

Research Article

Antimicrobial activity of *Origanum vulgare* and *Cinnamomum verum* essential oils separately and in combination against *Streptococcus iniae*

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Keywords

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Abstract

The study investigates the antimicrobial effect of two herbal essential oils, the leaves of *Origanum vulgare* and the bark of *Cinnamomum verum*, separately and in combination against *S. iniae*. The essential oils were extracted and their chemical composition was analyzed using gas chromatography-mass spectrometry. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the broth microdilution method. The fractional inhibitory concentration (FIC) method was employed to analyze the combined effect of the essential oils. The MIC of *O. vulgare* and *C. verum* against *S. iniae* were 0.976 mg/L and 100 mg/L, respectively, and the MBC value of oregano and cinnamon essential oils against *S. iniae* were 125 mg/L and 6400 mg/L, respectively. The FIC index was 0.625, which shows that the combined performance of the two essential oils is additive. The major compounds in *Origanum* and *Cinnamomum* essential oils were phenol-2-methyl-5 (1-methylethyl) (48.61%), thymol (16.76%) and cinnamaldehyde (64.5%), respectively. Overall, the results demonstrate that both essential oils exhibit antibacterial effects against *S. iniae*. In conclusion, the combined essential oils of *O. vulgare* and *C. verum* may hold promise as a novel antibacterial agent for the potential treatment and control of streptococcosis caused by *S. iniae*.

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Introduction

The most important bacterial disease in aquaculture industry is streptococcosis, known for its septicemic infectious nature (Karsidani *et al.*, 2010). It has been reported in various types of freshwater, saltwater, and brackish water fish and can even infect humans, making it a potential zoonotic disease (Soltani *et al.*, 2005). The most common cause of *streptococcosis* is *S. iniae*, which has emerged as one of the most critical pathogens in recent decades (Kamgar and Ghane, 2012). Conventionally, antibiotics have been the primary approach to control *S. iniae* infections in aquaculture. However, there are many reports of severe antibiotic resistance among aquaculture antibiotics (Caputo *et al.*, 2023; Shin *et al.*, 2023; WU *et al.*, 2023). Furthermore, *S. iniae* has gradually developed resistance to multiple antibiotics, such as kanamycin, neomycin, cephalixin, ampicillin, furazolidone, and sulfamethoxazole (Rattanachaikunsopon and Phumkhachorn, 2010; Zhang *et al.*, 2012). The use of antibiotics to combat fish diseases has drawbacks, including the risk of generating bioaccumulation and environmental pollution; Also, the development of fish vaccines is associated with obstacles such as high prices, time constraints, and a focus on specific pathogens (Ardó *et al.*, 2008; Mondal and Thomas, 2022). Given these challenges, have led to more attention being given to alternatives to antibiotic treatment.

Plant-based therapies have garnered attention due to their established therapeutic efficacy, supported by rigorous scientific investigations (Yang *et al.*, 2020). Essential oils derived from various plants

have shown promising antimicrobial properties, with the ability to inhibit the growth of many microorganisms by affecting their plasma and cell membranes (Álvarez-Martínez *et al.*, 2021).

Cinnamomum verum belongs to the Lauraceae family (Jayaprakasha and Rao, 2011). The genus *Cinnamomum* comprises over 250 recognized aromatic species, predominantly distributed across Asia and Australia (Thomas and Kuruvilla, 2012). Among the various species within the genus *Cinnamomum*, *Cinnamomum zeylanicum* Blume (Ceylon cinnamon; also known as *Cinnamomum verum* J. Presl) and *Cinnamomum cassia* J. Presl (*Cassia cinnamon*) stand out as the most economically significant species. (Barceloux, 2008; Muhammad and Dewettinck, 2017). In recent years, the antibacterial properties of *Cinnamomum verum* essential oil have been proven through many studies (Nematollahi *et al.*, 2020; Wijesinghe *et al.*, 2021; dos Santos Franciscato *et al.*, 2022).

Origanum, a genus within the Lamiaceae family, encompasses over 50 diverse species, with *Origanum vulgare* L. being the most famous. A prominently acknowledged plant extensively employed for medicinal purposes (García-Beltrán and Esteban, 2016; Alekseeva *et al.*, 2020). The essential oil isolated from *Origanum vulgare* L. has been shown to have biological and pharmacological activities such as antibacterial, fungicidal, and antiviral (Coccimiglio *et al.*, 2016; Leyva-López *et al.*, 2017; Vinciguerra *et al.*, 2019; Simirgiotis *et al.*, 2020; Picoli *et al.*, 2021). Moreover, Studies are demonstrating the effectiveness of *Cinnamomum verum* and

Origanum vulgare L. in controlling and preventing bacterial diseases in fish (Mabrok and Wahdan, 2018; Beltrán *et al.*, 2020; Susanti *et al.*, 2021; Alnahass *et al.*, 2023).

The combined antibacterial effects of these essential oils against *S. iniae* have not been previously reported. This study aims to analyze the chemical composition of essential oils from *Cinnamomum verum* bark and *Origanum vulgare* leaves and evaluate their individual and combined antimicrobial effects against *S. iniae*.

Materials and methods

Plant material and isolation of the essential oils

The bark of *Cinnamomum verum* and air-dried leaves of *Origanum vulgare* were purchased from an Herbalist in The Tohid Square in MASHHAD City, IRAN, in March 2023. The species were identified by comparison with voucher specimens kept at the Herbarium of Botany, Faculty of Agriculture, University of Tabriz, IRAN (Code 12906-TBZAH for *Cinnamomum verum* and Code 14567-TBZAH for *Origanum vulgare*). The leaves of *O. vulgare* and *C. verum* bark were submitted separately to a hydrodistillation in a Clevenger-type apparatus for four hours. Then, the essential oil was isolated and kept at four °C until used for antibacterial testing. Figure 1 shows the Clevenger apparatus and extracting essential oils. The extraction of essential oil from *O. vulgare* utilized 600 grams of plant material, resulting in the production of 1 ml of essential oil. Similarly, for *C. verum* bark, 500 grams were used, yielding a notable 3 mL of essential oil.

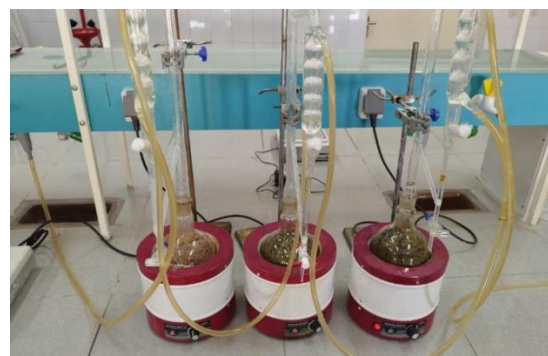


Figure 1: Extracting essential oils with Clevenger apparatus.

This essential oil was used to prepare stock dilution. The stock solution of each plant was prepared by dissolving in dimethyl sulfoxide (DMSO; Sigma-Aldrich). The stock concentration of *C. verum* essential oil was 12800 mg/L, and *O. vulgare* essential oil 2000 mg/L was prepared in DMSO (final concentration of 10%).

(Approvals of the research ethical committee:

IR.IAU.TABRIZ.REC.1402.187, 2023.07.04)

Chemical analysis

Gas chromatograph-mass spectrometry (GC/MS) was used to identify and detect the effective chemical composition in the essential oils of *Cinnamomum verum* and *Origanum vulgare* plants (Gas chromatograph model 7890B and mass spectrometer model 5977A manufactured by Agilent USA, equipped with split/splitless injection system and electron bombardment ionization model and has NIST and WILEY mass libraries). To analyze and identify the desired compounds, the HP5-MS column (60 m × 0.25 mm; film thickness 0.25 µm) was

used. The carrier gas was helium at 1.1 mL/min. The injection, interface, and ionization source temperatures were set to 270, 290, and 230 °C, respectively. The temperature program of the column started with an initial temperature of 70°C and was kept at this temperature for 3 min; then, the temperature of the column reached 180°C with a gradient of 10°C/min and remained constant at this temperature for 2 min. Finally, with a gradient of 20°C/min, it reached a temperature of 290°C and

remained at this temperature for 5 min. The split mode was set as 1:20 and the injection volume was 0.5 µL. The essential oils components were identified by comparison of their retention indices and mass spectra with a NIST/ WILEY mass spectral library. The percentage content was calculated by peak area normalization. Figure 2 shows the GC/MS spectrometer and its injection method.



Figure 2: Gas chromatography/mass spectrometer (right side) and how to inject into it (left side).

Bacterial strain

S. iniae (PTCC 1887) was tested. This strain was obtained from the Persian Type Culture Collection (PTCC¹) a subset of the Biotechnology Department at the Iranian Research Organization for Science and Technology (IROST). It was maintained on Trypticase Soy Agar with 5% defibrinated sheep blood at 30°C and supplied in a freeze-dried form. Figure 3 shows the bacterial strain.



Figure 3: *Streptococcus iniae* bacterial strain.

¹ PTCC is a member of World Federation for Culture Collections (WFCC) and the UNESCO Microbial Resources Centers Network (MIRCEN). The Site Link: <https://irost.org/ptcc/en/>

Antimicrobial sensitivity tests

The minimal inhibitory concentration (MIC) test

The Broth microdilution method was used to determine the minimal inhibitory Concentration (MIC) of *C. verum* and *O. vulgare* essential oils (Sarker *et al.*, 2007; Ahn *et al.*, 2012; Bazargani and Rohloff, 2016).

At first, 100 μ L of Mueller Hinton Broth medium (Scharlau) was added into each well of a 96_well microtiter microplate, then 100 μ L of *C. verum* and *O. vulgare* oils dissolved in DMSO (final concentration of 10%) were added to the first well, and It was diluted two-fold. In the next step, 100 μ L of bacterial suspension equivalent to 0.5 McFarland (OD 600 nm=0.8) was added to each of the wells. Afterward, the plate was covered with parafilm tape to prevent dehydration and incubated at 37°C for 24 hours. After incubation, 30 microliters of resazurin 0.01% (Sigma-Aldrich) were added to each of the wells and incubated for 2 hours at 37°C. After incubation, the last well with the lowest concentration of the plant material, indicated by a blue color, was recorded as the MIC value. This test was repeated three times for each sample. A control series was also considered for each microplate, which includes the positive control: the row in which 100 μ L of ciprofloxacin (1 mL/mg) was added instead of the essential oil and dilution was performed (culture medium+ antibiotic + bacteria); Negative control: the row in which instead of essential oil, 100 μ L of solvent (DMSO 10%) was added in the first well and dilution was done (culture medium + DMSO + bacteria); Controlling the

sterility of plant materials: the row in which 100 μ L of essential oil was added in the first well and dilution was done (culture medium + essential oil); Control of bacterial growth: the row in which 100 μ L of the standard strain of *S. iniae* (PTCC1887) was added in the first well and dilution was done (culture medium + bacteria).

The minimal bactericidal concentration (MBC) test

MBC is the lowest concentration of plant material that can kill 99.9% of inoculated bacteria during 24 hours of incubation at 37°C (Sasidharan *et al.*, 2014). 100 μ L of the bacterial suspension from the MIC well and the wells containing essential oil concentrations higher than the MIC (all blue wells) were removed. Subsequently, the suspension corresponding to each of the mentioned dilutions was inoculated on different Mueller Hinton agar mediums (Ibresco). The plates were examined for bacterial growth after 24 hours of incubation at 37°C. The concentration of the well that killed 99.9% of the bacteria inoculated on the Mueller Hinton agar medium was recorded as MBC. This test was repeated three times for each sample.

The Fractional Inhibitory Concentration (FIC) method

The combined effect of two essential oils, *C. verum* and *O. vulgare*, against *S. iniae* bacteria was investigated and implemented based on the methods described by Pei *et al.* (2009) and Mulyaningsih *et al.* (2010). After determining the MIC of each essential oil separately, concentrations above and below the MIC (usually seven wells along

with the MIC well) were used to determine the FIC.

In this method, the final volume of each microplate well was 200 μ L. Subsequently, 100 μ L of bacteria, previously measured using a spectrophotometer, was added to each well in the first row. Following this, 100 μ L of various concentrations of *C. verum* oil was added to each well in the same row. In the first column, different concentrations of *O. vulgare* were added to each well. This method allowed for the simultaneous determination of the single MIC and combined MIC of each compound. The remaining wells received a mixture of different dilutions of both essential oils in the first column, with 100 μ L of bacteria and 50 μ L of various dilutions of the oils added to each well in the first row. The microplate was then shaken for 30 seconds and incubated for 24 hours at 37°C. Following incubation, 30 μ L

of 0.01% resazurin was added to every well and incubated for an additional 2 hours at 37°C to determine the FIC.

Results

Chemical composition of the essential oils

The chromatograms related to the active ingredients in *C. verum* and *O. vulgare* essential oils are shown in Figures 4 and 5. Table 1 lists the constituents of *C. verum* essential oil in the order of the peaks shown in the chromatogram. According to the table, 17 compounds have been identified. The 'Area sum %' indicates the percentage composition of each compound within the total oil content. Cinnamaldehyde, representing 64.5% of the oil content and corresponding to the highest peak in the chromatogram, emerges as the most prominent compound.

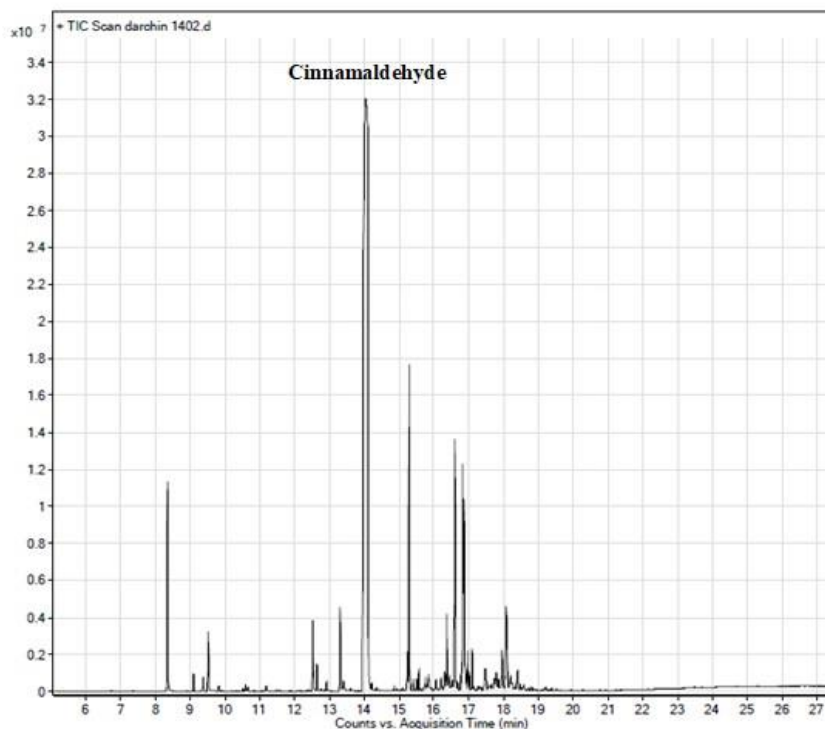


Figure 4: Chromatogram of *Cinnamomum verum* essential oil.

Table 2 lists the constituents of *O. vulgare* essential oil according to peak order in the chromatogram. This table identifies 20 effective compounds. The most prevalent compound in *O. vulgare* oil, as indicated by the percentage composition, is Phenol, 2-

methyl-5 (1-methylethyl), also known as carvacrol, at 48.61%. Thymol follows at 16.76% and constitutes the highest peak in the chromatogram. The molecular structures of these compounds are depicted in Figure 6.

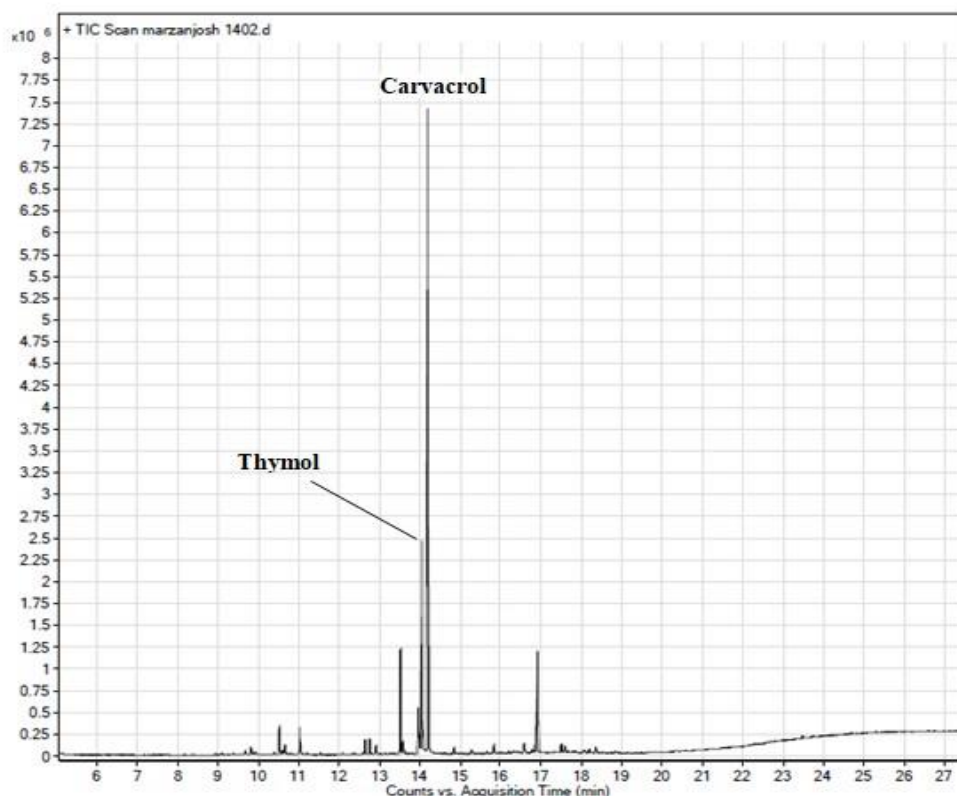


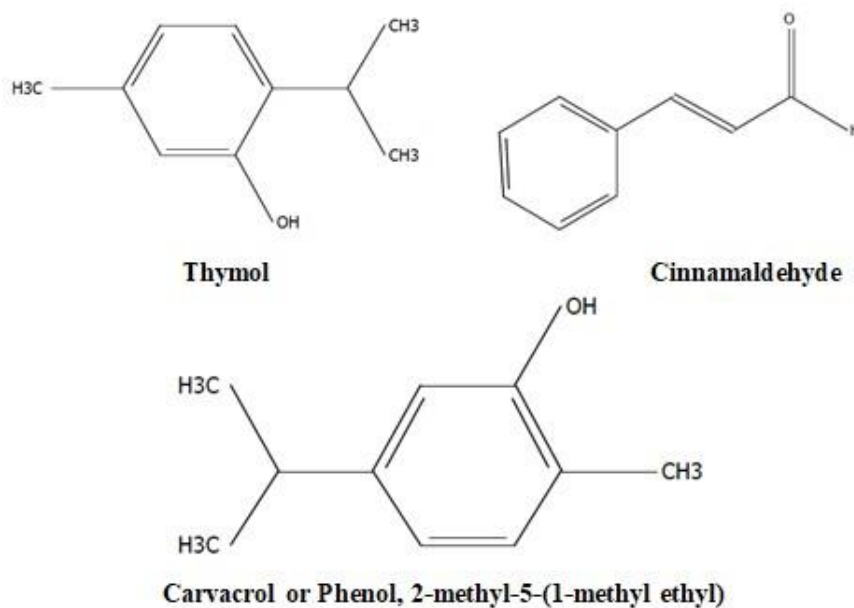
Figure 5: Chromatogram of *Origanum vulgare* essential oil.

Table 1: Compound of *Cinnamomum verum* essential oil.

Compound Label	Area sum %
Styrene	3.89
Benzaldehyde	1.18
Benzenepropanal	1.29
(Z)-3-Phenylacrylaldehyde	1.64
Cinnamaldehyde, (E)-	64.51
1,2,4-Metheno-1H-indene, octahedron-1,7adimethyl- 5-(1-methyl ethyl)-, [1S-(1.alpha.,2.alpha.,3a.beta.,4.alpha.,5.alpha.,7a. beta.,8S*)]-C15H24 10 Cpd 7: .alfa.-Copaene 15.284	0.65
.alfa.-Copaene	5.62
.gamma.-Muurolene	1.3
.alpha.-Muurolene	5.35
Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7- dimethyl-1-(1-methyl ethyl)-, (1S-cis)-	3.85
cis-Calamenene	4.3
.alpha.-Calacorene	0.68
Caryophyllenyl alcohol	0.67
Di-epi-1,10-cubenol	1.09
.tau.-Muurolol	2.67
.tau.-Cadinol	0.66
Naphthalene, 1,6-dimethyl-4-(1-methylethyl)-	0.66

Table 2: Compound of *Origanum vulgare* essential oil.

Compound Label	Area sum %
1,2:4,5:9,10-Triepoxydecane	0.53
3-Octanone	0.65
o-Cymene	2.13
Eucalyptol	0.7
.gamma.-Terpinene	2.05
Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, (1Sendo)-	1.24
3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-	1.2
.alpha.-Terpineol	0.84
Benzene, 1-methoxy-4-methyl-2-(1-Methylethyl)-	7.83
Pulegone	1.51
Cinnamaldehyde, (E)-	4.69
Thymol	16.76
*Phenol, 2-methyl-5-(1-methyl ethyl)-	48.61
2-Cyclohexen-1-one, 3-methyl-6-(1-methylethylidene)-	0.56
Caryophyllene	0.77
.beta.-Bisabolene	1.04
Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methyl ethyl)-, (1S-cis)-	0.53
Cyclohexene, 4-[(1E)-1,5-dimethyl-1,4-hexadien-1-yl]-1-methyl-	7.14
1H-Cycloprop[e]azulen-7-ol, decahedron-1,1,7-trimethyl-4-methylene-, [1ar-(1a.alpha.,4a.alpha.,7.beta.,7a.beta.,7b.alpha.)]	0.67
.alpha.-Bisabolol	0.53

**Figure 6: Chemical structures of the main antimicrobial identified in *Cinnamomum verum* and *Origanum vulgare*.**

* Carvacrol

MIC and MBC of *Cinnamomum verum* and *Origanum vulgare* essential oils

Based on the results, the MIC level for *C. verum* oil in the *S. iniae* strain was 100 mg/L. Also, the MIC value of *O.*

vulgare oil in the *S. iniae* strain was 0.976 mg/L. The final Figure of MIC adjusted using the broth microdilution method and using resazurin is shown in Figure 7.

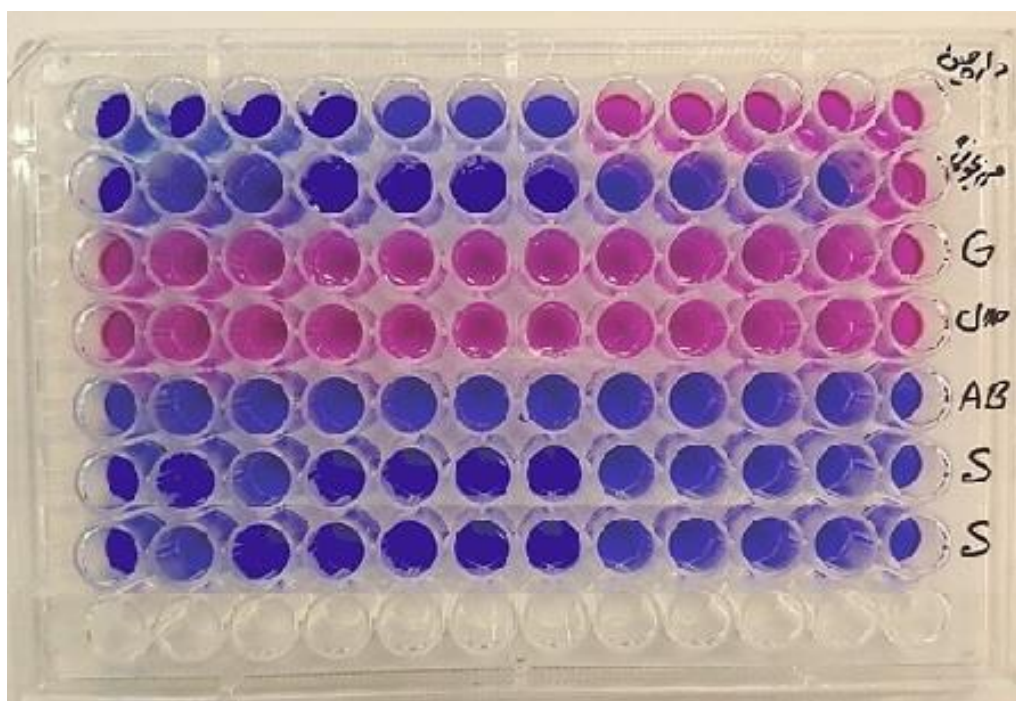


Figure 7: Minimum inhibitory concentration (MIC) test using *Cinnamomum verum* and *Origanum vulgare* essences on the *Streptococcus iniae* strain under study after adding the rejuvenator of resazurin. Row one: *Cinnamomum verum* essential oil, row two: *Origanum vulgare* essential oil, row G: bacterial growth control, fourth row: solvent control or DMSO 10%, row AB: positive control and row S: sterility control of essential oils.

The MBC of *C. verum* and *O. vulgare* essential oil in *S. iniae* was 6400 mg/L and 125 mg/L, respectively (Table 3). The final

photo taken from the cultures carried out to determine the MBC of the studied materials is shown in Figures 8 and 9.

Table 3: Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of *Cinnamomum verum* and *Origanum vulgare* essential oils against *S. iniae*.

Essential oil	MBC (mg/L)	MIC (mg/L)
<i>Cinnamomum verum</i>	6400	100
<i>Origanum vulgare</i>	125	0.976

FIC index results

Table 4 shows the result of evaluating the combined antibacterial properties of *C. verum* and *O. vulgare* essential oils using the Broth microdilution method against *S. iniae*. As shown in the table, the FIC index was 0.625, which shows that the combined

performance of the two essential oils of *C. verum* and *O. vulgare* has an additive effect.

Also, the final Figure taken from the FIC result is shown in Figure 10.



Figure 8: Minimum bactericidal concentration (MBC) test against *Streptococcus iniae* strain by *Cinnamomum verum* essential oil. The numbers inside the plates indicate the number of the well of the microplate containing essential oil and bacteria.



Figure 9: Minimum bactericidal concentration (MBC) test against *Streptococcus iniae* strain by *Cinnamomum verum* essential oil. The numbers inside the plates indicate the number of the well of the microplate containing essential oil and bacteria.

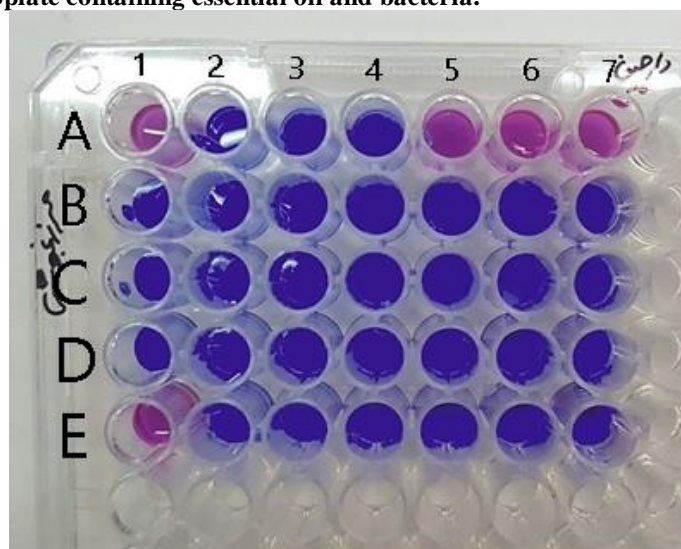


Figure 10: Fractional inhibitory concentration (FIC) index of the combination of *Cinnamomum verum* and *Origanum vulgare* essential oils. Well A1 is used as a growth control.

Discussion

Our study specifies the antimicrobial activity of *Origanum vulgare* and *Cinnamomum verum* oils against *S. iniae*, proving their individual effectiveness through MIC and MBC determinations. These findings align with prior studies (Pathirana *et al.*, 2019; Fakharzadeh *et al.*, 2020). Genetic factors, agro-climate conditions, cultivation methods, soil attributes, and harvesting timing influence the production and quality variations of essential oils. Consequently, these oils' chemical composition and predominant compounds exhibit diversity across different environments. This diversity significantly influences their bioactivity, closely correlating with alterations in their composition (Teles *et al.*, 2019). The MIC of *O. vulgare* oil against *S. iniae* is notably low (0.976 mg/L), indicating its potent inhibitory effect on bacterial growth.

Additionally, the MBC value of 125 mg/L underscored the bactericidal capacity of this essential oil against the target pathogen; in contrast, the efficacy of *C. verum* oil against *S. iniae* exhibited higher MIC and MBC values (100 mg/L and 6400 mg/L, respectively), its inhibitory and bactericidal concentrations were comparatively higher than those of *O. vulgare*. These findings underscore the differing efficacy and suggest varying degrees of potency in inhibiting and eradicating bacterial growth; hence, an interpretation arises suggesting the superior antibacterial efficacy of *O. vulgare* over *C. verum*. The significance of this finding receives emphasis from the Ebani *et al.* (2020) study, where they observed heightened antimicrobial efficacy in *O.*

vulgare compared to *Cinnamomum* against multiple strains of *Staphylococcus* bacteria. The chemical constituents of essential oils play a pivotal role in determining their antibacterial efficacy.

The GC/MS analysis revealed prominent compounds within *C. verum* and *O. vulgare* oils with *Cinnamaldehyde* identified as the primary constituent in *Cinnamomum* (Echegoyen and Nerín, 2015; Procopio *et al.*, 2018). Consistent research findings have established a strong connection between Cinnamaldehyde and its potent antibacterial properties, highlighting its effectiveness against a diverse range of bacterial strains (Silva *et al.*, 2016; Friedman, 2017; Vasconcelos *et al.*, 2018). According to the review article by Doyle and Stephens (2019), Cinnamaldehyde exhibits action by several antibacterial mechanisms: 1- It disrupts cell division by binding to FtsZ proteins, 2- Degrades the extracellular matrix of biofilms, and 3- Interferes with quorum sensing, curtailing coordinated bacterial behaviors. Moreover, 4- It compromises cell membrane integrity, 5- Alters cell morphology, 6- Cinnamaldehyde reduces intracellular ATP levels in bacteria like *E. coli* and *Listeria* (Oussalah *et al.*, 2006), *Salmonella* (Silva *et al.*, 2018), *Mycobacterium avium* subsp. *Paratuberculosis* (Nowotarska *et al.*, 2017) disrupts vital energy processes crucial for their survival. 7- Its inhibition of membrane-bound ATPases disrupts the proton motive force, leading to compromised cellular functions and decreased ATP levels. Collectively, these actions impede bacterial replication and

survival, showcasing the multifaceted antibacterial potential of Cinnamaldehyde. The antimicrobial activity of *O. vulgare* essential oils could be associated with Carvacrol and thymol, the main component of *O. vulgare* oil (Hou *et al.*, 2020). Researchers have shown that Thymol and Carvacrol employ diverse mechanisms to combat bacterial activity. Based on the review article by Kachur and Suntres (2020), 1- Their actions involve disrupting bacterial membranes (It has been proven as a significant mechanism), 2- Inhibiting efflux pumps crucial for resistance, 3- preventing and dismantling biofilms, 4-impeding bacterial motility, and 5- Inhibiting membrane-bound ATPases vital for bacterial function. In essence, Thymol and Carvacrol's diverse antibacterial strategies highlight their potential as effective agents against bacteria.

To the best of our knowledge, this research is the pioneering exploration into the joint effects of essential oils from *C. verum* and *O. vulgare* on *S. iniae*. The combination of essential oil compounds can result in four potential effects: synergistic, additive, indifferent, or antagonistic interactions. Specifically, an additive effect occurs when the combined impact equals the cumulative sum of the individual effects observed (Bassolé and Juliani, 2012). This report has shown that an FIC index of 0.625 indicates an additive effect when the essential oils are combined. In the study of Pouyan *et al.* (2021), a combination of *Cinnamomum camphora* and *O. vulgare* essential oils against bla CTX-M Producing *Escherichia coli* isolated from poultry *colibacillosis* demonstrated an additive effect in 40% of the samples, aligning with findings in our

investigation. In addition, half of the samples demonstrated a synergistic effect, while the rest were ineffective.

Pie *et al.* (2009) examined the antibacterial characteristics of essential oil components against *E. coli*, noting both additive and synergistic effects. Similarly, El Atki *et al.* (2020) showed that mixing *cinnamon* essential oil with Carvacrol and thymol compounds resulted in an additive effect against *E. coli* and *S. aureus* pathogens, a finding supported by our study. Despite the limited number of studies investigating the combined antibacterial mechanism of essential oils, El Atki *et al.* (2020) mentioned that thymol or Carvacrol might increase cell membrane permeability, facilitating the penetration of *cinnamon* compounds into cells and enabling their interaction with proteins and nucleic acids. Furthermore, the interaction could involve the simultaneous disruption of multiple bacterial targets, such as cell division and membrane integrity, and the inhibition of efflux pumps. This coordinated attack on various bacterial processes results in an enhanced antibacterial effect against *S. iniae*.

The observed antimicrobial activities of both essential oils, individually and in combination, against the studied microorganism, advocate for their potential use in treating and preventing *Streptococcus* diseases. However, further investigations into the safety and toxicity of these essential oils are crucial for their clinical evaluation and application in the treatment of infectious diseases.

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Conflicts of interest

The Authors declare that they have no conflict of interest.

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