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Effects of methyl jasmonate and phloroglucinol on thebaine and sanguinarine production in cell suspension culture of Persian poppy (*Papaver bracteatum* Lindl.)

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Abstract: The biosynthesis path engineering could be very promising for mass production of alkaloids by applying elicitors in the cell suspension culture of Persian poppy (*Papaver bracteatum* Lindl.). In this work, the effects of different concentrations of methyl jasmonate (MJ) and phloroglucinol (PG) on thebaine and sanguinarine productions in vitro were investigated. Roots as explant and supplementing 3 mg L^{-1} 2,4-Dichlorophenoxyacetic acid with 0.5 mg L^{-1} Benzyl amino purine to modified MS medium were selected to achieve the most efficient combination for callus induction and production of callus fresh and dry weights. At 48 h after treatment, the addition of PG and MJ individually and in combination together significantly increased both thebaine and sanguinarine contents than the control. The results of high-performance liquid chromatography (HPLC) detection indicated that the highest production rate has been achieved through a synergic effect of two elicitors after 48 h. Results revealed that adding 200 µM of MJ and 100 mg L^{-1} PG increased thebaine and sanguinarine contents by 56.36 and 107.71–fold than control cells, respectively.

Key words: Cell suspension culture; Methyljasmonate; Phloroglucinol; Sanguinarine; Thebaine; *Papaver bracteatum* Lindl.

Introduction

Persian poppy (*Papaver bracteatum* Lindl.) is a diploid species and known as the Iranian poppy (1). This species belongs to section *Oxytona* Bernh. in the core of *Papaver* sensustricto (2) and has outcrossing pollination with gametophytic self-incompatibility. This medicinal plant naturally is scattered and distributed in 1500 up to 2500 meters from sea level of Alborz Mountains, Iranian Kurdistan and the northern slope of the Caucasus (3–4). *P. bracteatum* is one of the important medicinal plants that it's known as the ideal source for morphine, thebaine, codeine and semi-synthetic derivatives such as oxycodone and naltrexone (5). Furthermore, this plant serves as an ideal source of benzylisoquinoline alkaloids with effective pharmacological properties including the cough suppressant and potential anticancer drug noscapine, vasodilator papaverine and the antimicrobial agent sanguinarine. Of these, thebaine and sanguinarine alkaloids are very important due to their useful medicinal potentials including antimicrobial, antifungal, and anti-inflammatory capacities (6–7). Thebaine is one of the important alkaloids that can be transformed into several opiates. This alkaloid due to their low enzyme's activity involved in demethylation to codeine and morphine could be employed as a non-addictive substitute for morphine as a medical painkiller (8). Sanguinarine is another form of benzophenanthridine which extracted from poppy plants (9). It has been reported that sanguinarine has the pharmacological and toxicological effects such as anti-inflammatory, antimicrobial, antioxidant and antitumor properties (9).

In vitro culture of plant cells seems to be a hopeful approach for enhance of production of key secondary metabolites from various medicinal plants. Plant cell cultures produce valuable secondary metabolites under controlled and defined conditions (10). Elicitation is one of the key strategies currently being deployed to increase the production of secondary metabolites in tissue or cell cultures. An elicitor could be defined as an external factor, a substance and a molecule. When a minor quantity of it is incorporated into an existing cellular background, it improves, the biosynthetic competence of specific compounds particularly secondary metabolites in the pathway (11–12). In plant cells, one of the defense response is the accumulation or even production of secondary metabolites. These responses can be activated by different endogenous and exogenous molecules called elicitors (13). One of the important elicitors, which naturally exist in higher plants is methyl jasmonate (MJ). Exogenous application of this elicitor causes the biosynthesis and accumulation of several secondary metabolites, such as anthraquinones, flavonoids, alkaloids and terpenoids (14). Phloroglucinol (PG, 1,3,5–trihydroxybenzene or phloroglucinol) is considered an organic component that is used in the synthesis of pharmaceuticals and explosives. PG had two isomers hydroxyquinol (1,2,4-benzenetriol) and

pyrogallol (1,2,3-benzenetriol). PG has important roles in tissue culture and it has been reported that this component as an elicitor can be increased shoot formation, root stimulating and somatic embryogenesis in several horticultural and grain crops (15). Berardi et al. (16) showed that PG in combination with Naphthalene Acetic Acid (NAA) or Indole-3-Butyric Acid (IBA) promoted the greatest increase in rooting of *Pyrus calleryana*. The addition of PG to the medium controlled the formation of poly-phenolics in vitro, leading to improved callus formation of *Aristolochia indica* (17). Rooting of British wild cherry *(Prunus savium* L.) was also enhanced by adding PG to the rooting medium (18). Previously improving production of secondary metabolites by elicitation in cell suspension culture of different medicinal plants such as benzophenanthridine alkaloids from *Eschscholtzia californica* (19); flavonoids from *Hypericum perforatum* (20); flavonolignans frome *Silybum marianum* (21) and protoberberine from *Tinospora cordifolia* (22) had been proven. In the present work, we examined the effects MJ and PG individually and in combination with for enhancement of thebaine and sanguinarine production in Iranian poppy (*P. bracteatum*) through cell suspension culture.

Materials and Methods

Plant material and sterilization

Persian poppy (*P. bracteatum* Lindl.) seeds used in this study were provided by Department of Biotechnology, Institute of Medicinal Plants, ACECR, Karaj, Iran. The seeds were surface-sterilized with 70% (v/v) ethanol for 1 min and sodium hypochlorite solution 1.5% (w/v) for 8 min, and then rinsed 3 times (5 min for each time) with sterile distilled water. The seeds were cultured on Murashige and Skoog (MS) medium (23) solidified with 6.5 g L^{-1} agar, and preserved in a growth chamber at 25 ± 2 °C and 55±5% relative humidity (RH) with a 16:8 h (light:dark) photoperiod cycle under 35 $\mu molm^{-2}s^{-1}$ flux rate. The maintained period between 14 and 20 days.

Callus induction and cell suspension culture establishment

The leaves, hypocotyls and roots (5 ± 1 mm segments) from 14-days-old in vitro grown plants were planted on solid 3:4 MS medium supplemented with different levels of BAP (0, 0.5, 1 and 2 mg L^{-1}), 2,4-D (0, 0.5, 1, 2 and 3 mg L^{-1}) and 30 g L^{-1} sucrose. The cultures were placed in the dark environment with a temperature of 25 ± 2 °C. After 21 days, the cultures were subjected to sub culture process. At the end of this period (42 days), several parameters such as the percentage of callus induction, callus morphology, callus fresh weights (CFW) and callus dry weights (CDW) were measured. Cell suspension culture was started according to protocol as descripted by Zare et al. (13). Briefly, friable root callus (80–100 mg) transferred into a 50 mL Erlenmeyer flask containing 15 mL of liquid 3:4 MS medium filled with 3 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP. After three sub-cultures, the established suspension cells were transferred into 250 mL Erlenmeyer flasks containing 50 mL of the same previous medium with an initial cell density of 4.3×10^5 cells m L^{-1}.

Elicitation of cell suspension cultures

Two elicitors including methyl jasmonate (MJ; Sigma-Aldrich, Germany) and phloroglucinol (PG; Duchefa Biochemie, Netherlands**)** were used to investigate the effect of exogenous elicitation on thebaine and sanguinarine production of *P. bracteatum*. MJ and PG were dissolved in 95% ethanol and water, respectively. Elicitation was applied on 21 and 22 days after culture, when the cell growth curve was at the peck of the number of produced cells (until 31 days after elicitation). The treatments were 100 and 200 µM MJ and100 and 200 mg L^{-1} PG individually and in combination together. Samples were taken 24 and 48 h after adding elicitor. In the control treatment, cultures received an equal amount of medium instead of elicitor. Finally, cells were harvested and subjected to alkaloid extraction.

Alkaloid extraction and high-performance liquid chromatography (HPLC) analysis

Alkaloid contents were extracted according to Cho et al. (24). Benzophenanthridine alkaloids were quantified by their optical density peaks at 280 nm, using authentic sanguinarine and thebaine (Temad Chemical Co. Tehran, Iran) as standard compounds for calibration. HPLC analysis was performed on a Knauer HPLC system (1200 series, UV detector K-2501). A volume of 50 µL of samples was injected in an 18 reverse-phase phenomenex column (Gemini NX-C18, 5mm, 4.6 × 250 mm). The mobile phase for alkaloid elution was 90% methanol and 5% deionized water (0.2% Triethylamine) (13).

Statistical analysis

The callus induction experiment was performed with four replications and the elicitation experiment was done in triplicate data that were expressed as the mean of samples with standard deviation. Statistical differences were assessed based on analysis of variance (ANOVA) using SPSS ver. 18 software. The significant tests between all treatment combinations were done by Duncan's multiple range tests at a probability level of 0.05.

Results

Effects of explants type and growth regulators combination on callus induction

MS medium modified with different levels of 2,4-Dichlorophenoxyacetic acid (2,4-D) (0, 0.5, 1, 2 and 3 mg L^{-1}) alone and in combination with Benzyl amino purine (BAP) (0, 0.5, 1, and 2 mg L^{-1}) investigated for callus formation from leaf, root and hypocotyl explants. For this purpose, Persian poppy seeds were cultured on hormone-free MS medium (Figure 1A). After one week, the seeds were germinated and after 20 days, the plantlets were ready to be cut for preparing different explants (Figures 1B–D). Callus induction was observed after three weeks with swelling and the formation of mass on the surface of the root explants (Figure 1E). Meanwhile, most of the leaf and hypocotyl explants produced callus later than root explants (Figure 1F). The color of calli from different explants ranged from shiny cream to dark brown and the texture was from flimsy to hard. The results showed that the cream and friable calli were more

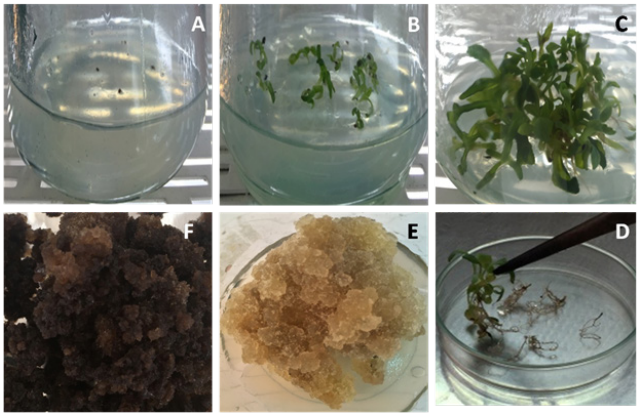

Figure 1. The steps from seed culturing to callus induction of *P. bracteatum.* (A) Seed culturing on MS medium without growth regulators; (B) Seed germination; (C) Ready plantlets to be cut for preparing explants; (D) Preparing different explants for callus induction; (E) Cream and friable callus obtained from root explants; (F) Brown and flimsy callus obtained from leaf explant.

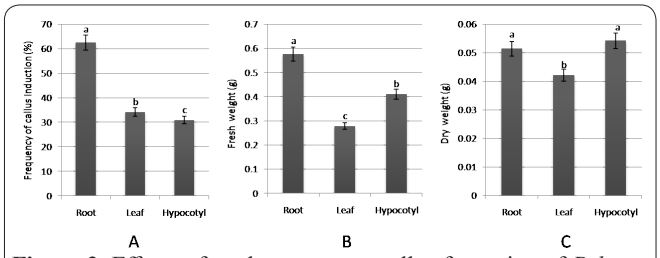

Figure 2. Effects of explants type on callus formation of *P. bracteatum.* (A) Frequency of callus induction (%); (B) Fresh weight (g); (C) Dry weight (g). Different letters in each bar show significantly different at $P \leq 0.05$.

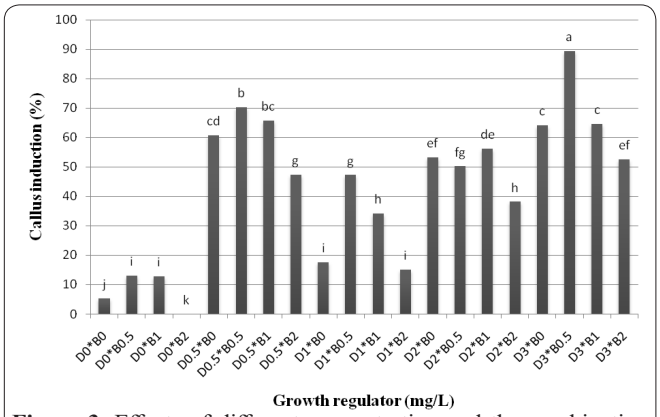

Figure 3. Effects of different concentration and the combination of 2,4-D and BAP on the frequency of callus induction of *P. bracteatum.* B and D indicated BAP and 2,4-D, respectively. Different letters in each bar show significantly different at $P \leq 0.05$.

generated on root explants while the dark colors and flimsy textures were mostly related to leaf and hypocotyls explants (Table 1).

Based on ANOVA, both parameters (explants type and growth regulators combination) influenced the frequency of callus induction, fresh weight and dry weight of callus (data not shown). According to the mean comparisons, the highest frequency of callus induction (Figure 2A), fresh (Figure 2B), and dry weight (Figure 2C) related to root explants. The means comparison results of dry weight showed not only the root explants, but also hypocotyls explants were considerably appropriate (Figure 2C). Therefore, due to the callus induction and fresh weight, the root has been chosen as the best explant. Furthermore, the highest percentage of callus induction (100%), callus fresh weight (CFW; 3.69 g) and callus dry weight (CDW; 0.36 g) were observed on root explants (data not shown). The means comparison of the growth regulators combinations on callus induction showed that the best treatment belonged to3 mg L^{-1} 2,4-D along with 0.5 mg L^{-1} BAP applied in MS medium (Figure 3). The same combination in cultural

Table 1. Effects of different plant growth regulator combinations on callus morphology of different explants in *P. bracteatum.*

Hormone (mg L^{-1})	Callus Morphology		
	Root	Leaf	Hypocotyl
2,4-D 0+BAP 0	Cream, flimsy	-	-
2,4-D 0+BAP 0.5	Cream, friable	Whitish brown flimsy	-
2,4-D 0+BAP 1	Cream, friable	Whitish brown flimsy	-
2,4-D 0+BAP 2	-	-	-
2,4-D 0.5+BAP 0	Cream, friable	Whitish brown, flimsy	Cream, friable
2,4-D 0.5+BAP 0.5	Brown, friable	Pale brown, flimsy	Pale brown, flimsy
2,4-D 0.5+BAP 1	Shiny Cream, friable	Pale brown, flimsy	Brown,flimsy
2,4-D 0.5+BAP 2	Cream, friable	Shiny cream, friable	Whitish brown, friable
2,4-D 1+BAP 0	Cream, friable	-	-
2,4-D 1+BAP 0.5	Cream, hard	Pale brown, hard	Cream, hard
2,4-D 1+BAP 1	Whitish brown, flimsy	Pale brown, hard	-
2,4-D 1+BAP 2	Cream, hard	-	-
2,4-D 2+BAP 0	Cream, brown, friable	Whitish brown, flimsy	Cream, hard
2,4-D 2+BAP 0.5	Shiny cream, friable	Whitish brown, hard	Cream, hard
2,4-D 2+BAP 1	Cream, hard	Whitish brown flimsy	Pale brown, friable
2,4-D 2+BAP 2	Cream, friable	Cream, friable	Cream, white, friable
2,4-D 3+BAP 0	Cream, friable	Whitish brown flimsy	Cream, brown, flimsy
2,4-D 3+BAP 0.5	Cream, friable	Cream, Brown, friable	Cream, friable
2,4-D 3+BAP 1	Cream, white, friable	Whitish brown flimsy	brown, flimsy
2,4-D 3+BAP 2	Cream, white, friable	Pale brown, flimsy	Shiny brown, flimsy

medium also led to the highest mean of fresh and dry weights (Figures 4–5). The calli obtained from root explants in modified MS medium supplemented with 3 mg L^{-1} of 2,4-D and 0.5 mg L^{-1} BAP were the most optimum in terms of quality (cream and friable) and quantity which then were used for the establishment of cell suspension cultures.

Effects of MJ and PG on biomass, thebaine and sanguinarine production

Application of different concentration of MJ and PG individually and in combination together increased the cell biomass after 48 h (Table 2). Since the best time of treatment with the highest cellular biomass was 48 h, the consideration of data respected to 24 hours has been waived. It has been observed that the treatment with Application of 100 and 200 mg L^{-1} PG cellular biomass increased 1.53 and 3.23-fold cellular biomass than control, respectively. With regard to results obtained by HPLC (Figures 6–7), adding MJ and PG increased significantly thebaine and sanguinarine contents of cells in relative to the control treatment and the highest amounts were recorded at 48 h after elicitation. That supplementing medium with 100 and 200 µM of MJ increased thebaine content by 3.18 and 4.81-fold than control. Also, these medium increased sanguinarine content by 10.91 and 20.38-fold than control. Furthermore, 100/200 mg L^{-1} of PG increased thebaine and sanguinarine contents by 1.68/2.25-fold and 2.04/3.61-fold than control treatment. The results collected from the analysis of variances related to the extracted thebaine and sanguinarine detecting by HPLC showed that the effect of each elicitor alone and in combination was significant. The highest accumulated amounts of thebaine at 24 and 48 h after elicitation were 31.55 and 65.36 mg L^{-1}, respectively (Figure 6). The maximum sanguinarine contents (74.6 and 291.92 mg L^{-1}) were obtained at medium consisted of 200 µM MJ and 100 mg L^{-1} PG for 24 and 48 h after elicitations, respectively (Figure 7). The results of HPLC chromatograms of thebaine and sanguinarine extracted from cell suspension culture on 48 h after treatment with 200 µM MJ and 100 mg L^{-1} PG are shown in Figure 8. At treatment 48 h after elicitations, the production of both thebaine and sanguinarine alkaloids increased by 56.34 and 107.71-fold more than

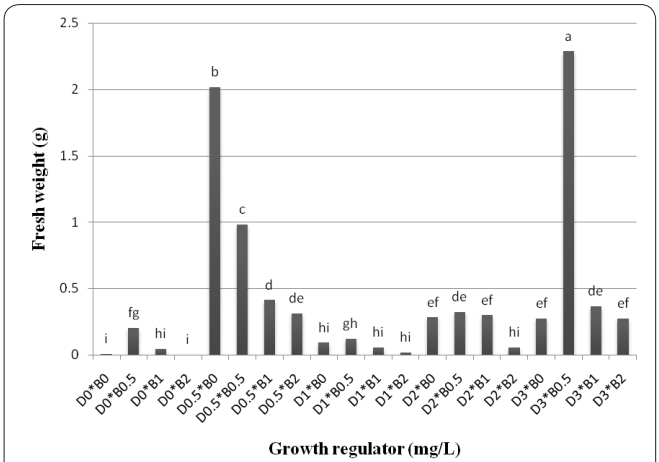

Figure 4. Effects of different concentration and the combination of 2,4-D and BAP on callus fresh weight of *P. bracteatum.* B and D indicated BAP and 2,4-D, respectively. Different letters in each bar show significantly different at $P \leq 0.05$.

Table 2. Effects of MJ (µM), PG (mg L^{-1}) on the growth of cell suspension culture of *P. bracteatum* after 48 h.

Treatment	freeze-dried cell weight (g l^{-1}) at 48 h
Control	8.667±0.13
100MJ	43.651±0.05
100PG	13.262±0.17
200MJ	52.600±0.11
200PG	28.046±0.20
100MJ+100PG	64.798±0.03
100MJ+200PG	58.225±0.15
200MJ+100PG	68.491±0.01
200MJ+200PG	66.041±0.17

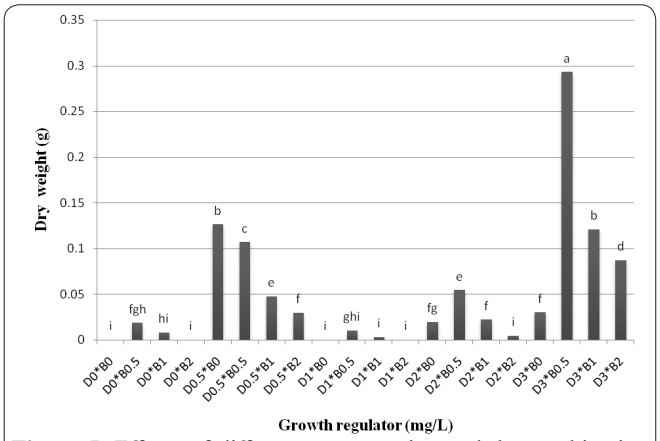

Figure 5. Effects of different concentration and the combination of 2,4-D and BAP on callus dry weight of *P. bracteatum.* B and D indicated BAP and 2,4-D, respectively. Different letters in each bar show significantly different at $P \leq 0.05$.

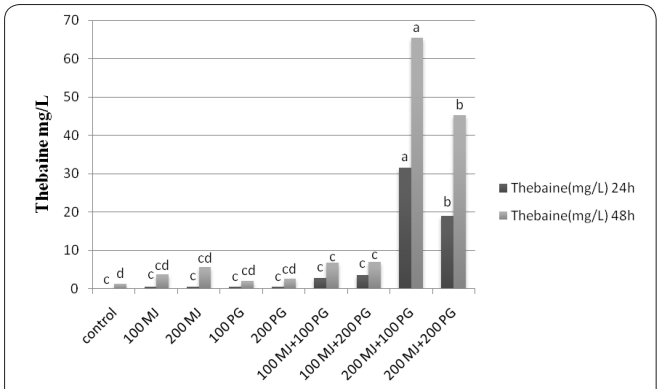

Figure 6. Effects of different concentration and combination of MJ (0,100 and 200 uM) and PG (0,100 and 200 mg L) on the production of thebaine 24 and 48 h after elicitation. Different letters in each bar show significantly different at $P \leq 0.05$.

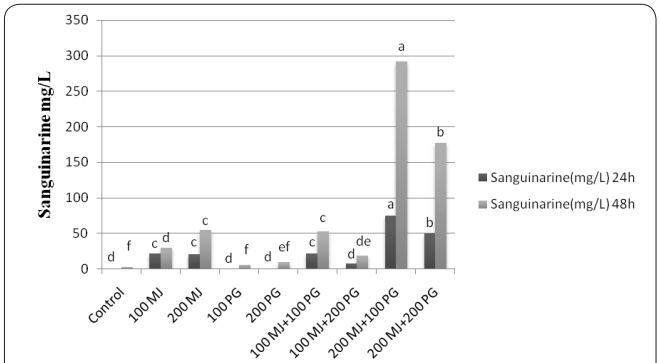

Figure 7. Effects of different concentration and the combination of MJ (0,100 and 200 uM) and PG (0,100 and 200 mg L) on the production of sanguinarine 24 and 48 h after elicitation. Different letters in each bar show significantly different at $P \leq 0.05$.

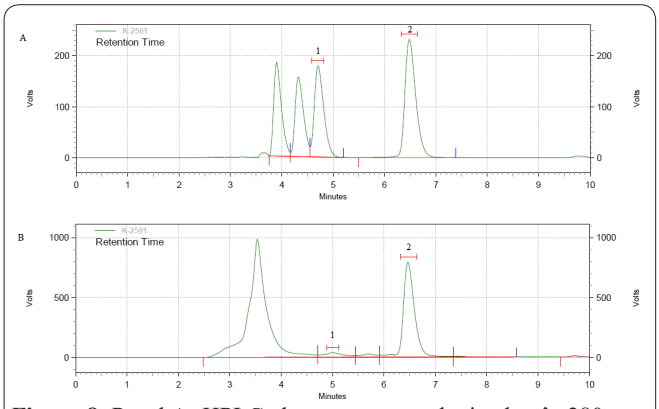

Figure 8. Panel A: HPLC chromatograms obtained at λ=280 nm: (1) and (2) show the thebaine and sanguinarine peaks in standard solution (the two first picks related to morphine and codeine standards which were not detected in all treatments and controls), respectively. Panel B: *P. bracteatum* cell on 48 h after treatment with2 00 µM MJ and 100 mg L^{-1} PG: (1) and (2) show the thebaine and sanguinarine picks, respectively.

non-elicited cells, respectively. Also, in this condition, the weight of cellular biomass increased 7.90-fold than to control (Table 2).

Discussion

Effects of explant types and different growth regulators concentrations in plant callus induction have been well studied. The type of explants tends to have a great effect on the induction of callus (25). This response has been observed at the present study just like the previous reports in other species of Papaveraceae such as *Hylomeconvernalis* (26) and *Papaver nudicaule* (27). Auxins and cytokinins play a crucially important role in regulating growth and morphogenesis in plant tissue culture, as was supported in this study. Many reports indicated that auxin is a key factor in callus (28). Among auxins, 2,4-D is considered to be the most common auxin one applied to initiate callus growth since it can revert explants cells to a dedifferentiated distinguished state and begin to actively proliferate thrive (29). Previous studies in this field were presented in various reports. For example, callus induced from *P. bracteatum* seedlings cultured on MS medium supplemented with 0.5 mg L^{-1} BAP and 1 mg L^{-1} NAA (30) and callus induction rate was the highest on MS medium containing 1.0 mg L^{-1}NAA and BAP (27). In a study conducted by Farjaminezhad et al. (31), the highest percentage of callus induction (86.67%) and CFW from *P. bracteatum* were observed in MS medium containing 0.1–0.2 mg L^{-1} kinetin, 1–2 mg L^{-1} 2,4-D and 15 mg L^{-1} ascorbic acid.

The main outcome of this study was the development of a reliable and well-defined protocol for increase thebaine and sanguinarine production by the profit of the synergic effect of MJ and PG. The role of plant growth regulators in growth and development, as well as inducing the production of secondary metabolites, has been widely reported. For instance, there are several works which show the effects different regulators on improving secondary products such as increasing alkaloid through auxins and cytokinins (32), influence of 3-Indoleacetic acid (IAA) on the vincristine content of *Catharanthus roseus* (33) and increased production of Terpenoid Indole Alkaloids (TIAs) in supplementing media with different PGRs (34); enhanced content of tannin, alkaloids and saponin by using IAA in medium for the growth of *Balanites aegyptiaca* (35). Phloroglucinol (PG) is an important plant growth regulator and has a considerable potentiality for application in tissue and organ cultures through its cytokinin-like and auxin-like activities (15, 36). It has been reported that PG enhances shoot (37) and root formation (18, 38) and somatic embryogenesis (39) in several horticultural and grain crops. Regarding the application of PG, the results showed that increase of sanguinarine and thebaine production after adding PG might be due to the growth of suspension cells by activating phosphorylation of ERK and subsequently the production of secondary metabolites. Also, PG has an antioxidant effect against reactive oxygen species (ROS) that are harmful in the products formed during aerobic metabolism (40, 41). This effect of PG may be caused the decrease of ROS levels in cell suspension culture (41) and deduction of death cell (42) by balance the production and metabolism of ROS and constraint of the production of active types of ROS (43).

Methyl jasmonate (MJ) has already been used in many plant cell suspension cultures to stimulate alkaloids. For example, in *Taxus canadensis* cell suspensions, the highest paclitaxel production obtained by 100 µM MJ (44). The excretion of secondary metabolites from *Scopolia parviflora* was significantly improved by treatment with MJ. In another study, the highest excretion of scopolamine was showed after 48 h from the application of 2.0 mM MJ compared with the control treatment. Also, application of 2.0 mM MJ stimulated the excretion of hyoscyamine after 12 h and the maximum level of hyoscyamine was detected after 72 h than control treatment (45). The elicitation of hairy roots in *Catharanthus roseus* with different concentrations of MJ, improved the accumulation of several important alkaloids such as serpentine, catharanthine, ajmalicine and ajmaline. The highest accumulation of alkaloids was observed after 48 h, when 100 and 250 µM of MJ was added (46). Accumulation of Silymarin in *Silybum marianum* significantly increased after 48 h of MJ (100 uM) application (21). Tropane alkaloids were induced by MJ in *Datura stramonium* and these results also indicated that MJ enhanced putrescine *N*-methyltransferase (PMT) activity and key alkaloid biosynthetic enzymes due to the increase of mRNA of PMT (47). MJ plays its role as an important signaling molecule to trigger gene expression in response to biotic and abiotic stresses (48). Increasing in thebaine and sanguinarine amount could be due to the synergic effects of elicitors applied to suspension culture of *P. bracteatum*. Accordingly, this noted combination might be caused by the enhancement of the production of alkaloid and cellular biomass. It has been reported that combination of different elicitors could be useful to improve of production of secondary metabolites in cell culture (24). The combination of MJ with salicylic acid and fungal elicitors lead to increasing the taxol accumulation in *Taxus baccata* suspension cultures (49). The synergistic effects of sequential treatment with MJ increased the accumulation of sanguinarine (5.5-fold than control treatment) in *E. californica* suspension cultures (24). Zare et al. (13) demonstrated that MJ and L-tyrosine elicitors had a

positive synergistic effect on thebaine production in suspension cultures of *P. bracteatum*, so that our findings were in accordance with this result. Accordingly, similar results have been reported in cell suspension cultures of *Vitis vinifera* (50) and *E. californica*cells (51).

In conclusion, the synergic effect of MJ along with PG was significantly recognizable. Our results showed that combination of external elicitors and PGRs is an effective strategy to improve morphine an alkaloid production in cell suspension cultures of *P. bracteatum*. So that, the highest productions of thebaine and sanguinarine were obtained by a combination of 200 μM MJ with 100 mgL^{-1} PG. The approach implemented in the present study displays itself as very promising for further steps in cell suspension culture of many other plants to produce various important secondary metabolites.

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Interest conflict
Authors declared no conflict of interest.

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