

Original Article

# Phytoestrogen constituents and estrogenic activity of *Pueraria candollei* var. *mirifica* callus and its extract preparation for removing cytotoxic constituents

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## Abstract

*In vitro* cultures of *Pueraria candollei* var. *mirifica* (PC) are a promising source of phytoestrogens, however, the estrogenic activity of PC callus has not been evaluated. In this study, the phytochemicals of PC callus were analyzed and compared with the estrogenic activities on MCF-7 cell proliferation. The ethanolic extract of PC callus contained isoflavonoids and deoxymiroestrol. The estrogenic activity of the extract (10 µg/mL) was equivalent to  $1 \times 10^{-10}$  M estradiol. When the crude extract was reconstituted with 10% (v/v) ethanol in water, most of the isoflavonoid and deoxymiroestrol contents were solubilized. The soluble fraction exhibited the estrogenic effect in a concentration-dependent manner, but treatment with the insoluble fraction at a high concentration (100 µg/mL) resulted in cell growth suppression. Overall, the crude ethanolic extract and 10 % (v/v) ethanol-soluble crude extract of PC callus were good sources of phytoestrogens. The reconstitution could remove toxic constituents, but the estrogenic activity was retained.

**Keywords:** *Pueraria candollei* var. *Mirifica*, isoflavonoids, chromenes, estrogenic activity

## 1. Introduction

*Pueraria candollei* var. *mirifica* (PC), known as *Pueraria mirifica* or locally as White Kwao Krua, is a Thai medicinal plant that has been used for a long time in folk medicine for rejuvenation. The phytochemical constituents of

this plant include isoflavonoids such as daidzein, genistein, daidzin, genistin, puerarin, and kwakhurin. The potent phytoestrogens of PC belongs to the family of chromenes, including miroestrol (MI), deoxymiroestrol (DMI), and isomiroestrol (ISO) (Chansakaow *et al.*, 2000). Recently, the tuberous root of PC has demonstrated strong estrogenic properties in MCF-7 cell proliferation assays (Cherdshewasart, Cheewasopit, & Picha, 2004; Cherdshewasart, Traisup, & Picha, 2008), yeast estrogen screen (YES) assays (Boonchird, Mahapanichkul, & Cherdshewasart, 2010),

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animal studies (Cherdshewasart, Kitsamai, & Malaivijitnond, 2007; Jaroenporn, Urasopon, Watanabe, & Malaivijitnond, 2014; Malaivijitnond, Kiatthaipipat, Cherdshewasart, Watanabe, & Taya, 2004; Trisomboon, Malaivijitnond, Watanabe, Cherdshewasart, & Taya, 2006; Trisomboon, Malaivijitnond, Watanabe, & Taya, 2004), and clinical studies (Chandeying & Sangthawan, 2007; Suwanvesh, Manonai, Sophonsritsuk, & Cherdshewasart, 2017). Regarding the estrogenic effect, PC is currently used as the main active ingredient in traditional medicine recipes, dietary supplements, and cosmetic products that are widely distributed in the global markets.

The conventional method of obtaining PC takes three to five years of growth as a crop before destructive harvesting of the root. The supply of PC from infield plantation is at a shortage because demands for PC for various health product industries are continuously increasing. Alternative sources for PC phytochemicals are of interest. Plant tissue culture techniques reduce the cultivation period and variations in the phytoestrogen content caused by uncontrollable environmental factors (Cherdshewasart *et al.*, 2007; Cherdshewasart & Sriwatcharakul, 2007). The PC callus established using Murashige and Skoog (MS) medium (0.1 ppm of thidiazuron (TDZ), 0.5 ppm of naphthaleneacetic acid (NAA), and 1.0 ppm of benzyladenine (BA)) accumulated isoflavonoids as the principal chemicals (20.72 mg/g dry weight) and 184.83 µg/g dry weight of the total miroestrol and deoxymiroestrol content (Udomsuk, Juengwattanarakul, Jarukamjorn, & Putalun, 2012). The PC callus initiated and cultured in MS medium containing 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.46 µM kinetin produced daidzein and genistein with contents of 5.12 and 2.77 mg/g dry weight, respectively (Thanonkeo & Sanha, 2006). Additionally, many reports have been published on the production of isoflavonoids by the callus, cell suspension, and hairy root culture of PC (Boonsnongcheep *et al.*, 2010; Udomsin *et al.*, 2019; Udomsuk, Jarukamjorn, Tanaka, & Putalun, 2009a, 2009b). Culture of PC cell suspensions was efficient for the large-scale production of isoflavonoids and deoxymiroestrol (Udomsin *et al.*, 2019). The phytochemical content of the PC callus was comparable to or higher than, in some cases, that in the intact plant. However, the estrogenic activity of the PC callus has not been investigated. Previously, the PC cell culture medium, which contains puerarin, daidzin, genistin, daidzein, and genistein, exhibited weaker estrogenic activity in ovariectomized rats than the tuberous root (Saisavoev *et al.*, 2014). However, the content of potent phytoestrogens such as deoxymiroestrol in the medium was not analyzed.

Until now, studies on the estrogenic activity and safety of PC phytochemicals have focused on puerarin, daidzin, genistin, isomiroestrol, miroestrol, deoxymiroestrol, daidzein, and genistein. Solvent optimization for the PC tuber extract indicated that ethanol is the most appropriate for MI and DMI extraction (Yusakul *et al.*, 2018). In another report, 95% ethanol showed the highest extraction yield of isoflavonoids, MI, and DMI from the PC tuber (Peerakam *et al.*, 2018), which decreased the ethanol concentration to lower than the extraction capacity. However, the extraction of PC using 95% and absolute ethanol produced an extract with

unknown nonpolar components, compromising the standardization methods due to too complicated compositions. When many chemical components of a plant extract are unidentified, the quality control for batch-to-batch consistency of the extract is more difficult. Therefore, simplification of the PC phytochemical composition by reconstitution of the ethanol extract in an aqueous solvent is a simple procedure to remove the unknown nonpolar constituents. Finally, most of the chemical components in the resultant PC extract were identified, resulting in simplicity for robust standardization of the PC extract.

In this study, we investigated the accumulation of bioactive compounds to include either the potent chromenes or isoflavonoids of the PC callus extract. Next, the estrogenic activities of MCF-7 cell proliferation were investigated. The simplification of extract chemicals was performed via reconstitution in 10% (v/v) aqueous ethanol. The chemical content and estrogenic activity were compared between 10% ethanol-soluble (10ES) and -insoluble (10EI) fractions. Thus, this research established the callus culture system and its extract preparation and then confirmed the estrogenic activity of the PC callus as an alternative source of PC phytoestrogens.

## 2. Materials and Methods

### 2.1 Chemicals and reagents

Puerarin was obtained from Sigma-Aldrich (MO, USA). Daidzin, daidzein, genistin, and genistein were obtained from Fujicco Co., Ltd. (Kobe, Japan). Miroestrol, deoxymiroestrol, isomiroestrol, and kwakhurin were isolated from the tuberous root of *P. candollei* and were identified by Nuclear Magnetic Resonance spectroscopy as previously described (Chansakaow *et al.*, 2000). All other chemical reagents in our experiment were of analytical grade.

### 2.2 Plant materials

The seeds of *P. candollei* var. *mirifica* (PC) were collected from Suranaree University of Technology (Nakhon Ratchasima province, Thailand). Voucher specimens (NI-PSKKU 007) were deposited at the Herbarium of the Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand.

The *in vitro* callus culture of PC was established from *in vitro* plantlets. The PC seeds were washed with sterile distilled water and then were sterilized with 3% (w/v) sodium hypochlorite for 20 minutes. The seeds were washed three times with sterile distilled water and then were immersed in 95% ethanol for 1 minute. The sterilized seeds were germinated on hormone-free and solidified MS medium with 3% (w/v) sucrose (pH 5.5). Plantlets were observed within 14 days of culture. The stems of the *in vitro* plantlets were cut into small pieces and were placed on MS medium supplemented with 0.1 ppm of TDZ, 1 ppm of NAA, and 0.5 of ppm BA for callus induction as described previously (Udomsuk *et al.*, 2012). Three-gram initial fresh weight of the callus was subcultured to the same fresh medium every four weeks. All *in vitro* cultures were cultured at 25 °C and for 16 hours/day in fluorescent light.

### 2.3 Sample preparation

*In vitro* stem-derived calluses were collected and dried in a hot-air oven at 50 °C until completely dry. The dried materials were ground into a fine powder. Ethanol (1 mL) was added to 200 mg of the sample powder. Absolute ethanol was selected to effectively extract compounds from plant material as described previously (Yusakul *et al.*, 2018). Next, the sample was extracted by sonication for 20 minutes. After centrifugation, the extract was collected. The extraction process was repeated four times. Finally, the extracts were combined and evaporated at room temperature to produce the crude ethanolic extract.

### 2.4 Reconstitution of the crude extract with aqueous ethanol

The ethanolic extract of the PC callus was prepared as described in the sample preparation section. The callus extract was collected and dried under vacuum in a rotary evaporator. The preliminary experiment using 5–20% of solvent for reconstitution found that 10% ethanol is the most appropriate to recover isoflavonoids and deoxymiroestrol (data not shown). The crude ethanolic extract (2 g) was dissolved in 400 mL of 10% (v/v) ethanol in distilled water. This ratio of water and ethanol was used to separate the polar compounds from the crude extract. The unknown nonpolar compounds were retained in the insoluble fraction. Sonication was applied for 60 minutes to accelerate solubilization, and then the mixture was transferred to a 50-mL tube, followed by centrifugation at 4,000 rpm for 5 minutes (25 °C). The soluble fraction of the extract was collected and concentrated under vacuum in a rotary evaporator to produce the soluble crude extract (10ES). The remaining precipitate in the tube was collected, combined, and dried to produce the insoluble fraction of the crude extract (10EI).

### 2.5 Determination of isoflavonoids by gradient HPLC

Six isoflavonoids (puerarin, daidzin, genistin, daidzein, genistein, and kwakhurin) were analyzed by HPLC using a gradient mobile phase system. This method was modified from a previously reported study (Udomsin, Juengwatanatrakul, Yusakul, & Putalun, 2015) and performed on a Shimadzu® equipped UV-vis detector (280 nm). A reversed-phase column (LiChroCart®; 250 mm × 4 mm, 5 µm) was utilized, and the column oven was set at 30 °C. The autosampler injected a sample solution (20 µL) of PC callus extract to the column. The gradient elution program was created by varying the proportions of solvent A (1.5% (v/v) acetic acid in water) and solvent B (100% acetonitrile). The flow rate was set at 1 mL/min. The column was eluted using a linear gradient program of 15–20% B over 0–15 minutes, 20–40% B over 15–40 minutes, 40–100% B over 40–45 minutes, and then maintaining 100% B for 5 minutes to elute the unwanted matrix. Later, the gradient elution program was changed from 100% to 15% B over 5 minutes and was maintained at 15% B for 5 minutes. Thus, the mobile phase system was returned to the initial conditions. The condition is suitable for isoflavonoids determination, as shown in the chromatograms (Figure 1).

### 2.6 Determination of chromenes by indirect competitive ELISA

All crude extract solutions of the PC callus were investigated using an indirect competitive ELISA (icELISA) to determine the chromene content (MI, DMI, and ISO); the method was modified from those in prior reports (Kitisripanya *et al.*, 2017; Yusakul *et al.*, 2013a, 2013b). First, ovalbumin conjugates of MI, DMI, or ISO were individually coated on the surface of the 96-well immune plate (100 µL, 2.5 µg/mL

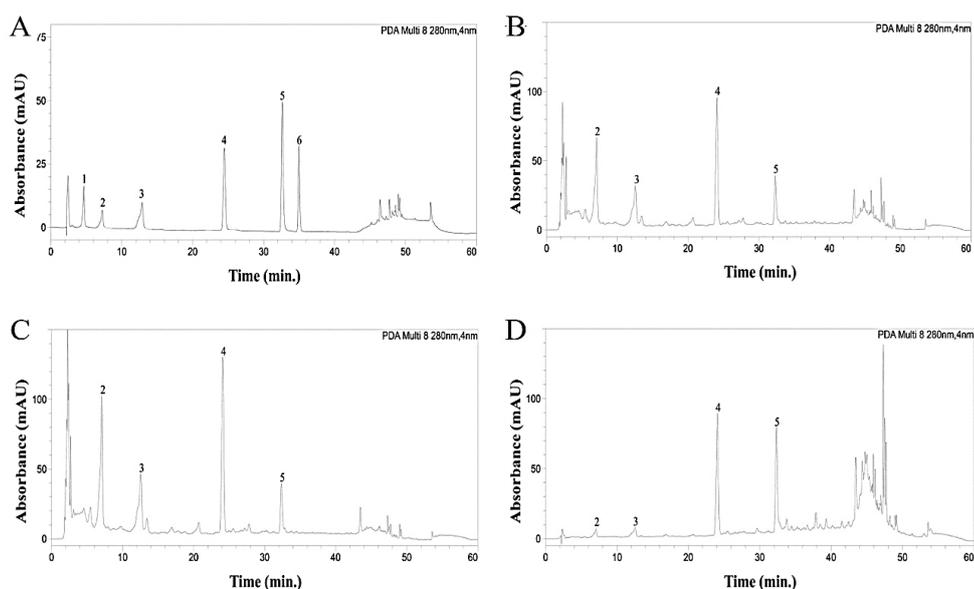


Figure 1. HPLC chromatograms of (A) the standard isoflavonoids (1, puerarin; 2, daidzin; 3, genistin; 4, daidzein; 5, genistein; 6, kwakhurin), (B) ethanol crude extract of PC callus, (C) 10% (v/v) ethanol-soluble fraction of the extract (10ES), and (D) 10% (v/v) ethanol-insoluble fraction of the extract (10EI)

in 50 mM sodium carbonate buffer at pH 9.6) for 1 hour at 37 °C. Next, the plate was washed with 0.05% (v/v) Tween 20 in 10 mM phosphate-buffered saline (T-PBS). Second, the plate surface was treated with 300 µL of 1% (w/v) gelatin in phosphate-buffered saline (PBS) for 1 hour at 37 °C, and then excess skim milk was washed out with T-PBS. Third, the solution of each serial concentration of authentic MI, DMI, ISO, or sample solution prepared in 20% (v/v) aqueous ethanol was added (50 µL) to each well. Additionally, 50 µL of antibody against MI, DMI, or ISO, prepared in T-PBS, was added in the well. After 1 hour of reaction, the complex of free MI, DMI, or ISO and its antibody were washed out with T-PBS. Next, a solution (100 µL) of secondary antibodies conjugated with the detection enzyme, specific for the antibodies reacted with ovalbumin conjugates of MI, DMI, or ISO, was added in the well for 1 hour. After washing with T-PBS, 100 µL of the substrate solution was added and incubated for 20 minutes. The absorbance was measured at 405 nm using a microplate reader (Model 550 Microplate Reader; BioRad Laboratories, CA, USA).

### 2.7 MCF-7 cell culture

Human breast adenocarcinoma MCF-7 cells (estrogen receptor alpha-positive) were obtained from the American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and antibiotics (100 U/mL of penicillin and 100 µg/mL of streptomycin) and were grown at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>.

### 2.8 Proliferation assay in MCF-7 cells

MCF-7 cells were suspended in estrogen-free medium (phenol red-free DMEM/F12 with 10% (v/v) charcoal-treated fetal bovine serum and antibiotics) and were seeded into 96-well plates at a density of  $7 \times 10^3$  cells/well. After 48 hours of culture, the cells were exposed to  $1 \times 10^{-10}$  M estradiol (E<sub>2</sub>) or different concentrations of test samples in 0.1% (v/v) ethanol in estrogen-free medium. Cell proliferation was assessed after 6 days of exposure, during which the medium was changed every 3 days. The cells were incubated with 10 µL/well of 5 mg/mL of tetrazolium 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution and 100 µL/well of estrogen-free medium for 2 hours at 37°C. Next, the solution (100 µL, 2% (v/v) glacial acetic acid, 40% (v/v) dimethylformamide, and 16% (w/v) sodium dodecyl sulfate in distilled water) were added for formazan solubilization under shaking for 15 minutes, and then the absorbance was read at 595 nm using a microplate reader. The cell proliferative response was defined as an increase in the frequency of proliferating cells (RPE%, relative proliferation effect):

$$RPE(\%) = (S/E) \times 100$$

where S and E are the cell proliferation levels as treated with the sample and with  $10^{-10}$  M estradiol, respectively.

### 2.9 Statistical analysis

Determination of the phytoestrogen content was performed in triplicates, and the assays of MCF-7 cell

proliferation were performed in six replicates. The results are expressed as the means±SDs. The differences in means were investigated using one-way analysis of variance (ANOVA) followed by Duncan's test ( $p < 0.05$ ).

## 3. Results and Discussion

### 3.1 Extraction and phytoestrogens of the PC callus

*In vitro* callus culture of plant tissue was performed in an aseptic and controlled environment. The callus is a promising source of important PC phytochemical production because the callus grows quickly. Therefore, this *in vitro* cultivation resolved the long period required for PC growth in a field plantation. Moreover, the technique provides high, consistent, and continuous production of PC phytoestrogens in a short period of culture (Boonsongcheep *et al.*, 2010; Korsangruang, Soonthornchareonnon, Chintapakorn, Saralamp, & Prathanturug, 2010; Udomsuk *et al.*, 2009b). Therefore, our research group developed the PC callus culture to study the possibility of using an alternative material for health products from PC. The callus culture was developed using MS medium supplemented with 0.1 ppm of TDZ, 1 ppm of NAA, and 0.5 of ppm BA, which produced 20.72 mg of total isoflavonoids per gram dry weight of callus, and 184.83 µg/g dry weight of deoxymiroestrol (Udomsuk *et al.*, 2012). This callus produced phytoestrogens at an amount exceeding that of an intact PC tuber. However, no report is available on the estrogenic effect of the PC callus induced using the plant growth regulators mentioned above. The ethanolic extract of the PC callus comprised isoflavonoids (26.81 mg/g crude extract) and deoxymiroestrol (383.73 µg/g crude extract). The crude ethanolic extract of the PC callus accumulated only four isoflavonoids (daidzin, daidzein, genistin, and genistein), whereas puerarin and kwakhurin could not be found (Figure 2). The chemical profile of the PC callus was similar to that reported previously (Rani, Meelaph, Kobtrakul, & Vimolmangkang, 2018; Udomsin, Yusakul, Kitisripanya, Juengwatanatrakul, & Putalun, 2019) where the principal isoflavonoids of the PC callus were daidzin, daidzein, genistin, and genistein. In the intact PC, however, puerarin was predominantly accumulated in the tuber (Cherdshewasart & Sriwatcharakul, 2007). Transcriptome analysis indicated that puerarin was synthesized by C-glycosyltransferase using daidzein as a substrate (Suntichaikamolkul *et al.*, 2019). The expression of C-glycosyltransferase in the PC callus might be low.

The crude ethanolic extract of the callus was dissolved with 10% (v/v) ethanol in distilled water, and then the yields of the soluble and insoluble fractions were recorded. Two grams of crude callus extract (100%) was divided by the process of reconstitution into soluble crude extract (1.44 g, 78.26%) and insoluble crude extract (0.40 g, 21.74%). The phytoestrogens and estrogenic activity of the extracts were evaluated. The results indicated that almost all the contents of daidzin, daidzein, genistin, and genistein, were dissolved in 10% (v/v) ethanol in water, especially polar isoflavonoids such as daidzin and genistin (Figure 2). However, the insoluble fraction of the crude extract was found to contain daidzein and genistein as the major chemicals. These results suggest that daidzein and genistein, the aglycone parts of daidzin and genistin, were sparingly soluble in 10% ethanol in

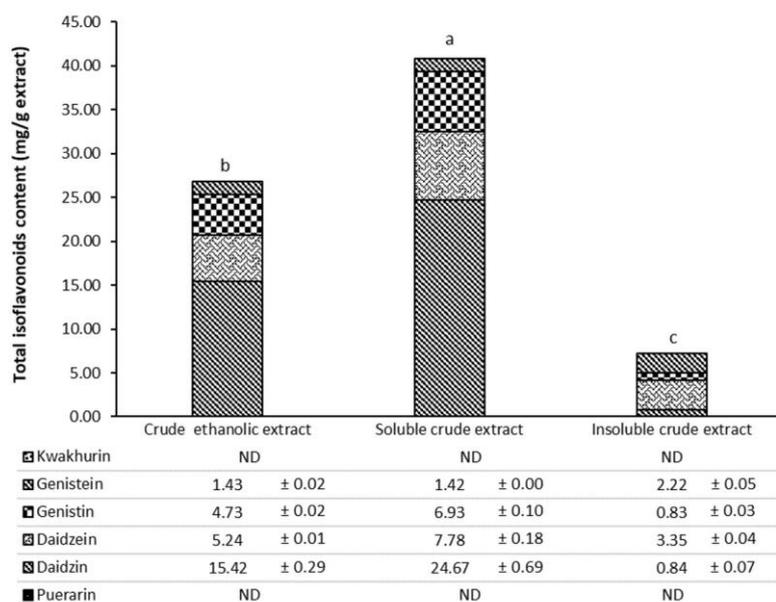


Figure 2. Isoflavonoid contents of the crude ethanolic extract of PC callus, and its 10% ethanol-soluble (10ES) and insoluble (10EI) crude extracts. Data are given as mean±SD from three replicates. Different letters indicate statistically significant differences in total isoflavonoids content between samples at  $p < 0.05$  by Duncan's multiple range test. ND indicates that the compound was not detected.

water. Thus, these components were not completely separated into 10ES fractions from the crude extract. The MI, DMI, and ISO contents of the crude ethanolic extract and its fractions of 10ES and 10EI components were determined using icELISA (Figure 3). The crude ethanolic extract of the callus accumulated only DMI, similar to that reported previously (Udomsuk *et al.*, 2012). When the crude ethanolic extract was dissolved in 10% ethanol, DMI could not be perfectly solubilized because the DMI contents in the soluble crude extract and insoluble crude extract were not significantly different. However, compared with the recovery percentage of DMI (Figure 4), the soluble fraction (79.02% yield) comprised more DMI than the insoluble fractions of the PC callus (20.98% yield) by about four-fold. Additionally, the insoluble crude extract showed a low content of MI, likely due to condensation of the crude extract in the precipitate, in which the concentration of MI was sufficiently high to be analyzed.

Previously, the solvents, including various concentrations of ethanol in water (50–95% (v/v)), were investigated for the effective extraction of PC phytochemicals. The results indicated that 95% ethanol was the most effective for puerarin, daidzin, daidzein, genistin, genistein, MI, and DMI extraction, but the extraction capacity of these compounds decreased when the concentration of ethanol in the solvent was lowered (Peerakam *et al.*, 2018). The extraction of MI and DMI by water also produced a low yield compared with ethanol (Yusakul *et al.*, 2018). However, the reconstitution procedure using 10% aqueous ethanol can recover both the isoflavonoid and DMI contents from the crude ethanol extract. Direct extraction of PC using 10% ethanol in water may not be effective and produced different results compared with the reconstitution procedure. For direct extraction, ethanol has a lower viscosity than water, and ethanol can disrupt the plant cell membrane to facilitate the penetration and extraction of phytochemicals. Water can dissolve PC chemicals; however, it penetrates less across the

cell membrane. Reconstitution of the PC callus ethanolic extract is an effective approach to remove nonpolar contaminants extracted using absolute ethanol. The resultant extract is less complicated with chemical constituents, and estrogenic retention can be investigated to ensure lasting efficacy.

### 3.2 Proliferation assay in MCF-7 cells

The estrogenic activities of the PC callus crude extract and its fractions were determined using MCF-7 cell proliferation. Both the crude ethanolic extract of the PC callus and its 10ES fraction showed estrogenic activity in a dose-dependent manner in the concentration range of 0.1–100  $\mu\text{g/mL}$  (Figure 5). By contrast, the 10EI fraction of the PC callus extract exhibited a dose-dependent proliferative effect at the concentration of 0.1–10  $\mu\text{g/mL}$ , and an antiproliferative effect on MCF-7 cells was observed at 100  $\mu\text{g/mL}$ . The 10EI fraction comprised various nonpolar components extracted using ethanol. The nonpolar constituents of the PC callus ethanolic extract were separated after reconstitution with 10% ethanol. Similar to that reported previously (Cherdshewasart *et al.*, 2008), the tuberous root extracts of PC at concentrations of 10, 100, and 1,000  $\mu\text{g/mL}$  with highly concentrated phytochemicals also exhibited cytotoxicity effects on MCF-7 cells. The highest stimulated MCF-7 cell proliferation was presented at 100  $\mu\text{g/mL}$  of the crude ethanolic extract. The 10ES fraction from the PC extract at 100  $\mu\text{g/mL}$  and 10EI (10  $\mu\text{g/mL}$ ) stimulated the growth and proliferation of MCF-7 cells more than  $10^{-10}$  M estradiol (100 RPE%). However, low concentrations (0.1 and 1  $\mu\text{g/mL}$ ) of all extracts showed less proliferation of MCF-7 cells than  $10^{-10}$  M estradiol. Overall, the reconstitution of the PC callus extract with 10% ethanol removed unknown and toxic components while retaining the estrogenic activity of the PC callus extract.

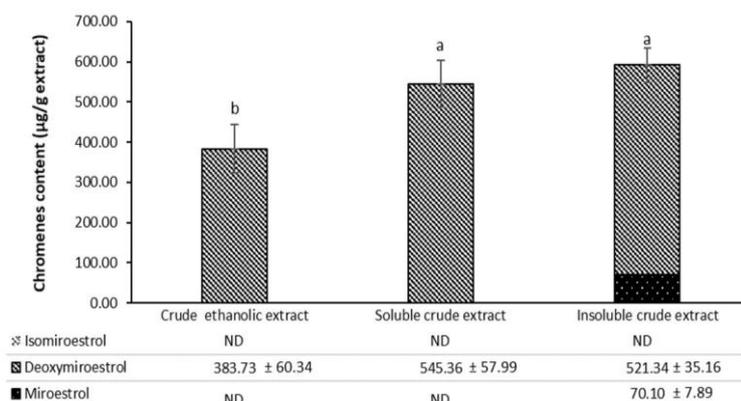


Figure 3. Chromene contents of the crude ethanolic extract of PC callus, and its 10% ethanol-soluble (10ES) and insoluble (10EI) crude extracts. Data are given as mean±SD from three replicates. Different letters indicate statistically significant differences in total chromene content between samples at  $p < 0.05$  by Duncan's multiple range test. ND indicates that the compound was not detected.

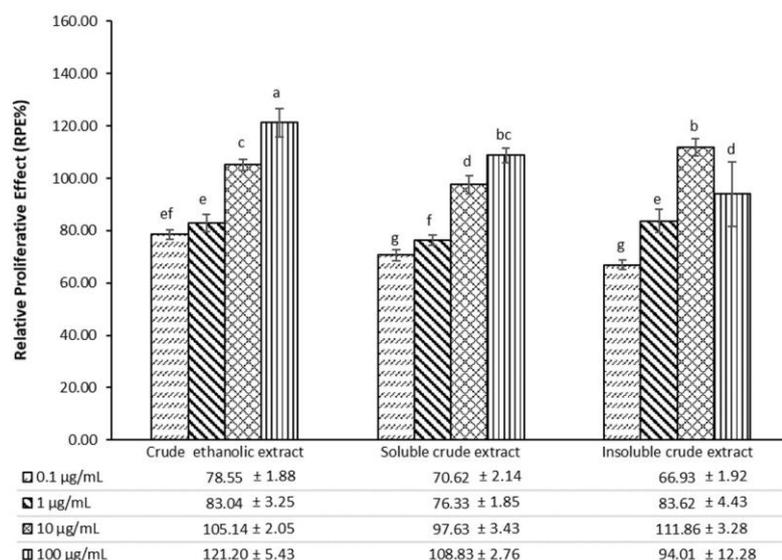


Figure 5. The MCF-7 cell proliferation effects of the crude ethanolic extract of PC callus, and its 10% ethanol-soluble (10ES) and insoluble (10EI) crude extracts. The proliferative effect is relative to  $E_2$  ( $1 \times 10^{-10}$  M estradiol, 100%), and is expressed as Relative Proliferative Effect (RPE). The results are expressed as mean±SD ( $n=6$ ). Different letters indicate statistically significant differences at  $p < 0.05$  by Duncan's multiple range test.

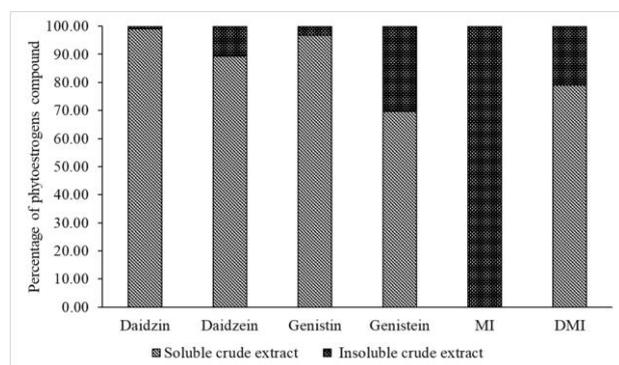


Figure 4. The percentage of phytoestrogen amounts in 10% ethanol-soluble (10ES) and insoluble (10EI) crude extracts calculated from the yields obtained after reconstitution of each phytoestrogen component, where the amount of these phytoestrogens in the ethanol crude extract was referred to as the 100% amount.

#### 4. Conclusions

The PC callus-derived crude extract comprised total isoflavonoids ( $26.81 \pm 0.32$  mg/g extract) and deoxymiroestrol ( $383.73 \pm 60.34$  µg/g extract). The most abundant isoflavonoid of the PC callus extract was daidzin, whereas only DMI was found as the chromene constituent. Reconstitution of the extract using 10% ethanol in water separated most of the isoflavonoids ( $40.80 \pm 0.89$  mg/g extract) and deoxymiroestrol ( $545.36 \pm 57.99$  µg/g extract) into the 10ES fraction. The unknown nonpolar components were retained in the 10EI fraction. The MCF-7 cell proliferation assay confirmed that the crude ethanolic extract and its 10ES extract (100 µg/mL) had estrogenic activities higher than  $10^{-10}$  M estradiol. Furthermore, the 10EI fraction of the PC callus at 100 µg/mL showed a toxic effect on MCF-7 cells. Hence, the crude ethanolic extract and its 10ES fraction from the PC callus might be useful alternative phytoestrogen sources for health products.

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