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The Antioxidant Potency and Hydrogen Peroxide Release Pattern in Some Libyan Floral Honeys

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ABSTRACT

The antioxidant and antibaterial properties of honey have been well documented. Hydrogen peroxide is the main antibacterial factor, whereas flavonoids and other phenolic contents are the main antioxidant components in honey. Different honeys have different charactaristics according to their botanical and geographical origin. In this study six different common Libyan honeys namely: Clover (C), Hanon (H), Nabk (N), Thyme (Th), Tamarix (Ta), and Multi-floral (Mf) honey samples were evaluated for their hydrogen peroxide (H_2O_2) level using Merckoquant peroxide test, and for their antioxidant capacity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) & ferric

reducing antioxidant power (FRAP) assays. The obtained data showed that the maximum levels of H_2O_2 accumulated in the tested honey samples were varied from 0.15 to 1.03mM. The anti-radical property (DPPH assay) expressed as IC_{50} for Hanon, Nabk, Tamarix, Clover, Multi-floral and Thyme honey were 25.2±3.6, 32.6±7.2, 34.6±6.8, 40.4±7.2, 48.3±10.2 and 53.9±4.8 mg/ml respectively; The total antioxidant power (FRAP) values (µM Fe II) were arranged descendingly as follow: 729±24(H), 577±61(Ta), 501±40(N), 435±23(Th), 390±13(Mf), 374±08(C). We can conclud that the local honeys examined in this study have antioxidant potential and H_2O_2 levels comparable to foreign honeys and could be used as a good natural antioxidant source and a powerful therapeutic agent.

Keywords: Honey, Antioxidant, Hydrogen peroxide, DPPH, FRAP, Libya.

1. INTRODUCTION

Honey has a long history of use as an effective medicine since ancient times for a wide range of illness and diseases. In the modern medicine, many practitioners have reported the effectiveness of honey in the healing of various types of infected wound, burnes, skin ulcers(1, 2), gastrities and duodenal ulcer(3). Much of the effectivness of honey in many of its therapeutic uses is attributed to its antibacterial activity. Honey has been shown to be effective againest bacterial strains including those that have become resisitant to synthetic antibiotics(4,5,6). The antibacterial mechanism of honey was attributed to its high osmolarity, low pH, hydrogen peroxide (H_2O_2) content(4,7,8), and non-peroxide components such as flavonoids(9), polyphenolic acids(10), aromatic acids(11), and methylglyoxal(12). Hydrogen peroxide was identified as the major source of antibacterial activity in honey(4). It is a by-product of glucose oxidation catalyzed by the bee-derived enzyme glucose oxidase.

The rate of the reaction increases with honey dilution, which facilitates access of the enzyme to its substrate (glucose). The continous production of H_2O_2 in the diluted honey produces a long-lasting antiseptic effect that is most sought after in fighting infections in wounds. In most honeys, the antibacterial activities strongly depend on the presence of H_2O_2(8,13,14) The level of hydrogen peroxide is proportional to relative levels of glucose oxidase enzyme(15), and catalase enzyme originating from pollen(16). Honey samples from different floral sources showed considerable varietion in their H_2O_2 content as well as in their antibacterial activity, which is significantly correlated with the H_2O_2 level(8,15).

Oxidative stress has been considerd a mechanism involved the pathogenesis of cancer, ischemic heart disease, diabetes, atherogenesis and other chronic diseases. The use of functional food that contains phytocompounds such as antioxidants that can act as prophylactics againest such diseases has received considerable attention by academia, food industry and the general public. İt is shown that the therapeutic potential of honey is highly associated with its antioxidant capacity againest reactive oxygen species(17). Honey contains a significantly high level of antioxidents, both enzymatic and non-enzymatic, including catalase, phenolic acids, flavonoids, carotenoids and ascorbic acid(18, 19, 20-22). According to Aljadi and Kamaruddin (18), the antioxident activity of honey is highly correlated with its phenolic acids and flavonoids content. Many researchers have indicated that the antioxident activity of honeys were widely varied depending on the floral sources and geographical origins(22-26). The potanical origin of honey is reported to have the greatest influence on its antioxidant activity, while processing, handling and storage have a low effect(22, 23, 26).

In recent years, Several studies on the identification and quantification of the biological properties of honey such as antibacterial, antioxidant,

And antiinflammatory activities have been reported all over the world (4, 15, 23, 27). However, there is limited data available for Libyan honey despite its high consumption rate by the general public. Thus, the aim of the present study was to evaluate the hydrogen peroxide level and the antioxidant capacity of some libyan honeys from different botanical sources and geographical origins, in an effort to predict their therapeutic values.

2. MATERIALS AND METHODS:

Honey Samples: Six different types of Libyan honey samples from different geographical regions were used in this study.They are named: Clover , Mountainy Hanon , Nabk , Thyme , Tamarix , and Multi-Floral honey, according to their floral sources (Table 1). They are of the most a bundant and widly consumed Libyan honeys. Samples were collected directly from beekeepers, and kept in air-tight containers at room temperature (22-25 ^{o}C) before use.

1.2. Determination of Hydrogen peroxide(H_2O_2) level: hydrogen peroxide level was determined using Merckoquant peroxide test procedure (1.1001; Merck, Darmstadt, Germany) as described by Cooke et al.(4). 10g honey was dissolved in 40ml of deionized water and then incubated at 37 ^{o}C. Accumulated hydrogen peroxide was measured according to the manufacturer's instructions at different time intervals.

2.2. Free radical scavenging activity: The method used by Chua et al.(25) and Tahmasbi et al.(23) for the assay of free radical scavenging activity of honey was follwoed in this study. İn the presence of an antioxidant, the purple colour of 1,1-diphenyl-2-picrylhydrazyle(DPPH) decays, and the change of absorbence can be measured spectrophotometrically at 517nm. Briefly; 0.75 ml of honey solution at different concentrations, ranged from 20-40 mg/ml**,** was mixed with 1.5 ml of 20 mg/L of DPPH(Sigma Aldrich, St. Louis,MO, USA) in methanol. The mixture was then incubated for 15 minutes at 25 ^{o}C; and the absorbance was measured spectrophotmetrically at 517 nm.

Ascorbic acid (0-10mg/L) in methanol was used as a positive control,wheras methanol was used as a blank sample. The percentage of DPPH scavenging activity(% inhibition) was calculated using the following formula: DPPH scavenging activity(%) = $[(A_{control} - A_{sample}) / (A_{control})] \times 100$. Where A_c is absorbance of blank sample, A_s is absorbance of sample. The concentration of honey sample required to scavenge 50% of DPPH(IC_{50}) was determined based on the ascorbic acid calibration curve. Measurements were done in triplicate.

Table 1. Details of the honey samples.

Honey Sample	Common or local Name	Botanical Source	Geographical Location of Collection
Clover	Alfalfa	*Trifolium spp.*	Southern region
Mountainy Hanon	Hanon(myrrh)	*Arbutus spp.*	Eastern region
Nabk	Sidir	*Ziziphus spina spp.*	Western region
Thyme	Zatter	*Thymus spp.*	Western region
Tamarix	Tamarisk(Athel)	*Tamarix spp.*	Southern region
Multi-floral	---	Mixed sources	Southern region

3.2. Total Antioxidant Power : The ferreic reducing antioxidant power (FRAP) assay that was originally developed by Benzie and Strain (28) and minoraly modified by Tahmasbi et al.(23) as a direct method for measuring the total antioxidant power was followed in this study. Basically, at acidic pH, reduction of a ferric 2,4,6-tri(2-pyridyl)-1,3,5-triazine (Fe^{3+}-TPTZ) complex to its ferrous coluord form (Fe^{2+}-TPTZ), in the presence of antioxidants, can be monitered spectrophotometrically by measuring the change of absorbance at 593 nm. The FRAP reagent prepared by mixing 2.5 ml of a 10 mM TPTZ (Sigma Aldrich, St. Louis, MO, USA) solution in 40 mM HCl, 2.5 ml of 20 mM $FeCl_3$ and 25 ml of 0.3 acetate buffer, pH3.6. It was prepared daily and was warmed to 37 ^oC before use.

Honey solutions (0.1 g/ml) in n-hexane-acetone mixture (6:4) were well prepared and filtered through whatman no. 4 filter paper. An aliquot of 400 ml of honey solution was mixed with 3.6 ml of FRAP reagent, and the absorbance was measured specrtophotometrically at 593 nm after incubation at 37^{o}C for 10 min. The total antioxidant capacity of samples was determined againest standard solution of $FeSO_4.7H_2O$ (1-10 mM)(Merck, Darmstadt, Germany), and the results were expressed as μM Fe(ll). For each sample, measurments were performed in triplicate.

4.2. Statistical Analysis: data is presented as mean ± Standard deviation of three determinations. The significant differences were obtained by T- test and a one-way analysis of variance (ANOVA). They were calculated using microsoft office excel 2007 and spss version 18.0. Differences at $P<0.05$ were considered statistically significant**.**

3. RESULTS AND DISCUSSION:

3.1. Hydrogen peroxide level: The pattern of hydrogen peroxide (H_2O_2) release in all honey samples was followed for a period of 24 hours as shown in table(2). Maximum accumulation occurred after 1 hour of incubation for all honey samples except for Tamarix honey which showed maximum accumulation after 2 hours of incubation. By comparison of the levels of H_2O_2 accumulated in each of the honey samples, Tamarix honey had the highest level (1.03mM), which was slightly decreased(0.8mM) after 24 hours of incubation; whereas M Hanone honey had the lowest level (0.15mM), which was returned to 0 time level (0.015mM) after 24 hours of incubation. Although there were differences between the six honeys tested, all honeys accumulated significant levels of hydrogen peroxide that are consistent with levels obtained for different honeys in other studies. In a screening of 90 honeys, levels ranging from 0 –2.12 mmol were recorded for samples diluted to 14% and incubated for 1 hour (29).

A similar study of 31 samples showed values ranging from 0–0.95 mmol (30). Another study showed a maximum levels of H_2O_2 in the range of 1–2 mM(31). Levels ranged from 30-240µmol/L were observed in some Canadian honeys(8). Maximum accumulation of 104µM and 57µM of H_2O_2 was observed in Malaysian Gelam and Coconut honeys respectively(32). Revamil honey, which is one the major medical grade honeys approved for clinical application, produces 22.5µg/ml of H_2O_2 in 30% dilution after 2 h and up to 148µg/ml after 24 h incubation, whereas manuka honey did not accumulate detectable amount (33).

Table 2. The release pattern of hydrogen peroxide (H_2O_2) in different honey samples diluted with deionized water (20% w/v) and incubated at 37°C. Results expressed as mM at the indicated time. values are means of 3 different measurements.

Honey Sample	Time of incubation (hours)					
	0 time	1(hr)	2(hr)	6(hr)	12(hr)	24(hr)
Clover	0.09	0.21	0.09	0.03	0.03	0.015
Mountainy Hanon	0.015	0.15	0.09	0.03	0.015	0.015
Nabk	0.03	0.3	0.3	0.3	0.21	0.09
Thyme	0.015	0.3	0.3	0.3	0.3	0.15
Tamarix	0.04	0.3	1.03	0.9	0.9	0.8
Multi-floral	0.09	0.3	0.3	0.09	0.04	0.015

Variations in the hydrogen peroxide level among different honeys is strongly related to the levels of glucose oxidase and catalase enzymes (34). Different honeys showed different glucose oxidase and catalase activities(15,16). Honeys from some floral sources with high levels of catalase showed low levels of H_2O_2, whereas those accumulating high levels of H_2O_2 had low levels of catalase(35).

A Strong correlation was found between the content of glucose oxidase and the level of generated H_2O_2 in honeys(15). On the other hand, other factors which have been found to reduce H_2O_2 accumulation in honey include vitamin C, flavonoids, phenolic compounds and some metals such as iron(36).

H_2O_2 is an essential factor in the total antibacterial activity of most honeys; A strong correlation was found between the antibacterial activity of honeys and their H_2O_2 contents(4, 8, 31). Despite this fact, to the best of our knowledge, there is no data about the H_2O_2 concentration in Libyan honeys. Results obtained in the present study showed that the H_2O_2 content in the tested Libyan honey samples varied from 0.015-1.03mM (at the indicated time intervals; table:2). These values are comparable to those reported on honeys from other countries especially those possess antibacterial activity(4, 8, 15); sugesting that the tested honeys especially "Tamarex honey" may have a great valuable antibacterial potency.

Bogdanove (30) reported that the 20% growth inhibition of S. aureus was observed after 16 hours incubation with H_2O_2 at 0.12mM. On the other hand, Hyslop et al.(37) found that the mean generation time of E. coli dramatically increased in the presence of continuous concentration of H_2O_2 between 25-50µM, and complete bacteriostasis was observed at continuous level of 100 µM; they also observed that exposure to continuous level of H_2O_2 at 500-1000µM resulted in time dependent killing of bacteria. Similarly, honey has been shown to be an effective antimicrobial agent by supplying low levels of hydrogen peroxide to wounds continuously over time rather than as a large amount at the time of dressing(31). Honey samples tested in this study, especially Tamarix honey, appear to offer effective hydrogen peroxide levels over at least 24 hours, suggesting a high antibacterial activity, however more studies need to be carried out to evaluate the antibacterial activity of these honeys.

3.2. Radical scavenging activity: Determination of the radical scavenging activity(RSA) of each honey sample was carried out based on the scavenging activity against the free radical 2,2-diphenyl-1-picryl-hydrazyl (DPPH˙) through the IC_{50} parameter, which represents the concentration of the material in question necessary to inhibit 50% of free radicals. Thus, the lower IC_{50} value the higher the scavenging activity of honey, because it requires lesser amount to reduce DPPH. This method was chosen because it is an easy, accurate (38), and is commonly used to evaluate the antioxidant activity of honey samples (17, 39 - 42). The mean values ± SD of the DPPH radical scavenging activity of different honey samples are shown in table(3). The IC_{50} values ranged from 25.2±3.6 to 53.9± 4.8 mg/ml. Mountainy Hanon honey showed the highest RSA activity(25.2mg/ml) followed by Nabk honey(32.6mg/ml), whereas Thyme honey showed the lowest activity(53.9 mg/ml). Our results are in agreement with that reported by some other researchers. The DPPH radical scavenging activity (RSA) expressed as IC_{50} was varied from7.2 to 53.8 mg/ml in Slovenian honey (42), from 5.99 to 89.66 mg/ml in Iranian honey (23), and from 4.2 to 106.72 mg/ml in Brazilian honey (43). Another study showed that the mean IC_{50} values for Malaysian Gelam, Acacia, and Tualang honeys were 15.7, 29.8 and 48.9 mg/ml respectively(25). In a similar study conducted by Beretta et al. (44), the antioxidant values ranged from 1.63 to 47.62 mg/ml. The present findings showed that the Libyan honey samples examined in this study have antioxidant potential comparable to honeys from other countries.

Table 3. DPPH radical scavenging activity, and ferric reducing antioxidant power (FRAP) of different honey samples.

Honey Sample	DPPH values mean ± SD (mg/ml)	FRAP values mean ± SD (µM Fe (II)/100g)
Clover	40.37±7.2[a]	374±08[a]
Mountainy	25.22±3.6[b]	729±24[b]

Hanon		
Nabk	32.63±7.2^{c}	501±40^{c}
Thyme	53.9±4.8^{d}	435±23^{d}
Tamarix	34.61±6.8^{ac}	577±61^{e}
Multi-floral	48.31±10,2^{d}	390±13^{a}

In each column different letters indicate significant differences ($p < 0.05$).

3.3. Ferric Reducing Antioxidant Power (FRAP): The FRAP assay is a simple, direct test that is widely used to test the total antioxidant potential; this test estimates the amount antioxidants or reductants present in a sample based on their ability to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) compounds (28). The total antioxidant power (FRAP)values for the investigated honey samples were between 374 and 729 µM Fe (II) (Table 3). Mountainy Hanon honey showed the highest total antioxidant potential (729 µM Fe (II)) followed by Tamarix honey(577 µM Fe (II)), whereas Clover honey showed the lowest activity(374 µM Fe (II)). The results obtained in the present study indicated that the FRAP values for the tested Libyan honeys are comparable with those obtained by other researchers. Similar studies on Malaysian honeys showed values ranged from 209.78 to 653.75 µM Fe (II) (18, 24). Another study on Malaysian Acacia honey conduted by Moniruzzaman et al. (21) obtained results ranged from 193.3 to 379.5 µM Fe (II). Honey samples from Iran had a total antioxidant potential ranged from 2.335 to 10.737 mM Fe (II)(23). The FRAP values for Algerian honey samples ranged from 287.5-403.5 µM Fe (II)(45). İt is noteworthy that the total antioxidant potential of Mountainy Hanon honey(729µM Fe (II)) is higher than that reported in Manuka honey(648.25 µM Fe (II)(24). Manuka honey from the manuka tree (*Lepto-spermum scoparium)*, a native of New Zealand, It is well known for various medicinal, antioxidant, antibacterial and antifungal properties(46).

The results obtained in the present study (Table 3) showed that all the tested samples were antioxidantively active, however the free radical scavenging activity and total antioxidant potential varied among the honey types. Some previous works have indicated that the antioxidant activity of honeys were widely varied depending on the floral sources and geographical origins (18, 21-23, 25, 26). The botanical origin of honey has the greatest influence on its antioxidant activity, while processing, handling and storage affect honey antioxidant activity only to a minor degree (22, 23, 26). The antioxidant activity is strongly correlated with the content of total phenolics (18, 25, 26). Beside that, a strong correlation was found between antioxidant activity and the color of honey. Many researchers found that dark honey has a higher total phenolic content and consequently a higher antioxidant capacity (21, 25, 45). It has been proposed that the antioxidant capacity of honey is due mainly to the phenolic acids and flavonoids they contain, and there is a high correlations between polyphenols and honey antioxidant capacity(18, 24, 26). There are more than 150 polyphenolic compounds that have been reported, including phenolic acids, flavonoids, flavonols, catechins and cinnamic acid derivatives (10, 17, 18). The composition and quantity of these components vary widely according to the floral and geographic origin of the honey, which explains the variations in the antioxidant activity of different honeys (24).

4. CONCLUSION

Numerous studies had been conducted on the antibacterial, antioxidant and anti-inflammatory properties of honey, but no similar study has been found on the hydrogen peroxide content or antioxidant capacity especially of Libyan honeys. This makes the present study, the pioneer in regards to locally harvested honeys. Both Mountainy Hanon honey and Tamarix honey were found to have strong antioxidant capacities, while Tamarix honey have the highest hydrogen peroxide levels among samples. Hydrogen peroxide is known to be a good predictor for the antibacterial property of honey, therefore,

Tamarix honey is a strong antibacterial because of its high H_2O_2 content. The results of the present study will promote beekeeping in Libya by increasing the commercial value of these honeys as a functional food and as a therapeutic agents. It is recommended however, that further studies be made in identifying and quantifying the antibacterial and antioxidant properties of Libyan honeys.

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Author Contributions

Conceived and designed the experiments: A. Aljadi, O. Almrhag. Performed the experiments: A. Aljadi, M. Hassan. Analyzed the data: N. Brekaw. Contributed reagents/ materials/analysis tools: M. Hassan, N. Brekaw. Wrote the paper: A. Aljadi. Critical review of the article: O. Almrhag.

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