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Antibacterial and Antioxidant Activity of Four Types of Honey with Different Floral Origion

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ABSTRACT

Honey is a popular food product produced by honey bees that it is a well-known antimicrobial and antioxidant activity. Floral origin of honey plays an important role on its biological properties. This research was carried out to evaluate the antibacterial and antioxidant activity of four sample honey with different floral origin collected from the bee hive in the Golestan province in north of Iran. Evaluation of antibacterial activity against four pathogenic bacteria was performed by agar well diffusion method. *Maple* honey with diameter of inhibition zone as 23.33,22,14.33 and 13.33mm against *Shigella dysenteriae, Staphylococcus aureus* , *Bacillus cereus* and *Escherichia coli* respectively showed significant antibacterial effects. MIC and MBC of honey samples were determined by broth macrodilution tube method. The total phenol content (TPC) and the total flavonoid content (TFC) of honey samples was determined by *Folin–Ciocalteu* reagent and with aluminum chloride method respectively. Also to assess potential antioxidant activity of honey samples was used from reaction with 1,1 diphenyl-2-picrylhydrazyl (DPPH) radical. MIC and MBC values of obtained for *linden*, *maple* and *astragalus* honeys were in the range of %6.25-25% (V/V). The highest values of TPC, TFC and DPPH radical scavenging activity was related to linden, citrus and maple honeys respectively. In this study was demonstrated antibacterial and antioxidant activity of honey samples especially *linden* honey. Statistical analysis showed that the total phenolic content correlated with its antioxidant activity ($P<0.001$). Overall, the results imply that biological activities of honey samples, according to the their floral origin are variable.

1. Introduction

Modern medicine despite apparent concessions into traditional medicine, overuse of chemical drugs has caused which unfortunately is becoming increasingly more acute and the spread of resistance to antibiotics is a major problem of the public health community (Monroe and Polk, 2000). Therefore, study in order to the introduction of new antimicrobial agents with natural origin in order to reduce of the antibiotic resistance and to elimination of adverse effects, chemical agents is an indispensable necessity. Honey as a natural product and a very popular food product according to the floral origin and geographical region has antimicrobial and antioxidant different properties (Basualdo et al., 2007). The antimicrobial activity of honey is attributed on the content of hydrogen peroxide produced by

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honey glucose oxidase(Allen et al., 1991), high osmolarity (Theunissen et al., 2001), low pH (Wahdan, 1998), the presence of chemical compounds such as methylglyoxal. Also existence of low levels of enzymes, particularly invertase, glucose oxidase, catalase and amylase in this activity is effective (Moussa et al., 2011; Boukraa and Amara, 2008). Many studies have showed the role of honey as a source of natural antioxidants (Kucuk et al., 2007; Aljadi and Kamaruddin, 2004).The main components of honey responsible for its antioxidant eefect are phenolic acids and flavonoid (Bertoncelj et al., 2007; Socha et al., 2009). Also antioxidant activity of honey depends on presence of compounds such as catalase, glucose oxidase, ascorbic acid, derivatives of carotenoids, organic acids, maillard reaction products, amino acids and proteins as well as a small amount of mineral content, particularly copper and iron (Aljadi and Kamaruddin, 2004; Meda et al., 2005). However, the amount and type of antioxidant that is largely dependent on floral origin, geographical region, seasonal and environmental factors, as well as the method of processing honey (Lachman et al., 2010; Fahim et al., 2014). The aim of this study was to evaluate and compare antibacterial and antioxidant activity of four types of honey with different floral origin including of, *Astragalus, linden, maple, citrus* collected from the bee hive in the Golestan province in north of Iran.

2. Materials and Methods

2.1. Honey samples

Four honey samples with different floral origin including of, *Astragalus, linden, maple, citrus* in February 2015 were collected from the bee hive in the Golestan province in north of Iran. All samples were transferred to the laboratory, and kept at 4°C and dark place, until used.

For agar well diffusion method, serial dilutions of honey samples were prepared aseptically in sterile double distilled water.

2.2 Bacterial Strains

The Bacterial strains used in this study were two species of gram-negative of *E. coli* PTCC 1338 and *S. dysenteriae* PTCC 1188 and the two species of gram-positive bacteria, including *S. aureus* PTCC 1112 and *B. cereus* PTCC 1154. These bacteria were provided in lyophilized form from Biotechnology Institute in Iranian Research Organization for Sciences and Technology, Tehran. These strains were used to antibacterial tests in agar well diffusion method and determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC).

Bacterial strains in brain heart infusion (BHI broth) (Merck) and 37°C for 18 to 24 hours were activated.

After activation were transferred to Muller Hinton agar (Merck) and incubated overnight at 37°C. Single colonies from plates were transferred into BHI broth and incubated at 37°C. The turbidity of the suspension was adjusted spectrophotometrically to the McFarland 0.5 turbidity standard (1.5×10^8CFU/ml).

2.3. Agar well diffusion method

Surface of Mueller Hinton agar were uniformly inoculated with bacterial suspension containing of 1.5×10^8 CFU/ml (0.5 McFarland) each of the bacteria. Then wells of 8 mm in diameter were prepared using sterilized cork borer. These wells were filled with different dilutions of honey samples. plates were incubated at 37°C for 24 hours. Zones of inhibition of microbial growth around the wells were measured using a scale ruler and recorded after the incubation time (mm). All experiments were performed in triplicate and the zone of inhibition was measured twice for each honey dilution (Tumin et al., 2005).

2.4. MIC and MBC determination

Minimum inhibition concentration (MIC) of honey samples were determined using macrodilution tube method or turbidimetric assay (Fritsche et al., 2007).

For this purpose, serial dilutions of honey samples were prepared in Mueller Hinton Broth (Merck) and 7 serial 1:1 dilutions were made, resulting in final concentrations of; 100%,50%, 25%, 12.5%, 6.3%, 3.1% and 1.6% v/v.

Then to each of the tubes from different dilutions of honey samples was added,

5×10^5CFU/ml from each of the tested pathogenic bacteria and incubated for 24 h at 37°C. There were also control tubes containing of various concentrations of honey samples diluted with the Muller hinton broth (without bacterial suspension) as negative controls and bacterial suspension of 5 $\times10^5$CFU/ml (without honey) as positive controls. The results after 24 h of incubation for microbial turbidity of visible were recorded. The last dilution (lowest concentration) in which microbial turbidity was not observed, as the minimum inhibitory concentration (MIC) was considered.

For the determination of MBC, from the tube that contained honey concentrations higher than the MIC were cultured onto the agar medium. The MBC was defined as the lowest concentration that allowed no visible growth on the agar (Cockerill et al., 2012).

2.5. Total Phenolic Content

Thetotal phenolic content (TPC) of honey samples was analyzed by using Folin-Ciocalteu reagent, based on the method described by Meda *et al*.(Meda et al., 2005) 0.5ml of Honey solution (0.1 g/mL) was mixed with 2.5mL of Folin-Ciocalteau reagent (2N) and incubated for 5min. Subsequently, 2mL of sodium carbonate solution (75g/L) was added into the honey solution and incubated for another 2 h at 25°C. After incubation, the absorbance of the solution was measured at 760 nm by using a UV-Visible spectrophotometer (Jenway-UK). The standard curve was produced for gallic acid within the concentration range from 100 to 1000 mg/L (R^2=0.9998, y=0.1254x-0.1424). The total phenolic content was reported as mean value of triplicate assays and expressed as mg gallic acid equivalent per 100 g of honey sample (mgGAE/100 g).

2.6. Total Flavonoid Content

The total flavonoid content of honeys was estimated by aluminium chloride (AlCl3) colorimetric method. A 5mL of honey solution (0.1g/mL) was mixed with 5mL of 2% aluminium chloride (AlCl3). A flavonoid-aluminium complex was formed after 10min of incubation time at 25°C. The formation of the complex was measured at 415nm by using an UV-Visible spectrophotometer (Jenway-UK). Quercetin (QE) (0–100mg/L) was used as a standard chemical for calibration curve preparation. The TFC was reported as mean value of triplicate assays and expressed as milligram of quercetin equivalent (QE) per 100 g of honey sample (mg QE/100g). (Chua et al., 2013; Pontis et al., 2014).

2.7. The free Radical Scavenging Activity

The free radical scavenging activity of honey samples was determined using the 2,2-diphenyl-1-picrylhydrazyl hydrate radical (DPPH).(Chua et al., 2013; Wilczyńska, 2010; Turkmen et al., 2006). The methtnolic DPPH· solution (20 mg/L) was prepared. A 0.75 mL of methanolic honey solution at different concentrations, ranging from 20 to 40 mg/mL, was added to 1.5 mL of DPPH· solution. The absorbance was measured at 517 nm after 15 min of incubation at 25°C. The ability to scavenge the DPPH· was calculated using (1), where $A_{control}$ and A_{sample} are the absorbances of control and sample, respectively. The control test was made with methanol in place of honey solution. The experiment was performed in triplicate. Antioxidant activity was expressed as a percent of inhibition of DPPH radical and calculated fromthe equation:

$$\text{DPPH scavenging activity (\%)} = A_{Control} - A_{Sample} / A_{Control} \times 100$$

2.9. Statistical analysis

All data were expressed as the mean ± standard deviation (n = 3). The results were analyzed statistically with One-way ANOVA using SPSS version 18.0 software. Correlations were established using Pearson's correlation coefficient (*r*) in bivariate linear correlations (*P* < 0.001). These correlations were calculated using Microsoft office Excel 2007 and SPSS version 18.0 differences were considered significant at levels of $p < 0.001$.

3. Results

Table 1 shows the antibacterial activity of honeys samples against tested bacteria using agar well diffusion method. In this method effect of antibacterial of honey samples was dose-dependent and this effects increases with increasing of concentrations. the honey *maple*, *linden*, *astragalus* and *citrus* were showed

highest diameter of inhibition zone against pathogenic bacteria respectively.

Maple honey with diameter of inhibition zone as 23.33,22,14.33 and 13.33mm against *S.dysenteriae*, *S. aureus*, *B. cereus* and *E. coli* respectively showed significant antibacterial effects and lowest antibacterial activity was recorded for *citrus* honey which only at concentration of 100% showed diameter of inhibition zone as 16.66 and 15.66 mm against *S. dysenteriae* and *S. aureus* respectively.

Table 1. Antibacterial Activity of Four Types of Honey with Different Floral Origion (Mean Zones of Inhibition)

Concentration	**100%v/v**				**50%v/v**				**25%v/v**			
Isolates	*Maple*	*Linden*	*Astragalus*	*Citrus*	*Maple*	*Linden*	*Astragalus*	*Citrus*	*Maple*	*Linden*	*Astragalus*	*Citrus*
S.dysenteriae	23.33 ± 1.15^{bcA}	20 ± 1.73^{efA}	19 ± 1^{fgA}	16.66 ± 1.15^{hi}	18.66 ± 1.52^{bB}	16.33 ± 1.52^{cdB}	14.33 ± 0.57^{efB}	_	13 ± 1^{bC}	_	10.33 ± 0.57^{cC}	–
S.aureus	22 ± 1^{cdA}	18.66 ± 0.57^{fgA}	18.66 ± 0.76^{fgA}	15.66 ± 0.57^{ij}	17 ± 1^{bcB}	14.33 ± 0.57^{efB}	15 ± 1^{defB}	_	_	_	11.33 ± 1.15^{bcC}	_
B.cereus	14.33 ± 0.75^{jk}	$12.33\pm0.57l$	14.66 ± 1.15^{jk}	_	_	_	_	_	_	_	_	_
E. coli	13.33 ± 0.57^{kl}	13 ± 0.5^{kl}	13 ± 0.5^{kl}	_	_	_	_	_	_	_	_	_

* diameter mm including well (8.2 mm)
** Those means corresponding to same alphabet are statistically non-significant at α=0.05
*** Those means corresponding to non-same alphabet are statistically significant at α=0.05
- no zone of inhibition was observed

The antibacterial analysis using by broth macrodilution tube method showed that MIC and MBC values of obtained for linden, maple and *astragalus* honeys were in the range of %6.25-25% (V/V). Low antibacterial activity of *citrus* honey in this method also confirmed as minimum inhibitory concentration of the this honey for *E. coli*, *S. aureus*, *B. cereus* and *S.dysenteriae* was , 75%, 75%, 50% and 25%, respectively.

In this method *S.dysenteriae* was the most sensitive bacteria to tested honeys and *E. coli* was the most resistant. As minimum inhibitory concentration of *linden*, *maple*, *astragalus* and *citrus* honey for *S. dysenteriae* was recorded by 6.25%, 6.25%, 12.5% and 25% respectively and for *E. coli*, 25%, 12.5%, 30% and 75% respectively (Table 2).

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) honey samples % v/v solution.

Honey sample	***Linden***		***Maple***		***Astragalus***		***Citrus***	
Bacterias	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. dysentery	%6.25	%6.25	%6.25	%6.25	%12.5	%12.5	%25	%25
S. aureus	%10	%12.5	%12.5	%12.5	%25	%25	%75	%75
B. cereus	%25	%25	%12.5	%12.5	%15	%25	%50	%50
E. coli	%25	%25	%12.5	%12.5	%30	%50	%75	%100

Table 3. Total phenols and total flavonoids and radical scavenging activities(DPPH) of tested honeys.

Honey (100 mg/ml)	TPC (mg GAE /100 g honey)	TFC (mg QE /100 g honey)	DPPH (EC_{50})
Linden	103.08 ± 4.93[a]	26.19±2.61[b]	31.46 ± 0.03[d]
Astragalu	89.91 ± 3.61[b]	23.57±2.61[bc]	33.72 ± 0.09[c]
Maple	76.64±4.93[c]	18.33±2.61[c]	40.07± 0.09[a]
Citrus	68.17±3.62[d]	43.73±3.92[a]	33.95± 0.18[b]

The values in table is Mean±SD

Table 4. Pearson Correlation total phenolics content, total flavonoid content, radical scavenging activity of hony sample

	[a]TPC	[b]TFC	[c]DPPH
[a]TPC	1		
[b]TFC	-.714**	1	
[c]DPPH	-.534	.017	1

**. Correlation is significant at the 0.01 level (2-tailed).

[a] Total phenolic content.

[b] Total flavonoid content.

[c] 2,2-diphenyl-1-picrylhydrazyl scavenging activity.

The total phenolic contents (TPC), total flavonoid contents (TFC) and The free Radical Scavenging Activity in four honey samples with different floral origin including of, a*stragalus, linden, maple, citrus* collected from the bee hive in the Golestan province in north of *Iran* are shown in Table3.The total phenolic content of honey samples in this study ranged from 68.17 to 103.08 mg GAE /100 g honey and their total flavonoid contents ranged from 18.33 to 43.73 mg QE /100 g honey. *linden* honey and *citrus* honey showed the highest levels of TPC and TFC respectively.

In order to assess antioxidant activity of honey samples was used from reaction with free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). As shown in Table 3 the scavenging ability of honey samples ranged from 31.46 to 40.07%.

Our results showed that all samples of honey have scavenge free Radical Scavenging Activity however, *Maple* honey exhibited higher antioxidant activity ($p < 0.001$) than other samples. Pearson correlation between total phenolic and flavonoid contents and antioxidant activity of honey samples were examined In Table 4, statistical analysis showed that the average correlation between antioxidant activity and total phenolic content is negative($r = -.534$ DPPH/TPC , $r= -.714^{**}$ TPC/TFC, $p<0.01$). The total flavonoid content and radical scavenging activity there is a low positive correlation.

4. Discussion

MIC and MBC values of obtained for *linden*, *maple* and *astragalus* honeys were in the range of 6.25-25% (V/V). Other studies also minimum inhibitory concentration for honey of different floral origion in this range were reported (Yavarpour et al., 2014; Mullai and Menon, 2007; Fidaleo et al., 2011).

E. coli showed significant resistance to different concentrations of honey samples. Resistance of *E. coli* and gram-negative bacteria to honey types have been reported in other studies (Sherlock et al., 2011; Fidaleo et al., 2011).

This resistance can be because the lower permeability of the outer membrane of gram-negative than gram positive bacteria that limits entry of antimicrobial agents into the bacterial cell (Nikaido, 2003).

Significant difference between *S. dysentery* and *E. coli*, while both of them are gram-negative and belong to enterobacteriaceae family has also been observed in other studies.

Tumin et al (2005) studied the antibacterial activity of honeys with Different Floral Origion against the bacteria *E. coli, S. aureus, S. typhi, S. sonnei, P. aeruginosa* and *S. pyogenes* were tested. In this study, *S. sonnei* the most vulnerable bacteria to honey samples were reported (Tumin et al., 2005). One possibility might be related to the differences in

susceptibility of each species of microorganism to the antibacterial activity of honey used. Similar observations are reported by others (Nzeako and Hamdi, 2000; Ceyhan and Ugur, 2001; Taormina et al., 2001).

Other possible explanation for these observations could be the differences in putative antibacterial agents present in honey samples. These agents may utilize hydrogen peroxide and non-peroxide antioxidant components.

The differences among honey samples in antibacterial and antioxidant activity could be attributed to the natural variations in floral sources of nectar and the different locations and the geographical factors like temperature, humidity where the honey was produced (Alzahrani et al., 2012; Al-Waili., 2005).

The variety inhibitory effects of different types of honey can be due to differences in plants that honey is obtained from them. In other words different species of plants in different regions have different compounds and honey of derived from them will not be the same and thus its biological effects also will be different.

This differences of antimicrobial activity is due to variation in the level of hydrogen peroxide (H_2O_2) , and in some cases to the level of non-peroxide factors such as phenolics that include cinnamic acid derivatives (mainly prenylatedcompounds) (Hamouda and Marzouk, 2011).

The levels of hydrogen peroxide and non-peroxide of honey samples is obviously related to the floral origion, and sometimes it can account for the major part of the antibacterial activity in a honey (Allen et al., 1991) and as components from some floral origion can affect both the production and the destruction of hydrogen peroxide (Olaitan et al., 2007). The main factor in the destruction of hydrogen peroxide is catalase. Catalase comes from the pollen and nectar of certain plants; more coming from the nectar (Olaitan et al., 2007). As a result, honey of different floral origion have different levels of hydrogen peroxide and different antibacterial activity.

The results showed that the total phenolic and flavonoid content in different honey samples collected from different botanical and geographical origin is very variable. Several studies in Croatia, Malaysia, Turkey, Portugal and Brazil show these differences (Kucuk et al., 2007; Aljadi and Kamaruddin, 2004; Piljac-Zegarac et al., 2009; Liberato et al., 2011).This differences in composition of honey, depending mainly on the floral source and also other external factors, including seasonal and environmental factors as well as processing.

Statistical analysis showed that the average correlation between antioxidant activity and total phenolic content is negative. The total flavonoid content and radical scavenging activity there is a low positive correlation.

Positive and negative correlation between total phenolic content and total flavonoid content with antioxidant activity in honey samples with different floral origion and geographical were observed in other studies (Pontis et al., 2014; Wilczyńska, 2010; Alzahrani et al., 2012; Al-Waili, 2005; Sarmento et al., 2013).

The differences in the antioxidant activity and correlation of this activity with total phenolic content and flavonoids of honeys related to differences in botanical and geographical origion and seasonal and environmental factors of honey (Alvarez-Suarez et al., 2012). Furthermore, the phenolic profile of honeys and consequently their antioxidant capacity depend on the floral sources used to collect honey.

Conclusion

Overall, the results imply that honey samples with different floral origin collected from the bee hive in the Golestan province in north of Iran have variable potential antibacterial and antioxidant activity. The total phenolic and flavonoid content varied between honey types and antioxidant activity of honey samples was correlated with this compounds. The variety antibacterial and antioxidant effects of different types of honey can be due to differences in plants that honey is obtained from them. In other words different species of plants in different regions have different compounds and honey of derived from them will not be the same and thus its biological effects also will be different.

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