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The Effects of Different Rates of Chicken Manure and Harvest Intervals on the Bioactive Compounds of Bitter Leaf (*Vernonia amygdalina* Del.)

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Abstract

Bitter leaf (*Vernonia amygdalina* Del.) is a valuable functional vegetable and traditional medicinal plant in many tropical countries including Indonesia. Bitter leaf is commonly used to remedy hypertension and diabetes among Indonesian people. The leaf extract comprises bioactive compounds such as sesquiterpene lactones, steroid glycosides, and flavonoids. Despite its growing market demand, there is insufficient data on agronomic practice in order to obtain optimum yield with high bioactive compounds. The experimental design used was a randomized complete block design with two factors namely chicken manure rates (0, 2.5, 5, and 7.5 kg per plant) and harvest intervals (2 and 3 months). There was a significant difference in fresh and dry weights of leaves, chlorophyll a and b, carotenoids, nitrogen concentration, anthocyanins, and flavonoids among different manure treatment. The highest concentration and production of bioactive compounds on the bitter leaf was found on the highest treatment of 7.5 kg chicken manure per plant. The harvest interval of three months significantly increased fresh and dry weights of leaves, concentration of anthocyanins, phosphorus, and potassium, uptake of phosphorus and potassium, and production of anthocyanins and flavonoids. Cultivation using chicken manure with frequent period of pruning can enhance the production of biomass as well as secondary metabolites in bitter leaf.

Keywords: flavonoids,functional vegetable, medicinal plant, nutrients uptake, secondary metabolites

Introduction

Vernonia amygdalina, commonly known as bitter leaf, belongs to the family Asteraceae. It is an evergreen tree that can grow up to 10 m but are commonly seen as shrub as a result of frequent pruning. *Vernonia amygdalina* is well known as a traditional medicinal plant across tropical countries (Grubben and Denton, 2004). Each part of the plant can be used for treating various diseases (Oyeyemi et al., 2017). The fresh leaf of the plant is usually consumed to treat diabetes, malaria, fever, and high blood pressure (Asante et al., 2016). Studies show that leaf extracts of *V. amygdalina* contain sesquiterpene lactones (Kupchan et al., 1969) saponins, flavonoids, steroids, and coumarins (Tona et al., 2004).

Limited data are available on effective cultivation of bitter leaf, and in order to comply to the Good Agriculture Practice for Medicinal Crops (Ministry of Agriculture Act Number 54, 2012), we aimed to determine the effects of organic fertilizer on bitter leaf quality and production. Application of organic fertilizers could potentially enhance both production of harvested plant parts and manufacture of secondary metabolites (Dumas et al., 2003; Abd-Alla et al., 1999). Antioxidant concentration in tomatoes was higher when treated with organic fertilizer than with inorganic fertilizer (Dumas et al., 2003). Organic fertilizer can change the chemical and physical properties of soil resulting in increased efficiency of nutrient absorption in faba bean, soybean, and lupin (Abd-Alla et al., 1999). Additionally, organic fertilizers, such as manure, is proven to improve physical, chemical, and biological soil properties, and to ensure the sustainability of soil health (Khaliq et al., 2006).

Application of organic fertilizer was found to promote vegetative growth, yield potential, and phytochemical content of *Corchorus olitorious* (Ghoneim and El-Araby, 2003). The use of chicken manure provided the best plant growth and yield results because it has higher macro- and micro-nutrients contents than compost and cow manure. Chicken manure was also found to increase secondary metabolite compounds. According to Ibrahim et al. (2013), total phenolics, flavonoids, ascorbic acid, saponins, and glutathione, and increase antioxidant activity in *Labisia pumila* increased after treatment with chicken manure. Karimuna et al. (2015) also showed that the application of chicken manure raised the fresh and dry weight of *Murraya paniculata*, however, it correlated negatively with the total flavonoid content. Additionally, in *Murraya paniculata*, the combination of chicken manure and rice-hull charcoal treatment resulted in a higher leaf production and total flavonoid content than without organic fertilizer application (Taufika et al., 2017).

Besides fertilizer application, harvest interval was also reported to influence the content of secondary metabolites on cultivated plants. Taufika et al. (2017) reported that *Murraya paniculata* contained the highest concentration of anthocyanins and flavonoids at 4-month harvest intervals compared to 2- and 3-month harvest intervals. Generally, the best harvest interval in *Talinum triangulare* that generates the highest anthocyanin concentration is at 10 days after planting (Susanti et al., 2011). However, the optimum harvest interval for bitter leaf to produce a high concentration of secondary metabolites is still unknown. In this study, we determined treatment rate and harvest interval that results in greater growth and yield in *V. amygdalina*. The results of this study will be useful in cultivating these plants efficiently in order to meet increasing demands of bitter leaves.

Materials and Methods

Study Location

The experiment was conducted at Cikarawang Organic Experimental Field Station, Bogor Agricultural University, Bogor, Indonesia from December 2017 to June 2018. The average temperature during the conduct of experiment was between 25.4 to 26.6°C, with the average monthly rain fall of 181.2 to 358.9 mm. The soil and chicken manure chemistry test are presented in Table 1. The analyses of nitrogen, phosphorus, and potassium of the leaves were carried out in the Testing Laboratory, Department of Agronomy and Horticulture at IPB University. The analyses of chlorophyll, carotenoids, anthocyanins, and flavonoids were performed in the Postharvest Laboratory, Department of Agronomy and Horticulture at IPB University.

Sampling and Treatments

A total of 120 *V. amygdalina* plants (Figure 1) for this study originated from stem cuttings with length of approximately 30 cm. The plants were grouped into 40 experimental units composed of 3 plants each and planted with a 1 m × 1 m planting distance.

Figure 1. *Vernonia amygdalina* one month after pruning

A randomized block design with two factors, i.e. chicken manure treatment rates (0 or control, 2.5, 5, and 7.5 kg per plant) and harvest intervals (2 and 3 months) was used in this study. Each treatment was repeated five times. The chicken manure treatment was applied a week before planting.

Data Collection

Harvesting was executed by cutting the main stem or branches from 50 cm above the ground. Leaves were collected, air-dried for 3 days at room temperature, then dried in the oven at 60 ℃ for 24 hours. The dried leaves were pulverized for nutrients and phytochemical analysis.

Nitrogen concentration of the leaf was analyzed using the Kjeldahl method (Eviati and Sulaeman, 2009). One milligram of leaf powder was mixed with 3 mL sulfuric acid at 350°C for 3–4 hours. The clear extract was diluted with ion-free water until 50 ml. Ten mL of the solution was mixed with 10 mL of 40% sodium hydroxide. Freed NH_3 was captured using 1% boric acid until the solution turned green from red using

Conway indicator. The green solution was then titrated with 0.05 N sulfuric acid until it turned light pink.

The wet ashing method (Eviati and Sulaeman, 2009) was used to analyze leaf phosphorus and potassium concentrations. Leaf powder of 0.5 mg was mixed with 5 ml HNO_3 and 0.5 ml $HClO_4$ overnight. The mixture was heated at 100°C for 1 hour, raised to 150°C for 2 hours, and to 200°C until white sediment or clear solution was formed. The solution was added with ion-free water and left overnight. Phosphorus concentration was further measured using Shimadzu UV-1800 Spectrophotometer, while potassium concentration was further measured using Atomic Absorption Spectrophotometry Agilent 240 FS AA.

Chlorophyll, carotenoid, and anthocyanin analysis followed the method described in Sims and Gamon's (2002). A total of 20 ± 2 mg fresh leaf observed as 1st to 3rd from apex (Tjhia et al., 2018) were collected, weighed and crushed with 2 mL of 85:15% acetone tris HCl, and centrifuged at 12000 rpm for 5 minutes. Three milliliters of acetone tris was added to 1 mL supernatant. The absorbance of the solution was measured using Shimadzu UV-1201 UV-VIS spectrophotometer at 457, 537, 647, and 663 nm. The formula to quantify pigment concentrations according to Sims and Gamon (2002) were used:

Chlorophyll a ($mg.g^{-1}$) = [((0.01373×A663)–(0.000897×A537)–(0.003046×A647)× df/fresh weight sample)×893.5]/1000

Chlorophyll b ($mg.g^{-1}$) = [((0.02405×A647)–(0.004305×A537)–(0.005507×A663) ×df/fresh weight sample)×907.5]/1000

Anthocyanin ($mg.100g^{-1}$) = [((0.08173×A537)–(0.00697×A647)–(0.002228 × A663) ×df/fresh weight sample)×0.20708×100]/100

Carotenoids ($mg.g^{-1}$) = [((A470–(17.1×(Chlorophyll a + Chlorophyll b) – 9.479× Anthocyanin))/119.26×df/fresh weight sample)×550]/1000

where
A= absorbance; df = dilution factor

Flavonoids were analyzed following the protocol of Chang et al. (2002). Extraction was made by mixing 100 mg of leaf powder with 1 mL of ethanol 100% and put into the a 60°C oven and left for 60 minutes. The mixture was centrifuged for 5 minutes at 12000 rpm. One milliliter of the supernatant was added to 1.9 mL ethanol, 0.1 mL 10% aluminum chloride, 0.1 1 M mL potassium acetate, and 2.8 mL distilled water. The mixture was incubated at room temperature for 30 minutes. The absorbance of the mixture was measured at wavelength 415 nm. A standard curve was constructed by 0–400 mg/L quercetin.

Statistical Analysis

Data were analyzed using the Fisher test, followed by the Duncan Multi-Range Test at a significance level of 5% using STAR (Statistical Tool for Agricultural Research) Nebula (IRRI, 2018).

Results

Data obtained from soil chemistry test using two months as harvest interval with four different rates of chicken manure treatment are shown in Table 1. Adding 7.5 kg of chicken manure to *V. amygdalina* plants results in highest retention of nitrogen, C-organic, K_2O and P_2O_5. These soil components decreased consistently as treatment of chicken manure was reduced (Table 1). These trends were also similar to those from the three-months harvest interval (Table 2).

The Effect of Chicken Manure Rates

As the rates of chicken manure were increased, the fresh and dry weight of the leaf, nitrogen concentration and uptake, and concentration of chlorophyll a, chlorophyll b, and carotenoids in the bitter leaf also increased (Table 3). Moreover, analysis of leaf anthocyanins showed that increasing the rates of chicken manure could increase the production of leaf anthocyanins. Meanwhile, concentrations of phosphorus, potassium, anthocyanins, and flavonoids, uptake of phosphorus and potassium were not affected by the chicken manure application.

The Effect of Harvest Interval

The effect of harvest interval on *V. amygdalina* plants are shown in Table 4. The fresh and dry weight of leaves, uptake of nitrogen, phosphorus, and potassium, concentration of anthocyanins, and production of flavonoids rose with longer harvest interval of 3-months. On the other hand, concentrations of phosphorus and potassium of bitter leaf were slightly higher at 2-month harvest interval. Concentrations of nitrogen, chlorophyll a, chlorophyll b, carotenoids, and flavonoids were not affected by the harvest interval treatment (Table 4).

Table 1. Soil chemical properties in set-up with harvest interval of two-months tested at the end of the experiment

Chemical indicator	Chicken manure	Soil chemistry before treatments	2 months harvest interval			
			0	2.5	5	7.5
pH_2O	7.97	5.92 slightly acidic	6.39 slightly acidic	6.64 neutral	6.9 neutral	6.9 neutral
pKCl	-	5.34 neutral	5.61 neutral	6.26 slightly alkaline	6.63 alkaline	6.6 alkaline
N (%)	1.64	0.25 moderate	0.25 moderate	0.35 moderate	0.41 moderate	0.42 moderate
C-organic (%)	19.12	2.17 moderate	2.05 moderate	2.68 moderate	2.92 moderate	3.35 high
K2O (mg K_2O/100g)	1.60%	31.4 moderate	12.43 low	94.93 very high	133.13 very high	144.08 very high
P (mg P_2O_5 /100g)	5.14%	82.1 very high	74.12 very high	406.74 very high	744.44 very high	816.98 very high

Table 2. Soil chemical properties in set-up with harvest interval of three-months tested at the end of the experiment

Chemical indicator	3 months harvest interval			
	0	2.5	5	7.5
pH_2O	6.4	6.38	6.71	6.76
	slightly acidic	slightly acidic	neutral	neutral
pKCl	5.58	5.83	6.46	6.54
	neutral	neutral	slightly alkaline	alkaline
N	0.24	0.29	0.32	0.38
(%)	moderate	moderate	moderate	moderate
C-organic	2.17	2.38	2.68	3.02
(%)	moderate	moderate	moderate	high
K_2O	2.28	50.27	137.83	141.55
(mg K_2O /100g)	very low	high	very high	very high
P	89.82	129.58	322.4	461.06
(mg P_2O_5/100g)	very high	very high	very high	very high

Discussion

Administering higher rate of chicken manure promoted higher leaf production in *V. amygdalina* as demonstrated in the increasing fresh and dry weight of leaves. According to Nwite and Alu (2017), the ability of chicken manure in improving the physical, chemical, and biological properties of soil can support the ideal growth of corn. The result of the soil chemistry test (Table 1 and 2) in this study proved that as the chicken manure rate increased, the soil acidity is reduced. The plant samples in this study grow better in the neutral than acidic soil. With the application of chicken manure, the soil acidity was reduced and the concentration of macronutrients was increase (Duruigbo et al., 2007) . The degree of soil acidity will affect the solubility and availability of ions for plants. If the pH of the soil becomes more acidic, aluminum, iron, manganese, copper, and zinc ions will dissolve even more (Duruigbo et al., 2007).

The increasing availability of macro nutrients in soil by application of higher chicken manure rates are shown in Tables 1 and 2. The nitrogen, phosphorus, and potassium concentration in the leaves (Table 3) were also increasing as the concentration of soil nutrients increased. Finally, the uptakes of three major nutrients, nitrogen, phosphorus, and potassium, also increased. Ewulo et al. (2008) study demonstrated that application of chicken manure reduces soil density, increases humidity, and increases nutrient concentration of the soil, which causes the uptake of N, P, and K nutrients to increase. A similar finding to Ewulo et al. (2008) was reported in tomato, i.e.

increasing soil density would reduce the absorption of N, P, K, Ca, and Mg (Adekiya and Ojeniyi, 2002). In addition to root morphology, soil structure and moisture are important factors in nutrient absorption from the soil (Baldwin, 1975).

The increasing nitrogen concentration and uptake is correlated with the increase in leaf production of bitter leaf. Nitrogen is a determinant element of plant growth. According to Evans (1989), nitrogen increases plant growth which in turn increases photosynthetic activity. Leaf photosynthetic capacity is associated with nitrogen concentrations as the proteins in the Calvin and thylakoid cycles represent most of the nitrogen in the leaves. Therefore, high nitrogen can increase photosynthesis which then promotes vegetative and generative growth, which ultimately increases crop production.

The increase of mineral concentration and uptake was accompanied by an increase in the concentration of chlorophyll a, chlorophyll b, and carotenoids. According to Tatjana et al. (2007), increase in pigment concentration is influenced mainly by an increase in the higher nitrogen concentration, along with the increase in the fertilizer rate found in wheat. Marschner (2012) also stated nutrients that are directly related to chlorophyll are nitrogen, because nitrogen is one of the main components of chlorophyll. This was supported also by findings of Suarez (2007) that the chlorophyll concentration in corn increases along with the increase use of nitrogen from chicken manure.

Higher chlorophyll concentrations indicate a higher rate of photosynthesis (Butterly and Buzzell, 1977), and high photosynthesis rates increases plant biomass (Arntz et al., 1998). In our study, the higher the rate of fertilizer treatment, the greater the concentration of pigments, thereby increasing the rate of photosynthesis. As a result, vegetative growth also increases.

The anthocyanin and flavonoids concentration were not significantly different among rates of treatment (Table 3). However, the production of anthocyanins and flavonoids was increasing as the rates increased because the production resulted from the multiplication

Table 3. The effects of chicken manure rates on leaf and dry weights, and metabolite contents of *Vernonia amygdalina* plants

Variables	Chicken manure rates (kg per plant)			
	0	2.5	5	7.5
Leaf fresh weight (g/plant)	397.11±63.41 a	661.53±265.42 b	828.32±295.92 c	850.53±247.46 c
Leaf dry weight (g/plant)	64.10±9.37 a	107.29±47.74 b	117.57±43.03 bc	136.37±54.10 c
Nitrogen concentration (%)	4.07±0.22 a	3.96±0.20 ab	4.18±0.27 ab	4.24±0.16 ab
Phosphorous concentration (%)	0.36±0.06 a	0.35±0.06 a	0.35±0.05 a	0.36±0.04 a
Potassium concentration (%)	3.21±0.75 a	3.59±0.56 a	3.50±0.82 a	3.93±0.37 a
Nitrogen uptake (g/plant)	3.22±1.98 a	3.75±2.60 ab	4.27±1.79 bc	4.88±2.20 c
Phosphorous uptake (g/plant)	0.28±0.13 a	0.34±0.23 a	0.38±0.19 a	0.45±0.24 a
Potassium uptake (g/plant)	2.40±1.35 a	3.17±1.74 a	3.77±2.44 a	4.56±2.27 a
Chlorophyll a ($mg.g^{-1}$)	1.53±0.19 b	1.60±0.12 ab	1.72±0.10 a	1.76±0.04 a
Chlorophyll b ($mg.g^{-1}$)	0.52±0.06 a	0.54±0.04 ab	0.58±0.04 bc	0.60±0.01 c
Carotenoids ($mg.g^{-1}$)	0.39±0.05 a	0.42±0.03 ab	0.46±0.02 bc	0.47±0.01 c
Anthocyanins ($mg.100g^{-1}$)	0.08±0.01 a	0.09±0.02 a	0.09±0.01 a	0.09±0.02 a
Flavonoids ($mg.g^{-1}$)	22.66±0.31 a	23.33±1.50 a	22.24±0.39 a	21.68±1.51 a
Flavonoids production (mg/plant)	1706.7±399.8 a	2357.1±1085.5 b	2360.1±693.8 b	2726.8±1073.6 b
Anthocyanins production (mg/plant)	0.51±0.24 a	0.72±0.44 b	0.81±0.43 b	0.88±0.39 b

Note: Values followed by different letters within the same row showed significant differences in Duncan Multiple Range Test (α= 5%)

of the concentration and the leaf production. Highest production of both anthocyanins and flavonoids and the highest leaf production were associated with the highest rate of chicken manure. Ibrahim et al. (2013) noted that the use of chicken manure could increase the production of phenolic compounds, especially flavonoids. This, according to Ibrahim and Jaafar (2011) and Weibel et al. (2000) is due to the fact that organic fertilizers, including chicken manure, generally contains several important micronutrients for plant metabolism. Moreover, according to Bímová and Pokluda (2009), some chemical reactions in plant metabolism involve micronutrients both directly and indirectly. Plants that are cultivated organically tend to produce high secondary metabolites.

In this study, we found higher leaf production in plants that had longer harvest interval i.e. 3 months. Longer harvest interval provided sufficient time for the plant to recover after pruning. Similar results have been reported in the continuously and regularly pruned *Murraya paniculata* (Taufika et al., 2017) and *Talinum triangulare* (Susanti et al., 2011). Susanti et al. (2011) also reported that the response of *Talinum triangulare* to harvest interval is related to process recovery, rejuvenation, and source-sink. Longer harvest intervals will allow the plants to regrow longer before the next harvesting.

Higher leaf production caused higher nutrient uptake because nutrient uptake was obtained from multiplication between dry weight and nutrient concentration per plant (Table 4). The longer harvest interval causes an increase in nutrient uptake, but decrease in the nutrient concentration. It was also confirmed by soil chemistry test that soil with harvest interval of 3-months had lower nutrient concentration (Table 2) than soil with harvest interval of 2-months (Table 1). Crop's dry weight and nutrient uptake is determined by harvest intervals, and dry weight and nutrient uptake for N, P, and K have a linear relationship with harvest interval (Allhands and Overman, 1995). While longer harvest intervals can increase dry weight, it can also cause a decline in the quality of leaves, reflected by a decreased nutrient concentration, protein, or fibers in the leaves (Allhands and Overman, 1995).

Harvest interval of three-month generated higher leaf anthocyanin concentration than the two-month harvest interval, whereas flavonoids concentrations in both harvest intervals were similar. However, both anthocyanins and flavonoids production tend to be higher in the longer harvest interval. According to Aziz (2015), bioactive compound production is the result of the bioactive compound concentration multiplied by the biomass of the harvested organ (e.g. leaves). The high production of bioactive compounds does not only depend on the high concentration of the bioactive compounds but also on the fresh or dry weight of the harvested organ. In this case, the longer

Table 4. The effects of harvest intervals on leaf fresh and dry weights and metabolite contents of *Vernonia amygdalina* plants

Variables	Harvest Intervals	
	2 months	3 months
Leaf fresh weight (g/plant)	494.4±114.9 b	874.3±284.9 a
Leaf dry weight (g/plant)	72.5±12.8 b	140.1±46.0 a
Nitrogen concentration (%)	4.1±0.2 a	4.0±0.1 a
Phosphorous concentration (%)	0.4±0.0 b	0.3±0.0 a
Potassium concentration (%)	3.8±0.7 b	3.2±0.7 a
Nitrogen uptake (g/plant)	2.3±0.7 b	5.7±1.5 a
Phosphorous uptake (g/plant)	0.2±0.0 b	0.5±0.2 a
Potassium uptake (g/plant)	2.1±0.6 b	4.8±2.0 a
Chlorophyll a ($mg.g^{-1}$)	1.6±0.1 a	1.6±0.1 a
Chlorophyll b ($mg.g^{-1}$)	0.5±0.0 a	0.5±0.0 a
Carotenoids ($mg.g^{-1}$)	0.4±0.0 a	0.4±0.0 a
Anthocyanins ($mg.100\ g^{-1}$)	0.08±0.0 b	0.1±0.0 a
Flavonoids ($mg.g^{-1}$)	22.5±1.2 a	22.4±1.2 a
Flavonoids production (mg/plant)	1595.0±218.6 b	2980.3±662.1 a
Anthocyanins production (mg/plant)	0.3±0.1 b	1.0±0.2 a

Note: Values followed by different letters within the same row showed significant differences according to Duncan Multiple Range Test (α= 5%)

growing duration resulted in more biomass, therefore, its bioactive compound production were also higher than those produced with the two-months harvest interval.

Conclusion

Our study demonstrated that the application of organic manures was essential in improving the growth and secondary metabolites production of *V. amygdalina* plants. The chicken manure rate that resulted in optimum growth and high production of secondary metabolites was 7.5 kg per plant. The harvest interval of 3 months produced a higher concentration of secondary metabolites than the harvest interval of 2 months.

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