Archives of Razi Institute, Vol. 77, No. 1 (2022) 57-64

DOI: 10.22092/ari.2021.353395.1602

Original Article

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

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Received 30 January 2021; Accepted 2 May 2021
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Abstract

Brucellosis is one of the most important zoonotic diseases in many regions worldwide. This study aimed to investigate the antimicrobial properties of hydro-ethanolic extracts of propolis (EEP) samples collected from six different regions of Iran against five *Brucella melitensis* (*B. melitensis*) clinical isolates causing human brucellosis and an antibiotic-resistant *B. abortus* vaccinal strain (RB51). *Brucella* clinical isolates were first carefully identified using conventional molecular typing and *Brucella* bio-typing methods. Different *Brucella* strains were then confronted with 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. It was found that all EEPs displayed significant antimicrobial activities against *Brucella* strains, though 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), 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 analysis. 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 open**s** 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

1. Introduction

Human brucellosis is a common zoonotic disease affecting many regions around the world, especially, Near and the Middle East regions, North and Sub-Saharan African countries, Western and Eastern Asia, as well as Latin America. *Brucella melitensis (B. melitensis*) is the main *Brucella* Species (spp.) that contaminates dairy products and is 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 (WHO) 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 socioeconomic 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 regimens, they may lead to an increased risk for antibiotic resistance. Considering the fact that some of these regimens are also used against tuberculosis (e.g., rifampicin), this resistance appears to be an important public health concern in brucellosis endemic regions. Another critical point in the treatment of brucellosis is the duration of therapy as a prolonged antibiotic administration is required to diminish the risk of relapse. These issues considerably increase the risk for the 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 is of prominent importance. Medicinal plants have long been considered potential sources of bioactive agents against a wide range of microorganisms while having fewer adverse effects and good affordability (4). Propolis is one of the most efficient and promising natural antimicrobial 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 which revealed an important bactericidal potential of propolis against a wide range of bacteria (7). Previously, these effects appeared to be species-dependent, and some earlier studies pointed out that Gram-negative bacteria were 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, exhaustively documented, and recently reviewed by (5). In addition, significant variations have been observed 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, as well as balsamic substances collected by bees.

The present study, therefore, aimed to investigate the antibacterial activity of hydro-alcoholic extracts of six propolis (EEP1-6) samples collected from different regions of Iran against *Brucella* Gram-negative bacteria isolated from five 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 also determined in this study.

2. Materials and Methods

2.1. Propolis Extracts

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

2.2. High-Performance Liquid Chromatography Analysis

For high-performance liquid chromatography (HPLC) analysis, one milliliter of each sample was added to 0.5 ml MilliQ water and centrifuged at 13000 rpm for 3 min. Afterward, 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 the HPLC analysis. Phenolic compounds were analyzed using HPLC (Unicam, Crystal-200, England), an instrument comprising a Diode Array Detector (DAD). For the need of this study, an SB-C18 model Zorbax column was used with a 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 in descending order. The auto-injection volume and the flow rate were 20 µl and 2 ml/min, respectively. Furthermore, 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.

2.3. Bacterial Clinical Isolates and RB51 Vaccine Strain

Prior to bacterial culture for *Brucella* isolation, the diagnosis of *Brucella* was performed for each patient 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 five *Brucella* strains were isolated in 2018 from the blood samples of patients with clinical diagnoses of brucellosis. Patients came from three 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 immediately used for bacterial culture. To this end, blood samples were inoculated on a *Brucella* selective media containing 12,500 IU of Bacitracin, Polymyxin B (2,500 IU), Cycloheximide (50.0mg), Vancomycin (10.0mg), Nystatin (50,000 IU), and Nalidixic acid (2.5mg) (Oxoid, UK) along with 5% inactivated horse serum prior to incubation at 37°C for 10 days under 10% CO2. Subsequently, 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 their full identity and 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 *Brucella abortus* strain, RB51, represents the official vaccine currently used to prevent bovine brucellosis worldwide. This vaccine has been produced and manufactured by the Razi Vaccine and Serum Research Institute since 2007.

2.4. 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 the supernatant containing genomic DNA was then collected and stored at -20°C until further analysis. The purity and concentration of DNA were evaluated by measuring the absorbance at wavelengths of 260 nm and 280 nm using a spectrophotometer (Nanodrop® spectrophotometer ND-1000, Germany). For the 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 López-Goñi, García-Yoldi (11). Finally, the amplified PCR products were separated by a 1.5% agarose gel electrophoresis.

2.5. 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 10^8 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, with a 70% v/v water-alcohol solution as a control. Furthermore, 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 three antibiotics are the most commonly used substances in 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

then compared with those obtained for negative and positive control disks, respectively. All tests were performed in triplicate.

3. Results

3.1. Isolation and Identification of Clinical Strains

All the isolated bacteria grew after incubation in 10% CO2 for 5 to 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 in both species and biovar levels using AMOS PCR and Bruce-ladder, respectively. According to the molecular typing results, both *Brucella* isolates collected from Alborz province were identified as *B. melitensis* biovar 3, while all other *Brucella* isolates came from Kerman and Kermanshah provinces belonged to the *B. melitensis* biovar 1.

3.2. Susceptibility to EEPs and Selected Antibiotics

Results of the evaluation of antimicrobial activities of six propolis samples obtained from disk diffusion susceptibility tests showed that all EEP possess significant antimicrobial action against tested *Brucella* clinical strains, based on the hydro-alcoholic extracts of propolis from Khorasan Razavi, Mazandaran, Alborz, Golestan, West Azerbaijan, and Markazi provinces (Table 1). However, these effects differed based on the various compositions of this substance across geographical locations.

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) did not show any 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 from Kerman province, 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 the three tested antibiotic disks (Table 1). The zones of inhibition around the disks of EEP6 were larger in all tested *Brucella* strains, except for *B. melitensis* biovar 1 (H5), as the inhibition zones induced by EEP1 and EEP6 were comparable (i.e., 30 mm in diameter).

3.3. Chemical Composition and Quantification of the Main Phenolic Compounds

HPLC analysis (Table 2) showed that Iranian EEP was rich in flavonoids, particularly in flavones (i.e., chrysin and apigenin) and flavonols (i.e., quercetin). Mean 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 most prominent phenolic compounds in all tested EEPs. With concentrations ranging from 510 to 1120 µg/g, chrysin was the most strongly represented compound in the 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) in descending order. EEP4 (from Golestan province) was the richest propolis extract in terms of flavones, followed by EEP5 (from West Azerbaijan province) and EEP2 (from Mazandaran province). The highest concentrations of flavonols, including quercetin, kaempferol, and myricetin, were found in EEP5 (from West Azerbaijan province), EEP1 (from Khorasan Razavi province), and EEP2 (from Mazandaran province) in descending order. 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.

Table 1. Anti-*Brucella* activity of ethanolic extracts of six 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

Samples	RB51	*B. melitensis* biovar 3 (Alborz) H1	*B. melitensis* biovar 3 (Alborz) H4	*B. melitensis* biovar 1 (Kermanshah) H3	*B. melitensis* 1 (Kerman) H2	*B. melitensis* 1(Kerman) H5
EEP1	20±0.5	20±0.3	25±0	25±0.5	20±0.3	30±0.5
EEP2	18±1	18±0.6	18±1	18±1	15±0	20±0.2
EEP3	2.3±0.5	18±0.5	22±0	23±0	20±0.5	15±0
EEP4	2.7±0.6	20±1	20±0.3	21±1	20±0	15±0.3
EEP5	25±1	19±0.5	25±0.3	26±1.3	18±0.2	16±0
EEP6	30±0.3	25±0.5	28±0.9	30±0	38±0.9	30±0.5
Doxycycline	23±0.3	40±1.3	35±0.5	45±0.8	30±0.3	30±0.3
Rifampicin	22±1	30±0.5	18±0.8	30±0.5	14±0.1	14±0.2
Gentamycin	21±0.3	37±1	30±0.3	30±0.3	32±0	32±0.2

Hydro-alcoholic extracts of propolis from Khorasan Razavi (EEP1), Mazandaran (EEP2), Alborz (EEP3), Golestan (EEP4), West Azerbaijan (EEP5), and Markazi (EEP6) provinces were evaluated for their capacity to inhibit the growth of five *Brucella melitensis* clinical isolates and RB51 vaccine strain

Table 2. HPLC analysis of 12 major phenolic compounds in Iranian propolis extracts (EEP). The concentration of active compounds is expressed as µg/g.

Samples	Chrysin	Kaempferol	Apigenin	Quercetin	Myricetin	Naringenin	Ferulic acid	p-Coumaric acid	Rutin	Caffeic acid	Chlorogenic acid	Gallic acid
	RT (min) 28.22	RT (min) 16.30	RT (min) 15.27	RT (min) 11.6	RT (min) 6.81	RT (min) 4.96	RT (min) 4.52	RT (min) 3.90	RT (min) 2.75	RT (min) 2.44	RT (min) 2.10	RT (min) 1.82
EEP1	536	42.6	390	96.3	--	39.1	48.3	69.3	32.6	26.1	--	60.7
EEP2	824	--	446	117.7	9.2	--	68.3	41.2	21.7	29.5	61.3	47.6
EEP3	510	18.6	419	72.4	--	--	31.7	40.6	17.6	19.1	36.9	27.6
EEP4	1120	30.6	631	69.2	21.4	20.3	40.0	112.6	19.1	24.8	64.4	73.3
EEP5	917	25.7	552	126.5	11.6	27.0	52.7	58.5	--	33.7	35.2	27.6
EEP6	546	--	327	53.1	14.2	25.5	42.1	29.8	22.2	23.3	42.0	34.5
Mean±SD	742.2±251	29.4±17	460.8±111.5	89.2±29.1	14.1±8.3	28±15.7	47.2±12.6	58.7±30	22.6±10.6	26±5	48±23.2	34.5±18.8

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

4. Discussion

Brucellosis, as one of the most important worldly zoonotic diseases, causes high morbidity in developing countries. The contamination of raw dairy products with *B. melitensis* and *B. abortus* strains are the primary reasons for the majority of infections in humans (12). In addition, the shedding of the RB51 vaccine strain in the milk of vaccinated livestock has recently caused public health concerns in North America (13).

Up to now, no food additive has been suggested for preventing the growth of pathogenic *Brucella* spp. in unpasteurized or post-contaminated milk products. The findings of the present study highlight the need for thoroughly investigating the anti-*Brucella* activity of propolis samples in different media and various

biochemical circumstances (e.g., in the presence of proteins, or alkaline pH). Several combinations of antibiotics have been proposed for the treatment of infected individuals; however, the results are still far to be optimal in clinical practice (14). For the purpose of this study, five *Brucella* strains were isolated in 2018 from the blood samples of patients with a clinical diagnosis of brucellosis. All five clinical isolates were identified as *B. melitensis* of either biovar 1 (n=3) or biovar 3 (n=2). This is in accordance with the results of previous studies showing that *B. melitensis* is the most common species responsible for human brucellosis in Iran (9, 14). Among *Brucella* clinical isolates, two *B. melitensis* biovar 1 strains, taken from patients living in Kerman province, 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 three antibiotics (i.e., rifampin, doxycycline, and gentamycin) conventionally used in brucellosis multi-drug therapy (Table 1). This finding 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 and showed no inhibitory effect against the RB51 vaccine strain. Overall, EEP6 was the most 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 and 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 the available resins used by honeybees to form propolis. Previous analyses performed by our teams showed that Iranian propolis samples were of poplar type with a high inhibitory potential against the advanced glycation end products (16). In temperate zones (i.e., Asia, Europe, and North America), poplar bud exudates are the main sources of propolis, although other local plant species may also contribute to its complex composition. Samples taken from these regions have similar chemical signatures and share some of their main constituents, mainly those belonging to the flavonoids and aromatic acid groups (17). HPLC analysis revealed that Iranian EEPs were rich in flavonoids, particularly in flavones (i.e., chrysin and apigenin) and flavonols (i.e., quercetin). The antibacterial activity of flavonoids 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, nucleic acid synthesis, as well as the reduction in biofilm formation and 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. The combination of apigenin and ceftazidime could damage the cytoplasmic membrane of *Enterobacter cloacae* strains which were initially resistant to ceftazidime (20). Flavonols, such as quercetin and rutin, 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 when combined with amoxicillin (21) and showed remarkable synergistic properties when accompanied by traditional antibiotics.

Among non-flavonoid compounds present in Iranian propolis samples, caffeic acid and ferulic acid are phytochemicals belonging to the 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 displays 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 diarrheal diseases (23).

Therefore, in light of the polyphenol-rich chemical composition and the 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 large clinical trials. The susceptibility of different *Brucella* spp. to different EEP supports preliminary findings on the antimicrobial activity of propolis against *B. melitensis* strain (24) and also reveals the potential of propolis to be used as a food additive in raw milk products in endemic regions. This is of particular importance given the increasing occurrence of rifampicin-resistant *Brucella* strains in recent years (25). However, different propolis samples showed different inhibitory potentials against *Brucella* growth. In addition, the pre-selection and *in vitro* screening of EEPs for their anti-*Brucella* activity appeared to be essential.

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

Authors' Contribution

Study concept and design: M. D., N. M., S. A., and Y. S.
Acquisition of data: M. D., N. M., and Y. S.
Analysis and interpretation of data: M. D., N. M., and Y. S.
Drafting of the manuscript: M. D., N. M., and Y. S.
Critical revision of the manuscript for important intellectual content: M. D., N. M., S. A., and Y. S.
Statistical analysis: M. D., N. M., and Y. S.
Administrative, technical, and material support: M. D., N. M., S. A., and Y. S.

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant Support

This study was supported by the Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO) (grant number 2-18-18-033-950404/3).

Acknowledgment

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

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