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Original Article

Production of Phenolic Compounds in *Iberis amara* L. Cell Suspension Culture under Chitosan Treatment

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ABSTARCT

Iberis amara L. medicinal herb is well-known for having pharmacological values although its use has been challenged by the low levels of secondary metabolites. For their bulk production, the present work aimed to investigate the effects of explants, different plant growth regulators, and photoperiod condition on the callus induction and cell suspension in *I. amara*, followed by investigating the chitosan effect on some secondary metabolites. A factorial experiment method based on completely randomized design with four replications was carried out. The optimum condition for induced callus achieved from the leaf explants in Murashige and Skoog (MS) media supplemented with 3 mg L^{-1} 6-benzylaminopurine (BAP) and 1 mgL^{-1} 1-naphthalene acetic acid (NAA) under 16-h light/8-h dark photoperiod. The MS enhanced with 3 mg L^{-1}BAP, 1 mg L^{-1}NAA, and 2% (w/v) sucrose appeared to be the optimum conditions for suspension establishment. Thus, the cells were exposed to different concentrations of chitosan (200, 100, 50, and 0 mg L^{-1}) in their exponential growth stage from day 8 to 12 and day 12 to 16 following sub-cultures (T1) and sub-cultures (T2), respectively. The contents of phenolics and malondialdehyde (MDA) were then examined by UV-Vis spectrophotometer. The results showed that 50 mg L^{-1} chitosan significantly improved the total phenol, flavonoid, flavonol, and anthocyanin content in the *I. amara* in a dose-dependent manner. The highest malondialdehyde (MDA) amount, as a result of lipid peroxidation, was observed under the 200 ppm chitosan elicitation. Overall, these novel findings demonstrated the possibility of applying the cell suspension of *I. amara* treated with chitosan as a helpful approach for improving synthesizing phenolic compounds under controlled and sterile conditions.

Keyword: Cell suspension culture, Chitosan, Medicinal herbs, Secondary metabolite

INTRODUCTION

Medicinal herbs have long been known for producing secondary metabolites, which are utilized as bioactive compounds in pharmaceutical applications. *Iberis amara* L. (*I. amara*), as a genus of the family Brassicaceae native to southern Europe, has been used by ancient humans in order to cure rheumatism and some other diseases. This medicinal herb is also well-known and beneficial for restoring health from bronchitis, asthma, and cardiac hypertrophy [1]. A phytomedicine (i.e, Iberogast®) has recently been provided from the *I. amara* herb extract and applied to relieve the symptoms of gastrointestinal disturbances related to irritable bowel syndrome and functional dyspepsia [2]. Such a therapeutic value has been attributed to the presence of some natural components available in the aerial part of medicinal herbs. *I. amara* has a large pool of bioactive ingredients including triterpenes, glucosinolates [3], amines, flavonoids, anthocyanins, flavones, and phenolic acids [2]. There are some reports on its use in traditional medicine as an anti-cancer [3], anti-inflammatory [4], antioxidant and antimicrobial properties [5].

Despite a large amount of information regarding the therapeutic value of *I. amara*, this herb meets low levels of bioactive products, which strongly depends on bio-physiological and environmental conditions [1]. Therefore, plant tissue and cell suspension culture appear as a viable biotechnological tool for the production of secondary metabolites in medicinal plants. These techniques have been applied for increasing the quantity and quality of drugs and provide a promising bio-production platform for a desired natural product [6, 7, 8]. A variety of elicitors

as chemical compounds stimulating the signaling pathways were suggested as an innovative tool for the improvement of natural product biosynthesis [9]. A number of elicitor compounds have so far been adopted to modulate cell metabolism and increase bioactive ingredients in cell culture media [10, 11]. Biotic elicitors with microorganism origins such as chitosan have been exhibited to increase the plant metabolites having therapeutic values [10]. Chitin and its deacetylated derivative (i.e, chitosan) also play a critical role in activating resistance against pathogens in plants [12]. It is considered as a biotic elicitor that induces phytoalexin accumulations in plant tissues [13]. Elicitors have so far been applied for improving the content of biologically valuable ingredients [14] and providing a high-throughput platform for producing natural products *in vitro* [15]. For instance, Khan, *et al.*, [16] observed that chitin and chitosan oligomers increased the activity of tyrosine/ phenylalanine ammonia-lyase (TAL and PAL) in soybean plants, resulting in a 50% increase in phenolic compounds. Similarly, Govindaraju and Arulselvi [17] reported a higher percentage of tannins, terpenoids, saponins, and alkaloids in *in vitro Coleus aromaticus* regenerated plants. Likewise, Putalun, *et al.*, [12] reported a dose-dependent 6-fold increase in artemisinin accumulations by subjecting *Artemisia annua* L. to 150 mg L^{-1} chitosan.

To the best of our knowledge, there are no reports on the establishment of cell suspension culture and the effect of chitosan on the accumulation of secondary metabolites in *I. amara*. This study is the first report to focus on optimizing a high-throughput procedure for inducing callus and establishing cell suspension in *I. amara*, as well as using chitosan to enhance the content of phenols, flavonoids, flavonols, and anthocyanins.

MATERIAL AND METHODS

Plant Materials and Optimization of Germination Medium

Iberis amara seeds were provided from the Gol Daroo Co. and then transferred to the lab for determining their best germination medium. To sterilize, the seeds were treated with 70% alcohol for 60 s as well as 2 % NaOCl solution for 20 min, and eventually washed in sterile water three times. After sterilization, the seeds were placed on MS base medium containing 5 mg L^{-1} GA3, 0.8% (w/v) agar and 3% (w/v) sucrose at pH 6 [18]. Eventually, the seeds were incubated at 25±1°C under the 16 h light/8 h dark photoperiod in the growth chambers.

Optimization of Callus Induction

For callus induction, two explants (stem and leaf) of *I. amara* were obtained from *in vitro* grown seedlings on the MS medium. Four weeks after the growth of buds *in vitro* in the completely sterile conditions, two explants including 6-7 mm stem and leaf sections were prepared and placed on MS medium with four treatments as follows: 2 mg L^{-1} 2,4 D; 1 mg L^{-1} NAA+ 3 mg L^{-1} BAP; 1 mg L^{-1} BAP + 3 mg L^{-1} NAA; 3 mg L^{-1} BAP. The explants cultured on MS medium without PGRs were considered as a control. The pH of the culture media was adjusted to 6 before autoclaving at 121°C and 1.3 kg cm^{-2} pressure for 20 min. All the operations of inoculation were carried out under strict aseptic conditions in a laminar airflow hood. Callus induction medium with explants was incubated in the dark and 16 h light/8 h dark photoperiod at 25°C in the culture chamber.

Relative Callus Growth Rate

We measured the callus growth rate (CGR) and callus induction percent (CI). Callus induction percentage was measured using the following equation: $[(n/N) \times 100]$ in which n is the total number of callus explants and N represents the total number of cultured explants. For calculating CGR, the means of CGRs (mm day^{-1}) at every two weeks was used in a two-month period [19].

Suspension Culture Establishment

To determine an optimum cell suspension culture, fragile callus (2.5 g) was moved into 100 mL flasks comprising 25 mL MS medium containing 1 mg L^{-1} NAA+ 3 mg L^{-1} BAP augmented by 2% (w/v) sucrose. Then incubated under 16-h light/8-h dark photoperiod on gyratory shakers at 125 rpm. The subculture of cell suspension was carried out at steady intervals of 14 days on the fresh media.

The Curve of Cell Growth

After being isolated from the suspension culture through filtration, 2.5 g of fresh cells were cultured in 25 mL MS within 100 mL flasks. Cell growth was followed with flasks harvested at two days intervals from the day of subculture (0^{th} day) until cell weight was stable (24^{th} day). The weight of the samples was measured and recorded by a digital scale. According to the plotted growth curve, the best time to harvest cells was selected.

Elicitor Treatment

To elicit the biosynthetic pathway of natural products in *I. amara*, the chitosan with 50k Da molecular weight (Sigma–Aldrich, Germany) was dissolved in 3% (v/v) acetic acid (0.1 M) through mild heating and continuing mixing at 55°C for 8 h. The eventual concentration of chitosan was adjusted to 10 mg. mL^{-1}. To further dissolve chitosan, the solution was mixed and autoclaved for 20 minutes at 121°C, and kept at 4°C. In the exponential growth stage, we exposed the cells to chitosan at the varying concentrations (0, 50, 100, and 200 mg L^{-1}), which was performed from day 8 to 12 following sub-cultures (T1) and day 12 to 16 following sub-cultures (T2). Suspensions were grown at 25 °C under 16-h light/8-h dark photoperiod on gyratory shakers at 125 rpm. Some empirical studies and reviewed literature were used as the basis for selection of the elicitation period and concentrations [20].

When elicitations were finished, we harvested the cells from the suspension cultures by filtration. For this purpose, a Buchner funnel was utilized with a nylon mesh in frozen and vacuum state in liquid N_2, and it was maintained at -80 °C for subsequent biochemical surveys.

Cell Growth Assay

In order to measure the cell growth, cells were collected after treatment and rinsed with distilled water twice. The fresh weight was then measured using a digital scale.

Cells Extraction Process

Firstly, cells were perfectly dried, and then 200 of them were ground in liquid N. Afterward, the cells were homogenized with methanol (3 mL), followed by centrifuging for 15 min at 10000 rpm. The supernatant was then gathered and maintained at -20°C for the subsequent analysis.

Total Phenol Content Determination

To estimate total phenol content, the methanolic extract (0.5 ml) was supplemented with 1.5 ml of 10% Folin–Ciocalteu reagent and 1.5 ml of 15% sodium carbonate, and eventually kept at 25°C for 90 min. The solution absorbance was read at 725 nm by using a spectrophotometer. The total content of phenolic compounds was estimated according to the gallic acid standard in mg gallic acid equivalent per g of cell dry weight [20].

Flavonoid and Flavonol Content Determination

Total flavonol and flavonoid contents were determined via the aluminum chloride procedure, as elucidated by Tahsili, *et al.*, [21]. For total flavonol, 3 mL of sodium acetate, 1 mL of methanolic extract, and 1 mL of aluminum chloride were mixed together. The absorbance for each mixture was determined at 445 nm. For total flavonoid, 250 µL of potassium acetate, 1 mL of methanolic extract, and 250 µL of aluminum chloride were mixed and remained at 25±1°C for 30 min to read the absorbance at 415 nm. Rutin was utilized as the standard for the calibration curve. The content of total flavonol and flavonoid was presented as mg rutin per g of dry weight.

Anthocyanin Content Determination

To measure the anthocyanin content, according to Hara, *et al.*, [22], 200 mg of dried cells was ground in 3 ml of acidic methanol (99: 1 ratio of ethanol to acetic acid), and then the resulted extract was carefully centrifuged at 12000 rpm for 20 min. The supernatant was placed in the dark overnight after filtration and its absorption was recorded by using a spectrophotometer at 511 nm. To calculate the concentration of anthocyanins, $M^{-1}cm^{-1}$ 33000 extinction coefficient was exerted and the anthocyanin accumulation was presented in nmol per g of dry weight.

Lipid Peroxidation Determination

MDA was measured for determining the cell membrane damage and lipid peroxidation. MDA was the end product of membrane lipids' peroxidation. Therefore, 0.1% trichloroacetic acid (TCA) solution was used for homogenizing the samples. We centrifuged the homogenate at 10000 rpm, and then collected supernatant. The process was followed by mixing supernatant (500 µL) with 0.5% thiobarbituric acid (TBA) (2 mL) and 20% TCA. Then, the sample was warmed for 30 min at 95 °C, and it was swiftly chilled in the ice bath. A spectrophotometer was used for measuring the sample's absorbance at 600 and 532 nm. The extinction coefficient used for quantifying the amount of MDA was 155 mM^{-1} cm^{-1}, and it was presented as µmol g^{-1}FW [23].

Statistical Analysis

The experiment was performed as a factorial experiment in a complete- randomized design (CRD) with two factors and four replications. Analysis of variance (ANOVA) and mean comparison (Least significant difference at $P \leq 0.05$) were accomplished through SAS software V. 9.1 (SAS Institute Inc.). The differences among means were represented as mean ± standard error. For callus induction, each replicate consists of four calli per replicate. The statistical significance of treatment effect was determined by using two-way analysis of variance (ANOVA). Next, the significant difference between treatments was evaluated using LSD test at $P \leq 0.05$ in SAS software (SAS 9.1 Institute Inc).

RESULTS AND DISCUSSION

Optimum Callus Induction Medium

Although *I. amara* is a significant medical herb, there is no study on using *in vitro* cultures of this plant. The signs of callus production were observed approximately 12-14 days after transferring stem and leaf explants to medium compositions.

The results of the analysis of variance (ANOVA) indicated the statistically significant impact (at $P \leq 0.05$) of plant growth regulators (PGRs), types of explants, photoperiod, and interactions with studied traits (data are not presented). Based on the findings, light significantly affected *I. amara* callus growth (CGR, CI) as compared to dark conditions. In addition, visual symptoms revealed that the induction, formation, and growth of callus in 16-h light/8-h dark photoperiod was highly better than the conditions of absolute darkness. As a result, further analyses were applied only on 16-h light/8-h dark photoperiod. According to previous reports, this phenomenon (i.e, light effect) may be associated with the crosstalk between light signaling and IAA hormone [24], photo-regulation modulated by phytochromes [25], and/or the effect of light on the photosynthetic pathway stimulation, chloroplast differentiation, ethylene evolution, and cell division rate [25]. These findings suggest that light modulates *I. amara* traits *in vitro* by physiological changes. Similar observations with 16-h light/8-h dark photoperiod for improving callus induction and callus growth rate were previously recorded for *D. polychaetum* and *D. kotschyi* [26].

The mean interactions in a completely randomized design were compared since the interactions of PGRs and explants were significant. Table 1 presents the effects of different PGRs (MS, MS+2 mg L^{-1} 2,4 D, MS+1 mg L^{-1} NAA+ 3 mg L^{-1} BAP, MS+1 mg L^{-1} BAP + 3 mg L^{-1} NAA, and MS+3 mg L^{-1} BAP) and explants (leaf and stem) on callus induction (%) and callus growth rate of *I. amara*. As previously mentioned, this is the first report regarding the optimized protocol for callus induction in *I. amara.* Our findings showed no callus induction on the MS medium, as a control, for two explants. Based on the results, 2 mg L^{-1} 2,4 D and 3 mg L^{-1} BAP represent an inhibitory influence on callus production thus they were excluded from further analyses. The completed MS medium with 3 mg L^{-1} BAP and 1 mg L^{-1} NAA was found to be the appropriate culture medium for the highest callus induction (85.16%) in the leaf explant of *I. amara* (Table 1). The least values for CI (27.17%) and (25%) were denoted to leaf and stem explants at the MS medium supplemented by 3 mg L^{-1} NAA + 1 mg L^{-1} BAP (Table 1). Leaf explants were detected to be suitable for callus induction so that they induced large calluses in the culture medium (Figure 1). As observed in the recent work, the callus induction from the leaf/ stem explant relied on the combination and concentration of PGRs. Accordingly, the optimal level of exogenous PGRs is vital for CI in *I. amara*. Four treatments were compared, and it was shown that BAP concentrations (as cytokinin)

were higher compared to NAA and 2,4 D (as auxins), which might promote a higher frequency in CI and growth in *I. amara*. Similar observations with NAA and BAP hormones for improving callus induction were previously recorded in some herbs [26, 27]. Based on the findings (Table 1), leaf explants with the enhanced MS medium with 3 mg L^{-1} BAP and 1 mg L^{-1} NAA demonstrated the largest CGR (0.32 mm day^{-1}) while leaf and stem explants at the enhanced MS medium with 3 mg L^{-1} NAA and 1 mg L^{-1} BAP indicated the smallest CGR (0.08 mm day^{-1}). Therefore, there was a positive interactive impact for NAA and BAP concentrations on the employed explant for the callus growth enhancement. PGR is a crucial factor that is responsible for CI and growth in plant cell cultures. PGR optimal concentration could rely on various factors including genotype of plant, endogenous PGR concentration of explants, and the origin of explants [26, 28]. According to previous reports, auxins and cytokinins are commonly employed in the culture of plant tissues for callus induction [25, 26].

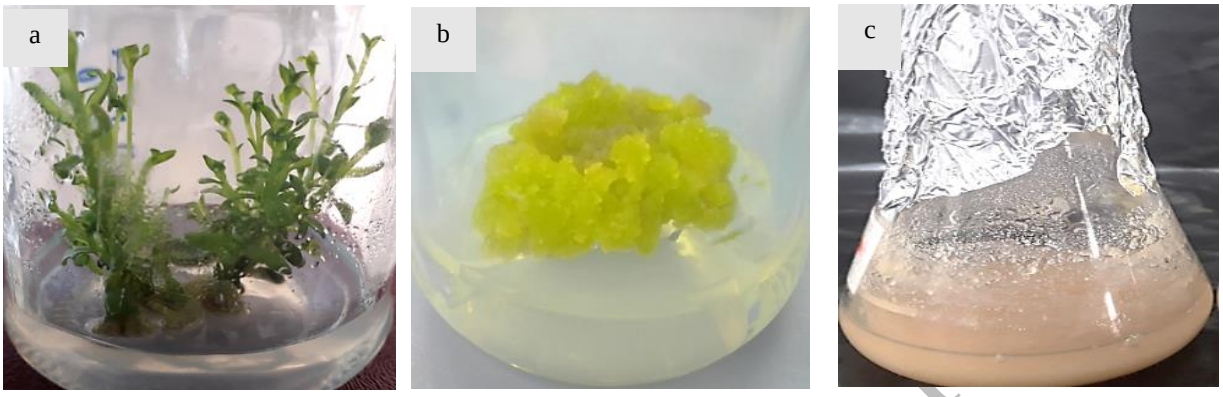

Fig. 1 Seedlings grown in Murashige and Skoog medium (a); Leaf explant-derived callus in MS medium completed with 1mg L^{-1} NAA and 3mg L^{-1} BAP (b); Establishment of suspension-cultured *I. amara* cells in MS medium with 1 mg. L^{-1} NAA, 3 mg. L^{-1} BAP (c).

Table 1 The effects of combination of different plant growth regulators (PGRs) on the callus induction (CI) and callus growth rate (CGR) of *I. amara.*

Explants	*PGRs*	*CI (%)*	*CGR ($mmday^{-1}$)*
Leaf	MS+NAA (1 mg L^{-1})+BAP (3 mg L^{-1})	¥85.16 a ±7.89	0.32 a±0.08
Stem	MS+NAA (1 mg L^{-1})+BAP (3 mg L^{-1})	70.13 b±6.75	0.21 b±0.05
Leaf	MS+NAA (3 mg L^{-1})+BAP (1 mg L^{-1})	25.00 c±14.02	0.08 c±0.06
Stem	MS+NAA (3 mg L^{-1})+BAP (1mg L^{-1})	25.17 c ±9.26	0.1 c±0.09

¥Data are the mean of four replicates; the values with the same superscript letters are not statistically different at $P \leq 0.05$ significance level according to LSD test.

Optimum Suspension Establishment Medium

According to our observations, *I. amara* cells could not grow on the suspension media having 3% (w/v) sucrose after transferring the callus. In similar studies Taghizadeh, *et al.*, 2020 observed that high sucrose levels in the culture medium generally led to low biomass accumulations. It seems that the cell growth is stopped through high osmotic pressure and/or relatively high initial concentrations of sucrose [28]. Based on this recommendation, the sucrose concentration was reduced to 2% (w/v). The MS medium supplemented with 1 mg L^{-1} NAA, 3 mg L^{-1} BAP, and 2% (w/v) sucrose at a pH of 6 appeared to be optimum conditions for suspension establishment according to visual symptoms. Regarding diverse responses to hormones, it can be concluded that PGRs are critical factors that are responsible for *I. amara* suspension establishment in cell culture. From previous reports, the advisable concentration of these hormones is dependent on explant type, genotype, and explant origin [26, 28].

Cell Growth Curve

The findings represented that cell growth was extremely low in the first five days (the delayed phase). The exponential growth of these cells was recorded from the 6^{th} to 16^{th} day after inoculation (the logarithmic phase).

The highest fresh weight of cells was observed on the 16th day, which increased approximately four folds. Then, the cells entered the dormant phase for about four days and the cell growth stopped (the dormant phase). Eventually, the weight of the cells decreased and the cells entered the death phase (Figure 2). Therefore, the times for treatment application were considered at the beginning (8-12th day) and middle (12-16th day) of the logarithmic growth stage. These findings open a time window toward the use of cell suspension culture to produce phenolic compounds, flavonoids, flavonols, and anthocyanins, which acquired a pharmacological value.

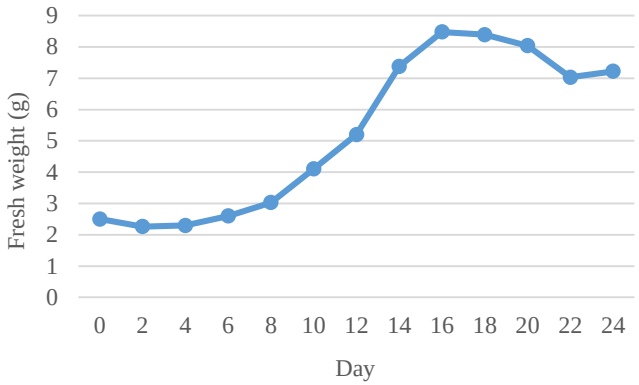

Fig. 2 Growth curve of suspension-cultured *I. amara* cells during 24 days of incubation in MS medium augmented with 1 mg L^{-1} NAA and 3 mg L^{-1} BAP.

Fresh Weight

The results showed that the chitosan elicitations, period time of elicitation, and different interactions had a significant effect on all the studied traits (data known show). Based on the results, the maximum fresh weight (10.3 g) was detected in both control cells and 50 mg L^{-1} chitosan-treated cells on the 12-16th day after the harvest (Figure 3a). The minimum fresh weight (6.22 g) was detected in 200 mg L^{-1} chitosan-treated cells on the 8-12th day (Figure 3a). The stressor effects of high concentrations of chitosan (100 and 200 ppm) reduced the cell fresh weight, which could be resulted from the inhibitory effects of elicitors on cell growth and the capacity of cell osmotic adjustment, increasing the requirement for maintaining the turgor of the growing cells, consuming energy, and decreasing cell growth. Additionally, other negative effects of chitosan in higher concentrations may be related to damaged cell division and cell membrane [24]. Likewise, higher concentrations of chitosan in safflower reduced the callus fresh weight [29]. Similar observations were reported by Udomsuk, *et al.*, [14], and Talukder, *et al.*, [30] on *Pueraria candollei* and *Plantago ovata*, respectively. Thereby, 50 mg L^{-1} chitosan was determined as the most favorable concentration for maintaining the cell fresh weight in *I. amara*.

Lipid Peroxidation

MDA is a product of lipid peroxidation that indicates free-radical accumulations and oxidative stress. Improved membrane permeability and disrupted membrane integrity were possible reasons for the induction of oxidative stress [26]. Further, the content of MDA increased remarkably in the cells that were elicited with 100 and 200 ppm chitosan compared with the control (Figure 3b). The largest increase in the MDA amount to 3.93 and 3.87 µmol. g^{-1} FW was observed under 200 ppm chitosan-treated cells on the 12-16th day and 8-12th elicitation, indicating a nearly 2-fold increase in comparison with that of the control cells. In the present research, the increment of MDA content (Figure 3b) indicated that high chitosan concentrations might have a direct impact on the cell membrane functions and structures. The oxidative stress also induced disruption in the integrity of the membrane, leading to increased permeability of the cell membrane [20]. Consequently, the cells could not hinder the peroxidation of membrane lipids. The increment of MDA content is in agreement with the decrease in fresh weight that was observed under 200 ppm chitosan-treated cells. In fact, high concentrations of chitosan caused the development of free oxygen radicals (reactive oxygen species, ROSs) and oxidative stress, and thus could directly disrupt cell membrane structures and eventually, caused cell death and fresh weight losses. The lipid peroxidation levels demonstrated no significant difference in control and cells with 50 ppm chitosan elicitation. The observed differences in the values of MDA at 50 ppm chitosan were not significant probably due to the

supplementary impacts of higher accumulations of phenolics at chitosan elicitation via ROS detoxification and membrane integrity protection. The effectiveness of secondary metabolites (e.g, phenolics) in the deceleration of lipid peroxidation is attributed to their free radical-scavenging ability [20]. The lipid peroxidation might also mediate signal transduction resulting in the increased generation of secondary metabolites. Other works indicated an increase of lipid peroxidation in *Acer pseudoplatanus* L. cultured cells under the chitosan elicitation [31].

Total Phenol Content

According to previous reports, elicitors can enhance the level of phenolic compounds through a rapid increment in the activity of key enzymes responsible for biosynthetic pathways, including PAL [17]. Thus, the interaction effect of different concentrations and treatment intervals of chitosan were investigated to retest this output in *I. amara* (Figure 3c). Based on the evidence, 50 mg L^{-1} chitosan on the 12-16th day after inoculation led to a greater increase in the total phenol accumulation when compared to other treatments and control. The total phenol compounds significantly increased approximately 1.22 fold from the control condition (1.22 mg GA g^{-1} DW) up to 37.1 (mg GA g^{-1} DW) when calli were treated with 50 mg L^{-1} chitosan (Figure 3c). Many studies demonstrated the strong relationship between plant secondary metabolism and plant defense responses [9]. The effectiveness of secondary metabolites such as phenolic compounds in the deceleration of lipid peroxidation is attributed to their free radical-scavenging ability [20]. Phenolic compounds could act as Fenton reaction inhibitors and iron chelators or could directly eliminate free radicals and reduce oxidative damage [32, 33]. The higher phenolic compounds might protect plant cells under the chitosan elicitation and show interference with the signaling cascade in plant cell responses. Consistent with the findings of the present research, it was reported that the phenol content derived from phenylpropanoid pathways because of *in vitro* chitosan application in *Coleus aromaticus* [17] and *Carthamus tinctorius* [29]. Khan, *et al.*, [16] also proved an about 50% augmentation in the total phenol amount in the soybean plants following chitosan treatment, displaying a positive correlation between PAL activity and the total phenol content. Considering that PAL is a vital enzyme in the phenylpropanoid biosynthetic pathway, it seems that its overactivity resulted in more phenol accumulations in chitosan-treated *I. amara* [16].

Flavonoid Content

Flavonoid accumulation in plants has a major role in protecting plants encountered with biotic and abiotic damages [34]. Flavonoids are the key components of the antioxidant system with subgroups as flavonols and anthocyanins [35]. The treatment of *I. amara* cells on the 12-16th day after inoculation with 50 mg L^{-1} chitosan resulted in a significant increase in the flavonoid accumulation (0.94 mg rutin. g^{-1} DW) in contrast to other treatments and the control (Figure 3d). In other words, the 50 mg L^{-1} chitosan caused an increase of about 2.19-folds flavonoid content rather than a non-elicited callus. Similarly, the increased content of flavonoid compounds was reported in *Plantago ovata* [30], *Carthamus tinctorius* [29], and *Pueraria candollei* using chitosan [14]. The increase in flavonoid content derived from the chitosan elicitor suggests a higher rate of flavonoid production owing to the possible positive effect of this elicitor on the expression of the gene coding enzymes engaged in the flavonoid biosynthesis [30]. In fact, increased phenolic compounds and flavonoids may protect plant cells against ROS generation in response to biotic and abiotic elicitors, and it will interfere with the signaling cascade involved in plant adaptation to environmental stresses. For instance, an improved flavonoid accumulation was reported due to the increased expression of the gene-coding PAL enzyme in the *Coleus aromaticus* plant [17]. Likewise, Chen, *et al.*, 2009 concluded that chitosan increases the expression of genes responsible for flavonoid and phenylpropanoid biosynthesis in soybean sprouts. Overall, the increased flavonoid content in *I. amara* may be related to the direct effect of 50 mg L^{-1} chitosan on gene expression, transcription factors, and activity of enzymes that are involved in the phenylpropanoid pathway.

Flavonol Content

The highest amount of flavonol (i.e. 0.8 mg rutin per dry weight) was detected in the cell suspension supplemented with 50 mg L^{-1} chitosan on the 12-16th day (Figure 3e), reflecting the favorable effect of low concentrations of the chitosan elicitor on the flavonol content in *I. amara* cells. Conversely, the lower level of

chitosan (50 mg L^{-1}) was found to further accumulate flavonols compared to its higher levels. These observations are in line with the results of Talukder, *et al.*, [30], and Govindaraju and Arulselvi [17] on *Coleus aromaticus* and *Coleus aromaticus*, respectively. Moreover, flavonol production increased in the cell suspension of the chitosan-treated *Cocos nucifera* herb [15]. Despite such reports regarding the beneficial effects of chitosan on flavonol content, the mechanism of action and biosynthetic or signaling pathways affected by this hormone remain unknown.

Anthocyanin Content

Given that, anthocyanins have been well-known for anti-inflammatory, anti-cancer, anti-microbial, and antioxidant properties, many studies have focused on these natural products. In the current experiment, the highest amount of anthocyanin (i.e. 12.27 µmol per dry weight), as a subgroup of flavonoids, was detected in the cell suspension treated with 50 mg L^{-1} on the 12-16th day (Figure 3f). Our findings on the chitosan-derived increased content of secondary metabolites corroborate with those of Park et al. [37] and Govindaraju and Arulselvi [17] on *Fagopyrum esculentum* and *Coleus aromaticus*, respectively. The change in anthocyanin content can be attributed to the modulated activity of transcription factors responsible for the anthocyanin biosynthesis pathway, which is affected by the chitosan elicitor and/ or partially by the applied plant growth regulators in the callus induction/ suspension establishment media [38]. Although the exact mode of action of chitosan is complicated, our findings verified the dependence of the elicitation process *in vitro* on elicitor concentrations. Some other factors including callus age, culture medium, plant species and genotype, and elicitor type and its exposure time can thus have a significant effect on elicitation processes [39].

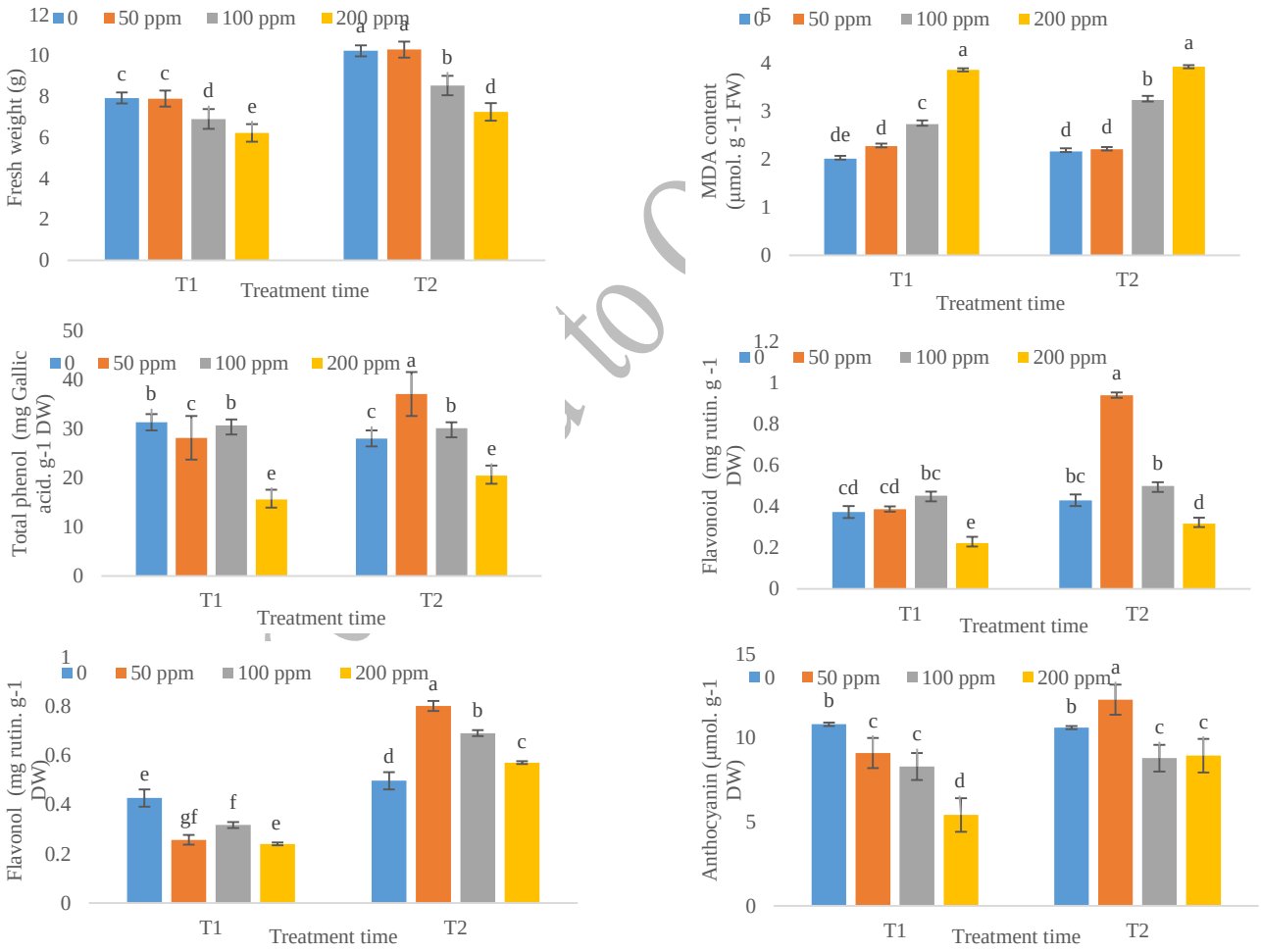

Fig. 3 Effect of treatment intervals and chitosan concentrations on fresh weight (a); MDA content (b); phenolic compounds (c); flavonoid (d); flavonol (e); anthocyanin (f) of *I. amara* cells. T1: A group of cells treated from 8th to 12th day after inoculation, T2: A group of cells treated from 12th to 16th day after inoculation. Different letters demonstrate significant differences at the P<0.05 level according to LSD test. The values were shown as mean± SE.

According to our data, the contents of the total phenolics, flavonoids, flavonol, MDA, and anthocyanins in chitosan-treated cells on the 12-16th day (T2) were significantly higher than those of cells treated with chitosan on 8-12th elicitation (T1). These observations suggest that the antioxidant system is significantly stimulated by increasing the age of cells elicited by chitosan.

Trait Correlations

Table 2 provides the obtained correlations among the evaluated traits. The total phenolic compounds indicated significant positive correlations with flavonoid and anthocyanin (0.702** and 0.762**) suggested at synchronization pathways for biosynthesis and gathering these compounds in chitosan and normal elicitation (Table 2). According to these results, with higher phenolic compounds, flavonoid and anthocyanin might provide protection for plant cells against stresses and show interference with the signaling cascades in plant responses. Furthermore, such a rise could be due to the elicitation of their biosynthetic pathways, the improved enzymatic activity, and the expression of pertinent genes. There was a significant negative correlation between MDA and the total phenolic compounds (-0.784**), flavonoid (-0.456*), anthocyanin (-0.722**), and fresh weight (-0.619**), implying that increases in all these metabolites caused a decrease in the MDA level. As mentioned earlier, MDA is a cytotoxic product of lipid peroxidation that indicates free-radical accumulations. Chitosan affects plant cells via inducing oxidative stress and increasing activity, lifetime, and concentrations of free radicals. The possible causing factor for the induction of oxidative stress disrupted membrane integrity, improved lipid peroxidation and cell toxicity, and then caused cell death [26]. In other words, these compounds have possibly a vital role as free radical scavenging in *I. amara* cells subjected to chitosan elicitation. In this condition, cell fresh weight decreased by an increase in MDA. Therefore, this correlation represented the strong antioxidant property of total phenolic, flavonoid, and anthocyanin in ROS scavenging or detoxifying the harmful effects of elicitation.

Table 2 Correlation coefficients among bioactive components in the *I. amara* studied under different concentration of chitosan.

	Total Phenolic	Flavonoid	*Flavonol*	*Antocyanin*	*Fresh weight*	*MDA*
Total Phenolic	1					
Flavonoid	0.752 **	1				
Flavonol	0.429 *	0.712 **	1			
Antocyanin	0.762 **	0.712 **	0.624 **	1		
Fresh weight	0.572 **	0.624 **	0.625 **	0.728 **		
MDA	-0.784 **	-0.456 *	-0.077	-0.722 **	-0.619 **	1

** and * Significant at 1% and 5% levels of probability, respectively.

CONCLUSION

In this study, the optimum *in vitro* conditions were obtained for inducing callus and establishing the cell suspension of the *I. amara* medicinal herb. The optimum medium for callus induction was provided from leaf and stem explants in the MS medium supplemented by 3 mg L^{-1} BAP and 1 mg L^{-1} NAA. The MS medium supplemented by 3 mg L^{-1} BAP, 1 mg L^{-1} NAA, and 2% (w/v) sucrose at a pH of 6 was found suitable for achieving rapid-growing suspension cells. Interestingly, 50 mg L^{-1} chitosan remarkably improved the phenol, flavonoid, flavonol, and anthocyanin content in the *I. amara* medicinal herb in a dose-dependent way. As a result, chitosan (50 ppm) was detected as an efficient elicitor for enhancing secondary metabolites in *I. amara*. The results of the present work can pave the way for improving the generation of beneficial phenolic compounds from undifferentiated cells in a vulnerable medicinal herb called *I. amara*.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contribution

M. T. and M. S. devised the project, the main conceptual ideas and proof outline. M. T. and M. S. N. developed the theory. M. T. carried out the experiment and collected data. M. T verified the analytical methods, M. T. and

M. S. analyzed and interpreted the data. M. T. wrote the manuscript. M. T, M. S. N. and M. S. contributed to the final version of the manuscript. All authors supervised the project, discussed the results, and contributed to the final manuscript.

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