Additive antimicrobial [corrected] effects of the active components of the essential oil of Thymus vulgaris–chemotype carvacrol

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Additive antimicrobial effects of the active components of the essential oil of *Thymus vulgaris* - chemotype carvacrol

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Abstract

Herbal remedies are multi component mixtures by their nature as well as by pharmaceutical definition. Being a multi component mixture is not only a crucial property of herbal remedies, it also represents a pre-condition for interactions such as synergism or antagonism. Until now, only few phytomedicines are accurately described concerning the interactions of their active components. The aim of this study was to search for interactions within such a naturally given multi component mixture and to discuss the pharmaceutical and clinical impact. The thyme oil chosen for the examination belongs to the essential oils with the most pronounced antimicrobial activity. Antibiotic activity of thyme oil and single active components were tested against six different strains of microorganisms. The checkerboard assay was used to search for interactions. Time-kill-assay was used to verify the observed effects and to get information about the temporal resolution of the antimicrobial activity. The degree of the detected interactions corresponded with the demarcating FICI-measure of 0.5, which separates the additive from the over additive (synergistic) effects. Therefore, the observed effect was called a "borderline case of synergism" respectively "partial synergism". Partial synergism was observed only in the presence of *Klebsiella pneumoniae.* Additive antimicrobial activity was observed for the combination of the two monosubstances carvacrol plus linalool and thymol plus linalool as well as with the combination of the two essential oils of the carvacrol and linalool chemotypes. An increase of the carvacrol-oil concentration from one to two times the MIC resulted in a considerable acceleration of the kill-rate. Thyme oil is composed of several different components that show antimicrobial activity (at least: carvacrol, thymol and linalool). The antimicrobial activity of thyme oil is partly based on additive effects, which might especially enhance the rapidity of the antimicrobial action. In addition, a mixture of several active ingredients that varies in its composition from year to year and from lot to lot as is the case with herbal remedies may be more stable concerning the antimicrobial activity than mixtures containing just a single active component.

Key words: *Thymus vulgaris L.* (Lamiaceae), *Klebsiella pneumoniae*, partial synergism, antibacterial activity, kill-rate

Abbreviations

Cve: carvacrol oil, Cvm: carvacrol monosubstance, Thm: thymol monosubstance, Loe: linalool oil, Lom: linalool monosubstance, Ac: artificial combination, FICI: fractional inhibitory concentration index, Gm: Gentamycin, MIC: minimal inhibition concentration, MBC: minimal bactericidal concentration

Introduction

Interactions between biologically active agents have become an important subject of scientific research since various synergistic and antagonistic effects of therapeutic importance have been discovered. In this paper, the focus concerning interactions lie on synergistic antimicrobial activities of the essential oil of *thymus vulgaris L.* and its active components. Herbal remedies are multi component mixtures by their nature as well as by definition. Being a multi component mixture is not only a crucial property of herbal remedies it also represents a pre-condition for interactions such as synergism or antagonism. Synergism roughly means “working together”, whereas antagonism means “working against each other”. In both cases, the effect of the mixture would be more than the pure sum of its single parts. Whereas the checkerboard method is the undisputable method to search for synergism respectively antagonism there exists a multitude of different definitions and concepts of synergism [1, 2]. Nevertheless, the fractional inhibitory concentration index (FICI) to calculate the degree of synergism respectively antagonism and the isobole method to visualize the data are well established by now [3-5]. The time-kill method was used to get information about the time dependent progression of the antimicrobial activity.

Until now, only few phytomedicines are accurately described concerning the interactions of their active components [6]. An eligible subject to study interactions is the essential oil of *Thymus vulgaris L.* because its strong antimicrobial activity is based on several well-known active components. Six strains of microorganisms and three different test methods were used to investigate the interactions between the active components of the essential oil of *thymus vulgaris*.

Materials and Methods

Two chemotypes of the essential oil of *Thymus vulgaris L.* were used, the chemotype thymol (Lot: 097144), which we called carvacrol-type (Cve) according to the GC-analysis identifying carvacrole as main component and the chemotype linalool (Loe; Lot: 10608). Both chemotypes were available through Farfalla (Zurich, Switzerland www.farfalla.ch/). The seven monosubstances representing the main components of the oils were purchased from Fluka: thymol (Thm; Lot: 1239340), carvacrol (Cvm; 1281965), linalool (Lom; 1230021), borneol (Lot: 1326329), gerani (Lot: 1330352)ol, p-cymene (Lot: 1282027) and γ-terpinene (Lot: 1327393).

Six different strains of microorganisms were chosen because of their clinical relevance as pathogens. They were purchased from "Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH" (DSMZ). The gram positive bacteria were: *Staphylococcus aureus ssp. aureus* (Sta) [DSM-Nr.: 799]; *Staphylococcus epidermidis* (Ste) [DSM-Nr.: 20044]; *Staphylococcus saprophyticus ssp. saprophyticus* (Sap) [DSM-Nr.: 20229]. The gram negative bacteria were: *Klebsiella pneumoniae ssp. pneumoniae* (Kl) [DSM-Nr.: 681]; *Escherichia coli* (Ec) [ATCC-Nr.: 25922]. One fungus was the yeast *Candida albicans* (Ca) [DSM-Nr.: 1386].

Müller-Hinton Broth from Oxoid (CM0405) was used as medium. Bacteriological Agar (REF: 4110302) was purchased from Biolife.

Ampicillin sodium salt (≥99.0%) (A0839.0025, Lot. 7V008524) and Gentamycin (Gm-sulfate with 600 μg/mL specific activity: A1492.0005, Lot. 5T002854, total impurities ≤10% water) were purchased from Applichem.

Solubility

The essential oils and monosubstances were diluted in 0.1% agar. Agar was added as a means for dispersion which inhibited respectively delayed the separation of the hydrophobic substrate from the aqueous phase of the medium [7, 8].

MIC and MBC

Determination of the minimal inhibition concentration (MIC) was carried out using 96-well-microtiter-plates and Mueller Hinton broth as medium. Inoculum size was diluted to obtain final inoculums of 10^5 – 10^6 colony-forming-units per mL [cfu/mL]. The highest concentration of essential oil tested was four times the MIC expected for the particular microorganism. The following rows contained twofold serial dilutions of the substance. The last row contained no essential oil and served as growth control. The essential oil concentration of the first well without visible growth represented the MIC. To determine the minimal bactericidal concentration (MBC) two times 20 μl were taken from the first three wells without visible growth, dropped on a Mueller Hinton agar plate and incubated for 24 hours at 37°C. The concentration which resulted in a reduction of the inoculum of ≥ 99.9% was defined as MBC. The antibiotic gentamycin (Gm) was used as positive control.

The checkerboard method

The checkerboard method was chosen to determine antimicrobial interactions such as synergism and antagonism between two different substances. The uppermost row (A) of a 96-well-microtiter-plate contained substance X in a concentration of about four times the expected MIC of the microorganism examined. Each following row (B-H) contained half the concentration of the previous one. The same procedure was carried out along the columns (1-12) with substance Y – but not necessarily with the same starting concentration. So, each well contained a unique combination of the two substances (X & Y). At last 100 μL Mueller Hinton broth containing about 10^5 [cfu/mL] was added to the wells and incubated at 37°C for 24 hours. The concentrations of the first wells without visible growth along the stepwise boundary between inhibition and growth were used to calculate the FICI-values.

The Fractional inhibitory concentration index

The Fractional inhibitory concentration index (FICI) of Elion et al. [9] is a simple mathematical approach to describe interactions quantitatively.

$$\mathbf{FICI} = \frac{\mathbf{MIC\ X\ in\ combination}}{\mathbf{MIC\ X\ alone}} + \frac{\mathbf{MIC\ Y\ in\ combination}}{\mathbf{MIC\ Y\ alone}}$$

Isobologram

An isobologram depicts the results of the checkerboard assay and the FICI-values. The x-axis of the isobologram represents substance X, the y-axis substance Y. The MIC-value of X is located on the x-axis, and the MIC of Y on the y-axis. The line connecting these two points represents the line of no interaction (line of indifference). Below the line of indifference we find the area of additive ($1 > FICI > 0.5$) and synergistic ($FICI \leq 0.5$) effects. Above the line of indifference are the combinations with subtractive ($1 < FICI < 4$) and antagonistic ($FICI \geq 4$) effects.

The time-kill method

The time-kill method was used to get information about the time dependent progression of the antimicrobial activity. Treatment of bacteria with different combinations of essential oils was performed in 2 mL Mueller Hinton medium and incubated at 37°C. At different time points (0, 15, 30, 60, 120, 240, 360 minutes and 24 hours) aliquots of 50 μL were taken and transferred to 450 μl of sodium chloride (0.9%) resulting in a 1:10 dilution. Further 10-fold dilutions were prepared (10^{-1} to 10^{-6}). Three times 20 μL of each dilution were dropped on Mueller Hinton agar plates and incubated for 24 hours at 37°C to determine the number of cfu/mL at the different time points.

Analysis of the essential oils

The essential oils were analyzed by gas-chromatography (GC) by the certified laboratory “Passafaro’s Analytic Lab” (Thayngen).

The GC analysis was carried out on a HP 5890 gas chromatograph. The GC column used was a DB-Wax fused silica capillary with a macrogel 20 000 R stationary phase (film thickness 0.25 μm), a length of 30 m and an internal diameter of 0.25 mm. The carrier gas was Hydrogen with a flow rate of 4.3 ml/min. The temperature of the oven was programmed as follows: 60°C, hold time 4 min, 5°C/min to 170°C, hold time: 15 min, runtime 40 min; injector temperature 220°C; detector temperature 270°C. Injection volume 1 μL; split ratio 1:100.

The identification of the components was based on reference solutions (monosubstances) from Fluka: thymol (>99.0%), carvacrol (>97.0%), linalool (>95.0%), borneol (>99.0%), geraniol (>99.0%), p-cymene (>99.5%) and γ-terpinen (>98.5%).

Statistical Methods

A double sided t-test of independent samples was used for the comparison of the MIC's (α = 0.05). Confidence intervals $CI_{(95\%, n)}$ of the FICI values were calculated to decide whether they differed from the threshold value of FICI < 0.5. To depict the trend of the FICI-values of the isobologram, a non linear regression curve was calculated. The most simple exponential equation was chosen as mathematical model $\{y(t) = a \cdot e^{-\lambda \cdot t}\}$.

Results

The GC-analysis was necessary for the comparison of the antimicrobial activities of the two chemotypes of the thyme oils (*Thymus vulgaris)* and their main components as monosubstances. The observed deviation of the main components between the two lots, which were derived from two consecutive years are consistent with a naturally occurring deviation (Tab. 1).

MIC's of the thyme oils (Cve and Loe) and the corresponding main monosubstances were determined (Tab. 2). The antibiotic activity of Cve was about 10 times stronger than that of Loe. Both chemotypes showed antimicrobial activity against all six examined microorganisms. In four out of the six bacterial strains, the antibiotic activity of the Cve was significantly higher than the activity of the monosubstances Cvm and Thm ($p \leq 0.05$). Loe which consists of more than 70% linalool did not show such superiority over Lom. Only weak antimicrobial activity against Klebsiella exhibited the monosubstances borneol (1250 μg/mL) and geraniol (2000 μg/mL).

The time-kill method yielded detailed informations about the temporal progression of the antimicrobial activity. Concentrations of 1 MIC Gm and Cve had only bacteriostatic effects. At concentrations of 2 MIC or more Gm and Cve reached bactericidal activity. A Cve concentration of two times the MIC resulted in a reduction of 10^3 [cfu/mL] within half an hour, whereas Gm at two times the MIC needed more than three hours (Fig. 1). Such an acceleration of the antimicrobial activity was observed with all six examined bacterial strains.

The combination of the monosubstances Cvm and Lom as well as Thm and Lom and the combination of the essential oils Cve and Loe demonstrated reproducible antimicrobial activities that almost reached the extent of synergism (Fig. 2 - 4). The interaction was called a "borderline case of synergism" respectively "partial synergism" because the confidence interval (CI: 95%, n) of the mean FICI value enclosed the threshold value of 0.5, which indicates the borderline between additive and over additive effects (synergism) [4, 10]. Most of the FICI values were slightly higher than the threshold-value of 0.5 (Tab. 3 - 5). Additive effects (FICI ~ 0.5) appeared only if the fraction of the weaker mono-substanc (Lom) dominated the mixture. However, mixtures of the essential oils exhibited additive effects even when the stronger Cve dominated the mixture ratios (Tab. 3).

To determine the contribution of the lesser substances to antimicrobial activity, Cve was compared with an artificial combination containing Cvm and Thm in identical concentrations as in the essential oil. The artificial

combination (Cvm & Thm) demonstrated a slightly reduced antimicrobial activity (MIC) against all microbial strains compared to Cve, the naturally occurring multi-component mixture (Tab. 6). Against Ste, Sta, Sap and Ec, the MIC's of the artificial mixtures were twice the MIC of the essential oil. For *Klebsiella pneumoniae* the difference in the MIC's of artificial and natural mixtures was four.

A more detailed picture was obtained by the time-kill method carried out with different concentrations of Cve and the artificial mixture (Ac). The Ac was composed of Cvm and Thm in identical concentrations like Cve (Fig. 5). No difference between Cve and Ac was detected at concentrations of 200 μg/mL Cve and Ac. At concentrations of 300 and 400 μg/mL the time dependent progressions of antimicrobial activities differed explicitly between Cve and Ac even though the cfu-count at 24 hours were similar. Cve at a concentration of 600 μg/mL produced a decrease of the inoculum of *Klebsiella pneumoniae* of 10^2 [cfu/mL] within 15 minutes whereas 600 μg/mL of the Ac needed approximately 3 hours (Fig. 6). The cfu-count after 24 hours was similar.

Discussion

The two chemotypes carvacrol and thymol of the essential oil of *Thymus vulgaris L.*, belong to the essential oils with the strongest antimicrobial activity [11]. It is well known, that the antimicrobial activity of these oils depends on more than one active ingredient. At least carvacrol, thymol and linalool exhibit considerable antimicrobial activity.

The first indication that Cve might be more effective than its main active components was obtained by the MIC's of Cve and the monosubstances Cvm, Thm and Lom (Tab. 2).

The checkerboard assay is the method of choice to search for antimicrobial interaction between two different substances [5]. By this method, additive interactions between Cvm and Lom and between Thm and Lom were documented against *Klebsiella pneumoniae*. Because the confidence interval ($CI_{95\%, n}$) enclosed the FICI threshold-value of 0.5, indicating the borderline between additive and over additive effects (synergism) [4, 5], the observed interactions were called "borderline cases of synergism" respectively "partial synergism". Most of the FICI values were slightly above the threshold-value of 0.5 (Tab. 3 – 5). Strong additive effects (FICI ~ 0.5) appeared only if the fraction of the weaker monosubstance (Lom) predominated in the mixture. Once this dominance of Lom was achieved, the FICI-value of 0.5 persisted over a broad range of concentration ratios. Mixtures of the two essential oils Cve and Loe exhibited additive effects even in ranges of concentration ratios where the stronger Cve predominated (Tab. 3). Minor components even without any antimicrobial activity might be responsible for this effect.

The results of the three different methods (MIC, checkerboard and time-kill) concerning the qualitative effect of the interactions examined were consistent to a large extent. Consistency between the checkerboard and the time-kill method has also been reported by White et al. 1996 [12, 13]. Although the time-kill method revealed a faster activity of Cve compared to the artificial combination of Cvm and Thm, no synergism was observed after 24 hours which may also be interpreted as a "borderline case of synergism".

The time-kill method reveals a more detailed insight into the antimicrobial activities of the essential oil and the artificial mixture. Providing a dynamic picture of antimicrobial activity, the time-kill method revealed that a doubling of the concentration of the Cve from 1 to 2 MIC caused a considerable increase of the kill rate. At concentrations of 600 μg/mL Cve decreased the inoculum of Klebsiella within 15 minutes of 10^2 [cfu/mL] whereas the same concentration of the Ac needed approximately 3 hours (Fig. 6). This rapid killing time might be an

important quality for therapeutic applications of thyme oils [14]. Comparable rapid killing times were also reported for tea tree oils [15].

For the time-kill method, synergism is defined as a 100-fold decrease in colony count at 24 hours, compared to the most active single drug. Unfortunately, this definition holds back valuable data of the progression with time which represent an enormous advantage of the method. The time dependent progression reveals big dynamic differences in antimicrobial activity between Cve, Ac and the monosubstances (Cvm, Thm and Lom). This documented rapidity of killing may be a great advantage of the essential oil in certain therapeutic applications such as gargling, inhalation [16] or topical application on wounds because in these cases direct physical contact with infected tissues lasts only a short time.

A further aspect concerning the multi component character of herbal medicinal products is the naturally occurring variability of their composition from year to year and from lot to lot (Tab. 1). Complete disappearance of one active part of a synergistic mixture might result in a considerable loss of activity. But, if changes in composition are rather quantitative than qualitative, a diversity of active ingredients might have a stabilizing effect on the overall activity. The chemical analysis of the two lots of Cve revealed quantitative differences in composition. However, the antimicrobial activities (MIC's) were not affected by these quantitative differences (data not shown). Whether this advantage prevails over a wider range of lots and essential oils produced in different environments and under varying conditions (soil and climate), deserves further attention.

In conclusion, the carvacrol oil is composed of several active components affecting the antimicrobial activity. The antimicrobial activity of thyme oil is partly based on additive effects, which especially increase the kill-rate. A high kill-rate might be of clinical importance in certain therapeutic applications such as gargling, inhalation or topical application. In addition, a mixture of several active ingredients that varies in its composition from year to year and from lot to lot as is the case with herbal remedies may be more stable concerning the antimicrobial activity than mixtures containing just a single active component.

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Legends for Tables

Tab. **1** Content of the main components of two lots of consecutive years of the carvacrol- and the linalool-type according to the GC-analysis.

Tab. **2** Minimal inhibition concentration (MIC-values) of the monosubstances carvacrol (Cvm) and thymol (Thm) compared to the MIC-values of the carvacroloil (Cve).
(*$p \leq 0.05$ and **$p \leq 0.005$), R = resistant.

Tab. **3** Results of the checkerboard assay: Concentration-ratios (Cv/Lo), concentrations in [µg/mL] and FICI-values.

CI (95%, n=10): 0.4832 – 0.6906.

Tab. **4** Results of the checkerboard assay: Concentration-ratios (Cvm/Lom), con-centrations in [µg/mL] and FICI-values.

CI (95%, n=6): 0.427 – 0.7196.

Tab. **5** Results of the checkerboard assay: Concentration-ratios (Thm/Lom), concentrations in [µg/mL] and FICI-values.

CI (95%, n=6): 0.4262 – 0.7195.

Tab. **6** The MIC values of carvacrol oil and artificial combinations containing the monosubstances in the same concentrations as carvacrol oil (monosubstances: Cvm = carvacrol; Thm = thymol; Lom = linalool).

Legends for Figures

Fig. **1** Time-kill curves of one and two MIC carvacroloil and gentamycin in the presence of *Klebsiella pneumoniae*.

The dotted line represents the MBC threshold. △ Cve-1-MIC; ▲ Cve-2-MIC; □ Gm-1-MIC; ■ Gm-2-MIC; O Control.

Fig. **2** Isobologram of the essential oils linalool and carvacrol. Each point represents one of the concentration-ratios of table 3.

Fig. **3** Isobologram of the monosubstances carvacrole and linalool. Each point represents one of the concentration-ratios of table 4.

Fig. **4** Isobologram of the monosubstances thymol and linalool. Each point represents one of the concentration-ratios of table 5.

Fig. **5** Composition of the thyme oil, chemotype carvacrol (Cve), versus the artificial combination (Ac).

Fig. **6** Time-kill curves of an artificial combination (Ac) and carvacroloil (Cve) in the presence of *Klebsiella pneumoniae*.

The dotted line represents a reduction of 10^2 cfu/mL of the inoculum. △ Cve [300 μg/mL], ▲ Cve [600 μg/mL], ◇ Ac [300 μg/mL], ◆ Ac [600 μg/mL], × Gm [0.3 μg/mL], O Control.

Tab. **1**

%	Carvacrol-Type		Linalool-Type	
	Lot 1	Lot 2	Lot 1	Lot 2
Carvacrol	**49.37**	**36.06**	**0.16**	**0.17**
Thymol	**16.21**	**16.70**	**3.11**	**3.74**
p-Cymene	**14.79**	**14.09**	**1.35**	**1.53**
Linalool	**2.96**	**4.40**	**78.95**	**72.33**
γ-Terpinene	**1.58**	**8.52**	**0.73**	**1.29**
Borneol	**1.02**	**1.12**	**0.47**	**0.41**
Geraniol	**0.13**	**0.12**	**0.79**	**3.47**

Tab. **2**

	MIC [μg/mL]					
Microorganism	**Ste**	**Sta**	**Sap**	**KI**	**Ec**	**Ca**
Antibiotics:						
Ampicillin (Ap)	**12.42**	**0.10**	**0.27**	**R**	**6.25**	**–**
Gentamycin (Gm)	**0.03**	**0.41**	**0.03**	**0.32**	**1.90**	**–**
Thyme oils:						
Carvacrol (Cve)	**375**	**208**	**313**	**169**	**313**	**176**
Linalool (Loe)	**3884**	**1832**	**2955**	**983**	**1563**	**1563**
Monosubstances:						
Carvacrol (Cvm)	**530***	**380***	**390***	**260**	**390***	**230**
Thymol (Thm)	**710****	**420****	**500****	**240***	**500****	**260***
Linalool (Lom)	**4630**	**2310**	**1690**	**840**	**1690**	**1690**

Tab. **3**

Cv / **Lo**	**[Cve]**	**[Loe]**	**FICI**
1 / 0	160	0	1
8 / 1	80	10	0.51
4 / 1	80	20	0.52
2 / 1	80	40	0.53
1 / 1	80	80	0.56
1 / 2	80	160	0.63
1 / 4	80	312	0.75
1 / 8	40	312	0.50
1 / 16	20	312	0.37
1 / 32	20	625	0.63
1 / 64	10	625	0.56
0 / 1	0	1250	1

Tab. **4**

Cvm / Lom	**[Cvm]**	**[Lom]**	**FICI**
1 / 0	50	0	1
1 / 6	25	160	0.56
1 / 12	25	310	0.63
1 / 25	25	625	0.75
1 / 50	12.5	625	0.50
1 / 100	6.3	625	0.38
1 / 200	6.3	1250	0.63
0 / 1	0	2500	1

Tab. **5**

Thm / Lom	**[Thm]**	**[Lom]**	**FICI**
1 / 0	80	0	1
1 / 4	40	156	0.56
1 / 8	40	312	0.63
1 / 16	40	625	0.75
1 / 31	20	625	0.50
1 / 63	10	625	0.38
1 / 125	10	1250	0.63
0 / 1	0	2500	1

Tab. **6**

	MIC [μg/mL]					
Microorganism	**Ste**	**Sta**	**Sap**	**KI**	**Ec**	**Ca**
Cve	600	300	300	150	600	300
Cvm & Thm	1200	600	600	600	1200	600
Cvm & Thm & Lom	1200	600	600	600	1200	300

Fig. **1**

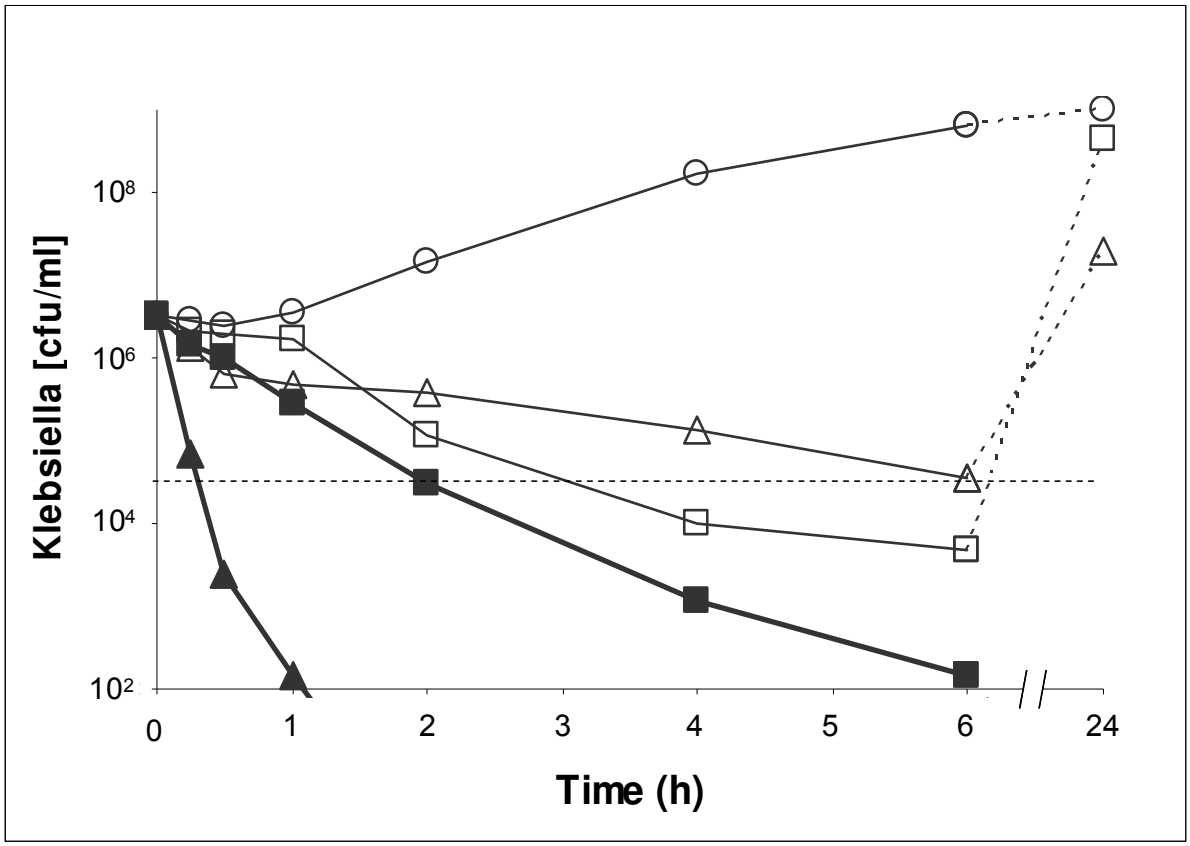

Fig. **2**

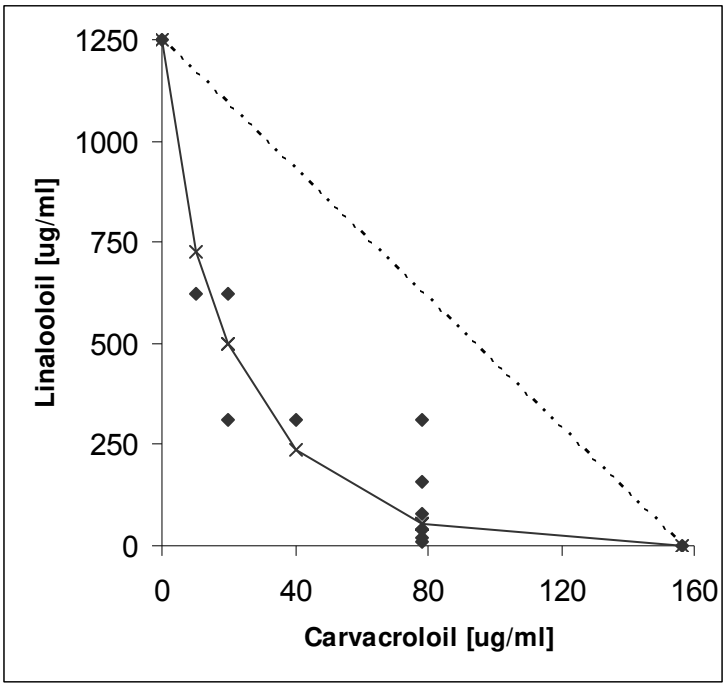
1250
1000
750
500
250
0
Linalooloil [ug/ml]
0
40
80
120
160
Carvacroloil [ug/ml]

Fig. **3**

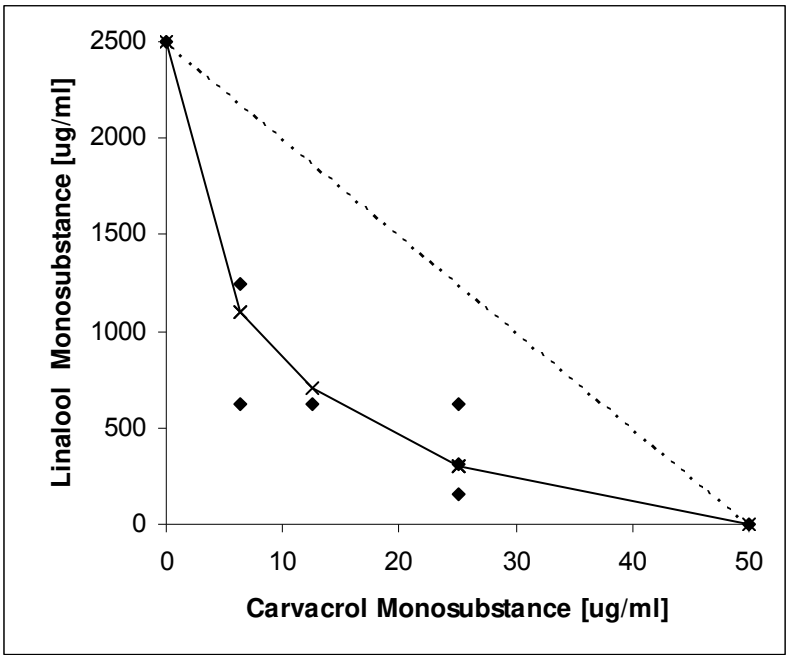
2500
2000
1500
1000
500
0
Linalool Monosubstance [ug/ml]
0
10
20
30
40
50
Carvacrol Monosubstance [ug/ml]

Fig. **4**

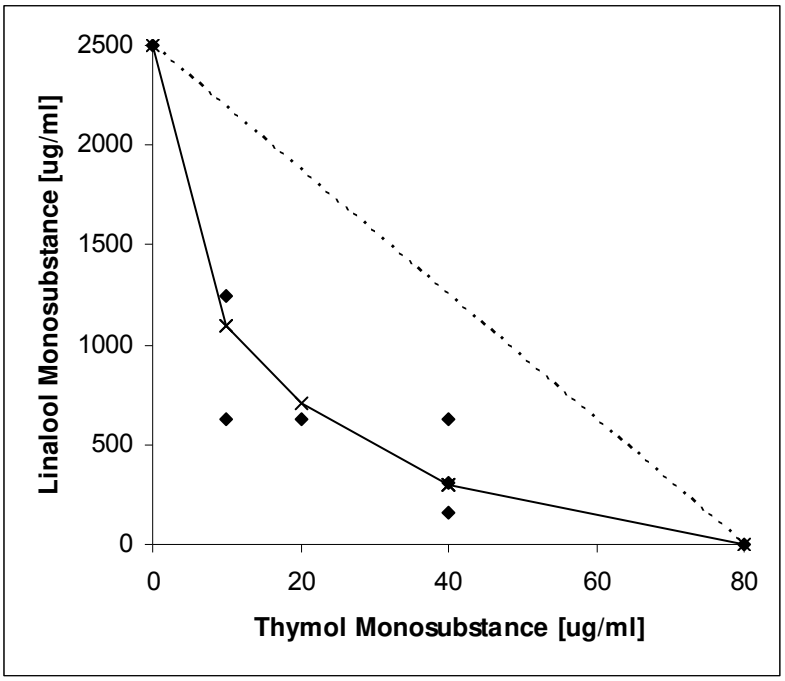
2500
2000
1500
1000
500
0
Linalool Monosubstance [ug/ml]
0
20
40
60
80
Thymol Monosubstance [ug/ml]

Fig. **5**

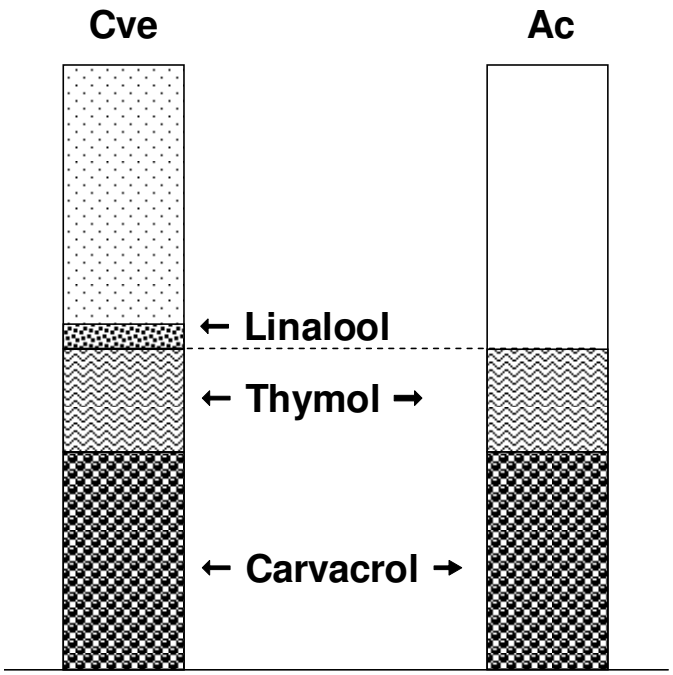

Fig. **6**