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In Vitro and in Silico Analysis on the Bone Formation Activity of *N*-Hexane Fraction of Semanggi (*Marsilea crenata* Presl.)

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ABSTRACT

Background: Estrogen deficiency in postmenopausal women causes various health problems, including osteoporosis. Osteoporosis is a result of imbalances between new bone formation and old bone resorption. Phytoestrogens can be used as an alternative to increase bone formation and overcome estrogen deficiency. Semanggi (*Marsilea crenata* Presl.) is a plant that contain phytoestrogens. The aim of this research was to investigate the bone formation activity of *n*-hexane fraction of *Marsilea crenata* Presl. leaves against hFOB 1.19 cells by observing the expression of osteocalcin and predicting the phytoestrogen contents of the fraction through metabolite profiling and in silico studies.

Methods: hFOB 1.19 cells were cultured in 24 well microplates and added the *n*-hexane fraction of *Marsilea crenata* Presl. leaves at doses of 62.5, 125, and 250 ppm. Genistein 1 µM was used as a positive control. Analysis of osteocalcin expression was conducted using immunocytochemistry with CLSM. Metabolite profiling was conducted using UPLC-QToF-MS/MS. In silico study of the compounds found in metabolite profiling was conducted using molecular docking with PyRx 0.8 software and 1ERE protein.

Results: *n*-hexane fraction of *Marsilea crenata* Presl. leaves increased osteocalcin expression with the optimum dose of 62.5 ppm and a value of 457.35 AU at $p < 0.05$. Metabolite profiling of *n*-hexane fraction found 26 known compounds, 14 unknown compounds. 10 of the identified compounds showed ER-β agonists activity.

Conclusions: The *n*-hexane fraction of *Marsilea crenata* Presl. leaves increased bone formation activity. Compounds of the fraction that showed ER-β agonists activity might be phytoestrogens.

Keywords: hFOB 1.19 cell, semanggi, *Marsilea crenata* Presl., osteocalcin, phytoestrogens.

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INTRODUCTION

Postmenopause is among the stages in a series of aging processes in women. Those in the postmenopausal stage experience decreased estrogen levels, which cause various health problems, such as an imbalance between resorption and bone formation rate. Furthermore, it leads to decreased bone mass accompanied by damage to bone microarchitecture, which in turn increases the risk of fractures and osteoporosis [1, 2, 3].

Hormone Replacement Therapy (HRT) is used in postmenopausal women to replace estrogen [5]. However, the long-term use of synthetic estrogen as a hormone replacement therapy poses various risks, such as uterine endometrial bleeding, disruption of the blood clotting process, interfering with enzymes in the liver, nausea, vomiting, and cancer [3].

The use of natural ingredients as hormone replacement therapy in postmenopausal women is an alternative to overcome estrogen deficiency. Phytoestrogens are compounds in plants whose structure or function is similar to estrogen. Therefore, they can replace the body's endogenous estrogens that are less or reduced by binding with free receptors [4, 5].

Semanggi (*Marsilea crenata* Presl.) is a traditional plant and food for people in Surabaya, East Java, and it is known to contain phytoestrogens. Furthermore, *n*-hexane and ethyl acetate fraction of *Marsilea crenata* Presl. leaves showed the ability to increase trabecular bone density of female mice's vertebrae [6, 7, 8]. In vitro studies, *n*-hexane extract could increase the proliferation and differentiation processes of MC3T3-E1 preosteoblast cells [9]. The presence of phytoestrogens contained in *Marsilea crenata* Presl. which is thought to play a role in these activities. The aims of this research were to investigate the bone formation activity of the *n*-hexane fraction of *Marsilea crenata* Presl. leaves against hFOB 1.19 cells by observing the expression of osteocalcin and predicting the phytoestrogen contents of the fraction through metabolite profiling and in silico studies.

Phytoestrogen content can increase osteocalsin production in osteoblasts, which is a marker for bone formation activity [10, 11]. In vitro bone formation activity was carried out by observing the added of *n*-hexane fraction of *Marsilea crenata* Presl. leaves on hFOB 1.19 cells and investigated the increased expression of osteocalcin as a marker [10, 11].

The great potential of *Marsilea crenata* Presl., has not been fully confirmed the phytochemical compounds that play a role in the resulting activity. So that the in vitro results were continued to confirm and predict the compounds that play a role in bone formation activity, by identifying the metabolite profiling of the *n*-hexane fraction of *Marsilea crenata* Presl. leaves, with Ultra Performance Liquid Chromatography - Quadrupole Time of Flight - Mass Spectrometry (UPLC-QToF-MS/MS). Meanwhile, the estrogen receptor that plays a role in bone formation activity is ER-β, and it is used as a molecular docking for the object, where the osteoblasts responsible for bone formation are strongly influenced by the bond between estrogen and ER-β [12].

The UPLC-QToF-MS/MS method is an excellent technique to identify metabolite profiles with high resolution, fast, more sensitive, as well as time and solvent saving [13]. Also, the use of the in-silico method aims to assist in preparation development with activity to increase bone formation based on the principle of observing physicochemical and the activity of compounds with certain target proteins. The protein target uses human ER-β which has a bond with 17β-estradiol with the code 1ERE obtained from protein data bank Research Collaboratory for Structural Bioinformatics (RCSB) [14, 15]. Through in silico studies, initial hypotheses and predictions related to the potential activity of compounds were obtained by correlating the structure and physicochemical properties using analysis software [14, 16].

MATERIALS AND METHODS

Material

Marsilea crenata Presl. leaves were collected in Benowo, Surabaya, Indonesia, and identified in UPT Materia Medica, Batu, with number 1a17b-18a-1. The leaves were dried and ground to obtain powdered form. Human fetal osteoblast cell, hFOB 1.19 (CRL-11372), was purchased from American Type Cell Culture (ATCC). *n*-hexane was purchased from Merck (Merck, Darmstadt, Germany). Bovine Serum Albumin (BSA), dimethyl sulfoxide (DMSO), paraformaldehyde (PFA), and Phosphate-Buffered Saline (PBS) were purchased from Sigma-Aldrich. Anti-mouse osteocalcin (OCN) were purchased from Abcam. Dulbecco's Modified Eagle's Medium (DMEM), G418, penicillin-streptomycin 1%, fetal bovine serum (FBS) were purchased from Aretha Laboratory, Bandung, Indonesia. Tween-80, paraformaldehyde, genistein were purchased from Laboratorium Sentral Ilmu Hayati (LSIH), Universitas Brawijaya, Malang, Indonesia. Dichloromethane (DCM), methanol, acetonitrile, and formic acid as solvent and mobile phase on UPLC-QToF-MS/MS were purchased from the Central Forensic Laboratory of the Indonesian National Police's Criminal Research Agency.

Extraction and fractination

A 1.6 kg of dry powder of *Marsilea crenata* Presl. Leaves were extracted with 96% ethanol using the ultrasonic-assisted methods (Soltec Sonica 5300EP S3, Italy). The extract was further separated using a liquid-liquid extraction technique by suspending in 700 ml of water, and fractionating using *n*-hexane in a ratio of 1: 1. The *n*-hexane fraction was further evaporated using a rotary evaporator (Heidolph Hei-VAP ML / G3, Germany).

Cell culture

The hFOB 1.19 cells were put in a 75 ml flask and cultured in a complete medium prepared from a mixture of DMEM, G418, 1% Penicillin-streptomycin, 10% FBS, and incubated in a 5% CO2 incubator at 37 °C for 6 days. During the process, cell development was observed for 24 hours, and the culture medium was replaced at any time if color is changed due to the depletion of nutrients. After reaching 80-90% of confluency, cells were seeded on a 24 well microplate.

Osteocalcin measurement

50 mg of *n*-hexane fraction was mixed with 0.5% Tween-80 and 0.5% DMSO in a volumetric flask to produce a 5000-ppm solution. The solution was sterilized using a 0.22-micrometer millipore filtration method, and diluted to produce solutions of 62.5, 125, and 250 ppm [7, 9, 18]. All solutions were then mixed with hFOB 1.19 cell in a 24 well microplate and incubated for 48 hours at 37 °C in a 5% CO2 incubator. 4% PFA was added to the mixture, followed by 10% BSA. PBS was used to rinse the cells in every step of the treatment. The Anti-mouse osteocalcin was added to the microplates and incubated at 4 °C for 24 hours. Microplates were then rinsed with PBS, and anti-mouse rhodamine was added, then incubated at 37 °C for 1 hour in a dark room. Immunofluorescence analysis was conducted using CLSM (fluoview Olympus FV1000) at wavelengths of 543 nm. The immunofluorescence of markers was analyzed using Olympus Fluoview Ver.4.2a. software to determine the value of Osteocalcin expression.

Metabolite profiling

The UPLC-QToF-MS/MS method has been developed and validated according to the regulator guidelines. The *n*-hexane fraction was prepared by solid-phase extraction, and 100 ppm n-hexane fraction extract insolvent. Then injected 5 µl each into the ACQUITY UPLC® H-Class System (Waters, USA), which was coupled to an MS detector Xevo G2-S QToF (Waters, USA). The sample was separated on an ACQUITY BEH C18 (1.7 µm 2.1 × 50 mm) with acetonitrile + 0.05% formic acid and water + 0.05% formic acid as the mobile phase, with a flow rate of 0.2 ml/minute. Also, the results of the UPLC-MS analysis were processed using MassLynx 4.1 software, to obtain the data of peak and m/z spectra of each detected peak. The Compounds of *n*-hexane fraction of *Marsilea crenata* Presl. leaves from the solvent can be predicted using the ChemSpider website.

In silico study

The receptor structure of ER-β used in this study was obtained from the Protein Data Bank (http://www.rcsb.org) with the code 1ERE (14, 15). Meanwhile, initial preparation was carried out to separate the internal ligand (17β-estradiol) from its protein using the Biovia Discovery Studio Visualizer 2016. Also, secondary metabolites from the *n*-hexane fraction of *Marsilea crenata* Presl. leaves were prepared using the SwissADME webtool and predicted components of phytochemical were tested using BOILED-Egg to determine the penetration of these compounds. Internal ligand determination and compound testing used Avogadro 1.90.0, and energy optimization used the MMF94s method. Also, molecular docking simulations were performed using PyRx 0.8 and the Autodock Vina. The receptor-ligand complex from the docking simulation results was then visualized using the Biovia Discovery Studio Visualizer 2016.

RESULTS

Extraction and fractination

The results of the extraction of *Marsilea crenata* Presl. leaves with 96% ethanol are 26.5 g and fractionation obtained 11.9 g *n*-hexane fraction of *Marsilea crenata* Presl. leaves.

Cell culture

hFOB 1.19 cell culture that has been carried out showed 80-90% confluent cells and used in in vitro activity tests.

Osteocalcin measurement

Osteocalcin measurements were done using immunocytochemistry (ICC) and Confocal Laser Scanning Microscopy (CLSM) instrument. The ICC makes it possible to evaluate the cells can produce the desired marker fluorescence, so that it can measure marker bone formation, namely osteocalcin expression on hFOB 1.19 cells [10, 11]. The visualization results using CLSM can be seen (Figure 1). The results of measuring the fluorescence intensity of osteocalcin were compared with the negative and positive control (genistein) to determine bone formation activity against hFOB 1.19 cells in the form of numbers with arbitrary units (AU). This can be seen in (Table 1) and the graph results are shown in (Figure 2).

Metabolite profiling

n-hexane fraction was analyzed using UPLC-QToF-MS/MS to obtain all its phytochemical components. The result of ion separation was detected by the detector and displayed as a chromatogram in dichloromethane and methanol as a solvent which can be seen in (Figure 3) and (Figure 4). The total data number of all the compounds characterized in the *n*-hexane fraction of *Marsilea crenata* Presl. leaves were 40 peak compounds. This can be seen in (Table 2) and (Table 3). Of all the peaks, only 26 can be known and 14 are unknown compounds.

In silico study

10 compounds can bind with at least two amino acids HIS (D: 524), ARG (D: 394), and GLU (D: 353), and their binding affinity is close to 17β-estradiol. This can be seen in (Table 4).

DISCUSSION

The hormone estrogen has an important role for women, including as a protection against osteoporosis. Therefore, therapies that can be used as prevention and treatment are principally drugs that act to reduce bone loss and anabolic agents that stimulate the formation of new bone [18, 19]. The critical pathogenic mechanism associated with aging-related skeletal fragility is impaired bone formation due to an insufficient number of osteoblasts [20, 21, 22]. This decreased osteoblast number may be from a reduction in the lifespan of osteoblasts, which is enhanced by estrogen deficiency [20, 22].

Osteoblasts are primarily known for their bone-building function, which involves the deposition of an organic matrix and its subsequent mineralization [22, 23]. One of the key players in bone endocrinology is osteocalcin or bone ɣ-carboxyglutamic acid protein, a factor expressed and secreted solely by osteoblasts and was used as a serum marker of osteoblastic bone formation [24, 25, 26]. The hFOB 1.19 cells in this study were given genistein as a positive control and showed increased expression of osteocalcin. Recently, phytoestrogens such as genistein have drawn significant attention as these compounds have been shown to increase bone formation through estrogen signaling pathways, without increasing cancer risk [22].

The *n*-hexane fraction of *Marsilea crenata* Presl. leaves can increase osteocalcin expression. In this study, increasing the dose was not accompanied by a linear increase in osteocalcin expression, which was due to the non-monotonic dose-response (NMDR). NMDR often occurs in research on hormone replacement samples and is due to differences in the level of affinities between hormones and hormone replacement compounds to target proteins such as receptors, cause responses to be difficult to predict despite increased doses of treatments [27, 28, 29]. The expression of osteocalcin in hFOB 1.19 cells after presenting the *n*-hexane fraction with various doses showed that at concentrations of 62.5 ppm and 125 ppm was significantly different from negative control and positive control (genistein), while at concentrations of 250 ppm it was not significantly different from negative control and positive control (genistein). The result expression of osteocalcin showed that 62.5 ppm was the optimal concentration in increasing osteocalcin in hFOB 1.19 cells.

Of the 40 compounds detected, not all chromatogram peaks can be identified in the metabolite profiling process which is indicated by the presence of 26 known compounds and 14 unknown compounds. The unknown compounds are those that cannot be identified by the database, which can be in the form of impurity or degradation compounds that are still detected by the instrument. In fact, they could be new and are not yet in the database, especially unknown compounds that have high concentrations [15, 30].

In silico study was performed by separating the 17β-estradiol ligand with 1ERE protein, the criteria for ER-β agonist compounds is the similarity of the compound to the internal ligan. The molecular docking results of 1ERE protein bonds with 17β-estradiol as internal ligands with amino acids HIS (D: 524), ARG (D: 394), and GLU (D: 353). If the compound can bind with at least two amino acids HIS (D: 524), ARG (D: 394), and GLU (D: 353) then it is an ER agonist, and the smaller the binding affinity value, the more stable the compound [30, 31, 32]. 10 compounds were predicted as ER-β agonists with proven similarities to 17β-estradiol. This can be seen in (Table 4).

The research was conducted to study the bone formation activity of the *n*-hexane fraction of *Marsilea crenata* Presl. leaves in hFOB 1.19 cells with in vitro studies, metabolite profiles, and in silico with 1ERE protein. The correlation of the results of these studies showed that the increase in bone formation activity in vitro was supported by the prediction of 10 secondary metabolites in the *n*-hexane fraction of *Marsilea crenata* Presl. which is proven to have activity in silico.

CONCLUSION

The *n*-hexane fraction of *Marsilea crenata* Presl. has a bone formation activity through the increased Osteocalcin expression and the best dosage at 62.5 ppm by 457.35 AU. There are 10 compounds predicted by 1ERE agonists which have been shown to have similarities with 17β-estradiol. It gives an indication, that phytoestrogen content of the *n*-hexane fraction of *Marsilea crenata* Presl. leaves have a high potential as a bone formation agent.

CONFLICT OF INTEREST

No conflict of interest associated with this work.

Contribution of Authors

Research activities are carried out by the authors mentioned in the article and everything related to the content of the article will be borne by the author.

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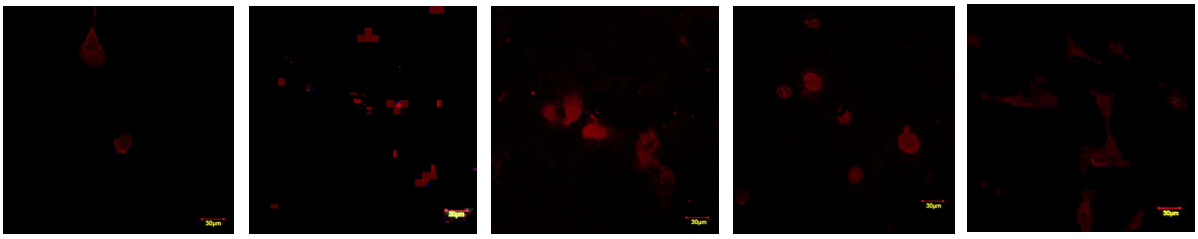


Figure 1: Fluorescence intensity of osteocalcin, (A) negative control, (B) positive control (genistein), (C) 62.5 ppm, (D) 125 ppm, (E) 250 ppm

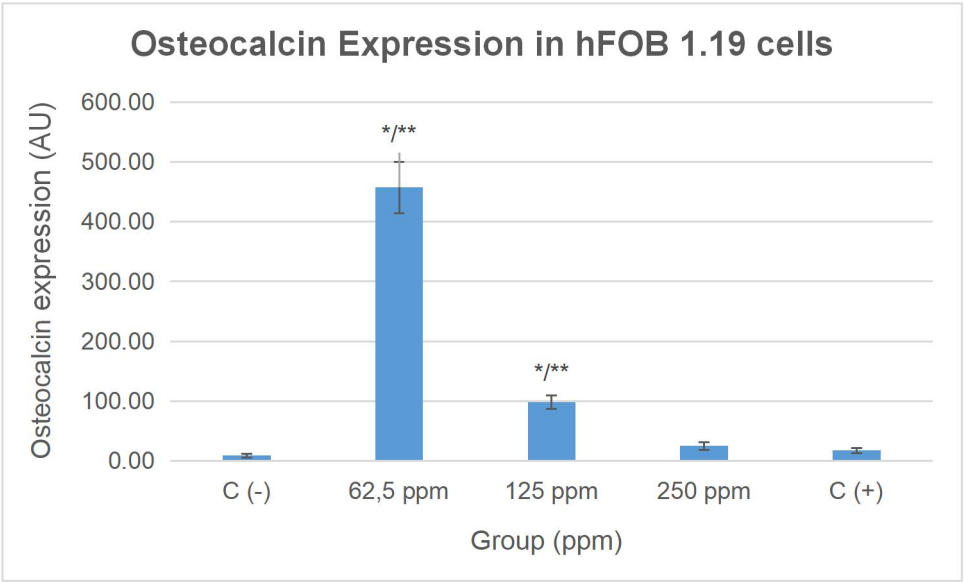


Figure 2: Osteocalcin expression in hFOB 1.19 cells. (*) significantly different from negative control and (**) significantly different from a positive control

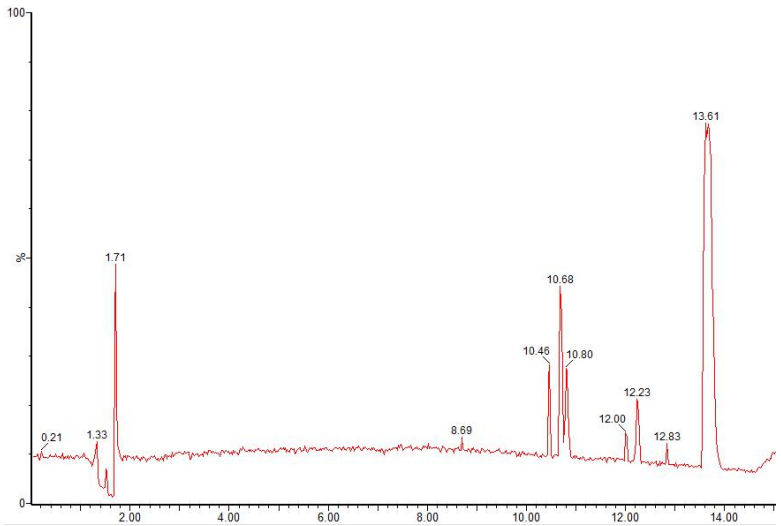


Figure 3: Chromatogram of *n*-hexane fraction of *Marsilea crenata* Presl. leaves in methanol solvent

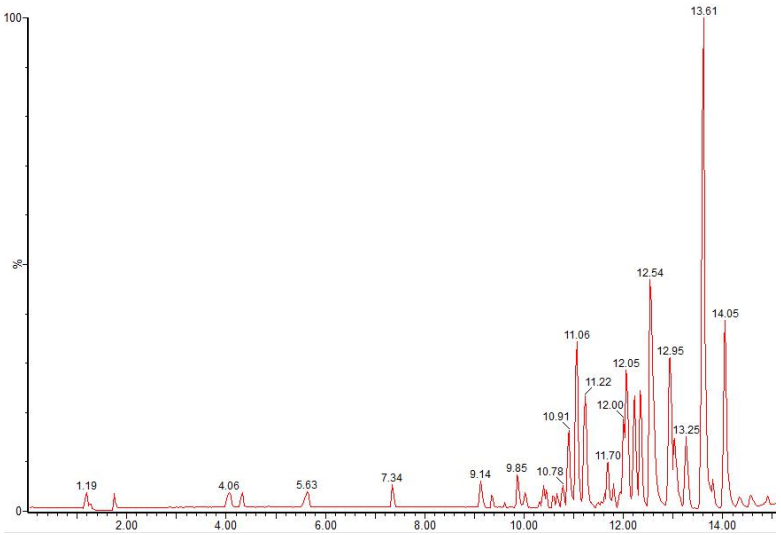
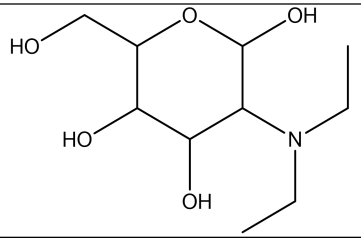
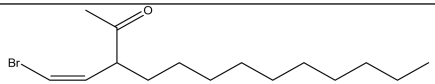
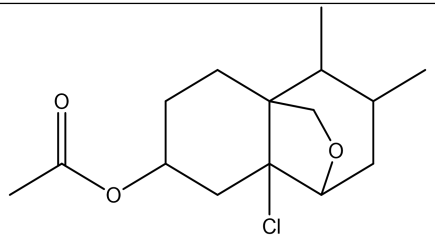
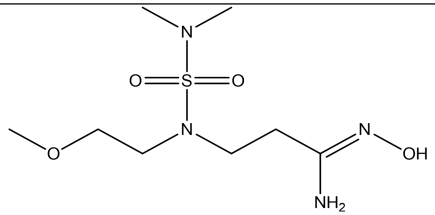
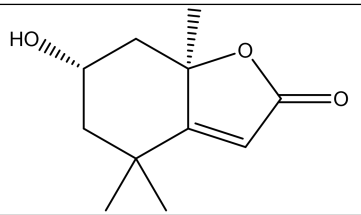


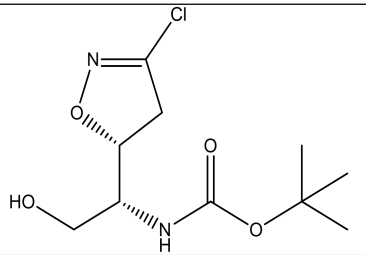
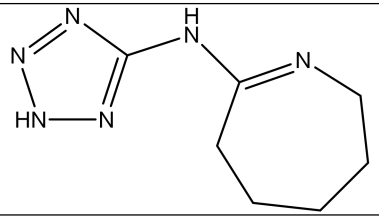
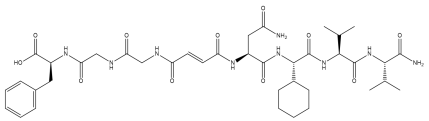
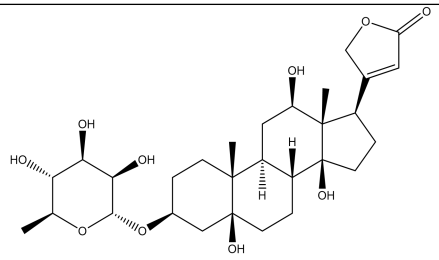
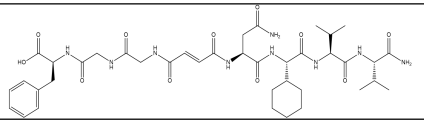
Figure 4: Chromatogram of *n*-hexane fraction of *Marsilea crenata* Presl. leaves in DCM solvent

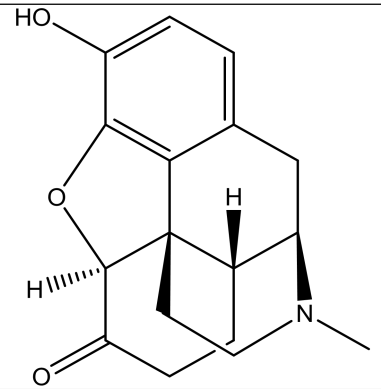
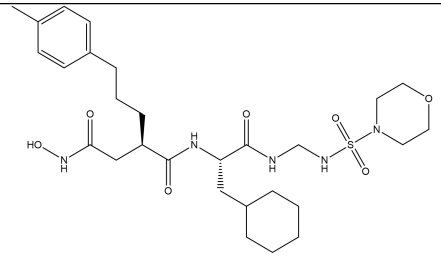
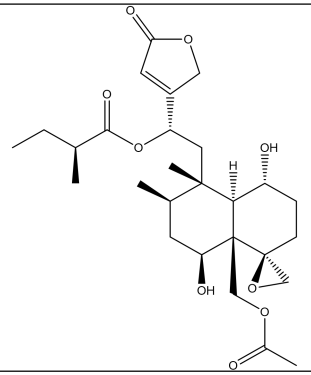
Table 1: Fluorescence intensity of osteocalcin in hFOB 1.19 cells

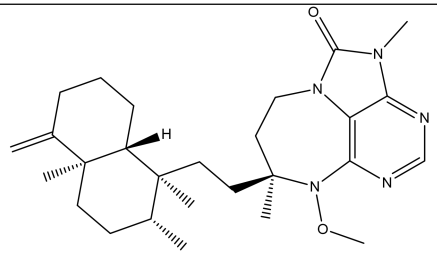
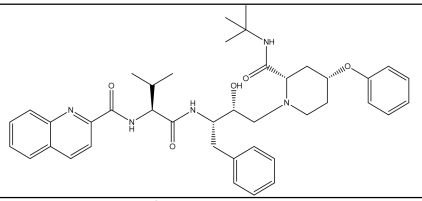
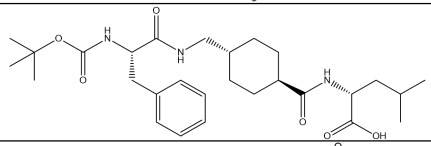
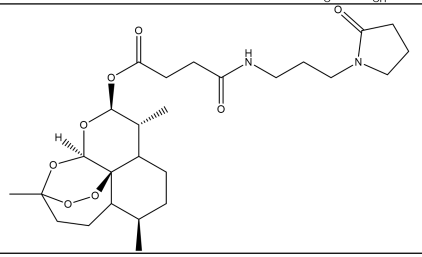
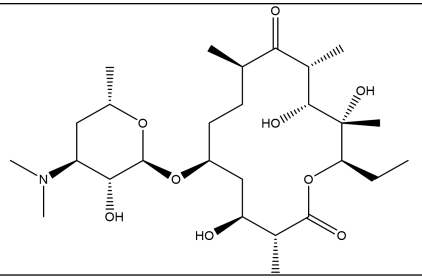
Tratment Dose	**Fluorescence intensity OCN (AU) ± SD**
Negative control	8.94 ± 3.15
Positive control	17.40 ± 4.20
62.5 ppm	457.35 ± 43.11
125 ppm	98.62 ± 11.27
250 ppm	25.01 ± 6.39

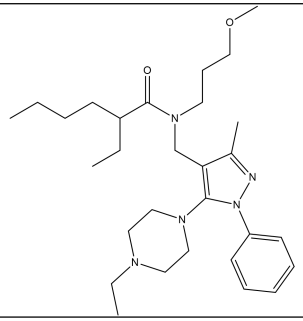
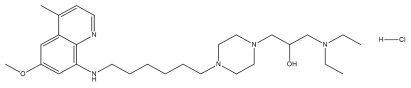
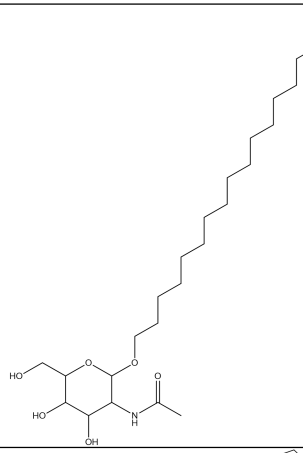
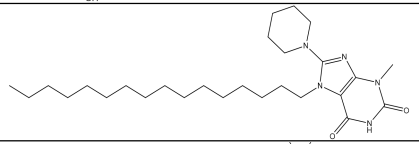
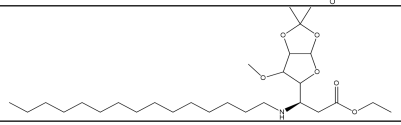
Table 2: Predicted compouds of *n*-hexane fraction of *Marsilea crenata* Presl. leaves in DCM solvent

No	Rt	% Area	*Measured Mass*	*Calculated Mass*	Molecular Formulas	Compound Name	Structure	Pub med	Activity
1	1.571	0.0067	235.1423	235.1420	$C_{10}H_{21}NO_5$	2-Deoxy-2-(diethylamino)hexopyranose		18	-
2	2.150	0.0103	191.1915	-	-	-	-	-	-
3	4.058	0.9439	302.1247	302.1246	$C_{15}H_{27}OBr$	3-[(Z)-2-Bromovinyl]-2-tridecanone		4	-
4	4.332	0.5735	286.1336	286.1336	$C_{15}H_{23}O_3Cl$	6-Chloro-9,10-dimethyl-12-oxatricyclo [5.3.2.01,6] dodec-4-yl acetate		10	-
5	4.869	0.0355	268.1206	268.1206	$C_8H_{20}N_4O_4S$	(1Z)-3-[(Dimethylsulfamoyl)(2-methoxyethyl) amino]-N'-hydroxypropanimidamide		2	-
6	5.628	1.0113	196.1100	196.1099	$C_{11}H_{16}O_3$	**Loliolide**		27	Antioksidan (Yang, 2011)

7	7.344	0.7962	264.0880	264.0877	$C_{10}H_{17}N_2O_4Cl$	2-Methyl-2-propanyl {(1R)-1-[(5R)-3-chloro-4,5-dihydro-1,2-oxazol-5-yl]-2-hydroxyethyl} carbamate		29	-
8	9.135	1.1763	180.1127	180.1124	$C_7H_{12}N_6$	N-(2H-Tetrazol-5-yl)-3,4,5,6-tetrahydro-2H-azepin-7-amine		25	-
9	9.347	0.3605	827.4181	827.4178	$C_{39}H_{57}N_9O_{11}$	(2S,11E,15S,18S,21S,24S)-15-(2-Amino-2-oxoethyl)-2-benzyl-24-carbamoyl-18-cyclohexyl-21-isopropyl-25-methyl-4,7,10,13,16,19,22-heptaoxo-3,6,9,14,17,20,23-heptaazahexacos-11-en-1-oic acid		4	-
10	9.610	0.1038	552.2933	552.2935	$C_{29}H_{44}O_{10}$	Antioside		17	-
11	9.852	1.2119	670.3174	670.3174	$C_{29}H_{46}N_6O_{12}$	N-Acetyl-L-α-aspartyl-L-α-aspartyl-L-isoleucyl-L-valyl-L-prolyl-L-alanine		9	-
12	10.010	0.4007	489.2860	489.2858	$C_{12}H_{31}N_{19}O_3$	Unknown	Unknown	-	-

13	10.389	1.3274	285.1365	285.1365	$C_{17}H_{19}NO_3$	Hydromorphone		914	-
14	10.568	0.3639	595.3035	595.3040	$C_{28}H_{45}N_5O_7S$	(2R)-N1-[(2S)-3-Cyclohexyl-1-({[(4-morpholinylsulfonyl) amino] methyl} amino)-1-oxo-2-propanyl]-N4-hydroxy-2-[3-(4-methylphenyl) propyl] succinamide		4	-
15	10.663	0.3964	508.2675	508.2673	$C_{27}H_{40}O_9$	Ajugamarin E2		3	-

16	10.780	0.5688	467.3263	467.3261	$C_{27}H_{41}N_5O_2$	asmarine E		10	-
17	10.905	2.6343	503.3061	503.3059	$C_3H_{29}N_{29}O_2$	-	-	-	-
18	11.063	5.8119	693.3891	693.3890	$C_{41}H_{51}N_5O_5$	N-[(2S)-1-{[(2S,3R)-3-Hydroxy-4-{(2S,4R)-2-[(2-methyl-2-propanyl)carbamoyl]-4-phenoxy-1-piperidinyl}-1-phenyl-2-butanyl]amino}-3-methyl-1-oxo-2-butanyl]-2-		4	-
19	11.222	4.6055	517.3152	517.3152	$C_{28}H_{43}N_3O_6$	N-[(trans-4-{[(N-{[(2-Methyl-2-propanyl) oxy] carbonyl}-L-phenylalanyl) amino] methyl} cyclohexyl) carbonyl]-D-leucine		18	-
20	11.496	0.1712	508.2781	508.2785	$C_{26}H_{40}N_2O_8$	**(5R,9R,10S,12R,13R)-1,5,9-Trimethyl-11,14,15,16-tetraoxatetracyclo[10.3.1.04, 13.08,13]hexadec-10-yl 4-oxo-4-{[3-(2-oxo-1-pyrrolidinyl)propyl]amino}butanoate**		6	-
21	11.696	2.4617	573.3256	573.3255	$C_{17}H_{39}N_{19}O_2S$	-	-	-	-
22	11.801	0.8108	495.3602	495.3605	$C_{18}H_{45}N_{11}O_5$	-	-	-	-
23	12.054	8.1891	495.3583	495.3579	$C_{14}H_{41}N_{17}O_3$	-	-	-	-
24	12.233	3.8649	531.3408	531.3408	$C_{27}H_{49}NO_9$	(3R,4S,6S,9R,11R,12R,13S,14R)-6-{[(2S,3R,4S,6S)-4-(Dimethylamino)-3-hydroxy-6-methyltetrahydro-2H-pyran-2-yl]oxy}-14-ethyl-4,12,13-trihydroxy-3,9,11,13-tetramethyloxacyclotetradecane-2,10-dione		4	-
25	12.338	4.1165	671.4105	671.4106	$C_{32}H_{57}N_5O_{10}$	-	-	-	-

26	12.538	13.0048	495.3336	495.3334	$C_5H_{37}N_{25}OS$	-	-	-	-
27	12.950	6.3419	497.3729	497.3730	$C_{29}H_{47}N_5O_2$	2-Ethyl-N-{[5-(4-ethyl-1-piperazinyl)-3-methyl-1-phenyl-1H-pyrazol-4-yl] methyl}-N-(3-methoxypropyl) hexanamide		10	-
28	13.034	3.7324	521.3493	521.3459	$C_{28}H_{48}N_5O_2Cl$	1-(Diethylamino)-3-(4-{6-[(6-methoxy-4-methyl-8-quinolinyl) amino] hexyl}-1-piperazinyl)-2-propanol hydrochloride		1	-
29	13.255	2.9255	473.3722	473.3517	$C_{26}H_{51}NO_6$	Octadecyl 2-acetamido-2-deoxyhexopyranoside		9	-
30	13.613	21.0825	473.3728	473.3730	$C_{27}H_{47}N_5O_2$	7-Hexadecyl-3-methyl-8-(1-piperidinyl)-3,7-dihydro-1H-purine-2,6-dione		48	-
31	14.045	9.2869	499.3872	499.3873	$C_{28}H_{53}NO_6$	Ethyl (3R)-3-(6-methoxy-2,2-dimethyltetrahydrofuro[2,3-d] [1,3] dioxol-5-yl)-3-(pentadecylamino)propanoate		4	-
32	14.329	0.5906	523.3642	523.364	$C_{14}H_{41}N_{19}O_3$	-	-	-	-
33	14.562	0.6677	341.3278	*Unknown*	*Unknown*	-	-	-	-

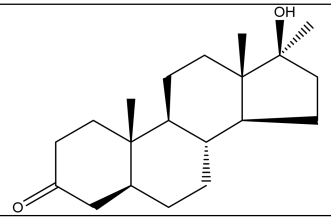
34	14.899	0.4148	304.2408	304.2403	$C_{20}H_{32}O_2$	Mestanolone		181	-

Table 3: Predicted compouds of *n*-hexane fraction of *Marsilea crenata* Presl. leaves in methanol solvent

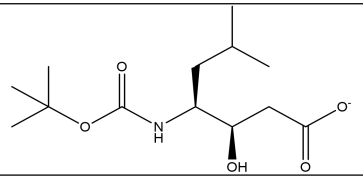
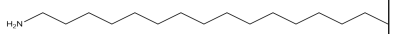
No	Rt	% Area	*Measured Mass*	*Calculated Mass*	Molecular Formulas	Compound Name	Structure	Pub med	Activity
1	1.076	11.7256	191.1621	-	-	-	-	-	-
2	1.508	6.9230	123.0915	-	-	-	-	-	-
3	8.693	14.4447	274.1654	271.1654	$C_{13}H_{24}NO_5$	(3R,4S)-3-Hydroxy-6-methyl-4-({[(2-methyl-2-propanyl) oxy] carbonyl} amino) heptanoate		2	-
4	10.801	47.6523	241.2773	241.2770	$C_{16}H_{35}N$	Cetylamine		776	Antibacteri, adjuvant for diphtheria, tetanus toxoid, and influenza (Schröckeneder, 2012)
5	12.001	9.3978	315.3144	-	-	-	-		
6	12.833	9.8565	436.2540	436.2542	$C_{19}H_{40}N_4O_3S_2$	-	-	-	-

Table 4: 17β-estradiol and Compounds of *n*-hexane fraction of *Marsilea crenata* Presl. leaves agonist with ER-β

No	Compound Name	Binding Affinity	% Area	Amino acid	Type of Bond
	17β-Estradiol	**-11**		**His 524; Arg 394; Glu 353**	**Hidrogen. Alkyl Pi-Pi T-shaped Pi-Alkyl**
1	(3R,4S)-3-Hydroxy-6-methyl-4-({[(2-methyl-2-propanyl) oxy] carbonyl} amino) heptanoate	-2.8	14.4447	His 524; Arg 394	Alkyl. Attractive Charge
2	Cetylamine	-5.9	47.6523	His 524; Glu 353	Hidrogen. Alkyl
3	6-Chloro-9,10-dimethyl-12-oxatricyclo [5.3.2.01,6]	-8.1	0.5735	His 524;	Hidrogen.

	dodec-4-yl acetate			Arg 349	Alkyl
4	(1Z)-3-[(Dimethylsulfamoyl)(2-methoxyethyl) amino]-N'-hydroxypropanimidamide	-5.2	0.0355	His 524; Glu 353	Hidrogen; Carbon
5	2-Methyl-2-propanyl {(1R)-1-[(5R)-3-chloro-4,5-dihydro-1,2-oxazol-5-yl]-2-hydroxyethyl} carbamate	-5.8	0.7962	His 524; Glu 353	Alkyl. Salt Bridge
6	asmarine E	2.3	0.5688	His 524; Glu 353	Alkyl; Unfavorable
7	(5R,9R,10S,12R,13R)-1,5,9-Trimethyl-11,14,15,16-tetraoxatetracyclo[10.3.1.04,13.08,13]hexadec-10-yl 4-oxo-4-{[3-(2-oxo-1-pyrrolidinyl)propyl]amino}butanoate	43.4	0.1712	His 524; Glu 353; Arg 349	Hidrogen. Pi-Alkyl; Unfavorable
8	Octadecyl 2-acetamido-2-deoxyhexopyranoside	2.0	2.9255	His 524; Glu 353	Hidrogen; Unfavorable
9	Ethyl (3R)-3-(6-methoxy-2,2-dimethyltetrahydrofuro[2,3-d] [1,3] dioxol-5-yl)-3-(pentadecylamino)propanoate	6.3	9.2869	His 524; Glu 353	Hidrogen; Unfavorable
10	Mestanolone	-9.5	0.4148	His 524; Arg 394	Hidrogen; Unfavorable