PHYTOTHERAPY RESEARCH
Phytother. Res. (2016)
Published online in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/ptr.5595

Neurotherapeutic Effects of *Pueraria mirifica* Extract in Early- and Late-Stage Cognitive Impaired Rats

Kanya Anukulthanakorn,[1,2] Ishwar S. Parhar,[3] Sukanya Jaroenporn,[2] Takashi Kitahashi,[3] Gen Watanbe[4] and Suchinda Malaivijitnond[2]*

[1]Biological Sciences Program, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[2]Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[3]Brain Research Institute, Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Bandar Sunway PJ46150, Malaysia
[4]Laboratory of Veterinary Physiology, Tokyo University of Agriculture and Technology, Tokyo, Japan

We determined the neurotherapeutic effects of *Pueraria mirifica* extract (PME) and pure puerarin (PU) in comparison with 17β-estradiol (E_2) in early- and late-stage cognitive impaired rats. Rats were ovariectomized (OVX), kept for 2 and 4 months to induce early- and late-stage cognitive impairment, respectively, and divided into four groups that were treated daily with (i) distilled water, (ii) 100 mg/kg of PME, (iii) 7 mg/kg of PU, and (iv) 80 μg/kg of E_2 for 4 months. The estrogen deficiency symptoms of OVX rats were abrogated by treatment with E_2 or PME, but not by treatment with PU. The mRNA level of genes associated with amyloid production (*App* and *Bace1*) and hyperphosphorylated Tau (*Tau4*) were upregulated together with the level of impaired cognition in the 2- and 4-month OVX rats. Treatment with E_2 reduced the level of cognitive impairment more than that with PME and PU, and 2-month OVX rats were more responsive than 4-month OVX rats. All treatments down-regulated the *Bace1* mRNA level in 2-month OVX rats, while PU and PME also decreased the *App* mRNA level in 2- and 4-month OVX rats, respectively. Only PU suppressed *Tau4* expression in 2-month OVX rats. Thus, PME and PU elicit neurotherapeutic effects in different pathways, and earlier treatment is optimal. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: Alzheimer's disease; amyloid plaques; estrogen receptor; menopause; neurofibrillary tangles; spatial memory.

INTRODUCTION

Estrogen is an important hormone with actions that are not limited to reproductive organs. Among the widely accepted non-reproductive functions of estrogen, it is recognized as one of the most important regulators of neuronal function, including neuronal proliferation, survival, and plasticity (Unal *et al.*, 2012), consistent with the fact that estrogen receptors are found in several brain regions (McEwen and Woolley, 1994). The importance of estrogen on cognitive actions in certain brain regions, such as the hippocampus, cortex, and striatum, is well known. Thus, the profound reduction in estrogen levels during menopause has been associated with the mnemonic decline in both normal aging and memory deterioration (Frick *et al.*, 2000).

With respect to estrogen replacement therapy (ERT) and cognitive function, estrogen treatment in postmenopausal women has provided inconsistent cognitive benefits (Yaffe *et al.*, 1998; Hogervorst *et al.*, 2000). Thus, the initiation of ERT after surgical menopause seemed to improve the memory (Henderson *et al.*, 2000), but initiation of ERT in elderly postmenopausal women (aged 65 and above) did not improve the impaired cognitive performance and may actually increase the risk of developing Alzheimer's disease (Shumaker *et al.*, 2003). One possible explanation for this discrepancy could be the presence of a critical window of time after menopause in which ERT is initiated (Whitmer *et al.*, 2011; Smith *et al.*, 2010).

In women, menopause shifts the balance of the hypothalamic-pituitary-ovarian axis feedback loop, which is attributed to the loss of negative feedback by estrogens, and results in a 3- to 4-fold increase in the concentration of serum luteinizing hormone (LH) and a 4- to 18-fold increase in the concentration of serum follicle-stimulating hormone (FSH) (Chakravarti *et al.*, 1976). As such, estrogen deprivation always happens together with increased LH and FSH concentrations. Generally, serum LH and FSH levels in rats significantly increase within 1 week after ovariectomy (Malaivijitnond *et al.*, 2004), peak at 6 or 8 weeks and remain at a plateau phase thereafter (Anukulthanakorn *et al.*, 2013). The effects of the increased circulating LH and FSH levels, because of the loss of negative feedback of estrogens, on the aging brain have been largely explored. However, Lei *et al.* (1993) indicated that only the LH receptor was found in the brain, with the highest density being found within the hippocampus. A relationship between high plasma LH levels and impaired cognitive performance was observed, and some reports have suggested that the LH peak following menopause might be a critical factor in brain damage (Casadesus *et al.*, 2006). Thus,

* Correspondence to: Suchinda Malaivijitnond, Department of Biology, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Bangkok 10330, Thailand.
E-mail: Suchinda.m@chula.ac.th

Received 01 November 2015
Revised 24 January 2016
Accepted 30 January 2016

treatment with chemicals that show both an estrogenic activity and a suppressive capacity on LH synthesis and secretion should provide a better neurotherapeutic alternative for neuronal dysfunction in postmenopausal women.

Using synthetic estrogens to treat neurodegenerative diseases has both pros and cons. While it can reduce the disease symptoms, it can also increase the risk of breast and endometrial cancer in women (Mayeauz and Johnson, 1996). Therefore, there has been some effort to try to find natural alternatives that minimize the negative impact of estrogens. Recently, research on natural-based agents that exhibit estrogenic activity with fewer side effects and that are cheaper, such as phytoestrogen-containing plants, has attracted much attention. *Pueraria mirifica* is a renowned endemic Thai plant that contains at least 17 different phytoestrogens that been isolated from its tuberous root, with puerarin (PU) as the major constituent (Cherdshewasart *et al.*, 2007a). The estrogenic activity of *P. mirifica* extracts (PME) has been tested widely in cell lines, laboratory animals, and humans (Cherdshewasart and Sriwatcharakul, 2008; Malaivijitnond *et al.*, 2006; Virojchaiwong *et al.*, 2011). Regarding its estrogenic activity, PME also exhibits neuroprotective actions, both *in vitro* in hippocampal primary cells (Chindewa *et al.*, 2008) and glutamate-insulted HT22 cells (Sucontphunt *et al.*, 2011) as well as *in vivo* in estrogen deficient mice (Monthakantirat *et al.*, 2014). Moreover, PME can also attenuate the increased serum LH levels in ovariectomized (OVX) rats (Malaivijitnond *et al.*, 2004) and aged female monkeys (Trisomboon *et al.*, 2006). Therefore, the aim of this study was to investigate if PME and PU could elicit neurotherapeutic effects in OVX rats and was evaluated in the early and late stages of cognitive impairment. The early stage is defined as the time of estrogen depletion and the LH peak with the upregulated mRNA expression level of genes associated with neurofibrillary tangles and amyloid plaques, but without signs of cognitive impairment, while the late stage is when cognitive impairment has occurred. A previous study indicated that cognitive impairment was observed at 4 months after ovariectomy (Anukulthanakorn *et al.*, 2013) and the increased expression levels of genes associated with neurofibrillary tangles (*Tau4*) and amyloid plaques (*App* and *Bace1*) were observed 1 and 4 months after ovariectomy, respectively, (Feng *et al.*, 2004; Anukulthanakorn *et al.*, 2013). Taken together with the LH peak, which occurred 2 months after ovariectomy (Anukulthanakorn *et al.*, 2013), the 2- and 4-month OVX rats were selected as representatives of the early and late stage of cognitive impairment, respectively, for this study.

MATERIALS AND METHODS

Animals. Two-month old adult female Sprague–Dawley rats were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. Animals were reared at five animals/cage in a room with controlled lighting (lights on 0600–1800 h) and temperature (25 ± 1 °C) at the Laboratory Animal Unit, Faculty of Science, Chulalongkorn University, Thailand. The animals were fed with a standard rat chow diet (Perfect Companion Group Co., Ltd., Samutprakarn, Thailand) and water *ad libitum*. The rats were reared until 4 months old and were then used following the protocols of Anukulthanakorn *et al.* (2013). The experimental protocol was approved by the Animal Ethics Committee in accordance with the guide for the care and use of laboratory animals prepared by Chulalongkorn University, Protocol Review No. 1123009.

Chemicals and PME. The tuberous roots of *P. mirifica* cultivar SARDI 190 (lot no. 0070317) were purchased from Kasetsart University, Kamphaeng Saen campus, Thailand. The PME was prepared from these tubers as described previously (Tiyasatkulkovit *et al.*, 2012), with a 5.74% yield. Five major isoflavones, genistin, genistein, daidzin, daidzein, and PU were detected in the PME by high-performance liquid chromatography analysis, with concentrations of 2.39, 2.03, 2.58, 4.17, and 18.14 mg/100 g PME, respectively (Fig. 1). The dried PME was stored at 4 °C until used.

A PME dose of 100 mg/kg/day and a synthetic PU (99% purity, COA no. Purechems 20110924, Pure Chemistry Scientific INC., TX, USA) dose of 7 mg/kg/day, were selected for this study based on previous reports of their estrogenic activity on the reproductive organs (Malaivijitnond *et al.*, 2006, 2010). A dose of 80 µg/kg/day of synthetic 17β-estradiol (E_2; 98% purity, Lot no. 060M0149V, Sigma-Aldrich Co., Ltd., MO, USA) was used in this study because it has previously been reported to prevent cognitive impairment in rats (Feng *et al.*, 2004).

Experimental design. The 4-month old rats were bilaterally OVX under sodium pentobarbital anesthesia (40 mg/kg, i.p.) and kept for 2 and 4 months (2- and 4-month OVX rats) to induce early- and late-stage cognitive impairment, respectively. The 2- and 4-month OVX rats were then each divided further into four treatment groups (10 rats in each group) for treatment with (i) distilled water (DW), (ii) PME, (iii) PU, and (iv) E_2. The OVX-DW group was fed daily with 1 mL of DW, a vehicle of PME; the OVX-PME group was fed with 100 mg/kg/day of PME; the OVX-PU group was

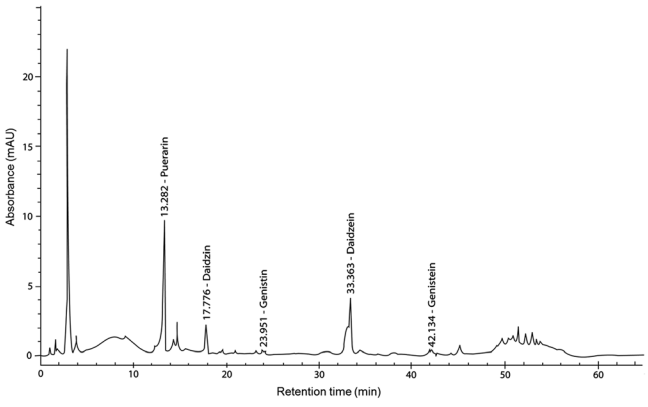

Figure 1. A chromatogram of *P. mirifica* extract determined by HPLC. The total retention times of five isoflavones, puerarin, daidzin, genistin, daidzein, and genistein, are presented.

subcutaneously injected with 7 mg/kg/day of PU, and the OVX-E_2 group was subcutaneously injected with 80 μg/kg/day of synthetic E_2, respectively, for 4 months. Sham control (SH) groups were also set up. Four-month old rats were operated on the same as the OVX rats, but their ovaries were kept intact for 2 and 4 months, as 2- and 4-month SH rats, respectively. The SH rats were fed with 1 mL of DW for 4 months. Generally, the 2- and 4-month SH rats were 10 and 12 months old at the end of the experimental periods, respectively (Fig. 2).

A spatial memory test was performed using the Morris water maze test for each group of rats for five consecutive days before the end of the experiment (Anukulthanakorn *et al.*, 2013). At the end of the experiment, the rats were euthanized and weighed for their body mass, blood sera were collected for the assay of E_2, LH and FSH levels, their uterus was dissected and weighed, and the hippocampus was collected for mRNA quantification. The mRNA levels of the *estrogen receptor* (*ER)α*, *ERβ*, *Tau4*, *App*, and *Bace1* genes were analyzed using quantitative real-time reverse transcriptase PCR (qrtRT-PCR).

Hormone assays. Serum E_2 levels were determined by a double-antibody RIA system using ^{125}I-labeled radioligands. The antiserum against E_2 (GDN 244) was provided by Dr. G. D. Niswender (Colorado State University, Fort Collins, CO, USA). Serum FSH and LH levels were determined using the National Institute of Diabetes and Digestive and Kidney disease kits for rat FSH and LH (Baltimore, MD, USA) as described previously (Jaroenporn *et al.*, 2011). The results obtained are expressed using rat FSH-RP-2 and rat LH-RP-2 as the reference standards.

Spatial memory test. The Morris water maze test was performed in a circular pool (180 cm diameter and 70 cm depth) as reported (Anukulthanakorn *et al.*, 2013). Each rat was given four trials per day for five consecutive days to find the hidden platform. The first trial was started by placing the rat in the water facing the pool wall in one of the four quadrants. The placing position was rotated clockwise to cover all four quadrants in the subsequent trials. From the second day onwards, the test was started from a different quadrant to the previous day. For each trial, the rat was allowed to swim for a maximum of 90 s to find the platform. When the rat successfully found the platform, it was allowed to have a 30 s rest on the platform. If the rat could not find the platform within the 90 s, it was guided to the platform manually and was given a 30 s rest on the platform and a score of 90 s was recorded. The latency to reach the platform and the swimming distance to find the hidden platform were measured using a video tracking system (Smart Junior, Panlab-Harvard Apparatus, Barcelona, Spain). Rats were trained for the first 4 days, and the average value of the four trials on the 5th day of each rat was calculated and counted as an individual value. The movement patterns of rats to find the platform were categorized into four strategies (line, taxis, random, and circular), based on the criteria of Feng *et al.* (2004). The frequency of each strategy accounted for by each rat was analyzed.

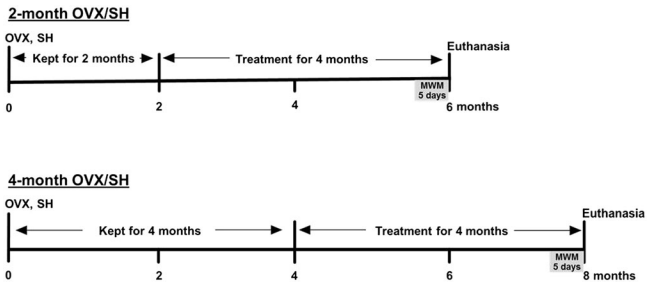

Figure 2. Schematic diagram showing the experimental design. The 4-month old rats were bilaterally ovariectomized (OVX) or sham operated (SH) and then kept for 2 or 4 months (2- and 4-month OVX rats, respectively) to induce early and late stage cognitive impairment. Rats were then divided into four groups and treated with distilled water (DW), 100 mg/kg/day of *P. mirifica* extract (PME), 7 mg/kg/day of puerarin (PU), or 80 μg/kg/day of 17β-estradiol (E_2), respectively, for 4 months. Neurocognitive testing, using the Morris water maze (MWM) to evaluate the spatial memory, was performed on five consecutive days before the end of the experiment.

Hippocampal RNA extraction and cDNA synthesis. The fresh brain of each rat was carefully removed from the rat's skull, frozen immediately on a dry-ice chip and stored at −80 °C for subsequent RNA extraction. The dissection of the brain was performed as a 40-μm thick transverse sections using a cryostat starting from the corpus callosum and continuing to the third ventricle, as defined by the rat brain atlas (Paxinos and Watson, 2007). Total RNA was extracted from the left hippocampus using 300 μL TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The quantity and purity of the RNA sample were checked by measuring the absorbance at a 260 and 280 nm wavelength. The total RNA sample (5 μg) was reverse transcribed to cDNA in a reaction mixture of 20 μL containing 2 μL of RT Buffer, 0.8 μL of dNTPMix, 2 μL of RT Random Primer, 1 μL of MultiScribe Reverse Transcriptase, and 1 μL of RNase Inhibitor using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA). The samples were incubated for 10 min at 25 °C, 2 h at 37 °C, and finally for 5 min at 85 °C.

Quantitative rtRT-PCR analysis. The expression level of each of the selected genes associated with neurofibrillary tangles (*Tau4*) and amyloid plaques (*App* and *Bace1*) were examined using qrtRT-PCR with primers following Anukulthanakorn *et al.* (2013): 5′-GATCTTAGCAACGTCCAGTCCAA-3′ (forward) and 5′-TCCCTAAGGAACCACACTTGGAG-3′ (reverse) for *Tau*4, 5′-CACACCCACATCGTGATTCCT-3′ and 5′-GTCCATCCGCTCCTGGTGTA-3′ for *App*, and 5′-TTGCCATGTGCACGATGAG-3′ and 5′-GCCGTGACAAACGGACCTT-3′ for *Bace1*, respectively. The primers for *ERα* were 5′-ACCAATGCA CCATCGATAAGAAC-3′ (forward) and 5′-TCTTTT CGTATCCCGCCTTTC-3′ (reverse) and for *ERβ* were 5′-GCGTTTGGTCATGTGAAGGA-3′ and 5′-GCCGGTTCTTGTCTATGGTACAC-3′. Amplification

of the S28 ribosomal RNA was performed as a reference housekeeping gene as reported (Anukulthanakorn *et al.*, 2013).

The qrtRT-PCR was performed using the StepOne™ Plus Real-Time PCR System (Applied Biosystems) in a 10 μL reaction mixture containing 1× POWER SYBR Green PCR Master Mix, 0.5 μM each of the forward and reverse primers and 1 μL of the cDNA sample. The reaction was performed at 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by a dissociation curve step. The relative expression levels of the target genes were then calculated by the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis. All data are presented as the mean ±1 standard error of the mean. One-way analysis of variance, with a LSD post-hoc test, was used to determine the significance of the difference between means. The significance of the difference of the movement patterns was tested by the Kruskal–Wallis and Mann–Whitney tests. The SPSS software program (version 17.0, SPSS Inc., Chicago, IL) was used for the analysis. Significance levels were set at $p < 0.05$.

RESULTS

Serum hormone levels

For the 2-month OVX-DW rats, which had been kept for 2 months after ovariectomy and fed with DW for another 4 months (total of 6 months), a low E_2 level (67.54 ± 9.54 pg/mL) and thus an estrogen-deficient stage were observed, compared with that of the 2-month SH rats (166.61 ± 48.03 pg/mL) (Fig. 3A). Similarly, in the 4-month OVX-DW rats, which had been kept for 4 months after ovariectomy and fed with DW for another 4 months (total of 8 months), the serum E_2 level was very low, but interestingly, it was not significantly different from the 4-month SH rats (65.30 ± 10.15 and 67.32 ± 13.67 pg/mL for the OVX-DW and SH groups, respectively) (Fig. 3D).

After 4 months of treatment with PME or E_2, the serum E_2 levels rose and reached the SH level ($p > 0.05$) in both the 2-month and 4-month OVX rats, but only the E_2 treatment groups had significantly higher serum E_2 levels than the OVX-DW groups ($p < 0.05$ and < 0.001 for 2- and 4-month OVX-DW rats, respectively) or even the 4-month SH rats ($p < 0.01$). It is worth noting that the serum E_2

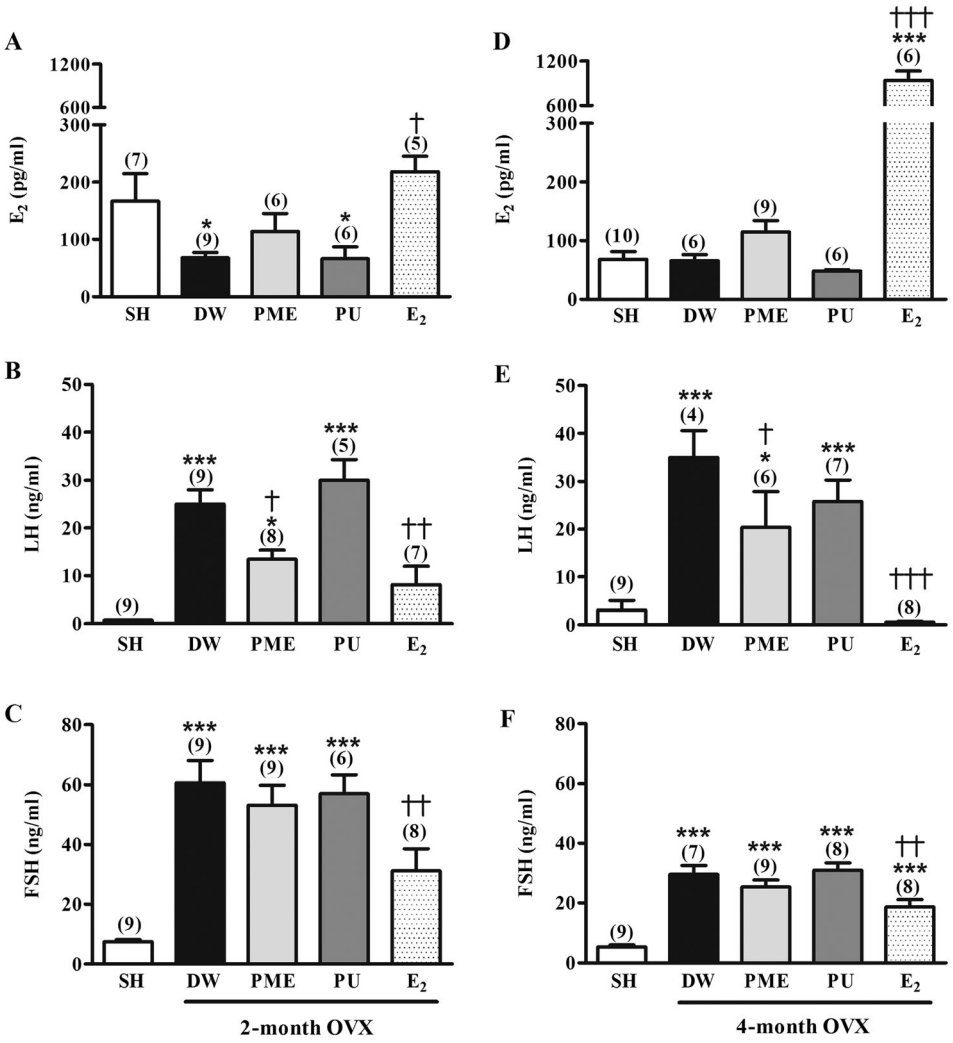

Figure 3. Serum levels of (A and D) 17β-estradiol (E_2), (B and E) luteinizing hormone (LH) and (C and F) follicle stimulating hormone (FSH) in the 2-month (left panel) and 4-month (right panel) ovary-intact rats (SH) and ovariectomized rats treated with DW, 100 mg/kg/day of *P. mirifica* extract (PME), 7 mg/kg/day of puerarin (PU), or 80 μg/kg/day of 17β-estradiol (E_2). * and *** represent $p < 0.05$ and 0.001, respectively, compared with the SH group, while †, †† and ††† represent $p < 0.05$, 0.005 and 0.001, respectively, compared with the DW group. The number in parenthesis indicates the number of rats used to determine that hormone in each group.

level in the 4-month OVX-E_2 rats was significantly higher than that in the 2-month OVX-E_2 rats, giving the highest E_2 level (935.10 ± 131.06 pg/mL; $p < 0.001$) among the five treatment groups.

After ovariectomy, serum LH levels in both the 2-month (24.97 ± 3.01 ng/mL) and 4-month (35.01 ± 5.55 ng/mL) OVX-DW rats were significantly higher than in the 2-month (0.72 ± 0.06 ng/mL) and 4-month (3.08 ± 2.04 ng/mL) SH rats, respectively ($p < 0.001$; Fig. 3B and E). Treatment with PME significantly decreased the serum LH level ($p < 0.05$) in both OVX-PME groups, while E_2 treatment significantly decreased the serum LH level compared with that in the OVX-DW group ($p < 0.005$ and $p < 0.001$ for 2- and 4-month OVX-E_2 rats, respectively) such that they were similar to the levels in the SH group ($p > 0.05$).

Similar to the serum LH levels, the serum FSH levels in both the 2-month (60.62 ± 7.40 ng/mL) and 4-month (29.58 ± 3.01 ng/mL) OVX-DW rats were significantly higher than in the 2-month (7.40 ± 0.61 ng/mL) and 4-month (5.30 ± 0.60 ng/mL) SH rats, respectively, (Fig. 3C and F). Treatment with PME had no effect on the serum FSH levels in either OVX-PME group, whereas E_2 highly and significantly decreased the serum FSH levels in both OVX-E_2 groups compared with that in the OVX-DW group ($p < 0.005$), but reaching the same level as in the SH group only in the 2-month OVX-E_2 rats ($p > 0.05$).

In contrast, treatment with PU had no effect on the serum E_2, LH or FSH levels ($p > 0.05$). Basically, the patterns of hormonal changes after treatment with PME, PU, and E_2 were not different between the 2-month and 4-month OVX rats, but the magnitude of the changes was greater in the 4-month rats.

Uterine weight

To corroborate the estrogen deficient stage in the OVX rats and the estrogenic activity of the PME, PU and E_2, the uteri were collected and weighed at six and eight months after ovariectomy in the 2- and 4-month OVX-DW rats. While serum E_2 levels were low, the relative uterus to body weights were significantly lower than in the SH rats ($p < 0.001$) (Table 1). Treatment with PME restored the uterus weight in OVX rats, but reached the level of the SH rates only for the PME treated 4-month OVX rats. In contrast, PU treatment had no effect on the relative uterus weights in either the 2- or 4-month OVX rats, while E_2 treatment significantly increased the relative uterus weight compared with both OVX-DW groups ($p < 0.005$) and, especially, in the 4-month OVX-E_2 rats, where it was significantly higher than that of the 4-month SH rats ($p < 0.005$). Generally, the uteri of rats were more sensitive to PME and E_2 after they had been kept for a longer estrogen deficient period.

Table 1. Relative uterus weights of the ovary-intact rats (SH) and ovariectomized rats treated with distilled water (DW), 100 mg/kg/day of *P. mirifica* extract (PME), 7 mg/kg/day of puerarin (PU), or 80 μg/kg/day of 17β-estradiol (E_2) in the 2- and 4-month OVX rats

Treatment group	Relative uterus weight (× 10^{-2})	
	2-month OVX	4-month OVX
SH	16.27 ± 1.29	16.05 ± 1.13
DW	4.47 ± 0.52**	5.70 ± 0.49**
PME	12.43 ± 0.66*††	19.37 ± 2.28††
PU	5.94 ± 0.55**	6.65 ± 0.67**
E_2	14.25 ± 0.63††	24.73 ± 1.28**††

* and ** represent $p < 0.05$ and 0.001, respectively, compared with the SH group, while † and †† represent $p < 0.05$ and 0.005, respectively, compared with the OVX group.

Cognitive performance

While ovariectomy of the rats led to their serum E_2 depletion and serum LH peak, it also induced cognitive impairment, as indicated by the increased numerical latency in the searching time for the hidden platform and the distance traveled on the 5th day of the Morris water maze test (Fig. 4A, B, D and E). However, these increases were only of significance in the 4-month OVX-DW group ($p < 0.05$) (Fig. 4D and E). Thus, the latency and distance traveled by 4-month OVX rats were higher than that of the 2-month OVX rats. The PME and E_2 treatment of both the 2- and 4-month OVX groups significantly decreased the latency and distance traveled ($p < 0.05$), while PU treatment showed a significant decrease in the latency ($p < 0.05$) and a marginal decrease in the distance traveled ($p = 0.055$) in only the 2-month OVX-PU group (Fig. 4A and B).

Although the circular and random strategies of movement were higher in both the 2- and 4-month OVX-DW rats compared with the SH rats ($p < 0.05$), the 4-month OVX-DW rats showed higher values than the 2-month OVX-DW rats (53% for 4-month and 39% for 2-month OVX-DW rats) (Fig. 4C). This indicated that eight months of estrogen deficiency in the 4-month OVX-DW rats induced a greater cognitive impairment. The percentages of circular and random strategies were both reduced in the PME (OVX-PME), PU (OVX-PU) and E_2 (OVX-E_2) treatment groups, but the efficacy of treatment in the 2-month OVX rats was higher than that in the 4-month OVX rats (12%, 18%, and 4% for 2-month and 25%, 28%, and 8% for 4-month OVX rats, respectively) ($p < 0.05$) (Fig. 4C). In addition, E_2 treatment could ameliorate the cognitive impairment better than PME or PU treatment.

Expression of ERα and *ERβ*

In the 2- and 4-month OVX-DW rats, the level of mRNA expression of the *ERα* and *ERβ* in the hippocampus tended to increase compared with that in the SH rats, although only the expression level of *ERα* in the 4-month OVX-DW rats reached a significantly higher level ($p < 0.05$; Fig. 5). The PME and E_2 treatments showed comparably similar effects on the mRNA expression level of *ERα* and *ERβ* in the 2-month OVX-DW rats, with a significant decrease in the *ERα* mRNA levels ($p < 0.05$) and no significant change in the *ERβ* mRNA levels ($p > 0.05$) compared with that in the OVX-DW groups. In contrast to the PME and E_2 treatments, treatment with PU significantly decreased the *ERβ* mRNA level ($p < 0.05$), but not the *ERα* mRNA level, of the 2-month OVX rats. For the 4-month OVX rats, a significant decrease was only detected in the PME treatment group ($p > 0.05$), although it was not

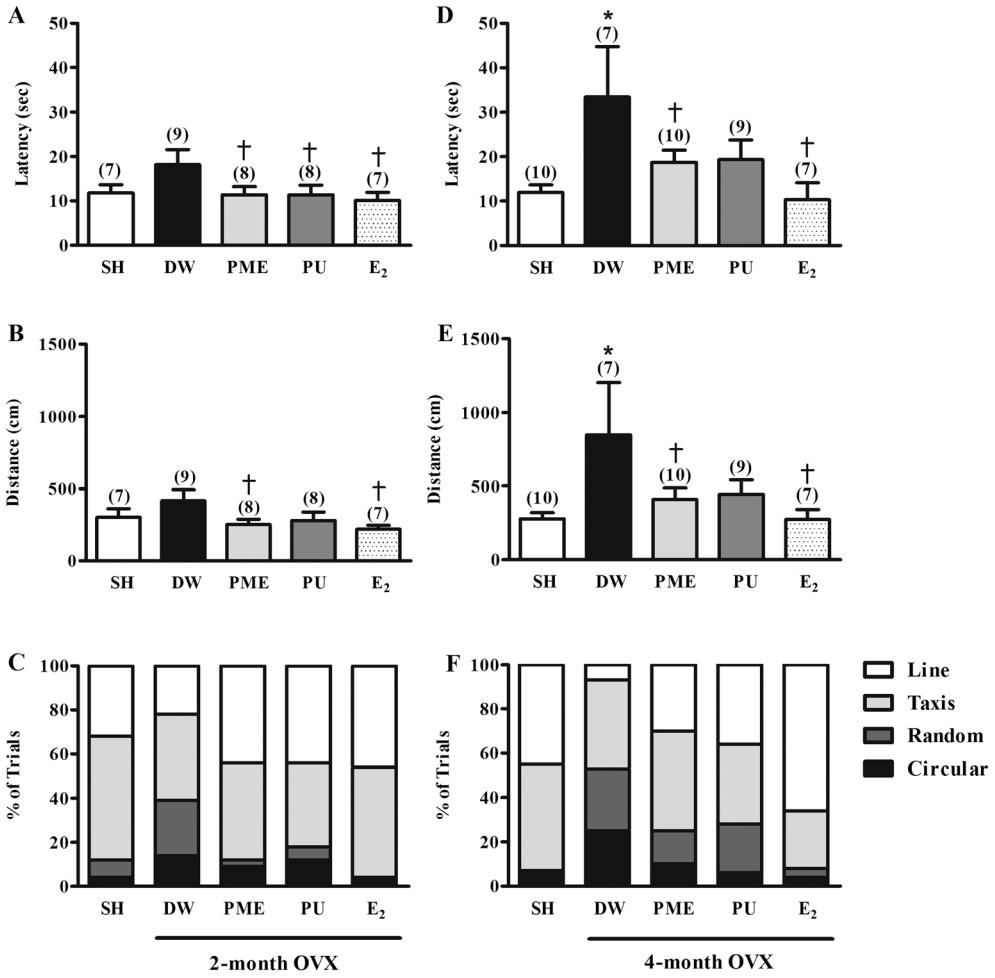

Figure 4. The (A and D) latency period, (B and E) distance and (C and F) strategy of movement patterns of searching for the hidden platform at the 5th day of the Morris water maze test in the 2-month (left panel) and 4-month (right panel) ovary-intact rats (SH) and ovariectomized rats treated with DW, 100 mg/kg/day of *P. mirifica* extract (PME), 7 mg/kg/day of puerarin (PU), or 80 μg/kg/day of 17β-estradiol (E_2). * and † represent $p < 0.05$ compared with the SH and DW groups, respectively. The number in parentheses indicates the number of rats used in each group.

significantly different from that in the OVX-DW groups. However, the *ERβ* mRNA levels of the OVX-PME and OVX-E_2 groups, and the *ERα* mRNA level of the OVX-PME group, were apparently comparable to those of the SH groups.

Expression level of the neurofibrillar-tangle-associated *Tau4* gene

Although the relative mRNA level of the *Tau4* gene in both the 2- and 4-month OVX-DW rats increased, there was no significant difference compared with that in the SH rats ($p = 0.411$ and 0.269 for 2- and 4-month OVX-DW rats, respectively) (Fig. 6). Treatment with either PME or E_2, either in 2- or 4-month OVX rats, had no effect on the *Tau4* expression level compared with that in the OVX-DW and SH groups. Only the PU-treated 2-month OVX rats showed a significantly decreased *Tau4* mRNA level ($p < 0.05$).

Expression level of the amyloid-plaque-associated genes (*App* and *Bace1*)

After ovariectomy, the relative mRNA levels of *App* and *Bace1* tended to numerically increase in the 2- and 4-month OVX-DW rats, but only the *Bace1* expression level was significantly upregulated in the 2-month OVX-DW rats ($p < 0.05$) (Fig. 7B). Treatment with PME, PU or E_2 all resulted in different patterns of changes in the expression of the two selected genes associated with amyloid plaques. Congruent with the decreased *Tau4* mRNA level (refer in the preceding texts), only the PU treatment significantly decreased the mRNA level of *App* in the 2-month OVX-DW rats. A decreased *App* mRNA level was also detected in the 4-month OVX-PME rats (Fig. 7A and C). However, the expression of *Bace1* compared with that in the OVX-DW group was only significantly decreased ($p < 0.05$) by estrogen (OVX-E_2) and the phytoestrogens (OVX-PME and OVX-PU) in the 2-month OVX rats (Fig. 6B). Generally, the reduction in the *App* and *Bace1* expression level after estrogen and phytoestrogen treatment was more evident in the 2-month OVX rats than in the 4-month OVX rats, especially for the *Bace1* gene.

DISCUSSION

Significantly, low relative uterus weights and serum E_2 levels, together with the high serum LH and FSH levels

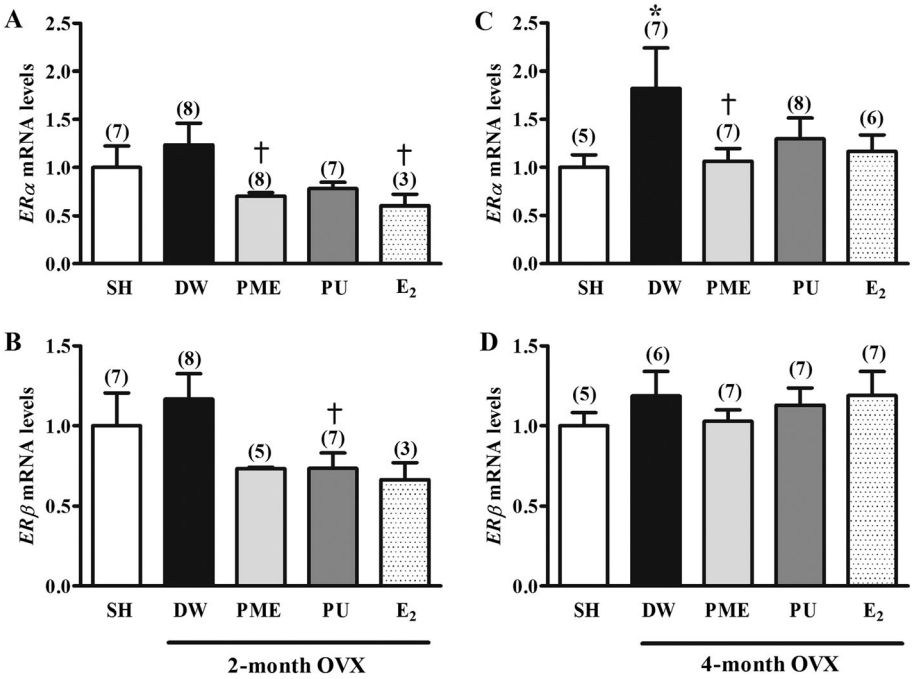

Figure 5. Relative mRNA levels of the (A and C) *ERα* and (B and D) *ERβ* genes in the 2-month (left panel) and 4-month (right panel) ovary-intact rats (SH) and ovariectomized rats treated with DW, 100 mg/kg/day of *P. mirifica* extract (PME), 7 mg/kg/day of puerarin (PU), or 80 μg/kg/day of 17β-estradiol (E_2). * and † represent $p < 0.05$ compared with the SH and DW group, respectively. The number in parenthesis indicates the number of rats used in each group.

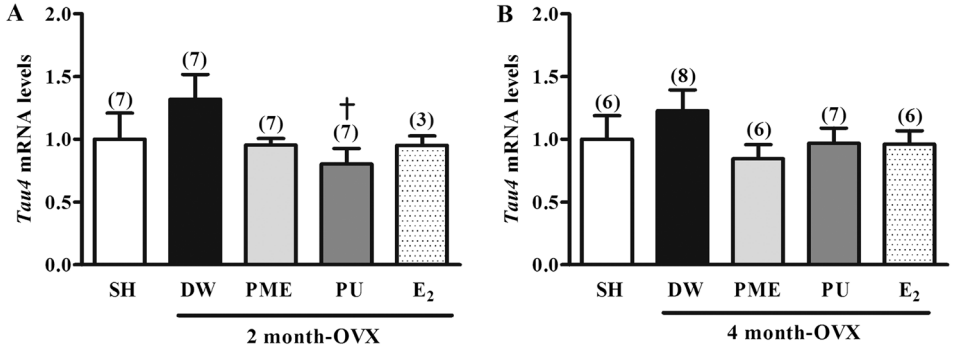

Figure 6. Relative mRNA levels of the genes associated with neurofibrillary tangles (*Tau4*) in the 2-month (left panel) and 4-month (right panel) ovary-intact rats (SH) and ovariectomized rats treated with DW, 100 mg/kg/day of *P. mirifica* extract (PME), 7 mg/kg/day of puerarin (PU), or 80 μg/kg/day of 17β-estradiol (E_2). † represents $p < 0.05$ compared with the DW group. The number in parenthesis indicates the number of rats used in each group.

in the OVX rats, confirmed their complete ovariectomy and development of an estrogen deficient stage. Unexpectedly, the serum E_2 level in the 4-month SH rats was very low and comparable to that of the 4-month OVX-DW rats. However, because low serum LH and FSH levels and a high uterus weight were detected in the SH rats, it is possible that the SH rats were euthanized during the diestrus stage when the serum E_2 level was low. Treatment with PME at a dose of 100 mg/kg/day elicited an estrogenic potency on the reproductive organs (stimulating the uterus proliferation) and anterior pituitary gland (attenuating the increased serum LH levels), as reported previously (Malaivijitnond *et al.*, 2004). Puerarin, the major and species-specific phytoestrogen of the *Pueraria* genus, has previously been reported to have a very weak estrogenic activity on the reproductive organs (Malaivijitnond *et al.*, 2010). In the present study, however, subcutaneous injections of 7.0 mg/kg/day of PU for 120 days (4 months) had no significant effect on the uterus weight or the serum LH and FSH levels in both the 2- and 4-month OVX-PU rats. The subcutaneous injection of 7.0 mg/kg/day of PU in the OVX rats was previously reported to stimulate vaginal proliferation, but without any effect on the uterus, when the duration of treatment was longer than 140 days (Malaivijitnond *et al.*, 2010). However, the estrogenic activity of a higher administered dose of PU, such as 3000 mg/kg/day, for 3 months should also be confirmed (Rachoń *et al.*, 2007). Regardless, although PU is the major phytoestrogen constituent of *P. mirifica* tubers, it appears to not be the main player in the estrogenic activity of PME. It has been reported that *P. mirifica* contains at least 17 different phytoestrogens and that miroestrol has the highest estrogenic activity (Malaivijitnond, 2012).

Post-menopausal women are in an estrogen-deprived state and are at risk of neurodegenerative disease with a decline in cognitive function (Farrag *et al.*, 2002;

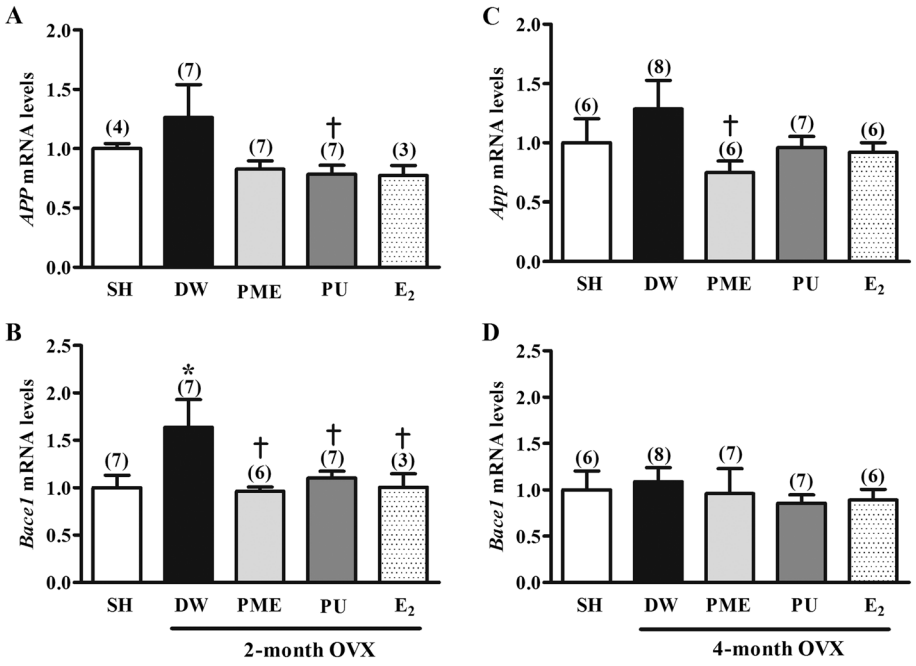

Figure 7. Relative mRNA levels of the genes associated with amyloid plaques, *App* (A and C) and *Bace1* (B and D), in the 2-month (left panel) and 4-month (right panel) ovary-intact rats (SH) and ovariectomized rats treated with DW, 100 mg/kg/day of *P. mirifica* extract (PME), 7 mg/kg/day of puerarin (PU), or 80 μg/kg/day of 17β-estradiol (E_2). * and † represent $p < 0.05$ compared with the SH and DW groups, respectively. The number in parenthesis indicates the number of rats used in each group.

Sherwin, 2006). Unfortunately, several studies have shown that ERT was generally unsuccessful in ameliorating the decline in cognitive function (Brenner *et al.*, 1994; Henderson *et al.*, 2000), and no verification of how this phenomenon happens has been reported. The other hormonal changes that are associated with the dysregulation of the hypothalamic-pituitary-ovary axis following menopause have been implicated in the pathogenesis of neurodegeneration (Bowen *et al.*, 2004). The 'gonadotropin LH peak' has recently attracted attention (Gregory and Bowen, 2005), because LH is known to cross the blood–brain barrier (Lukacs *et al.*, 1995), and LH receptors are expressed in the hippocampus (Lei *et al.*, 1993). Thus, treatment with PME, which elicits estrogenic activity and can also suppress serum LH levels, as mentioned in the preceding texts, in women who are in the early- or late-climacteric stages should better alleviate the cascade of neurodegeneration.

As expected, the 2- and 4-month OVX rats, used in this study as a representative animal model of the early and late stages of cognitive impairment, respectively, showed different degrees of cognitive impairment, as determined by the Morris water maze test, being greater in the 4-month OVX rats. As such, treatment with PME, PU, or E_2 could better improve the impaired cognition in the 2-month OVX rats than in the 4-month OVX rats. Different degrees in the magnitude of the improved cognitive impairment after treatment with PME, PU, or E_2 were also detected, where E_2 was more effective than PME and PU, respectively. Treatment with E_2, PME, or PU decreased the combined circular and random strategies of rat movement to find the hidden platform by 4%, 12%, and 18%, respectively, for the 2-month OVX rats and by 8%, 25%, and 28%, respectively, for the 4-month OVX rats. Thus, as noted earlier, the estrogenic activity of PU on the uterus weight or serum LH and FSH levels was not detected, and so the improvement in the impaired cognition in rats is likely to occur via other pathways, such as anti-oxidant (Xie *et al.*, 2014). The observed improvement in cognitive ability after E_2 or PU treatment, in terms of the higher latency and distance to find the platform, agreed with that previously reported in OVX mice (Xu *et al.*, 2004) and Aβ-induced cognitive impaired rats (Li *et al.*, 2010).

The common pathology of neurodegenerative diseases is characterized by the deposition of the Aβ peptide and neurofibrillary tangles (Maccioni *et al.*, 2001), where the elevated level of Aβ in the brain is believed to play a critical role in cognitive dysfunction (Li *et al.*, 2010). As known, Aβ is generated from the amyloid precursor protein (APP) by β-secretase (Bace1) and γ-secretase activity (De Strooper *et al.*, 2010). Estrogen deficiency is well known to induce neurodegenerative disease by increasing Aβ deposition (Yue *et al.*, 2005). The level of Aβ plaques was reported to increase during the 3 months after ovariectomy in 18-month old triple-transgenic mice (Palm *et al.*, 2014), while Bace1 activity was elevated together with brain estrogen depletion in the transgenic mice model of Alzheimer's disease (Yue *et al.*, 2005). Disregulation of Bace1 expression at both the transcription and translation levels was recently conceived as a major cause of the pathogenesis of Alzheimer's disease (Lei *et al.*, 2015; Mei *et al.*, 2015). In accord, the treatment of mixed neuronal/glial cell cultures with E_2 was observed to downregulate the Bace1 protein expression level (Nord *et al.*, 2010). In accord, the upregulated *Bace1* mRNA expression in the 2-month OVX-DW rats observed in the present study was decreased by E_2 treatment and to a comparable degree by PME and PU treatment. Additionally, PU treatment at a dose of 7 mg/kg/day decreased the relative mRNA expression of *App*. It was previously shown that PU treatment ameliorated the Aβ (1–42)-induced cognitive impairment and reduced the elevated level of apoptosis in the hippocampus (Li *et al.*, 2010). Taken together, it can be postulated that PME and PU are

potential candidates to prevent the estrogen deficiency-induced neurodegeneration and protect the early stage of memory loss in OVX rats by inhibiting the production of Aβ in the hippocampus brain area.

In addition, the late-stage cognitive impairment animal model (4-month OVX-DW rats) showed a tendency to have higher *App* and *Bace1* mRNA expression levels compared with the SH rats, which indicated a possible increased Aβ production. Interestingly, no alterations in the expression level of those two genes were observed after treatment with PME, PU, or E_2 compared with the OVX-DW rats, and only PME treatment in the 4-month OVX rats significantly decreased the *App* mRNA expression level. The lack of response to E_2 treatment in the OVX rats might be explained by the shortage of brain estrogen, which alters the sensitivity of response to E_2 treatment (Li *et al.*, 2013). Additionally, this indicates that PME could be a better neurotherapeutic agent for recovering the late-stage cognitive impairment via an Aβ pathway compared with synthetic estrogens.

Hyperphosphorylation of the microtubule-associated Tau leads to its aggregation into neurofibrillary tangles and related neurodegeneration diseases (Shi *et al.*, 2011). An imbalanced regulation of the relevant protein kinases and phosphatases in the affected neurons has been proposed to account for the abnormal Tau hyperphosphorylation (Pant *et al.*, 1999). In a previous study, *Tau4* mRNA expression was found to significantly increase in rats from 1 month after ovariectomy (Anukulthanakorn *et al.*, 2013). However, only a numerical tendency of increased *Tau4* mRNA expression, compared with the SH rats, was observed in the 2- and 4-month OVX-DW groups in this study. Moreover, only the PU treatment decreased the *Tau4* mRNA level in the 2-month OVX rats, with no significant increase in *Tau4* mRNA levels after E_2 or PME treatment in both 2- and 4-month rat groups, or after PU treatment in the 4-month OVX rats. In addition to a weak estrogenic activity (Rachoń *et al.*, 2007; Malaivijitnond *et al.*, 2010; this study), PU has also been reported to elicit a strong anti-oxidant capacity on neuronal cells (Mahdy *et al.*, 2014). The reduced level of cognitive impairment in APP/presenilin-1 mice induced by PU was likely because of the significant decrease in the levels of lipid peroxidation without any changes in Aβ deposition (Zhou *et al.*, 2014). Taken together, these results suggest that PU ablates neurodegenerative disease primarily by a reduction in the production of neurofibrillary tangles and may possibly not involve the amyloid pathway. Interestingly, only PME treatment could decrease the *App* mRNA level in the 4-month OVX rats or in the late stage of neurotherapeutic action, while E_2 treatment did not change the expression level of any of the evaluated genes associated with Aβ and the production of neurofibrillary tangles, although the cognitive performance of the OVX rats was improved. Thus, the mechanism of neurotherapeutic action of E_2 should be determined further.

As seen in the preceding texts for the different responses in terms of the mRNA expression level of genes associated with hyperphosphorylated Tau and Aβ production, and the reduction in the level of cognitive impairment after E_2, PME, or PU treatment, it is of interest to understand the mechanism of action of these three chemicals in the brain. Estrogens and phytoestrogens are known to act after binding with one of two subtypes of ERs (α and β), where *ERα* and *ERβ* are both differentially expressed throughout the hippocampus (Bliss and Collingridge, 1993). In the hippocampus of rats, it was reported that *ERα* expression increased during low estrogen levels together with a low memory performance (Foster, 2012), which is in accord with the results of the OVX rats in this study. Although the mRNA expression level of *ER*α and *ER*β in the 2-month OVX-DW rats was numerically, but not significantly, upregulated (this study), treatment with E_2 or phytoestrogens (PME or PU) significantly downregulated the expression level of *ER*α and *ER*β mRNA. Still, the effects of PME or E_2 were mainly on the *ER*α expression level, while PU mainly affected *ER*β expression. In contrast, only PME treatment decreased the *ERα* mRNA level in the 4-month OVX-PME rats. Previous studies have indicated that the ERα mediates the effects of estrogenic chemicals on neuroprotection during the process of Alzheimer's disease (Hu *et al.*, 2003), while the ERβ enhances the cognitive performance of female mice in the object recognition and placement tasks (Walf *et al.*, 2008). This might explain why only the PME-treated rats showed a reduced *App* expression level together with the recovery of cognitive performance in the 4-month OVX rats. However, whether changes in the mRNA and protein levels of *ER*α and *ER*β are linked to the Aβ and neurofibrillary tangle production and cognitive impairment needs to be investigated further.

In conclusion, treatment with E_2, PME, or PU could elicit a neurotherapeutic effect in the OVX rats either at the early or at the late stage of cognitive impairment. The mechanism of action could be mediated via the ERs and be associated partly with the suppression of expression of genes associated with amyloid plaques and Tau hyperphosphorylation. Comparing the neurotherapeutic effects of PME and PU with E_2, different pathways of mechanism of action between PME and PU are proposed. Thus, PME acts mainly through the *ERα* and elicits estrogenic activity on hippocampal neurons, while PU, because of its weak estrogenic activity, may act primarily via the anti-oxidative pathway. In addition, PME is comparable to E_2, which mainly acts on the amyloidogenic pathway, while PU affects the production of neurofibrillary tangles. The results of this study also suggest the importance of the timing of neurotherapy after menopause or estrogen deficiency, where the earlier the treatment, the better the level of restoration of cognitive impairment. It has recently been reported that PME treatment decreased the incidence of breast tumor development and growth (Cherdshewasart *et al.*, 2007b). Thus, using this natural-based agent for the treatment of neurodegenerative diseases should be safe. Although the results of this study corroborate the high potential of developing *P. mirifica* and PU as a therapeutic agent for neurodegenerative diseases in humans, confirmation of the alteration of these genes at the protein level and histopathological levels in the brain are still required, and this will be our future study.

Acknowledgments

This study was supported by a Grant for International Research Integration: Chula Research Scholar, Ratchadaphiseksomphot

Endowment Fund (to S. Malaivijitnond) and the 90th Anniversary of Chulalongkorn University Fund, Thailand (to K. Anukulthanakorn). The authors thank Dr. Robert Butcher and the Office of Research Affairs, Chulalongkorn University for having improved the English in this manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the reported research.

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