Introduction

Honey is a natural compound synthesized by bees that have been consumed by people from ancient times. *Apis mellifera* is a bee, which produces honey from the sweet liquid in flower or from secretions of trees and plants (1). Honey prevents lipid oxidation in meat (2) and light, heat, and metal-induced oxidation reactions in food (2).

The other research immunologically confirmed the presence of α-glucosidase in stomach, α-glucosidase II in stomach, blood and lymph, and α-glucosidase III beneath the hypopharyngeal apparatus that the enzyme may be released into nectar and collected by honeybees (3). However, α-amylase inhibitory capacities of phenolic extracts of honey reveal its anti-diabetic potential. Diabetes occurred when oxidative stress is more than antioxidants production (4). The most important management for treatment of diabetes is to decrease blood glucose after meal, which is performed through inhibition of α-amylase and α-glucosidase known as carbohydrate hydrolyzing enzymes (5).

Moreover, flavonoids (such as kaempferol, catechin, and quercetin) and phenolic acids (such as caffeeic acid and gallic acid) are the most significant components of honey.
which have anti-cancer properties (6).

Objectives
In this study, phenolic extracts of four Iranian honey were evaluated to determine the antioxidant potentials using DPPH (2,2-diphenyl-1-pircyldihydrzaly) radical scavenging, nitric oxide (NO) radical scavenging, and reducing potential was evaluated by FRAP (ferric reducing ability of plasma) method. Besides, determination of phenolic and flavonoid contents was performed. Moreover, anti-diabetic potentials of four kinds of Iranian honey and their phenolic extracts were evaluated by determination of α-amylase and α-glucosidase inhibition.

Materials and Methods
Materials
Butylated hydroxy toluene (BHT), α-amylase, and bovine serum albumin (BSA) were obtained from Sigma-Aldrich. The other materials were bought from Merck Chemical Co.

Collection of samples
The honey samples were purchased from local stores in Shiraz and Meymand, Iran.

Preparation of phenolic extracts of honey
In order to prepare phenolic extracts of honey, 30 g of Amberlite XAD-2 resin was immersed in methanol for 24 hours. Afterwards, most of the methanol was discharged and substituted with distilled water. After 5–10 minutes, the glass column (25 cm × 2 cm) was filled with this mixture. Then, honey (50–100 g) was dissolved in 70 mL of distilled water. The mixture was passed gradually through the column of Amberlite XAD-2 resin. The column was washed with 350 mL of mixture of water and hydrochloric acid (pH=2) and afterward washed with 400 mL of distilled water to separate carbohydrates as polar compounds from honey. By using 400 mL of methanol, the phenolic components were eluted from the sorbent. The methanol extracts were concentrated under vacuum by using a rotary evaporator at 40°C (7).

Evaluation of DPPH radical scavenging
The DPPH scavenging was performed according to the method of Bruits et al with some modifications (8). Before transferring to a 96-well microplate, different concentrations (12.5-3200 µg/mL) of honey samples (3.2 mg) were mixed with 200 µL of DPPH (100 mM). Negative controls contained 20 µL of methanol and 200 µL DPPH in triplicate. The microplate was held for 30 minutes at 25°C and the absorbance was detected by using a microplate reader (Biotek, ELX 800) at 495 nm (8). Quercetin was used as the antioxidant standard. The percent inhibition was plotted against the concentrations of the honey and the IC50 was estimated by the fitted linear curve. The results were exhibited at the mean ± SD of three replicates.

Evaluation of NO radical scavenging
For evaluation of NO radical scavenging, 50 µL of nitroprusside (10 mM nitroprusside dissolved in phosphate buffer, 20 mM, pH 7.4) was blended with 50 µL of honey extract (200 µg/mL) and the mixture was put for 150 minutes at 27°C (9). After that, 100 µL Griess solution was added and the absorbance was determined at 542 nm. The blank contained honey extract without any reagent. The control contained 50-µL methanol, 100-µL Griess solution, and 50 µL of nitroprusside. Inhibition of NO activity was determined as follows (9):

Inhibition: A0 – A / A0 × 100
A0: is the absorbance of control
A: is the absorbance of test - absorbance of blank (9)

Evaluation of ferric-reducing antioxidant power
In this method, 20 mmol/L of FeCl3, 0.3 mol/L of acetate buffer (pH = 3.6), and 10 mmol/L of TPTZ (2, 4, 6-tripyridyl-S-triazine) solution were mixed in HCl (40 mmol/L). In a 96-well microplate, 20 µL of each sample and 180 µL of FRAP reagent were mixed and put for 10 minutes at 37°C. The absorbance of the mixture was determined at 593 nm (10).

Determination of phenolic compound
Phenolic compounds levels were dedicated as stated by Miliauskas et al (8). Around 0.15 mL of the sample (10 mg/mL) was blended with 0.75 mL of Folin-Ciocalteu (diluting 1:10 with distilled water) and after 3 minutes, 0.6 mL of 7.5% w/v of sodium carbonate was added to the mixture. Then, the combination was held for 1 hour in the dark and the absorbance was read at 765 nm (8). Various concentrations of Gallic acid (0.0003–0.0024 mg/mL) were used for plotting the standard curve. Phenolic compounds of the samples were estimated by the subsequent Equation

\[ C = \frac{c \cdot v}{m'} \]

While C is the phenolic contents (mg/g), c is the concentration of GA obtained from the standard curve (mg/mL), v is the extract volume (mL), and m’ is the extract weight (g).

Determination of flavonoid compounds
Flavonoid compounds of the samples were determined through the colorimetric method (8). In this method, 0.5 mL solution of the sample was blended with 2 mL of distilled water and with NaNO2 15% solution. After 6 minutes, 2 mL of 4% NaOH solution was added to the combination. Then, water was added and the final volume became 5 mL. The combination was blended and permitted to stand for extra 15 minutes. At 510 nm, the absorbance of the combination was detected (8). Quercetin was used as a standard and all values were stated as milligram of quercetin equivalent per 1 g of
extract. Data were presented as mean± SD.

**Evaluation of α-amylase inhibition**

Alpha-amylase inhibition was performed by Ademiluyi et al method (11). Solution of enzyme (4 unit/mL) was made by blending 0.001 g of α-amylase (EC 3.2.1.1) in sodium phosphate buffer (pH = 6.9, 20 mM), comprising 6.7 mM sodium chloride. The samples were dissolved in DMSO and blended with starch solution. The reagent (DNS) solution contained 96 mM of 3, 5-dinitrosalicylic acid (20 mL) and deionized water (12 mL) and 5.31 M potassium tartarate sodium in 2 M sodium hydroxide (8 mL).

On the other hand, 0.04 mL of the enzyme solution and 0.56 mL of the extract-starch solution were blended and held for 15 minutes at 37°C. Then, 0.6 mL of DNSA was added, and the test tube was incubated in the water bath (85°C, for 15 minutes), after that, samples absorbance was read at 540 nm (11). The control presented 100% enzyme activity and replaced extracts with DMSO. Acarbose was used as a standard. All experiments were carried out 3 times. The percentage of α-amylase inhibition was determined by the subsequent equation:

\[
\text{α-amylase inhibition} \% = 100 - \left(\frac{\Delta A_{\text{control}} - \Delta A_{\text{sample}}}{\Delta A_{\text{control}}}\right) \times 100
\]

\[
\Delta A_{\text{control}} = A_{\text{test}} - A_{\text{blank}}
\]

\[
\Delta A_{\text{sample}} = A_{\text{test}} - A_{\text{blank}}
\]

**Evaluation inhibition of α-glucosidase**

Alpha-glucosidase inhibitors were assessed by the method stated by McCue et al (12). The enzyme solution is composed of phosphate buffer (125 μL, pH 6.9, 0.1 M) and 5 μL of α-glucosidase (25 unit/mL). 4-Nitrophenyl α-D-glucopyranoside in the mentioned buffer (pH 6.9) was applied as a substrate. Twenty microliters of the various concentrations of the samples were blended in microplate wells with enzyme solution and hold at 37°C for 15 minutes. The reaction was begun by adding 20 μL of substrate solution and incubated for an extra 15 minutes. The reaction was retarded by adding 0.2 M of sodium carbonate solution (80 μL).

The absorbance of the samples was determined at 405 nm by a microplate reader. The system without extracts was used as a control. The reaction system without α-glucosidase was applied as a blank, and acarbose was used as a positive control. All measurements were performed 3 times. The rates of enzyme inhibition of the samples were estimated by the subsequent equation:

\[
\text{Inhibition} \% = \left[\frac{\text{control absorption-sample absorption}}{\text{control absorption}}\right] \times 100
\]

**Statistical analysis**

The obtained results were presented as the mean±SD of three replicates. ANOVA (one-way analysis of variance) and post test Tukey were applied to calculate the differences among the means. P values ≤ 0.05 are considered as significant differences.

**Results**

Among honey samples, Gavan honey had the highest DPPH scavenging (IC$_{50}$=2±3.09 mg/mL). In this regard, the IC$_{50}$ of quercetin as an antioxidant standard was shown in Table 1. Besides, Bahare honey had the highest NO scavenging (IC$_{50}$= 0.04±0.0009 mg/mL) among honey samples and quercetin (IC$_{50}$=0.07±0.0016 mg/mL, Table 1).

In FRAP method, Meymand honey had the highest antioxidant potential (IC$_{50}$=0.0018±0.000003 mg/mL) in comparison with quercetin (IC$_{50}$=0.009±0.00003 mg/mL).

As shown in Table 2, Gavan honey had the highest amount of phenolic (3817±1.52 mg GAE/100 g) and flavonoids (3.1±0.005 mg QE/100 g honey, Table 2). Bahare honey possessed the lowest amounts of phenolic (58±1.06 mg GAE/100 g honey) and flavonoids (1±0.0015 mg QE/100 g honey).

All of the samples, except Bahare, inhibit α-amylase. By increasing the concentration of honey, α-amylase inhibition was decreased. Moreover, as shown in Table 3, Gavan honey revealed the highest inhibition of α-amylase (23±0.1 % in 2.5 mg/mL). After isolation of sugar, as shown in Table 3, Gavan honey presented the highest inhibition of α-amylase (31.2±0.1% in 25 mg/mL); however after isolation of sugar, α-amylase inhibition was increased in honey samples depended on the concentrations. As shown in Table 3, acarbose as a standard inhibits α-amylase (100±0.18 %) in 2.5 mg/mL.

<table>
<thead>
<tr>
<th>Samples</th>
<th>DPPH radical scavenging (IC50, mg/mL)</th>
<th>Nitric oxide scavenging ability% (200 mg/mL)</th>
<th>Antioxidant potential by FRAP method (IC50, mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gavan, bee</td>
<td>2 ± 0.003</td>
<td>0.054 ± 0.002</td>
<td>0.652 ± 0.002</td>
</tr>
<tr>
<td>Zataria</td>
<td>&gt;3 ± 2.00</td>
<td>0.045 ± 0.0017</td>
<td>0.294 ± 0.0014</td>
</tr>
<tr>
<td>Bahare</td>
<td>&gt;3 ± 2.00</td>
<td>0.0403 ± 0.0009</td>
<td>&gt;3 ± 2.00</td>
</tr>
<tr>
<td>Meymand</td>
<td>&gt;3 ± 2.00</td>
<td>0.05 ± 0.0014</td>
<td>0.0018 ± 0.000003</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.0265 ± 0.00006</td>
<td>0.07 ± 0.0016</td>
<td>0.009 ± 0.00003</td>
</tr>
</tbody>
</table>

Results are given as mean± SD values.
Table 2. Phenolic and flavonoid compounds of four honeys phenolic extracts

<table>
<thead>
<tr>
<th>Samples</th>
<th>Phenolic content (mg GAE/100 g honey)</th>
<th>Total flavonoids (mg QE/100 g honey)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gavan</td>
<td>3817 ± 1.52</td>
<td>3.1 ± 0.005</td>
</tr>
<tr>
<td>Zataria</td>
<td>102 ± 1</td>
<td>2.3 ± 0.015</td>
</tr>
<tr>
<td>Bahare</td>
<td>58 ± 1.06</td>
<td>1 ± 0.0015</td>
</tr>
<tr>
<td>Meymand</td>
<td>866 ± 1.15</td>
<td>2.7 ± 0.005</td>
</tr>
</tbody>
</table>

GAE: Gallic acid equivalent; QE: Quercetin equivalent. All values are expressed as mean ± SD of three parallel measurements (P < 0.05).

Table 3. Percentage of α-amylase inhibition by crude extracts and phenolic extracts of four honeys in comparison to acarbose

<table>
<thead>
<tr>
<th>Samples</th>
<th>2.5 mg/mL</th>
<th>5 mg/mL</th>
<th>10 mg/mL</th>
<th>15 mg/mL</th>
<th>20 mg/mL</th>
<th>25 mg/mL</th>
<th>30 mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gavan (crude)</td>
<td>23±0.1</td>
<td>19.6±0.1</td>
<td>15.4±0.2</td>
<td>11±0.22</td>
<td>5.2±0.14</td>
<td>2.3±0.1</td>
<td></td>
</tr>
<tr>
<td>Zataria (crude)</td>
<td>-</td>
<td>15±0.2</td>
<td>10.6±0.18</td>
<td>5.2±0.1</td>
<td>2.3±0.32</td>
<td>1±0.22</td>
<td></td>
</tr>
<tr>
<td>Bahare (crude)</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Meymand (rude)</td>
<td>11.2±0.2</td>
<td>10±0.1</td>
<td>8.4±0.24</td>
<td>6.3±0.15</td>
<td>4.7±0.3</td>
<td>3.2±0.16</td>
<td></td>
</tr>
<tr>
<td>Gavan (phenolic extract)</td>
<td>5±0.1</td>
<td>7.2±0.14</td>
<td>15.4±0.22</td>
<td>18.7±0.22</td>
<td>22±0.16</td>
<td>31.2±0.1</td>
<td></td>
</tr>
<tr>
<td>Zataria (phenolic extract)</td>
<td>1.7±0.22</td>
<td>3.2±0.32</td>
<td>5.2±0.1</td>
<td>7±0.18</td>
<td>11.4±0.2</td>
<td>13.2±0.24</td>
<td></td>
</tr>
<tr>
<td>Bahare (phenolic extract)</td>
<td>5.2±0.16</td>
<td>7.6±0.3</td>
<td>9.2±0.15</td>
<td>11.4±0.24</td>
<td>15±0.1</td>
<td>17.3±0.5</td>
<td></td>
</tr>
<tr>
<td>Meymand (phenolic extract)</td>
<td>0.125 mg/mL</td>
<td>0.25 mg/mL</td>
<td>0.5 mg/mL</td>
<td>1 mg/mL</td>
<td>1.5 mg/mL</td>
<td>2 mg/mL</td>
<td>2.5 mg/mL</td>
</tr>
<tr>
<td>Acarbose</td>
<td>0±0.1</td>
<td>14±0.15</td>
<td>28±0.14</td>
<td>44±0.2</td>
<td>56±0.1</td>
<td>75±0.25</td>
<td>100±0.18</td>
</tr>
</tbody>
</table>

Discussion

The therapeutic capacity of honey is usually related to antioxidant property against free radicals. The use of the whole extract rather than special antioxidants allows evaluating interactive effects of varied phenolic complexes that exist in the extracts. Moreover, this property is more simply observed in the phenolic extracts rather than in the total honey. For isolation of sugar and purification phenolics of honey, Amberlite XAD-2 has been used as the solid phase (10).

Antioxidant potentials of 4 kinds of honey after isolation of sugar were evaluated by DPPH radical scavenging, NO radical scavenging, and FRAP method. Furthermore, the amounts of antioxidant compounds such as phenolic and flavonoid compounds were measured.

Following the separation of sugar from four honey samples, DPPH radical scavenging radicals were evaluated in lower proportion than quercetin as a standard (Table 1). DPPH radicals scavenging measured the reduction in the absorption of DPPH after exposure to antioxidant...
Phenolic extract of Gavan sample presented the highest potential (IC\textsubscript{50} = 2 ± 0.003 mg/mL, Table 1) of DPPH radical scavenging. Moreover, in other research, the IC\textsubscript{50} of DPPH radical scavenging was reported 90.78 ± 5.10 mg/mL, 168.44 ± 1.99 mg/mL, and 204.24 ± 0.63 mg/mL in phenolic extracts of light honey, amber honey, and dark honey, respectively (13).

However, all of the honey samples could scavenge NO radical and in this regard, Bahare honey (IC\textsubscript{50} = 0.0403 ± 0.0009 mg/mL in 200 μg/mL) presented the highest antioxidant potential which was more than quercetin as an antioxidant standard (IC\textsubscript{50} = 68.73 ± 1.6 μg/mL, P < 0.001, Table 1).

In the determination of reducing power by FRAP method, meymand extract has the greatest antioxidant potential (IC\textsubscript{50} = 0.0018 ± 0.000003 mg/mL) which was more than quercetin (IC\textsubscript{50} = 0.009 ± 0.0003 mg/mL, P < 0.0001, Table 2). However, in FRAP method the conversion of a Fe\textsuperscript{3+}/ferri cyanide complex to the ferrous form was evaluated. The phenolics was detected using Folin-Ciocalteu method (8).

In the present study, phenolic extract of Gavan honey showed the highest amounts of phenolic (3817 ± 1.52 mg GAE/100 g, P < 0.001, Table 2) and flavonoid compounds (3.1 ± 0.005 mg QE/100 g, Table 2). The correlation coefficient between phenolic levels and scavenging of NO was 1; it means may be phenolic compounds were responsible for scavenging of NO radical. Also, it was reported that phenolics of honey may be responsible for its different biological activities (14).

For determination of flavonoids, a spectrophotometric method based on the formation of an aluminum chloride compound was used (8).

A proportionately high flavonoids compound was existed in rosemary honey (2.35 mg QE/100 g) from Spain (16). However, in the present study, Gavan honey and Meymand honey possessed 3.1 ± 0.005 mg QE/100 g and 2.7 ± 0.005 mg QE/100 g of flavonoids respectively, which were higher than Spanish rosemary honey (Table 2).

In this study, a significant difference was noted between phenolic and flavonoid contents of different samples (P < 0.001).

To evaluate the anti-diabetic effects of honey samples, inhibition of α-amylase and α-glucosidase were evaluated. After isolation of sugar, Gaven honey possessed the highest inhibition of α- amylase (Table 4).

In other research, the inhibition rate of α-amylase was reported between 88.8% and 30.5% from the greatest to the smallest concentration (4 μg/mL) (17).

By increasing the honey concentrations, inhibition of α-glucosidase also increased. Accordingly, Bahare sample presented the most inhibition of α-glucosidase (46 ± 0.1% in 20 mg/mL, Table 4). In addition, after isolation of sugar, inhibition of α-glucosidase was increased by increasing the concentrations. Consequently, Zataria honey with 30 mg/mL concentration presented the highest inhibition of α-glucosidase (54±0, 6%, Table 4).

In Gavan honey, after isolation of sugar in 1.25, 2.5, 5, 10, and 15 mg/mL, α-glucosidase was activated in amounts of 5.1%, 7.2%, 7.2%, 11.4%, and 80.1% respectively (Table 4). It was reported that increase of α-glucosidase activity decreased ROS generation (18), and ROS was the origin of different diseases such as diabetes.

The α-glucosidase inhibitors, which include acarbose and miglitol (12), reduce the absorption rate of carbohydrate in the small intestine.

The results presented that inhibition mode of Zataria honey (after isolation of sugar) may be un-competitive (mixed) inhibitions (Table 5). Un-competitive inhibition may demonstrate an increase in Km value while the inhibitor favors binding to the free enzyme. It is probable that Zataria honey cannot bind the active site of the enzyme and presents a broader of inhibition in comparison with acarbose as a competitive inhibitor (19). It means that higher concentrations of acarbose (20) are needed to present the same effect of Zataria honey.

Conclusion

The samples of honey possessed antioxidant properties especially NO radical scavenging and anti-diabetic effects by inhibition of α-amylase, and α-glucosidase. Zataria honey, after isolation of sugar, presents the highest α-glucosidase inhibition with uncompetitive mode of inhibition compared to acarbose as a competitive inhibitor.

Authors’ contribution

MM and SM designed the study. FF, SR and ZS performed the experiments. SM wrote and revised the final version of the manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest for publication of this manuscript.

Ethical issues

The Ethics Committee of Faculty of Medicine, Shiraz University of Medical Sciences, Iran approved this study. Moreover, ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

Table 5. Kinetic parameters of α-glucosidase inhibition by Zataria honey after separation of sugars

<table>
<thead>
<tr>
<th>Samples</th>
<th>Km mM</th>
<th>Vmax μM/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-glucosidase without inhibitor</td>
<td>2.29±0.005</td>
<td>0.0016±0.0000</td>
</tr>
<tr>
<td>Zataria</td>
<td>3.56±0.001</td>
<td>0.0008±0.00001</td>
</tr>
</tbody>
</table>

IC\textsubscript{50} α-glucosidase inhibition= 11±0.19 mM.
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Shiraz University of Medical Sciences, Shiraz, Iran (Grant # 10346).

References
15. Voravuthikunchai S, Howe P. Report on the Fifth International Conference on Natural Products for Health and Beauty (NATPRO 5); May 6–8; Multidisciplinary Digital Publishing Institute; Thailand; 2014.