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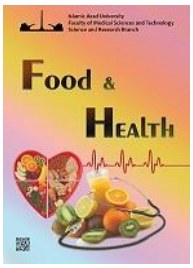

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Study of the combined effect of *Trachyspermum ammi* and *Lavandula officinalis* essential oils on some foodborne pathogenic bacteria

Asal Ahmadi[1], Hassan Gandomi[1*], Negin Noori[1], Melika Farzaneh[2], Mahdie Shabanizade[3]

[1] Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
[2] Department of Food Science and Technology, Shahr-e-Qods Branch, Islamic Azad University, Tehran, Iran.
[3] Faculty of Medicine, Kashan University of Medical Sciences, Kashan, Iran

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ABSTRACT

In this study, the effects of different concentrations of *Trachyspermum ammi* and *Lavandula officinalis* essential oils (EOs) were investigated individually and in combination against certain foodborne pathogens, including *E. coli*, *Salmonella typhimurium, L. monocytogenes*, *Bacillus cereus*, and *Staphylococcus aureus*. The MIC of *T. ammi* essential oil alone against the studied bacteria was as follows: *E. coli* (1000 ppm), *S. typhimurium* (2000 ppm), *L. monocytogenes* (1000 ppm), *B. cereus* (2000 ppm), and *S. aureus* (2000 ppm). On the other hand, the MIC of *L. officinalis* alone against the studied bacteria was higher: *E. coli* (>4000 ppm), *S. typhimurium* (>4000 ppm), *L. monocytogenes* (1000 ppm), *B. cereus* (1000 ppm), and *S. aureus* (2000 ppm). Interestingly, the combined effect of *T. ammi* and *L. officinalis* EOs demonstrated that a combination of 500 ppm *T. ammi* EO and 1000 ppm *L. officinalis* EO could effectively inhibit the growth of *E. coli*. Similarly, a combination of 500 ppm *T. ammi* EO with 500 ppm *L. officinalis* EO and 125 ppm *T. ammi* EO with 1000 ppm *L. officinalis* EO effectively inhibited the growth of *S. aureus*. Furthermore, the combined MIC values for specific bacteria were 1000 ppm *T. ammi* EO with 3000 ppm *L. officinalis* EO for *Salmonella typhimurium,* 500 ppm *T. ammi* EO with 500 ppm *L. officinalis* EO for *L. monocytogenes*, and 1000 ppm *T. ammi* EO with 500 ppm *L. officinalis* EO for *Bacillus cereus*. Overall, the combination of these two essential oils led to an increase in the lag phase and a decrease in the growth rate of the target bacteria, which is significant in food microbiology. In conclusion, the study highlights the importance of the combination application of *T. ammi* and *L. officinalis* EOs as natural antimicrobial agents in food safety.

1. Introduction

Foodborne diseases resulting from consumption of food contaminated with pathogenic bacteria are of great importance to public health. Annually, substantial financial and human losses are attributed to such diseases. One way to control the growth of pathogenic bacteria in food products is through the use of chemical preservatives and antimicrobial compounds (1). However, public concerns about the side effects of chemical preservatives have led consumers to prefer products that are either free of preservatives or use natural preservatives (2). Consequently, extensive research has been conducted in recent years on natural preservatives. Among these, plant essential oils stand out. Plant essential oils and their constituents have well-known antibacterial effects (3). Their diverse applications include controlling the growth of foodborne pathogens and preventing spoilage (4). *Trachyspermum ammi* L. is a medicinal plant mostly found in arid and semi-arid regions in various regions in India and Iran, particularly in Baluchestan, Tabriz, and Isfahan (5). Locally, it is known as "Ajowan" and "Espar Kai." The plant is traditionally used as a dietary supplement due to its health-related properties such as antioxidant, antimicrobial, sedative, stimulant, carminative, antifungal, anti-inflammatory agent, liver refresher, and for removing paralysis and antispasmodic effects (6-9). Ajowan seeds are widely used as a spice in curry powder because of their aromatic odor and spicy taste (10). *Lavandula officinalis* is a fragrant plant that belongs to the

*Corresponding author: Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

E-mail address: gandomih@ut.ac.ir (Hassan Gandomi).

Lamiaceae family (11). The distribution of this plant spans across the European continent, including the Mediterranean region, southwestern Asia, North Africa, and the Canary Islands (12). Its primary use lies in the perfume industry. In medicine, it is attributed with various properties including natural antibiotic, antiseptic, antispasmodic, sedative, and detoxifier properties (13). In general, higher concentrations of plant essences are necessary in food compared to laboratory environments to effectively exhibit their antibacterial effects (14). However, using high concentrations of essential oils could adversely affect the taste of food. On the other hand, the economic impracticality of using a single natural food preservative in large quantities has limited its application. Consequently, the simultaneous use of two or more preservative combinations has gained attention in terms of Hurdle technology (15). Hurdle technology combines plant EOs and other natural preservatives at lower concentrations to target important foodborne pathogens. The goal is to achieve a reliable and suitable synergistic combination of natural preservatives with the maximum antimicrobial effect and minimal adverse effects on organoleptic properties. The aim of this study was to investigate the antibacterial effects of using a combination of *T. ammi* and *L. officinalis* on certain foodborne bacteria.

2. Materials and methods

2.1. Preparation of T. ammi and L. officinalis EOs

The *T. ammi* plant was collected from Sistan and Baluchestan province, and the *L. officinalis* plant was collected from Fars province during the spring season. The essential oils (EOs) were extracted using steam distillation with a Clevenger apparatus, and their moisture was removed using sodium sulfate.

2.2. Preparation of bacterial inoculum

Five foodborne bacterial strains were investigated: *Escherichia coli* O157:H7, *Salmonella typhimurium*, *Listeria monocytogenes* ATCC 19118, *Bacillus cereus* ATCC 11778, and *Staphylococcus aureus* ATCC 6538. Initially, stock cultures maintained at -20°C were refreshed at least twice in Brain Heart Infusion (BHI) broth for 16-18 hours at 37°C. To prepare the inoculation dose using the optical absorption method, an appropriate volume of bacterial suspension was transferred to BHI broth in a cuvette to adjust the optical density (OD) of the bacterial suspension to 0.1 at a wavelength of 600 nm. Bacterial counts were determined by plate count method through serial dilutions and plating aliquots on BHI agar, followed by incubation at 37 °C.

2.3. Determination of the individual and combined minimum inhibitory concentration (MIC) of the EOs

The minimum inhibitory concentration (MIC) was determined using the broth microdilution method. A 96-well round-bottom microplate was used. Sequential concentrations of *T. ammi* EO (including 0, 125, 250, 500, 1000, and 2000 ppm) and *L. officinalis* EO (including 0, 125, 250, 500, 1000, 2000, and 4000 ppm) were prepared in BHI broth supplemented with 10% dimethyl sulfoxide (DMSO). Then, 100 microliters of the various EO concentrations were transferred to each well, followed by the addition of 20 microliters of each bacterial suspension (with a final bacterial concentration of 10^5 CFU/ml). Contents were mixed using a shaker for 2 minutes. Microplates were incubated at 37 °C for 24 hours. After incubation, turbidity in the wells was visually observed. The MIC was the lowest concentration with no observed turbidity.

2.4. Evaluation of the combined effect of T. ammi and L. officinalis EOs

The combined effect of *T. ammi* and *L. officinalis* EOs was assessed using the fractional inhibitory concentration index (FIC). This index is based on MIC values and assesses interaction type between oils. The FIC index was calculated using the following formula: FIC index= (MIC of *T. ammi* EO in combination with *L. officinalis* EO/ MIC of *T. ammi* EO alone) + (MIC of *L. officinalis* EO in combination with *T. ammi* EO/ MIC of *L. officinalis* EO alone). An FIC of <1 indicates synergism, =1 indicates additivity, 1-2 indicates indifference, and >2 indicates antagonism. The interaction was also analyzed by constructing isobologram curves from MIC values.

2.5. Evaluation of the effects of T. ammi and L. officinalis EOs on the bacterial growth curve

Different sub-inhibitory concentrations of *T. ammi* and *L. officinalis* EOs, alone or in combinations, were prepared in BHI broth. The broth was inoculated with a bacterial suspension at a final concentration of 5×10^5 CFU/ml. Cultures were incubated for 24 hours at 37 °C. Optical density (OD) of cultures was measured at 1, 2, 4, 6, 8, 10, 12, and 24 hours after incubation using spectrophotometry at 600 nanometers. Bacterial growth curves were constructed by plotting OD versus incubation time.

3. *Results*

The results of determining the minimum inhibitory concentration (MIC) of essential oils (EOs) from *T. ammi* and *L. officinalis*, alone or in combination, against five bacterial strains are shown in Table 1. The MIC of *T. ammi* EO against *E. coli* was 1000 ppm, while the MIC of *L. officinalis* EO was >4000 ppm. The combined effect of *T. ammi* and *L. officinalis* EOs showed that concentrations of 500 ppm of *T. ammi* EO and 1000 ppm of *L. officinalis* EO were able to inhibit the growth of *E. coli*. For *S. typhimurium*, the MICs were 2000 ppm of *L. officinalis* EO and 4000 ppm of *L. officinalis* EO, individually. The combination of these two EOs at a concentration of 1000 ppm of *L. officinalis* and 3000 ppm of *L. officinalis* EOs was also able to inhibit bacterial growth. *T. ammi* EO and *L. officinalis* at 1000 ppm, individually, and their

combination of 500 ppm of *T. ammi* EO with 500 ppm of *L. officinalis* EO led to inhibition of the growth of *L. monocytogenes*. *T. ammi* EO at 2000 ppm and *L. officinalis* at 1000 ppm, individually, and their combination with 1000 ppm of *T. ammi* EO and 500 ppm of *L. officinalis* EO prevented the growth of *B. cereus*.

Table 1. The individual and combined minimum inhibitory concentration (MIC) of essential oils of *T. ammi* and *L. officinalis* on studied foodborne bacteria.

Bacteria	MIC (ppm)			FIC index	Interaction effect
	T. ammi	*L. officinalis*	*T. ammi* + *L. officinalis*		
E. coli	1000	> 4000	500 + 1000	0.75	synergistic
S. typhimurium	2000	> 4000	1000 + 3000	<1.25	indifferent, additive, synergistic
L. monocytogenes	1000	1000	500 + 500	1	additive
B. cereus	2000	1000	1000 + 500	1	additive
S. aureus	2000	2000	500 + 500 125 + 1000 1000 + 125	0.54	synergistic

The MIC of *T. ammi* EO alone and *L. officinalis* alone against *S. aureus* was 2000 ppm, and the MIC of their combination was determined as 500 ppm of *T. ammi* EO with 500 ppm *L. officinalis* EO, 1000 ppm of *T. ammi* EO with 125 ppm of *L. officinalis* EO, and 125 ppm of *T. ammi* EO with 1000 ppm of *L. officinalis* EO. The type of combined effect of the EOs was investigated by calculating the fractional inhibitory concentration (FIC) index. The FIC index of the combination was 0.75 and 0.54 for *E. coli* and *S. aureus*, respectively, indicating a synergistic effect. The FIC index for *B. cereus* and *L. monocytogenes* was 1, showing an additive effect, and for *S. typhimurium*, it was > 1.25, which is ineffective. The type of combined effect was also analyzed by drawing isoblogram diagrams (Figures 1-5). The combined MIC of *T. ammi* EO and *L. officinalis* for *E. coli* and S. aureus was lower than the horizontal line, presenting a synergistic effect (Fig.1 and Fig.2).

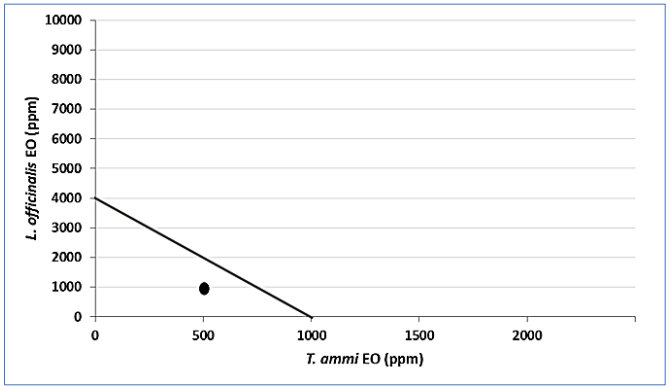

Fig. 1. Isobologram curve related to the combined effect of *T. ammi* and *L. officinalis* EOs against *E. coli*. Diagonal line demonstrated the isobologram line connecting the individual MIC of the EOs, and the solid circle shows the combination MIC.

For *L. monocytogenes* and *B. cereus*, the combined MIC was located on the horizontal line, indicating an additive effect (Fig.3 and Fig.4). For *S. typhimurium*, the combined MIC is located above the isoblogram line, demonstrating no effect (Fig. 5). The effect of sub-inhibitory concentrations of *T. ammi* and *L. officinalis* EOs alone and in combination on the growth curve of *S. aureus* is shown in Fig. 6. In the control group, rapid growth of *S. aureus* was observed in the early hours of incubation, reaching a peak at 8 h. The growth of *S. aureus* in the presence of *T. ammi* EO alone was significantly lower than the control, as the delay phase increased until 8 h, after which logarithmic growth began and reached a maximum at 24 h. *L. officinalis* EO alone increased the delay phase until 12 h.

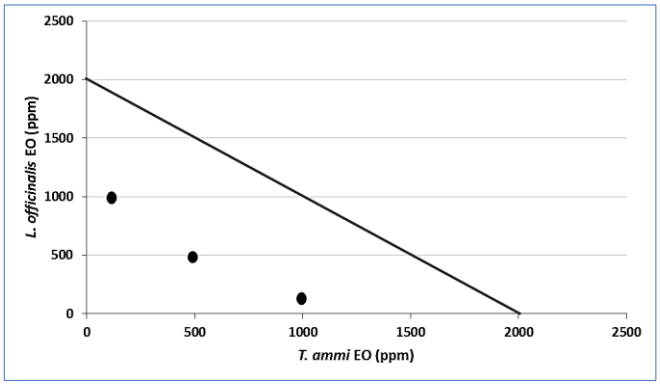

Fig. 2. Isobologram curve related to the combined effect of *T. ammi* and *L. officinalis* EOs against *S. aureus*. Diagonal line demonstrated the isobologram line connecting the individual MIC of the EOs, and the solid circle shows the combination MIC.

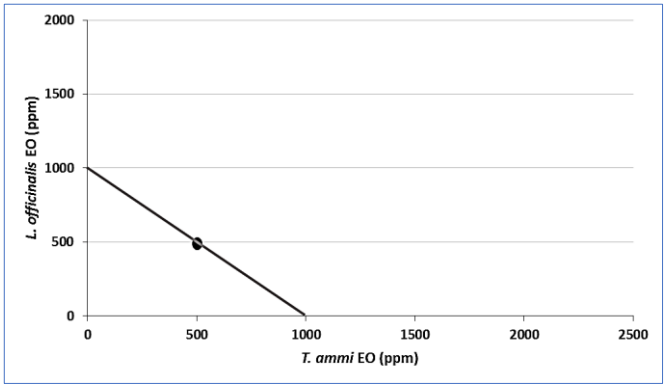

Fig. 3. Isobologram curve related to the combined effect of *T. ammi* and *L. officinalis* EOs against *L. monocytogenes*. Diagonal line demonstrated the isobologram line connecting the individual MIC of the EOs, and the solid circle shows the combination MIC.

The combination of *T. ammi* and *L. officinalis* EOs completely inhibited the growth of bacteria, with no growth observed until 24 h. Similarly, the effect of sub-inhibitory concentrations of *T. ammi* and *L. officinalis* EOs alone and in combination on the growth curve of *E. coli* is shown in Fig. 7.

In the control group, as with Staphylococcus bacteria, logarithmic growth of *E. coli* was observed in the early hours, reaching a peak at 6 h. The inhibitory concentration of *L. officinalis* EO could not prevent the growth of bacteria, and the OD level of this group was not significantly different from the control group. *T. ammi* EO delayed the growth of bacteria until 6 h, after which bacterial growth began, reaching a maximum at 12 h. The combination of *T. ammi* and *L. officinalis* EOs completely inhibited the growth of bacteria, with no growth observed during 24 h.

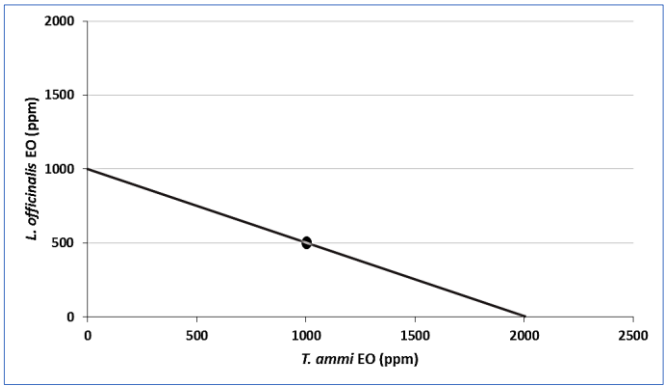

Fig. 4. Isobologram curve related to the combined effect of *T. ammi* and *L. officinalis* EOs against *B. cereus*. Diagonal line demonstrated the isobologram line connecting the individual MIC of the EOs, and the solid circle shows the combination MIC.

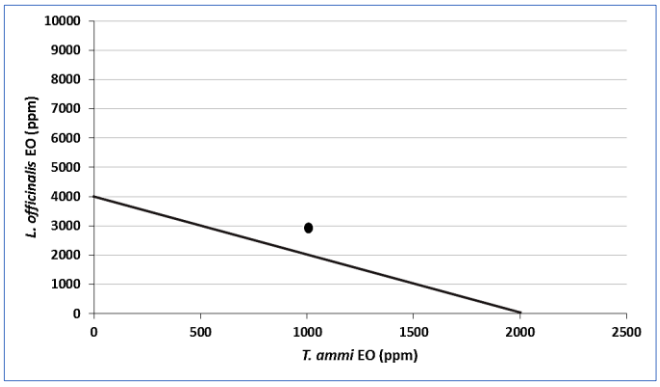

Fig. 5. Isobologram curve related to the combined effect of *T. ammi* and *L. officinalis* EOs against *S. typhimurium*. Diagonal line demonstrated the isobologram line connecting the individual MIC of the EOs, and the solid circle shows the combination MIC.

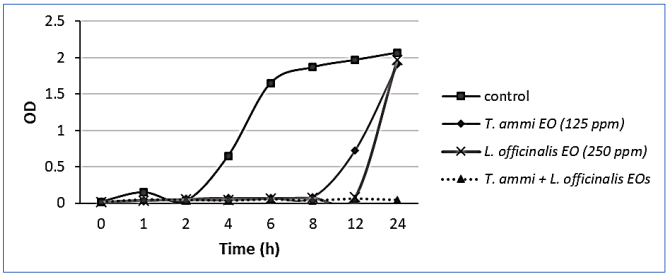

Fig. 6. Growth curve of *S. aureus* affected by sum-inhibitory concentrations of *T. ammi* and *L. officinalis* EOs used individually or in combination.

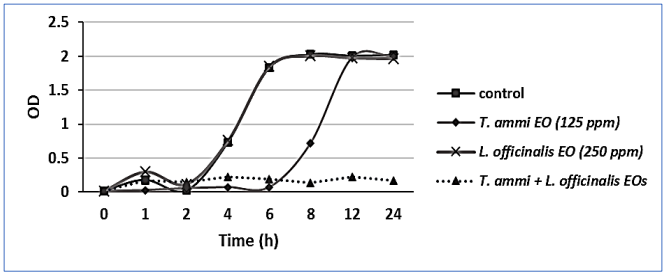

Fig. 7. Growth curve of *E. coli* affected by sum-inhibitory concentrations of *T. ammi* and *L. officinalis* EOs used individually or in combination.

4. Discussion

In this study, the effect of *T. ammi* and *L. officinalis* EOs on some foodborne pathogenic bacteria including *E. coli*, *S. typhimurium*, *L. monocytogenes*, *B. cereus* and *S. aureus* was investigated. The MIC of *T. ammi* EO against both *E. coli* and *L. monocytogenes* was 1000 ppm, while the MIC value of 2000 ppm was obtained for *S. typhimurium*, *B. cereus* and *S. aureus*. The antibacterial effect of *T. ammi* EO has been investigated in several studies. Kumar et al. (16) evaluated the antibacterial activity of *T. ammi* EO against water pathogens. In this research, the broth microdilution method was used and the MIC of *T. ammi* EO for *E. coli* and *S. typhimurium* was obtained as 0.087 and 0.128 (two different strains of *E. coli*) and 0.109 mg/ml respectively. Mahboubi et al. (17) studied the antimicrobial properties of *T. ammi* EO against several microorganisms using the broth microdilution method and found that the MIC value for *S. aureus*, *E. coli* and *S. typhimurium* was 1 µl/ml, 0.5 µl/ml, and 1 µl/ml, respectively. Paul et al. (18) in a study on the permeability of EO and various extracts of *T. ammi* EO to the cell membrane in inhibiting foodborne pathogenic bacteria, showed the MIC of *T. ammi* EO for *S. aureus*, *E. coli*, and *S. typhimurium* was 162.5- 175 µl/ml, 450- 462.5 µl/ml, and 225 µl/ml, respectively. The MIC values of *T. ammi* EO for *E. coli*, *S. aureus,* and *S. typhimurium* reported by Guderzi et al. (19) was 0.031%, 0.31 0.0%, and 0.015%, respectively. Furthermore, in the present study, the antimicrobial effect of *L. officinalis* EO on the studied bacteria was determined, and the MIC value calculated for *E. coli* and *S. typhimurium* was <4000 ppm, 1000 ppm for *L. monocytogenes* and *B. cereus*, and 2000 ppm for *S. aureus*. Similarly, Rota et al. (20) reported the antimicrobial properties of several essential oils obtained from aromatic plants, including *L. officinalis*, against food pathogens such as *S. aureus*, *L. monocytogenes*, *E. coli*, and *S. typhimurium* using the tube dilution method. Bayob et al. (21) investigated the antibacterial effects of 13 ethanol extracts, including *L. officinalis*, on *L. monocytogenes*, finding a MIC of 11.5 mg/mL. Their research supports the potential use of these plants and their components as food preservatives to inhibit the growth of foodborne bacteria and extend the shelf-life of processed foods. Hamer et al. (22) assessed the antimicrobial activity of EOs and extracts from 52 plants, including *L.*

officinalis, and reported the MIC values of 0.5% for *S. aureus*, >2% for S. typhimurium, and 0.25% for *E. coli*. Lu et al. (23) investigated the effects of *L. officinalis* and nine other EOs on *E. coli*, *B. cereus*, *S. aureus*, and S. *cerevisiae* through the agar disk diffusion method, demonstrating a moderate antimicrobial effect. The discrepancies in MIC levels between the current research and those reported in other studies for *T. ammi* and *L. officinalis* EOs may be attributed to variations in the chemical composition of the essential oils. These differences could be due to the geographic location and harvesting season of the plants, the extraction method of the essential oil, and the type and strain of the microorganism, as well as the methodology used to determine the antimicrobial effect. Moreover, the results of this study showed that the effect of the studied EOs on gram positive bacteria is greater than on gram negative bacteria. This effect difference has also been proven in previous studies (24). The reason for this difference is the presence of an outer membrane around the cell wall in gram-negative bacteria, which limits the penetration and diffusion of hydrophobic compounds in the lipopolysaccharide coating of the bacteria (25). Also, in this study, *L. monocytogenes* was found to be the most sensitive and *S. typhimurium* the most resistant among the studied bacteria. It is stated that the use of high concentrations of EOs causes adverse effects on the taste of food and the use of a preservative with high amounts is not economical. Therefore, it is suggested that some preservatives should be used in small amounts, in combinations (26). So far, various studies have been conducted on the combined effect of EOs and plant extracts on important foodborne pathogens (27-30). Gutierrez et al. (31) in a study investigated the combined effect of marjoram, thyme and marjoram essential oils on different bacteria and reported their additive effect in the presence of the combination. In this study, the type of combined effect of the studied essential oils on different bacteria was different, and this difference has been proven in previous studies. For example, it was reported in the research conducted by Fu et al. (32) on the combined effect of clove and rosemary EOs. Depending on the type of microorganism, the combination of these two EOs had an additive, synergistic or antagonistic effect. In the present study, the lower concentrations of EO were used in combination which indicates the synergistic or additive effect of these two EOs. Also, the results of the growth curve study show that the combination of these two EOs increased the lag phase, an effect that has been seen in other studies. In a study conducted by Valero and Salmeron (33) on the antimicrobial activity of 11 essential oils, including mint, oregano, thyme, cinnamon, rosemary, etc., against B. cereus INRA L2104, it was found that thyme and oregano EO prolonged the lag phase of bacterial growth.

5. Conclusion

In conclusion, the current study elucidated the antibacterial activities of *T. ammi* and *L. officinalis* EOs individually and in combination against important foodborne bacteria. The combined application of sub-inhibitory levels achieved increased antimicrobial effects compared to when applied alone, demonstrating a synergistic interaction. Growth curve analysis confirmed the inhibitory effects on the lag and logarithmic phases. Thus, these results emphasize the natural preservative potential of appropriately combined EOs, which could find application in minimally processed or fresh food matrices as alternatives to chemical preservatives. Further research evaluating their impact on sensory and functional qualities of foods is warranted to establish their practical usefulness. Overall, the study highlights the importance of exploring synergistic interactions between safe natural antimicrobial agents in the development of innovative food preservation strategies utilizing hurdle technology principles.

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