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Research Article

Characterization of Secondary Metabolites Profile from Methanol Fraction of Temurui (*Murraya koenigii* (Linn.) Spreng) Leaves Using UPLC-MS

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Abstract

The leaves of the temurui (*Murraya koenigii* (Linn.) Spreng) plant have long been known and used by Indonesian and even Asian people as a traditional medicine to treat stomach aches and diabetes. This study aimed to determine the secondary metabolite profile of the methanol extract of *M. koenigii* leaves. *Murraya koenigii* leaves fine powder was extracted in stages using *n*-hexane, ethyl acetate, and methanol. Each extract was prepared using a mixture of water, formic acid, acetonitrile, and formic acid, then injected into the UPLC-MS, then analyzed with MassLynx and ChemSpider. The results showed that the metabolite profile of the methanol extract of *M. koenigii* leaves contained 13 compounds, including phenolic, steroid, and alkaloid groups. Those compounds could be tested to identify their bioactivity.

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INTRODUCTION

After Brazil, Indonesia is the second largest country with tropical and subtropical forests[1,2]. The diversity and richness of these biological sources provide opportunities for researchers to investigate nutritious chemical compounds that can be processed as raw materials for industry, food, and medicine[3,4]. The community still uses plants as traditional medicine, particularly in rural areas where plant diversity is still abundant[5,6]. This is because plants contain chemical compounds that result from both primary and secondary metabolism[7,8].

Temurui (*Murraya koenigii* (Linn.) Spreng) is one of the plants that has been used for a long time. *Murraya koenigii* fruit has medicinal properties. The root component protects the liver and cures kidney disease[9,10]. The bark of *M. koenigii* has been shown in rats to be anti-diabetic[11]. The leaves of *M. koenigii* have long been used as a flavoring spice by the people of Indonesia, India, and Bangladesh. Aside from that, *M. koenigii* leaves are used as a stomachache remedy[12].

Murraya koenigii, particularly its leaves, has been the subject of extensive research to determine the active substance content and test its bioactivity. *Murraya koenigii* leaves contain phenolic compounds with various bioactivity properties, including antioxidants[13,14], antihyperglycemic[15], anticancer[16], and antimicrobial[17,18]. These active compounds are mostly flavonoids, saponins, terpenoids, steroids, and carbazoles[19-21]. Amna *et al.*[22] successfully conducted phytochemical screening and testing of anticancer properties of M. *koenigii* leaves *n*-hexane extract. This study shows that *M. koenigii* leaves *n*-hexane extract

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contains active terpenoid and steroid compounds. Aside from that, the study discovered that *M. koenigii* leaves have anticancer properties against HeLa cancer cells. Chakraborty[21] determined the active compound content of *M. koenigii* stem bark methanol extract, known to contain mumunin and mahanimbin, which were carbazole alkaloids. Arunkumar[23] examined *M. koenigii* leaves extracted with methanol. The study found that methanolic *M. koenigii* leaves extract had anti-inflammatory properties when tested on albino rats. Mathur *et al*.[24] observed the compound isolated from *M. koenigii* and its bioactivity using the GC-MS analysis. The profile of secondary metabolites in the methanol extract of *M. koenigii* leaves were 1-methyl-pyrrolidine-2-carboxylic acid (69.00%), ethyl α-d glucopyranoside (13.36%), isolongifolene (3.86%), c-himachalene (2.88%), 1,2-ethanediol monoacetate (2.79%), and 1,2-benzenedicarboxylic acid diisooctyl ester (2.55%).

The secondary metabolite profile in the methanol extract of *M. koenigii* leaves using the UPLC-MS analysis still needed to be done. As a result, this study was carried out to identify the secondary metabolites present in the methanol extract of *M. koenigii* leaves via phytochemical screening, followed by the addition of secondary metabolite profiles to the methanol extract via UPLC-MS. Compared to the GC-MS analysis, the UPLC-MS technique is effective and highly selective in preparing ions in mixtures based on mass ratios to provide ionic fragments of a chemical structure. It is also susceptible and requires a small sample[25]. These findings will be used to test the bioactivity of *M. koenigii* leaves.

MATERIALS AND METHODS

Materials

The materials used include fresh *M. koenigii* leaves, *n*-hexane (Merck), ethyl acetate (Merck), methanol (Merck), Mayer's reagent, Liebermann-Burchard reagent, chloroform (Merck), distilled water, iron (III) chloride (Merck), potassium iodide (Merck), concentrated sulfuric acid (Merck), sodium hydroxide (Merck), magnesium powder (Merck), and ammonia (Merck). The instrument used includes UPLC-MS (Acquity UPLC®H-Class System). The extraction was done at the Politeknik ATI Padang, while the Jakarta Forensic Laboratory Center tested the chemical composition of *M. koenigii* leaves extract with UPLC-MS.

Methods

Plant identification

The plant was collected in the Loung Bata, Banda Aceh, Indonesia. Plant identification was performed at the Taxonomy Laboratory, Universitas Andalas, Padang. Based on the findings, the plant (**Figure 1**) was classified as belonging to the Rutaceae family, with the species *M. koenigii* (L) Spreng. The number of the plant determination certificate was 285/K-ID/ANDA/XI/2014.

Figure 1. *Murraya koenigii* leaves.

Extraction

The fresh plant was dried and crushed into powder. The fine powder was weighed at 5 kg and extracted using the maceration method with *n*-hexane solvent thrice in 24 hours. The mixture was then filtered to extract *n*-hexane and residues. The residues were macerated with ethyl acetate solvent to obtain ethyl acetate extract. Finally, methanol was used to macerate the extracts, which were then concentrated using a rotary evaporator. The yield was calculated after weighing the resulting extract.

Phytochemical tests[26]

Fresh *M. koenigii* leaves were weighed to 2 g, placed in a test tube, and extracted with methanol, chloroform, and distilled water in a 1 : 1 ratio for 15 mL each. The mixture was then shaken to form two layers. Terpenoid and steroid compounds were examined in the chloroform layer, while flavonoids, phenolic, and saponin compounds were examined in the water layer.

Flavonoid analysis (cyanidin test): A layer of 1 mL of water, concentrated hydrochloric acid, and a few grains of magnesium powder were placed in a test tube. The transformation of orange to red indicated the presence of flavonoids.

Phenolic analysis: A layer of 1 mL of water was placed in a test tube, and iron (III) chloride solution was added. A positive phenolic group was indicated by the formation of a blue or dark purple color.

Saponin analysis: A layer of water up to 1 mL was placed in a test tube and shaken vigorously afterward. If a foam was formed that did not disappear when a few drops of concentrated hydrochloric acid were added, this indicated the presence of saponins.

Steroid and triterpenoid analysis: The layer of chloroform was dripped onto the drip plate. Then, acetic anhydride was added. The mixture was then shaken and dripped with concentrated sulfuric acid. The formation of a brick-red color indicated the presence of triterpenoids, and the presence of steroids was indicated by the formation of a blue ring.

Alkaloid analysis: A 10 mL chloroform layer containing ammonia and 2 N sulfuric acid was added. Mayer's reagent was added after the acid layer was separated. The presence of alkaloids was indicated by the formation of a white precipitate.

Secondary metabolite analysis using UPLC-MS

The metabolite profile of *M. koenigii* leaves extracts in methanol, ethyl acetate, and *n*-hexane was investigated using UPLC-MS with a BEH C18 column (1.8 µm 2.1x50 mm), with column temperature 50°C. The mobile phase in this study consisted of two mixed solutions: water and 0.1% formic acid, as well as acetonitrile and 0.1% formic acid. With an isocratic system, the flow rate was 0.2 mL/minute. This experiment lasted 23 minutes. The UPLC-MS data were then analyzed as a chromatogram with the MassLynx 4.1, allowing the molecular formula to be obtained and analyzed via the web ChemSpider so that the compound names could be known. Then, using the ChemDraw, the compound's structure was determined.

RESULTS AND DISCUSSION

Table I shows the results of testing the phytochemical profile of *M. koenigii* leaves with *n*-hexane, ethyl acetate, and methanol solvents. The phytochemical profile was tested to detect the presence of phytochemicals in different extracts of *M. koenigii* leaves. Our results highlight that all the extracts contain phenolics, steroids, and terpenoids. The Liebermann-Burchard method is used to test steroids and triterpenoids. After reacting with Liebermann-Burchard reagent (glacial acetic acid-concentrated H_2SO_4), the extract crude methanol fractions showed positive results for steroids with the formation of blue ring while the extract crude methanol fractions showed positive results for terpenoids with the formation of brick-red color. The hue of the solution created by steroids and triterpenoids differs due to the differences in groups on the C-4 atom[27]. However, flavonoids, alkaloids, and saponins were absent in the *n*-hexane extract. It may be due to the poor solubility of these phytochemicals in *n*-hexane. Steroids, flavonoids, and alkaloids are extracted in semi-polar and polar solvents; the flavonoid group is found in ethyl acetate and methanol extracts.

Table I. Phytochemical profile of *M. koenigii* leaves.

No	Chemicals content	Reagents	*n*-hexane extract		Ethyl acetate extract		Methanol extract	
			Result	Conclusion	Result	Conclusion	Result	Conclusion
1	Phenolic	Iron (III) chloride	Dark purple color	+	Dark purple color	+	Dark purple color	+
2	Flavonoid	Cyanidin test	Red color is not presented	-	Red color	+	Red color	+
3	Steroid	Liebermann-Burchard	Blue ring	+	Blue ring	+	Blue ring	+
4	Alkaloid	Mayer's	White precipitate is absent	-	White precipitate	+	White precipitate is absent	-
5	Terpenoid	Liebermann-Burchard	Brick-red color	+	Brick-red color	+	Brick-red color	+
6	Saponin	Aquadest	Not foamy	-	Foamy	+	Foamy	+

Note: + = Detected, - = Not detected.

Further analysis was carried out to measure the extract results to understand the solvent's effect on the extraction results. The weight of each extract with its yield obtained from a multilevel maceration process starting with *n*-hexane, ethyl acetate, and methanol solvents can be seen in **Table II**. According to this data, methanolic extracts had the maximum extraction yield. Because the yield of methanol extract generated is more significant than that of ethyl acetate and *n*-hexane extracts, the chemicals in *M. koenigii* leaves may be more polar. Furthermore, the biological activity of *M. koenigii* leaves methanolic extracts has been examined[14]. These findings indicate that the methanolic extract was the most effective antioxidant because it had the highest quantity of phenolic and flavonoid compounds. Methanol is the optimum solvent for extracting bioactive compounds from *M. koenigii* leaves.

Table II. Multilevel extraction with *n*-hexane, ethyl acetate and methanol solvents on a 1500 g sample.

No	Extracts	Weight (g)	Yield (%)
1	*n*-hexane	52.96	3.53
2	Ethyl acetate	61.49	4.09
3	Methanol	63.22	4.21

Further research was performed using UPLC-MS to identify the chemical content of the methanolic extract from *M. koenigii* leaves. Furthermore, as indicated in **Figure 2** and **Table III**, the chromatogram examination of the phytochemical profile of metabolites from *M. koenigii* leaves methanol extract yielded 21 peaks.

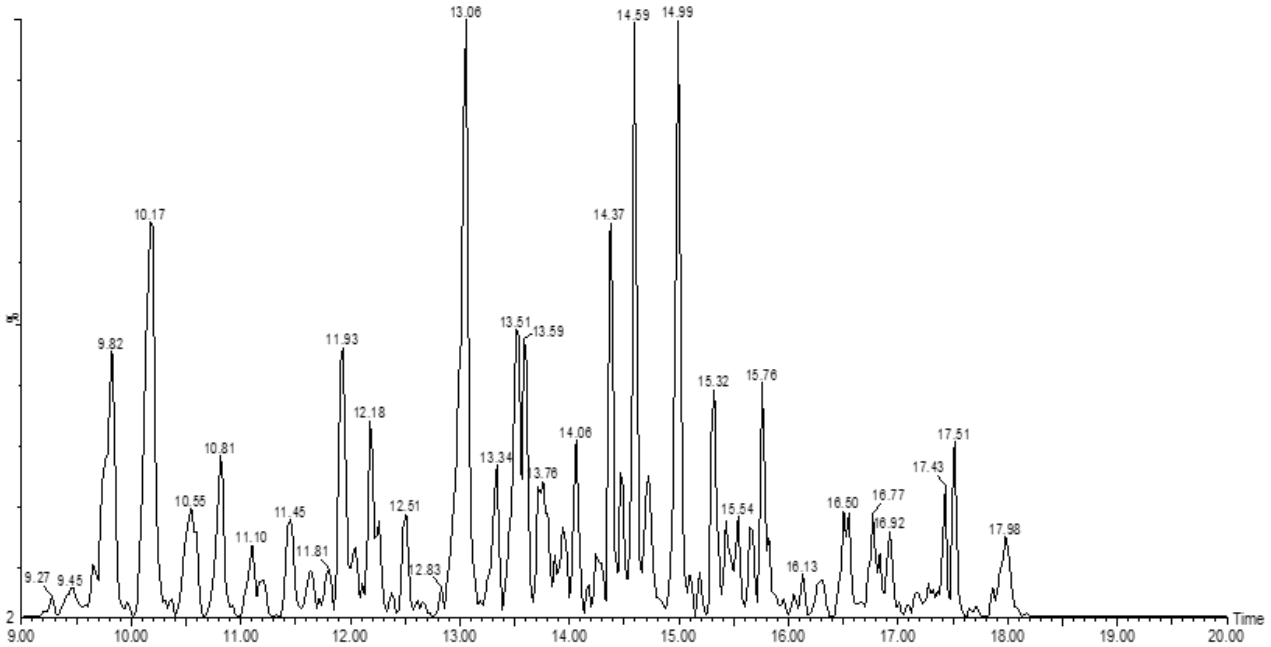


Figure 2. Chromatogram of *M. koenigii* leaves samples with methanol extract.

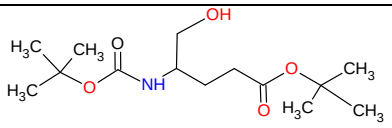
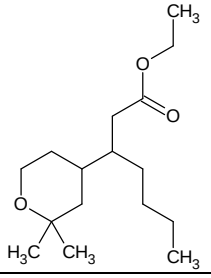
Table III. UPLC-MS chromatogram peaks methanol extract of *M. koenigii* leaves.

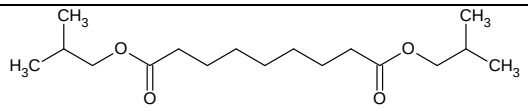
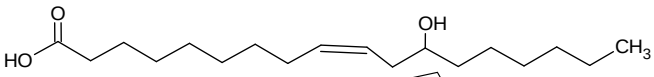
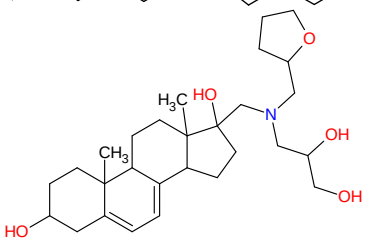
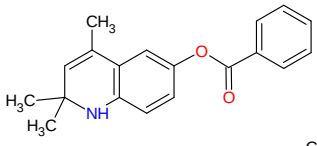
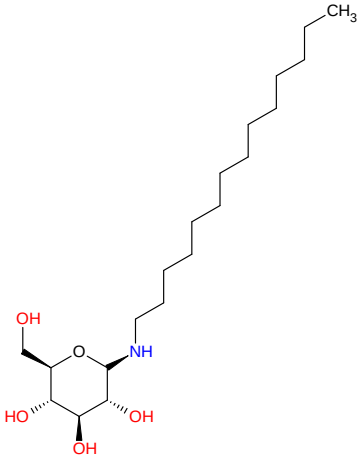
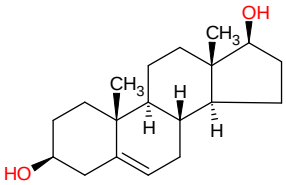
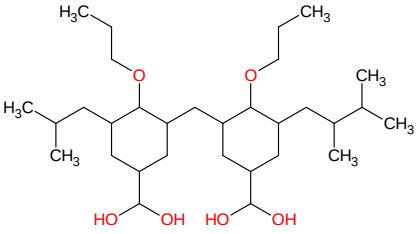
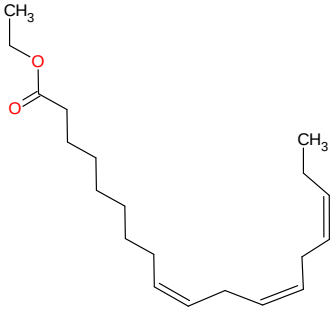
No.	Peak	R_t (minute)	% Area
1	1	9,80	6,74
2	2	10,17	9,12
3	3	10,55	2,15
4	4	10,81	2,47
5	5	11,12	0,98
6	6	11,45	1,72
7	7	11,93	6,88
8	8	12,20	4,16
9	9	12,51	1,56
10	10	13,04	11,58
11	11	13,54	10,97
12	12	14,04	1,67
13	13	14,37	4,20
14	14	14,59	8,25
15	15	14,99	7,02
16	16	15,32	4,35
17	17	15,76	4,01
18	18	16,52	4,01
19	19	16,81	2,74
20	20	17,49	3,80
21	21	17,96	2,42

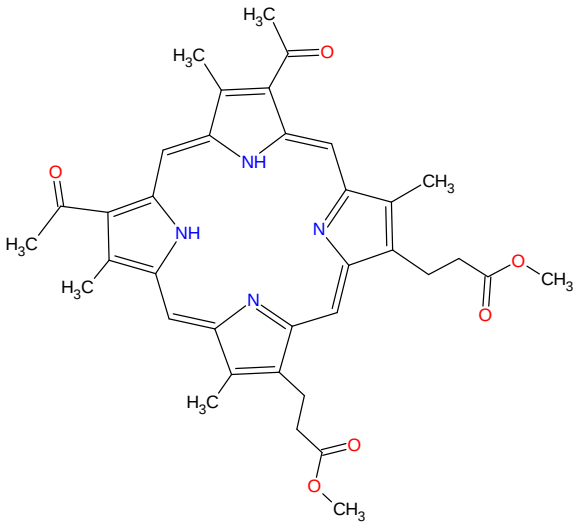
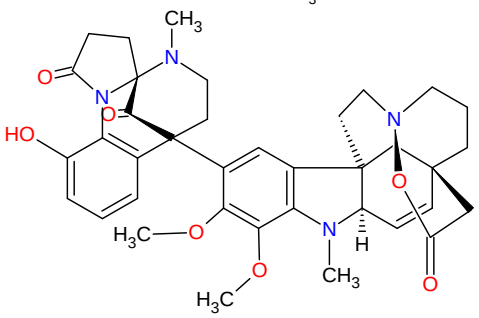
The obtained chromatogram was then processed using the MassLynx 4.1 to determine the retention time (R_t), peak height, area, and spectra, allowing the molecular formula of the compound to be interpreted. Each peak chromatogram represents a single compound[28]. The ChemSpider can help predict the molecular formula based on the compound's name. Because the ion source used by ESI (+) will add one H charge to the compound in question, the molecular formula written on the ChemSpider reduces the number of H molecules by one, and the measured m/z value must be reduced by the atomic mass of H, which is 1.0078[29].

According to **Table IV**, 13 compounds can be identified using the MassLynx and the ChemSpider from the 21 peak chromatograms produced from the methanol extract of *M. koenigii* leaves. The other compounds not found may have occurred due to the imperfect and coinciding peak. This is due to the UPLC-MS limited capabilities in this study. The dominant chemical compounds in the methanol extract is 6.74% of (*S*)-tert-butyl-14-(tert-butoxycarbonyl)amino)-5-hydroxypentanoate, 9.12% of ethyl-3-(2,2-dimethyltetrahydro-2H-pyran-4-yl)-6-methylheptanoate, 11.58% of 3β,17β-dihydroxy-5-androsten, 8.25% of dimethyl-3,3′-(7,12-diacetyl-3,8,13,17-tetramethyl-2,18-porpyridyl)dipropanoate, and 7.02% of halopitin. Previous research[30] has found the compound dimethyl-3,3′-(7,12-diacetyl-3,8,13,17-tetramethyl-2,18-porpyridyl)dipropanoate in persimmon leaves methanol extract, which is an active compound capable of inhibiting or preventing substrate oxidation at a low concentration of 100 µL. Meanwhile, the compound 3β,17β-dihydroxy-5-androsten can be anti-inflammatory and anti-diabetic[31].

Table IV. UPLC-MS chromatogram peaks methanol extract of *M. koenigii* leaves.

Peak	R_t (minute)	% Area	m/z measured	Molecular formula	2D Structure
1	9,80	6,74	290,196	$C_{14}H_{28}NO_5$	
2	10,17	9,12	285,2430	$C_{17}H_{33}O_3$	

3	10,55	2,15	301,2379	$C_{17}H_{33}O_4$	
4	10,81	2,47	299,2586	$C_{18}H_{35}O_3$	
5	11,12	0,98	476,3376	$C_{28}H_{46}NO_5$	
6	11,45	1,72	nd	-	-
7	11,93	6,88	nd	-	-
8	12,20	4,16	294,1494	$C_{19}H_{20}NO_2$	
9	12,51	1,56	376,3063	$C_{20}H_{42}NO_5$	
10	13,04	11,58	291,2324	$C_{19}H_{31}O_2$	
11	13,54	10,97	293,2481	$C_{33}H_{65}O_7$	
12	14,04	1,67	307,2637	$C_{20}H_{35}O_2$	

13	14,37	4,20	nd	-	-
14	14,59	8,25	623,2870	$C_{36}H_{39}N_4O_6$	
15	14,99	7,02	653,2975	$C_{37}H_{41}N_4O_7$	
16	15,32	4,35	nd	-	-
17	15,76	4,01	nd	-	-
18	16,52	4,01	nd	-	-
19	16,81	2,74	nd	-	-
20	17,49	3,80	nd	-	-
21	17,96	2,42	nd	-	-

CONCLUSION

The UPLC-MS analysis of *M. koenigii* leaves methanol extract yielded 21 peaks, and after analysis with the MassLynx and the ChemSpider, 13 compounds with the structures listed were identified. These compounds may be tested further to determine their bioactivity.

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AUTHORS' CONTRIBUTION

All authors have an equal contribution in carrying out this study.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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