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Cytotoxic activity of Bulgarian *Linum* species

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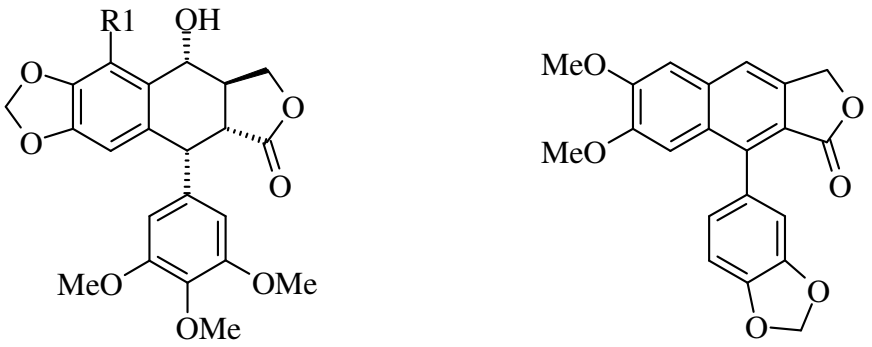
ABSTRACT - As part of our ongoing program on the investigation of lignans and their cytotoxic activity in Bulgarian *Linum* species, extracts of the aerial parts of *L. tauricum* ssp. *linearifolium* and *L. elegans* both endemic in the Balkan area were examined. The aryltetralin lignan 6-methoxypodophyllotoxin (MPTOX) is the main lignan in the extract from *L. elegans* with about 2 mg/g dry weight. Podophyllotoxin (PTOX) is the main lignan in the extract from *L. tauricum* ssp. *lineraifolium* with about 8 mg/g dry weight. The cytotoxicity of the extracts from both species was measured using the MTT–dye assay on human leukemic cell lines. Both showed moderate cytotoxic activity.

KEY WORDS - Cytotoxic activity, podophyllotoxin, 6-methoxypodophyllotoxin, justicidin B, *Linum tauricum* ssp. *linearifolium*, *Linum. elegans*

INTRODUCTION

The aryltetralin lignan podophyllotoxin is used as precursor for semisynthetic derivatives like Etoposide, Teniposide or Etopophos® which are used in the treatment of cancer. However, continued supply of podophyllotoxin is not compatible with the conservation of the wild *Podophyllum* plants, the commercial source for this compound. Therefore, the identification of other sources of this rare natural lignan is required (1). Other plants from which the accumulation of podophyllotoxin is described are: *Juniperus* species, *Callitris drummondii*, *Diphylleia sinensis*, *Polygala polygama*, *Hugonia tomentosa*, *Anthriscus sylvestris*, *Hyptis verticillata* and *Linum* species. Especially the *Linum* species can be well cultivated *in vitro* and show accumulation of lignans (2, 3). Interestingly, *Linum* species of the section Syllinum accumulate 6-methoxypodophyllotoxin as main lignan (4). Only in two species, *L. album* and *L. persicum*, podophyllotoxin is the main lignan (5). Another lignan, justicidin B, was in the genus *Linum* only detected in plants and *in vitro* cultures from species of section Linum (Fig. 1) (3, 6).



R1=H: (-)-podophyllotoxin
R1=OMe: (-)-6-methoxypodophyllotoxin

justicidin B

Fig. 1: Chemical structures of some of identified lignans

There is no report in the literature related to the cytotoxic activity of extracts from *L. tauricum ssp. linearifolium* and *L. elegans,* which are endemic in the Balkan area and belong to the Section Syllinum. The objective of this study is to determine the lignan content in the extracts of *L. tauricum* ssp. *linearifolium* and *L. elegans* (Linaceae) and to examine the cytotoxic activity of these extracts.

MATERIALS AND METHODS

Plant material

The plant material was collected and identified by A. Petrova (Institute of Botany, Bulgarian Academy of Sciences) - *L. tauricum ssp. linearifolium* (Lindem.) near the town Pleven and *L. elegans* Sprun. ex Boiss.- mountain Slavianka near the town Sandanscy (Bulgaria). Voucher specimens are deposited in the herbarium of the Institute of Botany, Bulgarian Academy of Sciences (SOM 130107 and SOM 126713, respectively).

Extraction and isolation of lignans

Lignans were extracted from powdered plant material (200 mg) with MeOH (2 ml). The mixture was homogenized in an ultrasonic bath (2 x 30 s) with intermediate cooling on ice. Distilled water (6 ml) was added and the pH was adjusted to 5.0 with 5% phosphoric acid. After adding β-glucosidase (1mg), the sample was incubated at 35°C for 1 h in a water bath. MeOH (12 ml) was added and the mixture was incubated for another 10 min at 70°C in an ultrasonic bath. After centrifugation for 7 min at 4500 rpm the volume of supernatant was determined. 1 ml of the supernatant was taken and centrifuged at 13000 rpm for 5 min at 25 °C. This final solution was used for HPLC analysis and the determination of its cytotoxic activity.

Quantitative analysis

The HPLC determination was performed on a Thermo Quest (Egelsbach, Germany) equipped with a Spectra SYSTEM UV6000LP detector. The separation column was a GROM-SIL 120 ODS-5 ST (250 x 4 mm, particle size 5 µm) supplied with a precolumn (20 x 4 mm, particle size 5 µm); the gradient system was water with 0.01% phosphoric acid (85%) (A) and acetonitrile (B) as follows: 0 to 25 min from 25% to 38% B, from 25 to 43 min to 43% B, from 43 to 46 min to 55% B, from 46 to 54 min to 70% B, until 56 min back to 25% B, holding that until 60 min. The flow rate was 0.8 ml/min between 0 and 25 min, 1 ml/min between 43 and 56 min and again 0.8 ml/min after 56 min, detector wavelengths 290 nm and 230 nm. The lignans were identified by comparison of the retention time and spectra with authentic standards (PTOX was from Xi'an Sino-dragon Import and Export Co. Ltd., Xi'an, China; 6MPTOX was isolated from *L. album* hairy roots, J. Windhövel, unpublished results; justcidin B was from callus and root cultures of *L. austriacum* (6), by using HPLC. The retention time for PTOX is about 30 min, for 6MPTOX about 37 min and justicidin B about 50 min.

Cytotoxicity study

Cell lines and culture conditions

The panel of malignant cell lines consisted of: the chronic myeloid leukemia – derived cell lines K-562 and LAMA-84, the Hodgkin lymphoma-derived HD-MY-Z and the human urinary bladder carcinoma-derived EJ cells. The leukemic cells were supplied from the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, Braunschweig, Germany), whereas the human urinary bladder carcinoma-derived cell line EJ was obtained from the American Type Culture collection (Rockville, MD, USA). The cells were maintained as suspension type culture (leukemias), semiadherent culture (HD-MY-Z) or monolayer culture (EJ) in a controlled environment: RPMI-1640 medium, supplemented with 10 % fetal calf serum and 2.5 mg/ml L-glutamine in an incubator with 5% CO_2 humidified atmosphere at 37 °C. The cells were kept in log-phase by trypsinization and consequent supplementation with fresh medium, 2-3 times per week.

Drug solutions, treatment and cytotoxicity determination

Stock solutions of the extracts were freshly prepared in ethanol water and were consequently diluted with RPMI-1640 medium to yield the final concentrations. Etoposide (as a commercially sterile available dosage form) was dissolved in water for injections and accordingly diluted in RPMI-1640. Cells were seeded into 96-well plates (100 µl/well at a density of 1×10^5 cells/ml) and exposed to the tested extracts or etoposide for 72 h. Cell survival was determined with the MTT dye-reduction assay as described by Mosmann (7), with some modifications (8). Briefly, after the incubation with the test-compound, MTT-solution (10 mg/ml in PBS) was added (10 µl/well). Plates were further incubated for 4 h at 37°C and the formazan crystals formed were dissolved by adding 100 µl/well of 5% formic acid in 2-propanol. Absorption was measured on an ELISA reader (Uniscan®Titertek, Helsinki, Finland) at 540 nm. For each concentration at least 8 wells were used. As a blank solution 100 µl RPMI 1640 medium with 10 µl MTT stock and 100 µl 5% formic acid in 2-propanol was used. Each MTT test was run in quadruplicate.

Table 1. Cytotoxicity of the tested extracts in a panel of human tumour cell lines after 72 h exposure (MTT-assay)

Extract/Compound	IC_{50} (μg/ml)[a]			
	LAMA-84	K-562	HD-MY-Z	EJ
L. elegans	0.016 ± 0.09	0.589 ± 0.062	0.512± 0.094	0.807 ± 0.022
L. tauricum ssp. linearifolium	0.031 ± 0.05	0.603 ± 0.019	0.616 ± 0.022	0.912 ± 0.031
Etoposide[b]	0.124 ± 0.102	0.311 ± 0.092	0.247 ± 0.04	0.379 ± 0.044

[a]*Data represent the arithmetic mean (± sd) of four separate experiments;* [b]*Positive control*

RESULTS AND DISCUSSION

The amounts of PTOX, 6MPTOX and justicidin B (Fig.1) in extracts from aerial parts of *L. tauricum* ssp. *linearifolium* and *L. elegans* were determined as aglycones after enzymatic hydrolysis with β-glucosidase. The main lignan in the extracts obtained from *L. tauricum* ssp. *linearifolium* was PTOX with 8.1 mg/g dry weight accompanied by 1.0 mg /g dry weight 6MPTOX, 0.1 mg/ g dry weight justicidin B and *β*-peltatin-A-methylether, an intermediate in 6MPTOX biosynthesis (2). The occurrence of the aryltetralin lignan 6MPTOX as main lignan has already been reported from *Linum* species from section Syllinum (1, 4). *L. tauricum* ssp. *linearifolium* is now beside *L. album and L. persicum* the third *Linum* species of section Syllinum with PTOX as the main lignan. (4). Since PTOX is the preferred precursor for the semi-synthesis of anti-cancer drugs like etoposide and etopophos®, the accumulation of predominantly PTOX in this subspecies is especially interesting. The occurrence of arylnaphthalenes has only been reported from intact plant species of section *Linum* (1, 3, 4). *L. tauricum* ssp. *linearifolium* to the best of our knowledge as the sole representative of section Syllinum studied so far, was found to contain arylnaphthalene lignan justicidin B together with aryltetralin lignans 6MPTOX and PTOX. The extract of *L. elegans* contained 2.1 mg/g dry weight 6MPTOX and trace amounts of *β*-peltatin. These lignans could already be detected in *L. elegans* before (1). PTOX and justicidin B were not detected.

The antiproliferative action of the extracts was tested against malignant cell lines (the chronic myeloid leukemia – derived cell lines K-562 and LAMA-84, the Hodgkin lymphoma-derived HD-MY-Z and the human urinary bladder carcinoma-derived EJ cells) with etoposide as a positive control. The tested extracts reduced the viability of tumor cells in a concentration-dependent manner, whereby their relative potency was comparable or even superior to that of the referent drug etoposide (Table 1).

The cell lines displayed differential sensitivity towards tested extracts whereby LAMA-84 cells were found to be most susceptible, K-562 and HD-MY-Z were less responsive and the urinary-bladder carcinoma EJ proved to be the most resistant cell line among the panel investigated. The juxtaposition of the IC_{50} values shows that notwithstanding the cell line and its respective cell type and origin the extract from *L. elegans* proved to exert the most prominent cytotoxic activity against all tested human cancer cells, especially to LAMA-84 cells. The extract from *L. tauricum ssp. linearifolium* showed a moderate cytotoxicity to all tested cell lines with IC_{50} in the range from 0,031 to 0,912 μg/ml.

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