Acta Poloniae Pharmaceutica – Drug Research, Vol. 67 No. 1 pp. 69–73, 2010 ISSN 0001-6837
Polish Pharmaceutical Society

NATURAL DRUGS

IMMUNOSTIMULATORY ACIVITY OF *CALOPHYLLUM BRASILIENSE*, *IPOMOEA PES-CAPRAE* AND *MATAYBA ELAEAGNOIDES* DEMONSTRATED BY HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS PROLIPHERATION

MARINA ELISA PHILIPPI, BRUNA MOMM DUARTE, CAROLINA VIEIRA DA SILVA, MICHEL THOMAZ DE SOUZA, RIVALDO NIERO, VALDIR CECHINEL FILHO and EDNEIA CASAGRANDA BUENO*

Programa de Mestrado em Ciências Farmacêuticas and Núcleo de Investigações Químico-Farmacêuticas, Universidade do Vale do Itajaí (UNIVALI), Rua Uruguai, 458, bloco 17, Itajaí-SC, 88.302-202, Brazil

Abstract: This study evaluates the effect of methanol extracts of three Brazilian medicinal plants on *in vitro* proliferation of human mononuclear cells. Lymphoproliferation assay was carried out by incubating human peripheral blood mononuclear cells from healthy donors (1 x 10^6 cells/mL) with extracts of *Calophyllum brasiliense* (roots), *Ipomoea pes-caprae* (whole plant) and *Matayba elaeagnoides* (bark), both at 10, 50, 100 and 200 µg/mL, alone or with phytohemagglutinin (PHA, 5 µg/mL), in 96-well microplates at 37°C with 5% CO_2, for 72 h. The quantification of cell proliferation assay was performed by blue tetrazolium (MTT) reduction with reading at 540 nm. Cells incubated with only the culture medium were used as negative control for cell proliferation, while the positive control consisted of cells and PHA. The results suggest that the extracts of all three studied plants induce T lymphocyte proliferation. *I. pes-caprae* showed immunostimulatory activity three times higher than the *C. brasiliense* extract, while that of the *M. elaeagnoides* extract was 1.5 times higher. The results demonstrate immunostimulatory effects of these three plants, therefore the continuity of these studies is recommended, in order to determine the active principles.

Keywords: *Calophyllum brasiliense,* immunostimularory activity, immunomodulatory activity, *Ipomoea pes-caprae*, *Matayba elaeagnoides*, medicinal plants, mononuclear cells

The use of medicinal plants as a therapeutic resource is common in Brazilian folk medicine (1, 2) and studies have been carried out to scientifically confirm their effects. The plants are recognized for their ability to produce a large amount of secondary metabolites, many of which have biological and pharmacological activities, used as a starting point for the development of modern drugs (3).

As part of a program to search for natural products with immunostimulatory activity, we have selected three Brazilian medicinal plants, *Calophyllum brasiliense, Ipomoea pes-caprae* and *Matayba elaeagnoides*. The first, *C. brasiliense* (Clusiaceae) is associated with ulcer treatment, inflammation and pain, and has been proven to have anti-secretory, cytoprotectant and antimicrobial activities, among others (4, 5). *I. pes-caprae* (L.) R. Br. (Convolvulaceae), popularly used to treat inflammation, colic, diuretic disorders, gonorrhea and pain, has demonstrated its usefulness for treating pain associated with local reaction after contact with jellyfish as well having antinociceptive and antispasmodic activities (6-9). For *M. elaeagnoides* RADLK (Sapindaceae), which is popularly used to treat inflammation, analgesia and liver cancer, studies have confirmed its antinociceptive, antimicrobial, antifungal and antiparasite activities (10).

Immunomodulatory activity has been a focus of study by researchers in the field of natural products. The lymphocyte proliferation assay, also known as lymphoblastogenesis or blastic transformation, is an *in vitro* test that shows the lymphocyte stimulation or suppression promoted by the extract or compound, whether or not this occurs simultaneously to mitogens as phytohemagglutinin (PHA) (11, 12). This study seeks to evaluate the activity of methanol extracts of the above-mentioned plants on the *in vitro* proliferation of human mononuclear cells.

* Corresponding author: e-mail: ecbueno@univali.br, phone: 55-47-3341-7540, fax: 55-47-3341-7501

EXPERIMENTAL

Plant material

The botanical material used was *C. brasiliense* roots, the whole plant of *I. pes-caprae*, and *M. elaeagnoides* bark, collected in Florianópolis-SC-Brazil, Jaguaruna-SC-Brazil and Caçador-SC-Brazil, respectively. Voucher specimens were deposited at the Barbosa Rodrigues Herbarium, Itajaí, Santa Catarina, Brazil under numbers VC Filho 007, VC Filho 009 and MTS 001, respectively. The extracts were prepared from dried and powdered plant material using methanol as liquid extractor by maceration. After filtration, the solvent was removed by rotary evaporation under reduced pressure, yielding the respective methanol extracts. The methanol extracts were stored in desiccators with silica, until use, when they were reconstituted with 2% of dimethylsulfoxide (DMSO) in the culture medium Dulbecco's Modified Eagle's Medium (DMEM, Sigma Inc., St. Louis, MO, USA) and then filtered (0.22 µm).

Biological material

Peripheral blood samples from 100 healthy volunteers were collected with heparin, and mononuclear cells were obtained by density gradient separation (Ficoll-PaqueTM Plus, Amersham Biosciences, Uppsala, Sweden). The cell viability was determined by Trypan blue (13), using only the samples with viability greater than 95%. This study was approved by the Research Ethics Committee/ UNIVALI under number 06/06.

Lymphocyte proliferation assay

The DMEM used in the assay was supplemented with 10% fetal bovine serum, 2% sodium bicarbonate at 10%, 1% L-glutamine at 200 mM, 1% HEPES at 10 mM and 110 mg/mL of sodium pyruvate. The assay was performed using 96-well cell culture plates (TTP – Techno Plastic Products, Trasadingen, Switzerland) at 37°C with 5% CO_2, for 72 h (12, 14). The effect of the extracts on mononuclear cell proliferation at 10, 50, 100 and 200 µg/mL (1×10^6 cells/mL) was evaluated in the presence and absence of mitogen PHA (5 µg/mL). The assay included internal controls in order to determine the presence of contamination. The cell culture alone with supplemented DMEM was used as negative control for cell proliferation, while the cell culture exposed only to PHA was used as positive control for cell proliferation. Cell proliferation was identified by MTT reduction (15) and the color intensity was quantified by optical density (OD) at 540 nm (Quick ELISA, Drake, São Paulo, Brazil). The results of the lymphocyte proliferation assay were presented as a percentage of average growth of four experiments performed in triplicate, eliminating the variation between the tests [% growth = 100 × (ODtest – ODnegative control) / ODnegative control] (16, 17).

Statistical analysis

The results were evaluated by the Kruskal-Wallis and Mann-Whitney tests, to determine their statistical significance ($p < 0.05$). The correlation between the concentration of the extract and its effect on cell proliferation was assessed by the growth curve obtained from the polynomial tendency line with order 3 and $R^2 = 1.0$.

RESULTS

The methanol extract of *C. brasiliense* induced human mononuclear cell growth, with average percentages of 20.6% to 47.4% in the absence of mitogen PHA, and 14.3 to 35.3% in the presence of PHA (Fig. 1A). The extract showed cell proliferation stimulation, and was significantly higher than the positive control for cell proliferation (9.7%) when incubated alone in culture at concentrations of 50 µg/mL, 100 µg/mL and 200 µg/mL ($p \leq 0.041$). The growth curve obtained from the polynomial tendency line with order 3 and $R^2 = 1.0$ showed a dose-response effect only at concentrations below 100 µg/mL, when the methanol extract of *C. brasiliense* was incubated together with the mitogen.

The methanol extract of *I. pes-caprae* promoted human mononuclear cell growth, with an average growth percentage of 90.6% to 140.8%, when the extract was incubated alone in cell culture, and 106.5% to 162.4% when it was added together with the PHA (Fig. 1B). This extract showed cell proliferation stimuli that was significantly higher than the positive control for cell proliferation (27.3%) when the extract (200 µg/mL) was maintained with mitogen in the cell culture ($p = 0.038$). The cell growth observed with methanol extract of *I. pes-caprae* showed no dose-response correlation.

The methanol extract of *M. elaeagnoides* also resulted in stimulus for the human mononuclear cells, both in the absence of PHA (29.0% to 95.6%) and in the presence of mitogen (71.0% to 153.5%) (Fig. 1C). The cell growth was significantly higher than the positive control for cell proliferation (15.4%) when the extract was incubated alone in the cell culture, at 100 µg/mL and at 200 µg/mL ($p = 0.029$), and also when it was incubated simultane-

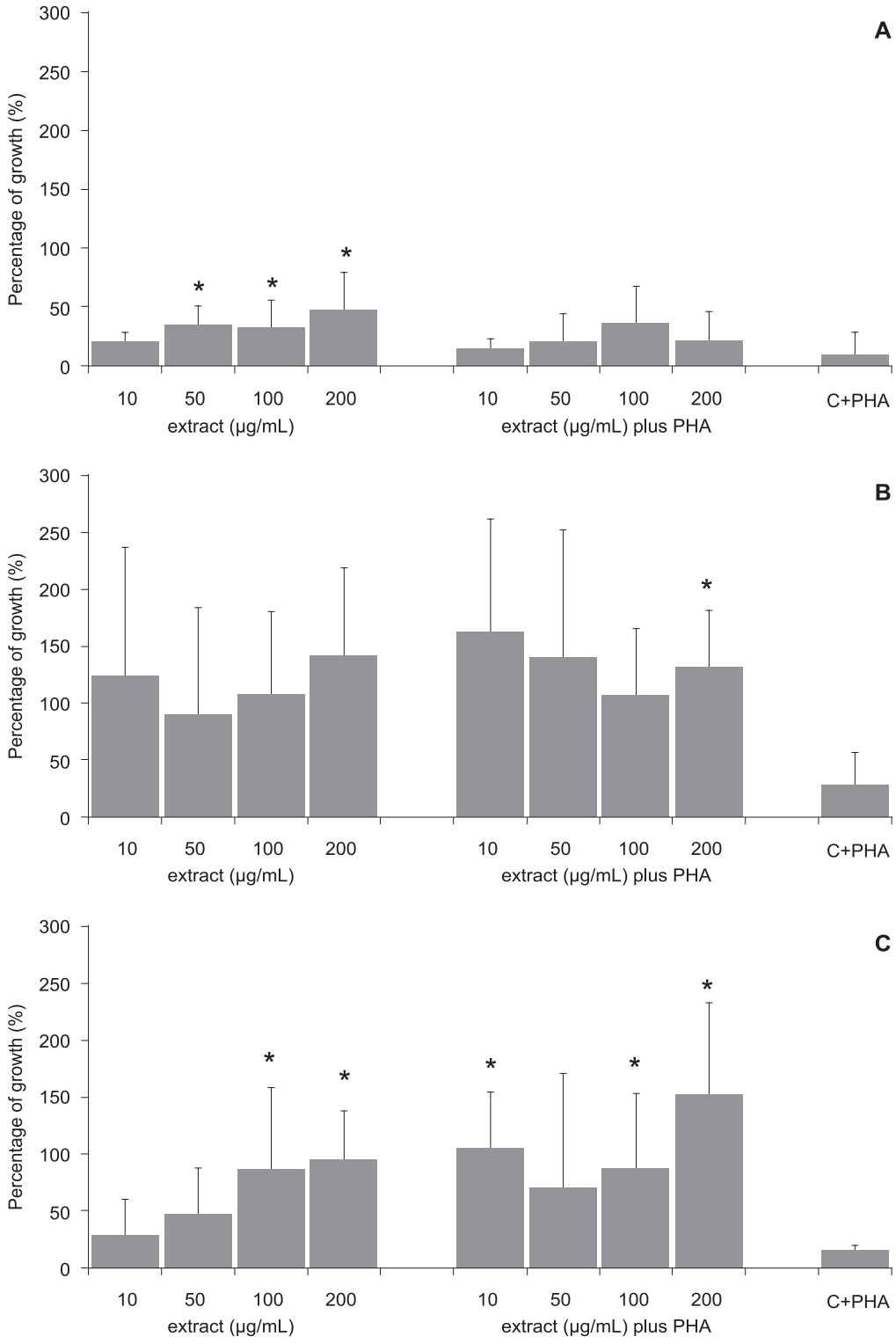

Figure 1. Human mononuclear cells proliferation by methanolic extracts of *Calophyllum brasiliense* (A), *Ipomoea pes-caprae* (B) and *Matayba elaeagnoides* (C), in the presence and absence of phytohemaglutinin (PHA, 5 µg/mL), for 72 h at 37ºC and 5% CO_2. C+ PHA: phytohemaglutinin positive control (5 µg/mL), *$p < 0.05$ compared to C+. Results presented as the average and standard deviation of four experiments performed in triplicate

ously with PHA, at concentrations of 10 µg/mL, 100 µg/mL and 200 µg/mL ($p = 0.029$). Considering only the results with significant difference when compared to positive control for cell proliferation, the methanol extract of *M. elaeagnoides* showed no dose-response effect on cell proliferation.

DISCUSSION AND CONCLUSION

Since the 1980s, the need for control and balance between the stimulatory and suppressor activities of the immune system has led to the identification and characterization of natural compounds with immunomodulatory activity. The modern immune therapy involves the use of immunomodulators for the treatment of diseases caused by hypersensitivity or immunodeficiencies, in addition to their traditional use in transplants. These drugs may exacerbate or reduce the immune response from topical or systemic use, and are divided into two main groups, according to their effect on the immune system: immunostimulating drugs, which increase innate and adaptive immunity, and immunosuppressants, which decrease the activity of the immune system (3).

Most of the immunomodulators emerged empirically, but the understanding of the pathophysiology of the immune system has facilitated the development of new immunomodulators and also the identification of their mechanisms of action (3). The identification and study of substances that act with the various components and mechanisms of the immune system, aimed at maintaining health, has been the focus of study by several research groups (11, 12, 18, 19).

The methanol extracts from *C. brasiliense*, *I. pes-caprae* and *M. elaeagnoides* evaluated here, acted as stimuli for *in vitro* mononuclear cell human proliferation. The dose-response effect was observed only at concentrations of less than 100 µg/mL when the methanol extract of *C. brasiliense* was incubated with mitogen in cell culture. Lymphoproliferation assay with 72 h of incubation and PHA as mitogenic stimuli is conventionally used to verify T lymphocyte activation (11, 12, 14, 20). Considering this use of the lymphoproliferation assay, the results obtained from all the plants suggest that both extracts induce the proliferation of T cells.

The phytochemical studies showed that *C. brasiliense* possesses a wide variety of compounds, including xanthones, coumarins, biflavonoids, chalcones, benzophenones and triterpenes (4, 5, 21-24). *I. pes-caprae* has indicated the presence of steroids, terpenoids, flavonoids and alkaloids (6, 7). The phytochemical evaluation of *M. elaeagnoides* allowed the isolation of substances including steroids, coumarins, triterpenes and flavonoids, among others (10).

The coumarin tricyclic GUT-70 isolated from bark of *C. brasiliense* was characterized as a natural agent against cancer, inhibiting the growth of six human leukemic cells lineage without causing inhibition of white blood cells and normal hepatocyte proliferation (25, 26). Added to this, the betulinic acid, common to the three species studied, was described as a mitogenic inducer with the ability to stimulate and modulate *in vitro* cytokine production by human cells (27).

In contrast, triterpenes and flavonoids have been described as inhibiting lymphocyte proliferation (12, 28). The triterpene friedelin had been reported as inhibiting human lymphocyte proliferation (12, 29) and also inhibiting the growth of human cancer cells (29). However, the results obtained here suggest that the presence of triperpenes and flavonoids in methanolic extracts of the plants studied are present in lower concentrations than other compounds that stimulate cell proliferation. Therefore, it seems that compounds such as coumarins and betulinic acid have an overlap activity in these extracts in cell culture, retaining the stimulation of lymphocyte proliferation. Furthermore, the substances present in *C. brasiliense* suggest a toxic effect on tumor cells (25, 26), but the results obtained here indicate a cytoprotecting effect on normal cells, as well as stimulating immune response.

The methanol extract of *I. pes-caprae* showed immune stimulatory activity 3.0 times higher than the extract of *C. brasiliense* in the absence of PHA, and 3.6 times higher when incubated with PHA. The *I. pes-caprae* extract stimulus was also 1.5 times higher than that of *M. elaeagnoides* extract in the absence of PHA, and similar when the extracts were incubated in the presence of PHA. The *M. elaeagnoides* methanolic extract showed stimulus of human mononuclear cell proliferation 2.0 times higher than that of *C. brasiliense* extract in the absence of PHA, and 3.3 times higher when incubated together with PHA.

The immune stimulating activity of *C. brasiliense*, *I. pes-caprae* and *M. elaeagnoides* methanolic extracts on *in vitro* human mononuclear cells observed in this study indicate the continuity of the biomonitored study with fractions and isolated compounds from these medicinal plants, in order to identify the compounds responsible for this effect and promote advances in the field of immunotherapy.

Acknowledgements

We are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Apoio à Pesquisa Científica e Tecnológica do Estado de Santa Catarina (FAPESC) and Universidade do Vale do Itajaí (UNIVALI) for financial support.

REFERENCES

1. Phillipson J.D.: Planta Med. 69, 491 (2003).
2. Wagner H.: in Química de produtos naturais, novos fármacos e a moderna farmacognosia, Yunes R.A., Cechinel Filho V. Eds., p. 35, Univali, Itajaí 2007.
3. Lima H.C.: An. Bras. Dermatol. 82, 207 (2007).
4. Gasparotto Jr A., Brenzan M.A., Piloto I.C., Cortez D.A.G., Nakamura C.V., Dias Filho B.P., Rodrigues Filho E., Ferreira A.G.: Quím. Nova 28, 575 (2005).
5. Noldin V.F., Isaias D.B., Cechinel Filho V.: Quím. Nova 29, 549 (2006).
6. Pongprayoon U., Baeckstrom P., Jacobsson U., Lindstrom M., Bohlin L.: Planta Med. 58, 19 (1992).
7. Krogh R., Kroth R., Berti C., Madeira A.O., Souza M.M., Cechinel-Filho V., Delle-Monache F., Yunes R.A.: Pharmazie 54, 464 (1999).
8. De Jesus R.A., Cechinel-Filho V., Oliveira A.E., Schlemper V.: Phytomedicine 7, 111 (2000).
9. de Souza M.M., Madeira A., Berti C., Krogh R., Yunes R.A., Cechinel-Filho V.: J. Ethnopharmacol. 69, 85 (2000).
10. de Souza M.T., Buzzi Fde C., Cechinel Filho V., Hess S., Della Monache F., Niero R.: Z. Naturforsch. 62, 550 (2007).
11. Pandima Devi K., Sai Ram M., Sreepriya M., Ilavazhagan G., Devaki T.: Biomed. Pharmacother. 57, 105 (2003).
12. Arokiyaraj S., Perinbam K., Agastian P., Balaraju K.: Indian J. Pharmacol. 39, 180 (2007).
13. Boyum A.: Scand J. Clin. Lab. Invest. Suppl. 97, 7 (1968).
14. Bueno E.C., Vaz A.J., Machado L.R., Livramento J.A., Avila S.L., Ferreira A.W.: Clin. Exp. Immunol. 126, 304 (2001).
15. Mosmann T.: J. Immunol. Methods 65, 55 (1983).
16. Risco E., Ghia F., Vila R., Iglesias J., Alvarez E., Canigueral S.: Planta Med. 69, 785 (2003).
17. Manosroi A., Saraphanchotiwitthaya A., Manosroi J.: J. Ethnopharmacol. 89, 155 (2003).
18. Souza-Fagundes E.M., Queiroz A.B., Martins Filho O.A., Gazzinelli G., Correa-Oliveira R., Alves T.M., Zani C.L.: Mem. Inst. Oswaldo Cruz 97, 1207 (2002).
19. Sriwanthana B., Treesangsri W., Boriboontrakul B., Niumsakul S., Chavalittumrong P.: Songklanakarin J.: Sci. Technol. 29, 17 (2007).
20. Bessler H., Salman H., Bergman M., Straussberg R., Djaldetti M.: Clin. Immunol. 117, 73 (2005).
21. Ito C., Itoigawa M., Miyamoto Y., Rao K.S., Takayasu J., Okuda Y., Mukainaka T., Tokuda H., Nishino H., Furukawa H.: J. Nat. Prod. 62, 1668 (1999).
22. da Silva K.L., dos Santos A.R., Mattos P.E., Yunes R.A., Delle-Monache F., Cechinel-Filho V.: Therapie 56, 431 (2001).
23. Abe F., Nagafuji S., Okabe H., Akahane H., Estrada-Muniz E., Huerta-Reyes M., Reyes-Chilpa R.: Biol. Pharm. Bull. 27, 141 (2004).
24. Isaias D.E., Niero R., Noldin V.F., de Campos-Buzzi F., Yunes R.A., Delle-Monache F., Cechinel-Filho V.: Pharmazie 59, 879 (2004).
25. Ito C., Itoigawa M., Mishina Y., Filho V.C., Enjo F., Tokuda H., Nishino H., Furukawa H.: J. Nat. Prod. 66, 368 (2003).
26. Kimura S., Ito C., Jyoko N., et al.: Int. J. Cancer 113, 158 (2005).
27. Zdzisińska B., Rzeski W., Paduch R., Szuster-Ciesielska A., Kaczor J., Wejksza K., Kandefer-Szerszeń M.: Pol. J. Pharmacol. 55, 235 (2003).
28. Brochado C.d.O., Almeida A.P.d., Barreto B.P., Costa L.P., Ribeiro L.S., Pereira R.L.d.C., Koatz V.L.G., Costa S.S.: J. Braz. Chem. Soc. 14, 449 (2003).
29. Moiteiro C., Justino F., Tavares R., Marcelo-Curto M.J., Florencio M.H., Nascimento M.S., Pedro M., Cerqueira F., Pinto M.M.: J. Nat. Prod. 64, 1273 (2001).

Received: 9. 02. 2009