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Phenylpropanoid biosynthetic gene expression in cell suspension culture of *Haplophyllum virgatum* Spach. under chitin treatment

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Abstract

Plants of the genus *Haplophyllum* produce various secondary metabolites, including lignans, which are the product of the phenylpropanoid pathway. This study investigated the effects of different concentrations of chitin (0, 50.0, 100.0, and 150.0 mg L^{-1}) on the gene expression pattern of the key biosynthetic enzymes in phenylpropanoid pathway and the production of podophyllotoxin, as a medicinal valuable lignan. The cell suspension culture of *Haplophyllum virgatum* variety *virgatum* was used as plant material. The effects of chitin were also studied on the changes of fresh weights of the cultured cells. The contents of produced H_2O_2, malondialdehyde, and the antioxidant enzymes activities (superoxide dismutase, peroxidase, and catalase) were measured under the treatments as well. Increasing the concentration of chitin and duration of elicitor exposure resulted in reducing the malondialdehyde content. Also, podophyllotoxin showed the highest accumulation in 150.0 mg L^{-1} after 120 h. Transcripts of the studied genes (*4CL*, *CCR*, and *CAD*) showed the highest level at 12 h after treatments, decreasing after 24 h. A time lag was observed between the maximum gene expression of biosynthetic enzymes and the measured lignan contents in different time courses. This study suggests that the introduced optimum concentrations of chitin in this research could be considered an effective biotic elicitor to improve the *in vitro* production of podophyllotoxin, in this medicinal plant.

Keywords Cell suspension culture · Chitin · Elicitation · *Haplophyllum virgatum* · Phenylpropanoid pathway · Podophyllotoxin

Introduction

Plants of the genus of *Haplophyllum* (Rutaceae), with 70 species, grow chiefly in warm, temperate, and subtropical areas of the northern hemisphere of the Old World. The Iran-Turanian region, especially Iran, Turkey, and Central Asia is the major center of diversity of this genus (Townsend 1986; Parhoodeh *et al.* 2011). This genus has 30 species in the flora of Iran, of which 14 are endemic, such as *H. virgatum* Spach. (Mozaffarian 1996; Joharchi 2008). The dominant compounds of *H. virgatum* essential oil include non-terpene hydrocarbons and monoterpene hydrocarbons such as β-pinene (Mohammadhosseini *et al*. 2021). The antimicrobial, insecticidal, and antitumor activities of the β-pinene derivatives were reported (Zielinska-Blajet and Feder-Kubis 2020). Rutaceae plants have been applied in traditional medicine to remedy herpes, warts, stomachache, toothache, skin diseases (Bessonova *et al.* 1989), and testicular cancer (Ea *et al*. 2008). Plants of this genus contain essential oils, alkaloids, fixed oils, coumarins, sterols, flavonoids, and lignans (Al-Burtamani *et al*. 2005; Karimi *et al*. 2013; Mechehoud *et al*. 2014; Rasulova *et al*. 2015). The presence of podophyllotoxin, as a lignan, in some *Haplophyllum* species (Shah *et al* 2021) prompted this study to investigate this compound in *H. virgatum*.

Plants produce a variety of organic compounds that do not function in growth and development. Plant secondary metabolites play important roles in the defense mechanism under biotic and abiotic environmental stress. Many secondary metabolites have pharmacological and therapeutic activities. In plants, the phenylpropanoid pathway is related to the biosynthesis of diverse secondary metabolites, including coumarins, flavonoids, lignins, and lignans (Alfermann *et al*. 2008; Kabera *et al*. 2014). Phenylpropanoids regulate

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various physiological processes, including pathogens resistance, environmental stresses, pigmentation, and structural compounds biogenesis (Tanaka *et al*. 2008; Vogt 2010; Valdes-Lopez and Hernandez 2014). The phenylpropanoid pathway converts phenylalanine to hydroxycinnamoyl-CoA thioesters. These compounds directly synthesize two groups of secondary metabolites, including flavonoids and monolignols. As one of the phenylpropanoid groups, monolignols are present in lignans and lignins. The monolignol biosynthesis pathway starts with the deamination of L-phenylalanine, which results in the formation of trans-cinnamic acid (Supplementary Fig.). Trans-cinnamic acid turns to cinnamaldehyde, *via* several successive enzymatic reactions (Hano *et al*. 2006). The enzyme 4-coumarate CoA ligase (4CL; EC6.2.1.12) converts hydroxycinnamic acids to hydroxycinnamoyl CoA thioesters by forming a bond between the carboxyl group of hydroxycinnamic acid and coenzyme A. It is the critical step in the biosynthetic pathway of phenylpropanoids. The cinnamoyl-CoA reductase (CCR; EC 1.2.1.44) catalyzes the reduction of hydroxycinnamoyl CoA thioesters to comparable aldehydes. CCR is an essential enzyme in the rate-limiting step of monolignol biosynthesis (Whetten and Sederoff 1995; Zhou *et al*. 2010). CAD (EC 1.1.1.195) has an important role in the last stage of monolignol biosynthesis. CAD is a major rate-limiting enzyme in monolignol biosynthesis and catalyzes the NADPH-dependent conversion of hydroxycinnamic aldehydes to hydroxycinnamyl alcohols (monolignols), which are used in lignin and lignan biosynthesis. Lignans, as a large class of secondary metabolites, are composed of phenylpropanoid dimers. These compounds play an important role in human pharmacological and nutritional value (Westcott and Muir 2003; Landete 2012). They also have a preeminent position in plant defense because of their antifeedant activity (Harmatha and Dinan 2003). Podophyllotoxin has shown antiviral activities, and its semisynthetic derivatives, such as teniposide, etoposide, and etopophose, are employed as anticancer drugs (Canel *et al*. 2001).

Cell suspension culture is an effective technique for studying the effects of different factors on the induction of valuable secondary metabolites (Georgiev *et al*. 2009; Murthy *et al*. 2014). In many instances, the chemical synthesis of metabolites is not possible or economically efficient. However, few valuable plant natural products have been commercialized using plant cell cultures (Yue *et al*. 2016). Thus, the different experimental strategies are used for increasing productivity in plant cell culture, such as elicitation (Murthy *et al*. 2014). The elicitation technique adds exogenous elicitors in plant cells and tissue cultures. This process triggers the stress response in the suspension culture resulting in a concomitant increase in secondary metabolite biosynthesis. Elicitors are biotic or abiotic based on their origin and can cause biochemical and physiological alteration in plants (Ramirez-Estrada *et al*. 2016). The fungal polysaccharides, including chitin, act as biotic elicitors. They affect the induction of different secondary metabolites (Namdeo 2007). Chitin is a polymer of N-acetyl glucosamine with the β-(1,4)-glycosidic bond and is effective in the induction of secondary metabolites in plant cell suspension cultures (Gadzovska Simic *et al*. 2015). Therefore, chitin was investigated to stimulate the lignan production in this study.

This research has determined whether chitin, as a biotic elicitor, is effective on the phenylpropanoid pathway and podophyllotoxin production in the Iranian endemic plant *Haplophyllum virgatum* variety *virgatum*. The influence of chitin on the relative expression of *4CL*, *CCR*, and *CAD*, the key enzymes acting in the phenylpropanoid pathway, was evaluated to determine how chitin might improve podophyllotoxin production. Supplementary physiological factors, including the activity of antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POX), and catalase (CAT), and the production of hydrogen peroxide and malondialdehyde were analyzed to evaluate antioxidant defense mechanisms of treated cells under stressful situations.

Materials and Methods

Plant Materials and Cell Suspension Cultures The seeds of *H. virgatum* variety *virgatum* were collected from the Geno area in the Hormozgan province of Iran (27°27′ N latitude and 56°18′ E longitudes at the altitude of 45 m). The seeds were sterilized with 70.0% (*v/v*) ethanol for 30 s and 10.0% sodium hypochlorite for 10 min and then washed with sterile distilled water three times. For initiation of callus culture, sterile crushed seeds were cultured as explants on solid B5 medium (Gamborg *et al*. 1968) supplemented with 0.2 mg L^{-1} kinetin (Kin), 0.5 mg L^{-1} α-naphthaleneacetic acid (NAA), and 0.5 mg L^{-1} indole-3-acetic acid (IAA) at pH 5.7 in darkness at 25°C. The final medium pH was adjusted using a diluted NaOH solution. Cell suspension cultures were established from 2-mo-old fresh friable calluses inoculated in 50 mL liquid B5 medium supplemented with the same explained plant growth regulators. The callus suspension cultures were incubated in darkness at 25°C, shaking 110 rpm. The separated cells (25-d-old) were used as the plant material for further investigation in elicitation experiments (Fig. 1).

Elicitor Treatment Treatments of cell suspensions with concentrations of 0, 50.0, 100.0, and 150.0 mg L^{-1} chitin (Sigma Aldrich, Steinheim, Germany, C7170) were performed when cells were in the mid-log phase of growth. The cells were separated from the liquid media after 8, 12, 24, 48, 72, and 120 h of treatments. The suspension was filtered on a Buchner funnel through screen nylon mesh to remove

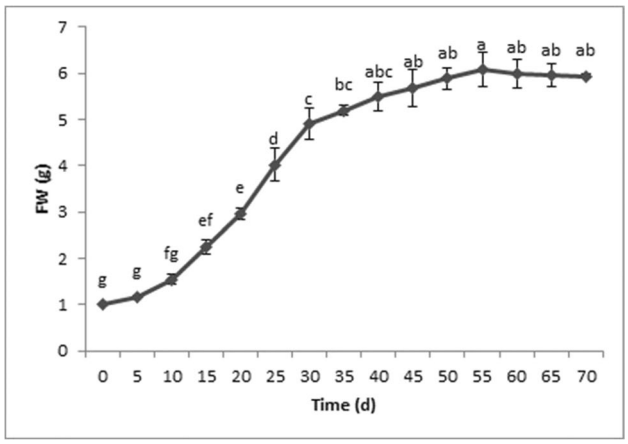

Figure 1. The growth curve of cell suspension culture obtained from *Haplophyllum virgatum* friable calluses. Data represent average values of three replicates ± SD. The *vertical bars* represent standard errors. Means with *different letters* are significantly different at $P \leq 0.05$ as determined by the Duncan test.

cell aggregates, then frozen in liquid nitrogen and preserved at − 80 °C for subsequent analyses. The fresh weight changes of treated samples were also compared with the control.

Hydrogen Peroxide Content H_2O_2 content was measured based on the procedure reported by Velikova *et al*. (2000). Approximately 0.5 g of fresh sample was homogenized with 1.0 mL 0.1% (*w/v*) trichloroacetic acid solution at 4°C. The mixture was centrifuged at 4°C (7300 × g) for 20 min, then 0.5 mL of the supernatant was mixed with 0.5 mL of 10 mM potassium phosphate buffer (pH 7) and 1.0 mL of 1.0 M KI. The absorbance of the mixture was recorded at 390 nm. H_2O_2 content was calculated using an extinction coefficient of 0.28 μM^{-1} cm^{-1} and expressed as μM g^{-1} FW.

Malondialdehyde Content The malondialdehyde (MDA) content was measured according Health and Packer (1968). A fresh sample (0.2 g) was homogenized with 0.1% trichloroacetic acid solution (*w/v*) (2 mL), and the mixture was centrifuged at room temperature (7300 × *g*) for 10 min. The supernatant (1.0 mL) was mixed with 1.0 mL of 0.5% (*w/v*) thiobarbituric acid dissolved in 20.0% (*w/v*) trichloroacetic acid. The resulting mixture was placed in a water bath at 95°C for 30 min. It was immediately cooled in an ice bath, then centrifuged at room temperature (7300 × *g*) for 10 min. The absorbance of the supernatant was recorded at 532 and 600 nm. The MDA content was measured using 155 mM^{-1} cm^{-1} as the extinction coefficient and was reported as μM g^{-1} FW.

Total Protein Content Total protein content was measured based on the method of Bradford (1976) using bovine serum albumin. For this, 0.3 g of fresh sample was homogenized at 4°C with 1.0 mL of 50.0 mM Tris–HCl (pH 7.5), then centrifuged at 4°C and 8600 × *g* for 20 min. The supernatants were used for the designation of protein content and enzyme activity. The protein content was reported as milligrams per gram of FW.

The Activity of Antioxidant Enzymes The activity of SOD (E.C. 1.15.1.1) was determined using the amount of enzyme that inhibited the photochemical reduction of nitro blue tetrazolium (NBT) (Giannopolitis and Ries 1977). The reaction mixture contained 50.0 mM sodium phosphate buffer (pH 7), 0.1 mM EDTA, 13.0 mM methionine, 10.0 µM riboflavin, 75.0 µM NBT, and 100.0 µL of enzyme extract. The reaction mixture was exposed to light for 15 min, and absorbance was recorded at 560 nm against the non-irradiated sample. The SOD activity was expressed as units per milligram of protein.

The activity of CAT (E.C. 1.11.1.6) was measured based on the decrease of absorbance at 240 nm due to H_2O_2 decomposition. Approximately 3.0 mL of 50.0 mM sodium phosphate buffer (pH 7), 100.0 µL of H_2O_2 (1%), and 100.0 µL of enzyme extract were mixed. The final activity of the enzyme is defined in units per mg of protein (Aebi 1984).

The activity of POX (E.C. 1.11.1.7) was measured based on Upadhyaya *et al*. (1985) method. The reaction mixture contained 2.5 mL of 50.0 mM sodium phosphate buffer (pH 7), 0.1 mL of 5.0 mM H_2O_2, 0.1 mL of 20.0 mM guaiacol, and 100.0 µL enzyme extract. The POX activity was expressed as a result of the oxidation of guaiacol with increasing absorbance at 470 nm and reported as units per milligram of protein.

Podophyllotoxin Extraction and Analysis Fresh cells (500 mg) were homogenized in 1.0 mL of ethyl acetate, followed by sonication for 10 min and shaking for 1 h. The supernatant was obtained after centrifugation at room temperature and 5000 × *g* for 10 min and evaporated until dry. The residue was dissolved with 1.0 mL methanol and filtered *via* a syringe filter with 0.2-µ pore size (SARTORIUS, Goettingen, Germany) followed by HPLC analysis. The instrument that was used was a Knauer GmbH HPLC system (Berlin, Germany) with a 5-µm C18 vertex column (250 mm × 4.6 mm ID). Column temperature was regulated at 25°C and 20.0 µL of extract injected. The mobile phase was prepared of two HPLC grade solvents (A, acetonitrile 100%; B, 0.01% aqueous phosphoric acid). The gradient elution program was as shown in Table 1. The flow rate was 0.9 mL min^{-1}, and UV detection was adjusted at 290 nm. The calibration curve (concentration against peak surface area) was drawn using standard podophyllotoxin (Sigma-Aldrich, Saint Louis, UK).

LC–MS analysis was used to confirm the presence of podophyllotoxin in *H. virgatum* cell suspension culture extracts. For this purpose, LC–MS analyses were performed

Table 1. The HPLC gradient elution program used for the analysis of extracts

Time (min)	Flow Rate (mL min^{-1})	Solvent A (%)	Solvent B (%)
0	0.9	0	100
10	0.9	80	20
30	0.9	80	20
37	0.9	100	0
45	0.9	0	100

on the standard podophyllotoxin and the lignan extracts. The device used was Waters Alliance 2695 HPLC-Micromass Quattro micro API Mass Spectrometer with positive-mode electrospray ionization (ESI) (Fig. 5).

Relative Expression of 4CL, CCR, and CAD Fresh samples were powdered with liquid nitrogen. Total RNA extraction was performed using the RNX-plus kit (SinaClon, Tehran, Iran) according to the constructor's instructions. The quantity of total RNA was assessed using spectrophotometer analysis (BMG Labtech Spectrostar, Ortenberg, Germany). Agarose gel electrophoresis (1.0%) was used to evaluate the quality of RNA. The cDNA Synthesis Kit (KiaGeneFanavar Aria, Tehran, Iran) was used for cDNA synthesis from 1.0 µg RNA in a final volume of 20.0 µL according to the instruction provided by the constructor.

Real-time quantitative PCR (RT-qPCR) (Rotor-Gene 6000, QIAGEN, Germantown, MD) was used to determine the expression of *4CL*, *CCR*, and *CAD* genes. RT-qPCR was performed by 0.5 µL of cDNA being added to the solution, including 0.5 µL of each primer, 5.0 µL of Green-2-Go qPCR Mastermix (Bio Basic, Toronto, Canada), and 3.5 µL double-distilled water for a final volume of 10.0 µL. The real-time program is a combination of the primary denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, followed by annealing temperature (*Tubulin*, 57.4°C; *4CL*, 58°C; *CCR*, 56.5°C; and *CAD*, 57.4°C) for 30 s, and 72°C for 30 s. To confirm the single amplified product of the gene, a single distinct peak of melting curve analysis was used at 72 to 95 °C. Tubulin was used as an interior control to normalize the RT-qPCR data. The $2^{-\Delta\Delta CT}$ method was used for calculating the relative gene expression. The design of primers based on conserved sequences in the Rutaceae family. The Oligo 7 program was used for primer designing (Table 2).

Statistical analysis All data were obtained from three independent replications. SPSS software (Ver.16) was used to compare the mean differences, and one-way analysis of variance (ANOVA) was done to analyze the data. The significance of differences was defined by Duncan's tests at the $P \leq 0.05$ level. A heatmap diagram was drawn using the Pearson correlation coefficient and Phyton 3.7.4 software (https://www.phyton.org) between all parameters.

Table 2. Sequence of primers used for the real-time PCR analysis

Gene name	Sequence
4CL	F: 5′-ATGGAATGACAGAGGCAGGG-3′
	R: 5′-TTGATTGTGGGGAAGGGAGG-3′
CAD	F: 5′-GGCTGAGAGGAGGAATCTTGG-3′
	R: 5′-AGCTGACAAGGTACTGATCGG-3′
CCR	F: 5′-CTCACCAGCTCCATTTCTTCC-3′
	R: 5′-TGCCACCACATCCGTTCC-3′
Tubulin	F: 5′-GTAGGCGGAGGAGATGATGC-3′
	R: 5′-GATGGAAGAGTTGGCGGTAAGT-3′

Result and Discussion

Effect of Elicitor on Growth The influence of the elicitor on the growth of *H. virgatum* cells was monitored by the fresh weight measurement of the cells after treatment with and without chitin (Fig. 2). An increase of fresh weights was observed in the control samples during the experiment (0 mg L^{-1} chitin between 8 and 120 h). In the concentration of 50.0 mg L^{-1} chitin, no significant increment of cell fresh weight was observed during the investigation. However, a substantial increase in the fresh weight was observed in the presence of 100.0 and 150.0 mg L^{-1} chitin after 120 h and 48 h, respectively. Gadzovska Simic *et al.* (2015) found that biomass production decreased by using chitin as an elicitor

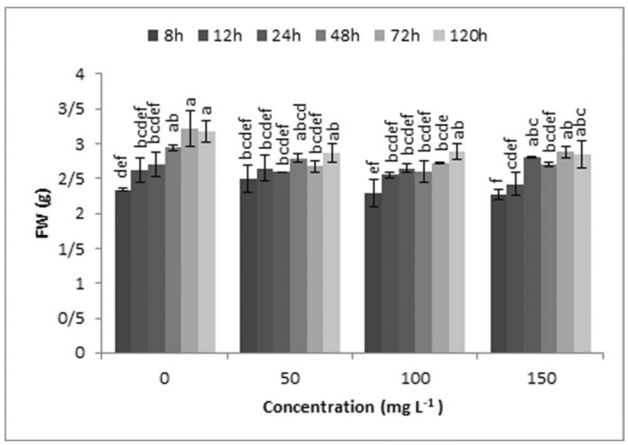

Figure 2. Fresh weight (FW) changes in cell suspension culture of *Haplophyllum virgatum* under different concentrations of chitin (0, 50.0, 100.0, 150.0 mg L^{-1}) after 8, 12, 24, 48, 72, and 120 h time courses. Data represent average values of three replicates ± SD. The *vertical bars* represent standard errors. Means with different *letters* are significantly different at $P \leq 0.05$ as determined by the Duncan test.

while it increased by pectin and had no change in the presence of dextran, which is contrary to the results of this study. The observed differences between reactions to different exogenous elicitors could depend on the difference in plant species, elicitor types, and elicitor concentrations (Baldi *et al*. 2008). Winkler *et al*. (2017) demonstrated that chitin in the form of tetramer induced the expression of genes related to vegetative growth, developmental processes, and carbon and nitrogen metabolism while its octamer form led to the activation of immune and defense responses of the plant. Supposedly, chitin oligomers bearing different lengths activated different responses in the plants. A challenge in synthesizing secondary metabolites through tissue culture is improving their products without reducing the biomass. In this study, treatment with chitin increased lignan production without affecting growth probably by changing the expression of the key genes in the phenylpropanoid pathway.

Effect of Elicitor on H_2O_2 Content Elicitors induce H_2O_2 production at the early stages of treatment (Lin *et al*. 2005). In this study, the H_2O_2 contents decreased initially (in the 8 and 12 h time courses) and then increased in the 24 and 48 h time courses. The decreasing trend was observed at 72-h time course again (Fig. 3(*a*)). At 120 h, no significant difference was observed between the control and different concentrations of chitin. Sharma and Dubey (2005) showed drought stress decreased H_2O_2 content in rice seedlings, which may be related to the scavenging of H_2O_2 by increasing guaiacol peroxidase activity. An increase in H_2O_2 production was observed when root suspension cultures of *Panax ginseng* (Ali *et al*. 2007), hairy root cultures of *Silybum marianum* (Khalili *et al*. 2014), and shoot cultures of *Melissa officinalis* (Fooladi vanda *et al*. 2019) were treated with salicylic acid and methyl jasmonate, Ag^+, and chitosan, respectively. Toghyani *et al*. (2020) reported that H_2O_2 content increased immediately after gamma irradiation in *Chlorella vulgaris* and decreased over 48 h after treatment. The reduction of hydrogen peroxide content and the high activity of antioxidants coincided, which led to a decrease in MDA levels. The balance between the activity of H_2O_2-producing enzymes and its scavenging enzymes in cells has an important function in the content of the reactive oxygen species (ROS) and defense mechanisms against oxidative stress (Creissen *et al*. 1994; Paciolla *et al*. 2016).

Effect of Elicitor on MDA Content The environmental stresses increase ROS production and thus cause peroxidation of membrane lipids and damage to the cell membrane (Ali *et al*. 2006). Lipid peroxidation is associated with MDA as the marker, which has been used to evaluate the sensitivity of the plant to oxidative stress (Blokhina *et al*. 2003; Li *et al*. 2011). The MDA content was decreased upon treatment with chitin in a concentration-dependent manner at 8- and 12-h time courses. However, no changes in lipid peroxidation were observed in the treated samples at 24, 48, 72, and 120 h in comparison to the control (0 mg L^{-1} chitin) (Fig. 3(*b*)). In contrast to the results in this study, the MDA content increased in *Allium cepa* L. and *Pisum sativum* under NaCl

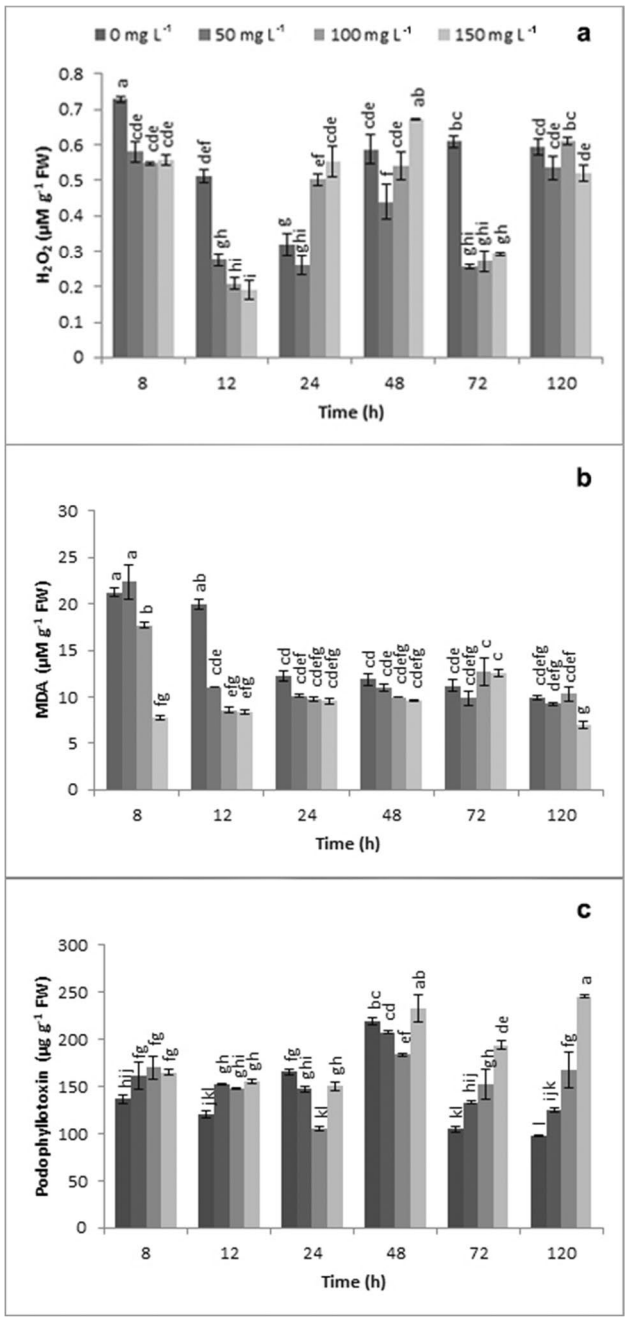

Figure 3. The content of (*a*) H_2O_2, (*b*) MDA, and (*c*) podophyllotoxin in cell suspension culture of *Haplophyllum virgatum* under different concentrations of chitin (0, 50.0, 100.0, 150.0 mg L^{-1}) after 8, 12, 24, 48, 72, and 120 h time courses. Data represent average values of three replicates ± SD. The *vertical bars* represent standard errors. Means with different *letters* are significantly different at $P \leq 0.05$ as determined by the Duncan test.

treatment (Ahmad *et al*. 2008; El-baky *et al*. 2003) and *Cnidium officinale* exposed to MeJA (Ho *et al*. 2020). The significant increase in MDA level appeared to be derived from the lower activity of the antioxidant enzymes. Salicylic acid (SA) increased the activity of the antioxidant enzymes, such as SOD, POX, and CAT, which protected plants against ROS generation and lipid peroxidation (Hayat *et al*. 2010). Similar to the results in this study, using chitosan under salinity stress decreased the MDA levels and increased the activity of antioxidant enzymes in *Oryza sativa* (González *et al*. 2015). Based on this study's findings, the decrease in MDA content under chitin treatment was caused by the effective antioxidant system.

Effect of Elicitor on Protein Content and Antioxidant Enzyme Activities Based on the results in this study, total protein content did not show a significant difference with the control in 8 to 48 h after chitin treatments. Still, after 72 h, the protein content at 150.0 mg L^{-1} of chitin was significantly decreased (Fig. 4(*a*)).

In 120-h time course, the protein contents increased in response to 50.0 and 100.0 mg L^{-1} chitin then decreased again in response to 150.0 mg L^{-1} chitin. It seems that the effect of chitin on protein content started at higher time courses. The increase in protein content in response to stress may be because new proteins' biosynthesis is related to defensive mechanisms (Poonam *et al*. 2013).

Decreased protein content might be because of reduced protein biosynthesis (Hall and Flowers 1973) or increased protein hydrolysis by catabolic enzymes, such as proteases (Davies 1987). The next cause is protein denaturation and oxidation, which leads to increased proteolysis (Mourato *et al*. 2012).

The elicitation affects the induction of ROS, such as O_2^- and H_2O_2. A variety of antioxidant enzymes for ROS depletion in plant cells were reported, including SOD, POX, and CAT (Foyer *et al*. 1991; Mittler 2002; Dynowski *et al*. 2008). SOD acts as the first line of defense against oxidative damage and scavenges superoxide radicals (Mittler 2002). SOD catalyzes the conversion of superoxide anion to H_2O_2 and molecular oxygen (Giannakoula *et al*. 2010). According to the results, the SOD activity significantly increased with increasing chitin concentration (Fig. 4(*b*)). Higher levels of SOD activity were related to the concentration of 150.0 mg L^{-1} chitin at the time courses of 72 and 120 h, which was about two- and threefold higher than control samples,

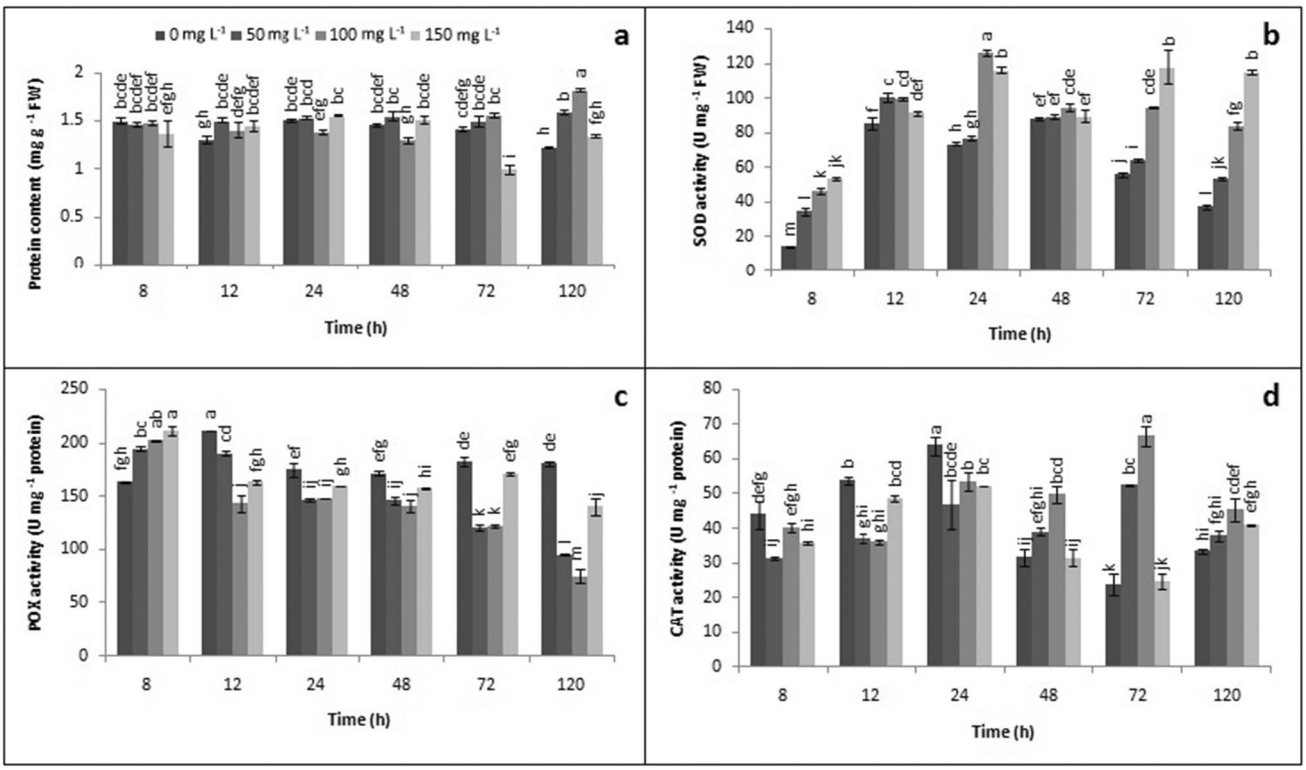

Figure 4. Total protein contents and antioxidant enzyme activities in cell suspension culture of *Haplophyllum virgatum* under different concentrations of chitin (0, 50.0, 100.0, 150.0 mg L^{-1}) after 8, 12, 24, 48, 72, and 120 h time courses. (*a*) Total protein contents; (*b*) SOD activities; (*c*) CAT activities; (*d*) POX activities. Data represent average values of three replicates ± SD. The *vertical bars* represent standard errors. Means with different *letters* are significantly different at $P \leq 0.05$ as determined by the Duncan test. SOD, superoxide dismutase; CAT, catalase; POX, peroxidase.

respectively. Increased SOD production and activity has led to improved plant oxidative stress tolerance (Gupta *et al*. 1993). Yin *et al*. (2002) and Sun *et al*. (2004) showed scavenging of the superoxide anions by chitosan and SOD. The scavenging ability of chitosan seems to be related to its structure because it contains hydroxyl and amine agents that react with ROS (Xie *et al*. 2001; Li *et al*. 2002; Sun *et al*. 2004). This study's results are in accordance with previous reports, which have shown that SOD activity increased when plants were under different environmental stresses, such as metal toxicity and drought (Sharma and Dubey 2005; Mishra *et al*. 2011). The flax plants treated with SA, mainly when applied with pathogen, increased SOD activity. This increment could prevent the attack of superoxide radicals to biological molecules (Belkadhi *et al*. 2013). Based on this study's findings, with increasing chitin concentration and duration of exposure, the MDA content decreased, which is consistent with an increase in SOD activity.

One group of antioxidant enzymes that scavenge hydrogen peroxide is peroxidases. POX catalyzes the decomposition of hydrogen peroxide by the oxidation of phenolic and indolic compounds (Giannakoula *et al*. 2010). In this study, POX activity declined when cells were exposed to chitin, especially in the concentration of 100.0 mg L^{-1} (Fig. 4(*c*)). There were no significant differences between the control samples at the examined times. However, different environmental stresses induced guaiacol peroxidase activity (Shah *et al*. 2001; Moussa and Abdel-Aziz 2008). Increased activity of POX *via* elicitation has been described in many studies (Xu *et al*. 2007, Gharechahi *et al*. 2013, Khataee *et al*. 2020). Fooladi vanda *et al*. (2019) did not report a significant increase in guaiacol peroxidase activity under higher doses of chitosan. It could be due to the scavenging of superoxide radicals in the presence of high doses of chitosan. Due to the role of peroxidase in the plant antioxidant system, its increase in stress is predictable. However, the decrease in the activity of this enzyme in some concentrations can be due to the excessive accumulation of oxygen radicals (Demiral and Turkan 2005).

Catalase is another group of antioxidant enzymes that decompose hydrogen peroxide (Sofo *et al*. 2005). In this study, catalase activity was enhanced at 50.0 and 100.0 mg L^{-1} chitin concentrations with increasing the exposure time. Still, such activity was reduced at 150.0 mg L^{-1} (Fig. 4(*d*)). Environmental stress conditions may increase or decrease catalase activity based on stress type, intensity, and duration (Sharma and Dubey 2005; Moussa and Abdel-Aziz 2008). Chitosan stimulated the catalase activity in *Triticum aestivum* (Ma *et al*. 2014) and *Melissa officinalis* (Fooladi vanda *et al*. 2019). Saisavoey *et al*. (2014) showed that treating the cell culture of *Pueraria mirifica* with methyl jasmonate decreased CAT activity. However, the glutathione peroxidase activity was increased. It could suggest that the peroxidase compensated H_2O_2 scavenge. H_2O_2 accumulation under stress can be one of the factors leading to catalase inactivation (Harinasut *et al*. 2003).

Effect of Elicitor on Podophyllotoxin Content The contents of produced podophyllotoxin in suspension cells under chitin treatment were measured by HPLC where podophyllotoxin (Sigma-Aldrich) was used as standard. The results show that chitin treatments could significantly affect the rate of podophyllotoxin accumulation (Fig. 3(*c*)). Analysis of standard podophyllotoxin (Sigma-Aldrich) by electrospray ionization mass spectrometry (ESI/MS) showed a mass of 416 (M + 1) for this compound (Fig. 5*a*). As shown in Fig. 5*b*, in a similar analysis of the lignan extract obtained from the *H. virgatum* suspension cells, the presence of this compound was confirmed.

This study's results showed that the contents of podophyllotoxin were increased in response to chitin 72 h and 120 h after the beginning of the treatment in a concentration dependent manner (Fig. 3(*c*)). The highest content of podophyllotoxin (245.886 ± 2.165 μg g^{-1} FW) was observed after 120 h under 150 mg L^{-1} chitin. Studies have shown that the treatment of *Linum album* with methyl jasmonate increases the amount of podophyllotoxin compared to the control (Fuss 2003; Van Fürden *et al*. 2005). Esmaeilzadeh Bahabadi *et al*. (2011) demonstrated the accumulation of podophyllotoxin was increased in *L. album* cell cultures. Chitosan and chitin oligomers increased the production of podophyllotoxin in *Linum album* cell culture by fungal extracts, such as chitin, chitosan, and methyl jasmonate (Esmaeilzadeh Bahabadi *et al*. 2014). In some reports, lignan compounds were metabolized and entered into another pathway (Renouard *et al*. 2014; Corbin *et al*. 2017). Tokunaga *et al*. (2005) demonstrated the incorporation of pinoresinol into lignin polymers in isolated *Zinnia elegans* mesophilic cells. Tashackori *et al*. (2019) showed that pinoresinol was increased in PLR-suppressed roots of *Linum album* after treatment with fungal cell wall. Under the same treatment of fungal cells, the content of podophyllotoxin was decreased. It appears that the metabolites of the phenylpropanoid pathway shifted to lignin accumulation. Factors, such as elicitor concentration, time to add elicitor in culture medium, and duration of elicitor exposure, affect the induction of secondary metabolites (Vasconsuelo and Boland 2007).

Effect of Elicitor on the Expression of 4CL, CCR, and CAD Genes As mentioned before, the effects of chitin on transcription levels of *4CL*, *CCR*, and *CAD* in *H. virgatum* cell suspensions were studied by qRT-PCR method (Fig. 6). The expression of *4CL* was increased in the treatments with 100.0 and 150.0 mg L^{-1} chitin after 12 h. However, *4CL* expression decreased at 24 h (Fig. 6(*a*)). The transcript level of *4CL* in treated cells with 50.0 mg L^{-1} chitin was similar to

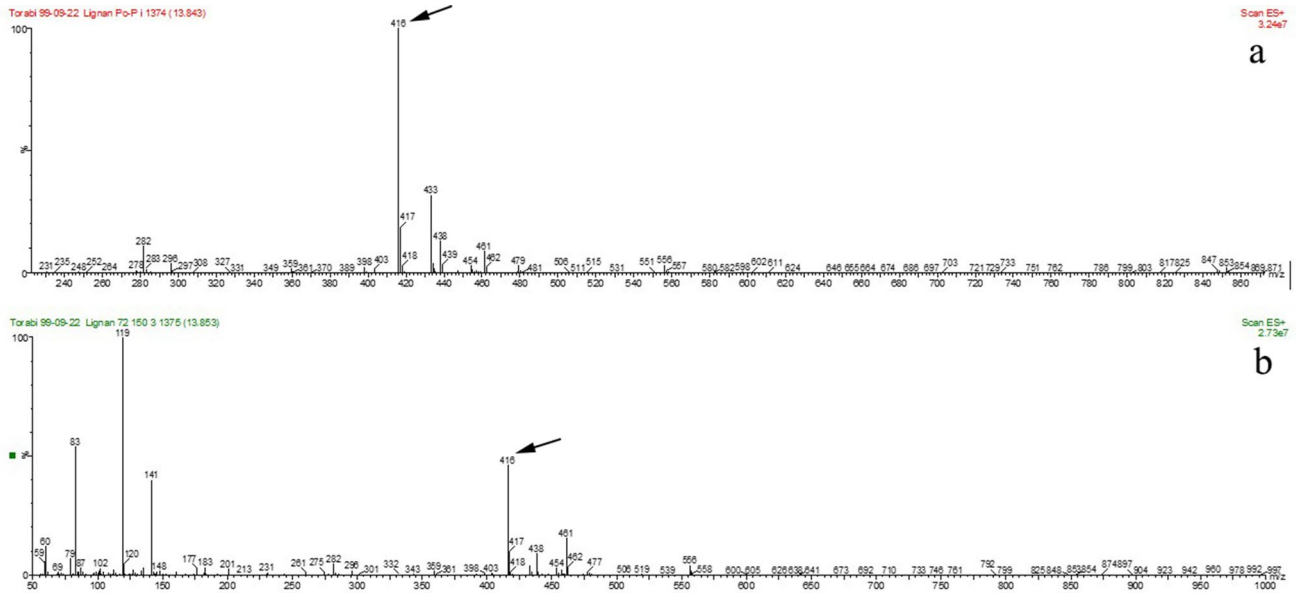

Figure 5. Results of LC–MS analysis. (*a*) mass spectrum of standard podophyllotoxin, (*b*) mass spectrum of lignan extract from suspension culture of *Haplophyllum virgatum.*

the control. The maximum *4CL* expression (2.806 ± 0.098) attained in the 100 mg L^{-1} chitin-treated samples after 12 h was nearly 2.7 times more than the control (1.050 ± 0.024). The transcripts of *4CL* were accumulated by wounding mature Arabidopsis leaves after 2 h. It increased after 6 h by infection of Arabidopsis leaves with a *Pseudomonas syringae pv. maculicola* strain containing avrB (Lee *et al*. 1995). Di *et al*. (2012) showed the increment of *4CL* expression under methyl jasmonate treatment until 8 h, and then decreased at 12 h, which is consistent with this current study's findings. Methyl jasmonate treatment also increased the relative expression of *4CL* in cell cultures of *Agastache rugosa* and hairy root cultures of *Mentha spicata* (Kim *et al*. 2013; Yousefian *et al*. 2020).

In *Nicotiana tabacum* (Piquemal *et al*. 1998) and *Arabidopsis thaliana* (Goujon *et al*. 2003), decreased lignin accumulation due to decreased *CCR* expression showed that CCR is a rate-limiting enzyme in the biosynthesis of monolignol. In this study, the *CCR* expression increased significantly in all used concentrations of chitin after 12 h. It decreased significantly after 24 h, yet they were still more than the control (Fig. 6(*b*)). The highest *CCR* expression observed after 12 h under 150.0 mg L^{-1} chitin concentration, which was 17 times more than the control. Hano *et al*. (2006) observed *Botrytis cinerea* and *Fusarium oxysporum* extracts activated the flax *CCR* gene expression. Esmaeilzadeh Bahabadi *et al*. (2014) showed that the expression levels of *CCR* increased by chitosan and chitin oligomers reaching a peak 3 d after treatment.

Similar to *4CL* and *CCR*, the *CAD* gene expression reached a peak at 12 h and decreased at 24 h (Fig. 6(*c*)). The *CAD* gene expression significantly increased in 100.0 mg L^{-1} (10.1-fold) and 150.0 mg L^{-1} (7.8-fold) chitin treatments in comparison with control after 12 h. The highest expression (2.565 ± 0.334) was recorded in the cells treated with 100.0 mg L^{-1} chitin at 12 h. Brill *et al*. (1999) showed induction of *CAD* expression after using salicylic acid and wounding in *Medicago sativa* leaves. The fungal elicitors upregulated *CAD* gene expression in *Linum usitatissimum* cell suspension cultures (Hano *et al*. 2006). Consistent with the current study's findings, Esmaeilzadeh Bahabadi *et al*. (2014) showed increased *CAD* expression under chitin treatment on day 3 and then decreased after 5 d in the cell culture of *Linum album*.

Heatmap Diagram Analysis The heat map diagram between all studied parameters in the cell suspension culture of *H. virgatum* under chitin treatment is in Fig. 7. H_2O_2 content showed the highest positive correlation with MDA content. This study's observations confirmed that increasing the content of reactive oxygen species could lead to cell damage. Among antioxidant enzymes, H_2O_2 showed the highest negative correlation with catalase activity. However, it had a positive correlation with peroxidase activity. High levels of H_2O_2 appear to deactivate the catalase enzyme. MDA content indicated a positive correlation with POX activity and a negative correlation with SOD and CAT activities. Like the current study's results, some studies have shown a direct correlation between increased SOD activity and decreased oxidative damage. When SOD activity is high, scavenging of reactive oxygen species, especially superoxide radicals, which damage the membrane, performs. There

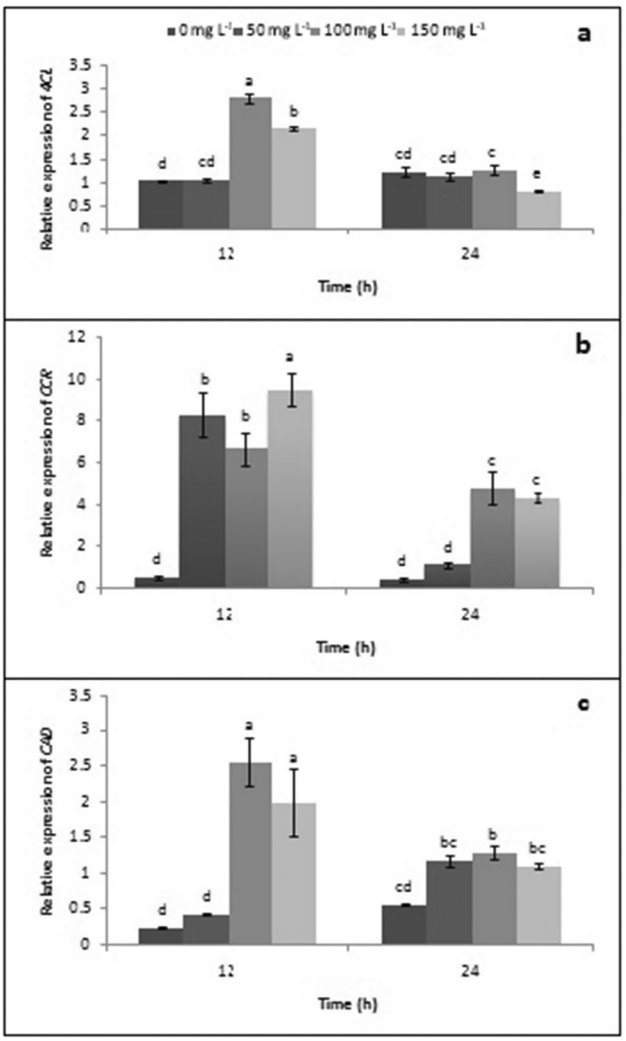

Figure 6. The relative expression levels of (*a*) *4CL*, (*b*) *CCR*, and (*c*) *CAD* genes in cell suspension culture of *Haplophyllum virgatum* under different concentrations of chitin (0, 50.0, 100.0, 150.0 mg L^{-1}) after 12 and 24 h time courses. Data represent average values of three replicates ± SD. The *vertical bars* represent standard errors. Means with different *letters* are significantly different at $P \leq 0.05$ as determined by the Duncan test. 4CL, 4-coumarate CoA ligase; CCR, cinnamoyl-CoA reductase; CAD, cinnamoyl alcohol dehydrogenase.

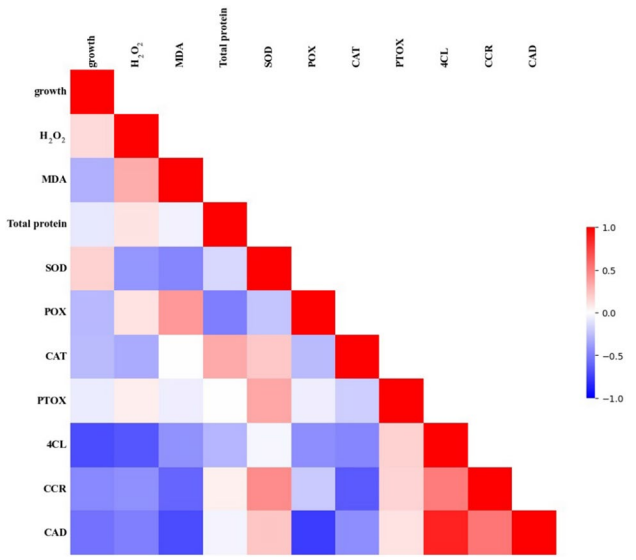

Figure 7. Heatmap diagram generated from obtained data in this study, which shows correlations between different studied parameters in cell suspension culture of *Haplophyllum virgatum* under chitin treatment. The *red* and *blue* parts indicate positive and negative correlation, respectively. MDA, malondialdehyde; SOD, superoxide dismutase; POX, peroxidase; CAT, catalase; PTOX, podophyllotoxin; 4CL, 4-coumarate ligase; CCR, cinnamoyl CoA reductase; CAD, cinnamoyl alcohol dehydrogenase.

was a positive correlation between podophyllotoxin content and the expression of studied genes, although the correlation coefficient is less than 0.5. Therefore, it seems that lignin production could be more than lignan production in *H. virgatum* suspension cells. Increasing the expression of upstream genes in the phenylpropanoid pathway can increase the podophyllotoxin content in the downstream pathway. The highest positive correlation was observed between the expression levels of *4CL* and *CAD* genes. All studied genes showed negative correlations with growth rate, POX and CAT activities, and H_2O_2 and MDA contents. *4CL* has no correlation with SOD activity while *CAD* and *CCR* have positive correlations with this enzyme activity. These findings can be considered proof of the role of phenylpropanoids in the defense mechanisms of this plant in the way that increasing biosynthesis of these substances could decrease the need for other defense mechanisms.

Conclusions

This current study showed chitin, as a biotic elicitor, affects the secondary metabolite production in *H. virgatum*. By comparing the time of lignans production with the time of gene expression, a time interval was recorded between maximum lignans contents and maximum gene expression. According to this study's results, the expression of genes *4CL*, *CCR*, and *CAD* involved in the upstream pathway of phenylpropanoid metabolism positively correlated with podophyllotoxin content. Study of downstream genes of the lignan biosynthesis pathway can provide more information on the relationship between chitin treatment and the content of other lignans. Chitin as an efficient ecofriendly biotic elicitor, at optimal concentrations, could improve the production of valuable secondary metabolites in this medicinal plant.

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Data Availability Detailed data are reserved by the authors and could not be made public. Detailed data will be provided upon request.

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