EFFECT OF ELICITORS ON THE ENHANCEMENT OF PODOPHYLLOTOXIN BIOSYNTHESIS IN SUSPENSION CULTURES OF *LINUM ALBUM*

[1]MOHAMMADREZA SHAMS-ARDAKANI, [2]SHIVA HEMMATI, [2]ABDOLALI MOHAGHEGHZADEH

[1]Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran , [2]Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran

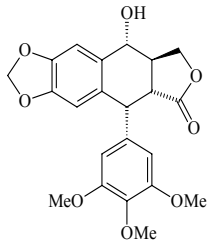
ABSTRACT

The possibility of enhancment accumulation of podophyllotoxin (PTOX) has been studied in cultures of cell suspension of *Linum album* Kotschy ex Boiss. (Linaceae) producing PTOX was studied. Attempts were made to manipulate the biosynthetic pathway of PTOX by some biotic (yeast extract) and abiotic (Ag^{+}, Pb^{2+} and Cd^{2+}) elicitors for 24 or 48 hr in Murashige and Skoog (MS) medium. Silver significantly enhanced PTOX production up to 0.24 % (mg/g cell dry wt.) in cultures. This effect could be attributed to the inhibitory role of silver on production of ethylene.

Key words: *Linum album*, Podophyllotoxin, Suspension culture, Elicitors

INTRODUCTION

About 400-600 years ago, both the native of Himalayas and the America, discovered that the resin produced from an alcoholic extracts of the roots and rhizomes of *Podophyllum* perennial plants had toxic effects which is now attributed to podophyllotoxin (PTOX) (I) a mitotic spindle agent that inhibits the polymerization of tubulin and stops cell division at the beginning of metaphase (1). This cytotoxic natural product was then semi-synthetically transferred to anticancer agents, etoposide (II), teniposide, etopophus®, NK-611 and GL 331 which are used in small cell lung and testis cancers, lymphoma and leukemia (1,2).



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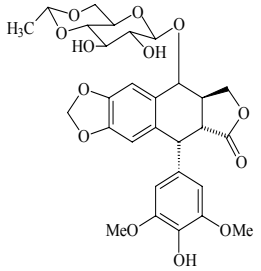

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Besides Podophyllaceae family, PTOX is also found in other plant species like *Linum album* Kotschy ex Boiss. (Linaceae) (3). As the native habitants endangered or disappear completely, and geographical and environmental instabilities make it difficult to acquire certain plant derived chemicals, it may become critical to develop alternative sources of important natural products. There have been considerable interests to investigate potential of the plant cell cultures as an alternative to traditional agriculture for industrial production of secondary plant metabolites (4). Although the undifferentiated cells of a plant suspension culture are generally totipotent, but many genes, including those involved in secondary metabolism, are rerepressed and as the consequence yields of the desired compounds in such cultures are very low. The maximal yield in *in vitro* production of suspension cultures of *L. album* is 0.2% of the dry weight (5, 6) and due to the high costs of cultivating plant cell cultures; their practical uses are far from being economic. Therefore it was decided to manipulate the biosynthetic pathway (Fig. 1) of this species to enhance PTOX accumulation. When plants are attacked by pathogens, they defend themselves against such invasion with several defence mechanisms, both active and passive, that consists of secondary metabolite production. Elicitors are signals triggering for formation of secondary metabolites (7). When cell cultures are subjected to biotic and abiotic elicitors, some genes are derepressed, and as a result, among other things of secondary metabolites are formed (8). In this paper, the effects of elicitors on the PTOX production in cell suspension culture of *L. album* were investigated.

Correspondence: A. Mohagheghzadeh, Department of Pharmacognosy, Shiraz Faculty of Pharmacy, Shiraz, PO Box: 71345-1583, Iran, E-mail: Mohaghegh@sums.ac.ir

MATERIALS AND METHODS

Chemicals

PTOX standard and β-glucosidase enzyme were purchased from Karl Roth (Germany); MS medium from Duchefa (The Netherlands); α-naphthalene acetic acid (NAA), gibberellic acid (GA_3), kinetin and 2,4-dichlorophenoxy acetic acid (2,4-D) were purchased from Sigma; yeast extract, silver nitrate, lead nitrate, cadmium acetate, agar and solvents were from Merck.

Plant material

Linum album Kotschy ex Boiss. (Linaceae) seeds were collected from Lashkarak road, Tehran, at an altitude of 1800 m. The plant material was identified by I. Mehregan and a voucher specimen was deposited at the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Shiraz University of Medical Sciences.

In vitro cultures

Sterile seeds were scarificated and treated with GA_3 (500 mg/l) in a sterile agar medium (0.9%). Callus cultures were initiated from seedlings (upper parts) on MS basal medium (10) supplemented with sucrose (3%), NAA (1 mg/l), kinetin (0.5 mg/l), 2,4-D (0.5 mg/l) and coconut milk (15%), and were solidified with agar (0.9%) at pH 5.6 before autoclaving. Callus cultures were subcultivated every 4 weeks. Suspension cultures were initiated by transfer of callus tissues into liquid MS medium (as above, but without agar; 50 ml in 300 ml Erlenmeyer flasks) and were incubated on a rotary shaker (120 rpm). Cells (5 g) were subcultured in the same medium every 9-10 days and cultures were maintained at 25 $\pm$ 2 °C under permanent darkness. Growth of a suspension culture was determined by sampling every other day during a cultivation period of 22 days. The cells were separated from the medium by filtration under suction and weighted immediately (Fig. 2).

Preparation of elicitors

In order to separate carbohydrate part of the yeast extract, 50 grams of the extract was dissolved in 250 ml to distilled water and after addition of ethanol(80 % v/v) it was incubated at 6 ºC for 4 days and then supernatant was decanted. This process was repeated and the final precipitate was dissolved in 200 ml of distilled water and the solution of final concentrations of 0.25 and 0.8 mg/ml were added to the cultures (9). The final concentration of Ag^{+}, Pb^{2+} and Cd^{2+} were 1, 0.5 and 0.5 mM, respectively (11).

Extraction

To extract PTOX, cells were separated from the liquid media and transferred to a freezer and afterward frozen cells were powdered. For each 200 mg of cells 2 ml ethanol were added and the suspension was put in an ultrasonic bath for 30 seconds and after cooling in ice bath for 90 seconds, again it was subjected to ultrasound for 30 seconds. After addition of water (6 ml) and β-glucosidase enzyme (1 mg) pH of the suspension was adjusted to 5 by addition of 80% o-phosphoric acid. The resulting suspension was incubated for 1 hour at 35 ºC and after addition of ethanol (12 ml), the cells were separated from the supernatant by centrifugation at 500 rpm 10 minutes (12).

Determination and quantification of PTOX by HPLC

The presence of PTOX in the samples was verified by comparison of the Rt and UV spectral peaks of the sample with those of an authentic sample. UV spectra were measured on-line using a Thermo Quest (Egelsbach, Germany) HPLC system was equipped with a Spectra System KO 6000 LP photodiode array detector. The optimum HPLC (Waters) conditions for quantification of PTOX was a water/acetonitril mixture (55:45) as mobile phase by a flow rate of 0.8 ml/min and a nucleosil column 100 (C18, 250 x 4.6 mm, 5 μm) as the stationary phase. The detector was set at 290 nm. Calibration curve for PTOX standards was linear in the range of 0.3-300 μg/ml with the regression factor of 0.9997 (Fig. 3).

Sampling and statistical analyses

Elicitors were added to the third generation of *L. album* suspension cells in triplicate. Cultures without elicitors were also included as control groups. Sampling was performed 24 and 48 hours after addition of the elicitors in triplicate. All statistical analyses were performed by one way ANOVA test, using SPSS 11.5 software; $p<0.05$ and $n=3$.

RESULTS AND DISCUSSION

In vitro cultures of PTOX-producing plant species have been shown to accumulate podophyllotoxin and related compounds. These systems might be developed as an alternative system for production of PTOX as a precursor for important semi synthetic anti-cancer agents (13). In the past 40 years, numerous strategies such as cell line selection, medium optimization, cell immobilization, the use differentiated cells, elicitation and more recently metabolic engineering have been developed to improve the productivity of plant cell culture (14).

As the impact of elicitors on *L. album* cell culture has not been studied, the possibility of increase in the production of PTOX by addition of elicitors to

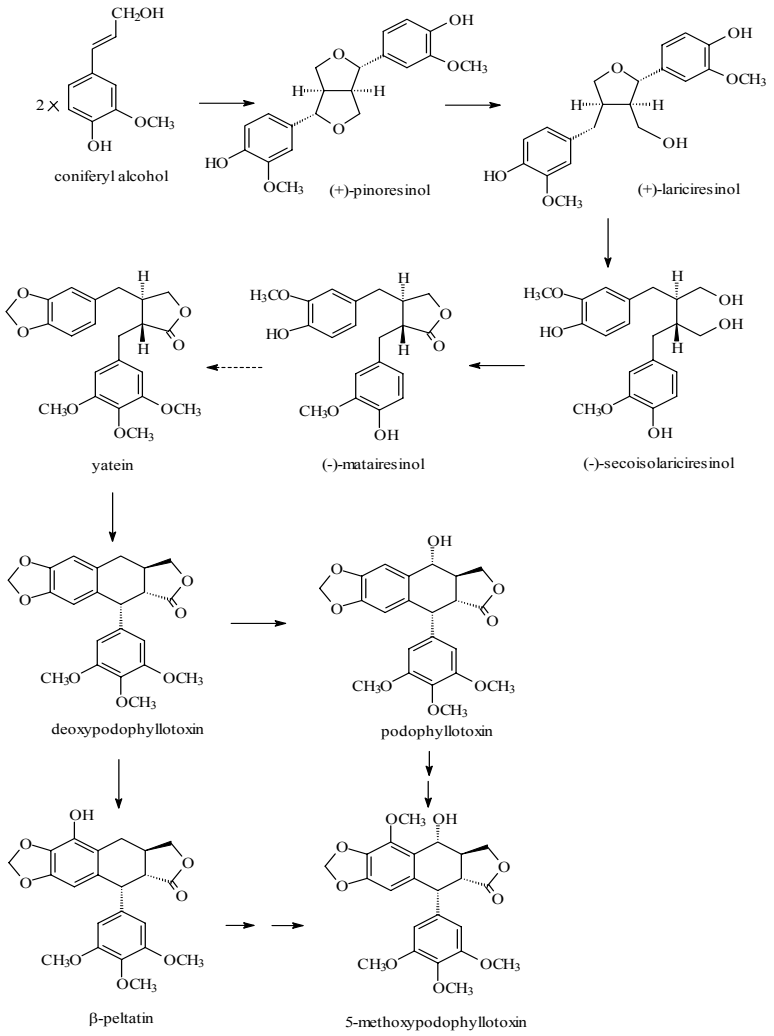

Figure 1. Hypothetic biosynthetic pathway of podophyllotoxin.

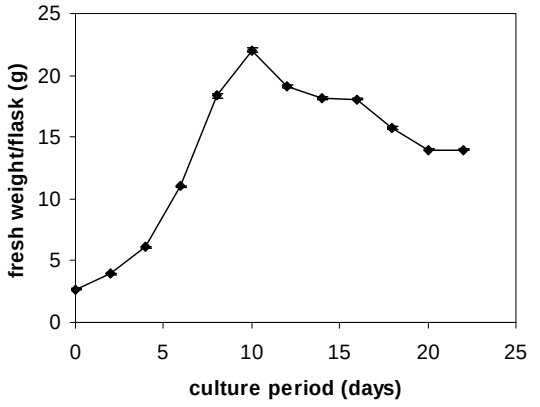

Figure 2. Growth of suspension culture of *Linum album* over a cultivation period of 22 days in MS liquid medium. The inoculum consisted of 5 g FW l^{-1}. Each value is the mean of 3 replications ± SD.

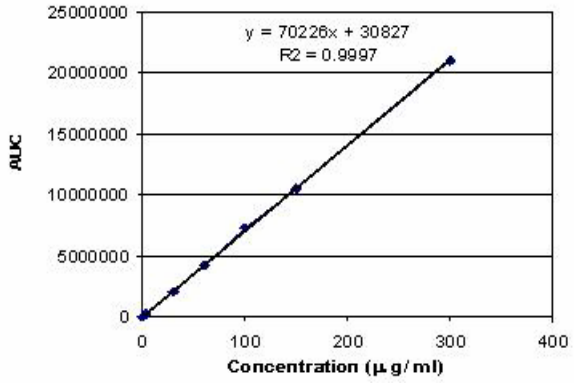

Fig. 3.

Figure 3. Podophyllotoxin calibration curve using HPLC. Each value is the mean of 3 replications

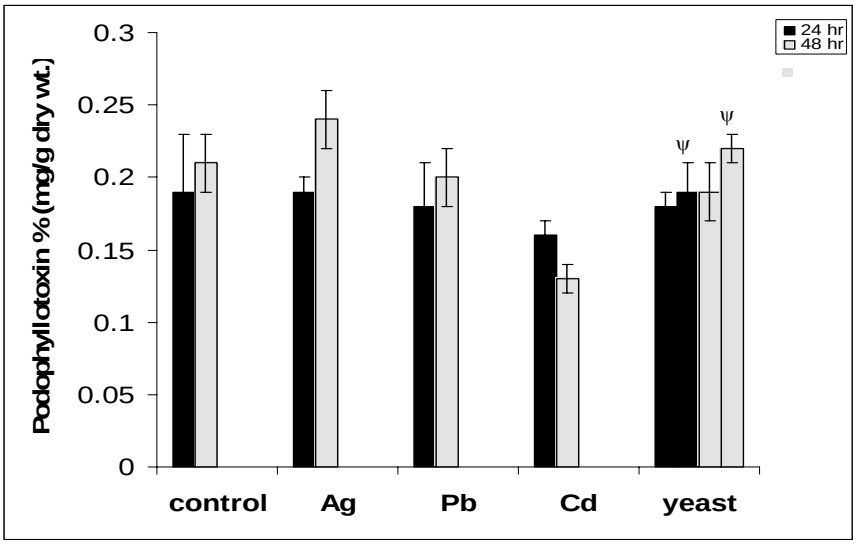

Figure 4. Effect of elicitors (0.25 or 0.8 mg/ml (ψ)) on the production of podophyllotoxin (mg/100 dry wt.) in cell suspension culture of *Linum album* (for 24 or 48 hr in MS medium) with or without Ag, Pb, Cd or yeast extract. Control, untreated cell lines. Each value is the mean of 3 replicates ± SD.

the culture medium was explored in this study. Results of the addition of different concentrations of elicitors to the third generation of the fast growing cell suspension (Fig. 2) cultures after 24 and 48 hours are shown in Fig. 4. These results showed that in comparison with other elicitors silver had the most significant effect on the accumulation of PTOX in suspension cultures of *L. album* and after 48 hours PTOX production reached up to 0.24 % (mg/g dry wt.). Lead didn't show any significant effect but Cd decreased both the fresh weight of the cells and production of PTOX. Yeast extract (0.8 mg/ml) had only a little effect after 48 hours. The effect of Ag may be attributed to its role on inhibition of ethylene production. Ethylene has both stimulatory and inhibitory activity on different genes. This compound blocks the genes encoding hydroxylase enzymes. Silver can be effective on metabolite production by inhibition of ethylene production (11). The effect of yeast extract is also due to its role in increase in phenylalanine ammonia lyase activity which is a key enzyme of phenylpropanoid pathway that catalyses L-phenylalanine deamination and trans cynamic acid production which links primary metabolism to the secondary one, and formation of vast secondary metabolites with phenylpropanoid skeleton (15). On the other hand, all these compounds can produce an osmotic pressure and acts as stress factors which causes rapid but transmitted activation of key enzymes in biosynthetic pathway (7). As it was mentioned in this study only some elicitors were screened and it is necessary to evaluate and screen various elicitors with different mechanisms on the production and accumulation of valuable metabolites for pharmaceutical industries.

ACKNOWLEDGMENT

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