

Short Communication:**LC-HRMS-Based Metabolomics Approach Reveals Antioxidant Compounds from *Centella asiatica* Leaves Extracts**Riva Silvia¹, Wulan Tri Wahyuni^{1,2*}, Eti Rohaeti^{1,2}, Siti Aisyah³, Dewi Anggraini Septaningsih⁴, Alfi Hudatul Karomah⁴, and Mohamad Rafi^{1,2,4}¹Department of Chemistry, Faculty of Mathematics and Natural Sciences, IPB University, Jl. Tanjung Kampus IPB Dramaga, Bogor 16680, Indonesia²Tropical Biopharmaca Research Center, IPB University, Jl. Taman Kencana No. 3, Kampus IPB Taman Kencana, Bogor 16128, Indonesia³Chemistry Program Study, Department of Chemistry Education, Universitas Pendidikan Indonesia, Jl. Dr. Setiabudi No. 229, Bandung 40154, Indonesia⁴Advanced Research Laboratory, IPB University, Jl. Palem, Kampus IPB Dramaga, Bogor 16680, Indonesia*** Corresponding author:**

email: wulantriws@apps.ipb.ac.id

Received: November 17, 2023

Accepted: September 4, 2024

DOI: 10.22146/ijc.90782

Abstract: *Centella asiatica* is a medicinal plant widely used as a traditional medicine due to several biological activities, such as antioxidants in Indonesia. This study aims to identify the active antioxidant compounds of *C. asiatica* leaves extract using a liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS)-based metabolomics approach. Extracts were prepared using different concentrations of ethanol p.a., i.e. 70% ethanol, 50% ethanol, 30% ethanol, and water. Antioxidant activity was tested using the 2,2-diphenyl-1-picrylhydrazyl method. The results showed the highest antioxidant activity was *C. asiatica* extracted by 70% ethanol with IC_{50} of 72.48 ± 2.42 $\mu\text{g/mL}$. The positive control was ascorbic acid, having an IC_{50} value of 3.38 ± 0.04 $\mu\text{g/mL}$. Ascorbic acid and 70% ethanol extract have strong antioxidant activity. Metabolite profiling using LC-MS/MS could identify 35 metabolites consisting of flavonoids, fatty acids, phenolics, terpenes, and several other groups of compounds. Orthogonal partial least squares-discriminant analysis classified the metabolites into active (ethanol 70% and ethanol 50%) and inactive (ethanol p.a., ethanol 30%, and water) antioxidants. Five metabolites have potential as antioxidants, namely 4,5-dicaffeoylquinic acid (11), kaempferol (13), and three unknown compounds.

Keywords: antioxidant; *C. asiatica*; LC-MS/MS; metabolomics

■ INTRODUCTION

Pegagan, with the Latin name *Centella asiatica*, is a medicinal plant in the Apiaceae family [1]. This plant is commonly found in watery areas and has tasteless and smells characteristics. *C. asiatica* is widely used as a vegetable or traditional medicine. *C. asiatica* has been traditionally used to slow down ageing symptoms, making it as a popular raw material for cosmetics. In addition, it has been used as a remedy for various health issues such as asthma, wounds, healing cysts, ulcers, high blood

pressure, varicose veins, and skin tuberculosis [2]. As per the World Health Organization, this plant can also be a brain tonic [3]. The biological activity *C. asiatica* that has been reported includes anti-inflammatory, antidiabetic, antibacterial, antifungal, and antioxidant [2,4-6]. Its bioactive metabolites, such as centelloids and chologenic acid [7], contribute to biological activity.

The most prominent metabolites in *C. asiatica* are 4 triterpene compounds, including asiatic acid, asiaticoside, madecassic acid, and madecassoside [8].

The composition of metabolites in medicinal plant extracts is affected by various factors, one of which is the polarity of the extraction solvent [9]. Apart from affecting the number of metabolites extracted due to different structures and polarities, it also affects the extraction quality and speed. Variations in the composition of extracted metabolites can influence biological activities, including antioxidant activity. Antioxidants are crucial for protecting cells from damage caused by free radicals, such as superoxide dismutase, tocopherol, glutathione peroxidase, and catalase [10].

C. asiatica contains various metabolites, but only some are potentially active as antioxidants. Therefore, it is essential to determine the active metabolites that act as antioxidants. Determination of active compounds that play a role in antioxidant biological activity in plant samples has previously been carried out using a bioassay guided fractionation approach [11]. However, this approach requires a long time to find out the active compounds in plants, so this research uses a metabolomics approach, which is considered more efficient. Metabolomics identifies and quantifies the overall metabolites contained in a sample at a particular time [12]. Metabolomics analysis is usually differentiated into targeted and non-targeted metabolomics. Targeted metabolomics aims to identify or quantify specific metabolites within a sample. Meanwhile, non-targeted metabolomics conducts a more comprehensive quantitative as well as qualitative evaluation of plant metabolite content. Measuring all metabolites in a biological system using non-targeted metabolomics allows for new hypotheses [13]. Non-targeted metabolomics has several stages, including sample collection and preparation, data acquisition, data processing and analysis, and identifying metabolites that enable biological interpretation.

liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) is one of the main instruments used to display metabolite profiles because it has high selectivity and sensitivity to simple sample preparation. LC-MS/MS are able to detect hundreds or even thousands of metabolites from samples in a short time [14]. LC-MS/MS-based metabolomic approaches provide a comprehensive understanding of the phytochemical

spectrum of plant constituents. This approach can help characterize and identify metabolites and evaluate herbal medicines [15]. Metabolite profiling is utilized not only to identify metabolites but also to evaluate the distribution of compounds. The study is suitable for showing chemical variability in different species, varying due to geographic region, planting age, and extracting solution polarity.

The combination of LC-MS/MS and chemometric methods have been widely used to determine the correlation between metabolite compounds from samples and their biological activity, such as in *Momordica charantia*, canary seeds, bee homogenates, *Artemisia annua*, and *Medicago sativa* [13,15-18]. Chemometrics methods for multivariate data analysis are necessary to lessen the complexity of the data produced by LC-MS/MS analysis. The multivariate data analysis used is principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA). PCA aims to classify each identified extract, and OPLS-DA predicts its antioxidant active compounds. However, no research has reported identifying active antioxidant compounds from *C. asiatica* extracts using an LC-MS/MS-based metabolomics approach. This research aims to identify active compounds from *C. asiatica* extract as antioxidants using an LC-MS/MS-based metabolomics approach.

■ EXPERIMENTAL SECTION

Materials

C. asiatica was collected and identified by the Biopharmaca Cultivation Conservation Center for Tropical Biopharmaca Studies LPPM IPB with collection number BMK00110082016. Pro analysis ethanol, water (LC-MS/MS grade), acetonitrile, and methanol (LC-MS/MS grade) were obtained from Merck, Darmstadt, Germany. Ascorbic acid (Sigma-Aldrich, Steinheim, Germany) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were utilized in this research.

Instrumentation

The instrument used in this research was ultrasonication (OVAN, Barcelona, Spain) and a set of

ultra-high-performance liquid chromatography (UHPLC) tandem Q Exactive Plus Orbitrab-High Resolution Mass Spectrometer (Thermo Scientific, Germany).

Procedure

Sample preparation and extraction

C. asiatica leaves were separated from the stems, washed, and dried for 48 h at 40 °C. The dried leaves were ground and sieved until an 80-mesh powder was obtained. The samples were macerated with ethanol p.a., ethanol 70%, ethanol 50%, ethanol 30%, and water, and 5 replications were performed. A total of 150 mL of solvent was used to extract 15 g of powder. The maceration was performed for 3 × 24 h at room temperature and filtered every 24 h. Then, the filtrate was concentrated with a rotary evaporator until a thick extract was obtained.

Determination of antioxidant activity DPPH method

The *C. asiatica* extracts were added to a 96-well plate, and 125 µM DPPH was added. It was incubated in the dark at room temperature for 30 min. A wavelength of 517 nm was used to measure the sample's absorbance. Ascorbic acid was used as a positive control for determining antioxidant activity expressed in %inhibition. The IC₅₀ value is determined through linear regression, which plots the concentration on the X-axis and the percentage inhibition on the Y-axis.

LC-MS/MS analysis

LC-MS/MS analysis for identifying metabolites refers to the method carried out by Alcazar Magana [19]. The column employed was Accucore (100 × 2.1 mm, 1.5 µm). The mass spectrometer utilized an electrospray ionization source with Q-Orbitrab mass analysis. The range of scanning values was from 100–1500 *m/z*, whereas ionization energies employed were 18, 35, and 53 eV. With a flow rate of 0.2 µL/min and an injection volume of 2 µL, samples were analyzed using two mobile phases: 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). We used a gradient elution system. Mobile phase composition: 0–1 min (5% B), 1–10 min (5–30% B), 10–20 min (30–100% B), 20–25 min (100% B), 25–30 min (5% B).

The concentrated *C. asiatica* leaves extract was dissolved in methanol LC-MS grade. For 30 min, the

extract was dissolved using an ultrasonicator. Then, the solution was filtered through a 0.22 µm filter membrane, and the filtrate was injected into LC-MS/MS. Compound Discoverer 3.2 software (Thermo Scientific, Germany) was used to evaluate the resultant data using an internal database that was already in place. Next, MS2 confirmation was conducted to predict the compounds in the sample.

Multivariate data analysis

Multivariate PCA and OPLS-DA data analysis was processed using Metaboanalyst 6.0 (<https://www.metaboanalyst.ca>). PCA analysis aims to classify based on the polarity of the extracting solvent using intensity variables throughout the chromatogram. Meanwhile, OPLS-DA analysis was carried out to determine the correlation between antioxidant activity and metabolites. Metabolites that have a major contribution to the antioxidant activity were predicted using S-plot and variable importance in the projection (VIP).

RESULTS AND DISCUSSION

Antioxidant Activity of *C. asiatica*

The antioxidant activity of *C. asiatica* leaves with different solvent extracts varied significantly ($p < 0.05$) (Table 1). In general, the antioxidant activity of *C. asiatica* was more potent in samples using ethanol solution than in pure ethanol. The antioxidant activity of 70% ethanol was the strongest, with IC₅₀ < 100 µg/mL. As the water content in the solvent increases, the antioxidant activity decreases. These results are consistent with previous studies that found *C. asiatica* to have more active antioxidant activity when a mixture of

Table 1. Antioxidant activity of *C. asiatica* leaves extract

Solvent	IC ₅₀ (µg/mL)
Water	256.46 ± 1.64 ^a
Ethanol 30%	238.28 ± 2.93 ^b
Ethanol 50%	146.17 ± 1.43 ^d
Ethanol 70%	72.48 ± 2.42 ^e
Ethanol p.a.	168.77 ± 4.29 ^c
Ascorbic acid	3.38 ± 0.04

*Note: Different numbers indicate significant differences by Tukey's test ($p < 0.05$)

ethanol and water was used as an extraction solvent [9].

Identified Metabolite Profiles

Metabolites in *C. asiatica* leaves extract were identified using LC-MS/MS. The LC-MS/MS analysis of the extract showed that the metabolite patterns of the *C. asiatica* leaves extracts were different (Fig. 1). The different chromatogram patterns explain the differences in the composition of the compounds detected in each solvent. All samples have a similar overall chromatogram pattern. Each solvent has differences in peak intensity. This difference in intensity indicates a difference in metabolite concentration in each extract.

Compound Discovered 3.2 software was used to process the raw data. The molecular weight tolerance of 5 ppm. The identification results were further confirmed using MS2 spectra [16]. As a result of data processing and MS2 confirmation, 35 metabolites were putatively identified. These metabolites include terpenes, phenolics, flavonoids, fatty acids, and other groups (Table 2).

The most metabolites contained in *C. asiatica* leaves extracts were in the terpenes group. Metabolites were identified from the terpenes group, one of which is madecassic acid (22), one of the characteristic compounds of *C. asiatica*. Compound (22) has a m/z of 503.3382 $[M-H]^-$ and fragments at a m/z of 459.0136 $[M-H-CHO_2]^-$. Apart from terpenes, phenolic groups were also identified in many *C. asiatica* leaves extract. Caffeic acid (6) is one of the phenolic groups that were also identified by

releasing m/z 135.0442 $[M-H-CHO_2]^-$ and m/z 92.9189 $[M-H-CHO_2-CH_2O]^-$. The next group is flavonoids, one of which is kaempferol (13) m/z 287.0545 $[M+H]^+$ fragmented into m/z 259.0585 $[M+H-CO]^+$, m/z 153.0180 $[M+H-C_6H_5O-C_2HO]^+$, m/z 121.0284 $[M+H-C_8H_6O_4]^+$, and m/z 107.0493 $[M+H-C_8H_4O_5]^+$.

The results of MS2 processing and confirmation revealed 35 putative metabolites (Table 2). The ethanol p.a. extract contained 24 metabolites, 70% ethanol extract 29 metabolites, 50% extract 24 metabolites, 30% extract 17 metabolites, and aqueous extract 11 metabolites. Luteolin (19), traumatic acid (20), 3-BHA (21), madecassic acid (23), asiatic acid (24), and oleamide (34) are metabolites found in all extracts. Meanwhile, some metabolites are only found in one of the extracts, namely homovanillic acid (4), which is in the water extract; 5-caffeylquinic acid (5), which is in the 50% ethanol extract; stearic acid (35) is in the 70% ethanol extract; caryophyllene oxide (30) and violaxanthin (33), which is in the ethanol p.a. extracts.

Discriminant Using PCA

PCA analyze the differences in metabolite profiles obtained from each extraction solvent. It is necessary to perform preprocessing to align peak shifts of chromatography data prior to PCA analysis. The segment length (m) and slack size (t) are critical parameters that must be optimized. The optimal segment length and slack size are compared to the highest

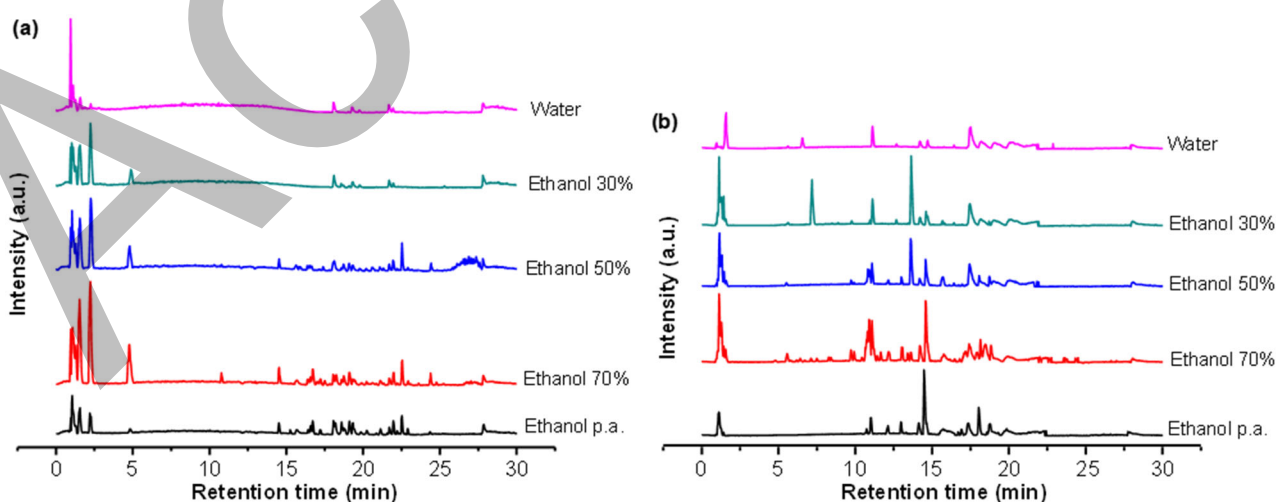


Fig 1. Base peak chromatogram of *C. asiatica* leaves extract in (a) positive and (b) negative ionization mode

Table 2. Putative identification *C. asiatica* leaves extract by LC-MS/MS

No	Compounds	Formula	RT	M.wt (g/mol)	Mode ion	MS/MS-MS	Solvents				
							Ep.a	E70	E50	E30	Water
<i>Terpenes</i>											
1	Madecassoside	C ₄₈ H ₇₈ O ₂₀	12.12	974.5069	[M+H] ⁺	975.5135, 215.1792	✓	✓	✓		
2	Methyl jasmonate	C ₁₃ H ₂₀ O ₃	12.62	224.1410	[M-H] ⁻	223.1337, 59.0128				✓	✓
3	Asiaticoside	C ₄₈ H ₇₈ O ₁₉	12.98	958.5119	[M+H] ⁺	959.5192, 85.0289, 71.0497	✓	✓	✓	✓	
4	Madecassic acid	C ₃₀ H ₄₈ O ₆	15.69	504.3451	[M-H] ⁻	503.3383, 459.0136	✓	✓	✓	✓	✓
5	Asiatic acid	C ₃₀ H ₄₈ O ₅	16.56	488.3488	[M+H] ⁺	489.3556, 235.1685, 217.1581, 187.1481, 107.0859	✓	✓	✓	✓	✓
6	Zerumbone	C ₁₅ H ₂₂ O	18.77	218.1668	[M+H] ⁺	219.1741, 135.0805, 121.0646, 81.0703	✓	✓			
7	2,4-tert-Butylphenol	C ₁₄ H ₂₂ O	19.14	206.1667	[M+H] ⁺	207.1741, 189.1637, 151.1118, 133.1012, 123.0806	✓	✓			
8	Farnesene	C ₁₅ H ₂₄	20.14	204.1875	[M+H] ⁺	205.1948, 161.1238, 149.1325, 121.1023, 93.0702	✓	✓			
9	Caryophyllene oxide	C ₁₅ H ₂₄ O	20.26	220.1824	[M+H] ⁺	221.1898, 203.1796, 161.1325	✓				
10	Violaxanthin	C ₄₀ H ₅₆ O ₄	21.55	600.4167	[M+H] ⁺	601.4233, 583.4141, 119.0858, 105.0703	✓				
<i>Phenolics</i>											
11	Homovanillic acid	C ₉ H ₁₀ O ₄	6.50	182.0573	[M-H] ⁻	181.0500, 94.9160, 59.0128					✓
12	Caffeic acid	C ₉ H ₈ O ₄	7.10	180.0415	[M-H] ⁻	179.0344, 135.0442, 92.9189		✓	✓	✓	✓
13	3-Feruloylquinic acid	C ₁₇ H ₂₀ O ₉	8.34	368.1107	[M-H] ⁻	367.1036, 191.0555, 134.0363, 93.0335		✓	✓		
14	3-BHA	C ₁₁ H ₁₆ O ₂	15.26	180.1148	[M+H] ⁺	181.1221, 163.1118, 135.1169, 107.0858, 95.0859	✓	✓	✓	✓	✓
15	Gingerol	C ₁₇ H ₂₆ O ₄	18.03	294.1827	[M+H] ⁺	295.1899, 137.0598, 109.0650, 95.0493	✓	✓			
16	<i>p</i> -Coumaric acid	C ₉ H ₈ O ₃	18.70	164.0471	[M+H] ⁺	165.0544, 121.0649, 91.0547	✓	✓	✓		
<i>Flavonoids</i>											
17	Quercetin glucuronide	3-O-C ₂₁ H ₁₈ O ₁₃	9.90	478.0745	[M-H] ⁻	477.0677, 301.0357, 178.9980, 151.0028, 107.0127, 59.0128	✓	✓	✓		
18	Kaempferol-3 galactoside	C ₂₁ H ₂₀ O ₁₁	10.76	448.1004	[M-H] ⁻	447.0938, 285.0404, 255.0299, 227.0347	✓	✓	✓		
19	Kaempferol	C ₁₅ H ₁₀ O ₆	10.78	286.0470	[M+H] ⁺	287.0545, 259.0585, 153.0180, 121.0284, 107.0493		✓	✓		
20	Quercetin	C ₁₅ H ₁₀ O ₇	13.37	302.0426	[M-H] ⁻	301.0357, 178.9975, 151.0027, 121.0286, 107.0129	✓	✓	✓	✓	
21	Luteolin	C ₁₅ H ₁₀ O ₆	14.53	286.0475	[M-H] ⁻	285.0406, 229.0503, 211.0397, 187.0393, 107.0129	✓	✓	✓	✓	✓
<i>Fatty acids</i>											
22	Glutamic acid	C ₅ H ₉ NO ₄	1.15	147.0526	[M+H] ⁺	148.0590, 130.0498, 102.0552, 84.0446		✓	✓	✓	
23	Traumatic acid	C ₁₂ H ₂₀ O ₄	14.66	228.1359	[M-H] ⁻	227.1285, 183.1385, 111.0802	✓	✓	✓	✓	✓
24	Ethyl palmitoleate	C ₁₈ H ₃₄ O ₂	20.17	282.2553	[M+H] ⁺	283.2627, 95.0860, 83.0860, 81.0704	✓				✓
25	Linolenic acid	C ₁₈ H ₃₄ O ₂	20.17	282.2553	[M+H] ⁺	283.2627, 95.0860, 83.0860, 81.0704	✓				✓
26	Juniperic acid	C ₁₆ H ₃₂ O ₃	21.23	272.2352	[M-H] ⁻	271.2800, 253.2193, 225.2219, 223.2062	✓	✓	✓	✓	
27	Oleamide	C ₁₈ H ₃₅ NO	21.97	281.2711	[M+H] ⁺	282.2786, 97.0115, 83.0859, 69.0704	✓	✓	✓	✓	✓
28	Stearic acid	C ₁₈ H ₃₆ O ₂	26.20	284.2708	[M+H] ⁺	285.2780, 89.0600, 71.0858		✓			
<i>Phenyl propanoids</i>											
29	3-Caffeoylquinic acid	C ₁₆ H ₁₈ O ₉	6.39	354.0949	[M-H] ⁻	353.0879, 191.0554, 93.0333		✓	✓		
30	5-Caffeoylquinic acid	C ₁₆ H ₁₈ O ₉	6.77	354.0952	[M-H] ⁻	353.0878, 191.0554, 173.0449, 135.0441, 93.03345			✓		
31	4,5-Dicaffeoylquinic acid	C ₂₅ H ₂₄ O ₁₂	10.70	516.1266	[M-H] ⁻	515.1195, 191.0554, 179.0342, 135.0442	✓	✓	✓	✓	
<i>Others</i>											
32	Citric acid	C ₆ H ₈ O ₇	1.38	192.0263	[M-H] ⁻	191.0191, 115.0024, 87.0077		✓	✓	✓	✓
33	Centellin	C ₁₅ H ₂₂ O ₃	10.43	250.1564	[M+H] ⁺	251.1635, 219.8749		✓	✓	✓	
34	Umbelliferone	C ₉ H ₆ O ₃	10.69	162.0314	[M+H] ⁺	163.0387, 145.0283, 135.0440, 117.0336, 89.0389	✓	✓	✓	✓	
35	Ferulic acid	C ₁₀ H ₁₀ O ₄	13.86	194.0574	[M-H] ⁻	193.0501, 121.0285, 108.0206, 93.0335	✓	✓			

similarity index value. The similarity index value, which demonstrates a higher correlation between other chromatograms, can minimize the shift in retention time [20].

C. asiatica leaves extract with different extraction solvents was classified based on the overall peak intensity of the chromatogram. The score plot grouped similar samples, with each point representing a single sample [21]. Fig. 2 shows a score plot explaining 65% of the total variation (PC1 = 42% and PC2 = 23%). Based on the resulting grouping pattern, the ethanol extract p.a, 70% ethanol, and 50% ethanol formed adjacent groups, which showed similar chromatogram patterns and distribution of metabolites in the extracts.

Antioxidant Active Compound Prediction Using OPLS-DA

The OPLS-DA model was used to predict active antioxidant compounds. The OPLS-DA model was created using peak area variables of all detected m/z and antioxidant IC_{50} values. The antioxidant activity of the samples was classified into two groups, namely active antioxidants ($IC_{50} \leq 150 \mu\text{g/mL}$) and inactive ($IC_{50} \geq 150 \mu\text{g/mL}$).

On the OPLS-DA score plot, the two groups were divided according to their antioxidant activity. The active antioxidant group is in the left quadrant, which consists of 70% ethanol extract and 50% ethanol extract, while the inactive group is in the right quadrant (Fig. 3).

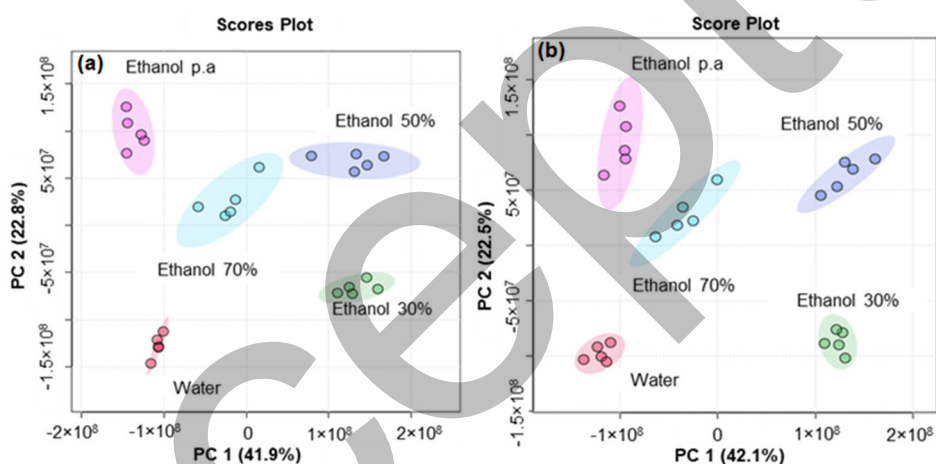


Fig 2. Plot of PCA score of *C. asiatica* leaves extract using the overall chromatogram intensity variable before (a) and after (b) COW

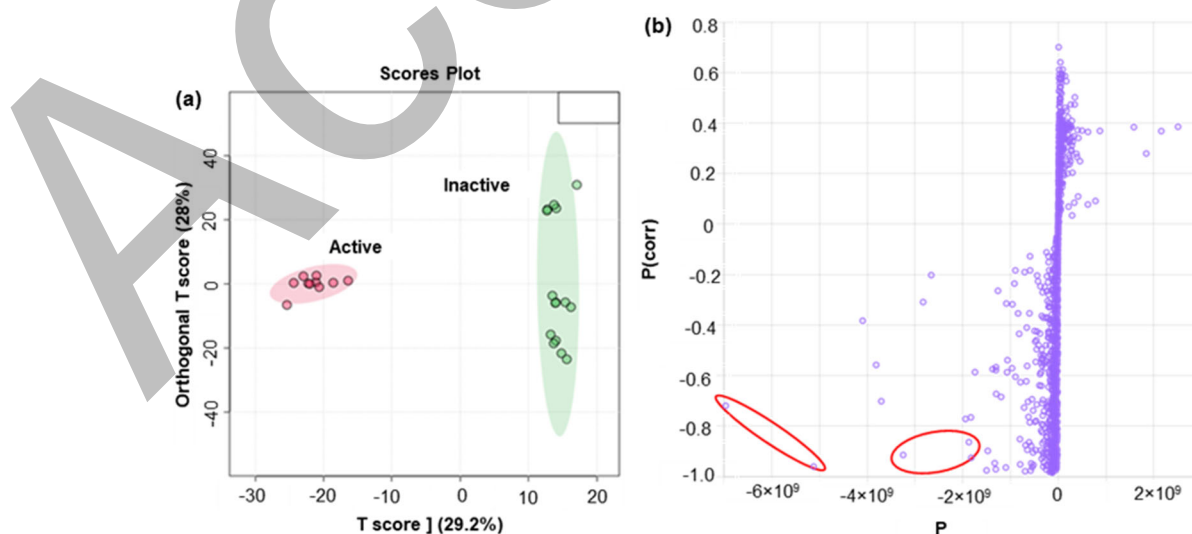


Fig 3. Plot of score (a) and S-plot (b) of OPLS-DA using the variable peak area of detected metabolites

Table 3. p-Value, fold change, and VIP for compounds that have potential as antioxidants

Name	Formula	RT	p-value	Fold change	VIP
4,5-Dicaffeoylquinic acid	C ₂₅ H ₂₄ O ₁₂	10.709	3.25 × 10 ⁻⁹	5.836	1.691
Kaempferol	C ₁₅ H ₁₀ O ₆	10.787	5.12 × 10 ⁻⁹	7.598	1.776
Unknown	C ₆ H ₁₄ N ₄ O ₂	1.061	1.86 × 10 ⁻⁹	4.302	1.597
Unknown	C ₁₁ H ₁₂ N ₂ O ₂	4.773	6.96 × 10 ⁻⁹	2.382	1.330
Unknown	C ₂₀ H ₃₄ O ₃	21.789	1.82 × 10 ⁻⁹	7.892	1.712

R² and Q² values evaluate model suitability and predictive ability. The model is acceptable if the R² > 0.5 and Q² > 0.4 [22]. The model created has a value of R² = 0.896 and Q² = 0.825, so it can be said that the resulting model has good predictive ability.

S-plot and VIP are used to determine the variables with the most potential as antioxidants. In an S-plot, the X-axis represents the contribution of variables to the observed variance. The Y-axis also shows the reliability of the results and the correlation between the samples. As a result, the plot's upper right or lower left corners typically contain variables that differ significantly between groups [22]. Variables were selected based on the criteria of fold change > 1, p-value < 0.05, and VIP > 1 [23]. Five compounds were identified as potential antioxidants based on these parameters.

Based on these criteria, 5 compounds were predicted to have potential as antioxidants (Table 3). Two of the five compounds were identified, namely 4,5-dicaffeoylquinic acid and kaempferol. The prediction of these compounds is following the research conducted by Maulidiani [24], who reported the compounds 4,5-dicaffeoylquinic acid (11) and kaempferol (13), including predictions of active antioxidant compounds in *C. asiatica* different varieties from 70% ethanol extract.

■ CONCLUSION

The antioxidant active compounds of *C. asiatica* leaves extract have been successfully identified using an LC-MS/MS-based metabolomics approach. The 70% ethanol extract had the more potent antioxidant activity, while the water extract had the weakest. Metabolite analysis using LC-MS/MS succeeded in identifying 35 putative metabolites. Multivariate PCA analysis succeeded in grouping *C. asiatica* leaves extract into 2 groups: antioxidant active and inactive. Five metabolites

are predicted to have potential as antioxidants from *C. asiatica* leaves extract, namely 4,5-dicaffeoylquinic acid (11), kaempferol (13), and three unknown compounds.

■ ACKNOWLEDGMENTS

The author would like to thank The Directorate General of Higher Education, Research, and Technology, The Ministry of Education, Culture, Research, Republic of Indonesia for financial support for this research, with contract number: 027/E5/PG.02.00.PL/2024.

■ CONFLICT OF INTEREST

We declare no conflict of interest regarding the publication of this manuscript.

■ AUTHOR CONTRIBUTIONS

Riva Silvia: Investigation, formal analysis, visualization, and original draft writing. Wulan Tri Wahyuni and Mohamad Rafi: Conceptualization, methodology, validation, data curation, writing review, and editing. Eti Rohaeti and Siti Aisyah: Conceptualization, methodology, validation, writing review, and editing. Dewi Anggraini Septaningsih and Alfi Hudatul Karomah: Investigation and validation.

■ REFERENCES

- [1] Belwal, T., Andola, H.C., Atanassova, M.S., Joshi, B., Suyal, R., Thakur, S., Bisht, A., Jantwal, A., Bhatt, I.D., and Rawal, R.S., 2019, "Chapter 3.22 - Gotu Kola (*Centella asiatica*)" in *Nonvitamin and Nonmineral Nutritional Supplements*, Eds. Nabavi, S.M., and Silva, A.S., Academic Press, Cambridge, MA, US, 265–275.
- [2] Yasurin, P., Sriariyanun, M., and Phusantisampan, T., 2015, Review: The bioavailability activity of

- Centella asiatica*, *KMUTNB Int. J. Appl. Sci. Technol.*, 9 (1), 1–9.
- [3] Arora, R., Kumar, R., Agarwal, A., Reeta, K.H., and Gupta, Y.K., 2018, Comparison of three different extracts of *Centella asiatica* for anti-amnesic, antioxidant and anticholinergic activities: *In vitro* and *in vivo* study, *Biomed. Pharmacother.*, 105, 1344–1352.
- [4] Ju Ho, P., Jun Sung, J., Ki Cheon, K., and Jin Tae, H., 2018, Anti-inflammatory effect of *Centella asiatica* phytosome in a mouse model of phthalic anhydride-induced atopic dermatitis, *Phytomedicine*, 43, 110–119.
- [5] Vasavi, H.S., Arun, A.B., and Rekha, P.D., 2016, Anti-quorum sensing activity of flavonoid-rich fraction from *Centella asiatica* L. against *Pseudomonas aeruginosa* PAO1, *J. Microbiol., Immunol. Infect.*, 49 (1), 8–15.
- [6] Rafi, M., Nomi, A.G., Septaningsih, D.A., Heryanto, R., and Putri, S.P., 2022, Quantitative HPLC and FTIR-based metabolomics for clustering *Centella asiatica* cultivation ages and evaluation of their radical scavenging activity, *Sains Malays.*, 51 (6), 1789–1797.
- [7] Ncube, E.N., Steenkamp, P.A., Madala, N.E., and Dubery, I.A., 2017, Metabolite profiling of the undifferentiated cultured cells and differentiated leaf tissues of *Centella asiatica*, *Plant Cell, Tissue Organ Cult.*, 129 (3), 431–443.
- [8] Kunjumon, R., Johnson, A.J., and Baby, S., 2022, *Centella asiatica*: Secondary metabolites, biological activities and biomass sources, *Phytomed. Plus*, 2 (1), 100176.
- [9] Mohapatra, P., Ray, A., Jena, S., Nayak, S., and Mohanty, S., 2021, Influence of extraction methods and solvent system on the chemical composition and antioxidant activity of *Centella asiatica* L. leaves, *Biocatal. Agric. Biotechnol.*, 33, 101971.
- [10] Kasote, D.M., Katyare, S.S., Hegde, M.V., and Bae, H., 2015, Significance of antioxidant potential of plants and its relevance to therapeutic applications, *Int. J. Biol. Sci.*, 11 (8), 982–991.
- [11] Yaermaimaiti, S., Wu, T., and Aisa, H.A., 2021, Bioassay-guided isolation of antioxidant, antimicrobial, and antiviral constituents of *Cordia dichotoma* fruits, *Ind. Crops Prod.*, 172, 113977.
- [12] García-Pérez, P., Miras-Moreno, B., Lucini, L., and Gallego, P.P., 2021, The metabolomics reveals intraspecies variability of bioactive compounds in elicited suspension cell cultures of three *Bryophyllum* species, *Ind. Crops Prod.*, 163, 113322.
- [13] Quansah, E., and Karikari, T.K., 2016, Potential role of metabolomics in the improvement of research on traditional African medicine, *Phytochem. Lett.*, 17, 270–277.
- [14] Perumal, V., Khatib, A., Uddin Ahmed, Q., Fathamah Uzir, B., Abas, F., Murugesu, S., Zuwairi Saiman, M., Primaharinastiti, R., and El-Seedi, H., 2021, Antioxidants profile of *Momordica charantia* fruit extract analyzed using LC-MS-QTOF-based metabolomics, *Food Chem.: Mol. Sci.*, 2, 100012.
- [15] Mukherjee, P.K., 2019, “Chapter 13 - Bioassay-Guided Isolation and Evaluation of Herbal Drugs” in *Quality Control and Evaluation of Herbal Drugs*, Elsevier, Amsterdam, Netherlands, 515–537.
- [16] Zhang, S., Liu, Z., Li, X., Abubaker, M.A., Liu, X., Li, Z., Wang, X., Zhu, X., Zhang, J., and Chen, X., 2022, Comparative study of three raspberry cultivar (*Rubus idaeus* L.) leaves metabolites: Metabolome profiling and antioxidant activities, *Appl. Sci.*, 12 (3), 990.
- [17] Sawczuk, R., Karpinska, J., Filipowska, D., Bajguz, A., and Hryniewicka, M., 2022, Evaluation of total phenols content, anti-DPPH activity and the content of selected antioxidants in the honeybee drone brood homogenate, *Food Chem.*, 368, 130745.
- [18] Sayed, A.M., Sherif, N.H., El-Gendy, A.O., Shamikh, Y.I., Ali, A.T., Attia, E.Z., El-Katatny, M.H., Khalifa, B.A., Hassan, H.M., and Abdelmohsen, U.R., 2020, Metabolomic profiling and antioxidant potential of three fungal endophytes derived from *Artemisia annua* and *Medicago sativa*, *Nat. Prod. Res.*, 36 (9), 2404–2408.
- [19] Alcazar Magana, A., Wright, K., Vaswani, A., Caruso, M., Reed, R.L., Bailey, C.F., Nguyen, T., Gray, N.E., Soumyanath, A., Quinn, J., Stevens, J.F., and Maier, C.S., 2020, Integration of mass spectral

- fingerprinting analysis with precursor ion (MS1) quantification for the characterisation of botanical extracts: Application to extracts of *Centella asiatica* (L.) Urban, *Phytochem. Anal.*, 31 (6), 722–738.
- [20] Kumar, K., 2018, Chemometric assisted correlation optimized warping of chromatograms: Optimizing the computational time for correcting the drifts in chromatographic peak positions, *Anal. Methods*, 10 (9), 1006–1014.
- [21] Wang, P., Zhong, L., Yang, H., Zhu, F., Hou, X., Wu, C., Zhang, R., and Cheng, Y., 2022, Comparative analysis of antioxidant activities between dried and fresh walnut kernels by metabolomic approaches, *LWT*, 155, 112875.
- [22] Kang, C., Zhang, Y., Zhang, M., Qi, J., Zhao, W., Gu, J., Guo, W., and Li, Y., 2022, Screening of specific quantitative peptides of beef by LC–MS/MS coupled with OPLS-DA, *Food Chem.*, 387, 132932.
- [23] Hrbek, V., Rektorisova, M., Chmelarova, H., Ovesna, J., and Hajslova, J., 2018, Authenticity assessment of garlic using a metabolomic approach based on high resolution mass spectrometry, *J. Food Compos. Anal.*, 67, 19–28.
- [24] Maulidiani, M., Abas, F., Khatib, A., Shitan, M., Shaari, K., and Lajis, N.H., 2013, Comparison of Partial Least Squares and Artificial Neural Network for the prediction of antioxidant activity in extract of *Pegaga* (*Centella*) varieties from ¹H Nuclear Magnetic Resonance spectroscopy, *Food Res. Int.*, 54 (1), 852–860.