Antineoplastic Activity and Genotoxicity of Organic Extracts and Barbatic Acid Isolated from the Lichen *Cladonia Salzmannii* Nyl.

ORIGINAL

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Abstract

Background: Secondary metabolites are responsible for most of the biological activities of lichens. Many of these compounds exhibit significant antineoplastic activity. The aim of the present *in vitro* study was to evaluate the antineoplastic and genotoxic activities of organic extracts and purified barbatic acid from the lichen *Cladonia salzmannii* Nyl.

Methods: The thallus of the lichen (22 g) was cleaned and dried with the solvents diethyl ether, chloroform and acetone. Organic extracts were obtained using the hot exhausted method in a Soxhlet apparatus. Barbatic acid (BAR) was purified from the ether extract (1.3 g). Chemical analysis of the organic extracts and purified BAR was performed using thin-layer chromatography. The purity of purified BAR was determined using high-performance liquid chromatography. The MTT method [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] and cytokinesis-block proliferation index (CBPI) were used to determine, respectively, the antineoplastic and genotoxic activities of the organic extracts and purified BAR. The micronucleus test and comet assay were used to determine genotoxic potential of the organic extracts and purified BAR. Dimethyl sulfoxide was used as the diluting solvent of the samples in all biological tests.

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Results: The IC_{50} results demonstrated significant antineoplastic potential of the ether extract against cell lines NCI-H292 (IC_{50} : 29.91 μ g/mL), HEP-2 (IC_{50} : 26.75 μ g/mL) and HL-60 (IC_{50} : 3.59 μ g/mL) as well as the purified BAR against cell lines HEP-2 (IC_{50} : 15.79 μ g/mL) and MCF-7 (IC_{50} : 18.28 μ g/mL). The CBPI demonstrated the cytotoxic activity of the purified BAR at all concentrations tested (5, 10, 20 and 40 μ g/mL) and all organic extracts (50 μ g/mL) against Ehrlich carcinoma cells. For sarcoma 180, only BAR purified at a concentration of 40 μ g/mL and the ether and chloroform extracts (50 μ g/mL) were considered cytotoxic. The micronucleus test revealed that the purified BAR at a concentration of 5 μ g/mL had no genotoxic potential against either tumor cell line. Furthermore, the chloroform extract and purified BAR at a concentration of 10 μ g/mL were not considered genotoxic for sarcoma 180. In the comet assay, all compounds tested induced DNA damage in both tumor lines.

Conclusion: Based on the present results, organic extracts and purified barbatic acid from *C. salzmännii* exhibit antineoplastic and genotoxic activity against of the tumor cell lines tested.

Keywords

Antitumor Drugs; Depsides; Micronuclei; Comet assay.

Introduction

Cancer is a worldwide public health problem. The World Health Organization estimates an incidence of 27 million cases and 17 million deaths in the year 2030, with an annual prevalence rate of 75 million people with cancer. The effect of this increase in cases of cancer will weigh heavily on low and middle-income countries. An estimate from 2012 demonstrated that more than 60% of all new cases occurred in developing countries, with projections of an increase to 80% of the more than 20 million new cases of cancer estimated for the year 2025 [1, 2].

The growing epidemiological relevance of neoplastic diseases has stimulated the investigation of substances that have potential treatment applications. Although radiotherapy and chemotherapy continue to be employed as treatment strategies,

recovery rates rarely surpass 50% of cases, which underscores the importance of adopting therapies based on the use of cytotoxic substances [3]. The primary target of chemotherapy is to destroy tumor cells. However, most chemotherapeutic agents have nonspecific action and cause harm to both malignant and normal cells. Moreover, the resistance of tumors to drug therapies is another obstacle related to chemotherapy that compromises patient survival [4].

The discovery of medications for the treatment of cancer is related to research involving natural products, as approximately 60% of medications have a natural origin. In recent decades, diverse natural compounds have been investigated as alternatives to the high cost no adverse effects of chemotherapeutic medications [5].

Lichens have been used in folk medicine since antiquity. Most secondary lichen metabolites have

a phenolic nature with important antimicrobial, healing, anti-inflammatory, antiseptic, anti-tumor and cytotoxic properties, making these compounds a potential source of new drugs [6]. The literature describes the anti-tumor [7, 8, 9] and genotoxic activity of lichen substances [10, 11].

Therefore, the aim of the present study was to evaluate the cytotoxic and genotoxic activity of organic extracts and purified barbatic acid obtained from the lichen *Cladonia salzmannii* Nyl. against human tumor cell lines.

Methods

Collection and identification of lichen material

Cladonia salzmannii Nyl. (100 g)

was collected from an area of sandy tableland in the municipality of Alhandra in the state of Paraíba, Brazil (latitude: 7° 24' 993" S; longitude: 34° 57' 734" W). The samples were identified based on morphological and chemical characteristics. Color reactions in the cortex and medulla were performed using 10% potassium hydroxide and 40% sodium hypochlorite (K test and C test, respectively). Identification keys were also used. A voucher was deposited at the Geraldo Mariz Herbarium of the Botany Department of the *Universidade Federal de Pernambuco*, Brazil (voucher n° 47.998).

Obtainment of organic extracts and isolation and purification of barbatic acid (BAR)

The organic extracts were obtained from 22 g of clean, dried lichen material with the solvents diethyl ether, chloroform and acetone, using the hot exhausted method in a Soxhlet apparatus in a hot bath at the ebullition temperature of each solvent for 12 h with 150 mL of each solvent obeying an eluotropic series. The ether (E), chloroform (C) and acetone (A) organic extracts were evaporated in a

rotary evaporator coupled to a hot bath at a temperature of 40°C and placed in a desiccator for the subsequent calculation of the yields. The previously dried ether extract (1.3 g) was submitted to successive washing in a G4 porous bottom funnel with 1 mL de chloroform (4x), as described by Asahina and Shibata [12], to obtain purified BAR.

Chemical analysis of organic extracts and purified BAR

Thin-layer chromatography (TLC)

The organic extract and the purity of the purified BAR were evaluated qualitatively using one-dimensional ascendant TLC. The organic extracts, BAR purified from *C. salzmannii* and standard BAR obtained from the Natural Product Laboratory of the *Universidade Federal de Pernambuco* (UFPE) were dissolved in 50 µL of diethyl ether, applied (1 µL) to plates of Merck® silica gel 60 F₂₅₄₊₃₆₆ and developed in the A solvent system (toluene/dioxane/acetic acid [45:12.5:2, v/v/v]). The chromatogram bands were viewed under UV light with short and long wavelengths (254 nm and 366 nm) and subsequently developed through vaporization with 10% sulfuric acid and heating at 50°C for 10 minutes on a hot plate. The results were evaluated based on the color reaction of the bands and the calculation of respective retention factors (R_f) in comparison to the barbatic acid standard [13].

High-performance liquid chromatography (HPLC)

The HPLC analysis of the organic extracts and purified BAR was performed in a HITACHI chromatograph (model 655A-11) coupled to a CG UV detector (model CG437-B). The chromatography conditions followed the method described by Legaz and Vicente [14]: MicroPack MCH-18 reverse phase column (250 × 4.6 mm) with an injection volume of 20 µL; isocratic mobile phase comprised of methanol, water and acetic acid (80:19.5:0.5 v/v/v); pressure 88 atm; room temperature (28°C ± 3°C); UV detector set to 254 nm in internal standards of 0.1 mg.mL⁻¹.

The purified BAR was diluted in diethyl ether until reaching a concentration of 0.1 mg.mL^{-1} and injected into the device. The results were analyzed through a comparison of the retention times of the purified and standard BAR.

Infrared (IR)

Proton nuclear magnetic resonance (NMR ^1H) and carbon 13 nuclear magnetic resonance (NMR ^{13}C): IR analysis was performed in a spectrophotometer with a Bruker Fourier transformer (model IF 566) using KBr pellets. The NMR ^1H and NMR ^{13}C spectra of the purified BAR were recorded in a Varian Unity Plus 300 MHz spectrometer. NMR ^{13}C was operated at 75 MHz and NMR ^1H was operated at 300 MHz, using DMSO- d_6 as the solvent in 5-mm tubes at room temperature.

In vitro antineoplastic assays

The following cells were used for the evaluation of cytotoxic activity: RAW 264.7 (murine macrophages), NCI-H292 (human lung mucoepidermoid carcinoma), HEp-2 (human larynx carcinoma), MCF-7 (human breast adenocarcinoma) and HL-60 (acute promyelocytic leukemia). All cell lines were obtained from the Cell Culture Laboratory of the Department of Antibiotics of UFPE, Brazil. The RAW 264.7, NCI-H292 and HEp-2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (SIGMA). The MCF-7 and HL-60 tumor cells were kept in the RPMI 1640 culture medium created by the Roswell Park Memorial Institute. The media were supplemented with 10% fetal bovine serum (GIBCO) and a 1% antibiotic solution (penicillin and streptomycin). The cells were kept at 37°C in a humid atmosphere enriched with 5% CO_2 .

The organic extracts and purified BAR were submitted to cytotoxicity assays using the MTT colorimetric method [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] [15, 16]. THE RAW 264.7, NCI-H292, MCF-7, HEp-2 (10^5 cells/mL) and HL-60 (0.3×10^6 cells/mL) cells were plated (190 μL) in 96-well plates and incubated for 24 h. Next, 10

μL of the samples dissolved in DMSO (0.5%) (organic extracts: 10 mg/mL; purified BAR: 5 mg/mL) were added to the wells at concentrations of 50, 25, 12.5, 6.25 and 3.12 $\mu\text{g/mL}$ for the organic extracts and concentrations of 25, 12.5, 6.25, 3.12 and 1.56 $\mu\text{g/mL}$ for the purified BAR. The drug doxorubicin at a concentration of 5 $\mu\text{g/mL}$ was used as the positive control and DMSO (0.5%) was used as the negative control. After 72 h of incubation, 25 μL of MTT (5 mg/mL) were added and the plates were incubated again for 3 h, after which the culture medium with the MTT was aspirated and 100 μL of DMSO were added to each well to solubilize the crystals that had formed.

Absorbance was read in a microplate reader at a wavelength of 560 nm. The experiments were conducted in quadruplicate. An intensity scale was used to evaluate the cytotoxic potential of the samples based on the percentage of growth inhibition: 95-100% = high potential; 70-90% = moderate potential; < 50% = no potential [17]. The concentration that inhibits growth by 50% in comparison to the negative control (IC_{50}) was determined considering moderate inhibitory activity (70%) or higher.

In vitro genotoxicity assays: micronucleus test and comet assay

Sarcoma 180 and Ehrlich carcinoma cells maintained in mice in their ascitic forms were used for the genotoxicity assays. The cells were obtained during the weekly maintenance of the tumors. The tumor lines were maintained through the intraperitoneal transference of the ascitic fluid of the donor animal to the receptor mouse. As only a suspension of 0.6 mL of aspirated ascitic fluid and 0.4 mL of 0.9% saline solution was inoculated into the receptor mouse, the remaining aspirated ascitic fluid was used for the *in vitro* tests. At the Nanotechnology, Biotechnology and Cell Culture Laboratory of the Academic Center of Vitória of UFPE, Brazil, the ascitic tumors sarcoma 180 and Ehrlich carcinoma

were transferred to Falcon tubes and centrifuged for 5 min at 2500 rpm. The supernatant was discarded and a 100- μ L sample of the cell precipitate was re-suspended in 900 μ L of DMEM culture medium (SIGMA) for the cell count in a Neubauer chamber. Based on the count, the concentration of cells was adjusted with the culture medium to 10^6 cells/mL.

For the micronucleus test, the tumor cells (10^6 cells/mL) were plated (900 μ L) in 24-well plates. The samples dissolved in DMSO (0.5%) were added to the wells (100 μ L) at a final concentration of 50 μ g/mL for the organic extracts and 20 and 40 μ g/mL for the purified BAR. The drugs cyclophosphamide and colchicine (50 μ g/mL) were used as the positive controls and DMSO (0.5%) was used as the negative control. Next, slides were prepared for the microscopic analysis. For such, the cells were stained with acridine orange 0.04 M.

The slides were analyzed in a blind test under a fluorescence microscope (Axio Imager.M2, Zeiss) with magnification of 1000 x and using an Alexa Fluor 488 filter. The analysis of micronuclei and bi-nucleated cells was based on Fenech [18]. The experiment was conducted in triplicate. Therefore, 3000 cells (1000 cells/slide) were evaluated for each treatment. The cytokinesis-block proliferation index (CBPI) was determined by the number of mononucleated, binucleated and multinucleated cells found among 1500 cells analyzed per treatment (500 cells/slide). The CBPI was calculated based on the OECD Guidelines for Testing of Chemicals [19], using the following formula:

$$\text{CBPI} = \frac{[(\text{N}^\circ \text{ of mononucleated cells}) + (2 \times \text{N}^\circ \text{ of binucleated cells}) + 3 \times \text{N}^\circ \text{ of multinucleated cells}]}{(\text{total number of cells})}$$

For the comet test, the tumor cells (10^6 cells/mL) were plated and treated under the same condi-

tions as those described for the micronucleus test. Cells in treated culture for 6 h with the samples were removed from the plate, centrifuged and re-suspended in new DMEM. The comet assay was performed based on Speit and Hartmann [20]. The stain was ethidium bromide and the analysis was performed using a fluorescence microscope (Axio Imager.M2, Zeiss) with magnification of 400 x and using an Alexa Fluor 546 filter. A total of 300 cells were analyzed per treatment (100 cells/slide).

The extent of the DNA that migrated from the nucleus was analyzed based on the visual classification described by Collins *et al.* [21]. From the classification of the cells analyzed, the results were evaluated according to two parameters: the damage index (DI: 0-400) and damage frequency (DF: 0-100%). The DI is the result of the sum of the number of cells found in each class multiplied by the value of the respective class. The DF is obtained from the total number of cells analyzed subtracted from the number of cells classified as grade 0, resulting in a percentage value of cells that exhibit any degree of detectable damage (1-4).

Statistical analysis

The percentage of inhibition (IC_{50}) and respective 95% confidence intervals (CI) obtained from the nonlinear regression were calculated with the aid of the *GraphPad Prism* 5.0 program. The level of significance was set to 5%. Fisher's exact test was used to determine differences in the frequencies of micronuclei between each treated group and the negative control as well as the group that received only DMSO. The Kruskal-Wallis with *a posteriori* analysis was used for the hypothesis tests referring to the CBPI, DI and DF, using the pairwise t-test strategy with the Bonferroni correction. For all hypothesis tests, the level of significance was set to 5%. All analyses were performed in the R software [22].

Results

Obtainment of organic extracts and chemical analysis of samples

The yield of the organic extract demonstrated the efficiency of diethyl ether in the hot exhausted extraction process, as the yield was higher (7.65%) in comparison to that achieved with chloroform (0.04%) and acetone (0.03%). The TLC analysis revealed the presence of BAR in all organic extracts (Rf: 0.57) (**Figure 1**). Two other bands were evidenced in the acetone extract (Rf: 0.15 and 0.18), corresponding to other compounds that may be present in *C. salzmännii*, such as the thamnolic acid and D-thamnolic acid described by AHTI, STENROOS and XAVIER-FILHO [23] (1993). Point 4, which corresponds to the purified BAR, had one band, which was compatible with the Rf of the BAR standard (0.57).

Figure 1: Schematic model of thin-layer chromatogram of organic extracts from *C. salzmännii* Nyl. and BAR purified from ether extract.

The organic extract, purified BAR and standard BAR were submitted to HPLC. A peak corresponding to the purified BAR (Tr: 19.09 min) demonstrated 96.3% purity (**Figure 2**). The HPLC analysis (**Figure 2A-D**) demonstrated the presence of barbatric acid in the chloroform and ether extracts (**Figure 2A & D**), with the higher level of this phenol found

Figure 2: HPLC chromatogram of purified BAR

A) diethyl ether (Tr: 4.15 min); B) purified BAR (Tr: 19.09min); C) Acetone extract; D) Chloroform extract.

in the ether extract. This component was not found in the acetone extract (**Figure 2C**), for which two peaks were found (Tr 3.45 and 12.38 min).

The infrared, NMR ^1H and NMR ^{13}C analyses confirmed the chemical structure of the purified BAR. The following data describe the results of these analyses: IR (KBr) cm^{-1} : 3100 (ν C-H) aromatic; 2984 (ν_{as} CH_3); 2932 (ν_{s} CH_3); 1720 (ν O=C-O) ester; 1660 (ν Ar- O=C-O) ester conjugated with aromatic ring; 1574 and 1505 (ν C=C) aromatic; 1444 (ν_{as} CH_3); 1390 (ν_{s} CH_3); 1233 and 1130 (ν two bands confirming ester). NMR ^1H (299 MHz, DMSO-d_6) δH : 1.99 (3H; s, Me-8'); 2.00 (3H; s, Me-8); 2.41 (3H; s, Me-9'); 2.56 (3H; s, Me-9); 3.86 (3H; s, MeO-4), 6.60 (1H; s, H-5); 6.69 (1H; s, H-5'), 10.73 (1H; s, HO-2). NMR ^{13}C (75 MHz, DMSO-d_6) δC : C-1: 110.0; C-2: 151.8; C-3: 106.3; C-4: 161.3; C-5: 107.1; C-6: 138.9; C-7: 168.6; C-8: 8.1; C-9: 22.8; C-4-OMe: 55.7; C-1': 159.4; C-2': 111.4; C-3': 161.1; C-4': 116.1; C-5': 139.0; C-6': 116.1; C-7': 173.2; C-8': 9.1; C-9': 23.8.

Cytotoxic activity of organic extract and purified BAR

Table 1 displays the inhibitory capacity of the organic extracts, purified BAR and doxorubicin (positive control) regarding the growth of the tumor cells. The ether extract demonstrated moderate inhibitory capacity (cell growth inhibition ranging from 70 to 90%) toward the RAW-264.7, NCI-H292, HEP-2 and HL-60 cell lines, but exhibited no cytotoxic poten-

tial with regard to MCF-7. The chloroform extract demonstrated moderate inhibitory capacity toward HEP-2 and MCF-7, but had no significant inhibitory effect on RAW-264.7, NCI-H292 or HL-60. The acetone extract demonstrated high inhibitory capacity (95-100%) toward NCI-H292 and moderate inhibitory capacity toward MCF-7, but had no significant effect on the growth of RAW-264.7, HEP-2 or HL-60. The purified BAR demonstrated moderate inhibitory capacity toward RAW-264.7, HEP-2 and MCF-7, but had no significant effect on NCI-H292 or HL-60.

The purified BAR and organic extracts that exhibited moderate to high inhibitory activity were tested at different concentrations (0.098 to 25 $\mu\text{g}/\text{mL}$ for purified BAR and 0.098 to 50 $\mu\text{g}/\text{mL}$ for organic extracts) for the determination of IC_{50} values. Doxorubicin (0.009 to 1.25 $\mu\text{g}/\text{mL}$) was used as the standard.

Table 2 displays the IC_{50} values of the organic extract and purified BAR. The purified BAR exhibited cytotoxic potential against all cell lines tested. The ether extract exhibited cytotoxic potential against the neoplastic cell lines testes, particularly HL-60 ($\text{IC}_{50} = 3.59 \mu\text{g}/\text{mL}$), but did not exhibit cytotoxic potential against RAW-264.7 ($\text{IC}_{50} = 36.54 \mu\text{g}/\text{mL}$). The chloroform extract exhibited no significant cytotoxic potential against the cell lines. The acetone extract exhibited cytotoxic potential against NCI-H292 and MCF-7.

Table 1. Percentage of inhibition to growth of human tumor cell lines and macrophage.

Samples	Concentration	% of inhibition				
	($\mu\text{g}/\text{mL}$)	RAW-264.7	NCI-H292	HEP-2	HL-60	MCF-7
Doxorubicin	1.25	96.5 $\pm$ 0.8	94.1 $\pm$ 2.0	79.4 $\pm$ 2.6	92.9 $\pm$ 0.6	74.8 $\pm$ 2.1
Purified BAR	25	79.2 $\pm$ 1.9	8.7 $\pm$ 0.4	86.5 $\pm$ 3.9	41.4 $\pm$ 3.1	71.1 $\pm$ 4.4
E extract	50	82.5 $\pm$ 1.1	80.1 $\pm$ 5.0	89.5 $\pm$ 4.2	83.9 $\pm$ 4.4	50.9 $\pm$ 2.3
C extract	50	23.3 $\pm$ 0.0	58.7 $\pm$ 2.1	73.4 $\pm$ 3.6	58.0 $\pm$ 3.1	72.0 $\pm$ 1.3
A extract	50	59.6 $\pm$ 1.2	98.4 $\pm$ 0.9	62.6 $\pm$ 1.3	38.0 $\pm$ 1.6	71.3 $\pm$ 1.0

BAR = barbatic acid; E = ether; C = chloroform; A = acetone; data expressed as mean and standard deviation. The tests were done in quadruplicate and in 3 different experiments.

Table 2. Cl_{50} of purified BAR and organic extracts .

Samples	Concentration	% of inhibition				
	($\mu\text{g/mL}$)	RAW-264.7	NCI-H292	HEP-2	HL-60	MCF-7
Doxorubicin	1.25	0.35	0.08	0.31	0.06	0.11
		0.26 – 0.47	0.06 – 0.12	0.26 – 0.36	0.05 – 0.07	0.08 – 0.15
Purified BAR	25	20.79	NT	15.79	NT	18.28
		18.54 – 23.32		13.59 – 18.34		13.05 – 25.62
E extract	50	36.54	29.91	26.75	3.59	NT
		33.85 – 40.26	26.20 – 34.14	22.13 – 32.35	2.38 – 5.41	
C extract	50	NT	NT	33.80	NT	36.5
				28.0 – 41.0		29.51 – 45.13
A extract	50	NT	16.60	NT	NT	7.55
			14.21 – 19.40			6.02 – 9.48

BAR = barbatic acid; E = ether; C = chloroform; A = acetone; NT = not tested; The tests were done in quadruplicate and in 3 different experiments. The values were expressed as IC_{50} (concentration that inhibited cell proliferation by 50%) and confidence interval using Graphpad Prima 8 Demo.

Genotoxic activity of organic extract and purified BAR

Table 3 displays the mean CBPI of the sarcoma 180 and Ehrlich carcinoma tumor lines. All samples tested had significantly different CBPI values from that of the negative control for Ehrlich carcinoma. For

Table 3. Mean cytokinesis-block proliferation index (CBPI) of cell lines treated with purified BAR and organic extracts ($\mu\text{g/mL}$) obtained from *C. salzmännii* Nyl.

Samples	Concentration	Mean CBPI	
	($\mu\text{g/mL}$)	Sarcoma 180	Ehrlich carcinoma
Purified BAR	5	1.39 $\pm$ 0.01	1.44 $\pm$ 0.01*
Purified BAR	10	1.39 $\pm$ 0.01	1.42 $\pm$ 0.01*
Purified BAR	20	1.38 $\pm$ 0.01	1.39 $\pm$ 0.01*
Purified BAR	40	1.17 $\pm$ 0.04*	1.21 $\pm$ 0.01*
E extract	50	1.09 $\pm$ 0.01*	1.12 $\pm$ 0.01*
C extract	50	1.23 $\pm$ 0.01*	1.19 $\pm$ 0.02*
A extract	50	1.36 $\pm$ 0.02	1.31 $\pm$ 0.03*
DMSO (NC)	0.5%	1.41 $\pm$ 0.01	1.53 $\pm$ 0.03

BAR = barbatic acid; E = ether; C = chloroform; A = acetone, DMSO = dimethyl sulfoxide; NC = negative control

*: significantly different from negative control ($p < 0.005$ - Sarcoma 180 / $p < 0.002$ - Ehrlich carcinoma)

sarcoma 180, however, only the purified BAR at a concentration of 40 $\mu\text{g/mL}$ and the ether and chloroform extracts at a concentration of 50 $\mu\text{g/mL}$ had significantly different CBPI values from that of the negative control. Therefore, considering cell proliferation, Ehrlich carcinoma demonstrated high sensitivity to all samples tested.

Table 4 displays the mean number of micronuclei (MN) per treatment in the sarcoma 180 and Ehrlich carcinoma cell lines. For sarcoma 180, statistically significant differences were found for all treatments in comparison to the negative control (DMSO), with the exception of the chloroform extract at a concentration of 50 $\mu\text{g/mL}$ and the purified BAR at concentrations of 5 and 10 $\mu\text{g/mL}$. For Ehrlich carcinoma, all treatments differed significantly from the negative control except the purified BAR at a concentration of 5 $\mu\text{g/mL}$.

According to the comet assay, all samples induced damage to DNA in both the sarcoma 180 and Ehrlich carcinoma tumor lines (**Table 5 & 6**). Considering the damage frequency and damage index, the purified BAR at a concentration of 40 $\mu\text{g/mL}$ exhibited the greatest damage to the DNA in both tumor lines.

Table 4. Mean frequency of micronuclei (MN) in binucleated cells treated with purified BAR and organic extracts (µg/mL) obtained from *C. salzmannii* Nyl.

Samples	Concentration	Mean frequency of MN	
	(µg/mL)	Sarcoma 180	Ehrlich carcinoma
Purified BAR	5	16.3 ± 4.04	9.7 ± 1.53
Purified BAR	10	18 ± 3.00	15.3 ± 3.51*
Purified BAR	20	32 ± 5.09*	19.7 ± 2.64*
Purified BAR	40	35 ± 6.37*	21.3 ± 3.23*
E extract	50	39.3 ± 6.21*	30.7 ± 3.42*
C extract	50	18.3 ± 4.46	14.3 ± 4.41*
A extract	50	24.7 ± 6.18*	20.3 ± 4.59*
Cyclo-phosphamide (PC)	50	54.7 ± 2.84*	35 ± 4.24*
Colchicine (PC)	50	69 ± 2.93*	45.7 ± 3.38*
DMSO (NC)	0.5%	9.3 ± 3.39	3.0 ± 1.61

MN = micronuclei; BAR = barbatic acid; E = ether; C = chloroform; A= acetone; DMSO = dimethyl sulfoxide; PC = positive control; NC = negative control
*: significantly different from negative control (p < 0.005 - Sarcoma 180 / p < 0.002 - Ehrlich carcinoma)

Table 5. Mean damage frequency for cell lines treated with purified BAR and organic extracts (µg/mL) obtained from *C. salzmannii* Nyl.

Samples	Concentration	Mean damage frequency	
	(µg/mL)	Sarcoma 180	Ehrlich carcinoma
Purified BAR	5	41.3 ± 2.51*	67.6 ± 4.51*
Purified BAR	10	71.7 ± 2.31*	68.6 ± 3.21*
Purified BAR	20	86.7 ± 1.41*	78.6 ± 2.51*
Purified BAR	40	92.3 ± 1.26*	91 ± 2.00*
E extract	50	93.3 ± 0.54*	87.3 ± 1.52*
C extract	50	79.3 ± 1.73*	80.6 ± 2.51*
A extract	50	45.7 ± 1.41*	38.6 ± 2.51*
Cyclo-phosphamide (PC)	50	100 ± 0.00*	98.6 ± 1.15*
Colchicine (PC)	50	99.7 ± 0.54*	99.6 ± 0.57*
DMSO (NC)	0.5%	17.0 ± 1.63	11.3 ± 2.08

BAR = barbatic acid; E = ether; C = chloroform; A= acetone; DMSO = dimethyl sulfoxide; PC = positive control; NC = negative control; *: statistically significant difference compared to negative control (p < 0.002).

Table 6. Mean damage index for cell lines treated with purified BAR and organic extracts (µg/mL) obtained from *C. salzmannii* Nyl.

Samples	Concentration	Mean damage frequency	
	(µg/mL)	Sarcoma 180	Ehrlich carcinoma
Purified BAR	5	82.7 ± 8.14*	149.3 ± 5.77*
Purified BAR	10	165.7 ± 5.50*	150.6 ± 11.92*
Purified BAR	20	237.7 ± 6.50*	172.3 ± 3.78*
Purified BAR	40	255 ± 7.81*	254.6 ± 12.50*
E extract	50	244.7 ± 8.38*	233.6 ± 3.51*
C extract	50	169.7 ± 11.80*	181.3 ± 5.85*
A extract	50	71.7 ± 4.50*	63.6 ± 5.50*
Cyclo-phosphamide (PC)	50	308.7 ± 6.65*	278 ± 2.00*
Colchicine (PC)	50	329.3 ± 7.57*	314 ± 4.00*
DMSO (NC)	0.5%	19 ± 2.64	12 ± 2.64

BAR = barbatic acid; E = ether; C = chloroform; A= acetone; DMSO = dimethyl sulfoxide; PC = positive control; NC = negative control; *: statistically significant difference compared to negative control (p < 0.002)

Discussion

The chromatographic analyses (TLC and HPLC) revealed the presence of barbatic acid in the ether and chloroform extracts obtained from *C. salzmanii*. This is the main compound found in the species, as described by AHTI, STENROOS and XAVIER-FILHO [23]. According to the authors, 4-O-dimethyl barbatic, thamnolic and D-thamnolic acids are also occasionally found in the species. The chromatographic analyses also demonstrated additional bands and peaks consistent with the presence of thamnolic and D-thamnolic acids.

Based on the IC_{50} values, considerable antineoplastic potential was found for the ether and acetone extracts and the purified BAR against the tumor cells investigated. According to the US National Cancer, extracts with an $IC_{50} \leq 4 \mu\text{g/mL}$ are considered promising drugs for cancer treatment [24]. Therefore, the ether extract demonstrated high antineoplastic activity against the HL-60 cell line ($IC_{50} = 3.59 \mu\text{g/mL}$). Considering the cytokinesis-block proliferation index (CBPI), the cytotoxic activity of the purified BAR at concentrations of 5, 10, 20 and 40 $\mu\text{g/mL}$ and the activity of the organic extracts at a concentration of 50 $\mu\text{g/mL}$ was relevant, specifically toward Ehrlich carcinoma cells. However, only the purified BAR at a concentration of 40 $\mu\text{g/mL}$ and the chloroform and ether extracts at a concentration of 50 $\mu\text{g/mL}$ were considered cytotoxic to sarcoma 180. This tumor is considered more resistant to chemotherapeutic drugs. Therefore, the findings show that Ehrlich carcinoma demonstrated high sensitivity to the samples tested.

In a previous study, Martins *et al.* [25] demonstrated the antineoplastic potential of the ether extract and purified barbatic acid from *C. aggregata* against the HEP-2, NCI-H292 and KB cell lines. The results demonstrated greater antineoplastic potential for the ether extract and the authors suggest that this effect is due to the synergic action of the components of the extract. Moreover, dose-dependent

antineoplastic activity was found for all samples. In the present study, no synergic effect was found on the cells lines tested.

In a study that has not yet been published, Tavares [26] evaluated the cytotoxicity of encapsulated BAR purified from *C. salzmännii* against the J774, sarcoma 180 and Ehrlich carcinoma cells lines. The results also demonstrate the dose-dependent cytotoxic potential of purified BAR.

The results of the comet assay demonstrated the genotoxic effect on sarcoma 180 and Ehrlich carcinoma of all samples tested. However, the micronucleus test demonstrated that the purified BAR at a concentration of 5 $\mu\text{g/mL}$ was not genotoxic to either cell line. Moreover, the micronucleus test demonstrated that the chloroform extract and purified BAR at a concentration of 10 $\mu\text{g/mL}$ were not genotoxic to sarcoma 180. In the literature, previous studies have evaluated the genotoxicity of substances stemming from the secondary metabolism of lichens. The *in vitro* and *in vivo* genotoxicity of usnic acid obtained from *Usnea steineri* was evaluated using the micronucleus test and comet assay [11]. V79 cells (Chinese hamster lung fibroblasts) were treated with usnic acid at concentrations of 15, 30, 60 and 120 $\mu\text{g/mL}$ and Swiss mice were treated with concentrations of 25, 50, 100 and 200 $\mu\text{g/kg}$. The *in vitro* results demonstrated damage to the DNA at concentrations of 60 and 120 $\mu\text{g/mL}$ in the comet assay, but none of the concentrations exhibited genotoxic potential against V79 cells on the micronucleus test. Moreover, the researchers found no genotoxic effects at any of the concentrations tested in the *in vivo* analysis.

Recently, the methanol extract obtained from *Cetraria islandica* increased the frequency of micronuclei in a dose-dependent manner in lymphocytes from peripheral blood, but the increase was only significant at the highest concentrations tested (50, 100 and 200 $\mu\text{g/mL}$) [10].

Conclusion

The present findings demonstrate the *in vitro* cytotoxic and genotoxic potential of organic extracts and purified barbatic acid obtained from the lichen *C. salzmannii*. The data regarding the *in vitro* cytotoxic potential of the samples tested corroborate descriptions found in the literature that demonstrate the promising antineoplastic profile of lichen substances against different tumor cell lines, which can lead to the development of drugs for the treatment of cancer. The evaluation of the genotoxicity revealed that the samples tested induced changes in the genetic material of the tumor cells investigated.

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