Volatile Constituents and Anti-Osteoporotic Activity of the \textit{n}-Hexane Extract From \textit{Homalomena gigantea} Rhizome

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Abstract

This study analyzed the chemical composition and anti-osteoporosis activity of the \textit{n}-hexane extract of \textit{Homalomena gigantea} rhizome. Sixty compounds, representing 92.0\% of the extract, were identified by gas chromatography-mass spectrometry. Linalool (15.3\%), oplopanone (9.8\%), (E)-\textalpha-\textit{atlantone} (5.6\%), khusinol acetate (5.4\%), bullatantriol (4.3\%), and \textbeta-sitosterol (3.8\%) were the main constituents. The anti-osteoporotic activity of the \textit{n}-hexane extract was determined by measuring alkaline phosphatase (ALP) activity, collagen content, and the mineralization of MC3T3-E1 cells. At concentrations of 4.0 and 20.0 \mu g/mL, the \textit{n}-hexane extract increased ALP activity by 8.2\% and 23.7\%, and increased collagen secretion by MC3T3-E1 cells by 114.9\% and 112.4\%, respectively. At 4 \mu g/mL, the extract significantly promoted the mineralization of MC3T3-E1 cells by as much as 133.2\% compared to the negative control. These results suggested that \textit{H. gigantea} rhizome contains a natural anti-osteoporotic compound.

Keywords

\textit{Homalomena gigantea}, sesquiterpenoid, linalool, oplopanone, bullatantriol, anti-osteoporotic activities

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Introduction

Osteoporosis is a condition typified by decreasing bone mass, degeneration of the bone microarchitecture, and fragility fractures. Elderly women and men, regardless of their ethnic origin, are susceptible to osteoporosis, which is associated with considerable morbidity and mortality.\textsuperscript{1,2} Worldwide, 1 in 5 men and 1 in 3 women aged >50 years will suffer a fracture due to osteoporosis.\textsuperscript{3–5} Dobbs et al.\textsuperscript{6} identified 2 basic forms of osteoporosis: primary, which includes postmenopausal (type I) and senile (type II) osteoporosis, and secondary osteoporosis. Because women have smaller, thinner bones and less muscle mass than men, they have a two-fold higher risk of developing primary type II osteoporosis.\textsuperscript{7} In the United States of America, over 34 million people have low bone mass, and thus are at elevated risk of osteoporosis. Furthermore, roughly 10 million people have osteoporosis.\textsuperscript{8,9} In Vietnam, both men and women are at high risk of osteoporosis.\textsuperscript{10–13}

Alendronate, etidronate, ibandronate, and risedronate are only a few of the medications being increasingly used to treat and prevent osteoporosis in postmenopausal women and those taking chronic corticosteroids. However, these drugs have numerous side effects, including hypocalcemia, kidney impairment leading to renal failure, and gastrointestinal symptoms (nausea, indigestion, and diarrhea).\textsuperscript{14} Moreover, prolonged use leads to increased blood triglyceride levels and a higher risk of stroke. For these reasons, medicinal plants have become the focus of many studies aiming to devise novel medications with improved safety and efficacy, including for osteoporosis.\textsuperscript{15,16} Alkaloids, phenolic compounds, saponins, terpenoids, lipids, and fatty acids are just a few of the many bioactive compounds found in medicinal plants; their biological properties include anti-inflammatory, anti-carcinogenic, anti-osteoporotic, anti-cancer, antimicrobial, and antioxidant activities.\textsuperscript{17–19}

The genus \textit{Homalomena} is distributed in Central America, South America, and Asia, and includes 250 species.\textsuperscript{20} Five species of \textit{Homalomena}, that is, \textit{H. cochinchinensis}, \textit{H. occulta}, \textit{H. gigantea}, \textit{H. pierreana}, and \textit{H. vietnamensis}, have been identified in Vietnam.\textsuperscript{21} The chemistry and activity of the volatile oils of

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one such species, *H. gigantea* Engl. (www.theplantlist.org), have not been investigated. This plant is found in Lam Dong province, Vietnam, where it has long been popular as a tonic, digestive stimulant, and for rheumatism treatment.22,23 Earlier research demonstrated its anti-inflammatory properties.24 In this work, we determined the chemical composition of the volatile oil by preparing an *n*-hexane extract of the plant, as well as its anti-osteoporotic characteristics associated with alkaline phosphatase (ALP) activity, collagen content, and mineralization. Our results show that *H. gigantea* stimulates osteoblast differentiation and proliferation, thus enhancing bone growth.

**Results and Discussion**

The *n*-hexane rhizome extract was analyzed by gas chromatography-mass spectrometry (GC-MS), which revealed 60 volatile compounds, representing 92.0% of all identified components. Alkanes and alcohols accounted for 2.6%, terpenoids (monoterpenes hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes) for 69.3%, aromatic compounds for 1.2%, fatty acids and fatty acid esters for 11.1%, and unidentified compounds for another 8.0%. No monoterpene hydrocarbons were detected in the volatile oil. Oxygenated sesquiterpenes (43.7%) were the most abundant class, represented mostly by oplopanone (9.8%), (*E*)-α-atlantone (5.6%), khusinol acetate (5.4%), bullatantriol (4.3%), β-chinenopodi (2.4%), α-cadinol (2.2%), homalomenol C (2.1%), homalolide B (2.0%), homalolide A (1.9%), spathulenol (1.7%) and τ-muurolol (1.2%), followed by oxygenated monoterpenes (23.1%), including linalool (15.3%), terpinen-4-ol (2.8%), α-linalyl acetate (1.7%) and terpineol (1.2%), and fatty acids and fatty acid esters (11.1%), including 2-chloroethyl linoleate (3.3%), citic acid (2.1%), and 2-monopalmi (1.5%). Steroid compounds, such as β-sitosterol (3.8%), stigmastesterol (2.7%), and campesterol (1.5%), were also detected in significant amounts in the *n*-hexane rhizome extract.

Previous studies showed that linalool and terpinen-4-ol were the major components of the rhizome oils of *H. aromatica, H. cochinchinesis, H. occulta,* and *H. sagittifolia.*25–32 These results demonstrate the close phylogenetic relationships between *H. aromatica, H. cochinchinesis, H. occulta, H. sagittifolia,* and *H. gigantea.*

The anti-osteoporotic effects of the *n*-hexane extract of *H. gigantea* were investigated. MTT assay was used to determine the toxicity of the extract on MC3T3-E1 osteoblasts. At doses of 0.8, 4.0, and 20.0 g/mL, the extract did not affect MC3T3-E1 cell growth (Figure 1). These doses were thus used in subsequent experiments.

This study is the first to identify homalolide A and homalolide B in the *n*-hexane extract of *H. gigantea* rhizomes. However, the presence of homalolide A may have been an artifact due to methylation during the methanolic extraction process.24,33–39 α-Cadinol,33 homalomenol C,34 homalomenol A, bullatantriol, and 1β,4β,7α-trihydroxyeudesmane,35 spathulenol,36 teucmosin,37 oplopanone,38 and 1α,4β,5α,6β,7β,10β-4,10-epoxy-6-guainol39 have also been isolated from other *Homalomena* species.

![Figure 1](image-url)  
**Figure 1.** Effects of *n*-hexane extract on the viability of MC3T3-E1 cells. Data are expressed as a percentage of control.
Osteoblasts produce ALP in the bone and play an important role in bone growth and quality. ALP is thus a critical biochemical marker for measuring MC3T3-E1 cell differentiation. After 5 days of incubation with the lowest dose (0.8 µg/mL) of the extract, the ALP activity of MC3T3-E1 cells was suppressed by 15.6% compared to the negative control cells. However, at concentrations of 4 and 20 µg/mL, the extract-treated cells had significantly higher (by 8.2% and 23.7%, respectively) ALP activity than determined in the negative control. ALP activity is increased in early differentiation osteoblasts. Our results demonstrate the ability of the n-hexane extract from *H. gigantea* to stimulate strongly osteogenic differentiation in MC3T3-E1 cells, based on its ability to increase ALP activity (Figure 2).

The extracellular matrix is another key indicator of MC3T3-E1 cell differentiation. Collagen is the most abundant extracellular matrix protein and a mineral deposition substrate. As an important component of the final extracellular matrix, it is required for bone development. In this study, the collagen content was measured using a Sirius Red-based colorimetric assay. Collagen secretion from MC3T3-E1 cells after 10 days of incubation in a medium containing vitamin C and β-glycerophosphate was comparable to that of differentiated cells (Figures 3 and 4). In cells treated with the n-hexane extract at concentrations of 4 and 20 g/mL, collagen secretion was increased by 14.9% and 12.4%, respectively, compared to the negative control. There was no enhancement of collagen secretion by MC3T3-E1 cells treated with the n-hexane extract at a concentration of 0.8 g/mL.

Mineral accumulation by the extracellular matrix begins at ~15 days after the differentiation of MC3T3-E1 cells into osteoblasts and is coupled with collagen formation. These events mark the final stage in the morphological growth of osteoblasts. Mineral salts in bones can retain up to 99% of the deposited calcium. We, therefore, used Alizarin Red staining to measure the calcium uptake ability of MC3T3-E1 exposed to the n-hexane extract of *H. gigantea*. The calcium granules stain bright red when incubated with Alizarin Red S, and stain purple when re-dissolved in 10% acetyl pyridinium chloride (Figure 5). At a dose of 4 µg/mL, mineralization was increased by 133.2% compared to the negative control. At doses of 0.8 and 20 g/mL, calcium mineralization was increased by 112.4% and 109.6%, respectively.

The ability of the n-hexane extract of *H. gigantea* to enhance ALP activity, collagen synthesis, and mineralization suggests that it possesses anti-osteoporotic potential. This can be attributed to its high content of terpenoids, such as oxygenated sesquiterpenes (43.7%), oxygenated monoterpenes (23.1%), and sesquiterpene hydrocarbons (2.5%). Similarly, a previous study reported that oplodiol, oplopanone, homalomenol C, bullatantriol, 1β,4β,7α-trihydroxyeudesmane, homalomenol E, 1β,4β,6α-trihydroxy-eudesmane, and homalomenol F were among the oxygenated sesquiterpenes isolated from the rhizomes of *H. occulta*. These compounds significantly stimulated the proliferation, differentiation, and mineralization of cultured osteoblasts in vitro. Sesquiterpenes, including bicyclolamellolactone A, lamellolactones A, and lamellolactones B, were also isolated from the Indonesian marine sponge *Lamellodysidea* sp. (cf *L. herbacea*) and showed inhibitory effects on BMP-induced osteoblastic differentiation.

**Figure 2.** Effect of n-hexane extract on the alkaline phosphatase (ALP) activity of MC3T3-E1 cells. Data are expressed as a percentage of control. **P < .01 versus control.**
the n-hexane extract may be a good candidate for use as an anti-osteoporotic agent, and thus merits further research.

## Conclusions

Phytochemical analysis of the n-hexane extract from *H. gigantea* revealed 60 volatile components, with most of the compounds being fatty acids and fatty acids esters, oxygenated sesquiterpenes, or oxygenated sesquiterpenes. The oxygenated sesquiterpenes included homalomenol A, homalomenol C, teucmosin, oplopanone, 1,4,5,6,7-H,10-epoxy-6-guainol, bullatantriol, and 14,7-trihydroxyeudesmane; these have also been found in the volatile oils of other *Homalomena* species. Our study is the first to examine the anti-osteoporotic activity, as reflected in ALP activity, collagen content, and bone mineralization, of the n-hexane extracts. Further investigation into the anti-osteoporotic properties of the n-hexane extract from *H. gigantea* may lead to the development of safe and effective treatments for osteoporosis (Table 1).

## Materials and Methods

### Materials

In January 2021, *H. gigantea* rhizomes (0.5 kg) were harvested in Lam Dong Province, Vietnam (geographical coordinates: 11° 25’25.3’’N; 108°03’28.9’’E). Dr Chinh Tien Vu verified the plant’s authenticity (Vietnam National Museum of Nature, VAST, Vietnam). A voucher specimen (TNK-LD-01) has been deposited at the Faculty of Pharmacy, Hue University of Medicine and Pharmacy, Vietnam.

### Solvent Extraction Process

*H. gigantea* dried rhizomes (0.5 kg) were powdered and extracted with n-hexane (2.5 L, 3 times) at room temperature. Under reduced pressure, the resulting extract was concentrated to provide 15 g of a dark solid extract. GC-MS was used to analyze the n-hexane extract. These tests were carried out 3 times.

### Analysis of the Volatile Compounds

The chemical composition of the volatile compounds was determined using a Shimadzu GC-MS-QP2010 Plus system (Kyoto,
Japan) with an Equity-5 capillary column (30 m 0.25 mm, 0.25 m film thickness) and a mass spectrometer (MSD QP2010 Plus) for GC/MS analysis. The \( n \)-hexane extract (1 mg) was diluted in a 1:100 ratio with dichloromethane, and 1 \( \mu \)L was used for analysis. The following were the analytical conditions: 1.5 mL/min carrier helium, 280 °C injector, 280 °C interface, and a column temperature controlled from 60 °C (2 min hold) to 240 °C at 3 °C/min (10 min hold) and subsequently increased to 280 °C at 5 °C/min (40 min hold). Splitless injection mode was used to inject the samples. The following were the MS conditions: Acquisitions scan, a mass range of 40–500 (m/z) at a sampling rate of 1.0 scan/s at a 70 eV ionization voltage. The retention indices (RI) of constituents were determined by co-injection under the same conditions with a homologous series of \( n \)-alkanes \((C_{8}–C_{38})\). The constituents were identified by matching their mass spectra with Wiley 7, NIST 11 library search data from the GC-MS system, primary component standards, and literature data. The relative peak area percentage was used to quantify the data.

**Anti-Osteoporotic Assay**

**Cell Culture.** American Type Cell Culture provided MC3T3-E1 cells (ATCC CRL-2593) (Manassas, Virginia, USA). At 37 °C in a 5% CO\(_2\) incubator, MC3T3-E1 cells were grown in \( \alpha \)-MEM (an \( \alpha \)-Modified Eagle Medium) with a supplement of 10% FBS (fetal bovine serum), 10 mM \( \beta \)-glycerophosphate, 50 g/mL ascorbic acid, and 1% penicillin/streptomycin. Cells were subcultured with Trypsin-EDTA (0.05%) solution after 2 days. An MTT assay was used to assess the extract’s cytotoxic effects on MC3T3-E1 cells. The MC3T3-E1 cells were seeded at 10 000 cells per well in a 96-well plate and cultivated for 48 h. After rinsing with PBS, the cells were incubated in new media for 24 h with varying doses of the selected extract. The viable cells were then incubated for 4 h in a CO\(_2\) incubator with a freshly prepared medium containing 20 \( \mu \)L of 5 mg/mL MTT and 80 \( \mu \)L of \( \alpha \)-MEM (10% FBS, penicillin/streptomycin). The living cells converted MTT to a purple formazan dye, which was dissolved in 100 \( \mu \)L DMSO after a 10-min shake. Finally, using an ELISA Plate Reader (BioTek ELx800) at a 490 nm wavelength, the relative colorimetric intensity of each well was determined. Without exposure to the extract, the control group’s cell viability was determined as 100%.48

**ALP Activity.** To begin differentiation, the cells were treated at 80% confluence with a culture medium containing 10 mM \( \beta \)-glycerophosphate and 50 g/mL ascorbic acid. The culture media with various concentrations of the extract was then added to each well. The medium was changed every 2–3 days, and the clear supernatant was tested for ALP activity using an ALP colorimetric assay kit (Abcam, Cambridge, England) according to the manufacturer’s procedure after 7 days of culture.49

**Measurement of Collagen Content.** Collagen content was measured using a colorimetric assay based on Sirius Red. Cultured osteoblasts were washed twice in PBS after 10 days, then fixed for 1 h

![Figure 5. Effects of \( n \)-hexane extract on the mineralization of MC3T3-E1 cells. Data are expressed as a percentage of the control. *\( P < .05 \) versus control.](image-url)
Table 1. Volatile Components From the n-Hexane Extracts of the Rhizomes of *Homalomena gigantea*.

| No | Compound* | RT     | RI     | %  | Identification method |
|----|-----------|--------|--------|----|-----------------------|
| 1  | cis-Linalool oxide (furanoid) | 11.84  | 1072   | 0.4 | MS, RI                |
| 2  | trans-Linalool oxide (furanoid) | 12.50  | 1088   | 0.4 | MS, RI                |
| 3  | Linalool | 13.21  | 1105   | 15.3| MS, RI                |
| 4  | Hotrienol | 13.29  | 1107   | 0.3 | MS, RI                |
| 5  | α-Phellandren-8-ol | 15.63  | 1160   | 0.1 | MS, RI                |
| 6  | Terpinen-4-ol | 16.44  | 1178   | 2.8 | MS, RI                |
| 7  | p-Cymen-8-ol | 16.60  | 1182   | 0.1 | MS, RI                |
| 8  | Cryptone | 16.79  | 1186   | 0.5 | MS, RI                |
| 9  | α-Terpineol | 16.98  | 1191   | 1.2 | MS, RI                |
| 10 | Verbenone | 17.78  | 1209   | 0.2 | MS, RI                |
| 11 | Not identified | 18.15  | 1217   | 1.1 |                      |
| 12 | Nerol | 18.68  | 1229   | 0.2 | MS, RI                |
| 13 | Linalyl acetate | 19.91  | 1257   | 1.7 | MS, RI                |
| 14 | 2,6-Dimethyl-1,7-octadiene-3,6-diol | 20.69  | 1274   | 0.6 | MS, RI                |
| 15 | p-Cymen-7-ol | 21.42  | 1291   | 0.2 | MS, RI                |
| 16 | p-Mentha-1,4-dien-7-ol | 23.09  | 1329   | 0.1 | MS, RI                |
| 17 | 3-Methyl-1,6-heptadien-3-ol | 23.48  | 1339   | 0.3 | MS                    |
| 18 | Geranyl acetate | 25.45  | 1384   | 0.3 | MS, RI                |
| 19 | β-Caryophyllene | 26.95  | 1420   | 0.2 | MS, RI                |
| 20 | α-Caryophyllene | 28.36  | 1454   | 0.3 | MS, RI                |
| 21 | α-selinene | 30.07  | 1496   | 0.4 | MS, RI                |
| 22 | α-Muurolene | 30.29  | 1501   | 0.3 | MS, RI                |
| 23 | γ-Cadinene | 30.83  | 1515   | 0.3 | MS, RI                |
| 24 | δ-Cadinene | 31.22  | 1525   | 1.0 | MS, RI                |
| 25 | 1α,4β,5α,6β,7βH,10β-4,10-Epoxy-6-guainol | 31.61  | 1535   | 0.5 | MS, Co-GC             |
| 26 | Nerolol | 32.78  | 1565   | 0.3 | MS, RI                |
| 27 | Spathulenol | 33.36  | 1580   | 1.7 | MS, Co-GC, RI        |
| 28 | Caryophyllene oxide | 33.56  | 1585   | 0.7 | MS, RI                |
| 29 | Not identified | 34.71  | 1615   | 0.6 |                      |
| 30 | τ-Muurolol | 35.81  | 1645   | 1.2 | MS, RI                |
| 31 | α-Cadinol | 36.31  | 1658   | 2.2 | MS, Co-GC, RI        |
| 32 | α-Bisabolol | 37.42  | 1688   | 0.5 | MS, RI                |
| 33 | Homalolide B | 37.60  | 1692   | 2.0 | MS, Co-GC             |
| 34 | Amorpha-4,9-dien-2-ol | 38.07  | 1705   | 0.8 | MS, RI                |
| 35 | Not identified | 38.67  | 1722   | 0.9 |                      |
| 36 | Oplopomone | 39.52  | 1746   | 9.8 | MS, Co-GC, RI        |
| 37 | Homalolide A | 39.81  | 1754   | 1.9 | MS, Co-GC             |
| 38 | (E)-α-Atlantone | 40.57  | 1776   | 5.6 | MS, RI                |
| 39 | Homalomenol C | 41.57  | 1804   | 2.1 | MS, Co-GC             |
| 40 | β-Chenopodiol | 41.94  | 1815   | 2.4 | MS, RI                |
| 41 | Khusisol acetate | 42.40  | 1829   | 5.4 | MS, RI                |
| 42 | Methyl palmitate | 45.69  | 1927   | 0.6 | MS, RI                |
| 43 | Palmitoleic acid | 46.26  | 1945   | 0.4 | MS, RI                |
| 44 | Teucemolin | 46.69  | 1958   | 1.9 | MS, Co-GC             |
| 45 | Bullatantriol | 47.10  | 1971   | 4.3 | MS, Co-GC             |
| 46 | Eicosane | 48.04  | 2000   | 0.7 | MS, RI                |
| 47 | 1β,4β,7α-trihydroxyeudesmane | 49.55  | 2049   | 0.2 | MS, Co-GC             |
| 48 | Methyl linolate | 50.97  | 2095   | 0.6 | MS, RI                |
| 49 | Methyl oleate | 51.15  | 2101   | 0.3 | MS, RI                |
| 50 | 2-Chloroethyl linolate | 52.35  | 2141   | 3.3 | MS                    |
| 51 | Civeic acid | 52.50  | 2146   | 2.1 | MS                    |
| 52 | Stearic acid | 53.10  | 2166   | 0.7 | MS, RI                |
| 53 | Docosane | 54.08  | 2200   | 0.2 | MS, RI                |
| 54 | Tetracosane | 59.64  | 2400   | 0.2 | MS, RI                |
| 55 | Cyclogallipharaol | 62.08  | 2492   | 0.2 | MS                    |
| 56 | 2-Monopalmitin | 62.54  | 2509   | 1.5 | MS, RI                |
| 57 | Not identified | 67.73  | 2668   | 2.2 |                      |
| 58 | Monolinolein | 68.37  | 2685   | 0.6 | MS                    |

(Continued)
with Bouin’s fluid (0.1%) dissolved in picric acid. The fluid was removed after fixation, and the culture dishes were washed for 15 min in running tap water. The culture dishes were air-dried and stained for 1 h in a shaker with Sirius Red dye reagent. The solution was then removed, and the cultures were rinsed with 0.01 N HCl to eliminate any remaining nonbound dye. The stained sample was dissolved in 0.1 N NaOH for 5 min, and absorbance was measured at 550 nm using an ELISA Plate Reader (BioTek ELx800) against 0.1 N NaOH as a blank.50

Mineralization. To begin in vitro mineralization, the cells were treated at 80% confluence with a culture media containing 10 mM β-glycerophosphate and 50 mg/mL ascorbic acid. The cells were washed twice with PBS, fixed with 70% ethanol (v/v) for 1 h, dried in the air, and then stained with 40 mM Alizarin red-S (pH 4.4) for 15 min with moderate shaking before being deionized water rising. The stain was solubilized with 10% cetylpyridinum chloride in 10 mM sodium phosphate buffer (pH 7.0) by shaking for 15 min while shielded from light to measure the bound dye. Microplate Reader optical densities (OD) were measured at 561 nm to evaluate cell staining in the samples (BioTek).51

Statistical Analysis
All data are presented as averages with standard deviations (SD). The statistical significance of group means was determined using the SAS statistical software and the Student’s t-test, F’s test, and one-way ANOVA (P < .05 or P < .01).

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Statement of Informed Consent
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Supplemental Material
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