

Preliminary Study on the Effect of Nitrogen and Potassium Fertilization on Phytochemical Content Quality of *Gynura procumbens*

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Abstract *Gynura procumbens* is an herbaceous plant. Despite the progressive reports on the pharmacological properties, many are overlooking at the importance of agronomic requirements, such as fertilization, to produce high phytochemical content which have not been conclusively concluded. The study was carried out to examine the effects of N and K interaction on physiological and phytochemical quality; to identify compositions of phytochemicals, and to determine marker compounds. Physiological and phytochemical attributes were recorded in three harvests of triplicate samples to exhibit the trend for plant quality, and statistically analyzed. Generally, N and K interaction have affected phytochemical content significantly ($p < 0.05$) with stronger effect on physiological and biochemical attributes ($p < 0.01$). The results have demonstrated that the following combination of fertilizer, 0 kg/ha N and 30 kg/ha K; and 90 kg/ha N and 0 kg/ha K are high and low, respectively affecting metabolite content in the plant. Lowest rate of N, moderate of K had produced significant phytochemical contents. Meanwhile, caffeic acid and kaempferol were demonstrated as marker compounds in this study. Thus, phytochemical content can be further established through the selection of appropriate N and K rates and proper abiotic stress interaction.

Keywords: agriculture, *Gynura*, nitrogen, phytochemical, potassium.

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Introduction

Nutrient that is taken up beyond plant's requirement would offer no further advantage for growth (Chen *et al.*, 2013). Little is known about the response in secondary metabolism effect. If nitrogen (N) and potassium (K) is taken up below the optimal level, it causes stunting, scleromorphism, and increased root to shoot ratio (Galieni *et al.*, 2015). However, the effects of N and K deficiency in secondary metabolism is less known. With limited amount of nutrient available in the environment, plant growth and secondary metabolism may compete for the nutrient, and a trade-off between growth and secondary metabolite synthesis can occur (Caretto *et al.*, 2015). The secondary metabolite synthesis may be at the cost of reducing plant growth, and *vice versa*. Thus, manipulation of N and/or K supply may obtain a balance between plant growth and metabolite synthesis, and provide chances to increase plant yield (Pal *et al.*, 2015), at the same time maintaining the secondary metabolite synthesis. Regulation in secondary

metabolites synthesis are frequently linked to many aspects of plant's interaction with environment (Figueiredo *et al.*, 2008). Therefore, agronomic factors which influence plant growth may also influence the secondary metabolism. It is important to understand how best to maximize biomass production and secondary metabolite to obtain optimal yield per plant of the active component. Therefore, good agronomic practice especially defining the best nutrient amount for highest yield and active substances is essential. According to Ali *et al.* (2012), increase in N supply, will increase flower yield, and amount and percentage of active substances. However, the study also reported that increase in N and P, will increase flower essence but addition of K will decrease it. The important role of K in metabolism was shown by K deficiency will affects the primary and secondary metabolites contents, with important consequences for both mechanical stability and pathogen/pest resistance in crops (Ibrahim *et al.*, 2012). Potassium is important and influencing transmembrane potentials, pH gradients, long-distance transport, ribosomal function, and changes of enzyme activities (Mohamad Bukhori *et al.*, 2015). The reports have explained various potential metabolic activity under K influence. However, while all these metabolic activities are depending on K availability, potential effect of K on secondary metabolite synthesis is yet to establish. In many aspects, K affects plants in an opposite way to nitrate, resulting in a complex interrelationship between these two nutrients and yield (Chen *et al.*, 2013). However, data are lacking on the mechanisms by which N and K interaction could affect plant growth and secondary metabolite synthesis. Therefore, considering the important of *Gynura procumbens* (*G. procumbens*) among local herbal plants and role of N and K on plant's active substances, the study was conducted to analyse the effect of selected rate of N and K on physiological, biochemical response, and phytochemical profile. The specific objective of the study is to determine the effects of different rates of N and K on physiological and biochemical attributes, and phytochemical profile (primary and secondary metabolites) at different age presented by the different harvesting time, to identify the optimum harvesting time for total content of secondary metabolite as established by the secondary metabolite harvest index, to examine the leaves secondary metabolite content under different N and K rates and harvest time, and to quantify the phenolic and flavonoid content and determine the phytochemical markers under different N and K rates and harvest time.

Materials and methods

Experimental Design and Treatments

The study was based on Randomized Complete Block Design. The data was processed using the analysis of variance (ANOVA) and mean differences analysed using Duncan Multiple Range Test (DNMRT) and Pearson's Correlation Coefficients (PCC), SAS® 9.4 software. This study was two-factorial experiment. The first factor was four rates of N at 30 (N30), 60 (N60), 90 (N90) kg N/ha and a control without N application (N0), which corresponds to respective g/plant applications of 0.36 g, 0.72 g, 1.08 g and 0 g/plant, applied in the form of urea (Hanudin *et al.*, 2012). The N was split into three fertilization phases (three months), and each phase (each month) was about 33.3% of total N applied at the first week of the month. The second factor was plant age at three harvest (H) time, viz. 4, 8 and 12 weeks after treatments (WAT).

Plant Preparation

The propagation was conducted using cutting from mature stem (10 cm, ≥ 2 internode) and placed in the seed tray containing uniform mixture of sand: coco-peat (1:1 v/v) for rooting purposes (Awang *et al.*, 2009). The regenerated plants (1 month old) were then transferred into polyethylene bags (25 cm diameter) containing uniform mixture of soilless medium of coco-peat: burnt paddy husk: well composted chicken manure (5:5:1 v/v/v) ratio, and left for a month to acclimatize until ready for the treatments (Jaafar *et al.*, 2012). The growing media was at pH value 5.5 to 7.0. The greenhouse average temperatures were at 22.9 to 27.2°C with relative humidity between 50 and 70%. Meanwhile, the average daytime irradiance was 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic active radiation.

Maintenance and Sampling

The plants were watered manually once a day in the morning or when necessary. During very hot, watering was conducted twice or thrice. Each watering was about 500 mL per plant. Non-chemical pest and disease control was conducted manually at the sight of their presence. Each combined treatment consisted of 27 plants totalling a sum of 108 plants used in the experiment. Three plants per treatment were sampled at each H time.

Photosynthetic Rate and Stomatal Conductance Rate

The measurement was obtained from LI-COR® Environmental with closed infrared gas analyzer. The measurements were carried out between 09:00 to 11:00 a.m. using fully expanded young leaves

numbered three and four from plant apex to record photosynthetic carbon assimilation rate (Photo) and stomatal conductance to water (Cond). The operation was automatic, and the data were stored in LI-6400 console and analyzed by the Photosyn Assistant software. Plants of the different treatments were measured alternately. Prior to use, the system was warmed for 30 min and calibrated with the ZERO IRGA mode. Two steps are required in the calibration process: first, the initial zeroing process for the built-in flow meter; and second, zeroing process for the infra-red gas analyzer. The measurements used optimal conditions set: 400 $\mu\text{mol/mol}$ CO_2 30°C cuvette temperature, 60% relative humidity with air flow rate at 500 $\text{cm}^3 \text{min}^{-1}$, and modified cuvette condition of 800 $\mu\text{mol/m}^2\text{s}$ photosynthetically photon flux density. Several precautions were taken to avoid errors during measurements such as leaf surfaces were cleaned and dried using tissue paper before enclosed in the leaf cuvette. (Ibrahim *et al.*, 2017).

Total Chlorophyll Contents

Total chlorophyll content (TChIC) was measured using fresh weight basis. Only fresh leaf tissues of the third fully expanded leaves from the top of the individual plant were used in the analysis. Leaf disk of 3 mm in diameter was obtained using puncher to acquire 15 consistent sizes per treatment per block. The leaf disks were immediately immersed and incubated in 20 mL of 80% acetone for homogenization in an aluminum foil-covered glass bottle for approximately 24 h at 5°C until all the green colour had bleached out. Finally, the solution (15 μL) was transferred into 96 well plate to determine absorbances of chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids (Car) using UV-vis Spectrophotometer at wavelengths of 645, 662 and 470 nm optical density (OD), respectively. Measurements were run in three replicates. The chlorophyll content was calculated as $\mu\text{g g}^{-1}$ fresh weight as per standard equations recommended (Ibrahim *et al.*, 2017).

$$\text{Chl a} = [(12.47 \times \text{OD at } 662) - (3.62 \times \text{OD at } 645) \times 10] / (1000 \times \text{wt})$$

$$\text{Chl b} = [(25.06 \times \text{OD at } 645) - (6.50 \times \text{OD at } 662) \times 10] / (1000 \times \text{wt})$$

$$\text{Car} = [(1000 \times \text{OD at } 4700) - (1.29 \times \text{Chl a}) - (53.78 \times \text{Chl b})] / 220$$

Total Carbohydrates Content

Total carbohydrates content (TCC) was measured using the method described by Anthrone and Hofreiter (Hansen & Moller, 1975). Samples were weighed 1 g into 50 mL conical tube. Sample were hydrolyse by keeping it in boiling Water Bath for 3 h with 5 mL of 2.5 M hydrochloric acid and cool to room temperature. Then, the sample were neutralise with solid sodium carbonate until the effervescence ceases. Next, the volume was made up to 50 mL and centrifuged at 5,000 rpm for 5 min. The supernatant separated and then filtered with filter paper. The 1 mL of aliquots was taken for analysis. Beforehand, the standards were prepared by taking 0.2, 0.4, 0.6, 0.8 and 1 mL of the working standard. The 0.0 mL serves as blank. The volume was made up to 1 mL in all tubes including the sample tubes by adding distilled water. Cool the contents of all tubes on ice before adding ice-cold anthrone reagent. Then, 4 mL of anthrone reagent were added and heated for 8 min in boiling water bath. The blanks used were absolute methanol. Finally, cool rapidly, then, the solution (15 μL) was transferred into 96 well plate and read the sample absorbance at 630 nm using UV-vis Spectrophotometer. Measurements were run in three replicates. The concentration of TCC was calculated according to the following equation that was obtained from the standard glucose graph: The TCC in the sample was expressed as mg glucose equivalent g^{-1} dry sample.

$$y = 0.01x + 0.1813$$

$$R^2 = 0.995.$$

All the reagents were prepared fresh prior to use. Anthrone reagent was prepared by dissolving 200 mg of anthrone in 100 mL of ice-cold 95% sulphuric acid. Meanwhile, standard glucose was prepared by preparing the stock of liquid chromatography-grade glucose (G) by dissolving 100 mg in 100 mL of distilled water. The working standard of glucose were prepared by taking 10 mL of glucose stock and diluted to 100 mL with distilled water. The solution was stored refrigerated after adding a few drops of toluene (Ibrahim *et al.*, 2017).

Total Protein Content

Total protein content (TPrC) was measured using the method described by Lowry (John, 1995). Sample were weight 1 g into 50 mL conical tube and extracted with 10 mL of 100% methanol at room temperature for 24 h and centrifuged at 7,000 rpm for 10 min. The supernatant was separated and then filtered with filter paper. Then, 0.2 mL of extract was pipette out and the volume was made to 1 mL with distilled water. The 5 mL of alkaline copper reagent was added to all tubes and allowed it to stand for 10 min. Then, 0.5 mL of Folin's Ciocalteu reagent was added and incubated in the dark for 30 min. The blanks used were absolute methanol. Finally, the solution (15 μL) was transferred into 96 well plates to determine the absorbances. The intensity of the colour developed was read at 660 nm using UV-vis Spectrophotometer. Measurements were run in three replicates. The concentration of TPrC was

calculated according to the following equation that was obtained from the standard bovine serum albumin graph: The TPrC in the sample was expressed as mg bovine serum albumin equivalent g^{-1} dry sample.

$$y = 0.0621x + 0.1554$$

$$R^2 = 0.9904$$

All the reagents were prepared fresh prior to use. Standard bovine serum albumin was prepared by preparing the stock of liquid chromatography-grade bovine serum albumin (BSA) by dissolving 20 mg BSA in 10 mL of the same diluents for the samples. Then dilute to 200, 400, 600, 800, 1,000 and 1,200 $\mu\text{g}/\text{mL}$ (Ibrahim *et al.*, 2011).

Total Lipid Content

Total lipid content (TLiC) was measured using the method described by Folch (Shams *et al.*, 2015). Sample were weighed 1 g into 50 mL conical tube and extracted with chloroform and methanol (2:1, v/v) (20 mL) for homogenization at room temperature for 24 h and centrifuged at 7,000 rpm for 10 min. Lipid extract was purified to eliminate some contaminants by pouring the extracts into a beaker through a filter paper containing activated charcoal to remove coloring matters. A clear supernatant was obtained which was then further purified with the use of 0.2 mL of aqueous 0.9% (w/v) sodium chloride. Purified lipids were transferred into evaporated and concentrated dryness at 40°C, and the residue was weighed. Measurements were run in three replicates. Quantification of crude lipid was performed based on the dry weight determination. The TLiC in the sample was expressed as mg g^{-1} dry sample. All the reagents were prepared fresh prior to use (Ibrahim *et al.*, 2017).

Total Phenolic Content

Total phenolic content (TPC) was measured using the method described by Folin-Ciocalteu colorimetric assay (Lowry *et al.*, 1951). Sample were weight 1 g into 50 mL conical tube and extracted with 10 mL of 100% methanol at room temperature for 24 h and centrifuged at 7,000 rpm for 10 min. The supernatant was separated and then filtered with filter paper. Briefly, 1 mL of extract was pipetted into 15 mL conical tube and 2 mL (10% v/v) of Folin-Ciocalteu reagent was added to the sample and incubated for 5 min. Later, 1.6 mL (7.5%) of sodium carbonate solution was added into the sample. The sample mixture was then vortexed and incubated in the dark for one hour at room temperature. The blanks used were absolute methanol. Finally, the solution (15 μL) was transferred into 96 well plates to determine the absorbances. Absorbance of the samples was then measured at 760 nm using UV-vis Spectrophotometer. Measurements were run in three replicates. The concentration of TPC was calculated according to the following equation that was obtained from the standard caffeic acid graph: The TPC in the sample was expressed as mg caffeic acid equivalents g^{-1} dry sample.

$$y = 0.0098x + 0.0427$$

$$R^2 = 0.9942$$

All the reagents were prepared fresh prior to use. Standard caffeic acid was prepared by preparing the stock of liquid chromatography-grade caffeic acid (CA) by dissolving 1 mg in 1 mL of the same diluents for the samples. Then, dilute to 0.1, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8 and 2.0 mg/mL (Ibrahim *et al.*, 2017).

Total Flavonoid Content

Total flavonoid content (TFC) was measured using the method of aluminum chloride complex colorimetric assay Mongkhonsin *et al.*, 2016). Sample were weighed 1 g into 50 mL conical tube and extracted with 10 mL of 100% methanol at room temperature for 24 h and centrifuged at 7,000 rpm for 10 min. The supernatant was separated and then filtered with filter paper. Briefly, 1 mL of extract was pipetted into 15 mL conical tube, mixed with 5 mL of distilled water and 0.3 mL of 5% sodium nitrite solution. Mixed well and the mixture allowed to stand for 6 min and then 0.6 mL of 10% aluminum chloride solution was added. After 5 min, 2 mL of 1M sodium hydroxide was added to the mixture and made up to total volume of 10 mL with distilled water. The blanks used were absolute methanol. Finally, the solution (15 μL) was transferred into 96 well plates to determine the absorbances. Absorbance of the samples was then measured at 510 nm using UV-vis Spectrophotometer. Measurements were run in three replicates. The concentration of TFC was calculated according to the following equation that was obtained from the standard kaempferol graph: The TFC in the sample was expressed as mg kaempferol equivalents g^{-1} dry sample.

$$y = 0.0108x + 0.0435$$

$$R^2 = 0.9933$$

All the reagents were prepared fresh prior to use. Standard kaempferol was prepared by preparing the stock of liquid chromatography-grade kaempferol (K) by dissolving 1 mg in 1 mL of the same diluents for

the samples. Then, dilute to 0.04, 0.20, 0.40, 0.70, 0.90, 1.10, 1.30, 1.50 and 1.80 mg/mL (Kaewseejan *et al.*, 2015).

Quantification of Phenolics and Flavonoid

Preparation of Plant Extract

Sample were weighed 1 g into 50 mL conical tube and extracted with 10 mL of 100% methanol at room temperature for 24 h, sonicated at normal mode for 5 min and centrifuged at 7,000 rpm for 10 min. The supernatant was separated and then filtered with filter paper. The methanolic-extract was transferred into evaporated and concentrated dryness at 40°C using Rotary Evaporator. It was weighed, redissolved in 1.5 mL liquid chromatography-grade methanol and filtered through sterile membrane filter, 0.45 µm; 25 mm in 2 mL amber glass vials and ready for further chromatographic analysis (Kaewseejan & Siriamornpun, 2015).

Thin Layer Chromatography

In thin layer chromatography (TLC) analysis, the stationary phase used was 20 x 20 cm, 0.25 mm plate pre-coated with silica gel 60 F₂₅₄ on aluminum sheets. The mobile phase used was a mixture of toluene, ethyl acetate and formic acid (5:4:1). Marker or reference compounds used were liquid chromatography-grade caffeic acid, cinnamic acid, chlorogenic acid, gallic acid, ferulic acid and vanillic acid for TPC. Meanwhile, kaempferol, quercetin, myricetin and rutin for TFC. Standard compounds were prepared by preparing the stock of compound by dissolving 1 mg in 1 mL of liquid chromatography-grade methanol (Ismail *et al.*, 2015). The 10 µL of sample and reference compound were applied as 6 mm band, 2 mm apart, 10 mm from the lower, upper, left, and right edges of the plate using microsyringe. In the glass tank, 50 mL of the developing solvent was poured and allowed to be saturated for 5 to 10 min in room temperature. Migration (as in linear ascending development) distance of the developing solvent on the plate is 80 mm from lower edge of the plate or equivalent to the time allowed for the development and maximal separation of the active compounds present in the samples was 15 to 25 min. The plates were then dried at 100°C using Forced Draft Oven for 3 to 5 min. Dried plates were visualised by observing under UV light at 254- and 366-nm. Spot detection was done qualitatively. The colour and distance of the unknown spots were compared with reference compound. The retardation factor (R_f) values were calculated using the formula of migration distance of spot/migration distance of solvent (Ismail *et al.*, 2015).

High-Performance Liquid Chromatography for TPC

The HPLC-grade purity chemicals and reference compounds such as methanol, acetonitrile, orthophosphoric acid 85%, caffeic acid, cinnamic acid, chlorogenic acid, gallic acid, ferulic acid and vanillic acid were used in the analysis. The linear standard curves were constructed by injecting range of 0.05, 0.07, 0.09, 0.11, 0.13, 0.15 and 0.17 mg of caffeic acid mL⁻¹ of methanol. Filtered water was obtained from a Milli-Q Ultrapure Water Purification System. All the reference compound stock solutions were prepared by dissolving 1 mg in 1 mL of liquid chromatography-grade methanol, filtered through sterile membrane filter, 0.45 µm; 25 mm in 2 mL amber glass vials, and stored at 5°C. Diluted working solutions were freshly prepared for each analysis (Giedyk, 2011). The system was equipped with Waters® 600E Multisolvant Delivery System, USA consisting of UV/Visible Detector (Waters® 2489), Degasser (Waters®), Autosampler (Waters® 2707) and Waters Empower 3 software was employed in this study. Separation was achieved on reversed phase column [RP-18e (100 x 4.6 mm i.d; 2 µm), Semiprep, Particle size 0 µm, Pore size 130 Å] preceded by C18 guard column at 40°C with diode array detector (DAD) set at 190 to 600 nm and (0.1%) orthophosphoric acid 85% (eluent A): acetonitrile (eluent B) (90.5:9.5, v/v isocratically) employed as the eluent. Column was washed with 100% methanol for 10 min, the initial conditions were again applied, the system was monitored for 30 min and the column allowed to equilibrate with (0.1%) orthophosphoric acid 85%: acetonitrile (90.5:9.5, v/v isocratically) for 35 min, being 75 min the total consuming time of run. The temperature of the oven was 40°C. The flow rate was kept constant at 0.7 ml min⁻¹ and the peaks were simultaneously identified using UV absorbance at 327 nm for caffeic acid. The injection volume was 1 µl (standard compound) and 10 µl (sample) for each technical repeat automatically. Quantification was carried out by integration of the peak using external standard method. The procedure was performed separately for each standard (Yi *et al.*, 2016). This method was linear in the range tested and had high linear regression coefficient of $r=0.9936$ between concentrations and peak areas.

High-Performance Liquid Chromatography for TFC

The HPLC-grade purity chemicals and reference compounds such as methanol, acetonitrile, orthophosphoric acid 85%, kaempferol, quercetin, myricetin and rutin were used in the analysis. The linear standard curves were constructed by injecting range of 0.2, 0.3, 0.4, 0.5 and 0.6 mg of kaempferol

mL⁻¹ of methanol. Filtered water was obtained from Milli-Q Ultrapure Water Purification System. All the reference compounds stock solutions were prepared by dissolving 1 mg in 1 mL of liquid chromatography-grade methanol, filtered through sterile membrane filter, 0.45 µm; 25 mm in 2 mL amber glass vials, and stored at 5°C. Diluted working solutions were freshly prepared for each analysis (Giedyk, 2011). The system was equipped with Waters® 600E Multisolvent Delivery System, USA consisting of UV/Visible Detector (Waters® 2489), Degasser (Waters®), Autosampler (Waters® 2707) and Waters Empower 3 software was employed in this study. Separation was achieved on reversed phase column [RP-18e (100 x 4.6 mm i.d; 2 µm), Semiprep, Particle size 0 µm, Pore size 130 Å] preceded by C18 guard column at 40°C with diode array detector (DAD) set at 190 to 600 nm and (0.03M) orthophosphoric acid 85% (eluent A): methanol (eluent B) (60:40, v/v isocratically) employed as the eluent. Column was washed with 100% methanol for 10 min, the initial conditions were again applied, the system was monitored for 30 min and the column allowed equilibrating with orthophosphoric acid 85%: methanol (60:40, v/v isocratically) for 35 min, being 75 min the total consuming time of run. The temperature of the oven was 35°C. The flow rate was kept constant at 1 ml min⁻¹ and the peaks were simultaneously identified using UV absorbance at 250 to 360 nm for kaempferol. The injection volume was 10 µl (standard compound and sample) for each technical repeat automatically. Quantification was carried out by integration of the peak using external standard method. The procedure was performed separately for each standard (Shoeva *et al.*, 2016). This method was linear in the range tested and had high linear regression coefficient of $r=0.9953$ between concentrations and peak areas.

Statistical Analysis

The data were subjected to ANOVA and correlation using SAS® 9.4 software. The analysis were done in triplicate and expressed as mean ($n=3$) ± standard error (SE) from the dependent treatments (Jaafar *et al.*, 2012). The variables from measurements were analyzed using General Linear Model with N and K supply managements. Any differences between treatment means were analysed by two-way analysis and compared using DMRT at p value ≤ 0.05 levels. The regression model that best fitted the data, evaluated by an F-test, was chosen.

Results

Photosynthetic Rate

Generally, Photo was statistically consistent in all treatments and significant in H time ($p \leq 0.05$) (Figure 1). The Photo in NOK0 was highest at Week 4. As treatment continued to Week 8, Photo has decreased lower than other treatments. Continuing the treatment to Week 12, the NOK0 recorded the lowest Photo than other treatment at almost 1.5 µmol CO₂ m⁻²s⁻¹. The Photo in NOK90 treatment was highest than other treatment at Week 12. Photosynthetic rate was highest in NOK0 at Week 4 when plants are young and this continued until Week 8 followed by NOK60, NOK30, N90K90, NOK90, N90K30, N90K0 and N90K60. Measurements of Photo are significantly correlated with Cond at $r=0.600$; $p \leq 0.0001$ by a linear function (Table 1). This is because Photo was decreased with continuing H time and increasing N and K rates. In this experiment, Photo was not significantly affected by N and K rates ($p \geq 0.05$). Plants in NOK0 displayed highest Photo (6.5 µmol CO₂ m⁻²s⁻¹) at Week 4 followed by NOK60 (5.6 µmol CO₂ m⁻²s⁻¹), NOK30 (5.5 µmol CO₂ m⁻²s⁻¹), N90K90 (5 µmol CO₂ m⁻²s⁻¹), NOK90 (4.5 µmol CO₂ m⁻²s⁻¹), N90K30 (4.4 µmol CO₂ m⁻²s⁻¹), N90K0 (2.6 µmol CO₂ m⁻²s⁻¹) and N90K60 (2.2 µmol CO₂ m⁻²s⁻¹). As plant aged at Week 8 and 12, Photo increased significantly ($p \leq 0.05$) especially at Week 8, following this order N90K60, NOK30, NOK60, N90K30, N90K0, NOK0, N90K90 and NOK90 with respective values 10.50 µmol CO₂ m⁻²s⁻¹, 10.40 µmol CO₂ m⁻²s⁻¹, 10.10 µmol CO₂ m⁻²s⁻¹, 10 µmol CO₂ m⁻²s⁻¹, 9.70 µmol CO₂ m⁻²s⁻¹, 9.50 µmol CO₂ m⁻²s⁻¹, 9.40 µmol CO₂ m⁻²s⁻¹ and 9 µmol CO₂ m⁻²s⁻¹ (Figure 1). The event was because the plants in NOK0 had enough N and K from the growth media for high photosynthetic capacity and light utilisation. Suggesting NOK0, NOK30 and NOK60 are highly respond to nutrient acquisition and affected by water availability and carbon use efficiency (Wang *et al.*, 2016). In this experiment, Photo decreased with continuing H time was probably because of K deficiency with continuing H time (Ali *et al.*, 2012). Potassium has influenced photosynthetic rate, stomatal conductance, water movements (turgor regulation and osmotic adjustment) and enzyme activation which affects adenosine triphosphate (ATP) production in plants (Chen *et al.*, 2013). Subsequently, the ATP will regulate the rate of photosynthesis (Torres & Lopez, 2010).

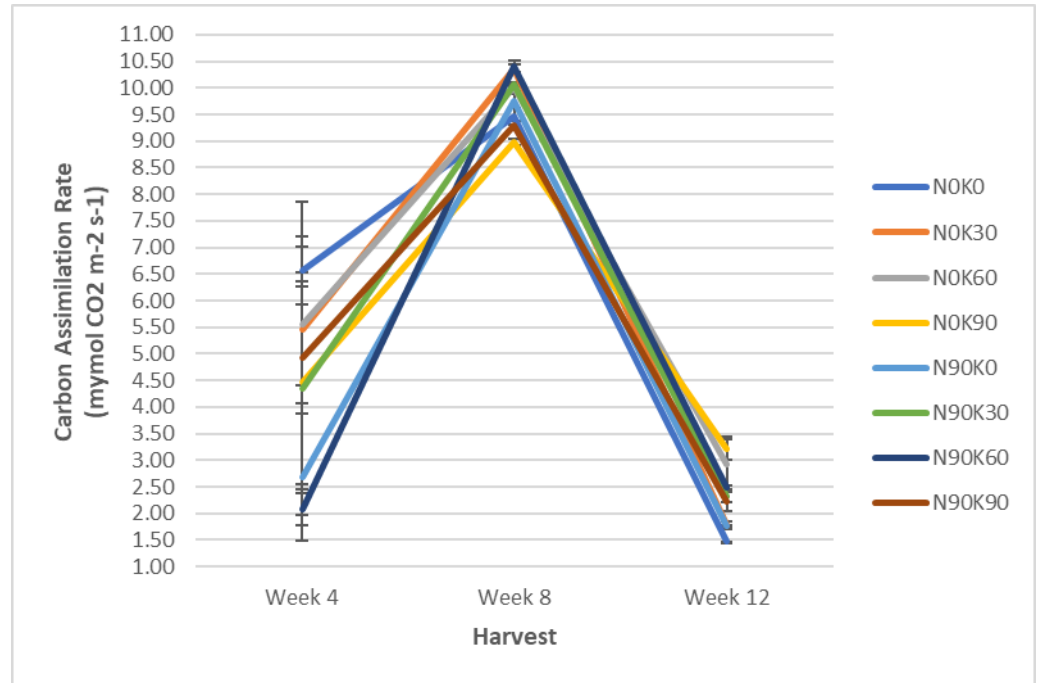


Figure 1. Changes in Photo ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) as affected by rates of N (0 and 90 kg N/ha), K [0 (Control), 30, 60 and 90 kg K/ha] and H times (4, 8 and 12 WAT). Bars represents standard error of differences between the means. N=72.

Stomatal Conductance Rate

Generally, Cond was affected in all treatments and statistically significant with increasing age indicated by H time ($p \leq 0.0001$) (Figure 2). The Cond of plants in N0K30 treatment recorded highest value ($p \leq 0.05$) from Week 4 to Week 12, meanwhile, N0K0 recorded lowest value. As plant aged, Cond in all treatments increased at Week 8 and decreased at Week 12. The Cond in N0K30 was significantly higher ($p \leq 0.0001$) than other treatments at longer H time (Week 12). The N0K0 has lowest Cond compared to N90K0. Stomatal conductance was highest in N0K30 at Week 8 compared to N90K60 at Week 8. Measurements of Cond are significantly correlated with Photo at $r=0.600$; $p \leq 0.0001$ by a linear function (Table 1). In this experiment, Cond seemed to fluctuate with increasing H time probably because of pre-adaptation, adaptation and post-adaptation during plant growth (Heydarizadeh, 2016). In early stage of growth, stomatal are in pre-adaptation during the acclimatization, therefore Cond was low, following adaptation during the continuous treatment, therefore, Cond was increased, and finally post-adaptation where Cond was decreased during the growth terminal (Wuyts, 2015). The Cond was highest in N0K30 at Week 8, and lowest in other treatments especially when plant aged at Week 12 (Figure 2). Plants in N0K0 probably had the lowest intercellular CO₂ concentration ($116 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) compared to N0K30 ($227 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), which represented higher carbon assimilation and could have directly contributed to active Cond ($0.094 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) (Figure 2). Meanwhile, plants in N0K0 were perhaps experiencing diffusion resistance along the experiments (Chen *et al.*, 2013). This finding suggested N0K30 is highly stimulated stomatal opening and closing.

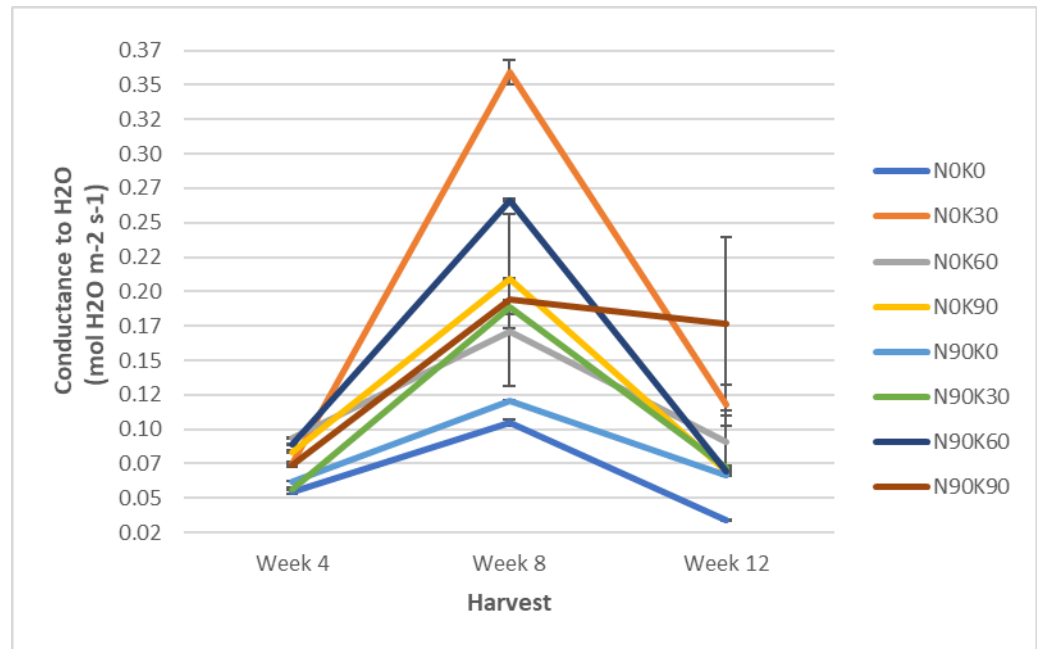


Figure 2. Changes in Cond (mol H₂O m⁻²s⁻¹) as affected by rates of N (0 and 90 kg N/ha), K [0 (Control), 30, 60 and 90 kg K/ha] and H times (4, 8 and 12 WAT). Bars represents standard error of differences between the means. N=72.

Total Chlorophyll Contents

Generally, TChIC was statistically affected in all treatments and age as indicated by the H time ($p \leq 0.0001$) (Figure 3). At Week 4, the N0K90 treatment recorded significantly ($p \leq 0.0001$) highest value of TChIC compared to other treatments which were not significantly different ($p \geq 0.05$) to each other with the lowest value recorded in N90K0. However, as the plant aged to Week 8 and 12, the N90K0 recorded increased TChIC ($9.00 \mu\text{g g}^{-1}$ FW) ($p \leq 0.0001$), followed by N90K30 ($8.70 \mu\text{g g}^{-1}$ FW). Meanwhile, other treatments were decreased, N0K0 ($8.10 \mu\text{g g}^{-1}$ FW), N0K30 ($8.80 \mu\text{g g}^{-1}$ FW), N0K60 ($8.60 \mu\text{g g}^{-1}$ FW), N0K90 ($7.70 \mu\text{g g}^{-1}$ FW), N90K60 ($8.70 \mu\text{g g}^{-1}$ FW), and N90K90 ($4.60 \mu\text{g g}^{-1}$ FW). In this experiment, TChIC was highest in N0K90 at Week 4 compared to N90K0 (Figure 3). However, measurements of TChIC are not significantly correlated with Photo at $r = -0.059$; $p \geq 0.05$ by a linear function (Table 1). The values are concurrent to Cond rates at Week 4 (Figure 2), where gas exchange rate leaf was low, and increased during Week 8 and subsequently decreased by 8% at Week 12. The event was because of pre-adaptation (acclimatisation), adaptation (growth) and post-adaptation (maturing) during plant growth cycle where N and K are vital component of plants energy metabolism and of activated intermediates in the photosynthesis following the growth cycle requirement accordingly (Pant *et al.*, 2015). Meanwhile, high N rate reported significantly decreased chlorophyll content ($p \leq 0.05$) (Rahimi *et al.*, 2013), and sufficient rates of K in N0K90 was determined the TChIC highest in the treatment where optimum K rate resulted in a maximum net Photo that could be associated with the highest chlorophyll content and fully open stomata conductance (Chen *et al.*, 2013) (Figures 1, 2 and 3).

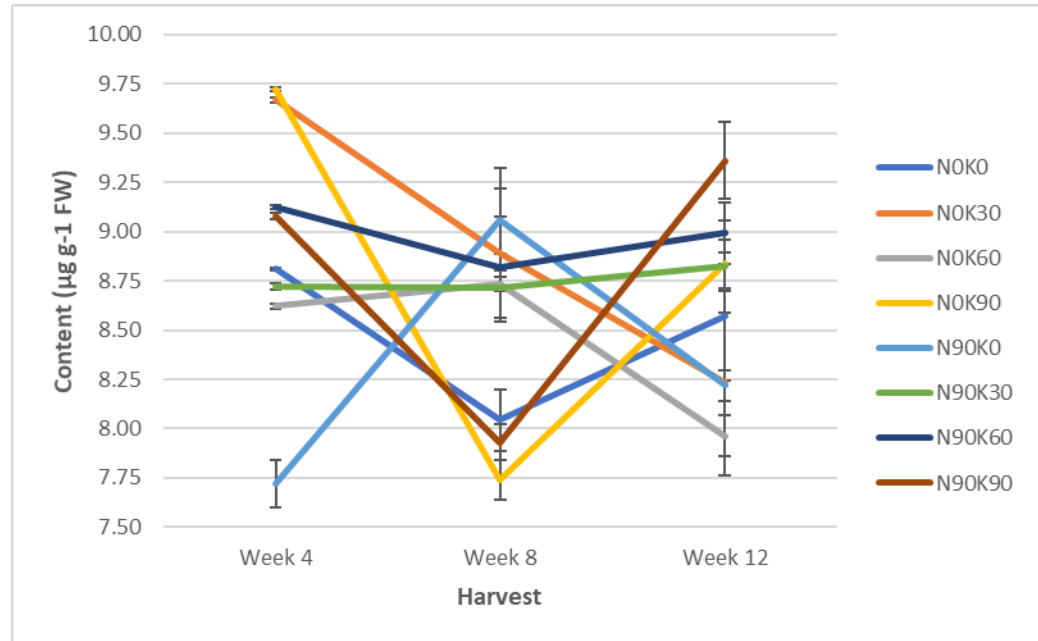


Figure 3. Changes in TChIC ($\mu\text{g g}^{-1}$ FW) as affected by rates of N (0 and 90 kg N/ha), K [0 (Control), 30, 60 and 90 kg K/ha] and H times (4, 8 and 12 WAT). Bars represents standard error of differences between the means. N=72.

Total Carbohydrates Content

Generally, TCC was statistically affected in all treatments at different plant age as represented by the H time ($p \leq 0.001$) (Figure 4). The N90K0 treatment has increased tremendously TCC followed by N0K60, N90K60, N0K90, N90K30, N90K90, N0K30, and N0K0 when applied during early growth at Week 4. As the growth progressed to Weeks 12, N90K0 continued to increase TCC from 105 to 250 mg GE/g DW compared to N0K0, which value did not continue to surpass all other treatments; instead the value was almost like N0K30, N0K90, N90K30, and N90K90 (Figure 4). Nitrogen is an essential element constituent of plant compounds that determine the quality of plant organic material (Macadam, Volenec, & Nelson, 1989). Meanwhile, K is involved in the activities of over 60 types of plant enzymes including metabolism of N and carbohydrates (Chen *et al.*, 2013). In this experiment, TCC was increased significantly with continuing H time and increasing N rate ($p \leq 0.05$) (Figure 4). Measurements of TCC are significantly correlated with TPrC at $r = -0.293$; $p \leq 0.05$. However, not significantly correlated with Photo, TLiC, TPC and TFC at $r = -0.226$; $p \geq 0.05$, $r = -0.078$; $p \geq 0.05$, $r = -0.208$; $p \geq 0.05$ and $r = -0.192$; $p \geq 0.05$, respectively by a linear function (Table 1). The event was because of high N rate would increase sugar content and promote the accumulation of soluble sugars of plants (Aminifard *et al.*, 2012). Meanwhile, when TCC decreased, the TPrC will increased (Figure 5), decreased photosynthetic capacity (Figure 1), and increased respiration rate (Figure 2) (Baby *et al.*, 2017).

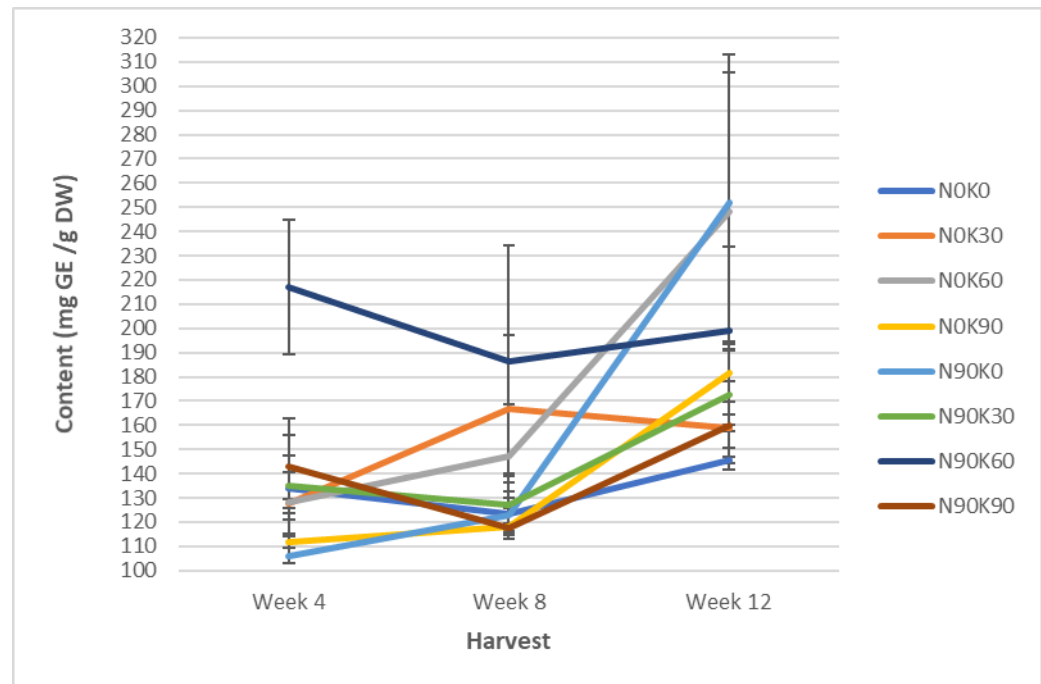


Figure 4. Changes in TCC (mg GE/g DW) as affected by rates of N (0 and 90 kg N/ha), K [0 (Control), 30, 60 and 90 kg K/ha] and H times (4, 8 and 12 WAT). Bars represents standard error of differences between the means. N=72. The calibration curves for TCC markers (glucose) were linear over the range of 0.0 to 100.0 mg/mL. The coefficient of determination (R^2) was greater than 0.995 for all standard curves.

Total Protein Content

Generally, TPrC was statistically affected in all treatments and H time ($p \leq 0.01$) (Figure 5). At early growth stage (Week 4), the N90K0 treatment recorded significantly the highest ($p \leq 0.0001$) TPrC value (11 mg BSAE/g DW) followed by NOK0 (10 mg BSAE/g DW), N90K90 (9.80 mg BSAE/g DW), NOK30 (9.50 mg BSAE/g DW), NOK60 (7.50 mg BSAE/g DW), N90K30 (7 mg BSAE/g DW), NOK90 (6 mg BSAE/g DW), and N90K60 (3.50 mg BSAE/g DW). However, during Week 12, TPrC has decreased in N90K0, which showed lower and almost similar with NOK30 value. Meanwhile, TPrC has increased tremendously in N90K30 and N90K90 with increasing H time from Week 8 to 12. In this experiment, TPrC was highest in N90K30 and N90K90, and lowest in N90K60 with continuing H time (Figure 5). Measurements of TPrC are significantly correlated with TCC at $r = -0.293$; $p \leq 0.05$. However, not correlated with Photo, TLiC, TPC and TFC at $r = -0.088$; $p \geq 0.05$, $r = -0.296$; $p \geq 0.05$, $r = 0.024$; $p \geq 0.05$ and $r = 0.086$; $p \geq 0.05$, respectively by a linear function (Table 1). The event was because of plants protein contents in each treatment are affected by N rates. Meanwhile, K may influence the metabolic processes, such as turgor driven movements, osmoregulation, control of membrane polarization and involved with enzyme activation which affects the protein and protein-related compound (Mohamad Bukhori *et al.*, 2015). Meanwhile, in N90K30 and N90K90 probably sufficient N and K rates has led to effective photosynthesis rates and trade-off with carbohydrate synthesis by increasing the proportion of soluble protein (Anulika *et al.*, 2016). In this experiment, high N and K rate exhibited higher TPrC could be related to value recorded in TChIC (Figure 3) and TCC (Figure 4) presented by N90K30 and N90K90. Plants that were supplied with enough K rate showed a greater capacity for nitrate assimilation. And N is crucial for plants to perform the routine and fundamental cellular activities. This include photosynthetic products which provide the energy and C-skeletons for amino acid synthesis during protein biosynthesis (Chen *et al.*, 2013).

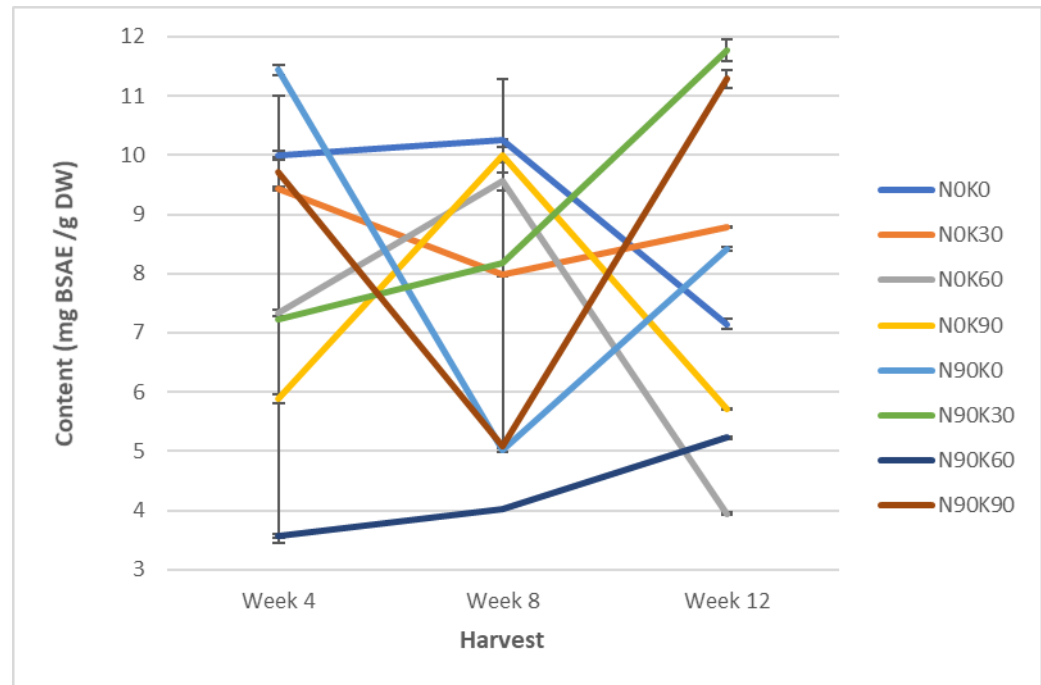


Figure 5. Changes in TPrC (mg BSAE/g DW) as affected by rates of N (0 and 90 kg N/ha), K [0 (Control), 30, 60 and 90 kg K/ha] and H times (4, 8 and 12 WAT). Bars represents standard error of differences between the means. N=72. The calibration curves for TPrC markers (bovine serum albumin) were linear over the range of 0.0 to 1.0 mg/mL. The coefficient of determination (R^2) were greater than 0.990 for all standard curves.

Total Lipid Content

Generally, TLiC was statistically affected in all treatments and H time ($p \leq 0.0001$) (Figure 6). At early growth, all treatments have started to influence the TLiC. With increase in age from Week 8 to Week 12, NOK30, NOK60, N90K0, N90K30, and N90K60 have increased the value of TLiC tremendously ($p \leq 0.05$) from the lowest value at 10 mg/g DW to 63 mg/g DW. Meanwhile, N90K90 treatment which recorded highest value at Week 8 (70 mg/g DW) compared to all treatments, upon reaching Week 12 has recorded significantly lower ($p \leq 0.05$) TLiC than that recorded by NOK30 and N90K0 but equal to NOK60 and N90K30 and higher than TLiC value in NOK90 and N90K60 (Figure 6). Measurements of TLiC are significantly correlated with Photo at $r = 0.286$; $p \leq 0.05$. However, not significantly correlated with TCC, TPrC, TPC and TFC at $r = -0.078$; $p \geq 0.05$, $r = -0.296$; $p \geq 0.05$, $r = 0.046$; $p \geq 0.05$ and $r = 0.042$; $p \geq 0.05$, respectively by a linear function (Table 1). In this experiment, TLiC has fluctuate with continuing H time and rising N and K rates. The event was because of metabolically, lipid synthesis may be related to development of intracellular membranous compartmentation and the availability of intracellular (non-plasmic and periplasmic) spaces as plant ages (Ora & Anekwe, 2013). Since vacuoles are intracellular spaces function as accumulation sites for plant metabolites, therefore, vacuolar membranes might play a pivotal role in compartmentalized acquisition, sequestration and retention of metabolites, including lipids (Mishra *et al.*, 2006). On the other hands, TLiC has increased during leaf expansion in order to attain optimum level at full maturity and substantial decline in senescing tissue of old plants (Mishra *et al.*, 2006). At the same time, K has influenced the metabolism of N, carbohydrates and protein, and the synthesis of lipid. This is because, K may exhibit changes in N:K ratio instead of N:P and/or in the N fractions proportions during lipid synthesis in the plant (Chrysargyris *et al.*, 2017).

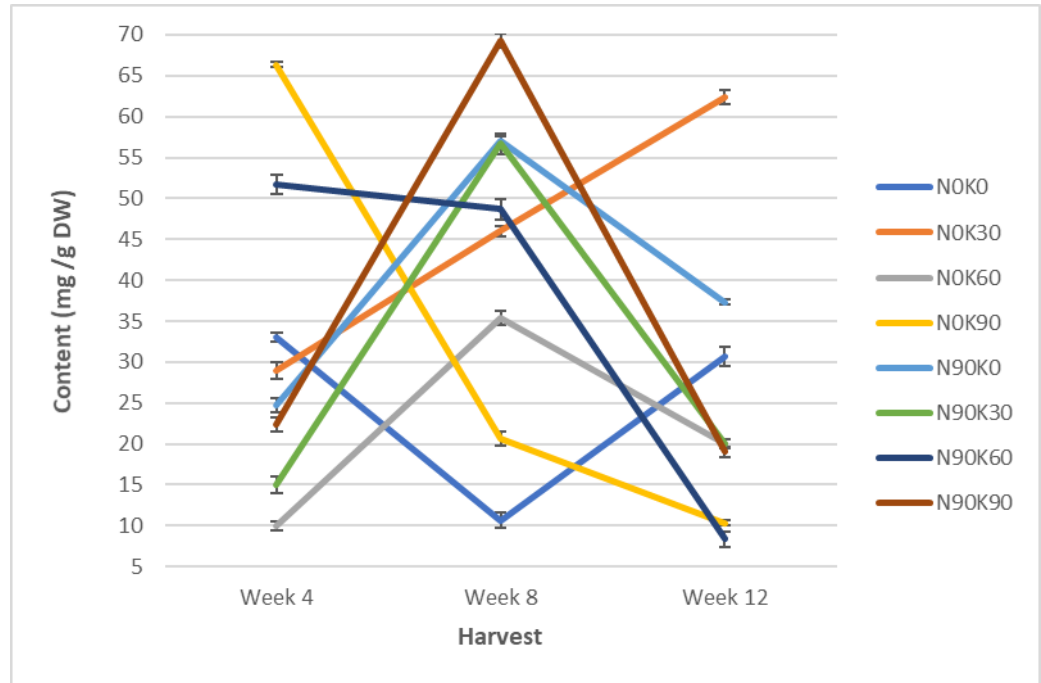


Figure 6. Changes in TLiC (mg/g DW) as affected by rates of N (0 and 90 kg N/ha), K [0 (Control), 30, 60 and 90 kg K/ha] and H times (4, 8 and 12 WAT). Bars represents standard error of differences between the means. N=72.

Total Phenolic Content

Generally, TPC was statistically affected in all treatments at different H time ($p \leq 0.0001$) (Figure 7). At Week 4, TPC value was significantly decreased ($p \leq 0.0001$) with different N and K treatment in descending manner N0K0 (104 mg CAE/g DW) > N0K30 (100 mg CAE/g DW) > N0K90 (88 mg CAE/g DW) > N90K30 (80 mg CAE/g DW) > N0K60 (78 mg CAE/g DW) > N90K0 (70 mg CAE/g DW) > N90K60 (60 mg CAE/g DW) > N90K90 (52 mg CAE/g DW). As the plant aged indicated by H time from Week 4 to Week 12, TPC has increased ($p \leq 0.05$) in N0K60 followed by N90K60 and N90K90 (Figure 7). Measurements of TPC are significantly correlated with TFC at $r=0.573$; $p \leq 0.0001$. However, not significantly correlated with Photo, TCC, TPrC and TLiC at $r=0.225$; $p \geq 0.05$, $r=-0.208$; $p \geq 0.05$, $r=0.024$; $p \geq 0.05$ and $r=0.046$; $p \geq 0.05$, respectively by a linear function (Table 1). The event was due to N supply rate. Low N treatment, resulted in an up-regulation of the phenolic synthesis, as demonstrated by an increase in the TPC. The amplitude of the increase in phenolic content did not depend on the Photo to which the plant was subjected, which indicates that the plants did not acclimate to low Photo (5 to $6 \mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$) (Figure 1) (Larbat *et al.*, 2012). However, successive K supply resulted in additive increases in phenolic content.

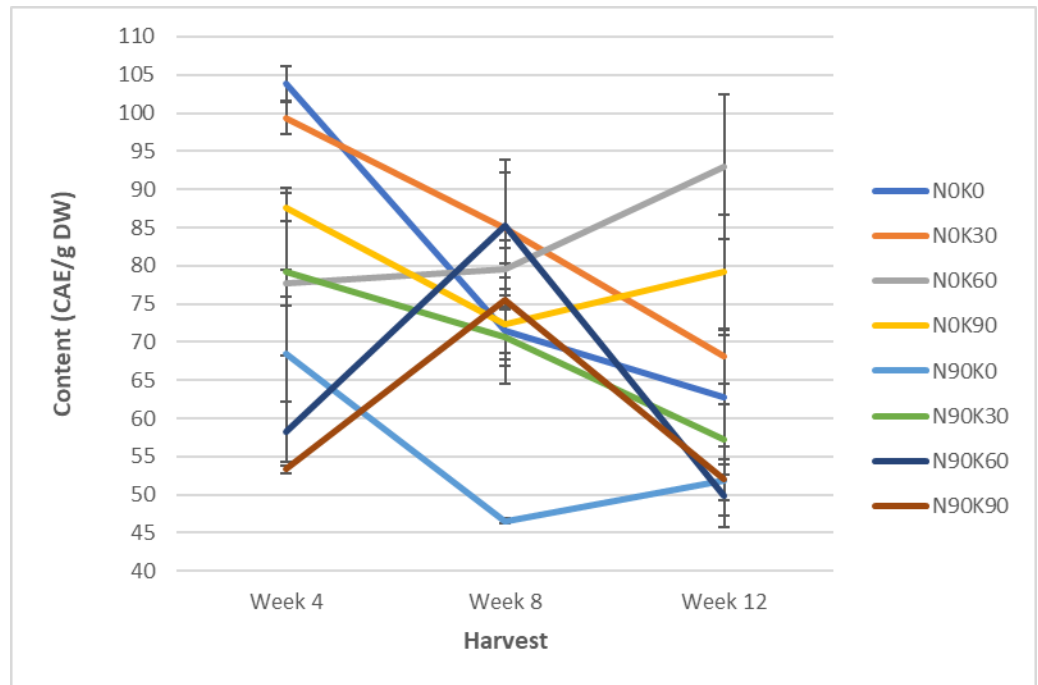


Figure 7. Changes in TPC (mg CAE/g DW) as affected by rates of N (0 and 90 kg N/ha), K [0 (Control), 30, 60 and 90 kg K/ha] and H times (4, 8 and 12 WAT). Bars represents standard error of differences between the means. N=72. The calibration curves for TPC markers (caffeic acid) were linear over the range of 0.1 to 2.0 mg/mL. The coefficient of determination (R^2) was greater than 0.994 for all standard curves.

Quantification of Phenolics

Thin Layer Chromatography

Qualitative analysis of methanol extract using TLC was performed using a mixture of toluene: ethyl acetate: formic acid (5:4:1 v/v) used as the mobile phase (Sasidharan *et al.*, 2011) yielded a good resolution of caffeic acid with bands at $R_f=0.66$ (Figure 8). Caffeic acid were identified as phytochemical marker in phenolic compounds analysis and quantified at visible light and visible wavelength of 254 and 366 nm before derivatisation (Figure 8). The results revealed that methanol extract at 1 g/10 mL of 100% methanol contained caffeic acid in accordance to TPC analysis, where highest in N0K30 at Week 4 (100 mg CAE/g DW), and lowest in N90K60 treatments at Week 12 (50 mg CAE/g DW) (Figure 7). This TLC procedure can be used as a fast screening method for *G. procumbens* leaf samples and herbal formulations.

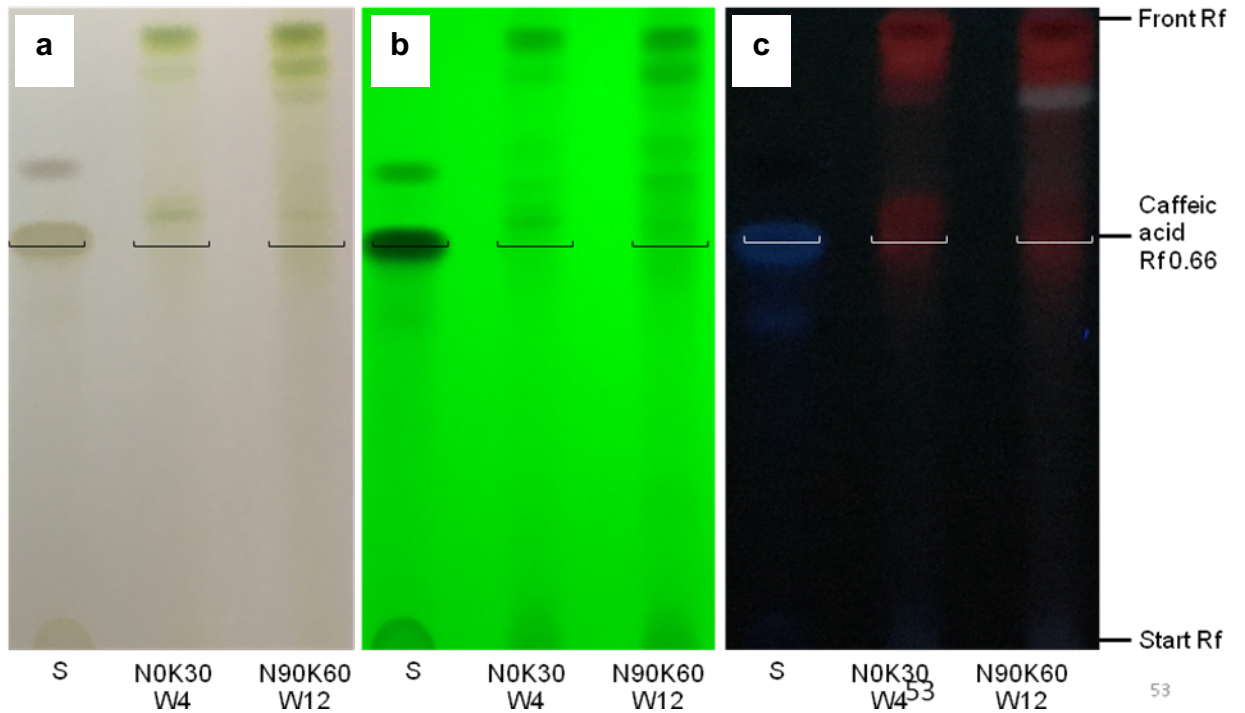


Figure 8. Thin layer chromatography developed-profiles of caffeic acid (S) of methanolic extract of dried leaves powder for N0K30 treatment Week 4 and N90K60 treatment Week 12 observed under various wavelength (visible light, a; 254 nm, b; and 366 nm, c) before derivatization.

High-Performance Liquid Chromatography

Quantitative analysis of methanol extract using HPLC was performed using a mixture of 0.1% of 85% orthophosphoric acid: acetonitrile (90.5:9.5, v/v isocratically) (Kaewseejan & Siriamornpun, 2015) used as the eluent yielded a good peak of caffeic acid at $t_r=6.6$ to 6.7 min, 327 nm (Figure 9). The results revealed that methanol extract at 1 g/10 mL of 100% methanol contained caffeic acid as phytochemical marker in accordance to TPC analysis, where highest in N0K30 at Week 4 (100 mg CAE/g DW), peak 4, $t_r=6.716$ min, 39.03% Area, and lowest in N90K0 treatments at Week 12 (50 mg CAE/g DW), peak 4, $t_r=6.649$ min, 19.61% Area) (Figures 7 and 9). This HPLC procedure can be used as a fast screening method for *G. procumbens* leaf samples and herbal formulations.

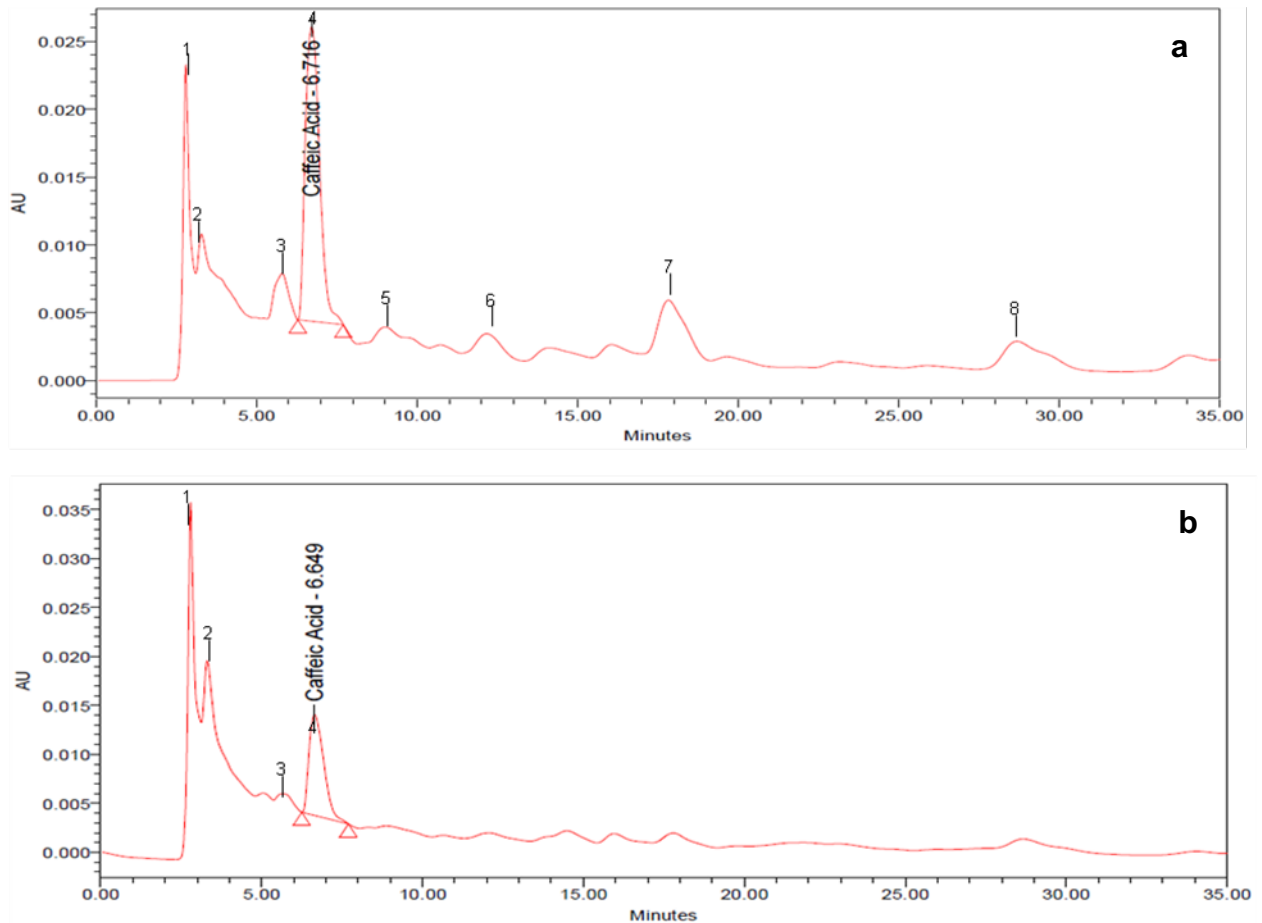


Figure 9. High-performance liquid chromatogram developed-profiles of caffeic acid standard solution (1.0 mg/mL) at $t_r=6.6-6.7$ min of methanolic extract of dried leaves powder for N0K30 treatment Week 4 (a) and N90K60 treatment Week 12 (b) observed under 327 nm derivatization. The calibration curves for caffeic acid were linear over the range of 0.05 to 0.17 mg/mL. The coefficient of determination (R^2) was greater than 0.999 for all standard curves.

Total Flavonoid Content

Generally, TFC was statistically affected in all treatments at different H time ($p \leq 0.0001$) (Figure 10). At Week 4, TFC value was significantly decreased ($p \leq 0.0001$) with different N and K treatment in descending manner N0K30 (72 mg KE/g DW) > N0K0 (71 mg KE/g DW) > N0K90 (63 mg KE/g DW) > N90K30 (60 mg KE/g DW) > N90K0 (55 mg KE/g DW) > N0K60 (47 mg KE/g DW) > N90K60 (36 mg KE/g DW) > N90K90 (25 mg KE/g DW). As the plant aged indicated by H time from Week 4 to Week 12, TFC has increased ($p \leq 0.05$) in N90K60 and N90K90, however, decreased in other treatments (Figure 10). Measurements of TFC are significantly correlated with TPC at $r=0.573$; $p \leq 0.0001$. However, not significantly correlated with Photo, TCC, TPrC and TLiC at $r=0.079$; $p \geq 0.05$, $r=-0.192$; $p \geq 0.05$, $r=0.086$; $p \geq 0.05$ and $r=0.142$; $p \geq 0.05$, respectively by a linear function (Table 1). The event was because of N availability has implicated phytochemical response in plants (Galieni *et al.*, 2015), where N deficiency induces the content of flavonoids and flavonols (Pal *et al.*, 2015). High N supply (N90 treatment) increases the content of nitrogenous compounds, such as free amino acids (protein) (Figure 5), which however, reduce the secondary metabolites content such as flavonoid (Larbat *et al.*, 2012). Meanwhile, low K rates has influenced enzyme activation metabolism which affects the protein and protein-related compound production. This compounds are precursors of secondary metabolites formation (flavonoids) (Ozidal *et al.*, 2013).

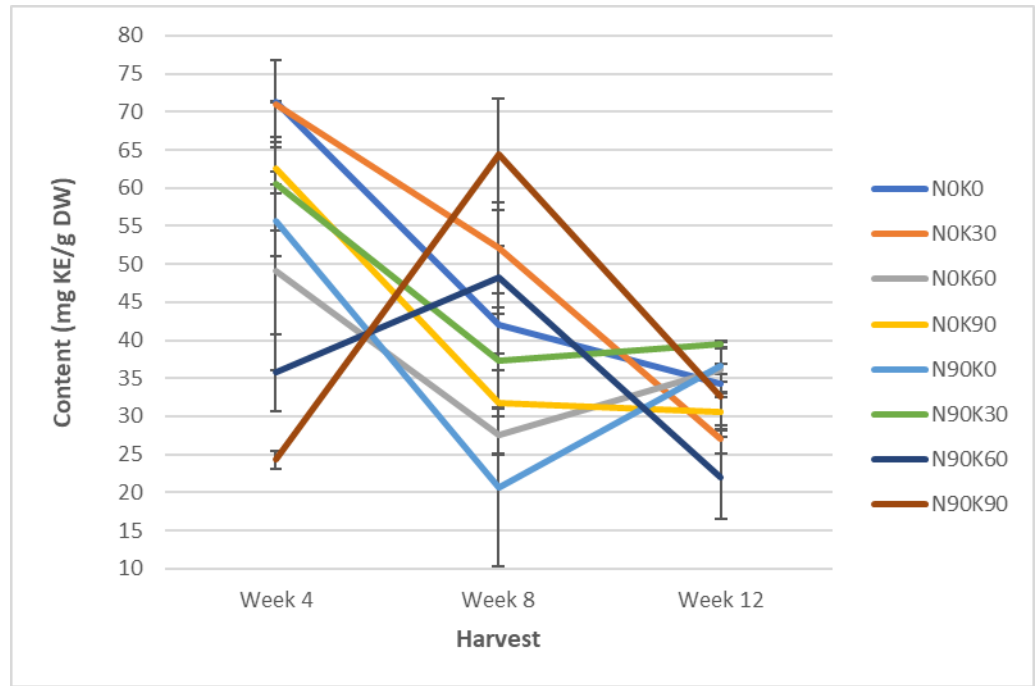


Figure 10. Changes in TFC (mg KE/g DW) as affected by rates of N (0 and 90 kg N/ha), K [0 (Control), 30, 60 and 90 kg K/ha] and H times (4, 8 and 12 WAT). Bars represents standard error of differences between the means. N=72. The calibration curves for TFC markers (kaempferol) were linear over the range of 0.04 to 1.80 mg/mL. The coefficient of determination (R^2) was greater than 0.993 for all standard curves.

Quantification of Flavonoids

Thin Layer Chromatography

Qualitative analysis of methanol extract using TLC was performed using a mixture of toluene: ethyl acetate: formic acid (5:4:1 v/v) used as the mobile phase (Sasidharan *et al.*, 2011) yielded a good resolution of kaempferol with bands at $R_f=0.71$ (Figure 11). Kaempferol were identified as phytochemical marker in flavonoid compounds analysis and quantified at visible light and visible wavelength of 254 and 366 nm before derivatisation (Figure 11). The results revealed that methanol extract at 1 g/10 mL of 100% methanol contained kaempferol in accordance to TFC analysis, where highest in NOK30 treatments at Week 4 (72 mg KE/g DW), and lowest in N90K60 at Week 12 (36 mg KE/g DW) (Figure 10). This TLC procedure can be used as a fast screening method for *G. procumbens* leaf samples and herbal formulations.

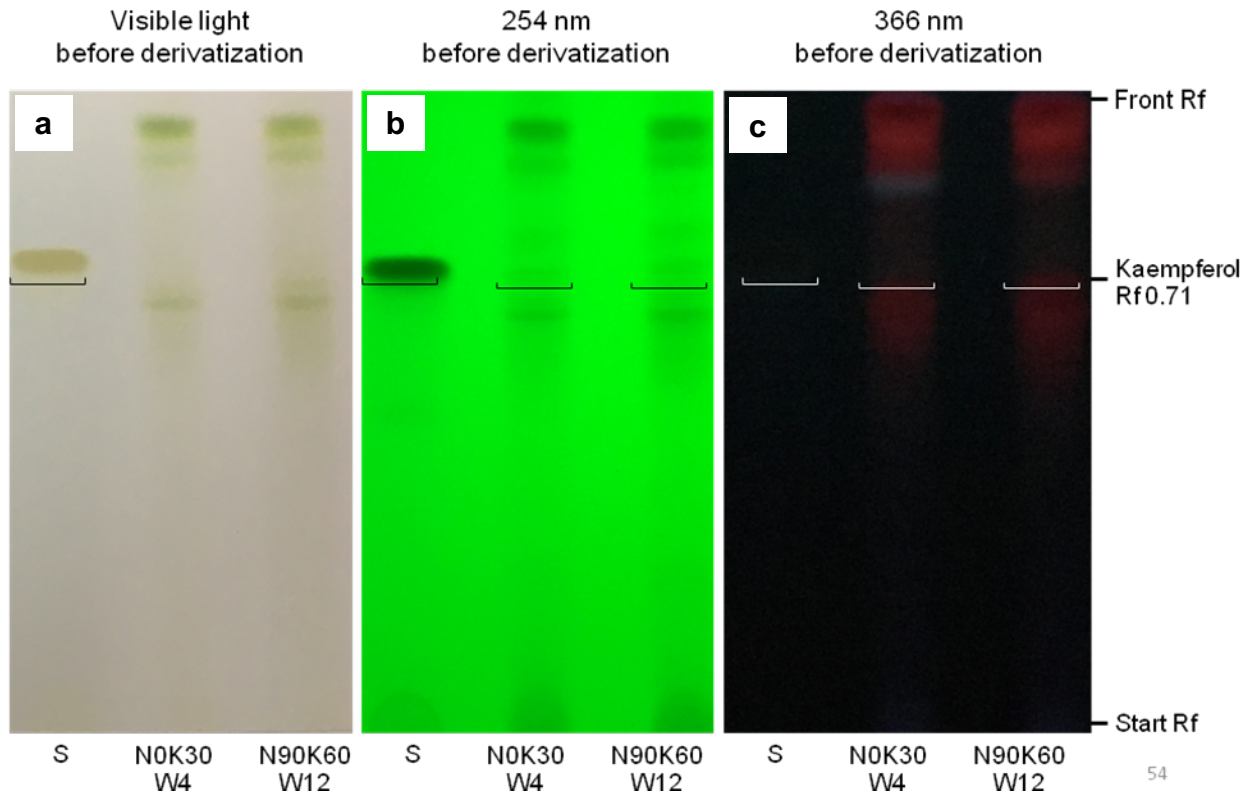


Figure 11. Thin layer chromatography developed-profiles of kaempferol (S) of methanolic extract of dried leaves powder for NOK30 treatment Week 4 and N90K60 Week 12 observed under various wavelength (visible light, a; 254 nm, b; and 366 nm, c) before derivatization.

High-Performance Liquid Chromatography

Quantitative analysis of methanol extract using HPLC was performed using a mixture of 0.03M of 85% orthophosphoric acid: methanol (60:40, v/v isocratically) (Olszewska, 2007; Yi et al., 2016; Yu et al., 2010) used as the eluent yielded a good peak of kaempferol at $t_r=11.0$ min, 250 to 360 nm (Figure 12). The results revealed that methanol extract at 1 g/10 mL of 100% methanol contained kaempferol as phytochemical marker in accordance to TFC analysis, where highest in NOK30 treatments at Week 4, peak 6, $t_r=11.067$ min, 39.03% Area, and lowest in N90K60 at Week 12, peak 2, $t_r=11.090$ min, 16.30% Area (Figures 10 and 12). This HPLC procedure can be used as a fast screening method for *G. procumbens* leaf samples and herbal formulations.

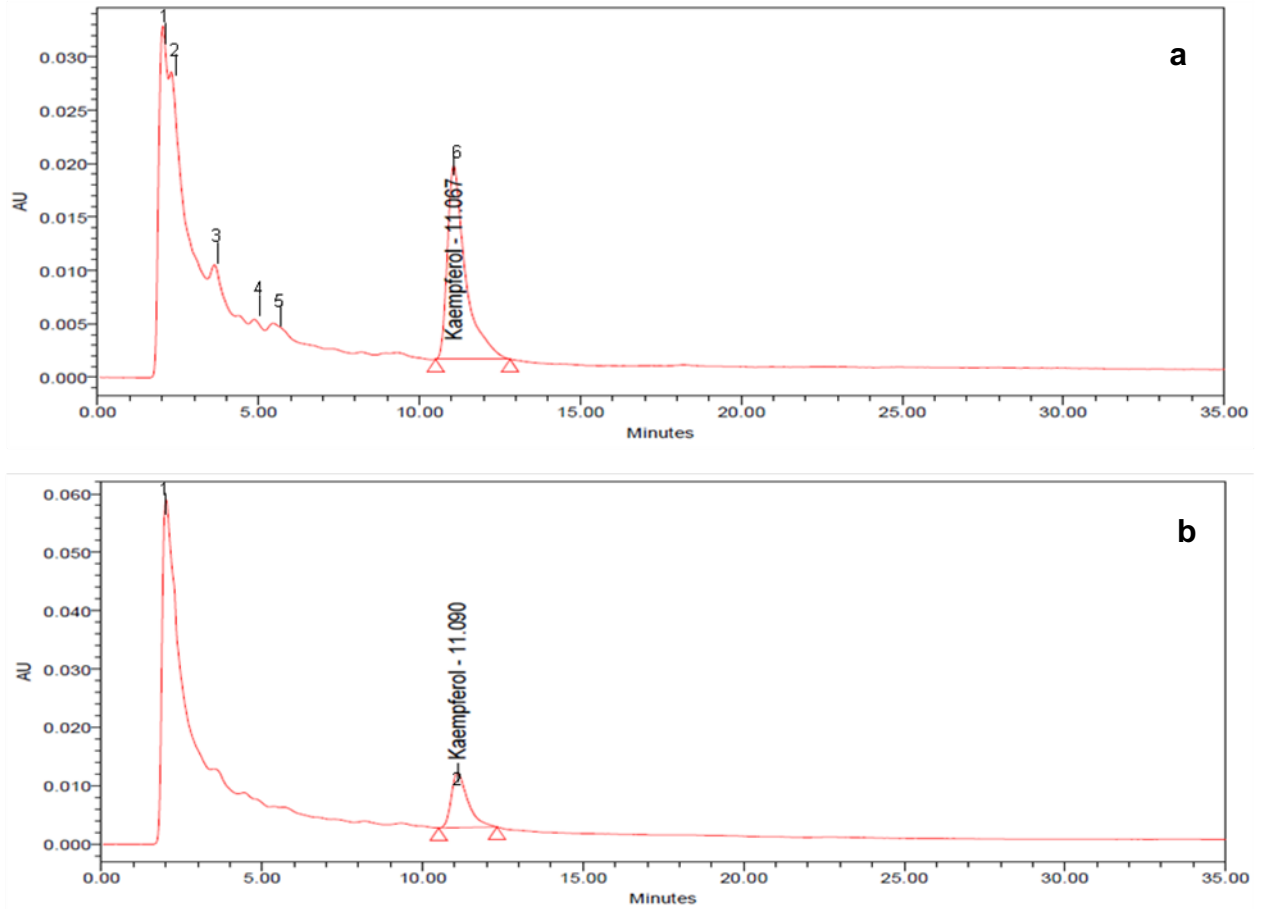


Figure 12. High-performance liquid chromatogram developed-profiles of kaempferol standard solution (1.0 mg/mL) at $t_r=11.0$ min of methanolic extract of dried leaves powder for N0K30 treatment Week 4 (a) and N90K60 treatment Week 12 (b) observed under 250 to 360 nm derivatization. The calibration curves for kaempferol were linear over the range of 0.2 to 0.6 mg/mL. The coefficient of determination (R^2) were greater than 0.999 for all standard curves.

Table 1. Correlation of Physiology and Phytochemical Assay.

	H	Photo	Cond	TChIC	TCC	TPrC	TLiC	TPC	TFC
H	1.000								
Photo	-0.258*	1.000							
Cond	0.068 ^{ns}	0.600****	1.000						
TChIC	-0.210 ^{ns}	-0.059 ^{ns}		1.000					
TCC	0.396***	-0.226 ^{ns}		-0.048 ^{ns}	1.000				
TPrC	-0.041 ^{ns}	-0.088 ^{ns}		-0.108 ^{ns}	-0.293*	1.000			
TLiC	-0.119 ^{ns}	0.286*		0.102 ^{ns}	-0.078 ^{ns}	-0.296 ^{ns}	1.000		
TPC	-0.332**	0.225 ^{ns}		0.063 ^{ns}	-0.208 ^{ns}	0.024 ^{ns}	0.046 ^{ns}	1.000	
TFC	-0.516****	0.079 ^{ns}		0.046 ^{ns}	-0.192 ^{ns}	0.086 ^{ns}	0.142 ^{ns}	0.573****	1.000

All analyses are mean \pm standard error of mean (SEM), N=72 using DNMR. * Significant at $p \leq 0.05$, ** Significant at $p \leq 0.01$, *** Significant at $p \leq 0.001$, **** Significant at $p \leq 0.0001$, and ^{ns} Not significant at $p \geq 0.05$, H=Harvest Time, Photo=Photosynthetic Rate, Cond=Stomatal Conductance Rate, TChIC=Total Chlorophyll Content, TCC=Total Carbohydrate Content, TPrC=Total Protein Content, TLiC=Total Lipid Content, TPC=Total Phenolic Content, and TFC=Total Flavonoid Content.

Discussion

This study was conducted with the main aim to examine the effects of rates of N and K with increasing plant age at H time on physiology responses and phytochemical contents of *G. procumbens*, it is of particular interest to determine the best harvesting time for the maximum total production of secondary metabolite. The interest was also to examine the presence of compound marker for *G. procumbens* in the selected treatment with high secondary metabolites.

From the analysis of variance, generally it was demonstrated that *G. procumbens* biochemical analyses of primary and secondary metabolites were significantly ($p \leq 0.01$) controlled by the interaction among all the factors studied (N, K, and plant age at H time) (Table 1). Photosynthesis as in physiology parameter was affected mainly ($p \leq 0.0001$) by plant age at H time, and N and H time interaction, whilst Cond was profoundly governed by the interactions of all the factors (N, K and plant age at H time) (Figures 1 and 2, and Table 1).

Plant growth was significantly affected by plant age at harvest time because there are mechanisms (growth, development, ageing) regulating phase change from juvenility to maturity which has directly influence the capacity for responding to growth signals. Different turnover patterns (supply of nutrient) of

structural modules can account for the range of plant growth curve (Thomas, 2013). Meanwhile, the biochemical analysis was governed by the interaction of all factors (N, K and plant age at H time) because as plant cells grow, they break down complex, high-molecular-weight carbon and energy sources through the action of catabolic enzymes. The end products of catabolism were reassembled to form primary metabolites (amino acids, carbohydrates, and fatty acids). These biosynthetic products are then assembled into the complex and essential metabolites that give structure and biological function to the plant. These regulations comprise the pathways that make up secondary metabolites. The nutrients and duration of growth serve basic, well-defined, and essential roles in supporting the synthesis and accumulation of metabolites (Hashiguchi *et al.*, 2017). Metabolic pathways of primary metabolism often supply the precursors for secondary metabolism, therefore, factors that influenced primary metabolism will affect secondary metabolism (Drew & Demain, 1977).

All the physiological (Photo, Cond and TChIC) and biochemical traits (TCC, TPrC, TLiC, TPC and TFC) were markedly influenced by N ($p \leq 0.05$) and K ($p \leq 0.01$) treatments, H time and the interactions ($p \leq 0.01$) between these three factors (Figures 1, 2, 3, 4, 5, 6, 7, and 10). Growth-differentiation balance and carbon-nutrient balance hypothesis indicated that N and K affected physiological traits and phytochemical content (primary and secondary metabolite synthesis) (Mohd Zain & Ismail, 2016). Based on harvest index analysis, N0K30 and N90K0 treatments represent the highest and least effect, respectively on *G. procumbens* physiology traits and phytochemical content.

The N0K30 was significantly increased TFC compared to control plants (N0K0) ($p \leq 0.05$) by 225%. However, in N90K0, the TFC was decreased by 25% (Figure 10). Nitrogen as a part of three important coenzymes in the plant cells; ATP, NADPH and Co-A, and K have influenced the photosynthesis rate and subsequently the metabolite biosynthesis by regulating the respiration for C skeleton production compounds (Davies *et al.*, 2009).

In quantitative HPLC analysis, phenolic and flavonoid content was high (0.047 to 0.179 mg/mL) under moderate rates of N and K treatments but decreased (0.042 to 0.165 mg/mL) under high rates of N and K (Figures 9 and 12). The metabolite content was decreased beyond the optimal point with increasing rate of N and K (Wuyts *et al.*, 2015) as shown in N0K30 and N90K0 (Figures 4, 5, 6, 7 and 10) (Fonseca *et al.*, 2006). A ratio of N:K at 0:30 (N0K30) indicated the plant has optimised for metabolite production at Week 4. However, in N90K0, with low K in relation to N, the metabolite production were decreased (Larbat *et al.*, 2012). Meanwhile, high K rates in N0K60 and N0K90 was not significantly affecting physiological traits and phytochemical content ($p \leq 0.05$) (Kaiser *et al.*, 2016). The event was due to total reduction of N content, and consequently K has lost interaction with N for metabolite differentiation balance (Pal *et al.*, 2015).

Meanwhile, qualitative and quantitative analysis developed using TLC and HPLC, respectively has markedly caffeic acid and kaempferol as phytochemical markers for phenolic and flavonoid content measurements (Figures 8 and 11).

Conclusions

The N and K affected metabolite content significantly ($p \leq 0.05$) with higher effect on physiological traits and biochemical attributes ($p \leq 0.01$). Harvest index analysis have shown the effect of N and K treatments in the following descending manner, N0K30 > N0K60 > N0K90 > N0K0 > N90K60 > N90K30 > N90K90 > N90K0 as explained in growth-differentiation balance and carbon-nutrient balance hypothesis. The results suggest that N0K30 and N90K0 were high and least, respectively, effective for Cond ($p \leq 0.0001$) and phytochemical content (TPrC and TLiC, $p < 0.01$; TPC and TFC, $p \leq 0.0001$). However, not significant in Photo ($p \geq 0.05$). These effects were because K has induced TFC, but decreased TPrC, especially in 0 kg/ha N interaction treatment. As compared to 90 kg/ha N interaction treatment, increases of TFC, and decreases of TPrC have led to plant's oxidative defense mechanism against temperature and pH stress. These were due to incorporation of K which alter the growth media and cells intercellular pH by internal K balance. Thus, high metabolites content of *G. procumbens* should be improved through the selection of appropriate N and K rates, and plant age at which high total metabolite production can be achieved.

Data availability

We have presented all our main data in the form of figures and tables. The data sets supporting the conclusions of this article are included within the article.

Conflicts of interest

The authors declare that they have no competing interests.

Funding statement

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