The anticonvulsant and anti-plasmid conjugation potential of *Thymus vulgaris* chemistry: an in vivo murine and in vitro study

Running title: Anticonvulsant activity of *T. vulgaris* essential oil

Krystyna SKALICKA-WOŹNIAK[a,*], Magdalena WALASEK[a], Tariq M. ALJARBA[b], Paul STAPLETON[b], Simon GIBBONS[b], Jianbo XIAO[c], Jarogniew J. ŁUSZCZKI[d,e]

[a] *Department of Pharmacognosy with Medicinal Plant Unit, Medical University of Lublin, Chodzki 1, PL 20-093 Lublin, Poland*

[b] *Research Department of Pharmaceutical and Biological Chemistry, UCL School of Pharmacy, London, WC1N 1AX, UK*

[c] *Institute of Chinese Medical Sciences, University of Macau, Taipa, Macau*

[d] *Department of Pathophysiology, Medical University of Lublin, Jaczewskiego 8b, PL 20-090 Lublin, Poland*

[e] *Isobolographic Analysis Laboratory, Institute of Rural Health, Jaczewskiego 2, PL 20-950 Lublin, Poland*

[*] Corresponding author: Department of Pharmacognosy with Medicinal Plant Unit, Medicinal University of Lublin, 1 Chodzki Str., 20-093 Lublin, Poland

Email address: kskalicka@pharmacognosy.org (K. Skalicka-Woźniak)

Tel.: +48 81448 7086, fax: +48 81448 7080

Highlights

- The first report on the direct separation of terpenoids from thyme oil by HPCCC
- MES test in mice used for evaluation of the anticonvulsant activities
- Borneol, thymol, eugenol exerted the strongest protection against induced seizures
- Linalool had 84% reduction on the transfer of *E. coli* plasmid pKM101

Formatted: Font: Italic

Abstract:

~~To assess the influence of *Thymus vulgaris* EO and its principal components on the antiepileptic activity, t~~The high-performance counter-current chromatography was used for the efficient purification of single constituents from *Thymus vulgaris* essential oil. Mixtures of *n*-heptane, ethyl acetate, methanol, and water (5:2:5:2 and 4:1:4:1 *v/v*), allowed purification of eugenol, 1-octen-3-ol, borneol, thymol, terpinen-4-ol, and camphor, while *n*-hexane, acetonitrile, and *tert*-butyl methyl ether (1:1:0.1 *v/v*) yielded carvacrol, borneol, linalyl acetate, caryophyllene oxide, *p*-cymene, and eucalyptol. The anticonvulsant activities were evaluated in the maximal electroshock-induced seizure test in mice model (systemic *i.p.* administration). The ~~EO~~ oil exerted protection against MES-induced seizures when administered 15 and 30 min before the tests (50 and 62.5%, respectively). Among the isolates, borneol, thymol, and eugenol exerted the strongest protection against seizures. Moreover, linalool had the ability to reduce the transfer of the pKM101 plasmid by 84%, what has the potential to reduce virulence and resistance spread in *E. coli*. No acute toxic effects towards the CNS were noticed either for the essential oil ~~EO~~ or for single compounds, in the chimney and grip-strength tests. The preclinical screening of *Thymus vulgaris* EO, as well as isolated terpenoids, provides evidence that the EO has partial protective activity against seizures and HPCCC technique is suitable for its large scale isolation.

Keywords: epilepsy, seizure, natural products, terpenes, isolation, purification, anti-conjugation, *Escherichia coli*

Abbreviations: EO, essential oil; HPCCC, high-performance counter-current chromatography; MES, maximal electroshock-induced seizure test.

*Manuscript for revision (track changes hidden)
Click here to view linked References

1. Introduction

Thymus vulgaris L. (Lamiaceae) is one of the major global plants of interest and as is one of the most often cultivated herbs. It is well-known for its many beneficial health effects, including antimicrobial, carminative, and antioxidant activities. It is used to manage diseases of the upper respiratory tract because of its antiseptic and expectorant activities. Spasmolytic effects on the gastrointestinal and increasing gastric juice secretion have also been reported. Thymol and carvacrol, the main compounds, can also act as strong anti-inflammatory agents.(EMA, 2009; Nabavi, Marchese, Izadi, Curti, Daglia & Nabavi, 2015).

It has been shown that plant-derived EOs exhibit a wide variety of activities toward the central nervous system (CNS) and may be considered as alternative options in the treatment of anxiety (Melo et al., 2010) and epilepsy, as they have been used in traditional medicine for these purposes (Nabavi et al., 2015). As small lipophilic molecules, the volatile compounds from EO are likely to cross the blood-brain barrier readily (Jukic, Politeo, Maksimovic, Milos & Milos, 2007). *Thymus vulgaris* EO has the ability to inhibit acetylcholinesterase in the CNS and thereby shows a neuroprotective effect (Youdim & Deans, 2000; Jukic et al., 2007). It was demonstrated that thymol, the principal component of the EO, is associated with anxiolysis, cessation of convulsions, and sedation as a positive allosteric modulator of the $GABA_A$ receptors (Priestley, Williamson, Wafford & Sattelle, 2003; Garcia, Bujons, Vale & Sunol, 2006). Studies by Mohammadi and colleagues (Mohammadi, Haeseler, Leuwer, Dengler, Krampfl & Bufler, 2001) demonstrated the ability of thymol to directly activate $GABA_A$ receptors in the absence of the natural agonist. Modulation of GABA receptors in the CNS has also been reported for eugenol (Reiner, Perillo & Garcĭa, 2013), which decreases the cytotoxic effects of amyloid β peptides towards neuronal cells and acts as an antidepressant agent in mice through the inhibition of MAO-A and B (Tao, Irie, Lia & Keunga, 2005). By interaction with the dopaminergic system, carvacrol shows anxiolytic effects

in the plus maze test (Melo et al., 2010) as well as antidepressant properties. Importantly no activity towards the serotonergic and noradrenergic systems was noticed (Melo et al., 2011). Linalool, which is also a main component of *Thymus vulgaris* EO, inhibited the occurrence of seizures in an experimental model of epilepsy *in vivo*, showing a sedative effect on the CNS system. It reduced the amount of quinolinic acid, which is responsible for the occurrence of seizures (Elisabetsky, Brum & Sousa, 2009).

According to Kannappan et al. (Kannappan, Gupta, Kim, Reuter, & Aggarwal, 2011) numerous lines of evidence have shown that modification of the diet can benefit patients with epilepsy, and the potential of many culinary spices awaits exploration. Based on the promising results described above, *Thymus vulgaris* EO should be evaluated as a possible antiepileptic agent. Moreover, it has been hypothesized that the enterotoxins of *Escherichia coli* have the ability to lower the seizure threshold by disturbing the balance between excitatory and inhibitory synaptic systems (Eloma, 1979). In imbalance of *E. coli* growth may lead to an increase of enterotoxin levels which could trigger seizures. Consequently, reducing the virulence of *E. coli*, for example by inhibiting the conjugation of its plasmids (which harbor its virulence genes), may have an impact on the aetiopathogenesis of epilepsy.Therefore the aim of our study was to assess the influence of the essential oil and its principal components, on the antiepileptic activity in mice with the maximal electroshock-induced seizure test (MES) and evaluate their anti-conjugation ability in *E. coli*. Such studies require access to the purified metabolites, which can be a time-consuming and an expensive process. In this study, high-performance counter-current chromatography (HPCCC) was used for the efficient purification of single constituents. This technique is based on partitioning between two liquid phases, where one of the liquids is a stationary phase, which eliminates the frequently observed irreversible process of sample absorption during chromatographic processing (Skalicka-Woźniak & Garrard 2015). The technique is effective for the efficient and rapid purification of active compounds from plant extracts, and more importantly, for the smooth isolation of pure

constituents from EO (Skalicka-Woźniak, Walasek, Ludwiczuk & Głowniak, 2013; Skalicka-Woźniak & Walasek 2014). The process was scaled from analytical to preparative format and allowed the preparation of sufficient quantities of purified terpenoids for our pharmacological studies.

2. **Materials and methods**

2.1. Chemical reagents

Analytical grade *n*-heptane, *n*-hexane, methanol, ethyl acetate, and acetonitrile were purchased from Avantor Performance Materials, (Gliwice, Poland), while *tert*-butyl methyl ether (MTBE) was from Acros Organics (part of ThermoFisher Scientific, Belgium). 99.999% pure helium 5.0 was used for GC/MS analysis (PGNiG, Poland). A Millipore purification system (France) was used to generate HPLC-grade water. Standard samples of p-cymene, eucalyptol, 1-octen-3-ol, camphor, terpinene-4-ol, linalyl acetate, thymol, borneol, carvacrol, carvone, linalool, amoxicillin, streptomycin and caryophyllene oxide were obtained from Sigma-Aldrich (Germany). Cell culture-grade DMSO was purchased from AppliChem Panreac. LB Broth (Miller; molecular biology grade), PBS (phosphate-buffered saline) and MacConkey agar were purchased from Fisher Scientific UK Ltd.

2.2. Plant material

Fresh herb of *Thymus vulgaris* L. (Lamiaceae) was collected in the summer of 2012 from the botanical garden of the Chair and Department of Pharmacognosy with Medicinal Plant Unit of the Medical University of Lublin, Poland, where a voucher specimen No KSW 1/27-30 is retained.

Identification was performed by specialists in botany, Mrs Krystyna Dąbrowska from The Botanical Garden of Marie Curie University. After drying, the plant was homogenised and a sample (50 g) was hydrodistilled with 400 mL of distilled water for 3 h in a Deryng apparatus. The EO was stored over anhydrous sodium sulfate at 4°C until analysis.

2.3. Equipment

A HPCCC Dynamic Extraction system (Slough, Berkshire, UK) with two coils: analytical (22 mL volume) and a semi-preparative system (137 mL volume), connected to an Alpha 10 pump and a Sapphire UV detector (both from Ecom, Czech Republic) were implemented in the study. Depending on the coil used, the sample injection valve was with either 1 mL or 6 mL loops.

The EO and the purified compounds were identified through a Shimadzu GC 2010 Plus gas chromatograph equipped with a Shimadzu QP2010 Ultra mass spectrometer. A Phenomenex (USA) fused silica capillary column ZB-5 MS (30 m, 0.25 mm *i.d.*) with a film thickness of 0.25 μm was used. The initial temperature was 50°C and set for 3 min followed by a rise of approximately 80°C min^{-1} up to 250°C. The flow rate of the carrier gas helium was 1 mL min^{-1}, and separation was 1:20 by the distribution ratio. A homologous series of *n*-alkanes (C_8-C_{24}) were used to determine the retention index and identification of compounds was performed using the computer-supported library (MassFinder) coupled with literature data (NIST Library).

2.4. Two-phase solvent systems selection

For the appropriate and efficient isolation from the EO, a number of immiscible, two-phase systems were evaluated, using methods described previously (Skalicka-Woźniak & Walasek, 2014). The main factor determining separation is the partition coefficient *K*, representing the concentration

of a target molecule in the stationary phase to the concentration of the same metabolite in the mobile phase. In this study, different solvent mixtures of *n*-hexane, ethyl acetate, methanol, water (HEMWat), or mixtures of *n*-hexane, acetonitrile, MTBE in different ratios were examined. From the chosen systems, solvent mixtures (4 mL each) were prepared in tubes, and to each was added a small amount of the EO. After mixing tubes were left for clear separation of the upper and lower phase, which were then analyzed separately by GC-MS analysis, and the *K* values of the particular metabolites of interest were calculated.

2.5. Separation procedure

Compound isolation was carried out on HPCCC analytical and semipreparative columns with a flow rate of the mobile phase of 1 mL min^{-1} and 6 mL min^{-1}, respectively. Conditions for the separation were initially optimized on the analytical scale and then adjusted for the semi-preparative by utilizing a scaling factor calculated on the basis of the respective volumes of the analytical and semipreparative columns. The previously selected two-phase systems were prepared. After filling the column with the (upper) stationary phase, rotation of the column was gradually increased to 1600 rpm. Then the mobile phase (lower) was introduced to the coil until a state of equilibrium was established. 25 mg of EO was dissolved in 1 mL of the selected solvent system for the analytical scale and 300 mg in 6 mL for the semi-preparative scale this mixture was injected and isolation was carried out at 30°C, monitoring at 210 nm. One-minute fractions were collected separately and subjected to GC-MS analysis.

2.6. In vivo experiments

Swiss albino male mice (20-23 g) delivered by the licensed breeder (Dr. J. Kolacz, Warsaw, Poland) were housed in cages with free access to food and tap water, and standard housing conditions (12 h light-dark cycle, 23 ± 1°C, humidity of 55 ± 5%). Animals were allowed to acclimatize for seven days. After this time, eight animals were randomly assigned to each test group. All tests were performed between 8:00 and 1500 hours. During the study, every effort to minimize the suffering of animals, and a minimum number of animals needed to obtain reliable scientific data was used. Procedures involving animal rights were according to the current European Community and Polish legislation on animal experimentation. The described procedure was approved by the Second Local Ethics Committee at the University of Life Sciences, Lublin, Poland (License Nos.: 85/2009, 42/2013, and 20/2014) and complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.7. Maximal electroshock seizure test

An alternating current stimulation (sine-wave, 25 mA, 50 Hz, 500 V, 0.2 s stimulus duration) delivered through ear-clip electrodes by a Rodent Shocker generator (Type 221, Hugo Sachs Elektronik, Freiburg, Germany) was used to produced electroconvulsions. The tonic hindlimb extension was the criterion for the occurrence of seizures in mice (Łuszczki, Andres-Mach, Gleńsk & Skalicka-Woźniak, 2010; Łuszczki, Włodarczyk, Gleńsk, Marzęda, Durmowicz & Florek-Łuszczki, 2013a, b).

A 1% solution of Tween 80 (Sigma, St. Louis, MO, USA) was used for the suspension of the EO, and the pure isolated compounds, in distilled water. Every time a single injection in a dose of 300 mg kg^{-1} (volume: 10 mL kg^{-1} of the body weight) was administered *i.p.* essential oil and single compounds were administered 15, 30, 60, and 120 min before the MES test and a percent

protection against maximal electroshock-induced seizures was calculated as a measure of the anticonvulsant activity.

2.8. Evaluation of acute neurobehavioral toxicity

2.8.1. Chimney test

The chimney test was used to quantify the adverse effects of both the essential oil and single compounds on motor performance in mice. The inability of the mice to climb backward up at transparent tube within 60 s was an indication of motor performance impairment (Boissier, Tardy, & Diverres, 1960).

2.8.2. Grip-strength test

The effects of both the essential oil and the isolated terpenoids on skeletal muscular strength (tone) in mice were quantified by the grip-strength test. Each mouse, which was forced to grasp the grid by forepaws, was gently pulled backward by the tail until the grid was released and the maximal force exerted by the mouse before losing grip was recorded. Substantial changes in skeletal muscular strength have been interpreted as evidence of motor neurotoxicity (Meyer et al., 1979)

2.9 *Bacterial plasmid transfer inhibition assay*

A plasmid assay was conducted to assess the ability of *Thymus vulgaris* essential oil and its isolated terpenoids (± linalool, eugenol, thymol, eucalyptol, *S*-carvone, *R*-carvone and borneol) to inhibit the conjugal transfer of plasmid-mediated antibiotic resistance in *Escherichia coli*.

Escherichia coli WP2 harboring plasmid pKM101 (conferring amoxicillin resistance) was used as the donor and *E.coli* ER1793 (streptomycin-resistant) used as a recipient in the bacterial conjugation assay. Both organisms were supplied by Dr. Paul Stapleton. Antimicrobial agents were used at the following concentrations to select for trans-conjugants; amoxicillin (30mg L^{-1}) and streptomycin sulfate (20 mg L^{-1}).

Both recipient *E.coli* ER1793 and plasmid-containing donor *E.coli* WP2 (pKM101) were incubated separately overnight (16h) at 37^{o}C in LB broth. Samples of the overnight cultures were taken for colony-forming unit (cfu mL^{-1}) determinations. Bacterial conjugation was performed as described by Rice and Bonomo (2005), with the exception that equal volumes of the donor and recipient were used. Compounds were assessed for anti-conjugative activity at a concentration of 100 mg L^{-1}. Transconjugants (recipient cells carrying the donated plasmid) and donor cells were recognized by inoculating 100 µL of serial dilutions of the overnight conjugation mixture on suitable antibiotic-containing selective MacConkey agar plates. The transfer rate was calculated as the ratio of total number of transconjugants (cfu mL^{-1}) to the total number of donor (cfu mL^{-1}). All experiments were performed in triplicate and the mean determined. The difference between the transfer frequencies in the absence and presence of the compounds were evaluated by Student's t-test. Values of $p< 0.05$ were statistically significant.

3. **Results and discussion**

The results of the GC-MS analysis of *Thymus vulgaris* essential oil are presented in Table 1 (in the order of elution from the column).

Fifty different metabolites, representing 94.05% of the total chromatographic peak area, were tentatively identified. The main compounds were thymol (34.78%), p-cymene (14.18%), carvacrol (6.16), *β*-caryophyllene (5.46%), linalool (3.83%), terpinen-4-ol (2.56%), caryophyllene

oxide (2.31%), and borneol (2.22%). Most of the identified compounds display a range of pharmacological activities, therefore the initial aim of this study was to elaborate efficient conditions for the definitive separation of the non-polar compounds from the *Thymus vulgaris* essential oil by HPCCC. A series of mixtures of different solvents were evaluated and the *K* values were calculated; the results are shown in Table 2. Optimum separation of the compounds takes place at partition coefficient values in the range 0.5-2. For this purpose, HEMWat systems in a ratio 5:2:5:2 (*v/v*) and 4:1:4:1 (*v/v*), and a system composed of mixtures of *n*-hexane, acetonitrile, and MTBE in the ratio 1:1:0.1 (*v/v*) were selected as having *K* values as close to this range as possible. Suitable analytical separation conditions were initially developed and re-scaled for the semipreparative studies. Fractions were collected at one minute intervals from the beginning of the run.

For the first time thymol, along with the other terpenoid metabolites, were isolated from *Thymus vulgaris* essential oil in a one-step run by means of the CCC method. Among the fifty compounds identified, six were isolated in less than 50 minutes with a purity level of greater than 96%. A HEMWat system in a ratio 5:2:5:2 (*v/v*) allowed isolation of eugenol (**1**; 1.58 mg), 1-octen-3-ol (**2**; 1 mg), borneol (**3**; 2.2 mg), thymol (**4**; 23 mg), terpinen-4-ol (**5**; 1.8 mg) and camphor (**6**; 3.6 mg) as pure compounds in fractions 19; 22; 26; 36-40, 41 and 42-46, respectively. Changing the solvent system to a HEMWat mixture 4:1:4:1 (*v/v*), facilitated the isolation of terpinen-4-ol (**5**, 1.2 mg) and 100% pure camphor (**6**, 2.78 mg) from fractions 19 and 20-23, respectively.

Additionally, a two-phase solvent system composed of *n*-hexane, acetonitrile, and MTBE (1:1:0.1 *v/v*) was used for further separation and permitted six compounds to be isolated quickly, with purities of 98-100%. Carvacrol (**7;** 4.45 mg), borneol (**3;** 2.45 mg), linalyl acetate (**8;** 1.05 mg), caryophyllene oxide (**9;** 2.47 mg), p-cymene (**10;** 8 mg) and eucalyptol (**11;** 1.58 mg) were obtained in fractions 14; 20-21; 26-28; 30-33; 40-43 and 49-50, respectively.

In the first system tested, eugenol (**1**), because of the low value of the partition coefficient *K* (0.64), was detected as the first pure compound, then the compounds **2** and **3** with *K* values in the range of 1.29-1.86. These compounds were isolated with a purity of 90, 99, and 100%, respectively. Finally, thymol (**4**), terpinen-4-ol (**5**), and camphor (**6**) with higher *K* values (1.58, 2.31, and 3.24) were obtained with a purity of 96, 97, and 100%, respectively. After changing the ratio of the HEMWat mixture from 5:2:5:2 to 4:1:4:1 the isolation time of terpinen-4-ol (**5**) and camphor (**6**) significantly decreased from 41 and 42-46 min to 19 and 20-23 min, respectively. This directly related to decreasing the *K* values of the metabolites from 2.31 and 3.24 in the first system to 1.34 and 2.03 in the second one, respectively. The use of a system composed of the n-hexane, acetonitrile and MTBE allowed the isolation of additional pure compounds like carvacrol (**7**), linalyl acetate (**8**), p-cymene (**10**), eucalyptol (**11**), and caryophyllene oxide (**9**), with virtually 100% purity. These developed methods led to the complete separation of *Thymus vulgaris* essential oil components in a very short time. Since essential oils, as well as their pure constituents, especially thymol, are very important, it is vital to search for new, easily applied, effective, sustainable, and economical methods for their separation. The literature refers to the availability of thymol (**4**) on an industrial scale as a result of synthesis. However, this method is long and requires toxic substrates and is relatively costly. In contrast, the HPCCC method proved to be more effective, less time-consuming, was more environmentally friendly, and thus represents an application of green chemistry (Cordell, 2015).

Epilepsy is a serious neurological disorder, which is characterized by recurrent spontaneous seizures which affects increasing numbers of people worldwide. Approximately 1% of the population suffers from epilepsy, and attacks are usually limited by either monotherapy or combination therapy, or in severe cases, by surgery (WHO, 2012). There are a number of synthetic drugs recommended for use, however, in large numbers of patients they are ineffective or cause serious side effects. Therefore, there is a continuing, significant need for new preparations based on

sustainable sources, such as plants, and essential oils and their main components have attracted the attention and encouraged scientists to study their pharmacological activity **(**de Almeida, Agra, Maior & de Sousa, 2011). In our research *Thymus vulgaris* essential oil and the isolated compounds were subjected to further study in order to determine their potential anticonvulsant activity and results are presented in Table 3.

Thymus vulgaris essential oil exerted protection against MES-induced seizures when administered 15 and 30 min before the test (50 and 62.5%, respectively). Prolongation of the pretreatment time resulted in the absence of anticonvulsant action, suggesting possible metabolism and elimination of the active metabolites. Among the single compounds, borneol, thymol, and eugenol at a fixed dose of 300 mg kg^{-1}, *i.p.*, exerted the strongest protection against MES-induced seizures. For borneol, the protection activity was observed at three time points tested (*i.e.,* 15, 30 and 60 min at the level of 75, 37.5 and 12.5%, respectively), while thymol and eugenol were inactive after 60 min. In contrast, any anticonvulsant activity in mice was noticed for eucalyptol only when administered 30 min before the MES-induced test, resulted in the anticonvulsant activity (one out of eight mice was protected). No protection against MES-induced seizures was noticed for linalool.

No acute adverse effects were noticed in the chimney and grip-strength tests. In our study, neither the essential oil nor any single compounds had a significant impact on muscular strength of the animals as assessed by the grip-strength test. Furthermore motor performance, as assessed by the chimney test, was also unaffected in experimental animals. However, some animals which received carvone alone and were subjected to the MES test, displayed mortality. This unpredicted situation can be explained by the application of a high dose of carvone, when administered alone. Of note, the animals were administered the same doses of the test compounds, equal to 300 mg/kg of body weight. This was based on information from the NIH Anticonvulsant Drug Development (ADD) Program, which evaluates the anticonvulsant action of novel compounds or agents in

preclinical studies in animals (Stables & Kupferberg, 1997). Since the concentration of the selected terpenoids in the essential oil was much more lower, no mortality was observed in experimental groups receiving essential oil. On the other hand, since the death of animals was noticed after electrically-evoked seizures, it can be hypothesized that carvone negatively influences the circulatory and respiratory center in the brain of the tested animals.

Essential oils are known for their pharmacological effects on the CNS. Volatile compounds are modulators of GABA receptors. The $GABA_A$ receptor possesses binding sites for the neurotransmitter GABA, and for other kinds of drugs, thus interaction with $GABA_A$ receptors results in different pharmacological effects ranging from sedative-hypnotic to anticonvulsant and anxiolytic. In experiments conducted by Mohammadi et al. (2001) thymol was determined to be an agonist of GABA receptors in a concentration-dependent manner. The ability for induction of chloride inward currents through GABA receptors in the absence of GABA is related to the structure of the compound (e.g. a phenolic group attached directly to the aromatic ring and an isopropyl group in the *ortho* position to the phenolic group). The half-maximum effect was observed at 200 μM (Mohammadi et al., 2001). Thymol (1–100 μM) potentiates the actions of GABA (both recombinant human $GABA_A$ and recombinant insect ionotropic GABA receptor) and modulation was dose-dependent, however weaker modulation was noticed towards mammalian $GABA_A$ receptors. It was concluded that the enhanced response to GABA is a result of a positive allosteric action of thymol (Priestley et al., 2003). Thymol also potentiated the agonist effects induced by pentobarbital, so that no competitive interaction was noticed. The mode of action of thymol is then different from that of pentobarbital as a GABA receptor agonist and probably uncharacterized, allosteric site on the $GABA_A$ receptor is involved (Priestley et al., 2003). Allosteric modulation of specific $GABA_A$ receptor recognition sites, without having any effect on non-specific binding, was reported by Sánchez et al. (Sánchez, Turina, Garcĭa, Nolan, & Perillo, 2004) who found that thymol is incorporated by membranes and exerts its activity by increasing the

surface curvature and polarity. Changes in the dipolar arrangement and in the molecular packing of the $GABA_A$ environment were discussed as possible mediators of the action mechanism of thymol (Sánchez et al., 2004). In further experiments, Garcia et al. (2006) provided evidence that thymol is a positive $GABA_A$ receptor modulator, and may produce conformational changes, which can lead to direct gating of the associated chloride channel. Thymol was able to enhance GABA action at concentrations which were significantly lower than those exhibiting direct activity in the absence of GABA (EC_{50}=12 µM and 135 µM, respectively).

Thymol and carvacrol were tested against cognitive deficits induced by amyloid β (Aβ) or scopolamine in rats, and both showed cognitive-enhancing activity with therapeutic doses equal to 0.5 mg kg^{-1} and 1 mg kg^{-1}, respectively. The acute toxicity for these compounds occurred at much higher doses (i.e., LD_{50} for thymol and carvacrol was 565.7 and 471.2 mg kg^{-1}, respectively) (Azizi, Ebrahimi, Saadatfar, Kamalinejad, Majlessi, 2012).

Eugenol, isolated and tested in these experiments, as well as carvacrol, one of the major compounds found in *Thymus vulgaris* essential oil, were studied on the $GABA_A$ receptor using primary cultures of cortical neurons, and their effects on the micro-viscosity of artificial membranes were investigated. They decreased the micro-viscosity of artificial membranes and enhanced the binding of [^{3}H]flunitrazepam to increase the GABA-evoked Cl- influx in a concentration-dependent manner, with EC_{50} values of 532 and 235 µM, for eugenol and carvacrol, respectively. Bicuculline, a competitive $GABA_A$ antagonist, completely inhibited these effects, which suggests that the tested compounds are positive allosteric modulators (Reiner et al., 2013).

Synthetic derivatives of eugenol, as well as natural methyleugenol, were tested in order to determine their antiepileptic activity and were compared to common antiepileptic drugs in the MES and PTZ tests. All eugenol derivatives were shown to be effective with protective indices similar to those of the standard drugs and were active in increasing the latency to the first minimal seizure, but were less active in prevention in the PTZ test (Dallmeier, Zelger, & Carlini, 1983). Eugenol (10–

100 µmol L^{-1}) on a free-Mg^{2+} model of epilepsy in rat neocortical and hippocampal tissues dose-dependently and reversibly suppressed both the epileptiform field potentials and spreading depression (Müller, Pape, Speckmann, & Gorji, 2006).

Eugenol, together with eucalyptol (1,8-cineole), both present in *Thymus vulgaris* essential oil and investigated in this study, shared responsibility for the anticonvulsant activity of essential oil of *Laurus nobilis* L. (Lauraceae) against PTZ and MES-induced seizures (ED_{50} values of 0.19 and 1 mL kg^{-1}, respectively), and additionally at the anticonvulsant doses, both sedation and motor impairment were observed (Sayyah, Valizadeh, & Kamalinejad, 2002). Previously, eucalyptol at a dose of 400 mg kg^{-1} significantly inhibited the locomotion of mice, by about 49% compared to the control group. Additionally, at the same dose a significant potentiation of pentobarbital sleeping time was observed (Santos & Rao, 2000). However in experiments conducted by Sayyah et al. (2002) eucalyptol was not active and no toxicity was observed. Rather, death of the test animals was caused by thymol and carvone (at 300 mg kg^{-1} injected *i.p.*).

Borneol, a bicyclic monoterpene, which can easily cross the blood brain barrier, was also found to possess gamma amino butyric acid (GABA) modulatory effect. The antiepileptogenic potential of borneol in the PTZ-induced kindling model of epilepsy was proved recently. At the dose of 25 mg/kg borneol significantly prevented the expression of kindling, while at the dose of 10 mg/kg protected the animals and inhibited the process of kindling. Overall, borneol pretreatment at the dose of 10 and 25 mg/kg significantly suppressed the progression of kindling (Tambe, Jain, Patil, Ghumatkar, & Sathaye, 2016).

The toxicity of thymol was observed previously. *In vitro* preincubation of thymol (7 mg w/v) gave a dose-dependent decrease in acetylcholinesterase (AChE) and lactic dehydrogenase (LDH) (91.02% and 74.0% of control, respectively) activities in the nervous tissue of the mollusc *Lymnaea acuminata.* At 12 mg (w/v) thymol reduced the LDH activity by 82.64%. Additionally, a significant reduction of the endogenous levels of 5-hydroxytryptamine and dopamine was observed,

which indicated that thymol adversely effects both the cholinergic and monoaminergic neurons in the snail (Singh, Singh & Singh, 1999). However, Garcia et al. (2006) excluded any harmful effect of thymol on cellular membrane integrity. In this assay, neuronal damage was measured either after short or long exposure times (30 min up to 24 h). No loss of cell viability and decrease of neuron mitochondrial activity was observed in the MTT assay (0-1 mM) (Garcia et al., 2006). Cytotoxicity towards CNS system was determined for both the *S*-(+)- and *R*-(-)-enantiomers of carvone, which caused depressant effects, including a decrease in touch and ambulatory response, an increase in sedation, as well as the potentiation of phenobarbital sleeping time, with LD_{50} values in the range of 400-500 mg kg^{-1}. The *S*-(+)-enantiomer at a dose of 200 mg kg^{-1} significantly increased the latency of convulsions induced by PTZ (De Sousa, de Farias Nóbrega, & de Almeida, 2007). Additionally, both the essential oil as well as the isolated compounds were previously evaluated for their toxic potential in in vitro toxicological tests as substances used in the active food packaging. Neither mutagenicity nor genotoxicity was noticed for the investigated compounds applied in the tested dose range (Llana-Ruiz-Cabello, Pichardo, & Maisanaba, 2015).

In our evaluation of the essential oil components to reduce plasmid conjugation, linalool (100 mg L^{-1}) had a significant inhibitory effect (84% reduction) on the transfer of plasmid pKM101 (Fig. 1), whereas eucalyptol was the least active agent where only a 31% reduction in plasmid transfer was observed. The remaining essential oil components, *R*-carvone, *S*-carvone, eugenol, borneol and thymol, showed only moderate inhibitory activities with 67%, 66%, 64%, 63% and 51% reductions in plasmid transfers being noted, respectively. Linalool as the most active plasmid transfer inhibitor was also the only acyclic compound tested. Our previous work in this area has shown that whilst inhibitors may contain phenyl and heterocyclic ring systems, they tend to be linear in shape, for example as seen in the imidazole alkaloids of *Lepidium sativum* (Kwapong, Stapleton & Gibbons 2018) or the acylphloroglucinols of *Mallotus philippinensis* (Mbaebie-Oyedemi, Shinde, Shinde, Kakalou, Gibbons, S. & Stapleton 2016).

4. Conclusion

This is the first report of a successful attempt to use HPCCC technique for the one-step purification of a series of terpenoids from *Thymus vulgaris* essential oil in a very short time. The method can potentially be used for the purification of compounds which are of importance in the food industry. The preclinical screening of *Thymus vulgaris* essential oil, as well as isolated terpenoids, provides evidence that the essential oil has partial protective activity against seizures. Additionally, borneol, thymol, and carvacrol are worthy of further investigation as potentially interesting therapeutic agents in epileptopathology, particularly in terms of synergistic effects with known active agents. The anti-plasmid transfer inhibitory activity of the simple monoterpene linalool is also noteworthy. As it has been hypothesized that *E. coli* enterotoxins may have an impact on seizure threshold, reducing the virulence of *E. coli* by interfering with plasmid transfer may reduce its pathogenicity. As mentioned above, linalool like previously reported plasmid transfer inhibitors, is structurally linear and the most active natural product reported in this study. This structural feature may well allow linalool to bind in to the major or minor groves of plasmid DNA making a structural change that inhibits its uptake by the type-IV secretion systems in *E. coli*, that are responsible for plasmid mobility between cells. Taking points made above, *T. vulgaris* can be considered as a source of biologically active compounds ready to use or to be submitted for further structure modifications.

Conflict of interest

There are no conflicts of interest to declare.

Acknowledgment

This work was supported by Medical University of Lublin.

References

Azizi, Z., Ebrahimi, S., Saadatfar, E., Kamalinejad, M., & Majlessi, N. (2012). Cognitive-enhancing activity of thymol and carvacrol in two rat models of dementia. *Behavioural Pharmacology*, *23*, 3, 241-249.

Boissier, J.R., Tardy, J., Diverres, J.C. (1960) Une nouvelle méthode simple pour explorer l'action «tranquillisante»: le test de la cheminée (in French). *Pharmacology*, 3, 81-84.

Cordell G. A. (2015). Ecopharmacognosy and the responsibilities of natural product research to sustainability. *Phytochemistry Letters*, *11*, 332-346.

Dallmeier, Z. K. R., Zelger, J. L., & Carlini, E. A. (1983). New anticonvulsants derived from 4-allyl-2-methoxyphenol (eugenol): comparison with common antiepileptics in mice. *Pharmacology, 27*, 40–49.

De Almeida, R. N., Agra, M. F., Maior, F. N. S., & de Sousa D. P. (2011). Essential oils and their constituents: Anticonvulsant activity. *Molecules, 16*, 2726-2742.

de Sousa, D. P., de Farias Nóbrega, F. F., & de Almeida, R. N. (2007). Influence of the chirality of (*R*)-(-)- and (*S*)-(+)-carvone in the central nervous system: a comparative study. *Chirality, 19*, 4, 264-268.

Elisabetsky, E., Brum, L. F. S., & Souza, D. O. (2009). Anticonvulsant properties of linalool in glutamate-related seizure models. *International Review of Neurobiology, 85*, 237-248.

Eloma, E. (1979). A New Look at the Aetiopathogenesis of Epilepsies. *Medical Hypotheses*, *5*, 347-349.

EMA/HMPC/131903/2009, Committee on Herbal Medicinal Products (HMPC).

Garcĭa, D. A., Bujons, J., Vale, C., & Sunol, C. (2006). Allosteric positive interaction of thymol with the $GABA_A$ receptor in primary cultures of mouse cortical neurons. *Neuropharmacology, 50*, 1, 25-35.

Jukic, M., Politeo, O., Maksimovic, M., Milos, M., & Milos, M. (2007). In Vitro acetylcholinesterase inhibitory properties of thymol, carvacrol and their derivatives thymoquinone and thymohydroquinone. *Phytotherapy Research, 21*, 259-261.

Kannappan, R., Gupta, S. C., Kim, J. H., Reuter, S. & Aggarwal, B. B. (2011). neuroprotection by spice-derived nutraceuticals: You are what you eat! *Molecular Neurobiology, 44*, 142-159.

Kwapong A., Stapleton P., & Gibbons S. (2018). A new dimeric imidazole alkaloid plasmid conjugation inhibitor from *Lepidium sativum*. *Tetrahedron Letters 59*, 1952-1954.

Llana-Ruiz-Cabello, M., Pichardo, S., Maisanaba, S., Puerto, M., Prieto, A., Gutiérrez-Praena, D., Jos, A., Cameán, A.M. (2015) In vitro toxicological evaluation of essential oils and their main compounds used in active food packaging: A review. *Food and Chemical Toxicology 81*, 9-27.

Łuszczki, J. J., Andres-Mach, M., Gleńsk, M., & Skalicka-Woźniak, K. (2010). Anticonvulsant effects of four linear furanocoumarins, bergapten, imperatorin, oxypeucedanin, and xanthotoxin, in the mouse maximal electroshock-induced seizure model: a comparative study. *Pharmacological Reports*, *62*, 6, 1231-1236.

Łuszczki, J. J., Włodarczyk, M., Gleńsk, M., Marzęda, E., Durmowicz, D., & Florek-Łuszczki, M. (2013a). Effects of various naturally occurring compounds (arbutin, borneol, esculetin, esculin, ellagic acid, gallic acid, hesperidine, piperitol, piperonal, quercetin, thymoquinone and ursolic acid) against maximal electroshock-induced seizures in mice. *Current Issues of Pharmacy and Medical Sciences, 26*, 2, 135-139.

Łuszczki, J. J., Włodarczyk, M., Gleńsk, M., Marzęda, E., Durmowicz, D., & Florek-Łuszczki, M. (2013b). Effects of alizarin, betulin, curcumin, diosmin, linalool, menthofuran, α-terpineol, theobromine, β-thujaplicin and vanillin against maximal electroshock-induced seizures in mice. *Journal of Pre-Clinical and Clinical Research*, *7*, 1, 40-42.

Mbaebie-Oyedemi, B.O., Shinde, V., Shinde, K., Kakalou, D., Gibbons, S. & Stapleton, P. (2016). Novel R-Plasmid Conjugal Transfer Inhibitory and Antibacterial Activities of Phenolic Compounds from *Mallotus philippinensis* (Lam.) Mull. Arg *Journal of Global Antimicrobial Resistance 5*, 15-21.

Melo, F. H. C., Venâncio, E. T., de Sousa, D. P., de França Fonteles, M. M., de Vasconcelos, S. M. M., Viana, G. S. B., & de Sousa, F. C. F. (2010). Anxiolytic-like effect of carvacrol (5-isopropyl-2-methylphenol) in mice: involvement with GABAergic transmission. *Fundamental and Clinical Pharmacology, 24*, 437-443.

Melo, F. H. C., Moura, B. A., de Sousa, D. P., de Vasconcelos, S. M., Macedo, D. S., Fonteles, M. M., Viana, G. S. & de Sousa, F. C. F. (2011). Antidepressant-like effect of carvacrol (5-Isopropyl-2-methylphenol) in mice: involvement of dopaminergic system. *Fundamental & Clinical Pharmacology, 25*, 3, 362-367.

Meyer, O.A., Tilson, H.A., Byrd, W.C., Riley, M.T. (1979) A method for the routine assessment of fore- and hindlimb grip strength of rats and mice. *Neurobehavioral Toxicology, 1*, 233-236.

Mohammadi, B., Haeseler, G., Leuwer, M., Dengler, R., Krampfl, K., & Bufler, J. (2001). Structural requirements of phenol derivatives for direct activation of chloride currents via $GABA_A$ receptors. *European Journal of Pharmacology, 421*, 2, 85–91.

Müller, M., Pape, H. C., Speckmann, E. J., & Gorji, A. (2006). Effect of eugenol on spreading depression and epileptiform discharges in rat neocortical and hippocampal tissues. *Neuroscience, 30*, 140, 2, 743-751.

Nabavi, S. M., Marchese, A., Izadi, M., Curti, V., Daglia, M., & Nabavi, S. F. (2015). Plants belonging to the genus *Thymus* as antibacterial agents: From farm to pharmacy. *Food Chemistry, 173*, 15, 339–347.

Priestley, C. M., Williamson, E. M., Wafford, K. A., & Sattelle, D. B. (2003). Thymol, a constituent of thyme essential oil, is a positive allosteric modulator of human $GABA_A$ receptors and a homo-oligomeric GABA receptor from *Drosophila melanogaster*. *British Journal of Pharmacology*, *140*, 1363-1372.

Reiner, G. N., Perillo, M. A., & Garcia, D. A. (2013). Effects of propofol and other GABAergic phenols on membrane molecular organization. *Colloids and Surfaces B: Biointerfaces*, *101*, 61-67.

Rice, L.B., & Bonomo, R.A. (2005). Genetic and biochemical mechanisms of resistance to antimicrobial agents. In: Lorian, V. (Ed.), Antibiotics in Laboratory Medicine. Lippincott Williams & Wilkins, USA, pp. 483–484.

Sánchez, M. E., Turina, A. V., Garcĭa, D. A., Nolan, M. V., & Perillo, M. A. (2004). Surface activity of thymol: implications for an eventual pharmacological activity. *Colloids and Surfaces B: Biointerfaces, 34*, 77-85.

Santos, F. A., & Rao, V. S. (2000). Antiinflammatory and antinociceptive effects of 1,8-cineole a terpenoid oxide present in many plant essential oils. *Phytotherapy Research*, *14*, 240–244.

Sayyah, M., Valizadeh, J., & Kamalinejad, M. (2002). Anticonvulsant activity of the leaf essential oil of *Laurus nobilis* against pentylenetetrazole- and maximal electroshock-induced seizures. *Phytomedicine*, *9*, 212–216.

Singh, V. K., Singh, S. & Singh, D. K. (1999). Effect of active molluscicidal component of spices on different enzyme activities and biogenic amine levels in the nervous tissue of *Lymnaea acuminata*. *Phytotherapy Research*, *13*, 649 – 654.

Skalicka-Woźniak, K., & Garrard, I. (2015). A comprehensive classification of solvent systems used for natural product purifications in countercurrent and centrifugal partition chromatography. *Natural Product Reports, 32*, 1556-1561.

Skalicka-Woźniak, K., & Walasek, M. (2014). Preparative separation of menthol and pulegone from peppermint oil (*Mentha piperita* L.) by high-performance counter-current chromatography. *Phytochemistry Letters, 10*, 94-98.

Skalicka-Woźniak, K., Walasek, M., Ludwiczuk, A., & Głowniak, K. (2013). Isolation of terpenoids from *Pimpinella anisum* essential oil by high-performance counter-current chromatography. *Journal of Separation Science, 36*, 2611-2614.

Stables J.P., Kupferberg H.J. (1997): Chapter 16 – The NIH Anticonvulsant Drug Development (ADD) Program: preclinical anticonvulsant screening project. In: Molecular and

cellular targets for anti-epileptic drugs. Eds. Avanzini G., Regesta G., Tanganelli P., Avoli M., John Libbey. London. 191–198.

Tao, G., Irie, Y., Lia, D-J. & Keunga, W. M. (2005). Eugenol and its structural analogs inhibit monoamine oxidase A and exhibit antidepressant-like activity. *Bioorganic & Medicinal Chemistry, 13,* 4777–4788.

Tambe, R., Jain, P., Patil, S., Ghumatkar, P., Sathaye, S. (2016). Antiepileptogenic effects of borneol in pentylenetetrazole-induced kindling in mice. *Naunyn-Schmiedeberg's Archives of Pharmacology, 389,* 467–475.

WHO, Epilepsy. (2012). Fact sheet N°999.

Youdim, K. A., & Deans, S. G. (2000). Effect of thyme oil and thymol dietary supplementation on the antioxidant status and fatty acid composition of the ageing rat brain. *British Journal of Nutrition, 83,* 1, 87-93.

Tables legend:

Table 1. GC-MS analysis of *Thymus vulgaris* essential oil and amount of isolated compounds (semipreparative scale).

Table 2. Partition coefficients (*K*) for isolated terpenoids.

Table 3. Anticonvulsant effects of *Thymus vulgaris* essential oil and isolated compounds (number of animals protected (p) against maximal electroshock (MES)-induced seizures out of eight animals per group (t) (and as % in parentheses).

Captions for figures

Fig. 1. Activity of *Thymus vulgaris* components on the inhibition of transfer of plasmid pKM101 (all samples were at the100 μg mL^{-1} concentration).

Table1

Table 1. GC-MS analysis of *Thymus vulgaris* EO and amount of isolated compounds (semipreparative scale).

Compounds	Retention Time	RRI[a)] (calcd)	RRM[b)] (REF)	Area (%)	Isolated compounds mg/purity	
					HEMWat 5: 2: 5: 2 (v/v)	*n*-hexane, acetonitrile, MTBE (1: 1: 0.1 v/v)
α-Pinene	7.21	935	936	0.20		
Camphene	7.59	952	950	0.17		
β-Pinene	8.23	980	978	0.05		
1-Octen-3-ol	**8.28**	**982**	**962**	**0.73**	**1 / 99%**	
β-Myrcene	8.46	990	987	0.52		
α-Phellandrene	8.86	1008	1002	0.07		
δ-3-Carene	8.92	1010	1010	0.03		
α-Terpinene	9.10	1019	1013	0.60		
p-Cymene	**9.29**	**1028**	**1015**	**14.18**		**8 /100%**
D-Limonene	9.37	1032	1025	0.24		
β-Phellandrene	9.41	1033	1023	0.29		
Eucalyptol (1,8-Cineole)	**9.44**	**1035**	**1024**	**0.58**		**1.58 /100%**
γ-Terpinene	10.00	1061	1051	5.29		
cis-Sabinene hydrate	10.29	1074	1082	0.14		
α-Terpinolene	10.58	1088	1082	0.15		
Linalool	10.88	1102	1086	3.83		
trans-Sabinene hydrate	10.94	1105	1053	0.12		
Camphor	**11.88**	**1153**	**1123**	**2.60**	**3.6 /100%**	
Borneol	**12.42**	**1180**	**1150**	**2.22**	**2.2 /100%**	**2.45 / 100%**
Terpinen-4-ol	**12.55**	**1186**	**1164**	**2.56**	**1.8 / 97%**	
p-Cymen-8-ol	12.68	1193	1169	0.21		
α-Terpineol	12.84	1201	1176	0.66		
n-Octyl acetate	12.99	1209	1188	0.14		

Isopropyl benzaldehyde	13.26	1223	1220	0.08		
Thymol methylether	13.41	1232	1215	1.45		
Carvacrol methylether	13.59	1241	1226	1.30		
Linalyl acetate	**13.62**	**1244**	**1236**	**1.10**		**1.05 / 98%**
Carvone	13.72	1249	1214	0.09		
Geraniol	13.80	1254	1235	0.17		
Bornyl acetate	14.43	1288	1276	1.12		
Thymol	**14.68**	**1301**	**1267**	**34.78**	**23 / 96%**	
Carvacrol	**14.81**	**1309**	**1278**	**6.16**		**4.45 / 100%**
Thymol acetate	15.49	1348	1329	1.12		
Eugenol	**15.66**	**1358**	**1346**	**0.48**	**1.58 / 90%**	
α-Copaene	16.09	1383	1373	0.36		
β-Burbonene	16.24	1392	1386	0.16		
β-Caryophyllene	16.87	1430	1421	5.46		
α-Humulene	17.45	1466	1455	0.24		
Germacrene D	17.71	1483	1479	0.56		
α-Muurolene	18.08	1506	1496	0.35		
β-Bisabolene	18.18	1512	1503	0.29		
γ-Cadinene	18.34	1522	1507	0.87		
δ-Cadinene	18.39	1526	1520	1.06		
trans-Calamanene	18.46	1530	1527	0.31		
Spathulenol	19.37	1590	1572	0.08		
Caryophyllene oxide	**19.44**	**1595**	**1578**	**2.31**		**2.47 / 100%**
Cubenol	19.91	1627	1630	0.16		
γ-Eudesmol	20.05	1636	1618	0.29		
tau-Cadinol	20.30	1654	1633	0.66		

a) RRI, relative retention index calculated.

b) RRM, relative retention index according to MassFinder.

Table 2. Partition coefficients (*K*) for isolated terpenoids.

	Eugenol	**1-Octen-3-ol**	**Borneol**	**Thymol**	**Terpinen-4-ol**	**Camphor**	**Carvacrol**	**Linalyl acetate**	**Caryophyllene oxide**	**p-Cymene**	**Eucalyptol**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
HEMWat 5:2:5:2	0.64	1.86	1.29	1.58	2.31	3.24	-	-	-	-	-
HEMWat 3:1:3:1	0.43	1.25	0.86	1.17	1.57	2.14	-	-	-	-	-
HEMWat 4:1:4:1	0.33	1.12	0.68	0.91	1.34	2.03	-	-	-	-	-
HEMWat 5:1:5:1	0.008	0.74	0.37	0.53	0.37	1.15	-	-	-	-	-
Eter 1:1:0.1	1.45	5.97	0.51	0.38	0.43	0.49	0.3	1.00	1.43	1.76	0.14

Table 3. Anticonvulsant effects of *Thymus vulgaris* EO and isolated compounds (number of animals protected (p) against maximal electroshock (MES)-induced seizures out of eight animals per group (t) (and as % in parentheses).

Time (min)	**15**	**30**	**60**	**120**
Substances	**p/t (%)**	**p/t (%)**	**p/t (%)**	**p/t (%)**
Thyme oil	4/8 (50)	5/8 (62.5)	0/8 (0)	0/8 (0)
Borneol	6/8 (75)	3/8 (37.5)	1/8 (12.5)	0/8 (0)
Thymol	4/7 (57.1)	3/7 (42.9)	0/6 (0) + 1 died	0/6 (0) + 1 died
Eugenol	4/8 (50)	6/8 (75)	0/8 (0)	0/8 (0)
Eucalyptol (cineole)	0/8 (0)	1/8 (12.5)	0/8 (0)	0/8 (0)
Linalool	0/8 (0)	0/8 (0)	0/8 (0)	0/8 (0)
Carvone	5/5 (100) + 2 died	4/4 (100) + 3 died	5/6 (83.3) + 1 died	1/6 (16.6) + 1 died

The MES test was performed at various pretreatment times (15, 30, 60, and 120 min) after systemic (*i.p.*) administration of the investigated compounds at a fixed dose of 300 $mg.kg^{-1}$.

Figure1

Click here to download high resolution image