Bone-protective effects of bioactive fractions and ingredients in Sambucus williamsii HANCE

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Abstract

Our previous study demonstrated that 60% ethanol crude extract of Sambucus williamsii HANCE (SWH) improved bone mass, bone strength and bone micro-structure in both ovariectomised (OVX) rats and mice. The present study aims to identify the bioactive fractions and ingredients in SWH that account for its osteoprotective effects. Bilateral sham-operated mice acted as controls. OVX C57BL/6J mice, aged 12 weeks, were orally administrated daily with vehicle or 17β-oestradiol (3.2 mg/kg), SWH (60% ethanol crude extract; 1.0 g/kg), SWA (water eluate; 0.570 g/kg), SWB (30% ethanol eluate; 0.128 g/kg) or SWC (50 and 95% ethanol eluates; 0.189 g/kg) for 12 weeks. The effects of the different fractions on bone properties in the OVX mice model were studied. In addition, their effects on osteoblast proliferation and differentiation were evaluated in UMR 106 cells. SWC significantly restored bone mineral density and improved bone size and bone content parameters in the femur and tibia as well as increased biomechanical strength at the tibia diaphysis in OVX mice. Similarly, SWC was the most potent fraction in stimulating cell proliferation and differentiation in UMR 106 cells. Also, SWC did not alter uterus weight in OVX mice. Nine major peaks, seven lignans and two phenolic acids, in the HPLC fingerprint of the SWC fraction were identified, isolated and characterised. In conclusion, the present study demonstrated that SWC was the most potent fraction in SWH that exerted anti-osteoporotic effects. Furthermore, lignans might be the potential bioactive components in SWC.

Key words: Osteoporosis: Lignans: Sambucus williamsii HANCE: Phyto-oestrogens

Osteoporosis is a skeletal disorder of reduced bone mass and disruption of bone architecture with a consequent increase in bone fragility and fracture risk(1). The decline of gonadal function following menopause and ageing plays a key role in the pathogenesis of osteoporosis. With a worldwide increase in ageing populations, osteoporosis has become a more serious global health problem. Hormone replacement therapy was a routine method to alleviate physical symptoms and to prevent the clinical consequence of postmenopausal osteoporosis(2). However, recent studies suggest that hormone replacement therapy is associated with an increased risk of developing breast, ovarian and endometrial cancers(3–6). This has led to increased interest in identifying natural oestrogen analogues that possess osteoprotective effects without any side effects on reproductive tissues. A class of plant-derived substances, the so-called ‘phyto-oestrogens’, has been reported to possess structures similar to mammalian oestrogens and display both oestrogenic and anti-estrogenic effects(7–8). Isoflavones, lignans and cumestans are the major classes of phyto-oestrogen(7). Numerous studies on isoflavones in preventing the loss of bone mineral density (BMD) have been reported(9,10), while those that focus on the bone-protective effects of lignans and cumestans are few.

Sambucus williamsii HANCE (SWH), one species of Sambucus distributed in various regions of China, Korea and Japan, has been used as a folk medicine to treat bone and

Abbreviations: BMD, bone mineral density; BV/TV, bone volume/tissue volume; 3D, three-dimensional; E2, 17β-oestradiol; OVX, ovariectomised; Sham, sham-operated vehicle-treated; SWA, Sambucus williamsii HANCE water eluate; SWB, Sambucus williamsii HANCE 30% ethanol eluate; SWC, Sambucus williamsii HANCE 50 and 95% ethanol eluates; SWH, Sambucus williamsii HANCE; Tb.N, trabecular bone number; Tb.Sp, trabecular bone separation.

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Table 1. The fractions of *Sambucus williamsii* HANCE* and their yield

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Isolated solvent (v/v)</th>
<th>Isolated content (g)</th>
<th>Percentage of total crude extract (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWA</td>
<td>Ethanol 50%</td>
<td>712·5</td>
<td>57·0</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWB†</td>
<td>Ethanol 30%</td>
<td>160·0</td>
<td>12·8</td>
</tr>
<tr>
<td>SWC†</td>
<td>Ethanol 95%</td>
<td>224·2</td>
<td>13·9</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>11·8</td>
<td>5·0</td>
</tr>
</tbody>
</table>

*The ethanol extract of *Sambucus williamsii* HANCE (1250 g) was applied to a D-101 macroporous adsorptive resin column (diameter 8·0 cm x height 96 cm), and eluted with water and ethanol in gradient to give its fractions.

† The 50% ethanol eluate and 95% ethanol eluate were combined together to give SWC.

Materials and methods

Plant materials and isolation

The stems and branches of SWH were collected in Shenyang, Liaoning Province in the northeast of China in April 2007 and authenticated according to a method listed in the Chinese Bencao with the help of Professor Zerong Jiang (Shenyang Pharmaceutical University in Shenyang, China). The identity of SWH was confirmed by analysing its major ingredients pentaacyl triterpenoid, phenol acid and derivatives and protein. A voucher specimen (HHXSWGZ-2007) was deposited in the Herbarium of the Institute of Traditional Chinese Medicine and Natural Products (Jinan University, Guangzhou, China).

Dry stems and branches of SWH (50 kg) were refluxed with 500 litres of 60% (v/v) ethanol three times, for 2 h each time. After filtration, the filtrate was concentrated under reduced pressure and then freeze-dried to obtain a total extract of 1·25 kg powder. As shown in Table 1, SWH (1250 g) was applied to a D-101 macroporous adsorptive resin column (diameter 8·0 cm x height 96 cm), eluted with water and ethanol in gradient, to give SWA (eluate: 712·5 g), SWB (30% ethanol eluate: 160·0 g) and SWC (50 and 95% ethanol eluates; 224·2 g). The 50% and 95% ethanol eluates were combined to give SWC after HPLC analysis because both fractions contain similar constituents and because the yield of the 95% ethanol eluate was too low. The HPLC fingerprint of SWC was established using the Agilent series 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA): a reversed-phase (RP)-HPLC column (Welch XB-C18, 4·6 x 250 mm; Welch Material, Shanghai, China) with a gradient of methanol–water (0:1% acetic acid) as the mobile phase (0–9 min, 25%; 9–39 min, 36%; 39–54 min, 50%; 54–59 min, 100%), at a flow rate of 0·8 ml/min. Detection wavelength was at 280 nm and column temperature was at 35°C. The major peaks of the fingerprint were characterised with the compounds isolated from SWC using the same HPLC conditions.

Animal study

A total of fifty-six virgin C57BL/6j specific-pathogen-free (SPF) female mice, aged 12 weeks, were purchased from Laboratory Animal Services Centre (Chinese University of Hong Kong, Hong Kong, China) and housed in cages under a 12 h light–12 h dark cycle at 20 ± 1°C. During the study, all animals were allowed free access to distilled deionised water, and OVX mice were pair-fed a phyto-oestrogen-free diet (D00031602; Research Diets, Inc., New Brunswick, NJ, USA) (Table 2) based on the average daily food consumption of the sham control group.

The mice were either sham-operated (Sham, n 8) or ovariectomised (OVX, n 48). The treatment dose of each fraction was deduced from the yield. The OVX mice were randomly divided into six groups: vehicle-treated group (OVX, n 8); 17β-oestradiol administration group (E₂; n 8; 3·2 µg/g body weight per d); SWH (ethanol extract group; n 8; 1·0 mg/g body weight per d); SWB (water eluate group; n 8; 0·570 mg/g body weight per d); SWW (30% ethanol eluate

Table 2. Formulations and estimated nutrient composition of experimental diets

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Diet (D00031602) (g/kg diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total energy (kJ/kg)</td>
<td>16 119·2</td>
</tr>
<tr>
<td>Protein</td>
<td>142·0</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>731·0</td>
</tr>
<tr>
<td>Fat</td>
<td>40·0</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>0</td>
</tr>
<tr>
<td>Maize oil</td>
<td>40·0</td>
</tr>
<tr>
<td>1-Butyldihydroquinone</td>
<td>0·008</td>
</tr>
<tr>
<td>Mineral mix S10022G*</td>
<td>35·0</td>
</tr>
<tr>
<td>Vitamin mix V10037†</td>
<td>10·0</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2·5</td>
</tr>
<tr>
<td>Casein</td>
<td>140·0</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>1·8</td>
</tr>
<tr>
<td>Maize starch</td>
<td>495·7</td>
</tr>
<tr>
<td>Maltodextrin 10</td>
<td>125·0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100·0</td>
</tr>
<tr>
<td>Cellulose, BW200</td>
<td>50·0</td>
</tr>
</tbody>
</table>

*The mineral mix composition was as follows (amount in 35 g): 5·0 g Ca, 2·0 g P, 0·5 g Mg, 3·6 g K, 0·3 g S, 1·0 g Na, 1·6 g Cl, 6·0 mg Cu, 0·2 mg I, 45 mg Fe, 10·5 mg Mn, 0·2 mg Se and 30·0 mg Zn.
† The vitamin mixture composition was as follows (amount in 10 g): 2·2 mg Vitamin A (all-trans-retinyl palmitate), 25 µg cholecalciferol, 33·75 mg vitamin E (all-rac-α-tocopheryl acetate), 0·75 mg phyloquinone, 0·2 mg biotin, 25 µg cyanocobalamine, 2 mg folic acid, 30 mg niacinamide, 16 mg calcium pantothenate, 7 mg pyridoxine-HCl, 6 mg riboflavin, 6 mg thiamin HCl.
group; n = 8; 0·128 mg/g body weight per d); SWC (50 and 95 %
ethanol eluates group; n = 8; 0·189 mg/g body weight per d).
The solutions of SWH and its fractions were freshly prepared
as SWH (100 mg/ml), SWA (57 mg/ml), SWB (12·8 mg/ml)
and SWC (18·9 mg/ml), and were given daily to mice. A phyto-
oestrogen-free diet was applied in the present study to
eliminate interactions between diet and fractions of SWH
that had been reported in our previous study[13]. Drug treat-
ment started 2 weeks after the surgery and was administrated
orally through a gastric tube for 3 months. At the end of the
treatment, the mice were killed, blood was withdrawn from
the abdominal aorta and serum was prepared. From each
animal, the uterus was collected, weighed and then stored at
−80°C. The left femur and tibia were dissected and cleaned
of all soft tissue, then wrapped in gauze saturated with PBS
buffer, sealed in Eppendorf tubes and stored at
20°C for further analysis. The animal study protocols were approved
by the Animal Ethics Committee of The Hong Kong Poly-
technic University.

Micro-computed tomography analysis of bone properties

The distal metaphysis of femur and the proximal metaphysis
of tibia were scanned with a high-resolution micro vivaCT
40 system (Scanco Medical, Basserdorf, Switzerland). The
distal and proximal site was defined as 2·0 mm away from
the femur end and tibia head. After images were captured,
twenty out of 100 slices were chosen and established as the
volume of interest. Trabecular bone was separated from cortical
bone by free drawing regions using the software provided
with the scanner. Then three-dimensional (3D) modelling
was generated and morphological date of the sample was
evaluated using a script-based 3D volume analysis tool IPL
(Image Processing Language; Scanco Medical; http://www.
scanco.ch/systems-solutions/software.html). 3D parameters for trabecular bone were obtained as follows: (1) bone
volume/tissue volume (BV/TV); (2) trabecular bone number (Tb.N); (3) trabecular bone separation (Tb.Sp); (4) BMD.

Three-point bending test

The left tibias were cleaned to remove their surrounding soft
tissue before the test. A three-point bending machine
(model H10KM; Hounsfield Test Equipment Limited, Redhill,
Surrey, UK) was used to determine the mechanical strength
on the mid-shaft of the left tibia[18]. The two supporting
points were fixed 4 mm apart with a single central loading
point. A load was applied on the mid-shaft with a deformation
rate of 2·0 mm/min and a load–deformation curve was plotted
simultaneously until the specimen was broken. Bending
stiffness was calculated as the slope of the linear part of the
loading–deformation curve.

Cell culture

UMR 106 cells were cultured in Dulbecco’s modified Eagle’s
medium with 10 % fetal bovine serum, which was pre-
supplemented with penicillin (100 units/ml) and streptomycin
(100 mg/ml). At 80–90 % confluence, the cells were seeded in
a ninety-six-well microtitre plate with 4000 cells/well. After
incubation for 48 h, the culture medium was changed to
phenol red-free Dulbecco’s modified Eagle’s medium with
1 % charcoal-stripped fetal bovine serum for 24 h. The cells
were then treated with SWH fractions at 0·1, 1·0, 10 or
100 μg/ml for 24 or 48 h. The cells were cultured at 37°C in
a humidified atmosphere of 95 % air and 5 % CO2.

Cell proliferation assay

The proliferation effects of the fractions of SWH on the UMR
106 cell line were determined by the MTS colorimetric assay.
After treating with SWH fractions (0·1 to 100 μg/ml) or vehicle
for 24 h or 48 h, the medium was removed, followed by
the addition of 0·2 mg/ml 3-(4,5-dimethylthiazol-2-yl)-5-(3-car-
boxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS;
Promega Corp., Madison, WI, USA) and 1 mg/ml phenazene
methosulfate (PMS; Sigma-Aldrich, St Louis, MO, USA) reagent.
The absorbance was detected on a microplate spectrophotometer (model 680; Bio-Rad, Hercules, CA, USA) at a wave-
length of 405 nm after 2 h of incubation at 37°C.

Alkaline phosphatase activity assay

Alkaline phosphatase activity of the treated cells was deter-
mined by the hydrolysis of p-nitrophenylphosphate (Promega
Corp.) to p-nitrophenol to assess the differentiation activity
of UMR 106 cells. The treated cells were rinsed with PBS
twice before 100 μl p-nitrophenylphosphate were added to
them. Absorbance of the tested samples was measured
at 405 nm. All results are expressed as a ratio normalised
to the corresponding total protein content (optical density at
405 nm/μg BSA).

Statistical analysis

The data from these experiments are reported as mean values
with their standard errors for each group. All statistical
analyses were performed using PRISM version 5.0 (GraphPad,
San Diego, CA, USA). Inter-group differences were analysed
by one-way ANOVA, and followed by Tukey’s multiple-
comparison test as a post hoc test to compare the group
mean if overall P < 0·05. Differences of P < 0·05 were
considered statistically significant.

Results

Body weight, uterine weight and serum biochemical
makers

The effects of SWH fractions on body weight, uterus index and
serum biochemical makers are summarised in Supplementary
Appendix 1 (available online at http://www.journals.
cambridge.org/bjn). As expected, body weight increased
significantly (P < 0·05) and uterus index decreased significantly
(P < 0·05) in OVX mice as a result of oestrogen deficiency.
Treatment of OVX mice with SWH or SWC extracts significantly
suppressed oestrogen deficiency-induced body-weight gain (\(v.\) O VX group, both \(P<0.001\)). Uterus indexes were significantly increased in O VX mice in response to treatment with E2 or SWH. However, uterus indexes of the mice treated with the fractions of SWH did not alter as compared with those treated with vehicle. Serum Ca level and P levels were not altered by O VX or fractions of SWH.

Micro-computed tomography analysis of left femur and tibia

As shown in Table 3, ovariectomy significantly reduced BMD at the distal metaphysis of femur (\(P<0.05\) \(v.\) sham) and the proximal metaphysis of tibia (\(P<0.05\) \(v.\) sham) in mice. Treatment of O VX mice with E2 prevented the decrease in BMD at both sites (\(P<0.001\) \(v.\) O VX). Similarly, treatment of O VX mice with SWH or SWC significantly restored BMD at the femur and tibia. Trabecular bone microstructure, as indicated by parameters such as BV/TV, Tb.N and Tb.Sp at the distal metaphysis of femur and at the proximal metaphysis of tibia deteriorated significantly in mice upon ovariectomy (Table 3; \(P<0.05\) in all parameters at both sites). The damage of bone microstructure induced by ovariectomy in mice at the femur were reversed significantly by treatment with SWH (\(P<0.01\) in BV/TV), SWA (\(P<0.05\) in BV/TV) or SWC (\(P<0.01\) in Tb.N and \(P<0.05\) in Tb.Sp) and at the tibia by treatment with SWH (\(P<0.001\) in BV/TV, \(P<0.05\) in Tb.N and \(P<0.01\) in Tb.Sp) or SWC (\(P<0.001\) in BV/TV, \(P<0.05\) in Tb.N and \(P<0.01\) in Tb.Sp). Among all the fractions of SWH, the bone-protective effect of SWC was the same as or even higher than that of the total ethanol extract SWH. There were no differences of the trabecular bone parameters between Sham and the fraction-treated groups. The administration of SWH fractions restored the bone loss induced by ovariectomy to the normal level and maintained the microarchitecture of trabecular bone.

Biomechanical measurement of left tibia

Table 4 shows the effects of SWH fractions on the biomechanical properties of the left tibia in O VX mice. Three-point bending experiments indicated that treatment of O VX mice with E2 significantly increased the ultimate load (\(P<0.001\)), breaking force (\(P<0.05\)) as well as stiffness (\(P<0.05\)) of the tibia mid-shaft. Ultimate load of the left tibia mid-shaft were significantly increased in O VX mice in response to treatment with different SWH fractions, namely SWA (\(P<0.05\)), SWB (\(P<0.05\)) and SWC (\(P<0.01\)). Breaking forces of the left tibia mid-shaft were increased significantly in O VX mice in response to treatment with SWC (\(P<0.01\)) while treatment with SWH did not show such effects on the breaking force. Stiffness of the tibia was only increased in O VX mice in response to treatment with SWA (\(P<0.05\)). Treatment of O VX mice with SWH fractions suppressed the OVX-induced reduction of ultimate load and stiffness in the tibia, resulting in an increase back to the Sham level. However, E2 and all fractions of SWH had no significant effects on changes of energy of breaking.

Table 3. Effects of Sambucus williamsii HANCE fractions on bone parameters as measured by micro-computed tomography at the distal metaphysis of femur and proximal metaphysis of tibia in ovariectomised mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sham</th>
<th>OVX</th>
<th>E2</th>
<th>SWH</th>
<th>SWA</th>
<th>SWB</th>
<th>SWC</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD (mg HA/cm²)</td>
<td>Mean</td>
<td>±SEM</td>
<td>Mean</td>
<td>±SEM</td>
<td>Mean</td>
<td>±SEM</td>
<td>Mean</td>
</tr>
<tr>
<td>Femur BV/TV (%)</td>
<td>0.043</td>
<td>0.003</td>
<td>0.06</td>
<td>0.004</td>
<td>0.072</td>
<td>0.006</td>
<td>0.05</td>
</tr>
<tr>
<td>Tibia BV/TV (%)</td>
<td>0.039</td>
<td>0.001</td>
<td>0.06</td>
<td>0.004</td>
<td>0.068</td>
<td>0.006</td>
<td>0.06</td>
</tr>
<tr>
<td>Femur Tb.N (1/mm)</td>
<td>0.04</td>
<td>0.003</td>
<td>0.06</td>
<td>0.004</td>
<td>0.072</td>
<td>0.006</td>
<td>0.05</td>
</tr>
<tr>
<td>Tibia Tb.N (1/mm)</td>
<td>0.039</td>
<td>0.001</td>
<td>0.06</td>
<td>0.004</td>
<td>0.068</td>
<td>0.006</td>
<td>0.06</td>
</tr>
<tr>
<td>Femur Tb.Sp (1/mm)</td>
<td>0.04</td>
<td>0.003</td>
<td>0.06</td>
<td>0.004</td>
<td>0.072</td>
<td>0.006</td>
<td>0.05</td>
</tr>
<tr>
<td>Tibia Tb.Sp (1/mm)</td>
<td>0.039</td>
<td>0.001</td>
<td>0.06</td>
<td>0.004</td>
<td>0.068</td>
<td>0.006</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Mean value was significantly different from that of the OVX group: *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\).

Mean value was significantly different from that of the Sham group: †\(P<0.05\), ††\(P<0.01\).

‡ Mice were subjected to the treatments for 12 weeks.
E2, SWH (Fig. 2(a)) and SWB (Fig. 2(c)) significantly promoted SWH, SWA, SWB and SWC were measured. As observed with in UMR 106 cells in response to different concentrations of on osteoblastic cell differentiation, alkaline phosphatase activi-
liferation. To determine the effects of different SWH extracts (Fig. 2(d)) significantly increased cell alkaline phosphatase SWA (Fig. 2(b)) had no effects on cell differentiation. SWC 1
100
of SWA (Fig. 1(b)) or SWC (Fig. 1(d)) for 24 or 48 h significantly (Fig. 1(a)). Treatment of UMR 106 cells with 0·1 to 100 proliferation were more potent than the effects of SWH differentia-

ties in UMR 106 cells in response to different concentrations of of SWA, SWB and SWC (Fig. 1(b), (c), (d)) on UMR 106 cell increased cell proliferation (\( \mu \text{g/mL} \) for 24 and 48 h significantly of SWH at 1

| Table 4. Effects of Sambucus williamsii HANCE fractions on biomechanical properties of the left tibia in ovariectomised mice (Mean values with their standard errors) |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Treatment†      | Ultimate load (N) Mean | SEM | Breaking force (N) Mean | SEM | Energy for breaking (\( \times 10^{-3} \) J) Mean | SEM | Stiffness (N/mm) Mean | SEM |
| Sham            | 20·83           | 0·87 | 18·57           | 0·59 | 9·82              | 0·67 | 54·31              | 3·14 |
| OVX             | 17·91†          | 0·48 | 15·60†          | 0·56 | 9·06              | 0·78 | 36·10†             | 2·33 |
| E\(_{2}\)        | 23·37***        | 0·47 | 18·34*          | 0·46 | 10·20             | 1·22 | 69·00*             | 9·43 |
| SWH             | 19·95           | 0·76 | 17·60           | 0·56 | 9·89              | 0·60 | 61·48              | 7·94 |
| SWA             | 20·90*          | 0·53 | 17·48           | 1·15 | 9·57              | 1·31 | 59·40              | 5·69 |
| SWB             | 20·97*          | 0·82 | 19·72**         | 1·16 | 9·22              | 0·88 | 61·63              | 10·15|
| SWC             | 21·81**         | 0·76 | 18·57           | 0·59 | 9·82              | 0·67 | 61·48              | 7·94 |

Sham, sham-operated, vehicle-treated; OVX, ovariectomised, vehicle-treated; E\(_{2}\), 17\-oestradiol (3·2 \mu g/g body weight per d); SWH, Sambucus williamsii HANCE ethanol extract (1·0 mg/g body weight per d); SWA, SWH water eluate (0·570 mg/g body weight per d); SWB, SWH 30 % ethanol eluate (0·128 mg/g body weight per d); SWC, SWH 50 and 95 % ethanol eluates (0·189 mg/g body weight per d). Mean value was significantly different from that of the OVX group: *\( P<0·05 \), **\( P<0·01 \), ***\( P<0·001 \). Mean value was significantly different from that of the Sham group: †\( P<0·05 \). ‡ Mice were subjected to the treatment for 12 weeks.

**Rat osteoblast-like UMR 106 cell proliferation and differentiation assay**

Fig. 1(a) shows that treatment of rat osteoblast-like UMR 106 cells with SWH at 1 \mu g/mL for 24 and 48 h significantly increased cell proliferation (\( P<0·001 \)). The stimulatory effects of SWA, SWB and SWC (Fig. 1(b), (c), (d)) on UMR 106 cell proliferation were more potent than the effects of SWH (Fig. 1(a)). Treatment of UMR 106 cells with 0·1 to 100 mg/mL of SWA (Fig. 1(b)) or SWC (Fig. 1(d)) for 24 or 48 h significantly increased cell proliferation at all concentrations. SWH eluate at 10 or 100 mg/mL (Fig. 1(c)) significantly increased UMR 106 cell proliferation. To determine the effects of different SWH extracts on osteoblastic cell differentiation, alkaline phosphate activities in UMR 106 cells in response to different concentrations of SWH, SWA, SWB and SWC were measured. As observed with E\(_{2}\), SWH (Fig. 2(a)) and SWB (Fig. 2(c)) significantly promoted cell differentiation activity at 0·1 mg/mL (both \( P<0·05 \)), while SWA (Fig. 2(b)) had no effects on cell differentiation. SWC (Fig. 2(d)) significantly increased cell alkaline phosphatase activity at all concentrations (0·1 to 100 mg/mL), especially at 1 mg/mL (122-8 \%), which was higher than the increased level upon treatment with E\(_{2}\) (112-4 \%).

**Identification of components in Sambucus williamsii HANCE 50 and 95 % ethanol eluate**

The peaks of the HPLC fingerprint of SWC (Supplementary Appendix 2; available online at http://www.journals.c
duction**

The present study systematically evaluated the osteoprotective effects of different fractions of SWH in OVX mice and in rat osteoblast-like UMR 106 cells. The present results clearly demonstrated that the 50 and 95 % ethanol eluates (SWC) of the ethanol extract of SWH suppressed OVX-induced loss in bone mass and bone strength and increase in body weight. Besides, SWC did not alter the uterus weight in OVX mice. Moreover, the present study showed that SWC effectively promoted proliferation and differentiation in UMR 106 cells. In addition, the present study identified that the major components isolated from SWC were lignans, which may account for the osteoprotective effects of this fraction or even this herb.

Our recent study\( ^{13} \) reported that a 60 % ethanol extract of SWH dose-dependently increased BMD but only a high-dose treatment group showed statistically significant differences compared with OVX mice. To investigate if further fractions of SWH may lead to more potent protective effects in bone, SWH was separated with a macroporous adsorptive resin column and three fractions were obtained. The bone-protective effects of the fractions and SWH were evaluated and were compared using in vivo and in vitro results.

Trabecular bone mass, trabecular bone microstructure and cortical bone biomechanical strength are three key aspects associated with bone properties and turnover\( ^{19} \). Micro-computed tomography measurement showed that SWH improved trabecular BMD and suppressed disruption of trabecular bone microstructure of the femur and tibia in OVX mice,
Bone-protective effects of Sambucus williamsii

Fig. 1. Effects of (a) Sambucus williamsii HANCE (SWH), (b) Sambucus williamsii HANCE water eluate (SWA), (c) Sambucus williamsii HANCE 30 % ethanol eluate (SWB) and (d) Sambucus williamsii HANCE 50 and 95 % ethanol eluates (SWC) on cell proliferation in rat osteoblast-like UMR 106 cells. (a), 24 h; (b), 48 h; OD, optical density. Results were obtained from three independent experiments. Values are means, with standard errors represented by vertical bars. Mean value was significantly different from that of the control (24 h): **P<0.01, ***P<0.001. Mean value was significantly different from that of the control (48 h): †††P<0.001.

a result that is in accordance with our previous study(12,13). The fractions obtained from further fractionations of SWH (i.e. SWA and SWC) did inhibit bone mineral loss induced by ovariectomy. In particular, the protective effects of SWC on trabecular bone in OVX mice were comparable with the effects of SWH. Moreover, micro-architectural parameters such as BV/TV, Tb.N and Tb.Sp of trabecular bone at the distal metaphysis of femur and the proximal metaphysis of tibia in OVX mice were significantly improved by treatment with SWC and the improvement was even higher than brought about by SWH. The results of three-point bending experiments on tibial cortical bone demonstrated that all fractions of SWH could significantly increase the ultimate load of the tibia diaphysis. Furthermore, the SWC treatment significantly increased breaking force and SWA treatment significantly increased stiffness in OVX mice. These results indicated that all fractions of SWH significantly improved the biomechanical strength of the long bone in OVX mice. The analysis of micro-computed tomography and three-point bending tests suggested that SWC may contain nearly all (87 %) of the active ingredients of SWH that account for its protective effects on bone mass and bone quality.

SWH extract inhibited bone resorption by suppressing osteoclastogenesis via modulation of the osteoprotegerin: receptor activator of NF-κB ligand (OPG:RANKL) ratio in UMR 106 cells(12). To investigate the effects of different SWH fractions on osteoblastic cell function, the proliferation and differentiation of osteoblast-like UMR 106 cells were determined in the present study. The studies showed that SWH promoted cell proliferation and differentiation at 1 and 0.1 µg/ml, respectively. Fractions obtained from further fractionation of SWH stimulated osteoblastic cell proliferation and differentiation. Among the fractions, SWC showed very high potency for increasing cell proliferation and differentiation at doses from 0.1 to 100 µg/ml. Our in vitro results confirmed the conclusion of the in vivo experiment that SWC was the fraction with the highest potency on bone protection and was enriched with most of the active ingredients in SWH that account for its ability to improve bone properties.

Our previous study found that the most effective dose (1000 mg/kg) of ethanol extract of SWH after conversion from animal to human dosage was 5.5 g/d in women with a body weight of 50 kg(13). Similarly, the treatment dose of SWC (0.189 g/kg) applied in the present study for the improvement of bone properties will be 1.0 g/d for postmenopausal women administered orally. The present finding revealed that a lower dose of SWC will be needed to obtain effects that are similar to or even better than SWH.

Oestrogen or substances with oestrogenic potential were previously shown to be able to attenuate ovariectomy-induced weight gain(19–21). The present results showed that SWH and SWC significantly suppressed the body-weight gain induced by OVX, suggesting that SWH and SWC might also contain phyto-oestrogens. Similar to our previous study(13), the present study confirmed that SWH stimulated the increase of uterus weight in OVX animals. In contrast to SWH, SWC did not alter uterus weight in OVX mice, suggesting that SWC did not exert undesirable oestrogen-like effects on the
Fig. 2. Effects of (a) *Sambucus williamsii* HANCE (SWH), (b) *Sambucus williamsii* HANCE water eluate (SWA), (c) *Sambucus williamsii* HANCE 30% ethanol eluate (SWB) and (d) *Sambucus williamsii* HANCE 50 and 95% ethanol eluates (SWC) on cell differentiation activity in rat osteoblast-like UMR 106 cells. OD, optical density; BSA, bovine serum albumin. Results were obtained from three independent experiments. Values are means, with standard errors represented by vertical bars. Mean value was significantly different from that of the control: *P<0.05, **P<0.01, ***P<0.001.

In conclusion, the present study revealed that SWC was the fraction of highest potency in SWH and that it could prevent bone loss, improve trabecular bone microstructure and increase cortical bone strength in OVX mice. Most importantly, the doses of SWC used in the present study can fully restore bone loss to the pre-OVX level. The *in vitro* experiments suggested that SWC might increase bone formation by promoting proliferation and differentiation of osteoblast-like cells. Most importantly, the effective dose of SWC to achieve similar bone-protective effects as SWH is found to be only one fifth of that of SWH. In addition, seven lignans and two phenolic acids, which are the major peaks of the HPLC fingerprint of SWC, were identified. Further studies are needed to characterise the mechanism by which the isolated lignans and phenolic acids exert anabolic effects on bone cells, presumably osteoblasts. The present study suggests that SWC needs to be investigated as a natural alternative for the management of postmenopausal osteoporosis.

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References

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