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Synergistic antibacterial effects of *Trachyspermum ammi* L. essential oil and sodium nitrite in combination on artificially inoculated food models

T. Malik[a,✉], O. Sarkar[b] and S. Pant[a]

[a]Department of Microbiology, Dolphin PG Institute of Biomedical & Natural Sciences, Dehradun, India.
[b]Department of Nursing, DSMS Group of Institutions, Durgapur, West Bengal, India.
[✉]Corresponding author: triptimalikahuja@gmail.com

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SUMMARY: The addition of carminative essential oils could be an approach for food preservation and would minimize or substitute chemical preservatives. In the present study, essential oils (n=11) namely, *Anethum sowa*, *Cinnamomum zeylanicum, Citrus bergamia*, *Cymbopogon flexuosus*, *Cymbopogon martini*, *Cymbopogon winterianus*, *Elettaria cardamomum*, *Mentha arvensis*, *Ocimum basilicum, Salvia sclarea* and *Trachyspermum ammi,* were screened against *Aeromonas hydrophila* and *Listeria monocytogenes*. The largest diameters of zone of inhibition, 19.9 ± 0.33 mm and 21.7 ± 0.58 mm, were exhibited by *T. ammi* essential oil *against Aeromonas hydrophila* and *Listeria monocytogenes,* respectively. Growth inhibition studies for *T. ammi* essential oil, sodium nitrite and their combinations were also carried out on cucumber, apple, gram flour soup and mutton broth models. The combination of *T. ammi* essential oil and sodium nitrite depicted synergism and was also effective in reducing the bacterial counts in artificially inoculated food systems.

KEYWORDS**:** *Chemical preservatives; Essential oil; Food-borne pathogens; Synergism; Trachyspermum ammi.*

RESUMEN: ***Efectos antibacterianos sinérgicos del aceite esencial de* Trachyspermum ammi *L. en combinación con nitrito de sodio empleando modelos de alimentos inoculados artificialmente.*** La adición de aceites esenciales carminativos puede tener una posible aplicación en la conservación de alimentos que podría minimizar o sustituir los conservantes químicos. En el presente estudio, los aceites esenciales (n=11): *Anethum sowa*, *Cinnamomum zeylanicum*, *Citrus bergamia*, *Cymbopogon flexuosus*, *Cymbopogon martini*, *Cymbopogon winterianus*, *Elettaria cardamomum*, *Mentha arvensis*, *Ocimum basilicum*, *Salvia sclarea* y *Trachyspermum ammi* se ensayaron frente a *Aeromonas hydrophila* y *Listeria monocytogenes*. El diámetro mayor de la zona de inhibición i.e 19,9 ± 0,33 mm y 21,7 ± 0,58 mm se mostraron para el aceite esencial de *T. ammi* frente a *Aeromonas hydrophila* y *Listeria monocytogenes* respectivamente. También se realizaron estudios de inhibición del crecimiento para el aceite esencial de *T. ammi*, con nitrito de sodio y sus combinaciones en modelos de caldo de pepino, manzana, sopa de harina de garbanzos y caldo de cordero. La combinación de aceite esencial de *T. ammi* y nitrito de sodio mostró sinergismo y también fue eficaz para reducir la proliferación de bacterias en sistemas alimentarios inoculados artificialmente.

PALABRAS CLAVE: *Conservantes químicos; Aceite esencial; Patógenos transmitidos por los alimentos; Sinergismo; Trachyspermum ammi.*

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1. INTRODUCTION

Food-borne illness and food spoilage encompass major public health problems globally but are more common in low and middle-income countries (Adley and Ryan, 2016). Contamination in foods with pathogenic and toxico-genic microorganisms causes food-borne diseases, resulting in 600 million cases of foodborne diseases and 420,000 deaths worldwide every year. *Campylobacter, Clostridium, Salmonella*, *Escherichia coli*, *Shigella*, *Cyclospora*, *Yersinia*, *and Vibrio* are the major etiological agents (Lee and Yoon, 2021). Traditionally, chemical preservatives are used for extending the shelf life and preventing the microbial deterioration of food. However, consumers are now being health conscious, and looking for alternative eco-friendly methods of food preservation (Sambu *et al.,* 2022). The development and application of natural products with both antioxidants and antibacterial activities in different food products can be an approach to prolonging their storage shelf life and potential for preventing food diseases. Essential oils (EOs), and their volatile compounds, have been used in the food industry for flavoring, in the cosmetic industry for fragrance, and the pharmaceutical industry for their functional properties (Malik, 2017; Malik and Rawat, 2021). The antimicrobial potential of these fragrant oils has been well proven against foodborne pathogens, and has been explained due to their volatile and bio-active properties (Malik, 2017; Wickramanayake *et al.*, 2023).

Anethum Sowa EO and its emulsion in water have been used in ayurvedic medicines for the treatment of gastrointestinal disorders, and is an important ingredient in gripe water, which is usually given to infants (Saleh-e-In *et al*., 2017). *Cinnamomum zeylanicum* EO contains cinnamaldehyde and *trans*-cinnamaldehyde (Cin) as active constituents, has been used in food items, perfumes, and various medicinal products (Rao and Gan, 2014). *Citrus bergamia* Risso et Poiteau (Bergamot) EO has a combination of bitter aromatic taste and a pleasant odor and has shown physiological, anti-inflammatory, and anti-cancerous properties (Navarra *et al.*, 2015). *C. flexuosus* (lemongrass), *C. martini* (ginger grass or palmarosa), and *C. winterianus* (citronella) are aromatic grasses of the genus *Cymbopogon.* The EO from different *Cymbopogon species* has medicinal and pharmacological significance in addition to its fragrant and cosmetic properties (Ganjewala and Gupta, 2016). Due to its characteristic gastronomic value and flavoring properties, *Elettaria cardamomum* EO has nutritional and pharmacological applications (Anwar *et al*.., 2016). *Mentha arvensis* EO has menthol as an active phytochemical, has been traditionally used for food seasoning, and also has antiseptic, carminative, refrigerant, stimulant, and diuretic properties (Thawkar *et al.*, 2016). The popular culinary herb *Ocimum basilicum* EO has antimicrobial, antifungal, insect-repelling, anticonvulsant, hypnotic, and antioxidant properties (Joshi, 2014; Rathore *et al*., 2023). *Salvia sclarea* L. (clary sage) EO has anti-inflammatory, antimicrobial, and cytotoxic properties, used for the treatment of stress, asthma, digestive and menstrual problems (Aćimović, *et al*., 2018). *Trachyspermum ammi* (ajwain) EO has diverse pharmacological activities, is used as a food flavoring, preservative, and remedial agent for gastrointestinal disorders (Vitali *et al*., 2016). Hence, various EOs have been used in food products for flavor enhancement and their antioxidant activity, whilst their antimicrobial potential for application as preservatives has been less explored. There is also a paucity of literature on studies on the antimicrobial activity of EOs in food systems. Therefore, in the present study, the antimicrobial potential of some food-grade EOs was investigated in comparison as well as in combination with the chemical preservatives against food-borne pathogens. Furthermore, the combination of the most effective essential oil and sodium nitrite was investigated for its possible synergistic antimicrobial potential.

2. MATERIALS AND METHODS

2.1. Microbial cultures

The following bacterial cultures were procured from the Microbial type culture collection, in Chandigarh, India:

I. *Listeria monocytogenes* MTCC 657.
II. *Aeromonas hydrophila* MTCC1739.

Bacterial cultures were kept on Luria Bertani agar and preserved at 4 °C in the refrigerator. The cultures were sub-cultured routinely once a month.

2.2. Essential oils

The essential oils (EOs) used were as follows:

i. Anethum sowa Roxb. ex Fleming (Dill seed)
ii. *Cinnamomum zeylanicum Linn.* (Cinnamon)
iii. *Citrus bergamia* Risso (Bergamot)

iv. *Cymbopogon flexuosus* (Nees ex Steud) Wats. (Lemongrass)
v. *Cymbopogon martini (*Roxb.) Will. Watson) (Ginger Grass)
vi. *Cymbopogon winterianus* Jowitt (Citronella)
vii. *Elettaria cardamomum* Maton (Cardamom)
viii. *Mentha arvensis* L. (Mint)
ix. *Ocimum basilicum* L. (Basil)
x. *Salvia sclarea* L. (Clary sage)
xi. *Trachyspermum ammi* L. (Ajwain)

EOs were of food grade, purchased from Sai Export India, Kannauj, India. The stock solution of EOs was prepared by using 10% Dimethyl sulphoxide (DMSO, HiMedia, India) and Tween-80 (0.005%, HiMedia, India), stored in McCartney vials, in the dark, below 25 °C.

2.3. Screening of antimicrobial activity of essential oils against bacteria according to the disc-diffusion method

For the preparation of the bacterial inoculum, two to three colonies of bacterial test organism were inoculated in Muller Hinton Broth (HiMedia, India) and incubated at 4 °C. The broth was incubated until the turbidity of the broth reached 0.5 McFarland (≈10^6 cfu/ml). The test culture was spread uniformly on a Muller Hinton Agar (HiMedia, India) plate using a sterile spreader. The plates were dried for 15 minutes. Three sterile discs (HiMedia, 6mm) were placed on each plate at equal distances. With the help of a micropipette, 10 µl of a particular EO were dropped onto the sterile discs. 10% DMSO was used as control.

The plates were incubated at 37 °C for 24 h. After the incubation, the diameter of the zone of inhibition (in mm) was measured using an 'Antibiotic Zone Scale' (HiMedia) caliper. Three readings were noted, finally calculated as average ± standard error (SE). An inhibition zone of 14 mm or greater was considered as high antibacterial activity (Prabuseenivasan *et al.*, 2006; Malik and Singh, 2010; Malik and Singh, 2015). A chloramphenicol disc (30 mcg, HiMedia) was taken as a positive control.

2.4. Assessment of minimum inhibitory concentration and minimum bactericidal concentration of essential oils and chemical preservatives

Dilution susceptibility testing was carried out for the determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). In brief, Muller Hinton Broth (HiMedia, India) and a bacterial inoculum were added to the wells of the microtiter plate (Greiner Bio-one, Germany). Subsequently, EO was diluted (from 8 to 0.01562% v/v). EO was diluted in series in the wells of the microtiter plates, and they were incubated at 37 °C for 18 h. After incubation, 20 µl of resazurin dye (HiMedia) were added to every well. The microtiter plates were incubated in the dark at 37 °C for 2 h, and the color was noted. The appearance of pink/violet color or colorless liquid indicated the inhibition of bacteria in the medium. The lowest concentration showing blue color in the wells was considered to be MIC. For the determination of Minimum Bactericidal Concentration (MBC), subculturing was performed, and the lowest concentration, in which growth was absent, was noted as the MBC value of that particular oil (Malik and Singh, 2010; Malik and Singh, 2015; Malik *et al*., 2015). Similarly, the MIC and MBC of chemical preservatives [sodium nitrite, sodium benzoate, and potassium sorbate (HiMedia, India)] were also determined.

2.5. Evaluation of the combined antimicrobial effect of essential oil and chemical preservatives

The EO and chemical preservatives showing the lowest MIC were selected for the evaluation of their combined antimicrobial effect. For the combination studies, the checkerboard method was adopted. In brief, dilutions (MIC, MIC/2, MIC/4 and MIC/8) of both EO and chemical preservatives were prepared following the same broth microdilution method which was also adopted to assess the MIC of these individual antimicrobial agents.

The analysis of the combination was obtained by calculating the Fractional Inhibitory Concentration (FIC) index in the following steps:

i. FIC EO = MIC of essential oil in combination/ MIC of essential oil alone
ii. FIC chemical =MIC of chemical in combination/ MIC of chemical alone
iii. FIC index (FICI) = FIC EO + FIC chemical

The interpretation of the FIC index is as follows:

i. A synergistic effect when ≤ 0.5,
ii. An additive or indifferent effect when › 0.5 and ‹ 1 and
iii. An antagonistic effect when › 1 (Malik *et al*., 2011; Karaca *et al*., 2020)

2.6. Inhibition studies in cucumber, banana, gram-flour, and mutton broth food models

A cucumber model media was prepared by taking two to three medium-sized fresh cucumbers which were washed with distilled water. The surface sterilization was done by soaking the cucumbers in 1.5% sodium hypochlorite for 20 min followed by 70% ethyl alcohol for 30 s and then allowed to air-dry inside a laminar airflow cabinet on sterile paper towels. The cucumbers were then peeled and chopped into fine pieces using a clean knife. Sterile de-ionized water (1:2; w/v) was added to the chopped cucumber and it was ground in a mixer for 2 to 3 minutes to make a smooth suspension. The suspension was filtered using muslin cloth; and the pH of the cucumber juice was adjusted to 5.5 using a pH meter (Eutech Instruments, India). 50 ml of the filtered juice were dispensed in a conical flask, then autoclaved at 121°C for 20 minutes. The Banana model was prepared by taking two to three ripe bananas, peeling, cleaning, and surface sterilizing them in the same manner as described above. The banana pulp was aseptically homogenized with sterile distilled water (1: 2, w/v), and the pH was adjusted to 4.7. 50 ml of the banana pulp were transferred to a sterilized 250 ml flask and autoclaved as described above. The gram flour (chickpea) soup model (10% w/v) was prepared by using the flour purchased from a local shop; the pH was adjusted to 5.6 using a pH meter. 50 ml of the soup were dispensed into a sterilized 250 ml flask and autoclaved. For preparing the meat broth model, mutton meat steaks were purchased from a local meat shop, external fat was removed and the meat was cut into pieces of uniform sizes (3 x 3 x 3 cm). The mutton pieces were boiled in distilled water (90 °C /20 min), the resulting broth was filtered using the Whatman filter paper no. 1 (HiMedia), and the filtrate was sterilized by autoclaving (121 °C/20 min) and stored in aliquots at -20 °C.

In all the food models, EO/chemical preservatives were added at different concentrations (MIC, 2MIC) and inoculated with 10^3 cells of the test bacterial culture, and incubated at 37 °C for 24 hours. The bacterial counts were determined at regular intervals and expressed as log cfu/ml (Gutiérrez *et al*., 2009; Catherine *et al.,* 2012).

2.7. Statistical analysis

All the experiments were carried out in triplicate. Using SPSS software package version 16.0, the results of the zone of inhibition were analyzed using analysis of variance (one-way ANOVA) followed by post hoc Tukey's test. The significance was checked at three p-levels, 0.01, 0.1 and 0.5 levels**.** The results of inhibition studies obtained in model food experiments were compared with the control by t-test, at both 0.1 and 0.5 levels.

3. RESULTS

3.1. Screening of antimicrobial activity of essential oils against bacteria

The screening of antimicrobial activity by disc diffusion methods showed the inhibition of bacteria in different magnitudes. All the EOs under study showed inhibition, although the highest inhibition was observed by *C. winterianus* EO against both *A. hydrophila* and *L. monocytogenes*. Both the bacteria *Aeromonas hydrophila* and *Listeria monocytogenes* were found to be sensitive to chloramphenicol (Table 1).

Table 1. Inhibition of bacteria by different essential oils (mean ± SE, in mm)

Diameter of zone of inhibition (mean ± SE, in mm)[a]		
	Aeromonas hydrophila	**Listeria monocytogenes**
Anethum Sowa	12.10^{M1}±0.33	13.90^{M1}±0.60
Cinnamomum zeylanicum	14.56^{M1}±0.60	15.80^{M1}±0.40
Citrus bergamia	16.56^{M1}±0.58	17.90^{M1}±0.33
Cymbopogon flexuosus	18.80^{M2}±0.15	19.40^{M2}±0.70
Cymbopogon martinii	18.70^{M2}±0.58	19.35^{M2}±0.58
Cymbopogon winterianus	17.33^{M1}±0.33	17.56^{M1}±0.33
Elettaria cardamomum	15.05^{M1}±0.58	16.70^{M1}±0.6
Mentha arvensis	17.16^{M1}±0.18	18.20^{M2}±0.58
Ocimum basilicum	15.10^{M1}±0.61	16.87^{M1}±0.44
Salvia sclarea	15.25^{M1}±0.58	16.25^{M1}±0.70
Trachyspermum ammi	19.90^{M2}±0.33	21.70^{M1}±0.58
Chloramphenicol	21.25±0.23	23.34±0.55
DMSO	nz	nz

[a] Values are given in mm with SE, each value represents the average of 3 readings.
The mean comparisons for different essential oils and chloramphenicol 30 mcg (control) were performed by one-way ANOVA followed by Tukey's HSD post-hoc multiple comparison tests. ^{M1} ($p < 0.05$), ^{M2} ($p < 0.01$). nz: no zone of inhibition. The experiment was performed in triplicate.

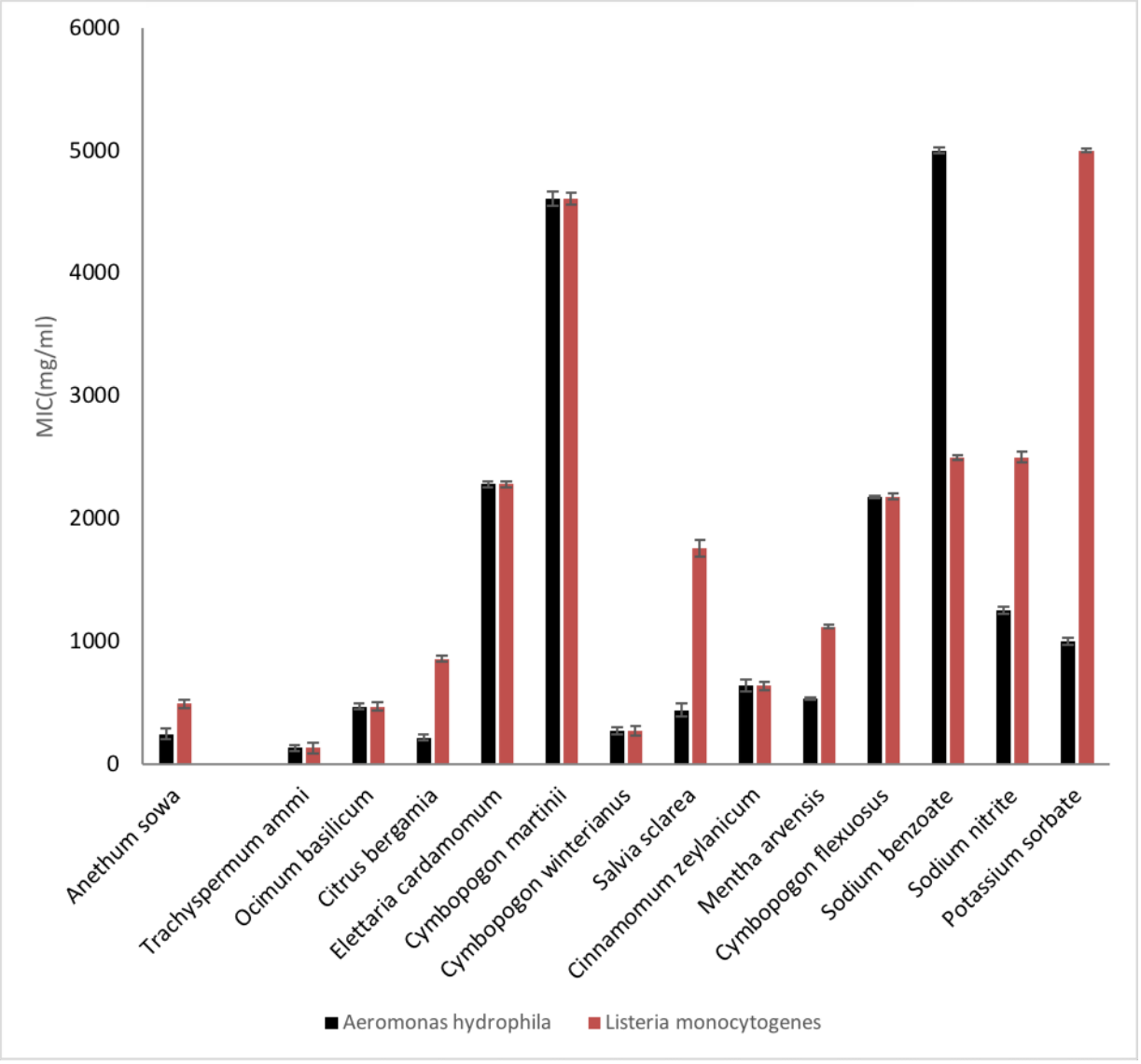

Figure 1. Minimum Inhibitory Concentration (MIC) of essential oils and chemical preservatives against *Aeromonas hydrophila* and *Listeria monocytogenes.*
*The values of Minimum Inhibitory concentration (MIC) are expressed in mg/ml. The bar represents average of three readings ± SE. The experiment was performed in triplicate.

3.2. Dilution susceptibility testing of essential oils and chemical preservatives

The MIC value (13 mg/ml) was found to be the lowest for EO against both *Aeromonas hydrophila* and Listeria *monocytogenes*. Among the chemical preservatives, sodium nitrite showed the lowest MIC as well as MBC (125 and 250 mg/ml) against both *Aeromonas hydrophila* and *Listeria monocytogenes* (Figure 1, sheet 4). For the chemical preservatives, the lowest MIC (125 mg/ml) and the lowest MBC (250 mg/ml) were determined for sodium nitrite against both *Aeromonas hydrophila* and *Listeria monocytogenes* (Figure 2).

3.3. Combined antimicrobial effect of *Trachyspermum ammi* EO oil and sodium nitrite

The results of the combined studies (*T. ammi* EO and sodium nitrite) depicted the values of FICI to be 0.50 and 0.375 against *A. hydrophila* and *L. monocytogenes,* respectively. Both values for FICI were found to be ≤ 0.5, which indicated synergism between the two tested agents (Table 2).

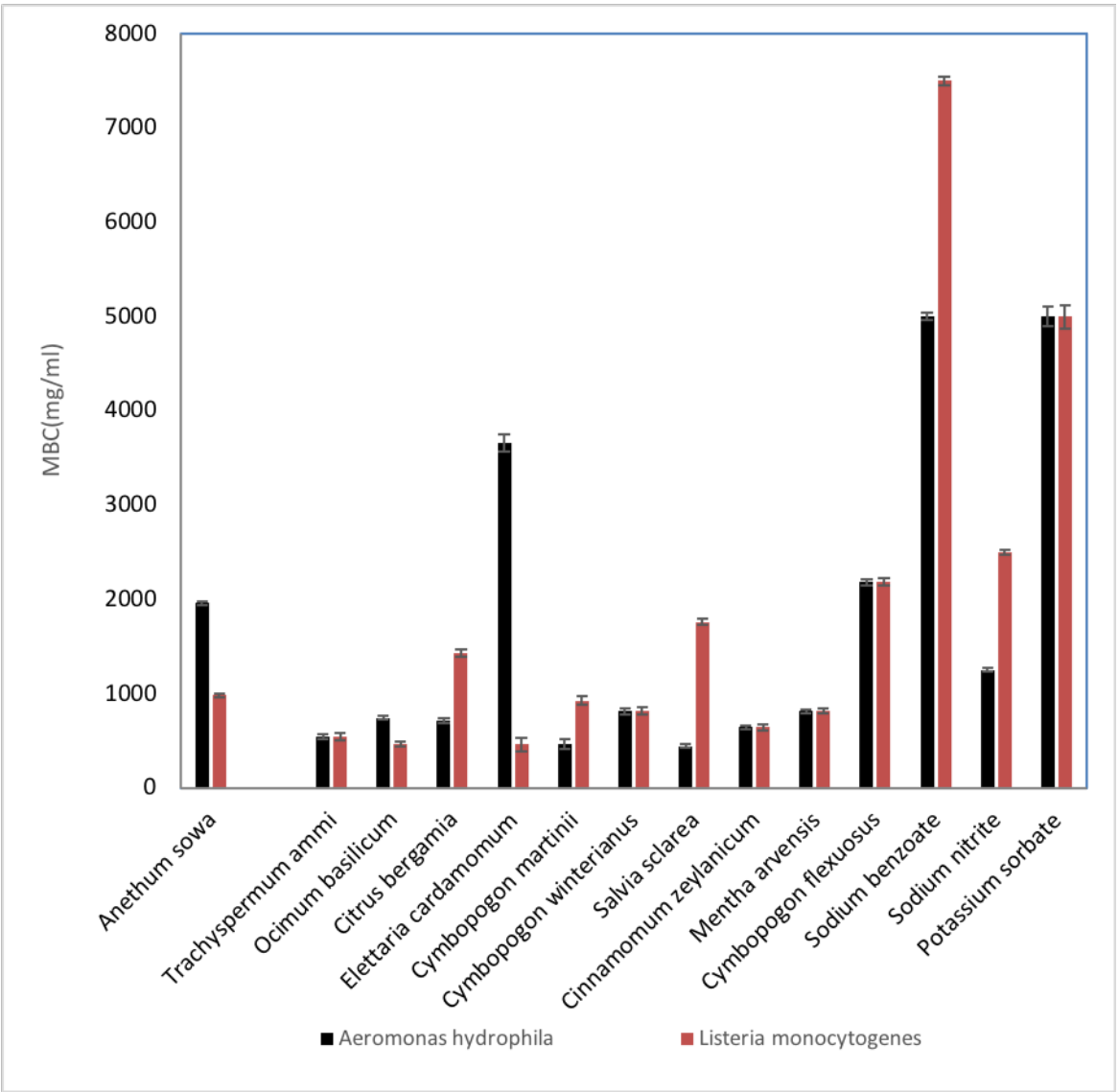

Figure 2. Minimum Bactericidal Concentration (MBC) of essential oils and chemical preservatives against *Aeromonas hydrophila* and *Listeria monocytogenes.*
*The values of Minimum Bactericidal Concentration (MBC) are expressed in mg/ml. The bar represents average of three readings ± SE. The experiment was performed in triplicate.

Table 2. FIC indices of T**.** *ammi* EO and sodium nitrite

Microorganism	MICT.a EO (mg/ml)	FICT.a EO (mg/ml)	MIC s.n. (mg/ml)	FIC s.n. (µg/ml)	FICI
A. hydrophila	1300	0.25	1250	0.25	0.50
L. monocytogenes	1300	0.25	2500	0.25	0.50

FICI ≤ 0.5, synergistic effect
MICT.a EO: Minimum Inhibitory Concentration of *Trachyspermum ammi* essential oil; FICT.a EO**:** Fractional Inhibitory Concentration of *Trachyspermum ammi* essential oil; MIC s.n.: Minimum Inhibitory Concentration of sodium nitrite
FICT.a EO**:** Fractional Inhibitory Concentration of *Trachyspermum ammi* essential oil; FICI: Fractional Inhibitory Concentration Index.
FIC and FICI are reported as means of three replicates.

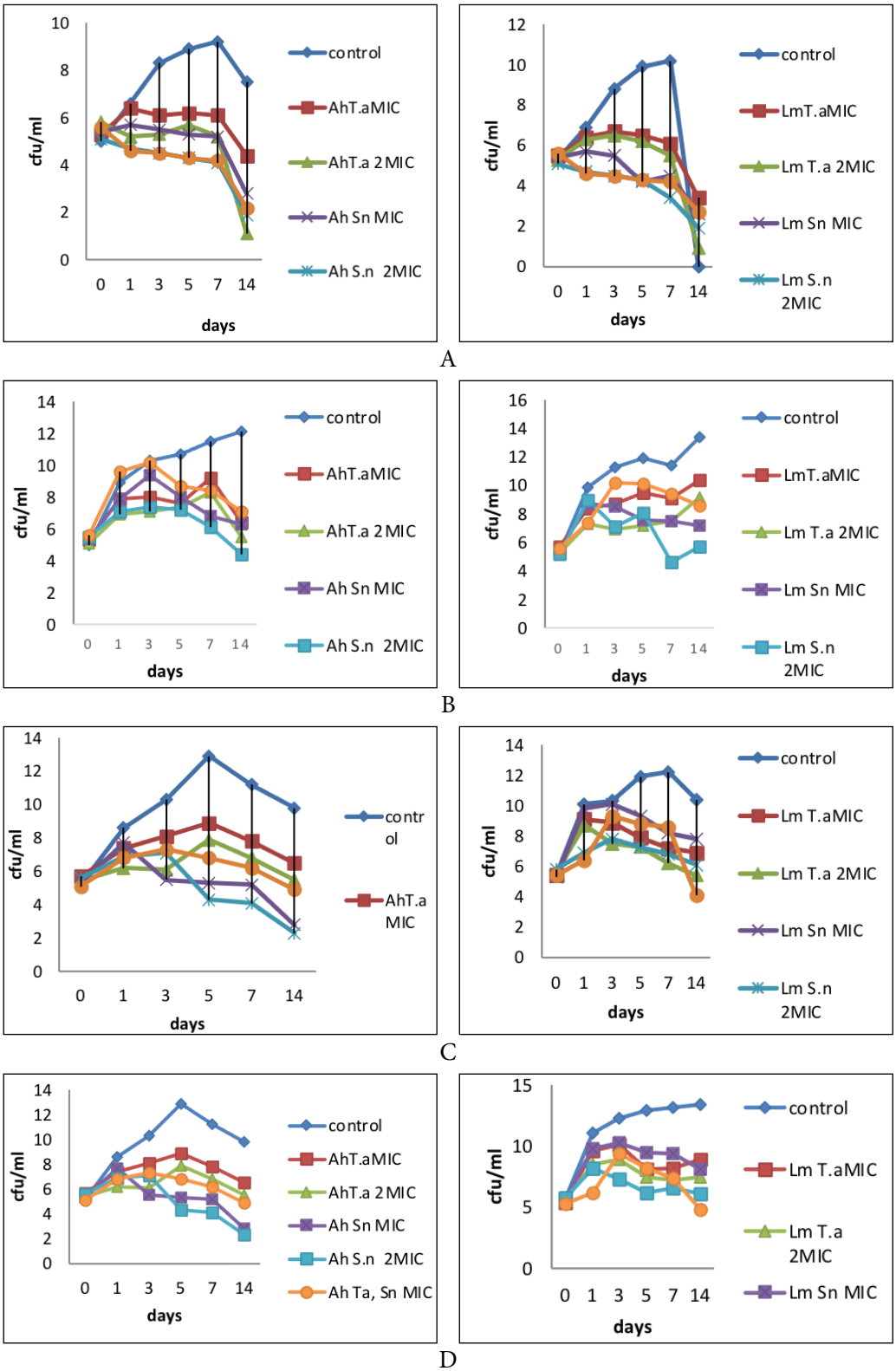

Figure 3. Effect of different concentrations (MIC and 2MIC) of *Trachyspermum ammi* essential oil (T.a) and sodium nitrite (Sn) on the populations (log_{10}cfu/ml) of *Aeromonas hydrophila* (Ah) and *Listeria monocytogenes* (Lm) in (A) Cucumber model (B) Banana model (C) Gram flour soup model (D) Mutton broth model

MIC: Minimal inhibitory concentration; 2MIC: Twice of Minimal inhibitory concentration; T.a: *Trachyspermum ammi* essential oil; Sn-Sodium nitrite; cfu/ml: colony forming units/ml; Ah: *Aeromonas hydrophila*; Lm: *Listeria monocytogenes*

3.4. Inhibitory effects of *T. ammi* EO and sodium nitrite in food models

Both *T. ammi* EO and sodium nitrite were effective in reducing the bacterial counts when added alone in the food-based broth media. However, a higher and more significant reduction in the bacterial counts was observed when *T. ammi* EO and sodium nitrite were added at twice the inhibitory concentrations ($p < 0.01$). A longer lag period was also observed in the presence of antimicrobial agents throughout the storage period as compared to the control ($p < 0.01$) (Figures 3 and 4).

4. DISCUSSION

Aeromonas hydrophila and *Listeria monocytogenes* are potential causes of food-borne illness which are commonly found in refrigerated ready-to-eat food items such as meats, poultry, and fresh produce (Callejón *et al.,* 2015). The highest values for the zone of inhibition (ZOI) and the lowest values for minimal inhibitory and bactericidal concentrations (MIC and MBC) of *T. ammi* EO were determined against the both-tested food-borne bacteria. Hence, it has been conferred to be most efficacious among the different food-grade essential oils under study, which could be corroborated in previous works (Vitali *et al*., 2016).

Nitrates are commonly used as food preservatives and colorants in processed meats and cheese but are carcinogenic (Chazelas *et al.*, 2022); hence should be reduced or replaced. The antimicrobial action of *T. ammi* essential oil has been previously reported to be due to the combined synergistic effects of its major constituents viz., thymol, nonadecane, and carvacrol (Moein et al. 2015). A synergistic effect of *T. ammi* essential oil and sodium nitrite based on the FIC index has been proven in combinatorial studies, which suggests the combination could be an alternative food preservatives or colorants.

The determination of the antimicrobial activity of most of the essential oils in previous studies has usually been carried out in synthetic growth media. The efficacy of EOs as preservatives could vary due to the presence of fats, carbohydrates, proteins, salt, and pH (Witkowska *et al.*, 2014). Hence it would be more relevant to conduct anti-microbial studies on real food models, where parameters such as nutrient availability, composition, and ionic environment could be mimicked (Al-Maqtari *et al*., 2021). In the present pioneer work, the cucumber, banana, gram-flour and mutton broth food models employed may assist in optimizing the final application of EOs. However, the synergistic antibacterial effect of EOs and chemical preservatives has been reported in few previous studies.

The efficacy of peppermint oil and eugenol reduced bacterial counts in cabbage and barley food systems as well as in a papaya pulp model (Catherine *et al*., 2012). In meat broth and a meat model, the combination of *Origanum vulgare* L. essential oil and lactic acid acted synergistically against *Staphylococcus aureus* (Barros *et al.*, 2012). In beef fillets, the combination of sodium nitrite (100 mg/kg) and essential oils *Satureja bachtiarica* (1.1 %v/w) inhibited the growth of *Clostridium* species, anaerobic bacteria responsible for other fatal food infections (Bakhtiary *et al*., 2018). Combinations of lemon balm essential oil with thyme essential oil yielded additive activity against *Listeria* strains in food model media based on lettuce, meat, and milk (Gutiérrez *et al.*, 2009).

5. CONCLUSIONS

Trachyspermum ammi essential oil proved to be inhibitory against both *Aeromonas hydrophila* and *Listeria monocytogenes,* and can be used for the preservation of foods. The addition of *T. ammi* essential oil in artificially inoculated food (broths made of cucumber, banana, oatmeal, and mutton) showed a reduction in bacterial counts. However, the addition of higher concentrations of *T. ammi* essential oil in complex foods would be required, which may alter their organoleptic characteristics and lead to an undesirable taste. The addition of an effective and minimal concentration of *T. ammi* essential oil in combination with sodium nitrite could be a possible solution. The antimicrobial efficacy in real systems and concurrent sensory analysis of the combination needs to be further investigated.

6. DECLARATION OF COMPETING INTEREST

The authors of this article declare that they have no financial, professional or personal conflicts of interest that could have inappropriately influenced this work.

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