PODOPHYLLOTOXIN AND RELATED LIGNANS: BIOTECHNOLOGICAL PRODUCTION BY IN VITRO PLANT CELL CULTURES OF LINUM UCRAINICUM

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Abstract. Podophyllotoxin has to be isolated from wild growing *Podophyllum* and *Linum* species, some of which are considered as endangered. The objective of this study is to establish cell cultures *in vitro* from *Linum ucrainicum* – an endemic rare plant species belong to the Section Syllinum, and to determine the lignan content in order to find alternative approach for production of podophyllotoxin.

We have established several callus, suspension and shoot cultures and checked for the occurrence of lignans. Ariltetralin lignans 6-methoxypodophyllotoxin (6MPTOX) and podophyllotoxin (PTOX) were identified in the cultures as main lignans. The both compounds, isolated for the first time from the in vitro cultures of this plant were identified by HPLC. The content of PTOX was 0.300 ± 0.029 mg/g DW and 6MPTOX was 2.026 ± 0.804 mg/g DW. The results of the study indicate that in vitro cultures of *L. ucrainicum* can serve as an alternative way of production of ariltetraline lignans.

Key Words: 6-methoxypodophyllotoxin, *Linum ucrainicum*, *in vitro* cultures

Introduction

Lignans are found in many plant species but only in low concentrations [1]. This structural diversity can be followed in the family of the Linaceae especially the genus Linum [2]. The lignans are a large and varied group of natural products with highly complex structures and their specific stereochemical requirements (a wide variety of structural types and enantiomeric forms) make the chemical synthesis not economically. Many plant species accumulate lignans with different general structures. The chemical diversity is even higher since most lignans are chiral compounds. Podophyllotoxin, in contrast, is a relatively rare natural product. The biotechnological part focuses on alternative production systems for these natural compounds, because the plant in vitro cultivation has several advantages over collecting plants from fields. Screening for rapid growth and high lignan yield showed that Linum species belonging to the Syllinum section are promising for exploitation in vitro [2].

In continuation of our research on lignans in Linum species [3], for the first time we have established several callus and suspension cultures from single sterile seedlings from *L. ucrainicum* and checked for the occurrence of lignans. The plant is endemic species in the Ukraine and in the European part of Russia. Here we report the identification of the 6-methoxypodophyllotoxin (MPTOX) and podophyllotoxin (PTOX) as the main lignans in the shoot, callus and suspension from this endemic plant species belong to the Section Syllinum. The objective of this study is to establish cell cultures *in vitro* and to determine the lignan content in these in vitro cultures in order to find alternative approach for production of podophyllotoxin. To our knowledge, there are no publications about the lignans in *in vitro* cultures of this plant species.

Experimental

Plant material

Seeds of *L. ucrainicum* were provided by the

botanical garden of Jardins Botaniques de Nancy, France, 2013

Germination of seeds and callus induction

Seeds of *L. ucrainicum* were surface sterilized with absolute ethanol and chlorine-releasing disinfectant and germinated aseptically in Petri dishes containing 25 ml of growth-regulator-free MS Murashige and Skoog medium [4], supplemented with 3% (w/v) sucrose and solidified with 0.9% agar (w/v) in the dark at 25 0C. The pH of MS medium was adjusted to 5.6 before sterilization by autoclaving on hormone free MS medium [4] in the dark at 25 ^{0}C.

Establishment of in vitro cultures

For callus induction, parts of seedlings were used. Seedlings were grown on MS medium supplemented with 1.1 mg/l naphthalene acetic acid (NAA), and 1.0 mg/l 6-benzylaminopurine (BAP). After 3 to 4 weeks, developed callus cells were subcultivated by transferring 5 g wet cells to 50 ml fresh medium without agar-agar in 300 ml Erlenmeyer flasks. The suspension cultures were placed on a gyratory shaker (100 rpm) in the dark at 25 ^{o}C. Suspensions (5 g frwt) were transferred every 12 days into 50 ml fresh medium.

Shoot explants were placed on MS medium with 0.4 mg l^{-1} naphtylacetic acid (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 rpm) in the dark at 25 ^{0}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 13000 rpm for 5 min at 25 °C. This final solution was used for HPLC analysis.

Quantitative analysis

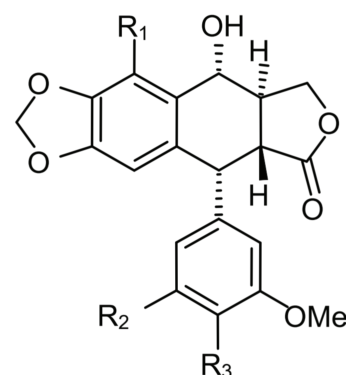
The HPLC determination was performed on Waters 1825 Binary HLPC Pump equipped with 2489 UV/VIS Detector, at 290 nm, on Luna C18 (25 cm x 4.6 mm, 5 µm) reverse phase column (Phenomenex, USA) with injection volume 20 µl. The mobile phase consist of 0.01% o-phosphoric acid (A) and acetonitrile (B). We create calibration curve, on the base of Breeze 2 software - Ptox (Rt=9.494 min) the reference standard of Ptox was used, 6-MPT (Rt=12.004 on the base of Ptox standard curve.

Results and discussion

Aryltetralin lignans (podophyllotoxin type) are the main lignan constituents of species belonging to Linum section Syllinum (Linaceae). Cell cultures from *L. ucrainicum*, a plant native to Ukraine and in the European part of Russia, have not yet been studied.

In the present study for the first time in vitro cell cultures from *L. ucrainicum* were established. Callus was induced from sterile grown seedlings (levels and stems) of *L. ucranicum* on MS medium solidified with agar (1%) in the dark. From single seedlings we established callus and suspension cultures different lines. The high-yielding callus line was isolated in medium MS-Li (0.4 mg l^{-1} naphthylacetic acid and 0.1 mg l^{-1} kinetin) and was further subjected to chemical analysis.

The lignan profile of aryltetralin lignans found in *L. ucrainicum*, callus and suspension cultures were PTOX, 6MPTOX – see Figure 1.



podophyllotoxin: R1 = H; R2 = OMe ; R3 = OMe
6-methoxypodophyllotoxin: R1 = OMe; R2 = OMe; R3 = OMe

Fig. 1. Structures of identified lignans in cell cultures of L. ucrainicum

Tabl.1 Content of Podophyllotoxin and 6-methoxypodophyllotoxin (mg/g DW) for different in vitro cultures of Linum ucrainicum

In vitro cultures of *L. ucrainicum*	**Medium**	**Podophyllotoxin (mg/g DW)**	**6-methoxypodophyllotxin (mg/g DW)**
Shoot culture	MS	0.104 ± 0.0016	2.026 ± 0.804
Callus culture	MS-Li	0.037 ± 0.005	0.952 ± 0.215
Suspension	MS-Li	0.300 ± 0.029	1.836 ± 0.164

The amounts of 6MPTOX and PTOX were determined as aglycone after enzymatic hydrolysis with β-glucosidase. The presence was verified using HPLC with authentic standards. As a result, after optimisations of growth media, a stable growth and production of the both compounds was achieved.

The maximal content of PTOX in suspension of L. ucrainicum is 0.300 ± 0.029 mg/g dry weight. The contents of MPTOX was in shoots cultures – 2.026 ± 0.804 mg/g dry weight – see Table 1.

Conclusion

To meet the growing demand by pharmaceutical industries of these anticancer compounds, the alternative strategy has been proposed in our study in order to improve the yield in batch cultivation of in vitro cultures of *L. ucrainicum* – an endemic rare plant species, which accumulate predominant 6-methoxypodophyllotoxin and podophyllotoxin. Since podophyllotoxin is the preferred precursor for the semi-synthesis of anti-cancer drugs like etoposide and etopophos, the accumulation of podophyllotoxin in this species is especially interesting.

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