Metabolite Profiling of Ethyl Acetate Extract from *Marsilea crenata* Presl. Using UPLC-QToF-MS/MS

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Abstract: *Marsilea crenata* Presl. is a plant that widely used as traditional food in Surabaya, Indonesia. Although in some research it was known contain phytoestrogens which have activity in bone formation, the phytochemical properties of *M. crenata* has not been completely confirmed yet. The aim of this research was to determine the metabolite profile of ethyl acetate extract of *M. crenata* using UPLC-QToF-MS/MS, which can be used as a reference for further research and utilization of *M. crenata*. Dried powder of *M. crenata* was extracted with *n*-hexane followed by ethyl acetate. The 100 ppm of ethyl acetate extract in DCM and methanol then injected 5 µl each into the UPLC-QToF-MS/MS. The results were analyzed by Masslynx 4.1 software, and showed various types of compounds, either detected compounds (36 compounds), or unknown compounds.

1 INTRODUCTION

Marsilea. crenata Presl. is an aquatic plant that widely used as an ingredient for traditional food in Surabaya, Indonesia (Nurjanah and Abdullah, 2012; Ma'arif *et al*., 2016).

Figure 1: *Marsilea crenata* Presl.

Some of the research that had been done showed that 96% ethanol extract, *n*-hexane extract, and ethyl acetate extract of *M. crenata* leaves can inhibit osteoporosis in female mouse (*mus musculus*) with mechanism of bone formation improvement (Laswati, 2011; Aemi, 2012; Adityara, 2017; Widiasari, 2017). Other studies were also showed that *n*-hexane extract of *M. crenata* leaves can increase the alkaline phosphatase production in MC3T3-E1 preosteoblast cell differentiation process, which indirectly also play a role in bone formation improvement (Ma'arif *et al*., 2018).

This activity appears to be suspected because of the phytoestrogens content in *M. crenata*, where phytoestrogens can bind to estrogen receptors (ERs) in osteoblasts to increase their activity (Cos *et al*., 2003; Villiers, 2009). Phytoestrogens are a group of compounds contained in plants which have estrogen-like structures or can replace the function of estrogen, both in association with estrogen receptors (ER-dependent) and not (ER-independent) (Ososki and Kennelly, 2003; Yang *et al*., 2012; Cui *et al*., 2013).

Although it has great potential as a medicinal plants, the phytochemical properties of *M. crenata* has not been completely confirmed yet. This research was done to identify the metabolite profile of ethyl acetate extract of *M. crenata* using UPLC-QToF-MS/MS, which can be used as a reference for further research and utilization of *M. crenata.*

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UPLC-QToF-MS/MS is a powerful technique used for metabolite profiling which has improved in performance of chromatographic resolution, speed and sensitivity analysis, saves time, also reduces solvent consumption (Patil *et al*., 2011),

The ethyl acetate extract was selected because in the preliminary study using TLC visualizer, this extract showed the best TLC profile (Figure 2). Whereas metabolite profiling of *n*-hexane extract has been done before (Ma'arif *et al*., 2016).

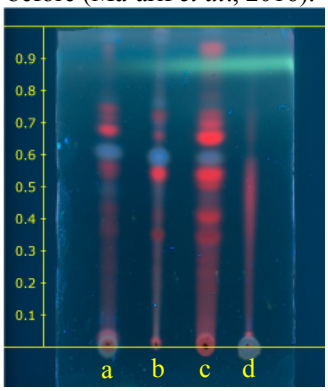

Figure 2: TLC profile of : a. 96% ethanol extract; b. n-hexane extract; c. ethyl acetate extract; and d. metanol extract; from *M. crenata* leaves at λ 366 nm.

2 MATERIAL AND METHODS

2.1 Material

2.1.1 Plant Material

M. crenata were collected in Benowo, Surabaya, Indonesia at November 2017, and identified in UPT Materia Medica, Batu, Indonesia at December 2017 with specimen number 1a-17b-18a-1. The leaves were prepared to get dry powder of *M. crenata*.

2.1.2 Chemical

All chemicals were grade of analytical reagent and used as received. N-hexane, and ethyl acetate as solvent were purchased from Pharmacy Department, Faculty of Medical and Health Science, Maulana Malik Ibrahim State Islamic University. Dichloromethane, metanol, acetonitrile, and formic acid as solvent and mobile phase on UPLC-QToF-MS/MS were purchased from Central Forensic Laboratory Badan Reserse Kriminal Kepolisian Negara Republik Indonesia.

2.2 Methods

2.2.1 Extraction

Dry powder of *M. crenata* leaves were extracted with *n*-hexane first. Its residue then re-extracted with ethyl acetate. In the preliminary study, the 96% ethanol extract was obtained by directly extracting dry powder of *M. crenata*, while methanol extract was obtained from re-extracting residue of ethyl acetate extract with methanol. All extraction process was using ultrasonic assisted extraction method (Sonica 5300EP S3). This process was repeated, collecting all the supernatants, which were finally evaporated in a rotary evaporator (Heidolph) to get ethyl acetate extract.

2.2.2 Analysis with UPLC-QToF-MS/MS

A simple, rapid, reliable and precise reversed phase UPLC-QToF-MS/MS method has been developed and validated according to the regulator guidelines. The ethyl acetate preparation was done using solid phase extraction, 100 ppm of ethyl acetate extract in DCM and methanol then injected 5 µl each into the an ACQUITY UPLC® H-Class System (Waters, USA) coupled to an MS detector Xevo G2-S QToF (Waters, USA). Sample were separated on an ACQUITY BEH C_{18} (1.7 µm 2.1x50 mm) with acetonitril + 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 was processed using the Masslynx Version 4.1 software, to obtain the data of peak and m / z spectra of each detected peak. The compound content can then be predicted using the chemspider website.

3 RESULTS AND DISCUSSION

A total of 300 g dry powder of *M. crenata* leaves were extracted with *n*-hexane and then ethyl acetate to produce 2.82 g extract. The dry powder need to be extracted first with *n*-hexane to remove impurities which may interfere with the identification process, such as fatty acid compounds. Ethyl acetate extract of *M. crenata* were analysed by UPLC-QToF-MS/MS to better interpret the diversity of available phytochemicals.

Table 1: Predicted compounds of ethyl acetate extract from *M. crenata* leaves in DCM solvent

No.	RT (min)	% Area	Measured m/z	Molecular Formula	Proposed Metabolite	Activity
1	1.272	0.3022	150.0280	Unknown	Unknown	-
2	1.420	0.2059	119.0944	Unknown	Unknown	-
3	2.118	1.0502	201.1728	$C_{11}H_{23}NO_2$	11-Aminoundecanoic acid	-
4	2.598	1.7620	122.0842	$C_7H_{10}N_2$	2-Pyridylethylamine	Histamine agonist (Kunkel and Dixon, 1984)
5	4.427	0.2680	301.1890	$C_{15}H_{27}NO_5$	Megalanthonine	Antifungal (Reina *et al*., 1998)
6	4.828	0.0245	378.1862	$C_{21}H_{30}O_4S$	Tixocortol	Corticosteroid, antiinflammatory (Friedman and Metcalfe, 1991), decongestant (Cuenant *et al*., 1986)
7	4.930	0.0063	299.1944	$C_{12}H_{29}NO_7$	Unknown	-
8	5.193	0.0799	315.1134	Unknown	Unknown	-
9	5.342	0.1373	149.1203	Unknown	Unknown	-
10	5.479	0.0713	431.2729	Unknown	Unknown	-
11	5.662	0.0830	210.1255	Unknown	Unknown	-
12	5.959	0.0335	519.3245	$C_{27}H_{45}N_5O_3S$	3,5-Isothiazoledicarboxamide, 4-amino-N^3,N^5-dicyclohexyl-N^5-[1-[[(3-methylbutyl) amino] carbonyl]butyl]-	-
13	6.211	0.0193	545.3508	Unknown	Unknown	-
14	6.623	0.0089	462.2615	$C_{13}H_{39}N_{10}O_4PS$	Unknown	-
15	7.206	0.4010	196.1099	$C_{11}H_{16}O_3$	1-carboxy-3-hydroxyadamantane	-
16	7.972	0.1522	271.1930	$C_{12}H_{26}N_5P$	Pyrrolidine, 1,1',1"-(hydrazinylidenephosphoranylidyne)tris-	-
17	9.733	0.0992	256,1936	$C_{17}H_{24}N_2$	1H-Benzimidazole, 1-(2-cyclohexylethyl)-5,6-dimethyl-	Antituberculosis, antibacterial (Gobis *et al*., 2015)
18	10.967	0.4997	191.1309	Unknown	Unknown	-
19	11.448	1.0321	241.2772	$C_{16}H_{35}N$	Hexadecylamine	Antibacterial, adjuvant for diphtheria, tetanus toxoid, and antiinfluenza (Attwood and Florence, 2012)

20	11.630	0.5779	386.1728	$C_{22}H_{26}O_6$	Benzophenone, 2-(1-ethylacetonyl)-3',4,4',5-tetramethoxy-	-
21	11.882	0.0066	310.1203	$C_{19}H_{18}O_4$	Benzylbutylphthalate	Estrogenic activity (Harris *et al*., 1997)
22	12.111	0.1000	310.1775	$C_{17}H_{26}O_5$	Portentol	Antioxidant, anticancer (Schröckenedeer, 2012)
23	12.842	0.1933	3032925	$C_{21}H_{37}N$	Pregnan-3-amine	-
24	13.345	0.0078	228.1152	$C_{15}H_{16}O_2$	Bisphenol A	Estrogenic activity (Hewitt and Korach, 2010)
25	13.940	0.1502	567.4201	$C_{36}H_{58}NO_2P$	Dibenzo[d,f][1,3,2]dioxaphosphepin-6-amine, N,N-dibutyl-2,4,8,10-tetrakis(1,1-dimethylethyl)-	-
26	14.077	0.1513	531.3416	$C_{28}H_{45}N_5O_5$	Glycine, N-[[(E)-2-(4-methoxyphenyl)diazenyl]carbonyl]leucyl-, compd. with N-cyclohexylcyclohexanamine (1:1)	-
27	15.038	3.7928	627.1884	$C_{33}H_{30}N_5O_6Cl$	1H,5H-Pyrrolo[3,4-g][1,2,4]triazolo[1,2-a]cinnoline-1,3,8,10(2H,7H,9H)-tetrone, 7-(3-chloro-4-hydroxy-5-methoxyphenyl)-7a,10a,11,11a-tetrahydro-2-methyl-9-[(4-methylphenyl)amino]-7a-phenyl-	-
28	16.970	36.4625	775.2261	$C_{38}H_{38}N_5O_{11}Cl$	(1R,13S,16S,17R,28R)-28-Amino-20-chloro-17,25-dihydroxy-5,8,10,24-tetramethoxy-N-methyl-15,29,31-trioxo-22-oxa-14,30,32triazahexacyclo [14.14.2.2^{18,21}.1^{2,6}.1^{23,27}.0^{7,12}]hexatriaconta-2(36),3,5,7,9,11,18,20,23(33),24,26,34-dodecaene-13-carboxamide	-
29	17.633	34.5167	592.2692	$C_{30}H_{33}N_{12}P$	Unknown	-
30	17.885	10.8884	849.2460	$C_{52}H_{41}N_5OPSCl$	Unknown	-
31	18.330	6.4550	701.2070	Unknown	Unknown	-

32	21.658	0.0608	156.9950	$C_4H_3N_3O_2S$	1H-Pyrazolo[3,4-d]thiazole-3,5(2H,6H)-dione	-

Table 2: Predicted compounds of ethyl acetate extract from *M. crenata* leaves in methanol solvent

No.	RT (min)	% Area	Measured m/z	Molecular Formula	Proposed Metabolite	Activity
1	0.581	0.0068	124.9797	$C_3H_5NCl_2$	3,3-Dichloro-2-propen-1-amine	-
2	1.500	1.0634	235.1421	$C_{10}H_{22}NO_5$	Nitromethanetrispropanol	-
3	2.266	0.1459	122.0478	$C_6H_6N_2O$	Nicotinamide	Activity of diphosphate (ADP) - ribosyltransferase (Maurer *et al*., 2012), anti-SIRT2 (Cui *et al*., 2014).
4	4.016	0.0642	124.9789	Unknown	Unknown	
5	5.045	0.1590	149.1201	$C_{10}H_{15}N$	(S)-(+)-Methamphetamine	Increase activity of neurotransmiter norepinefrin and dopamine, reduce appetite (Ward *et al*., 2016).
6	5.228	0.1070	431.2733	$C_{18}H_{41}NO_{10}$	Unknown	
7	5.445	0.0977	466.2989	$C_{33}H_{37}N_3$	(1E)-1-(2,2",4,4",6,6"-Hexamethyl1,1':3',1"-terphenyl-2'-yl)-3-mesityl-1-triazene	-
8	5.662	0.0169	519.3256	$H_{34}N_{31}OCl$	Unknown	
9	7.206	4.6301	196.1102	$C_{11}H_{16}O_3$	1-carboxy-3-hydroxyadamantane	-
10	8.006	0.2579	125.1882	$C_{12}H_{25}NO_2$	12-Aminododecanoic acid	Hamper expression of CD_{40} (Albertshofer *et al*.,2005)
11	8.886	0.0908	119.0941	Unknown	Unknown	
12	10.533	1.4306	180.1148	$C_{11}H_{16}O_2$	2-tert-butyl-4-methoxyphenol	Antioxidant (Lee *et al*., 2006)
13	11.013	0.6199	224.1886	$C_{13}H_{24}N_2O$	Ethyl (4S)-5-cyclohexyl-2,2-difluoro-4-{[(2S)-2-{[N-(4-morpholinylsulfonyl)-L-phenylalanyl]amino}-4-pentenoyl]amino}-3-oxopentanoate	-

14	11.379	0.2271	340.1314	$C_{20}H_{20}O_5$	Morachalcone A	Tyrosinase Inhibitors (Nguyen *et al*., 2012), inhibition of nitric oxide (Joo *et al*., 2014), pancreatic lipase inhibitory (Jeong *et a*l.,2015)
15	11.562	3.0017	310.1200	$C_{14}H_{19}N_4O_2Cl$	Lintopride	Treatment of gastrointestinal reflux, nausea and vomiting (Delvaux *et al*., 1995)
16	11.996	0.1281	332.1961	$C_{16}H_{24}N_6O_2$	8-(4-Ethyl-1-piperazinyl)-3-methyl-7-(2-methyl-2-propen-1-yl)-3,7-dihydro-1H-purine-2,6-dione	-
17	12.431	4.2855	503.3096	$C_{25}H_{45}NO_9$	Pederin	Antioxidant, anticancer (Ghoneim, 2013)
18	12.614	0.6065	693.3941	$C_{33}H_{59}NO_{14}$	Methyl {[(9Z)-17-{[(2R,3R,4S,5S,6R)-4,5-dihydroxy-6-(hydroxymethyl)-3-{[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl]oxy}tetrahydro-2H-pyran-2-yl]oxy}-9-octadecenoyl]amino}acetate	-
19	12.911	0.2985	363.3127	$C_{18}H_{42}N_5Cl$	Unknown	
20	13.208	0.7061	276.2087	$C_{18}H_{28}O_2$	Phenyl laurate	Antimicroba, antihipertensio n (Edwin and Edmund,1940)
21	13.460	0.3641	495.3566	$C_{24}H_{46}N_9Cl$	Unknown	
22	13.791	2.6389	531.3408	$C_2H_{37}N_{29}O_2S$	Unknown	
23	14.306	0.8403	698.5889	$C_{30}H_{75}N_{14}O_2Cl$	Unknown	
24	15.541	21.6948	698.5885	$C_8NO_{15}S_6Br_2$	Unknown	
25	16.718	11.5201	698.5896	$C_{43}H_{86}S_3$	Unknown	
26	17.153	11.2271	592.2689	$C_{35}H_{36}N_4O_5$	Pheophorbide A	Antiinflamatio n, antioxidant (Vencl *et al*., 2009), anti-HIV (Zhang *et al*., 2003)

27	17.370	0.6928	592.2694	$C_{36}H_{40}N_4S_2$	1,1'-(1,4-Butanediyl)bis{2,6-dimethyl-4-[(3-methyl-1,3-benzothiazol-2(3H)-ylidene)methyl]pyridinium}	-
28	18.330	33.0776	698.5885	$C_8NO_{15}S_6Br_2$	Unknown	-

Table 1 and Table 2 summarise all the compounds characterized in ethyl acetate extract of *M. crenata*, including retention times, % area, measured m/z, molecular formula, putative compounds, and its activity based on references.

In total there were 32 peak of compounds identified in the DCM solvent, and 28 peak in the methanol solvent. The use of two types of solvent aimed to elute the ethyl acetate extract optimally. From all the peaks, only 36 peaks can be identified, while the rest are unknown compounds.

Unknown compounds may be identified as impure compounds which are still detected by the instrument, or they may be a new compounds, which is undetectable in chemspider database, especially unknown compounds with high concentrations.

Based on the results of this study, it is not yet known which compounds are likely to have activity as phytoestrogens, but when viewed from the activity data in Table 1 and Table 2, it is known some compounds have activity as antioxidants. Where antioxidants is one form of phytoestrogens activity, the ER-independent pathway. Phytoestrogens can work through two pathways, both ER-dependent and ER-independent pathway. Although most biological actions of phytoestrogens are mediated through ERs in cells (ER-dependent), its can exert antioxidant effects and suppress oxidative stress through an ER-independent pathway. Phytoestrogens effectively prevent pro-oxidant stress by limiting ROS release from damaged mitochondria, and provides antioxidant activity in cells (Cui *et al*., 2013).

4 CONCLUSIONS

From UPLC-QToF-MS/MS analysis, it is concluded that ethyl acetate extract of *M. crenata* leaves contain various types of compounds, either detected compounds (36 compounds), or unknown compounds. The unknown compounds still need to be investigated further, especially those with high concentrations.

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