Evaluation of *In vitro* Anti-Brucella Activity and Chemical Composition of Different Geographically-Distinct Propolis from Iran

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Abstract

Brucellosis is one of the most important zoonotic diseases in many regions worldwide. The aim of this study was to investigate antimicrobial properties of hydro-alcoholic extracts of 6 propolis (EEP) samples collected from different regions of Iran against 5 *Brucella melitensis* clinical isolates causing human brucellosis and an antibiotic-resistant *Brucella abortus* vaccinal strain (RB51). *Brucella* clinical isolates were first carefully identified using conventional molecular typing and *Brucella* biotyping methods. Different *Brucella* strains were then confronted to EEPs using the disk-diffusion agar method to evaluate the antimicrobial activity of each propolis extract. Chemical composition of EEPs was then determined using HPLC-DAD and the main phenolic compounds were quantified. We found that all EEPs displayed significant antimicrobial activities against *Brucella* strains, although to varying extents. All tested clinical strains were susceptible to different EEPs with inhibition zones ranging from 18 to 38 mm diameter. Interestingly, the RB51 vaccine strain was more susceptible to EEP6 (from Markazi province) when compared to conventional antibiotics used in the treatment of brucellosis. Substantial differences observed in EEP antimicrobial activity could be due to their distinct botanical origins and chemical compositions as confirmed by our HPLC analyses. The promising inhibitory effect of some propolis preparations against a broad spectrum of *Brucella* strains points to the need for further studies in the context of systematic clinical investigations and opens up the way for the development of natural complements in support of conventional antibiotic therapy.

**Keywords:** antimicrobial activity; *Brucella melitensis*; propolis; HPLC-DAD; RB51; resistance
Introduction

Human brucellosis is a common zoonotic disease affecting many regions around the world, especially, Near and Middle East regions, North and Sub-Saharan African countries, Western and Eastern Asia as well as Latin America. *B. melitensis* is the main *Brucella* spp. contaminating dairy products and the principal cause of human brucellosis worldwide. Nowadays, the treatment of brucellosis mainly relies on a combination of at least two antibiotics, as suggested by the World Health Organization since 1985. Unfortunately, the WHO recommendations for the treatment of human brucellosis are not always properly implemented in clinical practice, particularly in countries with low socio-economic status (1). In addition, these regimens sporadically lead to therapeutic failures and relapses (2). Although the majority of these relapses are not severe and can be treated with the same drug regimen, they may lead to an increasing risk for antibiotic resistance. These resistances appeared to be an important public health concern in brucellosis endemic regions considering the fact that some of these regimens are also used against tuberculosis (e.g. rifampicin). Another critical point in brucellosis treatment is the duration of therapy as a prolonged antibiotic administration is required to diminish the risk of relapse. All these facts considerably increase the risk for emergence of multi-drug mycobacterial resistance in regions where brucellosis is an endemic zoonotic disease (3). Therefore, the development of complementary preventive strategies against brucellosis appeared to be of rising importance.

Medicinal plants have long been considered as potential sources of bio-active agents against a wide range of microorganisms, while having less adverse effects and good affordability (4). Propolis is one of the most efficient and promising antimicrobial natural substances due to its rich content in flavonoids, phenolic aldehydes and terpenoids (5). This resinous substance is collected by bees from various plants as a defense against various microorganisms, insects or other predators (6). The anti-bacterial activity of various propolis extracts was the subject of numerous studies revealing an important bactericidal potential against a wide range of bacteria (7). However, these effects appeared to be species-dependent and some earlier studies pointed out that Gram-negative bacteria are less susceptible to the antimicrobial activity of propolis than Gram-positive bacteria (7). However, the susceptibility of several pathogenic Gram-negative bacteria, such as *Escherichia coli* and *Salmonella* spp., is now well recognized and exhaustively documented (recently reviewed by (5)). In addition, significant variations have been shown in the antibacterial activity of propolis samples originating from different regions (8). Geographical and botanical origins were found to have a major influence on the quality and chemical composition of the gummy and balsamic substances collected by bees.

The present study aimed to investigate the antibacterial activity of hydro-alcoholic extracts of 6 propolis (EEP1-6) samples collected from different regions of Iran against *Brucella* Gram-negative bacteria isolated from 5 clinical samples and the RB51 *Brucella abortus* vaccinal strain which is known to be resistant to rifampicin and penicillin. The chemical composition of different propolis extracts was compared and the concentration of 12 major phenolic compounds was determined.
Methods

Propolis extracts

Iranian propolis samples were obtained from six geographically distinct areas located in Alborz, Markazi, West Azerbaycan, Khorasan Razavi, Mazandaran and Golestan provinces. The samples were grounded, homogenized and extracted with 70% of ethanol (1/20, w/v) at ambient temperature. For all samples, 1 g of propolis was incubated in 20 mL of 70% EtOH overnight. The resulting mixtures were filtered and the filtrate stored at 4 °C until use.

HPLC analyses

For HPLC analyses, one milliliter of each sample was added to 0.5 ml MilliQ water and centrifuged at 13000 rpm for 3 minutes, and the supernatant was evaporated to near dryness. The derived dry extract was then reconstituted in a 4/1 v/v hydro-alcoholic solution prior to HPLC analyses. Phenolic compounds were analyzed using HPLC (Unicam, Crysta1-200, England), an instrument comprising a Diode Array Detector (DAD). For the need of this study, we used a Zorbax column model SB-C18 with particle size of 3.5 μm in diameter. The solvents used for elution were aq. 0.25% orthophosphoric acid + 1.5% tetrahydrofuran and 100% methanol, respectively. The auto-injection volume and the flow rate were 20 μl and 2 ml/min, respectively. The injector and the column temperatures were fixed at 20°C and 30°C respectively. The HPLC analysis was performed at 220 and 320 nm and commercial standards were used to quantify phenolic compounds.

Bacterial clinical isolates and RB51 vaccine strain

For each patient, prior to bacterial culture for Brucella isolation, the diagnosis of Brucella was performed according to a precise anamnesis confirmed by a battery of in vitro tests including serum agglutination test (above 1:160 in selected cases) along with the Coombs and the 2-mercaptoethanol (2-ME) tests (titers ≥1:40). A total of 5 Brucella strains were isolated in 2018 from blood samples drawn from patients with clinical diagnosis of brucellosis. Patients came from 3 different Iranian provinces of Alborz (n=2), Kermanshah (n=1) and Kerman (n=2). After the confirmation of clinical diagnosis by conventional serological tests, all blood samples were rapidly used for bacterial culture. For this purpose, blood samples were inoculated on a Brucella selective media (containing 12,500 IU of Bacitracin), polymyxin B (2,500 IU), Cyclohexamide (50.0mg), Vancomycin (10.0mg), Nystatin (50,000 IU), and Nalidixic acid (2.5mg) (Oxoid, UK) along with 5% inactivated horse serum prior incubation at 37°C for 10 days under 10% CO2. Then, the passage of grown bacteria was carried out on Brucella-specific agar (Himedia, India) and incubated for 7 days at 37°C. Suspected colonies of Brucella spp. were then sub-cultured and further analyzed to obtain full identity and their related biotype (9). For the purpose of this study, a full-dose of the RB51 vaccine was randomly sampled and used for propolis susceptibility testing. The live attenuated B. abortus strain RB51 represents the official vaccine currently used to prevent
bovine brucellosis worldwide. This vaccine is produced and manufactured by the Razi Vaccine and Serum Research Institute since 2007.

**Molecular typing**

Crude DNA extraction was prepared by heating a loopful of bacterial material dissolved in 300 µl of molecular biology-grade water for 15 min at 100 °C. The bacterial suspension was vortexed and centrifuged at 13,000 g for 5 min and then, the supernatant containing genomic DNA was collected and stored at –20 °C until further analysis. The purity and concentration of DNA were evaluated by measuring absorbance at wavelengths 260 nm and 280 nm using a spectrophotometer (Nanodrop® spectrophotometer ND-1000, Germany). For identification of the *Brucella* spp., the extracted DNA was subjected to IS711-based PCR. The thermal PCR steps were 1 cycle at 95 °C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing temperature at 55°C for 30 sec and extension at 72°C for 1 min (10). In addition, multiplex PCR (Bruce-ladder) was performed for species-level molecular identification as previously described by Lopez-Goñi et al. (2008)°(11). The amplified PCR products were separated by a 1.5% agarose gel electrophoresis.

**Susceptibility to propolis extracts**

The primary quantitative screening of the anti-*Brucella* activity of different EEP was based on measuring the diameter of inhibition zones around the disks. The preparation of bacterial solution was performed in 0.5 McFarland units to spread in the Muller-Hinton agar plates supplemented with 5% sheep’s blood. The bacterial plates were incubated at 37°C in the presence of 10% CO2 and the antimicrobial results were read after 48 h. The inoculation of bacterial suspensions containing 10⁸ cells/ml onto plate surfaces was performed using a sterile cotton swab. Disk diffusion susceptibility tests for different propolis extracts were performed by adding 10 µL EEP to a cotton-based paper disk of 6.5 mm in diameter, while a 70% v/v water-alcohol solution was added as control. Also, the following antibiotic disks were placed on the seeded plates as positive controls: rifampin (5 µg per disk), doxycycline (30 µg per disk) and gentamycin (10 µg per disk). These 3 antibiotics are the most commonly used substances in the multi-drug therapy against brucellosis worldwide. The effect of EEP was defined by measuring the diameters of the growth inhibition zones around the disks and compared with those obtained for negative and positive control disks, respectively. All tests were performed in triplicate.

**Results**

**Isolation and identification of clinical strains**

All isolated bacteria grew after incubation in 10% CO₂ for 5–21 days at 37°C and showed typical phenotypic properties of *Brucella* spp. Clinical isolates were visible as Gram negative shiny and translucent colonies with a smooth surface of small honey colored. *Brucella* isolates were then successfully identified at both species and biovar levels by using AMOS PCR and Bruce-ladder, respectively. According to molecular typing results, both *Brucella* isolates originating from Alborz
were identified as *B. melitensis* biovar 3, while all other *Brucella* isolates coming from Kerman and Kermashah provinces belonged to the *B. melitensis* biovar 1.

**Susceptibility to EEPs and selected antibiotics**

Results of the evaluation of antimicrobial activities of 6 propolis samples obtained by disk diffusion susceptibility tests showed that all EEP displayed significant antimicrobial action against tested *Brucella* clinical strains according to different hydro-alcoholic extracts of propolis from Khorasan Razavi, Mazandaran, Alborz, Golestan, West Azerbaycan and Markazi provinces (Table 1). However, these effects were different based to the various composition of this substance on the geographical location.

As depicted in Table 1, all tested *B. melitensis* clinical strains were susceptible to different EEPs with inhibition zones ranging from 18 to 38 mm diameter. However, two EEPs, i.e. EEP3 and EEP4, showed no noticeable inhibitory effect against the RB51 vaccine strain. Overall, RB51 was the most resistant strain to tested antibiotics with zones of inhibition not exceeding 23 mm for rifampin, doxycycline and gentamycin. The two isolates originating from Kerman, identified as *B. melitensis* biovar 1, showed the lowest susceptibility to rifampicin with inhibition zones of 14 mm in diameter. Interestingly, these two strains showed the highest susceptibility to EEP6 among *Brucella* isolates with inhibition zones reaching 30 and 38 mm in diameter. Similarly, RB51 was more susceptible to EEP6 when compared to other EEPs and 3 tested antibiotic disks (Table 1).

The zones of inhibition around the disks of EEP6 were larger in all tested *Brucella* strains, expected for *B. melitensis* biovar 1 (H5), as the inhibition zones induced by EEP1 and EEP6 were comparable (i.e. 30 mm in diameter).

**Chemical composition and quantification of main phenolic compounds**

HPLC analyses (Table 2) showed that Iranian EEP were rich in flavonoids, particularly in flavones (i.e. chrysin, and apigenin) and flavonols (quercetin). Means values obtained from the quantification of 12 major components showed that flavonoids of the flavone family i.e. chrysin and apigenin were by far the prominent phenolic compounds in all tested EEPs. With concentrations ranging from 510 to 1120 µg/g, chrysin was the most strongly represented compound in tested EEPs, followed by apigenin (327-621 µg/g), quercetin (53.1-126.5 µg/g), p-coumaric acid (29.8-112.6 µg/g), chlorogenic acid (35.2-64.4 µg/g), ferulic acid (31.7-68.3 µg/g), gallic acid (27.6-73.3 µg/g), kaempferol (25.7-42.6 µg/g), naringenin (20.3-39.1 µg/g), caffeic acid (19.1-33.7 µg/g), rutin (19.1-32.6 µg/g) and myricetin (9.2-21.4 µg/g). EEP4 (Golestan) was the richest propolis extract in terms of flavones followed by EEP5 (West Azerbaycan) and EEP2 (Mazandaran). The highest concentrations of flavonols, including quercetin, kaempferol and myricetin, were found in EEP5 (West Azerbaycan), EEP1 (Khorassan Razavi) and EEP2 (Mazandaran), respectively. Iranian EEPs also contained high amounts of non-flavonoid phenolic compounds belonging to the hydroxycinnamic acid group, including p-coumaric acid, chlorogenic acid, ferulic acid and caffeic acid. EEP4 had the richest content in terms of hydroxycinnamic acids with a p-coumaric acid concentration reaching 112.6 µg/g.
Discussion

Brucellosis is one of the most important zoonotic diseases worldwide, causing high morbidity in developing countries. The contamination of raw dairy products with *Brucella melitensis* and *Brucella abortus* strains is responsible for the majority of infections in humans (12). In addition, the shedding of the RB51 vaccine strain in milk of vaccinated livestock has been occasionally reported and caused recent public health concerns in North America (13).

Up to now no food additive has been suggested for the prevention of the growth of pathogenic *Brucella* spp. in unpasteurized or post-contaminated milk products. The present results point the need of deeper studies investigating the anti-*Brucella* activity of propolis samples in different media and various biochemical circumstances (presence of proteins, alkaline pH). Several combinations of antibiotics have been proposed for the treatment of infected individuals but the results are still far to be optimal in clinical practices (14). For the purpose of this study, 5 *Brucella* strains were isolated in 2018 from blood samples collected from patients with clinical diagnosis of brucellosis. All 5 clinical isolates were identified as *B. melitensis* of either biovar 1 (n=3) or biovar 3 (n=2). This is in accordance with previous studies showing that *B. melitensis* is the most common species responsible for human brucellosis in Iran (Alamian et al., 2019; Dadar et al., 2019). Among *Brucella* clinical isolates, two *B. melitensis* biovar 1 strains, originating from patients living in Kerman, showed the lowest susceptibility to rifampicin. Nonetheless, EEP6 appeared to be surprisingly effective against these two strains, inducing zones of inhibition exceeding 30 mm in diameter. Similarly, RB51 showed high susceptibility to EEP6 with an inhibition zone exceeding those obtained for the 3 antibiotics (rifampin, doxycycline and gentamycin) conventionally used in brucellosis multi-drug therapy (Table 1). This result is of particular importance as the risk of human infections through this resistant vaccine strain, shedding in the milk of sporadic vaccinated cattle, has been recently emphasized (13). However, significant differences were observed in the antimicrobial activity of different propolis extracts as EEP3 and EEP4 showed no inhibitory effect against the RB51 vaccine strain. Overall, EEP6 was the more efficient propolis extract against different *Brucella* strains inducing inhibition zones ranging from 25 to 38 mm in diameter. These substantial variations in the antimicrobial activity of EEP could be due to the differential propolis compositions due to their origin as depicted in Table 2.

The complex chemical composition of propolis depends on the flora surrounding honeybee colonies (15). Therefore, differences in the anti-*Brucella* activity according to the propolis origin could be explained by different botanical sources and available resins used by honeybees to form propolis. Previous analyses performed by our teams showed that Iranian propolis samples are of poplar type with a high inhibitory potential against advanced glycation end-products (16). In the temperate zones (Asia, Europe and North America), poplar bud exudates are the main source of propolis, although other local plant species may contribute to its complex composition. Samples originating from these regions have similar chemical signatures and share main constituents,
mainly belonging to the flavonoids and aromatic acids groups (17). HPLC analyses showed that Iranian EEPs were rich in flavonoids, particularly in flavones (i.e. chrysin, and apigenin) and flavonols (quercetin). The antibacterial activity of flavones has been exhaustively studied and is now well documented. Different mechanisms of action have been proposed such as alteration in cytoplasmic membrane properties and functions, inhibition of energy metabolism and nucleic acid synthesis as well as the reduction in biofilm formation and in bacterial cell attachment (18, 19).

It was shown that flavones, such as chrysin and apigenin, are able to bind cell wall components and inhibit further adhesions and growth of pathogenic bacteria. For example, the combination of apigenin and ceftazidime damaged cytoplasmic membrane of Enterobacter cloacae strains which were initially resistant to ceftazidime (20).

Flavonols such as quercetin and rutin, also present in Iranian propolis samples, showed antimicrobial activity against various bacterial strains including Staphylococcus aureus and Staphylococcus epidermidis. Interestingly, quercetin appeared to be quite more effective in combination with amoxicillin (21) and showed remarkable synergistic properties when used in complement of traditional antibiotics.

Among non-flavonoid compounds present in Iranian propolis samples, caffeic acid and ferulic acid are phytochemicals belonging to phenylpropanoid class, widely distributed over the plant kingdom (22). The use of these phenolic compounds in combination or separately led to promising results in the control and prevention of certain chronic diseases such as cardiovascular troubles and cancer. It has been also shown that phenylpropanoid displayed synergistic action with antibiotics against bacteria (22). The combination of ferulic acid, caffeic acid and chlorogenic acid (among the major components of Iranian EEPs) had a dose-dependent bactericidal effect on Shigella sonnei (group D), the pathogen responsible for diarrhoeal diseases (23).

Hence, in light of the polyphenol-rich chemical composition and bactericidal properties of the Iranian propolis against different Brucella strains, these extracts merit to be further studied as safe complements to conventional antibiotics commonly used in the treatment of brucellosis in the context of large clinical trials. The susceptibility of different Brucella spp to different EEP supports preliminary findings on the antimicrobial action of propolis against a B. militensis strain (24) and also revealed the potential of propolis to be used as food additive in raw milk products in endemic regions. This fact is of particular importance given the increasing occurrence of rifampicin-resistant Brucella strains in recent years (25). However, different propolis samples showed differential inhibitory potential against Brucella growth and the pre-selection and in vitro screening of EEPs for their anti-Brucella activity appeared to be essential.

In this paper, the polyphenolic composition of 6 propolis preparations in the form of ethanolic extract (EEP) has been determined by means of HPLC-DAD. Flavonoids such as chrysin, apigenin and quercetin were the major constituents of all Iranian EEPs, despite their different geographical origins. All 6 EEPs were able to inhibit the growth of the tested Brucella strains, although to a different extent. One important finding of this study is the susceptibility of rifampicin-resistant strains to propolis extracts, particularly to EEP 6, originating from the semi-arid region of Markazi.
The anti-*Brucella* activity of this propolis is thus not influenced by the level of the antibiotic susceptibility of different strains. These results open new possibilities for brucellosis treatment and prevention in endemic regions as a potential complement to conventional antibiotics. Such approaches require systematic clinical investigations to further determine efficacy, dose ranging and safety of propolis-based preparations.

**Authors' Contribution**

All authors have made substantial contributions to the conception and design of the study (M.D., N.M., S.A., Y.S.), or acquisition of data analysis (M.D., N.M., Y.S.) and interpretation of data (M.D., N.M., Y.S.), drafting the article (M.D., N.M., Y.S.) revising it critically for important intellectual content (M.D., N.M., S.A., Y.S.), final approval of the version to be submitted (M.D., N.M., S.A., Y.S.).

**Ethics**

Ethics approval was not required for this study.

**Conflict of Interest**

The authors declare no competing financial interests regarding the present study.

**Grant Support**

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**Acknowledgment**

The authors would like to thank their respective institutions and departments.

**References**


Table 1: Anti-Brucella activity of ethanolic extracts of 6 propolis samples (EEP 1-6) collected from different regions of Iran. The diameter of the zone of inhibition is indicated in mm. Data are expressed as the mean ± SD.

<table>
<thead>
<tr>
<th>Samples</th>
<th>RB51</th>
<th>B. melitensis biovar 3 (Alborz) H1</th>
<th>B. melitensis biovar 3 (Alborz) H4</th>
<th>B. melitensis biovar 1 (Kermanshah) H3</th>
<th>B. melitensis1 (Kerman) H2</th>
<th>B. melitensis1 (Kerman) H5</th>
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<tbody>
<tr>
<td>EEP1</td>
<td>20 ± 0.5</td>
<td>20 ± 0.3</td>
<td>25 ± 0</td>
<td>25 ± 0.5</td>
<td>20 ± 0.3</td>
<td>30 ± 0.5</td>
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<tr>
<td>EEP2</td>
<td>18 ± 1</td>
<td>18 ± 0.6</td>
<td>18 ± 1</td>
<td>18 ± 1</td>
<td>15 ± 0</td>
<td>20 ± 0.2</td>
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<tr>
<td>EEP3</td>
<td>2.3 ± 0.5</td>
<td>18 ± 0.5</td>
<td>22 ± 0</td>
<td>23 ± 0</td>
<td>20 ± 0.5</td>
<td>15 ± 0</td>
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<td>EEP4</td>
<td>2.7 ± 0.6</td>
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<td>25 ± 1</td>
<td>19 ± 0.5</td>
<td>25 ± 0.3</td>
<td>26 ± 1.3</td>
<td>18 ± 0.2</td>
<td>16 ± 0</td>
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<tr>
<td>EEP6</td>
<td>30 ± 0.3</td>
<td>25 ± 0.5</td>
<td>28 ± 0.9</td>
<td>30 ± 0</td>
<td>38 ± 0.9</td>
<td>30 ± 0.5</td>
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<td>Doxycycline</td>
<td>23 ± 0.3</td>
<td>40 ± 1.3</td>
<td>35 ± 0.5</td>
<td>45 ± 0.8</td>
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<tr>
<td>Rifampicin</td>
<td>22 ± 1</td>
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<tr>
<td>Gentamycin</td>
<td>21 ± 0.3</td>
<td>37 ± 1</td>
<td>30 ± 0.3</td>
<td>30 ± 0.3</td>
<td>32 ± 0</td>
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</table>

Hydro-alcoholic extracts of propolis from Khorasan Razavi (EEP1), Mazandaran (EEP2), Alborz (EEP3), Golestan (EEP4), West Azerbeycan (EEP5) and Markazi (EEP6) provinces were evaluated for their capacity to inhibit the growth of 5 B. melitensis clinical isolates and RB51 vaccine strain.
Table 2: HPLC analyses of the 12 major phenolic compounds in Iranian propolis extracts (EEP). The concentration of active compounds is expressed as µg/g.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Chrysin</th>
<th>Kaempferol</th>
<th>Apigenin</th>
<th>Quercetin</th>
<th>Myricetin</th>
<th>Naringenin</th>
<th>Ferulic acid</th>
<th>p-Coumaric acid</th>
<th>Rutin</th>
<th>Caffeic acid</th>
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<tr>
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<td>Mean SD</td>
<td>742.2 ± 251</td>
<td>29.4 ± 17</td>
<td>460.8 ± 111.5</td>
<td>89.2 ± 29.1</td>
<td>14.1 ± 8.3</td>
<td>28 ± 15.7</td>
<td>47.2 ± 12.6</td>
<td>58.7 ± 30</td>
<td>22.6 ± 10.6</td>
<td>26 ± 5</td>
<td>48 ± 23.2</td>
<td>34.5 ± 18.8</td>
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</table>

Hydro-alcoholic extracts of propolis from Khorasan Razavi (EEP1), Mazandaran (EEP2), Alborz (EEP3), Golestan (EEP4), West Azerbayejan (EEP5) and Markazi (EEP6) provinces were evaluated for their polyphenol contents. RT indicates the retention time.