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Cytotoxicity of Alkyl Derivatives of Protocetraric Acid

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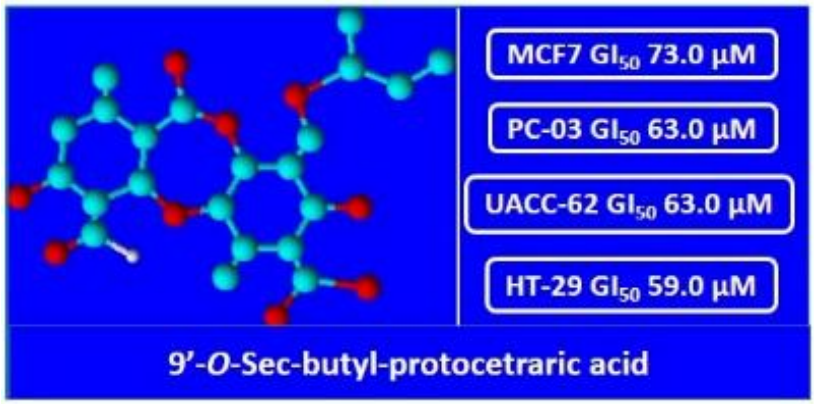
The protocetraric acid **(1)** is a depsidone produced by countless species of lichens and has shown potential biological activity. In this work, we evaluated through the sulforhodamine B (SRB) assay the cytotoxic activity of seven alkyl ether derivatives of protocetraric acid (9´-O-alkyl protocetraric acid) against the following cell lines: HT-29 (colon carcinoma), 786-0 (kidney carcinoma), MCF7 (breast carcinoma), HEP2 (laryngeal carcinoma), PC-03 (prostate carcinoma), B16F10 (murine melanoma), UACC-62 (human melanoma), and NIH/3T3 (mouse embryonic fibroblast). Most compounds were cytotoxic to tested cells, with GI50 < 100.0 µM, and did not show selectivity over them.

Graphical abstract



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1. Introduction

The compounds of the secondary metabolism of lichens, such as depsides, depsidones, dibenzofurans and others, have been extensively studied with regard to biological activities and have shown promising results [1-3]. The protocetraric acid **(1)** has been evaluated against numerous microorganisms, as an antioxidant and as an antitumor agent in some cells, in addition to genotoxic activity [4 – 11]. The protocetraric acid **(1)** is a potent inhibitor of human melanoma cells UACC-62 and did not show genotoxic activity when tested on *D. melanogaster* cells [11, 12]. Recently, Fagnani et al [13] showed that salazinic and protocetraric **(1)** acids are potential inhibitors of SARS-COV-2 3CL protease.

Substances of the depsidone class evaluated for antitumor activity have shown potential action. For example, the depsidone derived from orcinol, lobaric acid, which was tested on seventeen cell lines and showed cytotoxic activity for fourteen of them [14 – 16]. Protocetraric acid **(1)** was evaluated by Manojlović et al [8] on human FemX melanoma cells and on human colon carcinoma LS174, however, the activity was not significant, whereas fumarprocetraric acid, a derivative of protocetraric acid **(1)**, was considered active on these same cell lines, with IC_{50} values of 64.9 ± 1.8 and 87.2 ± 0.8 µM, respectively [9]. Bézevin et al [17] evaluated the effect of fumarprocetraric acid on a panel of tumor cells and the IC_{50}

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values were >100.0 μM. Another derivative of protocetraric acid **(1)**, 9´-*O*-methyl protocetraric acid **(2)**, evaluated on the same panel of cells, was also inactive (IC_{50} > 100.0 μM) [17].

Depsidones containing lactol ring, such as, norstictic, hypostictic, salazinic and stictic acids were also evaluated on several tumor cell lines. The activities of norstictic acid on MDA-MB-231 and MDA-MB-468 (breast cancer) cell lines with IC_{50} values of 14.9 ± 1.4 and 17.3 ± 1.6 μM stand out [18]. Some authors have included normal cell lines in the tests, such as NIH/3T3 (mouse embryonic fibroblast) and MRC-5 (human normal fetal lung fibroblast). The results of the activity of a given substance on these cells allow the selectivity index of the substance on the tumor cells to be defined. Bézevin et al [19] considers substances of interest to be those with a selectivity index equal to or greater than 3. Thus, stictic acid, although not potentially active on HT-29 cells (Human colon adenocarcinoma - 75.8 μM), showed high selectivity on these cells when compared to activity on normal cells MRC-5 (SI 84.6) [20]. Hypostictic acid also showed excellent activity and selectivity against several tumor cell lines [21]. Micheletti et al [22] evaluated the cytotoxic activity of alkyl derivatives of salazinic acid on cell lines MDA/MB-435 (human breast), HCT-8 (human colon) and SF-295 (human glioblastoma) and the results showed an increase in cytotoxic activity with the elongation of the alkyl chain. These results led us to evaluate, in this work, the cytotoxic activity of alkyl ether derivatives of protocetraric acid **(1)** against a panel of cancer cell lines: HT-29 (colon carcinoma), 786-0 (kidney carcinoma), MCF7 (breast carcinoma), HEP2 (laryngeal carcinoma), PC-03 (prostate carcinoma), B16F10 (murine melanoma), UACC-62 (human melanoma), and also on a normal cell line, NIH/3T3 (mouse embryonic fibroblast).

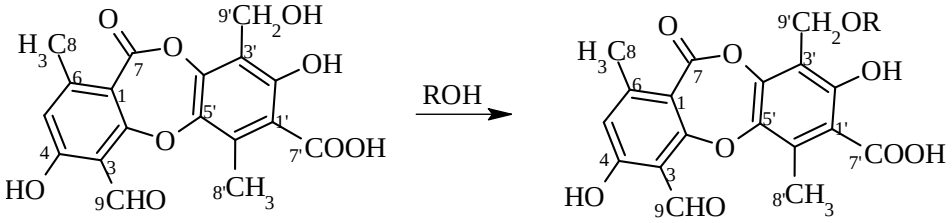
2. Material and Methods

2.1 Lichen

Lichen *Parmotrema dilatatum* (Vain.) Hale, was collected near Piraputanga village in Aquidauana county, Mato Grosso do Sul state, Brazil (20°27'21.2"S, 55°29'00.9"W; alt. approx. 200 m; on corticicolous substrate in open forests). The identification was conducted by Prof. Dr. Mariana Fleig of the Federal University of Rio Grande do Sul. Voucher specimens are deposited at the Campo Grande Herbarium of the Federal University of Mato Grosso do Sul (CGMS 49840). This species is registered at SisGen platform (entry A4CE261).

2.2 Structural modification of protocetraric acid (1)

The reactions of structural modification of protocetraric acid **(1)** were carried out according to the methodology described by Micheletti et al [22]. The derivatives: 9'-O-methyl **(2)**, 9'-O-ethyl **(3)**, 9'-O-n-propyl **(4)**, 9'-O-n-butyl **(5)**, 9'-O-isopropyl **(6)**, 9'-O-sec-butyl **(7)**, and 9'-O-tert-butyl protocetraric acid **(8)** were obtained **(Fig 1)**. The structures were confirmed through 1H, 13C and DEPT-135 NMR analyses [7].



Protocetraric acid (**1**) 9'-*O*-alkyl-protocetraric acid (**2** - **8**)

R = CH_3 (**2**); CH_2CH_3 (**3**); $CH_2CH_2CH_3$ (**4**); $CH_2CH_2CH_2CH_3$ (**5**); $CH(CH_3)_2$ (**6**); $CH(CH_3)CH_2CH_3$ (**7**); $C(CH_3)_3$ (**8**).

Fig. 1. Schematic representation of the reaction of protocetraric acid with alcohols.

2.3 *In vitro* cytotoxic activity

MCF7 (ATCC-HTB-22, breast adenocarcinoma), 786-0 (ATCC-CRL-1932, renal cell adenocarcinoma), PC-3 (ATCC-CRL 1435, prostatic adenocarcinoma), B16F10 (ATCC—CRL-6475, murine melanoma), UACC-62 (human melanoma) and HEP2 (ATCC-CCL-23, laryngeal carcinoma) cells donated by J.E.C (CPQBA – UNICAMP), and NIH/3T3 cells (ATCC-CRL 1658, mouse embryonic fibroblast) purchased from the Rio de Janeiro Cell Bank, were used for evaluation of cytotoxic activity. Cell maintenance and treatment were performed as described by Freshney [23]. The cytotoxicity assay and statistical analysis for GI_{50} determination are described in Bogo et al [24]. The selectivity index (SI), a measure of the ability of a given compound to target a neoplastic rather than a normal cell line, indicating the compound's potential for use in clinical trials, 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.

2.4 Chemometric treatment

Principal Component Analysis (PCA) was applied on biological activity data for dimensionality reduction of results using the Pirouette 4.5 (Infometrix) software. Two chemometric matrices were evaluated: first, GI_{50} values were interpreted to outline the general behavior of each compound against the cancerous cell lines. Finally, the selectivity index (SI) for the compounds on all cell lines were used to understand the selectivity of the cytotoxicity. PCA was performed using mean-centered preprocessing.

3. Results and Discussion

The compounds **(1)** – **(8)** were evaluated against a panel of tumor cells. **Table 1** shows growth inhibition (GI_{50}) and selectivity index (SI) values for alkyl protocetraric acid derivatives and doxorubicin, against the cells tested. Thus,

according to Brandão et al [12] protocetraric acid showed a potent activity of inhibiting the growth of UACC -62 cells and less pronounced activity on B16 F10, while activity on HEP2 cells could be considered moderate (GI_{50} 41.4 μM), as described by Bogo et al [24]. Among the derivatives, compounds **(4)**, **(5)**, **(7)** and **(8)** were active against four cell lines each (GI_{50} < 100 μM), and the best result was achieved for compound **(7)** against HT-29 cells, with GI_{50} of 59 μM. Methyl and ethyl protocetraric acids **(2)** and **(3)** were active only on MCF7 and HEP2 cells.

The comparison of the activities of a given compound on a tumor cell and on normal cells, defines the degree of selectivity of the compound. Thus, if the selectivity index, that is the quotient between the GI_{50} of the substance over a given tumor cell and the GI_{50} of that same substance over normal cells (in this case, NIH/3T3), is equal to or greater than 3.0, the substance is considered a promising antitumor agent [19]. All derivatives **(2)** – **(8)** showed SI (selectivity index) values of less than 3.0; therefore, considering this criterion, these compounds do not have the characteristics to be considered promising antitumor agents. However, it is important to note that compounds **(2)** – **(8)** were potential cytotoxic for most of the cells tested here. The trials were carried out using the drug Doxorubicin as a comparison standard. Although the GI50 values presented by Doxorubicin are much lower than those presented by the tested substances, the SI values calculated for cells 786-0, HT-29 and PC-03 indicate the high degree of toxicity of this drug on normal cells.

Table 1. Values of growth inhibition (GI_{50}) and selectivity index (SI) for alkyl protocetraric acid derivatives and doxorubicin against tested cell lines.

Substance	Cell lines														
	786-0		MCF7		HT-29		PC-03		HEP2		B16F10		UACC-62		NIH/3T3
	GI_{50} (μM)	SI	GI_{50} (μM)	SI	GI_{50} (μM)	SI	GI_{50} (μM)	SI	GI_{50} (μM)	SI	GI_{50} (μM)	SI	GI_{50} (μM)	SI	
(1)	457.5[a]	0.3	103.5[a]	1.2	99.5[a]	1.3	651.1[a]	0.2	41.4[a]	3.1	64.0[b]	2.0	1.4[b]	93.3	129.7[b]
(2)	215.0	0.25	88.0	0.61	121.0	0.44	598.0	0.09	78.0	0.69	638.0	0.08	114.0	0.47	54.0
(3)	208.0	0.54	93.0	0.91	357.0	0.24	209.0	0.41	84.0	1.02	627.0	0.13	148.0	0.57	86.0
(4)	165.0	0.54	88.0	1.02	66.0	1.37	77.0	1.17	155.0	0.58	500.0	0.18	85.0	1.06	90.0
(5)	138.0	1.07	66.0	2.24	75.0	1.95	88.0	1.67	102.0	1.44	190.0	0.77	89.0	1.65	148.0
(6)	141.0	0.76	86.0	1.25	70.0	1.53	188.0	0.57	156.0	0.69	298.0	0.36	105.0	1.02	108.0
(7)	102.0	0.76	73.0	1.06	59.0	1.18	63.0	1.24	180.0	0.43	188.0	0.41	63.0	1.24	78.0
(8)	129.0	0.78	116.0	0.86	76.0	1.33	153.0	0.65	92.0	1.09	71.0	1.42	64.0	1.58	101.0
Doxorubicin	0.41	2.29	0.10	9.16	0.43	2.2	0.44	2.1	0.03	27.5	0.03	27.5	0.03	27.5	0.94

[a] Bogo et al [23], [b]Brandão et al [12].

Chemometric analysis showed a relation of the activities presented between the tested compounds and doxorubicin on HT-29, 786-0, HEP2, PC-03, B16F10, UACC-62 and NIH/3T3 cell lines. For protocetraric acid derivatives **(2)** – **(8)**, a treatment was performed using PCA; it revealed that their compounds were more distant to the doxorubicin standard in the score plot (Figure 2A). In general, protocetraric acid derivatives showed GI_{50} values higher than those found for the standard doxorubicin, against the same cancerous cell lines **(Fig 2)**.

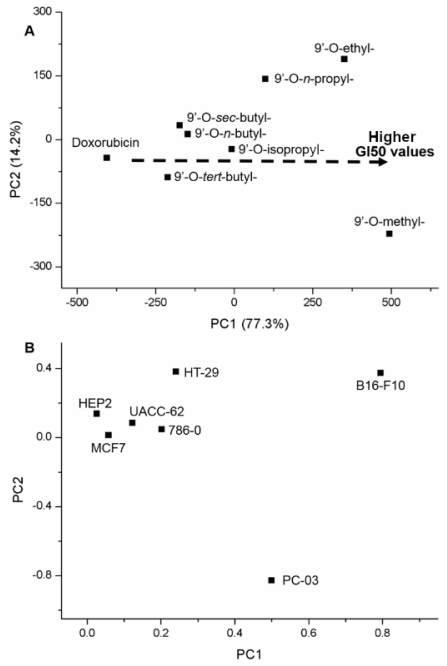


Fig. 2. PCA score (A) and loading (B) plots from growth inhibition values (GI_{50}) for all protocetraric acid derivatives against the cancerous cell lines tested.

4. Conclusions

Alkyl derivatives of protocetraric acid **(2 – 8)** showed cytotoxic activity on a panel of seven tumor cells and a normal cell line (NIH/3T3). The most active compound was 9´-*O*-sec-butyl protocetraric acid (7) on HT-29 (GI_{50} 59.0 μM), PC-03 (GI_{50} 63.0 μM) and UACC-62 (GI_{50} 63.0 μM) cells. Most of the tested compounds showed GI_{50} values on the tested cells in the range <100.0 μM. However, they did not show selectivity for the tested tumor cells (SI> 3). Although these compounds were cytotoxic, but not selective for the cells tested, the possibility of being tested on another panel of cells and respond satisfactorily as far as cytotoxicity and selectivity are concerned cannot be excluded.

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Author Contributions

DB and NKH conceived the study. NKH and ACM were responsible for isolation and structure modification. DB and MFCM realized the biological assays. GBA worked on chemometrics. NKH and ACM drafted the manuscript. All authors commented on drafts on the paper. All authors have approved the final draft of the manuscript.

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