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ANTITUMORAL ACTIVITY AND *IN VIVO* TOXICITY OF *CNIDOSCOLUS QUERCIFOLIUS* POHL (EUPHORBIACEAE)

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ABSTRACT

Ethnopharmacological relevance *Cnidosculos quercifolius* Pohl, commonly known as faveleira, is a plant widely used as a folk medicine used as phytotherapy. From the plant roots of this genus, some activity is reported in the literature as antioxidant potential, inhibition of cellular carcinogenic and antimicrobial agents, among others. **Purpose:** This work was designed to investigate the faveline rich fraction (FRF) obtained from the root bark for their acute toxicity when administered orally, *in vivo* antitumor activity evaluated in mice inoculated with sarcoma 180 tumor cells. To further investigate, to identify major chemical compounds in FRF by the HPLC method. **Materials and methods:** Therefore, acute toxicity study was investigated through oral administration of FRF in mice for 14 days and lethality was monitored daily, to verify if its administration could be considered safe, followed by the antitumor activity were performed against the experimental solid tumor Sarcoma 180 in Swiss albino mice, for 7 days. The identification of major chemical compounds in FRF was assessed by using a high-performance liquid chromatography-Diode Array detector (HPLC-DAD). **Results:** In the acute toxicity study, a single oral dose of FRF did not result in any behavioral changes or mortality, indicating non-toxicity. Biochemical assays showed differences in (AST, ALT), but no morphological changes were detected. In the HPLC fingerprints were obtained four peaks corresponding to four major faveline, as well as one single peak of coumarin. Conclusion: The results derived from in vivo antitumoral experiments, confirm the potential of faveline giving additional scientific support to the subjective use of *Cnidoscolus quercifolius* Pohl in traditional medicine to cancer treatment.

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INTRODUCTION

Currently, new cancer incidence worldwide will numbers up to 18 million by 2019, with approximately 10 million cancer-related deaths. Fortunate patients with cancer who end up in remission face the sobering reality of possible recurrence which varies widely between cancer types (Sen *et al*., 2019). Cancer is a disease at the cellular level that causes abnormal and uncontrolled cell proliferation and growth. Cancer cells also need to adapt their metabolism to survive and multiply under the metabolically compromised conditions provided by the tumor microenvironment. They alter your metabolism to support its rapid proliferation and expansion throughout the body through circulation (Hakim *et al*., 2015). In Brazil, cancer is the second leading cause of death. By 2025, the cancer burden is expected to increase by 50% due to population growth and aging (Rezende *et al*., 2019). Today, in medical science, the most significant challenges are cancer treatment. Conventional treatments consist of radiotherapy, hormone therapy, chemotherapy, stem cell transplantation, surgery, immunotherapy, and precision medicine. Possibly personalized combination therapies using new techniques will be the next promising strategies for the

future direction of cancer treatment (Zhang and Chen, 2018). However, the search for new therapeutical techniques based on plant structures has been rising significantly. Natural products have been considered a promising source of new anticancer compounds (Oliveira Júnior *et al*., 2019). Around 50% of the pharmaceutical drugs existent in the market today are derived from plants. Herbal medicines and medicinal plants have been traditionally used throughout the world for centuries, and more recently are used by many people with various types of cancer disease, as an additional treatment (Greenwell and Rahman, 2015; Safarzadeh *et al*., 2014; Tavakoli *et al*., 2012). The specialized literature has been demonstrating that such plants, rich in bioactive compounds, can be useful in cancer treatments by various mechanisms, like apoptosis induction, epigenetic modulation, and cell differentiation induction (Schnekenburger *et al*., 2014; Tavakoli *et al*., 2012). Plant native from northeast Brazilian's Caatinga biome, *Cnidoscolus quercifolius* Pohl (Euphorbiaceae), popular known as favela or faveleira, is an endemic plant very well adapted to the climate type of the semi-arid region, being used for animal feed and human consumption, besides other uses (Ribeiro ***et al***., 2017). In faveleira has been identified many secondary metabolites, including various di- and tri-terpenes, some of them only found in this species until the present moment as neofavelanone, favelanone, and faveline (Lemos ***et al***., 1991). These compounds are notable for their biological and pharmacological activity against different diseases, like cancer, cardiovascular, and gastrointestinal disorders (Gomes ***et al***., 2014).

Interestingly, previous studies revealed that species from genus *Cnidoscolus* present a range of pharmacological activities, such as antitumoral (Paredes *et al*., 2016) and anti-inflammatory activities (Peixoto Sobrinho *et al*., 2012a), besides hypoglycemic (Lira *et al*., 2017), antibacterial (Peixoto Sobrinho, 2012b) and antinociceptive (Gomes *et al*., 2014) properties. New bioactive molecules with antioxidant activity have also been discovered in the faveleira seed oil (Ribeiro *et al*., 2017), also present on leaves, branches, and roots (MORAIS *et al*., 2016) evidencing its potential as a new source of bioactive products. Importantly, other studies describe antitumor activity and toxicity of species of this genus. Peixoto Sobrinho *et al*. (2012b) demonstrated that the ethanolic extract of aerial parts showed the cell growth inhibition of Hep-2, NCI-H292, and HT-29T. Besides, Paredes *et al*. (2018) investigated the chemical composition of *C. quercifolius* root bark extract through HPLC analysis and evaluated its cytotoxic effect on the different cancer cell lineages (OVCAR-8, SF-295, HCT-116, HL-60 strains). The presence of faveline compounds showed more toxicity towards cancer cell lines in comparison with normal cell lines, being a potential new source of anti-cancer compounds. Phytochemical characterization studies have shown the presence of triterpenoids, flavonoids and catechins in leaves (Lira *et al*., 2017; Otitolaiye and Asokan, 2016), tannins, flavonoids, coumarins, phenolic compounds and terpenoids in leaves and roots (Peixoto Sobrinho *et al*., 2011, 2012b), among others. Based on this, this work aims to contribute with the growing data associated with the toxicology of *C. quercifolius* testing the faveline-rich fraction (FRF) obtained from a chloroform root extract by evaluation of the acute toxicity, in-vivo anti-tumor activity, and assess the weight and histological characteristics of the organs.

MATERIALS AND METHODS

Plant material: The procedures of collection and herborization of the vegetal sample were carried out based on the methodologies (Cartaxo *et al*., 2010). The plant was collected in the Inhamuns micro-region (Tauá, Ceará, 040 ° 18'05.4 "W, 06 ° 01'03.6" S), with authorization from SISBIO, as proof of registration for collection of botanical material, and microbiological of No. 6729103. The shells of roots of the *faveleira* were collected and stored in plastic bags. After the collection, all plant material was transferred to the Laboratory of Biotechnology and Molecular Biology (LBBM) at State University of Ceará (UECE) to perform the preparation of dried specimens. For herbage, parts of the plants that were in the breeding stage were collected. The identification and deposition of the dried sample was carried out in the Herbarium Prisco Bezerra. Exsiccata: EAC 56043 - *Cnidoscolus quercifolius* Pohl, being the date of deposit October 8, 2014.

Preparation of extracts and fractions: To obtain the dry crude extract of *C. quercifolius*, the fresh root bark was washed; after that, the root was air-dried at 45-48 °C for three days. Then, the dried roots of *C. quercifolius* were grounded and macerated in commercial methanol for one week. After, the excess solvent from the extract was removed by roto-evaporation and, subsequently, submitted to a liquid-liquid fractionation, eluted with hexane and chloroform, separating the chemical constituents (Matos, 1997). The fraction eluted with chloroform rich in faveline (fraction C) was lyophilized and stored at 4 °C until use. The fraction C (1.5 g) was mixed with 1.5 g of silica to form a flour-based material that was loaded on a silica gel column chromatography for the concentration of faveline. 15 g of silica gel (0, 063-0.200 mm, 70-230 mesh) previously rinsed with hexane was used as the stationary phase and a gradient elution method was used to separate fractions from fraction C by using solvents from low polarity to high polarity: hexane (1L) and ethyl acetate (1L), interspersed with Q binary mixtures. The fractions obtained were compared by CCD, being visualized in UV light, showing a blue coloration, characteristic of favelines, and coumarins. After that, the fractions collected and compared by thin-layer chromatography (TLC), were once more analyzed under UV light. The fractions which presented blue spot under UV light were joined for chemical analysis. Altogether, 21 fractions of the analyzed sample were obtained, although, after CCD analysis, fraction 6 was chosen for further analysis. The fraction was named faveline- rich fraction (FRF) (Paredes *et al*., 2018).

Characterization of FRF through High Performance Liquid Chromatography (HPLC): HPLC-DAD was performed with a Shimadzu Prominence AutoSampler (SIL-20A) CLAE system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT alternative pumps connected to a DGU 20A5 degasser with integrator CBM 20A, UV-VIS detector SPD-M20A and Software LC solution 1.22 SP1. Chromatographic analyses were performed using a reverse-phase column (Phenomenex®) Luna C18 (4.6 × 250mm, 5µm). The mobile phase was a mixture of acetonitrile-water acidified with 0.5% glacial acetic acid (25:75, v/v), 0-30min. The flow rate was 1.0 mL/min, with an injection volume of 20 µL. Chromatography peaks were confirmed by comparing the retention time with the reference standards and the DAD spectrum (200 to 400 nm).

The FRF was analyzed by dissolution in methanol at the concentration of 5.0 mg/mL. All chromatographic operations were carried out at room temperature and in triplicate.

Animals: Adult male and female Swiss mice (*Mus musculus*) (8–12 weeks, 20–30 g) were provided by Animal House of the Federal University of Ceará (UFC), Brazil. The animals were maintained under controlled temperature (23 °C), under 12 h light– 12 h dark cycle and free access to food and water. The experimental procedures were carried out to the Ethical Principles in Animal Research and approved by the Committee for Ethics in Animal Experimentation at the State University of Ceará approved the experimental protocol (No 12640972-2.)

Acute toxicity: The acute toxicity assay was conducted following OECD Guideline 420 (OECD, 2001). In this study, 28 Swiss albino mice (male and female, 25-30 g weight), were divided into 4 groups of 14 animals, 7 males and 7 females each: control group, treated orally with water (1mL/kg b. w.) and groups of mice treated orally with different doses of FRF dissolved in distilled water (300, 1000 and 2000 mg/kg). The rats were fasted overnight before dosing and 3h after treatment. During the experiment, the general behavior of mice, such as: heart rate, respiratory rate, number of deaths and side effects (e.g., piloerection, diarrhea, sialorrhea, hypnosis and seizures) were monitored continuously during the first 24h (30, 60, 120, 240, and 360 min), and for a 14-days period. After behavioral observation, and at the end of this period, all animals were euthanized, and the kidney and the liver were collected for subsequent analysis.

Biochemical parameters: Blood samples of the animals were collected from the retro-orbital plexus under light ether anesthesia. Biochemical analyses were performed in serum samples obtained after centrifugation of total blood without anticoagulants, at 600 × *g* for 3 min, and then the procedure was repeated at 600 × *g* for 1 min. The determination of the concentrations of the biochemical parameters aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine and urea were done using commercially available test kits based on kinetic, enzymatic and colorimetric methods through spectrophotometry according to the manufacturer's guidelines (Bioclin®, Brazil), using the MindrayBS-200 automatic device.

Assessment of body weight and relative organ weight: The assessment bodyweight of each mice was recorded once a week throughout the experiment. On day 14 of the dosing period, the animals were sacrificed and subjected to thorough necropsy inspection of the exterior of the whole body and vital organs. To determine the relative weight of the organs, the animals were euthanized, organs were removes (liver and kidney), measured, and compared to the saline group.

Histopathology: Histological analysis was performed following routine analysis at the Laboratory of Pathology and Legal Medicine of Federal University of Ceará - UFC. When the mice were sacrificed, tumors and spleen were excised. In brief, the tumor and spleen tissues were fixed in 10% formalin for 24 h. The tissue was embedded in epon resin for processing conventional histological analysis, and the paraffin sections were cut and mounted on glass slides, stained with hematoxylin and eosin (HE). Histological analyses were captured by the digital camera (Moticam 3.0) coupled to the optical microscopy (Nikon-E200), and representative of each organ were captured with a digital camera (Nikon COOLPIX L14 7.1 megapixels), obtaining 10 fields per slide with a final magnification of × 400.

Assay of antitumor activity: Sarcoma 180 tumor cells were maintained in the peritoneal cavities of the Swiss mice, obtained from the central animal house of the State University of Ceará (UECE). Ten-day-old sarcoma 180 (S180) ascites tumor cells were removed from the peritoneal cavity, and 1.0×10^6 cells/animals were implanted subcutaneously into the left posterior groin of the mice. On the following day to inoculation, animals were divided into 5 groups (n = 8 each). FRF was dissolved in distilled water according to the different treatment doses (250 and 500 mg/kg/day) and administered orally. Positive control and negative control groups received, respectively, 5-FU (25 mg/kg/day *i.p.*), and distilled water p.o. Healthy group, composed of S180 non-inoculated animals, was used as an additional comparison parameter, receiving only distilled water through oral administration. Administration of substances started one day after inoculation and was carried out daily, once a day, for 7 consecutive days. On day 7, the animals were anesthetized with ketamine (90mg/kg)-xylazine (4.5mg/kg) for blood collection from each animal via retro-orbital plexus (Waynforth,1980) using sterile tubes and heparinize pipettes. Afterward, all mice were sacrificed by cervical dislocation and tumors and spleens were extirpated, weighed and fixed in formaldehyde 10%. The following formula calculated inhibition ratio (%): inhibition ratio (%) = $[(A - B)/A] \times 100$, where A is the tumor weight average of the saline group, and B is that of the treated group.

Statistical analysis: The data are expressed as the means ± standard error of the means (SEM). The significance of differences between animals from the groups was assessed using an analysis of variance (ANOVA), followed by the Newman-Keuls test ($p<0.05$) using Graph Pad Prisms software.

RESULTS

Quantification of the FRF of C. quercifolius: The chromatogram obtained for the FRF from HPLC-PDA showed the presence of five peaks at around 30.0 min retention time (Fig 1A) corresponding to four faveline, as well as one coumarin (Fig 1B). The yield of these compounds in the FRF extract were confirmed by comparisons of retention time (Rt) and UV profile with previously published reports. The peaks were assigned at retention times of 6.894, 7.485, 11.391, 12.659 and 14.209 min with ultraviolet spectra in the range 202-348 nm corresponding to faveline (5.42%), methyl-faveline (0.46%), deoxofaveline (35.09%), neofavelanone (10.36%) and coumarin (1.24%), respectively (Table 1).

Evaluation of the acute toxicity test: After administration of the FRF doses (300, 1000, and 2000 mg/kg), no death nor behavioral changes were observed for the animals, such as diarrhea, vomiting, motor difficulties, and morbidity. Regarding the relative weight of the organs, there was a significant change, only in the group treated with the dose of 2000 mg/kg/day, between the females, where the relative weight of the kidneys presented a significant increase when compared to the control group (Table 2). There was no change in biochemical parameters, as can be observed in Table 3.

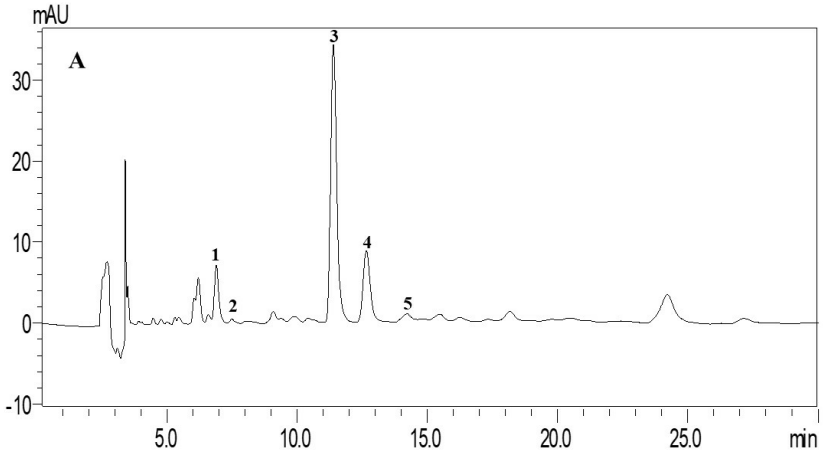
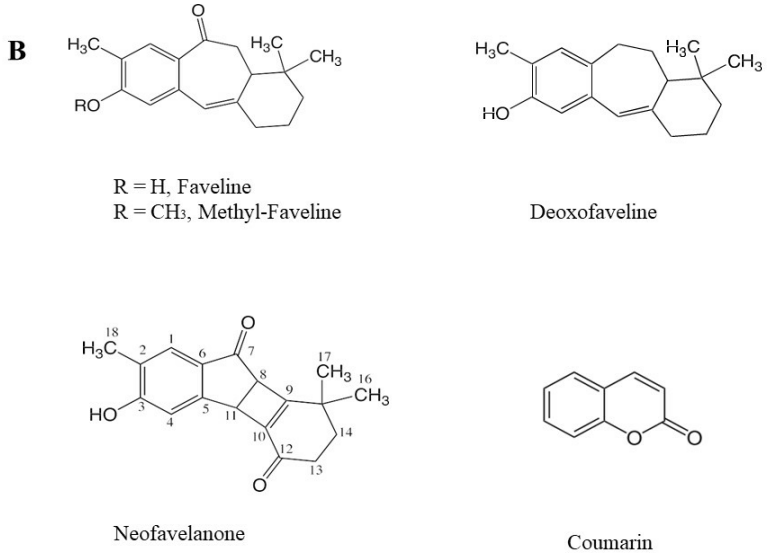
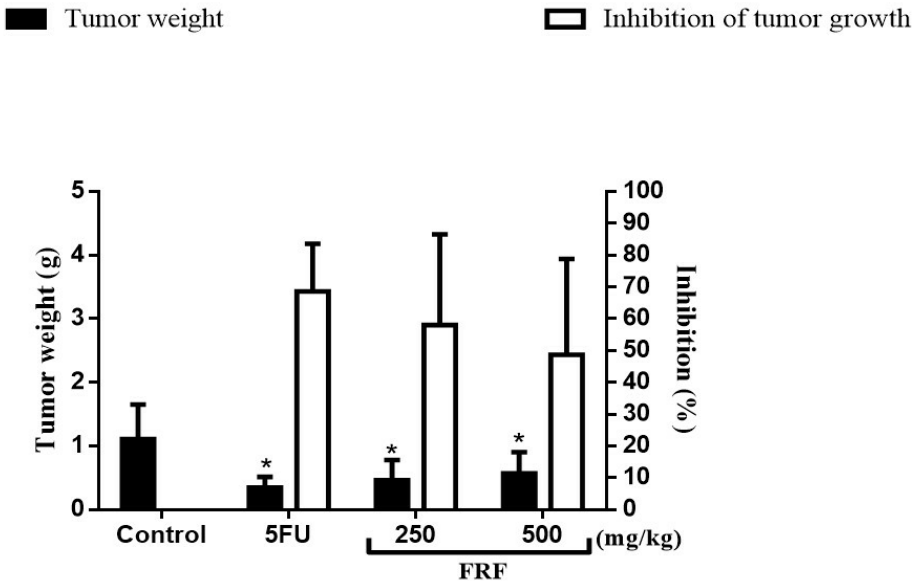


Figure 1. A-High efficiency liquid chromatogram of FRF from the root bark extract of faveleira
B- Chemical structures of faveline and coumarin



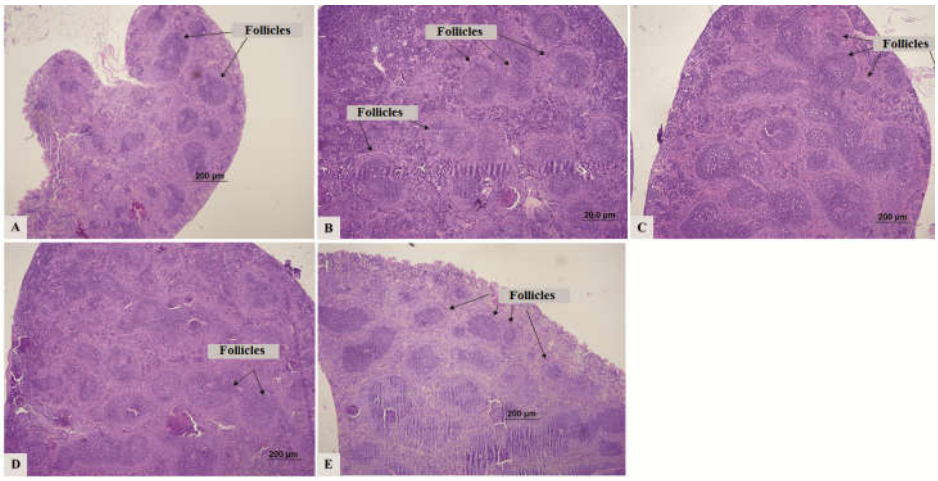
Peak assignment: 1. Faveline, 2. Faveline methyl ether, 3. Deoxofaveline, 4. Neofavelanone. 5.Cumarine.

Figure 2. Effect of FRF on weight and inhibition of Sarcoma 180 tumor in transplanted mice



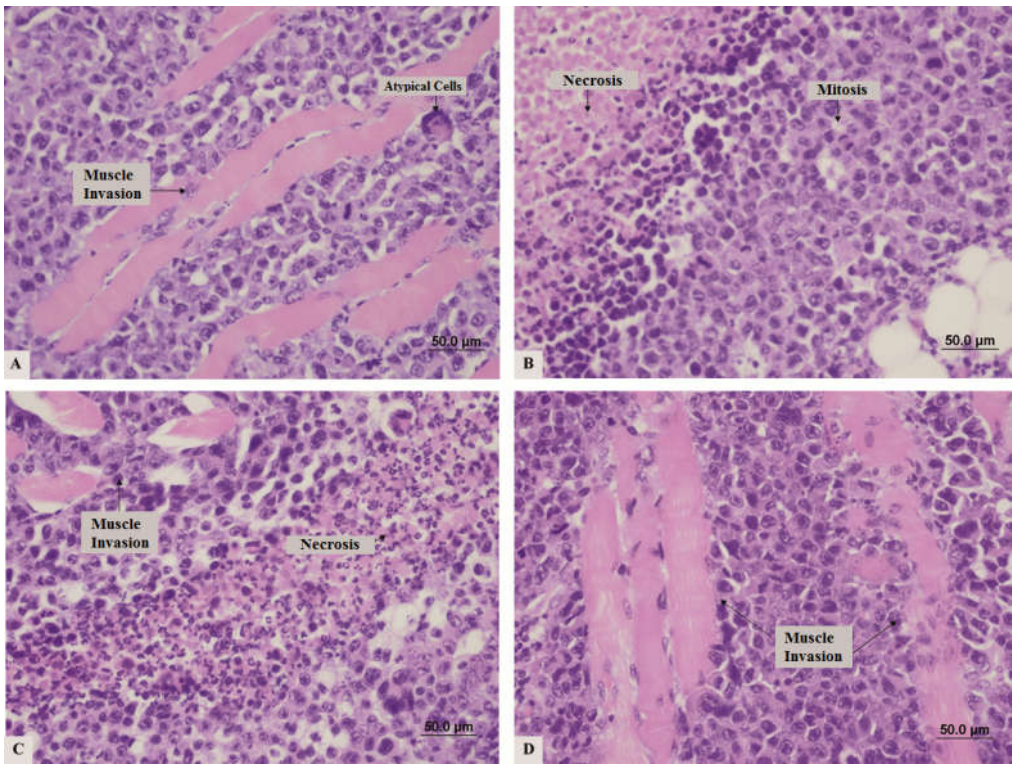
Values were represented by the mean ± SEM of 8 animals per group. * p <0.05 compared to the Control group (negative control) by ANOVA followed by the Newman-Keuls test.

Figure 3. Histopathology of spleen cells after treatment



The tissue sections (spleen) were stained with hematoxylin and eosin and analyzed by light microscopy (400x). Arrow show the presence of follicles. A - Control (negative control); B - Healthy group; C - FRF250 (250mg/kg/day); D - FRF500 (500mg/kg/ day); E - 5-FU (25mg/kg/day *i.p*), positive control. (n=8)

Figure 4. Histopathology of Sarcoma 180 tumor cells



The tissue sections (tumor) were stained with hematoxylin and eosin and analyzed by light microscopy (400x). A - Control (negative control); B - FRF250 (250mg/kg/day v.o); C - FRF500 (500mg / kg/ day v.o); D - 5-FU (25mg/kg/day *i.p*), positive control. (n=8)

Table 1. Chromatographic and spectrometric data with the relative percentage of compounds identified in FRF *C. quercifolius* root bark extract

Peaks	Retention time (min)	Description	Absorbance bands and wavelength (nm)	Relative percentage
1	6.894	Faveline	230; 247; 280; 295; 312.	5.42
2	7.485	Methyl- faveline	239;258;280;300;322.	0.46
3	11.391	Deoxofaveline	202;226;256; 291.	35.09
4	12.659	Neofavelanone	200;214;222;235;261;280; 295;322.	10.36
5	14.209	Cumarin	219;242;276;300;312;348.	1.24

Table 2. Animal weight (g) and relative organ weight (mg/g) of treated mice at the dose of FRF *C. quercifolius* (300, 1000 e 2000mg/kg)

	Male				Female			
Treatment	Body weight (g)		Organs (mg/g)		Body weight (g)		Organs (mg/g)	
	Day 0	Day 14	Liver	Kidney	Day 0	Day 14	Liver	Kidney
Negative control	25.8±0.6	31.9±0.5	4.8±0.1	1.7±0.1	25.2±0.7	29.2±1.1	3.5±0.1	1.0±0.0
FRF1	24.4±0.9	31.6±0.7	4.4±0.1	1.6±0.0	26.3±0.6	27.0±0.6	3.6±0.1	1.1±0.1
FRF2	27.0±0.7	32.9±0.9	4.5±0.1	1.5±0.0	28.5±1.3	29.4±1.4	3.6±0.1	1.1±0.0
FRF3	26.0±1.3	33.0±1.1	4.3±0.1	1.6±0.1	27.3±0.5	28.6±0.5	3.9±0.0	1.2±0.0[a]

Results were expressed as Mean ± SEM. n = 7. ap < 0.05 vs. Negative control. Data were analyzed by ANOVA followed by New man-Keuls test.

Table 3. Serum levels of AST and ALT in U/L and Urea and Creatinine in mg/dL of male and female mice treated with FRF of *C. quercifolius* at doses of 300, 1000, and 2000mg/kg toxicity

Treatment	Dose (mg/kg/day)	Male				Female			
		AST (U/L)	ALT (U/L)	Urea (mg/dL)	Creatinine (mg/dL)	AST (U/L)	ALT (U/L)	Urea (mg/dL)	Creatinine (mg/dL)
Control	-	126.3±3.63	50.5±3.75	0.65±0.06	62.93±6.47	162.3±5.13	54.17±4.4	0.7±0.04	67.07±1.14
FRF1	300	122.7±13.64	53.14±3.46	0.60±0.04	61.47±7.39	131.0±13.55	48.5±3.09	0.65±0.09	68.15±9.31
FRF2	1000	121.6±7.80	49.71±5.64	0.59±0.04	55.66±3.03	133.3±7.48	55.67±4.79	0.59±0.05	54.9±6.02
FRF3	2000	118.9±9.34	46.0±5.52	0.68±0.04	55.94±3.09	141.8±15.88	47.67±3.12	0.62±0.07	62.95±9.07

Values were represented by mean ± SEM of 7 animals per group. For analysis of the significance of the difference between the samples, we used variance analysis (ANOVA) followed by Newman-Keuls test.

Table 4. Effect of FRF in mice transplanted with Sarcoma 180 in serum levels of AST and ALT in U/L and Urea and Creatinine in mg/dL.

Treatment	Dose (mg/kg)	AST (U/L)	ALT (U/L)	Ureia (mg/dL)	Creatinina mg/dL)
Healthy	-	96.50±10.45	40.57±2.15	47,08±3,47	0.54±0.02
Control	-	233.4±17.89[a]	34.43±3.49	42.29±3.0	0.46±0.03
5FU	25 *	178.5±16.0[a]	15.17±2.12[a]	35.45±2.69	0.46±0.03
FRF250	250**	244.0±37.40[a]	42.33±4.33	40.83±4.92	0.54±0.03
FRF500	500**	265.2±22.93[a]	37.80±2.80	36.36±2.17	0.51±0,02

* 5-Fluorouracil (5 FU 25mg / kg, i.p.) was used as positive control; ** Faveline rich fraction (FRF; 250 and 500 mg / kg). Values were represented by mean ± SEM of 8 animals per group. (-) not detected. For analysis of the significance of the difference between the samples, we used variance analysis (ANOVA) followed by Newman-Keuls test. at p <0.05 vs Healthy.

Table 5. The relative weight of organs in g of mice treated with FRF of *C. quercifolius* at the dose of 250 and 500 mg/kg and with 5-FU at a dose of 25 mg/kg

Tratament	Dose (mg/k)	Body weight (g)	Liver (mg/g)	Kidney (mg/g)	Spleen (mg/g)
Healthy	-	27.16±0.26	3.88±0.13	1.12±0.05	0.26±0.03
Control	-	31.74±1.23	4.01±0.15	1.30±0.03	0.50±0.05[a]
5FU	25 *	28.06±0.39[b]	4.40±0.09 [b]	1.22±0.06	0.36±0.03[b]
FRF250	250**	29.60±0.72	3.95±0.09	1.14±0.02	0.48±0.04[a]
FRF500	500**	32.83±0.69	4.30±0.17	1.15±0.05	0.57±0.04[a]

Values were represented by mean ± SEM of 8 animals per group. (-) not detected. For analysis of the significance of the difference between the samples, we used variance analysis (ANOVA) followed by Newman-Keuls test. at [a]p <0.05 vs Healthy; [b] p<0.05 vs Control (used as negative control and treated with distilled water).

Effects of FRF on mice transplanted with sarcoma 180 tumors: The effects of a fraction on sarcoma 180-transplanted mice are shown in Fig 2. There was a reduction of tumor weight in animals that belonged to FRF250 and FRF500 groups (P < 0.05) when compared to the Control group. On day 8, the mean tumor weight of the Control group was 1.01±0.23 g, while in the 5-FU, mice it was 0.35±0.06 g. Moreover, for the FRF250 and FRF500 groups, the mean tumor weights were 0.46±0.11 and 0.57±0.12 g, respectively. Overall, inhibition rates of tumor growth were 58.08 and 48.71% for FRF250 and FRF500, respectively, while treatment with 5FU reduced tumor weight in 68.66%. In other words, FRF at the dose of 250 mg/kg does not differ from the standard drug used in the antineoplastic treatment. Blood biochemistry analysis of Sarcoma 180 tumor-transplanted mice showed that FRF250, FRF500, as well as 5-FU, treated animals, did not present alterations in serum levels of renal function indicators analyzed (urea and creatinine) about the healthy group. Based on serum levels of the enzyme aspartate aminotransferase (AST), all treatment groups, including the negative control group, showed significant changes when compared to the healthy group. Regarding serum levels of hepatic alanine aminotransferase (ALT), there was a significant change only in the group treated with 5-FU, where levels were lower when compared to all groups tested. These results can be seen in Table 4. Regarding the relative weight of the organs evaluated at the end of the treatment, there was no significant difference in the negative control group about any treated group, as can be seen in Table 5.

Histopathology: For a more detailed evaluation of the doses of the FRF of *C. quercifolius* on the organs of treated animals, histopathological evaluation was performed, as only the spleens were altered relative to weight. In the analysis of spleen, it was evidenced that comprised lymphoid follicles numbers were more evident in FRF 250, FRF500, and 5-FU groups, as well as the negative control when compared with the healthy group (Fig 3). Besides, tumors presented malignant characteristics, such as the presence of atypical mitosis, necrosis, and pleomorphisms, in all experimental groups. In microscopy, transplanted tumors were identified as malignant neoplasms with marginal, irregular, and infiltrative borders with invasion of surrounding adipose tissue, reaching and dividing into skeletal muscle tissue shown in Fig 4. In tumor, large, round, and polygonal cells, with pleomorphic shapes, hyperchromatic nuclei, and binucleation (Fig 4. D) were observed. Several degrees of cellular and nuclear pleomorphism was seen (Fig 4. D). Mitosis, muscle invasion, and coagulation necrosis were observed (Fig 4. A, B, C, D). In the tumors, extirpated from animals treated with 5-FU extents areas of coagulative necrosis were observed.

DISCUSSION

It has been well known that flavonoids, diterpenoids, tannins, and coumarins represent the main classes of secondary metabolites isolated from genus *Cnidoscolus* (Euphorbiaceae family). Dinorditerpenoids, particularly *abeo-abietne*, showed significant practical effects with activity against P-388 murine leukemia cells (Ghosh *et al*., 1994).

According to Gomes *et al*. (2014), the presence of several classes of secondary metabolites was verified in *C. quercifolius*, such as coumarins, flavonoids, monoterpenes, diterpenes, among others, which corroborates with the results found through HPLC-PDA analysis in the present study. Previous studies with the species also identified tricyclic benzocycloheptene diterpenes derivative substances in the root bark, as well as faveline ($C_{18}H_{22}O_2$, MW. 270.16), deoxofaveline ($C_{18}H_{24}O$, MW. 256.18), and faveline methyl ether ($C_{19}H_{24}O_2$, MW. 284.17) (Endo *et al*., 1991; Ohta *et al*., 1994). Likewise, terpenoids were recognized, as 3β-O-dihydrocinnamoyl-lupeol and phyllacanthone, 3β-O-cinnamoyl-lupeol (Lemos *et al*., 1991). Fig 1 A, B demonstrated the compounds isolated from *C. quercifolius* in this extract. This result adds value to our previously published works with different biological activities of *Cnidoscolous* sp. with (Lira *et al*., 2017; Paredes *et al*., 2016, 2018). Although the compound coumarin (Fig 1; Table 5) was a minor constituent of the plant extract, this metabolite was already reported by Numa *et al*. (2015) in plants of this genus, as *C. aconitifolius*.

To investigate the effect of oral administration of FRF the *C. quercifolius*, it was carried out a study of acute toxicity in mice. Oral administration of the FRF (300, 1000, 2000 mg/kg), did not cause any behavioral changes in the animals during the experiment for any tested dose, besides those alterations on the other evaluated parameters were also absent. Based on the results, the FRF did not present toxicity in the experimental conditions evaluated. Concerning the change in the relative weight of the kidneys in females at a dose of 2000 mg/kg, shown in Table 2, it may be described as a characteristic of low toxicity which withdrawal or dose adjustment usually leads to rapid improvement and reversal of damages (Montenegro *et al*., 2008). In this study, no weight loss was observed in treated animals. Such a finding is relevant since studies show that changes in body weight have been taken as a measure of adverse effects (Santos *et al*., 2009; Tofovic and Jackson, 1999). Bodyweight loss is a hallmark feature observed in most animals exposed to toxic substances, usually manifesting within a few days after exposure and resulting in a substantial reduction of adipose and muscle tissues (Sangeetha *et al*., 2013).

Also, in this study, FRF showed tumor-inhibiting activity towards Sarcoma 180, with an inhibitory rate up to 58.08 and 48.29% at the doses of 250 and 500 mg/kg, respectively. This result corroborates with a previous study, which showed that *C. quercifolius* presented antitumor activity against several cell lines (OVCAR-8, SF-295, HCT-116, HL-60 cell strains) (Paredes *et al*., 2018). Such a property may be related to the presence of molecules as Lupeol-3β-O-nanoate, deoxofavelin, and methyl-favelin. The cytotoxic activity of those molecules against the HL-60, MCF7, and NCI-H292 cell lines was already reported, with IC_{50} values between 1.6 and 15.6 µg/mL, indicating that *C. quercifolius* can be considered an important source of anticancer molecules (Peixoto-Sobrinho *et al*., 2012a). Additionally, a recent another study with this specimen also demonstrated the cytotoxic potential against human pro-myelocytic leukemia (HL-60), human lung carcinoma (NCI-H292), and human breast adenocarcinoma (MCF-7) cells (Paula *et al*., 2016). On the other hand, 5-FU, a clinically useful chemotherapeutic agent, induced a significant decrease in body weight and relative spleen weight.

In addition to the atrophy of the spleen white pulp, these findings are indicative of the immunotoxicity of drugs (Dória *et al*., 2016). FRF250, FRF500, and 5-FU groups presented a decrease in the mean tumor weight about the negative control group (Fig 2). Regarding the antitumor potential, the FRF has shown other promising results. The liver and the kidneys are the most important organs for detoxification and excretion, respectively. To investigate liver function alterations, the effect of FRF on the biochemical enzymatic parameters (AST and ALT) was evaluated, whereas creatinine and urea were measured as renal function parameters. The AST values presented by all groups of mice inoculated with S-180 are higher than the values of the healthy mice group (Table 4). This result is justified by the fact that AST is also found in skeletal and cardiac muscles, kidneys, pancreas, and erythrocytes (Mincis, 2008). S-180 tumor, in solid form, grows in axillary regions, possibly causing tissue damages in the skeletal muscle and liver, and consequently leading to changes in the AST and ALT enzymatic biochemical parameter results. The loss of body mass is a major effect of antineoplastics, resulting in severe nausea and vomiting often caused by the medication, which may cause intense discomfort to the patient. In this study, no weight loss was observed in the treated animals at any of the FRF doses. However, a significant difference in the animals treated with 5FU could be seen about all treatments. This result is relevant because studies show changes in body weight may be taken as a measure of adverse effect since 5 FU is clinically used as the standard chemotherapeutic agent (El-Sayyad *et al*., 2009). Several studies have shown that terpenes have *in vitro* and *in vivo* antitumor activity. These results of FRF are important and provide insights as to their use for cancer treatments (Sobral *et al*., 2014).

The histological analysis was performed on the tumors and spleen, mainly due to the spleen alterations in relative weight, when compared to the healthy group. The FRF treatment reduced the tumor weight in solid form of S180, despite failing to resolve lesions at a histological level. The analysis of solid tumors showed extensive damage provoked by tissue invasion of malignant cells. FRF250, FRF500, 5-FU, and negative control groups all revealed progressive destruction of parenchyma and stroma. Tumor histology showed that all tumors analyzed had malignant characteristics: intense cellular and nuclear pleomorphism, muscular invasion, mitosis, and necrosis areas. Among the mutations present in malignant tumors, self-sufficiency in growth, insensitivity to growth-inhibitory factors, apoptosis, irregular mitosis, tissue invasion, and metastasis (Hanahan and Weinberg, 2011). Considering the characteristics found in the present study, it affirms the malignancy of the transplanted tumor in the animals.

Conclusion

The results of the present study suggested that the root bark extracts of *C. quercifolius,* up to the dose of 2000 mg/kg, are relatively safe when administered orally to mice. This can be deduced from the fact that the extract at the different treatments did not show any acute adverse effects on the mice, as well as did not induce significant alterations in all the biochemical parameters, which predicts low toxicity of the studied fraction. FRF showed efficient *in vivo* antitumor activity against Sarcoma 180 tumor cells. However, future research is needed to reveal further the unexploited pharmacological potential of this plant, which can be a source

of new bioactive compounds with functional properties, beneficial to restore health.

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Abbreviations

5-FU – Group treated with Fluorouracil
ALT – Alanine aminotransferase
ANOVA – Analysis of variance
AST – Aspartate aminotransferase
FRF – Faveline-rich fraction
FRF250 – Group treated with faveline-rich fraction at dose of 250 mg/kg/day
FRF500 – Group treated with faveline-rich fraction at dose of 500 mg/kg/day
HE – Hematoxylin and eosin
HPLC-High performance liquid chromatography
DAD -Diode array detector
i.p. – Intraperitoneal
OECD – Organization for Economical Co-operation and Development
p.o. – *Per os*
S180 – Sarcoma 180
SEM – Standard error of the means

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