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Original Paper

Phytochemical investigation of the active constituents from *Caesalpinia sappan* on stimulation of osteoblastic cells

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Abstract Heartwood of *Caesalpinia sappan* L. has been traditionally used to many diseases such as homoptysis, syphilis, eye disease, dysentery, depurative and prevention of osteoporosis. Our previous in vitro screening of Indonesian plants revealed that an ethanolic extract of the heartwood of *C. sappan* exhibits a proliferation stimulating activity against primary osteoblastic cells. In our continued interest to this plant, we further fractionated the extract and isolated active constituents on the basis of the stimulating activity in the osteoblastic cells. The fractionation and isolation were carried out with various chromatography methods and the structure of isolated compounds was elucidated based on NMR, IR, UV and MS spectroscopic data. From an active fraction, a new biphenyl dimer, namely caesappanin C (**1**), along with two known compounds, protosappanin A (**2**) and sappanchalcone (**3**), were isolated. Among them, the new compound **1** exhibited the strongest activity and significantly increased the cell viability up to 276±5%. The other two compounds **2** and **3** also stimulated the cell proliferation and increased the cell viability up to 233±8% and 187±4%, respectively.

Key words: *Caesalpinia sappan*, caesalpiniaceae, biphenyl dimer, osteoblast.

Caesalpinia sappan L. is a medicinal and dye yielding plant that belongs to Caesalpiniaceae family. This plant is widely distributed in Southeast Asia including Indonesia. Its heartwood, also known as Sappan Lignum, has been used as a traditional medicine for a long time to many diseases such as homoptysis, syphilis, eye disease, dysentery, depurative and prevention of osteoporosis (PT Eisai 1995). Previous phytochemical studies on the heartwood of *C. sappan* indicated the presence of homoisoflavanoids, triterpenoids, steroids and other phenolic compounds such as brazilin and brazilein (Badami et al. 2004; Namikoshi et al. 1987). Among the reported compounds, the major compound, brazilin, in the heartwood of *C. sappan* has been utilized as a dye. This compound also reported to show a pharmacological effect as hypoglycemic and antiproliferative agent for treatment of vascular diseases (Guo et al. 2013). Various biological activities, such as antibacterial (Xu and Lee 2004), anti-hepatotoxic (You et al. 2005), antioxidative (Badami et al. 2003) and anticonvulsive (Baek et al. 2000) effects have also been reported in the extracts of the heartwood of *C. sappan*.

Osteoporosis is the most frequent bone-remodeling disease that enhances bone fragility and increases the risk of fracture by both the loss of bone mass and the micro-architectural deterioration of the skeleton (Baylink et al. 1999). Patients who lost a substantial amount of bone are thus necessary to increase bone mass by stimulating new bone formation. For the formation of the bone, osteoblast plays a crucial role in creating the new bone and maintaining the bone structure. Osteoblast covers the resorption area and begins the process of new bone formation by secreting osteoid. This osteoid and the adjacent bone cells are eventually mineralized and developed into the new bone tissue (Manolagas 2000). The hormone preparation such as calcitonin and estrogen preparations is one of the drugs that have been used for osteoporosis treatment. However, recent studies have begun to reveal that the long-term use of calcitonin-containing medicines increases the risk of cancer (European Medicines Agency 2012). Therefore, non-hormonal or alternative therapies are more acceptable for

Abbreviations: HR-ESI-MS, high-resolution electron impact mass spectrometry; MPLC, medium pressure liquid chromatography; α-MEM, alpha-modified minimal essential medium; PBS, phosphate-buffered saline; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide; COSY, ^{1}H–^{1}H correlation spectroscopy; HMQC, the heteronuclear single quantum coherence; HMBC, the heteronuclear multiple bond correlation.

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preventing osteoporosis than the hormonal replacement therapy. Natural products such as volatile compounds, resveratrol, daidzein and glabridin from licorice root have been reported to increase the function of the osteoblastic MC3T3-E1 cell (Choi 2005; Mizutani et al. 1998; Sugimoto and Yamaguchi 2000; Wu et al. 2012).

Primary osteoblast cultures reflecting more phenotypic properties of normal osteoblast than osteoblastic cell lines can be used as an experimental tool for investigating the osteoblastic function in vitro (Ho et al. 1999). Interestingly, in our previous investigation, the ethanolic extract of heartwood of Indonesian *C. sappan* has exhibited the activity against primary osteoblastic cells in vitro (Subehan et al. 2013). To the best of our knowledge, this is the first demonstration of the in vitro osteoblast activity by the extract from *C. sappan*. In our continuous interest to this plant, this study is thus performed and reports the isolation and structural elucidation of three constituents including a new compound from this plant, as well as their in vitro effect that stimulates the proliferation of osteoblastic cells prepared from the neonatal mouse calvaria of male mice.

Materials and methods

General experimental

The optical rotations were measured using a JASCO DIP-140 digital polarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan). The IR spectra were measured using a Shimadzu IR-408 spectrophotometer (Shimadzu Co., Kyoto, Japan) in KBr pellet (Jasco, Tokyo, Japan). High-resolution electron impact mass spectrometry (HR-ESI-MS) measurement was carried out using a FT-MS-ESI LTQ Orbitrap XL (Thermo Fisher Scientific, Waltham, MA, USA). The ^{1}H, ^{13}C, and 2D NMR spectra were recorded using a JEOL JNM-LA400 spectrometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan) with tetramethylsilane (Wako, Osaka, Japan) as an internal standard. The column chromatography was performed using silica gel 60 (Nacalai Tesque, Inc., Kyoto, Japan). Analytical and preparative TLCs (Merk, Darmstadt, Germany) was conducted using precoated Merck Kieselgel $60F_{254}$ and RP-$18F_{254}$ plates (0.25- or 0.50 mm thick).

Chemicals and biochemicals

Alpha-Modified minimal essential medium (α-MEM), phosphate-buffered saline (PBS) and fetal bovine serum (FBS) was purchased from Gibco BRL Products (Gaithersburg, MD, USA). Penicillin G potassium salt, streptomycine sulphate, 17β-estradiol and 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). A WST-1 cell counting kit was purchased from Dojindo (Kumamoto, Japan). Cell culture flasks and 96-well plates were from Corning, Inc. (Corning, NY, USA).

Plant material

C. sappan was collected from the rain forest in South Sulawesi Province, Indonesia and was authenticated by Ms. Sri Suhadiyah, Yayasan Keragaman Hayati Sulawesi, Indonesia. This plant was selected based on its ethnopharmacological use as a treatment for osteoporosis. A voucher sample (SL-11-002) is preserved at the Biofarmaca Research Center of Faculty of Pharmacy, Hasanuddin University, Makassar, Indonesia.

Extraction and isolation

The stems of *C. sappan* were greater than 8 cm in diameter. The heartwood was then separated, cut into small pieces, and dried in the room at room temperature. The dried heartwood (100 g) was extracted three times by sonication with 500 ml of 70% ethanol for 3 h. All the extracts were combined and lyophilized to yield the ethanolic extract. The extract (5 g) was subjected to a medium pressure liquid chromatography (MPLC) of normal-phase silica gel (4.5 cm×30 cm) with gradient system at a flow rate of 100 ml min^{-1}. Gradient elution was performed with *n*-hexane–EtOAc (0–15 min, linear gradient from 0 to 30% EtOAc; 15–23 min, linear gradient from 30 to 40% EtOAc; 23–32 min, linear gradient from 40 to 55% EtOAc; 32–44 min, linear gradient from 55 to 75% EtOAc; 44–54 min, linear gradient from 75 to 90% EtOAc; 54–64 min, linear gradient from 90 to 100% EtOAc; 64–70 min, 100% MeOH) to give six fractions (Fr.1: 0–30% EtOAc, 1200 mg, Fr.2: 30–40% EtOAc, 570 mg, Fr.3: 40–55% EtOAc, 710 mg, Fr.4: 55–75% EtOAc, 750 mg, Fr.5: 75–90% EtOAc 630 mg, and Fr.6: 90–100% EtOAc 670 mg). Each fraction was tested for their in vitro activity against the osteoblastic cells. The active fraction (Fr.4, 500 mg) was rechromatographed by a MPLC of normal-phase silica gel (4.0 cm×15 cm) with a $CHCl_3$–MeOH gradient system (0–40 min, linear gradient from 0–100% at a flow rate of 25 ml min^{-1}) and three subfractions (Fr.4.1: 0–5% MeOH, 52 mg, Fr.4.2: 5–80% MeOH, 320 mg, and Fr. 4.3: 70–100% MeOH, 70 mg) were obtained. Fr. 4.2 was then purified with normal- and reversed-phase preparative TLCs (*n*-hexane–EtOAc, 8:2 and CH_3CN–MeOH–H_2O, 4:4:2, respectively) to give a new biphenyl dimer, namely caesappanin C (**1**, 8.0 mg), along with two known compounds, protosappanin A (**2**, 12.2 mg) and sappanchalchone (**3**, 15.6 mg).

Caesappanin C (**1**). Yellow powder; $[\alpha]_D^{24}$ 0° (*c* 0.05, CH_3OH); IR (KBr) ν_{max} 3350, 1610, 1500 cm^{-1}; HR-ESI-MS *m/z*: 631.1783 $[M+Na]^+$ (Calcd. for $C_{23}H_{22}O_9Na$: 631.1791). ^{1}H NMR (CD_3OD, 400 MHz) δ_H 6.97 (2H, d, *J*=8.8 Hz, H-12, 12′), 6.73 (1H, s, H-6), 6.72 (1H, s, H-3), 6.71 (1H, s, H-6′), 6.66 (1H, s, H-3′), 6.56 (1H, dd, *J*=8.8, 2.2 Hz, H-11′), 6.53 (1H, dd, *J*=8.8, 2.2 Hz, H-11), 6.50 (1H, d, *J*=2.2 Hz, H-9′), 6.44 (1H, dd, *J*=2.2 Hz, H-9), 4.38 (1H, d, *J*=12 Hz, H-15′), 4.14 (1H, d, *J*=12 Hz, H-15), 3.85 (1H, d, *J*=12 Hz, H-15), 3.56 (3H, m, H-15′, 16, 16′), 3.46 (1H, d, *J*=12 Hz, H-16′), 3.39 (1H, d, *J*=12 Hz, H-16), 2.68 (2H, s, H-13′), 2.57 (1H, d, *J*=13.6 Hz, H-13), 2.49 (1H, d, *J*=13.6 Hz, H-13). ^{13}C NMR (CD_3OD, 100 MHz) δ_C 159.3 (C-8), 158.1 (C-8′), 157.9 (C-10′), 157.8 (C-10), 143.7 (C-4, 4′, 5′), 143.6 (C-5), 132.0 (C-12), 131.5 (C-12′), 131.3 (C-1), 130.8 (C-1′), 126.9 (C-2′), 126.2 (C-2), 124.0 (C-7′), 122.8 (C-7), 118.7 (C-3), 117.8 (C-3′), 116.6 (C-6′), 116.3 (C-6), 110.8 (C-11′), 110.2 (C-11), 107.6 (C-9′), 106.9 (C-9), 75.6 (C-15′), 75.3 (C-15), 72.0 (C-14′), 71.7 (C-14), 67.2 (C-16), 64.6 (C-16′), 41.5 (C-13′), 38.7 (C-13).

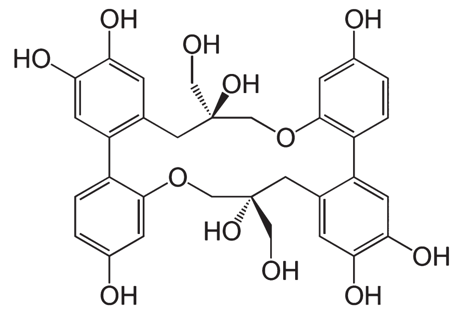
Assay for stimulation of osteoblastic cell proliferation

Mouse primary osteoblasts were isolated from neonatal mouse calvaria of male mice (2–3 d old) using the reported method (Takahashi et al. 1998). Briefly, the isolated osteoblasts were suspended in α-MEM, and 8000 cells well^{-1} were plated in 96-well plates in a total volume of 198 μl. The cells were preincubated in α-MEM containing 10% FBS for 24 h at 37°C under a humidified atmosphere of 5% CO_2 to allow the attachment, and then subsequently incubated in α-MEM without FBS. After 24 h, the cells were exposed to the test compounds at a final concentration of 100 μM for 48 h. MTT was then added to each well, and were incubated for 4 h. The formation of formazan was measured at 590 nm in a plate reader. The samples were dissolved in 5% DMSO and diluted with the medium. The proliferation was calculated based on the mean of three wells. The cell viability without any treatment was set as 100%.

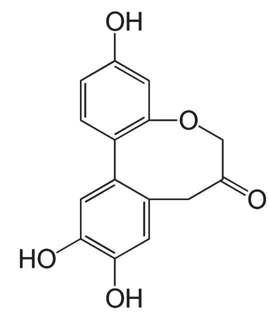
Results and discussion

In our previous in vitro screening for the osteoblastic activity, the extract of heartwood of *C. sappan* has exhibited the stimulating activity on proliferation of osteoblastic cells by 164±5% in a concentration of 100 μg ml^{-1} (Subehan et al. 2013). The active extract was thus fractionated with the silica gel in the gradient system to give 6 fractions. Their stimulation activity revealed that the fraction 4 exhibits the strongest activity in the osteoblastic cells by 200±2% at a concentration of 100 μg ml^{-1}. Finally, further purification of chemical constituents in this fraction afforded three compounds, a new biphenyl dimer, caesappanin C, (**1**) and two known compounds, protosappanin A (**2**) (Nagai et al. 1986; Fu et al. 2008) and sappanchalcone (**3**) (Namikoshi et al. 1987) (Figure 1). Spectral data of the known compounds has been confirmed with the reported data. The purity of each compound was determined by TLC and NMR, which showed purities greater than 95%.

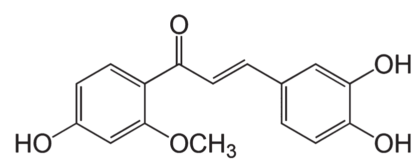
New compound caesappanin C (**1**) was obtained as yellow powder, having $[\alpha]_D^{24}$ 0° (c=0.05, CH_3OH). The IR spectrum showed absorption bands corresponding to hydroxyl group (3350 cm^{-1}) and aromatic ring (1610 and 1500 cm^{-1}). The ^{1}H NMR spectrum showed the presence of ten aromatic protons, four oxygenated methylene protons, and two methylene protons. The ^{13}C NMR spectrum showed 32 carbon signals with eight oxygenated aromatic carbon, sixteen aromatic carbons, four oxygenated methylene carbons, two methylene



Caesappanin C (**1**)



Protosappanin A (**2**)



Sappanchalcone (**3**)

Figure 1. Structures of compounds **1**–**3**.

carbons, and two oxygenated quatenary carbons. Double pair peaks pattern was observed in the ^{13}C NMR spectrum. Its molecular formula was determined to be of $C_{32}H_{32}O_{12}$ using HR-ESI-MS from its positive HR-ESI-MS m/z: 631.1783 $[M+Na]^+$ (Calcd. for $C_{23}H_{22}O_9Na$: 631.1791). MS/MS (positive) displayed the high intensity at m/z 327 [$(M/2+Na)^+$, 63%] of the parent ion peak m/z 631 $(M+Na)^+$. These spectral features suggested the possibility of symmetrical nature of **1**.

The ^{1}H NMR and ^{1}H ^{1}H correlation spectroscopy (COSY) spectra showed the presence of two ABX-type coupling systems at δ_H 6.97 (d, J=8.8 Hz), 6.53 (dd, J=8.8, 2.2 Hz), and 6.44 (d, J=2.2 Hz) and at δ_H 6.97 (d, J=8.8 Hz), 6.56 (dd, J=8.8, 2.2 Hz), and 6.50 (d, J=2.2 Hz). Furthermore, the heteronuclear single quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) connectivity of the proton and carbon signals between δ_H 6.97, 6.53, and 6.44 and δ_C 159.3, 157.8, 132.0, 122.8, 110.2, and 106.9 and between δ_H 6.97, 6.56, and 6.50 and δ_C 158.1, 157.9, 131.5, 124.0, 110.8, and 107.6, respectively, suggested the presence of two tri-substituted aromatic moieties (rings

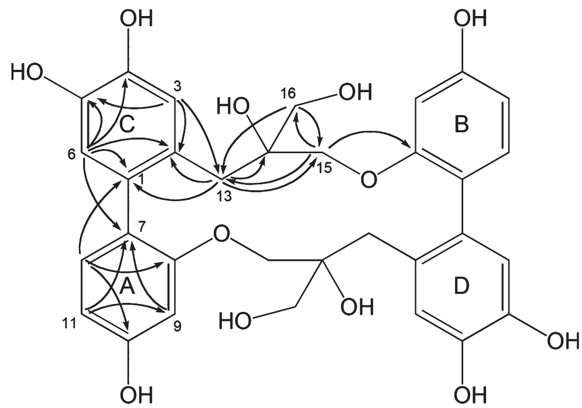


Figure 2. The selected key HMBC correlations of **1**.

A and B) (Figure 2). On the other hand, the two aromatic protons at δ_H 6.73 (s) and 6.72 (s) showed correlations with δ_C 143.7, 143.6, 131.3, 126.2, 118.7, and 116.3. The remaining two aromatic protons at δ_H 6.71 and 6.66 also connected with the carbon signals at δ_C 143.7 (2C), 130.8, 126.9, 117.8, and 116.6 indicated the presence of two 1,2,4,5-subtituted aromatic moieties (rings C and D) in addition to the two tri-substituted aromatic moieties in the structure. Moreover, the HMBC long range correlations of δ_H 6.97 on the ring A to δ_C 131.3 on the ring C and δ_H 6.73 on the ring C to δ_C 122.8 on the ring A indicated that these two aromatic moieties directly link each other. The same case was also observed at δ_H 6.97 on the ring B to δ_C 130.8 on the ring D and δ_H 6.71 on the ring D to δ_C 126.9 on the ring B, suggesting that **1** has two biphenyl skeletons.

Further analyses of the HMQC and HMBC spectra revealed that the methylene protons at δ_H 2.49 (d, J=13.6 Hz) and 2.57 (d, J=13.6 Hz) attach to the methylene carbon at δ_C 38.7 with germinal coupling, and connect to the aromatic carbons at δ_C 131.3, 126.2, and 118.7 on the ring C. They also correlated with the oxygenated quaternary carbon at δ_C 71.7 and the oxygenated methylene carbon at δ_C 75.3. The methylene protons at δ_H 3.56 (d, J=12.0 Hz) and 3.39 (d, J=12.0 Hz) showed an attachment to the oxygenated carbon at δ_C 67.2. These two protons also correlated with the methylene carbon at δ_C 38.7 and the oxygenated methylene carbon at δ_C 75.3. On the other hand, the methylene protons at δ_H 3.85 (d, J=12.0 Hz) and 4.14 (d, J=12.0 Hz) showed an attachment to the oxygenated carbon at δ_C 75.3 and displayed connectivity with the aromatic carbon at δ_C 158.1 on the ring B, together with correlations between oxygenated carbon at δ_C 67.2 and methylene carbon at δ_C 38.7. These observations suggested that **1** contains a $-CH_2-C(OH)(CH_2OH)-CH_2-O-$ partial structure linked to the rings B and C. A very similar connectivity including the rings A and D was observed in the remaining proton and carbon signals. This supported the presence of other $-CH_2-C(OH)(CH_2OH)-CH_2-O-$ partial structure linked to the rings A and D.

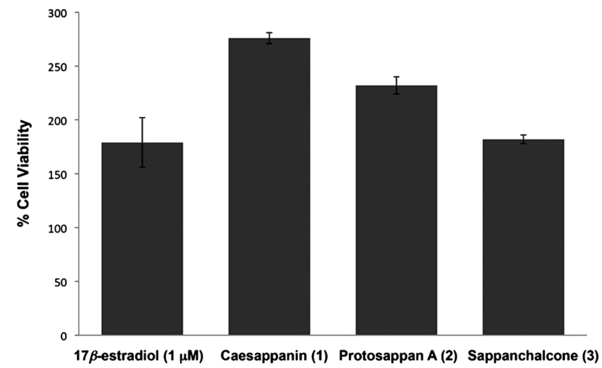


Figure 3. Effect of the isolated compounds on proliferation of osteoblastic cells in vitro. The osteoblastic cells were prepared from the neonatal mouse calvaria of male mice (2–3 d old). The cells were seeded in 96-well plates and treated for 48 h with 100 μM of the indicated compounds, except for 17β-estradiol. The cell viability was measured using the MTT assay. Percentage of the cell viability control (without treatment) was set as 100% cell viability. Data are presented as the mean±SD (n=3).

However, the chemical shifts of the proton and carbon signals neighboring the chiral center showed significant differences between two partial structures. In case of the positions of C13 and C13′, the methylene proton signals at δ_H 2.49 and 2.57 with germinal coupling were observed at the position of C13, while only one methylene protons signal (δ_H 2.68) was shown at the position of C13′. The methylene proton signals of δ_H 3.85 and 4.14 at the position of C15 were shown by δ_H 3.56 and 4.38 at the position of C15′. Furthermore, the methylene proton signals at the position of C16 were δ_H 3.39 and 3.56, while at the position of C16′ were observed by the methylene proton signals of δ_H 3.46 and 3.56. The carbon signals of δ_C 38.7 and 67.2 at the positions of C13 and C16 were also shifted to δ_C 41.5 and 64.6 at the positions of C13′ and C16′ in the other partial structure, respectively. In contrast, although biphenyl dimer caesappanin B has been isolated from *C. sappan*, no any differences are observed in chemical shifts of respective dimers of this compound, which has the same stereochemistry at its chiral centers (Shu et al. 2011). These findings suggest that **1** might represent different stereochemistry at the each chiral center. On the basis of these data, we concluded the structure of **1** to be a novel biphenyl dimer, and named as caesappanin C, according to the structure of caesappanin B.

All the isolated compounds were tested for their stimulation activity on proliferation of primary osteoblastic cells isolated from the fetal calvaria bone at the concentration of 100 μM (Figure 3). The cell viability was measured using the MTT assay. The stimulation activity of 17β-estradiol as a positive control (1 μM) was 179±23%. All the isolated compounds showed the stimulation effect. Among them, **1** exhibited the strongest activity of 276±5% and significantly increased the proliferation of the osteoblastic cells. **2** also showed the significant activity in the cells by 233±4%. On the other hand, **3** exhibited the proliferation stimulating activity in the osteoblastic cells by 187±5%. It has been reported that natural products such as daidzein, glabridine and resveratrol exhibit the stimulation effect on osteoblastic cell at concentration less than 10 μM (Choi 2005; Mizutani et al. 1998; Sugimoto and Yamaguchi 2000; Wu et al. 2012). The isolated compounds **1**–**3** thus showed moderate osteoblastic in vitro proliferation stimulating activity.

Conclusion

In this research, we isolated three compounds, caesappanin C (**1**), protosappanin A (**2**), and sappanchalcone (**3**) from the ethanolic extract of the heartwood of Indonesian *C. sappan* that showed the proliferation stimulating activity against the primary osteoblastic cells in vitro. All the isolated compounds exhibited the moderate activities, in which the new compound **1** showed the strongest in vitro proliferation stimulating activity. These observations suggest that *C. sappan* and the isolated compounds may have the potential to stimulate bone formation and regeneration.

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References

Badami S, Moorkoth S, Rai SR, Kannan E, Bhojraj S (2003) Anti oxidant activity of *Caesalpinia sappan* heartwood. *Biol Pharm Bull* 26: 1534–1537

Badami S, Moorkoth S, Suresh B (2004) *Caesalpinia sappan* A medicinal and dye yielding plant. *Nat Prod Radiance* 3: 75–82

Baek NI, Jeon SG, Ahn EM, Hahn JT, Bahn JH, Jang JS, Cho SW, Park JK, Choi SY (2000) Anticonvulsant compounds from the wood of *Caesalpinia sappan* L. *Arch Pharm Res* 23: 344–348

Baylink DJ, Strong DD, Mohan S (1999) The diagnosis and treatment of osteoporosis: future prospects. *Mol Med Today* 5: 133–140

Choi EM (2005) The licorice root derived isoflavan glabridin increases the function of osteoblastic MC3T3-E1 cells. *Biochem Pharmacol* 70: 363–368

European Medicines Agency (EMA) (2012) European medicines agency recommends limiting long-term use of calcitonin medicines. Press release 20 July 2012. http://www.ema.europa.eu/docs/en_GB/document_library/Press_release/2012/07/WC500130122.pdf

Fu LC, Huang XA, Lai ZY, Hu YJ, Liu HJ, Cai XL (2008) A new 3-benzylchroman derivative from Sappan Lignum (*Caesalpinia sappan*). *Molecules* 13: 1923–1930

Guo J, Li L, Wu YJ, Yan Y, Xu XN, Wang SB, Yuan TY, Fang LH, Du GH (2013) Inhibitory effect of Brazilin on the vascular smooth muscle cell proliferation and migration induced by PDGF-BB. *Am J Chin Med* 41: 1283–1296

Ho ML, Chang JK, Chuang LY, Hsu HK, Wang GJ (1999) Characteristics of primary osteoblast culture derived from rat fetal calvaria. *Kaohsiung J Med Sci* 15: 248–255

Manolagas SC (2000) Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocr Rev* 21: 115–137

Mizutani K, Ikeda K, Kawai Y, Yamori Y (1998) Resveratrol stimulates the proliferation and differentiation of osteoblastic MC3T3-E1 cellls. *Biochem Biophys Res Commun* 1253: 859–863

Nagai M, Nagumo S, Lee SM, Eguchi I, Kawai K (1986) Protosappanin A, a novel biphenyl compound from Sappan Lignum. *Chem Pharm Bull* 34: 1–6

Namikoshi M, Nakata H, Nuno M, Ozawa T, Saitoh T (1987) Homoisoflavonoids and related compounds. III. Phenolic constituents of *Caesalpinia japonica* SIEB. Et ZUCC. *Chem Pharm Bull* 35: 3568–3575

PT Eisai Indonesia (1995) In Medicinal Herb Index in Indonesia, Jakarta, p. 106

Shu S, Deng A, Li Z, Qin H (2011) Two novel biphenyl dimers from the heartwood of *Caesalpinia sappan. Fitoterapia* 82: 762–766

Subehan S, Rifai Y, Mufidah (2013) The characterization and anti-osteoporotic activity of Sappan Lignum (*Caesalpinia sappan* L.) extracts. *International Journal of Phytomedicine* 5: 7–13

Sugimoto E, Yamaguchi M (2000) Stimulatory effect of Daidzein in osteoblastic MC3T3-E1 cells. *Biochem Pharmacol* 59: 471–475

Takahashi N, Akatsu T, Udagawa N, Sasaki T, Yamaguchi A, Moseley JM, Martin TJ, Suda T (1998) Osteoblastic cells are involved in osteoclast formation. *Endocrinology* 123: 2600–2602

Wu YB, Wu JG, Yi J, Chen TQ, Lin XH, Wu JZ (2012) Osteoblastic activity of ethanolic extract and volatile compounds from Er-Zhi-Wan, a famous traditional Chinese herbal formula. *Afr J Pharm Pharmacol* 6: 1733–1740

Xu HX, Lee SF (2004) The antibacterial principle of *Caesalpinia sappan. Phytother Res* 18: 647–651

You EJ, Khil LY, Kwak WJ, Won HS, Chae SH, Lee BH, Moon CK (2005) Effect of brazilin on the production of fructose-2,6-bisphosphate in rat hepatocytes. *J Ethnopharmacol* 102: 53–57