Plant Cell, Tissue and Organ Culture (PCTOC) (2021) 145:29–41
https://doi.org/10.1007/s11240-020-01990-4

ORIGINAL ARTICLE

Elicitation of *Pueraria candollei* var. *mirifica* suspension cells promises antioxidant potential, implying antiaging activity

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Received: 29 September 2020 / Accepted: 12 December 2020 / Published online: 6 January 2021

Abstract

Elicitors that trigger the defense mechanism of plants could be a promising approach for elevating the bioactive compounds in tissue-cultured plant suspension cells. The objective of this study was to determine the effects of salicylic acid (SA), chitosan (CHI) and methyl jasmonate (MeJA) elicitation applied at different concentrations to suspension cells of *Pueraria candollei* var. *mirifica* (PM) on the contents of isoflavonoid, antioxidant, and antiaging activities. Six standards of isoflavonoids, namely, daidzein, genistein, daidzin, genistin, khwakhurin and puerarin, were determined by reversed-phase high-performance liquid chromatography (HPLC). To our knowledge, SA elicitation was first reported in this study and showed the highest accumulation of daidzein (2.37 mg/g DW), khwakhurin (1.05 mg/g DW), genistin (0.14 mg/g DW) and puerarin (5.13 mg/g DW). CHI elicitation gave favorable results, and the amounts of daidzein, genistein, khwakhurin and puerarin quantified were 2.24, 0.29, 1.04, and 1.80 mg/g DW, respectively. MeJA elicitation was the least effective method of all three elicitors. The results from DPPH-based assays suggested that extract from SA elicitation had the greatest antioxidant activity, followed by nonelicited cells (control), CHI, and MeJA elicitation. We also further evaluated the biological activities of PM extracts with elicitors on human dermal fibroblasts. We found that salicylic acid is the most promising elicitor to enhance the biological activities of PM extracts. The PM extract from SA-elicited cells promoted the proliferation of fibroblasts by approximately 20%. Furthermore, pretreatment with SA-elicited extract can prevent fibroblasts from experiencing oxidative insults, such as H_2O_2. The pharmacological data on fibroblasts support the antioxidant activities, implying the antiaging activities of PM extract. Taken together, our study clearly supports the potential utility of cell suspension culture of PM in the pharmaceutical and cosmeceutical industries to enhance the production of isoflavonoids and other active components.

Key message

Elicitation of suspension cell cultures of *Pueraria candollei* var. *mirifica* by salicylic acidenhances the isoflavonoid content with promising antioxidant and antiaging activities.

Keywords *Pueraria mirifica* · Kwao Krua Kwao · Isoflavonoids · Pharmaceutical · Cosmeceutical

Introduction

Skin aging is a complex biochemical process due to various intrinsic and extrinsic factors, such as age, hormones, and exposure to UV light (Gragnani et al. 2014). The level of reactive oxygen species (ROS) increases over time in all human tissues, including skin; thus, additional sources of antioxidants or other supplements are required to promote the activity of the ROS-detoxifying system of skin cells (Limtrakul et al. 2016). The antioxidants present in plant extracts rejuvenate the skin and improve the skin condition (Farris et al. 2014). Moreover, knowledge of the antioxidant

Communicated by Christophe Hano.

Supplementary information The online version of this article (https://doi.org/10.1007/s11240-020-01990-4) contains supplementary material, which is available to authorized users.

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activities responsible for the antiaging potential of molecules obtained from plants is continuously increasing. Some of the plants explored for their antiaging potential are *Terminalia chebula* (Manosroi et al. 2010), *Cassia fistula* (Limtrakul et al. 2016), and *Panax ginseng* (Shin et al. 2017), among many others. Recently, the callus extract of *Leontopodium alpinum* exhibited antiaging activity by reducing inflammation and wrinkling and increasing moisturizing activity (Cho et al. 2020).

Pueraria candollei var. *mirifica* (PM) is an economically important medicinal plant endemic to Thailand. For ages, PM has been used individually or in combination with other herbs in traditional practices. The roots of this plant, because of its rejuvenating qualities, are used by local people in Thailand to treat menopausal women and andropausal men (Malaivijitnond 2012). Due to the antioxidant, anti-collagenase and anti-elastase properties of PM, it is presently used as an active ingredient in numerous nutraceuticals and cosmeceuticals (Chattuwatthana and Okello 2015; Dorni et al. 2017). Isoflavonoids are a broad group of naturally occurring polyphenolic compounds that have a wide range of bioactivities (Karwasara and Dixit 2012). Isoflavones are diverse secondary metabolites that are abundantly produced in leguminous plants and have several biochemical and pharmacological activities (Kim et al. 1998). A high variation (tenfold) in the isoflavonoid content in PM is reported due to season, location and time of collection (Cherdshewasart et al. 2007). Chromenes, coumestrans, and isoflavones such as daidzein, genistein, and puerarin are the major chemical constituents of PM, and all of them have estrogenic activity (Chansakaow et al. 2000). Various other bioactive compounds, such as miroestrol, deoxymiroestrol, daidzin, genistin, and khwakhurin, have also been reported in PM (Chansakaow et al. 2000; Cherdshewasart et al. 2007).

Isoflavones also exhibit promising antioxidant effects and thus prevent oxidative stress, which includes aging and cancer (Slavin et al. 2009). Natural antioxidant compounds contribute notably to the food, pharmaceutical, and cosmeceutical industries (Miliauskas et al. 2004). In contrast to the wide applicability of *P. mirifica* var. *candollei* in the pharmaceutical and cosmeceutical industries, few reports on the antioxidant activity of suspension cell cultures of *P. mirifica* var. *candollei* can be found. The antioxidant activity of suspension cell cultures of *P. mirifica* var. *candollei* was higher than that of the tuber extract (Saisavoey et al. 2014).

Plants serve as a storehouse for the discovery and development of natural products. Plant species have been used to produce cell suspension cultures for molecular farming rather than whole-plant production (Hellwig et al. 2004). Suspension cell cultures represent the best alternative to conventional methods for raising the yields of bioactive compounds to cater to the escalating industrial demands for such natural, low-molecular-weight molecules. Traditional plant tissue cultures are a feasible system for obtaining commercially important secondary metabolites and help to conserve plant bioresources in their native environment; however, low, unstable metabolic yields have been a major impediment to the further development of this technology (Murthy et al. 2014). Suspension cell cultures raised from *P. mirifica* var. *candollei* reported more total isoflavonoids than native plants (Udomsuk et al. 2009, 2012; Boonsnongcheep et al. 2010; Rani et al. 2018, 2020).

An elicitor is a compound, either natural or synthetic, that initiates or enhances the biosynthesis of specific metabolites when introduced at modest concentrations to a living cell system (Zhao et al. 2005). Both biotic and abiotic elicitors induce the accumulation and excretion of secondary metabolites into cell cultures. MeJA and SA have been widely used to stimulate flavonoid and polyphenol production in suspension cells, calli, and tissue cultures of various plant families (Mendoza et al. 2018). In a study conducted by Liu et al. (2018), both MeJA and SA were effective in increasing the amount of chlorogenic acid in suspension cell cultures of *Gardenia jasminoides*. CHI is derived from fungal cell walls and has been studied because of its effects on enzymes in the phenylpropanoid pathway (Chakraborty et al. 2009). Elicitation by yeast extract significantly improved the amount of isoflavonoids in the suspension cell culture of PM (Udomsin et al. 2019; Rani et al. 2020).

As mentioned above, the contents of isoflavones vary in naturally growing PM, and growing plants in Thailand is limited by law. We have recently established a suspension culture of single cells of PM that provide a good source of plant material (Rani et al. 2018), and only yeast extract was tested for its potential to increase the bioactive compounds of PM cells in our previous study (Rani et al. 2020). However, it is possible that the amount of isoflavonoids in the cell could be increased by other elicitors. Therefore, the cell culture would potentially be a reliable source of industrial scale production. The antioxidant and antiaging activities of the elicited cells were also determined for their potential use in cosmeceuticals. Hence, the objectives of this study were to elicit a cell suspension culture of PM and to investigate their isoflavonoid content, antioxidant potential and antiaging activity.

Materials and methods

Chemicals and reagents

Murashige and Skoog media, Agargellen™, and 2,4-dichlorophenoxyacetic acid were obtained from PhytoTechnology Laboratories (Kansas, USA). Puerarin (98%), daidzein (98%), daidzin (95%), genistein (98%), genistin (95%), thiamine hydrochloride, methyl jasmonate (1 g/mL in water),

chitosan (from crab shells), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were procured from Sigma-Aldrich (MO, USA). Khwakhurin (≥ 90%) was isolated from the tuberous roots of PM according to a previously described method (Gorawit et al. 2019), and the compound was confirmed using ^{H}NMR. Khwakhurin was kindly provided by Dr. Gorawit Yusakul, Walailak University, Nakhon Si Thammarat, Thailand. Salicylic acid was purchased from Ajax FineChem (Sydney, Australia). Glacial acetic acid, toluene, acetonitrile and ethyl acetate were obtained from J.T. Baker (Fisher Scientific, USA). HPLC-grade acetonitrile was procured from RCI Labscan (Bangkok, Thailand). Minimum essential medium (MEM), FBS, pyruvate, DMSO, and antibiotics were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All other chemicals that are not mentioned are commercial analytical grade products.

Callus induction and maintenance of cell suspension cultures

In vitro germinated PM seedlings were used as explants for the induction of calli. Seeds were collected from PM plants grown in the open field of Khaolaor Laboratories, Samut Prakan, Thailand. The plant was identified as *Pueraria candollei* var. *mirifica* by comparison with herbarium specimens deposited at the herb museum at the Faculty of Pharmaceutical Sciences, Chulalongkorn University. Aseptic seedlings were inoculated on Murashige and Skoog medium (MS) (Murashige and Skoog 1962) supplemented with 0.5% Agargellan™ (Phytotechnology Laboratories, USA) with 3% sucrose. Calli were obtained from PM stem explants using the protocol established in our previous report (Rani et al. 2018). Briefly, approximately 1-cm-long explants of 20-day-old PM seedlings were excised and inoculated on MS medium supplemented with 200 mg/L KH_2PO_4, 1 mg/L thiamine HCl, 100 mg/L myo-inositol and 0.2 mg/mL 2,4-dichlorophenoxyacetic acid for callus induction. The callus obtained from a single explant was used in suspension cell cultures (Fig. 1a). The cultures were incubated at 100 rpm on an orbital shaker. The suspension cells obtained after 21 days were subcultured onto fresh medium (20% seed volume). After 4–5 generations, suspension cell cultures were used to quantify the amount of daidzein produced over time (Fig. 1b). Cells were collected every 7 days after subculturing until a decline in cell growth (35 days) was observed.

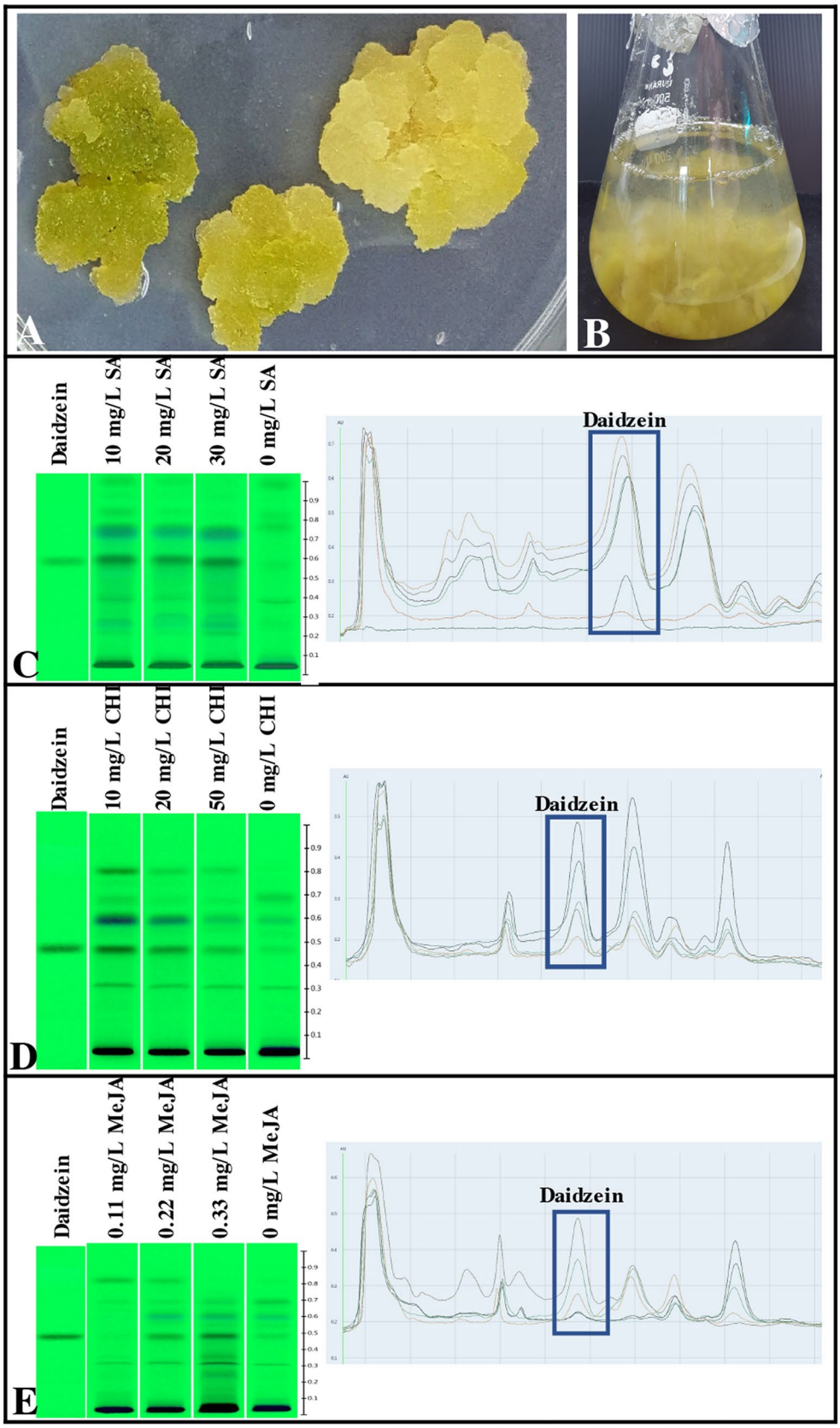
Elicitor treatments

Six days after the initiation of the suspension cell cultures, elicitors were introduced to suspension cells. The elicitors used in the present study were MeJA, SA, and CHI. The elicitors were filter sterilized (0.22 µm) prior to their addition to the medium. MeJA was introduced to suspension cell cultures to achieve final concentrations of 0.11, 0.22, and 0.33 mg/L. The stock of SA (Merck, Germany) was prepared using ultrapure water to achieve concentrations of 1, 2, and 3 mg/L. The control cells for MeJA and SA received 1% ultrapure water. The stock solution of CHI (minimum 85% deacetylated) was prepared in glacial acetic acid by gentle heating at 60 °C and bringing the solution to the final volume with ultrapure water. The stock solution of CHI was autoclaved before use. CHI was used at concentrations of 10, 20, and 30 mg/L. The control for CHI elicitation was 1% acetic acid.

Cell growth and quantification of daidzein

Twenty-one days after the addition of the elicitors to the cell suspensions, cells from the three treatment flasks in triplicate and control cells were collected in accordance with an earlier report by Korsangruang et al. (2010), who reported that the maximum yield of the isoflavonoid content occurred after 21 days. The cells were dried at 50 °C for 48 h in a hot air oven to obtain the dried biomass. The dried callus sample (100 mg) was extracted in methanol, sonicated for 30 min at 25 °C and then filtered through Whatman No. 42 filter paper. The filtrate was concentrated to dryness using a SpeedVac (Savant SpeedVac SC100 Centrifugal Evaporator, Canada). The dried samples were dissolved in methanol. Aliquots of the samples were filtered through 0.22-µm filter membranes for further use. A stock solution of daidzein (0.1 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) was prepared in methanol. Different volumes (2, 4, 6, 8, and 10 µl) of the stock solution, equivalent to 200, 400, 600, 800, and 1000 ng, were applied to high-performance thin layer chromatography (HPTLC) plates.

The amount of daidzein was quantified by using HPTLC. The standard and sample solutions were loaded on precoated 20 × 10 cm silica gel (60 F254, Merck, Germany) plates in the form of bands with a 100-µl syringe using an automatic Linomat 5 (CAMAG, Switzerland) applicator. The mobile phase used was toluene:acetonitrile:ethyl acetate:water at a ratio of 55:31:13:0.5, and 250 µL of glacial acetic acid was added to the solution. The plates were developed in an ADC 2 automatic developing chamber (twin trough chamber CAMAG, 20 × 10 cm). After the plates had been developed, they were dried in the same ADC 2 unit for 5 min, and the results were documented using a CAMAG TLC visualizer. Scanning of daidzein containing the stationary phase was performed on a CAMAG TLC scanner 3 controlled by VisionCATS software in absorbance mode at 254 nm. Daidzein was also evaluated under white light and UV 366 nm. The resulting peak of the daidzein standard was used as a reference for


A
B
Daidzein
10 mg/L SA
20 mg/L SA
30 mg/L SA
0 mg/L SA
Daidzein
C
Daidzein
10 mg/L CHI
20 mg/L CHI
50 mg/L CHI
0 mg/L CHI
Daidzein
D
Daidzein
0.11 mg/L MeJA
0.22 mg/L MeJA
0.33 mg/L MeJA
0 mg/L MeJA
Daidzein
E

◂**Fig. 1** Cells *of P. candollei* var. *mirifica* after 56 days of culture obtained from stem explants. **a** Healthy calli from modified medium (MS + 0.2 mg/L 2,4-D + 200 mg/L KH_2PO_4 + 1 mg/L thiamine HCl + 100 mg/L myo-inositol) and **b** suspension cell culture after 20 days of culture. HPTLC fingerprint at 254 nm showing the effects of different treatments on cell suspension cultures of *P. candollei* var. *mirifica*: **c** salicylic acid, **d** chitosan, **e** methyl jasmonate

the identification of daidzein in the various samples by comparing the sample peaks to the peak of the standard.

Preparation of elicited suspension cell extracts for HPLC analysis and cell-based assay for antioxidant activity

The concentrations of SA (30 mg/L), CHI (10 mg/L) and MeJA (0.33 mg/L) that gave the highest amount of daidzein in the HPTLC quantification were further used for elicitation. They were extracted in a similar way as described earlier and used for HPLC analysis, antioxidant, and antiaging activities.

HPLC analysis

Six isoflavonoid standards, namely, puerarin, daidzein, daidzin, genistin, genistein, and khwakhurin, were used for the analysis. Standards were used at a concentration of 1 mg/mL in methanol, and linear 5-point calibration curves were plotted using 15.625–250 ng concentrations of the standards. The analysis was performed using a Shimadzu HPLC (LC-20A) connected with a PDA detector. A C-18 column (100 mm × 4.6 mm, 5 µm particle size, Phenomenex, USA) was used. The mobile phase used was a gradient of 1% acetic acid (A) and acetonitrile (B) at a flow rate of 1 mL/min with the following linear gradient HPLC solvent program: solvent B was increased from 0 to 45% over 30 min and further decreased to 0% over 40 min and then held at 100% A for 5 min before returning to the initial state. The column temperature was controlled at 30 °C, and chromatograms were recorded at 254 nm.

Conventional method for utilizing PM in traditional preparations

Three-year-old, mature tubers of PM were collected from a cultivated PM field at Kasetsart University, Thailand. The tubers were washed, dried and ground to powder (Fig. S1). The dried powder was extracted in methanol as described above and used for HPLC analysis. The amount of daidzein was quantified using the same conditions as discussed above.

Antioxidant activity

To determine the antioxidant activity, 10 µL/band of test extract was applied on the TLC plate by using an automatic Linomat 5 (CAMAG, Switzerland) applicator using 8-mm bands at a distance of 11 mm from the lower edge. The development of the plate was performed in a 20 cm × 10 cm saturated twin trough chamber using mobile phase toluene:acetonitrile:ethyl acetate:water at a ratio of 55:31:13:0.5 and 250 µL of glacial acetic acid up to a migration distance of 80 mm. The chromatogram was documented by the TLC Visualizer Documentation system (CAMAG) at 254 and 366 nm. For DPPH activity, the developed chromatogram was sprayed with 0.2% methanolic DPPH solution and incubated in the dark for 30 min. The chromatogram was observed under white light. The IC_{50} DPPH values were also calculated using GraphPad Prism (version 8.4.3). To determine the scavenging activity, 100 µL of DPPH reagent (8 mg DPPH dissolved in 100 mL of methanol) was mixed with 100 µL of varying concentrations of callus extract in a 96-well microplate and incubated at 25 °C for 30 min. After incubation, the absorbance was measured at 517 nm using an ELISA reader (CLARIOstar using MARS software), and 100% methanol was used as a control. The percent (%) inhibition of free radicals by DPPH was calculated using the following formula:

$$\%Inhibition = \frac{(OD517_{Control} - OD517_{sample}) \times 100}{OD517_{Control}}$$

$OD517_{Control}$ Optical density at 517 nm of 100% methanol; $OD517_{sample}$ Optical density at 517 nm of suspension cells of PM extract.

Cell culture

The normal human fibroblast cell line BJ (ATCC number, CRL-2522) was purchased from American Type Culture Collection (ATCC, USA). BJ cells were cultured in minimum essential medium (MEM) supplemented with 10% FBS, 1 mM pyruvate, and 100 U/mL penicillin/100 µg/mL streptomycin. Cells were maintained in a humidified atmosphere of 95% air/5% CO_2 at 37 °C and passaged routinely for experiments until passage 20.

Cell proliferation assay

For cell culture experiments, each PM extract was dissolved in DMSO to produce a 25 mg/mL stock solution. BJ cells were seeded into 96-well culture plates at 20,000 cells per well in 200 µL of medium and allowed to attach for 24 h. Cells were incubated with *Pueraria* extracts

(12.5–100 µg/mL) for 18 h. Control groups were exposed to equivalent amounts of DMSO (0.5%; vehicle control). After treatments, exposure media were discarded, and cells were washed twice with PBS. The cell viability was then evaluated with the MTT assay. Briefly, washed cells were incubated with a solution of MTT (200 µL; 1 mg/mL in serum-free medium) for 3 h in the dark. Subsequently, the MTT solution was removed, and reduced formazan crystals were dissolved by the addition of DMSO (200 µL) into each well. The absorbance of the formazan solution was determined at 570 nm using a CLARIOstar microplate reader (BMG Labtech, Germany).

The percentage of increased cell proliferation was calculated according to the following formula:

$$\%Increased\ cell\ proliferation = \frac{(OD570_{Ex} - OD570_{Ct}) \times 100}{OD570_{Ct}}$$

$OD570_{Ex}$ optical density at 570 nm of extract groups; $OD570_{Ct}$ optical density at 570 nm of vehicle control.

Cell survival assay

Cells were seeded into 96-well culture plates at 20,000 cells per well in 200 µL of medium and were allowed to attach for 24 h. Cells were pretreated with *Pueraria* extracts (12.5–100 µg/mL) for 18 h. Following pretreatment with extracts, cells were exposed to H_2O_2 (50 µM in serum-free MEM) for 1 h, and the MTT assay was immediately performed using conditions previously described in the "Cell proliferation assay" section.

The percentage of increased cell survival was calculated according to the following formula:

$$\%Increased\ cell\ survival = \frac{(OD570_{EH} - OD570_{H}) \times 100}{OD570_{H}}$$

$OD570_{EH}$ optical density at 570 nm of extracts + H_2O_2 groups; $OD570_{H}$ optical density at 570 nm of the H_2O_2-alone group.

Statistical analysis

HPTLC and HPLC analyses of samples were performed by using 3 independent sets of samples and were repeated two times. The values obtained are presented as the mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA), Tukey's post hoc test, Python (v. 2.7.15) stats models, and stats libraries. Statistically significant differences were considered at $p < 0.05$.

Results and discussion

Growth and daidzein accumulation of native PM suspension cells

The growth pattern of the dried biomass of suspension cells coincided with the daidzein accumulation trend in the cells. Initially, until 21 days of culture, the cells exhibited slow and steady growth of both cells and the daidzein content. Steep increases in cell biomass (5.70 g) and daidzein content were observed after 28 days (1.28 mg/g DW) of subculture (Fig. 2a). Maintaining the cells in the same medium without subculturing was detrimental to growth and daidzein accumulation. The cell biomass decreased to 4.65 g, and daidzein was quantified at 1.03 mg/g DW. Based on this and previous studies, we concluded that suspension cells should be subcultured after 25–28 days to reach the maximum biomass and daidzein accumulation. The used medium was also checked for daidzein accumulation, but it was not detected, suggesting that the suspension cells retained all the daidzein and were not exuded in the medium.

Quantification of daidzein in tubers of PM

In the methanol extract of tubers of PM obtained from Kasetsart University, Thailand, the amount of daidzein quantified was 0.22 mg/g DW (Fig. S1). The results suggest that suspension cells were more effective in daidzein production than wild plants.

Effects of MeJA, SA and CHI on daidzein content

The elicited cells were checked through HPTLC to determine the concentration that would be used for further studies. SA is an important elicitor of hormones in triggering defense-related genes and enhancing metabolism, especially in medicinal plants (Zhao et al. 2004). SA is generally a signal for the immune response in plants, but its effect on each species may vary. The addition of SA to the suspension cells produced more daidzein than that to the control cells at all tested concentrations (Figs. 1c, 2b). When SA was used at concentrations of 10 mg/L and 20 mg/L, the accumulation of daidzein in cells increased by 3.3-fold (2.26 mg/g DW) and 4.2-fold (2.87 mg/g DW), respectively. With the increase in SA to 30 mg/L, the amount of daidzein significantly increased to 3.56 mg/g DW, which was 5.2-fold higher than that in the control cells. The low solubility of CHI in neutral and alkaline solutions limits its use in pharmaceutical applications (Cheung et al. 2015). The highest concentration used in this study was 50 mg/L, and at this concentration, the amount of daidzein quantified was

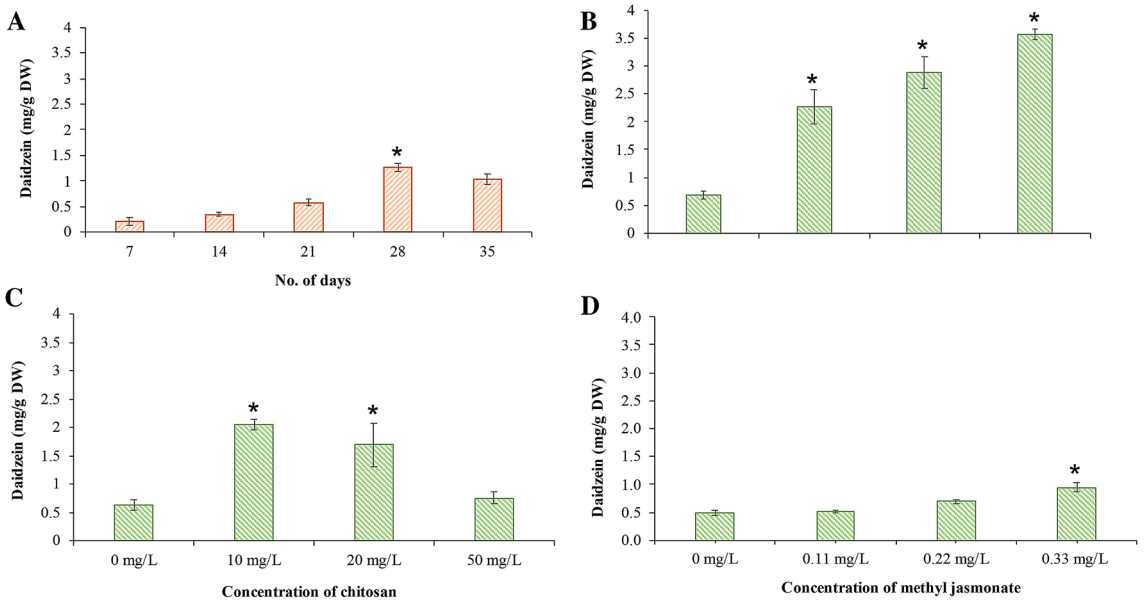


Fig. 2 Daidzein content in cell cultures of *P. candollei* var. *mirifica*. **a** Daidzein accumulation over time in nontreated suspension cells. Daidzein in suspension cell cultures after treatment with salicylic acid (**b**), chitosan (**c**), and methyl jasmonate (**d**). The values are expressed as the mean ± SD. Asterisks indicate significant differences ($P < 0.05$)

almost equal to that of the control cells (Figs. 1d, 2c). The lowest concentration of CHI used in this study (10 mg/L) led to a 3.2-fold (2.053 mg/g DW) increase in the amount of daidzein compared with the control cells (0.63 mg/g DW). The addition of MeJA resulted in a slight increase in the daidzein content in the present study, particularly at concentrations of 0.22 and 0.33 mg/L MeJA (Fig. 2d). The amounts of daidzein recorded were 0.69 mg/g DW and 0.95 mg/g DW when MeJA was used at concentrations of 0.22 mg/L and 0.33 mg/L, respectively (Figs. 1e, 2d).

Based on these results, the suspension cells of PM were again elicited with the concentration of elicitors that gave the maximum amount of daidzein. The concentrations used were 0.33, 30, and 10 mg/L MeJA, SA, and CHI, respectively.

Effects of MeJA, SA, and CHI on isoflavonoid content

Six isoflavonoid standards, namely, daidzein, daidzin, genistein, genistin, puerarin, and khwakhurin, were evaluated in the elicited suspension cells of PM by RP-HPLC analysis, and the values were expressed as mg/g DW. The HPLC chromatograms of the standards are indicated in Fig. 3, and the amount of each isoflavonoid is summarized in Table 1. Of all three elicitors used in the present study, SA was the most potent.

Four isoflavonoids, namely, daidzein (2.37 mg/g DW), khwakhurin (1.05 mg/g DW), genistin (0.14 mg/g DW) and puerarin (5.13 mg/g DW), were detected in the suspension cells elicited with 30 mg/L SA (Table 1). Of all the elicitors tested in the present study, the amount of the above four isoflavonoids quantified was highest in the SA-elicited cells. To our knowledge, this is the first report of SA being used as an elicitor in suspension cell cultures of PM. Goyal and Ramawat (2008) reported that SA was effective in inducing higher amounts of isoflavonoids at all concentrations in *Pueraria tuberosa*. The amount of daidzein quantified in the current study is 16 times greater than the amount quantified by Goyal and Ramawat (2008).

The next elicitor tested was 10 mg/L CHI, which also gave promising results. Four isoflavonoids, daidzein (2.24 mg/g DW), genistein (0.29 mg/g DW), khwakhurin (1.04 mg/g DW), and puerarin (1.80 mg/g DW), were detected. In a similar report, Korsangruang et al. (2010) reported that higher concentrations of CHI were not effective in enhancing isoflavonoid production in PM. They also reported that 10 mg/L CHI was the most effective amount and increased the amount of isoflavonoids by 1.13-fold. In contrast, the hairy roots of PM treated with as high as 200 mg/L CHI resulted in the highest deoxymiroestrol accumulation of 111 µg/g of roots, which was just 1.4-fold higher than that in the control hairy roots (Udomsin et al. 2019). This indicated that although a high concentration of CHI reduced the accumulation of isoflavonoids, it had a positive effect on the production of deoxymiroestrol, which is biosynthesized later in the metabolic pathway. Investigation of the CHI mechanism should be further studied.

In the present study, MeJA elicitation was not as effective as SA and CHI elicitation. The isoflavonoids found in cells elicited with 0.33 mg/L MeJA were daidzein (0.80 mg/g DW), genistin (0.12 mg/g DW) and puerarin (1.64 mg/g

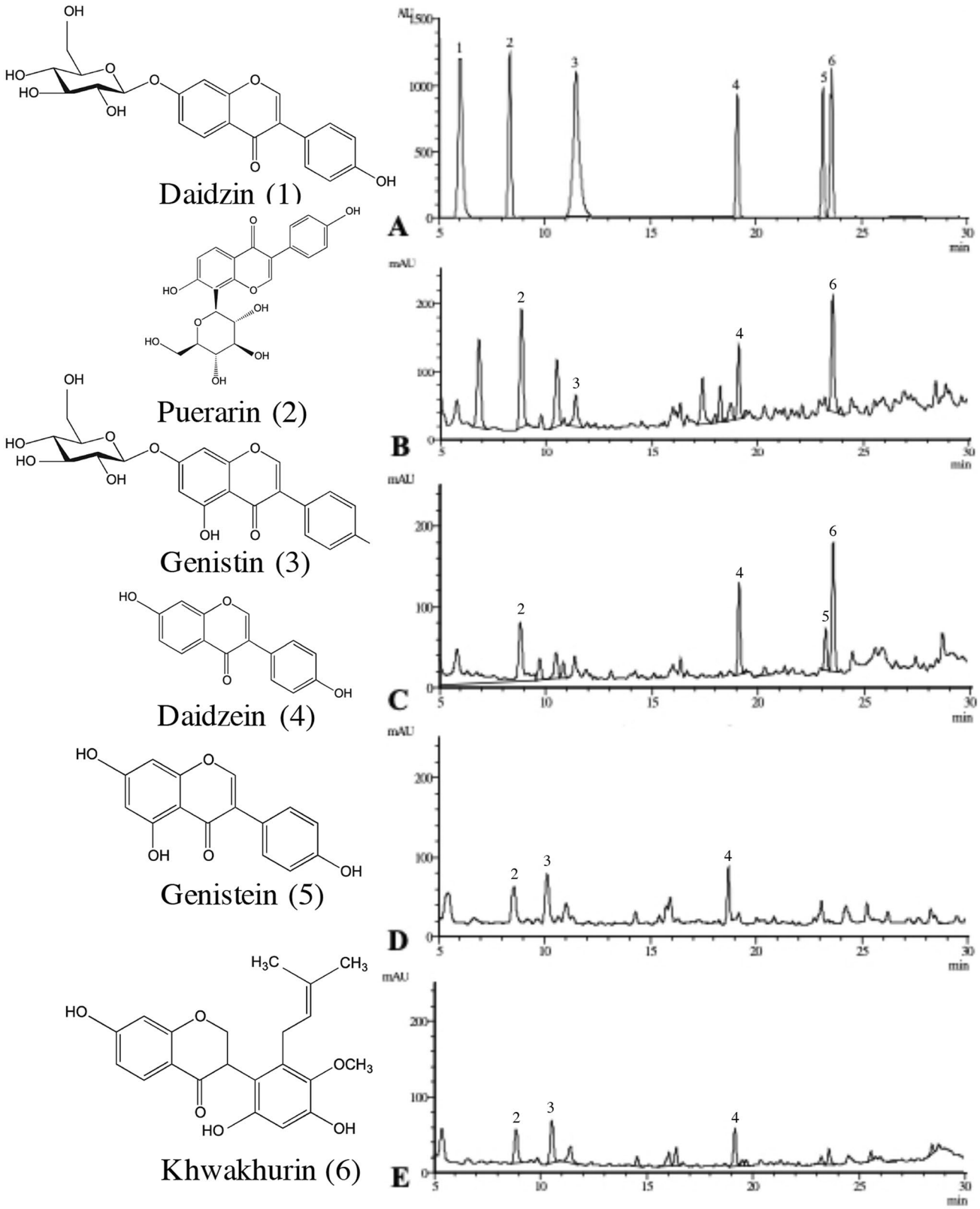


Fig. 3 HPLC profiles of the suspension cell cultures after elicitation. **a** Chromatograms of the standards and the cells after elicitation: **b** salicylic acid, **c** chitosan, **d** methyl jasmonate, and **e** control (1 Daidzin, 2 Puerarin, 3 Genistin, 4 Daidzein, 5 Genistein, 6 Khwakhurin)

Table 1 Isoflavonoid contents of *P. candollei var. mirifica* suspension cell cultures

Elicitor used	Daidzein (mg/g DW)	Genistein (mg/g DW)	Khwakhurin (mg/g DW)	Daidzin (mg/g DW)	Genistin (mg/g DW)	Puerarin (mg/g DW)
SA	2.37 ± 0.07	ND	1.05 ± 0.08	ND	0.14 ± 0.01	5.13 ± 0.14
CHI	2.24 ± 0.02	0.29 ± 0.0.01	1.04 ± 0.03	ND	ND	1.79 ± 0.06
MeJA	0.80 ± 0.07	ND	ND	ND	0.12 ± 0.09	1.69 ± 0.03
CL	0.59 ± 0.08	ND	ND	ND	0.131 ± 0.07	0.68 ± 0.09

The values are expressed as the mean ± SE (n = 3) *ND* not detected, *SA* salicylic acid, *CHI* chitosan, *MeJA* methyl jasmonate, *CL* control

DW). Saisavoey et al. (2014) reported the highest isoflavonoid contents (6.27 mg/g DW) in *P. mirifica* cells when MeJA was used at a concentration of 1 mg/L and elicited for 1 day, which was lower than the amount found in the present study using a lower concentration of MeJA.

The control cells (nonelicited) gave the least amount of isoflavonoids, and three of them were detected: daidzein (0.59 mg/g DW), genistin (13 mg/g DW), and puerarin (0.68 mg/g DW). Thus, it can be assumed that in the present study, elicitation of PM yielded favorable results by enhancing the amount of isoflavonoids. In our previous report (Rani et al. 2020), we suggested that yeast extract elicitation of suspension cells of PM significantly increased the amount of daidzein (5.12 mg/g DW), which was 11 times higher than that of the control cells. In our continuing effort to find a suitable elicitor that would give the highest amount of isoflavonoids and thus could be used at the industrial scale, the present study was one step forward, as SA elicitation yielded better results on the overall amount of isoflavonoids.

DPPH (2,2-diphenyl-1-picrylhydrazyl) activity

Estimating the antioxidant potential of elicited suspension cell cultures is crucial in evaluating the potential of these cells to be used at an industrial scale. Two methods to determine the antioxidant potential based on DPPH assays were used. The extract of elicited cells when developed on TLC plates derivatized with DPPH reagent exhibited the presence of antioxidant compounds as bands on a purple background (Fig. 4c). The intensity of whitish bands was highest in the SA-elicited samples, followed by CHI- and MeJA-elicited cells, and the least intense bands were observed in the control cells.

Among the elicitor treatments, the strongest radical scavenging capacity was observed in the cells elicited with SA (89.60%), followed by CHI (89.25%), MeJA (79.02%), and control cells (72.41%) at a concentration of 500 mg/mL. The antioxidant potential of each extract was represented as IC_{50} values and was found to be 1.64, 2.10, 2.18 and 1.91 mg/mL for SA, CHI, MeJA and control cells, respectively (Fig. 5). Few reports on the antioxidant potential of PM suspension cells can be found. Saisavoey et al. (2014) reported that the antioxidant potential of callus cells was higher than that of tubers of PM. The difference in antioxidant potential in different elicited suspension cells could be related to the difference in the triggering of antioxidant activity of the elicitors, which could also be related to the amount of major components present in the suspension cells. Earlier reports suggest that there is a positive correlation between phenolic and flavonoid contents and antioxidant activity (Liaudanskas et al. 2014; Sarkate et al. 2017).

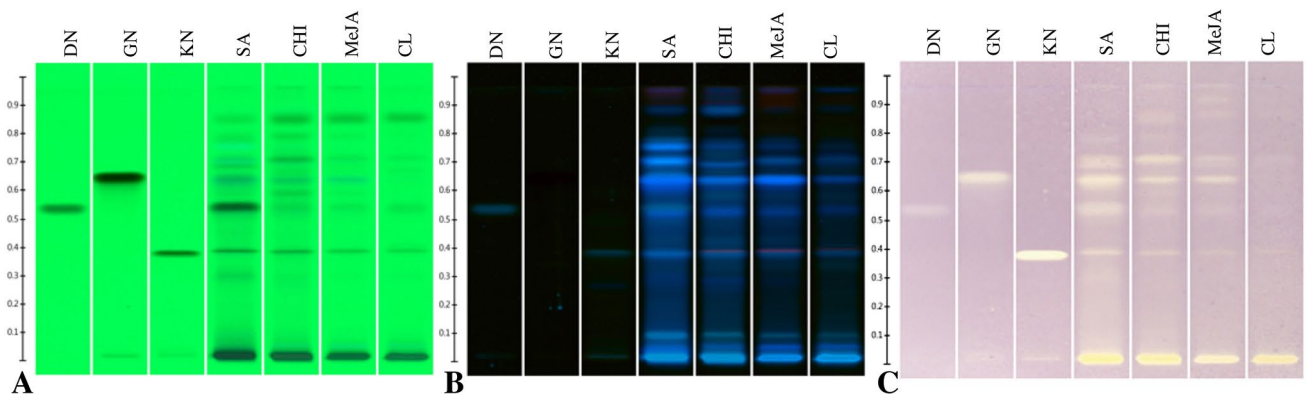


Fig. 4 TLC fingerprints of standards and suspension cell extracts. **a** 254 nm, **b** 366 nm **c** after derivatization with DPPH *DN* daidzein, *GN* genistein, *KN* Khwakhurin, *SA* salicylic acid, *CHI* chitosan, *MeJA* methyl jasmonate, *CL* control

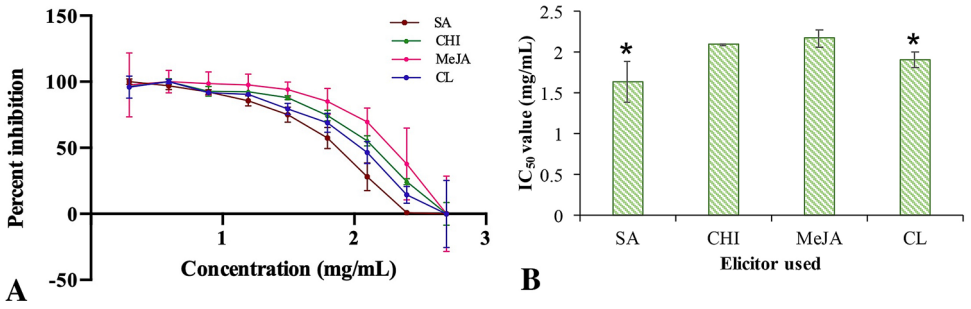


Fig. 5 Antioxidant capacity of callus of *Pueraria candollei* var. *mirifica* according to the IC_{50} values using the DPPH assay. **a** Percent inhibition after treatment with different concentrations of extract. **b** IC_{50} values. The values are expressed as the mean ± SE. Asterisks indicate significant differences ($p < 0.05$) (*SA* salicylic acid, *CHI* chitosan, *MeJA* methyl jasmonate, *CL* control)

Protective effects of PM extracts against oxidative damage in human dermal fibroblasts

Dermal fibroblasts play a pivotal role in the maintenance of the structural integrity of skin. Deregulation of proliferative and metabolic activities of fibroblasts can lead to skin aging processes (Varani et al. 2006). In this study, we used BJ cells as a model for human dermal fibroblasts to investigate the biological activities of each of the elicited suspension cells of PM extracts. We demonstrated that none of the extracts at the selected concentrations (12.5–100 μg/mL) caused toxicity to fibroblasts, supporting the safety of using elicited suspension cells of PM extracts on skin cells (Fig. 6a, d and S2). Interestingly, SA-elicited PM cells significantly promoted the proliferation of dermal fibroblasts. The proliferation of BJ cells supplemented with SA-elicited PM cells was approximately 20% greater than that of the vehicle-control group (Fig. 6b, S2). The results from the proliferative study of dermal fibroblasts highlight the contribution of SA elicitors to the biological activities of suspension cells of PM.

Oxidative stress is strongly associated with the aging processes of human skin (Rinnerthaler et al. 2015). Thus, several investigators have focused on ameliorating skin aging by enhancing the capacity of skin cells to combat oxidative stress. Here, we used H_2O_2 as an inducer of oxidative stress and observed the protective activities of each PM-elicited

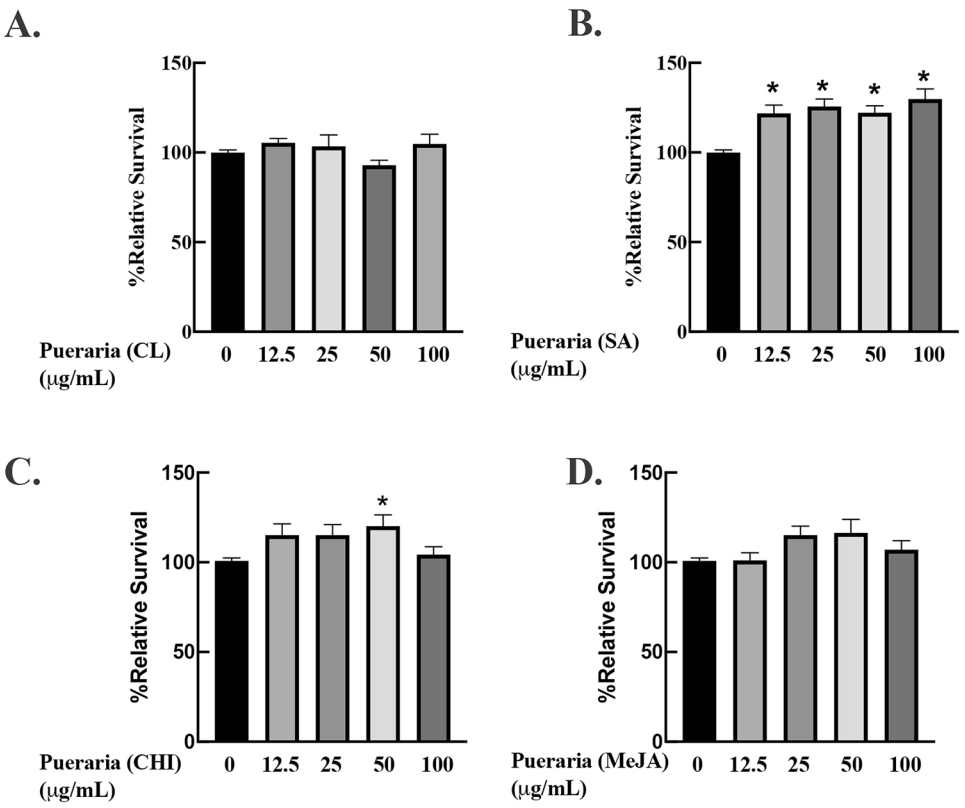


Fig. 6 Safety of extracts from suspension cells of *Pueraria candollei* var. *mirifica* on human dermal fibroblasts. Treatments with **a** CL-, **b** SA-, **c** MeJA-, and **d** CHI-elicited cell extracts demonstrated no cytotoxicity on dermal fibroblasts ($n = 3$; mean ± SEM; $^*p < 0.05$ vs. nontreated control; *CL* control, *SA* salicylic acid, *MeJA* methyl jasmonate, *CHI* chitosan)

extract against this oxidative insult. As demonstrated in Figs. 7 and S3, preincubation with elicited suspension cells of PM extracts prevented oxidative damage following exposure to H_2O_2. These results suggest that the protective effects of suspension cells of PM extracts are possibly due to an increase in the detoxifying capacity of fibroblasts against oxidative stress. Over similar dose ranges, we clearly demonstrated that suspension cells of PM with SA elicitors and nonelicitors had the strongest activity against oxidative insults (Fig. 7A and B and S3). These data strongly support the application of the SA elicitor to enhance the biological activities of PM suspension cells. The extracts of suspension cells of PM elicited with CHI and MeJA did not show promising results when compared with nonelicited suspension cells (Figs. 7c, d, S3).

The protective effects of PM extracts from our study were consistent with several lines of *in vitro* and *in vivo* evidence. For instance, miroestrol, an active phystoestrogen in PM, is reported to have the ability to improve the capacity of the antioxidant system to counterbalance oxidative stress *in vivo* (Chatuphonprasert et al. 2013). Similarly, puerarin, an isoflavone glycoside from the roots of *Pueraria*, protected neuronal cells from xenobiotic-induced cell death through an elevation in the activity of cellular antioxidant systems (Liu et al. 2014; Zou et al. 2013; Cheng et al. 2016). Moreover, puerarin has also shown protective activities in several *in vivo* models, e.g., arthritis (Wang et al. 2016), intestinal injury (Li et al. 2020) and ulcerative colitis (Jeon et al. 2020), through attenuation of the inflammatory response as well as increases in both the activity and expression of the antioxidant defensive system. Taken together, these results suggested that puerarin, which is highly present in SA-elicited cells, is a potential active constituent that contributes to protective activity against oxidative insults. However, we noticed that the isoflavonoid pattern of MeJA-elicited cells was similar to that of the control cells, but the activity was different. This suggested that not only isoflavonoids but also other compounds were involved in the biological activity. Thus, we can assume that the ability of SA-elicited suspension cell cultures of PM may come from the presence of puerarin and other compounds, which should be explored further for its antiaging potential, as it was nontoxic at the studied concentrations and was helpful in reducing oxidative stress induced in fibroblasts.

Conclusions

The results of this study showed that the use of elicitors on the suspension cell culture of PM enhanced the accumulation of isoflavonoids in the culture period of 20 days. Elicitation of suspension cells of PM by SA, being reported

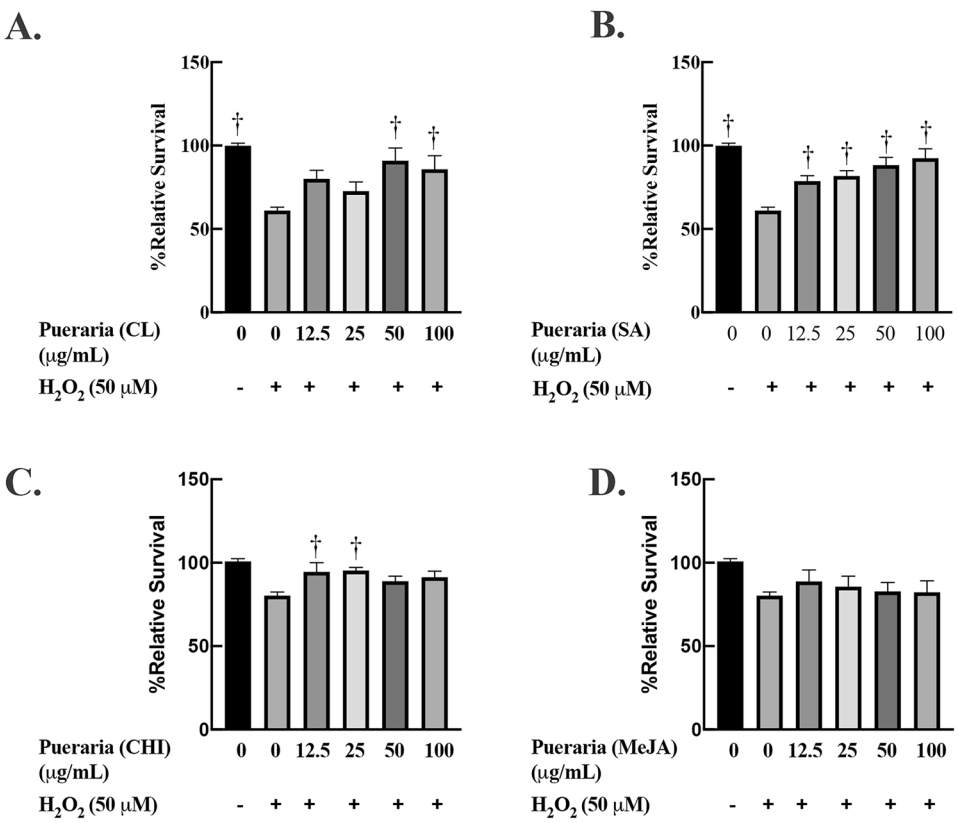


Fig. 7 Antioxidant activities of extracts from suspension cells of *Pueraria candollei* var. *mirifica* on human dermal fibroblasts. Preincubation with **a** CL- and **b** SA-elicited cell extracts attenuated H_2O_2-induced cell death, while pre-exposure to **c** MeJA- and **d** CHI-elicited cell extracts slightly prevented oxidative damage ($n = 3$; mean ± SEM; $^{\dagger}p < 0.05$ vs. H_2O_2-treated control; *CL* control, *SA* salicylic acid, *MeJA* methyl jasmonate, *CHI* chitosan)

for the first time, was most effective in producing isoflavonoids, namely, daidzein (2.37 mg/g DW), khwakhurin (1.05 mg/g DW), genistin (0.14 mg/g DW), and puerarin (5.13 mg/g DW), when compared with elicitation by MeJA and CHI. Knowledge of these effects will be useful when optimizing the medium composition suitable for plant cell culture, which can be adjusted for bioreactor-based scaled production for commercial purposes. The suspension cells showed promising antioxidant activity, especially elicitation with SA, which also correlated with the protective activity against oxidative damage conducted on BJ cells, in which cell proliferation was enhanced by 20% compared with the vehicle-control group. The substitution of synthetic with natural antioxidants may be beneficial because they are free of health implications and are cost effective, and elicited suspension cell culture of PM can be one such candidate.

Acknowledgements Tuber of soil-grown PM was kindly provided by Dr. Siriporn Wannachart, Kasetsart University, Thailand. This research was supported by the Rachadapisek Sompote Fund for Postdoctoral Fellowships from the Graduate School and Research Unit for Plant-Produced Pharmaceuticals from Chulalongkorn University, Thailand. This research was also supported by PMU-C of the Office of the National Higher Education Science Research and Innovation Policy Council.

Author contributions DR performed the experiments, analyzed data and wrote the manuscript. VB performed the antiaging assays and revised manuscript. KK helped in the experiment's preparation. WD supervised the project. SV designed the experiment, analyzed data and revised the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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