Physico-chemical properties, antioxidant, and antimicrobial activity of five varieties of honey from Saudi Arabia

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Abstract. Wild honey is believed to have plentiful nutritional and health benefits. Numerous studies dealing with the characteristics and biological activities of honey have proved its biological activities. However, little is known about Saudi wild honey. In the current study, the physico-chemical properties, antioxidant and antimicrobial activity of five varieties of wild honey collected from different locations in Saudi Arabia were examined. Results showed that, the physico-chemical characteristics of all samples were in compliance with international standards. Qualitative and quantitative analysis of sugar (glucose, fructose and sucrose) was determined by high-performance liquid chromatography (HPLC). The analysis exhibited that fructose is the major sugar type in all examined wild honeys, which was found in highest concentration in flowers honey (46 g/100 g), whereas, the lowest fructose concentration was found in Ziziphus honey (36 g/100 g). All monofloral honey showed significant antioxidant activity. Alfalfa honey recorded the highest antioxidant activity, followed by Ziziphus honey, respectively. However, Tamarisk honey recorded the lowest antioxidant activity among honey samples. Honey's antibacterial activity against five microorganisms displayed differing degrees of inhibition. The overall findings indicated that no antifungal activity was seen against *Candida albicans*. The antibacterial examination of honey samples revealed non-significant activity against all tested bacteria, except with *Staphylococcus aureus* ATCC BAA 1026, where the Acacia honey exhibited significant activity against this bacterium ($p < 0.05$), with a mean of inhibition zone of 12.0 ± 0.0 mm and a minimum inhibitory concentration of 12.5%. The results of the other types of honey were statistically non-significant.

Keywords: antimicrobial, antibacterial, honey, chemical, physico-chemical, Saudi Arabia

INTRODUCTION

Honey is a natural product that has been commonly used for its medicinal effects since ancient times. Historic literature has shown that honey and bee products were utilized in medicine by ancient Egyptians, Greeks, and Romans, particularly for wound treatment and intestinal diseases (Eteraf-Oskouei & Najafi, 2013). Honey is basically a saturated sugar solution, consisting of 82.4% carbohydrates (fructose, glucose, and other sugars), 17.1% water, and only 0.5%

bioactive compounds like enzymes, vitamins, minerals, amino acids, phenolic compounds, flavonoids, carotenoids, ascorbic acid, α -tocopherol, and tannins (Rossi & Marrazzo, 2021). This 0.5% possibly carrying two health-promoting effects: a curative effect, such as wound-healing, antioxidant, antimicrobial, anti-parasitic and antidiabetic, and a protective effect on gastrointestinal, cardiovascular, nervous, and respiratory systems (Cianciosi *et al.*, 2018) Honey

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sugars are also extremely advantageous. As an example, fructo-oligosaccharides are indigestible compounds and may improve intestinal health since they are a decent source of prebiotics that help the probiotics in our intestines (Ramsay *et al.*, 2019). Nevertheless, honey varies considerably in its properties depending on the plant species, its nectar, the climate, the environment, and the geographical distribution, and thus the chemical and therapeutic properties of honey also vary, and not all honey samples have the same medicinal properties (Abdallah, 2016). Depending on the plant origin, honey can be differentiated into two types: polyfloral (or multifloral) and monofloral (or unifloral) kinds of honey; the presence of pollen grains may provide information on plant composition, and it can also be useful in obtaining indications of geographical origin due to the spatial distribution of different plants (Utzeri *et al.*, 2018). In the 21st century, global comprehensive health schemes have been established based on synthetic compound libraries with apparent neglect of natural products (Lam, 2007). The human body has a diverse set of natural enzymatic and non-enzymatic antioxidant defenses that resist the formation of free radicals and other oxidants' harmful effects. A wide variety of illnesses, including cancer, neural disorders, cardiovascular diseases, atherosclerosis, Alzheimer's disease, Parkinson's disease, liver diseases, ulcerative colitis, and even ageing are caused by free radicals (Alam *et al.*, 2013). In literature, honey as folk medicine is recommended as a natural antioxidant by many scientific reports (Al-Mamary *et al.*, 2002; Bogdanov *et al.*, 2008; Ahmed *et al.*, 2018). The world continues to face the threats of ancient, emerging and renewable infectious diseases and that modern health system stands helpless in the face of recent outbreaks such as Ebola, dengue fever, Zika, influenza, Middle East respiratory syndrome, and severe acute respiratory syndrome, as well as the looming danger of increasing antimicrobial resistance (Bloom & Cadarette, 2019). Therefore, it is expected that scientific research on natural products will witness a revival in search of good, holistic, and more effective treatment alternatives. The current study aimed to evaluate the physico-chemical properties and antimicrobial activity of five brands of Saudi wild honey, one polyfloral and four monofloral honey.

MATERIALS AND METHODS

Honey samples

Five honey samples from different botanical origins were generously provided by Honey Jarbou stores, Unaizah city, Saudi Arabia. This company is committed to selling only wild kinds of honey which are raw and unpasteurized honey collected manually by the beekeepers from different localities in Saudi Arabia. Four monofloral and one polyfloral honey samples were investigated, namely Acacia honey (H1), Ziziphus honey (H2), Flowers' honey (H3), Alfalfa honey (H4), and Tamarisk honey (H5). Samples were acquired in June 2019 and were kept in well-tighten dark glass bottles under refrigeration at 4-5°C until analysis.

Microorganisms

Four referenced bacterial strains and one yeast strain were used in this investigation. Bacterial strains are two Gram-positives (*Staphylococcus aureus* ATCC BAA 1026 and *Bacillus cereus* ATCC 10876), two Gram-negatives (*Pseudomonas aeruginosa* ATCC 10145 and *Escherichia coli* ATCC 1637), and the yeast strain was *Candida albicans* ATCC 10231. All microbial isolates were obtained from the stock culture of the Department of Laboratory Sciences, College of Sciences and Arts at Al-Rass, Qassim University, Saudi Arabia.

Physico-chemical analyses

Five honey samples solution 50% (w/v) were prepared using distilled water at room 20°C. The resulting solution was used to determining the physico-chemical parameters such as pH, electrical conductivity, density, ash content, and element analysis.

pH values

The pH of the samples was measured by using a pH meter (Martini, Mi 180 Bench Meter) according to the harmonized methods (Abdecellah *et al.*, 2020).

Electrical conductivity

The electrical conductivity (EC) of samples was measured at 20°C with the aid of a conductivity meter (Thermo Scientific, Orion Star A212) (Svečnjak *et al.*, 2019).

Density

The pycnometry method (Lullah-Deh *et al.*, 2018) was used to determine the density of the honey samples using the expression below:

$$D = M_2 - M_1 / V$$

M_1 = mass of the pycnometer when empty

M_2 = mass of the pycnometer filled with honey sample

V = volume of the pycnometer

Ash content

Ash content was determined according to the dry-ash method (Sajid *et al.*, 2020; Ahmed *et al.*, 2007). Ten grams of each sample was placed in the burning crucibles. The crucibles were placed in Electric Muffle Furnace (JISICO J-FM38, South Korea) at 500°C for two hours. The proportion of ash WA in g/100 g honey is calculated using the following formula:

$$WA = M_1 - M_2 / M_0$$

M_0 = weight of honey taken,

M_1 = weight of crucible + ash

M_2 = weight of crucible

Element analysis

Elemental analysis was carried out using Thermo scientific EDXRF Analyzer QTX-1024. 1 g of five raw honey samples were placed in special cups (Lazaridou *et al.*, 2004; Aljohar *et al.*, 2018).

Quantitative analysis of sugar by LC

The sugar of honey samples was analysed by injecting the sample on an injector with a 20 L loop on the system. The column was Water Radial-pak silica (7:100 mm), reagents (glucose, fructose and sucrose) standards are from Sigma-Aldrich. Methanol, acetonitrile and water all HPLC grad sensitive balances were used. Acetonitrile and water (75:25) were mixed, sonicated and used as mobile phase. HPLC conditions flow rate: 1.5 mL/min; mobile phase: acetonitrile: water (75:25, v/v); column and detector temperature: 30°C, and sample volume: 20 µL were used for HPLC separation. Peaks were identified on the basis of their retention times.

Preparation of sample

Selected honey samples were weighed (2.5 g) in two a 250 ml flask dissolved in 20 ml of HPLC grade water, 12.5 ml of methanol was added after that honey solution was filtered through a nylon membrane filter syringe (Bogdanov, 2009).

DPPH radical scavenging capacity

The DPPH radical scavenging activity of honey samples was assayed as a method described (Elsharkawy & Shiboob 2017; Brand-Williams 1995). Ascorbic acid was used as the positive control. the radical scavenging activity was expressed as % DPPH inhibition calculated from the graph of inhibition percentage plotted.

Antimicrobial disc-diffusion susceptibility method

Kirby-Bauer Disk Diffusion Susceptibility Test Protocol has been used as described by (Bakht *et al.*, 2011) to evaluate the potential antimicrobial activity of honey samples. Briefly, under aseptic conditions, plates containing nutrient agar were prepared and left to solidify. Five sets of filter paper discs (6 mm in diameter) were cut from Whatman No. 1 filter paper and each set was saturated separately with the desired honey solution at 50% v/v using sterile distilled water. The solidified agar plates were inoculated with 18 to 24 h cultures of microbial inoculums (about 1 to 2×10^7 CFU ml⁻¹), using sterile cotton swabs. Then, the saturated discs were put over the nutrient-agar plats. Discs saturated with chloramphenicol (1.25 mg/ml) for bacteria and Clotrimazole (2.5 mg/ml) for yeast were also loaded and served as positive controls. In separate inoculated plates, discs saturated with the solvent (80% methanol) were evaluated and showed no inhibitory effects. Plates were incubated at 35°C for 18 to 24 h for bacteria, or for 48 h for yeast and then inspected for inhibition zones around the discs, which was recorded in millimeter using a ruler. The test was repeated twice and the mean zone of inhibition $\pm$ standard error of means was calculated. Zones have been interpreted as weak if it was less than 10 mm, moderate of the zone of inhibition was between 10 and 13 mm, and high if the zone of inhibition was greater than 13 mm (Abdallah, 2020).

Minimum inhibitory concentration method

Microorganisms that showed susceptibility to honey samples from the 10.0 mm inhibition zone and above were subjected to minimum inhibitory concentration (MIC) using the agar well diffusion method (Gonelimali *et al.*, 2018). Under aseptic conditions, two-fold serial dilutions were prepared to get decreasing concentrations starting from 25, 12.5, 6.3, 3.1, and 1.6% v/v using distilled water. 1 ml of each prepared inoculum of tested bacteria was transferred to a nutrient-agar Petri-dish and spread over the plate using a sterile cotton swab. Then, 5 wells (size 6 mm) were made on each plate by a sterile cork-borer and each honey dilution was loaded into the wells in order. Inoculated plates were then incubated at 35°C for 18 h (for bacteria) for up to 48 h (for yeast). The MIC was described as the lowest concentration that inhibited the growth of the microorganisms.

Statistical analysis

The findings of this study are stated as mean $\pm$ SEM (Standard Error of Means). One-Way ANOVA (analysis of variance) test was used to determine the important variations between the examined microorganisms, for statistical analysis, SPSS program 14.0 (SPSS Inc., Chicago, USA) was used.

RESULTS AND DISCUSSION

Physico-chemical properties

The results of the physicochemical parameters analyzed are shown in Table 1 and the Element analysis is shown in Table 2. As for the colour, it was noted that honey colours vary between dark amber, light amber, and white. The difference in colour is due to the difference in the percentages of dissolved substances in the origin of the plant, such as the pigment compounds that are transported through nectar and are rich in chlorophyll and carotene. The effect is also due to the proportion of minerals and mineral salts that vary according to the type of plant. In addition, the temperature and storage conditions affect the honey colour (Bouhlali, *et al.*, 2019). The pH values of honey samples correspond to

international specifications; the values ranged between 3.44 to 4.83 honey is considered an acidic medium and the difference in acidity values between honey samples is due to several reasons, including the period of maturity, the type of flowering, as well as the chemical composition of honey, the concentration of minerals, the phenols in it, the percentage of acids in addition to mineral concentrations (Laaroussi *et al.*, 2020). Generally, the density values obtained for honey samples comply with international specifications (Lullah-Deh *et al.*, 2018). The values ranged between 1.1 to 1.16; the lowest value for honey sample 1 and the highest value for sample 5, and the variation of these values is due to the density being affected by storage or neglecting to close storage vessels (Perez-Arquillué *et al.*, 1994). According to the results in Table 1, the conductivity of the samples values ranged between 0.4 to 8.63 mS/cm, and the highest value of the conductivity is for sample 1, and the reason is that bees graze on the flowers of sample 1 that are located in salty areas. Compared to the areas where other samples are located, in addition, the mineral concentration for sample 1 is much higher than the other samples, and this directly affects its conductivity. Ash content is a quality measure for plants and the geographical origin of honey. In this study, honey sample 2 has a higher ash content (0.19%) compared with the other samples. Ash content of honey samples was within the acceptable range (0.6-1.2%) mentioned in previous research (Alqarni, *et al.*, 2014). The results of element analysis of honey samples by XRF instrument showed that potassium was the major mineral ranged between 48.9% and 10.1%, followed by aluminum (between 42.9% and 10.47%), calcium (between 3.4% and 1.6%), and iron in the lowest amount (between 0.05% and 0.08%). The mineral contents in this study were within the range reported in honey (Solayman *et al.*, 2016; Adugna *et al.*, 2020). The study also showed that some of the honey samples contain non-metal such as chlorine, bromine, sulfur, and phosphorus. In addition to minerals, honey sample 2 contains heavy metals. The mineral content in honey is an important pointer of potential environmental pollution, especially for heavy metals, and it is a possible indicator of honey's geographical origin (Ribeiro *et al.*, 2014; Otaka *et al.*, 2014).

Table 1. Physico-chemical properties of the five honey samples.

Honey Samples	Colour	pH	Density (g/cm ³)	Electrical conductivity (mS/cm)	Ash (%)
Sample 1	Dark amber	4.8	1.10	8.63	1.15
Sample 2	Light Amber	4.83	1.11	1.78	0.19
Sample 3	Light Amber	3.85	1.12	1.07	0.1
Sample 4	Extra White	3.44	1.11	0.4	0.09
Sample 5	Light Amber	4.26	1.16	3.2	0.1

Table 2. Results of element analysis of the five honey samples.

Honey samples	The percentage of elements analysis (m/m) %											
	K	Al	Ca	Fe	Cl	Br	S	P	Zn	Ni	Mn	Rb
Sample 1	48.9	10.47	3.4	0.05	4.47	0.07	2.14	2.8	0.07	0.02	-	0.02
Sample 2	32	28.2	2.1	0.08	4.9	-	-	-	0.09	-	0.06	-
Sample 3	10.1	42.9	1.6	-	3.1	-	0.6	-	-	-	-	-
Sample 4	9.0	38.67	5.57	-	6.81	-	0.53	0.09	0.04			
Sample 5	21.8	30.4	2.8	-	6.1	0.03	1.1	-	-	-	-	-

Quantitative analysis of sugar in honey samples by HPLC

Sugar is a major compound of Carbohydrates in the honey bee that gives honey its quality and it is related to maturity and its origin. The results of the analysis of five samples of different content (glucose, fructose, and sucrose) are shown in (Table 3). The maximum concentration of glucose was found in Tamarisk honey (38.5 g/100 g) and minimum concentration (24 g/100 g) was found in sample H1 (Acacia honey), while fructose showed high concentration (46 g/100 g) in sample H3 (Flowers honey) and minimum concentration reached to (36 g/100 g) in sample H2 (Ziziphus honey).

The present study pointed to the mean total sugar content of examined honey ranging from 60.0 to 80.3 g/100 gm. Fructose was found in higher concentrations than glucose. These results agreed with the findings of previous studies on monofloral honey (Ouchemoukh *et al.*, 2010; León-Ruiz *et al.*, 2011). Sucrose was considered as an indicator of honey quality; the percentage of sucrose to total sugar in honey increased when honey was harvested before ripening. During the ripening process, in the cell, the sucrose level is reduced by the action of enzyme invertase (Bogdanov *et al.*, 1999).

Table 3. Sugar composition (g/100) for the five tested honey samples.

Sample	Glucose	Fructose	Sucrose	Glucose + Fructose
Sample (H1)	24.0	36.84	0	60.84
Sample (H2)	26.60	39.72	0	66.30
Sample (H3)	31.00	46.00	0	77
Sample (H4)	36.00	40.00	0	76
Sample (H5)	38.5	41.8	0	80.3

Antioxidant

Many natural sources can be used to overcome on effects of oxidative stress underlying the pathogenic disease, the DPPH radical scavenging activity of honey was assayed by DPPH radical scavenging methods, the results were illustrated in (Table 4). In comparison among the honey samples, the highest antioxidant activity was

shown in sample H4 (Alfalfa honey), being the best in terms of antioxidant activities among the 5 types of honey samples. This is followed by sample H2 (Ziziphus honey) and minimum scavenging activity was determined by sample H5 (Tamarisk honey). All honey samples were statistically significant compared to the ascorbic

acid (AC), except for sample H5 (Tamarisk honey).

The results also showed that Alfalfa honey has the highest antioxidants (95.42 mg/ml) and the lowest DPPH antioxidant activity was 10.70 mg/ml of Tamarisk honey; while Ziziphus honey determined by DPPH activity was 63 mg/ml. Another study by Aziz & Tuba (2018) reported that the DPPH radical scavenging of pine honey was 65.52 mg/ml and strawberry honey recorded 54.25 mg/ml. This agrees with our study where

most of the honey samples demonstrated high values of DPPH and the antioxidant potential is correlated to the mineral content in honey samples (Zn, Al, and Fe) and also the phenolic content, where natural honey is well known of its high amounts of phenolic compound, salicylic, and gallic acid. Chua *et al.* (2013) and Gül & Pehlivan (2018) found a positive correlation between the antioxidant and chemical content of honey-like phenolics and vitamins, which is in agreement with the current study.

Table 4. Mean DPPH scavenging activity of the five honey samples (Mean $\pm$ S.D.).

Test	H1	H2	H3	H4	H5	AC
DPPH (mg/ml)	88.01 $\pm$ 0.01	63.21 $\pm$ 0.01	78.22 $\pm$ 0.02	95.4 $\pm$ 0.012	10.72 $\pm$ 0.01	66.33 $\pm$ 0.001

AC: Ascorbic acid; S.D.: Standard deviation

Table 5. Antimicrobial activity of tested honey samples.

Samples	Zone of inhibition (mm)				
	Gram-positive bacteria		Gram-negative bacteria		Yeast
	Sa	Bc	Pa	Ec	Cand
Sample H1	12.0 $\pm$ 0.0	8.5 $\pm$ 0.5	9.0 $\pm$ 0.0	9.0 $\pm$ 0.0	6.0 $\pm$ 0.0
Sample H2	8.5 $\pm$ 0.5	9.0 $\pm$ 1.0	8.5 $\pm$ 0.5	8.0 $\pm$ 0.0	6.0 $\pm$ 0.0
Sample H3	8.0 $\pm$ 0.0	8.0 $\pm$ 0.0	8.5 $\pm$ 0.5	8.5 $\pm$ 0.5	6.0 $\pm$ 0.0
Sample H4	8.5 $\pm$ 0.5	8.5 $\pm$ 0.5	9.0 $\pm$ 0.0	8.5 $\pm$ 0.5	6.0 $\pm$ 0.0
Sample H5	9.5 $\pm$ 0.5	8.0 $\pm$ 0.0	8.5 $\pm$ 0.5	7.0 $\pm$ 0.0	6.0 $\pm$ 0.0
Chloramphenicol (1.25 mg/ml)	26.5 $\pm$ 0.5	30.5 $\pm$ 0.5	11.5 $\pm$ 0.5	27.0 $\pm$ 0.0	-
Clotrimazole (2.5 mg/ml)	-	-	-	-	23.5 $\pm$ 0.5

Sa=*Staphylococcus aureus* ATCC BAA 1026; Bc=*Bacillus cereus* ATCC 10876; Pa=*Pseudomonas aeruginosa* ATCC 10145; Ec=*Escherichia coli* ATCC 1637; Cand=*Candida albicans* ATCC 10231

Table 6. Minimum inhibitory concentration (MIC) of the honey sample 1.

Microorganism	Minimum inhibitory concentration (MIC) %				
	25%	12.5%	6.3%	3.1%	1.6%
<i>Staphylococcus aureus</i> ATCC BAA 1026	+	+	-	-	-

Antimicrobial properties

Regarding the results of the antimicrobial disc-diffusion susceptibility test, unlike antibiotics, there are no standard criteria for interpreting the inhibition zones for raw natural compounds. However, Philip *et al.* (2009) suggested that inhibition zones larger than 10 mm have good antibacterial activity. As shown in Tables 5 and 6, the disc diffusion test showed that honey samples have no antifungal activity against the yeast *Candida albicans*. Whereas, various degrees of antibacterial activity (moderate to weak) were recorded with bacteria and this moderate to weak activity was non-significant at $p \leq 0.05$. Amongst

the five honey varieties, the honey sample H1, which was Acacia variety, showed the highest antibacterial activity (significant at $p \leq 0.05$). It recorded a 12.0 $\pm$ 0.0 mm zone of inhibition against *Staphylococcus aureus*. Based on the methodology, bacteria that showed susceptibility to honey samples from the 10.0 mm inhibition zone and above were subjected to minimum inhibitory concentration (MIC). Accordingly, only *Staphylococcus aureus* was tested for MIC against Acacia honey. The MIC of this bacterium was 12.5%. It is believed that peroxide and other bioactive constituents are the main cause of the antibacterial activity of honey (Zainol *et al.*, 2013)

The current findings are in agreement with previous studies which showed that Acacia honey has remarkable antibacterial activity against *Staphylococcus aureus*, in particular (Hegazi *et al.*, 2017; Mohd *et al.*, 2020). However, the other honey samples showed various antibacterial activity (less than 10.0 mm zone of inhibition). Hence, it is important to mention that honey is a sensitive product and many factors could affect the antibacterial potential of honey, such as the handling processes, transportation, season of collection, and storage conditions (Abdallah, 2016).

Finally, the current study revealed that honey samples showed diverse activity in terms of mineral contents, pH, density, sugar contents, and antioxidant and antibacterial activity. This could be related to the difference in properties of the nectar source, the climate, the environmental conditions, and geographical distribution (Alghamdi *et al.*, 2020; Al Kiyumi *et al.*, 2021; Pauliuc *et al.*, 2021).

CONCLUSION

In conclusion, the honey samples have revealed a significant antioxidant potential of all tested honey samples, except for the Tamarisk honey. In addition, the samples have shown a degree of antibacterial activity, which was statistically non-significant, except with one bacterium (*Staphylococcus aureus*). The physico-chemical analysis of honey samples was also discussed and uncovered many interesting constituents, which indicates various health benefits. Accordingly, honey is a valuable food not only for its nutritious value, but also for its physical, chemical, and biological capabilities. These bioactive functions are mostly due to the biochemical compounds found in honey, which may be used alone as a functional food or as potential additives in a variety of foods.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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