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Effect of Light Intensities and Nitrogen Fertilizer Doses on Growth, Phenolic, and Flavonoid Production of *Adenostemma lavenia*

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ABSTRACT

Adenostemma lavenia (Asteraceae) is a medicinal plant considered a weed, consisted a lot of secondary metabolites, including phenolic and flavonoid. This species has been widely distributed in various countries but has yet to be widely cultivated. Thus, this study aimed to determine the highest plant growth, phenolic, and flavonoid production from A. lavenia cultivated under different shade of nitrogen fertilizers. The experiment used a nested design consisting of 2 factors. The first factor was shade with different intensities (0, 25, 50, and 75%) and the second was nitrogen fertilizer with different doses (0, 45, and 90 kg/ha). The highest plant was achieved at 50% shade level, while the highest leaf number and branch were 25% and 0%, respectively. Leaf thickness and stomata number increased in no-shade conditions. The production of phenolics and flavonoids was high under shaded conditions, supported by significantly high phenolics and flavonoid total under shade conditions, i.e., 35.94 mol gallic acid equivalent/plant and 21.76 mol quercetin equivalent/plant, respectively. A 90 kg/ha nitrogen fertilizer dose produced the best plant growth, phenolic, and flavonoid production.

1. INTRODUCTION

Indonesia is a megadiversity country because it is a country that has the highest biological wealth in the world. One of the plant families in Indonesia is the *Asteraceae* family, the second largest plant in the kingdom Plantae (Pertiwi *et al*., 2015). One of them is *Adenostemma lavenia*, a potential medicinal plant spread across Pakistan, India, China, Taiwan, Australia, and almost all of Southeast Asia (Chen *et al*.,, 2019). *A. lavenia* has been used for various traditional treatments in the Asian and Pacific Islands, such as curing pneumonia, fever, hepatitis, lung congestion, pulmonary embolism, and edema (Batubara & Prastya, 2020). This plant has been reported to contain high levels of flavonoids, polyphenols, alkaloids, terpenoids, and essential oils (Batubara *et al*., 2020). In addition, several chemical compounds of *A. lavenia* had been reported, such as p-coumaric acid (Chen *et al*., 2019), 4-Allyl-2,6-Dimethoxyphenol, levoglucosan, and coniferyl alcohol (Fauzan *et al*., 2017). Therefore, this plant produces various biological activities, such as antioxidant, antibacterial, antiaging, anti-melanogenic, and anti-inflammatory (Nurlela *et al*., 2022).

Flavonoids and other phenolic compounds are a group of secondary metabolites that are abundant and widespread in plants (Mutha *et al*., 2021). Moreover, they contribute to various activities, such as antioxidant, anticancer, anti-inflammatory, antibacterial, and cardioprotective agents (Tungmunnithum *et al*., 2018). One component of the phenolic group was found in *A. lavenia*, namely p-coumaric acid. It is known that these components have anti-inflammatory and antioxidant activities (Chen *et al*., 2019). Unfortunately, research on the effect of *A. lavenia* cultivation on phenolic and flavonoid production has still not been reported. Even though the synthesis of phenolics and flavonoids in plants is influenced by several factors, such as growing region, environmental conditions, age, plant parts, geographic variations, and fertilizers used (Fauzan *et al*., 2017).

Shading was chosen because the light is an environmental factor affecting plant growth, development, and secondary metabolites at specific intensities (Mawardy & Karyawati, 2021). According to Xie *et al*., (2006), phenolic biosynthesis requires light, and the biosynthetic rate of flavonoid formation depends on light density. Supported by Wang *et al*., (2020), the leaves of the four types of fern species can synthesize more phenolics and flavonoids at higher light intensities. However, different plants respond differently to changes in light intensity and total phenolic and flavonoid production. As in previous studies, higher light intensity reduced phenolic compounds in lettuce leaves (*Lactuca sativa* L.) (Pérez-López *et al*., 2018).

In addition to the factors above, cultivation also needs fertilization activity to support growth and plant production (Pramuhadi *et al*., 2023), especially its metabolites. N fertilizer was chosen because element N is one of the most important elements needed by plants worldwide. In previous studies, adding N elements increases total phenolic and Quercetin-3-βD-glucoside in *Allium fistulosum* L. (Zhao *et al*., 2021). Increases the phenolics because there is an increase in the precursor phenylalanine ammonia-lyase (PAL). In biosynthesis, this precursor converts phenylalanine to cinnamic acid in the shikimic acid pathway. Furthermore, several phenolic compounds are converted into flavonoids (Ghasemzadeh *et al*., 2010). Nevertheless, information on how nitrogen fertilizer affects phenolics and flavonoids is scarce. Therefore, this study aimed to combine the shade factor and nitrogen fertilizer to obtain the best growth, phenolic production, and flavonoids from *A. lavenia* cultivated with variations in shading and different doses of nitrogen fertilizer.

2. RESEARCH METHOD

2.1. Cultivation

Cultivation was carried out in the Biopharmaca Conservation and Cultivation Station, Tropical Biopharmaca Research Center, IPB University, Bogor, West Java, Indonesia, from Desember 2021 to January 2022 (6°32’25.47” N and 106°42’53.22” E). The research was carried out experimentally using a nested plot design method. The main treatment (main plot) was shade with 4 different densities, namely 0 (control), 25, 50, and 75%, on a 2 m × 1.5 m area. The sub-plot treatment was three different doses of N in urea fertilizer, namely 0 kg/ha (N: 0 kg/ha), 100 kg/ha (N: 45 kg/ha), and 200 kg/ha (N: 90 kg/ha). This research focuses on the effect of N on plants, so this writing used a dose of nitrogen in urea (0, 45, and 90 kg/ha).

Before being cultivated, the *A. lavenia* plant was determined (Nurlela *et al*., 2022). *A. lavenia* is propagated by stem cuttings from plants eight weeks after planting (WAP), with cuttings about 10 cm long. The cuttings were planted in 10 cm x 10 cm polybags and given a homogeneous treatment for 3 weeks, they are SP-36 100 kg/ha, KCl 100 kg/ha, and cow manure 20 ton/ha. After that, the plant cuttings are transferred to the land, which has been given different shading and nitrogen fertilizers. The soil is latosol type with pH 4.69:4.06, 0.20% N, and 2.05% organic C.

2.2. Field observation

Observations were made non-destructively and destructively. In non-destructive, the observed variables were plant height, leaf number, and branch primary number at intervals of a week, starting from one week after planting (WAP) for six weeks. In addition, light intensity measurements were also carried out for each shading at 3 and 6 WAP using a Lux Meter (LX-1010B) 3 times a day, namely in the morning, afternoon, and evening. While the destructive observations, such as leaf thickness and stomata number observed three days before harvest, and relative growth rate (RGR) and net assimilation rate (NAR) observed every two weeks, were carried out using a microscope and IMAGE J software. Plants were harvested after six WAP.

2.3. Sample preparation

All samples were cleaned, then the stems and leaves were separated. Furthermore, it is dried (45℃) and mashed. After that, the dry powder samples were determined for Phenolics, flavonoid total, and thin-layer chromatography (TLC).

2.4. Determination of phenolics and flavonoid total, phenolics, flavonoid production, and TLC

Before the total phenolics and flavonoids were determined, the extraction process was accomplished. First, 1 g dry leaf *A. lavenia* was put into a 50 mL vial, and methanol as the solvent was added at a ratio of 1:20 (w/v). After that, macerated *A. lavenia* leaf with the help of a shaker for 3 hours. These extracts were used to determine the total phenolics and flavonoids. The total phenolic and flavonoid content was determined using Batubara *et al*., (2020) method. Quantification was done concerning to the standard curve of gallic acid equivalent (GAE) for phenolic and quercetin equivalent (QE) for flavonoid. Phenolic production is obtained by multiplying the total phenolic content and the dry leaf weight per plant, as well as for the production of flavonoids. Thin layer chromatography was determined using a method used by Safitri *et al*., (2017), with chloroform as the eluent and H2SO4 10% for derivatization.

2.5. Statistical analysis

Data obtained from each variable were analyzed statistically using variance (ANOVA) at the 5% confidence level. The treatment results that had a significant effect would be continued with Duncan's Multiple Range Test with the SAS program version 9.0. Heatmap processing and data correlation were carried out using Metaboanalyst 5.0. The analysis was done to find out which variables had the most influence on the growth and productivity of phenolics and flavonoids.

3. RESULTS

3.1. General condition

Based on the Indonesian Agency for Meteorological, Climatological, and Geophysics (BMKG), Dramaga, Bogor (2022), the maximum temperature is from December 2021 to January 2022 25.0–25.2℃ and the relative humidity is 86.9–90.0%. The rainfall is 7.8–14.4 mm, and the duration of solar radiation is 1.6–2.7 hours. Plant growth in the early days of cultivation tends to be slow. Plants start flowering at 3–4 WAP. The relationship between light intensity and time in the ante meridiem (AM) and post meridiem (PM) is presented in Figure 1. The highest and lowest average light is at 12:30 PM and 02.30 PM, respectively. There was a decrease in light intensity at 25% shading by 24.63%, 50% shading by 52.53%, and 75% shading by 75.98% at 12:30 PM.

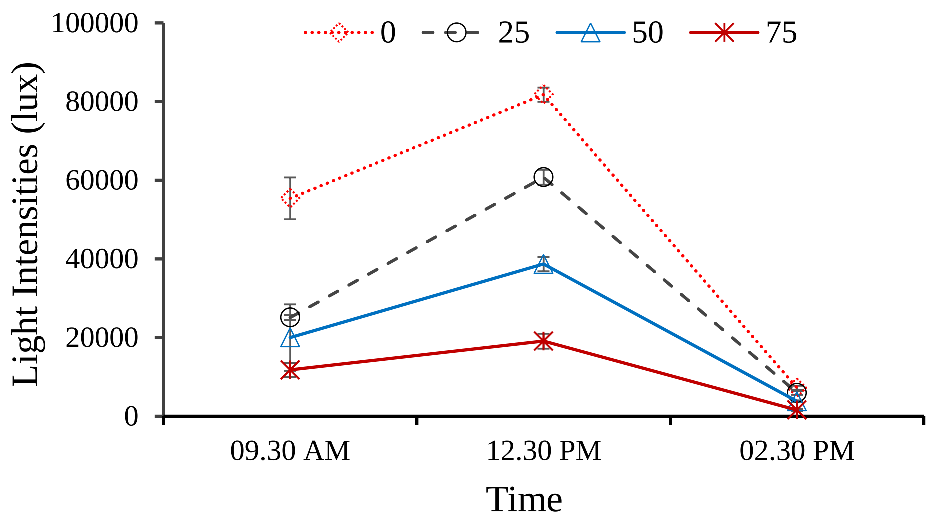


Figure 1. The average intensity of light entering different shading (0, 25, 50, and 75%) at different measurement

3.2. The effect of shading or nitrogen fertilizer on the growth of *A. lavenia*

The plant height of *A. lavenia* was affected by shading but not by applying N fertilizer. At 3 WAP, 75% shading treatment was not significantly different from 50%, but significantly higher than 25% and no shading. Furthermore, at 6 WAP, the height of plants in shade 50% tends to increase by 1.63% compared to the control (Figure 2A). Nitrogen fertilization did not affect plant height, but fertilization with a 90 kg/ha at 6 WAP tends to be higher by 9.40% than without shade (Figure 2B). At 4–6 WAP, plant height was not significantly different in each treatment because growth had started to switch to the generative phase (Figure 2).

The leaf number and branches of *A. lavenia* were affected by shading but not by applying N fertilizer. Overall, at the age of 1, 5, and 6 WAP, the leaf number treated with 25% shading was significantly higher than the other treatments. The branch number of *A. lavenia* was affected by shade at 1, 4, 5, and 6 WAP. At 6 WAP plants without shading was significantly higher than the other treatments. Nitrogen fertilization did not affect the leaf and branch number, but N fertilization dose 90 kg/ha at 6 WAP tends to be higher for leaf and branch number, namely 29.54 and 33.62%, respectively, of the control (Figure 3 and 4).

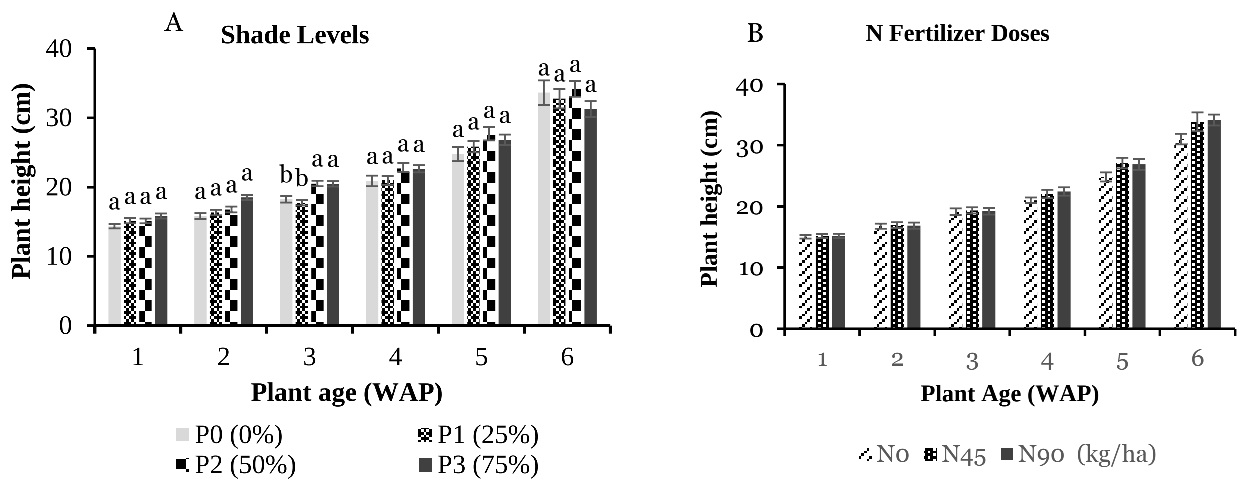


Figure 2. Bar graphs *A. lavenia* plant height at various levels of shade (A) or fertilizer (B). Note: Different letters in each column indicate significant differences results ($P < 0.05$; Duncan's test)

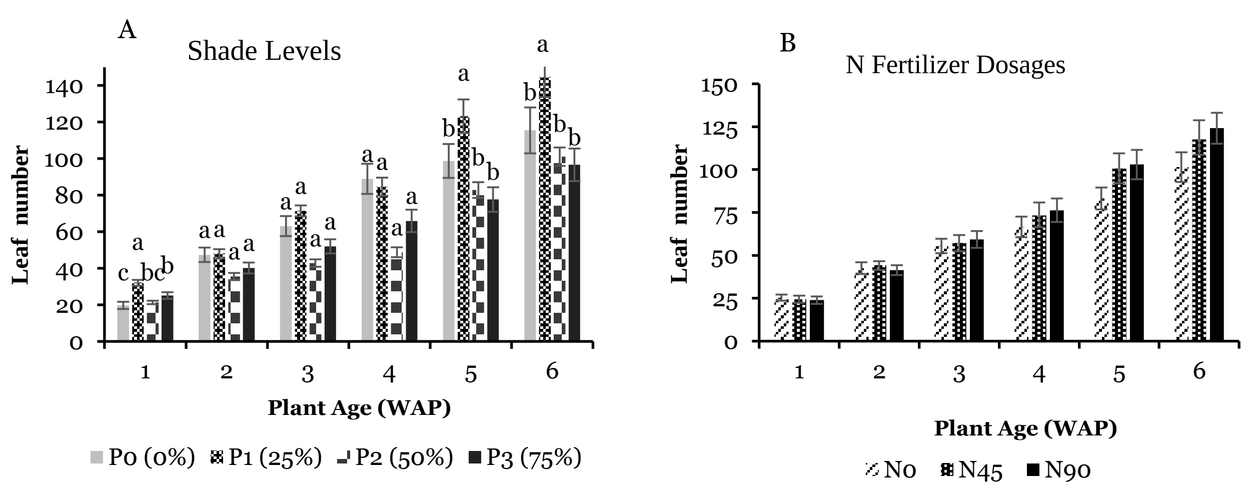


Figure 3. Bar graphs leaf number of *A. lavenia* at various levels of shading (A) or fertilizer (B). (Note: Different letters in each column indicate significant differences results ($P < 0.05$; Duncan's test))

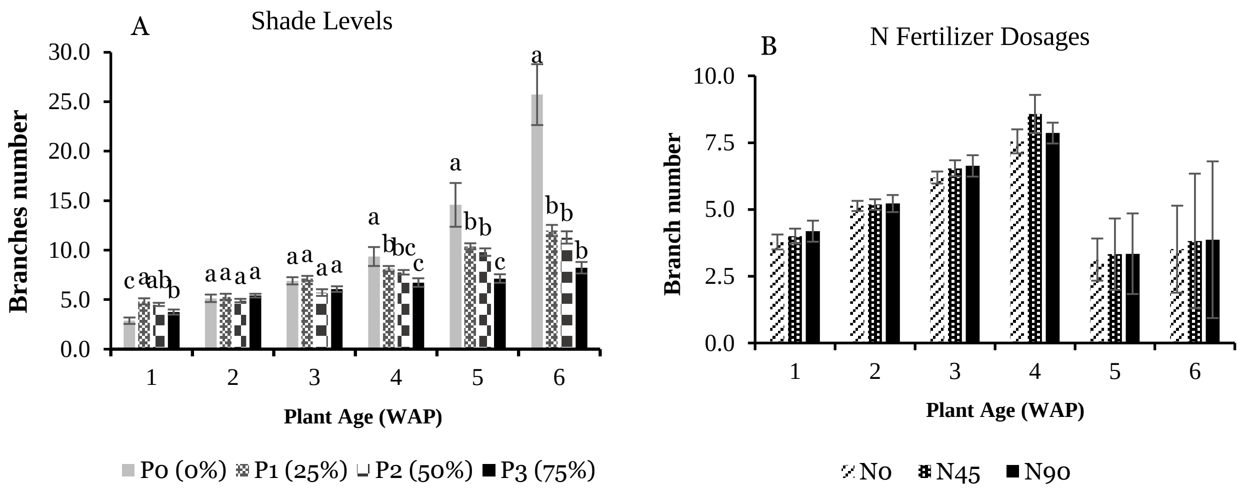


Figure 4. Bar graphs branch number of *A. lavenia* at various levels of shading (A) or fertilizer (B). (Note: Different letters in each column indicate significant differences results ($P < 0.05$; Duncan's test))

In Figure 5, the RGR value did not significantly affect by the shade and the different N fertilizers. In addition, the curve tends to decrease at 6 - 8 WAP, which indicates the plant has entered the maximum vegetative growth step. In accordance with Tustiyani *et al*., 2023, increasing plant age reduces the relative growth rate (RGR) and net assimilation rate (NAR) in *Psophocarpus tetragonolobus* (L.), which indicates that it is starting to enter the generative growth step.

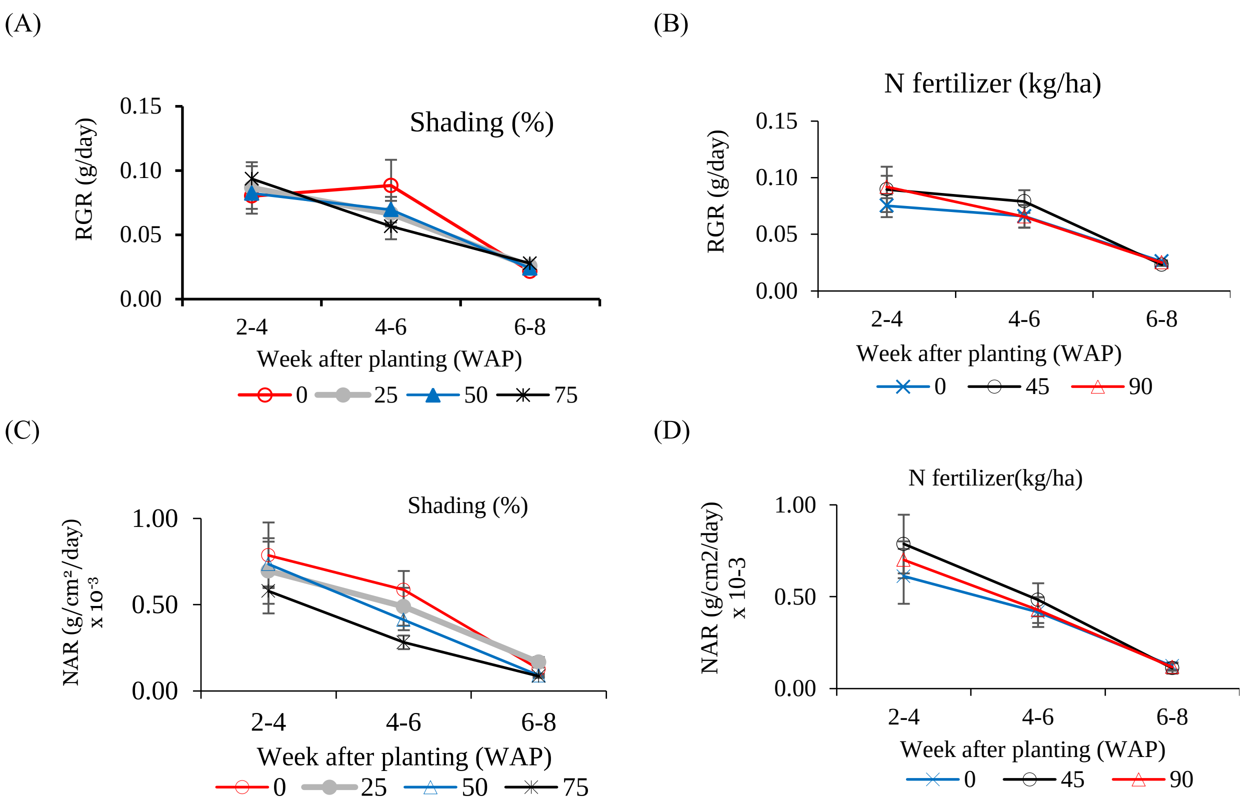


Figure 5. Average relative growth rate (RGR) (top row) and net assimilation rate (NAR) (bottom row) of *A. lavenia* due to different treatment: (A) and (C) effect of shade levels; (B) and (D) effect of N fertilizer doses.

3.3. Effect of shading or nitrogen fertilizer on the anatomy of *A. lavenia* leaves

Observations at harvest (6 WAP) showed that shading and N fertilizer did not affect dry leaf weight, leaf thickness, and stomata numbers. However, conditions without shade tended to increase leaf thickness and stomata number by 20.50 and 43.93%, respectively compared to 75% shading. The application of N fertilizer also had no significant effect on leaf/plant dry weight, leaf thickness, and stomata number (Table 1).

Table 1. Dry weight, leaf thickness, and stomata number *A. lavenia*

Treatments	Dry leaf weight (g/plant)	Leaf thickness (µm)	Stomata number (unit)
Shading (%):			
0	1.60 ± 0.22	185.16 ± 8.38	52.78 ± 4.44
25	1.88 ± 0.14	176.16 ± 9.71	49.11 ± 5.02
50	1.55 ± 0.12	170.71 ± 6.42	45.22 ± 3.22
75	1.42 ± 0.14	153.66 ± 6.23	36.67 ± 2.28
Fertilizer dosages (N kg/ha):			
0	1.30 ± 0.08	170.94 ± 8.56	47.58 ± 3.06
45	1.68 ± 0.16	169.90 ± 5.74	45.08 ± 4.65
90	1.83 ± 0.13	173.43 ± 7.91	45.17 ± 3.37

Note: Different letters in each column indicate significant differences results (P < 0.05; Duncan's test)

Table 2. Total phenolic and flavonoid content of *A. lavenia*

Treatments	Phenolics (μmol GAE/g dry leaf)	Flavonoids (μmol QE/g dry leaf)	Phenolic Production[t] (μmol GAE/ plant)	Flavonoid Production[t] (μmol QE/ plant)
Shading (%):				
0	15.83 ± 1.13b	10.81 ± 0.60b	21.41 ± 3.43b	15.50 ± 3.35
25	23.17 ± 2.87a	12.78 ± 1.12ab	35.94 ± 6.15a	21.76 ± 2.71
50	18.91 ± 2.64ab	12.16 ± 0.89b	25.49 ± 5.54ab	17.20 ± 2.42
75	24.58 ± 1.40a	15.60 ± 1.05a	30.61 ± 4.02ab	19.79 ± 2.52
Fertilizer dosages (N kg/ha):				
0	21.95 ± 2.12	12.71 ± 1.08	22.95 ± 3.84b	15.01 ± 2.00
45	17.32 ± 1.34	12.46 ± 0.84	26.28 ± 3.52ab	18.67 ± 2.32
90	22.59 ± 2.35	13.35 ± 0.91	35.87 ± 5.44a	22.00 ± 2.52

Note: Different letters in each column indicate significant differences results (P < 0.05; Duncan's test)

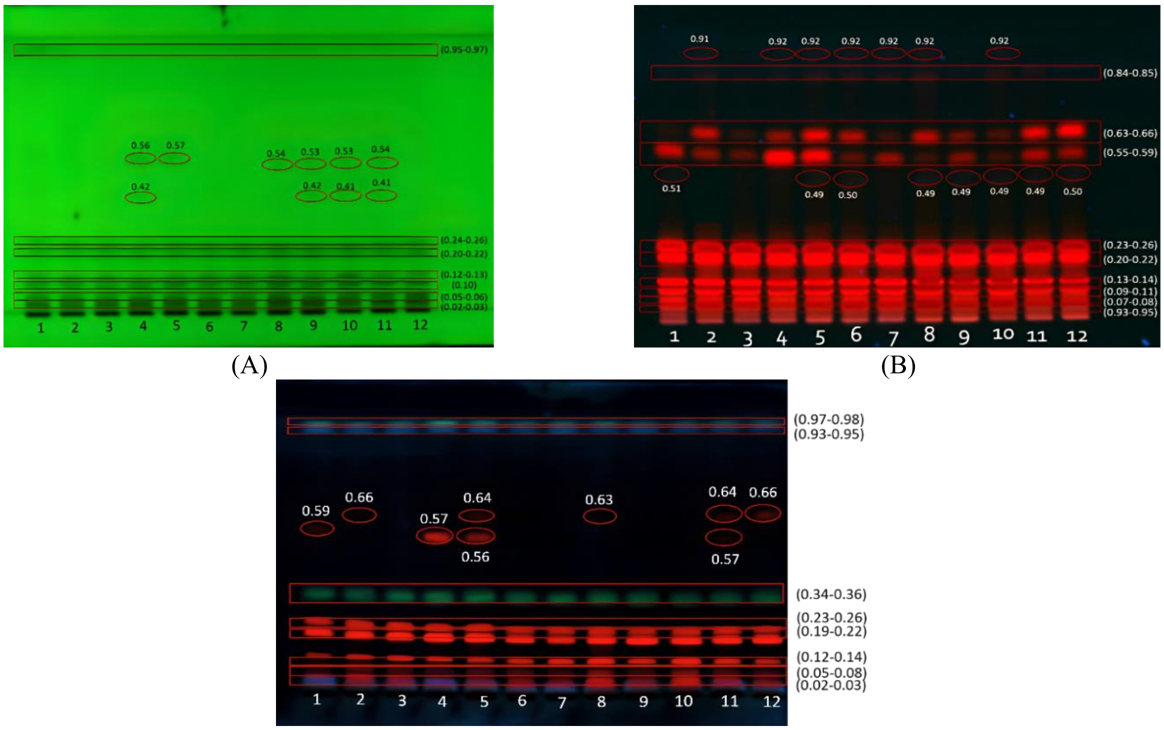
3.4. Phenolics and flavonoids production

A 25% shading increased phenolics and flavonoids total by 46.37 and 44.31%, respectively compared to without shading. The application of N fertilizer did not affect plants' total phenolics and flavonoids. However, applying N fertilizer at 90 kg/ha tended to increase phenolics and flavonoids total more than the other treatments, namely 2.92 and 5.04%, respectively of the control (Table 2). A 25% shading and 90 kg/ha N fertilizer significantly increased phenolic production by 67.87 and 40.39%, respectively from the control. Shading 25% and N 90 kg/ha also showed better production of flavonoids than control, namely 56.30 and 46.57%, respectively (Table 2). Significant differences in the production of phenolics and flavonoids highly depend on the dry weight per plant produced or their total content.

3.5. Thin layer chromatography

Figure 6 showed that the possibility of the entire methanol extract of the *A. lavenia* dry leaf contains flavonoid compounds indicated by the brown band at UV 254 nm and the green band at UV 366 (Yusnawan & Inayati, 2018). The retention factor (Rf) bands and ranges can be seen in Figure 6 C, for flavonoids green bands appear in the Rf range 0.34–0.36 after spraying with sulfuric acid under UV 366.

Methanol extract in certain Rf ranges has different band thicknesses. Thick bands indicate abundance or higher the concentration. The analysis of variance showed that neither shading nor N fertilizer gave a significant difference. However, based on the area under the curve (AUC) value of the compounds, it is suspected that the flavonoids tended to be high in conditions without shade or when N fertilizer was applied at 90 kg/ha (Figure 7).



(C)

Figure 6. Chromatogram of *A. lavenia* (A) UV 254 nm; (B) UV 366 nm; and (C) H2SO4 at UV 366. Note: (1) P0N0, (2) P0N45, (3) P0N90, (4) P1N0, (5) P1N45, (6) P1N90, (7) P2N0, (8) P2N45, (9) P2N90, (10) P3N0, (11) P3N45, (12) P3N90. (P = shading (1=0%, 2=25%, 3=50%, and 4=75%), N = Nitrogen (0, 45, and 90 kg/ha)

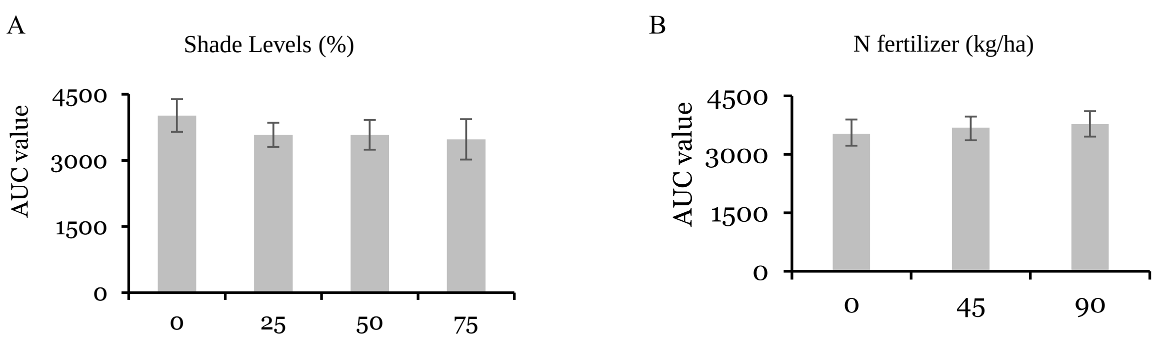


Figure 7. Bar chart of AUC values suspected to be flavonoids (Rf 6 range 0.34 – 0.36), shade treatment (A), or N fertilizer application (B)

4. DISCUSSION

In the growth of *A. lavenia*, shading at a 25% density was better than other shading treatments because it showed a higher dry weight per plant, accompanied by higher leaf and branch numbers (Figure 9). An increase in shade density causes plants to experience etiolation by stimulating growth hormones such as auxin and gibberellins in triggering the elongation of plant stem cells (Ningrum *et al*., 2020). In contrast, the leaf number and branch increased with high light intensity. This is because sufficient light for plants increases photosynthetic yields (Mawardy & Karyawati, 2021). Application of N fertilizer increases plant height, leaf number, and branch number because it affects plant growth, such as the formation of chlorophyll, cells, tissues, and plant organs (Wang *et al*., 2020). This is appropriate to Abbaspour *et al*., (2012), that plant height, branch, and leaf number can increase the dry leaf weight.

Leaf thickness and stomata number increased in no-shade conditions. According to Setiawati *et al*., (2018), leaves exposed to full sun adapt by thickening their tissue layers to minimize excessive light interception. The stomata number increased to adapt to the arid environment (Daningsih *et al*., 2022). A strong enough correlation exists between leaf thickness and stomata number marked in pink (Figure 9). Both play an essential role in photosynthesis and transpiration (Daningsih *et al*., 2022).

Application of N fertilizer in this study increased dry leaf weight/plant, plant height, leaf number, and branch number at 6 WAP. This is due to the optimal availability of nutrients, such as nitrogen, which can increase chlorophyll synthesis because nitrogen is a constituent element. The higher the N was given (up to the optimum limit), causing the increase in the amount of chlorophyll and also photosynthetic activity. The rate of photosynthesis can increase plant growth and size, which is closely related to the increase in plant dry weight (Sitorus *et al*., 2014).

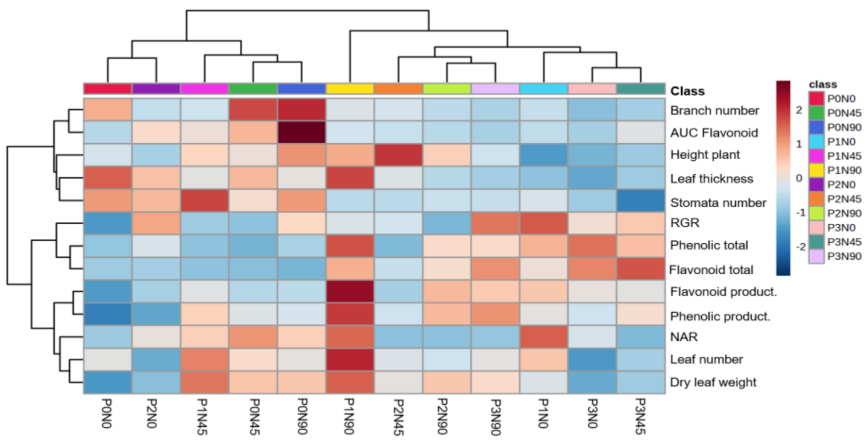


Figure 8. Correlation between variables and treatments with heatmap (Note: P = shading (1=0%, 2=25%, 3=50%, and 4=75%), N = Nitrogen (0, 45, and 90 kg/ha)

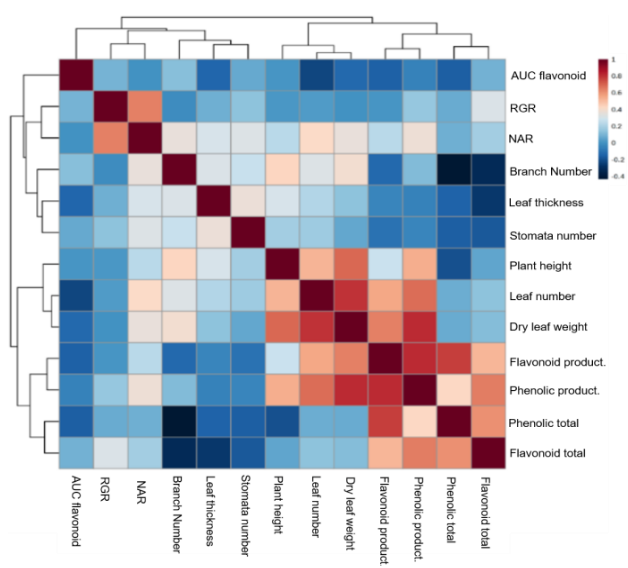


Figure 9. Correlation of research variables with heatmap. Noted: Total phenolic content (TPC) and total flavonoid content (TFC)

The increase in phenolics and flavonoids is related to the presence of the enzyme phenylalanine ammonia-lyase (PAL). PAL is one of the important enzymes in the biosynthesis process because it is the first enzyme that plays a role in metabolite biosynthesis (Gusmawan *et al*., 2022). After that, the biosynthesis process proceeds through the shikimic acid pathway to produce phenolics and flavonoids with phenylalanine as a precursor (Ekawati *et al*., 2013). According to the shikimic acid pathway, phenolic components are produced first, then phenolic, hydroxycinnamic, lignin, and flavonoids, so phenolics are higher than flavonoids (Ghasemzadeh *et al*., 2010). In addition, phenolics and flavonoids generally accumulate in the epidermal cells in the leaves (Yang *et al*., 2020).

Total phenolics and flavonoids correlate very strongly with phenolic and flavonoid production. This is because the production of metabolites is obtained by multiplying the total metabolites obtained by the dry weight used. Thus, in this study, the production of phenolics and flavonoids was also correlated with dry leaf weight, leaf number, and plant height. The best treatment of flavonoids with the AUC method with TLC sprayed with sulfuric acid and aluminum chloride reagent was different. The results of total flavonoids with aluminum chloride were better because each oxo and hydroxyl group in the methanol extract of *A. lavenia* flavonoids could form Al(III)-flavonoid chelates and were identified as total flavonoids (Shraim *et al*., 2021). While the chromatogram photo results can provide different light intensities and affect the AUC value in each band produced. The correlation results support that flavonoids with the TLC method have no relationship with other data marked in blue (Pearson correlation coefficient value <0). The treatment most affected each variable was P1N90 (25% shade and 90 kg/ha N) application (Figure 8). Different from that, based on the results of the ANOVA test because there was no interaction between shading and N fertilizer, the treatment that affected the growth, production of phenolics, and flavonoids was 25% shading or 90 kg/ha N fertilizer.

5. CONCLUSION

The results of the current study indicated that the more shade density or the application of N fertilizer 90 kg/ha increased the total phenolics and flavonoids. Plant height, leaf number, dry leaf weight, total phenolic, and flavonoids can increase the productivity of phenolics and flavonoids.

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