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# ANTI-INFLAMMATORY EFFECTS OF CHAMOMILE ESSENTIAL OIL IN MICE

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## ABSTRACT

Essential oils are plant secondary metabolites with positive pharmacological properties, e.g. anti-oxidative, antimicrobial or immunomodulative, but they can have toxic and allergic effects as well. The aim of this study was to analyze anti-inflammatory effects of chamomile essential oil dietary administration in carrageenan paw oedema and trinitrobenzene sulfonic acid (TNBS) colitis. Mice received chamomile essential oil in three concentrations (5000, 2500 and 1250 ppm) in the standard rodent diet starting two weeks before induction of carrageenan paw oedema and TNBS colitis. Dietary supplementations with 5000 ppm of chamomile essential oil significantly reduced both the oedema and the weight of mice paws compared with control. The same dose of chamomile essential oil showed protective effect on colonic mucosa and improved macroscopic signs of TNBS-induced colonic inflammation. Bacterial translocation from the lumen into the mesenteric lymph nodes was significantly reduced in mice treated with 5000 ppm and 2500 ppm concentrations of chamomile essential oil. Overall our data indicate that chamomile essential oil is able to improve some parameters of murine experimental inflammatory models depending on the concentration used.

**Key words:** inflammation; carrageenan paw oedema; TNBS colitis; chamomile essential oil

## INTRODUCTION

The primary role of essential oils is to protect plants against bacteria, viruses, fungi and insects. Since the middle ages essential oils have been known for their health-beneficial properties including anti-oxidative, anti-inflammatory and antimicrobial effects, and they are widely used in medical prevention and therapy. Currently it is seen as important to gain a better understanding of the principles of their biological actions, with the aim of developing new applications for human and animal health.

Anti-inflammatory effects of various plant extracts and essential oils have been tested in carrageenan-induced mouse paw oedema (e.g. Fernandes *et al*., 2007). Experimental colitis in rodents induced by the administration of irritating chemicals has been frequently used to evaluate various bioactive compounds as possible therapeutic agents for inflammatory bowel disease (e.g. Jagtap *et al*., 2004). The intrarectal application of trinitrobenzene sulfonic acid (TNBS) produces severe necrotic lesions that heal after several weeks leaving scars and fibrosis as sequelae.

Currently-used therapies for inflammatory bowel disease are often suboptimal, and various complementary and alternative approaches have been employed for symptom relief and improved quality of life. Accordingly, many of these alternative therapies are able to modulate the immune system and disrupt the proinflammatory cascade through a variety of mechanisms, including antioxidant effects, alterations in cell signaling (in particular the NF-κB pathway), cytokines, proinflammatory mediators, and changes in intestinal microflora (Clarke and Mullin, 2008). Different herbal therapies based on administration of plant extracts with anti-oxidant, anti-inflammatory and antimicrobial effects are also used as complementary

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remedies. We have described very recently that dietary supplementations with thyme or rosemary essential oils have anti-inflammatory effects as they are able to suppress carrageenan-induced mouse paw oedema and to attenuate murine TNBS-induced colitis depending on the concentration used (Juhás *et al*., 2008; 2009).

Chamomile has a long tradition as a folk remedy used for a wide variety of purposes. Chamomile tea, decoction or tincture have been extensively employed for treating colic, convulsions, croup, diarrhea, fever, indigestion, insomnia, infantile convulsions, toothache, bleeding or swollen gums, even as a folk cancer remedy. Some chamomile biological effects have been confirmed in different experimental models and clinical studies as well (Yamada *et al*., 1996; Van Dross *et al.*, 2003). Moreover, the chamomile extracts flavonoid-apigenin and chamazulene, like most examined constituents of chamomile, have been shown to exert anti-inflammatory activity in vivo and in vitro (Shipochliev *et al.*, 1981; Verbeek *et al.*, 2004; McKay and Blumberg, 2006), and chamomile and its components possess also antioxidative effects (McKay and Blumberg, 2006).

To our knowledge, no study concerning the effects of chamomile or its essential oil on the gastrointestinal apparatus has so far been published. Our aim therefore was to examine whether dietary addition of chamomile essential oil could have positive effects on experimental intestinal inflammation induced by TNBS administration in mice.

## MATERIALS AND METHODS

### Animals and treatment

Weight-matched ICR mice (5-6 week old males from the Institute breeding facility, weighing 28-32 g) were randomly put into groups. The organization of the experiment, the investigations conducted and the related documentation complied fully with legislative regulations governing the protection of experimental animals in the Slovak Republic. All animal experimentation was reviewed and approved by the Ethical Committee of the Institute of Animal Physiology.

Chamomile essential oil (Ph.Eur. 4.05) purchased from Calendula, a.s. (Nová Ľubovňa, Slovakia, lot 5-001-004-04-06, containing approx. 25 % bisabolol, 4 % chamazulene and 2.5 % bisabololoxide) was added to powdery commercial rodent diet (Diet for laboratory mice and rats SPF, M1; František Machal, Ricmanice, Czech Republic) in 1% edible soya oil (Brölio, Germany) at the following concentrations: 5000 ppm (wt/wt) - group A; 2500 ppm (wt/wt) - group B; 1250 ppm (wt/wt) - group C. The diet for control and sham groups was prepared similarly using only 1% edible soya oil. The diets were fed ad libitum during the experiment period, starting 14 days before administration of TNBS enemas. The mice of experimental group D were treated with dexamethasone (DEXAMED, Medochemie Ltd., Limassol, Cyprus) at a dose of 3 mg/kg b.w. s.c. 2 h before carrageenan application (paw oedema model) or 0.25 mg/kg b.w. s.c. starting 2 h before TNBS instillation, and then every 24 h until the sacrifice of the animals. Dexamethasone has anti-inflammatory effects and attenuates the enhanced neutrophil infiltration in many animal inflammatory models including carrageenan paw oedema and TNBS colitis.

### Carrageenan paw oedema

Male ICR were anaesthetized with mixture [ketamine 5% (42.5 %) / xylasine 2% (7.5 %) / NaCl 0.9% (50 %)]; 60 µl/20 g body weight i.p. Each group of animals received subplantar administration of 50 µl of saline to the left paw or 50 µl of carrageenan 1% (w /v) (Sigma-Aldrich, Steinheim, Germany) in saline to the right paw (Juhás *et al*., 2009). The thickness was measured using a Mitutoyo thickness gauge (Mitutoyo, No. 7313, Japan) immediately before subplantar injection, and 2, 4, 24 h thereafter. The increase in paw volume (swelling) was calculated as the difference between the right paw thickness (carrageenan) and the left paw thickness (saline) measured at each time point. (Data obtained at 2, 4 and 24 h were corrected considering initial difference evaluated at time 0 h.) Mice were killed by cervical dislocation after the last time point (24 h) and the carrageenan paws were rapidly amputated at the tarsocrural joint, weighed on an analytical balance and were used for myeloperoxidase activity evaluation.

### Induction of TNBS colitis

Mice were anesthetized with mixture [ketamine 5% (42.5 %) / xylasine 2% (7.5 %) / NaCl 0.9% (50 %)]; 60 µl/20 g body weight i.p. and colitis was induced by intrarectal administration of 120mg/kg of the hapten reagent TNBS (Fluka, Steinheim, Germany) in 50% ethanol (the total volume: 40µl), and they were then kept in a vertical head-down position for 30 seconds. The sham group received solvent alone through the same technique (Juhás *et al*., 2009). During the development of colitis, body weight was assessed daily. Relative weight (%) represented the relation of actual weight to weight measured at the day of colitis induction. The mortality rate was observed during this study. Mice were killed by cervical dislocation 3 days after TNBS administration. Their abdomens were soaked with 70% ethanol and an incision was performed through the skin and peritoneum using sterile scissors. The mesenteric lymph nodes draining the cecum and colon were excised with another set of sterile instruments for the determination of bacterial translocation. Afterwards the colons were removed, opened longitudinally and cleared of fecal

material with gentle spray of 0.9% saline solution. The extent of mucosal damage was assessed using the colon macroscopic scoring system (Bukovská *et al*., 2007; adapted from Wallace *et al*., 1989). *Ulceration:* 1 - focal hyperemia, no ulcer; 2 - ulceration, no hyperemia/bowel wall thickening; 3 - ulceration, inflammation at one site; 4 - ulceration, inflammation at 2 or more sites; 5 - major injury > 1cm; 6 - 10 major damage > 2 cm. *Adhesion*: 1 - minor (colon easily separated from other tissue); 2 - major. *Diarrhea*: 1. *Bowel wall thickening*: 1. After scoring, the detached colon was blotted dry and weighed. The colon weight/body weight ratio was calculated as a marker of colonic inflammation. Immediately after weighing, the macroscopically most intensively affected segment was cut for assessment of myeloperoxidase activity.

**Bacterial translocation**

The mesenteric lymphatic nodules were removed, weighed separately and placed in a sterile grinding tube. The samples were homogenized with 1000 µl of PBS. After mechanical grinding, 100 µl aliquots were placed onto Mc agar plates (Imuna, Šarišské Michaľany, Slovakia). All agar plates were incubated aerobically for 24 h at 37 °C. Quantitative culture results were determined as the logarithm of the number of colony-forming units (cfu) per 0.01 g of tissue, calculated with the following formula: number of cfu × reciprocal of dilution × 10 / weight of tissue.

**Myeloperoxidase (MPO) analysis**

Myeloperoxidase activity correlates directly to the degree of neutrophil infiltration in the tissues. Samples (40-60 mg) of paws (carrageenan) or colonic samples (TNBS colitis) were homogenized on ice in 50 mM of phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. After three cycles of freezing and thawing, samples were sonicated (90 sec) and then centrifuged at 12 000 g at 4 °C for 30 min. The supernatant was diluted with distilled water (1:10). The MPO (myeloperoxidase) activity of the diluted supernatant (50 µl) was assessed in 96-well plates (Gama group a.s., Trhové Sviny, Czech Republic). Supernatant aliquots were added to 50 µl of the reaction mixture, which contained citrate buffer (1.8405 g $Na_2HPO_4$, 0.511 g citric acid, 0.01 g menthiolate in 100 ml of distilled water), 4.62 mM of 1, 2 phenylenediamine, and 4.76 mM of $H_2O_2$ solution. MPO activities are presented as arbitrary units per g of paws or mg of colonic tissue. The enzyme activity was determined spectrophotometrically at 490 nm on a Microplate Spectrophotometer µQuant (BioTek Instruments, Winooski, VT, USA).

**Statistical analysis**

The results are expressed as means ± SD. Kruskal-Wallis and Mann-Whitney U tests were used for macroscopic damage scores. The Chi-square test was used to analyse differences in mortality rate. Student's t-test was used for differences in paw width and weight, the colon weight/body weight ratio and MPO enzyme activities. Values of $P < 0.05$ were considered as significant.

## RESULTS

**Mouse paw oedema**

As early as at the first measurements, 2 h after induction of paw oedema, we detected a significant decrease of paw oedemas in mice fed the diet with 2500 ppm chamomile essential oil (ChEO) compared to control mice ($P < 0.01$, Table 1). In contrast to this, at the next measurement (4 h) we observed significant increase

**Table 1: Effects of chamomile essential oil dietary administration on carrageenan-induced paw oedema in mice**

| | n | right paw weight ($10^{-2}$ g) | MPO activity (absorbance 490nm/g × 100) | swelling thickness ($10^{-2}$ mm) | | |
|---|---|---|---|---|---|---|
| | | | | 2 h | 4 h | 24 h |
| Paw oedema | 10 | 25.0 ± 3.0 | 94.45 ± 20.1 | 55.1 ± 14.4 | 57.7± 17.1 | 89.1 ± 32.6 |
| DEX | 11 | 20.9 ± 1.6*** | 73.45 ± 16.95* | 42.3 ± 18.4 | 39.4 ± 12.7** | 39.7 ± 10.0*** |
| ChEO 1250 | 12 | 25.0 ± 2.5 | 97.9 ± 28.71 | 45.9 ± 13.9 | 73.4 ± 11.6* | 98.0 ± 21.0 |
| ChEO 2500 | 12 | 26.6 ± 3.0 | 101.37 ± 21.20 | 36.1 ± 15.7** | 62.5 ± 27.1 | 95.1 ± 32.7 |
| ChEO 5000 | 12 | 22.8 ± 1.8* | 88.25 ± 20.92 | 48.9 ± 7.9 | 74.9 ± 15.6* | 64.5 ± 12.6* |

Values are arithmetical means ± SD. MPO activity - myeloperoxidase activity; DEX - animals treated with 3 mg/kg dexamethasone; ChEO 1250 - animals fed with 1250 ppm chamomile essential oil; ChEO 2500 - animals fed with 2500 ppm chamomile essential oil; ChEO 5000 - animals fed with 5000 ppm chamomile essential oil. Statistical differences between paw oedema and other experimental groups (Student's t- test): * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$

of paw swelling in mice fed with 5000 and 1250 ppm chamomile essential oil ($P < 0.05$). The application of 3 mg/kg dexamethasone induced the reduction of paw oedemas compared to control group 4 hours ($P < 0.01$) and 24 hours ($P < 0.001$) after carrageenan application. The last measurement (24h) demonstrated significant decrease of paw swelling in mice treated with 5000 ppm ChEO compared to control mice ($P < 0.05$). Significantly lower paw weights ($P < 0.05$) were detected in the group fed with the highest concentration of ChEO (Table 1). Significant reductions in paw weight ($P < 0.001$) and MPO enzyme activity in paws ($P < 0.05$) were observed in mice treated with dexamethasone (Table 1).

**TNBS-induced colitis**

The macroscopic damage scores and colon weight/body weight ratio of intact mice and mice in the sham goup were significantly lower than those of mice in the TNBS group ($P < 0.001$, Table 2). Colon weight/body weight ratios in mice treated with all concentrations of chamomile essential oil in the diet were comparable with mice in the TNBS group. Treatment with dexamethasone induced significant decrease in the colon weight/body weight ratio in comparison with the control TNBS group ($P < 0.001$). However, dexamethasone medication seemingly does not protect the colon mucosa, as we observed a moderate non-significant increase in macroscopic scores. The addition of ChEO to the diet induced a dose-dependent decrease in macroscopic scores, but we did not observe significant changes in groups on 1250 ppm and 2500 ppm ChEO diet compared with the control TNBS group. However, the macroscopic scores of the group on 5000 ppm ChEO diet were significantly lower ($P < 0.05$) than those of the control mice (TNBS group).

Sham mice receiving only 50% ethanol intrarectally and intact mice did not show any bacterial translocation to mesenteric lymph nodes, and there were significant differences between the sham or intact mice and the TNBS-treated mice ($P < 0.001$, Table 2). The bacterial translocation in mice on the lowest amount of ChEO in the diet or treated with dexamethasone was not significantly different from the control TNBS group, but we observed a highly significant reduction of bacterial translocation to the mesenteric lymph nodes ($P < 0.001$) in mice on 2500 and 5000 ppm ChEO in the diet.

Intact mice and sham mice (receiving 50% ethanol intrarectally) showed significantly lower activity of MPO ($P < 0.001$, $P < 0.01$) in colon samples compared with the TNBS group. Dexamethasone treatment as well as diets with higher concentrations of ChEO (5000 and 2500 ppm) did not change colon MPO activity in comparison with the control TNBS group. However, we observed

**Table 2: Effects of chamomile essential oil dietary administration on TNBS-induced colitis in mice - mortality, body weight changes, macroscopic score, colon weight /body weight ratio, bacterial translocation, MPO activity**

| | Treated mice (n) | Surviving mice (n) | Mortality (%) | Relative weight on day 3 (%) | Colon weight (% of b.w.) | Macroscopic score | Bacterial translocation | MPO activity[1] |
|---|---|---|---|---|---|---|---|---|
| Intact | 11 | 11 | 0 | 102.71±1.29$^{***}$ | 1.03 ± 0.11$^{***}$ | 0.09 ± 0.30$^{***}$ | 0 ± 0$^{***}$ | 1.57± 1.3$^{***}$ |
| Sham | 12 | 12 | 0 | 99.73±3.82$^{***}$ | 1.24 ± 0.10$^{***}$ | 1.5 ± 2.02$^{***}$ | 0 ± 0$^{***}$ | 7.72 ±7.0$^{**}$ |
| TNBS | 14 | 13 | 7.14 | 83.56 ± 1.41 | 2.14 ± 0.24 | 8.23 ± 0.93 | 2.97 ± 0.83 | 15.47 ±3.0 |
| DEX | 12 | 8 | 33.3 | 81.84 ± 2.31 | 1.83 ± 0.12$^{***}$ | 9.13 ± 1.46 | 3.45 ± 0.73 | 15.43 ±5.8 |
| ChEO 1250 | 12 | 11 | 8.33 | 82.13 ± 2.10 | 2.21 ± 0.24 | 8.45 ± 0.82 | 3.18 ± 1.52 | 12.49 ±2.8$^{*}$ |
| ChEO 2500 | 12 | 10 | 16.7 | 81.08 ± 3.45 | 2.26 ± 0.43 | 7.60 ± 1.26 | 0.38 ± 0.67$^{***}$ | 14.92 ±8.7 |
| ChEO 5000 | 12 | 12 | 0 | 77.85 ± 2.26$^{***}$ | 2.11 ± 0.20 | 7.25 ± 1.22$^{*}$ | 1.49 ±0 .92$^{***}$ | 17.35 ±4.6 |
| | | | | | KW: $^{***}$ | KW: $^{***}$ | | |

Values are arithmetical means ± SD. Statistical differences between untreated colitic animals and other groups of animals [weight and MPO (myeloperoxidase) activity t-test; bacterial translocation and macroscopic score Mann-Whitney test; mortality Chi-square test]: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; KW, Kruskal-Wallis test; ([1]absorbance 490nm/mg × 10000); Intact - control animals (without ethanol); Sham - control animals (50% ethanol); TNBS - untreated colitic animals (TNBS+ethanol); ChEO 1250 - colitic animals fed with 1250 ppm chamomile essential oil; ChEO 2500 - colitic animals fed with 2500 ppm chamomile essential oil; ChEO 5000 - colitic animals fed with 5000 ppm chamomile essential oil

significant reduction of colon MPO activity in mice on the diet with 1250 ppm ChEO compared with control mice in the TNBS group ($P < 0.05$).

## DISCUSSION

Chamomile essential oil contains a complex mixture of sesquiterpenes (alpha-bisabolol, bisabolol-oxides A and B, farnesene), sesquiterpenelactones (chamazulene) and acetylene-derivatives (spiroethers) (Ganzera *et al*., 2006). The antiinflammatory, antioxidative and sedative effects of chamomile and its major components (apigenin, azulene and bisabolol) were reviewed recently by McKay and Blumberg (2006). Bisabolol has a weak sweet floral aroma and is known to have anti-irritant, anti-inflammatory and anti-microbial properties. Chamazulene is a deep blue oil extracted from the chamomile flower, and as a potent antioxidant it is comparable to well-known antioxidants such as quercetin or propyl gallate, which have therapeutic value in the anti-inflammatory processes. Both above-mentioned compounds were the principal effector components of the chamomile essential oil preparation used in our experiment. We analysed the anti-inflammatory effects of chamomile essential oil dietary administration in mice using carrageenan-induced mouse paw oedema and TNBS-induced colitis. A single dose of carrageenan or TNBS administered at the start of the experiment resulted in an acute local inflammatory response. Our results indicate that chamomile essential oil in the diet was able to attenuate paw inflammation, but the dose response was not fully evident, as the 2500 ppm dose was effective after 2 h and the 5000 ppm dose only after 24 hours. Similarly to this observation, the highest dose of chamomile essential oil (5000 ppm) was also able to significantly attenuate colon inflammation, as proved by the decreased macroscopic score, as well as decreasing bacterial translocation to the mesenteric lymph nodes. These results could be hypothetically related to a synergy of biologically active compounds of chamomile essential oil, in particular their anti-inflammatory as well as anti-microbial effects, because luminal bacterial microflora plays an important role in the pathogenesis of TNBS-induced colitis in mice.

There are only a few previous experimental studies concerning the anti-inflammatory effects of chamomile and its extracts. Al-Hindawi *et al*. (1989) showed that i.p. applicaton of ethanol chamomile extract (400 mg/kg) significantly decreased carrageenan-induced paw oedema in rats. These data are in accordance with the results of our study showing anti-oedematic effect of chamomile even after its dietary application in mice. Some in vitro studies have revealed a possible molecular basis of the anti-inflammatory effects of chamomile and its extracts. Chamazulene in vitro is able to block the chemical peroxidation of arachidonic acid and the formation of leukotriene B4 in intact neutrophilic granulocytes, and consequently chamazulene may contribute to the anti-inflammatory activity of chamomile extracts by inhibiting leukotriene synthesis, with additional antioxidative effects (Safayhi *et al*., 1994). Abe *et al*. (2003) assessed the anti-inflammatory activities of essential oils including chamomile essential oil on neutrophil activation examined in vitro. Interestingly, chamomile essential oil tested at 0.1% concentration suppressed TNF-α-induced neutrophil adherence. Similarly, in our experiment, anti-inflammatory effects of 1250 ppm of chamomile essential oil were confirmed by the apparent decrease of neutrophil infiltration at the site of colonic inflammation, reflected in the lower MPO activity. Very recently Srivastava *et al.* (2009) have shown in vitro that chamomile extract interferes with the COX-2 pathway and they suggest that chamomile works by a mechanism of action similar to that attributed to non-steroidal anti-inflammatory drugs.

On the other hand, there are numerous publications dealing with the antibacterial effects of chamomile and its extracts (e.g. Weseler *et al*., 2005; Cervenka *et al*., 2006). These well-known antiseptic effects of chamomile could also explain the significant reduction of translocated bacteria to the mesenteric lymph nodes and the protection of intestinal mucosa in our TNBS colitis. From this point of view, plants with antibacterial properties could also have positive effects on pathological processes in intestinal inflammation. Recently we have shown that thyme or rosemary essential oil dietary supplementation (Juhás *et al*., 2008; 2009) could attenuate TNBS-induced colitis, not only due to their proved anti-inflammatory effects but hypothetically also due to their antibacterial impacts.

Our study indicates that chamomile essential oil dietary application is able to affect murine experimental inflammatory models depending on the concentration used. To elucidate mechanisms of its action, it is necessary to study in greater detail the immunomodulatory properties of chamomile extracts. We conclude, moreover, that the anti-inflammatory effects of chamomile essential oil should be interpreted with caution, due to its contradictory dose-related effects.

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