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ANTI-OSTEOPOROSIS ACTIVITY OF EUCOMMIA, CUSCUTA AND DRYNARIA EXTRACTS IN THE OVARIECTOMIZED RATS

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Keywords:

E.C.D, Osteoporosis, Ovariectomized rat, Phytoestrogens, BMP/Smads, Wnt/β-catenin

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ABSTRACT: To observe the effect of Eucommia, Cuscuta, and Drynaria (E.C.D.) extracts in ovariectomized rats and explore the possible mechanisms. Forty-five female Sprague Dawley rats were randomly divided into 5 groups: sham operation group (Sham), ovariectomized group (Model) and ovariectomized rats + treatment group (estradiol valerate of 104.17 mg/kg/day (EV), E.C.D. extracts of 50.08 mg/kg/day (E.C.D.-L), or E.C.D. extracts of 104.17 mg/kg/day (E.C.D.-H), and all the rats were treated by gavage for 8 weeks). Bone metabolism, estrogen index, phosphorus and calcium were compared, and the right femur structural parameters were evaluated by micro-CT. The rat femoral bone specimens were stained using hematoxylin and eosin for pathological examination, and mRNA expression of related genes in bone tissue was detected by real-time quantitative polymerase chain reaction (PCR). We observed E. C. D.-H groups significantly increased serum estrogen levels, and significantly elevated the maximum load and bone mineral density (BMD) levels in bone tissues. EV group and E. C. D.-H groups improved bone metabolism, biomechanical properties, serum phosphorus and calcium indexes. Compared with the model group, bone morphogenetic protein 2 (BMP2), small mothers against decapentaplegic 5 (Smad5), runt-related transcription factor 2 (Runx 2), Wnt3a of the E. C. D.-H groups were significantly increased ($p<0.05$), the NF-κβ was significantly decreased ($p<0.05$). In conclusion, E.C.D. extracts can increase the content of serum estradiol E2, improve the BMD and promote bone formation in ovariectomized rats, and the mechanism may be related to the regulation of BMP/Smads pathway and Wnt/β-catenin pathway.

INTRODUCTION: Osteoporosis is a common and frequently occurring disease in post-menopausal women, the elderly and chronic diseases [1, 2, 3].

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The main cause of osteoporosis is decreased bone mass and the deterioration of the fine bone structure, resulting in increased bone fragility and fracture risk [1, 2].

It is well known that an in-depth understanding of the pathogenesis of diseases is the key to the study of prevention and treatment measures [1]. The incidence of osteoporosis is closely related to vitamin D receptors and type I collagen. At present, the research on the pathogenesis of osteoporosis mainly focuses on receptor activator for nuclear

factor-κβ (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG), which determines the dynamic balance between osteoblasts and osteoclasts *in-vivo* [1]. Once the dynamic balance is broken, the differentiation direction of osteoblasts and osteoclasts can be determined [2]. Recent research reveals that the role of the immune system can regulate bone cells, and participate in the pathogenesis of the disease; bone protection system and Wnt signaling pathways in the differentiation and activation of osteoclasts and osteoblasts, is crucial in the course of treatment is divided into three aspects [2]: first is a preventive treatment, through the functional exercise prescription, *etc*. [3]; Second, use drugs that inhibit bone resorption, such as estrogen, calcitonin, and bisphosphonates. Finally, drugs that promote bone formation, such as calcium supplements, vitamin D derivatives, and fluoride, are used [2, 3].

So, far, there is no very effective method to prevent and treat osteoporosis. Most of them are integrated therapy, which provides an opportunity to prevent and treat osteoporosis with traditional Chinese medicine (TCM). E.C.D. are widely usedas a combination and play a synergy in TCM decoction for osteoporosis treatment [2, 3].

Total lignans (TL) extracted from Eucommia (*Eucommia ulmoides* Oliv.) barks treatment can effectively suppress the loss of bone mass induced by ovariectomy [2]. Cuscuta (*Cuscuta chinensis* Lam) showed promising effects in the protection against glucocorticoid-induced osteoporosis through protecting osteoblasts and suppressing osteoclastogenesis [2]. Drynaria (*Drynaria fortunei* (Kunze ex Mett.) J. Sm.) showed significant activity on both the proliferation of UMR106 cells and promoting bone mineral density in ovariectomized (OVX) mice [2].

Although traditional Chinese medicine does not have the name "post-menopausal osteoporosis (PMOP)", there are many similar symptoms described in historical literature, PMOP should be under the category of osteoporosis, post-menopausal symptoms. In fact, PMOP should be a disease with kidney deficiency as the root cause [2]. There are many methods for osteoporosis building the most commonly used method is to remove ovaries and testes, collectively known as castration [2]. We chose the ovariectomized method because of its single construction factor and good reproducibility [2]. In this study, SD female rats with bilateral ovaries removed by surgery were used as the research objects, and the bone tissue morphology, bone biomechanics, bone mineral density index, *etc*. of the rats after E.C.D. extract drug intervention was observed. The protective effect of drugs on bone metabolism was evaluated from the overall and molecular levels to guide the clinical treatment of drugs better.

MATERIALS AND METHODS:

Plant Species Identification: Eucommia (*Eucommia ulmoides* Oliv.) was purchased from Anhui Zhiliang Traditional Chinese Medicine Decoction Pieces Co., LTD. Cuscuta (*Cuscuta chinensis* Lam) and Drynaria (*Drynaria fortunei* (Kunze ex Mett.) J. Sm.) were purchased from Bozhou Traditional Chinese Medicine Commodity Trading Center Co., LTD. HPTLC identified raw medicinal materials according to the method developed by Alkemist Labs (Hoover St, Garden Grove, CA)IDTSOP-72-01 in the U. S.

Prepare of E.C.D. Extracts: The combination of E.C.D. has been developed as an adjunct to dietary supplements for treating osteoporosis (EuBone, Batch No. BH201-20201013-01, Product code: T-4005-1). It is also applying for a patent of novel formula to promote bone repair (patent application number: 2020105750230).

The E.C.D. extracts were made from ground Eucommia, Cuscuta and Drynaria at a ratio of 6:1:3 (theratio is based on the pharmacopoeia recommended dosage and extracts calculation rate) into powder and sieved through a 20-mesh sieve. The extraction process of counter-current extraction (CCE) was based on a previous publication [13]. Briefly, the raw material of *E. ulmoides*, *C. chinensis* and *D. fortune* was extracted with 5 L of 95% ethanol 2 times, and the extraction time was 1 h, respectively.

The extract was then filtered and condensed undervacuum and then further dried using a freeze-dryer. To confirm the identity of ingredients, manufacturers use high-performance liquid chromatography (HPLC) to check for the presence of active ingredients, as shown in **Table 1.**

TABLE 1: THE ACTIVE INGREDIENTS OF E.C.D. EXTRACTS

Herb	Mark Compound	Acceptance Criteria	Analytical Method
Eucommia ulmoides Oliv.	Chlorogenic acid	NLT 0.3%	HPLC
	Pinoresinol Diglucoside	NLT 0.15%	HPLC
Drynaria fortunei (Kunze ex Mett.) J.Sm.	Naringin	NLT 0.50%	HPLC
Cuscuta chinensis Lam	Hyperin	NLT 0.10%	HPLC

Animals and Treatments: A total of 40 SPF healthy female SD rats (215.6 ± 14.7g) were purchased from Jinan Pengyue Experimental Animal Breeding Co. LTD (certification number: SCXK (Shandong) 2019-0003). The rats received *ad libitum* access to standard chow pellets and water in 22±3℃, 40-70% humidity. 12 hours of alternating light and dark. After adaptation for 1 week, rats were randomly divided into five groups (n = 8 per group).

Rats were weighed and anesthetized by 10% chloral hydrate and 0.3mL/100g intraperitoneal injection. After anesthesia until there was no blink reflex, the rats were fixed in the abdominal position and the rats were shaved to expose the surgical site under the lowest ribs on both sides and at the intersection of the midaxillary line and about 1-2cm from the lateral spine. After sterilizing the towel with 75% alcohol, the skin, back muscles, and myolemma were cut, and the white glowing cellulite was gently pulled out of the incision with a camera. The cellulite was separated, and pink ovaries were visible.

The lower fallopian tube of the ovary was ligated and then the ovary was removed completely. The other side of the ovary was further removed by the same method and the inner muscle and outer skin were sutured successively and the iodine tincture was applied for disinfection. In the sham operation group, only the ovaries were exposed, but no resection was performed. After operation, the animals were kept in cages and fed normally. Three days after modeling, the animals were given intramuscular penicillin injection with a daily dose of 800,000 units for each animal. Post-surgery, animals were treated to estradiol valerate (PROGYNOVA®; Bayer Healthcare Co., LTD. Guangzhou Branch; Approval No. J20171038) of 104.17 mg/kg/day (EV group), E.C.D. extracts of 52.08 mg/kg/day (E.C.D.-L group), or E.C.D. extracts of 104.17 mg/kg/day (E.C.D.-H group) by oral gavage for 8 weeks. E.C.D. extracts were dissolved in normal saline, the sham operation group and the modelcontrol group were given the same volume of normal saline by gavage.

Serum Chemistry Analysis: After centrifuging at 3000rpm for 10min, serum samples were collected and stored at -20℃ until the detection of enzyme-linked immunosorbent assay (ELISA). The level of serum osteocalcin (BGP), bone-specific alkaline phosphatase (BALP), type I collagen carboxyl end before the peptide (PICP), collagen type I N terminal peptide (NTX), serum estradiol E2 and follicle stimulating hormone (FSH) (Jingmei Biotechnology, Yancheng) were determined by commercially ELISA kits according to manufacturer's instruction using an Multiskan microplate reader (Thermo Fisher, USA). The serum calcium and phosphorus concentration was measured by Siemens ADVIA 2400 blood biochemical analyzer.

Biomechanical Testing: The attached muscle and connective tissue were removed from the left femur and the Instron E10000 universal material mechanical testing machine was used for on-machine testing. During the test, the samples were placed at the two support bars, and the loading rod was located in the center. All samples were loaded at a uniform speed of 3 mm/min. Load until the specimen breaks and record the maximum load that the tested femur sample bears before breaking.

Micro-Computed Tomography (Micro-CT) Detection: Micro-CT of left proximal medial metaphyseal tibia were acquired using Scanco Mct35 scanner (Scanco, Switzerland) at 70KVp, 114µA of 800ms. Bone mineral density, bone volume fraction (BV/TV), bone trabecular number (Tb.N) and trabecular separation degree (TB. Sp) was evaluated based on the micro-CT results.

Hematoxylin and Eosin (H & E) and Toluidine Blue O Staining: Bone demineralization: EDTA decalcified bone tissue specimens with 10% liquid under test for decalcified 8 weeks, replace a piece every 3 days EDTA decalcified liquid, until 1 mL syringe needle stab to the bone tissues, no

resistance, decalcified fluid decalcified specimens is washed with water after the completion of several hours to remove attached to the bone tissue of redundant EDTA decalcified liquid and then the femur bones were fixed in 10 neutral buffered formalin solution for 48h, dehydrated in graded ethanol(70%-0%), cleared in xylene, embedded in paraffin, sectioned into 5 m. For H & E stained with hematoxylin for 3-8 min and eosin for 1-3min. For toluidine blue O staining, sections were rinsed in toluidine blue O staining, sections were rinsed in toluidine blue O solution for 1 min. The images were observed by Olympus BX51 light microscopy (Olympus, Japan).

RT-qPCR Detection: Total RNA from bone specimens were extracted using Trizol (Sangon, Shanghai). The RNA quality and quantity were examined using Nanodrop 2000 spctrophotometer (Nanodrop, USA). RT reaction was performed using qPCR RT kit (Transgene, Beijing) according to the manufacturer's instruction on the ABI9700PCR system (ABI, USA).

The PCR reaction was performed using SYBR Green PCR kit (Transgene, Beijing) on Stratagene Mx3000P Real-time PCR system (Agilent, USA) according to manufacturer's instruction. The primers were shown in **Table 2**; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control. Relative expression was calculated using $2^{-\Delta\Delta C_t}$ method and normalized to sham group.

TABLE 2: THE PRIME SEQUENCE OF RT-QPCR

Primer	Sequence（5'-3'）
RAT-BMP2-F	GCGATGGACAGCACAGGGACA
RAT-BMP2-R	TTGGTGCAAAGACCTGCTAATCCTC
RAT-Smad5-F	ACCACCGACAGGATGTAACCA
RAT-Smad5-R	GTCAGGACTTTATCCAGCCACT
RAT-Runx2-F	GGTACTTCGTCAGCGTCCTATCAG
RAT-Runx2-R	CCATCAGCGTCAACACCATCA
RAT-Wnt3a-F	GAACTGCACCACTGTCAGCAACA
RAT-Wnt3a-R	CCCTGGCATCGGCAAACTC
RAT-β-catenin-F	GGACTCTAGTGCAGCTTCTGGGTT
RAT-β-catenin-R	CAGATGGCAGGCTCGGTAATG
RAT-NF-κβ-F	GGTGGGCAAGCACTGTGAGGA
RAT-NF-κβ-R	GGCAAGGTCAGAATGCACCAGAA

Statistical Analysis: Data were expressed as mean ± standard deviation and compared using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Statistical analyses were performed using the SPSS 10.0 software (SPSS, USA). Significance was considered at $p < 0.05$.

RESULTS:

HPTLC Determination: The testing report of plant species identification is shown below **Fig. 1**. Results of the HPTLC **Fig. 1A** showed that lanes 5 are the test sample of *E. ulmoides* extract. Lanes 2, 3, 6, 7 and 8 are the reference samples used for comparison.

This test sample of *E. ulmoides* extract consistent with the chromatographic profile of the reference samples of *E. ulmoides*, used above. This test sample of *E. ulmoides* extract has characteristics of the *E. ulmoides* herb. Results of the HPTLC **Fig. 1B** showed that lanes 4 and 5 are the test sample of *C. chinensis* extract Lanes 2, 3, 6, and 7 are the reference samples used for comparison.

This test sample, *C. chinensis* extract, has characteristics of the chromatographic profile of *C. chinensis* reference samples used above. This test sample of *C. chinensis* extract indicates the presence of *C. chinensis* seed.

Results of the HPTLC **Fig. 1C** showed that lanes 4 and 5 are the test sample of *D. fortunei* extract Lanes 2, 3, 6 and 7 are the reference samples used for comparison.

This test sample, *D. fortunei*extract has characteristics of the chromatographic profile of *D. fortunei* reference samples used above. This test sample of *D. fortunei* extract indicates the presence of *D. fortunei* rhizome.

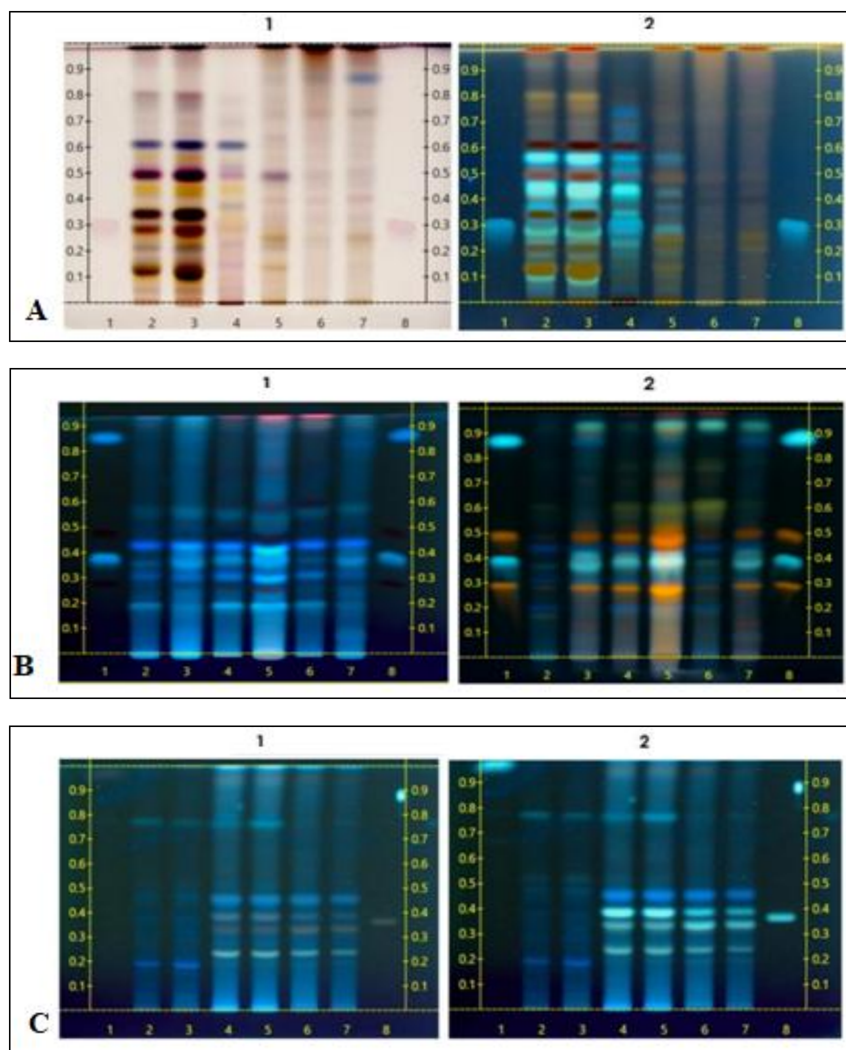

FIG. 1: HIGH-PERFORMANCE THIN LAYER CHROMATOGRAPHY TEST RESULTS OF *E.ULMOIDES, D. FORTUNEI* AND *C. CHINENSIS*. (A) The left-side portion 1 is the *E. ulmoides* standard and the right-side portion 2 is the extract of *E. ulmoides*. (B) The left-side portion 1 is the *C. chinensis* standard and the right-side portion 2 is the extract of *C. chinensis*.(C) The left-side portion 1 is the *D. fortunei* standard and the right-side portion 2 is the extract of *D. fortunei*.

Body Weight: The animals in the sham operation group were generally lightweight before modeling. During the modeling administration, the animals in the model group and the drug group gained weight slowly. After 8 weeks, the mean weight of the sham operation group was significantly higher than that of other groups ($p<0.05$). The weight of animals in drug group 3 increased significantly compared with that in the model group ($p<0.05$); See **Fig. 2** for details.

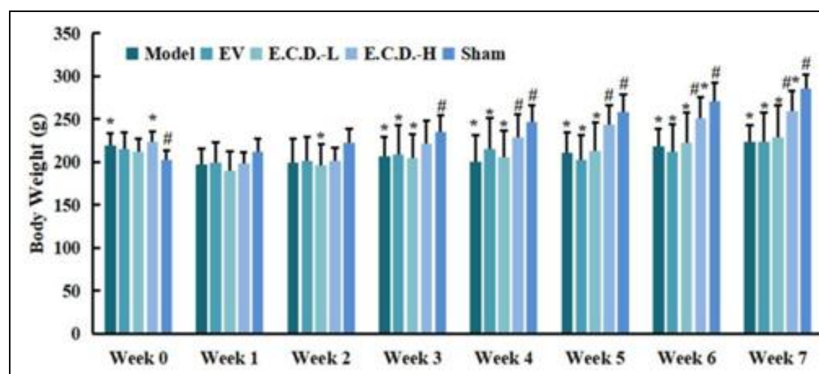

FIG. 2: WEIGHT MONITORING RESULTS OF ALL GROUPS OF RATS. The data are expressed as means ± SD, n = 9 rats/group. Model, ovariectomized; EV, OVX + estradiol vale rate 1000 mg; E.C.D.-L, OVX + E.C.D. extracts 500 mg; E.C.D.-H, OVX + E.C.D. extracts 1000 mg; Sham, sham-operated; * *p*< 0 .05 *vs* the sham group; # *p*< 0.05 *vs* model group.

Bone Metabolism and Estrogen Index Test Results: Animal building 8 weeks after the treatment, corresponding to the four bone metabolic index: BGP, BALP, PICP, and NTX, ELISA test results showed that the E.C.D. extract showed an increasing trend compared with the model group, but there was no significant change trend in all groups and no significant difference between groups. The content of E2 in E.C.D.-H group were significantly higher than those in the model group (p<0.05); Compared with the sham group, the content of E2 in the model group and other drug groups was significantly decreased (p<0.05) **Fig. 3E** and the content of FSH was significantly increased compared with the sham group (p<0.05) **Fig. 3F**. The results are shown in **Fig. 3**.

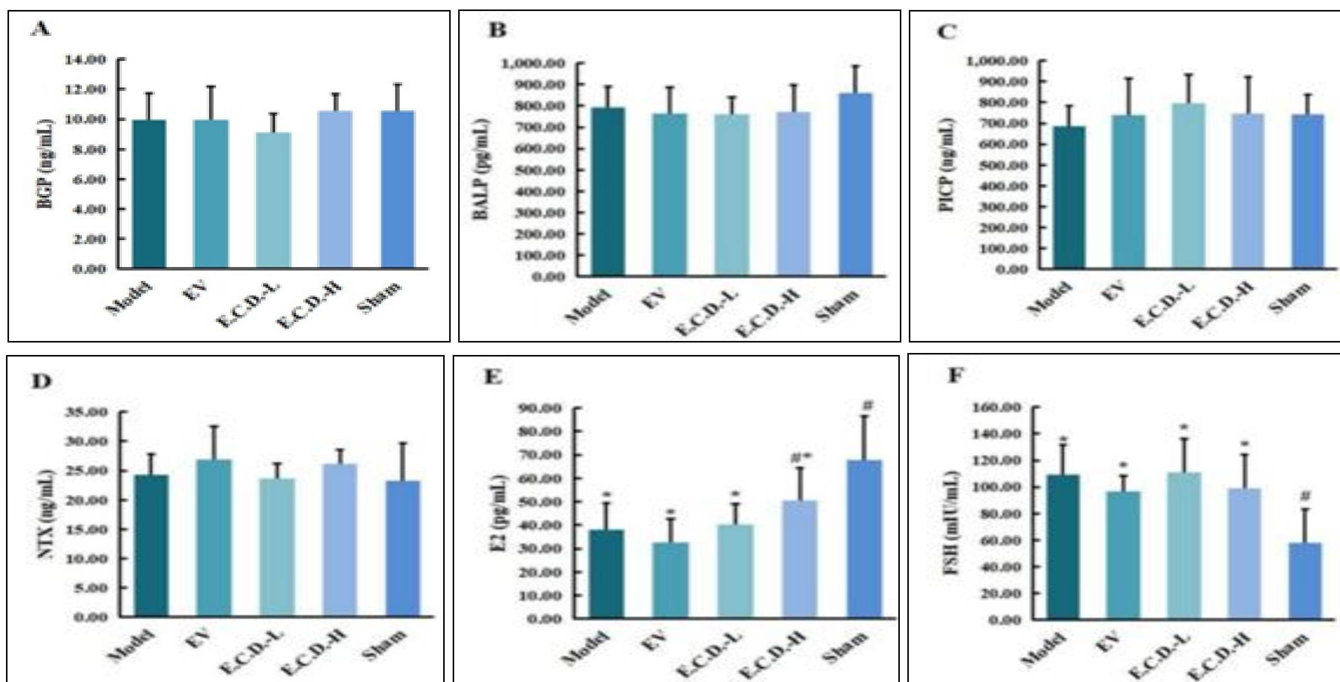

FIG. 3: BONE METABOLISM AND ESTROGEN INDEX TEST RESULTS OF ALL GROUPS. (A) BGP levels; (B) BALP levels; (C) PICP levels; (D) NTX levels; (E) E2 levels; (F) FSH levels. The data are expressed as means ± SD, n = 9 rats/group. Model, ovariectomized; EV, OVX + estradiol vale rate 1000 mg; E.C.D.-L, OVX + E.C.D. extracts 500 mg; E.C.D.-H, OVX + E.C.D. extracts 1000 mg; Sham, sham operated; * p< 0 .05 *vs* the sham group; # p< 0.05 *vs* model group.

Serum Phosphorus and Calcium Detection Results: After 8 weeks of animal modeling administration, blood phosphorus levels in estradiol vale rate group were significantly higher than those in the model group and the sham group (p<0.05), compared with the sham group, the blood phosphorus content in E.C.D.-L group was significantly lower (p<0.05), there was no significant difference in blood phosphorus content in other groups **Fig. 4A**.

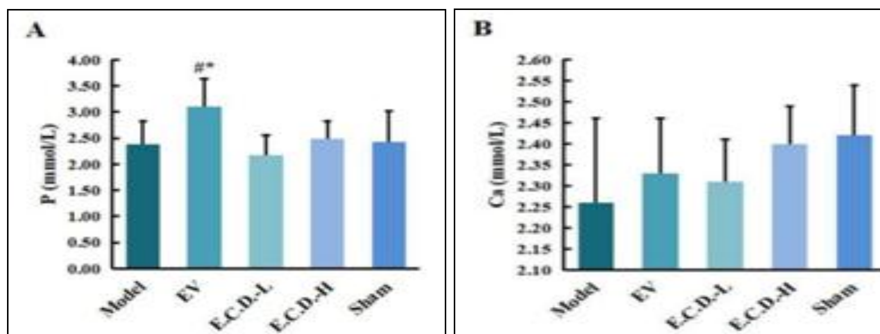

FIG. 4: SERUM PHOSPHORUS AND CALCIUM DETECTION RESULTS OF ALL GROUPS. (A) Blood phosphorus content; (B) Blood calcium content. The data are expressed as means ± SD, n = 9 rats/group. Model, ovariectomized; EV, OVX + estradiol valerate 1000 mg; E.C.D.-L, OVX + E.C.D. extracts 500 mg; E.C.D.-H, OVX + E.C.D. extracts 1000 mg; Sham, sham operated; * p< 0 .05 vs the sham group; # p< 0.05 vs model group.

There was no significant difference in blood calcium content between all experimental groups **Fig. 4B**. There was no difference in blood calcium and phosphorus content between E.C.D-H group and Sham group, as shown in **Fig. 4**.

Biomechanical Test and Micro-CTScan Results of Femur: The biomechanical test results of the femur of rats showed that the maximum load of the femur of rats in the model group was significantly reduced compared with the sham operation control group ($p<0.05$). Compared with the model group, the maximum load of E.C.D.-H was significantly higher ($p<0.05$) **Fig. 5A**.

Analysis of bone microstructure parameters in the metaphyseal region of the femur showed that BMD, bone volume fraction (BV/TV) and bone trabecular number (Tb. N) were significantly decreased in the model group compared with the sham control group ($p<0.05$), the trabecular separation degree (TB. Sp) was significantly increased ($p<0.05$); Compared with the model group, BMD of the E.C.D.-H groups was significantly increased **Fig. 5B**, and there was no significant difference in all index parameters compared with the control group. See **Fig. 5** for the results.

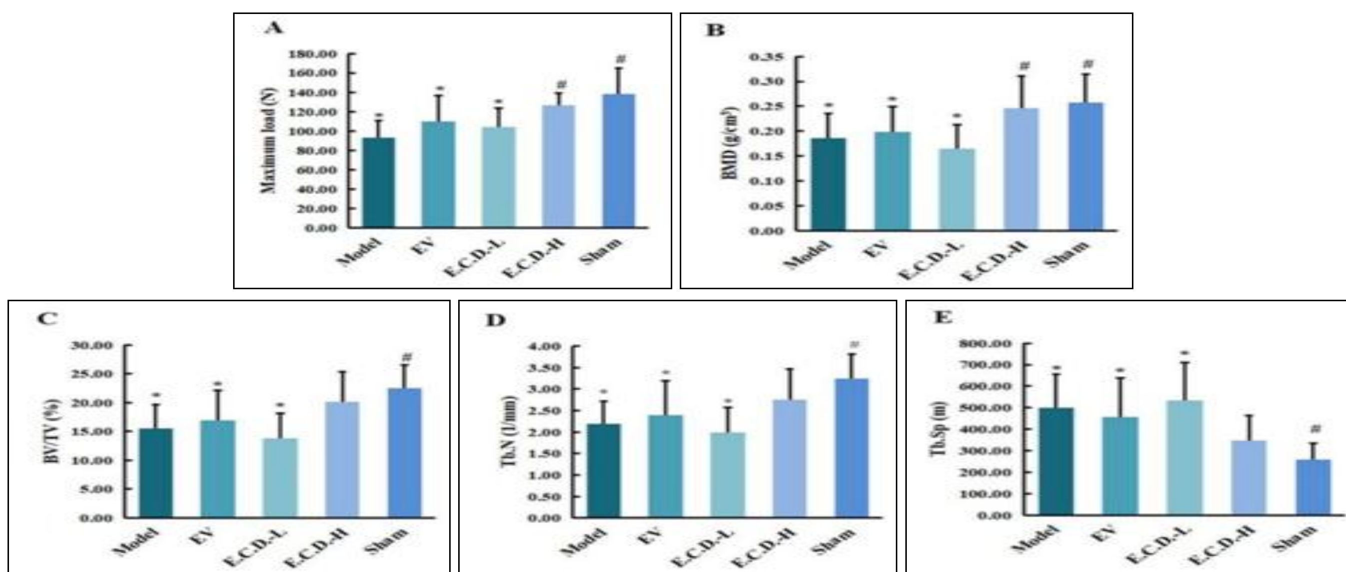

FIG. 5: BIOMECHANICAL TEST AND MICRO-CT SCAN RESULTS OF ALL GROUPS. (A) Maximum load; (B) BMD levels; (C) BV/TV levels; (D) Tb. N levels; (E) Tb.Sp levels. The data are expressed as means ± SD, n = 9 rats/group. Model, ovariectomized; EV, OVX + estradiol valerate 1000 mg; E.C.D.-L, OVX + E.C.D. extracts 500 mg; E.C.D.-H, OVX + E.C.D. extracts 1000 mg; Sham, sham operated;* $p< 0 .05$ vs the sham group; # $p< 0.05$ vs model group.

Hematoxylin and Eosin (H&E) and Toluidine Blue O Staining Results: The nuclei are purplish blue with HE staining, and the cytoplasm and extracellular matrix are redor pink. The cytoplasm of the osteoclasts is wine-red and the nuclei are pale blue with TRAP staining. No osteoclast positive staining was found in the sham operation group and the osteoclast positive staining results were found in both the administration group and the model group. See **Fig. 6** for the results.

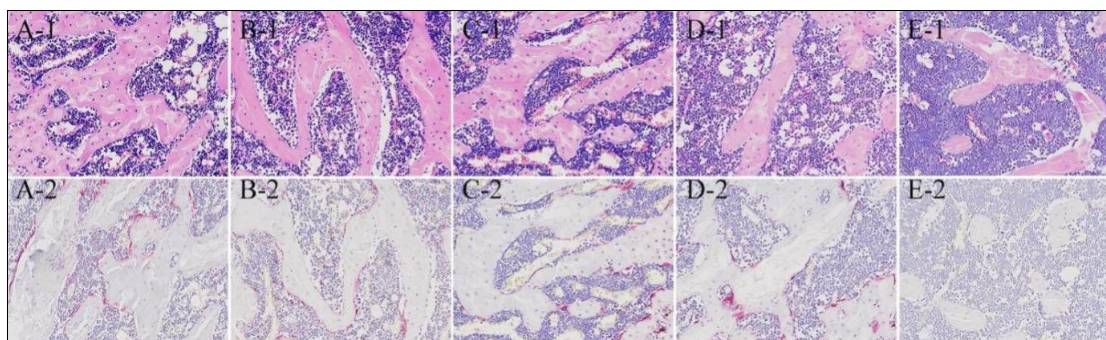

FIG. 6: H & E AND TOLUIDINE BLUE O STAINING RESULTS. (A) Model, ovariectomized; (B) EV, OVX + estradiol valerate 1000 mg; (C) E.C.D.-L, OVX + E.C.D. extracts 500 mg; (D) E.C.D.-H, OVX + E.C.D. extracts 1000 mg; (E) Sham, sham operated.

Fluorescence Quantitative PCR Analysis: Total RNA was isolated and RT-PCR was performed to determine the mRNA expression. Results showed that Wnt3a was significantly decreased in the EuBone-H group compared with the sham control group ($p<0.05$) **Fig. 7C**; Compared with the model group,BMP2, Smad5, Runx 2, Wnt3aof the E.C.D.-H groups was significantly increased($p<0.05$), the NF-κβ was significantly decreased ($p<0.05$) **Fig. 7F**.

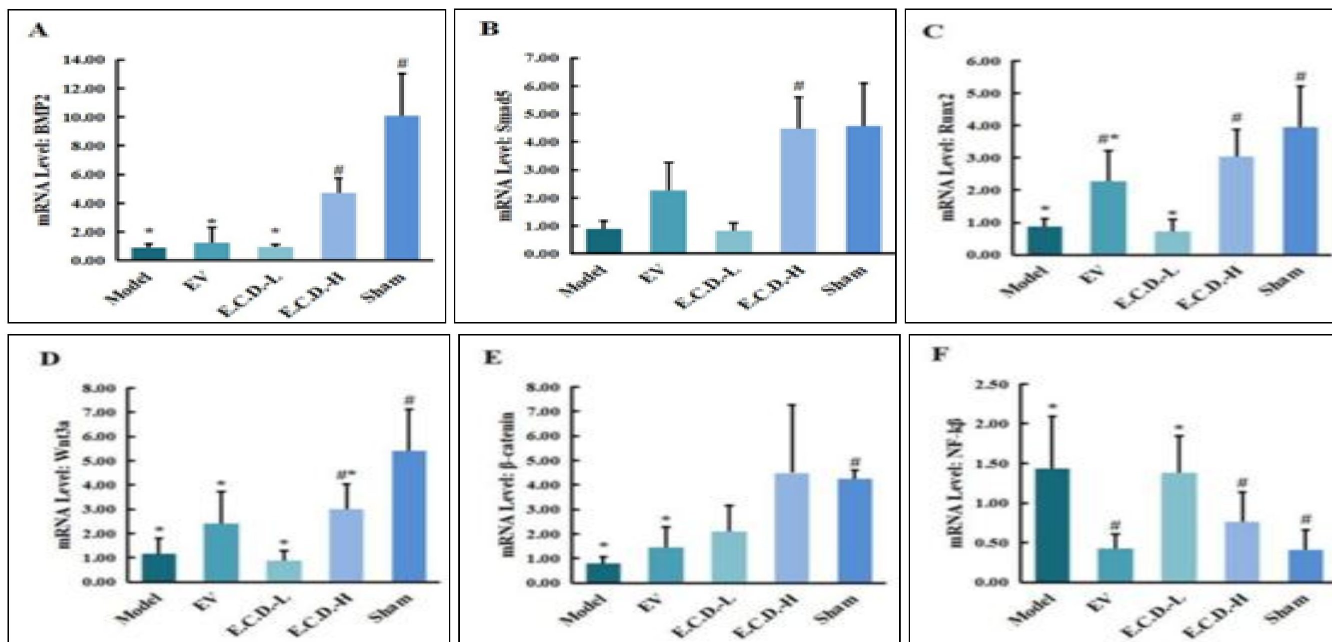

FIG. 7: MRNA EXPRESSION RESULTS OF RELATED GENES IN BONE TISSUE. (A) BMP 2; (B) Smad5; (C) Runx 2; (D) Wnt3a; (E) β-catenin; (F) NF-κβ. The data are expressed as means ± SD, n = 9 rats/group. Model, ovariectomized; EV, OVX + estradiol valerate 1000 mg; E.C.D.-L, OVX + E.C.D. extracts 500 mg; E.C.D.-H, OVX + E.C.D. extracts 1000 mg; Sham, sham operated;* $p< 0.05$ vs the sham group; # $p< 0.05$ vs model group.

DISCUSSION: TCM formulas not only reduce bone loss by decreasing bone resorption and increasing bone formation through multi-component and multi-targets [21], but also regulate the body's function in overall and relieve the pain in back and lumbago [22]. The herbal medicine that possess activity of replenishing kidney are often shown to have estrogen-like [23], antioxidant activity or regulating the function of hypothalamus-pituitary axis to enhance the estrogen level in serum and the herbal medicine that reinforce spleen can intensify the effects of to nifying kidney and herbal medicine activating blood can help active chemical constituents to arrive at the skeletonsite and regulate bone metabolism [24]. Furthermore, TCM formulas modulate bone metabolism networks modestly and then alleviate the symptom of osteoporosis at low concentrations through exerting synergistic effects of multiple components [25]. Therefore, a rationally designed TCM formula can also be considered an option for multitarget therapeutic and prophylactic applications. The development of a standardized, synergistic, safe, and effective TCM formula with robust scientific evidence can offer faster and more economical alternatives.

Aging, estrogen deficiency, chemical drugs, and decreased mechanical loading may cause bone loss leading to osteoporosis [26]. The corresponding animal models are respectively castrated osteoporotic models, osteoporotic models caused by drugs, and disused osteoporotic models [27]. The investigations of TCM formulas on osteoporotic animals focus on osteoporotic rats induced by ovariectomy, glucocorticoids, and retinoic acid. According to previous studies [13], administration of E.C.D. extracts (Eucommia, Cuscuta, and Drynaria) in experimental GIO rats resulted in decreased expression of RANKL, CTX in serum, increased serum calcium, phosphorus, and OPG level, improved bone density, structural integrity, and biomechanical function in experimental GIO rats. Some specific parameters associated with the animal model should be analyzed to highlight the antiosteoporotic characteristic of TCM formulas.

Antiosteoporotic effects of TCM formulas are attributed to active chemical constituents of their herbs. These active constituents are diverse inchemical structure, including flavonoids, saponins, lignans and coumarins, and have the potential to be developed as antiosteoporotic leads [28]. Hence, phytochemicals from TCM formulas and the composition of their herbs is of great potentials for the development of novel antiosteoporotic drugs [29]. Phytoestrogens can selectively combine with estrogen receptors (ER) *in-vivo* and have estrogen-like effects by regulating estrogen receptors [30]. Phytoestrogens can promote bone formation, inhibit bone resorption and play an important role in bone metabolism of bone marrow mesenchymal stem cells (BMSCs) [31]. Fructus Psoraleae (FP) has strong estrogen-like activity and anti-osteoporosis activity by activating the ER-Wnt-β-catenin signaling pathway [32]. JIA Min's findings suggest that osthole and imperatorin may represent new pharmacological tools for the treatment of osteoporosis by displaying estrogenic properties or by stimulating osteoblast activity through the ER pathway. Estrogen can inhibit the oxidative stress response and promote the proliferation of osteoblasts through the positive regulatory protein Wnt/β-catenin (the gene-β-catenin of the Drosophila gene Wingless combined with the mouse gene Int1) signaling pathway of osteoblasts [33]. The results of this study found that E.C.D. extracts can increase the content of estrogen serum estradiol E2, indicating that it has an estrogen-like effect on ovariectomized rats and promotes bone formation. In addition, the E.C.D. extracts group of ovariectomized rats significantly increased BMD and maximum load, suggesting that E.C.D. extracts can alleviate osteoporosis symptoms in ovariectomized rats by regulating estrogen. The BMP/Smads pathway plays an important regulatory role in the process of bone formation. BMP-2 is a member of the TGF-β superfamily and is highly expressed in bone tissue under normal circumstances. Smad 1/5/8 are downstream signaling molecules of BMP, and Runx2 is an Osteocyte-specific transcription factor that promotes bone formation. When the BMP signal is activated, the downstream Smad1/5/8 will be phosphorylated, causing the nuclear displacement of p-Smad 1/5/8, and further regulating the expression level of the downstream target gene Runx 2 [34]. Wnt/β-catenin signaling can stimulate osteoblast proliferation and differentiation, and Runx 2 can be activated by Wnt/β-catenin signaling pathway [35]. Wnt/β-catenin and BMP-2/Smads signaling pathways are two important signaling pathways [36] that regulate the growth, development and differentiation of osteoblasts, and play an important role in osteoblast differentiation and bone formation [37].

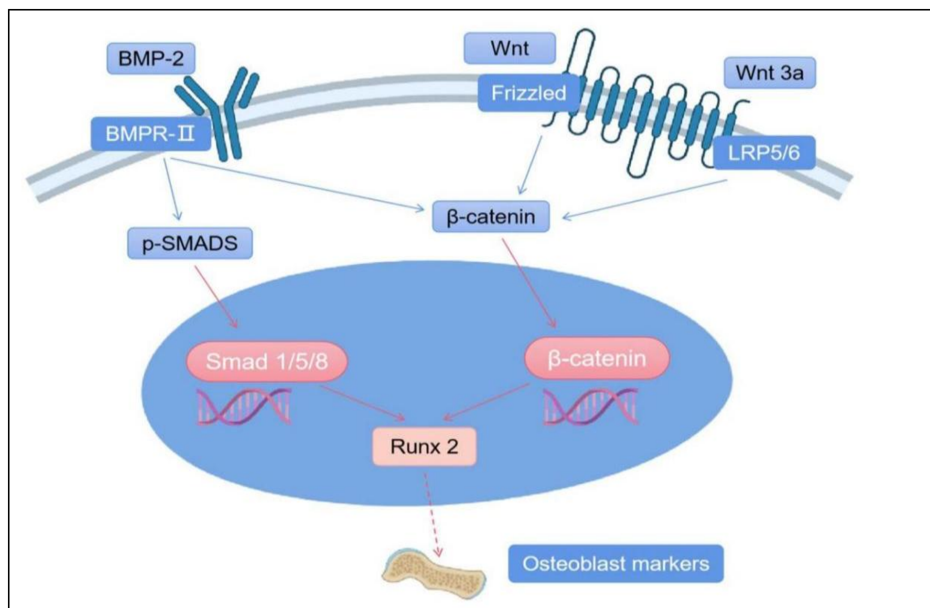

FIG. 8: SCHEMATIC ILLUSTRATING THE MECHANISM BY WHICH E.C.D. EXTRACTS INDUCED THE OSTEOGENIC DIFFERENTIATION. E.C.D. extracts enhanced the activity of the BMP signaling pathway and increased the accumulation of β-catenin by activating the Wnt pathway. Both BMP/Smads and Wnt/β-catenin signaling pathways can up-regulate the expression of Runx 2, a key bone factor, to differentiate osteoblasts.

The results of this study found that E.C.D. extracts could up-regulate the mRNA expression levels of BMP-2, Smad 5, Runx 2 and Wnt 3a in bone tissue and down-regulate the expression of nuclear factor (NF)-κβ, presumably by regulating the BMP/Smads pathway and the Wnt/β-catenin pathway to regulate bone metabolism. Both BMP/Smads and Wnt/β-catenin signaling pathways can up-regulate the expression of Runx 2, a key bone factor, to differentiate osteoblasts [38], thereby significantly increasing bone mineral density and maximum load in ovariectomized rats, as illustrated in **Fig. 8.**

CONCLUSION: The present study's findings demonstrated that E.C.D. extracts can enhance the bone mineral density and increase serum estrogen levels in ovariectomized rats. It is speculated that the mechanism may be related to the regulation of BMP/Smads pathway and Wnt/β-catenin pathway.

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