

ORIGINAL ARTICLE

Evaluation of the antioxidant potential of *Copaifera multijuga* in Ehrlich tumor-bearing mice

Ana Paula Simões da CUNHA¹, Luana BALDISSERA¹, Débora Linsbinski PEREIRA², Lucineia Reuse ALBIERO¹, Lindsey CASTOLDI¹, Adilson Paulo SINHORIN², Valéria Dornelles Gindri SINHORIN^{2*}

¹ Universidade Federal de Mato Grosso, Instituto de Ciências da Saúde, Campus de Sinop, Sinop, Mato Grosso, Brazil

² Universidade Federal de Mato Grosso, Instituto de Ciências Naturais, Humanas e Sociais, Programa de Pós Graduação em Ciências Ambientais (PPGCAM), Laboratórios Integrados de Pesquisas em Ciências Químicas (LIPEQ), Campus de Sinop, Sinop, Mato Grosso, Brazil

* Corresponding author: valeriadgindri@gmail.com

ABSTRACT

Copaifera multijuga, commonly known as copaiba, is popularly used in the form of tea for various conditions due to the presence of antioxidant substances in its composition, which protect cells against damage caused by free radicals. Its oleoresin is also used as an anti-inflammatory and antitumoral agent. The present study investigated the antioxidant effect of the ethanolic extract of copaiba stem bark on *Swiss* mice inoculated with solid Ehrlich tumors. Mice were inoculated subcutaneously with 1×10^6 Ehrlich's tumor cells and treated via gavage with ethanolic extract of copaiba for thirty days, with doses varying between 100 and 200 mg kg⁻¹. Biochemical analyses of enzymatic antioxidants [superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST)], non-enzymatic antioxidants [reduced glutathione (GSH) and ascorbic acid (ASA)], substances reactive to thiobarbituric acid (TBARS) and protein carbonylation (carbonyl) in different tissues were significantly affected. The extract administered at 200 mg kg⁻¹ presented higher antioxidant capacity in the liver, increased CAT, GST, GSH and decreased TBARS, as well as increased CAT activity and protein carbonylation in brain tissue. The results showed that the copaiba extract was able to reverse the oxidative stress caused by solid Ehrlich tumor, probably due to the presence of antioxidant compounds, and had potential antineoplastic effect after a 30-day treatment.

KEYWORDS: solid tumor, oxidative stress, free radicals, copaiba, antineoplastic

Avaliação do potencial antioxidante de *Copaifera multijuga* em camundongos com tumor de Ehrlich

RESUMO

Copaifera multijuga, ou copaíba, é utilizada popularmente como chá para o tratamento de diversas afecções, o que se deve à presença de substâncias antioxidantes em sua composição, que protegem as células contra danos causados pelos radicais livres. O óleo-resina da árvore é usado como antiinflamatório e antitumoral. O presente estudo avaliou o efeito antioxidante do extrato etanólico da casca da copaíba sobre camundongos *Swiss* machos inoculados com tumor sólido de Ehrlich. Os camundongos foram inoculados subcutaneamente com 1×10^6 células de tumor de Ehrlich e foram tratados, via gavagem durante 30 dias, com doses de extrato etanólico de copaíba variando de 100 a 200 mg kg⁻¹. Realizou-se análise bioquímica dos antioxidantes enzimáticos [superóxido dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST)], antioxidantes não-enzimáticos [glutathione reduzida (GSH) e ácido ascórbico (ASA)], substâncias reativas ao ácido tiobarbitúrico (TBARS) e carbonilação proteica (carbonil) em diferentes tecidos e apresentando resultados significativos. O extrato administrado na concentração de 200 mg kg⁻¹ apresentou melhor capacidade antioxidante no fígado, aumentando a CAT, GST, GSH e diminuindo TBARS, além de aumentar a atividade da CAT e da carbonilação proteica no tecido cerebral. Os resultados mostram que o extrato de copaíba foi capaz de reverter o estresse oxidativo causado pelo tumor sólido de Ehrlich, provavelmente por conter compostos antioxidantes, e possivelmente teve um efeito antineoplásico após 30 dias de tratamento.

PALAVRAS-CHAVE: tumor sólido, estresse oxidativo, radicais livres, copaíba, antineoplásico

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INTRODUCTION

The increasing incidence of cancer in the world calls for the continuous search for new treatments that are both efficient and cause minimal side effects to normal cells. This includes the research on active components in plants, and the development of phytopharmaceuticals with anticancer activity (Loaces and Cabrera 2003).

The trees of the genus *Copaifera* are popularly known in Brazil as copaiba, with 26 species distributed throughout north and northeastern Brazil (Costa 2018). Copaiba oleoresin, which is extracted from the trunk of the tree and is rich in terpene compounds, including sesquiterpenes and diterpenes, is very popular as a medicinal plant compound in northern Brazil (Veiga and Pinto 2002; Santiago *et al.* 2015; Silva *et al.* 2017; Furtado *et al.* 2018). The oleoresin of 17 species of *Copaifera* has been chemically studied (Veiga *et al.* 2007; Gramosa *et al.* 2010; Leandro *et al.* 2012), and has been described as having anticancer activities (Gomes *et al.* 2008), antiparasitic activity against Chagas disease (Izumi *et al.* 2012), and anti-inflammatory and neuroprotective activity (Guimarães-Santos *et al.* 2012). The leaf extract has protective effects against colon carcinogenesis (Senedese *et al.* 2013), as well as antioxidant activity and neuroprotective effects (Botelho *et al.* 2015).

Copaifera multijuga Hayne (Fabaceae, Caesalpinioideae) is a copaiba species endemic to the Amazon region (Costa 2018; Furtado *et al.* 2018). The oleoresin of *C. multijuga* has long been explored by indigenous and traditional Amazonian peoples by tapping the trunk to obtain the exuded oleoresin, which is used as an anti-inflammatory and wound-healing agent, as an urinary antiseptic, and to treat ulcers, bronchitis and tumors (Veiga and Pinto 2002). The leaves of *C. multijuga*, which are used as tea, are rich in phenolic compounds (Pereira *et al.* 2018), including two flavonoid heterosides and 16 galloylquinic acid derivatives (Furtado *et al.* 2018), which are compounds with strong antioxidant potential. *In vitro* and *in vivo* tests have shown that the ethanolic stem bark extract of *C. multijuga* reduces tumor growth at a concentration of 200 mg kg⁻¹ (Albiero *et al.* 2016).

Antioxidant compounds have an important role in the metabolism of oxidative stress, which has been implicated in the development of neurodegenerative diseases, epileptic seizures, aging, and the promotion of certain types of cancer (Pinent *et al.* 2006). Oxidative stress is involved in carcinogenesis due to the generation of reactive oxygen species (ROS) (Noda and Wakasugi 2000). Several human tumors, including melanoma, leukemia, gastric, prostatic, mammary and colon carcinomas, have high levels of ROS (Reuter *et al.* 2010). ROS are molecules formed during mitochondrial respiration that play important roles in cellular signaling (Kronek and Sosa-Torres 2015). They originate from endogenous or exogenous sources, and trigger biochemical

reactions which lead to the formation of new reactive molecules that are capable of attacking membranes and other cell parts (Lushchak 2014). ROS are produced naturally in our bodies through oxidative metabolic processes, important as effector mechanisms of immune system cells (Schneider and Oliveira 2004). The increased production of ROS during oxidative stress, such as the formation of superoxide and hydroxyl radicals during pathophysiological conditions, reduces the generation of antioxidant resources, unbalancing and harming healthy tissues, and leading to lipid peroxidation (Grivennikov *et al.* 2010).

The Ehrlich tumor method is an efficient tool in the evaluation of the antioxidant effect of test compounds on carcinom development in animal models is. Ehrlich's tumor is a spontaneous murine mammary adenocarcinoma adapted to ascitic form and carried in mice by serial intraperitoneal passages (Cassali *et al.* 2006; Calixto-Campos *et al.* 2013). This tumor was described for the first time by Ehrlich and Apolant (1905) and is used for testing antineoplastic drugs, cancer pain and cachexia (Calixto-Campos *et al.* 2013; Frajacomo *et al.* 2016; Albiero *et al.* 2016). The Ehrlich's tumor model is widely used in experimental cancer studies due to its versatility. It evolves into an ascitic form when inoculated by intraperitoneal route, and into a solid form when inoculated subcutaneously, and is able to grow in any type of mice (Calixto-Campos *et al.* 2013; Albiero *et al.* 2016; Frajacomo *et al.* 2016).

Considering the known presence of antioxidant compounds in *C. multijuga* leaves and bark, its traditional use for treatment of tumors in popular medicine, and the findings by Albiero *et al.* (2016) that copaiba stem bark extract was able to interfere in the development of tumor cells as well as their viability, we hypothesized that the antioxidants present in the extract (Pereira *et al.* 2018) may interfere in the generation of oxygen reactive species produced by the Ehrlich tumor. Thus, our objective was to evaluate the ethanolic extract of *C. multijuga* stem bark for possible antioxidant and antineoplastic effects on Ehrlich tumor-bearing mice using three extract concentrations.

MATERIAL AND METHODS

Collection and extract preparation

Stem bark of *Copaifera multijuga* was collected from one tree in the city of Guarantã do Norte (Mato Grosso state, Brazil) (9°48'31.0"S, 54°53'18.0"W). Voucher specimens were deposited in the Herbarium of the Federal University of Mato Grosso (UFMT), Sinop campus, under registration number 4801. The samples were dried and milled for extraction of the ethanolic extract (Albiero *et al.* 2016) for the *in vivo* test.

In vivo test procedures

Male *Swiss* mice with an average weight of 45 g were obtained from the Central Animal Facility at Federal University of Mato

Grosso (UFMT) and were kept in controlled conditions of temperature ($22 \pm 20^\circ\text{C}$), relative humidity ($55 \pm 10\%$), light (12 hours light / dark), and received commercial pelleted feed (Purina, Brazil) and filtered water *ad libitum*. All procedures were conducted in accordance with the recommendations of the Brazilian College of Animal Experimentation and were approved by the Ethics Committee on Animal Use (Comitê de Ética no Uso de Animais - CEUA) of Federal University of Mato Grosso (UFMT) (CEUA Protocol nº 23108.700603/14-3).

The animals were acclimated for 14 days, and were then divided into four groups of eight animals per group. All animals were inoculated subcutaneously with 1×10^6 Ehrlich tumor cells. After 24 hours, the control group (C) started to receive phosphate buffer saline (PBS). Three treatment levels were determined based on Albiero *et al.* (2016). Each treatment group received 100 mg kg^{-1} , 150 mg kg^{-1} and 200 mg kg^{-1} of ethanolic extract dissolved in PBS, respectively. The solutions were administered intragastrically by gavage ($100 \mu\text{L}/\text{animal}/\text{day}$) during 30 consecutive days. After the treatment period, the animals were sacrificed by cervical dislocation and the liver, brain and kidneys were removed. The tissues were stored in an ultra-freezer at -80°C .

Ehrlich tumor cells were kindly provided by Dr. Rondon Tosta Ramalho, from Federal University of Mato Grosso do Sul - UFMS, Brazil). Ehrlich tumors were maintained through intraperitoneal inoculation (ascitic form) in *Swiss* mice, every seven days. Tumor cell suspensions were prepared in sterile PBS to the final concentration of 1×10^7 viable cells mL^{-1} . Mice were inoculated subcutaneously in the right flank region (0.1 mL per animal). Viability, assessed by the Trypan Blue dye exclusion method, was always found to be at least 70%.

Biochemical analyses

In order to assess the effect of the copaíba stem bark extract on the oxidative stress produced by the Ehrlich tumors we determined the levels of up to seven biochemical parameters (enzymatic and non-enzymatic antioxidants and lipid and protein damage biomarkers).

Superoxide dismutase (SOD) activity was assessed in the liver tissue by inhibition of adrenaline oxidation, measured spectrophotometrically at 480 nm, using the UV-VIS spectrophotometer according to Misra and Fridovich (1972) and expressed as UI SOD mg protein^{-1} . Catalase (CAT) activity was determined in liver, kidney and brain tissue according to Nelson and Kiesow (1972). The principle is based on decomposition of H_2O_2 that is measured spectrophotometrically at 240 nm and expressed in $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg protein}^{-1}$. Glutathione-S-transferase (GST) activity was determined in the liver tissue according to Habig *et al.* (1974), the enzymatic activity was measured based on the

formation of GS-DNB adduct, and the result was expressed in $\mu\text{mol GS-DNB min}^{-1} \text{ mg protein}^{-1}$.

Reduced glutathione (GSH) was measured in liver, kidney and brain tissue using the colorimetric method consisting of a reaction of sulfhydryl groups developed by Sedlak and Lindsay (1968), and quantified at 412 nm. The result was expressed in $\mu\text{mol GSH mg protein}^{-1}$ and compared to a standard GSH curve. Ascorbic acid (ASA, vitamin C) levels in the liver tissue were determined according to Roe (1954) by colorimetric method and read at absorbance of 520 nm. The result was expressed in $\mu\text{mol ASA g}^{-1}$ of tissue and compared to a standard curve of ascorbic acid.

Lipid peroxidation levels in liver tissue were evaluated according to Buege and Aust (1978) by determining the levels of substances reactive to thiobarbituric acid (TBARS). TBARS concentration was expressed in $\text{nmol MDA mg protein}^{-1}$ following the calibration curve for MDA. The protein carbonyl content in liver, kidney and brain was determined by spectrophotometry after DNPH derivation according to Yan *et al.* (1995), with some modifications. The total carbonyl content was assessed using a molar extinction coefficient of $22.000 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as $\text{nmol carbonyl mg protein}^{-1}$.

Protein content (except ASA) was estimated by spectrophotometry according to Bradford (1976) using bovine serum albumin as a standard. Absorbance of the samples was measured at 595 nm.

Statistical analysis

Biochemical parameters were compared among treatments and control using one-way ANOVA, followed by the *post hoc* Tukey test. The results were considered statistically significant at $P < 0.05$.

RESULTS

In the hepatic tissue, relative to the control, there was a significant increase in SOD activity in the 150 mg kg^{-1} treatment (Figure 1A), CAT and GST activity in the 200 mg kg^{-1} treatment (Figure 1B, 1C), and GSH in the 200 mg kg^{-1} treatment (Table 1). There was no significant difference among groups in ASA levels (Table 1). There was a significant decrease in TBARS in the 200 mg kg^{-1} treatment (Figure 1A). Protein carbonylation did not differ significantly among groups (Table 1). In renal tissue, there was a significant decrease in GSH in the 100 mg kg^{-1} treatment in relation to the control, while CAT, ASA and carbonyl did not differ among groups (Table 2). In brain tissue, there was a significant increase in CAT activity in the 200 mg kg^{-1} treatment, and in carbonyl in the 200 and 100 mg kg^{-1} treatments, all relative to the control. GSH did not vary significantly among groups (Table 3).

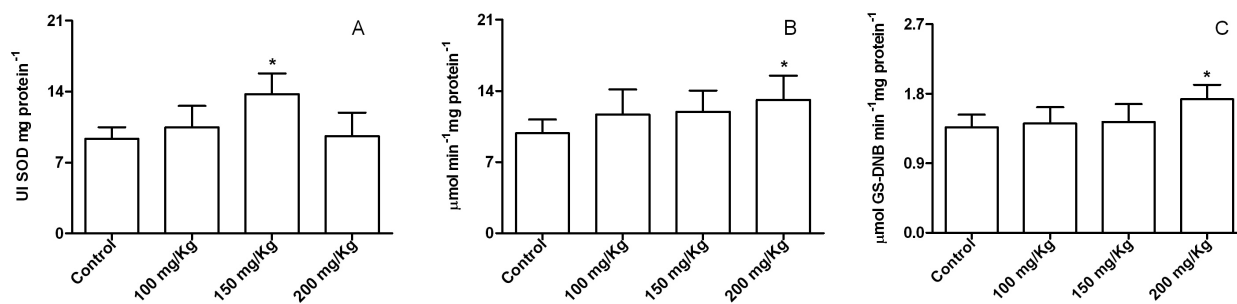


Figure 1. Effect of different concentrations of the ethanolic extract of *Copaifera multijuga* stem bark on hepatic tissue of mice after 30 days of inoculation with Ehrlich subcutaneous carcinoma, as indicated by SOD (A), CAT (B) and GST (C). Asterisks indicate significant differences in relation to the control according to ANOVA followed by *Tukey* test ($P < 0.05$); $N = 8$.

Table 1. Effect of different concentrations of the ethanolic extract of *Copaifera multijuga* stem bark on indicator parameters in hepatic tissue of mice after 30 days of inoculation with Ehrlich subcutaneous carcinoma. Values are the mean \pm standard deviation. Asterisks indicate significant differences in relation to the control according to ANOVA followed by *Tukey* test ($P < 0.05$); $N = 8$.

Treatments	GSH ($\mu\text{mol GSH mg protein}^{-1}$)	ASA ($\mu\text{mol ASA g}^{-1}$ tissue)	TBARS ($\text{nmol MDA mg protein}^{-1}$)	CARBONYL ($\text{nmol carbonyl mg protein}^{-1}$)
Control	420.7 \pm 88.52	1.254 \pm 0.17	1.175 \pm 0.29	4.600 \pm 0.80
100 mg kg ⁻¹	452.2 \pm 80.11	1.250 \pm 0.07	0.952 \pm 0.14	5.134 \pm 0.89
150 mg kg ⁻¹	359.8 \pm 65.67	1.095 \pm 0.14	1.060 \pm 0.23	4.189 \pm 0.64
200 mg kg ⁻¹	616.4 \pm 130.60*	1.230 \pm 0.09	0.847 \pm 0.13*	5.551 \pm 1.21

Table 2. Effect of different concentrations of the ethanolic extract of *Copaifera multijuga* stem bark on indicator parameters in renal tissue of mice after 30 days of inoculation with Ehrlich subcutaneous carcinoma. Values are the mean \pm standard deviation. Asterisks indicate significant differences in relation to the control according to ANOVA followed by *Tukey* test ($P < 0.05$); $N = 8$.

Treatments	CAT ($\mu\text{mol min}^{-1}$ mg protein ⁻¹)	GSH ($\mu\text{mol GSH mg protein}^{-1}$)	ASA ($\mu\text{mol ASA g}^{-1}$ tissue)	CARBONYL ($\text{nmol carbonyl mg protein}^{-1}$)
Control	15.86 \pm 2.04	184.6 \pm 31.76	0.9163 \pm 0.15	8.580 \pm 1.75
100 mg kg ⁻¹	14.93 \pm 3.00	134.1 \pm 28.39*	0.8688 \pm 0.15	7.630 \pm 1.62
150 mg kg ⁻¹	16.40 \pm 1.81	162.4 \pm 39.94	0.8313 \pm 0.12	8.257 \pm 1.54
200 mg kg ⁻¹	16.60 \pm 1.16	140.6 \pm 27.43	0.9025 \pm 0.11	9.088 \pm 1.08

Table 3. Effect of different concentrations of the ethanolic extract of *Copaifera multijuga* stem bark on indicator parameters in brain tissue of mice after 30 days of inoculation with Ehrlich subcutaneous carcinoma. Values are the mean \pm standard deviation. Asterisks indicate significant differences in relation to the control according to ANOVA followed by *Tukey* test ($P < 0.05$); $N = 8$.

Groups	CAT ($\mu\text{mol min}^{-1}$ mg protein ⁻¹)	GSH ($\mu\text{mol GSH mg protein}^{-1}$)	CARBONYL ($\text{nmol carbonyl mg protein}^{-1}$)
Control	1.224 \pm 0.30	452.8 \pm 98.35	4.348 \pm 0.89
100 mg kg ⁻¹	1.610 \pm 0.36	448.5 \pm 109.70	6.503 \pm 1.57*
150 mg kg ⁻¹	1.513 \pm 0.30	440.2 \pm 95.55	3.648 \pm 0.73
200 mg kg ⁻¹	2.156 \pm 0.14*	423.1 \pm 42.61	6.092 \pm 1.57*

DISCUSSION

Several human tumors present high levels of reactive oxygen species (ROS) (Reuter *et al.* 2010). Although, ROS are produced naturally in the body through oxidative metabolic processes, they are also important as effector mechanisms of the cells of the immune system (Schneider and Oliveira 2004). In this context, the immune system is activated through the inoculation of mice with Ehrlich tumor. The continuous production of free radicals during metabolic processes has led to the development of many antioxidant defense mechanisms to limit and prevent cell damage (Vasconcelos *et al.* 2014). The antioxidants present in the *C. multijuga* stem bark extract (Pereira *et al.* 2018) may be interfering in the generation of oxygen reactive species produced by the Ehrlich tumor.

SOD is considered one of the most important enzymes in the antioxidant process (Ighodaro and Akinloye 2017). In Ehrlich ascitic tumor-bearing mice a decrease in the activity of SOD, CAT and GSH occurs, as well as an increase in malondialdehyde levels, the end product of lipid peroxidation (Samudrala *et al.* 2015). In our study, the copaiba extract at 150 mg kg⁻¹ was able to reverse the oxidative stress caused by the Ehrlich tumor, increasing SOD activity in the liver. At 200 mg kg⁻¹ the activity of CAT and GST increased, and TBARS decreased. The increased activity of these enzymes in presence of oxidative stress is an adaptive response, aiming at detoxifying the organism of oxygen free radicals and prevent damage to macromolecules (Ballesteros *et al.* 2009). The ethanolic extract of *C. multijuga* stem bark is rich in antioxidants, containing a high concentration (250 mg kg⁻¹) of total phenols and flavonoids, more specifically phenolic compounds in the form of tannins (Pereira *et al.* 2018). Rajeshwar *et al.* (2005) obtained similar results with mice inoculated intraperitoneally with 2x10⁶ Ehrlich tumor cells and treated for 14 days with 125 and 250 mg kg⁻¹ methanolic extract of *Mucuna pruriens* (Fabaceae).

Our results for increased CAT activity in the liver are in accordance with Ali *et al.* (2015), who observed an increase of CAT in the liver and blood of tumor-inoculated mice fed grape

bark and seed supplemented diet for 30 days. This supports our results, suggesting that copaiba bark extract, when administered for 30 days, has a positive effect on the damage generated by Ehrlich tumor, probably by helping to remove ROS.

GST is the enzyme responsible for the xenobiotic detoxification of the organism, and promotes protection against electrophilic compounds and oxidative stress products (Nathiya and Nandhini 2014). Our results for increased GST activity agree with Bhattacharya *et al.* (2011), who inoculated mice intraperitoneally with 2×10^6 Ehrlich tumor cells and treated them for 9 days with 5 and 10 mg kg⁻¹ ethanolic extract of *Trichosanthes dioica* root, showing that this dose was one of the most effective against oxidative stress generated by tumor growth, increasing both CAT and GST.

GSH is the only non-protein thiol and an important non-enzymatic antioxidant in the process of keeping the body in homeostasis against free radicals and detoxifying xenobiotics, and performs an important role as coenzyme to GST and glutathione peroxidase (GPx) (Goulart *et al.* 2007). Although GSH decreased in renal tissue with lowest extract concentration, the increase of GSH in hepatic tissue at 200 mg kg⁻¹ of extract suggests that, at the highest concentration tested, the copaiba extract might have a protective effect, probably due to its high concentration of tannins (Pereira *et al.* 2018), which can interfere positively with some antioxidants in the liver. Ali *et al.* (2015) also observed an increase of GSH in hepatic tissue.

Lipid peroxidation (TBARS) occurs through malondialdehyde, a physiological acetaldehyde produced by the decomposition of unsaturated lipids from the metabolism of arachidonic acid, and its excess can lead to tissue damage, in addition to the carbonylation of proteins, that can form carbonyl compounds, a general marker used to prove the severe oxidation of proteins (Sellés *et al.* 2016). The decrease in TBARS in hepatic tissue in the 200 mg kg⁻¹ treatment indicated that there was no increase in lipid peroxidation, and the extract reduced the damage caused by the tumor. Ali *et al.* (2015) and Bhattacharya *et al.* (2011) also observed a decrease in lipid peroxidation in hepatic tissue. Protein carbonylation was not altered in the liver and kidney in our study. Although carbonylation increased in brain tissue at the lowest and highest treatment dose, the increase in CAT in the brain suggests that the extract compounds may act to some extent to protect against damage in this tissue.

Overall, our results suggest that the treatment with copaiba stem bark extract is able to reverse the pro-oxidant effect induced by the Ehrlich tumor, improving the antitumor immune response, supporting the results of Samudrala *et al.* (2015) and Albiero *et al.* (2016), who showed that the ethanolic extract of copaiba bark is able to reduce the viability of Ehrlich tumor cells *in vitro*, as well as their development *in vivo*.

The cellular proliferation of tumors is inversely proportional to lipid peroxidation and is involved in the decrease of GPx and GST activity (Das *et al.* 2014). The decrease in TBARS and the increase in GST in the liver of mice treated with 200 mg kg⁻¹ of copaiba thus indicates that this dose was effective in hepatic tissue, as it affected most of the evaluated parameters. Decreased SOD, CAT, and tumor-related GSH concentrations are considered malignant transformation markers (Kavitha and Manoharan 2006), therefore our results for GSH and CAT in the 200 mg kg⁻¹ treatment equally suggest that the copaiba extract protected the animals from the damage by the Ehrlich tumor at the highest test dose.

Albiero *et al.* (2016) used the same experimental model and found reduced tumor growth in mice treated with copaiba extract at a concentration of 200 mg kg⁻¹. The same study also evaluated *ex vivo* cytokine production in ConA or SAC-stimulated spleen cell culture supernatants, resulting in an increase of IL-12p70, TNF- α and IFN- γ in mice treated for seven days with the 200 mg kg⁻¹ copaiba ethanolic extract, demonstrating a proinflammatory profile in response to the immune system against the tumor. Our results support these authors in that copaiba stem bark extract contains compounds that act as biological response modifiers.

CONCLUSIONS

Our results showed that a concentration of 200 mg kg⁻¹ of ethanolic extract of *Copaifera multijuga* stem bark administered during 30 days had significant effects on some biomarkers of oxidative stress in *in vivo* models inoculated with Ehrlich tumor cells, suggesting that this plant part contains substances capable of reducing the damage generated by free radicals. This is the first study to evaluate the pharmacological potential of *C. multijuga* stem bark extract. Future research should further explore the phytochemical potential of *C. multijuga* stem bark compounds.

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