Combined effect of CO₂ enrichment and foliar application of salicylic acid on the production and antioxidant activities of anthocyanin, flavonoids and isoflavonoids from ginger

Ali Ghasemzadeh*, Hawa ZE Jaafar*, Ehsan Karimi and Mohd Hafiz Ibrahim

Abstract

Background: The increase in atmospheric CO₂ concentration caused by climate change and agricultural practices is likely to affect biota by producing changes in plant growth, allocation and chemical composition. This study was conducted to evaluate the combined effect of the application of salicylic acid (SA, at two levels: 0 and 10⁻³ M) and CO₂ enrichment (at two levels: 400 and 800 μmol·mol⁻¹) on the production and antioxidant activities of anthocyanin, flavonoids and isoflavonoids from two Malaysian ginger varieties, namely Halia Bentong and Halia Bara.

Methods: High-performance liquid chromatography (HPLC) with photodiode array detection and mass spectrometry was employed to identify and quantify the flavonoids and anthocyanins in the ginger extracts. The antioxidant activity of the leaf extracts was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and thiobarbituric acid (TBA) assays. The substrate specificity of chalcone synthase, the key enzyme for flavonoid biosynthesis, was investigated using the chalcone synthase (CHS) assay.

Results: CO₂ levels of 800 μmol·mol⁻¹ significantly increased anthocyanin, rutin, naringenin, myricetin, apigenin, fisetin and morin contents in ginger leaves. Meanwhile, the combined effect of SA and CO₂ enrichment enhanced anthocyanin and flavonoid production compared with single treatment effects. High anthocyanin content was observed in H Bara leaves treated with elevated CO₂ and SA. The highest chalcone synthase (CHS) activity was observed in plants treated with SA and kept under ambient CO₂ conditions showed the lowest CHS activity. The highest free radical scavenging activity corresponded to H Bara treated with SA under high CO₂ conditions, while the lowest activity corresponded to H Bentong without SA treatment and under atmospheric CO₂ levels. As the level of CO₂ increased, the DPPH activity increased. Higher TBA activity was also recorded in the extracts of H Bara treated with SA and grown under high CO₂ conditions.

Conclusions: The biological activities of both ginger varieties were enhanced when the plants were treated with SA and grown under elevated CO₂ concentration. The increase in the production of anthocyanin and flavonoids in plants treated with SA could be attributed to the increase in CHS activity under high CO₂ levels.

Keywords: CO₂ enrichment, Salicylic acid, Chalcone synthase, Flavonoids, DPPH activity, Ginger

*Correspondence: upmali@yahoo.com; Hawazej@gmail.com
Department of Crop Science, Faculty of Agriculture, University Putra Malaysia, 43400 University Putra Malaysia (UPM), Serdang, Selangor, Malaysia

© 2012 Ghasemzadeh et al; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
Background
Phytochemicals and antioxidants from plant sources are of increasing interest to consumers because of their roles in the maintenance of human health. Plants are a rich source of various phytochemicals, proteins, enzymes and other products of immense biotechnological value. Most of the secondary metabolites of herbs and spices are commercially important and are used in a number of pharmaceutical products. Flavonoids are the most important group of secondary metabolites and bioactive compounds in plants [1]. Flavonoids are known for their health-promoting properties, which include protective effects against cardiovascular disease, cancer and other diseases. They also have antioxidant properties, being capable of scavenging free superoxide radicals, as well as having anti-aging and anticancer activities [2]. It was found that flavonoids reduce blood lipid and glucose, and enhance human immunity [3]. The effect of flavonoids on human health is the result of their ability to induce human protective enzyme systems [4]. Several studies have suggested that flavonoids such as catechin and quercetin are able to control cancer cell growth in the human body [5-7]. The flavonoid biosynthetic pathway starts with the condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA, yielding naringenin chalcone. This reaction is carried out by chalcone synthase (CHS). Chalcone is isomerised to a flavanone by the enzyme chalcone flavanone isomerase (CHI). From these central intermediates, the pathway diverges into several side branches, each resulting in a different class of flavonoids. Flavanone 3-hydroxylase (F3H) catalyses the stereospecific 3ß-hydroxylation of (2S)-flavanones to dihydroflavonols. In the biosynthesis of anthocyanins, dihydroflavonol reductase (DFR) catalyses the reduction of dihydroflavonols to flavan-3,4-diols (leucoanthocyanins), which are converted to anthocyanidins by anthocyanidin synthase (ANS). The formation of glucosides is catalysed by UDP glucose-flavonoid 3-O-glucosyltransferase (UFGT), which stabilises the anthocyanidins by 3-O-glycosylation [8,9]. The overview of the flavonoid pathway is presented in Figure 1. There is evidence that the enzymes involved in the flavonoid metabolism act as membrane-associated multi-enzyme complexes, which has implications on the overall efficiency, specificity, and regulation of the pathway [10]. Anthocyanin is the water-soluble pigment which imparts the red, purple, and blue coloration to many fruits, vegetables, and cereal grains. This pigment is largely responsible for the colour characteristics of raw and processed products. Anthocyanin is frequently used as a food additive and it has been recognised that procyanidin has anti-carcinogenic and anti-oxidant activities [11].

Salicylic acid (SA) is a phenolic compound capable of enhancing plant growth and yield in some plants [12]. SA acts as a potential non-enzymatic antioxidant, as well as a plant growth regulator, and plays an important role in the defense mechanisms of plants against biotic and abiotic stresses.

![Flavonoid biosynthetic pathway](image_url)
role in regulating a number of plant physiological processes, including photosynthesis [13,14]. Previous reports showed that exogenous SA could ameliorate the damaging effects of heavy metals in rice [15], drought stress in wheat [14], and salt stress in wheat [12]. These observations suggested that SA could be linked to oxidative stress. Furthermore, other reports indicated that CO2 enrichment increased the production of secondary metabolites [16,17] and the antioxidant activity of plants [18]. Enrichment with high CO2 levels has been shown to enhance the medicinal properties of some plants, including *Labisia pumila* Blume (known in Malaysia as Kacip Fatimah) [19], oil palm [20], ginger [21], and strawberry [17]. According to the carbon-nutrient balance theory, as the carbon to nitrogen ratio increases under an elevated atmospheric CO2 environment, a greater amount of the plant's carbohydrates can be allocated to the plant's secondary metabolism, resulting in the production of greater amounts of carbon-based secondary metabolites [22]. Ginger (*Zingiber officinale* Roscoe) is a famous and widely used herb, especially in Asia, which contains several interesting bioactive constituents possessing health-promoting properties. In Malaysia, ginger has been used as a food and medicinal plant for over 2000 years to treat diabetes, high blood pressure, cancer and many other illnesses [23]. However, there has been little discussion about the combined effect of SA and CO2 enrichment on the production of secondary metabolites generated by the phenylpropanoid pathway and their antioxidant activity in plants.

Hence, the aim of the present study was to examine the combined effect of SA and CO2 enrichment on the production and antioxidant activity of anthocyanin, flavonoids, and isoflavonoids from two Malaysian ginger varieties. We also measured the CHS activity with salicylic acid stimulation.

### Methods

**Plant materials**

Rhizomes from two ginger varieties (Halia Bentong and Halia Bara) were collected from a village in Bentong, Pahang, Malaysia. Rhizomes were soaked in a Mancozeb solution for 30 min to give a final concentration of 0.3%. Pots filled with about 1 kg peat moss were prepared. Rhizomes were cut into 3–5 cm pieces containing 2 to 3 buds, and were planted 6 cm deep into the peat moss, with the buds facing upward. Rhizomes were grown in a glasshouse for two weeks. Afterward, when the young leaves reached a height of 5 cm, seedlings were transplanted into polyethylene bags filled with a soilless mixture composed of burnt rice husk and coco peat (1:1).

### Extraction of anthocyanin and flavonoids

Approximately 1 g of plant powder was extracted with 5 mL of methanol containing 0.1% HCl (pH 2.8) at 4°C for 24 h in a dark room, vortexed every 6 h. The liquid was separated from the solid matrix by filtration through sheets of qualitative filter paper (Hangzhou Special Paper Industry, Zhejiang, China). The filtrate was further passed through 0.22 µm reinforced nylon membrane filters (Shanghai ANPEL, Shanghai, China) before HPLC analysis. Three replicates were performed for each sample.

### HPLC analysis of anthocyanin and flavonoids

Reversed-phase HPLC was used to assay flavonoid composition. The HPLC system (Agilent Technologies Inc., Palo Alto, CA) consisted of a Model 1100 pump equipped with a multi-solvent delivery system and an L-7400 ultraviolet (UV) detector. The analytical column was an ODS-80Ts QA C18 column (150 mm × 4.6 mm
After incubation, oxidation was terminated by adding 2 mL of TBA reagent (0.67% TBA). The mixture was heated at 80°C for 1 h and then cooled down in an ice bath for 10 min. A blank was prepared following the same procedure but without a test sample. The TBA-MA adduct formed was measured using a spectrophotometer at 532 nm. Known antioxidants (BHT and \( \alpha \)-tocopherol) were used as positive controls [27].

**Antioxidant activities**

**1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay**

Free radical scavenging activity was determined according to the method described by Mensor et al. [26]. A DPPH alcohol solution (3 mL) was added to 1 mL samples containing different concentrations of extracts originating from different ginger parts. Samples were first kept in a dark place at room temperature and their absorbance was read at 518 nm after 30 min. The antiradical activity was determined using the formula below:

\[
\text{Percent} \% \text{ inhibition of DPPH activity} = \left( \frac{(A_0 - A_1)}{A_0} \right) \times 100 \%
\]

Where \( A_0 \) is the absorbance value of the blank sample or control reaction, and \( A_1 \) is the absorbance value of the test sample. The optic densities of the samples, controls and blanks were measured in comparison with ethanol. BHT (butylhydroxytoluene) and \( \alpha \)-tocopherol were used as positive controls. The parameter IC\(_{50}\) was calculated graphically. A lower IC\(_{50}\) value indicates greater antioxidant activity.

**Thiobarbituric acid (TBA) assay**

Various concentrations of test samples (10–500 \( \mu g \cdot mL^{-1} \)) were added to an aqueous solution (2 mL) containing 200 \( \mu L \) of Tris buffer (pH 7.4), 300 \( \mu L \) of 1 M KCl, 400 \( \mu L \) of 1% SDS (sodium dodecyl sulfate), 10 \( \mu L \) of linolenic acid, 40 \( \mu L \) of 1.0 mM FeCl\(_2\), and 20 \( \mu L \) of 0.5 mM \( \text{H}_2\text{O}_2 \) in a brown non-transparent vial (to avoid any oxidation caused by UV irradiation). The sample vial was then incubated for 18 h at 37°C while being shaken. After incubation, oxidation was terminated by adding 50 \( \mu L \) of 4% solution of BHT in ethanol, followed by the addition of 2 mL of TBA reagent (0.67% TBA). The mixture was heated at 80°C for 1 h and then cooled down in an ice bath for 10 min. A blank was prepared following the same procedure but without a test sample. The TBA-MA adduct formed was measured using a spectrophotometer at 532 nm. Known antioxidants (BHT and \( \alpha \)-tocopherol) were used as positive controls [27].

**Chalcone synthase (CHS) activity**

CHS activity was assayed spectrophotometrically, as described by Obinata et al. [28]. Enzyme was extracted at 4°C by homogenising the harvested frozen cells (0.4 g) in 1 mL of 0.1 M borate buffer (pH 8.8) containing 1 mM 2-mercaptoethanol. The homogenates were treated with 0.1 g of Dowex l×4 for 10 min and the cell debris and resin were removed by centrifugation at 15000 rpm for 10 min. Dowex l×4 resin (0.2 g) was added to the supernatant and treated for another 20 min. Then, the resin was removed by centrifugation at 15000 rpm for 15 min. The resultant supernatant was used for the CHS assays. For this, 100 \( \mu L \) of enzyme extract was mixed with 1.89 mL of 50 mM Tris-HCl buffer, pH 7.6, containing 10 mM KCN. The enzyme reaction was allowed to proceed for 1 min at 30°C after adding 10 mg of chalcone in 10 \( \mu L \) ethylene glycol monomethyl ether. The activity was determined by measuring the absorbance at 370 nm.

**Statistical analysis**

All analytical values shown represent the means of three replicates. Data were analysed using analysis of variance by Statistical Analysis System (SAS 9.0). Mean separation test between treatments was performed using Duncan multiple range test and a \( P \)-value \( \leq 0.05 \) was regarded as significant.

**Results and discussion**

**HPLC analysis of anthocyanin, flavonoids and isoflavonoids**

Carbon dioxide levels had a significant \( (P \leq 0.01) \) impact on the production of anthocyanin and other flavonoids in both ginger varieties (Table 1). As \( \text{CO}_2 \) levels increased from 400 to 800 \( \mu \text{mol} \cdot \text{mol}^{-1} \), flavonoid production was enhanced. High \( \text{CO}_2 \) conditions significantly enhanced the anthocyanin, rutin, naringenin, myricetin, apigenin, fisetin, and morin contents in ginger leaves. Leaves from plants grown under ambient \( \text{CO}_2 \) conditions had the lowest content of these flavonoids. Plants treated with SA produced higher concentrations of anthocyanin and flavonoids compared with plants kept under high \( \text{CO}_2 \) concentration but without SA treatment. The combined effect of SA and \( \text{CO}_2 \) enrichment resulted in significant enhancement of anthocyanin and flavonoid production compared with the single individual treatments. High anthocyanin content was observed in H Benta leaves (0.355 mg.g\(^{-1}\) DW) treated with elevated \( \text{CO}_2 \) and \( 10^{-3} \) M SA. The lowest content of anthocyanin was observed in H Bentong (0.245 mg.g\(^{-1}\) DW) grown under ambient \( \text{CO}_2 \) with no SA treatment. An interesting finding was that plants treated with SA exhibited a lower content of apigenin compared...
with untreated plants. According to the results in Table 1, both ginger varieties treated with 10^{-3} M SA and kept under ambient and elevated CO2 conditions had significantly lower concentrations of apigenin. A high content of apigenin (0.644 mg·g^{-1} DW) was detected in H Bara without SA treatment and kept at 800 \mu mol·mol^{-1} CO2 while a low content (0.204 mg·g^{-1} DW) of this isoflavonoid was detected in H Bentong treated with 10^{-3} M SA and kept under ambient CO2 conditions (400 \mu mol·mol^{-1}CO2). A similar trend of increasing concentrations of flavonoids with increasing CO2 concentration was observed in Scutellaria species [29]. Wang et al. [17] reported that strawberry plants under CO2 enrichment conditions (950 \mu mol·mol^{-1}CO2) had a significantly increased \textit{p}-coumaroylglucose, dihydroflavonol, quercetin 3-glucoside, quercetin 3-glucuronide, and kaempferol 3-glucoside contents in fruit, as well as increased cyanidin 3-glucoside, pelargonidin 3-glucoside, and pelargonidin 3-glucoside succinate contents.

Fisetin is a rare yet well-known flavonoid compound in plants. Previous studies have shown that fisetin has anti-inflammatory [30,31], anti-carcinogenic [32] and strong antioxidant effects. Ginger leaves contained relatively high levels of this flavonoid. It was apparent that fisetin content could also be improved by increasing CO2 concentration coupled with SA treatment in both ginger varieties, and especially in H Bara (2.47 mg·g^{-1} DW increased to 3.23 mg·g^{-1} DW). Morin also belongs to the flavonol group, and acts as a chemopreventive agent \textit{in vitro} and \textit{in vivo} against oral carcinogenesis [33]. The importance of morin and related compounds as anti-tumour drugs has been widely recognised [34]. In comparison with old fustic (\textit{Chlorophora tinctoria}), osage orange (\textit{Maclura pomifera}), and almond (\textit{Prunus dulcis}) [35], the local ginger varieties showed good levels of morin when grown under 800 \mu mol·mol^{-1} CO2 levels coupled with 10^{-3} M SA treatment, indicating that the plant is naturally a good source of morin, although the content was variable. The content of morin in leaves decreased in both varieties with increasing CO2 concentration. However, a high content of morin was detected in H Bara (0.849 mg·g^{-1} DW) treated with SA under ambient CO2 condition. Thus, in ginger treated with SA under ambient CO2 conditions, the concentration of morin in leaves was enhanced, while under elevated CO2 levels, the concentration of this flavonoid decreased in both varieties (Table 1). Figure 2 shows the HPLC chromatogram of H Bara leaf extracts from plants treated with 10^{-3} M SA under elevated CO2 condition (800 \mu mol·mol^{-1} CO2).

### Table 1 The concentrations of anthocyanin and some flavonoid compounds in two varieties of ginger, treated with SA and grown under different CO2 concentrations (400 and 800 \mu mol·mol^{-1}CO2)

| Variety | CO2 (\mu mol·mol^{-1}) | SA (M) | Anthocyanin | Rutin | Naringenin | Myricetin | Apigenin | Fisetin | Morin |
|---------|------------------------|--------|-------------|-------|------------|-----------|----------|---------|-------|
| H.Bentong | 400 | 0 | 0.245±0.007 ^{3} | 0.68±0.023 ^{3} | 0.058±0.0014 ^{de} | 0.119±0.0016 ^{a} | 0.32±0.0148 ^{d} | 0.78±0.017 ^{b} | 0.545±0.007 ^{a} |
| | 800 | 0 | 0.297±0.0027 ^{de} | 0.924±0.0098 ^{b} | 0.093±0.0023 ^{cd} | 0.184±0.0014 ^{d} | 0.593±0.024 ^{ab} | 2.16±0.144 ^{d} | 0.503±0.027 ^{f} |
| H.Bara | 400 | 0 | 0.273±0.0035 ^{f} | 0.773±0.015 ^{c} | 0.039±0.0013 ^{f} | 0.127±0.0033 ^{e} | 0.571±0.0041 ^{b} | 1.44±0.063 ^{f} | 0.686±0.0035 ^{b} |
| | 800 | 0 | 0.325±0.0155 ^{bc} | 1.04±0.0551 ^{a} | 0.133±0.0056 ^{b} | 0.201±0.013 ^{d} | 0.644±0.0077 ^{a} | 2.47±0.021 ^{c} | 0.592±0.013 ^{d} |

All analyses are the mean of triplicate measurements ± standard deviation; Results expressed in mg g^{-1}DW; Means not sharing a common letter were significantly different at P ≤ 0.05.

### Chalcone synthase (CHS) activity

CHS activity was influenced by SA and CO2 concentrations (P ≤ 0.01; Figure 3). In both ginger varieties treated with SA, the highest CHS activity was consistently found in plants kept under 800 \mu mol·mol^{-1} CO2, with values ranging between 8.5 and 10.2 nkat-mg protein^{-1}. In contrast, plants subjected to the 400 \mu mol·mol^{-1} CO2 treatment had a CHS activity between 6.2 and 6.8 nkat-mg protein^{-1}. Plants kept under ambient CO2 conditions and not treated with SA showed the lowest CHS activity, with values between 4.7 and 5.4 nkat-mg protein^{-1}. The present study shows that CHS activity was enhanced with an increase in CO2 levels coupled with the application of SA. This is mainly because CHS is a precursor to flavonoid biosynthesis [36,37]. The increase in CHS activity is usually followed by an increase in the C/N ratio derived from the enhanced growth rate under elevated CO2. Recent studies have indicated that an increase in the C/N ratio in plants corresponded to an increase in the synthesis of secondary metabolites, especially flavonoids [20,38]. An increase in PAL activity (another enzyme involved in flavonoid synthesis) under high CO2 has also been observed in tobacco and \textit{Spergula avensis} [39-41].
Thus, the increase in the production of anthocyanin and flavonoids reported in the present work could be attributed to an increase in CHS activity under high CO₂ levels. Ozeki et al. [42] pointed out that changes in CHS activity, rather than PAL activity, were correlated with changes in anthocyanin accumulation under various culture conditions. CHS is the first enzyme to branch off from phenylpropanoid metabolism to flavonoid metabolism and is believed to be a key enzyme of this system [10]. These findings, together with evidence for channeling between CO₂ enrichment and CHS activity in the general phenylpropanoid pathway, indicate that the organisation of these systems is important for understanding how plant metabolism is regulated.
Antioxidant activity

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

The DPPH stable free radical method is an easy, rapid and sensitive way to evaluate the antioxidants that scavenge free radicals. As shown in Table 2, DPPH activities in ginger leaf were influenced by CO2 levels and SA treatment ($P \leq 0.01$). Generally, the highest DPPH activity was recorded in H Bara (70.27%) treated with SA and kept under 800 $\mu$mol·mol$^{-1}$ CO2 and the lowest activity was observed in H Bentong (38.29%) not treated with SA and kept under 400 $\mu$mol·mol$^{-1}$ CO2. As the levels of CO2 increased, the DPPH activities were enhanced. DPPH activities corresponding to elevated CO2 treatment (800 $\mu$mol·mol$^{-1}$ CO2) were in the range 61.88% to 70.22%. In contrast, DPPH activities corresponding to 400 $\mu$mol·mol$^{-1}$ CO2 treatment ranged from 38.29% to 45.35%, suggesting that elevated CO2 conditions were able to enhance the antioxidant properties of the ginger leaves. It was interesting that the antioxidant activity in CO2-treated plants increased significantly when $10^{-3}$ M SA was applied. The increase in the DPPH activity of H Bentong and H Bara might be due to the high anthocyanin and flavonoid content in these varieties. Significant positive correlations between DPPH activity and flavonoids ($P \leq 0.01$) were observed, implying that the enhanced antioxidant activity detected in plants kept under high levels of CO2 coupled with SA treatment could be related to the increased hydrogen donating abilities of the plants [43,44].

For both ambient and elevated CO2 conditions, ginger leaves treated with $10^{-3}$ M SA exhibited higher radical scavenging activity than leaves from untreated plants (Table 2). For H Bara kept under 400 $\mu$mol·mol$^{-1}$ CO2 treatment, the antioxidant activity was enhanced by about 28.8% for plants treated with SA. The IC$_{50}$ (fifty percent free radical scavenging) value changed significantly after SA treatment. In both varieties, the IC$_{50}$ in SA-treated gingers was observed to be lower compared with untreated ginger. Also, the IC$_{50}$ was observed to be lower in CO2-enriched plants. The H Bara variety treated with SA and grown under elevated CO2 concentration (800 $\mu$mol·mol$^{-1}$ CO2) exhibited a lower IC$_{50}$ value (21.46 $\mu$g·ml$^{-1}$).

The DPPH values of leaf extracts of both ginger varieties treated with SA and grown under two different CO2 concentrations (400 and 800 $\mu$mol·mol$^{-1}$ CO2) were significantly lower than those of $\alpha$-tocopherol (93.47%) and BHT (89.27%) (Table 2). It was evident that CO2 enrichment significantly enhanced flavonoid content in both ginger varieties, and the high flavonoid content was associated with high antioxidant activity.

Table 2 DPPH scavenging activities and IC$_{50}$ values of the methanolic extracts of two varieties of Zingiber officinale treated with SA and grown under different CO2 concentrations (ambient: 400 $\mu$mol·mol$^{-1}$ CO2 and elevated: 800 $\mu$mol·mol$^{-1}$ CO2)

| Variety | CO2 ($\mu$mol·mol$^{-1}$) | SA (M) | DPPH (%) | IC$_{50}$ ($\mu$g·g$^{-1}$) |
|---------|--------------------------|--------|----------|-----------------|
| H.Bentong | 400 | 0 | 38.29±0.55 $^a$ | 37.4 |
|          |     | $10^{-3}$ | 52.98±1.209 $^a$ | 35.8 |
|          | 800 | 0 | 61.88±0.636 $^d$ | 34.2 |
|          |     | $10^{-3}$ | 68.07±0.403 $^a$ | 30.55 |
| H.Bara   | 400 | 0 | 45.35±0.82 $^a$ | 28.2 |
|          |     | $10^{-3}$ | 63.7±2.52 $^cd$ | 25.6 |
|          | 800 | 0 | 65.91±0.19 $^bc$ | 23.71 |
|          |     | $10^{-3}$ | 70.27±0.431 $^a$ | 21.46 |
| Positive controls | $\alpha$-tocopherol | 93.47±0.77 | 16.2 |
|                 | BHT | 89.27±1.04 | 20.4 |

All analyses are the mean of triplicate measurements ± standard deviation; Means not sharing a common letter were significantly different at $P < 0.05$. 

Figure 3 CHS activity in two ginger varieties treated with SA and CO2 enrichment (SA1=non SA, SA2=$10^{-3}$ M SA, ambient=400 $\mu$mol·mol$^{-1}$ CO2, elevated=800 $\mu$mol·mol$^{-1}$ CO2). Error bars represent the standard error of means.
Our results agree with the findings of previous studies. A positive relationship between phenolics and flavonoids with free radical scavenging has been reported in previous studies [45-48]. Our results showed that DPPH activity had a significant positive correlation with most flavonoids ($P \leq 0.01$ and $P \leq 0.05$; Table 3).

**Thiobarbituric acid (TBA) assay**

In comparison to control plants, the extracts analysed showed strong antioxidant activity when treated with SA and CO2 enrichment (Figure 4). Higher TBA activity was recorded in extracts of H Bara (79.84%) treated with SA ($10^{-3}$ M) and grown under elevated CO2 conditions (800 $\mu$mol·mol$^{-1}$ CO2). The leaf extracts of H Bara and H Bentong treated with SA and exposed to elevated CO2 conditions were observed to have medium antioxidant activity (79.84 and 68.96%, respectively) compared with the positive controls $\alpha$-tocopherol (94.2%) and BHT (82.7%), while plants with no SA treatment and grown under ambient CO2 conditions showed the lowest antioxidant activity (51.04 and 50.1%, respectively). Under CO2-enriched conditions (800 $\mu$mol·mol$^{-1}$ CO2) the TBA activity of H Bara (70.5%) with no SA treatment was not significantly different from that of H Bentong (68.96%) treated with SA. Therefore, these results need to be interpreted with caution.

Wang et al. [17] reported that the free radical scavenging power of strawberry increased at elevated CO2 concentrations (950 $\mu$mol·mol$^{-1}$ CO2). Similarly, a high CO2 content may have enhanced the antioxidant activity of the ginger extracts. Correlation analyses showed that the increase in antioxidant activity might be up-regulated by an increase in the flavonoid and anthocyanin content of plants treated with SA under elevated CO2 (Table 3).

Furthermore, CHS showed a positive and significant correlation ($P \leq 0.05$) with anthocyanin, fisetin, morin and naringenin, although no significant correlation was observed between this enzyme and myricetin, apigenin and rutin. This study has shown that ginger has a remarkable free radical scavenging ability, and therefore

| Table 3 Correlation between measured parameters in two ginger varieties |
|---------------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
|                           | Anthocyanin | Fisetin | Morien | Myricetin | Apigenin | Rutin | Naringenin | CHS | DPPH | TBA |
|----------------------------|------------|---------|---------|-----------|------------|-------|------------|------|-------|-----|
| Anthocyanin                | 1          |         |         |           |            |       |            |      |       |     |
| Fisetin                    | 0.785**    | 1       |         |           |            |       |            |      |       |     |
| Morien                     | 0.473**    | 0.505*  | 1       |           |            |       |            |      |       |     |
| Myricetin                  | 0.334**    | 0.248** | 0.56*   | 1         |            |       |            |      |       |     |
| Apigenin                   | 0.577**    | 0.761** | 0.141** | 0.201**   | 1         |       |            |      |       |     |
| Rutin                      | 0.978**    | 0.833** | 0.394** | 0.19**    | 0.665**   | 1     |            |      |       |     |
| Naringenin                 | 0.58*      | 0.809** | 0.402** | 0.495**   | 0.446**   | 0.628** | 1         |      |       |     |
| CHS                        | 0.55*      | 0.587*  | 0.447** | 0.304**   | 0.134**   | 0.368** | 0.517*    | 1    |       |     |
| DPPH                       | 0.881**    | 0.886** | 0.525*  | 0.377**   | 0.636**   | 0.901** | 0.793*    | 0.441**| 1     |
| TBA                        | 0.642**    | 0.714** | 0.462** | 0.235**   | 0.635**   | 0.465** | 0.404**   | 0.276**| 0.401**| 1   |

n.s = non significant; * = significant at $p \leq 0.05$; ** = significant at $p \leq 0.01$.

Figure 4 TBA activity of ginger varieties treated with SA and CO2 enrichment (SA1=non SA, SA2=10$^{-3}$ M SA, ambient=400 $\mu$mol·mol$^{-1}$ CO2, elevated=800 $\mu$mol·mol$^{-1}$ CO2). Error bars represent standard error of means.
can be used as a radical inhibitor or scavenger, acting possibly as a primary antioxidant.

**Conclusion**

The results of this study suggest that rising atmospheric concentrations of carbon dioxide could have a major impact on the antioxidant capacity of ginger. Anthocyanin and flavonoid compounds are largely responsible for the antioxidant activity in plant tissues [25]. Anthocyanin is known to reduce the damage caused by free-radical activity, including low-density lipoprotein oxidation, platelet aggregation, and endothelium-dependent vasodilation of arteries. The anthocyanin and flavonoid contents of ginger treated with SA and grown under 800 μmol·mol⁻¹ CO₂ treatments were significantly higher than those of untreated plants grown under 800 μmol·mol⁻¹ CO₂ or treated with SA and grown under 400 μmol·mol⁻¹ CO₂. Increases in the C/N ratio of plants were an indication of increases in the synthesis of secondary metabolites, especially phenolics and flavonoids [49]. Our results also indicated that CHS activity in ginger can be enhanced by CO₂ enrichment in a controlled environment (CE). Thus, the production of anthocyanin, flavonoids and isoflavonoids in young ginger could also be increased. The increase in anthocyanin and flavonoids in elevated CO₂-treated gingers is associated with increased antioxidant capacity in both ginger varieties. The two ginger varieties contain flavonoids with potent antioxidant properties, and under CO₂ enrichment conditions, both the anthocyanin concentration and the CHS activity increased. The impact of cultural conditions and CO₂ concentration on bio-pharmaceutical production in herbs will enable the optimisation of herb chemistry. The composition of flavonoids will be an important consideration in the development of any CE production system for medicinal plants. The present findings have important implications for the development of ginger plantations in Malaysia.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

Study design and experimental work was by A Ghasemzadeh under the supervision of H Jaafar. The first draft of the paper was written by A Ghasemzadeh and reviewed by H Jaafar. E Karimi and M Ibrahim participated in extraction. All authors reviewed and approved the final version.

**Acknowledgements**

The authors are grateful to the Research Management Centre of University Putra Malaysia for financing this work.

Received: 19 July 2012 Accepted: 17 October 2012
Published: 23 November 2012

**References**

1. Kim DO, Jeong SW, Lee CY: Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. *Food Chem* 2003, 81:321–326.

2. Karimi E, Oskouei E, Hendra R, Jaafar HZ: Evaluation of Crocus sativus L. stigma phenolic and flavonoid compounds and its antioxidant activity. *Molecules* 2010, 15:6244–6256.

3. Atoei K, Mansouri A, Bosku G, Kefalas P: Tea and herbal infusions: their antioxidant activity and phenolic profile. *Food Chem* 2005, 89:21–36.

4. Larouette AG, Guillamon E, Villares A, Rostagno MA, Martinez JA: Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. *Infa Res* 2009, 58:537–552.

5. Shukla Y, Prasad S, Tripathi C, Singh M, George J, Kalta N: In vitro and in vivo modulation of testosterone mediated alterations in apoptosis related proteins by 6-gingerol. *Mol Nutr Food Res* 2007, 51:1492–1502.

6. Arts IC, Jacobs DRJ, Gross M, Harrak L, Folsom AR: Dietary catechins and cancer incidence among postmenopausal women: the Iowa Women’s Health Study (United States). *Cancer Cause Control* 2002, 13:373–382.

7. Davis W, Lamson MS, Matthew S, Brignall ND: Antioxidants and cancer III: quercetin. *Alter Med Rev* 2000, 5:196–208.

8. Harborne JB: The Flavonoids, Advances in Research since 1986. London: Chapman & Hall; 1994.

9. Bohm B: Introduction to Flavonoids. Singapore: Harwood Academic Publishers; 1998.

10. Winkel-Shirley B: Flavonoid biosynthesis. A colorful model for genetics. *biochemistry cell biology and biotechnology. Plant Physiol* 2001, 126:485–493.

11. Yi W, Fischer J, Akoh C: Study of antioxidant activities of muscadine grape phenolics in vitro. *J Agric Food Chem* 2005, 53:8804–8812.

12. Afan M, Atthar HR, Ashraf M: Does exogenous application of salicylic acid through the rooting medium modulate growth and photosynthetic capacity in two differently adapted spring wheat cultivars under salt stress? *J Plant Physiol* 2007, 6(4):685–694.

13. Fariduddin Q, Hayat S, Ahmad A: Salicylic acid influences net photosynthetic rate, carbohydrate efficiency, nitrate reductase activity and seed yield in *Bosvicka juncea*. *Photosynthetica* 2003, 41:281–284.

14. Waseem M, Atthar HR, Ashraf M: Effect of salicylic acid applied through rooting medium on drought tolerance of wheat. *Pak J Bot* 2006, 38:1127–1136.

15. Mishra A, Choudhuri MA: Effects of salicylic acid on heavy metal-induced membrane degradation mediated by lipoxigenase in rice. *Biopl Plant* 1999, 42:409–415.

16. Broadmeadow MJ, Jackson SB: Growth response of *Quercus petraea, Fraxinus excelsior and Pinus sylvestris* to elevated carbon dioxide, ozone and water supply. *New Phytol* 2000, 146:437–451.

17. Wang YSH, Bunce AJ, Maas LJ: Elevated carbon dioxide increases contents of antioxidant compounds in field-grown strawberries. *J Agric Food Chem* 2003, 51:4315–4320.

18. Mattson WJ, Julkunen-Tiitto R, Herrs DA: CO₂ enrichment and carbon partitioning to phenolics: Do plant responses accord better with the protein competition or the growth-differentiation balance models? *Oikos* 2005, 111:337–347.

19. Ibrahim MH, Jaafar HZ: Increased carbon dioxide concentration improves the antioxidative properties of the Malaysian herb *Kacip Fatmah (Labisia pumila Blume)*. *Molecules* 2011, 16:6086–6081.

20. Ibrahim MH, Jaafar HZ: Impact of elevated carbon dioxide on primary, secondary metabolites and antioxidant responses of *Elaeis guineensis Jacq. (oil palm)* seedlings. *Molecules* 2012, 17:5195–5211.

21. Ghasemzadeh A, Jaafar HZ: Effect of CO₂ enrichment on synthesis of some primary and secondary metabolites in ginger (*Zingiber officinale Roscoe*). *Int J Mol Sci* 2011, 12:1101–1114.

22. Tisserat B, Vaughn SF: Essential oils enhanced by ultra-high carbon dioxide levels from Lamiaceae species grown in vitro and in vivo. *Plant Cell Rep* 2001, 20:361–368.

23. Ghasemzadeh A, Jaafar HZ, Rahmat A: Synthesis of phenolics and flavonoids in ginger (*Zingiber officinale Roscoe*) and their effects on photosynthesis rate. *Int J Mol Sci* 2010, 11:4539–4555.

24. Jaafar HZ: Carbon dioxide enrichment technology for improved productivity under controlled environment system in the tropics. *Acta Hort* 2006, 742:353–363.

25. Wang H, Race EJ, Shrikhande AJ: Characterization of anthocyanins in grape juices by ion trap liquid chromatography–mass spectrometry. *J Agric Food Chem* 2003, 51:1839–1844.

26. Mensor LL, Menezes FS, Lettao GC, Reis AS, Santos TS, Coube CS: Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. *Phytother Res* 2007, 11:127–130.
Ghasemzadeh et al. BMC Complementary and Alternative Medicine 2012, 12:229
http://www.biomedcentral.com/1472-6882/12/229

27. Kosar M, Goeger F, Can Baser KH. In vitro antioxidant properties and phenolic composition of Salvia virgata Jacq. from Turkey. J Agric Food Chem 2008, 56:2369–2374.
28. Obinata N, Yamakawa T, Takamiya M, Tanaka N, Ishimaru K, Kodama T. Effects of salicylic acid on the production of procyanidin and anthocyanin in cultured grape cell. Plant Biotechnol 2003, 20:105–111.
29. Stutte GW, Eraso I. Carbon dioxide enrichment enhances growth and flavonoid content of two Scutellaria species. J Am Soc Hortic Sci 2008, 133:631–638.
30. Park HH, Lee S, Son HY, Park SB, Kim MS, Choi EJ, Singh TS, Ha JH, Lee MG, Kim JE, Hyun MC, Kwon TK, Kim YH, Kim SH. Flavonoids inhibit histamine release and expression of proinflammatory cytokines in mast cells. Arch Pharm Res 2008, 31:1393–1311.
31. Geraets L, Haegens A, Brauers K, Haydock JA, Vernooy JH, Wouters EF, Bast A, Hageman GJ. Inhibition of LPS-induced pulmonary inflammation by specific flavonoids. Biochim Biophys Acta 2009, 1825:598–603.
32. Lin DOY, Park JH. Induction of PS3 contributes to apoptosis of HCT-116 human colon cancer cells induced by the dietary compound fisetin. Am J Physiol-Gastro L 2000, 279:1060–1068.
33. Brown J, Prey J, Harrison PR. Enhanced sensitivity of human oral tumours to the flavonol, morin, during cancer progression: involvement of the Akt and stress kinase pathways. Carcinogenesis 2003, 24:171–177.
34. Song YM, Kang JW, Zhou J, Wang ZH, Lua XQ, Wang LF, Gao JZ. Study on the fluorescence spectra and electrochemical behavior of ZnL2 and morin with DNA. Spectrochim Acta A Mol Biomol Spectrosc 2000, 56:2491–2497.
35. Wijeratne SSK, Abou-Zaid MM, Shahidi F. Antioxidant polyphenols in almond and its coproducts. J Agr Food Chem 2006, 54:312–318.
36. Muzika RM. Terpenes and phenolics in response to nitrogen fertilization: A test of the carbon/nutrient balance hypothesis. Chemoecology 1993, 4:3–7.
37. Fajer ED, Bowers MD, Bazaaz FA. The effects of nutrients and enriched CO2 environment on the production of carbon based allelochemicals in Plantago: A test of the carbon/nutrient balance hypothesis. Am Nat 1992, 140:707–723.
38. Winger A, Purdy S, Maclean A, Pourtau N. The role of sugars in integrating environmental signals during the regulation of leaf senescence. New Phyto 2006, 161:781–789.
39. Hartley SE, Jones CG, Couper GC, Jones TH. Effects of salicylic acid on the production of procyanidin and anthocyanin in cultured grape cell. Plant Biotechnol 2003, 20:105–111.
40. Miliuskas G, Venskutonis PR, Beek TA. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. Food Chem 2004, 85:231–237.
41. Ghasemzadeh A, Jaafar HZE, Rahmat A. Identification and concentration of some flavonoid components in Malaysian young ginger (Zingiber officinale Roscoe) varieties by a high performance liquid chromatography method. Molecules 2010, 15:6231–6243.
42. Karimi E, Jaafar HZE, Ahmad S. Phytochemical analysis and antimicrobial activities of methanolic extracts of leaf, stem and root from different varieties of Labiisa pumila. Molecules 2011, 16:4438–4450.
43. Karimi E, Oskouieian E, Hendra R, Oskouieian A, Jaafar HZE. Phenolic compounds characterization and biological activities of Citrus aurantium. Molecules 2012, 17:1203–1218.
44. Karimi E, Jaafar HZE. HPLC and GC-MS determination of bioactive compounds in microwave obtained extracts of three varieties of Labisia pumila Benth. Molecules 2011, 16:6791–6805.
45. Ibrahim MH, Jaafar HZE. The relationship of nitrogen and C/N on secondary metabolites and antioxidant activities in three varieties of Malaysian Kacip Fatimah (Labisia pumila Blume). Molecules 2011, 16:5514–5526.

Cite this article as: Ghasemzadeh et al.: Combined effect of CO2 enrichment and foliar application of salicylic acid on the production and antioxidant activities of anthocyanin, flavonoids and isoflavonoids from ginger. BMC Complementary and Alternative Medicine 2012, 12:229.