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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