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Comparison of different extraction methods for the determination of podophyllotoxin and 6-methoxypodophyllotoxin in *Linum* species

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Abstract

Three extraction methods for analysis of podophyllotoxin and its derivatives from *Linum* species were compared. No statistical difference on the percentage of recovery were found between the methods. The "glycosidase-method" showed the best result with respect to the accuracy studies; the "acetone-method" has an advantage compared to the other methods due to its capability to calculate the aglycone, lignan glycoside and total lignan. The content of podophyllotoxin and 6-methoxypodophyllotoxin in *Linum mucronatum* subsp. *mucronatum* Bertol, *Linum arboreum* L., and the endemic Turkey species of *Linum flavum* subsp. *scabrinerve* Davis were determined. This is the first report on the analysis of podophyllotoxin and 6-methoxy podophyllotoxin of natural collected *Linum flavum* subsp. *scabrinerve* and *Linum arboreum.*

Keywords: Podophyllotoxin; 6-Methoxy podophyllotoxin; *Linum*; *Extraction*

1. Introduction

Podophyllotoxin is a natural lignan used as a precursor for the semi-synthetic anti-cancer drugs etoposide, teniposide and Etopophos®. The main commercial source of podophyllotoxin is *Podophyllum emodi* Wall. (syn. *P. hexandrum*, Berberidaceae) found in alpine and sub-alpine areas of the Himalayas, which has become an endangered species as a result of over-collection [1,2]. The resin content of rhizomes of *P. emodi* from the Himalaya is at 10–18% with podophyllotoxin as the predominant compound (ca. 40%). *P. peltatum* originates from North America and the resin content of the rhizomes is at 3–5% with podophyllotoxin as the main compound (ca. 20%) besides α- and β-peltatin [3]. Plant cell cultures of different *Linum* species have been shown to accumulate substantial amounts of cytotoxic lignans, mainly podophyllotoxin (PTOX) and 6-methoxypodophyllotoxin(6-MPTOX) (Fig. 1.)

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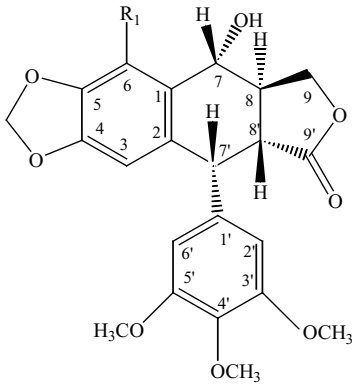

Fig. 1. Podophyllotoxin ($R_1 = H$) and 6-methoxypodophyllotoxin ($R_1 = OCH_3$).

Although lignan levels might not be sufficient to use these cell cultures as biotechnological production systems, but after understanding the biosynthesis of cytotoxic lignans and the regulation on enzyme and gen levels, which might help to optimize the biotechnological production of lignans by plant cell cultures [4].

The genus *Linum* contains about 230 species mainly annual or perennial herbs with some small shrubs, and they are distributed all over the world in a very wide variety of habitats [5]. The genus *Linum* is represented by 39 species (51 taxa) in the Flora of Turkey and East Aegean Islands. Twenty-four taxa of these are endemic [6,7].

Our interest is to evaluate the content of podophyllotoxin and related compounds in different *Linum* species with respect to a possible use as an alternative source instead of *Podophyllum*. Here, we compare the extraction methods [3,8,9] for the determination of lignans in plants of *Linum mucronatum* subsp. *mucronatum* Bertol, *Linum arboreum* L., and the endemic Turkey species of *Linum flavum* subsp. *scabrinerve*.

2. Experimental

2.1. Chemicals

Analytical grade acetone, ethyl acetate, methanol, *o*-phosphoric acid, KH_2PO4, K_2HPO_4, HPLC-grade acetonitrile (E. Merck) and chromatographic grade-double distilled water were used. Podophyllotoxin (Sigma; P-4405) and β-glucosidase from almonds (Sigma; G-4511), 6-methoxypodophyllotoxin (6-MPTOX) was isolated from *L. mucronatum* ssp. *armenum* by Konuklugil et al. [9]. Identity and purity of the isolated 6-MPTOX were confirmed by chromatographic (TLC, HPLC) and spectral (^{1}H NMR) method.

2.2. Plant materials

Aerial and root parts of *Linum mucoratum* subsp. *muconatum* Bertol (AEF 22945), *L. flavum* subsp. *scabrinerve* Davis (AEF 19566), and *L. arboreum* L. (AEF 22946) were collected and plant specimen has been deposited at the herbarium of the Faculty of Pharmacy, Ankara University, Ankara, Turkey.

2.3. Apparatus

The assays were performed with a LC system comprised of a Thermo Finnigan Spectra System P2000 pump, SCM1000 degasser, UV6000LP photodiode-array detector and AS1000 autosampler. The system was controlled with Spectra System SN4000 control module and data analyses were performed with the ChromQuest 4.0 software. The detector was set at 290 nm and separation was carried out at 25 °C using Jetstream 2 Plus Column thermostate. A Grom-Sil 120 ODS-5ST, 5 μm C_{18} (250 mm × 4.6 mm i.d.) column. A guard column Grom-Sil 120 ODS-5ST, 5 μm C_{18} (50 mm × 4.6 mm i.d.) was used to safeguard the analytical column. In the present work all injection volume was 20 μl. All the calculations for quantitative analysis were performed with linear regression external standardization by measurement of peak areas.

2.4. Chromatographic conditions

A gradient system with acetonitrile (A) and 0.01% (v/v) H_3PO_4 in water (B) were used as follows.

Minutes	A (%)	B (%)	Flow rate (ml/min)
0	40	60	0.8
17	67	33	1.0
18	40	60	1.0
24	40	60	0.8

This method has been used for a long time with modifications by Empt et al. [3].

2.5. Linearity and limit of quantitation

Five concentrations of PTOX and 6-MPTOX were subjected to linear regression analysis to calculate the calibration equation. Calibration ranges for PTOX was from 0.055 to 22.0 μg/ml, with a regression line equation $Y = 3497.7 + 96436.4X$; $n = 5$; $r = 0.9999$; Vxo = 2.1% [10]; F-calculated value for ANOVA-linear testing = 12449, for $P < 0.0001$. For 6-MPTOX, the range concentrations were 0.750–60.0 μg/ml, the line equation was $Y = 2633.9 + 12761.8X$; $n = 5$; $r = 0.9999$; Vxo = 1.0% [10], F-calculated = 42482.8 for $P < 0.0001$. Quantitation limit (QL) were established at a signal-to-noise ratio (S/N) of 10. QL were calculated to be 0.550 μg/ml for PTOX and 0.750 μg/ml for 6-MPTOX (injection volume 20 μl). The amount of PTOX and 6-MPTOX were determined in μg/g of dried aerial and root parts. Results are expressed as the mean of three determinations.

2.6. Extraction methods

2.6.1. Method 1: aqueous extraction

Five hundred milligrams of powdered plant material was mixed with 20 ml 25 mM potassium phosphate buffer at pH 7.0 and extracted 1 h in a 100 ml Erlenmeyer by magnetic stirrer. Stirring continued for another 1 h after addition of 20 ml Ethyl acetate. After centrifugation, aqueous and organic phases were separated and ethyl acetate layer was evaporated to dryness under reduced pressure. The residue was dissolved with methanol and completed to 25.0 ml in a volumetric flask then injected to HPLC [1].

2.6.2. Method 2: glucosidase extraction

Five hundred milligrams of plant material was suspended in 5 ml methanol and incubated two times for 30 s in an ultrasonic bath. In order to prevent excessive heating, the samples were cooled on ice in between. Fifteen milliliters distilled water was added to the sample and the pH was adjusted 5.0 with *o*-phosphoric acid. After adding 2.5 mg β-glucosidase to each sample, they were incubated at 35 °C for 1 h. The samples were diluted with 30 ml methanol and were incubated in an ultrasonic bath at 70 °C for 10 min and centrifuged for 15 min at 5000 rpm. The supernatant was filtered under vacuum and completed to 50.0 ml in a volumetric flask with methanol then injected to HPLC [3].

2.6.3. Method 3: acetone extraction

Five hundred milligrams of plant material was suspended with 20 ml acetone in a 100 ml erlenmayer and incubated in an ultrasonic bath one time 30 s. Extraction was continued for 1 h by using a magnetic stirrer. After centrifugation for 15 min at 5000 rpm, the supernatant was filtered and evaporated. Then completed to 25.0 ml with methanol in a volumetric flask and injected to HPLC to measure lignan aglycon in plant material (fraction A). The extraction was continued after adding 20 ml water:methanol (16:4) and the pH was adjusted to 5.0 with *o*-phosphoric acid. β-Glucosidase (2.5 mg) was added to each sample and incubated at 35 °C for 1 h. Twenty milliliters of ethyl acetate was added and vortexed for 10 min. After centrifugation for 15 min at 5000 rpm, aqueous and organic phases were separated and the ethyl acetate layer was evaporated to dryness under reduced pressure. The residue was dissolved with methanol and completed to 25.0 ml in a volumetric flask. By this procedure, lignan glucosides are hydrolyzed and then extracted as agylcone (fraction B). For recovery studies, the total content of lignans was calculated by combining 5 ml of two fractions (fractions A and B) in a 10.0 ml volumetric flask and injected to HPLC to measure total lignan content. In Tables 1 and 2, content of lignans are given in acetone extraction method as aglycone before the enzymatic treatment and together with aglycone and glucosides as total lignan after the treatment.

2.7. Selectivity

The selectivity of the extraction methods and chromatographic method were tested by injecting three different extract from *Linum* species and Standard solution of PTOX and 6-MPTOX (Fig. 2.). The chromatograms at 290 nm, the contour plot and the three dimensional of the chromatograms showed a complete resolution of all peaks. The UV spectra of all the analyte-peaks were identical to the UV-spectra of the standards (Using PDA detector). These proved that the methods are selective enough.

Table 1
PTOX content in the aerial and root part of *Linum* species using different extraction method

	Aqueous	β-Glucosidase	Acetone
PTOX content (μg/g) in aerial part (mean ± S.D.)			
Linum mucronatum subsp. *mucronatum*	159.19 ± 10.57	182.77 ± 5.29	174.98 ± 11.02 25.22 ± 0.49[a]
Linum flavum subsp. *scabrinerve*	237.39 ± 5.55	268.19 ± 0.23	224.21 ± 0.61 42.06 ± 2.09[a]
Linum arboreum	58.66 ± 2.50	38.69 ± 1.63	41.49 ± 0.01 2.77 ± 0.01[a]
PTOX content (μg/g) in root part (mean ± S.D.)			
Linum mucronatum subsp. *mucronatum*	31.83 ± 2.22	39.61 ± 4.08	44.40 ± 1.68 4.05 ± 0.17[a]
Linum flavum subsp. *scabrinerve*	98.90 ± 4.02	98.38 ± 8.17	97.60 ± 9.99 26.11 ± 0.11[a]
Linum arboreum	45.45 ± 3.04	24.57 ± 0.62	38.31 ± 2.82 5.42 ± 0.13[a]

[a] PTOX as a lignan aglycon in plant material with acetone extraction (fraction A).

2.8. Recovery studies

Accuracy studies on the extraction methods were performed by using the standard addition method. Different volumes of a standard solution (containing 453.32 μg/ml PTOX in methanol) were added to 500.0 mg of plant materials. Fifty microliters (22.67 μg) for aerial and root part of *L. mucronatum*, 100 μl (45.33 μg) for aerial and root part of *L. flavum*, and 200 μl (90.66 μg) for aerial and 150 μl (68.0 μg) for root part of *L. arboreum* were added and allowed to dry in room temperature for 12 h. Three extraction methods were applied to all samples after addition of PTOX. Recovery rate was calculated in % for each extraction method of the herbal and root part of *Linum* samples, and evaluated

Table 2
6-MPTOX content in the aerial and root part of *Linum* species using different extraction method

	Aqueous	β-Glucosidase	Acetone
6-MPTOX content (μg/g) in aerial part (mean ± S.D.)			
Linum mucronatum subsp. *mucronatum*	637.33 ± 2.60	545.59 ± 5.71	464.86 ± 19.23 42.59 ± 1.17[a]
Linum flavum subsp. *scabrinerve*	1351.87 ± 1.36	1262.98 ± 69	970.41 ± 42.67 579.39 ± 17.96[a]
Linum arboreum	1648.26 ± 122.27	1504.86 ± 22.41	1144.31 ± 10.01 124.70 ± 0.35[a]
6-MPTOX content (μg/g) in root part (mean ± S.D.)			
Linum mucronatum subsp. *mucronatum*	3151.68 ± 122.11	2492.37 ± 139.36	755.95 ± 5.47 287.15 ± 3.82[a]
Linum flavum subsp. *scabrinerve*	6071.73 ± 117.14	8903.01 ± 22.87	8127.71 ± 43.06 2182.15 ± 79.27[a]
Linum arboreum	6099.08 ± 66.62	5632.53 ± 13.44	5186.23 ± 95.78 1243.43 ± 5.23[a]

[a] 6-MPTOX as a lignan aglycon in plant material with acetone extraction (fraction A).

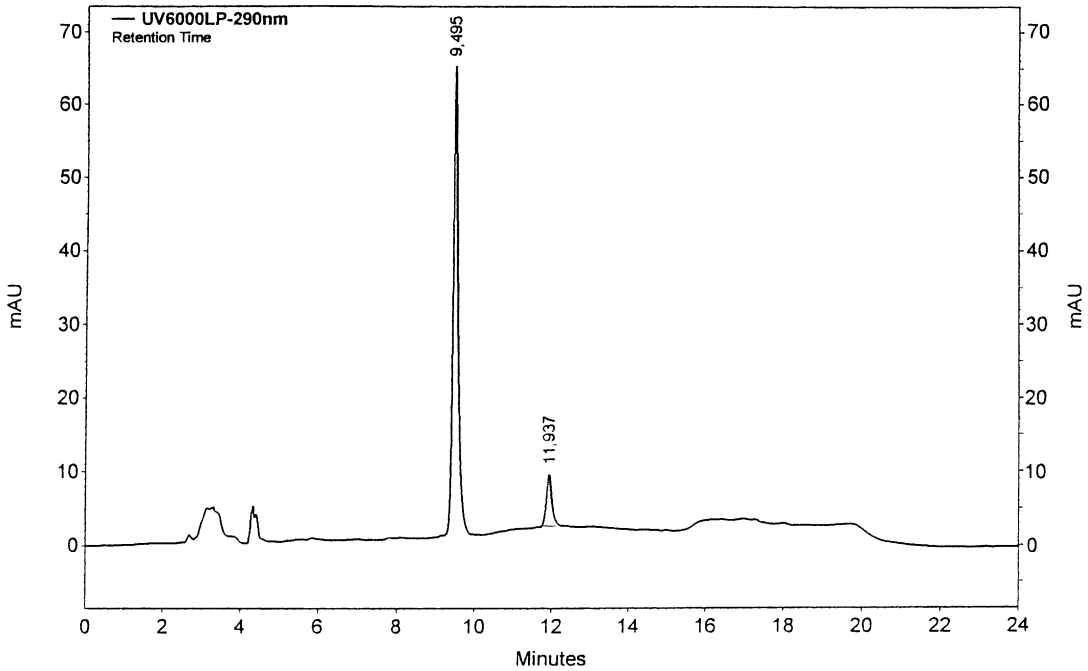

Fig. 2. HPLC chromatogram of the mixture of PTOX (9495) and 6-MPTOX (11,937).

according to method of Funk et al. [10] and Buick et al. [11].

2.9. Precision

The precision of the extraction methods (repeatability) were evaluated by the analysis of three determination of PTOX and 6-MPTOX in the aerial and root part of *Linum* species (Tables 4 and 5).

3. Results and discussion

Different extraction methods have been published for the extraction of podophyllotoxin from plant material [1,4,12,13]. Commercial production of podophyllotoxin involves its purification from an ethanolic extract of the dried roots and rhizomes of *P. emodii*. An *aqoueous extraction* method for podophyllotoxin from *Podophyllum* spp. was developed by Canel et al. [1] (method 1). The use of this extraction method allows substantial in situ enyzmatic conversion of podophyllotoxin glucosides to podophyllotoxin. *β-Glucosidase extraction* (method 2) [3] method is mostly used for podophyllotoxin extraction from plant cell cultures of *Linum* spp.; in this method β-glucosidase converts the lignan glycosides to aglycone which are easily extracted by methanol without loss. Unfortunately, methods 1 and 2 do not differentiate between genuine lignan aglycone and lignan glycosides. Using the *acetone extraction method* (method 3), the lignan agylcone, lignan glucosides and total lignan can be determined separately.

Tables 1 and 2 summarise the results of PTOX and 6-MPTOX analysis from three species of *Linum*. The genus *Linum* and especially section *Syllinum* is known to contain aryltetralin lignans mainly PTOX and 6-MPTOX [8,14]. The results reported here agree with other published work that PTOX and 6-MPTOX content in *Linum* species ranging trace to 1900 μg/g and trace to 23,800 μg/g, respectively [8,15]. The aerial parts of *Linum* species have the higher amounts of PTOX, the roots a much higher amounts 6-MPTOX.

The results of recovery studies are summarized in Table 3. Statistical calculation using one-way ANOVA did not show significant differences between

Table 3
Results of the accuracy determinations according to recovery (%).

Samples	Recovery (%)		
	Method 1	Method 2	Method 3
L. mucronatum subsp. *mucronatum*			
Aerial-parts	101.0	103.9	106.2
Root-parts	94.0	101.4	95.8
L. flavum subsp. *scabrinerve*			
Aerial-parts	104.5	100.5	107.6
Root-parts	100.5	89.6	92.1
L. arboreum			
Aerial-parts	108.1	99.6	104.9
Root-parts	93.5	102.3	96.8
Mean recovery (R.S.D.)	100.3 (5.72)	99.6 (5.11)	100.6 (6.52)
Recovery curve	$X_f = -17.2 + 1.10X_c$	$X_f = -1.7 + 1.01X_c$	$X_f = -16.9 + 1.11X_c$
Vb(af)[a]	-17.2 ± 20.6	-1.7 ± 18.5	-16.9 ± 22.3
Vb(bf)[a]	1.09 ± 0.07[b]	1.01 ± 0.07	1.11 ± 0.09[b]

[a] For $p = 0.05$.
[b] Showed slightly proportional systematic error.

the three methods of PTOX determination ($P = 0.95279$). Analysis using recovery curve according to the method of Funk et al. [10] showed that the method 2 yielded the best results. Methods 1 and 3 show a slightly proportional systematic error. Tables 4 and 5 show that precision (repeatability studies) between three extraction methods for PTOX and 6-MPTOX analysis were almost satisfactory (all less than 10%, except two data in Table 4) [11].

This present work proved that all the methods tested here were satisfactory for the determination of lignan in plant material due to their recovery studies were less than 10% [11]. Comparing the three extraction methods, it was found that one can be used instead of other according to researcher's objectives, if the results of the validation studies showed good results.

Table 4
Repeatability of Extraction Methods for PTOX Analysis

Samples	R.S.D. % ($n = 3$) of PTOX analysis		
	Method 1	Method 2	Method 3
L. mucronatum subsp. *mucronatum*			
Aerial-parts	6.63	2.89	6.30
Root-parts	6.97	10.30	3.78
L. flavum subsp. *scabrinerve*			
Aerial-parts	2.34	0.09	0.27
Root-parts	4.06	8.30	10.24
L. arboreum			
Aerial-parts	4.26	4.21	0.02
Root-parts	6.69	2.52	7.36

Table 5
Repeatability of extraction methods for 6-MPTOX analysis

Samples	R.S.D. % ($n = 3$) of 6-MPTOX analysis		
	Method 1	Method 2	Method 3
L. mucronatum subsp. *mucronatum*			
Aerial-parts	0.41	1.05	4.14
Root-parts	3.87	5.59	0.72
L. flavum subsp. *scabrinerve*			
Aerial-parts	0.84	0.77	4.40
Root-parts	1.93	0.26	0.53
L. arboreum			
Aerial-parts	7.42	1.49	0.87
Root-parts	1.09	0.24	1.85

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