

Effects of *Pueraria mirifica* and miroestrol on the antioxidation-related enzymes in ovariectomized mice

Waranya Chatuphonprasert^{a,c}, Latiporn Udomsuk^d, Orawan Monthakantirat^b, Yaowared Churikhit^b, Waraporn Putalun^{a,b} and Kanokwan Jarukamjorn^{a,b}

^aResearch Group for Pharmaceutical Activities of Natural Products using Pharmaceutical Biotechnology (PANPB), and ^bAcademic Office for Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, ^cFaculty of Medicine, Mahasarakham University, Mahasarakham, and ^dCollege of Medicine and Public Health, Ubon Ratchathani University, Ubon Ratchathani, Thailand

Keywords

catalase; glutathione peroxidase; miroestrol; *Pueraria mirifica*; superoxide dismutase

Correspondence

Kanokwan Jarukamjorn, Research Group for Pharmaceutical Activities of Natural Products using Pharmaceutical Biotechnology (PANPB), Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand. E-mail: kanok_ja@kku.ac.th

Received August 7, 2012

Accepted October 16, 2012

doi: 10.1111/jphp.12003

Abstract

Objectives The influences of *Pueraria candollei* var. *mirifica* (PM), a Thai medicinal plant with long tradition of medicinal consumption among menopausal women for rejuvenation and estrogen hormone replacement, on oxidative status in ovariectomized (OVX) mice were determined.

Methods The crude extract of PM and its active phytoestrogen, miroestrol (MR), were given to OVX mice. The effect of them on antioxidation enzymes and glutathione (GSH) levels in livers and uteri were examined in OVX mice and compared with the synthetic estradiol hormone.

Key findings Ovariectomy significantly decreased total GSH content, reduced GSH content, and the ratio of GSH to oxidized glutathione (GSSG) in both the livers and the uteri of mice. Moreover, an ovariectomy reduced the activities of glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT). The crude extract of PM as well as MR significantly increased levels of GSH, levels of reduced GSH, and the ratio of GSH/GSSG in both the livers and the uteri, while estradiol did not. In addition, the potential of PM and MR to return the activities of GPx, SOD, and CAT to normal levels was noted.

Conclusions These observations support using PM and MR as promising alternative medicine candidates for hormone replacement therapy of estradiol because of their ability to improve GSH levels and the activities of antioxidative enzymes, especially in OVX mice.

Introduction

Pueraria mirifica (synonym: *Pueraria candollei* Wall. Ex Benth var *mirifica* (Airy Shaw & Suvat.) Nyomdham), of the family Leguminosae, is a Thai medicinal plant with a long tradition of medicinal consumption among menopausal women for rejuvenation and oestrogen replacement.^[1,2] The tuberous roots of *P. mirifica* contain several phytoestrogens, including miroestrol (Figure 1), deoxymiroestrol, and isoflavonoids.^[3,4] Antioxidant properties are one of the most important claims for food ingredients, dietary supplements, cosmetics, and natural anticancer products. Phytoestrogen-rich plants have an established antioxidant activity.^[5] Reactive oxygen species (ROS) cause damage to cells and organs when the rate of ROS generation exceeds the rate of their decomposition by antioxidant defence systems. These

defence systems are composed of both the enzyme system – with enzymes glutathione peroxidase (GPx), catalase, and superoxide dismutase (SOD) – and a nonenzyme system of reduced glutathione (GSH).^[6,7] Levels of GPx, catalase, SOD, and GSH were decreased in ovariectomized animals.^[8,9] Several studies have demonstrated the antioxidant activity of *P. mirifica* and miroestrol. For example, the root-cultured extract of *P. candollei* inhibited lipid peroxidation in mouse brain.^[10] *P. mirifica* tuberous extracts have shown antioxidant activity by DPPH assay, compared with α -tocopherol, and by the β -carotene bleaching method.^[11,12]

Oestrogen (estradiol) is considered the first-line drug for the prevention and treatment of multiple conditions affecting women's health. It has been widely recommended for

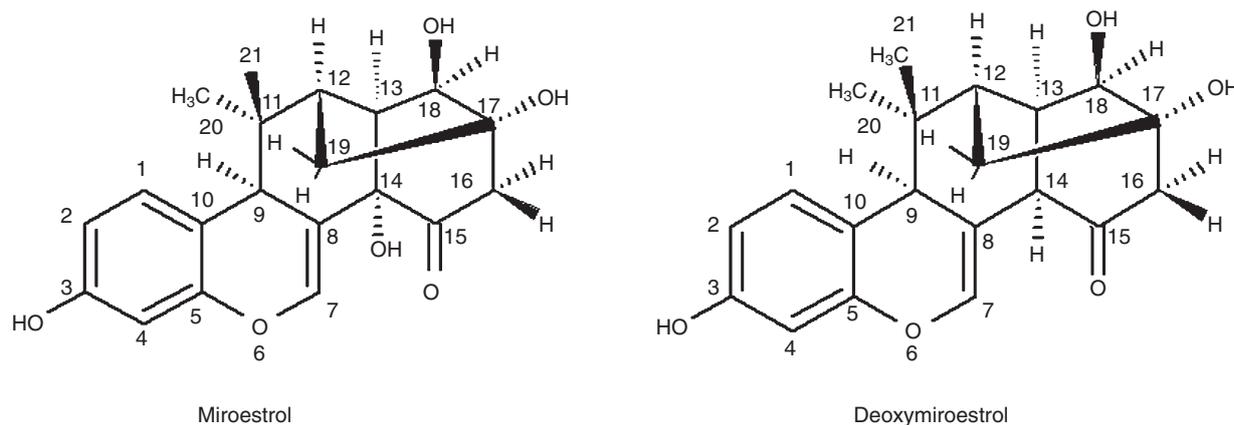


Figure 1 Chemical structures of miroestrol and deoxymiroestrol.

the prevention of osteoporosis, reduction of the risk of mortality from cardiovascular disease, maintaining lipid profiles, improvement of the signs and symptoms of menopause, and possible protection against the development of Alzheimer's disease.^[13,14] However, the current issue of debate is whether oestrogen therapy might increase the risk of carcinogenesis, especially breast cancer and endometrial adenocarcinoma in the uterus.^[15] Therefore, the search for natural products possessing oestrogenic activity with benefits on cancer prevention continues.

Butea monosperma, a herb of the family Papilionaceae containing miroestrol in the stem bark, showed a significant recovery in the level of GSH, and elevated levels of hepatic GPx and SOD in thioacetamide-treated rats.^[16] Daidzin is a major isoflavone from *Puerariae radix*, a plant in the same family as *P. mirifica*, that increased the level of hepatic reduced GSH in mice with lipopolysaccharide-induced hepatic failure.^[17] Therefore, *P. mirifica* extract or miroestrol might possess the potency to modify the level or activity of GSH, GPx, SOD, and catalase. However, the effects of *P. mirifica* or miroestrol on the levels of GSH and on the activity of GPx, SOD, and catalase in ovariectomized mice have undergone less research.^[18] Therefore, our study has determined the influences of *P. mirifica* extract and miroestrol on antioxidation systems, including the levels of GSH, GPx, SOD, and catalase, in the liver and uterus of ovariectomized mice.

Materials and Methods

Chemicals

Miroestrol was isolated from the tuberous roots of *P. mirifica* plants as described previously.^[4] NMR identification was performed, and the results were compared with authentic standards from Dr Chaiyo Chaichantipyuth, Faculty of

Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. Estradiol benzoate (E2), reduced glutathione (GSH), oxidized glutathione (GSSG), superoxide dismutase (SOD), xanthine, xanthine oxidase, nitroblue tetrazolium (NBT), ammonium molybdate, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and 4-vinylpyridine were products of Sigma (St Louis, MO, USA). All other laboratory chemicals were of the highest purity available from commercial suppliers.

Plant materials

The tuberous roots of *P. mirifica* were collected in Ubon Ratchathani, Thailand, in March 2010. Plant materials were identified by Dr Thaweesak Juengwatanatrakul, Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, Ubon Ratchathani, Thailand. Reference specimens (NI-PSKKU 007–010) were deposited at the Herbarium of the Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand.

Preparation of the *P. mirifica* crude extract and miroestrol

Dried tuberous root bark of *P. mirifica* was powdered and extracted three times with hexane, and the maceration was extracted three times with ethyl acetate, following the protocol of Yusakul *et al.*^[19] The ethyl acetate crude extracts were combined, evaporated, and fractionated by column chromatography (Silica gel 60 with hexane: ethyl acetate 3:1, 3:2, 1:1, and 0:1). The miroestrol fraction (compared with the referenced standard) continued to be purified using a Sephadex LH-20 column (ethyl acetate: methanol, 7:3). Miroestrol was recrystallized and identified using high-performance liquid chromatography (HPLC) (Figure 2) and ¹H NMR spectrum. The ethyl

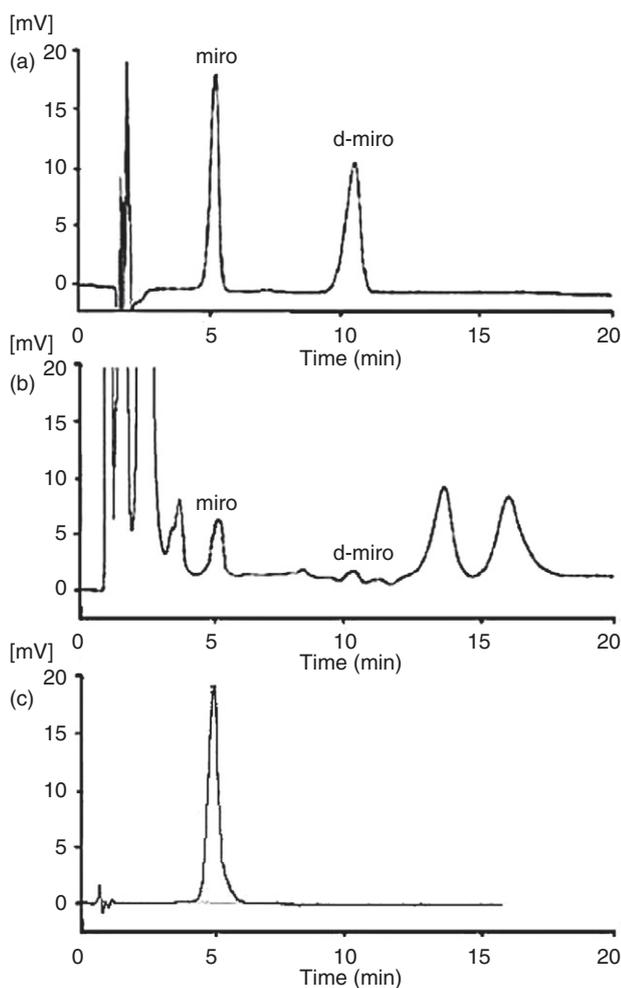


Figure 2 HPLC chromatogram of (a) standards of miroestrol (miro) and deoxymiroestrol (d-miro), (b) the crude extract of *Pueraria mirifica*, and (c) the pure compound of miroestrol.

acetate crude extract (the *P. mirifica* crude extract) was suspended in corn oil, while miroestrol was dissolved in 15% Tween 20 for the animal treatments.

Determination of miroestrol and deoxymiroestrol by high-performance liquid chromatography analysis

Dried powder of the *P. mirifica* crude extract (1 g) was washed with 5 ml hexane for 1 h with sonication and then extracted four times with 5 ml ethyl acetate-chloroform (3 : 1, v/v) with sonication for 1 h. The extracts were combined and evaporated at 60°C. The residual solid was dissolved in 1 ml ethanol for HPLC analysis. The mobile phase for HPLC consisted of 20% acetonitrile containing 1.5% acetic acid at a flow rate of 1.0 ml/min. The detection wavelength was set at 254 nm. HPLC was performed

using a PerkinElmer Series 200 LC pump coupled with a PerkinElmer 785A UV/vis detector. A RP-18 column (LiChroCART, 125 mm × 4 mm, 5 µm particle-size, Merck, Germany) was employed. A series of standard solutions was prepared by mixing standards of miroestrol and deoxymiroestrol to obtain the solutions with final concentrations of 25.00, 12.50, 6.25, 3.13, 1.56 and 0.78 µg/ml for each compound. Triplicate injections were made for each of the six concentrations. Calibration curves for miroestrol and deoxymiroestrol were constructed. The linearity of each standard curve was confirmed by plotting the peak area vs the concentration.

Animal design and treatments

Seven-week-old female ICR mice were supplied by the National Laboratory Animal Center (Mahidol University, Nakhon Pathom, Thailand) and were housed in the Laboratory Animal Unit of the Faculty of Pharmaceutical Sciences (Khon Kaen University, Khon Kaen, Thailand) under the supervision of the Animal Ethics Committee for Use and Care, Khon Kaen University, Khon Kaen, Thailand (Approval No. AEKKU 01/2555). The mice were intraperitoneally anaesthetized with pentobarbital sodium (100 mg/kg; Nembutal, Ceva Sante Anamale, France) and underwent a bilateral ovariectomy via dorsolateral incision. The ovaries were excised and the oviducts replaced; the incisions were closed. Three days after the operation, mice ($n=5$) received intraperitoneal injections of either E2 (1 µg/kg per day) in oil vehicle, or the crude extract of *P. mirifica* (2.5 or 25 mg/kg per day) in corn oil, or miroestrol (0.1 or 1.0 mg/kg per day) in 15% Tween 20 daily for two months. The sham control was simply left untreated to exhibit the constitutive levels of the enzymes. The control groups were intraperitoneally injected with corn oil (0.2 ml per mouse per day) or 15% Tween 20 in distilled water (0.2 ml per mouse/day) daily for the same time. The animals were killed 24 h after the last treatment, and the livers and uteri were immediately excised for further analysis.

Determination of protein content

The livers and uteri were homogenized in cold 1.15% KCl (1 g sample per 3 ml 1.15% KCl) with a hand-homogenizer in an ice bath. The protein content in the homogenized samples was determined according to the Bradford method.^[20] As per this method, 50 µl of the diluted sample was mixed with 200 µl Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA, USA). The formation of protein-dye complexes was measured at a wavelength of 595 nm and compared with the standard, bovine serum albumin.

Measurement of glutathione/oxidized glutathione content

GSH/GSSG content in liver and uterus was determined using the glutathione assay kit, according to the manufacturer's instructions (Sigma, St Louis, MO, USA and Trevigen, Gaithersburg, MD, USA). Liver or uterus homogenates were extracted in 5% sulfosalicylic acid. After centrifugation at 10 000g at 4°C for 10 min, the supernatant was collected and diluted for GSH/GSSG content analysis using DTNB. To measure GSSG content, the supernatant was treated with 4-vinylpyridine and incubated for 1 h at room temperature before performing the assay. The formation of thiol anions was measured at a wavelength of 405 nm at 1-min intervals over a 5-min period. The GSH or GSSG contents were determined as mmol/mg protein or nmol/mg protein, by comparison with the slope of the GSH or GSSG standard curve, respectively.^[21]

Determination of glutathione peroxidase activity

The activity of GPx in liver and uterus was determined according to the methods of Pinto and Bartley^[22], with some modifications. The assay mixture sequentially contained 3.0 ml of a homogenate of either liver or uterus, 0.5 ml 0.25 M phosphate buffer at pH 7.4, 0.1 ml 25 mM ethylenediaminetetraacetic acid (EDTA), 0.1 ml 0.4 M sodium azide (NaN₃), 0.3 ml 50 mM GSH, and 0.1 ml 50 mM hydrogen peroxide (H₂O₂). The reaction was stopped using 1.0 ml 5% sulfosalicylic acid 10 s after the addition of the H₂O₂. After centrifugation at 10 000g at 4°C for 10 min, the supernatant was taken for GSSG measurement as described above. The amount of GSSG contained in this reaction was an accumulation of GSSG that was created in this reaction as well as GSSG that was endogenous to the sample, in addition to the GSSG that was contained in the GSH solution. One unit of GPx activity was calculated from 1 μmol GSSG formed/min in the reaction at 30°C and pH 7.4.

Determination of superoxide dismutase activity

For the sample's preparation, 0.5 ml of liver or uterus homogenate was extracted using 0.3 ml chloroform and 0.5 ml ethanol, vigorously vortex-mixed for 1 min, and subjected to centrifugation at 13 000g at 4°C for 30 min.^[23] The supernatants were used for the SOD assay. SOD activity was determined by the inhibition of NBT reduction due to the O₂^{•-} generated by the xanthine/xanthine oxidase system.^[24] Either 12.5 μl supernatant or bovine Cu-Zn SOD standard (10–100 μg/ml) was added to a 96-well microplate. Then, 107.5 μl reagent mixture (1.2 mM xanthine, 140 μM EDTA, 140 mM NBT, 56 mM Na₂CO₃, and 70 μg/ml BSA) was

added to each well, followed by the xanthine oxidase solution (1.5 μU/well). The reaction was incubated at 25°C for 20 min and stopped by adding 25 μl 0.8 mM copper chloride (CuCl₂). The production of formazan was measured at a wavelength of 550 nm. The percentage of formazan inhibition was determined and compared with the standard curve of bovine Cu-Zn SOD.^[23]

Determination of catalase activity

Catalase activity was determined by Goth's colorimetric method^[25] with some modifications. Sample was incubated in a H₂O₂ substrate, and the enzymatic reaction was terminated by the addition of ammonium molybdate. Specifically, 15 μl homogenate sample was incubated in 50 μl 130 mM H₂O₂ substrate at 37°C for 1 min. The reaction was stopped using 65 μl 32.4 mM ammonium molybdate, and the yellow complex of molybdate and H₂O₂ was measured at a wavelength of 405 nm.^[23] The percentage of inhibition of the molybdate-H₂O₂ complex was collected and compared with the standard curve of the hepatic bovine catalase.

Statistical analysis

The data was presented as the mean ± SD and analysed by one-way analysis of variance followed by the Tukey post-hoc test (SPSS ver. 17.0). *P* < 0.05 was considered to be statistically significant.

Results

Identification and quantitative determination of miroestrol

Miroestrol was isolated as a white powder: ¹H NMR (CD₃OD, 400 MHz): 6.99 (d, H-1), 6.50 (dd, H-2), 6.30 (d, H-4), 6.29 (s, H-7), 3.31 (s, H-9), 2.74 (m, H-12), 2.43 (d, H-13), 2.58 (dd, H-16a), 2.89 (bd, H-16b), 3.70 (s, H-18), 1.87 (dd, H-19a), 1.99 (1H, br t, H-19b), 0.56 (s, H-20), 1.23 (s, H21), according to a previous report of Chansakaow *et al.*^[4] The contents of miroestrol and deoxymiroestrol in the crude extract of *P. mirifica* were quantified as previously described using the HPLC method of Yusakul *et al.*^[19] The *P. mirifica* crude extract contained 69.37 ± 1.91 μg/g dry weight miroestrol and 15.41 ± 0.71 μg/g dry weight deoxymiroestrol (Figure 2b). The purity of miroestrol employed in this study was > 99% as shown in the HPLC chromatogram (Figure 2c).

Glutathione content in liver and uterus of ovariectomized mice

Total GSH, reduced GSH, and oxidized GSH (GSSG) levels were measured in the livers (Table 1) of ovariectomized mice. The hepatic total GSH content, the reduced GSH

Table 1 Effect of the *Pueraria mirifica* crude extract and miroestrol on total glutathione, reduced glutathione, and oxidized glutathione content in the liver of the ovariectomized mouse

Treatment group	Total GSH content (mmol/mg protein)	Reduced GSH content (mmol/mg protein)	GSSG content (mmol/mg protein)	Ratio of GSH/GSSG
Sham	8.64 ± 0.56	6.55 ± 0.46	2.36 ± 0.20	2.78 ± 0.03
Ovariectomized-corn oil	5.55 ± 0.80 ^A	2.87 ± 0.36 ^A	3.03 ± 0.15	0.95 ± 0.18 ^A
Ovariectomized-Tween	3.39 ± 0.07 ^A	1.21 ± 0.30 ^A	2.19 ± 0.60	0.55 ± 0.05 ^A
Ovariectomized-E2	4.88 ± 0.47 ^A	2.55 ± 0.32 ^A	2.33 ± 0.16	1.09 ± 0.06 ^A
Ovariectomized-PM2.5	5.41 ± 0.90 ^A	4.19 ± 0.97 ^{A,B}	1.27 ± 0.19 ^{A,B}	3.30 ± 0.04 ^{A,B}
Ovariectomized-PM25	6.11 ± 0.49 ^{A,B}	4.41 ± 0.72 ^{A,B}	1.70 ± 0.12 ^{A,B}	2.59 ± 0.02 ^B
Ovariectomized-MR0.1	6.97 ± 0.71 ^C	4.24 ± 0.61 ^{A,C}	2.73 ± 1.20	1.55 ± 0.07 ^{A,C}
Ovariectomized-MR1	8.72 ± 0.36 ^C	6.52 ± 0.93 ^C	2.20 ± 1.40	2.96 ± 0.10 ^C

E2, estradiol benzoate 1 µg/kg per day; GSH, glutathione; GSSG, oxidized glutathione; MR, miroestrol (mg/kg per day); PM, *P. mirifica* crude extract (mg/kg per day); Sham, sham operated mice. ^A*P* < 0.05 vs sham; ^B*P* < 0.05 vs ovariectomized-corn oil; ^C*P* < 0.05 vs ovariectomized-Tween.

Table 2 Effect of the *Pueraria mirifica* crude extract and miroestrol on total glutathione, reduced glutathione, and oxidized glutathione content in the uterus of the ovariectomized mouse

Treatment groups	Total GSH content (nmol/mg protein)	Reduced GSH content (nmol/mg protein)	GSSG content (nmol/mg protein)	Ratio of GSH/GSSG
Sham	68.89 ± 12.65	66.16 ± 11.49	3.78 ± 0.69	22.75 ± 5.31
Ovariectomized-corn oil	14.17 ± 4.85 ^A	11.77 ± 9.64 ^A	2.62 ± 1.25 ^A	5.77 ± 0.53 ^A
Ovariectomized-Tween	12.41 ± 2.34 ^A	9.22 ± 4.01 ^A	3.36 ± 2.20	3.37 ± 1.97 ^A
Ovariectomized-E2	12.24 ± 1.42 ^A	10.51 ± 0.96 ^A	2.30 ± 0.33 ^A	5.84 ± 1.79 ^A
Ovariectomized-PM2.5	23.75 ± 2.59 ^{A,B}	22.12 ± 2.56 ^{A,B}	1.63 ± 0.23 ^{A,B}	13.74 ± 2.36 ^{A,B}
Ovariectomized-PM25	17.13 ± 3.07 ^A	16.66 ± 4.11 ^A	1.47 ± 0.43 ^{A,B}	19.21 ± 5.96 ^{A,B}
Ovariectomized-MR0.1	24.77 ± 3.61 ^{A,C}	23.63 ± 3.92 ^{A,C}	1.26 ± 0.59 ^{A,C}	16.15 ± 3.19 ^{A,C}
Ovariectomized-MR1	15.25 ± 3.50 ^A	14.82 ± 3.61 ^A	1.12 ± 0.41 ^{A,C}	18.68 ± 6.26 ^{A,C}

E2, estradiol benzoate 1 µg/kg per day; GSH, glutathione; GSSG, oxidized glutathione; MR, miroestrol (mg/kg per day); ovariectomized, ovariectomized mice; PM, *P. mirifica* crude extract (mg/kg per day); Sham, sham operated mice. ^A*P* < 0.05 vs sham; ^B*P* < 0.05 vs ovariectomized-corn oil; ^C*P* < 0.05 vs ovariectomized-Tween.

content, and the ratio of GSH/GSSG were markedly decreased in both groups of control ovariectomized mice. E2 did not improve any of the levels of the various GSH measures in the liver. The crude extract of *P. mirifica* significantly increased the levels of hepatic total GSH and the reduced GSH, while decreasing the GSSG content in ovariectomized mice treated with corn oil. However, only a high dose of *P. mirifica* (25 mg/kg per day) restored GSH/GSSG to a sham control level. All miroestrol injections restored the hepatic total GSH and GSSG to sham control levels, whereas the reduced GSH content and the ratio of GSH/GSSG reached their normal values only at the high dose of miroestrol (1 mg/kg per day). On the other hand, in uteri (Table 2), the total GSH content, the reduced GSH content, the GSSG content, and GSH/GSSG were all strongly reduced post-ovariectomy. E2 did not improve any of the levels of the various GSH measures in uterus. Administration of either *P. mirifica* or miroestrol significantly increased the total GSH, the reduced GSH, and the ratio of GSH/GSSG, while the amount of GSSG was suppressed.

Glutathione peroxidase activity in liver and uterus of ovariectomized mice

Liver and uterus glutathione peroxidase activity (GPx) was suppressed after ovariectomy and treatment with corn oil or Tween 20 (Figure 3). E2 numerically increased, but not significantly, the GPx activity in liver, and caused a further reduction of GPx activity in uterus, compared with the ovariectomized control groups. The crude extract of *P. mirifica* increased hepatic GPx activity in a dose-dependent pattern (Figure 3a). Miroestrol showed a dose-dependent effect of increasing hepatic GPx activity, though the activity was still less than the sham control level (Figure 3b). In the uteri of post-ovariectomy mice, *P. mirifica* significantly increased the activity of GPx (Figure 3c). Miroestrol restored the activity of GPx, but not dose-dependently, whilst the higher dose exhibited the lower increase to the sham control (Figure 3d). The dose-dependent pattern of *P. mirifica* and miroestrol on liver GPx level was not observed in their effect on uterus.

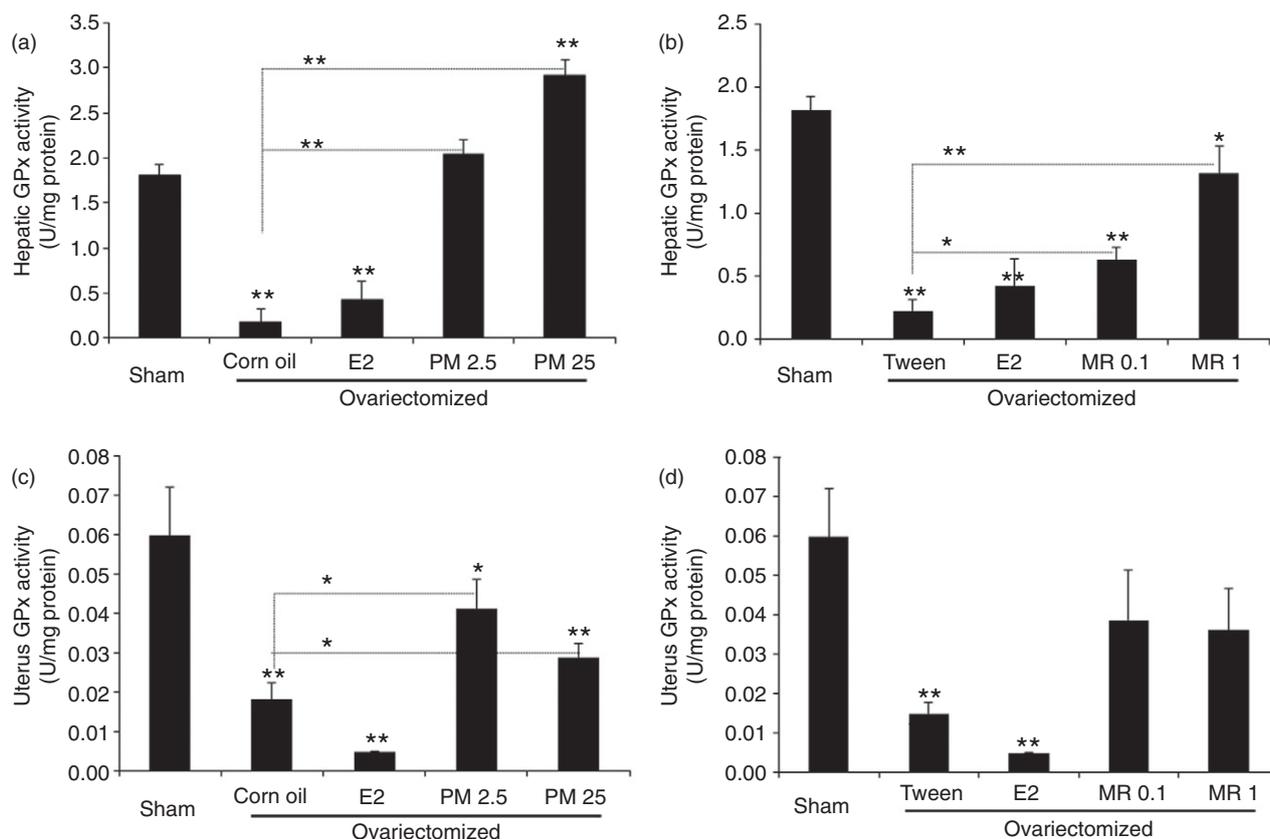


Figure 3 Glutathione peroxidase (GPx) activity in the liver and uterus of ovariectomized mice. Ovariectomized mice were intraperitoneally injected with estradiol benzoate (E2, 1 µg/kg per day), the crude extract of *Pueraria mirifica* (PM; 2.5 or 25 mg/kg per day), or miroestrol (MR; 0.1 or 1.0 mg/kg per day) daily for two months. The ovariectomized control groups were intraperitoneally injected with either corn oil or 15% Tween 20 daily for two months. The sham control mice were left untreated. The data are presented as the mean ± SD from duplicate ($n = 4-5$) independent experiments. A significant difference was determined by one-way analysis of variance followed by a Tukey post-hoc test. * $P < 0.01$, ** $P < 0.001$ compared with sham group or ovariectomized control.

Superoxide dismutase activity in liver and uterus of ovariectomized mice

Superoxide dismutase (SOD) activity was measured in liver and uterus (Figure 4). Ovariectomy significantly reduced the SOD activity in both organs (Figure 4). E2 numerically increased the activity of SOD in liver and uterus compared with the corn oil ovariectomized group, but the increase was significant compared with the Tween 20 ovariectomized group (Figure 4b and d). The crude extract of *P. mirifica* improved the SOD activity in the liver of ovariectomized mice, following a dose-dependent pattern (Figure 4a). The hepatic SOD activity in ovariectomized mice was significantly elevated by miroestrol, but no dose-dependent effect was observed (Figure 4b). The crude extract of *P. mirifica* also increased the uterus SOD activity (Figure 4c). Miroestrol extensively increased the activity of SOD in

uterus (Figure 4d), again with no dose-dependent pattern observed.

Catalase activity in liver and uterus of ovariectomized mice

Catalase activity was significantly decreased in both liver and uterus of mice post-ovariectomy (Figure 5). E2 could not recover the catalase activity in either the liver or uterus of these ovariectomized mice, where it tended to suppress the hepatic catalase activity (Figure 5). Interestingly, the crude extract of *P. mirifica* increased the catalase activity in liver and uterus of ovariectomized mice up to the levels of the sham group, but the *P. mirifica* crude extract at 25 mg/kg per day exhibited less potential than at 2.5 mg/kg per day (Figure 5a and c). Miroestrol showed the potential to enhance the activity of catalase in only uterus

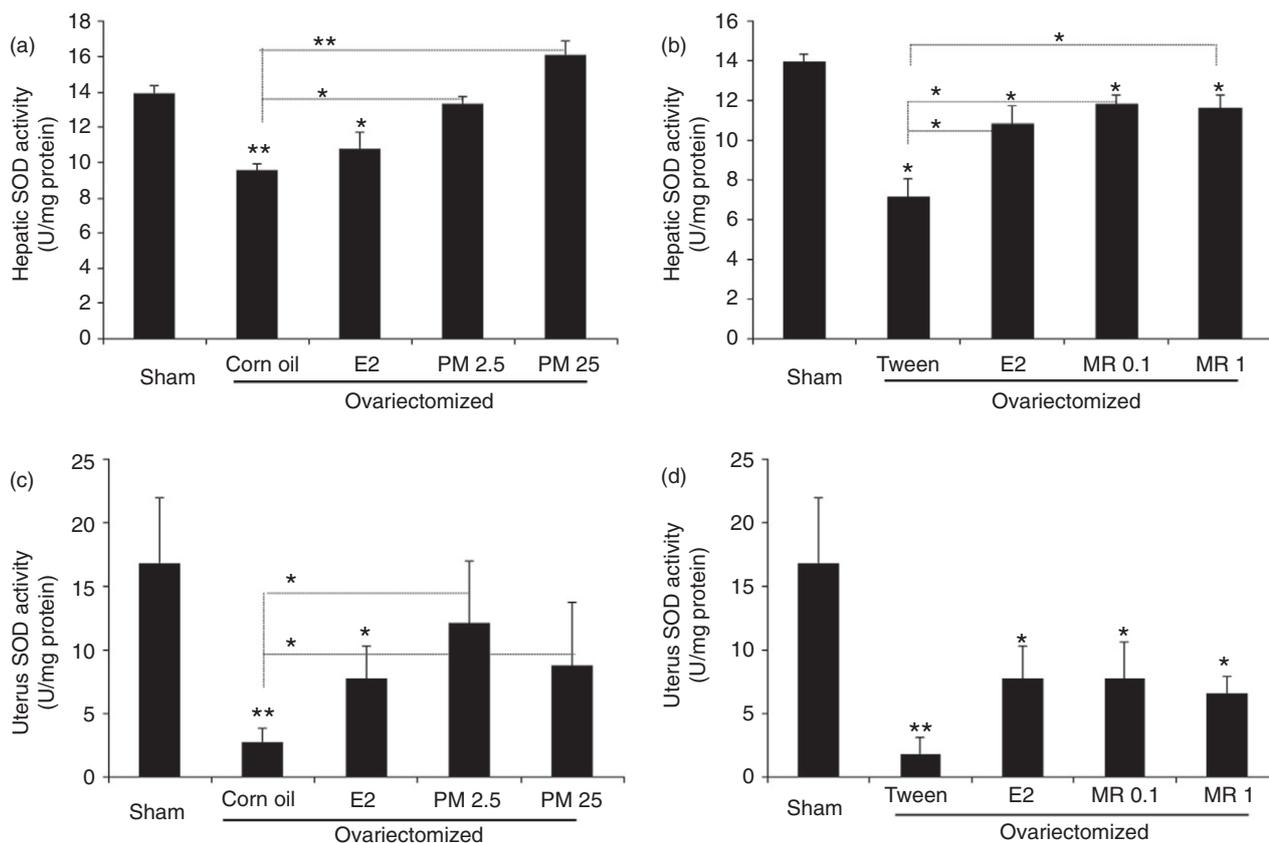


Figure 4 Superoxide dismutase (SOD) activity in liver and uterus of ovariectomized mice. Ovariectomized mice were injected with estradiol benzoate (E2, 1 $\mu\text{g}/\text{kg}$ per day), the crude extract of *Pueraria mirifica* (PM; 2.5 or 25 mg/kg per day), or miroestrol (MR; 0.1 or 1.0 mg/kg per day) daily for two months. The ovariectomized control groups were injected with vehicle for two months. The sham control mice were left untreated. The data are presented as the mean \pm SD from duplicate ($n = 4\text{--}5$) independent experiments. A significant difference was determined by one-way analysis of variance followed by a Tukey post-hoc test. * $P < 0.01$, ** $P < 0.001$ compared with the sham group or ovariectomized control.

ovariectomized mice, without dose-dependency (Figure 5b and d).

Discussion

The level of oestrogen in ovariectomized mice is greatly decreased, leading to body weight gain, abdominal fat stores, increased liver fat, and osteoporosis.^[13,14] Therefore, ovariectomized mice were used as a model to reflect the pathological changes in perimenopausal and postmenopausal women.^[26,27] Oestrogen (estradiol) is considered as the first-line drug for the prevention and treatment of multiple conditions affecting menopausal women.^[13,14] However, the current issue of debate is whether oestrogen therapy might increase the risk of carcinogenesis, especially breast cancer and endometrial adenocarcinoma in the uterus.^[15] Mechanisms of hormone therapy performing on the carcinogenesis process are various such as DNA damage induction, enhancement of DNA adduct in chronic oestrogen exposure, and free radical generation

via the catecholestrogen pathway.^[28] Some studies have focused on the involvement of ovarian steroid hormones, especially oestrogens, in the phenomena of oxidative stress.^[28,29] The oxidative stress in uterus was increased by E2, resulting in a significant increase in malondialdehyde.^[30] The oestrogenic potential of *P. mirifica* has been demonstrated by both its crude extract and the potent phytoestrogen, miroestrol, in several experimental models, including immature mice, female mice, ovariectomized rats, female monkeys, and human clinical trials.^[31–36]

In this study, E2 did not vary any levels of the various GSH measured in either liver or uterus, while *P. mirificai* extract and miroestrol improved the GSH level. These observations suggested that *P. mirifica* extract and miroestrol were more effective than E2 for restoring the GSH status in both liver and uterus of ovariectomized mice. This is the first report revealing the effects of the *P. mirifica* crude extract and miroestrol on the antioxidation-related enzymes and the GSH content in the liver and uterus of the ovariectomized mouse.

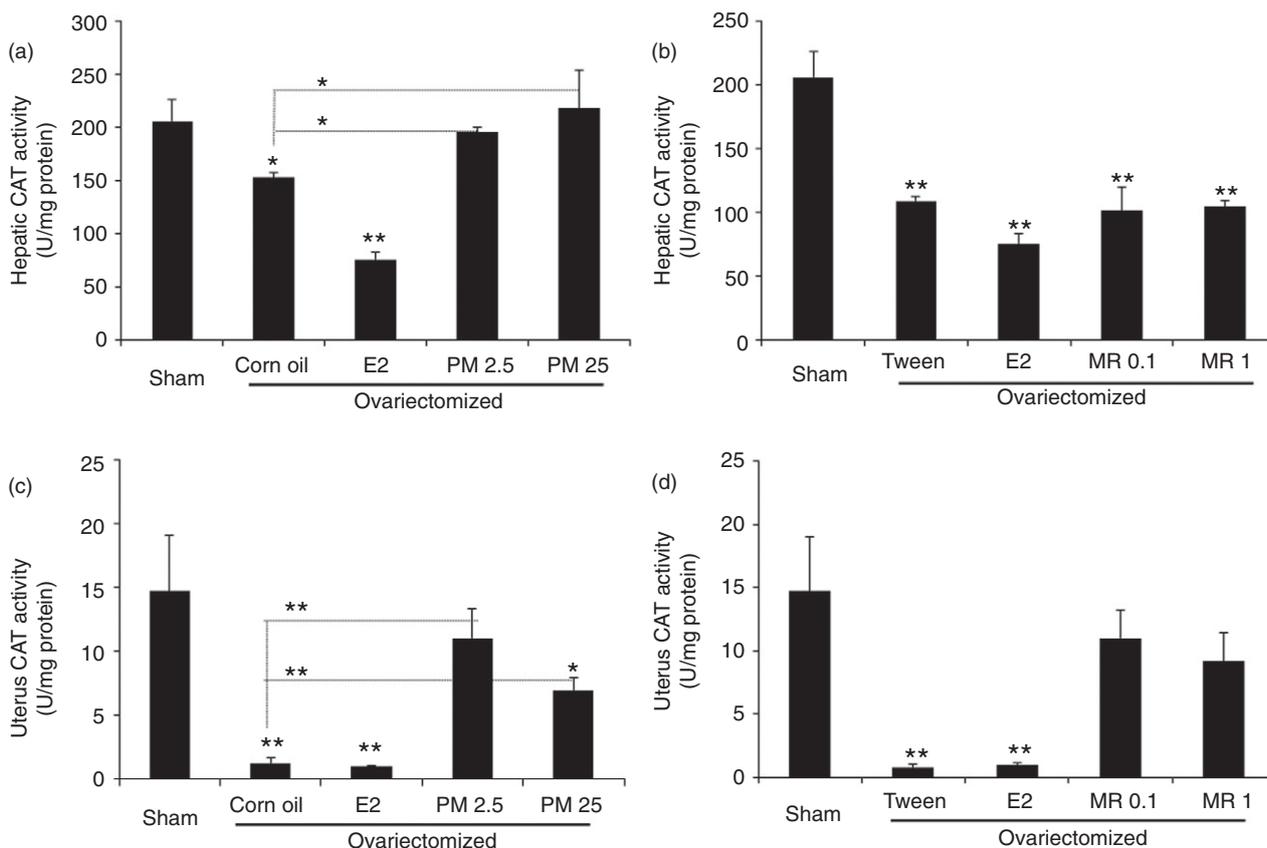


Figure 5 Catalase (CAT) activity in the liver and uterus of ovariectomized mice. Ovariectomized mice were injected with estradiol benzoate (E2, 1 $\mu\text{g}/\text{kg}$ per day), the crude extract of *Pueraria mirifica* (PM; 2.5 or 25 mg/kg per day), or miroestrol (MR; 0.1 or 1.0 mg/kg per day) daily for two months. The ovariectomized control groups were injected with vehicle for two months. The sham control mice were left untreated. The data are presented as the mean \pm SD from duplicate ($n = 4\text{--}5$) independent experiments. A significant difference was determined by one-way analysis of variance followed by a Tukey post-hoc test. * $P < 0.01$, ** $P < 0.001$ compared with the sham group or ovariectomized control.

From the HPLC chromatogram (Figure 2), we showed that the crude extract of *P. mirifica* consisted of both miroestrol and deoxymiroestrol (in which deoxymiroestrol was usually metabolized to miroestrol). Moreover, *P. mirifica* actually contains other compounds such as chromenes, flavonoids, and isoflavones, while miroestrol is the pure compound of miroestrol.^[37] Hence, a difference between the *P. mirifica* crude extract and miroestrol might be observed. In addition, the liver is the most abundant organ of antioxidative enzymes, while the uterus contains less of these enzymes than the liver (< 10-fold) (Figures 3–5). Therefore, the constitutive levels of enzymes might reflect the dose response of the *P. mirifica* crude extract or miroestrol, namely the dose-dependent pattern was observed in the liver, but not in the uterus.

The free radical tissue-defence mechanisms including SOD, GPx, and GSH, are the important antioxidative enzymes against ROS and, particularly, against superoxide anion radicals in mammals.^[38,39] The isoform of SOD has Cu

and Zn in its catalytic centre and is localized in intracellular cytoplasm compartments (CuZn-SOD or SOD1). Another isoform of SOD has manganese (Mn) as a co-factor and has been localized in the mitochondria of aerobic cells (Mn-SOD or SOD2).^[38] GPx exists in the cytosol as a homotetramer, with each subunit containing a selenium atom incorporated within a catalytically active selenocysteine residue.^[40] This amino acid is sterically exposed on a flat lipophilic segment of the protein, allowing it to be oxidized by an approaching hydroperoxide.^[41] GSH has long been used as a defensive agent against the action of toxic xenobiotics, including drugs, pollutants, and carcinogens. As a prototypical antioxidant, GSH has been involved in protecting cells from the noxious effect of excessive oxidative stress.^[42] Hence, the decrease in levels of these defensive enzymes including GSH might affect the defence mechanisms of organs, allowing any abnormality or disease to progress.

SOD and catalase activity were significantly decreased in ovariectomized rats.^[8] Moreover, the activity of GPx and

SOD were decreased in the femur of ovariectomized albino rats.^[43] Ovariectomy reduced the GSH levels in the bone marrow of rats.^[44] We correspondingly found a decrease in the total GSH content, the reduced GSH content, and the ratio GSH/GSSG, as well as decreased activity of GPx, SOD, and catalase enzymes in both the liver and the uterus of mice after undergoing ovariectomy. A previous study by Tiwari *et al.*^[45] reported that *Butea monosperma*, a plant containing miroestrol, increased the levels of GSH in carbon tetrachloride-induced hepatotoxicity in rats.^[46] The effects of either *P. mirifica* or miroestrol on GPx, SOD, and catalase activity have not been reported previously.

In this study, the crude extract of *P. mirifica* and the isolated miroestrol showed their potential to return the levels of total GSH and GSSG to nearly those of the sham control mice. Therefore, the potential of the *P. mirifica* crude extract and miroestrol to improve the activity of the GPx, SOD, and catalase enzymes to those of the normal levels might help counterbalance the endogenous ROS and oxidative stress induced by the oestrogen deficient state, and halt the progression of diseases related to oxidative stress and menopause.

References

- Kashemsanta MLC *et al.* A new species of *Pueraria* (Leguminosae) from Thailand, yielding an oestrogenic principle. *Kew Bull* 1952; 7: 263–266.
- Suntara A. *The Remedy Pamphlet of Kwao Krua Tuber of Luang Anusorn-suntarakromkarnpiset*. Chiang Mai: Upatipongsa Press, 1931.
- Ingham JL *et al.* Minor isoflavones from the root of *Pueraria mirifica*. *Z Naturforsch C* 1989; 44c: 724–726.
- Chansakaow S *et al.* Identification of deoxymiroestrol as the actual rejuvenating principle of 'Kwao Keur', *Pueraria mirifica*, the known miroestrol may be an artifact. *J Nat Prod* 2000; 63: 173–175.
- Lee CH *et al.* Relative antioxidant activity of soybean isoflavones and their glycosides. *Food Chem* 2005; 90: 735–741.
- Mates JM *et al.* Antioxidant enzymes and human diseases. *Clin Biochem* 1999; 32: 595–603.
- Datta K *et al.* Reactive oxygen species in health and disease. *Nat Med J India* 2000; 13: 304–310.
- Yalin S *et al.* Acute effect of single-dose cadmium treatment on lipid peroxidation and antioxidant enzymes in ovariectomized rats. *Ecotoxicol Environ Saf* 2006; 65: 140–144.
- Konyalioglu S *et al.* The potential antioxidant effect of raloxifene treatment: a study on heart, liver and brain cortex of ovariectomized female rats. *Cell Biochem Funct* 2007; 25: 259–266.
- Udomsuk L *et al.* Impact of *Pueraria candollei* root cultures on cytochrome P450 2B9 enzyme and lipid peroxidation in mice. *J Health Sci* 2010; 56: 182–187.
- Cherdshewasart W, Sutjit W. Correlation of antioxidant activity and major isoflavonoid contents of the phytoestrogen-rich *Pueraria mirifica* and *Pueraria lobata* tubers. *Phytomedicine* 2008; 15: 38–43.
- Chanwitheesuk A *et al.* Screening of antioxidant activity and antioxidant compounds of some edible plants of Thailand. *Food Chem* 2005; 92: 491–497.
- Grady D *et al.* Hormone therapy to prevent disease and prolong life in postmenopausal women. *Ann Intern Med* 1992; 117: 1016–1037.
- Grodstein F *et al.* Postmenopausal hormone therapy and mortality. *N Engl J Med* 1997; 336: 1769–1775.
- Go'mez-Zubeldia MA *et al.* Influence of estradiol on oxidative stress in the castrated rat uterus. *Gynecol Oncol* 2001; 80: 227–232.
- Sehrawat A *et al.* *Butea monosperma* and chemomodulation: protective role against thioacetamide-mediated hepatic alterations in Wistar rats. *Phytomedicine* 2006; 13: 157–163.
- Kim SH *et al.* Protective effect of daidzin against d-galactosamine and lipopolysaccharide-induced hepatic failure in mice. *Phytother Res* 2009; 23: 701–706.
- Sindhia VR, Bairwa R. Plant review: *Butea monosperma*. *Int J Pharm Clin Res* 2010; 2: 90–94.
- Yusakul G *et al.* Comparative analysis of the chemical constituents of two varieties of *Pueraria candollei*. *Fitoterapia* 2011; 82: 203–207.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72: 248–254.
- Kondo S *et al.* Cellular glutathione content modulates the effect of andrographolide on β -naphthoflavone-induced CYP1A1 mRNA expression

Conclusion

These findings support the view that *P. mirifica* crude extract or miroestrol could be candidates as alternative medicine in oestrogen replacement therapy, based on their oestrogenic activity and ability to improve menopausal syndromes. Moreover, their potential to improve the level of oxidative stress-related enzymes and to maintain the level of GSH content in ovariectomized mice over estradiol should be promoted.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

- in mouse hepatocytes. *Toxicology* 2011; 280: 18–23.
22. Pinto RE, Bartley W. The effect of age and sex on glutathione reductase and glutathione peroxidase activities and on aerobic glutathione oxidation in rat liver homogenates. *Biochem J* 1969; 112: 109–115.
 23. Ozmen B *et al.* Lens superoxide dismutase and catalase activities in diabetic cataract. *Clin Biochem* 2002; 35: 69–82.
 24. Sun Y *et al.* A simple method for clinical assay of superoxide dismutase. *Clin Chem* 1988; 34: 497–500.
 25. Goth L. A simple method for determination of serum catalase activity, and revision of reference range. *Clin Chim Acta* 1991; 196: 143–152.
 26. Wang J *et al.* Effect of Radix Puerariae flavones on liver lipid metabolism in ovariectomized rats. *World J Gastroenterol* 2004; 10: 1967–1970.
 27. Bolon B *et al.* Adenoviral delivery of osteoprotegerin ameliorates bone resorption in a mouse ovariectomy model of osteoporosis. *Mol Ther* 2001; 3: 197–205.
 28. Roy D, Liehr JG. Estrogen, DNA damage and mutations. *Mutat Res* 1999; 424: 107–115.
 29. Huh K *et al.* Effect of sex hormones on lipid peroxidation in rat liver. *Arch Pharm Res* 1994; 17: 109–114.
 30. Gomez-Zubeldia MA *et al.* Influence of estradiol and gestagens on oxidative stress in the rat uterus. *Gynecol Oncol* 2002; 86: 250–258.
 31. Jones HEM, Pope GS. A study of the action of miroestrol and other oestrogens on the reproductive tract of the immature female mouse. *J Endocrinol* 1960; 20: 229–235.
 32. Jaroenporn S *et al.* Assessment of fertility and reproductive toxicity in adult female mice after long-term exposure to *Pueraria mirifica* herb. *J Reprod Dev* 2007; 53: 995–1005.
 33. Benson GK *et al.* Mammogenic activity of miroestrol. *J Endocrinol* 1961; 21: 401–409.
 34. Cherdshewasart W *et al.* Estrogenic activity of the wild *Pueraria mirifica* evaluated by vaginal cornification assay. *J Reprod Dev* 2007; 53: 385–393.
 35. Trisomboon H *et al.* Assessment of urinary gonadotropin and steroid hormone profiles of female cynomolgus monkeys after treatment of *Pueraria mirifica* herb. *J Reprod Dev* 2007; 53: 395–403.
 36. Muangman V, Cherdshewasart W. Clinical trial of the phytoestrogen-rich herb, *Pueraria mirifica* as a crude drug in the treatment of symptoms in menopausal women. *Siriraj Hosp Gaz* 2001; 53: 300–309.
 37. Malaivijitnond S *et al.* Different effects of *Pueraria mirifica*, a herb containing phytoestrogens, on LH and FSH secretion in gonadectomized female and male rats. *J Pharmacol Sci* 2004; 96: 428–435.
 38. Zelko IN *et al.* Superoxide dismutase multigene family: a comparison of the CuZn-Sod (Sod1), Mn-Sod (Sod2), and Ec-Sod (Sod3) gene structures, evolution, and expression. *Free Radic Biol Med* 2002; 33: 337–349.
 39. Morel Y, Barouki R. Repression of gene expression by oxidative stress. *Biochem J* 1999; 342: 481–496.
 40. Sukenaga Y *et al.* Nucleotide sequence of a human gene for glutathione peroxidase. *Nucleic Acids Res* 1987; 15: 71–78.
 41. Li R *et al.* Effect of vitamin E on human glutathione peroxidase (GSH-px1) expression in cardiomyocytes. *Free Radic Biol Med* 1996; 21: 419–426.
 42. Pompella A *et al.* The changing faces of glutathione, a cellular protagonist. *Biochem Pharmacol* 2003; 66: 1499–1503.
 43. Muthusami S *et al.* Ovariectomy induces oxidative stress and impairs bone antioxidant system in adult rats. *Clin Chim Acta* 2005; 360: 81–86.
 44. Lean JM *et al.* A crucial role for thiol antioxidants in estrogen-deficiency bone loss. *J Clin Invest* 2003; 112: 915–923.
 45. Tiwari P *et al.* Hepatoprotective potentials of *Butea monosperma* stem bark extract against carbon tetrachloride induced hepatotoxicity in albino rats. *Int J Med Med Sci* 2011; 3: 252–255.
 46. Sharma AK, Deshwal N. An overview: on phytochemical and pharmacological studies of *Butea monosperma*. *Int J Pharm Tech Res* 2011; 3: 864–871.