Screening and Fractionation of Plant Extracts with Antiproliferative Activity on Human Peripheral Blood Mononuclear Cells

Elaine M Souza-Fagundes , Ana BR Queiroz*, Olindo Assis Martins Filho, Giovanni Gazzinelli*, Rodrigo Corrêa-Oliveira*, Tânia MA Alves , Carlos L Zani/+**

Laboratório de Química de Produtos Naturais *Laboratório de Imunologia Celular e Molecular **Laboratório de Doença de Chagas, Centro de Pesquisas René Rachou-Fiocruz, Av. Augusto de Lima 1715, 30190 002 Belo Horizonte, MG, Brasil

Three hundred and thirteen extracts from 136 Brazilian plant species belonging to 36 families were tested for their suppressive activity on phytohemaglutinin (PHA) stimulated proliferation of human peripheral blood mononuclear cells (PBMC). The proliferation was evaluated by the amount of [^{3}H]-thymidine incorporated by the cells. Twenty extracts inhibited or strongly reduced the proliferation in a dose-dependent manner at doses between 10 and 100 µg/ml. Three of these extracts appeared to be non-toxic to lymphocytes, according to the trypan blue permeability assay and visual inspection using optical microscopy. Bioassay-guided fractionation of Alomia myriadenia *extract showed that myriadenolide, a labdane diterpene known to occur in this species, could account for the observed activity of the crude extract. Using a similar protocol, an active fraction of the extract from* Gaylussacia brasiliensis *was obtained. Analysis of the ^{1}H and ^{13}C NMR spectra of this fraction indicates the presence of an acetylated triterpene whose characterization is underway. The extract of* Himatanthus obovatus *is currently under investigation.*

Key words: plant extracts - *Alomia myriadenia* - *Gaylussacia brasiliensis* - *Himatanthus obovatus* - lymphocyte proliferation - immunomodulators

Plants are recognized for their ability to produce a wealth of secondary metabolites and mankind has used many species for centuries to treat a variety of diseases (Cragg et al. 1999). Secondary metabolites are biosynthesized in plants for different purposes including growth regulation, inter and intra-specific interactions and defense against predators and infections. Many of these natural products have been shown to present interesting biological and pharmacological activities and are used as chemotherapeutic agents or serve as the starting point in the development of modern medicines (Verpoorte 1998, 2000). In mammals, the immune system plays a vital role as the main line of defense against infections. Its integrity and efficiency is important during chemotherapeutic intervention for the treatment of many diseases (Ishizuka et al. 1995). Besides primary metabolites with immunomodulatory activity, several plant secondary metabolites were found to interfere with different immune system functions, including the activation of cell mediated immunity (Wagner 1993, Wong et al. 1994). Plant species such as *Viscum album, Panax ginseng* and *Tripterygium wilfordii* are known to present immunomodulatory activity and are being subjected to detailed studies to elucidate the active components and their mechanisms of action (Davis & Kuttan 2000). The immunosuppressive effect of *T. wilfordii*, for example, is due to the inhibition of T cell receptor signaling pathway (Ho et al. 1999). Almeida et al. (2000) showed that an enriched saturated fatty acid fraction from *Kalanchoe pinnata* presents inhibitory activity on lymphocyte proliferation. *Cissampelos sympodialis* (Piuvezam et al. 1999) inhibited peripheral blood mononuclear cells (PBMC) proliferation due to the presence of bisbenzylisoquinoline alkaloids and flavonoids. Rayward et al. (1997) showed that the inhibitory effect of an alcoholic extract of the fern *Polypodium leucotomos* in this assay was related to a direct effect on T lymphocytes and/or macrophages.

Knowing the potential of plant extracts as sources of immunomodulators we report herein the results of our screening of 313 extracts from 136 plant species using the bioassay based on the inhibition of proliferation of PBMC stimulated with phytohemaglutinin A (PHA).

MATERIALS AND METHODS

Plant collection - The plants (Table I) were collected in the states of Minas Gerais and Espírito Santo, Brazil. After botanical identification, voucher specimens were deposited in the BHCB Herbarium at the Department of Botany, Federal University of Minas Gerais.

Extracts preparation - The plant material was dried in the shade, ground in a knife mill or in a homogenizer and extracted twice (24 h) with an organic solvent (hexane, dichloromethane, methanol, ethanol 95%) or water at room temperature. The solvent was removed by rotary evaporation under reduced pressure at temperatures below 45°C. Water was removed by freeze-drying. The resulting crude extracts were stored at -20°C until assayed. Immediately before running the bioassay, sufficient amounts of ex-

Financial support: Fiocruz, CNPq, Pronex
+Corresponding author. Fax: +55-31-3295.3566. E-mail: zani@cpqrr.fiocruz.br
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TABLE I

Families represented in the collection and the respective number of species

Entry	Family	Species
1	Anacardiaceae	1
2	Annonaceae	4
3	Apiaceae	1
4	Apocynaceae	5
5	Asteraceae	5
6	Bignoniaceae	10
7	Bixaceae	1
8	Cecropiaceae	7
9	Clusiaceae	4
10	Combretaceae	2
11	Crassulaceae	1
12	Cucurbitaceae	1
13	Ericaceae	1
14	Euphorbiaceae	19
15	Fabaceae	17
16	Flacourticaceae	1
17	Icacinacea	2
18	Lamiaceae	4
19	Magnoliaceae	1
20	Malpighiaceae	3
21	Melastomataceae	1
22	Meliaceae	5
23	Moraceae	1
24	Myristicaceae	1
25	Myrtaceae	2
26	Piperaceae	1
27	Proteaceae	2
28	Rubiaceae	5
29	Sapindaceae	6
30	Sapotaceae	1
31	Scrophulariaceae	1
32	Simaroubaceae	4
33	Solanaceae	3
34	Sterculiaceae	3
35	Verbenaceae	5
36	Vochysiaceae	4
Total		136

tract were transferred to a vial and the residual solvent removed under high vacuum for at least 24 h. Stock solutions and serial dilutions of extracts and fractions were prepared in dimethylsulphoxide (DMSO). These solutions were diluted in RPMI-1640 ("Roswell Park Memorial Institute" medium, GIBCO, Grand Island, NY) and added to the culture so as to attain the desired final concentrations. Control experiments were performed using DMSO at the same concentration used to test the extracts (≤ 0.1%).

Cell preparations - PBMC were obtained from healthy adult volunteers by centrifugation of heparinized venous blood over Ficoll/Hypaque cushion (Histopaque, Sigma, St Louis, MO). Mononuclear cells were collected from the interphase after Ficoll separation and washed three times in RPMI before further processing. The cell suspensions were adjusted to $1.5x10^6$ cells/ml.

Viability assays - PBMC viability was determined by staining using a modification of the protocol described by Slezak and Horan (1989). Cells were treated with different plant extracts concentrations, pure substance and controls (DMSO at ≤ 0.1%) for 72 h at 37ºC in 95% humidity atmosphere with 5% CO_2. Following incubation, cells were incubated for 10 min with Trypan blue (0.4% in normal saline – NaCl 0.9%). For the flow cytometric analysis gated lymphocytes were analyzed based on their fluorescence properties. Viable lymphocytes were identified as non-fluorescent cells whereas dead lymphocytes presented higher fluorescence intensity on FL2 channels.

PBMC culture - All cultures were carried out in RPMI-1640 medium (GIBCO, Grand Island, NY), supplemented with 5% (v/v) heat-inactivated, pooled human sera type AB (Flow Laboratories, Royaune, UN) and L-glutamine (2 mM, GIBCO, Grand Island, NY). An antibiotic/antimicotic solution containing 1000 U/ml penicillin, 1000 µg/ml streptomycin and 25 µg/ml fungisone (SIGMA, St. Louis, MO) was added to control fungal and bacterial contamination.

Lymphocyte proliferation assay - The in vitro cellular proliferation (blastogenesis) was performed as previously described (Gazzinelli et al. 1983). Briefly, $1x10^6$ cells/ml ($1.5x10^5$ cells per well) were cultured in complete RPMI-1640 in flat bottomed microtiter plates (Costar, Tissue Culture Treated Polystyrene # 3512, Corning Inc., NY, USA). The cultures were stimulated with 2.5 µg/ml of PHA (SIGMA, St Louis, MO) and incubated for 72 h at 37°C in a humidified atmosphere containing 5% CO_2. Six hours before harvesting, 0.5 µCi [^{3}H]-thymidine (Amersham, England) in 25 µl RPMI-1640 was added to each well. The cells were harvested by collection onto glass fiber filters (Whatman Inc.) with an automated cell harvester (model M.245, BRANDEL). The filters were air dried and added to vials containing 3 ml scintillation fluid, constituted by 0.26 g of POPOP [1,4 - bis(5-phenyl-2-oxazolyl)-benzene, 2,2'-*p*-phenylene-bis(5-phenyloxazole)] and 2.1g of PPO (2,5-diphenyl-oxazole) (Sigma , St Louis, MO) per liter of toluene for scintillation counting. The [^{3}H]-thymidine incorporation was expressed as counts per minute (cpm).

Chromatographic fractionation - The extract (2 g) was subjected to MPLC on silica gel (2 x 40 cm) using gradient from 100% dichloromethane to 100% ethyl acetate over 4 h at 5 ml/min. Sixty fractions of 25 ml were collected and grouped according to their behavior on TLC to afford 19 groups. The fraction with the highest activity in the bioassay yielded crystals of myriadenolide (Fig. 3), identified by direct comparison (TLC, HPLC, and mixed melting point) with an authentic sample previously isolated and fully characterized in our laboratory (Zani et al. 2000). The *Gaylussacia brasiliensis* extract was subjected to a similar procedure using a gradient from 100% hexane to 100% dichlorometane. Fraction 8 of the *G. brasiliensis* extract was the most active in the assay (Fig. 5) and its ^{1}H and ^{13}C-NMR spectra were obtained.

Statistical analysis - Each experiment with PBMC was run in triplicate and the results are expressed as their mean ± SD.

RESULTS AND DISCUSSION

Samples of different parts from 136 plant species were collected in the Brazilian savanna (Cerrado) and the coastal rain forest (Mata Atlântica). The families to which they

belong are listed in Table I . The plant materials were extracted with organic solvents or water to yield 313 extracts.

The extracts were dissolved in DMSO and evaluated for their ability to inhibit PBMC proliferation at concentrations between 12.5 and 100 µg/ml. After stimulation with PHA and incubation for 72 h, several extracts inhibited the PBMC proliferation as measured by [^{3}H]-thymidine incorporation by the DNA from these cells. Twenty extracts from 16 species presented a dose-dependent inhibitory activity (data not shown), halting proliferation at the highest concentration (100 µg/ml, Table II).

Based on visual inspection of the cells using an optical microscope, we found that from the 20 active extracts only three were able to suppress the proliferation without any apparent harm to the lymphocytes at 12.5 µg/ml (Table III). This finding was confirmed by flow cytometry analysis, using a viability test based on the fluorescence of the dead cells after incubation with 0.4% trypan blue for 10 min (Slezak & Horan 1989). Fig. 1 indicates the gate, based on the granularity and size of the cells, selected for analysis. Fig. 2 shows typical histograms resulting from the assay with a cytotoxic and a non-cytotoxic extract.

The present report shows that several organic extracts from different species exert inhibitory effects on human PBMC proliferative response. Extracts of *A. myriadenia, G. brasiliensis* and *H. obovatus* were capable of inhibiting the in vitro replication of human PBMC when stimulated with PHA. The reduced incorporation of tritiated thymidine into lymphocyte DNA was not due to a toxic effect of these extracts on these cells since we observed that the number of viable cells in treated and control cultures remained constant according to the trypan-blue exclusion method (Table III). These results agree with those obtained by Gharagozloo and Ghaderi (2001), Benecia et al. (2000) and Rayward et al. (1997), in their study of the in vitro immunomodulatory activity of some medicinal plants extracts. Knowing that the inhibition of the proliferative response of human PBMC to mitogens may involve, among other things, the suppression of cytokine production (Shan et al. 1999) and direct effects on T lymphocytes and/or macrophages (Rayward et al. 1997), our preliminary results encourage us to further investigate the immunomodulatory properties of these extracts and its components.

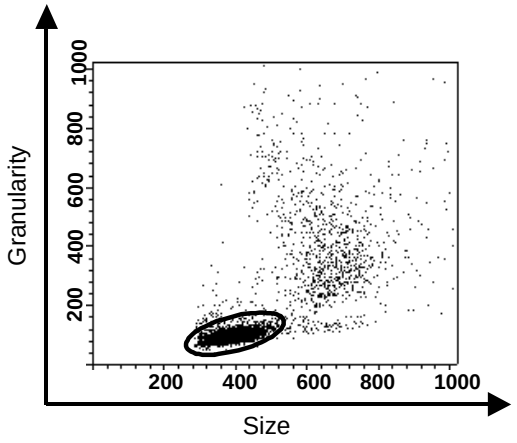

Fig. 1: representative forward scatter/side scatter dot plot distribution of peripheral blood mononuclear cells after in vitro incubation for 72 h at 37ºC in 95% humidity atmosphere with 5% CO_2. The gate highlights the lymphocytes selected for analysis.

TABLE II

Extracts with inhibitory activity on phytohemaglutinin A-induced peripheral blood mononuclear cells proliferative response

No.	Species	Family	Part	Solvent	Inhib [a]
1	*Alomia myriadenia* Schultz-Bip. ex Baker	Asteraceae	Aerial parts	EtOH	99
2	*Andira humilis* Mart.	Fabaceae	Leaves	DM-MeOH	95
3	*Cabralea polytricha* Juss.	Meliaceae	Fruit	MeOH	99
4	*Eucalyptus globulus* Labill.	Myrtaceae	Leaves	MeOH-H_2O	98
5	*Gaylussacia brasiliensis* (sp.) Meisn.	Ericaceae	Stem	DM-MeOH	98
6	*Guarea penningtoniana* Pinheiro	Meliaceae	Leaves	DM-MeOH	99
7	*Himatanthus obovatus* (Müll. Arg.) Woodson	Apocynaceae	Leaves	DM-MeOH	97
8	*Himatanthus obovatus* (Müll. Arg.) Woodson	Apocynaceae	Stem	DM	97
9	*Pilocarpus jaborandi*	Piperaceae	Root	DM-MeOH	97
10	*Pilocarpus jaborandi*	Piperaceae	Leaves		
11	*Joannesia princeps* Vell.	Euphorbiaceae	Pericarp	DM-MeOH	94
12	*Maprounea guianensis* Aubl.	Euphorbiaceae	Stem	DM-MeOH	93
13	*Michelia champaca* Linn	Magnoliaceae	Seed	H_20	99
14	*Sapium glandulatum* (Vell.) Pax.	Euphorbiaceae	Stem	DM-MeoH	99
15	*Simarouba amara* Aubl.	Simaroubaceae	Flowers	DM-MeOH	99
16	*Simarouba amara* Aubl.	Simaroubaceae	Stem-wood	DM-MeOH	99
17	*Simarouba amara* Aubl.	Simaroubaceae	Leaves	DM-MeOH	98
18	*Trixis vauthieri* DC	Asteraceae	Leaves	EtOH	95
19	*Vitex* sp.	Verbenaceae	Flowers/fruits	DM-MeOH	100
20	*Vitex* sp.	Verbenaceae	Leaves	DM-MeOH	96

DM: dichloromethane; EtOH: ethanol; MeOH: methanol; *a*: percent inhibition of the proliferation after 72 h incubation at 37°C, 95% relative humidity and 5% CO_2 atmosphere. The proliferation was estimated by measuring [^{3}H]-thymidine incorporation into DNA and compared with the control without extracts.

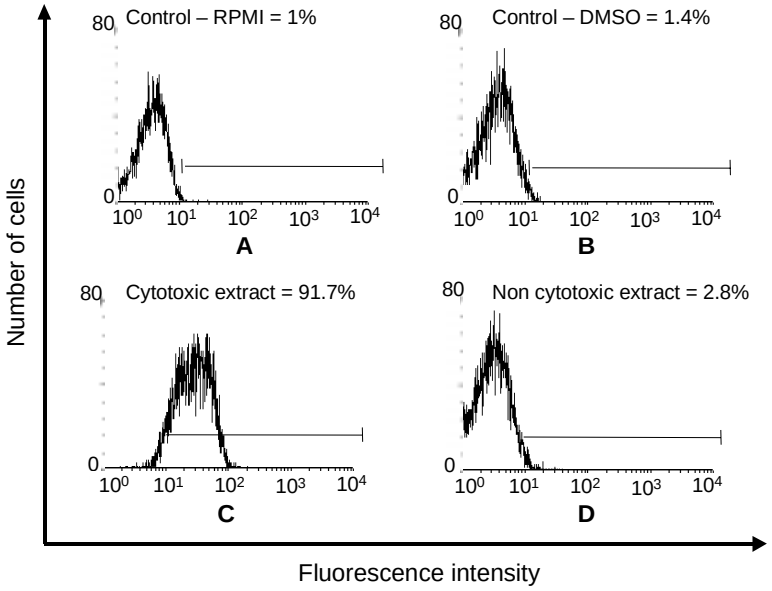

Fig. 2: representative histograms showing the effect of the incubation with plant extracts on cell viability. Cells were incubated in a parallel study with RPMI medium and dimethylsulphoxide as negative control (A and B) or cytotoxic (C) and non cytotoxic (D) plant extracts for 72 h at 37ºC in 95% humidity atmosphere with 5% CO_2. Following incubation, cells were treated for 10 min with trypan blue (0.4% in normal saline – NaCl 0.9%) prior to the flow cytometric analysis. After incubation with plant extract, gated lymphocytes were analyzed based on their fluorescence properties. Viable lymphocytes were identified as non-fluorescent cells (A, B and D) whereas dead lymphocytes presented high fluorescence intensity on FL2 channel (C).

TABLE III

Extracts selected on the basis of their high inhibitory activity on peripheral blood mononuclear cells (PBMC) proliferation and low toxicity towards lymphocytes

Species	Part	% Inhibition [a]	% Viability [b]
Alomia myriadenia	Aerial parts	98 ± 2	> 92 ± 5
Himatanthus obovatus	Leaves	71 ± 0	> 96 ± 4
Gaylussacia brasiliensis	Stems	68 ± 9	> 94 ± 5

a: PBMC was treated with the extract at 25 µg/ml and stimulated with phytohemaglutinin A (2.5 µg/ml) for 72h. Proliferation was evaluated by [^{3}H]-thymidine incorporation; *b*: viability was determined by staining with trypan blue and flow cytometry analysis. The results are expressed as the average of three experiments ± SD.

A. myriadenia is an invasive herb from the family Asteraceae without any medicinal or economic uses. Previous work with this plant in our group disclosed the presence of cytotoxic diterpenes (Zani et al. 2000). Re-investigation of this extract using column chromatography and crystallization afforded myriadenolide (Fig. 3) (Souza-Fagundes et al. manuscript in preparation), a labdane type diterpene already known to occur in this plant (Zani et al. 2000). This compound completely inhibits PBMC proliferation at concentrations above 7.5 µM (Fig. 4). Interestingly, we found that this compound is not cytotoxic to lymphocytes at this dose, as deduced by the trypan blue assay (Souza-Fagundes et al. manuscript in preparation).

The genus *Gaylussacia* is known to present antifungal activity, probably due to the presence of phenolic compounds (Cipollini & Stiles 1992). Other known compounds in the genus include stilbene (Askari et al. 1972) and anthocyanin derivatives (Ballington et al. 1988). An extensive literature search showed that none of the compounds present in this genus were evaluated for their activity in the in vitro blastogenesis assay. Column chromatography over silica gel of this extract afforded 16 fractions (Fig. 5), from which fraction 8 was the most active, inhibiting in 98% the lymphocyte proliferation at 25 µg/ml. Analysis of the ^{1}H and ^{13}C-NMR spectra of this fraction indicate the presence of an acetylated triterpene. The

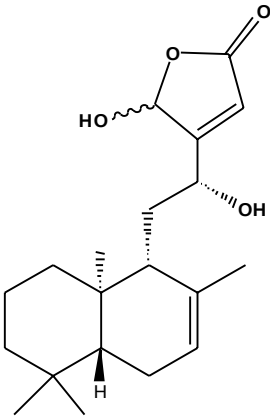

Fig. 3: structure of myriadenolide, a labdane diterpene previously isolated from *Alomia myriadenia* (Zani et al. 2000). It is the major component of the extract and presents strong antiproliferative activity in the peripheral blood mononuclear cells assay and low toxicity toward lymphocytes at 7.5 µM.

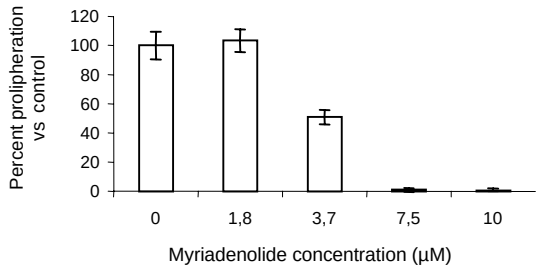

Fig. 4: effect of myriadenolide on phytohemaglutinin A-induced proliferative response of human peripheral blood mononuclear cells. The cells were stimulated with phytohemaglutinim A and cultivated for 72 h in the presence of the compound followed by 6 h incubation with [^{3}H]-thymidine. The values represent percent proliferation inhibition compared to control without drug ± SD (n = 9).

re-isolation of this compound in larger amounts will be necessary to complete its characterization. Investigation of the other fractions is also being carried out using the bioassay to guide the isolation of further active components.

Several species from the genus *Himatanthus* are used in folk medicine in South America for malaria, snakebites, tumors, arthritis, gastritis, anthelmintic and other ailments (Elisabetsky & Castilhos 1990, Schultes & Raffauf 1994, Milliken 1997). Among the members of this genus, some species are reported to present moluscicidal activity (Perdue & Blomster 1978, Souza et al. 1984, Endo et al. 1994, de Miranda et al. 2000). Previous phytochemical investigation of this genus disclosed the presence of triterpenes, steroids, iridoids and lignans (Endo et al. 1994, Abdel-Kader et al. 1997, Barreto et al. 1998). A literature search indicates that none of the compounds reported to be present in this genus have been evaluated in the lymphocyte proliferation assay, indicating that this plant deserves further investigation to identify the active compound(s).

In conclusion, our screening yielded three active species, corresponding to about 2% of our collection. The active extracts showed dose-dependent activity in the range between 10 and 100 µg/ml, and their initial fractionation yielded natural products with strong and interesting activity. Further investigation may disclose other active compounds that may serve as leads to develop new drugs that interfere in disease process related to the immune system (Luk et al. 2000).

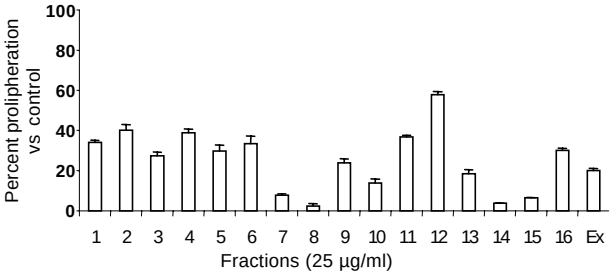

Fig. 5: effect of the crude extract (Ex) and fractions of *Gaylussacia brasiliensis* (1-16) at 25 µg/ml on the lymphocyte proliferation after phytohemaglutinin A stimulation. The cells were stimulated with phytohemaglutinin A and cultivated for 72 h in the presence of the compound followed by 6 h incubation with [^{3}H]-thymidine The control experiment was run with medium containing 0.1% dimethylsulphoxide.

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