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Original article

Enhancement of Antibiotic Activity and Reversal of Resistance in Clinically Isolated Methicillin-Resistant *Staphylococcus aureus* by *Trachyspermum ammi* Essential Oil

Mahdi Vazirian[1,2], Khadijeh Hamidian[3], Mehrzad Noorollah[1], Azadeh Manayi[2], Nasrin Samadi[3,4*]

[1]Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.
[2]Medicinal Plants Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.
[3]Department of Drug and Food Control, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.
[4]Pharmaceutical Quality Assurance Research Center, The Institute of Pharmaceutical Sciences, Tehran University of Medical Sciences, Tehran, Iran.

Abstract

Background and objectives: Methicillin-resistant *Staphylococcus aureus* (MRSA) has resulted in a worldwide threat due to the virulence and broad distribution in the hospital and community. Novel antibiotics are required to combat the emergence of multidrug-resistant bacteria such as MRSA. In the present study, the antibacterial activity of *Trachyspermum ammi* essential oil alone and in combination with fifteen antibiotics of different classes against a standard and five clinical strains of MRSAs was investigated. **Methods:** Chemical composition of the essential oil was investigated by using gas chromatography-mass spectrometry (GC-MS). The possible synergistic interaction of several antibiotics in combination with essential oil was screened by disc diffusion method. Interaction of the essential oil and the candidate antibiotic was investigated by checkerboard assay. **Results:** The essential oil was rich in thymol (74.2%), *p*-cymene (16%), and γ-terpinene (7.1%). Combination of sub-inhibitory concentrations of essential oil with vancomycin or gentamicin increased their inhibition zones against MRSA ATCC 33591 and clinically isolated MRSAs. All of the clinically isolated MRSAs were resistant to gentamicin, while combination of gentamicin with the essential oil caused augmentation of the antibacterial activity and 4 to 520-fold decrease in gentamicin minimum inhibitory concentrations was observed against different MRSA strains with fractional inhibitory concentration indices ranging from 0.50 to 0.75. Combination of essential oil with ciprofloxacin or imipenem increased the inhibition zones against some clinically isolated MRSAs. **Conclusion:** Combination of sub-inhibitory concentrations of *T. ammi* essential oil and gentamicin could be considered as a new choice for treatment of infectious diseases caused by MRSA strains.

Keywords: essential oil; gentamicin; MRSA; synergism; *Trachyspermum ammi*

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Introduction

Infections caused by resistant microorganisms involve high morbidity and mortality rates as well as increased treatment costs due to limitation of the effectiveness of existing drugs and treatment failure. One of the most widespread multidrug resistant (MDR) bacteria is methicillin-resistant *Staphylococcus aureus* (MRSA) which causes community-acquired (CA) infections. The incidence of MRSA infection has increased significantly in different

*Corresponding author: samadin@tums.ac.ir

countries. In this respect, control of MRSA infections can be helpful in reducing morbidity, mortality and health care costs [1-6].

Medicinal plants are potential reservoir of several effective antimicrobial molecules and may serve as alternative sources to conventional antibiotic therapies [7-10]. Many researchers have shown that essential oils of some medicinal plants containing active phytochemicals including flavonoids, terpenoids, carotenoids, coumarins and curcumines can exhibit significant antibacterial activity against a wide range of resistant microbial strains [3,11]. Also, synergistic interactions of essential oils with antibiotics might be a new way for treatment of infectious diseases of drug-resistant pathogens [12]. According to the clinical studies, using combination of two or more pharmaceuticals is more effective than a single drug therapy. In antibacterial synergistic interactions, bacterial resistance is partially or completely inhibited [5,13]. There are several reports about synergistic effects of essential oils or plant extracts with antibiotics against different bacteria and fungi. For instance, the essential oil of *Thymus maroccanus* and *Thymus broussonetii* showed synergism with amphotericin B and fluconazole against *Candida albicans* [12]. Combinations of *P. graveolens* oil with norfloxacin was synergistic against *Staphylococcus aureus* and *Bacillus cereus* [8]. Enhanced anti-MRSA activity was found in combination of *Thymus kotschyanus* essential oil with oxacillin or methicillin [14].

Trachyspermum ammi (L.) Sprague ex Turrill (Apiaceae family), commonly known as Ajowan, grows in Afghanistan, Pakistan, India, north of Africa and in the center, south and southeast of Iran [4,11]. Fruit is the most used part of this plant which is small, brown and is popular for its dietary and traditional applications such as antispasmodic, stimulant, tonic, and carminative properties [15]. Antimicrobial and antioxidant activities of its essential oil were demonstrated in previous studies [16]. Thymol, as one of its essential oil phenolic compounds, has been reported as a membrane permeabilizers which facilitate penetration of antibiotics into Gram-negative bacteria [4].

The aim of present study was to determine the chemical composition and antibacterial activity of the fruit essential oil of *T. ammi* alone and in combination with fifteen antibiotics of different classes against MRSA ATCC 33591 and clinically isolated MRSA strains.

Material and Methods

Ethical considerations

This study was approved by the Ethics Committee of the Tehran University of Medical Sciences, Tehran, Iran (ethics committee reference number: IR.TUMS.REC.1394.942, 2015).

Plant material and isolation of the volatile oil

The fruits of *T. ammi* were identified by Professor Gh. Amin with the voucher number of PMP-775 at the Pharmacognosy Department, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran. The fruits (100 g) were hydro-distilled using a Clevenger-type apparatus with 1000 mL of distilled water for 4 h at a temperature of 100 ºC. The extracted oil was dried using anhydrous sodium sulfate (Merck, Germany) and then stored in a sealed dark glass vial in a refrigerator at 4 ºC.

Essential oil analysis

GC-MS analysis

The chemical identification and quantification of the essential oil were done by gas chromatography-mass spectrometry (GC-MS) using an Agilent 6890 and Agilent 5973 MS instrument equipped with a BPX5 fused silica column (30 × 0.25 mm, film thickness 0.25 µm). The oven temperature was held at 50 °C for 5 min and raised from 50 °C to 300 °C with a gradient of 3 °C/min for 83 min. Helium was used as the carrier gas at a flow rate of 0.8 mL/min. Injection was performed in the split mode ratio of 1:30. The injector temperature was set 290 °C. The quadrupole mass spectrometer ionizing voltage was 70 eV with an ionization current of 150 µA, ion source temperature was 220 °C, the mass range 50-550 m/z.

The retention indices of all components were calculated to the retention times of C_8-C_{25} n-alkanes injected at the same temperature and conditions. Identification of the compounds was carried out by (i) comparison of their retention indices, Kováts Indices, with those reported in the literature and by (ii) comparison of their mass spectra with those recorded in the Wiley7n and NIST (National Institute of Standards and Technology) libraries and by (iii) co-injection of available reference compounds [17]. The percentage composition of the

essential oil was computed by the normalization method (the percentage of peak area relative to the total peak area of all compounds) from the GC peak areas.

Evaluation of antibiotic susceptibility of *S. aureus* isolates to oxacillin, methicillin, and cefoxitin discs

Agar disc diffusion method was applied for evaluation of susceptibility pattern of the isolated strains to oxacillin (1 µg/disc), methicillin (5 µg/disc), and cefoxitin (30 µg/disc). Mueller-Hinton agar (MHA) plates were seeded individually with bacterial suspensions (1.5×10^8 CFU/mL) using a sterile cotton swab and then antibiotic discs were put in triplicate on the surface of agar plates. The plates were incubated at 37 °C for 24 h. After incubation, the mean inhibition zone diameter for each antibiotic disc was determined to demonstrate antibiotic resistance of *S. aureus* isolates [14].

Determination of minimum inhibitory concentration (MIC)

MICs were obtained by both agar dilution and broth micro-dilution methods [18,19]. Briefly, for the agar dilution method, serial two-fold dilutions of the essential oil and antibiotics were prepared with 1 mL of dimethylsulfoxide (DMSO) and sterile distilled water, respectively. Each dilute was added to molten MHA (19 mL) at 50°C. Control plates containing 1 ml of DMSO or distilled water were also prepared. All plates were spot-inoculated with 3 µL (10^4 CFU/spot) of each bacterial suspension. The plates were incubated at 37 °C for 24 h. The MIC was determined as the lowest concentration of the essential oil that completely inhibited visible growth of the microorganism.

In broth micro-dilution method, 100 µL of essential oil was mixed with 100 µL DMSO and 10 µL of Tween 80. Then sterile Mueller-Hinton broth (MHB) was added to the mixture to reach to the final volume of 1 mL. The antibiotics were dissolved in 1 mL sterile distilled water. Then, 200 µL aliquot of the prepared solution was transferred into the first well in each row of microtiter plate and serially two-fold diluted by mixing with 100 µL of MHB in subsequent wells. Three control wells each containing 100 µL of the mixture of solvents (100 µL of DMSO, 10 µL of Tween 80, and 890 µL of sterile MHB) were also considered. Then 100 µL of bacterial suspension in MHB was added to each well to attain the final bacterial concentration of 10^5-10^6 CFU/well. After 24 h incubation at 37 °C, bacterial growth in comparison with positive control (the medium without antibacterial agents) was monitored. The MIC was defined as the lowest concentration of the antibacterial agent which completely inhibited visible growth of the microorganism.

Synergy testing

Screening for synergistic interactions

The possible synergistic interaction of several antibiotics in combination with essential oil against MRSA strains was screened by disc diffusion test. Sterile cotton swab was used to spread MRSA suspension (1×10^8 CFU/mL) on MH agar plates containing sub-inhibitory concentrations (1/2 and 1/4 × MIC) of essential oil and control MH plates without essential oil. Then, the antibiotic discs were placed on the surface of the plates. After 24 h incubation of the plates at 37 °C, the inhibition zone diameters (IZDs) around discs on essential oil containing plates and control ones were measured [20].

Determination of combined effects of gentamicin and *T. ammi* essential oil

The checkerboard broth micro-dilution method was used to assess the interactions of the candidate antibiotic (gentamicin) and *T. ammi* essential oil [4,21]. Two-fold dilutions of the two agents were mixed in a 96-well microtiter plate so that each row or column contained a fixed amount of the first agent and increasing amounts of the second one. Aliquots of 100 µL of the essential oil dilutions in MHB broth were added to the wells of 96-well plate in horizontal orientation. Then, aliquots of 100 µL of gentamicin dilutions (in MHB broth) were added in vertical orientation. The wells of microtiter plate were inoculated by 100 µL of each bacterial suspension in MHB broth to reach the final concentration of 10^5 CFU/well. The inoculated trays were incubated at 35 °C for 24 h and then evaluated for bacterial growth. The fractional inhibitory concentration indices (FICIs) for the clear wells with no visible bacterial growth were calculated for gentamicin-essential oil combination by the method reported by Didry et al. [22] according to the following formula:

$$\text{FIC index} = FIC_A + FIC_B$$

For each clear well, FIC_A or FIC_B was calculated by dividing the concentration of drug A or drug B in combination by the MIC of drug A or drug B alone, respectively.

The FICIs were interpreted as follows: Total synergism: FIC index of <0.5; partial synergism: FIC index of 0.5-0.75; additive: FIC index of 0.75-1; no interaction: FIC index of 1-4; antagonism: FIC index of >4 [21,22]. Moreover, the MICs and FICs of the antimicrobials agents, were plotted to form isobolograms to confirm synergism interactions [23]. The MIC of the antibiotic and the essential oil are located on the x and y-axes, respectively. These two points are connected by the line of additively. Then, FICs of each compound are pointed in the plot. The interaction between two antimicrobials is additive when the isobologram lies along the line of additivity. In antagonistic and synergistic interactions, the isobologram lies above and below the line of additivity, respectively.

Results and Discussion

The fruits of *T. ammi* yielded 4% (v/w) of essential oil with a pungent smell. GC-Mass analysis of the total essential oil of *T. ammi* revealed that thymol (74.2%), *p*-cymene (16%), and γ-terpinene (7.1%) were the major components. Table 1 shows that 13 compounds were identified, representing 99.1% of all compounds with aromatic monoterpenes (90.7%), monoterpene hydrocarbons (7.9%), and oxygenated monoterpenes (0.5%).

As per updated CLSI Guidelines, the cut-offs of resistance for IZDs of oxaciclline (1 µg/disc), methicillin (5 µg/disc), and cefoxitin (30 µg/disc) were ≤10 , ≤9 mm, and ≤21, respectively [24]. In this study, no inhibition zones were detected around the antibiotic discs and all of the clinically isolated strains were considered resistant to oxacillin, methicillin, and cefoxitin.

The MIC value of the essential oil, rich in thymol (74.2%), against the standard (MRSA ATCC 33591) and clinically isolated MRSAs were 0.42 µL/mL using both agar dilution and broth micro-dilution methods. No growth inhibition was observed in control plates or wells in both methods. Previous studies similarly reported that the antibacterial activity of *T. ammi* essential oil is attributed to the amount of thymol, carvacrol, *γ*-terpinene and *p*-cymene [25,26]. The IZDs of MRSA strains in the presence of different antibiotic discs and the essential oil at sub-inhibitory concentrations (1/2 and 1/4 × MIC) have been represented in table 2.

Table 1. Chemical composition of the essential oil of *Trachyspermum ammi*

NO	Compounds	Calc. KI[a]	Rep. KI[b]	Percent (%)
1	α-Thujene	926	926	0.1
2	β-Pinene	981	981	0.3
3	β-Myrcene	992	992	0.1
4	α-Terpinene	1021	1021	0.1
5	*p*-Cymene	1031	1031	16
6	Limonene	1033	1033	0.1
7	β-Phellandrene	1036	1036	0.1
8	γ-Terpinene	1063	1063	7.1
9	Terpinen-4-ol	1190	1190	0.2
10	α-Terpineol	1206	1206	0.1
11	Carvone	1260	1260	0.2
12	Thymol	1306	1306	74.2
13	Carvacrol	1312	1312	0.5
Monoterpenes hydrocarbons				7.9
Oxygenated monoterpenes				0.5
Aromatic monoterpenes				90.7
Total				99.1

[a]kovats index values represent the retention indices calculated against C_8-C_{25} n-alkanes on the mentioned column, [b]kovats index values were extracted from reference 16

Comparison of IZDs of antibiotics alone and in combination with the *T. ammi* essential oil showed that IZDs for vancomycin against all MRSA strains in the presence of essential oil were moderately increased. For ciprofloxacin and imipenem increase in IZDs was observed in the plates of MRSA2, MRSA3, and MRSA5. All of the clinically isolated MRSAs except the standard one were resistant to gentamicin, while combination of essential oil with gentamicin disc remarkably increased gentamicin activity against all the MRSA strains. Susceptibility of MRSAs did not show any difference when other antibiotics were combined with *T. ammi* essential oil.

The MIC values of gentamicin against the standard and clinical strains using broth micro-dilution and agar dilution methods were as follows: standard MRSA, 3.12 µg/mL; MRSA2, 50 µg/mL; and 25 µg/mL for other MRSA strains. The results of synergy testing between gentamicin and *T. ammi* essential oil against six MRSA strains including one standard MRSA ATCC 33591 and five clinically isolated strains have been given in table 3. The combined effects of gentamicin and essential oil against all MRSAs resulted in FIC indices ranging from 0.50 to 0.75, which showed partial synergistic interactions. In figure 1, isobolograms representing interactions of *T. ammi* essential oil and gentamicin have been shown.

Table 2. Inhibition zones (mm) of different antibiotics against MRSA strains (in presence or absence of sub-inhibitory concentrations of *Trachyspermum ammi* essential oil)

Antibiotic (µg/disc)	MRSAs			MRSA1			MRSA2			MRSA3			MRSA4			MRSA5		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Vancomycin (30)	20± 0.5	24± 0.8	24± 0.3	20± 0.7	24± 0.2	23± 0.6	19± 0.4	24± 0.4	24± 0.3	20± 0.2	24± 0.4	23± 0.1	20± 0.5	24± 0.6	23± 0.5	20± 0.2	23± 0.4	23± 0.1
Doxycycline (30)	14± 0.9	12± 0.4	10± 0.4	19± 0.8	19± 0.5	18± 0.7	11± 0.4	10± 0.5	9± 0.8	12± 0.7	9± 0.4	9± 0.2	11± 0.1	10± 0.5	9± 0.5	13± 0.2	9± 0.3	9± 0.3
Gentamicin (10)	20± 0.4	27± 0.9	25± 0.2	NZ	15± 0.4	13± 0.8	NZ	15± 0.5	15± 0.6	NZ	12± 0.0	10± 0.2	NZ	13± 0.3	11± 0.2	NZ	12± 0.5	10± 0.6
Ciprofloxacin (5)	35± 0.6	35± 0.8	35± 0.5	19± 0.1	19± 0.7	18± 0.8	11± 0.3	18± 0.6	20± 0.8	13± 0.6	19± 0.3	19± 0.8	20± 0.2	20± 0.3	19± 0.5	12± 0.2	19± 0.2	18± 0.3
Imipenem (10)	33± 0.2	32± 0.8	33± 0.3	14± 0.4	13± 0.5	14± 0.5	NZ	14± 0.6	14± 0.9	9± 0.1	11± 0.2	11± 0.1	10± 0.2	11± 0.5	10± 0.4	10± 0.3	11± 0.8	11± 0.3
Amoxicillin (25)	10± 0.4	10± 0.4	10± 0.2	10± 0.5	11± 0.6	10± 0.3	10± 0.5	10± 0.9	10± 0.6	9± 0.3	9± 0.8	8± 0.7	10± 0.1	10± 0.4	9± 0.3	9± 0.5	8± 0.2	8± 0.6
Co-trimoxazole	30± 0.5	30± 0.6	30± 0.8	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Clindamycin (2)	NZ	NZ	NZ	33± 0.3	45± 0.9	40± 0.8	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Methicillin (5)	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Oxacillin (1)	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Cefoxitin (30)	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ

Table 2. Continued

Antibiotic (µg/disc)	MRSAs			MRSA1			MRSA2			MRSA3			MRSA4			MRSA5		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Cefazolin (30)	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Cefuroxime (30)	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Erythromycin (15)	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Piperacillin (100)	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ

Abbreviation: NZ: No zone, MRSA: Methicillin resistant *S. aureus*, $MRSA_S$: Standard MRSA ATCC 33591, A: IZDs for antibiotics alone, B: IZDs for the combination of antibiotic with one-half the MIC of the *T. ammi* essential oil, C: IZDs for the combination of antibiotic with one-fourth the MIC of the *T. ammi* essential oil. Data are represented as mean±SD (mm) from the experiments in triplicate

The best synergistic interaction was observed against MRSA1 at the concentration of 0.048 µg/mL for gentamicin and 0.21 µL/mL for the essential oil which resulted in FIC index of 0.50 due to 520-fold reduction in MIC of gentamicin.Gentamicin is a bactericidal antibiotic that irreversibly binds to the 30S subunit of the bacterial ribosome and interrupts protein synthesis. It is revealed that resistance to gentamicin can be either chromosomal or plasmid-borne [27]. Several reports have described the antimicrobial action of secondary metabolites from plants [7,28]. *Thymus maroccanus* and *Thymus broussonetii* essential oils were found to interact synergistically with antibiotics against resistant bacteria [3]. Synergistic interaction of galangin, a flavonol, plus gentamicin was reported against MRSAs [29]. Similar to our study, Veras et al. showed that combination of essential oil of *Lippia sidoides* and thymol with aminoglycosides considerably decreased MICs of the antibiotics [30]. In addition, the activity of gentamicin has increased against *Pseudomonas aeruginosa* after contact with volatile compounds of essential oil of *Croton zehntneri* [31]. Various mechanisms are involved in the modification of antibiotic activity in the presence of essential oils like interaction of chemical compositions of the essential oil and the bacterial lipid membrane. The antibacterial mechanism that has been reported for thymol is damage to membrane integrity through the change of pH hemostasis and equilibrium of inorganic ions [26]. Antimicrobial activity of thymol is improved with *p*-cymene, a hydrophobic compound, by enhancement of thymol dissolution in the cytoplasmic membrane of the bacterial cell [32]. Another potential mechanism of action could be impairment of enzymes that are achieved by combination of natural products and chemotherapeutic or antibacterial agents [31].

In summary, we have shown that essential oil of *T. ammi* which was rich in thymol and *p*-cymene exhibited high antibacterial activity against MRSA strains. The current findings showed that the combination of *T. ammi* essential oil with gentamicin reduced resistance of MRSA strains to this antibiotic. The essential oil synergistically enhanced the antibacterial activity of gentamicin against clinically isolated strains of MRSA and about 520-fold reduction in MIC of gentamicin in combination with essential oil was observed against MRSA1; therefore, this combination could be considered as a new choice for treatment of infectious diseases caused by MRSA strains.

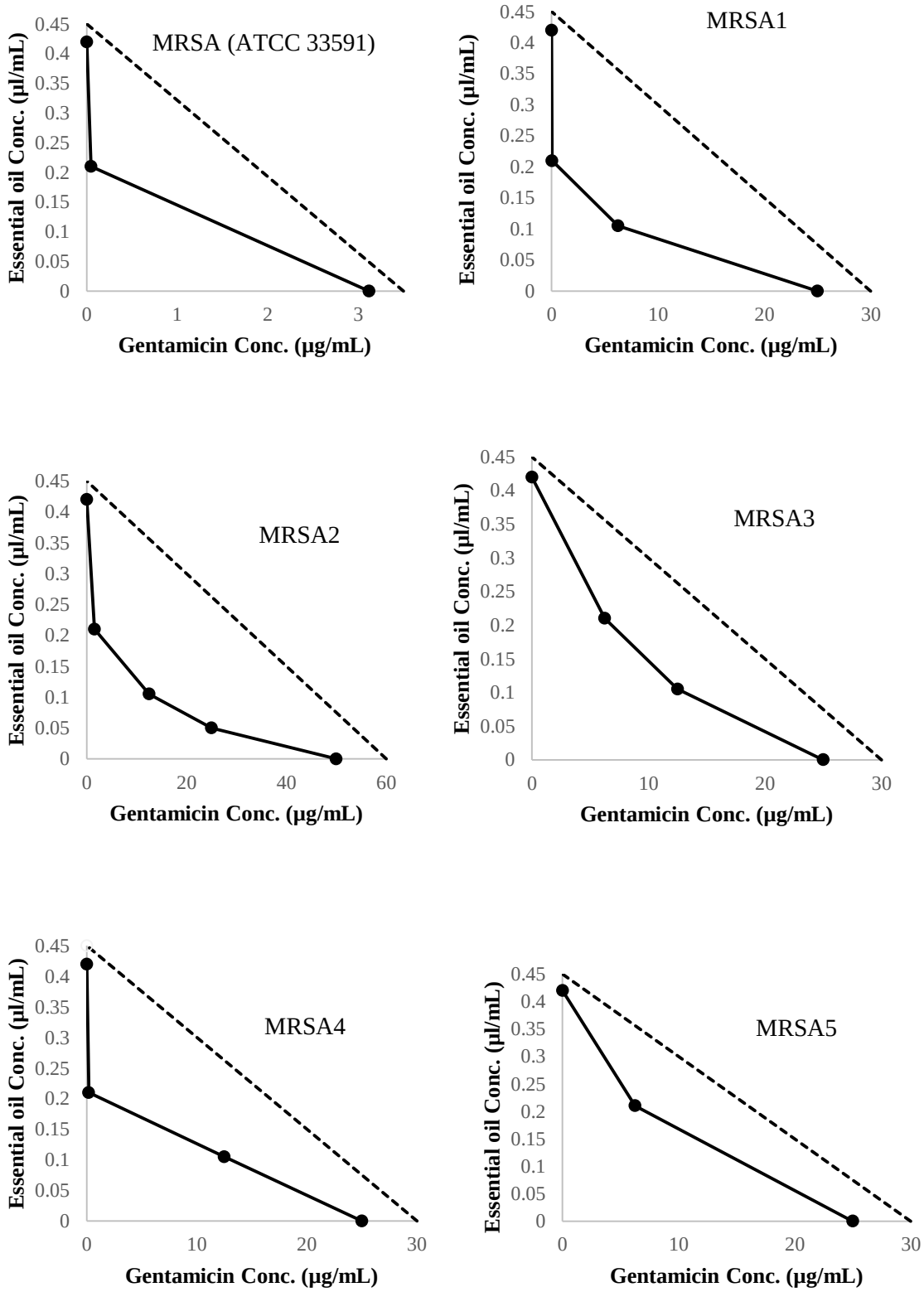

Figure 1. Isobologram analysis for the combinations of gentamicin and the essential oil of *Trachyspermum ammi* against MRSA strains

Table 3. Interaction of gentamicin with the essential oil of *Trachyspermum ammi* using checkerboard micro-dilution assay against MRSA strains

MRSA strain	Essential oil (µL/mL)		Gentamicin (µg/mL)		ΣFIC indices	Inference
	MIC_a	MIC_c	MIC_a	MIC_c		
MRSAs	0.42	0.21	3.12	0.048	0.51	Partial synergism
MRSA1	0.42	0.21	25	0.048	0.50	Partial synergism
MRSA2	0.42	0.21	50	1.56	0.53	Partial synergism
MRSA3	0.42	0.21	25	6.25	0.75	Partial synergism
MRSA4	0.42	0.21	25	0.19	0.51	Partial synergism
MRSA5	0.42	0.21	25	6.25	0.75	Partial synergism

MIC_a: minimum inhibitory concentration of alone, MIC_c: minimum inhibitory concentration of the most effective combination, FIC: fractional inhibitory concentration, MRSA: methicillin resistant *S. aureus*, $MRSA_S$: standard MRSA ATCC 33591; partial synergism: FIC index of 0.5-0.75; additive: FIC index of 0.75-1; no interaction: FIC index of 1-4

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Author contributions

Mahdi Vazirian: conception; Khadijeh Hamidian: experimental studies, analysis of data and manuscript preparation; Mehrzad Noorollah: literature search and experimental studies; Azadeh Manayi: conception and design; Nasrin Samadi: design of study, manuscript preparation, editing and review

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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Abbreviations

CA: Community-Acquired; DMSO: dimethylsulfoxide; FIC: fractional Inhibitory concentration; GC-MS: gas chromatography-mass spectrometry; IZDs: inhibition zone diameters; MDR: multidrug resistant; MRSA: methicillin-resistant *Staphylococcus aureus*; MIC: minimum inhibitory concentration; MHA: Mueller-Hinton Agar; MHB: Mueller-Hinton Broth; NIST: National Institute of Standards and Technology