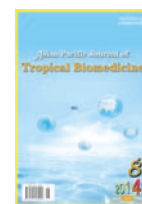


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Physico–chemical analysis and antimicrobial potential of *Apis dorsata*, *Apis mellifera* and *Ziziphus jujube* honey samples from Pakistan

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PEER REVIEW

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Comments

It is an ethnopharmacological research on a locally available honey samples from Pakistan. New data on the properties of studied samples can be seen in the present report. Implication on microbiology is interesting and can be further useful. Further referencing on this work is possible.

Details on Page 640

ABSTRACT

Objective: To evaluate physico–chemical properties and antimicrobial potential of indigenous honey samples against different reference strains including *Escherichia coli* ATCC 8739, *Enterobacter aerogenes* ATCC 13048, *Pseudomonas aeruginosa* ATCC 9027, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Salmonella typhi* ATCC 14028, *Klebsiella pneumoniae* ATCC 13883, *Aspergillus niger* ATCC 16404, *Rhizopus oligosporus* PCSIR1, *Candida albicans* ATCC 14053 and *Candida utilis* ATCC 9950.

Methods: By using standard methods samples were evaluated for their antimicrobial properties including additive effect of starch and non–peroxidase activity, antioxidative properties (phenol contents, flavonoid contents, 1,1–diphenyl–2–picrylhydrazyl radical scavenging activity). Prior to this evaluation, complete physico–chemical properties including pH, color, ash contents, protein contents, moisture contents, hydroxymethyl furfural contents, total sugar contents, reducing sugar and non–reducing sugar contents were analyzed.

Results: Relatively higher ash contents were found in the Siddar honey *i.e.* (0.5900±0.0336)% and small honey showed relatively higher protein contents *i.e.* (777.598±9.880) mg/kg. The moisture contents of tested honey samples ranged between 13.8%–16.6%, total sugar contents from 61.672%–72.420% and non–reducing sugar contents from 1.95%–3.93%. Presences of phenolic contents indicate higher antioxidant potential of these honey samples. All bacteria showed clear inhibition zones in response to tested honey samples whereas fungi and yeast showed inhibition at higher concentrations of these honey samples. For *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhi*, *Pseudomonas aeruginosa* and *Aspergillus niger*, overall the small honey showed the higher activity than other honey samples.

Conclusion: Physico–chemical analysis of honey samples confirmed good quality of honey according to the standards set by European Union Commission and Codex Alimentarius Commission. Evaluation of these honey samples confirms antimicrobial potential of particular types of honeys indigenous to Pakistan.

KEYWORDS

Ziziphus jujube honey, Antimicrobial properties, Non–peroxidase activity, Antioxidative properties

1. Introduction

Honey is a heterogeneous mixture of proteins, flower nectar sugars and glandular secretions produced by honey bees[1]. Honey contains significant antioxidant

contents including glucoseoxidase, catalase, ascorbic acid, flavonoids, phenolic acids, carotenoid derivatives, organic acids, Maillard reaction products, amino acids and proteins[2–4]. Generally, the darker the honey is, the higher its phenolic content and its antioxidant power

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is contained^[5,6]. According to a study honey increases antioxidant agents, vitamin C concentration by 47%, β -carotene by 3%, uric acid by 12% and glutathione reductase by 7% in human body^[7]. Antioxidant activity depends on the botanical origin of honey and shows variations in different honeys acquired from different sources^[4,6,8].

Furthermore, previous studies showed that honey had remarkable antimicrobial activity against fungi, bacteria, viruses and protozoa^[9]. This activity of honey has been reported against dermatophytes, some yeast, *Aspergillus* spp. and *Penicillium* spp.^[9]. Moreover, it has been shown that honey has inhibitory effects on rubella virus, herpes virus and three species of the *Leishmania* parasite^[9,10]. Antimicrobial activity of honey is linked to hydrogen peroxide which is produced by glucose oxidase especially when honey is diluted. In addition, hydrogen peroxide has antibacterial activity and at the same time it is not tissue damaging^[11].

In the diluted form of honey, produced hydrogen peroxide is an important stimulant of the growth of tissues and has the potential for wound healing^[12]. The hydrogen peroxide activity of most of the honeys can be destroyed by heat or by the presence of catalase. However, some honeys retain their antimicrobial activity even in the presence of catalase which are known as “non-peroxidase honeys”^[9]. This activity is important especially in the context of topical antimicrobial and wound dressings fluids^[13].

Recently some progress has been made regarding the treatment of infections caused by methicillin-sensitive *Staphylococcus aureus*. However, due to different methodologies and variety of honey samples used data are inconsistent, which suggests the need to further evaluate potency of different honey samples. To our understanding, little is known about the antimicrobial potential of a particular type of honeys indigenous to Pakistan. This study focused on the analysis of different honey samples. Prior to antimicrobial activity testing, the physico-chemical properties (such as pH, color, ash contents, protein contents, moisture contents, hydroxymethyl furfural contents, total sugar contents, reducing sugar and non-reducing sugar contents), antimicrobial properties (such as additive effect of starch and non-peroxidase activity) and antioxidative properties (such as phenol contents, flavonoid contents and 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity) of various honey samples were analyzed to assess the quality of these honey samples.

2. Materials and methods

2.1. Honey samples

Three different types of honey samples including *Apis*

dorsata (locally called big honey), *Apis mellifera* (small honey), and *Ziziphus jujube* (Siddar Honey) were collected from a local honey centre in Lahore, Pakistan and the experimental work was conducted in PCSIR laboratories Lahore, Pakistan.

2.2. Physico-chemical analysis of honey

The pH was determined with a digital pH meter by using the method described by the Association of Official Analytical Chemist^[14]. The color of the honey samples was determined by spectrophotometric measurement of the absorbance of 50% (w/v) honey solution at 635 nm according to the method of White^[15]. The color of honey samples were classified according to the Pfund scale after conversion of the absorbance values.

2.3. Ash, moisture and total protein content measurements

Total ash contents were measured by incinerating honey samples in a muffle furnace at a temperature of 550 °C according to AOAC Official Method^[16]. The total protein content was determined spectrophotometrically by using Bovine Serum Albumin (BSA) standard curve according to Lowry's method (1951). The moisture content was determined by using refractometric method given in the harmonised methods of the European Honey Commission^[17]. The refractive index values were further corrected for a standard temperature of 20 °C by adding the correction factor of 0.000 23/°C. The moisture values corresponding to the corrected refractive indices values were calculated using the Chataway Table^[18].

2.4. Effect of heat treatment on hydroxymethyl furfural (HMF) production

The HMF contents were estimated spectrophotometrically by using the method of White at different temperatures (60 °C, 70 °C and 80 °C)^[19].

2.5. Estimation of total sugar and reducing sugar content

Total sugar content was determined spectrophotometrically according to previously described method of Dubois *et al.* by using sucrose to plot calibration curve^[20]. The estimation of reducing sugar content was done spectrophotometrically according to dinitrosalicylic acid method proposed by Miller^[21]. Glucose was used as a standard for preparing the calibration curve. The amount of non-reducing sugars, such as sucrose content, was measured by subtracting the reducing sugar content from total sugar content as previously described^[22].

2.6. Estimation of total phenol and flavonoid contents

The total phenol contents were determined spectrophotometrically by using Folin–Ciocalteu reagent according to the method of Singleton *et al.*[23]. Vanillin was used as a standard for preparing the calibration curve. The blue complex was formed by the reduction of the Folin–Ciocalteu reagent by phenolic compounds present in honey. The total flavonoid contents were determined spectrophotometrically according to the method of Zhishen *et al.*[24]. Catechin was used as a standard for preparing the calibration curve.

2.7. Measurement of DPPH radical scavenging activity and non-peroxidase activity

The scavenging activity of honey samples for the radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was measured as described by Velazquez *et al.*[25]. The non-peroxidase antimicrobial activity of honey was determined spectrophotometrically by using 0.2% (w/v) catalase solution according to the method of Sherlock *et al.*[26], using 96-well microtitre plate. The minimum bactericidal concentration (MBC) was determined by sub-culturing a loopful of the culture media on sterilized nutrient agar plate (from each test well that showed no apparent growth). After incubation, the MBC was read as the least concentration showing no growth on the nutrient agar plates. After that, 0.2% (w/v) catalase solution was added in each honey dilution and it was incubated in dark at room temperature for 24 h. Honey dilution containing catalase was mixed with microbial culture. Control wells include negative control (wells containing nutrient broth), positive control (wells containing inoculums and nutrient broth) and the corresponding negative control (wells containing honey dilution and nutrient broth). After incubation (2 d at 37 °C for bacteria and 5 d at 25 °C for yeast and fungal strains), growth was observed by visual inspection and by measuring the optical density at 620 nm with the help of plate reader (Asys, VUM-340). The MBC was determined by sub-culturing a loopful of the culture media on sterilized nutrient agar plate (from each test well that showed no apparent growth). After incubation, the MBC was read as the least concentration showing no growth on the nutrient agar plates.

2.8. Antimicrobial activity of honey

For well diffusion assay, different honey concentrations against selected microbes were used according to the method of Al-Somal *et al.*[11]. The honey samples were tested at 20 different concentrations ranging from 5% to 100% (v/v). Sterilized agar media was inoculated with 24 h old microbial culture and was poured in sterilized plates. After solidification, wells were made by using sterilized borer. Honey dilutions were poured in each well aseptically. After incubation, zones of inhibition were noted in millimeter with the help of a scale. The synergistic effect of starch on the antimicrobial activity of honey was performed spectrophotometrically by using 10% (w/v) starch solution.

3. Results

3.1. Estimation of pH, colour ash, protein moisture and sugar content

The pH of tested honey samples remained 3.14 to 4.19 throughout this study. As is shown in Table 1 that the Siddar honey had the highest pH *i.e.* 4.190±0.707 in comparison to other honey samples. The color of the tested honey samples falls between amber to dark amber, particularly Siddar honey showed the dark amber color (Table 1). The total ash contents in tested honey samples ranged between 0.411%–0.590%. The highest ash contents were found in the Siddar honey *i.e.* (0.5900±0.0336)%. The total protein contents in three types of honey samples used in this study ranged from 571.272 to 777.598 mg/kg (Table 1). The small honey had the highest protein contents *i.e.* (777.598±9.880) mg/kg. However lowest protein contents were found in the big honey *i.e.* (571.272±6.020) mg/kg. The moisture contents in tested honey samples ranged from 13.800%–16.600% (Table 1). The highest moisture contents were found in the Siddar honey *i.e.* (16.600%±0.283)%. It was observed that the HMF contents increases with the increase in temperature (Table 2). The range of HMF contents in unheated honey samples was from 27.69–36.08 mg/kg. The highest HMF contents were found in the small honey *i.e.* (36.08±1.06) mg/kg. It was observed that when the temperature was increased, the HMF contents were also increased (Table 2).

Table 1

Physico-chemical characteristics of three different types of honey samples (mean±SD).

| Physico-chemical parameters | pH | Color (mm) | Ash contents (%) | Total protein (mg/kg) | Moisture contents (%) | Total sugar (%) | Reducing sugar (%) | Non-reducing sugar (%) |
|-----------------------------|-------------|---------------|------------------|-----------------------|-----------------------|-----------------|--------------------|------------------------|
| Small honey | 3.140±0.424 | 109.260±2.626 | 0.4970±0.0515 | 777.598±9.880 | 13.800±0.283 | 72.420±1.486 | 70.467±1.458 | 1.950±0.028 |
| Siddar honey | 4.190±0.707 | 128.950±0.262 | 0.5900±0.0336 | 764.532±8.590 | 16.600±0.283 | 61.672±1.284 | 57.748±1.944 | 3.930±0.658 |
| Big honey | 3.440±0.566 | 94.410±2.626 | 0.4110±0.0374 | 571.272±6.020 | 14.200±0.566 | 67.357±0.811 | 64.967±1.458 | 2.390±0.636 |

The total sugar contents range from 61.672%–72.420% (Table 1). In this study, the highest sugar contents were found in the small honey *i.e.* (72.420±1.486)%. The reducing sugar contents ranges from 57.748%–70.467% (Table 1). Regarding reducing sugars (fructose and glucose), EU Directive[27], imposes values $\geq 60\%$ and according to the obtained results, only one sample (Siddar honey) showed a value that was lower (57.748%) in comparison to the standard value set for reducing sugars by EU Directive[27]. The non-reducing sugar contents were found to be in the range of 1.950%–3.930% (Table 1). Finally, the highest non-reducing sugar contents were found in the Siddar honey *i.e.* (3.930±0.658)%.

Table 2

Effect of temperature on HMF production in different honey samples.

| Temperature | Heating time (min) | HMF formed (mg/kg) (mean±SD) | | |
|-------------|--------------------|------------------------------|--------------|------------|
| | | Small honey | Siddar honey | Big honey |
| Control | 0 | 36.08±1.06 | 27.69±1.69 | 34.81±0.74 |
| | 1 | 41.92±1.03 | 29.94±0.98 | 35.93±0.69 |
| | 3 | 44.31±1.14 | 35.93±0.85 | 41.92±0.17 |
| 60 °C | 5 | 49.10±0.96 | 41.92±0.36 | 47.90±1.66 |
| | 1 | 62.28±0.36 | 53.29±1.68 | 59.88±0.98 |
| | 3 | 68.86±0.11 | 56.89±2.14 | 65.87±3.14 |
| 70 °C | 5 | 70.66±0.29 | 59.28±2.88 | 67.66±2.55 |
| | 1 | 97.60±0.74 | 71.86±1.98 | 83.83±0.93 |
| | 3 | 101.20±1.99 | 77.84±0.84 | 89.82±0.15 |
| 80 °C | 5 | 110.18±1.25 | 84.83±0.66 | 93.41±3.09 |

3.2. Phenolic and flavonoid content and DPPH radical scavenging activity

The total phenolic contents in all honey samples were determined and it was observed that the total phenolic contents in our tested honey samples range from 460.260 to 540.720 mg/kg (Table 3). The highest phenolic contents were present in the Siddar honey *i.e.* (540.720±3.035) mg/kg. Similarly, the total flavonoid contents were observed in the range of 46.519–64.396 mg_{catechin}/kg (Table 3). The highest flavonoid content was found in the Siddar honey *i.e.* (64.396±

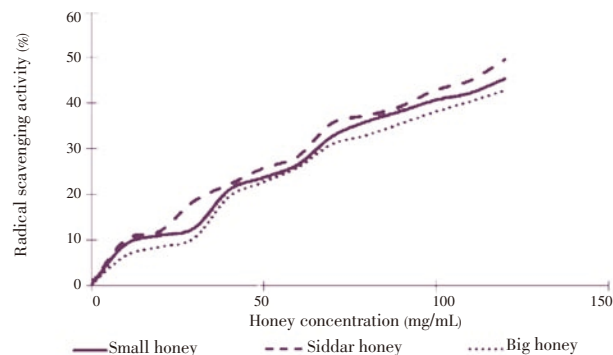
3.534) mg/kg. The DPPH radical scavenging activity of honey at different concentrations was studied and it was observed that the Siddar honey has slightly higher radical scavenging activity as compared to other two honey samples (Figure 1).

Table 3

Total phenol and flavonoid contents in some Pakistani honey samples.

| Antioxidant properties | Total phenol contents (mg _{vanillin} /kg) | Total flavonoid contents (mg _{catechin} /kg) |
|------------------------|--|---|
| | Small honey | 484.930±5.310 |
| Siddar honey | 540.720±3.035 | 64.396±3.534 |
| Big honey | 460.260±4.552 | 46.519±3.806 |

The values in table is Mean±SD.

**Figure 1.** DPPH radical scavenging activity of different honey samples.

3.3. Antimicrobial activity of honey

The well diffusion assay was performed to measure the zones of inhibition produced by using different concentrations of honey samples against various pathogenic microorganisms and results are shown in Figure 2. It was observed that all bacteria showed clear inhibition zones in response to all honey samples whereas fungi and yeast showed inhibition at higher concentrations of honey. It is shown in Figure 2 that for *Escherichia coli* (*E. coli*), *Bacillus subtilis* (*B. subtilis*), *Salmonella typhi* (*S. typhi*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Aspergillus niger* (*A. niger*),

Table 4

Visual MIC, spectrophotometric MIC₉₅ and MCB values of different Pakistani honey samples with and without adding starch in them.

| Microorganisms | Small honey | | | | | | Siddar honey | | | | | | Big honey | | | | | | |
|-------------------------------------|----------------|--------|-----------------------|--------|----|--------|----------------|--------|-----------------------|--------|----|--------|----------------|--------|-----------------------|--------|----|--------|--|
| | Visual MIC (%) | | Spectrophotometric | | | | Visual MIC (%) | | Spectrophotometric | | | | Visual MIC (%) | | Spectrophotometric | | | | |
| | | | MIC ₉₅ (%) | | | | | | MIC ₉₅ (%) | | | | | | MIC ₉₅ (%) | | | | |
| | No | With | No | With | No | With | No | With | No | With | No | With | No | With | No | With | No | With | |
| strach | | starch | | strach | | starch | | strach | | starch | | strach | | starch | | strach | | starch | |
| <i>E. coli</i> [*] | 35 | 15 | 60 | 30 | 70 | 55 | 40 | 20 | 55 | 40 | 70 | 55 | 35 | 25 | 60 | 35 | 70 | 55 | |
| <i>E. aerogenes</i> [*] | 35 | 15 | 55 | 35 | 70 | 50 | 30 | 15 | 50 | 35 | 60 | 50 | 35 | 20 | 50 | 40 | 65 | 55 | |
| <i>B. subtilis</i> [*] | 55 | 35 | 70 | 50 | 80 | 65 | 65 | 45 | 70 | 55 | 80 | 70 | 50 | 35 | 70 | 55 | 75 | 65 | |
| <i>S. aureus</i> [*] | 60 | 40 | 75 | 55 | 80 | 70 | 50 | 35 | 70 | 50 | 85 | 70 | 55 | 40 | 75 | 60 | 85 | 70 | |
| <i>K. pneumoniae</i> [*] | 40 | 20 | 55 | 30 | 70 | 40 | 35 | 20 | 55 | 35 | 65 | 55 | 25 | 15 | 50 | 30 | 70 | 45 | |
| <i>S. typhi</i> [*] | 35 | 25 | 50 | 30 | 65 | 45 | 40 | 25 | 60 | 30 | 75 | 50 | 40 | 20 | 55 | 40 | 65 | 55 | |
| <i>P. aeruginosa</i> [*] | 40 | 25 | 55 | 35 | 65 | 50 | 45 | 25 | 55 | 35 | 75 | 55 | 40 | 25 | 55 | 35 | 75 | 50 | |
| <i>C. albicans</i> ^{**} | 70 | 55 | 85 | 70 | 90 | 80 | 75 | 60 | 80 | 70 | 85 | 75 | 80 | 70 | 85 | 75 | 90 | 85 | |
| <i>C. utilis</i> ^{**} | 80 | 65 | 90 | 75 | 95 | 85 | 80 | 65 | 90 | 75 | 90 | 85 | 85 | 65 | 90 | 70 | 95 | 80 | |
| <i>R. oligosporus</i> ^{**} | 80 | 70 | 85 | 75 | 90 | 80 | 75 | 60 | 80 | 70 | 85 | 80 | 80 | 65 | 85 | 75 | 90 | 80 | |
| <i>A. niger</i> ^{**} | 80 | 60 | 85 | 75 | 90 | 80 | 80 | 70 | 85 | 75 | 90 | 85 | 85 | 70 | 90 | 75 | 95 | 85 | |

^{*}: Incubation period, 24 h; Incubation temperature, 37 °C. ^{**}: Incubation period, 96 h; Incubation temperature, 25 °C.

Table 5

Visual MIC, spectrophotometric MIC₉₅ and MCB values of different Pakistani honey samples with and without adding catalase in them.

| Microorganisms | Small honey | | | | | | Siddar honey | | | | | | Big honey | | | | | |
|--------------------------|----------------|----------|--|----------|----------|----------|----------------|----------|--|----------|----------|----------|----------------|----------|--|----------|----------|----------|
| | Visual MIC (%) | | Spectrophotometric MIC ₉₅ (%) | | MBC (%) | | Visual MIC (%) | | Spectrophotometric MIC ₉₅ (%) | | MBC (%) | | Visual MIC (%) | | Spectrophotometric MIC ₉₅ (%) | | MBC (%) | |
| | No | With | No | With | No | With | No | With | No | With | No | With | No | With | No | With | No | With |
| | catalase | catalase | catalase | catalase | catalase | catalase | catalase | catalase | catalase | catalase | catalase | catalase | catalase | catalase | catalase | catalase | catalase | catalase |
| <i>E. coli</i> * | 35 | 60 | 60 | 80 | 70 | 90 | 40 | 60 | 55 | 70 | 70 | 85 | 35 | 65 | 60 | 75 | 70 | 85 |
| <i>E. aerogenes</i> * | 35 | 55 | 55 | 75 | 70 | 85 | 30 | 55 | 50 | 70 | 60 | 80 | 35 | 55 | 50 | 65 | 65 | 80 |
| <i>B. subtilis</i> * | 55 | 70 | 70 | 90 | 80 | 95 | 65 | 85 | 70 | 85 | 80 | 95 | 50 | 75 | 70 | 85 | 75 | 90 |
| <i>S. aureus</i> * | 60 | 75 | 75 | 85 | 80 | 95 | 50 | 75 | 70 | 90 | 85 | 95 | 55 | 80 | 75 | 90 | 85 | 95 |
| <i>K. pneumoniae</i> * | 40 | 60 | 55 | 70 | 70 | 80 | 35 | 60 | 55 | 70 | 65 | 85 | 25 | 50 | 50 | 70 | 70 | 80 |
| <i>S. typhi</i> * | 35 | 50 | 50 | 70 | 65 | 85 | 40 | 60 | 60 | 75 | 75 | 90 | 40 | 55 | 55 | 75 | 65 | 85 |
| <i>P. aeruginosa</i> * | 40 | 55 | 55 | 75 | 65 | 90 | 45 | 65 | 55 | 70 | 75 | 90 | 40 | 60 | 55 | 75 | 75 | 90 |
| <i>C. albicans</i> ** | 70 | - | 85 | - | 90 | - | 75 | - | 80 | - | 85 | - | 80 | - | 85 | - | 90 | - |
| <i>C. utilis</i> ** | 80 | - | 90 | - | 95 | - | 80 | - | 90 | - | 90 | - | 85 | - | 90 | - | 95 | - |
| <i>R. oligosporus</i> ** | 80 | - | 85 | - | 90 | - | 75 | - | 80 | - | 85 | - | 80 | - | 85 | - | 90 | - |
| <i>A. niger</i> ** | 80 | - | 85 | - | 90 | - | 80 | - | 85 | - | 90 | - | 85 | - | 90 | - | 95 | - |

*: Incubation period, 24 h; Incubation temperature, 37 °C. **: Incubation period, 96 h; Incubation temperature, 25 °C.

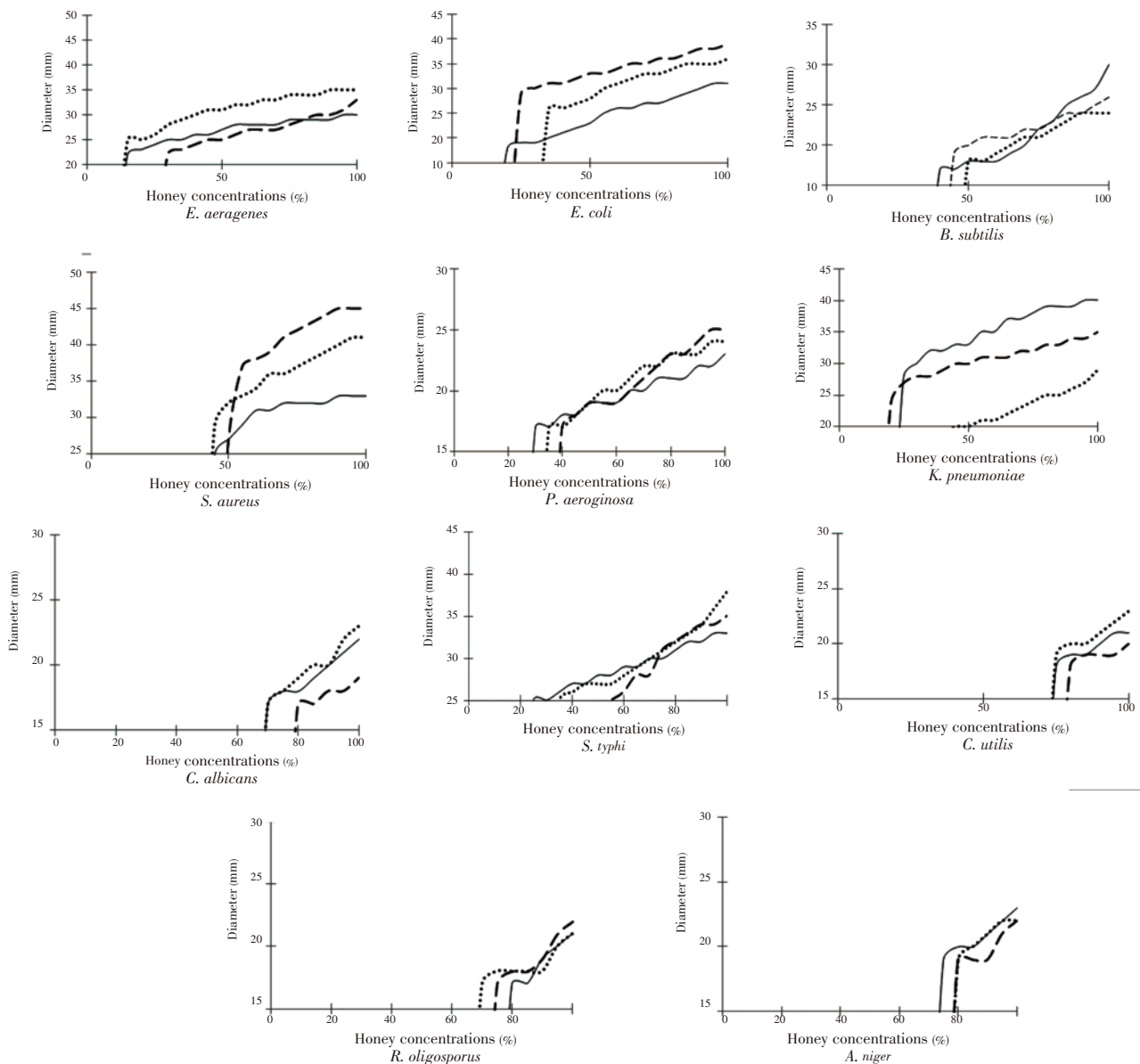


Figure 2. The inhibition zone diameters of tested microorganisms.

Straight line: Small honey; Dotted line: Siddar honey; Dashed line: Big honey.

the small honey showed the higher activity than other honey samples. For *Enterobacter aerogenes* (*E. aerogenes*), *Staphylococcus aureus* (*S. aureus*), *Candida albicans* (*C. albicans*), *Candida utilis* (*C. utilis*), both small and Siddar honey samples showed high activity than big honey. For *Klebsiella pneumonia* (*K. pneumonia*), big honey showed comparatively higher activity. For *Rhizopus oligosporus* (*R. oligosporus*), Siddar honey showed higher activity. Overall, it was noticed that Gram-negative microbes were more susceptible to honey as compared to Gram-positive microbes.

Upon addition of starch the antimicrobial potential was increased (Table 4). However, when starch solution was added in honey, the MIC values were decreased. Upon mixing honey samples with starch, small honey showed more effectiveness against *E. coli* and *B. subtilis*. Both small and Siddar honey seems to be more effective against *E. aerogenes*, *S. typhi* and *C. albicans*. Similarly, Siddar honey showed more effectiveness against *S. aureus* and *R. oligosporus*. However, big honey showed more effectiveness against *C. utilis*. Both small and big honey samples showed more effectiveness against *K. pneumonia*. All honey samples showed same activity against *P. aeruginosa* and *A. niger*.

3.4. Non-hydrogen peroxidase activity of honey sample

The non-hydrogen peroxidase activity of all honey samples were studied spectrophotometrically (Table 5). Upon removal of hydrogen peroxide from the honey samples, the MIC values were increased. In the absence of hydrogen peroxide, Siddar honey showed more effectiveness against *E. coli* and *P. aeruginosa*. Big honey seems to be relatively more effective against *E. aerogenes* and the small honey against *S. aureus* and *S. typhi*. Both Siddar and big honey samples showed more effectiveness against *B. subtilis* and all honey samples showed same activity against *K. pneumonia*.

4. Discussion

Physico-chemical analysis of all honey samples confirmed acidic pH ranging from 3.14 to 4.19. Low pH of honey is attributed to the presence of organic acids such as gluconic, pyruvic, malic and citric acids[28], and the variation in pH of different honey samples is described to be due to floristic composition and floral diversity of the regions. Observed values in this study are lower than those previously reported for other honey samples from India, which had pH values between 3.7 and 4.4[29]. Published data suggests that pH of honey may range between 3.2 and 4.5 and our finding is approximately within this range[30]. Overall however, the Siddar honey has the highest pH *i.e.* (4.190±0.707) that

might be due to the presence of alkaline contents in Siddar extracts. Similar findings were made in Saudi Arabia for sidr honey by Abu-Tarboush[31].

In this study the colors of all samples fall between amber to dark amber. The Siddar honey showed the dark amber color. Batrusaityte *et al.* reported that the color of honey is usually related to the mineral, pollen and phenolic contents of honey[32]. Jasicka-Misiak *et al.* also reported that the higher levels of polyphenols in honey resulted in the darker color of honey[33]. The total phenolic contents of the honey samples used in this study are higher than those of two reported Malaysian honey samples as well as that of Gelam and Coconut honeys, which are lighter in color[3]. Total ash contents in tested honey samples ranged between 0.411% and 0.590%. These findings meet the standards of European Union Commission[34], and Codex Alimentarius Commission[35], and are comparable with other studies like Salim *et al.*[36], in which ash contents ranged up to 0.09–0.54. Relatively higher ash contents were found in the Siddar honey *i.e.*, 0.5900%±0.0336%. Similar findings were made by Abu-Tarboush *et al.*[31] who found the highest ash contents in sidr honey. Furthermore, total protein contents in three honey samples used in this study ranged between 571.272 and 777.598 mg/kg. Comparatively, small honey showed highest protein contents *i.e.* (777.598±9.880) mg/kg and the lowest protein contents were found in the big honey *i.e.* (571.272±6.020) mg/kg. These findings are much more different than those observed by Islam *et al.*[37], who found the range of protein contents in Bangladeshi honey samples remained between 900 mg/kg and 8600 mg/kg. The moisture contents of tested honey samples ranged between 13.8% and 16.6%, which is in the range defined by Egyptian Organization Standards[38], European Union Commission[34], and Codex Alimentarius Commission[35]. Almost similar finding was reported by Khalil *et al.*[22], who found the moisture contents in their tested honey samples ranging between 11.59% and 14.13%. In this study, the highest moisture contents were found in the Siddar honey *i.e.* (16.600±0.283)% which is lower than the maximum suggested moisture contents by international standards. The percent moisture contents of analyzed honey samples tends to be less than other investigated honeys, such as 17.19%–19.19% for samples from Bangladesh[37], 17.2%–21.6% for samples from India[29] and 17.0%–19.4% for samples from Turkey[8]. Water content is very important for the shelf-life of honey during storage and can lead to undesirable honey fermentation due to osmotolerant yeasts, which form ethyl alcohol and carbon dioxide[17].

In this study a direct increase in the HMF contents was observed by increasing temperature. The range of HMF contents in unheated honey samples was from 27.69–36.08 mg/kg. This result was within the recommended range set by the Codex Alimentarius at 80 mg/kg[39]. The values

are also within the allowed maximum limit of 40 mg/kg, as recommended by the Turkish Alimentarius Codex for honey samples from tropical countries^[40]. However in some studies tested honey samples showed very low HMF contents *i.e.* 0.2–22.8 mg/kg^[41]. On contrast, Khalil *et al.* found that Malaysian Tualang honey samples have very high HMF concentrations *i.e.* 118.47–1139.95 mg/kg^[42]. Overall, the low HMF concentrations of these tested honey samples confirmed good quality of the samples. It was observed that when the temperature was increased, the HMF contents were also increased. Similar observations were made by Tosi *et al.* who recorded that HMF increased from 10.1 ppm to 32.8 ppm by heating of honey for 1 min at 100 °C and 140 °C respectively^[43]. In addition, This increase in HMF at high temperatures is associated with the decomposition of labile fructose particularly in the presence of acid^[44]. For the analyzed honey samples total sugar contents range from 61.672% to 72.42%. None of the samples exceeded the highest limit set for total sugar content by the European community directive^[27]. Regarding reducing sugars (fructose and glucose), EU Directive imposes values $\geq 60\%$ and according to the obtained results^[27], only one sample (Siddar honey) showed a value lower (57.748%) as compared to the standard value set for reducing sugars by EU Directive^[27]. Borsato *et al.* also found lower reducing sugar content in their one honey sample *i.e.* 58.75%^[45]. Overall our results are comparable to the results obtained by Gomes *et al.* who found the range of reducing sugar contents in organic honey from the northeast of Portugal that ranged between 65.60% and 68.90%^[46]. These finding are also comparable with the results obtained by Borsato *et al.* who found the range of reducing sugar contents of honeys of Campos Gerais region of Paraná, Brazil that ranged between 58.75% and 82.37%^[45]. The non-reducing sugar contents were found to be in the range of 1.95%–3.93% the highest non-reducing sugar contents were found in the Siddar honey *i.e.* (3.930±0.658)%. This might be due to the early harvest of the honey. Gomes *et al.* reported that the higher sucrose contents could be the result of an early harvest of honeys^[46], *i.e.* as sucrose has yet to be converted to fructose and glucose.

Like non-reducing sugar content the highest phenolic contents were present in the Siddar honey *i.e.* (540.720±3.035) mg/kg which indicates its high antioxidant potential. The highest flavonoid content was reported in the Siddar honey *i.e.* (64.396±3.534) mg/kg. The flavonoid contents of our tested honey samples were higher than those of Turkish^[47], and Malaysia honey samples^[48], which ranged from 4.80–22.80 mg_{catechin}/kg and 11.52–25.31 mg_{catechin}/kg respectively, which indicates Siddar honey samples have higher antioxidant potential as compared to Turkish and Malaysian honey samples. The DPPH radical scavenging

activity of honey at different concentrations was checked and it was observed that the Siddar honey has slightly higher radical scavenging activity as compared to other two honey samples. Possibly, observed high radical scavenging activity of the Siddar honey is due to the high phenolic and flavonoid contents in it, thus indicating high antioxidant potential. Furthermore, it was observed that the highest radical scavenging activity is linked with the highest honey concentration tested of all honey samples. Recently, similar findings were reported by Khalil *et al.* who found the highest scavenging activity at highest honey dilution^[22]. The percentage of inhibition exhibited by tested honey samples were similar to that of Algerian honey samples^[22], some Malaysian honey samples and Indian honey samples^[29,48].

The non-hydrogen peroxidase activity of all honey samples was checked that showed removal of hydrogen peroxide increases the MIC values. In the absence of hydrogen peroxide, Siddar honey showed more effectiveness against *E. coli* and *P. aeruginosa*. Big honey showed more effectiveness against *E. aerogenes*. Small honey showed more effectiveness against *S. aureus* and *S. typhi*. Overall, both Siddar and big honey samples showed more effectiveness against *B. subtilis*. All honey samples showed same activity against *K. pneumonia*. However, all honey samples showed no non-peroxidase activity against *C. albicans*, *C. utilis*, *R. oligosporus* and *A. niger*.

All bacteria showed clear inhibition zones in response to all honey samples whereas fungi and yeast showed inhibition at higher concentrations of honey. For *E. coli*, *B. subtilis*, *S. typhi*, *P. aeruginosa* and *A. niger*, the small honey showed higher activity than other honey samples. For *E. aerogenes*, *S. aureus*, *C. albicans* and *C. utilis*, both small and Siddar honey samples showed higher activity than big honey. For *K. pneumonia*, big honey showed comparatively higher activity. For *R. oligosporus*, Siddar honey showed higher activity. Allen *et al.* reported the antibacterial properties of honey against two laboratory isolates *e.g.* *P. aeruginosa* and *E. coli*^[1]. El-Toum and Yagoub found through well diffusion method that sidr honey showed antimicrobial activity against *S. aureus*^[49], *K. aerogenes*, *P. aeruginosa* and *C. albicans*, and the zone of inhibition ranged between 9 mm and 50 mm, and sunflower honey showed markedly sensitivity towards *E. coli*, *S. aureus* and *K. aerogenes*, and the inhibitions zone were between 15 mm and 50 mm, and sunut honey showed antimicrobial activity toward *S. aureus*, *E. coli*, *K. aerogenes* and *C. albicans*, and the inhibition zone range between 10 mm and 42 mm. It was also noticed that Gram-negative bacteria were more susceptible to honey as compared to Gram-positive bacteria.

This study confirms antimicrobial properties of these honey samples against *E. coli*, *E. aerogenes*, *P. aeruginosa*, *B. subtilis*, *S. aureus*, *S. typhi*, *K. pneumonia*, *A. niger*, *Rhizopus oligosporus*, *C. albicans* and *C. utilis* ATCC 9950.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

It is an ethnopharmacological research on a locally available honey sample made from local species that can be seen in some tropical area of Pakistan.

Research frontiers

A standard ethnopharmacology study on locally available honey samples. It can be a good data for further pharmaceutical and pharmacological research.

Related reports

Not much on the studied samples. However, there are some previous publications from Pakistan and Middle East on the locally available honey such as ones from Iraq.

Innovations and breakthroughs

New data on the properties of studied samples can be seen in the present report. Implication on microbiology is interesting and can be further useful.

Applications

Data can be further referred and used in further pharmaceutical and pharmacological research. Also, the test on microbiology aspect is helpful in tropical medicine work.

Peer review

It is an ethnopharmacological research on a locally available honey samples from Pakistan. New data on the properties of studied samples can be seen in the present report. Implication on microbiology is interesting and can be further useful. Further referencing on this work is possible.

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