Article

Therapeutic Potential of Water Chestnut Fruit Extract (*Trapa bicornis*) against Ovariectomy-Induced Climacteric Symptoms in Mice

Su Shin [1,†] , Md. Mohibbullah [2,†] , Ki-Young Kim [1], Eun-Jin Hong [1], Jae-Suk Choi [3,*] and Sae-Kwang Ku [4,*]

[1] Research Institute, Bio Port Korea Inc., Busan 46034, Republic of Korea; sshin@bioportkorea.com (S.S.); kyk1967@bioportkorea.com (K.-Y.K.); hej0557@naver.com (E.-J.H.)
[2] Department of Fishing and Post Harvest Technology, Sher-e-Bangla Agricultural University, Dhaka 1207, Bangladesh; mmohib.fpht@sau.edu.bd
[3] Department of Seafood Science and Technology, The Institute of Marine Industry, Gyeongsang National University, Tongyeong-si 53064, Republic of Korea
[4] Department of Anatomy and Histology, College of Korean Medicine, Daegu, Haany University, Gyeongsan-si 38610, Republic of Korea
* Correspondence: jsc1008@gnu.ac.kr (J.-S.C.); gucci200@hanmail.net (S.-K.K.); Tel.: +82-55-777-9142 (J.-S.C.); +82-53-819-1549 (S.-K.K.)
† These authors contributed equally to this work.

Abstract: Climacteric symptoms, as well as postmenopausal estrogen deficiency, have been associated with many psychological problems and the risk of osteoporosis and heart disease. Therefore, in this study, we aimed to evaluate, for the first time, the dose-dependent effect of water chestnut (WC), also known as *Trapa bicornis*, a fruit extract, on ovariectomy (OVX)-induced menopause in ICR mice. After bilateral OVX surgery, 200, 100, and 50 mg/kg of WC and 200 mg/kg of pomegranate concentrate powder (PCP) were administered orally for 84 days from 4 weeks after OVX operation. Then, anti-climacteric activities were evaluated in five groups: (1) estrogenic, (2) anti-obesity, (3) hypolipidemic, (4) hepatoprotective, and (5) anti-osteoporosis effects. Different biochemical assays, histopathological and morphological inspections, and mRNA expression findings showed that OVX-induced estrogen deficiency-related AMPK decrease was associated with climacteric symptoms such as obesity, hyperlipidemia, hepatic steatosis, and osteoporosis in ICR mice. However, these climacteric effects were reversed in OVX rats by treating them with WC at a dose relative to the same dose of PCP in OVX-ICR mice (200 and 100 mg/kg). Water chestnut fruit extract demonstrated promise as a complementary treatment for menopausal symptoms, indicating possible uses in the health of women through supplements or prescription drugs.

Keywords: ovariectomy; climacteric symptoms; water chestnut; anti-obese; hepatoprotective; anti-osteoporotic; functional food

1. Introduction

Climacteric symptoms mark the phase in a woman's life when reproductive capacity may gradually diminish due to aging [1]. As stated by the World Health Organization [2], this stage usually occurs between the ages of 40 and 65 and is associated with a decrease in ovarian follicular activity and subsequent estrogen deficiency. Approximately 70% of women have symptoms associated with estrogen deficiency, including vasomotor problems, night sweats, and urogenital problems that affect their quality of life [2,3]. An increased risk of cognitive and psychiatric symptoms, osteoporosis, and cardiovascular diseases has also been observed [1,4]. Postmenopausal status is likely associated with estrogen deficiency and the risk of obesity-related metabolic diseases [5,6]. Ovariectomy-induced animal models can demonstrate menopausal symptoms, highlighting the importance of

hormone therapy and the utilization of alternative treatments such as phytoestrogens to reduce menopausal symptoms [1,7].

Water chestnut (WC), scientific name *Trapa bicornis*, is a famous aquatic plant belonging to the Rhombaceae family. Commonly found in China, Japan, India, and Southeast Asia, is characterized by two thick horns. WC grows in shallow paddy fields, reservoirs, or lakes and has a history of use in ethnomedicine to treat diseases such as stomach ulcers and diarrhea [8]. Its key components include starch, dietary fiber, and polyphenols such as trapain and ellagic acid. Investigations into WC from the *T. japonica* species have demonstrated its anti-adipogenic and anti-diabetic properties, as well as antioxidant and anti-inflammatory activities [9–12]. Considering concerns about the safety of conventional treatments for women, developing supplementary food or functional food items from natural sources appears to be a great promise for resolving this issue.

Water chestnut has high levels of carbohydrates, proteins, lipids, minerals, and vitamins (B1, B2, B5, B6, E, A, and C). Water chestnut includes dietary fibers, polyphenols including phenolic acids and flavonoids, and hydrolyzable tannins. These bioactive chemicals are thought to have anti-diabetic, antioxidant, antibacterial, and anticancer properties [13]. According to Malviya et al. [14], water chestnut peels have significantly more phenolic chemicals than their kernels. Water chestnut contains a number of beneficial components, including fibers, vitamins, fatty acids, minerals, proteinase inhibitors, cysteine, lectin, and quercetin; starch (85–97.4% dry basis) is the primary component of this crop, indicating its potential as a nutritious food [15].

By examining the potential of water chestnut (*Trapa bicornis*), a plant recognized for its rich phytochemical profile, as a safer, plant-based therapy for menopausal symptoms, this study fills a major gap in the literature. The precise benefits of water chestnut on menopausal symptoms have not been adequately explored, despite some knowledge of its nutritional and antioxidant qualities [14,15]. The study sheds light on the effectiveness of water chestnut extract in reducing climacteric symptoms using an ovariectomy-induced mouse model to mimic menopause. This could lead to the development of a new, natural treatment option for women looking for alternatives to hormone replacement therapy. The findings of this study may aid in the development of safer, plant-based medications that carry fewer dangers than traditional ones.

However, WC from the *T. bicornis* species has not been the subject of any known pharmaceutical studies, with the exception of WC from the *T. japonica* species. Therefore, we, for the first time, intended to observe the dose-dependent effects of the fruit of *T. bicornis* of a WC extract on OVX-induced climacterium in ICR mice, a representative animal model that can resemble human postmenopausal symptoms [16–18], as compared to those of the fruit of *Punica granatum* L., or pomegranate concentrate powder (PCP), which has been extensively reported as possessing favorable anti-climacteric effects [7,17,18]. Therefore, a study on interventions for climacteric conditions was structured around five distinct categories: (1) the evaluation of estrogenic effects, (2) the prevention of obesity, (3) the evaluation of hypolipidemic effects, (4) the investigation of hepatoprotective effects against hepatic steatosis, and (5) the exploration of anti-osteoporosis effects.

2. Materials and Methods

2.1. Animals and Husbandry

A total of 90 female 6-week-old ICR mice, weighing approximately 25–28 g, were used for bilateral OVX surgery after a 6-day acclimatization period. After 27 days post-OVX operation, 10 mice in each group were selected based on the body weight deviations (OVX mice—average 34.14 ± 1.37 g and ranged between 32.10 and 38.50 g, and sham-operated mice—average 30.35 ± 1.26 g and ranged between 27.90 and 32.00 g, respectively). Four female mice in each polycarbonate cage were housed in a controlled environment (20–25 °C, 50–55% humidity, 12 h light/dark cycle), with free access to food and water. Experimental procedures were approved by the Animal Care and Use Committee of Daegu Haany

University (Gyeongsan City, Gyeongsangbuk-do, Republic of Korea) on 23 April 2021, with the approval number DHU 2021-029.

2.2. Preparation of Test Samples

WC was provided by Bioport Korea, Yangsan, Republic of Korea (supervisor: Dr. Shin S.) and stored at -20 °C before use. The water chestnut extract was extracted at 80 °C for 6 h using 20% fermented ethanol, 10 times the weight of the raw material (water chestnut), concentrated under reduced pressure, and after adding 20% maltodextrin, it was spray-dried to obtain a powder. A total of 160 kg of water chestnut was added and 32.4 kg of powder was recovered, showing a yield of approximately 20.3%. Selected WC samples were deposited in the herbarium of the Herbal Medicine Research Center for Liver Diseases at Daegu Haany University, South Korea with the identification number WC2020Ku01. WC was prepared by extracting with distilled water to a minimum concentration of 20 mg/mL. Pink-colored PCP powders (Lot number 13NT12-4) supplied by HL Science (Uiwang, Republic of Korea) were used and stored at 4 °C.

2.3. Experimental Groups and Treatment

A total of 6 groups, with 10 animals per group, were subjected to experiments as follows: (1) sham vehicle control = distilled water (DW) orally, 10 mL/kg, once daily; (2) OVX control = bilateral OVX + DW orally, 10 mL/kg, once daily; (3) PCP200 = bilateral OVX + PCP in DW orally, 200 mg/kg, once daily; (4) WC200 = bilateral OVX + WC in DW orally, 200 mg/kg, once daily; (5) WC100 = bilateral OVX + WC in DW orally, 100 mg/kg, once daily, and (6) WC50 = bilateral OVX + WC in DW orally, 50 mg/kg, once daily. Treatments were performed on mice 4 weeks after OVX surgery and were continued for 84 days.

2.4. Climacteric Inducement

Mice were anesthetized using 2–3% isoflurane, and the OVX treatment group underwent open surgery, including bilateral OVX through a midline incision along the linea alba, following the protocol of previous studies [16–18].

2.5. Body Weight Measurement

Body weight gain during the 28-day OVX recovery/induction period and after the 84-day baseline measurement was calculated using the following formula:

The OVX recovery/induction period (28 days): weight gain (g) = weight on the day of the first dose − weight on the day of OVX surgery.

After administration (84 days): weight gain (g) = weight at sacrifice − weight at the time of first administration.

2.6. Food Consumption Evaluation

Each mouse was kept in a separate cage, and each mouse was given 150 g of feed and the amount of additional feed was measured 24 h after feeding. This included the daily food intake (g/24 h/mice). A total of six measurements were made during each dosage, especially on days 1, 3, 5, 7, 28, 56, and 83 after the start of the dose.

2.7. Measurement of BMD and Body Fat Density

The bone mineral density (BMD) of the total body and the right femur were evaluated with live dual-energy X-ray absorptiometry (DEXA) (Hologic, Marlborough, MA, USA). This analysis was performed once at the end of the 84 days of the test administration, and the average amount of fat on the body and the abdominal cavity of each mouse was evaluated.

2.8. Gross Inspection and Organ Weight Measurement

After sacrifice, abdominal wall fat, the entire liver, and the uterus (including the genital area) were collected from the abdominal cavity and examined using a digital camera. The organs were weighed at wet weights. The relative body weight (% of body weight) was calculated with the following formula: [(weight of abdominal wall fat, uterus, or liver/body weight at sacrifice) $\times$ 100].

2.9. Bone Weight Measurement

The right femur was collected after 84 days of continuous treatment, which began 28 days after bilateral OVX surgery. The weight of the femur was measured as its wet weight. The femurs were then dried at 120 °C for 8 h to measure the dry bone weight. To obtain the weight of the ash, the dry bones were carbonized in an oven at 800 °C for another 6 h. The relative bone weight (% of body weight) was calculated using the following formula: [(bone weight/body weight at sacrifice) $\times$ 100].

2.10. Bone Strength Measurement

The bone strength, expressed as the load to failure (FL), was evaluated by performing a three-point bending test in the mid-shaft region of the right dry femur. Measurements were made using a computerized measurement system (SV-H1000, Japan Instrumentation System Co., Yokohama, Japan) and the results were expressed in Newtons (N).

2.11. Blood Collection

For blood biochemistry, 1 mL of whole blood was obtained from the vena cava at the time of sacrifice. Blood was separated by centrifugation at 15,000 rpm and 4 °C for 10 min. All blood samples were then frozen at −15 °C until further analysis.

2.12. Serum Biochemistry

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triacylglycerides (TGs) were analyzed using an automatic hematology analyzer. Additionally, serum osteocalcin levels (ng/mL), serum bone-specific alkaline phosphatase (bALP) activities (U/L), and estradiol content (pg/mL) were determined according to the company's instructions (ELISA kit, MyBioSource, San Diego, CA, USA).

2.13. Real-Time RT-PCR Analysis

A real-time fluorescence quantitative method was used to analyze the mRNA expression of acetyl-CoA Carboxylase 1 (ACC1), 5′ adenosine monophosphate-activated protein kinase (AMPKα1), and AMPKα2. The peroxisome proliferator-activated receptor (PPARα and PPARγ), leptin, the mitochondrial uncoupling protein 2 (UCP2), adiponectin, the CCAAT enhancer binding protein (C/EBPα), C/EBPβ, fatty acid synthase (FAS), and the sterol regulatory element bonding protein (SREBP) 1c were analyzed based on previous studies [19–22]. Data were normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. PCR oligonucleotide primer sequences are shown in Table S1.

2.14. Abdominal Wall Fat Pad, Uterus, and Liver Histological Procedures

Tissues were fixed in 10% neutral buffered formalin for 24 h and processed in serial sections of 3–4 μm using an automated microtome. Representative sections were stained with hematoxylin and eosin (HE) for light microscopy using an automated photomicroscope (Eclipse model 80i, Nikon, Tokyo, Japan). Liver cells were dehydrated in a 30% sucrose solution and cryosectioned, followed by oil red staining [16–18]. Additionally, the left side of the femur of each mouse was fixed, decalcified in formic acid and 0.5 N sodium hydroxide solution for 3 days (changed daily), trimmed, embedded in paraffin, and sectioned (3–4 μm).

These sections were stained with HE for bone histomorphometry to measure bone size and structure.

2.15. Statistical Analyses

The results of 10 mice in the experiment are shown as the mean ± standard deviation (SD). Levene's test showed a difference in homogeneity. Dunnett's T3 (DT3) was then used to identify specific pairs with significant differences. For groups that did not show significant differences, further analyses were performed using a one-way analysis of variance (ANOVA) followed by the Tukey difference test (THSD) using SPSS (version 18.0, SPSS Inc., Chicago, IL, USA). The significance of the comparison between treatment groups was determined as $p < 0.05$.

3. Results

3.1. Effects on Body Weight and Gain

The body weight of OVX control mice increased significantly against the sham control ($p < 0.01$ or $p < 0.05$). In contrast, OVX mice administered 200 and 100 mg/kg WC and 200 mg/kg PCP showed a significant decline in body weight 49 days after the first dose compared to the OVX control group ($p < 0.01$ or $p < 0.0.05$) (Figure 1A). Figure 1B shows the body weight gains during 4 weeks of the OVX-induced period and also during 84 days of the treatment/administration period. Additionally, weight gain decreased in the WC 200 (−53.41%) and 100 mg/kg (−41.23%) and PCP 200 mg/kg (−54.36%) control groups during the 84-day treatment period. In contrast to the significant increase in the OVX control group (278.10%) mice.
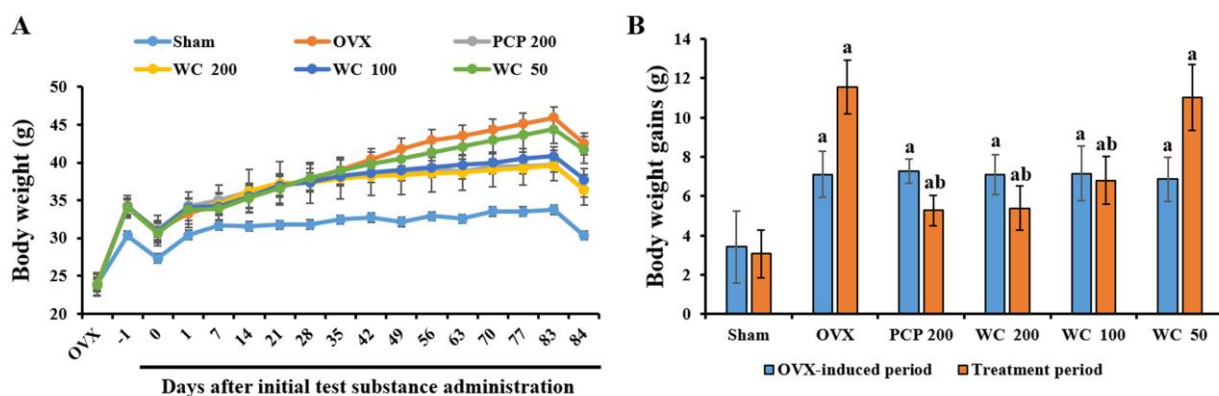

Figure 1. Effect of WC on body weight changes (**A**) and gains (**B**) in OVX mice. Arrow ($p < 0.01$) and dot arrow indicated statistical significance vs. OVX control by ANOVA. Values are expressed as mean ± SD of 10 mice. Different letters indicate significantly different.

3.2. Effects of Feeding

OVX control mice showed a significant ($p < 0.01$) increase in food intake in all six time periods (1, 3, 7, 28, 56, and 83 days) against the sham control. In contrast, there was no significant change in daily food intake of mice using the tested substances (Table S2).

3.3. Effect on Abdominal Wall Fat, Uterus, and Liver Weight, and General Inspections

The absolute and relative increase in abdominal wall fat in the OVX control mice was compared to the sham control, with a significant difference ($p < 0.01$) in weight (2967.51% and 2120.87%, respectively). On the contrary, both parameters were decreased with 200 mg/kg of WC (−45.76% and −36.75%, respectively), 100 mg/kg of WC (−37.20% and −29.15%, respectively), and 200 mg/kg of PCP (−44.91% and −35.70%, respectively) given to OVX mice compared to the OVX control group (Figure 2).

The absolute and relative weights of the uteruses of the OVX control mice (−77.10% and 83.68%, respectively) were significantly reduced ($p < 0.01$) against the sham control,

indicating a change in estrogen deficiency. However, both did not increase with 200 mg/kg of WC (101.07% and 135.51%, respectively), 100 mg/kg of WC (70.36% and 91.57%, respectively), and 200 mg/kg of PCP (102.68% and 136.21%, respectively) given to OVX mice compared to the OVX control group (Figure 2).
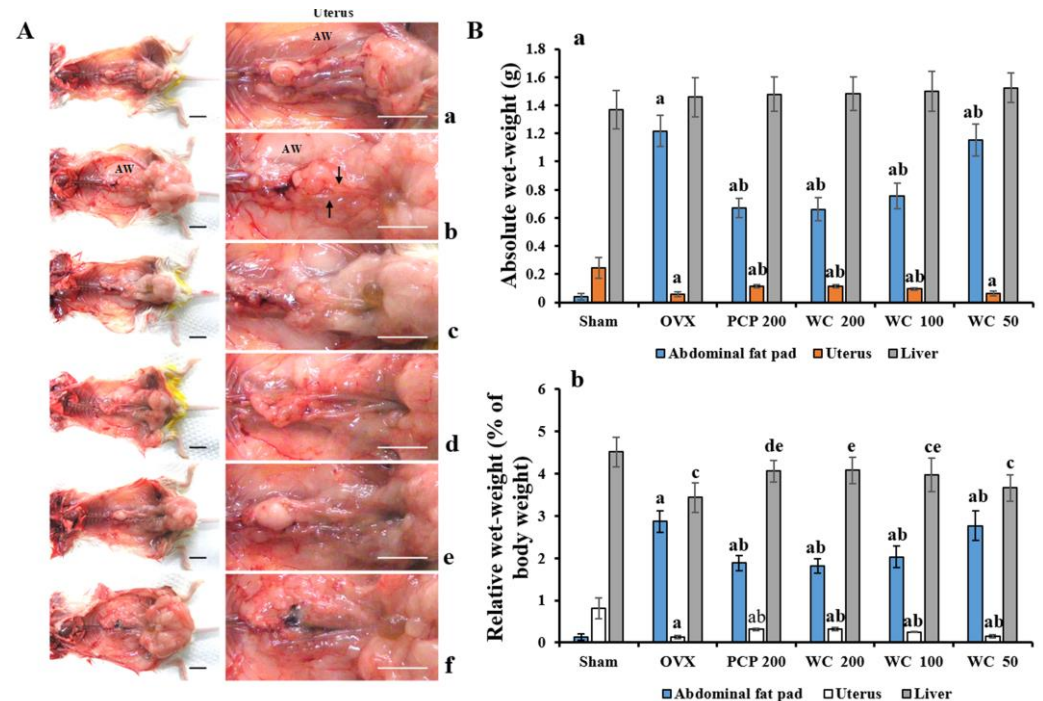

Figure 2. Changes in gross observation (**A**) in sham control (**a**), OVX control (**b**), PCP 200 (**c**), WC 200 (**d**), 100 (**e**) and 50 (**f**) mg/kg administered OVX mice. Analyses of organ weights (**B**) for abdominal fat pad, uterus, and liver in absolute wet weight (g) (**a**) and relative wet weight (% of body weight) (**b**). Statistical significance: a $p < 0.01$ vs. sham control and b $p < 0.01$ vs. OVX control by DT3 test, and c $p < 0.01$ and d $p < 0.05$ vs. sham control and e $p < 0.01$ vs. OVX control by THSD test. Values are expressed as mean ± SD of 10 mice. Different letters indicate significantly different. Scale bar = 10.00 mm. AW = abdominal wall fat pads deposited.

The liver relative wet weight of the OVX control mice (−23.90%) decreased against the sham control ($p < 0.01$). On the contrary, the relative liver of OVX mice administered with 200 mg/kg of WC (18.72%), 100 mg/kg of WC (15.78%), and 200 mg/kg of PCP (18.26%) was significantly increased ($p < 0.01$), against the OVX control group. Additionally, mice administered with 200 mg/kg of PCP and 200, 100, and 50 mg/kg of WC did not show any significant changes in liver absolute weight against the sham control and the OVX control group (Figure 2).

3.4. Effect on Femur Weight

The femur relative wet weight and absolute, relative dry, and ash weights of the OVX control mice were significantly reduced ($p < 0.01$) against the sham control. In contrast, the femur wet/dry/ash content in OVX mice administered 200 and 100 mg/kg of WC increased compared to the OVX control group. A total of 200 mg/kg of WC showed good inhibitory activity on the OVX-induced reduction in relative femur wet weights and absolute, relative dry, and ash weights when compared with the same dose of PCP. Additionally, there were no significant differences between the OVX control mice and those treated with 50 mg/kg of WC (Table S3).

3.5. Effect of Biochemistry on AST, ALT, TC, LDL, HDL, and TG

Serum AST (124.01%), ALT (178.53%), TC (179.55%), LDL (202.24%), and TG (414.36%) levels increased significantly ($p < 0.01$), and the serum HDL (−63.49%) level decreased significantly ($p < 0.01$) in the OVX control mice when comparing to the sham control. On the contrary, a consistent decrease in serum AST, ALT, TC, LDL, and TG levels was observed from 200 mg/kg of PCP ($p < 0.01$), while an increase in serum HDL was observed (−33.45%, −36.43%, −34.01%, −34.88%, −33.03%, and 100.51%, respectively) from 200 mg/kg of WC (−34.63%, −38.44%, −34.39%, −35.94%, −34.93%, and 106.84%, respectively) and 100 mg/kg of WC (−22.61%, −32.42%, −24.94%, −23.57%, −25.76%, and 76.96%, respectively) given to the OVX mice compared to the OVX control mice (Table 1).

Table 1. Effect of WC on AST, ALT, TC, LDL, HDL, and TG levels in OVX mice.

Groups	Serum Biochemistrical Values					
	AST (U/L)	ALT (U/L)	TC (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	TG (mg/dL)
Sham	75.80 ± 1.54	34.00 ± 10.12	75.30 ± 20.69	62.60 ± 11.93	108.20 ± 14.98	36.90 ± 14.99
OVX	169.80 ± 20.45 [c]	94.70 ± 19.41 [a]	210.50 ± 30.95 [a]	189.20 ± 23.04 [a]	39.50 ± 10.22 [a]	189.80 ± 23.60 [c]
PCP 200	113.00 ± 11.18 [cd]	60.20 ± 12.64 [ab]	138.90 ± 15.72 [ab]	123.20 ± 23.99 [ab]	79.20 ± 13.71 [ab]	127.10 ± 17.75 [cd]
WC 200	111.00 ± 11.52 [cd]	58.30 ± 11.53 [ab]	138.10 ± 17.28 [ab]	121.20 ± 18.14 [ab]	81.70 ± 12.98 [ab]	123.50 ± 22.29 [cd]
WC 100	131.40 ± 8.11 [cd]	64.00 ± 10.19 [ab]	158.00 ± 16.28 [ab]	144.60 ± 9.14 [ab]	69.90 ± 11.57 [ab]	140.90 ± 10.64 [cd]
WC 50	159.70 ± 15.87 [c]	87.20 ± 14.95 [a]	199.50 ± 22.88 [a]	178.50 ± 25.59 [ab]	41.40 ± 13.07 [a]	179.70 ± 32.94 [c]

Values are expressed mean ± SD of 10 mice. Statistical significance: [a] $p < 0.01$ vs. sham control and [b] $p < 0.01$ vs. OVX control by THSD test, and [c] $p < 0.01$ vs. sham control and [d] $p < 0.01$ vs. OVX control by DT3 test.

3.6. Effect on Serum Estradiol Levels

The OVX control mice (−86.60%) had a decreased serum estradiol concentration against the sham control ($p < 0.01$). In contrast, 200 mg/kg of PCP (154.15%) and 200 mg/kg (161.83%) and 100 mg/kg of WC(98.59%) given to OVX mice resulted in an increased trend of estradiol levels compared to the OVX control mice (Figure 3).
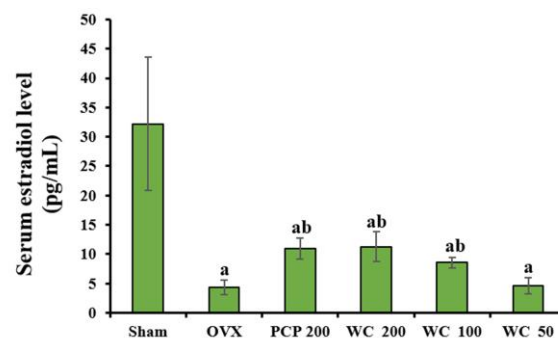

Figure 3. Effect of WC on serum estradiol levels in OVX mice. Statistical significance: a $p < 0.01$ vs. sham control and b $p < 0.01$ vs. OVX control by DT3 test. Values are expressed mean ± SD of 10 mice. Different letters indicate significantly different.

3.7. Effects on Serum Osteocalcin and bALP Levels

Significant ($p < 0.01$) increases in serum osteocalcin levels (144.59%) and significant ($p < 0.01$) decreases in serum bALP levels (64.80%) were observed in the OVX control mice against the sham control. Conversely, significant ($p < 0.01$) decreases in serum osteocalcin and non-significant increases in bALP levels were demonstrated in 200 mg/kg of PCP (−45.33% and 54.23%) and 200 mg/kg (−45.51% and 57.91%) and 100 mg/kg of WC (−36.18% and 33.01%) administered OVX mice against the OVX control (Figure 4).
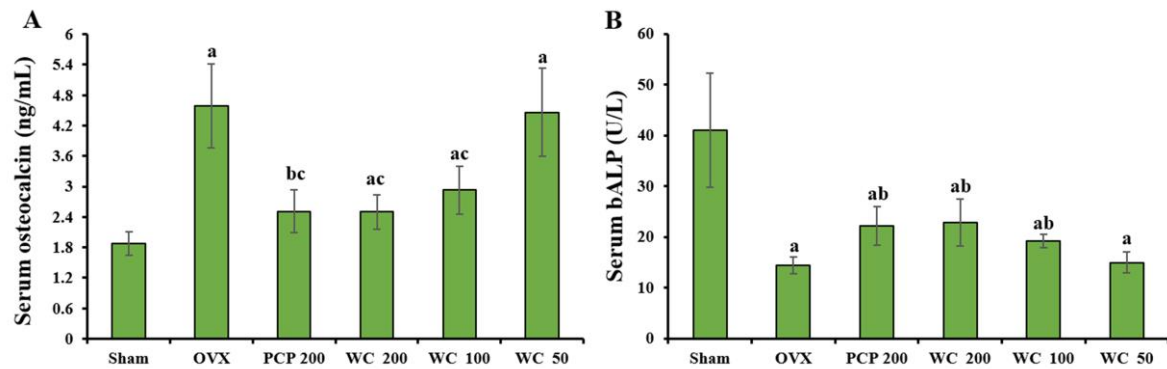
Figure 4. Effect of WC on serum osteocalcin (**A**) and bALP (**B**) levels in OVX mice. Statistical significance: a $p < 0.01$ and b $p < 0.01$ vs. sham control, and c $p < 0.01$ vs. OVX control by DT3 test. Values are expressed mean ± SD of 10 mice. Different letters indicate significantly different.

3.8. Effects on Hepatic mRNA Expression Related to Lipid Metabolism

A significant ($p < 0.01$) increase in hepatic ACCI mRNA expression and a decrease in hepatic AMPKα1 and AMPKα2 mRNA expressions were detected in the OVX control mice (382.65%, −70.61%, and −71.91%, respectively) against the sham control mice. However, these expressions were significantly ($p < 0.01$ or $p < 0.05$) normalized by 200 mg/kg of PCP (−35.97%, 71.48%, and 63.12%, respectively), 200 mg/kg of WC (−36.18%, 73.49%, and 58.87%, respectively), and 100 mg/kg of WC (27.97%, 45.64%, and 43.26%, respectively) administered to OVX mice compared to the OVX control mice (Figure 5). Additionally, there were no significant differences between the OVX control mice and those treated with 50 mg/kg of WC.
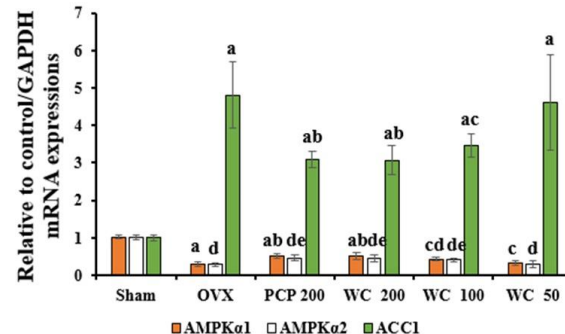

Figure 5. Effect of WC on the lipid metabolism-related hepatic AMPKα1, AMPKα2, and ACC1 mRNA expressions in OVX mice. Statistical significance: a $p < 0.01$ vs. sham control, and b $p < 0.01$ and c $p < 0.05$ vs. OVX control by DT3 test; d $p < 0.01$ vs. sham control and e $p < 0.01$ vs. OVX control by THSD test. Values are expressed the mean ± SD of 10 mice. Different letters indicate significantly different.

3.9. Effect of mRNA Expression on Lipid Metabolism-Related Genes in Adipose Tissue

Significant ($p < 0.01$) increases in leptin, C/EBPα, C/EBPβ, FAS, SREBP1c, and PPARγ mRNA expressions and decreases in UCP2, adiponectin, and PPARα mRNA expressions in adipose tissue were detected in the OVX control mice (560.49%, 202.65%, 294.90%, 1071.68%, 241.44%, 588.81%, −68.64%, −79.20%, and −79.60%, respectively) against the sham control. In contrast, these expressions were significantly ($p < 0.01$ or $p < 0.05$) normalized by 200 mg/kg of PCP (−41.58%, −34.31%, −31.55%, −30.11%, −41.78%, −38.95%, and 50.32%, 95.69%, and 62.56%),200 mg/kg of WC (−42.02%, 34.60%, −34.79%, −32.01%, −41.40%, and −39.04%, and 53.85%, 91.87%, and 67.49%), and 100 mg/kg of WC (−33.58%, −26.87%, −21.02%, −20.80%, −33.83%, and −26.84%, and 33.01%, 46.41%, and 35.47%) when given to OVX mice compared to the OVX control group (Table 2). Furthermore, no significant

differences were seen between the OVX control mice and those treated with 50 mg/kg of WC.

Table 2. Effect of WC on lipid metabolism-related mRNA expression in abdominal wall adipose tissue in OVX mice.

Relative to Control	Sham	OVX	PCP 200	WC 200	WC 100	WC 50
Leptin	1.03 ± 0.09	6.77 ± 1.23 [d]	3.96 ± 0.71 [de]	3.93 ± 0.64 [de]	4.50 ± 0.65 [de]	6.17 ± 1.58 [d]
UCP2	1.00 ± 0.05	0.31 ± 0.04 [d]	0.47 ± 0.07 [de]	0.48 ± 0.11 [df]	0.42 ± 0.04 [de]	0.32 ± 0.09 [d]
Adiponectin	1.01 ± 0.08	0.21 ± 0.03 [a]	0.41 ± 0.09 [ab]	0.40 ± 0.07 [ab]	0.31 ± 0.04 [ac]	0.22 ± 0.05 [a]
C/EBPα	1.02 ± 0.05	3.08 ± 0.39 [d]	2.02 ± 0.26 [de]	2.01 ± 0.21 [de]	2.25 ± 0.19 [de]	2.93 ± 0.74 [d]
C/EBPβ	1.00 ± 0.05	3.95 ± 0.42 [d]	2.70 ± 0.49 [de]	2.58 ± 0.55 [de]	3.12 ± 0.29 [de]	3.81 ± 0.80 [d]
SREBP1c	1.00 ± 0.07	3.41 ± 0.95 [d]	1.99 ± 0.14 [df]	2.00 ± 0.22 [df]	2.26 ± 0.17 [df]	3.28 ± 0.64 [d]
PPARα	1.00 ± 0.04	0.20 ± 0.03 [d]	0.33 ± 0.06 [de]	0.34 ± 0.06 [de]	0.28 ± 0.02 [de]	0.21 ± 0.03 [d]
PPARγ	1.01 ± 0.06	6.96 ± 1.08 [d]	4.25 ± 0.77 [de]	4.24 ± 0.47 [de]	5.09 ± 0.64 [de]	6.64 ± 1.26 [d]
FAS	1.02 ± 0.05	11.92 ± 1.11 [d]	8.33 ± 0.85 [de]	8.10 ± 0.95 [de]	9.44 ± 0.67 [de]	11.39 ± 1.61 [d]

Values are expressed mean ± SD of 10 mice. Statistical significance: [a] $p < 0.01$ vs. sham control, and [b] $p < 0.01$ and [c] $p < 0.01$ vs. OVX control by THSD test; [d] $p < 0.01$ vs. sham control, and [e] $p < 0.01$ and [f] $p < 0.01$ vs. OVX control by DT3 test.

3.10. Effect of In Vivo DEXA on BMD and Body Fat Density

The whole body and femur mean BMDs were significantly ($p < 0.01$) decreased in the OVX control mice (−15.92% and −14.96%) compared to the sham control mice. However, with 200 mg/kg of PCP (9.19% and 10.83%, respectively), 200 mg/kg of WC (9.33% and 11.61%, respectively), and 100 mg/kg of WC (6.44% and 8.05%, respectively), the whole body and femur mean BMDs were significantly ($p < 0.01$) increased in OVX mice against the OVX control group, exhibiting a dose-dependent trend (Figure 6). The total body and abdominal fat densities were increased significantly ($p < 0.01$) in the OVX control mice (122.66% and 129.36%, respectively) compared to the sham control group. However, with 200 mg/kg of PCP (−28.72% and −27.41%, respectively), 200 mg/kg of WC (−28.80% and −27.96%, respectively), and 100 mg/kg of WC (−19.59% and −18.56%, respectively), the total body and abdominal fat densities were significantly ($p < 0.01$) decreased in OVX mice against the OVX control group, showing a dose-dependent response (Figure 6).
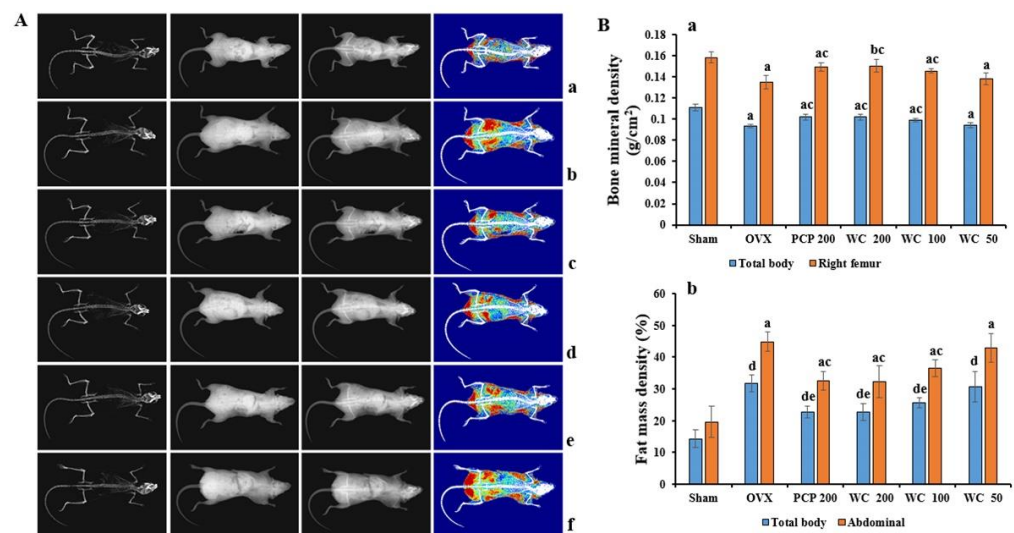

Figure 6. Representative whole-body DEXA images (**A**) of sham control (**a**), OVX control (**b**), 200 mg/kg of PCP (**c**), 200 mg/kg of WC (**d**), 100 mg/kg of WC (**e**), and 50 mg/kg of WC (**f**) administered to OVX mice. Analyses (**B**) for bone mineral density of the total body (the average density across the entire body) and the right femur (**a**) and fat mass density in the percentage of the total body and abdominal cavity (**b**). Statistical significance: a $p < 0.01$ and b $p < 0.05$ vs. sham

control, and c $p < 0.01$ by THSD test; d $p < 0.01$ vs. sham control and e $p < 0.01$ vs. OVX control by DT3 test. Values are expressed as the mean $\pm$ SD of 10 mice. Different letters indicate significantly different. DEXA = dual-energy X-ray absorptiometry.

3.11. Effect on Bone Strength

The strength (FL) of the femur midshaft region in the OVX control (-55.70%) mice was significantly reduced ($p < 0.01$) against the sham control. In contrast, an increase in the femoral FL was observed in OVX mice administered with 200 mg/kg of PCP (52.39%), 200 mg/kg of WC (53.73%), and 100 mg/kg of WC (39.37%) compared to the OVX control group, exhibiting a dose-dependent pattern (Figure 7).

Figure 7. Effect of WC on bone strength changes in OVX mice. Statistical significance: a $p < 0.01$ vs. sham control and b $p < 0.01$ vs. OVX control by DT3 test. Values are expressed mean $\pm$ SD of 10 mice. Different letters indicate significantly different.

3.12. Effects on Abdominal Wall Fat Pad, Liver, and Uterus Histopathology–Histomorphometry

There was a significant ($p < 0.01$) increase in abdominal wall fat pad thickness and the mean adipocyte diameter in the OVX control mice (235.86% and 185.02%), which was attributed to significant tissue accumulation and adipocyte hypertrophy, respectively. However, there were significant differences in abdominal wall fat thickness and the mean adipocyte diameter with 200 mg/kg of PCP (-46.11% and -46.04%), 200 mg/kg of WC (-46.61% and -47.78%), and 100 mg/kg of WC (-33.96% and -35.68%) administered to OVX mice against the OVX control mice, respectively (Figures 8 and S1).

It was determined that the percentage of fat transfer and the average hepatocyte diameter (1437.10% and 76.49%) increased significantly ($p < 0.01$) in the OVX control mice, respectively, due to lipid accumulation and steatosis. However, OVX mice given 200 mg/kg of PCP and 200 and 100 mg/kg of WC showed a decrease of -55.07%, -54.36%, and -38.96% in fat area and -31.32%, -31.47%, and -22.37% in the mean hepatocyte diameter compared to the OVX control group, respectively (Figures 8 and S2).

In the OVX control group, the total uterine thickness, mucosal thickness, and the percentage of uterine glands in the mucosa were decreased ($p < 0.01$) (-76.90%, -76.26%, -69.25%, and -78.53%, respectively), due to estrogen-depletion-related atrophic changes. However, significant ($p < 0.01$ or $p < 0.05$) increases in all these parameters with 200 mg/kg of PCP (58.99%, 46.81%, 105.18%, and 144.61%), 200 mg/kg of WC (56.34%, 51.64%, 113.57%, and 162.50%), and 100 mg/kg of WC (38.03%, 27.92%, 61.01%, and 98.39%) administered to OVX mice compared to the OVX control group, respectively, showing a dose-dependent pattern (Figures 8 and S3).

3.13. Effects on Bone Mass, Structures, and Resorption in Femur Histopathology

The femur TV/BV, Tbn, Tbl, and Tbt were significantly ($p < 0.01$) decreased in the OVX control group by -74.85, -65.57, -48.44, and -51.88%, respectively, against the sham control group. These reductions were significantly ($p < 0.01$) affected when doses of 200 mg/kg

of PCP and 200 and 100 mg/kg of WC were given to OVX mice, resulting in 150.86, 154.28, and 113.21% of femur TV/BV, 56.98, 56.53, and 31.96% of femur Tbl, 88.10, 92.86, and 47.62% of femur Tbn, and 64.98, 69.33, and 36.80% of femur Tbt compared to the OVX control mice, respectively (Figure 9). The femur Ocn and OS/BS were increased by 18.60 and 381.36%, respectively, in OVX control mice, against the sham control group ($p < 0.01$). However, these increases were reduced ($p < 0.01$) in mice given doses of 200 mg/kg of PCP and 200 and 100 mg/kg of WC, showing reductions of −39.44, −38.89, and −28.33% of femur Ocn, and −44.78, −44.65, and −29.74% of femur OS/BS, respectively (Figure 9).

Figure 8. Effect of WC on the abdominal wall fat pad (**A**,**B**), liver (**C**,**D**), and uterus (**E–H**) histopathology–histomorphometry changes in OVX mice. Statistical significance: a $p < 0.01$ and b $p < 0.05$ vs. sham control, and c $p < 0.01$ vs. OVX control by DT3 test; d $p < 0.01$ vs. sham control and e $p < 0.01$ and f $p < 0.05$ vs. OVX control by THSD test. Values are expressed as the mean ± SD of 10 mice. Different letters indicate significantly different.
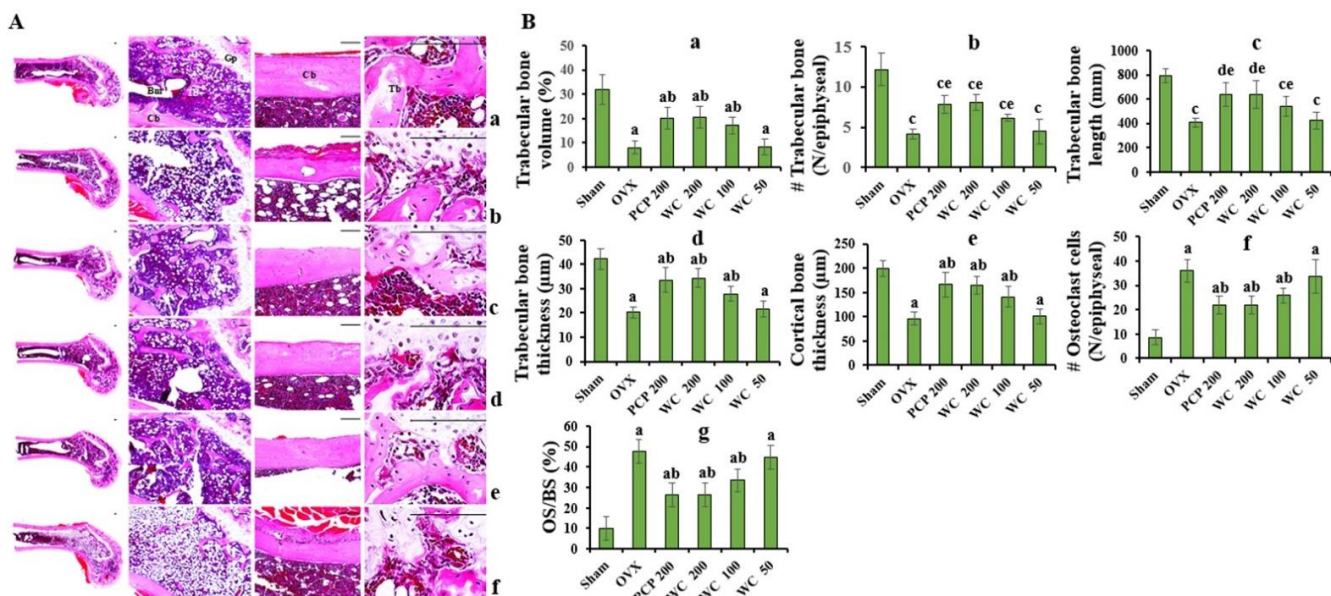

Figure 9. Effect of WC on histopathological profiles (**A**) of sham control (**a**), OVX control (**b**), PCP 200 (**c**), WC 200 (**d**), 100 (**e**) and 50 (**f**) mg/kg administered OVX mice for trabecular bone volume, structures and resorption analyses (**Ba–g**). Statistical significance: a $p < 0.01$ vs. sham control and b $p < 0.01$ vs. OVX control by THSD test; c $p < 0.01$ and d $p < 0.05$ vs. sham control, and e $p < 0.01$ vs. OVX control

by DT3 test. Values are expressed mean ± SD of 10 mice. Different letters indicate significantly different. Hematoxylin and eosin stain. Scale bars = 80 μm. Cb = cortical bone; Tb = trabecular bone; Bm = bone marrow; Gp = growth plate; and OS/BS = osteoclast cell surface/bone surface.

4. Discussion

Climacteric symptoms associated with estrogen deficiency in the postmenopausal period include depression, insomnia, cognitive impairment, irritability, fatigue, psychological problems, and an increased risk of osteoporosis and cardiovascular disease [3,4]. Postmenopausal status is correlated with an increased risk of metabolic diseases, including obesity, cardiovascular disease, diabetes, and hypertension [5,6]. Although hormonal therapy is often recommended to reduce the effects of ovarian failure on women's health, safety concerns have been reported, particularly the risk of heart disease and long-term cancer [23]. Researchers have investigated alternative treatments such as phytoestrogens to reduce menopausal symptoms [1,7,24,25]. Phytohormones are obtained from plants and contain pure compounds that increase their bioavailability and activity in the body [24]. Phytoestrogens, such as coumestrol and isoflavones found in soybeans and alfalfa, bind to ER- α and ER- β and are estrogen-like due to their structural similarities to estrogens [26,27]. Isoflavones are known for their distinct biological activities and have been associated with improvements in metabolic symptoms [28] and bone protection during pregnancy [29]. Additionally, water olive extract is known for its anti-inflammatory and antioxidant potential and shows many medicinal activities [8,10–12,30]. In particular, previous experiments have shown that peanut regulates and controls blood sugar enzyme activity, gene expression related to lipid metabolism, the antioxidant defense system, and pancreatic lipid digestive enzyme activity with AMPK upregulation and modulation [11,12], and those effects have collectively shown anti-diabetic activity, as compared to metformin.

In our experiment, OVX surgery induced notable increases in various parameters such as body weight, food intake, fat mass densities, abdominal wall fat weights, serum markers, and adverse histopathological changes, while causing reductions in uterine, liver, and femur weights, bone mineral density, femoral strength, and hormone levels. The observed histological alterations included hypertrophic adipocytes, a fatty liver, an atrophic uterus, and compromised bone mass. From a molecular perspective, the OVX control mice exhibited altered gene expressions related to AMPK, leptin, and other factors. This aligns with estrogen deficiency-induced climacteric symptoms, such as obesity, hyperlipidemia, hepatic steatosis, and osteoporosis. Isoflavones can bind to estrogen receptors (ERs), particularly ERβ, and modulate gene expression similarly to estrogen. In the context of WC, isoflavones could act on ERs in the adipose tissue and bone, leading to reduced fat accumulation and increased bone density [31]. Moreover, lignans are another class of phytoestrogens, including enterodiol and enterolactone; they may influence fat distribution and bone health by modulating estrogenic activities in target tissues [32]. The exact content of these compounds in WC extract should be analyzed to understand their contribution in this study. Continuous 84-day treatment with WC (at 200 and 100 mg/kg doses) significantly mitigated these OVX-induced symptoms. Notably, 200 mg/kg of WC showed superior efficacy compared to an equal dose of PCP. However, no significant effects were observed with 50 mg/kg of WC. Previous studies demonstrated that pomegranate extract alone or mixed with other natural products was shown to be able to lessen climacteric symptoms in OVX mice by decreasing blood lipid levels, body weight growth, and the deposition of belly fat [17,18]. These results are supported by the current investigation, which found that WC extract also significantly improved blood lipid profiles (AST, ALT, TC, LDL, and TG levels) and decreased obesity indicators in a dose-dependent way. These research works emphasize how phytoestrogens and natural antioxidants influence lipid metabolism and decrease obesity via similar pathways. These results indicate that WC holds promise as a potential herbal formulation for managing menopausal symptoms. While further research is essential for comprehensive evaluation and potential applications, these findings suggest

WC as a candidate for pharmaceutical or nutraceutical development pending additional animal studies and clinical trials.

The fruit extract of water chestnut (*Trapa natans*) includes considerable levels of phenolic, flavonoid, and tannin components, measured in equivalents per gram of dry matter [33]. The total phenolic content is calculated as 63.81 mg of gallic acid equivalents per gram of dry material, showing a high concentration of phenolic substances recognized for their antioxidant qualities. The flavonoid concentration is 21.34 mg of rutin equivalents per gram, indicating the presence of chemicals that can promote anti-inflammatory responses and improve cardiovascular health. Additionally, the tannin concentration is 17.11 mg per gram, indicating the extract's astringent characteristics and probable antibacterial action. The methanolic leaf extract of *Trapa natans* L. contains a wide range of phenolic chemicals, including phenolic acids and flavonoids [34]. Gallic acid and its derivatives, such as gallic acid hexoside, protocatechuic acid, and ellagic acid, are among the phenolic acids that are known for their potent antioxidant properties. Flavonoids like quercetin, naringenin, and rhamnetin promote anti-inflammatory responses and improve cardiovascular health. The extract also contains flavonoid glycosides such as rutin and astragalin, which increase bioavailability and therapeutic efficacy. These polar phytochemicals are expected to be present in WC water extract and this emphasizes its potential health advantages for managing climacteric symptoms in women and applications of WC extract in functional foods and nutraceuticals.

Moreover, WC extract is particularly abundant in antioxidants such as flavonoids and phenolic compounds, which play crucial roles in combating oxidative stress and reducing inflammation in the body [35]. These bioactive compounds have been shown to scavenge free radicals, thereby protecting cells from damage and lowering the risk of chronic diseases such as cardiovascular disorders and cancer [36,37]. Moreover, WC fruit extract is packed with essential vitamins and minerals, including vitamin C and potassium, which are vital for maintaining immune function and electrolyte balance [35]. Studies have also indicated the presence of phenolic and flavonoid compounds in WC extract, which exhibit antimicrobial antibiofilm properties and may help in combating various pathogens [38]. Furthermore, research suggests that the phytochemicals present in this WC root extract could have potential antidiabetic effects in rat models through suppressing hyperglycemic and hepatotoxic effects, aiding in the management of diabetes, and improving metabolic health [39]. All of these findings were significantly justified by the present study investigated in an in vivo obese climacteric mice model, making it a valuable natural resource for promoting human health. However, future studies should focus on conducting comprehensive phytochemical analyses to elucidate the chemical composition of WC extract and its potential synergistic interactions, providing a more robust foundation for its therapeutic application in treating climacteric symptoms and related health complications.

5. Conclusions

This study reveals that WC, at doses of 200 and 100 mg/kg, consistently exhibits inhibitory effects on estrogen deficiency-induced climacteric symptoms in OVX-ICR mice. These symptoms include obesity, hyperlipidemia, hepatic steatosis, and osteoporosis, commonly associated with AMPK downregulation. The beneficial effects of WC are found to be comparable to the effects of the same dose of PCP. However, this study indicates that the 50 mg/kg dose did not show significant effects in the given experimental conditions. This suggests that WC, particularly at 200 and 100 mg/kg oral dosages, holds promise as a potential herbal preparation for refining agents or medicinal food ingredients targeting various menopausal symptoms.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app14177464/s1, Figure S1: Representative histological images of the adipocytes; Figure S2: Representative histological images of the left lateral lobes of the liver; Figure S3: Representative histological images of the left uterus horn; Table S1: Oligonucleotides for

realtime RT-PCR used in this study; Table S2: Effect of WC on food consumptions in OVX mice; Table S3: Effect of WC on right femur weights in OVX mice.

References

1. Del Giorno, C.; Da Fonseca, A.M.; Bagnoli, V.R.; De Assis, J.S.; Soares, J.M., Jr.; Baracat, E.C. Effects of Trifolium pratense on the climacteric and sexual symptoms in postmenopausal women. *Rev. Assoc. Med. Bras.* **2010**, *56*, 558–562.
2. Speroff, L.; Barnhart, K.T.; Gonzalez, J. The menopause: A signal for the future. In *Treatment of the Postmenopausal Woman*, 2nd ed.; Elsevier: Amsterdam, The Netherlands, 2007; pp. 1–13.
3. The, N. The 2017 hormone therapy position statement of The North American Menopause Society. *Menopause* **2017**, *24*, 728–753.
4. Zhang, G.-Q.; Chen, J.-L.; Luo, Y.; Mathur, M.B.; Anagnostis, P.; Nurmatov, U.; Talibov, M.; Zhang, J.; Hawrylowicz, C.M.; Lumsden, M.A. Menopausal hormone therapy and women's health: An umbrella review. *PLoS Med.* **2021**, *18*, e1003731. [CrossRef] [PubMed]
5. Choi, J.S.; Koh, I.-U.; Song, J. Genistein reduced insulin resistance index through modulating lipid metabolism in ovariectomized rats. *Nutr. Res.* **2012**, *32*, 844–855. [CrossRef]
6. van Seumeren, I. Weight gain and hormone replacement therapy: Are women's fears justified? *Maturitas* **2000**, *34*, S3–S8. [CrossRef] [PubMed]
7. Kang, S.J.; Choi, B.R.; Kim, S.H.; Yi, H.Y.; Park, H.R.; Kim, D.C.; Choi, S.H.; Han, C.H.; Park, S.J.; Song, C.H. Dried pomegranate potentiates anti-osteoporotic and anti-obesity activities of red clover dry extracts in ovariectomized rats. *Nutrients* **2015**, *7*, 2622–2647. [CrossRef]
8. Kim, Y.-S.; Hwang, J.-W.; Jang, J.-H.; Son, S.; Seo, I.-B.; Jeong, J.-H.; Kim, E.-H.; Moon, S.-H.; Jeon, B.-T.; Park, P.-J. Trapa japonica pericarp extract reduces LPS-induced inflammation in macrophages and acute lung injury in mice. *Molecules* **2016**, *21*, 392. [CrossRef]
9. Lee, D.; Lee, O.-H.; Choi, G.; Dai Kim, J. Antioxidant and anti-adipogenic activities of *Trapa japonica* shell extract cultivated in Korea. *Prev. Nutr. Food Sci.* **2017**, *22*, 327. [CrossRef] [PubMed]
10. Park, C.; Hwang, Y.; Hwang, B.S.; Shin, S.Y.; Cho, P.Y.; Lee, S.Y.; Choi, K.-M.; Lee, K.W.; Kim, G.-Y.; Cho, Y.H. Trapa japonica inhibits adipocyte differentiation and adipogenesis through AMPK signaling pathway in 3T3-L1 pre-adipocytes. *Int. J. Food Sci. Nutr. Res.* **2019**, *1*, 1008. [CrossRef]
11. Kang, M.-J.; Lee, S.-K.; Song, J.-H.; Kim, M.-E.; Kim, M.-J.; Jang, J.-S.; Lee, J.-H.; Kim, J.-I. Water chestnut (*Trapa japonica* Flerov.) exerts inhibitory effect on postprandial glycemic response in rats and free radical scavenging activity in vitro. *Food Sci. Biotechnol.* **2009**, *18*, 808–812.
12. Kim, B.; Kim, J.E.; Choi, B.-K.; Kim, H.-S. Anti-inflammatory effects of water chestnut extract on cytokine responses via nuclear factor-κB-signaling pathway. *Biomol. Ther.* **2015**, *23*, 90. [CrossRef] [PubMed]
13. Huang, H.-C.; Chao, C.-L.; Liaw, C.-C.; Hwang, S.-Y.; Kuo, Y.-H.; Chang, T.-C.; Chao, C.-H.; Chen, C.-J.; Kuo, Y.-H. Hypoglycemic constituents isolated from Trapa natans L. pericarps. *J. Agric. Food Chem.* **2016**, *64*, 3794–3803. [CrossRef] [PubMed]
14. Malviya, N.; Jain, S.; Jain, A.; Jain, S.; Gurjar, R. Evaluation of in vitro antioxidant potential of aqueous extract of *Trapa natans* L. fruits. *Acta Pol. Pharm.* **2010**, *67*, 391–396. [PubMed]

15. Kaur, K.; Kaur, G.; Singh, A. Water chestnut starch: Extraction, chemical composition, properties, modifications, and application concerns. *Sustain. Food Technol.* **2023**, *1*, 228–262. [CrossRef]

16. Cho, C.-S.; Jeong, H.-S.; Kim, I.-Y.; Jung, G.-W.; Ku, B.-H.; Park, D.-C.; Moon, S.-B.; Cho, H.-R.; Bashir, K.M.I.; Ku, S.K. Anti-osteoporotic effects of mixed compositions of extracellular polymers isolated from *Aureobasidium pullulans* and *Textoria morbifera* in ovariectomized mice. *BMC Complement. Altern. Med.* **2018**, *18*, 295. [CrossRef]

17. Kang, S.J.; Choi, B.R.; Kim, S.H.; Yi, H.Y.; Park, H.R.; Song, C.H.; Ku, S.K.; Lee, Y.J. Selection of the optimal herbal compositions of red clover and pomegranate according to their protective effect against climacteric symptoms in ovariectomized mice. *Nutrients* **2016**, *8*, 447. [CrossRef]

18. Kang, S.J.; Choi, B.R.; Kim, S.H.; Yi, H.Y.; Park, H.R.; Song, C.H.; Ku, S.K.; Lee, Y.J. Anti-climacterium effects of pomegranate concentrated solutions in ovariectomized ddY mice. *Exp. Ther. Med.* **2017**, *13*, 1249–1266. [CrossRef] [PubMed]

19. Jacobs, A.J.; Roskam, A.L.; Hummel, F.M.; Ronan, P.J.; Gorres-Martens, B.K. Exercise improves high-fat diet-and ovariectomy-induced insulin resistance in rats with altered hepatic fat regulation. *Curr. Res. Physiol.* **2020**, *3*, 11–19. [CrossRef]

20. Sung, Y.-Y.; Kim, D.-S.; Kim, S.-H.; Kim, H.K. Anti-obesity activity, acute toxicity, and chemical constituents of aqueous and ethanol *Viola mandshurica* extracts. *BMC Complement. Altern. Med.* **2017**, *17*, 297. [CrossRef]

21. Sutjarit, N.; Sueajai, J.; Boonmuen, N.; Sornkaew, N.; Suksamrarn, A.; Tuchinda, P.; Zhu, W.; Weerachayaphorn, J.; Piyachaturawat, P. *Curcuma comosa* reduces visceral adipose tissue and improves dyslipidemia in ovariectomized rats. *J. Ethnopharmacol.* **2018**, *215*, 167–175. [CrossRef]

22. Veiga, F.M.S.; Graus-Nunes, F.; Rachid, T.L.; Barreto, A.B.; Mandarim-de-Lacerda, C.A.; Souza-Mello, V. Anti-obesogenic effects of WY14643 (PPAR-alpha agonist): Hepatic mitochondrial enhancement and suppressed lipogenic pathway in diet-induced obese mice. *Biochimie* **2017**, *140*, 106–116. [CrossRef]

23. Kaari, C.; Abi Haidar, M.; Júnior, J.M.S.; Nunes, M.G.; de Azevedo Quadros, L.G.; Kemp, C.; Stavale, J.N.; Baracat, E.C. Randomized clinical trial comparing conjugated equine estrogens and isoflavones in postmenopausal women: A pilot study. *Maturitas* **2006**, *53*, 49–58. [CrossRef] [PubMed]

24. Knight, D.C.; Eden, J.A. A review of the clinical effects of phytoestrogens. *Obstet. Gynecol.* **1996**, *87*, 897–904. [PubMed]

25. Setchell, K.D. Phytoestrogens: The biochemistry, physiology, and implications for human health of soy isoflavones. *Am. J. Clin. Nutr.* **1998**, *68*, 1333S–1346S.

26. Hedelin, M.; Klint, Å.; Chang, E.T.; Bellocco, R.; Johansson, J.-E.; Andersson, S.-O.; Heinonen, S.-M.; Adlercreutz, H.; Adami, H.-O.; Grönberg, H. Dietary phytoestrogen, serum enterolactone and risk of prostate cancer: The cancer prostate Sweden study (Sweden). *Cancer Causes Control* **2006**, *17*, 169–180. [CrossRef] [PubMed]

27. Kuiper, G.G.J.M.; Carlsson, B.O.; Grandien, K.A.J.; Enmark, E.; Häggblad, J.; Nilsson, S.; Gustafsson, J.-A.k. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. *Endocrinology* **1997**, *138*, 863–870. [CrossRef] [PubMed]

28. Taku, K.; Umegaki, K.; Sato, Y.; Taki, Y.; Endoh, K.; Watanabe, S. Soy isoflavones lower serum total and LDL cholesterol in humans: A meta-analysis of 11 randomized controlled trials. *Am. J. Clin. Nutr.* **2007**, *85*, 1148–1156. [CrossRef] [PubMed]

29. Ma, D.-F.; Qin, L.-Q.; Wang, P.-Y.; Katoh, R. Soy isoflavone intake increases bone mineral density in the spine of menopausal women: Meta-analysis of randomized controlled trials. *Clin. Nutr.* **2008**, *27*, 57–64. [CrossRef]

30. Yasuda, M.; Yasutake, K.; Hino, M.; Ohwatari, H.; Ohmagari, N.; Takedomi, K.; Tanaka, T.; Nonaka, G.-i. Inhibitory effects of polyphenols from water chestnut (*Trapa japonica*) husk on glycolytic enzymes and postprandial blood glucose elevation in mice. *Food Chem.* **2014**, *165*, 42–49. [CrossRef]

31. Vitale, D.C.; Piazza, C.; Melilli, B.; Drago, F.; Salomone, S. Isoflavones: Estrogenic activity, biological effect and bioavailability. *Eur. J. Drug Metab. Pharmacokinet.* **2013**, *38*, 15–25. [CrossRef]

32. Rietjens, I.M.C.M.; Louisse, J.; Beekmann, K. The potential health effects of dietary phytoestrogens. *Br. J. Pharmacol.* **2017**, *174*, 1263–1280. [CrossRef] [PubMed]

33. Rajput, J.D.; Singh, S.P. Water Chestnut (*Trapa natans* L.): Functional characteristics, nutritional properties and applications in food industry: A review. *J. Phytopharm.* **2023**, *12*, 119–126. [CrossRef]

34. Aleksic, I.; Ristivojevic, P.; Pavic, A.; Radojević, I.; Čomić, L.R.; Vasiljevic, B.; Opsenica, D.; Milojković-Opsenica, D.; Senerovic, L. Anti-quorum sensing activity, toxicity in zebrafish (Danio rerio) embryos and phytochemical characterization of *Trapa natans* leaf extracts. *J. Ethnopharmacol.* **2018**, *222*, 148–158. [CrossRef]

35. Garg, S.; Anvar Hussain, N.A.; Syed, I.; Asaithambi, N.; Mundhada, S. Water Chestnut (*Trapa natans*). In *Antioxidants in Vegetables and Nuts—Properties and Health Benefits*; Nayik, G.A., Gull, A., Eds.; Springer: Singapore, 2020; pp. 453–465.

36. Kris-Etherton, P.M.; Lichtenstein, A.H.; Howard, B.V.; Steinberg, D.; Witztum, J.L. Antioxidant vitamin supplements and cardiovascular disease. *Circulation* **2004**, *110*, 637–641. [CrossRef] [PubMed]

37. Serafini, M.; Bellocco, R.; Wolk, A.; Ekström, A.M. Total antioxidant potential of fruit and vegetables and risk of gastric cancer. *Gastroenterology* **2002**, *123*, 985–991. [CrossRef] [PubMed]

38.   Radojevic, I.D.; Vasic, S.M.; Dekic, M.S.; Radulovic, N.S.; Delic, G.T.; Durdevic, J.S.; Comic, L.R. Antimicrobial and antibiofilm effects of extracts from *trapa natans* L., evaluation of total phenolic and flavonoid contents and gc-ms analysis. *Acta Pol. Pharm.* **2016**, *73*, 1565–1574. [PubMed]

39.   Kharbanda, C.; Alam, M.S.; Hamid, H.; Bano, S.; Haider, S.; Nazreen, S.; Ali, Y.; Javed, K. *Trapa natans* L. root extract suppresses hyperglycemic and hepatotoxic effects in STZ-induced diabetic rat model. *J. Ethnopharmacol.* **2014**, *151*, 931–936. [CrossRef] [PubMed]