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INFLUENCE OF ELEVATED CO_2 ON THE GROWTH AND PHENOLIC CONSTITUENTS PRODUCTION IN *HIBISCUS SABDARIFFA* VAR. UKMR-2

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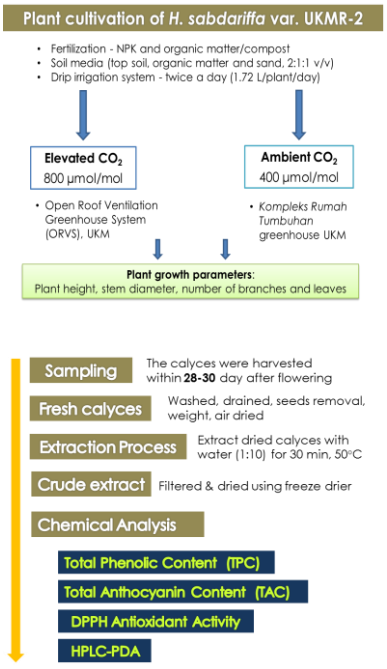
Graphical abstract



Abstract

The impact of global climate change on plants which has been widely reported can exhibit significant changes on the growth, yield and metabolite production. Studies on the impact of elevated carbon dioxide concentration, [CO_2] on plant growth and production of phenolic constituents in *Hibiscus sabdariffa* var. UKMR-2 has not been reported in any previous studies. This study investigated the growth quality and production of phenolic constituents of UKMR-2 under different [CO_2]. The cultivation was subjected to two atmospheric [CO_2]; ambient (400 µmol/mol), and elevated (800 µmol/mol). Selected parameters for growth performance were recorded throughout the plant development. UKMR-2 calyx extract was analysed for total phenolic, total anthocyanins, antioxidant activity, and evaluated based on HPLC-PDA method. The results revealed that UKMR-2 responded differently to the [CO_2] treatments. The results clearly showed that exposure to elevated [CO_2] increased calyx yields, production of phenolic constituents, and antioxidant activity. Furthermore, different [CO_2] had significant interaction on the production of phenolic constituents, and antioxidant activity ($p < 0.05$), except for plant growth. The HPLC-PDA showed the presence of delphinidin-3-*O*-sambubioside, cyanidin-3-*O*-sambubioside, ascorbic acid, caffeic acid, and chlorogenic acid. Therefore, increased [CO_2] may have significant effects on UKMR-2 to not only produce higher production yields, but also on the production of phenolic constituents with potential physiological impact to human health.

Keywords: Phenolic constituents, *H. sabdariffa* var. UKMR-2, elevated [CO_2], antioxidant activity, HPLC-PDA

Abstrak

Kesan perubahan iklim global terhadap tumbuh-tumbuhan telah dilaporkan mampu menunjukkan perubahan yang ketara terhadap kadar pertumbuhan, jumlah hasil pengeluaran dan penghasilan metabolit. Kajian mengenai kesan peningkatan kepekatan karbon dioksida, [CO2] terhadap kadar pertumbuhan dan penghasilan sebatian fenolik daripada *Hibiscus sabdariffa* var. UKMR-2 masih belum dilaporkan dalam mana-mana kajian. Oleh itu, kajian ini merungkai kualiti pertumbuhan dan

penghasilan sebatian fenolik UKMR-2 pada [CO_2] yang berbeza. Penanaman dilakukan pada dua [CO_2] atmosfera; ambien (400 µmol/mol) dan peningkatan (800 µmol/mol). Parameter terpilih bagi kadar pertumbuhan direkod sepanjang pertumbuhan pokok. Ekstrak kaliks UKMR-2 dianalisis bagi penentuan jumlah fenolik, jumlah antosianin, aktiviti antioksida dan dinilai berdasarkan HPLC-PDA. Keputusan kajian menunjukkan bahawa UKMR-2 memberi respon yang berbeza terhadap rawatan [CO_2]. Dapatan kajian dengan jelas menunjukkan bahawa pendedahan kepada peningkatan [CO_2] mampu meningkatkan penghasilan kaliks, sebatian fenolik dan aktiviti antioksida. Selain itu, [CO_2] yang berbeza mempunyai interaksi yang signifikan terhadap penghasilan sebatian fenolik dan aktiviti antioksida ($p < 0.05$), kecuali bagi kadar pertumbuhan pokok. HPLC-PDA menunjukkan kehadiran delfinidin-3-*O*-sambubiosida, sianidin-3-*O*-sambubiosida, asid klorogenik, asid kafeik dan asid askorbik. Oleh itu, peningkatan [CO_2] boleh memberi kesan yang ketara ke atas UKMR-2 bukan sahaja dalam meningkatkan hasil pengeluaran, tetapi juga untuk penghasilan sebatian fenolik yang mempunyai potensi fisiologi kepada kesihatan manusia.

Kata kunci: Sebatian fenolik, *H. sabdariffa* var. UKMR-2, peningkatan [CO_2], aktiviti antioksida, HPLC-PDA

1.0 INTRODUCTION

Global atmospheric carbon dioxide concentration, [CO_2] is rapidly increasing and threatening global food security. Global background [CO_2] has already approached 400 parts per million (ppm) and expected to reach 900 ppm by the end of 2100 [1-2]. At present, research interest related to climate impacts on the growth and plant secondary metabolites production, especially dietary phenolics due to their biological activities is aggregating. Exposure of plants to elevated carbon dioxide concentration, [CO_2] has been shown to exhibit significant changes in plant growth, yield and their chemical composition. However, different plant species respond differently to CO_2 enrichment. The high level of CO_2 has frequently been reported to increase the rates of photosynthesis, plant growth, biomass accumulation, carbohydrate content, and carbon-based secondary metabolites such as phenolics in many plant species [3-5].

In contrast, other reports showed no change or even led to a decrease in secondary metabolites production [6-8]. For instance, recent studies on *Sorghum bicolor* revealed elevated [CO_2] reduced the stomatal conductance and improved protein content by 60% [9]. Moreover, elevated [CO_2] did not significantly affect the photosynthetic rate, relative growth rates, and biomass of *Shorea platycarpa* [10].

Roselle (*Hibiscus sabdariffa*) from Malvaceae family is a subtropical plant and an exotic crop in Malaysia. Roselle, most commonly known as 'asam paya' or 'asam susur' in Malaysia [11], has enormous potential for food colorant, nutraceuticals, pharmaceuticals, and many other uses [12]. The red calyx juice has been claimed to be a pro-health drink due to its high contents of anthocyanins and ascorbic acid [13], and it is also used to make jams, syrups, puddings and ice cream due to its pleasant acidic taste [14-15]. Roselle is widely used in health associated products and has many therapeutic potential. This herbaceous shrub is used as antioxidant, antimicrobial, anti-hypertensive, antidiabetic, and anti-cancer agents, and useful for cardio protective action [16-18]. The calyces extract has high amount of anthocyanins and phenolic acids including chlorogenic acid, hydroxycitric acids, hibiscus acid, caffeic acid, protocatechuic acid, oxalic acid, and ascorbic acid as well as minerals, alkaloids, flavonoids, saponins, steroids, sterols, and tannins [13, 19-20].

Hibiscus sabdariffa var. UKMR-2 was produced by mutation breeding, is cultivated in Universiti Kebangsaan Malaysia (UKM). UKMR-2 is a medium-sized plant that has a deep red colour of calyces, broad leaves, and has several unique characteristics such as a shorter life cycle, produces high yield of calyces per plant, higher lodging resistance compared to their parent variety 'Arab' and other local varieties [21].

Extensive studies in major food crops showed that atmospheric CO_2 enrichment has significant influence on plant growth, yield productivity, and plant primary and secondary metabolites [2, 9, 22]. While the impact of elevated CO_2 on yield, plant growth and quality of important beverage crops such as roselle remained mostly unknown. Moreover, no information is available on the effect of CO_2 concentration on phenolic content and antioxidant activity of roselle.

This study investigated the influences of elevated atmospheric CO_2 treatments on the growth, yield, antioxidant activity, and the production of phenolic constituents in *H. sabdariffa* var. UKMR-2 cultivated under controlled environmental conditions. This information was useful to assess the implications of future changes with increasing [CO_2] and provided the basis for recommendation regarding the

suitability for roselle cultivation in order to produce higher phenolic contents.

2.0 METHODOLOGY

2.1 Chemicals and Reagents

The standard compounds such as gallic acid, ascorbic acid, chlorogenic acid, caffeic acid, delphinidin-3-*O*-sambubioside, and cyanidin-3-*O*-sambubioside were purchased from Merck (Darmstadt, Germany) and Extrasynthese (Genay Cedex, France). Formic acid, Folin-Ciocalteu reagent, methanol, sodium carbonate, potassium chloride, and sodium acetate were acquired from Merck (Darmstadt, Germany). 2,2-diphenyl-1-picrylhydrazyl (DPPH) for the antioxidant assay was purchased from Sigma Aldrich (USA). Acetonitrile HPLC grade used for preparing mobile phases were purchased from Fisher Scientific (USA). Acetonitrile HPLC grade was degassed in an ultrasonic bath (Branson, USA) before use. As for vortex mixer, G560E Vortex-Genie 2 (Scientific Industries, Bohemia, NY, USA) was used. Distilled water was purified and deionized by EVOQua water system (Water Technologies, Fahrenberg, Germany).

2.2 Plant Samples and Treatments

Plant cultivation was conducted in *Kompleks Rumah Tumbuhan* greenhouse and Open Roof Ventilation Greenhouse System (ORVS), Universiti Kebangsaan Malaysia (UKM), Bangi from August until December 2017. Healthy seeds of UKMR-2 hybrid were initially sown in a seed tray filled with organic soil. After 2 weeks, the seedlings (6-8 cm in height) were selected and transplanted into nursery polyethene bags (20 x 20 cm) containing 20 kg of soil mixture of top soil, organic matter, and sand (2:1:1 v/v). Irrigation treatment with 1.72 L/plant/day using drip irrigation systems was applied based on the recommended option for better roselle growth as described by Nur Amirah *et al*. [23], twice a day over 120 days after transplanting (DAT). All plants received similar commercial fertiliser treatment per polyethene bag (NPK Green 15:15:15, NPK + Mg Blue 12:12:17:2), and organic matter/compost. Fertilisers were applied at an interval of two weeks after transplanting. Manual weeding was practiced regularly, and pesticides were applied when necessary.

Carbon dioxide elevated treatment was started after transplanting by exposing them to two levels of [CO_2]; ambient [CO_2] at approximately 400 µmol/mol as the control in greenhouse and elevated [CO_2] with approximately 800 µmol/mol in the open roof ventilation greenhouse system (ORVS). Elevated [CO_2] with 800 µmol/mol value intended to represent the predicted range of atmospheric CO_2 concentration at the end of 21st century from a global climate model under WGIII scenario [1]. The elevated [CO_2] treatment was done by daily automated continuous injection of pure CO_2 for 2 hours until the desired concentration was reached. CO_2 gas cylinder was connected to air delivery system and air blower of the ORVS. The concentration was regulated by dilution with air stream generated by the blower. CO_2 concentration in the chamber was monitored and administered using CO_2 analyser.

2.3 Growth Measurements

The growth parameters such as plant height (cm), stem diameter (mm), number of branches and leaves (indicate physiological age of the plant) were observed and measured every seven-day interval, after plant transplanting. Plant height (cm) was measured from the main stem from the ground level to the shoot tip of the plant using a measuring tape. The primary stem diameter (mm) was measured at a consistent point using callipers. The number of branches was determined by counting the primary reproductive branches. Also, the number of leaves was calculated and recorded for every visible foliage on the plant, including the tips of new leaves just beginning to emerge.

2.4 Samples Preparation

Calyces were collected from six individual plants per treatments as biological replicates. The calyces were harvested within 28-30 day after flowering. The collection of calyces started at the end of November until December 2017. The fresh calyces were placed in a zip lock bag, which was set in a cooler for transportation to the laboratory. Each calyx was washed with water, drained, followed by seed removal and air dried at room temperature for three days. The percentage yield of fresh and dried weight calyces was determined using the weight of fresh calyces before and after removing the seed [24]. Dry calyces were ground with mortar and pestle under liquid nitrogen, packed in a zip lock bag, and stored in a refrigerator at -20°C until subjected to extraction. The UKMR-2 calyces extract was prepared according to the method described by Chumsri *et al.* [24]. In brief, the extraction of dried calyces with water at a ratio of 1:10 was conducted in a water bath at a constant temperature of 50°C for 30 minutes. The extracts were filtered through Whatman No.1 filter paper, and filtrates were dried using Alpha 1-2 LDplus freeze dryer (Martin Christ, Germany).

2.5 Total Phenolic Contents (TPC) and Total Anthocyanin Contents (TAC) Quantification

Folin-Ciocalteu assay was used to determine the TPC as described by Waterhouse [25]. 20 µL of sample solution (10 mg/mL in water) or gallic acid (standard) mixed with 1.58 mL water, followed by 100 µL Folin-Ciocalteu reagent (2N) and 300 µL sodium carbonate solutions (20%, w/v). After 2 hours of

incubation at room temperature, the absorbance was measured at 765 nm. The TPC was expressed as mg gallic acid equivalent (mg GAE) /g of dry weight (g DW). For TAC, two buffer systems: potassium chloride buffer, pH 1.0 (0.025 M) and sodium acetate buffer, pH 4.5 (0.4 M) were used as described by Giusti and Wrolstad [26]. Essentially, 3 mL test dilutions were prepared separately by mixing 300 µL extract solution (10 mg/mL in water) with buffer pH 1.0 and pH 4.5 (1:10 dilution factor). After 15 minutes of incubation, the absorbance was measured at 520 nm and 700 nm for each solution. TAC was calculated and expressed as mg cyanidin-3-glycoside equivalent per gram of dried weight. All of the absorbance was measured using Epoch Microplate Spectrophotometer (BioTek, USA). All measurements were performed in triplicate.

2.6 DPPH Radical Scavenging Assay

The modified method was used for DPPH radical scavenging activity of roselle extract and ascorbic acid (standard reference) as described by Kouakou *et al.* [15]. Briefly, 100 µL from different concentrations of water extract solutions were added to 100 µL 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol (0.1 mM) in a 96-well plate. The mixture was shaken and left to stand for 30 minutes in a dark environment. The absorbance was measured using Epoch Microplate Spectrophotometer (BioTek, USA) at 517 nm. Each sample was measured in triplicate and averaged.

2.7 High Performance Liquid Chromatography with Photodiode Array Detector (HPLC-PDA) Method

HPLC analyses were performed with the HPLC Waters e2695 separation module equipped with a degasser, an autosampler automatic injector, and a Waters 2998 Photodiode Array Detector (PDA) at multiple wavelengths. HPLC separation was conducted using Purospher STAR RP-18e LichroCART column (250 mm x 4.6 mm x 5 µm). 10 mg freeze-dried extracts were dissolved in 1 mL of 0.1% formic acid in water. Samples were sonicated for 5 minutes and filtered through a 0.45 µm PTFE membrane syringe filter (Gema Medical, Spain), and directly injected into the HPLC system.

HPLC separation and identification were carried out at a flow rate of 1 mL/min, injection volume 30 µL and 30°C column oven temperature. 0.1% formic acid in water and 0.1% formic acid in acetonitrile were employed as mobile phases A and B, respectively, in gradient elution as follows: 10 - 15% B (0-15 minutes), 15 - 90% B (15-25 minutes), 90-10 % B (25-30 minutes) and 10% B (30-35 minutes). The chromatograms were monitored at 520 nm, 265 nm, and 320 nm. All compounds were determined by standard reference calibration curves and were expressed as mg per g dried weight (mg/g DW). Linear correlation co-efficient was > 0.996 for each compound.

2.8 Statistical Analysis

Data was expressed as a mean ± standard deviation of three parallel measurements. Statistical calculations were carried out using Statistical Package for Social Science (SPSS) for Windows version 25.0 software (SPSS Inc., Chicago, USA). One-way analysis of variance (ANOVA) was performed to determine the significant differences between treatments. Significant different values were defined at the 5% level ($p < 0.05$). Correlations between TAC, TPC and antioxidant potential were analysed by Pearson's correlation.

3.0 RESULTS AND DISCUSSION

3.1 Growth Characteristics

Morphological traits, including plant height, stem diameter, number of branches and leaves as well as fresh and dry weights of calyces were determined. The result of the effect of carbon dioxide treatments for every growth parameter for a 7-day interval on UKMR-2 is presented in Figure 1.

In general, all plant growth parameters showed an increasing trend throughout 70 DAT for all treatments. However, as the weeks increased, no significant effect was recorded on the growth parameters with different carbon dioxide treatments ($p > 0.05$). Plant growth in ambient CO_2 (400 µmol/mol) tends to be higher compared with elevated CO_2 (800 µmol/mol) treatments, except for plant height. Plant height was more responsive to CO_2 treatment compared to other plant growth parameters. At 70 DAT, UKMR-2 with elevated [CO_2] showed an increase in plant height by 15.4% compared to ambient [CO_2]. Similar results had previously been reported on green tea (*Camellia sinensis* L.) with an increase in plant height by 13.5% after exposure to elevated CO_2 conditions for 24 days [27].

For stem diameter, the number of branches and leaves, ambient [CO_2] treated plant showed increased growth development compared to elevated [CO_2] at 70 DAT by 6.9%, 19.6%, and 8.4%, respectively. This was probably due to the influence of individual growth performance. Nor Lailatul *et al.* [10] stated that elevated CO_2 (800 ± 50 µmol mol^{-1}) did not enhance stem diameter and number of leaves growth rate of *S. platycarpa* with $p > 0.05$ after seven months of treatment.

Previous studies showed that CO_2 enrichment improved photosynthesis and respiration towards the increase of biomass accumulation, which helps the plant to optimise carbon and nutrient allocation towards secondary metabolism and plant growth [27, 28]. Other considerable variations of growth responses were partly dependent on the duration of exposure to elevated CO_2, plant species, and the availability of primary resources [29].

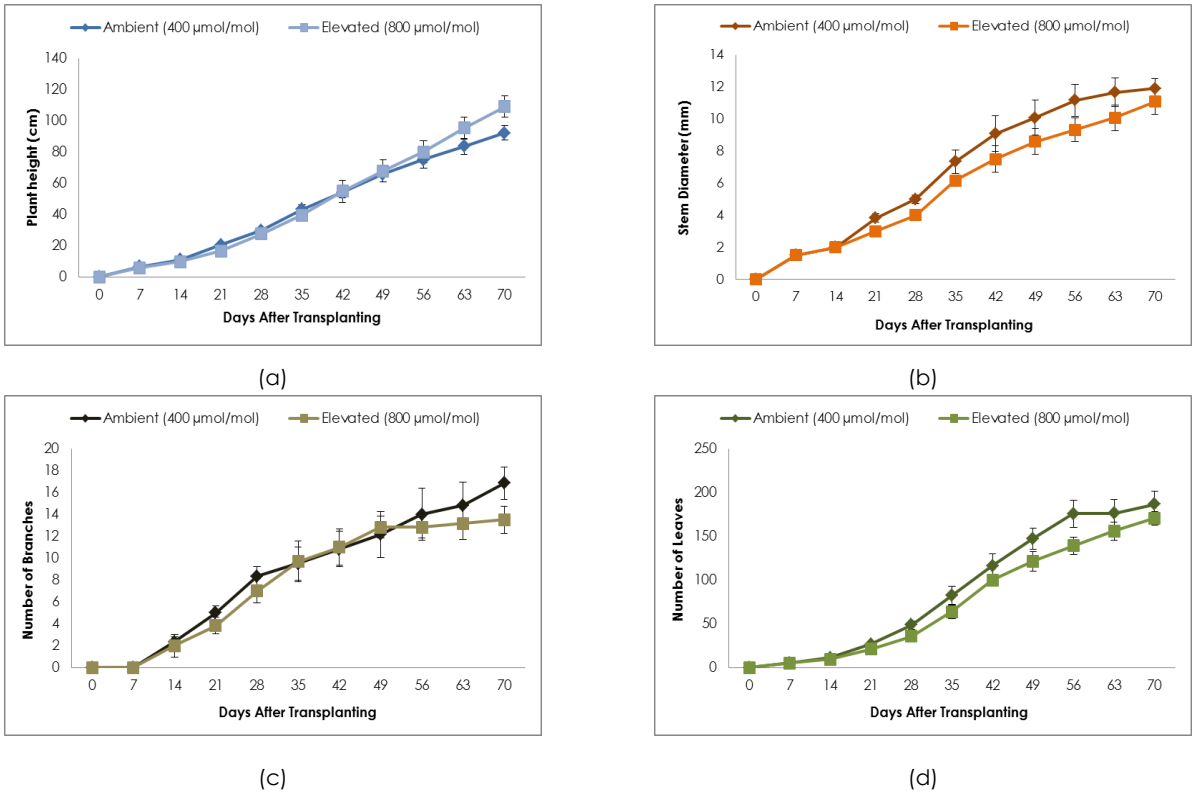


Figure 1 Effect of CO_2 treatments (µmol/mol) on plant growth with different parameters: a) Plant height; b) Stem diameter; c) Number of branches and d) Number of leaves. Data points are means of biological replicates (n = 6). Vertical bar represents standard deviation

Fresh weight of UKMR-2 calyces was enhanced with elevated [CO_2]. The highest yield of fresh weight roselle calyces (with seed) harvested were obtained from elevated [CO_2] treated plants with 511.0 g compared to ambient [CO_2] with 123.8 g. These results showed that elevated [CO_2] plant harvestable yields have significant differences ($p < 0.05$) with 75.8% higher (fresh weight) than ambient [CO_2]. These results were similar to broccoli (*Brassica oleracea* var. *italica* Plenck), where elevated CO_2 (685-820 ppm) instigated an increase in fresh weight by approximately 7% [30]. Moreover, it was reported that the total plant biomass (expressed in g/plant) of leaves, stems and rhizomes from two ginger varieties were also enhanced to 47.6 - 76.3% with rising CO_2 [31]. According to Wittwer [32], much scientific research had been conducted to measure the effects of elevated CO_2 up to 1,000 ppm and above in most green plants. These greenhouse-grown fruits and vegetables showed earlier maturity, larger fruit size, greater number of fruits, and yield increase ranging from 10 to 70% [32].

The percentage of cumulative yield of fresh (without seed) and dried roselle calyces are shown in Figure 2. It was observed that both [CO_2] treatments produced a similar percentage of fresh and dried yields. However, elevated [CO_2] treated plants presented a higher percentage of fresh and dry calyx yields were 60.2% and 9.5%, respectively.

Chumsri *et al.* [24] reported that 10% moisture content of dried roselle calyces provided better extraction capability compared to fresh ones, enhanced stability during storage and resistant towards high humidity that may lead to degradation. Therefore, air-dried roselle calyx for both [CO_2] treatments should be stable when maintained under proper storage conditions.

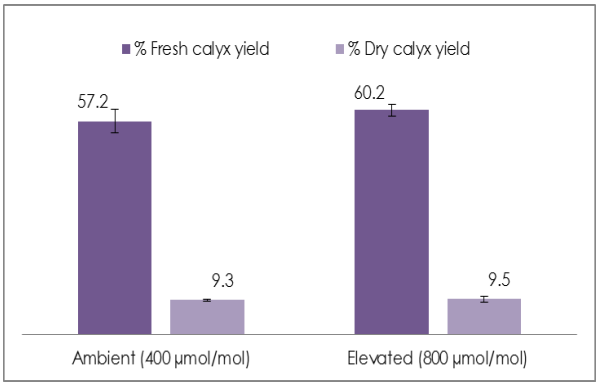


Figure 2 Percentage yield of fresh (without seed) and dried roselle calyces from CO_2 treatments. The results are expressed as the mean values ± SD, n = 6

3.2 Chemical Analysis

Initially, the total phenolic contents, total anthocyanin contents and antioxidant activity were measured to assess the influence of elevated [CO_2] concentration on UKMR-2 calyces. Although numerous works had reported on the phenolic contents on roselle calyces, this work revealed for the first time regarding phenolic constituents' in UKMR-2 cultivated under different atmospheric CO_2 concentrations. In this study, the overall range of TPC and TAC for all plants from both treatments was 2.11 to 3.72 mg/g DW, and 5.63 to 14.01 mg C3G equivalent/g DW, respectively. Meanwhile, the overall range of antioxidant activity represented by IC_{50} value was 0.056 to 0.157 mg/mL. Phenolic and anthocyanin contents in UKMR-2 calyces under elevated [CO_2] were higher than those of the ambient condition. The increase in phenolic and anthocyanin contents for elevated [CO_2] treated plant was associated with an increase in antioxidant activity.

The overall TAC and TPC values in UKMR-2 calyx were significantly affected by the applied CO_2 treatments ($p < 0.05$), with the highest levels of TAC and TPC found in elevated [CO_2] plot (see Table 1). Therefore, there was an interaction between CO_2 concentration with anthocyanins and phenolic contents in comparison to the elevated [CO_2] treatment. Hence, the exposure of UKMR-2 plant to high CO_2 concentration showed an increasing value of TAC and TPC contents.

Based on carbon-nutrient balance theory, CO_2 enrichment affects the C/N ratio and total non-structural carbohydrates that may stimulate the secondary metabolites production in plants as a result of a metabolic excess of carbon with no physiological cost on growth [33]. Li *et al.* [27] also stated that increased carbon supply towards secondary metabolic pathway can be a potential reason for increased production of carbon-based secondary metabolites under elevated CO_2 condition.

The increase in phenolic and anthocyanins contents with elevated CO_2 was in agreement with Mohd Hafiz *et al.* [33], Ghasemzadeh and Jaafar, [31], Veteli *et al.* [34] and Wang *et al.*'s [35]findings. Mohd Hafiz *et al.* [33] stated that elevated CO_2 (from 400 to 1200 µmol/mol) enhanced total phenolic and flavonoid productions in three varieties of *Labisia pumila* leaves, but there was no statistical significance between varieties. Besides, Ghasemzadeh and Jaafar [31] found that total flavonoids and total phenolic contents increased significantly in all parts of ginger (*Zingiber officinale*) varieties under elevated CO_2 (800 µmol/mol). Moreover, strawberry fruits [35] and Boreal tree [34] also demonstrated an increase in total anthocyanin and phenolic contents due to elevated [CO_2] treatment.

Table 1 Effect of elevated carbon dioxide treatments on total phenolic, total anthocyanins content and antioxidant activity in UKMR-2 calyces

CO_2 Treatment	TPC (mg GAE/g DW)	TAC (mg C3G/g DW)	IC_{50} (mg/mL)
Elevated (800 µmol/mol)	3.15 ± 0.43 [a]	11.05 ± 1.94 [a]	0.108 ± 0.028 [a]
Ambient (400 µmol/mol)	2.67 ± 0.56 [b]	8.11 ± 1.13 [b]	0.139 ± 0.020 [b]

Values represent the mean of three replicates ± standard deviation. Mean denoted by different letters indicate significant differences between the treatments ($p < 0.05$). DW, dry weight

In contrast with the present results, total phenolic content was found to reduced in brown rice, *Oryza sativa* L. [22] and *Ribes nigrum* roots [36] grown under elevated [CO_2] conditions. Time exposure of the plant to elevated [CO_2] may have had a potential effect on plant allocation to carbon-based compounds such as phenolic production. Acclimatization of plants to elevated [CO_2] over several weeks may change the plant chemical composition [37].

A Pearson correlation was run to determine the relationship between total phenolic contents with total anthocyanin contents for both CO_2 treatments. There was a very strong, positive linear correlation between TPC and TAC in roselle water extracts with a range coefficient, r of $0.650 < r < 0.925$ ($N = 6$, $p < 0.01$) for both treatments. This study suggested that there is an association between TAC and TPC in UKMR-2 calyx's growth under elevated [CO_2] treatments.

The antioxidant activities of roselle calyces from different [CO_2] treatment were investigated using the DPPH scavenging assay and compared with ascorbic acid as the reference standard (see Figure 3). The antioxidant activity expressed as IC_{50} (mg/mL) where IC_{50} is the extract concentration required to inhibit 50% radical-scavenging activity. The lower IC_{50} value corresponds to the higher antioxidant activity of studied extract [15]. It was observed that elevated [CO_2] treated plants showed higher DPPH inhibition percentage (lower IC_{50} value) compared to ambient [CO_2]. However, IC_{50} for those calyxes grown under two different [CO_2] treatments were lower than ascorbic acid.

The IC_{50} value for elevated [CO_2] were lower than ambient [CO_2] with 0.108 ± 0.028 mg/mL and 0.139 mg/mL ± 0.020, respectively. Data from DPPH assays

indicated that the elevated [CO_2] significantly influenced the antioxidant activity of UKMR-2 calyx ($p < 0.05$). The lower IC_{50} value suggests that elevated [CO_2] treatment pronounces the antioxidant activities of UKMR-2 calyx, hence, possibly enhancing the medicinal properties of this plant. These results were in agreement with Ghasemzadeh and Jaafar [31] on two *Zingiber officinale* (leaves, rhizomes and stems) which displayed increased antioxidant activity under elevated [CO_2] (expressed as ferric reducing antioxidant potential (FRAP) activity) but significantly lower than those of vitamin C. Similar results were also observed in strawberry fruits grown in elevated [CO_2] conditions based on the highest oxygen radical absorbance capacity [35]. In contrast, Goufo *et al.* [22] found that exposure of white and brown rice (*Oryza sativa* L.) to high CO_2 concentration reduced antioxidant activity with a strong correlation between the total phenolic contents and the DPPH radical scavenging capacity ($r = 0.928$). Moreover, Goncalves *et al.* [37] also indicated that elevated [CO_2] did not significantly change the total antioxidant capacity of the grapevine (*Vitis vinifera* L.) based on the DPPH and ABTS assays.

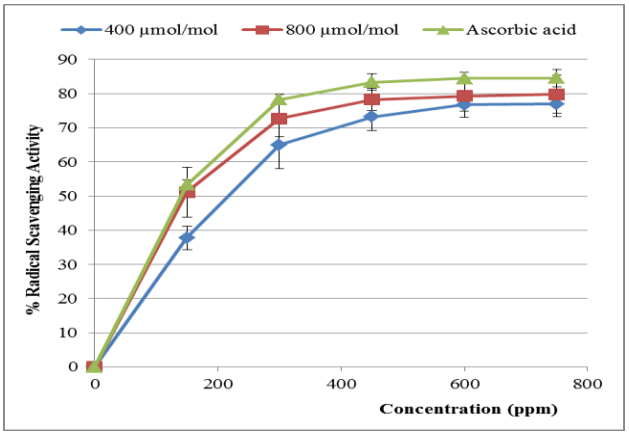


Figure 3 DPPH radical scavenging activities of UKMR-2 water extracts. Ascorbic acid was used as a reference standard. The data represent the mean of three replicates ± standard deviation

In this study, the Pearson correlation also showed that there was no correlation between total anthocyanins and total phenolics with antioxidant activity of UKMR-2 calyx in both CO_2 treatments. No correlation between antioxidant activity levels with TPC and TAC showed that there were other components besides phenolic compounds in calyx such as ascorbic acid and strong water-soluble antioxidants which were likely to contribute to its antioxidant activity [38, 39]. These findings were the same as Mohd-Esa *et al.*'s findings [40], which indicated no correlation between TPC and antioxidant activity in roselle water extract from Terengganu. In contrast, several studies have reported a strong correlation between antioxidant activities and phenolic contents in different roselle varieties [15, 41, 42]. They documented the antioxidant ability derived from the contribution of phenolic compounds in roselle calyx.

3.3 Chromatography Profiling

The influence of enhanced CO_2 on the phenolic profiles of UKMR-2 calyces was studied by HPLC-PDA analysis of water extracts. Phenolic compounds were identified and quantified based on their retention times, which were compared with the standard reference. The methodology employed enabled identification and quantification of five compounds as shown in Figure 4 and Table 2.

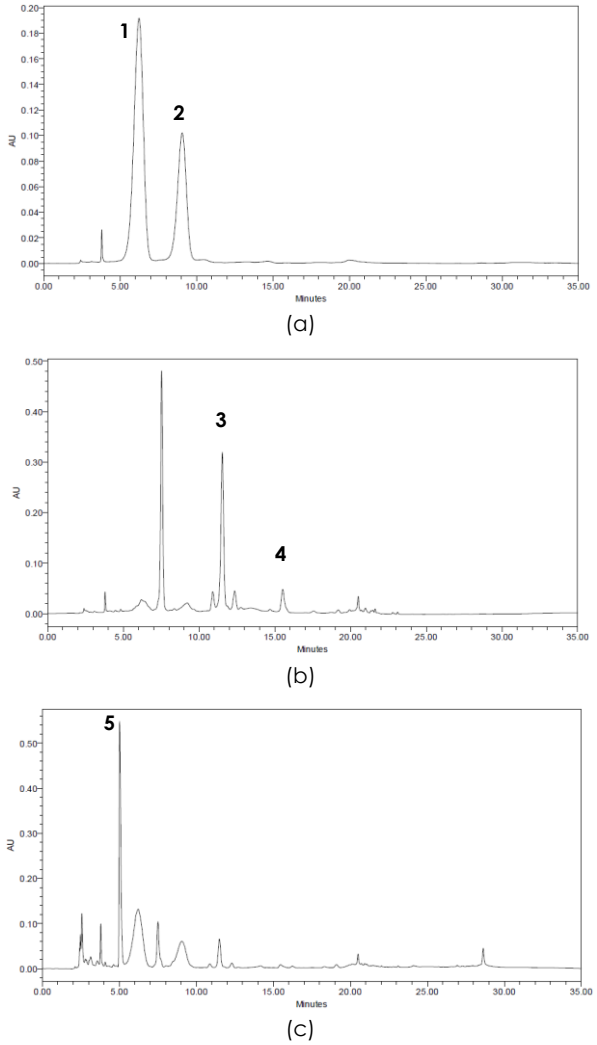


Figure 4 Representative HPLC-PDA profiles of phenolic constituents' in UKMR-2 calyx extracts at (a) 520 nm, b) 320 nm and (c) 265 nm

Two main anthocyanins were detected at 520 nm with a retention time of 6.30 minutes and 9.17 minutes. The peaks were identified as delphinidin-3-*O*-sambubioside (peak 1) and cyanidin-3-*O*-sambubioside (peak 2). At 320 nm, two peaks were identified as chlorogenic acid (peak 3) and caffeic

acid (peak 4) with retention times of 11.65 and 15.61 minutes, respectively. Meanwhile, at 265 nm, only ascorbic acid (peak 5) was identified with a retention time of 5.10 minutes. The presence of all compounds agreed with previous reports on other *H. sabdariffa* varieties worldwide [15, 19, 43, 44].

Table 2 Concentrations of phenolics and ascorbic acid detected in UKMR-2 calyx extracts

Peak	Compound	(mg/g DW)	
		Elevated [CO_2]	Ambient [CO_2]
1	Delphinidin 3-*O*-sambubioside	3.18 ± 0.62 [a]	2.42 ± 0.36 [b]
2	Cyanidin 3-*O*-sambubioside	1.39 ± 0.19 [a]	0.97 ± 0.13 [b]
3	Chlorogenic acid	0.63 ± 0.07 [a]	0.62 ± 0.11 [b]
4	Caffeic acid	0.20 ± 0.04 [a]	0.09 ± 0.02 [b]
5	Ascorbic acid	1.03 ± 0.52 [a]	5.91 ± 2.64 [b]

Values represent the mean of three replicates ± standard deviation. Mean denoted by different letters in the same row indicate significant differences ($p < 0.05$). DW, dry weight

The HPLC analysis demonstrated an overall increase for all compounds detected in UKMR-2 grown under elevated [CO_2] except for ascorbic acid. The amount of caffeic acid in elevated [CO_2] plants was two times higher than ambient [CO_2] although its presence was considered minute compared with others compound detected. However, Ifie *et al*. [45] reported that caffeic acid content in roselle which originated from Nigeria was much higher with 2.98 mg/g DW. Caffeic acid was mainly found in the form of ester in fruits, vegetables and herbs [46], and is a well-known antioxidant, which boosts immunity, controls lipid levels in blood and anti-mutagenic.

For anthocyanins, delphinidin-3-*O*-sambubioside concentration was higher compared to cyanidin-3-*O*-sambubioside for both treated plants. However, the results indicated that the content of anthocyanins for both treatments were lower than in previous findings. Kouakou *et al*. [15] reported that delphinidin-3-*O*-sambubioside and cyanidin-3-*O*-sambubioside contents in *H. sabdariffa* L. originated from Côte d'Ivoire is 21.38 mg/g DW and 17.11 mg/g DW, respectively. Therefore, the content and distribution of anthocyanins in roselle are believed to be influenced by the type of cultivation, environmental conditions, the degree of fruit maturation, postharvest storage condition, genetic factors, and the variety of the plant [47, 48]. It was observed that both treatment plants produced similar concentrations of chlorogenic acid with an overall range between 0.50 to 0.74 mg/g DW. On the contrary, Alarcon-Alonso *et al*. [49] reported that the amount of chlorogenic acid in roselle which originated from Mexico was much higher with 2.70 mg/g DW. Chlorogenic acid has a potential protective effect on human health, act as an antioxidant by scavenging radicals, and chelating metals [50, 51]. Thus, this compound could contribute significantly to the antioxidant activity of roselle calyces.

In contrast, low concentration of ascorbic acid was observed in elevated CO_2 treated plants. A remarkable amount of ascorbic acid was found in UKMR-2 from ambient [CO_2] condition, which was 5 times greater than UKMR-2 treated with elevated [CO_2]. The result is in agreement with Zhang *et al.* [52] and Wu *et al.* [53] on tomato and carrot. According to Wu *et al.* [53], the content of ascorbic acid in two carrot cultivars 'Kurodagosun' and 'Deep purple' in elevated [CO_2] was lower than ambient [CO_2]. Moreover, Zhang *et al.* [52] stated that elevated [CO_2] increased the synthesis of soluble sugar and total solid in tomato towards maturity development. Thus, it reduced the production of organic acids and ascorbic acid when it reached maturity. Generally, organic acids and ascorbic acid content elevated when the tomato reached maturity and then reduced when it exceeded its maturity [54, 55]. Ubiquitous of ascorbic acid content in fruits and vegetables can be influenced by various factors such as genotype variety, pre and post-harvest conditions, cultivation practices and fruit maturity [56, 57]. Although ascorbic acid is widely used in pharmaceutical and cosmetic industries, only a few studies have quantified the effect of elevated [CO_2] on the production of ascorbic acid in fruits.

In this study, interaction was observed between the CO_2 treatment and the detected compounds ($p < 0.05$). The present result suggests that elevated [CO_2] is able to enhance the production of anthocyanins and phenolic acids in UKMR-2 calyces.

4.0 CONCLUSION

In summary, our findings showed that elevated [CO_2] on UKMR-2 cultivation significantly affected the phenolic constituents and antioxidant activity ($p < 0.05$) except for plant growth. UKMR-2 growth under elevated [CO_2] produced greater plant height and higher fresh yields than ambient [CO_2]. UKMR-2 treated with elevated [CO_2] also had higher concentrations of TAC, TPC, and antioxidant activity. Therefore, elevated CO_2 treatment with approximately 800 µmol/mol may have significant effects on UKMR-2 cultivation into producing higher yields and higher phenolic constituents.

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