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Original Article

Phytochemical screening of the dichloromethane–ethanolic extract of *Eriosema campestre* var. *macrophylum* roots and its antiproliferative effect on human peripheral blood lymphocytes

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ABSTRACT

Eriosema campestre var. *macrophylum* (Grear) Fortunato, Fabaceae, is a native plant of the Brazilian Cerrado and the decoction of its roots has been used by folk medicine for the therapy of inflammatory diseases. In this study we aimed to investigate the effect of the dichloromethane–ethanolic extract of *E. campestre* roots on the proliferative response of lymphocytes and to examine the profile of IL-2 production. The effect of dichloromethane–ethanolic extract of *E. campestre* on the proliferation of phytohemagglutinin-stimulated lymphocytes was evaluated by using flow cytometry and the cell supernatants were assayed for IL-2 concentrations by using an enzyme-linked immunosorbent assay. The phytochemical screening of *E. campestre* roots was performed to determine the main secondary metabolites through chromogenic and precipitation reactions and by using HPLC-PAD. In addition to the presence of subclasses of flavonoids (flavones and flavonols) in dichloromethane–ethanolic extract of *E. campestre*, we observed that the extract induced a concentration-dependent decrease in IL-2 levels on the supernatant of the cell cultures as well as an antiproliferative effect on T lymphocytes, including CD4+ and CD8+ cells. The anti-inflammatory effects attributed to *E. campestre* by folk medicine may partly be explained by its antiproliferative action on T lymphocytes.

Introduction

Inflammation is a complex immune response that involves multiple factors. The initial events result in the activation of innate immune responses and involvement of enzyme systems (Nathan, 2002; Medzhitov, 2008). In late events, cooperation between T and B lymphocytes contributes to antigen-specific antibody production; T lymphocytes initiate a cell-mediated immune-inflammation process *in situ* and maintain the activation of phagocytic cells by transforming them into tissue-destructive effector cells (Barton, 2008). In chronic inflammatory diseases, the permanent activation and proliferation of T lymphocytes amplify tissue damage and contribute to the clinical outcome observed in these diseases, in addition to the cellular interactions occurring in the affected sites (Cai et al., 2012; Chimenti et al., 2013).

Since chronic inflammatory disorders are characterized by the high activation and proliferation of T lymphocytes, most immunosuppressive drugs aim to block the cell cycle progression of these cells (Macián, 2005). In addition to the drugs registered for first-line therapy, which may have many and sometimes severe side effects (De Mattos et al., 2000), there are numerous alternative herbal treatments with promising, but not yet proven, efficacy (Reuter et al., 2010).

Eriosema campestre var. *macrophylum* (Grear) Fortunato, Fabaceae, is a plant popularly known as "pustemeira" and is native to the Brazilian Cerrado (Grear, 1970; Fortunato, 1999). This species consists of small and erect herbs ranging from 14 to 17.5 cm/high, with yellow flowers and fusiform roots (Rogalski and Miotto, 2011). The decoction of *E. campestre* roots has been described and used by folk medicine for the therapy of inflammatory diseases including inflammatory skin disorders such as psoriasis. However, no study of the chemical composition of this plant or any biological analysis has yet provided evidence regarding the effectiveness of this plant as an anti-inflammatory.

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We decided to perform a phytochemical screening to characterize the main classes of secondary metabolites present in the plant extract as well as to investigate the profile of its chemical composition. Moreover, in addition to the role of T lymphocytes in the pathological process of chronic inflammatory diseases, we also investigated the effect of the dichloromethane–ethanolic extract of *E. campestre* roots (DEEC) on the proliferative response of lymphocytes as well as the profile of IL-2 production, the cytokine essential for the expansion of these cells during the adaptive immune response.

Materials and methods

Plant material

Fresh *Eriosema campestre* var. *macrophylum* (Grear) Fortunato, Fabaceae, plants were collected in the city of Datas, Minas Gerais, Brazil (S 18°27.318′, W 43°39.764′, 1223 m altitude). Botanical identification was performed by Dr. Ana Paula Fortuna Perez, Curator of Herbario BOTU, Department of Botany, Universidade Estadual Paulista, Botucatu, SP, Brazil, and a specimen was deposited under voucher number 881 at Jeanine Felfili Dendrologic Herbarium of the Federal University of Jequitinhonha and Mucuri Valleys.

Preparation of plant extracts

The roots of *E. campestre* were dried to a constant weight at room temperature. The dried material (450 g) was ground and macerated in a mixture of dichloromethane and ethanol (1:1 v/v) for 72 h at room temperature. The macerate was separated by filtration and concentrated under vacuum on a rotatory evaporator (Fisatom, model 801, Brazil) at 40 °C to furnish a brown residue (12.1 g). A 5.0 mg/ml solution of the residue (DEEC) in DMSO (Sigma, USA) was prepared. Small stock aliquots were kept at −20 °C until the time of use. New dilutions of the extract in DMSO were performed to obtain the required concentration in the cell culture medium.

Chromatographic profile using high performance liquid chromatography (HPLC)

Reagents

HPLC-grade methanol was obtained from J. T. Baker (Ecatepec, Mexico), HPLC-grade acetonitrile was obtained from Tedia High Purity Solvents (Fairfield, USA), trifluoroacetic acid was purchased from Synth (São Paulo, Brazil) and HPLC-quality ultrapure water was prepared by using a Millipore Milli-Q Direct-8 System (Billerica, USA).

Chromatographic analysis

Samples of DEEC diluted to 10 mg/ml were dissolved in HPLC-grade methanol, filtered through a 0.2 μm membrane and analyzed on a Thermo Accela HPLC system (Thermo Fisher Scientific Inc., Waltham, USA) with a solvent delivery unit, on-line degasser, column oven and autosampler, and equipped with a photodiode array detector (PAD). For data analysis, we used ChromQuest software (Version 5.0, Thermo Fisher Scientific Inc., Waltham, USA). A Luna C18(2) analytical column (150 × 4.6 mm; particle size 3 μm; Phenomenex, USA) was used. The sample was eluted by a gradient elution employing ultrapure water (Solvent A) and acetonitrile (Solvent B), both containing 0.1% v/v trifluoroacetic acid: 0–5 min, 90% of A, 50 min, 100% B; after 10 min the column was re-equilibrated with 90% A. The column temperature was maintained at 40 °C. Analysis was performed at a flow rate of 1.0 ml/min and was monitored at 288 nm.

Biological samples and preparation of peripheral blood mononuclear cells (PBMC)

Peripheral blood was obtained from 22 healthy adult donors. Volunteers with any infectious, autoimmune diseases or making use of antibiotics, anti-inflammatory medication, corticosteroids or other immunosuppressive drugs were not considered for blood donation. Informed written consent was obtained from all participants. The study was approved by the Ethical Committee at the UFVJM, Diamantina, Minas Gerais, Brazil (register code 569.313/2014).

PBMC was isolated from heparinized human peripheral blood samples (15 ml) by using the Ficoll-Paque (specific gravity 1.077) gradient density method, as described previously by Bicalho et al. (1981). Peripheral blood was diluted with phosphate-buffered saline (PBS; pH 7.2) and centrifuged in a Ficoll-Histopaque (Sigma, USA) discontinuous gradient at 400 × *g* at room temperature for 30 min for obtaining the characteristic layer containing the mononuclear cells. PBMC was collected, washed with PBS and centrifuged (240 × *g* at room temperature for 7 min). Cells were suspended at a concentration of 1×10^7 cells/ml in PBS or RPMI-1640 medium (Sigma, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco, Invitrogen Corporation, USA), 2 mM L-glutamine (Sigma, USA) and an antibiotic/antimycotic cocktail (100 UI/ml penicillin G, 100 μg/ml streptomycin and 250 ng/ml amphotericin B – Sigma, USA). The PBMC from each research subject was obtained separately and used for cell culture analysis individually.

Cell viability analysis

PBMC (5×10^5) were cultured with 0.5% dimethyl sulfoxide (DMSO), as solvent control, or DEEC (100, 50, 25 or 12.5 μg/ml) at 37 °C in a humidified incubator with a 5% CO_2-air atmosphere for 24 h or 5 days. Untreated PBMC was used as the unstimulated cell culture control (Ctrl). In the end of the incubation, cells were washed with PBS, centrifuged (240 × *g*, room temperature, 7 min) and resuspended with 0.5 ml PBS. Then, 10 μl of cell suspension was mixed with an equal volume of 0.4% trypan blue (Sigma, USA). Total, viable and nonviable cell numbers were counted under the microscope by using a hemocytometer Neubauer chamber.

Lymphocyte proliferative response

PBMC (1×10^7 cells) were resuspended in PBS and labeled with 1 μM of BD Horizon™ Violet Proliferation Dye 450 (VPD 450, BD Biosciences, USA) for 15 min at 37 °C (Lyons, 1999). The VPD 450-stained PBMC (5×10^5 cells/well in 24-well plate) were cultured in RPMI-1640 containing 10% FCS (Gibco, Invitrogen Corporation, USA), 2 mM L-glutamine and the antibiotic–antimycotic cocktail (Sigma, USA), with or without phytohemagglutinin (PHA 5 μg/ml). Cells were also stimulated with PHA in combination with 0.5% DMSO (solvent control) or different concentrations of DEEC (25, 12.5 or 6.25 μg/ml). Cells stimulated with PHA in combination with dexamethasone at 8 μg/ml (Aché, Brazil) were used as the inhibition control (DEXA). The plate was kept in a humidified incubator with a 5% CO_2 air atmosphere for five days at 37 °C. After incubation, cells were harvested, washed in PBS and stained with specific monoclonal antibodies (mAB) for human CD4 (CD4 mAb phycoerythrin conjugated – CD4/PE) and CD8 (CD8 mAb conjugated with fluorescein isothiocyanate – CD8/FITC) (both from BD Biosciences, USA). Samples were analyzed on a BD FACSCanto II with BD FACSDiva software, and 50,000 events were acquired for each tube. Proliferation was measured based on the dilution of VPD 450 (diminished staining intensity). The proliferative index (PI) was then calculated from the violet

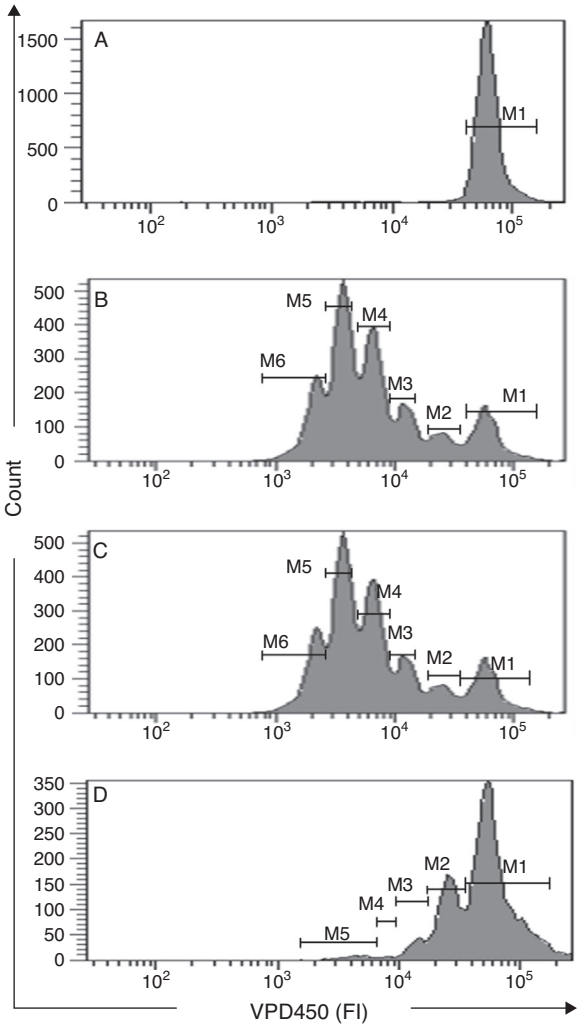

Fig. 1. Analysis of cell proliferation using Violet Proliferation Dye (VPD) 450 in flow cytometry. A, VPD 450-stained cells derived from unstimulated cultures; B, VPD 450-stained cells in cultures stimulated with phytohemagglutinin (PHA), C, VPD 450-stained cells in cultures stimulated with PHA and treated with DMSO (solvent control), D, VPD 450-stained cells in cultures stimulated with PHA and treated with DEEC 25 μg/ml.

fluorescence histograms (Fig. 1) by using the following formula, as described by Angulo and Fulcher (1998): $PI = (100 - Y)/Y$, where $Y\ (\%) = X0 + X1/2 + X2/4 + X3/8 + X4/16 + X5/32$; $X0$ represents the percentage of T-cells that did not divide (located in M1), and X1–X5 represents the peaks of gradual division (located in M2–M6).

IL-2 production

PBMC (5×10^5 cells/well on 24-well plates) were cultured in RPMI-1640 containing 10% FCS (Gibco, Invitrogen Corporation, USA), 2 mM L-glutamine, the antibiotic–antimycotic cocktail (Sigma, USA) and PHA (5 μg/ml) with (i) PBS (control), (ii) 0.5% DMSO (solvent control) or (iii) different concentrations of DEEC (25, 12.5 or 6.25 μg/ml). Cells stimulated with PHA in combination with dexamethasone at 8 μg/ml were used as the inhibition control (DEXA). The plate was maintained at 37 °C in a humidified incubator with a 5% CO_2 air atmosphere for 36 h, followed by centrifugation for 10 min, at 240 × *g* and at room temperature. The cell supernatants were collected and assayed for IL-2 concentrations by using an enzyme-linked immunosorbent assay (ELISA; R&D Systems, USA) according to the manufacturer's instructions.

In brief, plates were coated with mouse capture antibody to human IL-2 and incubated overnight at room temperature. Wells were washed five times with PBS containing 0.05% Tween 20 (PBST) and incubated with 1% bovine serum albumin in PBS containing 0.05% sodium azide. Different concentrations of recombinant human IL-2 diluted in PBS (standard curve) or cell supernatants were added and incubated at room temperature for 2 h. After washing with PBST, goat biotinylated antibodies to human anti-IL-2 were added, and the plate was incubated at room temperature for 2 h prior to adding streptavidin horseradish peroxidase. After detection with tetramethylbenzidine (Sigma, USA), sulfuric acid was added to block the enzymatic reaction. The absorbance at 450 nm was determined in a microtiter plate reader (SpectraMax 190, Molecular Devices, USA). Concentrations of IL-2 in samples were estimated by comparison with a standard curve.

Statistical analysis

GraphPad Prism, version 5.0 for Windows (GraphPad Software, USA) was used for the statistical analysis, and *p*-values □0.05 were considered to be statistically significant. Data are reported as the mean ± standard deviation (SD). The Shapiro–Wilk test was used to evaluate the normality of the data. Analysis of variance (one-way ANOVA) was employed, followed by Tukey post hoc pairwise comparisons.

Results and discussion

E. campestre is a plant native to Brazil and its traditional use as a therapy against chronic inflammatory disorders is widespread in the Brazilian Cerrado. However, there are no reports of any chemical or biological-toxicological studies for this plant thus far. Thus, the current study provided the first investigative contribution to the chemical characterization and biological analysis of this plant species.

The phytochemical analysis (Farnsworth, 1966; Bustamante et al., 2010) of DEEC showed the presence of flavonoids and terpenes.

A complex chemical profile for DEEC was observed in the HPLC-PAD analysis, indicating the presence of substances of high, medium and low polarity (Fig. 2A). High polarity compounds are represented by peaks with shorter retention times, eluted between 1 and 20 min, because these substances have a higher affinity for the mobile phase. Some peaks were eluted between 20 and 35 min, a fact that demonstrates the presence of medium polarity molecules. The late eluting peaks observed between 35 and 47 min, which represent a higher affinity for the non-polar stationary phase (C18), are distinguished by a higher intensity and they seem to constitute majority compounds compared with the other peaks. This observation indicates that the substances of low polarity predominate in the extract.

Flavonoids have two absorption bands in the ultraviolet–visible region: band II with a maximum absorption between 240 nm and 285 nm, corresponding to the benzoyl group, and band I with a maximum absorption region from 300 to 550 nm, which corresponds to the cinnamoyl group. The analysis of bands I and II (absorption maximum and intensity) can assist in identifying the type of flavonoid (Marston and Hostettmann, 2006). The HPLC-PAD spectra showed an absorption profile in specific regions similar to those observed for flavonoids. This result agrees with the data observed in the chromogenic and precipitation reactions. Moreover, the absorption and intensity analysis of bands I and II in the UV/VIS spectra suggests the presence of ten flavones (Fig. 2B) and four flavonols (Fig. 2C) in DEEC. This result is in agreement with the phytochemical screening performed by chromogenic and precipitation reactions.

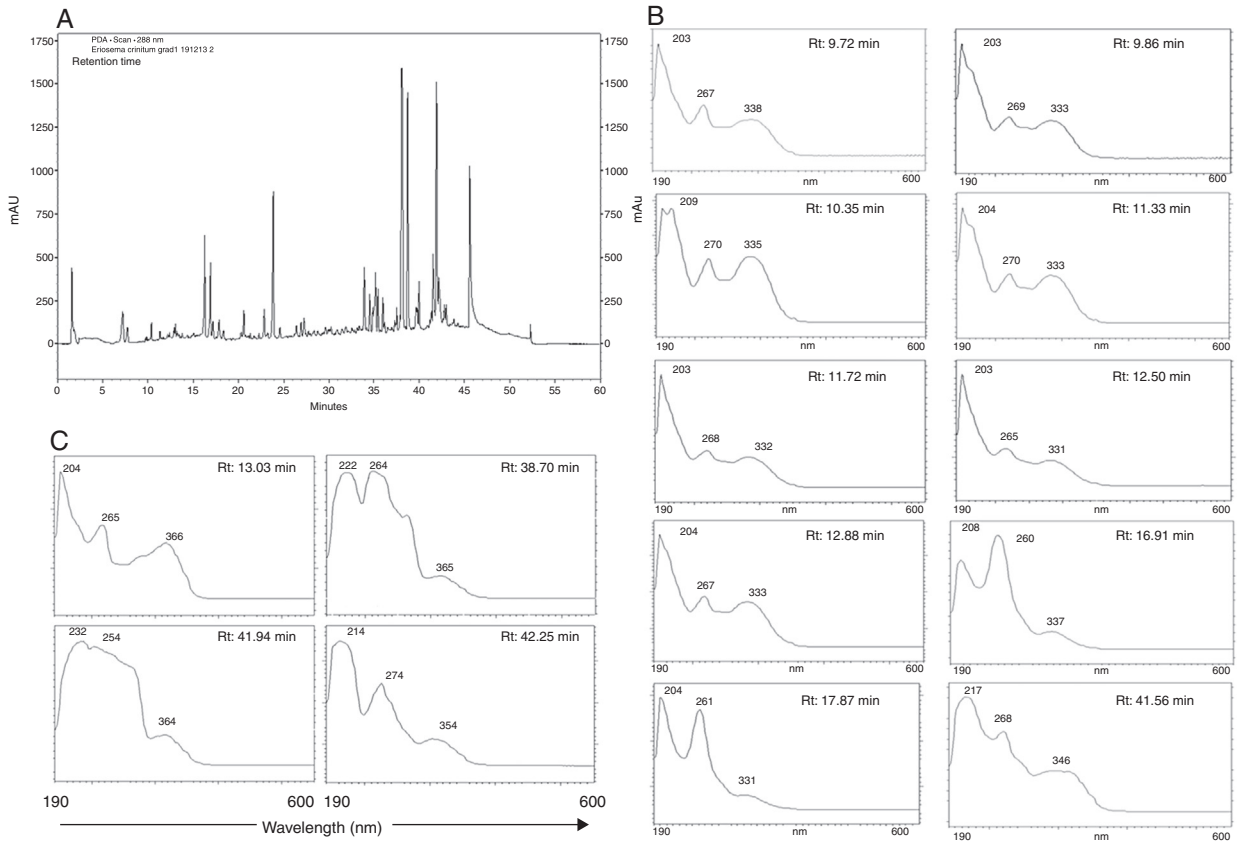

Fig. 2. Chemical analysis of DEEC. HPLC-PAD chromatogram of DEEC, monitored at 288 nm (A) and UV/VIS spectra (190–600 nm), indicating the presence of flavones (B) and flavonols (C).

Several studies with other species of the *Eriosema* genus have reported that flavonoids are responsible for the biological activity attributed to these plants. In some studies, authors have reported the presence of several novel flavonoids such as Kraussianones 1–7, observed in the roots of *E. kraussianum* (Drewes et al., 2002, 2004), Erioschalcones A and B, isolated from *E. glomerata* (Awouafack et al., 2008), Robusflavones A and B, extracted from the aerial parts of *E. robustum* (Awouafack et al., 2013) and Khonklonginols A–H, isolated from the roots of *E. chinense* (Sutthivaiyakit et al., 2009). It is possible that *E. campestre* contains novel molecules. This idea supports the necessity for further chemical studies of its bioactive compounds.

The substances present in plant extracts might include those with cytotoxic activities (Veiga-Júnior et al., 2005), and the toxicity of these compounds may define the use of the plant as a phytomedicine. To investigate this issue, the first trials in our study aimed to evaluate the toxicity of DEEC in human PBMC by using the trypan blue exclusion test. After 24 h of culture in the presence of different concentrations of the extract, we observed that DEEC at 12.5, 25 and 50 μg/ml did not decrease cell viability (12.5 μg/ml = 97.56 ± 0.32%; 25 μg/ml = 95.09 ± 1.71%; 50 μg/ml = 93.88 ± 1.46%) compared with the control cultures (96.75 ± 1.49%) and solvent control (DMSO = 95.66 ± 1.56%). Data showed cytotoxicity only when PBMC was treated with DEEC at 100 μg/ml (56.60 ± 7.24%) (Fig. 3A).

After five days of cell culture, DEEC in concentrations lower than 25 μg/ml showed no cytotoxic effect on PBMC (12.5 μg/ml = 93.56 ± 3.03%; 25 μg/ml = 83.75 ± 6.67%) compared with the untreated (Ctrl = 91.50 ± 2.86%) and solvent control cultures (DMSO = 85.47 ± 7.75%). However, a significant reduction in cell viability was observed in cultures treated with DEEC at 50 (65.47 ± 10.37%) and 100 μg/ml (54.70 ± 2.90%) compared with the DMSO-treated and untreated cell cultures (Fig. 3B).

These results allowed us to choose concentrations of DEEC lower than 25 μg/ml as the optimum concentrations for use in cell proliferation assays since the cells are kept in contact with an extract for five days.

In order to understand the molecular basis for its traditional use in the treatment of chronic inflammatory diseases and considering the pivotal role of T-cells on the maintenance of the inflammatory process, we decided to characterize the influence of DEEC on the cell division of activated human lymphocytes by using VPD 450-labeled cells. VPD 450 is inherited by daughter cells after cell division, and the intensity of the fluorescence of each dividing cell decreases proportionally along the mitosis. The dye does not influence the viability of the stained cells. VPD 450 fluorescence in lymphocytes was analyzed by using flow cytometry in the presence of a control medium or various concentrations of DEEC (6.25, 12.5 and 25 μg/ml) (Fig. 4A and B). The PI of total lymphocytes (12.5 μg/ml = 1.21 ± 0.37; 25 μg/ml = 0.46 ± 0.25) and T-CD8 lymphocytes (12.5 μg/ml = 1.56 ± 0.39; 25 μg/ml = 0.45 ± 0.15) in cell cultures treated with DEEC at 12.5 and 25 μg/ml was lower than those observed in cell cultures treated only with PHA (total lymphocytes = 2.12 ± 0.65, T-CD8 lymphocytes = 2.99 ± 1.53). The proliferative response of T-CD4 lymphocytes decreased in cultures treated with DEEC at 25 μg/ml (0.47 ± 0.29) in comparison to cell cultures treated only with PHA (2.07 ± 0.77). This antiproliferative effect observed was due to the chemical constituents presented in the extract, since DMSO did not alter lymphocyte cell proliferation, as shown in the solvent cell cultures of total lymphocytes (2.11 ± 0.77) as well as the CD8 (2.56 ± 1.47) and CD4 (2.26 ± 1.09) T lymphocyte subsets.

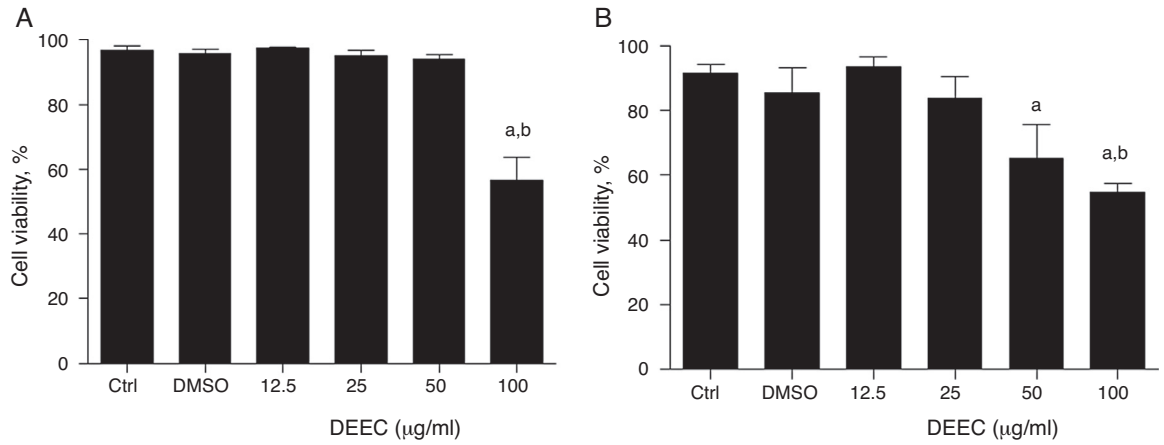

Fig. 3. Effect of DEEC on cell viability after 24 h (A) and five days (B) of culture. Percentages of viable PBMC in PBS-treated (Ctrl), DMSO-treated and DEEC-treated cells were assessed by using the trypan blue exclusion test ($n = 8$). The results are expressed as the mean ± SD of the percentage of cell viability in different groups. Significant differences ($p \square 0.05$, ANOVA with post hoc Tukey test) are identified by the letters "a" and "b" for comparisons relative to the control and DMSO cell cultures, respectively.

The influence of DEEC on the proliferation of human lymphocytes by using cell-based assays was compared with pharmacological doses of dexamethasone, which is the benchmark of immunosuppressive pharmaceuticals to define its immunotherapeutic activity. DEEC exhibited a concentration-dependent inhibition of the cell division of the activated T lymphocytes, including CD4 and CD8 T-cells. In comparison with dexamethasone, the inhibition promoted by DEEC at 25 μg/ml on T lymphocytes and CD4 T-cells presented efficacy levels of 71.65% and 53.14%, respectively. In the CD8 T-cell analysis, DEEC exhibited an efficacy of

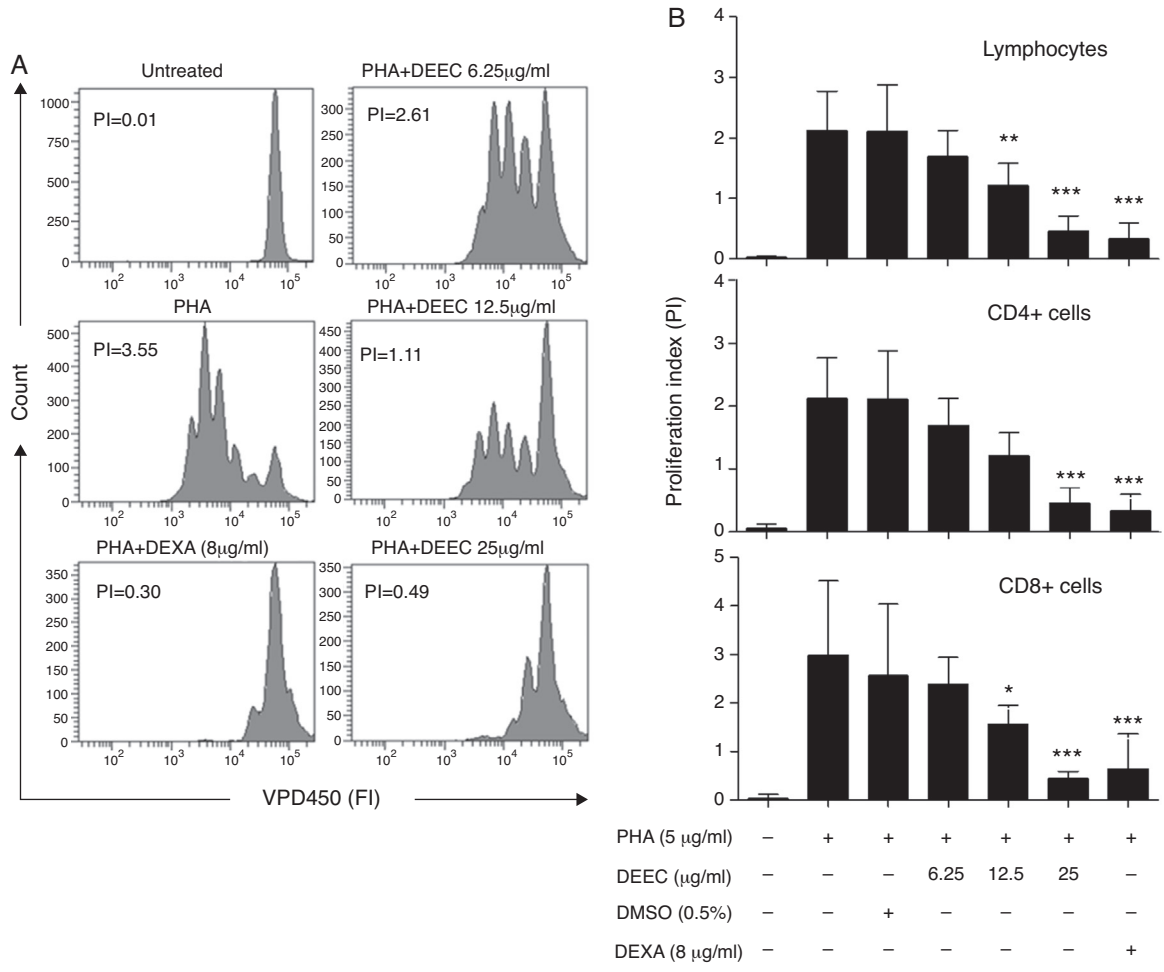

Fig. 4. Influence of DEEC on the proliferation of activated lymphocytes. VPD 450-labeled human lymphocytes (5×10^5 cells) were cultured in the presence of medium, dexamethasone (DEXA, 8 μg/ml) or different concentrations of DEEC (6.25, 12.5 and 25 μg/ml) and activated with PHA (5 μg/ml) for five days. The lymphocyte proliferation was analyzed by using flow cytometry. The representative histograms indicate the proliferation index (PI) into gated lymphocytes (A). The results are expressed as the mean ± SD of the PI in all human lymphocytes, CD8+ and CD4+ cells (B) ($n = 8$). The statistical method used was ANOVA followed by the Tukey test.

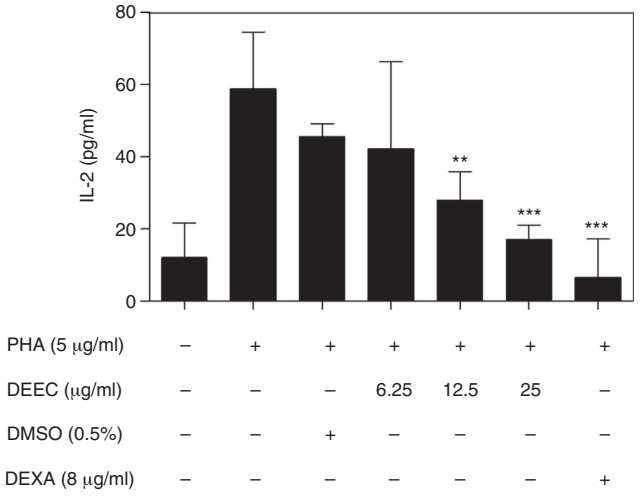

Fig. 5. Analysis of IL-2 production in the supernatant human PBMC cultures activated by PHA and treated with DEEC. Human PBMC (5×10^5 cells) were cultured in the presence of medium, DMSO (0.5%), dexamethasone (DEXA, 8 μg/ml) or different concentrations of DEEC (6.25, 12.5 and 25 μg/ml) and activated with PHA (5 μg/ml) for 36 h. The amount of IL-2 was measured in the supernatant of the cell cultures by using an ELISA-based cytokine detection method. The results are presented as the mean ± SD of six independent experiments. The statistical method used was ANOVA followed by the Tukey test.

167.94%, an antiproliferative effect higher than those presented by dexamethasone. T-CD8 lymphocytes play an important role in viral infections, cancer and transplant rejection as well as in skin lesion and tissue damage maintenance in autoimmune and degenerative inflammatory diseases characterized by a heightened cellular adaptive immune response (Gao et al., 2009). The inhibitory effect of DEEC could thus be a promising therapeutic strategy in these inflammatory situations, opening new perspectives for research.

T lymphocyte proliferation is initiated by bonding the T-cell receptor to external stimuli that initiate the secretion of the autocrine growth factor IL-2, which, in turn, promotes the interaction with the CD25 receptor that is upregulated on the surface of activated T-cells (Ganguly et al., 2001; Malek, 2008). The effect of DEEC on IL-2 production in the supernatant of PBMC cultures was analyzed by using ELISA (Fig. 5). DEEC inhibited the production of IL-2 in the PHA-stimulated cultures treated with the extract at 12.5 μg/ml (28.01 ± 7.95 pg/ml) and 25 μg/ml (17.02 ± 4.04 pg/ml) compared with the cell cultures stimulated only with PHA cultures (58.77 ± 15.70 pg/ml). Once again, DMSO was not able to inhibit the production of IL-2 (DMSO = 39.79 ± 15.7 pg/ml).

These results indicated that the antiproliferative action exhibited by the extract on T lymphocytes was due to its inhibitory action on the production of IL-2. The inhibitory effect of DEEC (25 μg/ml) on the secretion of IL-2 presented an efficacy of 38.33% in comparison to those observed in cultures treated with dexamethasone. The expression of IL-2 involves the activation of transcription factors, mainly NF-kB. The recognition of an antigen by the T lymphocyte receptor/CD3 complex, as well as co-stimulatory stimuli initiates the activation of the signaling pathway related to NF-kB. This transcription factor is essential for T-cell proliferation, cytokine production and the development of a well-established cell-mediated immune response, cytokine production and consequently an increase of inflammatory mediators such as prostaglandins and leukotriene (Kane et al., 2002; Li and Verma, 2002).

The inhibition of IL-2 production is the target for several immunosuppressive drugs (Schreiber and Crabtree, 1992). Because of the inhibitory action of DEEC on cell proliferation and its inhibitory effect on the secretion of IL-2, in addition to the predominant presence of flavonoids in the *E. campestre* extract, molecules belonging to this chemical group might be the active compounds responsible for the effects presented here. Several studies have identified flavonoids as the agents responsible for the inhibition of lymphocyte proliferation, and this effect has been shown to be associated with lower levels of IL-2 in cell cultures (Gao et al., 2009; Chang et al., 2008; Gharagozloo et al., 2010). Further studies using fractionated DEEC will be performed to elucidate the structure of the substances responsible for the effect demonstrated here as well as the performance of the active fractions in inflammatory models *in vivo*.

The results suggest that the anti-inflammatory activity attributed to *E. campestre* by folk medicine may be, at least in part, the result of the action of its flavonoids on the activation and proliferation of T lymphocytes. This finding means that *E. campestre* is a promising natural source for the development of new effective phytomedicines for the treatment of the damage observed in autoimmune and inflammatory degenerative diseases, especially those mediated by T lymphocytes.

Authors' contributions

MGS collected plant sample, performed the experiments, interpreted the results and drafted the manuscript. VGA and BAAF contributed to biological studies and to critical reading of the manuscript. CFFG contributed to prepare the dichloromethane–ethanolic extract of *E. campestre* roots. LEG contributed to chromatographic analysis. WFP contributed to draft and critical reading of the manuscript. GEABM 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.

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