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Article

Evaluation of the proliferative activity of methanol extracts from six medicinal plants in murine spleen cells

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A number of natural compounds have been used as immunomodulatory agents, enabling the function of the immune system to be modified by stimulating or suppressing it. There has been increasing interest in the study of therapeutic action of plant extracts regarding their immunomodulatory activity. The aim of this study was to identify and evaluate the action of extracts of the medicinal plants *Calophyllum brasiliense*, *Ipomoea pes-caprae*, *Matayba elaeagnoides*, *Maytenus robusta*, *Rubus imperialis* and *Vernonia scorpioides* on the development of spleen cells from mice, using the *in vitro* cellular proliferation assay. The cells, obtained by mechanical rupture of mice spleen ($5x10^4$ cells/mL), were incubated with methanol extracts (10, 50, 100 and 200 µg/mL) and phytohemagglutinin (PHA, 5 µg/mL). The basal control for proliferation consisted of cells alone, while the positive control consisted of cells and PHA. The cell culture was kept at 37 °C in 5% CO_2 for 72 hours, and cell proliferation was revealed by the blue tetrazolium reduction assay (MTT). The results were expressed as percentage of growth and were analyzed using the Kruskal-Wallis and Mann-Whitney tests. The *C. brasiliense*, *I. pes-caprae* and *M. elaeagnoides* extracts showed dose-dependent induction of cell proliferation, with a significant increase in cell proliferation ($p<0.03$) and percentage growth of 88.2%, 73.1% and 52.7%, respectively, suggesting T lymphocyte stimulation. By contrast, *M. robusta*, *R. imperialis* and *V. scorpioides* extracts showed significance only with a negative percentage of growth, suggesting inhibition of cell proliferation ($p<0.04$). Further biomonitoring studies will enable the fractions and isolated substances responsible for the immunomodulatory activities to be identified.

Uniterms: Medicinal plants. Natural products/immunomodulatories. Immunomodulation. *Calophyllum brasiliense/*pharmacognosy. *Ipomoea pes-caprae/*pharmacognosy. *Matayba elaeagnoides/* pharmacognosy. *Maytenus robusta/*pharmacognosy. *Rubus imperialis/*pharmacognosy. *Vernonia scorpioides/*pharmacognosy.

Várias substâncias de origem natural têm sido utilizadas como agentes imunomoduladores, permitindo modificar a função do sistema imune e propiciando o estudo de atividades terapêuticas de extratos de plantas. Este trabalho objetivou identificar a atividade imunomodulatória dos extratos de seis plantas medicinais da flora brasileira, *Calophyllum brasiliense*, *Ipomoea pes-caprae*, *Matayba elaeagnoides*, *Maytenus robusta*, *Rubus imperialis* e *Vernonia scorpioides*, sobre a proliferação de células esplênicas de camundongos. As células esplênicas murinas obtidas por ruptura mecânica do baço ($5x14^3$ células/mL) foram incubadas com os extratos metanólicos das plantas (10, 50, 100, 200 µg/mL) e fito-hemaglutinina (PHA, 5 µg/mL). O controle basal de proliferação foi constituído de células apenas e o controle positivo formado por células e PHA. O cultivo celular foi mantido a 37 °C, 5% de CO_2, 72 horas, com quantificação da proliferação celular pelo ensaio de redução do azul de tetrazólio. Os resultados expressos em percentagem de crescimento foram analisados pelos testes de Kruskal-Wallis e Mann-Whitney. Os extratos de *C. brasiliense*, *I. pes-caprae* e *M. elaeagnoides* mostraram indução dose-dependente da proliferação celular ($p<0,03$), com percentagem de crescimento de, respectivamente, 88,2%, 73,1% e

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52,7%, sugerindo estímulo de linfócitos T. Contrariamente, os extratos de *M. robusta*, *R. imperialis* e *V. scorpioides* apresentaram significância apenas com percentagem de crescimento negativa, indicando inibição da proliferação celular ($p<0,04$). A continuidade no estudo biomonitorado permitirá a identificação das frações e substâncias isoladas responsáveis pelas atividades imunomoduladoras.

Unitermos: Plantas medicinais. Produtos naturais/imunomoduladores. Imunomodulação. *Calophyllum brasiliense*/farmacognosia. *Ipomoea pes-caprae*/farmacognosia. *Matayba elaeagnoides*/farmacognosia. *Maytenus robusta*/farmacognosia. *Rubus imperialis*/farmacognosia. *Vernonia scorpioides*/farmacognosia.

INTRODUCTION

The identification of therapeutic activities of plant extracts by means of chemical, pharmacological and toxicological studies is an area of growing research interest. The plant kingdom is a large reservoir of pharmacologically active molecules, and the target of investment by pharmaceutical companies searching for new active substances (Hostettmann, Queiroz Vieira, 2003), which is reflected by the large number of plant-derived medicines now commercially available (Newman, Cragg, 2007; Wagner, 2007).

The ethnopharmacological knowledge has culminated in the development of substances that have had a significant impact on current therapeutics, such as salicylic acid, atropine, pilocarpine, quinine, artemisinin, taxol, digoxin and morphine (Verotta *et al.*, 2000; Viegas *et al.*, 2006). Several plants found in the State of Santa Catarina, Brazil, have popular medicinal use. These include homemade alcohol, infusions, baths and homogenized preparations of fresh plant, such as: *Calophyllum brasiliense* – used to treat bronchitis, kidney and gastric diseases, inflammation, diabetes, varicose, diarrhea, herpes, rheumatism, hemorrhoids and chronic ulcers (Noldin, Buffon, Cechinel Filho, 2006); *Ipomea pes-caprae* – used to treat dermatitis caused by jellyfish, cramps, diuretic disorder, gonorrhea, inflammation and pain (Pongprayoon *et al.*, 1989; Souza *et al.*, 1998); *Matayba eleagnoides* – used to treat inflammation, pain and liver cancer (Lorenzi, 2000; Souza *et al.*, 2007); and *Maytenus robusta* (Niero *et al.*, 2006; Andrade *et al.*, 2007) and *Rubus imperialis* – used in the treatment of peptic ulcer, diabetes and pain (Cechinel Filho, 2000), and also *Vernonia scorpioides* – used to treat skin disorders and varicose ulcers and to combat parasites (Monteiro *et al.*, 2001).

Studies of phytochemical and biological activity have identified several compounds present in these plants, showing several activities such as antinociceptive activity against *C. brasiliense* (Silva *et al.*, 2001; Isaias, *et al.*, 2004; Niero *et al.*, 2006), *I. pes-caprae* (Krogh *et al.*, 1999; Souza *et al.*, 2000), *M. eleagnoides* (Souza *et al.*, 2007), *M. robusta* (Niero *et al.*, 2001) and *R. imperialis* (Niero *et al.*, 1999; Niero *et al.*, 2002; Ardenghi *et al.*, 2006), and also molluscicidal activity against *C. brasiliense* (Gasparotto Jr. *et al.*, 2005) and cytotoxic activity against *V. scorpioides* (Buskühl, 2007).

The use and development of drugs that suppress the immune system - known as immunosuppressors - has broadened due to transplantation and advances in knowledge of Clinical Immunology, which has revealed the pathophysiology of diseases caused by both exacerbation and deficiency of the immune response (Lima, 2007). The analysis of immunostimulating or immunosuppressive activity promoted by natural compounds has been determined by a host of different methodologies and models by means of *in vivo* and *in vitro* assays. The lymphocyte proliferation assay is one of the models used to assess the activity of these compounds on cell proliferation, revealing the stimulation or suppression of the activation of this cell population (Pandima Devi *et al.*, 2003). In this model, the *in vitro* proliferative function of murine spleen cells and human mononuclear cells can be evaluated in the presence or absence of stimulators such as phytohemagglutinin (PHA), concanavalin A, pokeweed mitogen, lipopolysaccharide, specific antigens derived from different aggressive agents and even extracts, purified natural substances and synthetic compounds (Pandima Devi *et al.*, 2003; Rocha, Gorescu, Beltrame, 2007).

In view of the phytochemical and biological activity studies carried out in other models, with some species obtained from Santa Catarina, as *C. brasiliense*, *I. pes-caprae*, *M. eleagnoides*, *M. robusta*, *R. imperialis* and *V. scorpioides*, and the lack of information on the immunomodulatory activity of these plants, this study aimed to evaluate the activity of methanol extracts of these six medicinal plants on the proliferation of murine spleen cells *in vitro*.

MATERIAL AND METHODS

Plant material collection

The studied plants – roots of *C. brasiliense*, whole plant of *I. pes-caprae*, shells of *M. elaeagnoides*, aerial

parts of *M. robusta* and *R. imperialis,* plus leaves and flowers of *V. scorpioides*, were previously collected in Santa Catarina, in the same place of origin and seasonal period as the specimens deposited in the Herbarium Barbosa Rodrigues, Itajaí-SC, under voucher numbers VC Filho 007, V.C. Filho 009, MTS 001, V.C. Filho 016, V.C. Filho 012, HBR and M. Biavatti 11, respectively. The methanol extracts were prepared from dried, triturated plant material. The material collected was macerated for 10 days in a closed container at room temperature, protected from light, using methanol as the fluid extractor. The material obtained was filtered and concentrated by evaporation and negative pressure. A preliminary investigation of the composition of the extracts were performed using spectroscopy methods such as infrared, ^{1}H and ^{13}C nuclear magnetic resonance, and bidimensional techniques (Krogh *et al.*, 1999; Niero *et al*., 1999; Silva *et al*., 2000; Niero *et al*., 2001; Souza, 2006; Buskühl, 2007). The methanol extracts obtained were kept in the dark in desiccators, together with silica to remove the moisture. The extracts were solubilized with 2% dimethylsulfoxide (DMSO, VETEC Química Fina LTDA, Duque de Caxias, RJ), resulting in a concentration of 1 mg/mL in DMEM culture medium (Dulbelccos's Modified Eagles Medium, Sigma Inc., St . Louis, MO, USA). These preparations were filtered at 0.22 µm, aliquoted and stored at -20 °C in the dark, until use.

Spleen cell collection

This study was conducted in accordance with the ethical standards for research involving animals, with the prior approval of the University of Vale do Itajaí (UNIVALI) Research Ethics Committee (No. 131/2006). A total of sixty male Swiss mice, weighing between 20 and 30 grams, were obtained from the vivarium of UNIVALI. The spleen cells were obtained aseptically by mechanical disruption of the capsule of the spleen of animals killed by cervical dislocation. The determination of cell concentration in a Neubauer chamber was performed after lysis of red blood cells with hypotonic shock, together with verification of cell viability by the trypan blue exclusion test (VETEC) (Boyum, 1968). Only cell suspensions with viability exceeding 90% were used.

Cell culture

The DMEM cell culture medium used was supplemented with 10% fetal bovine serum (CULTILAB, Campinas, SP), 2% sodium bicarbonate at 10% (Dinâmica, São Paulo, SP), 1% L-glutamine at 200 mM (Sigma), 1% HEPES at 10 mM (Sigma) and 110 mg/mL sodium pyruvate (VETEC). The cell culture was performed in microtiter plates (TTP, Techno Plastic Products, Trasadingen, Switzerland) with incubation of the cells (50,000 cells/mL) and methanol extracts (10, 50, 100 and 200 µg/mL) in the presence or absence of PHA (Sigma, 5 µg/mL) for 72 hours at 37 °C and in 5% CO_2 (Jouan, model IG150, Saint Herbain, France) (Yasni *et al.*, 1993; Roseghini *et al.*, 2006). The cell and mitogen concentrations were determined by the growth curve (data not shown), while the concentrations of the extracts were defined according to the literature (Wilasrusmee *et al.*, 2002; Manosroi, Saraphanchotiwitthaya, Manosroi, 2003; Mehrotra *et al.*, 2003). The assay revelation was performed by blue tetrazolium reduction assay (MTT), with the addition of 10 µL of MTT at 5 mg/mL (Amreco, Solon, Ohio, USA) in 0.9% NaCl to each well of the microplate three hours before the end of the incubation period. At the end of the incubation period, 100 µL of sodium dodecyl sulfate 10% was added (SDS, Amreco) in HCl 0.001 N (VETEC) to each well. The plate was incubated overnight and optical density (OD) determined at 540 nm (Quick ELISA, Drake, São Paulo, SP) (Mosmann, 1983; Denizot, Lang, 1986).

Cell response to PHA was used as a positive control for cell proliferation, while the absence of contamination and residual staining of cells and extracts (basal control) were obtained when they were kept alone in the culture medium. The colorimetric method of MTT reduction can be used to measure cytotoxicity, proliferation and even cell activation, since the determination of the activity of mitochondrial dehydrogenase of living cells directly and proportionally represents the number of cells (Mosmann, 1983). The results were presented as a percentage of growth, according to Manosroi, Saraphanchotiwitthaya and Manosroi (2003) and Risco *et al.* (2003), through equation 1. This indicator eliminates the residual reduction of MTT by cells not exposed to the extract or mitogen, as well as the background color of the methanol extract and the inter-assay variations. All the results were expressed as the mean and standard deviation of four experiments performed in triplicate.

$$\% \text{ of growth} = \frac{(\text{OD test} - \text{OD basal control})}{\text{OD basal control}} \qquad (1)$$

Analysis and discussion of data

The dose-response effect was evaluated by the coefficient of determination, obtained from the polynomial trend line in the order 3, with a value of $R^2 = 1.0$ for all the extracts. The data were also analyzed by the Kruskal-Wallis and Mann-Whitney tests.

RESULTS AND DISCUSSION

Studies with natural products involve biological activity and pharmacological assays for the screening of plant extracts, with subsequent evaluation of the fractions and isolated compounds responsible for the activity observed (Cechinel Filho, Yunes, 1998). In this context, there are several aspects to be considered when choosing a plant to be studied, one of which is its use in folk medicine (Yunes, Cechinel Filho, 2001; Yunes, Cechinel Filho, 2007). The immunomodulatory effect in the cell proliferation model has been a target of study in the search for new therapeutic agents of natural origin (Souza-Fagundes *et al.*, 2002; Pandima Devi *et al.*, 2003; Arokiyaraj *et al.*, 2007; Sriwanthana *et al.*, 2007). The activation of the immune response in this model promotes cell proliferation with an increase in the number of cells present in the culture within a defined period (Rocha, Goresco, Beltrame, 2007) and can be identified through MTT reduction by mitochondrial dehydrogenase of living cells (Mosmann, 1983).

The methanol extract of *C. brasiliense* assessed in this study induced an increase in cell proliferation (Figure 1A) in a dose-dependent response (Figure 2A). The average percentage of cell growth ranged from 5.8 to 88.2% and from 15.3 to 96.3%, for the extract alone and extract together with PHA in the cell culture, respectively. The extract at 100 and 200 µg/mL under both cell culture conditions showed significantly higher values ($p = 0.02$) than the positive control of cell proliferation (12.2%).

PHA is the most applicable lectin in the activation of T lymphocytes *in vitro*, and enables the evaluation of immune response mediated by this cell type (Rocha, Gorescu, Beltrame, 2007). Thus, the results obtained with the methanol extract of *C. brasiliense* suggest that the component(s) present in the plant stimulate T lymphocyte proliferation, supposedly activating the cellular immune response. It should be noted that the study of this model against lipopolysaccharide (LPS) and other lectins such as concanavalin A and pokeweed mitogen, enable the evaluation of the effect of the extract on other cell types involved in the defense process. However, the preliminary data obtained in our study provides guidance for biomonitored follow up studies with fractions and isolated substances from plants that have shown promising results.

In previous studies, phytochemical investigations of the *Calophyllum* genus have shown the presence of a large variety of compounds, such as xanthones, phenolic acids, coumarins, flavonoids, biflavonoids, chalcones, benzophenones and triterpenes (Noldin, Buffon, Cechinel Filho, 2006), quercetin, gallic and protocatetic acid, hyperoside, amantoflavona (Silva *et al.*, 2001) and betulinic acid (Buffon, 2005). The tricyclic coumarin GUT-70, isolated from the bark of *C. brasiliense*, was characterized as a natural agent against cancer by inhibiting the growth of six strains of human leukemia cells, without causing inhibition of proliferation of white blood cells and normal hepatocytes (Ito *et al*., 2003; Kimura *et al*., 2005).

Immunomodulatory activity has been reported for other compounds present in *C. brasiliense,* when extracted from other species. For instance, the gallic acid present in other species prevents DNA oxidation, preventing cell death by accumulation of nitric oxide in the culture medium (Kuhlmann *et al*., 1998). The coumarin extracted from *Hydrangeae dulcis folium* had no effect on the response of human T lymphocytes stimulated with PHA (Shimoda *et al*., 1998), while triterpenes from the bark of *Quercus suber* proved a potent inhibitor of human lymphocyte proliferation, as well as reducing the growth of the human cancer cell strain (Moiteiro *et al*., 2001). Triterpenes isolated from the leaves of *Justicia gendarussa* also showed inhibition of human lymphocyte proliferation, with maximum inhibition of 85%,when using a methanol extract of the plant (Arokiyaraj *et al*., 2007). Although the triterpenes reported in the literature as inhibitors of lymphocytes proliferation are also present in *C. brasiliense*, their concentration in the extract used in this study may be lower than the other compounds that stimulated cell proliferation. This finding strongly indicates a need for further studies of fractions and isolated compounds obtained from *C. brasiliense*, to identify those responsible for the toxic effects on tumor cells and cytoprotective effect in normal cells, and also those responsible for stimulating the proliferation of T lymphocytes, activity observed in this study.

The methanol extract of *I. pes-caprae* induced dose-dependent proliferation of murine spleen cells (Figures 1B and 2B). The average rate of cell growth ranged from 13.4 to 73.1% when the cells were treated with the extract alone, and 2.9% to 63.9% for cells treated with extract and PHA combined. Under both culture conditions, the extract at 200 µg/mL showed significantly higher cell growth in comparison to the positive control of cell proliferation (12.2%) ($p = 0.02$). As observed for the methanol extract of *C. brasiliense*, the results suggest that the component (s) present in the methanol extract of *I. pes-caprae* stimulate the proliferation of T lymphocytes.

Phytochemical studies of *I. pes-caprae* have identified the presence of steroids, terpenoids, alkaloids and flavonoids (Souza *et al*., 2000), β-damascenone, E-phytol, betulinic acid, triperpenos, α- and β-amyrin, acetates, isoquerticinas (Pongprayoon, Bohlin, Wasuwat, 1991; Souza *et al.*, 2000) and isoprenoids (Krogh *et al*., 1999). Although triterpenes and flavonoids have been described

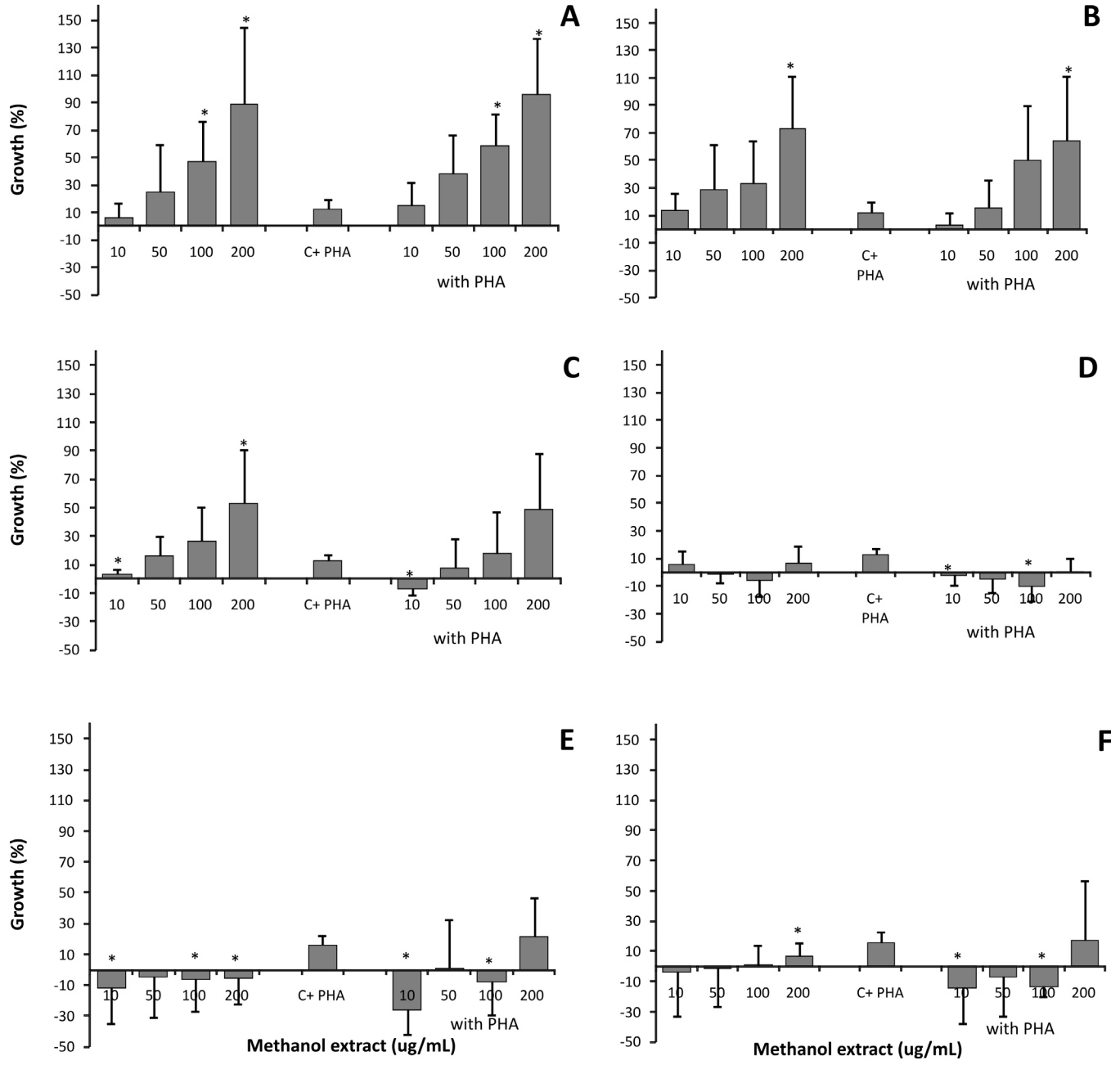

FIGURE 1 - Percentage growth of murine spleen cells treated with methanol extracts from different medicinal plants, in the presence or absence of mitogenic stimulation with phytohemagglutinin (PHA), for 72 hours at 37 °C and 5% CO_2. A: *Calophyllum brasiliense*; B: *Ipomea pes-caprae*; C: *Matayba elaeagnoides*; D: *Maytenus robusta*; E: *Rubus imperialis*, F: *Vernonia scorpioides*; C+PHA: positive control phytohemagglutinin (PHA), * $p < 0.05$ compared to C+PHA. Results are presented as the mean and standard deviation of four experiments performed in triplicate.

in the literature as inhibitors of human lymphocytes proliferation (Moiteiro *et al.*, 2001; Brochado *et al.*, 2003; Arokiyaraj *et al.*, 2007), the action of these compounds appears to be inhibited by other compounds with immunostimulating effects, such as alkaloids (Sheng, Bryngelsson, Pero, 2000), since the results obtained showed an increase in cell proliferation and possibly activation of T lymphocytes. Given the finding of the stimulating lymphocyte response and that the literature describes the betulinic acid, also present in this species, as a promising anticancer agent against melanoma by inhibiting the growth of cancer cells and inducing apoptosis (Pettit, 1996), the continuity of studies with fractions and isolated compounds of this plant can contribute to advances in the field of immunotherapy.

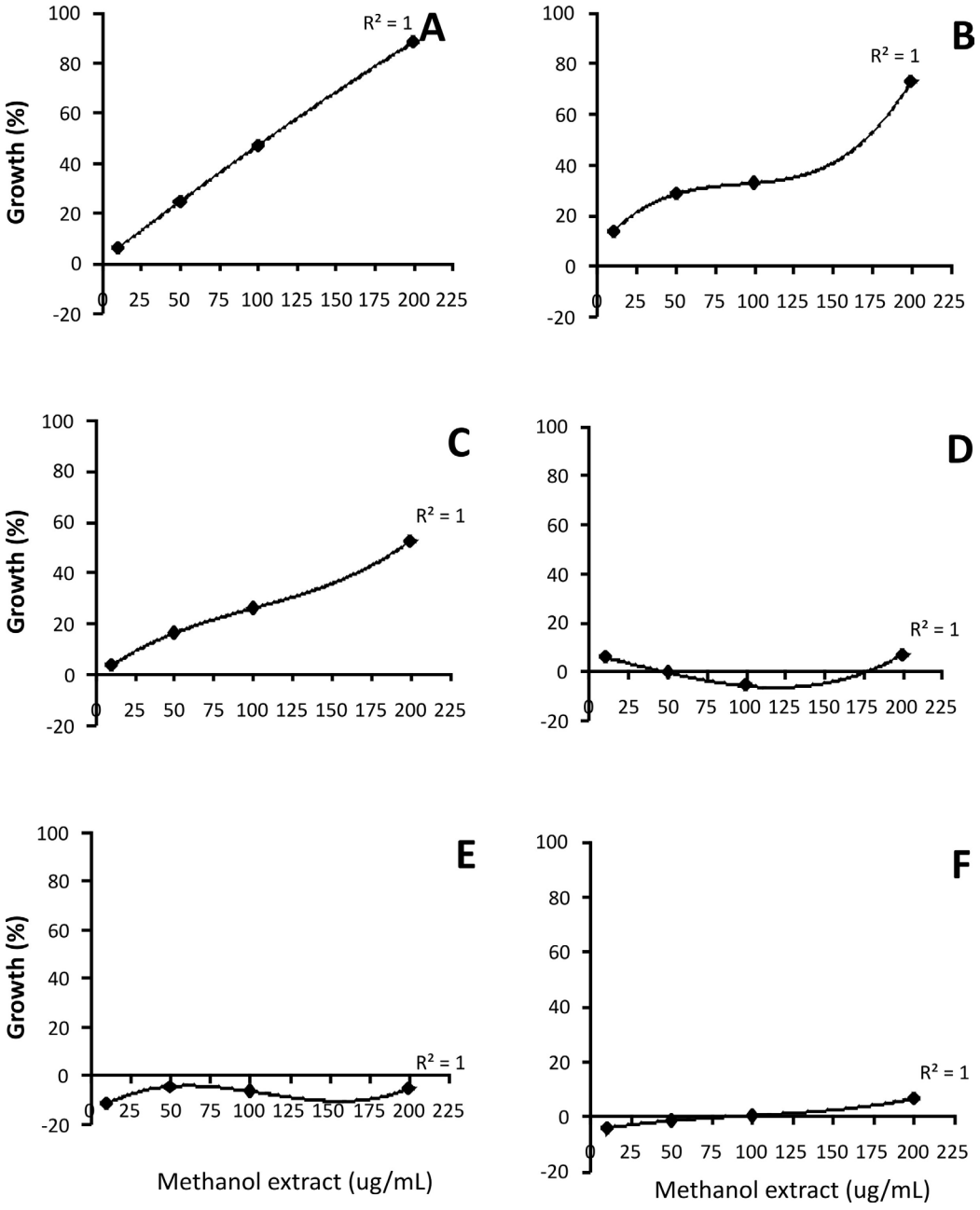

FIGURE 2 - Dose-response curve of methanol extracts from different medicinal plants in the proliferation assay with murine spleen cells for 72 hours at 37 °C and 5% CO_2. A: *Calophyllum brasiliense*; B: *Ipomea pes-caprae*; C: *Matayba elaeagnoides*; D: *Maytenus robusta*; E: *Rubus imperialis*, F: *Vernonia scorpioides*; R^2: coefficient of determination. Results are presented as the mean of four experiments performed in triplicate.

The methanol extract of *M. elaeagnoides* showed dose-dependent induction of spleen cell proliferation (Figures 1C and 2C). The average percentage of cell growth was from 3.4 to 52.7% and -7.0 to 48.8%, for extract alone and extract combined with PHA in cell culture, respectively. Under both culture conditions, the extract at 10 µg/mL induced significantly lower cell growth than the positive control PHA (12.5%) ($p = 0.03$), while at 200 µg/mL the extract induced higher cell growth than the positive control ($p < 0.02$). These data suggest that there is greater activation of T lymphocytes in the presence of high concentrations of the extract.

Several substances were previously isolated from the methanol extract of the bark of *M. elaeagnoides*, including steroids, coumarins, triterpenes (lupeol, α- and β- amyrin, betulin), sitosterol, scopoletin, and flavonoids, among

others (Souza, 2006). Although the coumarins extracted from other medicinal plants have shown no influence on the proliferation of human lymphocytes (Shimoda *et al*., 1998) whereas flavonoids and triterpenes inhibited the proliferation of these cells (Patocka, 2003; Moiteiro *et al.*, 2001; Reyes *et al.*, 2006; Arokiyaraj *et al.*, 2007), betulin, another compound present in *M. elaeagnoides*, has shown mitogenic activity and the ability to induce and modulate cytokine production in cultured human cells (Zdzisińska *et al*., 2003).

The methanol extract of *M. robusta* induced the average rate of cell growth by -5.8% to 7.0% for the extract alone and -10.2% to 0.6% for the extract plus PHA in cell culture (Figure 1D) yielding a dose-dependent response (Figure 2D). The positive control of cell growth was 12.5%, differing significantly between the extract at 10 and 200 µg/mL, with both concentrations being responsible for the inhibition of cell growth induced by the mitogen ($p = 0.02$). These results suggest that the *M. robusta* extract has compounds that inhibit the proliferative activity of murine spleen cells, while also inhibiting the mitogenic activity of PHA.

Phytochemical analysis of *M. robusta* demonstrated that it contains triterpene and phenolic compounds, as does *Maytenus ilicifolia*, popularly known in Brazil as "espinheira-santa" (Niero *et al.*, 2001; Jorge *et al.*, 2004). The triterpene friedelin appears to be the main component responsible for the action of this species against ulcers and gastritis, while in other plants it has been associated to inhibition of human lymphocyte proliferation (Moiteiro *et al.*, 2001; Arokiyaraj *et al.*, 2007). The presence of this triterpene in *M. robusta* suggests that the biological activity of this compound is predominant over other compounds in the murine spleen cell culture.

The inhibition of murine spleen cell proliferation was also observed with methanol extract of *R. imperialis*, with average growth of -11.8% to -4.4% for extract alone and -26.2% to 21.6% for extract with the mitogen in cell culture (Figure 1E). No dose-response relationship was found under either of the culture conditions (Figure 2E), with results reaching significance versus the positive control of cell proliferation (15.9%) when the extract was used at both 10 and 100 µg/mL ($p < 0.04$).

R. imperialis has triterpene constituents including ichigosídeo niga-F-1, tormentic and 23-hydroxy-tormentic acid (Niero *et al*., 1999). The inhibitory effect on the proliferation of murine spleen cells treated with *R. imperialis* methanol extract in this study confirmed the reports in the literature regarding the inhibitory activity of triterpenes on human lymphocyte proliferation (Moiteiro *et al.*, 2001; Brochado *et al.*, 2003; Arokiyaraj *et al.*, 2007). Further studies with fractions and substances isolated from this species could identify those responsible for this inhibitory activity.

The last plant analyzed was *V. scorpioides*, which also showed inhibition of cell proliferation. The average growth obtained was -4.1% to 6.7% with the methanol extract alone and -13.9% to 16.9% with combined extract and PHA in cell culture (Figure 1F). Again, no dose-response relationship was observed under either of the culture conditions (Figure 2F), with a significance result only for the extract at 100 µg/mL versus the positive control of cell proliferation (15.9%) ($p < 0.04$).

Although it was not possible to confirm the cytotoxic activity of the *V. scorpioides* extract in this study, the literature has reported this activity in different models, typically related to the presence of sesquiterpene lactones (Pagno *et al.*, 2006; Buskühl, 2007), as well as another compound isolated from the plant, polyacetylene 5-octa-2,4,6-triinyl-furan-2(5H)-one (Buskühl, 2007). The cytotoxic activity of sesquiterpene lactones and polyacetylene obtained from the dichloromethane fraction of *V. scorpioides* on Hela cells has recently been reported, with induction of apoptosis and increased expression of caspase 3 by polyacetylene (Buskühl, 2007). The dichloromethane fraction of this species increased the influx of neutrophils into the peritoneal cavity in mice with Ehrlich tumor, completely inhibiting the development of the tumor (Pagno *et al.*, 2006).

The study of suppressor or stimulatory effects on cell proliferation using specific methodologies and molecular techniques may contribute to the elucidation of the mechanisms involved in the effects observed. The evaluation of immune stimulation can be determined by markers of cellular activation and proliferation, such as CD69 (Maino, Suni, Ruitenberg, 1995; Craston *et al.*, 1997) and KI67 (Starborg *et al.*, 1996; Scholzen, Gerdes, 2000; Kausch *et al.*, 2003). In contrast, inhibition of cell proliferation and cytotoxic effect may assessed by the use of specific methodologies for the identification of necrosis and apoptosis such as LDH dosage in the supernatant of cell culture (Cruz-Chamorro *et al*., 2006) and detection of caspase 3 by immunocytochemistry (Krajewska *et al*., 1997) or through the release of p-nitroalinine (Posmantur, Wang, Gilbertsen, 1998).

In short, the need for control and equilibrium between the activities of suppression and stimulation of immune response for proper functioning of the immune system, has promoted the identification and characterization of natural compounds with immunomodulatory activity as an area of research interest (Phillipson, 2003). The characterization of medicinal plants from the Brazilian flora presented in this study should guide further studies

on the theme which enable identification of compound(s) responsible for the most promising activities observed, i.e. the stimulating action on the proliferation of murine spleen cell by methanol extracts of *C. brasiliense*, *I. pes-caprae* and *M. elaeagnoides*, and the inhibitory action of extracts of *M. robusta R. imperialis* and *V. scorpioides*, as well as the mechanisms underlying these biological properties.

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