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In vitro assay for the anti-brucella activity of medicinal plants against tetracycline-resistant *Brucella melitensis**

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Abstract: Brucellosis, a zoonosis caused by four species of brucella, has a high morbidity. *Brucella melitensis* is the main causative agent of brucellosis in both human and small ruminants. As an alternative to conventional antibiotics, medicinal plants are valuable resources for new agents against antibiotic-resistant strains. The aim of this study was to investigate the usage of native plants for brucellosis treatment. For this purpose, the anti-brucella activities of ethanolic and methanolic extracts of *Salvia sclarea*, *Oliveria decumbens*, *Ferulago angulata*, *Vitex pseudo-negundo*, *Teucrium polium*, *Plantago ovata*, *Cordia myxa*, and *Crocus sativus* were assessed. The activity against a resistant *Br. melitensis* strain was determined by disc diffusion method at various concentrations from 50–400 mg/ml. Antibiotic discs were also used as a control. Among the evaluated herbs, six plant (*Salvia sclarea*, *Oliveria decumbens*, *Ferulago angulata*, *Vitex pseudo-negundo*, *Teucrium polium*, and *Crocus sativus*) showed anti-brucella activity. *Oliveria decumbens* was chosen as the most effective plant for further studies. A tested isolate exhibited resistance to tetracycline, nafcillin, oxacillin, methicillin, and colistin. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values for *Oliveria decumbens* against resistant *Br. melitensis* were the same (5 mg/ml), and for gentamicin they were both 2 mg/ml. Time-kill kinetics for a methanolic extract of *Oliveria decumbens* was 7 h whereas for an ethanolic extract it was 28 h. Also, *Oliveria decumbens* extracts showed a synergistic effect in combination with doxycycline and tetracycline. In general, the similar values of MIC and MBC for *Oliveria decumbens* suggest that these extracts could act as bactericidal agents against *Br. melitensis*. In addition to *Oliveria decumbens*, *Crocus sativus* and *Salvia sclarea* also had good anti-brucella activity and these should be considered for further study.

Key words: Brucellosis, Antibiotic resistance, *Brucella melitensis*, Medicinal plant, *Oliveria decumbens*
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1 Introduction

Brucellosis, a bacterial zoonosis, is an important public health concern due to its high morbidity. The prevalence of infection in humans is directly related to the prevalence in animals, particularly in domestic ruminants (Grilló *et al*., 2006). Furthermore, considering the economic loss from animal infections, in terms of decreased milk production, abortions, weak offspring, weight loss, infertility, and lameness, brucellosis is one of the most serious diseases of livestock, and hence a major problem for the trade (Gul and Khan, 2007). Among brucella species, *Brucella melitensis*, *Br. abortus*, *Br. suis*, and *Br. canis* are pathogenic for human, but *Br. melitensis* causes the highest morbidity with severe complications, including endocarditis as the main cause of mortality. While brucellosis occurs worldwide, it is endemic in the Mediterranean basin, the Middle East, Western Asia, Africa, and Latin America (Valenza *et al*., 2006). For treatment, a combination of antibiotics that penetrate the macrophage should be used. The choice treatment for human brucellosis caused by *Br. melitensis* field strains is a combination of long-acting

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tetracyclines and streptomycin. As well, studies have shown that for treatment of patients with *Br. melitensis* vaccine strains Rev1, a gentamicin/doxycycline combination may be the first choice (Grilló *et al*., 2006). In general, tetracycline/aminoglycoside combinations are the most common antibiotics used for brucellosis treatment. However, because of high rates of treatment failure or relapses due to emerging resistance, the treatment of brucellosis is still problematic. Thus, new antibacterial compounds are becoming necessary for brucellosis treatment. Medicinal plants have always been sources for new drug discovery. Plants readily synthesize substances for their defense against insects, herbivores, and microorganisms (Aboaba *et al*., 2006). Moreover, they may produce secondary antimicrobial metabolites as a part of their normal growth and development or in response to stress (Mirjana *et al*., 2004). Evidence for use of these natural resources in Iran is longstanding and there are a number of historical, scientific documents in this area, such as the famous 'Laws of Medicines' of Ibn Sina (Avicenna, 980-1037), which contains sections that discuss the herbal medicines in detail (Lothfipour *et al*., 2008). *Salvia sclarea*, *Oliveria decumbens*, *Ferulago angulata*, *Vitex pseudo-negundo*, *Teucrium polium*, *Plantago ovata*, *Cordia myxa*, and *Crocus sativus* are traditional medicinal plants used in Iran for many purposes, particularly for gastrointestinal disorders and analgesia. The object of this study was to screen for antibacterial activity of hydro-alcoholic extracts of the above-mentioned plants against resistant *Br. melitensis* strain in vitro.

2 Materials and methods

2.1 Plant collection and identification

Salvia sclarea, *Oliveria decumbens*, *Vitex pseudo-negundo*, *Teucrium polium*, *Plantago ovata*, and *Cordia myxa* were collected from hills around Behbahan (southeast of Khuzestan Province, Iran) from April to August, 2008 (Table 1), whereas *Crocus sativus* and *Ferulago angulata* were purchased from the market in Behbahan. The taxonomic identification of these plants was done by the Herbarium of Agricultural Science Faculty, Shahid Chamran University, Iran.

Table 1 Plants and their families, parts used, and time of collection in this study

Plant	Family	Part used	Month of collection
Oliveria decumbens	Umbelliferae	Aerial	May
Salvia sclarea	Labiatae	Aerial	April
Teucrium polium	Labiatae	Aerial	May
Ferulago angulata	Apiaceae	Aerial	−
Vitex pseudo-negundo	Verbenaceae	Seed	August
Plantago ovata	Plantaginaceae	Seed	May
Crocus sativus	Iridaceae	Stigma	−
Cordia myxa	Boraginaceae	Fruit	July

−: not collected (purchased)

2.2 Plant extract preparation

The seeds of *Vitex pseudo-negundo* and *Plantago ovata*, the fruit of *Cordia myxa*, the stigmas of *Crocus sativus*, and aerial parts of *Salvia sclarea*, *Oliveria decumbens*, *Teucrium polium*, and *Ferulago angulata* were shade dried at room temperature for 10 d. The aforementioned parts were ground to a fine powder. One gram of powder was extracted using 10 ml of alcohol (ethanol or methanol)-distilled water solution (alcohol:water=8:2, v/v), with centrifugation at 3000 r/min for 15 min, and then the supernatant was collected. This process was repeated three times. Solvents were then removed by evaporation (Seyyednejad *et al*., 2001; Moazedi *et al*., 2007).

2.3 Bacterial strain

The *Br. melitensis* strain used in this study was isolated from aborted sheep fetus with acute brucellosis. It was identified to the species level by conventional methods (the requirement for CO_2 for growth, production of H_2S, urease production, sensitivity to fuchsin and thionin, and agglutination with specific antiserum). A class II biological safety cabinet was used. This strain was stored in skim milk at −70 °C and twice subcultured before starting the study.

2.4 Antibacterial susceptibility assay

The test isolate was grown in Muller-Hinton Broth (MHB, Merck) medium at 37 °C for 22 h. Final inoculum bacterial numbers were adjusted to 10^8 CFU/ml with reference to the McFarland turbidometry (Burt and Reinders, 2003; Zakaria *et al*., 2007). A total of 0.1 ml of bacterial suspension was poured on each plate containing Muller-Hinton Agar (MHA, Merck). The lawn culture was prepared by sterile cotton swab and allowed to remain in contact for 1 min. Four concentrations of ethanolic and methanolic extracts (50, 100, 200, and 400 mg/ml) from each plant were prepared. The sterile filter paper discs (6-mm diameter) were saturated by 50 μl of different concentrations of each extract and then were placed on lawn cultures (Hsieh *et al*., 2001; Cermelli *et al*., 2008). The Petri dishes were subsequently incubated at 37 °C for 24 h and the inhibition zone around each disc was measured in mm. As positive controls, discs (Difco, USA) containing streptomycin 10 μg, tetracycline 30 μg, gentamicin 10 μg, doxycycline 30 μg, oxacillin 1 μg, colistin 10 μg, nafcillin 1 μg, and methicillin 5 μg were used. Different discs impregnated with 80% ethanol and 80% methanol were also included to test if they had an inhibitory effect on the test bacteria.

2.5 MIC and MBC determination

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of ethanolic and methanolic extracts of the most effective plant were determined against *Br. melitensis*. MIC was determined by the macro broth dilution assay method (NCCLS, 1993; Forbes *et al*., 1998). In the tube dilution assay, standard bacterial suspension and different concentrations of extracts (5, 10, 20, 40, 80, 160, and 200 mg/ml) were added to tubes containing 1 ml MHB. These tubes were incubated at 37 °C for 24 h. The first tube in the above series with no sign of visible growth was considered as the MIC. MBC was determined by culturing one standard loop of the tubes with no apparent growth on MHA and subsequent incubation at 37 °C for 24 h. The least concentration that inhibited colony formation on agar was taken as the MBC for these extracts.

2.6 Time-kill kinetic study

The time-kill kinetics was surveyed by culturing one standard loop of the suspension from the tube possessing MIC on MHB from 0 to 36 h after inoculation of extracts into the tube containing 1 ml MHB. This was performed at 1-h intervals for the first 18 h, and then at 2-h intervals for the later 18 h.

2.7 Study of the synergistic effect

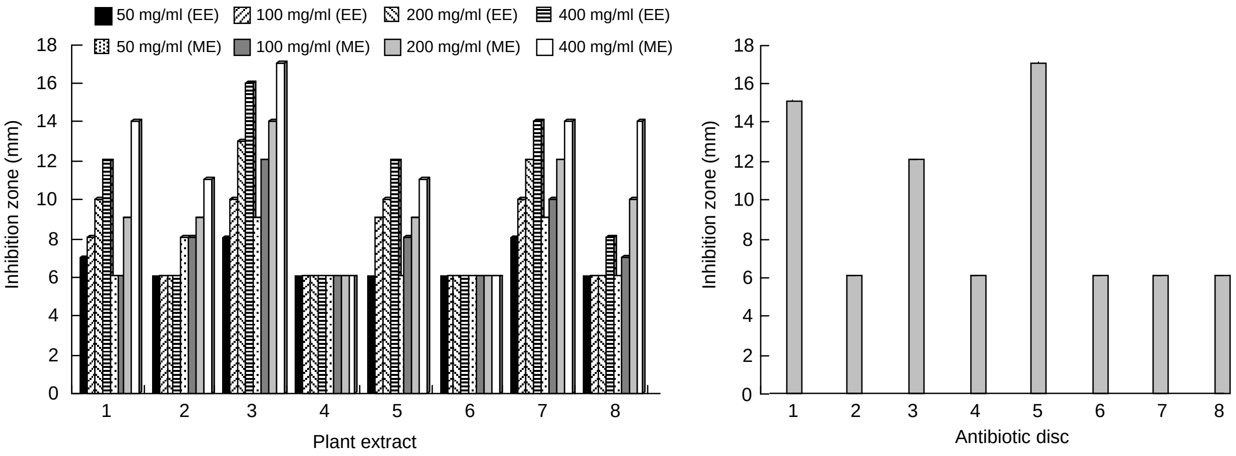
To determine the synergistic effect of the most effective plant with synthetic antibiotics, 400 mg/ml of its extracts were added to the discs containing gentamicin, streptomycin, tetracycline, and doxycycline, and their effects were evaluated by the disc diffusion method (Mahboobi *et al*., 2006).

3 Results

On the basis of the primary screening results (Fig. 1a), *Cordia myxa* and *Plantago ovata* did not have antibacterial activity against *Br. melitensis*, even at the highest concentration, whereas *Oliveria decumbens* and *Crocus sativus* extracts were effective even at the lowest concentration. Also, ethanolic extract of *Salvia sclarea* and methanolic extract of *Vitex pseudo-negundo* showed anti-brucella activity, even at the lowest concentration. Ethanolic extract of *Teucrium polium* exhibited an inhibitory effect at all concentrations except at 50 mg/ml. The *Br. melitensis* isolate was resistant to tetracycline, nafcillin, methicillin, oxacillin, and colistin (Fig. 1b). Considering the diameter of the inhibition zone, *Oliveria decumbens*, which showed the highest anti-brucella activity, was chosen for further study. MIC and MBC values for ethanolic and methanolic extracts of *Oliveria decumbens* were the same (5 mg/ml), and these values for gentamicin were both 2 mg/ml (Table 2). The time-kill kinetics for methanolic extract of *Oliveria decumbens* was 7 h whereas for ethanolic extract it was 28 h (Table 3). The study of the synergistic and antagonistic effects of *Oliveria decumbens* with standard antibiotics showed that the combination of extracts of this plant with doxycycline, tetracycline, and oxacillin have a synergistic effect against *Br. melitensis*. In contrast, antagonistic effects were observed for streptomycin and gentamicin (Table 4).

Table 2 MIC and MBC for *Oliveria decumbens* aerial part extracts and gentamicin against *Br. melitensis*

Antibiotic or extract	MIC (mg/ml)	MBC (mg/ml)
Methanolic	5	5
Ethanolic	5	5
Gentamicin	2	2



(a) (b)

Fig. 1 Inhibition zones of ethanolic and methanolic extracts of some medicinal plants (a) and antibiotic discs (b) against *Br. melitensis*
ME: methanolic extract; EE: ethanolic extract. Plant extracts: 1. *Salvia sclarea*; 2. *Vitex pseudo-negundo*; 3. *Oliveria decumbens*; 4. *Cordia myxa*; 5. *Teucrium polium*; 6. *Plantago ovata*; 7. *Crocus sativus*; 8. *Ferulago angulata*. Antibiotic discs: 1. streptomycin; 2. tetracycline; 3. doxycycline; 4. oxacillin; 5. gentamicin; 6. methicillin; 7. nafcillin; 8. colistin. The diameter of disc is 6 mm

Table 3 Time-kill kinetics of *Oliveria decumbens* extracts at 5 mg/ml against *Br. melitensis*

Extract	Time (h)																										
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	20	22	24	26	28	30	32	34	36
Methanolic	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Ethanolic	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−

+: growth; −: growth inhibition

Table 4 Results of the synergistic and antagonistic effects of *Oliveria decumbens* extracts with antibiotic discs

Antibiotic discs	Inhibition zone (mm)			Result
	None	ME	EE	
Oxacillin	0	17	14	Syn
Doxycycline	12	21	19	Syn
Streptomycin	15	11	12	Ant
Tetracycline	0	17	16	Syn
Gentamicin	17	14	13	Ant

ME: methanolic extract; EE: ethanolic extract; Syn: synergism; Ant: antagonism

4 Discussion

Nowadays, the treatment of brucellosis remains a major public health concern, especially in countries with low socio-economic status (Turkmani *et al*., 2006). In order to increase the treatment efficacy and avoid disease relapse, a classic combination of tetracycline and aminoglycoside has been recommended. Multiple drug resistant strains of brucella have developed, and unfortunately, bacteria have the ability to transmit and acquire resistance to drugs (Nascimento *et al*., 2000). NorMI multidrug efflux protein in *Br. melitensis* confers resistance to gentamicin and other antimicrobial agents (Grilló *et al*., 2006). In order to explore new agents, medicinal plants are a good choice because these natural resources commonly have fewer adverse effects, are less costly and more effective against broad-spectrum antibiotic resistant bacteria. In many parts of the world, extracts of medicinal plants are used because of their antibacterial, antifungal, and antiviral activities (Hassawi and Kharma, 2006). The majority of the plants that were used in this study are applied in traditional medicine in southern regions of Iran as antipyretic, and also in order to cure gastrointestinal disorders. Thus, these natural sources were evaluated in a pilot effort to explore antibacterial compounds against *Br. melitensis*. The primary quantitative screening of the anti-brucella activity of the hydroalcoholic extracts obtained from the tested plants was done based on the presence or absence of inhibition zone and measuring the diameter of inhibition zones around the discs (Fig. 1). In total, 75% of the evaluated herbs inhibited the growth of the tested bacterium. In general, the anti-brucella activity of ethanolic and methanolic extracts of the active plants was decreased at lower concentrations. The MIC and MBC values for ethanolic and methanolic extracts of *Oliveria decumbens* were the same. It is generally held that for bactericidal agents, the MIC and MBC are often near or equal values, so one can say that the extracts of *Oliveria decumbens* have a bactericidal effect on *Br. melitensis* (Reuben *et al*., 2008). Determination of the time-kill kinetics for hydro-alcoholic extracts of *Oliveria decumbens* showed that the methanolic extract of this plant had a better efficacy than its ethanolic extract against *Br. melitensis*.

5 Conclusions

The best synergistic activity was observed for the combination of *Oliveria decumbens* extracts and doxycycline, while a slightly antagonistic effect was observed in combination with streptomycin and gentamicin. Thus, use of doxycycline and extracts of *Oliveria decumbens* can be suggested as an alternative for tetracycline/aminoglycoside combination for the treatment of brucellosis in human. However, while in vitro results are encouraging and merit further clinical study of *Oliveria decumbens*, the in vivo efficacy remains to be confirmed. In general, the results revealed significant anti-brucella activity of medicinal plants, which could be a potential source of new antibacterial agents.

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