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Estrogenic and acetylcholinesterase-enhancement activity of a new isoflavone, 7,2′,4′-trihydroxyisoflavone-4′-*O*-β-D-glucopyranoside from *Crotalaria sessililflora*

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Abstract

A new isoflavone, 7,2′,4′-trihydroxyisoflavone-4′-*O*-β-D-glucopyranoside has been isolated from the aerial part of *Crotalaria sessiliflora*. The isoflavone glucoside enhanced the proliferation of the MCF-7 human breast cancer cell line, which possesses estrogen receptor (ER) and responds to estrogen in culture.The estrogenic property of the isoflavone glucoside was blocked by the known ER antagonist tamoxifen, indicating the involvement of the ER. Furthermore, the isoflavone glucoside was found to enhance the acetylcholinesterase (AChE) activity of the rat neuronal cell line PC12 at low concentrations of nerve growth factor (NGF).

Indroduction

Estrogenic compounds isolated from plants are commonly known as phytoestrogens. They are found in a variety of plants, including vegetables, fruits and medicinal plants. Although their chemical structures are different, they have structural similarity. All of them contain similar pharmacophores as found in estradiol and diethylstilbestrol (Tham et al. 1998). Among them, isoflavones are typical phytoestrogens that are found mostly in leguminous plants, especially in soybeans. Two major isoflavones found in soybeans are daidzein and genistein.

Phytoestrogens have received much attention due to their involvement in human health. Epidemiological studies have shown that intake of dietary phytoestrogens is correlated with lower risks of cardiovascular diseases, osteoporosis and cancer (Knight and Eden 1996). In addition, in vitro studies showed that isoflavones inhibit proliferation of some human cancer lines established from the gastrointestinal tract and prostate (Peterson and Barnes 1993; Yanagihara et al. 1993). Isoflavones were also reported to reduce the development of mammary carcinoma in rats and had preventive effects on experimental myocardial ischemia-reperfusion injury (Lamartiniere et al. 1995; Deodato et al. 1999). Discovery of new non-steroidal estrogens may help to develop alternative treatments for estrogen-dependent diseases. New phytoestrogens may also serve as model compounds for designing new estrogenic compounds with potential estrogen activity and minimum side effects.

We have been screening for new antioxidative compounds, and isolated a new isoflavone

glucoside, 7,2′,4′-trihydroxyisoflavone-4′-*O*-β-D-glucopyranoside **1** from a BuOH soluble fraction obtained from the aerial part of *Crotalaria sessiliflora* (Leguminosae). Although this compound did not show antioxidative activity, its structure resembled that of a phytoestrogen, daidzein. For these reasons, we investigated the estrogenic activity of the compound by E-screen assay using human breast cancer cell line MCF-7. The MCF-7 cell line possesses estrogen receptors (ERs) and responds to estrogens in culture. In contrast to ER binding assay, this method has the advantage of being a biological response, which can be taken as a measure of the direct interaction of agonists with the ER and can be equated to estrogenic potential (Karin 1991). This method has been widely used as a rapid and straight-forward assay to determine estrogenic activity of chemicals (Soto et al. 1995).

Isoda et al. (2002) have reported that genistein and daidzein enhanced the acetylcholinesterase (AChE) activity of the rat neuronal cell line PC12 by binding to the ER. This enhancement was effectively blocked by the known ER antagonist tamoxifen, indicating the involvement of the ER in AChE induction. The effect of compound **1** on the AChE was therefore studied using rat neuronal cell line PC12.

Furthermore, we examined whether the estrogenic activity of the compound **1** on the MCF-7 cell line was blocked by tamoxifen and bound to the classical estrogen response element (ERE).

Materials and methods

Apparatus

The following instruments and experimental conditions were used in this study: ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded using a JEOL α400 spectrometer in DMSO-d_6 with TMS as an internal standard. UV spectra were measured using a Shimadzu UV-365 spectrophotometer. IR spectrum was recorded on a Shimadzu FTIR-4300. ESI-MS spectrum was obtained using Platform LC (Micromass, UK). Preparative HPLC was carried out on a Gasukuro Kogyo model 576 equipped with a JASCO Uvidex 100-II detector (column: TSK gel ODS-80Ts, 250 × 10 mm). The elution was performed with a gradient of MeOH from 20 to 70% containing 0.01% TFA (trifluoro acetate) for 80 min and with an isocratic of 70% MeOH containing 0.01% TFA for the next 20 min. The flow rate was 2.0 ml min^{-1}. Analytical HPLC was performed on a Tosoh CCPS equipped with a Tosoh UV-8020 detector (column: TSK gel ODS-80Ts, 250 × 4.6 mm). The elution was performed with a gradient of 20–70% MeOH (0.01% TFA) for 60 min and with an isocratic of 70% MeOH (0.01% TFA) for 20 min, at a flow rate of 0.6 ml min^{-1}.

Plant materials

Plants were cultivated at the Agriculture and Forestry Research Center, University of Tsukuba, Japan. The seeds collected from the wild plants were scoured with sand paper and sown in soil on May 2000. After two months, the young plants were transferred to the field. Maturated plants were harvested in October 2000.

Extraction and isolation

The freshly harvested aerial part (ca. 40 kg) was refluxed with 80% MeOH. After evaporation of MeOH under reduced pressure, the aqueous concentrate was filtered to remove chlorophyll. The filtrate was successively partitioned with EtOAc and *n*-BuOH. The organic layers were concentrated under reduced pressure to give EtOAc extract (96.9 g) and BuOH extract (132.9 g). BuOH extract was subjected to a Sephadex LH-20 column (42 × 7 cm) and eluted with 50% MeOH. The eluate, which was collected in each 18 ml, was monitored with UV at 280 and 350 nm. Based on the elution pattern, the eluate was separated into nine fractions. Fraction 5 (test tube nos. 155–178) was concentrated and subjected to preparative HPLC under the same conditions as described above to give three compounds.

Cell line maintenance

The MCF-7 cell line was obtained from Dr H. Shinmoto of the National Food Research Institute (Tsukuba, Japan). Cells were routinely maintained in phenol-red free RPMI-1640 (Sigma Chemical Co. Ltd., MO, USA) supplemented with 10%

fetal bovine serum (Sigma Chemical Co. Ltd.) and 1% streptomycin (5000 IU ml^{-1})–penicillin (5000 $\mu g\ ml^{-1}$) solution (ICN Biochemical Inc. Aurora, OH, USA) in 75 cm^2 tissue culture flasks. Cells were incubated at 37 °C in 95% air–5% CO_2 incubator. Cell passage was carried out at 80% confluence at a split ratio of 1–3 every 3 or 4 days using trypsin. The rat pheochromocytoma PC12 cell line was obtained from the National Institute of Bioscience and Human-Technology (Tsukuba, Ibaraki, Japan) and routinely maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co. Ltd.) supplemented with 5% fetal bovine serum (Sigma Chemical Co. Ltd.), 10% horse serum (Sigma Chemical Co. Ltd.), and 1% streptomycin (5000 IU ml^{-1})–penicillin (5000 $\mu g\ ml^{-1}$) solution (ICN Biomedicals, Inc.) in tissue culture flasks.

E-screen assay

MCF-7 cells were trypsinized and plated onto 96-well plates at an initial concentration of 3×10^3 cells per well. After allowing the cells to attach for 24 h, the seeding medium (10% FBS in phenol-red free RPMI) was removed and replaced with the experimental medium (10% charcoal-treated FBS in phenol-red free RPMI). Test compound, with or without tamoxifen (Wako Pure Chemical Ind. Ltd., Osaka, Japan) was then added to the cells to obtain the required final concentration. Daidzein and genistein (Wako Pure Chemical Ind. Ltd.) were used as positive control. The plates were incubated for 6 days, after which 10 μl of 50 $\mu g\ ml^{-1}$ MTT {3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide)} (Wako Pure Chemical Ind. Ltd.) solution was added to each well and then incubated for 4 h. Sodium dodecyl sulfate (10%) was then added at 100 μl per well and the plates were incubated for 24 h. The absorbance was then measured at 570 nm using a microplate reader (BIO-RAD, Model 550).

Acetylcholinesterase assay

The AChE assay was performed as follows. PC12 cells were inoculated onto 96-well microplates at 1×10^4 cells per well in 100 μl of the medium. The cells were treated with 5 μM of genistein, daidzein, and compound **1** for 7 days, then incubated with 0–50 μM of nerve growth factor 2.5S (NGF) (Funakoshi, Tokyo, Japan) for 24 h. After treatment, the medium was carefully removed and the cells carefully washed twice with 200 μl of PBS. Then, 20 μl of 5.6 mM acetylthiocholine iodide and 180 μl of buffer solution, pH 7.5 (0.12 M NaCl, 0.2% Triton X-100, 1 mM EDTA, 50 mM Hepes) were added into each well. After incubating for 2 h at room temperature, 20 μl of the cell lysates were transferred to another multiwell plate and incubated for 1 h with 160 μl buffer solution, pH 5.0 (1 mM EDTA, 0.2% Triton X-100, 50 mM acetate buffer) and 20 μl of 0.4 mM 7-diethylamino-3-(4′-maleimidyl-phenyl)-4-methylcoumarin in acetonitrile. The absorbance in each well was then measured using a fluorescence plate reader (Fluoroskan Ascent FL) at 460 nm.

Results and discussion

Isolation and structural elucidation

Fraction 5 obtained from BuOH extract of *C. sessiliflora* after separation on Sephadex LH-20 column was separated using preparative HPLC. Figure 1 shows elution pattern of fraction 5 on the preparative HPLC. After concentration of fraction A, the precipitate was filtered and recrystallized from MeOH to give compound **1** (64 mg). Two known substances, 8-*C*-rhamno-glucosyl-apigenin (258 mg) and 6-*C*-rhamnoglucosyl-luteolin (249 mg) were isolated from fractions B and C, respectively.

Compound **1** was obtained as a pale yellow crystal. On analytical HPLC, the retention time of this compound was 21.3 min and the purity was 100%. The structure was determined on the basis of the following spectral data. ESI-MS (positive ion mode): m/z 455 $[M + Na]^+$, ESI-MS (negative ion mode): 431 $[M - 1]^-$, 269. UV λ_{max} (MeOH) nm (log ε): 289 (4.08), 262 (4.21), 247 (4.24); +NaOH: 289; +$AlCl_3$: 375 (sh), 298, 268; +$AlCl_3$ + HCl: 375 (sh), 290; +NaOAc: 290, 263, 248; +NaOAc + H_3BO_3: 290, 263, 248. IR ν_{max} (KBr) cm^{-1}: 3471, 1633, 1612. ^1H and ^{13}C NMR spectral data are presented in Table 1.

The molecular formula of compound **1** was deduced as $C_{21}H_{20}O_{10}$ from MS, ^1H and ^{13}C NMR spectral data. The ESI-MS spectrum gave the

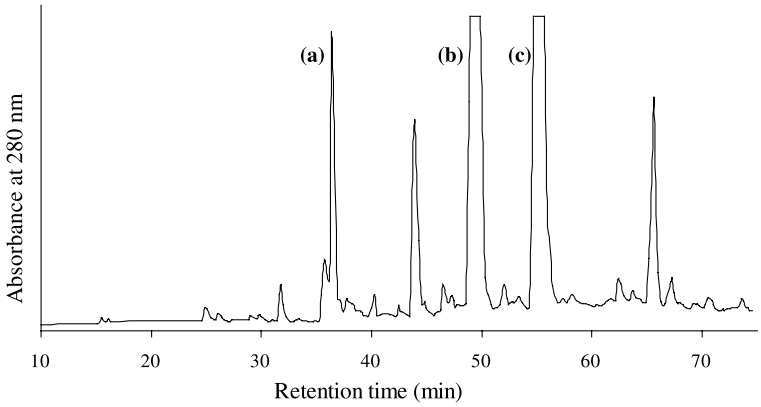

Figure 1. Elution pattern of fraction 5 obtained from BuOH extract after separation on Sephadex LH-20 column. (a) Compound **1**; (b) 8-*C*-rhamoglucosyl-apigenin and (c) 6-*C*-rhamnoglucosyl-luteolin.

Table 1. NMR data and HMBC correlation of compound **1**

Position	δ_C (ppm)	δ_H (ppm)	HMBC correlations
2	156.2	8.18 (1H, s)	C-3, C-4, C-8a
3	123.2		
4	177.3		
5	133.3	7.05 (1H, d, $J = 8.5$ Hz)	C-4a, C-7
6	107.9	6.40 (1H, d, $J = 8.5$ Hz)	C-4a, C-7, C-8
7	159.0		
8	104.6	6.41 (1H, s)	C-6, C-8a
4a	157.1		
8a	111.4		
1′	119.3		
2′	158.3		
3′	104.6	7.29 (1H, d, $J = 1.8$ Hz)	C-2′, C-4′, C-5′
4′	162.3		
5′	116.8	7.23 (1H, dd, $J = 8.5$ and 1.8 Hz)	C-1′
6′	128.1	8.09 (1H, d, $J = 8.5$ Hz)	C-4, C-2′, C-4′
1″	100.7	5.16 (1H, d, $J = 7.3$ Hz)	C-4′
2″	74.0	3.46 (1H, m)	C-1″, C-3″
3″	77.6	3.57 (1H, m)	C-2″
4″	70.6	3.34 (1H, m)	C-5″
5″	76.9	3.48 (1H, m)	
6″	61.6	3.83 (1H, d), 3.63 (1H, d)	

negative molecular ion peak at m/z 431 $[M - 1]^-$. The IR spectrum exhibited absorbances at 3471 and 1633 cm^{-1}, indicating the presence of hydroxyl and carbonyl groups, respectively. The UV spectrum of compound **1** showed absorption maxima at 289 and 262 nm, and a bathochromic shift by addition of 1 M NaOH. Addition of $AlCl_3$/HCl did not cause any shift. These suggested that compound **1** was isoflavone bearing free hydroxyl group, but not at C-5 position (Mabry et al. 1970). Furthermore, signal at δ 8.18 (1H, s, H-2) in the ^{1}H NMR, as well as δ 156.2 (C-2), 123.2 (C-3) and 177.3 (C-4) in the ^{13}C NMR spectra were typical of an isoflavone derivative (Woodward 1980).

In the low magnetic field of the ^{1}H NMR spectrum, six signals due to aromatic protons were observed. The signal at δ 7.05 (1H, d, $J = 8.5$ Hz), which showed long range connectivity with δ 157.1 (C-4a) and 159.0 (C-7) in the HMBC spectrum (Table 1), was assigned to H-5. This assignment confirmed the absence of a free hydroxyl group at C-5 position. The signal at δ 7.05 was coupled with signal at δ 6.40 (1H, d) with coupling constant of 8.5 Hz, indicating that they are vicinal aromatic protons. The singlet signal at δ 6.41 (1H, s), which showed long-range correlation with C-6 and C-8a in the HMBC spectrum, was assigned H-8. The three remaining signals were assigned to the protons on the B-ring. The signal at δ 8.09 (1H, d, $J = 8.5$ Hz) was coupled with δ 7.23 (1H, dd, $J = 1.8$ and 8.5 Hz), which correspond to H-6′ and H-5′, respectively. The signal at δ 7.29 (1H, d, $J = 1.8$ Hz) showed cross peaks with C-2′, C-4′ and C-5′ in HMBC spectrum, and was assigned to H-3′. These indicated that the B-ring was substituted at C-2′ and C-4′. On the basis of data explained above and fragment ion peak at m/z 296 in ESI-MS spectrum, the aglycone of compound **1** was determined as 7,2′,4′-trihydroxyisoflavone.

The ^{1}H NMR spectrum also showed signals in the range of δ 3.34–5.16, which gave cross peaks with six carbon signals (δ 61.6–100.7) in the C–H COSY spectrum. These signals were determined to arise from glucose (Agrawal 1992). The anomeric

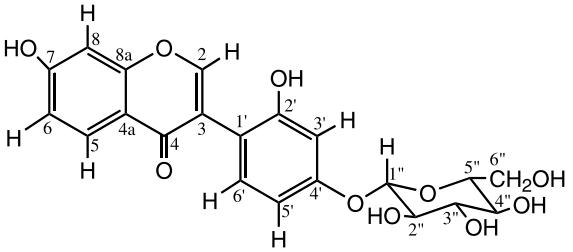

Figure 2. The structure of compound **1**.

proton signal in the ^{1}H NMR spectrum was observed as a doublet signal at δ 5.16 with a coupling constant of 7.3 Hz, indicating β-configuration (Agrawal 1992). The position of sugar linkage on the aglycone was determined by HMBC spectrum. The anomeric proton of glucose showed a long range coupling with a δ 162.3 (C-4′) on the ^{13}C NMR spectrum. This indicated that glucose was linked to the aglycone at the 4′-position. Compound **1** was therefore identified as 7,2′, 4′-trihydroxyisoflavone-4′-*O*-β-D-glucopyranoside (Figure 2).

Woodward (1980) isolated 7,2′,4′-trihydroxyisoflavone from *Phaseolus vulgaris* infected with *Monilinia fructciola*. The presence of this compound has been also reported in *Phaseolus lunatus* and *Phaseolus mungo* treated with $CuCl_2$ (Adesanya et al. 1984; O'Neill et al. 1986). 7,2′,4′-trihydroxyisoflavone-7,4′-di-*O*-β-D-glucopyranoside has been isolated as a stress metabolite from *Vigna angularis* induced by actinomycin D or nigeran (Kobayashi and Ohta 1983; Hattori and Ohta 1985). However, compound **1** has not previously been reported.

Estrogen activity

Phytoestrogens or hormonally active agents can recognize the estrogen receptor and trigger cell proliferation in estrogen responsive cells (Soto et al. 1991; White et al. 1994). Such compounds can also antagonize the effect of natural hormones, react directly or indirectly with the receptor, alter the pattern of synthesis of natural hormones, and even alter the hormone receptor level (Soto et al. 1995).

The estrogenic activity of compound **1** was investigated using the MCF-7 human breast cancer cell line. Daidzein and genistein, the best known naturally occurring non-steroidal estrogens, were used as positive controls. The MCF-7 cell line possesses ER and responds in culture to estrogens. Cell proliferation was measured by a colorimetric MTT assay to estimate the number of cells as the end point (Mosmann 1983).

Results in Figure 3 show that when the MCF-7 cells were incubated for 6 days in the presence of compound **1**, there was a significant increase in cell proliferation compared to control ($p < 0.01$, t test). The MCF-7 cell proliferation activity responds to compound **1** in a concentration-dependent manner. The isolated isoflavone glucoside in low concentration showed lower cell proliferation activity than did genistein and daidzein. Genistein showed an increased cell count up to 1.99-fold over untreated cells (control) at 0.3 μM, whereas compound **1** at the same concentration caused cell to proliferate at 1.14-fold compared to the control. However, at 5 μM, compound **1** led to an increase of 1.74-fold over the control. This was nearly equal to that of genistein. The maximum proliferation by daidzein seems to be at a concentration of 5 μM.

In this experiment, proliferation activity of daidzein was lower than that of genistein. This is in contrast with the result of Han et al. (2002). It is

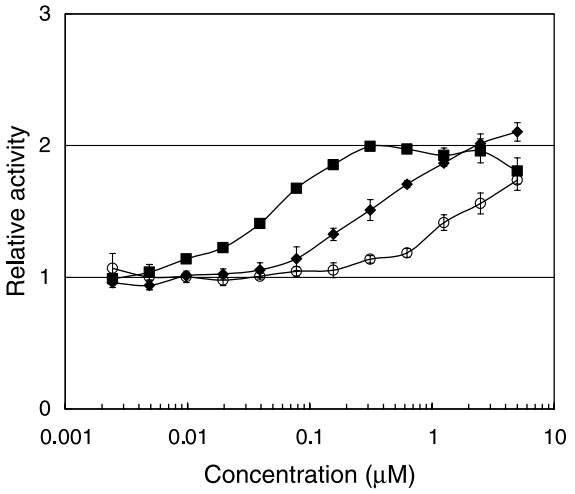

Figure 3. Dose–response effect of (○) compound **1**, (◆) daidzein and (■) genistein on the proliferation of MCF-7 cells after 6 days incubation. Results are expressed as relative estrogenic activity, which is obtained by dividing the proliferative activity of cell incubated with each compound by the proliferative activity of control (untreated cells); data points represent the mean value of four independent experiments and vertical bars represent standard errors.

known that the behavior and responds to 17β-estradiol of MCF-7 cells grown in different laboratories varies. The lack of standard protocol, difference in conditions and the strain of MCF-7 cell in different laboratory may lead to significant inter-laboratory variability (Jones et al. 1998; Payne et al. 2000).

To confirm whether the estrogenic activity of the new compound was due to the binding to ER, the cells were incubated with various concentrations of tested compounds in the presence of 1.5 μM tamoxifen. Figure 4 shows the effect of the addition of tamoxifen on the estrogenic activity of tested compounds. The proliferation activities of the tested compounds at all concentrations were blocked by addition of tamoxifen. Tamoxifen showed no proliferation activity on MCF-7 cells at 1.5 μM (data not shown).

Tamoxifen is a non-steroidal antiestrogenic compound that is a triphenylethylene derivate of stilbene. It has been used extensively for treatment of hormone-dependent breast cancer (Ratna 2002). The antiestrogenic activity of tamoxifen, which has agonist and antagonist activity, may be explained by the competition with estradiol for ER, which exists as two subtypes, denoted ERα and ERβ. The estrogen agonist activity of tamoxifen was mediated by ERα, and behaves as a complete antagonist through ERβ (Katzenellenbogen et al. 2000). Morito et al. (2001) have shown that tamoxifen has a higher affinity for ERα than ERβ, but did not induce transcription. Since the proliferation activity of compound **1** was blocked by the tamoxifen, the estrogenic activity was due to the binding to the classical ERα. In addition, the structural similarity between compound **1** and soy isoflavones, genistein and daidzein, suggests that they have identical mechanism.

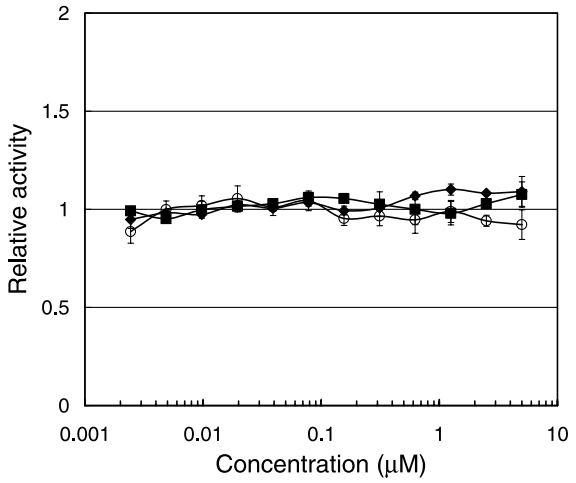

Figure 4. Dose–response effect of (○) compound **1**, (◆) daidzein and (■) genistein on the proliferation of MCF-7 cells after 6 days incubation in the presence of 1.5 μM tamoxifen. Tamoxifen showed no proliferation activity on MCF-7 cells at 1.5 μM. Results are expressed as relative estrogenic activity, which is obtained by dividing the proliferative activity of cell incubated each compound in the presence of tamoxifen by the proliferative activity of control (untreated cells); data points represent the mean value of four independent experiments and vertical bars represent standard errors.

Acetylcholinesterase activity

AChE activity is known as a marker for the neuronal differentiation in rat pheochromocytoma cell line PC12. Greene and Rukenstein (1981) hypothesized that NGF may work via parallel or branching pathways so that its effect on AChE activity can be dissociated from its effect on neurite outgrowth and proliferation. In the neuromuscular junction, the neurotransmitter acetylcholine binds to the acetylcholine receptor inducing a conformational change that opens this transmitter-gated ion channel. This short-lived opening is followed by a closed state, whereby the acetylcholine dissociates from the receptor and is hydrolyzed by a specific enzyme called AChE. Since AChE plays an important role in cholinergic synaptic transmission, its activity in neural cells is of particular interest.

Sohrabji et al. (1994) reported that NGF-treated PC12 cells express mRNA for the ER. Using *in situ* hybridization histochemistry, RT-PCR and a modified nuclear exchange assay, they have found both ER mRNA and estrogen binding in PC12 cells. They also observed that while estrogen binding was relatively low in naive PC12 cells, long-term exposure to NGF enhanced estrogen binding in these cells by 6-fold.

In this study, we exposed pheochromocytoma cell line PC12 to 5 μM isoflavones for 7 days, and then treated with 0–50 μM NGF in short-term to determine their effect on acetylcholine activities through ER mediation or expression enhancement. Figure 5 shows that 5 μM of compound **1** and daidzein enhanced the AChE activity of PC12 cells in lower concentration of NGF (< 0.1 μM) significantly, suggesting that at low dose of NGF,

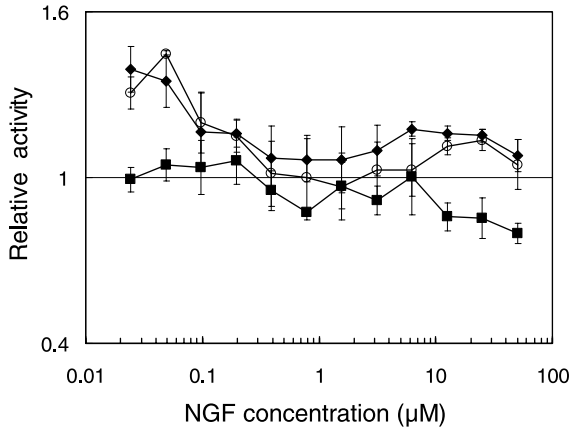

Figure 5. Effect of (○) compound **1**, (◆) daidzein and (■) genistein on the AChE in rat neuronal PC12 cells. The cells were pretreated with 5 μM tested compounds for 7 days and then incubated for 24 h with varying concentration of NGF. Result based from three independent replicates are expressed as relative AChE activity, which is obtained by dividing the mean AChE activity of cells incubated with the test compounds and NGF by the mean AChE activity of cells incubated with NGF alone (positive control). Vertical bars represent standard errors. Relative AChE activity of positive control is therefore 1.

a larger proportion of tested compounds enhanced the AChE activity through their binding to ER than NGF did. At higher concentrations of NGF (>20 μM), the AChE activity was slightly enhanced by 5 μM of compound **1** and daidzein. This results suggest that compound **1** and daidzein do not only bind to the ER to enhance AChE activity in PC12 cells but may also potentiate the effect of NGF or enhance the NGF-receptor-mediated pathway through its effect on the enzymes involved in AChE production. Genistein did not enhance AChE activity at the tested NGF concentrations. The reason why genistein did not enhance AChE activity, although genistein showed strong estrogenic activity in MCF-7 cell line, remains unclear.

The present data demonstrate that compound **1** increases the AChE activity in low concentration of NGF-treated PC12 cells due to the its binding to ER, supporting the estrogenic activity in MCF-7 tumor cells.

Considering that compound **1** enhanced AChE activity in neuronal cells as well as estrogenic activity, it may useful in cases of AChE deficiency (Kohara et al. 2002). However, further studies on the physiological function of this compound are required.

Conclusion

In conclusion, this study has shown that a new isoflavone glucoside, 7,2′,4′-trihydroxyisoflavone-4′-*O*-β-D-glucopyranoside **1** from BuOH soluble fraction obtained from the aerial part of *C. sessiliflora* (Leguminosae) induced estrogenic activity in a cell proliferation assay using the human breast cancer line MCF-7, and the proliferation was blocked by ER antagonist tamoxifen, indicating the involvement of the ER. The new isoflavone also enhanced the AChE activity of the rat neuronal cell line PC12 at low concentrations of NGF, suggesting the involvement of ER.

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