

Article

Antioxidant Activity and Metabolite Profiling of *Xylocarpus granatum* Extracts Using Gas Chromatography–Mass Spectrometry

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Abstract: The potential application of *Xylocarpus granatum*, a mangrove species, as traditional medicine has been widely linked to its high secondary metabolite and antioxidant contents. However, few studies have been reported to identify and classify active metabolites responsible for such excellent biological activities. Therefore, the aim of this work was to determine the antioxidant activity, identify the metabolite profiles, and predict the metabolites acting as antioxidants in *X. granatum* extract using a gas chromatography–mass spectrometry (GC-MS)-based metabolomics approach. The seeds, stems, fruit peel, pulp, leaves, and twigs of *X. granatum* were macerated with ethanol. Each extract was analyzed with GC-MS, and the data were processed using mass spectrometry data-independent analysis (MS-DIAL) software to identify the metabolites. The IC₅₀ value of plant parts of *X. granatum* ranged from 7.73 to 295 ppm. A total of 153 metabolites were identified and confirmed in the *X. granatum* extracts. Among the identified metabolites, epicatechin and epigallocatechin were the two most abundant in the stem extracts and are expected to have the greatest potential as antioxidants. Principal component analysis (PCA) succeeded in grouping all parts of the plant into three groups based on the composition of the metabolites: group 1 (stems, fruit peel, and twigs), group 2 (seeds and pulp), and group 3 (leaves).

Keywords: antioxidant; GC-MS; metabolite; PCA; *Xylocarpus granatum*



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1. Introduction

Mangrove is a type of plant that grows in coastal areas and is spread across Indonesia. Extracts and raw materials from mangrove have been widely utilized by coastal people for traditional medicines [1]. One such mangrove species widely used as material for traditional medicine is *X. granatum*. This species is a sea mangrove from the Meliaceae family with a majority of plants spread across Southeast Asia and along the Indian Ocean. In Indonesia, this plant can be found in the Kalimantan and Sulawesi regions [2]. Coastal people still use parts of *X. granatum* plants as traditional medicine, owing to their wide range of biological activities.

Parts of *X. granatum* such as leaves, stems, twigs, and fruit have been reported to contain several secondary metabolites such as alkaloids, flavonoids, monoterpenes, triterpenoids, tetraterpenoids, limonoids, proanthocyanidins, and phenolic acids [3,4]. These compounds have the potential to exert anticancer, antihyperglycemic, antidyslipidemic,

antidepressant, and neuroprotective activities [5–7]. Additionally, the extracts of the seeds, stems, fruit peel, and leaves of *X. granatum* can be used as antioxidants and antidiabetics, owing to their polyphenol contents [2,8,9]. The antioxidant activities of *X. granatum* extracts make this species a potential source of one of raw materials for cosmetic skin lightening, which work by inhibiting tyrosinase activity [10]. The biological activities of *X. granatum*, e.g., antioxidant activity, are greatly influenced by its contents of active compounds, which is a crucial factor affecting the quality of *X. granatum* as an antioxidant [2].

One method to identify the contents of active compounds in *X. granatum* extracts is by analyzing the metabolite profiles. Metabolite profiling is one method associated with metabolomic approaches that can be used to comprehensively identify primary or secondary metabolites in plants, both qualitatively and quantitatively, and is generally associated with specific metabolite pathways [11,12]. Metabolite profiling facilitates the efficient activity determination and use of active compounds utilization and can be used as a plant quality control process [13].

Comprehensive metabolite profile identification from a complex sample requires a high-resolution analysis method, such as liquid chromatography—mass spectrometry (LC-MS), gas chromatography—mass spectrometry (GC-MS), liquid chromatography—mass spectrometry—mass spectrometry (LC-MS/MS), or capillary electrophoresis—mass spectrometry (CE-MS) [14]. GC-MS analysis is often used for metabolite profiling, owing to its high sensitivity and high resolution, in addition to providing good reproducibility. Another advantage of the GC-MS technique is that it is easy to use and relatively inexpensive in terms of operational cost [15].

In this study, the metabolites in several parts of *X. granatum* plants, i.e., leaves, stems, twigs, fruit peel, pulp, and seeds, were identified using GC-MS. The resulting data were processed with the assistance of MS-DIAL version 4.20 software to identify metabolites; additionally, the compounds acting as antioxidants were predicted based on a comparison of the obtained profile to that of known antioxidant compounds reported in the literature. The result of GC-MS data processing include information on m/z values, retention time, retention index, area, and the peak intensity of the identified metabolites. The result of data normalization, which performed with MS-DIAL, was evaluated by PCA (principal component analysis) with SIMCA version 13 software (Umetrics, Umea, Sweden). PCA was used to discriminate every part of *X. granatum* based on similarities in metabolite content.

2. Materials and Methods

2.1. Samples and Instruments

The plant sample used in this study was *X. granatum* (leaves, stems, twigs, fruit peel, pulp, and seeds) from Togean Islands, Central Sulawesi. All parts of the plant were dried in an oven at 40 °C and ground before extraction.

The following instruments and software were used: GC-MS QP2010 Ultra (Shimadzu, Kyoto, Japan), MS-DIAL version 4.24 software, SIMCA version 13 software (Umetrics, Umea, Sweden), Abf Converter, AMDIS, MORPHEUS, and ChemSketch. The following chemicals were used: N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), pyridine, alkane mixture (C10–C31), ribitol (internal standard), methanol, chloroform, Milli-Q water, methoxyamine HCl, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2-(N-morpholino)ethanesulfonic acid (MES) buffer.

2.2. Extraction

The extraction method used was maceration with ethanol. Samples from each plant part (1 g) were soaked in 5 mL ethanol for 24 h, then filtered. The filtrate was concentrated using a rotary evaporator. This extract was used for antioxidant activity tests.

2.3. Antioxidant Activity

The antioxidant activity of every plant part of *X. granatum* was determined by DPPH method with referent to the method performed by Batubara et al. (2010) [10]. Each extract was diluted in ethanol to final concentrations of 1.67, 3.33, 6.67, 10.00, 13.33,

16.67, 33.33, 66.67, 100.00, 133.33, and 166.67 µg/mL. The sample aliquot, 100 µL 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 7.4), and 100 µL DPPH solution (11.8 mg DPPH in 100 mL ethanol) were added to each well of a 96-well plate. The mixtures were incubated for 30 min; then, the absorbances were read at 514 nm. Vitamin C was used as positive control and ethanol was used as the blank. Inhibition activity was calculated using the following formula:

$$\text{Inhibition (\%)} = [1 - (A_{\text{sample}} - A_{\text{control}}) / (A_{\text{blank}} - A_{\text{control}})] \times 100\%$$

where A_{sample} is the sample absorbance, A_{control} is the vitamin C absorbance as the control, and A_{blank} is the ethanol absorbance as the blank. The concentration of each sample and a positive control were tested in triplicate.

2.4. Sample Preparation for GC-MS Analysis

First, 1 mL methanol:chloroform:water (5:2:2) and 100 µg/mL ribitol as an internal standard were added to 10 mg of ground herbal material of every part of *X. granatum* (stems, leaves, pulp, fruit peel, twigs, and seeds). The mixture was homogenized by vortexing and incubated in a shaker for 30 min, then centrifuged at $12,298 \times g$ at 4 °C for 3 min. The supernatant (600 µL) was transferred into a new tube and mixed with 300 µL Milli-Q water, then recentrifuged under the same conditions described above. Subsequently, 400 µL (sample) and 200 µL (QC sample) of the supernatant were transferred into separate new tubes and dried under vacuum using a centrifugal concentrator at room temperature for 2 h, then dried with a freeze dryer overnight (12 h) before derivatization. The QC (quality control) sample was a mixture of all samples. After drying, derivatization was performed by adding 100 µL methoxyamine HCl in pyridine (20 mg/mL), followed by incubation at 30 °C for 90 min, the addition of 50 µL MSTFA, and reincubation at 37 °C for 30 min in order to induce silylation before being injected into the GC-MS. Sample derivatization was performed to improve the volatility of the compound for GC-MS analysis.

2.5. GC-MS Analysis

The GC-MS analysis performed in this study is a standard procedure used to analyze natural products and was conducted at Fukusaki Lab, Osaka University, Japan [16]. GC-MS QP2010 Ultra (Shimadzu, Japan) with ab InertCap 5 MS/NP column was used, with an injection temperature at 230 °C and an injection volume of 1 µL in split mode (25:1 *v/v*). The carrier gas (He) flow was 3.0 mL/minute, with a linear speed of 39 cm/s. The column temperature was held at 80 °C for 2 min, then increased at a rate of 15 °C/min to 330 °C and held for 6 min. The temperature of the transfer line and the ion source were 250 and 200 °C, respectively. The ions were generated by electron ionization (EI) at 1 kV, and the spectrum was recorded in the mass range of 85–500 *m/z*.

Then, 1 µL pyridine was injected into the GC-MS to check the background, and 1 µL of an alkane compound mixture was used to obtain the n-alkane retention time, which was further used for retention index calculation. Afterwards, pyridine was injected to clean the remaining n-alkane compounds, and 1 µL of the sample blank was injected to check for any contamination. Then, the samples or extracts from every part of *X. granatum* were analyzed. Every sixth sample injection was followed by a QC sample, a sample blank, and pyridine injections in order to check for any contamination and clean the column. For every plant part of *X. granatum*, injection was performed in quintuplicate, with 2 blank injections and 10 QC sample injections.

2.6. Data Analysis with MS-DIAL

The resulting data from GC-MS analysis were obtained as .QGD files. Peak conformation, peak filtering, and annotation were processed using MS-DIAL ver. 4.20 (Riken, Kanagawa, Japan). The output data from GC-MS were converted into CDF files, which were subsequently converted into Abf files with Abf Converter, then imported to MS-DIAL software. Data processing steps and compound identification in MS-DIAL included inputting

data as a new project, setting some parameters before analysis (data collection, peak detection, deconvolution, identification, alignment, and filtering), and metabolite identification.

Compound identification at the peak was performed by comparing the conformity of the retention time (RT), retention index (RI), and mass spectrum values from the results of analysis with those of known metabolites in the available library from AllPublic-KovatsRI-VS2. MS-DIAL software identified the metabolites according to the predetermined parameters of analysis. The resulting accuracy of compound identification was confirmed by examining the total score of each compound in the compound search.

After identification was completed, data normalization was performed before the data were used in PCA analysis. Data normalization was performed by determining the ID number of a compound used as an internal standard (ribitol); then, the ID number of the standard compound was input in the ID column of the identified compound.

2.7. Data Analysis

All determinations were conducted in triplicate, and the data are reported as average values $\pm$ standard deviation (SD).

PCA analysis was achieved using SIMCA version 13 software (Umetrics, Umea, Sweden). The data were exported from MS-DIAL as .txt files, which were then converted into Microsoft Excel files. The Microsoft Excel data were then transposed. PCA classification was expected to be able to group every plant part of *X. granatum* extract based on the metabolite content.

3. Results

3.1. *X. granatum* Antioxidant Activity

In this study, the antioxidant activity of every part of *X. granatum* was determined using the DPPH method. As a free radical scavenging method, DPPH, has been widely used to evaluate the antioxidant activity of plant extracts, owing to its rapidity, sensitivity, simplicity, and reproducibility. The results of antioxidant activity are reported as IC₅₀, indicating the number of antioxidants needed to lower 50% of the initial concentration of DPPH. Higher IC₅₀ values indicate lower antioxidant activity.

The IC₅₀ values of every part of *X. granatum* are presented in Table 1. The antioxidant activities of each part of *X. granatum* vary, with IC₅₀ values ranging from 7.73 to 295 ppm. In this study, vitamin C was used as a standard, with an IC₅₀ value of 4.18 ppm. Among all plant parts, the stem extract showed the highest antioxidant potential, with an IC₅₀ value of 7.73 ppm (Table 1).

Table 1. Antioxidant activity of *X. granatum* ethanol extract.

Plant Part	IC ₅₀ (ppm)
Seeds	104.64 ^e
Fruit peel	9.02 ^c
Stems	7.73 ^b
Pulp	44.48 ^d
Leaves	295.08 ^f
Twigs	9.86 ^c
Vitamin C	4.18 ^a

The same letter indicates non-significant differences at the 95% confidence interval.

3.2. *X. granatum* Metabolite Profiling Using GC-MS

The results of profiling were obtained after confirming the metabolites and performing data curation. One metabolite identified in MS-DIAL was confirmed by conforming the retention index value and the mass spectrum of the compound identified with a known compound found in the AllPublic-KovatsRI-VS2 library. Data curation involves reducing the data on the compounds with the same retention time values and the same compounds with different retention time values. The compound with the highest total score value (750

to 1000) was selected for profiling. The total score shows indicates the similarity of the identified compound relative to a compound in the database.

Metabolite identification from every part of *X. granatum* resulted in 153 identified compounds (Table 2). Every part of *X. granatum* is primarily composed of simple sugar (monosaccharides), as reflected by the relative area. The sugar compounds contained in *X. granatum* are sucrose (12.02%), glucose (7.59%), fructose (7.58%), and epicatechin (2.00%), with a chromatogram pattern as shown in Figure 1.

Table 2. Metabolites in the extract of *X. granatum*.

No	Rt (minutes)	RI	Ion (m/z)	Formula	Compound	Area (%)
1	4.33	1003	117	C ₃ H ₈ O ₂	propylene glycol	0.028
2	4.60	1028	174	C ₃ H ₉ N	n-propylamine	0.006
3	4.71	1038	117	C ₄ H ₁₀ O ₂	butane-2,3-diol	1.084
4	4.73	1039	152	C ₅ H ₅ NO	2-hydroxypyridine	0.035
5	4.94	1058	147	C ₃ H ₈ O ₂	propane-1,3-diol	0.006
6	4.95	1058	130	C ₃ H ₄ O ₃	Pyruvic acid	0.047
7	5.02	1064	147	C ₃ H ₆ O ₃	Lactic acid	0.125
8	5.07	1069	131	C ₄ H ₈ O ₃	2-Hydroxyisobutyric acid	0.341
9	5.18	1078	147	C ₂ H ₄ O ₃	Glycolic acid	0.356
10	5.39	1097	174	C ₄ H ₁₁ N	n-Butylamine	0.003
11	5.55	1111	131	C ₄ H ₈ O ₃	2-hydroxybutanoic acid	0.003
12	5.87	1141	152	C ₅ H ₅ NO	4-Hydroxypyridine	0.007
13	5.93	1146	147	C ₄ H ₈ O ₃	3-hydroxybutyric acid	0.317
14	5.94	1147	116	C ₃ H ₇ NO ₂	Sarcosine_2TMS	0.007
15	5.95	1148	142	C ₄ H ₇ NO	butyrolactam	0.036
16	6.68	1216	131	C ₄ H ₈ O ₃	3-Hydroxyisovaleric acid	0.005
17	6.76	1224	144	C ₅ H ₁₁ NO ₂	Valine_2TMS	0.063
18	6.89	1237	189	CH ₄ N ₂ O	Urea	0.005
19	6.91	1238	116	C ₄ H ₈ O ₃	4-hydroxybutyric acid	0.011
20	6.95	1242	174	C ₂ H ₇ NO	Ethanolamine	0.008
21	7.01	1249	175	C ₆ H ₁₀ O ₄	ethylsuccinate	0.007
22	7.04	1252	179	C ₇ H ₆ O ₂	Benzoic acid	0.017
23	7.07	1255	147	C ₃ H ₆ O ₃	Dihydroxyacetone	0.013
24	7.17	1264	116	C ₃ H ₇ NO ₃	Serine_2TMS	0.003
25	7.29	1276	174	C ₂ H ₇ NO	2-Aminoethanol	0.070
26	7.33	1280	158	C ₆ H ₁₃ NO ₂	Leucine_2TMS	0.033
27	7.35	1282	147	C ₃ H ₈ O ₃	Glycerol	0.880
28	7.50	1297	180	C ₆ H ₅ NO ₂	Nicotinic acid	0.008
29	7.53	1300	158	C ₆ H ₁₃ NO ₂	Isoleucine_2TMS	0.033
30	7.69	1317	147	C ₄ H ₆ O ₄	Succinic acid	0.068
31	7.80	1328	254	C ₆ H ₆ O ₂	Catechol	0.007
32	7.91	1340	147	C ₃ H ₆ O ₄	Glyceric acid	0.075
33	7.97	1347	241	C ₄ H ₄ N ₂ O ₂	Uracil	0.010
34	7.99	1349	254	C ₄ H ₄ O ₄	Fumaric acid	0.027
35	8.14	1365	240	C ₅ H ₅ NO ₂	pyrrole-2-carboxylic acid	0.013
36	8.21	1372	188	C ₃ H ₇ NO ₂	Alanine_3TMS	0.020
37	8.25	1376	156	C ₆ H ₁₁ NO ₂	DL-Pipecolic acid	0.158
38	8.32	1384	147	C ₄ H ₆ O ₄	erythronic acid lactone	0.032
39	8.46	1398	218	C ₄ H ₉ NO ₃	Threonine_3TMS	0.007
40	8.55	1408	239	C ₆ H ₆ O ₂	hydroquinone	0.021
41	8.55	1409	147	C ₅ H ₈ O ₄	Glutaric acid	0.069
42	8.61	1415	255	C ₅ H ₆ N ₂ O ₂	Thymine	0.001
43	8.66	1422	103	C ₄ H ₉ NO ₃	homoserine	0.016
44	8.83	1440	233	C ₄ H ₈ O ₄	2-deoxytetronic acid	0.004
45	8.86	1443	174	C ₄ H ₉ NO ₂	3-aminoisobutyric acid	0.080

Table 2. Cont.

No	Rt (minutes)	RI	Ion (m/z)	Formula	Compound	Area (%)
46	8.96	1456	117	C ₁₀ H ₂₀ O ₂	Decanoic acid	0.004
47	9.17	1480	158	C ₅ H ₉ NO ₃	trans-4-hydroxy-L-proline	0.012
48	9.23	1487	247	C ₅ H ₈ O ₅	Citramalic acid	0.003
49	9.33	1498	147	C ₄ H ₆ O ₅	Malic acid	0.751
50	9.34	1499	223	C ₇ H ₆ O ₂	p-Hydroxybenzaldehyde	0.011
51	9.43	1509	244	C ₄ H ₈ N ₂ O ₃	asparagine dehydrated	0.004
52	9.45	1512	117	C ₄ H ₈ O ₅	isothreonine acid	0.022
53	9.48	1516	147	C ₄ H ₁₀ O ₄	Threitol	0.031
54	9.53	1521	174	C ₄ H ₁₂ N ₂	putrescine 3tms	0.018
55	9.54	1523	267	C ₉ H ₈ O ₄	Acetylsalicylic acid	0.014
56	9.55	1524	217	C ₄ H ₁₀ O ₄	Meso erythritol	0.109
57	9.59	1528	232	C ₄ H ₇ NO ₄	Aspartic acid_3TMS	0.010
58	9.63	1534	156	C ₅ H ₇ NO ₃	Pyroglutamic acid_2TMS	0.168
59	9.68	1539	230	C ₅ H ₉ NO ₃	Hydroxyproline	0.187
60	9.70	1542	174	C ₄ H ₉ NO ₂	4-Aminobutyric acid	0.455
61	9.81	1556	263	C ₆ H ₆ O ₄	5-hydroxymethyl-2-furoic acid	0.002
62	9.84	1560	239	C ₆ H ₆ O ₃	Pyrogallol	0.004
63	9.96	1574	267	C ₇ H ₆ O ₃	3-Hydroxybenzoate	0.007
64	9.99	1577	147	C ₄ H ₈ O ₅	Threonine acid	0.097
65	10.01	1580	179	C ₈ H ₁₀ O ₂	4-Hydroxyphenethyl alcohol	0.012
66	10.04	1584	129	C ₅ H ₈ O ₅	2-hydroxyglutaric acid	0.015
67	10.12	1594	147	C ₇ H ₁₂ O ₅	2-Isopropylmalic acid	0.124
68	10.28	1614	147	C ₆ H ₁₀ O ₅	3-Hydroxy-3-methylglutarate	0.032
69	10.31	1617	217	C ₅ H ₈ O ₅	xylonolactone	0.009
70	10.35	1622	117	C ₄ H ₆ O ₆	L-(+)-Tartaric acid	0.034
71	10.41	1630	246	C ₅ H ₉ NO ₄	Glutamic acid_3TMS	0.007
72	10.46	1636	267	C ₇ H ₆ O ₃	4-Hydroxybenzoic acid	0.094
73	10.50	1642	200	C ₆ H ₁₁ NO ₂	Pipecolic acid	0.037
74	10.55	1648	179	C ₈ H ₈ O ₃	4-Hydroxyphenylacetic acid	0.004
75	10.74	1672	103	C ₅ H ₁₀ O ₅	Xylose	0.013
76	10.82	1683	103	C ₅ H ₁₀ O ₅	Lyxose	0.273
77	10.84	1686	271	C ₆ H ₆ O ₄	Kojic acid	0.285
78	10.98	1703	103	C ₅ H ₁₀ O ₅	Ribose	0.095
79	11.14	1726	204	C ₆ H ₁₀ O ₅	1.6-Anhydroglucose	0.087
80	11.20	1734	217	C ₅ H ₁₂ O ₅	Xylitol	0.016
81	11.22	1736	219	C ₆ H ₁₄ O ₅	diglycerol	0.005
82	11.29	1746	117	C ₆ H ₁₂ O ₅	Rhamnose	0.252
83	11.30	1746	217	C ₅ H ₁₂ O ₅	Arabitol	1.275
84	11.34	1752	117	C ₆ H ₁₄ O ₅	6-deoxyglucitol	0.971
85	11.48	1771	147	C ₆ H ₁₂ O ₅	2-Deoxy-D-glucose	0.065
86	11.51	1775	297	C ₈ H ₈ O ₄	vanillic acid	0.020
87	11.52	1777	231	C ₆ H ₁₀ O ₅	3.6-anhydro-D-galactose	0.021
88	11.60	1787	156	C ₅ H ₁₀ N ₂ O ₃	Glutamine_3TMS	0.007
89	11.62	1790	129	C ₆ H ₁₄ O ₅	3-deoxyhexitol	0.077
90	11.65	1794	147	C ₅ H ₁₀ O ₆	Xylonic acid	0.056
91	11.84	1821	204	C ₇ H ₁₀ O ₅	Shikimic acid	0.434
92	11.93	1834	193	C ₇ H ₆ O ₄	3.4-Dihydroxybenzoate	0.267
93	11.98	1841	204	C ₆ H ₁₂ O ₅	1.5-Anhydro-D-glucitol	0.067
94	12.26	1881	159	C ₆ H ₁₃ NO ₅	D-(+)-Galactosamine	0.268
95	12.29	1886	103	C ₆ H ₁₂ O ₆	Psicose	0.118
96	12.30	1887	103	C ₆ H ₁₂ O ₆	Tagatose	0.084

Table 2. Cont.

No	Rt (minutes)	RI	Ion (m/z)	Formula	Compound	Area (%)
97	12.33	1891	345	C ₇ H ₁₂ O ₆	Quinic acid	1.910
98	12.46	1910	104	C ₆ H ₁₀ O ₆	L-Gulcono-1.4-lactone	3.503
99	12.50	1916	103	C ₆ H ₁₂ O ₆	Fructose	6.983
100	12.53	1921	319	C ₆ H ₁₂ O ₆	Mannose	0.179
101	12.56	1925	204	C ₇ H ₁₄ O ₆	1-methylgalactose	0.121
102	12.57	1927	319	C ₆ H ₁₂ O ₆	Galactose	0.328
103	12.58	1928	217	C ₆ H ₁₀ O ₆	Glucono-1.5-lactone	0.998
104	12.62	1935	319	C ₆ H ₁₂ O ₆	Glucose	7.042
105	12.71	1947	203	C ₆ H ₁₃ NO ₅	Glucosamine	0.023
106	12.74	1953	293	C ₉ H ₈ O ₃	p-Coumaric acid	0.035
107	12.78	1959	218	C ₉ H ₁₁ NO ₃	Tyrosine	0.086
108	12.80	1962	275	C ₆ H ₁₂ O ₇	galactonic acid	0.014
109	12.82	1963	204	C ₆ H ₁₂ O ₆	hexose	1.079
110	12.84	1968	319	C ₆ H ₁₄ O ₆	Mannitol	0.181
111	12.89	1975	281	C ₇ H ₆ O ₅	Gallic acid	0.034
112	12.90	1977	217	C ₆ H ₁₄ O ₆	Galactitol	0.025
113	13.02	1994	318	C ₆ H ₁₀ O ₅	conduritol-beta-expoxide	2.131
114	13.10	2006	147	C ₆ H ₁₄ O ₆	hexitol	0.099
115	13.31	2040	353	C ₅ H ₄ N ₄ O ₂	Xanthine	0.006
116	13.33	2043	147	C ₆ H ₁₂ O ₇	Gluconic acid	0.154
117	13.35	2046	117	C ₁₆ H ₃₂ O ₂	palmitic acid	0.103
118	13.44	2059	204	C ₈ H ₁₅ NO ₆	N-Acetyl-D-glucosamine	0.363
119	13.48	2067	147	C ₆ H ₁₂ O ₆	myo-inositol	0.019
120	13.53	2073	129	C ₈ H ₁₅ NO ₆	N-acetyl-D-mannosamine	0.007
121	13.73	2105	338	C ₁₀ H ₁₀ O ₄	Ferulic acid	0.016
122	13.84	2124	204	C ₈ H ₁₅ NO ₆	n-acetyl-d-hexosamine	0.114
123	13.89	2132	217	C ₆ H ₁₂ O ₆	Inositol	1.004
124	14.00	2149	219	C ₉ H ₈ O ₄	Caffeic acid	0.011
125	14.07	2160	319	C ₈ H ₁₅ NO ₆	N-Acetyl galactosamine	0.035
126	14.30	2198	204	C ₉ H ₁₆ O ₉	beta-mannosylglycerate	0.027
127	14.57	2245	117	C ₁₈ H ₃₆ O ₂	Stearic acid	0.196
128	14.61	2251	202	C ₁₁ H ₁₂ N ₂ O ₂	Tryptophan_3TMS	0.011
129	14.69	2266	204	C ₉ H ₁₈ O ₈	glycerol-3-galactoside	0.021
130	14.79	2282	361	C ₁₀ H ₁₇ NO ₉ S ₂	Sinigrin	0.004
131	15.12	2341	217	C ₁₁ H ₁₅ N ₅ O ₄	1-methyladenosine	0.013
132	15.18	2352	204	C ₆ H ₁₃ O ₉ P	galactose-6-phosphate	0.056
134	15.85	2474	217	C ₉ H ₁₂ N ₂ O ₆	Uridine_4TMS	0.010
135	16.19	2538	204	C ₁₂ H ₂₂ O ₁₁	Leucrose	0.130
136	16.89	2674	361	C ₁₂ H ₂₂ O ₁₁	beta-Lactose	0.015
137	17.03	2702	361	C ₁₂ H ₂₂ O ₁₁	Sucrose	5.839
138	17.05	2706	204	C ₁₂ H ₂₂ O ₁₁	D-(+)-Maltose	0.236
139	17.16	2729	217	C ₁₂ H ₂₂ O ₁₁	D-(+)-Turanose	0.361
140	17.36	2770	217	C ₁₂ H ₂₄ O ₁₁	lactitol	0.105
141	17.53	2806	217	C ₁₂ H ₂₂ O ₁₂	lactobionic acid	0.047
142	17.56	2814	361	C ₁₂ H ₂₂ O ₁₁	Trehalose	0.137
143	17.76	2855	355	C ₂₁ H ₂₂ O ₁₀	4.5-dihydroxy-7-glucosyloxyflavanone	0.012
144	17.92	2889	361	C ₁₂ H ₂₄ O ₁₁	Palatinitol	0.009
145	17.94	2893	368	C ₁₅ H ₁₄ O ₆	(-)-Epicatechin	0.422
146	18.08	2925	361	C ₁₂ H ₂₂ O ₁₁	Gentiobiose	0.033
147	18.14	2937	204	C ₁₂ H ₂₂ O ₁₁	Melibiose	0.051
148	18.31	2975	456	C ₁₅ H ₁₄ O ₇	(-)-Epigallo catechin	0.032
149	18.74	3072	204	C ₁₂ H ₂₂ O ₁₁	Galactinol	0.021
150	18.87	3100	219	C ₁₆ H ₁₈ O ₉	Chlorogenic acid	0.008
151	18.93	3109	487	C ₁₅ H ₁₀ O ₆	Kaempferol	0.005
152	20.61	3349	204	C ₁₈ H ₃₂ O ₁₆	Maltotriose	0.003
153	20.93	3396	361	C ₁₈ H ₃₂ O ₁₆	Kestose	0.010

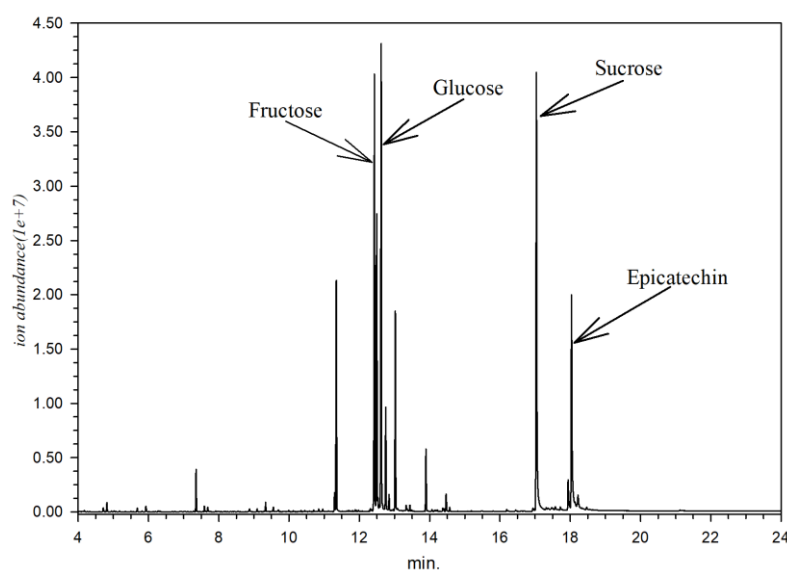


Figure 1. Chromatogram of *X. granatum* stem extract according to GC-MS analysis.

3.3. Prediction of Active Antioxidant Compounds in *X. granatum*

A total of 15 compounds with potential as antioxidants were identified in the various parts of *X. granatum*, as depicted in the form of a heat map in Figure 2. The heat map is intended to visualize the peak area percentage in a simpler way using the intensity of colors. In Figure 2, the intensity of red color indicates high compound content, whereas the intensity of green color shows indicates low compound content.

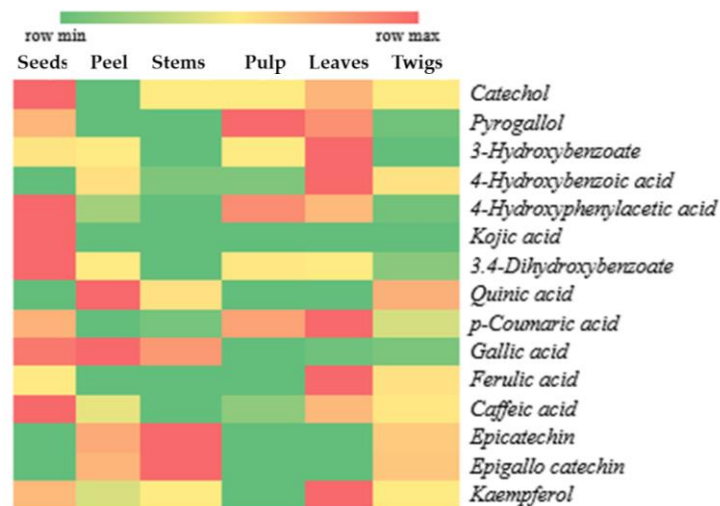


Figure 2. Distribution of antioxidant compounds in plant parts of *X. granatum*.

According to the results of antioxidant activity testing of *X. granatum* plant parts using the DPPH method, the ethanol extract of *X. granatum* stems has a very high potential compared to the other parts of the plant, as reflected by the lowest IC₅₀ value (Table 1). This result indicates that the antioxidant compounds dominantly found in the stems have higher antioxidant activity compared to those found in the other parts of the plant. Figure 2 shows that the dominant compounds in the stems are epicatechin and epigallocatechin. Besides the stems, these two compounds can be found in the fruit peel and twigs, making the antioxidant activity in the fruit peel and twigs higher than that in the seeds, pulp, and leaves (Table 1).

3.4. Discrimination of *X. granatum* Plant Parts with PCA Based on Metabolite Composition

Discrimination is a process used to differentiate one sample from another by identifying similarities between samples. Samples with many similarities are grouped together; in other words, samples with many differences are separated into different groups [17]. The PCA technique is a method of analysis of double variables with the intention of simplifying the observed variables by reducing the dimensions to facilitate the visualization of data grouping and evaluation of similarities among the groups [18]. The application of PCA to the chromatogram enables a reduction in large-sized GC-MS data into several primary components (PCs) so that a two-dimension score plot can show severability among samples. The score plots of the first two components (PC1 and PC2) are usually used in the analysis because these PCs contain the most data variation.

The result of PCA analysis is considered acceptable if a small number of primary components can describe a large number of total variations. The result of PCA analysis was visualized as a score plot depicting the grouping of each plant part of *X. granatum* based on the metabolite composition contained in each plant part. Every point in the score plot represents a single sample, and samples with similarities are categorized in the same group [19]. Figure 3 shows the obtained score plot, which explains 91% of total variation (PC1 = 51%, PC = 40%). Samples with the same labels are grouped in adjacent positions. However, some samples did not provide a satisfactory grouping representation, so those data were considered outliers, such as teed 1, seed 5, and twig 3. Therefore, outlier reduction was performed in order to visualize a clearer and better data grouping.

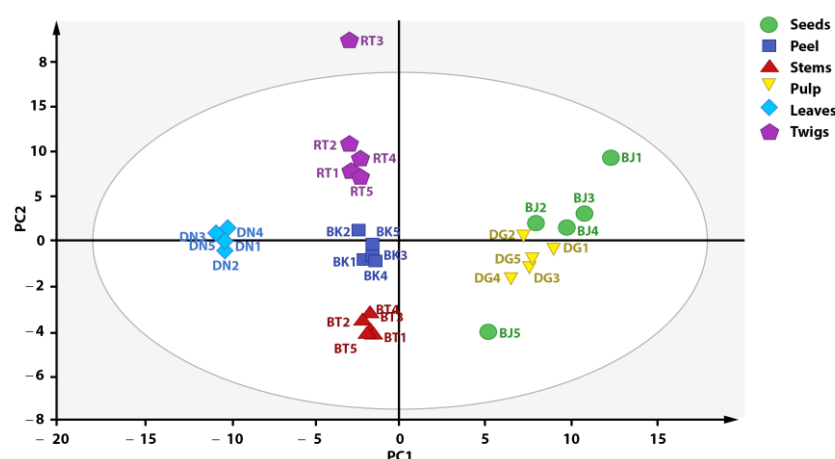


Figure 3. Score plot of PCA analysis before outlier reduction.

Outlier reduction resulted in the grouping presented in Figure 4, with an increase in the PC1 value of 1%. Figure 4 shows a plot score that can explain 92% of total variation (PC1 = 52%, PC = 40%). The result of score plot after outlier reduction shows that the metabolite profile of each *X. granatum* plant part can be differentiated into three groups based on metabolite composition, i.e., group 1 (stems, twigs, and fruit peel), group 2 (pulp and seeds), and group 3 (leaves). According to the PCA score plot (Figure 4), the metabolite characteristics of the fruit peel, stem, and twig parts of *X. granatum* are similar, as reflected by the antioxidant activities, with similar IC₅₀ values: 9.02, 7.73, and 9.83 ppm, respectively (see, Table 2). The samples of the pulp and the seeds parts belong to one group, whereas the leaves are separated from the groups containing the other plant parts, showing that there are metabolite composition differences in the leaves. This is also supported by the antioxidant properties of the leaf extract, which has the highest IC₅₀ value (295.08 ppm), showing that it has the lowest antioxidant activity. The association between the results of grouping and the antioxidant activity shows that the compounds with antioxidant activity play significant roles in the grouping of *X. granatum* plant parts.

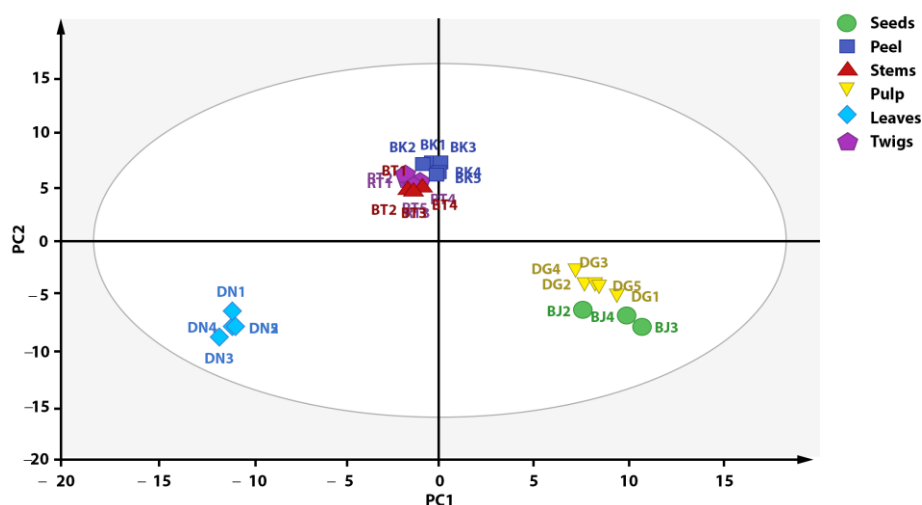


Figure 4. Distribution of antioxidant compounds in plant parts of *X. granatum*.

4. Discussion

Metabolite profiling is a technique of analysis used to determine the metabolite profile or the chromatogram pattern of chemical components of an extract with pharmacological activities or chemical components characterizing a plant with the objective of controlling the quality [12]. Metabolite profiling and antioxidant compound prediction of *X. granatum* extracts consisted of four stages, i.e., information gathering through GC-MS data analysis using MSDIAL and antioxidant compound prediction through a literature study, data reduction and compilation to convert the spectrum into data that can be processed statistically, PCA multivariate analysis, and review and interpretation of the results of chemometric processes.

An antioxidant is an electron-donor compound that can lower the free radical level and help to reduce or prevent the impact of oxidative stress because of free radicals [20,21]. Common natural antioxidant compounds include vitamin C, vitamin E, carotenoids, phenolic compounds, and polyphenols, which can be grouped as flavonoids, cinnamate acid derivatives, coumarins, tocopherol, and polyfunctional organic acids, respectively [22]. Our literature review of several studies on the properties of antioxidant compounds, as well as the characteristics and applications thereof, indicated that natural metabolite compounds that are usually found in plants commonly include compounds in the group of polyphenols with active groups of hydroxy (-OH) and double-bonded carbons (-C=C-) and function as scavengers and inhibitors of free radicals reactions. Polyphenol secondary metabolites, such as flavonoids, polyenes, and compounds containing many -OH groups, can react with free radicals as reducing agents, free radical scavengers, metal-chelating agents, and oxygen-singlet-forming suppressors [23–25].

According to our literature review of the properties and the general structures of antioxidant compounds, several compounds contained in *X. granatum* extracts have potential as antioxidants. These compounds mostly belong to the phenolics and polyphenols groups, including catechol [26], pyrogallol [24], 3-hydroxybenzoate, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, 3,4-dihydroxybenzoate [27], kojic acid [28], quinic acid [29], p-coumaric acid, gallic acid, ferulic acid, caffeic acid [30], epicatechin, epigallocatechin, and kaempferol [23].

Simple phenolic compounds such as catechol and pyrogallol are antioxidant compounds with the ability to lower the number of reactive oxygen species (ROS). Pyrogallol is a compound used widely to inhibit oxidation reactions in biodiesel [25]. On the other hand, a catechol-containing polyphenol, epicatechin, is a strong in vitro antioxidant compound, owing to its ability to rapidly lower the level of ROS, bind metallic ions, form inert complexes, and arrest the chain reaction of radical compounds [31].

Another phenolic compound identified as an antioxidant is phenolic acid. Phenolic acid is a strong antioxidant and exhibits antibacterial, antiviral, anticarcinogenic, and anti-inflammatory activities, as well as vasodilatory activity. Phenolic acid may further function as an anticancer agent and prevent heart diseases. Phenolic acid identified in *X. granatum* can be divided into two groups: benzoic acid derivatives and cinnamic acid derivatives. In this study, the identified compounds of benzoic acid derivatives were 3-hydroxybenzoate, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, and 3,4-dihydroxybenzoate, whereas the identified compounds of cinnamic acid derivatives were ferulic acid, gallic acid, p-coumaric acid, and caffeic acid. These compounds were reported to function as effective active antioxidants in radical scavenging of 2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), and superoxide anions, as well as metal chelation activity in iron ions [27,30,32]. Antioxidant activities of these phenolic acid compounds are influenced by the number of hydroxyl groups (OH-) in the phenyl ring. The length of conjugated double bonds, types of substituents, and the form of the molecules also contribute to the antioxidant activity [20].

In addition to the compounds in the phenolic group, other compounds were identified acting as antioxidants, such as quinic acid and kojic acid. Quinic acid is a carboxylic acid with many hydroxy groups (-OH), with potential as an antioxidant by inhibiting oral pathogens [29]. Kojic acid, on the other hand, is widely used in cosmetic products, especially as a skin-lightening agent [28,33].

5. Conclusions

The plant parts of *X. granatum* exhibit varying antioxidant activities. Metabolite profiling in *X. granatum* extracts using GC-MS succeeded in identifying 153 compounds. The compounds expected to have important roles in antioxidant activities are those compounds from the flavonol group, i.e., epicatechin and epigallocatechin, which are dominantly found in the stem of *X. granatum*. Multivariate analysis using PCA succeeded in grouping the plant parts of *X. granatum* into 3 groups based on metabolite composition: group 1 (stems, twigs, and fruit peel), group 2 (seeds and pulp), and group 3 (leaves). The grouping results of *X. granatum* plant parts using PCA can be associated with the antioxidant activities.

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