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Original Article

Antitumor activity and toxicity of volatile oil from the leaves of *Annona leptopetala*

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ABSTRACT

Annona leptopetala (R.E.Fr.) H. Rainer, Annonaceae, is used in folk medicine like antitumor and anti-inflammatory. The aim of this study was to determine chemical composition, toxicity and antitumor activity of *A. leptopetala* leaves volatile oil. Fresh leaves were hydrodistilled and then the volatile oil chemical composition was assessed by gas chromatography and mass spectrometry. Toxicity was assessed using haemolysis, micronucleus and acute toxicity protocols. Antitumor effects were determined *in vitro* and *in vivo*, using sulforhodamine B assay and sarcoma 180 murine tumor model, respectively. Spathulenol was the major component identified (12.56%). The volatile oil showed *in vitro* antitumor activity mainly in leukemia cell line (K-562), with Total growth inhibit (TGI) (concentration producing TGI) of 0.64 μg/ml. In other hand, the volatile oil <250 μg/ml did not inhibit HaCat non-tumor cell line growth. The concentration that produced 50% haemolysis was 372.8 μg/ml. The 50% lethal dose in mice was approximately 447.2 mg/kg intraperitoneally. Sarcoma 180 tumor growth inhibition rates were 59.29% and 58.77% at 100 and 150 mg/kg intraperitoneally, respectively. The volatile oil presented moderate gastrointestinal toxicity and no genotoxicity was observed at 350 mg/kg. Thus, the volatile oil shows antitumor activity with moderate toxicity.

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Introduction

Annona leptopetala (R.E.Fr) H. Rainer, Annonaceae, commonly known as "pinha-brava" is a tree or shrub endemic in Brazil used in folk medicine like antitumor and anti-inflammatory (Agra et al., 2007; David et al., 2007). Antioxidant and *in vivo* antitumor activities for extracts from *A. leptopetala* have been reported (David et al., 2007; Costa et al., 2012), in addition to antispasmodic effect in guinea pig ileum (Monteiro et al., 2008).

Different compounds has been obtained from *Annona* genus, such as flavonols (Júnior et al., 2016; Novaes et al., 2018), terpenes (Santana et al., 2017), tannins, saponins, cardiac glycosides, monosaccharides, aromatic and phenolic amino acids, steroids (Agu and Okolie, 2017), isoquinolonic and indolic alkaloids (Kuo et al., 2001), lignoids (Fevier et al., 1999) and acetogenins (Mangal et al., 2016). Regarding volatile oils from *Annona* species, monoterpenes and sesquiterpenes were isolated, including β-elemene (Kossouoh et al., 2007), bicyclogermacrene (Siqueira et al., 2011), α-copaene (Costa et al., 2013) and α-phellandrene (Meira et al., 2014). In addition, literature data showed antitumor activity for other components of *Annona* volatile oil, such as α-terpineol (Hassan et al., 2010), spathulenol (Bomfim et al., 2016), *trans*-caryophyllene (Hadri et al., 2010) and germacrene-D (Salvador et al., 2011),

Considering that the theory of synergistic action associated with the antitumor activity of volatile oils appears to be rather than its components separately (Bhalla et al., 2013), this study determined the chemical composition, antitumor activity and toxicity of the volatile oil from *A. leptopetala* leaves (ALO).

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Materials and methods

Drugs and reagents

RPMI 1640 culture medium, glutamine, penicillin, streptomycin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and fetal bovine serum (FBS) were obtained from GIBCO (Carlsbad, CA). 5-Fluorouracil (5-FU), Triton X-100, Tween-80, cyclophosphamide, Trizma base and sulforhodamine (SRB) were purchased from Sigma–Aldrich (St. Louis, MO). Dimethylsulfoxide (DMSO) was purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). Kits for biochemical analysis were purchased from LABTEST (Lagoa Santa, MG, Brazil). Sodium thiopental (Thiopentax®) was purchased from Cristália (Itapira, SP, Brazil) and heparin (Parinex®) from Hipolabor (Sabara, MG, Brazil). Doxorubicin (DOX) was from Tecnofarma International (Uruguay) and trichloroacetic acid (TCA) from Merck (Darnstadt, Germany).

Plant material

Annona leptopetala (R.E.Fr.) H. Rainer, Annonaceae, leaves were collected in August 2016 in Serra Branca, Paraíba State, Brazil. The plant material was identified by Dr. Maria de Fátima Agra. Voucher specimen number AGRA 3567 was deposited at the Herbarium Lauro Pires Xavier of Federal University of Paraíba (UFPB), Brazil.

Hydrodistillation of the volatile oil

Fresh leaves (1000 g) were collected over ice and hydro-distilled using Clevenger type apparatus for 4 h, at a temperature of 40 °C, yielding 400 mg of the volatile oil (yield of 0.04% relative to the weight of fresh material used). The resulting oil was dried with anhydrous sodium sulfate, stored in amber bottle and kept at 4 °C lower temperature. Thereafter, ALO was submitted for Gas Chromatography with Mass Spectrometry (GC-MS) analysis.

GC-MS analysis

Analysis of the oil was carried out on a Shimadzu GC-MS instrument under the following conditions: DB-5 ms (30 m × 0.25 mm internal diameter, film thickness 0.25 μm), fused-silica capillary column, programmed temperature of 60–240 °C (3 °C/min), injector temperature at 220 °C, helium carrier gas adjusted to a linear velocity of 32 cm/s (measured at 100 °C), splitless injection (2 μl of hexane solution 1:1000), split flow adjusted to yield a 20:1 ratio, septum sweep constant at 10 ml/min, Electron Ionization Mass Spectrometry (EIMS) electron energy of 70 eV, ion source and connections at 200 °C. The quantitative data for the volatile constituents were obtained by peak-area normalization using a Focus Gas Chromatography with Flame Ionization Detector (GC/FID), operated under GC-MS similar conditions except for the carrier gas, which was nitrogen. The retention index was calculated for all the volatile constituents by volatile oil co-injection using an *n*-alkane (C8-C20, Sigma–Aldrich) homologous series applying the equation of Van den Dool and Kratz (1963). Individual components were identified by comparison of both mass spectrum and Gas Chromatography (GC) retention data with previously analyzed authentic compounds stored in our private library, as well as with the aid of commercial libraries containing mass spectra, and retention indices of volatile compounds commonly found in volatile oils (Adams, 2001).

Cell lines

The tumor cell lines used were: U251 – glioma, MCF-7 – breast, NCI/ADR-RES - multidrug-resistant ovarian, 786-0 – kidney, NCI-H460 – non-small cell lung cancer, PC-3 – prostate, OVCAR – ovarian, HT29 – colon and K562 – leukemia, and HaCaT human keratinocytes served as the normal cell line. The cells lines were cultivated in RPMI-1640 supplemented with FBS 10%, glutamine 2 mM, penicillin 100 U/ml, streptomycin 100 μg/ml and HEPES 2 mM, at 37 °C with CO_2 5%, in the Chemical, Biological and Agricultural Pluridisciplinary Research Center, State University of Campinas, Campinas, Brazil. Sarcoma 180 tumor cells were maintained in the peritoneal cavity of Swiss mice.

Animals

Swiss albino mice (*Mus musculus*), females (36–42 g), were obtained from the Dr. Thomas George Bioterium of Research Institute in Drugs and Medicines of Federal University of Paraíba, Brazil. The animals were randomly housed in cages containing six animals with free access to food and water. All animals were kept on a 12 h/12 h off light-dark cycle (lights on at 6 am). All procedures were previously approved by the Animal Studies Committee (CEUA) of UFPB, n°. 0912/10.

Haemolysis assay

The haemolytic activity of ALO was tested using mouse erythrocytes according to Kang et al. (2009). Erythrocytes from fresh blood samples were suspended in phosphate buffered saline (PBS) to make a 1% (v/v) solution. Red blood cell suspension was incubated with various concentrations (0–750 μg/ml) of ALO dissolved in DMSO (5%, v/v, in PBS) in plates on a shaker for 60 min and then centrifuged. The absorbance of the supernatants was read at 540 nm using a UV–vis spectrophotometer (UV-1650PC Shimadzu) to measure the extent of red blood cell (RBC) lysis, and the concentration producing 50% haemolysis (HC_{50}) was determined. Positive controls (100% haemolysis) and negative controls (0% haemolysis) were also determined by incubating erythrocytes using Triton X-100 1% in PBS and DMSO 5% in PBS, respectively. The haemolysis assay was performed in quadruplicate and repeated three times.

In vitro *antitumor activity*

The sulforhodamine B assay (SRB) was performed as described by Monks et al. (1991). This assay relies on the ability of SRB to bind to protein components of cells that have been fixed to tissue-culture plates by TCA. SRB is a bright-pink aminoxanthene dye with two sulphonic groups that bind to basic amino acid residues under acidic conditions and dissociate under basic conditions.

Cells in 96-well plates (100 μl cells/well) were exposed to different concentrations of ALO (0.25, 2.5, 25 and 250 μg/ml) in DMSO/RPMI/FBS 5% at 37 °C and CO_2 5%, for 48 h. Final DMSO concentration did not affect cell viability. Cells were then fixed with TCA solution (50%, v/v), and cell proliferation was determined by spectrophotometric quantification (540 nm) of cellular protein content. DOX (0.025–25 μg/ml) was used as the positive control. Three measurements were obtained: at the beginning of incubation (T0) and 48 h post-incubation for compound-free (C) and exposed (T) cells. Cell proliferation was determined according to the equation: cell proliferation = $100 \times [(T - T0)/C - T0]$. The cytostatic effect was observed when $T0 \leq T < C$, while cytocidal effect occurred when $T < T0$. The experiments were done in triplicate to calculate the total growth inhibition (TGI) (concentration that produces TGI).

Acute preclinical toxicity

The evaluation of acute preclinical toxicity of ALO was performed according to the Guide for the conduct of non-clinical toxicology studies and safety pharmacology necessary for the

development of drugs, published by the Brazilian Health Surveillance Agency (Anvisa, 2013), with some modifications. Mice (six females) were given doses of 250, 375 and 500 mg/kg ALO intraperitoneally (*i.p.*), and the control group received vehicle alone (Tween-80 5% in saline). For detection of toxic signs suggestive of activity on the central nervous system (CNS) or autonomic nervous system (ANS) of a general nature, the animals were closely observed at the following times: 0, 15, 30 and 60 min, 4 h, and daily for 14 d (Almeida et al., 1999). For 50% lethal dose (LD_{50}) determination, the number of death animals was observed during 14 days after the treatments.

Genotoxicity

For the micronucleus assay, groups of six mice were treated intraperitoneally with different doses of ALO at 110, 230 and 350 mg/kg (25, 50 and 80% of the LD_{50} value, respectively) (Ribeiro, 2003). A group treated with a standard drug (cyclophosphamide, 50 mg/kg, *i.p.*), and a control group (Tween-80 5% in saline) were included. After 24 h, the animals were anesthetized with sodium thiopental (40 mg/kg), and peripheral blood samples were collected from the retro-orbital plexus for blood smears. Three blood smears were prepared for each animal, and a minimum of 2000 erythrocytes were counted to determine the number of micronucleated erythrocytes (OECD, 1997).

In vivo *antitumor activity*

Eight-day-old sarcoma 180 ascites cells (0.2 ml–25 × 10^6 cells/ml) were implanted subcutaneously into the left subaxillary region of female mice (n = six/group) (Bezerra et al., 2008). One day after inoculation, ALO (50 or 100 mg/kg) was dissolved using Tween-80 5% (v/v) and administered intraperitoneally for 7 days to mice transplanted with sarcoma 180 tumor. 5-FU (25 mg/kg) was used as the standard drug. The healthy group (healthy mice) and tumor control group (mice bearing sarcoma 180) were inoculated with Tween-80 5% in 0.9% (w/v) NaCl. On the eighth day, peripheral blood samples from all mice were collected from the retro-orbital plexus under light sodium thiopental anesthesia (40 mg/kg). The animals were then sacrificed by cervical dislocation. The tumors were excised and weighed, and then fixed in formaldehyde 10% and submitted to histopathological analysis. The tumor growth inhibition in per cent was calculated by the following formula: Inhibition (%) = $[(A - B)/A] \times 100$, where A is the average of the tumor weights of the tumor control group, and B is that of the treated group.

Toxicity in transplanted mice

Body weights were recorded at the beginning and end of treatment, while consumption of water and feed was evaluated daily for the 7 days of treatment. The liver, spleen, thymus and kidneys were excised and weighed for determination of their organ index. For biochemical analysis, levels of urea, creatinine and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined. For hematological analysis, the following parameters were determined: hemoglobin (Hb) level, red blood cells (RBC) count, haematocrit (Hct) and the red cell indices mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), as well as total and differential leukocyte counts. After weight determination and fixation using formaldehyde 10% (v/v), portions of the livers and kidneys were cut into small pieces and then into sections of 5 μm thickness, which were stained with hematoxylin-eosin and Masson's trichrome for the liver.

Statistical analysis

Data are presented as mean ± SEM. The HC_{50} value and their 95% confidence intervals (CI 95%) were obtained by nonlinear regression. From the concentration-response curve for each tumor cell line, TGI was determined through non-linear regression analysis using the software Origin 8.0 (OriginLab Corporation, Northampton, MA, USA). For *in vivo* assays, the differences between experimental groups were compared by analysis of variance (ANOVA), followed by Tukey's test (p <0.05) using the Graphpad program (Intuitive Software for Science, San Diego, CA, USA). We used Kolmogorov smirnov and Levene tests as prerequisites for the ANOVA test.

Results and discussion

It was identified 37 compounds in ALO, corresponding 98.1% of total oil, being 44.1% monoterpenes and 55.9% sesquiterpenes, corroborating to literature about Annonaceae species (Tavares et al., 2007). Previously, chemical composition for volatile oils from the leaves of *A. leptopetala* was described (Costa et al., 2008; Feitosa et al., 2009). Nevertheless, our data show spathulenol as major component (12.56%) followed by α-limonene (9.06%), both with reported antitumor activity (Bicas et al., 2011; Bomfim et al., 2016). Similarly, (*E*)-caryophyllene was also identified, since it was reported as a marker in Annonaceae (Valter et al., 2008; Palazzo et al., 2009). Other components identified in ALO are shown in Table 1.

Regarding to antiproliferative assay, ALO showed greater selectivity for leukemia cell line K-562 with TGI of 0.64 μg/ml. In addition, ALO showed no cytotoxic effect to non-tumor cell line HaCat, suggesting specificity to tumor cells. Date for TGI on other tumor cell lines are shown on Table 2.

Antitumor agents are limited due to their serious side effects. The incidence of anemia increases with the administration of chemotherapy/radiation therapy due to the destruction and/or inability of the bone marrow to produce red blood cells and hemoglobin. Moreover, studies have documented that volatile oils and their chemical constituents may have hemolytic effects (Mendanha et al., 2013). ALO exhibited a concentration-dependent hemolytic effect, with HC_{50} of about 372.8 μg/ml (344.8–403.1 μg/ml) (Fig. 1). Considering much lower concentrations that produced antiproliferative effect in SRB assay, higher concentrations were required to induce hemolysis, suggesting low toxicity to erythrocytes.

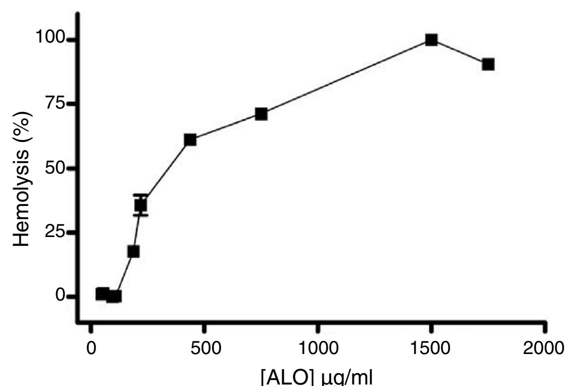

Fig. 1. Percentage of haemolysis in red blood cells of Swiss mice upon treatment with volatile oil from *Annona leptopetala* leaves (ALO) (μg/ml). Data presented as mean ± SEM of three experiments with three replicates, with a 95% confidence interval.

Table 1
Chemical composition (%) of volatile components from volatile oil from *Annona leptopetala* leaves.

Constituints[a]	Retention time (min)	RI[b]	RI[c]	%
α-Thujene	5.757	928	930	4.16
α-Pinene	5.968	935	939	3.16
Camphene	6.398	950	954	0.55
Sabinene	7.087	975	975	0.66
β-Pinene	7.214	979	979	0.77
Myrcene	7.563	991	991	2.75
α- Phelandrene	8.057	1007	1003	0.33
Iso-silvestrene	8.271	1012	1009	2.35
α-Terpinene	8.475	1018	1017	0.27
p-Cymene	8.754	1025	1025	0.84
α-Limonene	8.915	1030	1029	9.06
Eucalyptol	9.029	1033	1036	0.7
trans-β-Ocimene	9.181	1037	1037	0.42
(*E*)-β-Ocimene	9.572	1047	1050	1.36
γ-Terpinene	10.022	1059	1060	0.5
Terpinolene	11.19	1090	1089	1.25
Linalol	11.599	1101	1097	5
Terpinen-4-ol	14.977	1179	1177	0.94
α-Terpineol	15.556	1193	1189	6.95
Geraniol	18.355	1256	1253	1.27
(*E*)-Cariophylene	25.706	1423	1419	7.55
Aromadandrene	26.532	1443	1441	2.15
α-Humulene	27.156	1457	1455	1.32
9-epi-(*E*)- Cariophyllene	27.479	1465	1466	0.49
Germacrene-D	28.326	1485	1485	0.77
Bicyclogermacrene	29.001	1501	1500	8.34
δ-Cadinene	30.069	1527	1523	0.74
Spathulenol	32.33	1582	1578	12.56
Globulol	32.577	158	1585	4.98
Viridiflorol	32.895	1596	1593	1.84
Guaiol	33.108	1601	1601	7.26
Rosifoliol	33.299	1606	1600	0.58
γ-Eudesmol	34.446	1635	1632	0.37
α-Muurolol	35.015	1650	1646	0.85
β-Eudesmol	35.17	1654	1651	0.45
α-Eudesmol	35.32	1658	1652	0.8
Bulnesol	35.84	1671	1672	3.76
Total identified				98.1

[a] Identification by comparison with the GC/MS spectrum and IR Image library internal NIST-62 and Adams (2001).
[b] RI (retention rate) obtained on a DB-5 capillary column based on a series of *n*-alkanes and calculated according to Van den Dool and Kratz (1963).
[c] RI: according to Adams (2001).

Table 2
Antiproliferative effect of volatile oil from *Annona leptopetala* leaves and doxorubicin against human cancer cell lines.[a]

Cell lines	TGI (μg/ml)[b]	
	DOX	ALO
Glioma (U251)	0.06	47.23
Breast (MCF-7)	0.21	49.91
Ovary Multidrug Resistance Phenotype (NCI-ADR/RES)	1.35	>250
Kidney (786-O)	0.04	101.52
Lung (NCI-H460)	0.01	75.53
Prostate (PC-3)	0.27	45.12
Ovary (OVCAR)	0.26	>250
Colon (HT-29)	0.22	75.26
Leukemia (K-562)	0.40	0.64
Skin (line of non-tumor cells) (HaCat)	0.23	>250

[a] Assessed by the SRB assay.
[b] TGI values represent the necessary concentration (μg/ml) for total inhibition of cancer cells proliferation. Dose range tested: 0.25–250 μg/ml to volatile oil from *Annona leptopetala* leaves, and 0.025–25 μg/ml to doxorrubicin.

Acute preclinical toxicity was used to determine safe doses to be used in pharmacological tests in mice. ALO caused animal death in a dose-dependent manner, with LD_{50} of about 447.2 mg/kg. Ptosis, sedation, and other CNS depressive effects were observed (Table 3). Only at 500 mg/kg these effects remained after 4 h of treatment. In general, if LD_{50} of test substance is three times more than the minimum effective dose, the substance is considered a good candidate for further studies (Auletta, 1995; Mangueira et al., 2017).

Micronucleus testing is widely used and accepted by international and governmental agencies as part of assays needed to evaluate genotoxicity of new chemicals (Choy, 2001). ALO did not increase number of micronucleated erythrocytes in peripheral blood (Table 4), suggesting no genotoxicity.

A significant reduction in tumor weight was observed in ALO 100 and 150 mg/kg-treated groups, being 0.93 ± 0.09 and 0.95 ± 0.10 g, respectively, in comparison to tumor control group. Inhibition rates of sarcoma 180 tumor were 59.3%, 58.8% and 67.6% at doses of 100 and 150 mg/kg ALO and 25 mg/kg 5-FU, respectively (Fig. 2).

In the histopathological analysis of tumors, tissue infiltration, areas of coagulative tumor necrosis, desmoplasia and asymmetric distribution of mitoses were observed, with variations between groups (Fig. 3A and B). In tumor control group, desmoplastic reaction presented moderate level with areas of necrosis corresponding to about 60% of neoplastic growth. Tumors of animals treated with 5-FU (25 mg/kg), in turn, showed areas of necrosis corresponding to about 60% of neoplastic growth (Fig. 3C). Asymmetric mitosis which accounted for about three per high power field (data not shown). Architectural and cytological aspects of tumor in ALO-treated groups were similar to those observed in tumor control group (Fig. 3B and D). ALO 150 mg/kg treated tumors had the characteristics infiltration of adipose tissue and muscle limited, areas of coagulative tumor necrosis, about 35%, and asymmetric mitosis count, about three per high power field (Fig. 3D).

Concerning to toxicity analysis, ALO caused reduction in food consumption when compared to tumor control and healthy groups

Table 3
Effect the single doses (*i.p.*) of volatile oil from *Annona leptopetala* leaves in mice ($n = 6$).

Groups	Dose (mg/kg)	D/T[a]	Symptoms
Control	–	0/6	None
ALO	250	0/6	Hyperactivity
			None
ALO	375	1/6	Ptosis, sedation, ataxia, analgesia, decreased touch response, abduction of paws hindquarters, constipation, labored breathing, decreased urination
ALO	500	4/6	Hypnosis, ptosis, sedation, ataxia, analgesia, decreased response to touch, loss of corneal reflex, loss of ear reflex, constipation, heavy breathing

[a] D/T = number of mice dead/number of mice treated.

Table 4
Number of micronucleated erythrocytes in peripheral boold of mice treated with different doses of the volatile oil from *Annona leptopetala* leaves (ALO) and cyclophosphamide (*i.p.*) ($n = 6$).

Groups	Dose (mg/kg)	Number of Micronucleated cells
Control	–	6.17 ± 0.83
Cyclophosphamide	50	26.00 ± 5.01[a]
ALO	110	6.00 ± 0.45
ALO	230	6.50 ± 1.04
ALO	350	8.00 ± 1.29

Data presented as mean ± SEM of six animals in absolute numbers.
[a] $p < 0.05$ compared to control by ANOVA followed by Tukey.

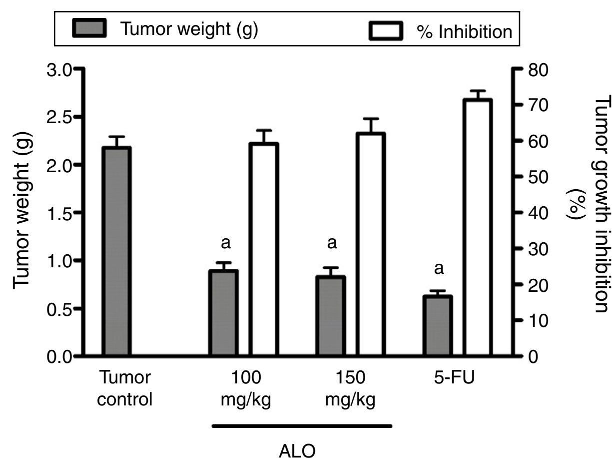

Fig. 2. Effects of volatile oil from *Annona leptopetala* leaves (ALO) and 5-FU on sarcoma 180 tumor growth in mice. The graph shows the tumor weight (g) and the inhibition rate of tumor growth (%) of the different experimental groups. Data are expressed as mean ± SEM of six animals analyzed by ANOVA followed by Tukey. [a]$p < 0.05$ compared to tumor control.

(Table 5), which was accompanied of decrease on body weights. It is already observed in literature for other chemotherapeutic drugs (Sánchez-lara et al., 2013; Caillet et al., 2016).

Main organ of metabolism, liver is susceptible to effects of antitumor drugs. Major markers of cell injury are cytoplasmic and mitochondrial enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT). ALO induced an increase on AST activity without changed ALT activity (Table 6), which may be related to non-severe liver damage (Henry, 2008) or damage to tissues near tumor, such as, skeletal muscle. On the other hand, ALO did not induce changes in urea and creatinine levels (Table 6), suggesting no renal toxicity.

To assess tissue damage induced by ALO, histopathological evaluation was performed. No histopathological changes were observed in kidneys of animals treated with ALO (data not shown).

In animals treated with ALO (100 mg/kg) there was hypertrophy of Kupffer cells (Fig. 4A), focal hepatic necrosis, and hepatocytes associated with lymph-histiocytic flow (Fig. 4B). At 150 mg/kg ALO, it was also observed microvesicular (Fig. 4C) and macrovesicular (Fig. 4D) steatosis, and hepatocellular regenerative atypia (Fig. 4F). In addition, there was cyst formation of bile nature, multilocular, with coating cylindrical epithelium simple cuboid and walls of fibrous connective tissue (Fig. 4E). Same changes, albeit more frequent, were observed in animals treated with 5-FU accompanied by periportal lymphocytes presence (data not shown).

Microgoticular steatosis observed is a lesion of metabolic etiology, usually reaction to drug therapy, but of reversible nature and rapid improvement after treatment discontinuation (Torti et al., 2001; De Vasconcellos et al., 2007; Montenegro et al., 2008). Another finding in livers were bile cysts, found in ALO treated group. There was no cholestatic histological effect in these animals neither to other bile ducts, nor to the hepatic terminal vein (HTV). These cysts are therefore a causal found and located.

Regarding to hematological parameters, there were only a decrease on MCV and MCHC, comparing with tumor control and health groups (Table 7). Nevertheless, these isolated changes have not any clinical significance. Animals transplanted with sarcoma 180 tumor showed a significant increase in total numbers of

Table 5
Feed and water consumption and weight of animals ($n = 6$) subjected to different treatments (7 days).

Groups	Dose (mg/kg)	Water consumption (ml)	Feed intake (g)	Initial weight (g)	Final weight (g)
Healthy group	–	37.1 ± 5.9	30.9 ± 1.4	36.8 ± 0.7	40.2 ± 0.8
Tumor control	–	40.9 ± 2.0	29.9 ± 0.8	36.7 ± 0.7	43.1 ± 0.8
5-FU	25	41.2 ± 4.4	25.8 ± 1.8	39.8 ± 1.7	39.8 ± 1.3
ALO	100	30.0 ± 3.7	16.2 ± 2.8[a,b,c]	42.4 ± 1.1	37.3 ± 1.5[a]
ALO	150	49.4 ± 6.0	20.6 ± 2.6[a,b]	36.5 ± 1.3	36.3 ± 1.5[a]

Data presented as mean ± SEM of the mean of six animals examined by ANOVA followed by Tukey.
[a] $p < 0.05$ compared to the tumor control.
[b] $p < 0.05$ compared to healthy group.
[c] $p < 0.05$ compared to 5-FU. ALO: volatile oil from *Annona leptopetala* leaves.

Table 6
Effects of 5-FU and volatile oil from *Annona leptopetala* leaves (ALO) on biochemical parameters of peripheral blood of mice ($n = 6$) subjected to different treatments (7 days).

Groups	Dose (mg/kg)	AST (U/l)	ALT (U/l)	Urea (mg/dl)	Creatinine (mg/dl)
Healthy group	–	183.0 ± 10.15	80.60 ± 10.18	51.40 ± 1.78	0.66 ± 0.11
Tumor control	–	279.0 ± 22.74	66.60 ± 7.90	35.33 ± 4.04	0.43 ± 0.06
5-FU	25	216.6 ± 13.11	61.40 ± 8.13	43.17 ± 5.12	0.45 ± 0.03
ALO	100	370.8 ± 30.38[a,b,c]	97.75 ± 5.01	48.80 ± 4.70	0.54 ± 0.04
ALO	150	412.0 ± 6.71[a,b,c]	78.00 ± 3.37	44.20 ± 3.25	0.33 ± 0.03[b]

Data presented as mean ± SEM of the mean of six animals examined by ANOVA followed by Tukey.
[a] $p < 0.05$ compared to the tumor control.
[b] $p < 0.05$ compared to the healthy group.
[c] $p < 0.05$ compared to compared to 5-FU.

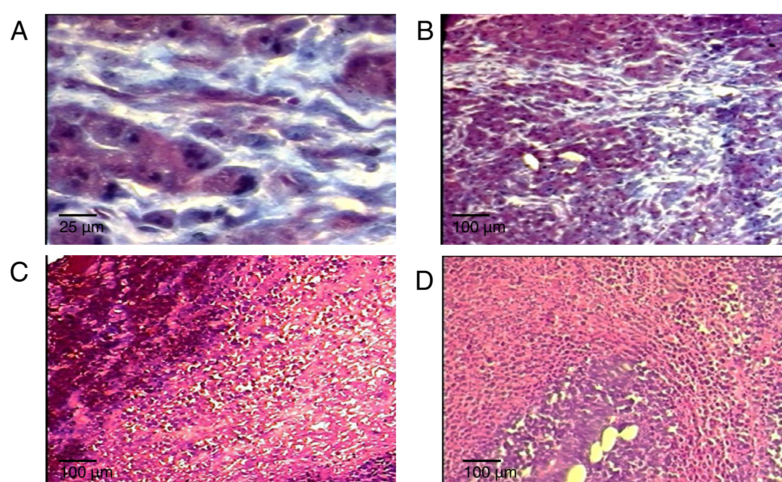

Fig. 3. Histopathology of tumors of the different experimental groups: peritumoral desmoplastic reaction in different treatments. A. 5-FU, moderate; B. ALO (150 mg/kg) discrete. Coagulative necrosis areas associated with the treatment in C 5-FU, D. ALO 150 mg/kg. A (×400), B (×100) – Masson; C, D: H.E. ×100, ALO: volatile oil from *Annona leptopetala* leaves.

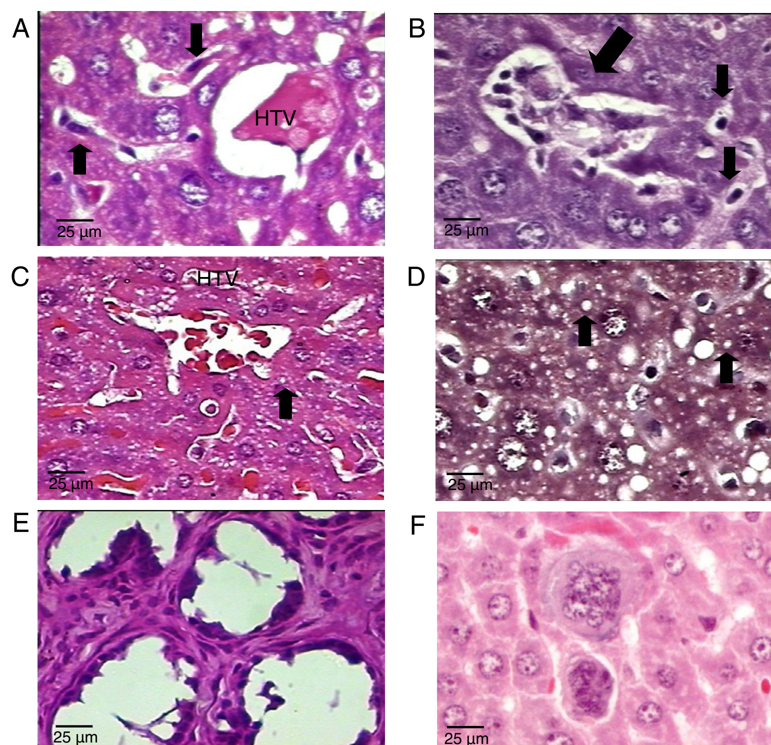

Fig. 4. Histopathology of liver of different groups: A. ALO (100 mg/kg), hypertrophy of Kupffer cells (short arrows) in zone 1; B. ALO (100 mg/kg), focal hepatic necrosis (thick arrow) hepatocyte associated with lymph-histiocytic flow in zone 2, accompanied by hypertrophy of Kupffer cells (short arrows); C. ALO (150 mg/kg) predominantly microvesicular steatosis in zone 3 (short arrow); D. and ALO (150 mg/kg) macrovesicular microvesicular steatosis in zone 2 (short arrows); E. ALO (150 mg/kg) cyst formation of bile nature, multilocular, with coating cylindrical epithelium simple cuboid and walls of fibrous connective tissue; F. ALO (150 mg/kg) Hepatocellular regenerative atypia. HTV: Hepatic Terminal Vein; A, B, C, E, F: H.E. ×400; D: Masson, ×400; ALO: volatile oil from *Annona leptopetala* leaves.

Table 7
Effects of 5-FU and volatile oil from *Annona leptopetala* leaves on hematological parameters of peripheral blood of mice ($n = 6$) subjected to different treatments (7 days).

Parameter	Healthy group	Tumor control	5-FU	ALO 100 mg/kg	ALO 150 mg/kg
Red blood cells (10^6 mm^{-3})	10.18 ± 0.07	11.31 ± 0.58	12.48 ± 0.42[b]	12.60 ± 0.32[b]	12.46 ± 0.11[b]
Hemoglobin (g/dl)	13.68 ± 0.11	15.47 ± 0.59[b]	15.63 ± 0.40[b]	15.06 ± 0.27	15.70 ± 0.13[b]
Hematocrit (%)	53.57 ± 0.42	51.13 ± 2.33	52.72 ± 1.53	51.44 ± 1.17	52.10 ± 0.43
MCV (fm^3)	52.50 ± 0.34	45.33 ± 0.71[b]	42.50 ± 0.62[a]	41.33 ± 0.56[a,b]	41.83 ± 0.31[a,b]
MCH (pg)	13.45 ± 0.10	13.70 ± 0.25	12.65 ± 0.19	12.23 ± 0.26[a,b]	12.62 ± 0.09[a,b]
MCHC (g/dl)	25.57 ± 0.24	30.23 ± 0.39[b]	29.63 ± 0.22[b]	29.65 ± 0.45	30.03 ± 0.10[b]
Total leukocytes (10^3 mm^{-3})	5.60 ± 0.77	11.50 ± 1.46[b]	2.02 ± 0.36[a]	7.98 ± 1.45	10.08 ± 0.92[b]
Lymphocytes (%)	82.83 ± 2.28	41.50 ± 2.74[b]	92.67 ± 1.52[a]	35.50 ± 3.52[b]	46.40 ± 4.15[b]
Neutrophils (%)	12.83 ± 1.74	53.67 ± 2.40[b]	6.00 ± 1.53[a,b]	61.50 ± 3.83[b]	49.80 ± 4.85[b]
Monocytes (%)	2.83 ± 0.54	4.50 ± 1.36	1.17 ± 0.40	2.67 ± 0.42	3.17 ± 1.08
Eosinophils (%)	1.17 ± 0.31	0.50 ± 0.22	0.16 ± 0.16[b]	0.33 ± 0.21	0.33 ± 0.21

Data presented as mean ± SEM of six animals examined by ANOVA followed by Tukey.
[a] $p < 0.05$ compared to the tumor control.
[b] $p < 0.05$ compared to the healthy group. ALO: volatile oil from *Annona leptopetala* leaves.

leukocytes compared to healthy animals. There was also an increase of neutrophils and decreased of lymphocytes in peripheral blood of animals transplanted with sarcoma 180, compared to healthy group (Table 7), corroborating to literature (Pita et al., 2012; Moura et al., 2016). 5-FU treatment led to a decrease in total leukocytes and neutrophils number, as well as an increase in lymphocytes count, when compared to tumor control group. ALO treatment induced no significant change in the leucogram parameters, when compared to tumor control group (Table 7). These data suggest that, unlike most chemotherapy, this volatile oil does not alter the number of hematopoietic cells, which represents one of main effects cancer chemotherapy treatment (Liu et al., 2013; Campos et al., 2015; Chopra et al., 2016).

In conclusion, ALO shows *in vitro* and *in vivo* antitumor activity, without major changes in toxicity parameters evaluated. The results provide essential information, making possible further investigations of antitumor activity and action mechanisms of ALO.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Author contributions

MTB, RCF, DMB, APGM, ALX, JCLRP, TMB, SGS, MFFMD, MVS, participated in study concept and design, acquisition of data, analysis and interpretation of data, and critical revision of the manuscript for important intellectual content. GBL, ALTGR, JEC participated *in vitro* antitumoral tests and participated in drafting the manuscript. JFT and VCOC carried out the extraction of volatile oil and participated in drafting the manuscript. KCPM performed the histophatological analysis.

Conflicts of interest

The authors declare no conflicts of interest.

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