

## Evaluation of the antibacterial activity of *Zataria multiflora* Boiss., *Rhus coriaria* L. (sumac), *Mentha piperita* L., and *Ocimum basilicum* L. extracts on *Brucella* strains isolated from brucellosis patients

Yousef MOTAHARINIA<sup>1</sup>, Mohammad Ali REZAEI<sup>1</sup>, Mohammad Saleh HAZHIR<sup>2</sup>,  
Farid ZANDI<sup>1</sup>, Reza SHAPOURI<sup>3</sup>, Mohammad Saied HAKHAMANESHI<sup>2</sup>,  
Ahmad RASHIDI<sup>1</sup>, Mohammad Reza RAHMANI<sup>1</sup>

**Aim:** Since brucellosis has been called a chronic illness and treatment of patients infected with this bacterium has not been successful, the present study aimed to evaluate the effects of several medicinal plant extracts on clinical *Brucella* strains.

**Materials and methods:** First, *Brucella* strains were isolated from brucellosis patients. Then an antibiotic resistance assay was performed for these strains, while the antibacterial activity of the above-mentioned extracts was evaluated.

**Results:** After performing accurate evaluations, the obtained results showed that all of the isolated *Brucella* strains were sensitive to tetracycline, doxycycline, and gentamicin, and the rates of antibiotic resistance to rifampin and streptomycin were 83.3% and 11.1%, respectively. The mean zone of growth inhibition for *Zataria multiflora* Boiss. was  $\geq 28.77$  mm, *Rhus coriaria* L. (sumac) was 22.55 mm, *Mentha piperita* L. was  $\geq 7.5$  mm, and the gentamicin disk was  $\geq 30$  mm. The mean minimum inhibitory concentration (MIC) for *Z. multiflora* was 1237  $\mu\text{g/mL}$ , and it was 3255.2  $\mu\text{g/mL}$  for sumac, and 5642  $\mu\text{g/mL}$  for *M. piperita* L. The minimum bactericidal concentration (MBC) rate for these herbs was 5900  $\mu\text{g/mL}$ , 9027  $\mu\text{g/mL}$ , and 12152  $\mu\text{g/mL}$ , respectively. Furthermore, no anti-*Brucella* activity was observed for *Ocimum basilicum* L.

**Conclusion:** The results obtained in this study prove that *Z. multiflora* extracts show high anti-*Brucella* activity and it can be used for better treatment of brucellosis.

**Key words:** *Brucella*, *Zataria multiflora* Boiss., *Rhus coriaria* L., *Mentha piperita* L., *Ocimum basilicum* L., antibacterial activity

### Introduction

*Brucella* spp. are obligate intracellular gram-negative bacteria that primarily infect animals, especially domestic livestock, but can be transmitted to humans. *Brucella* bacteria are also responsible for brucellosis. These bacteria can be transmitted

through contaminated dairy products or directly through the skin or respiratory tract (1,2). Brucellosis is a zoonotic disease and its prevalence is more than 500,000 cases annually worldwide. Brucellosis is endemic in the Middle East, where 5 countries in this area experience high prevalence of brucellosis.

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<sup>1</sup> Department of Microbiology and Immunology, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj - IRAN

<sup>2</sup> Department of Biochemistry and Nutrition, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj - IRAN

<sup>3</sup> Department of Microbiology, Islamic Azad University, Zanjan-IRAN

**Correspondence:** Mohammad Reza RAHMANI, Department of Microbiology and Immunology, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj - IRAN  
E-mail: rahmany191@gmail.com

The number of annual cases of human brucellosis in Turkey is substantially high, exceeding 15,000 cases in 2004 (3). Moreover, Iran is considered an endemic area for this disease and its prevalence ranged from 1.5 to 107.5 cases per 100,000 people in 2003 (4). *Brucella* infects host macrophage cells and proliferates inside of them.

For treatment to be successful, high penetration rate antibiotics should be used to get into the cell cytoplasm, as well as the infected macrophage. Tetracycline, doxycycline, rifampin, trimethoprim-sulfamethoxazole, streptomycin, and other amino glycosides are used separately or combined to treat patients (5,6). The resistance of *Brucella* to some antibiotics such as rifampin and streptomycin has been reported from different countries, including Iran. Thus, studying the antibacterial activity of medicinal healing plants and herbs on *Brucella* seems important (7,8). Since medicinal herbs are cheaper than drugs and cause fewer side effects, their application has been increased in recent years (9). Studies done on the antibacterial effect of *Rhus coriaria* L. (sumac) extract showed that this herb contains tannin, which can be dissolved easily in ethanol and has high antimicrobial activity on both gram-positive and gram-negative intestinal bacteria (10). *Zataria multiflora* Boiss. contains phenolic compounds that play an effective role against various bacteria. Moreover, another effective combination that can be dissolved very easily in ethanol and other organic solvents is carvacrol, which has an ethanolic extract that is also known to possess strong antibacterial effects (11). *Mentha piperita* L. is a type of herb growing in different areas of Iran and has several properties, such as disinfectant activity, antifatulence and antidiarrhea (12). *M. piperita* extract is used to prevent or treat irritable bowel syndrome, inflammatory bowel disease, biliary disorders, and liver problems (13,14). It has been shown that *Ocimum basilicum* L. has an antibacterial effect against *Salmonella* and *Bacillus cereus* (15,16). Reports indicate high prevalence of brucellosis, and this disease is considered to be chronic, while there is an increasing rate of antibiotic resistance of *Brucella* strains. Therefore, the purpose of this study was to determine the antibacterial activities of *Z. multiflora*, sumac, *M. piperita*, and *O. basilicum* extracts on *Brucella* bacteria isolated from brucellosis patients.

## Material and methods

### Samples

Blood samples were collected from 60 patients referred to Towhid hospital, located in Sanandaj, Kurdistan Province, Iran. The patients presented with symptoms such as fever, chills and night sweats, anorexia, and some cases with a previous history of brucellosis also had  $\geq 1/80$  Wright titre in their serum. From each of these patients, a 10 mL blood sample was collected (17,18).

### Biochemical test and polymerase chain reaction (PCR)

Immediately added to the BACTEC culture system (BD Diagnostic Systems, Sparks, MD, USA) were 8 mL of each blood sample and ethylenediaminetetraacetic acid was added to the other 2 mL of blood sample, which was then stored at  $-20^{\circ}\text{C}$ . The BACTEC culture was incubated at  $37^{\circ}\text{C}$  for 7 days. After that, each sample was cultured on *Brucella* agar (Merck, Whitehouse Station, NJ, USA) and was then incubated in aerobic and microaerobic conditions with 5%  $\text{CO}_2$ , for more than 48 h at  $37^{\circ}\text{C}$ . The grown colonies were evaluated by gram-stain, urease, catalase, and oxidase tests, and the production of hydrogen sulfide (17,18). In the polymerase chain reaction (PCR) method, the specific primer forward: 5'-CCAGCGCACCATCTTTTCAG-3' and reverse: 5'-TCGTTGCGCGTAAGGATGC-3' were used for the beta-conglycinin storage protein gene expressing a 31 kDa of outer membrane protein of *Brucella abortus*. This sequence is common among all *Brucella* species (19).

### Determination of antibiotic resistance

In the disk diffusion method, tetracycline, streptomycin, doxycycline, and rifampin antibiotic disks (Himedia, India) were used. During this experiment, Mueller-Hinton agar (Merck), enriched with 5% sheep blood was used. A 0.5 McFarland standard was prepared from each *Brucella* isolate and inoculated with a sterile swab on the culture medium. Then, the antibiotic disks were placed on the culture medium and all of the plates were incubated with 5%  $\text{CO}_2$  for 48 h at  $37^{\circ}\text{C}$ . The zone of growth inhibition for each antibiotic disk was calculated and compared with the National Committee for Clinical Laboratory Standards (NCCLS) fastidious bacteria table (20).

## Extraction

While preparing the *Z. multiflora*, sumac, *M. piperita*, and *O. basilicum*, and confirming them in the Faculty of Agriculture at the University of Kurdistan, Iran, 100 g of each plant was pulverized and soaked in 300 mL of 80% ethanol (Merck) and incubated for 48 h. Then the extracts were filtered and dried in a shaker incubator at 40 °C. After drying, the extracts were collected and kept at 4 °C (11).

## Disk diffusion

Applying this method, the dilutions of 10, 20, and 40 mg/mL of each extract in sterile distilled water were prepared and filtered using a 0.4 µm membrane filter. After that the blank disks were floated in these dilutions. A 0.5 McFarland standard of each isolated *Brucella* strain was prepared and inoculated with a sterile swab on Mueller-Hinton agar enriched with 5% sheep blood. After this process, the disks containing each extract were dried and a blank disk was floated in 80% ethanol. It was then dried and used as the negative control and a gentamicin antibiotic disk was used for the positive control. Subsequently, all of the plates were incubated at 37 °C for 48 h. The zone of growth inhibition was measured and compared with the NCCLS fastidious bacteria table (20,21).

## Serial dilution

In this method, a serial dilution of 48.5 to 25,000 µg/mL of each extract was prepared in Mueller-Hinton broth (Merck). Next, the media culture was sterilized using a 0.4 µm membrane filter. A 0.5 McFarland standard of each isolated *Brucella* strain was prepared and 100 µL of each solution of bacteria was added to each tube. They were then incubated at 37 °C for 48 h. The minimum inhibitory concentration (MIC) level for each extract of the *Brucella* strain was measured by growth failure (no turbidity). In order to evaluate the minimum bactericidal concentration (MBC) of the mentioned extracts on *Brucella* strains, the dilution containing the MIC and other dilutions were cultured on Mueller-Hinton agar enriched with 5% sheep blood, and later incubated at 37 °C for 48 h (20,21).

## Statistical analysis

The results of this study were analyzed using SPSS (SPSS Company, Chicago, IL, USA), as well as the Mann-Whitney U test.

## Results

After collecting 60 blood samples and culturing these samples, 18 *Brucella* strains were isolated and confirmed by PCR (Figure 1) and biochemical methods.

The results of the disk diffusion method for determining the antibiotic resistance of 18 *brucella* strains showed that the mean rate of resistance to rifampin and streptomycin was 83.3% and 11.1%, respectively. Furthermore, no antibiotic resistance to tetracycline and doxycycline was observed. The mean rate of antibacterial effects of each extract on the *Brucella* strains was evaluated (Table 1). The *Z. multiflora* (Figure 2), sumac, and *M. piperita* extracts had highly antibacterial effects on the *Brucella* strains. No anti-*Brucella* activity was observed for *O. basilicum*.

Statistical analysis of the serial dilution results showed that the antibacterial effect of the *Z. multiflora* extract on the *Brucella* strains was greater than that of the sumac and *M. piperita* extracts ( $P < 0.05$ ). When compared with the *M. piperita* extract, the sumac extract showed significant antibacterial effect on the *Brucella* strains ( $P < 0.05$ ) (Table 2).

The MIC and MBC levels of each extract on the *Brucella* strains were evaluated. The mean MIC and MBC of the *Z. multiflora* extract was 1237 µg/mL and 5902 µg/mL. Furthermore, for the sumac extract, the mean MIC and MBC was 3255.2 µg/mL and 9027 µg/mL and for *M. piperita* it was 5642 µg/mL and

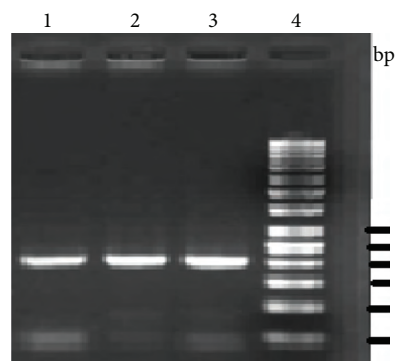


Figure 1. Agarose gel stained with ethidium bromide. Column 1: Bacteria that were separated with a 197 bp product, columns 2 and 3: *Brucella melitensis* Rev-1 strain with a 197 bp product, and column 4: DNA 50 bp weight marker.

Table 1. The mean rate of antibacterial activity of the mentioned extracts on *Brucella* strains using the disk diffusion method.

| Extract \ Disk concentration     | The mean zone of growth inhibition for disks that contained 10 mg/mL | The mean zone of growth inhibition for disks that contained 20 mg/mL | The mean zone of growth inhibition for disks that contained 40 mg/mL |
|----------------------------------|----------------------------------------------------------------------|----------------------------------------------------------------------|----------------------------------------------------------------------|
| <i>Zataria multiflora</i> Boiss. | 11.11 mm                                                             | 18.88 mm                                                             | 28.77 mm                                                             |
| Sumac                            | 6.77 mm                                                              | 15.72 mm                                                             | 22.55 mm                                                             |
| <i>Mentha piperita</i> L.        | –                                                                    | 6.66 mm                                                              | 7.5 mm                                                               |
| <i>Ocimum basilicum</i> L.       | –                                                                    | –                                                                    | –                                                                    |

Table 2. Comparison of the statistical analysis results of the antibacterial activity of the disk of each extract that contained 40 mg/mL and the antibiotic disks ( $P < 0.05$ ).

| Extracts \ Antibiotics                                                 | The mean inhibition zone of gentamicin: 26.05 mm | The mean inhibition zone of tetracycline: 39.16 mm | The mean inhibition zone of doxycycline: 40 mm | The mean inhibition zone of rifampin: 11.38 mm | The mean inhibition zone of streptomycin: 22.22 mm |
|------------------------------------------------------------------------|--------------------------------------------------|----------------------------------------------------|------------------------------------------------|------------------------------------------------|----------------------------------------------------|
| The mean inhibition zone of <i>Zataria multiflora</i> Boiss.: 28.77 mm | $P = 0.14$                                       | $P < 0.001$                                        | $P < 0.001$                                    | $P < 0.001$                                    | $P = 0.03$                                         |
| The mean inhibition zone of Sumac: 22.55 mm                            | $0.001 > P$                                      | $P < 0.001$                                        | $P < 0.001$                                    | $P < 0.001$                                    | $P < 0.001$                                        |
| The mean inhibition zone of <i>Mentha piperita</i> L.: 7.50 mm         | $0.001 > P$                                      | $P < 0.001$                                        | $P < 0.001$                                    | $P = 0.29$                                     | $P < 0.001$                                        |
| <i>Ocimum basilicum</i> L. disk without inhibition zone                | –                                                | –                                                  | –                                              | –                                              | –                                                  |

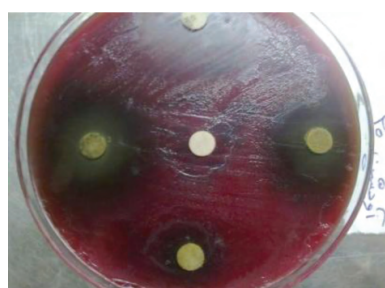


Figure 2. Antibacterial effect of *Zataria multiflora* Boiss. extract on *Brucella* strains using the disk diffusion method. The upper disk contained gentamicin (positive control), the lower disk contained 10 mg/mL, the right disk contained 20 mg/mL, the left disk contained 40 mg/mL, and the middle disk was the negative control.

12152  $\mu\text{g/mL}$ , respectively. No MIC and MBC for the *O. basilicum* extract on the *Brucella* strains was observed (Table 3) (Figure 3).

Statistical comparison of the mean MIC and MBC between the *Z. multiflora* extract and sumac showed that the *Z. multiflora* MIC and MBC levels were less than the MIC and MBC levels of the sumac extract ( $P < 0.001$ ). Furthermore, the MIC and MBC levels of the *Z. multiflora* extract were less than those of the *M. piperita* extract ( $P < 0.001$ ). The mean MIC of the sumac extract was less than that of the *M. piperita* extract ( $P < 0.005$ ) and the MBC of the sumac extract was not significantly different than the MBC level of the *M. piperita* extract ( $P = 0.1$ ).

Table 3. Results of the serial dilution method for the extracts, as observed in the study.

| Extracts                         | The mean MIC based on $\mu\text{g/mL}$ | The mean MBC based on $\mu\text{g/mL}$ |
|----------------------------------|----------------------------------------|----------------------------------------|
| <i>Zataria multiflora</i> Boiss. | 1237                                   | 5902                                   |
| Sumac                            | 3255.2                                 | 9027                                   |
| <i>Mentha piperita</i> L.        | 5642                                   | 12,152                                 |
| <i>Ocimum basilicum</i> L.       | -                                      | -                                      |

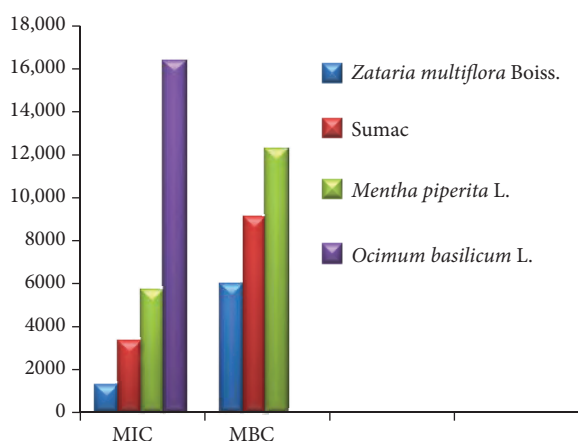


Figure 3. Comparison of MIC and MBC effects of the *Zataria multiflora* Boiss., sumac, *Mentha piperita* L., and *Ocimum basilicum* L. extracts on *Brucella* bacteria. The vertical column represents the concentration of the extracts based on  $\mu\text{g/mL}$ .

## Discussion

The antibiotic treatment recommended by the World Health Organization includes 200 mg of doxycycline and 600-900 mg of rifampin daily, for a period of less than 6 weeks (5). A study in 2008 by Vrioni et al. demonstrated that at 36 months after the first sample collection from the brucellosis patients, and also after completion of the antibiotic treatment, *Brucella* DNA was detected in the blood of these patients and the rate of *Brucella* DNA bacteria in their blood was  $56 \pm 74$  copies/5  $\mu\text{L}$  DNA extract (22). The purpose of this study was the evaluation of the antibacterial activity of *Z. multiflora*, *R. coriaria*, *M. piperita*, and *O. basilicum* extracts on *Brucella* strains isolated from the brucellosis patients, according to the high prevalence of brucellosis, the failure of treatment of the disease, chronicity of the disease, and the

possibility of antibiotic resistance to the bacteria. During this study, 18 *Brucella* strains were isolated from blood samples of brucellosis patients and were confirmed using biochemical techniques and the PCR method. The results of this study showed that the rate of resistance of *Brucella* strains to rifampin and streptomycin was 83.3% and 11.1%, respectively. Moreover, no antibiotic resistance was observed for tetracycline and doxycycline. These results indicated that most of the *Brucella* strains were resistant to rifampin, which is one of the selective antibiotics for the treatment of brucellosis. In a study by Baykam et al. in 2004, the antibiotic resistance of *Brucella* strains isolated from patients infected to brucellosis was studied. The results of that study indicated that *Brucella* strains were sensitive to doxycycline, while less sensitive to rifampin (23). The obtained results were significantly similar to the results of the present study. In preparing the ethanolic extracts of *Z. multiflora*, sumac, *M. piperita*, and *O. basilicum*, the disk diffusion and serial dilution methods were applied to evaluate the rate of sensitivity of *Brucella* strains to these extracts. The serial dilution results showed that the *Z. multiflora* extract, when compared with the gentamicin antibiotic, had no significant difference in anti-*Brucella* activity, but the antibacterial activity of this extract was significantly more than that of the antibiotics rifampin and streptomycin. This study also showed that the *Z. multiflora* and sumac extracts had high anti-*Brucella* activity compared with antibiotics such as rifampin and streptomycin. Furthermore, the *M. piperita* extract had a high concentration of anti-*Brucella* activity and the *O. basilicum* extract had no antibacterial effect on the *Brucella* strains. In a study by Motamedi et al. in 2010, the antibacterial activity of several medicinal plants against a tetracycline-

resistant strain of *Brucella* isolated from aborted sheep fetuses was investigated (21). In that study, in the ethanol and methanol extraction of the plants, the serial dilution and disk diffusion methods were used to evaluate the antibacterial effect of these plants. The results showed that the plant extracts of *Oliveria decumbens*, *Crocus sativus*, and *Salvia sclarea* had high anti-*Brucella* activity, but the plant extracts of *Cordia myxa* and *Plantago ovata* had no antibacterial activity on *Brucella melitensis*, even at high concentrations. The present study, which was similar to the study by Motamedi et al., for the ethanolic extracts, the disk diffusion and the serial dilution methods were applied to determine the antibacterial activity of the mentioned herbs on *Brucella* strains. In their study, 1 *Brucella* strain isolated from aborted fetal sheep was used, but, in the present study, 18 *Brucella* strains isolated from brucellosis patients

were used. The results of this study showed that the ethanol extractions of *Z. multiflora*, sumac, and *M. piperita* had high antibacterial activity on the 18 *Brucella* strains. In addition, the high concentration of the ethanol extraction of *O. basilicum* showed no antibacterial activity on the *Brucella* strains.

## Conclusion

The results of the present study showed that the *Z. multiflora* plant extracts had high antibacterial activity on all 18 *Brucella* strains isolated from brucellosis patients in comparison with such conventional antibiotics as rifampin and streptomycin, in vitro. It is hoped that the results of this study contribute to a new way to make use of medicinal plants for effective treatment and the prevention of the chronic form of the brucellosis disease.

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