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The effect of *Minthostachys verticillata* essential oil on the immune response of patients allergic to dust mites

[Efecto del aceite esencial de *Minthostachys verticillata* sobre la respuesta inmune de pacientes alérgicos a ácaros del polvo]

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Abstract

The essential oil from leaves of *Minthostachys verticillata* was obtained by steam distillation and analyzed by gas chromatography. Peripheral blood mononuclear cells from allergic patients were stimulated with essential oil. By application of MTT or 3,(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide colorimetric test the proliferation was assayed. In lymphocyte cultures $CD4^+$, $CD8^+$ and B cells were quantified, and in supernatants, IFN-γ and IL-13. The liberation of β-hexosaminidase enzyme was determined for basophils with essential oil added and challenged with allergen and its effects were compared with those of anti-allergic drugs. The main constituents identified were pulegone, menthone and limonene. Essential oil increased absolute values of $CD4^+$, $CD8^+$ and B cells ($p<0.002$), stimulated IFN-γ synthesis and reduced IL-13 levels. Essential oil diminished β-hexosaminidase liberation by basophils ($p<0.0001$), with effects majors to those of the drugs tested. Essential oil stimulated the Th1 deviation and reduced β-hexosaminidase enzyme liberation by basophils from allergic patients.

Keywords: *Minthostachys verticillata;* Essential oil; Immunomodulator; Basophil degranulation; β-hexosaminidase.

Resumen

El aceite esencial de hojas de *M. verticillata* fue obtenido por destilación en corriente de vapor y analizado por cromatografía gaseosa. Células mononucleares de sangre periférica de pacientes alérgicos, fueron estimuladas con aceite esencial. La proliferación fue ensayada mediante método colorimétrico del MTT o 3,(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide. Se cuantificaron células $CD4^+$, $CD8^+$ y B en cultivos de linfocitos y en sobrenadantes, IFN-γ e IL-13. Se determinó la liberación de la enzima β-hexosaminidasa de basófilos adicionados de aceite esencial, y desafiados con el alergeno. Los efectos del aceite esencial fueron comparados con los de drogas antialérgicas. Los principales constituyentes identificados fueron pulegona, mentona y limoneno. Aceite esencial incrementó los valores de células $CD4^+$, $CD8^+$ y B ($p<0.002$), estimuló la síntesis de IFN-γ y redujo los niveles de IL-13. El aceite esencial disminuyó la liberación de β-hexosaminidasa de basófilos ($p<0.0001$) con mayores efectos que los de las drogas ensayadas. Aceite esencial estimuló la desviación Th1 y redujo la liberación de la enzima β-hexosaminidasa de basófilos de pacientes alérgicos.

Palabras Clave: *Minthostachys verticillata;* Aceite esencial; Inmunomodulador; Desgranulación de basófilos; β-hexosaminidasa.

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INTRODUCTION

Allergic pathologies occur at an incidence of up to 30% in the population. Allergic patients are generally treated with corticoids and/or antihistamines, which constitute the anti-inflammatory therapies of choice because they block many inflammatory routes that are abnormally activated in this type of pathology (Barnes, 2001). Nevertheless, these methods are not always effective and can produce several adverse effects. Corticoids suppress both the innate inflammatory response and cellular immunity, inhibiting synthesis of IL-1, on behalf of macrophages, and of IL-2, on behalf of T cells. A correlation has also been discovered between their anti-lymphoproliferative effects and capacity to produce apoptosis in lymphocyte populations (Winiski et al., 2007).

Antihistamines can produce diverse adverse effects such as anemia or reduced platelets, reduced white cells and bone marrow failure (Leonardi et al., 2007).

In addition, some allergic patients display no response to treatment with these drugs, which, in conjunction with the fact that this resistance has also been observed in other inflammatory diseases (Barnes, 2001), justifies the search for new immunomodulators. A quarter of the drugs used in industrialized countries are obtained from the Plant Kingdom. Current investigations aim to find plant/herbal medicines whose therapeutic indices: permit phytomedicinal application, are low-priced and do not bring about adverse reactions or undesirable indirect effects, in order to be administered as an alternative or complementary therapy for conventional treatments.

Several *in vitro* or *in vivo* investigations have demonstrated that different extracts or pure compounds derived from medicinal plants can modulate the Th1/Th2 response, towards the Th1 profile, producing a beneficial effect for allergic patients (Na et al., 2002; Ko et al., 2004; Lee et al., 2006; Park et al., 2009).

Minthostachys verticillata (Griseb.) Epling, (Labiatae) commonly referred to as 'peperina', has a wide geographical distribution and is known for its ethno-medicinal properties. It is used as a digestive, a sedative, an anti-spasmodic, a stimulant and a bronchial-dilating agent (Ratera & Ratera, 1980).

Previous studies have revealed that the essential oil derived from this herb presents antimicrobial activity against some staphylococcal strains and antiviral properties against HSV-1, strain RC/79 of PrV and Herpes Suis Virus (Primo et al., 2001). Furthermore, in an *in vitro* study carried out on patients allergic to environmental fungi, essential oil, in concentrations of mg/mL, revealed mitogenic properties similar to those of PWM on human lymphocytes (Cariddi et al., 2005), were able to stimulate $CD8^+$ T cells, increased levels of IFN-γ and demonstrated anti-allergic properties majors to those of dexamethasone and theophylline (Cariddi et al., 2006).

The purpose of this investigation was to determine if the essential oil from *M. verticillata*, at concentrations smaller than previously evaluated, can modulate the Th1/Th2 response in allergic patients with history of allergies and asthma, and to verify if it maintains its inhibiting properties on basophil degranulation in these patients, comparing its effects with those of conventional anti-allergic drugs.

MATERIALS AND METHODS

Plant samples

Green leaves and thin stems of *Minthostachys verticillata* (Griseb.) Epling (Labiatae) were collected, during morning hours, from the city Santa Rosa in the Córdoba province, Argentina, in April 2004. The plant was identified by Dr. Margarita Grosso, professor in the Area of Botany of the Universidad Nacional de Río Cuarto, and a voucher specimen was stored in the RCV (Río Cuarto Vasculares) herbarium as file #1955. The morphological characterization of the plant was executed macro-and microscopically to confirm the identity of these specimens. The aerial parts of the plant were made up of the leaves and parts of the stem. The oil was isolated from the aerial parts.

Oil isolation

To prepare the essential oil (EO), 60 g of ground plant material were hydro-distilled for 3 h using a Clevenger-type apparatus, yielding 4.8% of the oil. The oil was separated from the aqueous phase, dried over anhydrous Na_2SO_4 and stored in the dark at -20 °C until use (Senatore, 1998). In order to perform the immunological *in vitro* assays, various concentrations were obtained, and the oil was emulsified in dimethylsulfoxide (DMSO) and diluted in Roswell Park Memorial Institute (RPMI-1640) medium.

Identification and quantification of EO compounds by Gas chromatography (GC)

Quantification of components present in the oil sample was made by measuring the area under each peak of the chromatogram (Zygadlo et al., 1996;

Cariddi et al., 2007). Briefly, analytical GC was performed on a Shimadzu GC-R1A gas chromatograph fitted with a DB5 capillary column (30 x 0.25 µm). Carrier gas N_2, flow rate 1.5 mL/min, split mode. Oven temperature programmed from 40–260 ºC at 3 ºC/min. Injector temp 280 ºC. Detector used FID, temperature 300 ºC. The identification of the compounds was made comparing their retention times against standard pure drugs injected in the same conditions.

Human blood sample

The sample included peripheral blood mononuclear cells (PBMCs) and basophils from 57 allergic patients, 27 male and 30 female (ages 1 to 30, average value: 17) with positive prick tests for allergy to dust mites (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*). This study was approved by the Universidad Nacional de Río Cuarto Institutional Review Board. In accordance with ethical standards, parents of underage children were properly informed of the study and signed an agreement authorizing the test. The characteristics of these patients are shown in Table 1. Skin prick testing was performed using a standard technique (single-headed lancet technique) and standard commercial solutions (International Pharmaceutical Immunology SA). All tests were performed by a single operator, an allergist doctor. Each patient underwent skin prick testing for allergens. Saline solutions and histamine were used as negative and positive controls, respectively. The size of the wheal was measured after 15 min and a positive result was noted if the wheal size was at least 3 mm greater than the negative saline control (Barbaud 2001). The allergen extracts were provided by International Pharmaceutical Immunology, S.A. (Alicante, Spain) Dropper vials were used, containing 3 mL of the antigen extract in 50% glycerin solution, plus 0.42% phenol each (concentration = 10.000 PNU/mL). The *in vitro* assays were performed with the same extracts. Ten milliliters of venous peripheral blood were obtained from each patient and stored in sterile tubes containing heparin.

PBMCs cultures

The effects of different concentrations (6 x 10^3, 600, 60, 6, 0.6, 0.06 and 0.006 µg/mL) of EO on the proliferation of lymphocyte from healthy individuals were evaluated. A dose-response curve was constructed in a previous *in vitro* assay. EO visibly demonstrated lymphoproliferative capacity, with dose-dependency. The concentration of 6 µg/mL obtained constituted the highest Proliferation Index (PI)

PBMCs were isolated from blood samples using Ficoll-Hypaque (Hystopaque®-1077; Sigma, St. Louis, US) centrifugation. The cellular proliferation assays were carried out with the Vybrant® MTT Cell Proliferation Assay Kit (Molecular Probes Invitrogen Detection Technologies, Eugene, Oregon, USA). Cells (2 x 10^5/mL), with a final volume of 200 µl, were cultured in 96-well sterile microplates containing RPMI-1640, to which was added 25 mM Hepes, 2 mM L-glutamine, 5% FCS, 50 mM 2-ME, 100 µg/mL streptomycin and 100 µg/mL penicillin (Merck). Cultures were stimulated with 10 µg/mL PHA, 5 µg/mL ConA and 6 µg/mL oil. Control lymphocyte cultures were performed using only media.

Cells were incubated during 72 h at 37 °C with 5% CO_2 in a humid atmosphere. An aliquot of 100 µL of fresh RPMI-1640 and 10 µL of MTT (3,(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide) solution (1 mg/mL of MTT in PBS 0.01 M pH 7.2) was added to each well and the plate was incubated at 37 °C with 5% CO_2 for 4 h. After incubation, the plate was centrifuged and supernatants, removed. DMSO (50 µL) was added to each well in order to dissolve the formazan crystals that resulted from the conversion of the salt (MTT). The plate was read in a UV spectrophotometer (Labsystems Multiskan MS) at 570/690 nm wave lengths.

The cellular expansion reached by the classic mitogens was compared with that produced by EO by calculating the PI according to the following formula: PI = stimulated cells/ non-stimulated cells > 1.20 (Tuchscherer et al., 2002).

$CD4^+$ and $CD8^+$ T cells and B cells quantification by immunofluorescence (IF)

The $CD4^+$ and $CD8^+$ T cells present in the EO-stimulated cultures were counted with the indirect IF technique using anti-human CD4 and anti-human (Sigma, St. Louis, US) CD8 monoclonal antibodies (Sigma, St. Louis, US), and B cells were counted with direct IF using anti-human γ-globulin-FITC (Sigma, St. Louis, US) (Edidin, 1989).

Table 1. Characterization of select allergic patients, including: age in years, symptoms, total IgE values and skin prick testing results.

				Skin prick testing with dust mite	
Nº clinical history	Age (years)	Symptoms	IgE (UI/mL)*	*D. pteronyssinus*	*D. farinae*
5	2	As Rh	618	++	+++
9	5	Rh Ecz	190	+++	+
10	5	As Rh	790	+++	++
12	6	As Rh Sin Pr	1133	++	+++
13	6	As Rh	1664	++	+
14	7	Rh, As	1236	++	+
15	8	Rh Si	365	+	+
16	8	As Rh	760	++++	++
18	9	As Rh	1000	+	+
20	11	Rh As	1321	++	++
21	11	As Rh	786	++	++
23	12	Rh Si	420	++	++
27	15	Rh	460	+++	+++
28	17	Rh Ecc	350	+	+
33	19	As Rh	512	++++	++
35	21	As Rh Ecz	122	+	+
36	21	As Rh Ecz	122	+	+
38	23	Rh Ecz	280	+	+
39	23	As Rh	139	+	+
41	26	As Rh	141	+	+
44	27	As	512	+	+
45	27	As RhEcz	123	+	+
47	28	Rh Ecz	790	+	+
50	29	As Ecz Rh	1560	+	+
51	29	As Rh Sin Pr	1133	++	+++
52	29	As Rh	355	+	+
53	29	Rh, As	236	++	+
54	30	As Ecz Rh	568	+	+
55	30	As Rh Sin Pr	1233	++	+++
56	30	As Rh	965	+	+
57	30	Rh, As	1236	++	+

As - Asthma; Rh - Rhinitis; Ecz - Eczema; Pr - Prurigo; Si - Sinusitis. *D. Pteronyssinus - Dermatophagoides pteronyssinus*; *D. Farinae - Dermatophagoides farinae.* Prick test: scale from + to ++++ according to Bousquet (1988).
* IgE was measurement by ELISA Kit (Iema Well de Radim, Rome, Italy)

IFN-γ and IL-13 measurement

IFN-γ and IL-13 synthesis was quantified in EO-stimulated supernatants of PBMC cultures from allergic patients, collected at 60 h incubation to optimize the detection of cytokine levels. IFN-γ detection was performed using a commercial Human Interferon Gamma (Hu- IFN-γ) ELISA kit (PBL Biomedical Laboratories, USA) and the lower limit of detection for the cytokine assay was less than 2 pg/mL (Kelder et al., 1986). IL-13 detection was performed using a commercial EHIL13 Human Interleukin-13 ELISA Kit (Pierce Endogen, France) and the lower limit of detection for the cytokine assay was less than 7 pg/mL (Ly et al., 2005). This assay was carried out with PBMC cultures stimulated with: a) allergen alone (a suspension of dust mite at 10.000 PNU/mL) and b) 6 μg/mL of EO, plus allergen. As a control, these cytokines were quantified in PBMC cultures with media alone. Each instance was triplicated.

β-hexosaminidase enzyme release assay

The effects of different concentrations (160, 80, 40, 20, 10 and 1 μg/mL) of EO on the release of β- hexosaminidase enzyme were evaluated constructing a dose-response curve in a previous assay. It was observed that EO reduced the levels of enzyme released in a dose-dependent way. The concentration of 10 μg/mL was that which produced the highest diminution of the release of enzyme.

Basophils of peripheral blood were obtained by density gradient using 6% of dextran 70 with NaCl 0.9%. The interphase that contained the cells was centrifuged and re-suspended in RPMI-1640. An

aliquot from the cellular suspension was taken and the cells were dyed with Toluidine Blue that it dyes only basophils. The cellular count was realized in camera Fuch Rosenthal in optical microscope. The concentration of basophils was increased 100 to 200 times after the treatment with dextran (from 1-2 to 128-256 cells in 64 fields). It had contamination by other PMN, such as neutrophils and eosinophils.

Fifty microliters of the basophil suspension at a concentration of 1 x 10^5 cells/mL were placed in each well of the 96-well microplate and pre-incubated for 15 min at 37 °C with the specific allergen alone (dust mite suspension), and the allergen plus: a) 0.04 mg/mL dexamethasone (Sidus); b) 0.2 mg/mL theophylline (Phoenix); c) 0.2 mg/mL disodium cromoglicate (Phoenix); d) 0.05 mg/mL ipratropium bromide (Altana Pharma), or e) 10 µg/mL essential oil. The negative control values were obtained using RPMI-1640 and the cells alone, without any allergen stimulation, in order to view unspecific spontaneous degranulation. In addition, montelukast (0.04 mg/mL) (Merck) was used as a negative control of β-hexosaminidase enzyme inhibition because it acts in the late phase of the allergic reaction, inhibiting leukotriene receptors. Blank values were obtained using just the media or DMSO. Following incubation, 50 µL of the chromogenic substrate for the enzyme β-hexosaminidase (4-nitrophenyl-N-acetyl-β-D-glucosaminide) (Sigma-Aldrich, Inc, St. Louis, USA) 1 mmol/L, was added to each well and dissolved in 0.1 mol/L of citrate buffer, pH 5. The system was incubated at 37 ºC for 1 h. The reaction was stopped with 200 µL of carbonate buffer 0.1 M, pH 10.5 per well. The product of the cleavage (4-nitrophenol) was interpreted by reader ELISA (Labsystems Multiskan MS) at 405 nm (Shibata et al., 1996). The percentage of β- hexosaminidase release inhibited was calculated according to the formula: Inhibition % = (A – B) x 100/A, where A is the amount of β-hexosaminidase released by basophils in the presence of the specific allergen alone and B, in the presence of the allergen aggregates from the anti-allergic drugs or oil (Na et al., 2002).

Statistical analysis

All of the values obtained in the assays carried out during this investigation were expressed in averages and standard deviation. The parameters were evaluated using the program GraphPad Prism version 4.0 (Inc. San Diego, USA, 2004) and compared with the parametric test *t*-Student for twin samples. Statistical differences were considered significant when the value was $p < 0.05$.

RESULTS

Identification and quantification of essential oil compounds

GC analysis revealed a composition not significantly different than those reported previously by the authors (Zygadlo et al., 1996; Cariddi et al., 2007). The main components were pulegone 63.0%, menthone 16.4% and limonene 1.9%, accompanied by other minor terpenoid components such as α-pinene (0.2%), β-pinene (0.3%) and 1,8-cineole (0.1%) were found in lower quantities in accordance with previous reports**.**

Lymphocyte response to stimulation with essential oil

EO (6 µg/mL) stimulated the proliferation of lymphocyte from allergic patients, revealing significant statistical differences in comparison with the cultures lacking stimulus ($p<0.0001$) and no differences when compared to PHA ($p=0.1396$) or ConA ($p=0.1749$). The PI calculated for EO, PHA or ConA, were > 1.20, indicating cellular proliferation (Fig. 1).

Absolute values of $CD4^+$ and $CD8^+$ T cells and B cells in cellular cultures stimulated with EO revealed an increase ($p<0.002$ to each one of cell types) in regard to the cultures without stimulus (Table 2).

In supernatants of EO-stimulated cultures, values of IFN-γ revealed an increase in regard to cultures without stimulus ($p<0.001$) and cultures stimulated with allergen alone ($p<0.0001$) (Fig. 2). IL-13 levels were greater in the cultures stimulated with allergen than in cultures without stimulus ($p<0.0001$). In allergen-stimulated cultures with EO, the levels of IL-13 were visibly reduced in regard to cultures stimulated with the allergen alone ($p<0.0001$) (Fig. 2).

Effects of essential oil on basophil degranulation

Basophils of all the allergen-challenged patients released high amounts of β-hexosaminidase enzyme in comparison with the values of enzyme released spontaneously ($p<0.01$) (Fig. 3). The anti-allergic drugs, excluding montelukast, reduced the *in vitro* levels of released enzyme in comparison with basophils challenged with the allergen alone ($p<0.02$) (Fig. 3). This result demonstrated that the plant fraction did not reveal allergenic effects *in vitro*. The

addition of EO to allergen-challenged basophils reduced β-hexosaminidase liberation to the same levels as those of the spontaneous release of the enzyme. The inhibiting activity of the oil at 10 µg/mL on the enzyme was higher than the one produced by standard drugs at concentrations: 0.04 mg/mL of dexamethasone, 0.2 mg/mL of theophylline, 0.2 mg/mL of disodium cromoglicate and 0.05mg/mL of ipratropium bromide ($p<0.05$ for each one) (Fig. 3). The percentage of inhibition of β-hexosaminidase released under different treatments is shown in Table 3 and the essential oil is significantly active.

Tabla 2. Average absolute values ± *SD* of total leukocytes from allergic patients and T $CD4^+$, T $CD8^+$ and B cells in cultures without stimulus (W/S) or stimulated with essential oil (EO).

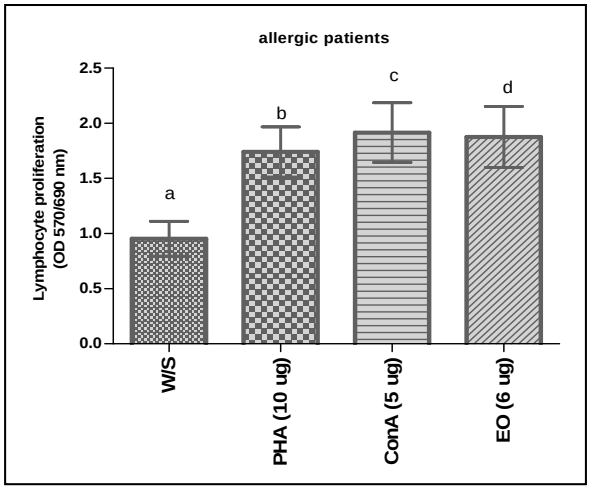
n	Total leukocytes/mm^3	W/S cultures			Cultures stimulated with EO		
		$CD4^+$	$CD8^+$	B cells	$CD4^+$	$CD8^+$	B cells
22	6432.6 ± 1321,6	1677 ± 65	714 ± 60	705 ± 57	2174 ± 82	988 ± 60	916 ± 61

Table 3. Percentage ± *SD* of inhibition of β-hexosaminidase enzyme release from basophils of allergic patients by anti-allergic drugs or essential oil.

n=57	Anti-allergice drugs or essential oil				
	dexa	theo	crom	Ip	EO
Inhibition (%)	44.8 ± 8	47.6 ± 10	44.7 ± 7	46.9 ± 5	54.2 ± 7

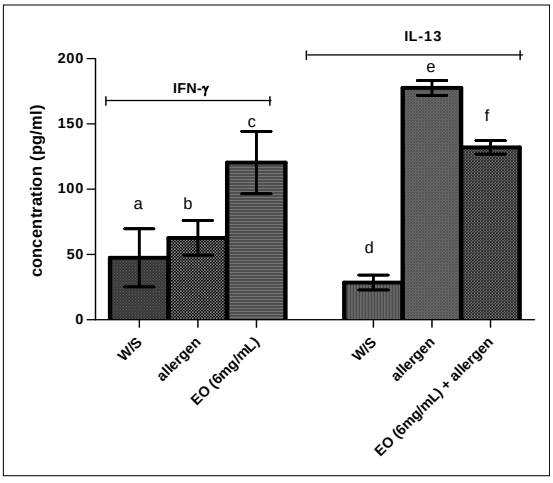
Dexa - dexamethasone; theo - theopylline; crom - disodium cromoglicate; Ip - ipratropium bromide; EO - essential oil.

Figure 1. Proliferation of lymphocytes from allergic patients (n=57), stimulated with 6 µg/mL essential oil (EO) from *M. verticillata*, according to MTT colorimetric assay.



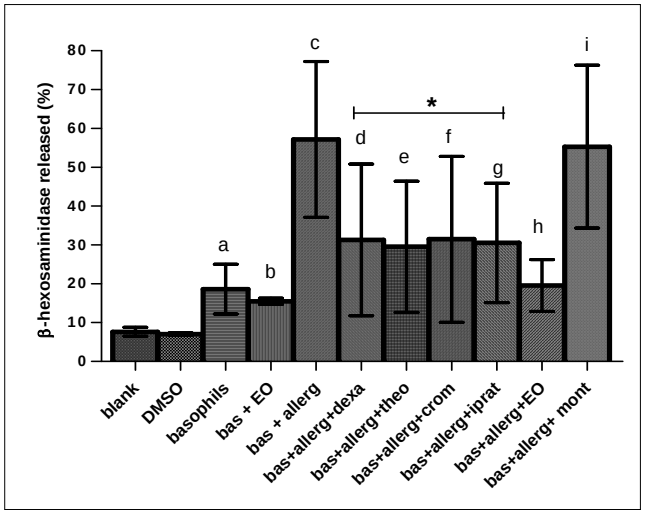
PHA (10 µg/mL) or ConA (5 µg/mL) were used as positive controls of proliferation, and lymphocyte cultures without stimulus, as negative controls. W/S, without stimuli; a *vs* d $p < 0.0001$; b *vs* d $p = 0.1396$; c *vs* d $p = 0.1749$; *PI* (PHA) = 1.81 ± 0.12; *PI* (ConA) = 1.90 ± 0.11; *PI* (EO) = 1.87 ± 0.10

Figure 2. Quantification of IFN-γ and IL-13 in supernatants of lymphocyte cultures from allergic patients (n=38).



IFN-γ was quantified in lymphocyte cultures without stimulus or stimulated with 6 µg/mL essential oil (EO) from *M. verticillata*. IL-13 was quantified in lymphocyte cultures without stimulus, stimulated with allergen alone or stimulated with allergen and with 6 µg/mL EO from *M. verticillata* added. W/S, without stimuli; a *vs* b $p= 0.1332$; a *vs* c $p<0.001$; b *vs* c $p<0.0001$, d *vs* e $p<0.0001$; d *vs* f $p<0.0001$, e *vs* f $p<0.0001$.

Figure 3. The amount of β-hexosaminidase enzyme released by allergen-challenged basophils from allergic patients (n=57) was reduced by essential oil (EO) and several anti-allergic drugs, excluding montelukast.



Basophils were incubated with EO alone, without allergen, and no significant liberation of the enzyme was observed. Cells without the addition of allergen and cells with addition of dimethylsulfoxide (DMSO) were also evaluated as controls.

a *vs* c $p< 0.0001$; a *vs* b $p = 0.3373$; b *vs* c $p <0.002$; c *vs* d $p < 0.01$; c *vs* e $p < 0.002$; c *vs* f $p< 0.01$; c *vs* g $p < 0.002$; c *vs* h $p < 0.0001$; c *vs* i $p = 0.8245$; h *vs* * p = ns; h *vs* i $p<0.0001$; d *vs* i $p < 0.01$; e *vs* i $p< 0.002$; f *vs* i $p< 0.02$; g *vs* i $p<0.003$.

blank, media alone; DMSO, dimethylsulfoxide; bas, basophils; allerg, allergen ; dexa, dexamethasone ; theo, theopylline ; crom, disodium cromoglicate; iprat, ipratropium bromide; EO, essential oil; mont, montelukast

DISCUSSION

In the present study, the essential oil from *M. verticillata* was composed mainly by mono-and sesquiterpenes. Zygadlo et al. (1996) analyzed oils of *M. verticillata* of different geographic areas from Argentina. They found that the main components are terpenes and seem to be divided in three chemotypes: carvone, thymol-carvacrol and predominantly pulegone-menthone. Our results agree those of De Feo et al. (1998), González Pereyra et al. (2005), Cariddi et al. (2007), Maldonado et al. (2007), who studied *M. verticillata* oil and reported the composition.

At optimal concentrations, EO from *M. verticillata* was able to stimulate *in vitro* the lymphocyte proliferation in patients allergic to dust mites with effects similar to those of PHA or ConA. These lectins bond specifically to carbohydrate components, including proteins of the TcR-CD3 complex. This produces the activation of several types of enzymatic routes which involve Ras-Map kinases, calcium-dependent PLCγl and PLCγl–DAG, key participants in cellular proliferation, differentiation and apoptosis (Crespo & Gutkind, 2004).

According to some authors *in vitro* studies of allergic patients have shown increased values of $CD4^+$ T cells (1500-1750 cells/mm^3), differentiating towards the Th2 profile; a decrease in $CD8^+$ T cells (370-520 cells/mm^3) (Bratke et al., 2006) and high values of B cells (480-650 cells/mm^3) (Mohamed et al., 2003) in comparison with non-allergic individuals. In this study EO (6 μg/mL) increased *in vitro* absolute values for both T $CD4^+$ and $CD8^+$ cells subpopulations in patients allergic to dust mites, indicating that this plant fraction stimulated cellular immunity. These data are compatible with those from a previous *in vitro* study in which high EO concentrations (mg/mL) increased values of $CD8^+$ T cells in both patients allergic to environmental fungi and healthy individuals alike (Cariddi et al., 2006).

The EO also increasing the absolute values of B cells. Previously we demonstrated that EO had effects similar to those of PWM, mitogen of B and T cells, on lymphocyte proliferation in both allergic and healthy individuals (Cariddi et al., 2005). These results correspond with those of this investigation, where we demonstrated that EO was able to stimulate both T and B cells.

Treatment with the EO increased the levels of IFN-γ, which is why it is suggested that the EO-activated $CD4^+$ T cells in this study could be Th1 cells, which produce IFN-γ. EO was able to stimulate *in vitro* $CD8^+$ T cells as well, also producers of IFN-γ. In addition, we observed that EO reduced IL-13 values in lymphocyte cultures co-stimulated with the allergen, as compared to cultures stimulated with allergen alone. These data demonstrated that EO modulated the *in vitro* Th1/Th2 response, favoring Th1 deviation. Anti-allergic therapies aim to redirect the profile of Th2 cells towards Th1, increasing IFN-γ synthesis and decreasing IL-4 and IL-13 values, thus reducing IgE levels (Bang & Plosker, 2004). It was confirmed that the vegetal derivative acts on the immune response by means of the stimulation of the Th1 profile. In previous studies this was deduced of the cuantification of IFN-γ and LT CD8+ (Cariddi et al., 2006, 2007; Maldonado et al., 2007).

β-hexosaminidase, an acid hydrolase, is released along with histamine when basophils are activated. Therefore, β-hexosaminidase is accepted as a degranulation marker (Shibata & Yagi, 1996).

During the β-hexosaminidase enzyme release assay, we confirmed that the anti-allergic drugs tested, excluding montelukast, were able to reduce the levels of enzyme released, as compared to basophils challenged with allergen alone. Dexamethasone, in addition to bonding to the cytoplasmic specific receptor that enters the nucleus and attaches itself to the DNA, works by stimulating cAMP production, inhibiting the degranulation process in basophils and mast cells (Fukui, 2008). Theophylline acts as inhibitor of phosphodiesterase. This enzyme deactivates cAMP forming icAMP, which favors cell degranulation (Lipworth, 2005). Cromoglycate is a basophil and mast cell membrane stabilizer, as it prevents their degranulation (Gotua et al., 2008). Ipratropium inhibits the liberation of chemical mediators, increased by acetylcholine, by blocking cholinergic receptors on the surface of basophils and mast cells (Spooner et al., 2003). In contrast, montelukast, a leukotriene receptor antagonist (Kawai et al., 2008), which was used in this study as a negative control for inhibition of the immediate reaction, did not decrease the levels of β-hexosaminidase released by basophils challenged with allergen, as expected in this model.

EO, at a concentration of 10 μg/mL, reduced the levels of β-hexosaminidase released by basophils, with effects majors to the anti-allergic drugs tested, excluding montelukast, which is why it has been suggested that this plant fraction could act upon cell membranes with a mechanism similar to that of one of these drugs. These results demonstrated that EO maintained its inhibiting properties on basophil degranulation at high (Cariddi et al., 2006) and small concentrations alike.

Some of main components of EO identified by GC could be responsible for its biological activity. Numerous investigations demonstrate the protective effects of terpenes on cell membranes. These substances also carry out a regulating activity on T cells, the synthesis of cytokines and inhibition of the apoptosis processes (Yeh et al., 2006; Ashibe et al., 2007).

Essential oils are complex mixtures of numerous molecules and their biological effects are the result of a synergy between all of them or the reflection of main molecules present at high levels according to analysis by GC (Bakkali et al., 2008).

According to the aforementioned, EO could have displayed protective effects on cell membranes when added to the basophils. This indicates that the increase in basophil membrane permeability may be an essential trigger for the release of β-hexosaminidase. The terpenes from EO may have acted upon basophil membranes, avoiding the disturbance induced by the specific allergen. There would not be modifications related at least to types of allergens both tried (fungi and dust mite) (González Pereyra et al., 2005; Cariddi et al., 2006, 2007; Maldonado et al., 2007). Moreover the active concentrations seem to be much lower than previously thought, as this work found activity in the range 6 mg/ml at 0.006 μg/mL, in contrast with our previous tests at only 150 mg/mL (Cariddi et al., 2005).

CONCLUSION

The EO from leaves of *Minthostachys verticillata* at small concentrations acted as a mitogen, increasing proliferation of T and B cells from allergic patients. As an immunomodulator, it favored Th1 deviation by increasing IFN-γ synthesis and decreasing IL-13 production. EO maintained its inhibiting effects on basophil degranulation, reducing the levels of β-hexosaminidase released.

Additional studies are being realized along the same line of investigation to reveal if the terpenes identified can have therapeutic applications in allergies. In the future, these products could be applied in alternative or complementary therapies with patients who do not respond to conventional treatment.

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