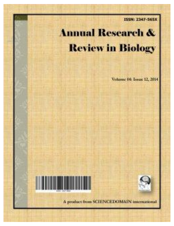

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Morphology, Leaf Gas Exchange and Quality of Pegaga (*Centella asiatica*) under Different Nitrogen Fertilization Rates

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Authors' contributions

This work was carried out in collaboration between all authors. Author MHI designed the study, author NM performed the statistical analysis, author AAI wrote the protocol and wrote the first draft of the manuscript. Author BZ managed the analyses of the study. Author NAMZ managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To investigate the physiological, leaf gas exchange and quality of *Centella asiatica* (pegaga) under different nitrogen fertilization rates.
Study Design: *Centella asiatica* were exposed to four different nitrogen fertilization rates (0, 50, 100, 150 kg/ha) using urea (46% N) as nitrogen sources. The experiment was conducted in a randomized complete block (RCBD) design with three replications. Each treatment consisted of eight plants making the total of plants used in this study is 96 plants.

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Place and Duration of Study: Department of Biology, Faculty of Science, Universiti Putra Malaysia From May 2016-June 2016.

Methodology: The growth parameters measured include total leaves numbers, leaf area, total chlorophyll content and total plant biomass. The carbon assimilation parameters were measured using LICOR 6400 XT Portable Photosynthesis System i.e net photosynthesis (A), Transpiration rate (E) and water use efficiency (WUE). Total phenolic and flavonoids contents from the leaves extracts were measured using Folin-Ciocalteu reagents.

Results: The growth parameters such as leaves number, chlorophyll content, leaf area and total biomass were significantly influenced by nitrogen fertilization ($P \leq 0.05$), However, there were no significant difference observed between 50, 100 and 150 kg N/ha suggesting that 50 kg N/ha was the efficient rates to apply to enhance the growth of this plant. Meanwhile, the net photosynthesis (A) and water use efficiency (WUE) were enhanced with the increasing rate of nitrogen from 0>150 kgN/ha. The production of total phenolics and flavonoids was found to be highest under 100 kg/ha. The harvest index of total phenolics also showed the applications of 100 kg/ha gave the highest harvest index compared to the other nitrogen treatments.

Conclusion: This study indicated growth and carbon assimilation parameters were enhanced under higher nitrogen fertilization and production of secondary metabolites was decreased with high rates of nitrogen. The recommended nitrogen fertilization for *C. asiatica* was at 100 kg N /ha, where it obtained the highest harvest index.

Keywords: Centella asiatica; physiological; leaf gas exchange; phenolic; flavonoid; nitrogen; fertilization.

1. INTRODUCTION

Centella asiatica or known by the Malaysians local as pegaga or commonly known as Gotu Kola, Indian Pennywort, Asiatic Pennywort and Thick-leaved Pennywort has been used as a medicinal plant since prehistoric times [1-6]. *C. asiatica* is a member of Apiaceae family. This small perennial herb is very popular as a traditional medicine among various cultures and it is widely distributed throughout the different parts of the world, especially in wet areas and humus rich soil [7]. This plant can naturally grow as weeds under a wide range of conditions. Originally known to be originated from Asian and East African regions, this herb has spread out to many countries including Malaysia [8]. Pegaga had become quite popular in Malaysia as a supplementary medicine [9]. Apart from that, *C. asiatica* also famous being eaten fresh as a salad or cooked as vegetables or simply made into healthy drinks such as tea or juice [7] among the locals. The Chinese, Indians and Malays use this herb for various ailments ranging from treatments of mental disorder, immune system deficiencies, circulatory problems, skin problems, liver ailments, epilepsy, asthma, hair lost and tetanus [10]. Other than that, it also can be used as blood purifier [11] anti-inflammatory [12] and many others. In Europe and United States the *C. asiatica* is widely used as part of the ingredient in the preparation of drugs where the active ingredients were extracted for making pills and creams [9].

Centella asiatica contains active ingredients such as ascorbic acid, centelloside, triterpene, flavanols, saponins, terpenoids, glycoside, alkaloids, calcium, iron, phosphorus and vitamins [13] which some have been considered as pharmacologically active ingredients that are beneficial in improving human health [10]. Due to the wide range of antioxidant properties and its vast availability locally, this herb is quite popular to be cultivated among the locals and had been produced in a mass production to meet the increase in demand and needs by the agricultural and even pharmaceutical based industries. Currently there are many herbal products being developed by using *C. asiatica* based. Up until the recent years, *C. asiatica* based drugs and cosmetic products are widely commercialized [14,15].

Due to the growing demand on herbal raw materials, a lot of method had been used to increase the yield of this potent herb based on previous and ongoing research. One of the most common methods employ among the farmers is through the application of the fertilizers. Nitrogen is known to be the most abundant nutrients in plants and often plays an important role in quality of crops [16]. Plants absorb nitrogen either as the nitrate ion (NO_3^-) or as the ammonium ion (NH_4^+). The sources of nitrogen are commonly used for crop production mainly ammonium nitrate and urea. Nitrogen is important as it is responsible for the dark green color on stem and leaves, leaf production, size enlargement and yield formation

[17]. Nitrogen fertilization has known to increase the yield of various types of medicinal plants [18]. There were many studies conducted on fertilization input on *C. asiatica* however, the previous study was more focused on the impact of compound fertilization on the growth of this particular herb. There was no study on the impact of single fertilizer especially nitrogen. The leaf gas exchange and quality of the plant were never documented. With that, there is a need to conduct experiments on the input of nitrogen on the growth, leaf gas exchange and secondary metabolites of *Centella asiatica*. The medicinal properties of the plant correspond to the production of secondary metabolites. The quality of medicinal properties of *C. asiatica* is influenced by many factors especially the fertilizer application. Therefore, an experiment was conducted to to determine the effect of different nitrogen (N) fertilization rates (0, 50, 100, 150 kg/ha) on the physiological and leaf gas exchange of *C. asiatica* and to find the best rates of nitrogen (N) to enhance the best growth and quality of *C. asiatica.*

2. MATERIALS AND METHODS

2.1 Plant Materials and Methods

The experiment was conducted at the Taman Pertanian Universiti, Universiti Putra Malaysia. *Centella asiatica* was planted by using the stolon cutting method. Several plants cutting of randomly individual plant of *C. asiatica* was collected from the same population. The stolon of the plant was propagated in a tray containing soil compost and coco peat as a medium and was left in the nursery for about one month. After one month, the plants that already develop some roots were transferred into a standard polybag filled with a mixture of topsoil and sand (ratio 3:1). The plants were left to grow under a natural light for 12 weeks and irrigated four times a day or when necessary. The experiment used in this study was the Randomized Complete Block Design (RCBD). In the experiment, four different types of nitrogen rates are used which are 0, 50, 100 and 150 kg/ha. The potassium and phosphorus were applied using muriate of potash (MOP) and trisodium phosphate (TSP) at fixed rates of 100 kg/ha (Table 1).

2.2 Number of Leaves

The number of leaves from each plant inside the polybags was counted manually including the tips of new leaf which just started to emerge.

2.3 Chlorophyll Content

The chlorophyll content of the *C. asiatica* leaves was measured using Chlorophyll Meter SPAD 502 Minolta. The readings were taken by clipping the meter on the leaves surface after the meter was calibrated. Three readings were randomly taken on the same leaf for every treatment in each replicate. The best time for measuring the chlorophyll content was between 9.00 am to 12.00 pm.

2.4 Total Leaf Area (cm^2)

The total leaf area of detached leaves of *C. asiatica* leaves was measured using the Automatic Leaf Area Meter (Model LI-3100) which located at the Faculty of Agriculture.

2.5 Total Biomass

The total biomass was calculated by adding the dry weight of leaves, stems and roots of *C. asiatica* together. Plant parts were separated and placed in paper bags and oven dried at 80°C until constant weight was reached before dry weights were recorded using the electronic weighing scale that have precision range of 0 g – 500 g.

2.6 Harvest Index

The harvest index was calculated by using the following formula [7]:

Harvest index = Total Biomass × Total phenolics / flavonoids

Table 1. Amount of fertilizers applied per polybag

Treatment (Nitrogen)	Urea N(kg/ha)	*MOP K (kg/ha)	**TSP P (kg/ha)
0	0	0.47	0.62
50	0.30	0.47	0.62
100	0.62	0.47	0.62
150	0.93	0.47	0.62

**MOP – muriate of potash; **TSP – trisodium phosphate*

2.7 Net Photosynthesis, Transpiration/ Stomatal Conductance Measurement

The leaf gas exchange was measured using a close infra-red gas analyzer (IRGA) of LICOR 6400 Portable Photosynthesis System (LI-COR Biosciences). The IRGA calculates the photosynthesis and cellular respiration of a plant by measuring the carbon dioxide uptake and release. This measurement allows us to see how the photosynthesis and respiration rates in plants changes in response to various environmental changes. In this case, the changes in response to different nitrogen fertilization rates. The IRGA also measured the stomatal conductance and water use efficiency. The instrument was warmed for 30 minutes before used and calibrated with ZERO IRGA mode. The measurement used optimal conditions sets of 400 µmol mol^{-1} CO_2 30°C cuvette temperature, 60% relatively humidity with air flow rate set at 500 cm^3 min^{-1}, and modified cuvette condition of 800 µmol m^{-2} s^{-1} photosynthetically photon flux density (PPFD). The measurement of gas exchange were carried out between 9.00 am to 11.00 am using fully expanded young leaves numbered three and four from plant apex to record net photosynthesis rate (A). The operation was automatic and the data was stored in the LI-6400 console and analyzed by the Photosyn Assistant software.

2.8 Total Phenolic and Flavonoids Quantification

The method of extraction and quantification for total phenolic and flavonoids contents followed after Ibrahim et al*.,* [19]. Two grams (dried plant sample) of ground tissue samples was extracted together with a 10 mL of 80% methanol. The mixture then was placed on an orbital shaker for 120 minute at 50°C. Next, the mixture was filtered using Whatman™ No.1 filter paper and the filtrate was used for the quantification of total phenolics and total flavonoids. After that, 200 µL of the sample extract was mixed with 1.5 mL of Follin–Ciocalteau reagent and left for 5 minute at 22°C before adding $NaNO_3$ solution (1.5 mL, 60 g L^{-1}). The Folin-Ciocalteu reagent (diluted 10-fold) was used to determine the total phenolics content of the leaf samples. After two hours, the absorbance was measured at 725 nm. The results were expressed as mg g^{-1} gallic acid equivalent (mg GAE g^{-1} dry sample). Moving onto the total flavonoids determination, 1 mL sample was mixed with 0.3 mL of $NaNO_3$ in a test tube covered with aluminum foil and left for 5 minute. Then 0.3 mL of 10% $AlCl_3$ was added followed by addition of 2 mL of 1 M NaOH. The absorbance then was measured using a spectrophotometer at 510 nm with rutin as a standard (results expressed as mg g−1 rutin dry sample).

2.9 Statistical Analysis

The statistical analysis was done by using IBM SPSS statistics software version 2.2. The data were analyzed using two-way Analysis of Variance (ANOVA) and the mean separation test between treatments was compared using Duncan Multiple Range Test (DMRT). The differences were accepted as significant at $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Leaves Numbers and Total Leaf Area

In this study, the leaves numbers of *Centella asiatica* was found to be influenced by the nitrogen treatments ($P \leq 0.05$; Fig. 1). A significant difference where $p \leq 0.05$ was observed in all week 2, 4, 6 and 8 after different nitrogen treatments being applied. Every two weeks, an increased in the number of leaves can be observed up until the final week (Week 8). In the 8 weeks after treatment, the 150 kg/ha of nitrogen treatments gave out the highest number of leaves followed by 100 kg/ha, 50 kg/ha and finally the lowest at 0 kg/ha (control). However, there were no significant difference between 50, 100 and 150 kg/ha N treatments on the leaf number was observed in all weeks of measurement.The leaf area (LA) was shown to be influenced by the nitrogen treatments where a significant difference ($P \leq 0.05$) was observed in all week 2, 4, 6 and 8. However, there were no significant differences were observed between nitrogen treatment in 2,6 and 8 WAT.

A vast plant growth towards the availability of nitrogen levels whether morphologically or physiologically has been demonstrated through several studies. Based on a study by Reddy et al., [20] deficiency in nitrogen will cause fewer numbers of leaves. The reason might ascribe to node addition rate which regulated by carbon supply. According to Brady and Weil [21], the influences of N deficiency will result in reduced chlorophyll, visible necrosis on older leaves and general poor development of leaf. On the other hand, increasing the nitrogen rate applied will result in an increase in the number of live leaves.

This was supported by many researches and one of them was by Westerveld et al. [22] where an increased in carrot leaves was observed along with an increased in N rate. The increase in number of leaves might be due to high photosynthetic rate [23]. While the lowest number of leaves were produced from the control nitrogen treatment which is 0 kg/ha, the levels of 100 kg/ha and 150 kg/ha nitrogen fertilizer produced the highest number of leaves. Simply said, the fertilized plants produced greater number of leaves and indicated that the nitrogen fertilizer had effect on number of leaves hence increased the leaves of *Centella asiatica* plants. Since nitrogen was known to promote leaf growth, increasing the level of nitrogen treatment will have stimulated the vegetative growth and probably helps the plant producing more leaves [24]. Meanwhile, leaf area was very responsive and sensitive to nitrogen supply [25]. The increased in leaf area was corresponded to to increases the number of and size of the plant cells [25]. Any nitrogen limitation will affect the leaf area in both cell production and cell expansion [26] and slower leaf enlargement thus reducing the size of the leaf. The cell expansion in response to N availability will result in an increase in LA due to improved nutrient availability [27].

3.2 Total Chlorophyll Content

From the result obtained, it is cleared that the production of chlorophyll content of *Centella asiatica* was affected by the nitrogen treatments ($P \leq 0.05$; Fig. 2). A significant difference where $p \leq 0.05$ was observed in all week 2, 4, 6 and 8. It was observed that the application treatments of 150kg/ha nitrogen produced the highest total chlorophyll content in all weeks measured followed by 100 kg/ha and 50 kg/ha. Although there was a decrease in total chlorophyll content

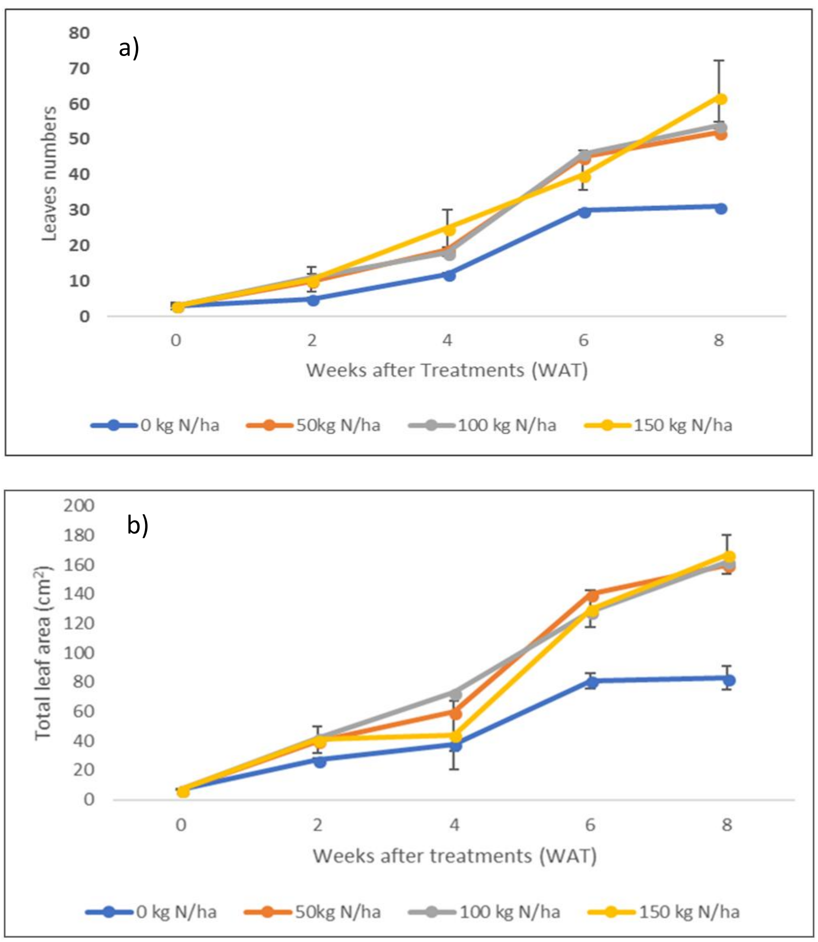

Fig. 1. Effect of nitrogen rates on the leaves numbers (a) and total leaf area (b) of *C. asiatica* during 8 weeks. Data are mean ± standard error of mean (SEM) of six replicates

in week 4, all treatments showed a steady increase in chlorophyll content up until the final weeks. The highest chlorophyll content recorded was at 46.43 in week 8 with 150kg/ha nitrogen applied. Nitrogen is known to be an essential compound of chlorophyll content as it is the primary structure of the primary light harvesting compound of photosynthesis [28]. Other than that, N is an important constituent of protoplasm protein, nucleic acid, preparation of starch in leaves and production of amino acids [28]. Additionally, nitrogen acts as structural compounds of chloroplast [29] thus any N deficiency will result in suppression of chlorophyll formation [30]. Previous result has reported an increase in nitrogen level will eventually result in an increase in the plant total chlorophyll content, this might be due to chlorophyll content was positively related to the plant nitrogen status [20]. The higher addition of nitrogen would have enhanced the production of chlorophyll content that was observed in the present study [21,22].

3.3 Total Dry Biomass

Fig. 4 showed that, the total biomass of *Centella asiatica* was affected by the nitrogen applications. There was a significant difference (P≤0.05) observed on week 2, 4, 6 and 8 of nitrogen treatments from the control (0 kg N/ha). From the graph below, the 50kg/ha nitrogen treatments came out the highest on week 2, 6 and 8 while the 100kg/ha N on week 4. On the final week, the 150kg/ha N came out as second highest in total biomass followed by 100kg/ha and control. Overall, the general trend of this result was the total biomass increased from week 2 until week 8. The result obtained was in agreement with Nasim et al*.,* [31] as the author observed an addition in biomass and yield with increased in nitrogen rates. According to Latiri et al. [32], the nitrogen application effects the dry matter production greatly due to increasing in leaf area development with high nitrogen . An increase in total biomass production is due to increase in leaf production caused by nitrogen supply [33] and a combination of N with plant matter produced during photosynthesis such as glucose, amino acids and protein [34].

3.4 Leaf Gas Exchange Characteristics

From Table 2, it was showed that the nitrogen treatment significantly influenced the net photosynthesis rate. As the level of nitrogen fertilization enhanced from 0 kg/ha to 150 kg/ha, the net photosynthesis rate was also enhanced. The highest net photosynthesis rate was 150 kg/ha (5.33 μmol/m^2/s) while the lowest is the control plant (2.43 μmol/m^2/s). This result was also in agreement with another study by Ivanova and Vassilev [35] where 100 kg/ha and 140 kg/ha nitrogen fertilization enhances the photosynthetic rate in *Chrysanthemum indicum* by 13%. Most of the plant leaf N was used in chloroplast production and important part of in Rubisco. As nitrogen level decline, it will cause the reduction in mesophyll activity, chlorophyll content, Rubisco activity [36] or amount of enzyme [37] affecting the photosynthesis rate. Meanwhile, adequate nitrogen level is necessary for stimulation of photosynthesis and may alter the Rubisco activity [38], so the higher rates of nitrogen would enhance net photosynthesis, that was observed in the present study.

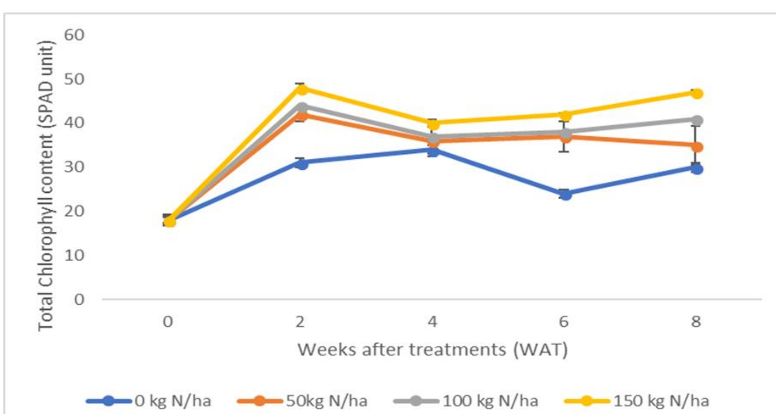

Fig. 2. Effect of nitrogen rates on total chlorophyll content of *C. asiatica* during 8 weeks. Data are mean ± standard error of mean (SEM) of six replicates

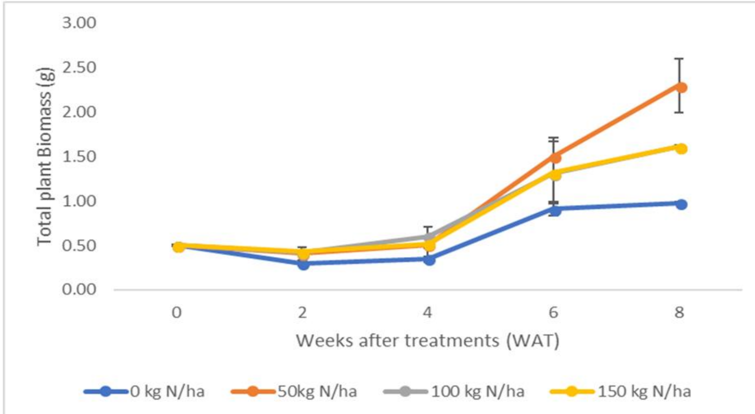

Fig. 3. Effect of nitrogen rates on the total biomass of *C. asiatica* during 8 weeks. Data are mean ± standard error of mean (SEM) of six replicates

Table 2. Plant leaf gas exchange characteristics of *C. asiatica*, as affected by different nitrogen rates. All analyses are mean ± standard error of mean (SEM). N = 6. Means not sharing a common alphabet were significantly different at *P* ≤ 0.05 using Duncan multiple range test (DNMRT)

	Nitrogen (kg N/ha)			
Plant characteristics	**0**	**50**	**100**	**150**
Photosynthesis, *A* (µmol $m^{-2}s^{-1}$)	2.51±0.32^d	3.72±1.32^c	4.47±1.56^b	5.42±3.11^a
Transpiration rate, *E* (mol m-$^2s^{-1}$)	2.28±0.76^d	2.03±1.23^c	1.44±2.04^b	1.17±0.87^a
Water use efficiency, WUE	1.08±0.05^d	1.82±0.54^c	3.17±0.77^b	5.00±0.10^a

It was observed that nitrogen rates has shown to influence the transpiration rate based on Table 2 (p≤0.01). In contrast to photosynthetic rate, the transpiration rate was higher in control treatment. Under high nitrogen supply, there was less water lost through transpiration compared to lower nitrogen supply. The highest transpiration rates were observed in the control plant (2.28 mmol/m^2/s), followed by 50 kg/ha (2.03 mmol/m^2/s), 100 kg/ha (1.44 mmol/m^2/s), while the lowest is 150 kg/ha nitrogen (1.17 mmol/m^2/s). There was no significant difference observed between treatments 0 and 50 kg/ha and between 100 and 150 kg/ha. The result obtained from this study was in agreement with Dordas & Sioulas [39] 40 study in safflower where transpiration rates were found to be 25% decreased as N level increased from 100>200 kg/ha. This is because nitrogen and transpiration showed a strong negative correlation at leaf level [40] thus any N toxicity will reduced the stomatal opening and transpiration rate [41] that was observed in the present study.

The water use efficiency (WUE) of *Centella asiatica* was observed to be influenced by the nitrogen rates(*P* ≤ 0.05; Table 2). The water use efficiency gradually increases as the nitrogen treatment increase from 0 kg/ha to 150 kg/ha. The 150 kg/ha nitrogen application produced the highest WUE (5.00 mmol CO_2/mol^{-1} H_2O) while 0kg/ha nitrogen treatment produced the lowest WUE (1.08 mmol CO_2/mol^{-1} H_2O). Water use efficiency is related to carbon dioxide (CO_2) assimilated by photosynthesis and water lost through transpiration [42]. The increase in photosynthesis rate was more influenced than reduction in transpiration rate in WUE [41]. On the other hand, nitrogen also enhances net assimilation rate which in turn improve the WUE. Nitrogen addition will cause more water uptake to assist in photosynthesis process and other cell metabolic activities. So this justifies why WUE was enhanced with increasing N levels in the present study.

3.5 Total Phenolics and Total Flavonoid Content

In this study, the nitrogen treatments had influenced the total phenolics content of *Centella asiatica* (Table 3). The highest total phenolics content was observed in 100 kg/ha nitrogen application (2.29 mg GAE/g) followed by both 50 and 150 kg/ha (1.50 mg GAE/g) and the lowest is 0 kg/ha (0.69 mg GAE/g). From the graph below, an increased in phenolics content can be observed up until the application of 100 kg/ha nitrogen and from there a decrease was detected at 150kg N /ha applications. This indicated that the 100kg/ha nitrogen applications were the most suitable rates for the phenolics production in *Centella asiatica* since the total phenolics content observed was at the highest. An increase in total phenolics content can only be displayed in low N supply [43] which might be due to improvement in the production of total non-structural carbohydrates (TNC) [44]. A high N supply causes a reduction in total phenolics content as shown in the present study which have the same findings with Nguyen and Niemeyer [45] in Basil. So it can be concluded that high nitrogen levels can reduce the production of total phenolics that observed in the present study.

From Table 3, the nitrogen application had an impact on the flavonoids production (P≤0.05). The highest total flavonoids content (TFC) was observed at 100 kg/ha nitrogen treatment (0.79 mg RE/g) while the lowest was 0kg/ha nitrogen treatment (0.22 mg RE/g). Similar to the total phenolics content, the TFC had a steady increased from 0 kg/ha to 100 kg/ha and starting from 150 kg/ha nitrogen application; a decrease in TFC was observed. The reason why the TFC showed an increased from 0 kg/ha to 100 kg/ha only is probably that the 100 kg/ha is the most optimum N rate for the synthesis of flavonoids in *C. asiatica.* This result was in agreement with a study by Ibrahim et al. [46] where the amounts of flavonoids in *Labisia pumila* plant were reduced as nitrogen increased up to 270 kg/ha. An increase in flavonoids production in plants can only be achieved when the nitrogen is limited which proven in a study by Stewart et al., [47]. The author described a nitrogen deficiency promotes an increased in flavonoids production in tomato seedlings. The synthesis of flavonoids was shown to be influenced by the nitrogen present based on above studies might due to low phenylalanine availability [48] and affecting it at gene level [49]. The harvest index (HI) shown to be influenced

Table 3. Plant secondary metabolites characteristics of *C. asiatica*, as affected by different nitrogen rates. All analyses are mean ± standard error of mean (SEM). N = 6. Means not sharing a common alphabet were significantly different at *P* ≤ 0.05 using Duncan Multiple Range Test (DNMRT)

Nitrogen (kg N/ha)	Total phenolics (mg GAE/g dry weight)	Total flavonoids (mg rutin/g dry weight)
0	0.69±0.05^{c}	0.20±0.03^{c}
50	1.49±0.02^{b}	0.37±0.01^{b}
100	2.29±0.12^{a}	0.79±0.03^{a}
150	1.49±0.05^{b}	0.71±0.04^{a}

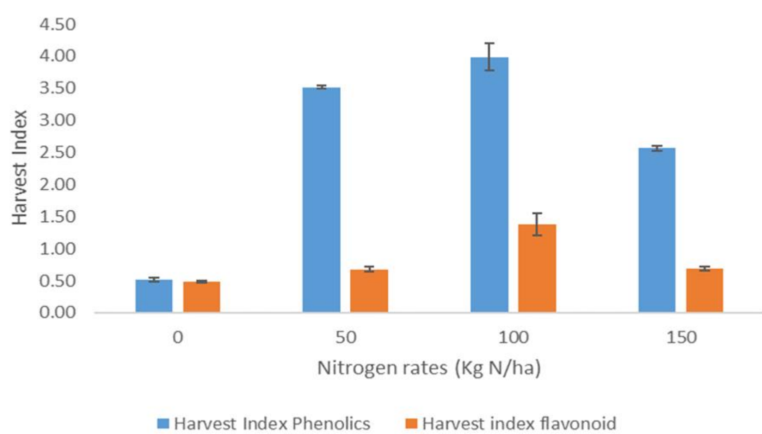

Fig. 4. Effect of nitrogen rates on the harvest index of *C. asiatica* during 8 weeks. Bars represent standard error of differences between the means (SEM) of six replicates

by the nitrogen treatment based on the analysis of variance (Fig. 4). An increase in harvest index can be observed up until the application of 100 kg/ha nitrogen and from there a decrease was detected at 150 kg/ha application. This showed that the 100 kg/ha nitrogen treatments was the recommended fertilization rate for this plant due to high HI in total phenolics and flavonoids. It can be concluded that application of nitrogen levels more than 100 kg N/ha would reduce the production of HI.

4. CONCLUSION

In this study, the result showed that nitrogen have influenced the physiological, leaf gas exchange and quality of *C. asiatica.* It was observed that high application of N enhanced and improved growth parameters of *C. asiatica* such as the number of leaves, total chlorophyll content, leaf area and total dry biomass of *C. asiatica.* However, there was no significant difference were observed between 50, 100 and 150 kg/ha N treatments in the growth parameters. As nitrogen rates increase from 0 to 150 kg N/ha it was observed that A and WUE were enhanced however transpiration (E) rate was reduced. It was found that the production of total phenolics and flavonoids content was highest under 100 kg/ha nitrogen fertilization. Also, the harvest index was also highest at 100 kg/ha treatment, this indicates that the recommended rate to enhance the growth and medicinal properties of this plant was at 100 kg N/ha.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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