American-Eurasian J. Agric. & Environ. Sci., 15 (6): 1147-1154, 2015
ISSN 1818-6769

DOI: 10.5829/idosi.aejaes.2015.15.6.9529

Evaluation of Functional Properties of Local and Imported Honey in Egypt

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Abstract: Honey is a sweet food made by bees using nectar from flowers. The aim of the present study is to evaluate the functional properties of honey to confirm its potency as a functional food. The total content of phenolic compounds in the honey samples varied from 24.88±0.57 to 67.66±0.58 µg GAE/g. The total flavonoids varied from 4.21±0.34 to 13.49±0.33 µg/g. A correlation coefficient was found between the phenolic compounds of honey and flavonoids ($P<0.05$). The obtained results confirmed the percentage inhibition of the honey samples associated with their phenolic and flavonoid content. Glucose oxidase activity in all samples varied from (02.07±0.02U/g in Egyptian honey to (37.67±0.14U/g) in Kashmiri honey. All types of honey samples showed growth inhibitory activity against *Clostridium botulinum ATCC 3584*; *Klebsiella pneumoniae ATCC12296; Staphylococcus aureus NCTC 10788; Staphylococcus epidermidis* and *Streptococcus dysgalactiae*. *Escherichia coli BA12296, Salmonella. senftenberg and Candida albicans* were resistant to Egyptian, Saudi, Yemeni and Kashmiri honey. A nutritional value and quality of different types of honey varied according to its source, handling and storage conditions during transportation.

Key words: Honey · Antioxidant · Glucose Oxidase Activity · Antimicrobial

INTRODUCTION

Honey is a sweet and flavorful natural product, which is consumed for its high nutritive value and for its effects on human health, with antioxidant, bacteriostatic and antimicrobial properties [1]. The healing effect of honey could be due to various physical and chemical properties [2]. The floral source of honey plays an important role on its biological properties [3]. The antibacterial action of honey was reported for the first time in 1982 [4]. Different aspects of the antibacterial properties of honey have recently been extensively reviewed [5]. There are two sorts of antibacterial agents, or so-called 'inhibines'. One of them is heat-and light-sensitive and has its origin in the H_2O_2 produced by honey glucose oxidase [6, 7]. Some workers believe that hydrogen peroxide is the main antibacterial agent in honey [6, 8, 9]. Hydrogen peroxide is an effective antimicrobial agent if present at a sufficiently high concentration [10], but at higher concentrations causes cellular and protein damage in tissues by giving rise to oxygen radicals [11, 12]. The antioxidant activity of different types of honey from different countries and different botanical origins has been carried out [13-17]. The antioxidant potential of natural honey is mainly correlated not only with total phenolics originating from the pollen of flowering plants and trees, but also with the dark colored honeys such honey types contain higher antioxidant capacities [18, 19]. The enzymes are among the most interesting aerials in honey. The origin of these enzymes is the hypopharyngeal (pharyngeal) glands of worker bees, which their secretion are rich in enzymes (invertase, diastase and glucose oxidase) used in elaborating honey. Extracted honey contained a rest of these enzymes, unless destroyed by heating [20]. Glucose oxidase is one the most important enzyme in honey. Its activity is an important property, because it produces hydrogen peroxide and glucone lactone by oxidizing glucose. It produces H_2O_2 in mature honey, when honey diluted with warm water. During this process,

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growth of most organisms may be inhibited [21, 22]. Heat treatment of honey killed yeast, inactivation of enzyme starts and antioxidant activity decline [21, 23]. Furthermore, natural honey has highly antimicrobial activity (24), which depends on different factors acting singularly or synergistically, the most salient of which are phenolic compounds, H_2O_2, pH of honey and osmotic pressure exerted by the honey itself [22, 24]. The aim of the present study is to evaluate the functional properties of different types of honey samples to confirm its quality and health benefits.

MATERIALS AND METHODS

Materials: Four types of honey collected from different sources were used for analysis in this study. These types were "Egyptian, Saudi, Yemeni and Kashmiri honey. In the antimicrobial activity, Eight microorganisms species known to be pathogenic to human such as *Candida albicans ATCCMYA-2876*; *Clostridium botulinum ATCC 3584*; *Escherichia coli BA 12296; Klebsiella pneumoniae ATCC12296; Salmonella senftenberg* ATCC 8400; *Staphylococcus aureus* NCTC 10788; *Staphylococcus epidermidis* ATCC12228 and *Streptococcus dysgalactiae* subsp. *Equisimilis strain 04/04* were used. The imported honey samples were collected from the market at Down town and Lauran districts, Alexandria Governorate, Egypt, While the local sample collected from *Ziziphus* sp. (Sidr trees) farm at El-Nobareya city, located on the Alexandria-Cairo desert Road. All samples were stored under freezing conditions (-28±2°C) till further analysis to keep its composition as it is and avoid any effect for the laboratory conditions.

Determination of Total Phenolic Compounds: The concentration of phenolic compounds in honey samples was determination using a modified spectrophotometric Folin-Ciocalteu method [25]. Briefly, 1 mL of honey extract was mixed with 1 mL of Folin-Ciocalteu's phenol reagent. After 3 min, 1 mL of 10% Na_2CO_3 solution was added to the mixture and adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm using a T-60 UV/VIS spectrophotometer (PG Instruments Ltd, UK). Gallic acid was used to calculate a standard curve (20, 40, 60, 80 and 100 µg/mL), ($r^2 = 0.9970$). The concentration of phenolic compounds was measured in triplicate. The results were reported as the mean ± standard deviation and expressed as µg of gallic acid equivalents (GAE) per g of honey.

Determination of Total Flavonoids Content: The total flavonoids content for each honey sample was determined using the colorimetric assay developed by Zhishen [26]. Honey extract (1 mL) was mixed with 4 mL of distilled water. At the baseline, 0.3 mL of $NaNO_2$ (5%, w/v) was added. After five min, 0.3 mL of $AlCl_3$ (10% w/v) was added, followed by the addition of 2 mL of NaOH (1 M) 6 min later. The volume was then increased to 10 mL by the addition of 2.4 mL distilled water. The mixture was vigorously shaken to ensure adequate mixing and the absorbance was read at 510 nm. A calibration curve was created using a standard solution of catechin (20, 40, 60, 80 and 100 µg/mL; $r^2 = 0.9880$). The results were expressed as µg catechin equivalents (CEQ) µg/g of honey.

DPPH Free Radical-Scavenging Activity: The antioxidant properties of each honey sample were also studied by evaluating the free radical-scavenging activity of the DPPH radical, which was based on the method proposed by Ferreira [27]. Briefly, honey extract (0.5 mL) was mixed with 2.7 mL of methanolic solution containing DPPH radicals (0.024 mg/mL). The mixture was vigorously shaken and left to stand for 15 min in the dark (until their absorbance stabilized). The reduction of the DPPH radical was determined by measuring the absorbance of the mixture at 517 nm [28]. Ascorbic acid was used as a reference. The radical-scavenging activity (RSA) was calculated as the percentage of DPPH discoloration using the following equation:

$$\%RSA = ([ADPPH - AS]/ADPPH) \times 100,$$

where,
AS is the absorbance of the solution when the sample extract has been added at a particular level and ADPPH is the absorbance of the DPPH solution.

Glucose Oxidase Activity: The activity of glucose oxidase was determined by Megazyme Assay Kit Glucose Oxidase K-GLOX 01/05 (Megazyme International Ireland Ltd., Ireland) based on the catalysis of oxidation of β-D-glucose to D-glucono- δ-lactone with the concurrent release of hydrogen peroxide. The solution (2.0 mL) of potasium phosphate buffer (60 mM, pH = 7) containing peroxidase (0.64 U/mL), p-hydroxybenzoic acid (12 mmol) and aminoantipyrine (0.4 mM) was mixed with β-D-glucose (0.5 M, 0.5 mL). The mixture in the cuvette was equilibrated to 25°C and the absorbance (A1) at

510 nm were measured using T-60 UV/VIS spectrophotometer (PG Instruments Ltd, UK) after 5 minutes. Then the reaction was started by the addition of 0.5 mL of honey sample. The samples were shaken in a water bath at 25°C during incubation time and the absorbance (A2) of a formed red quinoneimine dye was read after exactly 20 minutes against blank. Determine the absorbance difference (A2-A1) for both blank and sample. Consequently, activity values (mU/0.5 mL) are obtained by reference to the standard curve and calculated as follows:

U/L of sample solution = mU/0.5 mL x 2000 x 1 x D 1000 = mU/0.5 mL x 2 x D

where,
mU/0.5 mL is obtained from the ΔA510 nm/20 min, by reference to the standard curve 2000 = conversion from 0.5 mL as assayed to 1 L, 1/1000 = conversion from mU to U D = dilution factor (*i.e.* if sample is diluted 10-fold, D=10)
The activity (U/g) is calculated from the amount weighed as follows:

$$\text{Glucose oxidase activity (U/g)} = \frac{GOX\,(U/L)}{weight\ of\ sample}$$

Antimicrobial Activity: The antimicrobial activity was performed by agar well diffusion method [29] for Honey extract. Eight species known to be pathogenic to human such as *Candida albicans ATCCMYA-2876*; *Clostridium botulinum ATCC 3584*; *Escherichia coli BA 12296;Klebsiella pneumoniae ATCC12296; Salmonella senftenberg* ATCC 8400; *Staphylococcus aureus* NCTC 10788; *Staphylococcus epidermidis* and *Streptococcus dysgalactiae subsp. Equisimilis* were used. One hundred µl of the inoculum (1 x 10^8cfu/ml) was mixed with Hi-media and poured into the Petri plate. A well was prepared in the plates with the help of a cork-borer (0.85 cm). One hundred µl of the test compound was introduced into the well. The plates were incubated overnight at 37°C. Microbial growth was determined by measuring the diameter of zone of inhibition. For each bacterial strain controls were maintained as pure solvents were used instead of the extract. The result was obtained by measuring the zone diameter (mm). The experiment was done three times and the mean values were presented.

Minimal Inhibitory Concentration (MIC): MIC for Honey samples were performed by modified agar well diffusion method. Two-fold serial dilution of the stock solution was prepared in sterilized distilled water to make a concentration range from 12.5-400 mg/ml [30, 31]. One hundred µl of the inoculum (1 x 10^8cfu/ml) was mixed with Hi-media and poured into the Petri plate. Different honey samples from Egypt, Saudi Arabia, Yemen and Kashmir (400, 200, 100, 50, 25, 12.5 mg/ml) were added to the wells. In each of these plates six wells were cut out using a standard cork borer (0.85 mm). Using a micropipette, 100 µl of each dilution was added in to wells. All the plates were incubated at 37°C for 24 hours. Antimicrobial activity of honey was evaluated by measuring the zone of inhibition. Experiment was carried out in triplicates for each test organism.

Statistical Analysis: All analyses were carried out in triplicates and the data were presented as means± standard deviations. Analysis of variance (ANOVA) was used to compare the quantified variables in the samples of honey. The significance was calculated for $P < 0.05$. The statistical analyses were performed with the SPSS Statistic.

RESULTS AND DISCUSSION

Total Phenolic Content and Flavonoids: The total content of phenolic compounds in the honey samples varied from 24.88±0.57 to 67.66±0.58 µg GAE/g (Table 1). The highest content of phenolic compounds was found in Saudi honey samples (the highest color intensity) (P>0.05), while the lowest amount of phenolic compounds was found in Yemeni honey sample (the lowest color intensity). While the total phenolic content in Egyptian honey sample was 39.66±0.59 µg GAE/g and was 41.66±0.71µgGAE/g in Kashmiri sample. The same trend was remarked in the total flavonoid content, where the Saudi honey showed the highest flavonoids content (13.49±0.33 µg/g) while Yemeni honey showed the lowest value (4.21±0.34 µg/g). The total flavonoids in Egyptian honey was (5.63±0.48 µg/g) and 8.99±0.41µg/g in the Kashmiri Honey. A correlation coefficient was found between the color of honey and the content of its phenolic compounds and flavonoids (P<0.05) (Table 2). The variations in the level of phenolic and flavonoids content may refer to the geographic origin of the honey.

Table 1: Total phenolic, flavonoids and glucose oxidase activity

Honey types	Total phenolicµg GAE/g	Flavonoidsµg/g	GOX (U/g)
Eg	39.66±0.59	5.63±0.48	24.60± 0.70
Yem	24.88±0.57	4.21±0.34	17.11±0.19
Sau	67.66±0.58	13.49±0.33	02.07±0.02
Kash	41.66±0.71	8.99±0.41	37.67±0.14

The values were expressed as mean of triplicates ± SD (P<0.05)

Table 2: Correlation coefficient

Parameters	Corr (r)	S.E. of r	P (r=0)
TP and IC_{50}	-0.95310936	0.21398895743	0.0469*
TF and IC_{50}	-0.95588289	0.20771121616	0.0441*

Total phenolic (TP), Total Flavonoids (TF) and (IC_{50})

Glucose oxidase

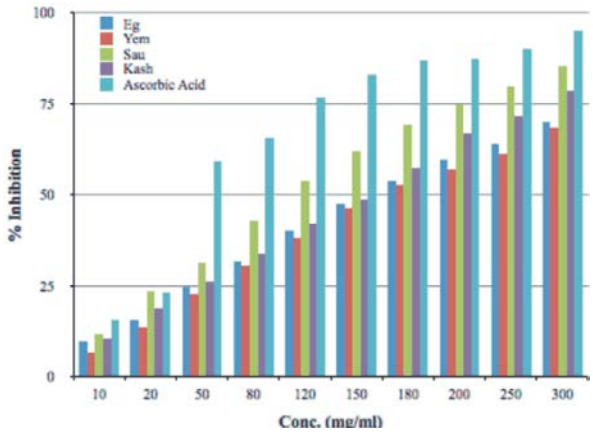

Fig. 1: DPPH-Antioxidant activity

DPPH Free Radicals Scavenging Activity: In evaluating the radical-scavenging potential of honeys, the DPPH assay is frequently used because the antioxidant potential of honey has been shown to be directly associated with its phenolic acids and flavonoids content [14], where high DPPH scavenging activity confers superior anti-oxidant activity. In this study the Saudi honey showed the highest percentage inhibition (IC_{50}=111.88mg/ml) followed by Kashmiri (IC_{50}= 157.40 mg/ml) and Egyptian (IC_{50}= 167.69 mg/ml), while the Yemeni samples showed the lowest percentage inhibition (IC_{50}=170.42 mg/ml) (Fig. 1). The obtained results confirmed the percentage inhibition of the honey samples associated with their phenolic and flavonoid content. These results agreed with the previously reported by Moniruzzaman [32] and Mohamed [17].

Glucose oxidase is an enzyme in honey that originates from bees. It plays a part in the formation of honey in the hive: It oxidizes glucose in the unripened honey. It yields gluconolactone, which equilibrates with gluconic acid, the principal acid of honey.

D-glucose + H_2O + O_2 → D-glucono-δ-lactone + H_2O_2: It also yields hydrogen peroxide, which contributes to the antibacterial properties of honey [33, 34]. Glucose oxidase is an active enzyme in nectar but is virtually inactive in honey. The enzyme may become active again if the honey is diluted. This enzyme is sensitive to light and heat. In the present study the glucose oxidase activity in Egyptian honey was (24.60±0.70 U/G), in Yemeni honey was (17.11±0.19 U/G), in Saudi honey was (02.07±0.02 U/G) and (37.67±0.14 U/G) in Kashmiri honey (Table 1). From the obtained results Egyptian and Kashmiri honey contained the highest glucose oxidase activity while Saudi honey is very low in glucose oxidase activity this may cousd by the effect of handling conditions. The production of gluconic acid and H_2O_2 is very slow in ripe honey and most of this production takes place, as the honey is being ripened and dried by the bees. If honey is diluted then this reaction speeds up again. This is an important factor that greatly affects the antibacterial property of a honey. At this time we have no published data on the half-life of GOX, but references to its stability indicate that this is highly variable. So, according our results the Saudi and Yemeni honey most ripened and dried than Egyptian and Kashmiri. On the other hand, Kashmiri and Egyptian released H_2O_2 and have antibacterial activity more than Yemeni and Saudi. Glucose oxidase catalyses a reaction in which produces hydrogen peroxide. This reaction effectively consumes the glucose while also exhibiting significant activity against harmful organisms due to the production of hydrogen peroxide. This is a crucial part of its digestive support, as well as its multiple health benefits [35].

Antimicrobial Activity: The inhibition zone diameter of different Egyptian Saudi Arabia, Yemen and Kashmir honey concentration (400 mg/ml) were determined for *Candida albicans ATCCMYA-2876*; *Clostridium botulinum ATCC 3584*; *Escherichia coli BA 12296; Klebsiella pneumoniae ATCC12296; Salmonella senftenberg* ATCC 8400; *Staphylococcus aureus* NCTC 10788; *Staphylococcus epidermidis* and *Streptococcus dysgalactiae subsp. equisimilis* by agar well diffusion (Figure 2). The highest inhibition zone (33±1.06 mm) was recorded for Kashmiri honey against *Staphylococcus epidermidis* at the concentration of 400 mg/ml while Egypt honey showed slightly lower inhibition zone (28±1.20mm) at the same concentration. The all type of honeys had more inhibitory growth effect against *Clostridium botulinum ATCC 3584* at 400mg/ml concentration with inhibition zone diameter 25±1.15, 20±0.82, 23±1.01 and 27?11.23 mm respectively (Fig. 2).

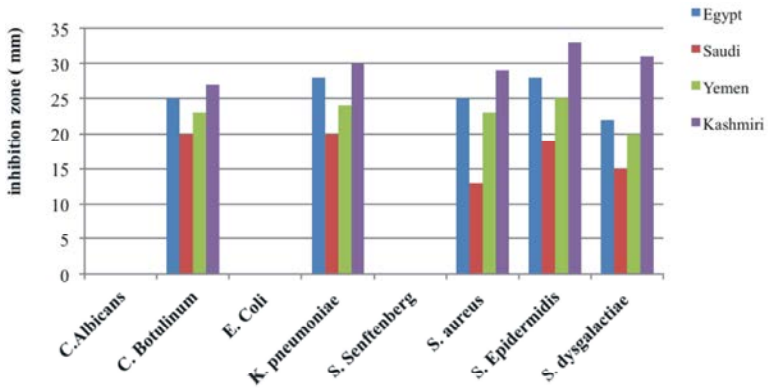

Fig. 2: Antimicrobial activity of different types of honey against gram-positive and gram-negative bacteria.

Table 3: Minimum inhibitory concentration (MIC) of honey against different microorganism.

Strains	Egyptmg/ml	Saudimg/ml	Yemenmg/ml	Kashmirimg/ml
*Candida Albicans*ATCCMYA-2876	0	0	0	0
Clostridium BotulinumATCC 3584	100±4.13	12.5±1.62	400±5.27	12.5±2.18
Escherichia Coli BA 12296	0	0	0	0
Klebsiellapneumoniae ATCC12296	400±3.74	400±6.35	12.5±1.89	12.5±2.27
Salmonella senftenberg ATCC 8400	0	0	0	0
Staphylococcus aureus ATCC 25923	100±2.34	200±4.28	400±4.86	0
Staphylococcus Epidermidis	400±6.25	12.5±1.68	200±3.19	25±2.56
Streptococcus dysgalactiae	0	100±2.34	400±5.23	12.5±2.45

The inhibition zone diameter of different Egyptian Saudi, Yemen and Kashmir honey against *Klebsiella pneumoniae* ATCC12296 ranged from 20-30 mm at concentration of 400 mg/ml while against *Staphylococcus epidermidis*, was ranged from 19-33 mm. *Candida albicans ATCCMYA-2876, Escherichia coli* BA 12296 and *Salmonella senftenberg ATCC 8400* were resistant to all kind of examined honey samples at 400 mg/ml concentration. The anti microbial activity showed a strong linear relationship with glucose oxidase activity; it may refer to production of H_2O_2 by glucose oxidase and caused a harmful effect against pathogenic bacteria.

Determination of Minimum Inhibitory Concentration (MIC): MICs of all the for Egyptian Saudi, Yemen and Kashmir honey brands were tested against *Candida albicans ATCCMYA-2876*; *Clostridium botulinum ATCC 3584*; *Escherichia coli BA 12296; Klebsiella pneumoniae ATCC12296; Salmonella senftenberg ATCC 8400; Staphylococcus aureus NCTC 10788; Staphylococcus epidermidis and Streptococcus dysgalactiae subsp. equisimilis*. It was observed that all the honey samples were having substantial antibacterial activity at least against five microorganism species. Based on the MICs, it was also observed that all the honey was sensitive to *Clostridium botulinum ATCC 3584*, *Klebsiella pneumoniae* ATCC12296, *Staphylococcus epidermidis* and *Streptococcus dysgalactiae* with Saudi and Kashmiri honey having the lowest MIC (Table 3). In our study, four types of honey samples were tested for their antimicrobial activity on *Candida albicans ATCCMYA-2876*; *Clostridium botulinum ATCC 3584*; *Escherichia coli BA 12296;Klebsiella pneumoniae ATCC12296; Salmonella senftenberg ATCC 8400; Staphylococcus aureus NCTC 10788; Staphylococcus epidermidis and Streptococcus dysgalactiae subsp*. equisimilis. The present study showed varying degree of *in vitro* growth inhibition activity of Egyptian, Saudi, Yemeni and Kashmiri honeys against tested organisms. These might be due to the osmotic effect, the effect of pH and the sensitivity of these organisms to hydrogen peroxide, which are unsuitable for bacterial growth, represented as an inhibition factor in honey [36, 37]. All the different types of honey samples showed growth inhibitory activity against *Clostridium botulinum ATCC 3584*; *Klebsiella pneumoniae* ATCC12296; *Staphylococcus aureus NCTC 10788; Staphylococcus epidermidis and Streptococcus dysgalactiae subsp. equisimilis*. *Escherichia coli BA* was resistant to Egyptian, Saudi, Yemeni and Kashmiri

honeys. This contrasts with the result reported by Hegazi [38] and Hegazi and Fyrouz [39] who reported that the different types of Saudi honey were less inhibitory against *E. coli* than other bacteria. Data obtained from the agar well diffusion and Minimum Inhibitory Concentration (MIC) has demonstrated, for the first time, that Kashmiri honey exhibits a stronger antimicrobial effect against three out of eight bacterial tested compared with Egyptian, Saudi and Yemeni honey. Using the agar diffusion method, on average, Kashmiri honey displayed largest zone of inhibition against *staphylococcus epidermidis* strain (Fig. 3). The different concentrations of the four honey samples had good growth inhibitory effect on the tested microorganisms. Similar result was previously reported by Alqurashi [40] for *Klebsiella pneumonia* [41] for *Staphylococcus aureus*, the MIC assay showed that a lowest MIC was observed with Kashmiri honey (12.5 mg/ml) for the tested microorganisms while other type of honey ranged from 100-400 mg/ml. The present findings supported by Kwakman [42]. Our results further show that there was an increase of inhibition zone for the tested microorganisms with increase in the concentration of honey but there is no relation between the antimicrobial activity and phenolic contents and flavonoids in examined honey samples.

CONCLUSION

The present study aimed to investigate and evaluate of functional properties of local and imported honey to confirm its economical and nutritional importance. All honey samples contained a reasonable concentration of phenolic content and flavonoids with considerable antioxidant activity. The Kashmiri honey showed the highest glucose oxidase activity, while Saudi honey showed the lowest activity. Antibacterial activity was increased with increasing the concentration of honey and glucose oxidase activity but there is no relation found between antimicrobial activity and phenolic compounds or flavonoids contents. These results confirmed the value of honey as functional food based on the source of honey.

ACKNOWLEDGEMENT

Authors are thankful to the Department of Food Technology, Arid Lands Cultivation Research Institute at City for Scientific Research and Technological Application, Alexandria, Egypt for providing the chemicals and facilities of this research work. The authors are thankful for the junior staff and technicians for their help in this work.

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