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EXPERIMENTAL PAPER

In vitro antibacterial activity of several plant extracts and essential oils against *Brucella melitensis*

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Summary

Medicinal plants are considered to be new resources for the production of agents that could act as alternatives to antibiotics in the treatment of antibiotic-resistant bacteria. The aim of this study was to evaluate the efficacy of some plants native to Syria in the treatment of brucellosis. *In vitro* activities of some essential oils and plant extracts of some medicinal plants against 89 *Brucella melitensis* isolates was determined by disc diffusion method at a concentration of 5%. The microdilution assay in the fluid medium was used to determine the MICs of essential oils and plant extracts. Among the evaluated herbs, only *Thymus syriacus* and *Cinnamomum zeylanicum* essential oils and *Laurus nobilis* plant extract showed a high activity against *B. melitensis* strains. Thus, minimal inhibitory concentration (MIC_{50}) values for *T. syriacus*, *C. zeylanicum*, and *L. nobilis* against *B. melitensis* were 6.25, 3.125 and 6.25 μl/ml, respectively. Among studied essential oils and plant extracts, *T. syriacus* and *C. zeylanicum* essential oils, and *L. nobilis* plant extract were the most effective ones. Moreover, *T. syriacus - C. zeylanicum* combination was more effective than use of each of them alone. Then, *T. syriacus* and *C. zeylanicum* essential oils and *L. nobilis* plant extract could act as bactericidal agents against *B. melitensis*.

***Key words**: brucellosis, antibacterial activity, Brucella melitensis, Cinnamomum zeylanicum, Thymus syriacus, Laurus nobilis, essential oils, plant extracts*

INTRODUCTION

Brucella melitensis is a facultative intracellular pathogen and one of the etiological agents of brucellosis which is a highly common bacterial zoonosis worldwide that can infect humans and animals and cause economic losses [1, 2]. It is considered as an endemic disease in Mediterranean basin, Middle East, Western Asia,

Africa, and Latin America [3]. Great efforts were being made to eradicate *B. melitensis* all over the world [4]. Despite the development of new antibiotics as well as new treatment regimens, only few modifications have been applied concerning the treatment strategies which used since half a century ago [5-7]. Quinolones were used as alternative drugs to conventional regimens [8-10]. However, the treatment of brucellosis is still problematic due to emerging resistance, which yields to high rates of treatment failure and relapses.

Essential oils and plant extracts serve as new sources of novel antibacterial agents [11]. They are also used as antimicrobial agents in food preservation [12], pharmaceuticals, alternative medicine and natural therapies [13, 14].

A strong activity was reported when applying *Satureja hortensis* essential oil against *B. abortus* A77 [15]. The components present in *Lamiaceae* family, particularly thymol and carvacrol, interfere with cellular metabolism after penetrating inside the cell [16]. Low concentrations (0.1 and 0.2 g/ml) of ethanolic extract of *Prunus mahaleb* seeds showed a good efficacy against *B. melitensis* [17]. Furthermore, the lowest concentrations of methanolic extract of *Oliveria decumbens* (50 mg/ml) as well as ethanolic extract of *Vitex pseudo-negundo* (50 mg/ml) found to have antibacterial activity against tetracycline-resistant *B. melitensis* [18,19]. In addition, almost all used concentrations, expect from 50 mg/ml (the lowest concentration that used), of ethanolic extract of *Teucrium polium* were found effective against *B. melitensis* [19].

Many plants oils and extracts are traditionally used as medicinal plants in Syria for many purposes, particularly for respiratory and gastrointestinal disorders. The aim of this study was to screen *in vitro* antibacterial activity of 7 essential oils and 10 plant extracts against *B. melitensis* isolates.

MATERIALS AND METHODS

Microorganisms and growth conditions

Eighty-nine *B. melitensis* isolates were collected prospectively between 2004 and 2007 from ovine milk from different Syrian provinces. Bacteria were isolated from milk cultures at the Immunology and Microbiology Laboratory, AECS [20]. *Brucella* was grown under optimal conditions in 2YT agar at 37°C in a water bath (Grant water, Cambridge, UK to ensure sufficient cell density. Following antibiotics (Oxoid, UK) were added to inhibit the growth of organisms other than *Brucella*: cycloheximide (100 mg), bacitracin (25,000 units), polymyxin B sulphate (5,000 units), vancomycin (20 mg), nalidixic acid (5 mg) and nystatin (100,000 units) [20]. In order to prepare solid selective media, basal medium is melted and then cooled to 56°C in a water bath and stock solutions of the antibiotics were added with 5% of horse serum (PAN-Biotech, GmBH, Germany). The biotyping of the bacteria was performed with use of the following tests: CO_2 requirement, H_2S 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) [21]. Isolates were stored in 2YT medium at –20°C.

Plant samples collection

Thymus syriacus Boiss.; *Citrus aurantium* L., *Citrus medica* L. (*Rutaceae*); *Myrtus communis* L., *Eucalyptus camaldulensis* Dehnk. (*Myrtaceae*); *Cinnamomum zeylanicum* L., *Laurus nobilis* L. (*Lauraceae*); *Juniperus foetidissima* Willd (*Cupressaceae*); *Pelargonium roseum* L. (*Geraniaceae)*; *Scilla maritima* Squilla.; *Pinus halepensis* Miller. (*Pinaceae*); *Artemisia herba-alba* Asso. (*Compositae*); *Anabasis haussknechtii* Boiss. (*Chenopodiaceae*); *Crataegus aronia* L. (*Rosaceae*); *Mercurialis annua* L. (*Euphorbiaceae*); *Matthiola crassifolia* Boiss. (*Brassicaceae*); and *Achillea fragrantissima* Forssk. (*Asteraceae*) samples were collected during the flowering season from different regions in Syria or purchased from local markets (tab. 1). Aerial parts and leaves were collected during the pre-blooming stage. The samples were cleaned from any strange plants, dust, or any other contaminants.

Table 1.

Plants and their families, collection sites, and extracted parts

Scientific name	Plant family	Collection site	Altitude (m)	Collection time	Extracted part	Extract or oil
Thymus syriacus Boiss.	*Lamiaceae*	Alsoja mountain – Damascus	840	July	Aerial parts	Oil
Citrus aurantium L.	*Rutaceae*	Latakia	300	April	Flowers	Oil
Citrus medica L.	*Rutaceae*	Latakia	300	April	Flowers	Oil
Myrtus communis L.	*Myrtaceae*	Latakia	300	June	Leaves	Extract
Eucalyptus camaldulensis Dehnk.	*Myrtaceae*	Tartous	300	June	Flowering branches	Oil
Cinnamomum zeylanicum L.	*Lauraceae*	Market			Barks	Oil
Laurus nobilis L.	*Lauraceae*	Latakia	300	July	Leaves	Extract
Juniperus foetidissima Willd	*Cupressaceae*	Dobaya-Damascus	800	June	Leaves	Oil
Pelargonium roseum L.	*Geranaceae*	Kodsaya-Damascus	916	May	Aerial parts	Extract
Scilla maritima Squilla.	*Liliaceae*	Tartous	300	March	Bulbs	Extract
Pinus halepensis Miller.	*Pinaceae*	Dobaya-Damascus	900	May	Leaves	Extract
Artemisia herba-alba Asso.	*Compositae*	Alsoja mountain – Damascus	840	March	Aerial parts	Extract
Anabasis haussknechtii Boiss.	*Chenopodiaceae*	Alkariatain-Homs	500	March	Aerial parts	Extract
Crataegus aronia L.	*Rosaceae*	Alkonaitera	1100	April	Flowering branches	Extract
Mercurialis annua L.	*Euphorbiaceae*	Kasab- Latakia	800	March	Aerial parts	Extract
Matthiola crassifolia Boiss.	*Brassicaceae*	Latakia	10	March	Aerial parts	Extract
Achillea fragrantissima Forssk.	*Asteraceae*	Palmyra	405	July	Aerial parts	Oil

Isolation of the essential oils

Aerial parts, flowers and leaves were cleaned and dried; whereas, fruits, barks and bulbs were dried, grounded and powdered using electrical blender prior to steam distillation. Isolation of essential oils was acquired using water steam distillation device (Clevenger-type apparatus) according to the European Pharmacopoeia method [22]. The device was attached to condenser and cold water recycler (Hydrodistillation technique). Briefly, 100 g of each plant was introduced in the distillation flask (1 l), which was connected to the steam generator via a glass tube and to a condenser to retrieve the oil. This was recovered in a funnel tube. Aromatic molecules of essential oils were released from plant material and evaporated into the hot steam. The hot steam forces the plant material to release the essential oil without burning the plant material itself. Then, steam containing the essential oil was passed through cooling system in order to condense the steam. Steam was applied for 3 hours. The recovered mixture was allowed to settle and the oil was withdrawn. The supernatant essential oils were dried over through anhydrous sodium sulphate (Na_2SO_4), filtered and stored in tighten vials and stored in refrigerator (4°C). The oil was diluted in dimethyl sulfoxide (DMSO) and used for the antimicrobial activity test.

Preparation of ethanolic extracts

Aerial parts, flowers and leaves were cleaned and dried, whereas bulbs were dried, grounded and powdered using electrical blender. Then, ethanolic extracts were prepared as follows: one gram of each sample was extracted using 10 ml of ethanol (10:1 *w*/*v*) and centrifuged at 10 000 × *g* for 15 min and then the supernatant was collected. This process was repeated three times. Finally, the ethanol was removed through evaporation by incubating at a room temperature.

Antibacterial susceptibility assay

The test isolates were grown in Muller-Hinton Broth (MHB, Merck) medium at 37°C for 22 h. Final inoculums bacterial numbers were adjusted to 10^6 CFU/ml. 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. The sterile filter paper discs (6 mm in diameter) were placed on lawn cultures, and 50 μl of 5% concentration of each essential oil or plant extract was added. The Petri dishes were subsequently incubated at 37°C for 24 h and the inhibition zone around each disc was measured.

Essential oils and plant extracts MIC determination

Microdilution broth susceptibility assay was used [23]. Three replicates of serial dilutions of each essential oil and plant extract were prepared in Brucella® broth medium in 96-well microtiter plates, using a range of concentrations for each essential oil or plant extract from 0.75 to 100 μl/ml. 100 μl of freshly grown bacteria standardized 10^6 CFU/ml in Brucella® broth were added to each well. Positive control was performed with the same conditions but without essential oils, negative control was also done with the same conditions but without adding the bacteria. The plate was incubated with shaking for 24 h at 37°C. The lowest concentration that completely inhibited visual growth was recorded and interpreted as MIC. Moreover, the lowest concentration that inhibited 90% of visual growth was (at the binocular microscope) recorded and interpreted as MIC_{90}, whereas, the lowest concentration that inhibited 50% of visual growth (at the binocular microscope) was also recorded and interpreted as the MIC_{50}.

Essential oil-essential oil and plant extract-plant extract combination effect

One *B. melitensis* isolate was chosen to evaluate the effect of several combinations between essential oils and ethanolic extracts. MIC was determined as described above.

RESULTS

On the basis of primary screening results (tab. 2), *T. syriacus* and *C. zeylanicum* essential oils, as well as *L. nobilis* plant extract have a good antibacterial activity against *B. melitensis.* Whereas, other studied essential oils and plant extracts were ineffective.

Furthermore, table 3 reveals that *T. syriacus* and *C. zeylanicum* essential oils and *L. nobilis* plant extract, showed the highest antibacteria activities, and the MIC_{50s} values were 6.25, 3.125 and 6.25 μl/ml, respectively. Whereas, only *C. zeylanicum* essential oil showed a good activity against 90% of isolates (MIC_{90}=6.25 μl/ml).

In addition, when a combination between two effective essential oils, *T. syriacus and C. zeylanicum* was used, the MIC_{50} of *T. syriacus* essential oils was 0.75μl/ml when the lowest concentration of *C. zeylanicum* essential oil (0.75 μl/ml) was applied, and vice versa. Whereas, when the combination between one effective ethanolic extract (*L. nobilis*) with one non-effective ethanolic extract (*M. communis*) was applied, the MIC_{50} level of *L. nobilis* plant extract was decreased to only 0.75 μl/ml when combined with 50 μl/ml of *M. communis* plant extract.

Table 2.

Number of *B. melitensis* isolates susceptible to essential oils or plant extracts

	Number of isolates susceptible to plant extracts (N=89)
Thymus syriacus Boiss.	79
Citrus aurantium L.	0
Citrus medica L.	0
Myrtus communis L.	0
Eucalyptus camaldulensis Dehnk.	0
Cinnamomum zeylanicum L.	81
Laurus nobilis L.	76
Juniperus foetidissima Willd	0
Pelargonium roseum L.	0
Scilla maritima Squilla.	0
Pinus halepensis Miller.	0
Artemisia herba-alba Asso.	0
Anabasis haussknechtii Boiss.	0
Crataegus aronia L.	0
Mercurialis annua L.	0
Matthiola crassifolia Boiss.	0
Achillea fragrantissima Forssk.	0

Table 3.

Minimal inhibitory concentrations of essential oils and plant extracts on *B. melitensis* strains

	Minimum inhibitory concentrations	
	MIC_{50} [μl/ml]	MIC_{90} [μl/ml]
Thymus syriacus Boiss.	6.25	NE
Citrus aurantium L.	50	NE
Citrus medica L.	25	NE
Myrtus communis L.	50	NE
Eucalyptus camaldulensis Dehnk.	50	NE
Cinnamomum zeylanicum L.	3.125	6.25
Laurus nobilis L.	6.25	NE
Juniperus foetidissima Willd	12.5	50
Pelargonium roseum L.	12.5	NE
Scilla maritima Squilla.	25	50
Pinus halepensis Miller.	50	NE
Artemisia herba-alba Asso.	NE	NE
Anabasis haussknechtii Boiss.	NE	NE
Crataegus aronia L.	50	NE
Mercurialis annua L.	25	50
Matthiola crassifolia Boiss.	50	NE
Achillea fragrantissima Forssk.	NE	NE

NE= no effect (>100 μl/ml)

DISCUSSION

Treatment of human brucellosis needs antibiotics that can act within the acidic intracellular environment. Despite a good diagnosis and treatment, many antimicrobial drug-resistant strains may be developed and lead on to treatment failure [24]. Multiple drug resistant strains of *brucella* have been developed. Then, it looks to be necessary to discover new antimicrobial agents capable to act against resistant strains and then could reduce cases of relapse or even cure the disease. Hereby, medicinal plants, which have fewer adverse effects and are cheaper than antimicrobial agents, seem to be good alternatives.

Since the ancient times, spices and their essential oils have been known to have various antibacterial effects [25-29]. More recently, plant extracts have been developed and used in foods as natural antioxidants [30] or antimicrobials [31]. Antimicrobial mechanisms of natural compounds found in herbs or spices have been discussed [32].

The majority of plants that were used in this study are applied in traditional medicine in all regions of Syria in order to cure respiratory and gastrointestinal disorders. Thus, these plants could be explored to evaluate their efficacy against *B. melitensis*. Essential oils and plant extracts efficacy of tested plants were determined quantitively, by measuring the diameter of inhibition zones around the discs (tab. 2). Only *T. syriacus and C. zeylanicum* essential oils *and L. nobilis* plant extract inhibited the growth of tested bacteria. The MIC_{50} values of *T. syriacus and C. zeylanicum* essential oils*, and L. nobilis* ethanolic extract were 6.25, 3.125 and 6.25 μl/ml, respectively.

Several studies reported a good effect of some plant extracts against *B. melitensis* including *Peganum harmala* L. [33], aqueous hops extract [34] and *Oliveria decumbens* [19].

Motamedi *et al.* [19] studied the effect of plant extract-antibiotic combination against *B. melitensis*. They observed the presence of synergistic effect in the combination of *Oliveria decumbens* extracts and doxycycline.

Ooi et al. [35] studied the antimicrobial effect of cinnamon against gram negative bacteria. They reported that *Cinnamonum verum* was effective against a broad spectrum of bacteria, such *E. coli*, *Enterobacter aerogenes*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Vibrio cholerae* and *Salmonella typhymurium*. It seems that the efficacy of *C. verum* oil related directly with the presence of active components, such as cinnamaldehyde cinnamyl acetate and cinnamyl alcohol, plus a wide range of other volatile substances.

Figueiredo et al. [36] found that the *T. capitata* essential oil, which is rich in carvacrol, was effective against *Salmonella* sp. and *E. coli*.

In another work performed in our laboratory, Almariri et al. [37] revealed that the main component of *T. syriacus* essential oil was carvacrol (36.73%), whereas the other major components were γ-terpinene (8.97%), β-caryophyllene (6.17%), farnesol (6.07%), ocimene (4.83%), thymol (4.00%), menthol (3.40%), myrcene (3.03%), and α-pinene (2.40%). They also found that the most effective components against

B. melitensis were carvacrol (<0.375 µl/ml), thymol (0.75 µl/ml) and dihydro-carvon (3.125 µl/ml), respectively.

In addition, in another work performed in our department, Tayoub et al. [38] found that the main components of *L. nobilis* essential oil were 1,8-cineol (50.3%), dihydrocarvone (5%), α-terpinenyl acetate (11.4%), sabinene (9.2%), spathulenol (3.4 %) and α-pinene (3.2%).

In our study, using the combination between *T. syriacus and C. zeylanicum* essential oils, only 0.75 µl/ml of each essential oil was enough to inhibit 50% of bacteria (MIC_{50} = 0.75 µl/ml for each essential oil).

In conclusion, *T. syriacus* and *C. zeylanicum* essential oils as well as *L. nobilis* plant extract were the most effective against *B. melitensis,* which could be a potential source of new antibacterial agents. Moreover, *T. syriacus – C. zeylanicum* essential oils combination was more effective than the use of each 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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AKTYWNOŚĆ ANTYBAKTERYJNA WYBRANYCH WYCIĄGÓW ROŚLINNYCH I OLEJKÓW ETERYCZNYCH PRZECIWKO *BRUCELLA MELITENSIS*

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S t r e s z c z e n i e

Rośliny lecznicze są uważane za nowe surowce do produkcji związków, które mogą być używane jako alternatywa dla antybiotyków w leczeniu chorób wywołanych przez bakterie oporne na antybiotyki. Celem niniejszej pracy było określenie skuteczności niektórych roślin rosnących w Syrii w leczeniu brucelozy.
Aktywność olejków eterycznych i wyciągów przeciw 89 szczepom *Brucella melitensis* badano metodą krążków bibułowych, stosując 5-cio% roztwory badanych substancji roślinnych. Do określenia wartości MIC olejków i ekstraktów zastosowano metodę mikrorozcieńczeń w podłożu płynnym.
Wśród badanych roślin tylko olejki eteryczne z *Thymus syriacus* i *Cinnamomum zeylanicum* i wyciąg z *Laurus nobilis* wykazały wysoką aktywność przeciwko szczepom *B. melitensis*. MIC_{50} dla *T. syriacus*, *C. zeylanicum* i *L. nobilis* wobec *B. melitensis* wynosiły odpowiednio 6,25, 3,125 i 6,25 μl/ml. Wśród badanych olejków eterycznych i wyciągów roślinnych, olejki eteryczne z *T. syriacus* i *C. zeylanicum* oraz wyciąg z *L. nobilis* były najbardziej aktywne. Co więcej, kombinacja *T. syriacus - C. zeylanicum* była bardziej skuteczna niż użycie każdego z tych olejków eterycznych osobno. Zatem olejki eteryczne z *T. syriacus* i *C. zeylanicum* oraz wyciąg z *L. nobilis* mogą być stosowane jako środki przeciwbakteryjne przeciwko *B. melitensis*.

***Słowa kluczowe**: bruceloza, aktywność przeciwbakteryjna, Brucella melitensis, Cinnamomum zeylanicum, Thymus syriacus, Laurus nobilis, olejki eteryczne, wyciągi roślinne*