

Original Research

The Effects of *Paeonia lactiflora* Pall. and *Astragalus Membranaceus* Single Extracts on Bone Metabolic Profile in Ovariectomized Mice

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Abstract

Background: Metabolic bone disease, associated with estrogen deficiency, is common condition in postmenopausal women. *Paeonia lactiflora* Pall. (PL) and *Astragalus membranaceus* (AM) have been known to have estrogenic activity and to improve postmenopausal osteoporosis symptoms when used as a mixture with other herbs. However, there have been no comparative studies on the effects of PL and AM on the bone metabolic profile. Thus, the present study aimed to investigate the effects of the single extracts of PL and AM on bone and metabolic profile, and further to compare the effects of the two herbs. **Method:** A total of 70 mice were randomly divided into seven groups (n = 10): six groups were bilaterally ovariectomized (OVX) and one group served as a sham-operated control (Sham). Two OVX groups received PL at 23.5 (OVX-PL-L) and 47 (OVX-PL-H) mg/kg bw/day. Another two OVX groups received AM at 38.5 (OVX-AM-L) and 77 (OVX-AM-H) mg/kg bw/day. The remaining two groups served as positive and negative controls and received estradiol valerate (OVX-E₂) and distilled water (OVX-vehicle). Vehicle (distilled water), PL, AM, and E₂ were administered to mice by oral gavage once a day for 6 weeks at a volume of 10 mL/kg of body weight. The body and uterine weights, serum lipid profiles, osteocalcin levels, and biomarkers of bone formation were measured. The expressions of the genes associated with the estrogen receptor (ER)-related downstream pathway were examined in the Michigan Cancer Foundation-7 (MCF-7) cells. **Results:** Treatment with PL and AM reversed OVX-induced body weight gain and changes to serum triglyceride, estradiol and osteocalcin levels at both doses. Evaluation of bone formation revealed that low-dose (23.5 mg) PL resulted in improvements in bone mineral density (BMD) and bone volume fraction (BV/TV), whereas AM improved all bone formation related parameters, BMD, BV/TV, trabecular number (Tb.N), and trabecular thickness (Tb.Th) at a high dose. There was no significant difference in the effects of PL and AM on changes in body weight and BMD, but high-dose AM was more effective than PL in restoring changes in osteocalcin levels and formation of the trabecular bone structure. Both PL and AM increased extracellular signal-regulated kinase and protein kinase B in MCF-7 cells. **Conclusions:** These results suggest that both low-dose PL and high-dose AM can be effective herbal alternatives to inhibit body weight gain and improve bone metabolism. This effect was more pronounced with AM than with PL.

Keywords: menopause; symptoms; *Paeonia lactiflora* Pall. (PL); *Astragalus membranaceus* (AM)

1. Introduction

Menopause refers to the cessation of menstruation and the decrease in estrogen production due to a decline in ovarian function in older women. This is a natural transition in life that is experienced by all women. Menopause is accompanied by a variety of symptoms that occur due to estrogen deficiency, including hot flashes, anxiety, difficult in sleeping, and vaginal dryness. The prevalence of menopause is on the rise due to the rapid increase in the life expectancy of women. According to some reports, the number of postmenopausal women worldwide is estimated to increase to

1.2 billion by 2030, and the proportion of postmenopausal women in the total female population is expected to increase to 59.8% by 2060 [1,2]. As a result, various healthcare systems are focusing on improving the quality of life (QoL) of postmenopausal women by addressing the physiological changes associated with obesity and lowered bone mineral density (BMD) [3].

Estrogen has many biological functions, and estrogen deficiency is known to have adverse effects on several physiological functions. Estrogen deficiency results in an increase in visceral fat, and imbalance in lipid metabolism, and rapid bone loss [4]. Hormone replacement therapy



(HRT) has been widely used as an the effective treatment of menopausal-related symptoms in postmenopausal women [5]. However, the long-term use of HRT is contraindicated in women with breast cancer, coronary heart disease, and stroke [4–7]. Phytoestrogens are estrogen-like compounds derived from plants. Their structure and/or functions are similar to mammalian estradiol (17- β -estradiol) and they are known to improve the health of postmenopausal women without the side effects associated with HRT [8]. Recently, several plants containing non-hormonal components with estrogen-like effects have emerged as therapeutic alternatives for the management of menopause-associated symptoms and the prevention of long-term health consequences post-menopause.

Paeonia lactiflora Pall. (PL) and *Astragalus membranaceus* (AM) are representative oriental herbs, which have been traditionally used in the treatment of female reproductive disorders including menstrual pain, infertility, and dysmenorrhea [9,10]. The root of PL, also referred to as Bai Shao in Chinese, is one of the most frequently prescribed herbs for the management of menopausal hot flushes and has shown estrogen-like activity in *in vitro* studies in Michigan Cancer Foundation-7 (MCF-7) cells [10,11]. In addition, PL is a component of the *TiaoGeng* decoction (TG-decoction), which is a Chinese herb mixture that has been used in the treatment of menopausal symptoms with significant benefits in China for more than 30 years [12]. Paeoniflorin, extracted from *Paeonia lactiflora* Pall., has been reported to affect bone remodeling by improving bone formation, thereby reducing osteoporosis in ovariectomized mice [13]. AM is known for its estrogenic activity and has been reported to improve postmenopausal osteoporosis symptoms when used as a mixture with *Rubus coreanus* Miquel (*R. coreanus* Miq) in ovariectomized (OVX) mice [14]. In a clinical trial in postmenopausal women, the ingestion of a mixture of AM and *R. coreanus* Miq significantly improved menopausal symptoms [15]. In addition, AM has been widely used in mitigating menopausal symptoms as a component of DangguiBuxue Tang (DBT), a traditional Chinese herbal, decoction also containing *Angelicae Sinensis Radix* [16].

Most *in vitro* and *in vivo* studies have used a mixture of extracts rather than a single extract based on the assumption that mixed extracts will have synergistic estrogen-like effects in treating menopausal symptoms compared to single extracts [10–12,14–16]. However, there are no comparative studies on the ameliorative effects of single extracts of PL and AM on menopause-associated estrogen decline, obesity, and bone metabolism. Therefore, in this study, we first investigated the estrogen-like activity of single extracts of PL and AM on estrogen receptor (ER)-positive MCF-7 human breast cancer cells. Subsequently, a comparative study of the effects of PL and AM single extracts was performed in improving the lipid profile, body weight loss, and bone metabolic profile in OVX mice.

2. Materials and Methods

This was a controlled experiment study using OVX mice.

2.1 Preparation of PL and AM Extracts

The PL and AM extracts were provided by Daehan Cell Pharm INC. (<http://www.dhcp.kr>). The PL and AM roots were purchased from Omni herb Co. Ltd. (Daegu, Korea). The plants were cultivated in field in the Gyeongsangbuk-do Province, Korea. The plants were identified by two botanical experts. The PL and AM roots were washed, dried naturally, and pulverized. Subsequently, the ethanol extracts of PL and AM were prepared using eight times the quantity of 30% (v/v) ethanol at 90 °C for 6 hours, and then subsequently vacuum dried at 60 °C and 50° Brix. The extracts were concentrated and dried under reduced pressure at 65 °C to obtain the PL extract (yield: 17.9%) and AM extract (yield: 38.5%). These steps were performed personnel from Daehan Bio Pharm Co., Ltd.

2.2 Cell Culture and Measurement of Estradiol Production

Estrogen receptor (ER)-positive MCF-7 human breast cancer cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) on September 8, 2021. We have contracted a Material Transfer Agreement (MTA) with the ATCC. We also obtained a certificate from Mycoplasma Testing Services (Hoechst DNA stain method, Agar culture method, and PCR-based assay) indicating the absence of mycoplasma contamination. No cell line infections were observed within 3 to 5 weeks of purchase. The MCF-7 cell lines purchased from ATCC were tested for short tandem repeat (STR) profiles and validated for human cross-species determination. The MCF-7 cell line is a human cell line, and as a result of analyzing the alleles present in each STR locus within the cell line, the specification and the STR result of the purchased MCF-7 cell line were identical.

This cell line was maintained and cultured in Dulbecco's modified eagle medium (DMEM; WelGENE, Daegu, Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS; WelGENE, Daegu, Korea), and 1% penicillin-streptomycin (P/S; Gibco, Brooklyn, NY, USA) at 37 °C in a humidified atmosphere with 5% CO₂. For quantification of estradiol production, the MCF-7 cells were treated with the single extracts of PL and AM at concentrations of 50, 100, and 200 µg/mL and cultured for 24 hours at 37 °C in a 5% CO₂ atmosphere. The medium was removed and centrifuged to obtain the supernatant. The estradiol levels in the supernatants were quantified using the Estradiol kit (Cusabio Technology Llc., Wuhan, China) according to the manufacturer's protocol.

2.3 Western Blot Assay

To investigate the downstream signaling mechanisms of the estrogenic activity of PL and AM, the protein acti-

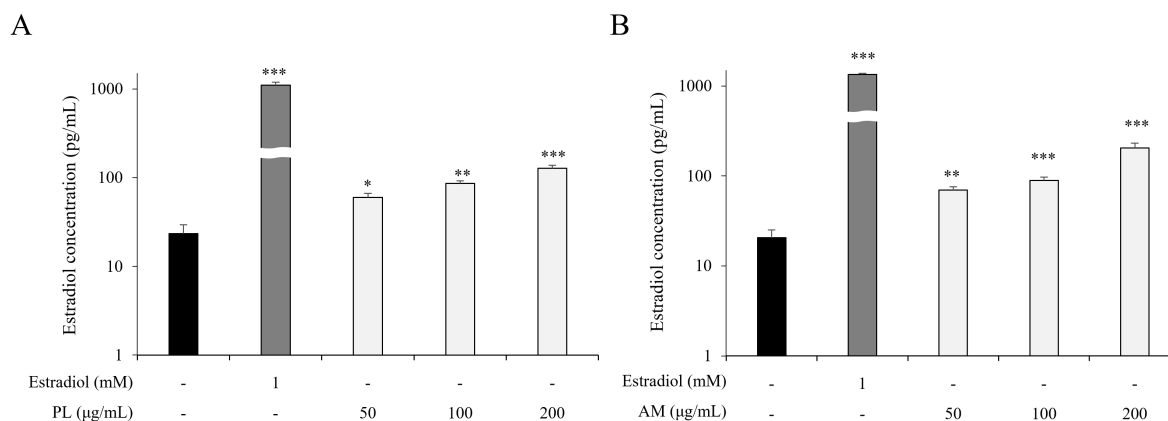


Fig. 1. Effects of PL and AM on estrogenic activity. MCF-7 cells were treated with different concentrations of PL (A) and AM (B) for 24 hours, respectively. The E₂ level was determined by an ELISA assay. Data are shown as mean $\pm$ SD. All experiments were performed in duplicate, with three replicates for each treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (vs. 0 μ g/mL). PL, *Paeonia lactiflora* Pall.; AM, *Astragalus membranaceus*; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation.

vation of extracellular signal-regulated kinase (ERK) and protein kinase B (Akt) was examined in the MCF-7 cell by western blot. The MCF-7 cells were cultured on 100 mm dishes until 70% confluency was reached. Then, the cells were replaced with serum and phenol red-free medium, and treated with PL or AM for 24 hours. Protein was extracted from the cultured cells using the CETi lysis buffer (#TLP-121CETi, TransLab, Daejeon, Korea) with a phosphatase inhibitor cocktail (#539131, Merck Millipore, MA, USA) according to the manufacturer's protocol. The protein content of the cell lysate was determined with a Bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Cell lysates were separated via 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Burlington, MA, USA). The membranes were blocked with a Phospho-BLOCK® solution (TransLab) for one hour at room temperature and washed using $1 \times$ Tris-buffered saline + Tween 20 (TBST) buffer (TransLab). After blocking, the membranes were incubated with the primary antibodies diluted in blocking reagent overnight at 4 °C. The membranes were washed using $1 \times$ TBST buffer and then incubated with suitable secondary antibodies for one hour at room temperature. The reaction was visualized using ProNATM ECL Ottimo Western Blot Detection Kit (TransLab) and detected using the Ez-CaptureII software version IV (ATTO, Daejeon, Korea). All antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA) and used at the indicated dilution for western blot analysis as follows: p-Akt1 (sc-293125, 1:100), Akt1 (sc-5298, 1:500), p-ERK1/2 (sc-136521, 1:100), ERK1/2 (sc-514302, 1:100), and β -Actin (sc-47778, 1:1000).

2.4 Animals

Inbred C57BL/6 mice aged 8 weeks were used in all the animal experiments. The mice were purchased from Koatech Inc. (Pyeongtaek, Korea) and bred under 12-hour light/dark cycle with free access to water and food in a specific pathogen-free (SPF) animal facility at a temperature of 21 ± 2 °C and relative humidity of $55\% \pm 10\%$. All animal experiments were conducted based on the Guide for the Care and Use of Laboratory Animals, funded by the National Institutes of Health, and approved by the Pusan National University Hospital Institutional Animal Care and Use Committee. This study was approved by the Institutional Review Board of Pusan National University Hospital (PNUH-2022-209).

2.5 Experimental Design and Treatment of Mice with PL and AM

A total of 70 mice were randomly divided into seven groups ($n = 10$). Six groups were performed bilaterally OVX using the dorsal approach under an intraperitoneal administration of Zoletil® (0.5 mg/kg; Virbac Laboratories, Carroscedex, France). Briefly, after the ovarian fat pad was lifted through the dorsal incision, the first ovary was visualized, and the ovary was exteriorized before distal uterine ligation and carefully removed. Then, the uterus was returned to the abdomen. The second ovary was removed in the same manner. One group served as a sham-operated control (Sham) and underwent the same procedure as the OVX mice but without resection of the ovaries. After two-week recovery period, two OVX groups received PL at two different doses (23.5 and 47 mg/kg bw/day, OVX-PL-L and OVX-PL-H, respectively). Another two OVX groups received AM at two different doses (38.5 and 77 mg/kg bw/day, OVX-AM-L and OVX-AM-H, respectively). The remaining two groups served as positive and negative con-

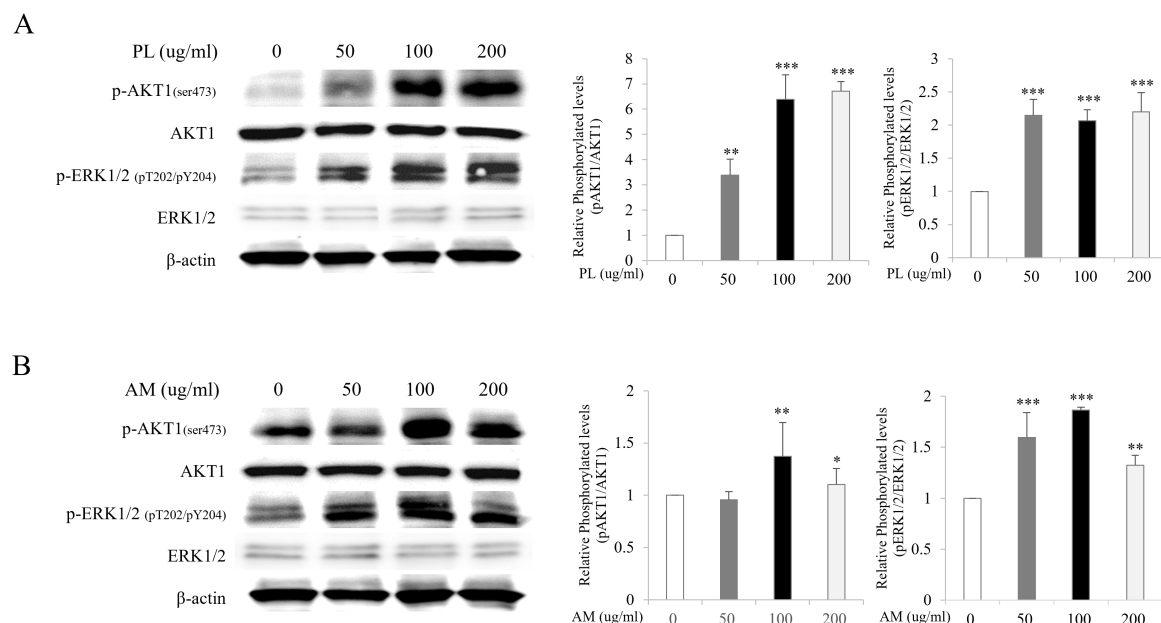


Fig. 2. Effects of PL and AM on the activation of ERK and Akt in the MCF-7 cells. MCF-7 cells were treated with PL (A) and AM (B) at concentrations of 50, 100, and 200 $\mu\text{g/mL}$, respectively, for 24 hours. The whole cell lysates were immunoblotted with anti-Akt1 (Ser473)/ERK1/2 (pT202/pY204) or Akt1/ERK1/2 antibodies. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (vs. 0 $\mu\text{g/mL}$). ERK, extracellular signal-regulated kinase; Akt, protein kinase B; MCF-7, Michigan Cancer Foundation-7.

trols and received estradiol valerate (OVX- E_2) and distilled water (OVX-vehicle), respectively. Vehicle (distilled water), PL, AM, and E_2 were administered to mice by oral gavage once a day for 6 weeks at a volume of 10 mL/kg of body weight. The mice were monitored daily for clinical signs, including behavioral abnormalities, general appearance, and mortality until they were sacrificed.

2.6 Measurement Body and Organ Weights and Blood Collection

Individual body weights were recorded on the first and last days of the experiment. Body weights were collected prior to the withdrawal of food for overnight fasting. Mice were sacrificed by CO_2 euthanasia, the day after the last treatment. Blood was collected using a cardiac puncture. Blood samples were allowed to clot at room temperature and the serum was separated by centrifuging at $1000 \times g$ for 15 min. The serum samples were stored at -80°C until use for further assays. Organs such as the liver, heart, spleen, kidney, and uterus were removed and weighed after the adhering fats were trimmed away. Total visceral fat mass was measured after separating perirenal, mesenteric, and periuterine adipose tissues using an electronic analytical balance.

2.7 Assessment of Serum Lipid Profile, Osteocalcin, and 17- β -Estradiol Levels

The serum concentrations of total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol

(HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured using an auto microplate chemiluminescence analyzer (Hitachi Chemical Diagnostics Systems Co., Ltd. Tokyo, Japan). The serum concentrations of osteocalcin and 17- β -estradiol ($E_2\beta$) were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (BioVision Inc., San Francisco, CA, USA and CUSABIO Technology LLC, Houston, TX, USA, respectively) according to the manufacturer's protocol.

2.8 Microcomputed Tomography (micro-CT) Analysis

The left femur from the mice of each group was fixed in 4% paraformaldehyde (PFA) for 24 hours, washed with phosphate-buffered saline (PBS) and then scanned using micro-computed tomography (CT) (Squantum FX, PerkinElmer, Hopkinton, MA, USA) with a source voltage of 90 kV and a source current 180 μA . For the 3-dimensional histomorphometric analysis of the trabecular and cortical bone, cross-sectional images of the distal femur were used. The region of interest (ROI) of the distal femur was 5% of the femoral length starting from 0.05 mm below the growth plate and was used to determine the trabecular and cortical bone mineral density (BMD), bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp).

2.9 Data Analysis

Results were presented as mean $\pm$ SD (standard deviation). The statistical analysis involved a normal distribution

of data. Data were analyzed using the Student *t*-tests in Microsoft Excel. A *p*-value < 0.05 was considered indicative of a significant difference.

3. Results

3.1 Effect of PL and AM on 17- β -Estradiol Production

To evaluate the estrogen-like activity of the single extracts of PL and AM, the estrogen-producing ability of PL and AM was measured in the MCF-7 cells. PL and AM increased estrogen production at all concentrations compared with the control group (Fig. 1A,B). This result implied that both PL and AM single extracts increase estrogen production in MCF-7 cells.

3.2 Estrogen-Like Effect of PL and AM on the ER-Dependent Downstream Signaling Pathway in MCF-7 Cells

To elucidate the estrogen-like activity of PL and AM via the ER-dependent downstream signaling pathway, the protein expressions of ERK and Akt were examined using the western blot on MCF-7 cells. The expression of phosphorylated ERK increased at all concentrations of PL and AM compared with the control. The expression of phosphorylated Akt increased at concentrations of 50, 100, and 200 μ g/mL of PL and at concentrations of 100 and 200 μ g/mL of AM compared with the control group (Fig. 2A,B). These results suggest that all the single extracts of PL and AM had estrogen-like activities and their effects are mediated through the activation of the ER-dependent signaling pathway.

3.3 Comparison of the Effects of PL and AM on Body Weight and Visceral Fat Mass in OVX Mice

The changes in the mean body weight, visceral fat mass, and organ weights at the experimental end point are presented in Tables 1,2. To elucidate the effects of the single extracts of PL and AM in OVX-induced body weight gain, visceral fat was collected from each mouse at necropsy and weighed. The body weight and visceral fat mass were significantly increased in the OVX-vehicle group compared to the Sham group. However, the oral administration of PL, AM and estrogen to OVX mice for 6 weeks significantly inhibited body weight and visceral fat mass gain (Table 1). The uterine weight significantly decreased in the OVX-vehicle group compared to the Sham group. However, the treatment of the OVX mice with PL and AM for 6 weeks did not affect uterine weight, unlike the estrogen treatment (Table 2). The weights of the other organs such as the liver, heart, spleen, and kidney did not change after ovariectomy and the oral administration of PL, AM and estrogen (Table 2). These results demonstrated the effects of the PL and AM single extracts in lowering body weight and visceral fat mass gain in OVX mice, and also revealed that, unlike estrogen, the extracts had no effect on uterine weight.

Table 1. Effects of PL and AM extract on the body weight gain in OVX mice.

Groups	Body weight (g)	Visceral Fat mass (g)
Sham	20.57 $\pm$ 0.36	0.34 $\pm$ 0.11
OVX-Vehicle	26.88 $\pm$ 1.41*	1.11 $\pm$ 0.25*
OVX-E ₂	22.15 $\pm$ 0.64**	0.70 $\pm$ 0.23**
OVX-PL-L	23.00 $\pm$ 1.34**	0.79 $\pm$ 0.26**
OVX-PL-H	24.60 $\pm$ 0.83**	0.94 $\pm$ 0.18
OVX-AM-L	23.32 $\pm$ 1.99**	0.93 $\pm$ 0.17
OVX-AM-H	22.14 $\pm$ 1.00	0.81 $\pm$ 0.19**

p* < 0.001 vs. Sham; *p* < 0.001 vs. OVX-Vehicle. OVX, ovariectomized; OVX-E₂, OVX group received estradiol valerate; OVX-PL-L, OVX group received PL at 23.5 mg/kg bw/day; OVX-PL-H, OVX group received PL at 47 mg/kg bw/day; OVX-AM-L, OVX group received AM at 38.5 mg/kg bw/day; OVX-AM-H, OVX group received AM at 77 mg/kg bw/day.

3.4 Comparison of the Effects of PL and AM on the Lipid Profile of the OVX Mice

A significant increase in TC, TG, and HDL-C levels was found in the OVX-vehicle group compared with the Sham group. After oral administration of PL and AM to the OVX for 6 weeks, there was no change in the TC levels (Fig. 3A), but the TG levels were significantly reduced by estrogen (OVX-E₂), a high dose of PL (OVX-PL-H), and a high dose of AM (OVX-AM-H) (Fig. 3B). The HDL-C levels were significantly reduced only by estrogen but were not affected by PL or AM (Fig. 3C). The LDL-C levels did not significantly change after ovariectomy, and even after treatment with estrogen, PL, or AM when compared with the Sham group (Fig. 3D). These results showed that PL and AM lowered TH levels at high doses. However, they did not affect other lipid parameters.

3.5 Comparison of the Effects of PL and AM on Estradiol Production in OVX Mice

Serum estradiol levels were significantly decreased in the OVX-vehicle group (4.8 $\pm$ 1.1 pg/mL) compared with the Sham group (15.5 $\pm$ 2.2 pg/mL). After 6 weeks of administration of estrogen, PL and AM, the serum estradiol levels were significantly increased (OVX-E₂: 13.0 $\pm$ 1.9, OVX-PL-L: 8.2 $\pm$ 1.0, OVX-PL-H: 7.4 $\pm$ 1.0, OVX-AM-L: 7.5 $\pm$ 0.3, OVX-AM-H: 8.0 $\pm$ 1.0 pg/mL) (Fig. 4). No significant difference in estradiol production were found between the PL and AM groups.

3.6 Comparison of the Effects of PL and AM on Bone Formation in OVX Mice

To investigate the effect of each extract of PL and AM on bone formation, serum osteocalcin levels, bone mineral densities (BMD) and trabecular structures of the right femur were measured. The OVX-vehicle group showed significant elevations of osteocalcin (26.1 $\pm$ 5.4 ng/mL) compared

Table 2. Effects of PL and AM single extracts on the weight of the liver, heart, spleen, kidney, and uterus in OVX mice.

Groups	Organ weight (g)				
	Liver	Heart	Spleen	Kidney	Uterus
Sham	1.38 ± 0.04	0.12 ± 0.01	0.09 ± 0.01	0.18 ± 0.02	0.11 ± 0.02
OVX-Vehicle	1.44 ± 0.06	0.11 ± 0.01	0.09 ± 0.01	0.19 ± 0.01	0.05 ± 0.05*
OVX-E ₂	1.42 ± 0.13	0.12 ± 0.01	0.12 ± 0.04	0.19 ± 0.01	0.12 ± 0.02**
OVX-PL-L	1.36 ± 0.08	0.11 ± 0.01	0.11 ± 0.03	0.18 ± 0.01	0.06 ± 0.03
OVX-PL-H	1.43 ± 0.09	0.11 ± 0.01	0.10 ± 0.01	0.17 ± 0.02	0.06 ± 0.02
OVX-AM-L	1.49 ± 0.17	0.12 ± 0.01	0.10 ± 0.02	0.18 ± 0.02	0.07 ± 0.03
OVX-AM-H	1.36 ± 0.09	0.12 ± 0.01	0.09 ± 0.01	0.18 ± 0.01	0.07 ± 0.04

* $p < 0.001$ vs. Sham; ** $p < 0.001$ vs. OVX-Vehicle. OVX-Vehicle.

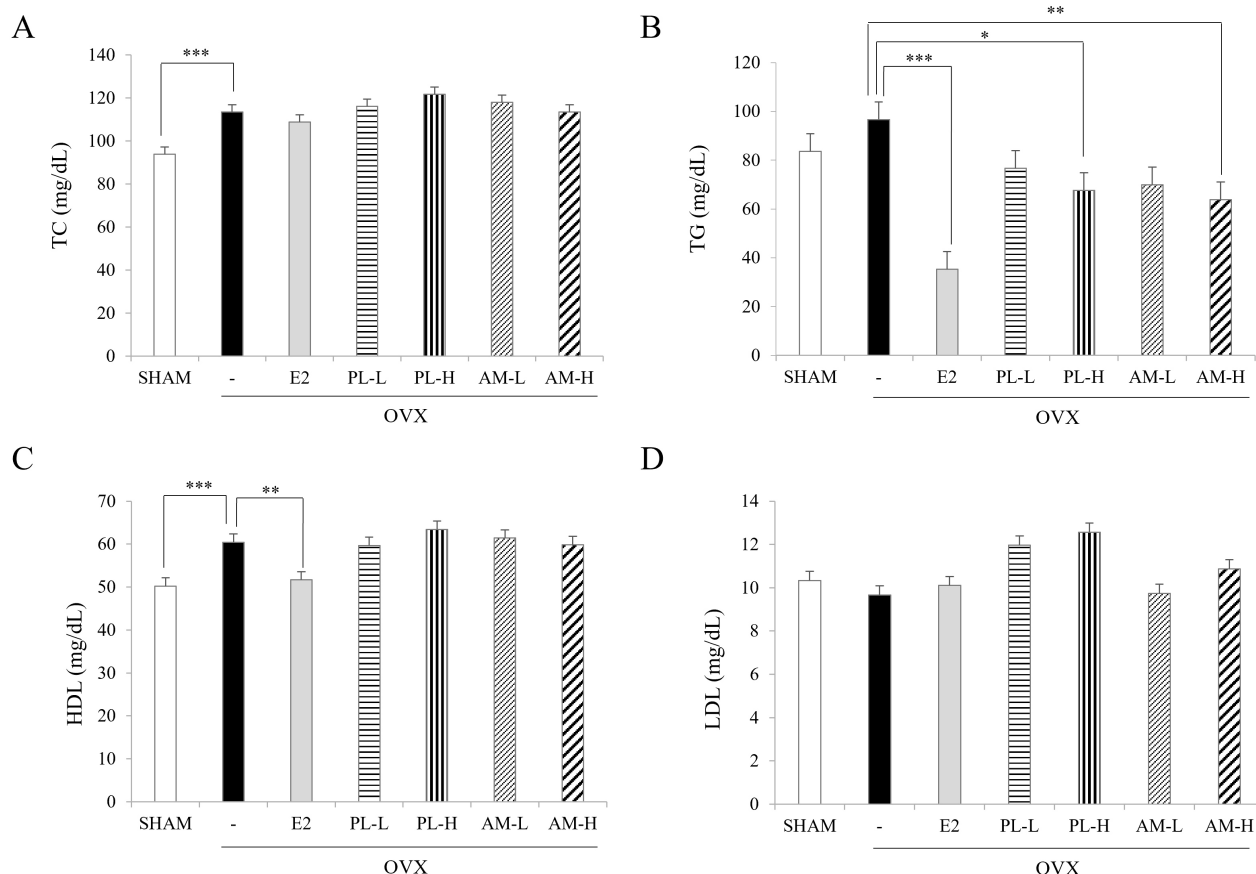


Fig. 3. Effects of PL and AM on serum lipids in OVX mice. (A) Total cholesterol. (B) Triacylglycerols. (C) HDL-cholesterol. (D) LDL-cholesterol. All the single extracts were administered by oral gavage. Data are shown as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (vs. OVX-Vehicle). TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; PL-L, OVX group received PL at 23.5 mg/kg bw/day; PL-H, OVX group received PL at 47 mg/kg bw/day; AM-L, OVX group received AM at 38.5 mg/kg bw/day; AM-H, OVX group received AM at 77 mg/kg bw/day.

with the Sham group (6.0 ± 1.0 ng/mL). The treatments of the ovariectomized mice with estrogen, PL, and AM for 6 weeks significantly decreased osteocalcin to 10.3 ± 2.6 (OVX-E₂), 12.1 ± 1.8 (OVX-PL-L), 10.7 ± 1.5 (OVX-PL-H), 15.4 ± 2.6 (OVX-AM-L), and 7.6 ± 2.0 ng/mL (OVX-AM-H). The OVX-AM-H group showed a more effective recovery from the OVX-induced osteocalcin increase compared to the OVX-PL-L and the OVX-E₂ groups (Fig. 5).

BMD also significantly decreased in the OVX-vehicle group compared with the Sham group, but was significantly elevated in the OVX-E₂, the OVX-PL-L, and the OVX-AM-H groups (Fig. 6). There were no significant differences between the OVX-AM-L and OVX-PL-H groups.

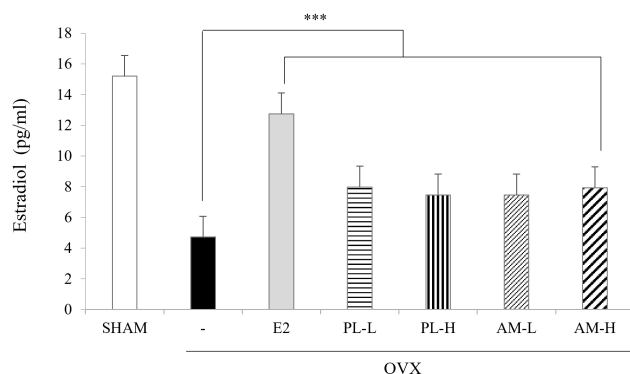


Fig. 4. Effects of PL and AM on the levels of serum estradiol in OVX mice. Data are shown as mean $\pm$ SD. *** $p < 0.001$ (vs. OVX-vehicle).

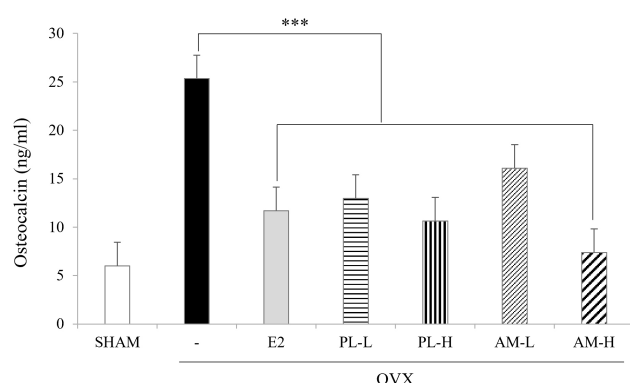


Fig. 5. Effects of PL and AM on the levels of serum osteocalcin in OVX mice. Data are shown as mean $\pm$ SD. *** $p < 0.001$ (vs. OVX-vehicle).

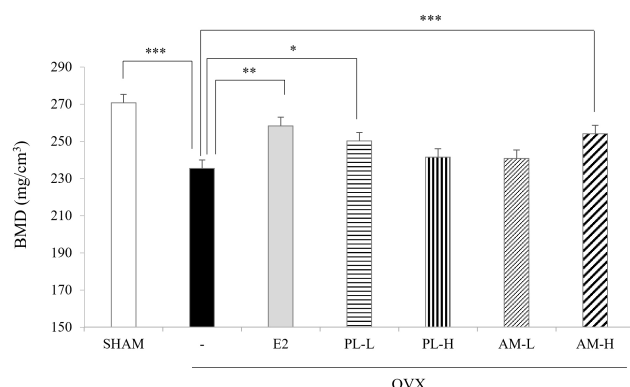


Fig. 6. Effects of PL and AM on bone mineral density (BMD) in OVX mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 7 showed the micro-CT image of the trabecular bone structure. Spongy (cancellous) bones were observed to be dense in the Sham group, but a decrease in density was observed in the OVX-vehicle group. The OVX-induced loss in spongy bone showed a significant recovery with the oral administration of estrogen, PL-L, and AM-H for 6 weeks. In addition, the OVX-vehicle group also showed

a significant decrease in BV/TV, Tb.N, and Tb.Th compared to the Sham group (Table 3). However, when the OVX mice were treated with PL and AM for 6 weeks, the OVX-PL-L group showed a significant increase in BV/TV, and the OVX-AM-H group showed significant increases in BV/TV, Tb.N, and Tb.Th. Conversely, trabecular separation (Tb.Sp) significantly increased in the OVX-vehicle group compared with the Sham group, but showed a significant decrease with all doses of PL and AM. These results suggested that PL and AM can effectively prevent OVX-induced bone loss, but AM is more effective than PL in the formation of trabecular bone structures.

4. Discussion

Several studies have shown that PL and AM have estrogenic activity and improve symptoms associated with postmenopause when used as a mixture with other herbs [10–16]. However, the present study revealed that single extracts of PL and AM had anti-menopausal effects by improving estradiol production and preventing bone loss in OVX mice, and that the effect was more pronounced with AM than with PL. To the best of our knowledge, this is the first study comparing the effects of single extracts of PL and AM with respect to their beneficial effects on bone loss due to estrogen deficiency.

Earlier *in vitro* studies have shown that MCF-7 cells are the best-characterized ER-positive cell line for investigating estrogen-like activity [17]. Our study also first examined the estrogenic potency of single extracts of PL and AM in MCF-7 cells, and then confirmed the estrogenic effect in OVX mice *in vivo*. The OVX mice model has been widely used as a representative model to study the anti-menopausal effects of various agents as the changes specific to menopause in human such as weight gain, increased serum osteocalcin levels, increased bone turnover, and decreased bone formation can be understood and extrapolated to menopause in human subjects [15–20]. In this respect, the present study also used OVX mice as a menopausal animal model.

The present study also showed that the OVX mice had a significant increase in body weight and visceral fat mass. The OVX mice treated with PL and AM showed significant recovery from the changes in body weight and visceral fat mass, which occurred due to ovariectomy. This effect was more remarkable in low-dose PL (OVX-PL-L group) and high-dose AM (OVX-AM-H group), and there was no significant difference observed between the PL and AM groups.

Various studies have reported the measurement of visceral fat mass. However, there has been no consensus regarding which fat deposit among retroperitoneal, perigonadal, perirenal and mesenteric adipose tissue is most appropriate for representing visceral fat mass [21,22]. Some studies have measured visceral fat mass by measuring only the perirenal fat pad [21]. For this reason, our present study

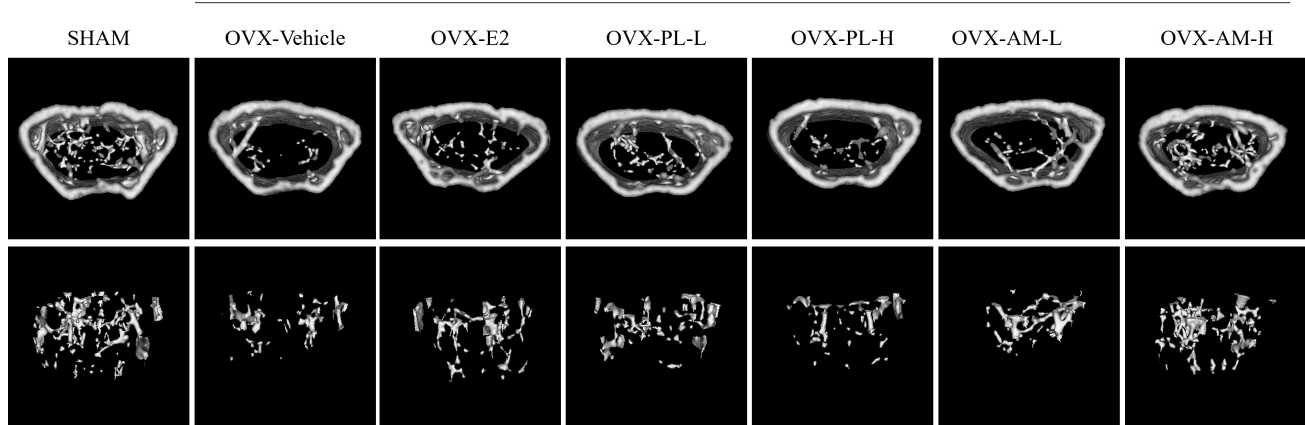


Fig. 7. Effects of PL and AM on bone formation in OVX mice. Micro-computed tomography (CT) images of trabecular bone. Upper: cortical bone + trabecular bone; Lower: trabecular bone.

Table 3. Effects of the PL and AM single extracts on bone volume/tissue volume (BV/TV) trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) in OVX mice.

Groups	Parameters			
	BV/TV (%)	Tb.N (1/mm)	Tb.Th (mm)	Tb.Sp (mm)
Sham	3.8 ± 0.3	0.8 ± 0.1	0.081 ± 0.005	0.23 ± 0.02
OVX-Vehicle	2.0 ± 0.4*	0.3 ± 0.1*	0.069 ± 0.003*	0.55 ± 0.12*
OVX-E ₂	2.7 ± 0.4**	0.5 ± 0.1**	0.074 ± 0.005***	0.33 ± 0.04**
OVX-PL-L	2.6 ± 0.2**	0.4 ± 0.1	0.071 ± 0.007	0.44 ± 0.07***
OVX-PL-H	2.1 ± 0.2	0.3 ± 0.1	0.072 ± 0.010	0.43 ± 0.05 [#]
OVX-AM-L	2.3 ± 0.3	0.4 ± 0.1	0.068 ± 0.004	0.39 ± 0.06***
OVX-AM-H	2.7 ± 0.4**	0.5 ± 0.1***	0.076 ± 0.005**	0.33 ± 0.05***

Data are expressed as means ± SD. [#] $p < 0.001$ (vs. Sham), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (vs. OVX-vehicle).

measured visceral fat mass as the accumulation of intra-abdominal fat, including perirenal, mesenteric, and periuterine adipose tissue.

Ovariectomy causes a decrease in uterine epithelial height, myometrial thickness, and uterine stroma expansion. As a result, The OVX mice showed significant atrophy of the uteri and a decrease in uterine weight [23]. The 17- β -estradiol treatment effectively prevents OVX-induced uterine weight reduction [24]. The present study also showed that estrogen significantly increased the uterine weight of the OVX mice. However, both PL and AM treatments did not increase uterine weight. This result is similar to that of Zierau *et al.* [25], who also reported that DBT treatment (AM + *Angelicae Sinensis Radix*) did not alter the weight of the uterus and liver in OVX rats. These results suggest that, unlike estrogen, both the PL and AM single extracts treatments did not result in an adequate recovery of uterine weight.

Another characteristic of menopause is the alterations in lipid metabolism. Estrogen deficiency affects lipid metabolism, resulting in increased abdominal obesity and an imbalance between the HDL and LDL cholesterol levels [26,27]. Indeed, some studies have reported that estro-

gen treatment in postmenopausal women increases serum TG levels [19], and the administration of estrogen in menopausal women with osteoporosis reduces serum LDL-C, but increases serum HDL-C levels [28]. In several studies, treatment with herbal extracts resulted in improvements in serum lipid levels in postmenopausal women [29–31]. Some studies reported that serum TG levels were negatively associated with serum estradiol levels in postmenopausal women [19,28]. In other studies, the gradual weight gain observed during the menopausal transition was strongly associated in an increase in the TC and LDL-C levels, with no change in the HDL-C levels [19,32]. Overall, the associations between menopause and serum lipid profiles have been highly inconsistent, probably due to differences in the menopausal status or aging [33]. The present study showed that estrogen treatment of the OVX mice reduced TG and HDL-C levels, but did not change TC and LDL-C levels. Both PL and AM effectively decreased the TG levels alone only at high doses (PL-H and AM-H), but did not affect the TC, HDL-C, and LDL-C levels. These results imply that the association between menopause and serum lipid profiles is very complex and further investigation is needed to understand the effects of PL and AM on the serum lipid profiles.

The development of osteoporosis due to increased bone turnover and decreased BMD is also another important consequence of estrogen deficiency in menopause [14]. Estrogen plays a critical role in skeletal homeostasis and the regulation of bone turnover [34], and estrogen deficiency is associated with bone loss in menopausal women [17]. The serum osteocalcin level is the representative biomarker of bone turnover. BMD is the amount of mineral (calcium hydroxyapatite) per unit of bone and is used to evaluate fracture risk and decreases in postmenopausal women with osteoporosis [17,35]. In the present study, the OVX mice showed decreased serum estradiol levels and BMD, but increased serum osteocalcin levels, a condition typical of menopause. The administration of PL and AM to the OVX mice increased the serum estradiol levels, while decreasing serum osteocalcin levels like that seen with estrogen. However, BMD was effectively increased only at a low dose of PL (PL-L) and a high dose of AM (AM-H). Changes in trabecular structure leading to bone loss are important factors in the progression of postmenopausal osteoporosis [36]. In the present study, the micro-CT image of the trabecular bone structure revealed that parameters such as connections to the cancellous reticulum, BV/TV, Tb.N and Tb.Th improved when the OVX-mice were treated with estrogen, PL, and AM. Specifically, a high dose of AM showed an effect similar to that of estrogen and was superior to the effect of PL. This effect of AM could be attributed to polysaccharides in AM, which have been reported, in an earlier study, to exert significant anti-osteoporotic activity by increasing the BMD in a dose-dependent manner [37]. Overall, there were no significant differences in the effects of the single extracts of PL and AM on estradiol production and BMD, but a high dose of AM was more effective in lowering the levels of osteocalcin and in the formation of trabecular bone structure than PL. These results suggest that AM could be superior to PL in preventing the risk of osteoporosis.

The present study confirmed effect of the single extracts of PL and AM in the production of estradiol in ER-positive MCF-7 cells, and then examined phosphorylation of ERK and Akt, ER-dependent downstream signaling pathway to understand whether these effects of PL and AM involved ER. ERKs are signal transducers of the mitogen-activated protein kinase (MAPK) family, which is a downstream signal for the activation of ERs [38,39]. The results of the present study showed that the expressions of phosphorylated-ERK and -Akt were increased at high concentrations (≥ 100 $\mu\text{g/mL}$) of PL and AM. Previously, Gao *et al.* [40,41] showed that DBT comprising a mixture of AM and *Angelica Sinensis Radix* activated the phosphorylations of ERK and ER- α in cultured MCF-7 cells as well as cultured T-lymphocytes. These results suggest that both PL and AM have estrogen-like properties and exert their action through the activation of the ER-dependent downstream signaling pathway.

5. Conclusions

The results of this study suggest that both PL and AM alone can be effective herbal medicines for improving estradiol production, inhibit body weight gain, and improve bone metabolism. These effects were more pronounced with AM than with PL. However, the present study has several limitations: first, it is uncertain whether 6 weeks of administration of PL and AM is sufficient. Treatment with PL and AM single extracts for 6 weeks was sufficient to improve bone metabolism. However, this period was probably insufficient to observe any significant changes in the uterine weight and lipid profile. In some studies, the duration of the herbal treatment was 8 weeks or longer. Therefore further studies are needed to examine the effect of PL and AM based on the treatment period. Second, the present study did not investigate whether treatment with a mixture of PL and AM had a synergistic effect on alleviating menopausal symptoms compared with single treatments. Therefore, if additional studies that can address these limitations could be conducted in the future, it is believed that we could have a better understanding of the beneficial effects of PL and AM on menopause-related obesity, and bone loss post-menopause.

Availability of Data and Materials

The ownership of the data belongs to the Korea Institute for Public Sperm Bank and Genoheal Co., Ltd. Upon request, the data used to support the findings of this study may be available from the corresponding author with permission of the Korea Institute for Public Sperm Bank and Genoheal Co., Ltd.

Author Contributions

MJP and CSK performed Conceptualization, Methodology, Writing—Original draft preparation. KTH help and advice on analysis and interpretation of data. JHB help on the experiments for acquisition of data. HC and YL analyzed the data and interpretation of data. CWK and BJS performed the conception or design of the work, the acquisition, analysis, interpretation of data for the work, critical revision of the manuscript, and provided obtaining research funding. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

All animal experiments were conducted based on the Guide for the Care and Use of Laboratory Animals, funded by the National Institutes of Health, and approved by the Pusan National University Hospital Institutional Animal Care and Use Committee. This study was approved by the Institutional Review Board of Pusan National University Hospital (PNUH-2022-209).

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Conflict of Interest

The authors declare no conflict of interest. Additionally, Bo Sun Joo is serving as one of the Editorial Board members of this journal. Declares that he was not involved in the peer review of this article and had no access to information regarding its peer review. Min Jung Park, Cha Soon Kim and Bo Sun Joo are from the Genoheal Co. Ltd. team. They are participating researchers and designed the research study and the acquisition of data. We have received no funding from Genoheal Co., Ltd.

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