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Phytochemical content and antioxidant activity of G*ynura procumbens* in response to shade levels and rates of nitrogen fertilizer

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Abstract

Gynura procumbens is one of the most common medicinal plants with extensive pharmacological properties. Light intensity and nitrogenous fertilisers are two main limiting factors in primary and secondary metabolism in plants. Information on the effects of shade levels and nitrogenous fertiliser rates on phytochemical content and antioxidant activity of *G. procumbens* is much scarce. This justifies an in-depth study to determine the effects of phytochemical content and antioxidant activity. This study was conducted with four levels of shade (0, 30, 50, and 70%) and four nitrogen fertilizer rates (0, 100, 200 and 300 kg N ha^{-1}). Data measurements were performed on protein content, C:N ratio, total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity (DPPH, FRAP) and flavonoid acids. The results showed that decreases in total phenol, flavonoid contents, C:N ratio and antioxidant activity were recorded with increasing shade levels and nitrogen rates. Protein content was increased with increases in shade levels and nitrogen fertilizer supplement. The highest flavonoid acids contents were observed from higher light intensity (no shade) and no nitrogen application. Enhancement in production of secondary metabolites and antioxidant activity were associated with high C:N ratio and low protein contents. The study demonstrated that high light intensity (0% shade) with a low N fertilizer rate (0 kg N ha^{-1}) was suitable for both phenolic compounds and antioxidants activity. The present study suggested that *G. procumbens* can achieve higher production of polyphenolic compounds and antioxidant activity under low nitrogen availability and open field conditions.

Keywords: *Gynura procumbens*, shade, nitrogen, growth, phytochemical, antioxidant.
Abbreviations: C:N_carbon: nitrogen ratio; CP_crude protein content; TPC_total phenolic content; TFC_total flavonoid content; DPPH_2, 2_Diphenyl_1_Picrylhydrazyl assay; FRAP_Ferric Reducing Antioxidant Power Assay; KMR_Kaempferol_3_ rotinoside; KMG_Kaempferol_3_ glucoside; MYR_Myricetin.

Introduction

Gynura procumbens is an annual evergreen medicinal herb belonging to the Asteraceae family. It is widely distributed in the African continent (Rahman and Al Asad, 2013; Sukadeetad et al., 2018) and tropical regions of South-east Asia including Malaysia, Indonesia, Thailand, Vietnam, Philippines and Myanmar, and China (Nasir et al., 2015; Mou and Dash, 2016; Tan et al., 2016). In Malaysia, the species is distributed mainly in the western part of the peninsula (Keng et al., 2009).

In countries like Malaysia, Indonesia and Vietnam, *G. procumbens* has been traditionally used in the treatments of various diseases and ailments such as rash, constipation, hypertension, migraines, diabetes mellitus, urinary infection, cancer and as an anti-inflammatory and anti-allergic agents (Perry and Metzger, 1980; Jiratchariyakul et al., 2000). The benefits of consuming *G. procumbens* are related to the presence of bioactive compounds such as saponins, flavonoids and terpenoids (Mou and Dash, 2016; Tan et al., 2016). Increase in knowledge of potential health benefits of the species as a medicinal plant, as an alternative to chemical remedies, has led to higher demand for the herb.

Plant growth and production of metabolites depend on changes in the ecosystem. It has been documented that biotic and abiotic factors influence secondary metabolite production and they have a bearing on enhancing the potential to produce phytochemicals for use in various applications. Thakur et al. (2018) stated that the accumulation of secondary metabolites in plants would increase as plants respond to stress conditions. They emphasized that enhanced synthesis of secondary metabolites, called elicitation, ensures survival, persistence and competitiveness of the plant. Enhanced production of secondary metabolites has significant economic benefits for the pharmaceutical and therapeutic industries.

The concentration of polyphenols in plants has been reported to be influenced by environmental conditions (abiotic factors) such as light intensity, temperature and fertilization, and biotic factors, which alter the

concentrations of the constituents (Fine et al., 2006). Irradiance is known to regulate not only plant growth and development, but also biosynthesis of both primary and secondary metabolites such as polyphenols (Hemm et al., 2004; Liu et al., 2002). Based on the effects of environmental conditions on carbon-nutrient balance, phenolic biosynthesis has been documented to require irradiance or is enhanced by irradiance (Zavala and Ravetta, 2001). Different plants have different responses to irradiance intensity that result in differences in their production of secondary metabolites depending on the species of plants. Gu et al. 2010 showed that differences in irradiance levels altered the production of secondary metabolites in plants. Consequently, the irradiance level affected the phytochemical content of these plants, besides changes in their morphology and physiology (Ferreira et al., 2007). These findings suggest that different irradiances have a direct effect on antioxidant activities in plants resulting in increased total phenolic and flavonoids contents (Ghasemzadeh et al., 2010; Nasiri, 2016).

In addition to sunlight, nitrogen is one of the main requisites for plant growth and is applied to crops in substantial amounts to ensure high yield. It strongly influences the use of other environmental resources such as water and light (Ibrahim et al., 2011). Nitrogen is generally accepted to be important in the biosynthesis of secondary metabolites which can also be altered by variations in environmental resources (Cai et al., 2009), and particularly plant nutritional status which could highly affect these metabolic compounds (El Gendy et al., 2015; Chrysargyris et al., 2016). Among the plant nutrients, nitrogen (N) is much demanded than any other mineral elements and heavily influence the chemical compounds and biomass of plants (Barickman and Kopsell, 2016). Nitrogen has been considered a plant's essential mineral contributing to the biosynthesis of a wide range of compounds such as nucleic acids, enzyme, pigments polysaccharides, as well as some secondary metabolites (Kováčik and Klejdus, 2014; Chrysargyris et al., 2016). The availability of nitrogen affects the content of secondary components in several ways. Nitrogen availability can change C:N balance in plants, as nitrogen deficiency promotes a shift from N-based secondary to C-based secondary compounds (Ibrahim et al., 2011). Consequently, this suggests that all levels of plant function, from metabolism to resource allocation, growth, and development are regulated and coordinated with nitrogen availability. Therefore, the manipulations of nitrogen fertilizer rates can affect the production and concentration of primary and secondary metabolites. Therefore, a study was conducted to examine the effects of shade level and nitrogen fertilizer rate on total phenolic content, total flavonoid content, antioxidant activity and flavonoid acids of *G. procumbens*.

Results

Carbon: nitrogen ratio (C:N)

Shade levels and nitrogen fertilizer rate had significant effects on carbon: nitrogen ratio (C:N) of *G. procumbens* plants. It was seen that C:N ratio increased with decrease in shade from 70% to full sunlight (0% of shade) and decreased with increase in nitrogen fertilizer rates from 0 to 300 kg N ha^{-1}. Figures 1-A and 1-B show that under full sunlight and 70% shade level *G. procumbens* plants achieve a maximum C:N ratio of 16.36 and 11.91, respectively. However, lower nitrogen fertilizer rate (0 kg N ha^{-1}) also produced a high C:N ratio. The percentage increase was 76.39% in no nitrogen application (0 kg N ha^{-1}) than the higher nitrogen fertilizer rate of 300 kg N ha^{-1} (Fig 1-B).

Crude protein content (CP)

Crud protein contents (CP) of *Gynura procumbens* in the present study were significantly affected by different levels of shade and nitrogen fertilizer rates. The plants showed high CP contents of 72.95 and 59.18% at 70 and 50%, respectively when compared with CP content under full sunlight (Fig 2-A). Similarly, CP content increased with increase in nitrogen fertilizer supplementation. The increment in CP percentage was 62.79% when the plants were grown at 300 kg N ha^{-1} compared to no nitrogen application (Fig 2-B).

Total phenolic content (TPC)

The total phenolic content of *G. procumbens* was markedly reduced in response to the increase in shade levels and nitrogen fertilizer rates (Fig 3). There were interaction effects between shade levels and nitrogen fertilizer rates. Mean TPC was 6.94 mg GAE/g under open field (0% shade) and no nitrogen application (0 kg N ha^{-1}), whereas the lowest TPC was 3.18, 3.05, 3.0 and 2.85 mg GAE/g under the 70% shade and 0, 100, 200 and 300 kg N ha^{-1}, without any significant difference with 3.02 mg GAE/g under 50% shade and 300 kg N ha^{-1} (Figure 3).

Table 1 shows that there was no significant interaction ($p>0.05$) between shade levels and nitrogen fertilizer rate in TPC of stem and root. However, the effects of both shade levels and nitrogen rates on stem and root TPC were significant. The maximum stem and root TPC were 2.29 and 1.99 mg GAE/g observed under open field (0% shade), and the minimum were 1.54 and 1.14 mg GAE/g under 70% shade. The maximum stem and root TPC under nitrogen fertiliser rates were 2.18 and 1.78 mg GAE/g, respectively, which observed under 0 kg N ha^{-1} of nitrogen rate and the minimum were 1.49 and 1.22 mg GAE/g under 300 kg N ha^{-1} of nitrogen.

Total flavonoid content (TFC)

There was a significant interaction effect between shade levels and nitrogen fertilizer rates on TFC concentration. Total flavonoid content (TFC) of *G. procumbens* was markedly reduced in response to an increase in nitrogen fertilizer rate (Fig 4). The average total of TFC produced was 5.29 mg CE/g under 0% shade and no nitrogen application (0 kg N ha^{-1}). The lowest TFC was 2.62, 2.51 and 2.32 mg CE/g at 100, 200 and 300) kg N ha^{-1}, respectively, under 70% shade, without any significant difference with 2.81 and 2.69 mg CE/g for 200 and 300 kg N ha^{-1} under 50% shade.

Table 2 shows that there was no significant interaction ($P>0.05$) between shade and nitrogen rates in stem and root TFC. However, the effect of both shade levels and nitrogen rates on the stem and root TFC was significant. The maximum stem and root TFC were 1.80 and 1.59 mg CE/g which observed under 0% shade. The minimums were 1.26 and 1.42 mg CE/g under 70 and 50% shade for stem and 0.97 and 0.90 mg CE/g under 50 and 70% shade for roots, respectively. The maximum stem and root TFC under nitrogen fertiliser rate was 1.84 and 1.43 mg CE/g which observed under no nitrogen application (0 kg N ha^{-1}) and the minimum were 1.19 and 0.91 mg CE/g under 300 kg N ha^{-1} of nitrogen, respectively.

Effect of shade levels and fertilizer rate on antioxidant activities

2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) assay

Increasing shade levels and nitrogen fertilizer rates had significant effects on the 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay of *G. procumbens* plants (P≤0.05). Figure 5-A shows that shade effects were more visible on DPPH assay at 80.36 and 78.71%, when plants were grown under 0 and 30% shade levels. However, the lowest was 69.20% under 70% shade. On the other hand, no nitrogen application (0 kg ha^{-1}) resulted in higher percentage of DPPH inhibition 78.83% than 74.13 and 73.20% under 200 and 300 kg N ha^{-1} of nitrogen rate, respectively (Figure 5-B).

Ferric Reducing Antioxidant Power (FRAP) assay

Shade levels and nitrogen fertilizer rates had significant effects on ferric reducing antioxidant power (FRAP), (P≤0.05). The percentage of inhibition of FRAP was decreased by 25.91% with increases in shade levels from 0 to 70% shade (Figure 6- A). Low nitrogen rates of 0 and 100 kg ha^{-1} recorded higher inhibition of FRAP of 74.47 and 72.21, in comparison with 69.61 and 67.35 at 200 and 300 kg N ha^{-1} of nitrogen supplementation (Fig 6- B).

Effect of shade levels and fertilizer rate on flavonoid compounds

Kaempferol-3- rotinoside (KMR)

There was a significant interaction effect between shade levels and nitrogen fertilizer rates on Kaempferol-3-rotinoside (KMR). Results showed that KMR considerably decreased with increase in shade levels and nitrogen fertilizer rates. No nitrogen application (0 kg N ha^{-1}) produced higher KMR (0.96 mg/g) under full sunlight without any difference with KMR (0.90 mg/g) at 100 kg N ha^{-1} under the same shade level. On the other hand, the lowest KMR was recorded at 0.079, 0.054 and 0.030 mg/g at 300, 200 and 100 kg N ha^{-1} of nitrogen supplementation under 70% shade (Fig 7).

Kaempferol-3- glucoside (KMG)

The kaempferol-3- glucoside (KMG) of *Gynura procumbens* plants was affected by the interaction effect of shade levels and nitrogen fertilizer rates. KMG decreased consistently with the increase in both shade levels and nitrogen fertilizer rates. The results indicated high KMG of 1.80 mg/g at no nitrogen application (0 kg N ha^{-1}) under full sunlight, whereas the lowest value of KMG was 0.19, 0.17 and 0.13 mg/g at 100,200 and 300 kg N ha^{-1} of nitrogen fertilizer, respectively, under 70% shade without any difference with 0.27 mg/g at 300 kg N ha^{-1} under 50% shade (Fig 8).

Myricetin (MYR)

Significant interaction (P≤0.05) was observed between shade levels and nitrogen fertilizer rates in myricetin (MYR) of *Gynura procumbens* plants. Results showed that MYR considerably decreased with increases in both shade levels and nitrogen rates. The shade of 0% yielded higher MYR at 0.26 and 0.25 mg/g when plants were supplemented with 0 and 100 kg N ha^{-1} of nitrogen fertilizer, respectively. On the other hand, the lowest MYR of 0.014 and 0.014 mg/g were recorded at 300 and 200 kg N ha^{-1} of nitrogen fertilizer supplementation under 70% shade, respectively (Fig. 9).

Discussion

Metabolism of primary and secondary metabolites in plants is known to be influenced by environmental conditions which alter the contents of these active constituents (Ibrahim et al., 2011). In Table 1, C:N ratio of *G. procumbens* was decreased to 27.26% and 43.3% when shade level was increased from 0 to 70% and nitrogen rate was increased from 0 to 300 kg N ha^{-1}, respectively. The increase in C:N ratio under high light intensity with low nitrogen supply was probably due to reduced nitrogen uptake which resulted in increased C:N ratio. The results were in agreement with studies of (Cronin and Lodge, 2003; Ibrahim and Jaafar, 2012; Oskoee et al., 2018). According to C:N balance hypothesis, when nitrogen is readily available, plants primarily make compounds with high nitrogen content (e.g., proteins for growth). However, when nitrogen availability is limited, metabolism changes more towards carbon-containing compounds such as starch, cellulose, and non-N-containing secondary metabolites such as phenolics and terpenoids (Gu et al., 2010). Rahmat et al. (2008) documented that plants allocate extra carbon that could not be used for growth to produce carbon based secondary metabolites. Conversely, crude protein content was increased 73% when the shade level increased from 0 to 70%, and 62.94% when nitrogen rate increased from 0 to 300 kg N ha^{-1}. The differences in protein content in plants under different light intensity are related to the effect of light intensity on leaf nitrogen content. Under high light irradiance, leaf nitrogen content reduces. However, at low light conditions, leaf nitrogen content is increased as a physiological response to increase light absorption by increasing chlorophyll content. A higher nitrogen content under low light may be associated with elevated levels of protein detected in plants under shade (Nasiri 2016). Nitrogen is a major structural component of protein. Therefore, increase in nitrogen rate can result in increase in protein concentration (Robredo et al., 2011; Nasiri, 2016). The soluble protein content may increase with increasing nitrogen fertilization levels from 0 to 270 kg N ha^{-1} (Ibrahim et al., 2013).

As shown in Figure 3, TPC was decreased 58.99% when compared with combined treatments of 0% shade and 0 kg N ha^{-1}) and 70% shade and 300 kg N ha^{-1}. Similarly, TFC was decreased to 56.14% under the same treatments of TPC (Fig 4). The reduced TPC and TFC with increasing shade level and nitrogen rate were related to the negative effect of these factors on plant defense compounds.

Light is one of the most extensively studied environmental factors in phenolic metabolism in plants (Macheix et al., 1990). Phenol and flavonoid biosyntheses require light or are enhanced by light. Flavonoid formation is absolutely light-dependent, and its biosynthetic rate is related to light intensity and density (Ghasemzadeh et al., 2010). Bussotti (2008) noted that ability of plants to produce defense compounds make them highly resilient to environmental stress. In the present study, there was a significant positive correlation between TPC and TFC with C:N ratio (r = 0.66 and 0.66) respectively (Table 2). The carbon/nitrogen balance hypothesis states that when nitrogen availability is limited in the soil, plant growth also limits the rate of photosynthesis, which results in allocation of the extra carbon that cannot be used for growth on the production of carbon- based secondary metabolites. Ibrahim et al. (2011) reported that the increase in production of TPC and TFC under low

Table 1. Effect of shade levels and nitrogen fertilizer rates on total phenol and total flavonoid content of stem and root of *Gynura procumbens*.

Treatment	TPC		TFC	
	Stem	Root	Stem	Root
Shade (S)%				
0	2.29 [a]	1.99 [a]	1.80 [a]	1.59 [a]
30	1.92 [b]	1.56 [b]	1.58 [b]	1.18 [b]
50	1.68 [bc]	1.25 [bc]	1.42 [bc]	0.97 [c]
70	1.54 [c]	1.14 [c]	1.26 [c]	0.90 [c]
LSD	0.264	0.389	0.166	0.080
Nitrogen (N) (kg N ha^{-1})				
0	2.18 [a]	1.78 [a]	1.84 [a]	1.43 [a]
100	2.03 [a]	1.54 [b]	1.63 [b]	1.23 [b]
200	1.73 [b]	1.40 [b]	1.40 [c]	1.06 [c]
300	1.49 [c]	1.22 [c]	1.19 [d]	0.91 [d]
LSD	0.194	0.147	0.114	0.090
SxN	ns	ns	ns	ns

Means with the same letters in same columns are not significantly different at $p < 0.05$ (LSD); ns = not significant.

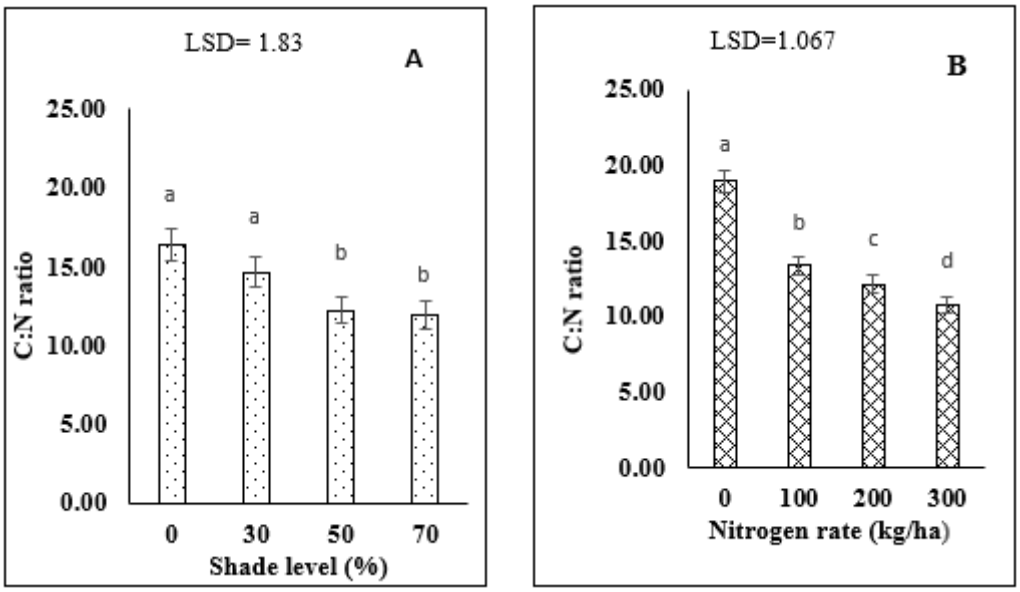

Fig 1. Effects of shade levels (A) and nitrogen fertilizer rates (B) on *G. procumbens* carbon: nitrogen ratio (C: N).

Table 2: Correlation coefficient (r) between carbon- nitrogen ratio (C;N), crude protein (CP), total phenolic content (TPC), total flavonoid content (TFC) , 2, 2-Diphenyl-1-picrylhydrazyl assay (DPPH), Ferric Reducing Antioxidant Power assay (FRAP), Kaempferol-3- rotinoside (KMR), Kaempferol-3- glucoside (KMG) and myricetin (MYR) of *G. procumbens* under different shade levels and nitrogen fertilizer rates.

	C:N	CP	TPC	TFC	DPPH	FRAP	KMR	KMG	MYR
C:N	-	-0.69 ***	0.66 ***	0.66 ***	0.63 ***	0.62 ***	0.63 ***	0.69 ***	0.67 ***
CP		-	-0.67 ***	-0.75 ***	-0.73 ***	-0.76 ***	-0.75 ***	-0.77 ***	-0.81 ***
TPC			-	0.88 ***	0.76 ***	0.89 ***	0.91 ***	0.92 ***	0.85 ***
TFC				-	0.75 ***	0.83 ***	0.86 ***	0.87 ***	0.84 ***
DPPH					-	0.83 ***	0.82 ***	0.82 ***	0.85 ***
FRAP						-	0.91 ***	0.90 ***	0.91 ***
KMR							-	0.97 ***	0.90 ***
KMG								-	0.91 ***
MYR									-

ns, not significant. *, significant at $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$.

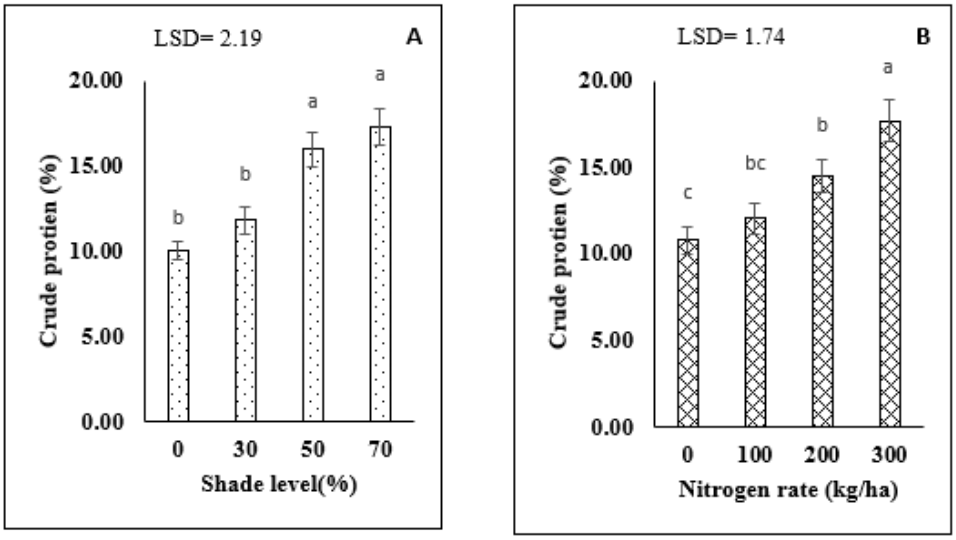

Fig 2. Effects of shade levels (A) and nitrogen fertilizer rates (B) on *G. procumbens* crude protein contents (%).

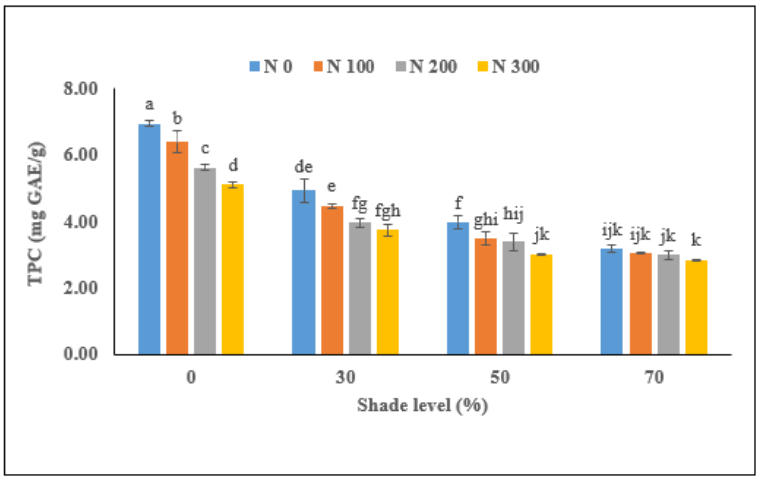

Fig 3. Effect of shade levels and nitrogen fertilizer rates on total phenolic content (TPC) (mg GAE/g) of *Gynura procumbens*.

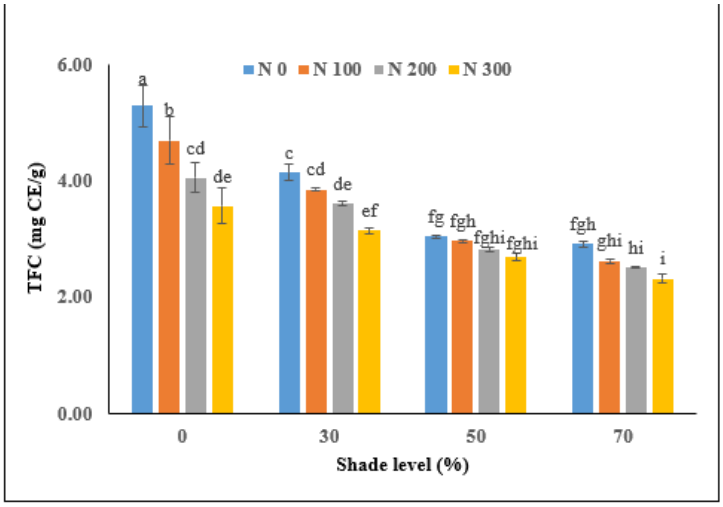

Fig 4. Effects of shade levels and nitrogen fertilizer rates on total flavonoid content (TFC mg CE/g) of *G. procumbens*.

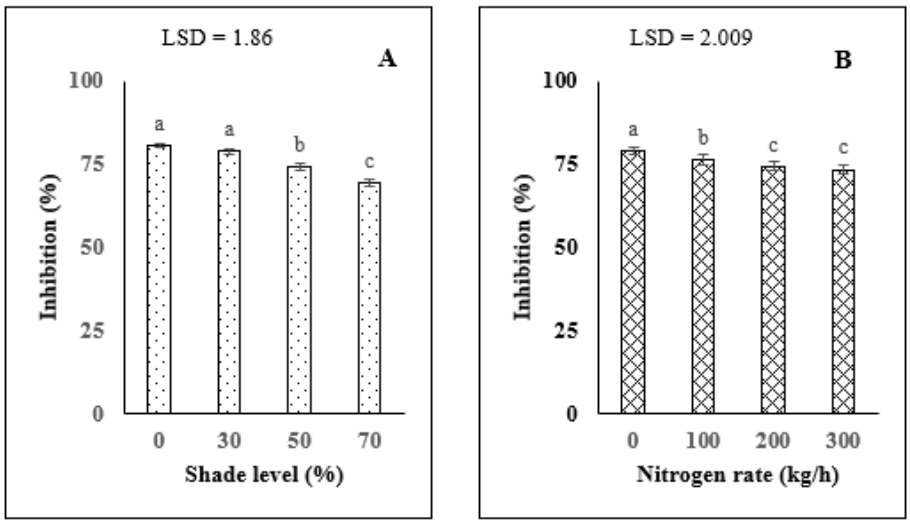

Fig 5. Effects of shade levels (A) and nitrogen fertilizer rates (B) on *G. procumbens* DPPH activity.

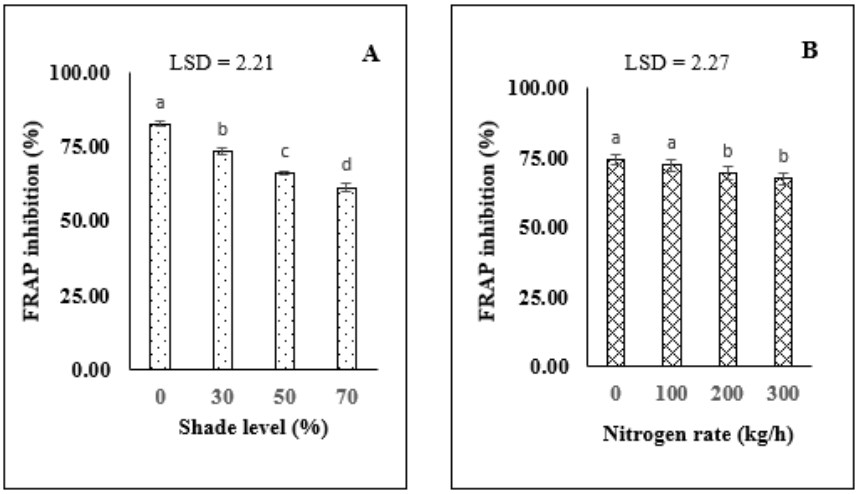

Fig 6. Effect of shade levels (A) and nitrogen fertilizer rates (B) on *G. procumbens* FRAP activity.

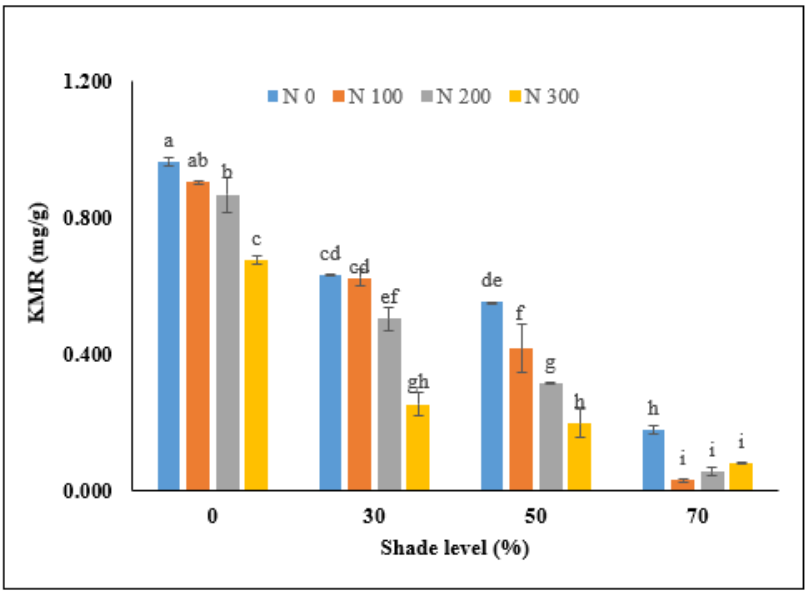

Fig 7. Effects of shade levels and nitrogen fertilizer rates on total Kaempferol-3- rutinoside content (KMR mg /g) of *G. procumbens.*

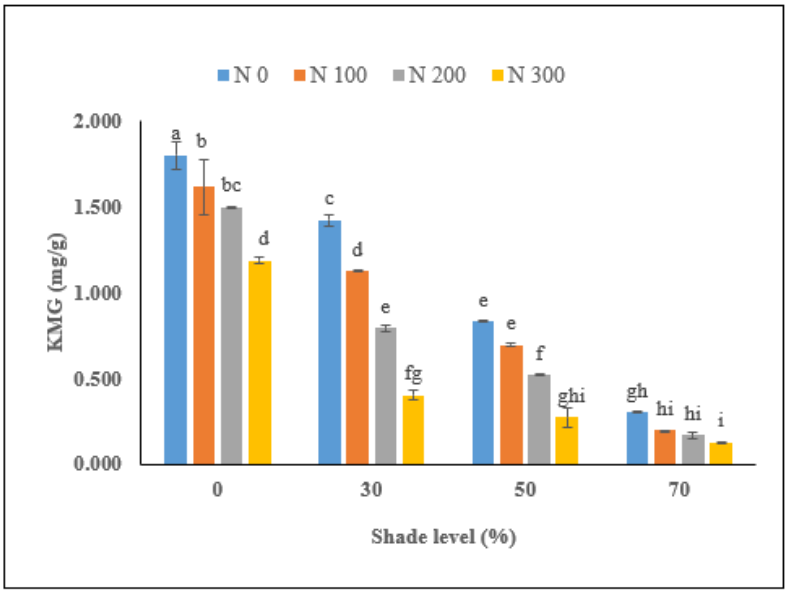

Fig 8. Effects of shade levels and nitrogen fertilizer rates on Kaempferol-3- glucoside content (KMG mg /g) of *G. procumbens.*

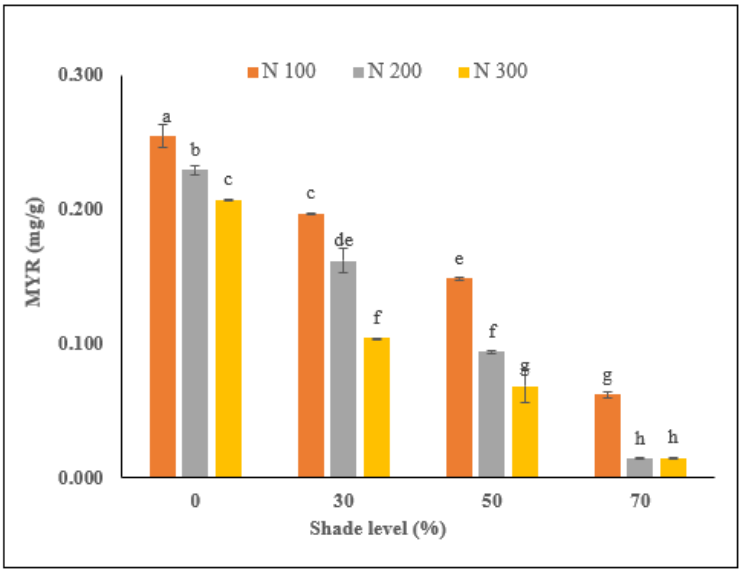

Fig 9. Effects of shade levels and nitrogen fertilizer rates on myrecetin content (MYR mg /g) of *G. procumbens*

The enhancement in PAL activity was followed by a reduction in the production of soluble protein under low nitrogen fertilization indicating more availability of amino acid phenylalanine (phe) under low nitrogen content that stimulates the production of carbon based secondary metabolites. The latter was manifested by high C:N ratio in *L. pumila* plants (Ibrahim et al., 2011).

Phenylalanine is a precursor of flavonoids and phenolics synthesis. It is assumed that biosynthesis of polyphenolic compounds may compete with protein synthesis for phenylalanine and that secondary metabolites synthesis may be inhibited because of limiting availability under conditions of rapid incorporation into protein.

The enhancement in secondary metabolites and antioxidant activity was associated with high C:N ratio and low protein contents (Oskoee et al., 2018). The inverse relationship between protein content and both TPC and TFC showed that there was a significant negative correlation between crude protein and TPC and TFC (r = -0.67 and -0.75, respectively, Table 2). The high content of flavonoid, phenols and antioxidant was characteristic for non-fertilized plants. Increase in the secondary metabolites at low nitrogen levels could be due to the increase in the nonstructural carbohydrates and enzymatic activity (Ibrahim et al., 2011). Similar to other medicinal plants, there was strong positive correlation between (TPC and TFC) with antioxidant activity (DPPH and FRAP) (r = 0.76 and 0.89) for TPC and (r = 0.75 and 0.83) for TFC respectively, in *G. procumbens* plant. This finding is in agreement with (Podsędek, 2007; Can et al., 2017) which elucidated that phenol and flavonoid contents directly affect antioxidant capacity. The high light intensity with low nitrogen rates increases total phenol, flavonoid and antioxidant activity of *G. procumbens*. It is well-known that different biotic and abiotic stresses cause variations in antioxidant activity (Bessada et al., 2016), as found in the present study where excessive light intensity with nitrogen

deficiency stress were accompanied by the higher antioxidant activity of the plants. Similar trends were observed that plant responded to different stresses by improving antioxidant activity (Zhao et al., 2016).
Kaempferol myrecetin are the rare flavonoid components in plants and they are the dominant flavonoids present in the *G. procumbens* extraction (Kim et al., 2011) showing significantly strong correlation between TFC and flavonoid acids (KMR, KMG and MYR) (r = 0.86, 0.87 and 0.84) respectively (Table 2). The highest content of flavonoid acids (Keampferol-3-glocuside, Keampferol-3-rutinoside and myrecetin) were at 0% shade under no nitrogen application (0 kg N ha^{-1}) due to the excessive radiation and nitrogen deficiency as a stress condition.

Materials and methods

Plant propagation
A total of 300 *G. procumbens* cuttings were initially propagated in Field 2, Faculty of Agriculture, Universiti Putra Malaysia (UPM), Serdang, Selangor. In order to reach the adequate number of homogenous seedlings twice the number of cuttings required for the experiment were multiplied to produce 1400 cuttings later to be used for the subsequent experiments in Field 15, Faculty of Agriculture, UPM. The cuttings were initially grown for three weeks in small paper cups which were later transferred to polybags filled with a thoroughly sieved mixture of topsoil, sand and peat moss in a ratio of 3:1:2 (v/v).

Experimental Design and Treatments
The experiment was based on Nested Design with four replicates. The treatments included four shade levels (0, 30, 50 and 70%) by using shading net (the average daily light intensity were 1375, 923, 527 and 312 µmol/m^2/s under the open field, 30, 50 and 70% shade respectively) with four nitrogen fertilizer rates (0, 100, 200 and 300 kg N ha^{-1}). The polybags with the plants were allotted randomly to the treatments by Randomized Complete Block Design (RCBD). Each treatment consisted of 15 plants, giving a total number of plants of 960. Plants were harvested at 12 weeks after transplanting.

Study Parameters
The present study involved the measurement of the following parameters to assess the effects of shade levels and nitrogen fertilizer rates:

Crude protein content and C:N ratio
Total carbon and nitrogen were measured to determine C:N ratio by using a CNS 2000 analyzer (Model A Analyst 300, LECO Inc., USA) according to Ibrahim et al. (2011). This was performed by placing 0.05 g of ground leaf samples in combustion boats. Successively, the combustion boats were transferred to a loader before the samples were burned at 1350 °C to obtain the reading of total carbon and nitrogen content of the samples. The C:N ratio was determined by divided carbon content to nitrogen content. The crude protein concentration was determined by multiplying nitrogen concentration with a constant 6.25.

Phytochemical Constituents

Extraction
Dry samples were extracted from *G. procumbens* following procedures of (Kaewseejan and Siriamornpun 2015) with some modifications. The dried ground samples of *G. procumbens* were extracted three times with 95% ethanol under constant stirring for 3 h at room temperature. The extract was then filtered using a sterilized cotton filter and Whatman No.1 filter papers. The solvent was completely removed by a rotary evaporator at 35°C in a vacuum and the crude ethanoic extract (CEE) was obtained. An amount of 1mg CEE was taken from each sample and dissolved in 1 ml ethanol and kept in a -80°C deep freezer.

Total Phenolic Content
The quantification of total phenolics content (TPC) from *G. procumbens* samples extracts was performed using Folin-Ciocalteu reagent method (Kaewseejan and Siriamornpun 2015). An amount of 200 µl of each extract was pipetted into test tubes and 1 ml (10% v/v) of Folin-Ciocalteu reagent added into each extract sample. Five minutes after incubation, 800 µl of 7.5% (w/v) sodium carbonate solution were added into the samples. The samples mixture was incubated for 30 minutes at room temperature. The absorbance of the solution was reached at 765 nm by a spectrophotometer. TPC was reported as mg Gallic acid equivalents per gram dry weight (mg GAE/g DW).

Total Flavonoid Content
Total flavonoid content (TFC) of *G. procumbens* leaf extracts was determined using aluminium chloride colourimetric method adapted from the procedures reported by (Kaewseejan and Siriamornpun, 2015). An amount of 250 µl of leaf extract solution was mixed with 1.25 ml distilled water and 75 µl of sodium nitrite (NaNO2) solution. After 6 minutes of incubation, 150 µl of 10% aluminium chloride solution (AlCl3) was added into the mixture solution. The mixture was allowed to stand for another five minutes before adding 500 µl of 1M sodium hydroxide (NaOH). Distilled water was added into the mixture solution to make up to a total volume of 2.5 ml. The absorbance was measured at 510 nm using UV-Visible spectrophotometer. TFC was expressed as mg catechin equivalent per gram dry weight (mg CE/g DW).

Antioxidant Activities

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Assay
The antioxidant activity of the ethanol extract was determined using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical base on the electron transfer reaction between DPPH reagent and the plant extracts. A 40 µL of plant extract was added to 195 µL of 0.1 mM ethanolic DPPH solution following the method described by (Wong et al., 2006) with some modification. The mixture then was incubated in room temperature for 30 min under dark conditions before measuring at 515 nm using a spectrophotometer. The per cent of inhibition was calculated using the following formula:

Percent inhibition (%) = [(A515 of control -A515 of sample)/A515 of control] x 100.

Ferric Reducing Antioxidant Power (FRAP) Assay
The FRAP assay was conducted using the method of (Wong et al., 2006). Two hundred microliter of extract were added to 3 ml of FRAP reagent that was prepared with a mixture of 300 mM sodium acetate buffer at pH 3.6, 10 mM 2, 4, 6-tri

(2-pyridyl)-s-triazine (TPTZ) solution and 20 mM FeCl.6H2O at the ratio of 10:1:1. The reaction mixture was incubated in a water bath at 37°C for 30 min. The increase in absorbance was measured using a spectrophotometer at 593 nm. The antioxidant capacity based on the ability to reduce ferric ions of the extracts was calculated as a percent of antioxidant. The percent of antioxidant was calculated using the following formula:

Percent antioxidant (%) = [(A593 of sample-A593 of control)/A593 of sample] x 100.

Flavonoid compounds (Kaempferol and Myricetin)

Kaempferol and Myricetin constituents were estimated using RP-HPLC (LC-20AC, Shimadzu, Japan), following the procedure reported by (Butsat, Weerapreeyakul, and Siriamornpun 2009). Extracted samples were dissolved in ethanol, followed by filtration through a 0.45 µm membrane filter, and a volume of dissolved from 20 µl samples was injected into an Inetsil ODS-3C18 column (4.6 mm×250 mm, 5 µm; Hichrom Limited, Berks, U.K.). The mobile phases were 1% acetic acid (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 0.8 ml/min. The bioactive constituents of the extract were isolated using gradient elution at 38°C and UV-diode array detector was used in detecting the eluted bioactive compounds in the flavonoids. The bioactive compounds were detected at 370 nm.

Data analysis

The data were analysed using analysis of variance (ANOVA), and means were separated by Least Significant Difference (LSD) post hoc test at significant level $p < 0.05$ using (SAS 9.4, Cary, NC, USA, 2009). Correlation analysis was carried out to indicate the strength of relationship among the parameters when the first-order interaction was found to be significant.

Conclusion

Considering the high medicinal value of *G. procumbens* for medicinal purposes, the optimization of the management strategies to enhance the content of phytomedicines are required. Different shade levels of 0% (full sunlight), 30%, 50% and 70% with different nitrogen fertilizer rates of 0 (no nitrogen application), 100, 200 and 300 kg N ha^{-1} significantly affected the TPC, TFC, antioxidant activities and flavonoid acids. The high light intensity (0% shade) and no nitrogen application (0 kg N ha^{-1}) stimulated the level of secondary metabolites (TPC and TFC) and antioxidant activity (DPPH and FRAP) of the *G. procumbens*. The low light intensity (70% shade) and high nitrogen application (300 kg N ha^{-1}) accompanied by reducing the phytochemical content and antioxidant activity. This implies the ability of *G. procumbens* to produce a higher value of secondary metabolites under different stress conditions.

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