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Pterodon polygalaeflorus Essential Oil Modulates Acute Inflammation and B and T Lymphocyte Activation

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Abstract: The increased life expectancy of the population has led to increasing incidences of cancer, chronic inflammatory and autoimmune diseases. Thus the continuous search for new drugs is necessary because ineffectiveness and adverse effects have been described for standard drugs. Essential oils are important sources of bioactive metabolites and several clinical trials have been developed using them. The Pterodon genus has been used in traditional medicine to treat rheumatic disorders, thus this work investigated the properties of essential oil from *Pterodon polygalaeflorus* fruits (EsOPpg) on acute inflammation and lymphocyte activation. The essential oil was obtained by hydrodistillation and its components were identified by GC/MS. The anti-inflammatory response was assessed using the air pouch model. Antinociceptive potential was evaluated using the writhing model. Lymphocyte phenotyping, cell cycle and apoptosis were analyzed by flow cytometry. EsOPpg promoted a reduction in leukocyte counts and protein concentration in the exudate, and reduced vasodilatation and inflammatory cell infiltrate in air pouch tissue. No antinociceptive effect was demonstrated for the doses tested. EsOPpg inhibited lymphocyte proliferation, arresting the cell cycle in G_1 phase, and induced apoptosis in these cells. EsOPpg downregulated both the total number of $CD8^+$ T cells and the activated subpopulation ($CD8^+CD69^+$), while promoting upregulation of the total number of $CD19^+$ and $CD19^+CD69^+$ B cells. In conclusion, *Pterodon polygalaeflorus* essential oil diminished the acute inflammatory response and inhibited lymphocyte proliferation, reducing neutrophil

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recruitment into the cavity and air pouch tissue and promoting distinct modulations of the activation level of each lymphocyte subpopulation.

Keywords: *Pterodon polygalaeflorus*; Medicinal Plant; Essential Oil; Inflammation; Lymphocyte.

Introduction

Although there is great interest in synthetic techniques such as combinatorial chemistry, natural product prospection is a continuous source of new drugs (Newman *et al.*, 2003). An essential oil is extracted from vegetal material by hydrodistillation. Its compounds are volatile, aromatic and considered secondary metabolites of the plant (Sánchez *et al.*, 2004; Tajkarimi *et al.*, 2010). Most plant essential oil constituents belong to the terpenoid class of compounds, including monoterpenes, sesquiterpenes, and their oxygenated derivatives (Chao *et al.*, 2005). These compounds are found abundantly in fruits, vegetables, aromatic and medicinal plants, exhibiting, in plants, a protective function against infections, parasites and other stress conditions (Bakkali *et al.*, 2008). Essential oils or their components are used in perfumes and make-up products, sanitary products, dentistry, agriculture, as food preservers, additives, and natural remedies (Perry *et al.*, 2003). Clinical trials with essential oils to treat anxiety and depression (Conrad *et al*., 2012), denture stomatitis (Sabzghabaee *et al.*, 2012), pain (Sasannejad *et al.*, 2012), wound healing (Vakilian *et al.*, 2011) and fungal (Mahboubi and Ghazian Bidgoli, 2010) and bacterial infections (Sienkiewicz *et al.*, 2012) have been performed.

Inflammation is the primary response of an organism against injury or infection and is characterized by vasodilatation, redness, exudation, pain and neutrophil migration to eradicate the agent and potentiate tissue repair. However, excessive or inappropriate inflammation can lead to tissue damage and chronic inflammation (Sherwood and Toliver-Kinsky, 2004). $CD4^+$ and $CD8^+$ T lymphocytes have an important function in the adaptive immune response and altered responses of these cells are observed in immunodeficiency syndrome (Atlan *et al.*, 1994) and autoimmune pathologies (Rodrigues *et al.*, 2009). Acquired immunodeficiency syndrome (AIDS) is characterized by suppressed lymphocyte proliferation and function (Atlan *et al.*, 1994), while rheumatoid arthritis, an autoimmune disease, is accompanied by the exacerbated role of these cells (Rodrigues *et al.*, 2009).

Studies of pharmacological properties have shown that the essential oil of *Casearia sylvestris* leaves reduces carrageenan-induced paw inflammation (Esteves *et al.*, 2005), while those of *Lippia gracilis* (Guilhon *et al.*, 2011) and *Hyptis pectinata* (Raymundo *et al.*, 2011) induce both anti-inflammatory and analgesic action. Altered responses of lymphocytes have also been described. The main components of *Minthostachys verticillata* essential oil: pulegone, menthone and limonene, have been shown to modulate the immediate-type hypersensitivity response, stimulating lymphocyte proliferation and inhibiting mast cell activation and degranulation (Cariddi *et al.*, 2011). *Melaleuca alternifolia* essential oil inhibited the proliferation and interleukin-2 secretion by mononuclear cells and increased the secretion of anti-inflammatory cytokines interleukin-4 and interleukin-10 (Caldefie-Chézet *et al.*, 2006).

The species *Pterodon polygalaeflorus* Benth. (Leguminosae/Fabacea) is a native tree, 8 to 18 m in height, found in the central region of Brazil, popularly known as "sucupira branca." The hydroalcoholic extract from its fruit is traditionally used in folk medicine to treat rheumatoid arthritis, cough and some anti-inflammatory disorders (Cruz, 1965). Some linear diterpenoid compounds, including geranylgeraniol and its derivatives (dos Santos Filho *et al.*, 1972), and the cyclic diterpenoid vouacapan and its derivatives (Fascio *et al.*, 1970, 1976; Arriaga *et al.*, 2000) have been described in *Pterodon sp.* extract, showing anti-inflammatory (Silva *et al.*, 2004), antinociceptive (Coelho *et al.*, 2005) and antiarthritic properties (Coelho *et al.*, 2004). This work investigated the properties of the essential oil from *Pterodon polygalaeflorus* fruits (EsOPpg) on the air pouch acute inflammatory response and lymphocyte proliferation and activation.

Material and Methods

Chemicals

Acetylsalicylic acid (ASA) was acquired from Merck, Brazil. Carrageenan λ, concanavalin A (ConA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), ribonuclease A, dimethyl sulfoxide (DMSO), indomethacin, propidium iodide (PI), RPMI 1640 medium, sodium dodecyl sulfate (SDS), Tween 20, were purchased from Sigma Chemical Co., St Louis, MO, USA and Fetal bovine serum (FBS) was from Vitrocell, Brazil. Annexin V-FITC apoptosis kit, Fluorescein (FITC)-conjugated anti-CD8α (clone 53.6.7), R-phycoerythrin (PE)-conjugated anti-CD69 (clone H1.2F3), cyanine dye (Cy7*)-conjugated anti-CD4 (clone H129.19), Fluorescein (FITC)-conjugated anti-CD19 (clone 1D3), were purchased from Beckton-Dickinson Biosciences, CA. USA. All other chemicals and reagents used were high-grade purity.

Plant Material and Essential Oil Extraction

The taxonomic identity of *Pterodon polygalaeflorus* fruits was confirmed by Haroldo Cavalcante de Lima, from the Department of Systematic Botany at the Botanic Gardens of the State of Rio de Janeiro, Brazil, where a voucher specimen has been deposited (RB 350278, July 1999). The fruits (100 g) were submitted to hydrodistillation three times for 2 h each, in a modified Clevenger-type apparatus, for essential oil extraction. The relative composition of the three extractions were determined by GC/MS and since they were identical, the extractions were mixed together and dried over anhydrous sodium sulphate, yielding 5.1% (w/w) of EsOPpg, which was stored in closed dark vials at 4°C for further analysis.

GC/MS Analysis

Qualitative analyses were conducted in a GC/MS-QP2010 PLUS Shimadzu with a ZB-5MS fused silica capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm film thickness).

The operating temperatures used were as follows: injector 260°C, detector 290°C and column oven 60°C up to 290°C (3°C/min). Hydrogen at 1.0 ml/min was used as a carrier gas. The essential oil components were identified by comparing the compounds elution order with their relative retention indices reported in the literature (Adams, 2001), and the mass spectra (MS) to published data and computer matching with WILEY 275 and the National Institute of Standards and Technology (NIST 3.0) libraries, provided by a computer-controlled GC/MS system. The retention indices were calculated for all the volatile constituents using the retention data of linear *n*-alkanes C_9–C_{30}.

Animals

Male Swiss Webster (SW) mice, weighing 25 to 35 g (b.w.) were maintained at 25°C under a 12-h light-dark cycle and provided access to water and food *ad libitum*. For each experiment, the mice were randomly selected into groups comprising five per cage. Studies were conducted in accordance with current guidelines for the care of laboratory animals and ethical guidelines for the investigation of experimental pain in conscious animals (Zimmermann, 1983) and under the consent and surveillance of the Ethics Committee for Animal Research of the Institute of Biology of the State University of Rio de Janeiro (CEA-IBRAG committee/protocol 05/2009).

Air Pouch Model

The pouch was created by a sterile air injection of 5 ml in the mouse's back and maintained by 3 ml injection of sterile air three days later. On day 6 post-injection, 1 h before the administration of 1.0% carrageenan (1 ml into the cavity), different doses (0.02 and 0.2 mg/kg) of EsOPpg (100 μl), prepared with the vehicle (ethanol 15% 1.25% Tween-20) were administered orally. One group received vehicle alone (control) and another was treated with the control drug indomethacin (10 mg/kg b.w.). Four hours after carrageenan injection, the mice were killed in a CO_2 chamber, their cavities were exposed, the exudates were collected and the volumes were measured, and the total number of leukocytes was determined using a hemocytometer. The air pouch tissues were removed, photographed and processed for histological analysis.

Acetic Acid-Induced Abdominal Constriction

Groups of mice were separated and food and water was removed 1 h before treatment. The mice were pretreated orally (p.o.) with different doses (1.5 ml/kg) of EsOPpg (0.1–5.0 mg/kg b.w.) 60 min before acetic acid injection. The abdominal constrictions induced by intraperitoneal (i.p.) injection of 0.6% acetic acid (10 ml/kg) were conducted according to Koster *et al.* (1959). For positive control of antinociception, a group of mice was treated with acetylsalicylic acid at 100 mg/kg b.w., p.o. (ASA group), 30 min before the noxious stimulus. The nociception control group received an equal volume of vehicle (ethanol 15% with 1.25% Tween 20). Control experiments showed no altered response due to oral

administration of the vehicle at the concentration used. Following the challenge, pairs of mice were separated in boxes and the number of constrictions was noted for 10 min, beginning 5 min after acetic acid injection (Loro *et al.*, 1999). Antinociceptive activity was detected as a reduction in the number of abdominal constrictions exhibited by treated mice compared with the nociception control group and was expressed as the percentage of pain inhibition.

In Vitro Biological Assays

The spleen cells were dissociated by pressing the tissue through a 200 mesh stainless steel grid, with aid of a rubber policeman, in RPMI 1640 medium supplemented with 5% FBS, glutamine 2 mM, β-mercaptoethanol 0.05 mM, penicillin (70 mg/l) and streptomycin (100 mg/l). The erythrocytes were lysed by treatment with cold hypotonic ACK buffer (NH_4Cl 0.15 M, $KHCO_3$ 10 mM, EDTA.Na_2 2 mM). The remaining cells were washed twice in 5 ml of RPMI 1640 medium supplemented with EDTA 1 mM, suspended in supplemented RPMI 1640, and counted by trypan blue exclusion. Cells were cultured for 72 h at 37°C with 5% CO_2, at 2×10^6/ml, with or without ConA 5 μg/ml, in the presence or absence of different EsOPpg concentrations. EsOPpg was stored in DMSO and then diluted with supplemented RPMI 1640 medium at different final concentrations. Control cultures received only 0.01% DMSO (Maximal DMSO concentration).

Cytotoxicity Assay

Cells were cultured in triplicate (final volume 100 μl), as described in the previous section, for 72 h in 96 flat-bottom-well plates (Becton Dickinson Labware, EUA), in the presence of ConA and EsOPpg or cyclosporine 5 μg/ml (final volume of 100 μl). Thereafter, cell survival was determined by incubation with 10 μl/well of MTT reagent for 2 h, according to Mossman (1983). The formazan crystal were dissolved adding 100 μl/well of 10% SDS solution with 0.01 N HCl and the 570 nm absorbance and viability determined (microplate reader μQuant, Bio-Tek Instruments, Inc.).

Cell Cycle Analysis

Cells were cultured for 72 h in the presence of ConA, with or without EsOPpg (100 μg/ml) or cyclosporine (5 μg/ml). Afterwards, the number of viable cells was determined by trypan blue exclusion to evaluate cell proliferation, and for cell cycle analysis, 1×10^6 cells were centrifuged (400 g, 5 min), suspended in 500 μl DNA staining solution (0.3% Triton X-100 and 50 μg/ml propidium iodide (PI) in 43 mM sodium citrate buffer pH 8.2) and maintained for 15 min at room temperature in the dark. Next, the samples were treated with 500 μl ribonuclease A (100 μg/ml, in 43 mM sodium citrate buffer pH 8.2) for 15 min at room temperature and PI fluorescence was measured in the Gallios flow cytometer (Beckmann Coulter, USA). Fifty thousand events per sample were acquired. The emission wavelength of PI fluorescence was 585 ± 15 nm (Dalmau *et al.*, 1999). Data were analyzed by the Summit v4.3 software. Hypodiploid nuclei and debris were discarded from the analysis.

Annexin V$^+$-FITC Apoptosis

Cells were cultured for 72 h in the presence of ConA, with or without EsOPpg (100 μg/ml) or cyclosporine (5 μg/ml). Phosphatidylserine (PS) exposure on the external layer of the plasmatic membrane, characteristic of apoptotic cells, was determined by Annexin-V-PI labeling, in accordance with the manufacturer's recommendations (Annexin V-FITC apoptosis kit). Briefly, cells (1×10^6) were washed twice with cold PBS and suspended in 1 ml binding buffer. Cells (1×10^5 cells, 100 μl) were treated with Annexin V (5 μl) and 50 μg/ml PI solution (10 μl) for 15 min at room temperature. Following the addition of 400 μl of binding buffer, FITC fluorescence was determined (5×10^4 events) at 535 ± 15 nm and PI at 585 ± 15 nm in a Gallios flow cytometer (Beckmann Coulter, USA). Normal size cells were analyzed using Summit v4.3 software. Annexin V$^+$PI$^-$ cells were considered as early apoptotic cells and Annexin V$^+$PI$^+$ as late apoptotic cells, while Annexin V$^-$/PI$^-$ and Annexin V$^-$/PI$^+$ were considered to be viable and necrotic cells, respectively.

Labeling of Cell Surface Antigens by Flow Cytometry

Following cell culture with ConA and EsOPpg (100 μg/ml) or cyclosporine (5 μg/ml) for 72 h, surface staining of 1×10^6 cells was conducted on melting ice, in the dark. Cells were incubated for 30 min with saturated concentration of Cy-chrome-conjugated anti-CD4, fluorescein (FITC)-conjugated anti-CD8α and R-phycoerythrin(PE)-conjugated anti-CD69 for triple labeling, and with R-phycoerythrin(PE)-conjugated anti-CD69 and fluorescein (FITC)-conjugated anti-CD19 for double labeling, in a 200 μl final volume of RPMI 1640 diluted twice with PBS containing 5% FCS. Then, cells were washed with 1 ml of PBS (1000 g, 3 min, 8°C), suspended in 500 μl of PBS and fluorescence was detected in a Gallius flow cytometer (Becton Dickinson Biosciences) using the 530 ± 15 nm (for FITC), 585 ± 15 nm (for PE) and 650 ± 15 nm (for Tricolor) band pass filters. A hundred events were captured per sample. The fluorescence overlap compensation was obtained from single antibody labeling. Viable cells were analyzed using the Summit v4.3 software.

Statistical Analysis

The variance between groups was evaluated by one-way ANOVA and the significance of difference determined by Dunnett's test or the Student's *t*-test, as appropriated, using the GraphPad Prism 5.0 software. Differences were considered significant when $p < 0.05$.

Results

Phytochemistry

The EsOPpg components (95.39%) were identified (Table 1) by comparing their retention rates and mass spectra (MS) with published data, and the WILEY program 275 + NIST

Table 1. Identified Compounds from *P. polygalaeflorus* Essential Oil.

	Compounds	[a]RI[Lit]	[b]RI	HD%	Identification
1	α-copaene	1377	1373	1.89	RI, GCMS
2	β-elemene	1391	1388	28.04	RI, GCMS
3	β-caryophyllene	1419	1417	34.95	RI, GCMS
4	α-humulene	1455	1452	4.20	RI, GCMS
5	alloaromadendrene	1460	1455	0.90	RI, GCMS
6	germacrene D	1485	1477	6.93	RI, GCMS
7	eudesmadiene	1490	1484	0.90	RI, GCMS
8	bicyclogermacrene	1500	1490	3.41	RI, GCMS
9	germacrene A	1509	1501	4.66	RI, GCMS
10	spathulenol	1578	1572	2.11	RI, GCMS
11	caryophyllene oxide	1583	1576	6.37	RI, GCMS
12	farnesol	1718	1712	0.71	RI, GCMS
13	farnesyl acetate	1834	1834	0.32	RI, GCMS

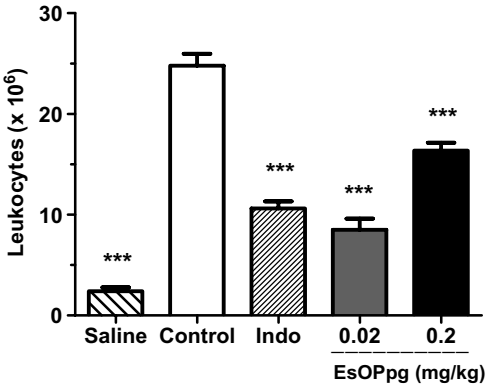
Note: % Peak Sum of Identified Compounds: 95,39; [a]RI[Lit]: Literature Retention Indexes (Adams, 2001); [b]RI: Experimental Retention Indices; HD: Hydrodistillation.

3.0. Beta-caryophyllene (34.95%) and β-elemene (28.04%) were verified as major compounds of EsOPpg. Six compounds were present in medium intensity: germacrene D (6.93%), caryophyllene oxide (6.37%), germacrene A (4.66%), α-humulene (4.20%), bicyclogermacrene (3.41%) and spathulenol (2.11%); and five compounds at low concentrations (α-copaene, 1.89%; farnesol, 0.71%; and farnesyl acetate, 0.32%).

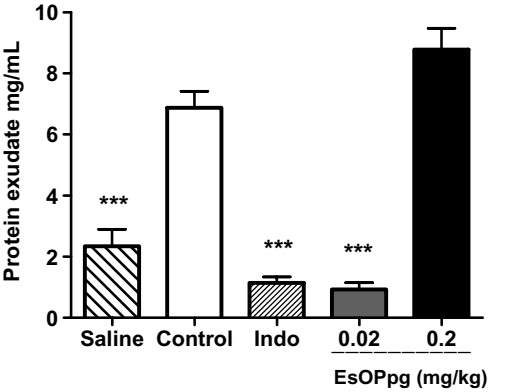
Effect of the EsOPpg on Carrageenan-Induced Air Pouch

Carrageenan induced significant infiltration of leukocytes into the cavity (Fig. 1A) and increased protein exudation (Fig. 1B) in the control group compared with the saline group. EsOPpg at 0.02 and 0.2 mg/kg promoted a decrease in the total leukocytes counts ($p \leq 0.001$) compared with the control group, after 4 h of inflammation induction, reducing it by 65.8% and 33.9%, respectively (Fig. 1A). A significant decrease (86.6%) in protein exudates was also observed following treatment with EsOPpg but only at the lowest dose (0.02 mg/kg) (Fig. 1B). The standard anti-inflammatory drug indomethacin inhibited the total number of leukocytes (57.2%) and protein exudation (83.5%), similar to a 0.02 mg/kg dose of EsOPpg.

Macroscopic analysis of the air pouch tissue of the control (vehicle) group (Fig. 2B) shows morphological signs of acute inflammatory response, such as redness and vasodilatation, compared with the saline group (Fig. 2A). The indomethacin and EsOPpg (Figs. 2C, 2D and 2E) reduced redness and vasodilatation. The inflammatory response in the pouch tissues was also examined histologically. The pouch internal wall usually consists of connective tissue with some adipocytes. The infiltration of numerous inflammatory cells, such as neutrophils, macrophages and lymphocytes, was observed in the pouch wall of the vehicle group (Figs. 3C and 3D), together with tissue edema that contributing to the enlargement of the wall compared with the untreated mice of the saline group (Figs. 3A



(A)



(B)

Figure 1. Effects of EsOPpg on the air pouch exudate. (A) Total leukocyte count. (B) Protein level. SW mice ($n = 5$/group) were treated (p.o.) with vehicle (Control), indomethacin (Indo, 10 mg/kg) or EsOPpg (0.02 or 0.2 mg/kg b.w.), 1 h before carrageenan injection into the pouch cavity. The saline group received only saline in the cavity. The results express the mean ± S.D. of three experiments. ***$p < 0.001$ compared with the control group (one-way ANOVA followed by Dunnett's test).

and 3B). In contrast, the pouch wall of the EsOPpg-treated mice, at doses 0.02 mg/kg (Figs. 3G and 3H) and 0.2 mg/kg (Figs. 3I and 3J), and of indomethacin-treated mice (Figs. 3E and 3F), consisted of thinner connective tissue than the vehicle group and displayed minimal evidence of inflammatory response, with predominance of resident cells (fibroblast), rather than leukocytes. These histological findings suggest that acute inflammatory response in the pouch wall was suppressed by EsOPpg treatment.

Effect of EsOPpg on Acetic Acid-Induced Abdominal Constrictions

The writhing model used to evaluate the antinociceptive potential of EsOPpg showed no significant antinociceptive effect at the doses tested (0.1, 1 and 5 mg/kg, maximal

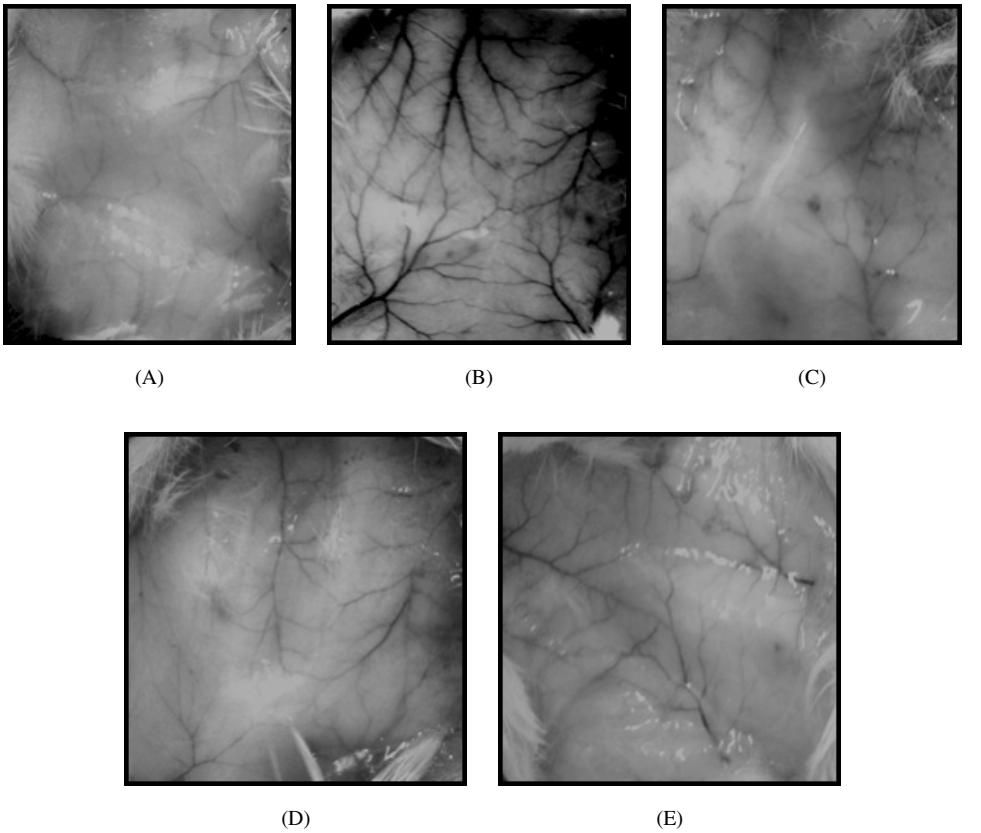


Figure 2. Effects of EsOPpg on macroscopic aspects of the air pouch tissue. The saline group (A) was injected only with saline instead of carrageenan into the cavity. Inflammation was induced by carrageenan injection into the cavity. The mice were treated p.o. with (B) vehicle (Control), (C) Indomethacin (10 mg/kg) or (D) EsOPpg 0.02 mg/kg b.w. and (E) EsOPpg 0.2 mg/kg b.w. 1 h before carrageenan injection ($n = 5$/group). Representative photographs from three experiments.

inhibition of 11.9%, data not shown). The mean number of abdominal constrictions in the control group was 47.06 ± 6.70. ASA (100 mg/kg) significantly ($n =$ ten/group, ANOVA followed by Dunnett's test) inhibited (43%) the number of constrictions compared with the control group.

Effect of EsOPpg on Spleen Lymphocyte Function

Having verified the anti-inflammatory effects of EsOPpg, its antiproliferative action was investigated. ConA-stimulated lymphocytes treated with EsOPpg showed a concentration dependent inhibition of MRA, with reductions of 37.7%, 42.5% and 86.2% ($p < 0.001$) when treated with 50, 70 and 100 μg/ml, respectively (Fig. 4A). The EsOPpg also reduced ($p < 0.01$) the total number of viable cells (Fig. 4B) following the incubation of

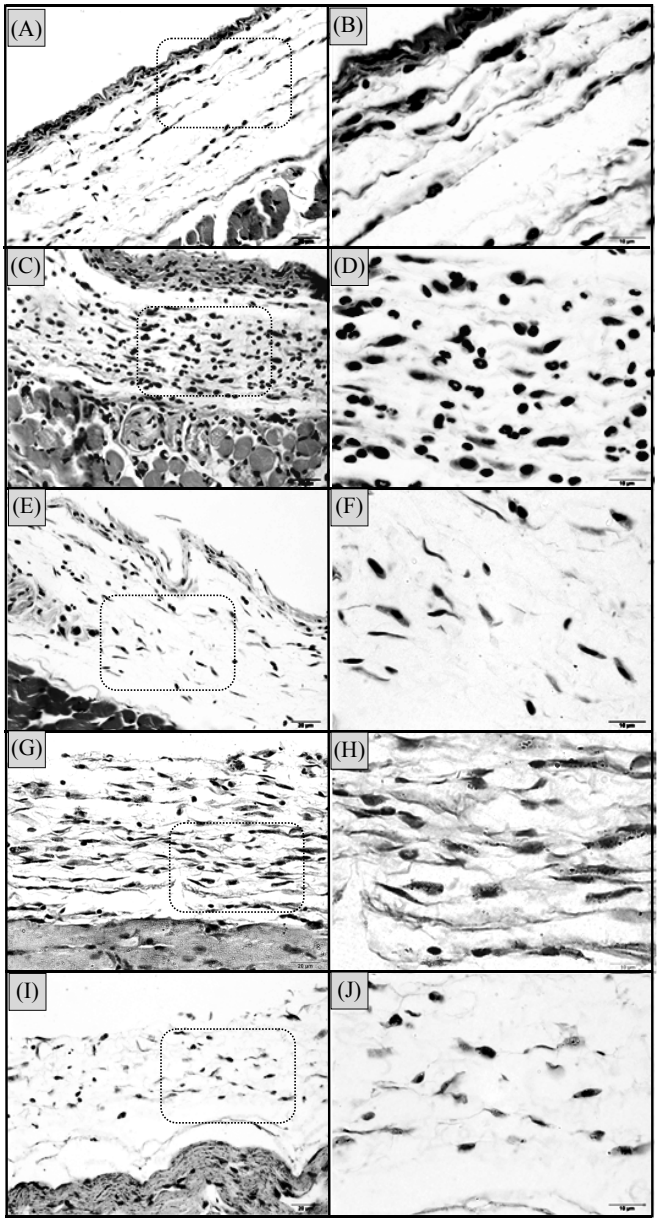
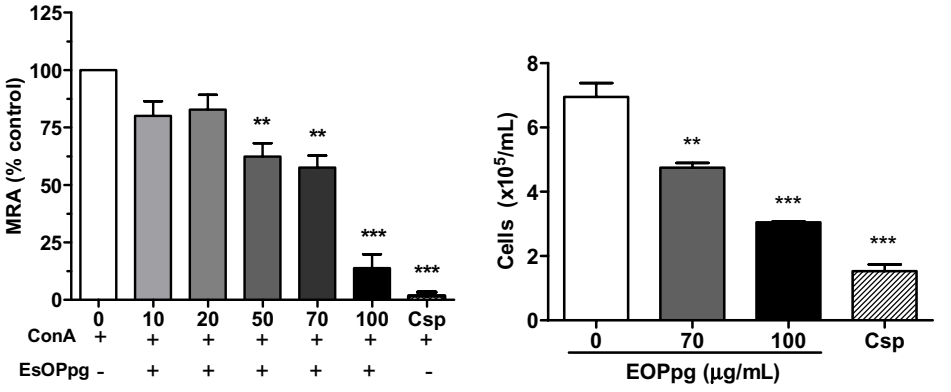


Figure 3. Histological examination of EsOPpg effects on the air pouch tissues. The saline group (A) was injected only with saline into the cavity. Inflammation was induced by carrageenan injection into the cavity. The mice ($n = 5$/group) were treated p.o. 1 h before carrageenan injection with (C, D) vehicle (Control); (E, F) Indomethacin (10 mg/kg); (G, H) EsOPpg 0.02 mg/kg; or (I, J) EsOPpg 0.2 mg/kg. Tissue sections were stained with hematoxylin/eosin and photographed under light microscopy. Inflamed pouch tissue shows intensive inflammatory infiltrate, best observed at the highest magnification. Treatment with EsOPpg and indomethacin reduced the infiltration of leukocytes. A, C, E, G and I (40× magnification). B, D, F, H and J (100× magnification). The amplified regions were delimited in the left column images.



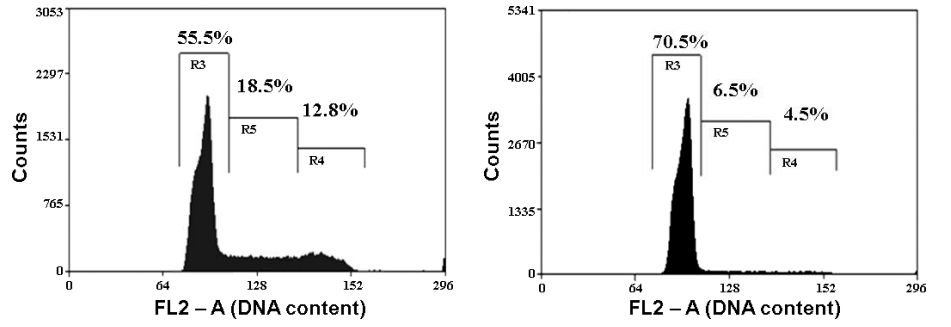
(A) (B)

Figure 4. Effect of EsOPpg on lymphocyte function. (A) Mitochondrial reduction activity (MRA). (B) Cell proliferation. Cells (2×10^6/ml) were cultured for 72 h with ConA (5 μg/ml), in the absence or presence of different EsOPpg concentrations or cyclosporine (5 μg/ml). MRA was determined by MTT assay and cell proliferation by trypan blue dye exclusion. Data represent mean $\pm$ S.D. of three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$, compared with cells cultured only with ConA (Student's *t*-test).

lymphocytes with ConA and EsOPpg at 70 or 100 μg/ml for 72 h. Cyclosporine reduced both lymphocyte MRA and proliferation.

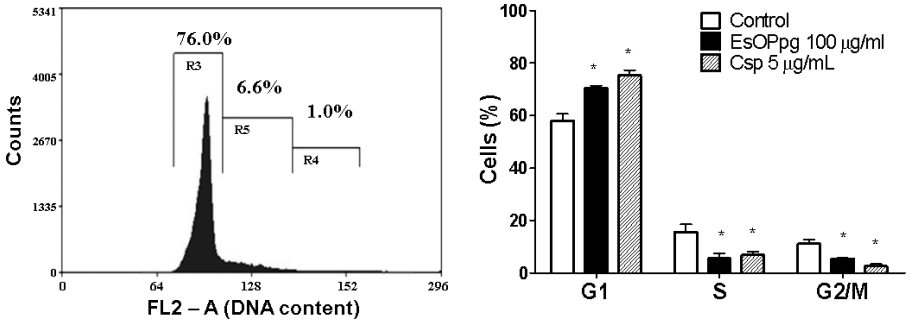
Effect of EsOPpg on Lymphocyte Cell Cycle

The effects of EsOPpg (70 μg/ml) on the cell cycle of spleen lymphocytes were also examined. Representative analysis of DNA content of a control culture (Fig. 5A) and EsOPpg (Fig. 5B) and cyclosporine (Fig. 5C) treated cultures showed an increase



(A) (B)

Figure 5. EsOPpg effects on lymphocyte cell cycle by flow cytometry. Representative results of ConA stimulated cells (%) in each phase of the cell cycle, after culture for 72 h without treatment (A) (control) or treated with (B) EsOPpg 100 μg/ml or cyclosporine 5 μg/ml (C). D. Mean data (mean $\pm$ S.E.M.) of three independent experiments. Cells were processed as described in "Material and Methods" and DNA content determined by PI fluorescence of stained nucleus, detected in the FL2 channel (FL2-A). $^{*}p < 0.05$ compared with control culture (Student's *t*-test).



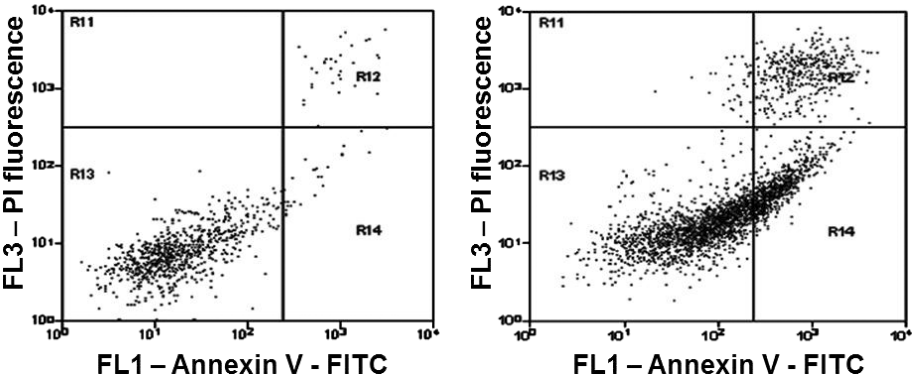
(C) (D)

Figure 5. (*Continued*)

($p < 0.05$) in cell numbers during the G_1 phase and decreases ($p < 0.05$) in S and G_2 phases in cells treated with EsOPpg, compared with the control culture. These results were confirmed by mean data (three independent experiments; Fig. 5D), suggesting that the EsOPpg arrests the lymphocyte cell cycle in G_1 phase, as demonstrated for cyclosporine.

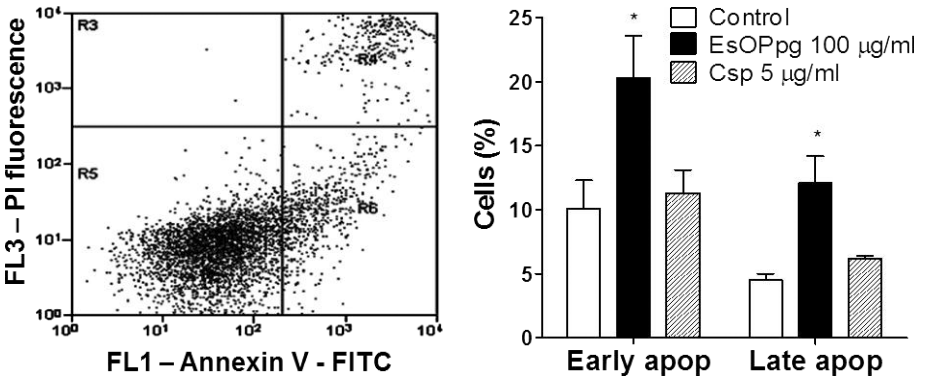
Effect of EsOPpg on Lymphocyte Apoptosis

Since the greatest MRA inhibition index was observed with 100 μg/ml of EsOPpg, this concentration was used to compare cell death susceptibility among control and EsOPpg-treated



(A) (B)

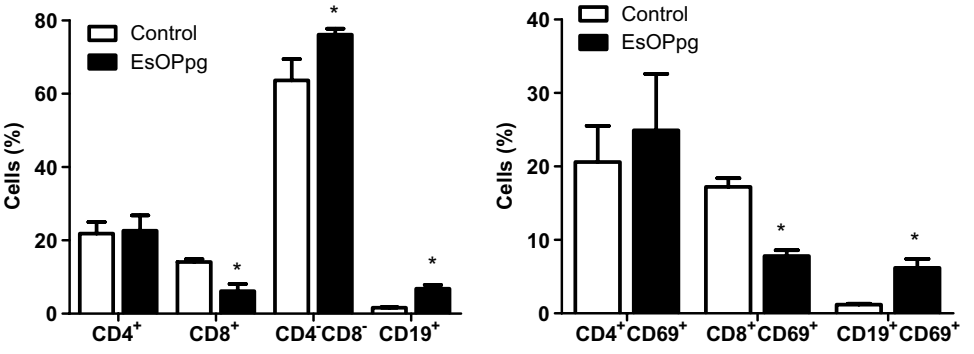
Figure 6. EsOPpg effects on lymphocyte apoptosis by flow cytometry. Annexin V-FITC/PI double staining representative cytograms of ConA stimulated lymphocytes for 72 h in the absence (A) or presence of (B) EsOPpg 100 μg/ml or (C) cyclosporine 5 μg/ml. (D) Mean data (mean ± S.E.M.) of early and late apoptotic cells following treatment of ConA-stimulated cells with or without EsOPpg 100 μg/ml from three independent experiments. Cells (2×10^6/ml) were processed as described in "Material and Methods." In the (A), (B) and (C) panels, the lower left quadrant contains the viable cells (Annexin V^-/PI^-), the upper left (Annexin V^-/PI^+) contains necrotic cells, the lower right quadrant (Annexin V^+/PI^-) contains early apoptotic cells and the upper right (Annexin V^+/PI^+) contains the late apoptotic cells. *$p < 0.05$ compared with control culture (Student's *t*-test).



(C) (D)

Figure 6. (*Continued*)

cells. Representative flow cytometry analysis is illustrated in dot plot cytograms for the control (Fig. 6A) and EsOPpg- (Fig. 6B) and cyclosporine-treated cultures (Fig. 6C). Double negative cells (Annexin$^-$PI$^-$) were considered viable cells, Annexin$^-$PI$^+$ cells were considered necrotic cells, Annexin positive and PI negative cells were considered "Early" apoptotic cells and cells positive for both Annexin V and PI were considered "Late" apoptotic cells. The results indicated an increase in cell death by apoptosis following treatment with EsOPpg, showing increases in the number of early and late apoptotic cells, which were confirmed ($p < 0.05$) by mean data (three independent experiments; Fig. 5D). In Fig. 6C (cyclosporine treatment), the increase in the cell numbers in the upper and lower right quadrant (late and early apoptosis, respectively) was proportional to



(A) (B)

Figure 7. EsOPpg effects on surface antigens of stimulated lymphocytes. (A) Counts of CD4$^+$, CD8$^+$, CD19$^+$ and CD4$^-$/CD8$^-$ cell subpopulations (%). (B) Counts of CD4$^+$/CD69$^+$, CD8$^+$/CD69$^+$ and CD19$^+$/CD69$^+$ cell subpopulations (%). Cells (2×10^6/ml) were stimulated with ConA in the absence (control) or presence of EsOPpg 100 μ/ml for 72 h. Cells were processed as described in "Material and Methods." Data represent mean $\pm$ S.E.M. of three independent experiments. *$p < 0.05$ compared with control culture (Student's t test).

that of the viable cells (lower left quadrant), which indicates that apoptosis induced by cyclosporine was not significantly different from the control culture.

Effects of EsOPpg on the Activation of Lymphocyte Subpopulations (*CD4, CD8 and CD19*)

The *in vitro* effects of EsOPpg on T and B cells were evaluated. EsOPpg down-regulated the total $CD8^+$ T cell subpopulation but not $CD4^+$ T cells, and promoted an increase in the total double negative cells ($CD4^-CD8^-$) and total $CD19^+$ B cells (Fig. 7A). Similar results were verified when the early activation level of these cells was determined by CD69 expression. $CD8^+$T cell early activation ($CD8^+CD69^+$ cells) was reduced by treatment with EsOPpg while early activation of B cells ($CD19^+CD69^+$ cells) was up-regulated compared with control culture, with no change in $CD4^+$ T cells early activation level (Fig. 7B).

Discussion

The increasing use and clinical investigation of essential oils in recent years (Mahboubi and Ghazian Bidgoli, 2010; Vakilian *et al.*, 2011; Conrad *et al.*, 2012; Sabzghabaee *et al.*, 2012; Sasannejad *et al.*, 2012; Sienkiewicz *et al.*, 2012) has made the public interest in alternatives to conventional medicines evident. Essential oil constituents belong to the terpenoid class, which includes monoterpenes, sesquiterpenes and their oxygenated derivatives (Chao *et al.*, 2005), and these low molecular weight (most below 300 g/mol) compounds easily diffuse across cell membranes to induce biological reactions (Chao *et al.*, 2005). CG-MS analysis of EsOPpg identified more than 95% of its compounds, and verified that the sesquiterpenes β-caryophyllene (34.5%) and β-elemene (28.04%) were the main constituents. Anti-inflammatory and immunoregulatory properties have already been described for these substances (Gertsch *et al.*, 2008; Wu *et al.*, 2011; Zhang *et al.*, 2011; Zhao *et al.*, 2012).

This work demonstrated that the EsOPpg inhibited the acute inflammatory response and lymphocyte proliferation, inducing apoptosis in these cells. One of the mechanisms that support the host's primary defense system is the recruitment of leukocytes from blood vessels to the site of inflammation (Lau *et al.*, 2005). The carrageenan efficiently triggered the inflammatory response in the vehicle group of mice, accumulating a high number of leukocytes in the air pouch cavity, in agreement with the literature (Selye, 1953; Morikawa *et al.*, 2003). The EsOPpg significantly inhibited this cell accumulation, similar to that observed for essential oils of other species, like *Lippia gracilis* (Guilhon *et al.*, 2011) and *Hyptis pectinata* (Raymundo *et al.*, 2011). However, EsOPpg inhibited cell migration at much lower doses (0.2 mg/kg b.w.) than *Lippia gracilis* (10–100 mg/kg b.w.) and *Hyptis pectinata* (10–100 mg/kg b.w.).

The acute inflammatory response is characterized by important changes in the micro-circulation structure, allowing plasma protein extravasation into the interstitium in the form of inflammatory exudate (Francischetti *et al.*, 2010). Vasodilatation and protein exudation are well-characterized features in inflammation triggered by carrageenan (Sedgwick and

Lees, 1986), which was demonstrated here in the air pouch of the vehicle group. EsOPpg not only inhibited leukocyte migration into the cavity but also reduced vasodilatation and protein exudation, in contrast with *Casearia sylvestris* essential oil, which presented anti-inflammatory properties without effecting vascular permeability (Esteves *et al.*, 2005). The essential oil of *Cordia verbenaceae* reduced both cell migration and exudation in carrageenan-injected paw edema (Passos *et al.*, 2007), but at much higher doses (300–600 mg/kg b.w.) than EsOPpg (0.02 mg/kg b.w.).

Furthermore, this investigation indicated that EsOPpg modulates the immune cell response. EsOPpg inhibited lymphocyte proliferation to mitogen, as described for the essential oil of *Melaleuca alternifolia* (Caldefie-Chézet *et al.*, 2006), arresting their cell cycle in the G_1 phase. Apoptosis is also an important mechanism for controlling the immune response (Dunkle and He, 2011). Early apoptotic cells externalize phosphatidylserine in the plasma membrane and this phospholipid specifically binds to annexin V-FITC. Apoptotic cells are rapidly phagocytized by macrophages, avoiding damage to adjacent tissues and inflammation (Dunkle and He, 2011).

The spleen cells treated with EsOPpg showed a reduction in the number of $CD8^+$ T cells and an increase in $CD19^+$ B cells. The increase in these cells could be related to the enhancement of $CD4^-CD8^-$ cells compared with the control group. These results further suggest that $CD4^+$ T lymphocytes are likely to show greater resistance to any EsOPpg cytotoxic effects than $CD8^+$ T cells. Interestingly, different sensitivities among lymphocyte subpopulations have also been described for oregano essential oil (Bimczok *et al.*, 2008).

The CD69 surface marker is an early activation antigen of leukocyte, expressed within hours of mitogenic stimulation. Research has shown that there is a positive correlation between lymphocyte proliferation and CD69 expression. This membrane protein is involved in costimulatory signals in lymphocytes and modulates the expression of various cytokines (Nielsen *et al.*, 1998).

When cell activation was evaluated in each lymphocyte subpopulation, observation verified that EsOPpg reduced the activation level of $CD8^+$ T cells (reduced $CD8^+CD69^+$ cells) and increased it in B ($CD19^+$) cells (increased $CD19^+CD69^+$ cells). The reduction in the number of activated $CD8^+$ T cells and the increase in the percentage of activated B cells could be related to a shift from Th1 to Th2 type immune response (Crane and Forrester, 2005; Elenkov *et al.*, 2000). This specific deviation of immune response implies a reduction in the response mediated by Th1 lymphocytes and macrophages and enhancement of the response mediated by Th2 lymphocytes, B cells and eosinophils (Shoenborn and Wilson, 2007; Kaiko *et al.*, 2008). The number of $CD4^+$ T cells or $CD4^+CD69^+$ cells did not change significantly in this work. The supposed increase in Th2 cells could be compensated by the supposed reduction in Th1 cells, since both are CD4 positive cells and the number of these cells did not change. Immune response modulation has been reported for plant essential oils (González Pereyra *et al.*, 2005; Caldefie-Chézet *et al.*, 2006). *Minthostachys vertcillata* essential oil stimulated lymphocyte proliferation, increasing the $CD8^+$ T cell subpopulation by 40% (González Pereyra *et al.*, 2005), which the authors assumed could be related to Th1 deviation of the immune response. According to Caldefie-Chézet *et al.* (2006), *Melaleuca alternifolia* essential oil also inhibited

lymphocyte proliferation and altered lymphocyte function, suggesting Th1-Th2 immunomodulation. In addition, a decrease in $CD8^+$ T cell frequency, reduction in CD69 expression on $CD8^+$ T cells and inhibition of the immune-inflammatory response are findings previously reported in another investigation (Woodworth *et al.*, 2010).

In conclusion, the *Pterodon polygalaeflorus* essential oil diminished the acute inflammatory response and inhibited lymphocyte proliferation, reducing neutrophil recruitment into the cavity and air pouch tissue and promoting distinct modulations of the activation level of each lymphocyte subpopulation.

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