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Inhibition of Immediate-Type Allergic Reaction by *Minthostachys verticillata* (Griseb.) Epling Essential Oil

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Abstract

An oil of *Minthostachys verticillata* (Griseb.) Epling was analyzed by GC. The main constituents identified were pulegone (63.0%) and menthone (16.4%). It was found that the oil inhibited β-hexosaminidase release from basophils. Forty-two percent of the cells present in lymphocyte cultures stimulated by the oil were CD8(+) T cells and showed higher IFN-γ levels than non-stimulated cultures ($p < 0.05$). According to these results, the oil was considered to be a Th1 deviation inducer, inhibiting the immediate-type allergic reaction.

Key Word Index

Minthostachys verticillata, Labiatae, essential oil composition, pulegone, menthone, anti-allergic effect.

Introduction

Minthostachys is a taxonomically complex South American genus represented by twelve species of aromatic herbs and green bushes, which belong to the Labiatae family. They grow widely in Colombia, Venezuela, Brazil, Ecuador, Peru, Bolivia and also in the northwest and central regions of Argentina (1).

The species that belong to this genus include: *Minthostachys glabrescens* Epling.; *Minthostachys verticillata* (Griseb.) Epling [syn. *Minthostachys mollis* (Griseb.)); *Minthostachys andina* (Brett.) and *Minthostachys spicata* (Benth.) Epling. *Minthostachys verticillata*, commonly known as 'peperina', 'pipirina', 'peperita' or 'piperita', has the widest geographical distribution and is known for its ethno-medicinal properties (2,3).

Minthostachys verticillata is an aromatic bush of 0.3 to 2 m in height, with oblong leaves from 1 to 5 cm in length, obtuse or sub-acute, which form 90° angles with the stem. Its white flowers are grouped in the leaves' axils and the stem has a quadrangular section. It's a pubescent plant that flowers in summer. The fruits are dry, small capsules with four seeds each. Its fructification period is in March and April and it is harvested in summer (4,5).

It is used mainly in infusions to diminish diarrhea and vomiting. It is also used as a digestive, a sedative, an anti-spasmodic and a bronchial dilating agent. It also has insecticidal, fungicidal and anti-parasitic properties (1,6). In the alimentary industry, it is used for the fabrication of liquors, cool drinks and mixed herb preparations (7,8).

Previous studies have shown essential oil derived from *M. verticillata* has antimicrobial activity against some staphylococcal strains and antiviral properties against *HSV-1*, strain RC/79 of *PrV* and *Herpes Suis Virus* (9). In addition, the oil has mitogenic properties on human lymphocytes as PWM (10).

For a long time, the corticoids represent the more effective anti-inflammatory therapy in allergy because they block many of the inflammatory routes that abnormally are activated in this type of pathologies as described by Jungsuwadee et al. (11). Nevertheless, an installed problem refers allergic patients who do not respond to the treatment with β-adrenergic steroids which justifies the search of new immunomodulators. It is known that the fourth part of drugs used in the industrialized countries has been obtained from the plant kingdom. Recent studies demonstrate that the homeopathy, acupuncture and

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the herbal medicine are the types of alternative medicine, but commonly used by patients with allergic diseases, is for that reason that the vegetal products are at the present time object from study at world-wide level (12,13).

The purpose of this study is to investigate the properties attributed to the oil derived from *M. verticillata.* Several objectives were established. First, we obtained the plant's essential oil and detected main constituents by gas chromatography. Second, we evaluated the modulating ability this has over basophils degranulation by β-hexosaminidase release assay, comparing their inhibitory properties with those of dexamethasone and theophylline. Third, we determined the % of CD8(+) T cells in lymphocyte cultures stimulated by the vegetal derivate and measured the IFN-γ level in the supernatants of these cultures in order to deduce if essential oil, can induce Th1 deviation.

Experimental

Plant samples: Green leaves and thin stems of *M. verticillata* were collected during morning hours, in April 2004. The original seedlings were from the Santa Rosa city in the Córdoba Province, Argentina. The plant was identified by Margarita Grosso, professor of the Area of Botany of Universidad Nacional de Río Cuarto (UNRC) and a voucher specimen was stored in RCV (Río Cuarto Vasculares) Herbarium under exsiccate Nº 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: For the preparation of the oil, 60 g of ground material was hydrodistilled for 3 h using a Clevenger-type apparatus, yielding 4.8% of oil. This was separated from the aqueous phase, dried over anhydrous Na_2SO_4 and stored at -20°C in the dark covered with aluminum foil, until its use, as described by Ratera et al. (6). In order to obtain various concentrations to perform the different *in vitro* assays, the oil was emulsified in dimethylsulfoxide (DMSO) and then diluted in Roswell Park Memorial Institute (RPMI-1640) medium.

Identification and quantification of essential oil compounds of M. verticillata by Gas chromatography (GC): 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 40-260ºC at 3ºC/min. Injector temp 280ºC. Detector used FID, temp. 300ºC. The identification of the compounds was made comparing their retention times against standard pure drugs injected in the same conditions. Quantification of components present in the oil sample was made by measuring the area under each peak of the chromatogram (14).

Population: Basophils and lymphocytes from 30 allergic patients (aged one to 20), with positive prick tests to environmental fungi *(Hormodendrum hordei, Rizophus arrhizus, Rizophus orizae, Aspergillus fumigatus, Alternaria tenuis* and *Mucor mucedo)* were studied. The characterization of these patients is shown in Table I. Skin prick tests were performed using a standard technique (single-headed lancet technique) and standard commercial solutions (International Pharmaceutical Immunology SA, Argentina). All tests were performed by a single operator. Each patient underwent skin prick tests to fungal allergens. Saline solution and histamine were used as negative and positive controls, respectively. The size of the wheal was measured 15 min after and a positive result was noted if the wheal size was 3 mm or more, greater than the negative saline control (15).

The fungic allergen extracts were provided by the International Pharmaceutical Immunology, S.A. Dropper vials were used, containing 3 mL of the antigen extract in 50% glicerine solution, plus 0.42% phenol each (concentration = 10.000 PNU/mL). With those same extracts, the *in vitro* tests were made. Ten milliliters of venous peripheral blood were obtained from each patient and collected in sterile tubes containing heparin.

According to ethics, parents of underage children were properly informed about the study and they signed conformity to make the test.

β-Hexosaminidase Assay: To obtain and concentrate basophils, the *Benveniste, J.* technique modified by Grinstein et al. (16) was used.

To evaluate the inhibitory effect of the vegetal fraction over basophils degranulation, the β-hexosaminidase release assay was performed. The basophils of the allergic patients were exposed to the environmental fungi single specific allergen and the allergen added of:

a) dexamethasone (0.04 mg/mL) (Montpellier S.A, Argentina).
b) theophylline (0.2 mg/mL) (Phoenix, Argentina).
c) essential oil (1; 0.8; 0.16 and 0.001 mg/mL).

The negative control values were obtained using RPMI-1640 and the cells alone, without any allergen stimulation, to visualize unspecific spontaneous degranulation. Blank values were obtained using RPMI-1640 medium alone. Other control was DMSO and the cells alone.

Fifty μL of the suspension of basophils, to a concentration of 1 x 10^5, was placed in the 96-well microplate and pre-incubated by 15 min to 37°C with the single specific allergen and the added specific allergen of a), b) or c) (17).

After the incubation was added 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, disolved in 0.1 mol/L of citrate buffer, pH 5. The system was incubated to 37ºC for 1 h. The product of the cleavage (4-nitrophenol) was interpreted by spectrophotometric reading to 405 nm as described by Shibata et al. (18). The reaction was stopped with 200 μL of buffer carbonate 0.1 M, pH 10.5 per well. The plate was read to 405 nm in a reader ELISA (Labsystems Multiskan MS). The percentage of inhibition of β- hexosaminidase released was calculated according to the equation:

$$\text{Inhibition\%} = (A - B) \times 100/A \quad (19).$$

In where **A** is β-hexosaminidase released by the basophils in the presence of allergen and **B** is β-hexosaminidase released by the basophils in the presence of the allergen with the aggregate of dexamethasone, theophylline or the oil.

Lymphocyte Cultures: Blood samples were diluted 1/3 with Hanks' balanced saline solution (HBSS) (Sigma, St. Louis, US), placed over Hystopaque - 1077 (Sigma, St. Louis, MO) and centrifuged at 2000 rpm for 20 min at room temperature. For obtaining lymphocytes, the interface was collected and washed three times using RPMI - 1640 (Sigma, St. Louis, MO).

The assays of cellular proliferation took place following the colorimetric method according to Mosmann (20) using the Vybrant MTT Cell Proliferation Assay Kit (Molecular Probes Invitrogen Detection Technologies, Eugene, OR). Cells (2 x 10^5/mL), in a final volume of 200 µL, were cultured in the 96-wells sterile microplates (NUNCLO, Delta Nunc Inter Med, Denmark) containing RPMI-1640 amended with 25 mM of Hepes (Gibco Laboratories, Life Technologies Inc. Grand Island, NY), 2 mM of L- glutamine (Parafarm, Industria Argentina) 5% fetal calf serum (FCS) (Gibco BDRL), 50 mM of 2-mercaptoethanol (2-ME) and 1% of antibiotics (100 µg/mL streptomycin and 100 µg/mL ampicylin). Cultures were stimulated with Phytohemagglutinin-M (PHA) (10 µg/mL), Concanavalina A (5 µg/mL) and different concentrations of the oil (1; 0.8; 0.16 and 0.001 mg/mL). Control lymphocyte cultures were performed using RPMI 1640 alone. Cells were incubated during 72 h to 37°C with 5% CO_2 in atmosphere humidified. After incubation, the plate was centrifugated and supernatants were placed in eppendof tubes to -80°C until use in the IFN-γ measurement assay. An aliquot of 100 µL of freshly RPMI-1640 and 10 µL of MTT solution (1 mg/mL of MTT in PBS 0.01 M pH 7.2) was added to each well and the plate was incubated to 37°C with 5% CO_2 for 4 h. Dimetilsulfoxide (DMSO, Sintorgan Industria Argentina) (50 µL) was added to each well, in order to dissolve the crystals of formazan that resulted from the conversion of the salt of tetramethyl-tetrazolium (MTT, or 3,(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide). The results were obtained using a UV spectrophotometer (Labsystems Multiskan MS) at 570/690 nm wave lengths. The cellular expansion reached about the classic mitogens was compared with the produced one by the different concentrations from the oil of *M. verticillata* calculating the Proliferation Index (PI) according to the following equation:

$$PI = \frac{\text{stimulated cells}}{\text{none stimulated cells}} \geq 1{,}20$$

According to Tuchscherer et al. (21) an IP ≥ 1,20 is indicative of cellular proliferation.

Table I. Allergic patient's characterization: age, symptoms, specific IgE values and prick test results.

				Prick Tests with fungi allergens				
Patients	**Age**	**Symptoms**	**IgE UI/ml**	***Altern***	***Asperg***	***Hormo***	***Rhizo***	***Mucor***
1	1	As[a] Ri[b]	89	+	+	+	+	_
2	1	As Ri	139	+	_	_	_	_
3	1	As Ri	41	+	+	+	+	+
4	2	AsRiEcz[c] Pr[d]	180	+	+	+	+	+
5	2	As Ri	512	++	++	+	+	+
6	2	As	43	+	+	+	+	++
7	2	As RiEcz	122	+	+	+	+	+
8	3	As Ri	68	+	+	+	+	_
9	3	Ri Ecz	280	+	+	+	_	_
10	4	As Ri	790	+++	++	++	++	_
11	4	As Ecz Ri	355	+	+	++	+	+
12	5	As Ri Si[e] Pr	1133	++	+++	+++	+++	+
13	6	As Ri	1560	+	+	+	+	+
14	6	Ri, As	1236	++	+	_	_	_
15	7	Ri Si	365	+	+	+	+	_
16	7	As Ri	760	++	++	+	+	_
17	9	As	292	+	+	+	+	+
18	9	As Ri	1000	+	+	+	+	+
19	10	Ri Si	193	++	_	_	_	_
20	11	Ri As	1131	++	++	+	++	+
21	11	As Ri	786	++	++	++	++	++
22	12	Ri Ot[f]	220	+	+	+	+	_
23	13	Ri Si	420	++	++	_	+	_
24	14	As Ri	325	+	_	_	_	_
25	14	Ri Si	542	+	_	+	+	_
26	20	Ri Si	122	+	+	+	+	+
27	20	Ri	360	+++	++	++	++	+
28	22	Ri Ecz	190	+	+	+	+	_
29	27	Ri Si	269	+	+	+	_	_
30	27	As	208	++	+	+	+	+

[a] As: Asthma, [b] Ri: Rhinitis, [c] Ecc: Eczema, [d] Pr: Prurigo, [e] Si: Sinusitis, [f] Ot: Otitis.

CD8(+) T Cells Score by Indirect Immunofluorescense (IF): CD8(+) T cells present in the cultures stimulated with the oil were scored by indirect IF technique. A 1 x 10^6 viable mononuclear cell/mL suspension was used. Five µL of anti-human CD8 monoclonal antibody (Sigma St. Louis, USA) were added to the cell suspension. Cells were incubated at 18º–22ºC for 30 min. Then, they were washed twice with 2 mL of Azide-PBS, suspended in 100 µL FITC-conjugated anti-γ-globulin (Sigma, St. Louis, MO) and diluted 1/15000 with Evans Blue stain. The suspension was incubated at 18º-22ºC for 30 min, in the dark. After this, cells were washed twice using 2 mL of Azide-PBS and the supernatant was thrown away. Cells were then suspended in 15 µL of glycine buffer and a drop (5-10 µL) of the cellular sediment was placed in a microscope slide. Cells were scored in an UV light epifluorescence microscope (1000X magnification).

The percentage of fluorescent cells was determined over 100 lymphocytes. CD8(+) T cells were distinguished by a bright fluorescence in their cellular membrane. CD8(+) T cells levels that were between 20 and 25% are considered as normal in peripheral blood (22).

IFN-γ Measurement: IFN-γ synthesis was quantified in allergic patients' sera and lymphocyte cultures' supernatants. The assay was performed using a commercial Human Interferon Gamma (Hu- IFN-γ) ELISA kit (PBL Biomedical Laboratories, USA) (23).

Results and Discussion

GC analysis revealed pulegone and menthone present as the main components in 63.0% and 16.4%, respectively. Other terpenoid components such as α-pinene (0.2%), β-pinene (0.3%), limonene (1.9%) and 1,8-cineole (0.1%) were found in lower quantities (Table II).

Basophils from all patients released the enzyme β-hexosaminidase when the specific allergen was present. This release was diminished when dexamethasone (0.36 mg/mL), theophylline (1.83 mg/mL) or different concentrations of the oil (1; 0.8; 0.16 and 0.001 mg/mL) were added to the wells along with basophils and allergen. The oil derived from *M. verticillata* independently of the tried dose inhibited the liberation of the *in vitro* β-hexosaminidase release. The effect of the oil was comparable with that of dexamethasone or theophylline (p=ns) (Figure 1).

Table II. Main constituents of the oil of *Minthostachys verticillata*

Compound	Percentage (%)
α-pinene	0.2
β-pinene	0.3
limonene	1.9
1,8-cineole	0.1
menthone	16.4
pulegone	63.0
Total identified	**81.9**

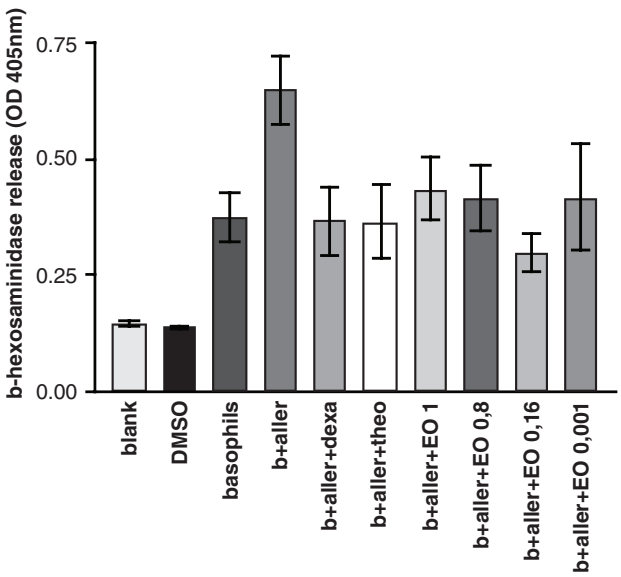

Figure 1. b-Hexosaminidase released by basophils (b) from allergic patients (n=30); the enzyme released was diminished when dexamethasone (dexa), theophylline (theo) or different concentrations of the oil (EO) were added along with allergen (aller); the blank well contained RPMI-1640 alone; cells without the addition of allergen and cells with addition of dimethylsulfoxide (DMSO) were evaluated as controls

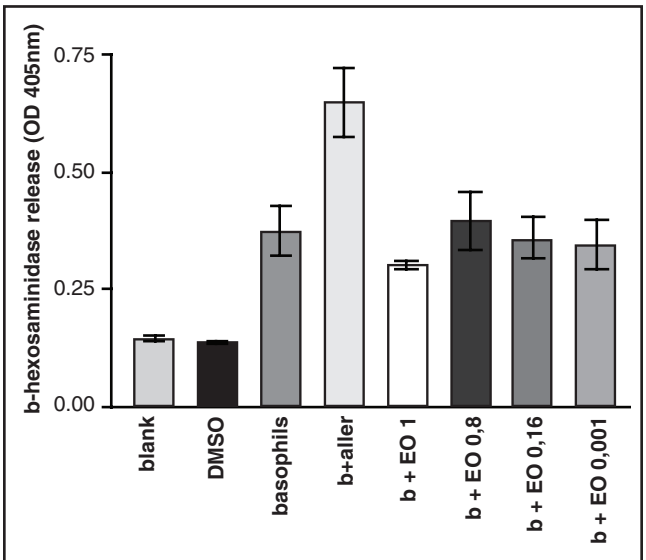

Figure 2. b-Hexosaminidase released by basophils (b) from allergic patients (n=30); the basophils were incubated with different concentrations (mg/mL) of the oil (EO) without specific allergen (aller) and a significant liberation of the enzyme was not observed; the blank well contained RPMI-1640 alone; cells without the addition of allergen and cells with addition of dimethylsulfoxide (DMSO) were evaluated as controls

When performing the β-hexosaminidase release assay using the different concentrations of the oil without the allergen, we observed no significant enzyme release in any case by the vegetal derivate themselves (Figure 2).

The expansion of lymphocytes in cultures stimulated with the vegetal fraction (1; 0.8 and 0.16 mg/mL) showed proliferation indexes comparable with those stimulated with the conventional mitogens PHA or ConA (p=ns). Greater doses to 1 mg/mL were toxic for the cells which showed to minors indices of proliferation and morphologic alterations. Smaller doses to 0.16 mg/mL were stimulating weak of the cellular expansion (Figure 3). Indirect IF technique revealed that 40% of the cells present in lymphocyte cultures stimulated by the oil derived from *M. verticillata* were CD8 (+) T cells.

The IFN-γ level in allergic patients' sera was very low (Figure 4 A). The values of this cytokine in serum of healthy individuals were also low (Figure 4 B). In allergic patients, the oil (1 and 0.8 mg/mL) did not increase the values of IFN-γ compared with the cultures without stimulus (p=ns). On the contrary, the oil (0.16 mg/mL) showed to significant difference respect to the cultures without stimulus ($p < 0.05$) (Figure 4 A). In healthy individuals the oil (0.8 and 0.16 mg/mL) increased the levels of the cytokine compared with the cultures without stimulus ($p < 0.05$) (Figure 4 B).

Zygadlo et al. (14) 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. In the present study, the essential oil from *M. verticillata* was composed mainly of mono- and sesquiterpenes; its main constituents (identified by GC) being pulegone, menthone, α-pinene, β-pinene, limonene and 1,8-cineole. Our results agree those of De Feo et al. (1) and González Pereyra et al. (24) who studied *M.verticillata* oil and reported the composition. This suggests that some of these components could be responsible for the biological activity of the oil.

Histamine, which is released from mast cells and basophils when they are stimulated by an antigen or degranulation inductor, is generally used *in vitro* as a degranulation marker in immediate type allergic reaction assays. β-Hexosaminidase is an acid hydrolase present in granules in the interior of basophils and mast cells and is released along with histamine when these cells are activated, as described Schwartz et al. (17). Therefore, β-hexosaminidase is accepted as a degranulation marker (25).

In the present study we have demonstrated that the oil derived from *M. verticillata* has inhibitory ability over the degranulation process in human basophils, inhibiting the

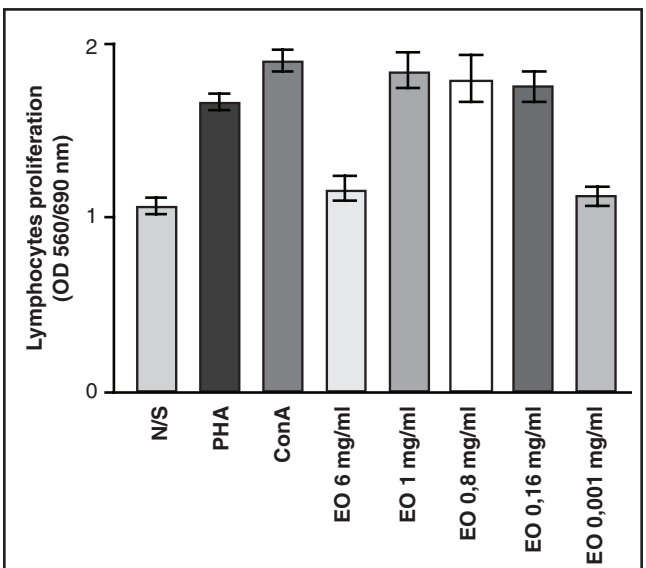

Figure 3. Lymphocyte proliferation from allergic patients (n=30); mononuclear cells were cultivated in four conditions: non stimulated (N/S), stimulated with phytohemagglutinin-M (PHA), concanavalina A (Con A) or different concentrations (mg/mL) of the oil (EO)

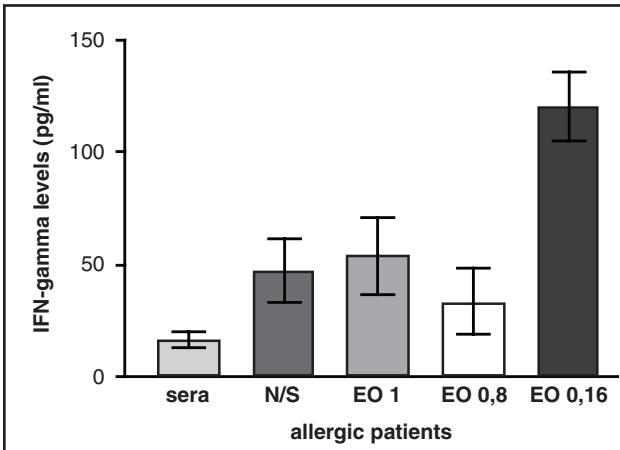

Figure 4 A. The IFN-g levels from allergic patients (n=30) present in sera and lymphocytes cultures supernatants none stimulated (N/S) and stimulated by different concentrations of the oil (EO)

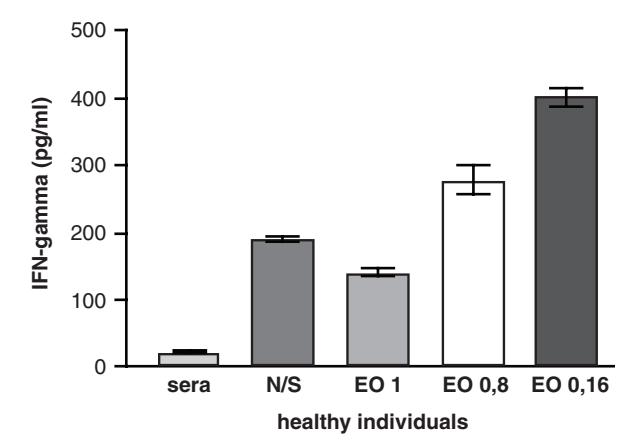

Figure 4 B. The IFN-g levels from healthy individuals (n=10) present in sera and lymphocytes cultures supernatants none stimulated (N/S) and stimulated by different concentrations of the oil (EO)

β-hexosaminidase release by 32.15% to 39.72%. These results are comparable with those of dexamethasone (39.75%) and theophylline (41.63%). Both drugs are still frequently used in the treatment of asthmatic disease. Dexamethasone, works by stimulating cAMP production, inhibiting the degranulation process in basophils and mast cells. It is a drug specifically prescribed for bronchial asthma attacks (11). Theophylline is a xanthine that acts as an inhibitor of the phosphodiesterase enzyme. Phosphodiesterase inactivates cAMP forming icAMP, which favors the degranulation of basophils and mast cells. Theophylline increases cAMP and inhibits the degranulation process. This drug is often used in the treatment of bronchial asthma (26). Our results concur with the work of Morikawa et al. (25) who studied the inhibitory effect of two constituents isolated from the Japanese herb *Acer nikoense*, on β-hexosaminidase release in RBL-2H3 cells. Both constituents showed higher activity than two frequently used anti-allergic compounds. In contrast, Na et al. (19) evaluated the inhibitory ability of *Tongkyutang*, an oriental herb-based preparation, on β-hexosaminidase release in sensitized rat cells. In this study, 13.7% inhibition was obtained; this was not considered significant.

The oil of *M. verticillata* added to the basophils exerted on cellular membranes protective effects similar to those of dexamethasone or theophylline. This result indicates that the membrane permeability increase may be an essential trigger for the release of the β-hexosaminidase mediator from basophils. The terpenes from *M. verticillata* oil might act on the membrane affecting the prevention of the perturbation being induced by specific allergen. Numerous investigations exist that demonstrate the effects of the terpenes on the protection of cellular membranes. These substances also have regulating activity on the T cells, synthesis of cytokines and inhibition of the apoptosis processes (27).

When analyzing proliferated cells stimulated by the oil derived from *M. verticillata*, indirect IF technique revealed that the vegetal derivates modulated the Th1 response, expanding the cytotoxic LT population. These results do not differ from those found by other authors who have tested similar products derived from different medicinal species as described by Ko et al. (28). In future studies, all cellular populations will be characterized in order to confirm this observation.

In most cases, we found that IFN-γ level in allergic patients' sera was not measurable what is normal because in these individuals, the lack of IL-12, produced by macrophages and NK cells, may have stimulated mast cells, basophils and LTh2 to produce IL-4, inhibiting IFN-γ synthesis as described by Janeway et al. (29). In healthy individuals the values of the cytokine were also low. These data are consistent because it has been demonstrated, except during an infection by intracellular bacteria or active viral infection that is very difficult to find IFN-γ levels significant as described by Moura et al. (30). The results obtained in this test suggest that essential oil, to have possible *in vivo* application, would harness the associate regulations physiological to IFN-γ such as: diminution of the allergic picture, increase of the capacity anti-viral, increase of the potential anti-cancer, etc. A therapy using non-toxic vegetal derivate like the one evaluated in this study - able to stimulate IFN-γ synthesis - would have a favorable application for the treatment of allergic disease in the future. It is worth the trouble to clarify that this is the first work in which the anti-allergic properties of the oil of *M. verticillata* have studied.

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