ESSENTIAL OILS AND THEIR MAIN COMPONENTS AS POTENTIAL ANTIMICROBIAL AND ANTI-INFLAMMATORY SUBSTANCES

Final report

Essential oils (EOs) are very interesting natural products and possess various biological properties. However, these plant extracts can be applied generally based upon long-standing use. The appearance of multidrug resistant bacteria and growing antibiotic resistance is leading to a continuous need for discovering new drugs and alternative treatments against infections. Respiratory tract diseases associated with bacterial infection and inflammation affect a large number of people from every age group worldwide. Because of volatility, EOs can easily reach the upper and lower parts of the respiratory tract via inhalation. Moreover, due to their antimicrobial and anti-inflammatory potency, they offer an effective treatment in respiratory tract infections. In our research, therefore, we focused on:

1) the phytochemical characterization of some EOs with different chromatographic techniques (TLC and GC-FID/GC-MS)
2) the microbiological investigation of the effect of EOs and their main components against microorganisms (bacteria and fungi) affecting humans with microbiological and Lab-on-a-chip technology and
3) the investigation of the anti-inflammatory effects of EOs in a mouse airway inflammation model *in vivo*.

(1) Phytochemical characterization of some EOs with different chromatographic techniques (TLC and GC-FID/GC-MS)

The EO was isolated from *Artemisia adamsii* Besser, a Mongolian medicinal plant. This plant sample was obtained from the University of Szeged, Faculty of Sciences, Department of Microbiology, who has been a Mongolian co-operation for many years. The air-dried plant material was pulverized and the EO was prepared by water-steam distillation in accordance with the Hungarian Pharmacopoeia, VIII. edition (2003). The EO yield was 0.56 mL/100 g of dried plant material. The color of the oil was pale yellow, which was interesting because *Artemisia* species (Asteraceae family) generally have blue EO due to the presence of sesquiterpenes. Later the gas chromatographic-mass spectrometric (GC-MS) analysis confirmed the presence of monoterpenes. α-Thujone was the main component (64.4%), while the amount of β-thujone was much lower (7.1%.). The presence of 1.8-cineole (15.2%) was also confirmed by GC-MS. Other components were: p-cymene (1.5%), terpinen-4-ol (1.5%), linalool (0.4%) and spatulenol (1.8%). The oil of this plant showed antibacterial activity in the bioautographic system, however, its medical application for internal use is not recommended because of the thujone content. The EO of *A. adamsii* might be useful as a disinfectant in controlling hospital infections caused by antibiotic-resistant bacteria, e.g. MRSA (Horváth et al. 2013b). Bouaziz et al. (2009) published the disinfectant properties of thujone-type EO obtained from *Salvia officinalis*.

We examined some other EOs obtained from a Hungarian Company (Aromax Ltd.). These EOs can be found in different herbal products, but their application is generally based upon long-standing use. Before the microbiological and pharmacological experiments, the chemical composition of these EOs (peppermint, lemon, citronella, sweet fennel, scots pine, eucalyptus, cinnamon bark, spearmint, thyme, chamomile, lavender, clary sage, rosemary, clove, tea tree) was investigated by GC-MS. We could detect every main (marker) compound of the EOs involved in our study and the quality of the EOs met the standards described in the Hungarian Pharmacopoeia VIII. The main compounds of the EOs were included: menthol (50.4%) and menthon (19.8%) in the peppermint, limonene (63%) in lemon, citronellal (32.8%) and geraniol (24.6%) in citronella, *trans*-anethol (78.9%) in sweet fennel, *α*-pinene (39.4%) and *β*-pinene (11%) in scots pine, eucalyptol (85%) in eucalyptus, *trans*-cinnamaldehyde (74%) and eugenol (2.7%) in cinnamon bark, carvone (69%) in spearmint, thymol (46.3%), *p*-cymol (22.1%) and carvacrol (3.2%) in thyme, *α*-bisabolol (67.4%) and *β*-farnesene (17.9%) in chamomile, linalyl acetate (41.1%) and linalool (36%) in lavender, linalool (57.4%) and linalyl acetate (27.3%) in cleary sage, 1,8-cineole (45.5%) and camphor (14%) in rosemary, eugenol (88%) and *β*-caryophyllene (8.6%) in clove, terpinen-4-ol (44%) and 1,8-cineole (24.9%) in tea tree. In GC-FID the identification of peaks was made by retention time and standard addition; percentage evaluation was carried out by area normalization. We made three parallel measurements, RSD percentages were below 4.5%. During GC-

MS the identification of the compounds was carried out by comparing retention times and recorded spectra with the data of authentic standards, and the NIST 05 library was also consulted.

(2) Microbiological investigation of the effect of EOs and their main components

- Determination of the *in vitro* antifungal and antibacterial activities of essential oils

The number of studies focusing on EOs, as well as on their applications as new potential antibiotic agents against plant and human pathogenic microorganisms has recently increased (Rota et al. 2004; Delamare et al. 2007; Tabanca et al. 2007; Rodrigues et al. 2009; Tserennadmid et al. 2010). Previous studies on the antimicrobial activity of EOs *in vitro* described a wide range of assays with different parameters (agar recipes, incubation time, solvents, microorganisms) (Griffin et al. 1999; Inouye et al. 2001), so the results from the assays are very different, sometimes their reliability is questionable. EOs are volatile, complex and viscous substances that are insoluble in water, so the common screening methods (disc diffusion, agar absorption) are not appropriate for their antimicrobial testing. Therefore, there is a need for optimized and reproducible assays for assessing the antibacterial effects of these oils.

We could successfully optimize the thin layer chromatography-direct bioautography (TLC-DB) method for detecting the antimicrobial activity of EOs involved in our study. In the TLC-DB method a developed TLC plate is dipped in the suspension of microorganisms growing in a suitable broth. The plate is incubated and microorganisms grow directly on it. For location and visualization of antibacterial substances, tetrazolium salts are usually used, which are converted by the dehydrogenases of living microorganisms to intense formazan (Rios et al. 1988; Botz et al. 2001; Choma-Grzelak 2011). After TLC-DB we could select EOs which have antimicrobial activity. In the microbiological experiments *Staphylococcus aureus* (SA; ATCC 29213), *S. epidermidis* (SE; 1118, isolated from human blood sample), methicillin-resistant *S. aureus* (MRSA 4262, isolated from human blood sample), *Escherichia coli* (EC; ATCC 25922), *Bacillus subtilis* (BS; ATCC 6633) and *Micrococcus luteus* (ML; ATCC 9341) were tested. These strains were maintained at the Department of Medical Microbiology and Immunology, Medical School, University of Pécs. **Fig. 1** demonstrates the TLC-DB detection of EOs without development of the plate. According to TLC-DB "screening results" we could select the effective antibacterial EOs and only these oils were included into our further experiments. The most effective oils were thyme, clove and cinnamon bark.

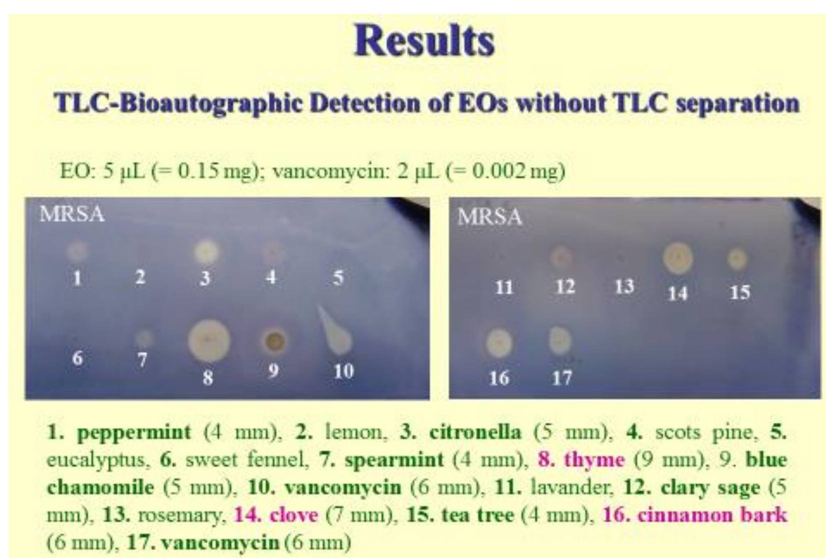

Fig. 1 TLC-DB detection of EOs used in our study without TLC separation.

The antibacterial activity was evaluated by the measurement of the diameter of inhibition zones (in mm, **Fig. 2**). The most effective EOs were highlighted with purple color.

Results - TLC-Bioautographic Detection of EOs without TLC separation

Essential oils	MRSA	ML	SA	SE	EC	BS
	Bacteria					
peppermint	4	4	-	3	6	8
lemon	-	-	-	5	-	8
citronella	5	5	5	-	5	10
scots pine	-	-	-	-	4	10
eucalyptus	-	-	-	-	7	5
sweet fennel	-	-	-	-	7	9
spearmint	4	-	-	-	7	8
thyme	9	8	9	8	12	13
blue chamomile	5	-	7	-	-	11
lavander	-	-	-	-	6	6
clary sage	5	6	4	-	5	5
rosemary	-	-	-	-	6	6
clove	7	6	8	7	9	15
tea tree	4	5	5	5	6	7
cinnamon bark	7	6	7	5-6	15	15
gentamicin	-	-	5	3-4	5-6	5-6
vancomycin	6-7	6-7	-	-	-	-

Fig. 2 TLC-DB detection of EOs used in our study without TLC separation. Diameters of inhibition zones in mm.

The main components (*trans*-cinnamic aldehyde, eugenol, thymol, carvacrol) of these EOs showed antibacterial activity after TLC development. On the whole, the antimicrobial activity of the EOs could be related to their main components, but the minor constituents may be involved in this process. The antibacterial activity of cinnamon bark oil obtained by TLC-DB can be seen in **Fig. 3**.

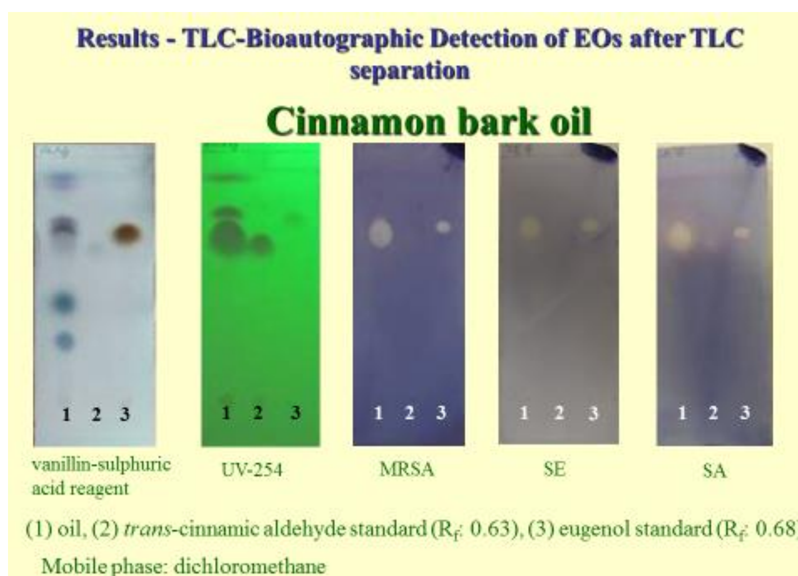

Fig. 3 Antibacterial activity of cinnamon bark oil and its main components by TLC-DB.

In another study the antibacterial activity of tea tree, clove, cinnamon bark, thyme, eucalyptus, spearmint and clary sage oils was investigated against the Gram-negative luminescence tagged plant pathogenic bacterium *Pseudomonas syringae* pv. *maculicola* and the naturally luminescent marine bacterium *Vibrio fisheri* (Horváth et al. 2013b, Móricz et al. 2015). The sensitivity of TLC-DB could be

improved by using luminescent test bacteria. Moreover, we could demonstrate the applicability of TLC-DB by testing three anaerobic *Clostridium perfringens* and three microaerophilic *Campylobacter jejuni* strains against two EOs, clove and thyme (Kovács et al. 2016). To the best of our knowledge, it was the first time when TLC-DB was optimized for anaerobic and microaerophilic bacteria.

What are the advantages of TLC-DB: (1) Effective and inexpensive technique for the study of biological activity of plant extracts, e.g. EOs, (2) Short analysis time, (3) Low cost per analysis, (4) No need to pre-treatment of samples, (5) Possibility of qualitative and semi-quantitative analysis of extracts. We concluded that TLC-DB is a directly combined application of an analytical method with an *in situ* bioassay that allows a rapid identification of the active compound or compounds in a complex mixture (Horváth et al. 2002, 2004, 2010).

EOs have traditionally been applied for respiratory tract infections via inhalation. Among *in vitro* methods, the vapor phase test (VPT) demonstrates the vapor activity of EOs in the most appropriate way and these results may be useful to understand the antimicrobial activity of them in the respiratory tract. In VPT, generally, a paper disc containing EO is placed on the inside surface of the upper lid of a Petri-dish. The lower lid contains agar, and a suspension of test microorganism containing approximately 10^6 cfu/mL is spread over this surface. The plate is immediately inverted on top of the lid and sealed with parafilm to prevent leakage of the vapor. After incubation an inhibition of bacterial growth on the agar plate can be detected, which is the measure of EO activity. There is a possibility to define the minimum inhibitory concentration (MIC) in atmosphere (MIC_{air}) by VPT (Tyagi – Malik 2010). In our experiments we evaluated the antibacterial activity of EOs of cinnamon bark, clove, thyme, citronella, peppermint, scots pine and eucalyptus against respiratory tract pathogens such as MRSA and *Pseudomonas aeruginosa* with VP and tube dilution (TD) techniques (Ács et al. 2016). Among the EOs, cinnamon bark was the most effective against all the investigated pathogens (MIC: 31.25-125 µL/L) in the VP assay, but clove oil presented the best inhibition against MRSA in liquid medium (MIC: 0.1 mg/mL). Thyme oil also showed antibacterial activity against MRSA and the antibiotic-sensitive strain of *P. aeruginosa* in both methods. In higher concentration, we found that peppermint oil was effective only in vapor form; contrarily, eucalyptus oil was more efficient in liquid medium. Surprisingly, scots pine did not show any activity in our test systems. These results suggest that the antimicrobial evaluation of EOs should be done in minimum two different test system, e.g. in VP and liquid phase. These findings may help in the drug development against respiratory diseases. However, more *in vivo* studies are necessary to calculate the effective dose of EOs in patients and determine their possible side effects and toxicity.

- Determination of the possible interactions between the EOs against the foregoing isolates *in vitro*

If we combine two antimicrobial constituents, we can distinguish different types of effects including indifferent, additive, synergistic, and antagonistic effects. The indifferent effect occurs when the combination of an antimicrobial agent and an inactive substance has an identical effect to that of the most effective compound. In case of additive effect, a mixture of antimicrobial compounds has an activity equal to the sum of the effects of each component. The synergistic effect occurs when a combination of antimicrobial constituents has a greater effect than the added activities of each compound. Antagonistic effect is observed when a reduced activity develops relative to the effect of the most efficient individual constituent (EUCAST 2000). These effects can be measured and quantified by the use of fractional inhibitory concentration (FIC) and fractional bactericidal concentration (FBC). If we have two antibacterial compounds, A and B, FIC can be calculated using the following expression: $FIC_A = MIC(_{A\text{ in the presence of B}})/MIC(_{A\text{ alone}})$; $FIC_B = MIC(_{B\text{ in the presence of A}})/MIC(_{B\text{ alone}})$. MIC is the minimum inhibitory concentration. The FIC index is the sum of FIC_A and FIC_B. The FBC index can be similarly calculated using FBC values of individual bactericidal compounds. If FIC index is < 0.5, it means synergistic effect, > 0.5-1 indicates additive effect, > 1-2 indifference, and ≥ 2 indicates antagonistic effect (EUCAST 2000).

The checkerboard assay can be used to determine the FIC index experimentally. In this case two antimicrobial agents are added to Mueller-Hinton broth in 96-well microtiter plates to give two-fold dilutions in the horizontal and vertical directions, respectively. Then bacterial cells (approx. 8×10^5/mL) are added, and plates are incubated (Osburne et al. 2006). In our study the anti-yeast and anti-mould activities of selected EOs were investigated, alone and in combinations against clinical isolates of *Candida albicans* (SZMC 1363, vaginal mycosis), *C. parapsilosis* (SZMC 1408, intestinal mycosis),

Aspergillus fumigatus (SZMC 2394, keratomycosis), *A. terreus* (SZMC 2394, keratomycosis), *Rhizopus microspores* (SZMC 13644, human mycosis), *Fusarium solani* (SZMC 11412, keratomycosis) and *Lichtheimia corymbifera* (FSU 9682, unknown origin). The fungal strains used in this study were from the Szeged Microbiological Collection. MICs were determined for the EOs of cinnamon bark, citronella, clove, spearmint and thyme. To investigate the combination effect of the EOs, FICs were defined by the checkerboard method and the type of interaction was determined by the FIC index (FICI). Strongest antifungal activity was showed by thyme EO with MIC values below 1.0 mg/ml. Combination of EOs resulted in additive or indifferent effect, with occasional "borderline synergism". The best combination was cinnamon with clove leading to additive effect in all cases (Horváth et al. 20016). Other research groups have demonstrated results similar to our findings (Hendry et al. 2009, Mulyaningsih et al. 2010, Hamoud et al. 2012). It can be concluded that the total EO is more active than its major compounds. In the future it is worth investigating the combination of EOs or EOs with antibiotics to reduce the development of antibiotic-resistance microbial strains. There are some promising results. In a study *Lippia origanoides* EO was assayed for its modifying drug activity against an MRSA strain by microdilution method. A significant potentiating activity between the EO and the aminoglycosides tested was proven. The high MIC values for neomycin (2500 µg/mL) and amikacin (788 µg/mL) were reduced to 248 and 78 µg/mL, respectively, when they were associated with the EO (Barreto et al. 2014).

- Determination of the effect of EOs and main components on outer membrane protein (OMP) composition

Currently, the knowledge about the mode of action of EOs against pathogens has been increasing. To date, studies have demonstrated that the bacterial cell targets of EOs include the cell wall and membrane, thereby disturbing ATP production and pH homeostasis (Guinoiseau et al. 2010, Bouhdid et al. 2010, Muthaiyan et al. 2012). Moreover, EOs can influence the cellular transcriptome, proteome, and the quorum-sensing system (Faleiro – Miguel 2013). It is well-known that Gram-negative bacteria are more resistant to EOs than Gram-positive bacteria because of the difference in their cell wall structure. In 2013 Nazzaro et al. published a review about the mode of action of EOs on pathogenic bacteria. They concluded that EOs and their components have single target or multiple targets during their antimicrobial activity (Nazzaro et al. 2013). Previously, our research group investigated the effect of thyme EO and their two components (thymol and carvacrol) on the OMPs of *Erwinia* strains with microfluid chip technology (Horváth et al. 2009). Changes in the composition of bacterial OMPs may result in antibiotic resistance and in altered invasive ability. Furthermore, certain members of the OMPs are parts of antibacterial efflux pumps in this way also contributing to the antibiotic resistance (Denyer – Maillard 2002). In our study streptomycin, gentamicin and thyme EO were capable of significantly changing the protein profile which may contribute to the explanation of antibacterial effect of thyme oil on pathogenic *Erwinia* strains. The antibacterial activity of the EO on the whole is supposed to be related with its most abundant components, but the effect of the minor components should be also taken into consideration (Horváth et al. 2009, Horváth et al. 2010).

During this study, our workgroup had demonstrated the effect of cinnamon bark EO and clove EO on OMP composition of *Pseudomonas aeruginosa* (Felső et al. 2013). The oils were administered to the culture at concentrations of 0.5 x MIC and 2 x MIC and incubated for 60 min. Some proteins (heat shock, flagellin) disappeared after the treatment of cinnamon and clove oils. Decreases in the amount of some proteins may be explained by the protein synthesis inhibiting effect of these oils. We received similar result to the study published first by Burt et al. (2007). We conclude that the mode of antimicrobial action of EOs is highly related to their chemical composition (Faleiro – Miguel 2013, Nazzaro et al. 2013). Lab-on-a-chip technique applied in this study for the analysis of outer membrane protein composition of bacterial strains ensures a fast, qualitative and quantitative method for studying the antibacterial effect of essential oils. Another effective possibility when the traditional SDS-PAGE or the lab-on-a-chip techniques are combined with modern MS methods. In this case we can correctly identify the questionable proteins.

(3) Investigation of the anti-inflammatory effects of essential oils *in vivo* in the endotoxin-induced mouse model

Respiratory tract diseases associated with bacterial infection and inflammation affect a large number of people from every age group worldwide. According to the data of World Health Organization

(2014), lower respiratory tract infections are responsible for 5% (3.1 million people) of deaths worldwide regarding both sexes. Therefore, it is worth investigating the potential therapeutic role of EOs in respiratory diseases. It is well known that the results coming from the *in vivo* animal models cannot be directly extrapolated to humans, but may provide valuable data about the mechanism of constituents tested. Before the full clinical application, further and more comprehensive *in vivo* studies are still required. Therefore, we investigated the anti-inflammatory effect of thyme, cinnamon bark, citronella, clove and scots pine in *in vivo* in the endotoxin-induced mouse model. We selected these EOs according to our microbiological findings and their traditional medicinal use. Because we have not published our results coming from animal study yet, therefore, we provide detailed description here due to the NKFI's rule. Our paper is under preparation.

- Endotoxin-induced acute airway inflammation model

Experiments were performed on female 8-12 week-old C57Bl/6 mice (18-25 g), 6-8 animals/group. Animals were bred and kept in the Laboratory Animal House of the Department of Pharmacology and Pharmacotherapy, University of Pécs, at 24-25°C, provided with standard chow and water *ad libitum*, maintained under 12 h light-dark cycle. All procedures were carried out according to the 40/2013 (II.14.) Government Regulation on Animal Protection and Consideration Decree of Scientific Procedures of Animal Experiments and Directive 2010/63/EU of the European Parliament. They were approved by the Ethics Committee on Animal Research of University of Pécs according to the Ethical Codex of Animal Experiments (licence No.: BA02/2000-5/2011).

Determination of airway responsiveness:

Interstitial acute lung inflammation was evoked by 60 µl intratracheal *Escherichia coli* (serotype: 083) endotoxin [lipopolysaccharide: LPS, 100 µg/ml dissolved in sterile phosphate-buffered saline (PBS)] (Helyes 2007, Elekes 2007). Intratracheal administration of LPS and PBS were carried out under ketamine-xylasine anaesthesia. Animals were divided into four groups: (1) mice receiving 60 µl PBS and exposed to paraffin oil inhalation, (2) mice receiving 60 µl PBS and exposed to EO inhalation, (3) mice receiving LPS and exposed to paraffin oil inhalation, (4) mice receiving LPS and exposed to EO inhalation. In a box (in which there was a paper disc containing EO on the lid of the box), mice inhaled EO 3 times for 30-30 min during the 24 h experimental period. After the first inhalation, mice received PBS or LPS. 24 hours after PBS/LPS administration airway responsiveness and pulmonary function were assessed by whole-body plethysmography (WBP) with Buxco instrument (PLY3211, Buxco Europe Ltd., Winchester, UK) in conscious, spontaneously breathing animals. We determined the breathing frequency (f), as well as tidal volume (TV), minute ventilation (MV), relaxation time (RT), inspiratory time (Ti), expiratory time (Te), peak inspiratory and expiratory flows (PIF, PEF) and enhanced pause (Penh) value, as an indicator of bronchoconstriction. Penh is a calculated parameter ((expiratory time/relaxation time)−1)/(max. expiratory flow/max. inspiratory flow), correlating with airway resistance measured by traditional invasive techniques using ventilated animals. Bronchoconstriction was induced by 11 and 22 mM of aerosolized carbachol (carbamoyl-choline; Sigma, St. Louis, MO, USA; dissolved in saline, 50 µl/mouse) following baseline measurements performed with aerosolized saline. The acquisition period was 2 minutes after the saline and 15 minutes after each carbachol solution. Breathing function parameters were calculated by the software component of the WBP system (Buxco, UK) (Elekes et al. 2008). At the end of the measurement mice were deeply anaesthesized by the mixture of ketamine-xylazine (100 mg/kg and 10 mg/kg i.p.) and the lungs were excised. Left lungs were fixed in 4% formaldehyde for 8 h and embedded in paraffin. The right lungs were cut into two halves and put in liquid nitrogen. Thereafter right lungs were stored at -80°C until further examination.

Histological studies and scoring:

According to WBP results we involved the EO of thyme (as effective EO), cinnamon bark (as effective EO) and citronella (as irritating EO) in histological studies. Formalin-fixed and paraffin-embedded lung sections were stained with hematoxylin-eosin and periodic acid-Schiff to precisely visualize mucus producing goblet cells. Semiquantitative scoring of the inflammatory changes was performed by an expert pathologist blinded from the study as described by Zeldin et al. (2001) on the

basis of perivascular edema (0-3), perivascular/peribronchial acute inflammation (0-3), goblet cell metaplasia of the bronchioles (0-2), and macrophages/mononuclear cells in the alveoli (0-2).

Measurement of myeloperoxidase (MPO) activity in the lungs:

The endotoxin-induced inflammatory reaction of the pulmonary tissues results in the recruitment of neutrophil granulocytes, monocytes and macrophages of myeloid origin expressing MPO enzyme (Rodrigues et al. 2002). MPO activity was determined from the homogenized and centrifuged lung samples (KH_2PO_4 20 mM and K_2HPO_4 20 mM buffer, pH=7.4) with spectrophotometry using H_2O_2-3,3',5,5'-tetramethyl-benzidine (H_2O_2/TMB, Sigma-Aldrich, Budapest) reaction and compared to a human standard MPO preparation (Sigma-Aldrich, Budapest). Reactions are performed in 96-well microtitre plates in room temperature, the optical density (OD) at 620 nm is measured at 5 min intervals for 30 min, using a microplate reader and plotted. The reaction rate (ΔOD/time) was derived from an initial slope of the curve. A calibration curve was then produced, with the rate of reaction plotted against the standard samples (Helyes et al. 2009).

Measurement of inflammatory cytokine concentrations in the lungs with Luminex technique:

According to the positive results obtained from WBP, histological and MPO experiments we involved only the EO of thyme in this method. The excised and frozen lung tissues were thawed and weighted, and immediately placed in cold PBS containing 10 mg/ml phenylmethanesulfonyl fluoride (PMSF, Sigma Aldrich, P7626) protease inhibitor, and were homogenized on ice with Miccra D-9 Digitronic device (Art-moderne Laborteknik, Germany). They were centrifuged then for 20 min (4°C, 4,000 rpm) and supernatants were kept and stored at -80°C until the measurement. Luminex Multiplex Immunoassay was performed to determine the protein concentrations of the following cytokines/chemokines using customized Milliplex Mouse Cytokin/Chemokine Magnetic Bead Panel (Millipore): 1. interleukin-1beta (IL-1β); 2. interleukin-6 (IL-6); 3. chemokine (C-X-C motif) ligand 1 (CXCL1) also called keratinocyte chemoattractant (KC); 4. chemokine (C-X-C motif) ligand 2 (CXCL2) also called macrophage inflammatory protein 2 (MIP-2); 5. chemokine (C-C motif) ligand 2 (CCL2) is also referred to as monocyte chemoattractant protein 1 (MCP-1); 6. tumor necrosis factor alpha (TNF-α). Following previous optimizations, all samples were tested undiluted in a blind-fashion and in duplicate. The experiment was performed according to the manufacturer's instructions. Briefly, 25 µl volume of each sample, control, and standard was added to a 96-well plate (provided with the kit) containing 25 µl of capture antibody coated, fluorescent-coded beads. Biotinylated detection antibodies and streptavidin-PE were added to the plate after the appropriate incubation periods. After the last washing step, 150 µl volume of sheat fluid was added to the wells, the plate was incubated and read on the Luminex100 instrument. Five-PL regression curve were used to plot the standard curves for all analyte by the xPonent 3.1 software analysing the bead median fluorescence intensity. Results are given in pg/mg wet tissue.

Statistics:

Values for pulmonary function measurements were expressed as the mean ±SEM of n= 6-8 mice in each group. Evaluation of the parameters was performed by one-way ANOVA with the exception of Penh analysed by Two-way ANOVA, both of them followed by Tukey's multiple comparisons test to see statistical differences between different data sets. MPO activity and cytokine concentrations were also expressed as the mean ±SEM of n= 6-8 mice in each group and analyzed with one-way ANOVA followed by Bonferroni's post test or unpaired t-test. Composite histopathological inflammatory score values (based on n=10 sections) were demonstrated in box plots, showing the median, upper/lower quartile and maximum/minimum values, statistically evaluated with Kruskal-Wallis analysis followed by Dunn's post test. In all cases $p<0.05$ was accepted as significant.

Results of determination of airway responsiveness:

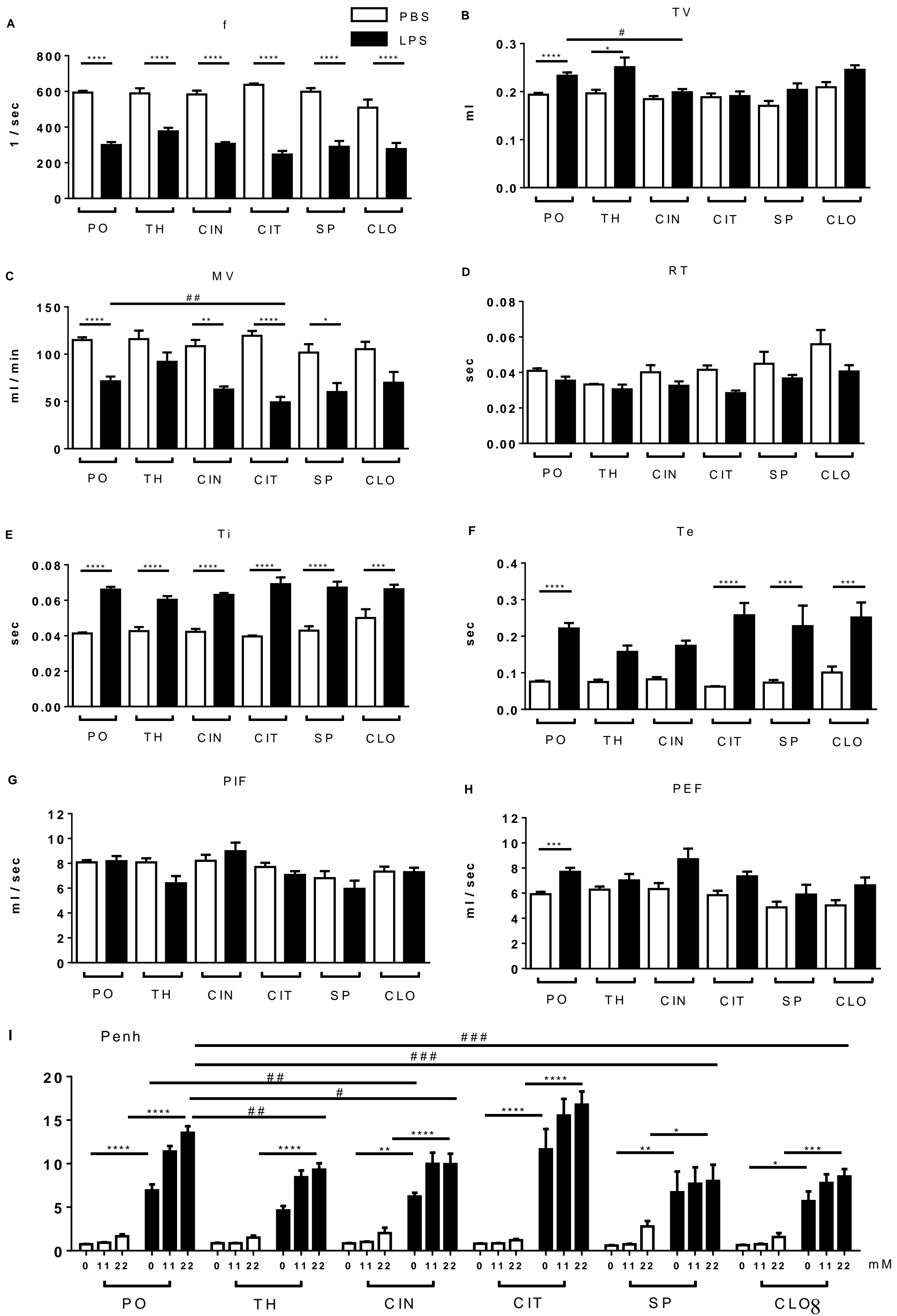
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Fig. 4 Unrestrained whole body plethysmography (WBP) parameters 24 hours after PBS and LPS administration (white and black columns, respectively) receiving paraffin oil (PO), thyme oil (TH), cinnamon oil (CIN), citronella oil (CIT), Scots pine (SP) and clove oil (CLO) inhalation. Panel A: frequency (f), B: tidal volume (TV), C: minute ventilation (MV), D: relaxation time (RT)., E: time of inspiration (Ti), F: time of expiration (Te), G: peak inspiratory flow (PIF) and H: peak expiratory flow (PEF) I: enhanced pause (Penh) measurements at baseline (0 mM), 11 and 22 mM aerosolized carbachol. n= 6-8 per group. (*p<0.05; **p<0.005; ***p<0,0005; ****p<0,0001 PBS and LPS groups inhaling the same oil, #p<0.05; ##p<0.005; ###p<0,0005; LPS + PO vs. LPS + essential oil groups).

Baseline Penh values were elevated 24 h following intratracheal LPS administration compared to the respective PBS-treated control mice. Inhalation of increasing concentrations of carbachol elicited concentration-dependent bronchoconstriction. The inhalation of thyme, cinnamon, scots pine and clove EOs reduced the airway hyper-responsiveness compared to LPS+PO group. However, citronella induced airway hyper-reactivity.

Results of histological studies and scoring:

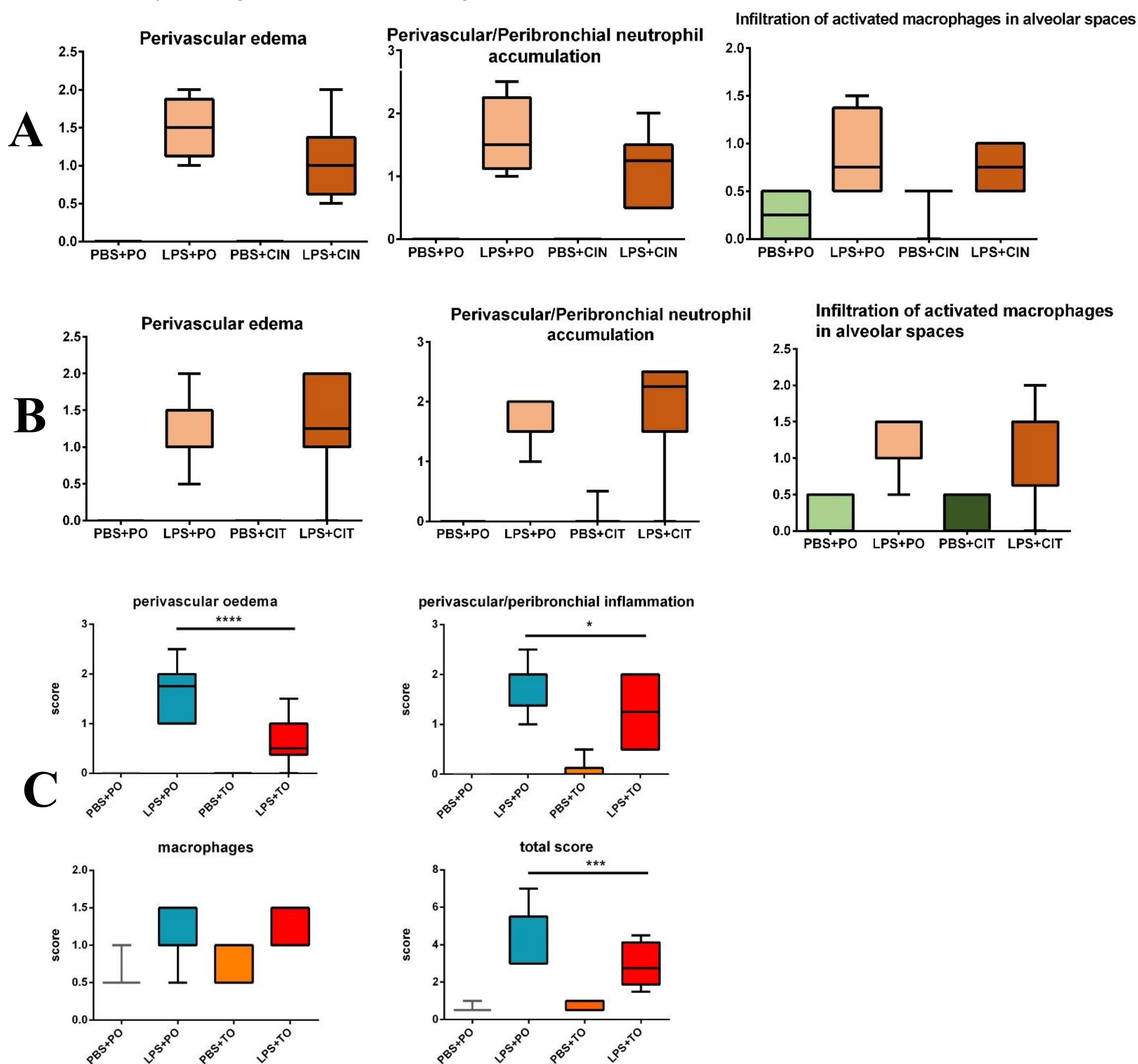
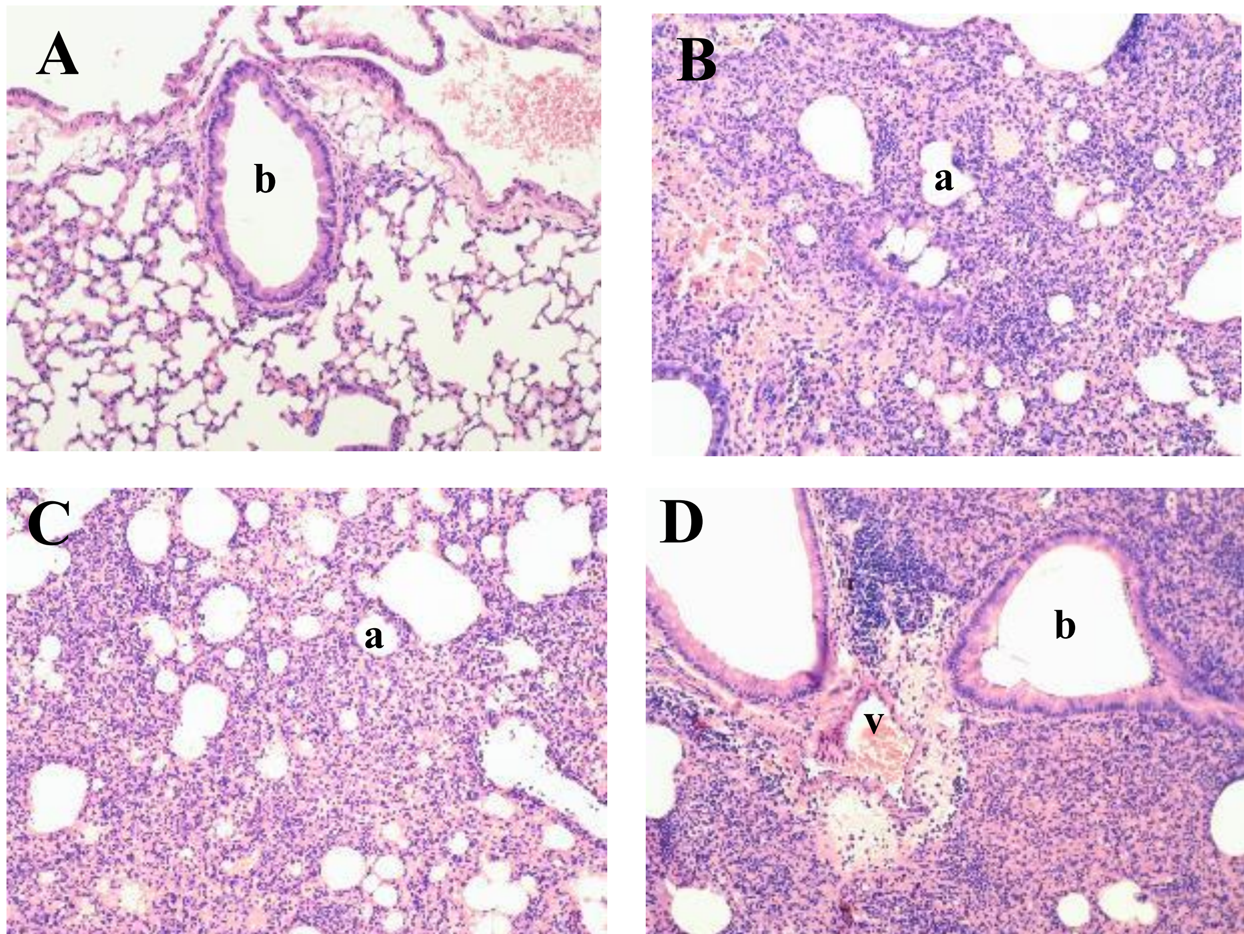


Fig. 5 Semiquantitative histopathological inflammation scores based on 3 parameters. (A) CIN: cinnamon bark EO, (B) CIT: citronella EO, (C) TO: thyme EO. n=10 sections, *p<0.05, ***p<0.05, ****p<0.0005. The hyperplasia of goblet cells which secrete mucus was not observed on histological sections. The treatment with cinnamon EO could decrease the edema formation around the bronchi and vessels, as well as the peribronchial neutrophil accumulation and infiltration of activated macrophages in alveolar spaces, but these changes were not statistically significant. However, the inhalation of thyme EO significantly decreased the edema formation around the bronchi and vessels, as well as the peribronchial neutrophil accumulation. Contrary, citronella increased the edema formation, peribronchial neutrophil accumulation and infiltration of activated macrophages in alveolar spaces.



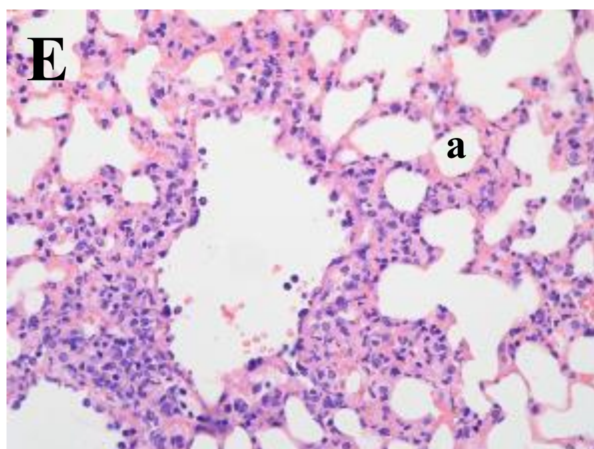


Fig. 6 Histopathological changes of the lungs. Representative histological pictures showing (A) PBS-treated mouse, (B) LPS+PO-treated mouse, (C) LPS+CIN-treated mouse, (D) LPS+CIT-treated mouse, (E) LPS+TO-treated mouse. HE staining. Magnification: 200x and 400x. a: alveoli, b: bronchiole, v: vessel. LPS-treatment induced edema and neutrophil accumulation. This inflammatory reaction could be decreased by TO-treatment. Neutrophil accumulaton was observed after cinnamon-treatment, but it was not so severe compared with LPS-treatment. Citronella-treatment caused edema and neutrophil accumulation around vessels and bronchi.

Measurement of myeloperoxidase (MPO) activity in the lungs:

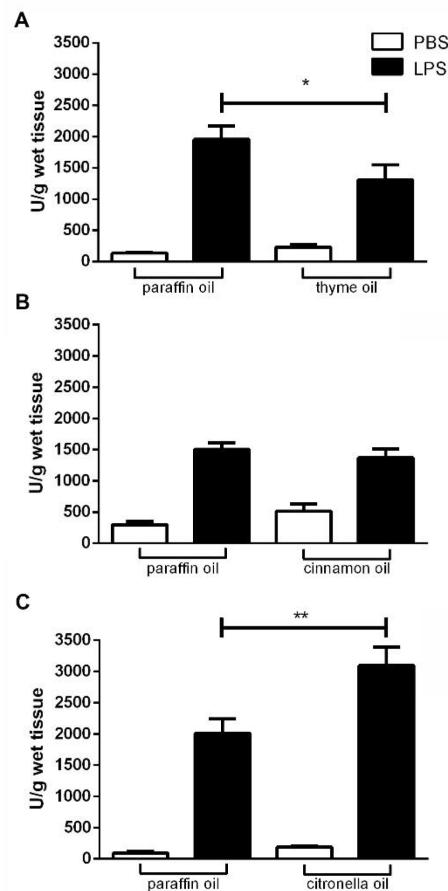


Fig. 7 MPO activity correlating with the number of accumulated granulocytes and macrophages in the lungs was significantly attenuated after thyme EO-treatment. *p<0.05, **p<0.005. Contrary, citronella EO increased the MPO activity which correlates with the histological findings. Fig. 7. also demonstrates that 24 h after LPS-treatment MPO activity was elevated in the inflamed pulmonary tissues of mice due to the recruitment of neutrophils and mononuclear cells in response to endotoxin stimuli. In case of clove and scots pine the MPO activity significantly increased vs. LPS+PO group (data not shown).

Measurement of inflammatory cytokine concentrations in the lungs with Luminex technique:

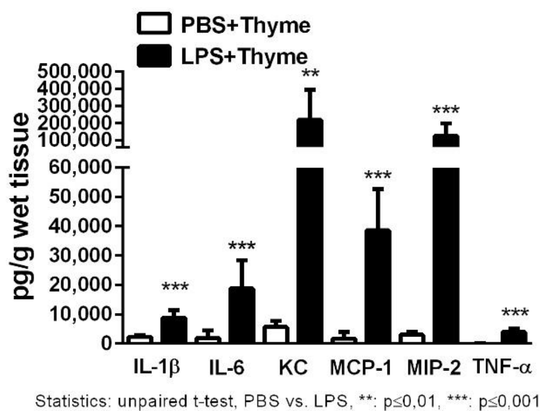


Fig. 8 Concentrations of the inflammatory cytokines in the lung homogenates. In the PBS+thyme EO group the level of cytokines was not elevated so high compared with LPS+thyme group. After endotoxin stimulus the level of each cytokine was significantly increased. Therefore, the thyme EO could not

reduce the endotoxin-induced inflammatory process. On the other hand, in the non-inflammed tissues (PBS+thyme group) the thyme EO did not evoke inflammation.

We conclude that a broad range of experimental protocols are used worldwide by different research groups. Although the basic pathophysiology is similar after certain inflammatory stimuli, the effects depend on the animal species, gender and age, the type and dose of the inflammatory agent, as well as the route of administration and the duration of exposure and investigation (Helyes – Hajna 2012). According to the animal results thyme EO can be regarded as effective agent against endotoxin-induced acute airway inflammation. Thyme oil inhalation could be a potentially promising supplementary treatment in respiratory diseases. The anti-inflammatory effect of EOs generally depends on their main compounds. Zhou et al. (2014) studied the effect of thymol constituents on allergic airway inflammation in an ovalbumin (OVA)-induced mouse asthma model. Animals were orally treated with thymol in a dose of 4, 8, and 16 mg/kg body weight 1 h before OVA challenge. Thymol reduced the level of IgE, IL-4, IL-5 and IL-13, as well as the number of inflammatory cells in the airways. Moreover, thymol decreased the airway hyper-responsiveness and blocked the activation of NF-κB pathway. Authors concluded that based on these results thymol may be involved in the treatment of allergic asthma.
On the other hand, inhalation of EO of scots pine and citronella is not recommended in case of respiratory inflammation based on the measurement of respiratory and histological parameters and MPO activity. To the best of our knowledge, we describe here first evidence about the effect of these three EOs in endotoxin-induced acute airway inflammation model.

Based on our *in vivo* findings, we can recommend and suggest the composition of products used for inhalation. E.g. in the official Formulae Normales (FoNo VII., standard prescription collection) there is a medicine called "Aetheroleum pro inhalatione" containing EOs of eucalyptus and scots pine as ingredients. Because of irritating property of scots pine EO proved in our studies we suggest the replacement of this oil to thyme EO. In the future we would like to reveal the pharmacological mode of action of these EOs. In a review published in 2011, Banner et al. summarized the evidence that modulation of selected TRP channels may have beneficial effects in targeting key features of several respiratory diseases including inflammation of the airways, hyper-reactivity of the airways, mucus secretion and cough. In 2015 we published a review about the EOs and their role in the treatment of respiratory diseases (Horváth – Ács 2015). Without doubt, further studies, principally human trials, are needed to assess the efficacy and tolerability of EOs in respiratory tract diseases.

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