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Original Article

Tannins, flavonoids, and lignin levels of *clitoria ternatea* L legumes in different levels of urea fertilizer and harvesting age

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Abstract

Objective: *Clitoria ternatea* L is a legume that has the potential as animal feed. This study determined to evaluate tannins, flavonoids, and lignin levels in different urea fertilizer applications and harvesting ages. **Methods:** This study used a split-plot design with three replications. The main plots were urea levels (0 kg/ha, 100 kg/ha, and 200 kg/ha), as subplots were harvesting ages (30, 45, and 60 days after planting (DAP)). Fresh weights of *Clitoria ternatea* L harvested at the ages of 30, 45 and 60 DAP were weighed and recorded. The weighed forage is put in newspapers and dried in a 55°C oven for three days to gain a constant dry weight. The dried samples were then ground using a willey mill with a sieve porosity of 1 mm. Furthermore, the sample is analyzed to determine the content of secondary metabolites. The parameters observed were tannins, flavonoids, and lignin levels. Analysis of variance (ANOVA) was used to analyze Research data, and Duncan's Multiple Range (DMRT) was used to analyze the differences between means. **Results:** The lowest lignin content of *Clitoria ternatea* L was prodiced in non-urea treatment (20.64± 2.53%), and significantly different compared to various levels of urea fertilization treatment (P<0.05). The highest tannin levels were obtained at 30 DAP harvest age (4.20±0.56%) and significantly different (P<0.05) from tannin levels at 45 and 60 DAP. The lowest flavonoid levels were obtained when the plants were harvested in 60 DAP (0.87±0.05%) and significantly different (P<0.05) compared to 30 and 45 DAP. **Conclusions:** The increase of urea fertilization level in *Clitoria ternatea* L only significantly affected lignin levels. The decrease of lignin content value is linear with the increase of harvesting age. In contrary, harvesting age of *Clitoria ternatea* L showed significant linear effects on tannins and flavonoids value.

Keywords: *Clitoria ternatea* L; Flavonoid; Harvest age; Lignin; Tannin; Urea

INTRODUCTION

Clitoria ternatea L is a type of leguminous plant that has the potential to be used as animal feed because of its high nutritional content, adaptive ability, and being favored by ruminants. *Clitoria ternatea* L could grow well on all soil types and is resistant to dry conditions [1]. Plants always carry out primary metabolism and secondary metabolism to maintain their survival. Therefore, *Clitoria ternatea* L generally has a high level of lignin, accompanied by secondary metabolites in flavonoids and tannins.

Tannins can be found in almost all green plants, both in higher and lower plants with different levels and qualities. Flavonoid compounds of plants are red, purple, blue, and yellow dyes [2]. Flavonoids are found in almost all parts of plants, including fruits, roots, leaves, and the outer bark of stems. Flavonoids in plants play a role in giving colour, taste, and aroma to seeds, flowers, and fruit, as well as protecting plants from environmental influences antimicrobials, and protectors from exposure to ultraviolet rays [3]. The function of flavonoids is for the physiological survival of the plant itself. Flavonoids have other benefits, such as antifungal agents and traditional medicine. Most flavonoids come from biosynthesis (about 2% of all carbon plants photo synthesize), which is converted into tannins, so these flavonoids are one of the largest natural phenols [4]. Plant productivity, nutrient content, lignin content, digestibility of feed ingredients, secondary metabolic substances, and antinutrient content are indicators to determine the quality of a feed ingredient. Numerous studies have investigated the effects of fertilization to Plant productivity, nutrient content, and secondary metabolic substances. Total yield and components were significantly affected by the application of nitrogen fertilizer rates [5]. It corresponds to the stage of plant development (ontogeny) from seed to maturing stages (flowering and fruiting). There are some specific characters associated with each stage [6].

Tannins, flavonoids, and lignin involve the main components of plants that support the existence of plants since their functions are vital as differentiators, protectors, and strengthen plants themselves. Contrary to animal feed, these three components are anti-nutritional, which can reduce the quality of roughage. However, certain amounts of tannins and flavonoids have a positive effect on digestibility, mainly if associated with the mitigation of greenhouse gases. The purpose of this study was to evaluate the content of tannins, flavonoids, and lignin of *Clitoria ternatea* L grown and fertilized at several levels of urea and harvested at different ages.

MATERIALS AND METHODS

Soil and climate information

The study was in Kembang Village, Nanggulan District, Kulon Progo Regency, Yogyakarta Special Region Province. This study was carried out in May until November 2021. The soil type in this study was grumusol, which has a very high coefficient and contraction. If there is no irrigation, it will dry up, expand and crack. Geographically, Kulon Progo Regency is between 7^o 38'42" to 7^o 59'3" south latitude and between 110^o 1'37" to 110^o 16'26" east longitude. The study site have an temperature 28 to 33^{o}C, humidity from 78 to 80%, rainfall 2.80 to 270.10 mm/monthly, and sunlight exposure 50.21 to 87.46% in average [7].

Experimental design

This study used a split-plot design consisting of three rates of urea fertilization (0 kg/ha, 100 kg/ha, and 200 kg/ha) as the main plot and three harvest ages (30, 45 and 60 days) as subplots, each performed with three replications, 3×3×3 so there are 27 plots. The size of each was a 75 cm × 75 cm plot. Each plot consists of four plants, so the total *Clitoria ternatea* L used was 108.

Plant cultivation

Land preparation begins with clearing and processing the land, using a hoe to loosen the soil. There were 27 plots, and its size were of 75 cm × 75 cm. 6 weeks aged *Clitoria ternatea* L seeds were planted with 7-10 cm depth planting holes. Each plot consist of four *Clitoria ternatea* L plants. Fertilization was carried out when it was planted, according to their respective treatments. The fertilizer level are 0 grams/plant, 5.6 grams/plant and 11.25 grams/plant. Fertilizer was sown around the plant (top dressing). *Clitoria ternatea* L plants maintenance was carried out by watering, and weeding. Watering was done once a day in the afternoon. Embroidery was done by replacing dead plants with new ones by looking at the conditions at the research site. *Clitoria ternatea* L harvesting age was carried out at 30, 45 and 60 days after planting.

Lignin, flavonoid, and tannin determination

The fresh weights of *Clitoria ternatea* L harvested at the ages 30, 45 and 60 DAP were weighed and recorded. The weighed forage is put in newspapers and dried in a 55°C oven for three days to gain a constant dry weight. The dried samples were then ground using a Willey mill with a sieve porosity of 1 mm. Furthermore, the sample is analyzed to determine the content of secondary metabolites. The tannin and flavonoid content analysis used the spectrophotometry method [8]. The lignin content analysis used the Chesson method [9]. Determination of the final content of tannins and flavonoids using a spectrophotometer and the three parameters are expressed in percent (%).

Statistical analysis

The data on tannins, flavonoids, and lignin levels were processed using analysis of variance (ANOVA). If there was a significant difference between treatments, further tests were carried out using Duncan's Multiple Range Test [10]. Data processing using SPSS 23 Software.

RESULTS

Levels of flavonoids, tannins, and lignin (%) in *Clitoria ternatea* L at different levels of urea fertilizer and harvest time are showed in the Table 1.

Tannin level

Different harvest age treatments showed a significant effect (P<0.05) on tannin levels. In figure 1, it can be seen that the tannin content tends to decrease along with increasing harvesting age. *Clitoria ternatea* L plants at 30 DAP harvest Time contained the highest tannin with an average of 4.20% and then decreased by 30.48% at 60 DAP and 20.95% at 45 DAP harvest Time. The tannin levels of the *Clitoria ternatea* L observed were not much different from those in other studies. Kumar observed several varieties of CT on the leaves and roots (1.01-3.19%) [11] but tended to be higher than the report by Sutedi, who reported the tannin content of *Clitoria ternatea* L is 1.11% [12]. Other legumes forage, koro beans have tannin levels of 1.37-6.53%, 1.26-1.31 in sword koro straw [13]. Compared to 30 DAP harvest Time, tannin content at 45 DAP harvest was decreased by 20.95% and became 30.48% at 60 DAP harvest Time. This condition may be caused by young plants defending themselves harder from pest predators.

Flavonoids levels

Harvest age significantly affected the flavonoid content of *Clitoria ternatea* L. The older the harvest, the lower content of flavonoids. The lowest flavonoid content was 0.87±0.05% at 60 DAP harvest age which was significantly different (P<0.05) with 45 DAP (1.19±0.11%) and 30 DAP (1.32±0,23%) harvest Time. The younger plant showed higher flavonoid levels. This condition is related to young plants, which tend to be more susceptible to environmental stress than older plants. The function of flavonoids is as a plant protection agent to fight pests and diseases. This is in line with Vittori, who stated that flavonoid levels are influenced by harvest age and the environment [14].

Based on figure 2, it can be seen that flavonoid levels in *Clitoria ternatea* L at different harvest ages ranged from 0.87-1.32%. This result is lower than the study of Styawan which states that the flavonoid content in *Clitoria ternatea* L flower extract is 4.65% [15]. This difference in result was

Table 1. Levels of flavonoids, tannins, and lignin (%) in *Clitoria ternatea* L at different levels of urea fertilizer and harvest ages

Variable	Harvest Time (DAP)	Urea levels			Average
		0 kg/ha	100 kg/ha	200 kg/ha	
Tanin (%)	30	4.14 ± 0.26	4.49 ± 0.86	3.96 ± 0.48	4.20 ± 0.56^{l}
	45	2.85 ± 1.38	3.69 ± 0.21	3.42 ± 0.01	3.32 ± 0.79^{k}
	60	2.95 ± 0.12	2.95 ± 0.17	2.86 ± 0.29	2.92± 0.18^{k}
	Average	3.32± 0.94	3.71± 0.81	3.41± 0.55	
Flavonoid (%)	30	1.33 ± 0.16	1.16 ± 0.19	1.48 ± 0.27	1.32 ± 0.23^{l}
	45	1.31 ± 0.08	1.17 ± 0.01	1.09 ± 0.07	1.19 ± 0.11^{l}
	60	0.83 ± 0.05	0.88 ± 0.06	0.91 ± 0.02	0.87± 0.05^{k}
	Average	1.16 ± 0.26	1.07 ± 0.17	1.16 ± 0.28	
Lignin (%)	30	17.94± 0.88	20.62± 2.06	21.23± 2.04	19.93 ± 2.4^{k}
	45	21.09 ± 1.77	22.28 ± 0.18	22.84 ± 0.95	22.07± 1.27^{l}
	60	22.88 ± 1.76	23.13 ± 0.86	25.76± 2.02	23.92 ±1.97^{m}
	Average	20.64 ±2.53^{a}	22.01 ±1.57^{b}	23.28 ± 2.49^{b}	

^{a,b,c} Different superscripts in the same column indicate differences (P<0.05).

^{k,l,m} Different superscripts on the same line indicate differences (P<0.05).

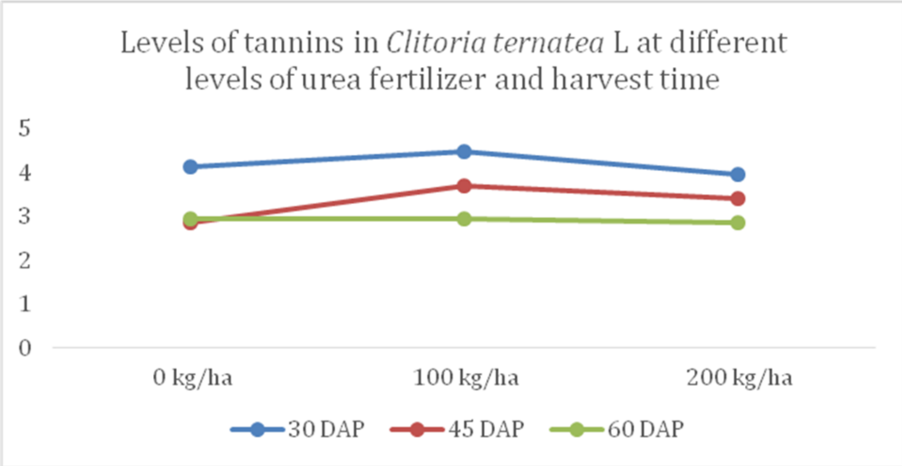

Figure 1. Levels of tannin in *Clitoria ternatea* L at different levels of urea fertilizer and harvest ages

due to the sample test using flowers with higher blue pigment accumulation. As is well known, the colour pigments in flowers indicate high flavonoid levels.

Lignin level

In figure 3, it can be seen that the level of urea fertilization and harvest age had a significant effect (P<0.05) on the lignin content of the *Clitoria ternatea* L plant. Lignin content without fertilization was 20.64±2.53%, significantly lower than which was fertilized with urea 100 kg/ha (22.01 ±1.57%) and 200 kg/ha (23.28±2.49%), although there was no difference between the doses given.

The levels of lignin were significantly different in each harvesting age, and the highest level was achieved at 60 DAP at 23.92±1.97%, followed by 45 DAP (22.07±1.275) and 30 DAP (19.93±2.4%). Compared to 30 DAP, the lignin content of 45 DAP increased by 10.74%, and 60 DAP increased by 20.02%.

Correlation and interaction

Based on the results of statistical analysis (Table 1) shows that there is no interaction (P>0.05) between the application of urea fertilizer levels and harvesting age to tannin, flavonoid, and lignin levels. The highest tannins level was at 100 kg/ha urea fertilisation with 30 DAP harvesting age (4.49±0.86%), and the lowest was 0 kg/ha at 45 DAP (2.85±1.38%). The highest flavonoid levels was at 200 kg/ha urea fertilisation at 60 DAP (1.48±0.27%) harvesting age and the lowest was at 0 kg/ha at 30 DAP (0.83%±0.05%). The highest lignin levels was at 200 kg/ha urea fertilisation with 60 DAP harvesting age (25.76± 2.02%), and the lowest was 0 kg/ha and 30 DAP (17.94±0.88%).

DISCUSSION

Metabolism in the high-level plantation is divided into two: primary metabolism, which

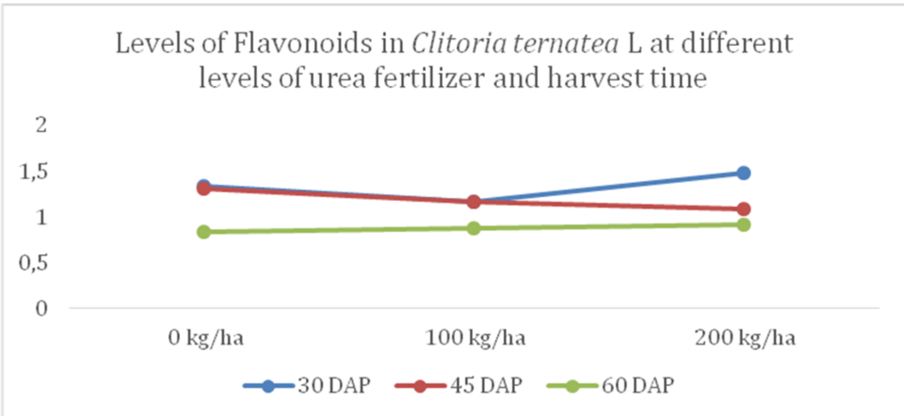

Figure 2. Levels of Flavonoids in *Clitoria ternatea* L at different levels of urea fertilizer and harvest ages

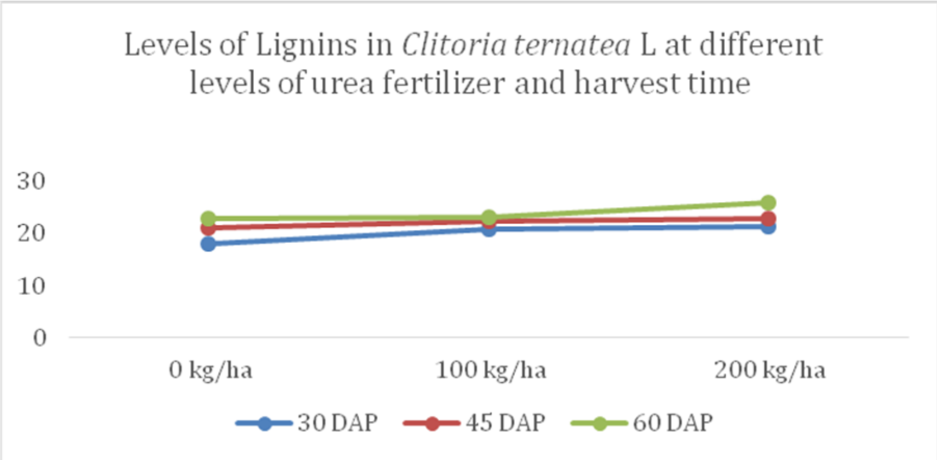

Figure 3. Levels of Lignins in *Clitoria ternatea* L at different levels of urea fertilizer and harvest ages

will produce primary metabolites, and secondary metabolism, which produces secondary metabolites. In plants, primary metabolism is carried out to maintain their survival.

The basic material of secondary metabolites is derived from primary metabolism, broadly divided into acetyl coenzyme-A, shikimic acid, mevalonate acid, and methylerythritol phosphate. Based on this introductory material, secondary metabolites are formed through the acetate malonate, shikimate, mevalonate, and methylerythritol phosphate pathways [16]. Plants use secondary metabolism to maintain their life from biotic and abiotic attacks. Secondary metabolism mainly functions to resist predators and pathogens [17]. Production of secondary metabolites occurs outside the biosynthesis of carbohydrates and protein pathways. Secondary metabolite compounds are also found in various types of single beans with phenolic and antioxidant content [18]. Secondary metabolites in *Clitoria ternatea* L include flavonoids and tannins. In addition, it also contains lignin, that is produced from primary metabolic processes.

Treatment of urea fertilizer level had no significant effect (P>0.05) on tannin levels. This is possible because the nature of the tannin compound is determined by the physiological conditions of the plant and environmental stresses, so it is not affected by the application of urea fertilizer. The accumulation of secondary metabolites of tannins in plants is influenced by environmental stresses such as salinity and drought [19].

Different harvesting age treatments showed a significant effect (P<0.05) on tannin levels (Table 1). Tannin levels decrease along with harvesting age. Young *Clitoria ternatea* L plants contain higher levels of tannins compared to older plants. This condition may be due to young plants carrying out self-defense from pest predator attacks, so tannin compounds produced in higher level than older age. The high tannin levels in pearl variety soybeans causing plants to be more resistant to Ophiomyia phaseoli seedling fly attacks [20]. Tannin levels in these plants are lower than in koro beans [21].

There was no interaction between the level of urea fertilizer application and harvest age to tannin levels. This is because the number of tannin levels in *Clitoria ternatea* L plants depends on plant age, pathogen attack, and environmental stress, as long as there is no pathogen attack during their life and environmental conditions support their growth. Condensed tannins are secondary metabolites with a broad spectrum against environmental stressors and predators. Tannins are also known as limiting factors for protein digestibility [22]. Tannins can bind protein and reduce protein availability [23]. Under natural conditions, the higher the protein content in the plant, the higher the tannin content and activity in the plant [24].

Urea fertilizer level had no significant effect on flavonoid levels (Table 1). This is due to the site conditions that are farmland. The irrigation is regular so that the soil water content is relatively high. Groundwater dramatically affects the levels of flavonoids in plants. The lower the soil water content, the lower the flavonoid and anthocyanin content in plants [28]. This condition is in line with a statement that said that humic acid addition in combination with urea showed that neither treatment nor interaction had a significant effect on the levels of flavonoids produced [25].

Different results were said that nitrogen dose can affect flavonoid content [26]. This due to nitrogen is one of the constituents of amino acids, where the amino acid is one of the flavonoid precursors through the shikimate pathway. This is presumably due to the nature of flavonoid compounds strongly influenced by plant type. Flavonoid levels are influenced by the type of plant, post-harvest handling, and processing. Flavonoids also include secondary metabolites, whose biosynthesis is genetically controlled and influenced by the environment, including temperature, light, water, habitat, and nutrients [4].

Plants that interact intensely with pathogens, pests, or biotic and abiotic stresses, will activate various defence mechanisms, including the induction of secondary metabolite biosynthesis such as flavonoids. One of the flavonoid functions is protecting plants from various biotic and abiotic stresses [4]. In addition, plant age affects the availability of primary metabolic products produced by plants, where primary metabolism is a precursor to the formation of flavonoids. It is suspected that one of the precursors is a protein contained in plants in the form of amino acids.

Harvesting ages have a significant effect ($P<0.05$) on flavonoid levels of *Clitoria ternatea* L plants (Table 1). Flavonoids decrease along with the harvest age. Younger plant ages show higher flavonoid levels. This condition is due to the function of flavonoids substances as plant protection from pests and diseases. Younger plants are still in the process of growing, so it is necessary to defend themselves from external threats. Plants that interact with pathogens, pests, or biotic and abiotic stressors, will activate various defense mechanisms, including bio synthesis induction of secondary metabolites such as flavonoids. Flavonoids, one of which serves to protect plants from various biotic and abiotic stresses [27]. The flavonoid levels are influenced by harvesting age and the environment [28].

The lignin content gave a positive response to the fertilization treatment. It is possible due to nitrogen content in the soil and its elements absorbed by plants, thus affecting the lignin content. Element of nitrogen, which is easily soluble in the soil, makes it easier for roots to absorb water in the soil, causing plants to contain more water which has an impact on slowing the lignification of plant parts [29]. In addition, fresh plants with higher water content automatically reduce lignin levels in their calculations.

As the age of harvest increases, the lignin content increases consistently. Cellulose and lignin are determinant factors of plant fibre strength; increased lignin content strengthens plant cell wall resistance. Cellulose is a component of crude fibre that blocks the process of breaking down the cell walls of feed ingredients by microbes or enzymes that work in the rumen, resulting in low digestibility [30]. The combination of lignin and cellulose (lignocellulose) is the most abundant component in older plants. So it is clear that the lignification process will be more massive to support plant growth.

Plant flowering decreased forage quality due to the translocation of dissolved carbohydrates from stems and leaves to inflorescences [30]. It can increase the relative proportion of cell walls and lignification in leaves and stems. Forage cut at old age will increase crude fiber concentration produced from the lignification process. This is in line with the opinion that old forage had high levels of lignin and crude fiber.

CONCLUSION

The increase in urea fertilization amount had effect on *Clitoria ternatea* L's lignin content. The greater the harvest age of these legumes, the more their tannin and flavonoid levels tend to decline while their lignin content continually increases.

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CONFLICT OF INTEREST

The authors declare no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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