

ДАРМАШНА

1-2/2005

TOM LII VOLUME



PHARMACIA

ФАРМАЦИЯ PHARMACIA

Том/Volume LII

2005

Книжка/Number 1-2

СПИСАНИЕ НА БЪЛГАРСКОТО НАУЧНО ДРУЖЕСТВО ПО ФАРМАЦИЯ

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JOURNAL OF THE BULGARIAN PHARMACEUTICAL SCIENTIFIC SOCIETY

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INFLUENCE OF THE COMPOSITION OF NUTRITION MEDIA ON THE PRODUCTION OF ARYLTETRALIN LIGNANS IN *LINUM TAURICUM* SSP. *TAURICUM* (WILLD) PETROVA CELLULAR AND TISSUE CULTURES

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ВЛИЯНИЕ НА СЪСТАВА НА ХРАНИТЕЛНАТА СРЕДА ВЪРХУ ПРОДУКЦИЯТА НА АРИЛТЕТРАЛИНОВИ ЛИГНАНИ В КЛЕТЪЧНИ И ТЪКАНИИ НА *LINUM TAURICUM* SSP. *TAURICUM* (WILLD) PETROVA

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Summary. Callus, suspension and shoot cultures were established, using conventional methods in sterile conditions from seeds of *L. tauricum* ssp. *tauricum* (Willd) Petrova. Two main lignans – 6-methoxypodophyllotoxin (MPTOX) and 4'-demethyl-6-methoxypodophyllotoxin, which differed from MPTOX in the absence of the 4'-O-methyl group, were isolated from the in vitro cultures of this species. They were identified by comparison of HPLC retention time and UV spectra with those of authentic samples. These two substances were previously isolated and identified from aerial parts of the intact plant. As a part of an experiment of optimization of lignan production, the influence of phytohormones content in growth medium was investigated. Here we present the results of these experiments on conventional cell and tissue cultures of *L. tauricum* ssp. *tauricum* (Willd) Petrova. It was shown that the maximum production of 4'-demethyl-6-methoxypodophyllotoxin was obtained in a liquid NAA media (2.16 mg/g dw), while 6-methoxypodophyllotoxin in G48 agar medium (3.99 mg/g dw).

Резюме. От семената на *L. tauricum* ssp. *tauricum* (Willd) Petrova, по конвенционални методи, при стерилни условия, бяха установени калус, суспензионни и надземни in vitro култури. От тях бяха изолирани два основни лигнана – 6-Метохуродопхилоксин (МРТОХ) и 4'-деметилподопхилоксин, които се различава от (МРТОХ) по отсъствието на 4'-О-метил група. Те бяха идентифицирани посредством сравнение на времената на задръжане при HPLC анализ и UV спектри с автентичен свидетел. Двете вещества предварително бяха изолирани и идентифицирани от надземни части на растението. Като част от експеримент по оптимизиране на лигнановата продукция, беше изследвано влиянието на фитохормоните в хранителната среда. В статията са представени резултатите от това изследване при конвенционални клетъчни и тъканни култури от *L. tauricum* ssp. *tauricum* (Willd.) Petrova. Беше установено, че максимална продукция на 4'-деметилподопхилоксин се постига в NAA течна хранителна среда (2.16 mg/g dw), а на 6-Метохуродопхилоксин – в мрърга G48 (3.99mg/g dw).

Key words: antineoplastic agents, phytogetic/chemistry; lignans/chemistry; podophyllotoxin/pharmacology; cultuce media, conditioned/chemistry (source: MeSH)

Lignans show a number of interesting biological properties and play a significant role in the treatment of different malignant diseases [14]. Their pharmacological activities include such effects as anti-HIV, immunosuppressive, hypolipidemic, anti-fungal, and antiasthmatic effects and antagonism of platelet-activating factor [4]. The aryltetralin lignans podophyllotoxin and 4'-demethylpodophyllotoxin are of great scientific value for their cytostatic properties. The podophyllotoxin blocks mitosis and keeps the cell in stage of metaphase. Thus it looses the ability to replicate. The mechanism of this process is explained by polymerisation of the tubulline in microtubules [11]. Podophyllotoxin however is extremely toxic [3, 5], so that is why semi synthetic derivatives are being sought, with lower toxicity and

higher solubility in water. Up to this moment good results are obtained with the substances etoposide and teniposide. It has been clarified that these molecules inhibit topoisomerasis during the late S- or G2-phase of the cell cycle [11]. The lignan podophyllotoxin (PTOX) is used for the semi-synthesis of the anti-cancer drugs etoposide, teniposide and Etopophos® [2]. The annual demand for etoposide and teniposide is approximately 500-800 kg worldwide. Podophyllotoxin is restricted to only a few plant species, mainly to the families Podophyllaceae, Cupressaceae and Linaceae. Chemical synthesis of PTOX is not economic. Therefore, the limited supply requires identification of other sources for this rare natural lignan. Literature data show encouraging results about in vitro cultures of *Linum* species,

Table 1. Occurrence of *Linum tauricum* (Willd) and its subspecies in Bulgaria (1)

SPECIES	Common occurrence	Habitat in Bulgaria	Subsp.	Habitat of subspecies
<i>L. tauricum</i> Willd.	South East Europe (Balkan peninsula, Krim)	Grassy, rocky, sandy chalky and silicate soils in plains, foothills and mountains, Black sea coast, SE Bulgaria, Danube Plain, West Balkan, Znepole region, East Rhodopes, Thracian lowland, Tundjan hilly Plain, from sea level to 1400m altitude.	serbicum (Podp.) Petrova	West Balkan, Znepole region
			bulgaricum (Podp.) Petrova	Black sea coast, East Rhodopes, Thracian lowland (region of Svilengrad), Tundjan hilly Plain (Derwentian highlands)
			tauricum Exs.: Pl. Bulg Exsicc. № 846	Northern Black sea coast, SE Bulgaria, Tundjan hilly Plain
			linearifolium (Lindem.) Petrova	Danube Plain

being useful for the production and accumulation of pharmaceutically important lignans [10]. Exploitation of other *Linum* species, characteristic for Bulgarian flora is of important value.

Monitoring the influence of nutrition media factors on the biosynthesis of aryltetralin lignans could lead to pathways for optimisation of growth conditions and achievement of higher production levels of cellular and tissue cultures of *Linum* species [8].

The objective of this study is to determine the influence of nutrition media on the synthesis of aryltetralin lignans in cellular and tissue cultures of *L. tauricum ssp. tauricum* (Willd) Petrova. In this paper we present our study results related to the influence of variation of growth hormones in growth media on lignan production of conventional cell and tissue cultures of *L. tauricum ssp. tauricum* (Willd) Petrova.

Materials and methods

Plant material. *Linum tauricum ssp. tauricum* (Willd.) belongs to the Section Syllinum of the genus *Linum* Linaceae (Table 1). It is a perennial herbaceous plant, with a branched root system, 9-40 cm high, the sterile shoots 0.5-13.0 cm high, may or may not form clumps. Flower-bearing shoots are 8-30 cm long; blossoms are yellow [1].

Seeds of *L. tauricum ssp. tauricum* (Willd) were germinated in sterile conditions [7] on hormone free MS-medium [9]. Sterile grown seedlings were used for the initiation of callus cultures on G48 - agar medium (Table 2). They were grown in 300ml Erlenmeyer flasks containing 100 ml medium, in light conditions at 25°C. A quantity of 1.0 g callus was subcultured into a fresh medium at a 21 days period. After more than one year of cultivation, callus was transferred on agar NAA medium. Cell suspension cultures were initiated, by means of transferring callus cells into liquid G48 growth media (Table 2). The cells were cultivated in 300 ml Erlenmeyer flasks, containing 50 ml medium. They

were grown in the dark, on a gyratory shaker at 120 rpm, at 25°C. A quantity of 5.0 g cells was transferred by means of a perforated spoon into fresh medium after a culture period of 14 days. After more than one year of maintenance on liquid G48 medium, suspension was transferred and cultivated in liquid NAA growth medium.

Table 2. Modifications of MS - nutrition media, obtained by supplementation of phyto-hormones and casein to main formula

Nutrition media	Growth factors					
	2,4-D [mg/l]	NAA [mg/l]	IAA [mg/l]	BAP [mg/l]	Kinetin [mg/l]	Casein [g/l]
HP 9	-	-	0.1	0.2	-	-
G48	0.1	-	0.2	-	2.0	1.0
NAA	-	4	-	-	-	1.0

Shoot cultures were initiated from sterile seedlings by means of transferring them into agar HP9 - medium (Table 2). They were grown into 300 ml wide neck Erlenmeyer flasks. At a period of 4 weeks, about 2cm from the upper parts of the best growing shoots were cut and 4-5 such cuttings were transferred into fresh medium.

Determination of fresh weight and dry weight was carried out, as cells were harvested and the weight measured on analytical scales every second day. The value of the growth index was also determined every second day after the formula [7].

$$GI = Mi/Mo$$

GI - Growth Index

Mi - mass of cells/callus, weighed at the day of harvest.

M0 - mass of cells/callus, subcultured in the flask on day 1.

Extraction and isolation of lignans. Lignans were extracted as described in [13], as the extraction method was enhanced by means of a final repeated extraction with Ethylacetate and concentration of the

whole extract. The dried extract was stored at -20°C . For HPLC analysis the pellet was resolved in 200 μl MeOH, diluted 25 times and centrifuged at 13 000 rpm in a microcentrifuge.

Quantitative analysis. The HPLC determination was performed on a Thermo Quest (Egelsbach, Germany), equipped with a Spectra SYSTEM UV6000LP detector [12]. The separation column was a GROM-SIL 120 ODS-5 ST (250 X 4 MM, particle size 5 μm); the analytical program was as follows with solvent A water with 0.05% of 85% phosphoric acid and solvent B acetonitrile: 0 min., 25% B, 0.8 ml/min; 25 min., 38% B, 0.8 ml/min; 43 min., 43% B, 1 ml/min; 46 min., 55% B, 1 ml/min; 48 min., 70% B, 1 ml/min; 50 min., 25% B, 1 ml/min; 55 min., 25% B, 0.8 ml/min. HPLC retention times for the two substances, detected in callus cultures, (agar G48 medium) – 6-Methoxy-podophyllotoxin and 4'-demethyl-6-methoxy-podophyllotoxin: 39.74 min and 28.82 min respectively, are shown on Fig. 1.

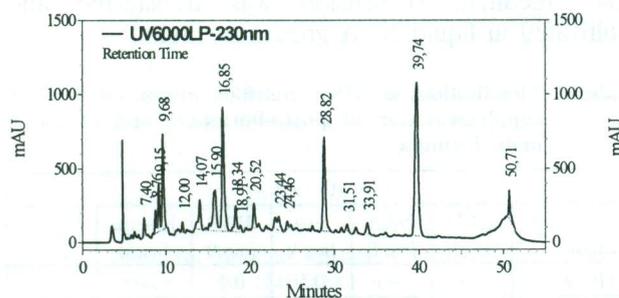


Fig. 1. HPLC retention times for lignan extract of callus culture of *L. tauricum* ssp. *tauricum* (4'-demethyl-6-methoxy-podophyllotoxin – 28.82 min and 6-Methoxy-podophyllotoxin – 39.74 min)

Results and discussion

Seeds of *L. tauricum* ssp. *tauricum* (Willd) were germinated in sterile conditions and suspension (G48), callus (G48) and shoot (HP9) cultures were initiated and maintained.

Callus, suspension and shoot cultures of *L. tauricum* ssp. *tauricum* (Willd) were thus maintained for more than one year, while stable growth was obtained (Fig. 2, 3, 4). The FW and DW curves show the phases of growth of the cultures: lag-phase, exponential phase, plateau and decay. Comparison of GI-curves of suspension and callus cultures shows that suspensions reach the maximum of growth faster than callus cultures, also their growth rate is higher than that of callus and they maintain a higher and longer plateau than callus. This characterizes suspensions as a faster growing and higher biomass producing than callus cultures. The DW curve, however, shows higher levels for the callus culture (although when subcultured, a fivefold higher mass is being taken from suspension than from callus).

This fact is explained with the higher water content in plant cells in a suspension culture, due to the higher turgor of the plant cell in a liquid medium.

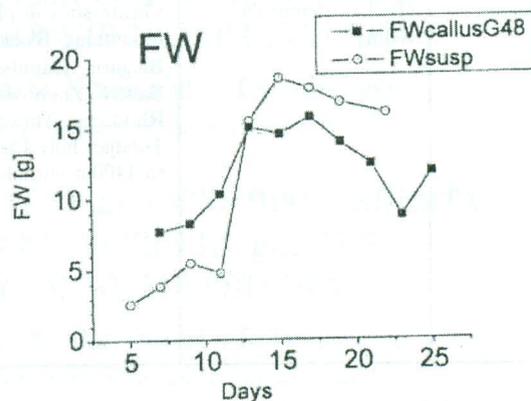


Fig. 2. Fresh weight curve of callus cultures grown in agar G48 and suspension cultures, grown in G48 liquid media

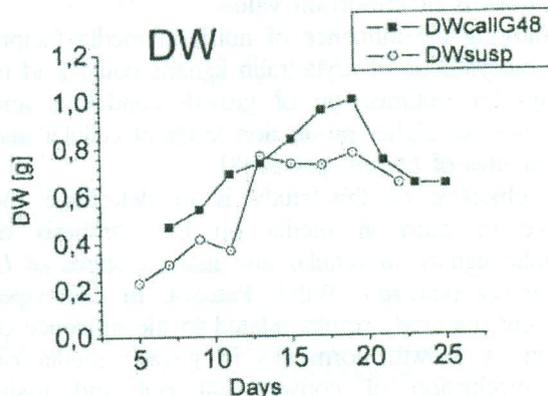


Fig. 3. Dry weight curve of callus cultures grown in agar G48 and suspension cultures, grown in G48 liquid media

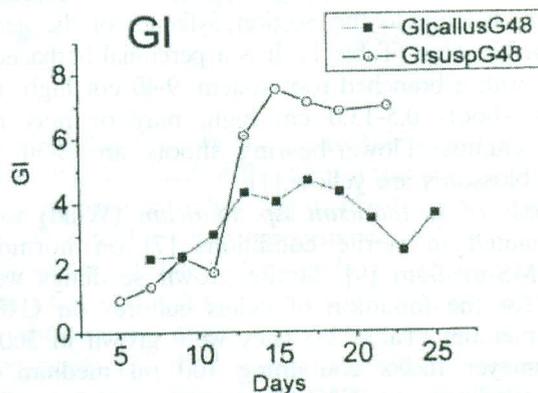


Fig. 4. Growth index curve of callus cultures grown in agar G48 and suspension cultures, grown in G48 liquid media

After more than one year of subcultivation, suspension and callus were transferred respectively to liquid and agar NAA media. The amounts of aryltetralin lignans were determined as aglycones after

Table 3. Concentration of aryltetralin lignans in *L. tauricum* ssp. *tauricum* (Willd) suspension, callus and shoots cultures, grown in variable phytohormones content media

	Suspension G48, content mg/g dw	Suspension NAA content mg/g dw	Shoots HP9 content mg/g dw	Callus G48 content mg/g dw
6-methoxypodophyllotoxin	traces	2.93	1.41	3.99
4'-demethyl-6-methoxypodophyllotoxin	traces	2.16	1.21	1.54

enzymatic hydrolysis with β -glycosidase. The identity of the two substances was verified using authentic samples and UV spectra. The contents of 4'-demethyl-6-methoxypodophyllotoxin and 6-methoxypodophyllotoxin in the respective growth media are presented in Table 3. Suspension cultures, grown in liquid NAA media yielded the highest content of the first lignan, while the second had maximal accumulation in callus cultures, maintained in agar G48 medium. The mg/g dw content in agar HP9 – shoot cultures showed medium levels, compared to the first mentioned. The two active compounds were present only in traces in suspension cultures, grown on agar G48 medium.

Conclusion

Two main lignans were identified in conventional in vitro cultures of *L. tauricum* ssp. *tauricum* (Willd): 6-Methoxypodophyllotoxin and 4'-demethyl-6-methoxypodophyllotoxin.

Maximum production of 6-Methoxypodophyllotoxin (3.99 mg/g dw) was obtained in agar growth medium, containing 0.1 mg/l 2,4-D, 0.2 mg/l IAA, 2.0 mg/l kinetin and 1.0 g/l casein (as protein source), while 4'-demethyl-6-methoxypodophyllotoxin (2.16 mg/g dw) in a liquid medium with 4 mg/ml NAA (naphthylacetic acid) and 1 g/l casein. As both substances were present only in traces in suspension cultures, grown in liquid G48 medium, while after transferring the culture into liquid NAA medium, their production increased, and even a maximum of production of 4'-demethyl-6-methoxypodophyllotoxin was observed, compared to the other media, a conclusion can be made about the decisive influence of NAA, compared to 2,4-D, IAA and kinetin on lignan accumulation in conventional in vitro cultures of *L. tauricum* ssp. *tauricum* (Willd).

Establishing of a high producing undifferentiated in vitro culture is very important, as suspension

cultures have faster growth rate than differentiated cultures, a shorter cycle (2 weeks) and thus a shorter period of harvest and a greater efficiency for the production of active substances.

Acknowledgements. The authors are grateful to Prof. Alfermann from Heinrich-Heine-Universität Dusseldorf for the helpful discussions, to Prof. Dr. A. Petrova from the Botanical Institute, Sofia, who has developed *Linum* genus for Flora of Bulgaria, for revising the plant material.

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