

The Antibacterial Activity of Selected Labiatae (Lamiaceae) Essential Oils against *Brucella melitensis*

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Abstract

Background: Brucellosis, a zoonosis caused by four species of *brucella*, has a high morbidity. The major cause of brucellosis worldwide is *brucella melitensis*. Medicinal plants are considered as new antibacterial sources that could replace conventional antibiotics in the treatment of antibiotic-resistant bacteria. The aim of this study was to evaluate the efficacy of some native plants, alone and in combination with some antibiotics, in the treatment of brucellosis.

Methods: The present experimental in vitro study was carried out to evaluate the anti-*brucella* activities of essential oils of *Rosmarinus officinalis* L., *Origanum syriacum*, *Thymus syriacus*, *Salvia palaestina* Benth, *Mentha piperia*, and *Lavandula stoechas* L., alone and in combination with some antibiotics. The activity against 16 tetracycline-resistant *B. melitensis* isolates was determined by disc diffusion method incorporating a concentration of 5%. Antibiotic discs were also used as a control. Microdilution *brucella* broth susceptibility assay was used in order to determine the MICs of essential oils and five antibiotics.

Results: Among all the herbs evaluated, only the essential oils of *O. syriacum* and *T. syriacus* plants demonstrated most effective anti-*brucella* activity, and were then chosen for MIC study. The minimal inhibitory concentrations (MIC₅₀) of essential oils of *O. syriacum* and *T. syriacus* against tetracycline-resistant *B. melitensis* were 3.125 µl/ml and 6.25 µl/ml, respectively.

Conclusion: Among the essential oils studied, those of *O. syriacum* and *T. syriacus* were most effective. Since a combination of levofloxacin and *Thymus syriacus* essential oil increased the efficacy of this antibiotic, *O. syriacum* and *T. syriacus* are recommended to be used as bactericidal agents against *B. melitensis*.

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Keywords • Brucellosis • Antibiotic resistance • *Brucella melitensis* • *Origanum*

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Introduction

Brucellosis is an endemic zoonosis in Syria, affecting large numbers of animals and an increasing number of cases in humans. It is considered as the most important public health problem due to its high morbidity. The severity of disease in humans correlates with its severity in animals, especially in domestic ruminants.¹ Furthermore, brucellosis continues

to have great economic importance considering decreased milk production, infertility, abortions, and weight loss.² *Brucella melitensis* remains the major cause of human disease worldwide, followed by *B. abortus* and *B. suis*. Rare cases of human infections caused by *B. canis* and pathogenic *brucella* of marine mammals have also been reported.^{3,4} Despite existing brucellosis worldwide, it is considered as an endemic disease in Mediterranean basin, Middle East, Western Asia, Africa, and Latin America.⁵ In spite of the development of new antibiotics as well as new treatment strategies, only few modifications have been applied to brucellosis treatment since its introduction half a century ago.⁶⁻⁸ Treatment of human brucellosis is still based on the World Health Organization (WHO) recommendations applied in 1986,⁹ suggesting the use of doxycycline, 100 mg twice daily for six weeks combined with either rifampicin, 600–900 mg daily for six weeks, or streptomycin, 1 g daily for 2–3 weeks. In addition, rifampicin monotherapy is the only viable option for treatment during pregnancy. Whereas a combined therapy with rifampicin and trimethoprim-sulfomethoxazole is recommended for children.^{10,11} Some disease complications, such as: endocarditis, meningitis, and spondylitis, were treated with triple-antibiotic combinations.^{12,13} Quinolones are an alternative to conventional treatment agents,¹⁴⁻¹⁶ and clinically similar results were obtained with rifampicin-ofloxacin and rifampicin-doxycycline combinations.¹⁷ However, the treatment of brucellosis is still problematic due to emerging resistance, which yield to high rates of treatment failure and relapses. Thus, it is necessary to develop new antibacterial drugs for treatment of brucellosis.¹⁸

The antimicrobial activity of medicinal plant extracts justifies them to be used in many fields such as food industry, pharmacy and medicine. Medicinal plants have always been sources for discovery of new drugs. Plants, easily protect themselves against various enemies such as insects and microorganisms by synthesizing special substances.¹⁹ Moreover, they maintain their normal growth and development by producing secondary antimicrobial metabolites.²⁰ *Brillantaisia lamium* extract is found to be of value against *Staphylococcus aureus*, *Enterococcus faecalis*, *Candida tropicalis* and *Cryptococcus neoformans*.²¹ Whereas, *Crimum pupurascens herb* extracts and its components showed antimicrobial activity against *Salmonella paratyphi* A and B.²² In addition, a strong anti *brucella abortus* A77 activity of *Satureja hortensis* essential oil has already been reported.²³ *Lamiaceae* family main components, thymol and carvacrol, interfere with cellular metabolism after penetrating into the cell.²⁴ Low concentrations of *Prunus mahaleb* seeds

ethanolic extract showed a good efficacy against *B. melitensis*.²⁵ Furthermore, the lowest concentration of ethanolic and methanolic extracts of *Oliveria decumbens* and *Vitex pseudo-negundo* found to have antibacterial activity against tetracycline-resistant *B. melitensis*.^{26,27} In addition, almost all concentrations of *Teucrium polium* ethanolic extract, a member of the *Lamiaceae* family, were found to be effective against *B. melitensis*.²⁷ *Rosmarinus officinalis* L., *Origanum syriacum*, *Thymus syriacus*, *Salvia palaestina Benth*, *Mentha piperia* and *Lavandula stoechas* L. are traditional medicinal plants used in Syria for many purposes, particularly for respiratory and gastrointestinal disorders. The aim of this study was to evaluate in vitro antibacterial activity of essential oils of the above-mentioned plants against tetracycline-resistant *B. melitensis* isolates.

Materials and Methods

Microorganisms and Growth Conditions

In this prospective study, 89 *brucella* species were isolated from bovine and ovine milk from different Syrian provinces between 2004 and 2007.

Bacteria were isolated from milk cultures at the Immunology and Microbiology Laboratory, atomic energy commission of syria (AECS).²⁸ *Brucella* was grown under optimal conditions in 2YT agar (Difco, BD, USA), containing peptone 10 g, sodium chloride 5 g, meat extract 5 g, agar 20 g, and distilled water 1 liter, and incubated at 37°C water bath (Grant water; Cambridge, UK.) to secure sufficient cell density. The growth of contaminating microorganisms was inhibited by supplementing the growth medium with cycloheximide 100 mg, bacitracin 25000 units, polymyxin B sulphate 5000 units, vancomycin 20 mg, nalidixic acid 5 mg, and nystatin 100000 units (Oxoid, UK).²⁸ The solid selective media were then prepared by melting the basal medium, cooling to 56°C in a water bath, adding appropriate amounts of stock solutions of the antibiotics and 5% horse serum (PAN-Biotech, GmbH, Germany). The biotyping of the bacteria was done by CO₂ requirement, H₂S production, urease and oxidase positivity, growth in the presence of dyes (thionine and basic fuchsine), and reaction with monospecific anti-A and anti-M sera (Arcomex, Jordan).²⁹ Strains identified as *B. melitensis* or *B. abortus* were stored in 2YT medium at -20°C. Only 16 isolates of *B. melitensis* resistant to tetracycline by susceptibility test were used in the present study.

Plant samples Collection

The arial parts of plant samples including the

leaves and buds of *Rosmarinus officinalis* L., *Origanum syriacum*, *Thymus syriacus*, *Salvia palaestina* Benth, *Mentha piperia* and *Lavandula stoechas* L. (Labiatae), were collected during the flowering season from their natural habitat in Syria (table 1). The samples were cleaned from impurities, such as contaminating plants, dust, and other pollutants. The collected plants were air dried and were cut to pieces.

Essential Oil Extraction

Extraction of essential oils was carried out using water steam distillation device (Clevenger-type apparatus) according to the European Pharmacopoeia method.³⁰ The device was attached to condenser and cold water recycler (Hydrodistillation technique). Distilled water was added (1:10 v/v) to each sample, and distilled for 2 h. The supernatant contained essential oil which was dehydrated by filtering through anhydrous Na₂SO₄. The essential oil thus prepared was collected in airtight vials and stored in refrigerator.

Antibacterial Susceptibility Assay

The test isolates was grown in Muller-Hinton Broth (MHB, Merck) medium at 37°C for 22 h. The bacterial number in the final inoculum was adjusted to 10⁶ CFU/ml. A bacterial lawn was prepared by pouring 0.1 ml of bacterial suspension onto each plate of Muller-Hinton Agar medium (MHA, Merck), spread by a sterile cotton swab, and allowed to remain in contact for 1 min. 5% concentration of each essential oil were prepared in order to impregnate the paper discs. The sterile filter paper discs containing tested essential oils (6-mm diameter) were then placed on the bacterial lawn. 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, BD, USA) containing tetracycline 30 µg, ciprofloxacin 5 µg, levofloxacin 5 µg, ofloxacin 5 µg and doxycycline 30 µg were used.

Antibiotics MIC Determination

Microdilution broth method in 96 microwell plates (TPP, Switzerland) was used to estimate the antibiotic's susceptibility. Two-fold dilutions of

antibiotics in *brucella* broth (acumedia, Michigan, USA) prepared in wells were inoculated with 10⁶ CFU of bacteria with final volume of 0.2 ml in each micro-well plate, and incubated for 48 hours at 37°C. The MIC was expressed as the lowest concentration that completely inhibited visual growth. Furthermore, the lowest concentration inhibiting 90% of visual growth was considered as MIC₉₀. MIC testing was performed according to the recommendations of the clinical laboratory standards institute.³¹ The concentrations assayed for each antibiotic ranged from 0.064 to 128 µg/ml. The absorbance was determined at 590 nm (Thermo-lab Systems Reader, Finland). All tests were performed in triplicate and then averaged. The antibiotics investigated included levofloxacin, ofloxacin, sparfloxacin, ciprofloxacin and doxycycline, along with a blank test containing no antibiotics.

Determination of MIC of Essential Oils

Microdilution broth susceptibility assay was performed using three replicates of each serial dilutions of essential oil prepared in *brucella*[®] broth medium in 96-well microtiter plates.³² The concentrations of each essential oil in serial dilutions ranged from 0.75 to 100 µl/ml. The content of each well was supplemented with 100 µl of freshly grown bacterial culture containing 10⁶ CFU/ml in *brucella* broth. The assay included positive control without essential oil and negative control lacking bacteria under the same conditions. The plate was incubated with shaking for 24 h at 37°C. The MIC was expressed as the lowest concentration that completely inhibited visual growth. Moreover, MIC₉₀ was the lowest concentration that inhibited 90% of visual growth with absorbance at 590 nm.

Essential Oil-Antibiotic Combination Effect

Two *B. melitensis* isolates were employed to evaluate the additive effects of various concentrations of *T. syriacus* essential oil on the MIC of levofloxacin. MIC was determined as described above. Two dilutions containing 3.125 and 6.250 µl/ml, of *T. syriacus* essential oil were then added to the 96-well microtiter plates to determine the MIC. The lowest concentration

Table 1: Plants and their families, collection sites, and parts used

No	Scientific name	Plant family	Collection site	Altitude	Collection time	Extracted part
1	<i>Rosmarinus officinalis</i> .L.	Lamiaceae	Latakia	300 m	June	Aerial parts
2	<i>Origanum syriacum</i> .L.	Lamiaceae	Kafr Nobol-Idlib	446 m	July	Aerial parts
3	<i>Thymus syriacus</i> . Boiss.	Lamiaceae	Alsoja mountain-Damascus	840 m	July	Aerial parts
4	<i>Salvia palaestina</i> Benth	Lamiaceae	Alyarmouk valley-Konaïtera	800 m	June	Aerial parts
5	<i>Mentha piperia</i> . L.	Lamiaceae	Latakia	300 m	June	Aerial parts
6	<i>Lavandula stoechas</i> L.	Lamiaceae	Tartous	300 m	June	Aerial parts

of levofloxacin that completely inhibited visual growth in presence of essential oil was recorded as the MIC.

Results

On the basis of the primary screening results (table 2), *O. syriacum* and *T. syriacus* essential oils showed a good antibacterial activity against *B. melitensis*. Whereas, no antibacterial activity was demonstrated by the essential oils of *R. officinalis* L., *S. palaestina Benth*, *M. piperia* and *L. stoechas* L (data not shown). In addition, *B. melitensis* isolates were resistant to tetracycline (data not shown), but susceptible to doxycycline, ciprofloxacin, levofloxacin and ofloxacin.

Also, *O. syriacum*, *T. syriacus* essential oils exhibited an inhibitory effect at a concentration of 50 mg/ml.

Considering the diameter of the inhibition

zone, *O. syriacum* and *T. syriacus*, which showed the highest anti-brucella activity, were chosen for further study. MIC₅₀ values for *O. syriacum* and *T. syriacus* essential oils were 3.125 and 6.25 µl/ml, respectively. Whereas, MIC₅₀ values for levofloxacin, ofloxacin, sparfloxacin, ciprofloxacin and doxycycline were 0.125, 0.5, 16, 64 and 0.5 µg/ml, respectively (table 3).

In addition, table 4 revealed that *T. syriacus* essential oil reduced the MIC₉₀ level of levofloxacin from 32 to 4 µg/ml in both isolates studied, whereas, it decreased the MIC₅₀ level from 0.125 to 0.064 µg/ml in only one isolate.

Discussion

Human brucellosis therapy requires antibiotics which are capable of penetrating the macrophages and act efficiently under acidic conditions. Antimicrobial drug resistant strains emerge frequently,³³ and lead

Table 2: Inhibition zones (mm) of effective essential oils of some medicinal plants and antibiotics against *B. melitensis*

Isolate number	<i>Thymus syriacus</i>	<i>Origanum syriacum</i>	Doxycycline	Ciprofloxacin	Levofloxacin	Ofloxacin
1	22	18	27	22	29	22
2	23	16	6	10	9	9
3	25	18	15	26	27	20
4	22	17	6	25	21	12
5	20	15	8	27	15	23
6	22	12	15	27	26	10
7	23	17	6	13	20	8
8	22	19	16	14	9	14
9	22	18	7	21	27	22
10	23	18	18	25	26	19
11	23	12	22	26	26	26
12	22	13	7	23	19	16
13	22	18	17	26	22	9
14	23	16	6	15	20	15
15	25	16	12	31	16	20
16	20	19	9	30	18	12

Table 3: MICs for *Thymus Syriacus*, *Origanum syriacum* and some antibiotics against *B. melitensis*

Essential oil or antibiotic	Mean of MIC ₅₀	Mean of MIC ₉₀
<i>Thymus syriacus</i>	6.25 (µl/ml)	NA
<i>Origanum syriacum</i>	3.125 (µl/ml)	NA
Levofloxacin	0.125 (µg/ml)	32(µg/ml)
Ofloxacin	0.5 (µg/ml)	32(µg/ml)
Sparfloxacin	16 (µg/ml)	32(µg/ml)
Ciprofloxacin	64 (µg/ml)	NA
Doxycycline	0.5 (µg/ml)	NA

Table 4: MICs of levofloxacin and *Thymus syriacus* essential oil combination

Isolate number	MIC (µg/ml) without E.o		MIC (µg/ml) with 3.125 µl/ml of E.o		MIC (µg/ml) with 6.250 µl/ml of E.o	
	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀
1	0.125	32	0.064	4	0.064	2
2	0.125	32	0.125	4	0.125	4

E.o=Essential oil

to treatment failure. Unfortunately, many strains of *brucella*, develop resistance to multiple conventional antibiotics. It is then necessary to discover new antimicrobial agents capable of acting against resistant strains, which could reduce relapsing cases or even cure the disease. In this context, medicinal plants which have fewer adverse effects and are less costly than antimicrobial agents, seem to be desired alternatives. Medicinal plants are found to be valuable for the treatment of infections caused by bacteria resistant to many antibiotics. Hassawi and Khama,³⁴ reported that the extracts of many plants worldwide, were suitable for treating bacterial, fungal or viral infections. Brul and co-worker highlighted the mechanisms of antimicrobial effects in certain plants.³⁵ In addition, phenolic and aromatic compounds of medicinal plants seems to possess an essential antibacterial role.³⁶ The growth of *B. melitensis* is affected by thymol and carvacrol. These are major phenolic components of thymus oil with prominent outer membrane disintegration activity that increased the permeability to ATP through cytoplasmic membrane.^{37,38} In this context, several in vitro experiments showed a wide spectrum of antimicrobial activity in thymus oil and its phenolic components.³⁹ Most of the plants used in this study are used in traditional medicine across Syria to cure respiratory and gastrointestinal disorders. Thus, these plants could be explored to evaluate their efficacy against. As demonstrated in table 2, the efficacy of antimicrobial activities of essential oil of tested plants was determined, quantitatively, by measuring the diameter of inhibition zones around the discs. Only *O. syriacum* and *T. syriacus* extracts inhibited the growth of *B. melitensis*. The MIC₅₀ values of *O. syriacum* and *T. syriacus* aqueous extracts were 3.125 µl/ml and 6.25 µl/ml, respectively. Reuben et al.⁴⁰ found that the MIC and MBC often had comparable or close values, concluding that the essential oils of *O. syriacum* and *T. syriacus* possessed bactericidal effect on *B. melitensis*. Darabpour et al. found that the methanolic extract of *Peganum harmala* L seed exhibited a broad antibacterial activity against *B. melitensis* even at lowest concentration (50 mg/ml).⁴¹ Shapouri and Rahnema reported the MIC of aqueous hops extract for *B. abortus* 544 and *B. melitensis* 16M, as 0.625 mg/ml, whereas that of acetonic and ethanolic extracts being 0.05 mg/ml.⁴²

Motamedi and his colleagues studied the effect of plant extract-antibiotic combination against *B. melitensis*, and observed a synergistic activity in the combination of *Oliveria decumbens* extracts and doxycycline. In our in vitro study of *T. syriacus* aqueous extract of essential oil, a good additive activity against two *B. melitensis* isolates was demonstrated when it was used in combination with levofloxacin.

Conclusion

Our study showed that *O. syriacum* and *T. syriacus* essential oils were most effective against *B. melitensis*. This could provide a potential source of new antibacterial agents which is worthy of clinical trials. In addition, doxycycline, levofloxacin and ofloxacin were the most effective antibiotics. Moreover, levofloxacin and *Thymus syriacus* essential oil combination was more effective than either antibiotic or the essential oil alone.

Further and more specific studies, *in vivo*, are recommended to determine the efficacy of these essential oils in the treatment of brucellosis infections.

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Conflict of interest: None declared

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