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Impact of Elevated CO_2 on Leaf Gas Exchange, Carbohydrates and Secondary Metabolites Accumulation in *Labisia pumila* Benth

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Authors' contributions

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

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ABSTRACT

Aims: The aim of this study was to investigate different levels of CO_2 availability alters total phenolic and flavonoid, total available carbohydrate (TAC) and to determine how elevated CO_2 influences gas exchange of *Labisia pumila* seedlings.
Study Design: The 3-months *Labisia pumila* seedlings of var *Alata,* var *Pumila* and var *Lanceolata* were put under 1 month to acclimatize in a nursery until ready for the treatment. Carbon dioxide enrichment treatments started when seedlings reached 4 months old by exposing them to three levels of CO_2, viz., ambient CO_2 (400 µmol/mol), twice ambient (800 µmol/mol) and thrice ambient CO_2 (1200 µmol/mol). The split plot 3 x 3 factorial experiment was designed using randomized complete block design with CO_2 levels being the main plot and varieties as the sub-plot replicated

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three times.
Place and Duration of Study: Ladang 2, Universiti Putra Malaysia Glasshouse complex between July to November 2011.
Methodology: The experiment was conducted for 15 weeks. The measurement of photosynthesis was obtained from a closed infra-red gas analyzer LICOR 6400XT Portable Photosynthesis System (IRGA, Licor Inc., USA). Total phenolics and flavonoid were determined using Follin–Ciocalteau reagent and total available carbohydrate using anthrone reagent.
Results: It was found that the treatment effects were contributed by CO_2 levels in all weeks measured in leaf gas exchange properties (Net photosynthesis (A), stomatal conductance (g_s), transpiration rate (E), intercellular CO_2 (C_i) and Instantaneous water use efficiency, WUE). A combination of increases rates of A and E was responsible for enhancement of WUE by 50% in elevated treatment (800 and 1200 µmol/mol). Total available carbohydrate, total phenolics and flavonoid were also influenced by elevated CO_2 in all weeks of measurement. At end of 15 weeks after treatment (WAT), 44% increase in total available carbohydrate had increased total phenolic and flavonoid by 56% and 149% respectively than ambient treatment. At end of 15 WAT It was found, that the photosynthetic capacity of *Labisia pumila* was enhanced under elevated CO_2 by significantly have higher maximum electron transfer rate, J_{max} and Rubisco CO_2 fixation capacity V_{cmax} than ambient seedlings.
Conclusion: In this work, it was observed that the increase in production of total phenolics and flavonoid in *L. pumila* might be due to increase in production of total available carbohydrate in the present study. The upregulation of photosynthesis in the present study was supported by enhancement of Maximum electron transfer rate, J_{max} and Rubisco CO_2 fixation capacity V_{cmax} than ambient seedlings that showed this plant has high sink strength to cope with high level of CO_2.

Keywords: Medicinal plant; carbon assimilation; carbohydrate accumulation; plant secondary metabolites.

ABBREVIATIONS

CO_2 : carbon dioxide;
A : net photosynthesis;
G_s : stomata conductance;
E : transpiration rate;
C_i : intercellular CO_2;
WUE : water use efficiency;
TAC : Total available carbohydrate;
WAT : Weeks after treatments;
J_{max} : maximum electron transfer rate,
V_{cmax} : Rubisco CO_2 fixation capacity

1. INTRODUCTION

Labisia pumila Benth, is a sub-herbacous plant with creeping stems from the family Myrsinaceae that is usually widespread in Indochina and throughout Malaysia forest. This beneficial herb is also popularly known as *Kacip Fatimah* and also referred locally as *Selusoh Fatimah, Rumput Siti Fatimah, Akar Fatimah, Tadah Matahari, Bunga Belangkas Hutan* and *Pokok Pinggang*. [1]. It has been used customly by Malay women to induce and facilitate childbirth as well as post-partum medicine [2]. Stone [3] have categorized three varieties of this herb in Malaysia namely *Labisia pumila* var. *alata*, *L. pumila* var. *pumila* and *L. pumila* var. *lanceolata*. Each of the varieties has different usage. The most universally utilized by the traditional healers are the first two varieties, *L. pumila* var *alata* and *L. pumila* var *pumila.* The other uses of this herb are for dysentery, dysmenorrhea, flatulence and gonnorhea treatments [4]. Because large uses in the herbal and commercial product this herb is highly demanded [5]. These plants are usually collected from the rainforest and the heavy demand for this herb might endangered the species. It is well known that the growth rate of *Labisia pumila* in the forest was very slow and the propagation from seed usually takes 20 – 24 months before this seedling can be used [6]. The use of CO_2 enrichment to the seedlings might reduce the time in the nursery and enhance the secondary metabolites of the plant [7]. The enhanced CO_2 under elevated levels is able to enhance photosynthesis, produce extra assimilates that are partitioned to plant organs for stimulation of growth [8]. Under these circumstances, the enhanced carboxylation process inhibiting photorespiration. These simultaneously increase the efficiency of the net carbon gain by decreasing photorespiratory CO_2 loss and diverting ATP and NADPH away from photorespiratory metabolism to photosynthetic assimilation [9].

Plant that exposed to elevated CO_2 often exhibit an increase in carbon assimilation rate, instantaneous water use efficiency and growth [10-12]. Total non-structural carbohydrates have been generally shown to increase under elevated CO_2 but this is a species-specific response and the responses may be affected by the nutrient levels [13,14]. Under condition of optimum CO_2 combined with nutrient resource limitation, which restricts growth to a greater extent than photosynthesis, plants tend to show an increase in C/N ratio and an excess of non-structural carbohydrates [15]. This excess may then be available for incorporation into C-based secondary compound such as phenolics and flavonoid [16]. The C-nutrient balance hypothesis predicts that the availability of excess C and a certain nutrient level leads to increased production of C- based secondary metabolites and their precursors [17]. Carbon dioxide enrichment often induce a reduction in nitrogen (N) concentration of plant tissues, that have been attributed to changes in plant N use efficiency [15]. The reduction in N tissue content of high CO_2 grown plant is a size dependent phenomenon resulting from accelerated plant growth. This may also affect plant-herbivore interaction that plays an important role in seedling survival and competitive ability [18].

The increase in plant productivity in response to rising CO_2 concentration is usually pronounced with photosynthesis, dark respiration, carbohydrate production and their differential allocation between plant organ and the subsequent incorporation into biomass [19]. This has increased studies regarding the effects of elevated CO_2 on the primary metabolism [20], but relatively few studies have investigated the response of plant secondary metabolites concentrations to increase CO_2 and its interaction with N availability [21]. The aim of this study was to investigate how CO_2 availability alters total phenolic and flavonoid, total available carbohydrate (TAC) and to determine how elevated CO_2 influences gas exchange of *Labisia pumila* seedlings. The null hypothesis in this study was: that elevated CO_2 would have no effect on gas exchange, phenolic, flavonoid and TAC of *Labisia pumila* seedlings; and that interaction of CO_2 levels with different varieties would have no effects on these variables. It is hypothesized, that elevated CO_2 would influence the gas exchange, production of secondary metabolites and TAC of *L. pumila* with differences in response with each varieties of this plant.

2. MATERIALS AND METHODS

The 3-months *Labisia pumila* seedlings of var *Alata,* var *Pumila* and var *Lanceolata* were left for 1 month to acclimatize in a nursery until ready for the treatment. Carbon dioxide enrichment treatments started when seedlings reached 4 months old by exposing them to three levels of CO_2, viz., ambient CO_2 (400 µmol/mol), twice ambient (800 µmol/mol) and thrice ambient CO_2 (1200 µmol/mol). The split plot 3 x 3 factorial experiment was designed using randomized complete block design with CO_2 levels being the main plot and varieties as the sub-plot replicated three times. Each treatment consisted of ten seedlings. Carbon dioxide at 99.8% purity was supplied from a high –pressure CO_2 cylinder and injected through a pressure regulator into fully sealed 2 m x 3 m growth compartment at 2h/day from 0800 to 1000 continuously. The CO_2 concentration at different treatments was measured using Air Sense ™ CO_2 meter inside every chamber during CO_2 exposition period. The microclimatic parameters. The microclimatic conditions under the chambers are presented in Table 1.

Table 1. Microclimatic condition under the growth compartment during the study

Microclimatic parameters	Quantification
Relative humidity	55.21-66.36%
Light intensity	50.21-270.31 µmol/m^2/s
Day temperature	27-35ºC
Night temperature	18-23ºC

Leaf gas exchange measurement was carried out every 3 weeks for 15 weeks after exposition with CO_2 enrichment. Measurements were taken using a closed system, infrared gas analyzer LICOR 6400 Portable Photosynthesis System (IRGA: LICOR Inc., Lincoln, NE, USA) by placing at the fully expanded leaves supported by a tripod stand and set with optimal growth conditions. The measurement used were standard optimal cuvette condition for *Labisia pumila* at 800 µmol/m^2/s photosynthetic photon flux density, 400 µmol/mol CO_2, 30ºC leaf temperature and 60% relative humidity. Fully expanded leaves were used to record net photosynthesis rate (A), leaf temperature, transpiration rate (E), stomata conductance (g_s) and intercellular carbon dioxide (C_i). Instantaneous water use efficiency (WUE) was calculated by dividing the net photosynthesis rate (A) by corresponding transpiration rate (E). The operation was automatic and the data were stored in the LI-6400 console and analyzed by "*Photosyn Assistant*" software (Version 3, Lincoln Inc, USA). The response of net CO_2 exchange (A) to changing intercellular CO_2 concentration (C_i) was conducted at 30ºC, 60% relative humidity and at light saturating conditions of 800 µmol/m²/s. The A/Ci measurements were made provided by a light source containing blue–red, light-emitting diodes (Li-Cor Model 6400-02B) mounted above the cuvette. External CO_2 partial pressures (*C*a) were supplied in 13 steps decreasing from 100 to 0 Pa. Measurements were automatically taken at each *C*a set- point when photosynthesis had equilibrated, which was typically 10–15 min after a stable *C*a set point had been reached. Leaves temperatures were maintained at 30℃ by means of thermoelectric coolers and air vapor pressure deficit was generally between 1.0 and 1.5 kPa, reflecting ambient water vapor conditions. Relative stomatal limitation (*R*), which is an estimate of the reduction in photosynthesis caused by the cumulative resistances to CO2 diffusion between the atmosphere and the site of carboxylation in the mesophyll, was calculated from *AC*i curves using by ACi curve fitting software version 10 (Davies Instruments, USA) to obtain the value of J_{max} and V_{cmax}.

Anthrone method was used to determine the content of total available carbohydrate in the samples as explained by [22]. The sample (leaves) was collected after CO_2 enrichment from 1100- 1200. One gram of dried ground sample was weighed into the 250 ml conical flask and added with 10 ml of distilled water and 13 ml of 52% Perchloric acid (Kanto Chemical; Japan). The mixture was then shaken using an orbital shaker for 20 minutes. Later, the mixture was transferred into a 100 ml volumetric flask and graduated to 100 ml with distilled water. After that, it was filtered into a 250 ml volumetric flask and graduated to 100 ml distilled water in another 100 ml volumetric flask. One ml of sample was mixed with 5 ml of Anthrone reagent (Merck; Germany) in a test tube. The tube was then placed in water bath at 100ºC for 12 minutes to obtain a dark green solution. The tube was immediately cooled under running tap water and the absorbance was read at 630 nm by a using spectrophotometer (Model UV-PC1; UV-VIS; USA).

The extraction for total phenolic and flavonoid used method proposed by [23]. An amount of 0.1 g of grounded samples were extracted with 10 ml of 80% ethanol on an orbital shaker for 120 minutes at 50ºC. The mixture were subsequently filtered (Whatman™ No.1;UK), and the filtrate was used for the quantification of total phenolics and flavonoid. Folin – Ciocalteu reagent (Kanto Chemical; Japan; diluted 10-fold) was used to determine the total phenolics content of the leaf samples. Two hundred µl of the sample extract was mixed with 1.5 ml of Follin –Ciocalteau reagent and allowed to stand at 22ºC for 5 minutes before adding it with 1.5 ml of $NaNO_3$ (Ajax Finechem; Australia; 60 g/L) solution. After two hours at 22ºC, absorbance was measured at 725 nm. The result was expressed as mg /g gallic acid equivalent (mg GAE/ g dry sample). For total flavonoid determination, 1 ml sample was mixed with 0.3 ml $NaNO_3$ (Sigma; USA) in a test tube with aluminum foil and left for 5 minutes. Then 0.3 ml 10% $AlCl_3$ (Sigma; USA) were added and 2 ml of 1 M NaOH (Sigma; USA) was added and the absorbance were measured at 510 nm using rutin as a standard (mg rutin/ g dry sample). Sample for total phenolics and flavonoids were replicated three times [24]. Data were analyzed using analysis of variance by SAS version 17 (SAS institute; USA). Mean separation test between treatments were performed using Duncan multiple range test and the standard error of differences with the assumption that data was normally distributed and equally replicated.

3. RESULTS

3.1 Carbon Dioxide Treatments

The two-hours daily mean of different CO_2 concentrations recorded values at 380.81, 858.23 and 1252.10 µmol mol^{-1} for CO_2 enrichment at levels of 400, 800 and 1200 µmol mol^{-1}, respectively. The measured values were sufficiently closed to the treatment levels assigned in each case, which indicated that method of CO_2 enrichment was correctly carried out (Table 2).

3.2 Leaf Gas Exchange Properties

3.2.1 Net Photosynthesis, A

The leaf gas exchange measurement showed that the effect of the treatment was contributed by CO_2 levels there were no varieties nor interaction effects were observed (Table 3). It was found that net photosynthesis (A) was higher for elevated treatment (800 and 1200 µmol/mol) compared to control (400 µmol/mol) throughout

15 WAT (Weeks after treatments). From 3 to 9 WAT, A was maintained between 7.42 – 7.73 µmol/m^2/s compared to control that record (5.16 – 6.67 µmol/m^2/s). Start at end of 9 WAT A was peaked until 15 WAT. At 15 WAT, 1200 µmol/mol treatment recorded highest A (13.04 µmol/m^2/s) followed by 800 µmol/mol (11.10 µmol/m^2/s) and 400 µmol/mol (5.73 µmol/m^2/s) and here were statistical significance difference ($P \leq 0.05$) observed between all of the treatment (Fig. 1).

Table 2. Two-hour (0800 – 1000am) daily means of CO_2 concentrations with the different treatments[1,2]

Carbon dioxide treatments (µmol mol^{-1},)	Two hour daily mean concentrations (µmol mol^{-1},)[3]
400	380.81 ± 23.15
800	858.23 ± 34.12
1200	1252.10 ± 98.20

Notes:
[1] Data were means of 120 points
[2] Value were presented over measurement period of four months
[3] µL L^{-1} = 1 microliter CO_2 per liter of air = 1 ppmv = 1 part per million by volume = 1 µmol mol^{-1}

3.3 Stomatal Conductance

The plant that enriched with high level of CO_2 was found to have lower stomatal conductance (g_s) than the control plants in 3, 6 and 15 WAT (Fig. 2; Table 4). At week 6, g_s of 400, 800 and 1200 µmol/mol was 0.071 mmol/m^2/s, 0.045 mmol/m^2/s and 0.011 mmol/m^2/s respectively. However, at 15 WAT, there were no significant difference was observed although 400 µmol/mol remained the highest g_s compared to the elevated levels.

3.4 Transpiration Rate

Fig. 3 gives the transpiration rate (E) of *Labisia pumila* seedlings under elevated CO_2. The E of elevated treatments was consistently and significantly lower ($P \leq 0.05$) in elevated treatments (800 and 1200 µmol/mol) start from 6 WAT(Table 5). At 15 WAT, the E at elevated treatment was 18% lower than the average ambient. The significantly lower g_s clearly reduce the E in elevated treatment.

3.5 Instantaneous Water Use Efficiency, WUE

There were evidently high water use efficiency (WUE) of *Labisia pumila* seedlings under high level of CO_2 (Fig. 4; Table 6). The increases in photosynthetic rate and decreases in stomatal conductance combined to increase WUE. Instantaneous WUE was found to be significant in week 3, 6, 9, 12 and 15 WAT. At 3 WAT, WUE for 400, 800 and 1200 µmol/mol was 5.24, 7.87, 7.26 µmol/mol CO_2 assimilated/ mmol H_2O transpired respectively, but at end of 15 WAT, WUE was low compared from all weeks of measurement. The elevated treatment was statistically higher ($P \leq 0.05$) than the ambient by 50% at 15 WAT (1.623 vs 1.012 µmol/mol CO_2 assimilated/ mmol H_2O transpired). The general relationship between WUE, gs and A was depicted in Fig. 5.

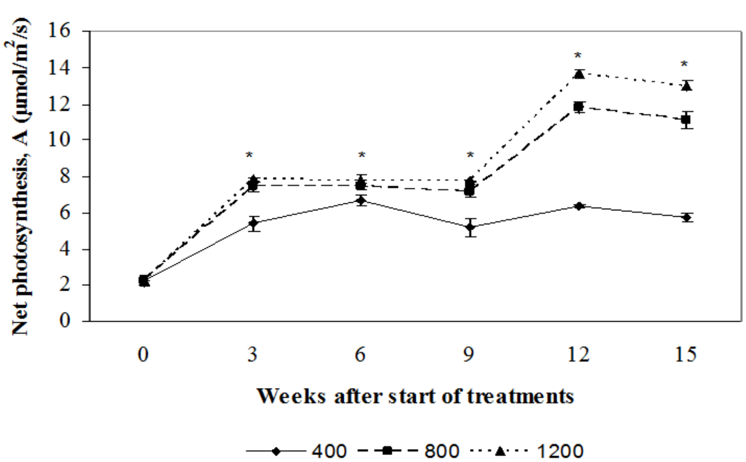

Fig. 1. Net photosynthesis (A) of *Labisia pumila* seedling as affected by different levels of CO_2 during 15 weeks of exposure. Data are mean ± SEM (standard error of mean) of 18 replicates

Table 3. Mean squares of net photosynthesis measured during the experiment

Source of variation	Df	Week 3	Week 6	Week 9	Week 12	Week 15
Carbon (C)	2	15.233**	2.8735*	16.278**	128.237**	128.014**
Block (B)	2	1.084	0.2035	0.8953	0.08733	0.6287
C x B	4	1.332	0.2000	1.3671	0.24528	1.4477
Varieties(V)	2	1.084	0.2035	0.8953	0.08733	0.6287
V x C	4	1.332	0.2004	1.3671	0.24528	1.4775
Error B	12	0.277	1.3631	0.7077	0.76111	0.8301
Total	26					
CV		7.64	15.98	12.55	8.219	9.158

**significant at 5% level; ** = significant at 1% level*

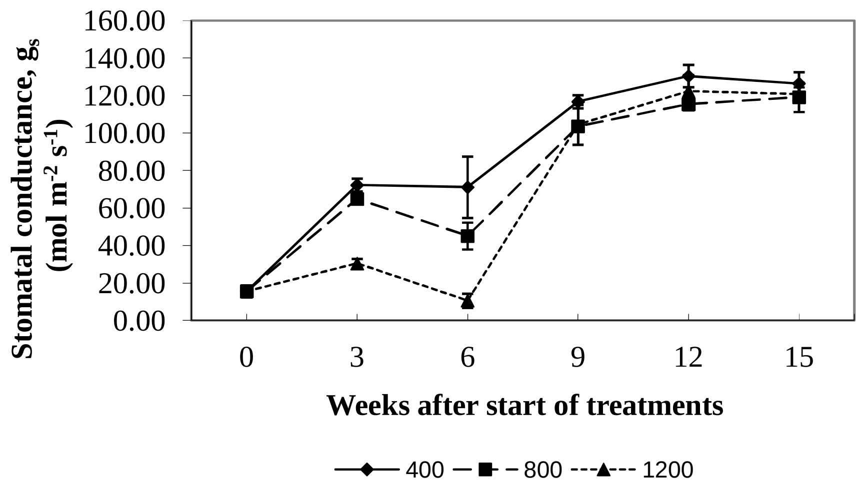

Fig. 2. Stomata conductance (gs) of *Labisia pumila* seedling as affected by different levels of CO_2 during 15 weeks of exposure. Data are mean ± SEM (standard error of mean) of 18 replicates

Table 4. Mean squares of stomatal conductance measured during the experiment

Source of variation	Df	Week 3	Week 6	Week 9	Week 12	Week 15
Carbon (C)	2	0.00414	0.3107*	0.04015**	0.02437**	0.01503**
Block (B)	2	0.0087	0.00134	0.000123	0.00031	0.00069
C x B	4	0.00636	0.00131	0.0005321	0.000124	0.00342
Varieties(V)	2	0.00877	0.00134	0.0001239	0.000311	0.00069
V x C	4	0.00636	0.00131	0.0000532	0.000124	0.00034
Error B	12	0.00274	0.16047	0.0018138	0.000522	0.00020
Total	26					
CV		0.67	0.167	29.85	13.43	8.75

**significant at 5% level; ** = significant at 1% level*

3.6 Intercellular CO_2

It was found that the intercellular CO_2 concentration (C_i) was influenced with CO_2 treatments (Table 7). Fig. 6 gives the C_i of *Labisia pumila* at various stages of plant growth in comparison to different levels of CO_2. Intercellular CO_2 concentration of 400 µmol/mol was the highest from 3, 6 and 9 WAT. However, from 12 -15 WAT C_i of 1200 µmol/mol remained the highest. At 15 WAT, C_i at 400 was 255.22 µmol/mol, 800 (255.71 µmol/mol) and 1200 (285.48 µmol/mol). At end of 15 WAT, C_i for 1200 µmol/mol was 12% higher compared to the ambient level.

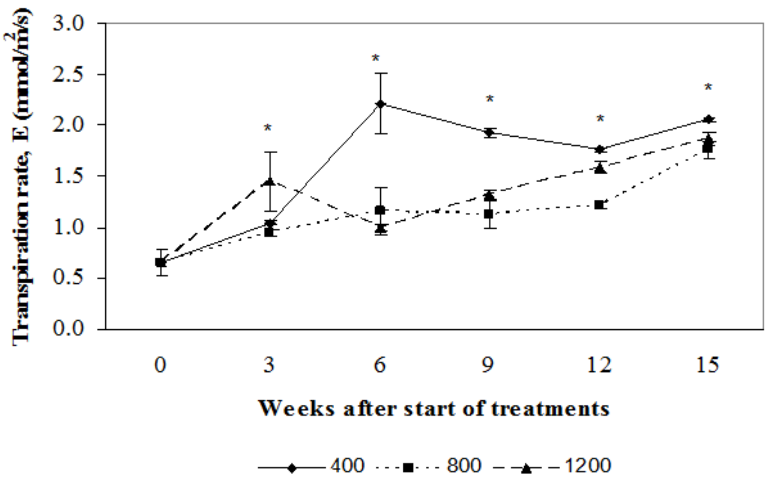

Fig. 3. Transpiration rate of *Labisia pumila* seedling as affected by different levels of CO_2 during 15 weeks of exposure. Data are mean ± SEM (standard error of mean) of 18 replicates

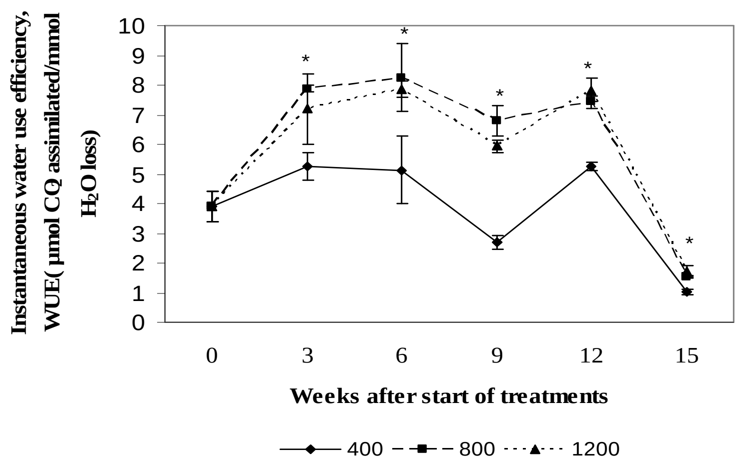

Fig. 4. Instantaneous water use efficiency (WUE) of *Labisia pumila* seedling as affected by different levels of CO_2 during 15 weeks of exposure. Data are mean ± SEM (standard error of mean) of 18 replicates

Table 5. Mean squares of transpiration measured during the experiment

Source of variation	Df	Week 3	Week 6	Week 9	Week 12	Week 15
Carbon (C)	2	0.644136**	3.9765	1.5765**	0.6949**	0.2054**
Block (B)	2	0.96633	0.00843	0.00558	0.0252	0.0498
C x B	4	0.97461	0.00996	0.00301	0.00711	0.00723
Varieties(V)	2	0.96631	0.00843	0.00558	0.02520	0.04988
V x C	4	0.97466	0.00996	0.00301	0.00711	0.0723
Error B	12	0.41459	2.4657	0.13191	0.0812	0.04154
Total	26					
CV		0.12	107.91	24.91	8.07	10.76

**significant at 5% level; ** = significant at 1% level*

Table 6. Mean squares of WUE measured during the experiment

Source of variation	Df	Week 3	Week 6	Week 9	Week 12	Week 15
Carbon (C)	2	14136.111*	15336.111*	24130.037**	5673.060**	2750.259**
Block (B)	2	351.777	353.777	71.8148	49.000	30.703
C x B	4	510.888	513.388	132.370	173.333	115.314
Varieties(V)	2	351.777	353.388	71.814	49.000	30.703
V x C	4	524.388	513.388	132.370	173.333	115.314
Error B	12	4119.685	4129.685	1130.074	442.000	404.944
Total	26					
CV		11.21	7.45	13.34	13.11	7.58

**significant at 5% level; ** = significant at 1% level*

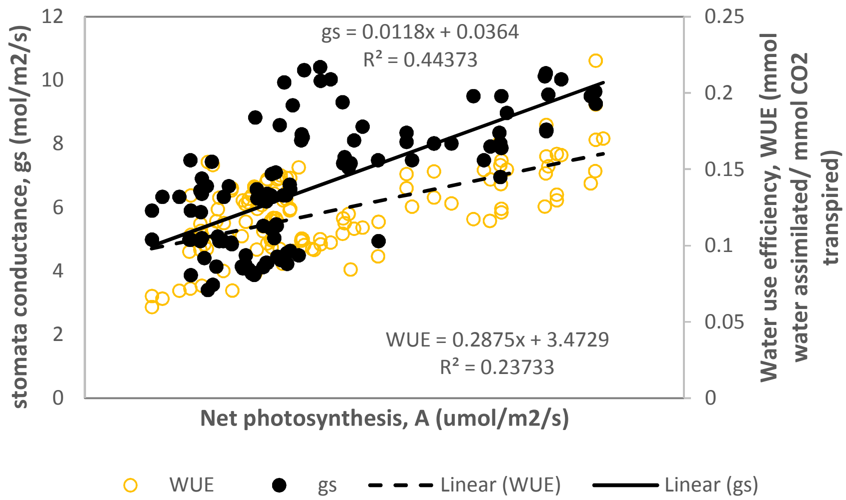

Fig. 5. The relationship between net photosynthesis (A), stomata conductance (gs) and water use efficiency

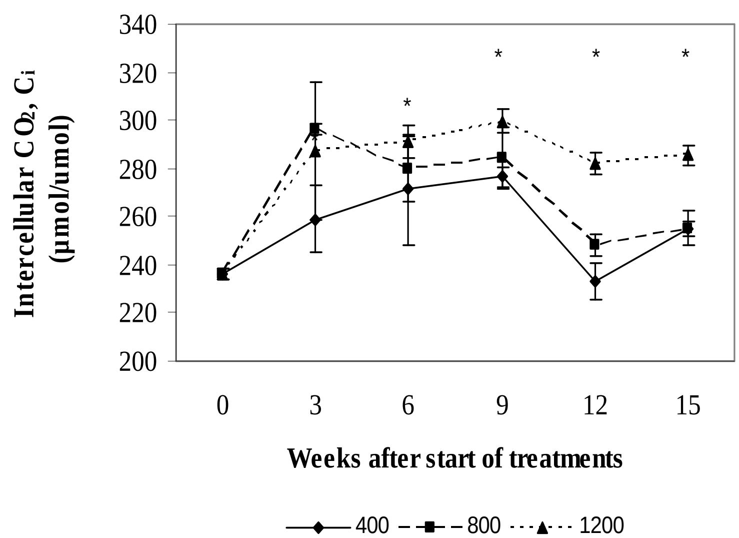

Fig. 6. Intercellular CO_2 (C_i) of *Labisia pumila* seedling as affected by different levels of CO_2 during 15 weeks of exposure. Data are mean ± SEM (standard error of mean) of 18 replicates

Table 7. Mean squares of intercellular CO_2 measured during the experiment

Source of variation	Df	Week 3	Week 6	Week 9	Week 12	Week 15
Carbon (C)	2	16.729**	26.056	42.1693**	17.412**	0.04076**
Block (B)	2	24.891	0.881	0.0876	0.4355	0.4822
C x B	4	14.647	0.6472	0.3901	0.1893	0.2274
Varieties(V)	2	24.89	0.8810	0.0876	0.4355	0.4822
V x C	4	14.647	0.6472	0.3901	0.1893	0.2274
Error B	12	7.964	15.573	1.8238	0.9744	0.189842
Total	26					
CV		0.123	55.75	26.27	14.43	0.34

**significant at 5% level; ** = significant at 1% level*

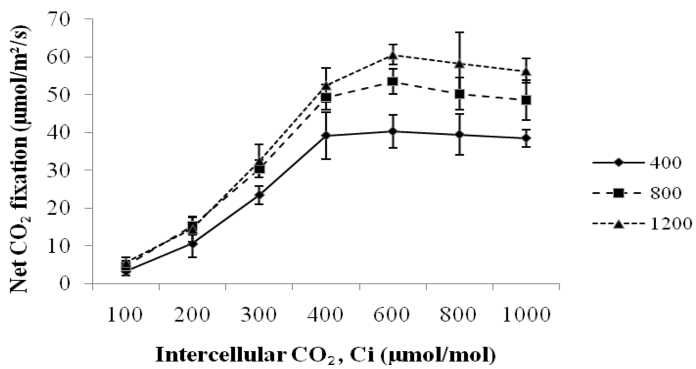

Fig. 7. The effects of different levels of carbon dioxide levels on A-Ci curves on *Labisia pumila* seedlings

N = 18

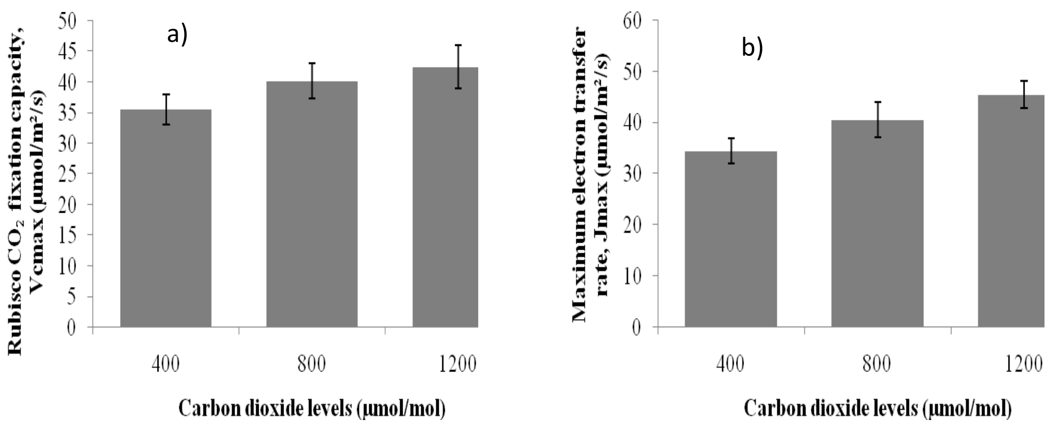

Fig. 8. Rubisco CO_2 fixation capacity, V_{cmax} (a) and Maximum electron transfer rate, J_{max} (b) of *Labisia pumila* seedling as affected by different levels of CO_2 at 15 weeks of exposure. Data are mean ± SEM (standard error of mean) of 18 replicates

3.7 CO_2 Fixation Rate Response to Intercellular CO_2 concentration (A-C_i Curves)

In Fig. 7, it is apparent that the CO_2 fixation rate, plotted against the intercellular CO_2 concentration (Ci) of the plant exposed at ambient CO_2 is lower than those plant exposed at elevated CO_2. There were no varietal effects was observed between the varieties of *Labisia pumila* seedlings. Further analysis on the A-Ci curves revealed that plant grown under elevated

CO_2 have higher Maximum electron transfer rate, J_{max} and Rubisco CO_2 fixation capacity (V_{cmax}). Plant under elevated treatment (800 and 1200 µmol/mol) have 16.5% and 24% higher V_{cmax} and J_{max} than ambient seedlings (Fig. 8). This implies that seedlings grown at elevated CO_2 had higher CO_2 fixation ability than the control seedlings.

3.8 Plant Secondary Metabolites

3.8.1 Total plant phenolics

The effect of CO_2 enrichment on *Labisia pumila* total phenolics is as shown in Fig. 9. After 3 WAT of exposure, plant total phenolics started to increase until end of week 15 (Table 8). From week 3 – 9, there were no statistical significance established between all of the treatments. The highest total phenols were observed in 1200 µmol/mol followed by 800 and 400 µmol/mol. At 15 WAT, total phenol for 1200 and 800 was 31% and 57% higher than the control respectively.

3.8.2 Total plant flavonoid

The high production of secondary metabolites in elevated CO_2 has subsequently resulted in higher total leaf flavonoid per plant (Fig. 10; Table 9). Total plant flavonoid at elevated treatment was significantly higher ($P \leq 0.05$) at all stages of plant growth. At 15 WAT, total plant flavonoid was 86% and 216% higher than the control plants. There was no statistical significance was observed between three varieties of *Labisia pumila* on total phenolics and flavonoid.

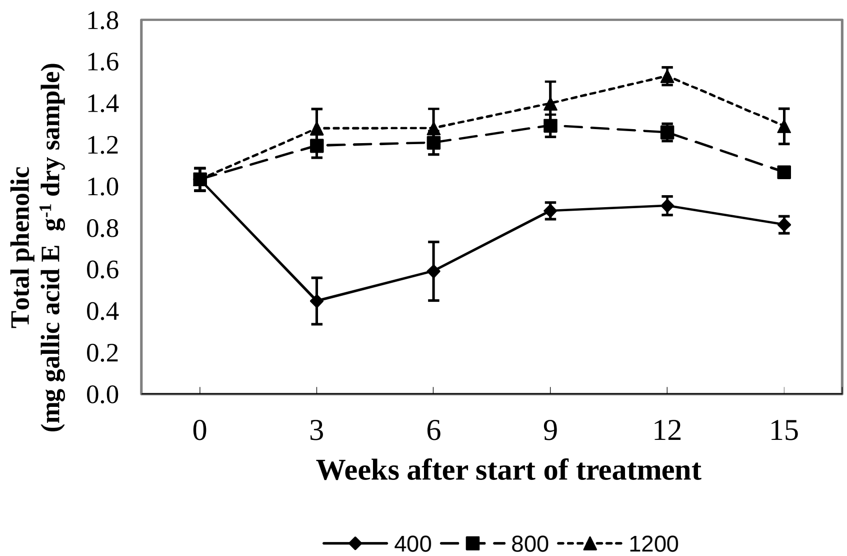

Fig. 9. Total phenolics of *Labisia pumila* seedling as affected by different levels of CO_2 during 15 weeks of exposure. Data are mean ± SEM (standard error of mean) of 18 replicates

Table 8. Mean squares of total phenolics measured during the experiment

Source of variation	Df	Week 3	Week 6	Week 9	Week 12	Week 15
Carbon (C)	2	1.8812**	1.2912**	0.78976**	0.8797**	0.50598**
Block (B)	2	0.01873	0.03571	0.00967	0.003439	0.00890
C x B	4	0.30714	0.43704	0.01670	0.010777	0.011063
Varieties(V)	2	0.01873	0.03571	0.00967	0.003439	0.00890
V x C	4	0.30714	0.43701	0.0167	0.010777	0.011063
Error B	12	0.00875	0.01236	0.007986	0.024300	0.04305
Total	26					
CV		0.12	0.75	23.08	12.64	19.62

**significant at 5% level; ** = significant at 1% level*

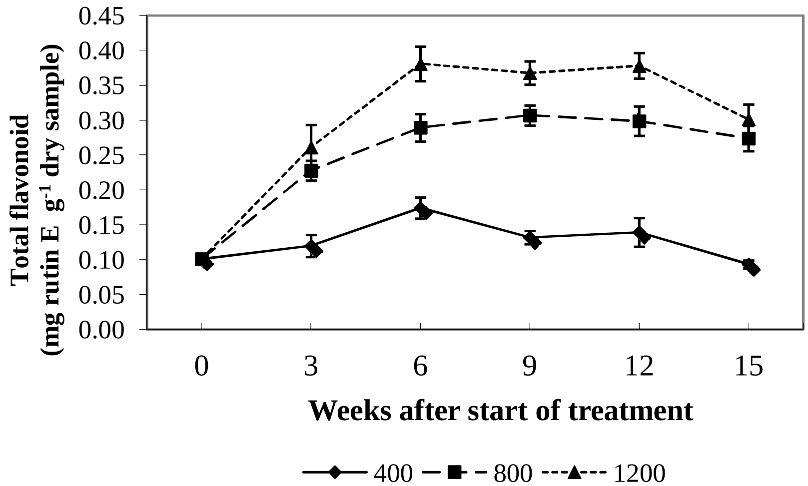

Fig. 10. Total flavonoids of *Labisia pumila* seedling as affected by different levels of CO_2 during 15 weeks of exposure. Data are mean ± SEM (standard error of mean) of 18 replicates

Table 9. Mean squares of total flavonoids measured during the experiment

Source of variation	Df	Week 3	Week 6	Week 9	Week 12	Week 15
Carbon (C)	2	0.049125*	0.0965161*	0.075981**	0.04985**	0.028305**
Block (B)	2	0.000865	0.007622	0.008993	0.01284	0.00665
C x B	4	0.001236	0.004275	0.000661	0.00151	0.000486
Varieties(V)	2	0.000865	0.007622	0.008993	0.01284*	0.00665
V x C	4	0.001236	0.004275	0.000661	0.00150	0.000486
Error B	12	0.007859	0.001949	0.002985	0.00194	0.002326
Total	26					
CV		43.69	15.69	3.07	19.57	30.88

**significant at 5% level; ** = significant at 1% level*

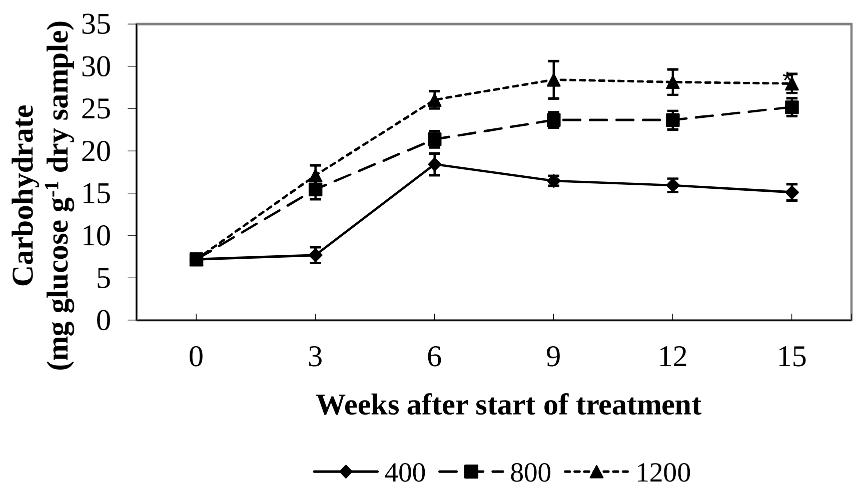

Fig. 11. Total available carbohydrate of *Labisia pumila* seedling as affected by different levels of CO_2 during 15 weeks of exposure. Data are mean ± SEM (standard error of mean) of 18 replicates

Table 10. Mean squares of total carbohydrates measured during the experiment

Source of variation	Df	Week 3	Week 6	Week 9	Week 12	Week 15
Carbon (C)	2	228.015**	133.0134**	325.379**	341.813**	330.1511**
Block (B)	2	10.2269	28.6979	6.840	25.482	2.5666
C x B	4	9.2501	7.5737	9.372	13.282	3.234
Varieties(V)	2	10.2269	28.697	6.840	25.482	2.506
V x C	4	9.2501	7.573	9.377	13.282	3.234
Error B	12	11.733	7.1045	27.54	7.2211	16.898
Total	26					
CV		25.51	12.54	22.98	11.98	18.61

**significant at 5% level; ** = significant at 1% level*

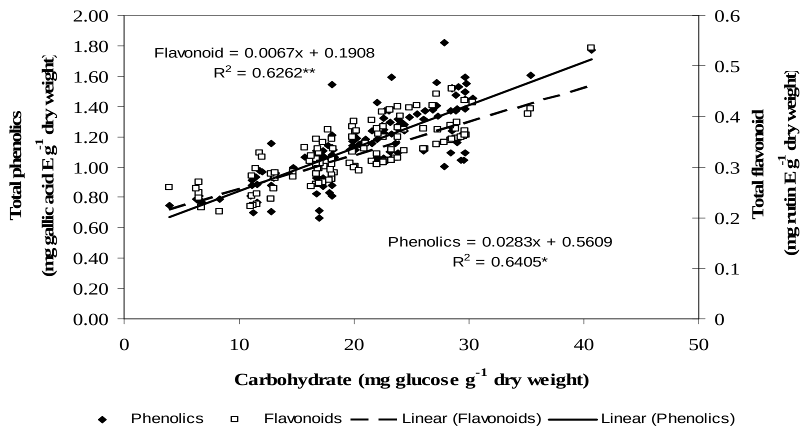

Fig. 12. Significant relationships between total phenolics and total flavonoid with carbohydrate content

** P≤ 0.05, **P ≤0.01, N = 140*

3.8.3 Total available carbohydrate

The increase level of CO_2 has shown to increase total available carbohydrate of the plant under elevated CO_2. Plant that enhanced with high level of CO_2 have shown to have significantly higher (P≤0.05) total available Carbohydrate in all weeks measured (Fig. 11; Table 10). There was significant difference between all the treatment in week 3, 6, 9, 12 but at end of 15 WAT elevated plants have shown to gain 56% more carbohydrate than the ambient. It was found that increase in exposure of *L. pumila* to elevated CO_2 have significantly enhanced the accumulation of carbohydrate. Significant positive relationship was also observed in total phenolics and flavonoid with total available carbohydrate (Fig. 12).

4. DISCUSSION

The aim of the present experiment was to investigate how CO_2 availability alters total phenolic and flavonoid, total available carbohydrate (TAC) and to determine how elevated CO_2 influences gas exchange of *Labisia pumila* seedlings. From the results, it was shown that carbon dioxide enrichment to the seedlings have shown to improve the leaf gas exchange properties of *Labisia pumila* especially net photosynthesis (A). The result showed that elevated plants had higher A than control plants. As the levels of enrichment increased, the higher A were observed (Fig. 1). Net photosynthesis was statistically higher (P≤0.05) in all weeks until 15 WAT. The same result was expected and obtained by [25,26]. Theoretically, a higher CO_2 should increase plant A by increasing the availability of the substrate (CO_2). In the present study, high A under elevated condition probably due to increase in intercellular CO_2 in the elevated leaf (Fig. 6). The increase in leaf intercellular CO_2 (C_i) might due to an increase in the thickness of the leaves under elevated CO_2 that contain high photosynthetic protein especially Rubisco that might up-egulate several enzyme related to carbon metabolism that simultaneously increase the C_i. This data implied that high A under elevated CO_2 are due to more efficient A due to more carbon fixed (high C_i) per area due increased into thickness of mesophyll layer [11,12].

Further enhancing the plant to 2X and 3X than ambient CO_2 have shown to reduce stomatal conductance (g_s) of seedlings exposed to elevated CO_2. Plant that grew under ambient CO_2 had the highest g_s from the elevated plants. The 3X than ambient CO_2 have shown to have the lowest g_s from 3 – 6 WAT (Fig. 2). The decreased g_s simultaneously reduced the transpiration rate (E) of plant under elevated CO_2. This phenomenon is usually reported in plant treated with high than ambient CO_2 [27,28]. It was believed that reduced g_s might be contributed to the plant acclimation to high intercellular CO_2 (C_i) [29].

Instantaneous water use efficiency (WUE) was found to be enhanced under elevated CO_2 (Fig. 4). This was observed in *Labisia pumila* seedlings under CO_2 enrichment. The 1200 µmol/mol and 800 µmol/mol treatments had shown to have statistically ($P \leq 0.05$) higher WUE recorded in 3, 6, 9,12 and 15 WAT. However, there was no statistical significance ($P \leq 0.05$) was observed between the elevated treatments. The enhancement of WUE in elevated plants are due to increase in A alone rather than reduction in E [30,31]. In the present study, in 15 WAT, increases of A by averagely by 97% and reduction of E by 18% have augmented WUE by 51% than control plants. According to [32] the enhancement of WUE in plant correlated with high turgor pressure in plant enriched with CO_2 thus explaining why higher rates of leaf expansion occurred under elevated CO_2.

In the present study, the photosynthetic capacity of leaves grown under in elevated CO_2 for 15 weeks has shown to be enhanced (Figs. 7;8). Thus, some studies reported the reduction in the photosynthetic capacity of seedling enriched under elevated CO_2 [33,34]. The high CO_2 fixation rate of plant under elevated CO_2 might due to high content of rubisco content of *Labisia pumila* Benth seedlings grown under elevated CO_2 than those grown under in ambient CO_2 [35]. This was supported by high V_{cmax} in elevated seedlings that showed high Rubisco fixation capacity than ambient treatment. However, there was no statistical significant difference between 800 and 1200 µmol/mol CO_2. In the present study, the accumulation of carbohydrate did not produce feedback inhibition of *Labisia pumila* seedlings that usually reduce the photosynthetic capacity of plants grown under elevated CO_2.

The enhancement of *Labisia pumila* seedling to CO_2 have successfully increased plant total phenolics (mg Gallic acid Equivalent / g dry sample). It was observed that as the levels of CO_2 enhanced total phenolics was increased. The 1200 µmol/mol CO_2 was found to produce high total phenolics in 3, 6, 9, 12 and 15 WAT (Fig. 9). There were no statistical significant ($P \geq 0.05$) between 2X and 3X in 3, 6, 9 WAT, but in 12 and 15 WAT each of the treatment were statistically significant ($P \leq 0.05$). Plant flavonoid that measured as (mg rutin Equivalent / g dry sample) are influenced by elevated CO_2 levels. It was observed, that there was a statistical significance ($P \leq 0.05$) between elevated CO_2 and control plants in all weeks measured (Fig. 10). The increase in secondary metabolite (phenolics and flavonoid) were observed in [36,37]. The increase in phenolics and flavonoid under high CO_2 are in agreement with Carbon Nutrient Balance (CNB) model that was proposed by [38]. In this model, plant that enriched with high CO_2 should have increased the production of secondary metabolites in the leaves tissue.

It was observed, that increased levels of CO_2 have increased the total available carbohydrate (TAC) of *Labisia pumila* seedlings (Fig. 11). As weeks increased, total available carbohydrate was found to increase significantly ($P \leq 0.05$) in 3, 6, 9, 12 and 15 WAT. From 3 -12 WAT, there was statistical significance between all of the treatments. As the level of CO_2 increase, TAC in the leaves increased. The enhancement of TAC in the leaf was found to contribute to increase in A under elevated CO_2. Net photosynthesis and TAC were statistically linearly related (Fig. 12). Regression analysis has shown that total phenolic and flavonoid have a linear positive relationship with TAC (Fig. 12). As the levels of CO_2 levels increase from 400 to 1200 the slopes of regression become high. This suggest that the increase in plant phenol and flavonoid in the study was due to high total carbohydrate produces in the leaves as levels of CO_2 increases the high carbohydrate produced under elevated treatments (800 and 1200 µmol/mol) were partitioned to the production of secondary metabolites [39].

The increase in A until the end of experiment indicated that there might no starch accumulation in the leaves that impair A although the plant has high total available carbohydrate in the leaves. Furthermore, at end of 15 WAT the elevated treatment was shown to have high photosynthetic capacity by having high J_{max} and V_{cmax} than ambient treatment. High TAC and no impaired A at the end proposed that the extra carbohydrate was partitioned to the production of secondary metabolites that increase the medicinal properties of *Labisia pumila*. The result was in agreement with the CNB hypothesis. [38,40] that stated when nitrogen availability was low, the low resource availability limits the growth of the plant more than photosynthesis and plant allocated their extra carbon that they cannot use for growth to carbon secondary metabolites.

5. CONCLUSION

This work was devoted to assessing the impact of elevated levels of CO_2 in the carbon assimilation of and accumulation of primary and secondary metabolites in medicinal plant *Labisia pumila*. Generally, it was observed that CO_2 solely contributed to treatment effects, there were no varietal and interaction effects were observed. As level of CO_2 increased from 400 to 1200 µmol/mol A, C_i, total phenolics, flavonoid and total available carbohydrate was enhanced and G_s and E was reduced in every week of measurements. In this work, it was observed that the increase in production of total phenolics and flavonoid in *L. pumila* might be due to increase in production of total available carbohydrate in the present study. The upregulation of photosynthesis in the present study was supported by enhancement of Maximum electron transfer rate, J_{max} and Rubisco CO_2 fixation capacity V_{cmax} than ambient seedlings that showed this plant has high sink strength to cope with high level of CO_2.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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