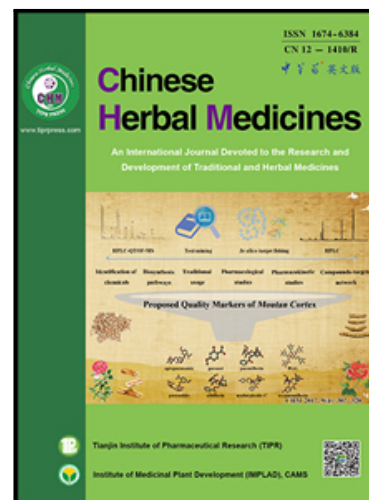


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Effects of *Davallia formosana* Hayata water extracts on ovariectomized mice

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Abstract

Objective: The Taiwanese native fern, *Davallia formosana* Hayata, is used to treat bone diseases in traditional Chinese medicine, but, very few animal tests to verify. To study how this fern and its active component alter bone metabolism.

Methods: It was examined that the effects of low and high doses of *D. formosana* water extracts (DFW, 50 and 200 mg·kg⁻¹·d⁻¹) and [-] epicatechin-3-O-D-allopyranoside isolates of DFW (ECAP, 0.06 and 2.5 mg·kg⁻¹·d⁻¹) on protein levels of important indicators of osteoclast and osteoblast activities. Ovariectomized mice were used as a model of osteoporosis. Bone morphogenetic protein 2 (BMP-2), collagen 1 (COL-1), alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX2), osteocalcin (OCN), and osteopontin (OPN) were analyzed using western blotting of femur tissue. Interleukin (IL)-1 β and tumor necrosis factor (TNF)- α were measured in the serum, and hematoxylin and eosin staining was used to assess bone morphology.

Results: Treatment with DFW or ECAP significantly induced BMP-2, COL-1, ALP, RUNX2, OCN, and OPN protein expression, indicating stimulation of osteogenesis. The treatments also reduced IL-1 β and TNF- α expression, indicating inhibition of osteoclastogenesis. In histological examinations, mice treated with DFW or ECAP had more bone trabeculae. The results demonstrate that DFW and ECAP inhibit osteoclast differentiation and promote osteoblast differentiation, and effectively ameliorate ovariectomy (OVX)-induced osteoporosis.

Conclusion: ECAP is a major component of DFW, which may have therapeutic potential for the treatment of diseases associated with excessive osteoclastic and insufficient osteogenic activity. ECAP may not be the specific active molecule because it is unstable in the stomach. However, the major active molecule needs to be confirmed using a relevant pharmacological model.

Keywords: *Davallia formosana* Hayata, osteoporosis, osteogenesis, osteoclastogenesis

1. Introduction

Osteoporosis development is attributable to various factors including hormones, aging, mechanical and metabolic factors, and postmenopause. Osteoporosis represents the greatest risk factor for bone fractures in the elderly, increasing medical and social costs. Therefore, preventing osteoporosis is an important public health challenge (Chen et al., 2004; Harada and Rodan, 2003).

Davallia formosana Hayata (DFH), a petrophilous fern, is a Taiwanese traditional medicine (called Gu-Sui-Bu), which differs from the traditional Chinese *Drynaria fortunei* (Kunze) J. SM. (Polypodiaceae), with a half-moon-shaped vascular bundle and an epidermis of rectangular cells (Lai et al., 2002). DFH is commonly used to treat bone injuries, inflammation, hyperlipidemia, and arteriosclerosis in traditional Chinese medicine in Taiwan (Chang et al., 2007; Jeong et al., 2005; Ko et al., 2012; Liu, 2001; Sun et al., 2003; Zhang et al., 2009). DFH contains several bioactive compounds including davallic acid, flavan-3-ol, and

proanthocyanidin allosides (Hwang et al., 1989; Lin et al., 1965). Recently, an in vitro study demonstrated that the (-)-epicatechin 3-O- β -d-allopyranoside (ECAP) constituent of DFH inhibited receptor activator of nuclear factor- κ B ligand (RANKL) involved in osteoclastogenesis protein expression and increased bone morphogenetic protein 2 (BMP-2), collagen 1 (CoL-1), alkaline phosphatase (ALP), and Runt-related transcription factor 2 (Runx 2), involved in osteogenesis protein expression (Lin et al., 2013; Wu et al., 2017).

The ovariectomized mouse is an excellent preclinical animal model that mimics the important clinical features of the estrogen-depleted human skeleton and the effects of therapeutic agents (Kimmel, 1996). In estrogen-deficiency, T cells produce elevated levels of proinflammatory cytokines including tumor necrosis factor (TNF)- α and interleukin (IL)-1 β . These cytokines promote increased receptor activator of nuclear factor- κ B ligand (RANKL) expression in osteoblasts, which increases osteoclastogenic potential (Riggs,

2000; Riggs et al., 2002). In addition, TNF- α can reduce mineralization and bone morphogenetic protein 2 (BMP-2) expression in MC3T3-E1 cells. BMPs are multifunctional growth factors belonging to the transforming growth factor (TGF)- β superfamily. During signaling, BMP interacts with various downstream proteins including Runt-related transcription factor 2 (RUNX2), which induces bone differentiation factors. Additionally, alkaline phosphatase (ALP) and type I collagen (COL-1) are produced as early osteogenic markers of matrix maturation while late osteogenic mineralization markers are osteocalcin (OCN) and osteopontin (OPN) (Chen et al., 2004; Gilbert et al., 2000; Rahaman et al., 2015; Stein et al., 1996).

This study aimed to examine the effects of low and high doses of distilled water and chromatography-purified extract of DFH to determine their effects on ovariectomy (OVX)-induced osteoporosis. The cytokines and osteogenesis markers mentioned above were used as indicators of osteoporosis severity.

2. Materials and methods

2.1. Plant material

The original names of *D. formosana* Hayata in the flora of Taiwan are *Davallia orientalis* C. Chr. (1932–1975) and *Davallia divaricata* B1 (1975–1991). Since 1991, the official nomenclature has been *D. formosana* (Ko et al., 2012). The DFH plant material is freely available as a commercial crop and was purchased locally at Sun Ten Pharmaceutical Co, Taipei, Taiwan, in July 2016. The plants were identified at the Institute of Chinese Pharmaceutical Sciences, China Medical University, where a plant specimen was deposited (no. CMCP 1253).

2.2. Extract preparation

Lyophilized DFH rhizomes (1 kg) were extracted twice with 100 °C distilled water at 28 °C for 2 d per extraction. The pooled distilled water (DFW) extracts were vacuum-concentrated and freeze-dried to obtain a powder.

2.3. Chromatography-purified extract preparation

Freeze-dried DFW powder (103.8 g) was dissolved in water and separated chromatographically using an HP20 column (Diaion, Nippon Rensui Co., Japan) and a step-gradient mobile phase (H₂O to methanol [MeOH]) to yield four fractions (Fr.1–4). Fr.3 (33.7 g) was dissolved in 20% methanol, and further separated using a Sephadex LH-20 column (Sigma-Aldrich St Louis, MO, USA), eluting with H₂O/methanol (80:20–0:100) to afford four subfractions (Fr.31–34). Two subfractions (Fr.321 and 322) were obtained by recrystallization of Fr.32. The major fraction, Fr.322 (5.1 g), was chromatographed using a CHP20 column (MCI, Mitsubishi Chemical Co., Japan), eluting with H₂O/methanol (80:20–60:40) to obtain the ECAP fraction ([-] epicatechin-3-O-D-allopyranoside, 2.1 g). ¹H NMR and ¹³C NMR of ECAP are [α]_D²⁵ – 34.5 (c=1.8, MeOH)

¹H NMR (DMSO-*d*₆, 400 MHz): δ _H 6.86 (1H, d, *J* = 2.0 Hz, H-2'), 6.67 (1H, m, H-6'), 6.59 (1H, d, *J* = 8.4 Hz, H-5'), 5.87 (1H, d, *J* = 2.0 Hz, H-6), 5.73 (1H, d, *J* = 2.4 Hz, H-8), 5.13 (1H, d, *J* = 2.4 Hz, H-2), 4.57 (1H, d, *J*

= 7.6 Hz, H-1"), 4.21 (1H, m, H-3), 3.79–3.09 (6H, m, H-2", H-3", H-4", H-5", H-6"), 2.68 (1H, dd, *J* = 16.4, 4.4 Hz, H-4), 2.32 (1H, dd, *J* = 16.0, 7.2 Hz, H-4).

¹³C-NMR (DMSO-*d*₆, 100 MHz): δ C 156.0(C-7), 156.2 (C-5), 155.1 (C-8a), 144.3 (C-4'), 144.3 (C-3'), 129.5 (C-1'), 118.5 (C-6'), 115.2 (C-2'), 114.7 (C-5'), 99.6 (C-1"), 98.5 (C-4a), 95.1 (C-8), 94.0 (C-6), 76.7 (C-2), 74.4 (C-5"), 72.4 (C-3), 71.5 (C-3"), 70.6 (C-2"), 67.6 (C-4"), 61.6 (C-6"), 22.9 (C-4).

2.4. Osteoporosis induced by OVX

Three-month-old female C57BL/6JNarl mice (20–22 g) were used in this study. The mice were ovariectomized bilaterally using Zoletil (20 mg/kg by inhalation, Virbac, Carros, France)/Rompun (Bayer Animal Health GmbH, Leverkusen, Germany) anesthesia, and control mice were sham operated for comparison. After a 3-d recovery, the mice were randomly divided into six groups (10 mice per group): sham-operated, OVX control, OVX treated with DFW/mineral oil (50 and 200 mg·kg⁻¹·d⁻¹; DFWL and DFWH, respectively) and OVX treated with ECAP/mineral oil (0.06 and 0.25 mg·kg⁻¹·d⁻¹; CPL and CPH, respectively). Extract/mineral oil mixtures were administered by gastric gavage daily for 20 weeks. All animals were kept under controlled conditions at room temperature [(22 ± 1) °C] and a 12-h light-dark cycle. All animals were treated in accordance with the Institutional Animal Care and Use Committee (IACUC) of Tajen University, and the study protocol was approved by the ethics committee of the Tajen University, Pingtung, Taiwan.

2.5. Enzyme immunoassay

Blood was collected in a serum separator tube (SST), allowed to clot for 30 min, and then centrifuged for 15 min at approximately 1000 × *g*. The serum was collected and assayed immediately, or aliquoted and stored at -20 °C. The serum content of TNF- α and IL-1 β was measured using the enzyme-linked immunosorbent assay (ELISA) kits (TNF- α : Cat number: 559732, IL-1 β : Cat number: ab100704, Abcam, Cambridge, MA, USA).

2.6. Western blot analysis

The right femur tissue was homogenized in phosphate-buffered saline (PBS) and then was centrifuged for 5 min at 10 000 × *g*. The precipitate was solubilized in radioimmunoprecipitation assay (RIPA) lysis buffer (Merck Millipore, Minneapolis, MN, USA) as described previously (Sun et al., 2014). Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to Immobilon®-P polyvinyl difluoride (PVDF) membranes (Bio-Rad, CA, USA). Membranes were blocked with 4% bovine serum albumin (BSA, Abcam) for 1 h at room temperature and then probed with primary antibody at room temperature for 1 h. Primary antibodies were from Santa Cruz Biotechnology Inc., TX, USA (anti-BMP-2, sc-6895, 1:350; anti- β -actin, sc-47778, 1:1000, sc-47778), from Abcam (anti-Col I, ab34710, 1:500; anti-ALP, ab34710, 1:500; anti-RUNX2, ab76956, 1:500; anti-RANKL, ab9957, 1:500; anti-OPN, ab8448, 1:500; anti-OCN, ab93876, 1:500) and from Proteintech Group anti

ALP 11187-1-AP. After three washes, the blots were incubated with donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 h at room temperature. The bands were visualized using the ChemiDoc™ MP imaging system (Bio-Rad) and quantified using ImageJ software (National Institutes of Health [NIH], Bethesda, MD, USA).

2.7. Histological examination

Left femur tissue samples were fixed by direct immersion in 10% formalin for 16 h. Following paraffin embedding, 4 µm sections were cut, stained with periodic acid-Schiff (PAS), and imaged with a light microscope (100×).

2.8. Statistical analysis

Statistical analyses were carried out using the statistical package for the social sciences (SPSS) 13.0 software program (SPSS Inc., IL, USA). Triplicate samples were analyzed twice, and differences between the means were analyzed using Duncan's multiple range tests.

3. Results and Discussion

3.1. DFW and ECAP reduce expression of osteoclastogenesis markers

TNF-α can be secreted by monocytes to activate osteoclasts. The study by Chen et al. (2012) showed an increased expression of TNF-α in estrogen-deficient animals, and TNF-α also plays a role in inflammation. As shown in Fig. 1a, OVX (121.8 pg/mL)-treated mice exhibited significantly higher TNF-α levels than the sham mice did (85.8 pg/mL). Treatment of OVX mice with DFW (50 and 200 mg/kg, DFWL and DFWH, respectively) or ECAP (0.06 and 0.25 mg/kg, CPL and CPH, respectively) significantly reduced

TNF-α concentrations (89.6, 86.6, 87.0, and 81.0 pg/mL, respectively).

IL-1β is secreted by macrophages and can stimulate the activation of osteoclasts. According to a previous study, IL-1β stimulates bone resorption and inhibits bone formation (Nguyen et al., 1991). In Fig. 1b, IL-1β levels in the OVX group (121.0 pg/mL) were significantly increased compared to those in the DFWL, DFWH, CPL, CPH, and sham groups (27.2, 28.1, 24.5, 25.1, and 29.2 pg/mL, respectively). Therefore, the extract treatment groups were not different from the sham group.

RANKL, via its interaction with RANK, induces pre-osteoclast differentiation and activation of osteoclasts, which leads to bone resorption and decomposition of the bone matrix (Boyle et al., 2003). According to a previous report, RANKL blockade can prevent bone loss in animal models caused by osteoporosis, chronic inflammatory diseases, and malignancies, which may be related to postmenopausal osteoporosis, myeloma bone disease, and osteolytic metastases (Hofbauer and Schoppet, 2004). RANKL protein expression was significantly increased in the OVX group (134.5%) relative to that of the sham (Fig. 1c). However, treatment with all doses of DFW and ECAP extracts reduced RANKL protein expression in the ovariectomized mice to a level below that of the sham mice. Therefore, DFW and ECAP effectively reduced IL-1β, TNF-α, and RANKL expression to prevent osteoclastogenesis marker expression in response to OVX.

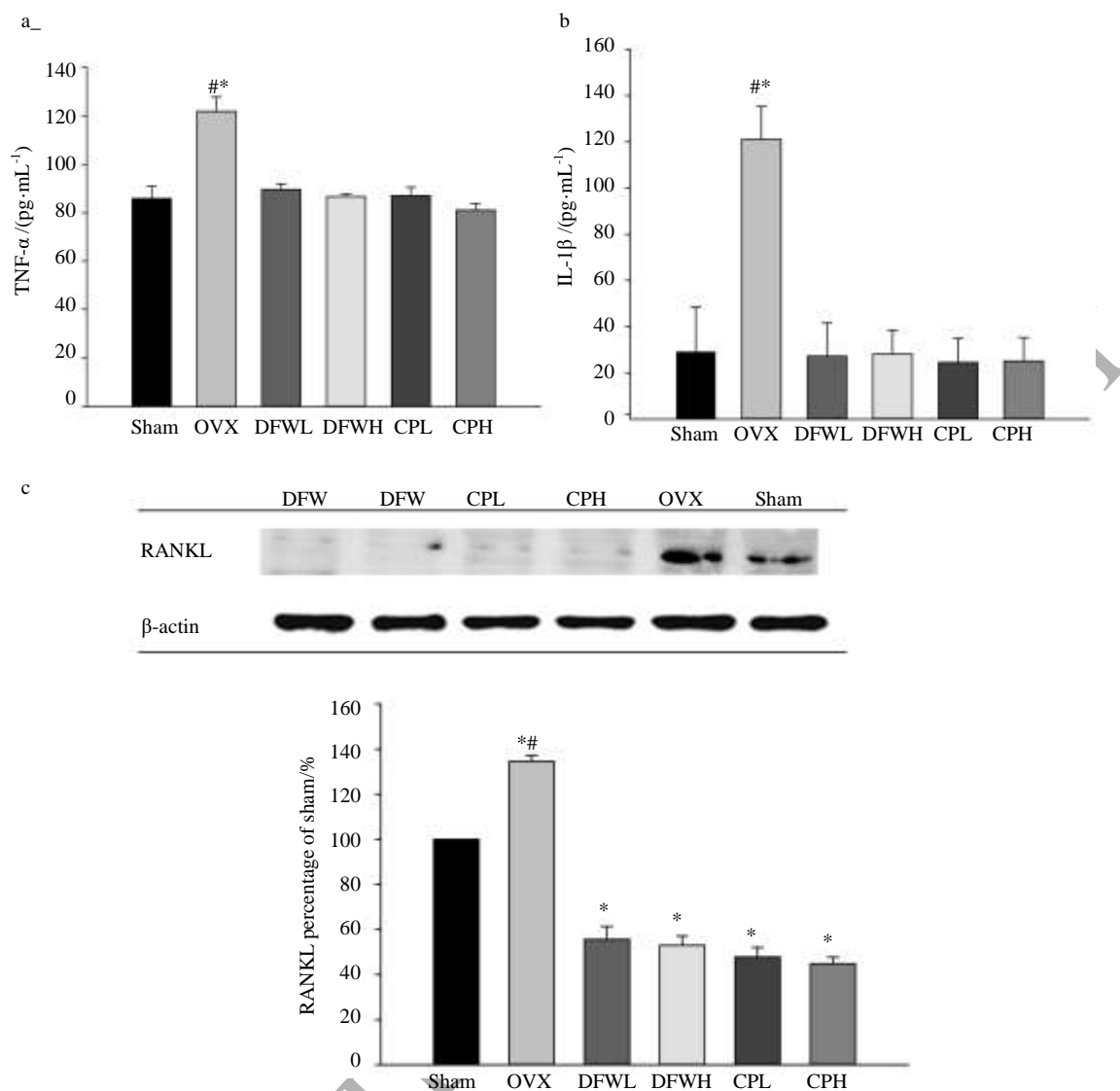


Fig. 1 Effect of *Davallia formosana* Hayata (DFH) water extract 50 and 200 mg·kg⁻¹·d⁻¹ (DFWL and DFWH, respectively) and [-] epicatechin-3-O-D-allopyranoside isolate (ECAP) 0.06 and 0.25 mg·kg⁻¹·d⁻¹ (CPL and CPH, respectively) on (a) TNF- α and (b) IL-1 β protein levels, (c) RANKL western blot analysis at 20 weeks of treatment.

Results are percentages of sham (vehicle); $n = 10/\text{group}$; * $P < 0.05$ vs sham and [#] $P < 0.05$ vs other groups

3.2. DFW and ECAP promote expression of osteogenesis markers

BMP-2 activates its receptor and induces mesenchymal stem cells to differentiate into pre-osteoblasts and osteoblasts to produce bone matrix, OCN, and ALP (Hsu et al., 2006). According to a prior report, mice lacking BMP-2 production in their limb bones have spontaneous fractures that do not heal. The lack of BMP-2 in the bone blocked the earliest stages of fracture healing, indicating that BMP-2 is a necessary endogenous mediator of fracture repair (Tsuji et al., 2006). As shown in Fig. 2a, BMP-2 protein expression levels were significantly reduced in the OVX mice (89.5%) compared to those of the sham mice (arbitrarily set to 100%). Treatment with all doses of DFW and ECAP extracts reversed the effects of OVX. In particular, the 0.25 mg/kg CPH-treated

group showed a higher increase in BMP-2 protein expression than the other groups did.

RUNX2, an important transcription factor, regulates the differentiation of mesenchymal stem cells into pre-osteoblasts and osteoblasts and promotes the expression of COL-I, OCN, and OPN; lack of RUNX2 leads to bone dysplasia or growth termination (Ducy et al., 1997; Komori et al., 1997). According to a previous study, which analyzed the role of RUNX2 in unloading-induced bone loss *in vivo*, maintaining an adequate RUNX2 dose is necessary to maintain the normal function of osteoblasts under mechanical unloading or nonphysiological conditions (Salingcamboriboon et al., 2006). The RUNX2 protein expression level was reduced in the OVX group (81.5%, Fig. 2b), compared to that in the sham group. Treatment with DFW or ECAP ameliorated these effects of OVX. In

particular, the ECAP extracts induced RUNX2 expression above that of the controls, in a dose-dependent manner.

ALP is a glycoprotein commonly found in tissues and secreted by osteoblasts. When mesenchymal stem cells gradually differentiate into mature osteoblasts, the ALP synthesis increases considerably, which can increase the differentiation of bone marrow cells (Aronow et al., 1990). ALP is involved in the matrix maturation and mineralization stages of bone development and is an important indicator of bone formation. ALP protein expression in the OVX group (74.9%) was significantly reduced compared to that of the other groups (Fig. 2c). Treatment of the OVX mice with DFWL, DFWH, CPL, or CPH significantly increased the expression of ALP to levels that were above those of the sham mice.

COL-1 is the main collagen of the bone, accounting for 90% of the bone matrix, and is an indicator of new bone and bone growth (Bedran-Russo et al., 2013). COL-1 protein expression was markedly reduced in the OVX mice (Fig. 2d). Treatment with all doses of DFW and ECAP extracts significantly ameliorated this effect of OVX. OCN can activate calcium absorption into the bone during osteogenesis.

During the mineralization stage of bone development,

osteoblasts secrete a large amount of OCN, which is a relatively late indicator of osteoblast maturation (Kavukcuoglu et al., 2009). The OCN protein expression level was slightly but significantly reduced in the OVX group (91.3%, Fig. 2e). Treatment with DFW and ECAP extracts at all doses ameliorated the effect of OVX, increasing the OCN expression level above that of the sham group. This was especially notable with the CPH-treated group (0.25 mg/kg ECAP), which exhibited the highest OCN protein expression levels among all groups.

OPN is synthesized by pre-osteoblasts and osteoblasts and can bind to calcium to regulate bone mineralization (Hunter et al., 1994). The OPN protein expression level was significantly reduced in the OVX group (81.4%), compared with that of the sham group (Fig. 2f). Treatment with DFW and ECAP extracts reversed the effect of ovariectomy on OPN expression, increasing expression levels above those of the sham mice. The 0.06 and 0.25 mg/kg ECAP extract doses (CPL and CPH-treated groups, respectively) exhibited the highest OPN levels among all of the groups. Therefore, DFW and ECAP effectively increased BMP-2, RUNX2, ALP, COL-1, OCN, and OPN to effectively promote the expression of these osteogenesis markers.

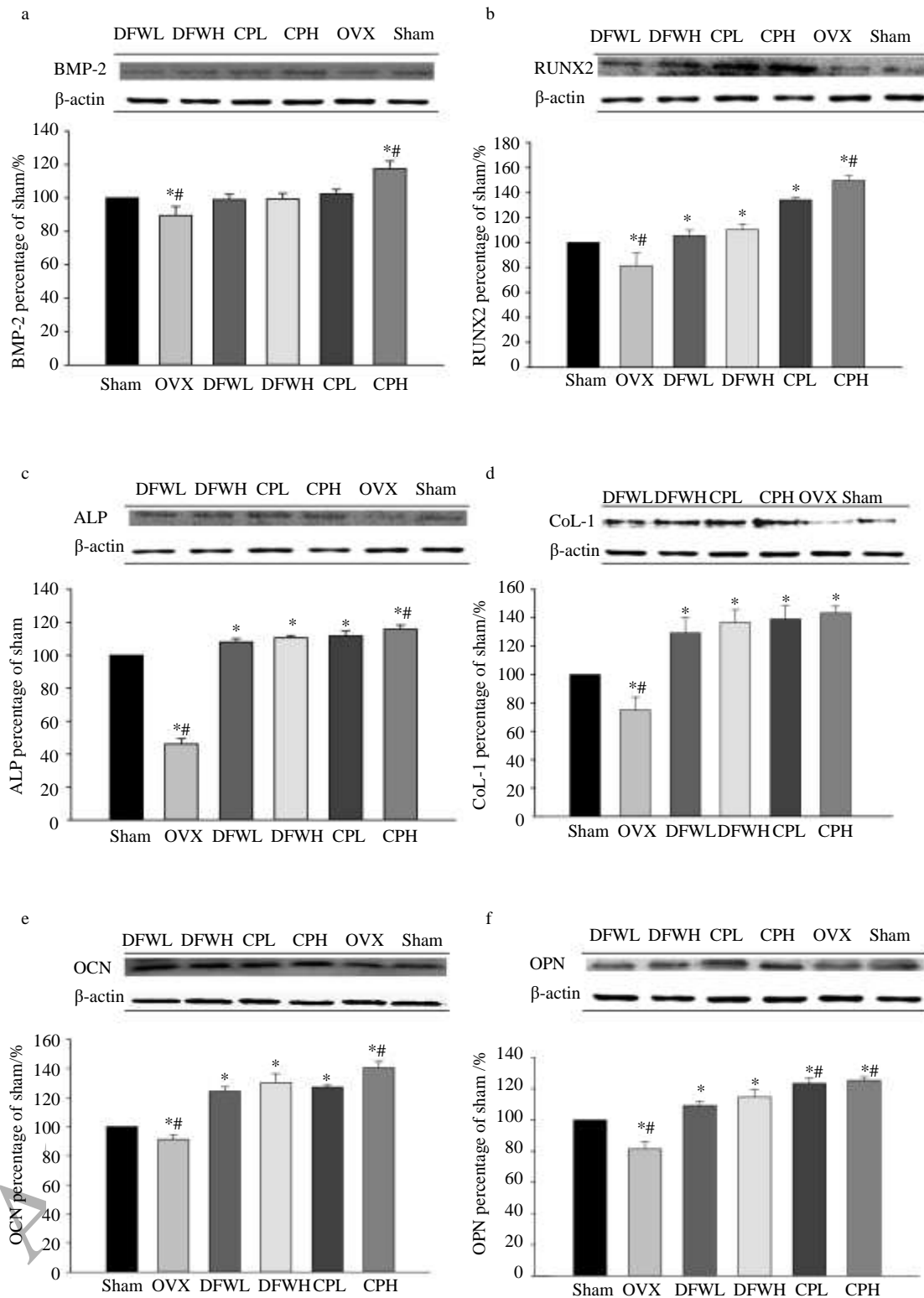


Fig. 2 Effect of *Davallia formosana* Hayata (DFH) water extract 50 and 200 mg·kg⁻¹·d⁻¹ (DFWL and DFWH, respectively) and [-] epicatechin-3-O-D-allopyranoside isolate (ECAP) 0.06 and 0.25 mg·kg⁻¹·d⁻¹ (CPL and CPH, respectively) on western blot analysis of (a) BMP-2, (b) RUNX2, (c) ALP, (d) COL-1, (e) OCN, and (f) OPN at 20 weeks of treatment

Results are percentages of sham (vehicle); $n = 10/\text{group}$; * $P < 0.05$ vs sham and # $P < 0.05$ vs other groups

3.3. Histological examination

Clinically, it has been demonstrated that increased adipogenesis and fat content in the bone marrow is correlated with decreased bone mineral density in the elderly and osteoporotic population. The increased number of adipocytes subsequently induces apoptosis in osteoblasts, which promotes the proliferation and differentiation of osteoclasts, resulting in increased bone resorption and reduced bone trabeculae numbers (Liu et al., 2011). The results show that the loss of bone trabeculae in the spongy bone of the left

femur and red bone marrow loss in OVX mice were significant (Fig. 3b).

Treatment with 50 and 200 mg/kg DFW extract (DFWL and DFWH, Fig. 3c and d, respectively) or 0.06 and 0.25 mg/kg ECAP extract (CPL and CPH (Fig. 3e and f), respectively) protected the bone trabeculae and bone marrow of OVX mice adequately. However, DFW and ECAP treatments did not completely restore these parameters to the levels seen in the sham group.

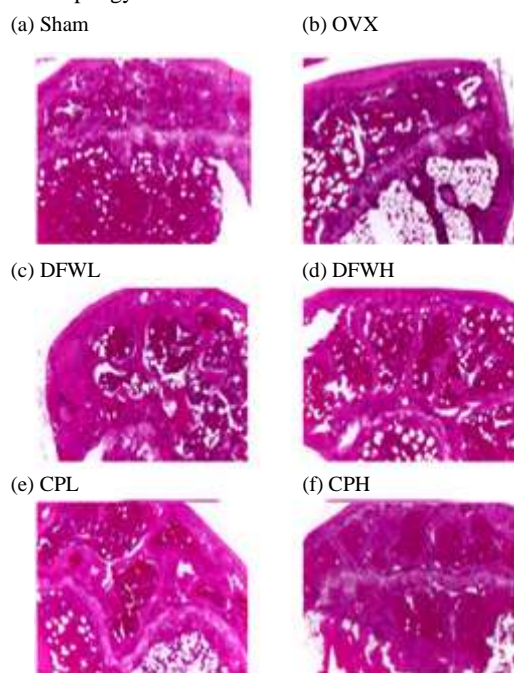


Fig. 3 Effect of *Davallia formosana* Hayata water extract (DFW) and (-)-epicatechin-3-O- β -D-allipyranoside (ECAP) on bone mass of C57BL/6JNarl mice after 20 weeks of treatment (a) Sham; (b) ovariectomized (OVX); (c) OVX and 50 mg·kg⁻¹·d⁻¹ DFW (DFWL); (d) OVX and 200 mg·kg⁻¹·d⁻¹ DFW (DFWH); (e) OVX and 0.06 mg·kg⁻¹·d⁻¹ ECAP (CPL); (f) OVX and 0.25 mg·kg⁻¹·d⁻¹ ECAP (CPH) with hematoxylin and eosin stain (100 \times).

Therefore, DFW and ECAP promoted the mechanisms of osteogenesis and inhibited osteoclastogenesis (Fig. 4). This effect was likely mediated by the reduction of IL-1 β , TNF- α , and RANKL expression to inhibit osteoclastogenesis. Increased BMP-2 protein expression likely promoted RUNX2 transcription, which induced the bone cell differentiation factors, ALP and COL-1, which are early osteogenic markers of differentiation. Moreover, DFW and ECAP prompted late mineralization, as indicated by OCN and OPN protein

expression. Therefore, we concluded that ECAP is a major active component of DFW extract, which may have therapeutic potential for the treatment of diseases associated with excessive osteoclastic and insufficient osteogenic activities. ECAP may not be the specific active molecule because it is unstable in the stomach. However, the major active molecule needs to be confirmed using a relevant pharmacological model.

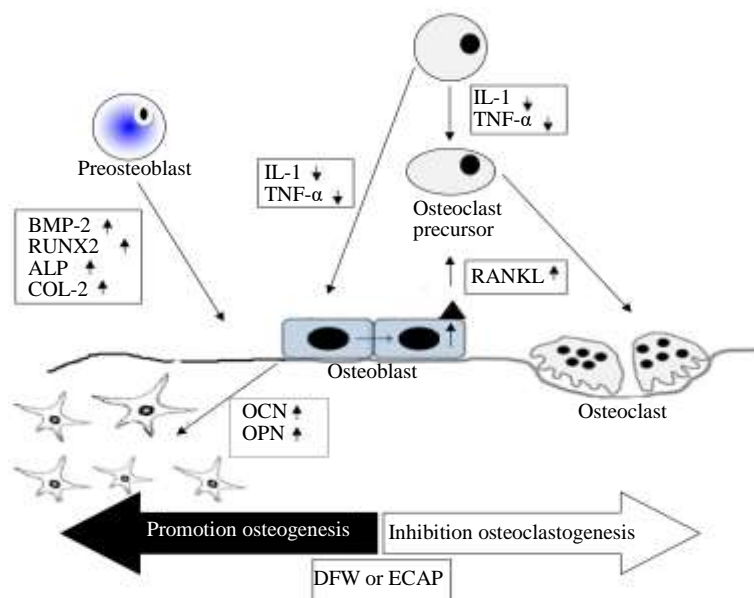


Fig. 4 Diagram showing how *Davallia formosana* Hayata (DFH) water extract (DFW) and (-)-epicatechin-3-O-D-allopyranoside (ECAP) can promote osteogenesis processes and inhibit osteoporosis processes.

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Conflict of interest

Yupintang Traditional Chinese Medicine Foundation is a non-profit organization that contributes immensely to the society, and, therefore, the co-authors have no conflict of interest to declare.

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