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Effects of *Davallia formosana* Hayata Water and Alcohol Extracts on Osteoblastic MC3T3-E1 Cells

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The Taiwanese native fern *Davallia formosana* Hayata (DFH) is used to treat bone diseases in classical Chinese medicine. We analyzed MC3T3E1 osteoblasts treated with different concentrations of water and ethanol extracts (10, 25, and 50 [both], and 100 μg/mL [DFE only]) using cell viability, expression of osteoblast differentiation markers [bone morphogenetic protein 2 (BMP-2), collagen 1 (CoL-1), alkaline phosphatase (ALP), and Runt-related transcription factor 2 (Runx 2)], and mineralization. These were significantly increased by DFW or DFE after 24-h incubation compared with the untreated controls. Compared with other treatments, DFW 50 and DFE 100 μg/mL significantly increased MC3T3E1 cell survival. DFW 25 and 50 μg/mL increased bone BMP-2, CoL-1, ALP, and Runx2 protein expression, ALP activity, and mineralization more than DFE did. Repeated chromatographic separation of DFW yielded compound (−)-epicatechin-3-O-d-allipyranoside (ECAP), which was characterized using ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. (−)-Epicatechin-3-O-d-allipyranoside (0.01 μg/mL) significantly increased cell survival (118.9%) and mineralization (218.7%) compared with that of the control treatment. We inferred that ECAP could mediate the main activity of DFW in bone formation, likely through BMP-2-induced Runx2 transcription, which increased bone cell differentiation factors ALP and CoL-1 and promoted mineralization. (−)-Epicatechin-3-O-d-allipyranoside could be an anti-osteoporotic agent. Copyright © 2017 John Wiley & Sons, Ltd.

Keywords: Davallia formosana Hayata; osteoporosis; BMP-2; CoL-1; ALP.

INTRODUCTION

Population aging is a progressing issue in Taiwan, and the occurrence of osteoporosis, a common disease of the elderly, is positively correlated with aging. It is the major risk factor for bone fractures in the elderly, increasing medical and social costs and risk of death. Therefore, preventing osteoporosis is an important public health challenge (Lin *et al.*, 2013). Osteoporosis is characterized by low bone mass and fragility, which promotes bone fracturing. Its development is attributable to various factors including hormones, metabolic and mechanical factors, aging, and the postmenopausal period (Chen *et al.*, 2004; Harada and Rodan, 2003; Riggs and Melton, 1992; Wu *et al.*, 2008).

The traditional medicine, Gu-Sui-Bu [Drynaria fortune (Kunze) J. SM., Polypodiaceae] (DFK) is listed in the Chinese Pharmacopeia (Chinese Pharmacopeia Commission 2005) as a typical 'kidney-tonifying' traditional Chinese medicine, which has been frequently used clinically for thousands of years to treat bone disorders. *Davallia formosana* Hayata (DFH), a petrophilous fern, is a Taiwanese traditional medicine also called Gu-Sui-Bu. The plant tissue characteristics of DFH differ from those of Drynaria fortune (Kunze) J. SM., Polypodiaceae, with a vascular bundle similar to a half moon, a base subsidence, and an epidermis similar to rectangular cells (Lai *et al.*, 2002). The DFH species is widely distributed in other countries including China, Vietnam, Thailand, and Laos, and grows on rocks and tree trunks. It is commonly used to treat bone injuries including inflammation, hyperlipemia, and arteriosclerosis in traditional Chinese medicine of Taiwan (Chang *et al.*, 2007; Jeong *et al.*, 2005; Ko *et al.*, 2012; Liu *et al.*, 2001; Sun *et al.*, 2003; Zhang *et al.*, 2009).

Osteoblast development involves proliferation, matrix maturation, and mineralization. Bone morphogenetic proteins (BMPs) are multifunctional growth factors belonging to the transforming growth factor (TGF)-β superfamily. The activities of BMP are regulated by different molecules including insulin-like growth factor-1 (IGF-1) in cell proliferation (Hung *et al.*, 2010). During signaling, BMP interacts with various downstream proteins including Runt-related transcription factor 2 (Runx 2), 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 the late osteogenic mineralization markers are osteocalcin (Oc) and osteopontin (Op) (Chen *et al.*, 2004; Rahaman *et al.*, 2015; Stein *et al.*, 1996).

In this study, ethanol and water extracts of DFH (DFE and DFW, respectively) were prepared and investigated for effects on bone cells. We obtained a pure active compound from DFW identified as (−)-

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epicatechin-3-O-β-D-allopyranoside (ECAP), which significantly increased osteoblast proliferation and mineralization.

MATERIALS AND METHODS

Plant material. The original names of *D. formosana* Hata 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 Dean Herbal Market, Taipei, Taiwan, in July 2015.

Paraffin mounting. The plant tissue specimens were paraffin wax-embedded and sectioned (Ruzin 1999). Briefly, the steps were sampling, fixative dehydration, vitrification, olefin immersion, olefin embedding, slicing, and paraffin removal. This was followed by afranin and fast green staining and vitrification after replacing the dye solution with a low to high ethanol gradient concentration. Finally, the mounted specimens were sealed with acacia gum (Sigma-Aldrich, St. Louis, MO, USA); the DFH characteristics were visualized and imaged using an inverted microscope (400× magnification).

Extract preparation. Lyophilized DFH rhizomes (1 kg) were extracted twice with 95% ethanol (Sigma-Aldrich) or 100°C distilled water at 28°C for 2 days. The total ethanol (DFE) and distilled water (DFW) extracts were vacuum-evaporated and freeze-dried to obtain a powder.

Cell culture. The murine osteoblast cell line MC3T3-E1 (Sigma-Aldrich) was grown in plastic cell culture dishes exposed to 95% air and 5% CO_2 in minimum α-essential medium (α-MEM, Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C for 24 h (Wu *et al.,* 2008).

Cell viability assay. The effects of DFW and DFE on cell survival were assayed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Sigma-Aldrich) as previously described (Lin *et al.,* 2013). Cells were treated with 0, 10, 25, 50, and 100 μg/mL DFE or 0, 10, 25, and 50 μg/mL DFW, cultured in fresh medium containing 0.5 mg/mL MTT for 3 h, the developed formazan crystals were dissolved in dimethyl sulfoxide (DMSO), and then the absorbance of the solution was measured at 550 nm (Hung *et al.*, 2010).

Alkaline phosphatase activity. The extract effects on ALP activity were examined in cells seeded in six-well plates. The ALP activity in the cellular fraction was measured using an assay kit (Abcam, Cambridge, MA, USA) with p-nitrophenol as the standard. An enzyme-linked immunosorbent assay (ELISA) reader was used for the measurements at 405 nm (Kim *et al.,* 2016).

Western blot analysis. Protein samples were prepared using radioimmunoprecipitation assay (RIPA) lysis buffer (Merck Millipore, Minneapolis, MN, USA) as described previously (Wu *et al.,* 2008). Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and transferred to Immobilon polyvinyl difluoride (PVDF) membranes (Bio-Rad, CA, USA), which were blocked with 4% bovine serum albumin (BSA, Abcam) for 1 h at room temperature and then probed with anti-BMP-2 (1:350, sc-6895), anti-β-actin (1:1000, sc-47778, both Santa Cruz Biotechnology Inc. Dallas, TX, USA), anti-Col I (1:500, ab34710), anti-ALP (1:500, ab34710), or anti-RUNX-2 (1:500, ab 76956, all Abcam) for 1 h at room temperature. 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 the ImageJ software (National Institutes of Health [NIH], Bethesda, MD, USA).

Osteoblast mineralization assay. Osteoblast mineralization was determined using the Alizarin red S dye (Fisher Scientific, Pittsburgh, PA, USA) staining protocol (Cooper *et al.*, 1998; Reseland *et al.*, 2001). Osteoblasts were treated with 0, 10, 25, 50, and 100 μg/mL DFE, 0, 10, 25, and 50 μg/mL DFW, 50 μg/mL ascorbic acid (Labscan Asia Co., Ltd., Bangkok, Thailand), and 10 mM β-glycerophosphate for 7 days. Then, the osteoblasts were washed with ice-cold phosphate-buffered saline (PBS, 0.14 M sodium chloride [NaCl], 2.6 mM potassium chloride [KCl], 8 mM sodium hydrogen phosphate [Na_2HPO_4], and 1.5 mM potassium dihydrogen phosphate [KH_2PO_4]) and fixed in ice-cold 95% ethanol for 20 min. For the staining, fixed osteoblasts were rinsed thoroughly, incubated in Alizarin red S for 10 min, rinsed thrice with distilled water, and the mineralized nodules were visualized and imaged using an inverted microscope (200× magnification). Mineralization was quantified by incubating Alizarin red S-stained cultured cells in 0.1 N sodium hydroxide (NaOH) for 30 min to release the calcium-bound Alizarin red S into the solution. The absorbance of the solution was measured at 405 nm. Each experiment was performed in duplicate wells and repeated thrice (Lee *et al.,* 2014; Wanachewin *et al.*, 2015).

Active compound isolation and activity determination. *Davallia formosana* Hayata (1 kg) was extracted with hot water at 100°C to prepare the DFW, which was vacuum-evaporated, and freeze-dried. Then, DFW (103.8 g) was dissolved in water and chromatographically separated using an HP20 column (Diaion, Nippon RssuiCo., Japan) using a step gradient system (H_2O to

methanol [MeOH]) to yield four fractions (Fr.**1**–**4**). Fr.**3** (33.7 g) was dissolved in 20% MeOH in water and further separated using a Sephadex LH-20 column (Sigma-Aldrich) eluted with H_2O/MeOH (80:20–0:100) to afford four subfractions (Fr.**31**–**34**). Two subfractions (Fr.321 and 322) were obtained by recrystallization from Fr.32. The major fraction, Fr.322 (5.1 g), was chromatographed using a CHP20 (MCI, Mitsubishi Chemical Co., Japan) eluted with H_2O/MeOH (80:20–60:40) to obtain compound H1 (2.1 g). The structure of compound H1 was analyzed using proton and C-13 (^1H and ^{13}C, respectively) nuclear magnetic resonance (NMR, Oxford As400, Bio-Surplus Co., Vista Sorrento Pkwy, San Diego USA), and its activity was analyzed using cell viability and mineralization assays.

Compound H-1: (−)-epicatechin-3-O-β-D-allopyranoside spectral characteristics. [α]D25–34.5 (c = 1.8, MeOH).

^1H NMR (DMSO-d6, 400 MHz)δH 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).

^{13}C-NMR (DMSO-d6, 100 MHz)δC 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).

Statistical analysis. The statistical analysis was 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.

RESULTS

Identification of *Davallia formosana* Hayata

According to Lai *et al.* (2002), plant tissue characteristics of DFH differ from those of other Gu-Sui-Bu forms, with a vascular bundle similar to a half moon (Fig. 1b), a base subsidence (Fig. 1a), and an epidermis resembling rectangular cells (Fig. 1a).

Cell survival assays

The results showed that 25 and 50 μg/mL DFW and 25, 50, and 100 μg/mL DFE significantly increased cell proliferation by 110% and 116%, and 105%, 111%, and 115%, respectively, compared with that of the untreated controls. In particular, the 50 and 100 μg/mL DFW-treated groups showed higher increases in cell proliferation than the other treated groups did.

Alkaline phosphatase activity

As shown in Fig. 2b, 25 and 50 μg/mL DFW and 25, 50, and 100 μg/mL DFE treatment significantly increased ALP activity (1.84, 1.85, and 1.95 U/mL, and 1.88, 1.85, 1.88, and 1.89 U/mL, respectively). The DFW 50 μg/mL-treated group exhibited a more significant increase in matrix maturation level than the other treated groups did.

DFW and DFE promoted expression of osteoblast differentiation markers

As shown in Fig. 2c and d, the protein expression of the osteoblast differentiation markers, BMP-2, RUNX-2, ALP, and CoL-1, increased significantly and dose-dependently following DFW and DFE treatment.

BMP-2 protein expression levels (Fig. 2e) increased markedly following 25 and 50 μg/mL DFW treatment

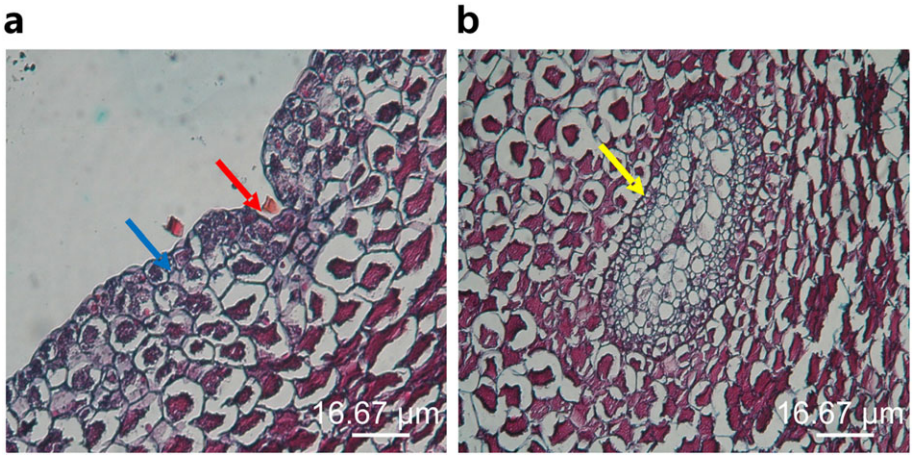

Figure 1. Identification of *Davallia formosana* Hayata (DFH) images. (a) Epidermal cell (blue) and base subsidence (red). (b) Vascular bundle (yellow). Morphologies of plant tissue were observed and photographed using a light microscope, 400× magnification. [Colour figure can be viewed at wileyonlinelibrary.com]

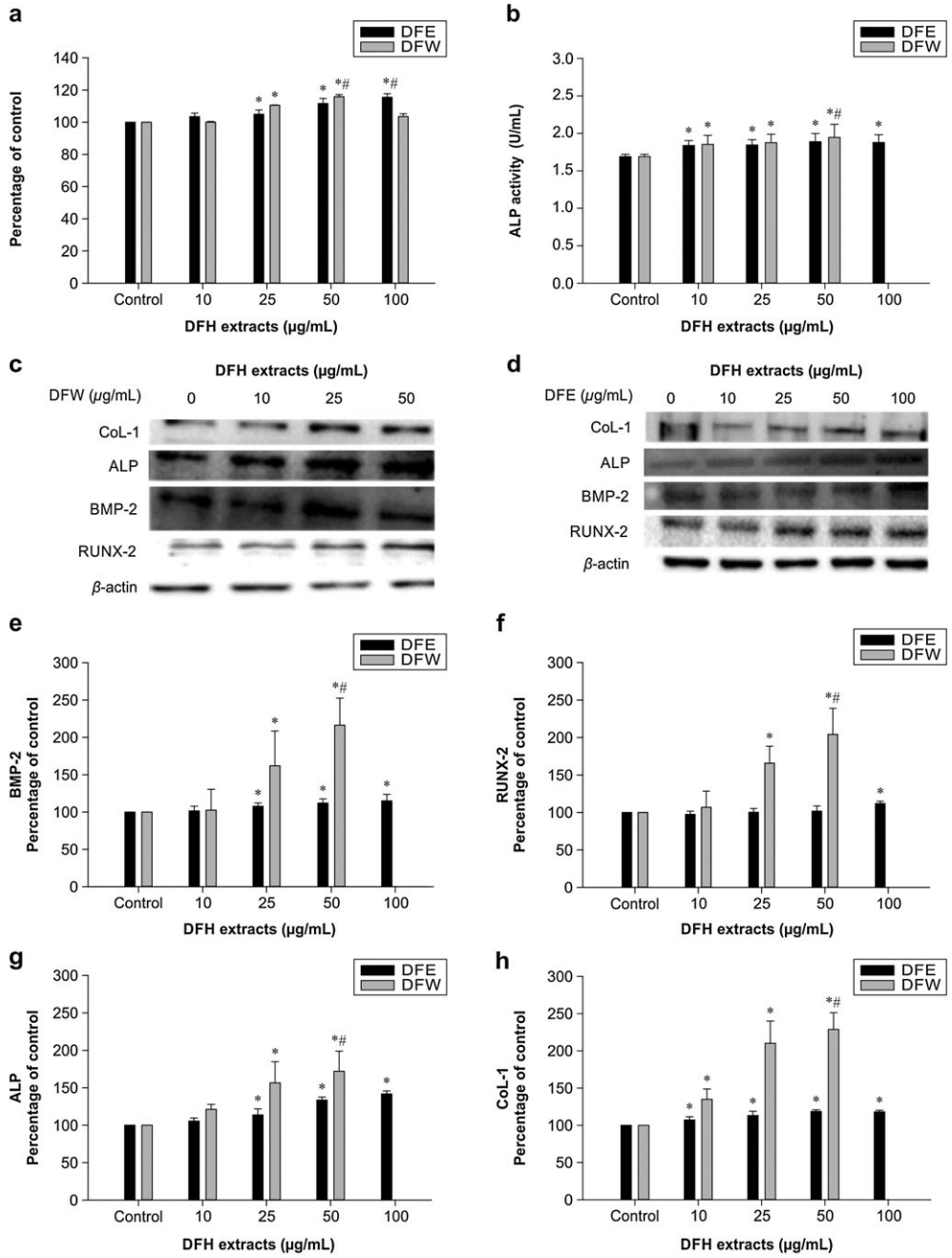

Figure 2. Effect of *Davallia formosana* Hayata (DFH) ethanol and water extract (DFE and DFW, respectively) on (a) proliferation, (b) alkaline phosphatase (ALP) activity, and (c and d) western blot analysis. Western blot analysis of (e) BMP-2, (f) RUNX-2, (g) ALP, and (h) CoL-1 for 24 h. Results are percentage of control (vehicle); $n = 3$/group; $^{*}P < 0.05$ vs. control and $^{\#}P < 0.05$ vs. other groups. BMP-2, bone morphogenetic protein 2; CoL-1, collagen 1; ALP, alkaline phosphatase; RUNX-2, Runt-related transcription factor 2.

(by 162.0 and 216.4%) and 25, 50, and 100 μg/mL DFE (by 107, 112, and 115%), respectively, compared with the control levels. In particular, the 50 μg/mL DFW-treated group showed a higher increase in BMP-2 protein expression than the other groups did.

RUNX-2 protein expression level (Fig. 2f) was markedly increased by 25 and 50 μg/mL DFW (165.7 and 204.2%, respectively) and 100 μg/mL DFE (111.9%) compared with the control values. Particularly, the 50 μg/mL DFW-treated group showed higher RUNX-2 protein expression levels than the other groups did.

Alkaline phosphatase protein expression level (Fig. 2g) increased markedly following treatment with 25 and 50 μg/mL DFW (156.6 and 172.1%, respectively) and 25, 50, and 100 μg/mL DFE (113.8, 113.5, and 142.0%, respectively) compared with the control levels. Especially, the 50 μg/mL DFW-treated group exhibited higher ALP protein expression levels than those of the other groups.

CoL-1 protein expression level (Fig. 2h) was markedly increased by 10, 25, and 50 μg/mL DFW (135.0, 210.2, and 228.9%, respectively) and 10, 25, 50, and 100 μg/mL DFE (107.2, 113.2, 118.6, and 118.1%, respectively) compared with the control value. Especially, the 50 μg/mL DFW-treated group showed higher CoL-1 protein expression levels than those of the other groups.

Osteoblast mineralization assay

Osteoblast mineralization was significantly and dose-dependently increased by DFW and DFE (Fig. 3a). The mineralization expression levels (Fig. 3b) were markedly increased by 10, 25, and 50 μg/mL DFW (142.0, 208.5, and 348.9%, respectively) and 25, 50, and 100 μg/mL DFE (142.4, 246.5, and 288.0%, respectively) compared with the control value. Especially, the 50 μg/mL DFW-treated group showed more pronounced mineralization than the other groups did.

Isolation, determination, and activity of active compound

DFH was extracted with hot water and repeatedly chromatographed to obtain compound H1, which was elucidated using physical and spectral data. Compound H1 was obtained as colorless needle-shaped particles. The ^{1}H and ^{13}CNMR data revealed that the compound showed a characteristic epicatechin structural feature at δ 5.13 (1H,d, J = 2.4 Hz, H-2), 4.21 (1H,m, H-3), 2.68 (1H,dd, J = 16.4, 4.4 Hz, H-4) and 2.32 (1H, d, J = 16.0, 7.2 Hz, H-4). The J2,3 coupling constant (J = 2.4 Hz) confirmed the cis arrangement of H-2 and H-3. The ^{1}H-NMR data showed the ABX-type resonance at δ 6.86 (1H,d, J = 2.0 Hz, H-2′), 6.67 (1H, m, H-6′), and 6.59 (1H,d, J = 8.4 Hz, H-5′), indicating the presence of 1,3,4-trisubstitutions in the B-ring. A pair of meta-coupled aromatic methines were found at δ 5.87 (1H,d, J = 2.0 Hz, H-6) and 5.73 (1H,d, J = 2.4 Hz, H-8) in the A-ring. Additionally, the ^{1}HNMR spectrum exhibited signals attributable to an anomeric proton at δ 4.57 (1H, d, J = 7.6 Hz) and four signals at δ 3.00–3.79 constituted a sugar moiety. The β-configuration of the sugar moiety was determined based on the coupling constant of an anomeric proton (J = 7.6 Hz). These results indicate that compound H1 was similar to (−)-epicatechin-3-O-β-D-allopyranoside and (−)-epicatechin-5-O-β-D-glucopyranoside (Cui *et al.* 1992). Therefore, compound H1 was compared with the literature and determined to be (−)-epicatechin-3-O-β-D-allopyranoside (Fig. 4d).

The activity of compound H1 (0, 0.01, 0.1, 1, and 10 μg/mL) was analyzed using cell viability and mineralization assays. The results showed that 0.01 and 0.1 μg/mL H1 significantly increased the cell viability by 118.9 and 116.4% compared with the control value (Fig. 4a). Osteoblast mineralization was significantly increased by H1 (Fig. 4b). Furthermore, treatment with 0.01 and 0.1 μg/mL H1 significantly increased the mineralization by 218.7 and 220.6%, respectively, compared with the control value (Fig. 4c).

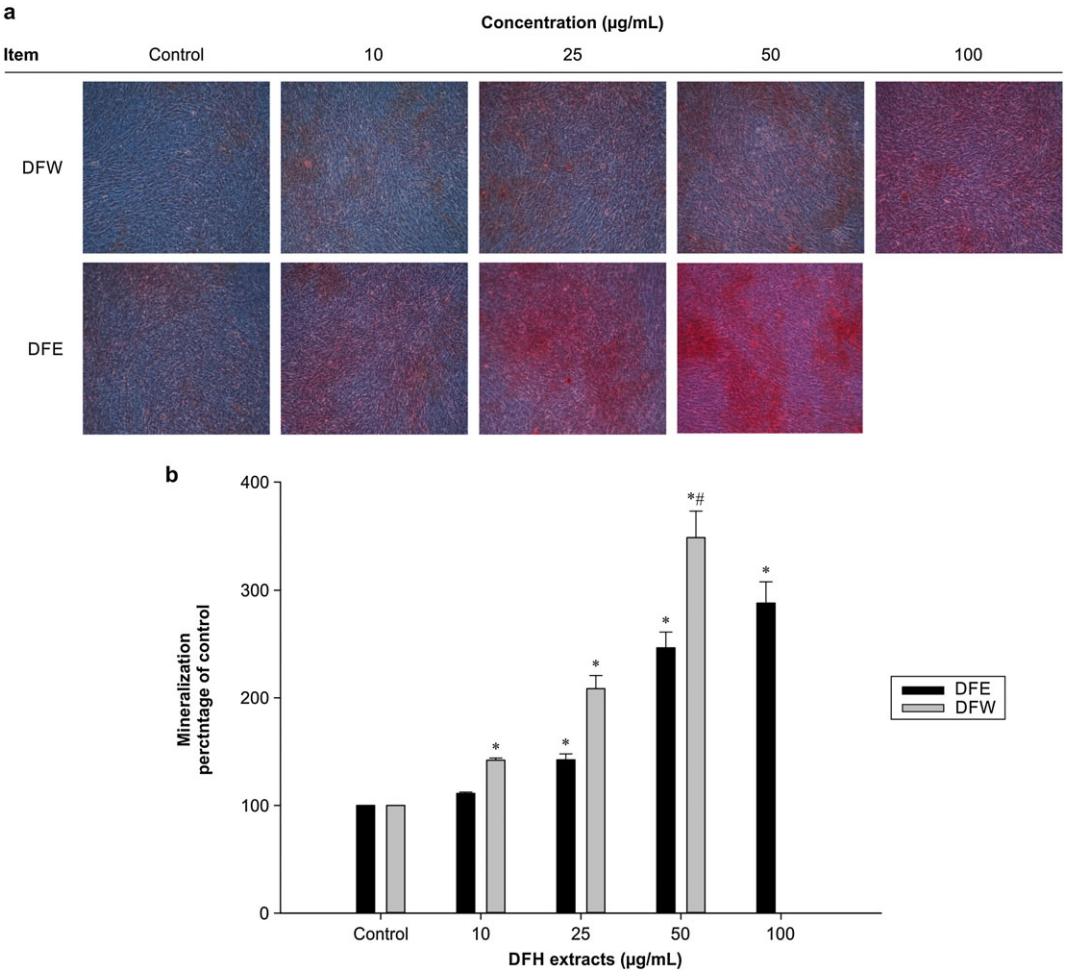

Figure 3. Effect of *Davallia formosana* Hayata (DFH) ethanol and water extract (DFE and DFW, respectively) on (a) mineralization detected using Alizarin red S staining and imaged using an inverted microscope (200× magnification) after 7-day treatment of MC3T3-E1 cells. (b) Mineralization was measured at 405 nm. Results are percentage of control (vehicle); n = 3/group; $^{*}P < 0.05$ vs. control and $\#P < 0.05$ vs. other groups. [Colour figure can be viewed at wileyonlinelibrary.com]

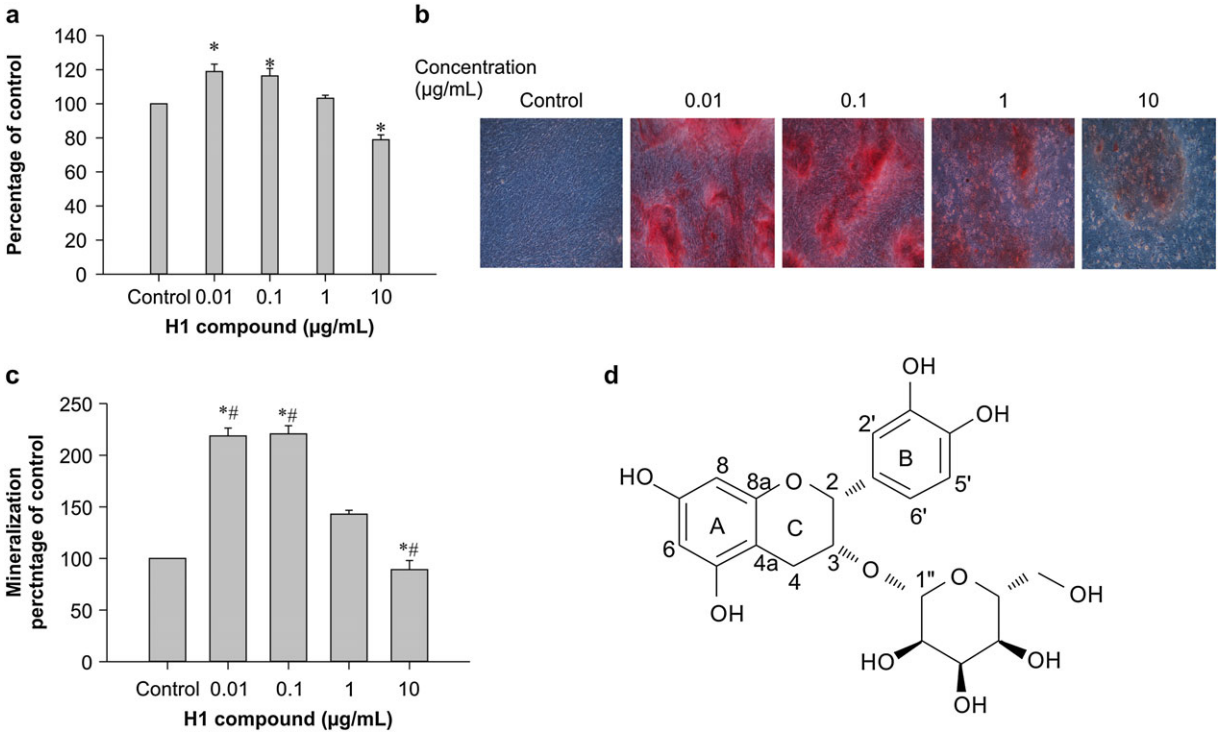

Figure 4. (a) Effect of H1 compound treatment on proliferation of MC3T3-E1 cells. (b) Mineralization was detected using Alizarin red S staining and imaged using an inverted microscope (200× magnification) after 7-day treatment of MC3T3-E1 cells. (c) Mineralization was measured at 405 nm. (d) Structure of compound H1 is (−)-epicatechin-3-O-β-D-allopyranoside. Results are expressed as percentage of control (vehicle). $^{*}P < 0.05$ vs. control and $^{\#}P < 0.05$ (both $n = 3$) vs. other groups. [Colour figure can be viewed at wileyonlinelibrary.com]

DISCUSSION

DFH and DFK are called Gu-Sui-Bus in Taiwan. We used paraffin mounting to clearly differentiate the plant tissue characteristics of DFH and DFK. Various studies have indicated that DFK possesses antiosteoporotic activity. However, the osteopenia-related studies of DFH are still few. Currently, this extract has not been studied for its effects on MC3T3E1 cells, and, therefore, we aimed to evaluate the potential effects of DFW and DFE extracts on the proliferation, differentiation, and mineralization of osteoblasts.

In cell survival analysis, the MTT colorimetric assay is used to assess cellular metabolic activity. Under defined conditions, reduced nicotinamide adenine dinucleotide (NADH)-dependent cellular oxidoreductase enzymes may reflect the number of viable cells present. The results showed that the DFW- and DFE-treated groups showed increased cell proliferation, especially the 50 and 100 μg/mL DFW-treated groups. Therefore, DFW promoted MC3T3-E1 cell proliferation at lower doses than DFE did (Fig. 2a).According to one of the only two recent studies on DFH extract effects on osteoclasts, DFE inhibited osteoclast differentiation *in vivo*, especially at 200 μg/mL (Ko *et al.,* 2012). A study reported that ethanol extracts of *D. formosana* (WL1101) concentration-dependently inhibited tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclast formation at 20, 60, and 200 μg/mL.

Alkaline phosphatase is an early cellular marker and essential enzyme for osteoblast differentiation. Bone ALP, a glycoprotein found on osteoblast surfaces, reflects the biosynthetic activity of bone-forming cells. Alkaline phosphatase is sensitive to bone metabolism (Kim *et al.,* 2014; Wang *et al.*, 2014; Zhang *et al.*, 2009). Therefore, DFW and DFE effectively stimulated osteoblast maturation markers, which enhanced osteoblast differentiation of MC3T3-E1 cells, especially in the 50 μg/mL DFW-treated groups (Fig. 2b).

Osteoblastic cells produce extracellular matrix proteins including BMP-2, RUNX-2, ALP, and CoL-1, which induce osteoblast proliferation, matrix maturation, and mineralization (Stein *et al.* 1996). The Wnt/β-catenin signaling pathway in osteoblasts activates BMP-2, suggesting that BMP signaling is controlled by functional crosstalk with the Wnt/β-catenin pathway (Zhang *et al.,* 2013). Skeletal development and remodeling require stringent control of gene activation and suppression in response to physiological cues. RUNX-2 is a key transcription factor of osteoblast differentiation. It controls the osteogenic process, as indicated by its activation of bone phenotypic genes in pluripotent cells, which convert committed premuscle cells to the osteoblast lineage (Stein *et al.,* 2004). *ALP* is an osteoblast differentiation-associated gene, and high ALP activity indicates enhanced cell proliferation, which may lead to matrix maturation. Therefore, ALP activity is used as an osteoblast differentiation and proliferation marker (Weyts *et al.*, 2003). CoL-1, an important component of the bone as the main extracellular matrix protein for calcification, plays a role in osteoblast differentiation. Therefore, CoL-1 is an osteoblast marker that participates in controlling osteoblast function and bone matrix mineralization (Guillerminet *et al.,* 2010). Mineralized nodule formation in osteoblastic cell cultures provides an index for assessing culture status. Currently, the von Kossa method is commonly used to visualize mineralized nodules in cell culture using Alizarin red S staining (Wang *et al.,* 2006). The results showed that the protein expression of BMP-2, RUNX-2, ALP, and CoL-1 and mineralization increased

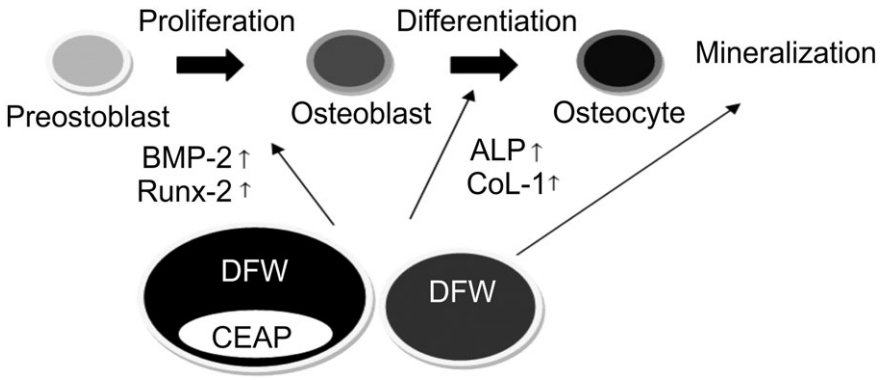

Figure 5. *Davallia formosana* Hayata (DFH) water and ethanol extracts (DFW and DFE, respectively) and [−] epicatechin-3-O-d-allipyranoside (ECAP) promoted bone formation processes.

following DFW and DFE treatment (Figs 2c–h and 3a and b). Therefore, DFW and DFE effectively stimulated osteogenesis of osteoblast, especially DFW treatment.

Using bioactivity-guided fractionation, we further isolated the active component, ECAP, from DFW. (−)-Epicatechin-3-O-d-allipyranoside promoted the proliferation and mineralization of osteoblast in MC3T3 E1 cell culture system at lower concentrations (0.01 and 0.1 μg/mL, Fig. 4a–c) than those of the crude extract.

Therefore, DFH extracts promoted mechanisms of cell proliferation, differentiation, and mineralization (Fig. 5). This was likely mediated through increased BMP-2 protein expression to promote Runx2 transcription, which induces the bone cell differentiation factors ALP and CoL-1, the early osteogenic markers of differentiation. Moreover, DFW and DFW also prompted late mineralization expression as observed in the mineralization assay.

CONCLUSIONS

Therefore, the results of our study suggest that DFW and DFE extracts of DFH promote cell proliferation, differentiation, and mineralization. The DFW exhibited better results than DFE did on bone formation. Therefore, the bone formation activity of DFW was proved to be mediated by ECAP, which may represent a potentially useful remedy for the prevention of osteoporosis. However, these results need to be confirmed using an ovariectomized rat model.

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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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None.

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