Anti-inflammatory and anti-osteoporotic lignans from Vitex negundo seeds

Cheng-Jian Zheng, Xiang-Wang Zhang, Ting Han, Yi-Ping Jiang, Jian-Yuan Tang, Dieter Brömme, Lu-Ping Qin

Chemical investigation of Vitex negundo seeds afforded four new lignans, including a phylindene-type lignan, vitexdoin F (1), and three phenylnaphthalene-type lignans, vitexdoin G, H, and I (2–4). Their structures were elucidated by detailed spectroscopic analyses on the basis of 

NMR, IR, and MS data. All compounds were evaluated for their anti-inflammatory and anti-osteoporotic activities, employing RAW264.7 macrophages, osteoblast-like UMR106 and osteoclastic cells, respectively. Compound I showed significant inhibition on the nitric oxide (NO) production (IC50 4.17 μg/mL) due to its down-regulation of the inducible nitric oxide synthase (iNOS) protein expression in LPS-stimulated RAW264.7 cells, which also exhibited potent stimulatory effects on the proliferation and ALP (alkaline phosphatase) activity of UMR106 cells, and significantly up-regulated the OPG/RANKL protein ratio.

1. Introduction

Rheumatoid arthritis (RA) is one of the most chronic destructive diseases to human health, which is also called “immortal cancer”, characterized by multi-system inflammation and bone destruction [1,2]. It can rapidly progress into irreversible joint damage thus causing premature mortality, disability and compromised quality of life in the industrialized and developing world [3,4].

Nowadays, disease-modifying antirheumatic drugs (DMARDs) supplemented with non-steroidal anti-inflammatory drugs (NSAIDs), steroid hormone and biologics (TNF-α antibody and the decoy TNF-α receptor, etc) remain the major strategy in the treatment of RA [4,5]. However, administration of these drugs is associated with severe adverse effects, including gastrointestinal lesions, cardiovascular complications, and reproductive toxicity [6,7]. Therefore, more and more attention has been paid to plant-derived anti-RA drugs with high efficacy and few side effects. Recent investigations have estimated that 60–90% of RA patients are very likely to use botanicals [8]. This growing interest in alternative medical practices clearly indicates the need for more safe and effective anti-RA botanicals used in the traditional medicine.

Vitex negundo L. (Verbenaceae) is a small aromatic plant with typical five foliolate leave pattern, being a native of China, Japan and India [9]. Its seeds are occasionally used as a condiment for edible purpose [10] and also find use for analgesia, rheumatism and joint inflammation in folk medicine [11,12]. In Ayurvedic Medicine, V. negundo seeds also found a good reputation for the treatment of rheumatoid arthritis [13]. Our study was therefore conducted to investigate the bioactive constituents with anti-inflammatory and anti-osteoporotic activities, which may be responsible for the potential Anti-RA activity of V. negundo seeds. We report

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herein the isolation, structure elucidation, anti-inflammatory and anti-osteoporotic activities of the isolated four new lignans, vitexoids F-I (1–4).

2. Experimental

2.1. General

Optical rotations were acquired with a Perkin-Elmer 341 polarimeter. IR spectra were recorded on a Bruker Vector 22 spectrometer with KBr pellets. NMR spectra were recorded on a Bruker Avance 600/400 NMR spectrometer with TMS as an internal standard. HRESIMS were measured using a Q-TOF micromass spectrometer (Waters, USA). Materials for column chromatography were silica gel (100–200 mesh; Huiyou Silical Gel Development Co., Ltd., Yantai, China), silica gel H (10–40 μm; Yantai), Sephadex LH-20 (40–70 μm; Amersharn Pharmacia Biochem AB, Upppsala, Sweden), and YMC-GEL ODS-A (50 μm; YMC, Milford, MA). HSGF254 silica gel TLC plates (Yantai) were used for analytical TLC. Preparative TLC (0.4–0.5 mm) was performed on glass plates precoated with silica gel GF254 (Yantai).

2.2. Plant material

V. negundo seeds were collected during October 2006 in Wanglang National Nature Reserve, Sichuan province, China. Prof. Lu-Ping Qin (School of Pharmacy, Second Military Medical University) identified the specimens and a sample (#2006-168) was deposited in the herbarium of the Department of Pharmacognosy, School of Pharmacy, Second Military Medical University, Shanghai, China.

2.3. Extraction and isolation

The air-dried and powdered seeds (25 kg) were extracted with hot aqueous ethanol (80% v/v) under reflux three times for 2 h each time. After removal of the solvent under reduced pressure, the residue was partitioned sequentially with petroleum ether, CH2Cl2, EtOAc, and n-BuOH, respectively. The CH2Cl2 extract (260 g) was subjected to silica gel chromatography using eluent mixtures of petroleum ether–EtOAc (50:1, 20:1, 10:1, 5:1, 3:1, 1:1, 0:1, 0:1 v/v) to afford fractions A–G. Fraction E (10.2 g) was subjected on ODS column chromatography using the gradient MeOH–H2O from 40% to 80% as eluent to provide five fractions (E.1–E.5). Fraction E.3 (190 mg) was rechromatographed on Sephadex LH-20 with MeOH–H2O (80%) to give 1 (40 mg). Fraction E.4 (210 mg) was rechromatographed on Sephadex LH-20 with MeOH–H2O (80%) followed by preparative TLC to give 2 (9 mg) and 3 (14 mg). Fraction E.5 (140 mg) was rechromatographed on Sephadex LH-20 with MeOH–H2O to give 4 (20 mg).

2.3.1. Vitexdoin F (1)

Yellowish amorphous powder; UV λmax (MeOH) nm: 223, 264, 364; positive HRESIMS [M + H]+ peak at m/z 313.1056 (calcd for C18H17O5, 313.1076); IR(KBr) cm−1: 3385, 1626, 1513, 1394, 1336, 1273, 1174; 1H-NMR (DMSO-d6, 400 MHz, Table 1) and 13C-NMR (DMSO-d6, 100 MHz, Table 1) spectra.

Table 1

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2.3.2. Vitexdoin G (2)

Colorless syrup; [α]D20 = -140.0° (c 0.2, MeOH); UV λmax (MeOH) nm: 223, 256, 358; positive HRESIMS [M + H]+ peak at m/z 595.3650 (calcld for C36H51O7, 595.3635); IR (KBr) νmax 3421, 2925, 2853, 1733, 1508, 1384, 1151; 1H-NMR (CDCl3, 400 MHz, Table 1) and 13C-NMR (CDCl3, 100 MHz, Table 1) spectra.

2.3.3. Vitexdoin H (3)

Colorless syrup; [α]D20 = -75.0° (c 0.2, MeOH); UV λmax (MeOH) nm: 223, 283, 338; positive HRESIMS [M + H]+ peak at m/z 595.3647 (calcld for C36H51O7, 595.3635); IR (KBr) νmax 3414, 2976, 2846, 1776, 1620, 1514, 1487, 1095, 1027; 1H-NMR (DMSO-d6, 600 MHz, Table 2) and 13C-NMR (DMSO-d6, 150 MHz, Table 2) spectra.

2.3.4. Vitexdoin I (4)

White amorphous powder; [α]D20 = -140.0° (c 0.2, MeOH); UV λmax (MeOH) nm: 262; positive HRESIMS [M + H]+ peak at m/z 397.1293 (calcld for C22H21O7, 397.1287); IR (KBr) νmax 3420, 2925, 1734, 1515, 1384, 1151; 1H-NMR (CDCl3, 500 MHz, Table 1) and 13C-NMR (CDCl3, 100 MHz, Table 1) spectra.

2.4. Biological assays

2.4.1. Cell culture

RAW 264.7 macrophages and rat osteoblast-like UMR106 cells were cultured at 37 °C in 5% CO2 in DMEM medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Primary osteoblastic cells and rat marrow cells were prepared according to the literature [14,15], derived from new-born Wistar rats (3-day-old) purchased from the Experimental Animal Center of the Second Military Medical University. Briefly, osteoblastic cells were isolated from the calvaria of new born rats. Four calvaria were collected and washed in sterilized Hanks’ balanced salt solution, which were then minced and subjected to sequential digestions of 15, 30, 45, and 90 min at 37 °C in a solution of phosphate buffered saline (PBS) containing 0.25% trypsin (Gibco, USA) and 1 mg/mL collagenase II (Biological Corp., USA). Cells of the third and forth digestions were collected by centrifugation and re-suspended in α-MEM containing 10% FCS. Rat marrow cells were collected as follows: the femurs of 3-day-old rats were disarticulated and the ends were removed. The bone marrow cells were flushed out using a 1-ml syringe. Then the primary osteoblastic cells (1 × 106/mL) and bone marrow cells (1 × 106/mL) were co-cultured in α-MEM medium containing 10% FCS, 1,25-dihydroxy vitamin D3 (10 nmol/L) and dexamethasone (100 nmol/L) at 37 °C in a humidified atmosphere of 5% CO2 for 10 days in 96-well culture dishes (100 μL per well). The formation of osteoclast-like multinucleated osteoclasts was confirmed by the staining of tartrate-resistant acid phosphatase (TRAP).

2.4.2. Assay for inhibition ability against LPS-induced NO production

RAW 264.7 macrophages were seeded in 96-well plates (105 cells/well). The cells were co-incubated with the isolated compounds and LPS (1 μg/mL) for 24 h. The amount of NO was assayed by determining the nitrite concentration in the cultured RAW 264.7 macrophage supernatants with Griess reagent. Aliquots of supernatants (100 μL) were incubated, in sequence, with 50 μL of 1% sulfanilamide and 50 μL of 0.1% naphthylethylenediamine in 2.5% phosphoric acid solution. The absorbance at 548 nm was read using a microplate reader (POLARStar).

2.4.3. Assay for osteoblast proliferation and ALP activity

UMR106 cells were plated into 96-well culture dishes at 2 × 104 cells/mL in α-MEM medium containing 10% FCS and incubated at 37 °C for 24 h. Then, the cells were treated with tested compounds or control for 24 h. Before the end of culture, 20 μL of MTT was added to each well and incubated for 4 h. Then the old medium was discarded, and 150 μL of DMSO was added to each well. The UV absorbance was recorded at 570 nm (ELx 800 universal microplate reader) and the results were expressed as a percentage of the mean proliferation with the control as 100%. Similarly, the osteoblast cells were treated with tested compounds or control for 6 days. The ALP activity was measured according to the literature [15]. The results were showed as a percentage of the mean ALP activity with the control as 100%.

2.4.4. Assay for TRAP (tartrate-resistant acid phosphatase) activity

The osteoclastic cells were cultured according to the method of “Section 2.4.1.” Then the cells were treated with or without the tested compounds for 48 h. The TRAP activity was measured according to the method of the literature [15,16]. The results were showed as a percentage of the mean TRAP activity with the control as 100%.

2.4.5. Assay for COX-2, iNOS, OPG and RANKL protein levels

The murine RAW 264.7 cells and rat UMR106 cells were seeded at an initial density of 2 × 105 and 2 × 105 cells/well, respectively, in 6-well tissue culture plates overnight. RAW 2013 was

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264.7 cells were exposed to *Escherichia coli* LPS (1 μg/mL; Sigma) for 24 h in the presence or absence of the tested compounds. UMR106 cells were treated with control or the tested compounds for 24 h. Beta-actin (β-actin) protein was used to monitor that equal amounts of protein were in each lane. Protein samples were collected and prepared as described previously [17], and the iNOS, COX-2, OPG and RANKL expression levels were investigated using western blot analysis. Briefly, samples containing equal quantities of protein (50 μg) were subjected to SDS/20%-polyacrylamide gel electrophoresis, and the separated proteins were electrophoretically transferred to nitrocellulose (NC) membranes. The resultant NC membranes were incubated with blocking solution and probed using antibodies specific to inducible nitric oxide synthase (iNOS; 1:1000 dilution; Cell Signaling), cyclooxygenase-2 (COX-2; 1:1000 dilution; Cell Signaling), osteoprotegerin (1:100, Santa Cruz Biotechnology) and receptor activator of nuclear factor kappa B ligand (1:200, Santa Cruz Biotechnology) protein and visualized using an ECL detection kit (PerkinElmer, Western Lightning Chemiluminescence Reagent Plus).

### 2.5. Statistical analysis

The data were analyzed using SPSS computer software Version 16.0. The data for multiple comparisons were performed by one way ANOVA followed by LSD t-test. A value of p < 0.05 was considered statistically significant and all results per presented as the means ± S.D.

### 3. Results and discussion

#### 3.1. Chemistry

The dichloromethane-soluble extract of *V. negundo* seeds was successively subjected to silica gel, Chromatorex ODS, and Sephadex LH20 column chromatography as well as preparative TLC to afford four new lignans, vitexdoins F-I (1–4) (Fig. 1). Compound 1, trivially named vitexdoin F, was obtained as yellowish amorphous powder and analyzed for the molecular formula C_{18}H_{16}O_{5} by positive HRESIMS [M + H]+ at m/z 313.1056 (calcd 313.1076). Its IR spectrum revealed absorption bands for hydroxyl (3386 cm⁻¹) and α, β-unsaturated aldehyde (1626 cm⁻¹) group. The 13C NMR spectral data of 1 showed 18 resonances attributable to two oxygenated methyls, six sp² methines, one methylene and nine non-protonated carbons (Table 1). Its 1HN NMR spectrum was characterized by the presence of one aldehyde proton, two aromatic proton singlets and three aromatic protons coupled in an ABX pattern, two methoxyl and one methylene group, which were similar to those of vitexdoin C, a phenylnaphthalene-type lignan previously isolated from *V. negundo* seeds [9], differing only in the appearance of a methylene group in 1 instead of a disubstituted olefinic group with two vicinal coupling protons in vitexdoin C. The molecular weight of 1 is only one less carbon atom than that of vitexdoin C. All these data led to the assumption that the only structural difference between 1 and vitexdoin C is in ring A and 1 is recognized as a phenylindene-type rather than a phenylnaphthalene-type lignan. In addition, 265...
the aldehyde group was established to be linked to C-2 by HMBC correlations (Fig. 2) observed from the aldehyde proton (δH 9.73, s) to C-2 (δC 137.9) and C-1 (δC 35.4), while the phenyl group was attached to C-3 deduced from HMBC correlations from H-2′ (δH 7.11, s), H-6′ (δH 6.99, d, J = 8.1 Hz) and H-4 (δH 6.98, s) to C-3 (δC 158.7). The structure of 1 was therefore defined as 5-hydroxy-3-(4-hydroxy-3-methoxyphenyl)-6-methoxy-1H-indene-2-carbaldehyde. Phenylnaphthalene-type lignans were characteristic constituents in Vitex plants and our study is the first report of phenylindene-type lignan in Vitex species. Since most phenylnaphthalene-type lignans consist of two units of phenylpropanoids (C6–C3) connected by the β-C of each C3 side chain with 8′–8/2′–7′ linkage, vitexdoin F (1) was therefore speculated to be a neolignan of two phenylpropanoids in 7′–8′/2′–7′ connection pattern with further 7-deethylation (Fig. 2).

Compound 2, trivially named vitexdoin G, was obtained as colorless syrup and the molecular formula was established as C36H50O7 by positive HRESIMS [M + H]+ at m/z 595.3650 (calcd 595.3635). The IR spectrum showed absorption bands at νmax 1733, 1151 and 2925 cm\(^{-1}\), suggesting the presence of an ester group of an aliphatic long-chain acid, which was supported by the resonance at δC 173.7 and a large number of signals between δC 29.1 and 29.7 and the appearance of a strong broad singlet at δH ca. 1.26 and a triplet at δH 2.23 due to a great number of protons in its NMR spectrum. Except for those signals corresponding to a long aliphatic chain, the other NMR data of 2 was extremely analogous to those of 6-hydroxy-4-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-7-methoxy-3,4-dihydro-2-naphthaldehyde [9,18], apart from the downfield shifted signals of a hydroxymethyl group [δH 4.04 (1H, m), 3.94 (1H, m) and δC 64.3 in 2; δH 3.31 (1H, m), 2.90 (1H, m) and δC 60.6 in analog]. All these data suggested that 2 was an ester formed from a long-chain fatty acid to the hydroxymethyl group at C-3α of 6-hydroxy-4-(4-hydroxy-3-methoxyphenyl)-

Table 3

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<td>2</td>
<td>40.44 ± 2.69</td>
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<tr>
<td>3</td>
<td>59.13 ± 4.14</td>
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<tr>
<td>4</td>
<td>23.40 ± 3.09</td>
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<tr>
<td>Indo*</td>
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* LPS: negative control; Indo: indomethacin, positive control.

Fig. 3. Key HBMC (→) correlations of compounds 1, 2 and 4.

![Fig. 3](image1.png)

Fig. 4. Effect of vitexdoin F (1) at 10 μg/mL on the LPS-induced pro-inflammatory iNOS and COX-2 protein expressions of RAW 264.7 macrophage cells by immunoblot analysis. Values are mean ± S.D. of three experiments with triplicate of each experiment. *p < 0.05 indicates statistically significant differences from control group. *p < 0.05 indicates statistically significant differences from LPS-stimulated group.

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3-hydroxymethyl-7-methoxy-3,4-dihydro-2-naphthaldehyde, evidenced by the HMBC connectivities (Fig. 3) from H-3α to the carbon atom of the carbonyl group at δC 173.7. Its molecular formula, C_{36}H_{50}O_{7}, further allowed the identification of the aliphatic chain as a hexadecanoic acid. In addition, the stereochemistry of the chiral carbons C-3 and C-4 was assigned in analogy with that of vitexdoin A and confirmed by the singlet of H-4 (δH 4.11 (s)) [9,19]. Finally, the structure of 2 was concluded to be 6-hydroxy-4-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2-aldehyde-3-naphthmethyl hexadecanoate.

Compound 3, trivially named vitexdoin H, was obtained as colorless syrup. The molecular formula of 3 was determined as C_{36}H_{50}O_{7} by positive HRESIMS [M + H]^+ at m/z 595.3647 (calcld 595.3635), which is the same as that of 2.

Further detailed comparison of the NMR data of 3 and 2 revealed that the major difference was the splitting patterns and chemical shifts of two aromatic protons [δH 7.16 (d, J = 8.2 Hz), 6.96 (d, J = 8.2 Hz) in 3; δH 6.88 (s), 6.77 (s) in 2]. In addition, a higher field signal due to the O-methyl protons was observed in 3 (δH 3.50) compared to 2 (δH 3.95). The 13C NMR spectrum was also similar to that of 2, apart from the significant low-field shift of an O-methyl group (δC 61.5 in 3; δC 56.2 in 2) on the B-ring. Therefore, 3 was recognized as a regiosomer of 2 with an O-methyl group at C-5 instead of C-7, resulting in considerable steric hindrance that accounts for the notable low-field shift of the 5-OMe carbon signal [9,18]. The structure of 3 was therefore deduced as 6-hydroxy-4-(4-hydroxy-3-methoxyphenyl)-5-methoxy-2-aldehyde-naphthmethyl hexadecanoate.

Compound 4, trivially named vitexdoin I, was obtained as white amorphous powder and analyzed for the molecular formula C_{22}H_{20}O_{7} by positive HRESIMS [M + H]^+ at m/z 397.1293 (calcld 397.1287). It was obtained as an inseparable mixture of two diastereoisomers in a 5:4 ratio, as determined by integration of well-resolved 1H NMR resonances for H-3α [δH 6.65, s in 4a (0.95); δH 6.53, s in 4b (0.80)] of each isomer. Exhustive efforts to separate this mixture employing column chromatography and HPLC using various stationary and mobile phases were unsuccessful. Therefore, the structure elucidation of 4 was performed on the mixture. The IR spectrum of 4, along with the 1H, 13C, and HMQC NMR spectroscopic data as discussed below, indicated that 4a and 4b are also phennaphthalen-type lignans, possessing a γ-lactone group.

The NMR data for the major and minor isomers were considered separately for structure elucidation purpose. The NMR data of the major isomer (4a) were similar to those of detetrahydroconidendrin [20], except for the appearance of an oxyethyl and an acetal group in 4a instead of an oxymethylene in detetrahydroconidendrin, which led to the assumption that 4 was 3α-ethoxy derivative of detetrahydroconidendrin. This was confirmed by the HMBC correlations from H-3α (δH 6.65, s) to C-2α (δC 168.9), C-2″ (δC 64.4) and from H-2″ (δH 3.57, m) to C-3α (δC 102.4). The β-configuration for the ethoxyl group at C-3α of the γ-lactone in 4a was deduced from the lower field resonance of H-3α (δH 6.65) than that of 4b (δH 6.53) [21,22].

Compound 4a was therefore determined as 3β-ethoxy-6-hydroxy-4-(4-hydroxy-3-methoxyphenyl)-7-methoxynaphtho[2,3-c]furan-1(3H)-one. The 1H and 13C NMR spectra of 4b together with DEPT and 1H-13C COSY analysis indicated the
presence of general structural features in common with 4a of the same molecular formula. The only difference was the signal corresponding to the acetal proton H-3\(\alpha\), of \(\beta\)-orientation, in the higher field than that of 4a. Thus, compound 4b was concluded to be 3α-ethoxy-6-hydroxy-4-(4-hydroxy-3-methoxyphenyl)-7-methoxynaphtho[2,3-c]furan-1(3H)-one.

### 3.2. Pharmacology

All compounds were evaluated for their anti-inflammatory and anti-osteoporotic activities, employing LPS-stimulated RAW264.7 macrophages and osteoblast-like UMR106 cells, respectively. Compound 1 was among the most potent NO production inhibitor, with IC\(_{50}\) value of 4.17 \(\mu\)g/mL, comparable to that of indomethacin (8.52 \(\mu\)g/mL), while compounds 2–4 exhibited moderate activity with IC\(_{50}\) values ranging from 20 to 60 \(\mu\)g/mL (Table 3). Further investigation revealed that compound 1 selectively inhibited the protein expression of iNOS in LPS-stimulated RAW264.7 macrophages at 10 \(\mu\)g/mL (Fig. 4), which was responsible for its ability of NO suppression.

In order to detect whether these compounds can also interfere the bone erosion process in rheumatoid arthritis (RA), the anti-osteoporotic capacity of the isolated compounds was examined using osteoblast-like UMR106 and osteoclastic cells. As a result, only compound 1 exhibited potent stimulatory effects on the proliferation (Fig. 5) and ALP activity (Fig. 6) of UMR106 cells, which also significantly up-regulated the OPG/RANKL protein ratio at 10\(^{-7}\) M (Fig. 7). In addition, only compound 3 showed inhibitory effects on the activity of osteoclastic TRAP (Fig. 8), at doses of 10\(^{-6}\) and 10\(^{-7}\) M, with inhibition of 11.4 and 8.0\%, respectively.

These results may, in part, explain the anti-RA potential of *V. negundo* seeds that is based on the anti-inflammatory and anti-osteoporotic activities of these isolated lignans. Thus, these constituents, especially, compound 1 might act as effective agent of suppressing inflammation and attenuating bone destruction.

In conclusion, our results indicated that vitexdoin F (1) can attenuate inflammation by down-regulating iNOS protein level and NO production and modulate the bone formation and resorption process through increasing osteoblast proliferation and production of alkaline phosphatase as well as increasing OPG/RANKL protein ratio, which exhibited potential anti-inflammatory and anti-osteoporotic activities *in vitro* and may be considered as a lead compound for promising anti-rheumatism agent.

### Acknowledgments

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at [http://dx.doi.org/10.1016/j.fitote.2013.12.006](http://dx.doi.org/10.1016/j.fitote.2013.12.006).

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**Fig. 7.** Effect of vitexdoin F (1) at 10\(^{-7}\) M on the RANKL and OPG protein expressions of UMR106 cells by immunoblot analysis. Values are mean ± S.D. of three experiments with triplicate of each experiment. *p < 0.05 indicates statistically significant differences from control group.

**Fig. 8.** Inhibitory effect of isolated lignans on the osteoclastic TRAP (trate-resistant acid phosphatase) activity. Each column represents the mean value ± S.D. (n = 6); *p < 0.05, **p < 0.01 compared with control.
References


