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Research Article

Productivity of Phenolic, Flavonoid, and Antioxidant in *Justicia gendarussa* Burm. f. by Different Shade and Dose of Nitrogen Fertilizer

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Phenolics

Abstract: *Justicia gendarussa* Burm.f., known as gendarussa, has been used as a traditional medicine to treat thrush, headaches, bronchitis, arthritis, jaundice, otalgia, indigestion, fever, cancer, male contraception, and UV protection. *J. gendarussa* can grow wild as a shrub, especially in forest areas and river embankments, which can make the production of secondary metabolites inconsistent, especially phenolics group, and change the biological activity. Therefore, the purpose of this study is to determine the optimal combination of shade and nitrogen fertilizer dose for maximizing phenolic, flavonoid, and antioxidant productivity in the aerial parts of *J. gendarussa*. This study employed a split-plot design, with shade (0, 25, and 50%) serving as the main plot and nitrogen fertilizer doses (0, 90, 180, and 270 kg ha^{-1}) serving as subplots. The highest productivities of phenolics, flavonoids, antioxidants, and dry weight were observed in the treatment with a nitrogen fertilizer dose of 270 kg ha^{-1} and no shading treatment. The dry weight of the plant's harvested aerial parts was 10.9 g $plant^{-1}$. The productivity of phenolics was 210 mg GAE $plant^{-1}$, while the productivity of flavonoids was 112 mg QE $plant^{-1}$. Using DPPH, ABTS, FRAP, and CUPRAC methods, antioxidant productivity was determined to be 63.5; 334; 171; and 525 mol TE $plant^{-1}$, respectively. Pearson correlation indicates that phenolic and flavonoid productivity is highly correlated with antioxidant productivity. Considering the research parameters of shading and nitrogen fertilizer dosage, 270 kg ha^{-1} nitrogen fertilizer application without shading was the optimum cultivation practice combination.

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1. Introduction

Justicia gendarussa Burm.f. belongs to the family Acanthaceae, known as gendarussa. *J. gendarussa* can be found widely distributed in Asian countries, such as China, Indonesia, Sri Lanka, India, and Malaysia (Ratih et al., 2019). *J. gendarussa* is a traditional medicine in Papua (Indonesia) for male contraception. In addition, Indians have used *J. gendarussa* to treat thrush, headaches, bronchitis,

arthritis, jaundice, otalgia, indigestion, fever, cancer, and UV protection (Putri et al., 2020). *J. gendarussa* contains secondary metabolites such as phenolics, alkaloids, saponins, steroids, and terpenoids (Widodo et al., 2019).

The secondary metabolite of *J. gendarussa* has the potential to have antioxidant activity. Antioxidant compounds are metabolites that can delay, inhibit, or prevent the oxidation of materials or compounds easily oxidized by free radicals and reduce oxidative stress (Chaudhary, 2015). Several diseases, including diabetes mellitus, neurodegenerative, cardiovascular, respiratory, cataract development, rheumatoid arthritis, and various types of cancer, have been linked to oxidative stress-induced free radicals (Di Meo and Venditti, 2020). Kuber's research (2021), the ethanol extract of gendarussa leaves had the highest antioxidant activity, with an IC_{50} value of 32 g mL^{-1} obtained through the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method.

J. gendarussa can grow wild as a shrub, especially in forest areas and river embankments (Akpriyanti et al., 2017). However, the wild growth conditions will lead to inconsistent production of secondary metabolites, especially phenolics, because the major compound in *J. gendarussa* is Gendarusin A, a flavonoid compound, which will lead to changes in biological activity. Hence, it is necessary to cultivate *J. gendarussa* to maintain a consistent content of secondary metabolites. Cultivation of plants can be done to maximize this potential, namely by providing shade and nitrogen fertilizer.

Providing shade is important for regulating the light intensity and a suitable temperature so that plant metabolic processes run optimally. Optimal shade can maintain optimal growth, development, and content of primary and secondary metabolites. However, this depends on the type of plant, such as sun plants or shade plants (Yustiningsih, 2019). Cassola et al. (2019) showed that Justicia (*J. brandegeeana*, *J. gendarussa*, and *J. pectoralis*) has higher phenolic and flavonoid contents for those grown in open fields than those grown in greenhouses, which is equivalent to 70% shade. This proves that shading affects the content of secondary metabolites in *J. gendarussa*.

One of the most important nutrients for plants is nitrogen. Nitrogen is important in plant growth, yield, and quality, affecting its chemical content (Warganegara et al., 2017). Nitrogen is an integral part of chlorophyll, the main light energy catcher needed in photosynthesis (Rai, 2018). Photosynthesis will increase the accumulation of primary metabolites, which can contribute to the shikimic acid and phenylpropanoid pathways for producing phenolic compounds and flavonoids (Ekawati, 2013). Shi et al. (2019) showed that adding nitrogen fertilizers in the 42.5-127.5 g to *Lycium barbarum* plant significantly affected chemical constituents, such as flavonoids, amino acids, and polysaccharides. In addition, the study identified 612 metabolites from *L. barbarum* fruit and 53 metabolites that were significantly affected by applying nitrogen fertilizers.

The cultivation of *J. gendarussa* must be evaluated to understand its impact on secondary metabolites, particularly phenolics, and their biological activity as antioxidants. Previous studies from Kuber (2021), Marliani et al. (2022), Putri et al. (2020), and Ratih et al. (2019) did not determine the productivity of phenolics, flavonoids, and antioxidants through DPPH, ABTS, FRAP, and CUPRAC methods in *J. gendarussa* aerial parts under different shade treatments (0, 25, and 50%) and nitrogen fertilizer doses (0, 90, 180, and 270 kg ha^{-1}). This study aims to identify the optimal shade and nitrogen fertilizer dose for the productivity of phenolics, flavonoids, and antioxidants in *J. gendarussa*.

2. Material and Methods

The research was carried out for plant cultivation at the Biopharmaca Cultivation Conservation Unit (6º3'49"S and 106º42'57"E) Bogor, West Java, Indonesia. The tools used were Minitab software version 17.0, GraphPad Prism software version 8.0, oven (Memmert UM 40), microplate nano spectrophotometer (Biotek, Winooski, USA SPECTROstar Nano, BMG Labtech, Germany), and a set of shading 25%; 50%. The main materials used were aerial parts samples of the *J. gendarussa* plant with a harvest age of 4 months.

2.1. Cultivation and sample preparation of *J. gendarussa*

The design of the cultivation experiment was carried out using a split-plot design with two factors, namely, treatment of shading intensity and N fertilization. The main plot was shading intensity, consisting of 3 levels, namely N0 (0% shading intensity) and N1 (25% shading intensity), and N2 (50%

shading intensity). The dosage of N fertilizer as subplots consisted of 4 levels, namely P0 (0 N kg ha^{-1}), P1 (90 N kg ha^{-1}), P2 (180 N kg ha^{-1}), and P3 (270 N kg ha^{-1}). All treatments were given a dose of P_2O_5 fertilizer at 100 kg ha^{-1}, KCl at 150 K_2O kg ha^{-1}, and cow manure at 20 tons ha^{-1}. To ensure optimal growth, the fertilizer is carefully blended and placed in small polybags (10x15 cm) around the plants for a month before being transferred to larger ones (30×30 cm) for further treatment. The experiment was conducted using 12 different treatment combinations, each of which was administered three times, resulting in 36 experimental units. Each unit comprised 10 plants, and the total number of plants used in the experiment was 360. The details of the treatment parameters can be found in Table 1.

Harvesting is done by pruning as high as 15 cm from the ground. The aerial parts are taken for harvesting in *J. gendarussa*, next the fresh weight of plants was determined using an analytical balance. The plants were cleaned of adhering soil, rinsed with running water, dried, and weighed. To determine the sample's dry weight, it was dried in an oven at 45°C for two 24-hour cycles.

Table 1. Parameters of the research treatment of *J. gendarussa* plants

The subplot – Nitrogen Fertilizer	**The main plot – Shade**		
	N0	**N1**	**N2**
P0	A1	B1	C1
P1	A2	B2	C2
P2	A3	B3	C3
P3	A4	B4	C4

2.2. Extraction *J. gendarussa*

The extraction method used refers to Marliani et al. (2022) with modifications. To extract the sample, 1 gram of powdered *J. gendarussa* aerial parts (leaves and stems) was mixed with 10 mL of ethanol p.a. at a 1:10 ratio. The mixture was macerated for 24 hours at room temperature in a dark room. After that, the sample was filtered using filter paper to obtain the sample extract. This extraction process was repeated twice (Diplo) for better results.

2.3. Phenolics productivity

The methods refer to Batubara et al. (2020) with a modification, where the total phenolic content was determined by 10 µL of sample extract mixed with distilled water (160 µL), 10% Folin-Ciocalteu reagent (10 µL), and 10% Na_2CO_3 (20 µL) in a 96-well clear polystyrene microplate. After 30 minutes of incubation at room temperature, the absorbance was measured at 750 nm using a microplate nano spectrophotometer. Productivity is measured in milligrams of gallic acid equivalents per plant (mg GAE $plant^{-1}$).

2.4. Flavonoids productivity

The method refers to Nurcholis et al. (2021) with a modification, where a sample solution from each extract (10 µL) was put into a microplate then added distilled water (120 µL), 10% aluminum chloride (10 µL), 1 M potassium acetate (10 µL), and ethanol p.a (60 µL), then incubated at room temperature for 30 minutes. The absorbance was measured with a microplate nano spectrophotometer at 415 nm. Productivity is expressed in mg of quercetin equivalent per plant (mg QE $plant^{-1}$).

2.5. Antioxidant DPPH productivity

The method refers to Batubara et al. (2020) with a modification, where the sample solution from each *J. gendarussa* extract (100 µL) was put into a microplate and added with 125 µM DPPH (100 µL). 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. The results are expressed with productivity as µmol Trolox equivalent per plant (µmol TE $plant^{-1}$).

2.6. Antioxidant ABTS productivity

ABTS reagent was prepared based on the method of Nurcholis et al. (2022) with a modification, sample extract (20 µL) was combined with ABTS (180 µL) reagent in a microplate. The mixture was left to incubate for 6 minutes before measuring the absorbance of the solution at a wavelength of 734 nm. The findings are presented as µmol TE plant^{-1}.

2.7. Antioxidant FRAP productivity

The method refers to Batubara et al. (2020) with modifications, samples from each extract (10 µL) were then added with FRAP (145 µL) in a microplate and then incubated in the dark for 4 minutes. The absorbance was read at a wavelength of 593 nm. The results are expressed with productivity as µmol TE plant^{-1}.

2.8. Antioxidant CUPRAC productivity

The method refers to Nurcholis et al. (2022) with a modification; the antioxidant test was carried out with 50 µL of the extracted sample added to 50 µL of 10^{-2} M $CuCl_2$ solution, 50 µL of NH_4Ac buffer pH 7, and 50 µL of 7.5×10^{-3} M neokuproin on a microplate. The mixture was then incubated for 30 minutes in a dark room at room temperature. The absorbance of the mixture was then determined at wavelength 450 nm. The results are expressed with productivity as µmol TE plant^{-1}.

2.9. Data analysis

Data analysis in this study used Minitab software with split plot analysis in a randomized complete block design, which was expressed by a p-value <0.05. Then, regression and correlation analysis were carried out using GraphPad Prism version 8.0 software to see the correlation between the productivity of phenolics and flavonoids and the productivity of antioxidants. A significant relationship occurs when the p-value is smaller than the significance level (α) used, the p-value <0.05.

3. Results

3.1. *J. gendarussa* cultivation

The cultivation results are presented in Figure 1, which show significantly different results from the split-plot test in a randomized complete block design at the 95% confidence level. In addition, different shade treatments and nitrogen fertilizer doses had interactions ($p<0.05$). Post hoc follow-up tests using Tukey showed that the treatment without shade with a dose of nitrogen fertilizer of 270 kg ha^{-1} had the highest weight of 10.9 grams plant^{-1}, which is the optimum condition for plant cultivation regarding the biomass produced.

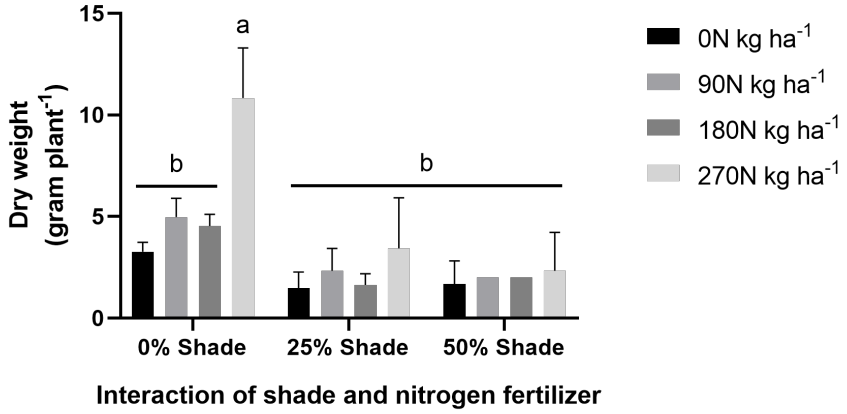

Figure 1. Harvest result as dry weight of each plant of *J. gendarussa*. Each value is presented as the mean ± standard deviation (SD). The mean value in each column marked with a different letter is significantly different at p <0.05 or the 95% confidence level.

3.2. Phenolics productivity of *J. gendarussa*

Productivity was carried out to find the most optimum treatment for plant cultivation and phenolic production. Productivity calculations are used for all tests conducted in the study. Productivity can be determined by multiplying the total phenolic content by the dry weight of the yield per plant. The phenolic productivity of *J. gendarussa* (Figure 2) had significantly different results and interactions between cultivation treatments ($p<0.05$). The highest phenolic productivity was found in the treatment without shade with a nitrogen fertilizer dose of 270N kg ha^{-1}, which was 210 mg GAE plant^{-1}. The study results are the same as those conducted by Cassola et al. (2019) where the phenolic content of *J. gendarussa* cultivated without shade had a higher phenolic content than that given shade.

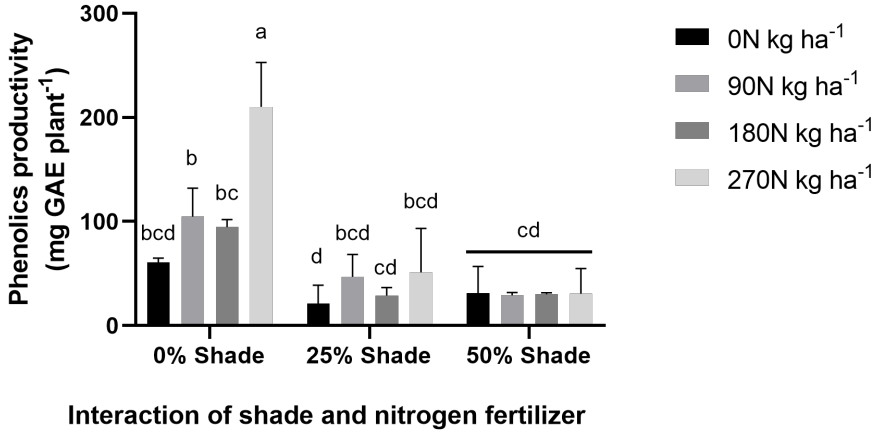

Figure 2. Phenolic productivity of *J. gendarussa*. Each value is presented as the mean ± SD. The mean value in each column marked with a different letter is significantly different at $p<0.05$ or the 95% confidence level.

3.3. Flavonoids productivity of *J. gendarussa*

The productivity of the flavonoids of *J. gendarussa* (Figure 3) had significantly different results and had interactions between cultivation treatments ($p<0.05$). The highest productivity of flavonoids was found in the treatment without shade with a dose of nitrogen fertilizer 270N kg ha^{-1}, which was 112 mg QE plant^{-1}. The study results are the same as those conducted by Cassola et al., (2019) where the flavonoid content of *J. gendarussa* cultivated without shade had a higher flavonoid content than that given shade.

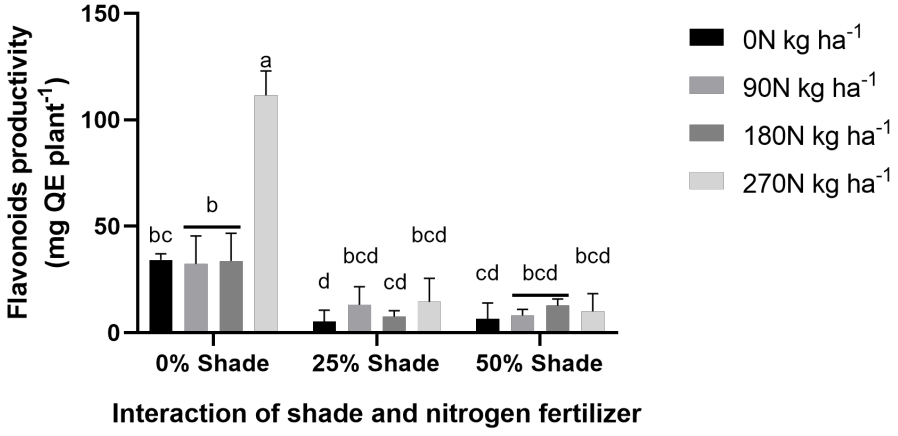

Figure 3. Flavonoids productivity of *J. gendarussa*. Each value is presented as the mean ± SD. The mean value in each column marked with a different letter is significantly different at $p<0.05$ or the 95% confidence level.

3.4. Antioxidant DPPH productivity of *J. gendarussa*

The antioxidant productivity of the DPPH *J. gendarussa* method (Figure 4) was significantly different and had interactions between treatments ($p<0.05$). The highest antioxidant productivity results were found in the treatment without shade with a dose of nitrogen fertilizer 270N kg ha^{-1} with a value of 63.5 µmol TE $plant^{-1}$.

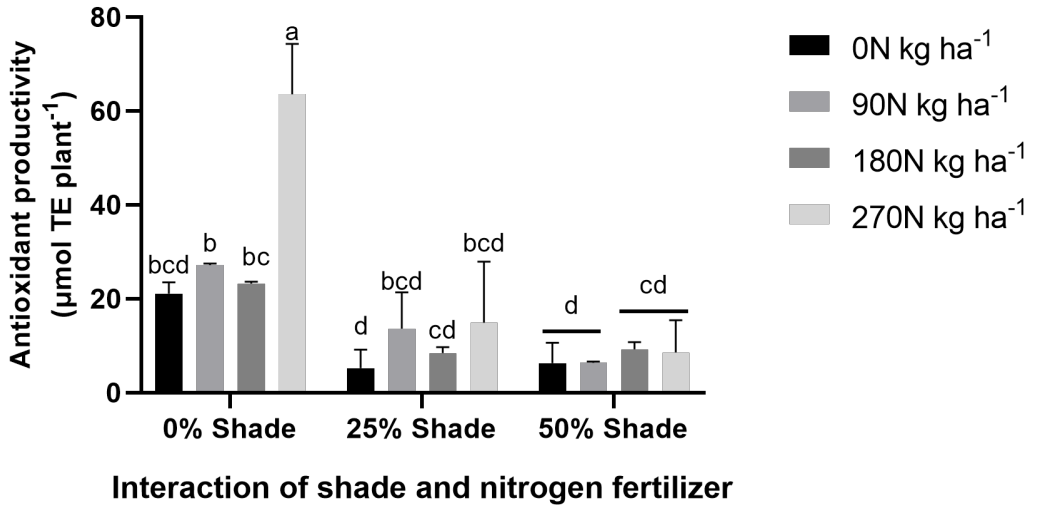

Figure 4. *J. gendarussa* antioxidant productivity of the DPPH method. Each value is presented as the mean ± SD. The mean value in each column marked with a different level is significantly different at $p<0.05$ or the 95% confidence level.

3.5. Antioxidant ABTS productivity of *J. gendarussa*

The antioxidant productivity of the ABTS *J. gendarussa* method (Figure 5) was significantly different and had interactions between cultivation treatments ($p<0.05$). The highest antioxidant productivity results were found in the treatment without shade with a dose of nitrogen fertilizer 270N kg ha^{-1} with a value of 334 µmol TE $plant^{-1}$.

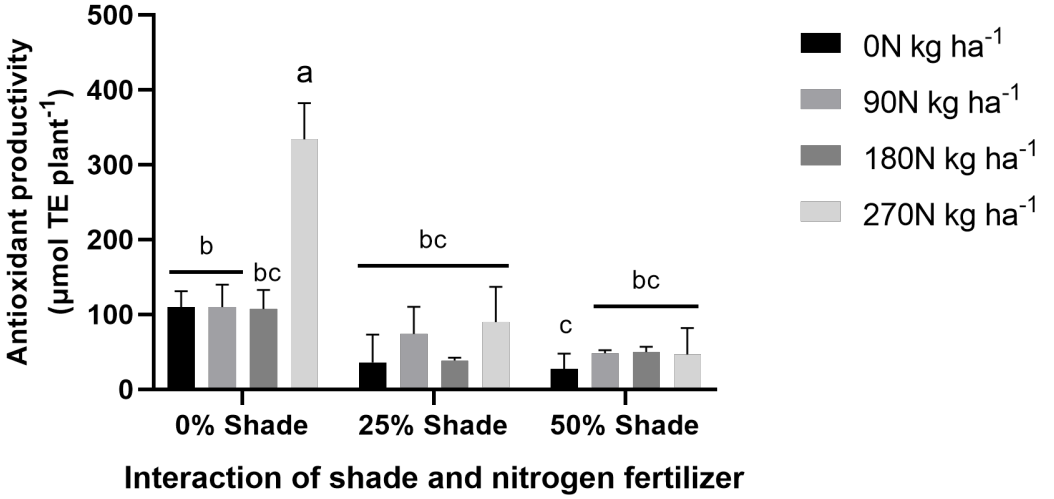

Figure 5. *J. gendarussa* antioxidant productivity of the ABTS method. Each value is presented as the mean ± SD. The mean value in each column marked with a different letter is significantly different at $p<0.05$ or the 95% confidence level.

3.6. Antioxidant FRAP productivity of *J. gendarussa*

The antioxidant productivity of the *J. gendarussa* FRAP method (Figure 6) had significantly different results and had interactions between treatments ($p<0.05$). The highest antioxidant productivity results were found in the treatment without shade with a dose of nitrogen fertilizer 270N kg ha^{-1} with a value of 171 µmol TE plant^{-1}.

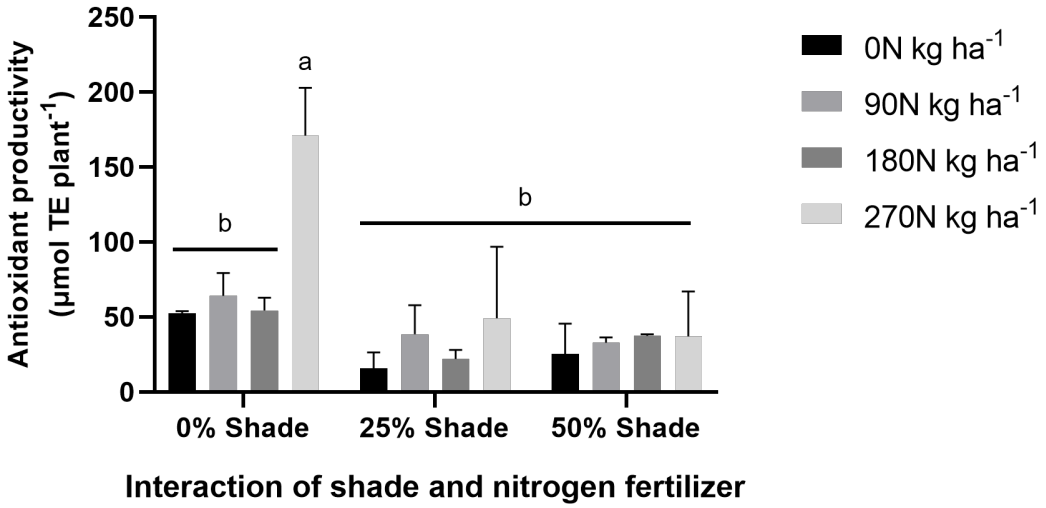

Figure 6. *J. gendarussa* antioxidant productivity of the FRAP method. Each value is presented as the mean ± SD. The mean value in each column marked with a different letter is significantly different at $p<0.05$ or the 95% confidence level.

3.7. Antioxidant CUPRAC productivity of *J. gendarussa*

The antioxidant productivity of the CUPRAC method of *J. gendarussa* (Figure 7) had significantly different results and had interactions between cultivation treatments ($p<0.05$). The highest antioxidant productivity results were found in the treatment without shade with a dose of nitrogen fertilizer 270N kg ha^{-1} with a value of 525 µmol TE plant^{-1}.

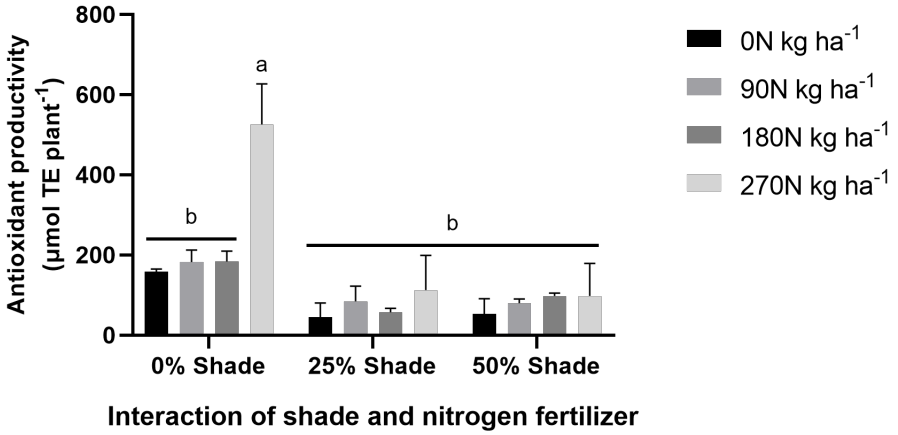

Figure 7. *J. gendarussa* antioxidant productivity of the CUPRAC method. Each value is presented as the mean ± SD. The mean value in each column marked with a different letter is significantly different at $p<0.05$ or the 95% confidence level.

3.8. Correlation between phenolic and flavonoid productivity against antioxidant productivity of *J. gendarussa*

In addition, a correlation test will be carried out between the productivity of the tested secondary metabolites and antioxidants to see the relationship through Pearson correlation. Pearson correlation is a test that analyzes the relationship between two variables and estimates the strength of the relationship (Schober et al., 2018). The basis for statistically significant Pearson correlations is determined based on the p-value generated from the analysis. A significant relationship occurs when the p-value is smaller than the significance level (α) used ($p < 0.05$), but this value does not indicate a correlation value between variables. The correlation value (r) divides the correlation into five groups: very strong correlation (r = 0.90–1.00), strong correlation (r = 0.70–0.89), moderate correlation (r = 0.40–0.69), weak correlation (r = 0.10–0.39), and negligible correlation (r = 0.00–0.10). In addition to correlated data, changes in the magnitude of one variable will affect changes in the magnitudes of other variables, both in the same direction (r is positive) or opposite (r is negative) (Schober et al., 2018).

All tests performed, including phenolic productivity with antioxidants and flavonoids with antioxidants, had a p-value of less than 0.05. In addition, the correlation results with all tests are in the range of relation coefficient values (r) at 0.90 – 1.00, indicating that between variables there is a very strong correlation, meaning that phenolic compounds and one of their groups, namely flavonoids have an important role in antioxidants (Figure 8).

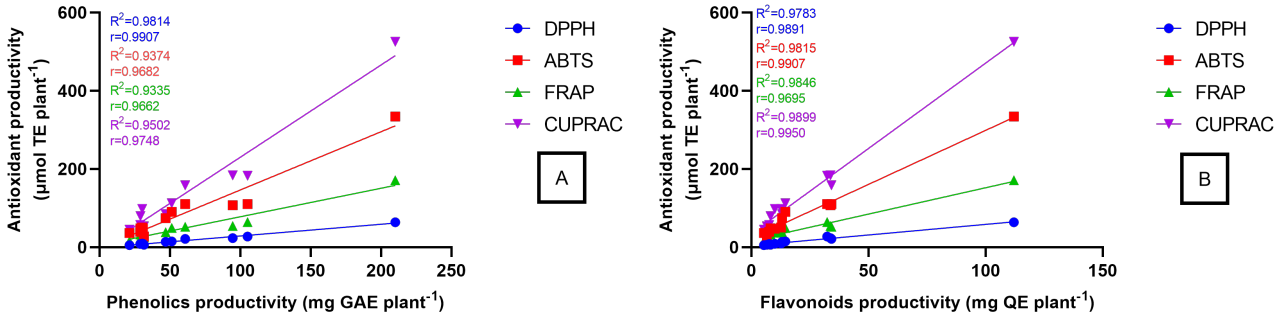

Figure 8. (a) Pearson correlation between phenolic productivity and *J. gendarussa* antioxidants productivity, (b) Pearson correlation between flavonoid productivity and *J. gendarussa* antioxidants productivity.

4. Discussion

Factors that can cause the treatment without shade to have a high dry weight content can be caused by the characteristics of the *J. gendarussa* plant. *J. gendarussa* is a sun plant that is tolerant and normally grows in 0% – 20% shade. In addition, *J. gendarussa* has C4 plant characteristics, which is a plant that can adapt to hot and dry environments. This is evidenced by the research of Cassola et al. (2019) who proved that members of the Justicia genus, namely *J. brandegeeana*, *J. gendarussa*, and *J. pectoralis* had high biomass yields when planted in conditions without shade, had a drastic decrease in biomass when planted in greenhouses (shade equivalent of 70%).

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

Nitrate ions (NO_3^-) and ammonium ions (NH_4^+) are the two forms of nitrogen that plants can absorb. The soil solution contains nitrate ions (NO_3^-), which are readily taken by plants but quickly washed away by running water. Nitrogen absorbed by plants is in the form of nitrate ions (NO_3^-) and ammonium ions (NH_4^+). Nitrate ion (NO_3^-) is available in the soil solution and is easily absorbed by plants but is easily washed away by running water. Soil colloids bind ammonium ions (NH_4^+) and can only be used by plants after a cation exchange process. Nitrogen in the soil can be lost due to evaporation,

leaching by water, or being carried away during harvest. Nitrogen loss can be caused by leaching (washing off rainwater or irrigation), runoff (carried away by surface water), erosion, and emissions (evaporation) (Arumsari, 2017).

The effect of nitrogen on the production of phenolic compounds can be seen in the biomass produced by plants. Based on the yields that high nitrogen fertilization can increase, the resulting biomass indicates that glucose production in plants will increase. Glucose is important in entering the shikimic acid pathway (Cassola et al., 2019). Therefore, the phenolic productivity value of the treatment without shade and the dose of nitrogen fertilizer 270N kg ha^{-1} has a high yield because the biomass produced is also high. This also applies to shade treatment, where shade causes small biomass for plants, causing low phenolic productivity. The effect of plant cultivation treatment on flavonoid productivity has the same discussion as phenolic productivity because nitrogen and shade can affect the production of primary metabolites from plants as biosynthetic precursors for secondary metabolites, which will cause differences in the value of flavonoid productivity based on biomass. The study results are the same as those conducted by Cassola et al. (2019) where the phenolics and flavonoids content of *J. gendarussa* cultivated without shade had a higher phenolic content than that given shade.

Antioxidant productivity with four different methods, namely DPPH, ABTS, FRAP, and CUPRAC, had different results, which could be due to the uniqueness of each reaction method carried out and the content of secondary metabolites contained in plants based on different cultivation treatments (Sadeer et al., 2020). However, the thing that causes the antioxidant productivity of CUPRAC to have the highest and the antioxidant productivity of DPPH to have the lowest result is due to the redox reaction principle, where the potential reduction value of each antioxidant reagent is different. Research by Sadowska-Bartosz and Bartosz (2022) shows that DPPH has a reduction potential of 0.3 V. According to Sadeer et al. (2020) showed that ABTS has a reduction potential of 0.68 V, while FRAP has a reduction potential of 0.70 V. CUPRAC based on the voltaic series is in the right position for Fe metal which means it has a greater reduction potential than FRAP. Reduction potential is the ability of an oxidizing agent (a substance that undergoes reduction) to capture electrons. The greater the reduction potential, the greater the ability of the substance to experience reduction. Therefore, the highest antioxidant productivity results are found in the CUPRAC method, and the lowest is in the DPPH method (Sadowska-Bartosz and Bartosz, 2022).

The Pearson correlation has been conducted, the result was the same as the one stated in the literature that phenolic compounds can be antioxidants based on their high redox potential to play a role in hydrogen donors, reducing agents, and neutralization of singlet oxygen (Chandra and Arora, 2018). *J. gendarussa* has main or major compounds in the flavonoid group, namely Gendarusin A with minor isomers (Figure 9) (Ratih et al., 2019). This correlates with the results of the Pearson correlation. The general mechanism of flavonoid compounds as antioxidants is through the delocalization of electrons in aromatic rings and resonance effects that stabilize the newly formed free radicals, which will further react with other free radicals to form stable compounds such as quinones (Speisky et al., 2022).

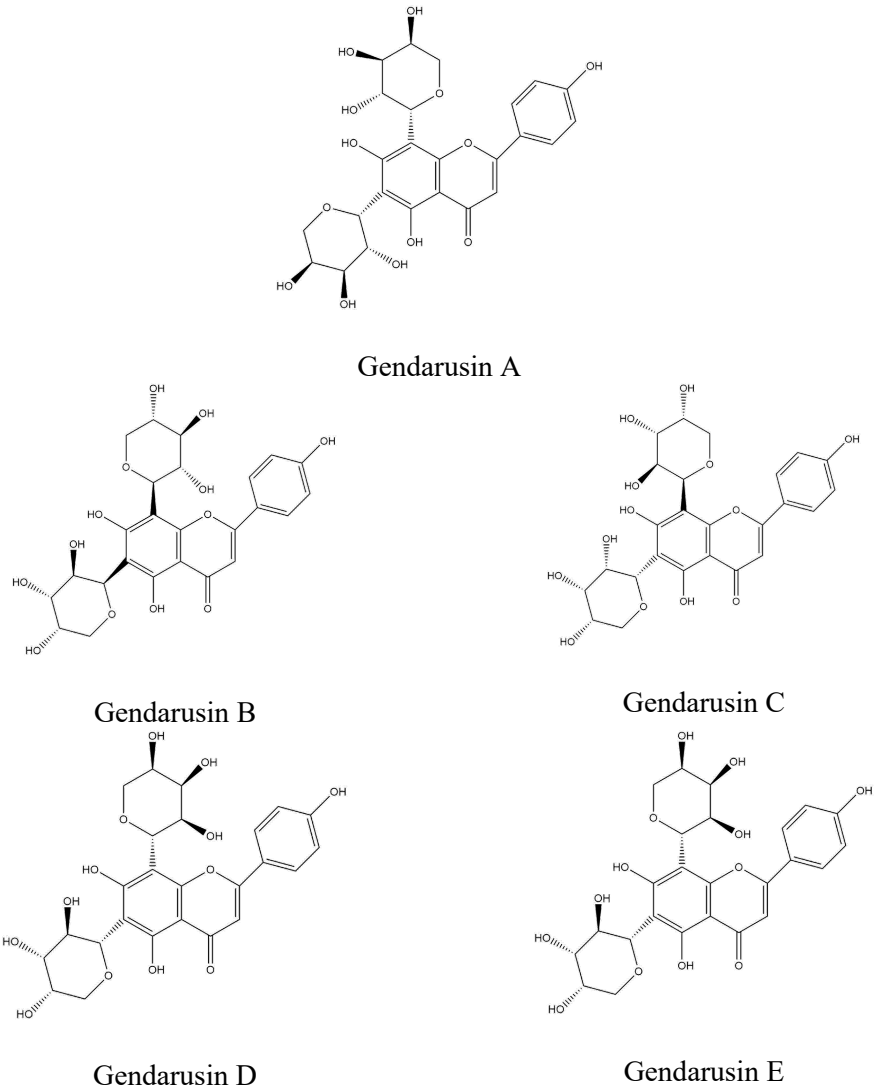

Figure 9. Structure of the main compound of *J. gendarussa* (Sinansari et al., 2018).

Conclusion

In this study, the cultivation of plants without shade and a nitrogen fertilizer dose of 270 kg ha^{-1} resulted in the highest productivity value. The plants yielded a dry weight of 10.9 g $plant^{-1}$, 210 mg GAE plant-1 of phenolic, 112 mg QE $plant^{-1}$ of flavonoids, and showed strong antioxidant activity in DPPH, ABTS, FRAP, and CUPRAC tests with values of 63.5, 334, 171, and 525 mol TE $plant^{-1}$, respectively. Pearson's correlation showed that the productivity of phenolics and flavonoids is highly correlated with antioxidant productivity, with a value of $r > 0.9$. Based on these findings, this study recommends the cultivation of plants without shade and a nitrogen fertilizer dose of 270 kg ha^{-1} as the most optimum method for the parameters in this study.

Ethical Statement

Ethical approval was not required for this study as it did not involve the use of animals or human subjects.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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Author Contributions

F.M. collected the data, conducted data processing and analysis, and prepared the initial draft of the manuscript. I.B. and W.N. designed the study and provided oversight throughout the research process. W.N. secured funding, contributed to drafting and revising the manuscript based on reviewer feedback, and assisted in the data analysis. All authors reviewed, edited, and approved the final manuscript.

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