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Beneficial effects of *Cirsium japonicum* var. *maackii* on menopausal symptoms in ovariectomized rats

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In women, menopause refers to a series of physiological and mental symptoms of distress that result from a decrease in 17β-estradiol. In addition to the loss of fertility, the symptoms include facial flushing, depression, osteoporosis, sexual dysfunction, and genitourinary atrophy. *Cirsium japonicum* var. *maackii* is a perennial herbaceous species found in the mountains and fields of Korea, China, and Japan. The medicinal uses of *C. japonicum* include antioxidant, antidiabetic, antitumor, antifungal, and anti-inflammatory activities. We investigated the effect of *C. japonicum* extract in a rat model of menopause that exhibited rapid estrogen decline induced by ovariectomy (OVX rats). The rats were treated with *C. japonicum* extract for 10 weeks and the following parameters were measured: food intake, feed efficiency, body weight, total cholesterol, triglyceride, LDL-cholesterol, HDL-cholesterol, liver weight, 17β-estradiol, uterus weight, AST, ALT, bone mineral density (BMD), bone alkaline phosphatase, calcitonin, and osteocalcin. In OVX rats, the administration of 50 and 100 mg kg^{-1} *C. japonicum* extract significantly decreased body weight, total cholesterol, triglyceride, HDL-cholesterol, and LDL-cholesterol and significantly increased 17β-estradiol and BMD. During the light/dark box test, the *C. japonicum* treatment group (100 mg kg^{-1}) spent more time in the light chamber than in the dark area, which was reflective of their diurnal nature. Using a molecular docking simulation, we predicted the plausible binding mode of the active compounds of *C. japonicum* with the ligand binding domain of estrogen receptor (ER)-α and ER-β. These results showed that *C. japonicum* extract can treat the symptoms before and after the menopause.

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Introduction

In women, menopause is a phase of life that begins between approximately 45 and 50 years old; therefore, many women live more than one-third of their lives after menopause. As the average life expectancy is increasing, the menopausal period is also increasing, and the incidence of postmenopausal women suffering menopausal disorders has emerged as a major worldwide health problem. Owing to a general improvement of living standards and an improvement of their education, women now pay more attention to the symptoms of menopause, quality of life after menopause, and health.[1,2]

Menopause refers to a transitional period in which the premenopausal gonadotropic state is altered to a postmenopausal state without fertility. In addition to a decrease in the secretion of the follicular hormone, menopausal processes, such as menstruation and ovulation, become irregular, and the period from when the reproductive ability is lost.[3,4] After menopause, the ovarian function markedly lowers, estrogen deficiency occurs, and changes are experienced throughout the body, including in the vascular, genitourinary, musculoskeletal, and cerebral nervous systems. These changes result in hot flushes, sweating, anxiety, headaches, urinary incontinence, irritability, dizziness, and memory impairment, which are classified as menopausal symptoms.[5]

Menopausal symptoms, especially osteoporosis and cardiovascular disease, can produce dangerous chronic aftereffects that directly affect the life of a woman.[6] Significant differences in the incidence of cardiovascular disease among men and women arise from the differences in cardiovascular risk factors and sex hormone status between men and women. In

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particular, the prevalence of atherosclerotic disease in women is lower before menopause than in men of the same age, but is known to increase rapidly to a frequency similar to that found in men after menopause.[7,8] The mechanism by which the female hormones inhibit arteriosclerosis and protects blood vessels is related to the improvement of blood lipid levels and the risk factors of cardiovascular disease, such as lipoprotein, fibrinogen, and homocysteine. In particular, it is related to a decrease in total cholesterol concentration and low density lipoprotein (LDL) cholesterol concentration, but it is known that lipoprotein cholesterol concentration increases.[9–12]

Another problem associated with postmenopausal women is osteoporosis that results from bone metabolic abnormalities. Osteoporosis is one of the most common types of metabolic bone disease, in which the bone mass significantly reduces in comparison with an average person of the same age and sex.[13] Osteoporosis in menopausal women is caused by estrogen deficiency, through which osteoelectricity increases bone loss and inhibits calcium absorption in the colon.[14,15]

Hormone replacement therapy (HRT) has been used to prevent menopausal diseases including osteoporosis, but increases the risk of cardiovascular disease, cervical cancer, and breast cancer.[16] Thus, interest in natural plants with similar structures and biological functions to estrogen has recently increased.[17]

Cirsium japonicum var. *maackii* (CJ), a member of the Compositae family, is a wild perennial herb found in Korea, China, and Japan. It is listed in the Korean and Chinese pharmacopoeias and has been used as an antihemorrhagic, antihypertensive, antihepatitis agent and as a diuretic in Korean traditional medicine.[18] In the present study, we investigated the effect of CJ extract on serum lipid metabolism, osteoporosis, and depression in ovariectomized (OVX) rats. In addition, we predicted a plausible binding mode for the active compounds of CJ extract with the ligand binding domains of estrogen receptor (ER)-α and ER-β by using molecular docking simulations.

Materials and methods

Plant materials

CJ was provided by Imsil Herbal Medicine, and the optimized CJ extract was named ICF-1. In brief, the dried aerial parts of CJ collected in May and November (3 : 2) 2015, supplied from Imsil Herbal Medicine, Korea, were extracted with 30% EtOH for 3 h (7 L × 3) under reflux at 65–75 °C. After the filtration and removal of solvent *in vacuo*, the dried extract (451.8 g) was collected. The plant sample was authenticated by the Korea National Arboretum, Pocheon, Korea and a voucher specimen (LEE16-01) was deposited at the Herbarium of the Department of Integrative Plant Science, Chung-Ang University, Korea.

Chemicals and reagents

Minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Co. (Grand Island, NY, USA). 17β-Estradiol was obtained from Sigma Aldrich (Saint Louis, MO, USA). TransFast™ reagent was purchased from Promega (Madison, WI, USA). A calcitonin ELISA kit (sensitivity: 0.27 ng mL^{-1}, linear range: 0.27–6.2 ng mL^{-1}, cross reactivity: calcitonin (rat) – 100%, calcitonin (human) – 80%, calcitonin (mouse) – 33%, intra-assay variation: <10%, inter-assay variation: <15%) for rat serum was purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA, Catalog No: EK-014-06). An osteocalcin ELISA kit (sensitivity: 0.78 ng mL^{-1}, linear range: 0.78–50 ng mL^{-1}, intra-assay variation: <6.38%, inter-assay variation: <7.14%) for rat serum was purchased from LifeSpan BioSciences, Inc. (Seattle, WA, USA, Catalog No: LS-F22801-1). An estradiol ELISA kit (sensitivity: 12.1 pg mL^{-1}, linear range: 71.7–91.5 pg mL^{-1}, cross reactivity: estriol – 86%, estrone – 26%, 17α-ethynylestradiol – 7%, progesterone – 6%, 17β-estradiol 3-benzoate – 3%, intra-assay variation: <5.4%, inter-assay variation: <7.6%) for rat serum was purchased from R&D Systems, Inc. (Minneapolis, MN, USA, Catalog No: KGE014).

HPLC analysis

The quantitative analysis of cirsimaritin was performed using a reversed-phase system with an INNO C18 column (250 mm × 4.6 mm, 5 μm) as described previously.[19] The mobile phase consisted of a gradient of acetonitrile and water plus 0.5% acetic acid. A UV detector was used to monitor the separation at an absorbance of 270 nm, and the flow rate was 1 mL min^{-1}.

Cell-based transactivation assay

MCF-7 cells were cultured in MEM supplemented with 10% FBS, 1× non-essential amino acid, 1% penicillin/streptomycin, and 1 mM sodium pyruvate at 37 °C under an atmosphere of 5% CO_2. MCF-7 cells were then seeded into 24-well plates and cultured for additional 24 h before transfection. The medium was replaced with MEM without phenol red and 10% charcoal dextran-treated FBS prior to transfection. After 4 h, the DNA mixture containing a ERE luciferase reporter plasmid (0.2 μg) and an internal control plasmid, pRL-SV-40 (4 ng), was transfected using the TransFast™ reagent. After transfection for 24 h, the cells were treated with 10 μM 17β-estradiol, the indicated concentrations of ICF-1 extract and cirsimaritin, or 1 μM ICI 182 780, and were incubated for 24 h. Luciferase activities of cell lysates were estimated using a Dual-Luciferase® Reporter Assay System in accordance with the manufacturer's instructions (Promega, WI, USA). The relative activity of luciferase was normalized to the corresponding luciferase activity of Renilla to determine the transfection efficiency.

Experimental animals and design

The experimental procedure and the number of animals were determined according to the literature[3] and opinions of the Institutional Animal Ethical Committee. 7-Week-old female Sprague-Dawley rats were purchased from Samtako Bio Co. (Osan, Korea). The animals were acclimatized to standard laboratory conditions with free access to water and food (Rod

feed, DBL Co., Republic of Korea) for 1 week before the experiment. The temperature was thermostatically regulated to 23 ± 2 °C and a 12 h light–dark schedule was maintained. All the animal experimental procedures were approved by the Animal Ethics Committee of Gachon University. At 8 weeks old, 32 and 8 Sprague-Dawley rats were bilaterally OVX and sham-operated (normal), respectively. The rats were anesthetized with a mixture of zoletil (25 mg kg^{-1}) and rompun (10 mg kg^{-1}) and performed ovariectomy. After a 1-week recovery from the surgery, the OVX rats were randomly divided into four groups with eight rats each and treated as indicated: control (OVX rats, water), positive control (OVX rats, 6.5 mg kg^{-1} red clover once per day), ICF-1 50 (OVX rats, 50 mg kg^{-1} ICF-1 once per day), and ICF-1 100 (OVX rats, 100 mg kg^{-1} ICF-1 once per day). ICF-1 was orally administered in distilled water (1 mL) for 10 weeks and the normal and control groups were administered an equal volume of distilled water.

Blood and organ dissection (liver, uterus, and femur)

At the end of the experimental period, the blood samples were collected *via* cardiac puncture, and the liver and uterus were excised from each rat, dried on filter paper and weighed. The rat femurs were dissected out and stored in a freezer for the measurement of bone mineral density (BMD).

Serum analyses

Serum calcitonin and osteocalcin levels were determined using ELISA kits with a Spectra Max 190 (Molecular Devices) at a wavelength of 450 nm. Serum 17β-estradiol levels were assayed using an ELISA kit and quantified with a Spectra Max 190. Serum total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-cholesterol), high-density lipoprotein cholesterol (HDL-cholesterol), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured with a Hitachi 7600 automatic analyzer (Hitachi Co., Tokyo, Japan).

BMD measurements

The BMD of rat femur bones was evaluated by dual-energy X-ray absorptiometry using a GE Lunar PIXImus system (GE Healthcare, Madison, WI, USA) equipped with software for BMD assessment, as recommended by the manufacturer.

Molecular docking study

Molecular docking simulation was accomplished using AutoDock 4.2.6 and AutoDock Tools 1.5.6.[20] The 3D structure of the ligand was prepared and minimized by MM2 using Chem 3D Pro 12.0 software. The structures of ER-α (PDB ID: 1A52) and ER-β (PDB ID: 2I0G) were downloaded from the RCSB protein data bank. The Chain A of each receptor was prepared for docking using UCSF Chimera 1.11 by the removal of other chains and all non-standard residues and the ligand and protein files were prepared in accordance with the AutoDock protocol.[21] The docking simulation was performed using a Lamarckian Genetic Algorithm. The hydrogen bonding analysis and visual investigation were performed using UCSF Chimera 1.11.[22]

Light/dark box test

The light–dark box test was performed as previously described. Briefly, square photo-beam activity arenas (26 × 26 × 38 cm) were divided into light and dark compartments with a black Plexiglas insert. The insert had a 4 × 4 cm opening in its center to allow rats free passage between the light and dark compartments. To begin the task, the rats were placed in the dark compartment through a lid in the top of the insert. The rats were allowed to explore the arena for 10 min and the time spent in the dark and the number of transitions between the dark and light sides were monitored by using Activity Monitor software (Med Associates Inc., Fairfax, Vermont). The innate anxiety of the rats during these approach avoidance conflicts was indicated by the time spent in the aversive portion of the conflict.

Statistical analysis

The results are expressed as mean ± SD. Statistical significance in all results was determined through the analysis of variance (ANOVA) following a multiple comparison test with a Bonferroni adjustment. P values <0.05 were considered statistically significant. Statistical analysis was performed using SPSS software (Version 20.0, SPSS Inc., Chicago, USA).

Results and discussion

When menopause begins, the loss of ovarian function results in irregular menstrual cycles. In addition, menstruation is finally terminated by the secretion of estrogen (follicle hormone) over a period of several months to 3 years, which acts on the autonomic nervous system of the genitourinary tract and causes the failure of the autonomic nervous system and menopausal disorders.[23] Estrogen has several effects on the cardiovascular system, including the modulation of the immune cell function and the inflammatory response. These effects are largely mediated through ERs, which are expressed in macrophages and monocytes.[24,25]

As shown in Fig. 1A, treatment with 10, 30, and 100 μg mL^{-1} of each extract showed ER transcription activity regardless of the extraction conditions. In particular, 30% ethanol extract showed an effect similar to red clover, which was used as the positive control at 100 μg mL^{-1}. Samples with high ethanolic extraction conditions were excluded from the results because they showed cytotoxicity at high concentrations. The *in vitro* experiments using MCF-7 cells showed that 30% ethanol extract was appropriate. Then, the extracts of each plant region were treated at 10, 30, and 100 μg mL^{-1} and the ER transcription activity was observed in flower and leaf regions, as shown in Fig. 1B. In particular, the extract of CJ leaf showed a higher efficacy than 17β-estradiol (E2, positive control) at 100 μg mL^{-1}.

The ER transcriptional activity of CJ leaf extracts based on the harvest period is shown in Fig. 2A. Stronger ER transcriptional activity was observed in CJ leaves harvested in spring than in autumn. In particular, 30% ethanol extract of spring-harvested CJ leaves showed an effect similar to

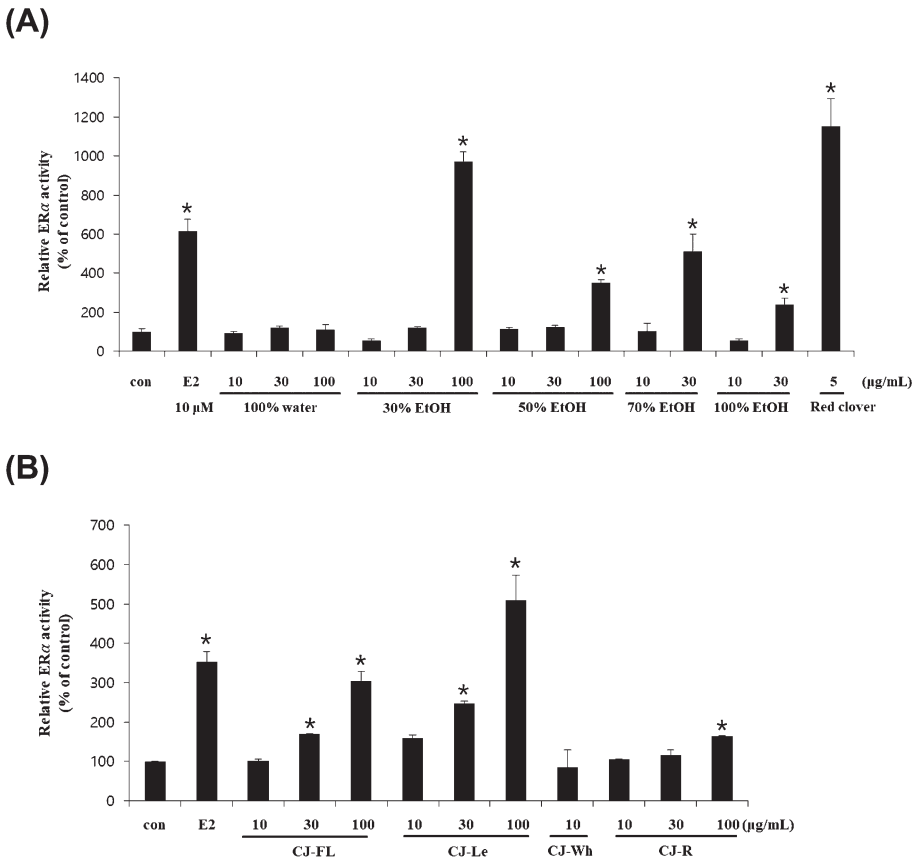


Fig. 1 Comparison of estrogenic activities of CJ extracts. (A) Estrogen receptor transcriptional activity of CJ extracts extracted with solvent containing 0, 30, 50, 70, and 100% ethanol. (B) Estrogen receptor transcriptional activity of CJ extracts by regions extracted with 30% ethanol. E2: 17β-estradiol, CJ: *C. japonicum* extract, FL: flower, Le: leaf, Wh: whole, and R: root.

17β-estradiol (E2) at 100 μg mL^{-1}. As shown in Fig. 2B, the ER transcriptional activities of CJ leaves were affected by the mixing ratio of CJL1 (spring harvest) and CJL3 (autumn harvest) (1 : 1, 2 : 3, 3 : 2, 1 : 4, and 4 : 1). The CJ extract with a 3 : 2 mixing ratio of CJL1 and CJL3 showed a stronger effect than 17β-estradiol (E2), the positive control, at 100 μg mL^{-1}. The amounts of cirsimarin, hispidulin, and cirsimaritin were 7.9, 1.4, and 13.1 mg g^{-1} DW, respectively, in CJL1 (Fig. 3B) and 6.7, 0.7, and 9.3 mg g^{-1} DW, respectively, in CJL3 (Fig. 3C). Therefore, the content of active ingredients was higher in CJL1 than in CJL3. This optimized extract with a 3 : 2 mixing ratio of CJL1 and CJL3 was named ICF-1 and the effects of a 10-week treatment period of ICF-1 extract in a rat model of menopause after a rapid decline in estrogen induced by ovariectomy (OVX) were assessed.

The effects of ICF-1 on the body weight, food intake, and feed efficiency are presented in Table 1; the initial body weights were similar among groups. After a 10-week treatment period, the body weight of the OVX control group was significantly higher than that of the normal group. However, the OVX-induced body weight increase was significantly inhibited in the ICF-1-treated groups (50 and 100 mg kg^{-1}), but ICF-1 did not significantly affect the food intake and feed efficiency at either dose.

High circulating concentrations of total cholesterol, LDL-cholesterol, and triglycerides, together with low levels of HDL-cholesterol, are proved to be biomarkers of risk factor for dyslipidemias and cardiovascular disease.[26] The total cholesterol, triglyceride, and HDL-cholesterol ratio significantly increased in the control group as compared with the normal group. LDL-cholesterol increased in the control group as compared with the normal group, but this difference was not significant. The treatment with 50 mg kg^{-1} ICF-1 resulted in significant reductions in the total cholesterol, triglyceride, and HDL-cholesterol ratio compared with the control group. LDL-cholesterol decreased in the ICF-1 50 mg kg^{-1} treatment group compared with the normal group, but this difference was not significant. The ICF-1 100 mg kg^{-1} treatment group resulted in significant reductions in the triglyceride, HDL-cholesterol, and LDL-cholesterol ratio compared with the control group. Total cholesterol decreased in the ICF-1 100 mg kg^{-1} treatment group compared with that in the normal group, but the difference was not significant. The liver weight of the treatment group was significantly lower than that of the control group, whereas it decreased significantly in ICF-1 and the red clover-administered groups (Table 2).

The female menopause progresses with depletion of the follicles of the ovaries and the concentration of 17β-estradiol

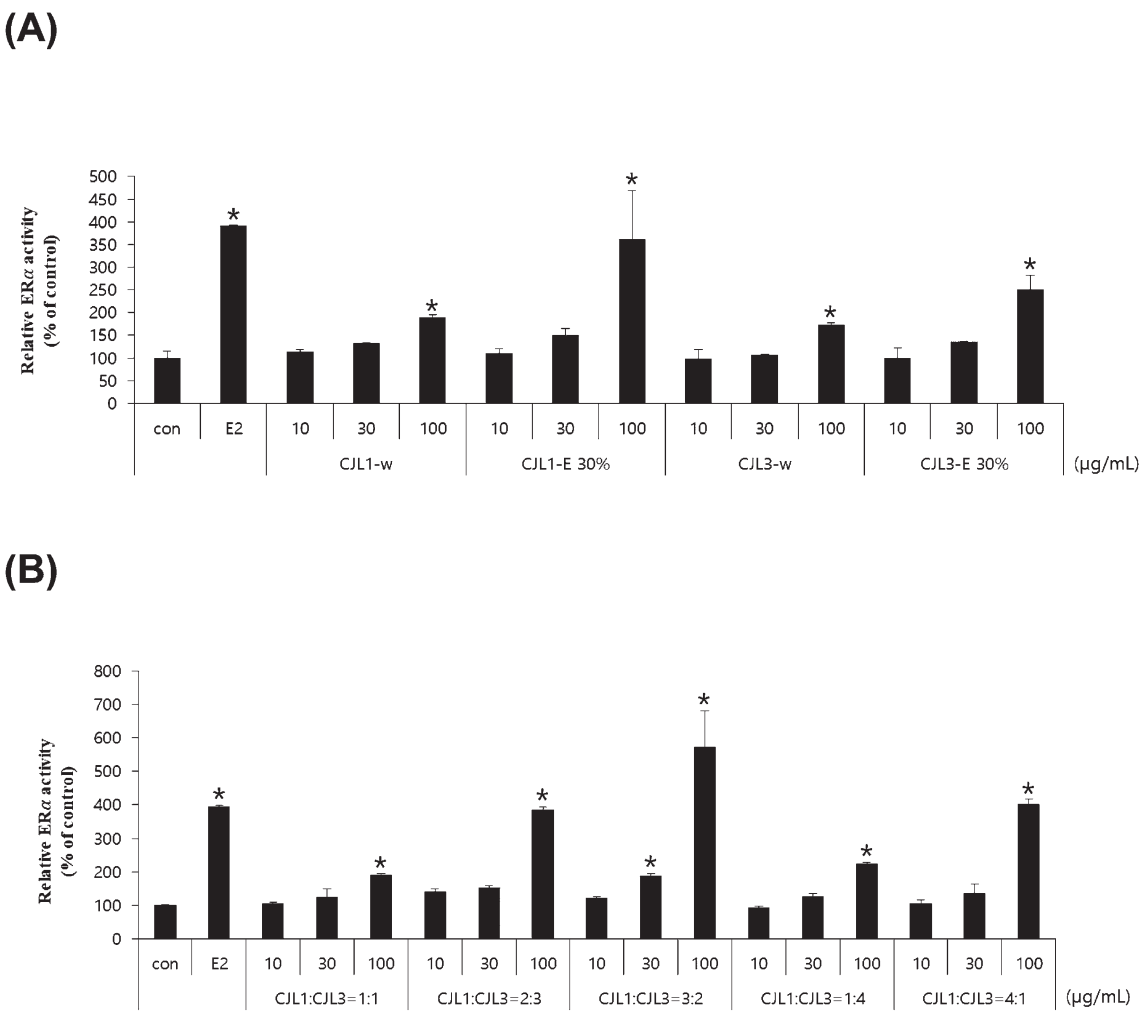


Fig. 2 Comparison in estrogenic activity of the mixture in accordance with the optimization conditions of the extract. (A) Estrogen receptor transcriptional activity of CJ extracts based on the harvest period. (B) Estrogen receptor transcriptional activity of CJ extract in accordance with the mixing ratio of both harvest periods. CJL1: spring harvest, and CJL3: autumn harvest.

secreted by the ovaries is reduced to approximately 15% of that found in healthy young women.[27] 17β-Estradiol is a type of estrogen that is important not only for reproductive function, but also for other organs, such as bones. It is mainly secreted directly from the ovaries and produced in small quantities from the adrenal cortex. This decrease in 17β-estradiol results in estrogen produced in adipose tissue as the most prevalent estrogen in menopause.[28,29] As shown in Table 3, there was a significant decrease in serum levels of 17β-estradiol in the control group in comparison with the normal group. There was a significant increase in the serum levels of 17β-estradiol in the OVX ICF-1 100 mg kg^{-1}-treated group in comparison with the control group. However, ICF-1 50 mg kg^{-1} did not significantly affect the serum levels of 17β-estradiol. The uterine weight of the treated group was not different from the uterine weight of the control group (Table 3).

The serum AST and ALT are enzymes found in hepatocytes that are released into the blood when liver cells are destroyed.[30,31] The serum AST and ALT levels of different groups of animals are shown in Table 4. These results indicated a significant increase in the serum AST and serum ALT levels in the OVX control group compared with the normal group. Treatment with ICF-1 (50 and 100 mg kg^{-1}) reduced the serum levels of AST and ALT, but the changes were not significant.

17β-Estradiol increases the calcium concentration in bones, aids osteoblast survival, and induces the apoptosis of osteoclasts, which leads to bone formation rather than bone resorption. This decrease in estrogen was thought to be the main cause of postmenopausal osteoporosis.[32,33] Alkaline phosphatase (ALP) is an enzyme present in the liver, bone, placenta, and small intestine. During the active progression of osteogenesis, the blood level increases. ALP is used as a biochemical marker for bone turnover and is strongly detected in OVX rats, suggesting that osteoporosis is linked with increased bone resorption and osteogenesis after ovariectomy.[34] The levels of BMD, ALP, calcitonin, and osteocalcin analyzed in the left

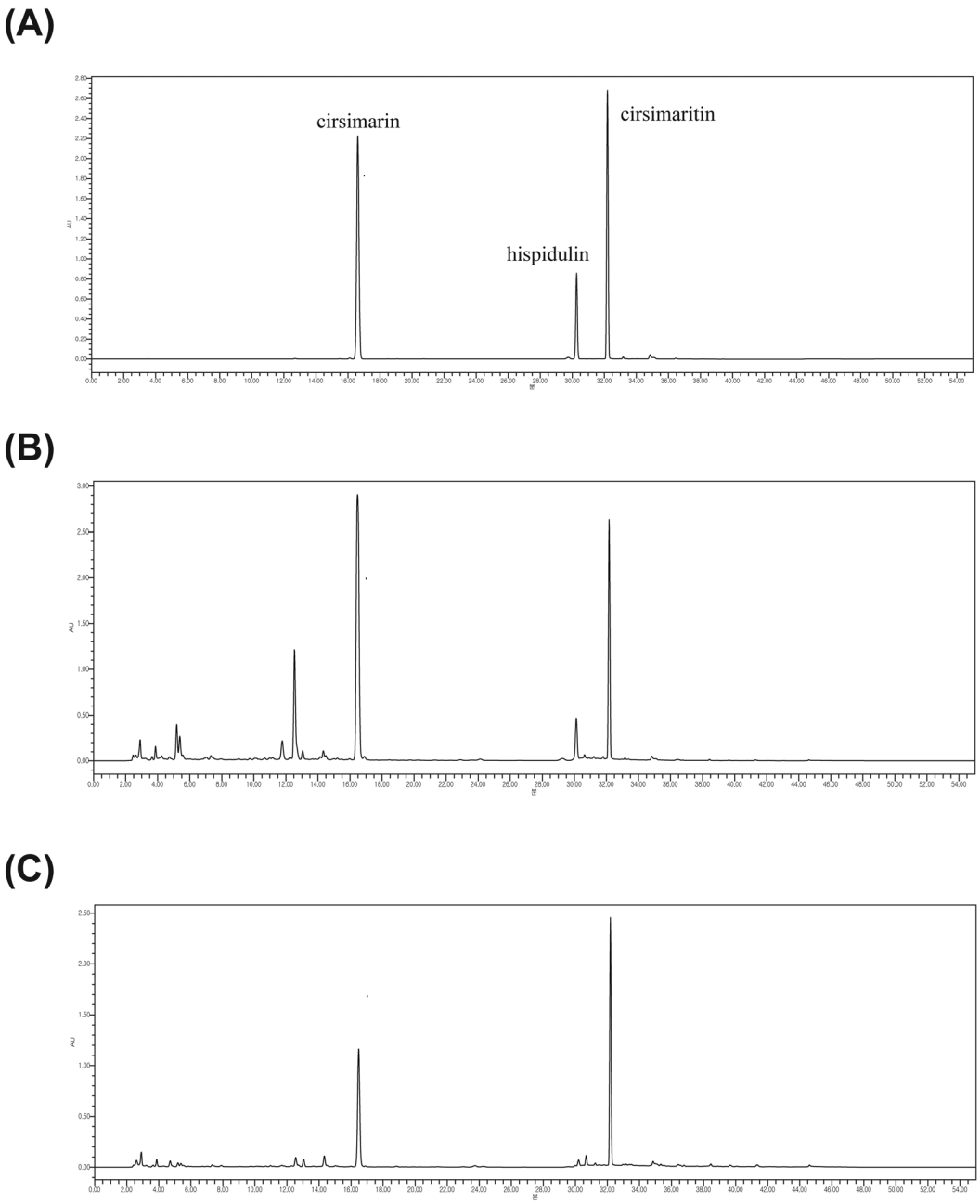


Fig. 3 Analysis of active ingredients using HPLC. (A) The HPLC analyses of standard compounds. (B) The HPLC analyses of CJ extracts harvested in spring. (C) The HPLC analyses of CJ extracts harvested in autumn.

femur and serum of OVX rats are listed in Table 5. Although the rats treated with ICF-1 (50 and 100 mg kg^{-1}) showed a higher BMD and ALP content than the control group, there were no significant differences between all groups. In addition, the ICF-1-treated group did not significantly affect the serum levels of calcitonin and osteocalcin.

The approach-avoidance conflict we used to measure the anxiety level was the light–dark box (Table 6). In this task, the time spent in the dark compartment was the main measure of anxiety. The treatment group (ICF-1 100 mg kg^{-1}) spent less time in the dark chamber than in the light area, which reflected its anxiolytic potential. There was also a significant increase in the light/dark preference of ICF-1 100 mg kg^{-1}-treated groups than those of the control when measured by the time spent in, and the number of visits to, the dark chamber.

A molecular docking simulation was performed to predict a plausible binding mode for the active compounds (Table 7, Fig. 4) present in *C. japonicum* leaf extract with the ligand binding domain (LBD) of ER-α and ER-β. The crystal structures of ER-α co-crystallized with estrogen (PDB ID: 1A52) and ER-β co-crystallized with SERBA-1 (PDB ID: 2I0G) were used to define

Table 1 Body weight, food intake, and feed efficiency of normal, control, positive control (red clover), and sample treatment (ICF-1 50 and 100 mg kg^{-1}) groups

		Group				
		Normal	Control	Red clover	ICF-1 50 mg kg^{-1}	ICF-1 100 mg kg^{-1}
Body weight	Initial	150.6 ± 2.1	158.3 ± 1.4	159.5 ± 2.1	156.6 ± 1.9	157.6 ± 2.6
	Final	286.3 ± 7.3	382.8 ± 7.7	368.6 ± 7.4	358.7 ± 8.8	361.0 ± 7.9
	Gain	135.7 ± 5.9*	224.5 ± 7.6	209.1 ± 6.8	202.1 ± 7.5*	203.4 ± 6.7*
Food intake		16.7 ± 1.1*	20.7 ± 0.9	19.6 ± 1.2	18.5 ± 1.4	18.9 ± 1.3
Feed efficiency		8.1 ± 0.8*	10.9 ± 1.0	10.6 ± 0.9	10.9 ± 0.8	10.8 ± 0.8

*$p < 0.05$ compared with the control value.

Table 2 Serum total cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol, and liver weight analysis

Group	Total cholesterol (mg dL^{-1})	Triglycerides (mg dL^{-1})	HDL-cholesterol (mg dL^{-1})	LDL-cholesterol (mg dL^{-1})	Liver weight (g)
Normal	77.2 ± 1.9*	81.5 ± 19.9*	42.5 ± 1.1*	7.5 ± 0.5*	9.2 ± 0.1*
Control	106.6 ± 1.0	108.6 ± 9.8	49.9 ± 0.8	9.3 ± 0.2	11.1 ± 0.8
Red clover	100.6 ± 1.3*	125.6 ± 15.8	46.3 ± 0.6*	7.1 ± 0.1*	10.2 ± 0.9*
ICF-1 50 mg kg^{-1}	98.4 ± 1.7*	77.6 ± 10.2*	45.5 ± 0.6*	8.9 ± 0.2	9.8 ± 1.1*
ICF-1 100 mg kg^{-1}	102.7 ± 1.7	90.7 ± 11.9	45.8 ± 0.4*	8.4 ± 0.2*	9.5 ± 0.7*

*$p < 0.05$ compared with the control value.

Table 3 17β-Estradiol concentration and uterus weight

Group	17β-Estradiol (pg mL^{-1})	Uterus weight (g)
Normal	142.4 ± 2.9*	0.76 ± 0.2*
Control	19.1 ± 9.1	0.10 ± 0.02
Red clover	194.9 ± 6.9*	0.09 ± 0.01
ICF-1 50 mg kg^{-1}	19.6 ± 3.5	0.08 ± 0.01
ICF-1 100 mg kg^{-1}	35.5 ± 6.9*	0.08 ± 0.02

*$p < 0.05$ compared with the control value.

Table 4 Serum AST and ALT analysis

Group	AST (SGOT) (U L^{-1})	ALT (SGOT) (U L^{-1})
Normal	51.2 ± 1.9*	26.2 ± 0.8*
Control	66.0 ± 1.6	33.7 ± 0.7
Red clover	62.0 ± 1.5	30.3 ± 0.6
ICF-1 50 mg kg^{-1}	64.6 ± 1.4	32.2 ± 0.9
ICF-1 100 mg kg^{-1}	62.3 ± 1.0	31.1 ± 0.7

*$p < 0.05$ compared with the control value.

Table 5 BMD, serum alkaline phosphatase, calcitonin, and osteocalcin analysis

Group	Bone density (BMD, g cm^{-2})	Alkaline phosphatase (ng mL^{-1})	Calcitonin (pg mL^{-1})	Osteocalcin (ng mL^{-1})
Normal	0.125 ± 0.005*	0.1221 ± 0.001	74.1 ± 2.1	0.094 ± 0.003
Control	0.118 ± 0.007	0.1202 ± 0.002	63.1 ± 3.4	0.091 ± 0.002
Red clover	0.117 ± 0.004	0.1225 ± 0.001	62.3 ± 3.8	0.092 ± 0.001
ICF-1 50 mg kg^{-1}	0.120 ± 0.003	0.1213 ± 0.001	62.3 ± 3.5	0.092 ± 0.001
ICF-1 100 mg kg^{-1}	0.120 ± 0.005	0.1214 ± 0.001	60.9 ± 2.8	0.092 ± 0.002

*$p < 0.05$ compared with the control value.

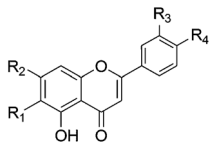
Table 6 Time spent in the dark and the number of transitions between the dark and light sides

Group	Time in the dark (s)	Number of transition between dark and light sides
Normal	428.2 ± 98.4*	6.6 ± 1.6
Control	358.7 ± 68.2	6.3 ± 1.4
Red clover	215.7 ± 67.2*	10.0 ± 1.1*
ICF-1 50 mg kg^{-1}	331.6 ± 75.8	4.0 ± 1.5
ICF-1 100 mg kg^{-1}	254.3 ± 68.2*	10.9 ± 1.9*

*$p < 0.05$ compared with the control value.

the LBD of each estrogen receptor.[35,36] The binding structures with the lowest AutoDock scores for the active compounds are presented in Table 7; luteolin had the highest binding affinity to ER-α, whereas hispidulin had the highest binding affinity to ER-β. Using the results of the docking simulation, possible hydrogen bonding interactions between the active compounds and LBD of ERs were checked by using UCSF Chimera. The highlighted residues, Glu353, Arg394, and His524 in ER-α

Table 7 Chemical structures of active compounds contained in ICF-1



Compound	R_1	R_2	R_3	R_4
Cirsimaritin	OCH_3	OCH_3	H	OH
Hispidulin	OCH_3	OH	H	OH
Cirsimarin	OCH_3	OCH_3	H	O-Glc
Linarin	H	O-Rut	H	OCH_3
Pectolinarin	OCH_3	O-Rut	H	OCH_3
Luteolin	H	OH	OH	OH
Apigenin	H	OH	H	OH
Diosmetin	H	OH	OH	OCH_3
Acacetin	H	OH	H	OCH_3

(Fig. 4A) and Glu305, Arg346, and His475 in ER-β (Fig. 4B), are required for the activation of receptors through the formation of hydrogen bonding networks.[35,37] Hispidulin was found to form hydrogen bonding interactions most similar to 17β-estradiol with the key amino acids in ER-β, which explained the high affinity of hispidulin for ER-β. However, the glycosylated flavones, cirsimarin, linarin, and pectolinarin, exhibited low binding affinities (Table 8). As shown in the modeling structures (Fig. 4A and B), they displayed highly distorted conformations, possibly because the glycoside chains are too bulky to fit in the LBD of ERs. Moreover, in ERs, most of the binding stability appears to come from hydrophobic van der Waals contacts within the lipophilic binding pocket,[36,37] but the hydrophilic properties of glycosides interfere in the

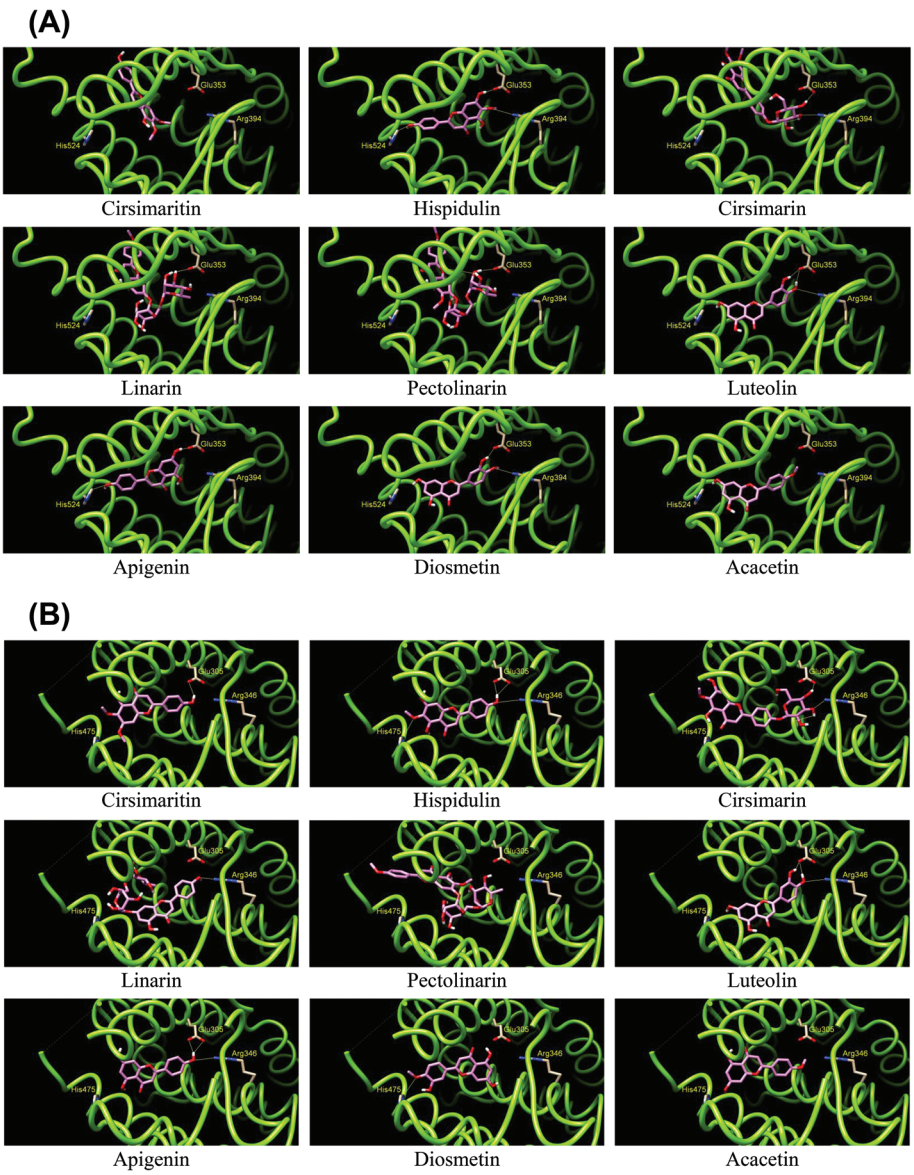


Fig. 4 Docking analysis results of active compounds. Putative hydrogen bonding was visualized by the yellow lines. Key amino acid residues are labeled. Molecular graphics were performed with the UCSF Chimera package. (A) Docking binding mode of active compounds over ER-α (PDB: 1A52). (B) Docking binding mode of active compounds over ER-β (PDB: 2I0G).

Table 8 Calculated lowest binding affinity of active compounds

Ligand	ER-α binding affinity (kcal mol^{-1})	ER-β binding affinity (kcal mol^{-1})
Cirsimaritin	−7.33	−8.28
Hispidulin	−7.95	−8.62
Cirsimarin	−7.72	−4.7
Linarin	−5.67	−3.17
Pectolinarin	−3.91	−2.2
Luteolin	−8.34	−8.48
Apigenin	−8.06	−8.32
Diosmetin	−7.77	−8.1
Acacetin	−7.54	−7.92
17β-Estradiol[a]	−10.21	—
SERBA-1[a]	—	−9.83

[a] Indicates the co-crystallized ligand of the original X-ray crystal structure.

hydrophobic interactions. Both reasons contribute to the observed low docking affinity of glycosylated flavones with the binding pocket of the active conformation of ERs.

Recently, studies on natural products that alleviate menopausal symptoms are increasing. Popular dietary supplements such as black cohosh and herbal medicines containing *Cynanchum wilfordii* extracts have been shown to attenuate a variety of menopausal symptoms.[38] The mechanism of action may be related to some forms of estrogen agonist actions that benefit menopausal symptoms, and *C. japonicum*, our research material, may have the same mechanism of action.

In summary, our results clearly showed that *C. japonicum* leaf extract improved the symptoms of menopause. Using a molecular docking simulation, we predicted a plausible binding mode of the active compounds of *C. japonicum* leaf extract with the ligand binding domains of ER-α and ER-β.

Conflicts of interest

There are no conflicts to declare.

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