Investigation of the effect of essential oils in an acute airway inflammation mouse model

PhD Thesis

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1. Introduction

We have a lot of evidence for the use of essential oils since ancient times. In the Middle Ages, in addition to their role in religious life, protection against epidemics and the alleviation of fever, headache and cough were also included among the possible uses [Burt 2004]. As natural remedies are becoming more and more popular today, interest in essential oils has also revived. The majority of them are used based on traditional use and experience gathered over the years, however, in order to use essential oils with sufficient efficiency and safety, a thorough understanding of their effects on the living organism is essential.

The use of essential oils in the case of cold and other respiratory diseases is increasing among people, either independently or as an adjunct to drug therapy. Respiratory diseases such as Chronic Obstructive Pulmonary Disease (COPD), lower respiratory tract infections and respiratory tract cancers lead the list of the most common causes of death [WHO], and their mortality rates are increasing, despite advances in treatment options. In most cases, these diseases are processes involving acute or chronic airway inflammation, and the traditional medical therapy of inflammatory conditions is based on the use of steroids (SAIDs) and non-steroidal anti-inflammatory drugs (NSAIDs). The use of steroid anti-inflammatory drugs (e.g. glucocorticoids) in this indication is already controversial, as they can be associated with many side effects (e.g. osteoporosis, metabolic disorders) [Schäcke et al. 2002]. Long-term use of non-steroidal anti-inflammatory drugs (e.g. aspirin, ibuprofen, diclofenac) can cause gastrointestinal, cardiovascular, liver, kidney, brain and lung problems [Buchanan and Bellamy 1991, Bindu et al. 2020]. That is why the development of safer anti-inflammatory drugs is necessary. The use of essential oils in problems affecting the respiratory tract is a logical step, since in such indications the volatility of their components prevails, thanks to which they easily enter the respiratory tract by inhalation, so they can have a local effect. Furthermore, after oral or systemic application, several of their components are excreted in the lungs, so they may even be suitable for achieving a delayed local effect. Their complex composition also offers the possibility of multiple points of attack/effect mechanisms, their increasingly frequently studied antibacterial properties can be useful, for example, in the treatment of respiratory tract infections. Their *in vivo* anti-inflammatory effect is less

well known, there is little animal and human data available, but based on the results so far, essential oils can also play a role in the prevention and treatment of respiratory diseases, even as a supplement to personalized therapies, and in improving the quality of life of patients. That is why it is necessary to study their effect and possible side effects. [Faleiro and Miguel 2013, Li et al. 2022]

2. Aims

In the course of our research work, we planned to investigate the anti-inflammatory effect of essential oils that often occur in practice, have a promising anti-inflammatory effect based on the literature, and have an antibacterial effect proved in our preliminary microbiological experiments, using *in vivo* methods as follows:

- Determination of the chemical composition of essential oils obtained from drug store using gas chromatography.
- *In vivo* investigation of the effect of inhaled essential oils and study of their mechanism of action in an animal model of acute respiratory inflammation induced by endotoxin (lipopolysaccharide, LPS). In addition to the respiratory function measurements, we also aimed at the histological examination, the measurement of myeloperoxidase activity (MPO activity) and the amount of inflammatory cytokines of the lung samples collected from the animals.

3. Material and methods

3.1. The experimental model

In our experiments, we used endotoxin-induced pneumonia mechanism model. All chemicals were obtained from Sigma-Aldrich Kft (Budapest, Hungary).

3.1.1. Essential oil samples

During our experiments, we applied commercially available (Aromax Zrt.) essential oils:

- lemongrass oil (source plant: *Cymbopogon nardus*, production number: E8912/1309)
- cinnamon oil (source plant: *Cinnamomum zeylanicum*, production number: H4991/1511)
- pine oil (source plant: *Pinus sylvestris*, production number: 69543/1509)
- thyme oil (source plant: *Thymus vulgaris*, production number: H3981/1509)
- clove oil (source plant: *Eugenia caryophyllata*, syn. *Syzygium aromaticum*, production number: J3481/1609)

The essential oils were purchased at the Herbária herbal shop and stored according to the regulations until use and during the experiments.

Shirazi thyme oil (source plant: *Zataria multiflora*) was obtained by distillation of the plant sample:

Packaged dried plant material (leaves) of *Zataria multiflora* were collected in spring 2018 in Shiraz, Iran and identified by Professor Younes Ghasemi (School of Pharmacy, Shiraz University of Medicine). The essential oil was obtained by steam distillation in 2018 according to the description of the distillation of thyme oil in the Hungarian Pharmacopoeia [Ph.Hg.VII]. 40.0 g of chopped (particle size <0.8 mm, V. sieve) dried leaves were used for one distillation. We performed a total of three distillation processes, the duration of each distillation was 3 hours. The essential oil content was measured using the volumetric method and expressed as a mass percentage of the plant material: dried *Zataria multiflora* contained 2.5% essential oil.

3.1.2. Animals

Adult (10-18 weeks old) female mice were used in the project. Male mice are more likely to develop severe LPS-induced lung inflammation, while females have lower mortality during the protocol [Chen et al. 2010, Helyes and Hajna 2012]. In order to determine the mechanism of action, including the role of TRP channels, C57Bl/6, TRPA1 wild type, TRPA1 knockout, TRPV1 knockout, and TRPV1/A1 knockout mouse strains were included in the experiment. The age and weight distribution of mice in each group was similar to avoid differences associated with these parameters.

The mice were kept in the animal houses of the Department of Pharmacology and Pharmacotherapy of the Medical School, University of Pécs in 325*170*140 mm cages, at 24-25°C, with a 12-hour dark-light cycle. Standard rodent chow and drinking water were freely available.

The experimental protocols, which comply with European legislation (Directive 2010/63/EU) and Government regulation (40/2013, II. 14.) and were drawn up according to the recommendations of the International Association for Research on Pain (IASP), were approved by the Ethics Committee dealing with animal experiments of the University of Pécs (BA02/2000 -26/2018).

3.1.3. Experimental protocol

During our experiments, we divided the mice into four groups of 8-10 elements each:

- PBS-PO: intratracheal 60 µl PBS control; inhaled paraffin oil control
- LPS-PO: intratracheal 60 µl LPS / PBS; inhaled paraffin oil control
- PBS-IO: intratracheal 60 µl PBS control; inhaled essential oil
- LPS-IO: intratracheal 60 µl LPS / PBS; inhaled essential oil

Two groups received the tested essential oil three times (at 0, 4 and 24 hours) for 30 minutes by dry inhalation, two groups received paraffin oil as a control. For inhalation, the animals were placed in a closed box measuring 33.5 x 19 x 12 cm. To model dry inhalation, 2 drops of essential oil were dropped onto an 8 cm diameter filter paper disk attached to the inner half of the top of the box. The maximum essential oil concentration in the air space can be calculated at 6.55 µl/l.

Under anesthesia (intraperitoneal 100 mg/kg ketamine and 5 mg/kg xylazine), each mouse in two groups received 60 µl of LPS (167 µg/ml, dissolved in sterile PBS), the other two groups received 60 µl of PBS solvent as a control intratracheally. LPS was isolated from *Escherichia coli* (serotype 083) bacterial culture at the Department of Medical Microbiology and Immunology of the Medical School, University of Pécs. Intratracheal LPS administration can induce more localized acute lower respiratory tract inflammation than intranasal administration, although symptoms of systemic inflammation such as fever, sedation, decreased appetite and weight loss may still occur [Chen et al. 2010, Helyes and Hajna 2012].

Respiratory parameters were measured 24 hours after the administration of LPS and pure PBS with a non-invasive whole-body plethysmograph, followed by termination with intraperitoneal ketamine-xylazine anesthesia. The lungs were excised, and the left lung lobe was placed in 6% formaldehyde for histological processing. The right one was cut into two pieces, frozen with liquid nitrogen and stored at -80ºC for further (MPO and cytokine) measurements.

3.2. Methods

3.2.1. Analysis of essential oil composition

The most commonly used methods for analyzing the composition of essential oils are the mass spectrometer (MS) and the flame ionization detector (FID). The former is usually preferred for qualitative measurements, the latter for quantitative measurements. We used a gas chromatography (GC) procedure with solid phase microextraction (SPME) sampling with a single quadrupole mass spectrometer (GC-MS) and a flame ionization detector (GC-FID).

Essential oil analysis with solid phase microextraction (sHS-SPME): Samples were put into vials (20 mL headspace) sealed with a silicon/PTFE septum prior to SPME-GC/MS analysis. Sample preparation using the static headspace solid phase microextraction (sHS-SPME) technique was carried out with a CTC Combi PAL (CTC Analytics AG, Zwingen, Switzerland) automatic multipurpose sampler using a 65 µM StableFlex polydimethyl siloxane/carboxene/divinyl benzene (CAR/PDMS/DVB) SPME fiber (Supelco, Bellefonte, PA, USA). After an incubation period of 5 min at 100ºC, extraction was performed by exposing the fiber to the headspace of a 20 ml vial

containing the sample for 10 min at 100ºC. The fiber was then immediately transferred to the injector port of the GC/MS, and desorbed for 1 min at 250ºC, split ratio was 1:50. The SPME fiber was cleaned and conditioned in a Fibre Bakeout Station in pure nitrogen atmosphere at 250ºC for 15 min.

Gas chromatography and mass spectrometry (GC-MS, GC-FID): 1 µl of essential oil samples diluted in ethanol (10µl/ml) were injected in split mode, the injector temperature was 250°C and the split ratio was 1:50. The analyses were carried out with an Agilent 6890N/5973N GC-MSD (Santa Clara, CA, USA) system equipped with an Supelco (Sygma-Aldrich) SLB-5MS capillary column (30 m × 250 µm × 0.25 µm) for GC-MS measurements and a J&W (Agilent) DB-5MS capillary column (25 m × 250 µm × 0.25 µm) for GC-FID measurements. The GC oven temperature was programmed to increase from 60ºC (3 min isothermal) –250ºC at 8 ºC/min (1 min isothermal). High purity helium (6.0) was used as carrier gas at 1.0 mL/min (37 cm/s) in constant flow mode.

The mass selective detector (MSD) was equipped with a quadrupole mass analyzer and was operated in electron ionization mode at 70 eV in full scan mode (41–500 amu at 3.2 scan/s).

The temperature of the flame ionization detector (FID) was 300ºC, He flow was 30mL/min, air flow was 400.0mL/min, the makeup gas was Nitrogen in constant flow mode (25 ml/min).

The data were evaluated using MSD ChemStation D.02.00.275 software (Agilent). The identification of the compounds was carried out by comparing retention times and recorded spectra with the data of authentic standards, and the NIST 2.0 library was also consulted. The percentage evaluation was carried out by area normalization.

3.2.2. Pulmonary function measurement

Respiratory function parameters were measured with a whole-body plethysmograph (Buxco Europe Ltd, Winchester, UK) 24 h after LPS administration in awake, freely moving animals. To measure Penh, the baseline was taken while nebulizing 50 µl of saline. Bronchoconstriction was induced by nebulizing a solution of the cholinergic agonist carbachol (50 µl/mouse, in 11.0 and 22.0 mM in saline) for 2 minutes. Each nebulization was followed by 15-minute measurement periods.

The measured parameters:

- breathing frequency (f),

- tidal volume (TV),
- minute ventilation (MV),
- relaxation time (RT),
- time of inspiration (Ti),
- time of expiration (Te),
- peak inspiratory flow (PIF),
- peak expiratory flow (PEF),
- Penh (Enhanced Pause).

Penh is a calculated parameter, the software calculates it based on the following formula:

$$\frac{\text{time of expiration / relaxation time - 1}}{\text{peak expiratory flow / peak inspiratory flow}}$$

This value correlates with airway hyperreactivity measured in animals ventilated with the common invasive technique [Helyes et al. 2009].

3.2.3. Histopathological assessment and semiquantitative scoring

From the lung samples fixed in 6% buffered formalin and embedded in paraffin, sections with a thickness of 5-7 μm were made with a microtome, and then stained with hematoxylin-eosin (HE) and periodic acid-Schiff (PAS) to visualize mucus-producing cells. The completed sections were evaluated using a light microscope based on a semiquantitative point system with the help of a pathologist colleague. Sections were made from each lung sample at 3 depths and these were averaged.

The following parameters were scored:

- perivascular edema (0-3)
- perivascular/peribronchial neutrophil accumulation (0-3)
- goblet cell hyperplasia of the bronchioles (0-2)
- infiltration of macrophages/mononuclear cells in the alveolar spaces (0-2) [Zeldin et al. 2001]

3.2.4. Measurement of myeloperoxidase activity

The quantitative marker of the accumulation of neutrophil granulocytes and macrophages is the activity of the myeloperoxidase (MPO) enzyme. Lung samples frozen at -80ºC were used to determine MPO activity. The samples were homogenized in phosphate buffer, thawed, and weighed, but continuously kept on ice, after centrifugation

the pellet contains the MPO. By homogenizing this sediment with an HTAB buffer solution (0.5% hexadecyltrimethylammonium bromide in phosphate buffer), and then collecting the clear supernatant after centrifugation, we obtained the measurable, properly prepared and cleaned samples. The MPO activity of the samples was measured using the reagent tetramethylbenzidine dihydrochloride (TMB, dissolved in phosphate citrate buffer) and hydrogen peroxide with a spectrophotometric method, using a plate-reader (Labsystems) at a wavelength of 620 nm, with two measurement points, 5 minutes apart. MPO from human leukocytes (Sigma "MPO from human leukocytes") was used as a standard.

Colorless tetramethylbenzidine participates as a proton donor in the reaction, during which in the first, step a blue (peak at 370 and 652 nm) and in the second step, a yellow (peak at 450 nm) product is formed [Josephy et al. 1982].

By calculating the averages of the optical density of the two parallel measurements, comparing the difference between the values measured at the two measurement points, i.e. the change (increase) of the optical density with the calibration curve calculated from the standard sample, we obtained the [unit MPO/ml] values. The MPO activity per gram was calculated using the dilution and tissue weights.

3.2.5. Inflammatory cytokine concentration

Another portion of lung tissue samples frozen at -80ºC were thawed and weighed, then immediately placed in cold PBS containing 0.01% phenylmethanesulfonyl fluoride (PMSF) protease inhibitor and kept on ice continuously. After homogenization and centrifugation, the clear supernatant was collected and the measurable, properly prepared and cleaned samples were obtained. Using these, we performed a Luminex Multiplex Immunoassay with a customized Milliplex Mouse Cytokine/Chemokine Magnetic Bead Panel (Millipore) to determine the protein concentrations of the following cytokines/chemokines:

- interleukin-1beta (IL-1β)
- interleukin-6 (IL-6)
- chemokine (C-X-C motif) ligand 1 (CXCL1), also called keratinocyte chemoattractant (KC)
- chemokine (C-C motif) ligand 2 (CCL2), also called monocyte chemoattractant protein 1 (MCP-1)

- chemokine (C-X-C motif) ligand 2 (CXCL2), also called macrophage inflammatory protein 2 (MIP-2)
- tumor necrosis factor alpha (TNF-α)

The six analytes were detected simultaneously on a 96-cell plate. All samples were tested undiluted, blind, and in duplicate. Based on the instructions for use, a mixture of the six antibody-coated bead populations was added to the plate in an amount of 25 µl/cell together with the standards and controls into the designated cells. After overnight incubation at 4°C, the plate was washed three times using a manual magnetic plate. After washing, 25 µl/cell detection antibody solution was added, and the plate was incubated for 60 minutes at room temperature with shaking at 500 rpm. After the following washing steps, 25 µl/cell streptavidin-phycoerythrin (SAPE) solution was added, and the plate was incubated for another 30 minutes at room temperature with shaking. After washing three times, 150 µl/cell drive buffer was added to the plate, and the assay was read on a Luminex MagPix (Merck Hungary Kft., Budapest, Hungary). A five-variable regression curve was used to edit the standard curves of all analytes with Belysa 1.1 software, analyzing the median fluorescence intensity of the beads. Results were given in pg/mg.

3.2.6. Statistical analysis of data

Statistical analysis was performed with the GraphPad Prism Version 8.01 program. One- and two-way ANOVA) analysis with Bonferroni's and Tukey's post-tests were performed for airway parameters, MPO activity and cytokine levels. Statistical analysis of the histopathological scoring was performed using Kruskal-Wallis analysis followed by Dunn's post-test. In all cases, $p<0.05$ was accepted as significant.

4. Results

4.1. Chemical compositions of the examined essential oils

The percentages of the components in the essential oils were determined using gas chromatography analysis.

The major components in lemongrass oil were citronellal (36.2%), geraniol (25.3%) and citronellol (13.6%). As expected, the main component of cinnamon oil was cinnamic aldehyde (74.0%), while eugenol (88.6%) was found to be in clove oil. The main components of pine oil were α-pinene (39.4%), limonene (14.3%) and β-pinene (11.0%). In the case of thyme oil and Shirazi thyme oil, thymol (46.3% and 53.6%) is the component present in the highest proportion, although in thyme *p*-cymol (22.1%), while in Shirazi thyme oil carvacrol (30 .9%) was still present in larger quantities.

4.2. Effect of the inhalation of the examined essential oils

4.2.1. Effect of examined essential oils on respiratory function parameters

The whole-body plethysmograph is suitable for registering many respiratory function parameters.

LPS treatment significantly decreased respiratory rate, minute ventilation, and relaxation time, but increased tidal volume, inspiratory and expiratory time, and peak expiratory flow 24 h after administration, while not altering peak inspiratory flow. Correlating with airway hyperreactivity, Penh in LPS-treated mice increased significantly already at baseline and was further aggravated by bronchoconstriction induced by carbachol inhalation.

Inhalation of cinnamon, pine, thyme and clove oil also significantly reduced carbachol-induced hypersensitivity in LPS-treated mice compared to the paraffin oil treatment used as a negative control, while lemongrass and Shirazi thyme oil increased it further, i.e. it worsened the values of Penh - at the base line, this change also became significant in the case of lemongrass.

Lemongrass oil, pine oil and thyme oil significantly reduced tidal volume compared to the LPS group treated with paraffin oil. Regarding the other respiratory parameters, although some essential oils modified certain values, these changes were no longer significant.

In the control group receiving PBS, the inhalation of essential oils significantly affected the respiratory function in only one case: clove oil increased the relaxation time. This oil and parameter was the only one in our studies where essential oil inhalation significantly affected the non-inflamed control group.

4.2.2. The effect of the examined essential oils on the histological parameters

Based on the examination of the prepared lung sections and the semiquantitative histopathological scoring, goblet cells appeared in only two cases, in the sections of one member of the LPS groups treated with paraffin oil and lemongrass oil.

Administration of LPS induced neutrophil granulocyte and macrophage infiltration, which was associated with significant perivascular and peribronchial edema and, in a single section, goblet cell hyperplasia.

The inhaled essential oils had no significant effect on inflammation in any of the examined parameters of the semiquantitative histopathological evaluation—this is partly due to the high variability observed between individuals, mainly in the case of lemongrass, pine and clove oil—but visible changes have occurred. Significant edema formation induced by LPS was prevented by thyme, pine and clove oil, and in these groups, it no longer increased significantly compared to the corresponding PBS-treated controls. The same can be said about clove oil in the case of perivascular and peribronchial granulocyte infiltration, and cinnamon oil in the case of macrophage accumulation. Cinnamon, thyme, and clove oil effectively prevented the significant increase in the total score of histological changes. Lemongrass oil did not affect the extent of the scored histopathological changes, but even in the control group (the group treated with PBS), it caused hemorrhages and the appearance of lymphocytes in several cases, while in the group treated with LPS we observed mild goblet cell hyperplasia on the sections, and increased lymphocyte influx and alveolar destruction in addition to granulocytic inflammation. In the case of Shirazi thyme oil, unfortunately, no histological evaluation was performed (the samples were lost due to a laboratory move).

4.2.3. The effect of the examined essential oils on myeloperoxidase activity

As a result of LPS, myeloperoxidase activity—correlating with granulocyte and macrophage activity—increased in accordance with the histopathological results.

Surprisingly, this increased value was significantly reduced only by thyme oil. Inhalation of cinnamon and Shirazi thyme oil did not affect it, but it increased significantly further under the influence of lemongrass, pine, and clove oil.

4.2.4. The effect of the examined essential oils on cytokine expression

In LPS-induced inflammation, the levels of all tested cytokines and chemokines increased significantly.

Among the inhaled essential oils, pine oil significantly aggravated LPS-induced interleukin-1beta (IL-1β), while thyme oil significantly aggravated macrophage inflammatory protein-2 (MIP-2). Unfortunately, in the case of cinnamon oil, cytokine levels were not measured (the samples were lost due to a laboratory move).

4.3. Effect of thyme inhalation in TRPA1/V1, TRPA1 knockout mice

As a next step, the essential oil with the greatest anti-inflammatory potential, thyme oil, was selected for further testing. Using TRPA1 and TRPV1 gene-deficient mice, we tried to determine whether at least one of the two ion channels has a role in the effect.

The strains participating in the study:

- TRPA1/V1$^{+/+}$ (WT, wildtype) and TRPA1/V1$^{-/-}$ (KO, knockout)
- TRPA1$^{+/+}$ (WT, wildtype) and TRPA1$^{-/-}$ (KO, knockout)

4.3.1. Effect of thyme oil on respiratory function parameters of knockout mice

We investigated the effect of thyme oil on the respiratory function of different strains of mice, TRPA1/V1 double knockout and TRPA1 single knockout, with whole-body plethysmograph. In the latter case, due to the unexpected failure of the plethysmograph device, we

unfortunately only managed to register the parameters of one animal per group. Of course, no conclusions can be drawn from these TRPA1 knockout respiratory function results.

LPS-induced acute lung inflammation significantly decreased respiratory rate and minute ventilation and increased inspiratory and expiratory times in both A1/V1 wildtype and A1/V1 knockout mice. The respiratory volume, relaxation time, maximum inspiratory and expiratory flow did not show significant changes. The concentration-dependent bronchoconstriction induced by carbachol is illustrated by the Penh, and it was also significantly increased by LPS for both baseline (0 mM) and the two different concentrations (11 and 22 mM) of carbachol nebulization, in the A1/V1 wildtype and A1 /V1 knockout groups to a similar extent. In the case of the A1 groups, due to the number of 1 element per group, it is unfortunately not possible to count on the respiratory function results.

Inhalation of thyme oil did not significantly affect the changes induced by LPS, except for the values of Penh: in A1/V1 wildtype mice it significantly improved, in contrast in A1/V1 gene-deficient individuals it significantly aggravated airway hyperreactivity to carbachol, which may indicate that the effect of thyme oil is mediated by TRPA1 and/or TRPV1, i.e. its protective role cannot prevail in gene-deficient mice.

4.3.2. Effect of thyme oil on histological parameters of knockout mice

According to the results of the histological examination and semiquantitative scoring, LPS administration caused the development of perivascular edema with diffuse granulocyte infiltration, as well as perivascular and peribronchial inflammation and infiltration of activated mononuclear cells in both A1/V1 wildtype and A1/V1 gene-deficient mice, and goblet cell hyperplasia was also observed in one section. The A1 groups also showed similar results in response to LPS, although the A1 knockout produced slightly elevated parameters even in the control group treated with PBS.

Inhalation of thyme oil did not cause significant changes in perivascular edema, perivascular and peribronchial inflammation, or the number of macrophages, but resulted in a milder histopathological picture, which was less pronounced in the A1/V1 knockout compared to the A1/V1 wild type. The same can be seen in the A1 groups, although the results of the A1 knockout group show a large individual

variability, and thyme also increased the values in the PBS control group.

4.3.3. Effect of thyme oil on myeloperoxidase activity of knockout mice

LPS massively increased the activity of myeloperoxidase in the A1/V1 and A1 groups in both wildtype and gene-deficient mice.

Thyme prevented LPS-induced elevation in A1/V1 wildtype mice, but no longer exerted this beneficial effect in A1/V1-deficient mice. In the case of the A1 groups, it did not affect the LPS effect in the wild type, but in the gene knockout mice it significantly worsened the value already raised by LPS.

4.3.4. Effect of thyme oil on cytokine expression of knockout mice

LPS treatment increased the levels of all six investigated inflammatory cytokines and chemokines in each paraffin oil control group, but this change was only significant in the case of interleukin-1beta (IL-1β), keratinocyte chemoattractant (KC) and macrophage inflammatory protein-2 (MIP- 2).

In the case of the A1/V1 groups, thyme oil further increased the level of all measured values except keratinocyte chemoattractant (KC) in the A1/V1 wild group, and this effect was even more observed in the A1/V1 gene-deficient group. Among them, it significantly aggravated the levels of interleukin-6 (IL-6) and macrophage inflammatory protein-2 (MIP-2) in A1/V1 gene-deficient mice compared to wildtype A1/V1. In the A1 groups, inhalation of thyme did not cause significant differences.

5. New results, conclusion

Nowadays, essential oils and preparations containing essential oils are becoming more and more popular among the population as possible complementary products to drug therapy, and in some cases as independent therapeutic agents. The essential oils are mainly recommended to relieve the symptoms of mild respiratory diseases and cold by vaporizing them. Thanks to their volatility, they easily reach the respiratory tract, and due to their complex composition, they can act on several points of action (antibacterial, expectorant, antitussive and anti-inflammatory). However, it is important to point out that the use of essential oils is currently based on knowledge of traditional use and folk medicine, so we do not always have accurate knowledge on their effects and possible side effects (e.g. allergies, bronchospasm). That is why, in addition to the determination and independent examination of their components, the examination of the essential oils themselves is essential. To fulfill the criteria of evidence-based medication, we therefore aimed to investigate the *in vivo* anti-inflammatory effect of essential oils that are widespread in practice and promising based on the literature. The chemical composition of the essential oils was determined by gas chromatography, and their effect on inflammation was investigated in a mouse model of LPS-induced acute airway inflammation.

Based on our results, the mouse model of LPS-induced acute pneumonia is a suitable model for investigating the effect of essential oil inhalation. The use of lemongrass and geraniol-predominant essential oils of Ceylon lemongrass has been shown to have an unfavorable effect on several inflammatory parameters, so it could be avoided in the case of diseases associated with respiratory tract inflammation. Although Shirazi thyme oil did not have a significant effect on inflammation, it somewhat aggravated respiratory responsiveness, so it should be used with caution in inflammatory respiratory diseases. It is characteristic of pine oil and clove oil that they improve the airway functions but increase certain inflammatory parameters. Therefore, we conclude that these essential oils may be beneficial in certain functional respiratory disorders but should be used with caution in inflammatory airway conditions. Cinnamon oil and thyme oil with a predominance of thymol and *p*-cymene may be suitable for adjunctive therapy of pneumonia—and although based on our results it is likely that the effect of thyme oil is related to the TRPV1 ion

channels—, however, regarding their safe medicinal use, further studies are of course necessary.

It is also important to mention that the results from *in vivo* animal models cannot be directly extrapolated to humans, but they still provide valuable data. Essential oils and their components can be effective members of the adjuvant treatment of respiratory diseases even on their own, although further research into the interactions between their main and minor components and the role of new drug targets related to them, such as TRP ion channels, can be even more promising for the development of more effective preparations with a unique composition.

Our new results:

- Inhalation of **lemongrass oil** (main components: citronellal and geraniol) aggravates airway hyperreactivity and MPO activity in a mouse model of endotoxin-induced lung inflammation.
- Inhalation of **cinnamon oil** (main component: cinnamic aldehyde) reduces inflammatory airway hyperreactivity and histopathological changes in a mouse model of endotoxin-induced pneumonia.
- Our work is the first study that demonstrates the effect of **pine oil** on inflammation *in vivo*. Inhalation of pine oil (main component: *α*-pinene) reduces inflammatory airway hyperreactivity and histological changes, but significantly aggravates MPO activity and increases the levels of several inflammatory cytokines in the LPS-induced acute lung inflammation model.
- Inhalation of **thyme oil** (main component: thymol and *p*-cymene) reduces inflammatory airway hyperreactivity, histopathological changes and MPO activity, but slightly increases the level of some cytokines in a mouse model of endotoxin-induced pneumonia.
- Inhalation of **clove oil** (main component: eugenol) reduces inflammatory airway hyperreactivity and histological changes, but significantly aggravates MPO activity and increases the levels of several inflammatory cytokines in the LPS-induced acute pneumonia model.
- Inhalation of **Shirazi thyme oil** (main component: thymol and carvacrol) slightly aggravates LPS-increased airway hyperreactivity, but has no significant effect on this, nor on MPO activity or the levels of the investigated cytokines in a mouse model of endotoxin-induced pneumonia.

- Under our experimental conditions, lemongrass, cinnamon, pine, thyme, and Shirazi thyme oil did not have a significant effect on respiratory function, histological parameters, MPO activity, or the level of measured cytokines in healthy animals treated with PBS control.
- For the first time, we investigated the *in vivo* mechanism of action of thyme oil in the LPS-induced lung inflammation model of **TRP** knockout mice. The protective effect of thyme oil in this model is presumably—at least partially—mediated by the TRPV1 channel.

Table 1: Our new results from our experiments with C57Bl/6 wildtype mice, summarized in tabular form
(↑: aggravated the effect of LPS, ↓: reduced the effect of LPS, –: no clear effect)

Parameter		**lemon-grass oil**	**cinna-mon oil**	**pine oil**	**thyme oil**	**clove oil**	**Shirazi thyme oil**
Respiratory parameters	Penh	↑↑	↓↓	↓↓	↓↓	↓↓	↑↑
Histo-pathology	perivascular edema	–	–	↓	↓	↓	no data
	perivascular/ peribronchial inflammation	–	–	–	–	↓	no data
	macrophages	–	↓	–	–	–	no data
	total score	–	↓	–	↓	↓	no data
MPO activity		↑↑	–	↑↑	↓↓	↑↑	–
Cytokine levels	IL-1β	–	no data	↑↑	–	–	–
	MIP-2	–	no data	–	↑↑	–	–

Table 2: Our new results from our experiments with knockout mice, summarized in tabular form
(↑: aggravated the effect of LPS, ↓: reduced the effect of LPS, –: no clear effect)

Parameter		**thyme oil**			
		TRP A1/V1$^{+/+}$ (wildtype)	TRP A1/V1$^{-/-}$ (knockout)	TRP A1$^{+/+}$ (wildtype)	TRP A1$^{-/-}$ (knockout)
Respiratory parameters	Penh	↓↓	↑↑	no data	no data
Histo-pathology	perivascular edema	↓	–	↓	–
	perivascular/ peribronchial inflammation	–	–	↓	–
	macrophages	–	–	↓	–
	total score	↓	–	↓	–
MPO activity		↓	↑	–	↑↑
Cytokine levels	IL-6	↑	↑↑	–	–
	MIP-2	↑	↑↑	–	–

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8. List of publications

8.1. Publications forming the basis of the thesis

Eszter Csikós*, Kata Csekő*, Amir Reza Ashraf, Ágnes Kemény, László Kereskai, Béla Kocsis, Andrea Böszörményi, Zsuzsanna Helyes Györgyi Horváth: Effects of *Thymus vulgaris* L., *Cinnamomum verum* J.Presl and *Cymbopogon nardus* (L.) Rendle essential oils in the endotoxin-induced acute airway inflammation mouse model. *Molecules* 2020, 25(15): 3553; https://doi.org/10.3390/molecules25153553
** The authors contributed equally to the present work.* **IF: 4,412**

Eszter Csikós, Kata Csekő, Ágnes Kemény, Lilla Draskóczi, László Kereskai, Béla Kocsis, Andrea Böszörményi, Zsuzsanna Helyes, Györgyi Horváth: *Pinus sylvestris* L. and *Syzygium aromaticum* (L.) Merr. & L. M. Perry Essential Oils Inhibit Endotoxin-Induced Airway Hyperreactivity despite Aggravated Inflammatory Mechanisms in Mice. *Molecules* 2022, 27(12): 3868. https://doi.org/10.3390/molecules27123868
IF: 4,927

8.2. Other publications

Ács Kamilla, Kocsis Béla, Balázs Viktória Lilla, Kerekes Erika, **Csikós Eszter**, Varga Adorján, Krisch Judit, Vágvölgyi Csaba, Horváth Györgyi: Illóolajok, illóolaj-komponensek és antibiotikumok együttes alkalmazásának lehetőségei légúti infekciók esetén. *Gyógyszerészet* 2018, 62(2): 73-79.

Györgyi Horváth, **Eszter Csikós**, Eichertné Violetta Andres, Tímea Bencsik, Anikó Takátsy, Gergely Gulyás-Fekete, Erika Turcsi, József Deli, Éva Szőke, Ágnes Kemény, Maja Payrits, Lajos Szente, Marianna Kocsis, Péter Molnár: Analyzing the Carotenoid Composition of Melilot (*Melilotus officinalis* (L.) Pall.) Extracts and the Effects of Isolated (all-*E*)-lutein-5,6-epoxide on Primary Sensory Neurons and Macrophages. *Molecules* 2021, 26(2): 503. https://doi.org/10.3390/molecules26020503
IF: 4,927

Eszter Csikós, Adrienn Horváth, Kamilla Ács, Nóra Papp, Viktória Lilla Balázs, Marija Sollner Dolenc, Maša Kenda, Nina Kočevar Glavač, Milan Nagy, Michele Protti, Laura Mercolini, Györgyi Horváth, Ágnes Farkas and on behalf of the OEMONOM: Treatment of Benign Prostatic Hyperplasia by Natural Drugs. *Molecules* 2021, 26, 7141. https://doi.org/10.3390/molecules26237141
IF: 4,927

Jana Pourová, Patrícia Dias, Milan Pour, Silvia Bittner Fialová, Szilvia Czigle, Milan Nagy, Jaroslav Tóth, Viktória Lilla Balázs, Adrienn Horváth, **Eszter Csikós**, Ágnes Farkas, Györgyi Horváth, and Přemysl Mladěnka: Suggested mechanisms of action of plant drugs and their active constituens in the treatment of cough: overview. *PeerJ* (kézirat beadás alatt)

Balázs Viktória Lilla, Ágnes Farkas, **Eszter Csikós**, Adrienn Horváth, Kamilla Ács, Marianna Kocsis, Martin Doseděl, Silvia Bittner Fialová, Szilvia Czigle, Milan Nagy, Jaroslav Tóth, Michele Protti, Laura Mercolini, Přemysl Mladěnka, Györgyi Horváth, and on behalf of the OEMONOM: Herbal drugs in chronic venous disease treatment: an update. *Plos One* (kézirat beadás alatt)

Györgyi Horváth, Adrienn Horváth, Balázs Viktória Lilla, **Eszter Csikós**, Kamilla Ács, Marianna Kocsis, Jana Pourová, Přemysl Mladěnka, Silvia Bittner Fialová, Szilvia Czigle, Milan Nagy, Jaroslav Tóth, Michele Protti, Laura Mercolini, Ágnes Farkas, and on behalf of the OEMONOM: Natural drugs in hypertension. (kézirat összeállítása folyamatban)

The summary of the conference presentations is included in the dissertation.