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Time-Dependent Accumulation of Phenolic Compounds in *Linum Album* Kotschy Cell Culture Exposed to Methyl Jasmonate

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Abstract

Linum album can produce many valuable phenolic compounds, especially lignans, potent anticancer agents. In the present study, we attempted to determine the inducing effect of Methyl Jasmonate (MeJa) (50 μ M) as an abiotic elicitor on the production of phenolics such as phenolic acids, flavonoids, and lignans in cell culture of *L. album* over a period of time. The content of cinnamic acid, p-coumaric acid, and ferulic acid increased at 72 h in response to elicitation. However, the level of caffeic acid increased at 48 h of elicitation, 1.53 times higher than the untreated sample. According to the results, the content of flavonoids, including catechin, quercetin, myricetin, and daidzein, also significantly increased during the last hours of the treatment with 50 μ M of MeJA in *L. album* cells. The maximum levels of quercetin and myricetin were observed at 48 h of elicitation, while catechin and daidzein contents peaked at 72 h, 2-fold and 2.42-fold over the control, respectively. Significant time-dependent changes also occurred in Lariciresinol (LARI) and 6-Methoxypodophyllotoxin (6MPTOX) contents under MeJa treatment, while there was no change in Secoisolariciresinol (SECO) content in treated cells compared to the control. The level of 6MPTOX reached 4 times the control sample after 72 h of elicitation. In conclusion, the induction of phenolic contents in response to MeJA in a time-dependent manner can suggest that treatment time adjustment is essential to increase and obtain specific phenolic compounds in the cell culture of *L. album* at a shake-flask scale.

Keywords: Cell culture, Flavonoids, Lignans, *Linum album*, Phenolic compounds.

1 | Introduction

To date, various bioactive agents are derived from plants as the main source of natural biocompounds, which can be used in the pharmaceutical, nutritional, and cosmetic industries [1], [2]. Among phytochemicals,

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phenolic compounds form a major group synthesized by the phenylpropanoid pathway, including phenolic acids, flavonoids, lignans, and lignins [3]. These substances play a pivotal role in preserving plant biological machinery, especially crucial macromolecules, including carbohydrates, proteins, lipids, DNAs, and RNAs, versus the harmful impacts of oxidative processes [4], [5]. On the other hand, phenolic substances have long been used for human health due to their antioxidant potential to prevent many chronic and degenerative disorders as protective dietary ingredients [6], [7]. Many in vitro and in vivo studies on polyphenols exhibit their anticancer, anti-inflammatory, antifungal, antiviral, and antibacterial properties [5], [8], [9]. Therefore, the biosynthesis of these compounds in various plants for their food and medicinal applications has been studied largely in recent years [10], [11].

Linum album Kotschy ex Boiss, a member of the Linaceae family, is a medicinal herb belonging to the endemic plant species of Iran [12]. This plant can produce many phenolic compounds, especially lignans [13], [14]. Podophyllotoxin (PTOX), 6-Methoxy Podophyllotoxin (6MPTOX), Secoisolariciresinol (SECO), and Lariciresinol (LARI) are the main lignans of this plant, which have antiviral and antineoplastic effects [15]. Based on the previous studies, it is evident that in vitro culture is an effective protocol to facilitate the production of large quantities of plants to isolate valuable metabolites while preventing the exploitation of wild plants [16]. In this case, in vitro studies have suggested that different elicitors can induce the accumulation of phenolic compounds such as lignans, lignin, phenolic acids, and flavonoids in *L. album* [17–19]. MeJa, as a potent elicitor, is a methylated derivative of jasmonic acid, which can act as a plant defense regulator involved in the induction of secondary metabolite production [20]. In our previous study, we represented the effect of MeJa on the production of lignans and the accumulation of amino acids and soluble sugars at only one-time point [21]. In the present work, we investigated the time-dependent production of phenolic compounds in *L. album* cell culture under MeJA elicitation by determining changes in phenolic acids, flavonoids, and lignans profiles.

2 | Materials and Methods

2.1 | Cell Culture and Elicitation

L. album seeds were obtained from wild plants in the Sohanak region (35°48'N, 51°32'E, and altitude of 1900 m) placed in Tehran province, Iran. Dr. Shahrokh Kazempour-Osaloo, a professor of plant systematics, identified this plant. Seeds were surface-sterilized and cultivated in a sterile MS medium jar [22] under dark conditions. After seed germination, seedlings as a source of explants were grown in a growth chamber at 16 h photoperiod under $45\mu\text{mol m}^{-2} \text{s}^{-2}$ of irradiance level, 21/17 °C (Day/night), and 50% humidity. A callus line was established from leaf explant grown on solidified MS medium supplemented with 2 mg L⁻¹ of NAA and 0.4 mg L⁻¹ Kin. After callogenesis, suspension culture was provided by crushing friable callus in liquid MS medium supplemented with NAA (2 mg L⁻¹) and Kin (0.4 mg L⁻¹). The samples were placed on a rotary shaker at 110 rpm and 25±2 °C under continuous darkness. Every 14 days, 2 g of cells were subcultured into the fresh media in a 100 mL flask. MeJA with a final concentration of 50μM was added to the cells after 7 days of culture [22]. The samples were harvested at 24, 48, and 72 h after elicitation.

2.2 | Quantification of Phenolic Acids

The samples were extracted based on [23] procedure for determining phenolic acids. Briefly, frozen cells (0.2 g) were ground in methanol with a mortar and pestle. After that, the extracts were centrifuged, and supernatants were collected. After evaporation of the solvent, the residual was resuspended in acetonitrile (4 mL), and the solution was extracted three times with 3 mL of n-hexane. Determination of phenolic acids was performed by HPLC system (Agilent Technologies 1260 infinity, USA) using a C-18 column (Perfectsil Target ODS-3 (5μm), 250 × 4.6 mm; MZ Analysentechnik, Mainz, Germany). A photodiode array (278-300 nm) was set on HPLC to detect UV spectral data. The mobile phase was aqueous acetic acid 2% (A) and methanol (B) [24].

2.3 | Determination of Flavonoids

To measure flavonoids, the cells (0.5g) were homogenized in 1.5 mL of methanol (40%, v/v) containing 0.5% acetic acid. All samples were shaken for 4 h and centrifuged at 13,000 rpm for 12 min. The detection of individual flavonoids was performed based on an HPLC system equipped with a C-18 column (Perfectsil Target ODS-3 (5 μ m), 250 $\times$ 4.6 mm; MZ Analysentechnik, Mainz, Germany). The UV spectra were 254, 280, 300, and 350 nm. Phosphoric acid (0.5%) in deionized water (Solvent A) and acetonitrile (solvent B) were utilized as the mobile phase in the gradient program [25]. Identification of flavonoids was achieved according to the retention time and peak area of their authentic standards.

2.4 | Analysis of Lignans

Lignans contents were determined by Agilent 1260 HPLC chromatography with a Diode Array Detector (DAD) based on [26] protocol. A total of 200 mg of dried cells were used to extract lignans, as described by Yousefzadi et al. [27], with a few modifications. Finally, HPLC-grade methanol (0.5mL) was mixed with the residues, and after centrifuging, 20 μ L of the solution was injected into the HPLC system. A C18-ODS3 (5 μ m, 250 $\times$ 4.6 mm) column was used as the stationary phase. The elution solvent was composed of acetonitrile and water with a standard gradient system, as described by [27].

2.5 | Statistical Analysis

The experiment was accomplished following a completely randomized design. All measurements were performed in triplicate, representing data as the mean $\pm$ standard error. To analyze data, SPSS software version 25 was used. Differences between groups were calculated using an Analysis of Variance (ANOVA) test followed by Duncan's multiple range test ($\alpha=0.05$).

3 | Results and Discussion

Today, the increasing demand for bioactive products has made cultured plant cells, tissues, and organs widely used as an important alternative source for producing and accumulating many valuable phytochemicals [28], [29]. In the last decade, *L. album* has also been introduced into different in vitro cultures, and their capacity for biosynthesis of desired phenolic compounds such as phenolic acids, flavonoids, and lignans has been evaluated in response to different biotic and abiotic elicitors [30], [18], [31–33]. In our previous work, the effect of several concentrations of MeJa elicitor was investigated on the accumulation of phenolic compounds, especially lignans, in *L. album* cell suspension [21]. Here, we detailed the accumulation of phenolics over a time when *L. album* cells were treated with the optimal concentration of MeJa (50 μ M).

The results showed that the contents of some phenolic acids, including cinnamic, p-coumaric, ferulic, and caffeic acids, changed in the treated cells. According to the results, cinnamic acid content altered in *L. album* cells during MeJa treatment, with its peak achieved after 72 h (*Fig. 1a*). On the other hand, changes in p-coumaric acid were found in the treated cells, which was up to 1.13-fold in comparison to the control after 72 h of exposure (*Fig. 1b*). Also, caffeic acid content increased at 48h of MeJa treatment, which was 1.53 times more than the control sample (*Fig. 1c*). As shown in *Fig. 1d*, the results indicated a decrease in ferulic acid content after 24h of MeJa treatment, while its content increased after 72h. Phenolic acids are a group of phenolic compounds with antimicrobial and antioxidant properties and play key roles in plant tolerance to cope with adverse conditions and decrease the toxic effects of Reactive Oxygen Species (ROS) [34]. Among the phenolic acids, cinnamic acid can also serve as a substrate to form other complex phenolic compounds with ubiquitous distribution in fruits and vegetables [35]. Furthermore, phenolic acids are commonly utilized as flavor and aroma compounds in the food, beverage, and perfumery industries [36]. Our observations were quite similar to the results reported by [18], where they considered higher concentrations of cinnamic, p-coumaric, ferulic, and caffeic acids in hairy roots of *L. album* exposed to chitosan in a time-dependent manner.

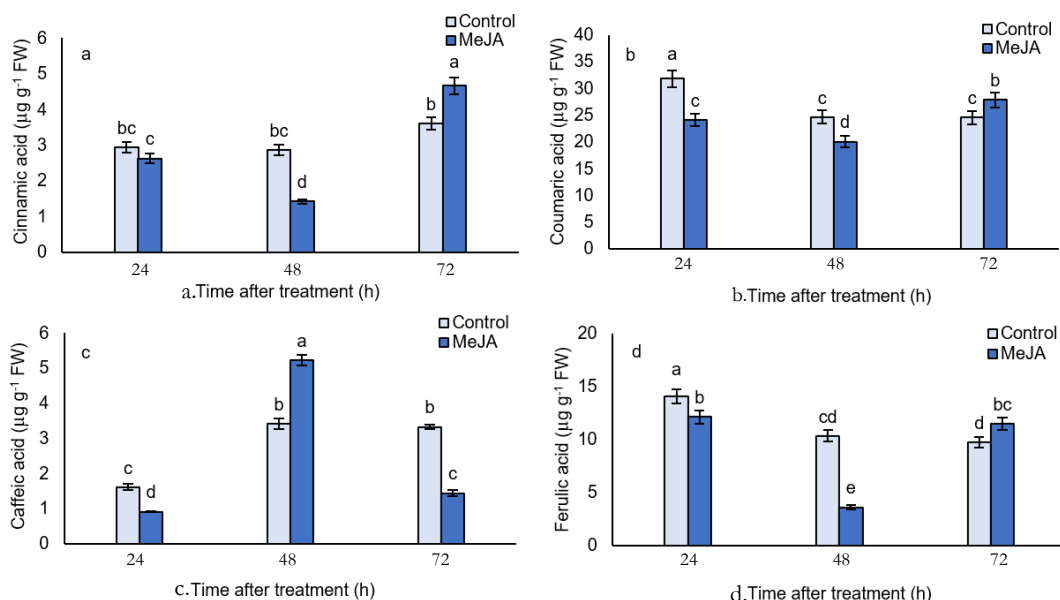
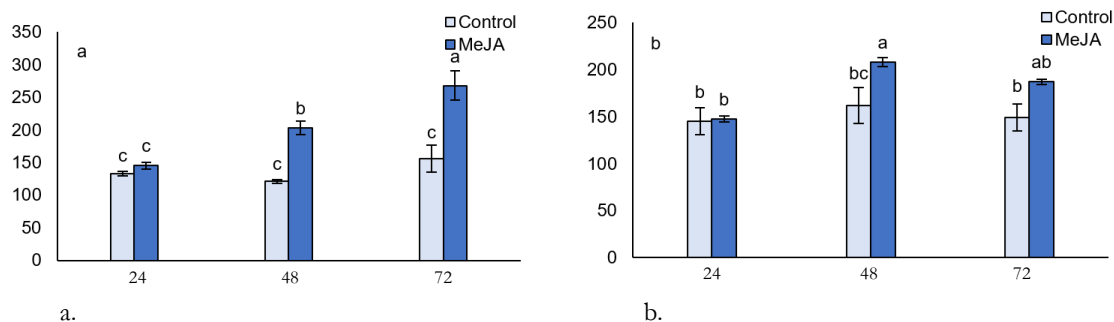


Fig. 1. Effect of MeJA elicitation on the contents of cinnamic acid; a. coumaric acid, b. caffeic acid, c. and Ferulic acid, d. in *L. album* cell culture.

Data are means $\pm$ SD. Significant differences ($P \leq 0.05$) are indicated by different letters.

The effect of MeJA elicitation on the contents of various flavonoids was tested by measuring the levels of individual flavonoids catechin, quercetin, myricetin, and daidzein, where HPLC chromatogram of the treated samples showed the significant changes in their contents (Fig. 2). According to the results, the content of catechin enhanced during MeJA treatment, which its peak achieved at 72h ($267.8 \mu\text{g g}^{-1}$ FW) (Fig. 2a). Quercetin content remained unaffected at 24 h of MeJA treatment compared to the control. However, its content showed a significant enhancement after 48h and 72h as a time-dependent response, where its content was up to approximately 1.26 and 1.28-fold, respectively (Fig. 2b). A significant increase was also observed for myricetin at 48h of treatment, which was 1.21 times higher than that of the control sample (Fig. 2c). Likewise, daidzein showed a linear increment in content during the treatment period, with a peak of 2.42 times that of the control sample after 72h of elicitation (Fig. 2d). It has been determined that flavonoids are abundant polyphenols that are involved in various plant life processes such as regulating cell growth, attracting pollinators, and protecting versus adverse conditions [37].

Likewise, these molecules can act as signaling agents, UV filters, and ROS scavengers, and thus, they can modulate the plant defense system against different stresses like drought, heat, and freezing [38]. In humans, flavonoids exhibit a broad spectrum of health benefits, which are dependent on their natural structures and bioactive functions, including anti-inflammatory, anticancer, anti-aging, cardio-protective, neuroprotective, immunomodulatory, antidiabetic, antibacterial, antiparasitic, and antiviral properties [39]. The hydroxyl group in flavonoids is well-known as a functional group for donating electrons through resonance, which deactivates free radicals and ameliorates the antioxidant system [40].



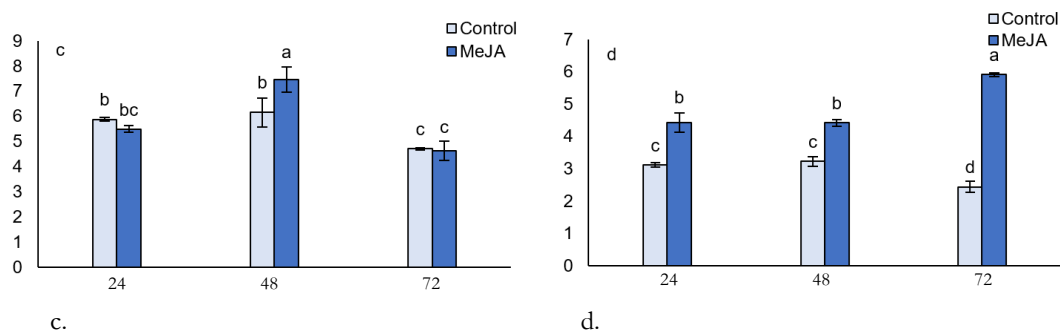


Fig. 2. Effect of MeJA elicitation on the contents of catechin (time after treatment), a. catechin, b. quercetin, c. Myricetin, d. daidzein.

Data are means $\pm$ SD. Significant differences ($p \leq 0.05$) are indicated by different letters.

On the other hand, we quantified 3 main lignans of 10-day-old cultured cells of *L. album* following MeJA elicitation at different time points. As shown in Fig. 3a, SECO content did not change significantly during MeJA elicitation. Moreover, LARI content significantly increased during MeJA elicitation over a period of 24 h in comparison with the untreated sample. However, its content was decreased at 48h, which was approximately 0.77-fold lower than the control (Fig. 3b). The content of 6MPTOX increased after MeJA elicitation so that a 4-fold increase was observed after 72h (Fig. 3c). Previous studies have been shown that lignans, as a subgroup of monolignols derived from phenylpropanoid route, are associated with various defense mechanisms in *L. album* that lead to the modulation of the effect of different biotic and abiotic elicitors [17], [18]. Clinician reports have shown that lignans and their glycosidic derivatives (e.g., etoposide, etopophos, and teniposide) have functional roles in treating several types of cancers as antineoplastic drugs [41], [42].

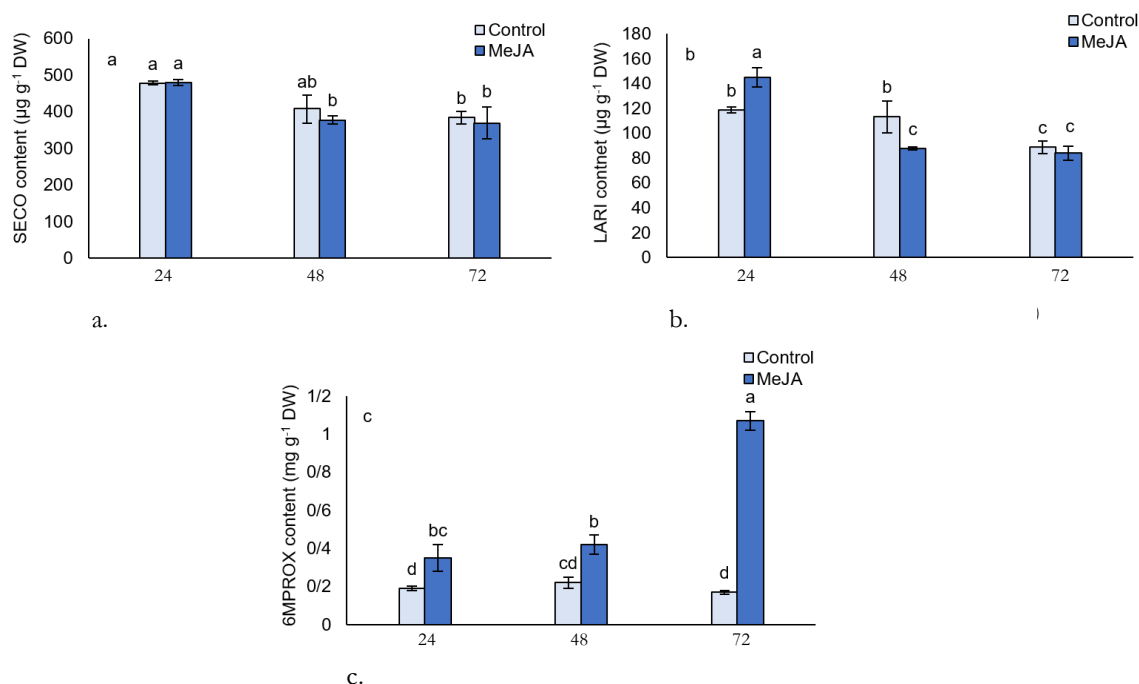


Fig. 3. Effect of MeJA elicitation on the contents of SECO (Time after treatment (h)); a. LARI, b. and 6MPTOX, c. in *L. album* cell culture. Data are means $\pm$ SD. Significant differences ($P \leq 0.05$) are indicated by different letters.

Fig. 4a shows a positive correlation between phenolic acids, flavonoids, and lignans was found. Cluster analysis of phenolic compounds revealed 4 distinct clusters: 1) myricetin to caffeic acid, 2) 6MPTOX to catechin, 3) ferulic acid to cinnamic acid, and 4) LARI to SECO. Principal Component Analysis (PCA) of the HPLC data

Author Contributions

All authors contributed to this research paper. The investigation, Methodology, Data curation, Resources, Formal analysis, Software, Performing the experiments, Analyzing the data and writing the manuscript, M.S., Design and Supervise the study, Project administration, Supervision, Methodology, Data curation, Manuscript review, and editing, M.SH., Advise the study, Manuscript review, and editing, E.S. All authors have read and agreed to the published version of the manuscript.

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Data Availability

The data supporting the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

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