Effect of *Pueraria candollei var. mirifica* and miroestrol on ovariectomized-induced emotion deficit and osteoporosis in ICR mice

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Abstract

Estrogen is an important steroid hormone which produced from female ovary. Estrogen take an actions via bind to estrogen receptors (ER), ERα and ERβ that distribute in whole body. Recently estrogens were found to effect on various systems especially in central nervous system (CNS) and musculoskeletal system. Deprivation of estrogens can cause depression and cognitive impairment and osteoporosis, a burden symptom in most of menopausal woman. Signal transducer and activator of transcription 3 (STAT3) which located in brain area can activated by estrogens to control of cognitive function. Ovariectomized (OVX) mice is the animal model use to represent estrogen deficiency conditions that can express the mood disorder and bone loss. *Pueraria candollei var. mirifica* (PM) known to be the popular herbs used as folk medicine for rejuvenating effects. PM contains various phytoestogens such as miroestrol (MR). The aim of this study was to investigate the protective effects of PM and MR on ovariectomy induced mood disorder and bone loss. OVX exhibits more number of immobility time in TST and FST, decrease ERβ and STAT3 mRNA expression in hippocampus and frontal cortex and also caused significantly decrease uterine weight and volume, femur and tibia dry weight, ash weight and calcium content compared with sham group. Giving hormone replacement therapy (HRT) normalized mood disorder and bone loss from estrogen deficiency as same as treated with PM extract and MR. This suggest that PM extract and MR has protective effects on ovariectomize induced depression and bone loss. The mechanism of action involved activation of estrogen receptor by estrogen-like structure of phytoestrogen. Our findings raise the possibility that MR and *Pueraria candollei var. mirifica*, the plant of origin of MR, may have a beneficial effect on depression and osteoporosis in which menopause/ovariectomy are implicated as risk factors.

Backgrounds

Menopause is defined by permanent cessation of the primary functions of the human ovaries. Emotional disorders such as postpartum and postmenopausal depression are the most common forms of mental illness associated with decreased serum levels of ovarian hormones [1]. High severity of depressionig can be a major cause of suicidal thinking. Estrogen deficiency is considered as the main determinant for bone loss in postmenopausal women [2]. Osteoporosis is caused by an imbalance in the normal bone remodeling process, in which there is excessive osteoclast resorption and adequate new bone formation by osteoblasts reduction. Hormone replacement therapy (HRT) has proven to be efficacious in depression and preventing bone loss and

reducing the incidence of skeletal fractures in postmenopausal women. However, long-term HRT increases the high risk of breast cancer, endometrial cancer, thromboembolic events and vaginal bleeding [3]. Traditional medicines have been used from long days in prevention and treatment of postmenopausal symptoms. Since these medicines are prepared from medicinal plants they have fewer side effects and are suitable for long-term use. Phytoestrogens and many other estrogen-like compounds in plants, due to fewer side effects, have been massively proposed to prevent menopause-related cognitive decline. Pueraria candollei var. mirifica (KwaoKrueKao : PM), an indigenous herb belongs to the Family Leguminosae is known to be the popular herbs used as folk medicine for increasing appetite, enlarging breasts, improving hair growth, and other rejuvenating effects [4]. The tuberous root of Pueraria candollei var. mirifica contains various compounds such as miroestrol, deoxymiroestrol, daidzein, genistin, and puerarin. Miroestrols are an isoflavonoid compound which has the chemical structure similar to female estrogenic hormones, estradiol [5]. It exhibited an estrogenic activity 0.25 times that of 17β-estradiol by a vaginal cornification assay [6].

To delineate the relationships between estrogen deficiency, depression, and cognitive impairment, we first examined the effects of chronic miroestrol administration as hormone replacement therapy on the depressive behavior and osteoporosis in ovariectomized mice as the model of menopause. Moreover, the effect of miroestrol on depression and osteoporosis-related gene expression will be examined to clarify the molecular mechanism of this phytoestrogen.

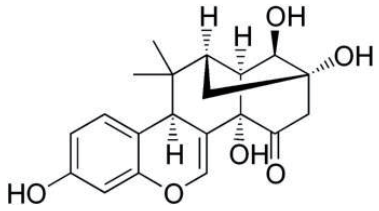

Fig.1 Chemical structure of miroestrol

Materials and Methods

Plant material preparation

Tuberous root bark of *P. candollei var. mirifica* (PM) was collected in Ubon Ratchathani, Thailand and was identified by Dr. Thaweesak Juengwatanatrakul, Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, Ubon Ratchathani, Thailand. Ten kilograms of dried tuberous root bark of PM was powdered and extracted by maceration method with hexane 3 × 20 l, and the product of maceration was extracted with ethyl acetate 3 × 20 l. The ethyl acetate crude extracts were combined, evaporated, and fractionated by column chromatography (Silica gel with hexane: ethyl acetate 3:1). The fractionated samples were combined and evaporated at 60 °C. The residual (PM) was stored at -20 °C until use.

Miroesterol was isolated from PM by high-performance liquid chromatography (HPLC). The HPLC separation was performed using a C18 reverse phase column and a mobile phase consisting of 16% acetonitrile: water with a flow rate at 45.0 ml/min. The UV detection wavelength was set at 205 nm.

Animals

Forty female ICR mice weighing 20-25 g were obtained from National Laboratory Animal Centre, Mahidol University, Nakhon Pathom, Thailand. At all times, the mice were housed on wood chip bedding in stainless steel cages with water and commercial mouse diet supplied ad libitum and acclimated for at least 7

days in housing with a 12-hour dark/light cycle under controlled temperature (22°C ± 2°C) and humidity (45% ± 2%) before dosing. The animal experiment in this study had been approved by Animal Ethics Committee of Khon Kaen University (AE 01/2556), and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, Khon Kaen University, Thailand. All experiments were performed during 08:00 a.m. to 4.00 a.m. and each animal was used only once.

Surgical procedure

Animals were divided into OVX and Sham group. OVX group were ovariectomized by the surgical removal of both ovaries under anesthesia with Nembutal® (Pentobarbital sodium) 60 mg/kg, i.p.. Sham operation was performed as the same conditions with ovaries intact. After a 3-day recovery period,the animals were divided into seven groups: (1) sham, (2) ovariectomy (OVX), (3) ovariectomy + 1 μg/kg 17β-estradiol, (OVX + E2),(4) ovariectomy + 0.1 mg/kg miroestrol (OVX + MR 0.1), and (5)ovariectomy + 1 mg/kg miroestrol (OVX + MR 1),(6) ovariectomy + 2.5 mg/kg PM extract (OVX + PM 2.5), and (7)ovariectomy + 25 mg/kg *PM* extract (OVX + PM 25). The drugs were given daily (during 08.00 and 12.00 a.m.) for eight weeks.

Tail suspension test(TST)

This test was carried out according to the method of Steru [7] TST was developed as a rodent screening test for potential (human) antidepressant drugs. The mice were individually suspended of the tail suspension test box, 60 cm above the surface of table. After treatment administration, each mouse was suspended on the edge of the table 60 above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Immobility duration was recorded for the last 4 min during 6 min period. Mice were considered immobile when they hanged passively and completely motionless [8].

Forced swimming test (FST)

This test demonstrates normal swimming and floating ability. FST used to examine 'learned helplessness' [9] Mice were placed individually in transparent glass cylinder (12 cm in diameter, height 25 cm) which was filled with water to a height of 10 cm of water at 25ºC. Twenty-four hours before the test, pre-test session for 15 minutes. In the test session, each mouse was administration with treatment 1 hour before test, Immobility time were recorded during 5 minutes period.

Serum corticosterone

Blood samples were immediately collected after decapitation and centrifuged at 3000 rpm, 4 ºC for 15 min to isolate serum from blood and store serum at -20 ºC until use. The serum corticosterone level was determined using Corticosterone (CORT) ELISA kit (Assay pro LLC. St. Charies, MO) follow ELISA kit instruction.

Uterus weight and volume

Uterus was immediately removed from mouse body after decapitation without uterine fate and pieces of oviduct and measure weight with 4-digit weighing machine, volume with venire.

Dry weight, ash weight and calcium content in femurs and tibias

Right femurs and tibias were dried in an oven at 60 °C after removed all tissues and wash with water. The bones were put in porcelain cups, weighed by using 5-digits weighing machine, and then incinerated in a furnace at 800 °C 8 hours. The bone was then powdered and ash weight was determined by using 5-digits weighing machine. The ash was dissolved in 15 ml of 6N HCl for 30 minutes before diluted to 1:100 with distilled water. The calcium content was measured by an Atomic absorption spectrophotometer at 422.67 nm.

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total DNA extraction

Total RNA from hippocampus and frontal cortex were extracted by using the TRIzol® reagent. Hippocampus and frontal cortex were homogenized with 1 ml of TRIzol® reagent. After homogenization, 0.2 ml of chloroform was add and mixed the sample vigorously for 15 seconds then incubated them at room temperature for 2 to 3 minutes. The samples were centrifuged at 12,000 x g for 5 minutes at 4oC. Following the centrifugation, the mixture was separated into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. Upper aqueous phase was transfer carefully into fresh tube without disturbing interphase. The volume of the aqueous phase was measure (The volume of aqueous phase is about 60% of the volume of TRIzol® reagent used for homogenization). The aqueous phase was mixed with 0.5 ml of isopropyl alcohol for precipitate the RNA. The sample was incubated at 15 to 30 °C for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4 °C. The RNA precipitate, often invisible before centrifugation, form a gel-like pellet on the side and bottom of the tube. The supernatant as completely removed. Then the RNA pellet was washed with 1 ml of 75%ethanol. The sample was mixed by vortexing and centrifuged at 7,500 x g for 5 minutes at 4°C. After that, This step was repeated again. Then all leftover ethanol was removed by air-dry or vacuum. It was important not to let the RNA pellet dried completely as this will greatly decreased its solubility. The RNA was dissolve in 40 µl RNase free water by passing solution a few times through a pipette tip. After that, the RNA was kept in -20°C refrigerator as stock RNA.

Estimation of RNA concentration

Stock RNA 1 µl was diluted with 99 µl of RNase free water. The sample concentration of total RNA was determined by using microcuvette in the absorbance at 260 nm and 280 nm. The A260/A280 ratio should be above 1.6 for purity. The convention apply that 1 OD at 260 nm equals to 40 µg/ml RNA. The RNA was calculated and adjusted to 0.5 µg/µl by RNase free water and approximate final volume was 60 µl.

Reverse transcription

RNA sample (0.5 µg/ µl) 4 µl was pipette to nuclease-free microcentrifuge tube. The master mix1 was prepared from 1 µl oligo (dT) 12-18, 1 µl 10 mM dNTP Mix (10 nM each dATP, dGTP ,dCTP and dTTP at neutral pH) and RNase free water adjust to 12 µl. The master mix 1 12 µl was added into microcentrifuge tube. The mixture was heated to 65 °C for 5 min and quick chill on ice. Then 8 µl of master mix2 [4 µl 5X First-Stand Buffer, 2 µl 0.1 M DTT, 0.2 µl RNase inhibitor, 0.8 µl RNase free water] was added. The tube was gently mixed and incubate at 37 °C for 2 min. One microlitter (200 units) of M-MLV RT was added, and mix by pipetting gently up and down. The tube was incubated at 37 °C for 50 min and inactivate the reaction by heating at 70 °C for 15 min. Reverse transcription-polymerase chain reaction (RT-PCR) Total RNA from hippocampus and frontal cortex were extracted. PCR amplification was carried out using gene-specific PCR primer setsas follows: β-actin: 5'-AAC GGT CTC ACG TCA GTG TA-3'(sense) and5'-GTG ACA GCA TTG CTT CTG

TG-3'(antisense); ER-β: 5'- CAG TAA CAA GGG CAT GGA AC -3'(sense) and5'- GTA CAT GTC CCA CTT CTG AC -3'(antisense); STAT3: 5'- CAC TAA CAA GGG CAT GGA AC -3'(sense) and5'- GTA CAT GTC CCA CTT CTG AC -3'(antisense) .PCR was performed with a preheating cycle at 95°C for 2 min, denaturation at 95°C for 30 sec, annealing at 60°C for 1 min, extension at 72 °C for 6 min. Aliquots of PCR products were analyzed by gel electrophoresis with 38% polacrylamide and 2% bispolyacrylamide gel stained in Novel juice®, photographed under UV light and analyzed by GeneSnap and ImageJ software.

Statistical analysis

Data were expressed as the mean ± SD for each group. The significant differences between SHAM and OVX group were analyzed using t-test. The significant differences between OVX and treatment groups were analyzed using one-way ANOVA and post hoc Tukey's test. The SigmaStat 3.5 (SYSTAT Software Inc.,Richmond, CA, USA) was used. The differences with p-value less than 0.05 were considered statistically significant.

Results

The effect of PM extract or miroestrol administration on depressive-like behavior

To determine whether miroestrol modulates the depression in an estrogen deprivation model of mice, the depressive-like behavior of OVX mice were elucidated in the tail suspension test (TST) and forced swimming test (FST), respectively. OVX mice received once daily administration of vehicle corn oil, 17β-estradiol, PM extract or miroestrol for 8 weeks before starting the behavioral experiments. The vehicle-treated OVX mice showed significantly longer immobility time than the sham-operated group, indicating depressive-like behavior caused by OVX. 17β-estradiol- and miroestrol-treated OVX groups showed significantly improved depressive-like behavior in the test compared with the vehicle-treated control OVX mice (Fig. 2A, 2B).

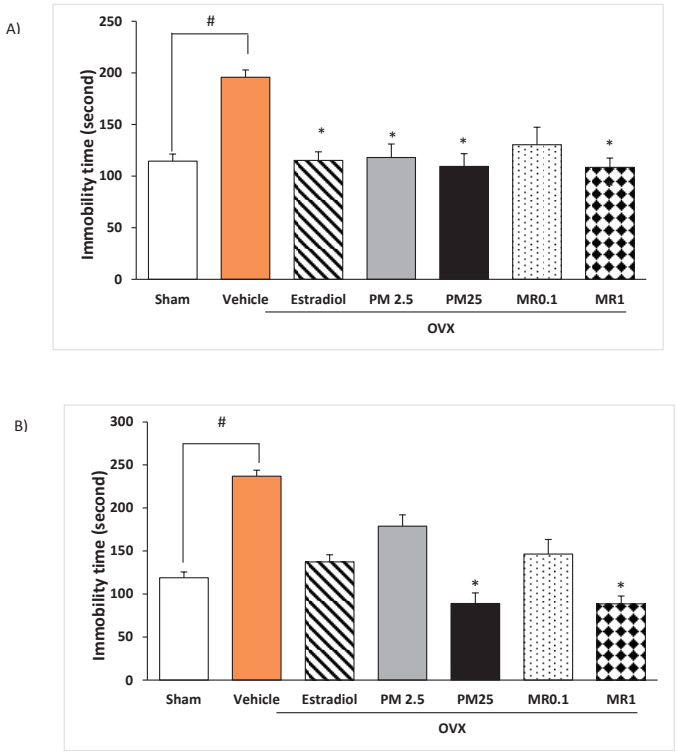

Fig 2. Effects of PM extract or miroestrol and 17β-estradiol (E2) on OVX-induced depressive-like behavior in tail suspension test (A) and forced swimming test (B). Each column represents the mean ± S.E.M. (n=10–12). #P<0.05 vs. sham. *P<0.05, ** P < 0.001 vs. vehicle-treated OVX group

Changes in uterus weight and volume after PM extract or miroestrol administration.

The OVX group showed a significant decrease in uterus weight and volume compared with the sham group (Table 1). Hormone replacement therapy of OVX mice with 17β-estradiol for 8 weeks significantly increased both the uterus weight and volume. Similarly, PM extract or miroestrol treatment significantly increased the uterus weight and volume in OVX animals.

Table 1

Group	Uterus weight (g/kg body weight)	Uterus volume (cm^3/kg body weight)
Sham	5.3728 ± 0.66	4.4961 ± 0.6
OVX	3.0115 ± 0.56#	1.3418 ± 0.42#
OVX + E2	4.9181 ± 0.71**	4.5268 ± 0.36**
OVX + PM 2.5	6.3510 ± 0.58*	4.0661 ± 0.27**
OVX + PM 25	6.5633 ± 0.59*	5.5860 ± 1.12**
OVX + MR 0.1	5.6207 ± 0.58**	4.5169 ± 0.17**
OVX + MR 1	7.4223 ± 0.78*	4.5056 ± 0.28**

Each datum represents the means ± SEM (n=5–6 in each animal group). #P<0.05 vs. sham. *P<0.05, **P<0.001 vs. vehicle-treated OVX group

Table 2 Effect of PM and MR on dry weight, ash weight, and calcium content in femurs.

Treatment	Dry weight (mg)	Ash weight (mg)	Calcium content (mg)
SHAM	51.80 ± 3.37	29.90 ± 2.30	4.90 ± 0.34
OVX	45.57 ± 3.47 #	24.71 ± 3.21 #	4.28 ± 0.28 #
OVX + PM 2.5	53.18 ± 1.38 *	30.98 ± 1.49 *	4.89 ± 0.18 *
OVX + PM 25	56.25 ± 5.48 *	33.53 ± 4.19 *	5.06 ± 0.38 *
OVX + MR 0.1	54.65 ± 2.42 *	30.15 ± 1.94	4.41 ± 0.22
OVX + MR 1	54.68 ± 4.33 *	31.23 ± 2.78 *	4.95 ± 0.34 *

Each datum represents the means ± SEM (n = 5-6 in each animal group). #P<0.05 vs. sham. *P<0.05 vs. vs. vehicle-treated OVX group.

Table 3 Effect of PM and MR on dry weight, ash weight, and calcium content in tibias

Treatment	Dry weight (mg)	Ash weight (mg)	Calcium content (mg)
SHAM	45.14 ± 1.46	27.38 ± 1.16	4.07 ± 0.11
OVX	39.51 ± 1.26 ##	23.69 ± 0.94 ##	3.56 ± 0.10 ##
OVX + PM 2.5	46.76 ± 2.36 **	28.30 ± 1.68 *	3.98 ± 0.16 *
OVX + PM 25	49.25 ± 3.46 **	29.85 ± 2.61 **	4.13 ± 0.16 **
OVX + MR 0.1	44.44 ± 1.65 *	26.75 ± 1.23	3.86 ± 0.16
OVX + MR 1	45.32 ± 1.59 *	28.13 ± 1.29 *	4.04 ± 0.27 *

Each datum represents the means ± SEM (n=5–6 in each animal group). ##P<0.05 vs sham. *P<0.05, **P<0.001 vs. vehicle-treated OVX group.

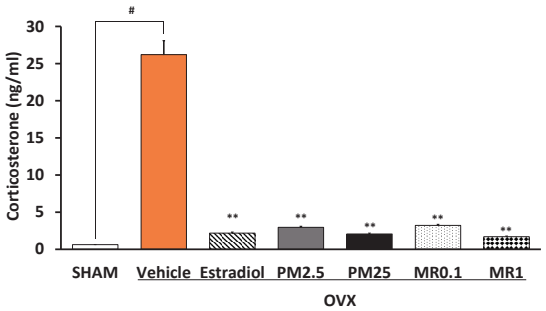

Figure. 3 Effects of PM and MR on OVX-induced elevation of serum corticosterone level.

Each data column represents the mean ± S.E.M. (n=3–5). #p<0.005 vs. sham and **p<0.001 vs. vehicle-treated OVX group.

Effects of PM extract or miroestrol administration on dry weight, ash weight and calcium content in femurs and tibias

The effect of PM and MR on femurs is consistent with the effect on tibias. Ovariectomy was found to cause significantly loss in dry weight, ash weight and calcium content as compared to sham operated group (Table 2 and 3). Dry weight was significantly increased in PM and miroestrol treated groups compared with OVX group. Ash weight and calcium content was significantly increased by both PM 2.5 and PM 25 compared with the OVX mice. For MR treated groups, only MR1 significantly increased ash weight and calcium content.

***PM* extract and miroestrol administration reduced serum corticosterone level in OVX**

Ovariectomy significantly decreased serum corticosterone level as compared to the sham-operated group (Fig.3). Serum corticosterone significantly decreased in PM and MR treated groups compared with OVX group.

***Effects of PM* extract and miroestrol on the expression levels of ERβ and STAT3 mRNA down-regulated in a OVX model**

The mRNA expression levels were evaluated by RT-PCR. Intensity of genes expression was measured using ImageJ and calculated into %relative expression. Administration of 17β-estradiol, PM and miroestrol

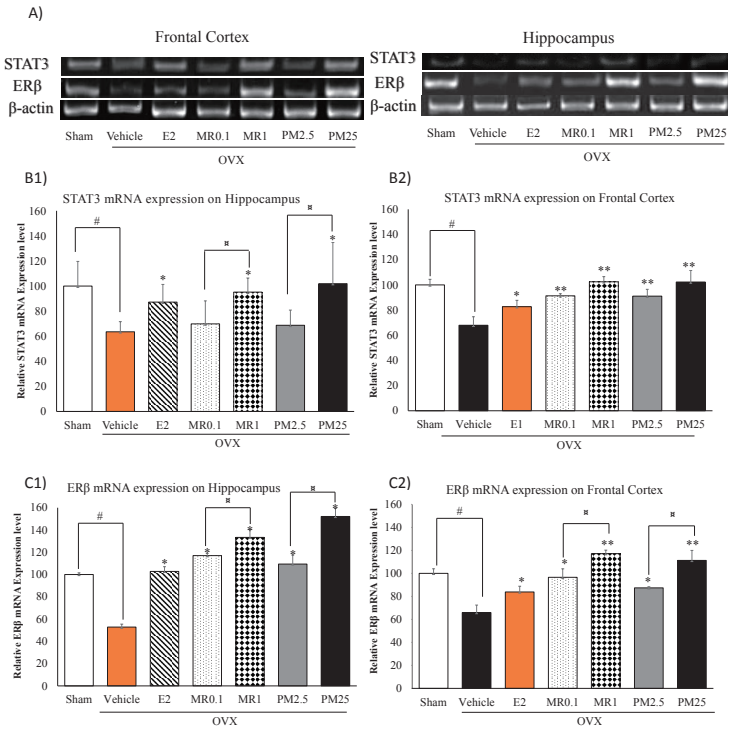

Fig. 4. *PM* extract- and miroestrol-induced reversal of down-regulated expression of ERβ and STAT3 mRNA expression in OVX animals. (A) Typical photo indicate effects of administration of 17β-estradiol (E2, 1 μg/kg/day) Pueraria mirifica crude extract (PM 2.5 and 25 mg/kg/day) and miroestrol (MR 0.1 and 1 mg/kg/day) on relative STAT3 and ERβ mRNA expression in hippocampus and frontal cortex. (B1-B2) Quantitative comparison expression levels of STAT3 and (C1-C2) ERβ against the expression level of housekeeping gene, β-actin. Results are presented as the mean ± S.E.M (n=5-6). Statistical analysis was undertaken by one-way ANOVA followed by the Turkey test. #P<0.001 vs. sham; *P<0.05, **P<0.001 vs. vehicle-treated OVX group; ¤P<0.001 vs. a low dose of each test drug.

led to activation of ERβ and STAT mRNA expression in hippocampus and frontal cortex as show in fig.4. There is significantly decreased ERβ mRNA expression of OVX + corn oil in hippocampus when compared to sham group ($p<0.001$). The result showed significantl increase in expression of ERβ and STAT mRNA after OVX mice received 17β-estradiol ($p<0.05$).

Discussion

The present study demonstrated that miroestrol ameliorated estrogen depletion-induced depressive-like behavior and bone loss suggested that these effects of miroestrol are attributable to the serum corticosterone, estrogen receptor-mediated facilitation of ERβ and STAT3 gene transcription in the brain and calcium content in bone. Our findings raise the possibility that MR and *Pueraria candollei var. mirifica*, the plant of origin of MR, may have a beneficial effect on depression and osteoporosis in which menopause/ovariectomy are implicated as risk factors.

Estrogens or estradiol is protective against ischemic brain damage and essential for growth and differentiation of the uterus, cervix, vagina, breast, and some mammary tumors, to promotion of this effect estrogen need to bind to estrogen receptors which acts as a transcription factor that binds to a sequence known as estrogen response element (ERE) to induce gene transcription. *Pueraria mirifica* can give the characteristic compounds are deoxymiroestrol and miroestrol which is the very potent phytoestrogen and believed to be the miracle plant to use in post-menopause women. Deoxymiroestrol is not stable compounds, it can be lose in the process of extraction so miroestrol is the candidate to use in this study. Estrogen receptor β (ERβ) is the specific receptor for mediated anxiety behavior by the activation of ERβ can decreases anxiety-related behavior, which is the problem of post-menopausal women. In estrogen deficiency condition, ERβ mRNA is down-regulated (Shima N. et. al., 2003) and can be normalized when giving hormone replacement therapy. Estrogen reduces bone turnover by decreasing RANKL, which a key stimulator of osteoclast-induced bone resorption. It also increases OPG, which works against RANKL, to induce bone formation. Previous studies showed that estrogen deficiency induced by ovariectomy lead to increasing RANKL and decreasing OPG, which indicate bone resorption. Treatment with PM extract and MR decreased the RANKL/OPG ratio by lowering level of RANKL (Udomsuk et al., 2012a; Tiyasatkulkovit et al., 2014). The results suggesting that their prevention effects on

ovariectomy induced bone loss may involve decreasing osteoclast-mediated bone resorption.

In conclusion, *Pueraria mirifica* crude extract and miroestrol are significantly increased uterus weight and volume, decrease serum corticosterone and immobility time of TST and FST, increase calcium content in femurs and tibias and also up-regulated the expression of ERβ and STAT3 mRNA as similar as giving synthetics estrogen in estrogen deficiency condition model due to the similar structure between phytoestrogen and estradiol. These can indicated that PM crude extract and miroestrol has estrogenic activity to use as hormone replacement therapy so PM crude extract and miroesterol may use as promising compound for alternative choice in hormone replacement therapy.

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