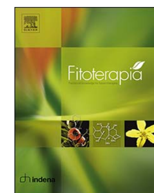
Fitoterapia 121 (2017) 146–151

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote



Cytochalasin E in the lichen *Pleurosticta acetabulum*. Anti-proliferative activity against human HT-29 colorectal cancer cells and quantitative variability

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ARTICLE INFO

Keywords:
Lichen
Pleurosticta acetabulum
Parmeliaceae
Cytochalasin E
Colorectal cancer cells

ABSTRACT

A biological screening of sixteen lichen extracts on human HT-29 colorectal cancer cells, led to the selection of *Pleurosticta acetabulum*, a lichen widely present in tree barks in Europe. Bioguided purification of the acetonic extract resulted in the isolation of cytochalasin E, a common fungal metabolite. This compound is responsible for the anti-proliferative activity of the extract. Its presence in lichens is reported here for the first time. LC-MS quantitation of cytochalasin E in different samples of *P. acetabulum* demonstrated quantitative variations of cytochalasin E production in the lichen and especially high concentrations in apothecia.

1. Introduction

Lichens result from symbiosis between a fungus and a photosynthetic organism, either an alga and/or a cyanobacterium. Recently, a third partner embedded in the lichen cortex was identified as a basidiomycete yeast [1]. Furthermore, it has been shown that the lichen thallus is a reservoir for many microorganisms (bacteria and fungi) [2]. This complex consortium led to the production of secondary metabolites endowed with many biological activities including anti-microbial, anti-proliferative and cytotoxic activities [3–6]. Colon cancer is one of the most prevalent forms of cancer. Mortality and incidence of colon cancer is third after prostate and lung cancer in men as well as breast and lung cancer in women [7,8]. In this context, sixteen acetone extracts were prepared from common lichens and evaluated against HT-29 cells using the MTT method. Among the tested extracts, *Pleurosticta acetabulum* displayed a strong anti-proliferative activity with an IC_{50} of 6 μg/ml. *Pleurosticta acetabulum* (syn. *Melanelia acetabulum* (Neck) Essl.; *Parmelia acetabulum* (Neck) Duby) is a foliose lichen having a greenish upper surface and a chlorococcoid photobiont. Its red-brown apothecia with a diameter of 5–15 mm, are among the largest in the lichen kingdom. *P. acetabulum* is a common lichen growing on nutrient-rich bark from trunks of broad-leaved trees, *Ulmus*, *Fraxinus*, *Acer pseudoplatanus* and *Sambucus* [9]. Its chemical content remains poorly studied and norstictic and connorstictic acids are the only two metabolites reported in the literature. Minor metabolites have been identified in some samples like atranorin, chloratranorin, salazinic acid, α-methylestersalazinic acid and stictic acid [10]. Bioguided fractionation of the acetone extract of *P. acetabulum* led to the isolation of cytochalasin E reported here for the first time. To confirm the presence of cytochalasin E in lichens, different samples of *P. acetabulum* were collected from several locations and at different seasons. Quantitation of cytochalasin in the different samples confirmed the lichenic origin of this molecule and justified the anti-proliferative activity of the acetone extract.

2. Results and discussion

Sixteen common lichens were collected from trees, soil or rocks. They were extracted with acetone to obtain sixteen extracts. All extracts were tested on human HT-29 colorectal cancer cells in order to determine their anti-proliferative activity (Table 1). Diosgenin, a common natural steroid well studied in the laboratory was used as positive control [11].

Ten extracts were inactive in the cancer cells with an $IC_{50} \geq 100$ μg/ml. Three of them displayed a moderate activity on the cancer cells with $20 < IC_{50} < 100$ μg/ml. The most active extracts were obtained from *Peltigera horizontalis*, *Nephroma parile* and

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http://dx.doi.org/10.1016/j.fitote.2017.07.006
Received 9 May 2017; Received in revised form 6 July 2017; Accepted 8 July 2017
Available online 10 July 2017
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Table 1
Anti-proliferative activity of lichen acetone extracts on HT-29 cells at 48 h.

Family	Lichens	IC_{50} at 48 h (µg/ml)
Cladoniaceae	*Cladonia coniocraea* (Flörke) Spreng.	> 250
	Cladonia glauca Flörke	> 250
	Cladonia parasitica (Hoffm.) Hoffm.	> 250
	Cladonia rangiformis Hoffm.	> 125[a]
	Cladonia squamosal (Scop.) Hoffm.	> 250
Lobariaceae	*Lobaria pulmonaria* (L.) Hoffm.	> 250
Nephromataceae	*Nephroma laevigatum* Ach.	20
	Nephroma parile (Ach.) Ach.	10
Parmeliaceae	*Evernia prunastri* (L.) Ach.	> 125[a]
	Platismatia glauca (L.) Culb.	250
	Pleurosticta acetabulum (Necker) Elix & L.	6
	Parmelia saxatilis (L.) Ach.	75
Peltigeraceae	*Peltigera horizontalis* (Huds.) Baumg.	10
Stereocaulaceae	*Leproloma membranaceum* (Dickson) Vainio	250
	Leprocaulon microscopicum (Vill.) Gams	20
Umbilliacariaceae	*Lasallia pustulata* (L.) Mérat	250
Positive control	Diosgenin	9

[a] Crystallization of compound for upper concentrations.

Pleurosticta acetabulum and exhibited an $IC_{50} \leq 10$ µg/ml. Nardemir et al. described the strong antimutagenic activity of *P. horizontalis* methanol extract against strains of *S. typhimurium* only [12]. While the antiproliferative activity of the chlorinated anthraquinone of *Nephroma laevigatum* has been reported [13,14], no biological activities have been described for *N. parile*.

The lichen *Pleurosticta acetabulum* (sample A, collected in Les Ulis, France), had the strongest effect on HT-29 cells with cytotoxic effects appearing 24 h after exposure (IC_{50} of 25 µg/ml) and the IC_{50} reached 6 µg/ml after 48 h of treatment. Microscopic observation of HT-29 cells treated with 10 µg/ml extract, showed cells with morphological differences compared to untreated control cells. Cell shrinkage and cytoplasm condensation appeared as early as 24 h after treatment (Fig. 1).

In order to determine whether apoptosis was responsible for the anti-proliferative effects, DNA fragmentation was studied. Results showed that DNA fragmentation was induced in HT-29 cells treated with *P. acetabulum* extract (6-fold versus control, $P < 0.05$) at 48 h suggesting that apoptosis was required for the anti-proliferative effects of the acetone extract (Fig. 2).

In order to identify the chemical compound(s) responsible for the pro-apoptotic effect, bioguided fractionation was performed on the acetone extract of *P. acetabulum*. The extract was first solubilized in acetone and submitted to several centrifugations. The resulting pure precipitate was analyzed by NMR and characterized as the depsidone, norstictic acid **1**, known to be biosynthesized by *P. acetabulum*. Norstictic acid was present in large amounts in the extract and represented > 70% of the extractible molecules. The supernatant was chromatographed on a Sephadex LH-20 column to yield eight fractions.

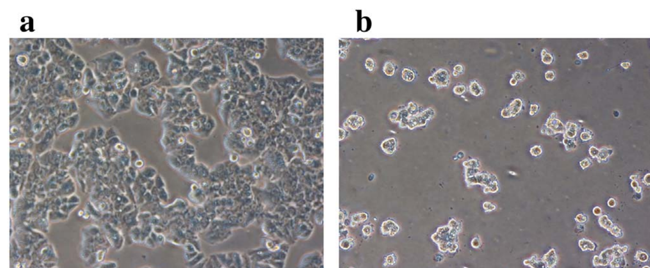


Fig. 1. Effect of *P. acetabulum* acetone extract on proliferation of human HT-29 colorectal cancer cells. Cells were incubated for 48 h without (a) or with 10 µg/ml of extract (b). Original magnification × 200.

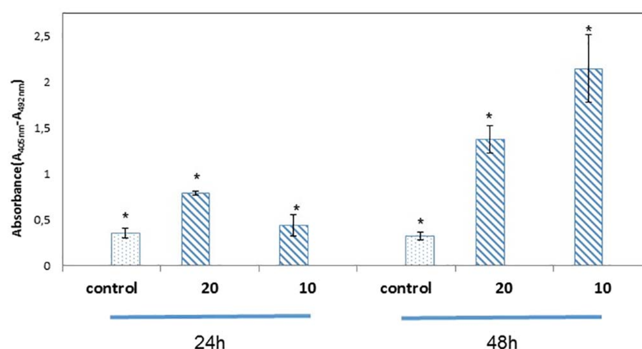
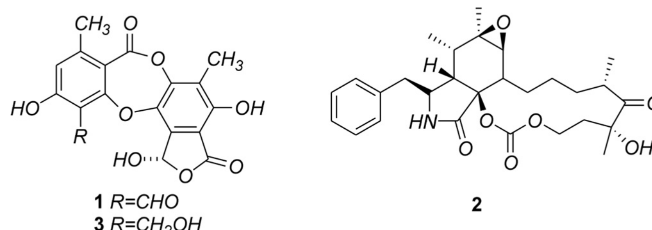


Fig. 2. DNA fragmentation after 24 h and 48 h treatment. Effect of *P. acetabulum* acetone extracts at 10 µg/ml and 20 µg/ml in HT 29 cells. Control. Results are expressed as means ± SD of three independent experiments. *$P < 0.05$ versus control.

Fractions F1 to F4 mainly contained chlorophylls, terpenes and sterols, which did not absorb under UV. Fractions F5 to F8 contained aromatic compounds (mainly depsides and depsidones including compound **1**) visible under UV. Anti-proliferative activities were evaluated for F3 and F5 as well for the precipitate corresponding to compound **1**. Results showed that **1**, is devoided of an anti-proliferative effect on HT-29 cells ($IC_{50} > 100$ µM). Previous studies demonstrated the effect of **1** on breast cancer cells, inhibiting the proliferation of cells in a concentration-dependent manner, and on melanoma cells (UACC-62) [15,16].

Fraction F3 had the best anti-proliferative activity, with an IC_{50} of 8 µg/ml compared to F5 with an IC_{50} of 75 µg/ml strongly suggesting that compounds responsible for the anti-proliferative activity were present in fraction F3. Purification of F3 by silica gel column resulted in the isolation of compound **2**. High resolution mass spectrometry (HR-MS) data coupled with 1D and 2D



NMR led to the characterization of **2**, an N-containing compound with a macrocyclic ring. Comparison with the published data in the literature [17] and the value of $[\alpha]_D$ led to the identification of cytochalasin E. This compound is a fungal metabolite produced by *Aspergillus clavatus*, *A. flavipes*, *Spicaria elegans* and *Xylaria* sp. [18,19]. Our work describes for the first time the isolation of a cytochalasin in lichens. This finding raised questions about the origin and biosynthetic pathway of cytochalasin by lichens. N-containing compounds are mostly biosynthesized in lichens with cyanobacteria as photosymbiote but in *P. acetabulum*, the photosymbiote is a chlorococcoid green algae. Anti-proliferative activity of cytochalasin E on HT-29 cells, confirmed the activity of this compound displaying a 10 µM IC_{50}. More than 80 cytochalasins have been described and are endowed with interesting biological properties. Cytochalasins are known to inhibit cell processes and to undergo apoptosis. Activity on HT-29 cancer cells has been evaluated for cytochalasin D and 100 nM causes the disruption of actin filaments [20]. Cytochalasin E displayed toxicity in brine shrimp and its mechanism of action was described as binding the barded end of F-actin and blocking polymerization [18,21]. Cytochalasin E specifically inhibits endothelial cell proliferation in vitro and angiogenesis and tumor growth in vivo [22]. Cytochalasin E has already been evaluated on other cancer cell lines (A549, HeLa, BEL-7402, RKO) with IC_{50} ranging between 37 and 64 µM. With an IC_{50} of 10 µM, HT-29 cells are particularly sensitive to this cytotoxic compound.

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