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Enhancing Leaf Flavonoid Production in Indian Camphorweed (*Pluchea indica* Less.) through the Provision of Chicken Manure

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Abstract

Indian camphorweed (*Pluchea indica* Less.) leaves exhibit antioxidant, antibacterial, anti-inflammatory, and antimicrobial activities, indicating significant potential for the pharmaceutical industry. This research aimed to determine the optimal rates of chicken manure for enhancing leaf and total flavonoid production in *Pluchea indica.* The study took place at the IPB Experimental Station in Bogor, Indonesia, spanning from July to October 2023. A completely randomized block design with a single factor (chicken manure doses) was employed: 0, 2.5, 5, or 7.5 kg per plant. Each treatment comprised three replications, each consisting of 20 plants. The results revealed that the application of chicken manure at 2.5 kg per plant led to significantly taller plants, more leaves, and tertiary branches compared to the control group. Specifically, the 2.5 kg dosage significantly increased the nitrogen content of the 7th leaf and the potassium content of the 3rd leaf. Meanwhile, the application of 5 kg of chicken manure per plant significantly boosted phosphorus content in the 3rd and 7th leaves and potassium content in the 5th and 7th leaves. However, no significant differences were observed in total flavonoid and antioxidant activity across all leaf positions with chicken manure application. Plants receiving 5 kg of manure demonstrated the highest fresh leaf weight (81.64 g) and dry weight (38.27 g), which were significantly greater than those receiving 2.5 kg per plant or no manure. Despite these variations, flavonoid production per plant did not show a significant difference with manure application.

Keywords: anti-bacterial, anti-inflammatory, antioxidant, functional food, organic

Introduction

Indian camphorweed, Indian fleabane, or Indian pluchea (*Pluchea indica* Less.) is an indigenous Asteraceae species utilized as a functional food, with a limited number of individuals incorporating its leaves as a food supplement. Recognized as a shrub capable of attaining a height of 2 meters, *Pluchea indica* is indigenous to a vast region encompassing much of Asia, India, and northern Australia (Parker, 2012). The leaf extract of *Pluchea indica* is rich in flavonoids (Yuliani et al., 2015; Tinrat, 2021), alkaloids (Mutrikah et al., 2018), saponins (Tinrat, 2021), and tannins (Lestari et al., 2020; Tinrat, 2021). Notable compounds include 1,3,4,5-tetra-O-caffeoylquinic acid and 3,4,5-tri-O-caffeoyl quinic acid (Ohtsuki et al., 2008). Within the flavonoid group of P. indica leaves, quercetin and kaempferol are the primary constituents (Suriyaphan, 2014), with the flavonoid concentration containing 81% quercetin (Andarwulan et al., 2010).

The diverse contents of *Pluchea indica* leaves include antibacterial properties (Srimoon and Ngiewthaisong, 2015; Lestari et al., 2020), anti-inflammatory effects (Pranata et al., 2021), as well as antimicrobial and antioxidant activities (Tinrat, 2021). The leaf extract of Pluchea indica finds applications in the formulation of products such as lotion bars (Tinrat, 2021), facial soaps (Komala et al., 2020), and anti-acne serums (Komala et al., 2021).

Best practices for the cultivation of *Pluchea indica* are not well-established, despite its extensive use as a medicinal plant with potential applications in the cosmetic and pharmaceutical industries. Minister of Agriculture Regulation Number 64/Permentan/OT.140/5/2013 recommends the cultivation of medicinal plants using organic fertilizer (Kementan, 2013). One such organic fertilizer is chicken manure,

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typically containing 1.64% total N, 5.14% total P, 1.6% K2O, and 19.12% C-organic (Betty, 2018). Application of chicken manure to ultisol has been shown to improve soil chemical properties, including soil pH, C-organic, total N, C/N ratio, P-available, and CEC (Walida et al., 2020). Chicken manure provides nutrients that enhance the physical and chemical properties of the soil, supporting the cultivation of plants.

The nitrogen content in chicken manure serves as a substrate for the enzyme phenylalanine ammonia-lyase (PAL), a key enzyme in the flavonoid biosynthesis pathway (Roy et al., 2022). Nitrogen is essential for regulating flavonoid biosynthesis by allocating carbohydrates for both primary and secondary metabolism (Deng et al., 2019). Phosphorus in chicken manure acts as a substrate for phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate (E4P) compounds, forming 3-Deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP) precursors in the Shikimate pathway, which participates in flavonoid biosynthesis (Tariq et al., 2023). Potassium serves as an activator for crucial enzymes involved in protein synthesis, sugar transport, and photosynthesis (Marschner, 2012).

Several studies have reported positive effects of chicken manure in increasing nutrient and flavonoid content in plants. For instance, chicken manure application to Moringa increased leaf production and total flavonoids (Rasmani, 2021). Research by Karimuna (2015) demonstrated that the highest levels of total flavonoids in orange jessamine leaves were obtained with chicken manure doses of 5 and 7.5 kg per plant. The optimal dose of chicken manure to increase the production of *Vernonia amigdala* leaf flavonoids was found to be 7.5 kg per plant (Betty, 2018). Previous research on orange jessamine plants indicated that while applying chicken manure increased the fresh and dry weight of leaves, it reduced the flavonoid concentration (Karimuna et al., 2015). Hence, this study aims to investigate if a similar effect might occur in *Pluchea indica*. Consequently, the research aims to determine the optimal application dose of chicken manure to increase leaf production and total flavonoids in *Pluchea indica*.

Material and Methods

The research was conducted from July to October 2023 at the Cikarawang experimental field, IPB, Bogor, West Java, Indonesia. Leaf tissue analysis was carried out at the AGH-IPB Testing Laboratory. The leaves were harvested at 8 WAP (week after planting). Fresh and dry leaf weights were measured. The total flavonoid analysis was carried out at the post-harvest laboratory, Department of Agronomy and Horticulture, IPB. Antioxidant activity analysis was carried out at the Biochemistry Laboratory, IPB.

The research used 2-month-old cuttings as planting materials. Rooted cuttings were planted in the field with a planting space of 1 m x 1 m in 4 m x 5 m plots. Each planting hole was applied with chicken manure according to the treatments.

Treatment

This research used a completely randomized block design with one factor/treatment, the rate of chicken manure was 0, 2.5, 5, and 7.5 kg per plant and repeated three times, totalling 12 experimental units; each the experimental unit consists of 20 plants.

Fresh and Dry Leaf Weight

Plants were harvested when having a minimum height of 45 cm from the ground surface and then pruned at 30 cm height. All leaves were separated from the branches to measure the leaf fresh and dry weights. The leaf positions of the 3rd, 5th, and 7th from the shoot of each pruned branch were separated for further analysis. The harvested leaves were air-dried and then placed in the oven at 70° C for 2 x 24 hours.

Antioxidant Activity Analysis

Analysis of antioxidant activity used dry powder leaves from the 3rd, 5th, and 7th leaf positions using 1,1-diphenyl-2-picrylhydrazyl (DPPH) as a free radical scavenging assay (Fukumoto and Mazza, 2000; Karimuna et al., 2015). Leaves were dried in an oven at 60° for 3 x 24 hours prior to analysis. The 0.1-g powder was extracted in 1 ml of MeOH in a microtube and then heated for 60 minutes at 60° C. Then the mixture was centrifuged for 5 minutes at 5° C at 12000 rpm and the supernatant was separated. DPPH was dissolved in 80% methanol 0.1 mM. The standard solution uses ascorbic acid in various concentrations (0-100 μg.ml^{-1}). The sample was placed in a 96-well microplate reader with 22 μl and added 200 μl of DPPH solution and triplicate. The blank used 200 μl of DPPH added with 22 μl of 80% methanol and carried out three times. The mixed solution was kept in a dark place for 20 minutes at 27° C, the solution was vortexed and measured at a wavelength of 517 nm using a nano spectrometer. Antioxidant activity was measured from the percentage of inhibition with the formula:

Percentage of inhibition of DPPH activity = (A – B) / A x 100%
Where A = control inhibition, and B= sample inhibition.

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Total Flavonoid Analysis

Analysis of total flavonoids used Vongsak et al. (2013) with a modification from Chang et al. (2002) using fresh samples of *P. indica* leaves in positions 3rd, 5th, and 7th on the secondary branch of each replication. Fresh leaves (0.001 g) were crushed and macerated with 2 ml of 70% ethanol. A sample of 0.5 ml was mixed with 0.5 ml of 2% aluminum chloride solution, potassium acetate (0.1 ml, 1 mM), and 2.8 ml of distilled air against the blank sample without aluminum chloride. The standard solution is 10 milligrams of quercetin dissolved in 80% ethanol which was diluted to 0, 2, 5, 25, 50, and 100 $\mu g.ml^{-1}$. The standard solution was mixed using a vortex, then incubated at room temperature 27° C for 30 minutes. The absorbance of the mixture was measured at 415 nm with a Shimadzu UV-160A spectrophotometer. The standard curve constructed was y = 0.03021 + 0.01006, R2 = 0.99977. The results were equivalent to mg of quercetin per gram of fresh sample. The total flavonoid per plant was obtained by multiplying the fresh leaf weight per plant with the total flavonoid concentration of the 7th leaf.

Leaf Tissue Analysis

Analysis of nitrogen, phosphorus, and potassium concentration was conducted in leaves at positions 3rd, 5th, and 7th from the apex of each plant. Total N concentration was determined using Kjeldhal method. Phosphorus and potassium concentration were extracted by wet ash method using a mixture of concentrated HNO_3 and $HClO_4$. Phosphorus concentration was determined using a spectrophotometer, whereas potassium using an atomic absorption spectrophotometer (AAS) (Balitttanah, 2005).

Plant Growth Measurement and Data Analysis

Growth variables consisted of plant height, leaf number, measured weekly at 2 until 8 weeks after planting (WAP); branch number data was collected at 6 until 8 WAP. Nutrient levels of leaves, total flavonoid, and antioxidant activity were measured at the 3rd, 5th, and 7th leaf positions from the apex. Yield was measured at 8 WAP or when plants reach a minimum height of 45 cm above the ground.

Data were analyzed using the F-test at a 5% significance level, followed by the Duncan Multiple Range Test. If the value of coefficient of variance was more than 30%, data was transformed using the formula $\sqrt{x+0{,}5}$. Statistical analysis used R Studio version 4.3.1 software.

Result and Discussion

Plant Height, Leaf Number, and Branch Number

Providing chicken manure affects the vegetative growth of *P. indica* (Table 1 and Table 2). Plants applied with 2.5 kg of chicken manure showed better growth compared to those without manures, specifically in plant height, leaf number, and number of tertiary branches. Increasing the dose of chicken manure significantly increased plant height ($P<0.05$) and leaf number ($P<0.05$) at 6 weeks after planting (WAP), but similar with 5 and 7.5 kg chicken manure per plant. Application of chicken manure also increased the number of tertiary branches ($P<0.05$) at 8 WAP.

Table 1. Effect of chicken manure application on plant height and leaf number of *Pluchea indica*

Chicken Manure (kg per plant)	Plant height (cm)				Leaf number			
	2 WAP	4 WAP	6 WAP	8 WAP	2 WAP	4 WAP	6 WAP	8 WAP
0	15.61	29	41.61 b	46.37 b	35.56	214.01	511.73 b	689.46 b
2.5	16.46	34.13	52.28 a	63.93 a	42.33	282.11	852.17 a	1,268.39 a
5.0	16.76	34.98	53.22 a	66.23 a	47.83	279.61	779.28 a	1,361.06 a
7.5	15.05	30.90	48.56 a	74.67 a	31.39	250.67	701.61 a	1,198.67 a
P value	0.2300	0.2020	0.0152	0.0031	0.0634	0.1890	0.0138	0.0055
Sig.	ns	ns	*	**	ns	ns	*	**

Note: Values followed by the different letters in the same column are significantly different in the Duncan test α=0.05; ns-nonsignificant; WAP: week after planting.

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Table 2. Effect of chicken manure application on branch number of *Pluchea indica*.

Chicken Manure (kg per plant)	Branch number					
	Primary		Secondary		Tertiary	
	6 WAP	8 WAP[1)]	6 WAP	8 WAP	6 WAP[1)]	8 WAP
0	4.60	5.97	27.71	34.91	21.67	29.08 b
2.5	4.28	6.94	41.11	65.22	35.33	98.47 a
5.0	4.89	6.17	32.17	66.50	32.54	86.50 a
7.5	4.56	5.22	25.33	45.11	27.24	109.10 a
P value	0.936	0.784	0.173	0.173	0.194	0.020
Sig.	ns	ns	ns	ns	ns	*

Note: Values followed by the different letters in the same column are significantly different in the Duncan test α=0.05; ns-nonsignificant; [1)] Data was transformed using the formula $\sqrt{x + 0{,}5}$; WAP: week after planting.

Leaf Fresh and Dry Weight

Chicken manures increased leaf fresh and dry weight of *P. indica* (Figure 1). Plants that received a dose of 5 kg of manure per plant had higher fresh (A) and dry (B) leaf weights compared to those received 2.5 kg per plant or without chicken manure.

per plant with the total flavonoid concentration at the 7th leaf position. Flavonoid production per plant also did not have a difference due to the application of chicken manure. In general, the total flavonoid concentration in the 3rd leaf position had a greater value than the 5th and 7th leaf positions as well as the antioxidant activity in the 3rd leaf position.

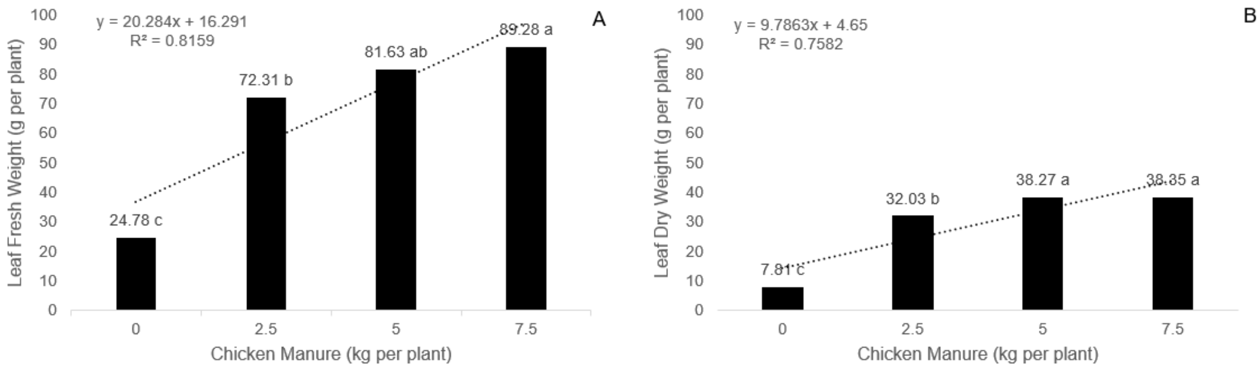

Figure 1. Effect chicken manure application on fresh and dry weight of leaves *Pluchea indica*

Leaf Nutrient Concentration

Provision of chicken manure increased the nutrient concentration in leaf tissue. At 2.5 kg per plant N levels of the 7th leaf and K levels of the 3rd leaf increased (Table 3). Application of 5 kg chicken manures per plant significantly increased the P levels of the 3rd and 7th leaves and the K levels of the 5th and 7th leaves (Table 3).

Flavonoid Production, Antioxidant Activity, and Flavonoid Production

The application of chicken manure did not affect the total flavonoid concentration and antioxidant activity at each leaf position (Table 4). There were no differences in total flavonoid levels and antioxidant activity in various leaf positions. Production of total flavonoids per plant was obtained by multiplying the fresh weight

Discussion

In another Asteraceae species, *Vernonia amygdalina,* chicken manure increased plant height, branch number, fresh leaf weight, and dry leaf weight (Tjhia et al., 2018). Chicken manure contains 1.64% N, 5.14% total P, 1.6% K2O, and 19.12% C-organic (Betty, 2018). All these nutrients have major roles in stimulating plant vegetative growth. Nitrogen is an essential element for the synthesis of amino acids, proteins, and nucleic acids (Taiz and Zeiger, 2002). Nitrogen increases the amount of chlorophyll thereby encouraging photosynthesis. Nitrogen increases the photosynthesis apparatus so that it can increase the chlorophyll content, the number and activity of carboxylation enzymes, total protein, sugar content, total N, and metabolites related to photosynthesis (Bassi et al*.,* 2018). Phosphorus is essential for energy transfer (Tian et al., 2019), photosynthesis,

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Table 3. Effect chicken manure application on concentration N, P, and K leaf of *Pluchea indica.*

Chicken manure (kg per plant)	Nutrient concentration (%)	Leaf position		
		3rd	5th	7th
0	N	3.87	3.92	3.78 b
2.5		4.11	3.97	4.08 a
5.0		4.14	4.06	4.08 a
7.5		4.26	4.24	4.25 a
P value		0.1627	0.1720	0.0192
Sig.		ns	ns	*
0	P	0.27 b	0.24	0.22 c
2.5		0.28 b	0.25	0.24 bc
5.0		0.31 ab	0.29	0.27 ab
7.5		0.34 a	0.30	0.29 a
P value		0.0328	0.0522	0.0328
Sig.		*	ns	*
0	K	1.04 b	1.00 c	0.98 c
2.5		1.48 a	1.30 b	1.26 b
5.0		1.62 a	1.71 a	1.48 a
7.5		1.59 a	1.55 ab	1.54 a
P value		0.0098	0.0030	0.0023
Sig.		**	**	**

Note: Values followed by the different letters in the same column are significantly different in the Duncan test α=0.05; ns-non significant; WAP: week after planting.

Table 4. Effect chicken manure application on antioxidant activity, flavonoid concentration, and flavonoid production in *Pluchea indica*.

Chicken manure (kg per plant)	Total flavonoid (mg QUE g^{-1} fresh weight)			Antioxidant activity (%)			Flavonoid production[2)] (mg QUE per plant)
	Leaf Position						
	3rd	5th 1)	7th 1)	3rd	5th	7th	
0	1.32	1.07	1.15	58.23	50.07	41.82	29.55
2.5	1.73	1.17	0.93	49.35	37.09	41.15	66.29
5.0	0.92	0.35	0.64	48.41	46.90	44.39	51.45
7.5	1.55	0.94	0.80	47.22	35.89	33.20	71.22
P value	0.100	0.274	0.527	0.147	0.196	0.383	0.382
Sig.	ns	ns	ns	ns	ns	ns	ns

Note: Values followed by the different letters in the same column are significantly different in the Duncan test α=0.05; ns-non-significant; [1)] Data was transformed using the formula $\sqrt{x+0{,}5}$; [2)]Data was transformed twice using formula $\sqrt{x+0{,}5}$; QUE: Quercetin equivalent

nutrient movement (Khan et al., 2023). Potassium in involved is enzyme activation of protein, starch, and adenosine triphosphate (ATP) (Sardans and Peñuelas, 2021). Chicken manure applied to okra resulted in taller plants, more leaves, higher harvest index and fresh weight per plant (Arifah et al., 2019). Other research showed that nitrogen in chicken manure plays a role in increasing *Vernonia amygdalina* leaf production as indicated by an increase in plant height, number of branches, fresh leaf weight, and dry leaf weight (Tjhia et al., 2018).

Chicken manure applied to alfisol significantly improved the soil nutrient status as shown by

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increasing C-organic, available P, exchangeable cations, and the effective cation exchange capacity. In addition, it caused increasing in N, P, and K concentrations in plant tissue, plant height and dry matter yield of soybeans (Soremi et al., 2017). In general, the use of 10 ton.ha^{-1} of chicken manure has a better effect on most growth variables and yield components, achieving the highest nutrient concentration and nutrient uptake for most macro and micronutrients in onion leaves and bulbs (Falodun and Egharevba, 2018). Similar results were shown when applying 10 tons.ha^{-1} of chicken manure to *Vernonia amygdalina* Del. produced a vigor plant and had the highest fresh and dry leaf weights (Ndukwe et al*.,* 2022). Long-term manure application is important for a sustainable environment increasing soil productivity and increasing crop yields (Du et al., 2020).

Provisions of chicken manures can increase soil C-organic and improve soil properties influencing plant growth through increasing soil nutrient cycles so that used as an alternative source of nutrients to increase crop yields, soil fertility, and plant nutrient uptake (Lin et al., 2016). Chicken manure increased the yield and nutrient composition of sweet basil (Yaldız et al*.,* 2019), sorghum (Aziz et al., 2020), and strawberry leaves (Sayğı, 2022). Chicken manure improves tomato performance and nutrient status and increases leaf N, P, K, Ca, and Mg levels (Adekiya and Agbede, 2009). Providing chicken manure made a significant difference to N levels in the 7th leaf position associated with the mobile N element in the tissue. The mature leaves close to the shoot play a role in providing photosynthate for growing shoots or juvenile leaves (Taiz and Zeiger, 2002). The highest nutritional concentration is found in the middle position of cassava leaves (Chaiareekitwat et al., 2022).

Leaf position affected the accumulation of secondary metabolites; phenolics, proanthocyanidins, and flavonoids in *Lantana camara* (Bhakta and Ganjewala, 2009). Younger leaves (positions 1st to 3rd) were most active in the biosynthesis and accumulation of secondary metabolites compared to mature leaves (position 4th to 5th) (Bhakta and Ganjewala, 2009). Extracts of younger leaf had stronger antioxidant activity than older or mature leaves. Different stages of leaf development had differences in secondary metabolites observed at different leaf positions. The 2nd leaf of *Coleus atropurpureus* L. in the vegetative phase had a positive correlation with pigment and total flavonoids (Respita et al., 2019). In general, the total flavonoid concentration in the 3rd leaf position had greater value than the 5th and 7th leaf positions in all treatments as well the antioxidant activity in the 3rd leaf position was supported by the statement (Vongsak et al., 2018) that the leaf shoots of *Pluchea indica* had a stronger activity and a higher number of bioactive components compared to mature leaves. Antioxidant properties of *P. indica* leaves were stronger than *Curcuma longa* rhizomes (Chan et al., 2022) and green tea *Camellia sinensis* (Sirichaiwetchakoon et al*.,* 2020). The study showed the total flavonoid concentration at each observed leaf position after the application of chicken manure were similar, and the flavonoid production per plant also showed a similar trend. However, the fresh weight per plant significantly increased by applying chicken manure so in general, the average value of flavonoid production per plant increased with the application of chicken manure. Providing chicken manure no longer showed effects when applied at 45-57 months after planting or after 4-6 harvests. However, heavy pruning at plant height of 60 cm stimulated growth, leaf production, and total flavonoid content of orange jessamine (Jati, et al., 2019). The best level of chicken manure to increase flavonoid production from *Vernonia amygdalina* was 7.5 kg per plant and increased total flavonoids production by 37% compared to without fertilization (Betty, 2018).

Conclusion

Application of 5 kg per plant of chicken manures significantly increased *P. indica* fresh leaf weight and dry weight over the lower dose of 2.5 kg per plant or without manures but had no effects on the flavonoid production.

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