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Canova medication and medicinal plants in south of Brazil

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Abstract

Brazil is one of the countries with the largest biodiversity of the planet. Its popular culture is particularly rich and the medicinal plants are used non-specifically for fitness and to improve the body function. In Brazil the population utilizes several plants, commonly prepared by infusion (tea), decoction, juice, bottled brew, and consumption of the plant as food. There is no predominance in the use of any specific part of the plant; in some cases, the whole plant is employed. Essential oils and ethanol extracts from the leaves and/or roots of medicinal plants are also commonly used. However the development of

phytotherapeutic agents using the Brazilian huge biodiversity allied to the popular knowledge is not satisfactory yet. Studies that scientifically confirm the effectiveness and the safety of products obtained from Brazilian biodiversity will allow their use as a remedy. Nowadays the research in this field is increasing with satisfactory results. The biological model used in our laboratory (cell culture), and microscopy techniques, are suitable to evaluate the mechanisms of action of some phytotherapeutic as well as homeopathic products. Canova was shown to be an effective immunomodulator. The results are very interesting since some of these substances and medicines activate the immune system allowing a natural defense against tumor cells as well as parasites and other infectious agents.

Introduction

Brazil is one of the countries with the largest biodiversity in the planet. In its extensive territory there are about 20% of approximately 250 thousand species that compose the world flora. Until today less than 10% of the world biodiversity was tested and up to year 2050, around 25% of flora will be extinct [1]. So far we are taken the risk of never know medicines that could come to be developed with these natural resources. Besides the great biodiversity, Brazil associates a great ethical and cultural diversity (Indigenous, African and European) that uses natural products as a tradition. It also presents social and economical characteristics that typify it as a developing country, where 80% of population depends on the use of plants for the primary health care [2].

In spite of gathered a series of favorable factors for the development of medicines starting from natural products, up to 2007 just a single genuinely Brazilian product has been developed. This new medicine is being sold by the name Acheflan®, a phytotherapeutic of the *Cordia verbenacea*. The lack of national products may be one of the consequences that characterize the Brazilian pharmaceutical market, which is predominantly composed by multinational companies (70%), that sell labeled products (90%) and all the research for new products is done in their origin countries. Studies that scientifically confirm the effectiveness and the safety of products obtained from Brazilian biodiversity will allow their use as a remedy. This will support the challenge of the HWO program, "health for all in the year 2000", released in 1993 that motivates the study and the application of medicinal plants in the primary health service. An example of success in Brazil was the study that validated the use of *Maytenus ilicifolia* Mart. ex Reissek and it made possible the devolution of the gained accurate and safe information to the population. The guided cultivation of *M. ilicifolia* influenced the ecosystem avoiding its decimation by the extractivism, fomented the internal and external trade, increased the income of agriculture/rural producer families, strengthened all of

the segments of the productive chain, and offered the population an effective, safe and cheaper medicine, mainly in the areas where the plant is cultivated. However, this investigation was an initiative of a few research groups, linked to public universities, without a normative government act.

The laboratorial researches with homeopathic medicines are very scarce; most of them are clinical reports, with varied methodologies, controversial and doubtful results. Even so the number of people that uses this therapeutic system is huge. In Europe, homeopathy is the most frequent complementary and alternative medical therapy. In Brazil, the general data of homeopathy use is unknown. In the medical school community 60% of the teachers admitted a low knowledge on this therapeutics, and 80% admitted a reasonable or a considerable usefulness of homeopathy [3, 4]. Since 1997, our group has been founding many important results with a study focused on a homeopathic medicine called Canova (CA). Its manipulation is based on Hahnemann's ancient homeopathic techniques that use highly diluted substances that are vigorously shaken (succussed) during the preparation. Clinical observation of patients confirmed the success of this treatment that seems to enhance the individual's own immunity to trigger a particular immunologic response against several pathological conditions. Several patients and doctors relate the same results: increase of the appetite, reduction of pains, and return to the daily activities.

The CA medication is a commercial product that represents a new form of immunomodulatory therapy. It is an aqueous, colorless and odorless solution produced and sold in authorized Brazilian drugstores. Mother tinctures are purchased from authorized agencies indicated by the Brazilian Health Ministry. These agencies assure the quality (endotoxin free) and physico-chemical composition of its products. Starting from the original mother tincture (in the case of a plant this is an ethanol extract) several dynamizations – succussion (shaking) and dilution in distilled water – are performed. The final commercial product is composed of *Aconitum napellus* (Ranunculaceae), *Thuja occidentalis* (Cupressaceae), *Bryonia alba* (Cucurbitaceae), *Arsenicum* (arsenic trioxide), *Lachesis* (Viperidae) and less than 1% ethanol in distilled water (www.canovado brasil.com.br). In our experiments we used the commercial product purchased from Canova do Brasil.

In our research we aimed to know what are the medicinal plants used in some communities at the south of Brazil, as well as laboratory research of Canova medicine, in order to elucidate how this homeopathic medicine improves the patient immune system.

Results and discussion

In 2006 the Brazilian Ministry of Health approved a National Politics of the Integrative and Complementary Practice in the “Sistema Único de Saúde”

(Single Health System), proposing the insertion of medicinal plants, phytotherapy, and homoeopathy among others, as therapeutic options complementing initiatives that began to be strengthened starting in the 80s. The city hall of Curitiba (Paraná state, Brazil) employs medicinal plants as a therapeutic option since 1990, through the program Green Health. In 1997, 17 pharmaceutical presentations were standardized, using 13 medicinal plants, for distribution to the population at the drugstores in the Health Units: *Achillea millefolium* L., *Arctium minus* (Hill) Bernh., *Chamomilla recutita* (L.) Rauschert, *Foeniculum vulgare* Mill., *Malva silvestris* L., *Maytenus aquilifolium* Mart., *Maytenus ilicifolia* Mart. ex Reissek., *Melissa officinalis* L., *Mikania glomerata* Spreng., *Peumus boldus* Molina, *Plantago lanceolata* L., *Plantago major* L. and *Salvia officinalis* L.

With the idea of rescuing and documenting the popular medicinal knowledge of culturally defined groups in the Paraná state, we began to collect ethnopharmacologic and ethnobotanic data in areas that present different social, economical and cultural characteristics. All the collected plants were identified to gender and/or species with the assistance of Professor Olavo Araújo Guimarães from Universidade Federal do Paraná (UFPR). The voucher herbarium specimens were collected and deposited at UPCB (Herbarium of the Botany Department at UFPR). All of natural resources used by different communities were described by the interviewed person and written down in charts standardized by the group of Pre-clinical Pharmacology and Toxicology of Natural Products of the Department of Pharmacology at UFPR. It was initially chosen a community distant from the urban centers (almost isolated). Between February of 2001 and January of 2002 we accomplished the rising of information about the natural resources used as medicines by the community from Rio Verde-Guaraqueçaba that is an area of environmental protection since 1985. It possesses an extension of 3134 Km² and is located at the north coast of Paraná. The district is isolated and of difficult access, still with additional difficulties such as altered geomorphology and rainy climate, that compose the socioeconomic dynamics, population and commercial flows of the location even more marginalized.

The ethnopharmacology and ethnobotany data were obtained through dialogue based on a preestablished script. During each family interview the information about all of the resources of vegetable, animal and mineral origin used in the preparation of home remedies was written down. Thus, 104 plants were mentioned, being the most cited the follow: *Mentha* sp., *Crocsmia crocosmiflora* (Nicholson) N.E.Br., *Allium sativum* L., *Aristolochia paulistana* Hoehne, *Phyllanthus urinaria* L. and *P. niruri* L., *Plantago australis* Lam., *Lippia alba* (Mill.) N.E.Br., *Aloysia pulchra* (Briq.) Moldenke and *Plectranthus barbatus* Andrews, also *Rosa* sp, *Persea americana* Mill. and *Musa* sp. The therapeutic property most mentioned was for the gastrointestinal

illness and among these: worms parasites, bellyache and diarrhea. From the 104 mentioned plants, 11 are used in the treatment of worms parasites: *Allium cepa* L., *Allium sativum* L., *Aristolochia paulistana* Hoehne, *Artemisia absinthium* L., *Carica papaya* L., *Chenopodium ambrosioides* L., *Cucurbita* sp., *Cuphea carthagenensis* (Jacq.) J.F.Macbr., *Menta* sp., *Plectranthus barbatus* Andrews and *Senna occidentalis* (L.) Link.

The homemade remedies against worms, influenza and colds are usually indicated only for children. According to the collected information, these plants teas calm down the worms without killing them, and for that they prefer the home remedies to the called allopathic. In the community's conception, it is not important to eliminate the worms, but to accommodate them and to calm them, because in Rio Verde it was stipulated that without the worms the individuals do not survive. Due to these concepts, specific conditions exist for the use of allopathic medicines and specific conditions to use of home remedies to treat worms symptoms, in other words, there is an appropriate time for home-made teas with the objective of calming the worms, and another suitable time to "drop them" through the administration of the allopathic medicines. Also, other interesting manipulation is the powder of the burned horn, which is one more product used against the worms, mixed with the mint tea.

A community, closer to the metropolitan area of Curitiba (Paraná), that also uses medicinal plants, is the Child's Pastoral, in the Anita Garibaldi section in Almirante Tamandaré (Paraná, Brazil). Among the "Basic Health Actions" developed under Sister Severina supervision, that is also the coordinator of the Pastoral works in the area for 19 years. This program teaches the communities leaders (women that are available to voluntarily help) and mothers, the correct form of preparation and use of medicinal plants, besides trying to explain the risks of the noxious effects for the inadequate use of these plants. According to the Sister Severina the Pastoral assists 60 communities, with 18-20 leaders that work separated among communities and each one is supervised by 2-3 leaders.

The ethnopharmacology and ethnobotany data were obtained in the years of 2003 and 2004 interviewing the community leaders, the mothers, and Sister Severina, through a project of the Department of Pharmacology of UFPR, involving biology, nursing, biochemistry and medicine graduating students. The botanical collection of the 72 plants collected and properly identified by the biology student Flavia Oliveira, were stored in two great pastes donated to Sister Severina and coworkers at the Child's Pastoral to serve as consultation material.

The mentioned plants more used were: *Achyrocline satureoides* (Lam.) DC., *Adiantum raddianum* C.Presl., *Ageratum conyzoides* L., *Aloe arborescens* Mill., *Alternanthera brasiliana* (L.) Kuntze, *Arctium minus* (Hill) Bernh.,

Aristolochia triangularis Cham., *Artemisia absinthium* L., *Bacharis trimera* (Less.) DC., *Bauhinia forficata* Link, *Bidens pilosa* L., *Bryophyllum pinnatum* (Lam.) Oken, *Calendula officinalis* L., *Centella asiatica* (L.) Urb., *Chelidonium majus* L., *Chenopodium ambrosioides* L., *Chrysanthemum leucanthemum* L., *Citrus limon* (L.) Burm.f., *Cordia curassavica* (Jacq.) Roem. & Schult., *Coronopus didymus* (L.) Sm., *Costus arabicus* L., *Cuphea calophylla* Cham. & Schltdl., *Cuphea calophylla subsp. mesostemon* (Koehne) Lourteig, *Cupressus sp.*, *Cymbopogon citratus* Stapf, *Cynara scolymus* L., *Equisetum giganteum* L., *Eriobotrya japonica* (Thunb.) Lindl., *Erythrina mulungu* Mart. ex Benth., *Eugenia uniflora* L., *Foeniculum vulgare* Mill., *Ipomoea batatas* (L.) Poir., *Leonurus sibiricus* L., *Lippia alba var. globiflora* (L'Her.) Moldenke, *Malva parviflora* L., *Matricaria chamomilla* L., *Maytenus ilicifolia* Mart. ex Reissek, *Melissa officinalis* L., *Mentha arvensis* L., *Mentha sp.*, *Mikania glomerata* Spreng., *Ocimum basilicum* L., *Origanum vulgare* L., *Passiflora alata* Dryand., *Petiveria alliacea* L., *Petroselinum crispum* (Mill.) A.W.Hill, *Phyllanthus niruri* L., *Piper solmsianum* C.DC., *Plantago australis* Lam., *Plectranthus neochilus* Schltr., *Psidium cattleyanum* Sabine, *Punica granatum* L., *Rorippa nasturtium-aquaticum* (L.) Hayek, *Rosmarinus officinalis* L., *Ruta graveolens* L., *Salvia microphylla* Kunth, *Salvia officinalis* L., *Sambucus nigra* L., *Schinus terebinthifolius* Raddi, *Sedum sp.*, *Sonchus oleraceus* L., *Sphagneticola trilobata* (L.) Pruski, *Stachys byzantina* K. Koch, *Symphytum officinale* L., *Tanacetum parthenium* (L.) Sch. Bip., *Taraxacum officinale* (L.) Weber, *Tropaeolum majus* L., *Urera baccifera* (L.) Gaudich. and *Verbena montevidensis* Spreng.

In the year of 2004 new data about the use of medicinal plants raised by a community living at Novo Mundo, a district in the city of Curitiba [5]. The teacher Sandra Crestani gave around 125 questionnaires to the 6th, 7th and 8th grade students of fundamental school. Children answered it together with their families. Around 56% have returned with the complete information. More than 70% learned how to use the medicinal plants with relatives (mainly grandparents), 10% with neighbors and friends, 2% at the health center, 6% with the physicians, 2% in TV and 5% in books. Half of the families that use medicinal plants declared that they cultivate them in the back yard and the other half declared that buy at specialized stores (drugstores, herbaria and markets). This research showed that the mostly used plants are *Plectranthus barbatus* Andrews, *Mentha sp.*, *Matricaria chamomilla* L., *Mikania glomerata* Spreng., *Foeniculum vulgare* Mill. and *Melissa officinalis* L. The predominant form of preparation is the tea (decoction or infusion - 84%). Less frequent are maceration (6%), syrup (6%), in natura (2%) and breaths or inhalation (2%). About the part of the plant used, they respond: leaf (56%), stem (15%), flowers (13%), root (8%) and seed (8%).

The mentioned therapeutic uses were mainly gastrointestinal problems (stomachaches, intestinal weakness, diarrheas, bellyaches, digestion, laxative, constipation, worms, gastritis, ulcer, nausea, vomit and intestinal gases - 39%), respiratory problems (flu, sore throat, catarrh, mucolithic, coughs, bronchitis, pneumonia, asthma and rhinitis - 22%), problems of the nervous system (depression, relaxing, sedative, stress and insomnia), cardiovascular problems (diabetes, heart, palpitations, to clean the blood, anemia, triglycerides, rheumatism, cholesterol and hemorrhage), dermatology problems (burns, erysipela, tumors and acne).

In addition to these three mentioned studies, there are countless data documenting the great acceptance and use of medicinal plants and natural products by the population. However, we did not have any document to quantify the studies accomplished with these products. That is why, in 2004, we did a new search, in order to be aware of all the investigations concerning medicinal plants in the last 10 years (1992-2002) in the Paraná state, Brazil [6]. The scientific areas that presented a larger number of studies having medicinal plants as the main scope were: biological activities (34%), phytochemistry (20%), agriculture (17.2%), botany (11%), quality control (4.7%), ethnopharmacology (3%), pre-clinic toxicology (3.2%), phytotherapy (1.9%), clinical studies (1.9%) and ethnobotany (1.5%). Other approached themes (biotechnology, clinical toxicology, legislation, teaching, pharmacopoeia, pharmaceutical formulation, organization and other) totalizing 3.41%. Although, the specific works of ethnopharmacology and of ethnobotany represented 4.5% of all studied areas, this perceptual was underestimated because almost 100% of the works somehow approached aspects of Ethnopharmacology and ethnobotany, attempting to indicate the importance of the studied plant. Therefore, this theme (Ethnopharmacology and Ethnobotany) is present in more than 90% of the published works with medicinal plants.

The medicinal plants that present some kind of study during the researched period were: *Achillea millefolium* L., *Ageratum conyzoides* L., *Annona glabra* L., *Arctium lappa* L., *Artemisia camphorata* Vill., *Averrhoa carambola* L., *Baccharis illinita* DC., *Baccharis trimera* (Less.) DC., *Bouchea fluminensis* (Vell.) Moldenke, *Calendula officinalis* L., *Casearia sylvestris* Sw., *Chamomilla recutita* (L.) Rauschert, *Chaptalia nutans* (L.) Polak, *Cissus sicyoides* L., *Coffea arabic* L., *Croton cajucara* Benth., *Curcuma zedoaria* (Bergius) Roscoe, *Cymbopogon citratus* Stapf, *Diodia brasiliensis* Spreng., *Eucalyptus citriodora* Hook., *Eucalyptus grandis* W.Hill. ex Maiden, *Heteropteris aphrodisiaca* O.Mach., *Hypericum perforatum* L., *Kielmeyera coriacea* Mart. & Zucc., *Kielmeyera variabilis* Mart., *Leonurus sibiricus* L., *Lippia alba* (Mill.) N.E.Br., *Lonicera japonica* Thunb., *Matricaria chamomilla* L., *Maytenus ilicifolia* Mart. ex Reissek, *Melissa officinalis* L., *Mikania glomerata* Spreng., *Ocimum basilicum* L., *Ocimum gratissimum* L., *Ocotea*

odorífera (Vell.) Rohwer, *Ocotea puberula* (Rich.) Nees, *Passiflora actinia* Hook., *Passiflora edulis* Sims, *Passiflora sp*, *Paullinia cupana* Kunth, *Pfaffia glomerata* (Spreng.) Pedersen, *Plantago major* L., *Plumeria lancifolia* Müll. Arg., *Porophyllum ruderale* Cass., *Rosmarinus officinalis*, *Siphocampylus verticilatus*, *Stevia rebaudiana* (Betoni) Bertoni, *Stryphnodendron adstringens* (Mart.) Coville, *Virola michellii* and *Zingiber officinale* Roscoe.

The great number of works investigating the general activities of medicinal plants, in comparison with the few ones that demonstrate the mechanisms of action, as well as with the great number that describe the characterization/composition (flavonoids, alkaloids etc.), in comparison to the small number of works that bring the identification/characterization of the isolated compounds, are a reflex of the limited infrastructure available for science in Brazil. The great diversity of research subjects, together with the great variety of medicinal plants (238 different species), suggests the existence of groups and/or researchers isolated that began their works more recently or that are located in distant centers without the interaction with other research group.

Scientific studies with medicinal plants in the state of Paraná added to the result of the existence of a low number of registered patents and of resulting products indicate that the improvement on installations and instrumental park of research, besides the concentration of efforts, will be of essential importance to the scientific and technical progress. The Associação Paranaense de Plantas Medicinas (ASPPM), Universidade Federal do Paraná, (UFPR), Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA - PR), Instituto Paranaense de Assistência Técnica e Extensão Rural (EMATER - PR), together with some general offices of the state and the municipal district, presented a plan for investigation of medicinal plants expansion. This initiative is an attempt to articulate in the state of Paraná, political actions of research financing in science and technology to make possible the consolidation of research groups, with the validation of medicinal plants and with the consequent increase of a number of patents and products generated from the Brazilian biodiversity.

Polysaccharides from various sources have been shown to have anti-tumoral activity and low toxicity and Electron Microscopy is one of the best tools for this kind of investigation. The polysaccharide α -D-glucan from the lichen *Ramalina celastri* has been studied against HeLa cells (*in vitro*) and against Sarcoma 180 (*in vivo*). It was shown that α -D-glucan was cytotoxic to HeLa cells with a dose of 80 μ g/ml, although the cell monolayer was similar to the control; at ultrastructural levels a large number of microvilli were substituted by cytoplasm blebs, indicating cellular injury. The Sarcoma-180 treated bearing mice indicated that α -D-glucan from the lichen *Ramalina celastri* could inhibit 36% of tumor growth and affect host defense and cell responses [7, 8].

Several microscopy techniques were utilized to demonstrate that the complex homeopathic medicine called Canova (CA) activates macrophages (M Φ). Piemonte and Buchi, in 2002, demonstrated both *in vivo* and *in vitro* that mice macrophages treated with CA were activated according to morphologic, biochemical and molecular criteria, namely $\alpha 5$ - $\beta 1$ integrins, FC receptors and α -actin filaments distribution were altered and TNF- α production was decreased [9]. Figure 1 shows images by differential interference contrast (DIC) of resident and activated M Φ after CA treatment. Over production of TNF- α plays an important role in a number of pathological conditions, including cachexia, septic shock, and autoimmune disorders. Our results showed that TNF- α release decreased after repeated doses of CA, justifying, in part, the clinical improvements of many patients.

The pools of lymphocytes co-cultured with untreated M Φ showed competence to eliminate the melanoma cells, and the CA treatment increased this capacity. Our results showed that CA treatment improves the activation of lymphocyte by macrophages interactions and increase the lymphocytes response against melanoma cells (Fig. 2).

Our assays have shown that the increase in nitric oxide (NO) production is accompanied by an increase in inducible nitric oxide synthase (iNOS) detection. The enzyme was found on the cytoplasm located mainly near vesicles and mitochondria. NO and reactive oxygen species (ROS) are produced, under

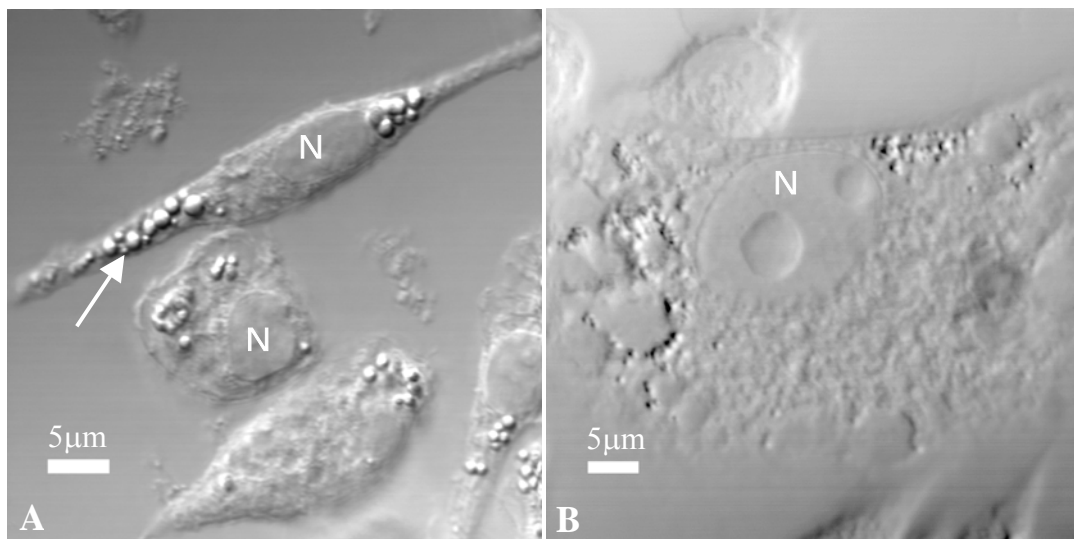


Figure 1. Spreading ability of macrophages after CA treatment and analyzed by DIC (differential interference contrast Microscopy), in a laser scanning confocal microscope (LSCM)—Radiance 2001 (Bio-Rad) coupled to an Eclipse E-800 (Nikon)—with a 60x NA oil plan-apochromatic objective lens and a 4x zoom. (A) Control group shows the characteristic morphology of resident macrophages with poor spreading. (B) Canova-treated macrophages exhibiting morphological characteristics of activated macrophages showing marked spreading. N – nucleus; arrow – lipid bodies; bars 5 μ m.

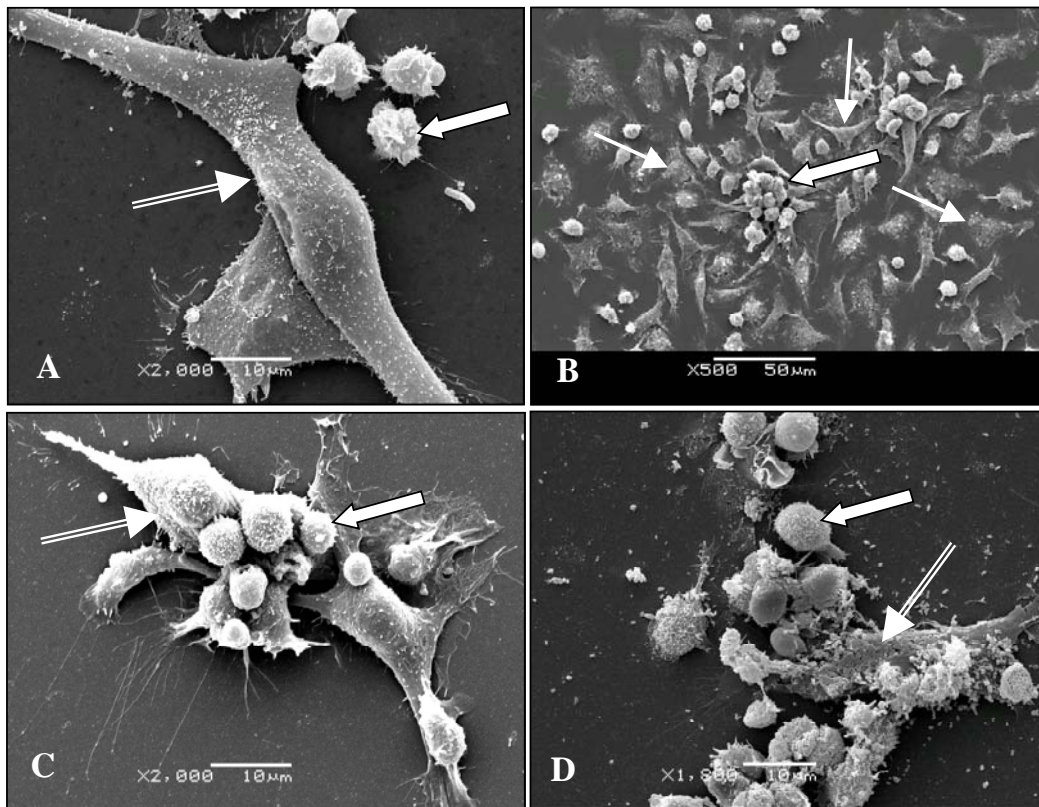


Figure 2. Scanning electron microscopy: co-culture systems between lymphocytes (Ly) previous co-cultured with macrophages (M Φ) and then plated with melanoma B16F10 lineage cells. The results showed high anti-melanoma capacity of murine Ly previous co-cultured with M Φ treated with Canova. **A** – Control group; **B** – Ly in contact with Canova treated M Φ ; **C** – Ly adhered over melanoma B16F10 lineage cells; **D** – Ly completed destroyed the melanoma cells. Lymphocytes – thick arrows; melanoma cells – open arrows; macrophages – thin arrows.

a variety of biological conditions and they are critical in host defense not only because they can damage pathogens and tumoral cells but also since they are immunoregulatory. Ultrastructural cytochemical detection of NADPH oxidase activity was performed and characterized by a local cerium precipitate. Ultrathin sections of the material were observed without stain, so that electrondense markers are indicative of a positive enzyme reaction. NADPH oxidase is normally dormant in resident macrophages, but can be rapidly activated by a variety of stimuli. When the phagocyte is activated, the cytosolic subunits migrate to the membranes, where they bind to the membrane-associated subunits to assemble the active oxidase resulting in the delivery of its products into vesicles and extracellular environment. The control group showed electrondense products in the few activated macrophages found. Canova, in some way, activates this pathway because in the treated cells we observed, mainly in vesicles, stronger positive reaction products indicative of

NADPH oxidase activity. The enhanced of nitric oxide synthase (iNOS), consequently producing ROS and NO respectively; causes the inhibition of the cytochrome oxidase activity [10], whereas Mg^{++} ATPase and AcPase activities were increased.

Acridine orange and confocal microscopy showed that the majority of Canova treated macrophages presented great amount of acid vesicles, also inside of the innumerable cytoplasmic projections (Fig. 3). CA stimulates an increase of the endosomal/lysosomal system as well as the phagocytic activity of macrophages when interacted with *Saccharomyces cerevisiae* and *Trypanosoma cruzi* epimastigotes [11]. The modulatory effects of CA were also observed both *in vivo* and *in vitro* in experimental infection by *Leishmania amazonensis* and *Paracoccidioides brasiliensis*, controlling infection progression and limiting its dissemination [12, 13]. Moreover, it is neither toxic nor mutagenic [14].

The results with CA treatment suggest an improvement in resistance against infectious and foreign agents and show an enhanced immunity favoring a specific immunological response to these microorganisms through the phagocytic pathway. The treatment induces morphological alterations of macrophages, including an increase in their spreading areas. This can be a key event for the enhancement of phagocytic ability of non-infective forms observed (Fig. 4).

To-date, several potent biological response modifiers, which are able to activate macrophages, has been extensively studied. Canova, a homeopathic medication with no side effects, provides a good alternative for macrophage activation. After these results, two master dissertations were concluded with

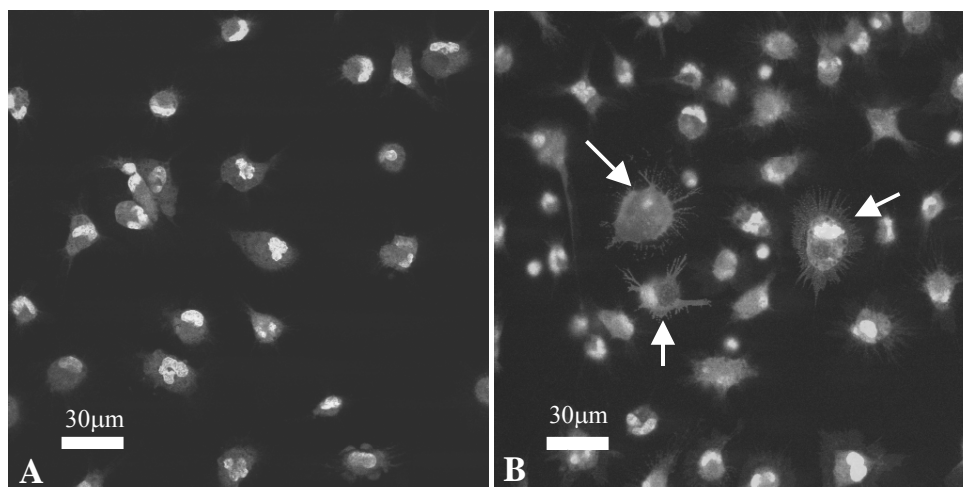


Figure 3. Confocal microscopy: Macrophages incubated with acridine orange; **A** – Control macrophage; **B** – Canova treated macrophage showing many projections and acid vesicles in red (arrows), and nucleus in green; bar 30 µm.

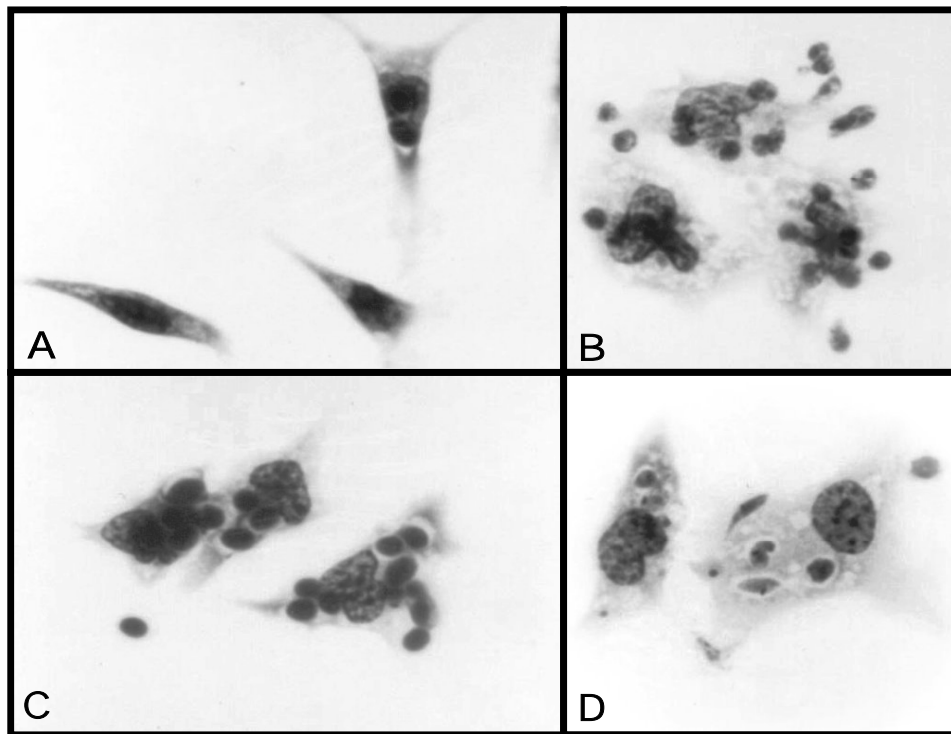


Figure 4. Light microscopy of phagocytosis by macrophages: **A** – *Saccharomyces cerevisiae* phagocytosed by resident (control) macrophages; **B** - *Leishmania amazonensis*, **C** - *S. cerevisiae* and **D** – *Trypanosoma cruzi* epimastigota phagocytosed by Canova treated cells respectively.

clinical results after the treatment of HIV/AIDS patients with Canova. The evolution of HIV/AIDS+ patients in Botswana, Africa, was evaluated in a prospective study. Participants were assessed on site, Gabane Home Care, prior to and after the completion of one and eighteen months period of Canova treatment, using a specific quality of life in HIV/AIDS questionnaire. The data indicate that the treatment is highly effective in reducing symptomatology and improving quality of life in individuals with HIV by recovering parameters like general pain feeling, appetite, capability to do small efforts and absenteeism among others. The evolution of HIV/AIDS+ patients was also evaluated in Brazil, before and after one and six months of continuous use of Canova. Patients were assessed linked to Non Governmental Organizations (NGO) and to a public institution of health in Curitiba, Paraná, Brazil. The patients were evaluated clinically, in a laboratory, and through a specific quality of life questionnaire, form 17 from The Measurement Groups. The data indicate that the treatment is effective in reducing opportunistic diseases, increasing the number of CD4 cells and erythrocytes number, and in improving the quality of life by parameters of recovering such as decreasing general pain and depression, and increasing appetite and energy to work [15, 16].

Similarly, the improvement in immune response of CA-treated mice was demonstrated in studies with Sarcoma 180. A reduction in sarcoma size was observed and a significant infiltration of lymphoid cells, granulation tissue and fibrosis occurred, surrounding the tumor. All animals from the treated group survived, and in 30% of them a total regression of the tumor was shown. The treatment with CA increased total numbers of leukocytes. Among lymphocytes, T CD4, B and NK cells increased [17]. These results suggested a direct or indirect action of the CA on hematopoiesis. So the bone marrow cells were treated and processed for light, transmission and scanning electron, and confocal microscopy, and also for flow cytometry. All microscopy techniques showed that monocytic lineage (CD11b) and stromal cells (adherent cells) were activated by treatment (Fig. 5). Canova also increased cell clusters over adherent cells, suggesting areas of proliferation and differentiation [18, 19].

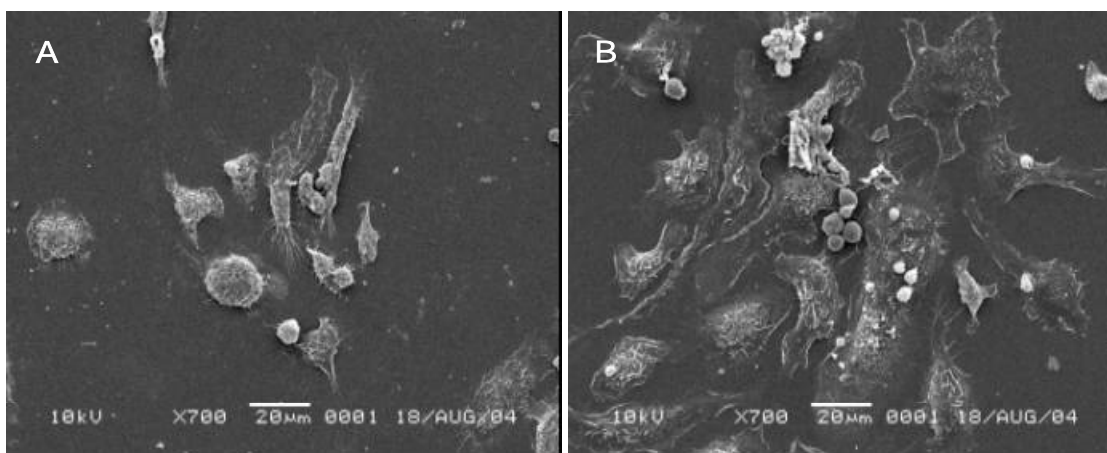


Figure 5. Scanning electron microscopy: A – bone marrow adherent control cells; **B** – bone marrow adherent cells CA treated showing activated morphology.

Conclusion

The vast biodiversity found in Brazil linked to a great ethical and cultural diversity (Indigenous, African and European) that uses natural products as a tradition, are the most promising sources of cure of several disease that affects people nowadays.

The great number of works investigating the general activities of medicinal plants, in comparison with the few ones that demonstrate the mechanisms of action, as well as with the great number that describe the characterization/composition (flavonoids, alkaloids etc.), in comparison to the small number of works that bring the identification/characterization of the isolated compounds, are a reflex of the limited infrastructure available for science in Brazil. The improvement on installations and infrastructure, from

transportation to the field to better equipments on the laboratory, are of essential importance to the scientific and technical progress in public health. Financial investments allied to political will of knowing the therapeutics potential of medicinal plants and homoeopathic medicines would improve the scientific studies, corroborating its effectiveness and allowing their use as a remedy. Our results point out to the need of a careful examination of the interplay between the immune system and homoeopathic medications as well as medicinal plants in the treatment of a disease. This will be an important area for future research. As this knowledge is extended, the ability to selectively influence the activation state of specific cells hopefully will allow us to manipulate these treatments in the malignant diseases.

Acknowledgements

To APES, CNPq, and SETI-PR for financial support; we are specially grateful to the laboratory team.

Experimental details

All recommendations of the National Law (number 6.638, November, 5th 1979) for scientific management of animals were respected and the Institutional Animal Care Committee at UFPR approved all related practices. Experiments were carried out at the Neoplastic and Inflammatory Research Laboratory, UFPR, which has a management program for produced residues. All experiments were performed at least three times with three control groups and the results analyzed in a double blind way.

Cell culture preparation

Primary cultured cells were obtained from washing mice peritoneal cavities (macrophages) or femurs (bone marrow cells). Lineage cells were purchased from specialized agencies. The cells were maintained in culture, incubated at 37°C under 5% CO₂ with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 50 µg/ml penicillin and 100 U/ml gentamicin, and handled according to the procedures required by each experiment.

Light microscopy

Cells were plated into culture plates with cover slips, maintained as described for each specific protocol, and then rinsed with phosphate buffer solution (PBS), fixed in Bouin, stained with Giemsa, dehydrated, and mounted with Entellan [20, 21]. Adhered cells were observed by light microscopy using a Nikon Eclipse E200 microscope. The morphological characteristics were analyzed.

Transmission electron microscopy

The cells were fixed with 2% glutaraldehyde, 4% paraformaldehyde, 5 mM CaCl₂, in 0.1 M cacodylate buffer (pH 7.2); post-fixed in 1% osmium tetroxide (OsO₄), dehydrated in acetone and embedded in Epon [20, 21]. Ultrathin sections were stained with lead citrate and uranyl acetate and observed with a Jeol-JEM 1200 EX II transmission electron microscope at the Electron Microscopy Center of UFPR. A GATAN CCD camera and GATAN digital micrograph software were used to obtain the digital images.

Scanning electron microscopy

The cells were fixed with 2.5% glutaraldehyde (0.1 M cacodylate buffer, pH 7.2), washed and post-fixed in 1% OsO₄ for 30 min in the dark at room temperature [20, 21]. After washing, the cells were dehydrated using increasing ethanol concentrations. Cells were CO₂ critical point dehydrated, metalized and observed using a JEOL JSM-6360 LV SEM scanning electron microscope in the Electron Microscopy Center at UFPR.

Confocal microscopy

Immunostaining: was performed according to standard protocols using commercially available antibodies for surface markers. The nuclei were stained with 300 nM DAPI (4,6 – diamidino – 2 – phenylindole, dihydrochloride) (Molecular Probes, Eugene, OR, USA). *Acridine orange*: the cells were incubated with 5 µg/ml acridine orange solution for 20 minutes at 37°C 5% CO₂. The fluorescence was observed in the Radiance 2001 laser scanning confocal microscope (BIO-RAD) coupled to an Eclipse E-800 (Nikon).

Flow cytometry

Immunophenotyping was performed using specific antibodies for each cell. Leukocytes were labeled using monoclonal antibodies anti-CD45. Among them monocyte/macrophage lineage (CD11b), granulocytes (Ly-6G), B lymphocytes (CD45R), dendritic cells (CD11c), T lymphocytes (CD3), erythrocytes (TER-119) were labeled. After incubation, the cells were washed, resuspended in PBS and the fluorescence was analyzed according to standard procedures on a FACSCalibur flow cytometer (Becton Dickenson – BD), equipped with an argon ion laser (488 nm). Data were analyzed in Cell Quest program (BD) and submitted to analysis of variance (ANOVA) and Tukey test ($p < 0.05$) to determine the statistical significance.

Ultrastructural cytochemistry

All ultrathin sections from ultrastructural cytochemistry were observed without stain using a Jeol 1200 EXII transmission electron microscope. A

GATAN image analyzer was used to acquire images. *Acid Phosphatase (AcPase)* and *Mg⁺⁺ATPase* activity techniques are based on the reaction of the phosphate generated by cells with cerium chloride, which results in an electron-dense precipitate of cerium phosphate. The reaction control was incubated in the same medium but without the substrate. *NADPH* oxidase activity technique is based on the reaction of H₂O₂ generated by cells with cerium chloride, which results in a precipitate of cerium perhydroxide (Ce-[OH]2OOH). The peroxisomal enzyme marker *catalase* and the *cytochrome oxidase* activity were detected based upon the oxidative polymerization of 3,30-diaminobenzidine (DAB) to an osmiophilic reaction product (DAB precipitation). Then the cells were processed according transmission electron microscopy protocol as described above.

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