Accumulation of Lignans by *in vitro* Cultures of Three *Linum* Species

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Z. Naturforsch. **64c**, 73–76 (2009); received January 14/April 21, 2008

Dedicated to Dr. Gholamreza Asghari, Professor of Pharmacognosy, Isfahan Faculty of Pharmacy, Isfahan on the occasion of his birthday

Justicidin B, an arylnaphthalene lignan, has strong cytotoxicity on chronic myeloid and chronic lymphoid leukemia cell lines. The first report of the production of justicidin B in a *Linum* species concerned *in vitro* culture of *Linum austriacum*. Therefore, culture characterization and presence of arylnaphthalene-type lignans in calli and plantlets of *Linum tenuifolium* from section *Linastrum*, *Linum bienne,* and *Linum glaucum* from section *Linum* were studied. Seed germination of *L. tenuifolium* in the light and darkness was significantly higher ($p < 0.05$) than of *L. bienne* in the light and *L. glaucum* in the darkness. *L. tenuifolium* seedling length in the darkness was significantly higher ($p < 0.01$) than under light conditions. There were no significant differences in the calli and shoot biomass weight, number and length of shoots in three species over one month, while the shoot diameter of *L. bienne* was significantly different ($p < 0.05$) from that of *L. glaucum*. Justicidin B was detected in *L. glaucum* callus and plantlet cultures by HPLC/MS/UV-DAD and HPLC coupled with a photodiode array detector. This finding is important from pharmaceutical point of view and shows the chemosystematic relation between *L. glaucum* and *L. austriacum* and this method will be a powerful tool for detecting natural products in interested and endangered medicinal plants.

Key words: Justicidin B, Lignan, *Linum*

Introduction

Lignans are monolignol-derived dimers (Hemmati *et al*., 2007). They form a large group of natural products, which show diverse biological effects. Lignans may serve as lead compounds for the development of new therapeutic agents with cytotoxic, antiangiogenic, antileishmanial, antifungal, hypolipidemic, antiasthmatic and antiviral activity (Vasilev and Ionkova, 2005).

Linum, the largest genus of the family Linaceae, comprises about 230 species distributed throughout the world (Heywood, 1985). The genus *Linum* is divided into the sections *Syllinum*, *Cathartolinum*, *Dasylinum*, *Linum* and *Linastrum* according to classical taxonomy based on morphological characters (Ockendon and Walters, 1968).

The occurrence of lignans in the genus *Linum* is documented since 1975 (Weiss *et al*., 1975). In the genus *Linum* the main types of lignans belong to the dibenzylbutane group (*e.g.* secoisolariciresinol diglucoside, SDG), aryltetralin group (*e.g.* podophyllotoxin, PTOX) and arylnaphthalene group (*e.g.* justicidin B, Fig. 1) (Bakke and Klosterman, 1956; Broomhrad and Dewick, 1990; Mohagheghzadeh *et al*., 2002). Justicidin B was isolated for the first time in the genus *Linum* from *Linum austriacum* and was previously known from *Justicia* spp. (Acanthaceae) and *Haplophyllum* spp. (Rutaceae). Since the structure of justicidin B is closely related to PTOX, it was hypothesized that justici-

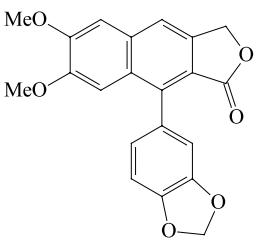

Fig. 1. Chemical structure of justicidin B.

din B may have similar cytotoxic effects as PTOX. Justicidin B was established to be the cytotoxic principle of *Justicia pectoralis* (Vasilev and Ionkova, 2005).

Nowadays, sustainable bioproduction of the compounds of interest may be achieved by plant *in vitro* cultures. For example PTOX – an anticancer compound – can be produced in cell suspension and root as well as hairy root cultures of *Podophyllum* and various *Linum* species (Wink *et al.*, 2005).

Herein, the presence of arylnaphthalene-type lignans in *in vitro* cultures of three species of *Linum*, evaluation of seed germination and shoot and calli cultures growth measurements were studied.

Results and Discussion

Seed germination was done successfully. The germination rate of *L. tenuifolium* seeds in the light and darkness was significantly higher ($p < 0.05$) than of *L. bienne* in the light and *L. glaucum* in the darkness. *L. tenuifolium* seedling length in the darkness was significantly higher ($p < 0.01$) than under light condition (Table I). However in the other two species such a difference was not observed. Calli and shoot formation occurred successfully. There were no significant differences in the calli fresh weight, number and length of shoots in three species over one month, while the shoot diameter of *L. bienne* was significantly different ($p < 0.05$) from that of *L. glaucum* (Table I).

Biomass analysis by HPLC coupled with a photodiode array detector showed the presence of justicidin B, an arylnaphthalene lignan (Fig. 1), in *L. glaucum* in comparison with the reference compound. However, in the two other species such a compound was not found. Analysis by HPLC/MS/UV-DAD also showed the presence of justicidin B. The ESI positive mode displayed a fragmentation into $[M+H]^+$ at *m/z* 365 in *L. glaucum*.

The identification of justicidin B or other arylnaphthalene lignans in *L. glaucum in vitro* cultures agrees with the morphological data. *L. glaucum* has many morphological similarities with *L. austriacum* (Mohagheghzadeh *et al.*, 2003). However, arylnaphthalene-type lignans were not found in *L. bienne* and *L. tenuifolium in vitro* cultures.

Justicidin B has cytotoxic effects that make it suitable to be used as a lead compound for the production of new anticancer drugs. Since, the chemical synthesis of justicidin B is not economically feasible, a plant cell culture is an alternative production system, which could be scaled up. These findings are important in the search for new sources for phytopharmaceuticals. It also shows the chemosystematic relation between *L. glaucum* and *L. austriacum*. Studying growth characteristics helps to optimize the growth conditions for pharmaceutically valuable plants to achieve significant amounts of chemicals.

Experimental

Plant material

L. tenuifolium L. seeds were collected in August 2001 at Ardabil, Khalkhal – Asalem road, east Azarbaijan province, Iran, at an altitude of 1900 m. *L. bienne* Mill. seeds were collected in May 2003 at Bostan mountain, Mamasani – Gachsaran road, Kohgiloie and Boir Ahmad province, Iran, at an altitude of 1000 m. *L. glaucum* Boiss. & Noein Boiss. seeds were collected in August 2001 at Chehel Cheshmeh mountain, Divan Dare, Kordestan province, Iran. The samples were identified by Iraj Mehregan (Institut für Spezielle Botanik und Botanischer Garten, Johannes Gutenberg-Universität, Mainz, Germany) and voucher speci-

Table I. *In vitro* characteristics of *Linum glaucum, L. tenuifolium* and *L. bienne.*

Species	Seedling length [mm]		Shoot diameter [mm]	Shoot length [mm]	Calli weight increase [g]
	Light	Dark			
L. glaucum	16.1	36.0	1.6	13.8	2.2
L. tenuifolium	15.3[a]	40.3	2.1	8.9	1.0
L. bienne	10.3	13.3	3.0[b]	8.6	8.4

Values are means ± S.D. ($n = 3$), ANOVA test.
[a] Significant different from light condition ($p < 0.01$).
[b] Significant different from *L. glaucum* shoot diameter ($p < 0.05$).

mens (nos. 240, 231 and 330) were deposited in the herbarium of Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.

In vitro cultures

After sterilization (1 min in 96% ethanol, then 9 min in 0.5% sodium hypochlorite and washing three times with sterilized distilled water) the seeds were incubated on agar and coconut milk medium for germination. Callus initiation was carried out from seedlings on MS medium (Murashige and Skoog, 1962) supplemented with α-naphthalene acetic acid (NAA, 1 mg/L), kinetin (kn, 0.5 mg/L) and 2,4-dichlorophenoxyacetic acid (2,4-D, 0.5 mg/L). Plantlets or shoots were initiated on MS medium without hormone or MS media supplemented with 2 mg/L kn, respectively. All the media were supplemented with 3% sucrose and solidified with agar (0.8%) at pH 5.6 before autoclaving. Cultures were subcultivated every 4 weeks. All mentioned cultures except for dark-growing calli cultures were maintained under permanent light at (25 ± 2) °C. For growth measurements of calli or shoots, the entire callus or shoot biomasses were transferred to a pre-weighted container and the fresh weight was determined. After 4 weeks their weight, length and diameter were measured. The length of the shoots was measured by a normal ruler. The diameter of the shoots was measured by a vernier caliper (accuracy 0.1 mm). All measurements were done three times.

Lignan analysis

Extraction and detection of arylnaphthalene lignans were performed as described by Empt *et al.* (2000). Arylnaphthalene lignans were detected by a Chromquest HPLC (Germany) photodiode array detector and a HPLC/MS/UV-DAD Thermofinnigan (Germany) instrument. The HPLC-PDA system from Thermoquest (Egelsbach, Germany) was equipped with a Spectra System KO 6000 LP photodiode array detector, an Spectra System AS1000 autosampler, a degaser and a Spectra System P2000 pump. Assay products were seperated on a C_{18} GROM-Sil (Grom, Rottenburg, Germany; particle size 5 μm, 2 cm × 4 mm and 25 cm × 4 mm) column. The elution system was water containing 0.01% phosphoric acid (A) and acetonitrile (B). The following gradient was used: hold, 25–38% B for the first 25 min, 38–43% B in 18 min, 43–55% B in 3 min, 55–70% B in 8 min, 70–25% B in 2 min, hold with 25% B for 4 min. The flow rate was 0.8 mL/min for the first 25 min, reaching 1 mL/min within 18 min, holding at 1 mL/min between 43–56 min, and 0.8 mL/min in the last 4 min. The LC-MS analysis was performed as mentioned by Schmidt *et al.* (2006).

Acknowledgements

The authors wish to thank Shiraz University of Medical Sciences and Health Services, Council of Research, Iran for providing financial support.

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