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Original Article

UHPLC-Q-Orbitrap-HRMS-based untargeted metabolomics reveal metabolites change in *Justicia gendarussa* and its antioxidant capacity at different doses of nitrogen fertilizer

Metabolômica não direcionada baseada em UHPLC-Q-Orbitrap-HRMS revela mudança de metabólitos em *Justicia gendarussa* e sua capacidade antioxidante em diferentes doses de fertilizante nitrogenado

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Abstract

This study delves into the antioxidant potential of *Justicia gendarussa*, commonly known as gendarussa, and its response to varying doses of nitrogen fertilizer. Gendarussa exhibits the potential for antioxidant activity. The diverse ecological conditions in which it thrives may influence its biological activity and lead to inconsistent production of secondary metabolites. Nitrogen, a pivotal factor in secondary metabolite production in plants, has become a focal point of this research. This research aims to determine the optimal nitrogen fertilizer dose on gendarussa antioxidant capacity and metabolites using a metabolomics approach. Employing a randomized block design for cultivation, the investigation revealed that a maximum harvest weight of 10.9 g/aerial parts of the plant was achieved with 270 kg/ha of nitrogen fertilizer. This study explored the DPPH, ABTS, FRAP, and CUPRAC assays of antioxidant effect, and found insignificant differences between the various nitrogen treatments. UHPLCQ-Orbitrap HRMS was employed to identify 30 metabolites in positive and 18 in negative ionization modes. Gendarusin A, a major metabolite in gendarussa, is identified in both positive and negative ionization. PCA and heatmap analysis successfully categorized these metabolites in the aerial parts of gendarussa at different nitrogen fertilizer dosages. Based on the metabolomics approach, variations in nitrogen fertilizer made metabolites at doses of 90 kg/ha had higher relative concentrations of metabolites compared to doses of 180 kg/ha and 270 kg/ha. So, 90 kg/ha are the optimal nitrogen fertilizer dose for cultivation and utilization strategies.

Keywords: cultivation, harvested weight, heatmap, gendarussa, PCA.

Resumo

Este estudo investiga o potencial antioxidante da *Justicia gendarussa*, comumente conhecida como gendarussa, e sua resposta a diferentes doses de fertilizante nitrogenado. A gendarussa apresenta potencial para atividade antioxidante. As diversas condições ecológicas em que prospera podem influenciar a sua atividade biológica e levar à produção inconsistente de metabólitos secundários. O nitrogênio, um fator crucial na produção de metabólitos secundários nas plantas, tornou-se um ponto focal desta pesquisa. Esta investigação tem como objetivo determinar a dose ótima de fertilizante nitrogenado na capacidade antioxidante e metabólitos de gendarussa utilizando uma abordagem metabolômica. Com um desenho de blocos casualizados para o cultivo, a investigação revelou que um peso máximo de colheita de 10,9 g/partes aéreas da planta foi alcançado com 270 kg/ha de fertilizante nitrogenado. Este estudo explorou os ensaios DPPH, ABTS, FRAP e CUPRAC de efeito antioxidante e encontrou diferenças insignificantes entre os vários tratamentos com nitrogênio. O UHPLC-Q-Orbitrap HRMS foi empregado para identificar 30 metabólitos nos modos de ionização positivos e 18 nos modos de ionização negativo. A *gendarusina A*, um metabólito importante da gendarussa, é identificada tanto na ionização positiva quanto na negativa. A análise de PCA e mapa de calor categorizou com sucesso esses metabólitos nas partes aéreas de gendarussa em diferentes dosagens de fertilizantes nitrogenados. Com base na abordagem metabolômica, as variações no fertilizante nitrogenado produziram metabólitos em doses de 90 kg/ha e tiveram concentrações relativas de metabólitos mais elevadas em comparação com doses de 180 kg/ha e 270 kg/ha. Portanto, 90 kg/ha é a dose ideal de fertilizante nitrogenado para estratégias de cultivo e utilização.

Palavras-chave: cultivo, gendarussa, mapa de calor, peso colhido, PCA.

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1. Introduction

Gendarussa (*Justicia gendarussa*) is a plant belongs to Acanthaceae family, that has medicinal properties. This plant is native to various Asian countries, including Sri Lanka, India, Malaysia, and Indonesia, and has been used medicinally for centuries (Ratih et al., 2019). The Sentani people in Papua, Indonesia use gendarussa as a natural contraceptive for males. Gendarussa is also frequently used in traditional Indian and Chinese medicine to treat fever, rheumatism, arthritis, headaches, earaches, muscle aches, respiratory problems, and digestive problems (Putri et al., 2020). Secondary metabolites found in gendarussa include phenolics, alkaloids, steroids, and terpenoids (Widodo et al., 2019).

The secondary metabolite of gendarussa possess antioxidant properties. Antioxidants compounds are metabolites that can delay, inhibit or scavenge free radicals and reduce the damage caused by oxidative stress (Chaudhary et al., 2015). Free radicals induced by oxidative stress have been known to be involved in various disease such as neurodegenerative disorders, diabetes mellitus, cardiovascular diseases, rheumatoid arthritis, cataracts development, respiratory diseases, and various types of cancer (Di Meo and Venditti, 2020). Using the DPPH method from previous research has extracted gendarussa leaves, found that the ethanol extract had the highest antioxidant activity, with an IC_{50} value of 32 µg/mL (Kuber, 2021).

Gendarussa can thrive as a shrub in the wild, particularly in forested setting and along river dams (Akpriyanti et al., 2017). However, uncontrolled growth conditions can cause variable secondary metabolite production. Therefore, plant cultivation is required to maintain the secondary metabolite content of gendarussa. The primary metabolites can act as precursors to affect secondary metabolite production. Plant development and growth can affect the quantity of primary metabolite production, which affects secondary metabolites production. The presence of light, nutrients, soil moisture, and substrate affects the growth and development of plants (Weng, 2013).

Nitrogen is one of the most essential nutrients that contributes significantly to plant growth (Warganegara et al., 2017). Nitrogen fertilizer have increasingly used to get a higher crop yields over the past three decades. Nitrogen is an essential component of chlorophyll, the primary light energy absorber required for photosynthesis. Photosynthesis will increase the accumulation of primary metabolites, which can contribute to producing secondary metabolites via the shikimic acid, phenylpropanoid, and other pathways (Rai, 2018). Changes in metabolite accumulation under the effect of nitrogen fertilizer can be observed using a metabolomics approach.

Research by Shi et al. (2019) reported that adding nitrogen fertilizer in the 42.5-127.5/g in *Lycium barbarum* plant significantly affected the chemical content, such as flavonoids, amino acids, and polysaccharides. In addition, this research succeeded in identifying 612 metabolites from *L. barbarum* fruit, and a total of 53 metabolites were significantly affected by applying nitrogen fertilizer in relative concentrations using a metabolomics approach. This research aims to determine the optimal nitrogen fertilizer dose on gendarussa antioxidant capacity and metabolites using a metabolomics approach. The benefit obtained from this research is providing information on the effect of different doses of nitrogen fertilizer on changes in gendarussa metabolites using a metabolomics approach. Then, this research can be helpful for industries engaged in developing herbal medicines in cultivation of gendarussa.

2. Materials and Methods

2.1. Cultivation and sample preparation of gendarussa

Biopharmaca Cultivation Conservation Unit (UKBB) Cikabayan Gardens Block C, IPB Dramaga Bogor Campus (6°3'49"S and 106°42'57"E) is 240 m above sea level and is where plant cultivation takes place. The cultivation experiment was designed using a Randomized Block Design (RBD) with four levels of N fertilizer doses: A (0N kg/ha), B (90N kg/ha), C (180N kg/ha), and D (270N kg/ha). All treatments received 100 kg/ha of P_2O_5 fertilizer, 150 kg/ha of KCl, and 20 tons/ha of cow manure. The fertilizer is mixed and distributed in polybags (10x15 cm) around plants grown for one month, then transferred to larger polybags (30x30 cm) for treatment. There were four treatments, and each treatment was conducted three times for a total of twelve experimental units. Each experimental unit consisted of 10 plants, totaling 120. The harvest is accomplished by pruning up to 15 cm from the ground. The leaves and stems of gendarussa, which had been fertilized for four months, were harvested. In addition, the sample's dried weight was determined by drying it in an oven at 45°C for 2 x 24 hours.

2.2. Extraction of gendarussa

The extraction method was 1 g of a sample of aerial parts (leaves and stems) of gendarussa in powder form was put into an Erlenmeyer flask, then added 10 mL of ethanol p.a with a ratio of 1:10. Samples were macerated for 24 hours in a dark room at room temperature. Furthermore, the sample is filtered using filter paper, and the resulting filtrate is sample extract. Extraction was carried out with two repetitions (diplo).

2.3. Antioxidant capacity

The DPPH method added 100 µL of the sample solution from the gendarussa extract to a 96-well clear polystyrene microplate, then 100 µL of 125 µM DPPH. Samples were incubated in a dark place at room temperature for 30 minutes. Absorbance was measured with a microplate nano spectrophotometer at a wavelength of 515 nm (Batubara et al., 2020).

The ABTS method added 20 µL of the extracted sample to 180 µL of ABTS reagent on a 96-well clear polystyrene microplate. Then, the mixture was incubated for 6 minutes. The absorbance of the solution was measured using a microplate nano spectrophotometer at a wavelength of 734 nm (Nurcholis et al., 2022).

The FRAP method added 10 µL samples of gendarussa extract were put into a 96-well clear polystyrene microplate,

then added 145 µL FRAP and then incubated in a dark place at room temperature for 4 minutes. The absorbance of the sample was read at a wavelength of 593 nm using a microplate nano spectrophotometer (Batubara et al., 2020).

The CUPRAC method added 50 µL of the extracted sample added to 50 µL of 10^{-2} M $CuCl_2$ solution, 50 µL of NH_4Ac buffer 1 M with pH 7, and 50 µL of 7.5×10^{-3} M neokuproin on a microplate. Then the mixture was incubated for 30 minutes at room temperature in a dark room. Then, the absorbance of the mixture was calculated at a wavelength of 450 nm using a microplate nano spectrophotometer. All used calibration curves with standard trolox solution with various concentrations. The results are equivalent to µmol trolox equivalent per gram dry weight (µmol TE/g dry weight) (Nurcholis et al., 2022).

2.4. Identification of metabolites using UHPLC-Q-Orbitrap HRMS

Metabolites were identified using the Vanquish UHPLC-Q Exactive Plus Orbitrap-High Resolution Mass Spectrometer. A 5 mg of sample was dissolved in 1 mL of LC-MS grade methanol, then sonicated for 10 minutes and filtered through a 0.2 µm filter membrane. Separation chromatography was performed using an AccucoreTM Vanquish C18+ column (1.5 µm, 120 Å, 2.1×100 mm). The mobile phases used were (A) water and 0.1% formic acid and (B) acetonitrile and 0.1% formic acid. The gradient system used is shown in Table 1. The column temperature is 30°C. The ionization source used is electrospray ionization (ESI) with a spray voltage of 3,800 volts (ESI+) and 3,200 volts (ESI-). The mass analyzer is a quadrupole orbitrap (tandem MS/MS). m/z range of 100-1,500 Da. The ionization energies used were 18, 35 and 53 eV. An injection volume of 2 µL. Identification of metabolites was carried out using the data in the form of RAW processed with MzMine software. Confirmation of the compound via MS and MS-MS was carried out based on the custom database and the MetFrag webserver.

2.5. Data analysis

The harvest weight data and antioxidant capacity were analyzed using the Minitab software with One Way ANOVA analysis, expressed with a p-value <0.05. Then Tukey's post hoc test was carried out. The analysis was carried out to find out the treatment that had the most effect on all the test results. All tests were visualized using GraphPad Prism software. Before multivariate analysis for UHPLC-Q-Orbitrap HRMS data, pre-processing of the chromatogram with correlation optimized warping (COW) using The Unscrambler X was carried out by determining the reference chromatogram, finding the optimum segment length and slack, and evaluating the shape of the chromatogram. The similarity index carries out the determination of the reference chromatogram. The chromatogram with the highest similarity index was used as a reference chromatogram. Optimum segment length and slack search with a ratio of 5:1. The comparison starts from 5:1 to 100:20. The highest chromatogram similarity index was chosen as the data to be used for multivariate analysis. PCA and heatmap were performed using the MetaboAnalyst webserver.

Table 1. Gradient system UHPLC-Q-Orbitrap HRMS.

Time (minute)	Flow rate (mL/minute)	%A	%B
0.0	0.2	95.0	5.0
1.0	0.2	95.0	5.0
18.0	0.2	55.0	45.0
30.0	0.2	5.0	95.0
33.0	0.2	5.0	95.0
33.1	0.2	95.0	5.0
35.0	*stop run*	*stop run*	*stop run*

3. Results

3.1. Cultivation

The research results shown in each plant's final dry weight yield (Figure 1) showed significantly different results from one-way ANOVA at the 95% confidence level. Post hoc follow-up tests using Tukey showed that the treatment with a nitrogen fertilizer dose of 270 kg/ha had the highest weight of 10.9 grams/plant, which is the ideal condition for plant cultivation regarding the biomass produced.

3.2. Antioxidant capacity

Antioxidant capacity was calculated using trolox as the standard curve for all methods. All aerial parts extract tests of gendarussa using the DPPH, ABTS, FRAP, and CUPRAC methods were not significantly different from the one-way ANOVA test, so they were not continued with the post hoc test. The highest antioxidant capacity among all methods was found in 0N kg/ha treatment with the DPPH, ABTS, FRAP, and CUPRAC methods, whose values were respectively 6.53, 33.6, 16.7, and 49 µmol TE/g dry weight (Figure 2).

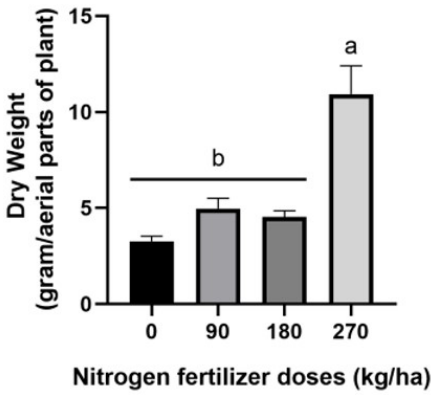

Figure 1. Dry weight of each plant of gendarussa. Each value is presented as the mean ± standard error of mean (SEM). The mean value in each column marked with a different letter is significantly different at p <0.05 or the 95% confidence level.

3.3. Metabolites identification of gendarussa extract at different nitrogen doses

The chromatogram positive and negative ionization modes produced for aerial parts extract of gendarussa with different doses of nitrogen fertilizer (Figure 3a and 3b). A total of 30 and 18 metabolites were identified in the positive and negative ionization mode, respectively. The distribution of identified metabolites can be seen in the Venn diagram (Figure 3c and 3d), with detailed results of identifying metabolites attached in Table 2 and Table 3. The results of compound identification using the UHPLC-Q-Orbitrap HRMS are putative.

3.4. PCA and heatmap of gendarussa extract metabolites at different nitrogen doses

PCA was used for all data from the peak intensity, while the heatmap was used for metabolites identified through the peak area. Before PCA, the resulting chromatogram was pre-processed in the form of COW. PCA was performed for both ionization modes (Figure 4). A heatmap using peak area data is the final visualization to explain the relative concentration metabolites identified in the extract of aerial parts of gendarussa treated with different nitrogen fertilizer doses (Figure 5).

4. Discussion

The soil conditions used for plant cultivation had a pH of 4.2, and the main nutrient concentration of carbon was 1.33%. The nutrient element nitrogen as a research factor in the treatment of plant cultivation was 0.21%. These nutrients are classified as low fertility (Rai, 2018). Therefore, additional fertilization was carried out in crop cultivation, such as KCl fertilizer, SP-36 fertilizer, and

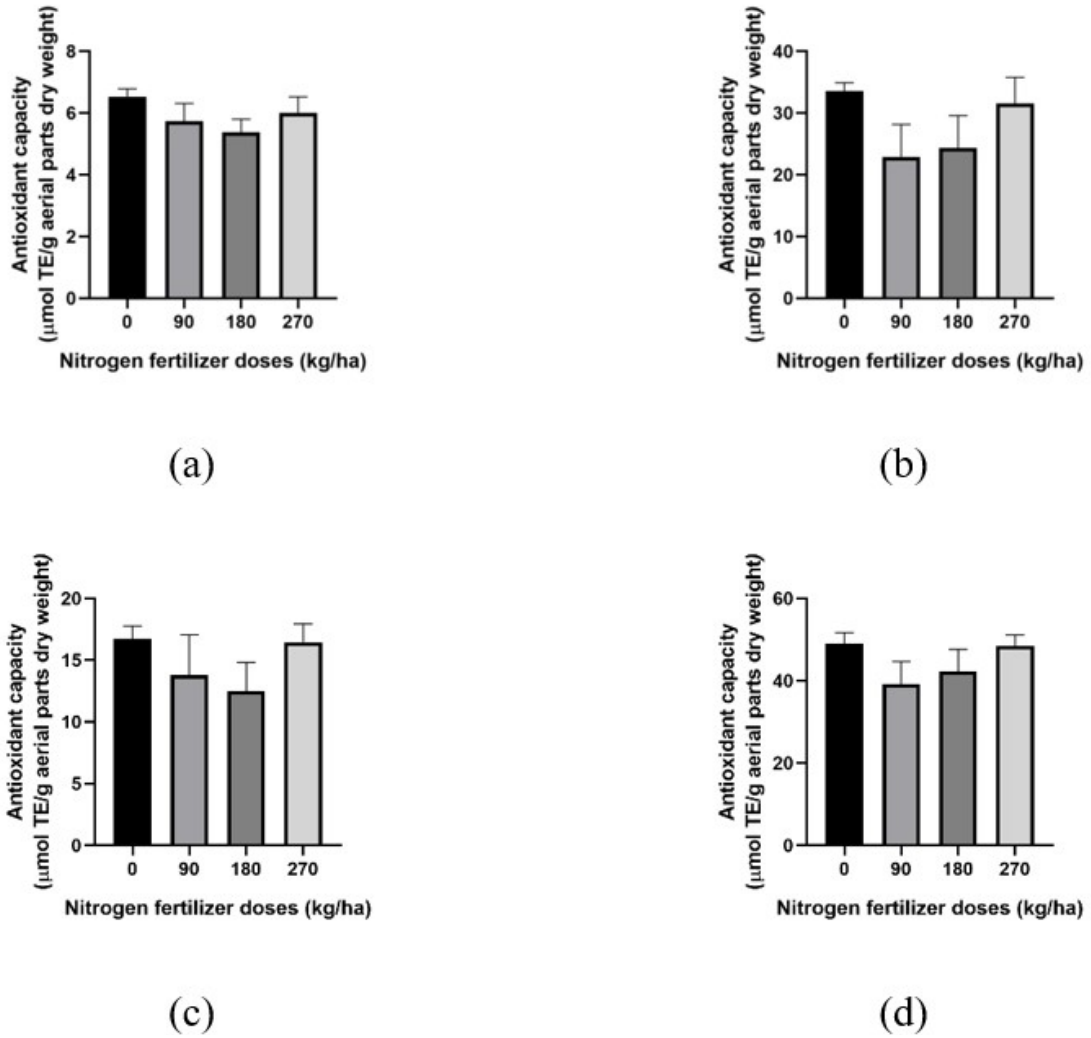

Figure 2. Antioxidant capacity of aerial parts extract of gendarussa (a) DPPH (b) ABTS (c) FRAP (d) CUPRAC. Each value is presented as the mean ± standard error of mean (SEM). The mean value in each column marked with a different letter is significantly different at $p < 0.05$ or the 95% confidence level.

Table 2. Identified metabolites with positive ionization in extracts of aerial parts of gendarussa.

No	Rt (minute)	Name	Molecular Formula	MS and MS-MS	Group	A	B	C	D
1	0.0246	2-methoxy-4-vinylphenol	$C_9H_{10}O_2$	$[M+H]^+$ 151.0350	Phenolic	√	√	√	
2	0.0666	Umbelliferone	$C_9H_6O_3$	$[M+H]^+$ 162.9842	Coumarin		√	√	√
3	0.1745	Syringic acid	$C_9H_{10}O_5$	$[M+H]^+$ 199.0353	Phenolic	√			
4	0.2524	Protocatechuic acid	$C_7H_6O_4$	$[M+H]^+$ 154.9896	Phenolic	√	√	√	√
5	0.9595	Homovanillyl alcohol	$C_9H_{12}O_3$	$[M+H]^+$ 169.0465	Phenolic		√	√	
6	1.0297	Chinensinaphthol	$C_{22}H_{18}O_7$	$[M+H]^+$ 381.0780	Lignan		√	√	
7	1.0645	Justicidone	$C_{20}H_{12}O_7$	$[M+H]^+$ 365.1039	Lignan	√	√	√	√
8	1.3368	Coumarin	$C_9H_6O_2$	$[M+H]^+$ 147.0438	Coumarin		√	√	
9	1.3479	Brazoide C	$C_{18}H_{26}N_2O_9$	$[M+H]^+$ 415.1699	Alkaloid	√	√	√	√
10	2.8528	Cinnamic acid	$C_9H_8O_2$	$[M+H]^+$ 149.0229 $[C_3H_5O]^+$ 57.0341 $[C_5H_5]^+$ 65.0392 $[C_7H_7]^+$ 91.0545 $[C_7H_5O_2]^+$ 121.0282	Phenolic	√	√		
11	6.0677	(8S,9R,9aS, 10aR)-5-Oxo-9-vinyl-1,2,3,8,9,9a,10,10aoctahydro-5H-imidazo[1,2-a] pyrano [4,3-d] pyridin-8-yl-α-D-glucopyranoside	$C_{18}H_{26}N_2O_8$	$[M+H]^+$ 399.1758	Alkaloid	√	√	√	√
12	6.4924	Rapanone	$C_{19}H_{30}O_4$	$[M+H]^+$ 323.1709 $[C_{10}H_9O_3]^+$ 177.0536	Quinone	√	√	√	
13	7.6232	Justridusamide A	$C_{17}H_{23}NO_8$	$[M+H]^+$ 370.1478	Nitrogenous compound	√	√	√	√
14	9.0710	5H,6H-quinindolin-11-one	$C_{15}H_{10}N_2O$	$[M+H]^+$ 235.1069	Alkaloid		√	√	
15	9.0817	1-Deoxy-1-{methyl[(2-oxo-2,3,4,5-tetrahydro-1H-1- benzazepin-7-yl) carbonyl] amino}hexitol	$C_{18}H_{26}N_2O_7$	$[M+H]^+$ 383.1802 $[C_4H_6NO]^+$ 84.0446 $[C_7H_8N]^+$ 106.0654 $[C_{10}H_{10}N]^+$ 144.0810 $[C_{12}H_{13}N_2O_2]^+$ 217.0953 $[C_{12}H_{15}N_2O_3]^+$ 235.1058	Alkaloid	√	√	√	√
16	9.1026	6-C-glusosyl-8-C-arabinosyl apigenin or Schaftoside	$C_{26}H_{28}O_{14}$	$[M-H]^+$ 565.1499	Flavonoid		√	√	
17	10.2072	Gendarusin A	$C_{25}H_{26}O_{13}$	$[M+H]^+$ 535.1432	Flavonoid	√	√	√	√
18	10.5471	3,4-Dihydroxy-6-(N-ethylamino) benzamide	$C_9H_{12}N_2O_3$	$[M+H]^+$ 197.1141	Phenolic	√	√	√	√
19	10.8425	Gendarusin B	$C_{25}H_{26}O_{13}$	$[M+H]^+$ 535.1430	Flavonoid	√	√	√	√
20	11.6116	6-{[(Benzyloxy) carbonyl]amino} -6-deoxy-1,2-O-isopropylidene-α-D-glucofuranose	$C_{17}H_{23}NO_7$	$[M+H]^+$ 354.1502	Nitrogenous compound	√	√	√	
21	18.4322	Sesamol	$C_7H_6O_3$	$[M+H]^+$ 139.0287	Phenolic	√			
22	21.6468	6-(2-Ethyl-5- hydroxy-hexoxy)-6-oxohexanoic acid	$C_{14}H_{26}O_5$	$[M+H]^+$ 275.1962	Fatty Acid			√	
23	24.4156	Palmitic acid	$C_{16}H_{32}O_2$	$[M+H]^+$ 257.1882 $[C_4H_9]^+$ 57.0702 $[C_5H_{11}]^+$ 71.0860 $[C_{12}H_{23}O]^+$183.1733	Fatty Acid		√		

Note: A = 0N kg/ha; B = 90N kg/ha; C = 180N kg/ha; D = 270N kg/ha.

Table 2. Continued...

No	Rt (minute)	Name	Molecular Formula	MS and MS-MS	Group	A	B	C	D
24	24.7143	(9 Z)1,9-heptadecadiene-4, 6-diyne-3,8,11-triol	$C_{17}H_{24}O_3$	$[M+H]^+$ 277.2160 $[C_5H_7]^+$ 67.0545 $[C_6H_7]^+$ 79.0544 $[C_{13}H_{19}O_2]^+$ 207.1370 $[C_{15}H_{23}O_2]^+$ 235.1704	Polyacetylenes	√	√	√	√
25	25.6183	12-Oxo-phytodienoic acid	$C_{18}H_{28}O_3$	$[M+H]^+$ 293.1936 $[C_4H_7]^+$ 55.0548 $[C_5H_7]^+$ 67.0549 $[C_6H_7]^+$ 79.0543 $[C_7H_9]^+$ 93.0703 $[C_8H_{11}]^+$ 107.0860 $[C_{10}H_{13}O]^+$ 149.0964 $[C_{12}H_{15}]^+$ 159.1160 $[C_{17}H_{25}]^+$ 229.1951 $[C_{18}H_{25}O]^+$ 257.1890 $[C_{18}H_{27}O_2]^+$ 275.2010	Fatty Acid		√	√	
26	25.9888	Gamma-Linolenic acid	$C_{18}H_{30}O_2$	$[M+H]^+$ 279.2309 $[C_4H_7]^+$ 55.0548 $[C_5H_7]^+$ 67.0549 $[C_6H_9]^+$ 81.0699 $[C_7H_{11}]^+$ 95.0857 $[C_8H_{13}]^+$ 109.1014 $[C_9H_{15}]^+$ 123.1163 $[C_{13}H_{21}O_2]^+$ 209.1539 $[C_{14}H_{23}O_2]^+$ 223.1700 $[C_{18}H_{30}]^+$ 243.2109 $[C_{18}H_{29}O]^+$ 261.2216	Fatty Acid	√	√	√	√
27	26.1801	(3aR, 4R, 7R)- 1,4,9,9- tetramethyl -3,4,5,6,7,8- hexahydro-2H-3a, 7-methanoazulen-2-one	$C_{15}H_{22}O$	$[M+H]^+$ 219.1718 $[C_4H_7]^+$ 55.0549 $[C_5H_9]^+$ 69.0704 $[C_6H_9]^+$ 81.0701 $[C_7H_{11}]^+$ 95.0859 $[C_8H_{13}]^+$ 109.1015 $[C_9H_{15}]^+$ 123.1164 $[C_9H_{11}O]^+$ 135.0798 $[C_{10}H_{15}O]^+$ 151.1109 $[C_{11}H_{15}O]^+$ 163.1114 $[C_{12}H_{17}O]^+$ 177.1269	Terpenoid	√	√	√	

Note: A = 0N kg/ha; B = 90N kg/ha; C = 180N kg/ha; D = 270N kg/ha.

Table 2. Continued...

No	Rt (minute)	Name	Molecular Formula	MS and MS-MS	Group	A	B	C	D
28	27.1271	9-OxoODE	$C_{18}H_{30}O_3$	$[M+H]^+$ 295.226	Fatty Acid	√	√	√	√
				$[C_4H_7]^+$ 55.0549					
				$[C_5H_7]^+$ 67.0544					
				$[C_5H_5O]^+$ 81.034					
				$[C_7H_9]^+$ 93.0703					
				$[C_{10}H_{15}]^+$ 135.1169					
				$[C_{10}H_{15}O]^+$ 151.1111					
				$[C_{12}H_{17}]^+$ 161.1322					
				$[C_{13}H_{19}O_2]^+$ 207.1370					
				$[C_{14}H_{21}O_2]^+$ 221.1530					
				$[C_{17}H_{27}]^+$ 231.2091					
				$[C_{18}H_{27}O]^+$ 259.2037					
				$[C_{18}H_{29}O_2]^+$ 277.2170					
29	27.8659	13(S)-HODE	$C_{18}H_{32}O_3$	$[M+H]^+$ 297.2426	Fatty Acid	√	√	√	√
				$[C_4H_7]^+$ 55.0548					
				$[C_5H_7]^+$ 67.0549					
				$[C_6H_9]^+$ 81.0700					
				$[C_7H_{11}]^+$ 95.0858					
				$[C_8H_{13}]^+$ 109.1014					
				$[C_8H_{13}O]^+$ 125.0962					
				$[C_{10}H_{15}]^+$ 135.1169					
				$[C_{12}H_{19}]^+$ 163.1466					
				$[C_{11}H_{19}O_2]^+$ 183.1378					
				$[C_{14}H_{23}O_2]^+$ 223.1692					
				$[C_{18}H_{29}O]^+$ 261.2216					
				$[C_{18}H_{31}O_2]^+$ 279.2329					
30	31.7902	Juniperic acid	$C_{16}H_{32}O_3$	$[M+H]^+$ 273.2569	Fatty Acid			√	
				$[C_4H_7]^+$ 55.0546					

Note: A = 0N kg/ha; B = 90N kg/ha; C = 180N kg/ha; D = 270N kg/ha.

Table 3. Identified metabolites with positive ionization in extracts of aerial parts of gendarussa.

No	Rt (minute)	Name	Molecular formula	MS and MS-MS	Group	A	B	C	D
1	0.9691	Methyl salicylate	$C_8H_8O_3$	[M-H]- 151.0603 $[C_2H_3O_2]$- 59.0127 $[C_3H_3O_2]$- 71.0129 $[C_5H_5O_3]$- 113.0236	Phenolic		√		
2	1.0722	Taiwanin E methyl ether	$C_{21}H_{14}O_7$	[M-H]- 377.085	Lignan	√	√	√	√
3	1.0804	Medioresinol	$C_{21}H_{24}O_7$	[M-H]- 387.1145 $[C_{12}H_{15}O_3]$- 207.1029	Lignan	√	√	√	√
4	1.3543	Brazoide C	$C_{18}H_{26}N_2O_9$	[M-H]- 413.1558	Alkaloid		√	√	
5	1.8866	Cinnamic acid	$C_9H_8O_2$	[M-H]- 147.0654	Phenolic		√	√	
6	6.0681	(8S,9R,9aS, 10aR)-5-Oxo-9-vinyl-1,2,3,8,9,9a,10,10aoctahydro-5H-imidazo[1,2-a] pyrano [4,3-d] pyridin-8-yl beta-D-glucopyranoside	$C_{18}H_{26}N_2O_8$	[M-H]- 397.1622	Alkaloid	√	√	√	√
7	7.6294	Justridusamide A	$C_{17}H_{23}NO_8$	[M-H]- 368.1358 $[C_4H_5O_3]$- 101.0230 $[C_4H_7O_4]$- 119.0345 $[C_6H_{11}O_5]$- 163.0603 $[C_{11}H_{10}NO_3]$- 204.067 $[C_{11}H_{12}NO_4]$- 222.0756	Nitrogenous compound	√	√	√	√
8	8.2833	(3S,5R,6R)-3-Allyl-3-[(S)-hydroxy(4-nitrophenyl) methyl]-5,6-dimethoxy-5,6-dimethyl-1,4-380,1339dioxan-2-one	$C_{18}H_{23}NO_8$	[M-H]- 380.1351 $[C_3H_5O]$- 57.0336 $[C_4H_5O_2]$- 85.0287 $[C_6H_{11}O_4]$- 147.0649 $[C_8H_{12}O_5]$- 188.0694 $[C_{12}H_{10}NO_4]$- 232.0609	Nitrogenous compound		√	√	
9	9.0761	1-Deoxy-1-{methyl[(2-oxo-2,3,4,5-tetrahydro-1H-1- benzazepin-7-yl) carbonyl] amino}hexitol	$C_{18}H_{26}N_2O_7$	[M-H]- 381.1664 $[C_3H_5O]$- 57.0337 $[C_5H_6NO_3]$- 128.0342 $[C_6H_{11}O_4]$- 147.0652 $[C_{12}H_{15}N_2O_4]$- 251.1028	Alkaloid	√	√	√	√
10	9.0976	6-C-glusosyl-8-C-arabinosyl apigenin or Schaftoside	$C_{26}H_{28}O_{14}$	[M-H]- 563.1419 $[C_8H_5O]$- 117.0338 $[C_{10}H_7O_4]$- 191.0357 $[C_{19}H_{13}O_7]$- 353.0684	Flavonoid		√	√	
11	10.2012	Gendarusin A	$C_{25}H_{26}O_{13}$	[M-H]- 533.1200 $[C_8H_5O]$- 117.0338 $[C_{20}H_{15}O_8]$- 383.0757	Flavonoid	√	√	√	√
12	10.8491	Gendarusin B	$C_{25}H_{26}O_{13}$	[M-H]- 533.1290	Flavonoid	√	√	√	√
13	11.6121	6-{[(Benzyloxy) carbonyl]amino} -6-deoxy-1,2-O-isopropylidene-Ã-D-glucofuranose	$C_{17}H_{23}NO_7$	[M-H]- 352.1406	Nitrogenous compound		√	√	

Note: A = 0N kg/ha; B = 90N kg/ha; C = 180N kg/ha; D = 270N kg/ha.

Table 3. Continued...

No	Rt (minute)	Name	Molecular formula	MS and MS-MS	Group	A	B	C	D
14	11.6796	Sesamol	$C_7H_6O_3$	$[M-H]^-$ 137.0454 $[C_6H_5O]^-$ 93.0336	Phenolic	√	√	√	
15	16.4111	Methyl 2-[cyclohex-2-en-1-yl(hydroxy) methyl]-3-hydroxy-4-(2-hydroxyethyl)-3-methyl-5-oxoprolinate	$C_{16}H_{25}NO_6$	$[M-H]^-$ 327.2183	Nitrogenous compound		√	√	
16	24.7523	(-)-Secoisolariciresinol	$C_{20}H_{26}O_6$	$[M-H]^-$ 361.1998	Lignan		√	√	
17	25.9558	13(S)-HODE	$C_{18}H_{32}O_3$	$[M-H]^-$ 295.2273	Fatty Acid	√	√	√	√
18	26.7698	9-OxoODE	$C_{18}H_{30}O_3$	$[M-H]^-$ 293.1756	Fatty Acid	√	√	√	√

Note: A = 0N kg/ha; B = 90N kg/ha; C = 180N kg/ha; D = 270N kg/ha.

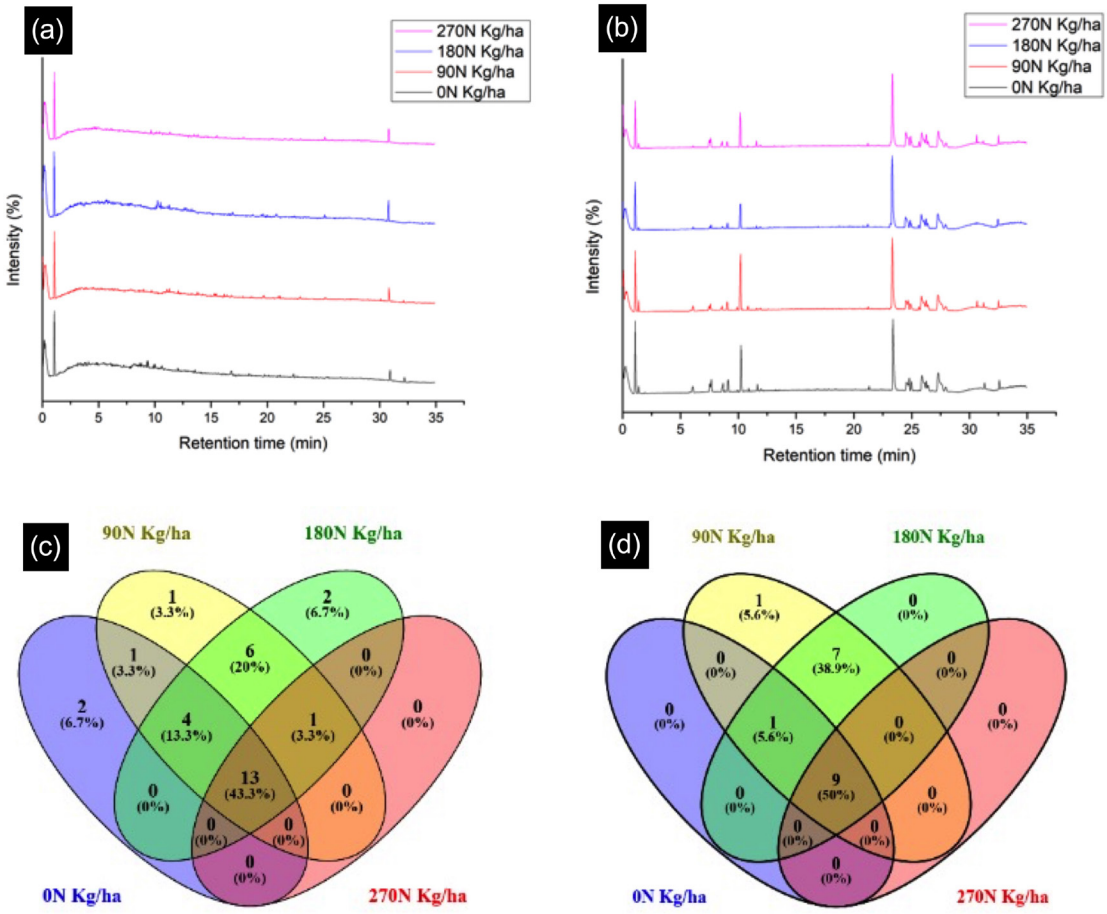

Figure 3. Chromatogram mode (a) positive ionization (b) negative ionization. A Venn diagram of the identified compounds (a) positive ionization and (b) negative ionization.

cow manure, to add nutrients to the soil and the primary research parameters. The crop cultivation design uses RBD, namely the crop cultivation design consisting of nitrogen fertilization doses of 0, 90, 180 and 270 kg/ha.

Higher doses of nitrogen fertilizers tend to yield higher biomass. Nitrogen is an essential element in the process of plant growth and development. The nitrogen source for plants can be taken from the atmosphere. Still, it cannot be absorbed directly by plants, except for symbiotic plants with several microbes, such as legumes, which have symbiosis with rhizobium bacteria for nitrogen capture (Rai, 2018).

The highest antioxidant capacity among all methods was found in 0N kg/ha treatment with the DPPH, ABTS, FRAP, and CUPRAC methods, whose values were respectively 6.53, 33.6, 16.7, and 49.0 µmol TE/g dry weight (Figure 2). Based on statistical tests, different doses of nitrogen fertilizer treatment did not affect the antioxidant capacity value. It may be because there is no change in metabolites concentration that affect antioxidant activity.

Metabolites identified in the positive ionization mode include phenolic, flavonoids, lignans, coumarins, quinones, terpenoids, fatty acids, alkaloids, and nitrogenous compound according to the literature on gendarussa metabolites that have been studied (Table 2). Identified metabolites in the negative ionization mode include phenolic, flavonoids, lignans, fatty acids, alkaloids, and nitrogenous compounds (Table 3). For example, fragmentation was taken from the major secondary metabolite found in gendarussa, namely gendarusin A. Fragmentation was carried out for metabolites with a high-intensity mass spectrum. Gendarusin A was found fragmented in the negative and positive ionization mode. The resulting fragmentation was $[C_{20}H_{16}O_8]^-$ with m/z 383.0757 and $[C_8H_6O]^-$ with m/z 117.0338 by previous literature (Ratih et al., 2019).

Before PCA, the resulting chromatogram was pre-processed in the form of COW. COW is a technique for correcting retention time shifts. This is because the chromatogram peaks are susceptible to shifts in retention time which can be caused by small changes in the composition of the mobile phase, the nature of the stationary phase, or the impact of the sample matrix, so an alignment process is needed to align the retention time. The step that must be carried out in COW is determining the reference chromatogram. The reference chromatogram is determined with the highest similarity index value (Jiao et al., 2018).

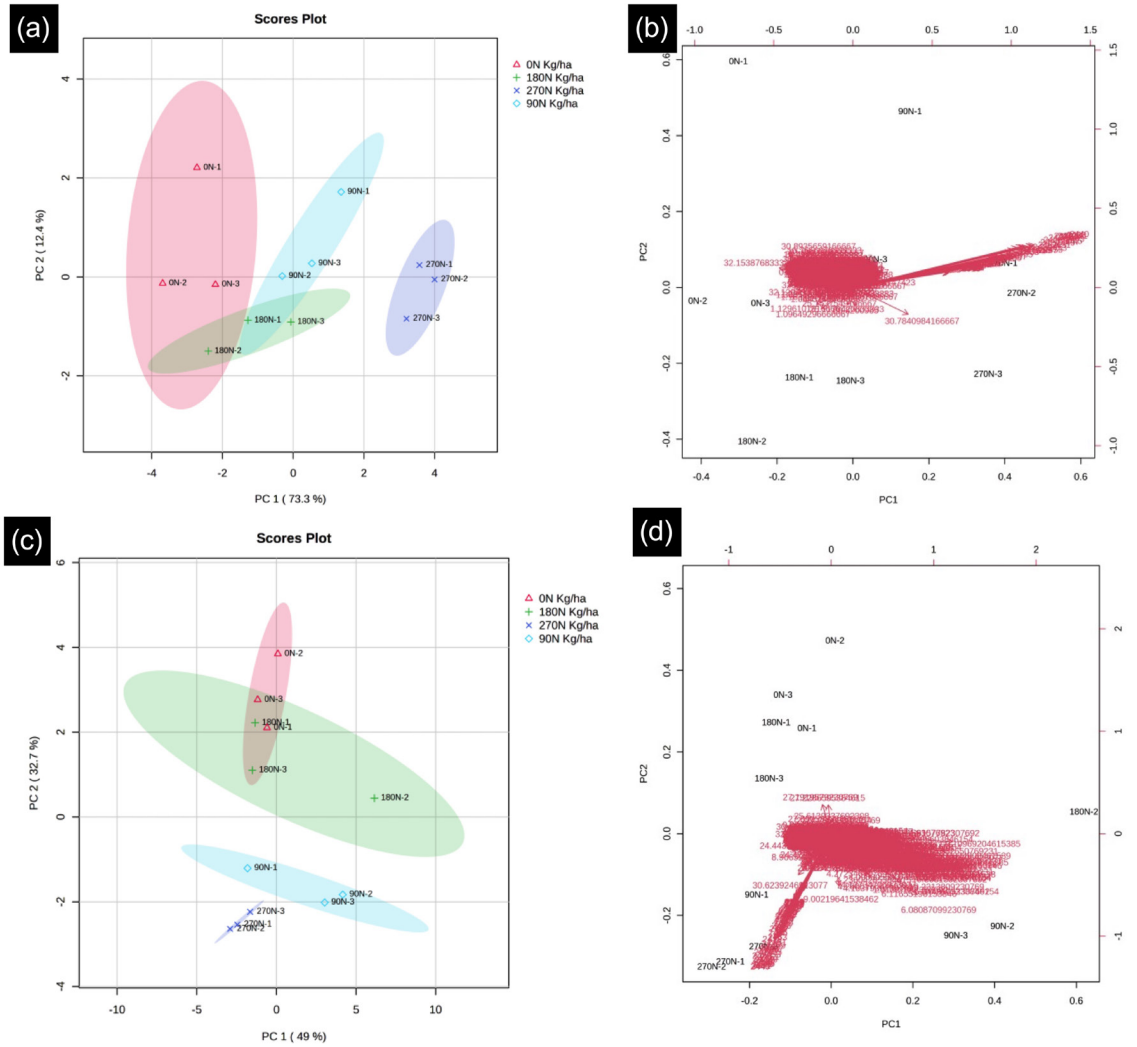

Figure 4. PCA (a) positive ionization score plot (b) positive ionization biplot (c) negative ionization score plot (d) negative ionization biplot.

After COW was carried out, the COW optimization results were used for PCA. Cluster formation on a particular PC in PCA is the most influential function in this analysis. The PCA score plot shows the grouping of each sample based on the variable peak area, sample absorbance, or peak intensity (Shafirany et al., 2018). This PCA analysis aims to differentiate metabolite profile of each sample based on the plant cultivation treatment with different doses of nitrogen fertilizer. The components most often used in PCA analysis are component 1 (PC1) and component 2 (PC2) (Nikzad et al., 2024).

The resulting score plot for the positive ionization mode yields a diversity of data from the two PCs of 85.7%, with a PC1 value of 73.3% and a PC2 of 12.4% (Figure 4a). Two PCs show that there is 85.7% of data variability, which can be explained by the peak intensity of extracts of aerial parts of gendarussa due to different doses of nitrogen fertilizer. Likewise, with the score plot from the PCA resulting from the negative ionization mode, the diversity of data from the two PCs was 82.6%, with a PC1 value of 49% and a PC2 of 32.7% (Figure 4b). The two PC values show a two-dimensional visualization sufficient for both positive and negative ionization modes because the diversity values of PC1 and PC2 are greater than 70% (Maulana et al., 2022). Based on the biplot Figure 4c and 4d, information was obtained that the metabolite's retention time around 20 minutes are the difference for administering nitrogen fertilizer doses. Based on PCA visualization in score plot, it can be concluded that adding different nitrogen fertilizer doses has no significant effect on gendarussa metabolites variability.

A heatmap is the final visualization to explain the metabolites identified in extracts of aerial parts of gendarussa treated with different doses of nitrogen fertilizer. The heatmap (Figures 5a and 5b) shows rows of colors representing the various metabolites, while the columns represent the analyzed nitrogen fertilizer doses. Based on the heatmap formed, metabolites that have a dark red color show a higher concentration than other

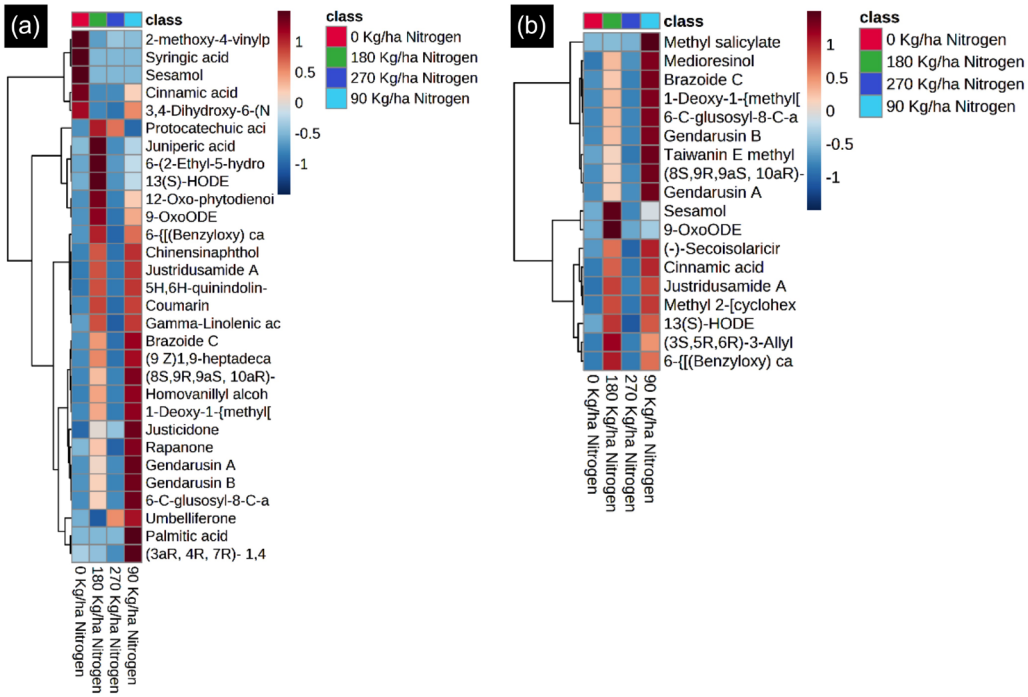

Figure 5. Heatmap of metabolites identified in gendarussa aerial parts extract (a) positive ionization (b) negative ionization.

metabolites, and this concentration decreases towards a dark blue color. The concentrations displayed on the heatmap are qualitatively relative. The research follows Shi et al. (2019), where the heatmap can provide the distribution of metabolites with relative concentrations from different nitrogen fertilizer doses to *L. barbarum*, but not the actual concentrations.

The distribution of metabolites through the heatmap with treatment with different doses of nitrogen fertilizer is shown by treatment without a dose of nitrogen fertilizer; five metabolites have relatively high concentrations (Figure 5a). At nitrogen fertilizer doses of 90 kg/ha and 180 kg/ha, the distribution of relative metabolite was quite similar, but higher in 90 kg/ha, whereas, at nitrogen fertilizer doses of 270 kg/ha, the relative concentration of metabolites tended to decrease, marked in blue (Figures 5a and 5b). This result follows the plant's dry weight, which was obtained that the dose of nitrogen fertilizer was 270 kg/ha with the highest dry weight of each plant, namely 10.9 grams/aerial part of the plant, showing a decrease in the relative concentration of metabolites. This means the nitrogen nutrients obtained are used for growth and development, not to produce secondary metabolites. This research follows Shi et al. (2019) and Shafira (2023), adding higher nitrogen doses, causing the relative concentrations of *L. barbarum* and *Adenostemma lavenia* metabolites to decrease, but the dry weight increased.

5. Conclusions

Variation of different nitrogen fertilizer doses treatment affects gendarussa's metabolites, based on metabolomics. The highest harvest weight was obtained in the nitrogen fertilizer dose of 270 kg/ha. The antioxidant capacity of DPPH, ABTS, FRAP, and CUPRAC methods did not affect the plant cultivating treatment with different nitrogen fertilizer doses. Then, 30 and 18 metabolites were successfully identified using UHPLC-Q-Orbitrap HRMS in positive and negative ionization, respectively. Based on PCA multivariate analysis and heatmap, it was found that the dose of nitrogen fertilizer influenced the metabolites of gendarussa. Nitrogen fertilizer doses of 90 kg/ha give a relatively higher concentration of metabolites rather than a dose of 270 kg/ha. So, the optimal nitrogen fertilizer dose is 90 kg/ha for cultivation and utilization strategies

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