

## THE CHARACTERIZATION OF BLOSSOM HONEYS FROM TWO PROVINCES OF PAKISTAN

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### ABSTRACT

This study characterized fifteen blossom honeys collected from eleven locations in the Punjab and Khyber Pakhtunkhwa (KPK) provinces of Pakistan. Mean values for physicochemical parameters (i.e., moisture, water activity, free acidity, pH, electric conductivity, diastase activity, total acidity, ash, total protein, hydroxymethylfurfural, fructose, glucose, sucrose, maltose, raffinose, reducing sugars, total sugars, and fructose/glucose ratio) were 18.09%, 0.57, 13.52 mEq kg<sup>-1</sup>, 4.27, 414.41 μS cm<sup>-1</sup>, 10.56 DN, 26.80 mEq kg<sup>-1</sup>, 0.15%, 313.30 mg 100 g<sup>-1</sup>, 15.39 mg kg<sup>-1</sup>, 35.49%, 30.77%, 4.41%, 2.12%, 0.11%, 66.25%, 72.88%, and 1.16, respectively. The sucrose content was slightly high in three honeys. In general, all of the remaining honey samples met the criteria for international honey standards.

*Keywords:* chemical analyses, comparison, honeys, qualitative properties

## 1. INTRODUCTION

Honey is a natural sweet material made by honeybees from floral nectar, plant secretion or excretion of insects (which suck the sap from living plant parts), that honeybees collect, modify and intermix with their own particular substances, deposit and leave it in the cells of the comb to ripen and mature (MENDES *et al.*, 1998).

Humans have used honey as a reliable sweetener for centuries. Honey holds a particular place in the food and medical industries, and it has been regarded as a highly nutritive food in many civilizations (FEÁS *et al.*, 2010 a).

Pakistan has diverse landscape, climate and environmental conditions, including sandy beaches, deserts, high mountains and pictorial valleys, each featuring specific vegetation. The country has a great potential for honey production because of its congenial climate conditions and variety of bee flora. Punjab, the most populous province of Pakistan, has the majority of its bee flora in the northern and central regions, where an ample amount of honey is harvested (IZHAR-UL-HAQ *et al.*, 2010). Khyber Pakhtunkhwa (KPK) also has environmental conditions that are conducive for the growth of high floral biodiversity that contributes to honey production, and its export to the Middle East and western countries (MAIRAJ *et al.*, 2008).

Variation in the composition of honey largely arises from the climate and environmental conditions of an area, its nectar and pollen, and the abilities of beekeepers (WHITE, 1978). The physicochemical properties of honey affect its honey storage, quality, granulation, texture, flavor, and nutritional characteristics as well as its medicinal qualities (IFTIKHAR *et al.*, 2011). The characterization of honey promotes the understanding of its medicinal properties as well as its antibacterial and antioxidant characteristics (ADEBIYI *et al.*, 2004). Most of its physicochemical properties can be used to reveal adulteration; therefore, studies of certain quality parameters are needed to ensure the purity of honey (KHAN *et al.*, 2006).

This study sought to characterize the quality parameters and discriminative properties of 15 different blossom honeys collected from diverse areas of two provinces of Pakistan. Previous work has mainly focused on a few samples from specific locality.

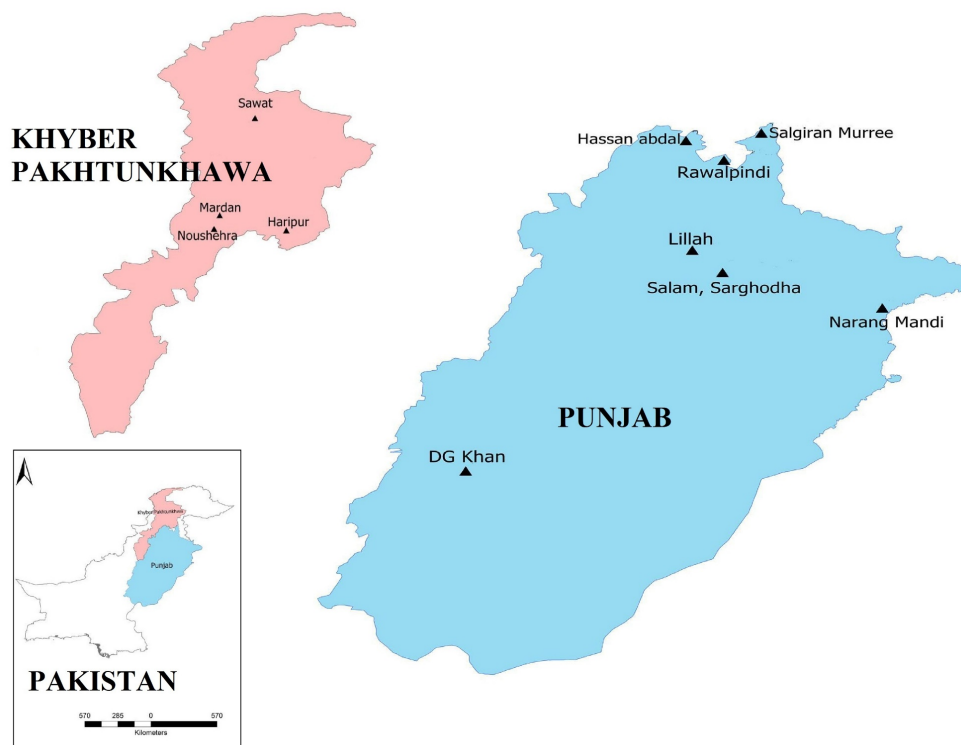
## 2. MATERIALS AND METHODS

All of the chemicals and reagents employed in this study were of analytical grade. Fructose, glucose, sucrose, maltose, raffinose, bovine serum albumin, Folin & Ciocalteu's phenol reagent, phosphoric acid and 5-(hydroxymethyl) furfural were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hiper Solv for HPLC™, water for high-performance liquid chromatography HPLC, was obtained from BDH Laboratory Supplies (Poole, UK). Acetonitrile (HPLC grade) was acquired from Fisher Scientific UK Ltd. (Leicestershire, UK).

### 2.1. Honey samples

In this study, fifteen samples of blossom honey were collected from different apiaries of Punjab and KPK provinces between March and April 2014. Nine samples, i.e., eucalyptus (*Eucalyptus* spp.), sunflower (*Helianthus annuus*), acacia (*Acacia* spp.), mustard (*Brassica campestris*), jujube (*Ziziphus mauritiana*), clover (*Melilotus officinalis*), citrus (*Citrus* spp.), currant bush (*Carissa opaca*), and multifloral honeys, were collected from seven locations of Punjab, whereas six honey samples, i.e., loquat (*Eriobotrya japonica*), eucalyptus (*Eucalyptus* spp.), jujube (*Ziziphus mauritiana*), acacia (*Acacia* spp.), citrus (*Citrus* spp.), and clover

(*Melilotus officinalis*), were collected from four locations of KPK (Fig. 1). These samples were brought to the Chair of Engineer Abdullah Ahmad Buqshan for Bee Research, Plant Protection Department, College of Food and Agriculture Sciences, King Saud University, Riyadh, Saudi Arabia and placed in a refrigerator at 4°C until analysis in May 2014.



**Figure 1:** Map of Pakistan showing the honey collection sites in Punjab and Khyber Pakhtunkhwa.

## 2.2. Moisture content

The moisture content (expressed as a percentage) of all honey samples was determined via refractometry using a refractometer (Abbe Mark II model 10480, Cambridge Instruments Inc., Buffalo, NY, USA) at 20°C according to the guidelines of the International Honey Commission (IHC) (BOGDANOV, 2002).

## 2.3. Water Activity ( $a_w$ )

Water activity in honey samples was measured using a bench-top Aqualab CX-2 water activity meter (Decagon Device, Inc., WA, USA) at 20°C (HABIB *et al.*, 2014).

## 2.4. Electrical conductivity (EC)

EC was measured by making a 20% (w/v) honey solution in distilled water according to IHC guidelines (BOGDANOV, 2002) via an HI-9835 EC/TDS/NaCl meter (HANNA Instruments, Woonsocket, RI, USA). The measurements were recorded in  $\mu\text{S cm}^{-1}$ .

## 2.5. Ash content

Ash content was measured using a predetermined EC value for the honey samples and by substituting those values into the following formula:  $X1 = (X2 - 0.143)/1.743$ , where X1 denotes the ash content and X2 represents the EC (in mS cm<sup>-1</sup>) at 20°C (PIAZZA *et al.*, 1991). The ash content is expressed as a percentage.

## 2.6. pH determination

To measure pH, 5 g of honey was mixed in 25 mL of distilled water, and a pH reading was taken using a professional benchtop BP3001 pH meter (Trans Instruments, Singapore).

## 2.7. Acidity (free and total)

Ten grams of honey was dissolved in 75 mL of CO<sub>2</sub>-free water (Micropure UV Tank-Thermo Scientific, Hungary) in a 250-mL beaker. This solution was titrated with 0.1 M sodium hydroxide solution until the pH reached 8.3. Then, the free and total acidity were measured according to IHC guidelines (BOGDANOV, 2002) and are expressed in mEq kg<sup>-1</sup>.

## 2.8. Diastase Activity (DA)

DA was determined according to FEÁS *et al.* (2010a). Five grams of honey was placed in a beaker and dissolved completely in 20 mL of distilled water and 2.5 mL of an acetate buffer solution. This mixture was transferred to a flask containing 1.5 mL of NaCl solution. Then, 10 mL of this solution was placed in a 50-mL volumetric flask and placed in a thermostatic bath (Thermolab Industries) at 40°C with a second flask containing 10 mL of starch solution. After 15 min, 5 mL of starch solution was pipetted into the honey solution and mixed. After the first 5 min, 1 mL aliquots from this solution were removed, and 5 mL of iodine solution was added at periodic intervals. The sample absorption was monitored at 660 nm against a water blank in a 1 cm cell using a PerkinElmer Lambda 25, UV/VIS/Spectrometer (Shelton, CT, USA). DA is expressed in Gothe units per gram of honey, i.e., the “diastase number” (DN). One Gothe unit is the amount of enzyme that converts 0.01 g of starch into a given point in 1 h at 40°C.

## 2.9. Sugar content

In this experiment, 5 g of honey were mixed in distilled water; to create a total volume of 100 ml, it was transferred to a 100 mL volumetric flask and adjusted with water. This honey solution was filtered using a 0.55 mm Whatman filter paper (Whatman International Limited, Maidstone, UK) and stored in vials at 4°C. The sugar content was determined using an HPLC system equipped with a refractive index (RI) detector (PerkinElmer Series-200a, USA). Sugar separation was performed at 85°C in a Sugar-Pak™ 1 column (6.5×300 mm) manufactured by Waters (USA). The HPLC pump, column oven, auto sampler, and RI detector were observed using a TotalChrom Workstation, version 6.3.1 (2006). The mobile phase consisted of 100% HPLC-grade water (HiprSolv for HPLC, BDH Laboratory Supplies, Poole, UK). The injection volume of the honey samples was adjusted to 1 µl accompanying a flow rate of 0.6 mL min<sup>-1</sup>. The peaks were recognized by matching respective retention times with those standards. Furthermore, honey samples were spiked with standards, to confirm the identity of the chromatographic peaks. The average peak areas of the triplicate injections were used for peak quantification. A calibration curve was generated for each sugar using standard solutions (10–30 mg ml<sup>-1</sup>).

The honey samples in the crystallized form were liquefied using a water bath (Thermolab Industries) at 40°C. The sugar results are expressed in g 100 g<sup>-1</sup> honey.

### 2.10. Total protein content

Lowry's method of protein estimation was used to determine the total protein content of the honey samples. The basic principle in this method was the formation of a copper-protein complex and the reduction of phosphomolybdate and phosphotungstate present in Folin & Ciocalteu's reagent to hetero polymolybdenum blue and tungsten blue, respectively. Bovine serum albumin (0–100 µg ml<sup>-1</sup>) was used as a standard to prepare the calibration curve (HABIB *et al.*, 2014). The results of the protein content were measured in mg 100 g<sup>-1</sup>.

### 2.11. Hydroxymethylfurfural (HMF)

One gram of honey was mixed in 10 mL of acetonitrile:water (1:1) solution. This mixture was homogenized via constant shaking for 10 min and then filtered using a 0.45-µm syringe filter into vials and set for injection into HPLC system equipped with Series 200 UV/VIS detector (PerkinElmer Series-200a, USA). The injection volume was 10 µl, and the separation was performed using a Symmetry® C18 5 µm (3.9 × 150 mm) column manufactured by Waters (Ireland), maintained at 22°C with a run time of 5 min. The mobile phase was 0.01 N phosphoric acid (86%) and acetonitrile (14%), with a flow rate of 0.2 mL min<sup>-1</sup> (CHINNICI *et al.*, 2003). The HMF found in the honey samples is expressed in mg kg<sup>-1</sup>.

### 2.12. Statistical analysis

All of the tests were conducted in triplicate. SPSS for Windows (version 17, IBM, Armonk, NY, USA) was used to analyze the data. The mean ± standard deviation differences among samples were ascertained using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The cluster analysis of 12 parameters (moisture content, free acidity, pH, EC, diastase activity, total acidity, ash, HMF, fructose, glucose, sucrose, and maltose) was applied to Pakistani (Punjab and KPK) and Mediterranean honey samples in order to determine the similarity among the honeys of different countries. For this analysis, each parameter data were represented by mean value and the fractions of zero were used for the parameters (free acidity, EC, diastase activity, and ash), which were not available (N.A.) for compared honeys. The dendrogram was constructed by Ward's Linkage method with Euclidean distances. Discriminant analysis was performed using Past v.3.12 software.

## 3. RESULTS AND DISCUSSIONS

The physicochemical analyses of honey samples from Punjab and KPK provinces are summarized in Tables 1 and 2 and their sugar profiles are mentioned in Tables 3 and 4. All the data were reported as mean values ± standard deviations. Table 5 gives a comparison of relevant published data of honey parameters from some Mediterranean countries to the present study.

Moisture content is one of the factors that determine the shelf life of honey during storage (PERÉZ-ARQUILLUE *et al.*, 1994). It can be as low as 13% or as high as 23%, depending on the source of the honey and the climate conditions (BRADBEAR, 2009). The moisture level

of honey can increase under high humidity permeability and high storage humidity because of its hygroscopic property (ÖZCAN and ÖLMEZ, 2014). Higher moisture content can promote honey fermentation during storage (IDRIS *et al.*, 2011). The moisture content values of all tested honey samples from both provinces varied from 16.32 to 19.91% (Tables 1 and 2) and were in acceptable range ( $\leq 20\%$ ) of international quality regulations according to the Codex Alimentarius Commission (2001). During the comparison of different Mediterranean honeys, the moisture values of Pakistani honeys were not statistically different from each other and from Turkish honeys reported previously (ÖZCAN and ÖLMEZ, 2014). Among the six types of compared honeys, the lowest mean moisture content of 17.01% was in Italian honeys (TRUZZI *et al.*, 2014) while the Moroccan honeys (AAZZA *et al.*, 2014) had the highest (18.97%). The variant moisture content might be the result of the diverse prevailing weather conditions across the disparate geographical locations of different countries or the different beekeeping practices that affect the degree of honey maturity.

The water activity of analysed honey samples varied from 0.51 to 0.66 (Tables 1 and 2). These results were in accordance with arid regions and Mexican honeys, with *aw* ranges of 0.52 to 0.64 (HABIB *et al.*, 2014) and 0.569 to 0.613 (MONDRAGO´N-CORTEZ *et al.*, 2013), respectively. The water activity is a key factor that controls food stability by limiting or preventing microbial growth. Osmotolerant yeasts can grow under minimal *aw* conditions of 0.6 (CHIRIFE *et al.*, 2006). The mustard honey from Punjab, which had the highest moisture content, had the highest *aw* and might be candidate for fermentation.

The presence of organic acids and inorganic ions in honey results in its acidity (TERRAB *et al.*, 2004).

The total acidity of honey samples from both provinces ranged from 15.90 to 39.06 mEq kg<sup>-1</sup> (Tables 1 and 2), both of which were acceptable (i.e., below 50 mEq kg<sup>-1</sup>; Codex Alimentarius Commission, 2001). The lowest total acidity was associated with citrus, whereas the highest value was found in multifloral and eucalyptus honeys. The difference in total acidity of honey is due to the harvesting season (HABIB *et al.*, 2014). The free acidity of the honey samples ranged from 3.97 to 24.77 mEq kg<sup>-1</sup>. The lowest free acidity was observed in clover and loquat honeys of Punjab and KPK, respectively, whereas the highest values were found in eucalyptus honey samples of both provinces. The total and free acidity of the analyzed honey samples approximated the values recorded at other geographical locations (HABIB *et al.*, 2014). A comparison between Pakistani and some Mediterranean honeys presented a substantial variation (Table 5). Nectar source, climatic conditions and soil properties of different countries might explain the acidity variation among the honey samples.

The pH values of tested honey samples of both provinces ranged from 3.22 to 5.18 (Tables 1 and 2). Lower pH was in acacia honey, whereas higher values were associated with the multifloral and jujube honeys of Punjab and KPK, respectively. The pH of honey is related to its storage and microbial growth and is responsible for its changes in texture and stability. The pH value of honey provides clues about its origin (i.e., floral vs. forest, where forest honey typically has a higher pH value; FEÁS *et al.*, 2010 a). The IHC has not described the limit of pH in honey; however, a low pH inhibits microbial contamination (HABIB *et al.*, 2014). The pH of the honey samples obtained from both provinces matched the values recorded by GULFRAZ *et al.* (2011) and RODRÍGUEZ *et al.* (2014). Honey samples from Pakistan showed a significant variation in pH from other Mediterranean honeys.

**Table 1:** Physicochemical properties of blossom honeys taken from the Punjab province of Pakistan.

| S. No. | Honey Type   | Location         | Moisture Content (%)     | Water Activity ( $a_w$ ) | Free Acidity (mEq kg <sup>-1</sup> ) | pH                     | EC ( $\mu\text{S cm}^{-1}$ ) | Diastase Activity (DN)  | Total Acidity (mEq kg <sup>-1</sup> ) | Ash (%)                | Total Protein (mg 100 g <sup>-1</sup> ) | HMF (mg kg <sup>-1</sup> ) |
|--------|--------------|------------------|--------------------------|--------------------------|--------------------------------------|------------------------|------------------------------|-------------------------|---------------------------------------|------------------------|---|----------------------------|
| 1      | Eucalyptus   | Hassanabad       | 18.64±0.04 <sup>b</sup>  | 0.59±0.00 <sup>c</sup>   | 19.62±0.08 <sup>a</sup>              | 4.31±0.02 <sup>b</sup> | 448.59±0.41 <sup>d</sup>     | 13.56±0.24 <sup>b</sup> | 36.19±0.72 <sup>a</sup>               | 0.17±0.00 <sup>c</sup> | 407.41±2.84 <sup>a</sup>                | 18.40±0.52 <sup>c</sup>    |
| 2      | Sunflower    | DG Khan          | 18.78±0.03 <sup>b</sup>  | 0.55±0.01 <sup>f</sup>   | 17.09±0.09 <sup>bcd</sup>            | 3.92±0.01 <sup>e</sup> | 319.17±0.41 <sup>f</sup>     | 9.12±0.53 <sup>cd</sup> | 29.59±1.42 <sup>bc</sup>              | 0.10±0.00 <sup>e</sup> | 241.72±1.69 <sup>f</sup>                | 29.16±0.73 <sup>b</sup>    |
| 3      | Acacia       | Rawalpindi       | 16.32±0.35 <sup>e</sup>  | 0.53±0.00 <sup>g</sup>   | 14.80±0.21 <sup>d</sup>              | 3.85±0.01 <sup>f</sup> | 467.87±0.41 <sup>c</sup>     | 10.15±0.24 <sup>c</sup> | 26.91±0.49 <sup>cd</sup>              | 0.18±0.00 <sup>c</sup> | 346.84±3.61 <sup>c</sup>                | 9.31±0.24 <sup>f</sup>     |
| 4      | Mustard      | Lillah           | 19.91±0.42 <sup>a</sup>  | 0.66±0.00 <sup>a</sup>   | 15.57±0.14 <sup>cd</sup>             | 4.34±0.01 <sup>b</sup> | 302.28±0.41 <sup>h</sup>     | 9.26±0.53 <sup>cd</sup> | 26.02±0.54 <sup>d</sup>               | 0.09±0.00 <sup>e</sup> | 242.37±2.46 <sup>f</sup>                | 37.25±0.86 <sup>a</sup>    |
| 5      | Jujube       | Lillah           | 17.88±0.06 <sup>d</sup>  | 0.56±0.01 <sup>e</sup>   | 17.05±1.81 <sup>bcd</sup>            | 5.16±0.02 <sup>a</sup> | 569.60±0.41 <sup>a</sup>     | 16.64±0.24 <sup>a</sup> | 28.00±0.76 <sup>cd</sup>              | 0.24±0.00 <sup>a</sup> | 289.40±1.21 <sup>d</sup>                | 9.10±0.17 <sup>f</sup>     |
| 6      | Clover       | Narang Mandi     | 18.58±0.03 <sup>b</sup>  | 0.61±0.00 <sup>b</sup>   | 3.97±0.21 <sup>e</sup>               | 4.12±0.01 <sup>d</sup> | 367.81±1.60 <sup>e</sup>     | 8.98±0.53 <sup>cd</sup> | 19.02±0.51 <sup>e</sup>               | 0.13±0.00 <sup>d</sup> | 207.07±0.80 <sup>g</sup>                | 13.19±0.35 <sup>e</sup>    |
| 7      | Citrus       | Salam, Sargodha  | 18.42±0.03 <sup>bc</sup> | 0.58±0.00 <sup>d</sup>   | 5.07±0.65 <sup>e</sup>               | 4.29±0.01 <sup>b</sup> | 308.03±1.55 <sup>g</sup>     | 5.73±0.75 <sup>e</sup>  | 18.92±0.50 <sup>e</sup>               | 0.09±0.00 <sup>e</sup> | 235.77±3.43 <sup>f</sup>                | 15.48±0.22 <sup>d</sup>    |
| 8      | Currant bush | Salgiran, Murree | 17.98±0.08 <sup>cd</sup> | 0.56±0.00 <sup>ef</sup>  | 17.29±1.49 <sup>abc</sup>            | 4.21±0.01 <sup>c</sup> | 446.56±0.41 <sup>d</sup>     | 8.72±0.53 <sup>d</sup>  | 31.50±2.19 <sup>b</sup>               | 0.17±0.00 <sup>c</sup> | 272.18±1.17 <sup>e</sup>                | 7.43±0.53 <sup>g</sup>     |
| 9      | Multifloral  | Rawalpindi       | 17.67±0.06 <sup>d</sup>  | 0.51±0.00 <sup>h</sup>   | 18.77±0.46 <sup>ab</sup>             | 5.18±0.02 <sup>a</sup> | 503.53±0.41 <sup>b</sup>     | 12.90±0.24 <sup>b</sup> | 36.59±0.61 <sup>a</sup>               | 0.21±0.00 <sup>b</sup> | 357.83±1.88 <sup>b</sup>                | 19.38±0.36 <sup>c</sup>    |

All analyses were performed in triplicate, and the mean value ± standard deviation (SD) are reported. Mean values in the same column but with different superscript letters differ significantly ( $P > 0.05$ ).

**Table 2:** Physicochemical properties of blossom honeys taken from the Khyber Pakhtunkhwa province of Pakistan.

| S. No. | Honey Type | Location  | Moisture Content (%)     | Water Activity ( $a_w$ ) | Free Acidity (mEq kg <sup>-1</sup> ) | pH                     | EC ( $\mu\text{S cm}^{-1}$ ) | Diastase Activity (DN)  | Total Acidity (mEq kg <sup>-1</sup> ) | Ash (%)                | Total Protein (mg 100 g <sup>-1</sup> ) | HMF (mg kg <sup>-1</sup> ) |
|--------|------------|-----------|--------------------------|--------------------------|--------------------------------------|------------------------|------------------------------|-------------------------|---------------------------------------|------------------------|---|----------------------------|
| 1      | Loquat     | Harripur  | 18.04±0.09 <sup>c</sup>  | 0.59±0.00 <sup>a</sup>   | 4.37±1.19 <sup>d</sup>               | 4.19±0.02 <sup>c</sup> | 322.11±2.15 <sup>e</sup>     | 9.51±0.53 <sup>cd</sup> | 20.42±2.13 <sup>bc</sup>              | 0.10±0.00 <sup>e</sup> | 391.40±2.12 <sup>b</sup>                | 11.48±0.31 <sup>d</sup>    |
| 2      | Eucalyptus | Mardan    | 17.37±0.05 <sup>de</sup> | 0.54±0.01 <sup>e</sup>   | 24.77±1.58 <sup>a</sup>              | 4.11±0.01 <sup>d</sup> | 439.14±0.41 <sup>c</sup>     | 11.51±0.24 <sup>b</sup> | 39.06±4.47 <sup>a</sup>               | 0.16±0.00 <sup>c</sup> | 377.60±1.08 <sup>c</sup>                | 23.33±0.43 <sup>a</sup>    |
| 3      | Jujube     | Noushehra | 17.49±0.07 <sup>d</sup>  | 0.55±0.00 <sup>d</sup>   | 12.70±0.25 <sup>c</sup>              | 5.01±0.01 <sup>a</sup> | 591.64±2.36 <sup>a</sup>     | 14.68±0.24 <sup>a</sup> | 25.89±1.54 <sup>b</sup>               | 0.26±0.00 <sup>a</sup> | 270.77±2.65 <sup>e</sup>                | 6.22±0.16 <sup>e</sup>     |
| 4      | Acacia     | Noushehra | 17.21±0.06 <sup>e</sup>  | 0.57±0.00 <sup>c</sup>   | 11.35±0.88 <sup>c</sup>              | 3.22±0.01 <sup>e</sup> | 487.53±0.41 <sup>b</sup>     | 9.43±0.53 <sup>cd</sup> | 18.65±0.87 <sup>c</sup>               | 0.20±0.00 <sup>b</sup> | 412.67±3.03 <sup>a</sup>                | 4.71±0.39 <sup>f</sup>     |
| 5      | Citrus     | Swat      | 18.29±0.08 <sup>b</sup>  | 0.58±0.01 <sup>b</sup>   | 5.25±0.95 <sup>d</sup>               | 4.27±0.02 <sup>b</sup> | 286.45±1.28 <sup>f</sup>     | 9.75±0.53 <sup>c</sup>  | 15.90±1.12 <sup>c</sup>               | 0.08±0.00 <sup>f</sup> | 311.36±5.65 <sup>d</sup>                | 13.98±0.26 <sup>c</sup>    |
| 6      | Clover     | Mardan    | 19.24±0.08 <sup>a</sup>  | 0.58±0.01 <sup>b</sup>   | 17.60±0.55 <sup>b</sup>              | 4.12±0.03 <sup>d</sup> | 357.03±0.41 <sup>d</sup>     | 8.44±0.53 <sup>d</sup>  | 33.16±3.30 <sup>a</sup>               | 0.12±0.00 <sup>d</sup> | 262.02±1.15 <sup>f</sup>                | 19.17±0.44 <sup>b</sup>    |

All analyses were performed in triplicate, and the mean value ± standard deviation (SD) are reported. Mean values in the same column but with different superscript letters vary significantly ( $P > 0.05$ ).

Tunisian and Italian honeys were statistically similar to average pH values, while the Turkish honeys had comparatively high values (Table 5). The difference observed in the pH values among the different honey samples might be because of their acidity and mineral concentrations (KAMAL *et al.*, 2002).

The EC results showed variations based on the floral origin of the honey samples from both provinces, and they ranged from 286.45 to 591.64  $\mu\text{S cm}^{-1}$ . Citrus honey was associated with less EC, whereas jujube honey with more EC (Tables 1 and 2). The EC of analysed honey samples matched the values reported by IDRIS *et al.*, (2011). TRUZZI *et al.*, (2014) reported a low average EC of Italian honey samples among the compared Mediterranean honeys while the Tunisian honeys (BOUSSAID *et al.*, 2014) showed high values (Table 5). The EC of honey is correlated with the intensity of its organic acids, mineral salts, and proteins; furthermore, it varies with changes in floral origin and is essential for differentiating the floral origins (HABIB *et al.*, 2014). The EC values of blossom honeys should be below 800  $\mu\text{S cm}^{-1}$ , whereas honeydew honeys have EC values above 800  $\mu\text{S cm}^{-1}$  (FEÁS *et al.*, 2010 b). In this study, the EC measurements were below 800  $\mu\text{S cm}^{-1}$ , which suggests that the origin of all the tested honey samples was floral.

The ash content is an important parameter to determine floral origin and differentiates nectar honey and honeydew honey (WHITE, 1978). Floral honey samples have a lower ( $\leq 0.6\%$ ) ash content than honeydew honeys ( $\leq 1.2\%$ ) (FEÁS *et al.*, 2010 b). The ash content of a honey is primarily due to certain nitrogen compounds, vitamins, minerals, aromatic substances and pigments (MAIRAJ *et al.*, 2008). The ash content of the tested honey samples ranged from 0.08 to 0.26% (Tables 1 and 2). The ash content in this study was below 0.6%, which indicates that the tested samples were nectar honeys (Codex Alimentarius Commission, 2001). The average ash content of analysed honeys was compared to some Mediterranean honeys. Turkish honeys (ÖZCAN and ÖLMEZ, 2014) had the minimum average ash content while the Moroccan (AAZZA *et al.*, 2014) and Tunisian honeys (BOUSSAID *et al.* 2014) reflected greater values than Pakistani honeys (Table 5).

The differences in the ash content of the tested honey samples might be because of soil type where the nectar plant was located (GÓMEZ-DÍAZ *et al.*, 2012), the atmospheric conditions, and plant physiology (KAMAL *et al.*, 2002).

Diastase is an enzyme found in honey, and its level changes based on the geography, plant source, and the freshness of honey. DA might indicate aging and point out the treated temperature during the processing of honey (FALLICO *et al.*, 2006). The lowest acceptable DA value is 8 on Gothe's scale according to international regulations (Codex Alimentarius Commission, 2001). The range of DA in the current study was 5.73 to 16.64 DN. In Punjab, the lowest DA was found in citrus honey, whereas the highest was in the jujube honey (Table 1). Similarly, the lowest and highest DA values were observed in the clover and jujube honeys of the KPK, respectively (Table 2). The DA of the honey samples corroborated previously reported values (FEÁS *et al.*, 2010 a; IFTIKHAR *et al.*, 2011).

The DA of Pakistani honeys was lower than Turkish and Moroccan honey samples (Table 5). The results indicate that the honey samples were natural because their DA was within the acceptable range.

The total protein content of the honey samples of both provinces ranged from 207.07 to 412.67 mg 100 g<sup>-1</sup>. These results are similar to the honey samples taken from arid regions, with the protein content ranging from 204.84 to 578.87 mg 100 g<sup>-1</sup> (HABIB *et al.*, 2014). The total protein content of the tested honey samples of both provinces was higher than that of samples from India, where it varied from 48 to 229.3 mg 100 g<sup>-1</sup> (SAXENA *et al.*, 2010). The protein content of honey depends on the presence of the enzymes introduced by honeybees as well as that putatively derived from floral nectar (SAXENA *et al.*, 2010); therefore, this value varies among the honey samples.



**Table 3:** Sugar content (expressed as a percentage) of blossom honeys taken from the Punjab province of Pakistan.

| S. No. | Honey Type   | Location         | Fructose (g 100 g <sup>-1</sup> ) | Glucose (g 100 g <sup>-1</sup> ) | Sucrose (g 100 g <sup>-1</sup> ) | Maltose (g 100 g <sup>-1</sup> ) | Raffinose (g 100 g <sup>-1</sup> ) | Reducing Sugars (g 100 g <sup>-1</sup> ) | Total Sugars (g 100 g <sup>-1</sup> ) | F/G ratio              |
|--------|--------------|------------------|-----------------------------------|----------------------------------|----------------------------------|----------------------------------|------------------------------------|--|---------------------------------------|------------------------|
| 1      | Eucalyptus   | Hassanabdal      | 35.71±0.02 <sup>e</sup>           | 30.79±0.01 <sup>d</sup>          | 7.23±0.02 <sup>a</sup>           | 3.15±0.03 <sup>b</sup>           | 0.111±0.006 <sup>c</sup>           | 66.50±0.02 <sup>g</sup>                  | 76.99±0.08 <sup>b</sup>               | 1.16±0.00 <sup>d</sup> |
| 2      | Sunflower    | DG Khan          | 37.13±0.05 <sup>b</sup>           | 32.93±0.02 <sup>a</sup>          | 2.52±0.02 <sup>g</sup>           | 1.47±0.03 <sup>f</sup>           | 0.043±0.002 <sup>f</sup>           | 70.06±0.07 <sup>b</sup>                  | 74.09±0.04 <sup>d</sup>               | 1.13±0.00 <sup>e</sup> |
| 3      | Acacia       | Rawalpindi       | 35.62±0.03 <sup>e</sup>           | 32.82±0.08 <sup>a</sup>          | 1.12±0.04 <sup>i</sup>           | 2.93±0.05 <sup>c</sup>           | 0.160±0.010 <sup>b</sup>           | 68.44±0.05 <sup>d</sup>                  | 72.65±0.13 <sup>f</sup>               | 1.08±0.01 <sup>f</sup> |
| 4      | Mustard      | Lillah           | 34.90±0.02 <sup>f</sup>           | 32.31±0.03 <sup>b</sup>          | 4.36±0.01 <sup>e</sup>           | 0.62±0.08 <sup>h</sup>           | N.D.*                              | 67.21±0.03 <sup>f</sup>                  | 72.19±0.05 <sup>g</sup>               | 1.08±0.00 <sup>f</sup> |
| 5      | Jujube       | Lillah           | 33.65±0.08 <sup>g</sup>           | 24.75±0.06 <sup>f</sup>          | 2.76±0.03 <sup>f</sup>           | 2.08±0.04 <sup>d</sup>           | 0.064±0.002 <sup>de</sup>          | 58.41±0.04 <sup>i</sup>                  | 63.30±0.09 <sup>h</sup>               | 1.36±0.01 <sup>a</sup> |
| 6      | Clover       | Narang Mandi     | 38.03±0.03 <sup>a</sup>           | 32.24±0.02 <sup>b</sup>          | 6.15±0.02 <sup>c</sup>           | 1.04±0.06 <sup>g</sup>           | 0.021±0.002 <sup>g</sup>           | 70.27±0.05 <sup>a</sup>                  | 77.48±0.03 <sup>a</sup>               | 1.18±0.00 <sup>c</sup> |
| 7      | Citrus       | Salam Sargodha   | 36.63±0.03 <sup>d</sup>           | 31.04±0.02 <sup>c</sup>          | 1.41±0.03 <sup>h</sup>           | 3.39±0.11 <sup>a</sup>           | 0.406±0.015 <sup>a</sup>           | 67.67±0.01 <sup>e</sup>                  | 72.88±0.06 <sup>f</sup>               | 1.18±0.00 <sup>c</sup> |
| 8      | Currant bush | Salgirran Murree | 36.55±0.01 <sup>d</sup>           | 29.45±0.03 <sup>e</sup>          | 6.41±0.01 <sup>b</sup>           | 1.17±0.04 <sup>g</sup>           | 0.084±0.002 <sup>d</sup>           | 66.00±0.02 <sup>h</sup>                  | 73.66±0.14 <sup>e</sup>               | 1.24±0.00 <sup>b</sup> |
| 9      | Multifloral  | Rawalpindi       | 36.93±0.03 <sup>c</sup>           | 32.35±0.04 <sup>b</sup>          | 5.18±0.01 <sup>d</sup>           | 1.69±0.05 <sup>e</sup>           | 0.052±0.007 <sup>ef</sup>          | 69.28±0.04 <sup>c</sup>                  | 76.20±0.07 <sup>c</sup>               | 1.14±0.00 <sup>e</sup> |

\* Not Detected ; Limit of detection (LOD) = 1.2 mg 100 g<sup>-1</sup>. All analyses were performed in triplicate, and the mean value ± standard deviation (SD) are reported. Mean values in the same column but with different superscript letters vary significantly (P > 0.05).

**Table 4:** Sugar content (expressed as a percentage) of blossom honeys taken from the Khyber Pakhtunkhwa province of Pakistan.

| S. No. | Honey Type | Location  | Fructose (g 100 g <sup>-1</sup> ) | Glucose (g 100 g <sup>-1</sup> ) | Sucrose (g 100 g <sup>-1</sup> ) | Maltose (g 100 g <sup>-1</sup> ) | Raffinose (g 100 g <sup>-1</sup> ) | Reducing Sugars (g 100 g <sup>-1</sup> ) | Total Sugars (g 100 g <sup>-1</sup> ) | F/G ratio              |
|--------|------------|-----------|-----------------------------------|----------------------------------|----------------------------------|----------------------------------|------------------------------------|--|---------------------------------------|------------------------|
| 1      | Loquat     | Harripur  | 36.40±0.02 <sup>b</sup>           | 31.30±0.02 <sup>d</sup>          | 4.10±0.02 <sup>c</sup>           | 3.67±0.06 <sup>a</sup>           | 0.097±0.003 <sup>b</sup>           | 67.70±0.03 <sup>b</sup>                  | 75.57±0.07 <sup>b</sup>               | 1.16±0.00 <sup>b</sup> |
| 2      | Eucalyptus | Mardan    | 37.03±0.06 <sup>a</sup>           | 32.86±0.01 <sup>a</sup>          | 9.71±0.01 <sup>a</sup>           | 2.06±0.05 <sup>d</sup>           | 0.082±0.002 <sup>cd</sup>          | 69.89±0.07 <sup>a</sup>                  | 81.74±0.05 <sup>a</sup>               | 1.13±0.00 <sup>c</sup> |
| 3      | Jujube     | Noushehra | 32.45±0.04 <sup>f</sup>           | 25.03±0.06 <sup>f</sup>          | 4.36±0.02 <sup>b</sup>           | 1.74±0.07 <sup>e</sup>           | 0.093±0.002 <sup>bc</sup>          | 57.48±0.06 <sup>f</sup>                  | 63.67±0.08 <sup>f</sup>               | 1.30±0.01 <sup>a</sup> |
| 4      | Acacia     | Noushehra | 34.74±0.01 <sup>d</sup>           | 32.72±0.04 <sup>b</sup>          | 1.97±0.06 <sup>e</sup>           | 2.43±0.05 <sup>c</sup>           | 0.072±0.004 <sup>d</sup>           | 67.46±0.05 <sup>c</sup>                  | 71.93±0.04 <sup>d</sup>               | 1.06±0.00 <sup>e</sup> |
| 5      | Citrus     | Swat      | 35.51±0.03 <sup>e</sup>           | 31.58±0.01 <sup>c</sup>          | 4.01±0.02 <sup>d</sup>           | 2.82±0.07 <sup>b</sup>           | 0.236±0.012 <sup>a</sup>           | 67.09±0.02 <sup>d</sup>                  | 74.16±0.11 <sup>c</sup>               | 1.12±0.01 <sup>c</sup> |
| 6      | Clover     | Mardan    | 32.99±0.03 <sup>e</sup>           | 29.92±0.02 <sup>e</sup>          | 3.97±0.01 <sup>d</sup>           | 0.96±0.08 <sup>f</sup>           | 0.050±0.002 <sup>e</sup>           | 62.81±0.11 <sup>e</sup>                  | 67.89±0.08 <sup>e</sup>               | 1.10±0.00 <sup>d</sup> |

All analyses were performed in triplicate, and the mean ± standard deviations (SD) are reported. Mean values in the same column but with different superscript letters vary significantly (P > 0.05).

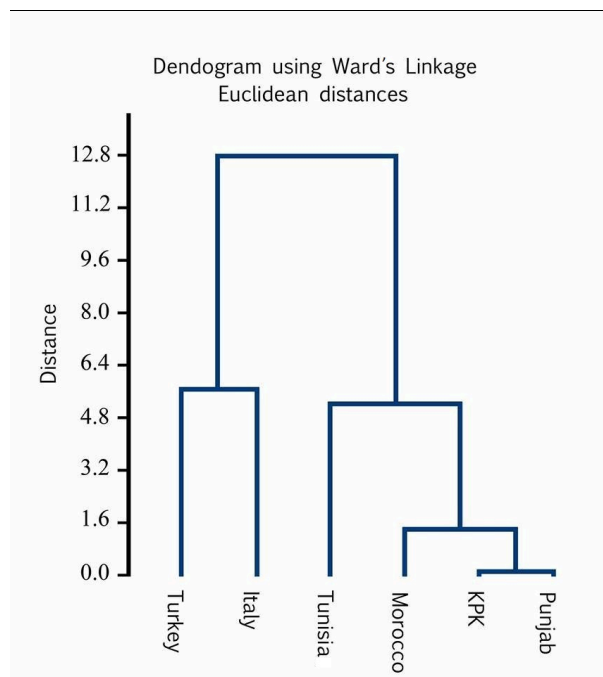
Honey freshness is widely judged based on its HMF content (BACANDRITSOS *et al.*, 2006; CORBELLA and COZZOLINO, 2006) as fresh honeys are mainly deprived of this compound therefore, HMF content might increase during honey processing and/or aging (HABIB *et al.*, 2014). The HMF values of the honey samples ranged from 4.71 to 37.25 mg kg<sup>-1</sup>. The lowest HMF values were in currant bush and acacia honeys, and the highest HMF values were observed in mustard and eucalyptus honeys (Tables 1 and 2). The HMF values of the honey samples in this study corroborated those of MAIRAJ *et al.* (2008), AKHTER *et al.* (2010), FEÁS *et al.* (2010 a), and FEÁS *et al.* (2010 b). Pakistan honey samples presented a substantial variation in average HMF from other Mediterranean honeys. Italian honeys (TRUZZI *et al.*, 2014) had the minimum average HMF while the Tunisian honeys (BOUSSAID *et al.*, 2014) had maximum values (Table 5). HMF values of Punjab honeys were statistically similar to Turkish honeys (ÖZCAN and ÖLMEZ, 2014). Many factors, including temperature and heat time as well as pH, storage environment, and floral origin, influence the levels of HMF in honey samples; therefore, HMF indicates overheating and poor storage conditions (FALLICO *et al.*, 2006). Long-term heat treatments of honey samples inactivate its natural enzymes, and increased HMF occurs due to fructose degradation (MAIRAJ *et al.*, 2008). All of the analyzed samples from both provinces of Pakistan showed HMF values within the acceptable range (< 80 mg kg<sup>-1</sup> for tropical or arid regions) according to the international quality regulations of the Codex Alimentarius Commission (2001).

Honey is primarily composed of sugars (RODRÍGUEZ *et al.*, 2014). The monosaccharides glucose and fructose are important components of honey while fructose is always the primary sugar, followed by glucose (HABIB *et al.*, 2014). Fructose and glucose were the major sugars in all of the tested honey samples. The amounts of fructose and glucose in the honey samples of both provinces ranged from 32.45 to 38.03 g 100 g<sup>-1</sup> and from 24.75 to 32.93 g 100 g<sup>-1</sup>, respectively, whereas other minor sugars containing sucrose, maltose and raffinose ranged from 1.12 to 9.71 g 100 g<sup>-1</sup>, 0.62 to 3.67 g 100 g<sup>-1</sup> and 0.021 to 0.406 g 100 g<sup>-1</sup>, respectively (Tables 3 and 4). Raffinose was not detected in the mustard honey sample from Punjab. The total sugar content of the honey samples ranged from 63.30 to 81.74 g 100 g<sup>-1</sup>. The fructose/glucose (F/G) ratio and the reducing sugars (fructose + glucose) of the honey samples ranged from 1.06 to 1.36 and from 57.48 to 70.27 g 100 g<sup>-1</sup>, respectively (Tables 3 and 4). Because glucose is comparatively less soluble in water than fructose, the F/G ratio can most likely be used to evaluate the granulation of honey (ANKLAM, 1998). The results of the current study were in line with the international quality regulations of the Codex Alimentarius Commission (2001). The reducing sugars of all of the tested honey samples were higher than 60%, except for the jujube honeys of both provinces. The comparison of examined Pakistani honeys with the Moroccan and Tunisian honeys (based on available data) revealed some variations. The fructose, glucose, sucrose and maltose contents were statistically different in compared honeys (Table 5). The average sucrose content of the examined Pakistani honeys slightly higher than Moroccan and Tunisian honeys but overall within the permissible limits (Codex Alimentarius Commission, 2001); however, the clover and currant bush honeys from Punjab were slightly high (Table 3). Early honey harvesting might explain the high sucrose content (AZEREDO *et al.*, 2003). Cluster analysis (Ward's Method- Euclidean distances) (Fig. 2) data categorized honeys into two main groups. The first group consisted of Turkish and Italian honeys and the second was comprised of Pakistani (Punjab and KPK), Moroccan and Tunisian honeys. It is interesting to note that in the second group, Pakistani honeys were quite similar even though these belonged to different provinces (Punjab and KPK). In second group, Moroccan honeys were close to Pakistani honeys while the Tunisian honeys were away from the remaining group members. Turkish and Italian honeys fell in first group and were close to each other.

**Table 5:** Comparison of physico-chemical properties of honeys from Pakistan (I & II) and some Mediterranean countries (III-VI).

| Honey origin  | Moisture Content (%)     | Free Acidity (mEq kg <sup>-1</sup> ) | pH                      | EC (μS cm <sup>-1</sup> ) | Diastase Activity (DN)   | Total Acidity (mEq kg <sup>-1</sup> ) | Ash (%)                | HMF (mg kg <sup>-1</sup> ) | Fructose (g 100 g <sup>-1</sup> ) | Glucose (g 100 g <sup>-1</sup> ) | Sucrose (g 100 g <sup>-1</sup> ) | Maltose (g 100 g <sup>-1</sup> ) |
|---|--------------------------|--------------------------------------|-------------------------|---------------------------|--------------------------|---------------------------------------|------------------------|----------------------------|-----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| I<br>Punjab, n=9<br>(Mean values of present study)    | 18.24±0.12 <sup>ab</sup> | 14.36±0.54 <sup>c</sup>              | 4.38±0.02 <sup>b</sup>  | 414.83±0.67 <sup>c</sup>  | 10.56±0.43 <sup>b</sup>  | 28.08±0.86 <sup>bc</sup>              | 0.15±0.01 <sup>c</sup> | 17.63±0.44 <sup>c</sup>    | 36.13±0.03 <sup>c</sup>           | 30.96±0.03 <sup>b</sup>          | 4.13±0.02 <sup>b</sup>           | 1.95±0.05 <sup>d</sup>           |
| II<br>KPK, n=6<br>(Mean values of present study)      | 17.94±0.07 <sup>ab</sup> | 12.67±0.88 <sup>c</sup>              | 4.15±0.02 <sup>c</sup>  | 413.98±1.17 <sup>c</sup>  | 10.55±0.44 <sup>b</sup>  | 25.51±2.24 <sup>cd</sup>              | 0.15±0.00 <sup>c</sup> | 13.15±0.32 <sup>d</sup>    | 34.85±0.03 <sup>d</sup>           | 30.57±0.02 <sup>c</sup>          | 4.69±0.03 <sup>a</sup>           | 2.28±0.06 <sup>c</sup>           |
| III<br>Morocco, n=17<br>(AAZZA <i>et al.</i> , 2014)  | 18.97±0.76 <sup>a</sup>  | 22.93±2.73 <sup>a</sup>              | 3.94±0.06 <sup>d</sup>  | 500.71±1.66 <sup>b</sup>  | 12.42±1.01 <sup>ab</sup> | 29.66±1.16 <sup>b</sup>               | 0.36±0.01 <sup>a</sup> | 44.80±0.78 <sup>a</sup>    | 38.47±0.04 <sup>a</sup>           | 30.76±0.05 <sup>bc</sup>         | 0.13±0.01 <sup>d</sup>           | 3.47±0.05 <sup>a</sup>           |
| IV<br>Tunisia, n=6<br>(BOUSSAID <i>et al.</i> , 2014) | 18.71±0.47 <sup>a</sup>  | N.A.                                 | 3.86±0.07 <sup>d</sup>  | 548.33±1.80 <sup>a</sup>  | N.A.                     | 22.59±0.92 <sup>d</sup>               | 0.26±0.02 <sup>b</sup> | 20.01±0.37 <sup>b</sup>    | 36.93±0.10 <sup>b</sup>           | 33.49±0.19 <sup>a</sup>          | 1.89±0.07 <sup>c</sup>           | 2.64±0.03 <sup>b</sup>           |
| V<br>Italy, n=43<br>(TRUZZI <i>et al.</i> , 2014)     | 17.01±0.80 <sup>b</sup>  | 18.16±1.01 <sup>b</sup>              | 4.55±0.05 <sup>a</sup>  | 87.00±0.17 <sup>d</sup>   | N.A.                     | 21.56±0.82 <sup>d</sup>               | N.A.                   | 1.67±0.21 <sup>e</sup>     | N.A.                              | N.A.                             | N.A.                             | N.A.                             |
| VI<br>Turkey, n=8<br>(ÖZCAN and ÖLMEZ, 2014)          | 18.30±0.26 <sup>ab</sup> | N.A.                                 | 4.24±0.07 <sup>bc</sup> | N.A.                      | 13.78±1.43 <sup>a</sup>  | 34.93±1.65 <sup>a</sup>               | 0.05±0.01 <sup>d</sup> | 18.36±0.50 <sup>c</sup>    | N.A.                              | N.A.                             | N.A.                             | N.A.                             |

The data are mean values ± standard deviation (SD). Mean values in the same column, but with different superscript letters differ significantly ( $P > 0.05$ ). NA: not available.



**Figure 2:** Dendrogram (Ward's Method- Euclidean distances) showing the comparison of honeys from different countries.

These observations indicated the key role of climate, floral origin and soil properties of a particular region that determine the physicochemical characteristics of the honey.

#### 4. CONCLUSIONS

The present study characterized nine Pakistani blossom honey samples from Punjab and six from KPK. A comparison was also made to some Mediterranean honeys. The results of this study allow us to assess the quality of Pakistani honeys and help to establish certain standards. Based on the studied quality parameters (i.e., moisture,  $a_w$ , acidity, pH, electric conductivity, diastase activity, ash, HMF, and sugar content) of the different honey samples from both provinces, Pakistani honeys meet international standards. However, the slightly higher sucrose content of certain honey samples and the lower values of reducing sugars in jujube honey indicate early harvesting by beekeepers.

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