SUPPLEMENTARY MATERIAL

The synergy effect of daidzein and genistein isolated from *Butea superba* Roxb. on the reproductive system of male mice

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Abstract

Butea superba Roxb. (BS) has been used in Thai men as an aphrodisiac, and prevent erectile dysfunction. Nevertheless, the active ingredients, dosages, have not been cleared. Hence, this study was to investigate the effect of compounds from the BS on the reproductive parameters of male mice.

The results revealed that BS was extracted to afford biochanin A, genistein, which were the first report on BS, and daidzein. The mice were treated by daidzein, genistein alone and in combination. The results showed that the sperm number and motility, cholesterol, and testosterone level of all isoflavones treated groups were significantly higher than controls ($p<0.01$). Obviously, daidzein plus genistein exhibited a synergistic effect, which is also the first report, resulted in significantly displayed higher levels of these parameters compared to others.

So, the synergistic activity of these isoflavones may useful in improving libido, erectile capacity, and assist infertility of poor spermatozoa in men.

Keywords: *Butea superba* Roxb; biochanin A; genistein; daidzein; sperm number; testosterone; synergistic effect

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1. Experimental

1.1 Instrumentation

The UV spectra were obtained with Varian CARY 1E UV-Vis spectrophotometer. The FT-IR spectra were recorded on Nicolet spectrophotometer. The ^1H -NMR and ^{13}C -NMR spectra were recorded on Varian INOVA 300 NMR spectrometer in acetone- d_6 solution for compound 1, $\text{CDCl}_3 + \text{CD}_3\text{OD}$ mixture for compound 2, the chemical shifts are expressed in δ (ppm) concerning the solvent signals. The Liquid Chromatography-Mass Spectrometry (LC-MS), 6400 Series Triple Quadrupole B.05.00 (B5027.0), quadrupole mass spectrometer equipped with the electrospray ionization (ESI) turbo ion interface was used to identify compound 3 in comparison with the standard compound. The mass spectrometer was recorded on Hewlett Packard 5989 HP mass spectrometer. Column chromatography was performed on silica gel 60 Art 7734 and 9385 and Preparative Thin Layer Chromatography (PTLC) silica gel GF254.

1.2 Plant materials

Extract preparation. Fresh tuberous roots of BS were collected from Chiang Rai province, Thailand. The plant specimens were authenticated by Associate Professor Yuthana Smitasiri who has many experiences in working with this plant and identification was done in comparison with the voucher specimen no. BCU 1046. The specimen was deposited at Forest Herbarium, National Park, Wildlife, and Plant Conservation Department, Ministry of Natural Resources and Environment, Thailand. The tuberous roots were washed thoroughly and dried in an oven at 60°C for 72 hours. The dried samples were ground to a fine powder.

1.3 Extraction, isolation, and identification

The methods of BS extraction, isolation and identification were modified from Cherdshewasart *et al.* (2007) and Kayano *et al.* (2012) (Cherdshewasart *et al.* 2007, Kayano *et al.* 2012). Briefly, dried powdered tuber roots of *B. superba* (2 kg) were extracted continuously with ethanol by Soxhlet extraction for 12 hours. The ethanol extracted was evaporated under reduced pressure to pass on the ethanol crude extract (30.5 g). The ethanol extract (25.0 g) was separated by silica gel column chromatography. The column was eluted sequentially with hexane, chloroform, acetone, and methanol. All fractions were concentrated to give hexane crude extract (0.5 g), chloroform crude extract (1.6 g), acetone crude extract (7.4 g), and methanol crude extract (8.6 g).

The acetone crude extract was subjected to silica gel column chromatography. The column was eluted successively with hexane-acetone (1:1), acetone, chloroform-methanol (1:1), and methanol. Every fraction was concentrated to a small volume to give four major fractions (Fr.1 1.7 g, Fr.2 1.8 g, Fr.3 1.1 g, Fr.4 2.0 g and Fr.5 4.0 g).

A portion of fraction 1 (1.0 g) was chromatographed on silica gel 60 column. The column was eluted successively with hexane, 1:4 hexane-acetone, 2:3 hexane-acetone, 3:2 hexane-acetone, 4:1 hexane-acetone, and acetone respectively. Every fraction was collected and concentrated to a small volume to give six fractions (Fr. 1-1 2.8 mg, Fr. 1-2 81.2 mg, Fr. 1-3 59.5 mg, Fr. 1-4 126.5 mg, Fr. 1-5 53.2 mg and Fr.1-6 54.8 mg).

Fraction 1-2 (81.2 mg) was further purified by preparative thin layer chromatography (hexane: acetone 2:1) to afford two fractions (A 57.6 mg, B 9.4 g). Fraction A (57.6 mg) was further purified by preparative thin layer chromatography using the same developer solvent to give the crude compound 1 (21.1 mg) which was recrystallized from chloroform-methanol mixed solvent to obtain pure compound 1 (18.1 mg) as a light yellow powder.

Fraction 1-5 (53.2 mg) was further purified by preparative thin layer chromatography (hexane: acetone 1: 1) to give pure compound 2 (8.5 mg) as a white powder.

A portion of fraction 5 (2.0 g) was chromatographed on silica gel 60 column. The column was successively eluted with a serial dilution of hexane:acetone, 1:1, separated by monitoring with TLC till compound 3 was collected. Structural identification of the isolated compounds was carried out following the method of Shaw *et al.* (2013) (Shaw et al. 2013) with a minor modification. Briefly, the LC-MS, 6400 Series Triple Quadrupole B.05.00 (B5027.0), quadrupole mass spectrometer equipped ESI turbo ion interface was performed to identify compound 3 in comparison with the standard compound. The mass spectrometer was completed in the positive ion detection mode. The analysis was quantified by multiple-reaction monitoring (MRM) mode performing with the precursor-to-product ion pair.

1.4 Experimental animals and procedures

Fifty adult male mice, aged about 130 days, weighing 30-40 g, were obtained from the National Laboratory Animal Centre, Thailand. The experimental protocol was approved in accordance with guidelines for the care and use of laboratory animal by animal care

and use committee (ACUC), Permit No. 13/2555, the Suranaree University of Technology, which was conducted in accordance with European community guidelines.

Mice were divided into five groups with 10 animals each. Before treatment (pre-treatment), all mice in these groups were collected blood and sperm for comparison with after treatment (post-treatment). These mice were fed with a diet that was a casein-based open formula purified diet with non-detectable levels of the estrogenic isoflavones genistein, daidzein or glycitein (Fielden et al. 2003). During the treatment period, the first group was treated by daily gavage with 0.1 ml of corn oil (Sigma, St Louis, MO, USA). The second group was treated by daily gavage with 0.1 ml of corn oil, plus sildenafil, a positive control, at 10.00 mg/kg BW/day. The third, fourth and fifth groups were orally gavaged with daidzein, genistein, and daidzein plus genistein in corn oil for a nominal dose of 1.0, 1.0 and 0.2 + 0.2 mg/kg BW/day respectively. The animal's weight was recorded daily throughout the experimental period. The experimentation was performed throughout 40 consecutive days. At the end of the treatment period, all mice were sacrificed under thiopental sodium anaesthesia and subjected to necropsy. The blood and sperm were collected to compare sperm motility, sperm number, cholesterol, and testosterone level with pre-treatment in each mouse (Cherdshewasart et al. 2008, Sharma et al. 2013).

1.5 Sperm motility and sperm count assays

Sperm motility was done following the method of Sharma et al., (2013) (Sharma et al. 2013).

Motility (%) = (number of motile spermatozoa / total number of spermatozoa) X 100

The cauda epididymis was cut and weighed. A cell suspension was prepared by macerating the cauda in 1.0 ml of 0.85% saline. The cell suspension was kept for 24 hours at four °C. The suspension was then filtered through a double gauze layer, and an aliquot of the sample was used for sperm count in an Makler counting chamber. An aliquot of the epididymis sperm suspension was smeared, stained with haematoxylin and Essen, and then examined under a light microscope (CH-2, Olympus, Japan) at a magnification of 100X. The head and tail abnormalities (200 sperms per animal) were recorded.

1.6 Serum testosterone assay

Concentrations of mice testosterone were measured by radioimmunoassay techniques of the World Health Organization and extracted with diethyl ether. The intra-assay

coefficients of variation were 7% for testosterone and reagents obtained from the National Hormone and Pituitary Program (Sharma et al. 2013).

1.7 Cholesterol analysis

Blood was collected for cholesterol analysis before and after treatment. At the end of the experiment, blood samples were collected by cardiac puncture under thiopental sodium anesthesia from 9.00 to 10.00 a.m. and blood serum was prepared by centrifugation at $1,000\times g$ for 30 minutes and kept at $-20\text{ }^{\circ}\text{C}$ for cholesterol analysis. The assays were performed with an automated analysis system at the service laboratory of Suranaree University of Technology Hospital (Cherdshewasart et al. 2008).

1.8 Effect on reproductive organs and body weight

At the end of treatment period as described previously, all mice were sacrificed and subjected to necropsy. The reproductive organs (testis, epididymis, vas deferens, seminal vesicle and prostate gland) were removed to examine relative organ weight and histopathology of seminiferous tubules was studied. These relative organ weights in each group were compared. The animal's weight was recorded every day throughout the experimental period (Sharma et al. 2013).

1.9 Synergy and Statistical Analysis

All data are presented as the mean $\pm$ S.E.M. The interaction between the two agents was estimated by calculating the fractional inhibitory concentration of the combination (FIC) index. The FIC of each agent was calculated by dividing the concentration of the compound present in that treated group in combination where post-treated group showed significantly higher levels (SHL) than a pre-treated group of that compound alone to increase that measured parameter. The FIC index was calculated using the following formula: FIC of daidzein = SHL daidzein in combination / SHL of daidzein alone; FIC of genistein = SHL of genistein in combination / SHL of genistein alone; hence FIC index = FIC of daidzein + FIC of genistein. When the FIC index of the combination is less than 1.0, the combination is termed as synergistic; when FIC index is equal 1.0, it indicates 'no interaction' between the agents, and a value above 1.0 indicates antagonism between the two compounds (Wagner & Ulrich-Merzenich 2009).

Significant differences between the relative selected reproductive organ weight or body weight control and treatment groups were analysed by ANOVA (Armstrong et al. 2002, Van Breukelen 2006). The differences of cholesterol, testosterone level, growth rate and sperm analysis between pre- and post-treatment groups were calculated

by paired student's *t-test*. Then, a significant difference between each group was compared using ANCOVA (Borm et al. 2007, Van Breukelen 2006, Winkens et al. 2007). The Tukey's HSD post hoc test at $p < 0.01$, means sharing the different superscript letters, were also considered statistically significant difference between each group.

Table S1. The ^1H -NMR and ^{13}C -NMR chemical shifts value of compound 1, 2, and 3 measured in acetone- d_6 , $\text{CDCl}_3 + \text{CD}_3\text{OD}$ mixture and DMSO respectively.

Atom position	^1H (J in Hz) of compound			^{13}C (J in Hz) of compound		
	1	2	3	1	2	3
2	8.15 (s)	7.92 (s)	8.30 (s)	153.82	153.23	153.36
3	-	-	-	123.20	122.18	123.04
4	-	-	-	180.94	181.24	179.82
5	12.99 (s)	-	-	163.31	162.49	135.56
5-OH	6.28 ($d, J = 2.1$)	-	-	99.28	-	-
6	9.60 (s)	6.28 ($d, J = 2.1$)	6.95 ($d, J = 8.7, 1.6$)	164.37	99.46	112.24
7	-	-	-		164.57	165.24
7-OH	6.40 ($d, J = 2.1$)	-	-	93.90	-	-
8	-	6.37 ($d, J = 2.1$)	6.90 ($d, J = 1.6$)	158.42	94.19	99.80
9	-	-	-	105.58	157.47	157.2
10	12.99 (s)	-	-	153.82	105.54	114.76
1'	-	-	-	153.82	123.86	126.62
2'	7.53 ($d, J = 8.4$)	7.35 ($d, J = 8.7$)	7.40 ($d, J = 8.4$)	123.20	130.37	130.14
3'	6.98 ($d, J = 8.4$)	6.89 ($d, J = 8.7$)	6.94 ($d, J = 8.4$)	180.94	115.57	115.02
4'	-	-	-	163.31	158.56	157.56
4'-OH	-	-	-	-	-	-
4'-OCH ₃	3.83 (s)	-	-	54.97	-	-
5'	6.98 ($d, J = 8.4$)	6.89 ($d, J = 8.7$)	6.94 ($d, J = 8.4$)	113.91	115.57	115.04
6'	7.53 ($d, J = 8.4$)	7.35 ($d, J = 8.7$)	7.40 ($d, J = 8.4$)	130.46	130.37	130.18

Table S2. Effects of daidzein, genistein isolated from the BS alone and in combination, and sildenafil on the reproductive organ weight of mice. Con = Control, Sil(10.0) = Sildenafil at 10 mg/Kg BW/day, Dai(1.0) = Daidzein at 1.0 mg/Kg BW/day, Gen(1.0) = Genistein at 1.0 mg/Kg BW/day, Dai(0.2)+Gen(0.2) = Daidzein 0.2 mg/Kg BW/day plus Genistein 0.2 mg/Kg BW/day. Data was displayed in mean±SEM (n=10). The significant difference between each group, means sharing the different superscript letters, was compared using ANOVA and Tukey's HSD post hoc test at $p<0.01$.

Reproductive organ (g)	Con	Sil(10.00)	Dai(1.0)	Gen(1.0)	Dai(0.2)+Gen(0.2)
Testis	0.18±0.023 ^b	0.30±0.045 ^a	0.29±0.014 ^a	0.25±0.008 ^{ab}	0.24±0.013 ^{ab}
Epididymis	0.08±0.002 ^b	0.08±0.01 ^c	0.09±0.01 ^c	0.16±0.003 ^a	0.14±0.007 ^{ab}
Vas deferens	0.03±0.004	0.02±0.003	0.03±0.003	0.03±0.004	0.02±0.004
Seminal vesicle	0.12±0.004 ^b	0.13±0.004 ^b	0.12±0.005 ^b	0.31±0.001 ^a	0.30±0.022 ^a
Prostate gland	0.06±0.005 ^a	0.04±0.005 ^b	0.06±0.003 ^a	0.02±0.002 ^a	0.02±0.002 ^b

Figure S1. Effects of daidzein, genistein isolated from the BS alone and in combination, and sildenafil on sperm count of mice. Con = Control, Sil(10.0) = Sildenafil at 10 mg/Kg BW/day, Dai(1.0) = Daidzein at 1.0 mg/Kg BW/day, Gen(1.0) = Genistein at 1.0 mg/Kg BW/day, Dai(0.2)+Gen(0.2) = Daidzein 0.2 mg/Kg BW/day plus Genistein 0.2 mg/Kg BW/day. The significant difference between pre- and post-test in each group was compared using paired Student t-test at $** p < 0.01$. A significant difference between ANCOVA adjusted post-treated level in each group, means sharing the different superscript letters, was compared using ANCOVA and Tukey's HSD post hoc test at $p < 0.01$.

Figure S2. Effects of daidzein, genistein isolated from the BS alone and in combination, and sildenafil on sperm motility (%) of mice. Con = Control, Sil(10.0) = Sildenafil at 10 mg/Kg BW/day, Dai(1.0) = Daidzein at 1.0 mg/Kg BW/day, Gen(1.0) = Genistein at 1.0 mg/Kg BW/day, Dai(0.2)+Gen(0.2) = Daidzein 0.2 mg/Kg BW/day plus Genistein 0.2 mg/Kg BW/day. The significant difference between pre- and post-test in each group was compared using paired Student t-test at $^*p<0.05$; $^{**}p<0.01$. A significant difference between ANCOVA adjusted post-treated level in each group, means sharing the different superscript letters, was compared using ANCOVA and Tukey's HSD post hoc test at $p<0.01$.

Figure S3. Micrographs of seminiferous tubules section of mice after treatment with daidzein, genistein isolated from the BS alone and in combination, and sildenafil; **a** = Control, **b** = Sildenafil at 10.00 mg/kg BW/day, **c** = Daidzein at 1.0 mg/Kg BW/day, **d** = Genistein at 1.0 mg/kg BW/day, **e** = Daidzein 0.2 mg/Kg BW/day plus Genistein 0.2 mg/Kg BW/day. All micrographs displayed at X100 magnification. Bar = 10 mm.

Figure S4. Effects of daidzein, genistein isolated from the BS alone and in combination, and sildenafil on the serum testosterone level of mice. Con = Control, Sil(10.0) = Sildenafil at 10 mg/Kg BW/day, Dai(1.0) = Daidzein at 1.0 mg/Kg BW/day, Gen(1.0) = Genistein at 1.0 mg/Kg BW/day, Dai(0.2)+Gen(0.2) = Daidzein 0.2 mg/Kg BW/day plus Genistein 0.2 mg/Kg BW/day. The significant difference between pre- and post-test in each group was compared using paired Student t-test at $** p < 0.01$. A significant difference between ANCOVA adjusted post-treated level in each group, means sharing the different superscript letters, was compared using ANCOVA and Tukey's HSD post hoc test at $p < 0.01$.

Figure S5. Effects of daidzein, genistein isolated from the BS alone and in combination, and sildenafil on cholesterol (mg%) of mice. Con = Control, Sil(10.0) = Sildenafil at 10 mg/Kg BW/day, Dai(1.0) = Daidzein at 1.0 mg/Kg BW/day, Gen(1.0) = Genistein at 1.0 mg/Kg BW/day, Dai(0.2)+Gen(0.2) = Daidzein 0.2 mg/Kg BW/day plus Genistein 0.2 mg/Kg BW/day. The significant difference between pre- and post-test in each group was compared using paired Student t-test at $**p < 0.01$. A significant difference between ANCOVA adjusted post-treated level in each group, means sharing the different superscript letters, was compared using ANCOVA and Tukey's HSD post hoc test at $p < 0.01$.

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