Phenolics Profiling and Free Radical Scavenging Activity of *Annona muricata*, *Gynura procumbens*, and *Typhonium flagelliforme* Leaves Extract

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Abstract: *The leaves of* Annona muricata *(sirsak),* Gynura procumbens *(sambung nyawa), and* Typhonium flagelliforme *(keladi tikus) have been used as traditional medicines in Indonesia. This study aims to determine the antioxidant capacity and putatively identified phenolics from the leaves of three medicinal plants forementioned above. We used the DPPH (2,2-diphenyl-1-picrylhydrazyl) method for measuring radical scavenging (antioxidant assay) while the phenolics profiling was determined using UHPLC-Q-Orbitrap HRMS. The results showed that the percentage of radical scavenging activity of* G. procumbens *leaves extract in ethanol was higher than the other two plants. Phenolics profiling of the three medicinal plants was identified with 38 compounds belonging to flavones and flavanols hydroxycinnamic acid, and several other groups. The number of metabolites identified putatively was 12, 31, and 19 metabolites in the extracts of* A. muricata, G. procumbens, *and* T. flagelliforme, *respectively. The results confirmed the correlation between the phenolics presence and the antioxidant capacity of three plants used in this study.*

Keywords: Annona muricata*; radical scavenging activity;* Gynura procumbens*; phenolics profiling;* Typhonium flagelliforme*; UHPLC-Q-Orbitrap HRMS*

■ INTRODUCTION

Medicinal plants from Indonesia need to be developed to find their specific biological activities for possible usage as herbal medicine. The medicinal plant extract's biological activity is strongly affected by its metabolite composition and concentration, especially its bioactive compound. Any information regarding the metabolites' composition and concentration gives different biological activity levels such as antioxidant activity [1-2]. It was reported that some medicinal plants from Indonesia are known to have antioxidant activity, such as *Annona muricata* [3], *Gynura procumbens* [4], and *Typhonium flagelliforme* [5]. These plants are also known to contain phenolic compounds, which are generally known as antioxidant agents.

Antioxidants play an essential role in inhibiting the oxidation process from protecting cells from harmful free radicals generated from the body's metabolism and other external factors [6]. In general, antioxidants derived from plants come from its phenolics, like flavonoids [7], hydroxamic acids derivatives [8-9], coumarins [10], vitamins (tocopherol) [11], and

phenolic acids (gallic acid) [12-13]. Phenolics are well known as primary active compounds as natural antioxidants and mostly found in plants [13-14]. Therefore, it is essential to have phenolics profiles in our sample to analyze and evaluate their antioxidant capacity.

To date, there is no reported paper regarding phenolic profiling of extract ethanol of *A. muricata* (*sirsak*), *G. procumbens* (*sambung nyawa*), and *T. flagelliforme* (*keladi tikus*). Therefore, we performed phenolic profiling of the three medicinal plant extracts and evaluated their percentage inhibition of DPPH radical as an antioxidant assay. Phenolics profiling was performed using UHPLC-Q-Orbitrap HRMS. This analytical instrument has high sensitivity and accuracy in the determination of the molecular weight of metabolites. Additionally, we analyzed the correlation of phenolic profile and its percentage inhibition of DPPH radical.

■ EXPERIMENTAL SECTION

Materials

The leaves *A. muricata, G. procumbens*, and *T. flagelliform*e were collected from the medicinal plant garden of Tropical Biopharmaca Research Center (TropBRC), IPB University, Bogor, Indonesia. Voucher specimens of *A. muricata* (BMK0091082016), *G. procumbens* (BMK 0310122016), and *T. flagelliforme* (BMK0175092016) were stored in TropBRC, IPB University. Ethanol pro analysis, acetonitrile, and water (LC-MS grade) were purchased from Merck (Darmstadt, Germany), while 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Sigma Aldrich (Palo Alto, USA). PTFE filter 0.22 μm was obtained from Anpel (Shanghai, China).

Instrumentation

The free radical scavenging assay by DPPH was performed using a microplate reader (Epoch BioTek, Winooski, USA). Separation and profiling of phenolics were conducted in Vanquish Flex UHPLC tandem Q Exactive Plus Orbitrap-High Resolution Mass Spectrometer. The chromatogram data were analyzed using the Thermo XCalibur, and the putative identification was performed using Compound Discoverer version 2.2 (Thermo Fisher, Waltham, MA, USA) with an in-house database, chemical literature, and spectral database.

Procedure

Extraction

The three samples' fresh leaves were separately washed and sun-dried in the open air and pulverized. The powder sample was macerated in ethanol with a ratio between the powder sample weight with solvent extraction of about 1:10 wt/v. The extraction was carried out for 24 h with three repetitions; the filtrates were concentrated using a rotary evaporator under low pressure [2].

Measurement of free radical scavenging activity

Ethanol extract stock solution was prepared with a concentration of 250 ppm. A sample solution of 100 μL was pipetted into the 96-well plate, added with 100 μL of 125 μM DPPH solution in ethanol, and incubated under light conditions and at room temperature for 30 min. The absorbance was measured using a microplate reader at a wavelength of 517 nm. We performed triplicate analysis, and the percentage of radical scavenging was determined using the equation below:

$$\%\ \text{Radicals cavenging} = \frac{(\text{Abs. of blank} - \text{Abs. of sample})}{\text{Abs. of blank}} \times 100\%$$

Profiling of phenolics using UHPLC-Q-Orbitrap HRMS

A total of 50 mg of each extract were dissolved in 5 mL methanol and sonicated for 30 min. The solution was filtered using a 0.2 μm PTFE filter into the vial and ready to be analyzed using UHPLC-Q-Orbitrap HRMS using a C18 column (100 mm × 2.1 mm × 1.8 μm). The mobile phase employed was 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) under a gradient of 5-95% (B) for 30 min. The flow rate was 0.2 mL/min, and the injection volume was 2.5 μL. The source of MS ionization used was ESI (–) with Q-Orbitrap mass analyzer, and the collision energy deployed for ionization was 18, 35, and 53 eV. The range of *m/z* of 100–1500 and automatic gain control (AGC) was set at 3×10^6, and the injection time was 100 ms. Other conditions were as followed: spray voltage 3.8 kV, the capillary temperature 320 °C, sheath gas, and auxiliary gas flow rate were 15 and 3 mL/min, respectively. The scan type used is full MS/dd MS2 and

full scan data in positive and negative were acquired at a resolving power of 70,000 FWHM.

Data Analysis

The statistical analysis was carried out using one-way analysis of variance (ANOVA) for free radical scavenging activity and followed with Tukey test ($p < 0.05$). The UHPLC-Q-Orbitrap HRMS chromatogram in raw data was processed using the X-Calibur 2.2 program to convert the data. Putative identification of metabolites was performed using Compound Discoverer 2.0 software by processing the data with spectra process, aligning retention time, detecting unknown compounds, group of unknown compounds, and predicting compositions. The work was continued with search the mass lists, fill gaps, normalized areas, and marked background compounds. Interpretation of mass spectra was used in-house database, chemical literature, and spectral database.

■ RESULTS AND DISCUSSION

Free Radical Scavenging Activity

Antioxidants stop or break the chain reaction of free radicals in the body; hence they could inhibit or prevent the body cells damage. DPPH is commonly used for measuring the free radical scavenging activity of a sample. DPPH is a stable free radical, dark purple color that can turn yellow if it accepts electrons or hydrogen from an antioxidant moiety, resulting in stable DPPH molecules [15].

Fig. 1 shows the free radical scavenging activity of ethanol extract of the three plants. At a concentration of 250 µg/mL, the inhibition of *A. muricata*, *G. procumbens*, and *T. flagelliforme* ethanol p.a. extract was 31.60±1.24%, 59.56±8.39%, and 35.06±3.05%, respectively. Among these extracts, *G. procumbens* has exhibited the highest free radical scavenging activity. The free radical scavenging activity of different extracts was in the following order: *G. procumbens* > *A. muricata* > *T. flagelliforme,* and no significant differences were found between *A. muricata* and *T. flagelliforme* leaves extract ($p > 0.05$). It means *G. procumbens* contained a high concentration of antioxidant compounds.

Phenolics Profiling of the *A. muricata*, *G. procumbens*, and *T. flagelliforme* Leaves Extract

Metabolite profiling in the context of drug development aims to identify and analyze a large group of metabolites, including the investigation's intermediate products, reflecting dynamic responses to physiological changes or developmental stimuli [16-17].

The phenolics in the ethanol extracts were identified using UHPLC-Q-Orbitrap HRMS. Fig. 2 shows a base peak chromatogram with negative ionization mode in the three studied medicinal plants' leaves extracts. The chromatograms give different patterns, indicating a difference in each species sample's metabolite composition and concentration.

A total of 38 compounds were putatively identified based on confirmation of the ion precursor values and MS2 fragmentation patterns with the available literature (Table 1). The number of metabolites identified in the leaves of *A. muricata*, *G. procumbens*, and *T. flagelliforme* is 31, 12, and 19 phenolics, respectively. These compounds belong to flavones, flavonols, hydroxycinnamic acids, and several other groups. Thus,

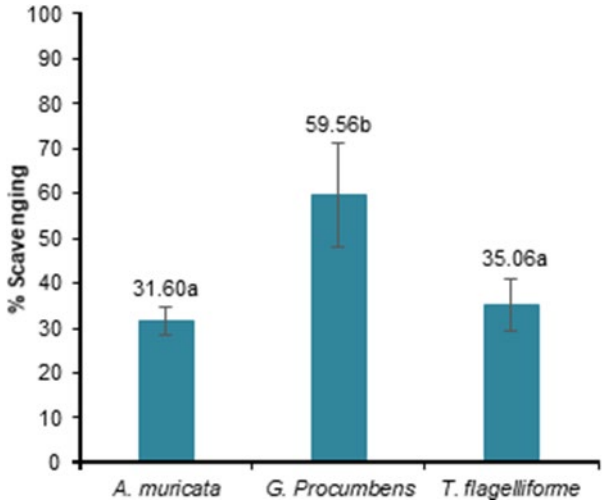

Fig 1. The *DPPH* radical scavenging activity of *A. muricata*, *G. procumbens*, and *T. flagelliforme* leaves ethanol extracts. All experiments were performed in triplicate. Data are expressed as mean ± SD (n = 3), and data marked with a different letter indicates a significant difference ($p < 0.05$)

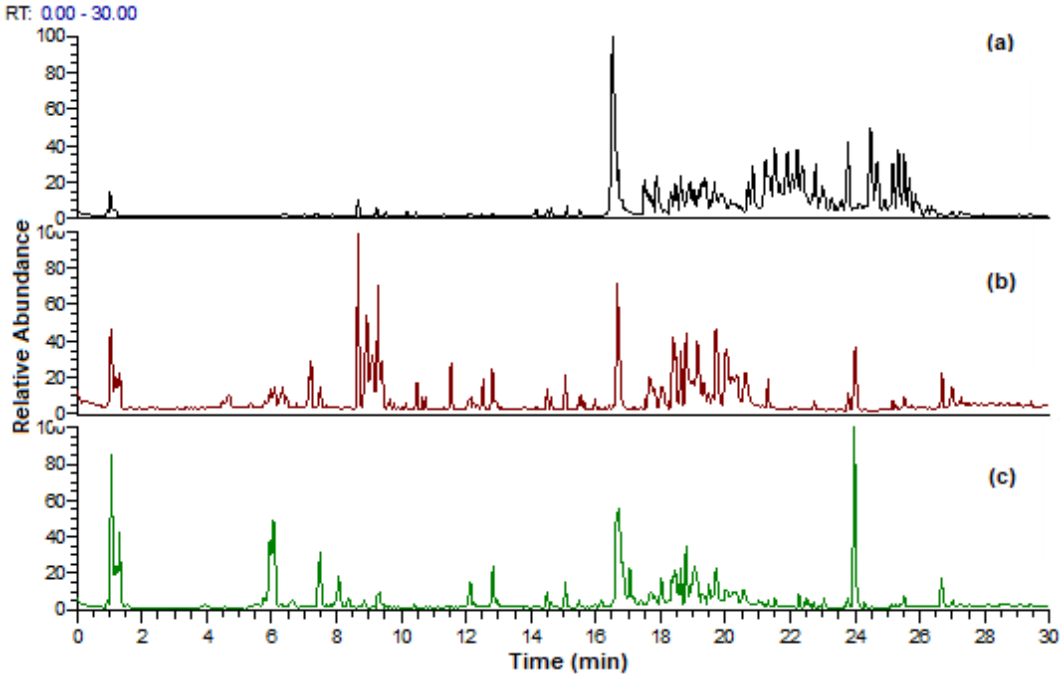

Fig 2. Base peak chromatograms of *A. muricata* (a), *G. procumbens* (b), and *T. flagelliforme* (c) ethanol extract in negative ionization mode

Table 1. Putative identification of phenolic from the ethanol extract of *A. muricata* (AM), *G. procumbens* (GP), and *T. flagelliforme* (TF) leaves extract by UHPLC-Q-Orbitrap HRMS

No	Compound	Formula	MW experimental	Rt (min)	MS2	Group	Sample		
							AM	GP	TF
1	Apigenin 7-O-rutinoside	$C_{27}H_{30}O_{14}$	578.16776	5.83	269; 225	Flavones		√	
2	Orientin (Luteolin 8-C glucoside)	$C_{21}H_{20}O_{11}$	448.10103	8.93	327; 285	Flavones		√	
3	Apigenin C-hexoside-C-pentoside	$C_{26}H_{28}O_{14}$	564.14873	7.29	545; 443; 431; 341; 311	Flavones			√
4	Cirsilineol	$C_{18}H_{16}O_7$	344.08898	14.38	345	Flavones		√	
5	Vitexin (Apigenin 8-C-glucoside)	$C_{21}H_{20}O_{10}$	432.10563	8.24	431; 341; 311; 269	Flavones			√
6	Kaempferol 3-O-rutinoside	$C_{27}H_{30}O_{15}$	594.15919	8.62	287	Flavonols	√	√	√
7	Kaempferol	$C_{15}H_{10}O_6$	286.04753	12.51	257; 153; 133; 121	Flavonols		√	√
8	Quercetin	$C_{15}H_{10}O_7$	302.04243	11.1	273; 179; 151; 121	Flavonols		√	
9	Quercetin 7-O-rutinoside	$C_{27}H_{30}O_{16}$	610.15386	7.99	301; 271; 255	Flavonols	√	√	
10	Kaempferol methyl ether	$C_{16}H_{12}O_6$	300.06375	1.31	284	Flavonols		√	
11	Myricetin	$C_{15}H_{10}O_8$	318.03826	8.23	271; 151; 137	Flavonols		√	
12	Quercetin 3-O-glucoside	$C_{21}H_{20}O_{12}$	464.09618	8.29	303; 205; 153	Flavonols		√	
13	Kaempferol 3-O-hexoside		448.10097	8.71	285; 284	Flavonols		√	
14	Kaempferol 3-O-(6"-acetyl-galactoside)-7-O-rhamnoside	$C_{29}H_{32}O_{16}$	636.16945	8.54	325; 337	Flavonols			√
15	2-Hydroxyformononetin	$C_{16}H_{12}O_5$	284.05013	8.30	287; 253	Isoflavonoids		√	
16	2,7-Dihydroxy-4,5-dimethoxyisoflavon	$C_{17}H_{14}O_6$	314.07878	15.74	315; 300; 137	Isoflavonoids		√	
17	4-Hydroxybenzaldehyde	$C_7H_6O_2$	122.04827	1.25	123	Hydroxybenzaldehyde			√
18	*p*-Anisaldehyde	$C_8H_8O_2$	136.05179	9.59	135; 93	Hydroxybenzaldehyde		√	√
19	Protocatechuic acid	$C_7H_6O_4$	154.02601	2.53	109	Hydroxybenzoic acids		√	
20	*p*-Coumaric acid	$C_9H_8O_3$	164.04701	7.48	119; 96	Hydroxybenzoic acids	√	√	√
21	Quinic acid	$C_7H_{12}O_6$	192.06303	1.08	146; 102	Hydroxybenzoic acids	√	√	√
22	3-Caffeoylquinic acid	$C_{16}H_{18}O_9$	354.09543	5.42	191	Hydroxybenzoic acids		√	
23	4-Caffeoylquinic acid	$C_{16}H_{18}O_9$	354.09543	6.04	191; 173; 135	Hydroxybenzoic acids		√	
24	3,5-Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	516.12664	10.07	353; 191; 179; 173; 161; 135	Hydroxybenzoic acids		√	
25	3,4-Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	516.12674	9.17	353; 191; 179; 173; 161; 135	Hydroxybenzoic acids		√	

Table 1. Putative identification of phenolic from the ethanol extract of *A. muricata* (AM), *G. procumbens* (GP), and *T. flagelliforme* (TF) leaves extract by UHPLC-Q-Orbitrap HRMS (*Continued*)

No	Compound	Formula	MW experimental	Rt (min)	MS2	Group	Sample		
							AM	GP	TF
26	4,5-Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	516.12677	8.92	353; 191; 179; 173; 135	Hydroxybenzoic acids		√	
27	Cinnamic acid	$C_9H_8O_2$	148.05234	1.66	128; 110	Hydroxybenzoic acids			√
28	Ferulic acid	$C_{10}H_{10}O_4$	194.05810	8.27	193	Hydroxybenzoic acids			√
29	*p*-Coumaric α-glucoside acid	$C_{15}H_{18}O_8$	326.10081	5.71	163; 145	Hydroxybenzoic acids			√
30	3-*p*-Coumaroylquinic acid	$C_{16}H_{18}O_8$	338.11543	16.11	255; 147	Hydroxybenzoic acids		√	
31	Dihydroxyferulic acid	$C_{10}H_{12}O_4$	196.07377	6.05	197; 151; 103	Hydroxybenzoic acids	√		
32	Rosmarinic acid	$C_{18}H_{16}O_8$	360.08525	9.48	197; 179; 161	Hydroxybenzoic acids		√	
33	Coumarin	$C_9H_6O_2$	146.03694	6.16	147; 103; 91	Coumarins		√	
34	Esculetin	$C_9H_6O_4$	178.02663	2.98	171; 153;135; 133	Hydroxycoumarins		√	
35	Mellein	$C_{10}H_{10}O_3$	178.06280	17.04	179; 161; 133	Dihydroxyisocoumarins	√	√	
36	Caffeic acid	$C_9H_8O_4$	180.89940	6.64	135	Hydroxy propanoic acids	√	√	√
37	Caffeic O-glucoside acid	$C_{15}H_{18}O_9$	342.09569	4.11	179	Hydroxy propanoic acids			√
38	Vanillin	$C_8H_8O_3$	152.04747	7.30	151; 136	Aldehyde phenolics	√	√	√

the phenolics in *G. procumbens* leaves are outnumbering the other two species. This phenomenon can be interpreted as correlating the metabolite content and the *G. procumbens* leaves' radical scavenging activity.

Flavones. Compounds **3-7**, identified as apigenin-7-O-rutinoside, orientin (luteolin 8-C glucoside), apigenin C-hexoside-C-pentoside, cirsilineol, and vitexin, belong to the flavones group. Apigenin-7-O-rutinoside (**1**) was identified from *m/z* 577 $[M–H]^-$ and MS2 *m/z* 269 $[M–H-308]^-$. The loss of 308 Da indicates rutinoside, so that this peak is identified as apigenin 7-O-rutinoside. The compound orientin or luteolin 8-C glucoside (**2**) yields *m/z* 327 $[M–H-120]^-$ and 285 $[M–H-162]^-$. Apigenin C-hexoside-C pentoside (**3**) is identified by the presence of the MS2 *m/z* fragment 545 $[M–H-18]^-$, 443 [M–H-120], 431 $[M–H-132]^-$, 341 $[M–H-132-90]^-$, and 311 $[M–H-132-120]^-$. The MS2 specimen pattern confirms the substitution of mono-C-hexoside-C-pentoside at positions 6 and 8 [18]. This compound was only detected in *T. flagelliforme* leaf extract. Vitexin (**5**) shows *m/z* 431 (deprotonated molecule), *m/z* 341 $[M–H-90]^-$ and *m/z* 311 $[M–H-120]^-$ as characteristic ions in the MS/MS negative mode [19].

Flavonols. Primarily, flavonols are identified in the three plants. The nine compounds detected were kaempferol 3-O-rutinoside (**6**), kaempferol (**7**), quercetin (**8**), quercetin 7-O-rutinoside (**9**), kaempferol methyl ether (**10**), myricetin (**11**), quercetin 3-O-glucoside (**12**), kaempferol 3-O-hexoside (**13**), and kaempferol 3-O-(6''-acetyl-galactoside) 7-O-rhamnoside (**14**). Kaempferol 3-O-rutinoside (**6**) is identified in all extract samples at a retention time of 16 min. The resulting fragment patterns *m/z* 593 $[M–H]^-$, and 285 $[M–H-308]^-$. Fragment with *m/z* 285 indicates kaempferol (**7**) and the loss of mass 308, indicating the loss of rutinoside. Kaempferol methyl ether (**10**) is detected with MS2 *m/z* fragment patterns 299 $[M–H]^-$ and 284 $[M–H-15]^-$.

Quercetin (**8**) is detected at 11.10 min with ion precursor *m/z* 301 $[M–H]^-$ with the MS2 fragment patterns are *m/z* 273 $[M–H-28]^-$, 179 $[M–H-122]^-$, and 151 $[M–H-122-28]^-$. Similar to compound **1**, compound **6** with the ion precursor 609 $[M–H]^-$ loses a mass of 308 Da. The resulting fragments are *m/z* 301 $[M–H-308]^-$, 300 $[M–2H-308]^-$, and 303, which shows quercetin; therefore, it is elucidated as quercetin 7-O-rutinoside. Similar results are for a compound at 8.29 min with *m/z* 463 and the appearance of *m/z* 301 at MS2. The mass loss of 162 Da indicates a loss of glucoside groups; thus, it is determined as quercetin 3-O-glucoside (**12**). Similarly, compound **13** is identified as kaempferol 3-O-hexoside with *m/z* 447 $[M–H-162]^-$.

Isoflavonoids. Compounds **15** and **16** are detected by precursor ion *m/z* 285 and 315, presumably 2'-hydroxyformononetin and 2,7-dihydroxy-4,5-dimethoxy isoflavones. The latter compound, $C_{17}H_{14}O_6$, except for ion [*m/z* 137], indicates that the compound is an isoflavone and characterized as dihydroxy-dimethoxyisoflavone [20].

Hydroxycinnamic acids. Compounds **23-35** are identified as a group of hydroxycinnamic acids. *p*-Coumaric acid (**20**), quinic acid (**21**), cinnamic acid (**27**), and ferulic acid (**28**) are detected with *m/z* 163 $[M–H]^-$, 191 $[M–H]^-$, 147 $[M–H]^-$, and 193 $[M–H]^-$, respectively. Compounds **22** and **23** have an ion precursor of *m/z* 353 $[M–H]^-$ and are identified as 3-caffeoylquinic acid with *m/z* 191 as the base peak [21]. Peak **23** in the presence of sufficiently high *m/z* 173 and 135 indicates 4-caffeoylquinic acid.

The compounds 3,5-dicaffeoylquinic acid (**24**), 3,4-dicaffeoylquinic acid (**27**), 4,5-dicaffeoylquinic acid (**26**) are detected only in the extract of *G. procumbens* leaves. The three compounds have the same ion precursor (*m/z* 515) but can be distinguished based on the intensity of the MS2 fragment. The 3,5-dicaffeoylquinic acid compound has a base peak MS2 *m/z* 191, whereas 3,4-dicaffeoylquinic acid is characterized by an *m/z* intensity of 179. The latter is shifted in higher *m/z* 173, distinguished from the three compounds by the absence of *m/z* 161 [17]. Compounds **29-31** show *p*-coumaroylquinic acid, 3-*p*-coumaroylquinic acid, and dihydroxy ferulic acid, with 326, 338, and 196 molecular weights, respectively. Rosmarinic acid (**32**) was identified with *m/z* 359 $[M–H]^-$ produces MS2 *m/z* 197 $[M–H-62]^-$, which is a radical ion of 3,4-dihydroxy phenylactic acid ($C_9H_{10}O_5$), and *m/z* 179 is a radical ion of caffeic acid ($C_9H_8O_4$) [22].

Other groups. Coumarin (**33**) is identified with precursor *m/z* 147 $[M+H]^+$ and give fragments 103 $[M+H-44]^+$ and 91 $[M+H-56]^+$ [23]. The esculetin (**34**) gives the 177 $[M–H]^-$ fragment pattern. In the MS/MS analysis, we found sequential CO losses, corresponding to $[M–H–CO]^-$ *m/z* 149 and $[M–H–2CO]^-$, *m/z* 119 fragment ions and $[M–OH–CO]^-$ *m/z* 133 fragment ions [24]. Esculetin belongs to the hydroxycoumarin group, whereas mellein (**35**) belongs to the dihydroxyisocoumarins.

Caffeic acid (**36**) and caffeic O-glucoside (**37**) with *m/z* are identified in the hydroxyphenyl propanoic acids group. Caffeic acid is identified as the presence of deprotonated molecular ions $[M–H]^-$ at *m/z* 179 $[M–H]^-$ and MS2 ions at *m/z* 135 $[M–H–CO_2]^-$ [25]. The caffeic acid O-glucoside shows an *m/z* of 179 and a loss of 162 Da. Compound **38** is vanillin with fragments of *m/z* 151 $[M–H]^-$ and 136 $[M–H–CH_3]^-$ [26].

■ CONCLUSION

Ethanol extract of *G. procumbens* leaves showed higher free radical scavenging activity than *A. muricata* and *T. flagelliforme* leaves. Phenolics profiling using UHPLC-Q-Orbitrap HRMS from the three medicinal plants showed about 38 metabolites were putatively identified belongs to flavones, flavanols, hydroxycinnamic acid, and several other groups. *G. procumbens* leaves extract has proven potential as an antioxidant supported by a higher content of phenolic compounds for about 31 metabolites. Further studies are needed to look at the correlation between the metabolites and the antioxidant activity.

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