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Osteoblastic activity of ethanolic extract and volatile compounds from Ee-Zhi-Wan, a famous traditional Chinese herbal formula

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Er-Zhi-Wan (EZW), a famous traditional Chinese formula, which is comprised of the aerial parts of Eclipta prostrata L. (EP) (Astraceae) and the fruits of Ligustrum lucidum Ait. (FLL)(Oleaceae) has been developed as a restorative formula for hundreds of years. It is widely used to prevent and treat various kidney diseases. This study aimed to investigate the effects of volatile components and ethanolic extract from EZW on the proliferation and differentiation of primary osteoblasts by the MTT method and measuring the activity of alkaline phosphatase (ALP). Both ethanolic extract and volatile components from EZW could significantly (p<0.01) stimulate the proliferation and increase the ALP activity of primary osteoblasts. The volatile components of EZW were analyzed by GC-MS. A total of 61 compounds, which were the major part (about 86.34%) of the volatiles were identified by matching mass spectra with a mass spectrum library (NIST 05.L) and retention indexes (RI) of the compounds reported on equivalent column.

Key words: Er-Zhi-Wan (EZW), volatile compounds, osteoblasts, GC-MS.

INTRODUCTION

Osteoporosis is a metabolic bone disease characterized by low bone mass and micro-architectural deterioration of bone tissues, resulting in increased bone fragility and susceptibility to fracture (Rodan and Martin, 2000). Post-menopausal osteoporosis is a major health problem with significant morbidity and mortality (Cummings et al., 1990). Classical hormone replacement therapy (HRT) has been recommended to prevent and treat post-menopausal osteoporosis for many years. However, in 2002, the American National Institute of Health stopped a clinical trial with HRT in healthy post-menopausal women due to the higher incidence of breast cancer, heart attack, and stroke and blood clots (Rossouw et al., 2002). Traditional Chinese medicines have been applied to prevent and treat post-menopausal osteoporosis in clinical practice for thousands of years, and these medicines with fewer side effects are more suitable for long-term use compared with chemically synthesized medicines. In addition, according to the law of compatibility of traditional Chinese medicines, a single herbal medicine usually exerts a limited therapeutic action. When several herbal medicines are mixed in a certain proportion, they...
will display their superiority over a single herb in the treat-
ment of a disease (Qin et al., 2008). As such, traditional
Chinese formulas will undoubtedly be a cost-effective
alternative to commercial pharmaceutical products. Er-
Zhi-Wan (EZW), a famous traditional Chinese formula
firstly recorded in “Yi Bian” written in Ming Dynasty, is
comprised of the aerial parts of Eclipta prostrata L. (EP)
(Astraceae) and the fruits of Ligustrum lucidum Ait. (FLL)
(Oleaceae). It possesses the actions of tonifying the liver
and kidney, nourishing the body’s essential fluid, and
arresting hemorrhages (Chinese Pharmacopoeia
Committee, 2005). According to the traditional Chinese
medicine theory, “kidney” controls bone. The “kidney-
tonifying” action of traditional Chinese medicine might
have relationship with bone formation (Wang et al., 2009;
Zhang et al., 2008). Moreover, our previous studies have
shown that both ethanolic extract and volatile compounds
from EP and FLL could significantly stimulate the
proliferation and increase the ALP activity of rat calvarial
osteoblasts (Lin et al., 2010; Wu et al., 2011). We also
found that the serum from rats treated with aqueous
extract of EZW did not facilitate proliferation of rat
calvarial osteoblasts and UMR106 cells, but evidently
inhibited both proliferation of RAW264.7 cells and
differentiation of osteoclasts from RAW264.7 cells
induced by receptor activator of nuclear factor κB ligand
(RANK-L) and macrophage-colony stimulating factor (M-
CSF) (Zhang et al., 2008). However, the effects of
ethanolic extract and volatile compounds from EZW on
the proliferation and differentiation of rat calvarial osteo-
blasts, and whether it has better effects than EP or FLL,
still have not been investigated.

Many researches showed that volatile compounds
obtained from many plants are responsible for their
pharmacological activities just as non-volatile compounds
in herbs (Lograda et al., 2010; Ho et al., 2010; Wei et al.,
2012; Sharma et al., 2012; Rahimi et al., 2011). Both EP
and FLL contain rich volatile compounds, from which we
could always smell the strong fragrance. However, as far
as our literature survey could ascertain, there is no report
on any investigation on the volatile compounds from
EZW.

According to the aforementioned knowledge and re-
searches in the present study, we investigated the effects
of ethanolic extract and volatile compounds from EZW on
the proliferation and differentiation of rat calvarial osteo-
blasts.

MATERIALS AND METHODS

Plant material

The aerial parts of Eclipta prostrata L. (20080710) and the fruits of
L. lucidum Ait. (Oleaceae) (20080601) were purchased from Fujian
Tianren Pharmaceutical Company and identified by Professor
Cheng-zi Yang of the Department of Pharmacy, Fujian University of
Traditional Chinese Medicine. The voucher specimens of these
fruits were deposited at the Herbarium of Department of
Pharmacognosy, Fujian University of Traditional Chinese Medicine,
Fuzhou, P. R. China.

Chemicals and reagents

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
(MTT) and dimethyl sulfoxide (DMSO) were purchased from
Sigma (U.S.A.), and n-Alkanes C10 to C40 were purchased from
Accustandard (U.S.A.). Phenol red-free Dulbecco’s modified
Eagle medium (phenol red-free DMEM) and fetal bovine serum
(FBS) was purchased from Hyclone (U.S.A.). Ethanol, diethyl ether,
anhydrous sodium sulphate, diethanolamine, disodium-4-
thiophenyl phosphate, and 4-nitrophenol were of domestic AR
grade. Luteolin and ursolic acid standards were purchased from the
National Institute for the Control of Pharmaceutical and Biological
Products with a purity of >98%, and n-Alkanes C10-C40
were purchased from Accustandard (U.S.A.). Deionized water was
purified by Milli-Q system (Millipore, Bedford, MA, USA).

Ethanolic extracts and volatile compounds preparation

EZW was extracted by the method described previously by Lin et al.
(2010), the same prepared procedures with EP and FLL. Briefly, the
powder of EZW 10g (EP: FLL = 1: 1, w/w) mixed with 100 ml of
75% (v/v) aqueous ethanol was loaded into a flask equipped with a
water condenser tube. The extraction solvent was boiled (80 ± 2°C)
and refluxed for a period of 120 min. Extraction was repeated twice.
The combined extracts were filtered through filter paper and
evaporated to dryness in a rotary evaporator (RE-52, Shanghai
splendor and biochemical instrument Co., China) at 45°C under
reduced pressure to yield the crude ethanolic extract. The dry ma-
terial of EZW 300 g (EP: FLL= 1: 1, w/w) was crushed (40 mesh),
then soaked in 3000 ml water for about 12 h before they were
subjected to hydro-distillation in a Clevenger type apparatus.
The contents were distilled for 3 h to obtain the volatile oils with yel-
lowish colour and a pleasant smell and the oils were then dried over
anhydrous sodium sulphate. The experiment was repeated thrice.
The mean recovery of volatile oils was 0.37 ± 0.032% (w/w). The
oils were stored at 4°C in the dark until tested and analyzed.

Preparation of test samples

Both ethanolic extract and volatile compounds from EZW were
dissolved in dimethylsulfoxide (DMSO) at concentration of 10
mg/ml, and diluted in culture medium to the working solution before
use. To avoid DMSO toxicity, the concentration of the solvent was
less than 1% (v/v). For effects of steroids on growth or
differentiation, culture media was charcoal stripped and without
phenol red.

Cell cultures

Sprague-Dawley rats, which were 2 to 3 days old, were purchased
from the Animal Center of the Fujian Medical
University, Fuzhou, P.R. China. Primary osteoblastic cells were
prepared from the calvarias of newborn rats following the sequential
enzymatic digestion method (Idris et al., 2008). Briefly, skull (frontal
and parietal bones) were dissected; then the endosteme and periosteum were stripped off, and the bone was cut into
approximately 1 to 2 mm² pieces and digested sequentially using
trypsin (0.25%, w/v) for 30 min and collagenase II (1.0 mg/ml)
containing 0.05% trypsin (w/v) for 2 h. The cells were collected and
cultured in phenol red free DMEM supplemented with 10% FBS and
1% penicillin/streptomycin for 24 h in a humified atmosphere of
5% CO₂ in air at 37°C and then, the media was changed.
Assay for osteoblast proliferation and ALP activity

Primary osteoblasts (2 × 10^4 cells/well) were subcultured into 96-well culture plates, and incubated 24 h before the addition of test samples or control (DMSO, final concentration was 1% v/v), and cultured again for another 48 h. Prior to the end of culture, MTT (20 μL and 5 mg/ml) was added to each well and incubated for 4 h, after which the medium was discarded, and 150 μL of DMSO was added to each well. The cells were incubated for 20 min. The UV absorbance was measured at 490 nm at a microplate spectrophotometer (Bio-rad Model 680, USA) with a reference at 630 nm and used as an indicator of osteoblast proliferation. Proliferation (%) was calculated as 100 × (OD of volatile compounds - treated / OD of control), where OD is the average absorbance of six experiments with 8 replicates. Primary osteoblasts were seeded at 2 × 10^4 cells/well in 96-well culture plates, and treated with test samples or control for 9 days (Media was changed per three days). The ALP activity was measured according to the literature (Owen, 1990). Total protein was assayed by the method of Bradford (Bradford, 1976). The ALP activity was expressed as micromoles of 4-nitrophenol liberated per milligram protein.

Quantification of the total triterpene acids (TTA) by the colorimetric method

After optimizing all experiment parameters, the content of the TTA was determined by the colorimetric method (Fan et al., 2006) with the following procedure. The suitable amount of each ethanolic extract was obtained as described in ethanolic extracts and volatile compounds preparation was dissolved in 50 ml of ethanol, respectively. Ethanol solution (0.2 ml) was added to the graduated test tube and evaporated to dry in a boiled water bath, and then 0.3 ml of 5% vanillin/glacial acetic acid (w/v) and 1 ml of perchloric acid solution were added to the tube successively. The sample solution was heated for 20 min at 70°C and then cooled in an ice-water bath to the ambient temperature. The absorbance of the sample was measured at 550 nm using an ultraviolet–visible spectrophotometer (Shanghai Laiptide Science Instruments Co., Ltd.) after 5 ml of glacial acetic acid was added. Ursolic acid was then used as the standard.

GC–MS analysis

GC–MS analysis was performed on an Agilent 6890N Network GC System, fitted with a HP-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; maximum temperature, 350°C), coupled to an Agilent 5975 inert XL Mass Selective Detector. Ultrahigh purity helium (99.999%) was used as carrier gas at a constant flow of 1.0 ml/min. The injection, transfer line and ion source temperatures were 250, 250 and 200°C, respectively. The ionizing energy was 70 eV. Electron multiplier (EM) voltage was obtained from autotune. All data were obtained by collecting the full-scan mass spectra within the scan range 35 to 500 amu. The splitless injection was employed for the analysis. The diluted sample (10 mg/mL, in redistilled diethyl ether) volume injected with an Agilent 7683B series injector was 1 μL. The oven temperature program was 90 to 2.5°C/min to 130 to 1.2°C/min to 170 to 2°C/min to 230 to 2°C/min to 250°C (5 min).

Identification and quantification of volatile compounds

Volatile compounds were first identified by comparing the spectra obtained with a mass spectrum library (NIST 05.L). Corroboration of the identification was then sought by matching the mass spectra of compounds with those present in the literatures and the retention indexes of the compounds reported on equivalent column (Cardile et al., 2010; Ogunbinu et al., 2009). Compounds relative percentages were calculated from the TIC from the automated integrator.

Statistical analysis

All data were presented as mean values of three determinations ± S.D. The results of all mean values were analyzed by one-way ANOVA and Turkey-HSD at p < 0.01 to detect significant differences among groups.

RESULTS

Proliferation and alkaline phosphatase activity assays

As the tested samples with different concentrations were added to wells for 48 h, both of which (1 μg/ml to 100 μg/ml) dose-dependently (p<0.01) stimulated the proliferation of rat calvarial osteoblasts, except ethanolic extract 1 μg/mL (Figure 1). The maximal effect was observed when cells were incubated with ethanolic extract 100 μg/ml. To ascertain whether EZW is capable of affecting osteoblastic cell differentiation, we examined the changes in ALP activity. As shown in Figure 2, both ethanolic extract and volatile compounds significantly (p<0.01) increased ALP activity in osteoblasts over the 9 days, and the maximal effects of them were observed when cells were incubated with 10 μg/ml and 1 μg/ml, respectively. Therefore, EZW could stimulate osteoblastic activity at least in part by enhancing synthesis of ALP.

Validation of the colorimetric method and the content of total triterpene acids (TTA) in EZW

Under the optimal colorimetric method condition used in this study, calibration curve was constructed in the range 0.02 to 0.08 mg. The regression equation of the curve was calculated as follows: y = 7.3786x−0.0091 (y is the absorbance of the sample solution after chromogenic reaction and x is the weight of ursolic acid or TTA (mg), the correlation coefficient of the regression equation (r²) was 0.9992. The precision and repeatability of this method were evaluated by replicate (n = 5) analysis of the same sample and by analysis of five independently prepared samples. Analysis of a sample after standing at room temperature for 0, 30, 60, 90 and 120 min was also conducted to assess sample stability. Both the RSD (%) of precision and repeatability were less than 1.0%. The sample was stable during 120 min (RSD% = 0.72%). The recovery assay of the TTA was carried out by adding the standard to the treated materials, and the recovery was in the range of 95.79 to 99.61%. The amount of TTA in raw materials of EZW, FLL and EP were 2.00 ± 0.011%, 3.98 ± 0.037%, 0.51 ± 0.006%, respectively.
Figure 1. Effect of ethanolic extract and volatile components from EZW on the proliferation of rat calvarial osteoblasts (n=8, X ± SD; * p<0.01, compared with control).

Figure 2. Effect of ethanolic extract and volatile components from EZW on the ALP activity of rat calvarial osteoblasts (n = 8 and X ± SD; * p<0.01, compared with control).
<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Component</th>
<th>RT (min)</th>
<th>RI*</th>
<th>Peak area (%)</th>
</tr>
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<tr>
<td>1</td>
<td>1-Methyl-4-(1-methylethyl)-benzene</td>
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<td>1027</td>
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<td>2</td>
<td>D-Limonene</td>
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<td>1032</td>
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<td>4-Methyl-1-(1-methylethyl)-3-cyclohexen-1-ol</td>
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<td>1181</td>
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<td>4</td>
<td>(S)-α, α, 4-Trimethyl-3-cyclohexene-1-methanol</td>
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<td>1195</td>
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<td>5</td>
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<tr>
<td>6</td>
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<tr>
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<td>n-Decanoic acid</td>
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<td>10</td>
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</tr>
<tr>
<td>11</td>
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<td>1419</td>
<td>0.07</td>
</tr>
<tr>
<td>12</td>
<td>Z,Z,Z-1,5,9,9-Tetramethyl-1,4,7,-cycloundecatriene</td>
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<td>1451</td>
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<td>14</td>
<td>(4aR-trans)- Decahydro-4a-methyl-1-methylene-7-(1-methylethylidene)-naphthalene</td>
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<td>1473</td>
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<td>15</td>
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<td>1518</td>
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<tr>
<td>21</td>
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<td>1528</td>
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<td>1561</td>
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<td>Cedrol</td>
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<td>31</td>
<td>[1S-(1α,4α,4β,8αβ)]-1,2,3,4,4a,7,8,8a-Octahydro-1,6-dimethyl-4-(1-methylethyl)-1-naphthalenol</td>
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<td>32</td>
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<td>1632</td>
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<tr>
<td>33</td>
<td>[1αR-(1α,4α,7α,7αβ,7βα)]-Decahydro-1,1,7-trimethyl-4-methylene-1H-cycloprop[e]azulene</td>
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</table>
Volatile compounds analysis

All of 61 compounds, which were the major part (86.34%) of the volatiles, were identified. GC–MS profile of the volatile compounds showed the presence of a wide range of compounds, including terpenoids, aromatics, long-chain hydrocarbons, alcohols, aldehydes, ketones, acids and esters. The retention indexes and percentage composition are given in Table 1, where the compounds were listed in order of elution from a HP-5MS column. The main compounds were as follows: (Z,Z)-9,12-octadecadienoic acid (23.11 ± 1.099%), n-hexadecanoic acid (20.58 ± 1.156%), (E)-9-octadecenoic acid methyl ester (1.82 ± 0.136%), 1,2-benzenedicarboxylic acid diisooctyl ester (1.44 ± 0.145%), 9,12-octadecadienoic acid methyl ester (1.38 ± 0.145%), (1S-cis)-1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1 methyllethyl)-naphthalene (1.29 ± 0.146%), tetradecanoic acid (1.12 ± 0.032%), and [1S-(1α,4α,4aβ,8aβ)-1,2,3,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylethyl)-1'-naphthalene (1.00 ±
DISCUSSION

The theory of traditional Chinese medicine believes that bones are governed and dominated by the “kidney”, which means that the “kidney” plays an important role in growth and formation of bones. Strong “kidney” can nourish bone and makes it flourish, but the weak “kidney” makes bone perish (Luo et al., 2006). In addition, according to the law of compatibility of traditional Chinese medicines, compound recipe often displays its superiority over a single drug in the treatment of a disease (Qin et al., 2008). EZW, a famous “kidney-tonifying” traditional Chinese medicine formula, which is widely used to prevent and treat various kidney diseases for its actions of nourishing the kidney, might be beneficial to bone formation. In EZW formula, there are various kinds of chemical constituents. The flavonoids from FLL and EP possess the estrogen-like activity (Lin et al., 2009). These kinds of compounds, which have aroused general concern, have the capacity to bind to the estrogen receptors and maybe decrease the bone loss like estrogen (Messina et al., 2000). This further verified the thoughts of traditional Chinese medicine that the bone could be strengthened by nourishing kidney.

Many studies have demonstrated that FLL could significantly improve the total, cortical and trabecular bone mineral density in lumbar spine and promote osteogenesis and suppress adipogenesis in MSCs as indicated by the elevated alkaline phosphatase activity, calcium deposition levels and decrease adipocyte number without cytotoxic effects (Ko et al., 2010). Moreover, FLL extract could inhibit high bone turnover, elevate intestinal calcium absorption and prevent calcium loss in young ovariectomized rats (Zhang et al., 2006). In addition, the methanol extract of aerial parts of EP was found to increase the ALP activity significantly in primary osteoblasts in vitro and stimulate bone mineralization of osteoblasts in vitro and stimulate bone-formation in vivo in a mouse calvarial bone formation model (Lee et al., 2008). In addition, some other triterpenoids were also shown to have anti-osteoporotic activity (Li et al., 2007). Therefore, quantification of the TTA is necessary for the quality control of EZW. Moreover, both EP and FLL contain rich volatile compounds, the volatile compounds analysis can be considered as a complementary measure of quality control of EZW.

Conclusion

EZW with the potential to stimulate osteoblast proliferation and differentiation might be used as an alternative therapeutic agent for prevention and treatment of osteoporosis and display its superiority over a single herb (EP or FLL). Further studies on the isolation of anti-osteoporotic fractions and constituents in EZW are in progress.

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