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Original Article

The Effect of Cellulase Enzyme on Some Biochemical Traits of Licorice (*Glycyrrhiza glabra* L.) under Cell Suspension Culture Conditions

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ABSTRACT

Licorice roots and rhizomes have numerous secondary metabolites, which are used as medicine. Cell and tissue culture of medicinal plants can be used for the production of secondary metabolites, and the use of elicitors, stimulates the production and increase of these valuable compounds. For this purpose, the effect of cellulase enzyme from *Aspergilus Nigare* as a fungal elicitor in cell suspension culture conditions as a completely randomized design with 3 replications, on traits such as phenol, flavonoid, antioxidant activity, carbohydrate, tannin, protein, nitrogen, and proline levels were tested in untreated, late-harvesting callus conditions, 24 hours, 48 hours and 72 hours after treatment with cellulase. Variance analysis showed that there is a statistically significant difference between the studied treatments in all traits. A comparison of the mean traits also showed that phenol, flavonoid, antioxidant activity, tannin, and proline traits showed the highest increase in the treatment 72 hours after the treatment, and the carbohydrate, nitrogen, and protein traits showed a decreasing trend compared to the control treatment. Regarding the increase in the amount of phenol, flavonoid, tannin, and antioxidant activity during the treatment with cellulase, it can be said that cellulase, like other fungal elicitors, stimulates and induces the production of secondary metabolites in cell suspension culture conditions and confirms the possibility of the presence of secondary metabolites in callus and cell suspension of this plant in addition to Licorice root and rhizome. A decrease in nitrogen and protein and an increase in proline have also been reported due to the role of nitrogen in the accumulation of amino acids and stimulating the accumulation of flavonoids in plants.

INTRODUCTION

Licorice is a Mediterranean plant widespread in Southeast Asia and also cultivated in large areas of England, Belgium, France, Germany, Italy, Greec e, and Turkey. In Iran, it is found in abundance almost in the north, east, west, and center of the country [1]. The root and rhizome of this plant have been used medicinally for about 4000 years and registered in the pharmacopeia of countries such as America, China, and other countries. Licorice is used in traditional Asian and European medicine to treat gastritis, respiratory infections, and peptic ulcers [2,3]. In traditional Chinese medicine, it is also used to treat hepatitis, tumor growth, and heart diseases [4]. In traditional Iranian medicine, it is also used as a treatment for gastritis and antitussive [5]. Other properties of licorice include anti-ulcer effects [6], anti-allergic effects [7], antiviral activities against DNA and RNA viruses, such as HIV [8] and anti-tumor [9].

In plants, saponins are used as defense molecules, and their production is often stimulated by biotic and abiotic stresses [10]. Several plant species such as licorice, and ginseng, as well as crops such as legumes and oats, synthesize triterpenoid saponins as part of their normal growth and development programs. They are usually plants that are used as medicinal sources [11].

Technologically, plant cell, tissue, and organ culture have been successfully used in recent years to produce valuable medicinal compounds and other commercially useful chemical compounds [12]. Suspension culture systems can be used for the large-scale cultivation of plant cells to extract secondary metabolites. Due to the limited availability and complexity of the chemical synthesis of secondary metabolites, plant cell culture has become an alternative route for the large-scale production of these desired compounds [13].

Plants that are damaged by pathogens and insects, or mechanically damaged, produce signal molecules, jasmonic acid (JA) and methyl jasmonate (MJ), which accumulate in the damaged parts of the plant [14]. One way to produce secondary metabolites is to stimulate the production of secondary metabolites by other molecules (elicitors). The optimal entry of elicitors in cell culture leads to a significant increase in the activity of enzymes involved in the synthesis of metabolism [15]. With the use of elicitors, in addition to increasing the concentration of the effective substance, the length of the cultivation period is also specially reduced [16].

Several plant pathogenic fungi produce cellulase [17]. It was first reported in 1988 that cellulase can act as an elicitor, in this report, cellulase derived from non-pathogenic *Trichoderma spp* induced the biosynthesis of phytoalexin, a sesquiterpenoid [18, 19]. Also, in another study, this cellulase induced capsidiol accumulation in *Capsicum annuum* [20] and wild tobacco [21]. Cellulase isolated from the fungus *Rhizoctonia solani* causes cell death in corn, tobacco, and Arabidopsis and also induces the accumulation of reactive oxygen species (ROS), alkalization of the environment, accumulation of Ca^{2+} and ethylene biosynthesis in cultivated tobacco cells and it has been proven that this enzyme can act as an elicitor [22].

In this research, the effect of cellulase enzyme obtained from *Aspergilus Nigare* fungi was investigated as a fungal elicitor on the levels of phenolic compounds, flavonoid, antioxidant activity, tannin, protein, nitrogen, and proline in the conditions of cell suspension culture. In this way, the elicitor role of this enzyme on licorice will be confirmed.

MATERIALS AND METHODS

Preparation of Seeds and Cultivation

Licorice seeds were collected from the Semirom region of Isfahan province. Then, it was placed in 50% sulfuric acid (Merck company) for 5 minutes to scarify and washed several times with sterile distilled water. To continue, it was placed in sodium hypochlorite solution for 15 minutes to disinfect and washed twice with sterile distilled water. Finally, it was disinfected under a sterile laminar airflow with 70% alcohol and cultured in a half-MS medium.

Callus Induction

About 4 weeks after cultivation, the hypocotyl and leaves of the plant were divided into small pieces and placed in a MS culture medium containing growth regulators (2 mg/L BA and half mg/L NAA) were cultivated with 3% sucrose and 0.7% agar [23]. Four weeks later, the produced calluses were transferred to the new culture medium containing the previous regulating hormones, and the harvesting was carried out in the new culture medium every three weeks.

Cell Suspension and Elicitor Treatment

After three consecutive stages of harvesting, half a gram of callus was transferred under laminar airflow to erlens containing 50 ccs of MS liquid culture medium with three percent sucrose, 2 mg/liter of BA hormone, and half mg/liter of NAA hormone, and placed in Shaker incubator with 150 rpm, temperature of 25 °C and in darkness. The experiment was conducted based on a completely randomized design with three repetitions and four treatments including different times of cell harvesting after treatment with cellulase. On the 18th day after cultivation, cellulase enzyme with a concentration of 200 µg/mL was added to each of the erlens (flasks) under a sterile hood and the samples were again transferred to the Shaker incubator. Then, harvesting was done at time intervals of 24 hours, 48 hours, and 72 hours after adding the elicitor. In this way, the cell suspension was passed through a strainer under a sterile hood, and after washing with sterile distilled water, the cells were placed in appropriate containers and dried in a freeze dryer for 48 hours.

Biochemical Analyses

Protein Content

Inside the test tube, 5 mL of Bradford's reagent was added to 100 microliters of plant extract. After 2 minutes, its absorbance was read at a wavelength of 595 nm. Based on the comparison with the standard curve, the protein concentration was expressed in mg/g wet weight [24].

Proline Concentration

The amount of 0.1 gram of wet plant material was rubbed well with 10 mL of 3% sulfosalicylic acid. After passing the homogenous substance through the filter paper, 2 mL of the extract was transferred to a closed tube and 2 mL of ninhydrin reagent and 2 mL of acetic acid were added to it. Proline standards were prepared from zero to 0.1 micromol/mL and the absorbance values of standard solutions and samples were read at 520 nm wavelength with a spectrophotometer. The control solution (blank) was toluene in this measurement [25].

Total Phenol Content

To 0.5 mL of each extract (10 mg/mL), 2.5 mL of Folin-Ciocaltio reagent 0.2 normal was added. After 5 minutes, 75 g/L of sodium carbonate was added to 2 mL of the solution. After 2 hours, the absorbance of the solution was read at a wavelength of 760 nm by a spectrophotometer. A mixture of reagents was used as a control (blank). Gallic acid was used as a standard to draw the calibration curve. The amount of total phenol was reported based on mg of gallic acid per gram of dry matter of the sample [26].

Total Flavonoid Content

Total flavonoid content was measured using an aluminum chloride reagent. 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride solution in ethanol, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water were added to 0.5 mL of each extract (10 mg/mL). The absorbance of the solution was read at a wavelength of 415 nm after 30 minutes of storage at room temperature. A mixture of reagents was used as a control (blank). Quercetin was used as a standard to draw the calibration curve. The amount of total flavonoid was reported based on mg of quercetin per gram of dry matter of the sample [27].

Antioxidant Activity

The antioxidant property was investigated by DPPH (2, 2-diphenyl-1-picrylhydrazyl) method. In this way, 0.1 mL of methanol extract was prepared in different concentrations and 5 mL of 0.1 mM DPPH methanolic solution was added to the samples. The mixture was shaken vigorously and kept in the dark at room temperature for 30 min. Then, the absorbance of the samples was read in a spectrophotometer at a wavelength of 517 nm. 80% methanol was used as a blank. For the control sample, a sample containing 0.1 mL of 80% methanol and 5 mL of methanolic DPPH solution was used. The inhibition percentage of the samples was calculated using the following formula.

$$100 \times \text{inhibition\%} = \frac{Ac-As}{Ac}$$

Where As and Ac are the absorption numbers of plant samples and control samples, respectively.

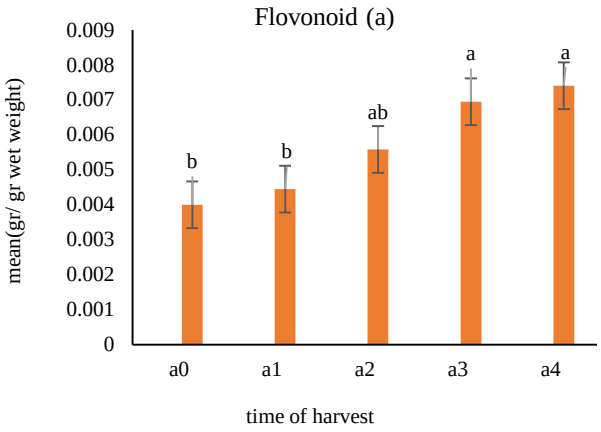
IC_{50} index was used to check DPPH radical scavenging activity. To better investigate this activity, the synthetic antioxidant BHT was used as a positive control [28].

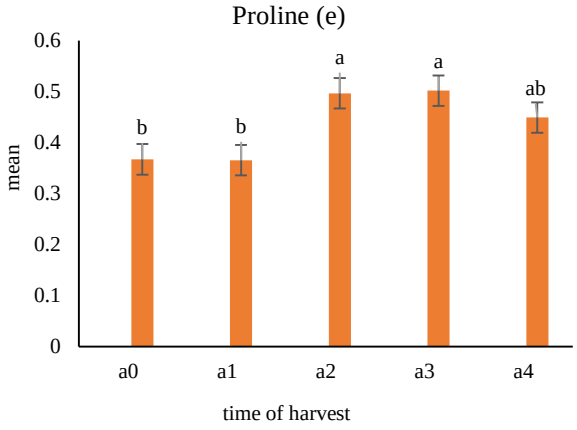
Estimation of Total Tannin

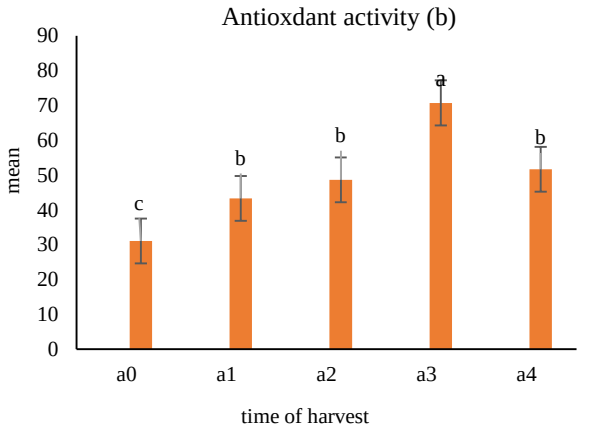
Total tannin was measured by the Copper Acetate method as follows: two grams of the samples were extracted separately for one hour with 100 ccs of acetone-water at a ratio of 1:1 and then filtered. The extract obtained in each case was transferred separately to a flask with a volume of 250 mL and its volume was adjusted with distilled water. Then, each of them was transferred to a 500 mL beaker and heated to a boil, then 30 mL of 15% copper acetate aqueous solution was added to each one while stirring. The copper tannate precipitate was collected on ashless filter paper and heated in a porcelain crucible. Then a few drops of nitric acid were added to them and heated again to a constant weight. The weight of copper oxide was determined and the percentage of tannin was calculated as follows: every 1 gram of copper oxide is equal to 1.305 grams of tannin [29].

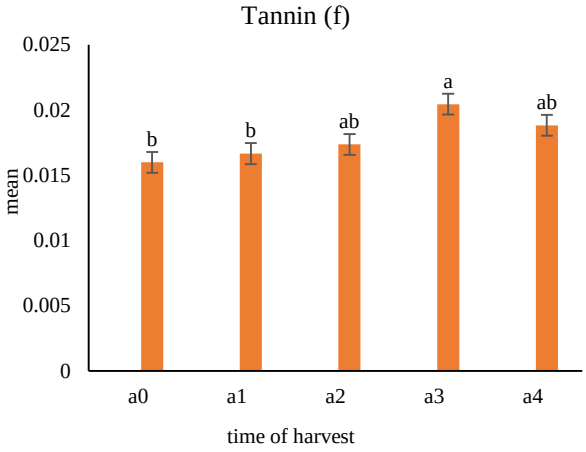
Nitrogen Percentage

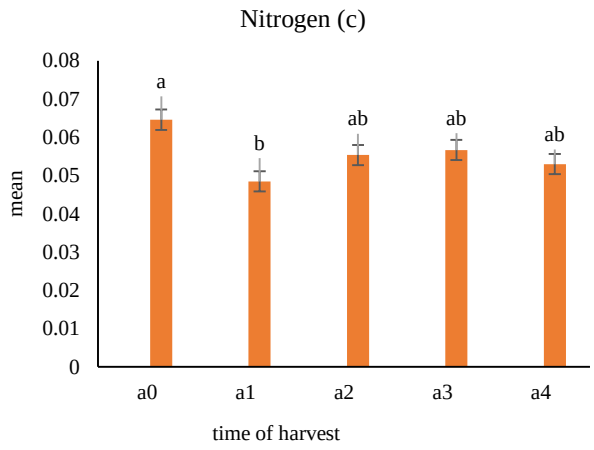
First, the desired sample is digested by sulfuric acid and the nitrogen is converted into ammonium sulfate. Then the nitrogen in the ammonium sulfate is converted into ammonium borate by boric acid as free ammonia and using sulfuric acid, and we titrated it using 0.1 normal sulfuric acids. Then, the amount of nitrogen was obtained by calculating the acid consumed [30].











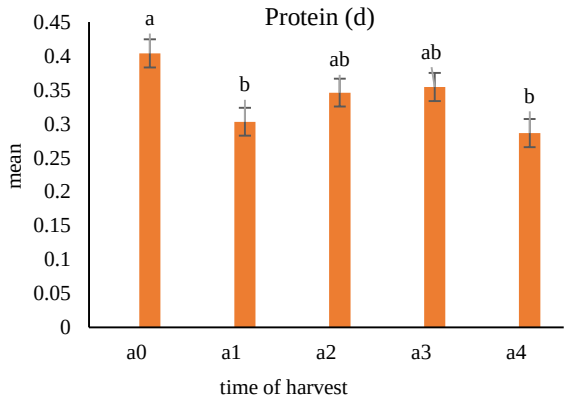


Fig. 1 Comparison of mean traits a: Flavonoid, b: Antioxidant activity, c: Nitrog en, d: Protein, e: Proline, f: Tannin, in cell suspension culture of *G. glabra* (Treatments with a common letter are placed in a group

Data Analysis

The treatments included the control (without elicitor), 24, 48, and 72 hours after treatment with the elicitor, as well as late-harvested callus were implemented as a completely randomized design with 3 replications. Variance analysis of the studied traits and mean comparisons with Duncan's level test 5% were done with SAS software version 9.2. Mean comparison charts were drawn with Excel software version 2016.

RESULTS

The Effect of Cellulase Enzyme on the Amount of Phenol, Flavonoid, and Antioxidant Activity

Although the addition of this biological elicitor caused a change in some factors even 24 hours after the treatment, at 72 hours after the treatment, most of the examined traits had increased. The results of

the analysis of variance for the traits of flavonoid and antioxidant activity were found to be significant at the level of 1% (Table 1). In examining the changes in the amount of phenol, the results of analysis of variance were not significant, but in the 72 hour treatment and the treatment of late harvested callus, the changes showed an increase compared to the control treatment, so that, compared to the control, it was 0.738 times in the 24-hour treatment (decrease), 0.0982 times in the 48-hour treatment, 1.157 times in the 72 hour treatment, and 1.24 times at late-harvested callus compared to the control (Table 2). The amount of flavonoid was increased 1.11 times in 24 hours after treatment compared to control, 1.39 times in 48 hours after treatment compared to control, 1.74 times in 72 hours after treatment compared to control, and 1.85 times in late-harvested callus compared to control (Table 2). The comparison results of the mean flavonoid also showed that the 72 hour treatment and stressed callus were in group a (the greatest increase), the 24 hour treatment was in group b, the 48 hour treatment was in group ab, and the control treatment with the lowest increase was in group b (Fig. 1-a). According to these changes, the cellulase enzyme caused severe stress within 72 hours of treatment and thus the rate of flavonoids increased. Similar changes were also observed in conditions where callus was harvested late and subjected to food stress. Therefore, it can be said that biotic stress and food stress have both stimulated the defense systems in the cell and increased the content of phenol and flavonoids.

The results of the analysis of variance for antioxidant activity showed a significant difference at the 1% level (Table 1). Antioxidant activity by using cellulase enzyme in cell suspension culture conditions 24 hours after treatment was 1.393 times, 48 hours after treatment was 1.565 times, 72 hours after treatment was 2.276 times and in late-harvested calluses had 1.662 times increase compared to control treatment (Table 2). So, the cellulase enzyme in this plant has increased the antioxidant activity. The results of the mean comparison showed the highest increase in 72 hour treatment in group a, 24 hour treatment, 48 hour treatment, and late-harvested callus in group b, and the control treatment with lowest antioxidant activity in group **c** (Fig. 1-b).

The Effect of Cellulase Enzyme on the Amount Tannin, Nitrogen, Protein, and Proline

The results of the analysis of the variance of proline were significant at the level of 1% and tannin at the level of 5% (Table 1). The amount of nitrogen and protein also showed a decreasing trend compared to the control treatment, despite the lack of significance between the treatments, so that this decrease in the 24 hour treatment was 0.75 times, the 48 hour treatment was 0.856 times, and the 72 hour treatment was 0.877 times for both nitrogen and protein, and the amount of this reduction in late-harvested callus was 0.82 times for nitrogen and 0.709 times for protein (Table 2; Fig 1-c, d).

The effect of the cellulase enzyme on the amino acid content of proline was significant at the level of 1% (Table 1). The amount of this amino acid 48 hours after the treatment and 72 hours after the treatment showed the greatest increase compared to the control treatment (Fig. 1-e). The amount of this increase was 0.997 times 24 hours after treatment, 1.352 times 48 hours after treatment, 1.367 times 72 hours after treatment, and 1.22 times in late-harvested callus compared to control treatment (Table 2).

Table 1 Results of variance analysis of biochemical traits in licorice plant under cell suspension culture conditions

		Mean of squares						
S.O.V	df	Phenol	Flavonoid	Antioxidant activity	Tannin	Protein	N	Proline
Treatment	3	2.9795 $^{n.s}$	0.00000541 **	637.44 **	0.00001296 **	0.00415369 $^{n.s}$	0.00010633 $^{n.s}$	0.01818473**
Error	8	5.703821	0.0000005	22.05	0.00000203	0.00205949	0.00005272	0.00223880
CV (%)	-	23.66	12.92	9.11	7.9	13.05	13.05	10.56

*,** and n.s are significant at 0.05, 0.01 and non-significant respectively

Table 2 The average of investigated traits and the amount of changes compared to the control treatment in cell suspension culture conditions of licorice

Traits	Treatment	Mean of traits	amount of changes compared to the control treatment
Phenol	control	0.001145	-
	24 hours after treatment	0.00079	0.6899
	48 hours after treatment	0.001125	0.9825
	72 hours after treatment	0.001325	1.157
	late-harvested callus	0.001427	1.246
Flavonoid	control	0.004	-
	24 hours after treatment	0.004445	1.11
	48 hours after treatment	0.005584	1.39
	72 hours after treatment	0.006945	1.736
	late-harvested callus	0.007407	1.851
Antioxidant activity	control	31.07	-
	24 hours after treatment	43.3	1.39
	48 hours after treatment	48.65	1.565
	72 hours after treatment	70.74	2.276
	late-harvested callus	51.65	1.66
Tannin	control	0.016	-
	24 hours after treatment	0.0166	1.041
	48 hours after treatment	0.0173	1.085
	72 hours after treatment	0.02045	1.278
	late-harvested callus	0.01883	1.177
Protein	control	0.4037	-
	24 hours after treatment	0.303	0.750
	48 hours after treatment	0.346	0.856
	72 hours after treatment	0.3543	0.875
	late-harvested callus	0.286	0.709
Nitrogen	control	0.0646	-
	24 hours after treatment	0.0484	0.750
	48 hours after treatment	0.0553	0.856
	72 hours after treatment	0.0566	0.877
	late-harvested callus	0.0529	0.802
Proline	control	0.367	-
	24 hours after treatment	0.365	0.995
	48 hours after treatment	0.496	1.352
	72 hours after treatment	0.501	1.367
	late-harvested callus	0.449	1.223

Table 3 Correlation between investigated traits of licorice in cell suspension culture conditions

Traits	Phenol	Flavonoid	Antioxidant activity	Tannin	Protein	Nitrogen	Proline
Phenol	1	-	-	-	-	-	-
Flavonoid	0.835 *	1	-	-	-	-	-
Antioxidant activity	0.514	0.801 *	1	-	-	-	-
Tannin	0.698	0.885 **	0.965 **	1	-	-	-
Protein	-0.044	-0.490	-0.286	-0.274	1	-	-
Nitrogen	0.287	-0.259	-0.304	-0.187	0.907 **	1	-
Proline	0.621	0.770 *	0.802 *	0.77 *	-0.095	-0.059	1

For the amount of tannin, it was showed an increasing trend compared to the control treatment, so that for the 24 hour treatment 1.041 times, 48 hours treatment 1.08 times, 72 hour treatment 1.27 times, and for late-harvested callus1.17 times increase was observed compared to the control treatment (Table 2). In this trait, the greatest increase was observed 72 hours after treatment with cellulase enzyme (Fig. 1-f).

The results of the correlation between the traits showed that the antioxidant activity has a correlation of 80% with the amount of total flavonoid and more than 96% with the amount of tannin. The correlation between protein and nitrogen was more than 90%. Amino acid proline showed a correlation between 77 and 80% with total flavonoid content, tannin content and antioxidant activity (Table 3).

DISCUSSION

Although little research is available on the elicitor role of cellulase enzyme, it has been reported that cellulase EG1 from fungi has been able to induce cell death, transcription of defense genes, production of ROS, and ethylene biosynthesis [31]. Therefore, this enzyme can be used as an elicitor. In this research, cellulase enzyme taken from *Aspergilus Nigare* fungus was used as an elicitor. This elicitor increased the content of total phenol, total flavonoid and antioxidant activity. In accordance with the results of this research, treatment with cellulase elicitor in the growth phase of *Capsicum annumm* L. suspension culture increased level capsidiol in 24 hours after treatment [32]. An increase in the production of flavonoids in the *Hypericum perforatum* plant has also been observed on the 15th day of cultivation by using methyl jasmonate at a concentration of 100 µM [33]. In the hairy root culture of the licorice plant, cellulase enzyme at a concentration of 200 µg/mL also caused an increase in the amount of glycyrrhizin 7 days after treatment [34]. In this research, the amount of flavonoid in all treatments showed an increase compared to the control treatment, but the amount of phenol showed a slight increase compared to the control treatment. Similar results were reported by Lashin *et al* [35] in the callus cultivation of the *Physalis peruviana* plant compared to the fruit, and they attributed these changes to the growth regulators used in the callus cultivation conditions compared to the field. Both plant growth regulators and multiple harvests in tissue culture conditions are effective in increasing flavonoid production [36]. In addition to plant growth regulators in tissue culture conditions, the use of elicitors also increases phenolic and flavonoid compounds. Al-Kheyri and Madhava Naik [37] reported an increase in the amount of phenol and flavonoid by using elicitors such as yeast extract, salicylic acid, $CdCl_2$, and silver nitrate in the cell suspension culture of date palm (*Phoenix dactylifera* L), Wang *et al* [33] reported an increase in phenol and flavonoid using methyl jasmonate elicitor in *Hypericum perforatum* cell suspension culture and Mendoza *et al* [38] using methyl jasmonate and salicylic acid in *Thevetia Peruviana* cell suspension culture.

The increase of antioxidant activity in other plants has also been observed by using elicitors in the conditions of cell suspension culture. Chung *et al* [39] reported an increase in antioxidant activity in the cell suspension culture of *Momordica dioica* Roxb by using jasmonic acid and salicylic acid. Al-Kheyri and Madhava Naik [37] observed an increase in phenol, flavonoid, and antioxidant activity at low concentrations of different elicitors such as salicylic acid and $cdcl_2$. Mamdouh and Smetanska [40] also used different plant growth regulators in the induction of callus and also in the culture of the cell suspension of the medicinal plant *Lycium schweinfurthii* showed the greatest increase in the amount of phenol, flavonoid, and antioxidant activity in the callus induced with the NAA hormone and also in cell suspension culture containing NAA hormone. Szczykutowicz *et al* [41] investigated the effect of different elicitors on the production of metabolites, phenol, flavonoid, and antioxidant activity of *Nasturtium officinale* and observed that the methyl jasmonate elicitor stimulated the production of *gluconasturtiin* and *glucobrassicin*, and the NaSA elicitor causes to increase the flavonoid level, but the elicitors did not increase the amount of phenol in this plant. Also, an increase in antioxidant activity (assessed by DPPH) was observed in this plant 24 hours after treatment with methyl jasmonate at a concentration of 100 µM. In this research, a positive and significant correlation was observed between the amount of phenol, flavonoid, and antioxidant activity with the use of cellulase elicitor (Table 3). The correlation between these

traits has been observed in other studies and other medicinal plants [40,41].

In this research, an indirect relationship between the amount of nitrogen and protein with phenol and flavonoid compounds was observed (Table 3). Despite the lack of significant difference between treatments, at 72 hours after treatment with cellulase, the amount of reduction in content of protein and nitrogen was significant. The content of soluble protein in the cell culture of *Betula platyphylla* Suk treated with fungal elicitor decreased 24 hours after treatment [42]. They attributed the decrease in nitrogen content to the carbon-nitrogen balance hypothesis. Secondary metabolism is directed toward carbon-rich metabolites in nitrogen-limited plants and nitrogen-rich metabolites in carbon-limited plants [43]. At high nitrogen levels, plant carbohydrates are more allocated to central metabolism, preventing the accumulation of flavonoids [44]. Allocation of carbon (C) flow from central metabolism to specialized metabolism of plants under N changes is considered an important step in the regulation of flavonoid biosynthesis [45]. Low nitrogen typically leads to the accumulation of secondary metabolites, including phenylpropanoids and flavonoids [46, 47].

In this research, the amount of proline amino acid increased under the treatment with cellulase enzyme. In accordance with the results of this research, an increase in amino acids of the glutamate family (Arg, Glu, and Pro) was observed with the use of a fungal elicitor in the cell suspension culture of *Betula platyphylla* Suk [42]. Nitrogen regulates the activities of enzymes related to nitrogen metabolism and affects the accumulation of amino acids. The relationship between nitrogen metabolism and amino acid levels is likely to stimulate changes in plant flavonoid accumulation [48]. The effect of nitrogen level on the cell suspension culture of the medicinal *Salvia nemorosa* plant was investigated and it was observed that the level of flavonoid *rosmarinic acid* decreases with the increase of nitrogen level in the culture medium [49], which is consistent with the results of this research regarding nitrogen and secondary metabolites (phenol, flavonoid and tannin).

The amount of tannin was also increased in 72 hours after treatment with cellulase elicitor. Consistent with the results of this research, an increase in saponin and tannin in the calluses obtained from the seeds of *Physalis peruviana* compared to other tissues was also observed [35]. Tannins have anticancer activity and can be used in cancer prevention [50]. The tannins in the plant are useful for treating inflamed or wounded tissues. They have also been used to treat intestinal disorders such as diarrhea and dysentery [51]. The antimicrobial activity of plant oils and extracts has been known for many years and shows that it may be attributed to alkaloids, saponins, flavonoids, tannins, glycosides, and phenols [52]. Antibacterial activity of ethanolic extract of the root of *glabra* species [53], antifungal activity of methanolic extract of the root of this species [54], and extract of this species [55], antiviral activity against viruses' hepatitis B, hepatitis C, AIDS [56-58], HIV and SARS-related coronavirus [59] have been reported. The presence of phenol, flavonoids, and tannins in the cell suspension culture of this plant and the increase of these biochemical compounds with the addition of an elicitor can confirm the antimicrobial activity in addition to the roots and rhizomes in the callus and cell suspension culture of this species.

Finally, considering that the fungal elicitor obtained from *Aspergilus Nigare* in cell suspension culture conditions has increased phenolic compounds, flavonoids, and antioxidant activity, and in past research, the use of cellulase derived from fungi in cell suspension culture conditions of different plants [20], cellulase enzyme derived from *Trichoderma viridae* fungus in *Capsicum annumm* L plant and Mialoundama *et al*. [60] in wild tobacco plant caused an increase in flavonoids and secondary metabolites, it can be said that in this research, cellulase enzyme in the cell suspension culture conditions of the medicinal licorice plant have played as an elicitor role.

CONCLUSION

Cellulase enzyme derived from *Aspergilus Nigare* fungi increased the compounds of phenol, flavonoid, antioxidant activity, amino acid proline, and tannin. The highest increase was observed 72 hours after treatment with cellulase in cell suspension culture conditions. Tannin was present in all treated and untreated cells and late-harvested callus and showed the most increase compared to the control treatment in the cells exposed to cellulase enzyme for 72 hours. The presence of tannin confirmed the antimicrobial activity of

licorice in callus in addition to roots and rhizomes. The cellulase enzyme also increased the induction of tannin in cell suspension culture of this plant. Regarding the decrease of nitrogen and protein and the increase of amino acid proline and flavonoid in the conditions of treatment with cellulase enzyme and the role of nitrogen in regulating the activities of enzymes related to nitrogen metabolism, it can be said that nitrogen affects the accumulation of amino acids and stimulates the accumulation of flavonoids in the licorice.

Conflict of Interest

The authors confirm that there are no conflicts of interest.

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