FULL PAPER

Cytotoxic Activity of Compounds from Lichens of the Cerrado Biome in Brazil

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Abstract:

Lichens are sources of numerous biologically active compounds and many of these have demonstrated antitumor potential. The purposes of this study were to evaluate the antiproliferative activity and selectivity of the following compounds isolated from lichens: atranorin and diffractaic, divaricatic, perlatolic, psoromic, norstictic, and protocetraric acids. Cytotoxicity tests based on sulforhodamine B were performed on normal cells (NIH/3T3, fibroblast) and cancer cell lines 786-0 (renal), MCF7 (breast), HT-29 (colon), PC-3 (prostate), and HEp2 (laryngeal). Diffractaic acid exhibited GI_{50} values in the 58.6-98.9 µM range. Divaricatic and perlatolic acids were the most active compounds, with GI_{50} values of 9.8 and 15.5 µM for PC-3 and MCF7 cells, respectively. Protocetraric acid proved active only against HEp2 cells (GI_{50} = 41.4 µM). Atranorin, psoromic acid, and norstictic acids were inactive against all the cells tested. Chemometrics was used to evaluate the effect of the compounds against the cell lines tested. PCA (Principal Component Analysis) based on GI_{50} values separated compounds into two groups compared to doxorubicin, while HCA (Hierarchical Cluster Analysis) separated them into three groups based on SI values.

Keywords: chemometric analysis; phenolic compounds; tumor cells

1. Introduction

Cancer constitutes a group of diseases characterized by uncontrolled growth of cells that can spread to multiple organs and tissues. Given the high death rate associated with cancer worldwide, the disease is currently viewed as a public health problem [1]. Estimates of the World Health Organization have projected 20 million new cases by 2025 [2]. These projections are alarming and require a concerted effort of solutions for prevention and treatment of cancer. Developing new anticancer drugs is currently a major goal of many research laboratories, for which synthetic or natural products have been evaluated. Several promising anticancer agents have been isolated not only from plants, but also from lichens [3]. Lichen extracts have been evaluated against several cell types, including P3X63-Ag8.653 (murine myeloma), HeLa, FemX (human melanoma), and LS194 (human colon carcinoma) lines [4-6]. Among the lichen compounds, usnic acid has been the most investigated [7-9]. Ambewelamide A extracted from the lichen *Usnea* sp. exhibited potential cytotoxicity *in vitro* and significant antineoplastic activity against P388 murine leukemia cells [10]. Salazinic, stictic, and psoromic acids displayed significant apoptotic activity, while divaricatic acid showed only moderate effects at subcytotoxic concentrations [11]. Usnic acid and atranorin act as activators of programmed cell death in A2780

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and HT-29 cells, probably through the mitochondrial pathway [12]. Vicanicin and protolichesterinic acids showed a dose–response behavior in the 6.25-50 µM range when tested against DU-145 and LNCaP cells, activating an apoptotic process that appears to be mediated, at least in part, by the inhibition of Hsp70 expression, which may be correlated with a modulation of redox-sensitive mechanisms [13]. Our group has investigated lichens from Brazil and Antarctica for the isolation, structural elucidation, and structural modification of phenolic substances to evaluate their biological activity [14-21]. In this work, we present the results of the activities of seven lichen substances against the cancer cell lines 786-0 (renal), MCF7 (breast), HT-29 (colon), PC-3 (prostate), and HEp2 (laryngeal) and a line of normal cells (NIH/3T3, fibroblast).

2. Material and Methods

General procedures

Silica gel (Merck, 230-400 mesh) was used in the chromatography columns. Nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker DPX-300 spectrometer using solvent residual signal as an internal reference. Thin-layer chromatography (TLC) was performed on plates pre-coated with silica gel 60 F_{254} (Merck). Spots were visualized by spraying the plates with 10% H_2SO_4-methanol solution, followed by heating.

Species selection and preparation

Parmotrema dilatatum (Vain.) Hale, *Usnea subcavata* Motyka, and *Dirinaria aspera* (H. Magn.) Awasthi were collected by Dra. Neli K. Honda near Piraputanga village, in Aquidauana county, Mato Grosso do Sul state, Brazil (20°27'21.2"S, 55°29'00.9"W; alt. *ca.* 200 m), on corticicolous substrate in open forest. *Ramalina anceps* Nyl., *Usnea jamaicensis* Ach., and *Cladina confusa* (Sant.) Folmm. & Ahti were obtained from home decor stores. Species identification was conducted by Prof. Mariana Fleig, of the Universidade Federal do Rio Grande do Sul, and Prof. Marcelo P. Marcelli, of the Instituto de Botânica de São Paulo. *Usnea jamaicensis* was identified by Dr. Philippe Clerc

of the Herbarium of Geneva, Switzerland. Voucher specimens were deposited at the Campo Grande Herbarium of the Universidade Federal de Mato Grosso do Sul (CGMS 49840 for *P. dilatatum*, CGMS 49843 for *U. subcavata*, CGMS 52964 for *D. aspera*, CGMS 49839 for *R. anceps*, CGMS 49838 for *U. jamaicensis*, and CGMS 40953 for *C. confusa*). All the species are registred on SisGen (Certificate of registration A4CE261).

Extraction and isolation of compounds

Lichen talli were powdered and exhaustively extracted with hexane followed by acetone, or with dichloromethane followed by acetone, at room temperature. The extracts were concentrated *in vacuo*. The hexane and dichloromethane extracts of *P. dilatatum*, *U. subcavata*, *D. aspera*, *C. confusa, R. anceps*, and *U. jamaicensis* were individually fractionated by silica gel (230-400 mesh) column chromatography and eluted with hexane:ethyl acetate or hexane:acetone mixtures in increasing polarity, to yield atranorin (**1**) (*P. dilatatum*), diffractaic acid (**2**) (*U. subcavata*), divaricatic acid (**3**) (*D. aspera*), and perlatolic acid (**4**) (*C. confusa*). From the acetone extracts of *U. jamaicensis*, *R. anceps*, and *P. dilatatum*, psoromic (**5**), norstictic (**6**), and protocetraric (**7**) acids were isolated, respectively. These compounds were purified by treatment with a small volume of acetone in an ice bath and centrifugation at 3000 rpm for 5 min. All the compounds thus obtained had purity degrees of over 95%, as indicated by TLC and NMR. Their structures were established by ^{1}H, ^{13}C, and DEPT-135° NMR spectrometric data (Figures1S – 14S) and comparision with published data [22-25].

Cytotoxic activity *in vitro*

Cytotoxic activity was evaluated in cultures of human MCF7 (ATCC-HTB-22, breast *a*denocarcinoma), 786-0 (ATCC-CRL-1932, renal cell adenocarcinoma), PC-3 (ATCC-CRL 1435, prostatic adenocarcinoma), HT-29 (ATCC HTB-38, colorectal adenocarcinoma), and HEp2 (ATCC-CCL-23, laryngeal carcinoma) cells. Cytotoxic activity was also evaluated in normal NIH/3T3 cells (ATCC-CRL 1658, mouse

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fibroblast) purchased from the Rio de Janeiro Cell Bank. Cell maintenance and treatment were performed as described by Freshney [26].

Cytotoxicity Assay

The sulforhodamine B (SRB) assay was performed as described by Skehan et al. [26]. Cells cryopreserved in liquid nitrogen were thawed and cultured in sterile flasks containing RPMI 1640 medium and Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum and gentamicin at 50 µg/mL (Europharma) (complete medium). The flasks were then placed at 37 °C in a humidified atmosphere containing 5% CO_2 [27].

Adherent cells were removed with trypsin (1 mM + 0.25% EDTA) in PBS at pH 7.4 and transferred to conical tubes containing complete culture medium. After low-speed centrifugation, the medium containing trypsin was discarded and the cells resuspended in a small volume of complete medium. The viable cells were counted using Trypan Blue in a Neubauer chamber. A cell suspension was prepared and 100 µL of medium containing 7,500-10,000 cells was deposited in each well of a 96-well plate. The plates were stabilized by incubation at 37 °C in a CO_2 incubator for 24 h. Subsequently, an aliquot of each fraction of the test samples previously dissolved in DMSO (0.25%) was added to the wells, resulting in four concentrations for each test sample (0.25, 2.5, 25, and 250 µg/mL), in triplicate. Doxorubicin (0.025, 0.25, 2.5, and 25 µg/mL) was used as the positive control. As a negative control, cells were cultured in the absence of any test sample. All plates remained exposed to the test samples in the incubator for 48 h and were subsequently fixed with 20% trichloroacetic acid at 4 °C for 30 min. The supernatant was discarded and the plate washed with water, dried for addition of 50 µL of 0.1% SRB in diluted acetic acid, and incubated at room temperature for 30 min. Excess dye was removed with 1% acetic acid followed by drying of the plates and addition of 10 mM Tris base to solubilize the dye bound to membrane proteins of the fixed cells. The plates were agitated for 10 min to dissolve the stained proteins and read in a microplate optical reader at 540 nm.

Selectivity index

The selectivity index (SI) is a measure of the ability of a given compound to target a neoplastic rather than normal cell line, indicating the compound's potential for use in clinical trials. In the present study, the SI of each substance was calculated as the quotient between its GI_{50} value for normal NIH/3T3 cells and the GI_{50} value for a neoplastic cell line. SI values greater than 3.0 were considered significant, indicating that the compound is three times more active on tumor cells than on normal cells [8].

Statistical analysis

The statistical analysis also took into account repetitions and treatments. For the *in vitro* assay, statistical analysis addressed the absorbance values obtained for the test samples (T), the negative control (NC), the blank test samples (B), and the start of incubation—*i.e.*, before addition of test samples (T0). Based on these data, cell response to incubation with the test samples can be categorized as either inhibition or absence of effect on cell growth. T ≥ T0 and T < NC indicate that growth was inhibited (cytostatic effect).

For these conditions, cell growth (%) is calculated as $100 \times [(T - T0)/(NC - T0)]$. For T < T0, the test sample induced cell death—a cytocidal effect, calculated as $100 \times [(T - T0)/T0]$ [28, 29]. The cell growth rates (%) calculated from these formulas were then subjected to nonlinear regression using Origin 6.0 software (OriginLab) for calculation of GI_{50} (drug concentration that inhibited cell growth by 50%), which expresses cytotoxic activity. The present results represent the means ± standard deviations of three independent experiments. Compounds with $GI_{50} > 100$ µM were considered inactive [30].

Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were applied on biological activity data for dimensionality reduction of the results. Two chemometric matrices were evaluated. First, GI_{50} values were interpreted to outline the general behavior of each compound against five cancerous cell lines: 786-0, MCF7, HT-29, PC-3, and Hep$_2$. Finally, the selectivity index (SI) for the compounds on all cell lines were used to understand the selectivity

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of the citotoxicity, which GI$_{50}$ values for the lichen substances on 3T3/NIH cells (healthy cell line) was divided by the GI$_{50}$ value for each compound on each cancerous cell lines. PCA and HCA were performed using mean-centered preprocessing, and HCA was carried out through Euclidean distance using a single linkage method.

3. Results and Discussion

Atranorin (**1**) and protocetraric acid (**7**) were isolated from *P. dilatatum*, diffractaic acid (**2**) from *U. subcavata,* and divaricatic (**3**) acid from *D. aspera.* These three species, common in many regions in Brazil, are also found in environments of the Cerrado biome in Mato Grosso do Sul state. Perlatolic (**4**), psoromic (**5**), and norstictic (**6**) acids were obtained from *C. confusa*, *U. jamaicensis*, and *R. anceps* (none native to Mato Grosso do Sul), respectively (Figure 1). The results are shown in Table 1.

Chemometric analysis from cytotoxicity assay

Chemometric analysis showed the relationship between all compounds and their biological activities. Considering the molar concentration required to inhibit 50% of the growth (GI$_{50}$) of cancerous cell lines, the PCA showed perlatolic, diffractaic, divaricatic and psoromic acids closer to doxorubicin standard than protocetraric and norstictic acids and atranorin (Figure 2A).

Regarding the arrangement of the compounds on the score plot, the similarities and differences between the biological activities expressed by the GI$_{50}$ (Figure 2B) revealed that atranorin is the less active compound against the cancerous cell lines, especially for HT-29 and HEp$_2$ cell lines, because it was allocated on the more positive PC1 axis, more distant from the doxorubicin standard.

Norstictic and protocetraric acids presented low biological activity against 786-0 and PC-3 cell lines, because although near to the doxorubicin in PC1, they were distant in PC2 (Figure 2A), which 786-0 and PC-3 loadings corresponded to high values of GI$_{50}$ for these cell lines. On the other hand, the compounds grouped together with the doxorubicin standard (diffractaic, divaricatic, perlatolic, and psoromic acids) showed lower loadings for cell lines and corresponded to lower GI$_{50}$ values.
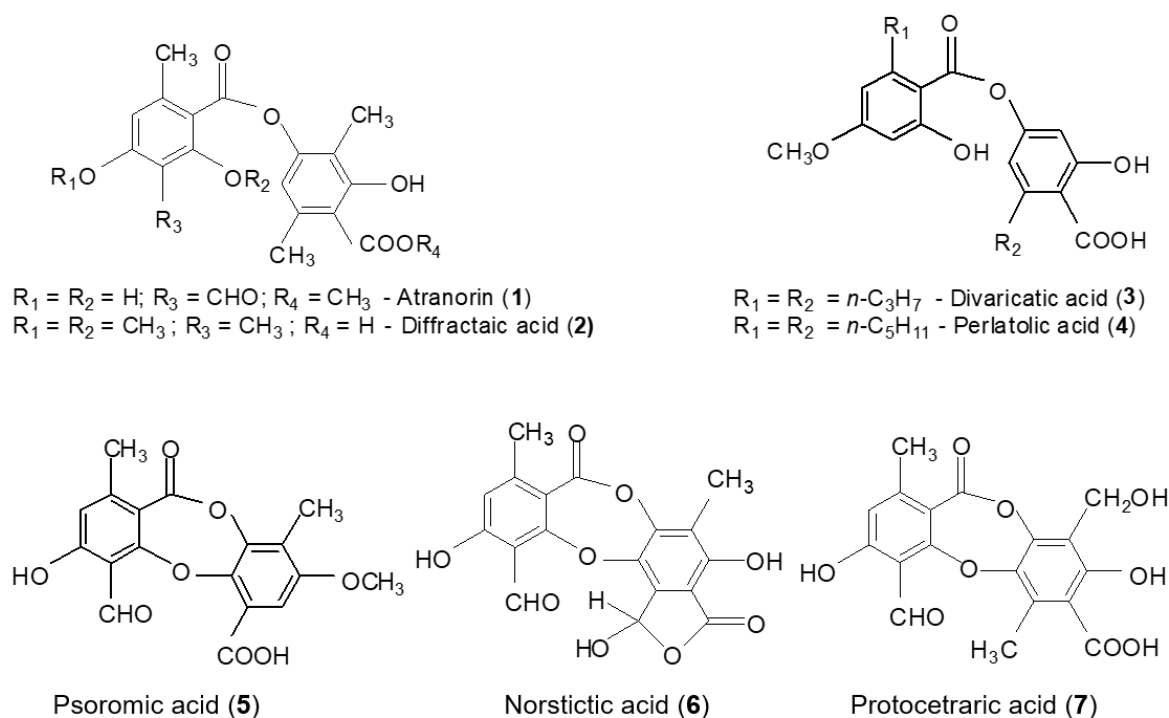

R$_1$ = R$_2$ = H; R$_3$ = CHO; R$_4$ = CH$_3$ - Atranorin (**1**)
R$_1$ = R$_2$ = CH$_3$; R$_3$ = CH$_3$; R$_4$ = H - Diffractaic acid (**2**)

R$_1$ = R$_2$ = *n*-C$_3$H$_7$ - Divaricatic acid (**3**)
R$_1$ = R$_2$ = *n*-C$_5$H$_{11}$ - Perlatolic acid (**4**)

Psoromic acid (**5**)   Norstictic acid (**6**)   Protocetraric acid (**7**)

Figure 1. Chemical structures of the compounds tested against tumor cells.

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Table 1. Growth inhibition (GI$_{50}$, in µM) and selectivity index (SI) values of the compounds **1-7** tested against cancer cells and normal cells.

Cell lines	786-0		MCF7		HT-29		PC-3		HEp2		NIH/3T3
Compounds	GI$_{50}$	SI	GI$_{50}$	SI	GI$_{50}$	SI	GI$_{50}$	SI	GI$_{50}$	SI	GI$_{50}$
Atranorin (**1**)	438.2 ± 0.67	1.7	754.6 ± 9.44	1.0	993.6 ± 13.41	0.8	526.2 ± 6.26	1.4	1093.8 ± 23.75	0.7	745.2 ± 14.84
Diffractaic acid (**2**)	81.3 ± 0.06	1	97.9 ± 1.05	0.8	74.1 ± 1.02	1.1	58.6 ± 1.52	1.4	98.9 ± 0.65	0.8	83.4 ± 4.76
Divaricatic acid (**3**)	72.2 ± 0.07	0.5	20.2 ± 1.68	1.8	16.6 ± 0.05	2.3	9.8 ± 0.35	3.8	14.2 ± 0.08	2.6	37.4 ± 5.98
Perlatolic acid (**4**)	23.6 ± 1.27	2.5	15.5 ± 1.05	3.8	62.0 ± 8.64	1.0	16.1 ± 1	3.7	29.8 ± 0.77	2.0	59.0 ± 0.04
Psoromic acid (**5**)	151.3 ± 3.51	4.6	124.6 ± 8.25	5.6	117.0 ± 3.85	5.9	132.6 ± 6.4	5.2	127.0 ± 0.18	5.5	694.1 ± 1.46
Norstictic acid (**6**)	758.9 ± 7.23	0.9	161.7 ± 10.21	4.3	915.6 ± 91.27	0.7	191.2 ± 1.19	3.6	156.9 ± 7.46	4.4	695.7 ± 3.06
Protocetraric acid (**7**)	457.5 ± 7.63	0.3	103.5 ± 3.52	1.2	99.5 ± 1.25	1.3	651.1 ± 0.73	0.2	41.4 ± 3.3	3.1	129.7 ± 0.62
Doxorubicin	0.4 ± 0.006	2.4	0.09 ± 0.002	11	0.1 ± 0.012	6.1	0.5 ± 0.01	2	1.0 ± 0.001	0.9	0.9 ± 0.001

GI$_{50}$: Concentration that inhibited 50% cell growth. Human cancer cell lines: 786-0 (renal), MCF7 (breast), HT-29 (colon), PC-3 (prostate), and HEp2 (laryngeal). Mouse non-tumoral cell line: NIH/3T3 (fibroblast). Doxorubicin was used as the positive control. Values represent means ± standard deviations for three independent experiments. Compounds with GI$_{50}$ > 100 µM were considered inactive [30].
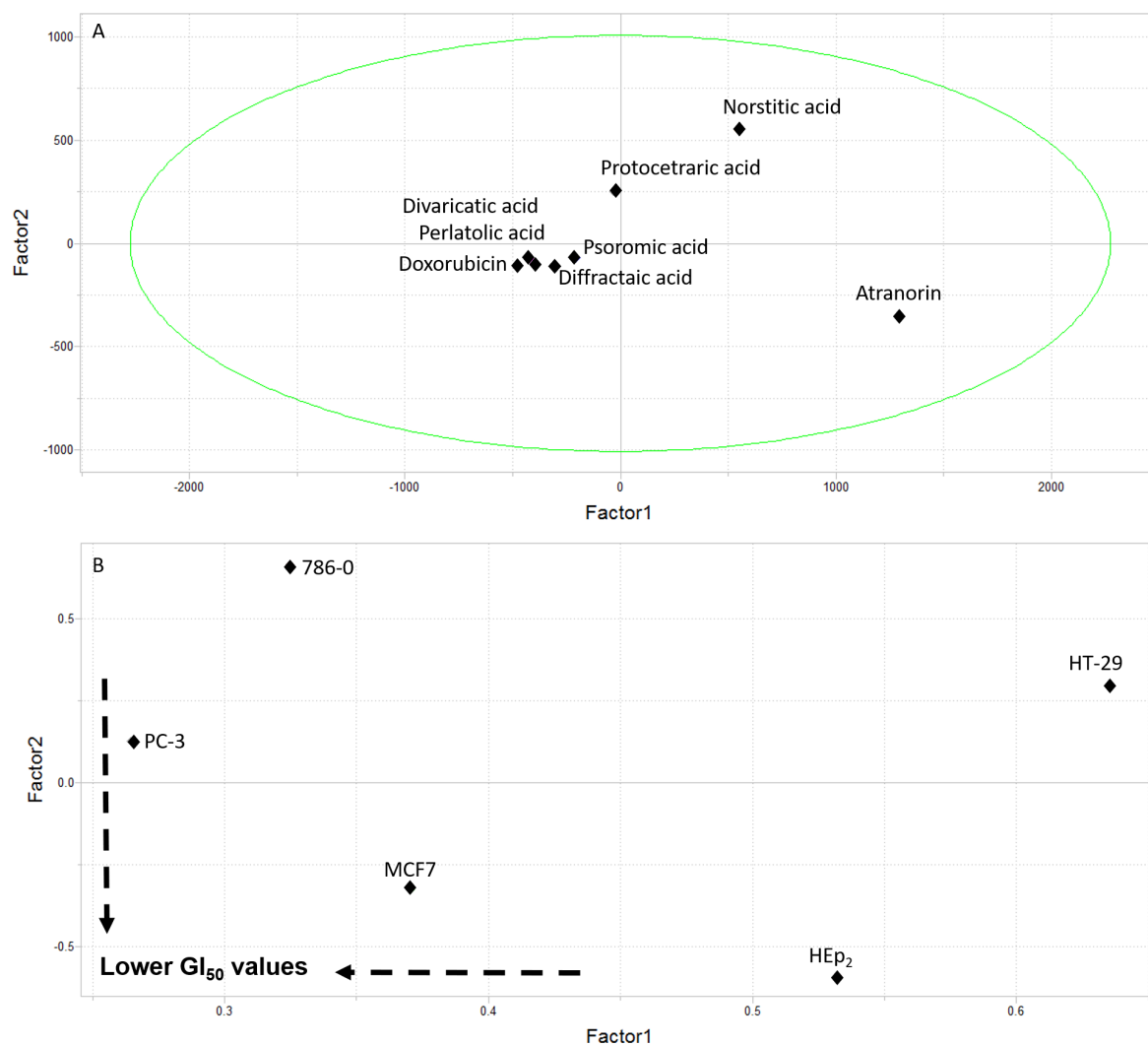
Figure 2. PCA score (A) and loading (B) plots from growth inhibition values (GI_{50}) for the studied compounds (doxorubicin standard, atranorin, diffractaic, divaricatic, norstitic, perlatolic, protocetraric, and psoromic acids) against the cancerous cell lines tested (MCF7, 786-0, PC-3, HT-29, and HEp2).

In addition, the selectivity index from compounds were evaluated using HCA method (Figure 3). HCA dendrogram showed that the selectivity index of the doxorubicin standard was distant to the lichen substances. However, interesting results for the selectivity of some lichen substances could be visualized, through the formation of three different groups: one containing atranorin, protocetraric and diffractaic acids, other with norstictic, perlatolic and divaricatic acids, and another group with psoromic acid. To understand the formation of these three groups, the bar plot from all SI values (Figure 4) revealed that atranorin, protocetraric and diffractaic acids have showed the lower SI against the cell lines, showing SI values up to maximum of 3.0. Medium values of SI (up to 4.4) could be observed for norstictic, divaricatic and perlatolic acids, while psoromic acid showed the selectivity for cancerous cell lines in comparison with healthy cell line (3T3/NIH) showing SI values between 4.4 and 5.9.

Medium values of SI (up to 4.4) could be observed for norstictic, divaricatic and perlatolic acids, while psoromic acid showed the selectivity for cancerous cell lines in comparison with healthy cell line (3T3/NIH) showing SI values between 4.4 and 5.9. However, the norstictic and psoromic acids are being considered inactive (GI >100 μM) according to the criteria established in this study.

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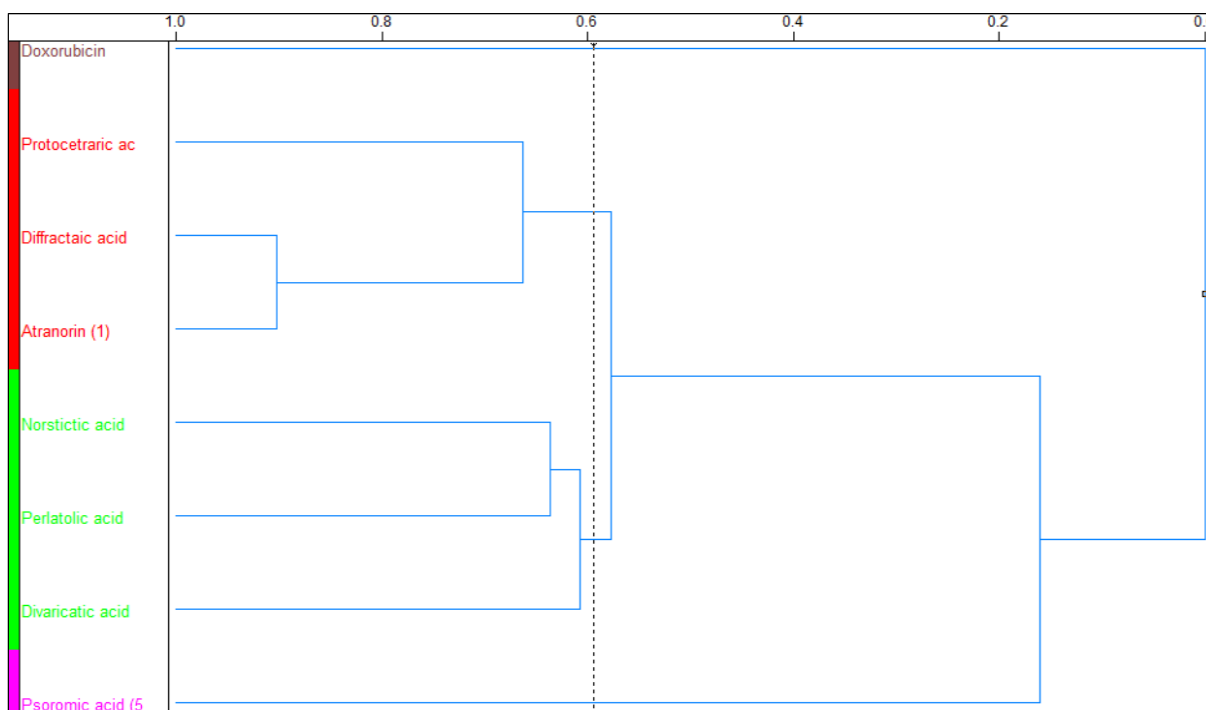*Orbital: Electron. J. Chem. 12 (1): 7-16, 2020*

Figure 3. Dendrogram of HCA from selectivity index (SI) data for the lichen substances tested against MCF7, 786-0, PC-3, HT-29, and HEp2 cancerous cell lines in comparison to 3T3 healthy cell line.
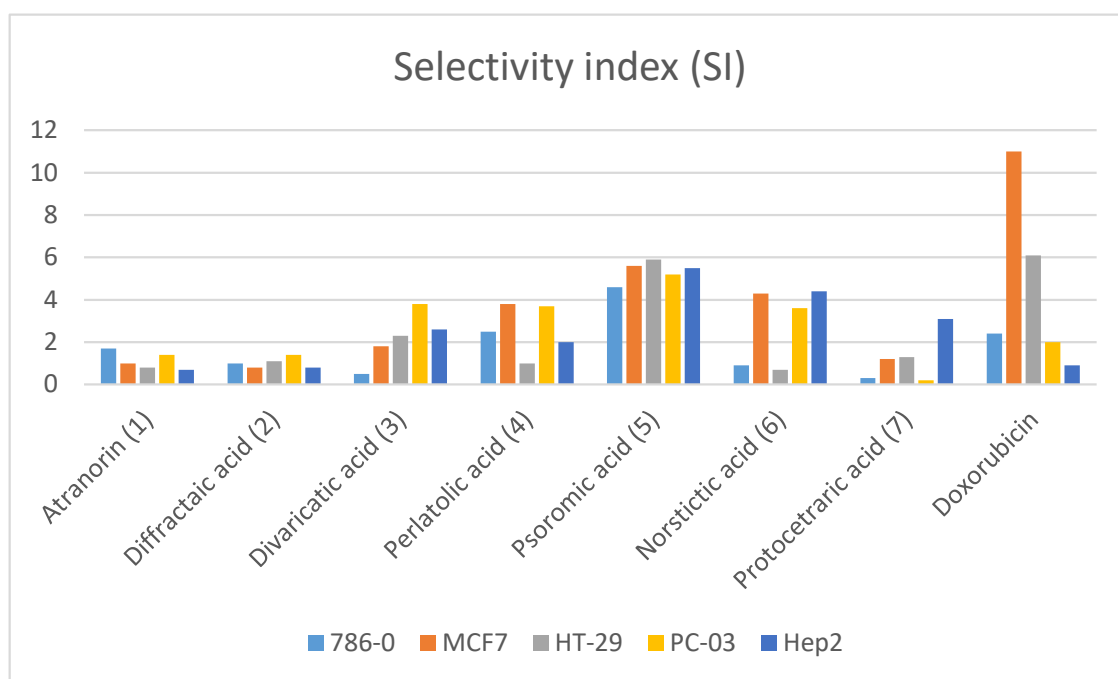

Figure 4. Bar plot from the selectivity index (SI) data for the lichen substances tested against MCF7, 786-0, PC-3, HT-29, and HEp2 cancerous cell lines in comparison to 3T3 healthy cell line.

Results of the SRB assay were expressed as GI_{50} and SI values (Table 1), revealing that the depside atranorin had no significant activity against any of the cells tested (GI_{50} in the 438.2-1093.8 µM range). Diffractaic acid was active, with GI_{50} values of 58.6-98.9 µM, albeit not sufficiently selective against the cells tested (SI in the 0.8-1.4 range). Another study found values of 93.4 µM for diffractaic acid in MCF7 cells [32], while the present investigation revealed a growth inhibition concentration of 97.9 µM.

Divaricatic and perlatolic acids, which differ only by two -CH_2 units in each alkyl chain linked to C-6 and C-6′ of the aromatic system, proved to

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be the most active against all the cells tested. A SI value of 3.8 was found for divaricatic acid against PC-3 cells, while against HEp2, HT-29, MCF7, and 786-0 cells GI_{50} values of 14.2, 16.6, 20.2, and 72.2 µM were obtained, respectively. Perlatolic acid proved highly active and selective against MCF7 and PC-3 cells, with GI_{50} of 15.5 and 16.1 µM and SI values of 3.8 and 3.7, respectively. Tested against 786-0, HT-29, and HEp2 cells, perlatolic acid exhibited significant activity (GI_{50} of 23.6, 62.0, and 29.8 µM, respectively), although this selectivity against these cells were not significant (IS < 3.0). SI values indicated that its selectivity against 786-0 and HEp2 cells (2.5 and 2.0, respectively) was similar to, or only slightly higher than, twice that measured against normal cells. Psoromic and norstictic acids presented GI_{50}> 117.0 and 156.9 µM, respectively. Although considered inactive, psoromic acid showed selectivity for all cells tested while norstictic acid was selective for HEp2, MCF7 and PC-3 cells.

Protocetraric acid was active and selective against HEp2 cells only (GI_{50} = 41.4 µM, SI = 3.13), but exhibited borderline activity against HT-29 and MCF7 cells (GI_{50} of 99.5 and 103.5 µM, respectively) and proved non-selective. Values of 60.2 µg/mL (160.9 µM) were obtained elsewhere against LS174 (human colon carcinoma) cells [5].

Testing protocetraric acid against FemX (human melanoma) cells, Manojlović et al. [5] obtained GI_{50} = 58.68 µg/mL (156.9 µM). Our group found GI_{50} = 1.4 µM against UACC-62 melanoma cells [19]. While the substances analyzed were less active than doxorubicin, the selectivity results were similar for some compounds, delimiting a set of promising substances less toxic to normal cells than the reference drug.

The cytotoxic (intracellular lactate dehydrogenase release) and apoptotic (caspase 3 activation and DNA fragmentation) effects of 15 lichen compounds have been evaluated in primary cultures of rat hepatocytes. At subcytotoxic concentrations that induce apoptosis before necrosis takes place, atranorin and divaricatic acid exhibited only a moderate apoptotic effect, while salazinic, stictic, and psoromic acids showed significant apoptotic activity [11]. In the presente study, the depsides

divaricatic and perlatolic acids showed activities on PC-3 (GI_{50} 9.8 µM , SI 3.8 and GI_{50} 16.1 µM, SI 3.7, respectively). Perlatolic acid was also active on MCF7 (GI_{50} 15.5 µM and SI 3.0). The depsidones psoromic, norstictic and protocetraric acids were considered inactive (GI_{50} >100 µM), except protocetraric acid on HEp2 (GI_{50} 41.4 µM and SI 3.1). Psoromic acid although considered inactive showed higher selectivity for all cells tested in this work. Evaluation of selectivity is important for conducting preliminary screening for cytotoxicity [33].

Although many of these compounds were isolated at relatively high yields (0.5% to 1.8% m/m), the slow growth of lichens is a limiting factor for large-scale production of drugs derived from these organisms. Nonetheless, these substances can serve as prototypes for laboratory synthesis and biotechnological approaches. Accordingly, several groups have been investigating the optimal conditions for production of secondary metabolites, either by immobilizing lichen cells [34-39] or culturing lichen mycobionts [40-42, 20].

4. Conclusions

Compounds originating from lichens of the Brazilian Cerrado biome showed promising results for use as cancer-fighting drugs, given the higher selectivity of these substances for neoplastic than for normal cells. Divaricatic and perlatolic acid depsides were potentially active on all evaluated tumor cells. Psoromic acid was moderately active, and fumaroprotetraric acid was moderately active only on HT-29 cells. Although these depsidones have a moderate antineoplastic effect, it is possible by structural changes of these substances to become potentially active compounds, serving as models for obtaining synthetic substances or modification products by semisynthesis. In addition, these compounds may represent novel candidates for studies in vivo, considering their cytotoxic activity.

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