Revista Brasileira de Farmacognosia 29 (2019) 449–456

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

Antitumor effect of depsidones from lichens on tumor cell lines and experimental murine melanoma

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ARTICLE INFO

Article history:
Received 5 October 2018
Accepted 3 April 2019
Available online 25 June 2019

Keywords:
Cytotoxicity
Cancer
Apoptosis
Depsidones
Phenolic compounds
Murine melanoma cells

ABSTRACT

Lichens have exhibited numerous biological activities, including growth inhibition of tumor cells. This study evaluated the antiproliferative activity of hypostictic and salazinic acids against tumor cell lines (B16-F10, PC-03, MCF7, HT-29, HEP-G2, K562 and 786-0) by the SRB assay *in vitro* and antitumor activity in experimental murine melanoma *in vivo*. Activation of caspase-3 was quantified by flow cytometry. The murine experimental melanoma model B16-F10 was used in BALB/c mice for evaluation of antitumor activity. Hypostictic acid showed significant antiproliferative activity in K562 cells (GI50 2.20 μM), B16-F10 (GI50 13.78 μM) and 786-0 (GI50 14.24 μM), whereas salazinic acid was more active against K562 cells (GI50 64.36 μM), HT-29 (GI50 67.91 μM) and B16-F10 (GI5078.64 μM). Quantification of capase-3 revealed that the test compounds did not increase the expression of that enzyme. In the *in vivo* antitumor evaluation in B16-F10 melanoma, the isolated compounds inhibited tumor growth in relation to weight and volume. Hypostictic acid (16.7 mg/kg) inhibited 72% and salazinic acid 88% of tumor volume ($p < 0.05$). The results indicated that, both in the *in vitro* and *in vivo* models, the compounds evaluated showed antiproliferative and antitumor activities.

Introduction

Cancer is among the leading causes of death worldwide. The estimate for the next two decades is 22 million new cases of cancer worldwide, of which 60% will occur in developing countries (NCI, 2015). This has attracted interest in the development of new drugs, and the search for new anticancer agents has increased in the last years (Newman and Cragg, 2012).

Skin cancer is considered to be an eminent global public health problem, and it is the most common malignant neoplasm in the world, with increasing incidence, covering all ethnicities, geographical regions and age groups (Gordon, 2013). Melanoma accounts for 4% of all cutaneous cancers, being less frequent than basal cell and squamous cell carcinomas. Although it has a low incidence, melanoma accounts for 80% of skin cancer-related deaths (Eggermont et al., 2014).

Natural products and their analogues play a significant role in the search for anticancer agents, since most of the drugs used in the therapeutic arsenal against cancer come from natural sources (Newman and Cragg, 2012; Bishayee and Sethi, 2016).

Lichens are promising sources of bioactive compounds. Currently more than 1000 substances produced by lichens are known, and among these, more than 100 are depsidones containing a rigid 11H-dibenzo[b,e][1,4] dioxepin-11-one ring with substituents at various positions. Although this class of compounds is responsible for innumerable biological activities, only about 15% of these

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https://doi.org/10.1016/j.bjp.2019.04.005

compounds have been evaluated for antitumor activity (Ögmundsdóttir et al., 1998; Haraldsdóttir et al., 2004; Bézevin et al., 2004; Brisdelli et al., 2013).

Therefore, the main objective of this work was to evaluate salazinic and hypostictic acids with antiproliferative activity against a panel of tumor cell lines and also their proapoptotic effects *in vitro* and their toxic and antitumor effects in vivo against melanoma cells.

Material and methods

General procedures

NMR spectra were obtained using a Brucker DPX 300(300 MHz for ^{1}H and 75 MHz for ^{13}C) spectrometer, and the solvent was used as an internal reference. Melting points were determined on a Uniscience Melting Point apparatus without correction. Absorbance in the cytotoxicity assay was read with a Molecular Devices Spectra Max 190® microplate reader and the caspase activation assay was carried with a BD Accuri 6 flow cytometer. Morphological alterations were determined using an Olympus BX41 fluorescence microscope.

Lichens

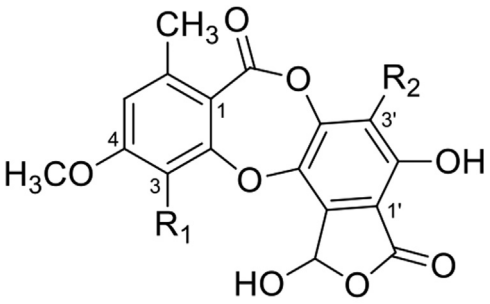
Pseudoparmelia sphaerospora (Nyl.) Hale and *Parmotrema cetratum* (Ach.) Hale were collected on bark and on rock, respectively, in open forests, near Piraputanga, Aquidauana Country, Mato Grosso do Sul State, Brazil (20°27′21.2″S, 55°29′00.9″W, altitude of approximately 200 m). *P. sphaerospora* was collected in November 1993 and was identified by M. P. Marcelli. IBT, Brazil; *P. cetratum* collected in April 1991 was identified by M. Fleig, Universidade Federal do Rio Grande do Sul. A voucher of each species was deposited at Universidade Federal of Mato Grosso do Sul Herbarium (CGMS – *Pseudoparmelia sphaerospora* 49837 and *Parmotrema cetratum* 37950).

Extraction and isolation

The extraction of hypostictic acid from *P. sphaerospora* (20 g) and salazinic acid from *P. cetratum* (25 g) was carried out according Honda et al. (2010) and Micheletti et al. (2009), respectively. The structures of these compounds were confirmed by ^{1}H, ^{13}C and Dept-135 NMR spectra and were in accordance with the literature (Eifler-Lima et al., 2000; Carvalho et al., 2004).

Hypostictic acid (1): white solid [yield 2.2 g (8.8%)]; mp: 287 °C (dec.); ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.18 (s, 3H, CH_3-8), 2.26 (s, 3H, CH_3-9′), 2.42 (s, 3H, CH_3-9), 3.87 (s, 3H, —OCH_3), 6.68 (s, 1H, lactol), 6.89 (s, 1H, H-5), 8.34 (sl, 1H, OH lactol). ^{13}C NMR (75 MHz, DMSO-d_6) δ 8.5 (C-8), 9.5 (C-9′), 20.7 (C-9), 56.1 (OCH_3), 95.7 (C-8′), 109.1 (C-1′), 110.8 (C-3), 112.6 (C-1), 114.6 (C-5), 120.3 (C-3′), 135.4 (C-6′), 138.1 (C-5′), 141.9 (C-6), 148.4 (C-4′), 151.5 (C-2′), 158.8 (C-7), 161.1 (C-7′), 161.5 (C-4), 166.5 (C-2).

Salazinic acid (**2**): white solid [yield 1.5 g (6%)]; mp: 200 °C (dec.); ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.41 (s, 3 H, CH_3-8), 4.65 (s, 2H, CH_2OH), 6.80 (s, 1H, H-5), 6.87 (s, 1H, lactol), 8.29 (sl, 1H, OH lactol), 10.44 (s, 1H, CHO), 12.04 (s, 1H, OH-4). ^{13}C NMR (75 MHz, DMSO-d_6) δ 21.6 (C 8), 52.9 (C 9′), 95.3 (C 8′), 109.8 (C 1′), 110.7 (C 3), 112.0 (C 1), 117.4 (C 5), 123.4 (C 3′), 137.3 (C 6′), 138.1 (C 5′), 146.5 (C 6), 148.1 (C 4′), 152.3 (C 2′), 160.4 (C 7), 163.7 (C 7′), 164.2 (C 4), 166.0 (C 2), 192.9 (C 9).



1 $R_1=R_2=CH_3$
2 $R_1=CHO$; $R_2=CH_2OH$

Cell culture

The tumor cell lines B16-F10 (ATCC – CRL-6475, murine melanoma), MCF7 (ATCC – HTB-22, breast cancer), 786-0 (ATCC – CRL-1932, renal cancer), PC-03 (ATCC – CRL-1435, prostate cancer), HT-29 (ATCC HTB-38, colon cancer), HepG2 (ATCC HB-8065, hepatocellular carcinoma) and K562 (ATCC CCL-243, chronic myelogenous leukemia) were donated by Professor João Ernesto de Carvalho (Center for Chemical, Biological and Agricultural Studies Unicamp). Also used was the murine fibroblast immortalized cell – NIH-3T3 (ATCC – CRL-1658, murine fibroblasts) obtained from the Cell Bank of Rio de Janeiro. All cells were maintained in the complete medium RPMI 1640, except for B16-F10 and NIH-3T3, which were maintained in DMEM, both from Sigma, USA. The media contained streptomycin (100 U/ml) and penicillin (100 μg/ml) from Sigma, USA and 10% fetal bovine serum (FBS) from Invitrogen.

Cell cytotoxicity assay

The cell cytotoxicity assay was performed by the colorimetric method with sulforhodamine B (SRB) (Sigma, USA), described by Skehan et al. (1990). Doxorubicin was used as a positive control. Cells from different lines were seeded in 96-well plates (7500/well) and maintained at 37 °C in a 5% CO_2 incubator. After 24 h incubation, the T0 plate was read which contained only the triplicate cell suspension for each line and complete medium, while the cells were treated with hypostictic and salazinic acids diluted in culture medium at concentrations of 0.25, 2, 5, 25 and 250 μg/ml in triplicate and incubated for 48 h. Treated cells were fixed with trichloroacetic acid (TCA) for 20 min and stained with 50 μl SRB. Absorbance was read at 540 nm, in triplicate. The percentage of growth of each test sample was calculated with SoftMax Pro 6.3, using the formulas according to Monks et al. (1991). The concentration that inhibited cell growth by 50% (GI_{50}) was determined by nonlinear regression in a program for graphs and data analysis (Origin v. 6.0).

Selectivity index

The selectivity index (SI) corresponds to the division of the compound GI_{50} in NIH-3T3 murine fibroblast cells and compound GI_{50} in tumor cells line (SI = GI50 NIH-3T3/GI50 tumor cells line). SI equal to or greater than 2.0 was considered significant (Suffness and Pezzuto, 1990).

Fluorescence microscopy of apoptotic morphology

B16-F10 cells were seeded in 96-well plates (7.5×10^3/well) and incubated for 24 and 48 h with hypostictic and salazinic acids at concentrations of 0.25, 2.5, 25 and 250 μg/ml. Doxorubicin was

used as the positive control. Acridine orange/ethidium bromide staining was used to visualize cell morphological changes (100) according to McGahon et al. (1995), with modifications.

Flow cytometry assay for caspase-3 activation

The assay was performed using PE Rabbit Anti-Active Caspase-3 (BD Pharmingen) following the manufacturer's protocol. A total of 30,000 events were acquired.

Acute toxicity test and experimental animals

Healthy male Swiss mice (*Mus musculus*) (6-7 weeks old) were obtained from the Biotério Central Animal House, Federal University of Mato Grosso do Sul (UFMS), Campo Grande. The study was approved by the Ethics Committee for Animal Experimentation – UFMS (Ethics No. 777/2016). To determine acute toxicity for hypostictic and salazinic acids, mice weighing 25-30 g, were fed with a standard rat pellet diet and tap water. Twenty-four mice were randomly assigned to seven groups: vehicle (5% DMSO/1 ml saline solution), low-dose (50 mg/kg), moderate-dose (300 mg/kg) and high-dose (2000 mg/kg) according to OECD (Organization for Economic Co-operation and Development) – guideline 423. Prior to dosing, the animals were fasted 6 h (*i.e.*, receiving water but not food). The animals were observed continuously for 30 min, they were monitored frequently (1, 2, 3, 4, 6, 12 and 24 h) for the onset of any clinical or toxicological symptoms. Mortality was checked over a period of 2 weeks. The animals were sacrificed on the 14th day. The animals were evaluated with respect to behavioral, motor and sensory functions to determine potential neurotoxic effects (systemic behavioral analysis), and weight gain and weight of the organs (liver and kidneys) were determined and compared to control, at the end of the experiment.

Evaluation of antitumor activity in vivo

Male BALB/c mice, 9 weeks old and weighing a mean of 30 g, from the UFMS after approval by the Commission of Ethics in the Use of Animals of the Federal University of Mato Grosso do Sul (CEUA – protocol No. 685/2015), as determined by the National Council of Control of Animal Experimentation. A subcutaneous nodule was formed using a suspension of B16-F10 cells (1×10^6 cells/0.2 ml PBS) inoculated subcutaneously into the interscapular region of the mice. On the 10th day after inoculation of the cells, the animals ($n = 5$) were treated with a single intraperitoneal dose (0.2 ml) with hypostictic acid at 16.7, 33.3 and 50 mg/kg and salazinic acid at 33.3, 66.7 and 100.0 mg/kg. The negative control group was treated with PBS + 5% DMSO and the positive control with doxorubicin (2 mg/kg). After 11 days of treatment, the animals were euthanized in a CO_2 chamber. The nodules were removed and the parameters weight (g), volume (mm^3) and area (cm^2) were determined (Gomes Neto et al., 2002). From the results, percentage of tumor growth inhibition was calculated for the treated groups.

Histopathologic evaluation

After euthanasia, the kidneys and liver were removed and subjected to histopathological evaluation. The parameters steatosis (liver), hydropic degeneration, hyaline degeneration, leukocytic infiltrate, fibrosis, apoptosis and necrosis (kidneys) were determined as to intensity of the lesion and a score was given according to the degree of change found. Afterwards, an arithmetic mean was determined for the lesion found.

Data analysis

Morphological changes and quantification of caspase-3 were determined using one-way ANOVA with Dunnett's post-test for comparison with the control. The results obtained in the *in vivo* anticancer activity assay were expressed as mean ± SE and compared to the controls by ANOVA followed by the Tukey post-test. In the histopathological evaluation the values were expressed as median and the Kruskal-Wallis test was performed to verify if there was a significant difference between the groups and Mann-Whitney test to determine where the statistical differences were $p < 0.05$ was considered significant.

Results

Cytotoxic effects of hypostictic and salazinic acid on cancer cells

The depsidones salazinic and hypostictic acids were evaluated against the tumor cell lines B16-F10, MCF7, 786-0, PC-03, HT-29, HepG2 and K562. In all cell lines, cell death induced by the depsidones occurred in a dose-dependent manner, and the compounds showed different degrees of cytotoxicity. Hypostictic acid exhibited GI_{50} values varying from 2.20 to 72.4 μM, while salazinic acid showed GI_{50} values of 64.4–756.7 μM. (Table 1). These compounds showed an SI greater than 2 for all lines, except for salazinic acid (**2**) with HepG2.

Caspase-3 independent induction of apoptosis in B16-F10 cells

Considering the results of the antiproliferative activity of hypostictic and salazinic acids against tumor cells, was evaluated whether cell death occurred *via* induction of apoptosis and/or necrosis. The evaluation of cellular morphology indicated that treatment with hypostictic and salazinic acids (25 μg/ml) and the chemotherapeutic drug doxorubicin (0.25 and 2.5 μg/ml) induced nuclear condensation and formation of apoptotic bodies. These compounds showed a significantly increased percentage ($p < 0.01$) of apoptotic cells at 24 and 48 h compared to the negative control (Fig. 1). Treatment with 250 μg/ml of either compound was lethal to B16-F10 cells for both exposure times. After induction of apoptosis by the test compounds was demonstrated, the effect of these compounds on the activation of caspase-3 was evaluated (Fig. 2). Hypostictic and salazinic acids did not cause a significant increase in cells with activated caspase-3 compared to the negative control. Cells treated with doxorubicin (positive control) showed 44% cells with activated caspase-3 protein.

Acute toxicity study

The results of the acute toxicity test showed no signs of systemic toxicity in the groups treated with hypostictic acid and no relevant changes in the toxicity screening test were observed, such as motor and/or sensory neurological alteration. For salazinic acid, the concentration of 2000 mg/kg was toxic, as two animals died (66.66%) in less than 24 h, and a surviving animal was euthanized three days after administration of the compound, since it exhibited signs of pain and suffering. In the other groups treated with salazinic acid (50 and 300 mg/kg) and hypostictic acid (50, 300 and 2000 mg/kg), weight gain was observed in all groups at the end of the experiment, ranging from 11.6% to 19.8% ($p > 0.05$). The analysis of the weight of the organs (liver and kidneys) did not show a significant difference between the groups ($p > 0.05$), according to Fig. 3. In light of the results, we can state that the median lethal dose (LD50) of hypostictic acid was over 2000 mg/kg and that of salazinic acid was between 300 and 2000 mg/kg.

Table 1
Values of growth inhibition (GI50) and selectivity index (SI*) for hypostictic and salazinic acids and doxorubicin on tumor and normal cells.

Substances		Cell lines							
		HT-29	786-0	MCF7	HepG2	PC-03	B16-F10	K562	NIH/3T3
Salazinic acid	μg/ml	26.4	88.3	62.1	293.8	38.0	27.2	25.0	396.8
	μM	68.0	227.5	159.7	756.7	97.8	78.6	64.4	1021.9
	SI	15.0	4.5	6.4	1.3	10.4	14.6	15.9	
Hypostictic acid	μg/ml	27.0	5.3	26.0	22.8	22.9	5.1	0.8	78.7
	μM	72.4	14.2	69.8	61.4	61.5	13.8	2.2	211.4
	SI	2.9	14.8	3.0	3.4	3.4	15.4	95.9	
Doxorubicin	μg/ml	0.2	0.03	0.02	0.2	0.02	0.02	2.3	1.6
	μM	0.4	0.05	0.04	0.4	0.04	0.04	9.9	2.9
	SI	6.6	33.6	63.2	6.6	63.2	63.2	0.7	

SI* was the GI50 value for a compound on 3T3 cells divided by the GI50 value for the compound on a line of cancer cells. Data are presented as GI50 values, in μg/ml and μM, obtained by non-linear regression performed in triplicate by SRB assay. Doxorubicin was used as the positive control.

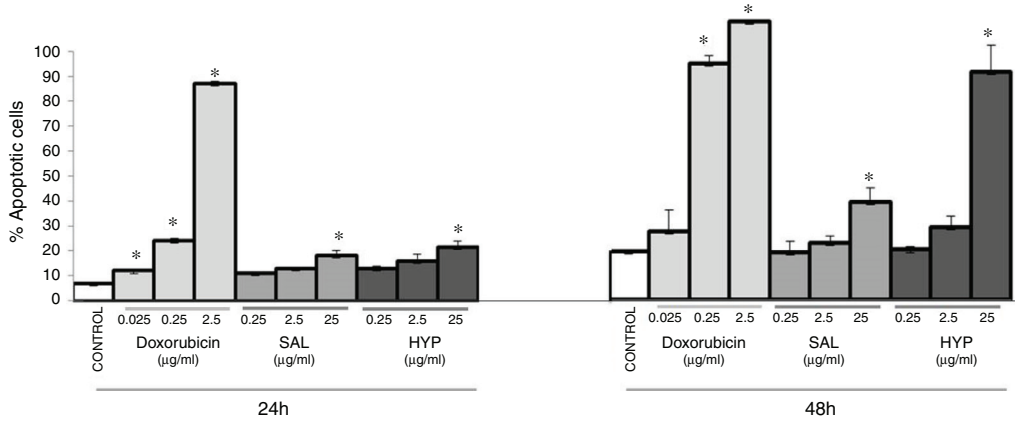


Fig. 1. Effect of salazinic (SAL) and hypostictic (HYP) acids on morphology of B16-F10 cells after 24 and 48 h of incubation, with both at concentrations of 0.25, 2.5 and 25 μg/ml. Doxorubicin served as the positive control. Values represent the mean ± standard error of the mean. *$p < 0.01$ (ANOVA, followed by Dunnett's post-test).

Inhibition of tumor growth in the murine melanoma model

Given that depsidones significantly inhibited B16-F10 cell proliferation *in vitro*, next the inhibitory effect was examined of hypostictic and salazinic acids on the B16-F10 murine melanoma tumor model. Hypostictic acid at a concentration of 16.7 mg/kg reduced tumor weight by 71.5% and tumor volume by 72.8%, and salazinic acid (33.3 mg/kg) reduced tumor weight and volume by 83.0% and 88.8%, respectively. These results were statistically similar to those for the positive control (2 mg/kg doxorubicin), which respectively caused a 70.8% and 68.6% reduction (Fig. 4).

Effects of treatment on histopathological analysis of the liver and kidneys

Hydropic degeneration (Table 2) of mild degree was observed in the groups treated with the hyposticitc acid (16.7; 33.3 and 50 mg/kg) and salazinic acid (100 mg/kg) presented a significant difference ($p < 0.05$) compared to the negative control group. No hepatic changes were observed in any experimental group.

Discussion

There is little information in the literature on the antiproliferative effect of salazinic acid. Correché et al. (2004) reported the apoptotic effect of this acid on rat hepatocyte primary cultures. Salazinic acid showed little significant activity against MM98 (malignant mesothelioma), A431 (vulvar carcinoma), HacaT keratinocytes, MDA-MB435 (human breast), HCT-8 (human colon) and SF-295 (human glioblastoma) (Micheletti et al., 2009; Burlando et al., 2009). Our results showed the potential of salazinic acid in the 50% cell growth inhibition of K562, HT-29, B16-F10 and PC-03 (with values from 24.99 to 37.96 μg/ml), results similar to that found by Manojlović et al. (2012) in human melanoma cells (FemX) and human colon carcinoma (LS174) (GI50 39.02 and 35.67 μg/ml, respectively). SI for this compound was high, ranging from 4.5 to 15.9 in the lines in which the compound was active. The antiproliferative effect of this acid on MCF7 and 786-0 cells was less significant; however, selectivity for tumor cells was significant (SI > 2.0). Regarding hypostictic acid, no information found on the antiproliferative or antitumor effect of this compound in the databases consulted. Thus, this is the first record of the antiproliferative and antitumor activity of hypostictic acid against HT-29, 786-0, MCF7, HepG2, PC-03, B16-F10, K562 and NIH/3T3 cells. Hypostictic acid (**1**) showed cell growth inhibition activity in all cells tested, with GI50 values of 2.2–72.4 μM, with emphasis on K562, B16-F10 and 786-0 (GI50 2.2, 13.8 and 14.2 μM and SI 95.95, 15.44 and 14.86, respectively). According to Silva et al. (2015), GI50 values can be used to classify the activity of substances as follows: inactive, >100 μM; moderate, between >10 and <100 μM; and active, <10 μM. Therefore, we can conclude that salazinic acid (**2**) had moderate activity against K562, HT-29, B16-F10 and PC-03 cells and that hypostictic acid was active in K562 and showed moderate activity against HT-29, MCF7, 786-0, HEPG2, PC-03 and B16-F10.

The compounds studied induced cell death by apoptosis at concentrations greater than 25 μg/ml at both 24 and 48 h of exposure. It was observed that the higher the dose, the greater the apoptotic effect. At doses of 250 μg/ml both compounds showed a potent cytocidal effect. These results corroborate other studies that have

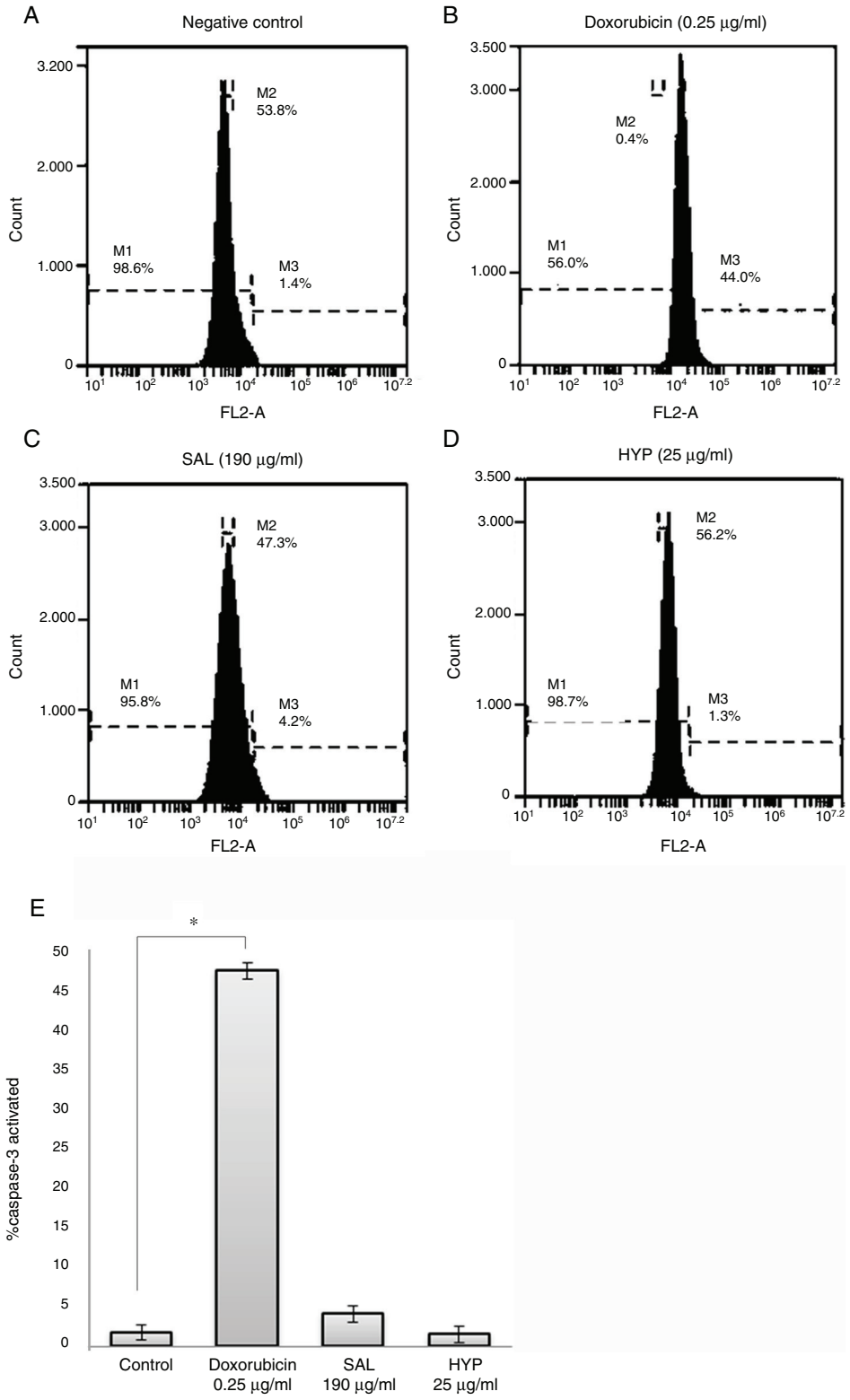


Fig. 2. Flow cytometry for the presence of activated caspase-3 in B16-F10 cells treated or not with salazinic acid (SAL) and hypostictic acid (HYP). The histograms show the percentage of activated caspase-3 for cells not treated (A) and treated with doxorubicin (0.25 µg/ml, B), salazinic acid (190 µg/ml, C) and hypostictic acid (25 µg/ml, D). (E) Bar graph compares the percentage of cells with activated caspase-3 for cells treated with test substances *versus* the control. (ANOVA, followed by Tukey's test). $^{*}p < 0.05$.

demonstrated that substances derived from lichens, from the class of depsides and depsidones, induce apoptosis against tumor cells (Russo et al., 2008; Bačkorová et al., 2011).

Hypostictic and salazinic acids do not activate the caspase-3 initiated apoptosis pathway. Caspase-3 is often activated in the mammalian apoptosis process, where it is required for chromatin condensation and DNA fragmentation in various cell types (Porter and Jänicke, 1999), but there are two other caspases that also lead to apoptosis, which are like caspases-6 and -7, in addition there is the caspase-independent intrinsic apoptosis pathway that

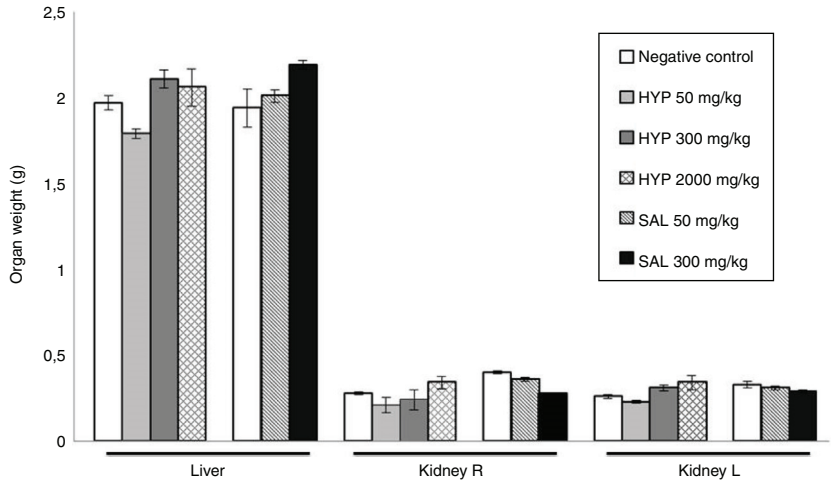


Fig. 3. Flow cytometry for the presence of activated caspase-3 in B16-F10 cells treated or not with salazinic acid (SAL) and hypostictic acid (HYP). The histograms show the percentage of activated caspase-3 for cells not treated (A) and treated with doxorubicin (0.25 μg/ml, B), salazinic acid (190 μg/ml, C) and hypostictic acid (25 μg/ml, D). (E) Bar graph compares the percentage of cells with activated caspase-3 for cells treated with test substances *versus* the control. (ANOVA, followed by Tukey's test). *$p < 0.05$.

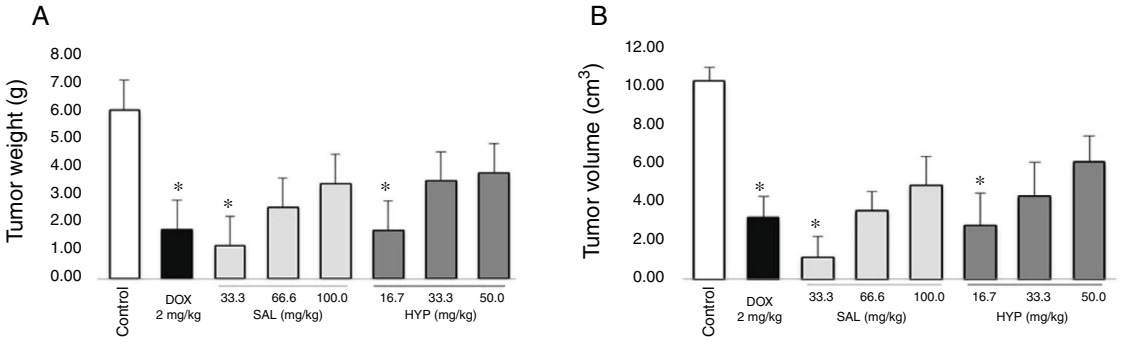


Fig. 4. Antitumor effect in relation weight (A) and volume (B) of the tumor nodules formed by B16-F10 cells after treatment with doxorubicin (2 mg/kg), salazinic acid (SAL) (33.3, 66.7 and 100 mg/kg) and hypostictic acid (HYP) (16.7, 33.3 and 50 mg/kg). Data are expressed as mean ± standard error of the mean. *$p < 0.05$ (ANOVA, followed by Tukey's test).

Table 2
Results of the analyses in the histopathological evaluations of the liver and kidney in the different treatments.

Organ	NC	DOX2.0 mg/kg	HYP16.7 mg/kg	HYP33.3 mg/kg	HYP50.0 mg/kg	SAL33.3 mg/kg	SAL66.7 mg/kg	SAL100.0 mg/kg
Kidney								
Hydropic degeneration	2	2	4*	4*	4*	4	4	4*

Values represented in medians.
*$p < 0.05$ relative to negative control.

occur through apoptosis inducing factors (AIF) and endonuclease G (Galluzzi et al., 2012; Dasgupta et al., 2016), so further studies to confirm the apoptosis induction pathway by the evaluated substances are required.

There are no studies in the literature on the acute toxicity of hypostictic and salazinic acid. Salazinic acid presented higher toxicity compared to hypostictic acid (categories 4 and 5, according to OECD, respectively).

Hypostictic and salazinic acids were able to inhibit tumorigenesis, and both showed statistically similar results and the same behavior, in which the best rate of tumor growth inhibition was found in the lowest concentrations tested, which may be related to several factors that make up the tumor microenvironment. According to Hanahan and Weinberg (2000), the tumor microenvironment plays a crucial role in tumor development. Recombinant cells, such as macrophages, granulocytes and lymphocytes, nitric oxide production and the activation of immune effectors, such as growth factors, cytokines, chemokines and kinins, may stimulate an effective antitumor response or may promote tumor growth and dissemination by promotion of angiogenesis (Wilson and Balkwill, 2002; Bisacchi et al., 2003). Signaling of apoptotic cells in the tumor microenvironment may have several pro-tumorigenic effects, including transformation of tumor-associated macrophages (TAM) to a pro-oncogenic state, these may stimulate angiogenesis and inactivate the immune responses by natural killers (NK) and T cells, preventing the attack on cancer cells (Ichim and Tait, 2016). According to Sun et al. (2011), B16-F10 melanoma cells produce higher levels of TDSF (tumor-derived soluble factors), including IL-10 (interleukin-10), transforming growth factor-β1 (TGF-b1), and vascular endothelial growth factor (VEGF), which suppress the immune system. Since melanoma cells are highly influenced by the tumor microenvironment, a high number of cells in apoptosis or infiltration of excess macrophages in the tumor microenvironment correlate with a poor prognosis for cancer patients (Villanueva and Herlyn, 2008; Ford et al., 2015). Therefore, treatments with the highest doses used in this study might have promoted the inhibition of factors responsible for the antitumor action, and consequently there was a greater action of factors promoting tumorigenesis. In

addition, in the histopathological analysis, only a slight degree of hydropic degeneration, also known as vacuolar degeneration, was observed, since it is the first manifestation of almost all forms of cellular damage and a reversible non-lethal alteration (Kumar et al., 2015). These treatments with the compounds at the doses tested do not cause expressive renal damage and no histopathological liver changes were found that could be associated with the toxicity of hypostatic and salazinic acid at the concentrations used.

Conclusions

Therefore, we conclude that hypostictic and salazinic acids presented antiproliferative effect *in vitro* against the cell lines tested, proapoptotic effect by inducing B16-F10 cells to undergo apoptosis, and antitumor effect *in vivo*. In addition, showed low toxicity and high selectivity essential feature in the search for new anticancer drugs and these results may be used in other studies for the application of these substances in the treatment of human melanoma.

Ethical disclosures

The study was approved by the Ethics Committee for Animal Experimentation – UFMS (Ethics No. 777/2016).

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.

Authors contribution

DB, NKH and MFCM designed and supervised the study; CAFA, MCBLS and PRBS performed the experiments and analyzed the data; LC contributed to the histological analyzes; MCTK and RCAG aided *in vivo* study and contributed to the interpretation of the results; RTP participated in the analysis of caspase; CAFA wrote the manuscript with support from MCBLS, NKH and DB. All authors reviewed the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors wish to acknowledge the Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul for the grant awarded. This investigation was funded by Fundect-MS and CAPES for financial support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2019.04.005.

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