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Metabolite profiling and activity study of ethanol extract of *Chrysophyllum cainito* L. leaves in increasing bone density in male mice

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ABSTRACT

Phytoestrogen is a group compound which has an estrogen-like structure or function. One of its roles was in the bone formation. *Chrysophyllum cainito* L. is a plant that contains phytoestrogen. The purpose of this research is to determine the metabolite profile and activity of ethanol extract of *C. cainito* leaves to increase trabecular femur bone density in male mice. The metabolite profiling analysis was done using ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry and analyzed using Masslynx 4.1 software. Four doses of ethanol extract of *C. cainito* leaves were prepared for treatment. Which are 2,14,48, and 316 mg/ body weight (BW) mice/day in 4 weeks, after the mice were induced by dexamethasone 0.0029 mg/BW mice/day as osteoporosis model, and induction of alendronate 0.026 mg/BW mice/day as a positive control. The metabolite profiling showed a total of 41 compounds, with some compounds have activity as estrogenic such as myricetin and dibutyl phthalate. The activity test showed that ethanol extract of *C. cainito* leaves in all doses can increase the trabecular femur bone density in male mice, with an optimum dose of 16 mg/BW mice/day. This activity is probably due to myricetin and dibutyl phthalate that act as phytoestrogens in *C. cainito*, that can replace the function of estrogen in its bond with bone-estrogen receptor.

Keywords: Metabolite profiling, *Chrysophyllum cainito* L., bone density, phytoestrogen

INTRODUCTION

Osteoporosis is a condition which is characterized by decreasing of bone mass that is accompanied by destruction of bone micro - architecture and leads to increase the risk of fracture.[1,2] Osteoporosis usually occurs in postmenopausal women, which is suffering from estrogen deficiency. Estrogen deficiency is known to become the most important factors in the increment imbalance of bone formation process.[3,4] Nowadays, hormone replacement therapy become one of the main choices to treat patients with estrogen deficiency. However, this therapy have unfavorable effects such as coronary events, venous thromboembolism, stroke, breast cancer, and dementia if used as long-term therapy.[5,6]

Phytoestrogens which have estrogen-like structure can replace estrogen function in the body, in its bond with (ER-dependent pathway) or without estrogen receptors (ER-independent pathway).[7,8] It is reported that phytoestrogen has an activity for treatment for estrogen deficiency[9,10] apart from no side effects and easy to obtain.[11]

Chrysophyllum cainito L. is known to contain phytoestrogens. In a previous study, *C. cainito*

leaves contain compounds such as alkaloid, phenol, flavonoid, triterpenoid, and sterol.[12] Isoflavones and sterols can be predicted as phytoestrogens compounds because their structure similarity with 17β-estradiol.[13]

The purpose of this research is to identify the metabolite profile of ethanol extract from *C. cainito* leaves using ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QToF-MS/MS) and determine the effective dose of ethanol extract of *C. cainito* leaves. Metabolite profiling using UPLC-QToF-MS/MS was carried out to obtain a comprehensive view of compounds in ethanol extract of *C. cainito* leaves, which can be used as a reference for further research and utilization of *C. cainito*[14] for estrogen deficiency treatment. Therefore, this study is appropriate for the development of new drugs from natural ingredients, especially from *C. cainito* as a potential antiosteoporosis agent in postmenopausal women with minimum side effects.

MATERIALS AND METHODS

Material

Plant material

C. cainito leaves were collected in September 2017. Identification process conducted in UPT Materia Medica, Batu, Indonesia. Before C. cainito leaves dried and ground to produce a dry powder, specimen then given a specific number to simplify the identification process. There are 1b-2b-3b-6b-7b-9b-10b-11b-12b-13b-14a-15a-109b-119b-120a-121b-124b-125a-126b-127a.

Chemical

Ethanol, dichloromethane, methanol, acetonitrile, and formic acid as solvent and mobile phase on UPLC-QToF-MS/MS were purchased from Merck. The 10% formaldehyde, dexamethasone 5 mg, CMC-Na 0.5%, alendronate, 10% formic acid, 3% nitric acid, chloroform, 5% NaCl, Hematoxylin and Eosin were purchased from Sigma-Aldrich.

Methods

Extraction

The ethanol used to extract dry powder of *C. cainito* leaves using ultrasonic assisted extraction method (Sonica 5300EP S3). Supernatants were formed from this process, then using rotary evaporator (Heidolph) to evaporate it.

Analysis using UPLC-QToF-MS/MS

The regulator guidelines had been validated a reversed phase UPLC-QToF-MS/MS method. This method has some benefit such as simple, rapid, reliable and precise. Solid phase extraction used to prepare ethanol extract. Each 5 µl 100 ppm of ethanol extract in DCM and methanol injected into the ACQUITY UPLC® H-Class System (Waters, USA) 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 mobile phase, with flowrate 0.2 ml/min. The results of UPLC-MS analysis were processed using the Masslynx Version 4.1 software, to acquire data of peak and m/z spectra of each detected peak.

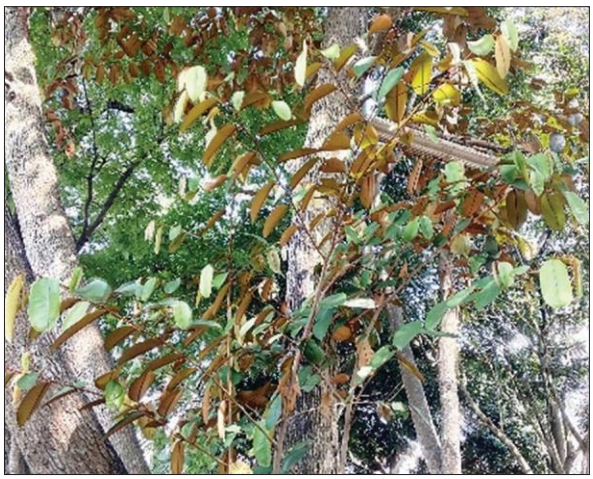

Figure 1: *Chrysophyllum cainito* L. (Personal Documentation)

The compound content then predicted using the ChemSpider database.

Bone density test

Bone density test was performed on male mice that have passed the ethical clearance, with ethics approval number 020/EC/KEPK-FKIK/2018. Bone loss by glucocorticoids such as dexamethasone can be induced in both genders. An advantage of using male mice is to avoid possible influences of the female endocrine system.[15]

The male mice were given dexamethasone 0.0029 mg/body weight (BW) mice/day in 4 weeks as osteoporosis model and randomly divided into six groups of treatment. The negative group was given CMC-Na 0.5% (without adding extract) after induced dexamethasone for 4 weeks. An alendronate 0.026 mg/BW mice/day was given as positive control group, and then treatment groups were given ethanol extract of *C. cainito* in CMC-Na 0.5% (2, 4, 8 and 16 mg mg/BW mice/day) in 4 weeks. In this study, analysis was carried out on the trabecular femur. As it was the most fragile part and often caused fractures due to aging or using of certain drug.[16] This make femur becomes appropriate variable to be analyzed.

Femur was observed using histomorphometry and HE staining methods to observed it's density. The histology of femur trabecular bones was observed with an Olympus CX23 microscope and OptiLab software.

Statistical Analysis

The results were analyzed by the *t*-test after ANOVA. Differences were considered significant at $P < 0.05$.

RESULT AND DISCUSSION

Metabolite profile of ethanol extract of *C. cainito* leaves was obtained using UPLC-QToF-MS/MS through methanol and DCM preparation. Two types of solvent used to eluate the extract optimally. The ethanol extract of *C. cainito* leaves have specific characters which summarized in Tables 1 and 2.

Any information including retention times, percentage area, measure in m/z, molecular formula, predicted

Table 1: Predicted compounds of ethanol extract of *Chrysophyllum cainito* leaves in methanol solvent

No.	RT (min)	% Area	Measured m/z	Molecular Formula	Proposed Metabolite	Activity
1	1.500	0.5829	359.1431	$C_{16}H_{26}N_3O_2SCl$	4-Amino-5-chloro-N-[2-(diethylamino) ethyl]-2-[2-(methylsulfanyl) ethoxy] benzamide	-
2	2.667	0.0996	1249790	UNKNOWN	UNKNOWN	-
3	4.382	0.1101	238.1420	$C_{10}H_{22}O_6$	Pentaethylene glycol	-
4	4.645	0.5183	299.1947	$C_{12}H_{29}NO_7$	UNKNOWN	-
5	4.896	3.0941	194.0808	$C_8H_{10}N_4O_2$	Caffeine	Neuroprotective,[17] increased estradiol production in ovarian.[18]
6	5.228	2.6907	431.2734	$C_{16}H_{29}N_{15}$	UNKNOWN	-
7	5.559	7.2157	318.0378	$C_{15}H_{10}O_8$	Myricetin	Estrogenic,[19] and renoprotective.[20]
8	5.891	0.7804	563.3516	$C_{37}H_{45}N_3O_2$	(4E)-N-[2,4-Bis (2-methyl-2-butanyl) phenyl]-4-{[4-(diethylamino) phenyl] imino}-1-oxo-1,4-dihydro-2-naphthalenecarboxamide	-
9	6.291	0.9429	386.1693	$C_{17}H_{26}N_2O_8$	1-(2,5-Dioxo-1-pyrrolidinyl) 4-(2-methyl-2-propanyl) N-{[(2-methyl-2-propanyl) oxy] carbonyl}-L-aspartate	-
10	6.691	0.6842	471.2680	$C_{33}H_{33}N_3$	4-{(E)-[(1,7-Diphenyl-2,3,6,7-tetrahydro-1H,5H-pyrido [3,2,1-ij] quinolin-9-yl) imino] methyl}-N, N-dimethylaniline	-
11	6.908	1.0304	515.2933	$C_{27}H_{41}N_5O_3S$	1-[(6,7-Dimethyl-2-oxo-1,2-dihydro-3-quinolinyl) methyl]-1,3-bis[3-(4-morpholinyl) propyl] thiourea	-
12	7.126	1.0018	559.3206	$C_{22}H_{37}N_{15}O_3$	UNKNOWN	-
13	7.274	1.4508	603.3464	$C_{39}H_{45}N_3O_3$	N, N', N''-Tris[1-(2,5-dimethylphenyl) ethyl] -1,3,5-benzenetricarboxamide	-
14	8.155	4.5071	474.2049	$C_{22}H_{30}N_6O_4S$	N-[4-Ethoxy-3-(1-pyrrolidinylsulfonyl) phenyl] -2-[4-(2-pyrimidinyl)-1-piperazinyl] acetamide	-
15	9.252	2.5179	245.2362	UNKNOWN	UNKNOWN	-
16	10.681	25.1743	273.2669	$C_{16}H_{35}NO_2$	Lauryldiethanolamine	Antimicroba[21]
17	11.379	1.8054	340.1307	$C_{12}H_{24}N_2O_7S$	N-[(2-Isopropoxyethyl) sulfonyl] glycyl-O,2-dimethylserine	-
18	11.928	8.3917	301.2980	$C_{18}H_{39}NO_2$	Safingol	Antioxidant, anticancer.[22]
19	12.294	33.3049	414.2037	$C_{19}H_{31}N_4O_4Cl$	4-[2-(Isopropylamino)-2-oxoethoxy]-3-methoxy-N-[2-(1-piperazinyl) ethyl] benzamide hydrochloride	-
20	13.094	3.5020	329.3296	$C_{20}H_{43}NO_2$	2,2'-(Hexadecylimino) diethanol	-
21	13.345	0.5948	355.3450	$C_{22}H_{45}NO_2$	N-(2-Hydroxyethyl) icosanamide	-

compounds, and activity sourced on references. There were 41 peaks of compounds found in ethanol extract of *C. cainito* leaves. From this step, not only several types of identified compounds found but also other unknown compounds. Unknown compounds were some impure compound that still detected in the instrument, which cannot identified by ChemSpider database.

Result shows, the specific compound is not yet known to have activity in bone formation. Viewed in Tables 1 and 2, based on activity data some compounds have activity as estrogenic. Estrogenic activity may explain the function of phytoestrogens as a substituent of estrogens in the ER-dependent pathway.[7]

To distinguish mice with osteoporosis from healthy mice, it can be seen from their backs. The differences in mice between before and after induced with dexamethasone are shown in Figure 2.

Osteoporosis model was made with induction of dexamethasone. Dexamethasone is one of the synthetic corticosteroids with the highest glucocorticoid content.[34,35] Glucocorticoids are estrogen agonists but have the same structure. Glucocorticoids can form bonds with ER by producing mRNA sulfotransferase (SULTE1).[36] Therefore, long-term therapy with glucocorticoids can inhibit bone formation.[36,37]

The retrieval of data was obtained from the histology of femur trabecular bone observation. All the obtained data were homogenous and normally distributed. Figure 3 shows the average result of reading specimen of femur trabecular bone for each test group.

The result of histopathology observation of femur trabecular bone of male mice in experimental groups can be seen in Figure 4. Measurement of bone mass density was done using the Motic Image Plus 3.0 software in the

Table 2: Predicted compounds of ethanol extract of *Chrysophyllum cainito* leaves in DCM solvent

No.	RT (min)	% Area	Measured m/z	Molecular Formula	Proposed Metabolite	Activity
1	2.084	4.1525	201.1724	$C_{11}H_{23}NO_2$	11-Aminoundecanoic Acid	-
2	3.215	0.1978	278.1516	$C_{16}H_{22}O_4$	Dibutyl phthalate	Antibacterial,[23] glicosidase inhibitor,[24] estrogenic.[25]
3	4.199	6.3960	122.0841	$C_7H_{10}N2$	2-(2-Pyridinyl) ethanamine	-
4	4.462	0.4560	301.1892	$C_{15}H_{27}NO_5$	Megalanthonine	Antifungal.[26]
5	4.930	0.2423	299.1939	$C_{12}H_{29}NO_7$	UNKNOWN	-
6	5.113	1.1702	343.2193	$C_{11}H_{25}N_{11}O_2$	UNKNOWN	-
7	5.342	3.3256	149.1201	$C_{10}H_{15}N$	N, N-Dimethylphenethylamine	TAAR1 agonist in human,[27]
8	5.708	1.6608	210.1253	$C_{12}H_{18}O_3$	Jasmonic acid	Antimalaria.[28]
9	6.508	0.1919	607.3782	$C_{26}H_{57}NO_{14}$	UNKNOWN	-
10	7.206	1.6855	196.1098	$C_{11}H_{16}O_3$	Loliolide	Antioxidant,[29] antipyretic, anti-inflammation, vasodilator.[30]
11	9.184	3.0011	763.5230	$C_{43}H_{73}NO_{10}$	1-[2,3,4,6-Tetrakis-O-(2,2-dimethylpropanoyl) hexopyranosyl] -5-undecyl-2-vinyl-3-pyrrolidinone	-
12	10.053	34.9810	331.0627	$C_{15}H_{13}N_3O_4S$	2-Methyl-1-[(2-methyl-5-nitrophenyl) sulfonyl]-1H-benzimidazole	-
13	10.567	0.2265	119.0939	UNKNOWN	UNKNOWN	-
14	10.967	8.1775	191.1311	$C_{12}H_{17}NO$	DEET	Insect repellent,[31]
15	11.482	15.2609	241.2771	$C_{16}H_{35}N$	Cetylamine	Antibacterial, adjuvant for diphteria, tetanus toxoid, and influenza.[32]
16	11.665	5.8381	287.2812	UNKNOWN	UNKNOWN	-
17	12.111	1.5154	310.1782	$C_{17}H_{26}O_5$	Portentol	Antioxidant, Anticancer.[33]
18	12.659	4.2821	315.3138	$C_{19}H_{41}NO_2$	3-(Hexadecylamino)-1,2-propanediol	-
19	12.877	5.5344	303.2935	UNKNOWN	UNKNOWN	-
20	13.940	1.7043	401.3496	UNKNOWN	UNKNOWN	-

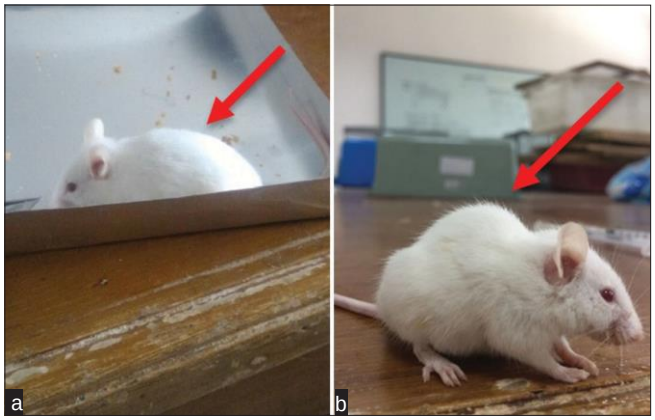

Figure 2: Normal mice (a) and mice with osteoporosis (b). Vertebrae changes to kyphosis are shown by arrows

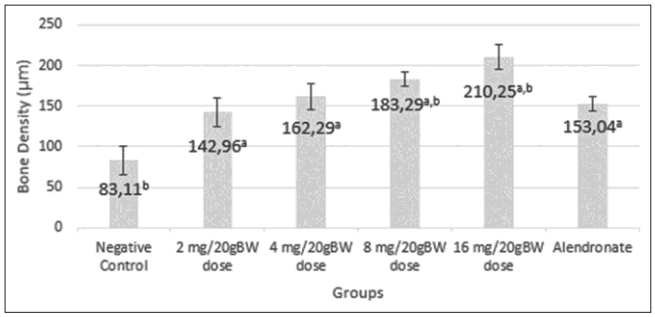

Figure 3: Trabecular Femur bone density value of mice after the administration ofi ethanol extract of *Chrysophyllum cainito* with dose variation. Each value is expressed as the mean ± SD. Significant differences in compared with negative control (a), and positive control (b), at $P < 0.05$

metaphysical section. Methapysis was the lower part of the epiphyse, which is an active part for bone growth and influences the formation of compact bone structure or bone cavity and easily measured in seeing the bone mass density and usually made to see the T-score in identifying osteoporosis. The metaphysical part is measured by 3 times replication on one side of the bone to get accurately identified parts and values.[38]

In one-way ANOVA statistical test with $P < 0.05$, to know the differences, post hoc test was done using the LSD method. The LSD test showed the significant difference between the bone density value of treatment groups in all dose and positive control group compare to the negative control group with $P = 0.000$ ($P < 0.05$). It showed that ethanol extract of *C. cainito leaves* in all dose could increase bone density value. The result of LSD test showed the significant difference

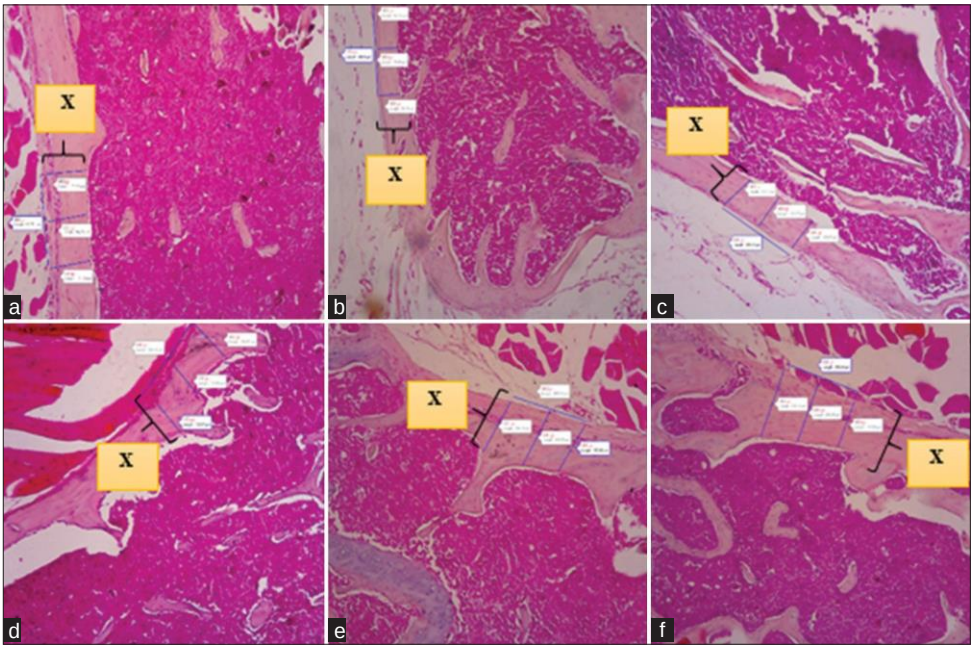

Figure 4: Histology of male mice trabecular femur bone density: (a) Positive control, (b) Negative control, (c) ethanol extract of *Chrysophyllum cainito* leaves dose 2 mg/BW mice/day, (d) 4 mg/BW mice/day, (e) 8 mg/BW mice/day, (f) 16 mg/BW mice/day. And X is ephifise part which is measured in trabecular femur bone

between the bone density value of extracted group with a dosage of 8 and 16 mg/BW mice/day compared to a positive control group with *P* value each is 0.009 and 0.000 ($p<0.05$). Meanwhile, for the extract with a dosage of 2 and 4 mg/BW mice/day with *P*-value each are 0.343 and 0.384 ($P > 0.05$) showed thati ethanol extract of *C. cainito* leaves on that dosage did not have significant difference compared to the positive control group. The data were analyzed using probit analysis to obtained effective dose (ED_{50} and ED_{99}) was given activity to increased trabecular femur bone density of male mice. The result of the probit analysis showed the effective dose value (ED_{50} and ED_{99}) were obtained 7.91 mg and 14.36 mg.

Phytoestrogens contain in ethanol extract of *C. cainito* leaves suspected this activity. Based on the results of metabolite profiling analysis, there are several compounds that have an estrogenic function, such as myricetin and dibutyl phthalate. Phytoestrogens will bind to ER in the nucleus of the bone cell. This will restore what happens to estrogen deficiency.[11] The process of osteoclastogenesis and bone resorption will decrease its activity due to the bond between phytoestrogens and ER but will increase the process of osteoblastogenesis and bone formation.[37,39-41]

In this research, we also find that phytoestrogens can affect male mice. This is because the hormone testosterone in male animals also has the same steroid core structure as estrogen. Phytoestrogens are not only ligands for ER but also androgen receptor (AR), where AR-coactivators will produce androgenic effects from phytoestrogens. So that the simple relationship between phytoestrogens and androgen activity can arise due to the close relationship with the ligand-receptor-cofactor system. Therefore, there is the possibility of phytoestrogens can also act as phytotestosterone, although it still needs to be proven by further research.[42]

CONCLUSION

Based on the analysis of UPLC-QToF-MS/MS, ethanol extract of *C. cainito* leaves contained many types of compounds, both detected compounds (41 compounds) and unknown compounds. Among the detected compounds, some compounds have activity as estrogenic such as myricetin and dibutyl phthalate. The activity test also showed that ethanol extract of *C. cainitox*leaves in all dose can increase the trabecular femur bone density in male mice, with an optimum dose of 16 mg/BW mice/day. This activity is probably due to myricetin and dibutyl phthalate that acts as phytoestrogens in *C. cainito*, that can replace the function of estrogen in its bond with ER inside the bone.

CONFLICT OF INTEREST

The author states that there is no conflict of interest with the parties involved in this study.

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