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Effect of *Arrabidaea chica* extracts on the Ehrlich solid tumor development

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Article

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Abstract: The aim of this study was to investigate the effect of *Arrabidaea chica* (Humb. & Bonpl.) B. Verl., Bignoniaceae, extracts on Ehrlich solid tumor development in Swiss mice. Leaves of *A. chica* were extracted with two distinct solvents, ethanol and water. The phytochemical analysis of the extracts indicated different classes of secondary metabolites like as anthocyanidins, flavonoids, tannins and saponins. Ethanol (EE) and aqueous (AE) extracts at 30 mg/kg reduced the development of Ehrlich solid tumor after ten days of oral treatment. The EE group presented increase in neutrophil count, α1 and β globulin values, and decrease of α2 globulin values. Furthermore, EE reduced the percentage of $CD4^+$ T cells in blood but did not alter the percentage of inflammatory mononuclear cells associated with tumor suggesting a direct action of EE on tumor cells. Reduced tumor development observed in AE group was accompanied by a lower percentage of $CD4^+$ T lymphocytes in blood. At the tumor microenvironment, this treatment decreased the percentage of $CD3^+$ T cells, especially due to a reduction of $CD8^+$ T subpopulation and NK cells. The antitumor activity presented by the AE is possibly related to an anti-inflammatory activity. None of the extracts produced toxic effects in animals. In conclusion, the ethanol and aqueous extracts of *A. chica* have immunomodulatory and antitumor activities attributed to the presence of flavonoids, such as kaempferol. These effects appear to be related to different mechanisms of action for each extract. This study demonstrates the potential of *A. chica* as an antitumor agent confirming its use in traditional popular medicine.

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Introduction

Cancer is one of the leading causes of mortality worldwide. Many of the chemotherapeutic agents used in the treatment of cancer injure rapidly dividing normal cells presenting substantial side effects. Therefore, the quest for effective anticancer drugs is an active research field. The plant kingdom is a potential source of chemicals with antitumor and cytotoxic activities (Kim et al., 2005). New drugs generated from secondary metabolites of plants represent an alternative source of anticancer agents, which frequently seem to be more effective and/or less toxic (Ma & Wang, 2009).

Arrabidaea chica (Humb. & Bonpl.) B. Verl., Bignoniaceae, is a shrub plant widespread from South Mexico to central Brazil, particularly in the Amazon basin (Correa, 1931; Takemura et al., 1995). The leaves of the plant have been traditionally used by Brazilians Indians as dye in ritual body painting, to protect the skin against the sunlight and to repel insects (Chapman et al, 1927). Very little is known about the chemical constitution of the leaves (Barbosa et al., 2008). Earlier studies of *A. chica* aimed to determine the composition of the dye (Chapman et al., 1927). Later, a flavone (Takemura, 1995) and three anthocyanidins (Zorn et al., 2001) were isolated. The content of phenolic and flavonoids in the leaf were

determined, ±10.2 mg/g and 0.06 mg/g, respectively (Silva et al., 2007). More recently the main classes of secondary metabolites in the ethanol extract was detected: anthocyanidins, anthocyanins, antraquinone, cathechins, organic acids, reducing sugars, steroids, xanthones, tannins, flavanonols and flavanone (Barbosa et al., 2008).

Nowadays *A. chica* is widely used in the popular medicine for wound healing, treatment of inflammation, intestinal colic, blood dysfunction, uterine inflammation and leukemia (Zorn et al., 2001; Jorge et al., 2008; Barbosa et al., 2008). Despite the wide use very little is known about the pharmacological properties of its extracts (Barbosa et al., 2008). The anti-inflammatory effect was already demonstrated *in vivo* (Oliveira et al., 2009) and *in vitro*, with the inhibition of the transcription factor NF-kB (Zorn et al., 2001). Also, healing and antioxidant activities were reported, possibly related to the presence of anthocyanidins (Jorge et al., 2008). Antitumor activity of the ethanol extract (25 µg/mL) was observed *in vitro* against Jurkat and HL60 cell lines (Ribeiro et al., 2010).

Therefore, based on the empirical ethnopharmacological use of *Arrabidaea chica* crude extract as an antitumor agent associated with the strong cytotoxic activity *in vitro* (Ribeiro et al., 2010) and the lack of *in vivo* studies with this plant and its constituents, this study examined the antitumor activity of the ethanol and aqueous extracts of *A. chica* on the Ehrlich solid tumor model.

Material and Methods

Plant material

Leaves of *Arrabidaea chica* (Humb. & Bonpl.) B. Verl., Bignoneaceae, with the common names: Pariri, Crajiru, Carajuru or Carajiru were collected in Maringá (State of Paraná, Brazil). The plant material was identified in Campinas-SP, Brazil, where a voucher specimen is deposited and registered under the number UEC 145.956 (Herbário da Universidade de Campinas).

Preparation of extracts

The leaves of *A. chica* were shade dried at room temperature, powdered, and extracted (40 g) with distillated water (0.5 L) originating a dark red crude extract. This extract was fractionated according to Matos (1988). Briefly, the pH was basified with ammonium hydroxide (pH 11.0). A solution of *n*-hexane and ethyl acetate (1:1) (0.5 L) was added to the crude extract leading to two distinct phases, one organic and one aqueous. After separation, hydrochloric acid (pH 1.5) and a solution of hexane: acetyl acetate (1:1) (0.5 L) were added to the organic phase leading to a new organic and a new aqueous phase. This last aqueous phase was concentrated under a rotary vacuum evaporator (65 ºC) originating the aqueous extract used in the experiment (AE). The pH was basified (pH 7.0) and the extract was submitted to lyophilization (1.6 g) and preserved in a freezer at -20 ºC until further use.

The ethanol extraction was performed as previously described (Leite et al., 2006) with slight modifications. Briefly, the ethanol extract (EE) was prepared by maceration of dry leaves (100 g) for exhaustive percolation with ethanol at room temperature. After filtration, the solvent (ethanol) was evaporated under reduced pressure to yield 1.8 g of a viscous red brown extract (yield, 1.8%). After, this extract was submitted to exhaustive extraction with hexane under reflux in a water-bath for 2 h, resulting in a hexane-insoluble fraction used in the experiment (EE) (1 g).

Phytochemical analysis

Chemical tests to detect the main classes of secondary metabolites were carried out in aqueous extract (AE) using classical specific color reactions (Matos, 1988).

The ethanol extract (EE) was dissolved in methanol (1 mg/mL) and filtered through Whatman paper filter and the filtrate was used for HPLC analysis. Chromatographic separation was performed on a Shimadzu® liquid chromatographic system equipped with a LC-10AT-vp solvent delivery system, SPD M-10 AVP photo diode array detector, and Rheodyne 7725i injector with 5 µL loop. A Phenomenex GEMINI C18 column (25cm×4.6mm i.d., 5 µm) was used for the separation. A mixture (75:25 v/v) of phosphate buffer (25 mM%) and acetonitrile was used as the mobile phase. It was delivered at a flow rate of 1.0 mL per min with detection at 360 nm. The retention time of kaempferol was found to be 28.34 min. The injection volume of the EE extract was 50 µL. Analysis was performed at room temperature.

Tumor cells

Ehrlich ascites carcinoma (EAC) cells were supplied by the Laboratório de Patologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Brazil. The cells were maintained *in vivo* in Swiss albino mice by intraperitoneal transplantation. EAC cells aspirated from the peritoneal cavity of mice were washed with saline and injected subcutaneously to develop solid tumor.

Antitumoral activity

This experiment was in agreement with the Ethical Principles in Animal Experimentation adopted by the Ethics Committee in Animal Experimentation of

Universidade Federal de Minas Gerais, Brazil (CETEA/UFMG) (Protocol 155/2009).

Fifteen female Swiss Albino mice with eight weeks of age were injected with EAC cells (2.5 x 10^6), subcutaneously in the left footpad to obtain the solid tumor. These animals were divided into three groups (n=5) and treated with aqueous extract (Group I), ethanol extract (group II) and distilled water (Group III).

The aqueous extract (AE) or the ethanol extract (EE) (30 mg/kg body weight) were suspended in distilled water (300 µL) and administrated to the animals orally, with the help of an intragastric catheter, once daily for ten days, beginning three days after the tumor inoculation. The selection of the dose was based on the work of Queiroz et al. (2008) who studied the antitumor properties of *Tabebuia avellanedae,* plant of the same family as *A. chica*. The control group received 300 µL of water by gavage, as describe to groups AE and EE.

Tumor growth was evaluated by measuring the inoculated footpad thickness with a graduate micrometer (Mitutoyo, model 7301, graduation 0.01 mm, range 0-10 mm, accuracy ±0.02 µm) as described (Silva et al., 2004; Silva et al., 2006; Pinto et al., 2009). This procedure was done at every 48 h. On the 10th day of treatment, *i.e.,* on the 12th day of tumor development, all mice were euthanized. The heart, lungs, kidney, liver, spleen, the inoculated footpad and the ipsilateral popliteal lymph node were collected, fixed in 10% buffered formalin and conventionally embedded in paraffin. The four micrometers sections were stained in hematoxilin and eosin for histological examination.

Blood samples were collected by retro-orbital puncture for hematological evaluation before the euthanasia. Total white blood cell (WBC), red blood cell (RBC) and haemoglobin (Hb) were determined by standard method using cell diluting fluid and haemocytometer (CELM DA 500®, Barueri-SP, Brazil). The packed volume cell (PVC) was measured in a microhematocrit centrifuge (Microspin, São Paulo, Brazil) with capillary tubes. The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated. Blood smears were stained by the May-Grünwald-Giemsa technique to obtain differential leukocyte count (Thrall, 2004).

Plasma protein concentration was measured using a refractometer (Ningbo Utech International CO Ltda, model 301). Plasmatic protein fractions were obtained by electrophoresis using an agarose gel electrophoresis system (CELM SDS-60, Brazil). A 1.0 µL sample of plasma was applied to the gel, which was exposed to 100V for 32 min. The gel was stained (Ponceau S, Vetec Química Fina Ltda, Duque de Caxias-RJ, Brazil), fixed and dried. Bands were scanned and measured using the software Celm SE-250. Percentage and absolute values (g/dL) for the proteins fractions were determined on the basis of total protein concentration. The albumin/globulin ratio was calculated as albumin/(a1+a2+b+y globulins).

Analysis of the mononuclear leukocyte subpopulations in blood and tumor tissue by flow cytometry

In this protocol the number of five types of mononuclear leukocytes on the blood and tumor tissue were evaluated: TCD_4, TCD_8 (CD3 PerCP, CD4 FITC, CD8 PECY5) and B lymphocytes (CD5 PerCP, CD19 FITC) and natural killer (NK1.1 FITC) cells. Total blood was collected and 50 µL were incubated with a monoclonal antibody labeled with fluorescein. The tumor tissue was squashed in RPMI-1640 (5 mL) media and filtrated on stainless steel gaze to obtain a single cell suspension. The cell suspension was kept on ice for 5 min and washed twice in RPMI-1640. After centrifugation (10000 x *g*, 10 min, 4 ^oC) the supernatant was ressuspended in 150 µL. Subsequently 30 µL of the supernatants were collected and incubated with a monoclonal antibody labeled with fluorescein. The protocols, antibodies and reagents employed were as per the manufacturer's recommendations (Sigma). The percentage of labeled cells was obtained by analysis of 10000 events with a FACSCalibur flow cytometer (Becton Dickinson. Palo Alto, CA, USA) using the Cell-Quest software.

Statistical Analysis

All data were checked for normality. Statistical significance between groups was performed by the application of one-way analysis of variance ANOVA followed by SNK (cv<25%) or Duncan (cv>25%) test when data were normally distributed. Kruskal-Wallis or Mann-Whitney U tests were used when data were not normally distributed. The difference was reliable with $p<0.05$. Data were expressed as means±SD.

Results and Discussion

In order to evaluate if *Arrabidaea chica* (Humb. & Bonpl.) B. Verl., Bignoneaceae, ethanol or aqueous extracts demonstrate antitumoral effect against a solid tumor, mice received intraplantar injection of 2.5 x 10^6 EAC cells. Measures of mice paws showed a continuous tumor growth in all experimental groups. Both groups treated with AE and EE showed similar patterns of tumor development - a steady growth without the occurrence of peaks during the experimental period, while the control group presented a peak seven days after the inoculation (Table 1).

In 12th day, the EE group presented a smaller tumor development (2.357±0.47µm), when compared to control group (3.237±0.75µm).

The growth curve of the Ehrlich tumor presents an exponential behavior from the third day post inoculation until day 30th (Dagli et al., 1992a). The control group showed significant differences in tumor growth between days 7th and 12th. The growth rate of Ehrlich tumor in mice differs in the site of implantation and in the form. In the solid form occurs a proliferation peak after the 7th day of implantation (Silva et al., 2006) like demonstrated with control group in our experiment.

There was a significant reduce in the tumor growth in mice treated with AE and EE at 12th day, different from control group (Figure 1).

Table 1. Measurement of Ehrlich solid tumor growth in mouse footpad (μm).

Group	Day of tumor development				
	3	5	7	9	12
Control	2.068±0.08 aA	2.092±0.06 aA	2.307±0.18 aAB	2.451±0.46 aAB	3.237±0.75 aB
AE	2.108±0.10 aA	2.053±0.08 aA	2.097±0.13 aAB	2.290±0.33 aAB	2.495±0.51 aB
EE	1.968±0.14 aA	1.935±0.13 aA	1.918±0.11 bA	2.067±0.22 aA	2.357±0.47 bA

*Data was presented as mean±standard deviation. Values with different letters are significantly different (p<0,05). Lowercase to columns and uppercase to lines.

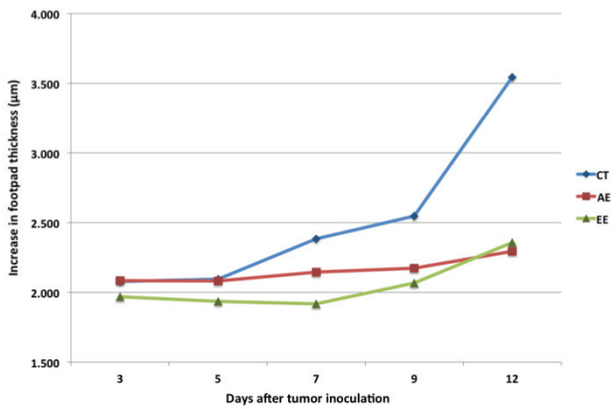

Figure 1. Measurement of Ehrlich solid tumor growth (μm) in the footpad of mice treated with *Arrabidaea chica* aqueous extract (AE) and ethanol extract (EE) during twelve days.

Cancer is a disease of misguided cells that have high potential of excess proliferation without apparent relation to the physiological demand of the process. It is the second largest cause of death in the world. Of all the available anticancer drugs used in the last decades, 40% were natural products per se or natural product derived, with another 8% being natural product mimics (Newman et al. 2003). The greatest recent impact of plant-derived drugs is observed in the area of antitumor research, where compounds such as taxol, vinblastine, vincristine, and camptothecin have dramatically improved the effectiveness of chemotherapy against some of the most dreaded cancers (Rates, 2001). Hence, there is a great potential for the development of anticancer drugs from the essentially untapped reservoir of the plant kingdom. A large number of plants possessing anticancer properties have been documented (Gupta et al., 2004, Aoki et al., 2005, Kim et al., 2005).

Earlier studies carried out in our laboratory have shown potent cytotoxic activity of *A. chica* ethanol extract (Ribeiro et al., 2010). Based on this observation, in the present study, the aqueous and ethanol extracts were evaluated for its in vivo antitumor properties. These results could indicate either a direct cytotoxic effect of A. chica extracts on tumor cells or an indirect local effect, which may involve macrophage activation and vascular permeability inhibition. The direct strong cytotoxic effect of ethanol extract was already demonstrated in the *in vitro* experiments against Jurkat and HL60 cell lines (Ribeiro et al., 2010) and in the present study, against Ehrlich solid tumor.

Preliminary phytochemical investigation of the ethanol extract of *A. chica* revealed the presence of phenolics, tannins, flavanols, anthocyanins, organic acids and reducing sugars. The antifungal and trypanocidal activities of *A. chica* ethanol extract appear to be related to the presence of quinones. Flavonoids could also be involved in the trypanocidal activity, since plants synthesized them in response to microbial infection (Barbosa et al., 2008).

We believed that the antitumoral activity of *A. chica* might be due to the presence of anthocyanins, flavonoids or both of them. Our HPLC study of the EE extract in comparison to the standard kaempferol indicated the presence of this compound (Figure 3). Barbosa et al. (2008) isolated other two flavonoids from the ethanol extract of *A. chica* besides kaempferol: 4'-hydroxy-3,7-dimethoxy flavone and vicenin-2. During the last decade, natural antioxidants, particularly phenolics, have been under very close scrutiny as potential therapeutic agents against a wide range of ailments including cancer, inflammatory diseases and also aging. The medicinal actions of phenolics is mostly ascribed to their antioxidant capacity, free radical scavenging, chelation of redox active metal ions, modulation of gene expression and interaction with the cell signaling pathways (Soobrattee et al., 2005).

Most chemotherapeutic agents induce collateral effects. Direct myelotoxicity is often observed in patients undergoing anticancer treatments. Hence, the evaluation of toxicity can be accomplished by blood count evaluation (Perez et al., 2005). None of the animals bearing Ehrlich Tumor (control group) and treated with *A. chica* extracts (AE or EE) showed alterations in total erythrocytes count, haemoglobin concentration, packed volume cell and haematimetric indices (Table 2).

The time of tumor development (12 days) was probably not sufficient to cause exhaustion of the bone

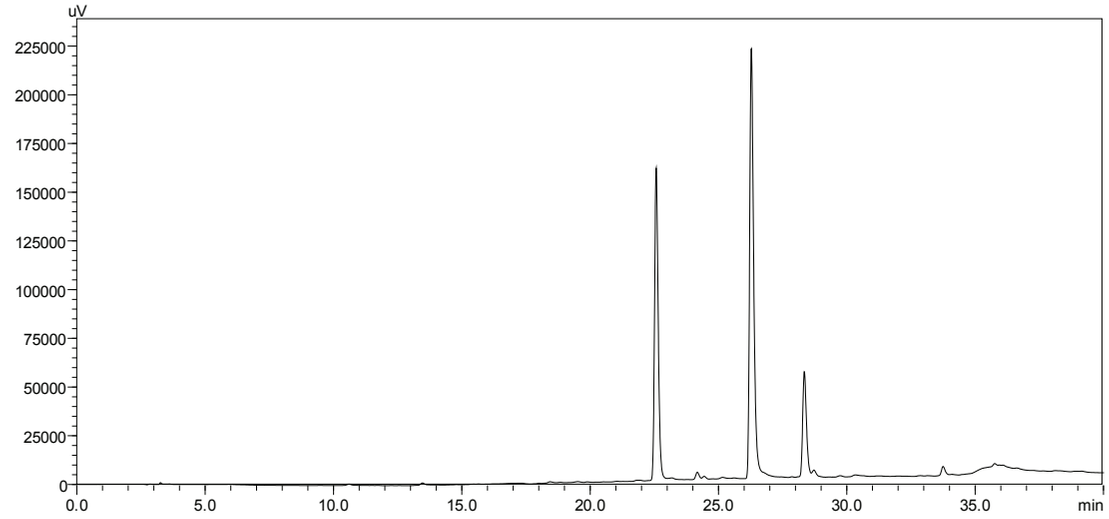

Figure 2. HPLC of standard flavonols (rutin RT= 22.56 min; quercetina RT= 26.28; kaempferol RT=28.34 min).

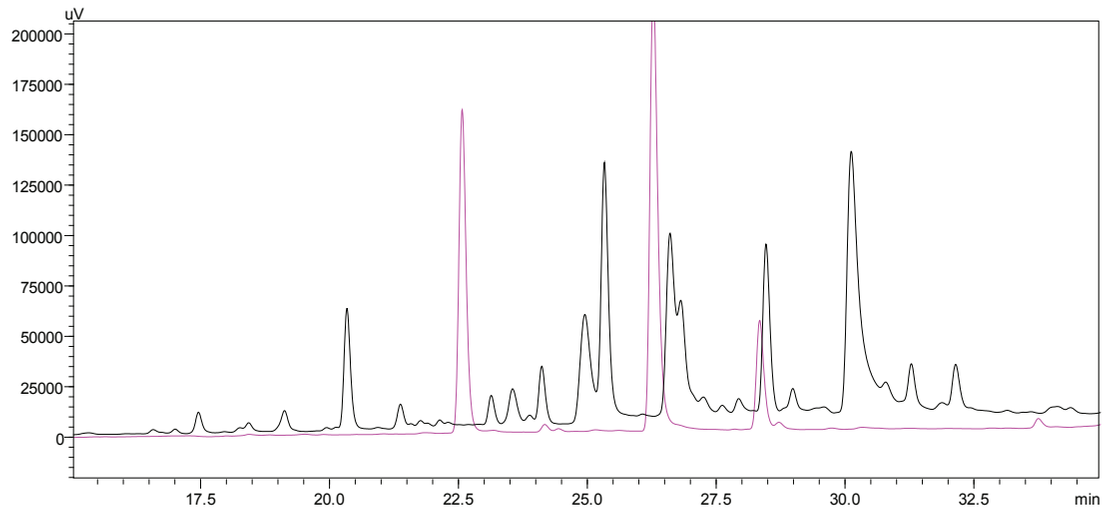

Figure 3. HPLC of standard flavonols (pink line) and EE extract (black line).

Table 2. Mean, standard deviation and statistical analysis results of erythrogram in mice bearing Ehrlich solid tumor treated with different extracts of *Arrabidaea chica.*

Haemogram	Control	AE	EE
RBC/µl	8300,000±600,000 a	8700,000±600,000 a	8600,000±800,000 a
Haemoglobin (g/dL)	12.7±0.9 a	13.3±0.7 a	14.2±0.4 a
Packed volume cell (%)	43.5±3.3 a	45.0±2.2 a	46.5±1.7 a
MCV (fL)	53.3±0.7 a	52.1±11.5 a	54.2±4.4 a
MCH (%)	15.3±0.3 a	15.4±0.3 a	16.6±1.3 a
MCHC (g/dL)	29.3±0.9 a	29.6±0.7 a	30.6±0.5 a

Data was presented as mean±standard deviation. Means with the same letters in the same line are not significantly different ($p \leq 0,05$).

marrow. Verçosa Junior et al. (2004) reported discreet anemia (RBC=7.23±1.74/µL; haemoglobin=10.7±2.55g/dl; PVC=30,86±7.78%) and leucopenia (2.96±1.22/µL) in mice bearing solid Ehrlich tumor. Also, it might be inferred that the extracts tested did not produce toxic effects in animals. Another report showed that the hidroalcoholic extract of *A. chica* with concentrations as high as 5g/kg had no acute toxic effects in mice (Catargenes, 2009).

There were no differences in the total leukocytes

between groups. However, the ethanol extract (EE) caused a significant increase in neutrophils count (Table 3).

Neutrophils play an important role in tumor angiogenesis releasing chemokines that cause endothelial cell invasion and vessel formation. Other studies showed that potent activation of infiltrating macrophages and granulocytes could lead to tumor destruction and tumor rejection (Albini et al., 2005).

In our study we observed that the reduction in the size of the tumor in the EE group was accompanied by an increase in the number of neutrophils in blood. Also, this group presented a larger number of neutrophils in the histology of the tumor. Bergami-Santos et al. (2004) showed that neutrophils from Swiss mice are cytotoxic against Ehrlich tumor cells but are not efficient in controlling tumor growth. The treatment with AE, which also reduced tumor growth, did not alter the number of neutrophils in blood or differ from control group in concern to the morphology of the tumor.

Tumor growth initiates a myriad of functional and phenotypic changes in macrophages and T-cells in association with alterations in cytokine synthesis and responsiveness (Walker et al. 1994). Several types of tumor also express receptors for granulocyte-macrophage colony stimulating factors or produces and uses granulocyte–macrophage colony stimulating factors as an autocrine growth factor (Fu et al., 1991).

Phagocytes, particularly macrophages and neutrophils, play a vital role in both the innate and the acquired immunity, exerting a key role in host defense against various infectious agents and tumor growth (Natan et al, 1983). One of the most important characteristics of these cells is their capacity to migrate into inflammatory sites (Spector & Ryan, 1979). Animal model studies have shown that a variety of tumor cells can produce factors that impair inflammatory responses, thus allowing tumor growth in vivo. Alternatively, the tumor cells can stimulate macrophage suppressor activities in host cells (Elgert et al., 1998). In addition to the deficient accumulation of phagocytes at the inflammatory sites, several other cellular parameters have been shown to be altered in the tumor-bearing state, in particular the formation of granulocyte–macrophage colonies in response to CSF (Baum & Fisher, 1972).

The treatment with ethanol extract (EE) caused an increase in α1 and β-globulins values compared to control group (Table 4). Increases in α-globulins can be accompanied by increased β-globulins. For example, α1-antitrypsin has antiprotease activity designed to inhibit proteases released by phagocytes and other cells of the immune system to minimize damage to normal tissues.

Acute phase proteins like β-lipoprotein and complement C3 were located in the β1 region, and transferrin and IgM were located in the β2 region. The increase of β-globulins values in the group treated with A. chica ethanol extract is likely to have happened due to the increase of IgM. The fact that flavonols were identified in *A. chica* ethanol extract raises the question of whether these substances could be responsible for an immunomodulatory effect.

Production of acute phase proteins (APP) is controlled by cytokines, with the pro-inflammatory cytokines interleukin-1, interleukin-6, and tumor necrosis factor-α released from the site of pathogenic or inflammatory damage stimulating the production of the APP. The mechanism of production involves cytokine receptor, signaling pathways, and induction of mRNA for the APP, which is released 6 to 12 h after stimulation. The liver is the main site of synthesis of the APP, but there have been recent reports of non-hepatic tissues such as lung, adipocyte, and intestine increasing expression of mRNA for APP following stimulation (Kaneko, 2008).

The treatment with ethanol extract (EE) caused a decrease of α2-globulins values when compared to control group. Haptoglobin and α2-macroglobulin were identified in the α2 fraction. Haptoglobin has scavenging activities and binds metabolites released from cellular degradation so they can reenter host metabolic processes rather than be utilized by pathogen. Therefore, another question is raised: whether the ethanol extract have caused a decrease in the tumoral area.

Tumor morphology was similar in all groups. The tumor cells formed solid pattern or cords invading the muscle and bone tissue. The tumor cells showed moderate

Table 3. Mean, standard deviation and statistical analysis results of leukogram in mice bearing Ehrlich solid tumor treated with different extracts of *Arrabidaea chica.*

Leukocytes	Control	AE	EE
WBC/μL	5.100±900 a	6.100±1.900 a	6.100±400 a
Lymphocytes/μL	3.570±439 a	4.538±606 a	3.904±299 a
Monocytes/μL	469±91 a	390±218 a	244±264 a
Neutrophils/μL	847±283 a	1.074±392 a	1.830±299 b
Bastonetes/μL	20±28 a	49±51 a	31±61 a
Eosinophils/μL	184±182 a	49±109 a	92±61 a
Basophils/μL	10±23 a	0±0 a	0±0 a

Data was presented as mean±standard deviation. Values with different letters in the same line are significantly different ($p \leq 0,05$).

Table 4. Mean, standard deviation and statistical analysis results of electrophoretic protein in mice bearing Ehrlich solid tumor treated with different extracts of *Arrabidaea chica.*

	Control	AE	EE
Total protein	6.2±0.7 a	5.5±0.3 a	5.7±0.3 a
albumin (%)	48.8±6.6 a	50.0±7.3 a	50.7±7.3 a
albumin (g/dL)	3.0±0.6 a	2.8±0.4 a	2.9±0.4 a
α1-globulin (%)	8.3±1.4 a	10.6±3.5 ab	14.1±2.6 b
α1-globulin (g/dL)	0.5±0.1 a	0.6±0.2 ab	0.8±0.2 b
α2-globulin (%)	28.2±7.7 a	20.1±3.4 ab	12.4±3.1 b
α2-globulin (g/dL)	1.7±0.5 a	1.1±0.3 ab	0.7±0.2 b
β-globulin (%)	10.9±1.5 a	16.1±6.5 ab	19.8±2.2 b
β -globulin (g/dL)	0.7±0.1 a	0.9±0.3 ab	1.1±0.2 b
δ-globulina(%)	3.8±2.1 a	3.3±1.7 a	3.0±1.4 a
δ-globulin (g/dL)	0.2±0.1 a	0.2±0.1 a	0.2±0.1 a
albumin/globulin ratio	1.0±0.2 a	1.0±0.2 a	1.1±0.3 a

Data as presented as mean±standard deviation. Values with different letters in the same line are significantly different ($p \leq 0,05$).

pleomorphism. There were groups of large and oval cells with abundant vacuolated faintly basophilic cytoplasm with indistinct borders. Oval basophilic nucleus was observed with multiple evident nucleoli. Other times the tumor cells were smaller, spindle shaped with basophilic cytoplasm. There were a moderate number of mitotic figures, some of them being atypical.

The inflammatory infiltrate was predominantly lympho-histiocytic moderate to mild with few neutrophils, except for the EE group, which presented lympho-histiocytic infiltrate with large amount of neutrophils. All groups presented extensive areas of necrosis scattered trough tumor tissue with discrete areas of hemorrhage.

Metastases were not seen in any of the organs studied (heart, lung, liver, spleen, kidney), or in the popliteal lymph nodes. The lymph nodes were enlarged with reactive hyperplasia of lymphoid follicles and active germinal centers.

The occurrence of metastases is not common in Ehrlich tumor. Exception is made to metastases to regional lymph nodes especially after the seventh day of tumor inoculation (Dagli, 1992a). In the present experiment there was no presence of metastasis in popliteal lymph nodes in all groups (including control group). Once started, neoplastic progression is usually accompanied by increased genetic instability. This may generate the phenotypic diversity manifested by different tumor cell clones (Dagli, 1992b).

Flow cytometric immunophenotyping of blood lymphocytes

There are no reports on literature that demonstrate the cell types involved in the inhibition of Ehrlich solid tumor growth. In this study we evaluated mononuclear cells in the blood of five animals per group. The frequency of NK cells, TCD3 and B-Lymphocyte as well as CD4 and CD8 T cell subpopulations is shown in Figure 4.

The percentage of CD4 T lymphocytes in blood was reduced by the treatments with EE and AE of *A. chica*. There were no changes in the percentages of CD8 T cells subpopulation, B-lymphocytes or NK cells (Figure 4). Further studies are necessary to clarify the mechanisms involved in the immunomodulatory regulation presented by the extracts over CD4 T cells subpopulation.

Flow cytometric immunophenotyping of tumor infiltrating lymphocytes

Determination of the cell types involved in the inhibition of a tumor is important because it allows one to infer whether the inhibition of the tumor growth is associated with a certain cell population and/or with the direct effect of the treatment on these cells (Santos et al., 2004). In this study we evaluated eight tumors: five from the control group, two from the AE group and two from the EE group. Three animals from the EE and three animals from the AE group were excluded from this study after the verification of insufficient number of inflammatory cells in the gate of lymphocyte population. Interestingly, all animals excluded were treated with *A. chica* extracts.

The frequency of CD3 T cells and B-Lymphocyte as well as CD4 and CD8 T cell subpopulations is are shown in Figure 5. The ethanol extract did not cause alteration on the percentage of tumor infiltrating lymphocytes subpopulations. Significant differences were observed between AE and control group. Data analysis demonstrated that the AE group had significantly lower percentage of CD3 lymphocytes ($p<0.05$) associated with the reduction of the CD8 T cell subpopulation without changes in CD4 T lymphocyte subpopulation. Also, the treatment with aqueous extract caused a reduction in NK

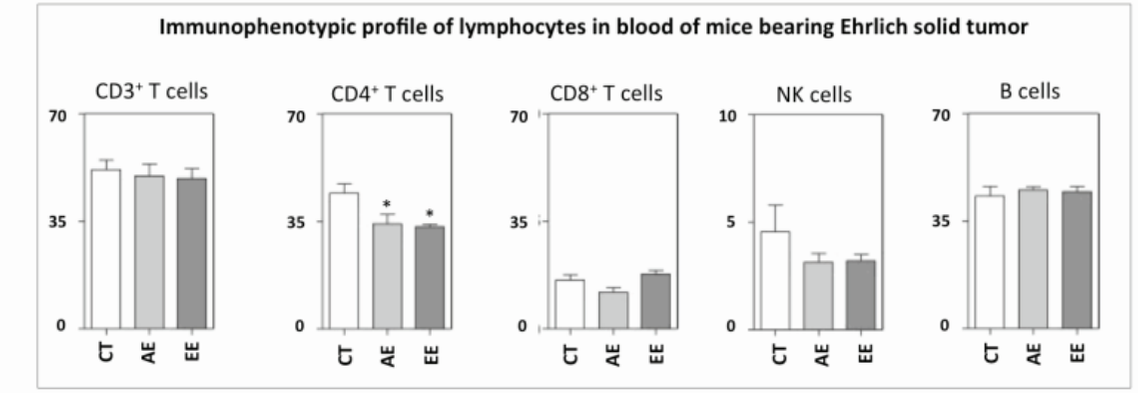

Figure 4. Immunophenotypic profile of lymphocyte in blood of mice bearing Ehrlich solid carcinoma. Lymphocyte populations were identified by flow cytometric immunostainning as described in Material and Methods. Data were expressed as percentage of positive cells within gated lymphocytes. Significant differences at $p<0.05$ are highlighted by asterisk.

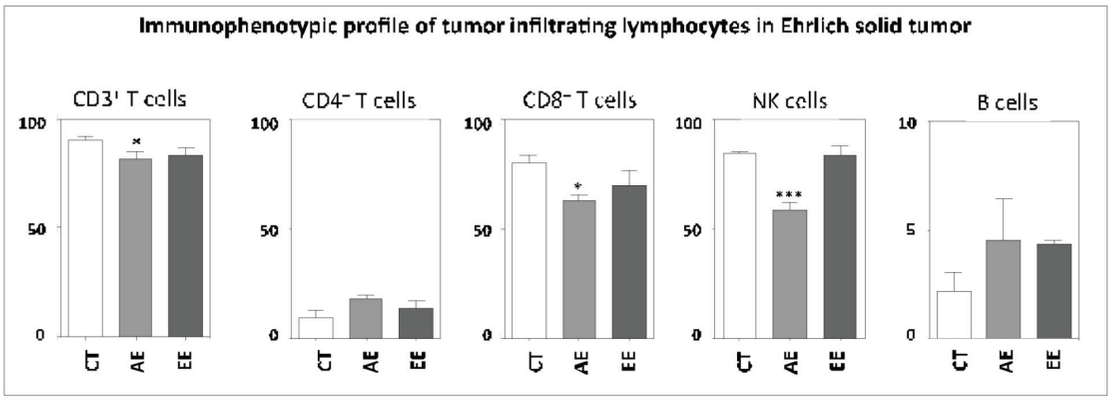

Figure 5. Immunophenotypic profile of tumor infiltrating lymphocytes in Ehrlich solid carcinoma. Lymphocyte populations were identified by flow cytometric immunostainning as described in Material and Methods. Data were expressed as percentage of positive cells within gated lymphocytes. Significant differences at $p<0.05$ are highlighted by asterisk.

cells population. These findings suggest that *A. chica* aqueous and ethanol extracts have immunomodulatory and antitumor activities attributed to the presence of flavonoids, such as kaempferol identified by High Performance Liquid Chromatography.

The role of inflammatory and immune responses in tumor development is unclear. Leukocyte infiltration of tumors has been described as playing either inhibitory or stimulatory roles in tumor growth (Bergami-Santos et al., 2004). Data suggest that AE antitumor action is related to its anti-inflammatory potential and the modulation of the immune system. The aqueous extract of *A. chica* caused a reduction in tumor growth associated with a reduction in CD8 T cells and NK cells. According to the literature, NK cells seems to be devoid of an important role in the resistance against Ehrlich ascites tumor (Gil et al, 1990). Apparently the same occurs here, suggesting that other nonspecific inflammatory mechanism might be relevant in tumor resistance. The Ehrlich tumor cells lack the expression of major histocompatibility antigens. This characteristic probably excludes a major role for cytotoxic T lymphocytes during tumor development, indicating that cellular immunity is not the main mechanism of host reaction to this tumor (Bergami-Santos et al., 2004).

The role of CD4 T cells in tumor immunity is not totally clear. Interestingly, the percentage of CD4 T cells infiltrated in tumor tissue in AE and EE group remained similar to the control group (17.85±2.72; 13.51±5.20; 9.54±5.82 respectively) despite the lower number present in blood. These results could suggest that CD4 T cells probably took part in the antitumor immunity against Ehrlich cells mediated by the production of cytokines like TNF-α (tumor necrosis factor-alpha) and INF-γ (interferon-gamma).

Most likely the two extracts tested here exhibit different mechanisms of action. The ethanol extract inhibited Ehrlich solid tumor development without changing mononuclear cell population infiltrating in the

tumor tissue. On the other hand, the morphologic study showed a large number of neutrophils infiltrating the tissue. It was already demonstrated that EE has potent cytotoxic effect against leukemic human cell lines (Ribeiro et al., 2010). This extract induces DNA fragmentation in Jurkat and HL60 cell lines as assessed by flow citometry assays (data not shown). Possibly the antitumor effect demonstrated here is also related to the induction of apoptosis of tumor cells. We do not discard the possibility that neutrophils exert cytotoxic effects against EAC cells contributing to the inhibition of tumor growth.

The mechanism of action of extracts on tumor cells should be elucidated in a detailed manner in the future. The association between cytokines and tumor growth inhibition has been described (Silva et al., 2002). It is possible that the antitumor effect of *A. chica* extracts is associated with the action of cytokines possessing antitumor properties. Whether cytokines may be involved in this process is currently under study in this laboratory.

Conclusions

Data showed that *Arrabidaea chica* (Humb. & Bonpl.) B. Verl., Bignoneaceae, aqueous extract (AE) and ethanol extract (EE) reduced the solid tumor growth in mice. No adverse side effects due to treatment were observed. Our findings may contribute to a better understanding of the beneficial effects of this folk remedie.

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