In Vitro and in Vivo Antineoplastic Activity of Barbatic Acid

ORIGINAL

Abstract

Background: Lichen compounds exhibit remarkable biological activity. Several of them are potent anticancer agent and some with low or none toxicity. The aim of this study was to perform *in vitro* and *in vivo* assessments of the antineoplastic activity of organic extract and barbatic acid (BAR) isolated from the lichen *Cladia aggregata* (Sw.) Nyl.

Methods: *In vitro* assays were performed with both extract and BAR against HEP-2 (Adenocarcinoma of the Larynx), NCI-H292 (Squamous Cell Lung Carcinoma) and KB (Nasopharyngeal Squamous Cell Carcinoma) cells. The tests were carried out on the Sarcoma-180 BAR, tumor and organs were analyzed by histopathological assays after 7 days of chemotherapy.

Results: Cytotoxic tests with BAR revealed 50% inhibitory concentration (IC_{50}) of $19.06 \mu\text{g mL}^{-1}$ for NCI-H292 and $12.0 \mu\text{g mL}^{-1}$ for KB and $6.25 \mu\text{g mL}^{-1}$ for HEP-2 cells. Tests with Sarcoma-180 demonstrated 46.3% inhibitory activity against the tumor by BAR. This substance showed no significant effect on the expression of argyrophilic nucleolar organizer regions proteins (AgNORs). The histopathology study of neoplastic tissue, demonstrated that cell proliferation was not affected by the antineoplastic action of the compound tested.

Conclusions: The results indicate that both barbatic acid and ether extract exhibits significant antineoplastic activity and low toxicity rate.

Background

The increase in the incidence of malignant tumors worldwide has stimulated the search for new drugs and therapies that may control the spread of cancer. The available pharmaceutical arsenal is composed of substances of natural or synthetic origin. However, the particularities of each tumor type and possible resistance to chemotherapeutic drugs

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constitute obstacles that underscore the need for new drugs with antineoplastic activity [1].

Lichens are biological structures formed by a combination of a mycobiont (fungus) and one or more photobionts (algae and or cyanobacteria). Specialists agree that the combination ranges from parasitism to a strict mutualism [2]. However, this combination is delicately balanced and any alteration in the growth and/or death rates of the components may lead to the death of the lichen. Lichen symbiosis is a successful evolutionary strategy that can result in a rich diversity of fungal species that produce exclusive substances of great biological potential from the depside, depsidone and dibenzofuran class (usnic acids), among others [3].

Lichen compounds commonly have two aromatic rings, the ideal orientation of which is approximately 125°. Carboxyl and hydroxyl are fundamental to biological activity in these substances [4]. Pharmacological use of lichen extracts includes antifungal [5], antibacterial [6, 7] and antitumor [8, 9] activity, as well as the prevention of cancer cells.

With regard to antineoplastic activity, there are promising reports associating compounds that are metabolically related to barbatic acid with the *in vitro* inhibition of cancer development. LIMA *et al.* [10] describe the inhibitory action of usnic acid (80% against Sarcoma-180) extracted from *Cladonia substellata*. SANTOS *et al.* [11] describe similar results with this in a nanoencapsulated form. Methanol extracts of *Umbilicaria esculenta* and *Usnea longissima* significantly reduce melanin formation in human melanomas [12]. Sphaerophorin (depside) and panarin (depsidone) have the same effect, inducing cell death by apoptosis [13]. The inhibition of tubulin polymerization has been described for substances from the lichen *Stereocaulon sasakii*, particularly lobaric acid [4]. Nevertheless, recent studies indicate that not all compounds have the same active site. For example, usnic acid inhibits the proliferation of breast tumors at a concentration of 29 μ M, but does not alter the formation and/or stability

of microtubules, which suggests that there is no association between microtubules and the action mechanism of this compound [14].

Due the potential activity of lichen compounds, the study of their extracts and purified substances constitutes a contribution to the knowledge of the natural products for treatment and cure of diseases. In addition, new compounds with low or no degree of toxicity are desirable, due to side and adverse effects of drugs used in chemotherapy. This way, the aim of the present study was to perform assays *in vitro* and *in vivo* the antineoplastic activity of barbatic acid and ethereal extract obtained from the lichen *Cladia aggregata*, in order to contribute to the search for new anticancer drugs with anti-tumor activity.

Methods

Collection and storage of lichen

Cladia aggregata (Sw.) Nyl. samples (200 g) were previously identified by Dr. E.C. Pereira through morphological and chemical analysis. The material was collected Bonito municipality, Brazil. The lichen was stored in paper bags and kept at room temperature ($28 \pm 3^\circ\text{C}$) until tests be performed. A specimen was deposited in the Herbarium UFP - Geraldo Mariz, of the Botany Department, Universidade Federal de Pernambuco (Brazil) voucher n° 36431.

Obtainment of extracts and isolation, purification and chemical characterization of barbatic acid (BAR)

Organic lichen extracts were obtained using Soxhlet extraction (30°C) using diethyl ether. BAR isolation and purification from ether extract, as well as chemical characterization/spectroscopic assays determined by HPLC, TLC, ^1H , ^{13}C NMR and UV spectra were based on the data described by MARTINS *et al.* [6].

Cytotoxic assays

Cytotoxic activity and cell viability cytotoxicity tests were performed with the HEp-2 (Adenocarcinoma of the Larynx), NCI-H292 (Squamous Cell Lung Carcinoma) and KB (Nasopharyngeal Squamous Cell Carcinoma) cell lines provided by the Cell Culture Laboratory, Department of Antibiotics, Universidade Federal de Pernambuco (Brazil). Cells were kept in Minimum Essential Medium, Dulbecco's Modified Eagle's Medium (SIGMA), supplemented with 10% Fetal Bovine Serum (GIBCO), 1% antibiotic solution (1000 U mL⁻¹ of penicillin + 250 mg mL⁻¹ of streptomycin) and 200 mM of 1% L-glutamine [15]. The cell suspensions (10⁵ cells mL⁻¹) were distributed in 96 culture plates (220 µL/plate). The plates were incubated at 37°C (Sedas, Milan-Italy) with a humid atmosphere supplied with 5% CO₂. After 24 h of incubation, 22 µL/plate of barbatic acid and/or ether extract were added, diluted in DMSO at final concentrations of 20, 10, 5 and 2.5 µg mL⁻¹ for BAR and 6.5, 12, 25 and 50 µg mL⁻¹ for ether extract. DMSO was used as the control. After 72 h of treatment, the cytotoxic effects of the samples were assessed using the MTT colorimetric method 3- (4.5-dimethylthiazol-2.5-diphenyltetrazolium) (MTT). Cell viability was determined using 0.4% Trypan Blue vital dye (Merck). The results were assessed in terms of cell inhibition in comparison to the control group, with the determination of the percentage of living and dead cells [16].

In vivo assays

Determination (LD₅₀) and antitumor activity

The antitumor activity assays were preceded by tests for the determination of the toxicity of the pyrimidine derivatives, following the methodology proposed by Karber and Berhrens [17] and modified by Berlion [18]. Male albino Swiss mice were used in the experiments, divided into two groups of ten animals. The administration technique was intraperitoneal injection.

The assays consisted of preliminary phase and definitive phases. In the preliminary phase, the animals were divided into three groups and received the test drug (BAR) in a single dose at increasing concentrations, following a geometric progression with a common ratio of 2.0. The aim was to determine the highest non-lethal dose (D₁) and the lowest 100% lethal dose (D₂). Then the animals received doses of the drug ranging between D₁ and D₂, following a geometric progression with a common ratio of 1.5. In the sequence, the animals were observed for 4 h (with notes recorded every 10 minutes) and monitored for periods of 24, 48 and 72 h. The final results were determined using the following formula: LD 50% = $Df - \sum (a.b)/n$. **Df** = lowest 100% lethal dose; **a** = difference between two consecutive doses; **b** = mean number of dead animals between two consecutive doses; **n** = number of mice per batch; **LD** = lethal dose.

Antitumor activity

Female albino Swiss mice (*Mus musculus*) with a mean weight of 48.25 g provided with chow (Labina®, Purina Brazil Ltd.) and water *ad libitum*. The mice were divided into two groups (n = 5) and subsequently inoculated in the right axillary region with a tumor cell suspension (0.2 mL at a concentration of 5x10⁶ cells mL⁻¹) developing a solid tumor (Sarcoma-180) provided by the Department of Antibiotics, Universidade Federal de Pernambuco (Brazil). The control group received doses of saline solution 0.9% and Tween 80 at 0.05% (v/v) corresponding negative control (C⁻) and 5-Fluorouracil (5-Flor) as positive control (C⁺) (3.5 mL kg⁻¹ of body weight) through intraperitoneal injection. The experimental group was treated with doses of 10% LD₅₀ (75.69 mg kg⁻¹), corresponding to 7.60 mg kg⁻¹ of BAR, every 24 h for seven days. At the end of the experiment, the mice were sacrificed through cervical dislocation and the tumors were surgically removed and weighed. The tissues and organs were analyzed to determine microscopic changes induced by

the treatment. The tumor inhibition was determined from the average weight of animals treated groups compared to the untreated group: $IT\% = CT/C \times 100\%$, where C is the average weight of the animals from the negative control group (C^-) and T is the average weight of the animals in the positive control ($C^+ = \text{BAR or 5-Flor}$). To calculate the average weight of the tumors of the control group was waived those whose weights corresponded to 0.39 g or less, called "no-takes". The entire experimental protocol received prior approval from the Research Ethics Committee of the Universidade Federal de Pernambuco (Brazil) n° 23076.016437/2013-18.

Histopathological analysis and argyrophilic nucleolar organizer regions (AgNOR) proteins staining procedure

Fragments of the tumor, kidneys, liver and spleen were washed with physiological serum and subsequently maintained in a fixative solution of 10% buffered formaldehyde for 24 h. The resulting biological material was dehydrated, clarified and embedded in paraffin. Semi-serial cuts with a thickness of 5 μm were performed. The cuts were stained using the hematoxylin and eosin technique for subsequent histopathological analysis under an optical microscope microscope Nikon E-200.

The technique recommended by Ploton et al. [19] was used to quantify the AgNORs, whereby histological cuts were incubated in a darkroom for 30 minutes at 37° C in a colloidal silver solution containing one part gelatin (2%) in formic acid (1%) and two parts silver nitrate (50%) aqueous solution. The slides were dehydrated, clarified and mounted with DPX. Histological images were captured with a digital camera (Moticam 2300) coupled to an optical microscope (Nikon E-200) at a final magnification of 400 X. The quantification was performed using the ImageJ 1.44 program (National Institute of Health, Bethesda, MD, USA). AgNORs were seen as black dots within the nuclei of the cells. The mean number of AgNOR per nu-

cleus was calculated, and for each animal, a total of 1200 cells were randomly assessed in the control and experimental groups.

Statistical analysis

The statistical analysis and deviations (SD) to organs and tumor weight were performed using GraphPad Prism 5.0 for windows (GraphPad Software San Diego, CA, USA) and the data were expressed as replicate means $\pm$ SD. Significant differences were established using one-way analysis of variance (ANOVA) and Turkey's test at $p < 0.05$ were considered significant. The mean number of AgNOR per nucleus was calculated using the SPSS 15.0 program and the Students test, with the significance level set at 5% ($p < 0.05$). The lethal concentrations (LC_{50}) was calculated using probit analysis with a reliability interval of 90% using the StatPlus 168 2006 software (AnalystSoft, Canada).

Results

Cytotoxicity tests on the carcinogenic cell lines submitted to the ether extract at concentrations of 6.5, 12, 25 and 50 $\mu\text{g mL}^{-1}$ achieved the following respective inhibition percentages: 60, 70, 72 and 78% for HEp-2; 36, 42, 61 and 63% for NCI-H292; and 47, 52, 80 and 80% for KB cells (**Figure 1A**). The BAR achieved lower inhibition percentages, suggesting synergism between the substances in the organic extract: 49, 54, 61 and 66% for HEp-2; 36, 7, 42, 61.1 and 63.4% for NCI-H292; and 43, 53, 71 and 77% for KB cells at concentrations of 2.5, 5.0, 10 and 20 $\mu\text{g mL}^{-1}$, respectively (**Figure 1B**).

The 50% inhibitory concentration (IC_{50}) of the purified substance and the ether extract is shown on **table 1**. The BAR was active against all the tested neoplastic cells. The most sensitive was the HE-p2 cells. The solid tumors reached 1.42 g in the experimental group and 2.65 g in the C^- group (**Figure 1A**). A reduction in weight ($**p < 0.05$, $F = 225.7$) was found in the kidneys (0.39 g) and liver (2.11 g), whe-

Figure 1: Adenocarcinoma of Larynx (HEp-2), Squamous Cell Lung Carcinoma (NCI-H292) and Nasopharyngeal Squamous Cell (KB) treated in vitro (% inhibition) with compounds from *Cladia aggregata*. A: ether extract; B: purified BAR.

reas a slight increase was found in the spleen (0.21 g) (**Figure 2 B**) (DL50= 765 mg kg⁻¹). When compared to the negative control, the reduction of organs weight was not significant, negative control vs liver (mean diff. 0.02368, 95% CI of diff. -0.2485 to 0.2959, ***p*<0.05), negative control vs kidney (mean diff. 0.04136, 95% CI of diff. -0.2309 to 0.3136, ***p*<0.05) and negative control vs spleen (mean diff. 0.1887, 95% CI of diff. -0.08352 to 0.4609, *p*>0.05). Despite of this, no histopathological changes were observed in these organs. BAR displayed

Table 1. IC₅₀ of ether extract and purified barbatic acid from *Cladia aggregata*, expressed as µg mL⁻¹.

Cells line	IC ₅₀ (µg mL ⁻¹)	
	Ether extract	Purified BAR
HEp-2	ud.	6.25
NCI-292	19.5	19.6
KB	8.9	12
ud: Undetermined		

Figure 2: A: Mean weigh of Sarcoma-180 (g); B: mean weigh of organs (g) after treatment with purified BAR (7.60 mg kg⁻¹) from *Cladia aggregata*.

***: *p*<0.001. C⁺, C: positive and negative controls, respectively. Letter "a" to indicate that the bars are no significantly different from each other (Tukey's test **: *p*<0.05) when compared control group (C).

Figure 3: Photomicrographs showing Sarcoma-180 tumor cells; AgNOR expression in (A) and (B), silver staining sheets of cells with marked nuclear atypical and irregular nuclear contours (C); Hematoxylin; magnification 400 X.

an inhibitory effect on tumor growth against Sarcoma-180, with a statistically significant ($p < 0.001$, $F = 89.10$) reduction in tumor weight (61%). This final inhibition percentage was calculated using the mean of the tumors measured seven days after the administration of the BAR.

The histopathological tumor analysis revealed groups of large, pleomorphic, anaplastic cells with an increased nucleus-cytoplasm ratio. The nuclei of these cells were notably hyperchromatic, with loose chromatin and evident nucleoli. A high mitotic index with atypical mitoses was also recorded (Figure 3 A, B, C). Neoplastic invasion of muscle and adipose tissue, hemorrhagic foci and coagulation necrosis were easily recognizable.

AgNORs were easily distinguished as black spots on the nucleus of tumor cells and were visible either as large round argyrophilic structures or as small

scattered units. The mean number of AgNORs per tumor cell was slightly higher in the control group (1.55 ± 0.35) than in the group treated with barbatolic acid (1.52 ± 0.24), although the difference between the groups was not statistically significant ($p = 0.612$).

Discussion

Recently, the screening of lichen substances against malignant mesothelioma MM98, vulvar carcinoma A431 and HaCat keratinocytes revealed sensitivity to usnic, salazinic, vulpinic and gyrophoric acids. The most pronounced cytotoxic activity was attributed to usnic acid. A combination of gyrophoric and usnic acids is reported to promote an increase in tissue regeneration [20]. Usnic acid is considered a potent inhibitor of DNA synthesis in breast cancer cells, with an IC_{50} of 4.2 and 5.0 $\mu\text{g mL}^{-1}$ when extracted from *C. arbuscula* and *Alectoria ochroleuca*, respectively [21]. BAR from *C. aggregata* showed cytotoxic activity against HEP-2 cells in low concentration (IC_{50} 6.25 $\mu\text{g mL}^{-1}$), a superior result when compared to methanol extract from *Cetraria islandica* and *Vulpicida canadensis* that possess fumarprotocetraric, protocetraric, vulpinic and usnic acids (148 $\mu\text{g mL}^{-1}$) [22].

Other species of Cladoniaceae (*Cladonia substellata*, *C. crispata* and *C. dendroides*), which occurs over sandy soils in tableland relieve, savannah like vegetation in the state of Paraíba (Brazil) have also been tested against adenocarcinomas. The most efficient were extracts from *C. substellata* due to the presence of usnic and stictic acids. The organic extracts of *C. substellata* at 50 $\mu\text{g mL}^{-1}$ achieved over 80% inhibition of prostatic (PC3) and mammary adenocarcinomas (MDA - MB 231) as well as over 90% inhibition of leukemia (P388 and L1210) [10]. The authors also analyzed the activity of lichens collected in Alhandra and Santa Rita (Paraíba, Brazil) and reported that extracts from *C. substellata* and *C. verticillaris* achieved 80% inhi-

bition of Sarcoma-180 as well as 73% and 64% inhibition for Ehrlich carcinoma, respectively. This activity has been attributed to the usnic, fumarprotocetraric acids and atranorin found in these species [10]. SANTOS *et al.* [11] also studied Sarcoma-180 and found that nanoencapsulated usnic acid reduced the tumor and considerably reduced its toxic action at the level of hepatocytes in comparison to the free form of this compound.

Different fractions, isolated from the lichen *Usnea fasciata* revealed that usnic acid is the main product from secondary metabolites, whereas the polysaccharides isolichenin and raffinose are the most abundant water-soluble carbohydrates. Fractions containing usnic acid, as well as those containing isolichenin, showed moderate activity against Sarcoma-180 and Ehrlich tumor cells. High antitumor activity, near 90% inhibition, was found associated with the fraction containing raffinose [23]. *Usnea esculenta* and *U. longissima* extracts, which contain usnic acid, have also been found to inhibit the growth of human melanoma at 31.2% and 34.9%, respectively, whereas the control group with ascorbic acid grew by 54.0% [7]. These previous studies with Cladoniaceae corroborate our results. They demonstrate the anticancer potential of lichens substances obtained from Cladoniaceae. Other metabolites, such as sphaerophorin and pannarin, are reported to be active against tumors (melanomas) by inducing apoptosis [13].

Nucleolar organizer regions (NORs) are chromosome regions containing rRNA (ribosomal RNA) genes. NORs also contain a set of acidic, non-histone proteins that bind silver ions and are selectively visualized by silver methods in histopathological samples. These proteins stained by silver are called AgNORs, whose quantity increases in parallel with ribosome biogenesis [24, 25]. The AgNORs proteins quantity represents a valuable parameter of cell proliferation, ploidy or protein synthesis [26]. Besides, these proteins play a fundamental role in the control of ribosomal

RNA transcription and are defined as markers of "active" ribosomal genes. The close relationship between the rate of cell proliferation and ribosomal biogenesis is well established [27]. In our study, the administration of barbatic acid did not influence significantly the expression *in vivo* of AgNOR proteins in Sarcoma-180 cells. However, the treatment occasioned a significant reduction in tumor weight. This fact suggests that other effects than reducing cellular proliferation or ribosomal synthesis appear to be of overriding importance in this tumor type control.

BAČKOROVÁ *et al.* [28] observed that atranorin induced a massive loss in the mitochondrial membrane potential along with caspase3 activation and phosphatidylserine externalization. In base of the obtained data the authors proposed that the mechanism of action this secondary metabolite was the activation of the programmed cell death through the mitochondrial pathway. Since the barbatic acid is a depside, like atranorin, is quite possible to have the same mechanism of action. Therefore, a possible explanation for the significant *in vitro* and *in vivo* antineoplastic effect provided by barbatic acid may be related to apoptosis triggering.

Conclusion

Both BAR and ether extract from *C. aggregata* showed inhibitory action against tumours and cancer cells, as well as low toxicity, and results were ratified by histopathological and mitotic analysis.

The remarkable *in vitro* action against tumour cells is also suggested by synergic effect between substances present in the ether extract.

These findings strongly suggest that the barbatic acid is a promising antitumor agent, being this study was first described regarding this kind of biological activity, opening new perspectives to develop new drugs with antitumor activity.

Author's contributions

MCBM contributed to experimental design, isolation and purification of barbatic acid, statistical analyses and writing the text.

TAR, TGS and TDSS contributed to bioassays with cells and tumours.

MPCN contributed to isolate and purification barbatic acid.

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NPS contributed to histopathological analysis.

FCAAJ contributed to histopathological analysis (Agnor).

EPSF collaborated in the planning of experimental design, chemical analysis of results, writing and revising the text.

ECP collaborated in the planning of experimental design, analysis of results, writing and revising the text.

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Competing interests

The authors declare no competing interests.

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