Maejo Int. J. Sci. Technol. **2025**, *19*(03), 232-248

Maejo International Journal of Science and Technology

e-SSN 2697-4746
Available online at www.mijst.mju.ac.th

Full Paper

Dioscorea polystachya extract treats menopause and osteoporosis in ovariectomised female mice

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Received: 6 February 2025 / Accepted: 2 October 2025 / Published: 27 October 2025

Abstract: Pathological osteoporosis (OP) reduces bone strength and density. Menopause is a significant factor contributing to the development of OP. Postmenopausal women lack oestrogen which boosts osteoclastic activity and reduces osteoblast cell activity. This research evaluates the impact of *Dioscorea polystachya* (DP) aqueous extract on ovarian function in ovariectomised (OVX) female mice. DP (5-40 µg/mL) was applied to MC3T3-E1 cells for 14 days. The impact on cellular changes was assessed using alkaline phosphatase (ALP) and alizarin red S (ARS) staining. The DP's effects on OP were evaluated in an OVX mouse model. Blood was withdrawn to measure oestradiol and osteocalcin concentrations. A micro-CT scan assessed bone mineral density (BMD), bone microstructure and femur tissue. OVX mouse femurs were histopathologically investigated. The DP group showed considerable improvement in ALP and ARS staining activity compared to the control group. Bone morphogenetic protein 2/Runt-related transcription factor 2 (BMP2/4/Runx2) expression was significantly greater in the DP groups compared to the control group. DP significantly raised serum oestradiol and osteocalcin levels in the tested groups. DP-treated groups expressed higher levels of runt-related transcription factor 2 (Runx2), bone morphogenetic protein 2/4, osteoprotegerin and phosphorylated Smad2/3 than the OVX group. DP enhances the bone tissue architecture and BMD of OVX mice. The study found that DP promotes osteoblasts via the BMP-2/4 and Runx2 pathway in OVX female mice, alleviating menopausal symptoms.

Keywords: *Dioscorea polystachya*, osteoporosis, osteoblasts, ovariectomy, menopause

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INTRODUCTION

Osteoporosis (OP) is a medical condition characterised by decreased bone strength and density. This results in a decrease in bone structure and makes them more prone to breaking due to loss of bone mass [1]. Menopause is a significant factor contributing to the development of OP. Postmenopausal women have an oestrogen shortage, resulting in heightened osteoclastic cell activity and decreased osteoblast cell activity. In a study performed in China in 2015 by the Chinese Centre for Disease Control and Prevention on 160 million OP patients, out of whom more than 12.4% are postmenopausal women, dysregulation of bone homeostasis in OP occurs due to hormonal imbalance and alteration of several signalling pathways [2]. In the process of bone formation, osteoblasts release alkaline phosphatase (ALP), Specificity Protein 7 transcription factor and Runt-related transcription factor 2 (Runx2), which stimulate osteoblasts, osteoprotegerin (OPG) and osteopontin, which inhibit the functions of osteoclats and lead to improvements in the process of bone formation. Reported studies suggest that oestrogen promotes bone morphogenetic protein 2/4 (BMP-2/4) and its receptor expression, which leads to the activation of Runx2 and promotes osteoblast cell action such as proliferation and mineralisation [3]. Menopause alters this pathway, contributing to the imbalance of osteoclast and osteoblast cell function and leading to the development of OP. The present therapy available for the management of postmenopausal OP includes hormone replacement therapy, which has several limitations. Thus, the development of alternative treatments is required for the management of OP among postmenopausal women.

Herbal therapy has shown promise in the treatment of chronic conditions such as OP. In China *Dioscorea* species are widely recognised for their health benefits particularly in alleviating degenerative conditions such as hypertension and osteoarthritis [4]. Research has also emphasised their relevance in lowering the risk of ovarian cancer, alleviating menopausal complications and addressing age-related disorders in women. Findings from animal studies highlight the potential of these species in preventing OP [5]. Extracts from *D. alata* leaves and roots were shown to promote the proliferation of spleen and bone marrow cells in mice, resulting in a marked increase in cell numbers [6]. In addition, Wu et al. [7] demonstrated that diosgenin reduced apoptosis in the hearts of ovariectomised mice, indicating its therapeutic value for postmenopausal cardiac health. Han et al. [8] reported that ethanol extracts of *D. spongiosa* alleviated glucocorticoid-induced OP in rats while Obidiegwu et al. [9] found that administering *D. alata* powder for two weeks to ovariectomised BALB/C mice prevented bone mineral density loss and improved bone calcium levels. Kim et al. [10] further demonstrated that treatment with *D. batatas* increased ALP activity in MC3T3-E1 cells, thereby counteracting the reduction typically associated with postmenopausal states. Similarly, Azam et al. [11] also observed that bark extracts of *D. batatas* stimulated osteogenesis by enhancing osteoblast proliferation and differentiation, along with increasing collagen synthesis, ALP activity and deposition of calcium and phosphorus.

Among *Dioscorea* species, *Dioscorea polystachya* (DP), commonly known as Chinese yam, has been traditionally used in China [12]. However, detailed investigations into its biomedical and pharmacological effects remain limited. Evidence suggests that DP may improve blood glucose regulation, reduce inflammation and alleviate oxidative stress by enhancing the growth of *Bifidobacterium* in the gut microbiota [13]. DP is also reported to prevent alcohol-induced osteopenia as it promotes bone mineral density (BMD) by enhancing bone resorption through activating osteoblast cell activity and attenuating the activity of ALP [14]. Moreover, Chinese yam is also reported to affect oestrogen receptors as it contains an arbutin compound that acts on

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estrogen receptor alpha and estrogen receptor beta [15]. However, no reliable experiments have been conducted to ascertain the extent to which DP extract contributes to the regulation of OP activity. Hence the present study aims to evaluate the impact of the DP on the BMP-2/4/Runx2 pathway in reducing menopausal symptoms and preventing OP in ovariectomised (OVX) female mice.

MATERIALS AND METHODS

Extraction of DP

DP was acquired from a local market in Zhejiang, China and taxonomically authenticated by the College of Agriculture and Biotechnology, Zhejiang University, China. The tuberous roots were washed, cut into small sections, air-dried and then coarsely powdered. The dried powder (750 g) was extracted with 70% ethanol by maceration for 24 hr at room temperature. The extract was filtered through Whatman® no. 1 filter paper and the solvent was evaporated under reduced pressure using a rotary evaporator (R-300, Büchi, Switzerland) at 28°C. The resulting residue (22.60 g) was stored at 4°C until further use.

Partial purification of the crude extract (20 g) was carried out by column chromatography. A standard glass column (120 cm × 2 cm i.d.) was packed with silica gel (200–400 mesh, 15 g). The crude extract was carefully loaded onto the top of the silica gel bed and elution was performed under gravity flow using 70% ethanol. This process facilitated partial purification of the extract by removing residual plant-derived impurities retained in the upper portion of the silica gel bed. The solvent was removed to afford the partially purified DP extract (1.89 g).

High Performance Liquid Chromatography (HPLC)

The chemical composition of the samples was analysed using HPLC (UFLC SHIMADZU CBM20A system, Shimadzu, Japan) coupled to a high-resolution mass spectrometer (AB Sciex TripleTOF 5600/5500 QTRAP, USA). Chromatographic separation was performed on a C18 analytical column (2.1 × 100 mm, 1.7 µm) maintained at 40 °C. The mobile phases consisted of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) delivered at a flow rate of 0.3 mL/min. using a gradient elution program. The injection volume was 2 µL and the autosampler was maintained at 8°C.

Mass spectrometric detection was carried out in both positive and negative electrospray ionisation modes. The instrument was operated under the following conditions: ion spray voltage +5500 V/–4500 V, source temperature 500 °C, curtain gas (nitrogen) 30 psi and ion source gases 1 (nebuliser gas) and 2 (heater gas) at 50 psi each. Full-scan spectra were acquired over the *m/z* range of 100–1200 and information-dependent acquisition was applied to obtain MS/MS spectra of the most intense precursor ions. Quantification of metabolites was performed using a predefined multiple reaction monitoring method [16]. Data acquisition and processing were performed using AB Sciex MultiQuant™ 2.1 software (research version) (AB Sciex, USA). Compound identification was achieved by comparing accurate mass measurements, retention times and fragmentation patterns with those of reference standards and databases.

Cell Culture and Proliferation

Mouse pre-osteoblast MC3T3-E1 subclone four cells were obtained from the American Type Culture Collection (Manassas, USA). Cells were maintained in alpha minimum essential medium,

which consists of inorganic salts (NaCl, KCl, $CaCl_2$, $MgSO_4$, $NaHCO_3$, NaH_2PO_4), a set of amino acids, vitamins (folic acid, riboflavin, thiamine, pyridoxal, nicotinamide, inositol and choline), D-glucose, sodium pyruvate and phenol red (Solarbio Life Sciences, China) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin–streptomycin. Cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 (Thermo Scientific, USA). The medium was refreshed every 2–3 days and cells were sub-cultured at 70–80% confluence using 0.25% trypsin-EDTA solution.

For proliferation assays, cells were seeded into 96-well plates at a density of 2×10^4 cells/well and allowed to adhere overnight. Subsequently, the cells were treated with different concentrations of DP extract (5, 10, 20 and 40 µg/mL) for 72 hr under standard culture conditions. After incubation, cell growth and viability were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay. Absorbance was measured at 570 nm using a multimode microplate reader (UB-DR-200B, Ybo Technologies Co., China). Cell proliferation was expressed as a percentage relative to the untreated control group.

RNA Extraction and qRT-PCR

MC3T3-E1 cells were seeded into 6-well plates at a density of 2×10^4 cells/well and allowed to adhere for 24 hr. Cells were then treated with DP extract at concentrations ranging from 5 to 40 µg/mL for 24 hr while control cells received only vehicle. After treatment, total RNA was extracted using a total RNA isolation kit (Tiangen Biotech Co., China) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesised using a reverse transcription kit (QIAGEN China Co., China). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SYBR Green master mix on an ABI 7500 Real-Time PCR System (Thermo Fisher Scientific, USA). Primers specific for osteocalcin (OCN), Runx2 and OPG were used, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control. Primers specific for OCN, Runx2, OPG and GAPDH were used for qRT-PCR analysis. The primer sequences were as follows: OCN forward 5′-CACTCCTCGCCCTATTGGC-3′ and reverse 5′-CCCTCCTGCTTG GACACAAAG-3′; Runx2 forward 5′-CCTGAACTCTGCACCAAGTCCT-3′ and reverse 5′-TCGT TGAACCTTGCTACTTGGG-3′; OPG forward 5′-AGCCTTGCCCTGACCACTCTTAT-3′ and reverse 5′-ACACTCAGTTATAGCCAAGGCAGT-3′ (163 bp); GAPDH forward 5′-AGGTCGGTGTGAACGGATTTG-3′ and reverse 5′-TGTAGACCATGTAGTTGAGGTCA-3′. Relative gene expression was calculated using the comparative cycle threshold method.

ALP Activity

MC3T3-E1 pre-osteoblastic cells were seeded into 12-well culture plates at a density of 2×10^4 cells/well and cultured under standard conditions. Osteogenic differentiation was induced by supplementing the medium with L-ascorbic acid (50 µg/mL) and β-glycerophosphate (10 mM) (Sigma-Aldrich, USA). After 7 days of induction, cells were treated with different concentrations of DP extract (5, 10, 20 and 40 µg/mL). In parallel, cells were also pretreated with inhibitors consisting of ICI182.780 (10 µM) and Noggin (200 ng/mL) for 1 hr prior to DP administration to evaluate pathway-specific effects. Treatments were refreshed every 3 days to maintain experimental conditions.

On the 7th day, cells were washed twice with phosphate-buffered saline (PBS) and lysed using the lysis buffer provided in the para-nitrophenyl phosphate (pNPP) ALP assay kit (SensoLyte®, USA). The supernatant was collected after centrifugation at 12,000 rpm for 10 min. at 4 °C. ALP

activity was then measured using the pNPP ALP assay kit according to the manufacturer's instructions. Briefly, pNPP substrate was added to the lysates and incubated at 37 °C until a yellow colour developed due to the enzymatic hydrolysis of pNPP. The reaction was terminated with stop solution and absorbance was measured at 405 nm using a multimode microplate reader **(**UB-DR-200B**,** Ybo Technologies Co., China). Total protein concentration in each sample was determined by the bicinchoninic acid method and ALP activity was expressed as U/mg protein.

Cell Mineralisation

To assess extracellular matrix mineralisation, MC3T3-E1 cells were induced to undergo osteogenic differentiation and subsequently treated with DP extract at concentrations of 5, 10, 20 and 40 μg/mL for 14 days. During this period, the culture medium was replaced every 2–3 days to ensure optimal cell growth and treatment efficacy. After 14 days of incubation, the cells were washed twice with PBS and fixed with 4% formalin for 15 min. at room temperature. Fixed cells were rinsed again with PBS and stained with 2% Alizarin Red S (ARS), pH 4.2 for 30 min. to visualise calcium-rich mineral deposits. Excess dye was removed by washing three times with distilled water until the background was clear. Stained cultures were qualitatively observed under a light microscope to evaluate calcium nodule formation. For quantitative determination, the bound ARS dye was extracted by incubating the stained cells with 10 mM sodium phosphate buffer containing 10% cetylpyridinium chloride (Synergy HT, China) for 30 min. at room temperature with gentle shaking. The released dye solution was transferred to a 96-well plate and absorbance was measured at 504 nm using the multimode microplate reader. The degree of mineralisation was expressed as relative absorbance values.

Animal Study

Female C57BL/6 mice, aged 8-9 weeks, were acquired from Beijing Weitong Lihua Experimental Animal Technology Co. in China. The mice were housed in a controlled environment with consistent temperature and humidity and were provided with unlimited access to food and water. The Ethics Committee of Wenzhou TCM Hospital, Zhejiang Chinese Medical University approved the entire experimental procedure (Registration no. TCM/ZCMU/85645/2022). The animals were categorised into five groups (n=6):

Control group. The mice did not undergo ovariectomy and received no treatments.

OVX group. These mice underwent ovariectomisation to induce estrogen deficiency and received saline orally at 10 mL/kg (approximately 0.25 mL per mouse) as a vehicle control.

β-E group (standard group). OVX mice received 30 μL of β-estradiol solution orally at a concentration of 1.67 mg/mL (equivalent to 2 mg/kg). This group served as positive control (standard treatment).

DP group (100 mg/kg). OVX mice were administered 30 μL of DP extract orally, formulated at 83.33 mg/mL to deliver a dose of 100 mg/kg daily for two weeks.

DP group (200 mg/kg). Similar to the low-dose group, these OVX mice received 30 μL of DP extract at 166.66 mg/mL to deliver a dose of 200 mg/kg daily for two weeks.

After 2 weeks, the animals were euthanised and blood was collected through a cardiac puncture. The femur and uterus were removed to estimate the effect of DP on biochemical parameters.

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Assessment of Biochemical Parameters

Blood samples were collected from experimental animals via cardiac puncture after treatment and were allowed to clot at room temperature for 30 min. and then centrifuged at 3000 rpm for 10 min. at 4 °C to obtain the serum. The serum concentrations of oestradiol and OCN were quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Biyotime Biotechnology, China), following the manufacturer's instructions. Serum samples and standards (synthetic 17β-estradiol and purified recombinant human OCN) were added to pre-coated 96-well ELISA plates and incubated with horseradish peroxidase-conjugated detection antibodies (Santa Cruz Biotechnology, China). After washing to remove unbound material, tetramethylbenzidine solution (100 µL, 0.4 mmol/L) was added to each well. The reaction was stopped with sulfuric acid and absorbance was measured at 450 nm using a multimode microplate reader. The concentrations of oestradiol and OCN in serum samples were calculated from standard calibration curves prepared using known concentrations of each analyte. Results were expressed as pg/mL for oestradiol and ng/mL for OCN.

Micro-Computed Tomography (µCT)

The distal femurs were carefully dissected from each animal, cleaned of soft tissues and fixed in 4% paraformaldehyde for 24 hr at 4 °C. Samples were then stored in 70% ethanol until further analysis. High-resolution bone microarchitecture was evaluated using a µCT scanner (CT9600, Matsusada Precision Inc., Japan). Scans were acquired at a voltage of 50 kV and a current of 200 µA with an isotropic voxel size of 9 µm and an exposure time of 300 ms per frame.

Reconstruction of cross-sectional images was performed using the manufacturer's software followed by 3D morphometric analysis of trabecular and cortical bone parameters within a standardised region of interest located 1 mm below the growth plate and extending distally for 1–2 mm. Trabecular bone indices include trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp). Cortical thickness (Ct.Th) was measured in the midshaft region. Additional parameters quantified included bone volume / total volume (BV/TV), bone surface / total volume (BS/TV), bone surface/bone volume (BS/BV) and BMD. All measurements were obtained using the integrated analysis software supplied with the µCT system and results were expressed according to standardised bonemorphometry guidelines.

Expression of Runx2, BMP2, OPG and p-Smad2/3 in bone tissue

Bone tissue was homogenised and total protein was extracted using radioimmunoprecipitation assay buffer with protease and phosphatase inhibitors (Beyotime Biotechnology, China). Protein concentrations were determined using a BCA protein assay kit (Beyotime Biotechnology, China). The expression levels of Runx2, BMP2, OPG and phosphorylated mothers against decapentaplegic homolog 2/3 (p-Smad2/3) were assessed by Western blotting. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Beyotime Biotechnology, China) and transferred to polyvinylidene difluoride membranes (Millipore, China). The membranes were blocked with 5% non-fat milk (Sangon Biotech, China) and incubated overnight at 4°C with primary antibodies against Runx2, BMP2, OPG, p-Smad2/3 and β-actin (Proteintech, China). After incubation with horseradish peroxidase-conjugated secondary antibodies (Beyotime Biotechnology, China), protein bands were visualised using an enhanced chemiluminescence kit (Beyotime

Biotechnology, China). Densitometric analysis was performed using ImageJ software (NIH, USA), and protein expression was normalised to β-actin.

Histopathological Study

Femoral tissues were carefully dissected, rinsed with PBS and immediately fixed in 10% (v/v) neutral buffered formalin for 48 hr at room temperature. Following fixation, tissues were dehydrated through a graded ethanol series (70%, 80%, 90%, 95% and 100%), cleared in xylene and subsequently embedded in paraffin wax blocks. Serial sections of 5 µm thickness were obtained using a rotary microtome (Minux® Rotary Microtome S712, RWD Life Science, China) and mounted on poly-L-lysine-coated glass slides.

For histological evaluation, sections were deparaffinised, rehydrated and subjected to hematoxylin and eosin (HE) staining to assess general tissue morphology. In addition, immunohistochemical staining was performed using a primary antibody against collagen type I (Thermo Fisher Scientific Inc., China) to evaluate bone matrix composition. After incubation with the primary antibody, sections were treated with horseradish peroxidase-conjugated secondary antibodies, visualised using diaminobenzidine substrate and counterstained with hematoxylin (Thermo Fisher Scientific Inc., China). Finally, stained slides were examined under a trinocular light microscope (BestScope BS-2074T, Beijing Bestscope Technology Co., China). Images were captured digitally and histopathological alterations including trabecular organisation, cortical thickness and collagen deposition were qualitatively assessed.

Statistical Analysis

The data is expressed as means ± SEM. One-way analysis of variance (ANOVA) was utilised for statistical assessment. For mean comparisons, Dunnett's post-hoc test was used. Graphpad Prism, version 6.1 (Graphpad Software Inc., USA) was used in the evaluation. The statistically relevant threshold was established at $p < 0.05$.

RESULTS AND DISCUSSION

In the present investigation, an in vitro study was conducted to evaluate the impact of DP on ALP activity and ARS staining in MC3T3-E1 cells. The effects of varying concentrations of DP (5, 10, 20 and 40 µg/mL) were assessed after 48 hr of incubation (Figures 1A and B).

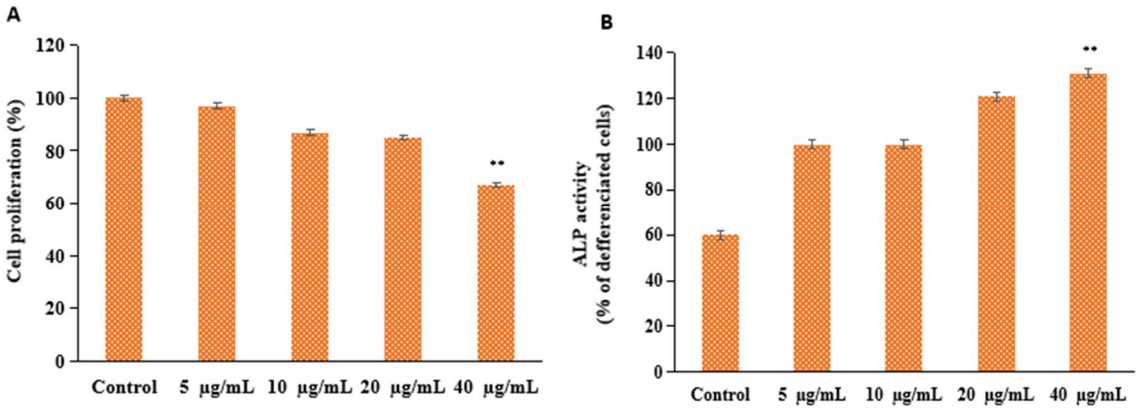

Figure 1. Cell proliferation assessment (A) and ALP activity (B) at various concentrations of DP extract on MC3T3-E1 cells**.** Values are mean ± SEM; **p<0.01 compared to control

Cell viability analysis indicates that DP treatment is non-toxic at concentrations ranging from 0 to 20 µg/mL, with viability maintained between 84% and 97%. ALP, a marker of osteoblast differentiation, is significantly upregulated by DP treatment. Specifically, DP extract at 10 and 20 µg/mL enhances ALP activity by 97–98% compared to control cells (Figure 1B). These results suggest that DP treatment promotes osteoblast activity, potentially contributing to bone health and regeneration.

Furthermore, ARS staining was performed to evaluate mineralisation (Figure 2). A marked increase in positively stained cells was observed in cultures treated with 10 and 20 µg/mL of DP extract, indicating enhanced calcium deposition. Collectively, these findings demonstrate that DP extract stimulates osteoblast differentiation and mineralisation in MC3T3-E1 cells, supporting its potential role in bone formation and regeneration.

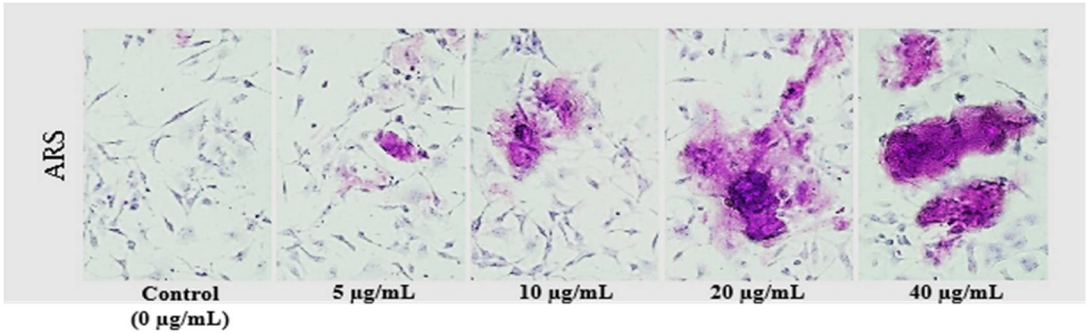

Figure 2. Assessment of effect of DP extract on ARS staining of MC3T3-E1 cells

Osteoblasts, originating from local stem cells within the mesenchyme, are the cells in charge of reconstructing lost bone. The inhibition of osteoclastogenesis has been a widely used approach in OP therapy for many decades [17]. The ALP activity serves as a crucial marker of the early stages of osteoblast differentiation. At the same time, the level of ARS staining is a phenotypic indicator of the later stages including osteoblast maturation and calcium deposition during matrix mineralisation. These markers play a vital role in elucidating the stages of bone formation and may provide insights into various bone disorders [18]. Further investigation into these indicators could enhance our ability to assess and manage bone-related conditions.

Bone-specific mammalian target of rapamycin knockout mice exhibits severely impaired bone development, characterised by a substantial reduction in bone mass and decreased expression of osteogenic genes such as Runx2 and OCN [19]. A recently identified novel protein from *D. opposita* Thunb has been shown to restore the expression of Runx2, bone-specific mammalian target of rapamycin complex 1, eukaryotic translation initiation factor 4E-binding protein 1, protein kinase B1 and ribosomal S6 kinase 1. It activates bone-specific mammalian target of rapamycin and 4E-binding protein 1, while also increasing the total protein levels of protein kinase B1, thereby promoting the differentiation of osteoblasts derived from human mesenchymal stem cells [20].

However, limited studies have been conducted in this area, highlighting the need for further investigation. This study investigates the effects of DP extracts on the expression levels of OCN, Runx2 and OPG in MC3T3-E1 cells (Figure 3). A significant increase ($p < 0.01$) in the expression levels of OCN (1 to 1.4), Runx2 (1 to 1.8) and OPG (1 to 4.8) was observed in MC3T3-E1 cells treated with DP extract at concentrations ranging from 5 to 40 µg/mL, compared to the control group (Figure 3). These findings suggest that DP extract enhances the expression of OCN, Runx2 and OPG, supporting its potential role in osteoblast differentiation.

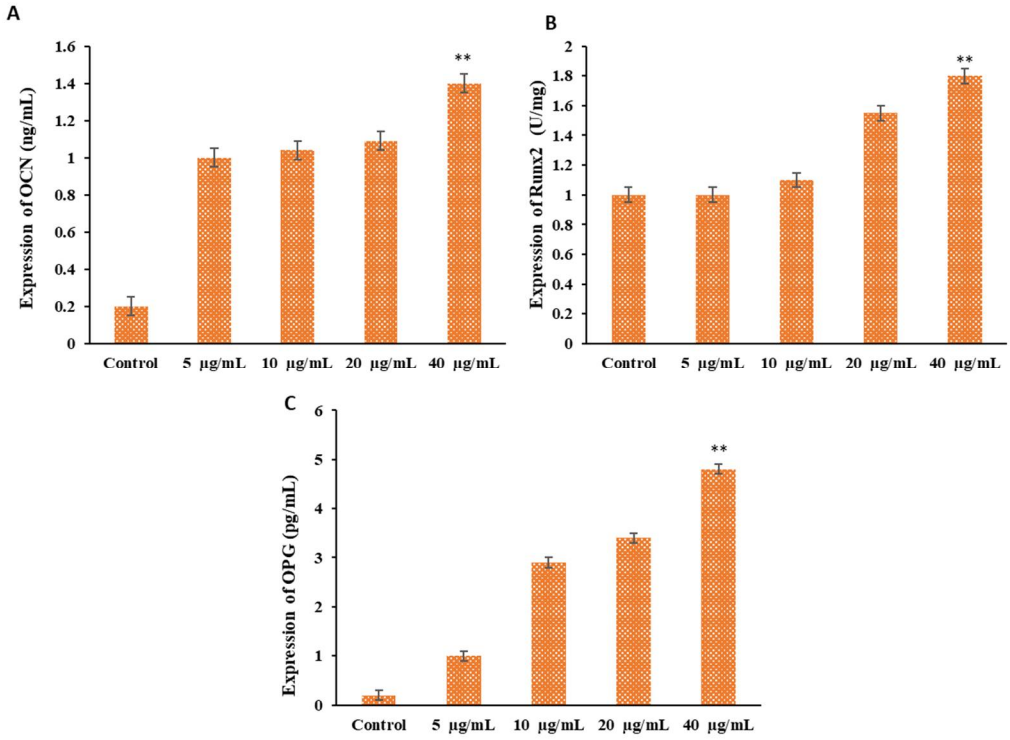

Figure 3. Assessment of effects of DP extract on expression of OCN (A), Runx2 (B) and OPG (C) of MC3T3-E1 cells**.** Values are mean ± SEM (n=6); **p<0.01 compared to control group

Numerous studies in both humans and animals have demonstrated that the administration of oestradiol can improve glucose tolerance, which is often impaired during the postmenopausal phase [21]. In earlier research, Ce et al. [22] reported an increase in serum OCN levels in OVX rats, both untreated and treated with oestrogen, while also observing a reduction in BMD in the left distal femur among the groups. The average values of serum OCN and BMD in the OVX rats showed statistically significant differences. The findings of Lu et al. [23] support the use of *Dioscorea oppositifolia* in traditional Chinese medicine for alleviating menopausal disorders. A Chinese anti-menopausal medicinal formulation containing rhizomes of *D. oppositifolia* has been shown to regulate serum levels of oestrogen, follicle-stimulating hormone and luteinising hormone, thereby reducing specific symptoms associated with menopause [24]. Park et al. [25] investigated the effects of yam (*Dioscorea japonica*) and gromwell (*Lithospermum erythrorhizon*) extracts on menopausal symptoms in OVX mice. Their findings demonstrated a significant reduction in both the frequency and severity of common menopausal symptoms. This study highlights the potential of these natural extracts as viable therapeutic alternatives for managing menopausal discomfort in various contexts. Silambarasan et al. [26] explored the cardioprotective effects of *D. alata* in a postmenopausal rat model. They reported a significant reduction in apoptosis markers, suggesting that *D. alata* may help mitigate cardiac apoptosis associated with menopause. In the present study the effects of DP extract on serum oestradiol and OCN levels in OVX mice are evaluated. The DP-treated groups (100 and 200 mg/kg) show serum oestradiol levels of 122 and 130 pg/m respectively while the OVX group records a level of 98 pg/mL (Figure 4). The values for control group is 132 pg/mL. In terms of serum OCN, the DP-treated groups exhibit levels of 42 and 47 ng/mL compared to 38

ng/mL in the OVX group. These findings indicate that DP extract enhances serum oestradiol and OCN levels in treated animals. The observed changes in serum OCN levels suggest a potential involvement in the alteration in bone metabolism associated with postmenopausal conditions.

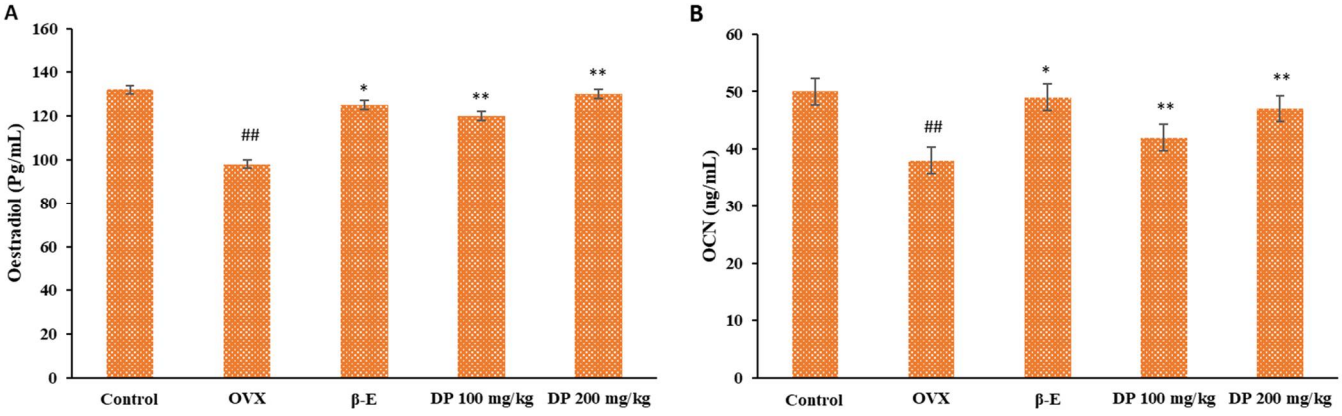

Figure 4. Assessment of effect of DP on level of oestradiol and OCN in serum of OVX mice**.** Values are mean ± SEM (n=6); $^{\#\#}p<0.01$ vs control group, $^{*}p<0.05$, $^{**}p<0.01$ vs OVX group.

The primary strategy for managing OP has traditionally involved the suppression of osteoclast activity. However, some investigations have also focused on activating osteoblasts to reverse bone loss. Despite its potential, limited research has been conducted on *Dioscorea* species. In our study the outcomes indicate that *D. spongiosa* extract effectively inhibits glucocorticoid-induced OP while improving bone tissue morphology, bone mineral content, BMD and various biomechanical parameters. Additionally, microscopic alterations in the cancellous and trabecular bones are found to be restored. The impact of DP extract (100 and 200 mg/kg) on bone microstructure, as assessed by micro-CT is presented in Figure 5.

The observed changes in biochemical indices suggest that these effects may be attributed to the yam extract's ability to suppress excessive bone resorption. In a related study, Kim et al. [27] reported that root and bark extracts of *D. batatas* stimulated osteogenic activity in osteoblasts by promoting bone matrix maturation, increasing collagen synthesis, ALP activity and matrix mineralisation. Similarly, Kubi et al. [14] evaluated the morphometric features of femoral and vertebral bone microarchitecture. They demonstrated that a novel protein derived from *D. opposita* significantly increased BV/TV, BS/TV, Tb.Th and Tb.N compared to the OVX control group. Previously, Song et al. [28] showed that treatment with diosgenin, derived from *Dioscorea* species, improved body weight and trabecular bone microstructure in rats. Zhang et al. [29] conducted a three-dimensional micro-CT analysis. They found that alveolar bone loss was reduced in rats treated with either raw *Dioscorea* extract or estradiol valerate, compared to the OVX group. Significant improvements in BV/TV, Tb.Th, Tb.N, Tb.Sp and the structural model index reflected the anti-osteopenic effects of *Rhizoma dioscoreae* extract and estradiol valerate. However, the latter exerted a more potent effect. Moreover, Peng et al. [30] conducted a micro-CT analysis which revealed that the treatment mitigated the deterioration of trabecular BMD, BV/TV and Tb.N in OVX mice.

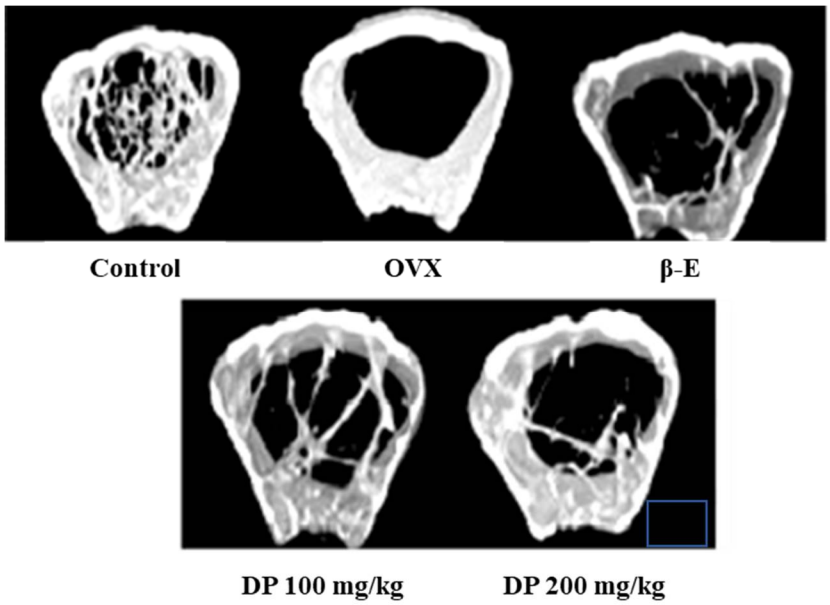

Figure 5. Effects of DP on microstructure of bone by micro-CT (image magnification × 100)

In this study Tb.N, Tb.Th, Tb.Sp, BV/TV, BS/TV, BS/BV and BMD are evaluated in OVX mice (Figure 6). The femoral tissue of the OVX group shows notable decreases in Tb.N, Tb.Th, BV/TV, BS/TV and BS/BV, as well as an increase in Tb.Sp, compared to the control group. Additionally, the OVX group exhibits a significant reduction in BMD (Figure 6). Administration of DP extract improves BMD, BS/TV, BV/TV, Tb.Th and Tb.N while simultaneously reducing BS/BV and Tb.Sp. These results demonstrate that DP extract improves bone microstructure. To further understand the underlying mechanism, the expressions of Runx2, BMP2, OPG and p-Smad2/3 in the bone tissue of DP-treated OVX mice were assessed by western blotting. Expression levels of all four markers are lower in the OVX group compared to the control group. However, DP administration significantly enhances the expression of Runx2, BMP2, OPG and p-Smad2/3 in a dose-dependent manner. Notably, treatment with 200 mg/kg DP extract results in the highest expression levels of these markers (Figure 7).

Histopathological changes in femoral tissue were assessed in DP-treated OVX mice using HE staining and type-I collagen immunostaining (Figure 8). HE staining reveals a loss of bone marrow cells in the femur tissue of the OVX group, whereas treatment with DP effectively suppresses this bone loss (Figure 8A). The number of type-I collagen-positive cells in the femur tissue is significantly reduced in the OVX group compared to the control group and this reduction is restored by treatment with DP at 200 mg/kg (Figure 8B). These findings suggest that DP extract can aid in the regeneration and repair of bone marrow injury.

The literature reports that constituents of DP include phenolic acids, flavonoids, polysaccharides, essential amino acids and sterols, particularly phytoestrogens [5, 31]. Previously, Zhang et al. [29] investigated seven distinct yam species (*D. alata*, *D. cayenensis*, *D. japonica*, *D. pseudojaponica*, *D. polystachya*, *D. opposita* and *D. batatas*), identifying significant amounts of various bioactive compounds including flavonoids, phenols, saponins, tannins and alkaloids. In a separate study the pharmacological functions of yam-derived peptides and proteins were described, highlighting their antioxidant, immunomodulatory, oestrogenic and enzyme-inhibitory activities [9]. These include inhibition of angiotensin-I-converting enzyme, carbonic anhydrase and trypsin, as

well as chitinase. The study also discussed potential clinical applications in managing inflammatory diseases, cardiovascular disorders, ageing-related conditions, menopause, cancers and OP [32].

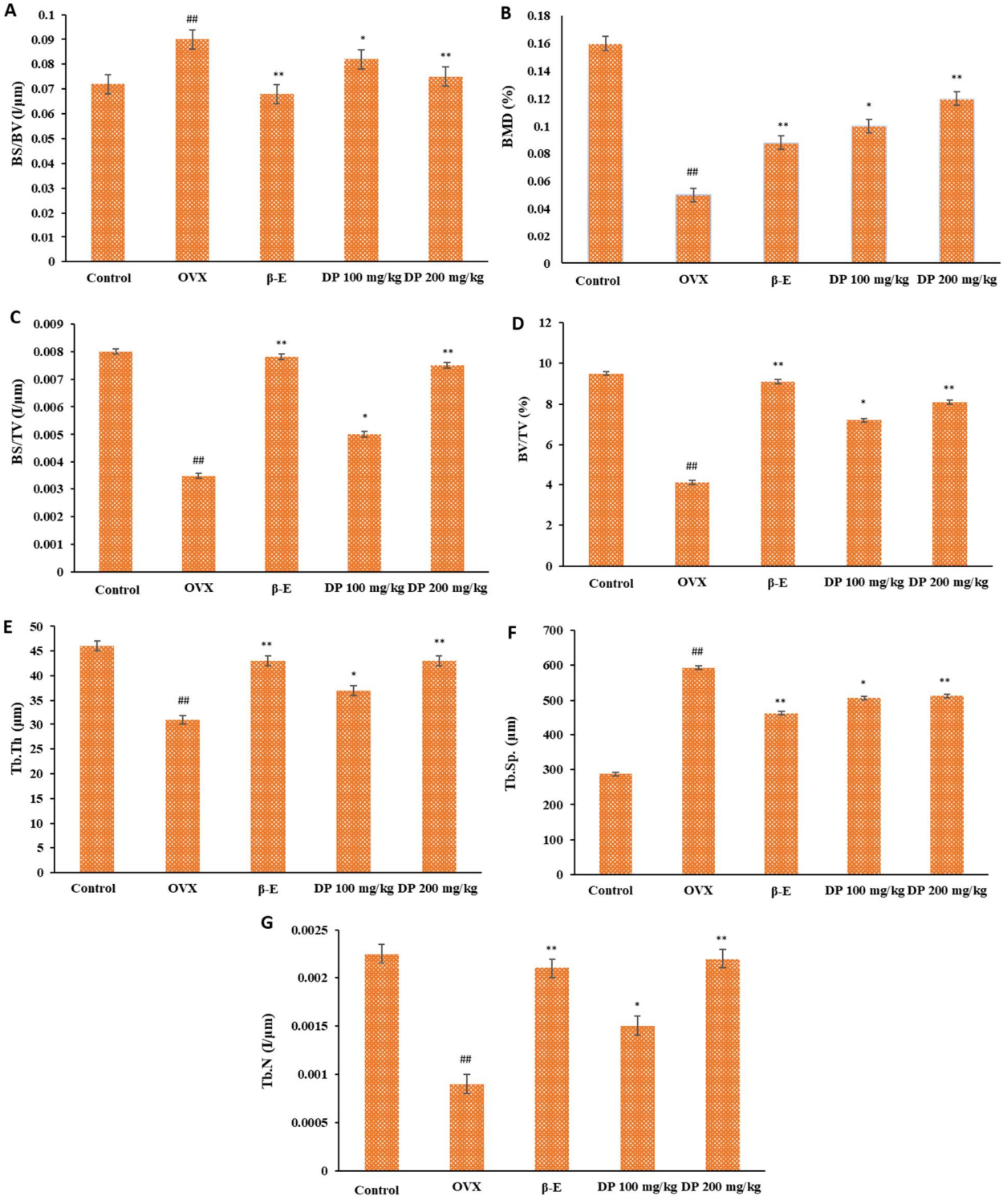

Figure 6. Assessment of BS/BV (A), BMD (B), BS/TV (C), BV/TV (D), Tb.Th (E), Tb.Sp (F) and Tb.N (G) in OVX mice. Values are mean ± SEM (n=6); $^{\#\#}p<0.01$ vs control group, $^{*}p<0.05$, $^{**}p<0.01$ vs OVX group

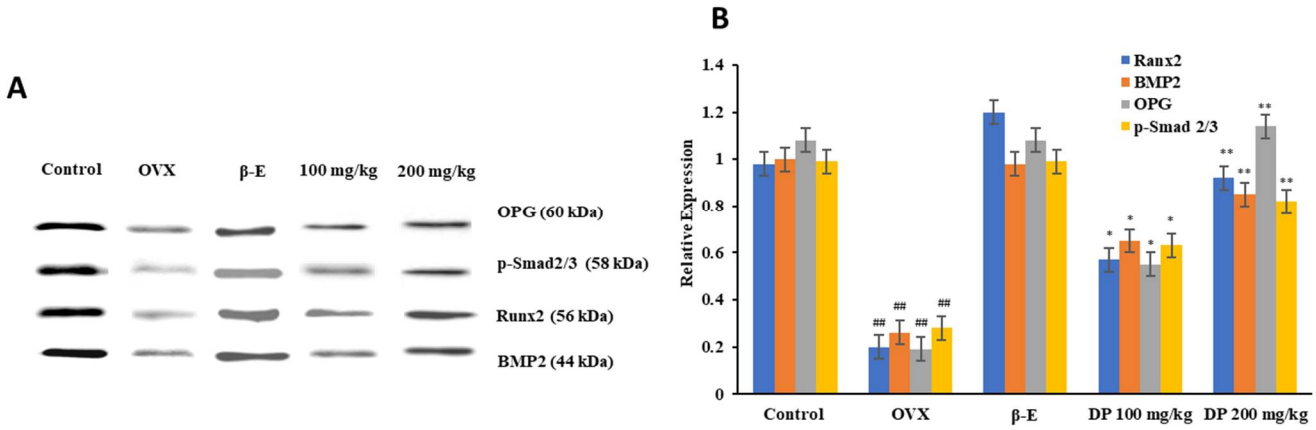

Figure 7. Assessment of effects of DP on expression of Runx2, BMP2, OPG and p-Smad2/3 in bone tissue of OVX mice by qRT-PCR**.** Values are mean ± SEM (n=6); $^{\#\#}p<0.01$ vs control group, *p<0.05, **p<0.01 vs OVX group.

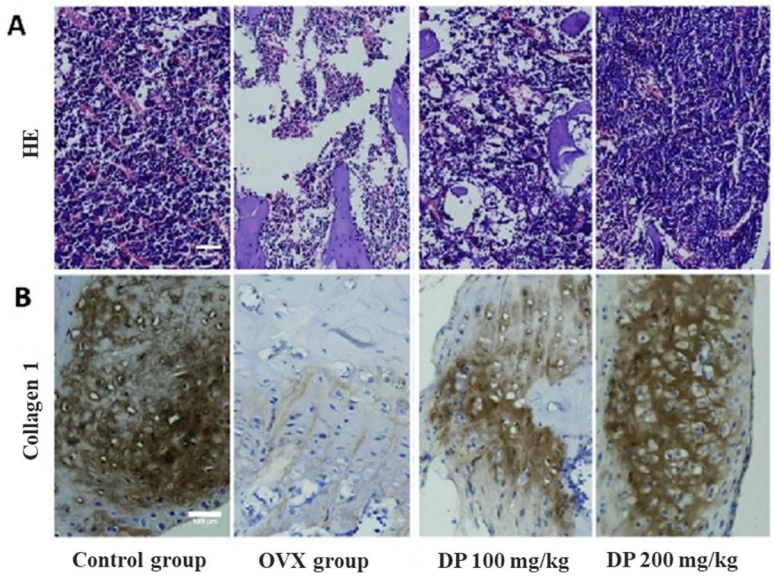

Figure 8. Assessment of effects of DP on histopathology of femur tissue of tested mice**:** (A) HE staining of femur tissue; (B) Type-1 Collagen antibody stained in femur tissue

In the present study Figure 9 displays the HPLC chromatogram of the ethanolic DP extract. The HPLC spectrum confirms the presence of several phytochemicals that match known reference standards and entries from the HPLC library. Identified compounds comprise gallic acid, protocatechuic acid, p-hydroxybenzoic acid, catechin, chlorogenic acid, vanillic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, sinapic acid, salicylic acid, naringin, rutin, ellagic acid, myricetin, quercetin, naringenin, apigenin and kaempferol. These results indicate that the DP extract contains a high concentration of bioactive phytochemicals, which may contribute significantly to its medicinal and therapeutic properties.

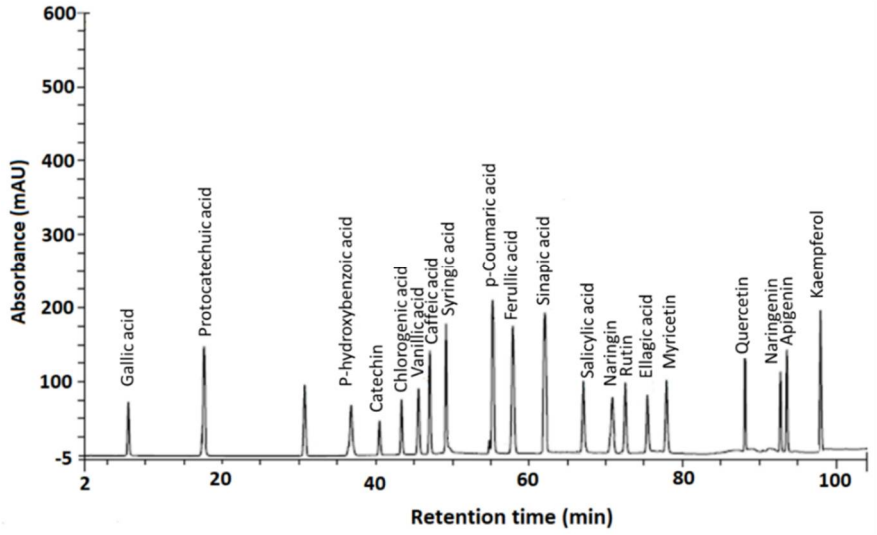

Figure 9. HPLC chromatogram of DP extract

CONCLUSIONS

DP-treated mice exhibit a noticeable reduction in menopausal symptoms. Collectively, these findings suggest that the beneficial effects of DP are mediated, at least in part, through activation of the BMP-2/4/Runx2 signalling pathway, which plays a critical role in osteoblast differentiation and bone formation. This research demonstrates that DP extracts have potential therapeutic value, contributing to a deeper understanding of the plant's pharmacological benefits. Despite its promising findings, this study has certain limitations related to data quality and representativeness. As the investigation is primarily based on cellular and animal models, further validation through well-designed human clinical studies is essential. Such studies not only would confirm the translational relevance of the current results but also provide deeper insights into the underlying mechanisms of action, paving the way for the development of novel therapeutic strategies for prevention and management of OP. Additionally, future research should aim to identify specific biomarkers associated with OP susceptibility, which could facilitate early diagnosis and targeted interventions. Bridging the gap between preclinical models and clinical applications will be crucial for advancing our understanding of bone biology and improving outcomes in individuals at risk of OP.

ACKNOWLEDGEMENTS

The authors appreciate the amenities provided by the institution.

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