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ORIGINAL ARTICLE

Stictic acid inhibits cell growth of human colon adenocarcinoma HT-29 cells

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Abstract The growth inhibition of stictic acid, a secondary metabolite isolated from the lichen *Lobaria pulmonaria* (L.) Hoffm. (Lobariaceae), was evaluated *in vitro* on three human cell lines for the first time. The cell lines HT-29 and MCF-7 were utilized for measuring the activity of stictic acid against cancer cells, while the cell line MRC-5 was selected for estimation of its effect on normal cells. The results suggest a moderate anticancer activity (IC_{50} value for the cell line HT-29 was 29.29 μg/ml) and a low growth inhibition on nonmalignant cells (IC_{50} value for the cell line MRC-5 was 2478.40 μg/ml) of stictic acid. This natural product can be considered as a promising lead compound for the design of novel human colon adenocarcinoma drugs.
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1. Introduction

Large numbers of different lichen substances can be generated from a limited set of monoaromatic units. Metabolites, which arise via phenolic coupling (Geissman and Crout, 1969) and form a substantial fraction of the chemical diversity of lichens, including *p*- or *m*-depsides and depsidones, consist of multiple phenolic units. Products of phenolic coupling can be modified by decarboxylation, halogenation, methylation, or esterification with short- and long-chain fatty acids. The polyketide biosynthetic pathway appears to be responsible (in whole or in part) for most of the classes of lichen compounds.

Their growth inhibition has been studied for more than three decades (Kupchan and Kopperman, 1975; Takai et al., 1979). However, the available sources of data are scarce. Since most studies have employed lichen extracts instead of chemically well defined compounds, only a small group of lichen compounds is tested (Ren et al., 2009; Zeytinoglu et al., 2008). On the other hand, the limited number of cancer cell lines has been utilized.

The most common colon cancer cell type is adenocarcinoma which accounts for 95% of cases. Other, rarer types include

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lymphoma and squamous cell carcinoma. Adenocarcinoma is a malignant epithelial tumor, originating from glandular epithelium of the colorectal mucosa. It invades the wall, infiltrating the muscularis mucosae, the submucosa and thence the muscularis propria (Danciu, 2010). Chemotherapy drugs may include combinations of agents including fluorouracil, capecitabine, UFT, leucovorin, irinotecan, or oxaliplatin, but such treatment can cause several side effects. Therefore, it is important to search for new drugs belonging to different structural classes compared to the agents in clinical use.

As part of the ongoing project on natural products of evolutionary lower organisms (Pejin et al., 2008, 2012a,b; 2013) a growth inhibition screening of stictic acid, an isolated β-Orcinol depsidone from the lichen *Lobaria pulmonaria* (L.) Hoffm. (Lobariaceae), has been performed.

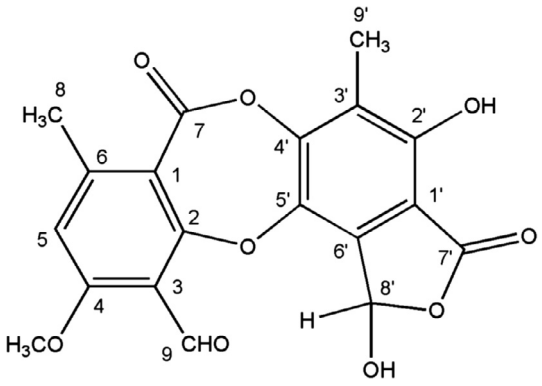

Figure 1 Stictic acid.

2. Experimental

2.1. General

^{1}H- and ^{13}C NMR spectra were recorded at the NMR Service of the Institute for Biomolecular Chemistry of National Council Research of Italy (CNR-ICB) on a Bruker Avance-400 spectrometer operating 400 and 100 MHz, respectively, using an inverse probe fitted with a gradient along the *Z*-axis, in $CDCl_3$, using the solvent signal as an internal standard. Thin-layer chromatography was carried out on pre-coated silica gel 60 F254 (0.25 mm, Merck, Darmstadt, Germany). LRMS and HRMS were recorded on a JEOL JMS D-300 and an AEI MS-50, respectively.

2.2. Plant material

The lichen *L. pulmonaria* (L.) Hoffm. (Lobariaceae) was collected from *Fagus sylvatica* on the mountain Zelengora (Bosnia and Herzegovina) in July 2009. Voucher specimen has been deposited in the Herbarium of the Institute of Botany, University of Belgrade, Serbia (BEOU 5997).

2.3. Extraction and isolation

Before extraction the lichen was carefully inspected for contaminants. Air-dried parts of *L. pulmonaria* (70 g) were ground and extracted three times with $CHCl_3$, $CHCl_3$-MeOH 1:1, MeOH, and MeOH-H_2O 1:1, respectively, (500 mL each) at room temperature, for up to 1 day each, with the extracts pooled and then evaporated in vacuo. The dried $CHCl_3$-MeOH (1:1) extract (5.81 g) was dissolved in H_2O (50 mL) and partitioned sequentially with $CHCl_3$ (3 × 50 mL) and *n*-BuOH (3 × 50 mL). The crude insoluble colored residue (0.46 g), obtained after the partition, was classified as fraction rich in epsilons, by means of its spectroscopic data and typical chromatographic profile. In order to further characterize the residue, it was chromatographed on Sephadex LH-20 column (20 mg) and eluted with the system of CH_2Cl_2 = MeOH 1:1 to yield stictic acid (5 mg, 0.0071% of dry weight).

Stictic acid (Fig. 1): ^{1}H NMR (DMSO-d_6, 200 MHz) δ 10.44 (1H, s, H-9), 7.10 (1H, s, H-5), 6.70 (1H, s, H-8′), 3.92 (3H, s, $0CH_3$-4), 2.50 (3H, s, H-8), 2.19 (3H, s, H-9′); ^{13}C NMR (DMSO-d_6, 50 MHz) δ 186.7 (CHO, C-9), 167.1 (COO, C-7′), 163.2 (C, C-4), 162.4 (C, C-2), 151.2 (C, C-2′), 150.88 (C, C-6), 147.9 (C, C-4′), 137.4 (C, C-6′), 135.8 (C, C-5′), 120.5 (C, C-3′), 114.4 (C, C-3), 109.8 (C, C-1′), 113.1 (C, C-1), 112.8 (CH, C-5), 95.5 (C, C-8′), 56.8 (C, $0CH_3$-4), 21.6 (CH_3, C-8), 9.6 (CH_3, C-9′). ESIMS *m*/*z* 385 $[M-H]^+$ (calcd. for $C_{19}H_{13}O_9$, 385.0559).

2.4. Cell lines

The cell lines used in the study were MCF-7 (human breast adenocarcinoma, ATCC HTB22), HT-29 (human colon adenocarcinoma, ATCC HTB38) and MRC-5 (human fetal lung fibroblasts, ATCC CCL 171). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5% of glucose, supplemented with 10% of fetal calf serum (FCS, NIVNS) and antibiotics: 100 IU/cm^3 of penicillin and 100 μg/cm^3 of streptomycin (ICN Galenika). The cells were sub-cultured twice a week and a single cell suspension was obtained using 0.25% trypsin in EDTA (Serva). All cell lines were cultured in flasks (Sarstedt, 25 cm^2) at 37 °C in 100% humidity atmosphere and 5% of CO_2. Exponentially growing cells were used throughout the assays. The cell density (number of cells per unit volume) and percentage of viable cells were performed as previously described (Bogdanović et al., 1994). Viability of cells used in the assay was over 95%.

2.5. MTT assay

Growth inhibition was evaluated by tetrazolium colorimetric MTT assay (SIGMA). Cells were plated into 96-well microtiter plates (Costar) in a volume of 90 μl per well, in the complete medium at an optimal seeding density of 5×10^3 cells per well to assure a logarithmic growth rate throughout the assay period. Tested substance at a concentration ranging from 10^{-8} μg to 10^{-4} M was added to all wells except to the control ones. Plates were incubated at 37 °C for 48 h. Three hours before the end of incubation period 10 μl of MTT solution (5 mg/ml) was added to all wells and plates were incubated for 3 h at 37 °C, after which medium and MTT were removed by suction. The formazan product was then solubilized in 100 μl of 0.04 M HCl of *iso*-PropOH. After a few minutes at room temperature, the plates were read on a spectrophotometer plate reader (Multiscan MCC340, Labsystems) at 540/690 nm. The wells without cells containing complete medium and MTT only acted as blank. Growth inhibition was expressed as a percent and calculated according to the formula: $\% C = 1 - (OD_{test}/OD_{control}) \times 100$.

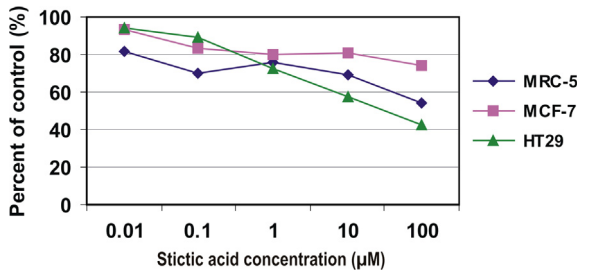

Figure 2 The effect of stictic acid on the growth of human cell lines. Human colon adenocarcinoma HT-29, breast adenocarcinoma MCF-7, or human normal fetal lung fibroblast MRC-5 were cultured for 48 h in the absence or presence of different doses of stictic acid as indicated and cell growth was measured using colorimetric MTT assay. Results are presented as % of death cells compared to untreated cells (100% viable).

2.6. Data analysis

Two independent experiments were set out with quadruplicate wells for each concentration of the compound. IC_{50} value defines the dose of compound that inhibits cell growth by 50%. The IC_{50} of the extract was determined by Median effect analysis (Mosmann, 1983).

3. Results and discussion

High-resolution mass spectrometry established the molecular formula of known depsidone stictic acid ($C_{19}H_{14}0_9$, Fig. 1). The structure followed from 1-D and 2-D NMR spectra. A combination of 2-D NMR experiments (COSY, NOESY, HSQC and HMBC) allowed assigning of all signals in the ^{1}H- and ^{13}C-NMR spectra. The spectral data are in good agreement with those previously reported in the literature (Elix et al., 1996).

The anticarcinogenic activity of stictic acid was evaluated by MTT assay against two human cancer cell lines (HT-29 and MCF-7) and one human normal cell line (MRC-5) for the first time. As depicted in Fig. 2, HT-29 human colon adenocarcinoma cells were found to be the most sensitive to stictic acid (IC_{50} value 29.29 μg/ml); compared to MCF-7 human breast adenocarcinoma, the tested compound was 689-fold more sensitive to the cell line HT-29. On the other hand, stictic acid showed a low growth inhibition on the nonmalignant cells (IC_{50} value for the cell line MRC-5 was 2478.40 μg/ml).

In comparison, the lichen substances such as atranorin, gyrophoric acid, parietin and usnic acid were less active toward the cell line HT-29 (IC_{50} values $>200, >200, >200$ and 99.70 ± 8.40 μg/ml, respectively) after 72 h exposition (Bačkorová et al., 2011). Furthermore, through investigation of the mechanisms of cytotoxicity of these four secondary metabolites, the same research group found that usnic acid and atranorin were more effective anticancer compounds on HT-29 cells compared to parietin and gyrophoric acid (Bačkorová et al., 2012). However, our results indicate that stictic acid, a widely distributed depsidone among lichens, can be considered as a more promising lead compound for the design of novel human colon adenocarcinoma drugs in relation to usnic acid and atranorin. Hence, structure–activity relationship (SAR) of stictic acid and its derivatives is worthy of investigation.

4. Conclusions

In summary, it may be concluded that stictic acid is more effective anticancer compound on the malignant HT-29 cell line than usnic acid, the most extensively studied lichen metabolite till date (Mitrović et al., 2011). Among the rest, further work should be focused on the molecular mechanism by which this depsidone induces the aforementioned growth inhibition. Taken together, it seems reasonable to assume that stictic acid may inspire new drugs for the treatment of colorectal tumors which are still among the most common and lethal types of cancer worldwide (Botteri et al., 2008).

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