




The Deoxymiroestrol and Isoflavonoid Production and Their Elicitation of Cell Suspension Cultures of *Pueraria candollei* var. *mirifica*: from Shake Flask to Bioreactor

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Abstract

To address the high demand for *Pueraria candollei* var. *mirifica* (PM) used as the active ingredient in health products and its difficulty to cultivate in the field, the growth and production of deoxymiroestrol (DME) and isoflavonoid (ISF) phytoestrogens in PM cell suspensions were studied. In a 125-mL shake flask, the cell suspension produced DME [78.7 ± 8.79 – 116 ± 18.2 $\mu\text{g/g}$ dry weight (DW)] and ISF (140 ± 6.83 – 548 ± 18.5 $\mu\text{g/g}$ DW), which are the predominant ISF glycosides. While ISF aglycones accumulated in the PM cell suspension cultured in the airlift bioreactor. The DME content was increased to 976 ± 79.6 $\mu\text{g/g}$ DW when the PM cell suspension was cultured in the 5-L scale bioreactor. The production of DME and ISF was enhanced by elicitors including methyl jasmonate (MJ), yeast extract (YE), and chitosan (CHI). MJ produced the highest induction of DME accumulation, while ISF accumulation was the highest with YE treatment. Analysis of catalase activity implied that the elicitors enhanced ROS production, which resulted in the enhancement of DME and ISF production and accumulation in PM cell suspension cultures. PM cell suspension culture is a promising source of beneficial PM phytoestrogens that exhibit bioactivity that may useful for the treatment of menopausal symptoms.

Keywords *Pueraria candollei* var. *mirifica* · Cell suspension · Bioreactor · Deoxymiroestrol · Isoflavonoid

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Introduction

White Kwao Krua (*Pueraria candollei* var. *mirifica*, PM) is a woody climbing plant that belongs to the Leguminosae family. Its root has been recognized as a rejuvenating herb, and several studies have demonstrated its estrogenic potency, which allows it to influence the prevention of bone loss and the reduction of postmenopausal symptoms [1]. Its anti-oxidant, anti-collagenase, and anti-elastase properties have also been noted [2, 3]. These pharmacological effects are due to the presence of major isoflavonoid (ISF) phytoestrogens, including puerarin (PUE), daidzein (DZE), daidzin (DZ), genistein (GTE), genistin (GT), kwakhurin (KWA), and mirificin, as well as minor, but potent estrogenic chromenes, including isomiroestrol, miroestrol, miroestrol glycoside, and deoxymiroestrol (DME). Coumestans, sterols, and pterocarpanes have also been found in this plant. The phytochemicals in PM have been demonstrated to have beneficial effects in preclinical and clinical trials and have been shown to be useful as health-promoting nutraceutical active agents, especially for older women suffering from estrogen deficiency. Within the aging world population, a large proportion (60–80%) of women are affected by vasomotor symptoms (VMS) during the menopausal transition [4], including hot flashes, night sweats, and other complications, that reduce quality of life. The use of hormone replacement for menopausal symptom treatment has been associated with an increased risk of various cancers [5] and cardiovascular disease [6]. The nutraceutical products of PM are attractive and beneficial for menopausal symptom alleviation. In addition, PM-phytoestrogens can be utilized as active compounds for cosmeceutical products that can be used to prevent skin aging.

The quantity and quality of PM phytochemicals were shown to be altered depending on the location of cultivation [7, 8] and the season of harvesting [9]. It is difficult to control these factors during field cultivation. Therefore, the phytoestrogen contents of *P. candollei* were dramatically varied. In addition, *P. candollei* cultivation in the field introduces the possibility of heavy metal, pesticide, herbicide, and pathogenic microbial contamination. Moreover, the supply of *P. candollei* is subject to shortages because the process of field cultivation requires at least three years. A sustainable and green source such as plant tissue culture would be useful for the production of important phytochemicals. Such techniques are a more efficient approach when compared with conventional cultivation due to the high and continuously production of plant secondary metabolites that is possible in the controllable conditions of plant tissue culture. Many techniques have been developed for further improvement of bioactive compound accumulation in plant tissue cultures, such as elicitation, immobilization, permeabilization, and genetic modification.

Elicitation is a simple and popular technique for enhancing plant secondary metabolite production. In addition, it does not require a genetic modification process. Stress conditions induced by elicitor(s) lead to the excessive production of ROS, such as O_2^- , H_2O_2 , and OH^\bullet , which cause oxidative damage and serve as signaling molecules. The oxidative burst also modulates plant defensive responses via secondary metabolite production. During the elicitation process, several nonenzymatic substances (ascorbate, glutathione, carotenoids) and enzymatic anti-oxidants are produced that scavenge and balance the effects of the ROSs [10]. Anti-oxidant enzymes such as catalase (CAT), peroxidase, and superoxide dismutase were produced in response to elicitor-mediated ROS production [11]. Therefore, the analysis of CAT activity can be used indirectly to monitor the effects of elicitors on plant cell responses.

Cell suspension culture in a bioreactor is suitable for the industrial-scale production of phytochemicals. The use of this system is highly effective for the production of vinblastine,

vincristine, paclitaxel, camptothecin, and podophyllotoxin, for which there is high demand that cannot be supplied by natural resources alone. The production of these compounds is high when using a bioreactor. Such a system is a sustainable source of phytochemicals, especially those obtained from plants requiring long cultivation, and it is appropriate for compounds for which synthetic approaches are not fully applicable.

Cell suspension culture of *P. candollei* has been previously reported. The cultured cells were reported to produce ISF, including PUE, DZ, GTE, DZE, and GTE [12, 13]. Interestingly, DME, which is potently estrogenic, was also produced by callus and cell suspension cultures derived from PM [14]. These results demonstrated that cell suspension culture of PM could serve as a useful approach. However, elicitation treatment has not yet been proven to be useful in enhancing DME accumulation in cell suspension cultures. The culturing of PM cell suspensions at the bioreactor scale has also not been well established. In this study, we generated a cell suspension culture of *P. candollei* and established an elicitation method. Moreover, the culturing of the cell suspension in the bioreactor improved the production of DME and ISF aglycones when compared with that obtained from shake flask culture.

Materials and Methods

Chemical and Immunological Reagents

Specifications of reagents used following experiments were explained in the files of supplementary information.

Callus Induction and Cell Suspension Establishment

The PM seeds were germinated in hormone-free Murashige and Skoog (MS) medium. Then, the stems of the plantlets were cut and placed on solidified MS medium containing 0.1 mg/L TDZ, 1 mg/L NAA, and 0.5 mg/L BA (MS-TNB) for callus induction. The PM cell suspension was established and maintained in the MS-TNB.

Effect of Inoculum Density on the Growth and Phytoestrogen Production of the Cell Suspension Culture

The inoculum density of the cell suspension was investigated at 1, 3, 6, and 9 g fresh weight (FW) of the initial cell mass, which was added to 30 mL MS-TNB contained in a 125-mL shake flask. The optimum inoculum density, i.e., that which produced the highest growth index (GI), DME, and ISF production, was used for further experiments. The growth index (GI) is a relative estimation of growth capacity that reflects the relationship of the biomass data at the sampling time to the inoculated cell density. The GI of the PM cell suspension was calculated using the following equation [15]:

$$GI = \frac{WF - W0}{WF}$$

where WF and W0 represent the final mass and initial mass, respectively.

Growth Rate and Phytoestrogen Production in Cell Suspension Culture

To investigate the growth pattern versus time course of the PM cell suspension culture, the cell suspension (3 g) was subcultured into fresh MS-TNB (30 mL). Three flasks of the cell suspension were then collected every week for 35 days after the culturing. The fresh and dry weights of the cell suspension were recorded. The phytoestrogen content of the dried PM cells was determined. The growth rate of the PM cell suspension was calculated using the following equation:

$$\mu = \frac{\ln X - \ln X_0}{t}$$

where X_0 represents the initial biomass, X represents the biomass at time t , and μ represents the specific growth rate. The specific growth rate (μ), which is reflected by the steepness of the growth curve, is the rate of the increase in biomass of a cell population per unit of biomass concentration [15].

Elicitation in Cell Suspension

Methyl jasmonate (MJ) was prepared as a 10 mM stock solution in 40% (v/v) ethanol. It was then sterilized via filtration under aseptic conditions. A YE stock solution at a concentration of 100 mg/mL was prepared in deionized water. The CHI stock solution was initially dissolved in glacial acetic acid with gentle heating at 60 °C for 15 min. After a clear solution of CHI was obtained, the pH of the solution was adjusted to pH 5.5, and the volume was adjusted to obtain a 10 mg/mL stock solution. Both the YE and CHI stock solutions were sterilized via autoclaving. The 18-day-old cell suspensions were treated with various concentrations of elicitors: 50–200 μ M MJ (MJ50, MJ100, and MJ200), 0.5–2 mg/mL YE (YE0.5, YE1, and YE2), and 50–200 mg/L CHI (CHI50, CHI100, and CHI200). All treatments were performed in triplicate. After the cells were exposed to the elicitors for one (1D), three (3D), six (6D), nine (9D), or 12 (12D) days, they were collected and dried. The ISF and DME contents were then determined.

Bioreactor

The PM cell suspension was scaled up in a large shake flask (2 L) and a bioreactor (5 L) using the optimized conditions. An FS-01-C series airlifter fermenter model, comprising a vessel (5 L) and a controller, was used. The cell suspension was maintained in MS-TNB medium with aeration at 1 LPM (L/min) at 25 °C in a 16 h light/day cycle. The growth rate of the cell suspension and the production of DME and ISF in the fermenter were measured. Cells were collected after 10, 20, 30, and 50 days of culture to evaluate growth over the time course. The ISF and DME contents in the collected cells were analyzed.

The production of ISF and DME was compared in the shake flasks (50 g of initial weight in 500 mL medium) and the bioreactors (200 g of initial weight in 2000 mL of medium). After 30 days of culture, the cell suspension was collected and dried. Subsequently, the ISF and DME contents were determined.

Extraction and Analyses of ISF and DME

Dried and powdered PM cell samples were extracted with 1 mL ethanol during sonication at room temperature. The clear extract was collected after centrifugation. The cell residue extraction was then repeated 3 times. The products of these extractions were combined, evaporated, and then re-dissolved in 1 mL ethanol. The ISF and DME contents in these extract solutions were determined by gradient HPLC [16] and indirect competitive ELISA [17], respectively. The analysis procedures were performed exactly as described in our previous report [16, 17]. The method was detailed in the files of supplementary information.

Determination of CAT Enzyme Activity

The crude protein in the PM cell suspension was extracted, and then the CAT activity was determined via the stable complexation between hydrogen peroxide and ammonium molybdate [18]. The protocol of the analysis was briefly described in the file of supplementary information.

Statistical Analysis

The values are expressed as the mean±SD. The level of significance was set at $p < 0.05$; one-way analysis of variance (ANOVA), followed by Tukey's HSD test, was used to determine the differences between the two groups.

Results and Discussion

Callus Induction and Cell Suspension Establishment

The PM callus was successfully dedifferentiated from the stems of *in vitro* plantlets. The semisolid MS-TNB medium was used to produce compact and green calluses. Subsequently, the PM cell suspension was obtained when the calluses were agitated in MS-TNB liquid medium. The cell suspension was green, friable, and exhibited rapid growth, as previously reported [14]. The results suggested that the use of MS-TNB medium produced PM cell suspensions with high capacities for DME production (65.0 ± 6.56 – 161 ± 1.40 $\mu\text{g/g DW}$) and total ISF production (103 ± 0.57 – 149 ± 1.01 $\mu\text{g/g DW}$) at various inoculum cell densities (Table S2). Previously, there were attempts to establish callus and cell suspension cultures of *P. candollei* that produced high levels of phytoestrogens. These reports indicated that MS medium containing a combination of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (KN) induced a callus that produced 34-fold more DZE (5.12 ± 0.04 mg/g DW) and GTE (2.77 ± 0.10 mg/g DW) than intact PM [19]. When the cell suspension was maintained in the modified MS medium containing only 2,4-D, the daidzein content (1.8 mg/g DW) was low [12]. The highest total ISF content (23.82 mg/g DW) was obtained from a stem-derived cell suspension that was maintained in MS medium containing a combination of 6-benzyladenine (BA, 0.56 μM) and 2,4-D (4.52 μM) [13]. The aforementioned reports did not investigate the production of DME, which is the most potent of the PM-derived phytoestrogens. The PM cell suspension that was initiated and maintained in MS-TNB produced high amounts of ISF (14.96 ± 2.82 mg/g DW) and DME (81.26 ± 15.09 $\mu\text{g/g DW}$) [14]. Therefore, the PM cell

culture established using the aforementioned combination of plant growth regulators is a promising system. During the current study of PM callus and cell suspensions, however, the production of ISF was much lower, which may have resulted from genetic variation in the PM seedlings. Overall, MS-TNB medium was shown to be suitable for PM callus initiation and culture for the purposes of phytoestrogen production.

Effect of PM Cell Inoculation Density on Growth and Phytoestrogen Production

The effects of cell density on growth and secondary metabolite production were investigated at densities of 1, 3, 6, and 9 g/flask of initial cell mass. The results showed that a lower initial cell density produced a higher GI (Table S1), which indicated a higher rate of increase in biomass compared with the initial biomass. However, the final cell masses were increased as the masses of the initial cell densities were increased. It is likely due to increases in the availability of nutrients and the aeration of the cultures. The higher initial cell density limited the available materials, which led to a lower GI. The initial inoculum density of the PM cells also influences the accumulation of secondary metabolites (Fig. 1, Table S2). The highest DME production (161 $\mu\text{g/g}$ DW) was achieved at 3 g/flask of initial cell mass. The PM cell suspension derived from all initial cell mass cultures produced amounts of ISF in the range of 103 ± 0.57 – 149 ± 1.01 $\mu\text{g/g}$ DW. When the inoculum cell masses were increased, DZ and GT accumulation were decreased, while the accumulation of DZE and GTE in their aglycone forms was increased. However, a small amount of PUE and KWA could not be detected. Therefore, 3 g/flask of initial cell mass was optimum for DME and ISF production (DZ, 60.5 $\mu\text{g/g}$ DW; DZE, 45.2 $\mu\text{g/g}$ DW; GT, 27.6 $\mu\text{g/g}$ DW; GTE, 10.3 $\mu\text{g/g}$ DW). Previous investigation, it has been demonstrated that paclitaxel production in *Taxus yunnanensis* cell cultures was increased when

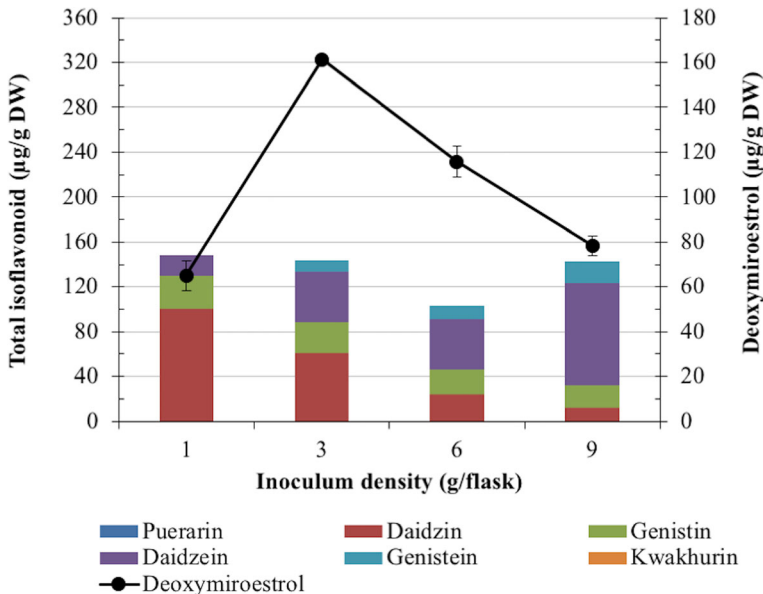


Fig. 1 Effect of PM cell inoculum density on the production of isoflavonoids and deoxymiroestrol. Initial cell masses of 1–9 g per 30 mL of medium were cultured for 35 days. The final masses and secondary metabolites were measured

the inoculum density was increased [20]. The same result was also observed during the production of jaceosidin in a *Saussurea medusa* cell suspension [21]. However, an inoculum cell density that is too high suppresses secondary metabolite production in these cell suspension cultures [20, 21]. Therefore, the optimal inoculum cell density must be determined for each plant cell culture.

Growth Rate, Secondary Metabolite Production, and CAT of the PM Cell Suspension Culture

The growth rate of the PM cell suspension culture gradually increased during the first 2 weeks. From the 2nd to the 3rd week, the cell growth increased rapidly. Then, the growth reached the deceleration phase in the 4th–5th week (Table S3). The production of secondary metabolites reached the highest level during the second week and then tended to decrease in long-term cultures (Fig. 2). The highest accumulation of compounds was observed during the 2nd week for DME ($116 \pm 18.2 \mu\text{g/g DW}$), PUE ($13.7 \pm 0.39 \mu\text{g/g DW}$), DZ ($260 \pm 12.8 \mu\text{g/g DW}$), GT ($246 \pm 2.34 \mu\text{g/g DW}$), DZE ($12.9 \pm 1.68 \mu\text{g/g DW}$), and GTE ($15.6 \pm 1.38 \mu\text{g/g DW}$). A PM cell suspension was found to produce the major ISF glycosides DZ and GT. DZ and GT were also found to be the predominant compounds in *P. candollei* var. *candollei* and PM cell suspension cultures that were reported previously [14, 22]. However, a small amount of KWA could not be detected in the PM cell suspension culture. Eighteen days of cell suspension culture (deceleration phase) was used for the elicitation of secondary metabolism. At this stage of growth, plant cells are in the optimal condition for secondary metabolism.

The CAT activity of the PM cell suspension culture was monitored over 5 weeks of growth (Table S4). The CAT activity peaked during the 2nd week and decreased during the 3rd week. However, the CAT activity again increased during prolonged culture. In plant cells, CAT is produced to compensate for radical production, which can damage cellular molecules. CAT is an anti-oxidant enzyme that plays an important role in scavenging ROSs produced during stress conditions [23, 24]. The production of ROSs is related to the modulation of secondary metabolite production. This implies that the peaking of the production of free radicals during the 2nd week induced secondary metabolite production, during which DME and ISF production was observed to be at its highest.

Effect of MJ on the Production of DME, ISF, and CAT in PM Cell Suspension Culture

All MJ treatments enhance DME and ISF production, and the enhancement of production is dose-dependent with equal MJ exposure time (Fig. 3, Table S5). The greatest enhancement in DME production was obtained when using MJ200 with three days of elicitation, which produced $282 \pm 16.3 \mu\text{g/g DW}$ DME (4-fold higher than that of the control). Of the different MJ treatments, MJ200 produced the highest total ISF accumulation ($417 \pm 8.42 \mu\text{g/g DW}$) in the PM cell suspension (two-fold higher than that of the control) on the 1st day after exposure. The enhancement of ISF accumulation by MJ in PM cell suspension culture has been previously reported [22]. The activity of the ISF biosynthetic pathway is increased effectively by MJ treatment. Exogenous MJ treatment was shown to increase intracellular ROS production [25]. These changes modulated the expression of biosynthetic enzymes, such as phenylalanine ammonia lyase, naringenin-chalcone synthase, isoflavone synthase, and chalcone isomerase [26, 27]. MJ treatment also increased ISF accumulation in legume

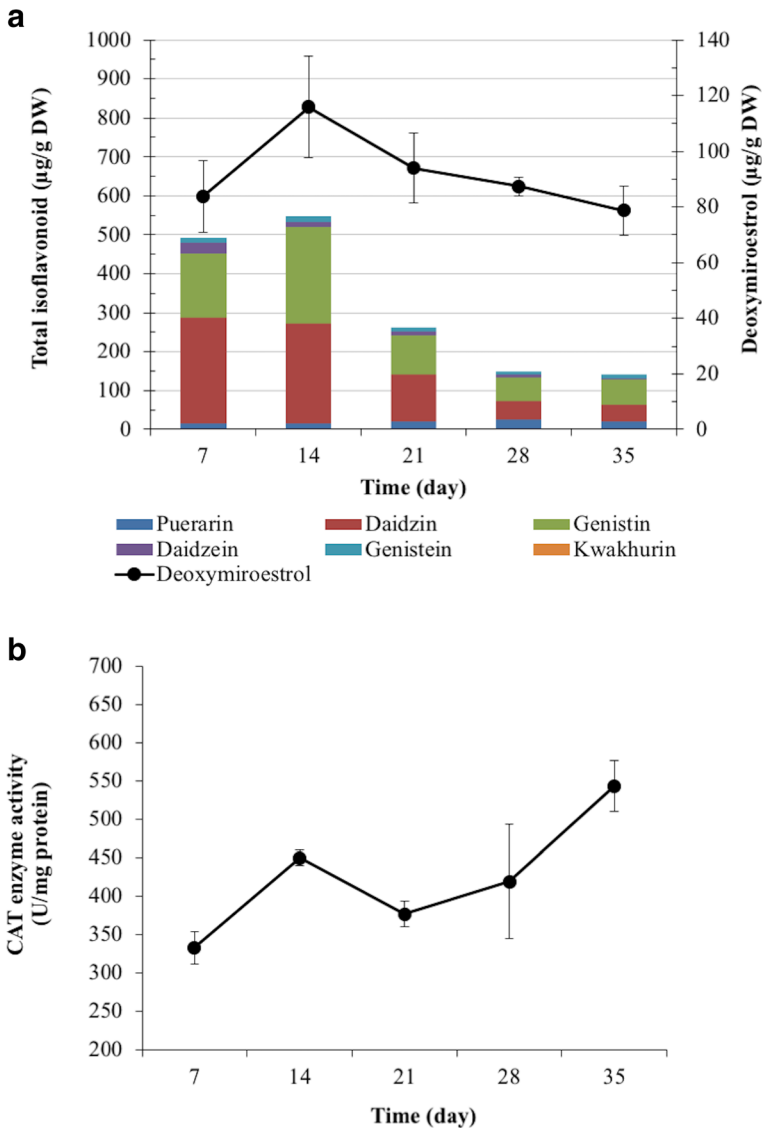


Fig. 2 Production of deoxymiroestrol and isoflavonoids using PM cell suspension culture over the time course (a), and CAT enzyme activity in PM cells during the same culture period (b). The PM cells (3 g) were cultured in 30 mL of medium and then collected every week over a 35-day culture period. The accumulation of secondary metabolites and CAT enzyme activity were determined

plants, including *Glycine max* (soybean) [27], *Pueraria montana* (kudzu) [28], and *Psoralea corylifolia* L. [29].

The CAT activity of the PM cell suspension responded to MJ treatment at almost all concentrations (Fig. 3, Table S5). Our results indicated that MJ treatment led to higher CAT activity. The CAT anti-oxidant enzyme protects cells against ROS-induced damage. Increased duration (1–9 days) of MJ exposure increased CAT and secondary metabolite production. However, longer exposure (12 days) tended to decrease the efficiency of the production of

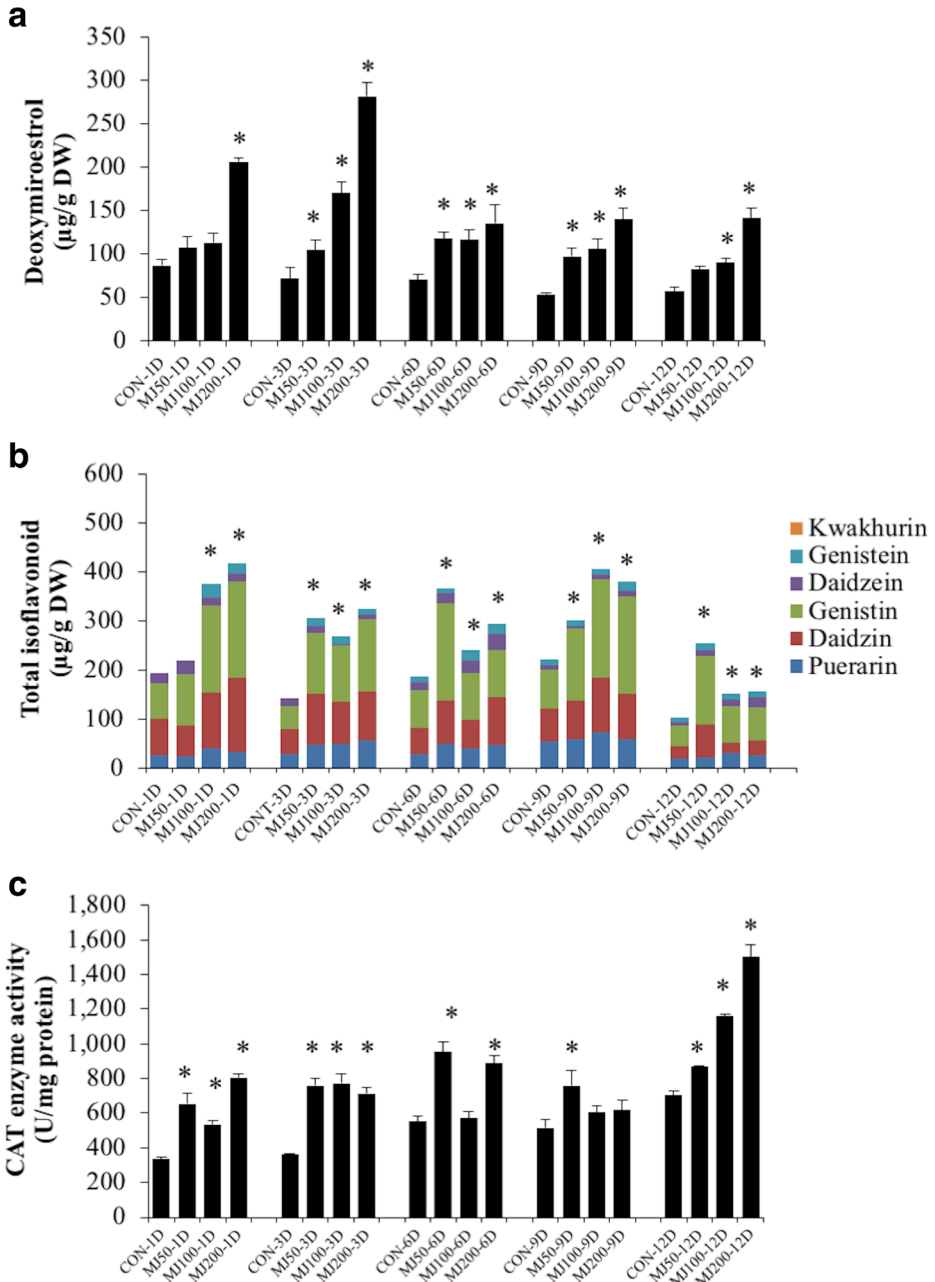


Fig. 3 Effect of the methyl jasmonate (MJ) concentration (50, 100, and 200 µM) and contact time (1, 3, 6, 9, and 12 days) on the production of deoxymiroestrol (**a**) and isoflavonoids (**b**) and CAT enzyme activity (**c**) in elicited PM cells. The statistical significance of different with control group (CON) was calculated using one-way analysis of variance (ANOVA) followed by Tukey’s HSD test ($p < 0.05$)

secondary metabolites DME and ISF. However, on the 12th day of MJ treatment, there was a sharp increase in CAT activity, which implied that higher production of ROSs was produced by

MJ treatment that was coupled with PM cell aging. This phenomenon may induce cell death [30] and suppress secondary metabolism.

Effect of YE on Production of DME and ISF and CAT Activity in PM Cell Suspension Culture

Lower concentrations of YE tended to enhance DME accumulation in PM cell suspension cultures (Fig. 4, Table S6). After one day of elicitation, 0.5 mg/mL of YE significantly enhanced DME accumulation ($191 \pm 27.3 \mu\text{g/g DW}$, 2-fold higher than that of the control). However, upon increasing the treatment duration, cells treated with 0.5 mg/mL of YE exhibited a lower degree of DME accumulation. In the same manner, all concentrations of YE (0.5, 1 and 2 mg/mL) enhanced the total ISF accumulation on the 1st day of treatment (334 ± 12.7 , 514 ± 2.57 and $488 \pm 3.99 \mu\text{g/g DW}$, respectively). Longer treatment duration (6, 9, and 12 days) decreased the effects of ISF induction. The effectiveness of YE-enhanced ISF in *P. candollei* has been observed in previous investigations [16, 22]. In addition, YE efficiently induced DZE and GTE production in suspension cultures of *P. corylifolia* L. [31].

The effect of YE on CAT activity in PM cell suspension cultures is shown in Table S6. CAT activity in PM cell suspension cultures was positively enhanced by almost all YE treatments. The response of CAT activity was similar to that observed during long-duration MJ treatment at high concentrations, during which YE2 produced the highest CAT activity ($1,811 \pm 80.4 \text{ U/mg protein}$) after 12 days of elicitation. However, the accumulation of secondary metabolites was not increased as a result of enhanced CAT activity. In the *G. max* L. cell suspension culture, YE also increased ROS production and ISF accumulation [32]. In addition, YE also induced oxidative burst and alkaloid biosynthesis in *Catharanthus roseus* cells; however, alkaloid biosynthesis is independent of ROS production [33]. The ROS accumulation observed after 12 days of treatment may not be at the appropriate level for the induction of DME and ISF production in PM cultures.

Effect of CHI on Production of DME and ISF and CAT Activity in PM Cell Suspension Culture

The effect of CHI on DME production in PM cell suspension culture is shown in Fig. 5 (Table S7). All concentrations (50, 100, and 200 mg/L) of CHI significantly enhanced DME accumulation that was observed after one day of treatment (143 ± 5.07 , 160 ± 1.63 and $134 \pm 8.26 \mu\text{g/g DW}$, respectively). CHI had a weaker effect on ISF production induction in PM cell suspension cultures. CHI50 significantly increased the highest total ISF accumulation that was observed after one day of treatment ($294 \pm 1.81 \mu\text{g/g DW}$). The effectiveness of CHI enhancement of ISF production in *P. candollei* has been observed in previous studies [22]. A study of soybean sprouts indicated that CHI treatment increases the transcription of genes involved in the ISF biosynthetic pathway [34]. In addition, CHI induced the accumulation of DZE and GTE in the hairy roots of *P. corylifolia* L. [29]

The effect of CHI on CAT activity in PM cell suspensions is shown in Fig. 5 (Table S7). The response of CAT activity to CHI treatment was observed similar to its response to MJ and YE treatment in a previous study. CHI200 induced the highest CAT activity ($1,242 \pm 19.9 \text{ U/mg protein}$) after 12 days of elicitation (Table S7). This indicates that the basic mechanism underlying the action of CHI may be involved in CAT activity. However, the enhanced induction of CAT also suppressed ISF accumulation in PM cell suspensions. In sycamore-

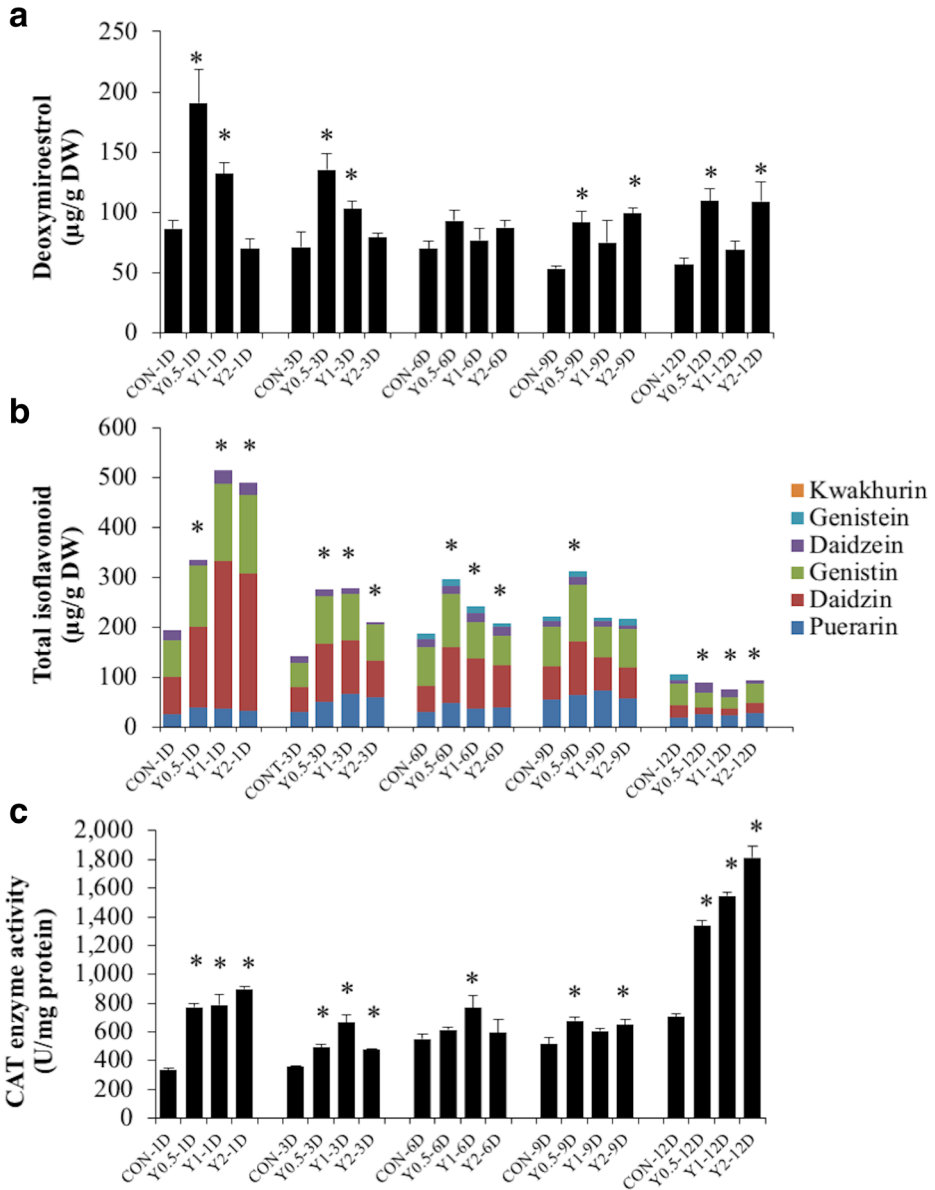


Fig. 4 Effect of yeast extract (YE) concentration (0.5, 1, and 2 mg/mL) and contact time (1, 3, 6, 9, and 12 days) on production of deoxymiroestrol (a) and isoflavonoids (b) and CAT enzyme activity (c) in elicited PM cells. The statistical significance of different with control group (CON) was calculated using one-way analysis of variance (ANOVA) followed by Tukey’s HSD test ($p < 0.05$)

derived cultured cells treated with CHI, concentrations of ROS species (H_2O_2 and O_2^-) were highly increased after 24 h, and this resulted in cell death due to increases in caspase-3-like activity and the release of cytochrome *c* [35]. Therefore, CHI may induce DME and ISF production temporarily, and cell death may be induced by high ROS production.

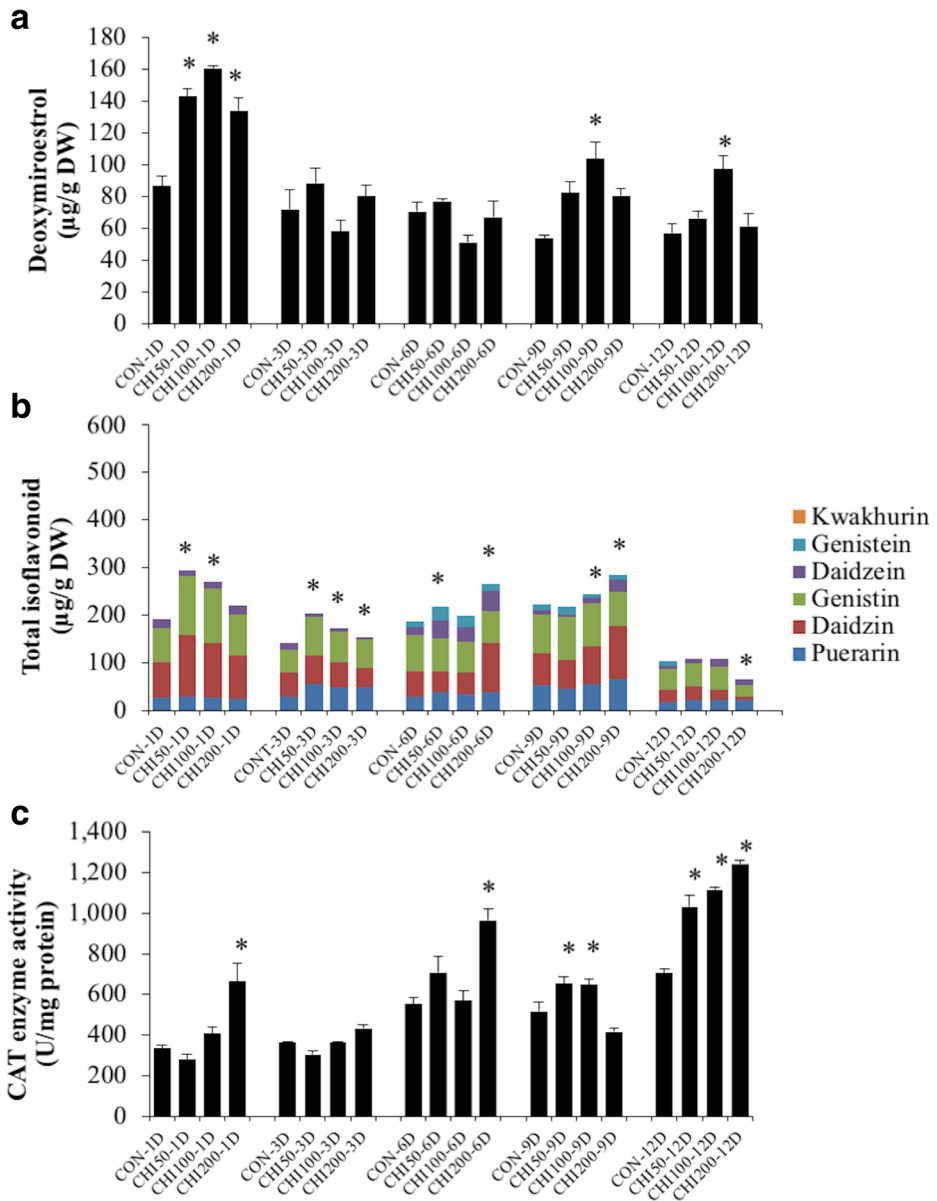


Fig. 5 Effect of chitosan (CHI) concentration (50, 100, and 200 mg/L) and contact time (1, 3, 6, 9, and 12 days) on the production of deoxymiroestrol (a) and isoflavonoids (b) and CAT enzyme activity (c) in elicited PM cells. The statistical significance of different with control group (CON) was calculated using one-way analysis of variance (ANOVA) followed by Tukey's HSD test ($p < 0.05$)

Growth Rate and Secondary Metabolite Production of the PM Cell Suspension Cultured in the Airlift Bioreactor

The growth rate and secondary metabolite production of PM cell suspensions cultured in airlift bioreactors were monitored for 50 days. The biomass and specific growth rates of the cell

suspensions cultured in the bioreactors are presented in Table S8. The PM cell suspension cultures were gradually grown and reached the highest growth rate after 30 days of culture. The cell suspension produced DME and ISF during cultivation in the bioreactor (Table S9). The highest DME accumulation was observed after 30 days of culture ($293 \pm 20.9 \mu\text{g/g DW}$; Fig. 6, Table S9). Surprisingly, DZE, an aglycone derived from DZ, was the only ISF that was detected in the cell suspension culture after 30 days in the bioreactor. GT and GTE could be detected in the cell suspension culture after 50 days in a bioreactor. The results indicated that the patterns of DME and total ISF accumulation in the PM cell suspensions in the bioreactor were quite different in the shake flask conditions that were described in the previous observation. Large-scale production of PM cell suspension resulted in higher DME accumulation but lower ISF accumulation. Due to the effects of stress from cell shock after inoculum introduction and/or the effects of aeration on the cell suspension, disruption of cells release of β -glucosidase which converts DZ to DZE [36, 37].

A comparative DME and ISF production of cultures grown in a 2-L shake flask and a 5-L bioreactor was demonstrated in Table S10. The optimum inoculum density ($3 \text{ g}/30 \text{ mL}$) was determined based on a previous experiment. The accumulation of DME and ISF in the PM cell suspension that was cultured in a 2-L shake was 410 ± 53.4 and $159 \pm 12.7 \mu\text{g/g DW}$, respectively, after 30 days of culture. The DME and total ISF contents of the PM cell suspension cultured in the bioreactor were 976 ± 79.6 and $587 \pm 21.6 \mu\text{g/g DW}$, respectively. Surprisingly, the cell suspension in both the large shake flask and the bioreactor produced higher DME accumulation than that produced during previous experiments in the 125-mL shake flask. Moreover, KWA ($312 \pm 18.1 \mu\text{g/g DW}$) could be detected in the modified bioreactor. Therefore, the culture conditions used for PM cell suspensions direct affect secondary metabolism and the accumulation of secondary metabolites. Larger scale cultures produced hydrodynamic shear stress and cell aggregation, which influenced secondary metabolism [38].

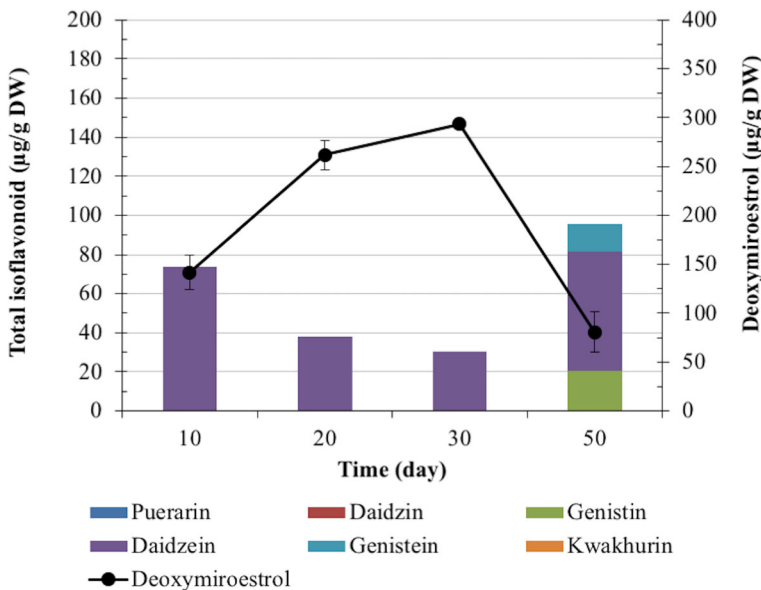


Fig. 6 Production of deoxymiroestrol and isoflavonoids in PM cells cultured in an airlift bioreactor over time. The PM cells (200 g) were cultured in 2-L of medium using a 5-L bioreactor and then collected on the 10th, 20th, 30th, 40th, and 50th days of culture. The accumulation of secondary metabolites was determined

The amounts of DME and ISF in intact PM have been reported to be in the range of 9.50–183.0 $\mu\text{g/g}$ DW and 0.01–3.91 mg/g DW, respectively [8]. PM cell culture resulted in greater production of DME; however, lower amounts of ISF were produced in the culture system. DME exhibits much more potent of estrogenic activity than ISF. In addition, ISF aglycones DZE and GTE exhibit higher potency and absorptivity in both the GI tract and skin than DZ and GT. However, intact PM predominantly accumulated ISF glycosides. Therefore, the bioreactor is a promising system for the production of PM materials containing high amounts of DME and absorbable DZE and GTE. DZE and GTE are potential sources of active ingredients that can prevent photo-induced skin aging. PM cells not only exhibited high potential as food supplements for menopausal relief but also as active ingredients that could be used in cosmeceutical products.

The PM cell suspension culture system that was established in this study has the ability to produce DME and ISF. Although the cell suspension produced lower ISF accumulation than has been previously reported, it was still able to produce a high accumulation of the most estrogenic compound DME (i.e.,). Our results suggest that 200 μM of MJ had a strong induction effect on both DME and ISF production in the elicited PM cell suspension, in which DME accumulated to a concentration of 282 ± 16.3 $\mu\text{g/g}$ DW (four-fold greater than that of the control) after three days of treatment. After one day of treatment, MJ at the same concentration produced the highest total ISF accumulation (417 ± 8.42 $\mu\text{g/g}$ DW; two-fold greater than that of the control). However, YE and CHI had weak effects on DME and ISF production. Elicitor treatment of the PM cell suspension in the present study showed its effect on CAT anti-oxidant enzymatic activity in terms of the scavenging of ROS produced during elicitation. This indicates that CAT may be involved in the basic mechanism that underlies the action of elicitors.

In summary, the present study demonstrated the use of cell suspension culture combined with elicitation techniques to ensure quality control of the PM cultivation process. Therefore, PM cell suspension culture may serve as an alternative source of plant secondary metabolites. These results would be useful for enhancing the large-scale production of bioactive compounds using PM cultures at the industrial level.

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Compliance with Ethical Standards

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

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