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Evaluation of the in vitro cytotoxic effects of carvoeiro leaf extracts [*Callisthene fasciculata* (Spr.) Mart.]

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Abstract. *Callisthene fasciculata* (Spr.) Mart. is a tropical tree belonging to the family Vochysiaceae and is popularly known as carvoeiro. In popular medicine, it is used for the treatment of hepatitis, stomach diseases, kidney problems, jaundice, and anemia; however, its effects have not been scientifically proven. Therefore, the objective of the present study was to conduct a preliminary study on the biological effects of carvoeiro by evaluating its in vitro cytotoxic effect in co-culture with different cell types. To prepare the extract, 70% ethanol (ratio, 1:5) was added to the dried and crushed leaves, which were incubated for 7 days in the dark. Next, the extract was filtered and evaporated in a rotavapor to reduce the volume and obtain a powder. The powder was resuspended in RPMI culture medium supplemented with 20% fetal bovine serum and added to Walker 256 tumor cells or splenic cells (rat or mice) at different concentrations (serial dilutions of 1000 µg/ml to 7.81 µg/ml). After 24 h, cytotoxicity analysis was performed by the colorimetric MTT reduction assay. The results demonstrated that the extract favored the viability of tumor and rat spleen cells at higher concentrations (Walker tumor: 1000 µg/ml, 0.685 ± 0.066, 500 µg/ml, 0.565 ± 0.067; 250 µg/ml, 0.329 ± 0.019; control, 0.168 ± 0.01; rat spleen: 1000 µg/ml, 0.334 ± 0.045; control: 0.219 ± 0.053) and showed a cytotoxic effect at lower concentrations, with the percentage of cell inhibition ranging from 7% to 19%. According to the results, we concluded that the leaf extract of *Callisthene fasciculata* (Spr.) Mart had a direct effect on the studied cells in vitro. This was the first study to report the biological effect of this plant; however, further study is needed to determine its effects on complex systems, such as those found in vivo.
Keywords: *Callisthene fasciculata* (Spr.) Mart., carvoeiro, cytotoxicity, Walker 256 tumor, spleen cell

Introduction

Callisthene fasciculata (Spr.) Mart. is a tropical tree belonging to the family Vochysiaceae (Mayworm et al., 2000). It is an endemic plant of the Cerrado biome and can also be found in semideciduous forests (Neto, 1991). The family Vochysiaceae comprises approximately 200 species of shrubs and trees (Mayworm et al., 2011), and the Callisthene genus has 10 forest species (Mayworm et al., 2002).

The tree *Callisthene fasciculata* (Spr.) Mart. is popularly known as carvoeiro (Neto, 1991) and white coal (Santos, 2009) and is of great regional economic importance as a source of wood and energy (coal) (Neto, 1991). Medicinally, it is used for the treatment of hepatitis, stomach problems, kidney problems, jaundice, and anemia (Carneiro, 2009).

The use of medicinal plants, in simple or complex formulations, is an integral part of popular cultures across the world and is accompanied by the development of civilizations (Akran et al., 2014). In the literature, we can find several studies on the biological effects of compounds extracted from plants, fungi, marine animals, amphibians, and microorganisms (Kaneno et al., 2004, Martins et al., 2009, Ferreira et al., 2013, Sultana et al., 2014, Albiero et al., 2016).

In fact, medicinal plants are the basis of several pharmaceutical products that are currently used, such as reserpine, deserpidine, vinblastine, and paclitaxel (Sultrana et al., 2014). The National Cancer Institute in the United States has evaluated approximately 114,000 natural extracts with anticancer activity (Sultrana et al., 2014). In Brazil, unconventional therapeutic activities are regulated by law, which legitimizes the use of unconventional therapies, such as acupuncture, phytotherapy, and homeopathy (BRASIL, 2013).

Apparently, no information is available in the scientific literature on the therapeutic use of *C. fasciculata* (Spr) Mart. Studies with ethnobotanical or therapeutic descriptions of the family Vochysiaceae are scarce and mainly include plants of the *Qualea* genus or *Callisthene minor* Mart species (Mayworn et al., 2000, Mazzolin et al., 2013, Bonacorsi et al., 2013, Carneiro et al, 2017).

Mazzolin et al. (2013) observed that methanolic extracts of *Qualea parviflora* Mart had antidiarrheal and antispasmodic actions and were able to reduce the influx of neutrophils and levels of

interleukin (IL)-1, tumor necrosis factor (TNF)-α, and myeloperoxidase (MPO) in animals subjected to the trinitrobenzenesulfonic acid (TNBS)-induced intestinal inflammation model. Bonacorsi et al. (2013) assessed the anti-inflammatory effect of *Q. parviflora* and *Q. multiflora* and demonstrated the antioxidant potential of these extracts against the oxidative burst of *Helicobacter pylori*-stimulated neutrophils. Regarding *C. minor*, the literature presents only the nutritional constituents of its seeds but not the evaluation of the biological effects of its compounds (Mayworm et al., 2000, Mayworm et al., 2002, Mayworm et al., 2011).

Thus, the objective of the present study was to conduct a preliminary study on the effects of carvoeiro leaf extracts on the viability of tumor and splenic cells in vitro.

Methods

Animals

Swiss male mice (n = 3) and Wistar rats (n = 3) were obtained from the Central Animal Facility of the Federal University of Mato Grosso - UFMT, Campus of Cuiabá - MT. They were housed in polypropylene boxes, with food (Purina, St. Louis, Missouri, USA) and water access *ad libitum* and xylan substrate (Suprimart Mercantil, Itaquaquecetuba, SP, Brazil) in a light/dark cycle of 12/12 h and controlled ambient temperature of 22°C ± 1°C.

Preparation of *C. fasciculata* (Spr.) Mart. (carvoeiro) Extracts

The leaves of *C. fasciculata* were collected from the Pantanal of Poconé - MT in the Pantanal region of Mato Grosso, between Bento Gomes river (16°18'55.01" S and 56°32'33.64" W) and Advanced Research Base of the Pantanal (6°30'3.41" S and 56°24'47.76" W), of the Federal University of Mato Grosso, located on the SESC Pantanal property near the Cuiabá River.

The collected leaves were conditioned in plastic bags to be transported to the Quality Control Laboratory of the Federal University of Mato Grosso, Campus of Sinop, where the sorting was carried out, discarding the damaged leaves or those infected with insects (gall-inducing insects) or fungi.

To prepare the extract, the leaves were dried in a forced air flow oven for 1 week at 40°C. They were subsequently ground in an analytical mill and subjected to extraction by maceration with ethanol: water (70:30) for 7 days. Then, the solvent was evaporated in a rotary evaporator to obtain the dried extracts. The powder was stored at −20°C until further use.

Walker 256 Tumor Cell Suspension

Cryopreserved aliquots of Walker tumor cells were kindly provided by Professor Dr. Eveline Aparecida Isquierdo Fonseca de Queiroz of the Institute of Health Sciences (ICS), Campus of Sinop (CUS) of the Federal University of Mato Grosso (UFMT). The aliquots were thawed in a water bath at 37°C, mixed with phosphate buffer saline (PBS), and intraperitoneally injected into male Wistar rats (n = 2) for the development of the tumor ascitic form. For tumor cell suspension, animal with the ascitic tumor form was euthanized under sedation with ketamine (4 ml, 10% solution, Syntec, Santana de Parnaíba, Sao Paulo, Brazil) and xilasin (2 ml of 2% solution, Syntec), and peritoneal lavage was performed with 20 ml of sterile PBS and 3% EDTA (Sigma, Saint Louis, USA). Subsequently, the cell suspension was centrifuged at 1,500 rpm for 10 min and resuspended in 1 ml of RPMI culture medium (Cultilab, Campinas, Brazil) supplemented with 20% fetal bovine serum (FBS; Cultilab, Campinas, Brazil). The final concentration was adjusted to 1×10^4 cells/ml (Colquhoun & Schumacher, 2001) using the trypan blue exclusion test, considering a minimum viability of 70%.

Suspension of total splenic cells

For the preparation of total splenic cell suspensions (Castoldi et al., 2006), the spleens of normal rats or mice were removed after the animals were sacrificed by cervical dislocation. Initially, the spleen was macerated using a nylon sieve and a rubber stopper in a Petri dish containing RPMI 1640 culture medium. To obtain the splenic cells, the cell suspensions were centrifuged at 1,500 rpm for 10 min and resuspended in 1 ml of RPMI 1640 culture medium supplemented with 20% FBS. The concentration of splenic mice cells was adjusted to 4×10^6 cells/ml (Albiero et al., 2016) and that of splenic rat cells to 2×10^6 cells/ml (Spinardi-Barbisan et al., 2004) using the trypan blue exclusion test, considering a minimum viability of 70%.

Evaluation of cellular cytotoxicity in vitro

For the analysis of the cytotoxic activity, the colorimetric MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium; MTT based cell growth determination kit, Sigma Aldrich, Saint Louis, USA) was used according to the manufacturer's instructions. Tumor and splenic cell suspensions were seeded onto sterile 96-well flat bottom plates (volume, 100 µl) in triplicate. Subsequently, the serially diluted crude extracts of *C. fasciculata* (Spr.) Mart. (1,000–7.81 µg/ml) were added. Cell culture in 100 µL of 20% RPMI 1640 FBS was used as the cell viability control (control group). The microplates were incubated in an incubator stove at 37°C with humidified atmosphere and 5% CO_2 for 24 h. Three independent experiments were performed for each cell type. Absorbance [optical density (OD)] was measured using the Thermo Plate Spectrophotometer, microplate reader (TP Reader) at a wavelength of 630 nm.

Statistical Analysis

OD values are presented as the mean ± standard deviation and compared using the analysis of variance (ANOVA), followed by the Tukey's test

(GraphPad InStat 3.06), considering a significance level of 5% ($p \leq 0.05$).

The percentage of cell inhibition (% CI) was calculated using the following equation: % CI = {[(absorbance of the control) − (absorbance of the sample)] / (absorbance of the control)} × 100 (GraphPad InStat 3.06).

Ethical Principles

This research was submitted to the Institutional Committee for Ethics for the Use of Animals (Federal University of Mato Grosso - UFMT) and was approved according to ethical principles and current legislation (Protocol No. 23108.704753/13-8).

Results and Discussion

Figure 1 shows the mean OD values of co-cultures of tumor or splenic cells with different concentrations of the carvoeiro extract. The OD values reflect the mitochondrial activity of living cells because their metabolites promote the reduction of MTT; in other words, they reflect the viability of the cells studied. We observed that higher concentrations of the carvoeiro extract promoted cell viability in the 24-h co-culture with tumor and splenic rat cells (Walker tumor: 1000 µg/ml, 0.685 ± 0.066; 500 µg/ml, 0.565 ± 0.067; 250 µg/ml, 0.329 ± 0.019; control, 0.168 ± 0.01; rat spleen: 1000 µg/ml, 0.344 ± 0.045; control, 0.219 ± 0.053).

In addition, we calculated the cell inhibition rate (%CI). Table 1 shows %CI of the Walker 256 tumor cell and splenic cell suspensions of normal rats or mice in 24-h co-culture with different concentrations of the leaf extract of *C. fasciculata* (Spr.) Mart.

According to the results, the extract had an inhibitory effect on the growth of all cells at concentration of 15.62 µg/ml (mice spleen: 10%, rat spleen: 18%, Walker tumor: 15%) and 7.81 µg/ml (mice spleen: 13%, rat spleen: 19%, Walker tumor: 18%). At higher concentrations (250–1000 µg/ml), there was no inhibitory effect of the extract on cell growth. Negative values indicate the opposite effect, i.e., cell growth.

In the literature, there are no reports about biological response modifiers of *C. fasciculata* species, and the present study is the first to present data on the cytotoxic action of the compounds present in the leaf extract.

In fact, the scientific community recommends that products of unknown action be initially evaluated for their biocompatibility through in vitro cell culture studies (Martins et al., 2009). In vitro cytotoxicity tests consist of the culture of mammalian cells in direct or indirect contact with a compound or material and observation of cellular alterations (Rogero et al., 2003).

One of the most commonly used parameters to evaluate cellular changes is cell viability, which can be shown with the help of vital dyes, such as neutral red or trypan blue (Rogero et al., 2003, Oliveira et al., 2009). Another alternative for the measurement of cell viability is the reduction of MTT to formazan crystals by mitochondrial NADPH of viable cells (Martins, 2009, Oliveira et al., 2009), which was the method used in the present study.

Table 1 – Percentage of cell inhibition (%CI) of splenic cells (rat and mice) and Walker 256 tumor cells cultured with different concentrations of the leaf extract of *Callisthene fasciculata* (Spr.) Mart. for 24 h at 37°C and 5% CO2.

Concentration (µg/mL)	*Leaf extract of Callisthene fasciculata (Spr.) Mart*		
	[a] Mice spleen	[b] Rat spleen	[c] Walker Tumor
1000	- 148	- 66	- 308
500	- 79	- 23	- 237
250	- 32	- 2	- 96
125	- 1	18	- 31
62,5	- 5	1	- 29
31,25	7	13	- 5
15,62	10	18	15
7,81	13	19	18

[a] r^2 = 0,9911, p<0,0001; [b] r^2 = 0,9569, P < 0,0001; [c] r^2 = 0,9370, P < 0,0001.

Thus, the main objective of in vitro cytotoxicity assays is to allow the study of cell behavior in a controlled environment, free of the complexity of the living organism, and in an easy, fast, and inexpensive way (Martins et al., 2009). After the in vitro studies, the material or compound may subsequently enter animal studies and human clinical trials (Rogero et al., 2003, Martins et al., 2009).

Studies already performed with plants of the genus *Callisthene* or those of the family Vochysiaceae have suggested the presence of cytotoxic compounds in the species *C. fasciculata*. Analyses carried out by Mayworm (Mayworm et al., 2000, Mayworm & Salatino, 2002) showed that the composition of fatty acids and polysaccharides in the seeds of plants of the family Vochysiaceae are potential taxonomic markers that allow the grouping of the species according to the composition of these compounds. In this case, the genera *Callisthene* and *Qualea* are grouped as plants with a predominance of arabinose, mannose, and oleic and palmitic acids in their seeds (Mayworm et al., 2000, Mayworm & Salatino, 2002).

Thus, a review of the studies on plants related to *C. fasciculata* indicated possible explanations for the effects observed in the present study.

Regarding the genus *Callisthene*, a study with *C. minor* showed the presence of 17% protein, 6.4% sugar (mainly arabinose and mannose), and 20% fatty acids (72% oleic acid and 13% palmitic acid) (Mayworm & Salatino, 2002, Mayworm et al., 2011). However, there were no reports on the biological effects of this plant.

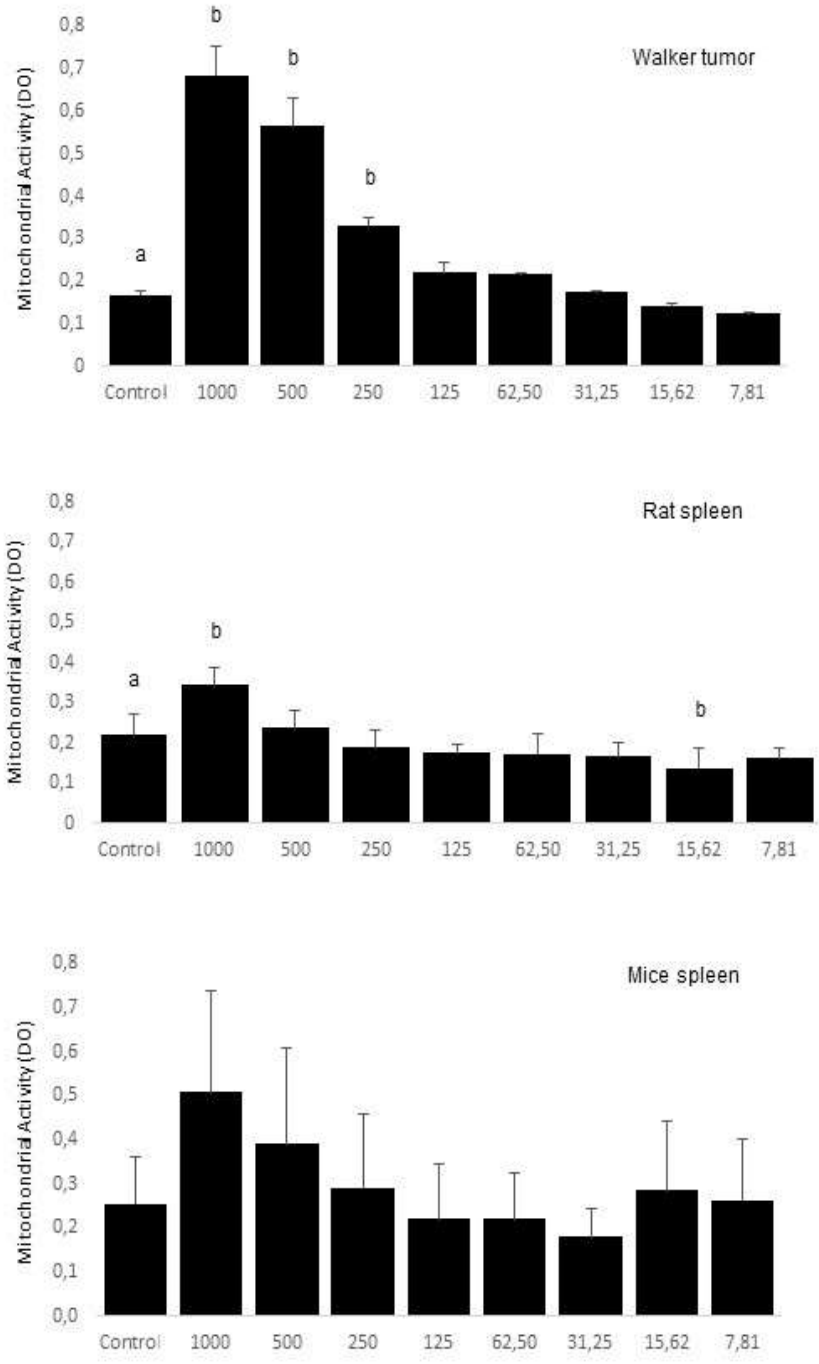

Figure 1. Cell viability of Walker 256 tumor cells and splenic rat and mice cells in 24-h co-culture with different concentrations of the extract obtained from the leaf of *Callisthene fasciculata* Spr. Mart. Cell viability is represented as the mean and standard deviation of the optical density of formazan crystals formed from the reduction of MTT by mitochondrial metabolites of living cells in vitro. The lowercase letters indicate the statistical difference between the control groups and those treated with different concentrations of the extract, with $a < b$, $p = 0.01$ for the evaluation of the effect on rat spleen cells at 15.62 µg/ml and $a < b$, $p = 0.001$ for other evaluations.

Regarding the genus *Qualea*, there are studies in the literature on the biological action of its compounds. Methanolic extracts of *Q. parviflora* and *Q. multiflora* resulted in the inhibition of *H. pylori* oxidative burst at the rate of 48% and 47.3% at 5 µg/ml and of 95% at 100 µg/ml, respectively (Bonacorsi et al., 2013).

The antioxidant and anti-inflammatory effects of *Q. parviflora* were also evaluated by Mazzolin et al. (2013) in the TNBS-induced colitis model. The results demonstrated that the methanolic extract (25 and 50 mg/kg/day) reduced the inflammatory damage in the colon of treated animals, and this effect was accompanied by the maintenance of the antioxidant mechanisms and glutathione levels and reduction of oxidative damage, with decreased levels of lipid peroxidation (Mazzolin et al, 13). In addition, the anti-inflammatory effect was a consequence of the reduction of the tissue infiltrate of neutrophils and production of inflammatory cytokines TNF-α and IL-13 (Mazzolin et al, 13).

Cordeiro et al. (2017) demonstrated the antiprotozoal effect of ethanolic extracts of *Q. gondiflora* against *Plasmodium falciparum* and *Trypanosoma brucei gambiensis* (IC50 < 100 µg/ml), without toxic effects on the myoblast cell line L6 (IC50, 28 µg/ml).

In the present study, we observed that the carvoeiro leaf extract showed concentration-dependent effects; it favored cell growth at higher concentrations and had an inhibitory effect at lower concentrations. This characteristic reflects the complexity of the unpurified extract, with a complex mixture of several compounds and therefore with several biological effects (Martel et al., 2017). We highlighted the phytotherapeutic potential of carvoeiro plant, because studies on related plants showed their anti-inflammatory, antioxidant, and antiprotozoal effects. Further studies are required to observe the effects of this extract in complex systems, such as those found in vivo.

Conclusion

The leaf extract of carvoeiro (*C. fasciculata* Spr. Mart) had an in vitro cytotoxic effect at low concentrations on the tumor and spleen cells in 24-h co-cultures. However, an exact opposite effect was observed at high concentrations, wherein the cell viability of tumor and splenic rat cells was maintained.

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