



Inhibitory Effects of Essential Oils of *Cinnamomum zeylanicum* and *Myristica fragrans* against *Brucella abortus* 544 Inoculated in Fresh Baladi Cheese

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HIGHLIGHTS

- Anti-*Brucella* activity of *Cinnamomum zeylanicum* essential oil was significantly more than that of *Myristica fragrans*.
- Due to the appropriate anti-*Brucella* activity, *C. zeylanicum* essential oil could be applied in production of Baladi cheese.
- Using *M. fragrans* essential oil could not protect the fresh Baladi cheese against *Brucella*.

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Acronyms and abbreviations

CFU=Colony Forming Unit

EO=Essential Oil

MIC=Minimum Inhibitory Concentration

ABSTRACT

Background: Essential Oils (EOs) are natural metabolic products of plants that contain a condensed chemical hydrophobic liquid compounds. The aim of this study was to evaluate inhibitory effects of EOs of *Cinnamomum zeylanicum* and *Myristica fragrans* against *Brucella abortus* 544 inoculated in fresh Baladi cheese.

Methods: Fresh Baladi cheese was manufactured from experimentally contaminated milk with *B. abortus* 544 in combination of EOs of *C. zeylanicum* or *M. fragrans*. Cheese samples were periodically subjected to further microbiological surveys at different storage times (0, 1, 24, 48, 72, and 96 h). The inhibition zone diameter and Minimum Inhibitory Concentration (MIC) against tested strain were also determined. Statistical analyses were conducted by GraphPad Prism Statistical Software.

Results: The inhibition zone diameter of the paper disk were 9.5 ± 0.5 and 16 ± 0.57 mm at 1% concentration of *M. fragrans* and *C. zeylanicum* EOs, respectively; and 15 ± 0.28 and 21 ± 0.76 mm at 5% concentration of *M. fragrans* and *C. zeylanicum* EOs, respectively. The values of inhibition zone diameters were significantly ($p < 0.0001$) different between the two selected concentrations of 1% and 5% for the studied EOs. Also, anti-*Brucella* activity of *C. zeylanicum* was significantly ($p < 0.0001$) more than that of *M. fragrans* EO.

Conclusion: Due to the appropriate anti-*Brucella* activity, *C. zeylanicum* EO could be applied as an effective natural preservative in the production of fresh Baladi cheese. Conversely, using *M. fragrans* EO could not protect the fresh Baladi cheese against *Brucella*.

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Introduction

Cheese is an ancient and popular fermented dairy products produced all around the world. This nutritious dairy is produced by adding an enzyme, rennet, to the milk

which causes the curds to separate from the whey. There are many kinds of cheese that differ from each other by the fat content of milk, salinity degree, length of ferment-

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tation, the percentage of water, the ratio of rennet, or the manufacturing temperature (Górska-Warsewicz et al., 2019; Johnson, 2017; Shakeel-ur-Rehman et al., 2008). In Syria, fresh Baladi cheese manufacturing industry is linked to spring, when milk yield increases, and air temperature decreases, which protects produced milk from decomposition and rancidity. It is preferable to use clean, fresh, and unpolluted milk from healthy animals for cheese production, and neglecting low pH milk (high acidity) which leads to milk coagulation (Kamleh et al., 2006).

Brucella spp. are among the pathogenic microbes that may contaminate dairy products (e.g. fresh cheese) and may endanger human health due to consumption of contaminated raw milk and dairy products. According to the classification of international organizations, brucellosis is considered as one of the most important bacterial zoonotic diseases in the world (Al-Mariri et al., 2012, 2015; Franc et al., 2018).

Plants Essential Oils (EOs) give new sources of antibacterial agents and are used in many domains, including food preservation, natural treatments, alternative medicine, and pharmacies. EOs or volatile oils are natural metabolic products of plants that contain a condensed chemical hydrophobic liquid compounds. These oils have been used as food preservatives, because of their antimicrobial compounds affecting a wide spectrum of Gram-positive and Gram-negative bacteria (Basavegowda et al., 2020; Hyldgaard et al., 2012; Moradi et al., 2014; Zhang et al., 2016).

Cinnamomum zeylanicum is a small evergreen tree belonging to the family *Lauraceae*, native to Sri Lanka. The inner bark of *Cinnamomum* species is used to make cinnamon. The trees are 10-15 meters tall and their leaves are ovate-oblong in shape and 7-18 cm long. The flowers arranged in panicles with a greenish color and a distinct odor. The fruit is a purple 1-cm drupe containing a single seed. The main constituents of cinnamon oil include cinnamaldehyde and minor components such as eugenyl acetate, linalool, and benzyl benzoate, each having antifungal and antibacterial activity (Christiany et al., 2021; El-Hack et al., 2020).

Myristica fragrans is an evergreen tree belonging to the family *Myristicaceae*, and it is indigenous to the Moluccas of Indonesia. It is widely grown across the tropics, including China, Taiwan, Indonesia, Malaysia, Caribbean, India, Sri Lanka, and South America. It is usually 5-15 m tall, but occasionally reaching 20 m or even 30 m. The alternately arranged leaves are dark green. The flowers are bell-shaped, pale yellow, and somewhat waxy and fleshy. Inside the fruit is a purple-brown shiny seed, with a red or crimson covering and the seed is the source of nutmeg. Some chemical components of *M. fragrans* EO are sabinene, terpinen-4-ol,

safrole, β -phellandrene, γ -terpinene, etc. (Jukić et al., 2006; Singh et al., 2005).

Consequently, the aim of this study was to evaluate inhibitory effects of EOs of *C. zeylanicum* and *M. fragrans* against *Brucella abortus* 544 inoculated in fresh Baladi cheese.

Materials and methods

Microorganisms and growth conditions

B. abortus 544 was obtained from the University of Namur (Belgium), it was grown in 2×YT broth (Difco, BD, Sparks, MD, USA) and incubated at 37 °C for 48 h (Dotreppe et al., 2011); after that, it was suspended in sterile Phosphate-Buffered Saline (PBS) (Jansen et al., 2020) and adjusted to 1×10⁶ Colony Forming Unit (CFU)/ml by monitoring the optical density at 590 nm.

Extraction of EOs

C. zeylanicum (*Lauraceae*) bark samples and *M. fragrans* (*Myristicaceae*) fruit samples were purchased from local markets in Damascus, Syria. The samples were grounded and powdered using electrical blender prior to steam distillation. The EOs extraction was carried out using the water-steam distillation device (Clevenger-type apparatus, Germany). Then, 100 g of the plant was used for extraction. After that, steam was condensed in a cooling vapor system to collect the EOs for 3 h, and then dried over anhydrous sodium sulfate (Na₂SO₄), filtered, and stored in resealable vials at 4 °C in the dark, but it was allowed to rest at room temperature prior to investigation. Oils were diluted in dimethyl sulfoxide (DMSO) at a final concentration of 4.4% (v/v) which had no growth inhibitory effects and used for antimicrobial activity tests. EO extraction yield (%) was 0.5 and 0.95 for *C. zeylanicum* and *M. fragrans*, respectively.

Preparation of fresh Baladi cheese

At first, milk was filtered and then pasteurized by heating at 72 °C for 15 s, and cooled quickly to 39 °C. CaCl₂ was added at the ratio of 20 g per 100 kg of milk, where Ca⁺² ions commenced coagulation of casein micelles in cheese milk. Rennet was then added at the ratio of 2.5 g to 100 kg of milk that will cause the milk to curdle. The mixture was incubated at a temperature of 39 °C for 40-60 min until the completed coagulation. The curd was destroyed and left for 15 min. Excess whey was then drained off, and the curd was cut off and put into cheesecloth for a few hours and pressed before being turned out.

Agar diffusion susceptibility test

To determine the antibacterial activity of the extracted EOs, a paper disk diffusion method was used. *B. abortus* 544 was grown in Mueller-Hinton Broth (MHB; Merck, Germany) medium at 37 °C for 48 h and the bacterial suspension was adjusted to 1×10^6 CFU/ml. Then, 0.1 ml of bacterial suspension was spread on Mueller-Hinton Agar medium (MHA; Merck, Germany) by a sterile cotton swab. After that, 2 µl of each EO with a concentration of 1% and 5% were prepared in order to be added to the 6 mm diameter sterile filter paper disks and then placed onto MHA plates previously inoculated with the suspension of the tested bacterium. DMSO was used as a negative control, whereas ciprofloxacin (5 µg/ml) was used as a positive control. The plates were then left at room temperature for 30 min and then incubated at 37 °C for 48 h. The antibacterial activity was evaluated by measuring the inhibition zones in mm (Al-Mariri and Safi, 2013). All tests were run in triplicate and the mean±SD result was calculated.

Minimum Inhibitory Concentration (MIC)

Microdilution broth susceptibility assay was performed using three replicates of each dilution of EO prepared in 2×YT medium in 96-well microtiter plates. In this study, two dilutions of EOs 1% and 5% were prepared. Each well was supplemented with 100 µl of freshly grown bacterial culture with a final concentration of approximately 1×10^6 CFU/ml in 2×YT. The assay included positive control without EO and negative control without bacteria under the same conditions. The plate was incubated with shaking for 24-48 h at 37 °C. The lowest concentration that completely inhibited visual growth of 50% was recorded as the MIC₅₀, whereas the lowest concentration that inhibited 90% of visual growth was recorded as the MIC₉₀. The absorbance was determined at 590 nm.

Anti-Brucella activity of EOs in cheese

A series population ranged between 0-10⁷ CFU/ml of *B. abortus* 544 was added to pasteurized milk during cheese manufacturing at the same time with the addition of *C. zeylanicum* or *M. fragrans* EOs at two concentrations (1% or 5%), and stored at 25 °C for 96 h. A patch of 25 g cheese was homogenized into a sterile strainer/filter-stomaching bags (Seward, stomacher lab system standard bags, England) with the addition of 225 ml of 2×YT and mixed in a pulsed stomacher (Seward, Stomacher 80 Biomaster, England) twice for 30 s. Ten fold serial dilutions of each sample at different storage time were cultured on 2×YT agar, followed by incubation at 37 °C for 48 h. Experimentally infected cheese without

the addition of EOs was considered as a positive control for bacterial growth. On the other hand, the cheese without the addition of bacteria was considered as a negative control.

Sensory evaluation

Sensory characteristics of cheeses are usually described using terms like appearance color, flavor, texture, and taste. These characteristics are outcome of interactions of the human sensory modalities of touch, vision, olfaction, mouthfeel, and gustation with stimulant-induced by structural, rheological, and chemical components of the cheese. Sensory evaluation was performed on the days of cheese processing and producing the supplemented cheese with EOs.

Statistical analysis

Antibacterial properties of antibiotics and oil extracts were analyzed by one-way repeated-measures analysis of variance (ANOVA) to compare the difference between each pair of means. Data were transformed into log₁₀ CFU. All analyses were conducted by using GraphPad Prism Statistical Software V5.03.

Results

The inhibition zone diameter of the paper disk were 9.5±0.5 and 16±0.57 mm at 1% concentration of *M. fragrans* and *C. zeylanicum* EOs, respectively; and 15±0.28 and 21±0.76 mm at 5% concentration of *M. fragrans* and *C. zeylanicum* EOs, respectively. The values of inhibition zone diameters were significantly ($p < 0.0001$) different between the two selected concentrations of 1% and 5% for the studied EOs. Also, antibacterial effect of *C. zeylanicum* was significantly ($p < 0.0001$) more than that of *M. fragrans* EO.

The MIC₅₀ values of *C. zeylanicum* and *M. fragrans* EOs were 1% and 5%, respectively. The MIC₉₀ of *C. zeylanicum* was 5%, whereas no inhibitory effect was found for *M. fragrans* EOs.

Although the log₁₀ population of *B. abortus* 544 in the cheese treated with *C. zeylanicum* EO (1% or 5%) ranged from 0 to 6.6 CFU/g at zero time of storage, no growth was found for any time of storage (from 1 to 96 h) for experimentally contaminated cheese. In Figure 1, the effects of *M. fragrans* EO on *B. abortus* 544 counts inoculated in cheese is shown.

Fresh Baladi cheese samples (control group) exhibited a bright white color, coherent texture, and cuttable curd without holes, rendering the taste of milk clearly. The treated cheese groups had sensory properties similar to the fresh cheese.

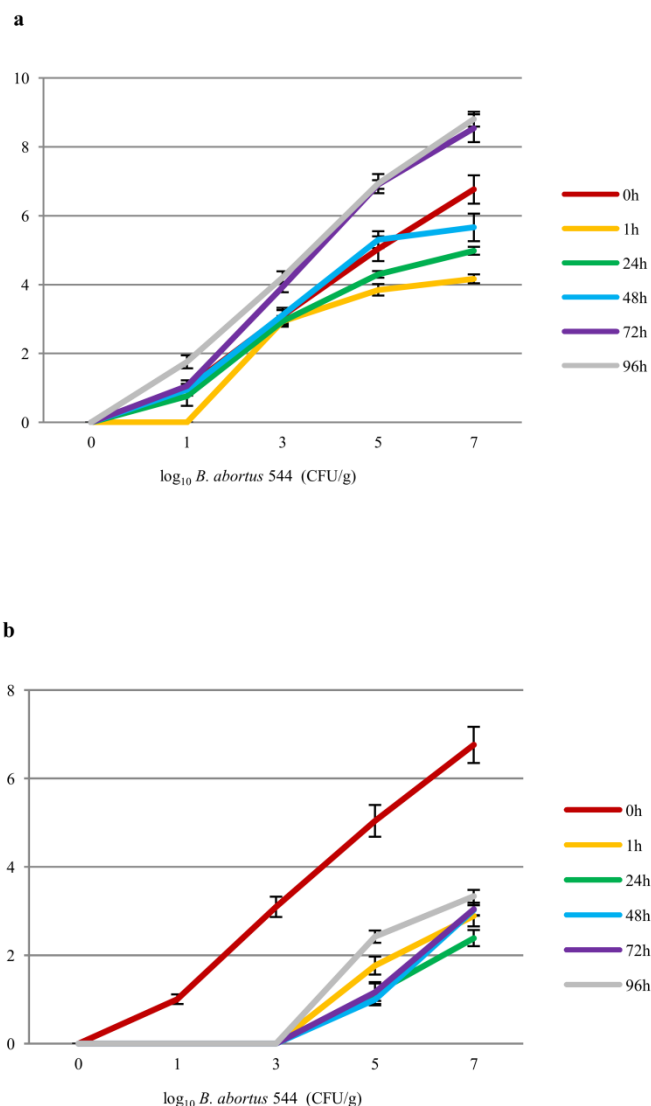


Figure 1: Effects of 1% (a) and 5% (b) levels of *Myristica fragrans* essential oil on *Brucella abortus* 544 counts inoculated in fresh Baladi cheese (\log_{10} of *B. abortus* 544 populations were conducted 0 to 96 h after infection)

Discussion

EOs have an important inhibiting function in food industry due to their antibacterial effects (Tayel et al., 2015). However, the critical requirements for this application are studying the properties of the EO, the MIC of the targeted microbes, the possible method of interaction between the EOs and food as a mixture, and also the quality of the product. EOs that are obtained from medicinal and aromatic plants, such as oregano, fennel, cumin, rosemary, dill, thyme, and pepper have shown good antimicrobial effects against food-borne pathogens commonly found in cheese, such as *Staphylococcus aureus*,

Listeria monocytogenes, *Escherichia coli*, and *Salmonella* (Caleja et al., 2015; Carvalho et al., 2018; De Carvalho et al., 2015; Hassanien et al., 2014; Moro et al., 2015). However, Amatiste et al. (2014) clarified that there was no effect of *Thymus vulgaris* and *Origanum vulgare* oils against *S. aureus* in fresh sheep cheese for 7 days of storage. Also, Hassanien et al. (2014) mentioned that a 0.1% concentration of black cumin EO resulted in the reduction of the growth of some bacteria, such as *L. monocytogenes*, *S. aureus*, and *E. coli* in a culture medium.

In this study, we observed inhibitory effects of two plants EOs on *B. abortus* 544 inoculated in fresh Baladi cheese. There are little data about the effect of plant EOs on *Brucella* sp. in cheese. However, there are many studies shown the inhibitory effects of plant EOs on the bacterial species *in vitro* and in food models. Similar to our finding, antimicrobial activity of extract of cinnamon has been reported against *L. monocytogenes*, *S. aureus*, and *Salmonella enterica* in cheese (Shan et al., 2011). Also, AL-Nabulsi et al. (2020) indicated that the addition of 2% cinnamon oil to Tahini reduced *E. coli* O157:H7 counts; after 21 days of storage at 10 °C. Matulyte et al. (2020) mentioned that *M. fragrans* EO with an excipient (1%) had a broader inhibitory effect on bacterial growth, whereas the pure oil was only efficient against *Pasteurella multocida*. In addition, Tayel et al. (2015) reported that plant extracts of cinnamon, cloves, garden cress, lemongrass, garlic, rosemary, sage, and oregano exhibited antibacterial effects against *L. monocytogenes* inoculated to cheese-based media. In a similar manner, Sadeghi et al. (2013) proved the inhibitory effect of cinnamon EO on the growth of *S. aureus* in white brined cheese which were in agreement with our result.

It is worth noting that the EOs can affect the growth of *Lactococcus lactis* as well as the pathogens with MIC of 1.25 µl/ml (Gouvea et al., 2017; Shan et al., 2011). This effect was also revealed by De Carvalho et al. (2015) who clarified that the MIC of thyme EO on *L. lactis* (as cheese starters), *Listeria* sp., and *S. aureus* in Coalbo cheese was 2.5 µl/ml. However, contrary to our result, according to another research in which *Zataria multiflora* EO was added to Gouda cheese milk at different concentrations (0.05, 0.2, and 0.4%), an increase in the Enterobacteriaceae and Lactococci count was observed from 1.23 log CFU/g on the first day to 2.96 log CFU/g after 90 days (Es'haghi Gorji et al., 2014).

Our findings revealed that both 1% and 5% levels of *C. zeylanicum* EO had more antibacterial effect than *M. fragrans* EO. This could refer to the presence of one (or more) of the *C. zeylanicum* EO antibacterial components, or its absence in *M. fragrans* EO (Weerakkody et al., 2011). The antibacterial activities of EO primarily belong to their various bioactive components, for example, cinnamaldehyde is the main components of cinnamon EO followed by eugenol (Christiany et al., 2021; El-Hack et al., 2020). While components of the *M. fragrans* EO are sabinene and myristicin (Maya et al., 2004). Ooi et al. (2006) demonstrated the strong antibacterial effect of cinnamaldehyde from cinnamon oil against *E. coli* O157, *S. aureus*, *Penicillium* spp., *Fusarium* spp., as well as *Aspergillus* spp. The antibacterial effect of cinnamaldehyde could be a result of the mechanism that

demands the inhibition of biosynthetic enzymes or the inhibition of cell wall synthesis because of the rapidity of ATP depletion or inhibition (Gill and Holley, 2006b). At deadly concentration, the cell membrane is destroyed, leading to an imbalance of cell division and motility reducing (Gill and Holley, 2006b). The antibacterial effect of *M. fragrans* L. EO is mainly due to its α -pinene component, which has antimicrobial effect on Gram-negative and Gram-positive bacteria (Takikawa et al., 2002) due to inhibiting the bacterial enzymes and protein synthesis of the cell wall (Gupta et al., 2008).

Furthermore, decreases in pH and an oxygen levels can lead to an increase in the antimicrobial effect of EOs. As an example, a decrease in pH can enhance the activity of the hydrophobic part of EOs, resulting in an increase in the degradation of the bacterial cell membrane (Gill and Holley, 2006a). The physical structure of cheese may reduce the antimicrobial effect of EOs on targeted microbe (Gutierrez et al., 2008). Despite the effect of EOs on limiting the growth and viability of microbes in cheese, EOs can interact with cheese components, such as proteins, fats, and carbohydrates (Burt, 2004). EOs can also influence sensory evaluation (Hyldgaard et al., 2012), and that is why Gutierrez et al. (2008) suggested incorporating the EO into films and coating. In such manner, the transmission of antimicrobial oil from the film or coating to food could be a slow and long-term effect, and the aim of eliminating food pathogens, if exist, could be achieved.

Conclusion

This study confirmed, for the first time, the appropriate anti-*Brucella* activity of *C. zeylanicum* EO in experimentally contaminated fresh Baladi cheese over the storage period. As conclusion, *C. zeylanicum* EO could be applied as an effective natural preservative in the production of fresh Baladi cheese. Conversely, it was found that using *M. fragrans* EO could not protect the fresh Baladi cheese against *Brucella*.

Author contributions

A.A-M. and R.I. designed the project of study; R.I., A.A., and B.A. conducted the experiments; A.A-M. and L.A. analyzed the data and wrote the manuscript. All authors revised and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest in this study.

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