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Identification of Antioxidant Compounds from *Gynura procumbens* Using LC-MS/MS-Based Metabolomics

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ABSTRACT

The antioxidant compounds in *Gynura procumbens,* known as *sambung nyawa,* one of Indonesia's medicinal plants, were identified by LC-MS/MS-based metabolomics. *G. procumbens* extracts were prepared by maceration using water, 70%, 50%, 30% ethanol, and ethanol p.a. and the antioxidant activity was measured using the DPPH method, with IC_{50} values ranging from 90.39 to 140.75 µg/mL. In total, 58 metabolites were identified in the extracts with flavonols being the most abundant group in all ethanol extracts. Five antioxidant compounds were predicted by correlating the extracts' metabolite profiles and the antioxidant activities using orthogonal partial least square-discriminant analysis, four of which were identified as kaempferol 3-O-rutinoside, 4-hydroxybenzaldehyde, 4,5-dicaffeoylquinic acid, and 3-dicaffeoylquinic acid. These five metabolites had a p-value <0.05, fold change >1.5, and VIP >1.46, indicating that they significantly contributed to the antioxidant activity of *G. procumbens* leaves.

Keywords: Antioxidant, metabolomics, *Gynura procumbens*, UHPLC Q-Orbitrap HRMS

INTRODUCTION

Gynura procumbens is a medicinal plant from Indonesia known as *sambung nyawa* (Ji *et al.,* 2019) and is reported to possess many biological activities, including antioxidant activity (Cao *et al.,* 2022). Antioxidant compounds can inhibit free radical attacks and slow the occurrence of chronic diseases caused by free radicals (Nurulita *et al.,* 2011), reducing the risk of cardiovascular disease and preventing cancer development caused by DNA damage. Antioxidants prevent free radical-induced tissue damage by preventing radical formation, scavenging them, or promoting their decomposition. There are two proposed mechanisms of action for antioxidants: the first involves the primary antioxidant donating an electron to the free radical, whereas the second mechanism removes ROS/reactive nitrogen species initiators (secondary antioxidants) by quenching the chain-initiating catalyst. Antioxidants affect biological systems through various mechanisms, such as electron donation, metal ion chelation, co-antioxidants, and gene expression regulation (Lobo *et al.,* 2010) and their biological activity is influenced by the chemical composition and concentration of the active compound. The active compounds of medicinal plants may vary based on growth locations, seasons, harvest times, etc. Furthermore, the type and amount of extracting solvent used can result in different compositions and concentrations of the extracted components (Ibrahim *et al.,* 2017), thereby affecting the biological activity (Ji *et al*., 2019). Chandradevan *et al.* [2020] reported that 100%, 20%, and 70% ethanol extracts of *G. procumbens* leaves had moderate antioxidant activity with an IC_{50} of 124.37, 317.41, and 131.92 µg/mL, respectively, whereas 50% and 100% ethanol extracts demonstrated strong antioxidant activity with an IC_{50} of 66.28 and 63.73 µg/mL, respectively. LC-MS/MS analysis revealed that these ethanol extracts contained major phenolic groups such as hydroxycinnamic acids, hydroxybenzoic acids, and flavonoids.

Most antioxidants are polyphenol metabolites (Budiarti *et al.,* 2019), therefore determining the plant extract's metabolite composition and concentration can yield initial insights regarding the potency of its biological activity. Typically, such compounds are identified by bioassay direct fractionation and an isolation approach, but this is time-consuming and costly. Alternatively, metabolomics (Fan *et al.,* 2020) can qualitatively and quantitatively evaluate compounds under certain conditions systematically and simultaneously (Li *et al.,* 2017).

The determination of active compounds by this method is more effective and efficient because it is less time-consuming and more economical (Pérez *et al*., 2021). LC-MS/MS is a commonly used instrument in metabolomics because of its high sensitivity and selectivity. It effectively separates many compounds to increase the number of compounds identified (Rafi *et al.,* 2022). It has also been combined with chemometrics analysis to evaluate the correlation between biological activity and the metabolite profile, such as in *Momordica charantia (Perumal et al., 2021), Artemisia annua (Alhadrami et al., 2021)*, *Daemonorops acehensis (Gioktavian et al., 2024)*, *Annona muricata* (Septaningsih *et al.*, 2024), etc. However, no reported paper identified antioxidant compounds from *G. procumbens* using the metabolomics approach. Therefore, in this study, LC-MS/MS was used to profile the metabolites contained in *G. procumbens* extracts. The metabolite profile was then correlated with antioxidant activities using chemometric analysis to predict the antioxidant compounds.

MATERIALS AND METHODS

Instrumentation and Materials

The following instruments were used in this study: rotary evaporator, ultrasonicator, UHPLC Tandem Q Exactive Plus Orbitrap-High Resolution Mass Spectrometer (Thermo Scientific, Waltham, USA), the unscrambler X 10.1 (CAMO, Oslo, Norway), compound discoverer 3.3 (thermos scientific, Germany) and a 0.22 µm PTFE filter (Anpel, Shanghai, China). *G. procumbens* leaves were obtained from the Tropical Biopharmaca Research Center, IPB University and Mr. Taopik Ridwan identified this sample with voucher specimen number BMK 0310122016. Ethanol and ascorbic acid were purchased from Merck (Darmstadt, Germany), and 2,2-diphenyl-1-picrylhydrazil (DPPH) was obtained from (Sigma-Aldrich-(Steinheim, Germany).

Sample Preparation and Extraction

The 4-month-post planting *G. procumbens* leaves were collected and washed to remove contamination such as dust, sand, etc. before being oven-dried at 40°C. The dry leaves were pulverized with a blender and sieved through 80 mesh before 20 g of *G. procumbens* powder was macerated using 200 mL of extracting solvent [water, 30%, 50%, 70% ethanol, and ethanol p.a.]. The extracts were incubated for 3 x 24 hours at room temperature and filtered every 24 hours. The filtrates were evaporated with a rotary evaporator until a concentrated extract was obtained. The extraction was repeated five times for each solvent.

LC-MS/MS Analysis and Metabolite Profiling

The extracts were dissolved in LC-MS grade methanol before being filtered through a 0.22 µm PTFE membrane and 2.5 µL of the filtrate was then injected into the LC-MS/MS for analysis. Quality control samples were prepared from a mixture of equal volumes (20 µL) of each sample. An Accucore™ C18 column (100×2.1 mm, 1.8 µm) was used to separate the extracted compounds at a column temperature of 25°C. The compounds were eluted in 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) as follows: 10-30% B for 12.0 minutes, followed by 30-100% B for 12.0-16.5 minutes, and 100% B for 16.5-22.0 minutes. Conditioning took place at 22.0-25.0 minutes with a 5% flow rate average of 0.2 mL/min. An electrospray ionization (ESI) source with Q-Orbitrap mass analysis was used with ionization energies of 18, 35, and 53 eV. The scanning range was set between 100 and 1500 m/z, with an AGC of 3106 and an injection duration of 100 ms. The spray voltage was set at 3.8 kV and the capillary temperature was kept at 320°C. The sheath flow rate was 15 mL/min, and the auxiliary gas flow rate was 3 mL/min. The scan type was complete MS/dd MS2, and full scan data with a resolving power of 70,000 FWHM was collected in both positive and negative modes. The data were analyzed and processed with Compound Discoverer 3.3 to determine the metabolites in the extracts. The metabolite identification process using a local database included selecting spectra, aligning retention time, detecting unknown compounds, grouping unknown compounds, predicting composition, searching mass lists, filling gaps, normalizing areas, and marking background compounds. Furthermore, MS2 confirmation was performed to predict compounds in the extracts.

Determination of Antioxidant Activity

Antioxidant activity was measured using the DPPH method according to the procedure described by Budiarti *et al*. (2019) using ascorbic acid as the positive control. The absorbance was measured at 517 nm to determine the antioxidant activity as follows:

$$\% \text{ Inhibition } \frac{\text{Blank absorbance} - \text{Sample absorbance}}{\text{Blank absorbance}} \times 100\%$$

The IC_{50} values (µg/mL) were determined from linear regression of the concentration on the X-axis and % inhibition on the Y-axis.

Chemometrics Analysis

The yield and antioxidant activity of the extracts were analyzed using ANOVA, followed by post-hoc analysis using the Tukey test. PCA was performed using the Unscrambler X 10.1 (CAMO, Oslo, Norway) to cluster samples based on the extracting solvent. MetaboAnalyst 5.0 was used to predict antioxidant compounds using OPLS-DA. The variable was the peak intensity of the chromatogram. The data were pre-processed using correlation-optimized warping (COW) to synchronize the retention time of each chromatogram before performing PCA and OPLS-DA analyses.

RESULTS AND DISCUSSION

Extraction Yield and Antioxidant Activity of *G. procumbens* Leaf Extracts

The water extracts gave the highest yield but the lowest antioxidant activity because water generally extracts polar compounds such as carbohydrates, proteins, and fatty acids which do not possess antioxidant activity. As per the findings of Mishra *et al.* [2021), an IC_{50} of less than 50 µg/mL is considered very strong antioxidant activity, an IC_{50} between 50 and 100 µg/mL is strong, and weak if the IC_{50} is more than 150 µg/mL. The ethanol extracts (50%, 70%, and 100%) had strong antioxidant activity. The third extract showed strong antioxidant activity using ANOVA but post hoc analysis with Tukey's test did not show significant antioxidant results or activity (Figure 1). The 30% ethanol and water extracts had medium antioxidant activity but higher yields than the other extracts (Figure 1). These results align with previous studies that the ethanol p.a extract of *G. procumbens* leaves has the best antioxidant activity compared to using varying concentrations of ethanol (Chandradevan *et al.,* 2020). Overall, when compared to the positive control (ascorbic acid), *G. procumbens* leaf extract has a lower antioxidant activity than ascorbic acid (IC_{50} of 4.91 µg/mL). The antioxidant activity was determined by the DPPH method because this method is simple, easy, fast, and sensitive. It only requires a small sample to evaluate the antioxidant activity of natural compounds, so it is widely used to test the ability of compounds that act as electron donors. The principle of this antioxidant activity test is to measure antioxidant activity quantitatively, namely by measuring the capture of DPPH radicals by a compound using UV-Vis spectrophotometry to determine the free radical scavenging activity which is expressed by the IC_{50} (inhibitory concentration). The IC_{50} value is defined as the concentration of the test compound that can reduce free radicals by 50%, the smaller the IC_{50} value, the higher the free radical scavenging activity. The working principle of this measurement is the presence of stable free radicals, namely DPPH, which are mixed with antioxidant compounds that have the ability to donate hydrogen, so that free radicals can be suppressed (Molyneux, 2003).

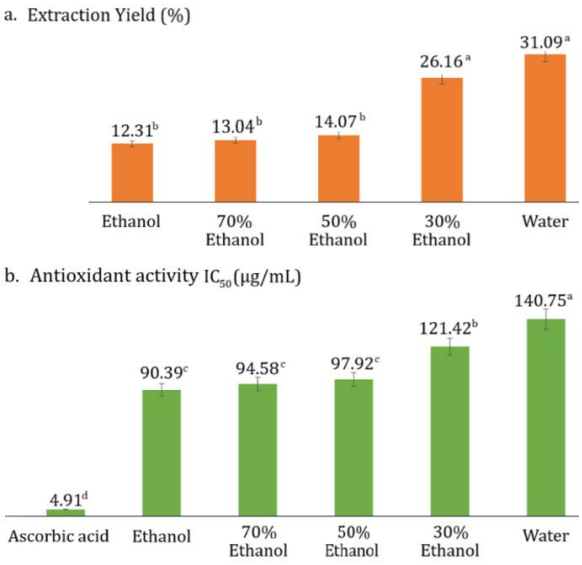

Figure 1. (a) Extraction yield and (b) antioxidant activity (IC_{50}),

The data obtained using the results of analysis of variance (ANOVA), each measurement was carried out 5 times with a maximum %RSD value of 2%. Symbols a, b, and c on the graph (next

to the numbers) indicate significant differences based on the results of the Tukey post hoc test. On the antioxidant activity graph, the water and 30% ethanol extracts have significantly different values from the other extracts so they are symbolized by the symbols a and b, while the 70%, 50% and p.a extracts have significantly different values from the water and 30% ethanol extracts so they are symbolized by the symbol c. For Ascorbic Acid, the value is very significantly different from the other extracts, so it is symbolized by the symbol d.

Water extracts have a high % yield but have low antioxidant activity values, and for ethanol extract the results are inversely proportional to water extract.

Metabolite Profiling of *G. procumbens* Leaf Extracts

Metabolic profiling of the extracts was performed using the UHPLC Q-Orbitrap HRMS and the base peak chromatograms achieved in negative ionization mode showcased comparable separation patterns (Figure 2).

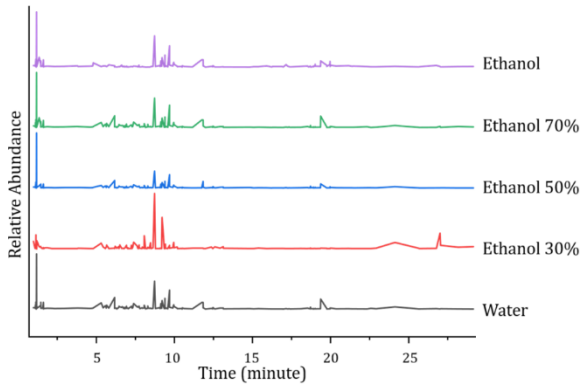

Figure 2. LC-MS/MS chromatogram of 100% Ethanol, 70% Ethanol, 50% Ethanol, 30% Ethanol, and water extracts

The raw data underwent processing via the Compound Discoverer 3.3 software utilizing molecular weight matches verified through internal database fragmentation patterns to identify 58 metabolites consisting of 1 flavanone, 16 flavonols, 2 flavonoid glycosides, 2 isoflavonoids, 7 fatty acids, 9 hydroxybenzaldehydes, 2 phenylpropanoids, 1 hydroxycumarin, 1 coumarin, 2 alkaloids, 1 amino acid, 1 dicarboxylic acid, 2 phenolic compounds, 3 polyphenols, 1 porphyrin, and 1 chlorophyll breakdown compound (Table I).

Discrimination of *G. procumbens* extracts with PCA

The sample chromatograms were pre-processed with correlation-optimized warping (COW) before performing principal component analysis (PCA). According to Perumal *et al.* (2021) using COW pre-processing resulted in score plots with clearer clustering patterns than those generated without COW. COW compresses or tests sample segments through linear interpolation to equate the retention time of the sample chromatogram with the reference chromatogram (Aziz *et al.,* 2020). PCA was used to visualize the relationships between samples, identify anomalies, establish patterns, and develop new hypotheses (Heryanto *et al.,* 2023). The peak intensity observed across the chromatogram was the variable used in PCA. The score plot demonstrates the ability to group each sample based on the extracting solvent with a combined PC1 and PC2 total variance of 90.8% (Figure 4a). The PC is defined as a score plot, which is a projection of multiple objects into PC1 and PC2, which are used to identify similarities and differences between samples. In addition to PCA, differences in the chemical compositions of each extract were analyzed and visualized using hierarchical cluster analysis (HCA). The resulting HCA heatmap depicts the compound variation in each sample and correlates with the antioxidant activity (Figure 3). According to the HCA heatmap, the water extract contains more fatty acids resulting in lower antioxidant activity compared to the ethanol extract, which has the dominant compound from the flavonoid class resulting in higher antioxidant activity. The polarity of the solvent used affects the composition of the extracted metabolites, as demonstrated by the variations in metabolites in each extract.

Prediction of Antioxidant Compounds Using OPLS-DA

OPLS-DA was used to predict antioxidant compounds. The variables for OPLS-DA were the peak area of all detected m/z and the IC_{50} value indicating antioxidant activity. The antioxidant activity was divided into two categories: active antioxidants (IC_{50} = 100 µg/mL) and non-active antioxidants (IC_{50} = >100 µg/mL). The score plot demonstrates that the model correctly classified the samples based on antioxidant activity.

Table I. Putative identification of metabolites from *G. procumbens* leaf extracts

No	Compound	Monoisotopic mass (Da)	Formula	RT (min)	Error (ppm)	MS2	Group	Solvent				
								Ep.a	E70%	E50%	E30%	Aq
1	Malic Acid	134.0215	$C_4H_6O_5$	1.1	-1.5	71, 115, 191	Flavonoid				ü	ü
2	Hydroxycitric acid	208.0219	$C_6H_8O_8$	1.13	-0.48	73, 125, 127, 189, 208	Flavonoid	ü	ü	ü		ü
3	Citric acid	192.027	$C_6H_8O_7$	1.13	-0.59	85, 192	Flavonoid	ü	ü	ü	ü	ü
4	Kaempferol methyl ether	300.0633	$C_{16}H_{12}O_6$	1.34	-1.99	284	Flavonoid					ü
5	4-Oxoproline	129.0425	$C_5H_7NO_3$	1.45	-4.64	128	Flavonoid	ü	ü	ü		ü
6	Methylmalonic acid	118.0266	$C_4H_6O_4$	1.56	-1.69	73, 118	Flavonoid	ü	ü	ü		ü
7	Glutaric acid	132.0422	$C_5H_8O_4$	1.56	-0.9	87, 132	Flavonoid	ü	ü	ü		ü
8	Rutin	610.1533	$C_{27}H_{30}O_{16}$	4.81	-0.49	303, 611	Flavonoid		ü	ü		ü
9	Apigenin-7-O-rutinoside	578.1635	$C_{27}H_{30}O_{14}$	5.8	-0.34	225, 269,8	Flavonoid				ü	
10	Kaempferol	286.0477	$C_{15}H_{10}O_6$	5.8	-2.34	107, 145, 185, 285, 287	Flavonoid				ü	
11	Quercetin-7-O-rutinoside	610.1533	$C_{27}H_{30}O_{16}$	7.8	-1.14	255, 271, 301	Flavonoid	ü			ü	
12	Quercetin-3-O-glucopyranoside	464.0954	$C_{21}H_{20}O_{12}$	8.3	-0.64	153, 205, 303	Flavonoid	ü	ü			
13	Myricetin	318.0375	$C_{15}H_{10}O_8$	8.3	-0.83	137, 151, 271	Flavonoid	ü	ü	ü		ü
14	2-hydroxy formononetin	284.0684	$C_{16}H_{12}O_5$	8.3	-1.72	253, 287	Isoflavonoids	ü	ü	ü		ü
15								ü	ü	ü		ü
16	Kaempferol 3-O-rutinoside	594.1584	$C_{27}H_{30}O_{15}$	8.74	-0.33	269, 311, 341, 594	Flavonoid				ü	ü
17	Kaempferol 3-O-hexoside	610.1533	$C_{27}H_{30}O_{16}$	8.74	-1.11	285, 448	Flavonoid	ü	ü	ü		ü
18	Hesperetin	302.079	$C_{16}H_{14}O_6$	8.74	-3.31	96, 302	Flavonoid	ü	ü	ü	ü	ü
19	Astragalin	448.1005	$C_{21}H_{20}O_{11}$	9.22	-0.44	68, 96, 227, 255, 284, 448	Flavonoid				ü	
20	Kaempferol-3-O-galactoside	448.1005	$C_{21}H_{20}O_{11}$	9.7	-2.1	353, 515	Flavonoid	ü	ü	ü	ü	ü
21	Azelaic acid	188.1048	$C_9H_{16}O_4$	9.7	-0.53	125, 188	Flavonoid	ü	ü	ü	ü	ü
22	Quercetin-3β-D-glucoside	626.1483	$C_{21}H_{20}O_{12}$	9.97	-3.44	69, 221, 271, 465	Flavonoid	ü	ü	ü		ü
23	Baicalin	446.0849	$C_{21}H_{18}O_{11}$	10.14	-2.38	271, 272	Flavonoid	ü				
24	2,7-Dihydroxy-4,5-dimethoxyisoflavon	298.0477	$C_{17}H_{14}O_6$	15.79	-0.95	137, 300, 315	Isoflavonoids	ü	ü	ü	ü	ü
25	Ostruthin	298.1568	$C_{19}H_{22}O_3$	17.59	-1.67	298	Flavonoid	ü	ü	ü	ü	ü
26	Oleate acid	282.2558	$C_{18}H_{33}O_2$	22.94	-0.71	282	Fatty acid	ü	ü	ü	ü	
27	16-Hydroxyhexadecanoic acid	272.2351	$C_{16}H_{32}O_3$	23.23	-1.1	59, 272	Fatty acid	ü				
28	α-9(10)-EpODE	294.2194	$C_{18}H_{30}O_3$	18.82	-2.79	293	Fatty acid	ü			ü	
29	13(S)-HOTrE	294.2194	$C_{18}H_{30}O_3$	17.59	-3.55	294	Fatty acid	ü	ü	ü	ü	ü
30	Traumatic Acid	228.1361	$C_{12}H_{20}O_4$	15.84	-0.43	183, 228	Fatty acid	ü				
31	(+/-)9-HpODE	312.23	$C_{18}H_{32}O_4$	16.97	-0.32	223, 312	Fatty acid	ü				
32	Corchorifatty acid F	328.2249	$C_{18}H_{32}O_5$	12.89	-3.04	91, 328	Fatty acid	ü	ü	ü		ü

Continue of Table I.

No	Compound	Monoisotopic mass (Da)	Formula	RT (min)	Error (ppm)	MS2	Group	Solvent				
								Ep.a	E70%	E50%	E30%	Aq
33	D-(-)-Quinic acid	192.0633	$C_7H_{12}O_6$	1.1	-1.04	102, 146	Hydroxybenzaldehyde				ü	ü
34	4-Hydroxybenzaldehyde	122.0367	$C_7H_6O_2$	1.34	-4.09	123	Hydroxybenzaldehyde				ü	ü
35	3-caffeoylquinic acid	354.095	$C_{16}H_8O_9$	4.81	-0.84	135, 179, 191, 354	Hydroxybenzaldehyde	ü	ü	ü	ü	ü
36	Caffeoylglucaric acid	372.0692	$C_{15}H_{16}O_{11}$	5.56	-0.26	85, 173, 179, 191, 209	Hydroxybenzaldehyde				ü	ü
37	p-Coumaric acid	164.0473	$C_9H_8O_3$	6.18	-1.82	120, 165	Hydroxybenzaldehyde				ü	ü
38	3,4-Dicaffeoylquinic acid	516.12677	$C_{25}H_{24}O_{12}$	9.22	-0.19	135, 161, 173, 179,	Hydroxybenzaldehyde				ü	ü
39	4,5-Dicaffeoylquinic acid	516.1267	$C_{25}H_{24}O_{12}$	9.7	-1.35	516	Hydroxybenzaldehyde				ü	ü
40	p-Anisaldehyde	137.0587	$C_8H_8O_2$	9.7	-5.14	93, 135	Hydroxybenzaldehyde				ü	ü
41	1,4-Dicaffeoylquinic acid	516.1267	$C_{25}H_{24}O_{12}$	15.79	-0.38	173, 179, 515	Hydroxybenzaldehyde				ü	ü
42	Chlorogenic acid	354.095	$C_{16}H_{18}O_9$	1.34	-0.9	85, 87, 111, 191	Phenylpropanoid					ü
43	Sinapinic acid	224.0684	$C_{11}H_{12}O_5$	7.3	-2.95	224	Phenylpropanoid		ü	ü		ü
44	Esculetin	178.0266	$C_9H_6O_4$	3.85	-4.89	105, 121, 133, 149, 177	Hydroxycoumarin	ü	ü	ü	ü	ü
45	Coumarin	146.0367	$C_9H_6O_2$	6.18	-1.9	91, 103, 147	Coumarins	ü				
46	3-hydroxydecanoic acid	188.1412	$C_{10}H_{20}O_3$	11.85	-4.53	59. 188	Alkaloids	ü	ü	ü		ü
47	vanilloloside	316.1158	$C_{14}H_{20}O_8$	6.51	-0.94	64, 69, 316	Alkaloids	ü			ü	
48	L-Isoleucine	131.0946	$C_6H_{13}NO_2$	1.56	-1.52	86, 131	Amino acid	ü				ü
49	Diethyl malonate	160.0735	$C_7H_{12}O_4$	6.51	-1.25	67, 102, 115, 130, 159, 160	dicarboxylic acids	ü			ü	
50	Vanillin	152.0473	$C_8H_8O_3$	7.3	-0.65	136, 151	Aldehyde phenolics		ü	ü		ü
51	Salicylic acid	138.0316	$C_7H_6O_3$	9.97	-6.5	93, 137	Phenolic	ü	ü	ü		ü
52	Caffeic acid	180.0422	$C_9H_8O_4$	9.97	-1.11	180	Polyphenol	ü	ü	ü		ü
53	Embelin	294.1831	$C_{17}H_{26}O_4$	13.1	-2.03	221, 236, 294	Polyphenol	ü	ü		ü	ü
54	5-O-methyl embelin	308.1987	$C_{18}H_{28}O_4$	14.86	0.64	308	Polyphenol	ü				
55	Harderoporphyrin	608.2634	$C_{35}H_{36}N_4O_6$	19.39	-5.74	609	Porphyrin		ü	ü		ü
56	Pheophorbide A	592.2685	$C_{35}H_{36}N_4O_5$	20.57	-5.74	593	Chlorophyll breakdown	ü			ü	
57	Unknown	584.3231	$C_{28}H_{50}N_4O_5P_2$	22.36	-4.59	584	-	ü				
58	Unknown	396.2148	$C_{21}H_{32}O_7$	11.16	-2.79	395	-	ü				

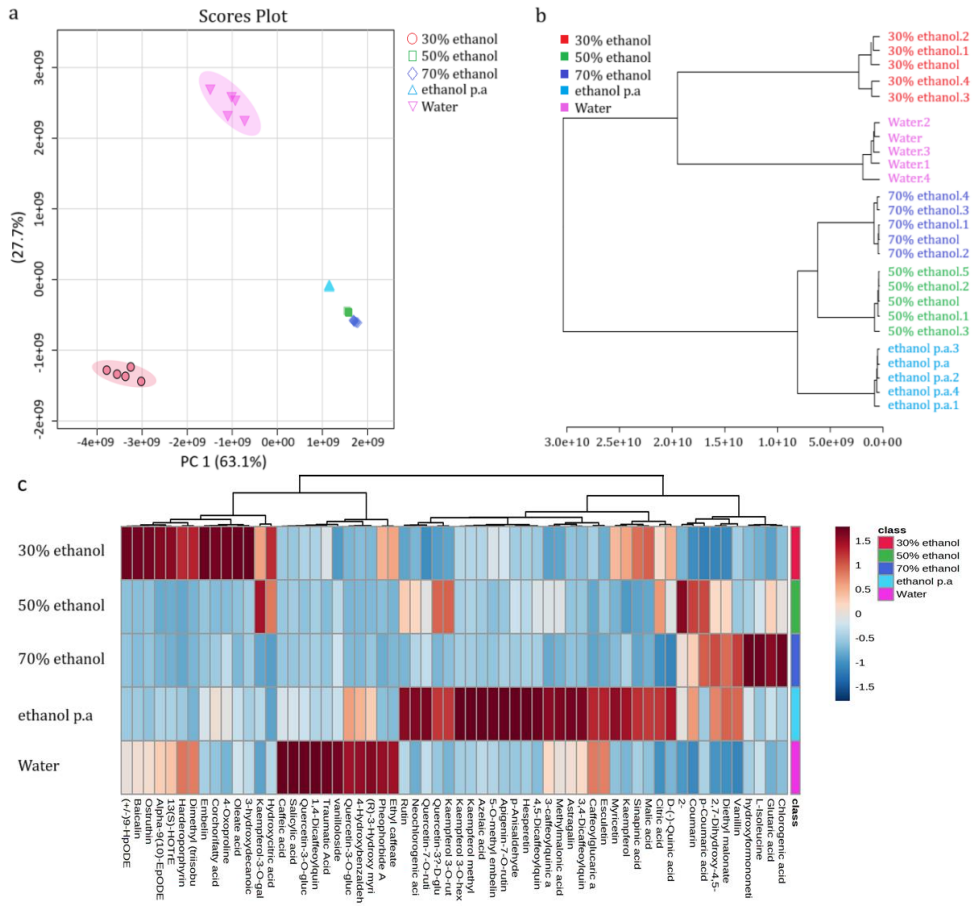

Figure 3. Grouping based on similarity of compounds used by (a) Plot score, (b) Dendogram, (c) Heatmap of HCA *G. procumbens* extracts

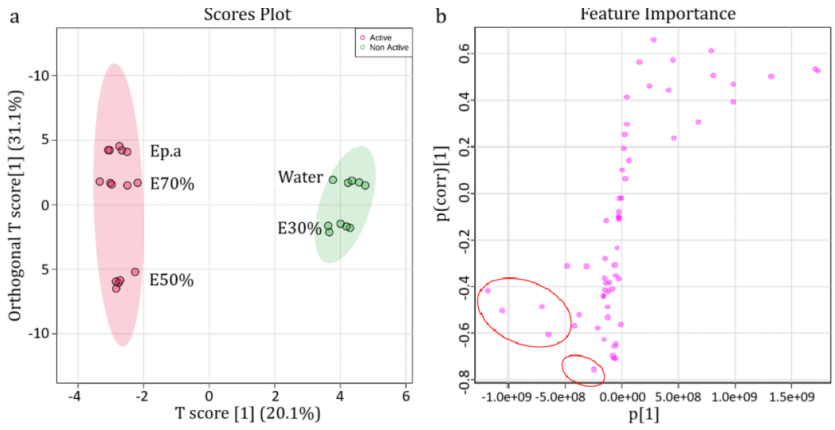

Figure 4. (a) Prediction of antioxidant compound using Score plot and (b)Evaluation of the suitability of the model for prediciting active antioxidant compunds using S-plot of OPLS-DA

The active antioxidant group comprised 50%, 70% ethanol, and ethanol p.a extracts in the left quadrant, while the non-active antioxidant group comprised water and 30% ethanol extracts in the right quadrant (Figure 4a). The model's suitability and ability to predict active antioxidant compounds was evaluated using a criterion of R2 > 0.5 and Q2 > 0.4, showing that the developed model (Figure 4b) has an R2 value of 0.898 (R2X = 0.822, R2Y = 0.991) and a Q2 value of 0.986, indicating its ability to differentiate between active and inactive compounds. S-plots can predict antioxidant activity in compounds, with the x-axis representing the variable's contribution to the observed variance and the y-axis representing the correlation between samples and the reliability of the results.
Variables that differ significantly between groups are typically placed at the top of the plot in the right corner, or the bottom left corner. The antioxidant variables were determined using a combination of S-plot and VIP with the variables including p-value (0.05), fold change (>1.5), and VIP (>1.46). Five compounds were predicted to have antioxidant potential based on these criteria (Table 2), four of which were identified as kaempferol 3-O-rutinoside **(1)**, 4-hydroxybenzaldehyde **(2)**, 4,5-dicaffeoylquinic acid **(3)**, and 3,4-dicaffeoylquinic acid **(4)**. The compound kaempferol 3-O-rutinoside was identified in all *G. procumbens* leaf extracts at retention time of 8.64 seconds.
The compound was predicted based on research conducted by Kim *et al.* (2011) who reported that kaempferol 3-O-rutinoside compounds were isolated from ethanol extracts of *G. procumbens* leaves. The compound was reported to have an IC_{50} value of 44.62 µg/mL based on research conducted by Afandi *et al.* (2014).

Kaempferol-3-O-rutinoside has a fragmentation pattern of flavonoids, the cleavage of the flavonoid B ring through a retro-Diels-Alder (RDA) releases hydroxyl $[M+H-OH]^+$ or methyl $[M+H-CH_3]^+$ groups (Stevenson & De Bo, 2017). This compound produces fragmentation patterns, namely m/z 285 $[M+H-C_{15}H_9O_6]^-$, 255 $[M+H-C_{14}H_7O_5]^-$, and 227 $[M+H-C_{13}H_7O_4]^-$ (March & Miao, 2004)

The unknown compound belongs to the sesquiterpenoid group due to the resemblance of its spectrum to that documented by González-Masís *et al*. (2020), with an ion mass of 395 m/z. Nonetheless, it is crucial to confirm this speculation by isolating the compound and determining its molecular structure.

CONCLUSION

The antioxidant compounds in *G. procumbens* leaf extracts were identified using an LC-MS/MS-based metabolomics approach. The ethanol p.a. extract had the highest antioxidant activity, while the aqueous extract had the lowest. A total of 54 compounds were identified with flavonols being the most abundant group in the ethanol p.a extract and hydroxybenzaldehyde in the water extract. The OPLS-DA model successfully grouped the extracts into active antioxidant (70%, 50% ethanol, and ethanol p.a extracts) and non-active (30% ethanol and water) groups, predicting five antioxidant compounds. Four compounds were successfully identified as kaempferol-3-O-rutinoside, 4-hydroxybenzaldehyde, 4,5-dicaffeoylquinic acid, and 3,4-dicaffeoylquinic acid. Kaempferol 3-O-rutinoside was identified in all *G. procumbens* leaf extracts.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Afandi, A., Halimhilmi Zulkiffli, M., Sadikun, A., & Ismail, S. (2014). Antioxidant properties of *Gynura procumbens* extracts and their inhibitory effects on two major human recombinant cytochrome P450S using a high throughput luminescence assay. *Asian Journal of Pharmaceutical and Clinical Research*, *7*(5), 36–41.

Alhadrami, H. A., Sayed, A. M., El-Gendy, A. O., Shamikh, Y. I., Gaber, Y., Bakeer, W., Sheirf, N. H., Attia, E. Z., Shaban, G. M., Khalifa, B. A., Ngwa, C. J., Pradel, G., Rateb, M. E., Hassan, H. M., Alkhalifah, D. H. M., Abdelmohsen, U. R., & Hozzein, W. N. (2021). A metabolomic approach to target antimalarial metabolites in the *Artemisia annua* fungal endophytes. *Scientific Reports*, *11*(1), 1–11. https://doi.org/10.1038/s41598-021-82201-8

Aziz, Z., Yuliana, N. D., Simanjuntak, P., Rafi, M., & Syamsudin. (2020). FTIR and HPLC-based metabolomics of yacon leaf extracts (*Smallanthus sonchifolius* [poepp & endl.] h.

robinson) from two locations in Indonesia. *Indonesian Journal of Chemistry*, *20*(3), 567–578. https://doi.org/10.22146/ijc.43453

Budiarti, E., Batubara, I., & Ilmiawati, A. (2019). Potential of Several Asteraceae Plant Extracts as Antioxidants and Antiglycation. Indonesian Herbal Medicine Journal, *4*(3), 103–111. https://doi.org/10.29244/jji.v4i3.161

Cao, M. Y., Wu, J., Xie, C. Q., Wu, L., Gu, Z., Hu, J. W., & Xiong, W. (2022). Antioxidant and anti-inflammatory activities of *Gynura procumbens* flower extracts through suppressing LPS-induced MAPK/NF-κB signaling pathways. *Food and Agricultural Immunology*, *33*(1), 511–529. https://doi.org/10.1080/09540105.2022.2098935

Chandradevan, M., Simoh, S., Mediani, A., Ismail, N. H., Ismail, I. S., & Abas, F. (2020). UHPLC-ESI-Orbitrap-MS analysis of biologically active extracts from *Gynura procumbens* (Lour.) Merr. and *Cleome gynandra L.* leaves. *Evidence-Based Complementary and Alternative Medicine*, *2020*. https://doi.org/10.1155/2020/3238561

Das, N., Mishra, S. K., Bishayee, A., Ali, E. S., & Bishayee, A. (2021). The phytochemical, biological, and medicinal attributes of phytoecdysteroids: An updated review. *Acta Pharmaceutica Sinica B*, *11*(7), 1740–1766. https://doi.org/10.1016/j.apsb.2020.10.012

Fan, M., Lian, W., Li, T., Fan, Y., Rao, Z., Li, Y., Qian, H., Zhang, H., Wu, G., Qi, X., & Wang, L. (2020). Metabolomics approach reveals discriminatory metabolites associated with the blue pigments from *Vaccinium bracteatum thunb.* Leaves at different growth stages. *Industrial Crops and Products*, *147*(January 2019), 112252. https://doi.org/10.1016/j.indcrop.2020.112252

González-Masís, J., Cubero-Sesin, J. M., Corrales-Ureña, Y. R., González-Camacho, S., Mora-Ugalde, N., Vega-Baudrit, J. R., Rischka, K., Verma, V., & Gonzalez-Paz, R. J. (2020). Nonirritant and cytocompatible *Tinospora cordifolia* nanoparticles for topical antioxidant treatments. *International Journal of Biomaterials*, *2020*. https://doi.org/10.1155/2020/3637098

Heryanto, R., Putra, C. A., Khalil, M., Rafi, M., Putri, S. P., Karomah, A. H., & Batubara, I. (2023). Antioxidant activity and metabolite profiling of *Xylocarpus granatum* extracts using gas chromatography-mass spectrometry. *Metabolites*, *13*(2). https://doi.org/10.3390/metabo13020156

Ibrahim, M. H., Kong, Y. C., & Zain, N. A. M. (2017). Effect of cadmium and copper exposure on growth, secondary metabolites, and antioxidant activity in the medicinal plant sambung nyawa (*Gynura procumbens* (Lour.) Merr). *Molecules*, *22*(10). https://doi.org/10.3390/molecules22101623

Ji, Y. Bin, Wang, Y. S., Fu, T. T., Ma, S. Q., Qi, Y. D., Si, J. Y., Sun, D. A., & Liao, Y. H. (2019). Quantitative analysis of pyrrolizidine alkaloids in *Gynura procumbens* by liquid chromatography-tandem quadrupole mass spectrometry after enrichment by PCX solid-phase extraction. *International Journal of Environmental Analytical Chemistry*, *99*(11), 1090–1102. https://doi.org/10.1080/03067319.2019.1616705

Li, J. E., Wang, W. J., Zheng, G. D., & Li, L. Y. (2017). Physicochemical properties and antioxidant activities of polysaccharides from *Gynura procumbens* leaves by fractional precipitation. *International Journal of Biological Macromolecules*, *95*, 719–724. https://doi.org/10.1016/j.ijbiomac.2016.11.113

Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). *Free radicals, antioxidants, and functional foods: Impact on human health*. https://doi.org/10.4103/0973-7847.70902

March, R. E., & Miao, X. S. (2004). A fragmentation study of kaempferol using electrospray quadrupole time-of-flight mass spectrometry at high mass resolution. *International Journal of Mass Spectrometry*, *231*(2–3), 157–167. https://doi.org/10.1016/j.ijms.2003.10.008

Molyneux P. (2003). The use of the stable free radical diphenylpicryl-hydrazyl (DPPH) for estimating antioxidant activity. *Songklanakarin Journal of Science and Technology*, *50*(June 2003), 211–219.

Nurulita, N. A., Meiyanto, E., & Sugiyanto, S. (2011). Selectivity of ethyl acetate fraction of *Gynura procumbens* on colon cancer and breast cancer. *Indonesian Journal of Cancer Chemoprevention*, *2*(3), 274. https://doi.org/10.14499/indonesianjcancchemoprev2iss3pp274-280

Perumal, V., Khatib, A., Uddin Ahmed, Q., Fathamah Uzir, B., Abas, F., Murugesu, S., Zuwairi Saiman, M., Primaharinastiti, R., & El-Seedi, H. (2021). Antioxidant profile of *Momordica charantia* fruit extract analyzed using LC-MS-QTOF-based metabolomics. *Food Chemistry: Molecular Sciences*, *2* (June 2020), 4–11. https://doi.org/10.1016/j.fochms.2021.100012

Rafi, M., Karomah, A. H., Septaningsih, D. A., Trivadila, Rahminiwati, M., Prama Putri, S., & Iswantini, D. (2022). LC-MS/MS-based metabolite profiling and lipase enzyme inhibitory activity of *Kaempferia angustifolia Rosc.* with different extracting solvents. *Arabian Journal of Chemistry*, *15*(11), 104232. https://doi.org/10.1016/j.arabjc.2022.104232

Rivera-Pérez, A., Romero-González, R., & Garrido Frenich, A. (2021). Application of an innovative metabolomics approach to discriminate geographical origin and processing of black pepper by untargeted UHPLC-Q-Orbitrap-HRMS analysis and mid-level data fusion. *Food Research International*, *150*. https://doi.org/10.1016/j.foodres.2021.110722

Stevenson, R., & De Bo, G. (2017). Controlling reactivity by geometry in retro-diels-alder reactions under tension. *Journal of the American Chemical Society*, *139*(46), 16768–16771. https://doi.org/10.1021/jacs.7b08895

Xu, H., Li, Z., Tong, Z., He, F., & Li, X. (2020). Metabolomic analyses reveal substances that contribute to the increased freezing tolerance of alfalfa (*Medicago sativa L.*) after continuous water deficit. *BMC Plant Biology*, *20*(1), 1–15. https://doi.org/10.1186/s12870-019-2233-9