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Essential Oils from Thyme (*Thymus vulgaris*): Chemical Composition and Biological Effects in Mouse Model

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ABSTRACT *Thymus* species are popular spices and contain volatile oils as main chemical constituents. Recently, plant-derived essential oils are gaining significant attention due to their significant biological activities. Seven different thymus-derived essential oils were compared in our study. First, we focused on their chemical composition, which was followed up by testing their effects on phagocytosis, cytokine production, chemotaxis, edema inhibition, and liver protection. We found limited biological activities among tested oils, with no correlation between composition and biological effects. Similarly, no oils were effective in every reaction. Based on our data, the tested biological use of these essential oils is questionable.

KEYWORDS: • *cytokines essential oils* • *hepatotoxicity* • *immunity* • *phagocytosis* • *thyme*

INTRODUCTION

THE USE OF VARIOUS HERBS is as old as the history of mankind. Herbal remedies are being used throughout the world, sometimes as the only available treatment and sometimes as an alternative or complementary medicine. About 5000 botanical species have been phytochemically studied, but their full pharmacological and biological activities have been rarely evaluated[1] and our knowledge is limited. Essential oils are becoming one of these botanical treatments, often with conflicting results.

Linalool (coriander essential oil) has some antitumorigenic potential via modulation of oxidative stress.[2] A comparative study showed that clove essential oil enhanced delayed-type hypersensitivity response and restored chemotherapy-damaged cellular and humoral immune responses, whereas ginger essential oil recovered only humoral immunity and ginger essential oil had no activity at all.[3] Cinnamon essential oil had some anti-inflammatory activity,[4] clove essential oil and extracts showed immunomodulatory and anti-inflammatory action on cytokine production by macrophages,[5] wild or southern marigold oil had anti-oxidative and anti-inflammatory effects,[6] and parsley essential oil suppressed immune response.[7] In some cases, essential oils had no effects.[8] Thyme essential oils had some anti-inflammatory and hepatoprotective properties,[9] whereas a direct comparison of carvacrol, thymol, and eugenol showed significant biological effects.[10] Detailed chemical composition is described in Teixeira *et al.*[11] For a recent review of anticancer activity of essential oils, see Bhalla *et al.*[12] With very few studies directly comparing either different oils or oils isolated from the same species, we decided to compare seven different thyme-derived essential oils.

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MATERIALS AND METHODS

Animals

Female, 8 week-old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All animal work was done according to the University of Louisville IACUC protocol. Animals were sacrificed by CO_2 asphyxiation. Nine mice/group were used.

Materials

Carrageenan, carbon tetrachloride (CCl_4), RPMI 1640, lipopolysaccharide (LPS), Giemsa, and *N*-formyl-methionyl-leucyl-phenylanaline (fMLP) were from Sigma (St. Louis, MO, USA); fetal calf serum (FCS) was from Hyclone (Ogden, UT, USA); and Biotrak cell proliferation kit was from GE Healthcare Bio-Sciences (Pittsburgh, PA, USA).

Essential oils

Individual thyme essential oils were purchased as follows: Sample No. 1 from Youngevity (Chula Vista, CA, USA), sample No. 2 from Nature's Alchemy (Twin Lakes, WI, USA), sample No. 3 from Aura Cacia (Norway, IA, USA), sample No. 4 from Edens Garden (St. Clemente, CA, USA), sample No. 5 from Rocky Mountains Oils (Orem, UT, USA), sample No. 6 from Young Living (Lehi, UT, USA), and sample No. 7 from doTERRA (Pleasant Grove, UT, USA). All were 100% pure essential oils from thyme.

Analysis

A Shimadzu single quadrupole GCMS-QP2010 ultra gas chromatograph-mass spectrometer was used for the identification of volatile components in essential oils. For this portion of the work, a gas chromatograph, equipped with a 30 m × 0.25 mm RP-5 non-polar column (Shimadzu) with 0.25 μm film thickness, was used. The mass spectrometer was operated in the electron impact ionization mode with an ionizing energy of 70 eV, scanning from *m/z* 35 to 650 at 0.3 scan/sec. The ion source temperature was 200°C, and the quadrupole temperature was 280°C. The electron multiplier voltage was maintained at 0.8 kV. The chromatographic conditions were identical to those used for gas chromatography analysis.

Helium was used as carrier gas, the flow through the column was 1 mL/min, and the split ratio was set to 400:1. The column was maintained at 45°C for 10 min, increased to 180°C at a rate of 2.5°C/min, and finally maintained at a rate of 20 min. Injection volume of the sample was 0.2 μL, and dilution was 1:1 with hexane. For the identification of the compounds, retention times and retention index were confirmed with commercially available standard compounds (Dr. Ehrenstorfer, GmbH), software NIST11 Mass Spectral Library, or Wiley registry of mass spectral data.

Percentage composition of samples was calculated according to the area of the chromatographic peaks (Table 1) or on the basis of calibration by using standards (Table 2).

Cells

Human breast cancer cell line ZR-75-1 (American Type Culture Collection, Manassas, VA, USA) was maintained in culture in RPMI 1640 medium that was supplemented with 10% FCS at 37°C in a humidified atmosphere supplemented with 5% CO_2.

Cell proliferation

A 100 μL of cells (1×10^6/mL in RPMI 1640 medium with 5% FCS) were added into each well of a 96-well plate in triplicate. After 72 h incubation in RPMI 1640 medium with 10% FCS, proliferation was evaluated by using Biotrak cell proliferation enzyme-linked immunosorbent assay (ELISA) system according to the manufacturer's instructions.

Hepatotoxicity

Hepatotoxicity was induced by CCl_4 (0.5 mL/kg body weight in olive oil, injected intraperitoneal [i.p.]) according to Ferreira *et al.*[13] Mice were randomly divided into several groups, and tested materials were administered orally by gavage

Table 1. Percentage Compositions of Samples According to the Area of the Chromatographic Peaks

		Samples						
Compound		*1*	*2*	*3*	*4*	*5*	*6*	*7*
α-Thujene	THC	0.04	0.76	1.00	0.06	0.06	0.74	0.90
α-Pinene	THC	0.11	1.06	1.06	3.55	0.67	0.92	0.91
Camphene	THC	0.00	0.85	0.73	0.00	0.11	0.71	0.57
Sabinene	THC	0.64	0.00	0.00	0.16	0.00	0.00	0.00
β-Pinene	THC	0.14	0.29	0.24	1.32	2.65	0.15	0.22
Myrcene	THC	0.60	1.35	1.45	0.86	0.37	1.35	1.32
α-Phellandrene	THC	0.07	0.06	0.07	0.00	0.00	0.10	0.00
3-Carene	THC	0.07	0.00	0.00	1.14	0.00	0.00	0.00
α-Terpinene	THC	0.93	1.50	1.63	0.20	0.46	1.32	1.52
p-Cymene	THC	3.96	18.03	20.62	27.15	27.62	18.87	17.65
Limonene	THC	1.00	0.61	0.48	3.38	0.00	0.38	0.57
γ-Terpinene	THC	2.00	8.58	8.86	0.57	42.08	6.70	8.56
E-Sabinene hydrate	THC	0.22	0.11	0.00	0.00	0.00	0.07	0.00
Terpinolene	THC	0.18	0.05	0.00	0.19	0.00	0.00	0.00
α-Humulene	THC	1.33	0.00	0.00	0.12	0.00	0.00	0.00
δ-Cadinene	THC	0.07	0.00	0.00	0.00	0.00	0.00	0.00
Caryophyllene oxide	THC	0.15	0.00	0.00	0.00	0.00	0.00	0.00
Linalool	PhC	54.39	4.72	4.54	3.24	0.00	4.60	4.06
Camphor	PhC	0.19	0.36	0.46	0.00	0.00	0.00	0.00
Borneol	PhC	0.00	1.26	1.07	1.02	0.00	0.82	0.82
4-Terpineol	PhC	2.95	1.34	1.18	1.03	0.00	1.18	1.11
α-Terpineol	PhC	0.75	0.44	0.52	1.69	0.00	0.51	0.49
Nerol	PhC	1.56	0.00	0.00	0.00	0.00	0.00	0.00
Thymol	PhC	16.90	52.57	49.49	51.69	25.70	55.47	55.98
Carvacrol	PhC	7.47	3.45	3.82	0.00	0.29	3.86	3.46
Caryophyllene	OHC	2.92	1.61	1.67	2.12	0.00	1.62	1.33
Carvacrol methyl ether	OHC	0.29	0.63	0.52	0.00	0.00	0.52	0.54
1,8-Cineole	OHC	1.08	0.36	0.60	0.50	0.00	0.09	0.00

OHC, oxygenated hydrocarbons; PhC, phenolic compounds; THC, terpenic hydrocarbons.

Table 2. Compositions of Samples Calculated from Calibration Using Standards

		Samples g/100 g Solution						
Compound		*1*	*2*	*3*	*4*	*5*	*6*	*7*
α-Terpinene	THC	1.95	9.94	7.50	0.51	44.32	7.30	6.17
p-Cymene	THC	2.98	16.10	13.45	18.99	22.41	15.82	9.81
Linalool	PhC	57.78	5.96	4.18	3.20	0.00	5.46	3.19
Thymol	PhC	17.39	64.28	44.20	49.50	28.55	63.71	42.61

for 10 days. At the end of the study, blood was collected and serum was prepared. After that, mice were sacrificed and livers were immediately excised and used for homogenates.

Biochemical analysis

The enzymatic activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were assayed spectrophotometrically (Antech Diagnostics, Louisville, KY, USA). Liver homogenates were prepared by the following technique: Livers were excised, rinsed in saline, frozen in liquid nitrogen, and stored at –80°C for later analysis. Frozen liver was ground to a fine powder, and 20–25 mg of powder was solubilized. The glutathione (GSH) levels were measured by the GSH test kit (Dojindo Labs, Kumamoto, Japan).

Phagocytosis

The technique employing phagocytosis of synthetic polymeric microspheres was described earlier.[14] Briefly, peritoneal cells were incubated with 0.05 mL of 2-hydroxyethyl-methacrylate (HEMA) particles (5×10^8/mL). The test tubes were incubated at 37°C for 60 min, with intermittent shaking. Smears were stained with Wright stain. The cells with three or more HEMA particles were considered positive. The same smears were also used for evaluation of cell types.

Cytokine studies

Mice were fed once a day with either the oils or phosphate-buffered saline (PBS) (control) for 3 days. At the end of the study, peritoneal cells were isolated from the peritoneal cavity by washing with cold PBS, centrifuged, and counted. The cells were incubated in RPMI 1640 that was supplemented with 10% FCS for 2 h. After 2 h, non-adherent cells were removed and adherent macrophages were reincubated with 5 μg/mL LPS for 24 h. Supernatants were collected, and IL-1β and IL-6 formation was measured by using Quantikine mouse kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Chemotaxis

To evaluate the effects on chemotaxis, neutrophils were isolated from the peritoneal cavity of mice 4 h after an i.p. injection of 1 mL of thioglycollate medium. Cell numbers were adjusted to 1×10^6 cells in RMPI 1640 medium with 1% FCS. Chemotaxis was evaluated by using a 48-well microchemotaxis plate (Neuro Probe, Gaithersburg, MD, USA), with chambers separated by a polyvinylpyrrolidone-free polycarbonate membrane. fMLP at a 10^{-6} M concentration was used as a chemoattractant and was placed in the lower chamber. Cells pretreated with essential oils (0.01, 0.1, or 1 μg/mL) were placed in the upper chamber and were allowed to migrate for 60 min at 37°C. Incubation was stopped by washing the membrane and staining with Giemsa. The area of each well was scored under a light microscope in five random fields. The results are expressed as the mean number of neutrophils/field.[15]

Paw edema test

The mouse paw edema test was originally described by Martins dos Santos *et al.*[16] Briefly, edema was induced by a subplatar injection of 0.02 mL of 1% carrageenan into one of the hind paws. As a control, the same amount of distilled water was injected into the contralateral paw. Edema was measured plethysmographically at 1, 2, 3, and 4 h after the injection of carrageenan, and the results were expressed as the increase in carrageenan-injected paw volume (μL) minus the volume of the water-injected paw.

Statistics

Statistical analyses between individual groups were performed by using analysis of variance followed by Fisher paired least-significant difference and the Student's *t*-test at the 95% confidence level.

RESULTS

Percentage composition of individual samples based on the area of the chromatographic peaks is given in Table 1. It is clear that with the exception of sample No. 1, the main component is thymol. In five samples, its percentage is almost identical, but in two (Nos. 1 and 5) samples, it is much lower. The second compound with the highest concentration found was *p*-cymene, and other important components were linalool and gamma-terpinene. When we calculated the composition of samples using standards, we got similar results (Table 2). The dose used in this study represents the most commonly used dose in literature. With rather low activity observed throughout this study, the lower dose would yield no activity at all and the higher dose would not be physiological.

First, we evaluated the effects of individual samples on proliferation of the human breast cancer cell line ZR-75-1. We found no effects on proliferation in serum-free conditions (Fig. 1); when used in serum-free conditions, no effects were found either (data not shown). As most natural immunostimulators affect cellular immunity, we measured the effects on phagocytosis by peripheral blood cells. Mice were fed once a day with either the oils or PBS (control) for 3 days with 100 μg of samples. The only significant stimulation was found for sample No. 4 (Fig. 2).

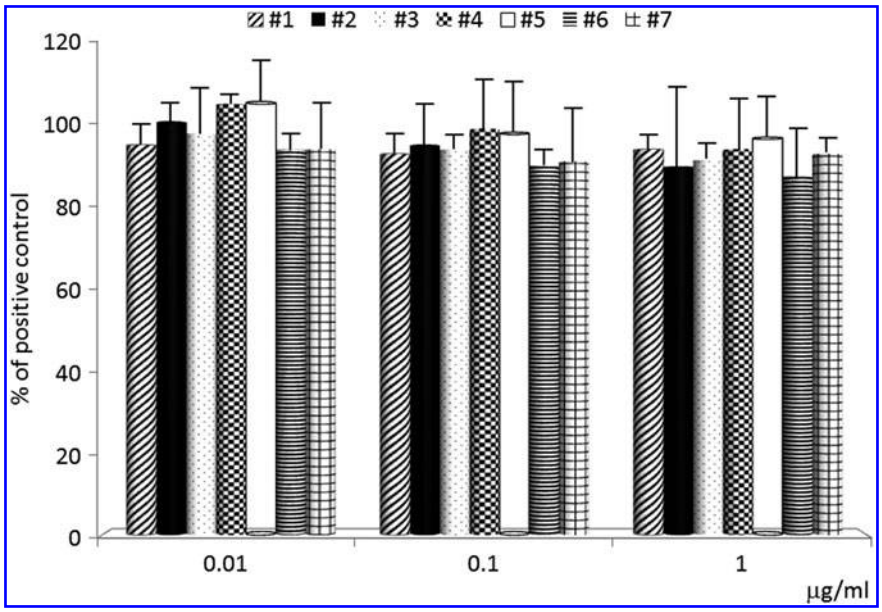

FIG. 1. Effects of individual samples on proliferation of ZR-75-1 cells. Each value represents the mean ± SD. All experiments were performed in triplicate. PBS, phosphate-buffered saline; SD, standard deviation.

Macrophages are the major source of IL-1β and IL-6. All samples except No. 4 caused strong inhibition of IL-1β in the LPS-stimulated model (Fig. 3). Compared with basal production (PBS), all oils stimulated the IL-1β production. On the other hand, IL-6 production was inhibited only in case of sample No. 6 (LPS model, Fig. 4).

Neutrophils are the first responders in an organism's fast attack on infectious agents. For our evaluation of oil's effects on chemotaxis, we employed an fMLP model. We found that four of the tested samples (Nos. 1, 3, 4, and 5) significantly reduced neutrophil migration in response to fMLP stimulation. Data summarized in Figure 5 used a 1 μg/mL concentration; however, 0.01 and 0.1 μg/mL concentrations showed similar results (data not shown).

In the paw edema test, oral administration of the individual oils in some cases reduced the edema induced by carrageenan (Fig. 6). However, the reduction differed with time of exposure—after 1 h, the active samples were Nos. 1, 3, 4, and 5; after 4 h, the active samples were only Nos. 1 and 5.

In the last part of our study, we focused on the role of essential oils in experimental liver injury. We used a well-established CCl_4 model. Effects of individual oils on levels of serum ALT, AST, ALP, and liver GSH are summarized in Table 3. The only sample consistently decreasing the damaging effects of CCl_4 was sample No. 7; however, with the exception of GSH levels, the changes did not reach control levels.

DISCUSSION

Various herbs and particularly essential oils of herbs are gaining attention of not only the general public seeking

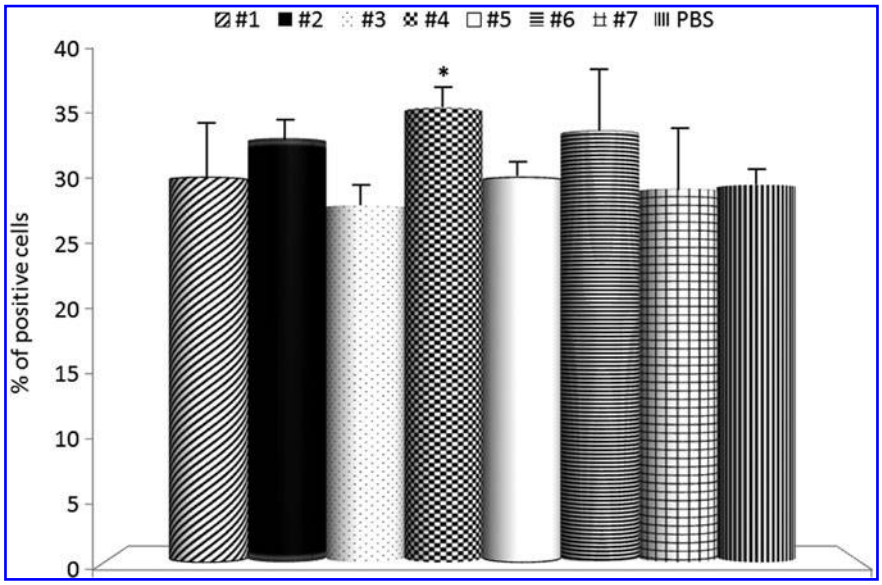

FIG. 2. Effect of an intraperitoneal administration of samples on phagocytosis by peripheral blood granulocytes. Each value represents the mean ± SD. *Represents significant differences between control (PBS) and tested samples at $P \leq .05$ level. All experiments were performed in triplicate.

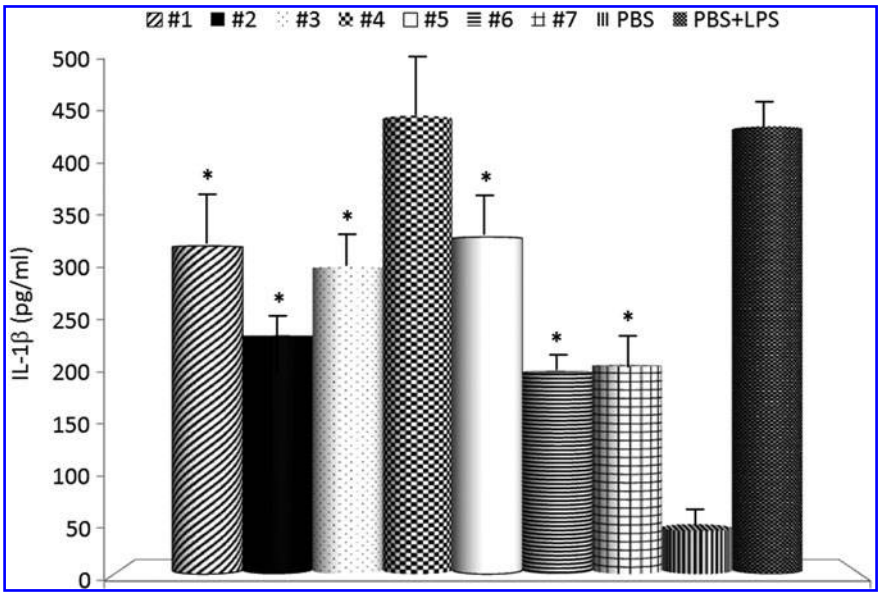

FIG. 3. IL-1β production by peritoneal macrophages of oil-treated mice. Macrophages were stimulated or not with LPS for 24 h. *Represents significant differences between the PBS-LPS group and tested samples at $P \leq .05$ level. All experiments were performed in triplicate. LPS, lipopolysaccharide.

alternative treatments but also the scientific community. Plant essential oils are complex mixtures of volatile and lipophilic organic substances that are synthesized as secondary metabolites.

Thyme (*Thymus vulgaris*) plant is a perennial shrub commonly grown throughout the Mediterranean and Caribbean regions. Essential oils from this plant are used as an aroma additive in food, pharmaceuticals, and cosmetics.[17] In addition, numerous health benefits based on antiseptic, antimicrobial, and antioxidative properties have been described.[18]

The differences in activities described in literature might be based on strong effects of drying methods on composition of thyme essential oil.[19] In addition, the composition differs widely based on area of harvesting[20] and on the thyme species used.[21] Due to these variations, we decided to directly compare biological effects of seven different commercial samples of thyme essential oils.

Chemical analysis revealed significant differences among all samples, with thymol and *p*-cymene being the most frequent components. Sample No. 1 had the highest level of linalool. When tested for direct effects on cell proliferation, no differences have been found in either serum-free (potential stimulation of proliferation) or serum-supplemented (potential inhibition of proliferation) conditions. In all cases, concentrations higher than 1 μg/mL started having direct toxic effects, revealed even on short-term incubation with cells. For the proliferation study, only the highest non-toxic concentration was used.

Every natural molecule able to stimulate the immune system directly affects cellular immunity, with macrophages and neutrophils being the most important targets. Therefore, we started our evaluation of essential oil activities by the testing of phagocytosis. We used the HEMA particles that have only a slight negative charge and, thus, do not nonspecifically adhere

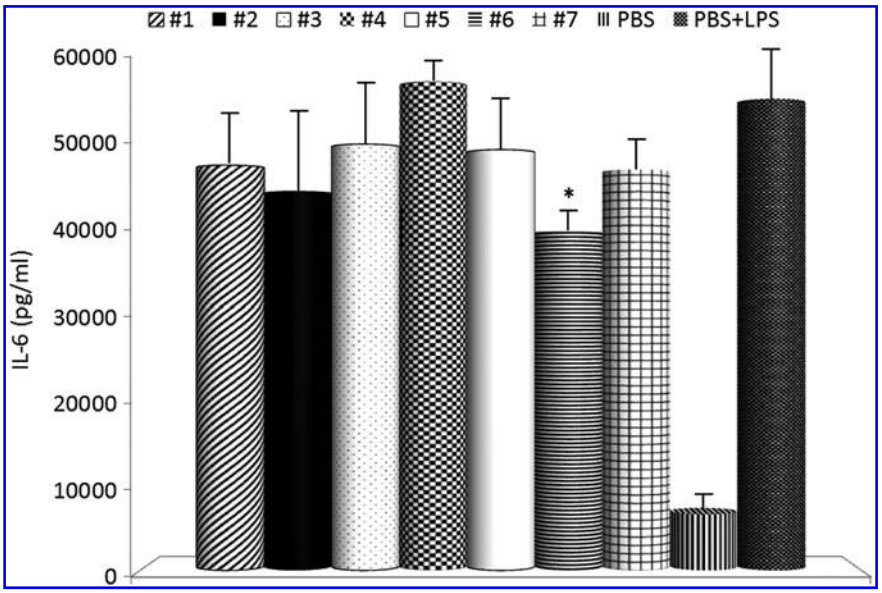

FIG. 4. IL-6 production by peritoneal macrophages of oil-treated mice. Macrophages were stimulated or not with LPS for 24 h. *Represents significant differences between PBS-LPS group and tested samples at $P \leq .05$ level. All experiments were performed in triplicate.

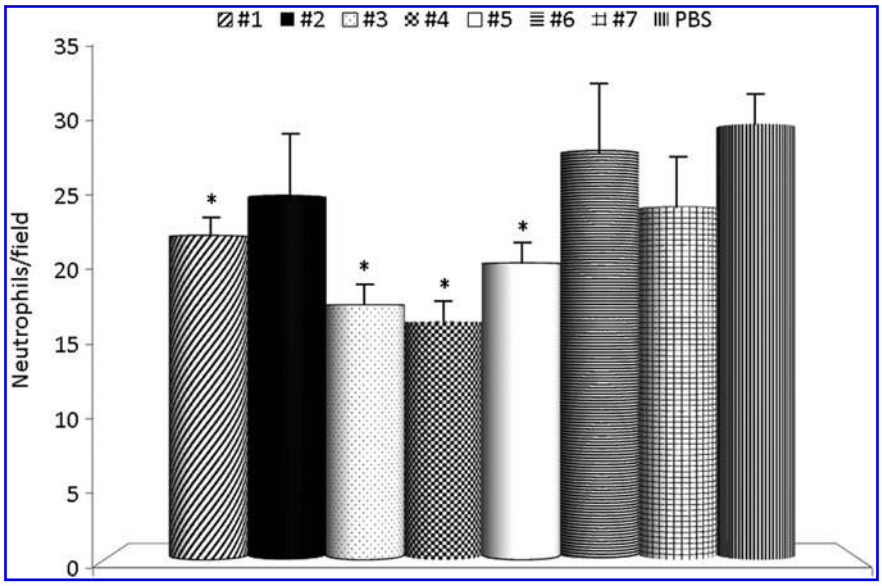

FIG. 5. Effects of tested oils on neutrophil chemotaxis *in vitro*. Mouse neutrophils were stimulated with fMLP after 30 min of treatment with oils at a dose of 1 μg/mL. *Represents significant differences between control (PBS) and tested samples at $P \leq .05$ level. All experiments were performed in triplicate. fMLP, *N*-formyl-methionyl-leucyl-phenylanaline.

to the cell surface. This guarantees that only phagocytosing cells will engulf these particles and it significantly lowers the chance of false negativity.[22] Our results show only limited effects on phagocytosis.

When we followed up with an evaluation of IL-1β and IL-6 production, we did not confirm the finding that the most responsible molecule is linalool.[23] Treatment of mice with thyme oils potentiated the production of both IL-1β and IL-6. Sforcin's group found that lemongrass oil inhibited the IL-1β production, but it stimulated the IL-6 production.[23] In both cases, the LPS stimulation was significantly stronger than oil-mediated production. Both cytokines are released by activated macrophages, and it is known that IL-1β can stimulate IL-6 synthesis.[24]

Besides macrophages, neutrophils represent another important aspect of cellular immunity. Their ability to start the fast response is based on fast migration to the place of attack. fMLP is a bacterial product that is well recognized by neutrophils on binding to its heterotrimeric ZG protein-coupled receptor. The binding starts the signaling cascade activating multiple pathways, including mitogen-activated protein kinase and phosphatidylinositol 3-kinase.[25] To directly evaluate the effects of essential oils on *in vitro* neutrophil chemotaxis, we used different concentrations. We found that some of the oils significantly reduce the neutrophil migration in response to fMLP stimulation.

Some essential oils are known for their anti-inflammatory properties.[16] Carageenan-induced edema is known for three different stages—histamine and serotonin release during the first hour, kinin release during the second hour, and prostaglandin release during the third and fourth hours.[26] In our study, some oils (Nos. 3 and 4) reduced edema only during the first phase (Nos. 1 and 4), some reduced it only during

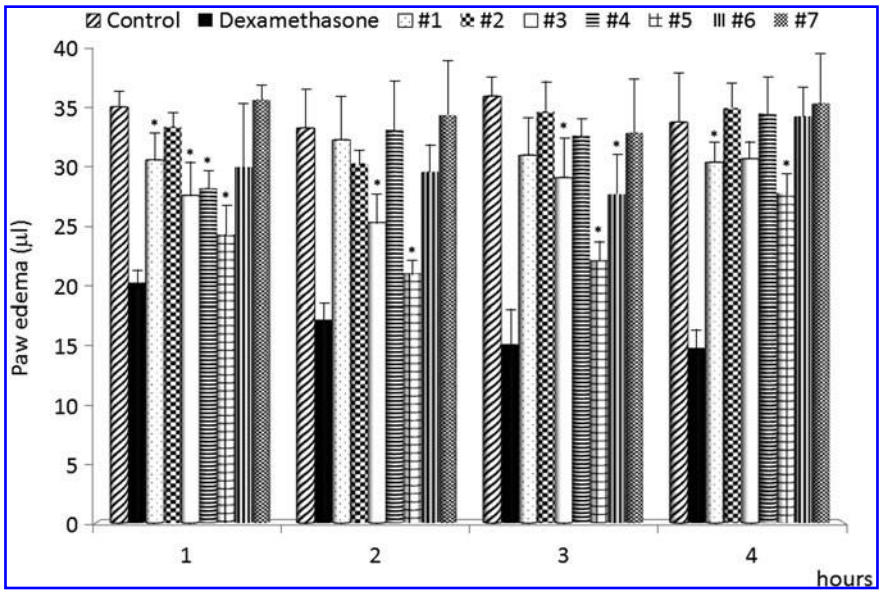

FIG. 6. Effects of orally administered essential oils in the paw edema test. Animals were treated with PBS, dexamethasone (2.25 mg/kg), or oils (100 μg/mL). *Represents significant differences between control (PBS) and tested samples at $P \leq .05$ level. All experiments were performed in triplicate.

Table 3. Effects of Individual Oils on Serum Alanine Aminotransferase, Aspartate Aminotransferase and Alkaline Phosphatase, and Liver Glutathione of Carbon Tetrachloride-Treated Mice

Sample	*AST (IU/L)*	*ALT (IU/L)*	*ALP (IU/L)*	*GSH (IU/L) (μmol/mg protein)*
PBS	72.1 ± 4.8	18.8 ± 2.5	20.7 ± 1.8	20.2 ± 1.9
CCl_4	170.5 ± 11.1	165.3 ± 16.4	180.6 ± 16.9	8.1 ± 2.2
Sample No. 1	141.4 ± 18.9	144.7 ± 21.7	170.1 ± 18.8	13.0 ± 3.3
Sample No. 2	166.9 ± 17.3	152.2 ± 17.0	166.4 ± 17.7	7.7 ± 4.1
Sample No. 3	150.1 ± 15.6	143.6 ± 15.9	145.2 ± 14.9*	14.4 ± 2.9*
Sample No. 4	166.3 ± 20.5	160.5 ± 16.9	165.9 ± 20.2	10.1 ± 3.4
Sample No. 5	142.2 ± 11.4*	156.1 ± 14.3	145.1 ± 12.7*	12.1 ± 2.1*
Sample No. 6	165.7 ± 18.7	152.8 ± 18.2	176.3 ± 18.5	12.9 ± 3.4
Sample No. 7	132.8 ± 16.2*	133.8 ± 13.6*	141.1 ± 15.2*	10.5 ± 1.9*

*Significant difference against CCl_4 group at $P \leq .05$ level.

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCl_4, carbon tetrachloride; GSH, glutathione; PBS, phosphate-buffered saline.

the second and third stages (sample Nos. 3, 5, and 6), and some reduced it during all stages (sample No. 5).

As the last part of our study, we evaluated the hepatoprotective effects of tested oils by using the hepatotoxicity model induced by CCl_4. In our work, hepatotoxicity was reflected by a marked elevation of the levels of serum marker enzymes AST, ALT, and ALP, and a strong decrease of liver GSH. These enzymes are well-established markers of the extent and type of hepatocellular damage.[27,28] After administration of individual oils, a small, but in some cases significant, reduction of these increases was observed. As previous results suggested, the effects are probably caused by the anti-inflammatory properties of essential oils. These effects, however, were much milder than those recently found for glucan and humic acids.[29]

In conclusion, the present study found that essential oils isolated from thyme plant have some biological activities on chemotaxis, IL-1β and IL-6 production, and reduction of experimentally induced liver damage. These oils had no effects on cell proliferation or on phagocytosis. No real conclusion with respect to the composition of these oils could be reached. At the same time, none of these oils showed superior effects. Compared with another popular natural immunomodulator β-glucan, these biological effects were very limited (for a review, see Vetvicka[30,31]). As these oils significantly differ in composition, no conclusion between chemical composition and biological activity could be made. Based on our results, the use of these essential oils as natural immunomodulators is questionable.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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