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CELLULAR AND MOLECULAR BIOLOGY

Medicinal Plants from Brazilian Cerrado Biome: Potential sources of new anti-inflammatory compounds and antitumor agents on Ehrlich carcinoma

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Abstract: This work describes a pharmacological screening of Brazilian medicinal plants through their anti-inflammatory and cytotoxicity activities. Cytotoxicity activity of *Mouriri elliptica* and *Alchornea glandulosa* as well as the drugs celecoxib and doxorubicin were evaluated in cultures of peritoneal macrophages. The immune system influence of these samples was analyzed by determining production/inhibition of NO, production of tumor necrosis factor-α and production of interleukin-10. Regarding the production/inhibition of NO, there was NO production by *M. elliptica* and NO inhibition when the cells were exposed to *A. glandulosa*; Macrophages generally produce more NO, plus TNF-α and less IL-10, when associated to the tumor phenomenon, characterizing the inflammation involved in cancer. *A. glandulosa* showed anti-inflammatory effect, inhibited NO production and it was associated with low TNF-α production, although not as low as the macrophages associated with celecoxib and doxorubicin. These cytokines were not different in animals with tumor. Celecoxib confirms its anti-inflammatory action by markedly inhibiting NO and TNF-α, but also inhibiting IL-10 which is an anti-inflammatory cytokine. Doxorubicin inhibited NO in a higher percentage in the group of animals with tumor, although the literature reports that this drug stimulates the production of NO and this collaborates with its cytotoxic effect.

Key words: Cytotoxicity, anti-inflammatory, *Mouriri elliptica*, *Alchornea glandulosa*, Ehrlich carcinoma.

INTRODUCTION

Short-term inflammatory activity usually brings benefits to the host organism in the presence of aggressive agents. However, the persistence of the inflammatory process often results in tissue and DNA damage, contributing to the development of cancer (De Visser et al. 2006). Cancer, being a pathological process, can provoke an immune response (Killion & Fidler 1994). Macrophages are the main elements present in the body to fight tumors (Nath et al. 2006) and are the first cells to be activated to participate in an immune response when the organism is exposed to exogenous factors, in an acute or chronic inflammation such as tissue trauma, infections, autoimmune disorders and cancer through the secretion of chemokines and cytokines. Macrophages exert anti-proliferative and cytotoxic activities, partially resulting from their ability to secrete reactive nitrogen and hydrogen species and pro-inflammatory cytokines (TNF, IL-1, IL-6) (Bonnotte et al. 2001).

Inflammation associated with cancer includes the presence of leukocyte infiltrate, expression of cytokines such as TNF or IL-2, chemokines such as CCL 2 and CXCL 8, active tissue remodeling and neo-angiogenesis (Allavena et al. 2008). Both inflammation and angiogenesis are exacerbated by increased production of cytokines, growth factors, proteolytic enzymes, lipid mediators and prostaglandins. Approximately 15 to 20% of malignancies are initiated or exacerbated by inflammation (Ono 2008). Among the antiinflammatories of natural origin, stand out those that come from plant sources due to their low toxicity and structural variety.

Kim et al. (2008) investigated the anti-inflammatory properties of 20 plants used in Chinese medicine. Three plants *Radix gentianae*, *Rhizoma coptidis* and *Citri unshiu* demonstrated anti-inflammatory effect in several experimental models, inhibiting NO and PGE2. Sharma et al. (2008) evaluated the cytotoxic effect of *Cymbopogon flexuosus* essential oil *in vitro* on twelve different cancer strains and *in vivo* on solid Ehrlich tumor and thesarcoma-180 model in mice. Intraperitoneal inoculation significantly inhibited the ascites and solid form of Ehrlich tumor and sarcoma-180, revealing promising anticancer activity. Uddin et al. (2009) analyzed the cytotoxic effect of 16 plants of traditional Bangladeshi medicine and found that 14 showed little or no cytotoxicity against healthy rat fibroblasts, and a selective cytotoxic effect against gastric, colon and breast cancer. Zhang et al. (2009) have shown that, in addition to the cytotoxic effect on tumor cells, *Ganoderma sinensis* lipid extract induces antitumor immune response, stimulating antitumor activity of tumor associated macrophages. Kováes et al. (2009) analyzed a fraction of *Xantinum italicum* and detected cellular and antiproliferative growth inhibitory activity in uterine cervix adenocarcinoma, skin carcinoma and breast adenocarcinoma.

Nogueira et al. (2008) evaluated the antitumor and antiangiogenic capacity of fractions of the *Synadenium umbellatum* Pax plant in Ehrlich solid tumor, whose results showed inhibition of the viability of tumor cells. *In vivo*, they showed a significant anti-tumor dosedependent capacity, being a strong candidate for anti-tumor agent in the future. Mesquita et al. (2009) evaluated the cytotoxic effect of 412 extracts of 50 species from Brazilian cerrado plants against colon, melanoma and brain tumor cell lines. 28 of the 412 extracts showed antiproliferative effect, with at least 85% inhibition of cell proliferation, in at least one of the cell lines.

Considering the potential of medicinal plants from Brazilian cerrado biome, this study proposed to determine the cytotoxicity from methanolic extract of *Mouriri elliptica*, the ethyl acetate fraction of *Alchornea glandulosa,* and the drugs celecoxib and doxorubicin in cultures of peritoneal macrophages as well as evaluate the influence of these samples on the immune system determining: Production/inhibition of NO, Production of tumor necrosis factor α and production of interleukin-10.

MATERIALS AND METHODS

Collection and preparation of extracts and fractions of plant species

The plant material from *M. elliptica* was collected at the Experimental Station of the Mogi-Guaçu Biological Reserve (São Paulo state, Brazil) and the plant material from *A. glandulosa* was collected at Ipueiras (Tocantins, Brazil). The exsiccates were deposited at the Herbarium in Tocantins (HTO) of the Federal University of Tocantins in Porto National city, under number 3341.

The plant material was dried at room temperature, and subjected to grinding using a knife mill (Willye - NL-226-02). The obtained powder was extracted by maceration using hexane and methanol. The methanolic extract was concentrated on a rotary evaporator (54-Rotavapor R-220, brand: Büchi, with vacuum pump Vacuum Contoller V-805, brand: Büchi and water circulator), yielding a crude, highly viscous extract which was diluted in a methanol: water mixture (1:9 v/v) and subjected to the liquid-liquid partition with ethyl acetate and *n*-butanol.

The drug celecoxib has been commercially available. In Brazil, only Pfizer (Celebra®) markets this drug. In addition, the drug doxorubicin has been commercially available. In Brazil, this drug is market by Libbs (Fauldoxo®) and by Pfizer (Adriblastine®). The solvents used in the preparation of extracts and fractions were all analytical grade obtained from Vetec.

Animals and obtaining peritoneal exudate cells (PEC)

In each experiment were used five male Swiss mice, with six weeks of life, weighing between 18 and 25g. They were provided by the Central Biotherm of the Faculty of Pharmaceutical Sciences - UNESP, located in Araraquara city, The animals were kept in polycarbonate cages with water and feed (Purina) *ad libitum* in an air-conditioned room (23 ± 2 ° C, 56 ± 2% relative humidity), with light and dark control at each period of 12 hours. All animal procedures were conducted according to the opinion of the local Ethics Committee (opinion no. 31/2008).

The animals were previously stimulated by intraperitoneal inoculation of 3.0 mL sodium thioglycolate (DIFCO Lab. LTDA) at 3.0 %, three days prior to collection of cells. After this period, the animals were euthanized by inhalation in a CO_2 chamber (M1-SBFF-1FM Chamber System). These animals had abdominal skin removed aseptically in laminar flow chamber, Class 100 (Veco) and exposed peritoneal. In the upper medial portion of the abdomen, 5.0 mL of sterile phosphate buffered saline (PBS) at pH 7.2 and at 4° C were injected with the aid of a syringe (BD) and sterile needles (BD). A mild manual massage was performed, and the peritoneal exudate cells were collected with the same syringe and dispensed into a sterile conical tube (Corning, Inc., USA) for preparation of the cell suspension. The cells of the peritoneal exudate were washed three times with 5 mL of PBS (pH 7.2) and centrifuged at 400x g for 5 minutes in a centrifuge (Fanem, Ind. Bras.) at room temperature. The pelleted cells were re-suspended in 1.0 mL RPMI-1640 ATCC® 30-2001™ culture medium (Sigma-Aldrich Brazil) containing $2x10^{-5}$ M of 2-mercaptoethanol (Sigma-Aldrich Brazil), 100 U/mL penicillin (Sigma-Aldrich Brazil), 100 U/mL streptomycin (Sigma), 2 mM L-glutamine (Sigma) and 5% fetal bovine serum (Cutilab-Campinas), the medium being composed of RPMI-1640. The number of cells was determined by counting in hemocytometric chamber of Neubauer (Boeco, Germany) using 10 µL of the diluted cell suspension in 90 µL of Lazarus Fluid (RAL Diagnostics). The cells were adjusted to the optimum concentration for each assay in RPMI-1640-C medium.

Evaluation of cell viability of peritoneal exudate cells

The colorimetric technique was used to determine the cell viability using solution of 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide (MTT), which is based on the verification of mitochondrial activity and integrity, interpreted as a measure of cell viability.

The Peritoneal Exudate Cells (PEC) suspensions, adjusted to the concentration of $5x10^6$ cells/mL, were used. 100 µL of the suspensions were added in 96-well plates

(Corning ® Costar®), and 100 µL of the *A. glandulosa* ethyl acetate fraction (100, 200 and 400 µg/mL), and *M. elliptica* methanolic extract (300, 150 and 75 µg/mL), celecoxib (100, 50 and 25 µg/mL), doxorubicin (12.5, 25, 50 and 100 µg/mL), LPS at 10 µg/mL, or only culture medium RPMI-1640. The plates were incubated for 24 hours in an oven at 37° C with constant tension of 5 % CO_2. After this period, 100 µL of a solution of 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) (Across Organics B.V.B.A.) was added to the cell culture at 0.5 mg/mL in RPMI-1640. The plate was incubated for an additional 3 hours under the same conditions as before. After this time, the contents of the plate were poured and 100 µL of isopropyl alcohol (Mallinckrodt Pharmaceuticals) were added to each bore to solubilize the formed formazan crystals. Only cells and RPMI-1640-C culture medium were used as a control, equivalent to 100 % viability of macrophages. The reading was done in a UV/Vis spectrophotometer (Multiskan Ascent, Labsystems Research Tech. Div., Helsinki, Finland) at 540 nm with reference filter at 620 nm (Mosmann 1983).

Determination of nitric oxide production

Nitric oxide was quantified by nitrite accumulation in culture medium and measured spectrophotometrically using Griess reagent with $NaNO_2$ as standard (Green et al. 1982).

The obtained cell suspension was adjusted to 5 x 10^6 cells/mL in RPMI-1640 medium. 100 µL of this cell suspension was distributed in 96-well sterile plate. 100 µL of the *A. glandulosa* ethyl acetate fraction (100, 200 and 400 µg/mL), and *M. elliptica* methanolic extract (75, 150 and 300 µg/mL), celecoxib (25, 50 and 100 µg mL), doxorubicin (12.5, 25, 50 and 100 µg mL). 100 µL from 10 µg/mL LPS solution as a positive (control) stimulant, and the other wells were filled with 100 µL of RPMI-1640 medium was added to the cell suspension as a control cell (negative control). The plate thus formed was incubated for 24 hours in an oven at 37° C with constant tension of CO_2 (5%). After incubation, 50 µL aliquots of each sample were withdrawn and transferred to another plate and another 50 µL/well Griess reagent, 0.1% N-1-naphthyl-ethylenediamine, 0.1% sulfanilamide in 3% phosphoric acid solution. After 10 minutes at room temperature in the dark, the plates were read in a UV/Visible microplate spectrophotometer (Multiskan Ascent, Labsystems Research Tech. Div., Helsinki, Finland) with a 540 nm filter. Concentrations of sodium nitrite were calculated from a standard curve previously established with known molar concentrations of sodium nitrite in RPMI-1640 medium. The tests were performed in triplicate and the values expressed in µmols of NO/5x10^5 cells.

Determination of inhibitory activity in cultures of mouse peritoneal cells in NO production

The test was performed from a cell suspension of the peritoneal exudate adjusted at 5x10^6 cells/mL in RPMI-1640 medium. 100 µL of cell suspension was distributed in 96-well sterile plate. 100 µL of the *A. glandulosa* ethyl acetate fraction (100, 200 and 400 µg/mL), and *M. elliptica* methanolic extract (75, 150 and 300 µg/mL), celecoxib (0.025, 0.05 and 0.1 mg/mL), doxorubicin (12.5, 25, 50 and 100 µg/mL) were used. 100 µL of 10 µg/mL LPS solution as positive (control) stimulant, and in other wells, only 100 µL of RPMI-1640 medium was added to the cell suspension as a control cell (negative control). 100 µL of LPS solution (10 µg/mL) was added in all wells where the compounds were tested. The plate thus formed was incubated for 24 hours in an oven at 37° C with constant tension of CO_2 (5%). After incubation, 50 µL aliquots of each sample were withdrawn and transferred to another plate and

another 50 µL/well Griess reagent, 0.1% N-1-naphthyl-ethylenediamine, 0.1% sulfanilamide in 3 % phosphoric acid solution (Green et al. 1982). After 10 minutes at room temperature in the dark, the plates were read in a UV/Visible microplate spectrophotometer (Multiskan Ascent, Labsystems Research Tech. Div., Helsinki, Finland) with a 540 nm filter. Concentrations of sodium nitrite were calculated from a standard curve previously established with known molar concentrations of sodium nitrite in RPMI-1640 medium. The tests were performed in triplicate and the values expressed in µmols of NO/5x10^5 cells.

Determination of cytokines TNF-α and IL-10

The cytokines TNF-α and IL-10 were quantified in the supernatant obtained from the macrophage culture through the ELISA immunoenzymatic assay using BD OptEIA kit (BD Biosciences Pharmingen) according to the manufacturer's instructions. The 96-well polystyrene microplates (Corning Inc., NY) were adsorbed with an anti-TNF-α or anti-IL-10 capture antibody and incubated overnight at room temperature. Plates were washed 3 times with PBS (pH 9.5) containing 0.05% Tween-20 (PBS-T). After washing, the plates were blocked with PBS containing 10 % FBS (PBS-FBS) and kept at room temperature for 60 minutes. The plates were washed three times with PBS-T. 100 µl of each standard cytokine or supernatants from the cell cultures to be tested were added to the plates. The plates were incubated at room temperature for 120 minutes, and washed five times with PBS-T. Then, 100 µL of biotin-labeled anti-TNF-α or anti-IL-10 monoclonal antibody was added and diluted to the appropriate concentration of each cytokine in PBS-FBS. The plates were incubated at room temperature for 30 minutes and washed 5 times with PBS-T, and then 100 µL of peroxidase-streptavidin conjugate diluted in PBS-FBS was added. The plates were incubated again at room temperature for 30 minutes. After this procedure, the plates were washed 7 times with PBS-T and 100 µL of the BD OptEIA™ ELISA sets TMB substrate (BD Biosciences Pharmingents) were added into each well. After further incubation at room temperature for 30 minutes, the reaction was quenched by adding 50 µL of 2N sulfuric acid (H_2SO_4). Absorbance was read at 450 nm in a UV/visible microplate spectrophotometer (Multiskan Ascent, Labsystems Research Tech Div., Helsinki, Finland) and cytokine concentrations were obtained using a standard curve previously established with known amounts of TNF-α and IL-10 standard. The tests were performed in triplicate and the results expressed in picograms/mL.

Statistical analysis

The statistical analyzes were performed using the software Graphpad Instat version 3.00 for windows (GraphPad Software, San Diego, CA). We compared the differences of the treatment groups and controls using one-way analysis of variance (ANOVA) and Tukey's multiple comparison *post hoc test*. Differences between groups were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Ehrlich's tumor, a transplantable tumor originating from a rapidly growing mammary adenocarcinoma and aggressive behavior (Segura et al. 2000), is able to grow in almost all the mouse strains, suggesting that recognition and immune response to this type of tumor are independent of the MHC (Chen & Watikins 1970). This feature suggests that Ehrlich tumor control is related to innate immunity. Hence, the participation of macrophages, especially with respect to the inflammatory response rather

than to an adaptive response mediated by T cells (Nascimento et al. 2006).

Compounds obtained from plants that inhibit the synthesis of NO may be important candidates for the development of anti-inflammatory drugs (Yunes & Calixto 2001) as observed in the present study with the fraction and extract of *A. glandulosa*.

Moleiro et al. (2009) observed inhibition of NO production, different from our findings, where both in the group of animals with Ehrlich tumor and in the group of healthy animals there was high NO production in the two tested concentrations (150 and 300 µg/mL). Several modern research points to a potential use of plant compounds in the treatment of inflammation. Spelman et al. (2006) highlights that a strategy in modulating cytokine expression may be a key factor. In this context, plant-derived immunomodulatory compounds could offer modern approaches in the treatment of a variety of diseases, making possible through the dynamic regulation of messenger molecules such as cytokines, a change in the activity of the immune system and/or control of the inflammatory process.

IL-1 and TNF-α are mainly derived from mononuclear cells and macrophages and induce the expression of several genes that stimulate the synthesis of innumerable proteins that contribute to inflammatory process (Carvalho 2004). TNF-α, initially discovered due to its antitumor activity, is one of the major mediators of inflammation. Induced by a wide range of pathogenic stimuli, this cytokine induces other inflammatory mediators and proteases that orchestrate the inflammatory response. TNF-γ is also produced by tumors and can act as an endogenous tumor promoter. This cytokine is related to the stages of tumorigenesis, including cell transformation, proliferation, invasion, angiogenesis and metastasis (Sethi et al. 2008).

Evaluation of macrophages viability

Considering that the cells of an animal with a pathological process may present different behaviors in relation to the cells of healthy animal, the first questioning of this research was to verify the viability of the cells obtained from normal and induced animals to tumor development. The viability is of fundamental importance during the development of experimental manipulations, ensuring the ability of a given cell population to respond to the substances to be tested (Freshney 1994). Viability assays ensures that the concentrations of the tested compounds are adequate and do not cause significant cytotoxicity to cell cultures in the presence and absence of substances to be tested. It still defines appropriate experimental conditions for *in vitro* assays and that the effects observed in the other NO and cytokine production tests would not be due to cytotoxicity.

For this, we used the test described by Mosmann (1983) based on the cell ability to cleave the tetrazole ring present in MTT (Figure 1) and healthy animals (Figure 2) presented compatible percentages of viability greater than 70 % when the cells were in contact with the substances to be tested. Regarding the cell viability tests, the results showed that the methanolic extract of *A. glandulosa* associated with the macrophages of the animals with Ehrlich tumor resulted in less cellular viability (slightly below 75 %) compared to healthy animals. Maybe the cells of the animals with tumor are less resistant to the cytotoxicity of the plant when compared to the cells of healthy animals. We can define the concentrations to be used in the other tests proposed here. The evaluation of macrophages viability was performed using MTT technique. Peritoneal exudate cells were incubated with the ethyl acetate fraction and the methanolic from *A. glandulosa*, and with the methanolic extract

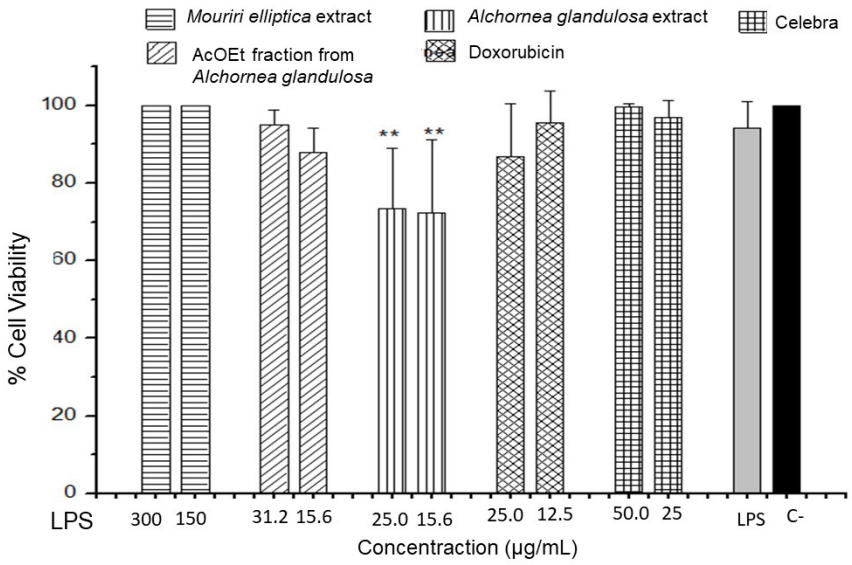

Figure 1. Viability of peritoneal macrophages of Ehrlich tumor-bearing mice in the presence of different concentrations of extracts, fractions and drugs. Cells in culture medium (RPMI-1640) were used as negative control (C-) and LPS solution as positive control. **$P < 0.001$ when compared to the negative control.

from *M. elliptica*, celecoxib and doxorubicin. Figure 1 shows the viability of macrophages of Ehrlich tumor bearing mice in the presence of all compounds.

In this study, satisfactory viability (around 70%) was observed in macrophages of mice bearing Ehrlich tumor in the presence of all compounds, the lowest viability being observed in those where *A. glandulosa* extracts were tested. Viability above 80% was observed in macrophages of normal mice in the presence of all samples including *A. glandulosa* extracts (Figure 2).

Determination of NO production/inhibition

In general, macrophages from Ehrlich tumor-bearing mice had a lower inhibition of NO production when compared to normal macrophages (5.56 to 52.23 % in the tumor group and 22.81 to 84.13 % in the group without tumor) (Figures 3 and 4). Exception was observed in macrophages associated with doxorubicin and celecoxib, which showed greater inhibition in the group of animals with tumor, with production around 30.0 to 36.0 µmol / $5x10^5$ cells. Only *M. elliptica* produced this mediator with results both in the animals with tumor and in the normal ones near the positive LPS control.

NO exerts modulatory effects on the inflammatory response and plays an important role in the regulation of immune responses. In Figure 3 we observed the results of NO production by *M. elliptica* and inhibition by *A. glandulosa*, doxorubicin and celebrated in animals bearing tumor. The highest percentages of inhibition were obtained with doxorubicin (52.23 %) and the drug celebrated (41.84 %) confirming the activity of these drugs related to NO. Aldieri et al. (2002) studying the production of nitrite by cardiac H9c2 cells from rats in contact with doxorubicin, showed that cardiotoxicity, a known effect of doxorubicin is due to an increase in the NO synthesis stimulated by the drug, which explains in part its cytotoxicity, used against tumors.

When we analyzed the dosage of this mediator in normal, tumor-free animals (Figure 4), we found that crude extract at 31.25 µg/mL and the *A. glandulosa* fraction had an inhibitory effect (65.48 % and 78.32 %, respectively) as much as it celebrates that it kept its inhibition result more pronounced (78.18 %).

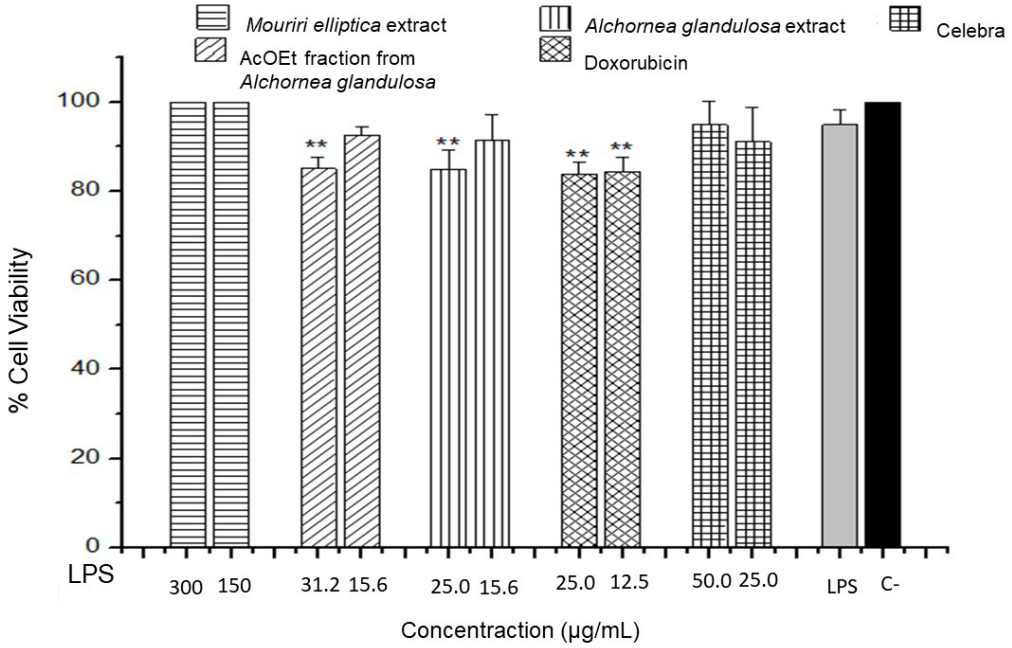

Figure 2. **Viability of peritoneal macrophages of normal mice in the presence of different concentrations of extracts, fraction and drugs. Cells in culture medium (RPMI-1640) were used as negative control (C-) and LPS solution as positive control. ** *P* <0.001 when compared to the negative control.**

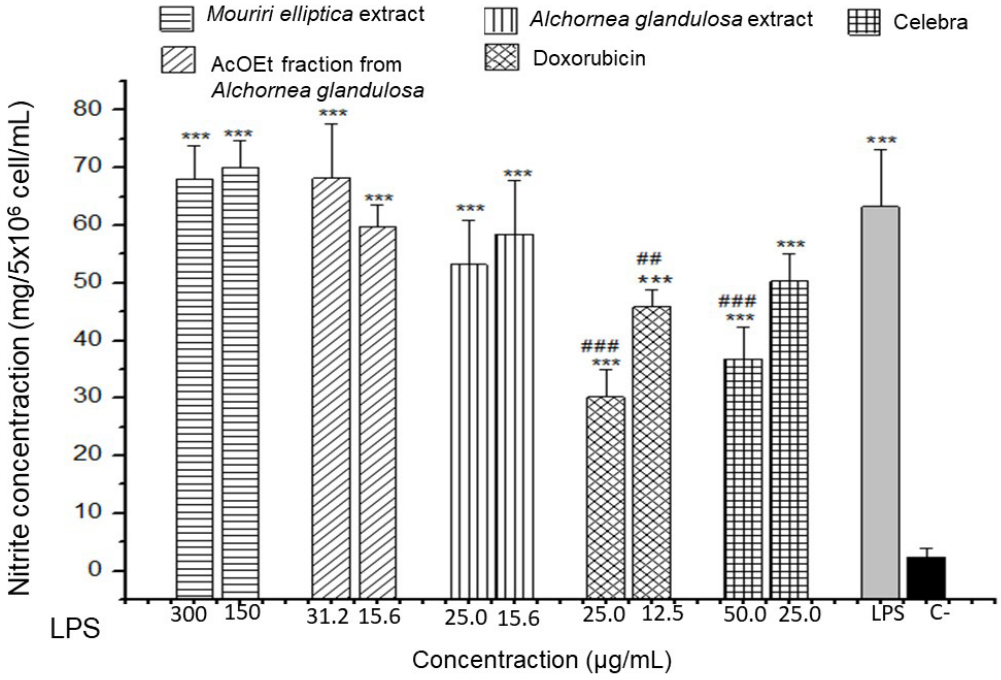

Figure 3. **Determination of NO production/inhibition in culture of peritoneal macrophages of mice bearing Ehrlich's tumor. The peritoneal exudate cells were incubated under the test conditions with the different extracts, fraction or commercial drugs concomitantly with LPS solution at 10 µg/mL. As a positive control, LPS solution (LPS) was used. As negative control (C-), only cells in culture medium (RPMI-1640-C) were used. The statistical analysis was performed through variance (ANOVA) using the Tukey post-test. *** *P* < 0.001 when compared to the negative control. ### *P* < 0.001, ## *P* < 0.01 when compared to the positive control.**

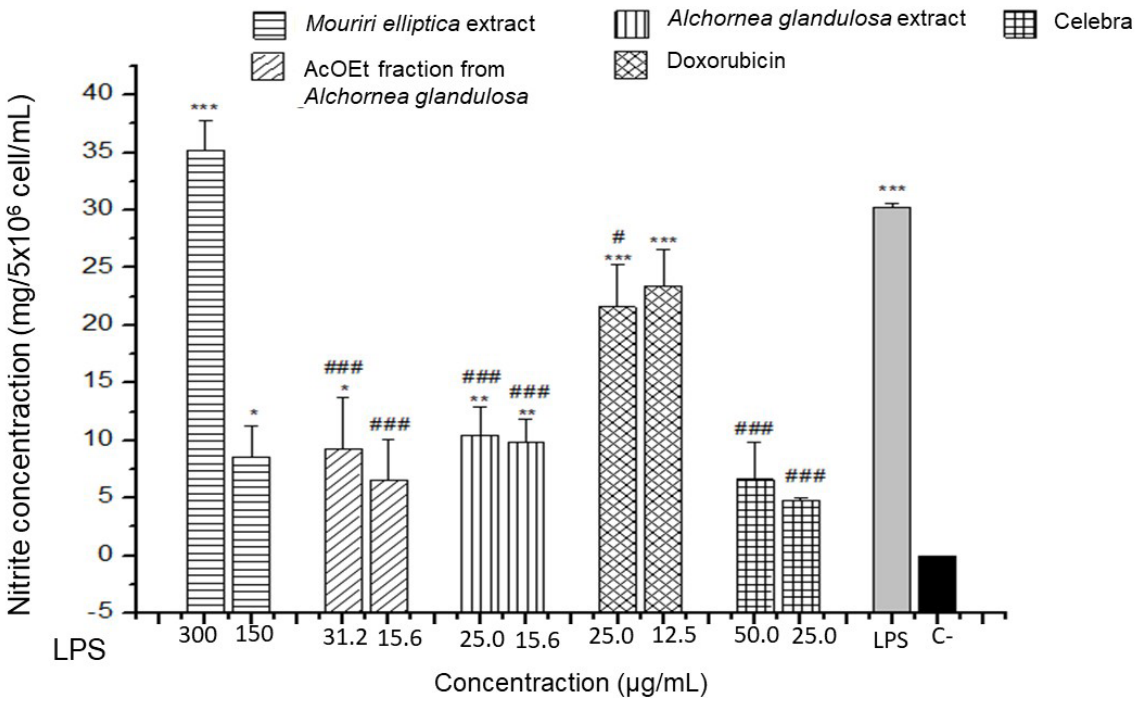

Figure 4. Determination of NO production/inhibition in culture of peritoneal macrophages of normal mice. LPS solution was used as positive control (LPS). As negative control (C-), only cells in culture medium (RPMI-1640-C) were used. The statistical analysis was performed through variance (ANOVA) using the Tukey post-test. * *P* <0.001, ** *P* < 0.01 and *P* < 0.05 when compared to the negative control. ### *P* < 0.001, # *P* <0.05 when compared to positive control.**

Determination of TNF-α production

Our TNF-α detection results demonstrate that *M. elliptica* in both tumor bearing animals (Figure 5) and healthy animals (Figure 6) induced the highest production of this mediator, data compatible with the of NO production, since this cytokine acts synergistically with the IFN-mediator in its production (Jang et al. 2003). The lower production of TNF-α by *A. glandulosa* observed in the present study is comparable with previous results and indicative of inhibition of the inflammatory process (Lopes et al. 2005). Since the presence of proinflammatory cytokines in the tumor microenvironment has been associated with angiogenesis, it is believed that the ethyl acetate fraction of *A. glandulosa* and the pure compounds afzelin and pteroginidine have a special role in that process. Such compounds inhibit simultaneously the production of proinflammatory cytokines by macrophages and the various stages of angiogenesis (Lopes et al. 2009). We must also consider that NO is a vasodilator, aiding angiogenesis and metastasis. (Cheng et al. 2014). Angiogenesis has been described as a key part of the cancer process, playing a fundamental role in tumor growth, tumor invasion and metastasis, thus promoting greater tumor irrigation and consequently favoring its growth (Sethi et al. 2008).

TNF-α can induce tumor necrosis through two mechanisms: the direct cytotoxic effect on tumor cells and stimulation of the immune system. TNF-α has been shown to induce death by necrosis or apoptosis in some primary human tumor cells and in tumor cell lines (Aggarwal & Natarjan 1996), including breast cancer strains (Ben-Baruch 2003), malignant melanoma, and sarcoma of soft tissues of the limbs (Lake 2003).

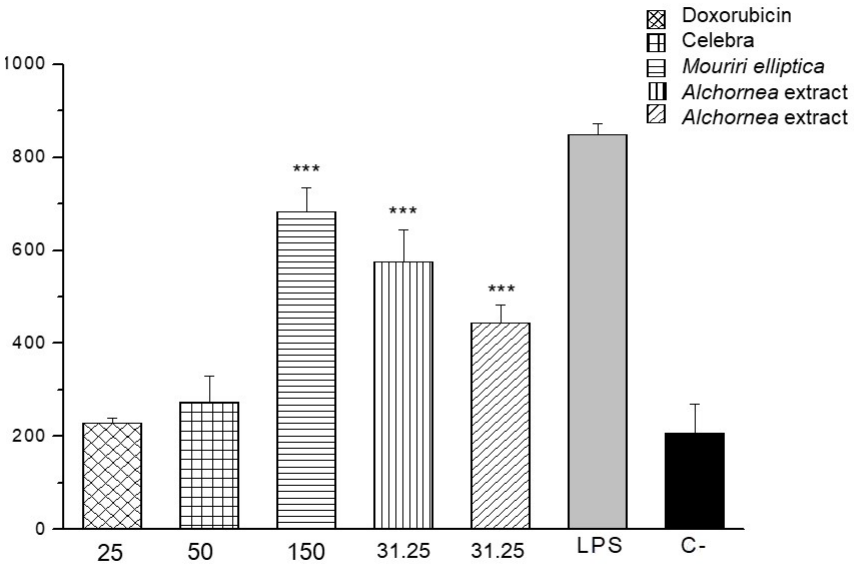

Figure 5. Determination of TNF-α production in culture of peritoneal macrophages of animals with tumor. Supernatants collected from macrophages incubated only in the presence of LPS (1µg/mL) or culture medium (RPMI-1640) were used respectively as positive (LPS) and negative (C-) controls. Each bar represents the mean ± standard deviation of seven independent experiments performed in triplicate. Statistical analysis was performed through analysis of variance (ANOVA) using the Tukey post-test. *** $P < 0.0001$ when compared to positive control (LPS).

Regarding the effect of *A. glandulosa* on the production of IL-10, a cytokine known as anti-inflammatory, there was very little production of IL-10 by the macrophages of animals with tumor (Figure 7). On the other hand, when we observed the production of this mediator by normal, non-tumor-bearing animals (Figure 8), there was a higher production of this mediator of the cells in contact with doxorubicin and with methanolic extract of *M. elliptica*, attracting attention because it is a mediator with an anti-inflammatory approach and the tumor process can be disadvantaged. It is important to note that NO and pro-inflammatory cytokines are the major targets in the treatment of inflammatory diseases. Since macrophages produce several biologically active molecules that participate in the inflammatory process, therapeutic interventions targeting these cells, or their products may create novel methods of controlling inflammatory diseases (Fujiwara & Kobayashi 2005).

In general, TNF-α production by macrophages of mice with tumor was higher than that of normal animal macrophages (Figures 5 and 6). Among the different compounds, the one that most stimulated the production of TNF-α was the methanolic extract of *M. elliptica*. The substances that least stimulated the production of this cytokine were doxorubicin and celecoxib. It should be noted that the stimulus of the extract and the ethyl acetate fraction of *A. glandulosa* were statistically lower than that observed in the positive control ($P < 0.05$).

The IL-10 production by the macrophages of mice with tumor was markedly lower when compared to the production by healthy animals with all the compounds tested (Figures 7 and 8). In fact, no compound stood out from the others in stimulating IL-10 production among animals with tumor.

Considering the healthy animals, the doxorubicin and the methanolic extract of *M. elliptica* showed production of this mediator higher than 50 % of the value found for the positive LPS control.

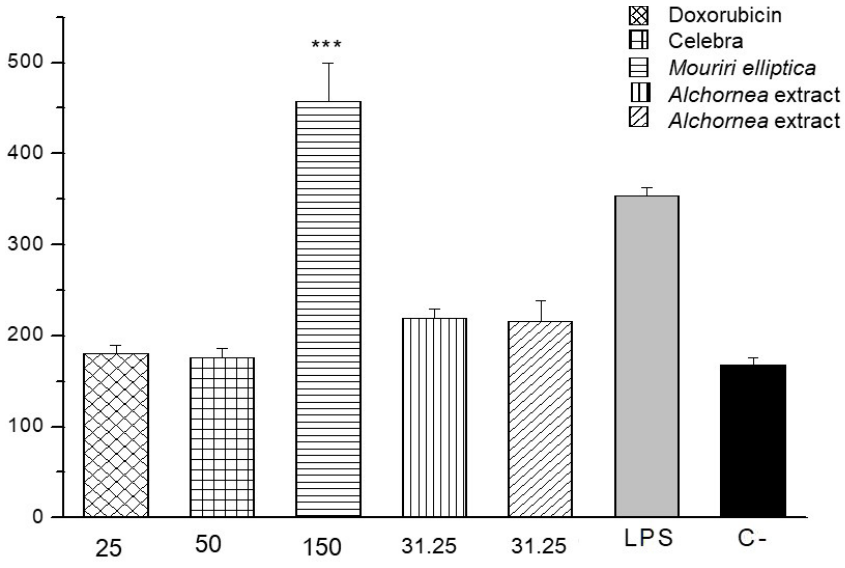

Figure 6. Determination of TNF-α production in culture of peritoneal macrophages of healthy animals. Supernatants collected from macrophages incubated only in the presence of LPS (1 µg/mL) or culture medium (RPMI-1640) were used respectively as positive (LPS) and negative (C-) controls. Each bar represents the mean ± standard deviation of seven independent experiments performed in triplicate. Statistical analysis was performed through analysis of variance (ANOVA) using the Tukey post-test. * *P* < 0.0001 when compared to the positive control (LPS).**

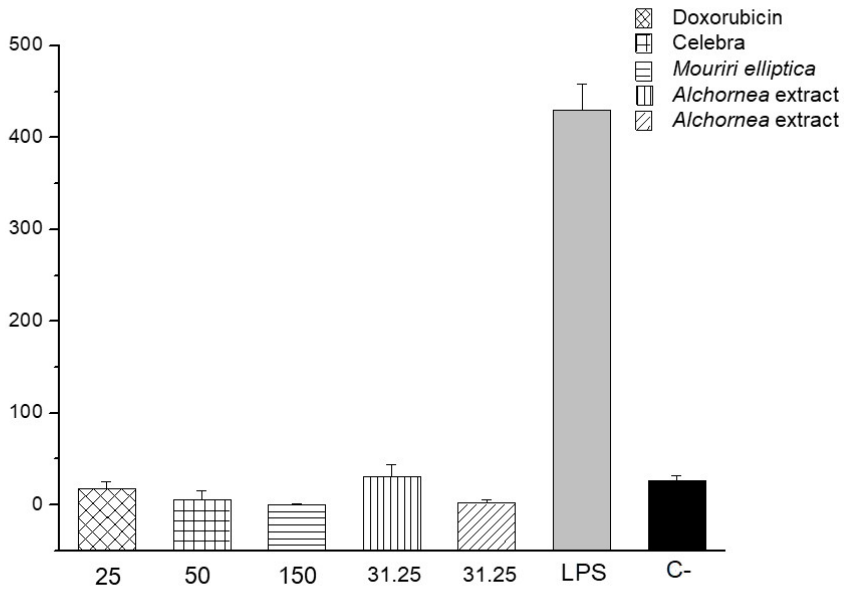

Figure 7. Determination of IL-10 production in culture of peritoneal macrophages of animals with tumor. Supernatants collected from macrophages incubated only in the presence of LPS (1 µg/mL) or culture medium (RPMI-1640) were used respectively as positive (LPS) and negative (C-) controls. Each bar represents the mean ± standard deviation of seven independent experiments performed in triplicate. Statistical analysis was performed through analysis of variance (ANOVA) using the Tukey post-test.

CONCLUSION

Thus, our results seek to contribute to a better understanding of the mechanisms by which natural products can act as new antitumor therapeutic compounds directed to the anti-inflammatory process. In addition, this study provided a better understanding of the pharmacological actions of *M. elliptica* and *A. glandulosa* with an immunological approach, emphasizing in this context the importance of biodiversity and medicinal plants.

Acknowledgments

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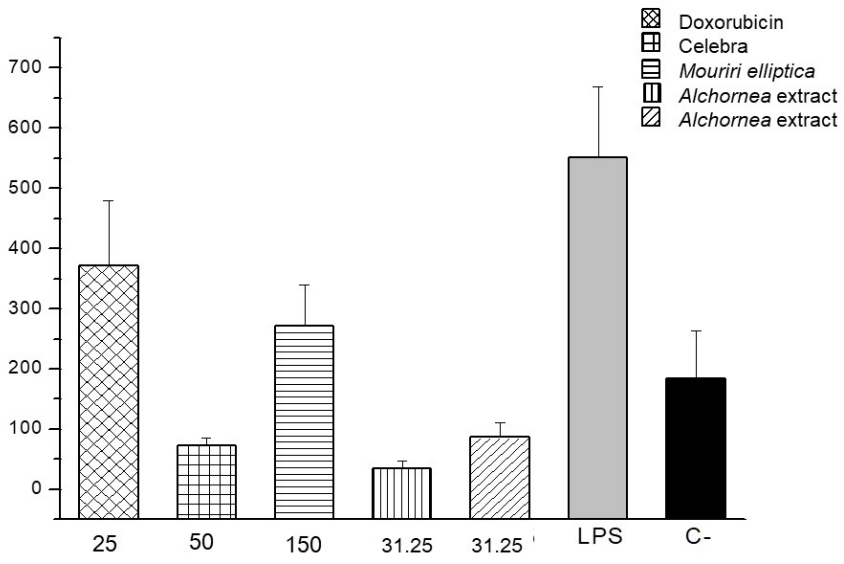

Figure 8. Determination of IL-10 production in culture of peritoneal macrophages of healthy animals. Supernatants collected from macrophages incubated only in the presence of LPS (1 µg/mL) or culture medium (RPMI-1640) were used respectively as positive (LPS) and negative (C-) controls. Each bar represents the mean ± standard deviation of seven independent experiments performed in triplicate. Statistical analysis was performed through analysis of variance (ANOVA) using the Tukey post-test.

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