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RESEARCH PAPER

Synergistic antibaterial activity of medicinal plants essential oils with biogenic silver nanoparticles

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ABSTRACT

Objective(s): Development of a nanobiosystem by using plant essential oils with green synthesized silver nanoparticles that present synergistic antibacterial activity for overcoming antibiotic resistance in pathogenic bacteria.

Material and Methods: Essential oils (EOs) of Kelussia odoratissima and Teucrium polium extracted by hydrodistillation were analyzed by gas chromatography-mass spectrometry (GC-MS). Then leaf aqueous extract of K. odoratissima prepared and used for green synthesise of silver nanoparticles (SNPs). The oils, and the colloidal preparations of silver nanoparticles, were then subjected to microdilution technique using ELISA reader to determine their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) on Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes, Escherichia coli O157: H7, Salmonella enterica and Pseudomonas aeruginosa. The type of interaction between EO and SNPs was also determined by calculating the fractional inhibitory concentration index and isibologram type.

Results: GC-MS analysis of K. odoratissima EO showed (Z)-ligustilide, (Z)-3-butylidene-phthalide, limonene and β-phellandren as main constiuents, while T. polium EO has β-caryophylene, germacrene D, γ-cadinene, (Z)-nerolidol, camphor, β-pinene, α- camphene, linalool and α-humulene. T. polium EO has more potent antibacterial property at MIC of 0.16-1.25 mg/ml compared to K. odoratissima (MIC of 0.3-2.5 mg/ml). Silver nanoparticles showed a potent antibacterial property (MIC of 0.006-0.025 mg/ml), and its colloidal suspension with plant EOs revealed a pathogen-dependent synergistic and additive effect based on calculated fractional inhibitory concentration index (FICi).

Keywords: *Antibacterial activity, Biogenic Silver nanoparticles, Essential oils, Medicinal plamts*

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INTRODUCTION

Foods contaminated with pathogenic bacteria can cause gastrointestinal disease. Inappropriate use of antibiotics and non-compliance with the recommended dose of antibiotics have led to increased bacterial resistance [1], which further emphasizes the need for discovery; and use of natural compounds with antimicrobial properties may be suitable against multi-drug resistant bacteria [2]. For this reason, pharmaceutical research has turned its attention to herbal products, looking for new leads to develop better drugs against multi-drug resistant microbial strains. In this study, two medicinal plants, Kelussia odoratissima and Teucrium polium, have been characterized for their essential oil content and antibiotic property.

Kelussia odoratissima Mozaff. belongs to family Apiaceae and is a sweet-smelling, self-growing plant native to Iran [3, 4]. Locally called ‘‘Karafse-Koohi”, it is a wild rebus, erect, glabrous, perennial aromatic herb, which grows to a height of 120 to 200 cm. It is known to have anti-inflammatory, sedative, anti-tussive and potent antioxidant activity.

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T. polium L. belongs to the family Lamiaceae and is found abundantly in South-Western Asia, Europe and North Africa. In Iranian folk medicine, the tea of T. polium is used for treating many diseases, such as abdominal pain, indigestion, the common cold and type 2 diabetes. It also has been found that this plant possesses a broad spectrum of pharmacological effects, including antioxidant, anticancer, antiinflammatory, hypoglycemic, hepatoprotective, hypolipidemic, antibacterial and antifungal properties. Antibacterial activity of its essential oils against E. coli O157:H7 has been confirmed [5].

Essential oils (EOs) and other forms of herbal extracts have been evaluated and it has been found that most of the essential oils extracted from the plants have insecticidal, anti-fungal, anti-parasitic, anti-bacterial, anti-viral and anti-oxidant properties [6-8]. EOs have been used to prevent the growth of bacteria and molds, thereby increasing the shelf life of foods [3, 9]. Antibacterial properties of EOs could be increased by the addition of nanoparticles, especially silver nanoparticles (SNPs). Antibacterial activity of a colloidal solution of silver was confirmed [10]. However, the antibacterial property of combined EOs from K. odoratissima or T. polium with SNPs has not been reported.

We herein report the antibacterial activity of K. odoratissima Mozaff. and T. polium EOs as a nanobiosystem. We further present the highest antibacterial activity at the lowest EO content by using the nanobiosystem (Fig 1).

MATERIALS AND METHODS

Plant materials

Dried leaves of K. odoratissima Mozaff. and fruits of T. polium were supplied from the Isfahan Research Center and the Ferdowsi University of Mashhad Research Field, respectively. The samples were authenticated in the FUM herbarium, and voucher specimens (Nos. 77242 and 33922 FUMH, respectively) were deposited in the herbarium.

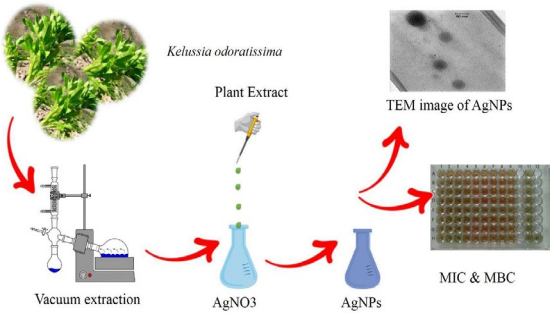

Fig 1. Schematic illustration of the present research

Essential oil extraction

Dried herbs (100 g) of K. odoratissima Mozaff. and fruits of T. polium were subjected to hydrodistillation using a modified clevenger-type apparatus for 4 hours. The EOs obtained were separated from water and dried over anhydrous Na_2SO_4, and stored in airtight containers prior to analysis by gas chromatography–mass spectrometry (GC– MS).

Chemical composition determination

GC and GC-MS analysis

Essential oil chemical components of the samples were identified by a Varian GC–MS spectrometer with a fused-silica column (DB-5, 30 m×0.25 mm i.d., J and W Scientific Inc.). The oven temperature was set at 60–240 ˚C with a ramp of 3 ˚C/min, injector temperature 280 ˚C, injector mode: split injection, with He as the carrier gas at a flow rate of 2 ml/min. The mass spectra ionisation potential was 70 eV, ion source temperature of 250 ˚C. The GC was a Shimadzu GC-17 equipped with a FID detector, fused-silica column (BP-5, 25 m×0.22 mm i.d.). The operating conditions were: oven temperature 60– 280˚C with a rate of 8˚C/min, injector temperature 280˚C, split ratio 1:10, with N_2 carrier gas, and detector temperature 300˚C.

Identification of components

The samples (1 µl) were injected neat and the components of essential oil were identified by using their retention indices (RI) obtained with reference to the n-alkane series (Sigma, UK) on the DB-5 column, mass spectra with those of authentic samples: [n-alkanes(C5-C28) composition of their mass spectra and fragmentation patterns using NIST database. Quantification of the relative amount of the individual components was performed according to the area percentage method.

Silver nanoparticles green synthesis

Preparation of K. odoratissima leaf aqueous extract

Dry leaves (20 g) of K. odoratissima were mixed in 200ml 60 °C water then concentrated in a rotary evaporator under 50 °C and reduced pressure for five minutes. The extract was filtered using cloth followed by a milli-pore filter (0.45 µm).

Synthesis of silver nanoparticles

The silver nanoparticles was synthesized according to previous report [11]. Briefly the 10^{-3} M silver nitrate solution was prepared and stored in amber bottles. Aqueous extract (10 ml) of K. odoratissima was added to 90ml $AgNO_3$ and was incubated at 37°C in a dark and stationary condition. The synthesis of Ag nanoparticles (NPs) was confirmed by using a UV–visible spectrophotometer (Cecil 1000 Series; Cecil Instruments, Cambridge, UK). The size and Zeta potential of the synthesized NPs were evaluated by the particle size analyzer (Nano–Zs[Zeta Sizer]) instrument (12-14) . The size distribution of AgNPs with maximum intensity was at 20-40nm with Zeta Potential(mV) equal to -19.9.

Antibacterial assay

Microbial strains

Representative strains of both Gram-positive and Gram-negative bacteria including S. aureus (ATCC 25923), B. cereus (ATCC 11778), L. monocytogenes (ATCC 19112), E. coli O157:H7 (ATCC 700728), Salmonella enterica (ATCC 9270) and Pseudomonas aeruginosa (ATCC 27853) were used. Bacteria were suspended in Tripticase Soy Broth (Merck, Darmstadt, Germany) at 37 °C and inoculated on Tripticase Soy (Merck, Darmstadt, Germany) plates for purity check.

Antibacterial activity assay

Determination of minimum inhibitory concentration

Antibacterial activity of the EOs and SNPs were evaluated by measuring minimum inhibitory concentration (MIC) using the broth microdilution method according to previous study [5]. Bacterial strains were cultured overnight at 37 °C in Muller Hinton Broth (MHB, Oxoid). Stock solutions of the EOs and antimicrobial standard (chloramphenicol) were prepared in 5.0% (v/v) dimethyl sulfoxide (DMSO). Dilution series, using MHB, were prepared from 10 to 0.01 mg/ml. Aliquots of 70 µl were transferred from each dilution into 96-well microtiter plates, followed by adding 70 µl of MHB and inoculating 70 µl of respective standardized microorganism suspensions containing approximately 10^8 colony forming units per ml (cfu/ml) (according to McFarland turbidity standards). Assays were performed in a volume of 210 µl. A well, consisting of MHB, 5.0% (v/v) DMSO and microorganisms, was the growth control; and a well containing MHB, 5.0% (v/v) DMSO and test oil or SNPs was the sterility control. After incubation at 37 °C for 22–24 h, the first well, without turbidity, was assigned as MIC (mg/ml). The microorganism growth inhibition was evaluated by measuring absorbance at 630 nm, using an ELISA reader (Statfax-2100, Awareness Technology Inc., USA). Chloramphenicol was used as the antibacterial standard against all pathogens. Experiments were performed in triplicate and at three different times.

Determination of minimum bactericidal concentration

The minimum bactericidal concentrations (MBC) of EOs and SNPs were determined according to the MIC values (11). Of each well showing complete absence of growth, 5 µl were transferred to agar plates (MHA) and incubated at 37 °C for 24 h. The lowest concentration of EOs where no viable bacteria were identified was the MBC.

Checkerboard assay to determine the interaction type EOs and SNPs against the studied microorganisms

In vitro assessment of combinations of antibacterial effects of EOs and SNPs were attempted by the modified dilution checkerboard technique previously described (5). Briefly, the MIC of each EO alone and in combination with SNPs was determined. Then, the FIC_{Index} of the EOs and SNPs was calculated by summing the separate fractional inhibitory concentrations (FICs) of EOs and SNPs by the following equation:

$$FIC_{index} = FIC_{EOs} + FIC_{SNPs} = \frac{MIC_{EOs}\ \text{in combination}}{MIC_{EOs}\ \text{alone}} + \frac{MIC_{SNPs}\ \text{in combination}}{MIC_{SNPs}\ \text{alone}}$$

Where (MIC_{EOs}) is the MIC of the K. odoratissima or T. polium EO in the presence of SNPs, MIC_{EOs} alone is the MIC of the K. odoratissima or T. polium EO alone. With this method, synergistic activities are defined in a range of $FIC_{Index} \leq 0.9$, additive effects in a range $0.9 < FIC_{Index} < 1.1$ and antagonistic effects in a range $FIC_{Index} > 1.1$.

The other method for determination of interaction type is the isobologram plot as shown in Fig. 2.

RESULTS AND DISCUSSION

Essential Oils' constituents

odoratissima

K. odoratissima leaf samples contained 0.64-0.75 %(V/W) EO. GC-MS analysis of the samples

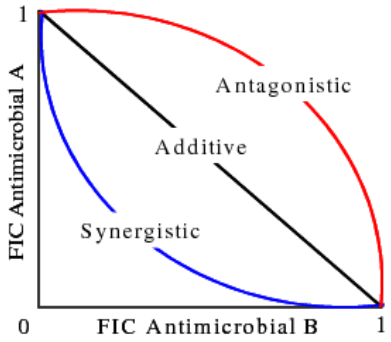

Fig 2. Schematic isobologram for determination of interaction between two antimicrobial agents

Table 1. K. odoratissima EO constituents and their retention indices by DB5 column

No	Compounds	RI	Content(%)
1	α-thujene	931	0.23
2	α-felchene	946	0.10
3	thuja-2,4(10)-diene	950	0.10
4	para-mentha-1(7),8-diene	1001	0.35
5	α-phellandrene	1004	0.14
6	o-cymene	1022	0.20
7	limonene+ β-phellandrene	1027	6.36
8	γ-terpinene	1054	0.11
9	n-pentyl isobutyrate	1056	0.61
10	Citronellol	1227	0.76
11	α-cubebene	1347	0.22
12	citronellyl acetatet	1353	0.61
13	γ-muurolene	1480	1.82
14	Unknown	1523	0.97
15	germacrene B	1556	2.80
16	Unknown	1651	2.45
17	(Z)-3-butylidene-phthalide	1672	14.37
18	Khusinol	1682	0.48
19	Unknown	1715	1.47
20	(E)-3-butylidene-phthalide	1726	0.55
21	(Z)-ligustilide	1738	54.11
22	14-hydroxy-α-muurolene	1765	0.26
23	(E)-ligustilide	1794	0.57
	Total		89.64

revealed 23 compounds (representing 89.46 % of the essential oil compounds) (Table 1). The main components of the EO of K. odoratissima were (Z)-ligustilide (54.11%) and (Z)-3-butylidene-phthalide(14.37%), but the percentage of (E)-ligustilide was very low (0.57%).

The sum of these three components was defined as Phthaleid derivatives and was near 69%**.** The content of limonene+β-phellandrene of the EO was 6.5%, and these two components were in the third place after (Z)-ligustilide and (Z)-3-butylidene-phthalide. The germacrene B content of the EO was 2.80%. Although some researchers report pharmacological acvtivity of K. odoratissima, there are only a few reports on the essential oils constitents and its antibacterial

Table 2. T. polium EO constituents and their retention indices by DB5 column

No	Compounds	RI	Content(%)
1	(E)-2-hexenal	852	0.23
2	α-pinene	929	2.52
3	α- camphene	952	5.73
4	Sabinene	965	0.64
5	1-octene-3-ol	978	2.97
6	3-octanal	982	3.29
7	β-pinene	987	6.09
8	Myrcene	995	2.61
9	p- cymene	1025	3.25
10	1,8-cineol	1032	3.60
11	Limonene	1036	1.89
12	(E)- β- Ocimen	1050	1.21
13	Camphor	1092	6.21
14	Linalool	1127	4.75
15	α-terpineol	1139	0.33
16	Bornyl acetate	1142	1.34
17	Terpinene-4-ol	1198	0.19
18	Carvacrol	1272	0.23
19	β-myrcene	1296	0.45
20	Camphene	1385	0.27
21	β-caryophylene	1421	7.94
22	α-humulene	1437	4.40
23	γ-cadinene	1478	6.26
24	Germacrene D	1482	7.36
25	Bicyclogermacrene	1494	2.02
26	Elemol	1519	3.26
27	(Z)- nerolidol	1534	6.23
28	Spathulenole	1552	3.30
29	Caryophyllene oxide	1578	3.69
30	α- cadinol	1702	1.68
31	Hexadecanoic acid	1896	0.75

activity [15, 16]. The main EO constituents of K. odoratissima was reported as ligustilide and (Z)-3-butylidene-phthalide [17]. Other research with a different species of the Kelussia genus reported that caryophylene, germacrene D and γ-cadinene were the main constituents [18].

polium

The results of EO analysis of T. polium is presented in Table 2. The data show that there are 32 compounds in the EO (representing 94% of the EO compounds). β-caryophylene(7.94%), germacrene D (7.36%), γ-cadinene (6.26%), (Z)-nerolidol(6.23%), camphor (6.21%), β-pinene (6.09%),α-camphene(5.73%),linalool(4.75%)and α-humulene (4.40%) are the main components and represent 55% of the total EO.

Antibacterial activity of EOs
kelussia odoratissima

Antibacterial activities were expressed as MIC and MBC values (Table 3). K. odoratissima EO exhibited varying levels of antibacterial activities against the bacteria investigated. The MIC of the EO against Staphylococcus aureus, Bacillus cereus and Listeria monocytogenes (Gram positive bacteria)

was between 0.31-1.25 mg/ml and was equal to the corresponding MBC. The most efficient antibacterial activity of the K. odoratissima EO was against S. aureus, with MIC=0.31 mg/ml, followed by L. monocytogenes and B. cereus with 0.62 and 1.25 mg/ml respectively.

The MIC of K. odoratissima EO against E. coli O157:H7 Salmonella enterica and Ps. aeruginosa (Gram negative bacteria) were higher than with the Gram positive bacteria (0.62 -2.5 mg/ml). The results confirm that among the Gram negative bacteria E. coli O157:H7 is the most tolerant (the highest MIC) and Ps. aerugenosa is the most sensitive (the lowest MIC) pathogenes to the essential oil. In Gram negative bacteria, regardless of the pathogen types, the MIC of the K. odoratissima EO were equal to the MBC. MIC comparison of the Gram positive bacteria with the Gram negative bacteria tested in this research showed that the former are more sensitive than the later (Table 3).

Teucrium polium

The results of MIC and MBC of the T. polium EO against pathogenic bacteria are shown in Table 3. The MIC of the EO against Gram positive bacteria were between 0.16-0.62 mg/ml. Similar to the K. odoratissima EO, the bacterium sensitive to the T. polium EO was S. aureus. The MBC evaluation of the T. polium EO against Gram positive bacteria showed that of the MBC is twice that of the MIC except in Bacillus cereus, whose MBC was equal to the MIC. In Gram negative bacteria the MIC of the EO was lower than with the Gram positive and determined as 0.31-1.25 mg/ml. Comparison of the T. polium EO with K. odoratissima (Table 3) confirms that the T. polium EO has a stronger antibacterial activity than K. odoratissima. The MIC of the Chloramphenicol (Table 3) against the tested pathogenic bacteria also confirm the results. Our finding on the content of the main EO constituents of T. polium (Table 2), especially 1,8 Cineol, p-Cymene, Camphore and Linalool, which have antibacterial activity, is in accordance with the results. Other researchers also confirm the mild to strong antibacterial activity of the EO of plants of the Apiaceae family [5, 19].

MIC comparison of the K. odoratissima and T. polium EOs confirms that the latter has higher antibacterial activity. This activity relates to the main constituents of T. polium EO, especially pinene, camphore, cadinene, caryophylene and germacren D (Table 2) in comparison to K. odoratissima, in which more than 68% of the EO were ligustilide and (Z)-3-butylidene-phthalide. Phenolic constituents of EOs are resposible for antibacterial activity (20-22). Previous research has also shown mild to strong antibacterial activity of the EOs of Apiaceae plants. According to other studies, the Gram negative bacteria are more resistant to EOs than are the Gram positive bacteria because there is an outer membrane surrounding the cell wall in former. In Gram positive bacteria direct contact of EOs' hydrophobic constituents

Table 3. Antibacterial activities (MIC and MBC mg/ml) of K. odoratissima (K. O) and T. polium (T. P) EOs alone and in combination with green synthesized SNPs

Pathogens	G	ATCC	Alone				In combination		SNPs		Cp.
			K. O		T. P		K. O	T. P			
			MIC	MBC	MIC	MBC	MIC		MIC	MBC	MIC
S. aureus	+	25923	0.31	0.31	0.24	0.16	0.15	0.09	0.025	0.050	0.24
B. cereus	+	11778	1.25	1.25	0.06	0.62	0.62	0.02	0.025	0.050	0.06
L. monocytogenes	+	19112	0.62	0.62	0.12	0.31	0.15	0.12	0.012	0.025	0.12
E. coli O157:H7	-	700728	1.25	1.25	0.24	0.62	0.62	0.18	0.006	0.012	0.24
S. enterica	-	9270	2.5	2.5	0.36	1.25	0.62	0.18	0.012	0.025	0.36
Ps. aeruginosa	-	9027	0.62	0.62	0.24	0.31	0.15	0.12	0.006	0.012	0.24

Table 4. Fractional inhibitory concentration (FIC) of SNPs and essential oils of K. odoratissima (K. O) and T. polium (T. P)

Pathogens	G	ATCC	MIC of K. O. EO in combination with SNPs					MIC of T. P. EO in combination with SNPs				
			SNPs	K. o	$FIC_{K.o}$	FIC_{SNPs}	FIC_{index}	SNPs	T. p	$FIC_{T.p}$	FIC_{SNP}	FIC_{index}
S. aureus	+	25923	0.012	0.15	0.48	0.48	0.96 [a]	0.012	0.08	0.5	0.48	1 [a]
B. cereus	+	11778	0.012	0.62	0.5	0.50	1 [a]	0.012	0.31	0.5	0.48	1 [a]
L. monocytogenes	+	19112	0.003	0.15	0.25	0.25	0.5 [b]	0.006	0.16	0.5	0.5	1 [a]
E. coli O157:H7	-	700728	0.003	0.62	0.50	0.5	1 [a]	0.001	0.16	0.25	0.25	0.50 <0.9 [b]
S. enterica	-	9270	0.006	0.62	0.25	0.5	0.75 [b]	0.003	0.62	0.5	0.25	0.75 <0.9 [b]
Ps. aeruginosa	-	9027	0.003	0.15	0.24	0.5	0.74 [b]	0.003	0.08	0.25	0.5	0.75 <0.9 [b]

with the cell membrane's phospholipid bilayers increases permeability and leakage of vital constituents and leads to cell death [23].

Antibacterial activity of slver nanoparticles

The antibacterial activity of the silvert nanoparticles (Table 3) was very high as the MIC in Gram positive bacteria determined 0.012-0.025 mg/ml, which was lower than the EOs tested in the experiment. SNPs' MIC against Gram negative bacteria measured between 0.006 and 0.012 mg/ml. In all the pathogenic bacteria the MBC of the SNPs were higher (two-fold) than the MIC. The antibacterial activity of SNPs' colloidal solution was studied and reported the MIC of the solution against S. aureus and E. coli to be 0.004 and 0.003 mg/ml, respectively [10].The differences may be attributed to the related ATCC of the pathogenes. The MIC of SNPs against S. aureus also reported 0.02 mg/ml [24].

Although the exact mechanism of the bactericidal effect of SNPs has not yet been elucidated, many possible mechanisms have been proposed. The SNPs have efficient antibacterial activity due to their extremely large surface area, which provides better contact with microorganisms. SNPs get attached to the cell membrane and also penetrate inside the bacteria and disturb the permeability and respiration functions of the cell. The SNPs preferably affect proton motive force, destabilization of the outer membrane and association with oxygen and SH groups on the cell wall to form R-S-S-R bonds, thereby blocking respiration and leading finally to cell death [25].

Interaction of EOs with silver nanoparticles

Because of the less efficient antibacterial activity of the EOs compared to that of chloramphenicol, assessment of possible synergistic and additive effects of the combination of EOs with SNPs was attempted. The results of integrated application of EOs with SNPs on pathogens is presented in Table 3. The MIC of chloramphenicol was also added for comparison. The results confirm that in most pathogens combined application of EOs with SNPs decreased the MIC as much as 50 percent (higher antibacterial activity). In T. polium (Table 3) the reduction of MIC in combined application was higher, especially in Gram negative bacteria. In other report, the interaction of SNPs with antibiotics was studied and showed that integrated

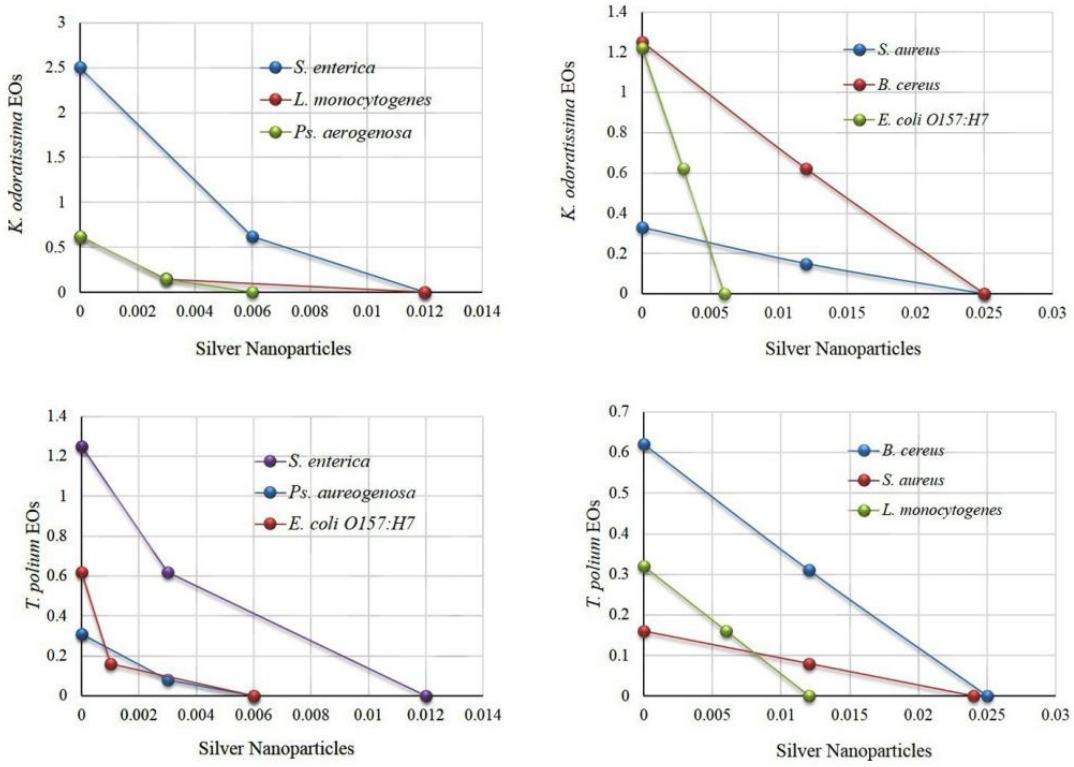

Fig 3. Isobologram of K. odoratissima (up), T. polium(down) EOs and SNPs. Left: Synergistic activities, Right: Additive activities

application of SNPs with antibiotics produces stronger antibacterial activity in comparison to the application of SNPs or antibiotics alone [26]. Increased antibacterial activity of integrated application of EOs with SNPs was also reported. The results presented a biosystem of the EO of Rosmarinus officinalis with SNPs that was very efficient in inhibiting biofilm formation by two Candida species and inhibition of antibiotic resistance, and they recommended combination of SNPs with plant EOs [27].

FIC_{index} of EOs and SNPs against phatogenic bacteria

The interaction type of two antibacterial agents is determined by evaluating fractional inhibitory concentration by using a modified dilution checkerboard or the isobologram chart. The results of FIC for two EOs and SNPs are presented in Tables 4 for K. odoratissima and T. polium EOs, respectively. The FIC_{index} in Table 4 shows the synergistic activity (FIC < 0.9) of the K. odoratissima EO with SNPs against L. monocytogenes (Gram positive) and S. enterica and Ps. aeruginosa (Gram negative). In S. aureus , B. cereus (Gram positive) and E. coli (Gram negative) additive activity ($0.9 > FIC_I < 1.1$) was recorded. Isobolograms also confirm the interaction between the K. odoratissima EO and SNPs (Fig. 2). In T. polium EO there was synergistic activity (FIC < 0.9) with SNPs against Gram negative bacteria and additive activity ($0.9 > FIC_I < 1.1$) against Gram positive bacteria when the EO were used in combination with SNPs (Table 4). There was no antagonistic activity ($FIC_{index} > 1.1$) (Fig. 3). The synergistic activity between lemon EO with SNPs was reported [28]. Other researchers confirmed the synergistic activity between Bunium persicum and Cuminum cyminum EOs [5]. The synergistic activity between Myrtus communis EO and Amphotericin B against Aspergillus niger and Candida albicanse was also reported [29]. Therefore, using combined SNPs and EOs decreases the MIC of the combination and provides a new strategy for overcoming antibiotic resistance in pathogenic bacteria.

CONCLUSION

Essential oil from T. polium appears to have a strong antibacterial activity that may be linked to its phenolic constituent. Synergism between EOs and SNPs was observed based on the calculated FICs. This finding points specifically to the potential use of colloidal suspension of EOs and SNPs in combating multidrug-resistant bacteria.

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CONFLICT OF INTEREST

Author has no received research grants. The author declares that he has no conflict of interest.

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