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The composition of chemicals and anti-osteogenic properties in the volatile extracts from *Homalomena gigantea* rhizome

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Abstract: In this study, the anti-osteogenic properties of the volatile oil extracted from *Homalomena gigantea* rhizome using ethyl acetate (EtOAc) and methanol (MeOH) were examined. Gas chromatography-mass spectrometry (GC-MS) was employed for the identification of volatile components. Following this, bioassays were performed to evaluate their effects on osteogenesis, encompassing parameters like cell viability, osteoblast differentiation, collagen synthesis and mineralization. The GC-MS analysis revealed 19 compounds in the EtOAc extract and 36 compounds in the MeOH extract. In the MeOH extract, major constituents included bis(2-ethylhexyl) terephthalate (13.83%), linalool (9.58%), palmitic acid (6.55%) and stearic acid (4.29%). The EtOAc extract contained bis(2-ethylhexyl) terephthalate (16.64%), palmitic acid (5.60%) and stearic acid (3.11%) as the predominant components. Both the EtOAc and MeOH extracts of *H. gigantea* exhibited promising potential for further investigation in anti-osteoporosis research. These findings contribute to the exploration of natural compounds with potential anti-osteoporotic properties, expanding our understanding of their therapeutic potential.

Keywords: Anti-osteoporotic activities, *Homalomena gigantea*, GC-MS, sesquiterpenoid.

INTRODUCTION

With the escalation of global aging, osteoporosis, recognized as a major public health concern, manifests as a condition typified by diminished bone mass and deterioration in bone tissue micro architecture. This ailment often results in excruciating pain, heightened susceptibility to bone fractures and potentially fatal outcomes, thereby imposing a substantial burden on both human health and socioeconomic aspects (Wang *et al.*, 2023). However, these pharmaceutical interventions often have undesirable side effects, including hypocalcemia, renal impairment, and gastrointestinal disturbances (Kennel and Drake, 2009). Prolonged use of these medications may also carry risks such as elevated blood triglyceride levels and an increased likelihood of stroke. As a result, there's been increasing curiosity surrounding the investigation of alternative methods, particularly those utilizing medicinal plants, as promising reservoirs for innovative osteoporosis therapies (Atanasov *et al.*, 2021). Medicinal plants harbor a plethora of bioactive compounds, encompassing alkaloids, phenolic compounds, saponins, terpenoids, lipids and fatty acids. These compounds manifest diverse biological properties, including anti-inflammatory, anti-osteoporotic, antioxidant activities antimicrobial, anti-carcinogenic and anti-cancer (Adeleye *et al.*, 2022).

The genus *Homalomena* approximately comprises 250 species found in tropical regions of Central America, South America and Asia (Boyce *et al.*, 2012). Vietnam is home to five known species: *H. cochinchinensis, H. gigantea, H. pierreana, H. vietnamensis* and *H. occulta*, (Van, 2017). However, there has been limited research on the volatile composition and associated biological activity of *H. gigantea* which is native to Lam Dong province, Vietnam. Traditional medicine has long utilized *H. gigantea* as a tonic, digestive stimulant and remedy for rheumatism (Chi, 2012). Previous investigations had demonstrated promising anti-inflammatory and anti-osteoporotic properties of this herb (Pham *et al.*, 2022; Nguyen *et al.*, 2023). In this work, the volatile chemicals of *H. gigantea* were recovered utilizing the procedures of EtOAc and MeOH extraction. Additionally, the anti-osteoporotic characteristics of this herb extracts were investigated by, evaluating key parameters such as alkaline phosphatase (ALP) activity, mineralization, and collagen content.

MATERIALS AND METHODS

Materials

Chemicals

Ethyl acetate (EtOAc), methanol (MeOH), Alkanes Calibration Standard, NaOH, *β*-glycerophosphate, FBS (fetal bovine serum), *α*-MEM (*α*-Modified Eagle Medium), ascorbic acid, penicillin/streptomycin, Trypsin, Ethylene Diamine Tetra Acetic (EDTA), Dimethylsulfoxide (DMSO), Bouin's fluid, were acquired from Sigma Aldrich, St. Louis, Missouri, USA, along with Sirius Red dye.

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Plant sample

A collection of 1.5kg of *H. gigantea* rhizomes was acquired from Lam Dong Province, Vietnam, in January 2021. The precise geographical coordinates of the collection site were 11°25′25.3″N and 108°03′28.9″E. Dr. Tran Minh Duc has authenticated the plant's identity (Faculty of Forestry, Hue University of Agriculture and Forestry, Vietnam). A voucher specimen (TNK-KS-01) has been archived at the Department of Biology, Hue University of Education, Vietnam.

Methods

The process of extraction

In order to conduct this investigation, 1.5 kg of dried *H. gigantea* rhizomes were powdered and extracted at room temperature using 1.5L each of EtOAc and MeOH. Under reduced pressure, the resultant extracts were concentrated, producing, for the EtOAc and MeOH extracts, respectively, around 15g and 17g of black solid extract. On both extracts, a GC-MS study was done. To guarantee accuracy and consistency of the results, the extraction and analytical procedures were carried out three times.

The GC-MS analysis

GC-MS analysis on an Aligent GC 7890B-MS 5975C (5301 Stevens Creek Blvd. Santa Clara, CA 95051, United States) was used to determine the chemical components of extracts from *H. gigantean* rhizomes. The HP-5MS column (30m x 250m x 0.25m) was utilized for the GC; The carrier gas, helium, was employed at 13 psi; the injection volume for each sample was 1L and the sample was injected in a split ratio of 1:50. 250^{o}C was the injection temperature. The program for the column temperature was as follows: it began at 70^{o}C and grew linearly to 280^{o}C at a rate of 10^{o}C min^{-1}. The GC column's effluent was immediately added to the MS source, and spectra in the EI mode with a 70eV ionization energy were produced. The sector mass analyzer was programmed to scan for 2s between 40 and 400 amu. Utilizing the C_8-C_{30} Alkanes Calibration Standard (Sigma Aldrich, St. Louis, Mo, USA), the retention indices of the compounds were determined. Numerous compounds were found by comparing mass spectra of different chemicals to those in the NIST14 database. For quantification, the relative peak area as a percentage was utilized.

The cell culture technique for the anti-osteoporotic assay was modified from a work carried out by Lee (Lee *et al.,* 2021). The assessment of alkaline phosphatase (ALP) activity was adopted from a work by Marchese (Marchese *et al.,* 2020), whereas Gentilini's (Gentilini *et al.,* 2018) approach was used to measure collagen content. Furthermore, the mineralization assay was modified from a study by Yun (Yun *et al.,* 2015).

Anti-osteoporotic assay

Culture of cells

The American Type Culture Collection (Manassas, Virginia, USA) provided the MC3T3-E1 cells (ATCC CRL-2593), which were grown in α-MEM (α-Modified Eagle Medium) supplemented with 10% fetal bovine serum, 10mM β-glycerophosphate, 1% penicillin/ streptomycin and 50g/mL ascorbic acid. The cells were incubated at 37°C in a 5% CO_2 environment and subcultured every 2 days using a 0.05% Trypsin-EDTA solution.

MTT assay

In a 96-well plate, 10,000 MC3T3-E1 cells were sown per well and treated for 48 hours to evaluate the cytotoxic effects. The MTT test was used to assess the vitality of the cells following a 24-hour treatment with various extract dosages. The formazan dye produced by viable cells was solubilized in DMSO and the absorbance was measured at 490 nm using an ELISA Plate Reader (Lee *et al*., 2022).

ALP activity

After the cells attained 80% confluence, they were placed in a differentiation medium containing 50g/mL ascorbic acid and 10mM β-glycerophosphate. The extract was given to each well and the medium was changed every two to three days. Following a 7-day period, ALP activity was assessed using a colorimetric assay kit (Marchese *et al.,* 2020).

Measurement of collagen content

Cultured osteoblasts were stained with Sirius Red dye and preserved with Bouin's solution after ten days. After dissolving the dyed samples in NaOH, an ELISA Plate Reader was used to assess absorbance at 550 nm (Gentilini *et al.,* 2018).

Mineralization

To induce mineralization, cells were fed with a differentiation medium containing 50mg/mL ascorbic acid and 10mM β-glycerophosphate. Following fixation and Alizarin red-S staining, the attached dye was dissolved and optical densities were determined with a microplate reader at 561 nm (Yun *et al.,* 2015).

STATISTICAL ANALYSIS

The mean values of the data are displayed together with the corresponding standard deviations (SD). Using IBM SPSS version 19.0 statistical software, one-way analysis of variance (ANOVA) was used for the statistical analysis. Duncan's multiple range tests were used to determine significant differences between means at a significance threshold of $p<0.05$.

RESULTS

A total of 36 chemicals were found in the MeOH rhizomes extract using GC-MS analysis, making up 60.10% of the extract composition (table 1). The major compounds belonged to the ester class (20.74%),

followed by the classes of diterpenoids (0.34%), sesquiterpenes (0.48%), ketones (0.56%), alcohols (1.38%), aromatics (2.27%), acids (10.99%), sesquiterpenoids (11.07%) and monoterpenoids (12.27%). Notably, palmitic acid (6.55%), oplopanone (7.32%), linalool (9.58%), and bis (2-ethylhexyl) terephthalate (13.83%) were identified as the principal compounds. Several other compounds were detected at percentages exceeding 1.0%, including methyl stearate (1.18%), *τ*-Muurolol (1.25%), terpinen-4-ol (1.28%), 1-Monostearin (1.72%), 3-((4*Z*,7*Z*)-Heptadeca-4,7-dien-1-yl)phenol (1.98%), 2-Monopalmitin (2.5%), and stearic acid (4.25%). In the case of the EtOAc extract, 19 compounds were identified, accounting for 30.10% of the extract. Esters (19.63%) and acids (9.02%) were the dominant chemical classes in this extract. Additionally, the MeOH rhizome extract contained trace amounts of aromatics (0.12%), alkanes (0.50%), ancols (1.95%), and monoterpenoids (2.79%). The principal compounds in the EtOAc rhizome extract were stearic acid (3.11%), palmitic acid (5.60%) and bis (2-ethylhexyl) terephthalate (16.64%). Several other compounds were present in lower quantities, such as methyl glycolate (0.04%), *p*-cymen-7-ol (0.06%), dimethyl malate (0.12%), 2-methoxy-4-vinylphenol (0.12%), myristic acid (0.31%), 2,3-butanediol (0.33%), (*E*)-2,6-dimethylocta-3,7-diene-2,6-diol (0.38%), hotrienol (0.42%), methyl palmitate (0.50%), eicosane (0.50%), *trans*-linalool oxide (pyranoid) (0.66%), 8-hydroxylinalool (0.67%), (*E*)-2,6-dimethylocta-1,7-dien-3,6-diol (0.82%), methyl stearate (0.82%), *cis*-Linalool oxide (pyranoid) (1.40%) and 2-monopalmitin (1.51%). Linalool and oplopanone were the main compounds present in the MeOH rhizome extract but they were noticeably lacking from the EtOAc rhizome extract. In contrast, the level of *cis*-linalool oxide (furanoid) in the EtOAc rhizome extract (1.40%) was higher than that of the MeOH rhizome extract (0.13%). These discrepancies in results could be explained by variables like geographic location, collecting period, and extraction techniques.

Our research showed that the MC3T3-E1 cells did not respond negatively to the MeOH extract at dosages of 0.8, 4.0 and 20.0µg/mL (table 2). The EtOAc extract had no effect on cell growth at dosages of 0.8, 4.0, 20.0 and 100 µg/mL. These doses were chosen for the subsequent treatment of MC3T3 cells based on these findings.

After 5 days of incubation, both the EtOAc and MeOH extracts significantly increased ALP activity compared to the negative control. The EtOAc and MeOH extracts showed a substantial increase in ALP activity of 23.43% and 29.72%, respectively, at a concentration of 20µg/mL. The EtOAc and MeOH extracts significantly increased ALP activity by 9.87% and 8.42%, respectively, in comparison to the negative control at a concentration of 4 µg/mL, showing a similar pattern (table 3).

With values of 130.25% and 91.75%, respectively, compared to the negative control, the EtOAc and MeOH extracts showed the greatest increase in mineralization-stimulating activity at a concentration of 20µg/mL. In comparison to the negative control, the calcium mineralization activity of the EtOAc and MeOH extracts was 104.84%-111.33% and 115.51%-117.05%, respectively, at dosages of 0.8 and 4µg/mL (table 3).

DISCUSSION

Earlier research has documented linalool and terpene-4-ol as primary constituents of rhizome oils in *H. aromatica*, *H. sagittifolia*, *H. occulta*, and *H. cochinchinesis* (Liu *et al.,* 2014; Tiwari *et al.,* 2022; Van *et al.,* 2022; Nguyen *et al.,* 2023). A number of volatile organic compounds was found as a result of our examination into the chemical make-up of the EtOAc and MeOH extracts of *H. gigantea* rhizomes.

According to these results, the early differentiation osteoblasts had increased ALP activity, which was a property of osteoblasts (Lai *et al.,* 2014). Consequently, it was demonstrated that *H. gigantea* extracts in EtOAc and MeOH might, at different concentrations, promote osteogenic differentiation in MC3T3-E1 cells, most likely by raising ALP activity (table 3).

Apart from ALP activity, the extra cellular matrix is essential for the differentiation of MC3T3-E1 cells. Collagen, an essential component of the extra cellular matrix and a substrate for mineral deposition, is necessary for bone formation (Alcantara *et al.,* 2011). Using a Sirius Red-based colorimetric assay, the collagen content was calculated in this study. Following a 10-day incubation period in circumstances containing glycerophosphate and vitamin C, the collagen released by MC3T3-E1 cells bore a striking resemblance to that of differentiated cells (table 3, fig. 1). These findings suggested that the pathways of collagen production in cells were affected by the presence of EtOAc and MeOH extracts. Both the EtOAc and MeOH extracts enhanced collagen secretion at doses of 0.8, 4 and 20µg/mL, resulting in increases of 12.45%-10.92%, 27.76%-20.09% and 22.11%-18.76%, respectively, in comparison to the negative control.

The possible anti-osteoporotic properties of the EtOAc and MeOH extracts from *H. gigantea* were suggested by their capacity to increase ALP activity, collagen synthesis, and mineralization. At doses of 4 and 20µg/mL, the EtOAc extract exhibited the highest calcium-inducing action, whereas the MeOH extract does so to a lesser amount. These qualities may be due to the terpenoids present in the EtOAc and MeOH extracts of *H. gigantea*, including monoterpenoids, sesquiterpenes, sesquiterpenoids and diterpenoids.

Table 1: Volatile components from the EtOAc and MeOH extracts of the rhizomes of *H. gigantean*

No	Compound	RT(min)	MeOH (%)	EtOAc (%)	Classification
1	Methyl glycolate	3.40	-	0.04	ester
2	2,3-Butanediol	5.06	-	0.33	alcohol
3	2-Hydroxy-2-cyclopenten-1-one	8.53	0.56	-	ketone
4	Hotrienol	13.72	-	0.42	alcohol
5	Linalool	13.73	9.58	-	monoterpenoid
6	Dimethyl malate	13.81	-	0.12	ester
7	*cis*-Linalool oxide (furanoid)	15.12	0.13	1.40	monoterpenoid
8	Terpinen-4-ol	15.41	1.28	-	monoterpenoid
9	(*E*)-2,6-Dimethylocta-3,7-diene-2,6-diol	15.50	0.58	0.38	alcohol
10	Terpineol	15.65	0.25	-	monoterpenoid
11	3,7-Dimethylocta-1,5-dien-3,7-diol	16.23	0.46	-	alcohol
12	*cis*-Linalool hydrate	16.30	0.13	-	monoterpenoid
13	*cis*-Geraniol	16.99	0.13	-	monoterpenoid
14	Linalyl acetate	17.12	0.50	-	monoterpenoid
15	(*E*)-2,6-Dimethylocta-1,7-diene-3,6-diol	17.18	0.34	0.82	alcohol
16	*p*-Cymen-7-ol	17.56	0.09	0.06	monoterpenoid
17	2-Methoxy-4-vinylphenol	17.95	-	0.12	aromatic
18	8-Hydroxylinalool	18.92	0.18	0.67	monoterpenoid
19	*trans*-Linalool oxide (pyranoid)	19.36	-	0.66	monoterpenoid
20	α-Humulene	21.11	0.19	-	sesquiterpene
21	δ-Cadinene	22.30	0.29	-	sesquiterpene
22	Spathulenol	23.32	0.77	-	sesquiterpenoid
23	Caryophyllene oxide	23.44	0.25	-	sesquiterpenoid
24	Ledol	24.02	0.40	-	sesquiterpenoid
25	Neointermedeol	24.19	0.20	-	sesquiterpenoid
26	*α*-Cadinol	24.89	0.64	-	sesquiterpenoid
27	*τ*-Muurolol	25.22	1.25	-	sesquiterpenoid
28	*α*-Bisabolol	26.19	0.24	-	sesquiterpenoid
29	Oplopanone	27.33	7.32	-	sesquiterpenoid
30	Myristic acid	28.20	-	0.31	acid
31	Isophytol	29.98	0.34	-	diterpenoid
32	Methyl palmitate	30.51	0.93	0.50	ester
33	Palmitic acid	30.92	6.55	5.60	acid
34	Methyl linoleate	32.00	0.39	-	ester
35	Methyl oleate	32.08	0.19	-	ester
36	Methyl stearate	32.31	1.18	0.82	ester
37	Oleic acid	32.35	0.15	-	acid
38	Stearic acid	32.58	4.29	3.11	acid
39	2-Monopalmitin	34.61	2.50	1.51	ester
40	Eicosane	34.76	-	0.50	alkane
41	3-((4*Z*,7*Z*)-Heptadeca-4,7-dien-1-yl)phenol	35.53	1.98	-	aromatic
42	1-Monostearin	35.75	1.72	-	ester
43	Bis(2-ethylhexyl) terephthalate	36.05	13.83	16.64	ester
44	*α*-Tocopherol	39.11	0.29	-	aromatic
Total			60.10	34.01	
Acids			10.99	9.02	
Alkanes			-	0.50	
Ancols			1.38	1.95	
Aromatics			2.27	0.12	
Diterpenoids			0.34	-	
Esters			20.74	19.63	
Ketones			0.56	-	
Monoterpenoids			12.27	2.79	
Sesquiterpenes			0.48	-	
Sesquiterpenoids			11.07	-	

[a]Elution order on Equity-5 column, [b]Retention Indices on Equity-5 column, MS: Mass spectrum, Co-GC: Co-injection with authentic compounds, RI: Retention Index literature comparison.

Table 2: Effects of the EtOAc and MeOH extracts on the viability of MC3T3-E1 cells.

Concentration (µg/mL)	Viability (Mean ± SE)	
	EtOAc extract	MeOH extract
100	91.48 ± 0.82a	68.31 ± 2.68b
20	94.45 ± 9.24a	98.84 ± 0.65a
4	95.22 ± 2.62a	101.26 ± 3.20a
0.8	99.69 ± 0.82a	108.48 ± 4.55a

Duncan's multiple range test indicates that, at a significance level of $p<0.05$, means ± standard errors within a column that share the same letter are not substantially different.

Table 3: Impact of the MeOH and EtOAc extracts on the collagen content, mineralization and alkaline phosphatase (ALP) activity of MC3T3-E1 cells.

Concentration (µg/mL)	ALP activity (Mean ± SE)		Collagen content (Mean ± SE)		Mineralization (Mean ± SE)	
	EtOAc	MeOH	EtOAc	MeOH	EtOAc	MeOH
20	123.4 ± 1.3a	129.7 ± 6.7a	122.1 ± 8.64a	118.8 ± 8.42a	130.3 ± 3.84a	91.8 ± 8.5b
4	109.9± 2.8ab	108.4 ± 7.7ab	127.8 ± 2.14a	120.1 ± 3.8a	115.5 ± 0.62b	117.1 ± 2.2a
0.8	99.4 ± 6.4b	93.7 ± 7.1b	112.5 ± 6.44b	110.9 ± 4.6b	104.8 ± 2.64c	111.3 ± 1.9b
0 (Control)	100.0 ± 5.7b	100.0 ± 5.7b	100.0 ± 3.7b	100.0 ± 3.7b	100.0 ± 4.2c	100.0 ± 4.2b

Using Duncan's multiple range test at a significance threshold of $p < 0.05$, means ± standard errors within a column that have the same letters do not show significant differences.

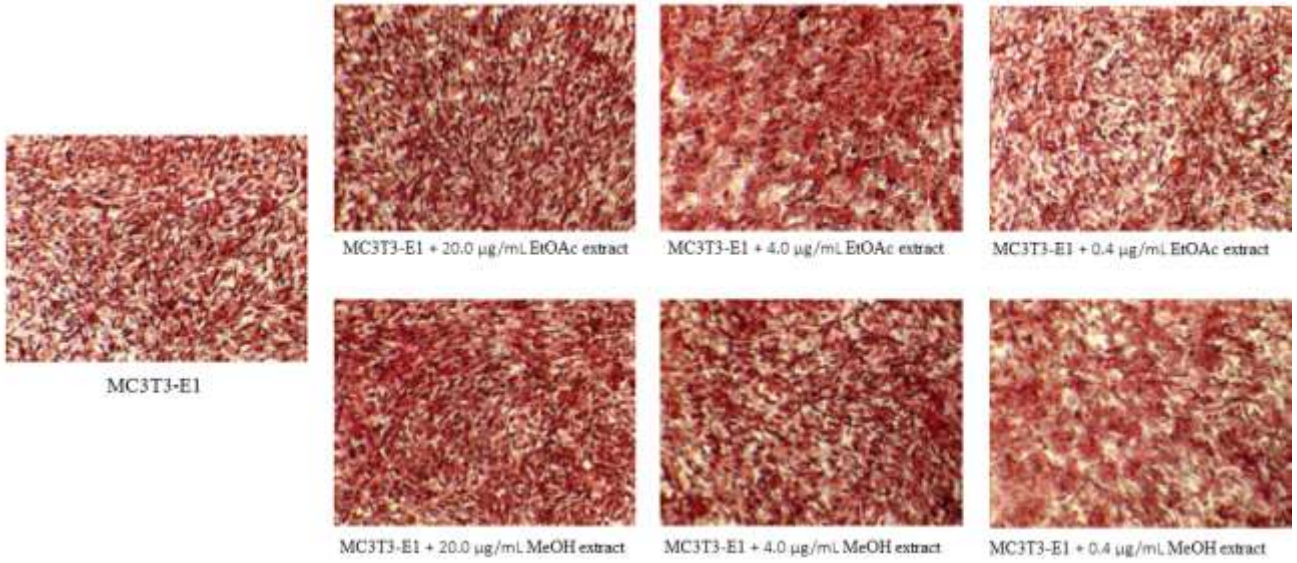

Fig. 1: Only MC3T3-E1, MC3T3-E1 + EtOAc extract and MC3T3-E1 + MeOH extract were stained with sirius red.

In a study by Hu *et al*. (2008), certain oxygenated sesquiterpenes extracted from the rhizomes of *H. occulta* were found to enhance the growth, differentiation and mineralization of cultured osteoblasts in vitro. Sesquiterpenes from the *Indonesian marine* sponge *Lamellodysidea* sp. have also been demonstrated to have inhibitory effects on BMP-induced osteoblastic development. These compounds include bicyclolamellolactone A, lamellolactones A and lamellolactones B. (Ohte *et al.*, 2021). All things considered, more research into the anti-osteoporotic properties of the EtOAc and MeOH extracts may produce encouraging findings.

CONCLUSION

The chemical analysis of the MeOH rhizome extract revealed 36 chemicals, constituting 60.10% of the extract, with notable compounds such as palmitic acid, oplopanone, linalool, and bis (2-ethylhexyl) terephthalate. Conversely, the EtOAc extract contained 19 compounds, making up 30.10% of the extract, highlighting stearic acid, palmitic acid and bis (2-ethylhexyl) terephthalate as principal components. The variations between these extracts, possibly attributed to geographical factors, extraction techniques and collection periods, were evident. Despite lacking certain key compounds present in

the MeOH extract, the EtOAc extract demonstrated higher levels of *cis*-linalool oxide (furanoid).

Moreover, the study indicated that both the MeOH and EtOAc extracts from *H. gigantea* rhizomes had no adverse effects on MC3T3-E1 cells at specific dosages. Interestingly, these extracts notably enhanced ALP activity, indicating the potential osteogenic differentiation stimulation. The increased collagen secretion and mineralization-stimulating activities further supported the osteogenic potential of these extracts. The higher calcium-inducing action of the EtOAc extract at certain doses suggested promising anti-osteoporotic properties, possibly linked to the presence of specific terpenoids.

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