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In vitro and *in vivo* antiproliferative activity of *Calotropis procera* stem extracts

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ABSTRACT

The cytotoxic potential of stem organic extracts from *Calotropis procera* (Asclepiadaceae) was firstly evaluated against cancer cell lines by MTT assay. Subsequently, samples considered cytotoxic were tested for antimitotic activity on sea urchin egg development and for *in vivo* antiproliferative activity in mice bearing Sarcoma 180 tumor. Among the five extracts (hexane, dichloromethane, ethyl acetate, acetone and methanol), ethyl acetate and acetone extracts displayed higher cytotoxic potential against tumor cells, with IC_{50} ranging from 0.8 to 4.4 μg/mL, while methanolic extract was weakly cytotoxic. Cytotoxic extracts also exhibited cell division inhibition capacity by antimitotic assay, revealing IC_{50} values lower than 5 μg/mL. In the *in vivo* antitumor assessments, ethyl acetate- and acetone-treated animals showed tumor growth inhibition ratios of 64.3 and 53.1%, respectively, with reversible toxic effects on liver and kidneys. Further studies are in progress in order to identify *C. procera* cytotoxic compound(s) and to understand the mechanism of action responsible for this tumor-decreasing potential.

Key words: antimitotic, antiproliferative, *Calotropis procera*, Sarcoma 180 tumor, stem extracts.

INTRODUCTION

Calotropis procera Aiton, 1811 (Gentianales: Asclepiadaceae) is a perene Asian shrub called "Ushar", being very common in adverse climate conditions and poor soils explaining its good adaptation to the Northeast Brazil, where it was introduced at the beginning of the century XIX, spreading within different biomes such as "Caatinga" and "Cerrado" (Kissmann and Groth 1999, Lorenzi and Matos 2002).

In Brazil, *C. procera* is popularly known as "algodão de seda", "leiteiro", "queimadeira" and "ciúme" (Kissmann and Groth 1999, Lorenzi and Matos 2002). Different parts of this tree have been used to treat and prevent diseases. In India, the leaves are prepared to ease pain from a variety of conditions (Satyavati et al. 1976). They possess anthelmintic (Iqbal et al. 2005), analgesic, antipyretic, antispasmodic (Mascolo et al. 1988, Dewan et al. 2000) and anti-inflammatory (Kumar and Basu 1994) properties. It is also notorious their antimalaric activity against *Plasmodium falciparum* (Sharma and Sharma 2000) and hepatoprotective action by the glutathione reduced decreasing prevention, an effect proba-

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bly reached due to flavonoids with antioxidant potentiality similar to vitamin C (Ahmed et al. 2003, Setty et al. 2007, Ferreira et al. 2008).

The latex found in the aerial parts (young leaves, predominantly) is utilized to treat diarrhea (Satyavati et al. 1976), possibly due to its desensitization of the gastrointestinal smooth muscle cells (Kumar and Shivkar 2004) and antimicrobial action (Jain et al. 1996). Moreover, latex possess biological activity against the coccidian protozoa *Eimeria ovinoidalis* (Mahmoud et al. 2001) and *Aedes aegypti* larvae (Ramos et al. 2006), protection against hyperglycemia induced by alloxan (Roy et al. 2005) and anti-inflammatory (Alencar et al. 2004) effects. Root methanolic extracts simulates oestrogenic actions, altering uterine endometrial and interfering on the blastocyst implantation (Kamath and Rana 2002), while stem bark extracts reduces bronchial inflammation, showing antitussigene activity by oral administration (Dieye et al. 1993). In this work, the great pharmacological potential of *C. procera* was evaluated aiming to study the *in vitro* cytotoxicity of stem extracts and their *in vivo* antitumor capacity on mice transplanted with Sarcoma 180 cells.

MATERIALS AND METHODS

Plant Material and Extract Preparation

Calotropis procera samples were collected in Sobral, Ceará, Brazil. A voucher specimen (34.706) was authenticated by Dr. Edson de Paula Nunes and deposited at Prisco Bezerra Herbarium (EAC), Department of Biology of Universidade Federal do Ceará, Brazil.

Stem samples (1000.0 g) were firstly grounded to a fine powder, weighed and subjected to successive extractions (4×) in 100% ethanol with a sample mass to solvent volume proportion of 1:5 at room temperature (25°C). Then, the material was concentrated under pressure until turn out a viscous residue. Afterwards, the ethanolic extract was partitioned using growing polarity organic solvents: hexane, dichloromethane, ethyl acetate, acetone and methanol. Each extraction procedure was performed under shaking for 24 h. The extracts were concentrated in a rotary vacuum evaporator and dissolved in sterile pure dimethylsulfoxide (DMSO, Sigma Aldrich) to stock solutions of 50 mg/mL.

Animals

Adult Swiss mice (*Mus musculus* Linnaeus, 1758) were obtained from the animal facilities of Universidade Federal do Ceará. They were kept in cages (ALESCO, São Paulo) under standard conditions of light (12 h with alternative day and night cycles) and temperature (25 ± 1°C), and were housed with access to commercial rodent stock diet (Purina, São Paulo, Brazil) and water *ad libitum*. The investigational protocol was approved by the local Ethical Committee in Animal Research at Universidade Federal do Ceará (Process No. 102/2007), and is in accordance with International Standard on the care and use of experimental animals (EEC Directive of 1986, 86/609/EEC).

Adult sea urchins of the *Lytechinus variegates* Lamarck, 1816 (Echinoidea: Toxopneustidae) species were collected at Pecém beach, northeastern coast of Ceará, Brazil. The urchins were maintained under standard laboratory conditions until the beginning of the experiments.

MTT Assay

The cytotoxicity against the tumor cell lines HL-60, CEM (human leukemia), HCT-8 (human colon cancer) and B-16/F10 (murine melanoma) was determined by MTT assay (Mosmann 1983), which analyzes the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to a purple formazan product. All lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penincillin and 100 μg/mL streptomycin, at 37°C with 5% CO_2. Briefly, cells were plated in 96 well plates (0.7×10^5 cells/well for adherent cells and 0.3×10^5 cells/well for suspended cells) and incubated to allow cell adhesion. Twenty-four hours later, stem extracts (hexane, dichloromethane, ethyl acetate, acetone and methanol) were added to each well (0.39–25 μg/mL). After 72 h of incubation, the supernatant was replaced by fresh medium containing 10% MTT. The formazan product was dissolved in DMSO to measure absorbance at 595 nm (DTX-880, Beckman Coulter). Doxorubicin (Sigma Aldrich) was used as positive control (0.009–5 μg/mL).

Antimitotic Assay

The antimitotic assay was performed in 24-well plates according with Moreira et al. (2007). Gamete elimination from *L. variegatus* was induced by injecting 3.0 mL of 0.5 M KCl into the urchin's coelomic cavity via the periostomial membrane. Concentrated sperm was collected with a Pasteur pipette and preserved under low temperature until the fertilization. Each well received 1 mL of fertilized egg suspension and extracts (ethyl acetate, acetone and methanol) were added immediately after fertilization (within 2 min) (0.1, 1, 5 and 10 μg/mL). Doxorubicin (0.3 μg/mL) and 1.6% DMSO were used as positive and negative controls, respectively. Plates were then shaken in a constant temperature water bath at 26 ± 2°C. At appropriate times, aliquots of 100 μL were fixed with 10% formaldehyde to obtain first and third cleavages and blastulae. One hundred eggs or embryos per well were counted in order to calculate IC_{50} values.

In vivo Antitumor Evaluation

Fifty healthy male mice (*M. musculus*) weighing 23–26 g were subcutaneously implanted with nine-day-old Sarcoma 180 ascites tumor cells (2×10^6 cells/0.5 mL) into the left hind groin of the mice. On the next day, they were randomly separated into five groups ($n = 10$ each) to receive stem extracts (ethyl acetate, acetone and methanol dissolved in 0.9% saline) at the dose of 250 mg/kg/day. In contrast, negative and positive controls received saline and 5-FU (50 mg/kg/day), respectively, all administered intraperitoneally for 7 days.

On day 8, mice were sacrificed by cervical dislocation and their organs (kidneys, spleens and livers) and tumors were dissected out, grossly examined for size, color changes and hemorrhage, weighed and preserved in 10% formaldehyde solution. The inhibition ratio of tumor growth (%) was calculated by the following formula: inhibition ratio (%) $= [(A - B)/A] \times 100$, where A is the average tumor weight of the negative control, and B is the tumor weight of the treated group. To examine morphological changes by light microscopy (Olympus, Tokyo, Japan), small pieces of organs and tumors were processed, embedded in paraffin and 3-5 μm thick sections were prepared and stained with hematoxylin-eosin.

Statistical Analysis

For cytotoxicity assays, the IC_{50} values and their 95% confidence intervals were obtained by nonlinear regression using the Graphpad program (Intuitive Software for Science, San Diego, CA). In order to determine differences among the treatments, data (mean $\pm$ standard error mean) were compared by one-way analysis of variance (ANOVA) followed by Newman-Keuls test ($P < 0.01$).

RESULTS AND DISCUSSION

Researches for antineoplasic compounds have demonstrated the great pharmacological relevance of the plant extracts (Cragg and Newman 2005, Costa et al. 2008, Buriol et al. 2009). At the last decades, *C. procera* received special attention, with lots of publications describing the biological activities of molecules and aqueous and organic extracts obtained from its distinct tissues (Jain et al. 1996, Kamath and Rana 2002, Ahmed et al. 2003, Iqbal et al. 2005, Ramos et al. 2006, Setty et al. 2007).

In the present work, we firstly determined the cytotoxic activity of organic extracts from *C. procera* stem using the MTT assay. According to the American National Cancer Institute, the IC_{50} limit to consider a promising crude extract for further purification is lower than 30 μg/mL (Suffness and Pezzuto 1990). Among the five extracts, ethyl acetate and acetone showed higher cytotoxic potential against tumor cells, with IC_{50} ranging from 0.8 to 4.4 μg/mL for colon (HCT-8) and melanoma (B-16) cells, respectively (Table I). Methanolic extract was weakly cytotoxic, despite the fact that it demonstrate moderately good activity on CEM line [IC_{50} value of 2.8 (2.1–4.1)μg/mL].

Previously, some reports showed that different parts of the plant exhibit cytotoxicity on cancer cells (Smit et al. 1995, Van Quaquebeke et al. 2005, Oliveira et al. 2007). Van Quaquebeke et al. (2005) isolated a natural cardiotonic steroid from the methanolic extract of *C. procera* root barks called 2′′-oxovoruscharin and developed a new hemisynthetic cardenolide derivative named UNBS1450 which display *in vitro* antiproliferative action comparable to taxol, while the latex shows cytotoxicity corroborated by morphological alterations in leukemia cells, such as chromatin condensation, DNA

TABLE I
Cytotoxic activity of extracts obtained from *Calotropis procera* stem on tumor cell lines after 72 h of exposure.

Substance	Yield (%)	Cell line IC_{50} (μg/mL)*			
		HL-60	CEM	B-16/F10	HCT-8
Hexane	5.3	> 25	> 25	> 25	> 25
Dichloromethane	22.7	> 25	> 25	> 25	> 25
Ethyl acetate	11.6	1.6	1.4	2.0	2.5
		1.4–1.9	1.1–3.8	1.0–3.9	2.3–2.6
Acetone	10.4	2.1	1.4	4.4	0.8
		2.1–2.2	1.3–2.8	2.1–9.0	0.6–1.0
Methanol	50.0	8.2	2.8	> 25	10.2
		5.4–12.4	2.1–4.1		7.2–14.2
Doxorubicin	—	0.02	0.02	0.002	0.01
		0.01–0.02	0.02–0.03	0.001–0.003	0.01–0.02

*Data are presented as IC_{50} values and 95% confidence intervals for human leukemia (HL-60, CEM), murine melanoma (B-16/F10), and human colon cancer (HCT-8) cells. Experiments were performed in triplicate.

fragmentation and cell volume reduction (Choedon et al. 2006, Oliveira et al. 2007). It is established that cardiotonic steroid glycosides (bufalin and digoxin, for instance) are capable to kill cancer cells through the activation of apoptotic pathways (McConkey et al. 2000, Kurosawa et al. 2001). On the other hand, additional and recent investigations propose autophagy as a probable kind of cell death caused by UNBS1450 in human glioblastoma lines (Lefranc et al. 2008). Autophagy is a singular self-destructive process in which injured, unnecessary or old parts of the cells, as mitochondria and endoplasmic reticulum, are degraded by enzymatic activity within lysossomes (Maiuri et al. 2007).

Sea urchin egg development shows some peculiarities that allows us to suggest how antimitotic substances act. Acetone, ethyl acetate and methanol extracts inhibited the division of sea urchin eggs since the first cleavage in a concentration-dependent way, revealing IC_{50} values lower than 5 μg/mL (Table II). The inhibition at the first cleavage is related to DNA and/or protein synthesis or microtubule assembly, given that RNA synthesis is very slow or absent after fertilization. At this time, the rapid increasing in the rate of protein synthesis is largely due to the recruitment of maternal mRNA into polysomes (Brandhorst 1985). On the other hand, when a substance blocks microtubule assembly, clear spots corresponding to nucleus duplication can be observed in the cytoplasm. Since zygotes treated with the extracts exhibited homogeneous cytoplasm, this process appears not to have been affected (Jacobs and Wilson 1986). So, the stem organic extracts acetone, ethyl acetate and methanol may affect DNA and/or protein synthesis, confirming their *in vitro* antiproliferative activity showed by MTT assay.

TABLE II
Inhibition of cell division of *Calotropis procera* stem extracts on embryos of the sea urchin *Lytechinus variegatus* on the first and third cleavage and blastulae stages.

Substance	IC_{50} (μg/mL)* First cleavage	Third cleavage	Blastulae
Ethyl acetate	3.2	2.0	1.1
	2.7–3.7	1.5–2.7	1.1–1.2
Acetone	3.7	2.7	3.5
	2.5–5.5	1.2–3.6	1.5–8.0
Methanol	4.1	3.7	4.7
	3.7–4.4	3.3–4.2	4.1–5.5
Doxorubicin	6.3	0.3	0.5
	4.3–9.1	0.2–0.7	0.3–1.0

*Data are presented as IC_{50} values and 95% confidence intervals for sea urchin eggs. Doxorubicin was used as positive control. Experiments were performed in duplicate.

TABLE III
Effects of the *Calotropis procera* stem extracts on mice transplanted with Sarcoma 180 cells.

Group	Dose (mg/kg/day)	Animal weight (g)	Liver	Kidneys	Spleen	Tumor (g)	Tumor inhibition (%)
			g/100g body weight				
Control	—	30.00 ± 0.50	5.94 ± 0.21	1.43 ± 0.07	0.69 ± 0.05	3.25 ± 0.47	—
5-FU	50	24.60 ± 0.40^{a}	4.86 ± 0.27	1.28 ± 0.03	0.22 ± 0.01^{a}	0.11 ± 0.05^{a}	96.5
Ethyl acetate	250	29.00 ± 0.94	4.93 ± 0.09	1.38 ± 0.02	0.46 ± 0.03	1.40 ± 0.35^{a}	64.3
Acetone	250	29.38 ± 0.82	5.20 ± 0.16	1.50 ± 0.06	0.49 ± 0.03	1.34 ± 0.22^{a}	53.1
Methanol	250	31.43 ± 0.92	5.94 ± 0.26	1.39 ± 0.05	0.52 ± 0.08	3.26 ± 0.52	3.22

*Data are means ± S.E.M., n = 10 animals/group, treated for seven days by intraperitoneal route. Positive and negative controls were treated by 5-Flouoruracil (5-FU) and saline 0.9%, respectively. $^{a}P < 0.01$ compared to control by ANOVA followed by Newman-Keuls test.

Herein, we also reported the antitumor activity of organic extracts (ethyl acetate, acetone and methanol) obtained from *C. procera* stem in mice bearing Sarcoma 180 tumor. Sarcoma 180 is a mouse-derived tumor very exploited in antitumor research *in vivo* (Sato et al. 2005, Magalhães et al. 2006). The effects of the ethyl acetate, acetone and methanol extracts on tumor growth are described in Table III. A significant reduction in tumor weight ($P < 0.01$) was found at 250 mg/kg/day in both ethyl acetate- and acetone-treated animals (1.40 ± 0.35 g and 1.34 ± 0.22 g, respectively) in comparison with negative control (3.25 ± 0.47 g), leading to tumor growth inhibition ratios of 64.3 and 53.1%, respectively. The dose of 50 mg/kg/day reduced tumor weight in 96.5% in 5-FU-treated mice. In contrast, methanol extract was unable to avoid tumor augmentation within identical experimental conditions.

The mice intraperitoneal treatment with the extracts was not able to interfere on the final body weight and in relative liver, kidneys and spleen weights of the experimental groups (Table III) when compared to negative control ($P > 0.05$). Moreover, neither mortality nor morbidity were recorded during the whole experiment.

Tumor morphological examination of the control group showed large and polygonal cells, with pleomorphic shapes, hyperchromatic nuclei, binucleation, mitosis and muscle invasion (Fig. 1A). Meanwhile, tumors excised from mice treated with 5-FU, ethyl acetate and acetone extracts exhibited extensive areas of coagulative necrosis alternated with pleormorphic cells (Figs. 1B, 1C and 1D).

Hepatic histopathological analysis of ethyl acetate-, acetone- and methanol-treated animals revealed Kupffer cell hyperplasia, ballooning degeneration of hepatocytes, portal tract and centrolobular venous congestion and discrete microvesicular steatosis, though no areas of necrosis were observed (Figs. 2C, 2D and 2E). Focal infiltrate of inflammatory cells was more evident in the methanol group. Regarding kidneys, it was observed glomerular and tubular hemorrhage and hydropic changes of the proximal tubular ephitelium in ethyl acetate- and acetone-treated groups (Figs. 3C and 3D, respectively), but the glomerular structure was essentially preserved. On the other hand, methanolic group did not present kidney alterations while 5-FU group presented hyaline cylinders (Fig. 3B). There was a considerable hyperplasia of the splenic white pulp and megakaryocytes in all treated groups in comparison to the negative control (data not shown), a result possibly caused by substances found in the extracts. In fact, ethanolic extracts of flowers, buds and roots of *C. procera* contain alkaloids, phenolic compounds/tannins, saponins and lectins, which may have immunomodulatory properties (Imboden 1988, Mossa et al. 1991, Melo et al. 2001, Ferreira et al. 2007, 2009).

The kidney and liver have been proposed as the major key organs to metabolize environmental toxic substances. Due to the good liver regeneration capacity, even when necrosis is found with conjunctive tissue preservation, generally there was a complete hepatic restoration (Kumar et al. 2004). Portal tract and centrolobular venous congestion were also visualized in the control group, suggesting that these effects are related to the hepatocyte metabolism (Scheuer and Lefkowitch 2000). Meanwhile, Kupffer cell hyperplasia and macrovesicular steatosis indicate a presence of a harmful agent

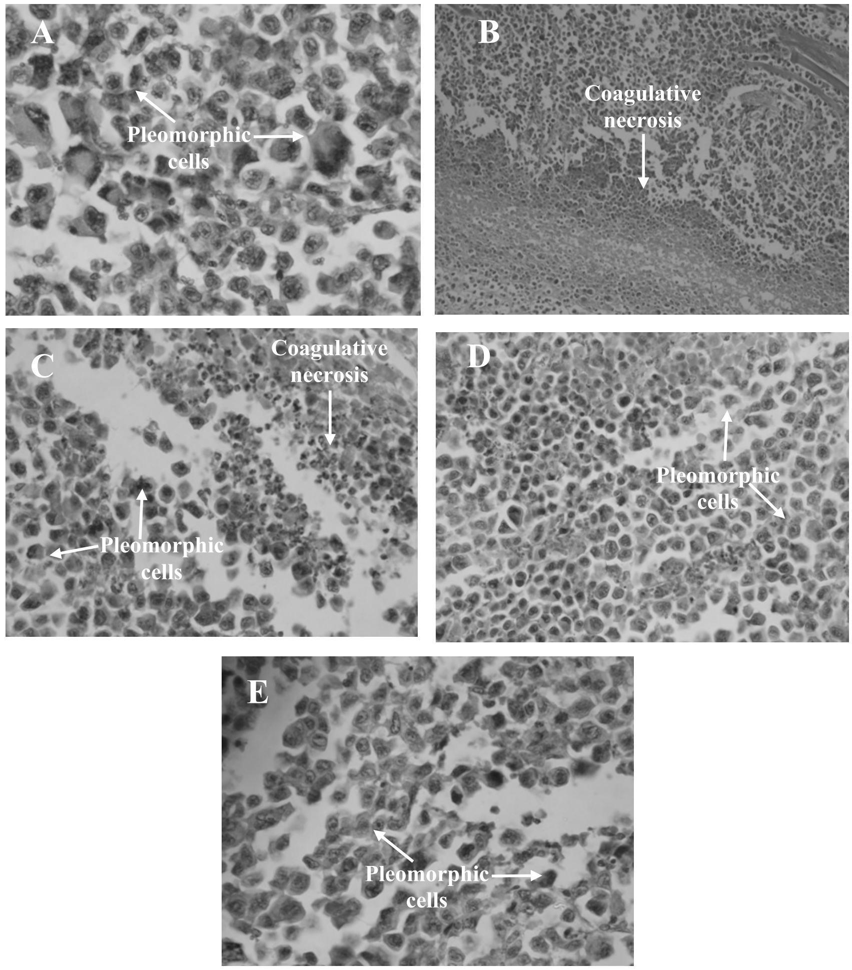

Fig. 1 – Histopathology of tumors excised from Sarcoma 180 transplanted mice after 7 days of intraperitoneal treatment with *Calotropis procera* stem extracts at the dose of 250 mg/kg/day (C – ethyl acetate; D – acetone; E – methanol). Negative control (A) and positive control (B) received 0.9% saline and 5-FU (50 mg/kg/day), respectively. Magnification, 400×.

(Kumar et al. 2004), as seen in 5-FU-treated mice. Although all alterations observed in liver and kidney-treated animals were considered reversible, the kidneys should be the target organ of the ethyl acetate and acetone extracts. Nevertheless, the reversible character of injuries proposes that treatment removal leads to quick improvement (Scheuer and Lefkowitch 2000). Melo et al. (2001) showed that goats subcronically fed with pulverized dehydrated aerial parts of *C. procera* (leaves and twigs) did not undergo suggestive biochemical modifications of liver damage, which could explain the light hepatotoxicity in the face of the dose administered (250 mg/kg). Safety evaluation studies revealed that ethanolic extracts of aerial parts in single high doses (up to 3 g/kg) do not produce any visible toxic symptoms or mortality, while extended treatment (90 days) causes significantly higher toxicity (Mossa et al. 1991).

The present work shows that ethyl acetate, acetone and methanol stem extracts from *C. procera* possess promising *in vitro* antiproliferative activity on cancer lines and sea urchin eggs. Meanwhile, ethyl acetate and acetone extracts are able to reduce *in vivo* tumor growth of Sarcoma 180 transplanted mice in the presence of liver and kidneys reversible toxic effects. Some investigations are in progress in order to identify *C. procera* cytotoxic compound(s) and to understand the mechanism of action responsible for this tumor-decreasing potential.

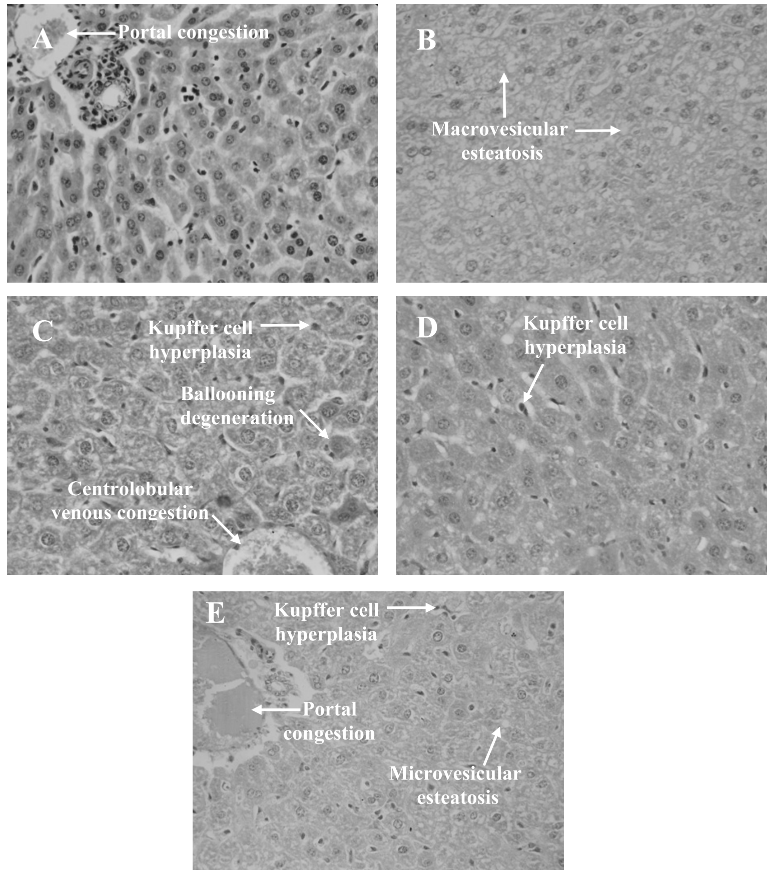

Fig. 2 – Histopathology of livers excised from Sarcoma 180 transplanted mice after 7 days of intraperitoneal treatment with *Calotropis procera* stem extracts at the dose of 250 mg/kg/day (C – ethyl acetate; D – acetone; E – methanol). Negative control (A) and positive control (B) received 0.9% saline and 5-FU (50 mg/kg/day), respectively. Magnification, 400×.

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RESUMO

O potencial citotóxico de extratos orgânicos do caule de *Calotropis procera* (Asclepiadaceae) foi primeiramente avaliado frente a linhagens de células tumorais através do ensaio de MTT. Aquelas amostras consideradas citotóxicas foram subsequentemente testadas para atividade antimitótica sobre o desenvolvimento de ovos de ouriço-do-mar e para atividade antiproliferativa *in vivo* em camundongos transplantados com tumor Sarcoma 180. Dentre os cinco extratos estudados (hexano, diclorometano, acetato de etila, acetona e metanol), os extratos acetato de etila e acetona mostraram maior potencial citotóxico contra células tumorais, com CI_{50} variando de 0,8 to 4,4 μg/mL, enquanto o extrato metanólico revelou ser fracamente citotóxico. Os extratos citotóxicos também exibiram capacidade de inibição da divisão celular com valores de CI_{50} menores que 5 μg/mL. Nas avaliações antitumorais *in vivo*, os animais tratados com os extratos acetato de etila e acetona mostraram taxas de inibição do crescimento tumoral de 64,3 e 53,1%, respectivamente, com efeitos tóxicos reversíveis sobre o fígado e os rins.

Palavras-chave: atividade antimitótica, antiproliferativa, *Calotropis procera*, Sarcoma 180, extratos de caule.

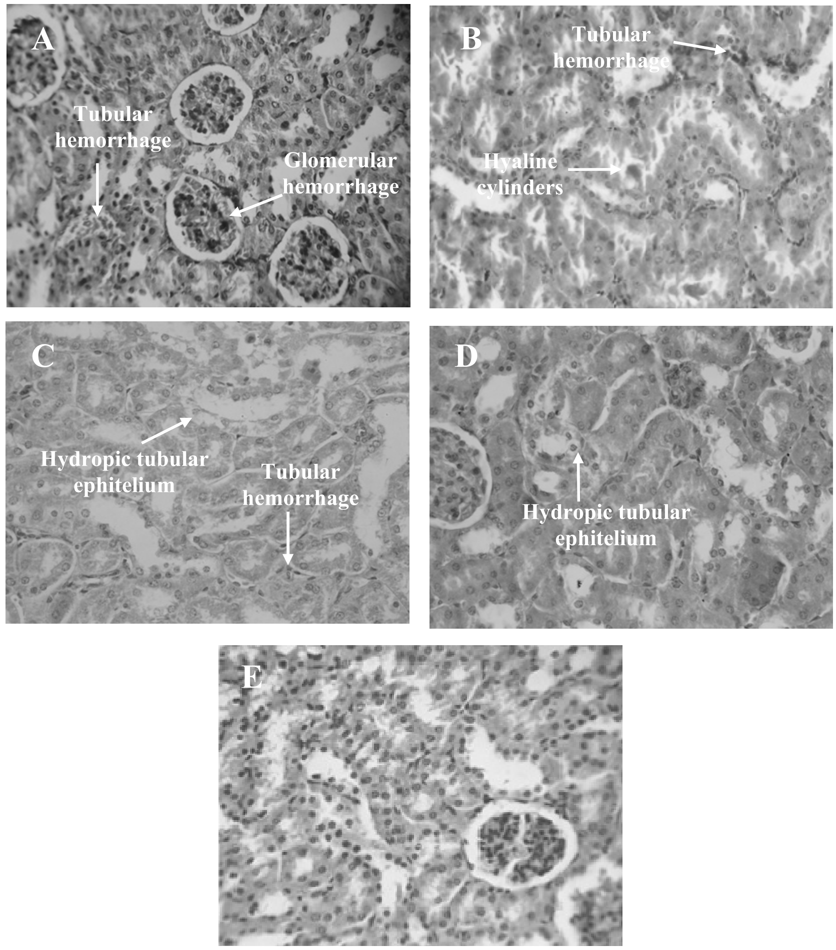

Fig. 3 – Histopathology of kidneys excised from Sarcoma 180 transplanted mice after 7 days of intraperitoneal treatment with *Calotropis procera* stem extracts at the dose of 250 mg/kg/day (C – ethyl acetate; D – acetone; E – methanol). Negative control (A) and positive control (B) received 0.9% saline and 5-FU (50 mg/kg/day), respectively. Magnification, 400×.

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