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Polarity Difference and the Presence of Phytoestrogen Compounds Affecting Estrogenic Activity of *Peperomia pellucida* Extracts

(Perbezaan Kekutuban dan Kehadiran Sebatian Fitoestrogen yang Mempengaruhi Aktiviti Estrogen Ekstrak *Peperomia pellucida*)

I Gusti Agung Ayu Kartika, Muhamad Insanu, Catur Riani, Kyu Hyuck Chung, I Ketut Adnyana*

ABSTRACT

Peperomia pellucida *(L.) Kunth has been studied as an anti-osteoporotic agent. However, there is no report about its estrogenic activity, which is important for its anti-osteoporotic activity. Thus, the aim of this research was to study the estrogenic potency of* P. pellucida *extracts. The estrogenic activity of* P. pellucida *extracts (n-hexane, ethyl acetate, ethanol, and water extracts) was studied using E-screen assay and confirmed with a molecular docking simulations. Further, the presence of phytoestrogen compounds was identified using thin layer chromatography (TLC), TLC densitometry, and high performance liquid chromatography. The n-hexane, ethyl acetate, and ethanol extracts at a concentration of 0.1 µg mL^{-1} exhibited a partial agonist effect, whereas the water extract showed full agonist effect at the similar concentration. This activity was produced through a classical ligand-dependent mechanism similar to estradiol. N-hexane and ethyl acetate extracts showed antiestrogenic activity. The TLC chromatogram evidently depicted the presence of quercetin and stigmasterol in the n-hexane and ethyl acetate extracts. Apigenin and apigetrin at concentrations of 0.239±0.076 and 1.063±0.156 µg mg^{-1} extract, respectively, were present in the water extract. A docking study on estrogen receptors confirmed that apigetrin prefer to produce estrogenic activity, whereas the other compounds can produce both estrogenic and antiestrogenic activity. Hence, we suggest that the bioactive compounds in the water extract are flavonoids, such as apigenin and apigetrin. In summary, the water extract is recommended to be used as an estrogenic agent.*

Keywords: Apigenin; apigetrin; docking; estrogenic; extract; Peperomia pellucida

ABSTRAK

Peperomia pellucida *(L.) Kunth telah dikaji sebagai agen anti-osteoporosis. Walau bagaimanapun, tiada laporan mengenai aktiviti estrogennya yang penting untuk aktiviti anti-osteoporosisnya. Oleh itu, tujuan penyelidikan ini adalah untuk mengkaji potensi estrogen ekstrak* P. pellucida*. Aktiviti estrogen ekstrak* P. pellucida *(n-heksana, etil asetat, etanol dan ekstrak air) telah dikaji dengan menggunakan ujian E-screen dan disahkan dengan simulasi dok pengimejan. Selanjutnya, sebatian fitoestrogen telah dikenal pasti menggunakan kromatografi lapisan tipis (KLT), KLT densitometri dan kromatografi cecair prestasi tinggi. Ekstrak n-heksana, etil asetat dan etanol pada kepekatan 0.1 µg mL^{-1} menunjukkan kesan agonis separa, manakala ekstrak air menunjukkan kesan agonis penuh pada kepekatan yang sama. Aktiviti ini dihasilkan melalui mekanisme ligan klasik yang sama seperti estradiol. Semua ekstrak juga menunjukkan aktiviti antiestrogenik. Kromatogram KLT jelas menggambarkan kehadiran quersetin dan stigmasterol dalam ekstrak n-heksana dan etil asetat. Apigenin dan apigetrin masing-masing pada kepekatan 0.239±0.076 dan 1.063±0.156 µg mg^{-1} ekstrak, hadir dalam ekstrak air. Kajian dok mengenai reseptor estrogen mengesahkan bahawa apigenin dan apigetrin lebih suka menghasilkan aktiviti estrogenik, sedangkan quersetin dan stigmasterol dapat menghasilkan kedua-dua aktiviti estrogenik dan antiestrogenik. Oleh itu, kami mencadangkan bahawa sebatian bioaktif dalam ekstrak air adalah flavonoid, seperti apigenin dan apigetrin. Ringkasnya, ekstrak air disyorkan untuk digunakan sebagai agen estrogen.*

Kata kunci: Apigenin; apigetrin; dok; ekstrak; estrogen; Peperomia pellucida

Introduction

Estrogen is classified as a class of steroid hormones. This hormone holds important roles in the human body and is implicated in many different diseases such as obesity, neurodegenerative diseases, cardiovascular disease, insulin resistance, lupus erythematosus, fibroids, endometriosis, osteoporosis, and cancer (Burns & Korach 2012).

Estrogen function is mediated by estrogen receptor (ERs), of which there are two distinct and functional ERs called ERα and ERβ. In many tissues, activations of ERα and ERβ are found to be antagonistic. For instance, in the prostate and the breasts, activation of ERα can promote cell proliferation and survival, whereas ERβ can suppress it (Nelson et al. 2014; Williams et al. 2008). Also, in the bones, estrogen can induce osteoclast apoptosis via ERα and not ERβ (Khalid & Krum 2016). Furthermore, several studies have shown that many chemicals that have estrogenic activity can bind to both types of estrogen receptors (Lecomte et al. 2017).

Phytoestrogens are polyphenolic compounds from plants that may exert estrogenic activity, primarily by binding to ERs. Phytoestrogens can have agonists, partial agonists, or antagonists action toward ERs (Mostrom & Tim 2018). They act as a selective estrogen receptor modulators (SERMs) similar to tamoxifen, the breast cancer drug. Tamoxifen is biologically active as a partial agonist on selected target tissues including bone, cardiovascular, and the uterus, but is mostly antagonistic in the breast (Peng et al. 2009). This special characteristic makes SERMs one of the drugs of choice in contemporary osteoporosis treatment.

Currently, natural products are widely studied in the search for a new, safe, and economically efficient antiosteoporosis agent. *Peperomia pellucida* (L.) Kunth is one of the medicinal plants that is of great interest worldwide and has been studied to treat osteoporosis. Recently, this plant was used as a candidate to find an antiosteoporosis agent (Florence et al. 2017; Kartika et al. 2018; Ngueguim et al. 2013; Putri et al. 2016). One compound that exerts estrogen-like properties was unexpectedly found together with some compounds that have the opposite effect from the ethanol extract of *P. pellucida* by Xu et al. (2006) in their study to find anticancer agents. The finding raises a great curiosity for further research on the estrogenic activity profile of this plant.

Thus far, there are no data available about the estrogenic effect from *P. pellucida* extracts. Considering the need of more supporting data for antiosteoporosis research specifically, the aim of this study is to investigate the estrogenic profile of various *P. pellucida* extracts with different polarities through a robust *in vitro* assay, detection of phytoestrogen compounds through Thin Layer Chromatography (TLC), Thin Layer Chromatography-Densitometry (TLC-D), and High Performance Liquid Chromatography (HPLC). Also, molecular docking simulations were performed to evaluate the binding affinity of the phytoestrogen compounds present in this plant to ERs. To the best of our knowledge, it is the first report of estrogenic activity potency of *P. pellucida* extracts.

Materials and Methods

Materials

P. pellucida plant was collected in March and April 2016 from Cagak and Ciater Region, West Java Province, Indonesia. The sample was authenticated by a botanist at the Herbarium Bandungense, Bandung Institute of Technology, Indonesia, referring to document number 705/I1.CO2.2/PL/2016. Extraction solvents including n-hexane, ethyl acetate, ethanol, and aquadest, were provided by CV Fadillah (Bandung Kulon, Indonesia). WST-1 reagent was purchased from Roche (Mannheim, Germany). Penicillin and streptomycin were obtained from GibcoBRL (Grand Island, NY, USA). Dulbecco's Modified Eagle's Medium (DMEM) and trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT). The other materials, i.e. dimethyl sulfoxide (DMSO), quercetin, stigmasterol, Na_2CO_3, 17β -estradiol and tamoxifen, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Extraction process

N-hexane, ethyl acetate, and ethanol extracts were prepared using sequential maceration for 3 × 24 h. The filtrate was collected and evaporated using a rotary evaporator. Meanwhile, water extracts were prepared separately using a similar method and concentrated using the freeze-drying method.

Cell culture

The estrogen-sensitive MCF-7 BUS human breast cancer cells used in the E-screen assay were kindly provided by Dr. Soto (Tufts University, MA, USA). Dulbecco's Modified Eagle's Medium supplemented with 5% fetal bovine serum, penicillin (100 units mL^{-1}), and streptomycin (100 μg mL^{-1}) were used as growth medium. The cells were incubated in an atmosphere of 5% CO_2/95% air and saturated humidity at 37 °C.

CYTOTOXICITY TEST

Cytotoxicity test using WST-1 cell proliferation assay was conducted prior to the E-screen assay as described by Kim et al. (2015b). At the end of the additional incubation period, all medium was removed and centrifuged at 8000 rpm, room temperature, for 4 min. The absorbance of 80 µL of the supernatant was measured at 440 versus 690 nm reference wavelength using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA).

E-SCREEN ASSAY

The E-screen assay was conducted according to previous studies (Oh et al. 2008; Perez et al. 1998). To determine the estrogenic activity of the extracts, each extract was added to the experimental medium at a range of concentrations from 0.1 to 10 µg mL^{-1}. In order to determine the mechanism of action of the samples, the extracts were combined with tamoxifen 10^{-6} M after which the decrease in activity was compared with estradiol. Also, in order to determine the antiestrogenic activity of the extract samples, estradiol (10^{-11} M, E_2) was treated with 0.1 µg/mL of samples. Absorbance was measured using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA).

PHYTOCHEMICAL SCREENING

Phytochemical analysis of the dry plant and extracts was done to evaluate the presence of alkaloids, flavonoids, saponin, tannins, quinones, steroids, and terpenoids. The tests were conducted according to the standard procedures described in the literature (Harborne 1998).

DETERMINATION OF TOTAL FLAVONOID CONTENT

The total flavonoid content was measured by colorimetric assay. One milliliter of aluminum chloride 10% and 8 mL of acetic acid 5% were added into 1 mL of sample with a certain concentration and was mixed thoroughly. After 18 min, the absorbance was determined at 413 nm. The mixture consisting of 1 mL sample and 9 mL aquadest was used as a control solution. The total flavonoid content was calculated by subtracting the absorbance of the control solution from the absorbance of the samples and then convert the value into µg QE/100 mg using the calibration curve.

DETECTION AND QUANTIFICATION OF PHYTOESTROGEN COMPOUNDS IN THE *P. pellucida* EXTRACT

Detection and quantification of phytoestrogen compounds in the *P. pellucida* extract was performed using TLC, TLC-D and HPLC. Phytosterol (stigmasterol) and flavonoids (apigenin, apigenin 7-O glucoside (apigetrin, cosmosiin), catechin, hyperoside, kaempferol, luteolin, luteolin 7-O glucoside, myricetin, naringenin, orientin, quercetin, and rutin) were used as standard compounds. First, a TLC analysis was performed on normal phase silica TLC plates (Merck) using certain mobile phases for each standard compound. One µL of 1 mg mL^{-1} methanolic solutions of standard phytoestrogen compounds and 10 µm/mL investigated extracts were spotted on the normal phase silica TLC plates, and one-dimensional TLC analysis was performed. The elution results were observed under UV light at 254 and 366 nm before and after spraying with 10% sulfuric acid, $AlCl_3$ 5% or sitroborat reagent.

A TLC-D analysis was performed on 4 × 8 cm aluminum plates coated with Si60 silica gel (Merck). Flavonoid standard and extract sample solutions were applied to the plates as 5-mm bands, 10 mm from the bottom edge of the plate, using a Linomat sample applicator. The application volume was 5 µL for the extract at 50000 ppm and 1 µL for the flavonoid standard at 500 ppm. Plates were developed to a distance of 6 cm at room temperature. Aluminium chloride was used as a spray reagent. Detection was performed using a densitometer Camag (Switzerland) at 366 nm.

HPLC was used to determine flavonoid compounds in the extracts. Each standard compound was prepared at 500 ppm and the extracts at 20000 ppm in methanol. Twenty µL samples were injected into the HPLC apparatus. Separation was carried out through column (5 µm; 4.6×250 mm, Kitenex) with diode-array detector. Solvent A (0.05% trifluoroacetic acid) and solvent B (0.038% trifluoroacetic acid in 83% acetonitrile (v/v) with the following gradient: 0-5 min, 15% B in A, 5-10 min, 70% B in A, 10-15 min, 70% B in A were used for separation. The flow rate was 1 mL min^{-1} and injection volume was 10 µL. The detection was performed at 280, 350, and 370 nm. The twelve standard compounds were run for comparable detection and optimized. All samples were assayed in triplicate. All quantitative data were explained by analysis software.

MOLECULAR DOCKING TEST

The conformation of ligands bound in the active site of estrogen receptor alpha (ERα) and beta (ERβ) were used as targets for the prediction of estrogenic activity of the phytoestrogen, which was determined using automated docking simulations. The protein structure files (PDB ID:1GWR for ERα and 3OLS for ERβ) were obtained from the Protein Data Bank (www.rcsb.org/pdb). The proteins were edited using Discovery Studio by removing the heteroatoms and water molecules. Phytoestrogen ligands were geometrically optimized using Avogadro. AutoDock Tools 4.0 was employed, using the Lamarckian genetic algorithm method, for docking preparation. Docking calculations were conducted using Command

Prompt with assigned Autodock4 and Autogrid4. All torsions were allowed to rotate during docking processes. The grid map was centered at particular residues of the protein which were generated using AutoGrid.

STATISTICAL ANALYSIS

The estrogenic activities were statistically analyzed using SPSS software version 22.0 for Windows. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) test or Student's t-test for normally distributed data, while Kruskal-Wallis test using Bonferroni adjustment or Mann Whitney U test was used to analyze the non-normally distributed data. Pearson product-moment correlation test was used to determine the correlation of the data. All values were expressed as mean±standard deviation (SD).

RESULTS AND DISCUSSION

CYTOTOXICITY TEST

The cytotoxicity test was conducted in order to choose the appropriate concentrations of extracts to be used in the E-screen assay. As seen in Table 1, the n-hexane extract at a concentration of 10 µg mL^{-1} showed significantly high toxicity ($p < 0.05$). Hence, the suitable maximum concentration for all of the extracts was 1 µg mL^{-1}. All of the samples at this concentration were considered non-toxic and were expected not to interfere with the results of the proliferative effect of the samples in the E-screen assay.

TABLE 1. Cytotoxicity test results of *P. pellucida* extracts using WST-1 cell proliferation assay

	% cytotoxicity (mean ± SD)		
Group of extracts	0.1 µg mL^{-1}	1 µg mL^{-1}	10 µg mL^{-1}
N-hexane extract	66.10 ± 1.30	100.21 ± 15.31	11.47 ± 1.26**
Ethyl acetate extract	100.62 ± 7.85	107.69 ± 4.75	130.40 ± 32.46
Ethanol extract	101.25 ± 33.16	105.45 ± 34.92	134.24 ± 48.14
Water extract	110.10 ± 20.37	133.23 ± 32.66	146.98 ± 38.30*

*($p < 0.05$), **($p < 0.01$) compared to control

E-SCREEN ASSAY

As depicted in Figure 1(a), each extract type has varying levels of estrogenic activity. Overall, the water extracts showed the highest estrogenic activity, while the n-hexane extracts the lowest. Estrogenic activity may support the antiosteoporosis activity of *P. pellucida*. Estrogen can downregulate TNF-α, IL-1, IL-6, and prostaglandin-E_2 that increase bone resorption by increasing receptor activator of NF-κB ligand or osteoclasts differentiating factor and decreasing osteoprotegerin secretion. Estrogen can also upregulate TGF-β, which acts as an inhibitor of bone resorption by decreasing activity and increasing apoptosis of osteoclasts (Riggs 2000).

The n-hexane, ethyl acetate, and ethanol extracts at a concentration of 0.01-1 µg mL^{-1} showed a partial agonist effect (RPE% 25-80%). Partial agonist estrogenic agents (antiestrogens) are an interesting topic. Partial agonists can work selectively, and can also play the role of an antagonist when a full agonist is present. This agent may either stimulate or block certain genes (MacGregor & Jordan 1998), resulting in phytoestrogens' ability to act not only as an estrogen-like compound but also able to antagonize the undesirable effect of estrogen such as proliferation in cancer. These properties make *P. pellucida* attractive targets for further characterization of their estrogenic activity.

Among the test samples, only the water extract showed a full agonist effect (RPE% > 80%). The estrogenic activity of the *P. pellucida* extracts occurred in a polarity-dependent manner. This finding suggests that the more polar the sample, the higher its estrogenic effect. The result supported a previous study that found the polar to mid-polar compounds mainly contribute to estrogenic activity through ERs (Creusot et al. 2013). This issue correlates with how a ligand binds to the ERs. Even

tamoxifen, a known antiestrogenic agent, undergoes bioactivation to become hydroxytamoxifen, which is more polar, giving it a higher affinity to the ERs.

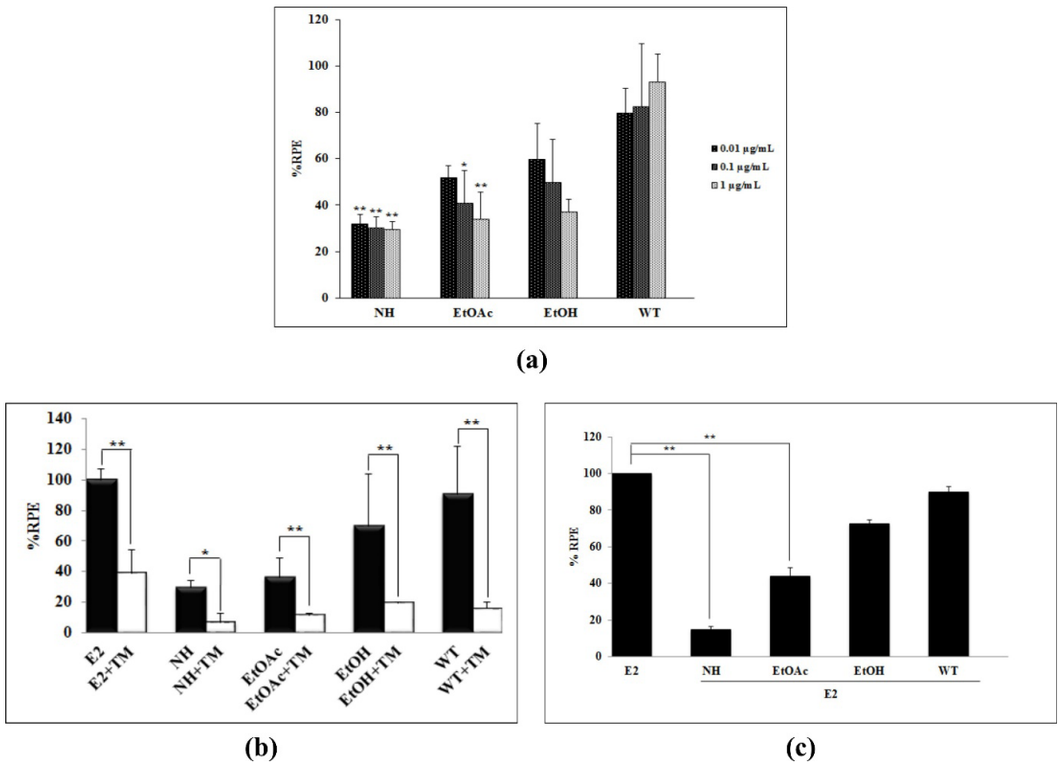

FIGURE 1. The estrogenic, mechanism, and antiestrogenic studies of *Peperomia pellucida* extracts. a: estrogenic test: estrogenic activity of four types of extracts from *P. pellucida* with E_2 10^{-9} M as standard control; b: mechanism study: estrogenic activity of the extracts alone and together with tamoxifen as antiestrogenic agent (extract 0.1 µg mL^{-1}; E_2 10^{-9} M; TM 10^{-6} M); c: antiestrogenic test: estrogenic activity of 17β-estradiol given alone and together with extracts at a concentration of 0.1 µg mL^{-1} (E_2 10^{-11} M). "*" ($p < 0.05$), "**" ($p < 0.01$). RPE, relative proliferative effect; NH, n-hexane extract; EtOAc, ethyl acetate extract; EtOH, ethanol extract; WT, water extract; E2, 17β-estradiol; TM, tamoxifen

The E-screen assay was also performed to analyze the estrogenic mechanism of the *P. pellucida* extracts. The proliferative effect of estradiol as a standard estrogenic agent and individual test extracts were compared with the proliferative effect of each of those estrogenic agents in combination with tamoxifen as the antagonist agent.

The result (Figure 1(b)) shows that the %RPE of estradiol decreased significantly when in combination with tamoxifen. The significant decrease of estradiol's proliferative effect was also shown in other test groups. This result indicates that the estrogenic activity of the extracts may be mediated by the same mechanism of action as estradiol's, which can be blocked by tamoxifen. It is a classical ligand-dependent mechanism. The classical ligand-dependent mechanism is one of the several mechanisms exerted by estrogen (Hall et al. 2001; Yaşar et al. 2016). In this mechanism, estrogen binds to

ERs, which induces the dimerization of the receptor and binding to specific DNA response elements (EREs) in the target genes' promoters. This finding is in line with the previous study. The compound named 7,8-trans-8,8′-trans-7′,8′-cis-7,7′-bis(5-methoxy-3,4-methylenedioxyphenyl)-8-acetoxymethyl-8′- hydroxy methyl tetrahydrofuran which was isolated from *P. pellucida* by Xu et al. (2006), demonstrated its estrogen-like activity through binding with ER and was entirely blocked by an ER antagonist.

The ligand-dependent mechanism of estrogen, especially in ERα, is mediated by two separate nonacidic activation domains, namely a constitutive activation function-1 (AF-1), located inside the amino-terminus (A/B domain), and also a hormone-dependent AF-2, located in the ligand-binding domain. In this case, tamoxifen inhibits AF-2 that contributes to its antagonist action in ERα. This agent also acts as an antiestrogen in the breast due to its competitive inhibition against estrogen and recruitment of corepressors (Berry et al. 1990; Peng et al. 2009; Shang & Brown 2002).

Besides estrogenic activity, antiestrogenic activity was also analyzed in this study. Antiestrogenic activity was identified by comparing the %RPE of each sample and the %RPE of a combination of sample and estradiol. In this case, estradiol was assumed to have 100% RPE. Hence, if the %RPE of the combination group is lower than 100%, the sample is considered to have antiestrogenic activity.

Based on Figure 1(c), the n-hexane and ethyl acetate extracts have antiestrogenic activity. The antiestrogenic levels of the n-hexane and ethyl acetate extracts are about 91.03 and 56.04%, respectively. This finding is similar to the results of the estrogenic activity test, i.e. the antiestrogenic activity occurs in a polarity-dependent manner.

The n-hexane extract showed low estrogenic activity and significantly high antiestrogenic activity compared with standard 17β-estradiol in 10^{-9} M (RPE% 100%). This high antiestrogenic activity is analogous to the activity of a pure antagonist agent. Estrogen antagonist agents block nuclear uptake of the receptor and then inhibit its nucleocytoplasmic shuttling. In this case, the antiestrogenic agent can bind to ER to form complexes and then bind to EREs, resulting in the inactivation of the transcriptional unit, provoking the destruction of the ER in breast cancer cells. A pure antiestrogenic agent can cause the accumulation of the newly synthesized receptor in the cytoplasm and inhibits its transport to the nucleus, whereas the receptor is retained in the nucleus in the presence of partial antiestrogens such as tamoxifen. The receptor complex that is paralyzed will be rapidly destroyed (Dauvois et al. 1993) and the complete destruction of the ERs will inhibit any estrogen-induced events from occurring.

PHYTOCHEMICAL SCREENING

Phytochemical screening was conducted to know the types of compounds present in the test extracts. The results show that the samples contained varying types of compounds. The dry plant sample had all types of compounds, except quinones, and steroids. It was observed that the n-hexane extract contained alkaloids, steroids and triterpenoids; the ethyl acetate extract contained only flavonoids and steroids; the ethanol extract contained all types of test compounds; while the water extract contained flavonoids, saponins, tannins, and triterpenoids. This finding suggests that there are many types of compounds that may have influenced the level of estrogenic activity of the test extracts. The compounds may work individually, antagonistically or synergistically as described in other studies (Khan et al. 2004; Mariotti et al. 2011).

DETERMINATION OF TOTAL FLAVONOID CONTENT

Since phytoestrogen mainly consists of flavonoid groups (Kühnau 1976), the total flavonoid content within each of the test extract was calculated. The total flavonoid content varied among the four types of extracts. The values of the n-hexane extract, the ethyl acetate extract, the ethanol extract and the water extract were 16.36 ± 0.23, 35.90 ± 0.49, 10.84 ± 0.11, and 6.70 ± 0.32 μg QE/100 mg extract, respectively. Statistical calculation showed that the level of total flavonoid content does not significantly correlate with either the estrogenic or the antiestrogenic activity of the samples. The activity may be produced not only by flavonoids but also by the interaction of two or more groups of compounds, as mentioned.

DETECTION AND QUANTIFICATION OF PHYTOESTROGEN COMPOUNDS IN THE *P. pellucida* EXTRACTS

Phytochemical tests were conducted to investigate the presence of specific phytoestrogen compounds in the extracts. As common and well-known phytoestrogen compounds, quercetin and stigmasterol were detected in the *P. pellucida* extracts (Figure 2). This finding supports previous studies that have successfully isolated these compounds from *P. pellucida* (Hartati et al. 2015; Kurniawan et al. 2016).

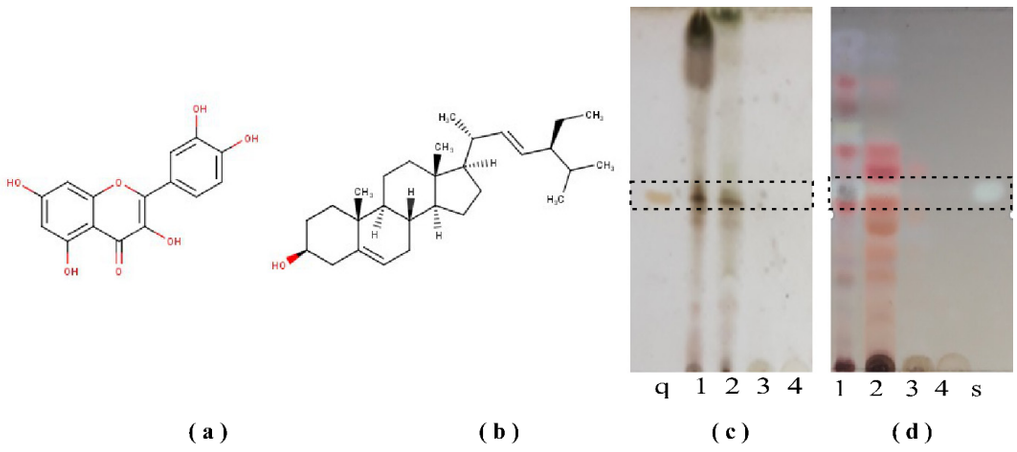

FIGURE 2. Detection of quercetin and stigmasterol in the test extracts using TLC. a) chemical structure of quercetin; b) chemical structure of stigmasterol; c) detection of quercetin using chloroform, ethyl acetate, and formic acid in the volume ratio 6:4:0.1 as the mobile phase; d) detection of stigmasterol using n-hexane and ethyl acetate in the volume ratio 7:3 as the mobile phase. Spots were detected under UV light at 366 nm after spraying with 10% sulfuric acid. The dotted-line show the possibility of quercetin and stigmasterol (q = quercetin, s = stigmasterol, 1 = n-hexane extract, 2 = ethyl acetate extract, 3 = ethanol extract, 4 = water extract)

As depicted on Figure 2(c) and 2(d), the presence of quercetin and stigmasterol were detected especially in the n-hexane and the ethyl acetate extract, which had high antiestrogenic activity. This finding suggests that the compounds may be correlated to the antiestrogenic activity of the extracts. Previous studies have reported similar results. Using the same method, quercetin was found to be able to significantly suppress the proliferation of MCF-7 cell as ERs antagonists (Resende et al. 2013). Another study also supports this finding (Miodini et al. 1999). An *in vivo* test has shown that it can exacerbate breast tumors induced by estradiol, however, this is not due to its estrogenic activity (Singh et al. 2010). The estrogenic and antiestrogenic activity of quercetin have been found to follow a dose-dependent manner, similar to genistein, a well-known phytoestrogen. Quercetin acts as an antagonist at high doses. Furthermore, other flavonoids, including daidzein and luteolin, have also been found to have an antiestrogenic effect (Han et al. 2002).

Stigmasterol as a phytosterol compound is known to be a weak SERM. β-sitosterol, a phytosterol, can competitively bind to both ERs with an equivalent affinity (Gutendorf & Westendorf 2001). It showed moderate stimulation toward the growth of the MCF-7 cell line, but inhibited the growth of estrogen-responsive human breast cancer xenografts in a test using mice and also failed to increase the uterine weight as a marker of estrogenic activity (Baker et al. 1999; Ju et al. 2004; Sakamoto et al. 2010).

As the extract with the highest estrogenic activity, the water extract was analyzed further to determine its active compounds. Among twelve standard compounds used in this study, the presence of apigenin and apigetrin was successfully confirmed in the water extract through the combination of TLC-D (Figure 3) and HLPC (Table 2) analysis. The presence of apigetrin in *P. pellucida* is reported in this study for the first time.

According to Figure 3(a), the standard apigenin had an Rf of 0.42 with an area of 5813.3 AU. The water extract was found to have same Rf, with an area of 2547.4 AU or 36.10% of the spotted solution. Meanwhile, the standard apigetrin has an Rf of 0.90 with area 13768.2 AU. The water extract was found to have the same Rf with an area of 9246.6 AU or 31.66% of the spotted solution (Figure 3(b)).

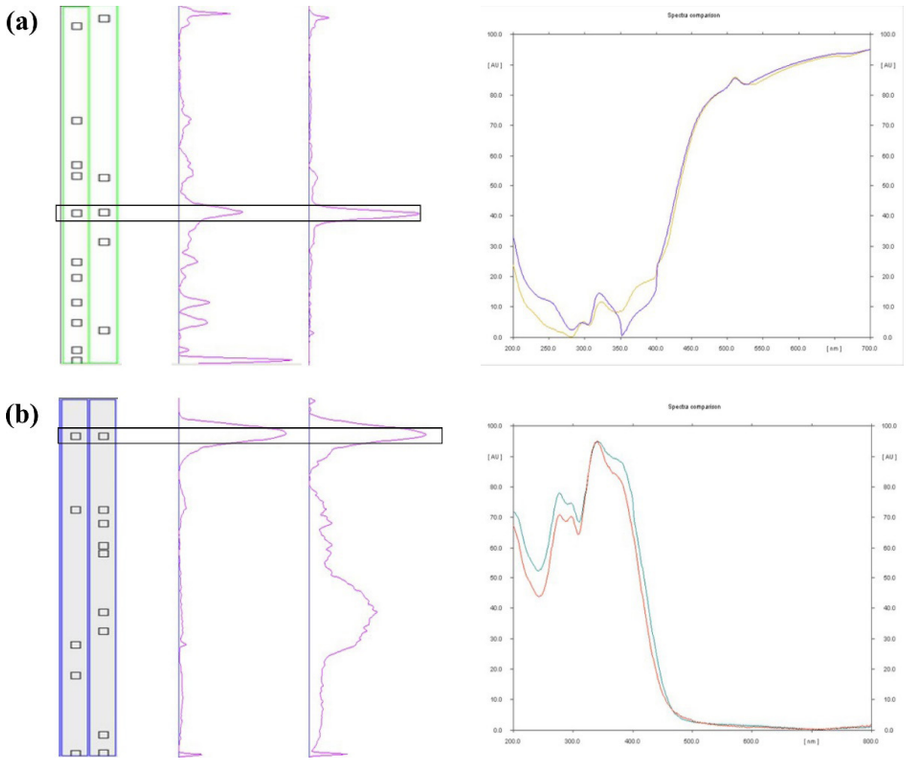

FIGURE 3. Detection of apigenin (a) and apigetrin (b) in water extract of *P. pellucida* using TLC-D: (1) reconstruction of all spots, (2) spectrums, and (3) overlay spectrums of standard and water extract in a certain Rf. Eluent for apigenin is toluen:formic acid:ethyl acetate (6:0.3:4) while for apigetrin 7-O glucoside is ethyl acetate:formic acid:water (5:1:0.5)

TABLE 2. HPLC quantification of flavonoids in *P. pellucida* extracts

Flavonoids		Standard	Water extract	
Name	Detection wave length (nm)	Retention time (min, mean ± SD)	Retention time (min, mean±SD);	Concentration (μg mg^{-1} extract, mean±SD)
Apigenin	350	7.663 ± 0.013	7.607 ± 0.057	0.239±0.076
Apigetrin	350	5.835 ± 0.001	5.764 ± 0.023	1.063±0.156

MOLECULAR DOCKING TEST

A molecular docking simulations were conducted in order to confirm the prediction regarding the involvement of phytoestrogens in the estrogenic and antiestrogenic activity of the *P. pellucida* extracts. The binding affinity of quercetin, stigmasterol, apigenin, and apigetrin for binding to ERα and ERβ were evaluated in the simulations. The affinities are represented by predicted free energy of binding (Table 3) and visualization of interactions between the ligands and amino acid residues in the binding site.

TABLE 3. Molecular docking data of estradiol, quercetin, and stigmasterol to estrogen receptors

Compounds	Hydrogen bonding (ER α)			Hydrogen bonding (ER β)		
	ΔG bind (kcal mol^{-1})	Amino acid residues	H-bond distance (Å)	ΔG bind (kcal mol^{-1})	Amino acid residues	H-bond distance (Å)
Estradiol	-9.55	GLU 353	3.16773	-9.55	HIS 475	3.14521
		HIS 524	2.94612		GLU 305	2.45984
Quercetin	-7.93	GLY 521	1.99724	-8.22	GLU 305	1.73588
		GLY 521	2.09053		GLU 305	1.86242
		GLU 353	1.83434		GLY 472	1.93541
		LEU 387	2.06028		LEU 476	3.07123
		ALA350	2.80111			
		HIS524	3.11883			
Stigmasterol	-9.96	ARG394	2.75854	-11.35	ARG 346	2.58623
		GLU353	2.06099		GLU 305	2.06616
Apigenin	-8.14	GLU353	1.99453	-8.21	GLU 305	1.81672
		GLY521	1.73517		GLU305	1.73669
		LEU387	2.1258		GLY472	1.91247
		GLU353	1.70145		ARG346	2.22906
		HIS524	2.01543			
		LEU525	2.42525			
Apigetrin	-6.81	GLU353	1.98727	-4.47	GLY472	1.98567
		ARG394	2.49958		GLU305	1.71836
		ARG394	2.30673		ARG346	2.42321
		HIS524	2.11942		ARG346	2.24630
		HIS524	2.07982		HIS475	1.85958

ΔG = Gibbs free energy

Based on the molecular docking results, each phytoestrogen compound has a specific preferable binding toward ERs. Both quercetin and stigmasterol, which are found in the n-hexane and ethyl acetate extracts, has a higher binding energy towards ERβ than towards ERα. These findings were in line with the previous studies (Powers & Setzer 2015; Triutomo et al. 2016). This finding suggests that both phytoestrogens have a higher antiestrogenic activity than estrogenic activity.

Apigenin, which was found in the water extract, showed the similar binding profile with quercetin and stigmasterol. This finding also in line with the previous studies (Kuiper et al. 1998; Powers & Setzer 2015). On the contrary, apigetrin, which was found and firstly reported presents in the water extract, has higher binding energy towards ERα than ERβ. The result was suggesting that the compound tends to have estrogenic activity. Even though the result related to apigetrin did not support the previous study (Powell et al. 2012), all of the compounds analyzed in this study have demonstrated their beneficial effects on osteoporosis condition (Gabay et al. 2010; Goto et al. 2015; Kim et al. 2015a; Oršolić et al. 2018). Further advance research needs to be performed to clarify these findings and find the other compounds that support the activity of water extract.

Conclusion

In conclusion, each type of extract from *P. pellucida* exerts differing estrogenic activity profiles depending on its polarity and the presence of certain phytoestrogens. The estrogenic activities may be mediated by a classical ligand-dependent mechanism. Since the ethyl acetate extract showed a partial agonist effect even though it contains phytoestrogen high flavonoids content, it may contain other interesting compounds that help boost its estrogenic activity. Meanwhile, the water extract showed promising estrogenic activity that can support its antiosteoporosis activity. It contains compounds such as apigenin and apigenin 7-O glucoside, which are phytochemical compounds that may be responsible for the estrogenic activity. Therefore, we have been doing further investigation related to these findings. Future studies in animal models are also warranted to better define the estrogenic efficacy and safety of extracts from *P. pellucida*.

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I Gusti Agung Ayu Kartika, Muhamad Insanu, Catur Riani & I Ketut Adnyana*
School of Pharmacy
Bandung Institute of Technology
40132, Bandung
Indonesia

Kyu Hyuck Chung
School of Pharmacy
Sungkyunkwan University
Suwon 16419
Republic of Korea

*Corresponding authors; email: ketut@fa.itb.ac.id

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