Original Article

In Vitro Antibacterial Effects of Five Volatile Oil Extracts Against Intramacrophage *Brucella Abortus* 544

Ayman Al-Mariri, George Saour, Razan Hamoud

Abstract

Background: *Brucella abortus* is a gram-negative facultative intracellular bacterium that can cause a highly contagious disease in sheep, goats, cattle and one-humped camels. It is responsible for one of the most important zoonosis in human. The aim of this study was to evaluate the role of *Mentha piperita*, *Origanum majorana*, *Citrus lemon*, *Cinnamomum verum* and *Myristica fragrans* essential volatile oil extracts on human macrophages infected by *B. abortus* 544.

Methods: Essential volatile oil extracts from *M. piperita*, *O. majorana*, *C. lemon*, *C. verum* and *M. fragrans* were extracted. Human macrophages were cultured at a density of 2×10^5 cells per well in sterile 96-well microtiter plates, and infected with *B. abortus* 544 at a ratio of 1:100 bacteria/cell. Then essential volatile oil extracts were added at a concentration of 1%. At specified times; cells were washed, lysed with 0.1% Triton, and plated on 2YT agar to determine the number of intracellular bacteria.

Results: *Cinnamomum verum* volatile oil at a concentration of 1% had the highest antibacterial activity against *B. abortus* 544 inside human macrophages. Its inhibitory effect observed from 24 h and continued till 144 h after the infection. Moreover, *C. verum* (0.1%) in combination with 1% concentration of *M. piperita*, *O. majorana*, *C. lemon* or *M. fragrans* volatile oil extracts produced a synergistic inhibitory effect against *B. abortus* 544.

Conclusion: The results indicate that, among the five selected oil extracts, *C. verum* volatile oil applied either separately or in combination with other oil extracts had the most effective antimicrobial activity against *Brucella*.

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Keywords ● *Brucella* ● macrophages ● essential oil extracts ● synergistic ● cinnamon

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Introduction

Brucellosis is a zoonotic disease with a worldwide distribution that is endemic in the world. *Brucella abortus* remains a major cause of morbidity in humans and domestic animals.[1] After invasion of the lymphoid system, the bacteria are developed within mononuclear phagocytes, and the infected cells play a crucial role in the dissemination of the bacteria in specific locations of the body such as spleen, brain, heart, and bones.[2] *Brucella* species virulence and chronic infections are thought to be due to their ability to escape killing mechanisms within macrophages, such as lysosomal enzymes and products of the

oxidative burst.[3] Food and pharmaceutical industries still need to find new and improved antimicrobial agents capable of being effective against brucellosis.

In spite of the improvements in food hygiene and food production techniques, food safety is an increasingly important public health issue.[4] For this reason, to produce safe foods new methods are still needed, to possibly in combination with the existing methods, reduce or inhibit foodborne pathogens.[5] Because of increasing pressure from consumers and legal authorities, food industry has tended to reduce the use of chemical preservatives in their products to either completely nil or to adopt more natural alternatives for the maintenance or extension of product shelf life.

Plants extracts have been traditionally used worldwide in the treatment of some diseases from long time ago, but more recently plants essential volatile oil extracts are becoming more important due to their proved antimicrobial effects. Consequently, they are extensively used in medicine, and food and cosmetic industries. In addition to their role as antimicrobial agents,[6] they have a role as antioxidant agents.[7] For instance, lemon *Citrus lemon* (L.) Burm. has been used as an antimicrobial,[8] anticoccidial,[9] and antifungal agent,[10] whereas, cinnamon *Cinnamomum verum* J. Presl has been used only an antimicrobial agent.[11] However, nutmeg *Myristica fragrans* Houtt., peppermint *Mentha piperita* L., and sweet marjoram *Origanum majorana* L. have been used as stimulating agents against bacteria,[6] and fungus.[12] There is, however, no information about the role of essential volatile oil extracts against intracellular bacteria such as *B. abortus* 544 inside the human macrophages. Thus, the aim of this study was to assess the efficacy of several essential volatile oil extracts from *C. verum*, *M. fragrans*, *M. piperita*, *C. Lemon* or *O. Majorana*. Such oil extracts arelargely used in Syrian traditional medicine for the treatment of respiratory and gastrointestinal diseases, against *B. abortus* 544, inside the human macrophages.

Materials and Methods

Bacterial Culture

For infection experiments, *B. abortus* 544 was grown for 48 h in 2YT (peptone; 16 g, sodium chloride; 5 g, meat extract; 10 g, distilled water; 1 litre, (Difco, BD, Spars, MD) with 5% sterile horse serum. Bacteria were suspended in a sterile phosphate-buffered saline (PBS). Abundance of *B. abortus* 544 in PBS was monitored by recording optical density (OD) at 590 nm. The exact number of bacteria colony forming units (CFU) was assessed by viable count on 2YT agar (20 g/L) plates. Plates were placed in an incubator for 48 h at 37°C with 10% CO_2 tension adjusted automatically. During the contact with the organism, laboratory personnel were wearing impermeable protective clothes, gloves, and face masks.

Plant Samples Collection

Leaves samples of *M. piperita* (Lamiaceae) and *O. majorana* (Lamiaceae), and peel samples of *C. lemon* (Rutaceae) were collected from their native growing regions in Syria, while *C. verum* (Lauraceae) bark samples and *M. fragrans* (Myristicaceae) fruit samples were purchased from the local markets. Plants characterizations were consigned in table 1.

Essential Volatile Oil Extraction

Aerial parts of *M. piperita* and *O. majorana* were cleaned and dried prior to steam distillation in a glass apparatus using double distilled water. The plant leaves, which were collected from one station, were separated from the steams and mixed thoroughly to ensure a good homogeneity. Seventy five grams of the dried leaves and 700 ml of water were placed into a distillation flask of one litre capacity, and were extracted for three hours. This process was applied on all plants collection. *Citrus lemon* peels of ripe fruit were separated from the whole sample and hydrodistilled directly (without drying) using the same steam distillation extraction method. Dried fruit of *M. fragrans* and the barks of *C. verum* were grounded, and the powdered materials were hydrodistilled into steam distillation apparatus, as mentioned above. Isolated volatile oil extracts collected from each distillation process were added to each other and dried over anhydrous sodium sulphate and stored in dark glass bottles in a fridge at 4°C until use.

Macrophage Infection

Healthy human macrophage cells were collected and cultured in RPMI. medium

Table 1: Characteristics of plants from which essential oils were derived

Scientific name	Plant family	Collection site	Altitude	Collection time	Extracted part	Essential oils content (%)
Mentha piperia	*Lamiaceae*	Latakia	350 m	May	leaves	1.5
Origanum majorana	*Lamiaceae*	Kafr Nobol- Idlib	750 m	June	leaves	2.08
Myristica fragrans	*Myristicaceae*	Market	–	–	fruits	9.5
Cinnamomun verum	*Lauraceae*	Market	–	–	Bark	0.5
Citrus limon	*Rutaceae*	Latakia	100 m	September	peel of ripe fruit	2.5

supplemented with 10% heat-inactivated fetal calf serum. For macrophage growth assays, 96-well microtiter plates were seeded with 2×10^5 macrophages/well and infected with *B. abortus* 544 at a ratio of 1:100 bacteria/macrophage. Cells were incubated for one h at 37°C in 5% CO_2. Extracellular bacteria were removed by three washes with PBS, followed by treatment with 25 µg/ml of gentamicin for 30 min. Then, the cells were maintained by the addition of medium containing 5 µg/ml of gentamicin. To evaluate the effect of plants volatile oil extracts on the ability of *Brucella* to invade human macrophage, 1% concentration of the five studied volatile oil extracts, or 0.1% of *C. verum* plus 1% of the other four volatile oil extracts, were added after 2, 4, 24, 48, 72, 96, 120 and 144 h of infection, the cells were washed three times by PBS, and lysed with 0.1% Triton. Five minutes after the incubation at room temperature, the lysates were plated on 2YT agar and incubated at 37°C for 48 h; in order to determine the intracellular bacterial count. All experiments were performed in triplicate. Macrophages infected with *B. abortus* 544 at a ratio of one bacteria/100 macrophage without adding any oil extract as a control.

Statistical Methods

Antibacterial properties of oil extracts were analyzed with one-way repeated measures analysis of variance (ANOVA) fallowed by Tukey's multiple comparison test to compare the difference between each pair of means. Data were transformed into $\log_{10}$ CFU prior to analysis to homogenize the variance. All analyses were conducted by using GraphPad Prism Statistical Software V5.03. Differences were considered statistically significant at $P<0.05$.

Results

Brucella abortus 544 $\log_{10}$ counts in human macrophages were significantly suppressed ($F_{5,35}=22.7$; $P<0.0001$) by volatile oil extracts treatments compared with the untreated control. For example, the inhibitory effect of *C. verum* at a concentration of 1% was started 24 h and continued till 144 h after the infection, and the $\log_{10}$ counts increased only from 3.11 to 4.9. The repeated measures ANOVA followed by Tukey's test of multiple comparisons revealed that *C. verum* volatile oil possessed the strongest antibacterial effect compared to all the other essential oil extracts (figure 1). It is worth pointing out that no significant difference occurred between the antibacterial activity of lemon, peppermint, sweet marjoram and nutmeg volatile oil extracts.

Cinnamon oil extract, when applied at a concentration of 0.1%, did not show any significant inhibitory effect against *B. abortus* 544 compared to the control group. In contrast, strong and statistically significant inhibitory effect was observed when 0.1% concentration of cinnamon volatile oil was applied in combination with 1% concentration of the other plants volatile oil extracts ($F_{5,35}=34.6$; $P<0.0001$). For instance, the $\log_{10}$ CFU did not exceed 4.6 and 4.9 for cinnamon (0.1%) and sweet marjoram (1%) or lemon (1%) mixture after 144 h of incubation, respectively (figure 2). Based on Tukey's multiple comparison test, we did not observe any significant differences between the four oil extracts used in combination with cinnamon volatile oil (0.1%) and when the

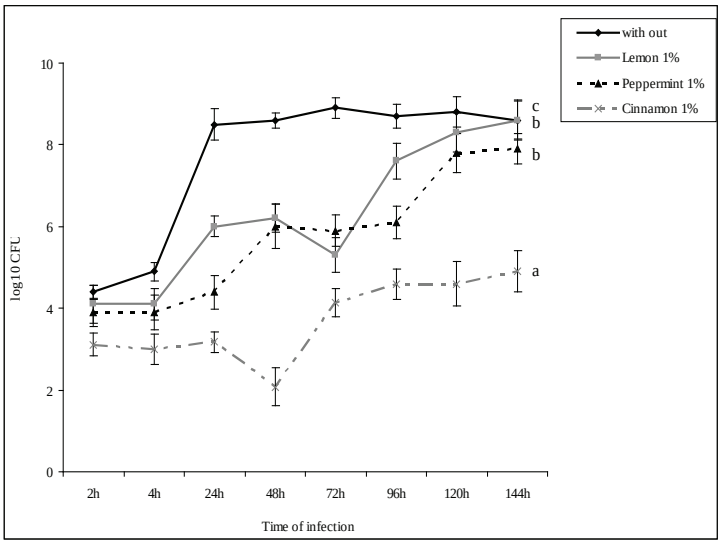

Figure 1: Inhibitory effects of 1% concentration of peppermint (*Mentha piperita*), lemon (*Citrus lemon*), and cinnamon (*Cinnamomum verum*) volatile oils on *Brucella abortus* 544 inside human macrophages. Intracellular bacterial counts were calculated 2, 4, 24, 48, 72, 96, 120 and 144 h after the infection. Different letters (a, b, c) at the end of each line represent a significant differences of an average response over times (h) at $P<0.05$ based on Tukey's multiple comparison test. The error bars represent the standard deviation in an average of $\log_{10}$ intracellular *B. abortus* 544 from triplicate platting taken at each mentioned time.

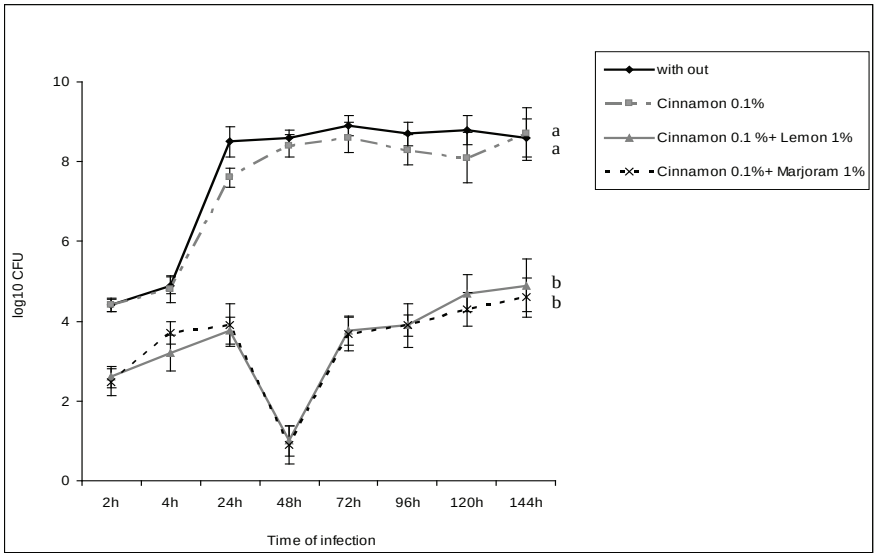

Figure 2: Inhibitory effect of 0.1% concentration of cinnamon (*Cinnamomum verum*) volatile oil used separately or in combination with 1% concentration of marjoram (*Origanum majorana*) or lemon (*Citrus lemon*) volatile oils on *Brucella abortus* 544 inside human macrophages. Intracellular bacterial counts were calculated 2, 4, 24, 48, 72, 96, 120 and 144 h after infection. Different letters (a, b) at the end of each line represent a significant differences of an average response over times (h) at P<0.05 based on Tukey's multiple comparison test. The error bars represent the standard deviation in an average of $\log_{10}$ intracellular *B. abortus* 544 from triplicate platting taken at each mentioned time.

above-mentioned oil mixtures were compared to cinnamon volatile (1%) oil used separately. However, a significant reduction in bacterial counts was recorded for each oil mixture and the cinnamon at 1% treatments compared to the untreated control ($F_{5,35}$=31.4; P<0.001).

Discussion

Nowadays, people worldwide try to avoid chronic stress, pollution and synthetic drugs. It is well documented that the number of pathogenic bacteria resistant to current antibiotics increases progressively, and the infections due to resistant strains of bacteria pose a serious clinical problem. All these negativities have brought natural agents to the fore and have brought alternative and complementary medicine up to date.[13]

Malta fever or Brucellosis is a disease found in the Middle East.[1] Most of the cases are not usually recognized, and fail to be classified. A low efficiency therapy system to eliminate *Brucella* is currently in use. That is the reason why there are lots of relapses and chronic infections.[14] Patients are usually medicated, thus, as being infected with other diseases; this would increase the odds of having some chronic cases.[15] With all in mind, it seems difficult to provide accurate estimates and numbers of *Brucella* infected patients. Estimates are usually lower than reality, especially in the case of children.[16] For all these reasons, good and new treatment regimens against *B. abortus* are urgently needed.

Our results showed that at a concentration of 1% *C. verum* volatile oil exhibited strong inhibitory effect against *B. abortus* 544 strain inside the human macrophages. This result concords with that found by Mayaud et al.[17] who reported that the *C. verum* bark volatile oil had an excellent antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* at concentrations ranging from 0.31% to 10% (v/v). The antimicrobial effect of cinnamon against gram negative bacteria was also reported by Ooi et al.[11] who concluded that *C. verum* was effective against a broad spectrum of bacteria, such *E. coli*, *Enterobacter aerogenes*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *V. parahaemolyticus* 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.[11] On the other hand, *C. verum* volatile oil at concentration of 0.1% revealed a minimum to nil inhibitory effect against *B. abortus* 544. Also, Ouafae et al.[18] also reported that viable bacterial counts decreased from 10^7 to 10^4 CFU/mL when *E. coli* O157:H7 cells were incubated at 37°C for 2 h in the presence of 0.025% concentration of cinnamon essential volatile oil. However, this bacteria was almost completely eliminated after 30 min of incubation in the presence of 0.05% concentration of cinnamon oil.

Our results revealed that *M. fragrans*, *C. lemon*, *O. majorana* and *M. piperita* volatile oil extracts had significant activities against *B. abortus* 544. Dabbah et al.[19] found that terpineol and

terpeneless fractions of *Citrus* volatile oil extracts to have greater inhibitory effects on gram-positive than gram-negative bacteria. However, Waikedre et al.[8] reported that the essential oil extracts from the leaves of *Citrus macroptera* and *C. hystrix* were inactive against 5 species of bacteria. Moreover, Baik et al.[20] reported that essential volatile oil extracted from 14 kinds of Korean endemic *Citrus* species did not have any activity against *S. epidermidis*, whereas O'Bryan et al.[21] found that *Citrus* essential volatile oil extracts at minimum inhibitory concentrations (MIC) ranging from 0.125% to 0.5% had a good inhibitory activity against the *Salmonella* spp. Barbosa et al.[22] found that the MIC_{90} of oregano (*Origanum vulgare* L.) essential volatile oil extracts was 5% and 0.46% v/v against gram-positive (*S. aureus* and *Listeria monocytogenes*) and gram-negative (*E. coli* and *S. Enteritidis*) bacteria, respectively. Whereas, López et al.[23] found that 8-10% (v/v) concentrations of *O. vulgare* essential volatile oil completely inhibited the growth of *E. coli*, *Y. enterocolitica*, *P. aeruginosa*, and *S. choleraesuis* bacteria.

Firouzi et al.[24] mentioned that the *M. fragrans* volatile oil extract had a moderate effect against *Y. enterocolitica*; while Mahady et al. [25] reported that the MIC of methanol extracts of *M. fragrans* seeds was 12.5 µg/mL against *Helicobacter pylori*. Al-Bayati,[26] reported that *Mentha longifolia* L. volatile oil had an antimicrobial activity against some gram positive pathogenic like as *S. aureus*, *Streptococcus mutans* but did not have any activity against *P. aeruginosa* bacteria. Mkaddem et al.[27] found that the *Mentha* essential volatile oil extracts were very active against *L. monocytogenes* and *Klebsiella pneumoniae* bacteria; whereas they were less effective against *E. coli*. Celikel and Kavas,[28] reported that essential oil of sweet orange (*Citrus sinensis* L.) at a concentration of 3% (v/v) exhibited the lowest antibacterial and bacteriostatic activity against *E. coli*, *L. monocytogenes*, and *S. aureus*. The results of Soković et al.[29] demonstrated that the essential oil of *M. piperita* possessed good activity against human pathogenic bacteria such as *E. coli* O157:H7, *S. typhimurium*, *S. aureus*, *P. aeruginosa* and *P. mirabilis*. Whereas, *C. lemon* was effective against only *E. coli* O157:H7, *S. typhimurium*, and *S. aureus*. These conflicting results are in agreement with reports indicating that essential oil extracts antimicrobial activities varied depending on the species, subspecies, variety or geo-ecological regions. Thus, it is not surprising to find that essential volatile oil extracts of some plants pertaining to the same species that were collected from different agricultural areas showed different levels of antimicrobial properties.[30-32]

Our data showed that the mixture of concentrations 1% of individual essential oil extracts and small amount of cinnamon oil (0.1%) was associated with enhanced antibacterial activity. In other words, the antibacterial property of the volatile oil extracts was apparently strengthened through the combination between cinnamon oil at low concentration and all the other essential oil extracts at high concentration. Thus, the results presented herein provide positive evidence regarding the synergism between different percentages of essential oil extracts as antibacterial agents against *B. abortus* 544. Our finding is in accordance with report of Probst et al.[33] findings, which showed that combinations of cinnamon with peppermint, ginger (*Zingiber officinale* Roscoe) and clove (*Syzygium aromaticum* L.) essential oil extracts produced synergistic antibacterial effects against gram-positive and gram-negative microorganisms. Moreover they are in agreement with Nanasombat and Wimuttigosol's,[34] results, which revealed that cinnamon oil in combination with nutmeg or makaen (*Zanthoxylum limonella* Alston) oil extracts showed a synergistic effect against *S. aureus*, *Pseudomonas fluorescens*, and *Salmonella* Rissen bacteria.

Conclusion

The goal of this study was to develop an effective and inexpensive therapy against *Brucella* inside human macrophages. *Cinnamomum verum verum* bark essential oil at a concentration of 1% used separately, or at a concentration of 0.1% in combination with a concentration of 0.1% *C. verum* with 1% *M. fragrans*, *M. piperita*, *C. Lemon* or *O. majorana* represents an alternative source of natural antimicrobial substances, and may replace conventional chemical antimicrobials. The high specific activity of cinnamon at low and non-toxic concentrations suggests that it could be used in clinical practice for the treatment of Brucellosis in animals and humans. More specific studies are recommended to examine this suggestion.

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Conflict of Interest: None declared

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