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Metabolite Profiling of Tabat Barito (*Ficus deltoidea*) Using UPLC-QTOF-MS/MS

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ABSTRACT

Tabat Barito (*Ficus deltoidea*) is known as a plant that has potency as an antioxidant because containing a significant phenolic compound. In this study, we performed metabolite profiling on *F. deltoidea* leaves by UPLC-QTOF-MS/MS to identify its phenolic compounds. Extraction by maceration and ultrasonication techniques with methanol (MM and UM) and ethanol (ME and UE) were used to extract the *F. deltoidea* metabolites. About 70 metabolites were identified by using UPLC-QTOF-MS/MS in negative ion mode. The amounts of metabolites found in each extract were different, i.e., 45 metabolites in MM, 64 metabolites in UM, 42 metabolites in UE and 41 metabolites in ME. MS/MS could further tentatively identify 16 metabolites. The identified compounds belonged to the class of flavonoids and phenolic acid. Also, we conducted an antioxidant activity by using DPPH method on each extract to determine its potency as an antioxidant. The highest antioxidant activity was exhibited by UM extract (IC50 71.93 ppm) may be due to the number of metabolites in UM extract which was higher than the other extract based on the detected metabolites.

Key word: *Ficus deltoidea*, metabolite profiling, UPLC-QTOF-MS/MS, antioxidant activity

INTRODUCTION

Ficus deltoidea is known as Tabat Barito in Indonesia, and it belongs to the Moraceae family. This plant widely distributed in Southeast Asia. *F. deltoidea* has been extensively studied for its biological activities such as antioxidant [1-3], antihypertensive [4], antiadipogenic [5], antimicrobial [6], antidiabetic [7], anti-cervical cancer [8], anti-inflammatory [9], and antinociceptive [10]. It is well known that *F. deltoidea* contains phenolic compounds which are in general mostly acts as an antioxidant [2]. The chemical components existing in *F. deltoidea* belong to polyphenols class (tannins, proanthocyanins), flavonoids, saponins, and triterpenoids [4, 5, 11]. Flavonoid compound that has been identified in *F. deltoidea* is (-)-epicatechin/(+)-catechin, (-)-epiafzelechin, (+)-afzelechin, (-)-epigallocatechin, luteolin, apigenin [12], vitexin, isovitexin [7], and caffeic acid [13].

Identification of chemical components in a plant could be identified through metabolomics approach. Metabolomics study can be used to observe the profile of secondary

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metabolites in plants which consist of tens or even hundreds of metabolites. Metabolite profiling is one of the strategies in metabolomics analysis that has been widely used to describe secondary metabolite profiles without going through lengthy processes of metabolite isolation [14]. This approach has been applied to *F.deltoidea* growing in Malaysia, and several compounds of flavonoid and proanthocyanidin class were identified [12]. Metabolite profiling also has been performed on other ficus species namely *F. lyrata*, and several compounds of flavonoid, phenolic acid, fatty acid, and sphingolipid were identified tentatively [14].

Metabolite profiling studies either using gas chromatography or liquid chromatography in tandem with mass spectrometry (MS). The tandem is intended to facilitate the process of identifying chemical compounds in a plant, which is a complex system of chemicals because the plant will contain tens to hundreds of metabolites. Chromatography is used to separate the compounds in the plant and to ease their identification by mass spectrometry. In many studies of chemical profiling, ultra-performance liquid chromatography quadrupole time of flight mass spectrometry/mass spectrometry (UPLC-QTOF-MS/MS) has been reported that it is more sensitive and selective in profiling than other chromatographic methods [15]. MS analysis in quadrupole time of flight (QTOF) as mass analyzer can be used to detect a broad range of compounds in a small number of samples required. In addition, QTOF-MS allows the establishment of mass information with high precision and accuracy so that the determination of possible structure is more accurate [16]. UPLC-QTOF-MS/MS has been reported for profiling of secondary metabolites in some plant species such as Curcuma [17], *Fragaria ananassa* [18] and *Merremia emarginata* [19].

In this study, we used UPLC-QTOF-(MS/MS) for metabolite profiling of *F. deltoidea* leaves from West Java, Indonesia. As additional information, we have determined the antioxidant capacity from a different extract of *F. deltoidea*. We also compared two different extraction modes (maceration and ultrasonication) with two solvents (ethanol and methanol) to know how many metabolites could be extracted and its effect on the antioxidant capacity of each extract.

EXPERIMENT

Plant Material and Chemicals

F. deltoidea leaves samples were obtained from Cikaniki village, Mount Halimun Sukabumi National Park collected in July 2014, methanol (Merck, Darmstadt, Germany), ethanol (Merck, Darmstadt, Germany), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Merck, Darmstadt, Germany).

Instrumentation

Multiplate reader (Epoch-Biotek, Winooski, USA) was used for measurement of antioxidant activity, whereas the metabolite profiling was conducted using LC-MS XEVO G2S-QTOF (Waters, Milford, USA), an ultrasonication US-3 38 kHz (As-one, Osaka-Jepang) and rotary evaporator (Heidolph WB 2000, Schwabach-Germany).

Procedure

Extraction of *F. deltoidea* leaves

Maceration was carried out by soaking *F. deltoidea* leaves samples in ethanol and methanol, respectively with a ratio of sample-solvent of 1:10 at room temperature for 24 hours and occasionally stirring. The filtrate was evaporated and obtained methanol extract (MM) and ethanol extract (ME). Ultrasonication was performed for 30 minutes by using 42

kHz ultrasonication wave. After that, the extract was filtered to obtain filtrate and residue. The filtrate was evaporated to obtain methanol extract (UM) and ethanol extract (UE).

Measurement of Antioxidant Activity with DPPH method

Antioxidant activity of the samples was measured using DPPH method described by Salazar-Arranda *et al*. [20]. Each extract was dissolved in ethanol to obtain a solution of each extract at a concentration of 100, 50, 25,12.5, 6.25 and 3.125 µg/mL. About 100 µL of each solution was added into 100 µL of DPPH 125 µM in ethanol, and then the mixtures were stirred and incubated at 37°C for 30 minutes. The absorbance of each solution was read in the multiplate reader at a wavelength of 517 nm. The sample was analyzed three times and data were evaluated using ANOVA.

Identification of Chemical Components by UPLC-QTOF-MS/MS

Identification of chemical components from *F. deltoidea* leaves was performed by using dissolving each extract into methanol and filtering with millipore 0.45 micron, then 5 µL of the filtrate was injected into UPLC-QTOFMS/MS. ESI ionization source and QTOF mass analyzer were used for MS analysis. Conditions in MS measurement were as follows: negative ion mode in a capillary temperature of 120 °C, gas atomizer with a flow rate of 50 L/hour, the source of voltage +2.9 kV, full scan mode from m/z 100-1000 at a temperature of 41ºC, an Acquity column HSS C18 1.8 µm x 2.1 x 150 mm (Waters, Germany) as stationary phase with mobile phase of 5 mm ammonium format (A) and 0.1% formic acid in acetonitrile (B) and eluent flow rate of 0.4 mL/min. Mode of elution was as follows: isocratic elution at 0-0.5 minutes with a ratio of 95:5, followed by linear gradient elution of solvent A from 95% to 5% at minutes 0.5-15, isocratic elution with a ratio of 5:95 at minutes 15-17 and linear gradient elution of solvent A from 5% to 95% at 17-20 minutes.

RESULT AND DISCUSSION

Identification of Chemical Components in *F. deltoidea* Leaves

In this study, to observe the differences in the chemical composition of *F.deltoidea* leaves, a non-targeted metabolite profiling of the samples was performed on two extracts from two different extraction techniques. The chemical components were separated and identified by using UPLC-QTOF-MS/MS. The data obtained from UPLC-QTOF-MS/MS was preprocessed with MZmine software. Filtering, baseline correction, peak detection, deisotope, alignment, gap filling, normalization, and identification were performed using this software.

The chromatograms obtained were converted into netCDF file extension for further process to separate the chromatogram peaks from signal and noise by using baseline correction. After this, we continue with peak detection to identify and quantify associated signal with molecules in the sample and also to reduce data complexity. Deisotoping was used to simplify data matrix for further analysis. Before statistical analysis, alignment should be applied to compare metabolites in the samples analyzed by matching and classifying the detected peaks. Gap filling was needed to observe very low peak intensity, poor quality form, or an error in detecting the peak, to prevent wrong conclusions. Data were corrected or eliminated to remove unwanted systematic bias in the measurement [21].

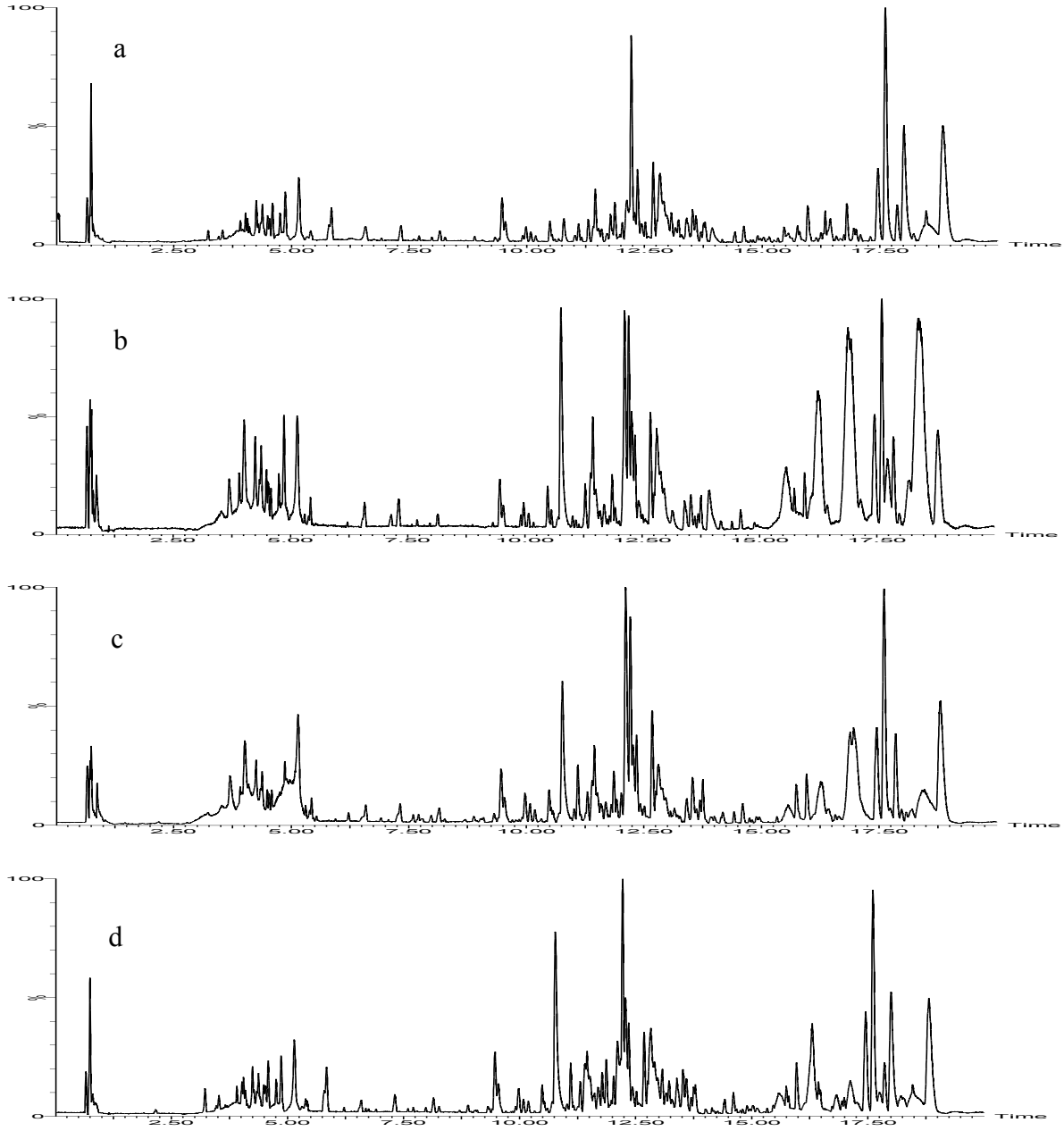


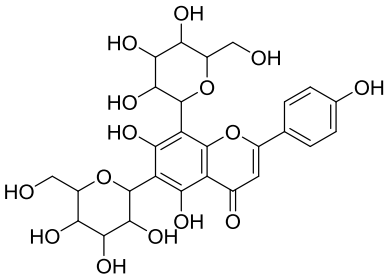
Figure 1. UPLC chromatograms of *F. deltoidea* leaves extracts: MM (a), ME (b), UM (c) and UE (d).

From the identification results obtained, metabolites number contained in each extract were different, *i.e.,* 45 metabolites in MM, 64 metabolites in UM, 42 metabolites in UE and 41 metabolites in ME. Fragmentation generated from MS^1 performs further confirmation of each metabolite. From the pattern of fragmentation in MS^1, we will obtain a base peak. After this, we compare it with the result obtained from Mzmine prediction results. The next step is confirmation using fragmentation pattern on MS^2 and compared it with the literature. A total of 16 peaks could be identified in 9 compounds in the class of flavonoids (*e.g.,* gallocatechin, catechin, epicatechin, vicenin-2), three compounds of phenolic acids (*e.g*., vanillic acid and

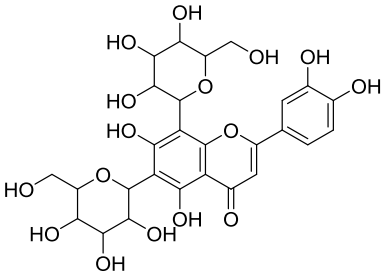
quinic acid) and 4 compounds of fatty acids (glutaric acid dimer) (Table 1). Some of the molecular structure of identified compounds was present in Figure 2.

Table 1. Determination of metabolite peak in *F.deltoidea* leaves extracts by using UPLC-QTOF-MS/MS in negative ionization mode

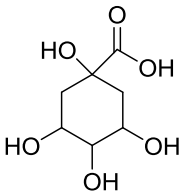
Peak	t_R	[M-H]-	Formula	MS-MS (*m/z*)	Compounds	MM	UM	UE	ME
2	0.73	191.0476	$C_7H_{11}O_6$	-	Quinic acid	-	-	+	+
8	3.22	305.0522	$C_{15}H_{14}O_7$	179,167,137	Galocatechin	+	+	+	-
9	3.53	289.0576	$C_{15}H_{14}O_6$	271, 245, 205, 179	Cathecin	+	+	+	+
10	3.91	593.1218	$C_{27}H_{30}O_{15}$	503, 473, 383, 353	Apigenin-6.8-*C*-Dihexoside (vicenin-2)	+	+	+	+
11	4.02	593.1218	$C_{27}H_{30}O_{15}$	503, 473, 383, 353	Apigenin-6.8-*C*-Dihexoside (vicenin-2)	+	+	+	+
12	4.05	289.0585	$C_{15}H_{14}O_6$	289, 245	Epicatechin	+	+	-	-
14	4.25	563.1138	$C_{26}H_{28}O_{14}$	443, 473, 545, 503, 383, 353	Apigenin-6-*C*-Glucoside-8-*C*-arabinoside (schaftoside)	-	+	+	+
15	4.39	447.0722	$C_{21}H_{20}O_{11}$	327, 357	Luteolin-8-*C*-glucoside (orientin)	+	+	+	+
18	4.86	431.080	$C_{21}H_{20}O_{10}$	431, 353, 341, 311,269	Apigenin-8-*C*-glukosida (vitexin)	+	+	+	+
20	5.39	167.0275	$C_8H_8O_4$	-	Vanilic acid	+	+	+	+
22	5.84	187.0892	$C_9H_{15}O_4$	125	Azelic acid	+	+	-	-
23	6.59	285.0270	$C_{15}H_{10}O_6$	285, 151, 133	Luteolin	+	+	+	+
27	8.15	245.0707	$C_{10}H_{14}O_7$	-	Glutaric acid dimer	+	+	+	+
28	8.89	245.0707	$C_{10}H_{14}O_7$	-	Glutaric acid dimer	+	+	+	+
29	9.47	245.0707	$C_{10}H_{14}O_7$	-	Glutaric acid dimer	+	+	+	+
30	9.55	245.0707	$C_{10}H_{14}O_7$	-	Glutaric acid dimer	+	+	+	+



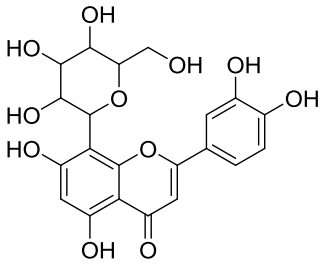
Apigenin-6.8-C-diglucoside



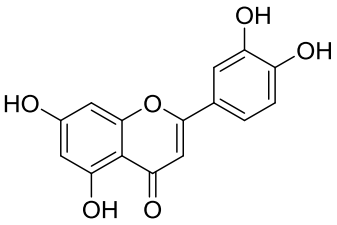
Luteolin-6,8-C-diglucoside



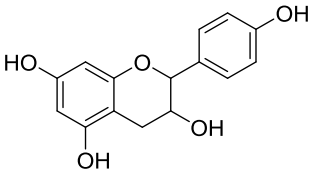
Quinic acid



Orientin



Luteolin



Epicatechin

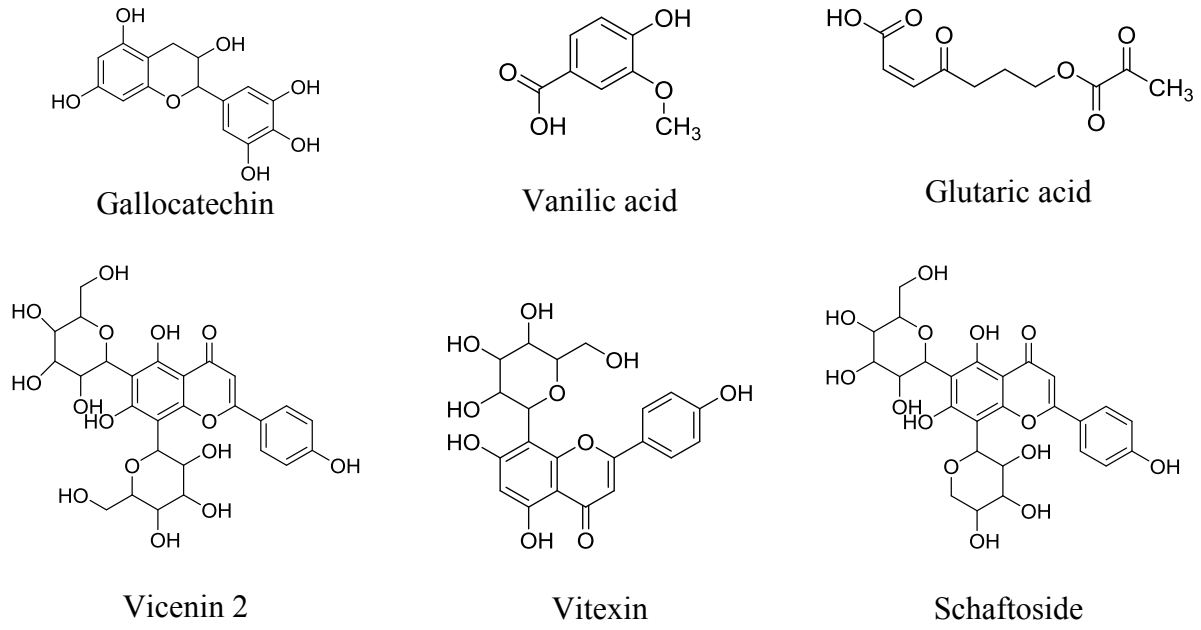


Figure 2. Structure of proposed compounds of flavonoid and phenolic acid in *F. deltoidea* leaves extracts

Identification of Flavonoid

Flavonoid has been reported for several Ficus species [12,14, 22,23]. Indication for the presence of several flavonoids in leaves samples comes from the molecular ion peak of spectrum MS^n with M-90 and M-120 fragments indicating for C-glycosides cleavage [14]. Previous studies also showed that luteolin and apigenin glycosides found in F. deltoidea were of the C-glycosides form [12]. From the Fig 1, peaks 10 and 11 were characterized [M-H]-at *m/z* 593 with the fragment ions *m/z* 503 ([M-H-90]-) and *m/z* 473 ([M-H-120]-) indicating the presence of apigenin-6,8-C-diglucoside (vicenin-2) [24]. In addition, typical fragmentation of C-glycosyl flavone is indicated by MS^2 peaks *m/z* 383 (aglycone + 113) and *m/z* 353 (aglycone + 83). The relative intensity of signal fragment with *m/z* 383 is higher than *m/z* 473, which showed the existence of apigenin aglycone [25]. Peak 10 and 11 identified the same compounds although their retention time was different, as one peak of UPLC MS/MS could consist of several compounds. Apigenin-6-C-glucoside-8-C-arabinoside (schaftoside) compound was identified at a retention time 4.25 minute (compound 14) with a base peak *m/z* 563. In general, 6-C-pentosyl-8-C-hexosyl substitution leads to a higher abundance of the ion *m/z* 473 [M-H-90]-¬ to *m/z* 443 [M-H-120]- [26]. Ion *m/z* 503 [M-H-60]- was derived from the cleavage of C-pentosyl, while ion [M-H-120]- was derived from cleavage of hexosyl, furthermore, glycosyl substitution at the C6 position of flavones produce the base peak.

The existence of luteolin at the retention time of 6.59 (peak 23) can be explained by comparing the product ion spectrum with luteolin standard mass spectrum [27]. Figure 3A shows the spectrum of *F. deltoidea* leaves extract at peak 23 and the spectrum for the standard (Figure 3B) to describe the presence of luteolin. MS/MS spectrum on precursor ion at *m/z* 151 [M-H-134]- losses of ring A of flavonoid molecules and m/z 133 [M-H-152]¯ losses of ring B flavonoid molecules [28].

Differences between orientin (luteolin-8-C-glucoside) and isoorientin (luteolin-6-C-glucoside) were shown a different pattern of fragmentation because of differentiation between C-glycosides at position 6 and 8. Peak 15 with retention time at 4.39 minutes was

obtained by base peak [M-H]- at *m/z* 447. The first compound was identified as isoorientin or orientin. Fragmentation pattern at *m/z* 357 [M-H-90]- and *m/z* 327 [M-H-120]- indicated that mono-C-glycosylation was in position 8. The losses of 120 u and 90 u corresponded to cross-ring cleavages in the sugar unit. Compound 15 was marked as luteolin 8-C-glucosyl known as orientin [29]. The difference with isoorientin was ion *m/z* 429 [M-H-18]- did not exist in the orientin spectrum [27]. The same method was used in identifying vitexin (peak 18). Vitexin (apigenin-8-C-glucoside) at m/z 431 showed *m/z* 341 [M-H-90] and *m/z* 311 [M-H-12] as characteristic ions in MS/MS. The absence of ion *m/z* 413 and 353 will distinguish the spectrum between vitexin and isovitexin. In addition, deprotonated aglycone (m/z 269) has the relative abundance of 5. It showed that the compound in peak 18 is a vitexin [27].

Gallocatechin belonging to the flavan-3-ol class is detected at peak 8 with a retention time of 3.22 (m/z 305). Cleavage pattern of MS^2 generates m/z 179 $[M-H-126]^-$, 167 $[M-H-138]^-$, and 137 $[M-H-168]^-$ [30]. Peak 9 and 12 are catechin and epicatechin compounds that produce a deprotonated molecule $[M-H]^-$on (*m/z* 289). Compound (epi) catechin with a base peak at m/z 289 where the ion peak deprotonated [M-H]- occurs at *m/z* 289 fragmented ions at *m/z* 271, 245, 205 and 179. There was a possibility that ion at *m/z* 205 was obtained due to the loss of observed flavonoids-ring A, while the ion at *m/z* 245 showed the presence of CO_2 or -CH_2-CHOH- divalent [27]. Fragments at *m/z* 271 are the result of water loss [M-H-$H_2$0], and *m/z* 179 of the lost ring B of flavonoid molecules.

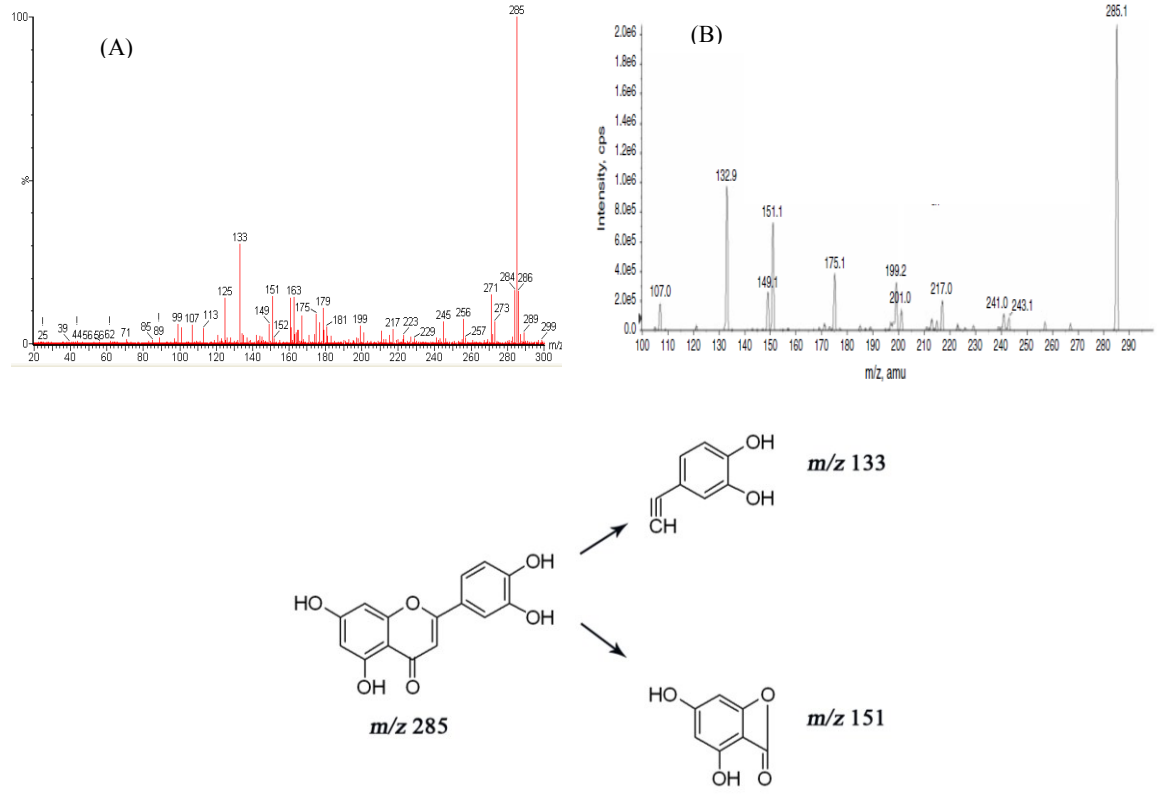


Figure 3. MS/MS from Peak 23 in *F. deltoidea* leaves extracts (A) and luteolin standard (B) [27]

Identification of Phenolic Acid

Derivatives formed from the interaction of hydroxycinnamic acids with quinic acids were commonly found in plants and contributed to the flavor [31]. Peak 2 is characterized by $[M-H]^-$at *m/z* 191.0476. The dominant fragment of 191 indicates the molecular formula of

$C_7H_{11}O_6$ for quinic acid in MS^n (phenolic acids) [14]. Quinic acid is identified in ethanol solvent in both extraction techniques. Other phenolic acids also have been identified in this study such as a vanillic acid (20) and azelaic acid (22), all of them are visible from the mass spectral data (Table 1). Vanillic acid has fragmentation pattern at 167, 152, 122 and 108 [32].

Antioxidant Activity

Free radical scavenging activity of *F. deltoidea* leaves extract with extraction technique and different solvent use DPPH method. The capacity of antioxidant activity of *F. deltoidea* leaves extracts indicated with IC_{50} values. IC_{50} of each extract was obtained at 85.98 µg/mL for MM, 80.75 µg/mL for ME, 71.93 µg/mL for UM, and 88.37 µg/mL for UE (Table 2). Table 2 showed there is no significant difference in the IC_{50} of the four extracts which means both extraction and solvent did not affect significantly by the antioxidant activity, but the four extracts have high antioxidant activity because the IC_{50} value obtained below 200 µg/mL. IC_{50} below 200 µg/mL is considered to have high antioxidant activity [33]. Methanol extract by ultrasonication method has the highest antioxidant activity with the lowest IC_{50} value. This result supported by the number of metabolites contained in the methanol extracts using ultrasonication also higher than the other extracts.

Table 2. Antioxidant activity of *Ficus deltoidea* leaves extract

Extraction	Solvent	IC_{50} (µg/mL) ± SD (n =3)
Maceration	Methanol	85.98 ± 3.26[a]
	Ethanol	80.75 ± 3.08[a]
Ultrasonication	Methanol	71.93 ± 0.86[a]
	Ethanol	88.37 ± 3.66[a]

CONCLUSION

This study demonstrated a metabolite profiling of *F. deltoidea* growing in Indonesia by using UPLC-QTOF-MS/MS. In the identification of *F. deltoidea* secondary metabolites, we found about 70 metabolites and 16 metabolites confirmed by MS/MS. The identified compounds belong to the class of flavonoids, phenolic acid, and fatty acid. The use of different extraction technique and solvent will affect the number of metabolites. Ultrasonication technique provides the most number of metabolites and the highest potential activity. Database development and further identification with complete structure elucidation are required to determine the presence of chemical components in *F. deltoidea*.

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