South African Journal of Botany 172 (2024) 710–719

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

LC—HRMS-based metabolomics on *Acalypha indica* with different altitude and antioxidant activity

Elok Kamilah Hayati[a,b], Aulanni'am[a], Akhmad Sabarudin[a,*], Mohamad Rafi[c,d], Alfi Hudatul Karomah[c]

[a] Department of Chemistry, Faculty of Mathematics and Natural Sciences, Brawijaya University, Jl. Veteran, Malang 65145, Indonesia
[b] Department of Chemistry, Faculty of Science and Technology, Maulana Malik Ibrahim State Islamic University of Malang, Jl. Gajayana No 50, Malang 65145, Indonesia
[c] Advanced Research Laboratory, IPB University, Jl. Palem raya IPB Dramaga Campus, Bogor 16680, Indonesia
[d] Department of Chemistry, Faculty of Mathematics and Natural Sciences, IPB University, Jl. Tanjung Kampus IPB Dramaga, Bogor 16680, Indonesia

ARTICLE INFO

Article History:
Received 5 March 2024
Revised 22 June 2024
Accepted 30 July 2024
Available online xxx

Edited by Dr G. Prinsloo

Keywords:
Acalypha indica
Metabolomics
Chemometrics
LC-HRMS
Antioxidant activity

ABSTRACT

Achalypha indica is a weed that has medicinal properties. This study aims to determine the metabolite profile and evaluate the antioxidant activity of *A. indica* from areas with different altitudes, Tuban (20 to 50 masl; TBN), Banyuwangi (60 to 90 masl; BB), Magetan (300 to 600 masl; MGT), and Malang (500 to 800 masl; MLG). The ethanol extract of *A. indica* was analyzed for total phenolics and antioxidant activity. Separation and identification of samples were carried out using liquid chromatography-high-resolution mass spectrometry (LC—HRMS), then classified by Principal Component Analysis (PCA). The total phenol value ranged from 6.83 to 10.67 mg GAE /g of dry powder, with an antioxidant IC_{50} of 86.09 to 118.96 μg/mL. LC—HRMS successfully identified 83 metabolites. PCA analysis was able to clearly distinguish each sample based on the altitude of the growing location with a total variance value reaching 82 % (PC-1: 57 %, and PC-2: 25 %). Heat map analysis that observes variations in metabolite composition in each sample shows an increase in the content of flavonoid compounds in the lowlands under certain geographical conditions. Therefore, the height of the growing location has a significant influence on the metabolite profile and antioxidant activity. These findings are very important for assessing the quality, safety, and efficacy of medicinal plant raw materials.

1. Introduction

Acalypha indica is a wild plant that can be used as a medicinal plant. In Indonesia, this plant is known as anting-anting. The biological activities of A. indica that have been reported are antioxidant (Syahiran et al., 2017), antibacterial (Batubara et al., 2016), antimalarial (Hayati et al., 2012; Pasumarthi and Sunita, 2018) and anticancer (Sanseera et al., 2015; Suresh et al., 2020; Chekuri et al., 2023). The active compounds found in this plant are responsible for its pharmacological activity such as kaempferol-3-O-rutinoside, apigenin, acalyphin, esculetin and 4-methyldaphneti, luteolin, catechin, dihydroartemisinin, artemotil/ *β-arteether,* peucenin, 13-oxo-ODE, azelaic acid, and traumatic acid (Fawzy et al., 2017; Sahukari et al., 2021; Ravi et al., 2017).

The composition and concentration of bioactive compounds could affect the level of biological activity (Suresh et al., 2020). One of the factors is the difference in altitude of the growing location. Related studies indicate that the composition of cannabis seeds in locations at different altitudes results in different amounts of metabolites and antioxidant potential. Plants at higher altitudes accumulate more amino acids, cannabinoids, and fatty acids (Rashid et al., 2021), the altitude of a plant's location significantly affects its secondary metabolism due to the combination of lower temperatures and increased UV-B radiation found at high altitudes. (Cirak et al., 2016). Another study explains that variations in altitude affect the levels of polyphenols in honey, honey from lowland areas has higher levels of total phenols and total flavonoids (Suleiman et al., 2020). Altitude is also a contributing factor, though it is notresponsible for all the variations. All differences in the types and contents of secondary metabolites can also be caused by different environmental and geographical conditions, including climate, temperature, air humidity and light intensity. climate, temperature, air humidity, and light intensity (Suresh et al., 2020; Zantar et al., 2015; Yaylı et al., 2016; Sanli and Karadogan, 2016). Therefore, it is important to evaluate variations in the metabolite composition in *A. indica* from different altitude regions and to determine their biological activities. Rapid and systematic tests are required to assess the level of medicinal plants with a

* Corresponding author.
E-mail address: sabarjpn@ub.ac.id (A. Sabarudin).

https://doi.org/10.1016/j.sajb.2024.07.064

Table 1
Sources of *A. indica* in East Java.

Location/ sample code	Collection Date	Altitude (MASL)*	Coordinates	Temperature (°C)	Relative Humidity (RH)	Air pressure (milibar)	Season
Banyuwangi (BYW)	22 January 2022	60 – 90	S08°30.440′ E114°15.416′	31.5 – 33.7	70.1 – 75.5	1002.1 – 10,004.9	Rainy
Magetan (MGT)	6 February 2022	300 - 600	S07°30.340′ 111°30.460′	27.7 – 30.1	73.7 – 82.8	953.9 – 966.2	Rainy
Malang (MLG)	16 January 2022	500 - 800	S07°55.620′ E112°39.049′	26.3 – 30.0	76.4 – 84.3	925.0 – 950.0	Rainy
Tuban (TBN)	30 January 2022	20 – 50	S06°53.930′ E112°05.085′	31.9 - 34.8	63.9 – 74.0	1008.1 –1008.4	Rainy

* MASL=meter above sea level.

comprehensive and integrated approach, such as metabolomics. In this study, non-targeted metabolomics using LC-MS/MS was used to evaluate the distribution of metabolites in samples.

The metabolomics approach and chemometric analysis can be used together as a quality control evaluation of medicinal plants. Metabolomics-chemometric analysis using PCA analysis can provide a quick overview of the clustering of *A. indic*a plant samples based on differences in their metabolite profiles. This approach aims to show the differences between samples more completely, enabling a better understanding of the composition of plant metabolites and changes in their biological activity. LC—HRMS-based non-targeted metabolomics and chemometrics techniques, through PCA analysis, can differentiate the two species *C. latifolia* and *C. orchioides* and their plant parts. Meanwhile, PLS analysis can predict the chemical compounds that contribute to antioxidant activity and α-glucosidase inhibition based on the growing location, which will cause differences in metabolite profiles, affecting antioxidant activity and α-glucosidase inhibition (Umar et al., 2021). In another study, geographic origin will influence the metabolite profile. LC—HRMS metabolomics technique succeeded in grouping metabolite profiles based on geographic origin in *A. paniculata* (Rafi et al., 2021) and *C. zanthorrhiza* (Klau et al., 2022).

Previous research on *A. indica* explained that the results of the isolation of water extracts had high flavonoid content and antioxidant activity in the DPPH assay (Syahiran et al., 2018). Hayati et al. (2012) explained that in vivo ethyl acetate extract has active potential against *P. bergei*, while hexane extract provides the best antibacterial potential (Batubara et al., 2016) and shows cytotoxic activity on breast cancer MCF-7 cell lines (Chekuri et al., 2017). The methanol extract showed anticancer activity against NCI-H187 and also demonstrated significant antioxidant activity (Sanseera et al., 2015), meanwhile, Ravi et al. (2017) identified several chemical compounds using HR-LC/Q-TOF/MS. However, the impact of different altitudes at growing sites on metabolite profiles and inhibitory activity against DPPH radicals, analyzed using metabolomic-chemometric methods, remains unexplored, which constitutes the novelty in this study. This study aims to determine the impact of the altitude at which *A. indica* grows on the content and composition of its active compounds, by analyzing metabolite profiles and classifying them using multivariate PCA analysis. In addition, evaluating antioxidant activity with the DPPH method. The findings revealed a significant correlation between variations in the altitude of the growth site and their effects on the content of active compounds and the activity of *A. indica*. These results offer new insights into the assessment of the control of raw materials for medicinal plants derived from this plant.

2. Materials and methods

2.1. Chemicals and instrumentation

Ethanol and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma Aldrich (St. Louis, USA). Ethanol, acetonitrile, and distilled water (LC-MS grade) were purchased from Merck (Darmstadt, Germany). Whatman membrane filters (0.22 μm pore size; PTFE; P/N E252) were sourced from Sigma Aldrich (St. Louis, MO, USA). Ultra high performance liquid chromatography-quadrupole-orbitrap high resolution mass spectrometry (UHPLC-Q-Orbitrap HRMS) (Thermo Scientific, Waltham, USA) was used to separate and identify metabolites. For in vitro antioxidant activity measurements, a microplate reader (Epoch BioTek, Winooski, USA) and UV–vis Spectrophotometer (Varian Cary 50 Bio, USA) were used.

2.2. A. indica *samples*

A. indica was obtained from different altitude locations, Tuban, Banyuwangi, Magetan, and Malang, with altitudes ranging from 500 to 800 m above sea level, and different geographical conditions (Table 1). Authentication of the species was carried out by taxonomists at the biology department of UIN Maulana Malik Ibrahim Malang. As well as specimen samples stored in the analytical chemistry laboratory of the chemistry study program of UIN Maulana Malik Ibrahim Malang.

2.3. *Extraction of* A. indica

A. indica samples were washed with running water to remove impurities and then dried in an oven at 40 °C. After the drying process, the herbs were ground into a powder of 90 mesh. A total of 20 g of sample powder was extracted in 300 mL of ethanol through maceration in five replications, each lasting 72 h. Furthermore, the separation of the metabolite ethanol extracts was analyzed using UHPLC-Q-Orbitrap HRMS (Thermo Scientific 2020), and the total phenolic and antioxidant activity were tested.

2.4. *Separation and identification of the* A. indica *metabolite using LC—HRMS*

The process of identifying metabolites through LC—HRMS corresponds to the technique developed by Ravi et al. (2017). In this method, 5 mg of samples were dissolved in 1 mL of LC-MS grade MeOH, followed by filtration using a 0.2 μm Polytetrafluroethylene (PTFE) membrane. Separation of metabolites from the extract of *A. indica* was carried out using a Vanquish Flex UHPLC-Q Exactive Plus Orbitrap-High Resolution Mass Spectrometer, equipped with an Accucore C18 column (100×2.1 mm, 1.5 μm, Thermo Scientific). The mobile phase consisted of 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B), employing a gradient elution system: 0.0 to 1.0 min (15 % B), 1.0 to 22.0 min (15 to 70 % B), 22.0 to 24.0 min (70 to 95 % B), 24.0 to 27.0 min (95 % B) and 27.0 to 32.0 min (15 % B). The flow rate was set at 0.25 mL/min, with an injection volume of 2.5 μL. The MS system used ESI as the ionization source, operating in both positive and negative ionization modes within the 100 to 1500 *m/z* range. Capillary temperature, spray voltage, and gas sheath and sweep gas speeds were configured at 320 °C, 3.8 kV, 15 mL/min and 3 mL/min, respectively. The automatic gain control (AGC) was established at 3×10^6, while the injection time (IT) was fixed at 100 ms. Impact energy levels of 18, 35, and 53 eV were applied, and the scanning approach employed was MS/MS2, with scan data acquired at a resolution of 70,000 full width at half maximum (FWHM).

2.5. Determination of the total phenolic content of A. indica

The Folin and Ciocalteu reagent was used to assess the total phenolic content of the extracts, following the method described by Ayele et al. (2022), with modifications. The readings for both the samples and standards were obtained using a spectrophotometer (Varian Cary 50 Bio UV–vis Spectrophotometer) at 765 nm, with reference to the reagent blank.

When 100 mg of the extract was weighed, it was then dissolved in 0.02 L water, resulting in a concentration of 50 mg/mL. Subsequently, 0.2 mL of the extract was pipetted and 15.8 mL of aquades and 1 mL of Folin–Ciocalteu reagent were added and stirred. The mixture was allowed to stand for 8 min before adding 3 mL of 10 % Na_2CO_3. The solution was left at room temperature in dark conditions for 2 h. Subsequently, the sample was measured using a UV–vis spectrophotometer at 765 nm, with the process repeated three times. The total phenolic content was calculated as the equivalent gallic acid (GAE) per gram of dry plant material according to the standard gallic acid curve (5–25 mg/L) with the equation $y = 0.0087x + 0.0004$.

2.6. Determination of antioxidant activity with the DPPH method of A. indica

The antioxidant activity was determined using the DPPH method developed by Khorasani Esmaeili et al. (2015). A 3 ml solution of the extract was mixed with 1 ml of DPPH (0.2 mM) in an ethanol solution with varying concentrations (2–10 mg/ml). Following the vortex, the mixture was incubated for 30 min at 37 °C. The decrease in absorbance of the test mixture was caused by quenching of DPPH free radicals at 517 nm.

The sample concentration required to scavenge 50 % of DPPH free radicals is denoted by the IC_{50} value. The following formula was used to calculate radical scavenging activity:

$$SCV\% = \frac{Absorbance\ Control - Absorbance\ sample}{Absorbance\ control} \times 100\%$$

Percentage scavenging is% SCV, the IC_{50} value was obtained by analyzing the graph depicting the scavenging activity at various extract concentrations. This value represents the total antioxidant concentration needed to reduce the initial DPPH radical concentration by 50 %. Measurements were made in triplicate, and scavenging effects were calculated on the basis of the percentage of DPPH scavenged.

2.7. Data analysis

Five duplicates were used to determine extraction, total phenol content, and antioxidant activity in DPPH. Data are shown as the mean ± standard deviation. A one-way analysis of variance (ANOVA) is performed to determine if there is statistical evidence of a significant difference. If $p < 0.05$, a Duncan test is performed. The relationship between identified metabolites and antioxidant activity was determined, the IC_{50} activity value and the peak area of each metabolite were the parameters used to calculate the Pearson correlation using Statistical Product and Service Solutions (SPSS, Version 25.0).

Compound Discoverer 2.2 processed LC—HRMS data using an internal database derived from the results of the study of *A. indica* metabolites. Various methods, including mass list search, retention time alignment, chemical identification (both found and not found), prediction procedures, and selected spectrum analysis, were employed for metabolite identification. The peak area of the metabolite is the variable in the PCA analysis, which might be different between samples. The Unscrambler X program version 10.1 was utilized to generate the PCA model. Meanwhile, MetaboAnalyst ver. 5.0 was used to analyze differences in metabolite distribution through hierarchical cluster analysis (HCA) with heat map visualization. (https://www.metaboanalyst.ca/).

3. Results and discussion

3.1. Extraction yields

The study was planned based on sample codes, sampling locations, and geographical conditions (Table 1). *A. indica* powder was subjected to extraction using ethanol solvent via the maceration technique. This method involves prolonged soaking to enhance the interaction between the sample and solvent, to optimize the extraction of metabolites. Furthermore, the maceration process uses room temperature to minimize the likelihood of compound degradation associated with elevated temperatures (Ponphaiboon et al., 2023).

In this study, the extraction yield of *A. indica* was found to range from 17.16±0.46 to 50.66±0.15 % (Table 2). The ANOVA results indicated a statistically significant impact of the altitude of the growing location on the extraction yield at a significance level of 95 % (p-value <0.05). The altitude of the growing location causes differences in the extracted metabolites, so the yield value is different. The diversity of secondary metabolites, or the composition of chemically active compounds. is influenced by soil conditions and the geographical environment (Giupponi et al., 2020). *A. indica* as samples came from four areas in East Java with different altitudes; BYW and TBN were classified as lowland areas, while MGT and MLG were classified as highland areas (Meybeck et al., 2001). This plant is a weed that is more common in the lowlands than in the highlands. Differences in altitude lead to variations in geographical conditions such as temperature, light intensity, humidity, air pressure and differences in altitude. So the difference in altitude is one of the factors that will influence the production of secondary metabolites (yield value). This difference in yield will also affect the total phenolic value and antioxidant activity.

3.2. Metabololite profiling of A. indica

LC—HRMS is widely used to separate and identify metabolites based on the location of the growth of *A. indica* plants in the ion-negative mode (loss of one proton $[M\text{-}H]^{-1}$). The chromatogram profiles of the samples were similar for all regions, as illustrated in (Fig. 1). The peak position (tR value) does not have a significant difference,

Table 2
Extraction yield, total phenolic content, and antioxidant activity of *A. indica* based on different altitudes.

Location*	Altitude (MASL)**	Extraction Yield (%)	Total Phenolic Content (mg GAE/g dried powder)	Antioxidant activity (IC_{50}, μg/mL)
BYW	60 – 90	17.72±0.59^{c}	10.67±0.42^{a}	91.23±0.88^{c}
MGT	300 - 600	49.19±0.52^{b}	6.83±0.57^{c}	115.02±0.29^{b}
MLG	500 - 800	17.16±0.46^{c}	8.10±0.36^{b}	118.46±1.723^{a}
TBN	20 – 50	50.66±0.15^{a}	7.72±0.45^{bc}	86.09±0.48^{c}

The values are mean ± SD of the 5 replicates test for each sample. The $^{a-d}$ mean ± SD of each extract in the same column followed by different superscript letters showed a significant difference at $p < 0.05$ base.

* BYW (Banyuwangi), MGT (Magetan), MLG (Malang), TBN (Tuban).

** MASL=meter above sea level.

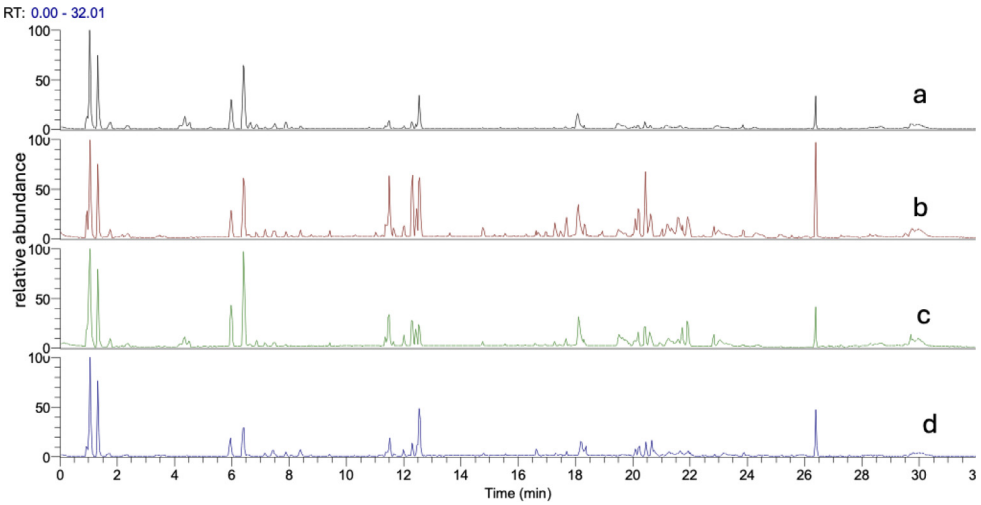

Fig. 1. Chromatograms of *A. indica* from (a) BYW, (b) MGT, (c) MLG, and (d) TBN in the negative ionization mode were obtained using LC—HRMS. The altitudes corresponding to each location can be found in Table 1.

but has a difference in intensity. So the difference in altitude does not affect the type of metabolite but affects the concentration of the metabolite. The similarity of the chromatogram profiles in each extract indicates the similarity of the metabolite composition, making it difficult to determine differences only from the chromatogram profiles. Therefore, multivariate chemometric analysis is needed to distinguish and classify chromatogram profiles. The analysis used was PCA.

The identification results using LC/MS-MS on ethanol extracts of *A. indica* from four regions showed the presence of a total of 83 metabolites. Identified metabolites include flavonoids, alkaloids, terpenoids, coumarins, lactones, amino acids, fatty acids, carboxylic acids, and other metabolites. According to the Venn diagram (Fig. 2), lowland areas (TBN and BYW) have a greater number of identified metabolites than highland areas. There are typical metabolites that are identified only in one area but not in other areas. A total of nine specific metabolites were identified in the BYW region and seven specific metabolites in the TBN region, while MGT and MLG each had two typical compounds identified. A total of forty metabolites were found in all regions, including flavonoids, phenolics, alkaloids, terpenoids, coumarins, fatty acids, amino acids, carboxylic acids and other metabolites.

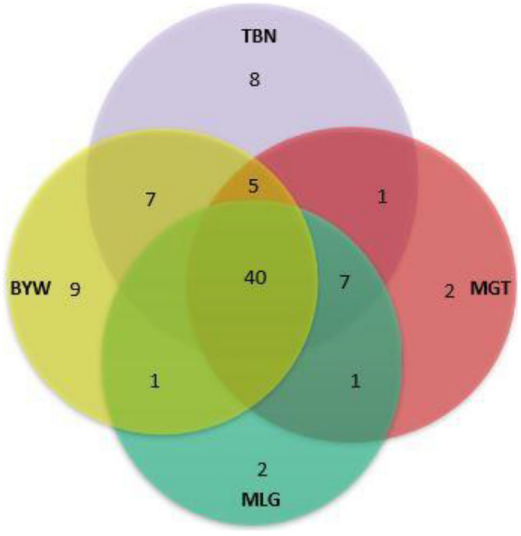

Fig. 2. Venn Diagram of the putatively identified compound of *A. indica* in different altitude. The altitude can be seen in Table 1.

Table 1 describes the environmental conditions of *A. indica* in the lowlands, where the temperatures are hotter, the humidity is lower and the air pressure is higher. This will affects the formation of secondary metabolites and confirm that air temperature, air humidity, and air pressure are important external factors that affect the bioactive components of *A. indica*. This finding is reinforced by research (Manukyan, 2019) on *Thymus transcaucasicus* Ronn plants, where the content of secondary metabolites of phenolic and carotenoid groups increased in areas with increased temperature, while essential oil groups increased in areas with low temperature.

Based on (Table 3), forty metabolites ware identified in all regions: five metabolites belonging to the flavonoid group, four metabolites of phenolic compounds, two metabolites of alkaloid compounds, two coumarin compounds, six fatty acid compounds, five amino acid compounds, nine carboxylic acid compounds, and four other groups of compounds.

Flavonoids and alkaloids are the most abundant groups in the extract of *A. indica.* Flavonoids are compounds that are antioxidants (Ravi et al., 2017) and anticancer (Chekuri et al., 2023). The results of the identification of about thirteen flavonoid compounds were identified in the ethanol extract based on the altitude region. Compounds kaempferol-3-O-rutinoside; apigenin (Fawzy et al., 2017); luteolin-6-C-glucoside; (+)-catechin-5-O-beta-D-glucopyranoside; luteolin; catechin (Sahukari et al., 2021) are the active compound identified in all BWY, TBN, MG and MGT regions. Catechin was identified based on its fragmentation in negative ionization mode with *m/z*. 289; 245; 205; 68. The rhamnetin O-rutinoside compound was only identified in the TBN extract, while brassicin was identified in the BYW area. Furthermore, there are compounds quercetin-7-O-p-coumaroylglucoside and rhamnetin ware identified in BYW, TBN MGT; 5,3′-dihydroxy-6,7,8,4′-tetramethoxyflavanone was identified in BYW, TBN; 2-geranyl-2′,3,4,4′-tetrahydroxydihydrochalcone and 6,8-diprenylnaringenin ware identified in TBN, MLG; MGT. In addition to flavonoids, alkaloids are metabolites that are also widely present in this plant. Three compounds were identified, namely thiazopyr, acalyphin (Fawzy et al., 2017), and senampeline A. The compounds thiazopyr and acalyphin were identified in all regions, while the compound senampeline A was only identified in TBN, MLG, and MGT. Acalyphin is the main compound, and it was identified based on its fragmentation in negative ionization mode with *m/z* 359, 300, 197, 181, 170, 169, 122, and 96 (Fawzy et al., 2017; Nahrstedt et al., 1982). Meanwhile, thiazopyr and senampeline A compound, we matched the MS fragmentation patterns from our research with online databases for these two compounds. There are similarities between the fragmentation patterns from our research and the online database.

Table 3
Identification of putative metabolite compounds of *A. indica* at different altitudes using LC—HRMS negative ion mode.

Putative compounds	RT [min]	Molecular formula	Error mass [ppm]	Exact MW	MS and MS2	Relative peak area (%)*			
						BYW	TBN	MLG	MGT
FLAVONOIDS									
Quercetin-7-O-p-coumaroylglucoside	5.314	$C_{27}H_{30}O_{16}$	−0.16	610.153	609; 463; 446; 301	4.68	3.50	–	3.46
5,3′-Dihydroxy-6,7,8,4′-tetramethoxyflavanone	5.768	$C_{19}H_{20}O_8$	−0.48	376.115	375; 329; 275; 107	2.53	3.78	–	–
Kaempferol-3-O-rutinoside	6.481	$C_{27}H_{30}O_{15}$	−0.90	594.158	593; 285; 255; 229; 68	0.001	0.44	1.90	0.71
Rhamnetin-O-rutinoside	6.558	$C_{28}H_{32}O_{16}$	−0.59	624.169	623; 315; 314; 285	–	0.20	–	–
Luteolin-6-C-glucoside	6.924	$C_{21}H_{20}O_{11}$	−0.47	448.100	447; 285; 73	0.31	0.51	0.56	0.44
Brassicin	7.152	$C_{22}H_{22}O_{12}$	−0.42	478.111	477; 315; 299; 285	0.48			
(+)-Catechin-5-O-beta-D-glucopyranoside	7.954	$C_{21}H_{24}O_{11}$	−0.56	452.132	451; 289; 219	0.33	0.13	0.08	0.13
Apigenin	11.686	$C_{15}H_{10}O_5$	−0.54	270.053	269; 227; 159; 117; 89; 65	0.32	0.12	0.26	0.30
Luteolin	12.061	$C_{15}H_{10}O_6$	−0.75	286.048	285; 229; 107; 63	0.19	0.09	0.06	0.09
Rhamnetin	12.381	$C_{16}H_{12}O_7$	−0.96	316.058	315; 283; 271; 109	0.12	0.14	0.09	0.10
Catechin	12.615	$C_{15}H_{14}O_6$	−1.36	290.079	289; 245; 205; 68	0.09	0.03	0.05	0.06
2-Geranyl-2′,3,4,4′-tetrahydroxydihydrochalcone	21.993	$C_{25}H_{30}O_5$	−1.20	410.209	409; 319; 267; 151; 135; 109	–	0.09	0.07	0.05
6,8-Diprenylnaringenin	22.901	$C_{25}H_{28}O_5$	−1.10	408.193	407; 389; 339; 333; 271; 217; 135; 107	–	0.07	0.05	0.07
PHENOLICS									
5-O-methyl embelin	14.841	$C_{18}H_{28}O_4$	−0.69	308.199	307; 289; 209; 83; 71	0.13	0.31	0.38	0.36
4-Hydroxy-3-nitrophenylacetic acid	1.467	$C_8H_7NO_5$	−3.68	197.032	196; 152; 124; 95	0.10	0.27	0.20	0.21
Salicylic acid	2.383	$C_7H_6O_3$	−7.48	138.031	137; 109; 93; 67; 65	0.04	0.10	0.06	0.05
3-Aminosalicylic acid	1.468	$C_7H_7NO_3$	−6.02	153.042	152; 124; 108; 65	0.04	0.09	0.06	0.04
ALKALOID									
Thiazopyr	1.116	$C_{16}H_{17}F_5N_2O_2S$	−0.32	396.093	395; 393; 359; 345	3.34	1.97	1.69	1.89
Acalyphin	1.138	$C_{15}H_{16}N_6O_5$	−4.79	360.116	359; 300; 197; 181; 170; 169; 122; 96	1.23	1.09	1.06	1.30
Senampeline A	21.995	$C_{25}H_{31}NO_8$	−1.54	473.204	472; 314; 67	–	0.14	0.30	0.18
TERPENOIDS (SESQUITERPENOIDS)									
Dihydroartemisinin	12.227	$C_{15}H_{24}O_5$	−0.58	284.162	283; 265; 239	–	–	0.18	0.17
Artemotil / β-arteether	15.616	$C_{17}H_{28}O_5$	−0.56	312.194	311; 309; 251; 223; 209	0.09	0.14	0.02	0.05
Peucenin	17.499	$C_{15}H_{16}O_4$	−1.22	260.105	259; 203; 175; 89	0.03	0.08	–	0.06
COUMARIN									
Marmin	2.081	$C_{19}H_{24}N_4O_5$	−3.88	388.173	331; 163; 161; 59	0.10	–	–	–
Esculetin	2.667	$C_9H_6O_4$	−4.50	178.026	331; 163; 161; 59	0.09	0.08	0.06	0.08
4-Methyldaphnetin	8.464	$C_{10}H_8O_4$	−4.11	192.041	177; 149; 135; 133; 121; 105; 93	0.05	0.11	0.02	0.06
Murrangatin	11.252	$C_{15}H_{16}O_5$	−0.45	276.100	191; 163; 149; 147; 123; 119; 107; 105; 91; 79; 65	0.03	0.08	–	0.04
Marmesin	12.616	$C_{14}H_{14}O_4$	−2.16	246.089	275; 257; 205; 203; 175	0.10	0.17	–	–
LACTONE									
2-Isocapryloyl-3R-hydroxymethyl-gamma-butyrolactone	4.137	$C_{13}H_{22}O_4$	−1.52	242.151	241; 183; 139; 69; 57	–	0.17	–	–
FATTY ACIDS									
Corchorifatty acid F	11.449	$C_{18}H_{32}O_5$	−0.96	328.225	327; 309; 229; 211; 171; 127; 125; 97; 85; 69	–	–	1.33	–
(15Z)−9,12,13-trihydroxy-15-octadecenoic acid	12.517	$C_{18}H_{34}O_5$	−0.89	330.240	329; 311; 29; 229; 211; 183; 171; 127; 125; 99; 69	0.51	0.86	1.11	0.98
10,16-Dihydroxyhexadecanoic acid	13.193	$C_{16}H_{32}O_4$	−0.29	288.230	287; 269; 141; 127; 113	–	0.37	0.43	–
13(*S*)-HpOTrE	17.026	$C_{18}H_{30}O_4$	−0.78	310.214	309; 291; 273; 185; 167; 139; 97; 67	0.15	0.13	0.10	0.16
9-HpODE	18.469	$C_{18}H_{32}O_4$	−0.73	312.230	311; 293; 275; 267; 249; 201; 171; 139, 127; 121; 97; 59	–	–	–	0.26
9(10)-DiHOME	18.481	$C_{18}H_{34}O_4$	−0.73	314.246	313; 295; 201; 199; 185; 171; 155; 127; 125; 67	–	0.19	–	–
9,10-Dihydroxystearic acid	20.178	$C_{18}H_{36}O_4$	−1.25	316.261	315; 297; 201; 171; 143; 141; 127; 125		0.08	0.09	0.10
13-Oxo-ODE	20.235	$C_{18}H_{30}O_3$	−1.06	294.219	293; 275; 257; 235; 221; 183; 177; 123; 113; 121; 97	0.08	0.13	0.12	0.08
12-Hydroxy-10-octadecynoic acid	21.081	$C_{18}H_{32}O_3$	−1.46	296.235	295; 277; 223; 209; 183; 151; 97; 59	0.12	0.10	0.07	0.12
13-Keto-9Z,11E,15Zoctadecatrienoic acid	21.52	$C_{18}H_{28}O_3$	−0.58	292.204	291; 273; 247; 221; 163; 135; 121; 97	0.08	0.11	0.11	0.08
2-Hydroxy lignoceric acid	26.432	$C_{24}H_{48}O_3$	−1.35	384.360	383; 339; 337	0.06	0.04	0.03	0,06
AMINO ACIDS									
4-Hydroxy-L-threonine	1.063	$C_4H_9NO_4$	2.32	135.053	134; 72; 59; 58	0.39	0.17	0.17	0.39
N-(1-Deoxy-1-Fructosylvaline	1.100	$C_{11}H_{21}NO_7$	−1.22	279.131	278; 188; 160; 158; 116; 114; 101; 89; 73	–	0.33	0.38	0,41
4-Oxoproline	1.395	$C_5H_7NO_3$	−8.97	129.041	128; 110; 84; 83; 82; 71	0.21	0.07	0.09	0.21
N-(1-deoxy-1-fructosyl)leucine	1.397	$C_{12}H_{23}NO_7$	−1.15	293.147	292; 232; 202; 172; 131; 130; 128; 126; 89; 73; 71	–	0.31	–	0.15
N(alpha)-citryl-N(epsilon)-acetyl-N(epsilon)-hydroxylysine	1.398	$C_{14}H_{22}N_2O_{10}$	−1.61	378.127	377; 335; 229; 59	0.29	–	–	–
N-(1-deoxy-1-fructosyl)phenylalanine	1.399	$C_{15}H_{21}NO_7$	−1.30	327.131	326; 236; 206; 164; 149; 147; 117; 101; 73	–	0.13	–	0.13
Shinorine	1.400	$C_{13}H_{20}N_2O_8$	−1.18	332.122	331; 155; 184	0.07	0.09	0.11	0.08

(*continued*)

Table 3 (*Continued*)

Putative compounds	RT [min]	Molecular formula	Error mass [ppm]	Exact MW	MS and MS2	Relative peak area (%)*			
						BYW	TBN	MLG	MGT
Glutaric acid	1.412	$C_5H_8O_4$	−8.60	132.041	131; 113; 87; 85 69; 59	–	0.10	–	–
4-(L-alanin-3-yl)−2-hydroxy-*cis*,cis-muconate-6-semialdehyde	1.466	$C_9H_{11}NO_6$	−2,16	229.058	228; 184; 166; 154; 152; 140; 138; 112 110; 95; 67; 65	0.09	0.11	0.04	0.06
DL-Tryptophan	1.746	$C_{11}H_{12}N_2O_2$	−3.19	204.089	203; 186; 142; 130; 116; 74; 72	0.03	0.06	0.06	0.09
Trp - Leu – Asp	2.414	$C_{21}H_{28}N_4O_6$	−3.91	432.199		0.04	0.08	0.04	0.07
Acetylphenylalanine	4.514	$C_{11}H_{13}NO_3$	−3.29	207.089	206; 164; 162; 147; 118; 103; 91; 58	0.05	0.07	–	–
N-Acetyl-L-phenylalanine	5.065	$C_{11}H_{13}NO_3$	−3.10	207.089	206; 164; 162; 147; 118; 103; 91; 58	–	0.03	–	–
DICARBOXYLIC ACID									
Azelaic acid	7.230	$C_9H_{16}O_4$	−4.12	188.105	187; 169; 143; 125; 97; 95; 57	0.55	0.91	1.30	1.53
Traumatic acid	12.367	$C_{12}H_{20}O_4$	−2.85	228.136	227; 209; 191; 183; 165; 111; 97; 63	0.16	0.32	0.28	0.29
CARBOXYLIC ACID									
Quinic acid	1.100	$C_7H_{12}O_6$	−4.58	192.063	191; 173; 131; 87; 85; 73; 71; 61; 59	1.12	0.42	0.25	0.28
Glucoheptonic Acid	1.117	$C_7H_{14}O_8$	−2.00	226.067	225; 89; 87; 71; 59	0.32	0.23	0.10	0.10
DL-Malic acid	1.123	$C_4H_6O_5$	−8.34	134.020	133; 115; 89; 87; 72; 71; 59	0.22	0.28	0.30	0.29
4-Oxo-L-proline	1.128	$C_5H_7NO_3$	−8.81	129.041	128; 84; 82; 55	0.20	0.08	0.08	0.09
Methylmalonic acid	1.135	$C_4H_6O_4$	−9.63	118.025	117; 73; 71; 55	0.15	0.18	0.25	0.23
Gentisic acid	1.756	$C_7H_6O_4$	−6.01	154.026	152; 124; 108; 65	0.11	0.08	–	–
D(+)-Phenyllactic acid	4.881	$C_9H_{10}O_3$	−5.64	166.062	153; 109; 107; 81; 68	0.06	0.11	–	–
6-Hydroxy-5-methyl-4,11-dioxoundecanoic acid	8.562	$C_{12}H_{20}O_5$	−1.65	244.132	137; 109; 93; 67; 65	0.06	0.11	0.08	0.06
5-Hexyl-2-oxotetrahydro-3-furancarboxylic acid	8.823	$C_{11}H_{18}O_4$	−2.51	214.120	213; 169; 125; 111; 97; 95; 71; 68; 57	0.09	0.10	0.04	0.04
2-(2-carboxyethyl)−4-methyl-5-pentylfuran-3-carboxylic acid (CMPentylF)	9.121	$C_{14}H_{20}O_5$	−0.65	268.131	267; 223; 219; 207; 71; 59	–	0.06	–	–
ESTER									
p-Coumaroylcaffeoyltartaric acid	1.395	$C_{22}H_{18}O_{11}$	−2.98	458.084	300; 180; 154; 138; 122; 71; 59	0.09	–	–	–
Di-n-Amyl phthalate	16.333	$C_{18}H_{26}O_4$	−0.54	306.1829	305; 249; 135; 125; 123; 79	0.08	0.04	0.03	0.04
LysoPE(0:0/18:3(6Z,9Z,12Z)	18.670	$C_{23}H_{42}NO_7P$	−0.88	475.270	474; 277; 214; 196; 171; 152; 140; 78	–	0.07	–	–
OTHER GROUPS									
D-Gluconic acid	1.097	$C_{12}H_{26}O_4S$	−4.20	196.057	195; 177; 87; 75; 72; 71; 61; 59	0.54	0.40	1.54	0.42
1-Pentofuranosyl-2,4(1H,3H)-pyrimidinedione	1.397	$C_{14}H_{30}O_4S$	−2.13	244.069	375; 243; 213; 200	0.04	0.16	–	–
D-Pantothenic acid	1.398	$C_{12}H_{18}O_4$	−3.28	219.110	218; 146; 128; 116; 88; 71; 59	0.33	0.43	–	0.30
4-Acetamidobutanoic acid	1.757	C_9H_7NO	−6.50	145.073	144; 102; 100	–	0.29	–	–
3-Phenyllactic acid	2.219	$C_9H_{10}O_3$	−5.24	166.062	165; 147; 91	0.29	0.29	0.29	0.29
Pyridinoline	2.385	$C_{10}H_{18}O_4$	−4.14	428.189	427; 381; 209; 126	0.29	–	–	–
Butopyronoxyl	3.511	$C_6H_{12}O_7$	−2.13	226.120	225; 97; 83; 71; 69; 57	0.21	0.12	0.14	0.11
Indole-3-acetic acid	5.268	$C_{22}H_{37}NO_3$	−4.82	175.063	174; 172; 156; 130; 128	0.05	0.09	–	–
4-Indolecarbaldehyde	6.651	$C_{10}H_9NO_2$	−6.54	145.052	144; 116	0.12	0.11	0.06	0.06
Picrotoxinin	8.261	$C_{15}H_{16}O_6$	−0.33	292.095	291; 273; 261	0.09	0.09	–	0.07
Kresoxim-methyl	9.110	$C_{18}H_{28}N_4O_8$	−0.44	313.131	312; 148; 134	0.09	–	–	–
3-tert-Butyladipic acid	9.484	$C_6H_{11}NO_3$	−3.27	202.120	201; 139; 111; 97	–	0.07	0.03	0.04
Dodecyl sulfate	18.449	$C_{18}H_{19}NO_4$	−1.48	266.155	265; 135; 110; 98; 96	0.07	–	–	–
Myristyl sulfate	22.037	$C_9H_{17}NO_5$	−0.14	294.187	293; 171; 98; 96	–	–	0.06	–
Leukotriene_B4 dimethylamide	23.774	$C_9H_{12}N_2O_6$	−0.42	363.277	362; 344; 67	–	0.04	0.03	0.03

$*Relative\ peak\ area = \frac{Area\ of\ individual\ peak}{Total\ peak\ area} x100\%$, relative peak area is equal with relative concentration. The (-) sign indicates not detected.

The identification results showed that the dominant metabolites were also found in the fatty acid, amino acid, and carboxylic acid classes. *A. indica* extract contains eleven of fatty acid compounds. For example, corchorifatty acid F was only identified in the MLG region, 9-HpODE was only identified in the MGT region, and 9(10)-DiHOME was identified in the TBN region. Meanwhile, other compounds were almost identified in all regions, such as (15Z)−9,12,13-trihydroxy-15-octadecenoic acid; 10,16-dihydroxyhexadecanoic acid; 13(*S*)-HpOTrE; 9,10-dihydroxystearic acid; 12-hydroxy-10-octadecynoic acid; 13-keto-9Z,11E,15Z-octadecatrienoic acid; 2-hydroxy lignoceric acid; and 13-oxo-ODE. The 13-oxo-ODE compound (Ravi et al., 2017) in this study was identified at a retention time of 20.235 in negative ion mode with an *m/z* value of 293; 275; 257; 177; 175; 121; 113; 97. In addition, there are thirteen compounds, including N(alpha)-citryl-N(epsilon)-acetyl-N(epsilon)-hydroxylysine which is only identified in the BYW region, glutaric acid and N-acetyl-L-phenylalanine which are identified in the TBN region. Meanwhile, other amino acid compounds were identified in almost all regions, such as 4-hydroxy-L-threonine; N-(1-deoxy-1-fructosyl)valine; 4-oxoproline; N-(1-deoxy-1-fructosyl)leucine; N-(1-deoxy-1-fructosyl)phenylalanine, shinorine, 4-(L-alanin-3-yl)−2-hydroxy-*cis*,cis-muconate; 6-semialdehyde; DL-tryptophan; Trp-Leu-Asp; and acetylphenylalanine. The extract of *A. indica* contained two dicarboxylic acid compounds, namely azelaic acid and traumatic acid (Sahukari et al., 2021; Ravi et al., 2017), which were present in all regions. Similarly, twelve compounds of carboxylic acid were also identified in all regions, such as quinic acid; glucoheptonic acid; DL-malic acid; 4-oxo-L-proline; methylmalonic acid; 4-hydroxy-3-nitrophenylacetic acid; 3-aminosalicylic acid; gentisic acid; salicylic acid; D(+)-phenyllactic acid; and 5-hexyl-2-oxotetrahydro-3-furancarboxylic acid. Only the compound CMPentylF was identified in the TBN region. There are three compounds identified as a class of Ester compounds, including di-n-amyl phthalate identified in all regions, compound p-coumaroylcaffeoyltartaric acid identified in the BYW region, while lyso PE(0:0/18:3 (6Z,9Z,12Z) identified in the TBN region.

3.3. *Classification of* A. indica *metabolites from different altitudes in East Java*

The distribution of metabolites in *A. indica* extracts was analyzed using multivariate PCA and HCA approaches to evaluate their differences. PCA can illustrate relationships between samples, such as

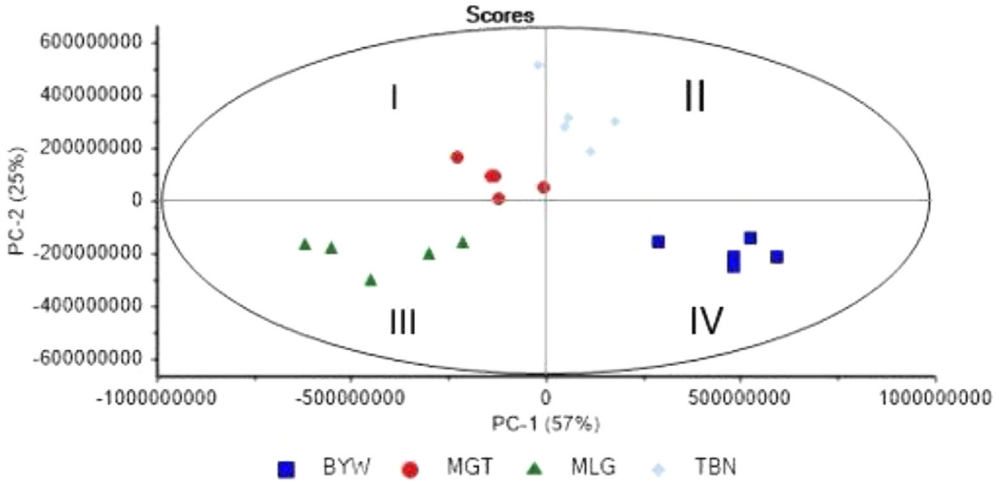

Fig. 3. PCA plot of *A. indica* using LC-MS/MS based altitude. The altitude can be seen in Table 1.

clustering, to differentiate samples based on altitude. PCA analysis, simplifying the observed variables by reducing their dimensions, produces a score plot of the principal components, resulting in the clustering of the sample.

Fig. 3 illustrates the PCA score plots of the samples. The results of the PCA analysis explain that the variations in the chemical components of *A. indica* are distributed into four groups: groups I-IV. Two main components, PC-1 and PC-2, contributed to the total variance of 82 % (PC-1: 57 %, PC-2: 25 %). Samples from BYW, MGT, MLG, and TBN were separated. MGT and MLG were closer in clustering and had similar metabolite profiles.

TBN and BYW have different clustering distances, even from MGT and MLG. They are suspected to have a different metabolite profile by differences in the amount and concentration of metabolites in *A. indica*. Therefore, differences in geographical conditions affect the production of secondary metabolites. Another study explained that a high-altitude environment with decreased temperature and increased UV-B radiation significantly affects the flavonoid and organic acid profiles of plants (Li et al., 2022, 2021). Conversely, the concentration of phenolic compounds increased with increasing temperature under high altitude geographical conditions. (Rivero et al., 2001) reported that increasing temperature could increase the enzymatic activity of PAL in tomato, and the concentration of total phenolic compounds increased with heat stress. The results showed that phenolic content differed significantly with altitude differences (Table 2), but did not have a distinctive pattern. As for antioxidant activity, *A. indica* at low altitude (TBN and BYW) has relatively higher antioxidant activity than at high altitude (MGT and MGL).

Differences in metabolite composition and concentration were identified in each extract and visualized in the HCA heatmap (Hierarchical Cluster Analysis). The HCA heatmap illustrates the variation of metabolites contained in each sample. Each row represents a compound, and each column represents a group of samples. The color scale on the right shows the relative concentration of the metabolite, with high concentrations in red and low concentrations in blue. The Fig. 4 explains that the metabolites in BYW and TBN are more varied in metabolite types, with the dominant metabolites being lactones, flavonoids, alkaloids, terpenoids and coumarins. However, the difference is the level of each metabolite.

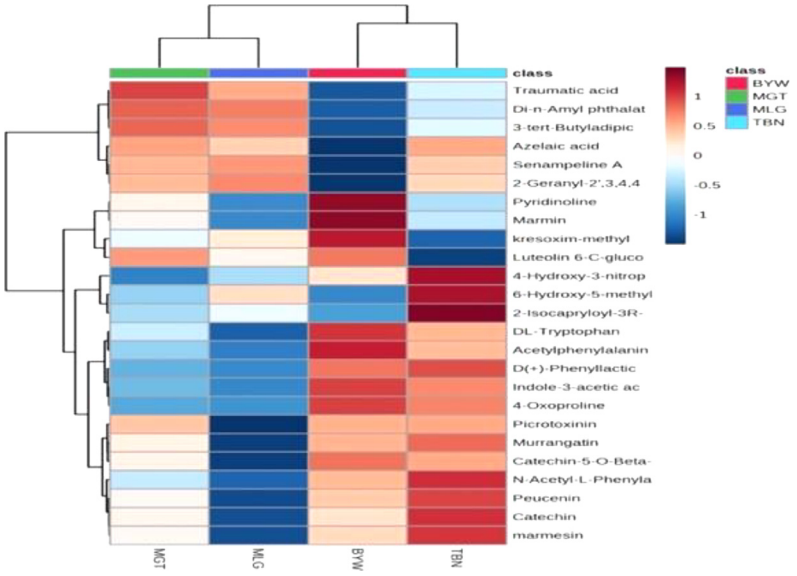

Fig. 4. Heatmap analysis of *A. indica* extracts based on altitude. The altitude can be seen in Table 1.

3.4. Total phenolic content and antioxidant activity of A. indica from different altitudes

The determination of the total phenolic content was carried out using the Folin-Ciocalteu method. In this method, phenolic compounds are oxidized in an alkaline atmosphere by the Folin-Ciocalteu reagent to produce a strong absorption of the blue molybdenum complex at 750 nm. The total phenolic content can be estimated based on the equivalent gallic acid concentration (Pandey et al., 2018).

The total phenolic content of *A. indica* in each region showed different values, range 6.83±0.57 to 10.67±0.42 mg GAE/g dry powder. The ANOVA result showed that differences in the altitude of the growing location significantly affected the total phenolic content at the 5 % significance level (p-value <0.05). The highest to lowest values are BYW, MLG, TBN, and MGT. The BYW area had the highest total phenolic content among other areas. Most of the extracts of *A. indica* from lower altitudes are more efficient in capturing free radicals than samples from higher altitudes (Table 2). The levels of total phenolic content in each region showed significant differences. Environmental and geographical factors such as altitude, soil nutrient content, rainfall, photosynthesis rate, and light intensity are suspected to have a strong influence on the variation of total phenolics and flavonoids in plants (Nataraj et al., 2022; Singh Rana et al., 2020; Rachmadiarti et al., 2019)

Antioxidant activity was determined using the DPPH method. A small value of IC50 is a very strong antioxidant activity and the compound is more effective in inhibiting free radicals (Gangwar et al., 2014). The extract of *A. indica* has an IC50 value ranging from 86.09± 0.48 to 118.46±1.723 μg/mL and is significantly different at the level of 95 % significance (p-value <0.05). Table 2, results of antioxidant analysis: The TBN and BYW samples have lower IC50 values than MGT and MLG. Statistical results show that TBN and BYW are not significantly different between the two, but significantly different from MGT and MLG. The small IC50 value in TBN and BYW is suspected because flavonoid compounds have a higher number and levels of metabolites than in MGT and MLG. Quercetin-7-O-p-coumaroylglucoside and 5,3′-Dihydroxy-6,7,8,4′-tetramethoxyflavanone compounds are suspected to contribute to neutralizing DPPH radicals. This result is also supported by and Lo et al., 2010, which mention the potential of both compounds as antioxidants. Differences in altitude where *A. indica* grows in TBN and BYW are caused by differences in environmental factors such as air temperature, relative air humidity, and air pressure, which affect the metabolites produced.

The higher the total phenolic content, the higher the antioxidant ability to donate electrons to reduce DPPH free radicals. The same results in strawberry and *Coleus forskohlii* plants have high levels of anthocyanin when growing in lowlands, so antioxidant activity is also high. This is due to geographical differences (temperature, humidity, average daily radiation, rainfall, and so on) at different altitudes (Singh Rana et al., 2020; Guerrero-Chavez et al., 2015; Saidan et al., 2015). The total phenol content of *A. indica* in this study was not linear with its antioxidant activity data. This is confirmed by research by Aryal et al. (2019) on wild vegetable plants in Nepal, which shows that the total phenol and antioxidant values are nonlinear. This is due to the antioxidant response in a biological system, which is influenced by various complex factors. Antioxidant activity is not only influenced by phenolic compounds that are antioxidants, but can also be influenced by non-phenolic metabolites in the extract that contribute to antioxidant activity (Saidan et al., 2015; Foti and Amorati, 2009). The results showed that *A. indica* contained non-phenolic groups, including lactone compounds, alkaloids, terpenoids, fatty acids, carboxylic acids, and amino acids. (Foti and Amorati, 2009) explained that these compounds interact with peroxyl radicals through several mechanisms: (i) formal H atom donation from weak X-H bonds (X = O, N), (ii) addition reactions on polyunsaturated systems with the formation of C radicals that are less reactive to O_2, (iii) co-oxidation processes involving rapid cross-termination reactions, and (iv) catalytic quenching of superoxide (O_2-).

Table 4
Correlation coefficients between metabolit in *A. indica* and antioxidant activity.

Metabolite	Antioxidant activity
4-hydroxy-3-nitrophenylacetic acid	−0.7534
3-aminosalicylic acid	−0.7431
4-(L-alanin-3-yl)−2-hydroxy-*cis*,cis-muconate 6-semialdehyde	−0.7015
4-oxoproline	−0.6829
glutaric acid	−0.6773
5,3′-dihydroxy-6,7,8,4′-tetramethoxyflavanone	−0.6541
3′-dihydroxy-6,7,8,4′-tetramethoxyflavanone	−0.6541
D(+) -phenylactic acid	−0.6492
peucenin	−0.6039
catechin	−0.5699

*Correlation is significant at the 0.05 level.

The potential of metabolites in *A. indica* as antioxidants was predicted using Pearson's correlation. (Table 4.) Pearson's correlation showed significant negative correlation between metabolite and antioxidant activity. Metabolites that were negatively correlated with correlation coefficients close to 1 were predicted to contribute to antioxidants. The Pearson's correlation coefficients of the nine compounds ranged from −0.5699 to −0.7534 ($p < 0.05$).

Nine compounds are metabolites that contribute to donate hydrogen atoms to stabilize DPPH free radicals. These nine compounds have been reported to have DPPH free radical scavenging activity (Martinovic et al., 2020; Yppolito et al., 2002; Dugas et al., 2000; Marti-Quijal et al., 2020; Hashem, 2007; Grzesik et al., 2018) while 4-oxoproline and glutaric acid play a role in amino acid metabolism and do not act directly as antioxidants. Of the nine metabolites that contribute as antioxidants, four are phenolic compounds and five are nonphenolic compounds.

4. Conclusions

The composition and concentration of metabolites in *A. indica* were influenced by the altitude of the growing location. *A. indica* from lowland areas had a higher number of identified metabolites and higher antioxidant activity values compared to those from highland areas. Utilizing LC—HRMS-based metabolomics, the metabolite profile showed variation in antioxidant activity at different altitudes. A 83 metabolites were identified in *A. indica* using LC—HRMS. PCA can explain the distribution of metabolites in *A. indica* extracts based on altitude differences. The compounds correlated to be active as antioxidant activity in the extracts of *A. indica* are 4-hydroxy-3-nitrophenylacetic acid; 3-aminosalicylic acid; 4-(L-alanin-3-yl)−2-hydroxy-*cis*,cis-muconate 6-semialdehyde; 4-oxoproline; glutaric acid; 5,3′-dihydroxy-6,7,8,4′-tetramethoxyflavanone; D(+) -phenylactic acid; peucenin and catechin.

Data availability

Data will be available upon request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Elok Kamilah Hayati: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Visualization. **Elok Kamilah Aulanni'am:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Akhmad Sabarudin:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing. **Mohamad Rafi:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing. **Alfi Hudatul Karomah:** Data curation, Investigation, Visualization.

Acknowledgments

This research was funded by World Class University Program 2021 of the Ministry of Education, Culture, Research, and Technology through collaborative research between Brawijaya University and Cluster 2 University Partners. We also thank Advanced Research Laboratory, IPB University, for the facility to use UHPLC-HRMS equipment.

References

Ayele, D.T., Akele, M.L., Melese, A.T., 2022. Analysis of total phenolic contents, flavonoids, antioxidant and antibacterial activities of Croton macrostachyus root extracts. BMC Chem. 16, 30. https://doi.org/10.1186/s13065-022-00822-0.

Batubara, I., Wahyuni, W., Firdaus, I., 2016. Utilization of anting-anting (Acalypha indica) leaves as antibacterial. IOP Conf. Ser. Earth Environ. Sci. 31, 012038. https://doi.org/10.1088/1755-1315/31/1/012038.

Chekuri, S., Panjala, S., Anupalli, R.R., 2017. Cytotoxic activity of Acalypha indica L. hexane extract on breast cancer cell lines (MCF-7). J. Phytopharm. 6, 264–268. https://doi.org/10.31254/phyto.2017.6502.

Chekuri, S., Vyshnava, S.S., Somisetti, S.L., Cheniya, S.B.K., Gandu, C., Anupalli, R.R., 2023. Isolation and anticancer activity of quercetin from Acalypha indica L. against breast cancer cell lines MCF-7 and MDA-MB-231. 3 Biotech 13, 289. https://doi.org/10.1007/s13205-023-03705-w.

Cirak, C., Radusiene, J., Jakstas, V., Ivanauskas, L., Seyis, F., Yayla, F., 2016. Secondary metabolites of seven Hypericum species growing in Turkey. Pharm. Biol. 54, 2244–2253. https://doi.org/10.3109/13880209.2016.1152277.

Dugas, A.J., Castañeda-Acosta, J., Bonin, G.C., Price, K.L., Fischer, N.H., Winston, G.W., 2000. Evaluation of the total peroxyl radical-scavenging capacity of flavonoids: structure-activity relationships. J. Nat. Prod. 63, 327–331. https://doi.org/10.1021/np990352n.

Fawzy, G.A., Al-Taweel, A.M., Perveen, S., Khan, S.I., Al-Omary, F.A., 2017. Bioactivity and chemical characterization of Acalypha fruticosa Forssk. growing in Saudi Arabia. Saudi Pharm. J. 25, 104–109. https://doi.org/10.1016/j.jsps.2016.05.004.

Foti, M., Amorati, R., 2009. Non-phenolic radical-trapping antioxidants. J. Pharm. Pharmacol. 61, 1435–1448. https://doi.org/10.1211/jpp/61.11.0002.

Gangwar, M., Gautam, M.K., Sharma, A.K., Tripathi, Y.B., Goel, R.K., Nath, G., 2014. Antioxidant capacity and radical scavenging effect of polyphenol rich mallotus philippenensis fruit extract on human erythrocytes: an in vitro study. Sci. World J. 13.

Giupponi, L., Leoni, V., Pavlovic, R., Giorgi, A., 2020. Influence of altitude on phytochemical composition of hemp inflorescence: a metabolomic approach. Mol. Basel Switz. 25, E1381. https://doi.org/10.3390/molecules25061381.

Grzesik, M., Naparło, K., Bartosz, G., Sadowska-Bartosz, I., 2018. Antioxidant properties of catechins: comparison with other antioxidants. Food Chem. 241, 480–492. https://doi.org/10.1016/j.foodchem.2017.08.117.

Guerrero-Chavez, G., Scampicchio, M., Andreotti, C., 2015. Influence of the site altitude on strawberry phenolic composition and quality. Sci. Hortic. 192. https://doi.org/10.1016/j.scienta.2015.05.017.

Hashem, F.A., 2007. Investigation of free radical scavenging activity by ESR for coumarins isolated from Tecoma radicans. J. Med. Sci. 7, 1027–1032. https://doi.org/10.3923/jms.2007.1027.1032.

Hayati, E.K., Jannah, A., Ningsih, R., 2012. Identifikasi senyawa dan aktivitas antimalaria in vivo ekstrak etil asetat tanaman anting-anting (Acalypha Indica L.). Molekul 7, 20. https://doi.org/10.20884/1.jm.2012.7.1.103.

Khorasani Esmaeili, A., Mat Taha, R., Mohajer, S., Banisalam, B., 2015. Antioxidant activity and total phenolic and flavonoid content of various solvent extracts from *in vivo* and *in vitro* grown *trifolium pratense* L. (red clover). BioMed. Res. Int. 2015, e643285. https://doi.org/10.1155/2015/643285.

Klau, M., Rohaeti, E., Rafi, M., Artika, M., Ambarsari, L., Nurcholish, W., 2022. Metabolite profiling of curcuma zanthorrhiza varieties grown in different regions using UHPLC-Q-orbitrap-HRMS and chemometrics analysis. Biointerface Res. Appl. Chem. 13, 26. https://doi.org/10.33263/BRIAC131.026.

Li, Y., Quan, H., Liang, L., Yang, T., Feng, L., Mao, X., Wang, Y., 2021. Nontargeted metabolomics reveals the discrimination of Cyclocarya paliurus leaves brewed by different methods. Food Res. Int. 142, 110221. https://doi.org/10.1016/j.foodres.2021.110221.

Li, Y., Xi, H., Feng, L., Liang, L., Yang, T., Mao, X., Wang, Y., 2022. Nontargeted metabolomics coupled with multivariate modelling techniques for discrimination of Cyclocarya paliurus (Batal.) Ijinskaja leaves from different geographic altitudes. Anal. Method. 14. https://doi.org/10.1039/D2AY00292B.

Lo, Y.-H., Pan, M.-H., Li, S., Yen, J.-H., Kou, M.-C., Ho, C.-T., Wu, M.-J., 2010. Nobiletin metabolite, 3′,4′-dihydroxy-5,6,7,8-tetramethoxyflavone, inhibits LDL oxidation and down-regulates scavenger receptor expression and activity in THP-1 cells. Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipid. 1801, 114–126. https://doi.org/10.1016/j.bbalip.2009.10.002.

Manukyan, A., 2019. Secondary metabolites and their antioxidant capacity of Caucasian endemic thyme (Thymus transcaucasicus Ronn.) as affected by environmental stress. J. Appl. Res. Med. Aromat. Plant. 13, 100209. https://doi.org/10.1016/j.jarmap.2019.100209.

Martinovic, N., Polak, T., Ulrih, N.P., Abramovič, H., 2020. Mustard seed: phenolic composition and effects on lipid oxidation in oil, oil-in-water emulsion and oleogel. Ind. Crops Prod. 156, 112851. https://doi.org/10.1016/j.indcrop.2020.112851.

Marti-Quijal, F.J., Tornos, A., Príncep, A., Luz, C., Meca, G., Tedeschi, P., Ruiz, M.-J., Barba, F.J., 2020. Impact of fermentation on the recovery of antioxidant bioactive compounds from sea bass byproducts. Antioxidants 9, 239. https://doi.org/10.3390/antiox9030239.

Meybeck, M., Green, P., Vörösmarty, C., 2001. A new typology for mountains and other relief classes. Mt. Res. Dev. 21, 34–45. https://doi.org/10.1659/0276-4741(2001)021[0034:ANTFMA]2.0.CO;2.

Nahrstedt, A., Kant, J.-D., Wray, V., 1982. Acalyphin, a cyanogenic glucoside from Acalypha indica. Phytochemistry 21, 101–105. https://doi.org/10.1016/0031-9422(82)80022-8.

Nataraj, N., Hussain, M., Ibrahim, M., Hausmann, A., Rao, S., Kaur, S., Khazir, J., Mir, B., Olsson, S., 2022. Effect of altitude on volatile organic and phenolic compounds of artemisia brevifolia wall ex Dc. from the Western Himalayas. Front. Ecol. Evol. 10, 864728. https://doi.org/10.3389/fevo.2022.864728.

Pandey, G., Khatoon, S., Pandey, M.M., Rawat, A.K.S., 2018. Altitudinal variation of berberine, total phenolics and flavonoid content in Thalictrum foliolosum and their correlation with antimicrobial and antioxidant activities. J. Ayurv. Integr. Med. 9, 169–176. https://doi.org/10.1016/j.jaim.2017.02.010.

Pasumarthi, B., Sunita, K., 2018. Phytochemical investigation and in vitro antimalarial activity of Acalypha indica (L.) and Cocculus hirsutus (L.) from Prakasam District, Andhra Pradesh, India. Biomed. Pharmacol. J. 11, 2123–2134. https://doi.org/10.13005/bpj/1592.

Ponphaiboon, J., Krongrawa, W., Aung, W.W., Chinatangkul, N., Limmatvapirat, S., Limmatvapirat, C., 2023. Advances in natural product extraction techniques, electrospun fiber fabrication, and the integration of experimental design: a comprehensive review. Molecules 28, 5163. https://doi.org/10.3390/molecules28135163.

Rachmadiarti, F., Kusuma Dewi, S., Asri, M., Soegianto, A., 2019. Total phenolic and flavonoid contents of Elephantopus scaber and Ageratum conyzoides (Asteraceae) leaves extracts from various altitude habitats. Ecol. Environ. Conserv. 25.

Rafi, M., Septaningsih, D.A., Karomah, A.H., Lukman, L., Prajogo, B., Amran, M.B., Rohman, A., 2021. Inhibition of ?-glucosidase activity, metals content, and phytochemical profiling of Andrographis paniculata from different geographical origins based on FTIR and UHPLC-Q-Orbitrap HRMS metabolomics. Biodiversit. J. Biol. Divers. 22. https://doi.org/10.13057/biodiv/d220359.

Rashid, A., Ali, V., Khajuria, M., Faiz, S., Gairola, S., Vyas, D., 2021. GC–MS based metabolomic approach to understand nutraceutical potential of Cannabis seeds from two different environments. Food Chem. 339, 128076. https://doi.org/10.1016/j.foodchem.2020.128076.

Ravi, S., Shanmugam, B., Subbaiah, G.V., Prasad, S.H., Reddy, K.S., 2017. Identification of food preservative, stress relief compounds by GC–MS and HR-LC/Q-TOF/MS; evaluation of antioxidant activity of Acalypha indica leaves methanolic extract (in vitro) and polyphenolic fraction (in vivo). J. Food Sci. Technol. 54, 1585–1596. https://doi.org/10.1007/s13197-017-2590-z.

Rivero, R.M., Ruiz, J.M., García, P.C., López-Lefebre, L.R., Sánchez, E., Romero, L., 2001. Resistance to cold and heat stress: accumulation of phenolic compounds in tomato and watermelon plants. Plant Sci. Int. J. Exp. Plant Biol. 160, 315–321. https://doi.org/10.1016/s0168-9452(00)00395-2.

Sahukari, R., Punabaka, J., Bhasha, S., Ganjikunta, V.S., Kondeti Ramudu, S., Kesireddy, S.R., Ye, W., Korivi, M., 2021. Phytochemical profile, free radical scavenging and anti-inflammatory properties of Acalypha Indica root extract: evidence from in vitro and in vivo studies. Mol. Basel Switz. 26, 6251. https://doi.org/10.3390/molecules26206251.

Saidan, N.H., Hamil, M.S.R., Memon, A.H., Abdelbari, M.M., Hamdan, M.R., Mohd, K.S., Majid, A.M.S.A., Ismail, Z., 2015. Selected metabolites profiling of Orthosiphon stamineus Benth leaves extracts combined with chemometrics analysis and correlation with biological activities. BMC Complement. Altern. Med. 15, 350. https://doi.org/10.1186/s12906-015-0884-0.

Sanli, A., Karadogan, T., 2016. Geographical impact on essential oil composition of endemic kundmannia anatolica Hub.-Mor. (Apiaceae). Afr. J. Tradit. Complement. Altern. Med. 14, 131–137. https://doi.org/10.21010/ajtcam.v14i1.14.

Sanseera, D., Niwatananun, W., Liawruangrath, B., Liawruangrath, S., Baramee, A., Trisuwan, K., Pyne, S.G., 2015. Antioxidant and anticancer activities from aerial parts of Acalypha indica Linn. CMU. J. Nat. Sci 11, 157.

Singh Rana, P., Saklani, P., Chandel, C., 2020. Influence of altitude on secondary metabolites and antioxidant activity of coleus forskohlii root extracts. Res. J. Med. Plant. 14, 43–52. https://doi.org/10.17311/rjmp.2020.43.52.

Suleiman, M.H.A., ALaerjani, W.M.A., Mohammed, M.E.A., 2020. Influence of altitudinal variation on the total phenolic and flavonoid content of Acacia and Ziziphus honey. Int. J. Food Prop. 23, 2077–2086. https://doi.org/10.1080/10942912.2020.1842445.

Suresh, M., Alfonisan, M., Alturaiki, W., Al Aboody, M.S., Alfaiz, F.A., Premanathan, M., Vijayakumar, R., Umamagheswari, K., Ghamdi, S.A., Alsagaby, S.A., 2020. Investigations of bioactivity of Acalypha indica (L.), Centella asiatica (L.) and Croton

bonplandianus (Baill) against multidrug resistant bacteria and cancer cells. J. Herb. Med., 100359. https://doi.org/10.1016/j.hermed.2020.100359.

Syahiran, N., Saidin, S., Zulkifli, R., Muhamad, I., Ya'akob, H., Nur, H., 2017. A review of Acalypha indica L. (Euphorbiaceae) as traditional medicinal plant and its therapeutic potential. J. Ethnopharmacol. 207. https://doi.org/10.1016/j.jep.2017.06.019.

Syahiran, N., Zulkifli, R., Muhamad, I., Ya'akob, H., Nur, H., Shariff, A., Saidin, S., 2018. Preliminary study of potential herbal tea, acalypha indica and comparison with domestic tea in Malaysia market. Food Sci. Technol. 6, 41–45. https://doi.org/10.13189/fst.2018.060105.

Umar, A.H., Ratnadewi, D., Rafi, M., Sulistyaningsih, Y.C., 2021. Untargeted metabolomics analysis using FTIR and UHPLC-Q-Orbitrap HRMS of two curculigo species and evaluation of their antioxidant and α-glucosidase inhibitory activities. Metabolites 11, 42. https://doi.org/10.3390/metabo11010042.

Yaylı, N., Tosun, G., Yaylı, B., Gündoğanc, Z., Coşkunçelebic, K., Karaoğlu, Ş.A., 2016. Altitude variation in the composition of essential oils, fatty acid methyl esters, and antimicrobial activities of two subspecies of primula vulgaris grown in Turkey. Nat. Prod. Commun. 11,. https://doi.org/10.1177/1934578×1601101020 1934578×1601101020.

Yppolito, R., Pappano, N., Debattista, N., Miskoski, S., Bertolotti, S.G., García, N.A., 2002. On the antioxidant properties of therapeutic drugs: quenching of singlet molecular oxygen by aminosalicylic acids. Redox Rep. 7, 229–233. https://doi.org/10.1179/135100002125000613.

Zantar, S., Garrouj, D.E., Pagán, R., Chabi, M., Laglaoui, A., Bakkali, M., Zerrouk, M.H., 2015. Effect of harvest time on yield, chemical composition, antimicrobial and antioxidant activities of thymus vulgaris and mentha pulegium essential oils. Eur. J. Med. Plants 69–77. https://doi.org/10.9734/EJMP/2015/17513.