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ORIGINAL ARTICLE

# Establishment and assessment of cell suspension cultures of *Matricaria chamomilla* as a possible source of apigenin under static magnetic field

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## Abstract

This study represents an optimized protocol for callus establishment and cell suspension culture of *Matricaria chamomilla*, and the impact of the static magnetic field (SMF) on flavonoid metabolism and antioxidant activity were examined for the first time. The effect of growth regulators was investigated to enhance biomass growth and apigenin production. Murashige and Skoog medium supplemented with 2,4-D (1.5 mg $l^{-1}$) and Kinetin (0.5 mg $l^{-1}$) showed the highest callus induction rate (100%), fresh weight, apigenin (0.82%) and apigenin-7-glucoside (1.57%) contents. Cell suspension culture was established, and the optimum subculture time was found to 13–15 days. SMF induced cell leaching and oxidative stress in all treated cells by an increase in $H_2O_2$ content and more stimulation of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) enzymes activities. Total phenolic, flavonoid and DPPH activity increased in cells treated to SMF, and the maximum content of apigenin (1.3%) and apigenin-7-glucoside (2.1%) were identified in cell treated to 4 mT. These results provided an effective method for the regulation of flavonoid biosynthesis in *M. chamomilla* cell suspension culture, and the use of SMF as a tool for the induction of apigenin production.

## Key message

Cell suspension cultures of *Matricaria chamomilla* contain valuable medicinal flavonoids. Static magnetic field promoted apigenin production and antioxidative enzyme activities in *M. chamomilla* cell suspension.

**Keywords** Cell suspension culture · Secondary metabolite · Antioxidant activity · Static magnetic field · *Matricaria chamomilla*

## Introduction

Chamomile (*Matricaria chamomilla* L.) is a medicinal plant of Asteraceae that exists all over the world. This plant is native to Iran and cultures in Europe, America, and Asia. Apigenin is a flavonoid compound that has detected in *M. chamomilla* seedling (Sayadiet al. 2014). Apigenin has extensive biological activities including anti-inflammatory, antioxidant, anti-cancer, neuroprotective, anti-microbial and anti-allergic impacts (Sebai et al. 2014; Zemestani et al. 2016; Patel et al. 2007; Ranpariya et al. 2011; Silvaet al. 2012). Apigenin exists in different plant species with low content such as *Capsicum annum*, *Allium sativum*, *Averrhoa bilimbi*, *Pisum sativum*, *Psidium guajava* and other plants (Miean and Mohamed 2001), while its content is higher in *M. chamomilla* than the mentioned plants. On the other hand, different populations of *M. chamomilla* from different countries showed various apigenin contents (Srivastava and Gupta 2007). Iranian *M. chamomilla* showed 0.74–1.11% apigenin content (Haghi et al. 2014).

Biotechnological approaches, especially plant cell and tissue culture as a supplement to traditional agriculture

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has potential for the high production of biological metabolites (Giri and Narasu 2000; Rao and Ravishankar 2002). Plants and cultivated cells produce secondary metabolites in qualitatively like pathways (Hellwig et al. 2004), and there are successful samples of tissue cultures to increase content of the secondary compounds in some medicinal plants. Besids, these contents were more as compared to wild plants (Estrada-Zuniga et al. 2009). In vitro cultures characterize a suitable tool for generating phenylpropanoids as a replacement for using wild plants (Estrada-Zuniga et al. 2009).

Numerous strategies are applied to improve the quality and quantity of secondary compounds generation in medicinal plant cell culture comprising a selection of suitable cell lines, immobilization of cells, alteration in compounds of the culture medium, various physical and chemical stresses, etc. (Tahsiliet al. 2014). Magnetic field (MF) is a physical stress has been recently proposed for plant secondary metabolite production and they have fewer dangerous for the environment (Aladjadjiyan 2010). MF effects on cells are related to the magnetic susceptibility, MF intensity, and gradient. Cells are containing various cellular components and combinations that could reply to the MF including mitochondria, cell membrane, protein and DNA (Pauling 1979). MF could modify electromagnetic characterizes of biomolecules, membrane permeability, and enzyme activity in biochemical pathways. Also, it has been stated MF is altered the concentration and activity of reactive oxygen species (ROS), membrane ionic currents, and cell growth and propagation (Çeliket al. 2009; Galland and Pazur2005; Payez et al. 2013; Sahebjamei et al. 2007). A few studies indicated that MF could induce various secondary metabolites in plants (Rezaei et al. 2010; Taghizadeh et al. 2019; Mansourkhaki et al. 2019), and its mechanism needs more investigation. Therefore, the purposes of this work were to investigate the impacts of the static magnetic field (SMF) on secondary metabolites and antioxidative responses in *M. chamomilla* cell suspension culture for the first time. Results from this study can aid us to develop our information about the mechanism(s) of the undifferentiated plant cells reactions to SMF. It too may aid us to induce the construction and secretion of the valuable flavonoid metabolites in undifferentiated plant cells.

## Materials and methods

### Plant material

*Matricaria chamomilla* L. plants were gathered in July 2018 from Dizin Mountains in the north-east of the Tehran province of Iran. The plants were identified with a voucher number to the Herbarium in the Department of Biological Science of Tehran University, Tehran, Iran.

### Callus induction for cell suspension culture

Seeds were put in sodium hypochlorite solution (10%) for 10 min and then washed three times with sterile distilled water. Surface sterilized seeds were placed in the Murashige and Skoog (MS) medium containing sucrose (3%) and agar (7%), temperature of 25 ± 2 °C (day/night), a 16-h photoperiod per day, 65% relative humidity and white fluorescent light (46 $\mu mol^{-1}\ m^{-2}\ s^{-1}$) (Murashige and Skoog 1962).

For callus induction, small pieces (1–1.5 cm) of hypocotyls were put on the MS medium culture with different 2,4-dichlorophenoxyacetic acid (2,4-D) (0, 0.5, 1.5 and 2 mg $l^{-1}$) levels and kinetin (Kin) (0, 0.5, 1 and 1.5 mg $l^{-1}$) at pH of 5.7. The cultured samples were placed in darkness at 25 ± 2 °C. Four explants were put on to the Petri dish, with five replicates, and all experiments were performed at least twice independently. The calli were emerged from the hypocotyl explants and were subcultured on MS medium supplemented with the same plant growth regulators after 3 weeks, and callus induction rate, fresh weight, callus index, callus status, and apigenin content were measured. The best friable callus derived from hypocotyl was selected for cell suspension culture. Callus induction rate of 30 replicates per treatment was evaluated based on Zeng et al. (2009).

Callus induction rate (%) = (Total number of explants produced callus / Total number of explants cultured) × 100.

For the determination of the callus index, callus induction rate was multiplied with growth score which was assessed by visual rating (poor = 1, medium = 2, good = 3, and profuse = 4). The mean score was expressed as a growth score (Hazeena and Sulekha 2008).

Cell suspension culture was initiated by transferring 2.5 g of friable-cream calli into 100 ml flasks containing 25 ml of MS liquid medium supplemented with Kin (0.5 mg $l^{-1}$), 2,4-D (1.5 mg $l^{-1}$) and sucrose (30 g $l^{-1}$) at pH 5.7 on a rotary shaker (120 rpm, 25 °C), and kept in darkness. The suspension cultures were subcultured in the fresh MS liquid medium at a two-week interval. The cells were homogenized after 2 months. For determination of cell suspension growth curve, fresh cells (1 g) from stock were separated from the medium by filtration under suction and then, were inoculated into 20 mL of the fresh liquid medium in a 100 mL Erlenmeyer flask. Growth of cell suspension culture and cell viability were measured with sets of flasks harvested at 2 day-intervals in a period of 21 days. Readings were taken

from three flasks for each parameter. Cells were separated from the medium by filtration using nylon mesh and weighed as fresh weight.

## Static magnetic field (SMF) treatment

Exposure to SMF was performed using locally designed homogenous SMF generator (MFG-13971, OFOGH, Iran) with a 220 V DC power supply (MP-6010, MEGATECH, Iran) for producing various SMF intensities in the range of 0.5 µT-15 millitesla (mT) (Mansourkhaki et al. 2019). This system consisted of a copper wire (1.1 mm in diameter), wrapped 1300 rounds around a polyethylene tube in 12 cm diameter and 50 cm length. Containers containing cell suspension culture of *M. chamomilla (*Erlenmeyer flask) were placed in the middle of the tube to get uniform intensity at all points of the container, and magnetic field intensities were measured by the Tesla meter (MG-3002) with a B-probe type of hall sound. The *M. chamomilla* cells in their exponential growth phase (from day 5 to 7 after subcultures) were treated with different intensities of the static magnetic field (0, 2, 4 and 6 mT) for one hour during three days. The cells were harvested for biochemical analyses on day 13 of the subculture. Different intensities of SMF were selected based on some literature studies (Aleman et al. 2014).

## Cell viability

Cells viability was determined by Evan's blue dye with a new method. Cell suspension (500 µL) from each flask was centrifuged at 1000 rpm at 25 °C for 5 min. The supernatant was separated, and 400 µL distilled water was added to the plate and centrifuged again. Then, 80 µL of 0.25% Evan's blue stain for 4 min was added to the tube, centrifuged, and the stain was separated. Finally, 200 µL distilled water was added to the tube and the cells were observed by ECLIPSE Nikon microscope, E-200. At least 550 cells were counted, and the experiment was repeated twice (n = 6). For cell leaching, cells were observed by a Nikon inverted microscope, MA100N after SMF treatment on day 10.

## DPPH radical scavenging

Reducing of 2,2-diphenyl-2-picryl hydrazyl (DPPH) solution was determined based on Patro et al. (2005). The fresh cells (100 mg) were homogenized in 2 ml of 80% methanol and then were centrifuged at 5000 rpm for 5 min. The 30 µL of extract solution was mixed with 1 mL of DPPH (0.1 mM in methanol). The absorbance was read at 517 nm at room temperature after 30 min incubation. The radical scavenging activities were calculated as the percent inhibition of the DPPH radical (%) = $100(A - B)/A$, where $A$ and $B$ are the absorbance of the control and the absorbance of the sample reaction mixture.

## Total phenolic and flavonoid contents

Total phenolic content was determined by a modified Folin-Ciocalteu procedure (Singleton and Rossi 1965). The 100 µL of the diluted extract was added to the 500 µL Folin–Ciocalteu reagent [previously diluted with water 1:9 (v/v)] and 400 µL sodium carbonate (0.7 mol $l^{-1}$) and then were incubated 90 min at room temperature in the dark. The absorbance was measured spectrophotometrically at 765 nm. The total phenolic content was measured by a calibration curve of gallic acid and expressed as per mg gallic acid equivalent.

Flavonoid content was estimated by using the aluminum chloride method with a minor modification (Hatamnia et al. 2014). Briefly, 500 µL of the plant cell extract, 50 µL of sodium nitrate solution (5%), 50 µL of aluminum chloride solution (10%) and 250 µL of sodium hydroxide solution (4%) were mixed and then, samples were put at room temperature for 30 min. The absorbance of the mixture was immediately measured at 415 nm. Total flavonoid content was expressed as mg of rutin equivalents in 1 g FW.

## Determination of apigenin by HPLC

Apigenin was extracted by callus or cell samples (0.5 g of dried samples) in methanol aqueous solution (20 ml), with bath ultrasonic and overnight incubation. The homogenated solution was centrifuged at 5000 rpm and then, the supernatant was collected, dried and dissolved in methanol (500 µL). For the quantitative and qualitative determination of apigenin and derivate in methanol, a HPLC program equipped with a UV–Vis photodiode-array detector (Agilent Technologies 1260 infinity, Santa Clara, CA). The chromatographic separation was obtained by a C18 column (MZ Analysentechnik, Mainz, Germany). The mobile phase (A solvent) consisted of deionized water with 0.1% (*v/v*) phosphoric acid and (B solvent) consisted of acetonitrile. The gradient system consisted of 18% B (0–30 min), 67% B (30–60 min) and 18% B (60–65 min). The flow rate was 1 mL $min^{-1}$at 25 °C, and the injection volume was 20 µL. The flavonoid apigenin was characterized by recording their absorbance peaks at 330 nm. The compounds were identified by comparing its retention time and UV λmax of peaks with those of standards, and its percentage was identified based on its standard calibration curve (Barreca et al. 2016). Peak identity was confirmed by comparing their retention times and absorption spectra with those of pure (> 99%) standards of apigenin and apigenin-7-glucoside from Sigma-Aldrich.

### $H_2O_2$ level

The content of $H_2O_2$ was measured using the method of Velikova et al. (2000). Samples were homogenized in an ice bath with 0.1% (w/v) trichloroacetic acid (5 mL). The homogenate was centrifuged at 12,000 rpm for 15 min and the supernatant (0.5 mL) was added to 10 mM potassium phosphate buffer (0.5 mL, pH 7.0) and 1 M potassium iodide (1 mL). The $H_2O_2$ level was evaluated by comparison of its absorbance at 390 nm with a standard calibration curve.

### Protein content and antioxidant enzyme activity

Soluble protein content was evaluated using the Bradford method (Bradford 1976) and bovine serum albumin as a standard. Fresh cells (0.2 g) were homogenized with 1 M Tris–HCl (pH 6.8) at 4 °C. Then, samples were centrifuged at 10,000 rpm for 15 min at 4 °C. The obtained supernatant was put at − 70 °C and used for enzyme assays.

The activity of superoxiddismutase (SOD; EC 1.15.1.1) was determined by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) as described by Giannopolitis and Ries (1977). The 80 µL of enzyme extract was added to the reaction mixture containing 75 µM NBT, 75 µM riboflavin, 50 mM sodium phosphate buffer (pH 7.5), 0.1 mM EDTA, and 13 mM methionine. The reaction mixture was irradiated for 14 min, and then, absorbance was measured at 560 nm against the non-irradiated blank.

Peroxidase (POX; E.C.1.11.1.7) activity was evaluated using the method of Abeles and Biles (1991). The 100 µL enzyme extract was added to the reaction mixture containing 0.2 mL $H_2O_2$ (3%), 2 mL of acetate buffer (0.2 M, pH 4.8), and 0.1 mL benzidine (40 mM). The absorbance was read at 530 nm.

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was evaluated by the method of Jebara et al. (2005). The 10 µL of enzyme extract was added to the reaction mixture containing $H_2O_2$ (0.1 mM), potassium phosphate buffer (50 mM, pH 7.0) and ascorbic acid (0.5 Mm) in a total volume of 1 mL. The content of oxidized ascorbate was determined at 290 nm, and calculated using the extinction coefficient ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$).

### Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) using SPSS. Duncan's multiple range test was used to determine statistical differences between treatments and control. Each experiment was repeated three to five times at the $P \leq 0.5$ level of confidence.

## Results

### Calli induction

Plant growth regulators are used to induce callus formation and growth in various plant species. In the present work, various concentrations of 2,4-D and Kin were applied in MS medium to detect the optimum concentration of plant growth regulators in hypocotyl explants of *M. chamomilla* (Table 1). Callus formation was successfully induced in all combination of 2,4-D and Kin (Fig. 1). No callus induction obtained in medium without hormone and showed that exogenous hormone was necessary to callus formation of *M. chamomilla.* Most calli were cream in color for 8 days, but some treatments started to get brownish and light green in color after 14 days which happened in treatments with high

**Table 1** Effect of different concentrations of growth regulators on callus induction, fresh weight, callus index and callus status of *M. chamomilla* hypocotyl explants

| Kin (mg $l^{-1}$) | 2,4-D (mg $l^{-1}$) | Callus induction rate (%) | Growth score | Fresh weight (g) | Callus index | Callus status |
|---|---|---|---|---|---|---|
| 0 | 0 | – | – | – | – | – |
| 0.5 | 0.5 | 100 | 3.2 | $0.32 \pm 0.045^{b}$ | 320 | Cream |
| | 1 | 100 | 4 | $0.39 \pm 0.031^{a}$ | 400 | friable and cream |
| | 1.5 | 100 | 3.9 | $0.38 \pm 0.042^{a}$ | 390 | friable and cream |
| 1 | 0.5 | 100 | 2.6 | $0.18 \pm 0.048^{cd}$ | 260 | Cream |
| | 1 | 100 | 2.98 | $0.21 \pm 0.019^{c}$ | 298 | Cream and compact |
| | 1.5 | 100 | 2.8 | $0.19 \pm 0.023^{c}$ | 280 | Brownish and cream |
| 1.5 | 0.5 | 34 | 1.7 | $0.08 \pm 0.003^{e}$ | 60 | Brownish and lightgreen |
| | 1 | 100 | 2.4 | $0.17 \pm 0.032^{cd}$ | 240 | Brownish and light green |
| | 1.5 | 100 | 1.8 | $0.14 \pm 0.039^{d}$ | 180 | Brownish and light green |

All data represent the mean ± SD (n = 5) with different concentrations of 2,4-D and Kin as a treatment. Means sharing the same letter in each column do not differ significantly at $P \leq 0.05$ (Duncan's test)

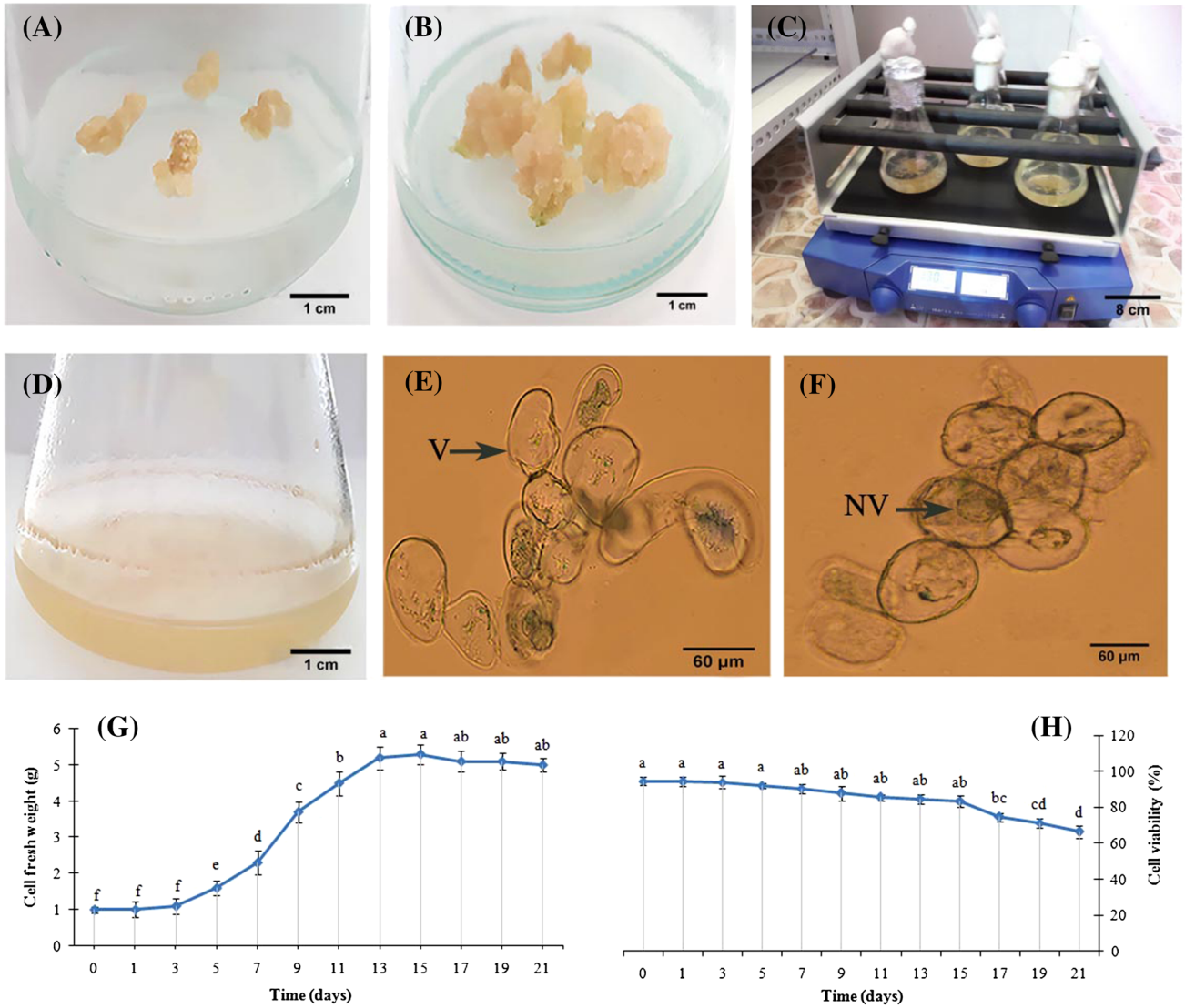


**Fig. 1** Establishment of *M. chamomilla* callus and cell suspension culture. **a** Callus induction (*scale bar* 1 cm), **b** optimization of callus culture (*scale bar* 1 cm), **c** cell culture on a shaker (*scale bar* 8 cm), **d** cell suspension culture (*scale bar* 1 cm), **e** and **f** cells on day 8 and 14 (*scale bars* 60 µm), and also **g** cell fresh weight and **h** cell viability after 21 days. *V* Viable cell, *NV* non-viable cell

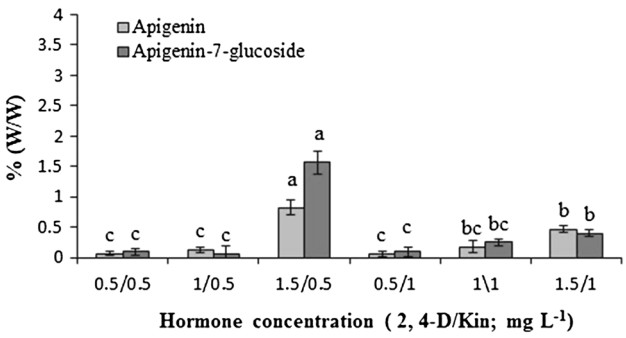


**Fig. 2** Apigenin and apigenin-7-glucoside content of *M. chamomilla* callus tissue under different concentrations of 2,4-D and Kin in MS medium after three weeks of subculture. (2,4-D) 2,4-dichlorophenoxyacetic acid and (Kin) kinetin

Kin concentration (1.5 mg $l^{-1}$). The lowest concentration of Kin (0.5 mg $l^{-1}$) showed the optimum concentration for *M. chamomilla* callus induction. Kin at 0.5 mg $l^{-1}$combined with 2,4-D (1 and 1.5 mg $l^{-1}$) show the highest fresh weight, fragile and cream calli in color and callus index of 390–400 after 3 weeks.

## Calli apigenin content

The content of apigenin and apigenin-7-glucoside were various between different levels of 2,4-D and Kin. A trace amount of apigenin was defined at an equal concentration of 2,4-D/Kin, and with raise of 2,4-D concentration, apigenin production increased in the *M. chamomilla* calli (Fig. 2). The

highest amount of apigenin (82%) and apigenin-7-glucoside (1.57%) were detected on the treatment of 2,4-D (1.5 mg $l^{-1}$) and Kin (0.5 mg $l^{-1}$). This treatment was selected for cell suspension culture and SMF treatment.

## Cell suspension culture, viability and SMF treatment

Friable and cream callus clumps with optimum growth and apigenin content were used for starting the cell suspension culture. Cell growth was evaluated in liquid MS medium supplemented with 2,4-D (1.5 mg $l^{-1}$) and Kin (0.5 mg $l^{-1}$) by measuring the fresh weight of the cells every 2 days (Fig. 1). The growth curve of suspension cultures showed that the growth rate was primarily slow during the first 3 days (lag phase). However, as the cultures continued it presented a noticeable rise from day 7 and accumulated a great amount of fresh weight over for 13 days (exponential phase) (Fig. 1g). Maximum growth in fresh weight was obtained on days 13 and 15 which was about fivefold over the primary fresh weight. The speed of growth was steady for 5 days (stationary phase). The stationary phase was followed by a decrease in cell growth (Fig. 1g). According to the growth curve, the time for subculturing to new fresh media was between days 13–15 of incubation, the finish of the exponential growth phase. Moreover, the cell viability as shown in Fig. 4b was around 84.5% throughout the 13 days of culture. Two kinds of cells (round and elongated shape) were identified in the cell suspension (Fig. 1e and f). Commonly in durations longer than 14 days (exponential phase), the number of round-shaped cells in the culture was augmented.

Different intensities of SMF changed the cell fresh weight, viability, and morphology of *M. chamomilla*. Observation of cells using an inverted microscope on day 10 of subculture appeared cell leaching with more round shape at 6 mT as compared to control. Cell fresh weight increased at 4 mT, but decreased at 2 and 6 mT SMF (Fig. 3a). Cell viability under SMF decreased as comparison to control, but there was no significant difference between treatments (Fig. 3b). The lowest cell viability was identified at 6 mT (71%) on day 13 of culture.

## Protein content

Cells treated to SMF showed different protein values under SMF (Fig. 3c). Protein content slightly decreased at 2 mT, and then significantly increased at 4 and 6 mT. The maximum protein content was observed at 4 mT with a 41.9% increase as compared to control.

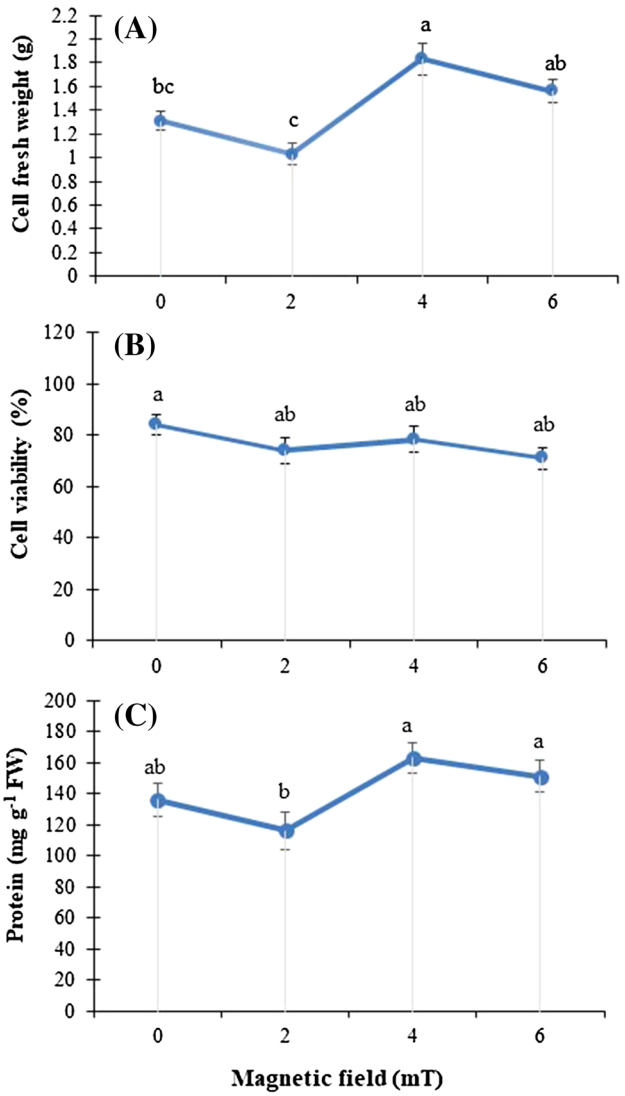


**Fig. 3** Effect of SMF at different intensities on *M. chamomilla* cell viability on day 7 of subculture (**a**), cell fresh weight (**b**) and protein content (**c**) on day 13 of subculture

## $H_2O_2$ level and antioxidant enzyme activity

The $H_2O_2$ level of SMF treated cells was more content than the control group. The maximum $H_2O_2$ content was witnessed at 6 mT (16.1 µmol $g^{-1}$ FW) (Fig. 4). The activities of antioxidant enzymes involved in reactive oxygen species (ROS) detoxification induced under different SMF treatments as compared to control. SOD as a scavenging enzyme of superoxide radicals increased with increasing of SMF intensity, and the highest activity was observed at 6 mT. POX and APX activities performed scavenging $H_2O_2$ in the cells were significantly increased at 4 and 6 mT, and their maximum activities were identified at 4 mT. The activity of antioxidant enzymes was similar to DPPH activity under SMF.

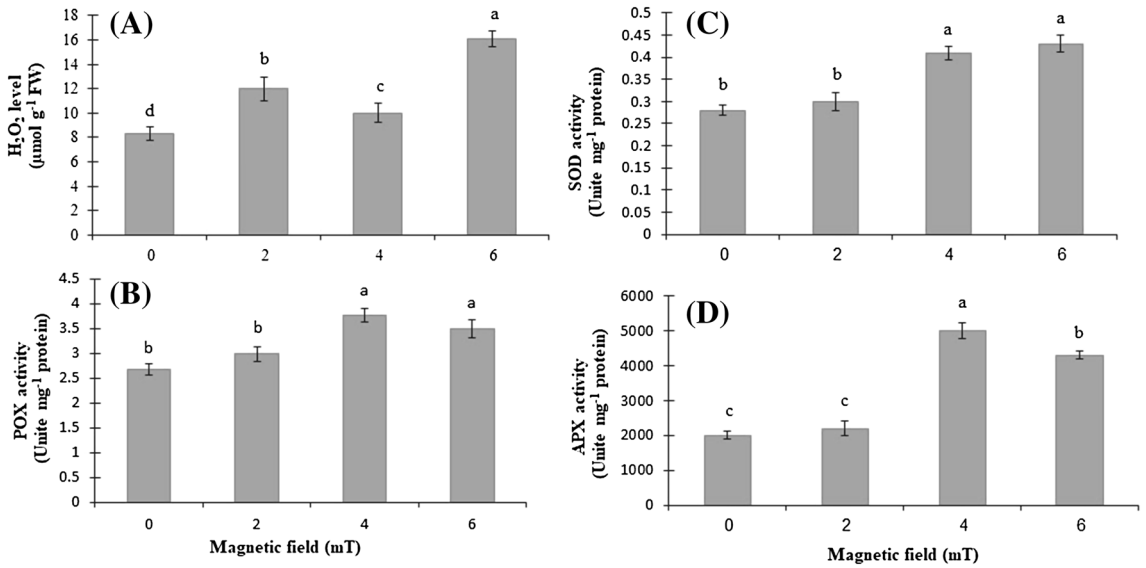


**Fig. 4** Effect of the SMF at different intensities on the **a** hydrogen peroxide ($H_2O_2$) level, **b** peroxidase (POX), **c** superoxide dismutase (SOD) and **d** ascorbate peroxidase (APX) activities of *M. chamomilla* cell suspension culture

**Table 2** Effect of different intensities of SMF on phenol, flavonoid contents and DPPH radical scavenging of *M. chamomilla* cell suspension culture

| SMF | Total phenol (mg GAE $g^{-1}$ DW) | Total flavonoid (mg GAE $g^{-1}$ DW) | DPPH scavenging activity (%) |
|---|---|---|---|
| 0 | $5.54 \pm 0.24^{c}$ | $2.71 \pm 0.10^{b}$ | $55.1 \pm 4.25^{c}$ |
| 2 | $8.01 \pm 0.39^{b}$ | $2.82 \pm 0.14^{b}$ | $59.65 \pm 3.19^{bc}$ |
| 4 | $9.51 \pm 0.29^{a}$ | $3.41 \pm 0.12^{a}$ | $76.72 \pm 2.78^{a}$ |
| 6 | $8.53 \pm 0.27^{b}$ | $3.54 \pm 0.17^{a}$ | $68.6 \pm 2.26^{b}$ |

All data represent the mean ± SD (n = 3) with different intensities of SMF. Means sharing the same letter in each column do not differ significantly at $P \leq 0.05$ (Duncan's test)

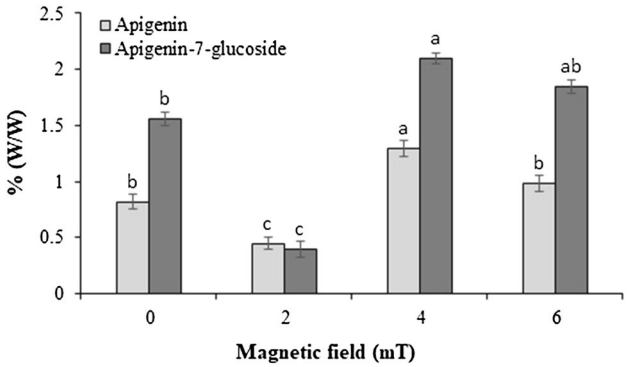


**Fig. 5** Effect of the SMF at the different intensities on apigenin and apigenin-7-glucoside of *M. chamomilla* cell suspension culture on day 13 of subculture

## DPPH radical scavenging

Overall, all treatments considerably improved the activity of DPPH relative to the control (Table 2). Cells treated with 4 and 6 mT SMF showed the highest value of the DPPH radical scavenging about 68 and 76%, respectively.

## Phenol, flavonoid and apigenin contents

Total phenolic and flavonoid contents of the *M. chamomilla* cells that were exposed to 0, 2, 4 and 6 mT significantly increased under SMF (Table 2). Cells treated with 4 mT displayed the highest phenolic content ($9.51 \pm 0.21$ mg GAE $g^{-1}$ DW) which showed a 1.73 fold rise as compared to control.

Cell responses to flavonoid production were various between different SMF treatments. Flavonoid content slightly decreased at 2 mT, but increased its content at 4 and 6 mT as compared to control. The highest value of total flavonoid content (3.54 mg RE $g^{-1}$ DW) was witnessed at 6 mT which showed a 1.3 fold increase compared to control (Table 2).

Treated cell to 4 and 6 mT showed a significant increase in apigenin and apigenin-7-glucoside as comparison to control (Fig. 5). The maximum apigenin (1.36%) and apigenin-7-glucoside (2.14%) induced in cell treated to 4 mT.

## Discussion

Growth regulators are essential for callus induction and stimulate cell lines growth. Various plant species require various phytohormones for callus formation and metabolites generation. Therefore, it is vital to choose the suitable growth regulators and identification of their optimal levels. Other investigators have performed callus induction in *M. chamomilla* previously. Sayadi et al. (2014) presented that the maximum callus formation was obtained on MS medium contained NAA (1 mg $l^{-1}$), Kin (1 mg $l^{-1}$) from axillary buds (89.68%), leaf (93.26%), and stem discs (80.75%) explants. In this study, callus formation successfully happened from hypocotyls explants, and the optimum levels of growth regulators were obtained at Kin (0.5 mg $l^{-1}$) and 2,4-D (1.5 mg $l^{-1}$) with maximum callus induction (100%), fresh weight, cream and friable calli (Table 1). 2,4-D causes the differentiation, elongation and cell division and belongs to phenoxy acetic acid herbicides. It commonly used for callus induction (Venkov et al. 2000). Cytokinin influences on the formation and function of mitotic apparatus and stimulation of cell division by regulation of proteins synthesis (Chawla 2002; George et al. 2008). In this study, the upper concentration of Kin (1.5 mg $l^{-1}$) changed the color from cream to green and brownish after 2 weeks (Table 1) which may be correlated to the effect of the high level of Kin on chlorophyll synthesis (George et al. 2008). Our data showed that exogenous hormone was necessary for callus induction of *M. pulegium*, and callus was not formed on basal MS medium. Several works showed that the formation of callus did not occur on MS medium without hormones (Mathur and Shekhawat 2013; Ray et al. 2011). Among the leaf, shoot and hypocotyl explants used, hypocotyl was more responsive for callus formation as a comparison to leaf and shoot explants (data not published).

Optimization of callus induction and cell suspension culture is the first stage to create the high contents of bioactive metabolites. Our results point out that MS medium with Kin (0.5 mg $l^{-1}$) and 2,4-D (1.5 mg $l^{-1}$) showed the maximum percentage of callus formation, callus growth and bioactive compounds including apigenin (0.82% w/w) and apigenin-7-glucoside (1.57% w/w) contents in *M. chamomilla* calli (Fig. 2), and it can be selected for cell suspension culture. Apigenin content in *M. chamomilla* has been previously reported in wild and in vitro plants, but not in callus tissue and cell suspension culture. Apigenin-7-glucoside content was 72.96 mg $kg^{-1}$ and 13.44 mg $kg^{-1}$ in in vivo and in vitro conditions in *M. chamomilla*, respectively (Banaz 2018). Haghi et al. (2014) presented the content of apigenin [0.74% (w/w)] and apigenin-7-glucoside [1.11% (w/w)] in wild *M. chamomilla* aerial part. There is a lack information about apigenin content in *M. chamomilla* callus. However, some reports have shown the impact of plant growth regulators on secondary compounds generation in callus tissues. Combination of 2,4-D and BAP increased saponin (4.8 mg $g^{-1}$ DW) content in *Centella asiatica* calli (Rao et al. 2015). Çolgecen et al. (2018) reported that 2,4-D and BAP induced aucubin (3.06 mg $kg^{-1}$) and verbascoside (0.74 mg $kg^{-1}$) in *Camptotheca acuminate*. Types and concentrations of plant growth regulators influence the in vitro creation of secondary compounds in medicinal plants (Palacio et al. 2012). Application of 2,4-D can induce some genes encoding stress, defense proteins, and also changes hormone hemostasis in the plant cells (Pazmiño et al. 2012). It should mention that the increase of apigenin and apigenin-7-glucoside might relate with the influence of a high level of 2,4-D on enzyme activities from flavonoid biosynthetic pathways.

Cell growth measurements of *M. chamomilla* showed the progression of cell growth over a 21-day culture period, and the subculture for new fresh media was performed between days 13 and 15 (Fig. 1g). The typical sigmoidal growth curve consists of three distinct phases including the lag phase, exponential phase, and stationary phase. The cells at the termination of the exponential stage are reliable to subculture, and after the logarithmic growth phase, the nutrients become limited or/and toxic materials are created by cells and increase the cell death (Stafford and Warren 1991; Bhojwani and Razdan 1983). In this study, cell viability reached to 84.5% throughout the 13 days of culture and then decreased to 66% after 21 days (Fig. 1h). The 50% cell viability considered that the suspension culture creation has unsuccessful (Qui et al. 2009). Our findings showed that the cell shapes in liquid medium changed from length to round during the culture (Fig. 1e and f). Changes of cell shape and size can relate to the production of secondary metabolites in medicinal plant cell suspension cultures (Forni et al. 1998). Based on our findings, cells treated to SMF showed various responses to growth and protein content. In contrast to 4 mT treatment, SMF at 2 and 6 mT decreased the fresh weight and protein content (Fig. 3a and c). Also, cell viability didn't show a significant response between different SMF intensities on day 7 of the subculture (Fig. 3b), while cell leaching induced with the increase of SMF intensity especially at 6 mT SMF on day 10 of subculture. It seems that SMF can induce ROS production and oxidative stress in cells, and following may decrease reaching time to stationary phase with more round shape cells especially at 6 mT (Fig. 3a and b). The first reaction of the cell to MF is varying the electrical conduct and permeability of cell membrane (Zhang et al. 2015). Moreover, MF affects the spins of paramagnetic molecules in cells and induces formation intracellular paramagnetic ROS (Durmuset al. 2015). So, cell proliferation and growth are affecting by SMF. However, the various impacts of SMF on cell proliferation and growth can be related to

cell type, MF intensity, cell density and plant species (Zhang et al. 2017).

Plant cells treated to MF were stimulated to the formation of ROS and oxidative damage (Maffei 2014; Payez et al. 2013; Sahebjamei et al. 2007). ROS can influence gene expression, cellular membrane integrity, and activity of enzymes (Serrano et al. 1999; Jithesh et al. 2006). Plant cells comprise various types of antioxidant enzymes that detoxify or inhibit the production of the destructive ROS, which maintain cells against oxidative stress (Matamoros et al. 2003). SOD, POX, APX, and CAT are commonly considered as the main components of the antioxidant mechanism of the plants (Xue et al. 2008). APX and CAT decompose $H_2O_2$ into oxygen and water. POX detoxifies $H_2O_2$ by oxidation of compounds include phenolic compounds and/or antioxidants (Apel and Hirt 2004; Demir and Kocaliskan 2001).

Based on our findings, SMF through excessive production of ROS such as $H_2O_2$ induced oxidative stress in SMF treated cells (Fig. 4a) and defense mechanisms for suppression of the harmful effects of ROS including SOD, POX and APX enzymes markedly increased in *M. chamomilla* cells. The maximum activity of POX and APX was identified at 4 mT, whereas SOD activity was more at 4 and 6 mT. It has also been previously shown that oxidative stress and some scavenging enzymatic mechanisms induced by MF treatment in suspension-cultured plant cells (Rajabbeigi et al. 2013; Çelik et al. 2009). It seems that SMF at especial intensity (4 mT) could protect *M. chamomilla* cells of oxidative damage by more induction of antioxidative enzymes, and promote cell growth.

The activity of DPPH-radical scavenging is one of the simplest manners used for the detection of antioxidant ability of plant cells in the short time (Khan et al. 2013). In this work, the DPPH activity markedly induced under different SMF intensities. Increased DPPH activity may be due to more accumulation of phenolic compounds in cell treated to SMF. Sharma and Ramawat (2013) proved a positive correlation between established DPPH concentration and phenolics in *Salvadora persic* callus tissue.

Our study indicated that SMF treatments at 4 and 6 mT increased the contents of flavonoid, total phenolic compounds and apigenin against the oxidative hurt induced by SMF (Table 2, Fig. 5). The positive correlation between the metabolism of secondary metabolites and defense processes has been presented in the other researches (Çelik et al. 2009; Maffei 2014). The oxidative stress induced by SMF can perform like an endogenous sign on increasing the defense mechanism of plants via alternation in bioactive compounds including phenol and flavonoid compounds which have multiple roles in plant cells, such as removing free radicals, inhibiting enzymatic systems producing free radicals, inducing the gene expression responsible for inhibition of oxidative damage, and enhancing the antioxidant enzymes activity. It has been published that various environmental stresses can improve the content of these metabolites in plants (Taghizadeh et al. 2019; Primiano et al. 1997; Złotek et al. 2014).

## Conclusion

In conclusion, this study is the first study about the formation of callus tissue and cell suspension culture of *M. chamomilla*, and identification of apigenin as an important flavonoid under different growth regulators and SMF treatments. The best callus growth and cell suspension culture, as well as the highest apigenin production were identified in MS medium with 2,4-D (1.5 mg $l^{-1}$) and Kin (0.5 mg $l^{-1}$). Overall, SMF-treated cells show an increase of total phenolics, flavonoids, and $H_2O_2$ levels. SMF at the higher level increase the cell leaching and decrease cell growth. The result of this study provides a new and efficient way to cell suspension culture and bioactive compounds production in *M. chamomilla*, and SMF can be a suitable tool for induction of antioxidative compounds in cell suspension culture.

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**Author contributions** HH and VN designed the project and discussed the results. HH participated in the bench experiments, and organized the manuscript.

### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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