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Original Article

Essential oil from *Ageratum fastigiatum* reduces expression of the pro-inflammatory cytokine tumor necrosis factor-alpha in peripheral blood leukocytes subjected to *in vitro* stimulation with phorbol myristate acetate

Bethânia A. Avelar-Freitas[a,b,*], Valéria G. Almeida[a], Michaelle G. Santos[a], Josué A.T. Santos[a], Poliana R. Barroso[c], Cristiane F.F. Grael[c], Luiz E. Gregório[d], Etel Rocha-Vieira[a], Gustavo E.A. Brito-Melo[a]

[a] *Laboratório de Imunologia, Universidade Federal dos Vales do Jequitinhonha e Mucuri, Diamantina, MG, Brazil*
[b] *Laboratório de Biologia Celular Instituto de Ciência e Tecnologia, Universidade Federal dos Vales do Jequitinhonha e Mucuri, Diamantina, MG, Brazil*
[c] *Laboratório de Farmacognosia e Fitoquímica, Universidade Federal dos Vales do Jequitinhonha e Mucuri, Diamantina, MG, Brazil*
[d] *Laboratório de Insumos Naturais e Sintéticos, Universidade Federal de São Paulo, Diadema, SP, Brazil*

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ABSTRACT

Ageratum fastigiatum (Gardner) R.M. King & H. Rob., a member of the Asteraceae family popularly known in Brazil as "matapasto", is indicated in folk medicine as anti-inflammatory and analgesic. Despite its popular use, little is known about its potential effect on the parameters involved in an inflammatory response. The objective of this study was to characterize the chemical composition of the essential oil from *A. fastigiatum* and to evaluate the frequency of tumor necrosis factor alpha and interferon gamma producing cells in peripheral blood lymphocytes stimulated with phorbol myristate acetate in the presence of essential oil from *A. fastigiatum*. Non-toxic concentrations of essential oil from *A. fastigiatum* were evaluated in cultures of peripheral blood leucocytes using the trypan blue exclusion assay by flow cytometry. GC–MS analysis revealed that the prevalent compounds identified in the essential oil from *A. fastigiatum* sample were α-pinene, limonene, trans-caryophyllene, α-humulene, caryophyllene oxide, 1,2-humulene-epoxide, 1,6-humulanodien-3-ol, and α-cadinol. Results showed that exposure to essential oil from *A. fastigiatum* at concentrations of 0.5×10^{-2} and 1×10^{-2} μl/ml caused no alterations in leukocyte viability as compared to the control group. Both concentrations lowered the percentage of tumor necrosis factor alpha (+)-lymphocytes and neutrophils. There were no changes in the percentage of lymphocytes positive for the interferon gamma cytokine. Our results suggest that part of the anti-inflammatory activity attributed to *A. fastigiatum* may be due to the effect of some of its components in decreasing the number of cells that produce the pro-inflammatory cytokine tumor necrosis factor alpha.

Introduction

Ageratum fastigiatum (Gardner) R.M. King & H. Rob., Asteraceae, popularly known in Brazil as "matapasto" or "enxota", is indicated in folk medicine as anti-inflammatory and analgesic (Del-Vechio-Vieira et al., 2009a). Local healers prepare the extract from freshly chopped leaves and branches (with or without inflorescences), which are then subjected to maceration or decoction. Following filtration, the aqueous extract is applied topically to treat pain and inflammation (Gonçalves et al., 2011). The anti-inflammatory effect of the essential oil of *A. fastigiatum* (EOAF) was demonstrated *in vivo* by the reduction of both paw edema and leukocyte migration induced by carrageenan in an animal model (Del-Vechio-Vieira et al., 2009a). However, the mechanisms involved in the anti-inflammatory effect of the plant have yet to be elucidated; components of the essential oil may interfere with mediators of the immune response, such as inflammatory cytokines. Inflammation aims to fight infection and promote tissue repair, but the exaggerated inflammation causes harmful effects to the body, as occurs in some autoimmune diseases (Sedger and McDermott, 2014). Cytokines are released in response to various forms of cellular stress including inflammation. Among the

* Corresponding author.
E-mail: bethania.avelar@ict.ufvjm.edu.br (B.A. Avelar-Freitas).

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pro-inflammatory cytokines, tumor necrosis factor alpha (TNF-α) plays a key role in leukocyte migration, central mediator of inflammation. Other cytokines, such as interferon gamma (IFN-γ), activate leukocyte populations. TNF-α is a pleiotropic cytokine that can amplify inflammatory responses by stimulating the release of chemotactic factors from macrophages and epithelial cells and by upregulating the expression of leukocyte and endothelial adhesion molecules (Braegger et al., 1992; Wilderman et al., 2006; Bibikova et al., 2014). IFN-γ is a major cytokine responsible for activating macrophages and stimulating greater histocompatibility complex expression, increases antigen presentation and activation of cells of the adaptive immune response (Kees et al., 1984; Amaldi et al., 1989; Alasandagutti et al., 2014). Considering the popular belief around the anti-inflammatory activity of *A. fastigiatum* and its potential effect on immune response, the objective of this study was to investigate the *in vitro* profile of cytokines in human leukocyte cultures treated with EOAF and stimulated with phorbol myristate acetate (PMA).

Methodology

Plant material

The aerial parts (branches with leaves and inflorescences) of *Ageratum fastigiatum* (Gardner) R.M. King & H. Rob., Asteraceae, were collected on December 2010 at the Juscelino Kubitscheck Campus – UFVJM (18°12.125′ S 43°34.367′ W, altitude 1392 m), located in the town of Diamantina, Minas Gerais. A voucher specimen was deposited in the Herbarium DIAM-UFVJM, under the registration number 1300. A technically proficient expert performed species identification. According to Normative Instruction No. 154/2007 by the Biodiversity Information System (SISBIO), proper authorization was obtained for the collection of plant material, with registration no. 5141849 issued by the Brazilian Institute of Environment and Renewable Natural Resources. Authorization to access and shipping sample component of genetic heritage no. 010 476/2012-1 (Conselho Nacional de Desenvolvimento Científico e Tecnológico).

Extraction of essential oil

The oil was obtained from fresh plant material, according to Method I of the Brazilian Pharmacopeia, 5th Edition (2010). This process is based on hydro-distillation with the extraction extending over a 4-h period. The essential oil was obtained and stored in flasks at −20 °C, protected from light. From 70 g of fresh plant material, 0.06 ml of oil was obtained (ml/70 g × 100) giving a ratio of 0.086%.

Analysis of the EOAF components by gas chromatography–mass spectrometry (GC/–MS)

GC/–MS analysis was performed using a Shimadzu GC–MS-QP2010 equipped with a DB-5-MS Agilent J and W capillary column (30 m × 0.25 mm × 0.25 μm). High purity helium was used as the carrier gas at a pressure of 57.5 kPa with a column flow of 1 ml/min. The injector temperature was 240 °C, and the oven temperature increased from 60 °C to 240 °C at 3 °C/min. Electron ionization at 70 eV was the selected ionization method. Under the same experimental conditions, the oil was co-injected with a homologous series of linear hydrocarbons (Alltech C9–C24), to calculate the retention index (RI) of each constituent of the essential oil (Van Den Dool et al., 1963). Compounds were identified by analysis and comparison of the mass spectra with the Wiley 7, NIST 62, and FFNSC 1.3 libraries and by comparison of the RI to those in literature (Adams, 1995) and web sites (<http://webbook.nist.gov/quimica> and <http://pherobase.com>). The relative areas (%) of each individual peak from the GC/MS chromatogram were proportional to the total ion current without standardization.

Analysis of the viability of peripheral blood leucocytes following exposure to different concentrations of essential oil

Blood samples were obtained from six healthy volunteers. Volunteers using corticosteroids or other immunosuppressant drugs were not considered for blood donation. The study was approved by the Ethics Committee of the UFVJM under registration number 146/10. Blood samples were collected in vacuum tubes containing heparin (Vacutainer; Becton Dickinson, San Jose, CA, USA). For cell viability analysis, 10 ml of venous blood obtained from six young blood donors was incubated for 10 min in the presence of 10 ml of red blood cell lysis solution (NH_4Cl 8.3 mg/ml, $NaHCO_3$ 1 mg/ml, ethylenediaminetetraacetic acid [EDTA] 1 mg/ml) followed by two washing steps and centrifugation (250 × *g* for 7 min) of the leucocytes in the presence of phosphate buffered saline (PBS, 0.015 M, pH 7.4). Cellular concentration was adjusted to 1×10^7 cells/ml. A 50-μl aliquot of the cell suspension (5×10^5 cells) was added to each culture containing RPMI medium and different concentrations of EOAF solubilized in dimethyl sulfoxide (DMSO; Sigma–Aldrich Corporation): 5×10^{-3}, 1×10^{-2}, and 2.5×10^{-2} μl/ml. Cell cultures treated with saline only or DMSO were used as the negative control and solvent control, respectively. After 24 h of incubation at 37 °C and 5% CO_2, the leukocytes were washed with PBS (centrifugation at 250 × *g* for 7 min) and resuspended in 50 μl saline. A 10 μl aliquot of the cell suspension was incubated in the presence of 190 μl of 0.4% trypan blue for cell counting with a Neubauer chamber. For flow cytometry analysis, trypan blue was used at a concentration of 0.002% (Avelar-Freitas et al., 2014). Cell viability was calculated as the number of viable cells divided by the total number of cells.

In vitro *effect of EOAF on the production of cytokines by human lymphocytes*

Blood samples were collected in vacuum tubes containing heparin (Vacutainer; Becton Dickinson, San Jose, CA, USA), and 500-μl aliquots were placed in polypropylene tubes (Falcon 2059, Becton Dickinson, San Jose, CA, USA). Samples were incubated in the presence of 500 μl of RPMI-1640 medium (Gibco, Grand Island, NY, USA) and 10 μg/ml Brefeldin (Sigma Chemical Co., St. Louis, MO, USA) in a humid CO_2 incubator (5% CO_2) at 37 °C for 4 h.

To analyze the effect of EOAF on the cytokine profile of leukocyte populations, leukocyte cultures were incubated in the presence of EOAF at concentrations of 5×10^{-3} and 1×10^{-2} μl/ml, and stimulated with 25 ng/ml PMA (Sigma, St. Louis, MO, USA). PMA activates leucocytes and induces production of pro-inflammatory cytokines (Bishayi and Samanta, 2002). Hence, we evaluated the inhibitory effect of EOAF on cytokine production by human leucocytes. Cells stimulated with PMA in combination with 8 μg/ml dexamethasone (disodium phosphate dexamethasone – decadron injection, 4 mg/ml, Aché, Brazil) were used as an inhibition control.

After incubation, samples were subjected to red blood cell lysis in the presence of red blood cell lysis solution and incubated at room temperature for 15 min. Subsequently, the samples were centrifuged, the supernatant was discarded, and the cells were resuspended in PBS-W (0.015 M PBS pH 7.4 containing 0.5% bovine serum albumin [BSA] and 0.1% sodium azide) and PBS-P (0.015 M PBS pH 7.4 containing 0.5% BSA, 0.1% sodium azide, and 0.5% of saponin), and incubated at room temperature for 10 min. Cells were then washed twice with PBS-W. The cell suspension was homogenized, and the volume of each cell culture was distributed into two new tubes containing monoclonal antibodies specific for the cytokines IFN-γ and TNF-α, diluted 1:10 with PBS-P, and incubated

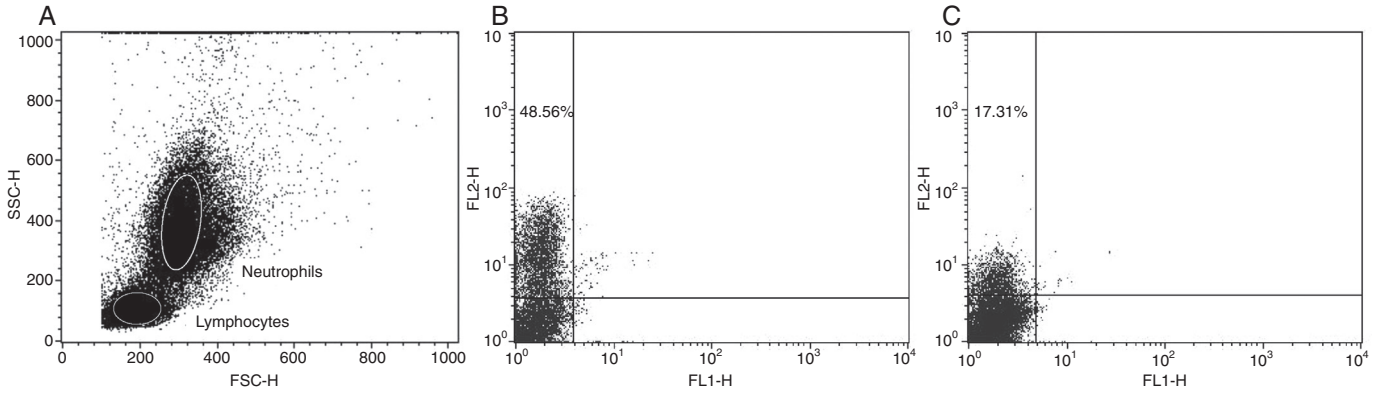

Fig. 1. Strategy for flow cytometry analysis. Sequence of procedures used for analysis of the percentage of cell populations by flow cytometry. Point distribution graph showing the variables, FSC (size) × SSC (granulosity), used for the selection of the lymphocyte and neutrophil populations (A), followed by the point distribution graph, FL1 × FL2, used to evaluate cytokine expression in leucocytes. The graph shown in (B) was obtained from a culture stimulated with phorbol myristate acetate (PMA) and labeled with monoclonal antibodies for tumor necrosis factor alpha (TNF-α). The graph shown in (C) was obtained from a culture stimulated with PMA in the presence of the essential oil of *Ageratum fastigiatum*, also labeled with monoclonal antibodies for TNF-α.

at room temperature protected from light for 30 min. The monoclonal antibodies were obtained from Pharmingen (San Diego, CA, USA) and were conjugated with phycoerythrin (PE). Following incubation, the cells were washed with PBS-W for evaluation of intracytoplasmic cytokine production by flow cytometry (FACScan – Becton Dickinson) at an acquisition rate of 30,000 events per tube.

The software CellQuest™ was used for data collection and analysis. Two-dimensional plots with point distribution were generated to show the relationship between variables cell size (FSC) and internal complexity (SSC). Based on these two variables, the lymphocyte and neutrophil cell populations was selected and percentage of cells positive for each evaluated cytokine was obtained from this cell population (Fig. 1).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA), version 5.0. p values <0.05 were considered statistically significant. Data are presented in the graphs as mean ± standard error. One-way analysis of variance (one-way ANOVA) and Tukey's post hoc test were the statistical tests used for analysis.

Results and discussion

Table 1 shows the components identified in the essential oil along with their retention indices and percentage values (relative areas of peaks in chromatogram). A total of 26 compounds were identified, corresponding to approximately 70% of the oil's composition. Fifteen of the identified compounds were sesquiterpenes and 11 monoterpenes. The unidentified compounds did not present mass spectra that were compatible with those found in the libraries (their mass spectra had a low similarity index [SI] when compared to those from the mass spectral libraries). Moreover, their retention indices were not compatible with those found in the literature or from websites; hence, precise identification of these compounds was not possible.

The monoterpenes, limonene, α- and β-pinene, and the sesquiterpenes *trans*-caryophyllene, α-copaene, and spathulenol were detected in previous studies performed with EOAF (Del-Vechio-Vieira et al., 2009a,b; Gonçalves et al., 2011). However, there were qualitative differences in the monoterpene and sesquiterpene components of EOAF when compared to the findings from previous studies. These differences reflect the variability among essential oils obtained from plants of the same species due to genetic, ontogenetic, and environmental factors (Lima et al., 2003; Gobbo-Neto and Lopes, 2007).

Table 1
Retention index (RI) and relative areas (%) of each individual peak (GC/MS chromatogram) of the compounds of the essential oil from aerial parts of *Ageratum fastigiatum*.

Compounds	RI[a]	RI_{calc}	%[b]
α-pinene	939	932	7.5
sabinene	976	971	0.3
β-pinene	980	977	0.6
myrcene	991	988	0.2
limonene	1031	1028	5.9
α-campholenic aldehyde	1127	1126	0.3
trans-pinocarveol	1139	1140	0.3
trans-verbenol	1144	1145	0.5
pinocarvone	1162	1161	0.2
verbenone	1204	1206	0.6
carvone	1242	1243	0.5
α-copaene	1376	1372	1.0
β-elemene	1391	1387	0.6
trans-caryophyllene	1418	1415	2.0
α-humulene	1454	1451	3.5
γ-muurolene	1477	1471	0.6
α-curcumane	1483	1478	0.4
α-muurolene	1499	1495	0.3
γ-cadinene	1513	1509	0.4
spathulenol	1576	1572	0.5
caryophyllene oxide	1581	1577	13.6
humulene 1,2-epoxide	1606	1604	8.4
1,6-humulanedien-3-ol	1619	1617	17.7
epi-α-cadinol	1640	1639	1.2
α-muurolol	1645	1642	0.5
α-cadinol	1653	1651	3.4

[a] Retention indexes reported in the literature or on websites.
[b] Percentage peak area proportional to the total ion current without standardization; RI_{calc}: retention index experimental/calculated.

Given that plant compounds and solvents are external factors capable of inducing cell death, our initial approach was to evaluate the effect of different concentrations of EOAF on the viability of leucocytes. Results showed that an EOAF concentration of 2.5×10^{-2} μl/ml reduced the viability of peripheral blood leucocytes to 87.9 ± 2.9% (Fig. 2), whereas EOAF concentrations of 0.5×10^{-2} and 1×10^{-2} μl/ml did not affect leukocyte viability (95.6 ± 1.4% and 95.1 ± 1.2%, respectively) compared to the control group (96.5 ± 2.1%). Based on these results, it was concluded that EOAF concentrations of 0.5×10^{-2} and 1×10^{-2} μl/ml were not toxic to leucocytes and were subsequently used for *in vitro* evaluation of the cytokine profile. Results from the viability tests obtained

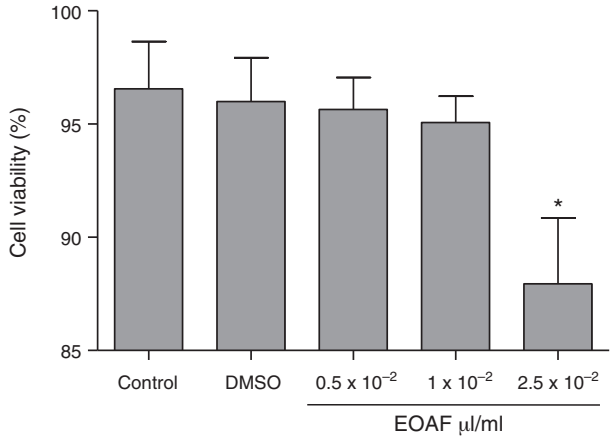

Fig. 2. Percentage of viable leucocytes as obtained for the negative control, solvent control (dimethyl sulfoxide [DMSO] 1%), and for cell cultures incubated in the presence of the essential oil of *Ageratum fastigiatum* (EOAF) at concentrations of 0.5×10^{-2}, 1×10^{-2}, and 2.5×10^{-2} μl/ml. The analysis was performed using the trypan blue exclusion assay by flow cytometry. Results are expressed as mean ± standard error. *Significant difference on comparison with the control. Statistical methods used: ANOVA followed by Tukey's test.

using the Neubauer chamber cell counts were consistent with those obtained by flow cytometry, as described by Avelar-Freitas et al. (2014). The values shown in the graph and mentioned in the text are related to the flow cytometry analysis.

The percentage of neutrophils positive for TNF-α was reduced in the presence of EOAF at concentrations of 0.5×10^{-2} and 1×10^{-2} μl/ml (9.6. ± 3.2% and 7.3 ± 2.5%, respectively), when compared to the control group, which was only subjected to stimulation with PMA (25.3 ± 3.3%) (Fig. 3A). Given that Neutrophils (Issekutz, 1981; Taylor et al., 2014) are the first cells to reach the site of inflammation, inhibition of this cytokine leukocyte population means the inhibition of acute inflammation. According to the results, PMA was not a good stimulus for IFN production in neutrophils and prevented assessment of the OEAF inhibitory effect on the production of this cytokine (Fig. 3B).

Similar to neutrophils, the percentage of lymphocytes positive for TNF-α was reduced in the presence of EOAF at concentrations of 0.5×10^{-2} and 1×10^{-2} μl/ml (23.28 ± 3.07% and 20.49 ± 4.24%, respectively), when compared to the control group, which was only subjected to stimulation with PMA (40.78 ± 3.46%) (Fig. 4A). The compounds present in the *A. fastigiatum* essential oil had no effect on the production of IFN-γ by lymphocytes, showing that the effect of the essential oil seems to be more efficient on the expression of immune mediators observed in the acute inflammation, instead chronic processes (Fig. 4B).

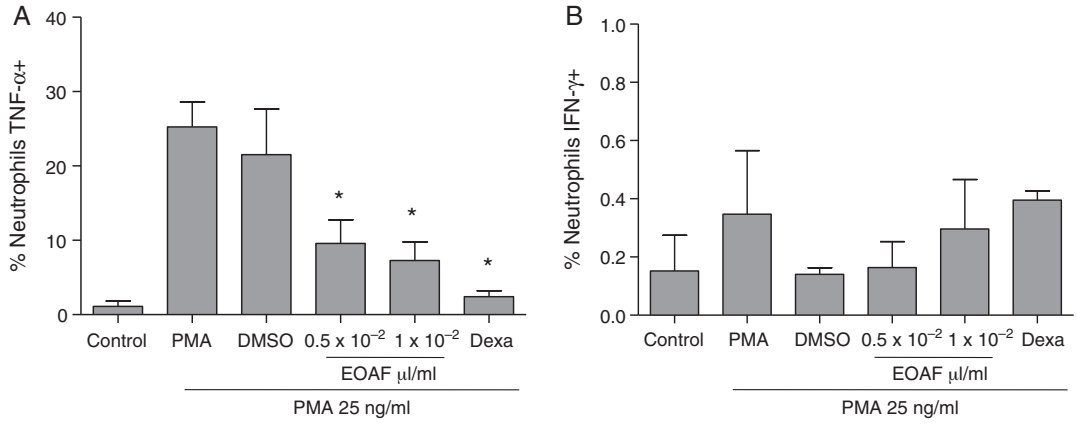

Fig. 3. Analysis of the percentage of tumor necrosis factor alpha (TNF-α)-positive (A) and interferon gamma (IFN-γ)-positive (B) neutrophils in leukocyte cultures stimulated with phorbol myristate acetate (PMA) in the presence or absence of the essential oil of *Ageratum fastigiatum* at concentrations of 5×10^{-3} and 1×10^{-2} μl/ml. dimethyl sulfoxide (DMSO) 1% (1%, v/v) was used as the solvent control and dexamethasone (8 μg/ml) was used as an inhibition control for cytokine production. Results are expressed as mean ± standard error. *Significant difference in comparison with the PMA group. Statistical methods used: ANOVA followed by Tukey's test.

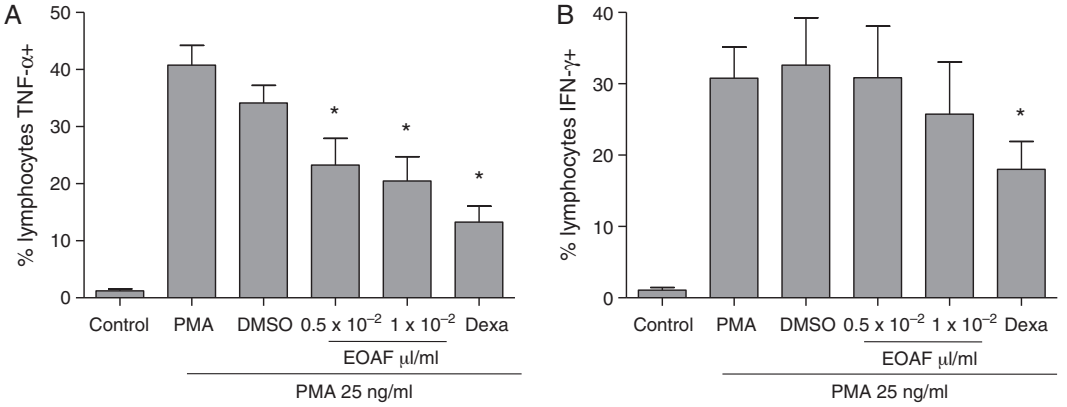

Fig. 4. Analysis of the percentage of tumor necrosis factor alpha (TNF-α)-positive (A) and interferon gamma (IFN-γ)-positive (B) lymphocytes in leukocyte cultures stimulated with phorbol myristate acetate (PMA) in the presence or absence of the essential oil of *Ageratum fastigiatum* at concentrations of 5×10^{-3} and 1×10^{-2} μl/ml. dimethyl sulfoxide (DMSO) 1% (1%, v/v) was used as the solvent control and dexamethasone (8 μg/ml) was used as an inhibition control for cytokine production. Results are expressed as mean ± standard error. *Significant difference in comparison with the PMA group. Statistical methods used: ANOVA followed by Tukey's test.

TNF-α is an important mediator of inflammatory response and is able to promote the expression of adhesion molecules on endothelial cells, inducing the recruitment of cells to the inflammation site (Braegger et al., 1992; Wajant, 2009; Apostolaki et al., 2010). Thus, a decrease in TNF-α levels may reduce leukocyte migration and lead to a lower number of leucocytes at the infection site. Further, TNF induces the expression of acute phase proteins of inflammation secreted by the liver, which enhances inflammation. This effect can be beneficial when there is severe inflammation and in some autoimmune diseases. In fact, Del-Vechio-Vieira et al. (2009a) showed reduced leukocyte migration to the site of inflammation induced by carrageenan in animals. Although the cytokines were not evaluated in the study of Del-Vechio-Vieira et al. (2009a), cytokine modulation may be involved in this effect.

Caryophyllene oxide, one of the compounds present in the essential oil (13.59%), also inhibited paw edema induced by carrageenan; in this case, cytokines were not shown to be involved, but it is possible that they acted by lowering the permeability of the endothelium in the inflamed tissue (Chavan et al., 2010). Other components of the oil such as α-pinene (7.51%), limonene (5.9%), *trans*-caryophyllene (2.04%), and α-humulene (3.52%) isolated from other plants inhibited the TNF-α cytokine in different models (Bae et al., 2012; Yoon et al., 2010; Fernandes et al., 2007; Medeiros et al., 2007). Limonene may interfere with the p38 signaling pathway (Hirota et al., 2010), whereas the compounds *trans*-caryophyllene and α-humulene may interfere with the nuclear factor-kappa B (NF-κB) pathway (Medeiros et al., 2007).

Our results suggest that part of the anti-inflammatory activity attributed to EOAF may be due to the effect of some of its components in decreasing the production of the pro-inflammatory cytokine TNF-α in activated lymphocytes and neutrophils.

Authors' contributions

BAA (PhD student) contributed in collecting plant sample and identification, confection of herbarium, running the laboratory work, analyzing the data and drafted the paper and contributed to biological studies. VGA, MGS and JATS contributed to biological studies. PRB, CFFG and LEG contributed in plant identification and herbarium confection and to chromatographic analysis. ERV and GEBAM designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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