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Preclinical anticancer effectiveness of a fraction from *Casearia sylvestris* and its component Casearin X: *in vivo* and *ex vivo* methods and microscopy examinations

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ABSTRACT

Ethnopharmacological relevance: *Casearia sylvestris* (Salicaceae) is found in South America and presents antiulcerogenic, cytotoxic, antimicrobial, anti-inflammatory and antihypertensive activities.
Aim of the study: To assess the *in vivo and ex vivo* antitumor action of a fraction with casearins (FC) and its main component - Casearin X-isolated from *C. sylvestris* leaves.
Materials and methods: Firstly, Sarcoma 180 bearing Swiss mice were treated with FC and Cas X for 7 days. Secondly, BALB/c nude animals received hollow fibers with colon carcinoma (HCT-116) or glioblastoma (SF-295) cells and were treated with FC for 4 days. On 5th day, proliferation was determined by MTT assay.
Results: FC 10 and 25 mg/kg/day i.p. and 50 mg/kg/day oral and Cas X 25 mg/kg/day i.p. and 50 mg/kg/day oral revealed tumor growth inhibition rates of 35.8, 86.2, 53.7, 90.0 and 65.5% and such tumors demonstrated rare mitoses and coagulation necrosis areas. Similarly, FC reduced multiplying of HCT-116 and SF-295 cells when evaluated by the Hollow Fiber Assay (2.5 and 5 mg/kg/day i.p. and 25 and 50 mg/kg/day oral), with cell growth inhibition rates ranging from 33.3 to 67.4% ($p < 0.05$). Flow cytometry experiments revealed that FC reduced membrane integrity and induced DNA fragmentation and mitochondrial depolarization ($p < 0.05$).
Conclusions: FC and Cas X were efficient antitumor substances against murine and human cancer cells and caused reversible morphological changes in liver, kidneys and spleens, emphasizing clerodane diterpenes as an emerging class of anticancer molecules.

1. Introduction

Casearia sylvestris Swartz (Salicaceae) is a plant widely distributed in Brazil and known as “guaçatonga” , “café silvestre“ and

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"cafezinho do mato". Ethanolic extracts of the leaves and their essential oils have antiulcerogenic activity and reduce gastric volume without altering the stomach pH, which corroborates their use in gastrointestinal disorders (Aboin et al., 1987; Basile et al., 1990; Esteves et al., 2005) and water extracts show phospholipase A_2 inhibitory activity that prevents damage effects on the muscular tissue after toxin inoculation (Borges et al., 2000, 2001; Cavalcante et al., 2007; Da Silva et al., 2008a).

Casearia sylvestris presents antimicrobial action against pathogens as fungi and bacteria, which explains its traditional use to treat wounds, skin ulcerations, diarrhea, flu and chest colds by the Brazilian Karajá Indian tribe and natives from the Shipibo-Conibo tribe (Peru) (Carvalho et al., 1998; Oberlies et al., 2002; Mosaddick et al., 2004; Da Silva et al., 2008b). Moreover, the antioxidant potential of distinct *Casearia* species was also shown by radical scavenging *in vitro* and *in vivo* assays. Among all bioactive compounds found in the ethanolic extract, the therapeutic effects of essential oils have been attributed to the biciclogermacrene, a sesquiterpene with anti-inflammatory activity (Menezes et al., 2004; Mosaddick et al., 2004; Araújo et al., 2015). Methanolic extracts obtained from *C. sylvestris* leaves reduced serum lipids and oxidative stress when orally administered in Swiss mice, prevented arterial thickening induced by high fat diet and diminished *in vitro* platelet aggregation (Brant et al., 2014).

Pharmacological properties of *C. sylvestris* are mainly attributed to the clerodane diterpenes, secondary metabolites derived from isoprene units especially found in the leaves (Itokawa et al., 1990; Santos et al., 2010; Ferreira et al., 2011a, 2014). Based on these extensive ethnopharmacological uses, this article analyzed the *in vivo and ex vivo* antitumor action of an ethanolic fraction with casearins (FC) and its main component - Casearin X-isolated from *C. sylvestris* leaves in murine and human tumor models.

2. Materials and methods

2.1. Chemicals, isolation of the compound and structure identification

Leaves of *C. sylvestris* were collected at Parque Estadual Carlos Botelho (São Miguel Arcanjo, São Paulo State) by researches of the Chemistry Institute of the São Paulo State University. Voucher specimens (AGS04, AGS05, AGS06, AGS13 and AGS19) were deposited at the Herbarium Maria Eneida P. Kaufmann of the Botanical Institute of São Paulo, Brazil. The ethanolic extract from the leaves and its fraction were obtained as described in Santos et al. (2010). Briefly, the extract was fractionated through a solid phase extraction using activated charcoal/silica gel 60–200 μm (1:1, m/m) as stationary phase and hexane/ethyl acetate (95:5, v/v), ethyl acetate and methanol as the mobile phase, providing three fractions, respectively. The second fraction obtained is named fraction with casearins (FC).

Clerodane diterpenes were identified at Núcleo de Bioensaios, Biossíntese e Ecofisiologia de Produtos Naturais - NuBBE, Institute of Chemistry, UNESP (Araraquara, São Paulo, Brazil) using high performance liquid chromatography (HPLC-DAD) as described in Claudino et al. (2013) and nuclear magnetic resonance considering literature data (Itokawa et al., 1990; Santos et al., 2010). These analysis displayed that FC presents 56.5% (mg/g) of the fraction, with Caseargrewiin F (Cas F) and Casearin X (Cas X, Fig. 1) being the majority molecules (9.9% and 14.2%, respectively) (Ferreira et al., 2014).

Fetal calf serum was purchased from Cultilab® (Campinas, SP), RPMI 1640 medium, trypsin-EDTA, penicillin and streptomycin were purchased from GIBCO® (Invitrogen, Carlsbad, CA, USA), 5-fluoruracil (5FU), propidium iodide (PI), ethidium bromide and rhodamine 123 was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and triton X-100 was purchased from Vetec Quimica (Rio de Janeiro, Brazil).

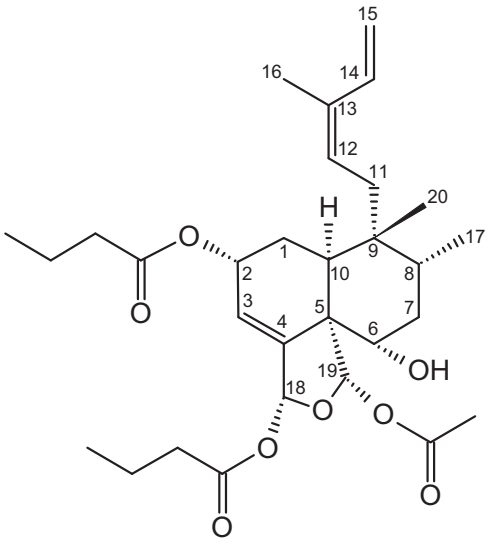

Fig. 1. Structure of Casearin X isolated from leaves of *Casearia sylvestris* Swartz (Salicaceae).

2.2. Animals

Adult female Swiss mice (*Mus musculus*) were obtained from Universidade Federal do Ceará (UFC), Fortaleza, Ceará, Brazil. They were kept in well-ventilated cages (Alesco®) under standard conditions of light (12:12 h light/dark cycle) and temperature (22 ± 1 °C) and were housed with free access to commercial rodent stock diet (Nutrilabor, Campinas, Brazil) and water. BALB/c nude (*nu/nu*) female mice were obtained from State University of São Paulo (USP), Faculty of Medicine, São Paulo, Brazil. They were kept in well-ventilated sterile cages (Tecniplast®, Germain) according to all international standards for production and maintaining of germ free animals. Similarly, they were housed under day and night cycles and with access to commercial sterile rodent stock diet and water *ad libitum*. All procedures were approved by the Committee on Animal Research at UFC (#102/2007) and they are in accordance with Brazilian (COBEA - *Colégio Brasileiro de Experimentação Animal*) and international guidelines on the care and use of experimental animals (Directive 2010/63/EU of the European Parliament and of the Council).

2.3. In vivo antitumor assays

2.3.1. Murine model - Sarcoma 180 tumor

Sarcoma 180 cells were maintained in the peritoneal cavity of mice. Ten-day-old sarcoma 180 (S180) ascite tumor cells were removed from the peritoneal cavity, counted and subcutaneously implanted into the right hind axillary of healthy Swiss animals (4×10^6 cells/mL). On the next day, they were randomly divided into six groups (n = 10 each) and the substances (FC and Cas X) dissolved in 4% DMSO were intraperitoneally administered by injection or orally by gavage for 7 days at the doses of 10, 25 or 50 mg/kg/day. Negative and positive controls received 4% DMSO (i.p. and oral) and 5FU (25 mg/kg/day, i. p.). On the 8th day, only FC-treated animals were anaesthetized with ketamine (90 mg/kg)-xylazine (4.5 mg/kg) for blood collection from each animal via retrorbital plexus (Waynforth, 1980) using sterile tubes and heparinize pipettes to determine the profile of circulating peripheral leukocytes and analyzed at 400x magnification in May-Grünwald-

Giemsa-stained blood smears (two per animal) to obtain differential amount of white blood cells (WBC). The absolute count of a leukocyte subtype was calculated as the product of its respective differential percentage and total leukocyte count (Biermann et al., 1999).

Afterwards, all mice were sacrificed by cervical dislocation and tumors, livers, spleens, kidneys and stomachs were dissected out, weighed and fixed in 10% formaldehyde for examination of size, color changes and hemorrhages. The inhibition ratio of tumor growth (%) was calculated as follows: inhibition ratio (%)=[(A−B)/A]x100, where A is the average tumor weight in the negative control, and B is the average for each treated group.

Tumors (from both FC and Cas X-treated groups) and livers, spleens, kidneys and stomachs (from Cas X-treated groups) were, subsequently, cut into small pieces to prepare histological Section (4–7 μm) and stained with hematoxylin and eosin (H&E). Histological analysis was performed under light microscopy.

2.3.2. Human model - Hollow Fiber Assay (HFA)

In this study, cultures of HCT-116 (colon carcinoma) and SF-295 (glioblastoma) were performed in RPMI 1640 medium supplemented with 20% fetal bovine serum, 2 mM glutamine, 100 U/mL penincillin and 100 μg/mL streptomycin, at 37 °C with 5% CO_2.

The *in vivo* HFA was carried out according to Hollingshead et al. (1995). Polyvinylidene fluoride (PVDF) HFs with a 1-mm internal diameter and a molecular weight cutoff point of 500 kDa were used (Spectrum Laboratories). The fibers were cut into pieces 12–15 cm long, washed 2x with sterile distilled water and kept in sterile conditions. Before use, under sterile conditions, the fibers were incubated in complete RPMI with 20% fetal bovine serum (FBS) overnight (packaging time). Viability of HCT-116 and SF-295 cells was assessed by trypan blue exclusion assay. A cell suspension of 1×10^7 cell/mL (equivalent to 200,000 cells/20 μL/2 cm of fiber) at 4 °C was injected into the fiber, with the ends thereof immediately heat-sealed. The fibers were cut into 2 cm each, transferred to petri plates and incubated in complete RPMI medium during 24 h prior to implantation in mice. Each line was injected into a fiber of different colors (HCT-116, yellow fibers; SF-295, blue fibers).

Afterwards, mice were anaesthetized with ketamine (90 mg/kg) and xylazine (4.5 mg/kg). A small incision was performed in the neck to permit subcutaneous (s.c.) implantation of two fibers in the dorsal region. All cuts were closed with surgical stapler. The treatments started in the following day and lasted four consecutive days. For this, mice were divided into six groups (n=7/group) as follows: negative control (4% DMSO i.p. and oral), positive control (5-FU, 20 mg/kg/day), FC i.p. (2.5 and 5 mg/kg/day) and FC oral by gavage (25 and 50 mg/kg/day). On 5th day, fibers were removed to measure the antiproliferative capacity.

Tumor cell proliferation was quantified using the MTT assay (Mosmann, 1983). For this purpose, fibers removed from animals and incubated with 1 mg/mL MTT in 6-well plates during 4 h at 37 °C and 5% CO_2. The MTT solution was aspirated, fibers were washed with saline solution containing protamine 2.5% sulphate and incubated in protamine solution overnight at 4 °C. Fibers were cut into 2 or 3 smaller pieces, transferred to 24 well plates, and put to dry. The formazan was dissolved in DMSO, aliquots (150 μL) were transferred to 96 well plates and quantification of cell proliferation was spectrophotometrically determined using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter). The antitumoral effect of the substances was quantified as the percentage of the control absorbance.

2.4. Analysis by flow cytometry in Sarcoma 180 cells

Once cytotoxicity of the FC and its compounds on S180 and other murine and cancer human cells was previously shown (Santos et al., 2010; Ferreira et al., 2011, 2014), we decided to performer biochemical assessments in a primary culture of S180 cells by flow cytometry. Ascite-bearing mice between 7 and 9 days postinoculation were sacrificed by cervical dislocation and a suspension of S180 cells was harvested from the intraperitoneal cavity under aseptic conditions. The suspension was centrifuged at $500 \times g$ for 5 min to obtain a cell pellet and washed three times with RPMI medium. Cell concentration was adjusted to 0.5×10^6 cells/mL in RPMI 1640 medium supplemented with bovine fetal serum and phytohemagglutinin (2%), plated in a 24-well plate and incubated with increasing concentrations of the FC (0.2, 0.4 and 0.8 μg/mL) for 24 h (Ferreira et al., 2011b). Doxorubicin was used as positive control (0.3 μg/mL). Subsequently, examinations were performed in a Guava EasyCyte Mine™ cytometry (Guava Express Plus CytoSoft 4.1 software, Guava Technologies Inc. Industrial Blvd. Hayward, CA, USA). Five thousand events were evaluated per experiment and cellular debris was omitted.

2.4.1. Membrane integrity

Cell membrane integrity was evaluated by the exclusion of PI after 24 h exposure. Briefly, 100 μL of treated and untreated cells were incubated with PI (50 μg/mL) for 5 min at 37 °C and membrane integrity was determined (Darzynkiewicz et al., 1992).

2.4.2. DNA fragmentation

Briefly, 24 h-treated and untreated cells were incubated at 37 °C for 30 min in the dark in a lysis solution containing 0.1% citrate, 0.1% triton X-100 and 50 μg/mL PI and fluorescence was subsequently measured (Ferreira et al., 2014).

2.4.3. Mitochondrial transmembrane potential

It was determined by rhodamine 123, a cell-permeable, cationic, fluorescent dye readily sequestered by mitochondria without inducing cytotoxic effects. After 24 h of treatment, cells were washed with PBS and incubated with rhodamine 123 at 37 °C for 15 min in the dark. Cells were incubated again in PBS at 37 °C for additional 30 min in the dark, and fluorescence was measured Cury-Boaventura et al., 2003.

2.5. DNA relaxation assay

The inhibitory effects of FC on human DNA topoisomerases (I and II) were examined using Topo I and Topo II Drug Screening Kits (TopoGEN, Inc.). Supercoiled plasmid DNA (250 ng) was incubated with human Topo I or II (4 UI) at 37 °C for 30 min in relaxation buffer (10 mM Tris buffer pH 7.9, 1 mM EDTA, 0.15 M NaCl, 0.1% BSA, 0.1 mM spermidine and 5% glycerol) in the presence or absence of FC (0.01, 0.1, 1 and 10 μg/mL). Camptothecin (1 μM) and etoposide (100 μM) were used as positive controls. The reaction was terminated by the addition of 10% SDS (2 μL) and proteinase K (50 μg/mL) and incubated at 37 °C for 30 min. The DNA samples were added to the loading dyes (2 μL) and subjected to electrophoresis on a 1% agarose gel for 90 min at room temperature and stained with ethidium bromide.

2.6. Statistical analysis

In order to determine differences between groups, data (mean ± S.E.M.) were compared by one-way analysis of variance (ANOVA) followed by Student Newman-Keuls test ($p < 0.05$) using GraphPad Prism® software.

3. Results

3.1. In vivo activity on Sarcoma 180 tumor

After the administration of the FC or Cas X [intraperitoneally (10 and 25 mg/kg/day) or orally by gavage (25 and 50 mg/kg/day)] for 7 consecutive days, mice were sacrificed on the 8th day by cervical dislocation and dissected out to remove tumor, liver, spleen, kidneys and stomach. Both substances – FC 10 and 25 mg/kg/day i.p. and 50 mg/kg/day oral and Cas X 25 mg/kg/day i.p. and 50 mg/kg/day oral – reduced tumor growth (1.3 ± 0.2, 0.3 ± 0.1, 1.1 ± 0.3, 0.2 ± 0.7 and 0.7 ± 0.1 g, respectively) in a significant way ($p < 0.05$) when compared to the negative control receiving DMSO 4% (FC: 1.9 ± 0.2 and 2.4 ± 0.3 g; Cas X: 2.3 ± 0.2 and 2.2 ± 0.3 g, for i.p. and oral administration, respectively) (Table 1). Then, FC 10 and 25 mg/kg/day i.p. and 50 mg/kg/day oral and Cas X 25 mg/kg/day i.p. and 50 mg/kg/day oral revealed tumor growth inhibition rates of 35.8 ± 8.9, 86.2 ± 7.2, 53.7 ± 10.9, 90.0 ± 2.9 and 65.5 ± 6.6%. Similarly, the positive control 5-FU also showed tumor reduction ranging from 52.7 ± 5.7 to 83.9 ± 2.3% ($p < 0.05$).

3.1.1. Macroscopic and microscopy examinations

There was a reduction in the weight of animals treated with FC (24.6 ± 0.8 g) and Cas X (22.5 ± 0.8 g) at 25 mg/kg/day i.p. (Table 1). Alterations in wet relative weight of organs were also seen in intraperitoneally FC- and Cas X-treated animals. There was a decreasing of the livers (4.52 ± 0.20 g) and spleens (0.26 ± 0.06 g) of animals treated with FC 25 mg/kg/day and similar findings were seen with spleens (0.44 ± 0.03 g) of Cas-X treated mice in comparison with their respective negative controls (0.51 ± 0.03 and 0.65 ± 0.05 g) ($p < 0.05$). 5-FU also strongly reduced spleen relative weights in both experimental protocols (0.22 ± 0.01 and 0.38 ± 0.03 g). On the other hand, oral doses of 50 mg/kg/day in both FC- and Cas X-treated groups caused increasing of stomachs (1.02 ± 0.11 and 0.93 ± 0.06 g) and FC 50 mg/kg/day also induced spleen decreasing (0.38 ± 0.04 g) ($p < 0.05$). No deaths were noticed during treatment of Swiss mice with FC or Cas X, though FC- and Cas X-treated animals at 25 mg/kg/day i.p. showed diarrhea from the second dose.

Tumors from negative control groups [i.p. and oral 4% DMSO (Fig. 2A and B, respectively)] and groups treated with FC and Cas X 10 mg/kg/day i.p. and FC and Cas X 25 mg/kg/day oral (Fig. 2D and F) showed characteristics of malignancy, consisting in round and polyhedral cells with anisokaryosis, binucleation, common mitoses, different degrees of cellular and nuclear pleomorphism, and large areas of muscle invasion and points of coagulation necrosis. Analysis of the groups that received 5-FU (Fig. 2C), FC and Cas X 25 mg/kg/day i.p. (Fig. 2E) and 50 mg/kg/day oral (Fig. 2G) showed, as described above, typical morphology of neoplastic cells, but they demonstrated rare mitoses and coagulation necrosis areas were more found.

Organs of the animals from the negative control group did not show signs of severe liver toxicity, though some points of hydropic degenation (Fig. 3A) and kidneys with light glomerular and tubular hemorrhage have been found (Fig. 4A). Spleens with visible follicles and many megakaryocytes were also observed. The stomachs showed no hemorrhage streaks, the cardia region had keratinized squamous covering without changes in the corium and parietal and zymogen cells and normal mucosa and submucosa were visualized. On the other hand, investigations showed that only Cas X 25 mg/kg/day i.p. was able to induce significant histopathological changes in livers, since toxicity was diagnosed by some points of inflammatory focus, portal and centrilobular vein congestion and evident strands of hepatocytes (Fig. 3D). Moreover, a more visible hydropic degenation was found in Cas X-treated animals with 50 mg/kg/day oral (Fig. 3F).

Table 1
Effect of a Fraction with Casearins (FC) and of Casearin X (Cas X) extracted from *Casearia sylvestris* leaves on relative weight of key organs and on tumor growth in Swiss mice bearing Sarcoma 180 after 7 days of intraperitoneal or oral treatment.

Substance		Dose (mg/kg/day)	Mice weight (g)	g/100g				Tumor (g)	Tumor inhibition (%)
				Liver	Kidney	Spleen	Stomach		
Intraperitoneal	**Control**	–	38.0 ± 1.2	5.31 ± 0.13	1.40 ± 0.04	0.51 ± 0.03	0.67 ± 0.07	1.9 ± 0.2	–
	5-FU	25	21.5 ± 1.0*	4.86 ± 0.27	1.28 ± 0.03	0.22 ± 0.01*	0.60 ± 0.04	0.3 ± 0.1*	83.9 ± 2.3*
	FC	10	36.3 ± 0.8	4.98 ± 0.15	1.37 ± 0.04	0.48 ± 0.04	0.59 ± 0.09	1.3 ± 0.2*	35.8 ± 8.9*
		25	24.6 ± 0.8*	4.52 ± 0.20*	1.43 ± 0.08	0.26 ± 0.06*	0.61 ± 0.05	0.3 ± 0.1*	86.2 ± 7.2*
Oral	**Control**	–	36.2 ± 1.2	5.28 ± 0.18	1.35 ± 0.07	0.53 ± 0.02	0.69 ± 0.04	2.4 ± 0.3	–
	FC	25	35.0 ± 1.1	5.22 ± 0.16	1.34 ± 0.05	0.45 ± 0.03	0.83 ± 0.04	1.8 ± 0.3	24.9 ± 10.6
		50	31.3 ± 1.5	4.88 ± 0.22	1.44 ± 0.05	0.42 ± 0.03	1.02 ± 0.11*	1.1 ± 0.3*	53.7 ± 10.9*
Intraperitoneal	**Control**	–	30.0 ± 0.8	5.62 ± 0.16	1.47 ± 0.07	0.65 ± 0.05	0.59 ± 0.09	2.3 ± 0.2	–
	5-FU	25	22.2 ± 1.0*	5.06 ± 0.15	1.51 ± 0.05	0.38 ± 0.03*	0.50 ± 0.08	1.0 ± 0.1*	52.7 ± 5.7*
	Cas X	10	30.9 ± 1.0	5.58 ± 0.27	1.58 ± 004	0.71 ± 0.03	0.63 ± 0.05	2.7 ± 0.3	-15.2 ± 13.7
		25	22.5 ± 0.8*	4.87 ± 0.31	1.61 ± 0.08	0.44 ± 0.03*	0.62 ± 0.06	0.2 ± 0.7*	90.0 ± 2.9*
Oral	**Control**	–	28.0 ± 0.7	4.86 ± 0.07	1.04 ± 0.02	0.55 ± 0.04	0.66 ± 0.02	2.2 ± 0.3	–
	Cas X	25	29.5 ± 1.2	4.97 ± 0.18	1.18 ± 0.03	0.57 ± 0.05	0.64 ± 0.02	2.1 ± 0.3	0.5 ± 12.1
		50	27.4 ± 1.5	4.53 ± 0.17	1.20 ± 0.08	0.38 ± 0.04*	0.93 ± 0.06*	0.7 ± 0.1	65.5 ± 6.6*

Values are means ± S.E.M., n = 10 animals/group. Negative control was treated with the vehicle used to dilute the drug (4% DMSO). 5-Fluorouracil (5-FU) was used as positive control.

* $p < 0.05$ compared with the negative control by ANOVA followed by Newman-Keuls test.

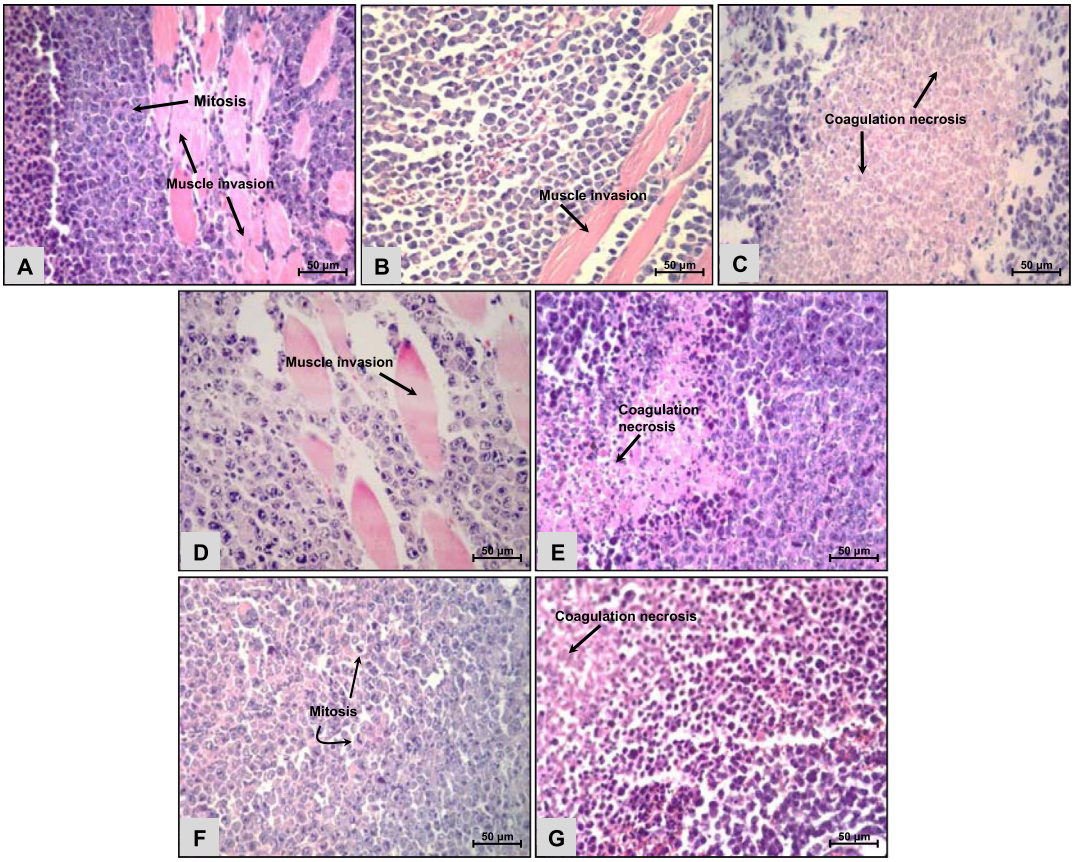

Fig. 2. Morphology of Sarcoma 180 tumor cells from Swiss mice after 7 days of treatment with a Fraction with Casearins and Casearin X extracted from *Casearia sylvestris*. Animals were treated by injection (10 and 25 mg/kg/dia: D and E) or gavage (25 and 50 mg/kg/dia: F and G, respectively). Negative control was treated with the vehicle used to dilute the substance (4% DMSO i.p. and oral; A and B). 5-Fluorouracil was used as i. p. positive control (C). Hematoxylin-eosin staining. Light microscopy magnification, 400x. Scale bar $=50\ \mu m$.

Kidneys of treated mice at 25 mg/kg/day i.p. showed macroscopically whitish appearance when compared with the negative control, and microscopically, there was glomerular and tubular hemorrhage with intense swelling of tubular epithelium and presence of hyaline cilinders (Fig. 4D). Hemorrhage focus and hyaline cilinders were also seen in all other groups (Fig. 4). Spleens of FC and Cas X groups at 25 mg/kg/day i.p. and FC 50 mg/kg/day oral showed decrease in size of follicles. Stomachs were similar to the negative control animals and morphological changes in both FC- and Cas X-orally treated groups were not detected in spite of the augment in their relative weights.

3.1.2. Profile of the peripheral blood white cells

Swiss mice bearing Sarcoma 180 and treated with FC were also analyzed regarding to the white blood cells' counting. It was found a decrease in the total number of leukocytes in the 5-FU group ($2.6 \times 10^3/\mu L$) due to the reducing of monocytes ($0.1 \pm 0.01 \times 10^3/\mu L$), neutrophils ($0.1 \pm 0.002 \times 10^3/\mu L$) and lymphocytes ($2.4 \pm 0.2 \times 10^3/\mu L$). On the other hand, there was an increasing of neutrophils ($5.0 \pm 0.3 \times 10^3/\mu L$) and reduction of lymphocytes ($2.0 \pm 0.1 \times 10^3/\mu L$) in animals intraperitoneally treated with FC 25 mg/kg/day i.p. (Table 2, $p < 0.05$), though no changes was noticed in relation to total number of white blood cells when compared to the control negative ($8.8 \pm 0.6 \times 10^3/\mu L$) presumably due to compensation in the overall score ($p > 0.05$).

3.2. Antiproliferative action on human cancer cells

In order to verify *in vivo* antitumor potential of FC on human cancer cells, HFA studies were performed. Then, after removing fibers, cell proliferation was spectrophotometrically quantified by MTT assay. As demonstrated in Table 3, FC reduced multiplying of neoplastic cells in all doses tested (2.5 and 5 mg/kg/day i.p. and 25 and 50 mg/kg/day oral), with inhibition rates of cell growth of 33.3%, 36.7%, 44.9% and 56.9% for SF-295 cells and 47.4%, 45.4%, 51.9% and 67.4% for HCT-116 cells, respectively ($p < 0.05$). One death was recorded in both i. p. groups and an additional one in the dose of 50 mg/kg/day oral.

3.3. Biochemical alterations on S180 cells

Outcomes revealed that FC at concentrations of 0.4 and 0.8 μg/mL reduced membrane integrity (63.5 ± 1.5 and $11 \pm 1.8\%$), caused DNA fragmentation (19.1 ± 1.1 and $48.4 \pm 1.8\%$) and induced mitochondrial depolarization (14.0 ± 0.5 and $18.3 \pm 0.3\%$) in primary cultures of S180 cells when compared to the negative control (80.0 ± 0.1, 6.7 ± 0.6 and $10.3 \pm 0.4\%$), respectively, after 24 h exposure (Fig. 5). Doxorubicin also caused similar changes ($p < 0.05$).

The bioactivity of FC on topoisomerases I and II was evaluated in supercoiled plasmid DNA relaxation experiments. None of the studied concentrations was capable of altering DNA topology (0.01, 0.1, 1 and 10 μg/mL). On the hand, camptothecin and etoposide, used as controls, inhibit the activity of topoisomerase I and II, respectively.

4. Discussion

Cancer is characterized as a collection of almost 200 kinds of complex diseases with proliferative, mutational, aberrant and

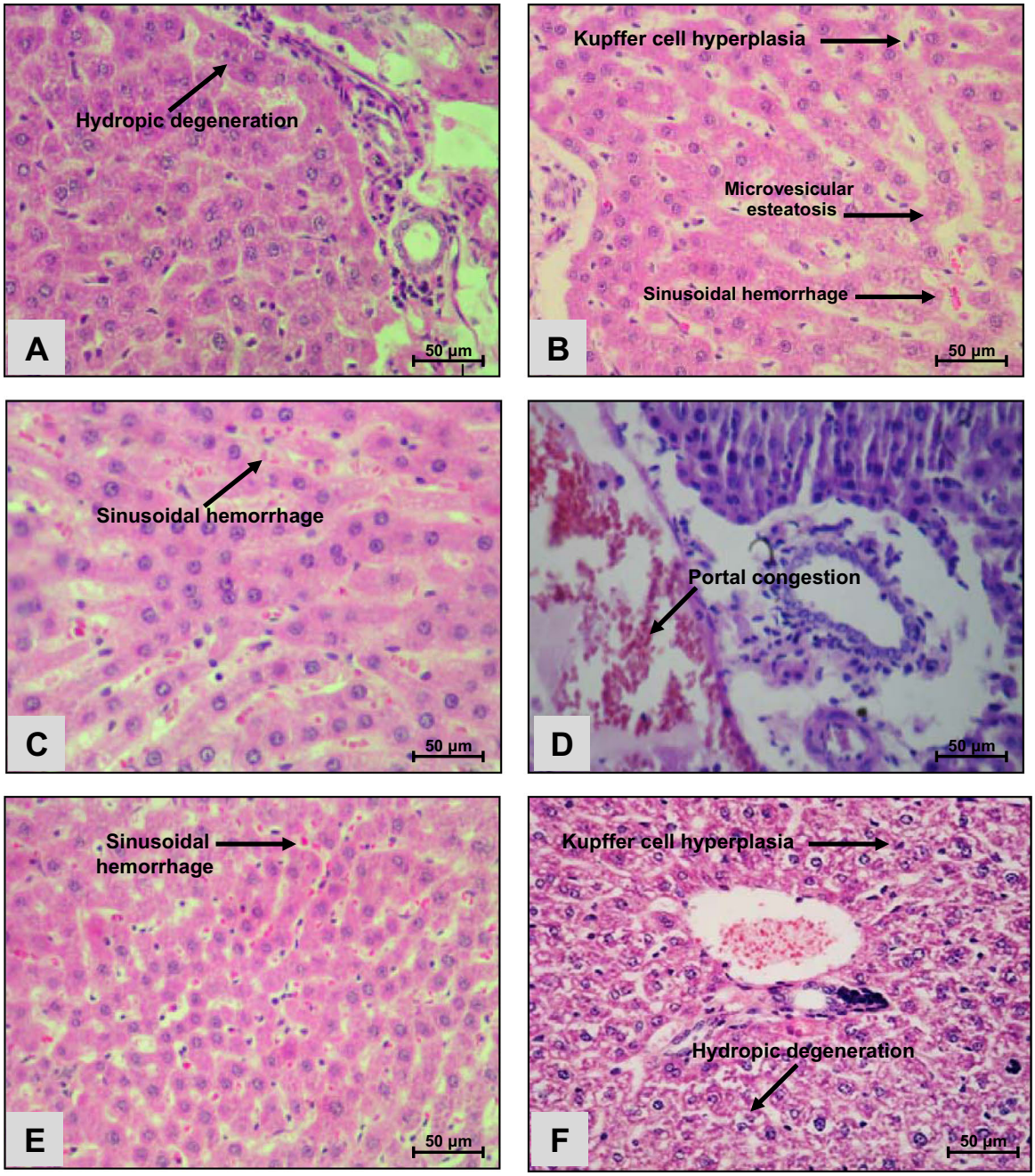

Fig. 3. Histological analyzes in livers of mice treated with Casearin X isolated from *Casearia sylvestris* leaves at doses of 10 (C) and 25 mg/kg/day i.p. (D) and 25 (E) and 50 mg/kg/day oral (F) during 7 days. Negative control (A) received DMSO 4%. 5-Fluorouracil (5-FU) was used as positive control (B). Hematoxylin-eosin staining. Light microscopy magnification, 400x. Scale bar =50 µm.

uncontrolled cell growth nature, wherein cells present in the same microenvironment, usually spread to adjacent tissues and organs (metastasize) and regions away from the primary tumor. These neoplasic properties are often caused by accumulation of mutations in oncogenes and in suppressor and DNA repair genes (Kumar et al., 2004; Grivicich et al., 2007; Hanahan and Weinberg, 2011). The genetic diversity and genomic instability in tumor cells contribute to the wide phenotypic heterogeneity, complicate treatment and facilitate the manifestation of resistance(s) and relapse(s) that can arise at any stage of the cancer development (Hanahan and Weinberg, 2011; McCubrey et al., 2015). In this scenario, natural compounds arise as alternative to overcome tumor resistance of aggressive forms of solid tumors, since about 60% of the drugs currently used in cancer chemotherapy has natural origin, including natural products or substances derived from natural prototypes, such paclitaxel, docetaxel, vinblastine, vincristine, vindesine, irinotecan and camptothecin. Then, instead of the development of chemical synthesis techniques, plant compounds remain essential to the discovery of new anticancer agents (Cragg and Newman, 2005; Srivastava et al., 2005; Bezerra et al., 2015; Monção et al., 2015; Simões et al., 2015).

In relation to the *Casearia sylvestris* plant, chemical studies have isolated clerodane diterpenes, whose molecules displayed remarkable *in vitro* and *in vivo* activity on different histological human and murine cancer lines, such as Ehrlich, Lewis ascite lung cancer, S180 cells, leukemias (HL-60, CEM, K-562), colon (HCT, HCT-8, HCT-116), breast (MDA/MB-231, Hs578-T, MX-1, MCF-7), melanoma (MDA/MB-435, A2058, B-16/F10, B16F10-Nex2),

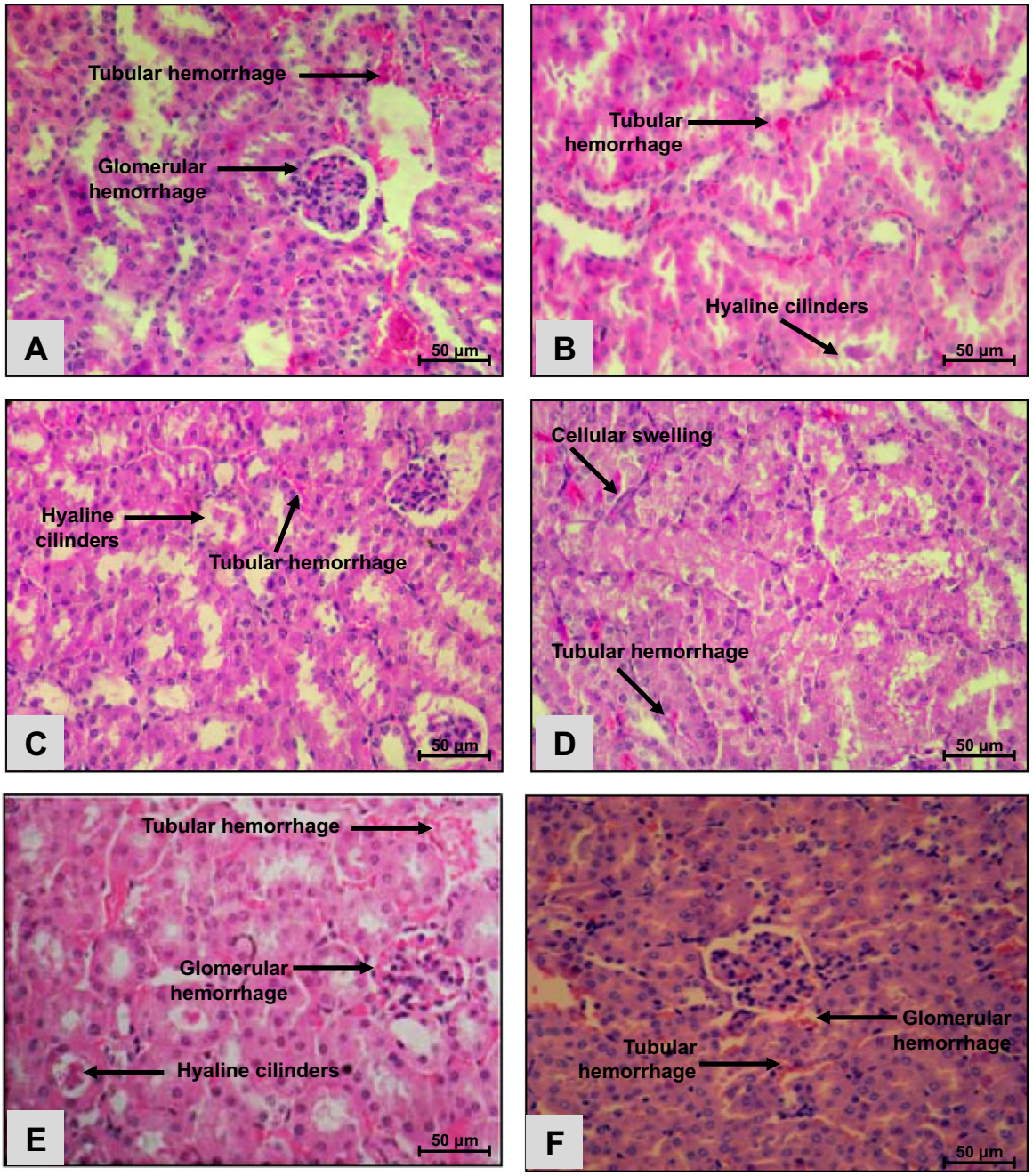

Fig. 4. Histological analyzes in kidneys of mice treated with Casearin X isolated from *Casearia sylvestris* leaves at doses of 10 (C) and 25 mg/kg/day i.p. (D) and 25 (E) and 50 mg/kg/day oral (F) during 7 days. Negative control (A) received DMSO 4%. 5-Fluorouracil (5-FU) was used as positive control (B). Hematoxylin-eosin staining. Light microscopy magnification, 400x. Scale bar = 50 μm.

prostate (PC-3, DU-145), ovarian (A-2780), lung (LX-1, A-549), cervical carcinoma (HeLa) and glioblastoma (SF-295) (Itokawa et al., 1990; Morita et al., 1991; Oberlies et al., 2002; Da Silva et al., 2008c, 2009; Wang et al., 2009; Ferreira et al., 2010; Santos et al., 2010; Felipe et al., 2014; Bou et al., 2015).

Based on the use of experimental tumors for the identification of substances with chemoterapeutic potential, the *in vivo* activity of the FC and Cas X was firstly assessed using Swiss mice transplanted with S180 tumor cells, a murine sarcoma experimental model extensively applied in the search for natural products with antitumor action (Itokawa et al., 1990; Bezerra et al., 2008; Magalhães et al., 2010; Ferreira et al., 2011b; Militão et al., 2012). Regression of tumors in animals, growth reduction of sensitive tumors and/or increase in life expectancy are factors directly related to the antineoplastic activity (Schabel, 1977; Da Silva et al., 2009). Schabel (1977) showed that better results depend on the treatment procedure, which shall be started within 48 h after tumor transplantation. At this time, tumor cells have already started the formation of tumor nodules. Based on these findings, the treatment was performed within 24 h after S180 inoculation. After 7 days of administration, FC and Cas X was capable of causing, approximately, until 90% of tumor mass reduction. In some animals, tumors were not macroscopically detected.

Since animals treated with FC caused amazing reduction in S180 tumors, *ex vivo* biochemical analysis by cytometry were carried out in attempting to understand this biological action. Previously, we have already demonstrated promising cytotoxic activities of the FC and Cas X on primary culture of S180 cells (IC_{50}

Table 2
Profile of the peripheral blood white cells of Swiss mice bearing Sarcoma 180 after intraperitoneal or oral treatment during 7 days with the Fraction with Casearin (FC) extracted from *Casearia sylvestris* leaves.

Substance		**Dose** (mg/kg/dia)	**Total leukocytes (10^3 /μL)**	**Leukocyte differential counting** (10^3/μL)			
				Eosinophils	Lymphocytes	Neutrophils	Monocytes
Intraperitoneal	**Control**	–	8.8 ± 0.6	0.1 ± 0.01	4.7 ± 0.7	3.7 ± 0.5	0.28 ± 0.1
	5-FU	25	2.6 ± 0.1*	0.0 ± 0.0	2.4 ± 0.2*	0.1 ± 0.002*	0.1 ± 0.01*
	FC	10	7.2 ± 0.3	0.0 ± 0.0	3.9 ± 0.1	3.0 ± 0.6	0.3 ± 0.1
		25	7.3 ± 0.7	0.1 ± 0.1	2.0 ± 0.1*	5.0 ± 0.3*	0.3 ± 0.1
Oral	**Control**	–	6.5 ± 0.9	0.1 ± 0.1	3.7 ± 0.9	2.5 ± 0.4	0.2 ± 0.1
	FC	25	8.4 ± 0.8	0.2 ± 0.1	4.1 ± 0.4	3.7 ± 0.5	0.4 ± 0.1
		50	6.9 ± 0.6	0.2 ± 0.1	3.4 ± 1.1	2.8 ± 0.6	0.4 ± 0.1

Values are means ± S. E. M., n = 10 animals/group. Negative control was treated with the vehicle used to dilute the drug (4% DMSO). 5-Fluorouracil (5-FU) was used as positive control.

* $p < 0.05$ compared with the negative control by ANOVA followed by Newman-Keuls test.

Table 3
Antitumor action of a Fraction with Casearins (FC) extracted from *Casearia sylvestris* leaves on human glioblastoma (SF-295) and colon carcinoma (HCT-116) cells determined by the *in vivo* Hollow Fiber Assay (HFA).

Substance	**Dose** (mg/kg/day)	**Administration**	**Survival**	**Proliferation** (absorbance)		**Inhibition rate** (%)	
				SF-295	**HCT-116**	**SF-295**	**HCT-8**
Negative control	–	i. p.	6/6	0.84 ± 0.10	1.02 ± 0.17	–	–
FC	2.5	i. p.	5/6	0.28 ± 0.08	0.48 ± 0.12	33.3	47.4
	5	i. p.	5/6	0.30 ± 0.10	0.50 ± 0.09	36.7	45.4
Negative control	–	oral	6/6	1.12 ± 0.14	1.01 ± 0.15	–	–
5-FU	25	i. p.	6/6	0.52 ± 0.08*	0.59 ± 0.10*	65.4	62.1
FC	25	oral	6/6	0.62 ± 0.17*	0.49 ± 0.11*	44.9	51.9
	50	oral	5/6	0.48 ± 0.10*	0.30 ± 0.06*	56.9	67.4

Values are means ± S. E. M. Negative control was treated with the vehicle used to dilute the drug (4% DMSO). 5-Fluorouracil (5-FU) was used as positive control.

* $p < 0.05$ compared with the negative control by ANOVA followed by Newman-Keuls test.

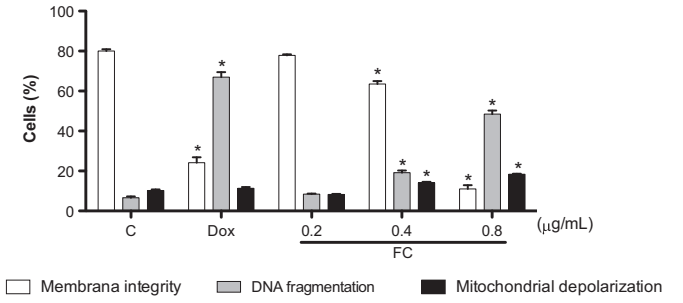

Fig. 5. Effects of the Fraction with Casearins (FC) on *ex vivo* primary cultures of Sarcoma 180 cells after 24 h exposure and analysis by flow cytometry. Cell membrane integrity was evaluated by the exclusion of propidium iodide. DNA fragmentation evaluation was performed by nuclear fluorescence using propidium iodide, triton X-100 and citrate. Mitochondrial transmembrane potential was determined by rhodamine 123 retention. Negative control (C) was treated with the vehicle used for diluting the tested substance. Doxorubicin (0.3 μg/mL) was used as positive control (Dox). Results are expressed as mean ± standard error of measurement (S.E.M.) from two independent experiments.*$p < 0.05$ compared to control by ANOVA followed by Student Newman-Keuls test.

values of 0.60 and 1.61 μg/mL, respectively) (Ferreira et al., 2014). Herein, this cytotoxicity was confirmed by flow cytometry, with reduction of membrane integrity and induction of DNA fragmentation and mitochondrial depolarization. Indeed, prior reports have indicated apoptosis as the main way by which clerodane diterpenes activate cell death and that Cas X causes DNA fragmentation as early as 6 h exposure (Huang et al., 2004; Ferreira et al., 2010, 2014), explaining, partially, the significant values of necrosis after 24 h of treatment, which may represent a secondary necrosis or late apoptosis.

Despite the antitumor oral activity of FC and Cas X, an i.p. action was notoriously superior with both substances on S180 bearing mice. Pharmacokinetic parameters may alter *in vivo* anticancer action when a substance is orally administered, since absorption is governed by several factors such as absorptive surface area, transit time, blood flow and gastric and intestinal pH (absorption barriers), and by the hepatic metabolism, through which all exogenous substances usually undergo biotransformation (first-pass metabolism) (Undevia et al., 2005). It is possible that gastric acid causes acid hydrolysis and, subsequently, opening of the diacetal ring of the clerodane diterpenes and structural changes. In fact, acid hydrolysis and other reactions that open this ring leads to molecular instability and loss of antiproliferative effects, as seen with Cas X dialdehyde and (-)-hardwickiic acid (Ferreira et al., 2010; Santos et al., 2010). So, such findings can explain, at least in part, the reduction or loss of the FC and Cas X antitumor activity when orally administered in S180 bearing mice.

Previously, we have shown that both FC and Cas X are *in vitro* cytotoxic substances on glioblastoma and colon cancer cells (Ferreira et al., 2014). Herein, this cytotoxicity against SF-295 and HCT-116 cells was also seen using the *in vivo* HFA. This technique is a new preclinical tool more suitable from a methodological and financial point of view when compared with xenograft assay. Moreover, it can predict the possibility of chemotherapy success in coming studies with tumor xenografts testing simultaneously two

different body compartments and six tumor lines (Hollingshead et al., 1995; Decker et al., 2004; Amaral et al., 2014; Bezerra et al., 2015). Some studies have shown a direct correlation between activity in HFA and xenograft models, increasing the likelihood of activity in clinical trials, at least for cytotoxic drugs (Johnson et al., 2001). In this method, lower intraperitoneal doses (2.5 and 5 mg/kg/day) were chosen because our previously studies with higher ones (10 and 25 mg/kg/day) were toxic for BALB/c nude animals, since some animals dead during treatment with FC, mainly at 25 mg/kg/day. Lower doses for i.p. treated groups in HFA can explain because such ones were less active in comparison with oral treatment, but these findings showed just a seeming (but not a comparative) antitumor activity since different doses were administered.

Prior investigations with ethanolic extract of *C. sylvestris* leaves at a dose of 100 mg/kg/day i.p. showed a potent antitumor activity in mice transplanted with S180 cells and tumor growth inhibition ranging from 87% to 98% (Itokawa et al., 1990). Bioguided studies of Itokawa et al. (1990) resulted in the purification of six new clerodane diterpenes (Casearin A, B, C, D, E and F) and showed casearin C as the most active molecule. Herein, such bioactivity was similar in this study, since both substances (FC and Cas X) intraperitoneally administered also presented inhibiting rates of tumor growth ranging from 86.2 to 90.0%. Almeida (2000) also demonstrated that the alcoholic extract of *C. sylvestris* leaves inhibited Ehrlich tumor growth and presented anti-inflammatory activity at a dose of 300 mg/kg/day. Similarly, two gallic acid-derived compounds isolated from *C. sylvestris* leaves – isobutyl gallate-3,5-dimethyl ether (IGDE) and methyl gallate-3,5-dimethyl ether (MGDE) – showed significant chemotherapeutic potential against Ehrlich and Lewis lung cancer ascite tumor cells and increased animals' survival (approximately 90%), though IGDE possesses a slightly superior activity when compared to MGDE (Da Silva et al., 2009). More recently, Felipe et al. (2014) showed that treatment with crude aqueous ethanolic extract (CAE) and chloroform fraction ((f-CHCl3)) of *C. sylvestris* leaves caused tumor growth decreasing in Ehrlich ascites-bearing mice (40% and 60%, respectively), whose *in vivo* antiproliferative effects were confirmed by ^{3}H-thymidine uptake and trypan blue exclusion methods.

Toxicity analyzes of key organs indicated liver and kidneys as main targets for FC and Cas X, especially in groups i.p. treated. Similar results were described by Araújo et al. (2015) about hepatic changes, since vascular congestion and hydropic degeneration were apparent in FC groups (2.5, 5 and 25 mg/kg/day) and slight perivascular infiltration associated with necrosis was detected with FC 2.5 mg/kg/day. Kidney and liver are proposed as major target organs for environmental contaminants, drugs and xenobiotics, since these substances usually have a low solubility in aqueous systems and require biotransformation in hepatocytes to metabolites that are more hydrophilic and more readily eliminated in the urine. Biotransformation reactions generally follow a detoxification process rendering metabolites inactive. Nevertheless, many drug intermediary products generated during metabolism are highly reactive and toxic, causing hepatotoxicity and nefrotoxicity (Williams et al., 2002; Ramaiah, 2007). However, liver and kidneys also possess a pronounced regenerative capacity and compensatory action: even when necrosis is found with conjunctive tissue preservation, there is often complete tissue restoration (Kumar et al., 2004). In this context, all observed morphological alterations in treated animals could be considered potentially reversible. Moreover, *in vivo* blood changes in neutrophils and lymphocytes were slighter in comparison with 5-FU-treated animals, which demonstrated decreasing in monocytes, neutrophils and lymphocytes. Unfortunately, anticancer drugs in clinical use usually cause hematologic and immunologic suppression, as observed with docetaxel (Ringel and Horwitz, 1991), methotrexate (Katzung et al., 2003) and 5-FU (Bezerra et al., 2008). Most of them are available for intravenous administration only, and this kind of treatment is very expensive due to hospitalization costs. Therefore, the development of new and effective oral cytotoxic agents has great social and economic interest (Ismael et al., 2008), but a substance with oral antitumoral capacity, as FC or Cas X, is uncommon primarily because the first pass effects (Undevia et al., 2005).

Despite toxicity findings, ethnopharmacological uses have encouraged the consumption of different parts of *C. sylvestris* because folk data and toxicological studies did not indicate unsafe clinical evidences for humans (Silva et al., 1988; Basile et al., 1990; Ferreira et al., 2011a; Araújo et al., 2014). However, Araújo et al. (2015) verified that FC-treated animals exhibited some alterations indicative of neurotoxicity and recommend precaution regarding the consumption of medicinal formulations based on *C. sylvestris.*

5. Conclusions

A fraction with casearins extracted from *C. sylvestris* leaves and Casearin X were efficient *in vivo* antitumor substances against murine cells and human glioblastoma and colon carcinoma neoplasms by intraperitoneal as well as oral routes, caused reversible morphological changes in the liver, kidneys and spleens. Additionally, *ex vivo* studies indicated apoptosis as the main way by which cell death is triggered. Taken together, these findings corroborate folk uses of *C. sylvestris* against different types of cancers and emphasize the reputation of clerodane diterpenes as an emerging class of anticancer molecules.

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