https://doi.org/10.31925/farmacia.2023.1.13 **ORIGINAL ARTICLE**

ANTIBACTERIAL SYNERGISTIC AND ANTAGONISTIC EFFECTS OF COMMERCIAL ESSENTIAL OILS FROM *THYMUS VULGARIS* L. AND *SYZYGIUM AROMATICUM L.* IN COMBINATION WITH *NIGELLA SATIVA* L.

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Abstract

The aim of this study was to assess how *Nigella sativa* L. (black cumin) essential oil (EO) modify the antibacterial activity of *Thymus vulgaris* L. (thyme) EO and *Syzygium aromaticum* L. (clove) EO. We used two bacterial strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. The micro atmosphere assay method was used. The tests were performed after prior incubation at room temperature for 30 and 60 minutes of the mixtures of black cumin EO with thyme EO and clove EO, respectively. After 60 minutes of incubating the mixture of thyme EO: black cumin EO (1:1.54) the maximal antibacterial activity of thyme EO against *E. coli* was reduced by 50%. Similarly, the RA of the clove EO against *E. coli* is decreased by 50% when clove EO and black cumin EO are mixed in 1:5.19 ratio. The present study demonstrated that mixture of EOs is not always beneficial.

Rezumat

Obiectivul acestui studiu a fost testarea modului în care uleiul esențial de *Nigella sativa* L. (chimen negru) influențează activitatea antibacteriană a uleiurilor esențiale de *Thymus vulgaris* L. (cimbru) EO și *Syzygium aromaticum* L. (cuișoare) EO. Am testat *Escherichia coli* ATCC 25922 și *Staphylococcus aureus* ATCC 25923. Am utilizat metoda microatmosferei. Testele au fost realizate după o prealabilă incubare la temperatura camerei timp de 30 minute sau 60 minute a mixturilor de EO chimen negru cu EO cimbru, respectiv EO chimen negru cu EO cuișoare. Incubarea timp de 60 de minute a mixturii EO cimbru:EO chimen negru (1:1,54) a dus la reducerea cu 50% a activității antibacteriene a EO cimbru față de *E. coli*. Similar, reducerea cu 50% a activității antibacteriene a EO cuișoare față de *E. coli* a fost obținută când EO cuișoare:EO chimen negru au fost in raport 1:5,19. Prezentul studiu a demonstrate că mixturile de uleiuri esențiale nu sunt întotdeauna benefice.

Keywords: essential oils, antibacterial activity, *Nigella sativa*, *Syzygium aromaticum*, *Thymus vulgaris*

Introduction

Herbal products are extremely diverse in term of their composition making its classification a great challenge. Essential oils (EOs) and other herbal extracts are used since ancient times for the curative purposes, as flavours to improve air conditions, in cosmetology, for food preservation, or alternative sanitizers to chlorine [12, 18, 27, 29, 35, 38, 42, 45]. Nowadays EOs are mostly used as fragrances, but the discovery of the biological effects of EOs alongside the characterization of their composition shifted the EOs investigation towards a wider range of potential applications. There are two divergent points of views regarding the use of EOs: either as biologically ineffective, but as well-being flavour, or as biologically effective compounds with antibacterial activity [15]. The numerous studies performed to date have shown that the answer lies between these extremes [44]. The composition of EOs, like any natural plant mixtures, is very complex and consists on near 60 components in different proportions, only a few components being prevalent (20 - 70%) [36]. Moreover, the composition of EOs depends on the variety of

plant species, the plants' origin, the cultivation conditions, etc. [1, 3, 22, 36, 39, 40, 46]. The high volatility of EOs is an important limitation of their use, but the identification and characterization of the active compounds encourage many research teams to develop technologies which improve EOs stability, such as encapsulation in nano-liposomes [45]. Other research teams demonstrate the enhancement of the antifungal activity of *Origanum majorana* L. EO in complex with polyamidoamine (PAMAM) G4.0 dendrimer or the improvement of antimicrobial activity of the *Zingiber officinale* EO in nanoemulsions [34, 43].

Because mechanisms of action of the EOs are very difficult to be assessed many studies are focused on elucidating the precise effect of the major components of EOs. *Nigella sativa* (black cumin) has an anti-tumoral effect, one mechanism being the interaction of thymoquinone with Polo-like kinase 1 – a regulator of cell division [2, 5, 28, 47]. According to Drug-Bank (www.drugbank.ca), eugenol (DB09086), a major component of some EOs, with unknown mechanism of action, has anti-inflammatory, neuro-protective, antipyretic, antioxidant, antifungal and analgesic properties. The analgesic effect may be mediated by opioidergic and α2-adrenergic receptors and the inhibition of TNF-α [9, 13, 37]. Studies that analysed the mechanisms of individual components of EOs shown that EOs could be promising candidates for the treatment of a wide range of clinical conditions associated with excessive smooth muscle contractility [17]. Among the biological effect of EOs, the anti-microbial effect is, by far, one of the most studied. The emerge of multiresistant bacterial and fungal species involved in life-threatening infections maintain the interest on the discovery of new antibacterial and antifungal compounds [7, 30]. The antibacterial effect of black cumin EO against *Staphylococcus aureus* is due to thymoquinone, carvacrol, and p-cymene [33].

Testing of biological activity of the EOs and the interpretation of the results are a major obstacle when analysing the results from different sources due to poor standardization, compared, for example, to the antibiotic testing methods described in Clinical and Laboratory Standards Institute (CLSI) procedural standards [8]. Presently many studies focus on routine quality-control analyses [31]. It is therefore an urgent need for standardized methods and procedures on analysis the EOs.

Because of huge number of components found in EOs composition, it is difficult to categorize and evaluate the interactions between different components. Still, the synergic effect was reported between carvacrol – a component of thyme EO and black cumin EO – and its precursor p-cymene [4]. To interpret the mechanism of action of a very complex natural mixture, it is necessary to know not only the biological activity of each compound but to identify the roles of the genes involved in the synthesis or degradation of these compounds. This approach requires substantial work to analyse.

Our previous work showed that some commercial EOs have good antibacterial activity against Gram-negative bacilli. Moreover, we detected synergism or antagonism between some commercial EOs when the disk diffusion method was made [21]. In the present study, we assessed the influence of the commercial black cumin EO on the antibacterial effect of the commercial thyme EO and commercial clove EO against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923.

Materials and Methods

Essential oils

The commercial samples of EOs included in the present study were black cumin oil (*Nigella sativa* L.), thyme oil (*Thymus vulgaris* L.), and clove oil (*S. aromaticum* L.). The EOs tested were purchased from two suppliers: thyme oil and clove oil from Steaua Divină (SD) and Fares (F), and black cumin oil from SD – the last EO was not manufactured by F at the time when the study was conducted.

The characteristics of essential oils tested

The qualitative GC-MS analysis of the essential oils was carried out using Shimadzu GC 2010 and MS QP 2010 PLUS system (Tokyo, Japan), EI ionization, operating in 70 eV mode. Samples of 1 μL (20 mg of oil dissolved in 1.5 mL of dichloromethane) were injected in a split mode at a ratio of 5:1. The compounds were separated HP-5MS on capillary column (a 12 m × 0.20 mm i.d., 0.33 μm) (Agilent J&W, Palo Alto, CA, USA) stationary phase (cross-linked 5% - phenyl)-methylpolysiloxane. The flow rate of helium through the column was kept at 0.8 mL/min. The initial temperature of the column was 50°C, then it was increased to 180°C at a rate of 35°C/min, and then heated up to a final temperature of 280°C at a rate of 20°C/min. The oven was kept at this temperature for 15 min. The injector temperature was 240°C, the transfer line temperature was 250°C, and the ion source temperature was 200°C. The solvent delay was 2 min. The scan range of the mass spectrometer was set at 50 to 650 m/z. Essential oil constituents were identified by comparison of their mass spectra with those stored in the Wiley 229 and NIST/EPA/NIH (NIST 05) mass spectral libraries [11, 41].

Antibacterial synergy and antagonism assay – the micro-atmosphere assay method

The tests were performed on the Mueller-Hinton agar inoculated with bacterial strain to be tested at a 0.5 McFarland standardized density according to CLSI procedural standards [8]. Ten microliters of essential oil were added in the middle of a sterile 90 mm paper

disc, the diameter of the size of Petri dish lid. After sealing the assemble, the volatile effect was tested. Because a combined antibacterial effect was tested in this study, the method was slightly modified as described below.

Before performing the antibacterial assay, the EOs to be tested were mixed in a sterile Eppendorf tube and incubated 30 minutes or 60 minutes at room temperature (RT). Then the EOs mixtures were added onto the paper filter stick on the Petri dishes lid on the range volumes 11 – 80 μL (Table III). The controls with thyme EO, clove EO and black cumin EO were made. In this study, the black cumin EO was considered the "diluent" and thyme EO and clove EO were considered the "aliquots". The dilutions were prepared in such way that the final volumes of thyme EO and clove EO were 10 μL.

Statistical analysis

The results were analysed in Excel under Microsoft Office package. The Student's *t*-test was used to evaluate the relationship between the samples. The p value < 0.05 threshold was considered to reject the null hypothesis. Because the p value calculated by the Student t-test is not focused on the effect size, our experimental results were subjected to further statistical analysis – 95% confidence interval (CI) and p value of the Mann-Whitney U test [19]. The DABEST ("data analysis with bootstrap-coupled estimation") was used to further compare the experimental results [19]. The graphical estimation of the results was further performed. The trendline equations, which best fit the results, were solved for subsequent results analysis.

Results and Discussion

The chemical composition of essential oils tested

The qualitative and quantitative composition of the tested commercial EOs was determined using GC-MS technique (Table I). The commercial clove EOs from the two suppliers analysed are similar, though slightly differing in the trans-caryophyllene concentrations. In contrast, the thyme EOs from the two suppliers significantly differ mainly in their thymol composition as this thyme-typical compound was detected only in the EO provided by SD.

Table I

The chemical composition of the tested EOs

Essential oil	Supplier	Main components	% Area [3]
T. vulgaris L. (thyme)	SD[1]	Thymol	42.84
		Benzene, 1,2,3,4-tetrametyl-(CAS)	28.44
		γ-terpinene	6.64
		linalool	4.52
		eucalyptol (1.8-cineole)	4.13
		trans-Caryophyllene	2.77
		α- terpinene	2.55
		camphene	1.81
		Hexadecanoic acid (CAS) palmitic acid	1.31
T. vulgaris L. (thyme)	F[2]	Benzene, 2 metoxy-1,3,4-trimetyl-(CAS)	42.69
		Benzene, 1,2,3,4-tetrametyl-(CAS)	22.45
		Phenol,5-methyl-2-(1-methylethyl)-(CAS)	7.04
		trans-Caryophyllene	6.94
		linalool	6.78
		γ-terpinene	3.17
		caryophyllene oxide	2.42
		borneol	2.10
		terpinen-4-ol	1.48
		camphor	1.40
		camphene	1.03
S. aromaticum L. (clove)	SD	Phenol	71.04
		Trans-Caryophyllene	20.19
		Eugenyl acetate	4.01
		α-humulene	2.71
S. aromaticum L. (clove)	F	Phenol	82.52
		Eugenyl acetate	9.64
		Trans-Caryophyllene	5.37
N. sativa L. (black cumin)	SD	9,12-Octadecadienoic acid	67.82
		Hexadecanoic acid (CAS) palmitic acid	11.13
		p-cymene	4.89
		cyclohexane	3.92

[1] Steaua Divină, [2] Fares, [3] the area of each component were calculated by normalization

We compared the major components of the commercial EOs reported in the literature with those similar in the EOs tested in the present (Table II). Although significant composition variability of the EOs from

the three studied plant species is documented, their major compounds (thymol, eugenol/eugenyl acetate and thymoquinone/linoleic acid, respectively) are fairly the same.

The major volatile compound of the black cumin EO is thymoquinone **[**16, 32, 33**]**, a volatile compound with a broad biological activity including antibacterial, anticonvulsant and antioxidant effects. The commercial EO from black cumin included in the present study lacks thymoquinone, the main compound being the 9,12-octadecadienoic acid (Table I) which is linoleic acid in the same concentration cited by other papers (Table II). Because black cumin EO was not available from other suppliers, the study was designed accordingly.

Table II

The main characteristics of the commercial EOs similar with EOs tested

EO	Species	Family	Major compounds (%)	Ref.
black cumin or black seed	*Nigella sativa* L.	*Ranunculaceae*	thymoquinone (0.72 - 21.03%), carvacrol (1.34 - 4.83%), ***t***-butylhydroquinone (0.3 - 11.5%), linoleic acid (22.4 - 61.85%), oleic acid (1.64 - 23.4%), p-cymene (0.09 - 14.8%)	[14, 16]
thyme	*Thymus vulgaris* L.	*Lamiaceae*	thymol (10 - 64%), carvacrol (2 - 11%), γ-terpinene (2 - 31%), p-cymene (10 - 65%)	[6, 14]
clove	*Syzygium aromaticum* L.	*Myrtaceae*	eugenol (75 - 85%), eugenyl acetate (8 - 15%)	[6]

Antibacterial activity of thyme EO and clove EO in the presence of black cumin EO

We performed the micro-atmosphere assay method in order to analyse the antibacterial effect of the mixtures of *N. sativa* EO and *T. vulgaris* EO or *S. aromaticum* EO against a Gram-negative bacteria strain and a Gram-positive bacteria strain [20, 24]. We tested the following bacterial strains: *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923.

The workflow used on the micro-atmosphere assay method is presented in Table III. Two independent experiments of the testing of the antibacterial effect were carried out on different days.

Table III

The workflow of the antibacterial testing

Eppi tube				Micro atmosphere test
Dilution	**T/C[2] (µL)**	**B[1] (µL)**	**Incubation**	**Volume (T/C[2] + B[1]) (µL)**
1:1.1	50	5	30 or 60 minutes at RT[3]	11 (10 + 1)
1:1.2	50	10		12 (10 + 2)
1:1.25	50	12.5		12.5 (10 + 2.5)
1:1.5	50	25		15 (10 + 5)
1:2	50	50		20 (10 + 10)
1:3	50	100		30 (10 + 20)
1:4	50	150		40 (10 + 30)
1:5	50	200		50 (10 + 40)
1:6	50	250		60 (10 + 50)
1:7	50	300		70 (10 + 60)
1:8	50	350		80 (10 + 70)

[1] B = black cumin EO; [2] T = thyme EO; [2]C = clove EO; [3] RT = room temperature

There was no inhibition when testing black cumin EO against *E. coli* in the volumes range 1 - 70 µL. The *S. aureus* was not inhibited by the black cumin EO at 1 µL, 2 µL, and 2.5 µL. Contrary, the *S. aureus* was inhibited by the black cumin EO at 5 µL (60 mm diameter of inhibition), and above 10 µL (85 mm diameter of inhibition).

The micro-atmosphere assay method allows loading great volume on the paper filter, compared with disc diffusion assay method (aromatogram) – when the paper disc was considerable smaller [10]. Also, the micro-atmosphere assay method is more adequate because the major active compounds of EOs are volatile compounds. Testing of different dilutions is adequate for the experimental measure of combination between two EOs and for comparison of the antibacterial effect of EOs from two suppliers. The % residual antibacterial activity (RA) of the various combinations comprising black cumin EO was compared with the antibacterial activity of thyme EO and clove EO.

$$\% \text{ RA} = \frac{(\text{diameter mixture} - \text{diameter black cumin EO}) \times 100}{\text{diameter thyme EO or clove EO}}, \quad (1)$$

The percentage of RA activity was calculated using the Equation 1 where the diameter of inhibition was recorded when testing 10 µL thyme EO and clove EO was positive control, and the diameter of mixture test was the diameter of inhibition when testing the thyme EO or clove EO diluted with black cumin EO.

Following reviewer's comments, we have adjusted the text as follows: we tested the thyme EO and clove EO at 10µl. Then we tested individual black cumin EO at volumes 1 µL, 2 µL, 2.5 µL, 5 µL, 10 µL, 20 µL, 30 µL, 40 µL, 50 µL, 60 µL and 70 µL. We

made the mixtures with black cumin EO. In these mixtures we increased only the black cumin EO volume. We maintained the thyme EO and clove EO at 10 µL each time. Therefore we have the positive control of the increasing or decreasing antibacterial activity of the thyme EO and clove EO in the presence of the black cumin EO.

Antibacterial activity of thyme EO and clove EO in the presence of black cumin EO against E. coli ATCC 25922

We tested the antibacterial activity of the individual EO and EOs mixture against *E. coli* ATCC 25922**.** We further analysed the antagonism between black cumin EO and thyme EO from the two suppliers (F or SD) by incubating the mixtures at 30 and 60 minutes, respectively (Figure 1). The mixture incubated for 30 minutes resulted in statistical significance between the thyme EOs from the two suppliers (p = 0.036418). An explanation of these results could reside in the different composition of the thyme EO from the two suppliers. The sample from Fares (F) supplier does not contain thymol (Table I).

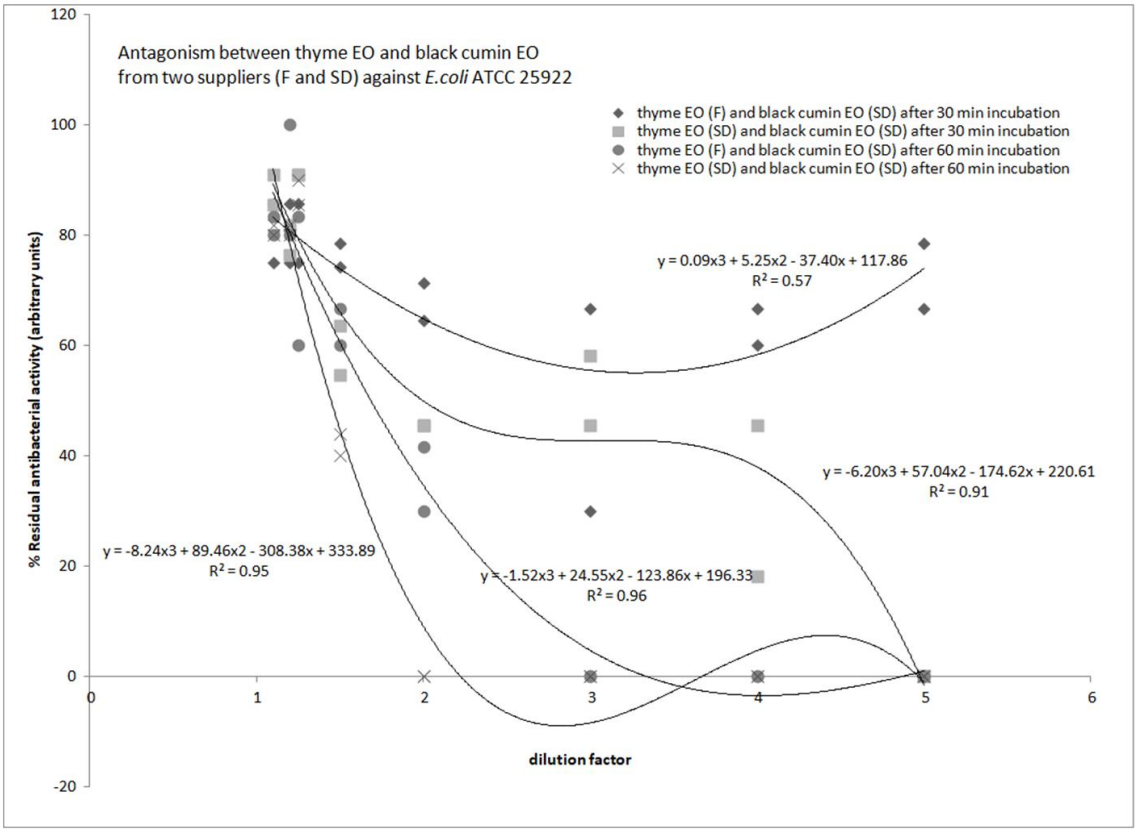


Figure 1.

The antibacterial activity of the thyme EO and black cumin EO mixture against *E. coli* ATCC 25922

The comparative % of the residual antibacterial activity (RA) of thyme EO from two suppliers, F = Fares SD = Steaua Divină, after dilution with black cumin EO (SD) and incubated for 30 minutes/RT (p = 0.036418) or 60 minutes/RT (p = 0.323533) before perform the antibacterial assay; RT= room temperature

In Figure 2 are shown the unpaired mean differences of the % residual antibacterial activity of the two samples of thyme EO in the presence of black cumin EO after different incubation time of the EOs mixtures. Analysing the graphical trendlines (Figure 1) and the two statistical analysis results, the results from the experiment carried out after incubation at 30 minutes could be explained differently. Contrary to the *t*-test when p value of 0.036418 could reject the null hypothesis H_0, the p value from *U* test (0.192) does not reject the H_0 of equal distributions. Because he two statistical analysis test different hypothesis, the analysis was correlate with graphical estimation. The diagrams from Figure 2 demonstrate that a prolonged incubation results in antagonism of the thyme EO by black cumin EO, regardless of the thyme EO origin, which matched with the trendlines form the Figure 1. Explication of these results could reside in time required by the different samples of thyme EO to interact with black cumin EO.

The comparative analysis of the synergism/antagonism between black cumin and clove EOs from the two suppliers reveals interesting results when the EOs mixtures were incubated at 30 minutes or 60 minutes at room temperature (Figure 3). It appears that, at 30 minutes incubation there is an increase of the anti-

bacterial activity of clove EO in the presence of black cumin EO, regardless of the origin of the clove EO samples. However, the synergy goes into antagonism when the time of interactions between clove EO and black cumin EO is prolonged at 60 minutes.

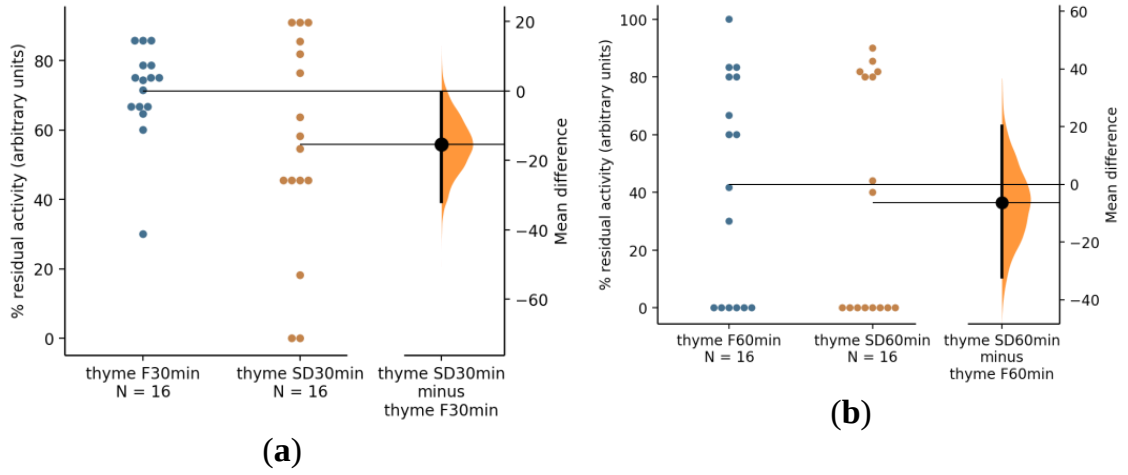


Figure 2.

The antibacterial activity of the thyme EO and black cumin EO mixture against *E. coli* ATCC 25922

The mean difference between the % of residual antibacterial activity of thyme EO from two suppliers (F = Fares and SD = Steaua Divină), after dilution with black cumin EO, is shown in the above Gardner-Altman estimation plot. Both groups are plotted on the left axes; the mean difference is plotted on a floating axis on the right as a bootstrap sampling distribution. The mean difference is depicted as a dot; the 95% confidence interval is indicated by the ends of the vertical error bar: (a) after the incubation of the mixtures for 30 minutes/RT, the unpaired mean difference between the two samples of thyme is -15.4 [95.0%CI -31.9, -0.578] and p = 0.192; (b) after the incubation of the mixtures for 60 minutes/RT, the unpaired mean difference between F60min and SD60min is -6.37 [95.0%CI -32.2, 20.1] and p = 0.738

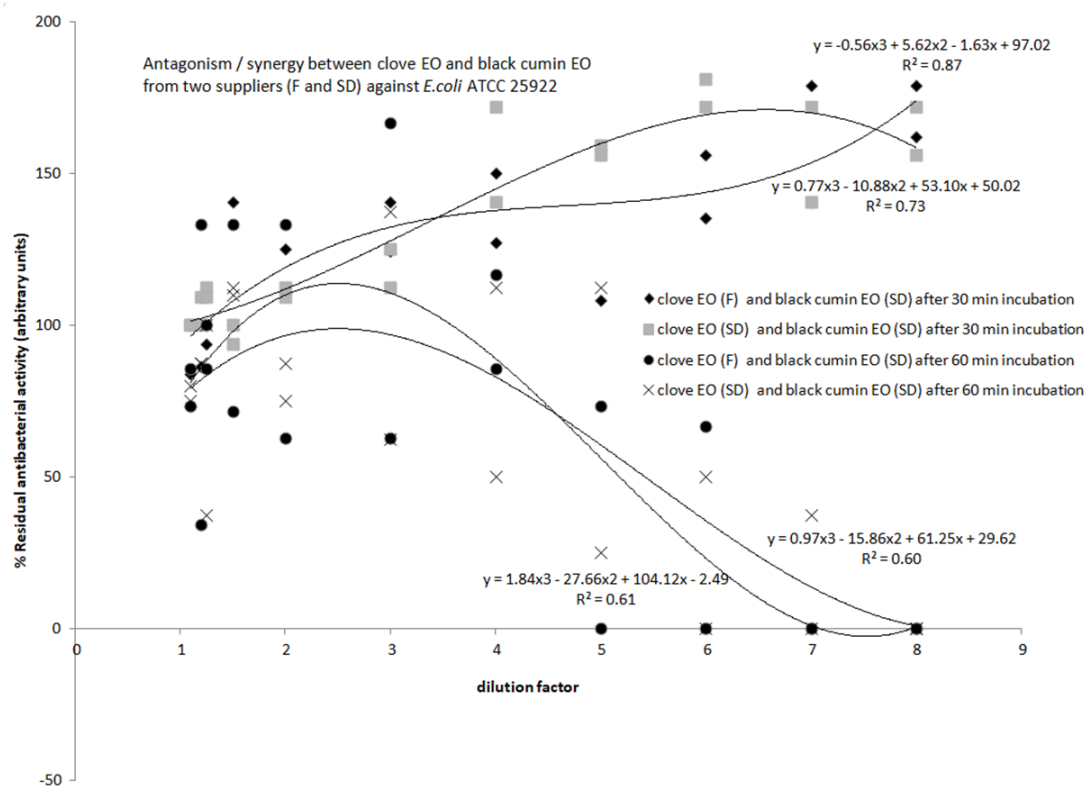
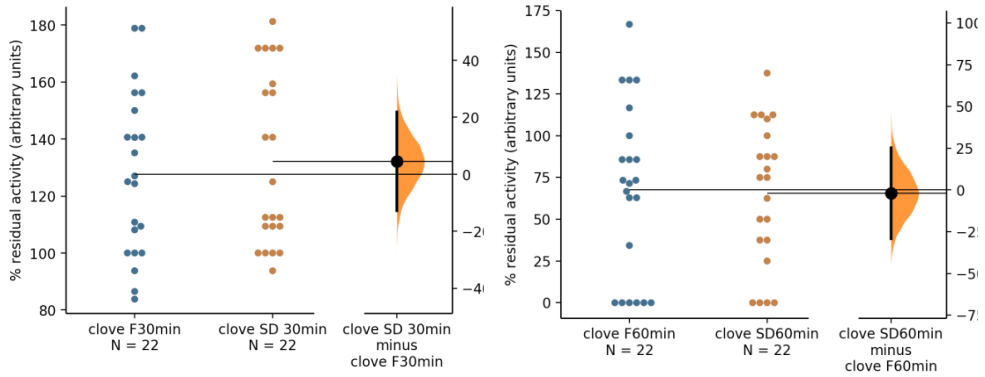


Figure 3.

The antibacterial activity of the clove EO and black cumin EO mixture against *E. coli* ATCC 25922

The comparative % of the residual antibacterial activity (RA) of clove EO from two suppliers, F = Fares SD = Steaua Divină, after dilution with black cumin EO (SD) and incubated for 30 minutes/RT (p = 0.308668) or 60 minutes/RT (p = 0.442908) before performing the antibacterial assay; RT= room temperature



(**a**) (**b**)

Figure 4.

The antibacterial activity of the EOs mixture against *E. coli* ATCC 25922

The mean difference between the % residual antibacterial activity of clove EO from two suppliers (F = Fares and SD = Steaua Divină), after dilution with black cumin EO is shown in the above Gardner-Altman estimation plot. Both groups are plotted on the left axes; the mean difference is plotted on a floating axis on the right as a bootstrap sampling distribution. The mean difference is depicted as a dot; the 95% confidence interval is indicated by the ends of the vertical error bar: (a) after the incubation of the mixtures for 30 minutes/RT, the unpaired mean difference between the two samples of clove is 4.47 [95.0%CI -12.9, 21.8] and p is 0.588; (b) after the incubation of the mixtures for 60 minutes/RT, the unpaired mean difference between the two samples of clove is -2.06 [95.0%CI -29.2, 25.3] and p is 0.943

The applied *t*-test indicates that the differences between the clove EO from the two suppliers are not statistically significant (Figure 3). The findings are confirmed by the second statistical analysis of the effects of combining clove and black cumin EOs (Figure 4). Still, estimation graphic reveals that the transition to antagonism occurs faster clove EO from SD supplier (Figure 4b).

Since there are no statistically significant differences between EOs from the two suppliers, but there are notable differences in the experiments performed after different incubation time of the EOs mixtures, the additional analysis of the overall residual activity was done. The overall % RA of thyme EO diluted with black cumin EO demonstrates that the antibacterial activity of thyme EO decreases in time by the presence of black cumin EO – p = 0.002136 (Figure 5). By solving the equations of the trendline that best fits the results, we could estimate the dilution where it reaches 50% of the RA. For instance, after 60 minutes of incubation, the maximal antibacterial activity of thyme EO is 50% reduced at the dilution 1:1.54 (thyme EO:black cumin EO). In the experiment conducted at 30 minutes of incubation, the 50% of the RA of thyme EO is when thyme EO:black cumin EO dilution is 1:2.71. An explanation might be the time it takes to interact with the active compounds of the two EOs. Since we do not know which the active compounds are involved in this interaction, we can only draw preliminary conclusions about thyme EO – black cumin EO interaction.

We tested the overall % RA of the clove EO diluted with black cumin EO, regardless the supplier (Figure 6). A comparison at the two incubation times of the mixture demonstrated that black cumin EO under specified conditions – the time of incubation – reduces or increases the antibacterial activity of the clove EO. More specifically, one hour of incubation reduces the antibacterial activity of clove EO, while the 30 minutes of incubation increases it (Figure 6). This behaviour of the clove EO in the presence of black cumin EO is more intriguing when analysing the trendlines of equations from Figure 6. The R-squared coefficient indicates that more results from the experiment after 30 minutes of incubation (about 76%) explained the synergy between clove EO and black cumin EO than in the experiment after 60 minutes of incubation (about 61% of results) when antagonism occurred. We can presume that at least two variables influence the antibacterial activity of clove EO – the concentration of black cumin EO and the time of interaction.

The antimicrobial antagonistic effect of combination between clove EO and black cumin EO is noticed after 60 minutes of incubation. Since the trendline equation is polynomial, there are three results for 50% of the RA, but only one value is in the range of dilutions tested experimentally. After 60 minutes of interactions of EOs mixture, 50% of the RA of clove EO is reached when dilution is 1:5.19 (clove EO:black cumin EO). In contrast, 30 minutes of interaction between clove EO and black cumin EO results in an enhanced antibacterial activity of the clove EO. This behaviour is confirmed by the second statistical analysis that supports the antibacterial activity of mixture resulted from thyme and clove EOs in the presence of black cumin EO (Figure 7). The antibacterial activity of thyme EO is lower in the presence of black cumin EO and needs a time of interaction for complete inhibition of the active compounds (Figure 7a). The

antibacterial activity of the clove EO in the presence of black cumin EO increases when the time of interaction is 30 minutes, but decreases after 60 minutes (Figure 7b).

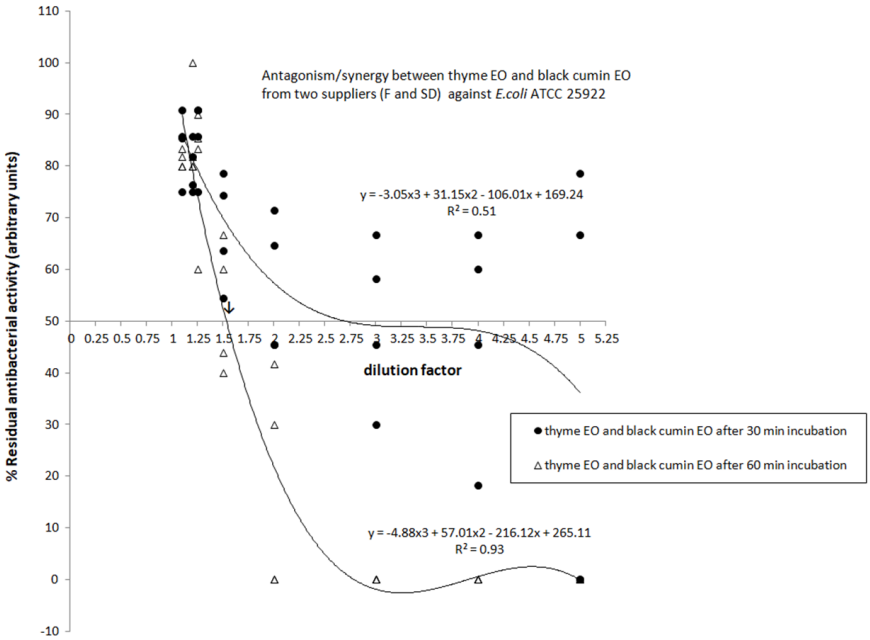


Figure 5.

The antibacterial activity of the thyme EO and black cumin EO mixture against *E. coli* ATCC 25922

The overall comparison of % of the residual antibacterial activity (RA) of thyme EO from F = Fares and SD = Steaua Divină suppliers after dilution with black cumin EO (SD) and incubated for 30 minutes or 60 minutes at RT before perform antibacterial assay ($p = 0.002136$). The arrow indicates the dilution factor 1.54 where 50% RA of thyme EO after 60 minutes of incubation at RT. The dilution factor was 50% RA of thyme EO after 30 minutes of incubation at RT is 2.71. The values estimated from the trendlines equations which best fit the results in the tested dilution range

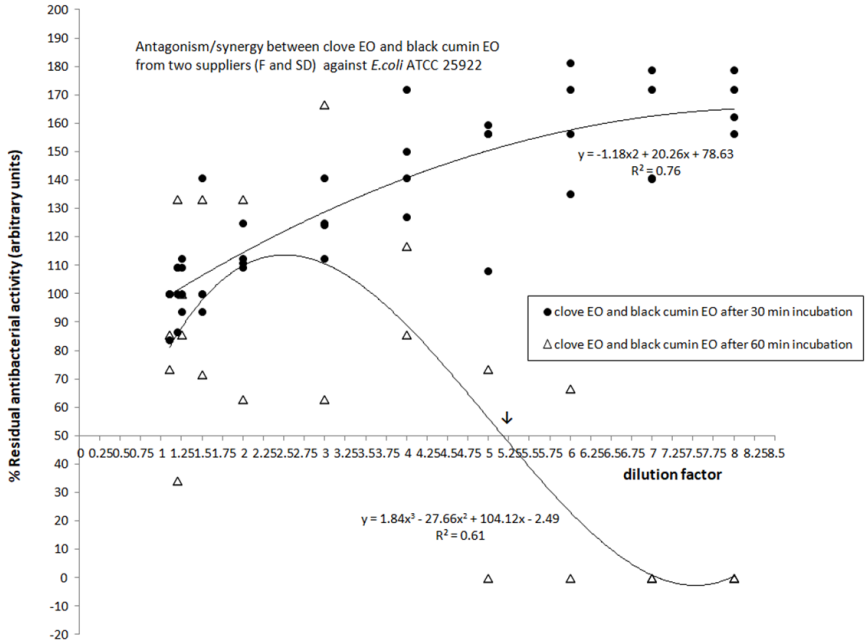


Figure 6.

The antibacterial activity of the clove EO and black cumin EO mixture against *E. coli* ATCC 25922

The overall comparison of % of the residual antibacterial activity (RA) of clove EO from F = Fares and SD = Steaua Divină suppliers after dilution with black cumin EO (SD) and incubated for 30 minutes or 60 minutes at RT before perform antibacterial assay ($p < 0.00001$). The arrow indicates the dilution factor 5.19 where 50% RA of clove EO after 60 minutes of incubation of the EOs mixture at RT. The values estimated from the trendlines equations which best fit the results in the tested dilution range

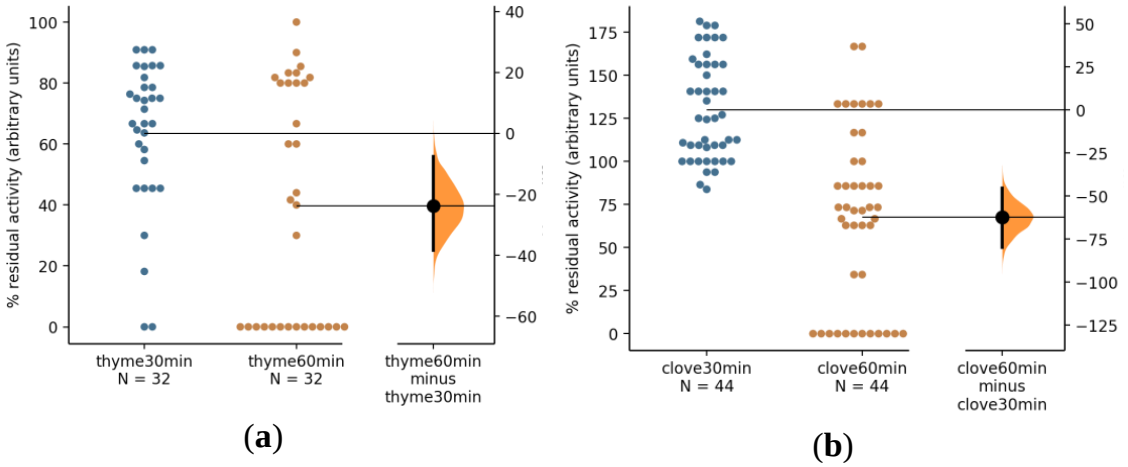


Figure 7.

The antibacterial activity of the EOs mixture against *E. coli* ATCC 25922

The overall comparison of % of the residual antibacterial activity (RA) of thyme EOs and clove EOs in the presence of black cumin EO after different time of incubation, regardless of the suppliers is shown in the above Gardner-Altman estimation plot. Both groups are plotted on the left axes; the mean difference is plotted on a floating axes on the right as a bootstrap sampling distribution. The mean difference is depicted as a dot; the 95% confidence interval is indicated by the ends of the vertical error bar: (a) the % RA of thyme EOs diluted with black cumin EO and incubated at 30 minutes/RT and 60 minutes/RT, respectively. The unpaired mean difference between thyme30min and thyme60min is -23.9 [95.0%CI -38.3, -7.56] and the p value is 0.0255; (b) the % RA of clove EOs diluted with black cumin EO and incubated at 30 minutes/RT and 60 minutes/RT, respectively. The unpaired mean difference between clove 30 min and clove 60 min is -62.4 [95.0%CI -79.7, -45.4] and the p value is 8.44e-09

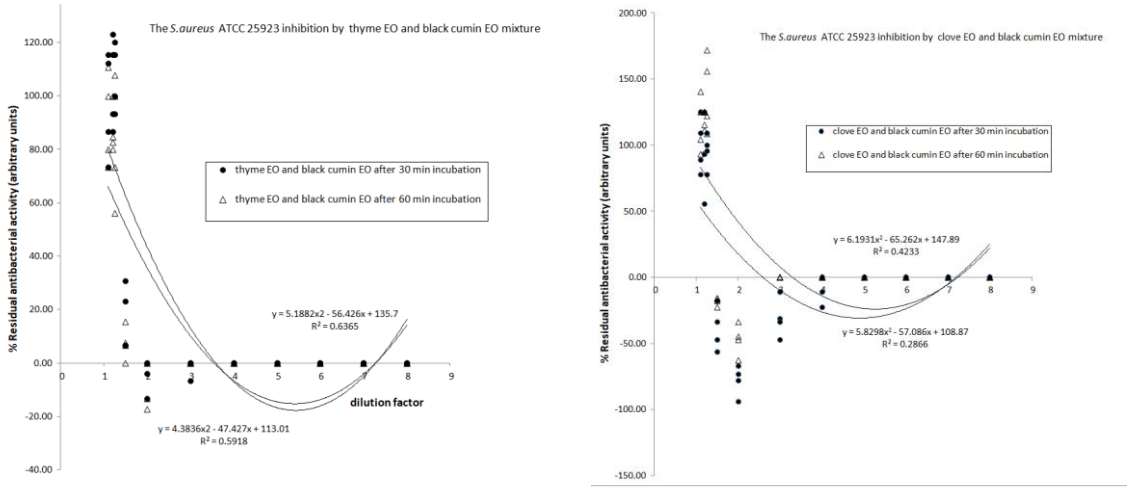
The mutual influence between the biologically active compounds requires the global analysis of experimental results to develop further tests. We do not know how the biological activity of *N. sativa* L. EO is influenced by the thyme EO and the clove EO. The black cumin EO commercial sample tested here does not have antibacterial activity against the *E. coli* ATCC 25922.

The method used in the present study permits the testing of quantities of EOs in a limited range. The mathematical analysis of the trendline equations, which best fits the results, gives some clues about what can happen beyond the experimental results.

Antibacterial activity of thyme EO and clove EO in the presence of black cumin EO against S. aureus ATCC 25923

We made the antibacterial testing of the thyme EO, clove EO, and black cumin EO against the Gram-positive strain *S. aureus* ATCC 25923. We tested the antibacterial activity of the individual EO and EOs mixture against *S. aureus* ATCC 25923**.** We tested individual thyme EO and clove EO at 10 µL. Then we tested black cumin EO at volumes 1 µL, 2 µL, 2.5 µL, 5 µL, 10 µL, 20 µL, 30 µL, 40 µL, 50 µL, 60 µL and 70 µL. In contrast to *E. coli*, the commercial black cumin EO shows antibacterial activity against *S. aureus*. Our study was designed to test a fix volume (10 µL) of thyme EO and clove EO in variable volume of black cumin EO. Thus, the antibacterial effect of black cumin EO against *S. aureus* did not allows the calculation of the residual antibacterial activity of thyme EO and clove EO at high dilutions. Contrary to *E. coli* we observed an increase of the thyme EO antibacterial activity in combination with low volume of black cumin EO when testing *S. aureus* (Figure 8).

The antibacterial activity of some EOs is largely investigated because the mechanism of action is not yet established. The aim of our study was to test the combination of some commercial EOs. The reason of selected commercial EOs instead of new synthesized EOs was to demonstrate that the EOs already existed on market should not be mixed without knowing the effect of these combinations. In a previous study, when testing the antibacterial activity of a series of commercial EOs by disc diffusing method (aromatogram) we noticed a synergy between clove EO and black cumin EO and antagonism between thyme EO and black cumin EO [21]. A major limitation of this method is that the paper disc could be loaded with a small volume of EOs. Because EOs are high volatile compounds the micro-atmosphere assay method is more suitable for testing larger volumes of EOs [38]. Because there is no standardized protocol for testing of different combination of EOs, many assays were made for designing the most suitable workflow. Regarding the time of incubation, a compromise had to be made. A shorter incubation time is not enough to allow the active compounds of the EOs to interact. We are aware of this limitation of the study, but the present protocol, with 30 and 60 minutes incubation at RT, was considered the best to analyse the anti-bacterial synergy/antagonism between tested commercial EOs.



(a) **(b)**

Figure 8.

(a) The overall comparison of antibacterial activity of thyme EO and black cumin EO mixture against *S. aureus* ATCC 25923; (b) The overall comparison of antibacterial activity of clove EO and black cumin EO mixture against *S. aureus* ATCC 25923

F = Fares and SD = Steaua Divină suppliers; the EOs were incubated 30 minutes or 60 minutes at RT after performing the antibacterial tests

The black cumin EO included in the present study does not have antibacterial activity against the Gram-negative bacteria strain *E. coli* ATCC 25922, but inhibited the Gram-positive bacteria strain *S. aureus* ATCC 25923 [4, 21, 33]. This study is not focused on characterization of EOs, because the comparison of different commercial EOs need extensive analysis ranged from the raw material to the analytical method used by each supplier [16, 23, 25, 26]. Moreover, the mechanism of antibacterial activity of the EOs analysed is not clarified. The aim of the present study was to investigate the antibacterial effect of the combination of commercial EOs samples. We were able to respond at the question of our study and demonstrate that black cumin EO could interferes with antibacterial effect of thyme EO and clove EO. In perspective, study with a black cumin EO containing thymoquinone could answer the question of how thymoquinone could influence the antibacterial effect of the thyme EO and the clove EO. Although many compounds from EOs have biological activity, due to the lack of studies on their mode of activity, there are no standardized treatment protocols. More, compounds derived from plants, despite their popularity, were withdrawn from the market by FDA (Food and Drug Administration). It is the example of terpin hydrate (DB13163) used to be as an expectorant in various pulmonary conditions, withdrawn because "based on evidence currently available, there are inadequate work to establish general recognition of the safety and effectiveness of these ingredients" (www.drugbank.ca). The n-alkane standards were not used to compare the retention index of the components of the EOs. Though, the work presented here offers a broader perspective of the mutual influence of some EOs. Further analysis of the major compounds of EOs could provide valuable insights of the topic of interaction between different components of EOs. The present study was focused on analysis of the antibacterial effect of combination of black cumin EO with thyme EO or clove EO. There is mutual relationship between these EOs, but we do not know the modification of biological effects of black cumin EO in the presence of other EOs. Our results showed that, under the experimental condition described in the present study, black cumin EO and clove EO initially had a synergistic antibacterial effect when the EOs were incubated 30 minutes at RT, but 60 minutes of incubation led to antagonism. We are still at the point when the exact mechanisms of the EOs are unknown and the complex connections between the active compounds require extensive experimental studies and theoretical analysis of the literature studies.

Conclusions

The antibacterial activity of two EOs – thyme EO and clove EO – in the presence of back cumin EO was evaluated. Although the present study has not investigated the mechanism of action of each active compound, it was clearly demonstrated that the association of the some EOs could have antagonistic antibacterial effect. The back cumin EO could enhance the clove EO antibacterial effect, but prolonged interaction led to antagonism. More, the activity of

the commercial EOs is different because the major active components are in different concentration or not present at all. Our present results suggest that despite a widespread perception of a harmless effect of commercially available herbal products mixing are not always beneficial.

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Conflict of interest

The authors declare no conflict of interest.

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