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RESEARCH ARTICLE

Estrogenic activity of a naringinase-treated extract of *Sophora japonica* cultivated in Egypt

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Abstract

The naringinase-treated methanol extract of *Sophora japonica* L. (Fabaceae) seeds showed potent estrogen agonist activity. Through bioassay-guided isolation of the main active constituents from the naringinase-treated methanol extract of *S. japonica*, the aglycones genistein and kaempferol were found to be the main phytoestrogens in the naringinase-treated extract. In addition, kaempferol was nearly equipotent to genistein as an estrogen agonist. Concerning the compounds isolated from the untreated methanol extract, sophoricoside showed weak estrogenic activity on ERβ only.

Keywords: *Estrogenic activity; ERα; ERβ;* Sophora japonica; *yeast two-hybrid assay*

Introduction

Estrogens are the key regulators of the cellular processes involved in development and maintenance of the reproductive function. There are two subtypes of estrogen receptor and several isoforms of each subtype. The first subtype is estrogen receptor α (ERα) (Green et al., 1986), and the second subtype is estrogen receptor β (ERβ) (Kuiper et al., 1996).

Phytoestrogens are plant-derived compounds that structurally or functionally mimic mammalian estrogens, and therefore are considered to play an important role in the prevention of cancers, heart disease, menopausal symptoms, and osteoporosis. There are several classes of phytoestrogens, such as steroidal estrogens found in a few plants; the more common phenolic estrogens are isoflavones, coumestans, and lignans (Ososki & Kenelly, 2003). Other classes of phytoestrogens that have been reported include: anthraquinones (Matsuda et al., 2001), chalcones (Rafi et al., 2000), flavones (Milligan et al., 1999), prenylated flavonoids (Kitaoka et al., 1998; Ahn et al., 2004b), naphthalenes, naphthopyrones, sesquiterpenoidal naphthoquinones (El-Halawany et al., 2007a, 2007b), and saponins (Chan et al., 2002).

Sophora japonica L. (Fabaceae) is a tree native to China and Korea. It is also named the Japanese pagoda tree or Chinese scholar tree. Flavones from the buds and pericarps were reported as hemostatic constituents (Tang et al., 2001). Triterpenes, phospholipids, alkaloids, polysaccharides, and fatty acids have been reported as the main chemical constituents of the seeds (Gorbachova et al., 1995; Mukhamedova & Glushenkova, 1997). Despite these discoveries, there is no previous report about the use of *S. japonica* as an estrogen replacement therapy (ERT). *S. japonica* was selected for this investigation due to its high flavonoid content.

The current study reports the biologically guided isolation of the major phytoestrogens from the methanol extract of *S. japonica* seeds before and after naringinase treatment.

Materials and methods

Chemicals

Naringinase was purchased from Sigma Co. (St. Louis, MO, USA). *O*-Nitrophenyl β-D-galactoside (ONPG) was purchased from Nacalai Tesque Co. (Kyoto, Japan).

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17β-Estradiol was obtained from Calbiochem Co. (Darmstadt, Germany). Tamoxifen was purchased from Wako Chemical Co. (Osaka, Japan). Zymolyase 20T was obtained from Seikagaku Kogyo Co. (Tokyo, Japan).

General experimental procedures

Thin layer chromatography (TLC) was carried out on pre-coated silica gel 60 F_{254} (0.25 mm; Merck) and RP-18 $F_{254}S$ (0.25 mm; Merck, Darmstadt). Column chromatography (CC) was carried out on BW-820MH silica gel and ODS DM 1020T (Fuji Silysia, Nagoya, Japan). Medium pressure liquid chromatography (MPLC) was performed on LiChroprep RP-18 (size A and B; Merck). ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were measured with a JHA-LAA 400 WB-FT (^{1}H, 400 MHz; ^{13}C, 100 MHz; Jeol, Tokyo) spectrometer, the chemical shifts being represented as ppm with tetramethylsilane as internal standard. Electrospray ionization mass spectrometry (ESI-MS) was carried out on an Esquire 3000 mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) system with ESI source.

Plant material

Seeds of *S. japonica* were collected from ripe fruits cultivated in the Medicinal Plant Station of the Faculty of Pharmacy, Cairo University, during December 2005. Authentication of the plant was established by Ass. Prof. Dr. Sherif El-Khanagry, Agriculture Museum, El-Dokki, Cairo, Egypt. A voucher specimen (No. S-1) is kept in the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Egypt.

Yeast strains

The genetically modified yeast strains were provided by Professor Tsutomu Nishihara, Faculty of Pharmaceutical Sciences, Hyogo College of Medicine, Kobe, Japan.

Extraction and isolation

The pulverized seeds of *S. japonica* (1.3 kg) were extracted with MeOH (1 L × 3) at room temperature and the combined extracts were evaporated *in vacuo*. The methanol extract (104 g) was suspended in 50% aqueous MeOH (600 mL) and partitioned with chloroform (500 mL × 3) to produce a chloroform-soluble fraction (4 g). The remaining solution was applied on a column of Diaion HP-20 (60 cm × 6 cm). Washing with H_2O (1 L) was followed by elution with 25% MeOH in H_2O (2 L), 50% MeOH in H_2O (2 L), and finally 100% MeOH (2 L). The eluates were evaporated under vacuum to give 8.5 g (fraction A), 14 g (fraction B), and 5.8 g (fraction C) of dry residue, respectively. All the fractions were screened for their estrogenic activity, before and after naringinase treatment, using yeast two-hybrid screen.

Most of fraction B (12 g) was applied to a silica gel column (300 g). Elution with $CHCl_3$–MeOH–H_2O (9:1:0.1, v/v/v) afforded eight fractions. Fraction 1 (735 mg) was purified on a silica gel column (25 cm × 2 cm) and eluted with $CHCl_3$–MeOH (9:1, v/v) to obtain compound **9** (13 mg). Fraction 3 (2 g) gave a yellow precipitate upon concentration. After filtration, the precipitate was washed several times with a mixture of chloroform–methanol (1:1) to give compound **3** (193 mg). The filtrate was evaporated under vacuum and the residue was purified on a MPLC RP-18 column (size A) using MeOH–H_2O (4:6, v/v) to afford compounds **7** (40 mg), **1** (2 mg), and **2** (5 mg). Fraction 4 gave compound **4** (358 mg) upon crystallization from MeOH, and the remaining supernatant was applied to a MPLC RP-18 column (size B) eluted with MeOH–H_2O (3:7, v/v) to give compound **10** (5 mg). Fraction 5 gave compound **5** (180 mg) upon crystallization from a chloroform–methanol mixture (1:1, v/v). Fraction 6 (1.5 g) was purified on a Sephadex LH-20 column (30 cm × 3 cm) and eluted with MeOH–H_2O (1:1, v/v), and sub-fractions (10 mL each) were collected. Sub-fractions 17–30 (150 mg) of this column were combined together and applied to a MPLC RP-18 column (size A) and eluted with MeOH–H_2O (3:7, v/v) to give compound **6** (37.4 mg). Fraction 7 (545 mg) was purified on a Sephadex LH-20 column (20 cm × 3 cm) and eluted with MeOH, followed by a MPLC column (size A) using MeOH–H_2O (1:4, v/v) to afford compound **8** (21 mg).

Naringinase treatment of 50% methanol fraction (fraction B) of S. japonica and isolation of aglycones

Part of fraction B (2 g) was dissolved in H_2O and incubated with naringinase enzyme (1 g) in 100 mL of 0.2 M acetate buffer (pH 4.7) at 37°C for 24 h. The resulting hydrolysate was extracted with EtOAc (250 mL × 3), and the combined extracts were evaporated under reduced pressure to give 650 mg of dry residue. The residue was applied to a silica gel column (25 cm × 2 cm) and eluted with $CHCl_3$–MeOH (9.5:0.5, v/v) to give **11** (25 mg) and **12** (10 mg) as the two major aglycones.

Yeast two-hybrid assay

The yeast two-hybrid assay was carried out according to the method of Nishikawa et al. (1999) and Kanayama et al. (2003). Briefly, yeast cells expressing ERα and ERβ were separately grown overnight at 30°C with shaking in a synthetic defined medium (SD) lacking tryptophan and leucine. Yeast cells were treated with 17β-estradiol and the test materials separately for 4 h at 30°C, and β-galactosidase activity was determined as follows. The growth of the yeast cells was monitored by measuring the

turbidity at 600 nm. The treated yeast cells were collected by centrifugation (8000 × *g*, 5 min) and re-suspended in 200 μL of Z-buffer (0.1 M sodium phosphate, pH 7.0, 10 mM KCl, and 1 mM $MgSO_4$) containing 1 mg/mL of zymolyase at 37°C for 15 min. The reaction was started by the addition of 40 μL of 4 mg/mL ONPG as substrate. When a yellow color developed (incubation time: *t*), 100 μL of 1 M Na_2CO_3 was added to stop the reaction. The absorbance of the solution (150 μL) was measured at 420 and 550 nm. The β-galactosidase activity (U) was determined using the following formula:

$$U = 1000 \times (A_{420} - 1.75 \times A_{550}) / (t \times 0.05 \times A_{600})$$

Anti-estrogenic assay

The antagonistic activity of various concentrations of test compounds was determined by measuring the inhibition of 17β-estradiol-induced β-galactosidase activity in the yeasts expressing ERα and ERβ.

Statistical analysis

Each set of experiments was repeated at least three times. Values are expressed as mean ± SEM. One-way analysis of variance followed by Dunnett's test was used for statistical analysis.

Results

The yeast two-hybrid assay expressing ERα and ERβ was used to investigate the estrogenic activity of the methanol extract and fractions of *S. japonica* before and after naringinase treatment. Naringinase enzyme is a mixed enzyme of β-glucosidase and α-rhamnosidase activities. This treatment was developed in our laboratory (Ahn et al., 2004a) as a partial mimic to the metabolism process (de-glucosylation) which takes place in the gastrointestinal tract (GIT). The naringinase-treated extract and fractions showed more activity than the original compounds.

Further fractionation of the methanol extract of *S. japonica* afforded a $CHCl_3$ soluble fraction, and 25% (fraction A), 50% (fraction B), and 100% methanol (fraction C) extracts. All the fractions were tested for their estrogenic activity before and after naringinase treatment (Table 1). The naringinase-treated 50% methanol fraction (fraction B-NT) exhibited the most potent estrogenic activity (Table 1). Both fraction B and fraction B-NT were chemically investigated and the isolated compounds were tested for their estrogenic activity.

Fraction B was purified several times over silica gel, ODS, and Sephadex LH-20 columns to produce 10 compounds. After comparing their NMR data with those reported in the literature, the compounds were identified as genistin (**1**) (Khalid et al., 2003), sophoricoside (**2**) (Min et al., 1999), sophorabioside (**3**), sophoraflavonoloside (**4**), genistein 7,4'-di-*O*-β-D-glucopyransoide (**5**) (Watanabe et al., 1993), kaempferol 3-*O*-α-L-rhamnopyranosyl (1→6) β-D-glucopyranosyl(1→2)β-D-glucopyranoside(**6**),kaempferol 3-*O*-β-D-sophoroside-7-*O*-α-L-rhamnopyranoside (**7**), kaempferol 3-*O*-α-L-rhamnopyranosyl (1→6) β-D-glucopyranosyl (1→2) β-D-glucopyranoside 7-*O*-α-L-rhamnopyranoside (**8**) (Tang et al., 2002), methylgallate (**9**), and rutin (**10**) (Figure 1).

Fraction B-NT afforded kaempferol (**11**) and genistein (**12**) as the major components in the extract (Figure 1).

Estrogenic activity of isolated compounds

In the yeast expressing ERβ (Table 2), an appreciable induction of β-galactosidase was found with sophoricoside (**2**) at a concentration of 10^{-4} M. Kaempferol (**11**) and genistein (**12**) showed estrogenic activity in a concentration-dependent manner.

In the yeast expressing ERα (Table 2), kaempferol (**11**) and genistein (**12**) showed a significant estrogenic activity at 10^{-4} M, while none of the tested glycosides showed any significant estrogenic activity.

Anti-estrogenic activity of isolated compounds

In the yeast expressing ERβ (Table 3), none of the compounds showed any anti-estrogenic activity. In the yeast expressing ERα (Table 3), sophoricoside (**2**), rutin (**10**), kaempferol (**11**), and genistein (**12**) exhibited weak anti-estrogenic activity at a concentration of 10^{-4} M.

Table 1. Induction of β-galactosidase in the yeast two-hybrid assay expressing ERβ by the methanol extract and fractions from *S. japonica* seeds before and after naringinase treatment (NT).

	β-Galactosidase activity (U)	
	ERβ	
Extract	100 μg/mL	10 μg/mL
MeOH extract	129.6 ± 1.6	127.2 ± 8.6
NT MeOH extract	1021.2 ± 40.1**	340.5 ± 6.8*
$CHCl_3$ fraction	170.5 ± 11.1*	130.0 ± 9.6
25% MeOH extract (fr. A)	30.2 ± 7.4	37.9 ± 33.1
50% MeOH extract (fr. B)	24.1 ± 2.5	17.8 ± 3.4
100% MeOH extract (fr. C)	45.2 ± 7.8	25.3 ± 4.1
NT 25% MeOH extract (fr. A-NT)	871.2 ± 50.8**	638.1 ± 44.7**
NT 50% MeOH extract (fr. B-NT)	1192.1 ± 129.9**	871.2 ± 50.8**
NT 100% MeOH extract (fr. C-NT)	531.5 ± 10.6**	280.8 ± 66.7*

Each value represents the mean ± SE of three independent experiments (n = 3). Asterisks denote significant differences from the control at $p < 0.05$ (*), $p < 0.01$ (**). In the yeast expressing ERβ: dimethylsulfoxide (DMSO) was 40.1 ± 1.6 (U) and 17β-estradiol was 695.0 ± 20.6 at concentration of 10^{-7} M. In the yeast expressing ERα (methanol extracts): DMSO was 40.0 ± 7.0 and 17β-estradiol was 786.8 ± 32.5 at a concentration of 10^{-7} M.

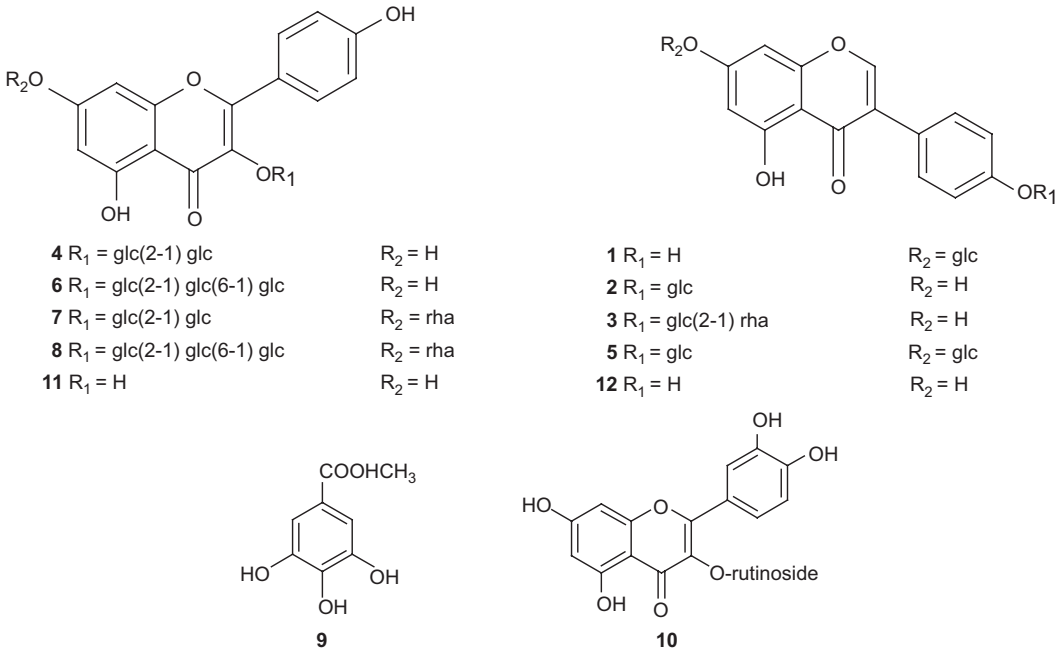

Figure 1. Chemical structures of the isolated compounds from *S. japonica* seeds.

Table 2. Induction of β-galactosidase in the yeast two-hybrid assay expressing ERα and ERβ by the isolated compounds from *S. japonica*.

	β-Galactosidase activity (U)					
	ERα			ERβ		
Compound	10^{-4} M	10^{-5} M	10^{-6} M	10^{-4} M	10^{-5} M	10^{-6} M
1	54.1 ± 16.5	71.6 ± 3.3	77.3 ± 4.1	56.2 ± 1.0	4.9 ± 5.9	39.2 ± 1.4
2	83.1 ± 5.9	80.5 ± 1.7	74.7 ± 5.9	184.2 ± 4.7*	58.8 ± 2.6	54.8 ± 4.9
3	73.3 ± 5.6	85.5 ± 9.0	83.4 ± 8.4	38.4 ± 5.8	37.9 ± 1.5	36.1 ± 1.5
4	99.7 ± 13.1	105.6 ± 1.9	109.9 ± 2.6	71.3 ± 24.5	90.5 ± 8.4	41.8 ± 6.21
5	66.1 ± 20.1	65.5 ± 4.8	55.6 ± 3.5	35.8 ± 2.5	31.2 ± 1.4	31.7 ± 1.3
6	117.2 ± 6.2	89.6 ± 3.7	71.5 ± 6.2	87.7 ± 3.4	88.9 ± 5.8	83.5 ± 4.1
7	79.2 ± 12.4	72.3 ± 5.5	56.2 ± 6.1	69.8 ± 6.1	75.2 ± 8.4	70.9 ± 7.4
8	76.8 ± 10.5	70.5 ± 9.5	110.3 ± 5.4	81.5 ± 9.1	58.1 ± 10.1	65.7 ± 4.1
9	55.1 ± 1.9	50.1 ± 1.7	48.5 ± 1.7	30.4 ± 1.7	29.2 ± 4.3	27.6 ± 3.9
10	132.4 ± 27.2	153.4 ± 29.8	152.1 ± 4.6	89.3 ± 23.3	57.6 ± 4.4	42.8 ± 2.2
11	228.6 ± 14.7**	70.3 ± 17.6	40.4 ± 23.4	2685.0 ± 145.4**	698.2 ± 38.8**	106.6 ± 11.2
12	390.3 ± 39.8**	73.4 ± 11.6	41.1 ± 12.5	3378.5 ± 284.3**	1956.2 ± 281.3**	526.2 ± 130.8**

Each value represents the mean ± SE of three independent experiments ($n = 3$). Asterisks denote significant differences from the control at $p < 0.05$ (*), $p < 0.01$ (**). In the yeast expressing ERβ: the negative control (DMSO) was 42.3 ± 3.0 and 17β-estradiol was 3121.7 ± 219.9 at a concentration of 10^{-7} M. In the yeast expressing ERα: DMSO was 63.6 ± 3.5 and 17β-estradiol was 1538.7 ± 44.4 at a concentration of 10^{-7} M.

Discussion

Through biologically guided isolation, kaempferol (**11**) and genistein (**12**) were found to be the major phytoestrogens of the naringinase-treated 50% MeOH fraction of *S. japonica* seeds, and are therefore responsible for the estrogenic activity. Chemical investigation of the 50% MeOH extract indicated that kaempferol and genistein are products of enzymatic hydrolysis of their corresponding glycosides (**1–10**).

Genistein (**12**) is known for its high activity as an ER agonist. Kaempferol (**11**) showed a nearly equipotent estrogenic effect to that of genistein on both ER subtypes. The abundance of kaempferol (**11**) in most medicinal herbs could make it a valuable source of phytoestrogens.

Among the tested glycosides, only sophoricoside (**2**) showed a weak estrogenic activity on ERβ. The activity of (**2**) and the inactivity of genistin suggest the importance of a free phenolic group at position 7, in genistein, for estrogenic activity.

It is also noted that the absence of quercetin in the 50% MeOH fraction can be attributed to the presence of rutin in a very low amount in the seeds, which is in accordance with the reported data (Balbaa et al., 1974).

Finally, the marked estrogenic activity of the *S. japonica* extract and compounds after naringinase

Table 3. Inhibitory effects of the isolated compounds from *S. japonica* on induction of β-galactosidase activity by 17β-estradiol in yeast two-hybrid assay (ERα and ERβ).

	β-Galactosidase activity (% of control)			
	ERα		ERβ	
Compound	10^{-4} M	10^{-5} M	10^{-4} M	10^{-5} M
1	93.5 ± 7.3	109.6 ± 3.1	94.9 ± 11.1	102.4 ± 1.1
2	81.8 ± 7.3*	112.5 ± 6.2	106.9 ± 6.8	94.8 ± 7.3
3	116.4 ± 10.2	117.2 ± 17.1	116.7 ± 7.1	102.2 ± 8.5
4	100.7 ± 2.2	91.6 ± 11.5	114.1 ± 5.4	121.4 ± 6.7
5	109.8 ± 2.8	122.5 ± 2.5	98.8 ± 6.5	105.2 ± 4.3
6	116.2 ± 6.8	97.8 ± 8.9	112.3 ± 6.3	120.8 ± 4.6
7	125.5 ± 2.2	117.6 ± 7.1	108.4 ± 5.5	99.6 ± 6.5
8	103.2 ± 1.5	111.1 ± 3.2	106.5 ± 4.3	100.5 ± 1.2
9	102.5 ± 10.2	103.1 ± 2.8	96.4 ± 0.8	96.1 ± 6.8
10	85.7 ± 3.5*	114.9 ± 3.1	108.6 ± 5.6	94.3 ± 3.2
11	85.7 ± 3.2*	114.9 ± 9.7	116.2 ± 4.5	111.8 ± 9.7
12	81.0 ± 7.6*	93.1 ± 2.2	93.4 ± 1.5	106.4 ± 7.6
Tamoxifen	28.2 ± 1.5**	91.9 ± 3.2	22.4 ± 2.5**	98.8 ± 10.9

β-Galactosidase activity (U) of 17β-estradiol was 483.1 ± 22.5 and 2333.4 ± 112.5 in yeast expressing ERα and ERβ, respectively, at a concentration of 10^{-7} M (considered as 100% activity). β-Galactosidase activity (U) of the tested compounds was calculated as a percentage of the 17β-estradiol activity (control). Asterisks indicate significant differences from the control at $p < 0.05$ (*), $p < 0.01$ (**) ($n = 3$).

treatment indicates the possible activation of this plant and compounds after its oral administration through the action of GIT bacterial enzymes.

***Declaration of interest*:** The authors report no conflicts of interest.

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