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Nikolay Vasilev & Iliana Ionkova

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# Lignan Production by Cell Cultures of *Linum setaceum* and *Linum campanulatum*

Nikolay Vasilev and Iliana Ionkova

Department of Pharmacognosy, Faculty of Pharmacy, Medical University, Sofia

## Abstract

Callus and suspension cultures of *Linum campanulatum* L. and *Linum setaceum* Brot. (Linaceae) were established to study accumulation of lignans. Justicidin B proved to be the main constituent in the callus and suspension cultures of *Linum campanulatum* L. Phytochemical analysis of cell cultures of *Linum setaceum* Brot. has demonstrated that callus produces yatein, desoxypodophyllotoxin, and β-peltatin, whereas the suspension cultures contain yatein, desoxypodophyllotoxin, β-peltatin, and 6-methoxypodophyllotoxin.

**Keywords:** Cell cultures, lignans, *Linum campanulatum* L., *Linum setaceum* Brot.

## Introduction

Lignans are a large class of phenolic compounds characterized by the coupling of two phenylpropane ($C_6C_3$) units (Moss, 2000). This group of natural products has drawn attention due to their tumor-inhibitory activity. Podophyllotoxin is the most used lignan. The strong cytotoxic effect of podophyllotoxin resulted in the introduction of three clinically useful medicines: etoposide, teniposide, and etopophos.

The main economic sources of podophyllotoxin-like lignans are the rhizomes and roots of *Podophyllum hexandrum* and the leaf blades of *Podophyllum peltatum* (Bedir et al., 2002). However, these two plants are endangered species now due to the intensive collection and lack of cultivation. Therefore, the supply problem forms the search for alternative sources of podophyllotoxin and related lignans.

*Linum* cell cultures and plants are well-known for their ability to produce and store relatively large amounts of aryltetralin lignans structurally related to podophyllotoxin (Broomhead & Dewick, 1990; Wichers et al., 1991). Hence, the biotechnological approach, aimed at the cell culture production from *Linum* plant species, might be a useful option for lignan supply (Petersen & Alfermann, 2001).

The biosynthesis of lignans starts with the formation of phenylpropane units via the shikimic acid pathway. Coniferyl alcohol is the phenylpropane precursor of podophyllotoxin-like lignans. Stereoselective coupling of two molecules of coniferyl alcohol then occurs to afford (+)-pinoresinol (Dinkova-Kostova et al., 1996; Davin et al., 1997). (+)-Pinoresinol goes through several sequential stereoselective reactions to give (−)-matairesinol (Ward, 1999). (−)-Matairesinol is considered to be the branch point leading to the formation of yatein, desoxypodophyllotoxin, podophyllotoxin, β-peltatin, β-peltatin-A-methyl ether, and 6-methoxypodophyllotoxin (Petersen & Alfermann, 2001).

In this study, we examine the occurrence of podophyllotoxin-like compounds in callus and suspension cultures of *Linum campanulatum* L. and *Linum setaceum* Brot. (Linaceae).

## Materials and Methods

### Plant material

The seeds of *Linum campanulatum* L. and *Linum setaceum* Brot. (Linaceae) were supplied by the botanical gardens of Nancy (France) and Universidade de Coimbra (Portugal), respectively.

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*Address correspondence to*: Iliana Ionkova, Department of Pharmacognosy, Faculty of Pharmacy, Medical University, 2 Dunav St., Sofia 1000, Bulgaria. E-mail: ionkova@pharmfac.acad.bg

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### Plant cultures

Seeds of *Linum campanulatum* L. and *Linum setaceum* Brot. were germinated under sterile conditions on hormone-free MS-medium (Murashige & Skoog, 1962) in continuous light. Shoot cultures from single seedlings were established on the same medium. These shoot cultures were used to initiate callus. Cell suspension cultures were derived from the callus cultures by transferring 5 g callus cells to 50 ml medium in a 300-ml Erlenmeyer flask. Standard medium for callus and suspension cultures was MS-medium containing kinetin 2 mg $l^{-1}$, 2,4-dichlorophenoxyacetic acid 0.1 mg $l^{-1}$ and indole–acetic acid 0.2 mg $l^{-1}$. Callus cultures were subcultivated every 3 weeks. Calli were grown under permanent light. Suspensions (5 g frwt) were transferred every 12 days into 50 ml of fresh medium in 300-ml Erlenmeyer flasks and incubated on a gyratory shaker at 120 rpm (suspension) in the darkness at 25°C.

### Extraction and isolation of lignans

Approximately 0.200 g fine powder obtained from the lyophilized cells was accurately weighed. The material was extracted with MeOH (2 ml) in an ultrasonic bath (two-times for 30 s with intermediary cooling on ice for 30 s). Distilled water (6 ml) was added and the pH was adjusted to 5.0 by adding a few drops of 5% phosphoric acid. After adding β-glucosidase (1 mg), the sample was incubated at 35°C for 1 h in a waterbath. 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. One ml of the supernatant was taken and centrifuged at 13,000 rpm for 5 min at 25°C. This final solution was analyzed by HPLC.

### HPLC determination of lignan contents

The content of justicidin B was measured using Thermo Quest (Egelsbach, Germany) equipped with a Spectra SYSTEM UV6000LP detector. Separation was conducted with a GROM-SIL 120 ODS-5 ST column (250 × 4 mm, particle size 5 μm) supplied with a precolumn (20 × 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 17 min from 40% to 67% B, from 17 to 18 min back to 40% B. The flow rate was 0.8 ml/min between 0 and 17 min, 1 ml/min between 17 and 24 min, and again 0.8 ml/min after 24 min. The detection wavelength was 290 nm. The retention times of the standards were as follows: β-peltatin about 9.9 min, 6-methoxypodophyllotoxin about 11.7 min, deoxypodophyllotoxin about 14.6 min, yatein about 15.7 min, and justicidin B about 16.0 min.

## Results and Discussion

Sterile grown seedlings of *Linum campanulatum* L. and *Linum setaceum* Brot. were used for initiation of callus and suspension cultures. The amounts of lignans were determined as aglycones after enzymatic hydrolysis with β-glucosidase. The levels of lignans were verified using HPLC by comparison with solutions of standard substances with known concentrations.

The contents of the major lignans detected during our research are summarized in Table 1. It was established the presence of only one lignan in the cultures from *Linum campanulatum*. The content of the arylnaphthalene lignan justicidin B in the suspension cultures of *Linum campanulatum* is 1.41 mg/g dwt, which is higher than the content in the callus: 0.40 mg/g dwt. Justicidin B is previously known from *Justicia* (Acanthaceae) and *Haplophyllum* (Rutaceae). We proved the existence of justicidin B in the cell cultures from *Linum campanulatum* L., which is a member of section *Syllinum*. The existence of justicidin B is established in three species of the genus *Linum* up to now that belong to the section *Linum*: *L. austriacum* (Mohagheghzadeh et al., 2002), *Linum narbonense* L, and *Linum leonii* F.W. Schulz. (Vasilev et al., 2004). Therefore, the presence of justicidin B in section *Syllinum* is reported for the first time. It was shown by the previous work of Konuklugil (1996) that justicidin B is not present in the intact plant of *Linum campanulatum* L. Consequently, the intact plants and cell cultures of *Linum campanulatum* L. have different biosynthetic pathways.

*Table 1.* The quantitative HPLC analysis of lignans in the callus and suspension cultures of *Linum campanulatum* and *Linum setaceum* (mg/g dwt).

| Species | Culture type | Justicidin B | Yatein | DOP | β-Peltatin | 6-MPTOX |
|---|---|---|---|---|---|---|
| *L. campanulatum* | Callus | 0.40 | — | — | — | — |
| *L. campanulatum* | Suspension | 1.41 | — | — | — | — |
| *L. setaceum* | Callus | — | 0.11 | 0.02 | 2.37 | — |
| *L. setaceum* | Suspension | | 0.19 | 0.09 | 3.34 | 2.72 |

DOP, desoxypodophyllotoxin; 6-MPTOX, 6-methoxypodophyllotoxin.

Callus cultures of *Linum setaceum* produced yatein, desoxypodophyllotoxin, and β-peltatin in the following concentrations, respectively: 0.11 mg/g dwt, 0.02 mg/g dwt, and 2.37 mg/g dwt. Suspensions of *Linum setaceum* yielded yatein (0.19 mg/g), desoxypodophyllotoxin (0.09 mg/g), β-peltatin (3.34 mg/g), and 6-methoxypodophyllotoxin (2.72 mg/g). Therefore, the suspension cultures from *Linum setaceum* produced one lignan more than the callus cultures: 6-methoxypodophyllotoxin.

Feeding experiments have shown that yatein is the common precursor of desoxypodophyllotoxin, which is the first aryltetralin lignan in the biosynthetic pathway. From desoxypodophyllotoxin, the lignan biosynthesis is split up into two pathways leading to podophyllotoxin and 6-mehtoxypodophyllotoxin (Van Uden et al., 1995, 1997). We now report for the first time the occurrence of three and four lignans, respectively, in callus and suspension cultures of *Linum setaceum* Brot. The occurrence of yatein, desoxypodophyllotoxin, β-peltatin, and 6-methoxypodophyllotoxin in the cell cultures of *Linum setaceum* Brot. proposes a biosynthetic interrelationship between them. This might be also concluded by the fact that yatein and desoxypodophyllotoxin are in the lowest amounts in the cell cultures of *Linum setaceum*. The possible reason for this fact is that yatein and desoxypodophyllotoxin are precursors of β-peltatin, and 6-methoxypodophyllotoxin and therefore they are easily converted into these lignans of the latter part of the biosynthetic pathway. The absence of podophyllotoxin is probably due to the deficiency of the enzyme desoxypodophyllotoxin 7-hydroxylase responsible for conversion of desoxypodophyllotoxin into podophyllotoxin.

It may be concluded that in general, the suspension cultures obtained from *Linum campanulatum* and *Linum setaceum* contain higher amounts of lignans. Consequently, cells cultures grown in continuous light produced higher amounts of lignans.

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