

Original Research Article

The characterization and anti-osteoporotic activity of Sappan Lignum (*Caesalpinia sappan* L.) extracts

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Abstract

The standardization and characterization of medicinal plants is important for establishing the scientific basis for the therapeutic effects of a traditional medicine. Sappan Lignum (*Caesalpinia sappan* L.) is a plant that is commonly used as a traditional medicine in Southeast Asia. In Indonesia, it is used to treat dysentery, hemoptysis, and ophthalmic diseases and as a depurative; the Buginese tribe add it to daily drinking water to prevent and treat osteoporosis. This study aimed to characterize the *Caesalpinia sappan* heartwood extracts and to evaluate the anti-osteoporosis activity of the characterized extracts to provide evidence for the efficacy of this traditional medicinal plant. The characterization was performed by chromatographic and spectrophotometric methods after treating the Sappan Lignum with n-hexane, methanol and 70% ethanol. Numerous high intensity peaks were detected on the spectrum from the methanol extract, indicating that the secondary metabolites of this plant were more soluble in methanol than in ethanol and n-hexane. The anti-osteoporosis activity was determined by evaluating the ability of the extracts to stimulate the proliferation of osteoblasts that were isolated from neonatal mouse calvaria and to inhibit the formation of osteoclast-like cells, which are responsible for bone absorption and resorption. The data revealed that all the extracts increased the proliferation of osteoblasts and inhibited the formation of osteoclast-like cells. In the osteoblasts, the ethanol extract was 64% more active than the untreated control and the other extracts at 100 µg/ml (0.01%). Compared with calcitonin, which was the positive control (2 U/ml), the extracts weakly inhibited the formation of osteoclast-like cells. Among the extracts, the ethanol extract most robustly stimulated the osteoblasts and inhibited the formation of osteoclast-like cells, suggesting that it has potential as a candidate anti-osteoporosis agent.

Keywords: *Caesalpinia sappan* L., Sappan Lignum, characterization, anti-osteoporosis

Introduction

Caesalpinia crista L., also known as Sappan Lignum, belongs to the Fabaceae family, grows primarily in hilly areas with clay soil and calcareous rock at low and medium altitudes, and does not tolerate excessively wet soil conditions [1]. The major constituent of the heartwood of this plant is brazilin, but the plant also contains protosappanins, chalcones, and other flavonoids [2,3]. It has been reported to have various biological activities, such as anti-inflammatory [4] antibacterial [5], anti-hepatotoxic [6], antioxidative [7], and anticonvulsive [8] effects. To the best of our knowledge, the extracts of this plant have not been evaluated for anti-osteoporotic activity.

Osteoporosis is the most frequent bone-remodeling disease and leads to both the loss of bone mass and the micro-architectural deterioration of the skeleton, resulting in enhanced bone fragility and an increased risk of fracture. Bone remodeling is the

physiological process by which old or damaged bone is removed and subsequently replaced with new bone. Osteoblasts create the new bone and osteoclasts remove the bone (resorption), but these two events are not independently regulated. In the remodeling of mature healthy bones, the balance between bone resorption and bone formation is tightly regulated to ensure that there are no major net changes in bone mass or mechanical strength after each remodeling cycle. This balance is maintained by coupling bone formation to bone resorption and involves numerous coordinated signaling mechanisms. The currently available treatments for osteoporosis, e.g., estrogen replacement therapy, are based on inhibiting bone resorption to prevent further bone loss. In patients who have already lost a substantial amount of bone, a treatment that increases bone mass by stimulating new bone formation is necessary. Recently, the use of calcitonin as an osteoporosis treatment received a warning from the European Medicines Agency in the European Union because the long-term injection or



infusion of calcitonin-containing medicines increases the risk for cancer. Therefore, non-hormonal or alternative therapies are more acceptable for preventing osteoporosis than hormonal replacement therapy, which has an increased risk of side effects.

The use of traditional medicines derived from plants has become more acceptable in modern medicine. Approximately one-third of adults in the western world use alternative therapies, including herbs [9]. In contrast to chemical drugs, herbs have sometimes been considered to be non-toxic because of their natural origin and long-term use in folk medicine. However, problems arise because of intrinsic toxicity, adulteration, substitution, contamination, misidentification, drug-herb interactions, and a lack of standardization. The standardization of plant material and extracts is important to ensure that qualified material is used to treat certain diseases.

The constituents of a plant extract can be characterized using spectrophotometric methods. Based on the absorption profile, each secondary metabolite that appears in the light-specific spectrum of an extract can be identified. Therefore, characterizing the plant extract is necessary before determining its activity and potential utility for treating certain diseases. In this study, we characterized the Sappan Lignum extracts and determined their potential as an anti-osteoporosis treatment.

Materials and Methods

Plant material

The heartwood of Sappan (*Caesalpinia 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.

Extraction

The plant material was obtained from stems that were greater than 8 cm in diameter. The heartwood was separated, cut into small pieces and dried in the room. The dried heartwood (100 g) was extracted three times by sonication with 500 ml n-hexane for 3 h. All the extracts were combined and lyophilized to yield the n-hexane extract. The same procedure was performed with methanol and 70% ethanol to prepare the methanol and ethanol extracts.

Characterization

The spectroscopic characterization was performed with a UV-Vis Spectrophotometer (Shimadzu), an FTIR Spectrophotometer (Bruker), a TLC Scanner (CAMAG), and an HPLC (Shimadzu) at various extract concentrations.

Anti-osteoporosis activity

The stimulation of osteoblast cell proliferation

Mouse primary osteoblasts were isolated from neonatal mouse calvaria and bone marrow cells were taken from the tibial marrow cavity of male mice (7 weeks old) using the method of Takahashi *et al.* [10]. Briefly, osteoblasts were suspended in -MEM, and 8000 cells/well were plated in 96-well plates in a total volume of 198 μ l. The cells were preincubated for 24 h at 37 C in a humidified atmosphere of 5% CO₂ in -MEM containing 10% FBS to allow for attachment; the cells were subsequently incubated in -MEM without FBS for 24 h. The cells were treated for 48 h with the test specimens at a final concentration of 0.1%, 0.05%, and 0.01% diluted in -MEM without FBS. MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well, and the plates 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.

Inhibiting the formation of osteoclast-like cells

Osteoblasts were plated at 104 cells/well in -MEM containing 10% FBS. The tibial marrow cavity of male mice (7 weeks old) was flushed with -MEM, and the isolated cells (105 cells/well) were cocultured with the osteoblasts in the presence of 1,25(OH)₂ vit-D₃ (10⁻⁷ M) and the test samples at various concentrations. Calcitonin (2 U/ml) served as a positive control. The medium was replaced with new medium containing the test sample and 1,25(OH)₂-vitamin D₃ every 2 days. All the cultures were maintained at 37 C in a humidified atmosphere with 5% CO₂. After 6 days of culture, the adherent cells were fixed with 10% formalin in phosphate-buffered saline (pH 7.2) for 10 minutes, dehydrated with ethanol-acetone (50:50, v/v) for 45 s, and strained for TRAP for 12 min at room temperature. The cells were observed under an inverted microscope, and those possessing three or more nuclei were counted as osteoclast-like multinucleated cells.

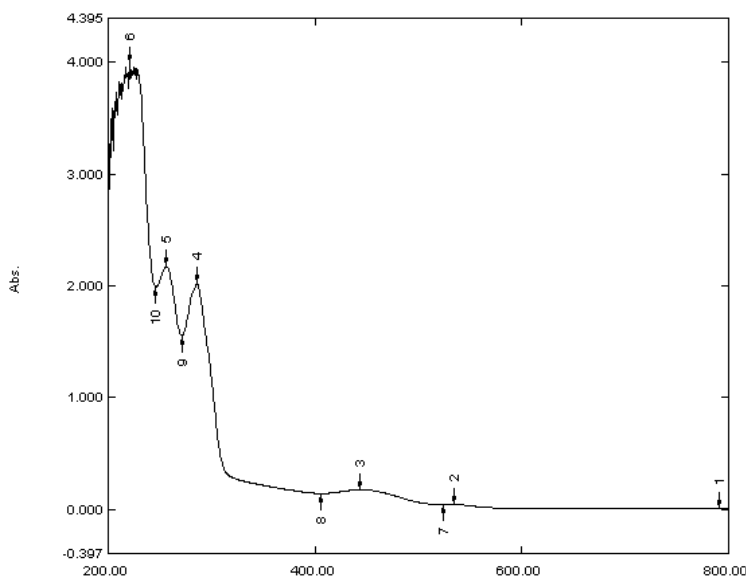
Result and discussion

The tradition of healing with medicinal plants has been passed on from generation to generation for hundreds of years. Empirical data or information provided by the older generation has guided the use of specific plants as remedies for particular symptoms or illnesses. In addition, the understanding of the utility of medicinal plants is combined with a traditional knowledge of healthcare. Sappan Lignum is a medicinal plant that is used to treat disease in numerous Southeast Asia countries. In Indonesia, it is believed to prevent illness when consumed daily in drinking water. We observed that the Buginese tribe utilized the heartwood of *Caesalpinia sappan* to treat osteoporosis. It has been reported that this plant is rich in flavonoids, and certain flavonoids that have been isolated from medicinal plants have anti-osteoporosis [11,12,13]. The extract of this plant is thought to have antioxidant activity [7], and antioxidants have been reported to promote osteoblast differentiation and to inhibit osteoclast activity [14]. Osteoporosis is prevalent worldwide and is considered to be a serious public health concern. Estrogen deficiency is the major

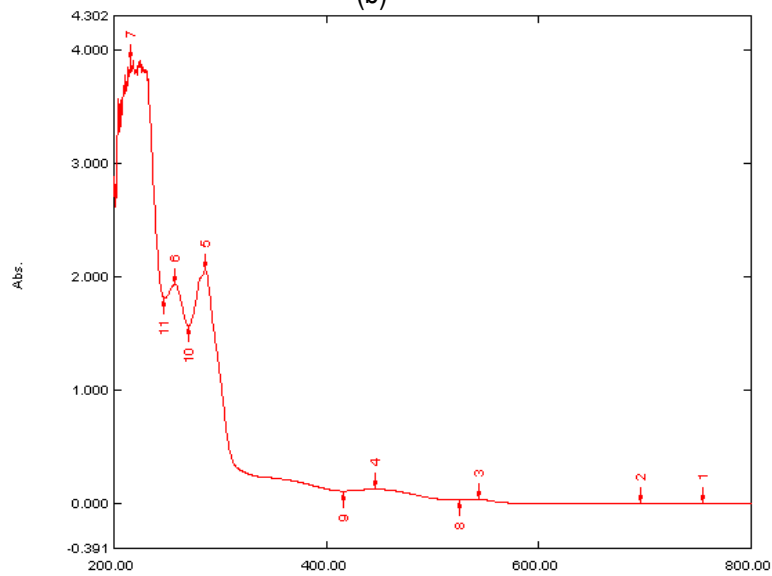
cause of postmenopausal osteoporosis. Because of the important role of estrogen in maintaining the homeostatic balance between osteoblasts and osteoclasts, estrogen replacement therapy has become the established regimen for preventing postmenopausal bone loss [15]. Osteoblasts are responsible for bone formation, and osteoclasts control bone resorption. An imbalance in these functions promotes disease. We characterized Sappan Lignum plant extracts and identified an anti-osteoporosis activity in these extracts. The heartwood of Sappan Lignum was extracted with n-hexane, methanol or 70% ethanol to produce three extracts with yields of 2.0%, 7.2% and 8%, respectively. In some cases, herbal extracts are more effective at treating a disease than a single isolated compound because synergism can occur between the constituents in the extract. The biologically active constituents in an extract are specified by the extraction solvent. Herbal medicine extracts have been reported to be inconsistent in terms of biological activity and therapeutic effects. This inconsistency may result from inadequate standardization and poor characterization of the evaluated extracts. For a particular plant, the same species or the same piece of the plant taken from different growing areas or at a different size, age and harvesting time will yield different amounts of bioactive constituents. This leads to variation in the biological activity. Therefore, in this study, we standardized the extraction methods and characterized the plant extract to provide a scientific rationale for the utility of the active extract. Future spectroscopic characterization will provide more data on the chemical identity of the constituents.

The UV-Vis spectrophotometry analysis demonstrated that the methanol and the 70% ethanol extracts had the same characteristic absorption at 221, 256, 286, 444, and 534 nm and that the hexane extract absorbed at 203, 254 and 445 nm (Fig. 1).

(a)



(b)



(c)

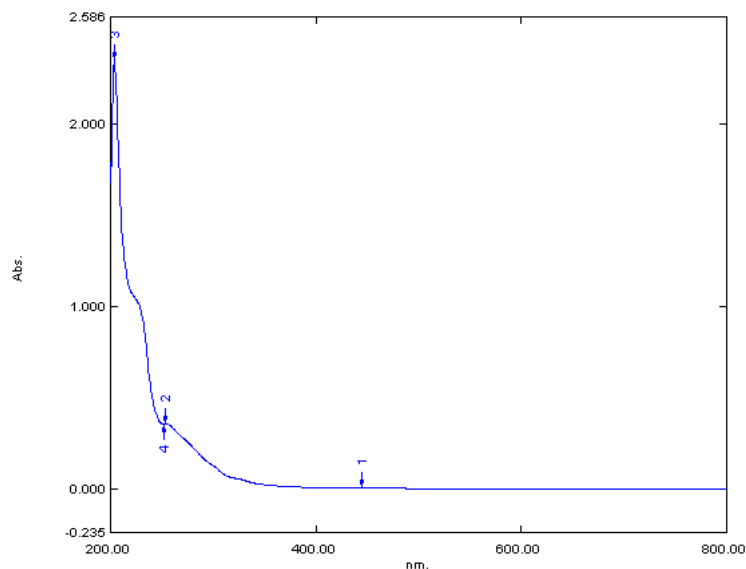


Figure 1. The UV-Vis spectra of the methanol (a), ethanol (b), and hexane (c) extracts (100 ppm) are presented.

Similar results were obtained with the TLC scanner at 245 nm: the methanol and ethanol extracts had similar absorption profiles, but the hexane extract had no absorption after running a F245 TLC plate with n-hexane:EtOAc (1:1) as the mobile phase (Fig. 2). A further comparison using FT-IR spectroscopy revealed different chromatographic profiles for each extract, and the methanol extract had more absorption bands, indicating the presence of additional constituents compared with the other extracts (Fig. 3). An HPLC chromatogram of the extracts indicated that the methanol and ethanol extracts were similar, with the exception of the presence of two additional peaks in the methanol extract at retention times of

6.99 and 11.29 minutes, and a unique profile was obtained from the n-hexane extract using an ODS column and eluting with

MeOH:H₂O (60:40) at a flow rate of 1 ml/min at 30 C (Fig. 4).

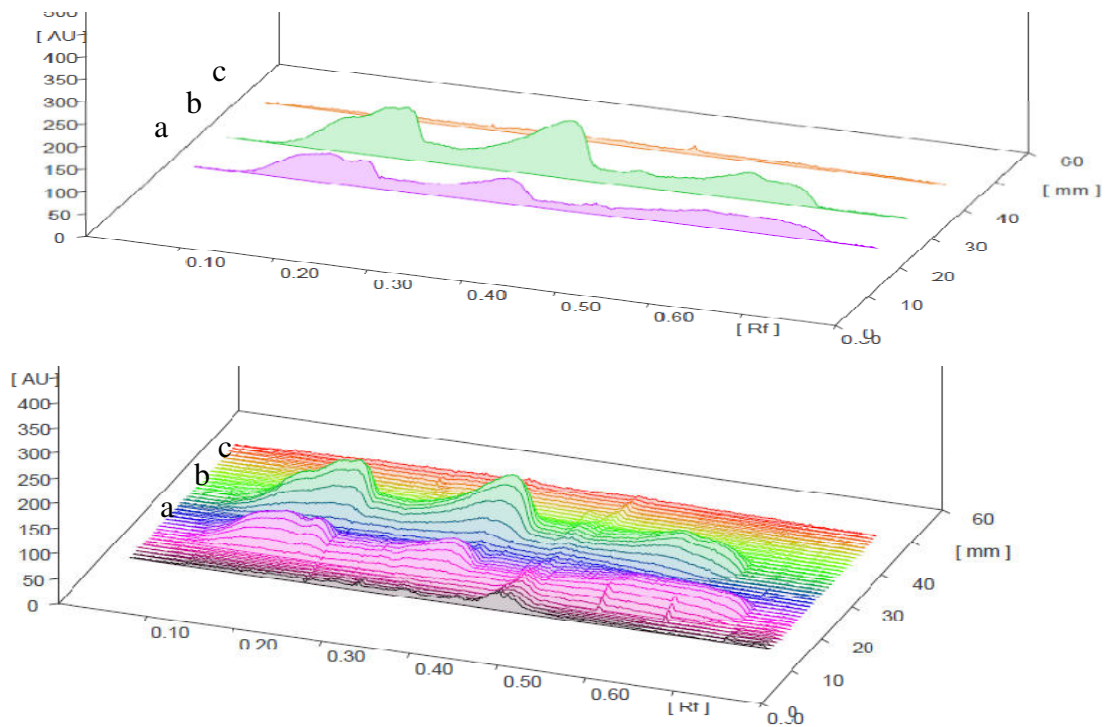
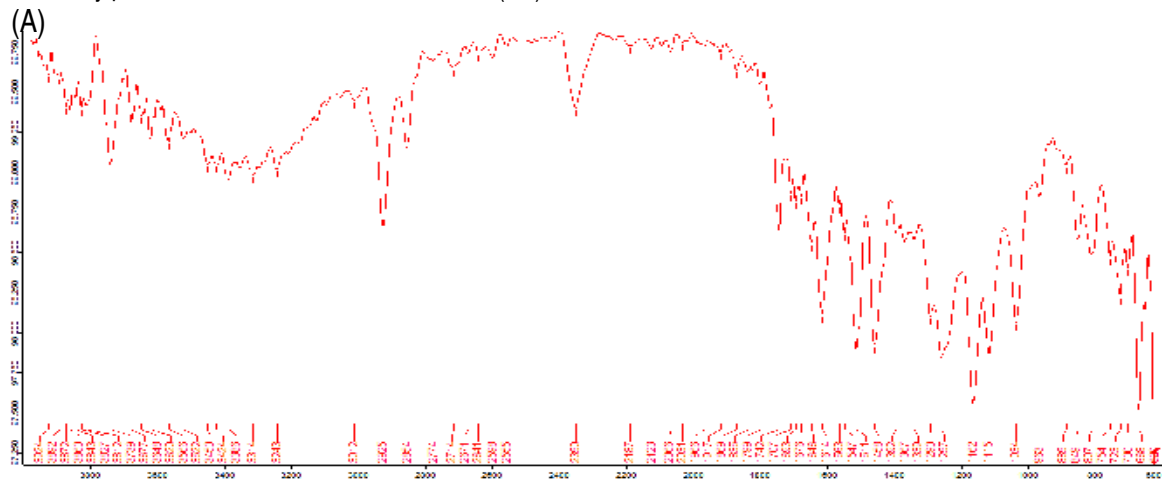


Figure 2. The densitometry profile is depicted for the methanol (a), ethanol (b) and hexane (c) extracts run on a silica gel F245 stationary phase and eluted with n-hexane:EtOAc (1:1).



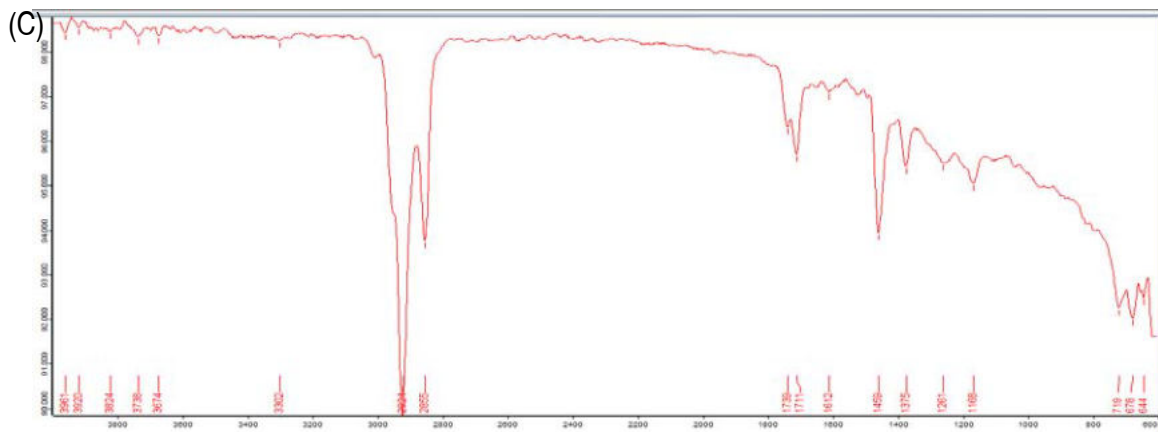


Figure 3. The FT-IR spectra of the methanol (a), ethanol (b), and hexane (c) extracts of Sappan Lignum (*Caesalpinia sappan* L.) are illustrated.

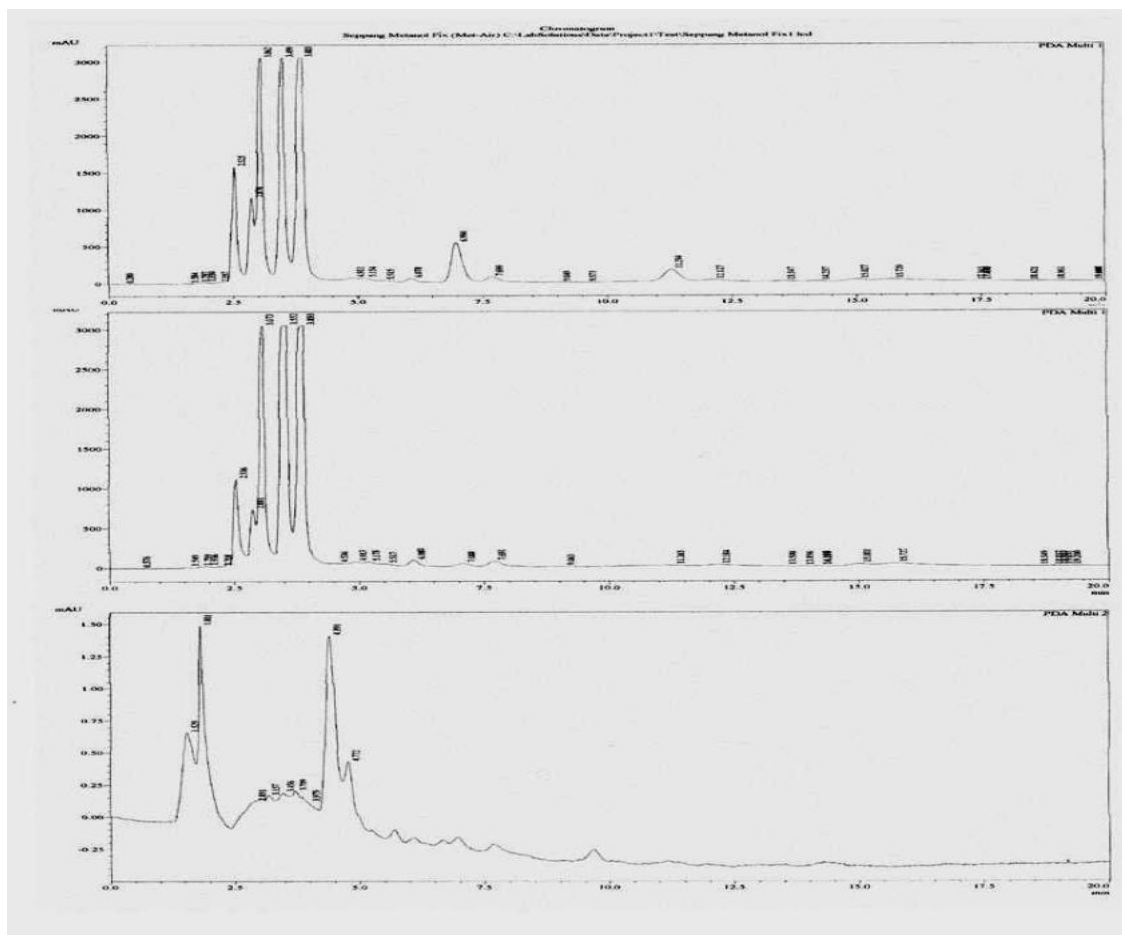


Figure 4. The HPLC chromatograms that were monitored at 190 nm are presented for the methanol, ethanol and n-hexane extracts (100 ppm). Shim-Pack VP-ODS columns (150x4.6 mm) were run at a flow rate of 1 ml/min in methanol:H₂O (60:40) at 30 C.

The extracts were tested for anti-osteoporosis activity by investigating their ability to stimulate the proliferation of osteoblasts. All the extracts stimulated the proliferation of osteoblasts at 100

µg/ml. The ethanol extract had 64% more activity than the other extracts (Fig. 5).

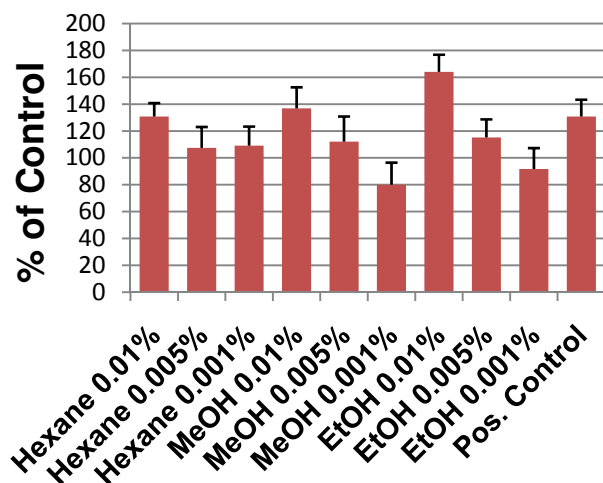


Figure 5. The effects of the Sappan Lignum extracts on the proliferation of osteoblasts are depicted.

Table 1. The inhibitory effects of the Sappan Lignum extracts on the formation of osteoclasts are presented. The percent inhibition was calculated with the control set as 0% inhibition.

No.	Extract	Concentration (µg/ml)	Inhibition (%)
1.	n-Hexane	10	2,50
2.		5	1,56
3.		1	2,50
4.	Methanol	10	6,43
5.		5	2,31
6.		1	-2,31
7.	Ethanol	10	8,75
8.		5	-1,50
9.		1	-4,68
10.	Calcitonin	2 U/ml	22,50

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All the extracts weakly inhibited (less than 10%) the formation of osteoclasts when compared with calcitonin, the positive control (Table 1). These data indicated that Sappan Lignum might be effective against osteoporosis because it enhanced bone absorption and inhibited the formation of osteoclasts, albeit weakly, suggesting that this traditional medicine may be a good candidate for the treatment of osteoporosis.

Conclusion

The spectrophotometric characterization of Sappan lignum extracts identified the extract that contained anti-osteoporosis activity. The ethanol extract was the most active in stimulating the proliferation of osteoblasts, whereas all the extracts weakly inhibited the formation of osteoclasts. In conclusion, this study demonstrated that the ethanol extract of Sappan lignum effectively stimulated the proliferation of osteoblasts and weakly inhibited the formation of osteoclasts.

Authors' contributions

SB carried out of the anti-osteoporosis activity and participated in sequence alignment and drafted of the manuscript. YR participated in instrumentation and helped to draft the manuscript and its design. MD carried out the instrumentation analysis data and collection of the material.

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