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Full Length Research Paper

Osteoblastic activity of ethanolic extract and volatile compounds from Er-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 prostrate* 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 treatment 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

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essential fluid, and arresting hemorrhages (Chinese Pharmacopoeia Committee, 2010). 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 $_K$B 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 osteoblasts, 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 researches in the present study, we investigated the effects of ethanolic extract and volatile compounds from EZW on the proliferation and differentiation of rat calvarial osteoblasts.

MATERIALS AND METHODS

Plant material

The aerial parts of *Eclipta prostrasta* 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 suphoxide (DMSO) were purchased from Sigma (U.S.A.), and n-Alkanes C_8 to C_{40} 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-nitrophenyl 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 C_8-C_{40} 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 material 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 yellowish 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 Experimental 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 endosteum and periosteum were stripped off, and the bone was cut into approximately 1 to 2 mm^2 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 humidified atmosphere of 5% CO_2 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 (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 and He, 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 Laipade 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 statistical analysis between groups was carried out using the Student's *t* test and $p < 0.01$ was considered to be statistically significant. All statistical analyses were performed using SPSS version 16.0 for Windows.

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 (r^2) 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.

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

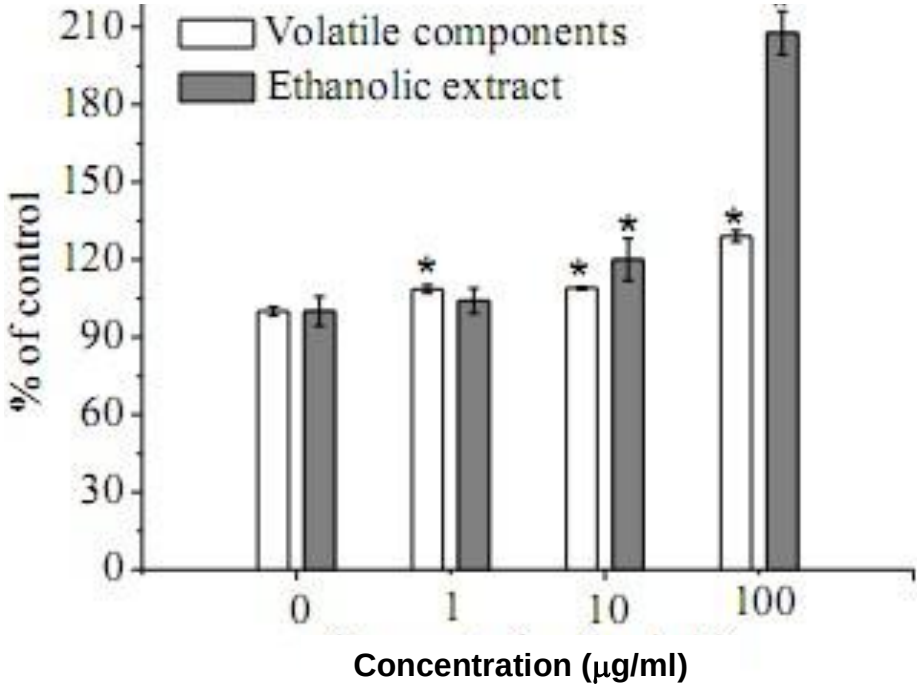

Figure 1. Effect of ethanolic extract and volatile components from EZW on the proliferation of rat calvarial osteoblasts (n = 8, $\overline{X}$ ± SD; * p<0.01, compared with control).

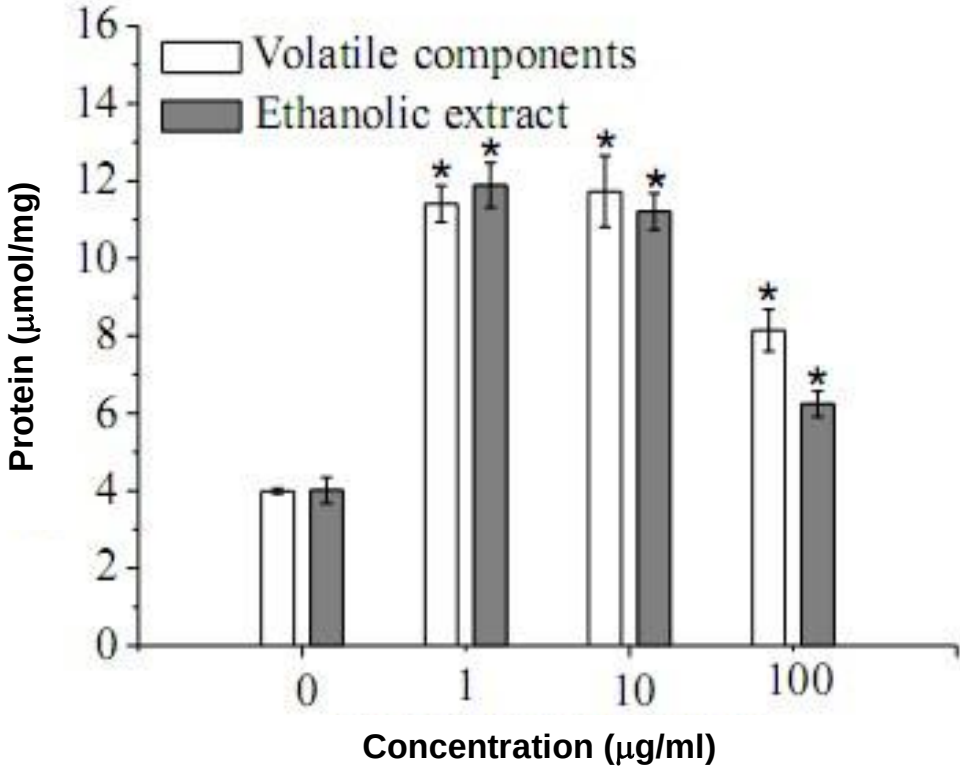

Figure 2. Effect of ethanolic extract and volatile components from EZW on the ALP activity of rat calvarial osteoblasts (n = 8 and $\overline{X}$ ±SD; * p<0.01, compared with control).

Table 1. Volatile components from EZW.

Peak no.	Components	RT (min)	RI[a]	Peak area (%)
1	1-Methyl-4-(1-methylethyl)-benzene	1027	1027	0.02
2	D-Limonene	1032	1032	0.10
3	4-Methyl-1-(1-methylethyl)-3-cyclohexen-1-ol	1181	1181	0.03
4	(S)-α, α, 4-Trimethyl-3-cyclohexene-1-methanol	1195	1195	0.03
5	6,6-Dimethyl-bicyclo[3.1.1]hept-2-ene-2-methanol	1202	1202	0.07
6	2-Methyl-3-phenyl-propanal	1241	1241	0.04
7	n-Decanoic acid	1365	1365	0.07
8	α-Cubebene	1377	1377	0.04
9	1,2-Dimethoxy-4-(2-propenyl)-benzene	1405	1405	0.05
10	[1R-(1R*,4Z,9S*)]-4,11,11-Trimethyl-8-methylene-bicyclo[7.2.0]undec-4-ene	1408	1408	0.09
11	Caryophyllene	1419	1419	0.07
12	Z,Z,Z-1,5,9,9-Tetramethyl-1,4,7,-cycloundecatriene	1451	1451	0.48
13	(Z)- 7,11-Dimethyl-3-methylene-1,6,10-dodecatriene	1454	1454	0.10
14	(4aR-trans)- Decahydro-4a-methyl-1-methylene-7-(1-methylethylidene)-naphthalene	1473	1473	0.11
15	(1α,4aα, 8aα)- 1,2,3,4,4a,5,6,8a-Octahydro- 7-methyl-4-methylene-1-(1-methylethyl)-naphthalene	1474	1474	0.09
16	[s-(E,E)]- 1-Methyl-5-met hylene-8-(1-methylethyl)-1,6-cyclodecadiene	1479	1479	0.14
17	Pentadecane	1500	1500	2.15
18	(S)-1-Methyl-4-(5-methyl-1-methylene-4-hexenyl)-cyclohexene	1507	1507	0.09
19	1,2,4a,5,6,8a-Hexahydro-4,7-dimethyl-1-(1-methylethyl)-naphthalene	1510	1510	0.24
20	(1S-cis)- 1,2,3,5,6,8a-Hexahydro-4,7-dimethyl-1-(1-methylethyl)-naphthalene	1518	1518	1.29
21	[1R-(1α,4aα,8aα)]- 1,2,4a,5,6,8a-Hexahydro-4,7-dimethyl-1-(1-methylethyl)-naphthalene	1528	1528	0.10
22	[S-(Z)]-3,7,11-Trimethyl-1,6,10-dodecatrien-3-ol	1551	1551	0.15
23	Dodecanoic acid	1556	1556	0.49
24	[1aγ-(1aα,4aα,7β,7aβ,7bα)]-Decahydro-1,1,7-trimethyl-4-methylene-1H-cycloprop[e]azulen-7-ol	1561	1561	0.19
25	Caryophyllene oxide	1565	1565	0.87
26	1-(4-Ethylphenyl)-3-methyl-pyrazol-(4H)-one	1570	1570	0.27
27	Cedrol	1580	1580	0.20
28	Hexadecane	1584	1584	0.32
29	1,2,3,4,4a,7-Hexahydro-1,6-dimethyl-4-(1-methylethyl)-naphthalene	1607	1607	0.78
30	α-Cadinol	1628	1628	4.85
31	[1S-(1α,4α,4aβ,8aβ)]-1,2,3,4,4a,7,8,8a-Octahydro-1,6-dimethyl-4-(1-methylethyl)-1-naphthalenol	1629	1629	1.00
32	[1S-(1α,7α,8aα)]- 1,2,3,5,6,7,8,8a-Octahydro-1,8a-dimethyl-7-(1-methylethenyl)-naphthalene	1632	1632	0.18
33	[1aR-(1aα,4aβ,7α,7aβ,7bα)]-Decahydro-1,1,7-trimethyl-4-methylene-1H-cycloprop[e]azulene	1657	1657	0.16
34	Z-1,6-Tridecadiene	1660	1660	0.50
35	8-Heptadecene	1669	1669	0.55
36	Heptadecane	1700	1700	4.59

Table 1. Contd.

37	2-Pentadecanol	1704	1704	0.12
38	Hexadecanal	1713	1713	0.52
39	3,7,11-Trimethyl-2,6,10-dodecatrien-1-ol	1719	1719	0.26
40	Phenanthrene	1753	1753	0.12
41	Tetradecanoic acid	1768	1768	1.12
42	6,10,14-Trimethyl-2-pentadecanone	1844	1844	3.20
43	2- Chloropropionic acid, octadecylester	1847	1847	0.30
44	Pentadecanoic acid	1867	1867	0.63
45	6,10, 14-Trimethyl-5,9,13-pentadecatrien-2-one	1914	1914	0.21
46	3-Methyl-2-(3,7,11-trimethyldodecyl) furan	1916	1916	0.10
47	Hexadecanoic acid methyl ester	1927	1927	0.76
48	9-Hexadecenoic acid	1943	1943	0.19
49	n-Hexadecanoic acid	1988	1988	20.58
50	9,12-Octadecadienoic acid methylester	2092	2092	1.38
51	(E)-8-Octadecenoic acid, methylester	2099	2099	1.82
52	Octadecanoic acid methyl ester	2128	2128	0.08
53	(Z,Z)-9,12-Octadecadienoic acid	2156	2156	23.11
54	(E)-9-Octadecenoic acid	2165	2165	8.89
55	2,2':5',2''-Terthiophene	2177	2177	0.29
56	Tricosane	2300	2300	0.22
57	Tetracosane	2400	2400	0.05
58	2,2'-Methylenebis[6-(1,1-dmethylethyl)-4-methyl-phenol	2408	2408	0.11
59	Pentacosane	2500	2500	0.27
60	1,2-benzenedicarboxylic acid diisooctyl ester	2544	2544	1.44
61	Heptacosane	2700	2700	0.07
	Total			86.34

[a] RI: Retention indexes relative to n-alkanes C_8-C_{40} on HP-5MS column.

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 (8.89 ± 0.579%), α-cadinol (4.85 ± 0.263%), heptadecane (4.59 ± 0.393%), 6,10,14-trimethyl-2-pentadecanone (3.20 ± 0.262%), pentadecane (2.15 ± 0.033%), (E)-8-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 methylethyl)-naphthalene (1.29 ± 0.146%), tetradecanoic acid (1.12 ± 0.032%), and [1S-(1α,4α,4aβ,8aβ)]-1, 2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylethyl)-1-naphthalenol (1.00 ± 0.016%).

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 and Wu, 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 and Messina, 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 cultures of mouse calvarial osteoblasts (Lee et al., 2008a). These findings give some insight into the antiosteoporotic mechanism of EZW.

In the present study, we investigated the effects of ethanolic extract and volatile compounds from EZW on the proliferation and ALP activity of rat calvarial osteoblasts. Our results showed that both ethanolic extract and volatile compounds could significantly ($p<0.01$) stimulate osteoblast proliferation in a dose-dependent manner and increased the ALP activity. By comparing the effects of ethanolic extracts and volatile compounds from EZW and every single herb (EP or FLL) we have studied (Lin et al., 2010; Wu et al., 2011), we found that the volatile compounds from EZW (compatibility of two herbs) displayed the similar effects on proliferation and ALP activity with those from every single herb. The ethanolic extract from EZW was also not superior to the ethanolic extract from every single herb on the enhancement of ALP activity. However, the ethanolic extract from EZW possessed a stronger osteoblastic proliferative activity than ethanolic extract from every single herb. This result displayed the advantage of compatibility of traditional Chinese medicine formula.

Total triterpene acids (TTA) such as ursolic acid might be main active compounds in EZW. Previous publication reported that ursolic acid could enhance differentiation and mineralization of osteoblasts *in vitro* and stimulate bone-forming *in vivo* in a mouse calvarial bone formation model (Lee et al., 2008b). 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-osteporotic fractions and constituents in EZW are in progress.

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