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Effect of *Pueraria mirifica* Subchronic Treatments on Ameliorating Depression by Increasing Tryptophan Hydroxylase Immunoreactive Neurons via Estrogen Receptors in Ovariectomized Mice

(Kesan Rawatan Subkronik *Pueraria mirifica* dalam Memperbaiki Kemurungan dengan Meningkatkan Neuron Imunoreaktif Triptofan Hidroksilase melalui Reseptor Estrogen dalam Tikus Terovariektomi)

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ABSTRACT

Estrogen depletion leads to menopause-associated depression. *Pueraria mirifica* (PM) contains phytoestrogen that has been used for rejuvenating in aged women. Therefore, this study aimed to investigate the effect of PM on depression-like behavior, density of tryptophan hydroxylase immunoreactive (TPH-ir) neurons and intensity of estrogen receptor α (ERα) and β (ERβ) in dorsal raphe nucleus (DRN) in ovariectomized (OVX) mice. Adult female IRC mice were divided into 7 groups: (1) Sham-operates (SHAM), (2) OVX and distilled water treated (PM0), (3) OVX and 40 μg/kg estradiol benzoate treated (E40), OVX and ethanolic extract of PM treated for 90 days at various doses (4) 25 mg/kg (PM25), (5) 50 mg/kg (PM50), (6) 100 mg/kg (PM100) and (7) OVX and 20 mg/kg fluoxetine treated for 20 days. The duration of immobility in both the FST and TST significantly prolonged in the PM0 group and significantly shortened in the E40, PM50, PM100, and fluoxetine groups ($p<0.05$). The density of TPH-ir neurons in the DRN was significantly reduced in PM0 and significantly increased in the E40, PM25, PM50, PM100, and fluoxetine ($p<0.05$). The ERβ immunoreactivity (ERβ-ir) was much stronger than the ERα immunoreactivity (ERα -ir). However, the intensity of the ERα-ir and ERβ-ir was significantly decreased in the PM0 and PM25 groups and significantly increased in the E40, PM50, PM100, and fluoxetine ($p<0.05$). Therefore, we suggested that subchronic treatments with 50 mg/kg and 100 mg/kg of PM played an effective role in improvement of depression by stimulating TPH via ERβ.

Keywords: Depression-like behavior screening test; estrogen receptors; ovariectomy; *Pueraria mirifica;* tryptophan hydroxylase

ABSTRAK

Kekurangan estrogen membawa kepada kemurungan yang berkaitan dengan menopaus. *Pueraria mirifica* (PM) mengandungi fitoestrogen yang telah digunakan untuk meremajakan wanita berumur. Oleh itu, penyelidikan ini bertujuan untuk mengkaji kesan PM terhadap tingkah laku seperti kemurungan, ketumpatan neuron triptofan hidroksilase imunoreaktif (TPH-ir) dan keamatan reseptor estrogen α (ERα) dan β (ERβ) dalam nukleus raphe dorsal (DRN) pada tikus terovariektomi (OVX). Tikus IRC betina dewasa dibahagikan kepada 7 kumpulan: (1) dioperasikan Sham (SHAM), (2) OVX dan air suling terawat (PM0), (3) OVX dan 40 μg/kg estradiol benzoat terawat (E40), OVX dan ekstrak etanol PM terawat selama 90 hari pada pelbagai dos (4) 25 mg/kg (PM25), (5) 50 mg/kg (PM50), (6) 100 mg/kg (PM100), dan (7) OVX dan 20 mg/kg fluoxetin terawat selama 20 hari. Tempoh imobiliti dalam kedua-dua FST dan TST berpanjangan dengan ketara dalam kumpulan PM0 dan dipendekkan dengan ketara dalam kumpulan E40, PM50, PM100 dan fluoxetin ($p<0.05$). Ketumpatan neuron TPH-ir dalam DRN berkurang dengan ketara dalam PM0 dan meningkat dengan ketara dalam E40, PM25, PM50, PM100 dan fluoxetin ($p<0.05$). Imunoreaktiviti ERβ (ERα -ir) adalah lebih kuat daripada imunoreaktiviti ERα (ERα -ir). Walau bagaimanapun, keamatan ERα-ir dan ERβ-ir telah menurun dengan ketara dalam kumpulan PM0 dan PM25 dan meningkat dengan ketara dalam E40, PM50, PM100 dan fluoxetin ($p<0.05$). Oleh itu, kami mencadangkan bahawa rawatan subkronik

dengan 50 mg/kg dan 100 mg/kg PM memainkan peranan yang berkesan dalam penambahbaikan kemurungan dengan merangsang TPH melalui ERβ.

Kata kunci: Ovariektomi; *Pueraria mirifica*; reseptor estrogen; triptofan hidroksilase; ujian saringan tingkah laku seperti kemurungan

INTRODUCTION

Depression is a common mental disorder that results in a persistent feeling of sadness and loss of interest. Moreover, depression is a leading cause of disability worldwide and can lead to suicide attempts. It is well known that more women are affected by depression than men. Depression in women occurs at the time of hormone flux such as premenstrual, postnatal, perimenopausal, and postmenopausal depression. These are called reproductive depression. Treatment with estrogen is very effective for reproductive depression (Studd *2020)*. However, long-term use of estrogen therapy leads to greater clinical risks and side effects (Grady et al. 2000). Therefore, phytoestrogen is preferred as a natural alternative in therapy. With crucial function in depression, the neurotransmitter serotonin (5-hydroxytryptamine, 5HT) is primarily released by the raphe nuclei. The main serotonin production is sourced by these raphe nuclei that are further classified into rostral and caudal groups. The largest population of serotonin neurons in the brain owns the rostral group, precisely with the dorsal raphe nucleus (DRN) (Hornung 2003). While ovarian hormones influence numerous factors regulating serotonin synthesis and serotonin levels in the central nervous system, estrogen enhances serotonergic transmission by increasing serotonin synthesis or reducing serotonin reuptake and therefore improves depressive symptoms. Ovarian hormones also stimulate the production of tryptophan hydroxylase (TPH) which is an enzyme involved in the synthesis of serotonin and decreases serotonin reuptake transporter (SERT) mRNA expression in raphe nuclei (Bethea et al. 1998). In further studies, the beta subtype of estrogen receptors was identified in the DRN, whereas the classic alpha subtype of estrogen receptors was not detected (Kuiper et al. 1997). These findings suggested that estrogen may facilitate serotonin synthesis via estrogen receptors.

Pueraria mirifica (PM) is a Thai herbal plant. It belongs to the family *Leguminosae*, subfamily *Papilionoideae* and has been used in Thai traditional medicine for its rejuvenating effect on aged women. Its tuberous root contains active phytoestrogen compounds which exhibit high estrogenic activity (Jaroenporn et al. 2006). Treatment with *P. mirifica* powder has stimulated the proliferation of the vaginal epithelium and uterus endometrium (Malaivijitnond et al. 2006). *P. mirifica* also decreased serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in ovariectomized rats (Malaivijitnond et al. 2004). *P. mirifica* was also reported to relieve climacteric symptoms such as hot flashes, frustration, sleep disorder, and skin dryness in post-menopausal women (Muangman & Cherdshewasart 2001). Treatment with *P. mirifica* obviated muscle atrophy and restored muscle strength in ovariectomized rats (Inthanuchit et al. 2017). However, the effect of *P. mirifica* on brain function has not been sufficiently researched, especially its effect on depression treatment. This study aims to investigate the effect of *P. mirifica* on depression-like behavior and its mechanisms on depression by investigating an alteration of TPH immunoreactive neurons and a change of ERα and ERβ of the DRN in ovariectomized mice.

MATERIALS AND METHODS

ANIMAL PREPARATION

Adult female IRC mice (60 days) weighing 25-30 g were purchased from Nomura Siam International (Bangkok, Thailand). Animals were maintained at 22 °C with 12/12 dark/light cycle (lighting on at 06.00 am) and housed in groups with food and water available *ad libitum*. The experimental protocols described in this study were approved by the Animal Ethical Committee of the Prince of Songkla University for the caring and use of experimental animals (MOE 0521.11/314). The sample size was calculated using program GPOWER (n=10 per group). The animals were randomly divided into 7 groups: (1) Sham-operates (SHAM), (2) ovariectomized and treated with distilled water (PM0), (3) ovariectomized and 40 μg/kg estradiol benzoate treated (E40), ovariectomized and ethanolic extract of *P. mirifica* treated for 90 days at various doses (4) 25 mg/kg (PM25), (5) 50 mg/kg (PM50), (6) 100 mg/kg (PM100), and (7) ovariectomized and 20 mg/kg fluoxetine treated for 20 days. The body weights of all groups were recorded daily. The dose of estrogen and PM treatments were modified from the study by Jaroenporn et al. (2007).

OVARIECTOMY PROCEDURES

The mice were anesthetized by an intraperitoneal injection of 10 mg ketamine - 1.5 mg xylazine/100 g of body weight (Islamov et al. 2002). One-centimeter vertical

skin, underneath subcutaneous tissue and muscle incisions were made bilaterally on the sides of the abdomen at the mid-axillary line, 1 cm below the lower edge of the costal cage. The ovaries were removed, and the uterine tubes were ligated. The muscle, subcutaneous tissue and skin were then sutured. Sham operation was performed by following a previous study. The protocol of operative steps included anesthesia treatment and bilateral incisions before the exposure of the ovaries and uterine horns outside of the abdominal cavity (without removing them) and closure of the wounds. Animals were housed for 7 days after ovariectomy before initiation of all experiments (Radenahmad et al. 2006).

PREPARATION OF PM SUSPENSION

The jet spray-dried *P. mirifica* was purchased from St. Herb Cosmetics International Co., Ltd., Thailand. The extract contained 31.73 mg puerarin, 5.81 mg daidzin, 7.22 mg genistin, 15.75 mg daidzein, and 8.35 mg/100g genistein as determined by HPLC analysis (Certificate of Analysis and Test Report Lot No. A55050501 from St. Herb Cosmetics International Co., Ltd., Thailand). It also comprised other compounds such as 3.34 ppm copper, 175 ppm iron, 19.1 ppm zinc, 38% calcium, 2% magnesium and 0.0176% sodium.

DEPRESSION-LIKE BEHAVIOR SCREENING TEST

Forced swimming test (FST)

The protocol of the forced swimming test was modified from the work of Porsolt, Bertin and Jalfre (1977). Briefly, mice were individually forced to swim in a vertical glass cylinder (height 40 cm and diameter 18 cm) with a depth of 15 cm of water at 25 °C. The time of immobility was scored during 6 min. Each mouse was considered to be immobile when it stopped struggling and continued floating with the absence of any movement except for those necessary for keeping the nose above water. The parameter acquired was the number of seconds spent immobile. The water in the containers was changed after each trial (Yankelevitch-Yahav et al. 2015).

Tail suspension test (TST)

The tail suspension test procedure was modified from the work of Steru et al. (1985). Each mouse was suspended 50 cm above the floor using an adhesive tape placed 20 mm from the extremity of its tail within its own three-walled rectangular compartment (5×15×11.5 cm). The total immobility period was scored manually during the 6 min test session. The scored parameter was the time measured in seconds that every mouse spent immobile. Immobility is defined as suspending passively without any movement of the head or paws.

SAMPLE PREPARATION

Blood samples were obtained from the retro-orbital sinus to evaluate serum estradiol levels using an electrochemiluminescence immunoassay (ECIA) kit (LKE 2026, DPT, Gwynedd, UK), serum glutamic oxaloacetic transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) levels and a complete blood count to investigate toxicity.

BRAIN SPECIMEN PREPARATION

At the end of the experimental period, the mice were deeply anesthetized by an intraperitoneal injection of 100 mg/kg of thiopental (Jagsonpal Pharmaceuticals, New Delhi, India). The transcardial perfusion was performed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, as a fixative. The brains were removed and post-fixed with the same fixative solution overnight at 4 °C. The brains then were transferred to 30% sucrose in 0.1 M PB, left until they sank to the bottom, and stored at -80 °C. The coronal serial sections (20 μm thick) were cut using a cryostat (LEICA CM 1850, Leica Biosystems, Germany) and mounted on 3-triethoxysilylpropylamine (TESPA) coated slides for staining with 0.1% cresyl violet to localize DRN.

IMMUNOFLUORESCENCE FOR TPH

Adjacent series of brain sections from each group were incubated in the following solution and between each of the steps the sections were rinsed with 0.1 M PB: (1) 10% normal rabbit serum (Vector Laboratories, Burlingham, CA, USA) with 0.3% Triton-X 100 (J.T. Baker Inc, Philipsburg, NJ) in 0.1 M PB; (2) sheep anti-TPH (AB1541, LOT #3764417, Merck Millipore, USA) at a dilution 1:1,000 for 24h at 4 °C; and (3) Texas Red anti-sheep IgG antibody (1:200 dilution, Vector Laboratories, Burlingham, CA, USA), for 1 h at room temperature, respectively. The sections were finally mounted with Vectashield (Vector Laboratories, Burlingham, CA, USA), The coded sections were examined by fluorescence microscopy (BX50, Olympus, Japan). Images were captured by a digital camera (DP73, Olympus, Japan). Controls were performed by either omitting the first or secondary. None of these controls showed any labeling. The number of TPH-ir neurons in each micrograph was counted and the area of each examined section was measured using Image-Pro Plus 6.0 software (Olympus, Japan). The results were expressed as a number of TPH-ir neuron per mm^2.

IMMUNOFLUORESCENCE FOR ERα AND ERβ

The immunofluorescence protocols for the study of the ERα and ERβ were modified from the work of Bunratsami et al. (2015). Briefly, the adjacent brain sections were incubated in 10% normal horse serum (Vector Laboratories, Burlingham, CA, USA) with 0.2% Triton-X 100 (J.T. Baker Inc, Philipsburg, NJ) in 0.1 M phosphate buffer (PB). The sections were then incubated with rabbit anti-ERα (MC-20, STCSC-542, LOT #A0716, Santa Cruz Biotechnology Inc, CA, USA) or rabbit anti-ERβ (H-150, SC-8974, LOT #D0615, Santa Cruz Biotechnology Inc, CA, USA) at a dilution of 1:50 in 0.1 M PB for 48 h at 4 °C. After washing with 0.1 M PB, the sections were incubated in Texas Red labeled anti-rabbit IgG for staining of the ERα or fluorescence- labeled anti-rabbit IgG secondary antibodies for staining of the ERβ (Vector Laboratories, Burlingham, CA, USA) at a dilution of 1:200 in 0.1 M PB for 2 h. The sections were finally washed with 0.1 M PB and mounted with Vectashield (Vector Laboratories, Burlingham, CA, USA), cover-slipped, and sealed with nail polish. Controls were stained by either replacing the first or secondary with 0.1 M PB. None of these controls showed any labeling. The sections from individual animals were analyzed for immunolabeling of the ERα and ERβ under an fluorescence microscope (BX50, Olympus). The intensity of the ERα and ERβ immunoreactivities were measured using Image-Pro Plus 6.0 software (Olympus, Japan).

STATISTICAL ANALYSIS

Data were expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) program version 23. The data distribution was tested and differences of means among groups were compared by one-way ANOVA, followed by a least significant difference test (LSD). Differences with a $p<0.05$ were considered to be statistically significant.

RESULTS

BODY WEIGHT, UTERINE WEIGHT, ESTROGEN LEVEL, CBC, SGOT, AND SGPT LEVELS

Body weight was significantly increased in the PM0 group, PM25, and fluoxetine groups compared to those of the SHAM ($p<0.05$). In contrast, the weight gain was significantly increased in only the PM0 group compared to those of the SHAM ($p<0.05$). The uterine weight and uterine weight/body weight were significantly decreased in the PM0 and fluoxetine groups compared to those of the SHAM ($p<0.05$). Whereas the uterine weight was significantly increased in the E40 and all PM treatment groups (PM25, PM50, and PM100) compared to those of the PM0 ($p<0.05$) (Table 1). The estrogen level was significantly reduced in the PM0, PM25, PM50, PM100, and fluoxetine groups but significantly increased in the E40 group ($p<0.05$) compared to those of the SHAM ($p<0.05$). Most of the complete blood count parameters in all groups (SHAM, PM0, E40, PM25, PM50, PM100, and fluoxetine) were normal. The SGOT level was high in the fluoxetine group, while the SGPT level was high in the E40 and fluoxetine groups (Table 1).

DEPRESSION-LIKE BEHAVIOR SCREENING TESTS (FST AND TST)

The duration of immobility of both the FST and TST was significantly increased in the PM0 (195.92±12.18 s, 195.38±16.16 s) and PM25 (171.21±19.53 s, 211.71±10.2 s) compared to those of the SHAM group (100.09±22.36 s, 145.9±11.1 s) ($p<0.05$). In contrast, the duration of immobility of the FST significantly decreased (122.69±19.72 s) and TST tended to reduce in the PM50 groups (166.61±17.94 s). Moreover, the duration of immobility of both the FST and TST was significantly decreased in the E40 (52.63±14.57 s, 116.72±17.53 s), PM100 (48.84±12.67 s, 140.53±18.18 s), and fluoxetine groups (144.07±14.34 s, 113.46±19.83 s) compared to those of the PM0 ($p<0.05$) (Figure 1(A) and 1(B)).

DENSITY OF TPH-ir NEURONS IN THE DRN

The sections were stained with anti-TPH showing the TPH-ir neurons in the DRN (Figure 2(A)) and the TPH-ir neurons were identified in their cytoplasm (Figure 2(B)). The TPH-ir neurons in the DRN were reduced in PM0 and PM25 but increased in the E40, PM50, PM100, and fluoxetine groups (Figure 2(C)-2(I)). The density of TPH-ir neurons in the DRN was significantly reduced in the PM0 group and the PM25 compared to those of the SHAM ($p<0.05$). In contrast, the density of TPH-ir neurons in the DRN was significantly increased in the E40, PM50, PM100, and fluoxetine groups compared to those of the PM0 group ($p<0.05$) (Table 2).

ERα-ir AND ERβ-ir IN THE DRN

The ERα-ir and ERβ-ir were identified in the nucleus of neurons in the DRN (Figures 3(A) & Figure 4(A)). However, the ERβ-ir was much stronger than the ERα-ir (Figures 3(B) & 4(B)). Both ERα-ir and ERβ-ir were reduced in the PM0 and PM25 groups but increased in the E40, PM50, PM100, and fluoxetine groups (Figures 3(C)-3(I) & 4(C)-4(I)). The intensity of the ERα-ir and ERβ-ir was sinificantly decreased in the PM0 and PM25 groups compared to those of the SHAM and significantly increased in the E40, PM50, PM100, and fluoxetine groups ($p<0.05$) (Table 2).

TABLE 1. Weight, weight gain, uterine weight, uterine weight/body weight, and estrogen level in all treatment groups

Parameter	SHAM	PM0	E40	PM25	PM50	PM100	Fluoxetine
Weight (g)	37.72±0.35	41.64±1.36*	39.99±0.70	41.63±1.10*	39.83±0.48	40.07±0.58	41.35±1.04*
Weight gain (g)	0.019±0.005	0.051±0.013*	0.023±0.004^{+}	0.027±0.005	0.024±0.007^{+}	0.019±0.009^{+}	0.026±0.014
Uterine weight (g)	0.40± 0.03	0.24±0.024*	0.60±0.043^{+}*	0.41±0.022^{+}	0.41±0.026^{+}	0.46±0.035^{+}	0.22±0.023*
Uterine weight/ body weight	1.081±0.069	0.588±0.066*	1.515±0.121^{+}	0.999±0.053^{+}	1.041±0.070^{+}	1.148±0.088^{+}	0.558±0.062*
Estrogen level (pg/mL)	14.40 ± 7.38	<5.00*	33.85±10.70*$^{+}$	<5.00*	<5.00*	<5.00*	<5.00*
Hb (13.0-16.3) (g/dl)	12.0±0.3	11.5±0.1	12.1±0.3	12.3±0.5	12.9±0.3	12.8±0.3	11.6±0.4
Hct (34.3-46.1) (%)	35.9±1.0	33.9±0.4	35.8±0.9	37.1±1.4	38.5±0.9	38.2±0.9	34.9±1.1
Platelet count (815-1358) ($\times 10^3/\mu L$)	1011±148.3	1013.44±76.7	1100±139.6	898.6±88.5	1024.1±89.0	1040.5±172.1	963.6±102.8
MCV (44.9-49.7)(fl)	50.1±0.9	47.6±1.1	45.2±1.2	50.2±1.0	50.3±1.2	49.0±1.5	47.8±0.8
WBC count (2.8-5.7) ($\times 10^3/\mu l$)	5.2±1.9	2.9±0.3	2.8±0.2	2.2±0.2	2.6±0.4	3.2±0.5	4.1±0.7
Lymphocyte (60.0-90.0)(%)	73.7±5.2	71.7±4.3	68.2±4.5	64.2±3.0	62.9±4.0	66.7±4.6	73.1±4.5
PMN (10.0-38.0)(%)	22.1±5.0	24.6±4.2	28.3±4.6	32.2±3.0	32.0±3.8	28.5±4.6	23.5±4.6
Monocyte (0.0-2.0) (%)	3.6±0.8	3.3±0.6	3.5±0.5	3.0±0.4	4.3±0.5	4.0±0.5	2.9±0.5
Eosinophil (0.0-4.0) (%)	0.3±0.2	0.3±0.2	0.0±0.0	0.4±0.2	0.7±0.3	0.5±0.2	0.3±0.1
Basophil (0) (%)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.1
Band	0.3±0.2	0.1±0.1	0.0±0.0	0.1±0.1	0.2±0.1	0.2±0.1	0.1±0.1
SGOT (103-329) (U/L)	368.55±43.942	318.78±49.839	299.80±32.407	328.5±30.716	281.85±25.766	271.67±27.587	423.83±121.676
SGPT (25-63) (U/L)	99.22±16.573	75.89±11.922	108.30±22.554	80.71±9.691	77.08±5.558	86.92±7.362	96.33±15.479

*Significant difference from SHAM, $P < 0.05$, $^{+}$Significant difference from PM0, $P < 0.05$, n = 10. SHAM: Sham-operates; PM0: ovariectomized and distilled water treated; E40: ovariectomized and 40 µg/kg estradiol benzoate treated; PM25: ovariectomized and 25 mg/kg *Pueraria mirifica* (PM) treated; PM50: ovariectomized and 50 mg/kg PM treated; PM100: ovariectomized and 100 mg/kg PM treated; Fluoxetine: ovariectomized and 20 mg/kg fluoxetine treated

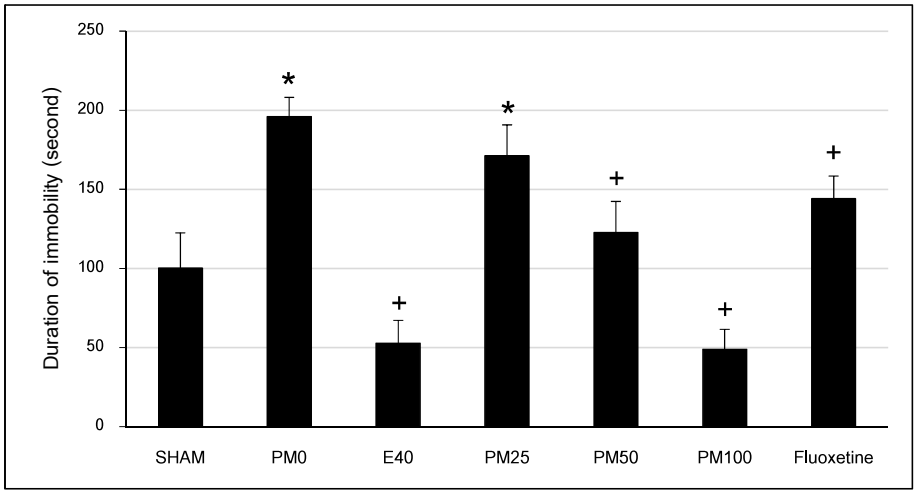

FIGURE 1(A). Bar graph of the FST of the SHAM, PM0, E40, PM25, PM50, PM100, and fluoxetine groups, n=10. *Significant difference from SHAM, $p < 0.05$. $^{+}$ Significant difference from PM0, $p < 0.05$. FST: Forced swimming test; SHAM: Sham-operates; PM0: ovariectomized and distilled water treated; E40: ovariectomized and 40 µg/kg estradiol benzoate treated; PM25: ovariectomized and 25 mg/kg *Pueraria mirifica* (PM) treated; PM50: ovariectomized and 50 mg/kg PM treated; PM100: ovariectomized and 100 mg/kg PM treated; Fluoxetine: ovariectomized and 20 mg/kg fluoxetine treated

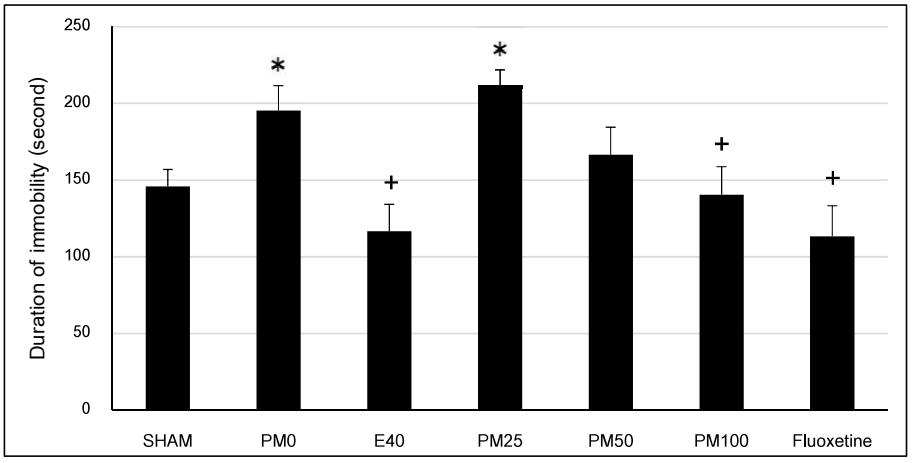

FIGURE 1(B). Bar graph of the TST of the SHAM, PM0, E40, PM25, PM50, PM100, and fluoxetine groups, n=10. *Significant difference from SHAM, $p < 0.05$. $^{+}$Significant difference from PM0, $p < 0.05$. TST: Tail suspension test; SHAM: Sham-operates; PM0: ovariectomized and distilled water treated; E40: ovariectomized and 40 µg/kg estradiol benzoate treated; PM25: ovariectomized and 25 mg/kg *Pueraria mirifica* (PM) treated; PM50: ovariectomized and 50 mg/kg PM treated; PM100: ovariectomized and 100 mg/kg PM treated; Fluoxetine: ovariectomized and 20 mg/kg fluoxetine treated

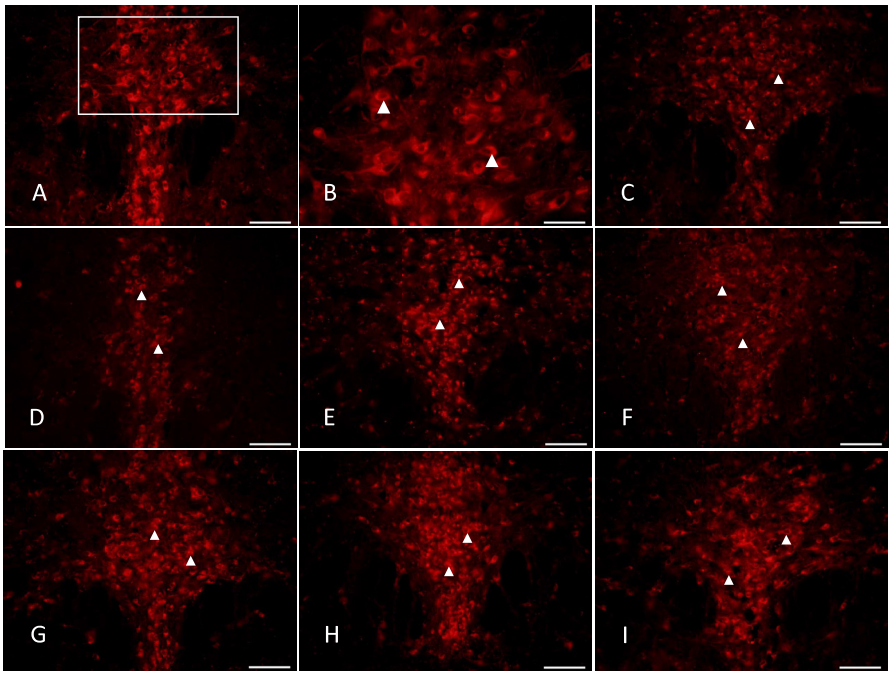

FIGURE 2. (A) Micrograph of TPH-ir (bright red) in the DRN, (B) high magnification of the area in the square frame from (A) showing TPH-ir (arrows) in the cytoplasm of neurons and micrograph of the TPH-ir (arrows) in the DRN of the SHAM (C), PM0 (D), E40 (E), PM25 (F), PM50 (G), PM100 (H), and fluoxetine (I); (A) scale bar = 100 μm (B) scale bar =50 μm, (C-H) scale bar = 200 μm. TPH-ir: tryptophan hydroxylase immunoreactive; DRN: dorsal raphe nucleus; SHAM: Sham-operates; PM0: ovariectomized and distilled water treated; E40: ovariectomized and 40 μg/kg estradiol benzoate treated; PM25: ovariectomized and 25 mg/kg *Pueraria mirifica* (PM) treated; PM50: ovariectomized and 50 mg/kg PM treated; PM100: ovariectomized and 100 mg/kg PM treated; Fluoxetine: ovariectomized and 20 mg/kg fluoxetine treated

TABLE 2. Density of TPH-ir neurons, intensity of ERα-ir and ERβ-ir

Quantification/ Measurement	SHAM	PM0	E40	PM25	PM50	PM100	Fluoxetine
Density of TPH-ir neurons (cell/ mm^2)	268.61±31.98	103.71±26.91*	223.87±33.12$^+$	160.25±26.75*	209.68±18.60$^+$	257.17±31.98$^+$	242.63±23.13$^+$
Intensity of Erα-ir	12.68±1.20	4.73±1.07*	11.16±1.05$^+$	7.62±1.01*	11.39±1.23$^+$	13.02±1.06$^+$	10.84±1.04$^+$
Intensity of Erβ-ir	47.53±5.98	16.61±4.47*	38.91±5.10$^+$	14.28±3.48*	40.88±3.36$^+$	38.66±5.46$^+$	35.40±6.84$^+$

*Significant difference from SHAM, $P < 0.05$, $^+$Significant difference from PM0, $P < 0.05$, n = 10. TPH-ir neurons: tryptophan hydroxylase immunoreactive neurons; ERα-ir: ERα immunoreactivity; ERβ-ir: ERβ immunoreactivity; SHAM: Sham-operates; PM0: ovariectomized and distilled water treated; E40: ovariectomized and 40 μg/kg estradiol benzoate treated; PM25: ovariectomized and 25 mg/kg *Pueraria mirifica* (PM) treated; PM50: ovariectomized and 50 mg/kg PM treated; PM100: ovariectomized and 100 mg/kg PM treated; Fluoxetine: ovariectomized and 20 mg/kg fluoxetine treated

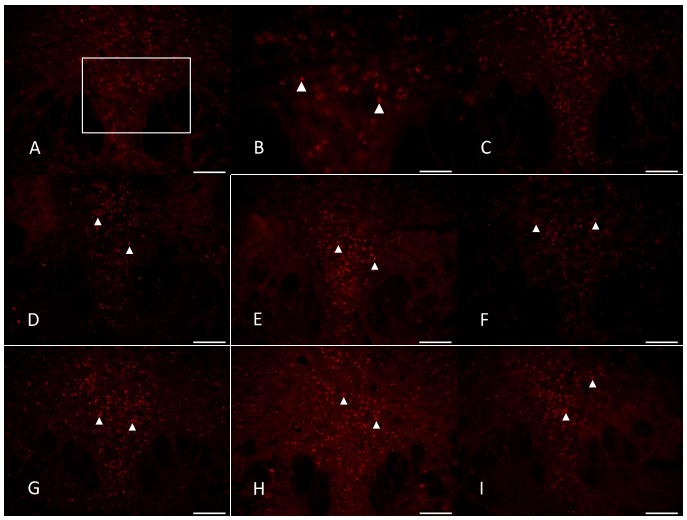

FIGURE 3. (A) Micrograph of ERα-ir (bright red) in the DRN, (B) high magnification of the area in the square frame from (A) showing ERα-ir (arrows) in the nucleus of neurons and micrograph of the ERα-ir (arrows) in the DRN of the SHAM (C), PM0 (D), E40 (E), PM25 (F), PM50 (G), PM100 (H), and fluoxetine (I); (A) scale bar = 100 μm (B) scale bar =50 μm, (C-I) scale bar = 200 μm. ERα-ir: ERα immunoreactivity; DRN: dorsal raphe nucleus; SHAM: Sham-operates; PM0: ovariectomized and distilled water treated; E40: ovariectomized and 40 μg/kg estradiol benzoate treated; PM25: ovariectomized and 25 mg/kg *Pueraria mirifica* (PM) treated; PM50: ovariectomized and 50 mg/kg PM treated; PM100: ovariectomized and 100 mg/kg PM treated; Fluoxetine: ovariectomized and 20 mg/kg fluoxetine treated

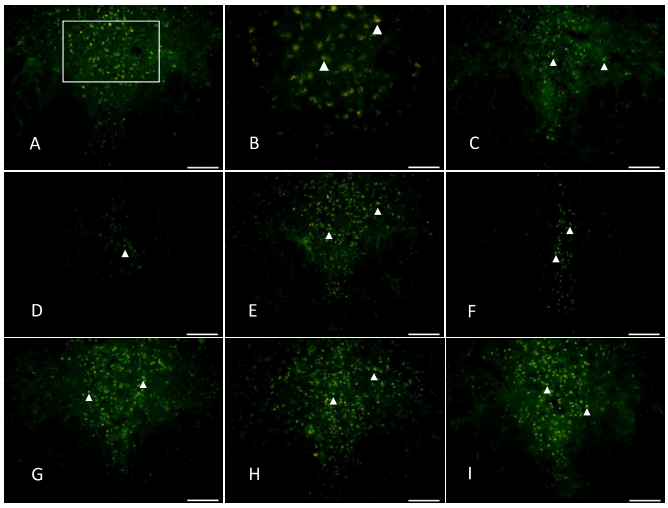

FIGURE 4. (A) Micrograph of ERβ-ir (bright yellow-green) in the DRN, (B) high magnification of the area in the square frame from (A) showing ERβ-ir (arrows) in the nucleus of neurons and micrograph of the ERβ-ir (arrows) in the DRN of the SHAM (C), PM0 (D), E40 (E), PM25 (F), PM50 (G), PM100 (H), and fluoxetine (I); (A) scale bar = 100 μm (B) scale bar =50 μm, (C-I) scale bar = 200 μm. ERβ-ir: ERβ immunoreactivity; DRN: dorsal raphe nucleus; SHAM: Sham-operates; PM0: ovariectomized and distilled water treated; E40: ovariectomized and 40 μg/kg estradiol benzoate treated; PM25: ovariectomized and 25 mg/kg *Pueraria mirifica* (PM) treated; PM50: ovariectomized and 50 mg/kg PM treated; PM100: ovariectomized and 100 mg/kg PM treated; Fluoxetine: ovariectomized and 20 mg/kg fluoxetine treated

DISCUSSION

Our data demonstrated that ovariectomy resulted in a decreased estrogen level in all groups treated with *P. mirifica* and fluoxetine. The reduction of estrogen level led to an increased body weight but decreased uterine weight in the PM0 and fluoxetine groups. The reduction of estrogen level led to an increased body weight but decreased uterine weight in the PM0 and fluoxetine groups. This was consistent with that of previous studies by Malinska et al. (2021). It was reported that loss of ovarian hormones resulted in an increases of food intake and body weight in ovariectomized rat and led to the increase in adipose tissue mass. According to the work of Marková et al. (2024), the elevated circulating leptin level was observed in the ovariectomized rats that would increase food intake and weight gain after ovariectomy. Basically, leptin was involved in regulating food intake and energy expenditure. It was found to play an important role in maintaining energy homeostasis. In addition, an increase of leptin levels was linked with obesity in association with the increase in volume of fat mass (Kozakowski et al. 2017). Overall, estrogen treatment improved uterine weight. Interestingly, treatment with PM25, PM50, and PM100 restored weight gain and uterine weight. Our results are according to previous research suggesting that *P. mirifica* has an estrogenic-like activity on reproductive organs by stimulating the proliferation of the epithelium at the vagina and uterus in female rats and women (Malaivijitnond et al. 2004; Pope et al. 1958). Treatment with all doses of PM (25, 50, and 100 mg/kg) for 90 days exhibited no abnormality of complete blood count parameters including SGOT and SGPT levels implying treatment with the PM may have no toxicity. This was associated with the work of Intranuchit et al. (2017) demonstrating that treated rats with 50 and 500 mg/kg for 90 days showed no signs of toxicity. In contrast, treatment with fluoxetine increased SGOT levels. Our results from the depression-like behavior screening tests indicated that a decreased estrogen level resulted in an increased immobility in both FST and TST implying an enhanced depression-like behavior. Whereas treatment with estrogen reduced depression-like behavior. According to the work of Khayum et al. (2020), 9 weeks after estrogen depletion by ovariectomy caused an increase in immobility time in the FST. The increased immobility time in the TST induced by ovariectomy was prevented by estrogen (Eid et al. 2020). Selective activation of ERα and ERβ were implications for depression-like phenotypes in female mice exposed to chronic unpredictable stress. Depression can disturb the hypothalamic-pituitary-adrenal axis (HPA) in humans. An enhanced level of adrenal hormones and corticotropin-releasing factor (CRF) in the blood has been identified in drug-free depressed patients (Banki et al. 1987). The glucocorticoid was involved in controlling serotonergic activity from the raphe nucleus (Meijer & de Kloet 1998). By modulating the HPA functions, estrogen replacement therapy (ERT) manifested anxiolytic and antidepressant effects in menopausal females (Kudielka et al. 1999; Patacchioli et al. 2006). Although treatment with *P. mirifica* at a low dose (25 mg/kg) did not reduce the duration of immobility in both the FST and TST, treatment with a higher dose of *P. mirifica* (50 mg/kg) tended to a declined immobility duration in both the FST and TST. Interestingly, treatment with 100 mg/kg of PM shortened immobility duration in both the FST and TST at almost the same level as observed in the estrogen treatment. Our data demonstrated that a high dose of *P. mirifica* alleviated depression-like behavior in ovariectomized animals, suggesting that the estrogenic activity of *P. mirifica* may ameliorate depression. Furthermore, our data demonstrated that treatment with fluoxetine, a well-known antidepressant, decreased the duration of both FST and TST. Fluoxetine improves depression by inhibited serotonin reuptake into presynaptic serotonin neurons via blocking the reuptake transporter protein located in the presynaptic terminal (Sohel, Shutter & Molla_2022). Ovariectomized IRC-mice treated with puerarin, a glycosyl isoflavone extracted from the root bark of *Pueraria candollei* var. *mirifica* and 17 β-estradiol, diminished ovariectomy-induced depression-like behavior as shown by reduced immobility times in the TST and FST (Tantipongpiradet et al. 2019). Puerarin is suggested to exhibit antidepressant-like effects in ovariectomized animals by decreasing ovariectomy-induced hyperactivation of the HPA axis and normalizing the downregulated transcription of the brain-derived neurotrophic factor (BDNF) and estrogen receptor (ERβ and ERα) mRNAs in the brain. The disturbances in HPA have been demonstrated to involve serotonergic dysfunction resulting in depression. In another study, estrogen facilitated serotonergic transmission by stimulating the production of TPH in the raphe nuclei (Bethea et al. 1998). Our data showed a decreased TPH-ir neuron density in the ovariectomized group (PM0) but an increased TPH-ir neuron density in all treated groups (E40; PM at 25 mg/kg, 50 mg/kg, and 100 mg/kg; and fluoxetine). Surprisingly, the increased TPH-ir neuron density was also found in the fluoxetine group. Whereas, previous studies reported sexual dysfunction as a side effect of fluoxetine, other studies demonstrated the side effect of sexual hyperactivity (Elmore & Quattlebaum 1997; Morris 1991). Our study showed that treatment with fluoxetine induced an increase of TPH-ir neuron, ERα-ir and ERβ-ir levels in DRN similar to estrogen and all doses of PM treatments while it not affected estrogen levels. Therefore, fluoxetine may have the same mechanism as

estrogen and *P. mirifica* which need further study. This correlation may explain the side effect of fluoxetine on sexual hyperactivity. Our data suggested that the increased TPH-ir neurons enhanced the serotonin synthesis level which led to a reduced immobility time of both FST and TST implying an improvement of depression-like behavior especially during treatments with estrogen, fluoxetine, and all doses of *P. mirifica*. Estrogen stimulates TPH to synthesize serotonin and reduce the serotonin reuptake transporter (SERT) mRNA expression in the raphe nuclei (Bethea et al. 1998). The beta subtype estrogen receptors and ERβ mRNA were identified in DRN (Kuiper et al. 1997; Shughrue, Lane & Merchenthaler 1997). Our data demonstrated both ERα-ir and ERβ-ir in nucleus of neurons in DRN. However, the intensity of ERβ-ir was considerably stronger than that of ERα-ir intensity. Our results correlated to the intense nuclear ERβ-ir of neurons in female mice substantia nigra and DRN but not ERα-ir (Mitra et al. 2003). Here, we suggest that ERβ functioned in DRN predominantly than ERα as a decrease of estrogen level resulted in reduced intensities of both ERα-ir and ERβ-ir. Even though all doses of *P. mirifica* and fluoxetine treatments did not increase estrogen levels as estrogen did, but treatment with estrogen, all doses of *P. mirifica* and fluoxetine increased the intensity of both ERα-ir and ERβ-ir. This correlated to the work of Tantipongpiradet et al. (2019) who reported that estrogen levels were associated with ERα and ERβ mRNA expression. We suggest that estrogen and *P. mirifica* stimulated the production of TPH via binding with ERα-ir or/and ERβ-ir but mainly ERβ. The induced TPH synthesis resulted in stimulating serotonin synthesis and therefore alleviated the depression. Based on this, the estrogenic activity of *P. mirifica* relieved depression-like behavior in ovariectomized mice by inducing TPH synthesis which led to stimulating serotonin synthesis. Furthermore, treatment at the dose of PM 100 mg/kg had greater effect than the dose of PM 25 or 50 mg/kg. *P. mirifica* contains various isoflavones such as puerarin, daidzein, daidzin (a glyco-conjugate form of *daidzein)*, genistein, and genistin (a glycoside form of genistein, genistein-7-O-beta-D-glucopyranoside, GT-glu). Puerarin, daidzein, and genistein have been reported to mitigate depression by different mechanisms in different stress conditions. Puerarin ameliorated the depression-like behaviors of rats induced with chronic unpredictable mild stress, and that mechanism may associate with antioxidant and anti-inflammatory effects (Zhang et al. 2018). Furthermore, puerarin modulated the depression-like behavior in ovariectomy-induced rats by activating the cAMP-CREB-BDNF signaling pathway (Liu et al. 2021). In addition, puerarin had been shown to relieve depression-like behaviors of chronic unpredictable mild stress in mice by remodeling their gut microbiota. There, puerarin reduced the spared nerve injury (SNI)-induced depression and pain via activating ERK, CREB, and BDNF pathways (Zhao et al. 2017). In contrast, daidzein attenuated depression-like behaviors by alleviating HPA axis hyperactivity, decreasing the levels of stress-related hormones, and partly rectifying some inflammatory cytokines imbalance in both rodent models of depression which were induced by learned helplessness and chronic mild stress (CMS) (Chen et al. 2021). Daidzein can antagonize depressive symptoms in mice with chronic stress increased content of BDNF in hippocampus, NPY protein and non-specific immune regulation (Tian et al. 2015). Whereas, genistein has been reported to alleviate major depression through down-regulating miR-221/222 by targeting Cx43 (Shen et al. 2018). Genistein functions as an antidepressant agent against chronic mild stress in depression-induced rats through the augmentation of brain-derived neurotrophic factors. (Chang et al. 2021). Furthermore, chronic genistein administration to mice resulted in antidepressant-like efficacy evidenced by lessened behavioral despair. The serotonergic system that preferentially couples with 5-HT_{1A} receptors may be crucially responsible for the anti-depressive effect of genistein (Hu et al. 2017). Therefore, we suggest that the three main components in *P. mirifica*, puerarin, daidzein, and genistein, play a crucial role to ameliorate depression. We propose that the mechanism of *P. mirifica* on alleviating depression is performed by stimulating TPH mainly via ERβ resulting in induced serotonin synthesis with no effect on plasma estrogen level. However, the mechanism of fluoxetine on increased TPH-ir neurons level was not explainable in this study and therefore requires further studies. There were some limitations of the present study. The bioavailability of P. mirifica is unknown and its molecular signaling pathways have not been characterized.

CONCLUSION

P. mirifica has an estrogenic-like activity on the female reproductive organ (uterus). Subchronic treatment with *P. mirifica* showed no abnormality of the complete blood count parameters and liver enzymes. Furthermore, subchronic treatments with 50 and 100 mg/kg of PM alleviated depression-like behavior by shorter immobility time of both FST and TST. *P. mirifica* stimulated an increase of TPH-ir neurons which may induce enhanced serotonin synthesis via ERβ with no effect on plasma estrogen levels, and therefore improved depression.

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