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Short Communication

Immunomodulatory activity of *Zingiber officinale* Roscoe, *Salvia officinalis* L. and *Syzygium aromaticum* L. essential oils: evidence for humor- and cell-mediated responses

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Abstract

Objectives The immunomodulatory effect of ginger, *Zingiber officinale* (Zingiberaceae), sage, *Salvia officinalis* (Lamiaceae) and clove, *Syzygium aromaticum* (Myrtaceae), essential oils were evaluated by studying humor- and cell-mediated immune responses.
Methods Essential oils were administered to mice (once a day, orally, for a week) previously immunized with sheep red blood cells (SRBCs).
Key findings Clove essential oil increased the total white blood cell (WBC) count and enhanced the delayed-type hypersensitivity (DTH) response in mice. Moreover, it restored cellular and humoral immune responses in cyclophosphamide-immunosuppressed mice in a dose-dependent manner. Ginger essential oil recovered the humoral immune response in immunosuppressed mice. Contrary to the ginger essential oil response, sage essential oil did not show any immunomodulatory activity.
Conclusions Our findings establish that the immunostimulatory activity found in mice treated with clove essential oil is due to improvement in humor- and cell-mediated immune response mechanisms.
Keywords delayed type hypersensitivity response; essential oils; humoral antibody response

Introduction

Aromatic plants are invaluable sources of new drugs. There is a growing interest in investigating plants that have potential therapeutic applications. Easy availability, low cost, efficacy and presumed safety are some of the reasons why plants are used as alternative medicines.[1] Plant essential oils and their constituents, products from the plants' secondary metabolism, have many applications in ethno-medicine. These oils have been widely used in the pharmaceutical, cosmetic, food and beverage industries.[2] Essential oils have been used in complementary therapies, such as aromatherapy.[3] Besides their flavour and aroma, it has been reported that essential oils have properties such as antioxidant, anti-inflammatory, antiviral, antibacterial, antidiabetic and anticancer activity.[4]

The biological activity of an essential oil depends on its composition. These oils are natural mixtures of terpenes obtained from aromatic and pharmaceutical plants, mainly monoterpenes and sesquiterpenes.[5] In folk medicine, ginger, *Zingiber officinale* Roscoe (Zingiberaceae), has been used for the treatment of pain, inflammation, arthritis, urinary infections and gastrointestinal disorders.[6] Preparations of sage (*Salvia officinalis* L., Lamiaceae) are used to alleviate stomatitis, periodontosis, gingivitis, abscesses and gingival bleeding.[7] The essential oil of *Syzygium aromaticum* L. (clove) has as its major constituent the phenolic compound eugenol (4-allyl-2-methoxyphenol), which is also present in many others oils, such as basil, cinnamon and nutmeg. Eugenol exhibits many pharmacological properties, including antiparasitic, antimicrobial, antioxidant, analgesic and anti-inflammatory activity.[2]

Immunomodulation is a procedure that alters the immune system of an organism by interfering with its functions. When this modulation results in an enhancement of immune

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response it is called immunostimulation and manly implies stimulation of a non-specific system (i.e. granulocytes, macrophages, complement, natural killer cells, lymphocytes and even the production of various mediators generated by activated cells (para-immunity)).[8] It is expected that these non-specific effects provide protection against different pathogens, including bacteria, fungi and viruses.[9]

Different plant compounds have demonstrated immunomodulatory activity. *Panax ginseng* is a classic example of a natural immunostimulant.[10] Many plants enhance the cellular and humoral immunity in normal organisms.[8,11] Others, however, restore immune system functions in immunosuppressed hosts.[12,13] Our previous studies have shown that clove, ginger and sage essential oils exert an inhibitory effect on inflammatory oedema formation and leucocyte chemotaxis.[14,15] Despite the fact that studies have been carried out on the plants' anti-inflammatory and immunomodulatory activity, few studies concerning essential oils and their compounds are available.[16] The aim of this study was to evaluate the immunomodulatory activity of clove, ginger or sage essential oils, through cellular and humoral immune responses in mice.

Materials and Methods

Drugs and chemicals

Levamisole was from Janssen-Cilag (São Paulo, Brazil) and cyclophosphamide was from Sigma (St Louis, USA).

Essential oils

Fresh rhizomes of *Zingiber officinale* Roscoe and fresh leaves of *Salvia officinallis* L. were collected in June 2007 from the Prof[a] Irenice Silva Medicinal Plant Garden on the campus of the State University of Maringá, Paraná, Brazil, identified and authenticated by botanist Maria Aparecida Sert. Voucher specimens were deposited in the Herbarium of the Department of Botany, State University of Maringá (No. 11612 and 13901, respectively). Ginger essential oil (GEO) and sage essential oil (SEO) were extracted by conventional steam distillation using a Clevenger-type apparatus for 3 h. The essential oil was kept at 4°C in dark vials, and then used in tests. Clove essential oil (CEO) was purchased from S.S. White dental products (Rio de Janeiro, Brazil).

Essential oil analysis

GC-MS analyses of GEO, SEO and CEO were performed using a Shimadzu QP-5000 (Shimadzu, Tokyo, Japan) instrument, equipped with an HP-5 cross-linked fused silica capillary column (25 m × 0.32 mm × 0.25 μm). Helium was used as carrier gas at 38 cm/s. The column total flow rate was 1 ml/min. General temperature conditions were: split/splitless injector at 280°C, transfer line at 280°C, source 230°C and column temperature programme of 80°C–310°C at 10°C/min. Mass detection limits were 50–700 Da.

^{1}H and ^{13}C NMR analyses were performed on SEO and CEO. ^{1}H (300 MHz) and ^{13}C NMR (75.5 MHz) spectra were recorded in $CDCl_3$ solution on a Varian Mercury Plus spectrometer, with δ (ppm), J in Hz and spectra referred to $CDCl_3$ (δ 7.27 for ^{1}H and 77.00 for ^{13}C) as internal standard.

Animals

Male Swiss mice, 25 ± 5 g, provided by the Central Animal House of the State University of Maringá were used in the experiments. The mice were housed at 22 ± 2°C under a 12-h light–dark cycle and had free access to water and food. Before the experiments, the mice were fasted overnight and had free access to water. The experimental protocols were approved by the Ethical Committee in Animal Experimentation of the State University of Maringá (CEAE/UEM No. 071/2007).

Antigen and mice immunization

Fresh blood was collected from a healthy sheep from the Central Animal House of the State University of Maringá and stored in Alsevier's solution. Sheep red blood cells (SRBCs) were washed three times in large volumes of sterile normal saline. Mice were immunized by injecting 0.1 ml of SRBC suspension containing 1×10^8 cells intraperitoneally on day 0, as previously described.[11]

Animal treatment

The doses of essential oils used in this study were similar to those in our previous studies of anti-inflammatory activity and toxicity. The non- and immunosupressed mice were treated orally (by gavage) with levamisole (50 mg/kg), CEO (100, 200 and 400 mg/kg), GEO (100, 200 and 400 mg/kg) or SEO (5, 10 and 25 mg/kg), once a day, for seven days. The control group received water orally (0.3 ml/mouse). For the experimentation, aqueous suspensions of levamisole, CEO, GEO and SEO were prepared. Immunossupression was induced in the mice by cyclophosphamide (50 mg/kg) administration on days 4, 5 and 6 after immunization, 1 h after treatment with levamisole, CEO, GEO, SEO or water (control group).

Assessment of immune response *in vivo*

Total white blood cell count before and after mouse immunization

Blood samples from the tails of mice were collected for determining the total white blood cell (WBC) count, before (on day 0) and seven days after mice immunization. The data obtained were expressed as mean ± SEM.

Humoral antibody response

After the immunization and treatment of mice, blood samples were collected in microcentrifuge tubes from individual mice from all the groups by tail vein puncture on day seven. The blood samples were centrifuged at 2500 rev/min for 10 min. The sera were separated and inactivated at 56°C for 30 min. Antibody levels were determined by the haemagglutination technique.[11] Briefly, a sample (50 μl) of serum from each mouse was two-fold serially diluted in sterile normal saline into microtitration plates (96 wells), and was challenged with 25 μl of 1% v/v SRBC suspension into each well. After mixing thoroughly, the plates were incubated at 37°C for 1 h and then visible haemagglutination was observed. The value of the highest serum dilution causing visible haemagglutination was taken as the antibody titre.

Delayed type hypersensitivity response

On day seven, the volume of the right hind paw was measured using a digital plethysmograph (Ugo Basile, Comerio, Italy). The mice were then challenged by injection of 50 μl of 5×10^8 SRBC suspension in the right hind paw. The paw volume was measured at 24 h and 48 h after challenge. The difference between the pre- and post challenge paw volume, expressed in μl, was taken as a measure of the delayed type hypersensitivity (DTH) response.

Statistical analysis

Data are expressed as the mean ± SEM for each group. Results were statistically analysed using one-way variance analysis followed by Tukey's test. Differences were considered significant when $P < 0.05$.

Results

Essential oil analysis

The results of CG-MS analysis on GEO showed a predominance of monoterpenes. The levels of five constituents, representing 96% of the components of GEO, were: ar-curcumene (59%), 1,8-cineol (8%), β-myrcene (14%), citral (7.5%) and zingiberene (7.5%) (Figure 1). SEO and CEO were analysed by GC-MS, ^{1}H and ^{13}C NMR. The major constituents of SEO were two monoterpene ketones, which were characterized as α-thujone (90%) and β-thujone (6%). The structures of both α-thujone and β-thujone were readily identified based on analysis of spectral data and comparison with those previously reported in the literature.[17] The major constituent of CEO was characterized and identified as eugenol (>98%) (Figure 2).[18]

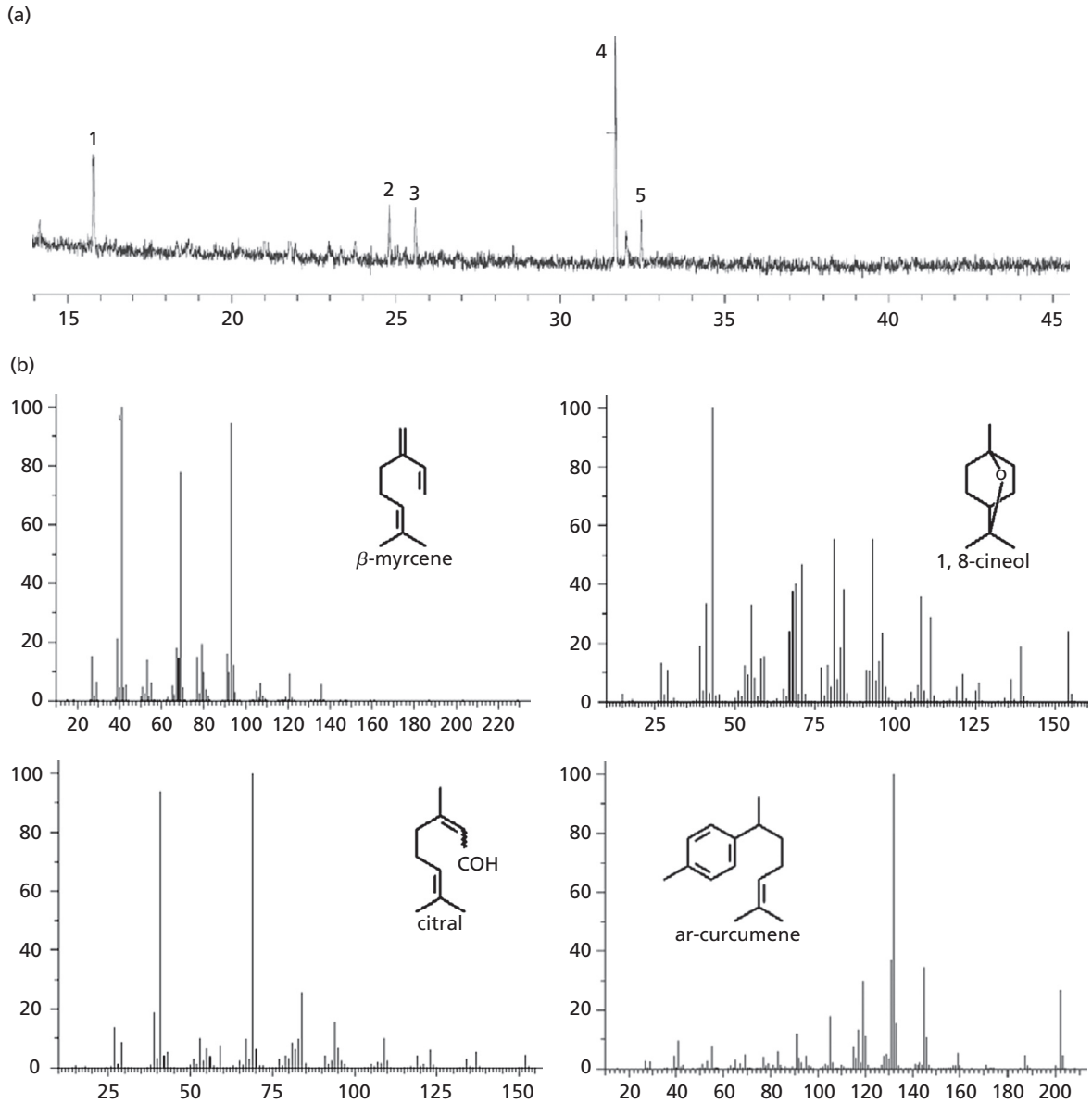

Figure 1 GC chromatogram of *Zingiber officinale* essential oil and mass spectra of some terpenoids present therein. (a) GC chromatogram of *Zingiber officinale* essential oil showing: peak 1 = β-myrcene (14%), 2 = 1,8-cineol (8%), 3 = citral (7.5%), 4 = ar-curcumene (59%), 5 = zingiberene (7.5%). (b) Mass spectra of some terpenoids present in *Zingiber officinale* essential oil.

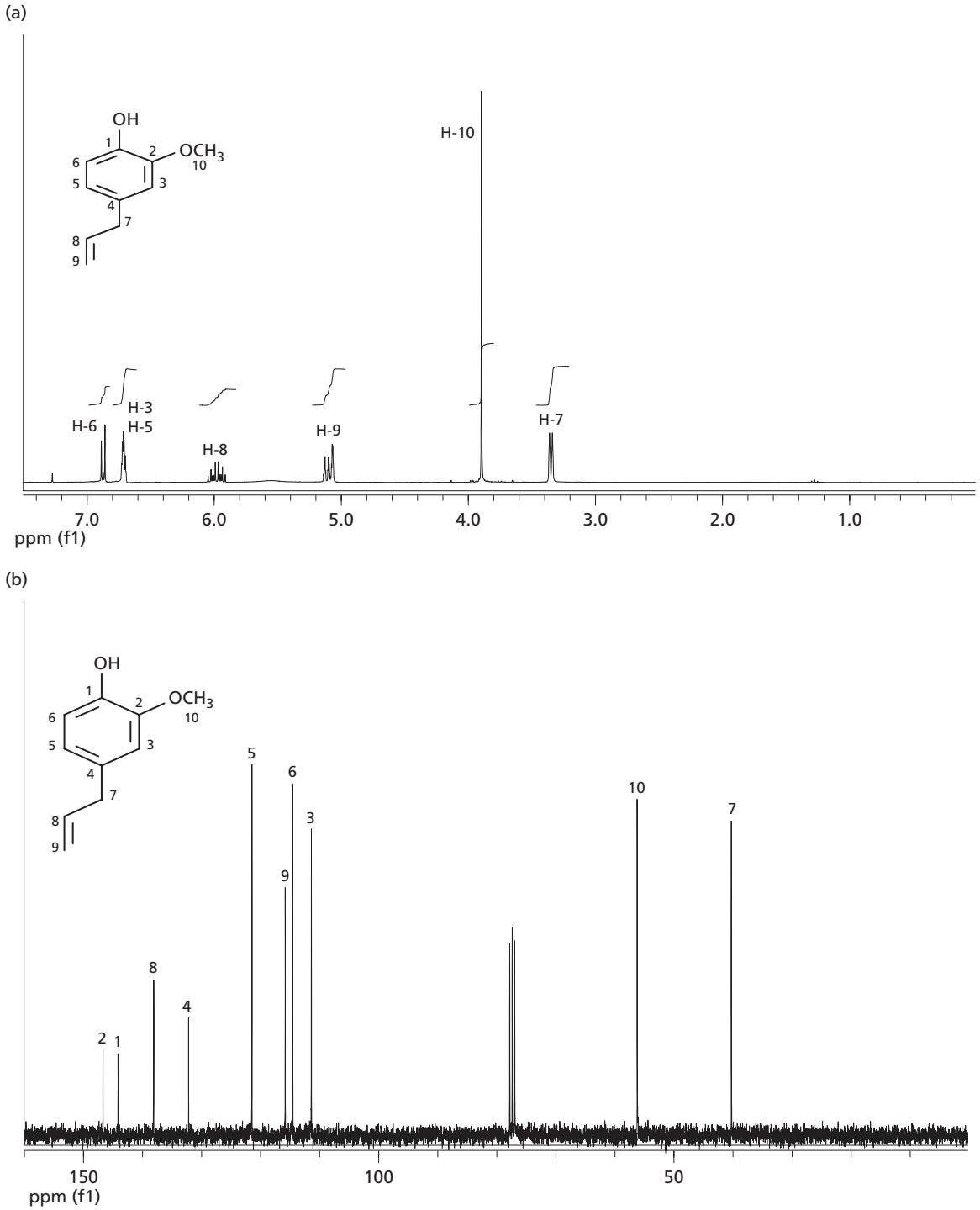

Figure 2 ^{1}H NMR (300 MHz, CDCl3) (a) and ^{13}C NMR (75.5 MHz, CDCl3) (b) spectra of eugenol

^{1}H and ^{13}C NMR spectra

α-Thujone: ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.12 (1H, dd, J = 5.4 and 4.0 Hz, H-6a); 0.94 (3H, d, J = 6.9 Hz, H-8); 1.00 (3H, d, J = 6.6 Hz, H-9); 1.15 (3H, d, J = 7.5 Hz, H-10); 1.34 (1H, sept, J = 6.9 Hz, H-7); 2,06 (1H, d, J = 18.9 Hz, H-2a), 2.21 (1H, qd, J = 7.5 and 0.9 Hz, H-4); 2.54 (1H, ddd, J = 18.9, 1.2 and 2.4 Hz, H-2b). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 18.4 (C-10); 18.9 (C-6); 19.9 and 20.2 (C-8 and 9); 25.7 (C-5); 30.0 (C-1); 33.1 (C-7); 39.8 (C-2); 47.5 (C-4); 221.6 (C-3).

Eugenol: ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.34 (2H, d, J = 6.9 Hz, H-7); 3.89 (3H, s, H-10); 5.08 (1H, dq, J = 10.2 and

1.5 Hz, H-9a), 5.11 (dq, J = 16.8 and 1.8 Hz, H-9b), 5.98 (1H, ddt, J = 16.8, 10.2 and 6.6 Hz, H-8); 6.71 (1H, dd, J = 8.4 and 1.8 Hz, H-5); 6.71 (1H, d, J = 1.8 Hz, H-3); 6.87 (1H, d, J = 8.7 Hz, H-6). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 40.1 (C-7); 56.0 (C-10); 111.3 (C-3); 114.4 (C-6); 115.7 (C-9); 121.4 (C-5); 132.1 (C-4); 138.0 (C-8); 144.0 (C-1); 146.6 (C-2).

The effects of levamisole, CEO, GEO and SEO on the total WBC count in non-immunosuppressed mice are shown in Table 1. An increase of total WBC count in peripheral blood seven days after immunization was observed. CEO treatment increased the total WBC count in a dose-dependent manner, similarly to levamisole treatment. This response was not observed when the mice were treated with GEO and SEO (Table 1).

With regard to humoral antibody (HA) levels, no difference among mice treated with levamisole, CEO, GEO and SEO was reported when compared with the control group (data not shown).

In the DTH response (cell-mediated immunity) assay in non-immunosuppressed mice, 24 h after the challenge, levamisole- and CEO-treated mice showed an increase in the paw oedema volume when compared with the control group, and in contrast to the GEO and SEO treatment groups. After 48 h the paw volume in the groups treated with levamisole, CEO and GEO was restored to initial values, differently to that observed in control mice and SEO groups. Increased paw volume, however, was still reported (Table 2).

Cyclophosphamide treatment induced a cellular and humoral immunossuppression in mice, as demonstrated in the control group. GEO and SEO treatment groups did not exhibit the cyclophosphamide-induced myelosuppression. On the other hand, treatment with levamisole and CEO (400 mg/kg) restored the total WBC count to initial values.

Observation of the humoral immune response (HA) showed that levamisole, CEO and GEO treatments were actually a protection against immunossuppression caused by cyclophosphamide (Table 3).

Table 1 Total white blood cell counts in non-immunosuppressed mice

Group	Dose (mg/kg)	Total WBC (cells/mm^3)		% Increase
		Day 0	Day 7	
Control	–	5317 ± 130	6542 ± 91	23.3 ± 3.3
Levamisole	50	5367 ± 472	9992 ± 743	87.3 ± 4.4**
CEO	100	5125 ± 264	7925 ± 442	54.8 ± 4.4*
	200	4750 ± 197	8142 ± 609	71.1 ± 9.4**
	400	4783 ± 243	9975 ± 716	107.8 ± 6.5**
GEO	100	4783 ± 169	5983 ± 233	25.0 ± 2.3
	200	4683 ± 181	5950 ± 252	27.5 ± 4.2
	400	4792 ± 154	5483 ± 168	14.6 ± 3.7
SEO	5	5217 ± 349	5783 ± 367	11.5 ± 5.4
	10	4742 ± 172	5500 ± 329	15.6 ± 4.0
	25	4625 ± 107	5342 ± 156	16.0 ± 4.4

WBC, white blood cells; CEO, clove essential oil; GEO, ginger essential oil; SEO: sage essential oil. Values are mean ± SEM, $n = 6$ mice in each group. *$P < 0.01$; **$P < 0.001$, when compared with the control group (analysis of variance).

Table 2 Delayed type hypersensitivity response in non-immunosuppressed mice

Group	Dose (mg/kg)	DTH response	
		24 h	48 h
Control	–	59.5 ± 4.7	19.6 ± 1.6
Levamisole	50	91.3 ± 6.2**	9.0 ± 4.2**
CEO	100	67.8 ± 9.9	0.6 ± 1.9***
	200	90.5 ± 5.4**	0.6 ± 2.6***
	400	108.5 ± 4.0**	1.6 ± 3.2***
GEO	100	46.6 ± 4.1	11.0 ± 2.6*
	200	38.17 ± 4.2*	9.1 ± 2.9*
	400	35.67 ± 3.7*	8.1 ± 2.3*
SEO	5	53.8 ± 8.7**	29.8 ± 9.4
	10	42.1 ± 2.1	34.8 ± 5.4
	25	51.8 ± 6.1	28.1 ± 5.4

DTH, Delayed type hypersensitivity; CEO, clove essential oil; GEO, ginger essential oil; SEO, sage essential oil. The values show the difference in mice paw volume before and after the antigen challenge in μl. Values are mean ± SEM, $n = 6$ mice in each group. *$P < 0.05$, **$P < 0.01$; ***$P < 0.001$, when compared with the control group (analysis of variance).

Discussion

Nowadays, there is a growing interest in identifying plant components with immunomodulatory activity that may be employed as alternative medicines in the future.[19] Immunosuppression associated with stress, auto-immune diseases and nutritional deficiency may respond favourably to treatment with immunomodulator agents.[20] Plant-based immunomodulators are often utilized as adjuvant therapy to overcome the undesired effects of cytotoxic chemotherapeutic agents. These plants help to restore health by enhancing the control of infections.[21]

In our study, the administration of different doses of CEO to non-suppressed mice increased total WBC count seven days after the immunization; a similar response was found with levamisole treatment. On the contrary, GEO and SEO treatments did not have any significant effect on WBC count. Studies *in vitro* have demonstrated that different concentrations of levamisole increased the blastogenic activity of bovine-stimulated lymphocytes.[22] Moreover, this drug was effective in promoting maturation of granulocytes and functioning of T-cells.[20] Our data suggest that CEO might act by activating the haematopoietic system along with increasing the amount of circulating leucocytes in non-suppressed mice.

The DTH reaction has a direct correlation with cell-mediated immunity and plays a role in many inflammatory disorders.[23] Such a reaction is characterized by large influxes of non-specific inflammatory cells. It is a type IV hypersensitivity reaction that develops when antigens activate sensitized T-cells, generally Th1 subsets, and promote the secretion of cytokines (i.e. interferon-γ). The overall effect of these cytokines is to recruit and activate macrophages, thereby promoting an increase in vascular permeability, vasodilatation, macrophage accumulation, activation of phagocytic activity and concentrations of lytic enzymes for more effective killing of foreign agents.[24] The

Table 3 Total white blood cell counts and humoral antibody titre in cyclophosphamide-immunosuppressed mice

Group	Dose (mg/kg)	Total WBC (cells/mm^3)		%	HA titre
		Day 0	Day 7		
Control	–	4850 ± 175	2592 ± 151	− 46.6 ± 1.8	29.3 ± 0.1
Levamisole	50	4750 ± 218	4925 ± 221	+ 4.3 ± 6.0**	100.7 ± 18.0*
CEO	100	4683 ± 242	3017 ± 208	− 35.5 ± 2.5*	28.4 ± 2.2
	200	5067 ± 210	3933 ± 182	− 22.1 ± 2.1**	56.8 ± 3.6**
	400	4983 ± 206	5042 ± 252	+ 1.1 ± 2.3**	120.7 ± 4.6**
GEO	100	4792 ± 325	2883 ± 210	− 38.6 ± 5.7	109.8 ± 11.7**
	200	4675 ± 190	2842 ± 220	− 39.1 ± 4.4	120.9 ± 4.5**
	400	4775 ± 281	2750 ± 195	− 41.8 ± 4.1	124.4 ± 3.5**
SEO	5	4817 ± 204	2758 ± 237	− 43.0 ± 3.4	35.5 ± 2.2
	10	4717 ± 162	2617 ± 186	− 44.8 ± 3.5	33.7 ± 1.8
	25	5150 ± 150	3017 ± 118	− 41.1 ± 1.8	31.3 ± 4.0

WBC, white blood cells; HA, antibody titre; CEO, clove essential oil; GEO, ginger essential oil; SEO, sage essential oil. Values are mean SEM, $n = 6$ mice in each group. Mice of all groups received cyclophosphamide on day 4, 5 and 6. *$P < 0.05$, **$P < 0.001$, when compared with the control group (analysis of variance).

DTH response was evaluated 24 and 48 h after challenge. The highest response was observed with 200 and 400 mg/kg of CEO; a similar response was found with levamisole treatment. GEO treatment induced a decrease in the DTH, while SEO failed to have any influence on this response. The effect of GEO could be related to its anti-inflammatory activity in inhibiting paw oedema development but not to the leucocyte migration.[14] In our study, an increase in the DTH response indicated that CEO, but not GEO or SEO, had a stimulatory effect on lymphocytes.

Humoral immunity involves interaction of B-cells with the antigen and their subsequent proliferation and differentiation into antibody-secreting plasma cells. Antibody production to T-dependent antigen SRBCs requires the co-operation of T- and B-lymphocytes and macrophages.[25] In our study, after essential oil treatment the anti-SRBC antibody titres were similar to all non-suppressed mice groups tested. It has been established that cyclophosphamide promotes a profound suppressive effect on all forms of cell-mediated immunity and antibody production.[26] After administration of cyclophosphamide the antibody levels were significantly reduced. CEO treatment enhanced the production of circulating anti-SRBC antibody in a dose-dependent manner. Equipotent effects were reported for levamisole and GEO, but these were not dose dependent. It has been demonstrated that levamisole does not affect B-lymphocytes directly, but it may influence humoral response indirectly by affecting macrophages and T-lymphocytes.[27] In fact, such immunoenhancement is pronounced in immunologically compromised hosts. Studies carried out in humans have shown that imidothiazoles might enhance the serum levels of thymic hormone-like factor.[28] The stimulation of the humoral response against SRBCs promoted by the treatments employed in our studies was demonstrated by an increase in the antibody levels in mice. This response indicates an enhanced responsiveness of macrophages and subsets of T- and B-lymphocytes involved in antibody synthesis.[25] Our results suggest that CEO and GEO were effective in restoring the decreased humoral immunity in cyclophosphamide-suppressed mice. Bone marrow is the most affected organ during any immunosuppressive therapy with cytotoxic drugs. Loss of stem cells and inability of the bone marrow to regenerate new blood cells result in leucopenia (i.e. as with cyclophosphamide treatment).[26] Cyclophosphamide-induced leucopenia was restored by CEO, as with levamisole treatment but not with GEO and SEO treatments. Under immunosuppressive conditions levamisole has been reported to be an immunorestorative agent and is able to restore normal functions of effector cells. Therefore, our data indicate that CEO treatment significantly increased the total WBC count and produced a protective action on the hematopoietic system.

Natural extracts have shown immunostimulating activity in immunocompromised animals.[11,12,29] Our data show that CEO is effective in increasing the total WBC count and in stimulating cell-mediated immunity (DTH) in non-immunosuppressed mice. The protective effect of CEO against immunosuppression induced by cyclophosphamide might be, partially, due to cell-mediated and humoral antibody-mediated activation of T- and B-cells. Although GEO treatment did not alter the cell-mediated response, it was able to restore the humoral immune response in immunosuppressed mice. On the other hand, SEO did not show any immunomodulatory activity. Further studies are needed to reveal the mechanisms involved in immunostimulatory response of CEO.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conlicts of interest to disclose.

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