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Full Length Research Paper

Volatile components of fruits of *Ligustrum lucidum* Ait. stimulate proliferation and differentiation of rat calvarial osteoblasts

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The fruits of *Ligustrum lucidum* Ait., (FLL), which contain rich volatile components, are commonly used as tonic for kidney and liver in the traditional Chinese medicine prescriptions. This study aimed to investigate the effects of volatile components of FLL on the proliferation and differentiation of rat calvarial osteoblasts by the MTT method and measuring the activity of alkaline phosphatase (ALP). Results showed that volatile components (1 to 100 µg/mL) of FLL significantly ($p<0.01$) stimulated the proliferation and increased the ALP activity of rat calvarial osteoblasts which indicated that volatile components of FLL played an important role in osteoblastic bone formation just as non-volatile components in FLL. Such finding accredited the FLL as a potential candidate that might be useful in bone engineering and in treating bone defects including osteoporosis. The volatile components were analyzed by GC-MS. A total of 67 compounds were identified and the main components included (Z,Z)-9,12-octadecadienoic acid (33.47%), n-hexadecanoic acid (15.02%), (E)-9-octadecenoic acid (9.03%), α-cadinol (6.51%), 4-hexyl-2,5-dihydro-2,5-dioxo-3-furanacetic acid (4.93%) and (E)-8-octadecenoic acid methyl ester (2.69%).

Key words: *Ligustrum lucidum*, volatile components, rat calvarial osteoblasts.

INTRODUCTION

Osteoporosis, a disease characterized by low bone mass and microarchictectural deterioration of bone tissues, is due to the persistent excess of osteoclastic bone resorption over osteoblastic bone formation (Rodan and Martin, 2000; Kong et al., 2000; Teitelbaum et al., 2000). Therefore, both stimulators of bone formation and specific suppressors of bone resorption are significant for the treatment of osteoporosis.

Many antiosteoporotic agents such as estrogen, bis-phosphonates, calcitonin, sodium fluoride and anabolic steroids have been developed to treat osteoporosis (Brixen et al., 2009; Watts 2003; Kapur et al., 2010). Ironically, data indicated that their long-term use is accompanied by potentially malignant effects (Davison and Davis, 2003; John 2010). In addition, their costs are too high to benefit a large population in the developing or even developed countries for the prevention and treatment of osteoporosis (Kaufman and Goemaere, 2008). For more than a millennium, Chinese herbal medicine has been extensively used, apparently safely and effectively, in Asian countries, especially in China, Japan and Korea, to alleviate various symptoms of diseases (Zhang et al., 2009; Wu et al., 2009; Chena et al., 2009). So, it will undoubtedly be a cost-effective alternative to commercial pharmaceutical products.

The fruits of *Ligustrum lucidum* Ait. (Oleaceae) (FLL, Chinese name, Nvzhenzi), are well known as tonic for kidney and liver in the traditional Chinese medicine. Previous papers reported that the aqueous and ethanolic extracts of FLL could improve bone properties by enhancing the mineralization process on osteoblast cells

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Abbreviation: **FLL**, Fruits of *Ligustrum lucidum.*

or maintaining the calcium balance, and accelerate the osteoblast differentiation of mesenchymal stem cells (MSCs) (Zhang et al., 2006, 2008; Li et al., 2008). Many researches showed that volatile components obtained from many plants are responsible for their pharmacological activities just as non-volatile components in herbs (Lograda et al., 2010; Ho et al., 2010; Da et al., 2010). Moreover, the quality and quantity of volatile components, related with pharmacological activities, are highly influenced by genetic and environmental factors (Cardile et al., 2010). FLL contain rich volatile components, from which we could always smell the strong fragrance. However, as far as our literature survey could ascertain, there is no report on any pharmacological investigation on the volatile components from FLL. Therefore, in this study, we investigated the effects of volatile components of FLL on the proliferation and alkaline phosphatase (ALP) activity (the expression of ALP is closely associated with osteoblastic differentiation) of rat calvarial osteoblasts and identified the composition of the volatile components of FLL.

MATERIALS AND METHODS

The fruits of L. lucidum Ait. (Oleaceae) (FLL, Chinese name, Nvzhenzi) (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 the Department of Pharmacognosy, Fujian University of Traditional Chinese Medicine, Fuzhou, P. R. China.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl suphoxide (DMSO) were purchased from Sigma (U.S.A.). n-Alkanes C8-C40 were purchased from Accustandard (U.S.A.). Phenol red-free Dulbecco's modified Eagle's medium (phenol red-free DMEM) and fetal bovine serum (FBS) were 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.

Extraction of volatile components

Dry fruits (200 g) were crushed (40 mesh), then soaked in 2000 ml water for about 12 h before they were subjected to hydrodistillation in a Clevenger type apparatus. The contents were distilled for 3 h to obtain the volatile oil in a 0.31% (w/w) yield (on a dry mass) of yellowish colour and with a pleasant smell. The oils were dried over anhydrous sodium sulphate and stored at 4℃ in the dark until they were tested and analyzed.

Preparation of test samples

Volatile components were dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mg/ml, and diluted in culture medium of 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. Rat calvarial osteoblasts 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℃, then the media was changed.

Assay for osteoblast proliferation and ALP activity

The rat calvarial 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.), then cultured for another 48 h. Prior to the end of the culture, MTT (20 µl, 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 components - 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 every 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.

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℃), 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℃, 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 of 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 – 2.5℃/min – 130 – 1.2℃/min – 170 – 2℃/min – 230℃ – 2℃/min – 250℃ (5 min).

Identification and quantification of volatile components

Volatile components 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; Lv, 2005; Zhang et al., 1993; Li and Li, 1990).

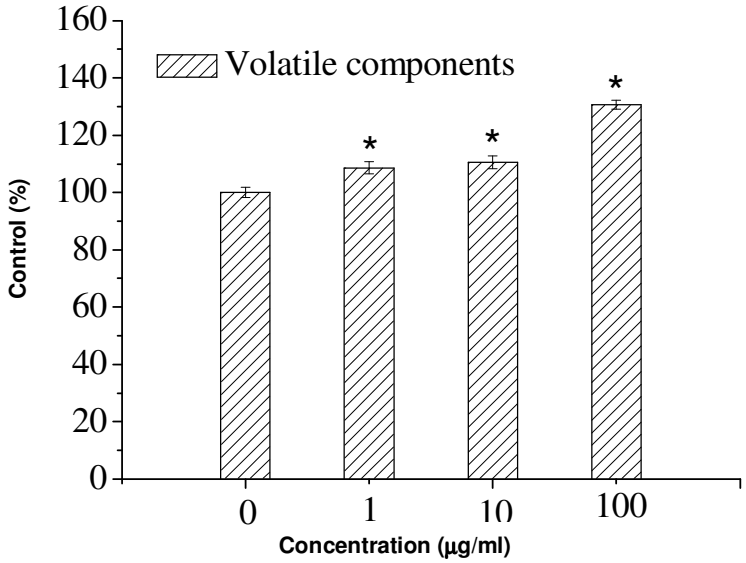

Figure 1. Effect of volatile components of FLL on the proliferation of rat calvarial osteoblasts (n = 8, $\bar{x}$±SD; *p<0.01, compared with control).

Components relative percentages were calculated from the TIC of the automated integrator.

Statistical analysis

Data were expressed as the mean ± standard deviation. Statistical significances were analyzed by using the Student's t-test. A value of $p<0.01$ was considered significant. Linear regression analysis was performed by the correlation coefficient.

RESULTS AND DISCUSSION

Volatile components with different concentrations (1 to 100 μg/ml) dose-dependently stimulated the proliferation of rat calvarial osteoblasts ($p<0.01$) (Figure 1). The maximal effect was observed when cells were incubated with volatile components (100 μg/ml). To ascertain whether FLL are capable of affecting osteoblastic cell differentiation, we examined the changes of ALP activity. As shown in Figure 2, volatile components significantly ($p<0.01$) increased ALP activity in osteoblasts over the 9 days, and their maximal effects were observed when cells were incubated with volatile components (1 μg/ml). Therefore, volatile components from FLL could stimulate osteoblastic differentiation at least in part by enhancing the synthesis of ALP. Such findings accredited the FLL as a potential candidate that might be useful in bone engineering and in treating bone defects including osteoporosis.

All of the 67 compounds, which were the major part (86.23%) of the volatiles, were identified. GC-MS profile of the volatile components 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 are listed in order of elution from a HP-5MS column. The main components were as follows: (Z,Z)-9,12-octadecadienoic acid (33.47%), n-hexadecanoic acid (15.02%), (E)-9-octadecenoic acid (9.03%), α-cadinol (6.51%), 4-hexyl-2,5-dihydro-2,5-dioxo-3-furanacetic acid (4.93%), (E)-8-octadecenoic acid methyl ester (2.69%), (Z,Z)-9,12-octadecadienoic acid methyl ester (1.86%), (1S-cis)- 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-naphthalene (1.57%), [1S-(1α,4α,4aβ,8aβ)]-1,2,3,4,4a, 7,8,8a-octa-hydro-1,6-dimethyl-4-(1-methylthyl)-1-naphthalenol (1.34%) and hexadecanoic acid methyl ester (1.13%). The results displayed notable difference between our data and those previously reported on FLL (Lv, 2005). The main components of the essential oils from FLL were 5-butyl-hexadecane (7.65%), α,α-diphenyl-benzene-methanol (6.63%), pentacosane (6.22%), benzophenone (5.12%), octacosane (4.98%) and eucalyptol (4.95%). These compounds were absent or only had low amount in our study. In comparison with the oils from FLL reported (Zhang et al., 1993; Li et al., 1990), the main components of the oils in this study also displayed significant difference. Hence, we thought that the differences of volatile components might arise from several environmental (climatical, seasonal and geographical) and genetic differences (Cardile et al., 2010), which were the important factors influencing the quality of medicinal herbs.

Essential fatty acids (EFAs) can be divided into two families viz. omega-6 or n-6 and omega-3 or n-3 families. Linoleic acid (LA) is the parent molecule of the n-6 series of EFAs metabolites while α-linolenic acid (ALA) is the

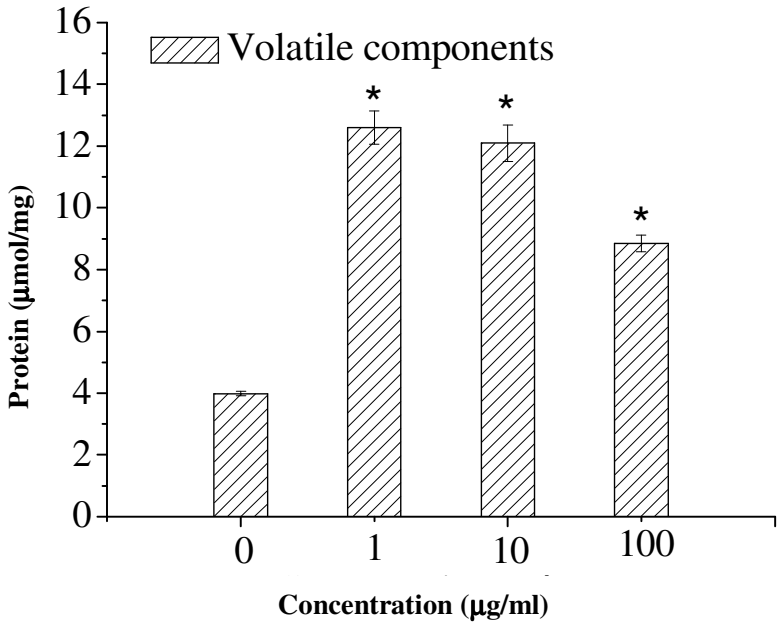

Figure 2. Effect of volatile components of FLL on the ALP activity of rat calvarial osteoblasts (n = 8, $\bar{x}$ ±SD; *p<0.01, compared with control)

Table 1. Volatile components of the fruits of *L. lucidum* Ait.

Peak number	Component	RT (min)	RI[a]	Peak area (%)[b]
1	β-pinene	3.269	981	0.04
2	2-pentyl-furan	3.330	991	0.02
3	D-limonene	3.912	1031	0.03
4	α-methyl-α-[4-methyl-3-pentenyl] oxiranemethnol	4.627	1075	0.02
5	(S)-α, α, 4-trimethyl-3-cyclohexene-1-methanol	7.402	1195	0.03
6	nonanoic acid	9.801	1268	0.12
7	(E, E)-2,4-decadienal	11.473	1316	0.02
8	5-ethenyl-3-pyridinecarboxylic acid methyl ester	12.842	1351	0.04
9	eugenol	13.060	1357	0.03
10	n-decanoic acid	13.363	1364	0.02
11	α-cubebene	13.836	1376	0.03
12	[E]-1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one	14.163	1385	0.03
13	1,2-dimethoxy-4-(2-propenyl)-benzene	14.951	1404	0.06
14	β-caryophyllene	15.581	1419	0.08
15	Z,Z,Z-1,5,9,9-tetramethyl-cycloundecatriene	16.975	1450	0.08
16	[1aR-(1aα,4aβ,7α,7aβ,7bα)]- decahydro-1,1,7-trimethyl-4-methylene-1H-cycloprop[e]azulene	17.302	1458	0.02
17	(1α,4aα, 8aα)- 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-naphthalene	18.029	1474	0.11
18	[s-(E,E)]- 1-methyl-5-methylene-8-(1-methylethyl)-1,6-cyclodecadiene	18.235	1479	0.35
19	4-hexyl-2,5-dihydro-2,5-dioxo-3-furanacetic acid	18.719	1490	4.93
20	(1α,4aα,8aα)-1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-naphthalene	19.180	1500	0.30
21	1,2,4a,5,6,8a-hexahydro-4,7- dimethyl-1-(1-methylethyl)-naphthalene	19.822	1510	0.38

Table 1. Continues

22	(1S-cis)-1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-naphthalene	20.343	1518	1.57
23	[1S-(1α,4aβ,8aα)]-1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-naphthalene	20.985	1528	0.11
24	Seychellene	23.385	1565	0.58
25	1-(4-ethylphenyl)-3-methyl-pyrazol-(4H)-one	23.651	1570	0.27
26	cedrol	24.318	1580	0.14
27	ledol	24.500	1583	0.28
28	2,6-bis(1,1-dimethylethyl)-2,5-cyclohexadiene-1,4-dione	24.694	1586	0.13
29	1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-naphthalene	25.239	1594	0.42
30	(1α,4aβ,8aα)-1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-naphthalene	26.208	1612	0.16
31	α-cadinol	27.057	1628	6.51
32	[1S-(1α,4α,4aβ,8aβ)]-1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylethyl)-1-naphthalenol	27.250	1631	1.34
33	[1aR-(1aα,7α,7aβ,7bα)]-1a,2,3,5,6,7,7a,7b-octahydro-1,1,4,7-tetramethyl-1H-cycloprop[e]azulene	28.268	1651	0.12
34	1,6-dimethyl-4-(1-methylethyl)-naphthalene	28.850	1662	0.11
35	heptadecane	30.801	1700	0.1
36	cis-11-tetradecen-1-ol	31.673	1712	0.09
37	3,7,11-trimethyl-2,6,10-dodecatrien-1-ol	32.134	1719	0.60
38	12-methyl-tridecanoic acid methyl ester	32.510	1724	0.04
39	(E,E)-3,7,11-trimethyl-2,6,10-dodecatrienal	33.430	1738	0.04
40	2-dodecen-1-yl(-)succinic anhydrid	33.600	1740	0.04
41	phenanthrene	34.497	1753	0.08
42	tetradecanoic acid	35.636	1770	0.68
43	6,10,14-trimethyl-2-pentadecanone	40.774	1843	0.65
44	15-methyl-oxacyclopentadecan-2-one	41.125	1848	0.09
45	1,2-benzenedicarboxylic acid bis(2-methylpropyl) ester	42.143	1862	0.14
46	pentadecanoic acid	42.422	1866	0.14
47	1,3,12-nonadecatriene	44.033	1889	0.02
48	nonadecane	44.761	1900	0.04
49	(E,E)-6,10,14-trimethyl-5,9,13-pentadecatrien-2-one	45.827	1914	0.05
50	hexadecanoic acid methyl ester	46.772	1927	1.13
51	9-hexadecenoic acid	47.911	1943	0.24
52	Z-11-hexadecenoic acid	48.323	1949	0.06
53	n-hexadecanoic acid	51.716	1997	15.02
54	(Z,Z)-9,12-octadecadienoic acid methyl ester	57.630	2093	1.86
55	(E)-8-octadecenoic acid methyl ester	58.090	2101	2.69
56	octadecanoic acid methyl ester	59.556	2129	0.19
57	(Z,Z)-9,12-octadecadienoic acid	60.114	2140	33.47
58	(E)-9-octadecenoic acid	61.410	2164	9.03
59	tricosane	68.063	2300	0.29
60	(Z)-9,17-octadecadienal	70.377	2353	0.03
61	(Z)-9-octadecenoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	70.644	2360	0.04
62	cyclotetracosane	72.159	2394	0.03

Table 1. Continues

63	2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-phenol	72.777	2409	0.04
64	pentacosane	76.485	2500	0.11
65	1,2-benzenedicarboxylic acid diisooctyl ester	78.206	2545	0.66
66	heptacosane	83.198	2700	0.02
67	(all-E)-2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene	86.676	2846	0.04
Grouped components (%)				
Monoterpene hydrocarbons				0.07
Oxygenated monoterpenes				0.10
Sesquiterpene hydrocarbons				4.34
Oxygenated sesquiterpenes				9.18
Fatty acid				62.91
Aliphatic hydrocarbons				0.56
Others				9.07
Total				86.23

[a]RI: Retention indexes relative to n-alkanes C_8-C_{40} on HP-5MS column; [b]Peak area (%)

parent molecule of the n-3 series. Both of them are converted by alternating desaturation and elongation reactions to their respective active metabolites (Schlemmer et al., 1999). There are some evidences that EFAs, as well as their metabolites play critical roles in regulating bone metabolism and may have potential in the treatment or prevention of osteoporosis. They may enhance the effectiveness of calcium, alter cell-to-cell signaling processes, and impact transcription factors *in vivo* (Poulsen et al., 2007, 2008; Das et al., 2000). Furthermore, EFAs might modulate other mechanisms also involved in the regulation of bone parameters. A recent study that examined dietary intake of the two families of EFAs reported that postmenopausal women with a high dietary ratio of n-6:n-3 fatty acids had the lowest bone mass density (BMD) (Poulsen and Kruger, 2006), which indicated that high n-6 EFAs intake rather than high total EFAs intake may be detrimental to bone mass. Hence, in light of this study, the effects of volatile components from FLL on the proliferation and ALP activity of rat calvarial osteoblasts might be not only related to the presence of (Z,Z)-9,12-octadecadienoic acid (LA) (33.47%), but the synergistic effects of the diversity of major and minor constituents present in the volatile components.

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REFERENCES

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Brixen K, Kassem M, Abrahamsen B (2009). Prevention and treatment of osteoporosis in women: an update. Obstetr. Gynaecol. Reprod. Med. 9: 157-163.

Cardile V, Russo A, Formisano C, Rigano D, Senatore F, Arnold NA, Piozzi F (2010) Essential oils of Salvia bracteata and Salvia rubifolia from Lebanon: Chemical composition, antimicrobial activity and inhibitory effect on human melanoma cells. J. Ethnopharmacol. 126: 265-272.

Chena MW, Feng WZ, Cao H, Zou LJ, Chen CG, Baatrup A, Nielsen AB, Li H S, Kassem M, Zou, XN, Bünger C (2009). A traditional Chinese medicine formula extracts stimulate proliferation and inhibit mineralization of human mesenchymal cells. J. Ethnopharmacol. 126: 265-272.

Da Silva JK, Andrade EH, Guimarães EF, Maia JG (2010). Essential oil composition, antioxidant capacity and antifungal activity of *Piper divaricatum*. Nat. Prod. Commun. 5: 477-480.

Das UN (2000). Essential fatty acids and osteoporosis. Nutrition, 16: 386-390.

Davison S, Davis SR (2003). Hormone replacement therapy: current controversies. Clin. Endocrinol. 58: 249-261.

Ho CL, Jie-Pinge O, Liu YC, Hung CP, Tsai MC, Liao PC, Wang EI, Chen YL, Su YC (2010). Compositions and *in vitro* anticancer activities of the leaf and fruit oils of *Litsea cubeba* from Taiwan. Nat. Prod. Commun. 5: 617-620.

Idris AI, Rojas J, Greig IR, van't Hof RJ, Ralston SH (2008). Aminobisphosphonates cause osteoblast apoptosis and inhibit bone nodule formation *in vitro*. Calcif. Tissue Int. 82: 191-201.

John CA (2010). Review of the cardiovascular safety of zoledronic acid and other bisphosphonates for the treatment of osteoporosis. Clin. Ther. 32: 426-36.

Kapur P, Wuttke W, Jarry H, Seidlova-Wuttke D (2010). Beneficial

effects of β-Ecdysone on the joint, epiphyseal cartilage tissue and trabecular bone in ovariectomized rats. Phytomedicine, 17: 350-355.
Kaufman JM, Goemaere S (2008). Osteoporosis in men. Best Pract. Res. Clin. Endocrinol. Metab. 22: 787-812.
Kong YY, Penninger JM (2000). Molecular control of bone remodeling and osteoporosis. Exp. Gerontol. 35: 947-956.
Li G, Chan CY, Zhang JF, Zhang XA, Yew DTW, He ML, Lin MCM, Leung PC, Kung HF (2008). The aqueous extract of Fructus Ligustri Lucidi regulates the differentiation of mesenchymal stem cells. Int. J. Integ. Biol. 3: 182-190.
Li KH, Li CS (1990). Study on chemical composition of essential oil from Fructus Ligustri Lucidi. Chin. Trad. Pat. Med. 12: 32-32.
Lograda T, Chaker AN, Chalchat JC, Ramdani M, Silini H, Figueredo G, Chalarde P (2010). Chemical composition and antimicrobial activity of essential oils of *Genista ulicina* and *G vepres*. Nat. Prod. Commun. 5: 835-838.
Lv JS (2005). Study on the chemical composition of the volatile oil in *Ligustrum lucidum*. Chin. Pharm. J. 40: 178-180.
Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, Kennedy MB, Pockwinse S, Lian JB, Stein GS (1990). Progressive development of the rat osteoblast phenotype *in vitro*: reciprocal relationship in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracelluar matrix. J. Cell. Physiol. 143: 420-430.
Poulsen RC, Kruger MC (2006). Detrimental effect of eicosapentaenoic acid supplementation on bone following ovariectomy in rats. Prostaglandins Leukot. Essent. Fatty Acids, 75: 419-427.
Poulsen RC, Moughan PJ, Kruger MC (2007). Long-chain polyunsaturated fatty acids and the regulation of bone metabolism. Exp. Biol. Med. 232: 1275-1288.
Poulsen RC, Wolber FM, Moughan PJ, Kruger MC (2008). Long chain polyunsaturated fatty acids alter membrane-bound RANK-L expression and osteoprotegerin secretion by MC3T3-E1osteoblast-like cells. Prostaglandins other Lipid Mediat. 85: 42-48.
Rodan GA, Martin TJ (2000). Therapeutic approaches to bone diseases. Science, 289: 1508-1514.
Schlemmer CK, Coetzer H, Claassen N, Kruger MC (1999). Oestrogen and essential fatty acid supplementation corrects bone loss due to ovariectomy in the female Sprague Dawley rat. Prostaglandins, Leukot. Essent. Fatty Acids, 61: 381-390.
Teitelbaum SL (2000). Bone resorption by osteoclasts. Science, 289: 1504-1508.
Watts NB (2003). Bisphosphonate treatment of osteoporosis. Clin. Geriatr. Med. 19: 395-414.
Wu YB, Zheng CJ, Qin LP, Sun LN, Han T, Jiao L, Zhang QY, Wu JZ (2009). Antiosteoporotic activity of anthraquinones from Morinda officinalis on osteoblasts and osteoclasts. Molecules, 14: 573-583.
Zhang JZ, Liu YQ, Qian RZ (1993). Analysis of volatile components from Fructus Ligustri Lucidi. J. Nanjing Inst. Chem. Technol. 15: 77-79.
Zhang R, Liu ZG, Li C, Hu SJ, Liu L, Wang JP, Mei QB (2009). Du-Zhong (*Eucommia ulmoides* Oliv.) cortex extract prevent OVX-induced osteoporosis in rats. Bone, 45: 553-559.
Zhang Y, Lai WP, Leung PC, Wu CF, Yao XS, Wong MS (2006). Effects of Fructus Ligustri Lucidi extract on bone turnover and calcium balance in ovariectomized rats. Biol. Pharm. Bull. 29: 291-296.
Zhang Y, Leung PC, Che CT, Chow HK, Wu CF, Wong MS (2008). Improvement of bone properties and enhancement of mineralization by ethanol extract of Fructus Ligustri Lucidi. Br. J. Nutr. 99: 494-502.