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UHPLC-Q-Orbitrap HRMS-based Untargeted Metabolomics of *Sida rhombifolia* Leaves and Stem Extracts

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ABSTRACT

***Sida rhombifolia*, also known as sidaguri in Indonesia, is a medicinal plant commonly used as a herbal medicine because of its metabolite and biological activities. One of the several factors that affect plant metabolite composition and concentration is the use of plant parts. In this study, the experiment aimed to identify the metabolite profile in the leaves and stem extracts of *S. rhombifolia* using UHPLC-Q-Orbitrap HRMS-based untargeted metabolomics. The samples were distinguished by principal component analysis (PCA). Extraction of metabolites was conducted by sonication for approximately 30 min with 70% ethanol as the extraction solvent; 28 metabolites were identified. Seven metabolites were identified only in the leaves, three were identified only in the stems, and 18 other metabolites were identified in both the leaves and stems. These metabolites were categorized as flavonoids, triterpenoids, alkaloids, coumarins, phenolic aldehydes, phenolic acids, ecdysteroids, fatty acids, and monoterpene lactones. Based on the classification results, PCA grouped the leaves and stem extracts of *S. rhombifolia* using the peak area variables of the identified metabolites.**

1. Introduction

Sida rhombifolia, known as sidaguri in Indonesia, is a medicinal plant used to treat diarrhea, malaria, gastrointestinal dysentery, fever, asthma, and inflammation (Mah *et al.* 2017). *S. rhombifolia* belongs to the Malvaceae family, grown widely in tropical and subtropical countries such as Indonesia, Malaysia, and India (Woldeyes *et al.* 2012). Furthermore, *S. rhombifolia* is known to have several biological activities, such as xanthine oxidase inhibition (Iswantini *et al.* 2014), anticancer, anticholinesterase (Mah *et al.* 2017), anti-inflammatory (Rodrigues and Oliveira 2020), antibacterial (Debalke *et al.* 2018), antihyperglycemic, antioxidant (Arciniegas *et al.* 2017), antiproliferation (Tumanggor *et al.* 2019), antimalarial, anti-diabetes (Singh *et al.* 2018), wound healing, and analgesic activities (Rohman *et al.* 2020). These biological activities originate from the bioactive compounds present in *S. rhombifolia*.

S. rhombifolia contains metabolites from various compound classes, such as phenolic acids, flavonoids, coumarins, steroids, porphyrins, alkaloids, and fatty acids. (Rohman *et al.* 2020). The content of these metabolites in the plant material may be varied depending on several factors, such as the growing environment (Zoyane *et al.* 2019), harvesting time (Rafi *et al.* 2018), drying method (Xiang *et al.* 2020), and extraction process (Llorent-Martínez *et al.* 2020). In addition, plant parts, such as the leaves and stems, may have different metabolite compositions and concentrations. A metabolomics approach can determine these differences because this analysis can determine qualitative and quantitative metabolites from plant materials that may vary or undergo changes under certain conditions. A typical analytical technique in metabolomics analysis is

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liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). LC combined with MS is used in metabolomic analysis employing the LC system's high resolution and sensitivity of the MS detector (García *et al.* 2020; Zeki *et al.* 2020).

Several studies describe *S. rhombifolia* metabolites and their biological activities (Chaves *et al.* 2017; Ferro *et al.* 2019; Wang *et al.* 2008). The aerial part of *S. rhombifolia* is mainly used. However, there has been no report on differences in metabolites between the stems and leaves of *S. rhombifolia*. Therefore, this study aims to identify the composition of metabolites in the leaves and stems of *S. rhombifolia* using Ultra-High Performance Liquid Chromatography (UHPLC)-Q-Orbitrap High-Resolution Mass Spectrometry (HRMS). The results of this study are expected to provide information about the parts of the *S. rhombifolia* plant that contain more secondary metabolites.

2. Materials and Methods

2.1. Materials

The materials used were the leaves and stems of *S. rhombifolia* (3 months old) taken from the Tropical Biopharmaca Research Center (TropBRC), IPB University, Bogor, West Java, Indonesia (collection number: BMK0070042016). All solvents used were obtained from Merck (Darmstadt, Germany) and were analytical or LC-MS grade. Filter papers and filter syringes of PTFE (0.2 µm) were obtained from Ambala Cantt (India).

2.2. Methods

2.2.1. Instrumentation and Software

In this experimant, to separate the metabolites of the *S. rhombifolia*, UHPLC-Q-Orbitrap HRMS (Thermo Fisher, Waltham, MA, USA) was used. Metabolite profiling was performed using Thermo XCalibur and Compound Discoverer ver 2.2 software (Thermo Fisher, Waltham, MA, USA). Principal component analysis (PCA) was performed using Unscrambler X ver 10.1 software (Camo, Oslo, Norway).

2.2.2. Identification of Metabolite by UHPLC-Q-Orbitrap HRMS

The leaves and stems of *S. rhombifolia* were extracted using the sonication method with seven replications. *S. rhombifolia* leaves, and stem powder (500 mg) was accurately weighed and separately sonicated using 5 ml 70% ethanol for 30 minutes. The filtrates were then filtered, and the solvent was added to the filtrate up to precisely 5 ml, then placed in a vial using a 0.2 µm PTFE filter.

The separation of metabolites was performed using the procedure described by Wang *et al.* (2008) with a modification. UHPLC-Q-Orbitrap HRMS was performed with an Accucore C18 separation column (100 × 2.1 mm, 1.5 µm). The mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) with a gradient elution system as follows: 0–3 min (5–20% B), 3–20 min (20–40% B), 20–26 min (40–75% B), 26–28 min (75–95% B), 28–30 min (95% B), 30.00–30.01 min (95–5% B), and 30.01–35 (5% B). The flow rate was 0.2 mL/min with an injection volume of 2.0 µL. The source of MS ionization was electrospray ionization (ESI) in the positive and negative ionization modes with an m/z range of 100–1500 Da. The other parameters were set: capillary temperature, 320°C; spray voltage, 3.8 kV; sheath and auxiliary gas, 15 and 3 ml/min, respectively; resolving power, 70,000 FWHM; scan type, full MS/dd MS2.

The UHPLC-Q-Orbitrap HRMS data were processed and analyzed using Compound Discoverer 2.2 software to putatively identify metabolites in the S. rhombifolia leaves and stem extracts. Putative identification of metabolites was achieved by spectra stage selection, retention time alignment, unknown compounds detection and grouping, composition prediction, mass list search, gap filling, area normalization, and background compound marking. *S. rhombifolia* and confirmatory in-house databases were used for the putative identification of metabolites.

2.2.3. Clustering of *S. rhombifolia* Leaves and Stem Extracts Using PCA

PCA was performed to discriminate *S. rhombifolia* leaves and stem extracts using the Unscrambler X version 10.1 (Camo, Oslo, Norway). The peak areas of the identified compounds were used as variables.

3. Results

3.1. Putative Identification of Metabolites in *S. rhombifolia* Leaves and Stem Extracts

The resulting chromatogram profile indicated the difference in the number of metabolites identified in the leaves and stem extracts. Based on the chromatogram profile, it can be observed that

the chromatogram of the leaves extract has more peaks than that of the stem extract (Figure 1). This indicates that leaves extract contains more diverse metabolites than the stem extract. Based on the chromatogram (Figure 1 and 2), 28 metabolites were putatively identified in the *S. rhombifolia* 70% ethanol extract. The metabolite was identified based on the in-house database from genus Sida, which was confirmed using the MS2 spectrum. Meanwhile, unidentified and unconfirmed (unknown) metabolites are not listed in Table 1. From the 28 compounds, seven were only present in the leaves, three were only in the stem, and 18 were identified in the leaves and extracts (Table 1).

3.2. Clustering *S. rhombifolia* Leaves and Stem Extracts

This study performed a multivariate analysis, PCA, for clustering *S. rhombifolia* leaves and stem extracts using the peak areas of the 28 identified compounds as variables. PCA was performed using a singular value decomposition algorithm and a 5% T^2 Hotelling to identify outliers. The PCA results indicated that *S. rhombifolia* leaves and stem extracts could be separated with two PC variants of approximately 85% (PC1 = 78% and PC2 = 7%). The total PC value indicates that the PCA could explain 85% of the variance in the data (Figure 3). The samples, which could be distinguished into two groups, showed differences in the peak area of each metabolite contained in the stems and leaves of *S. rhombifolia*. For example, based on Figure 4, scopoletin (12) is a compound that can be used to distinguish stems from leaves of *S. rhombifolia* because the peak area of scopoletin (12) on stems is higher than that on leaves. The small scale on PC1 is owing to the data used in the form of the peak area of each metabolite compound, so as not to cause significant data differences from the leaves and stem extracts of *S. rhombifolia.* Furthermore, the grouping with PCA illustrates that the sample analysis, repeated seven times, did not result in an outlier in the data of the metabolites, as indicated by the absence of samples outside the Hotelling T^2 circle.

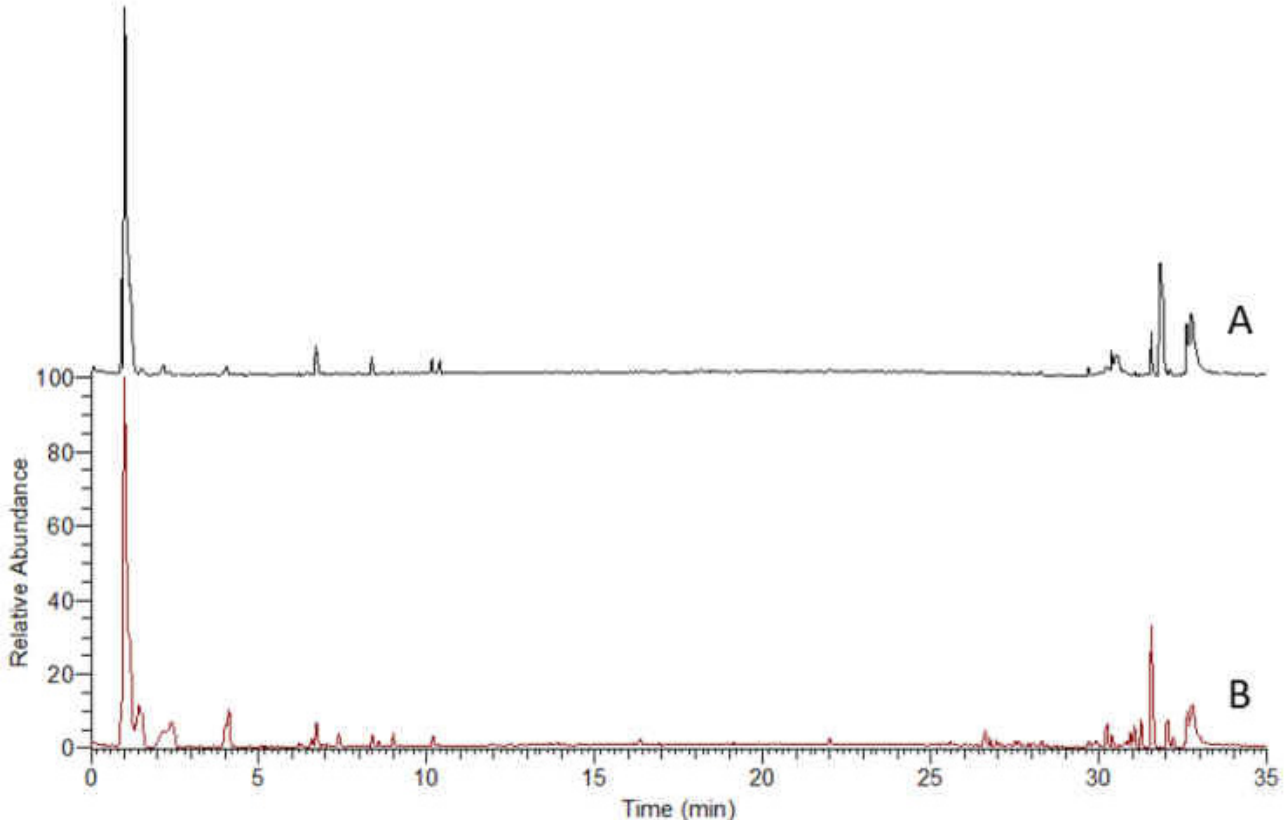

Figure 1. UHPLC-Q-Orbitrap HRMS chromatogram of stem (A) and leaves (B) extracts of S. rhombifolia in the positive ionization mode

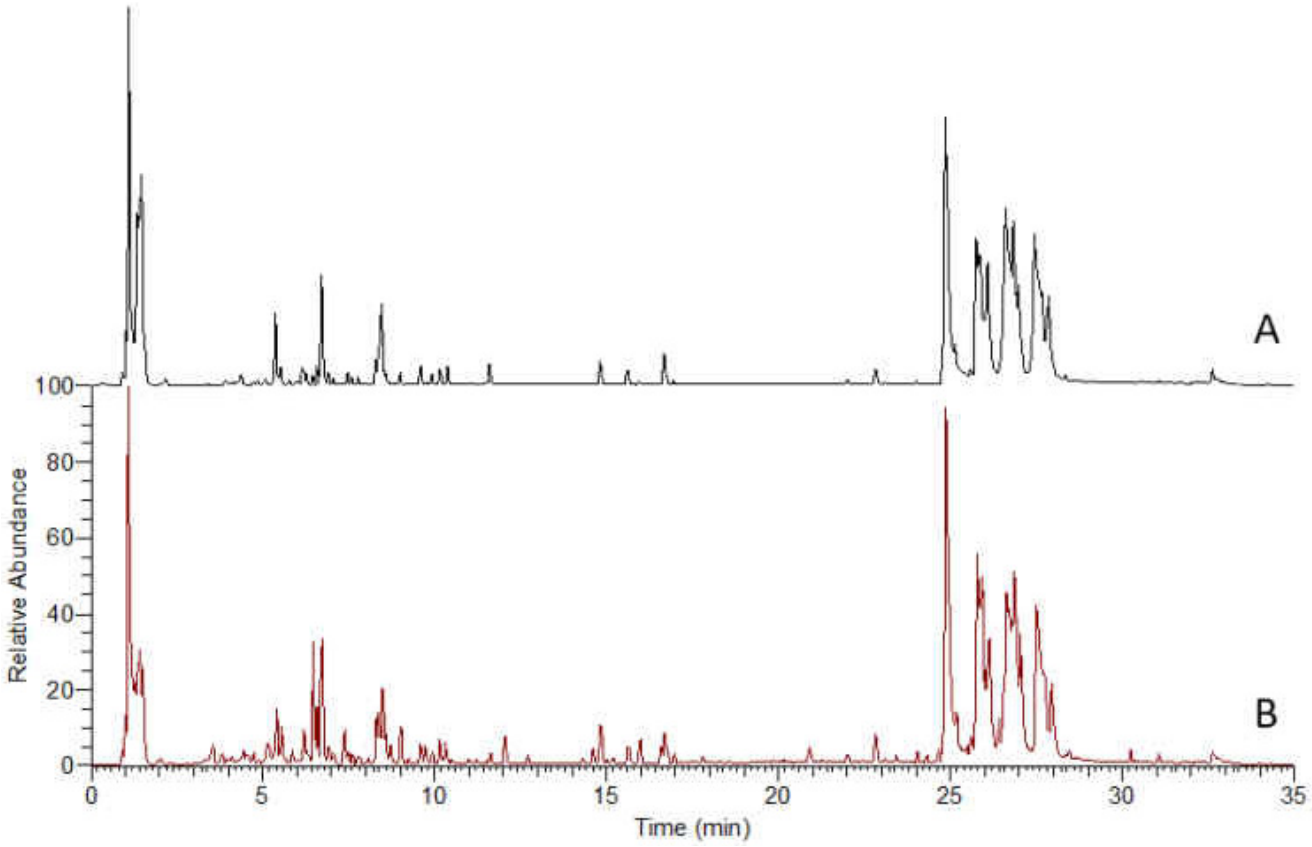

Figure 2. UHPLC-Q-Orbitrap HRMS chromatogram of stem (A) and leaves (B) extracts of *S. rhombifolia* in the negative ionization mode

Table 1. Putatively identified metabolites from the leaves and stem extracts of *S. rhombifolia*

Metabolites	Retention time (min)	Formula	MW	Error (ppm)	MS and MS/MS	Leaves	Stem
Flavonoid							
Quercetin	6,429	$C_{15}H_{10}O_7$	302.04265	–1.52	303, 285, 257, 229, 201, 165, 153, 137, 121	√	
Kaempferol	7,224	$C_{15}H_{10}O_6$	286.04774	–1.40	287, 258, 153, 133	√	√
Glutinoside	11,975	$C_{30}H_{26}O_{13}$	594.13734	–1.20	595, 287, 165, 147	√	√
Epicatechin	5,395	$C_{15}H_{14}O_6$	290.07904	0.07	291, 273, 163, 139,123	√	
Kaempferol-3-O-D-glucose-6""-D-rhamnose	7,219	$C_{27}H_{30}O_{15}$	594.15847	–1.09	595, 287	√	√
Terpenoid							
Vomifoliol	5,985	$C_{13}H_{20}O_3$	224.14124	0.04	225, 207, 189	√	
Taraxasterone	32,526	$C_{30}H_{48}O$	424.37052	–0.85	425, 407, 271, 217, 215, 187, 147, 119, 67	√	
Alkaloid							
Vasicinol	3,890	$C_{11}H_{12}N_2O_2$	204.08988	–1.32	205, 187, 146	√	√
N-trans-feruloyltyramine	10,310	$C_{18}H_{19}NO_4$	313.13141	–1.31	314, 177, 163, 145, 117	√	√
Betaine	1,048	$C_5H_{11}NO_2$	117.07898	–0.43	118, 58, 59	√	√
Coumarin							
Umbelliferone	3,549	$C_9H_6O_3$	162.03169	–0.12 0.31	163, 145, 135, 117, 107, 89	√	
Scopoletin	5,257	$C_{10}H_8O_4$	192.04226		193, 133	√	√

Table 1. Continued

Metabolites	Retention time (min)	Formula	MW	Error (ppm)	MS and MS/MS	Leaves	Stem
Phenolic aldehydes							
Syringaldehyde	6,406	$C_9H_{10}O_4$	182.05791	0.66	183, 165, 155, 141, 138, 121, 123		√
Sinapaldehyde	7,982	$C_{11}H_{12}O_4$	208.07356	−0.62	209, 191, 180, 177, 149	√	√
Coniferaldehyde	7,974	$C_{10}H_{10}O_3$	178.06299	0.17	179, 161, 148, 147, 119		√
Phenolic acid							
Salicylic acid	4,235	$C_7H_6O_3$	138.03169	0.36	139, 121, 94	√	√
p-Coumaric acid	6,182	$C_9H_8O_3$	164.04734	−3.84	163, 119	√	
Chlorogenic acid	4,558	$C_{16}H_{18}O_9$	354.09508	1.02	353, 191	√	
Caffeic acid	5,107	$C_9H_8O_4$	180.04226	−2.78	179, 135	√	√
4-Aminobenzoic acid	1,053	$C_7H_7NO_2$	137.04768	−0.44	138, 120, 94	√	√
Ecdysteroid							
Polypodine B	5,574	$C_{27}H_{44}O_8$	496.30362	−2.24	497, 461, 443, 425, 371	√	√
Ecdysone	8,906	$C_{27}H_{44}O_6$	464.31379	−2.33	465, 447, 429, 411, 393, 355	√	√
2-Deoxy-20-hydroxyecdysone-3-O-D- Glucopyranoside	6,929	$C_{33}H_{54}O_{11}$	626.36661	−2.28	627, 447, 429, 411, 355	√	√
20-Hydroxyecdysone-3-O-D- Glucopyranoside	7,477	$C_{33}H_{54}O_{12}$	642.36153	−1.96	643, 445, 427, 371	√	√
20-Hydroxyecdysone	6,579	$C_{27}H_{44}O_7$	480.30870	−2.77	481, 463, 445, 427, 409, 391, 371	√	√
Fatty acid							
Oleic acid	29,481	$C_{18}H_{34}O_2$	282.25588	−0.14	283, 265		√
Linoleic acid	28,180	$C_{18}H_{32}O_2$	280.24023	−0.36	281, 263	√	
Monoterpene lactone							
Loliolide	7,238	$C_{11}H_{16}O$	196.10967	−1.38	197, 179	√	√

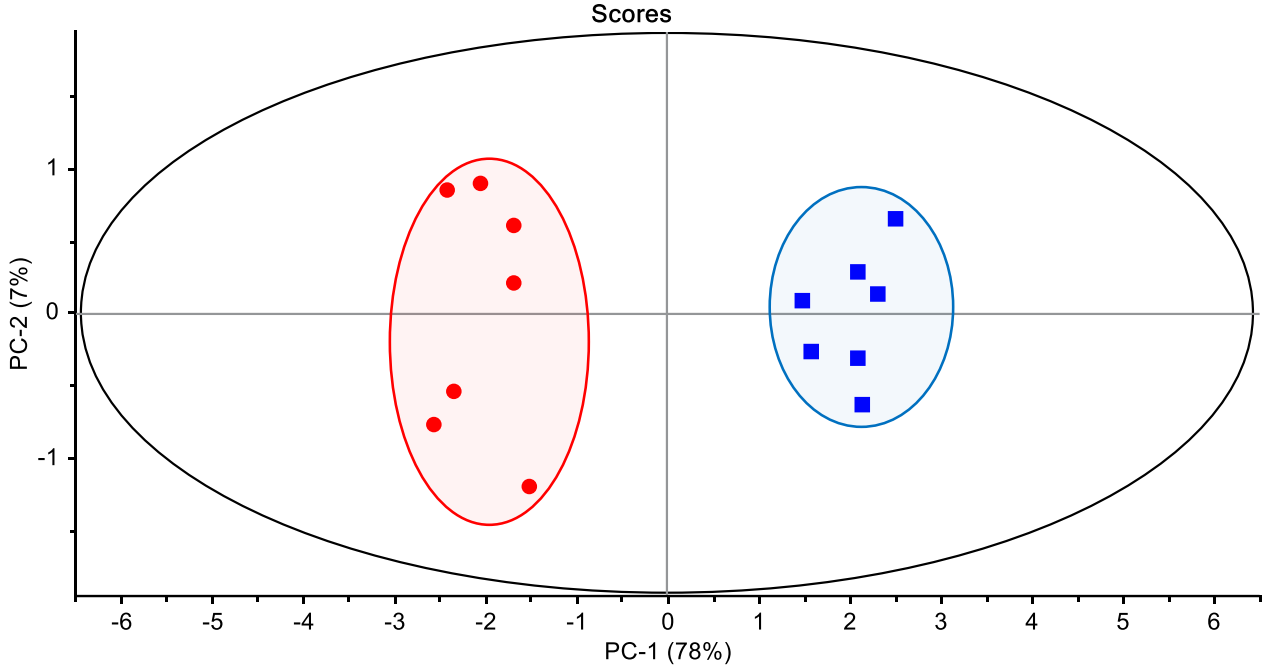

Figure 3. PCA plot score of *S. rhombifolia* leaves (red) and stem (blue) extracts using peak area of identified metabolites as variable

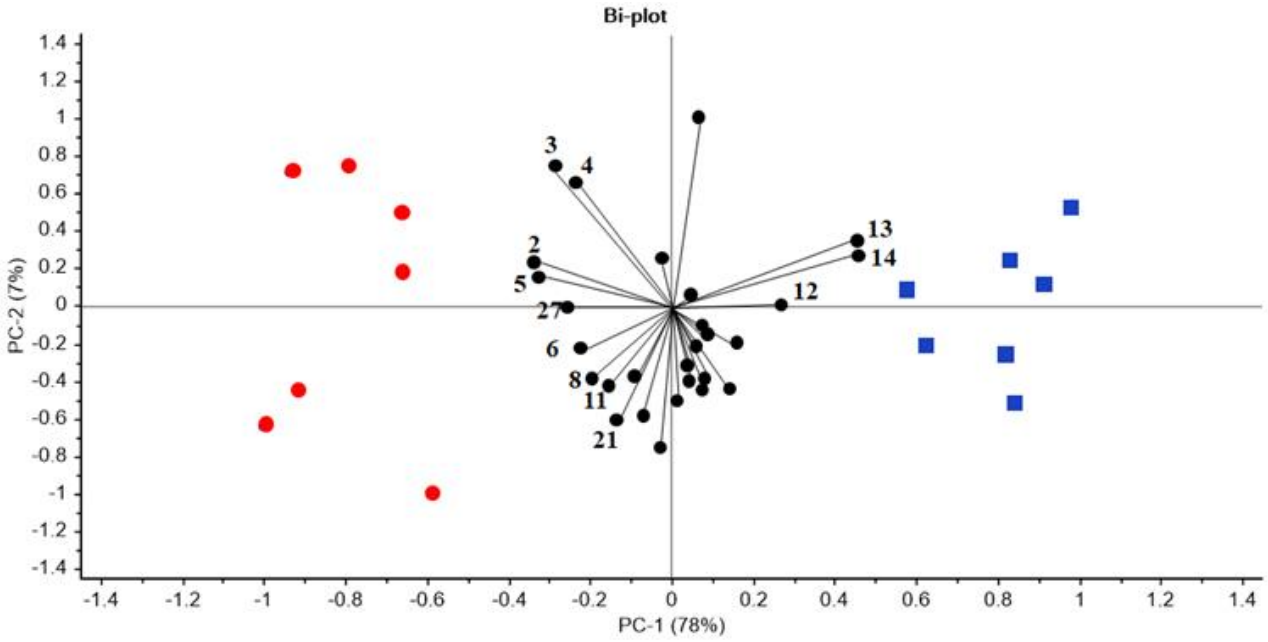

Figure 4. Biplot of PCA of *S. rhombifolia* leaves and stem extracts. Loading plot (black) and score plot (leaves (red) and stem (blue)). The numbers on the graph indicate the number of metabolites based on Table 1

4. Discussion

An untargeted metabolomics approach with UHPLC-Q-Orbitrap HRMS was used to putatively identify metabolites in the leaves and stem extracts of *S. rhombifolia*. The data shows that identified metabolites belong to several groups: five flavonoids, two terpenoids, three alkaloids, two coumarins, three phenolic aldehydes, five phenolic acids, five ecdysteroids, two fatty acids, and one monoterpene lactones. Five flavonoids were identified in the leaves and stem extracts of *S. rhombifolia* in the positive ionization mode (Table 1). The metabolites from this flavonoid group were typically fragmented by releasing H_2O, rhamnosyl (–146 Da), and glucosyl (–162 Da) (Wang *et al.* 2020). Quercetin (1) and epicatechin (4) were fragmented by releasing H_2O molecules $[M+H-18]^+$ at m/z 285 and 273, respectively. In addition, quercetin also underwent a retro Diels-Alder (RDA) reaction to produce fragment ions at m/z 153 $[M+H-C_8H_6O_3]^+$ and 137 $[M+H-C_8H_6O_4]^+$.

Kaempferol (2) was fragmented by releasing CO, which produced fragment ions at m/z 258, and underwent an RDA reaction to produce fragment ions at m/z 153 $[M+H-C_8H_5O_2]^+$ (March and Miao 2004). The MS2 spectrum of glutinoside (3) produced a peak at m/z 287 indicating [aglycone+H]$^+$, 165 [coumaric acid+H]$^+$, and 147 [coumaroyl]$^+$ (Das *et al.* 2011). Kaempferol-3-O-D-glucose-6"-D-rhamnose (5) was fragmented by releasing glucoside and rhamnose characterized by the presence of m/z 287 $[M+H-146-162]^+$.

A compound from the terpenoid class identified in the *S. rhombifolia* extracts consisted of vomifoliol (6) and taraxasterone (7), and was only identified in the leaves extract (Table 1). Vomifoliol (6), a sesquiterpenoid group, was fragmented by releasing 2 H_2O molecules indicated by peaks at m/z 207 and 189 $[M+H-18-18]^+$. Moreover, taraxasterone (7) from the triterpenoid group was identified in positive ionization mode at m/z 425 $[M+H]^+$.

Three compounds from the alkaloid class were identified in the leaves and stem extracts of *S. rhombifolia*. Vasicinol (8) was fragmented by releasing H_2O and C_3H_6O molecules at m/z 187 and 146, respectively. N-trans-feruloyltyramine (9) was fragmented by releasing a $C_8H_{11}NO$ molecule at m/z 177 $[M+H-137]^+$, followed by the loss of CH_3 molecules at m/z 163 $[M+H-136-15]^+$ and H_2O molecules at m/z 145 $[M+H-136-15-18]^+$. Betaine (10) was fragmented at m/z 58 and 59 by releasing CH_2COOH $[M+H-59]^+$ and CH_3COOH $[M+H-60]^+$ molecules, respectively.

Two coumarin compounds were identified in the *S. rhombifolia* extracts, namely umbelliferone (11), which was identified only in the leaves extract, and scopoletin (12), which was identified in both the leaves and stem extracts (Table 1). Umbelliferone (11) was fragmented by releasing an H_2O molecule which produced a fragment ion at m/z 145 $[M+H-18]^+$,

followed by the loss of 2 consecutive CO molecules at m/z 117 $[M+H-18-28]^+$ and 89 $[M+H-18-28-28]^+$. Simultaneously, scopoletin (12) was fragmented by releasing a CH_3COOH molecule at m/z 133 $[M+H-60]^+$ (Zheng *et al.* 2015).

Three phenolic aldehyde compounds were identified in the leaves and stem extracts of *S. rhombifolia*, namely syringaldehyde (13), sinapaldehyde (14), and coniferaldehyde (15). Syringaldehyde (13) underwent fragmentation by releasing CO molecules at m/z 155 $[M+H-28]^+$, COOH molecules at m/z 138 $[M+H-45]^+$, and H_2O molecules at m/z 165 $[M+H-18]^+$. Sinapaldehyde (14) underwent fragmentation by releasing H_2O molecules at m/z 181 $[M+H-18]^+$, COH molecules at m/z 180 $[M+H-29]^+$, and OCH_3 molecules at m/z 149 $[M+H-29-31]^+$. Simultaneusly, coniferaldehyde (15) was fragmented by releasing H_2O molecules at m/z 161 $[M+H-18]^+$, followed by the release of CH3 at m/z 147 $[M+H-18-15]^+$ Furthermore, OCH3 was released at m/z 148 $[M+H-31]^+$ followed by COH molecules at m/z 119 $[M+H-31-29]^+$.

Several phenolic acid compounds were identified in the *S. rhombifolia* extract. These compounds were identified in the positive and negative ionization modes. Salicylic acid (16) was fragmented by releasing H_2O molecules at m/z 121 $[M+H-18]^+$ and COOH at m/z 94 $[M+H-45]^+$. p-Coumaric acid (17) and caffeic acid (19) were fragmented by releasing CO_2 molecules $[M+H-44]^-$ in the negative ionization mode as indicated by the presence of peaks at m/z 119 and 135, respectively. Chlorogenic acid (18) produced fragments by releasing glucoside molecules at m/z 191 $[M+H-162]^-$. 4-Aminobenzoic acid (20) was fragmented by releasing CO_2 molecules at m/z 94 $[M+H-44]^+$ and H_2O at m/z 120 $[M+H-18]^+$ (Kumar *et al.* 2020).

Polypodine B (21), ecdysone (22), 2-deoxy-20-hydroxyecdysone-3-O-D-glucopyranoside (23), 20-hydroxyecdysone-3-O-D-glucopyranoside (24), and 20-hydroxyecdysone (25) compounds included in ecdysteroids were identified in the *S. rhombifolia* leaves and stem extracts. The fragmentation pattern in this group was characterized by the release of several H_2O molecules $[M+H-nH_2O]^+$ and the breaking of the chain at C22–C23 (Stevens *et al.* 2008). Polypodine B (21) and ecdysone (22) were fragmented by releasing 4 successive H_2O molecules observed by the peaks at m/z 425 and 393 $[M+H-4H_2O]^+$ and breaking of bonds at C22–C23, followed by the release of three H^2O molecules as indicated by the peaks at m/z 371 and 355 $[M+H-C_4H_9O-3H_2O]^+$. 2-Deoxy-20-hydroxyecdysone-3-O-D-glucopyranoside (23) and 20-hydroxyecdysone-3-O-D-glucopyranoside (24) were fragmented by releasing glucopyranose, followed by the release of two H_2O molecules at m/z 429 and 445 $[M+H-162-2H_2O]^+$, respectively. 20-Hydroxyecdysone (25) produced fragments by releasing five H_2O molecules in a row as indicated by the peaks at m/z 391 $[M+H-5H_2O]^+$. The cleavage at C22–C23 was followed by the release of two H_2O molecules at m/z 371 $[M+H-C4H9O-2H_2O]^+$.

Only two fatty acids and one monoterpene lactone were identified in the leaves and stem extracts of *S. rhombifolia*. Oleic acid (26) was identified only in the stem extract, whereas linoleic acid (27) was identified only in the leaves extract. These two compounds were identified at m/z 283 and 281, respectively, in the positive ionization mode. However, loliolide (28), a monoterpene lactone, was identified in the *S. rhombifolia* leaves and stem extracts. This compound was fragmented by releasing H_2O molecules at m/z 179 $[M+H-18]^+$.

Furthermore, untargeted metabolomics approach based on UHPLC-Q-Orbitrap HRMS was used to determine the differences in the metabolite composition and concentration in the *S. rhombifolia* leaves and stem extracts. PCA is an unsupervised multivariate analysis that can visualize sample grouping based on the proximity of the metabolites. In addition, PCA can also describe outliers among all samples and explore compounds that have potential as characteristic features in grouping leaves and stem extracts (Ghallab *et al.* 2020; Rafi *et al.* 2020).

It is evident from Figure 3, that the metabolites of *S. rhombifolia* could be separated into leaves and stem extracts. Several metabolites could influence this grouping as characteristic metabolites in the leaves or stems. The biplots of the leaves and stem extracts could be used to determine the metabolites that affect the grouping of samples, as can be observed from the proximity of the metabolites to each group (Figure 4). Based on the biplot, the metabolites that affected the grouping of the leaves extract were kaempferol (2), glutinoside (3), epicatechin (4), kaempferol-3-O-D-glucose-6"-D-rhamnose (5), vomifoliol (6), vasicinol (8), umbelliferone (11), polypodine B (21), and linoleic acid (27). Among these metabolites, kaempferol (2), epicatechin (4), vomifoliol (6), umbelliferone (11), and linoleic acid (27) were only identified in leaves (Table 1), so these metabolites have a significant contribution

to differentiate the leaves from the stem extract. The other four metabolites, glutinoside (3), kaempferol-3-O-D-glucose-6"-D-rhamnose (5), vasicinol (8), and polypodine B (21), were identified in both the leaves and stem extracts; however, these four metabolites had higher peak areas in the leaves extract compared to that of the stem extract. Furthermore, the stem extract grouping was influenced by scopoletin (12), syringaldehyde (13), and synapaldehyde (14). Of the three compounds, syringaldehyde (13) is a potential marker compound from stem extracts because these compounds were only identified in the stem extracts.

In conclusion, untargeted metabolomic analysis by UHPLC-Q-Orbitrap HRMS of *S. rhombifolia* leaves and stem extracts identified 28 metabolites. Seven metabolites were identified only in the leaves, three were identified only in the stems, and 18 were identified in both the leaves and stems. The metabolites putatively identified from the samples belonged to several groups: flavonoids, triterpenoids, alkaloids, coumarins, phenolic aldehydes, phenolic acids, ecdysteroids, fatty acids, and monoterpene lactones. PCA successfully distinguished *S. rhombifolia* leaves and stem extracts using the peak area variables from the identified metabolites.

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