutic potential of honey has been evaluated in large number of ailments like cardiovascular diseases, atopic dermatitis, eye infection, diabetes, hypertension, ulcers, cold cough, cancer and wound healing[6].

Use of honey as a medicinal remedy has been known throughout the history of human race. Sugar adulterants in honey has been linked to diabetes, obesity and hypertension. The common organs that can be affected by adulterants in honey are liver, kidney, heart and brain as proved by number of in-vivo studies[7]. Not only the adulterants in honey can pose the harmful effects on health but sometime honey itself can be the source of microbes, heavy metals and pesticides residues from certain geographical areas. Keeping in view how these environmental contaminants can become part of the honey composition, the maximum limits for heavy metals and pesticides residues have been added by Codex Alimentarius for honey standards. The application of honey as therapeutic is increasing so the determination of these metals should be taken into consideration. Arsenic, lead and cadmium are among the top 20 hazardous heavy metals compiled by Agency for Toxic Substances and Disease Registry (ATSDR)[8].

Similarly, pesticides residue such as insecticides, fungicides, antibiotics and herbicides can become a part of bee products such as honey as the nectar is being collected by the bees from plants. Pesticides have been shown to cause genetic mutation in humans. Legislative bodies such as the European Union have regulated maximum residual levels (MRLs) for pesticides in honey[9]. An evaluation of the presence of veterinary drug residues and pesticides is important to recognize honey as organic. Other than the safety purpose, evaluation of pesticides in honey can act as a bioindicator in well-developed agriculture and apiculture area to ensure safe food for the consumers.

The microbes that are identified and characterized in honey are bacteria, fungi and molds. Generally, the microbial load in honey is low, below 10^2 CFU/g and can even reach 10^3–10^4CFU/g[10]. Literature review showed zero to tens of thousands of bacterial counts[11]. If the bacteria count is way more than it should be taken to next step of bacterial isolation and identification to find the probable cause of the contamination and remove it from the source. Other than the bacteria, as the honey is acidic food the growth of fundi and mold is potentially possible. The maximum legislative limit for yeast and mold is 100CFU/g in honey[12]. Literature shows no yeast/mold or can contain up to 4×10^2 -2.6×10^5 thus making such honey unacceptable for human consumption[13].

The TPC in honey in literature can vary from as low as only 2.54 to 183 mg GAE/100g[14, 15]. TPC and TFC content is often correlated to its anti-oxidant potential. Higher the anti-oxidant present high radical scavenging activity[16] but this correlation was not found consistent among other studies. Thus, pointing towards other components that may paly role as antioxidants.

Sugar profile of honey samples had been extensively used specifically fructose and glucose ratios (F/G) and their concentration, plays an important role in checking the authenticity of honey. It indicates whether the bees have been fed with sugar syrup such as glucose, fructose or have been adulterated with sucrose syrup by the beekeepers[17]. Adulteration with either of the sugars 1in honey, affect the ratio of fructose and glucose. The permissible average F/G ratio is 1.2/1. Codex Alimentarius standards (CAS) of honey recommends minimum 1.12/1(F/G) for non-labeled natural honey sample[18]. However, in numerous studies honeys with F/G ratio more than 1 is considered

acceptable[19]. Apart from F/G ratio, the sugar composition can also be beneficial to identify the floral source of honey[20]. So, the evaluation of all these parameters is important before its clinical application.

2. Materials and methods

Commercial honeys samples were obtained from the market in Faisalabad, Pakistan (Table 1). All honey samples were kept in freezer at -18℃ for further analysis. Before each sampling honey was ensured its homogenized and no sugar crystals were present. All honey analysis was done in triplicates.

Table 1. Types of honey samples used in study

Honey samples	Scientific name	Code	Year
Orange Honey	*Citrus sinensis*	A	2020
Shesham (Rosewood) Honey	*Dalbergia*	B	2021
Ajwain (Carom) Honey	*Trachyspermum ammi*	C	2021
Alaichi Infused (cardamom) Honey	*Elettaria cardamomum*	D	2021
Black Forest Honey	--	E	2021
Blossom Honey	--	F	2021
Poly-floral Honey	--	G	2021
Poly-floral Honey	--	H	2020
Poly-floral Forest Honey	--	I	2022
Forest Sidr Honey	*Ziziphus spina-christi*	J	2022

2.1. Compositional analysis

Honey samples were analyzed for moisture, crude protein, total ash, total soluble solids (TSS), reducing sugars (RS), non-reducing sugars (apparent sucrose) (AS), proline and water-insoluble content (WIC) by using their respective methods [21,22,23].

2.1.1. Moisture and Total soluble solids

Moisture and total soluble solids (TSS) were measured by using automatic digital refractometer (RA-600 Kyoto Electronics Manufacturing Co., Ltd. Shanghai, China). The refractometer was thermostated at 20℃ and calibrated with water. The refractive index (*n*) was noted and used to measure water content from the refractive index and water content correlation standard table[21]. TSS expressed as Brix (°Bx).

2.1.2. Crude protein

The protein percentage in the samples was estimated using the Kjeltech (Technick GmbH D-40599, Behr Labor, Germany) following method No. 46-10 (AACC, 2010). The sample was digested in the digestion tube for 3-4 hours with 30mL conc. H_2SO_4 and 5g of digestion mixture till end point achieved (transparent or light green color).

The digested material was then transferred to 250mL volumetric flask and volume was made up to the mark with distilled water. 10mL of diluted sample was distilled with 10mL of 40% NaOH solution with the help of distillation apparatus. The ammonia released was collected in 4% boric acid having methyl red indicator. The solution was then titrated against 0.1N H_2SO_4. Crude protein was calculated by using the following formula:

$$\text{Nitrogen}(\%) = \frac{\text{Volume of 0.1N } H_2SO_4 \text{ used (mL) x Volume of dilution (mL) x 0.0014x100}}{\text{Weight of sample (g) x Volume of aliquot sample (mL)}} \text{x6.25}$$

2.1.3. Total ash

Ash content was determined by incineration of sample as inorganic matter[21]. The sample (5g) was taken in a pre-weighed crucible and charred on burner before incinerating in the Muffle Furnace (MF-1/02, PCSIR, Pakistan).

Ash was calculated by the following formula:

$$\text{Ash (\%)} = \frac{\text{weight before ashing (g)} - \text{weight after ashing (g)}}{\text{weight of sample (g)}} \text{x}100$$

2.1.4. Reducing and non-reducing sugars

Honey apparent reducing and sucrose content was measured using the harmonized methods of the International Honey Commission[21]. Briefly, 5mL of both Fehling solution A and B were taken in flask and titrated with honey diluted solution (2g dissolved in 200mL of D.W, 50mL from this solution further diluted with 100mL D.W) having aqueous 0.2% methylene blue solution acting as an indicator. Note the volume of honey solution used. To measure the reducing sugars, the following formula was used:

$$\text{C} = \frac{2}{\text{W2}} \text{x} \frac{1000}{\text{Y2}}$$

Where: C = total reducing sugar/gram; W2= weight of sample (honey) taken in grams; Y2= volume of diluted honey used in mL.

For the estimation of sucrose content, 50mL of honey solution was mixed with 25mL of D.W and heated in a water bath at 65°C. The solution was allowed to cool down at room temperature for 15 minutes followed by addition of 10mL of HCL. The temperature was set to 20°C and neutralize the pH using NaOH. Titrate the honey solution with Fehling solution A and B and 0.2% methylene blue solution.

The sucrose content (%) was estimated using the following expression:

$$\text{Sucrose content (\%)} = (\text{R.A.I} - \text{R.B.I}) \text{ x } 0.95$$

Where,

R.A.I= reducing sugar after inversion

R.B.I= reducing sugar before inversion

2.1.5. Proline

The total proline content in honey was measured according to the method AOAC Method No. 979.20[23]. About 0.5mL of honey solution was taken and 0.25mL of 80% formic acid and 1.0mL of 3% ninhydrin solution was added and placed in boiling water bath for 15 minutes and then transferred to water bath for 10 minutes at 22°C. 5mL of 2-propanol-water solution was added and allowed to cool for 45 minutes at room temperature. Absorbance was taken at 520nm. Proline standard curve was used for the calculation ($y=0.0379x-0.0202$, $R^2= 0.99$).

2.1.6. Water insoluble content

Water insoluble content (WIC) was measured following the gravimetric method. 10g honey was dissolved in distilled water (D.W) and filtered through filter paper. After several washings with D.W, the filter paper with water insoluble content was dried in a hot air oven. Weight of filter paper with WIC was determined using analytical balance until reach the constant value.

2.2. Mineral analysis

All honey sample were analyzed for its major minerals such as sodium (Na), potassium (K) and calcium (Ca) by using flame photometer (Model 410, Sherwood Scientific, UK) following methods as described in AOAC (2023)[23]. All honey samples (0.5g) were wet digested using 10mL of nitric acid and 5mL of perchloric acid, solution was made up to 50mL using distilled water.

2.3. Quality analysis

Honey samples were evaluated for refractive index (*n*), pH, Fiehe's test, hydroxy-methyl furfural (HMF), electrical conductivity (EC) and free acidity (FA) by using their respective methods[21, 23,24].

2.3.1. Refractive index

The refractive index (*nd*) of honey was measured by using automatic digital refractometer (RA-600, Kyoto Electronics Manufacturing Co., Ltd. Shanghai, China) thermostated at 20°C and calibrated with water prior sample reading.

2.3.2. pH

The pH level of the honey samples was measured by using a calibrated pH meter (HI9814 GroLine meter, Hanna Instruments Ltd. USA) with buffers of pH 7.00 and 4.00. The respective samples (2g of honey in 15mL) were stirred up in distilled water, to prepare solution for the measurement of pH value.

2.3.3. Fiehe's test

Fiehe's test was performed by following the method of Pakistan Standards and Quality Control Authority[25]. Briefly 10mL of honey was mixed in 5mL ether, mixture was allowed to separate and 2mL of this ethereal layer was taken in separate crucible. Allow ether to evaporate and add 1 drop of freshly made resorcinol, resublimed in HCL. Instant cherry/brick red color indicates positive test.

2.3.4. Hydroxy methyl-furfural

HMF was measured as the method described in harmonized methods of the International Honey Commission[21]. 5g of honey was transferred to 50mL volumetric flask, add 25mL of distilled water and dissolved. Add 0.5mL of carrez solution I and mixed. Afterward, same amount of carrez II solution was added and filled up to mark. The solution was filtered and 5mL was put in a test tube. Reference solution of sodium bisulphite (0.2%) was taken in another test tube and mixed well. Carrez I, II and sodium bisulphite solutions. Absorbance of reference and sample solution was measured at 284 and 336nm. To calculate HMF (mg/Kg), the following formula was used:

$$\text{HMF}\left(\frac{\text{mg}}{\text{Kg}}\right) = (\text{A}_{284} - \text{A}_{336}) \text{ x } 149.7 \text{ x } 5 \text{ xD/w}$$

Where,

A_{284} = absorbance at 284nm

A_{336} = absorbance at 336nm

149.7= Constant

5 = theoretical nominal sample weight

D = dilution factor (in case dilution is necessary)

W = Weight of honey sample in grams

2.3.5. Electric conductivity

Electrical conductivity (EC) was measured in 20% (w/v) honey solution using EC meter (HI9814 GroLine meter, Hanna Instruments Ltd. USA). The EC was measured in milli Siemens per centimeter ($mS.cm^{-1}$).

2.3.6. Free acidity

Acidity of the honey was determined by titration method[21]. About 2g of each sample was added in titration flask, along with 15mL of distilled water. The mixture was titrated against 0.1N NaOH to pH 8.3. The initial and final volume used from burette was noted. Acidity was measured as:

$$\text{NaOH used} = \text{Initial reading} - \text{Final reading}$$

$$\text{Free Acidity}\left(\frac{\text{meq}}{\text{Kg}}\right) = \text{mL of 0.1N NaOH used x } 10$$

2.4. Safety parameters

2.4.1. Microbial analysis

Microbial analysis such as total plate count (TPC) and total yeast and mold count was performed using serial dilution conventional method[12]. 1mL of honey was diluted in 9mL of 0.1% buffered peptone water (Sigma-Aldrich, St. Loise, MO, US) giving 10-fold dilutions. Further 100-, 1000- and 100,000-fold dilutions were made. 1mL of dilutions were used for inoculation on respective medias. For the total plate count, nutrient agar (Oxoid-LabMal) was prepared according to manufacturer instructions. The petri dishes were inverted and incubated for 24 hours at 37ºC. Petri dishes with countable colonies (30-300 colony per plate) using colony counter (Galaxy 230, Utech products INC. Albany, NY, US). For total yeast and mold count, potato dextrose agar (PDA) was prepared, autoclaved and acidified to pH of 3.5 using tartaric acid for the growth of fungi and inhibiting the growth of other bacteria present in honey. 1mL of each dilution were spread over the solid agar. The PDA plates were incubated at 25ºC for 5 days without inverting. At the end of incubation, plates with countable colonies (15-150) were selected. The results for both total plate count and total yeast and mold count were expressed as cfu/mL.

2.4.2. Pesticides residues

Pesticides residue analysis was done at analytical laboratory of Qarshi Research Internationals Pvt. Ltd, Hattar Pakistan by following AOAC Method No. 2007.01. The residues of commonly used pesticides such as tebuconazole, chlorpyrifos, endosulfan, carbofuran and acetamiprid were checked using GC-MS/MS (EVOQ GC-TQ, Bruker Daltonics, Germany). The experimental conditions for equipment were as follow:

Column: Scion 5MS- 15 m x 0.25 mm, 0.25 µm
Injection volume: 1µL
Mobile Phase: Helium
Flow rate: 1mL/minute
Temperature: 75-280 @ 25˚C/minute.
Detector: MS/MS
Mobile phase conditions: Acetonitrile, run time 15 minutes.
The detection of limit (DOL): 0.005mg/Kg
Detection of quantification (DOQ): 0.010mg/Kg.
Unit of measurement: mg/Kg.

2.4.3. Anti-bacterial activity

Honey anti-bacterial activity was checked against three clinical bacterial isolates such as *Klebsiella pneumonia*, *Staphylococcus aureus* and *Escherichia coli* using disc diffusion method[26]. 100mL of nutrient agar was prepared and autoclaved at 121°C for 15 minutes. Agar was allowed to cool in laminar flow and 100ul of bacterial suspension containing 10^7 colony-forming unit (CFU/mL) was added and poured into sterile petri plates. After the agar solidification, the wells were made into agar using back of sterile blue tip. Ciprofloxacin was used as positive control for all bacterial strains. Honey samples (undiluted) and ciprofloxacin were poured into wells and incubated for 24 hours at 37ºC. The diameter of zone of inhibition was measured in mm.

2.4.4. Heavy metal analysis

Honey samples were checked for heavy metals such as lead (Pb), cadmium (Cd) and arsenic (As) using atomic absorption spectrophotometer (AA240 Agilient Varian Agilent Technologies, Inc. CA. USA). The wet digestion for the sample was done following method mentioned in[23].

2.5. Therapeutic potential

Honey extract's total phenolic (TPC) was measured using Folin-Ciocalteau reagent method and total flavonoid contents (TFC) aluminum chloride ($AlCl_3$) colorimetric method as the protocol described by Anand[16] and Combarros-Fuertes[5] with some modifications.

Methanolic extract of honey was prepared,1g of honey dissolved in 10mL of 99% methanol. The mixture was put on continuous shake for 5 hours filtered using filter paper to remove particles. The filtrate was aliquoted and frozen at 20°C to avoid freezing and thaw cycle for further analysis.

The absorbance for TFC was taken at 510nm. The TFC was measured by using the standard curve ($y=0.0034x-0.076$, $R^2= 0.99$) of catechin and expressed as mg catechin (0-500µg/mL) equivalent per 100g of honey. The absorbance for TPC was taken at 765nm and expressed as mg gallic acid equivalent (GAE) per 100g of the honey extract using the standard curve ($y = 0.054x + 1.3842$, $R^2= 0.98$). Gallic acid standards were prepared with a concentration of 0-100mg/mL.

2.6. Antioxidant activity

Methanolic extract of honey was prepared for measuring total antioxidant capacity by diphenyl picrylhydrazyl (DPPH) radical scavenging activity assay and Ferric-reducing antioxidant power (FRAP) methods[27,16] with minor modifications.

2.6.1. DPPH assay

Honey filtrate (50µL) mixed with freshly prepared DPPH (3mL) in methanol (0.1mM) solution and placed for 30 minutes. Absorption of the samples and control was taken at 517nm. The following equation was used to measure scavenging activity (%).

$$\text{DPPH activity (\%)} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.6.2. FRAP assay

FRAP reagent was prepared by mixing 0.3M acetate buffer (pH 3.6) and 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 0.04M HCl and 0.02M $FeCl_3$ solutions (in ratio 10:1:1). The sample solution (200µL) and FRAP reagent (2mL) were mixed at 37°C for 10 minutes. The absorbance was taken at 593nm. 100-1000µM of $FeSO_4$ solution was prepared for standard curve. The results were presented as µM FeII/100g of honey sample.

3. Results

3.1. Compositional Analysis

The results for compositional analysis are presented in Table 2 and compared to international and national legislative standards. All compositional analysis remains within the permissible limits set for different honey samples. The moisture content for all commercial honey remains within the range of 13.6-18.4g/100g and fall under the <21g/100g. While other parameters such as CP, ash, TSS, RS, AS, proline and WIC have been found in the range of 0.38-1.58%, 0.04-0.5%, 79.8-84.6 °Bx, 61.7-78.8 g/100g, 1.32-10.82 g/100g, 579-2120 mg/Kg and 0-0.057 % respectively.

The lowest protein content (0.38±0.01%) was found in sample E (black forest honey) followed by sample G (0.46±0.02%) of honey. Honey is not a good source of protein. The protein content in honey samples can vary from as low as 0.26% as found in Nigerian honey[28] to high as 1.67% as reported in Ethiopian honey[29]. The honey samples used in current study showed slightly higher protein content than in most honeys from different countries but was in accordance with the Pakistani multi-flower honey from different regions (0.98-1.67g/100g) assessed by Tahir[30].

The lowest brix level was found in orange honey (Sample A) 79.8±0.01°Bx followed by poly-floral honey (Sample F) 80.1±0.05°Bx. The TSS content of honey varies from 68.5 °Bx as reported in forest honey from Indonesia[31] to 86.5°Bx from state of Campeche having different honey from 2 bee species[32]. The results of the honey samples are in accordance with the study of hetro- and mono-floral honey from different arid and non-arid regions of UAE[33].

Table 2. Compositional analysis of honey samples

Code	M (g/100g)	CP (%)	Ash (%)	TSS (°Bx)	RS (g/100g)	AS (g/100g)	Proline (mg/Kg)	WIC (%)
A	18.4±0.01	0.55±0.02	0.15±0.00	79.8±0.01	69.8±0.13	9.45±0.17	700±4.7	0.012±0.0
B	15.6±0.05	0.71±0.01	0.24±0.02	82.3±0.05	76.5±0.20	5.84±0.15	932±18.3	0.001±0.0
C	17.0±0.00	0.47±0.03	0.04±0.00	81.2±0.00	69.5±0.17	3.41±0.16	579±2.6	0.001±0.0
D	17.3±0.00	0.64±0.02	0.50±0.00	80.7±0.01	74.4±0.15	4.38±0.25	1559±28.1	0.002±0.0
E	13.6±0.05	0.38±0.01	0.09±0.00	84.6±0.05	61.7±0.07	10.82±0.1	689±8.7	0.021±0.0
F	18.8±0.05	0.60±0.01	0.01±0.00	80.1±0.05	76.1±0.11	2.68±0.17	888±46.54	-
G	17.0±0.05	0.46±0.02	0.15±0.00	81.2±0.05	67.2±0.15	9.23±0.13	712±1.96	0.013±0.0
H	16.2±0.05	0.67±0.03	0.22±0.00	82.1±0.05	78.8±0.16	2.31±0.13	796±4.6	0.012±0.0
I	16.5±0.05	0.85±0.00	0.11±0.00	83.4±0.05	76.3±0.14	2.58±0.17	714±8.4	0.017±0.0
J	17.8±0.05	1.58±0.02	0.21±0.01	83±0.05	72.7±0.25	1.34±0.09	2120±38.3	0.057±0.0
Min-Max	13.6-18.4	0.38-1.58	0.04-0.5	79.8-84.6	61.7-78.8	1.32-10.82	579-2120	0-0.057
Mean	16.82	0.691	0.172	82.1	72.1	5.02	968.9	0.0136
Standards*	≤ 21	-	≤ 0.6	-	≥ 60/65	≤ 5-15	≥180	≤0.1

***** Codex Alimentarius, European Union, Pakistan Standards

M=Moisture; CP= Crude protein; TSS=Total Soluble Solids; RS=Reducing Sugars; AS=Apparent Sucrose; WIS=Water-Insoluble Solids; °Bx=Brix.

Values represent mean ±SEM

4.2. Mineral Analysis

The results for major minerals such as Na, K and Ca are presentede3 in Table 3. It is evident from the results that sodium contents were ranged from 42 to 206 mg/Kg. The highest sodium content was found in sample A (orange blossom honey) (206±2.3mg/Kg). The lowest sodium (42±0.5mg/Kg) was in sample B (rosewood honey). In previous studies, similar quantities of sodium content (97-304.31mg/Kg) have been reported. The highest sodium content was found in honey from mustard plant (*B. campestris)*[34]. However, the sodium content in honey samples can be quite low *i.e.*, 4.77 mg/Kg in honey samples from Estonia obtained from "Cruciferae" plants[35].

Potassium content in honey samples was not much different from sodium. The range for potassium was 42 to 228 mg/Kg with mean 116.2±11.18mg/Kg. The lowest values (42±0.5mg/Kg) were in sample B. The higher potassium (1150mg/Kg) has been reported in Portuguese honey, accounting for 76% of total minerals[36]. Studies from other geographical locations also revealed potassium to be the most abundant minerals *i.e.,* eucalyptus honey from Italy (112-372mg/Kg)[37], *ziziphus spec.* from Algeria (1569.3–476.40mg/Kg)[38], *Citrus sinensis* from Portugal (170.07mg/Kg)[39].

The calcium content in honey samples ranged from 11 to 185mg/Kg. The lowest values (11±1.03mg/Kg) was found in sample B and the highest (185±4.5mg/Kg) in sample C (Carom honey). These results are with accordance to a study, honey from Portugal showed calcium in range of 6-134mg/Kg[36]. The calcium content in other natural poly-floral honey samples from Pakistan ranged from 80.49±0.83 to 149.11±24.00 µg/g, very near to calcium content of this study[40]. In another study, very low (0.43mg/Kg) calcium values in different mono-floral honey samples *i.e.,* thyme, orange, rosemary, strawberry tree, locust, eucalyptus and heather were found[41].

Table 3. Major minerals (mg/Kg) in honey samples

Honey samples	Sodium	Potassium	Calcium
A	206±2.3	192±3.1	18.7±1.02
B	42±0.5	42±0.5	11±1.03
C	114±1.4	78±0.7	185±4.5
D	82±0.9	112±0.6	20.3±2.4
E	166±2.4	200±2.3	18.2±2.7
F	146±0.3	84±0.5	24.1±0.8
G	108±0.2	76±0.6	20±0.68
H	138±0.4	66±2.1	20.2±1.2

I	122±0.5	84±0.5	17.7±2.1
J	104±2.6	228±2.5	19.5±0.1
Min-Max	42-206	42-228	11.0-185
Mean ± SEM	122.8±4.15	116.2±11.18	35.47±2.21

**Values are given as means (triplicates) and (±) standard error mean (SEM)*

4.3. Quality Analysis of Honey

There are certain legislative parameters such as refractive index (*nd*), pH, Fiehe's test, Hydroxy-methyl furfural (HMF), electrical conductivity (EC) and free acidity (FA) to check the quality of honey are given in Table 4.

The *nd* of honey samples ranged from 1.491-1.503. The highest refractive index (1.503±0.00) was found in sample E. These results are supported by other Pakistani honey ranging from 1.47-1.49 *nd*[42]. The mean pH of honey samples was 3.63±0.06. The pH value of Pakistani blossom honey (multi-flower origins) was ranged from 3.29 to 4.05[43]. The highest pH in the study was found in forest sidr honey (4.44±0.03).

Four samples such as orange honey (Sample A), rosewood honey (Sample B), blossom honey (Sample F) and poly-floral honey (Sample H) showed positive Fiehe's test.

HMF ranged from 9.55-214mg/Kg. The highest HMF content (214±2.06mg/Kg) was found in orange honey (Sample A) followed by Sample F (124±2.47mg/Kg) while others were in permitted range (40-80mg/Kg). National and international brands from Pakistan also showed great variability in HMF (14-112mg/Kg)[44] and 509.8±82mg/Kg indicating adulteration or mishandling affecting the freshness in imported multi-floral honey samples[43].

Electrical conductivity (EC) of all honey samples was ranged from 0.15-0.53 mS/cm. The highest EC value was found in Cardamom honey (Sample D) *i.e.,* 0.53±0.02mS/cm like other Pakistani national and international commercial honeys (0.1-0.6mS/m)[44]. The free acidity of honey samples has a mean value of 26.94 meq/Kg. The free acidity of all honey samples remained in permissible range *i.e.,* 50meq/Kg. These results are in accordance with the Pakistani honey sample (23.2-27meq/Kg)[44].

Table 4. Quality analysis of honey samples

Code	RI	pH	Fiehe test	HMF	EC	Free acidity
A	1.491±0.001	3.71±0.05	Positive	214.30±2.60	0.23±0.02	32.0±0.8
B	1.498±0.001	3.45±0.02	Positive	92.20±4.32	0.38±0.01	16.6±0.8
C	1.494±0.002	3.63±0.01	Negative	17.07±0.78	0.18±0.00	28.0±0.5
D	1.493±0.002	3.64±0.00	Negative	9.55±0.65	0.53±0.00	24.7±0.8
E	1.503±0.001	3.82±0.0	Negative	104.30±1.91	0.21±0.01	33.7±0.9
F	1.496±0.002	3.49±0.01	Positive	124.60±2.47	0.15±0.00	21.3±0.9
G	1.494±0.003	3.45±0.01	Negative	126.30±4.48	0.33±0.00	24.2±1.2
H	1.496±0.001	3.29±0.01	Positive	51.73±1.83	0.38±0.01	19.7±0.8
I	1.493±0.002	3.73±0.02	Negative	42.80±1.79	0.53±0.03	31.5±0.6
J	1.492±0.002	4.44±0.03	Negative	10.83±0.71	0.45±0.02	37.7±0.8
Min-Max	1.491-1.503	3.29- 4.44	-	9.55-214.3	0.15-0.53	16.6-37.7
Mean	1.495	3.665	-	79.36	0.337	26.94
Standards	-		Negative	≤ 40-80	≤ 0.8	≤ 50

*RI= Refractive index, HMF= Hydroxy-methyl furfural, EC=electrical conductivity.

Values shows mean ±SEM

*****Codex Alimentarius, European Union, Pakistan Standards

Min=Minimum; Max=Maximum; SEM= Standard error mean; HMF= Hydroxy-methyl furfural; ND=Not detected; mS/cm= milliSiemens per centimetres.

4.4. Safety Analysis of Honey Samples

The mean total plate count (TPC) of honey samples is given in Table 5. The highest bacterial load ($21x10^2$CFU/mL) in honey sample was found in black forest honey. The sample with bacterial load fall in range of 1 to $21x10^2$CFU/mL Yeast and mold in honey weren't detected in most of the samples

except two samples L (Blossom honey) with mold count of 200CFU/mL and sample E (black forest honey) with 100CFU/mL mold and 100CFU/mL yeast count. The maximum legislative limit for yeast and mold is 100CFU/g in honey[12], it can be up to 4×10^2 -2.6×10^5 in commercially available honeys thus making such honey unacceptable for human consumption[13].

Table 5. Safety analysis of honey samples

Parameters	**Min-Max**	**Mean ± SEM**
Total plate count (CFU/mL)	ND-21x10^2	-
Total yeast and mold count (CFU/mL)	ND-2x10^2	-
Pesticides residue (mg/Kg)		
Tebuconazole	ND	-
Chlorpyrifos	″	
Endosulfan	″	
Carbofuran	″	
Acetamiprid	″	
Anti-bacterial activity (mm)		
Staphylococcus aureus	3-22	12.20±1.95
Escherichia coli	ND-21	8.20±1.96
Klebsiella pneumoniae	ND-26	13.50±1.89
Heavy metals (mg/Kg)		
Lead (Pb)	0.0-2.22	0.378±0.12
Cadmium (Cd)	0.0-0.18	0.034±0.04
Arsenic (As)	0.0-0.20	0.045±0.01

*ND=Not detected; CFU/g= colony forming unit per gram; mg/Kg= milligram/kilogram; mm= millimeter.

Honey anti-bacterial activity is given in Table 5 and 6 as mean zone of inhibition (mm) which showed highest antimicrobial activity against *Klebsiella pneumoniae* and *Staphylococcus aureus* followed by and *Escherichia coli.* It is apparent from the results that some sample like black forest honey (Sample D), poly-floral honey (Sample F) showed very little or no anti-bacterial activity at all. While the control drug, Ciprofloxacin, showed zone of inhibition of 42±0.1mm. The honey results are in accordance with other Pakistani mono-floral honey samples *i.e., Ziziphus*, *Citrus* and *Brasica* (27±0.5mm) against *Staphylococcus aureus*[45]. Honey around the world have shown variable results for example honey from Pakistani sources shown no activity against *Escherichia coli* or other bacteria such as *Pseudomonas aeruginosa* and *Enterobacter*[46] and some multi-floral honey from Cameron showed inhibition zone up to 17-35mm for *Escherichia coli*[47]. For *Klebsiella pneumoniae*, the highest zone of inhibition (26±0.7mm) was shown by forest sidr honey (Sample J). The zone of inhibition for *Klebsiella pneumoniae* are in accordance with the Indian poly-floral honey from different regions showing 11-18mm[48] and to Pakistani honey *i.e.,* 25mm[45]. Overall, the forest sidr honey (Sample J) samples showed good zone of inhibition against all three bacteria.

Table 6. Anti-bacterial activity of honey samples

Honey sample	***Staphylococcus Aureus* (mm)**	***Escherichia coli* (mm)**	***Klebsiella pneumoniae* (mm)**
A	15±0.89	-	14±0.57
B	15±1.20	21±0.89	-
C	10±0.58	-	21±0.58
D	9.0±0.00	-	-
E	14±0.33	16±0.58	-
F	3.0±0.00	-	15±1.45
G	4.0±0.67	6±0.89	16±0.68
H	9.0±0.33	10±0.89	18±0.59
I	22±0.88	16±0.67	25±0.33
J	21±0.00	13±0.58	26±0.67
Mean	12.20±1.95	8.20±1.96	13.50±1.89
Control (CP)	42±0.12	40±0.51	40±0.23

*mm= Millimeter; CP= Ciprofloxacin

Data is presented as Means ± SEM values.

Means, minimum and maximum values for heavy metals in honey samples such as Pb, Cd, As are given in Table 5 and 7. The lead in honey samples was ranged from 0.0 to 2.22mg/Kg of honey. The maximum lead (2.22±0.04mg/Kg) was found in poly-floral honey (Sample G) followed by (0.86±0.04mg/Kg) cardamom honey (Sample D). Many honey samples did not show any lead traces at all. The range of lead from literature varies from 0.001-0.03mg/100g[8]. The maximum permissible concentration of cadmium is 200µg/Kg or 0.2mg/Kg[49]. The arsenic content in honey was ranged from 0 to 0.20mg/Kg, with mean 0.045±0.013mg/Kg. Arsenic maximum allowable level is 10-500µg/Kg or 0.01-0.5mg/Kg as per regulations of the Codex Alimentarius[49]. All 3 heavy metals in honey falls under permissible limits.

Table 7. Heavy metal content (mg/Kg) in honey samples

Honey samples	Lead (Pb)	Cadmium (Cd)	Arsenic (As)
A	0.16±0.02	0.00±0.00	0.03±0.03
B	0.14±0.01	0.00±0.00	0.10±0.01
C	0.13±0.01	0.00±0.00	0.20±0.00
D	0.86±0.04	0.12±0.01	0.00±0.00
E	0.00±0.00	0.00±0.00	0.06±0.00
F	0.22±0.02	0.00±0.00	0.14±0.01
G	2.22±0.04	0.15±0.01	0.01±0.00
H	0.00±0.00	0.18±0.00	0.01±0.00
I	0.05±0.03	0.00±0.00	0.01±0.00
J	0.00±0.00	0.00±0.00	0.00±0.00
Min-Max	0.00-2.22	0.00-0.18	0.00-0.2
Mean± SEM	**0.378±0.12**	**0.034±0.01**	**0.045±0.01**

Data is presented as Means ± SEM values

4.5. Phytochemical and Antioxidant Analysis of Honey

TPC of honey falls in the range of 21.9-165.4 mgGAE/100g (Table 8). The lowest TPC was found to be 21.2±2.85, 22.0±2.90 and 26.0±5.01mgGAE/100g in poly-floral honey (Sample H), cardamom honey (Sample D) and multi-flower forest honey (Sample I) respectively. The TPC in honey can be as low as only 2.54[14] to 183mgGAE/100g[15]. While TFC in honey was ranged from 3.24-5.57 mg/100g with mean 3.95±0.29mg/100g. The sidr honey (sample J) showed highest TPC as well as TFC content. DPPH radical scavenging activity of honey ranged between 18 to 64% with mean of 38±3.25%. The highest DPPH activity (64±1.3%) was found in forest sidr honey (Sample J). While the lowest scavenging activity (18±0.1%) was shown by multi-flower forest honey (Sample I). The %RSA of honey samples were in accordance with other Pakistani honey 23 to 66.57%[30]. The literature has shown DPPH activity of honey as less as only 2% in honey from arid regions of UAE[33] to as high as 80% in artisanal honey from Mexico[50].

FRAP analysis showed the highest content was found in sample M (2138±10.62µM). Pakistani honey samples have FRAP ranging from 213.78 to 1780.74 µM[15], Georgian honeys *i.e.,* 30–1353.50µM[52]. The FRAP activity of same kind of floral sourced honey can vary greatly.

Table 8. Phytochemical and antioxidant analysis of honey samples

Honey sample	TPC (mgGAE/100g)	TFC (mgCat/100g)	DPPH (%)	FRAP (µMFeII/100g)
A	41.76±4.35	4.216±0.085	36.62±3.83	1402±26.72
B	110.9±4.705	4.27±0.026	42.91±0.83	1076±18.87
C	64.97±5.39	4.334±0.13	34.95±2.72	954.0±25.52
D	22.01±2.96	4.58±0.08	19.73±1.62	1086±37.73
E	44.0±3.73	2.97±0.06	36.52±1.47	1097±18.13

F	51.23±2.88	4.599±0.11	32.74±4.28	1276±3.183
G	43.41±6.24	2.765±0.11	37.16±2.98	1445±54.18
H	21.9±2.85	3.245±0.14	20.86±3.90	1323±5.04
I	25.96±5.103	2.97±0.06	18.3±0.09	1239±39.02
J	165.4±1.132	5.57±0.16	44.92±0.18	1249±21.38
Min-Max	21.9-165.4	2.76-5.57	18.3-44.92	954-1445
Mean	59.154	3.9519	32.471	1214.7

***** TPC= Total Phenolic Contents (TPC); TFC= Total Flavonoid Contents; DPPH= 2, 2-diphenyl-1-picrylhydrazyl; FRAP= ferric reducing antioxidant potential: µM FeII= Micro mole ferrous ion; mg GAE= milligram gallic acid equivalent; Cat= Catechin equivalent.

5. Discussion

The sources of protein in honey are either the nectar, pollen from plants or the salivary or glandular secretions from bee. The most common proteins are major royal jelly protein (MJRP-1), shown to have immunostimulatory effects[53]. Total protein is not a quality parameter, but a very low percentage can predict adulteration along with other parameters.

The variations among the mineral content can be due to difference in the kind, types and sources of honey or due to harvesting processes, beekeeping techniques and the material collected by the bees during the foraging on the flora. Usually, the honey darker in color is found to be high in minerals[54]. Literature showed high ash content in honey collected from agriculture regions due to agriculture practices such as use of fertilizer[55]. But ash content falls under permissible set limits.

TSS along with other parameters can be used as an adulteration marker for authenticity. Honey is a super-saturated solution containing at least or more than 70% sugars in it. Honey having more than > 80°Bx and <20% water is considered high quality honey and represent better shelf stability. There is an inverse relation between moisture and TSS or brix, lower the brix means higher the moisture level and ultimately fermentation. However, the storage condition, harvesting time, maturing stage of honey in honeycomb and weather conditions can also affect the brix. The honey stored at higher temperatures have higher brix because of the evaporation of water than stored at relatively lower temperatures[56].

Low RS content has been linked with adulteration in honey. Nevertheless, low RS content doesn't always mean adulteration, it can also be affected by the temperature in which honey is being harvested. In general, the higher RS content depicts better quality of honey representing the efficient conversion of sucrose into reducing or invert sugars[57]. Apparent sucrose can also be used as an important parameter to find indirect adulteration such as bees fed on sucrose syrup which convert the sucrose fed to them into glucose and fructose. However, the decrease in proline content and electrical conductivity of such honey has been reported. The ability of honey crystallization is dependent on sucrose content and ratio of reducing sugars such as fructose and glucose. However, these two factors cannot be solely responsible for crystallization, other factors such as insoluble components (dextrin, pollen, bee parts), storage conditions and temperature can also influence crystallization.

The proline content is considered one of the quality parameters <180 mg/Kg proline is considered adulterated but can vary greatly depending on the source, origin and year[58,59]. It also reflects the nectar and pollens present in honey, as pollens are the major source of amino acid in the honey[60]. Some researchers think that IS set for proline need to be re-evaluate as these are too low to determine the authenticity of honey and literature showed higher proline than the legislative standards. The proline content of pure and adulterated honey ranged from 772.83-249.33 mg/Kg, the adulterated honey samples showed higher proline than the set standard (≥180mg/Kg)[61]. WIC is an important hygiene and quality parameter. The objective for this parameter is to estimate the impurities such as dirt, comb debris, bee particles and beeswax.

The highest minerals were potassium and sodium followed by calcium in the study. The potassium and sodium ratio are sometime considered as a marker for adulteration ≥1 indicates non-adulteration. But this parameter for adulteration is not commonly used[62]. The ratio for most of the honey samples is ≥1. Honey is not considered as the source of minerals in diet. As it contains less than 0.5% of ash.

The presence of minerals is associated with botanical origin, soil characteristics and agriculture practices. Honey from Saudia Arabia collected from agricultural areas showed mineral content[55]. The quantification of minerals is not a quality but rather to determine the composition of honey to determine nutritional value or to find the floral source of certain mono-floral honey although not a common. The literature for botanical origin differentiation from minerals content is available but unfortunately honey sampling cannot be the representative of one country. For the current study, the data related to minerals content of Pakistani honeys is not available readily so cannot be compared with.

The measurement of refractive index is important for the measurement of moisture. Although there is no set standard for honey pH, but honey is considered as acidic food because of presence of organic acids with pH between 3.2 to 6. The pH of honey is not only crucial for its stability and shelf life but also for its therapeutic potential *i.e.*, anti-bacterial, immune-modulatory and wound healing[63].

Fiehe's test is a preliminary test used for the detection of adulteration in honey with only invert sugar. It is a qualitative test which shows the presence of HMF. This test has been replaced with HMF, a quantitative analysis. But still this test is used as standard to detect the adulteration along with HMF and fructose-glucose ratio in honey[25,64].

HMF is usually formed due to breakdown of fructose in the presence of acid. HMF production can naturally increase in honey if stored for a longer period or in warmer temperatures. The process of HMF production speeds up as honey is heated for example during adulteration when sugars are added and heated to evaporate excess water. Detection of HMF is not only important to check the freshness of honey but also a safety parameter as HMF is toxic to human beings. Because of its safety concerns, the HMF limits have been developed by EU for different food such as dry fruits, fruit concentrates and juices. The HMF is proved to be cytotoxic for skin and upper respiratory tract, but most recently optimum quantity (30-150mg/day) of HMF in diet can proved to be beneficial such as anti-allergic, anti-hypoxic and anti-inflammatory. However, more research is required to establish safe levels[65]. Thus, the detection and quantification of this compound in honey is necessary before it can be used in clinically. Free acidity in honey is because of organic acids and their lactone and some inorganic ions. Many studies use free acidity or total acidity (free acidity + lactone acid) with other physicochemical parameters to determine the authenticity of honey.

Bacterial load in honey can be zero to tens of thousands of bacterial counts[11] showing secondary comtamination, however, it shouldn't be more than 9500CFU/g[10]. In current study, all the honey sample showed load in permissible limits. None of the pesticide residue was detected in honey. Pesticides that include insecticides, fungicides, antibiotics and herbicides can become part of honey Pesticides have been shown to cause genetic mutation in humans. Legislative bodies such as the EU have regulated maximum residual levels (MRLs) for pesticides in honey[9].

The anti-bacterial activity of honey is due to its physical and chemical attributes such as low moisture, high acidity and presence of hydrogen peroxide in honey. Many studies have shown positive correlation of hydrogen peroxide content with its anti-microbial activity, but this correlation wasn't consistent for all the honey, hence showing other factors responsible for this activity.

Arsenic, cadmium and lead are top among the top 20 hazardous heavy metals compiled by Agency for Toxic Substances and Disease Registry (ATSDR)[8]. The heavy metals in honey sometimes also used as markers of environmental contamination. The major cause of heavy metals or trace minerals in honey is generally due to exposure to certain pesticides, industrial waste and environmental pollution. Honey from Croatia have shown As as high as 499mg/Kg due to the presence of bee hives near railway lines and highways[66]. The application of honey as therapeutic is increasing so the determination of these metals should be taken into consideration.

Total flavonoids (TF) along with phenols are also considered an important group of antioxidants. However, both can vary greatly even in the same geographical origin. In honey, other anti-oxidative compounds can be the enzymes or reducing sugars that constitute about 65% of total composition. It is worth mentioning that honey samples with high HMF or sucrose content than permitted values showed much lower phenolic contents than other honey samples. Flavonoids are also known to

possess the properties such as antiulcer, antiangiogenic, estrogenic, anticancer, anti-allergic, anti-inflammatory and antibacterial activity[63]. TFC content is usually less than TPC. So, the comparison of phenolic and flavonoid content to its antioxidant property is rather difficult due to absence of standardized methods for honey analysis. However, honey itself is variable in its composition because of amalgamation of nectar from different floral sources and origin. Overall, the TFC in Pakistani honey samples were lower but were in accordance with Spanish mono- and poly-floral honey[5].

Honey with DPPH activity is richer in antioxidants. The positive correlation of DPPH scavenging activity to that of TPC or ascorbic acid (AA) is found hinting that phenolic compounds are mainly responsible for the antioxidant activity[16]. But this correlation wasn't consistent in some research[5] showing that other than phenolic compounds, the largest known class of antioxidant in nature, other compounds like reducing sugars and enzymes in honey play better role in its scavenging activity.

FRAP is another antioxidant measuring quantitative analysis. A single test is not able to give true picture of antioxidant potential. So multiple tests are done. Like other parameters, FRAP activity is highly variable among different honey samples based on its floral source, geographical origin and bee species. The antioxidant activities such as DPPH and FRAP are sometimes considered more important variable to discriminate between mono-floral honey from multi-floral honeys along with other parameters. Similarly, DPPH, FRAP is also linked to its phenolic and flavonoid content.

6. Conclusion

The database related to Pakistani mono- as well as poly-floral sourced honey isn't readily available. The quality and safety parameters are less reported that make it difficult to directly use the honey from a known source. Therefore, commercial honey samples were screened for compositional, quality, safety, phytochemical, and antioxidant analysis and further compared with global standards. The phytochemical and antioxidant analysis were performed to check its therapeutic effects. Like other honey samples around the world commercial honey samples also showed great variability for its *in-vitro* anti-bacterial, phytochemical content or antioxidant activity. Thats showed that pre-clinical screening must be rigorous before any clinical or efficacy trial.

Funding information

HEC scholarship under Indigenous 5000 PhD Fellowship (Phase II Batch VI).

Conflict of Interest

The authors do not have any competing interests.

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