Evaluation of Antioxidant and Antibacterial Activities and Essential Elements Content of Locally Produced Honey in Saudi Arabia

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Abstract: The aim of this study was to characterize antioxidant and antibacterial activities and essential elements content of Saudi honey marketed in Jeddah, Saudi Arabia. The different contents of flavonoid and phenolic compounds correspond to honey sample. The evaluation of antioxidant activity of methanol extract of honey samples was conducted by several methods. Honey samples extracts showed a concentration dependent on the scavenging of DPPH, ABTS radicals and the formation of phosphate/ molybdenum complex. Honey samples showed inhibitory activity with MIC values ranged between 2×10^{-5} to 2×10^{-2} µg honey phenolic content when tested against multi-drug resistant bacteria, namely, vancomycin resistant *enterococci*, methicillin resistant *Staph. aures* 4 and methicillin resistant *Staph. aures* 2. Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Sr and Zn were determined in all tested honey samples. The highest level of elements in all tested honeys are in the order of Al > Pb > Cu. In conclusion, the antioxidant and antimicrobial properties in addition to the essential elements content make Saudi honey as high added value product.

Keywords: Honey, Phenolics, Flavonoids, Antioxidant, Antibacterial, Elements, Saudi Arabia

1. Introduction

Honey is considered as a complex matrix, which consists mostly of a mixture of carbohydrates (e.g., fructose, maltose, sucrose), and several other substances, such as traces of other sugars, organic acids, enzymes, amino acids, pigments, pollens, and wax (Al *et al*., 2009). The composition of honey is largely depending on the floral source and maturation. Honey also contains several mineral substances up to 0.17%. Some of these are present at trace levels and being toxic (e.g., As, Cd and Pb) (Kücük *et al*., 2007).

It has been reported that the presence of flavonoids may contribute to the antioxidant effects observed in some honeys [Aljadi and Kamaruddin, 2004; Al-Mamary *et al*., 2002]. Some studies have shown that flavonoids have a scavenging effect on free radicals by different mechanisms (Cos *et al*., 1998). Phenols are very efficient scavengers of peroxyl radicals (Aruoma, 1994) because of their molecular structures which include an aromatic ring with hydroxyl groups containing mobile hydrogen. Moreover, the action of phenolic compounds can be related to their capacity to reduce and chelate ferric ions which catalyze lipid peroxidation (Gazzani *et al*., 1998). Honey has been known to have therapeutic and antimicrobial properties. The main factors responsible for the antimicrobial activity are phenolic compounds including phenolic acids and flavonoids [Allen *et al*., 1991; Isla *et al*., 2011; Molan, 1992; Weston, 2000; Weston and Brocklebank, 2000). Phenolic antioxidants are known to inhibit growth of a wide range of gram-negative and gram-positive bacteria (Davidson, 1993). The antioxidant and antibacterial capacities of different honeys exhibit a high variation according to their floral sources (Frankel *et al*., 1998).

Honey is locally produced in different regions in Saudi Arabia, hence there are few physicochemical and bioactivity studies of Saudi honeys in the literature. In this work, we characterize the Saudi marketed honeys in respect to their antioxidant and antibacterial activities and their elements content.

2. Materials and Methods
2.1. Honey samples

Ten locally produced honey samples of multi-floral and uni-floral origins were collected from local market in Jeddah, Saudi Arabia. These samples were Baha Sidr, Konfthah Sidr, Aga Gabal, Shokp Wady, Hegaz Rabia, Tanhat, Aswad Kors Sidr, Asir Mahyl Shoka, Abyd Kors Sidr and Qaseem Rabia honeys.

2.2. Preparation of honey extract

Two grams of each honey sample was extracted by shaking at 150 rpm at 25 ºC for 24 h with 80 % methanol and filtered through filter paper Whatman No. 1. The filtrate was designated as methanol extract.

2.3. Determination of the total phenolic and total flavonoid contents

Total phenolic content was measured according to Velioglu *et al*. (1998). Fifty µL of the methanol extract was mixed with 900 µL Folin-Ciocalteu reagent and

allowed to stand for 5 min at ambient temperature. An aliquot 500 μL of 20 % sodium carbonate was then added and allowed to react for 30 min. Absorbance was measured at 750 nm. Total phenols were quantified from a calibration curve obtained by measuring the absorbance of known concentrations of gallic acid. The results were expressed as mg gallic acid equivalent / gram honey.

The total flavonoid content of the samples was determined using a modified colorimetric method described previously by Zhishen *et al*. (1999) and used catechin as a standard. Methanol extract and standard solution (250 μL) were mixed with distilled water (1.25 mL) and 5 % $NaNO_2$ solution (75 μL). After standing for 6 min, the mixture was combined with 10 % $AlCl_3$ solution (150 μL). 1 M NaOH (0.5 mL) and distilled water (275 μL) were added to the mixture 5 min later. The absorbance of the solution was then measured at 510 nm. The results were expressed as mg catechin equivalent / gram honey.

2.4. DPPH radical scavenging activity

Free radical scavenging activity of crude methanol extract was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Ao *et al*., 2008). A methanol solution (100 μL) containing the methanol extract was added to 900 μL of freshly prepared DPPH methanol solution (0.1 mM). An equal amount of methanol was used as a control. After incubation for 30 min at room temperature in the dark, the absorbance was measured at 517 nm. Activity of scavenging (%) was calculated using the following formula:

DPPH radical scavenging (%) = [(OD control – OD sample) / OD control] x 100

The results were plotted as the (%) of scavenging activity against concentration of the sample. The inhibition concentration (IC_{50}) was defined as the amount of crude methanol extract required for 50 % of free radical scavenging activity. The IC_{50} value was calculated from the plots as the antioxidant concentration required for providing 50 % free radical scavenging activity.

2.5. ABTS radical cation decolorization assay

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) or ABTS also forms a relatively stable free radical, which decolorizes in its non-radical form. The spectrophotometric analysis of $ABTS^{*+}$ scavenging activity was determined according to the method of Re *et al*. (1999). In this method, an antioxidant was added to a pre-formed ABTS radical solution and after a fixed time period the remaining $ABTS^{*+}$ is quantified spectrophotometrically at 734 nm. $ABTS^{*+}$ was produced by reacting 7 mM ABTS in H_2O with 2.45 mM potassium persulfate ($K_2S_2O_8$), stored in the dark at room temperature for 16 h. The $ABTS^{*+}$ solution was diluted to give an absorbance of 0.750 ± 0.025 at

734 nm in 0.1 M sodium phosphate buffer (pH 7.4). Then, 1 mL of $ABTS^{*+}$ solution was added to the crude methanol extract. The absorbance was recorded 5 min after mixing and the percentage of radical scavenging was calculated in relative to a blank containing no scavenger. The extent of decolorization was calculated as percentage reduction of absorbance. The scavenging capability of test compounds was calculated using the following equation:

$ABTS^{*+}$ scavenging (%) = (1- AS / AC) x 100

AC is absorbance of a control (blank) lacking any radical scavenger and AS is absorbance of the remaining $ABTS^{*+}$ in the presence of scavenger.

The results were plotted as the (%) of scavenging activity against concentrations of the sample. The inhibition concentration (IC_{50}) was defined as the amount of crude methanol extract required for 50 % of free radical scavenging activity. The IC_{50} value was calculated from the plots as the antioxidant concentration required for providing 50 % free radical scavenging activity.

2.6. Phosphomolybdenum complex assay

Spectrophotometric evaluation of antioxidant activity through the formation of a phosphomolybdenum complex was carried out according to Prieto *et al*. (1999). Samples solutions were combined in Eppendorf tubes with 1 ml of reagents solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95 ºC for 90 min. After the samples had cooled down to room temperature, the absorbance of aqueous solution of each was measured at 820 nm against a blank. The antioxidant activity was expressed as the absorbance of the sample. EC50 value (μg phenolic compound) is the effective concentration at which the absorbance was 0.5 for the formation of phosphomolybdenum complex.

2.7. Human pathogenic test bacteria

Vancomycin resistant *enterococci* (VRE), Methicillin resistant *Staph. aures* 4 (MRSA 4) and Methicillin resistant *Staph. aures* 2 (MRSA 2) were obtained from King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia.

2.8. Measurement of the antibacterial activity of honey phenolic extract

In this investigation agar well diffusion method was employed (Weston *et al*., 1999). Suspensions of bacterial isolates were prepared to turbidity of McFarland Standard No. 0.5. Aliquots of 100 μL of these suspensions were inoculated onto Mueller-Hinton agar plates using the spread plate method. Five wells were made on the inoculated agar medium using a sterile cork borer (diameter 5 mm). 90 μL of the honeys phenolic extracts dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) were pipetted into their designated wells on

the agar plates. All inoculated plates were then incubated at 37 °C for 24 h. After incubation, each formed inhibition zone's diameter was measured in millimeters (mm) using a ruler.

2.9. Elements content determination

Five grams of each honey sample was heated in a porcelain crucible at 600 ºC for 16 h. 10 mL of distilled water was added and the mixture was filtered by filter paper Whatman No. 1. The filtrate was analyzed by ICPE-9000 spectrometer (Shimadzu) for elements determination.

3. Results and Discussion

The total phenolic and total flavonoid contents of different honeys, which were from different regions of Saudi Arabia, are shown in Table 1. A great variability in terms of total phenolic and total flavonoid contents was observed among the different honeys. Generally, phenolic contents were higher than flavonoid contents, except some honeys. The phenolic contents ranged from 0.1 to 0.53 mg gallic acid equivalent / gram honey. Aga Gabal honey had the highest phenolic content, where Abyd Kors Sidr had the lowest content. The flavonoid contents ranged from 0.006 to 0.441 mg catechin equivalent / gram honey. Similar to phenolic contents, Aga Gabal honey had the highest flavonoid content, where Abyd Kors Sidr had the lowest content. Great variations in terms of phenolic contents have also been reported among different honeys from different countries (Meda *et al.*, 2005; Vit *et al.*, 2008).

The evaluation of antioxidant activity of methanol extract of honey was conducted by several methods. Scavenging the stable DPPH radical model is a widely used method to evaluate antioxidant activity. The degree of discoloration indicates the scavenging potential of the antioxidant extract, which is due to the hydrogen donating ability (von Gadow *et al.*, 1997). The Honey extracts showed a concentration dependent scavenging of DPPH radical, which may be attributed to its hydrogen donating ability (Figure 1). The DPPH assay IC_{50} (inhibition concentration of the test sample that decrease 50% initial radical) values were found to be 3.0, 3.0, 2.15, 2.36, 3.0 and 2.68 µg phenolic content / ml Baha Sidr, Konfthah Sidr, Aga Gabal, Shokp Wady, Hegaz Rabia, and Tanhat honeys extracts, respectively (Table 1). The highest antioxidant activity was exhibited by MMS 401 honey with SC_{50} value of 10 µg / ml for DPPH compared to other honeys collected from Northwestern Argentina (Isla *et al.*, 2011).

The Trolox equivalent antioxidant capacity assay was also used to evaluate free radical scavenging capacities of Honey. The assay is based on the ability of antioxidant to scavenge ABTS radicals. It is a simple and usually used method for the evaluation of antioxidant capacity (Cai et al., 2004; Gan *et al.*, 2010).

The phenolic contents of honeys showed a concentration dependent scavenging of ABTS radical (Figure 2). The ABTS assay IC_{50} values were found to be 0.72, 0.94, 0.36, 0.725, 1.2 and 0.73 µg phenolic content / ml Baha Sidr, Konfthah Sidr, Aga Gabal, Shokp Wady, Hegaz Rabia, and Tanhat honeys extracts, respectively (Table 1). The results indicated that the honeys extracts had two to three-fold free radical scavenging capacity for ABTS radical assay greater than DPPH radical assay. Isla *et al.* (2011) reported that all honey samples collected from Northwestern Argentina showed a concentration-dependent pattern. The order of $ABTS^{*+}$ antioxidant activity for honey extracts was as follows: MMS 401 > MASE 101 > MCMJ 301 > lemon honey with SC_{50} values of 2.73, 3.32, 3.62, and 3.94 µg / mL, respectively.

The total antioxidant capacity is also based on the reduction of molybdenum (VI) to molybdenum (V) by honey extract and subsequent formation of a green phosphate/ molybdenum (V) complex at acidic pH. The high absorbance values indicated that the samples possessed significant antioxidant activity. The phenolic content of all tested honeys had significant total antioxidant activity and the effect increased with increasing the concentration (Figure 3). The phosphate / molybdenum complex EC_{50} (the efficient concentration of phenolic content that increases O.D 0.5) values were ranged from 0.3 to 0.46 µg phenolic content / ml (Table 1).

Screening of inhibitory activity of phenolic extracts of locally produced honeys against multi-resistant bacteria was investigated on Mueller-Hinton agar medium. Some honeys with different dilutions had inhibitory effects against three human pathogenic test bacteria. Table (2) shows that Baha Sidr, Konfthah Sidr, Asir Mahyl Shoka, Abyd kors Sidr and Qaseem Rabia honeys inhibited VRE (Vancomycin resistant *enterococci*). MRSA 4 (Methicillin resistant *Staph. aures* 4) was inhibited by Baha Sidr, Aga Gabal, Shokp Wady, Hegaz Rabia, Tanhat and Qaseem Rabia. MRSA 2 (Methicillin resistant *Staph. aures* 2) was also inhibited by Baha Sidr, Konfthah Sidr, Tanhat, Asir Mahyl Shoka and Abyd Kors Sidr Honeys. The inhibition zones caused by the extracts of honeys samples ranged from 6 to 10 mm inhibition zones. Aswad Kors Sidr had no effect on the three bacteria tested. Similarly, the antimicrobial activity of Cuban honeys was screened using two Gram-positive and Gram-negative bacteria. *Staph. aureus* was the most sensitive microorganism while *Pseudomonas aeruginosa* presented higher minimum active dilution values. *Bacillus subtilis* and *Escherichia coli* were both moderately sensitive to honey antimicrobial activity (Alvarez-Suarez *et al.*, 2010). On the other hand, minimum inhibition concentration (MIC) is the most

basic parameter in pharmacokinetics and pharmacodynamics. Therefore, MIC was detected for honey extracts as shown in Table (2). MICs for honey extracts ranged from 2×10^{-5} to 2×10^{-2} μg phenolic content.

Table 1. Total Phenolic and total flavonoid contents and antioxidant effect of phenolics content of honey samples on reduction of DPPH and ABTS radical scavenging, and formation of phosphomolybdenum complex (PMC).

Honey	mg phenolic as gallic acid equivalent / gram honey	mg flavonoid as catechin equivalent / gram honey	DPPH IC_{50}	ABTS IC_{50}	PMC EC_{50}
Baha Sidr	0.285±0.01	0.138±0.008	3.0±0.18	0.72±0.04	0.37±0.015
Konfthah Sidr	0.2±0.008	0.074±0.005	3.0±0.2	0.94±0.04	0.36±0.018
Aga Gabal	0.538±0.025	0.441±0.025	2.15±0.11	0.36±0.015	0.3±0.01
Shokp Wady	0.285±0.012	0.2±0.01	2.36±0.16	0.725±0.05	0.32±0.02
Hegaz Rabia	0.16±0.006	0.033±0.001	3.0±0.17	1.2±0.06	0.39±0.016
Tanhat	0.26±0.011	0.067±0.003	2.68±0.15	0.73±0.035	0.37±0.022
Aswad Kors Sidr	0.114±0.004	0.026±0.001	> 3.0	> 1.2	0.46±0.025
Asir Mahyl Shoka	0.12±0.007	0.016±0.001	> 3.0	> 1.2	0.34±0.018
Abyd Kors Sidr	0.1±0.003	0.006±0.0005	> 3.0	> 1.2	0.37±0.015
Qaseem Rabia	0.13±0.005	0.0235±0.0015	> 3.0	> 1.2	0.41±0.02

IC_{50} (μg phenolic content) is the inhibition concentration of the test sample that decrease 50% initial radical, EC_{50} (μg phenolic content) is the efficient concentration of the test sample that increases O.D 0.5. Each value is expressed as mean (n=3) ± SD

Table 2. Antibacterial activity of honey samples phenolic extracts on the growth of VRE (Vancomycin resistant *enterococci*), MRSA 4 (Methicillin resistant *Staph. aures* 4) and MRSA 2 (Methicillin resistant *Staph. aures* 2). Results expressed are means of the experiment carried out in triplicate.

Honey	Inhibition zone (mm)			MIC μg/ 90μl		
	VRE	MRSA 4	MRSA 2	VRE	MRSA 4	MRSA 2
Baha Sidr	8	7	8	2×10^{-3}	2×10^{-2}	2×10^{-3}
Konfthah Sidr	7	-	7	2×10^{-2}	-	2×10^{-2}
Aga Gabal	-	6	-	-	2×10^{-1}	-
Shokp Wady	-	7	-	-	2×10^{-3}	-
Hegaz Rabia	-	7	-	-	2×10^{-2}	-
Tanhat	-	7	6	-	2×10^{-3}	2×10^{-5}
Aswad Kors Sidr	-	-	-	-	-	-
Asir Mahyl Shoka	7	-	7	2×10^{-4}	-	2×10^{-4}
Abyd Kors Sidr	7	-	7	2×10^{-4}	-	2×10^{-4}
Qaseem Rabia	7	10	-	2×10^{-2}	2×10^{-2}	-

Table 3. Elements content in individual honey sample (mg / kg) determined by ICPE method after dry decomposition.

Honey	Al	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Sr	Zn
1	2.82	0.1056	0.3192	0.24	0.732	0.322	0.0772	0.3292	1.02	0.222	0.1084
2	5.92	0.086	0.2992	0.1924	0.688	0.152	0.0636	0.2568	1.076	0.2604	0.0888
3	2.5	0.09	0.294	0.19	0.632	0.208	0.05	0.2388	0.992	0.2164	0.1448
4	1.208	0.0876	0.2672	0.1772	0.476	0.1156	0.0464	0.1932	0.928	0.2596	0.0612
5	1.316	0.0784	0.2592	0.17	0.484	0.118	0.0548	0.2032	0.9	0.216	0.062
6	1.412	0.0868	0.2664	0.1776	0.468	0.1176	0.0452	0.1964	0.928	0.3752	0.0684
7	1.164	0.0924	0.2672	0.1704	0.512	0.1	0.0448	0.2036	0.936	0.4	0.0848
8	1.22	0.0888	0.2668	0.1736	0.48	0.1084	0.042	0.192	0.916	0.304	0.058
9	0.952	0.0708	0.2472	0.1684	0.262	0.092	0.0412	0.1628	0.852	0.2292	0.0736
10	0.804	0.0532	0.2016	0.128	0.1648	0.0706	0.0352	0.1588	0.782	0.204	0.0676

Honeys: (1) Baha Sidr, (2) Konfthah Sidr, (3) Aga Gabal, (4) Shokp Wady, (5) Hegaz Rabia, (6) Tanhat, (7) Aswad Kors Sidr, (8) Asir Mahyl Shoka, (9) Abyd Kors Sidr, (10) Qaseem Rabia.
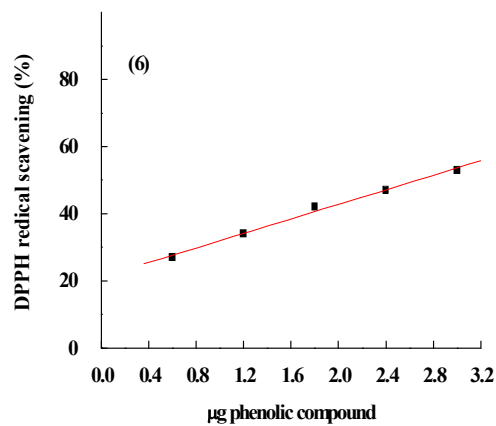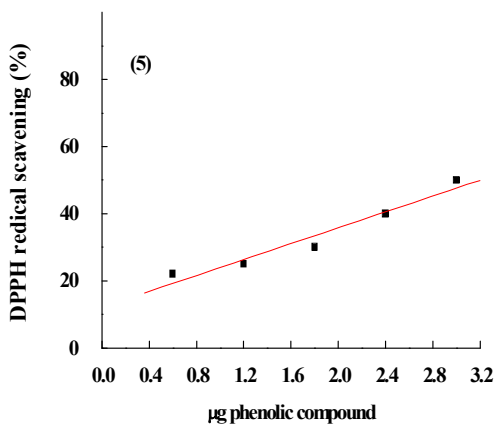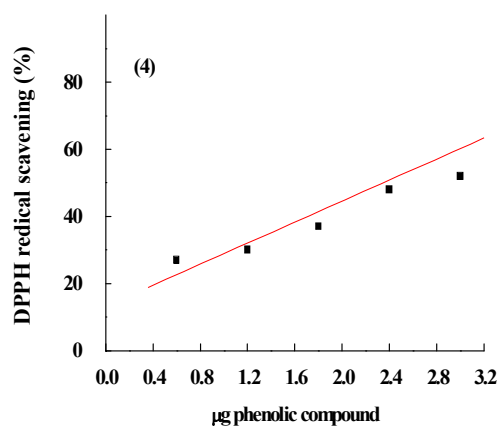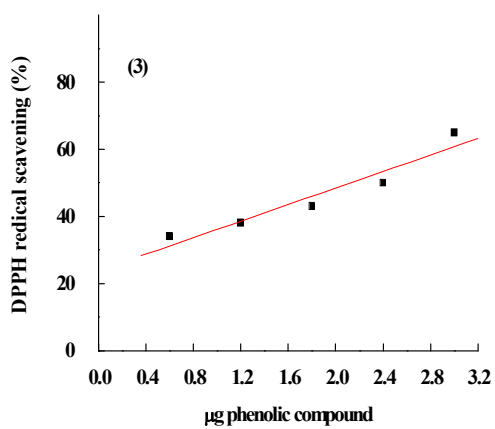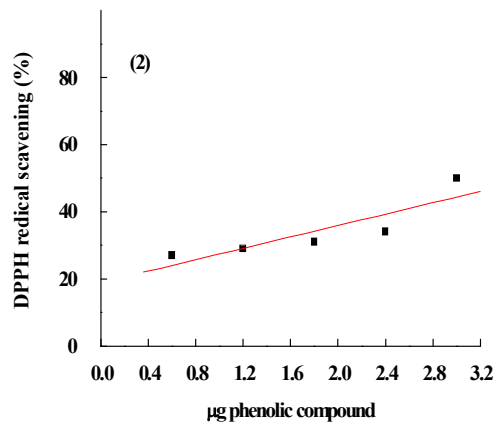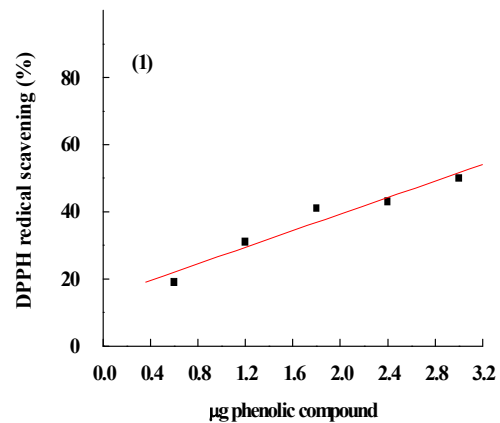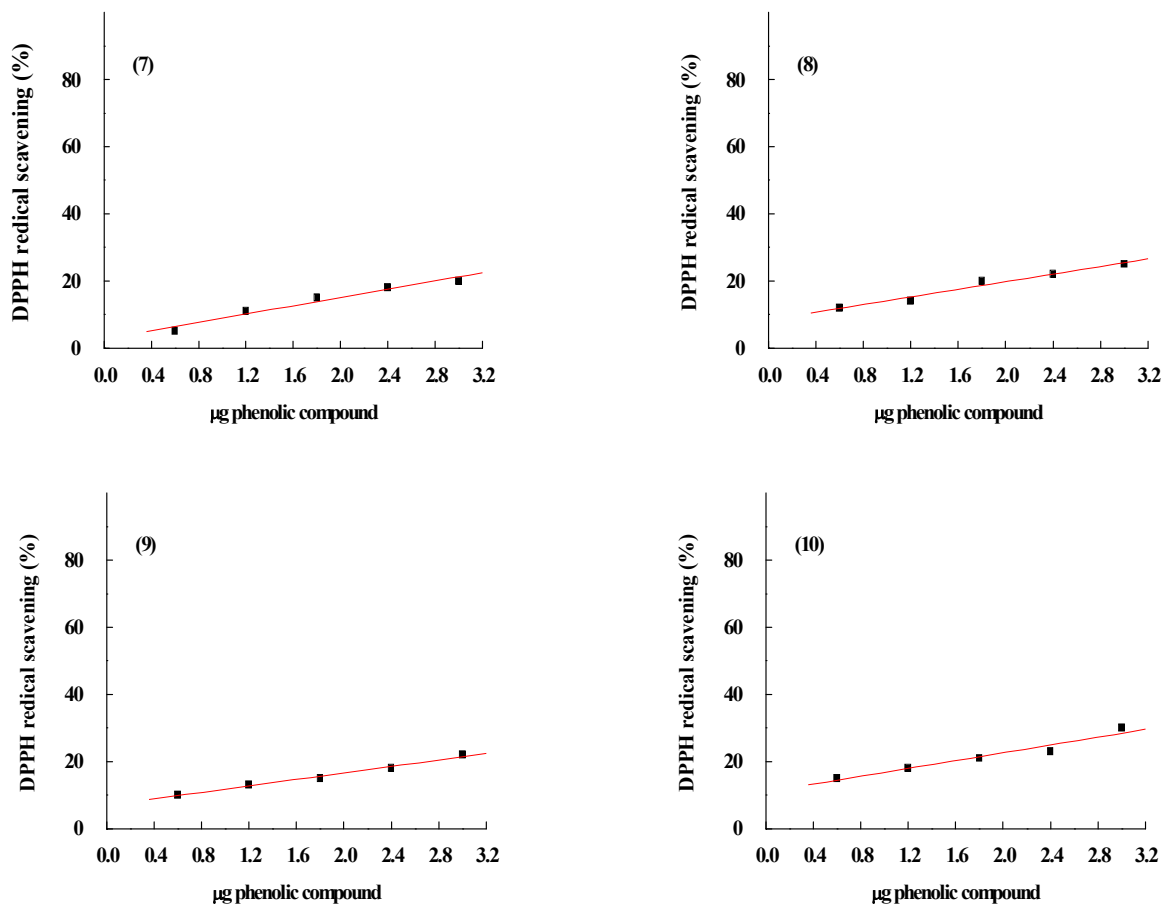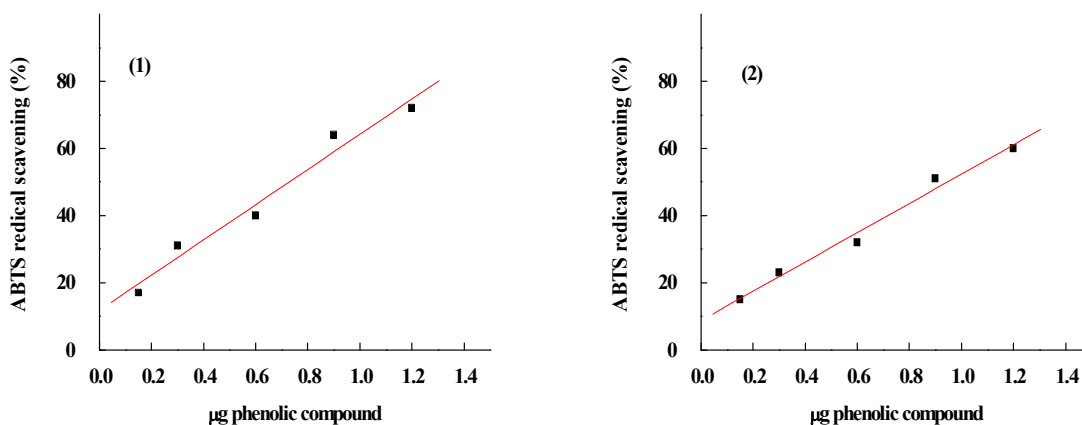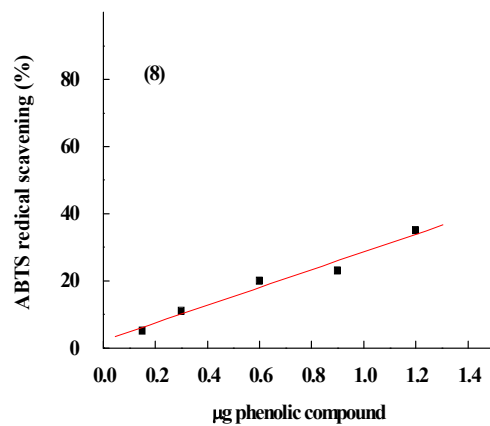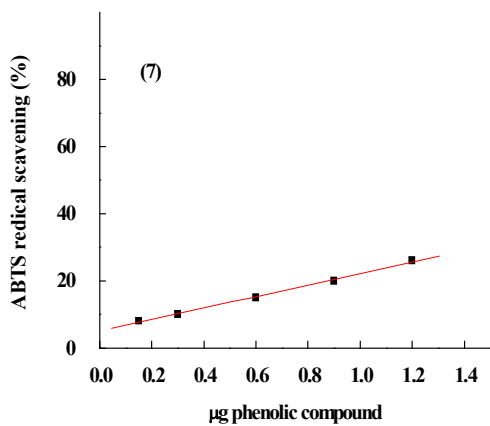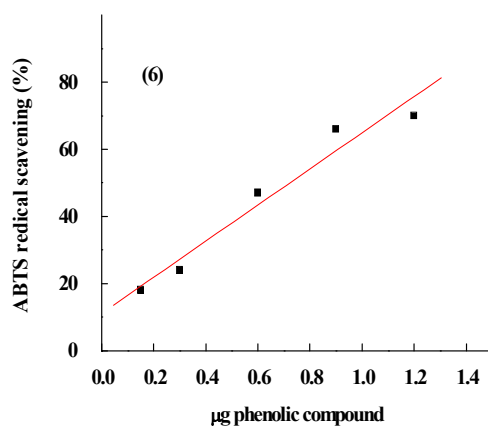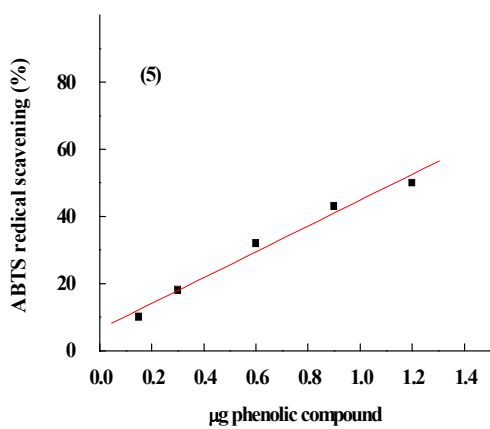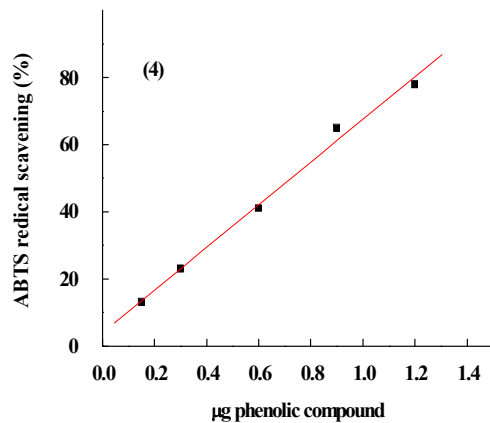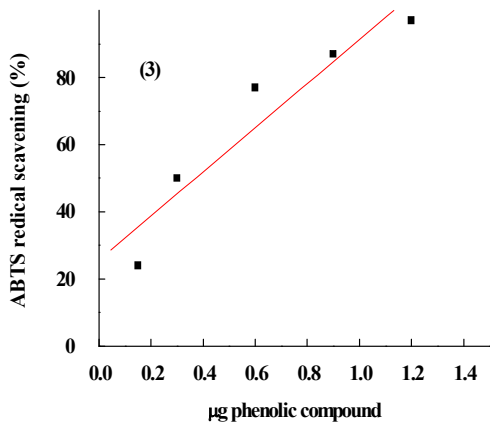
Figure 1. Correlation between concentrations of phenolic compounds of (1) Baha Sidr, (2) Konfthah Sidr, (3) Aga Gabal, (4) Shokp Wady, (5) Hegaz Rabia, (6) Tanhat, (7) Aswad Kors Sidr, (8) Asir Mahyl Shoka, (9) Abyd Kors Sidr, (10) Qaseem Rabia honeys and their antioxidant capacity as determined by DPPH assay.
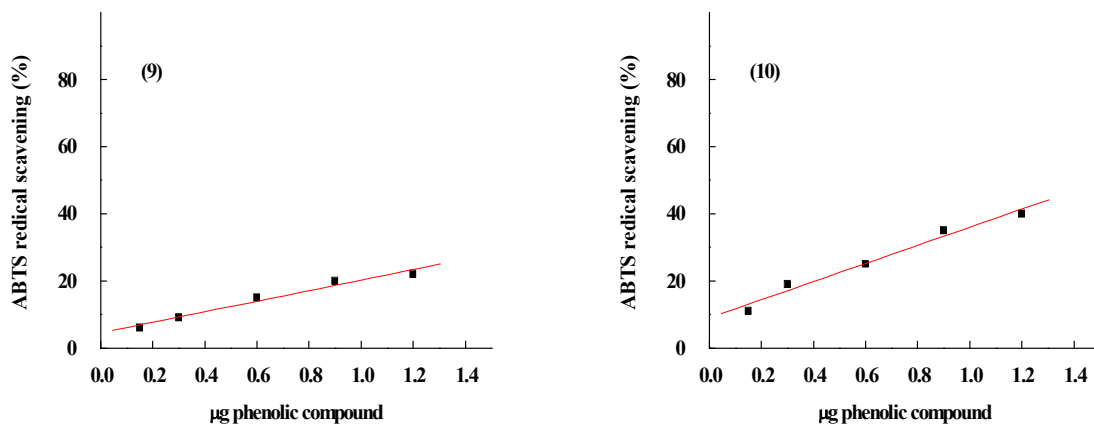
Figure 2. Correlation between concentrations of phenolic compounds of (1) Baha Sidr, (2) Konfthah Sidr, (3) Aga Gabal, (4) Shokp Wady, (5) Hegaz Rabia, (6) Tanhat, (7) Aswad Kors Sidr, (8) Asir Mahyl Shoka, (9) Abyd Kors Sidr, (10) Qaseem Rabia honeys and their antioxidant capacity as determined by ABTS assay.
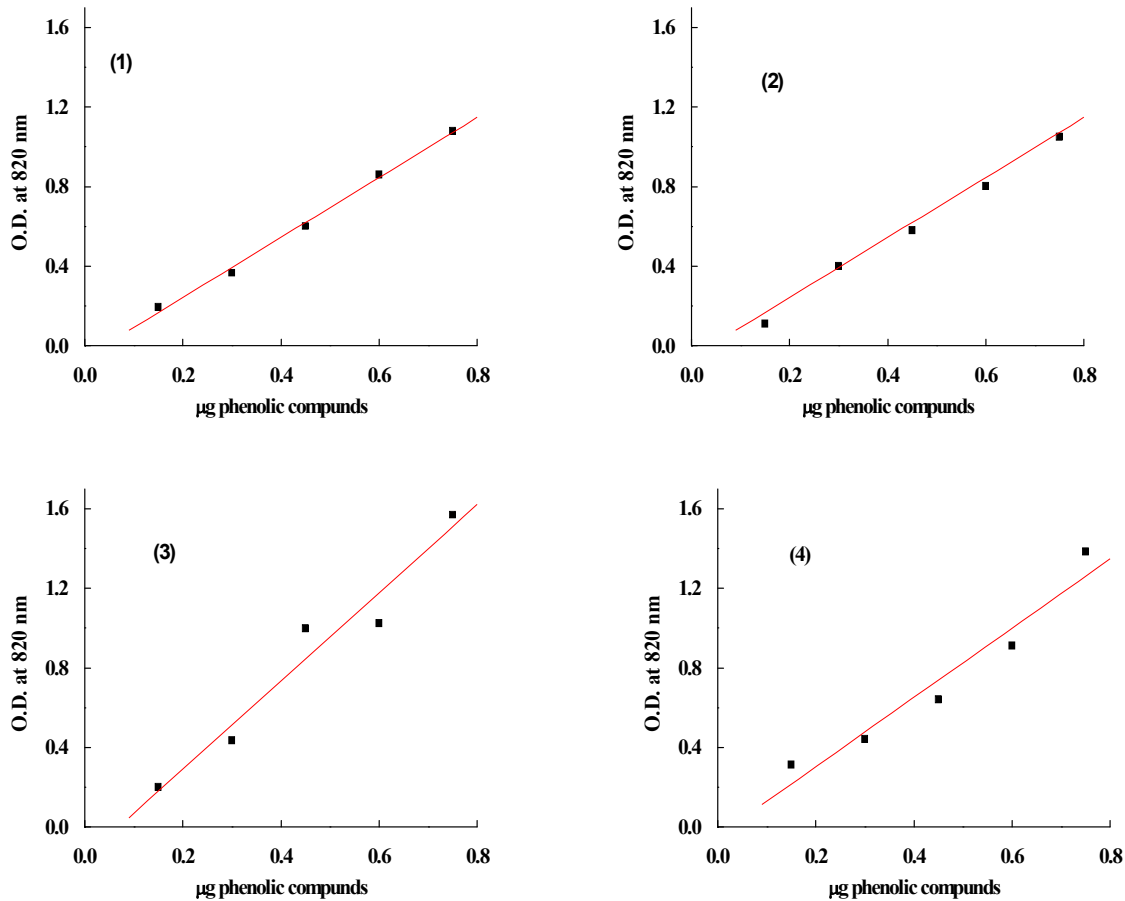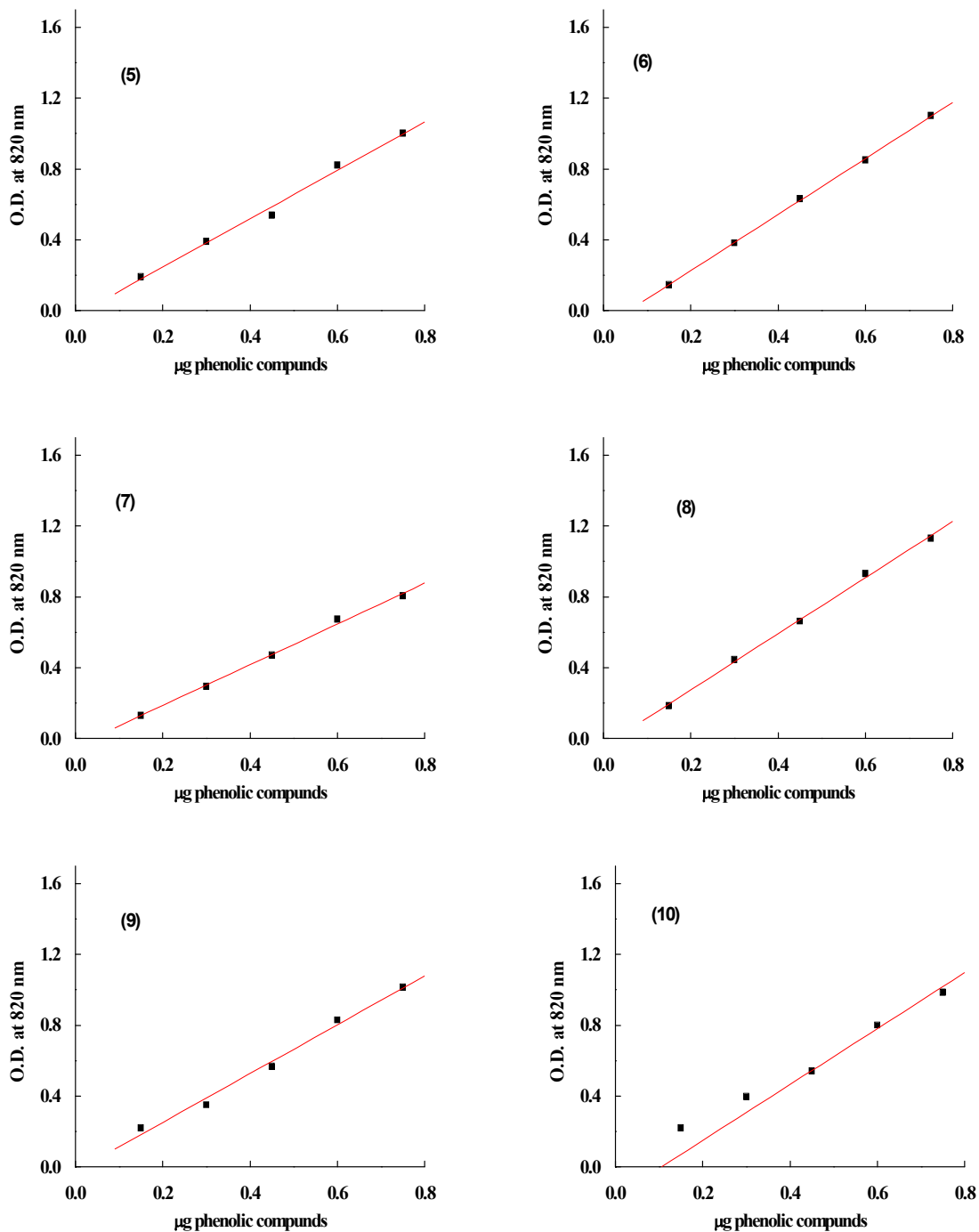
Figure 3. Correlation between concentrations of phenolic compounds of (1) Baha Sidr, (2) Konfthah Sidr, (3) Aga Gabal, (4) Shokp Wady, (5) Hegaz Rabia, (6) Tanhat, (7) Aswad Kors Sidr, (8) Asir Mahyl Shoka, (9) Abyd Kors Sidr, (10) Qaseem Rabia honeys and their antioxidant capacity as determined by the formation of phosphomolybdenum complex assay.

It is very important to analyze the content of elements in honey samples in terms of the fact that they can be transported through the root system into nectar or leave surface of the plant. Therefore, we determined the elements levels in samples of locally produced honeys in Saudi Arabia (Table 3). By means of emission spectrometry with inductively coupled plasma (ICPE), Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Sr and Zn

were determined. The highest level of elements in all tested honeys (mg / kg honey) were in the order of Al (0.8 - 5.92) > Pb (0.78 - 1.07) > Cu (0.16 - 0.73). On the other hand, the least level content was determined for Zn, Fe and Cd. Despite honey samples were collected from different regions of Kingdom of Saudi Arabia, no remarkable difference of the elements contents was observed. Similarly, the maximum contents of elements in honey samples from Kesan (polluted area) were found as 0.46, 0.82, 1.98, 14.0, 9.86 µg / kg, 0.48 mg / kg, 137, 115 and 290 µg / kg for Cu, Mn, Zn, Fe, Cd, Pb, Cr, Ni and Se, respectively (Citak *et al.*, 2012).

4. Conclusion

The antioxidant and antimicrobial properties and essential elements content of locally produced honeys in Kingdom of Saudi Arabia make them high added value products. Results obtained for elements in analyzed honey samples were acceptable to human consumption at nutritional and toxic levels.

5. Acknowledgement

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3/11/2014