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HPTLC densitomeric determination of justicidin B in *Linum in vitro* cultures

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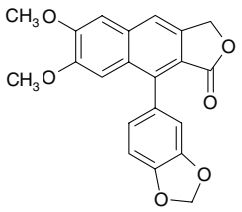
A convenient, sensitive and accurate method for the separation and determination of justicidin B was created. The values were calculated by linear calibration under standard conditions. The method can be applied for the quantification of justicidin B in plants or in vitro cultures.

1. Introduction

The lignans comprise a class of natural products, which are derived from cinnamic acid derivatives, and which are related biochemically to phenylalanine metabolism (Moss 2000). Lignans are found in more than 55 plant families (Dewick 1989) and show several physiological activities: cytotoxic, antiviral, antifungal, immunosuppressive, hypolipidemic, antiasthmatic and antiplatelet effects.

Cell cultures of different *Linum* species were shown to produce considerable amounts of arylnaphthalene lignans (Petersen 2001). Podophyllotoxin is the main lignan in the cell cultures of *L. album* (Van Uden et al. 1990) and 6-methoxypodophyllotoxin is predominantly accumulated in cell lines of *L. flavum, L. nodiflorum* and *L. mucronatum* (Wichers et al. 1990; Konuklugil et al. 1999, 2001). Justicidin B and isojusticidin B are isolated for the first time in the genus *Linum* from cultures of *L. austriacum* (Mohagheghzadeh et al. 2002).



During our preliminary research we proved the presence of justicidin B in *in vitro* cultures of *L. narbonense* L., *L. leonii* F. W. Schulz and *L. campanulatum* L. by means of HPLC and UV-spectroscopy (Ionkova et al. 2003; Krivshiev et al. 2003). Subsequently, we developed a simple and convenient method for the densitometric determination of justicidin B.

2. Investigations, results and discussion

The influence of the following key factors was studied in order to optimize the chromatographic performance: direct scanning and the type of developing system. It was established during our studies that the justicidin B aglycone gave good fluorescence when exposed to UV light at 366 nm. Therefore, direct scanning of fluorescence was selected. As a result of direct scanning, spraying with ceric sulfate/20% nitric acid solution and plate toasting were avoided in distinction from the existent methods (Ma 1992). This improved the result accuracy and convenience during the experimental work. Several developing systems were tested to obtain a better chromatographic separation: I. $CH_2Cl_2/(CH_3CH_2)_2O$ (4 : 1), II. $CHCl_3/CH_3COCH_3$ (65 : 35) III. $CHCl_3$/MeOH (90 : 10) IV. $CHCl_3$/MeOH (95 : 5) V. $CHCl_3$/MeOH (98 : 2) and VI. $CHCl_3$/MeOH (99 : 1). The system containing $CHCl_3$ and MeOH in the ratio 99 : 1 showed a favorable separation of justicidin B

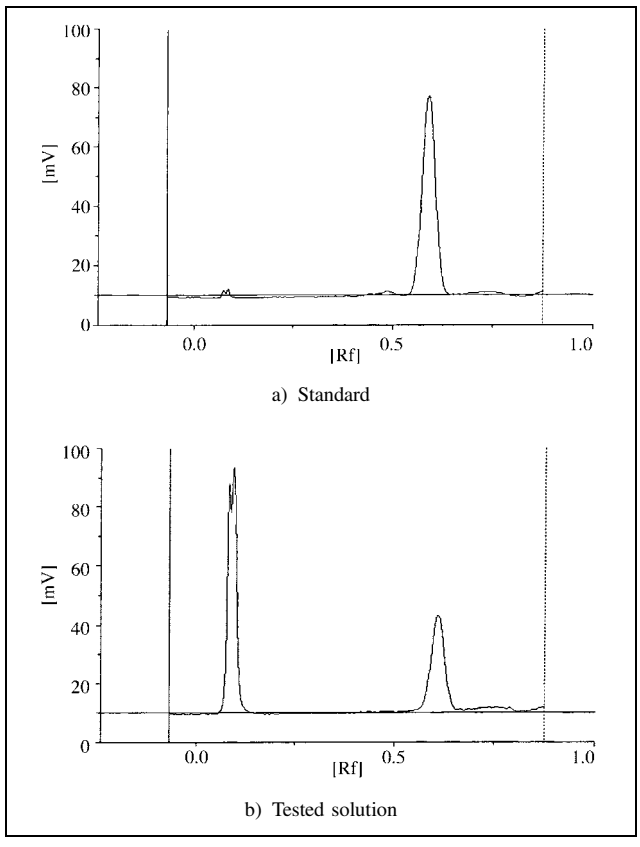


Fig.: Densitogram by 366 nm of justicidin B

Table: Content of justicidin B in *Linum* cultures *in vitro* calculated on a dry weight basis

Culture	Content of justicidin B aglycones (mg/g)*	RSD** (%)
L. narbonense (callus)	0.83	2.1
L. narbonense (suspension)	2.31	2.4
L. campanulatum (callus)	0.44	1.2
L. campanulatum (suspension)	5.22	2.1
L. leonii (callus)	1.02	2.5

* Mean (n = 4)
** Relative standard deviation

($R_f = 0.6$) from the accompanying compounds (Fig.). Therefore, system VI was selected for HPTLC developing. The calibration plots obtained with standard solution of justicidin B were linear over the range of 0.0185–0.111 μg.

Justicidin B was originally found in *Justicia* (Acanthaceae) and *Haplophyllum* (Rutaceae). We also proved the presence of justicidin B in *L. narbonense* L., *L. leonii* F. W. Schulz and *L. campanulatum* L. Justicidin B was not previously found in these three species. As justicidin B was already found in several *Linum* species, phylogenetic relation of these *Linum* species with *Justicia* and *Haplophyllum* genera might be supposed. The results for justicidin B content are presented in the Table. The cultures from *L. campanulatum* suspensions contain the highest amount of justicidin B: 5.2 mg/g dwt, followed by *L. narbonense* suspension and *L. leonii* callus cultures: 2.3 mg/g dwt and 1.0 mg/g dwt respectively.

The described method was applied for the determination of justicidin B in conventional cultures from *L. narbonense* L., *L. leonii* F. W. Schulz and *L. campanulatum* L. The procedure is simple and convenient for routine determination of justicidin B in plant material.

3. Experimental

3.1. Apparatus

Spots were applied using Camag Linomat IV. Camag TLC Scanner II was used for the spots scanning. Scanning, peak integration and calculations were made via Camag CATS software.

3.2. Chemicals and materials

Justicidin B was supplied by Dr. Abdolali Mohagheghzadeh Shiraz, Iran, who identified it by mass spectrometric (GC–EIMS) and NMR spectroscopic analysis (^{1}H, ^{13}C, COSY, HMQC and HMBC) (Mohagheghzadeh et al. 2002). *Linum* callus and suspension cultures grown *in vitro* were used as explants. Shoot cultures of *Linum narbonense* L *Linum campanulatum* L. and *Linum leonii* F. W. Schulz were developed from the corresponding seeds and were used to initiate callus and cell suspension cultures. The preliminary investigations proved that MS-medium with 0.2 mg · l^{-1} IAA, 0.1 mg · l^{-1} 2,4D and 2.0 mg · l^{-1} kinetin showed the most intensive growth of the cultures (Ionkova et al. 2001). Cells (5 g fr.wt) were transferred every 12 days into 50 ml medium in 300 ml Erlenmeyer flasks and incubated on gyratory shaker at 120 rpm in the darkness (suspension).

3.3. Preparation of samples

Approximately 0.200 g fine powder of the dried cells was accurately weighed. The material was extracted with MeOH (2 ml) in an ultrasonic bath (two times for 30 s with cooling on ice for 30 s between). 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 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. 1 ml of the supernatant was taken and evaporated to dryness. The residue was dissolved in 1 ml MeOH. This final solution was used for the densitometric analysis.

3.4. Chromatographic conditions

The sample solutions were applied at a point 1 cm from the bottom edge of the Merck silica gel TLC plate 60 F_{254} (20 × 10 cm), $CHCl_3$–MeOH (99 : 1) was used as a mobile phase. The plate was saturated for 5 min and then developed to 5 cm front using ascending technique. The spots of justicidin B were scanned by fluorescence at 366 nm. A filter Camag K400 was used to cut off UV light.

3.5. Assay procedure

The justicidin B levels in the cultures were measured by comparison with standards applied on the same plate. Quantification was performed by an integral calculation of peak areas.

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