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Estrogenic and Anti-Estrogenic Activities of the Thai Traditional Herb, *Butea superba* Roxb.

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This study evaluated the estrogenic and antiestrogenic activities of native and *in vitro* hepatic metabolized tuberous extracts of wild *Butea superba* collected from 23 out of the 76 provinces in Thailand by yeast estrogen screening (YES). The YES screen used consisted of the human estrogen receptors hERα and hERβ and the human transcriptional intermediary factor 2 or human steroid receptor coactivator 1, respectively, together with the β-galactosidase expression cassette as the reporter. The relative potency, effectiveness and relative inductive efficiency were evaluated by determining the β-galactosidase activity (EC_{50}) of each tuberous extract in relation to that induced by 17β-estradiol. Six pure compounds isolated from *B. superba* were tested in parallel and exhibited a maximum relative potency compared to 17β-estradiol of 15.5% and 5.27% in the respective hERα and hERβ assays. Eighteen and seventeen plant extracts were respectively found to interact with the hERα and hERβ receptors in the YES assays with higher relative potency and relative inductive efficiency with hERβ than with hERα. The selected plant extracts tested exhibited antiestrogenic activity. Coincubation with the rat liver S9 mixture also elevated the estrogenic potency of these plant extracts.

Key words: phytoestrogen; *Butea superba*; yeast estrogen screening; estrogen receptor; antiestrogenic

There are a few medicinal plant species that exhibit a significant level of estrogenic activity including red clover (*Trifolium pretense* L.), *Erythrina variegata* L., Kudzu (*Pueraria lobata*) and "white kwao krua" (*Pueraria mirifica* Airy shaw et Suvatabhantu). Red clover, a common perennial herb in Europe, has been shown to exhibit strong estrogenic activity in an evaluation by a yeast estrogen screening (YES) assay.[1,2] *Erythrina variegata* L., a traditional herb in Southeast Asia, India and China has been shown to have estrogenic activity by expressing an anti-oesteoporotic effect in ovariectomized rats.[3] Kudzu, the common tuberous vine of China, Korea and Japan contains a high amount of isoflavonoids[4–6] whilst "white kwao krua," the common tuberous vine of Thailand, Myanmar and Laos is traditionally consumed for rejuvenating and for treating menopausal symptoms.[7] The tuberous plant materials from *P. mirifica* have exhibited stronger estrogenic activity than that of Kudzu in ovariectomized rats that was evaluated by both the vaginal cornification assay[8] and uterotrophic assays.[9]

Butea superba Roxb., family Leguminosae, with the domestic Thai name of "red kwao krua," is commonly found in deciduous Thai forests and is an indigenous Thai herb popularly used for promoting male potency[7] as well as constituting a minor component of traditional medicines made with *P. mirifica* for treating menopausal symptoms. It has exhibited androgen disruption in male rats.[10] The efficacy of *B. superba* powder has been demonstrated in a human clinical trial resulting in the effective treatment of erectile dysfunction in Thai males.[11] *B. superba* products have recently been becoming popular as traditional medicines, dietary supplements and for topical applications to promote male vigor. The traditional used of this plant ingredient needed to be scientifically evaluated to aid in an understanding of the bioactivity of the plant recipes. We therefore set up a YES-based study to evaluate the estrogenic activity of *B. superba* tuberous samples collected from available sources in Thailand. The results could make it possible not only to discover why the plant is used in the recipes for treating menopausal symptoms but also to establish the first evaluated record of estrogenic activity in this plant species.

Materials and Methods

Plant materials. The tuberous roots of *B. superba* were collected from 23 out of the 76 provinces of Thailand during the summer (March-April) of 2000. The plant samples were identified by Cherdshewasart with reference to the herbarium voucher no. BCU 11046.[12] The tuberous roots were cleaned, sliced, dried in a hot-air oven at 70 °C, subsequently ground into powder and filtered through a 100-mesh-size sieve. The sieved powder was extracted with absolute ethanol at a ratio of 1:10 (w/v) for 3 d at room temperature, and then the sediment was discarded. The ethanolic supernatant was passed through Whatman no. 3 filter paper, and the filtrate was evaporated in a rotary evaporator to leave a solid ethanolic tuberous extract. Each crude plant extract was kept in a tightly-capped tube, in a light-protected container and stored at −70 °C until needed. A stock solution of each extract was freshly prepared in DMSO at concentrations ranging from 1×10^{-3} to 1×10^{3} μg/ml.

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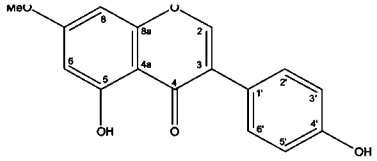

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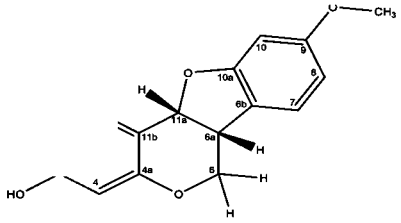
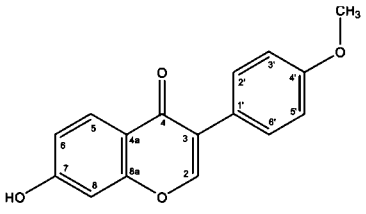
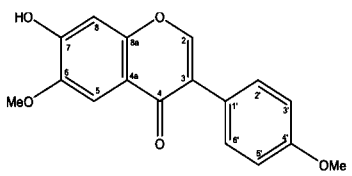
Pure compound no. 1



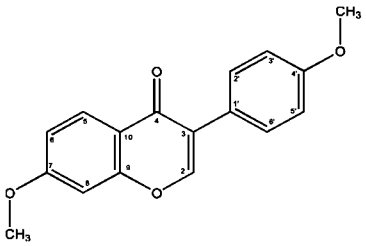
3-Hydroxy-9-methoxypterocarpan (Medicarpin)
Pure compound no. 2



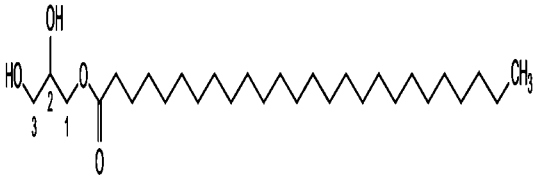
7-Hydroxy-4'-methoxy-isoflavone (Formononetin)
Pure compound no. 3



7-Hydroxy-6-4'-dimethoxyisoflavone
Pure compound no. 4



7,4'-Dimethoxyisoflavone
Pure compound no. 5



Hexacosanoic acid 2,3-dihydroxy-propyl ester
Pure compound no. 6

Fig. 1. The Chemical Structure of the Six Pure Compounds Isolated from *B. superba* (Pure Compounds 1–5 are from Ngamrojanavanich *et al.*, 2007).

Estrogenic activity testing of the six chemicals isolated from B. superba *tubers.* Five previously isolated chemicals[13] plus hexacosanoic acid 2,3-dihydroxy-propyl ester (Fig. 1), which had all been isolated from tubers of *B. superba* collected from Lampang Province, Thailand, were used as reference chemicals in the evaluating the estrogenic activity of the plant extracts. These were presented by Dr. N. Ngamrojanavanich.

Construction of yeast estrogen screening strains ERα + hTIF2 and ERβ + hSRC1. The YES assay was based on a yeast two-hybrid system and constructed by inserting the human estrogen receptor (hER) and coactivator into the yeast cells which allowed specific binding of suitable ligands with ER prior to their interaction with the yeast transcription machinery. The estrogenic activity was quantitatively evaluated by the level of expression of the reporter gene encoding the β-galactosidase enzyme.[14] Since hER exists in the two subtypes of hERα and hERβ that are distributed within specific tissues, only human transcriptional intermediary factor 2 (hTIF2) and human steroid receptor coactivator 1 (hSRC1) which have exhibited the greatest respective effectiveness to hERα and hERβ in the presence of 17β-estradiol[14] were chosen for evaluating the estrogenic activity of the *B. superba* tuberous ethanolic extracts and their six isolated chemicals.

Yeast *Saccharomyces cerevisiae* strain Y190 (MATa, ura3-52, his3-D200, ade2-101, trp1-901, leu2-3,112, gal4Dgal80D, URA3::GAL-LacZ, cyhr2, LYS2::GAL-HIS3; Clontech®, USA) was used as the host for constructing the two yeast strains, YES-hERα + hTIF2 and YES-hERβ + hSRC1, respectively harboring the plasmid pGBT9-hERαLBD and pGAD424-hTIF2 coactivator, and plasmid pGBT9-hERβLBD and pGAD424-hSRC1 coactivator. Plasmids pGBT9-hERαLBD, pGBT9-hERβLBD and pGAD424-hSRC1 were constructed by using genes hERα/β LBD and hSRC1 kindly provided by Dr. A. Ohta of the Department of Biotechnology, the University of Tokyo, Japan.[15] Plasmid pGAD424-hTIF2 was presented by Dr. Y. Masamune of the Department of Cellular and Molecular Biology, Faculty of Pharmaceutical Science, Kanazawa University.[16]

Yeast culture conditions. S. cerevisiae host strain Y190 was maintained on yeast peptone dextrose agar (YPD: 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose and 2% (w/v) agar). The yeast cells were incubated at 30 °C for two days and then preserved at 4 °C for routine use. Recombinant *S. cerevisiae* yeasts were usually selected and grown on a synthetic complete medium (SC) lacking tryptophan and leucine (SC agar: 0.67% (w/v) yeast nitrogen base w/o amino acid, 2% (w/v) glucose, 2% (w/v) dropout mix (lacking tryptophan and leucine) and 1.5% (w/v) agar). Long term preservation was achieved by growing them in SC broth lacking tryptophan and leucine at 30 °C to the mid to late logarithmic phase of growth (24 h), when an equal volume of 30% (v/v) glycerol was added and mixed and the yeast samples preserved in 1-ml aliquots at −80 °C. In the case of the β-galactosidase assay, the recombinant *S. cerevisiae* yeast samples were grown in a synthetic dextrose minimal medium (SD) broth supplemented with 0.02 mg/ml of adenine at 30 °C while vigorously shaking overnight for 14–16 h.

Recombinant yeast assay. The assays were performed by incubating 50 µl of an overnight yeast culture and 2.5 µl of one of (i) an ethanolic

tuberous extract (10^{-3}–10^{3} μg/ml final concentration), or (ii) an individual isolated chemicals from *B. superba* at 10^{-6}–1 μg/ml final concentration, or (iii) 17β-estradiol, E2 (10^{-2}–10^{-10} M final concentration, as a positive control) or (iv) DMSO (the vehicle) in each tube containing 200 μl of a fresh selective medium. After incubating at 30 °C while shaking for 4 h, a 150 μl aliquot of each cultured cell suspension was allocated to each well of a 96-well microtiter plate for measurement of the optical density (OD) at 660 nm, and 100 μl was used to harvest the cells by centrifugation at 12,000 g_{max} for 5 min. The resulting cell pellet was then resuspended in 200 μl of a Z-buffer (0.1 M sodium phosphate at pH 7.0, 10 mM KCl, 1 mM $MgSO_4$ and 3.5 mM β-mercaptoethanol) containing 1 mg/ml of zymolyase 20T, and the mixture incubated at 37 °C for 15 min. The resulting cell lysate was incubated with 40 μl of a substrate (4 mg/ml of *o*-nitrophenyl-β-D-galactoside (ONPG) in a 0.1 M sodium phosphate buffer at pH 7.0) at 30 °C for 30 min, the reaction being terminated when the yellow color of *o*-nitrophenol (ONP) had developed by adding of 100 μl of 1 M Na_2CO_3. To remove all the cell debris, the reaction tube was clarified by centrifugation at 12,000 g_{max} for 5 min, then 150 μl of the clarified supernatant was transferred into each well of a 96-well microplate and the absorbance levels at 420 nm and 550 nm were measured by a microplate reader.[14] The experiment was done in triplicate for each sample.

β-Galactosidase assay. The β-galactosidase activity is defined in terms of Miller units and calculated by the following equation:

$$\text{Miller unit} = 1000[OD_{420} - 1.75(OD_{550})]/[OD_{660}(t)(v)]$$

where OD_{420} is the absorbance of yellow ONP, OD_{550} is light scattering from the cell debris at the end of reaction, OD_{660} is the cell density at the start of the assay, t is the time of reaction (min) and v is the volume of culture used in the assay (μl).

Calculation of the estrogenic EC_{50} values for each extract. The yeast cells were incubated with none (control) and with 10^{-3} to 10^{3} μg/ml of the *B. superba* tuberous ethanolic extract before the β-galactosidase activity was subsequently analyzed. The relative potency, efficiency and relative inductive efficiency were evaluated and then compared with the values obtained from yeast cells incubated with 17β-estradiol at a dose of 10^{-2} to 10^{-10} M as a standardized reference for the estrogenic activity amongst the *B. superba* plant extracts.

The data obtained from the β-galactosidase assays were each fitted with a four-parameter logistic dose-response model. Calculations were performed with Sigmaplot software for Windows, version 9.0 (Microsoft Inc., USA), using the following function;

$$Y = \frac{[A - D]}{1 + [C/X]^{B}} + D$$

where Y is the response value (β-galactosidase activity), X is the sample concentration in the test, A is the maximum response of β-galactosidase activity (ligand efficiency), B is the relative slope of the middle region of the curve as estimated from a linear/log regression of the linear part of the dose–response curve, C is the sample concentration that resulted in 50% efficiency, and D is the detection limit. The EC_{50} value is the value of C in the equation and represents the ligand potency.

Preparation and use of the S9 mixture. The *in vitro* metabolic activation of each sample resulting from the addition of a freshly prepared rat liver S9 mixture, was tested against the tuberous ethanolic extracts from two plant samples that exhibited no detectable estrogenic activity in either of the two YES assays. Thus the plant samples were selected on the basis of their likely sensitivity and ease of detection, since they had a low endogenous estrogenic activity level which would otherwise potentially saturate the assay, masking the newly formed activity by the *in vitro* hepatic metabolic activation.

A 10-μl aliquot of a plant extract dissolved in DMSO was incubated with a 990-μl aliquot of freshly prepared S9 mixture as described[17] (containing 0.5 mg of the rat liver S9 fraction (Wako Pure Chemical Industries, Ltd., Japan), 0.16 M $MgCl_2$, 0.1 M NADP, 0.1 M G-6-P, 0.5 M sodium phosphate buffer at pH 7.4, and 1 M KCl) at 37 °C for 4 h. The negative control consisted of the S9 fraction heat-inactivated at 95 °C for 5 min. Each S9-treated plant extract was stored at −80 °C until needed. The final respective concentrations of S9-treated *B. superba* and puerarin in the assay were 1000 and 42 μg/ml.

Evaluation of the antiestrogenic activity. The antiestrogenic activity of *B. superba* was evaluated by examining the tuberous ethanolic extracts that showed no detectable estrogenic activity were examined for anti-estrogenic activity based on their ability to inhibit the β-galactosidase induction by 17β-estradiol in the YES-hERα + hTIF2 and YES-hERβ + hSRC1 assay systems. These two samples were accordingly selected based on their likely sensitivity and ease of detection of anti-estrogenic activity, since they would either have had too low an estrogenic activity level, relative to any anti-estrogenic activity, to mask overcoming the exogenous 17β-estradiol activity.

Statistical analyses. The β-galactosidase unit activity presented in this study is presented as the mean ± standard deviation (SD) of three independent experiments. An unpaired Student's *t*-test and Duncan's multiple-range test were used for analyzing of the test results with $p < 0.05$ being considered significant, by using SPSS Version 11 statistical software program.

Results

ERα- and ERβ-estrogenic activities

The ERα- and ERβ-estrogenic activities, as respectively determined with the YES-hERα + hTIF2 and YES-hERβ + hSRC1 based assays of the six pure compounds isolated from *B. superba* are summarized in Table 1. The relative ERα-estrogenic potency of these six isolated *B. superba* chemicals formed three broad groupings with, in order of potency, group 1 (7-hydroxy-6,4′-dimethoxyisoflavone) > group 2 (medicarpin > formononetin) > group 3 (prunetin > hexacosanoic acid 2,3-dihydroxy-propyl ester > 7,4′-dimethoxyisoflavone). However, although the ERβ-estrogenic activity showed the same three groupings, with group 1 also showing the highest potency, that of groups 2 and 3 was reversed such that the actual order in terms of relative potency was; 7-hydroxy-6,4′-dimethoxyisoflavone > 7,4′-dimethoxyisoflavone > hexacosanoic acid 2,3-dihydroxy-propyl ester > medicarpin > formononetin > prunetin.

To evaluate the ERα- and ERβ-estrogenic activities of the *B. superba* tuberous ethanolic extracts, the relative potency, efficiency and relative inductive efficiency of the plant extracts derived from the YES-hERα + hTIF2 and YES-hERβ + hSRC1 YES assays were compared (Tables 2 and 3). The results for the 23 geographical samples of *B. superba* are ranked in order of the highest relative potency observed for ERα (Table 2) and ERβ (Table 3). The mean value for the relative potency of each plant extract by the YES-hERβ + hSRC1 assay was found to be approximately six-fold greater than that by the YES-hERα + hTIF2 assay, this difference being statistically significant ($p < 0.05$). Presentation in terms of the efficiency and relative inductive potency showed a similar pattern for both. Of some note is the significant variation in both ERα and ERβ activities between the different samples. Whether this was attributable to different geographical locations (cultivars and or cultivation conditions) or occurred with any given cultivation remains to be ascertained.

Anti-estrogenic activity

The plant extracts that expressed very little or no detectable estrogenic activity in the YES-hERα + hTIF2 assay (collected from Chaiyaphum Province) and YES-hERβ assay (collected from Saraburi Province)

Table 1. Apparent *in Vitro* ERα and ERβ Activating Estrogenic Activities of *B. superba* Derived Pure Compounds Evaluated by the YES-hERα + hTIF2 and YES-hERβ + hSRC1 Assays

Pure compound	YES-hERα + hTIF2		YES-hERβ + hSRC1	
	EC_{50} (μg/ml)	Relative potency (%)	EC_{50} (μg/ml)	Relative potency (%)
Prunetin	$63.5 \pm 0.1^{d*}$	0.02	$349.1 \pm 27.9^{c*}$	0.03
Medicarpin	$1.13 \pm 0.36^{b*}$	1.27	$142.6 \pm 31.6^{b*}$	0.06
Formononetin	$1.58 \pm 0.32^{c*}$	0.91	$316.0 \pm 29.1^{c*}$	0.03
7-Hydroxy-6,4′-dimethoxyisoflavone	$0.09 \pm 0.35^{a*}$	15.47	$1.73 \pm 0.18^{a*}$	5.27
7,4′-Dimethoxyisoflavone	$220.2 \pm 0.2^{f*}$	0.01	$22.6 \pm 4.4^{a*}$	0.40
Hexacosanoic acid 2,3-dihydroxy-propyl ester	$162.3 \pm 0.04^{e*}$	0.01	$27.3 \pm 3.4^{a*}$	0.33
17β-Estradiol (E2)	$0.01 \pm 0.43^{a*}$	100	$0.09 \pm 0.24^{a*}$	100

Relative potency (EC_{50} of E2/EC_{50} of plant chemical) × 100.

Expressed as mean ± SD, n = 3. means not sharing a common superscript letter in the same column are significantly different ($p < 0.05$), as determined by Duncan's multiple range test.

$^{*}p < 0.05$ significantly different from the negative control (DMSO) by Student's *t*-test.

were selected to screen for the potential anti-estrogenic activity. These two plant extracts clearly demonstrated an anti-estrogenic effect in both these assays, showing clear inhibition of the 17β-estradiol effect. The apparent anti-estrogenic activity of both plant extracts was slightly higher in the ERβ (YES-hERβ + hSRC1) assay than in the ERα assay (YES-hERα + hTIF2) assays (Fig. 2). Regardless of this, these results are consistent with the notion that at least these two, if not all the unmetabolized *B. superba* tuberous ethanolic extracts harbored anti-estrogenic activity.

In vitro *metabolic activation*

One of the plant samples that exerted very little detectable estrogenic activity in the YES-hERα + hTIF2 assay (collected from Chaiyaphum Province) and the one plant showing negative in the YES-hERβ + hSRC1 assay (collected from Saraburi Province), were tested for potential *in vitro* hepatic metabolism by coincubating with a fresh S9 mixture prior to assaying for the estrogenic activity. Both the ERα and ERβ estrogenic activities of these two tuberous ethanolic plant extracts were significantly, but differently increased by the S9 mixture treatment, with an enhanced ERα- but not ERβ-estrogenic activity for the sample from the Chaiyapum Province and, conversely, enhanced ERβ- but not ERα-estrogenic activity for the sample from the Saraburi Province (Fig. 3). These results are consistent with the notion that hepatic metabolism, in this case attained *in vitro* by incubating with the S9 mixture, could elevate the net estrogenic activity (*i.e.*, that observed above any level of inhibitory anti-estrogenic activity also present) of the *B. superba* tuberous ethanolic extracts and furthermore, that such enhanced ERα- or ERβ-estrogenic activity may be different in different plant extracts, suggesting differing chemical constituents. However, the bioactive compounds so metabolized and the new bioactive compounds so formed remain to be elucidated.

Discussion

We tested in this study, a modified yeast two-hybrid system based upon the human estrogen receptors (ERα and ERβ, separately) coupled to the β-galactosidase reporter gene to separately monitor the ERα- and ERβ-estrogenic activities in the *B. superba* tuberous ethanolic extracts as well as those in six pure chemicals that had previously been isolated from *B. superba* tubers.

The YES-hERα + hTIF2 based assay revealed highly potent ERα-estrogenic activity with 7-hydroxy-6-4′-dimethoxyisoflavone at a level broadly comparable to that of the 17β-estradiol positive control. Moderately good ERα-estrogenic activity, and thus assumed binding and or activation, was also apparent with medicarpin and formononetin. However, the other three compounds each showed a much weaker ERα-estrogenic activity, the weakest ERα-estrogenic activity being observed with 7,4′-dimethoxyisoflavone. This highlights the diverse variation in estrogenic activity amongst the compounds.

With respect to the ERβ-estrogenic activity, 7-hydroxy-6-4′-dimethoxyisoflavone also exhibited by far the strongest activity of the six compounds, and thus could bind strongly and activate both ERα and ERβ. However, its ERβ-estrogenic activity was less than that of the 17β-estradiol positive control. Moreover, medicarpin and formononetin, which had moderate ERα-estrogenic activity, showed only a weak level of ERβ-estrogenic activity, whilst 7,4′-dimethoxyisoflavone and hexacosanoic acid 2,3-dihydroxy-propyl ester, which both showed weak ERα-estrogenic activity, revealed a moderate ERβ-estrogenic activity. This means that the *B. superba* tuberous ethanolic extracts would have been likely to initiate binding to both the human ERα and ERβ receptors with different affinity depending upon their relative chemical constituents.

Importantly, variation between the *B. superba* samples in their estrogenic activity was apparent in this study. The majority of the plant extracts expressed a detectable, but low level of both ERα- and ERβ-estrogenic activity. However, the plant estrogenic activity was classified as of low efficiency in all 23 samples, this was especially true for the ERα-estrogenic activity which was some four- to six-fold lower than the corresponding ERβ-estrogenic activity, depending on which parameter was used for calculating the estrogenic activity. The clear suggestion is that the non-metabolized phytochemicals in the *B. superba* tuberous ethanolic extracts could bind to and activate ERβ more strongly than ERα, at least with these two different YES constructions. However, it should be noted with these YES assays that 17β-estradiol bound or activated ERα more strongly than ERβ with the same YES constructions. This correlates with a previous finding in a study

Table 2. ERα-Estrogenic Activity of *B. superba* Tuberous Ethanolic Extracts Evaluated by the YES-hERα + hTIF2 Assay

No.	Province	EC_{50} (μg/ml)	Relative potency (%)	Efficiency (β-galactosidase unit/μg plant extract)	RIE (%)
1	Sisaket	83.25 ± 3.03^{b}	0.017	211.11 ± 19.43^{fg}	5.55
2	Ratchaburi	88.55 ± 18.05^{bc}	0.016	244.09 ± 9.40^{g}	6.42
3	Khon Kaen	124.66 ± 4.15^{cd}	0.012	454.76 ± 14.28^{i}	11.97
4	Nakorn Ratchasima	150.00 ± 1.27^{d}	0.010	379.19 ± 9.48^{h}	9.98
5	Lopburi	153.83 ± 15.97^{d}	0.009	123.55 ± 2.95^{de}	3.25
6	Loei	223.02 ± 61.35^{e}	0.006	70.61 ± 1.37^{abcd}	1.86
7	Chiang Rai	232.09 ± 5.48^{e}	0.006	45.99 ± 4.12^{abc}	1.21
8	Nong Bua Lam Phu	292.11 ± 6.24^{f}	0.005	128.74 ± 7.94^{de}	3.39
9	Lampang	387.69 ± 34.18^{g}	0.004	101.33 ± 7.62^{cd}	2.67
10	Chachaengsao	399.93 ± 4.63^{g}	0.004	87.55 ± 5.98^{bcd}	2.30
11	Saraburi	417.85 ± 7.72^{g}	0.003	22.96 ± 13.32^{ab}	0.60
12	Nakhon Sawan	463.07 ± 35.57^{h}	0.003	177.10 ± 14.32^{ef}	4.66
13	Phrachin Buri	508.83 ± 56.60^{i}	0.003	53.34 ± 6.33^{abc}	1.46
14	Mae Hong Sorn	510.05 ± 4.71^{i}	0.003	32.49 ± 1.21^{abc}	0.85
15	Tak	522.82 ± 39.23^{i}	0.003	98.45 ± 8.42^{cd}	2.59
16	Uttraradith	620.78 ± 7.70^{j}	0.002	6.11 ± 2.99^{a}	0.16
17	Phetchabun	666.92 ± 7.84^{k}	0.002	11.94 ± 3.16^{a}	0.31
18	Chonburi	823.83 ± 6.81^{l}	0.002	35.87 ± 5.54^{abc}	0.94
19	Chaiyaphum	$>1000^{m}$	<0.001	NA	NA
20	Chantaburi	$>1000^{m}$	<0.001	NA	NA
21	Chiang Mai	$>1000^{m}$	<0.001	NA	NA
22	Kanchanaburi	$>1000^{m}$	<0.001	NA	NA
23	Phitsanulok	$>1000^{m}$	<0.001	NA	NA
	17β-Estradiol (E2)	0.01 ± 0.00^{a}	100	$3{,}800.40 \pm 179.32^{j}$	100

NA, not applicable

Relative potency; (EC_{50} of E_2/EC_{50} of plant extract) × 100.

The plateau of the curve per μg plant extract designated as the ligand efficiency.

Relative inductive efficiency (RIE): (Efficiency of plant extract/Efficiency of E_2) × 100.

Expressed as mean ± SD, n = 3. means not sharing a common superscript letter in the same column are significantly different ($p < 0.05$) as determined by Duncan's multiple range test.

Table 3. ERβ-Estrogenic Activity of *B. superba* Tuberous Ethanolic Extracts Evaluated by the YES-hERβ + hSRC1 Assay

No.	Province	EC_{50} (μg/ml)	Relative potency[a] (%)	Efficiency (β-galactosidase unit/μg plant extract)	RIE (%)
1	Khon Kaen	82.99 ± 3.14^{b}	0.11	225.55 ± 15.81^{g}	47.74
2	Nakhon Ratchasima	83.42 ± 4.54^{b}	0.11	261.29 ± 17.89^{h}	55.30
3	Lopburi	99.96 ± 0.12^{b}	0.09	150.30 ± 2.72^{f}	31.81
4	Chiang Rai	99.96 ± 0.12^{b}	0.09	58.85 ± 7.47^{d}	12.46
5	Tak	199.76 ± 5.73^{c}	0.05	103.42 ± 8.21^{e}	21.89
6	Nong Bua Lam Phu	354.23 ± 19.50^{d}	0.03	59.46 ± 5.70^{d}	12.58
7	Lampang	371.75 ± 45.03^{d}	0.02	68.20 ± 10.45^{d}	14.43
8	Phrachin Buri	399.73 ± 0.17^{e}	0.02	21.43 ± 8.90^{abc}	4.54
9	Loei	400.11 ± 0.21^{e}	0.02	13.84 ± 7.02^{ab}	2.93
10	Chantaburi	413.50 ± 2.26^{e}	0.02	9.13 ± 0.85^{ab}	1.93
11	Sisaket	413.50 ± 13.38^{e}	0.02	35.65 ± 1.45^{c}	7.55
12	Nakhon Sawan	538.59 ± 13.84^{f}	0.02	25.31 ± 2.52^{bc}	5.36
13	Uttraradith	599.59 ± 0.15^{g}	0.02	28.45 ± 2.45^{bc}	6.02
14	Phetchabun	599.70 ± 0.42^{g}	0.02	21.18 ± 4.98^{abc}	4.48
15	Ratchaburi	619.02 ± 5.15^{g}	0.01	108.17 ± 5.35^{e}	22.89
16	Kanchanaburi	694.31 ± 17.01^{h}	0.01	9.91 ± 1.52^{ab}	2.10
17	Chaiyaphum	733.08 ± 8.78^{i}	0.01	15.31 ± 0.95^{abc}	3.24
18	Saraburi	0^{a}	0	0^{a}	0
19	Chonburi	$>1000^{j}$	<0.001	NA	NA
20	Chachaengsao	$>1000^{j}$	<0.001	NA	NA
21	Mae Hong Son	$>1000^{j}$	<0.001	NA	NA
22	Chiang Mai	$>1000^{j}$	<0.001	NA	NA
23	Phitsanulok	$>1000^{j}$	<0.001	NA	NA
	17β-Estradiol (E2)	0.09 ± 0.002^{a}	100	472.48 ± 46.09^{i}	100

NA, not applicable.

Relative potency; (EC_{50} of E_2/EC_{50} of plant extract) × 100.

The plateau of the curve per μg plant extract designated as the ligand efficiency.

Relative inductive efficiency (RIE); (Efficiency of plant extract/Efficiency of E_2) × 100.

Expressed as mean ± SD, n = 3. means not sharing a common superscript letter in the same column are significantly different ($p < 0.05$) as determined by Duncan's multiple range tests.

on soy isoflavonoids that those phytoestrogens could bind to or activate ERβ more strongly than ERα whilst estrogen bound to ERα more strongly than to ERβ.[16] The results of this present study also showed variation in the estrogenic activity of the plant samples collected from different provinces in Thailand although there was not a sufficient number of samples from within each of the 23 localities to distinguish intra-regional variation from inter-regional variation. Nevertheless, the results were not directly related to the region of the existent plant samples. This implies the likely combined influence of the environment, plant age/development stage and plant genetics on such bioactive difference. There was no assay on the concentration of the six isolated compounds in all analyzed samples, except for the one collected from Lampang Province. It is correspondingly not possible to correlate the estrogenic activity of the plant samples with the six chemicals isolated in this study.

The tuberous ethanolic extracts from *B. superba* that did not exhibit estrogenic activity in the YES-hERα + hTIF2 and YES-hERβ + hSRC1 based assays, did exhibit anti-estrogenic activity in the incubation assay with 17β-estrodiol. However, this result was obtained with non-metabolized samples whereas the oral administration of herbs like *B. superba* would be likely to result in hepatic metabolism. Such hepatic metabolism may enhance or reduce any anti-estrogenic activity, an aspect which was not assayed here. The potential importance of this point is illustrated by the estrogenic activity, whereby the addition of an *in vitro* hepatic metabolizing S9 system clearly increased the net observed estrogenic activity of the two *B. superba* samples tested and differentially increased the ERα- but not ERβ-estrogenic activity in one plant extract, but *visa versa* in the other sample.

Various methods have been used to evaluate the estrogenic activity of phytoestrogens or plant materials. The uterotrophic assay with ovariectomized rats is a classical method and has been used to establish the estrogenic activity in phytoestrogen-rich plants.[18] The vaginal cornification assay is a simpler method that is performed with ovariectomized rats, and has also enabled the successive differential evaluation of estrogenic activity for the phytoestrogen-rich *Pueraria mirifica* and *P. lobata* populations.[8,19] The MCF-7 screening test (E-assay), based on the estrogen-dependent *in vitro* proliferation of the human mammary adenocarcinoma cell line in the presence of a tested phytoestrogen, has been able to demonstrate the biphasic estrogenic response of the phytoestrogen-rich *B. superba* and *P. mirifica* extracts[12,20] and has also revealed that this was dependent on where the plant samples were collected. Our previous analysis using an MCF-7 proliferation assay of the same plant samples of *B. superba* collected from Lampang Province as those used in this study, found no proliferative effect but instead an antiproliferative effect in the tested dose

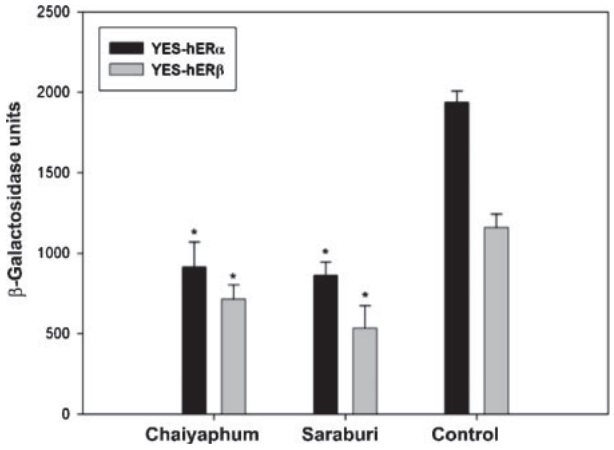


Fig. 2. The Anti-Estrogenic Activity of *B. superba* Tuberous Ethanolic Extracts at a Final Concentration of 1000 µg/ml as Determined by the YES-hERα + hTIF2 and YES-hERβ + hSRC1 Assays.

A plant sample at a 10^3 µg/ml, final concentration of was mixed with 17β-estradiol at a 10^{-9} M final concentration and determined against the same concentration of 17β-estradiol (as a control). Data are expressed as the mean ± SD, n = 3. $^*p < 0.05$; significantly different from the 17β-estradiol treatment by the Student's *t*-test.

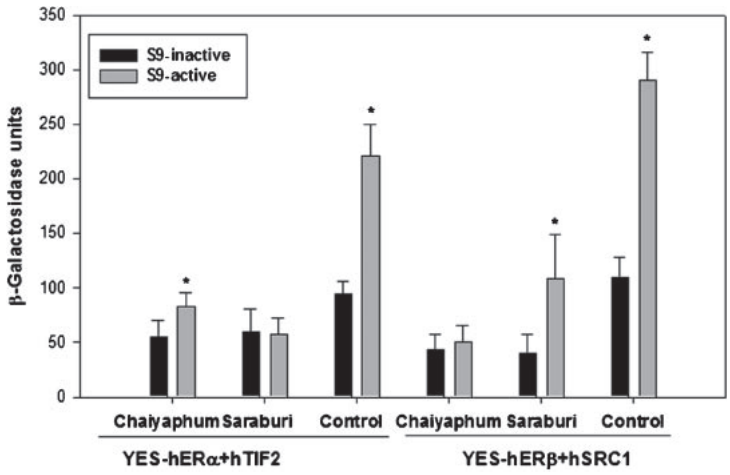


Fig. 3. Estrogenic Activity of the *B. superba* Tuberous Ethanolic Extracts after Treating with the Rat Liver S9 Fraction Evaluated by the YES-hERα + hTIF2 and YES-hERβ + hSRC1 Assays.

Abbreviation: S9-inactive is the heat-inactivated S9 fraction (negative control): S9-active is the active S9 fraction. Each value is the mean of three independent experiments. The final concentration of each S9-treated *B. superba* tuberous ethanolic extracts at 1000 µg/ml and of puerarin (control) at 42 µg/ml was tested. Data are expressed as the mean ± SD, n = 3. $^*p < 0.05$; significantly different from the S9-inactive treatment by Student's *t*-test.

range of 10–1000 μg/ml.[12] In contrast to the results with the MCF-7 assay, most of the *B. superba* samples in this YES-based study, including those from Lampang Province, exhibited estrogenic activity in the yeast two-hybrid system suggesting this hybrid system to be more sensitive than the standard MCF-7 cell proliferation test in determining the potential net estrogenic activity, especially of plant materials. However, these two test systems involve no hepatic metabolic activation and are theoretically unlikely to represent the real situation following an oral administration, indeed, the estrogenic activity of *P. mirifica* experimentally assayed with HepG2 cells has suggested the requirement for hepatic activation to demonstrate maximal estrogenic activity.[21] In contrast, the establishment of the YES test system in the presence of an S9 mixture comprising rat liver enzymes that was used in this study supports our previous finding that *in vitro* metabolic activation could enhance the estrogenic activity of the plant extracts.[22]

Even though the *in vitro* YES assay is a rapid method and could be quantitatively applied, it may not have the high level of sensitivity for detecting estrogenic activity that the *in vivo* vaginal cornification assay offers. Moreover, it may not be possible to reliably apply the results from the same plant materials in tests with animals to predict what would be the case with human consumption. Moreover, the successful detection of estrogenic activity in tuberous ethanolic extracts from diverse *B. superba* samples provides additional support for the efficacy of the *in vitro* yeast two-hybrid system for detecting estrogenic, and perhaps anti-estrogenic, activity. This system has previously been used to study estrogens in such other plants as *Moghania philippinensi,*[23] the traditional Chinese herb, *Polygonum cuspidatum*,[24] several Mediterranean plants[25] and *Millettia pachycarpa*.[26] We demonstrated in this study that most of the Thai *B. superba* populations showed detectable estrogenic activity and also potentially anti-estrogenic activity. The anti-estrogenic activity of the *B. superba* tuber extract is in accordance with the study of *B. superba* samples collected from Lampang Province by a hormonal assay and histological assay of the uterus in ovariectomized rats.[27] This might be one of the reasons why the plant is used in the recipes for treating menopausal symptoms. The YES test systems could provide useful information in screening potential *B. superba* material for further developing phytoestrogen-rich products, especially in conjugation with the S9 *in vitro* hepatic metabolic activation.

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