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ARYLTETRALIN LIGNANS FROM IN VITRO CULTURES OF *LINUM ELEGANS* AND THEIR CYTOTOXIC ACTIVITY

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Summary. In continuation of our research on lignans in Bulgarian Linum species, for the first time we have established several callus and suspension cultures from single sterile seedlings from *L. elegans*, endemic species in the Balkan area and checked for the occurrence of lignans. Here we report the identification of the 6-methoxypodophyllotoxin (MPTOX) as the main lignan in the callus and suspension from this endemic plant species belonging to the section Syllinum. The content of MPTOX is 1.9 mg/g dry weight. The extracts showed moderate cytotoxic activity on human malignant cell lines K-562, LAMA-84, HD-MY-Z and EJ compared with etoposide as a positive control.

Key words: 6-methoxypodophyllotoxin, *Linum elegans*, in vitro cultures

АРИЛТЕТРАЛИНОВИ ЛИГНАНИ ОТ IN VITRO КУЛТУРИ НА *LINUM ELEGANS* И ТЯХНАТА ЦИТОТОКСИЧНА АКТИВНОСТ

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Резюме. В продължение на нашите проучвания върху лигнани от български видове лен, за първи път създадохме калус и суспензионни култури от балканския ендемит *L. elegans* и доказахме продукцията на лигнани в тях. В калус и суспензионните култури от вида, принадлежащ към секция Syllinum, идентифицирахме 6-метоксиподофилотоксин (MPTOX) като главен лигнан. Съдържанието на MPTOX е 1.9 mg/g спрямо сухото тегло. Екстракти от култивираните in vitro клетки показват значителна цитотоксична активност, изследвана върху човешки туморни линии K-562, LAMA-84, HD-MY-Z и EJ при сравнение с етопозид като положителна контрола.

Ключови думи: 6-methoxypodophyllotoxin, *Linum elegans*, in vitro култури

The industry currently lacks sufficient methods for producing all of the desired plant-derived pharmaceutical molecules. Some substances can only be isolated from extremely rare plants. The majority of high-valuable plant secondary metabolites are still isolated from wild or cultivated intact plants. Due to overharvesting, many of these plants becomeing endangered. Lignans are highly complex structures and their specific stereochemical requirements (a wide variety of structural types and enantiomeric forms) make the chemical synthesis uneconomical. Biotechnological production in plant cell cultures is an attractive alternative production system.

The aryltetralin lignan podophyllotoxin is used as precursor for semisynthetic derivatives like Etoposide or Teniposide which are used in the treatment of cancer.

The supply of podophyllotoxin depends mainly on its extraction from roots and rhizomes of *Podophillum hexandrum* Royle (from Himalayas region) and *Podophillum peltatum* L. (North America). However, those resources are limited because of the intensive collection of the plants, lack of cultivation and the long juvenile phase and poor reproduction capacities of the plant. Therefore the identification of other sources of this rare natural lignan is required. Screening for rapid growth and high lignan yield showed that *Linum* species belonging to the *Syllinum* section are promising for exploitation in vitro [1].

Suspension cultures are of special interest due to their high growth rate and short cycle of reproduction. Another advantage is the fact that undifferentiated plant cells, maintained in a liquid medium, possess a high metabolic activity due to which considerably high yields of secondary products can be achieved in short terms (from one to three weeks of cultivation). This raises the question of investigation

of in vitro cultures of new plant species for the production of podophyllotoxin derivatives.

In continuation of our research on lignans in Bulgarian Linum species [6, 7], we have established several callus and suspension cultures from single sterile seedlings from *L. elegans*, endemic species in the Balkan area, and checked for the occurrence of lignans. There is no report in the literature related to in vitro cultures from *L. elegans*. Here we report the identification of the 6-methoxypodophyllotoxin (MPTOX) as the main lignan in the suspension from this endemic plant species belonging to the section Syllinum.

Experimental

Plant material

Plant material (seeds) of L. elegans (Sprun. ex Boiss.) was collected in Bulgaria near to Slavyanka mountain, South-Western Bulgaria, July 2004. Voucher specimens were deposited in the herbarium of the Faculty of Pharmacy, Medical University of Sofia (FAF 0407).

Germination of seeds and callus induction

Seeds from *L. elegans* were surface-sterilized in 80% alcohol for 1 min, then in 10% commercial bleach (Domestos) for 10 min, followed by three rinses with sterile distilled water. Seeds were germinated aseptically in Petri dishes containing 25 ml of growth-regulator-free MS Murashige and Skoog medium [5], supplemented with 3% (w/v) sucrose and solidified with 0.9% agar (w/v) in the dark at 25°C. The pH of MS medium was adjusted to 5.6 before sterilization by autoclaving.

For callus induction, parts of seedlings were used. Seedlings were grown on MS medium supplemented with 1.1 mg/l naphthaleneacetic acid (NAA), and 1.0 mg/l 6-benzylaminopurine (BAP)

Establishment of in vitro cultures

Callus and suspension cultures were established using standard methods [1]. Shoot explants were placed on MS medium with 0.4 mg l^{-1} (NAA) solidified with 1% agar-agar. After 3 to 4 weeks, developed callus cells were subcultivated weekly by transferring 5 g wet cells to 50 ml fresh medium in 300 ml Erlenmeyer flasks. The suspension cultures were placed on a gyratory shaker (100 ppm) in the dark at 25°C. Suspensions (5 g frwt) were transferred every 12 days into 50 ml fresh medium.

Extraction and isolation of lignans

Lignans were extracted from powdered plant cell 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 13 000 rpm for 5 min at 25°C. This final solution was used for HPLC analysis.

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, by using HPLC. The retention time for PTOX was about 30 min, for 6MPTOX about 37 min.

Cell lines and culture conditions for cytotoxicity study

The antiproliferative action of the extracts was tested against panel malignant cell lines (chronic myeloid leukemia-derived cell lines K-562 and LAMA-84, Hodgkin lymphoma-derived HD-MY-Z and the human urinary bladder carcinoma-derived EJ cells) with etoposide as a positive control. 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 incu-

bator with 5% CO_2 humidified atmosphere at 37°C. The cells were kept in logphase 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 x 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 [4], with some modifications [3]. 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 spectrophotometer (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.

Results and discussion

From 20 *Linum* species, spread in Bulgaria, most of them intensively studied now, 4 are Balkan endemits (*L. elegans, L. thracicum, L. extraaxillare, L. tauricum*). Aryltetralin lignans (podophyllotoxin type) are the main lignan constituents of species belonging to *Linum* section *Syllinum* (Linaceae). Cell cultures from *Linum elegans*, a plant native to Bulgaria and Balkan region, has not yet been studied.

Plants of *L. elegans* were collected near to Slavyanka mountain, South-Western Bulgaria, and divided into different parts. Collected materials were pressed, dried and stored as annotated specimens at the herbarium of the Faculty of Pharmacy, Medical University of Sofia. The native plant extracts were analyzed previously by HPLC-UV/DAD and HPLC-ESI/MS. The main aryltetralin lignan found in *L. elegans* plant parts were 6-methoxypodophyllotoxin (MPTOX) and trace of 4'-demethyl-6-methoxypodophyllotoxin, podophyllotoxin (PTOX), β-peltatin and α-peltatin [7].

In the present study for the first time in vitro cell cultures from *L. elegans* were established. The main lignan found in *L. elegans*, callus and suspension cultures was 6-methoxypodophyllotoxin (MPTOX) (fig.1). The amounts of MPTOX were determined as aglycone after enzymatic hydrolysis with β-glucosidase. The presence was verified using HPLC with authentic standards.

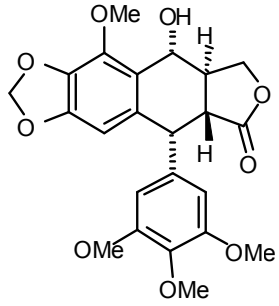

Fig. 1. *Structures of identified lignan in cell cultures of L. elegans*

As a result of more than 2 years maintenance of the cultures, and optimization of growth media, a stable growth and production of both compounds was achieved The amount of MPTOX was determined using HPLC. The maximal content of MPTOX in suspension of *L. elegans* is 1.9 mg/g dry weight. The cultural procedure and conditions used are fully defined and reproducible. Due to their high growth rate and short cycle of reproduction, suspension cultures are of special interest for industry. The selected suspension line is capable of producing 6-metoxipodophylotoxin (within 14 days) in yield and quality comparable to that of 2-3 years old field-grown plants [2].

MTT-dye assay has been used to characterize the cytotoxic effect of methanolic extracts. The antiproliferative action of the suspension extracts was tested against malignant cell lines: chronic myeloid leukemia-derived cell lines K-562 and LAMA-84, Hodgkin lymphoma-derived HD-MY-Z and 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. The extract from suspension of *L. elegans* showed a moderate cytotoxicity to all tested cell lines with IC_{50} in the range from 0,015 to 0,802 µg/ml (Table 1). The cytotoxic activity correlated with the content of podophyllotoxin derivative MPTOX, which was determined by HPLC.

Table 1. *Cytotoxicity of the tested extracts in a panel of human tumor 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	0.015 ± 0.041	0.431 ± 0.082	0.504 ± 0.056	0.802 ± 0.025
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

The juxtaposition of the IC_{50} values showed 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. The cytotoxic activity correlated with the content of podophyllotoxin derivative MPTOX, determined by HPLC. Taken together, our results give us reason to conclude that the extract from cell cultures of *Linum elegans*, possesses profound cytotoxic potential, which may be directly linked to the content of MPTOX.

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