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Elevated CO_2 induces a global metabolic change in basil (*Ocimum basilicum* L.) and peppermint (*Mentha piperita* L.) and improves their biological activity

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ABSTRACT

Many studies have discussed the influence of elevated carbon dioxide (eCO_2) on modeling and crop plants. However, much less effort has been dedicated to herbal plants. In this study, a robust monitoring for the levels of 94 primary and secondary metabolites and minerals in two medicinal herbs, basil (*Ocimum basilicum* L.) and peppermint (*Mentha piperita* L.), grwon under both ambient (aCO_2, 360 ppm) and eCO_2 (620 ppm) was performed. We also assessed how the changes in herbal tissue chemistry affected their biological activity. Elevated CO_2 significantly increased herbal biomass, improved the rates of photosynthesis and dark respiration, and altered the tissue chemistry. Principal Component Analysis of the full data set revealed that eCO_2 induced a global change in the metabolomes of the two plants. Moreover, Hierarchical Clustering Analyses showed quantitative differences in the metabolic profiles of the two plants and in their responsiveness to eCO_2. Out of 94 metabolites, 38 and 31 significantly increased in basil and peppermint, respectively, as affected by eCO_2. Regardless of the plant species, the levels of non-structural carbohydrates, fumarate, glutamine, glutathione, ascorbate, phylloquinone (vitamin K1), anthocyanins and a majority of flavonoids and minerals were significantly improved by eCO_2. However, some metabolites tended to show species specificity. Interestingly, eCO_2 caused enhancement in antioxidant, antiprotozoal, anti-bacterial and anticancer (against urinary bladder carcinoma; T24P) activities in both plants, which was consequent with improvement in the levels of antioxidant metabolites such as glutathione, ascorbate and flavonoids. Therefore, this study suggests that the metabolic changes triggered by eCO_2 in the target herbal plants improved their biological activities.

1. Introduction

Being sessile organisms, plants have evolved mechanisms to adjust their growth and development to the environmental conditions. Therefore, climate change will have a strong impact on growth and development of plants. One important climatic variable linked to global change is the increase in atmospheric CO_2 (Houghton et al., 2001). Due to anthropogenic activities, declining carbon sinks and natural global cycles, the concentration of atmospheric CO_2 is going to be doubled by the onset of the next century (IPCC, 2013). Many investigations have pointed to the fertilization effect of elevated CO_2 (eCO_2), within a physiological limit, on plant growth and productivity (Pérez-López et al., 2009; Azam et al., 2013; Vicente et al., 2016). More specifically, CO_2 and O_2 compete for ribulose-1,5-bisphosphate carboxylase/

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oxygenase (rubisco), and eCO_2 therefore suppresses the oxygenation reaction of rubisco, leading to more efficient C fixation and accumulation of non-structural carbohydrates (NSC) (Tuba et al., 2003; Pérez-López et al., 2009; Watanabe et al., 2014; Misra and Chen 2015; Vicente et al., 2016; Li et al., 2017). Such higher availability of NSC would account for higher energy production through an increased respiration rate (Wang and Curtis 2002; Li et al., 2013). These increases in respiration processes (*e.g.*, glycolysis, tricarboxylic acid (TCA) cycle and mitochondrial electron transport chain) were recorded at both the metabolic and gene levels (Leakey et al., 2009). The synchronous enhancement of photosynthesis and respiration provides the required intermediates and energy for the synthesis of a vast array of primary and secondary metabolites. In this context, there is much evidence for the close coordination between C and N metabolisms at both the biochemical and transcriptional levels (Nunes-Nesi et al., 2010; Vicente et al., 2017). Fatty acid biosynthesis is initiated by the ATP-dependent carboxylation of acetyl-CoA, mainly derived from the glycolytic pathway, to produce malonyl-CoA, which provides the carbon atoms required for fatty acid elongation (Brown et al., 2010). Moreover, the widely accepted growth-differentiation balance (GDB) and carbon-nutrient balance (CNB) hypotheses suggest that the excess availability of NSC are diverted for the synthesis of different classes of carbon-based secondary metabolites (Bryant et al., 1983; Herms and Mattson 1992). Moreover, eCO_2 has been found to increase the water use efficiency of plants through the reduction of transpiration rate and stomatal conductance (Teng et al., 2006; Li et al., 2017). Therefore, applying eCO_2 as a growth and metabolic improving factor and understanding its effect at the growth and metabolomics levels is a major concern.

In the past two decades, several metabolomic studies have been conducted to examine the effect of eCO_2 on growth and productivity as well as on the levels of primary and secondary metabolites in model and crop plants (Misra and Chen 2015; Noguchi et al., 2015). However, much less effort has been dedicated to medicinal and herbal plants, and the available literature mainly focus on the CO_2-induced changes in secondary metabolites and antioxidant properties of a few members of this important group of plants. In this regard, a significant improvement in net photosynthetic rate accompanied with reduction in stomatal conductance and transpiration rate was reported in leaves of *Isatis indigotica* as affected by eCO_2 (Li et al., 2017). Wang et al. (2003) reported an enhancement in the levels of anthocyanins, pelargonidin, quercetin, kaempferol, ascorbate, glutathione and total antioxidant capacity in fruits of strawberry grown under eCO_2. Similarly, CO_2 was reported to stimulate the accumulation of total soluble sugars, phenolics, flavonoids, glutathione and/or antioxidant potential of the Malaysian herb *Labisia pumila* (Ibrahim and Jaafar 2011; Jaafar et al., 2012). The impact of eCO_2 on the accumulation of individual flavonoids and antioxidant capacity was also assayed in ginger (Ghasemzadeh et al., 2010; Ghasemzadeh and Jaafar 2011). Therefore, more work is needed to actively investigate the influence of the progressively increased atmospheric CO_2 concentration on the biosynthesis of primary and secondary metabolites in medicinal and herbal plants and the triggered modifications in their biological activities.

With respect to their effectiveness, widespread availability and low cost, there is a growing demand for medicinal plants (Jusu and Sanchez 2013). Among the herbs commonly utilized in traditional medicine are the two lamiaceae members, basil (*Ocimum basilicum* L.) and peppermint (*Mentha piperita* L.). In addition to their culinary uses, these two medicinal plants are well recognized for their antioxidant, antimicrobial, anti-inflammatory, anti-allergenic and anticancer properties (Lv et al., 2012; Riachi and De Maria 2015; Szymanowska et al., 2015). The pharmaceutical potential of peppermint and basil is mainly ascribed to their vitamins and secondary metabolites such as terpenoids, phenolics, flavonoids and steroids (Figueroa Pérez et al., 2014; Ghasemzadeh et al., 2016). Thus, improving the levels of these bioactive phytochemicals could improve the medicinal and functional food values of basil and peppermint, and herbal plants in general. In this regard, eCO_2, by its impact on C assimilation and allocation, could modulate the entire metabolism of medicinal plants and consequently affect their biological activities. To the best of our knowledge, there is no metabolomic study available addressing the response of basil or peppermint to eCO_2. Previous studies have investigated the effect of environmental stresses, such as salinity and drought on the accumulation of phenolics, flavonoids, essential oils, osmorgulators and antioxidants in basil and peppermint (Figueroa-Pérez et al., 2014; Bekhradi et al., 2015; Çoban and Göktürk Baydar 2016; Ghasemi Pirbalouti et al., 2017). However, the current understanding of the underlying metabolic implications in these two medicinal herbs, and generally in medicinal plants, is still fragmentary. To understand how eCO_2 could improve target basil and peppermint growth and medicinal value, we employed a robust monitoring for the levels of 94 primary and secondary metabolites and mineral profile in their shoot tissues. Moreover, the impact of eCO_2 on antioxidant, antiprotozoal, antimicrobial and anticancer activities of these two medicinal herbs was assessed.

2. Materials and methods

2.1. Experimental setup, plant materials and growth conditions

Basil (*Ocimum basilicum* L.) and peppermint (*Mentha piperita* L.) seeds were stratified in darkness at 4 °C for 5 days, and then sown in potting mix (Tref EGO substrates, Moerdijk, The Netherlands, 15 × 15 cm pots), filled with a mixture (1:1, v/v) of a loamy soil and organic compost at a humidity of 0.30 g water/g dry soil. The soil nutrient composition was controlled to avoid any nutrient deficiency. The soil initially contained 1.2% carbon, 21 mg nitrate-nitrogen, 1.2 mg ammonium-nitrogen, 13 mg phosphorus and 0.8 g of potassium k^{-1} air dry soil. Pots were transferred to a controlled-growth cabinet under two CO_2 scenarios, viz: 1) ambient CO_2 (aCO_2) (360 ppm), 2) elevated CO_2 (eCO_2) (620 ppm). For each treatment, twenty pots, six plants each, were distributed in two growth chambers. Other growth conditions included: 150 µmol PAR m − 2 s − 1, 16/8 h day/night photoperiod, 21/18 °C air temperature and 60/70% humidity. All pots were watered equality to stabilize soil water content to 65%. Shoot materials were harvested after 4 weeks from the two treatments at the same time of the day and immediately frozen in liquid nitrogen. The experiment was replicated twice and similar results were obtained.

2.2. Measurement of stomatal conductance and photosynthesis and respiration rates

Light saturated photosynthetic and respiration rate (Asat, µmol $CO2\,m^{-2}\,s^{-1}$) were determined (LI-COR LI-6400, LI-COR Inc., Lincoln, NE, USA) on the youngest fully expanded leaves (Abdelgawad et al., 2015). A minimum of 5 min of leaf equilibration was set at each step before data were logged. LI-COR leaf chamber conditions were set at 360 or 620 ppm CO_2, and 22 °C (block temperature), according to the climate treatments, saturated photon flux density (1500 µmol $m^{-2}\,s^{-1}$). Dark respiration was measured as the absolute CO_2 exchange rate measured at photosynthetic photon flux density (µmol $m^{-2}\,s^{-1}$). Stomatal conductance (gs, mol $CO_2\,m^{-2}\,s^{-1}$) was measured on the abaxial side of fully developed leaves with a Leaf Porometer (Model SC-1, Decagon Devices, Inc., Hopkins, Pullman, WA USA). The average vapor pressure deficit and leaf temperature were 0.37 ± 0.02 and 20 ± 2.02, respectively.

2.3. Metabolite profiling

2.3.1. Quantitative estimation of individual sugars

High-performance liquid chromatography (HPLC) was employed to assess the concentration of individual sugars following the protocol described in Hamad et al. (2015). Briefly, dried plant samples were vigorously homogenized in 50% (v/v) acetonitrile. The mobile phase,

consisted of acetonitrile and HPLC-grade water (75:25 v/v), was applied at flaw rate of 1 ml min^{-1} and column temperature was adjusted at 30 °C. The existing sugars were identified by comparing their retention time with those of the standard mixture. Quantification was performed based on peak areas and comparison with a calibration curve of the corresponding standards.

2.3.2. Organic acids analysis

The quantitative determination of individual organic acids was performed according to Hamad et al. (2015). A known weight of dried plant samples was extracted in 0.1% phosphoric acid supplemented with butylated hydroxyanisole at the rate of 3 g/l. Organic acids were detected by HPLC using a SUPELCOGEL C-610H column coupled to UV detection system set at 210 nm (LaChrom L-7455 diode array, LaChrom, Tokyo, Japan). The mobile phase, phosphoric acid (0.1% v/v), was applied at a flow rate of 0.45 ml min^{-1}. The concentrations of each organic acids was calculated using a calibration curve of a corresponding standard.

2.3.3. Amino acids quantification

Plant samples were extracted in 80% aqueous ethanol using a MagNA Lyser (Roche, Vilvoorde, Belgium) (Sinha et al., 2013). After centrifugation at 14,000 rpm for 20 min, the clear supernatant was evaporated under vacuum to dryness, and the pellet was re-suspended in 5 ml chloroform (E1). Immediately, the residue was re-extracted with HPLC grade deionized water and centrifuged again, afterthought the supernatant was re-mixed with E1 and centrifuged for 10 min. The aqueous phase was isolated and filtered using Millipore micro filters (0.2 µM pore size). The amino acids were quantified using a Waters Acquity UPLC-tqd system (Milford, Worcester County MA, USA) equipped with a BEH amide 2.1 × 50 column.

2.3.4. Analysis of fatty acid

Fatty acids were quantified using gas chromatography following the methods described by Torras-Claveria et al. (2014). Briefly, 0.2 g dried plant samples were used to obtain lipophilic fractions via extraction in 50% aqueous methanol at 25 °C until tissue discoloration. Nonadecanoic acid was used as an internal standard. GC/MS analysis was carried out on a Hewlett Packard 6890, MSD 5975 mass spectrometer (Hewlett Packard, Palo Alto, CA, USA), with an HP-5 MS column (30 m × 0.25 mm × 0.25 mm). Fatty acids were identified using NIST 05 database and Golm Metabolome Database (http://gmd.mpimp-golm.mpg.de).

2.3.5. Phenolics and flavonoids determination

The protocol described in Hamad et al. (2015) was followed for quantification of individual phenolics and flavonoids. The plant sample was extracted in acetone-water solution (4:1 v/v) at room temperature for 24 h on orbital shaker. The extract was filtered, centrifuged and the resulted supernatant was evaporated under vacuum, afterthought the residue was resuspended in methanol (HPLC grade). Phenolic compounds were quantified using a Shimadzu HPLC system (SCL-10 A vp, Shimadzu Corporation, Kyoto, Japan), and consisted of a diode-array detector and a Lichrosorb Si-60, 7 µm, 3 × 150 mm column. The mobile phase composed of water-formic acid (90:10, v/v); and acetonitrile/water/formic acid (85:10:5, v/v/v). The concentration of each compound was calculated using a calibration curve of a corresponding standard.

2.3.6. Quantitative analysis of vitamins

Reversed phase HPLC was employed for determination of ascorbate and glutathione content following the method of Potters et al. (2004). Ascorbate and glutathione were separated on Polaris C18-A column (100 × 4.6 mm, 3 µm particle size; 40 °C). The components were detected using a custom-made electrochemical detector and in-line DAD (SPD-M10AVP, Shimadzu). Total ascorbate and glutathione concentration was determined after reduction with DTT (0.04 M) at room temperature for 10 min. For extraction of tocopherols, a known weight of the dried plant samples was homogenized in hexane (Siebert 1999). After centrifugation, the supernatant was concentrated under vacuum (CentriVap concentrator, Labconco, KS, USA) and the residue was resuspended in hexane. Separation and quantification of tocopherols were conducted by HPLC (Shimadzu, Hertogenbosch, Netherlands) using normal phase conditions (Particil Pac 5 µm column material, length 250 mm, i.d. 4.6 mm). 5,7-dimethyltocol (DMT; 5 ppm) was used as an internal standard. Carotene content in the samples was analyzed by using an autosampler (HPLC, Shimadzu SIL10-ADvp). The separation of the carotenoids is a reversed phase (a silica-based C18 column). The mobile phase consists of solvent A (acetonitrile:methanol:water; 81:9:10) and solvent B (methanol:ethyl acetate 68:32). The solvent flow rate is 1.2 ml/min (approx.70 bar) and the injection volume of the sample was 20 µl. The detection of the carotenoids was done by a diode array detector (Shimadzu SPD-M10Avp) at four different wavelengths (420, 440, 462, 660 nm) and integrated via the software program (Shimadzu Lab Solutions Lite) in which the concentration is determined using a calibration curve. Phylloquinone were detected by HPLC (reversed phase RP18 column, Eurosphos-100, 250 × 4.6 mm, Germany) according to the method outlined by Jakob and Elmadfa (1996). The eluent consisted of 90% methanol and 10% dichloromethane and supplemented with 5 ml of a methanolic solution containing $ZnCl_2$ (1.37 g), sodium acetate (0.41 g), acetic acid (0.30 g) per liter of mobile phase. Phylloquinone was detected by fluorescence detector (excitation, 243 nm; emission, 430 nm) and menaquinone-4 was used as internal standard.

2.3.7. Colorimetric determination of total sugars, phenolics and flavonoids

Soluble, insoluble and total sugars were extracted according to the method mentioned in Saleh et al. (2015) and determined by the Nelson's colorimetric method adopted by Clark and Switzer (1977). For extraction of total phenolics and flavonoids, a known weight of dried plant material was homogenized in 80% aqueous ethanol followed by centrifugation at 5000 rpm for 15 min. After that, the clear supernatant was used directly for determination of total flavonoids or subjected to acid hydrolysis before quantification of total phenolics (aglycones and glycosides). Folin–Ciocalteu assay was employed to determine total phenolic content, whereas gallic acid was used to set up the standard curve (Saleh et al., 2015). The modified aluminum chloride colorimetric method described in Hamad et al. (2015) was followed to assess the total flavonoid content using quercetin as a standard.

2.4. Determination of minerals contents

For extraction of inorganic nutrients, a known weight of dried plant tissue was digested in a HNO_3/H_2O solution (5:1 v/v) in an oven (Agusa et al., 2005). Standards were also prepared in 1% nitric acid. Thereafter the concentrations of macro-minerals and trace elements were determined (ICP-MS, Finnigan Element XR, Scientific, Bremen, Germany).

2.5. Assessment of biological activities

2.5.1. In vitro antioxidant activity

The widely accepted DPPH and FRAP methods were used to determine the free radical scavenging activity of plant extracts. Plant extracts were prepared by grinding 300 mg of shoot tissues in 3 ml of 80% ice-cold ethanol. For DPPH assay each plant extract was mixed with 0.5 ml of DPPH solution (0.25 mM in 95% ethanol) (Hamad et al., 2015). After shaking, the mixture was allowed to stand at room temperature for 30 min. Thereafter, 2 ml of dd H_2O was added and the absorbance of the mixture was measured at 517 nm to calculate the inhibition percentage. For FRAP assay, 180 µl of freshly prepared prewarmed FRAP reagent, was dispensed to each well of the micro-titration plate with 20 µl diluted ethanol extracts. After 30 min of incubation

at 37 °C the absorbance was measured at 593 nm using a micro-plate reader (Synergy Mx, Biotek Instruments Inc., Vermont, VT, USA) (Abdelgawad et al., 2015).

2.5.2. Antibacterial assay

The disc diffusion method was used to determine the antibacterial activity of plant extracts using 100 µl of suspension containing 10^8 cfu/ml of bacteria spread on Muller Hinton agar (MHA) (Selim et al., 2013). Sterilized dried ethanol plant extract were loaded on filter paper discs to obtain final concentration of 7.5 mg/disc. Loaded filter paper discs were placed on the top of Mueller-Hilton agar plates, plated then incubated at 35 °C for 24 h to allow the diffusion of herbal plant extracts. The presence of inhibition zones were measured by Vernier caliper, recorded and considered as indication for antibacterial activity. Negative controls were prepared using the same solvent employed to dissolve extract. After incubation for 48 h at 37 °C, the diameter of inhibition zones was recorded.

2.5.3. Antiprotozoal activity

The methods outlined by Räz et al. (1997) were used to assess *in vitro* the antiprotozoal activity of the plant extracts (5 mg/ml) against *Trypanosoma cruzi*. Viability measurements of trypomastigotes of *T. cruzi* (Tulahuen C4) were conducted using rat skeletal myoblasts (L-6 cells) that were seeded in the microtiter plates using chlorophenol red-β-D-galactopyranoside (CPRG)-Nonidet as substrate. The microtiter plates were incubated at 37 °C under a 5% CO_2 environment for 4 days and the developed color reaction was read photometrically at 540 nm. The results were expressed as the percentage reduction in parasite load compared to control.

2.5.4. Anticancer activity

The human cell lines, hepatocellular carcinoma (HepG2), colon carcinoma (Colo205), embryonic kidney adenocarcinoma (293) and urinary bladder carcinoma (T24P) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (10% fetal calf serum, Na-pyruvate, 1 mg/0.1 ml streptomycin, 1U/0.1 ml penicillin) with and without the plant extracts (2 mg/ml). The cell cultures were incubated at 37 °C and 5% CO_2 until a confluent growth was achieved. Then, cells were collected by trypsinization trypsin solution (0.25% trypsin w/v) and the vital cells determined by CellTiter-Blue reagent (Promega, Madison, WI, USA) as described by Solowey et al. (2014).

2.6. Statistical analyses

Data analyses were performed using the procedures provided in Statistical Analysis System (SPSS Inc., Chicago, IL, USA). Data normality and the homogeneity of variances were checked using the KolmogoroveSmirnov test and Levene's test, respectively. All the data were subjected to one-way Analysis of Variance (ANOVA). Tukey's Test ($p < 0.05$) was carried out as the post-hoc test for mean separations. The number of replicates for each experiment was three ($n = 3$). OriginLab software (9, OriginLab, Northampton, MA, USA) was used for performing Principal Component Analysis (PCA) of the full dataset. Cluster analysis was performed by using Pearson distance metric of the MultiExperiment Viewer (MeV)™ 4 software package (version 4.5, Dana-Farber Cancer Institute, Boston, MA, USA).

3. Results

3.1. Elevated CO_2 improves growth, photosynthesis and respiration of basil and peppermint

The data presented in Table 1 show that basil and peppermint have comparable fresh masses, stomatal conductance, and photosynthesis and respiration rates. Elevated CO_2 improved the biomass production in basil and peppermint by about 40 and 48%, respectively. The photosynthetic rate was significantly improved in both plants. However, the increase was more pronounced in basil. Moreover, eCO_2 significantly improved the respiratory rate in both medicinal herbs by about 80%. On the other hand, no significant impact of eCO_2 was observed on stomatal conductance in basil or peppermint.

3.2. CO_2 enrichment induces changes in the primary metabolome of basil and peppermint

Hierarchical clustering of primary metabolites revealed variations in their relative levels in basil and peppermint and in their responsiveness to eCO_2 (Fig. 1a). Primary metabolites could be grouped into four major groups including: those are higher in peppermint under both aCO_2 and eCO_2 conditions and unaffected by eCO_2 (group1); those higher in basil under aCO_2 conditions but increased in peppermint and mainly decreased in basil in response to eCO_2 (group 2); those higher in basil under both aCO_2 and eCO_2 conditions and mainly improved in basil and slightly increased in peppermint as affected by eCO_2 (group 3); and those comparable in the two plants and accumulated under eCO_2 (group 4). Sugars and TCA intermediates were mainly confined to groups 3 and 4 and one-half of the fatty acids clustered within group 4, while amino acids were distributed all over the groups. This variability in response of primary metabolites suggests that the effect imposed by eCO_2 was dependent on the plant species, metabolite type and the cellular activity involved.

3.2.1. Elevated CO_2 improves the accumulation of photosynthetic products and induces its metabolism

Glucose, fructose, sucrose starch and total sugars and some TCA organic acids were analyzed in shoot tissues of basil and peppermint grown in a CO_2 enriched environment (Table 2). Basil shoot contained higher levels of all the analyzed sugars as compared with peppermint. However, the two plants possessed comparable amounts of organic acids, except for malic acid, which was higher in basil, while citric acid was higher in peppermint. In both plants, starch was the most dominant NSC, while malic acid was the abundant organic acid. CO_2 enrichment had enhanced the accumulation of glucose, fructose, sucrose, starch and their totals in both plants by about 40%, as compared with their respective control. On the other hand, levels of all the measured organic acids, excepting fumarate, were not affected by eCO_2.

3.2.2. Individual amino acids in basil and peppermint respond differently to eCO_2

Amino acid profiles of basil and peppermint under aCO_2 were qualitatively similar but quantitatively different (Table 2). Among the detected amino acids, seven were higher in basil, including glutamine, histidine, arginine, ornithine, leucine, methionine, and glycine, but three amino acids were higher in peppermint: alanine, asparagine, and threonine. Glutamine was the predominant amino acid in both plants, representing about two thirds of the total amino acids content. The level of glutamine was significantly improved in both plants in response to eCO_2, as compared with their respective controls. It is notable that eCO_2 significantly induced the accumulation of ornithine and leucine in basil, and that of asparagine, proline and cystine in peppermint as compared with aCO_2 grown plants. The level of total proteins was significantly enhanced in peppermint but not in basil, as affected by eCO_2.

3.2.3. CO_2 enrichment affects fatty acids accumulation in a species dependent manner

Twelve saturated and eight unsaturated fatty acids were detected in both basil and peppermint, as revealed by GCMS analysis (Table 3). Under aCO_2, the levels of individual fatty acids were statistically similar in both plants, except for dodecanoic (C12:0), pentacosanoic (C25:0) and eicosadienoic (C20:2), which were significantly higher in peppermint. The effect of CO_2 enrichment on fatty acid accumulation was

Table 1
Fresh mass (g/shoot), rates of photosynthesis and respiration (μmol CO_2 m^{-2} s^{-1}), stomatal conductance (mol m^{-2} s^{-1}) and levels of non-structural carbohydrates, total carbohydrates and TCA organic acids (mg g^{-1} dry weight) in basil and peppermint grown under ambient (aCO_2) and elevated (eCO_2). Values are mean ± standard error of three independent replicates. Means followed by the same lower-case letter in a row do not differ significantly at the 0.05 probability level.

	Basil		*Peppermint*	
	aCO_2	eCO_2	aCO_2	eCO_2
Fresh mass	5.12 ± 0.12a	7.89 ± 0.38b	4.41 ± 0.17a	6.39 ± 0.34b
Photosynthesis	7.87 ± 1.53a	14.27 ± 3.11b	8.73 ± 2.31a	14.93 ± 2.64b
Stomatal conductance	0.150 ± 0.033a	0.142 ± 0.038a	0.183 ± 0.058a	0.178 ± 0.046a
Respiration	0.050 ± 0.016a	0.096 ± 0.018b	0.046 ± 0.015a	0.083 ± 0.010b
Sugars				
Glucose	0.85 ± 0.00c	1.14 ± 0.03d	0.41 ± 0.01a	0.55 ± 0.02b
Fructose	1.01 ± 0.03c	1.36 ± 0.05d	0.56 ± 0.00a	0.74 ± 0.02b
Sucrose	0.29 ± 0.00c	0.37 ± 0.01d	0.18 ± 0.00a	0.23 ± 0.01b
Soluble sugars	2.47 ± 0.00c	3.29 ± 0.11d	1.30 ± 0.01a	1.78 ± 0.05b
Starch	26.16 ± 0.03b	36.18 ± 2.13c	21.68 ± 0.01a	29.97 ± 1.75b
Total carbohydrates	48.56 ± 0.13b	67.44 ± 3.21c	38.87 ± 0.03a	54.68 ± 2.60b
Organic acids				
Oxalic acid	1.08 ± 0.06a	1.38 ± 0.21a	1.05 ± 0.06a	1.26 ± 0.12a
Malic	10.08 ± 0.61b	11.89 ± 0.66b	4.95 ± 0.50a	5.41 ± 0.62a
Succinic	1.04 ± 0.05ab	1.37 ± 0.28b	0.61 ± 0.07a	0.64 ± 0.03a
Citric	0.53 ± 0.03a	0.45 ± 0.22a	1.22 ± 0.15b	1.03 ± 0.14b
Fumaric	0.15 ± 0.01a	1.73 ± 0.54b	0.09 ± 0.01a	3.05 ± 0.93b
α-keto glutaric acid	0.32 ± 0.04a	0.45 ± 0.03ab	0.36 ± 0.02ab	0.56 ± 0.06b

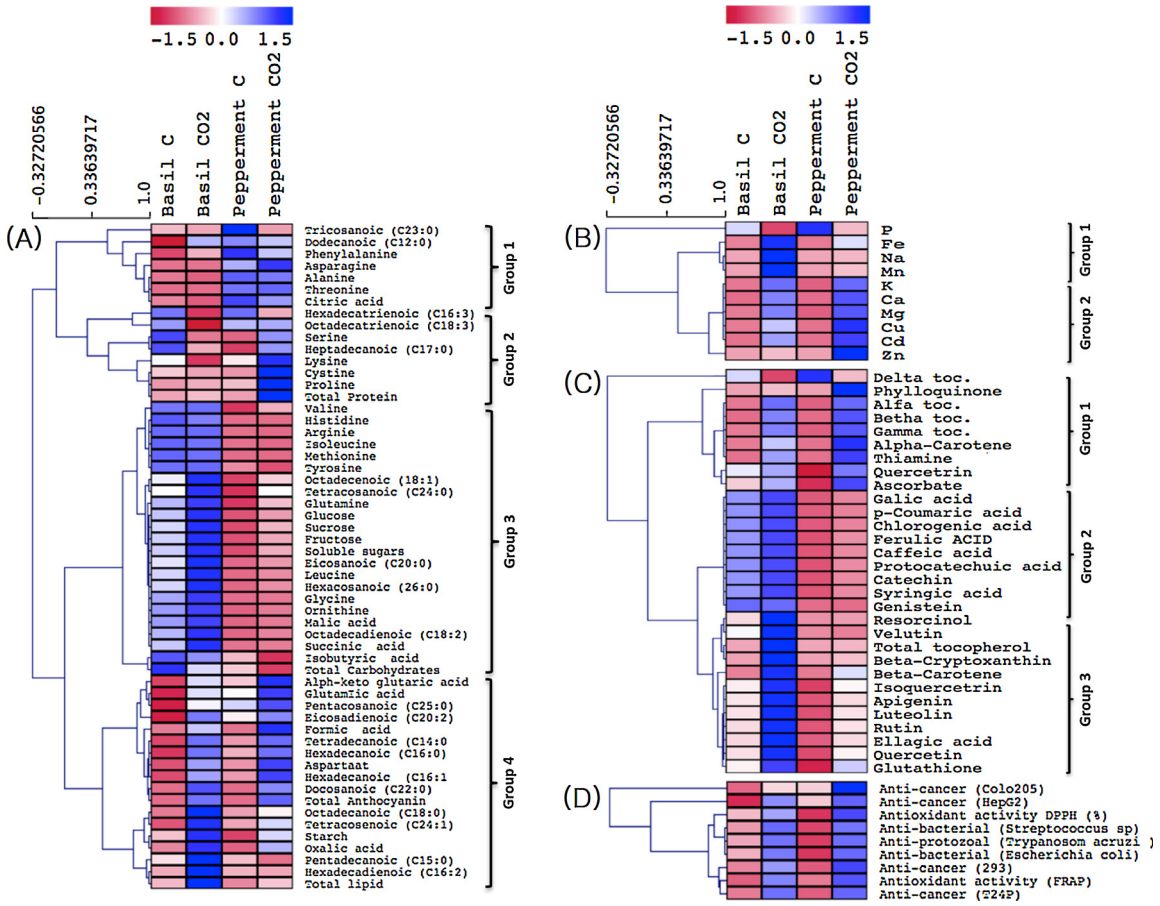

Fig. 1. Heatmaps for the levels of primary metabolites (A), minerals (B) antioxidant metabolites (C) and biological activities (D) in basil and peppermint grown under aCO_2 or eCO_2. The relative accumulation patterns are shown in the heatmap based on the average value (n = 3) for each metabolite. Red and blue colors indicate lower and higher concentrations, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

more obvious in basil than peppermint. Among the twenty detected fatty acids, six were significantly accumulated in basil in response to eCO_2. These include the saturated fatty acids dodecanoic (C12:0), and octadecanoic (C18:0) and the unsaturated fatty acids hexadecenoic (C16:1), hexadecadienoic (C16:2), eicosadienoic (C20:2) and hexadecatrienoic (C16:3). On the other hand, levels of the individual fatty acids in peppermint were not significantly affected by eCO_2, except for hexadecenoic (C16:1) and hexadecatrienoic (C16:3), which were

Table 2
Concentrations of individual amino acids (μg g^{-1} dry weight) and total protein (mg g^{-1} dry weight) in basil and peppermint grown under ambient (aCO_2) and elevated (eCO_2). Values are mean ± standard error of three independent replicates. Means followed by the same lower-case letter in a row do not differ significantly at the 0.05 probability level.

	Basil		*Peppermint*	
	aCO_2	eCO_2	aCO_2	eCO_2
Glutamic acid	20.58 ± 1.77a	22.46 ± 1.45a	22.16 ± 1.69a	23.64 ± 1.73a
Glutamine	126.12 ± 3.00b	151.91 ± 8.66c	69.19 ± 7.33a	97.45 ± 9.83b
Lysine	4.93 ± 0.36a	4.40 ± 0.27a	4.85 ± 0.30a	5.46 ± 0.34a
Histidine	1.96 ± 0.72b	1.75 ± 0.32ab	0.09 ± 0.01a	0.10 ± 0.00a
Alanine	12.25 ± 4.01a	11.43 ± 1.18a	21.99 ± 3.20b	21.14 ± 2.18b
Arginine	0.70 ± 0.13b	0.70 ± 0.07b	0.33 ± 0.11a	0.33 ± 0.03a
Ornithine	0.16 ± 0.02b	0.21 ± 0.00c	0.03 ± 0.00a	0.04 ± 0.01a
Proline	0.43 ± 0.03a	0.44 ± 0.02a	0.45 ± 0.04a	0.68 ± 0.03b
Asparagine	0.09 ± 0.01a	0.10 ± 0.01a	1.06 ± 0.03b	1.57 ± 0.15c
Isoleucine	0.22 ± 0.07a	0.22 ± 0.01a	0.13 ± 0.04a	0.13 ± 0.01a
Leucine	0.27 ± 0.01b	0.44 ± 0.03c	0.09 ± 0.01a	0.12 ± 0.01a
Methionine	0.36 ± 0.12b	0.35 ± 0.04b	0.10 ± 0.03a	0.10 ± 0.01a
Threonine	0.01 ± 0.00a	0.01 ± 0.00a	0.15 ± 0.05b	0.16 ± 0.01b
Valine	0.84 ± 0.09a	0.84 ± 0.09a	0.73 ± 0.05a	0.77 ± 0.03a
Serine	0.17 ± 0.02a	0.13 ± 0.01a	0.13 ± 0.02a	0.16 ± 0.01a
Phenylalanine	0.39 ± 0.04a	0.46 ± 0.02a	0.67 ± 0.13a	0.56 ± 0.03a
Glycine	1.47 ± 0.22b	1.80 ± 0.28b	0.09 ± 0.02a	0.15 ± 0.04a
Aspartate	0.04 ± 0.01a	0.05 ± 0.01a	0.04 ± 0.01a	0.05 ± 0.01a
Cystine	0.01 ± 0.00a	0.01 ± 0.00a	0.01 ± 0.00a	0.05 ± 0.01b
Tyrosine	0.96 ± 0.15a	0.96 ± 0.01a	0.64 ± 0.10a	0.57 ± 0.01a
Total proteins	5.72 ± 0.08a	5.87 ± 0.13a	5.70 ± 0.34a	7.67 ± 0.10b

significantly increased. CO_2 enrichment resulted in increment in the total lipid content of basil but not of peppermint.

3.3. Metabolic changes in antioxidant metabolites and minerals in response to eCO_2

Generally, antioxidant metabolites (phenolics, flavonoids and vitamins) were clustered into three main groups, as revealed by hierarchical clustering analysis (Fig. 1c). Metabolites in group 1 were comparable in the two plants, while the levels of metabolites in groups 2 and 3 were relatively higher in basil than in peppermint. With respect to their responsiveness to eCO_2, group 2, mainly phenolic acids, was not influenced by eCO_2; however, metabolites in groups 1 and 3, flavonoids, vitamins and glutathione, tended to accumulate under CO_2 enrichment. On the other hand, minerals were clustered into two groups (Fig. 1b). The first group, Fe, Na and Mn, were induced in basil but not in peppermint in response to eCO2, while the second group, K, Ca, Mg, Cd and Cu, was induced in both plants as affected by CO_2 enrichment.

3.3.1. Elevated CO_2 increases the levels of major antioxidants in basil and peppermint

Under aCO_2, both plants contained statistically similar amounts of total ascorbate. However, a significantly higher level of total glutathione was detected in basil (Table 5). CO_2 enrichment significantly improved the accumulation of total ascorbate and glutathione in basil

Table 3
Profiles of saturated and unsaturated fatty acids (μg g^{-1} dry weight) and total lipid content (mg g^{-1} dry weight) in basil and peppermint grown under ambient (aCO_2) and elevated (eCO_2). Values are mean ± standard error of three independent replicates. Means followed by the same lower-case letter in a row do not differ significantly at the 0.05 probability level.

	Basil		*Peppermint*	
	aCO_2	eCO_2	aCO_2	eCO_2
Dodecanoic (C12:0)	539.05 ± 2.17a	754.00 ± 12.46b	784.28 ± 26.63b	743.03 ± 20.16b
Tetradecanoic (C14:0	357.63 ± 11.81a	489.99 ± 56.35a	390.65 ± 10.60a	492.43 ± 86.08a
Pentadecanoic (C15:0)	75.67 ± 4.82ab	101.05 ± 12.56b	72.47 ± 3.62ab	65.71 ± 2.57a
Hexadecanoic (C16:0)	545.96 ± 13.36a	646.24 ± 22.78a	581.68 ± 36.99a	647.09 ± 23.80a
Heptadecanoic (C17:0)	264.41 ± 17.20a	244.69 ± 20.05a	236.26 ± 9.05a	259.03 ± 20.21a
Octadecanoic (C18:0)	43.19 ± 1.50a	64.35 ± 4.92b	45.19 ± 2.45a	49.87 ± 5.32ab
Eicosanoic (C20:0)	146.51 ± 15.76a	182.34 ± 23.87a	115.02 ± 7.35a	123.07 ± 6.71a
Docosanoic (C22:0)	93.72 ± 6.63a	121.98 ± 15.43a	93.69 ± 11.93a	117.11 ± 17.03a
Tricosanoic (C23:0)	55.36 ± 0.29a	47.15 ± 2.29a	61.16 ± 13a	44.18 ± 2.27a
Tetracosanoic (C24:0)	110.53 ± 8.50a	136.80 ± 24.46a	84.71 ± 7.03a	110.36 ± 18.34a
Pentacosanoic (C25:0)	168.39 ± 13.19a	309.41 ± 39.97ab	328.80 ± 62.33b	405.09 ± 67.14b
Hexacosanoic (26:0)	8.16 ± 0.12ab	10.12 ± 0.95b	6.04 ± 0.39a	6.46 ± 0.67a
Hexadecenoic (C16:1)	222.85 ± 16.68a	315.15 ± 31.59b	249.42 ± 7.55ab	346.96 ± 38.63c
Octadecenoic (18:1)	112.46 ± 9.71a	135.39 ± 17.87a	89.50 ± 10.73a	105.26 ± 15.14a
Tetracosenoic (C24:1)	350.95 ± 55.09a	438.04 ± 67.95a	368.28 ± 75.15a	398.30 ± 70.13a
Hexadecadienoic (C16:2)	237.72 ± 28.90a	326.42 ± 30.92b	257.41 ± 14.50ab	219.47 ± 11.39a
Octadecadienoic (C18:2)	122.96 ± 0.29ab	129.17 ± 2.91b	112.43 ± 2.75a	113.67 ± 4.34a
Eicosadienoic (C20:2)	146.81 ± 10.84a	209.75 ± 18.56b	183.60 ± 24.80b	207.24 ± 35.89b
Hexadecatrienoic (C16:3)	219.05 ± 4.97a	289.90 ± 12.30b	243.58 ± 5.50a	291.45 ± 11.19b
Octadecatrienoic (C18:3)	338.15 ± 30.86a	286.59 ± 35.21a	333.78 ± 12.31a	335.54 ± 16.89a
Total lipids	4.97 ± 0.30a	6.44 ± 0.31b	4.75 ± 0.21a	5.02 ± 0.13a

Table 4
Concentrations of anthocyanins and individual phenolic acids, flavonoids and their totals (mg g^{-1} dry weight) in basil and peppermint grown under ambient (aCO_2) and elevated (eCO_2). Values are mean ± standard error of three independent replicates. Means followed by the same lower-case letter in a row do not differ significantly at the 0.05 probability level.

	Basil		*Peppermint*	
	aCO_2	eCO_2	aCO_2	eCO_2
Caffeic acid	0.025 ± 0.01a	0.028 ± 0.01a	0.016 ± 0.01a	0.018 ± 0.01a
Ferulic acid	2.757 ± 0.25b	3.036 ± 0.31b	1.745 ± 0.43a	1.967 ± 0.53a
Protocatechuic acid	1.178 ± 0.23a	1.288 ± 0.25a	0.732 ± 0.31a	0.821 ± 0.34a
Catechin	0.509 ± 0.10a	0.561 ± 0.11a	0.317 ± 0.13a	0.355 ± 0.15a
Galic acid	13.523 ± 2.71b	14.921 ± 2.00b	8.409 ± 1.55a	9.227 ± 1.90a
p-Coumaric acid	1.401 ± 0.28b	1.541 ± 0.21b	0.871 ± 0.17a	0.938 ± 0.20a
Resorcinol	0.014 ± 0.00a	0.036 ± 0.01b	0.009 ± 0.00a	0.010 ± 0.00a
Chlorogenic acid	0.086 ± 0.02a	0.094 ± 0.02a	0.053 ± 0.02a	0.058 ± 0.03a
Syringic acid	0.798 ± 0.16b	0.882 ± 0.18b	0.496 ± 0.11a	0.554 ± 0.13a
Quercetin	1.197 ± 0.14b	2.029 ± 0.20c	0.744 ± 0.12a	1.265 ± 0.24b
Quercetrin	0.133 ± 0.03b	0.146 ± 0.03b	0.083 ± 0.04a	0.154 ± 0.03b
Luteolin	0.040 ± 0.01a	0.068 ± 0.01a	0.025 ± 0.01a	0.040 ± 0.02a
Apigenin	0.124 ± 0.03b	0.212 ± 0.02c	0.077 ± 0.02a	0.121 ± 0.03b
Isoquercetrin	0.187 ± 0.03b	0.286 ± 0.04c	0.116 ± 0.03a	0.191 ± 0.05b
Rutin	0.838 ± 0.17b	1.507 ± 0.20c	0.521 ± 0.12a	0.861 ± 0.27b
Ellagic acid	0.310 ± 0.05b	0.599 ± 0.06c	0.189 ± 0.05a	0.314 ± 0.13b
Velutin	0.368 ± 0.06a	0.633 ± 0.07b	0.225 ± 0.10a	0.212 ± 0.17a
Naringenin	0.005 ± 0.00a	0.009 ± 0.00b	0.003 ± 0.00a	0.005 ± 0.00a
Anthocyanins	1.87 ± 0.05a	9.21 ± 1.90b	1.85 ± 0.02a	9.65 ± 1.36b
Total flavonods	7.14 ± 0.27a	10.67 ± 0.37b	6.66 ± 0.16a	12.12 ± 1.57c
Total Phenols	49.49 ± 1.66b	83.04 ± 5.26c	35.58 ± 1.25a	75.22 ± 2.30c

and peppermint relative to their corresponding controls. Similar phenolic profiles were obtained for basil and peppermint (Table 4); however, the concentrations of most of the detected compounds were higher in basil. For both plants, gallic acid was the most abundant phenolic acid, followed by ferulic acid, whereas the flavonol quercetin and its glycoside rutin were the predominant flavonoids. In response to eCO_2, the levels of individual phenolic acids did not significantly change, except for resorcinol that increased (2.5 fold) in basil only. By contrast, most of the detected flavonoids had significantly accumulated (1.5–1.9 fold) in both plants as affected by eCO_2. Anthocyanins showed about 5-fold increase in both basil and peppermint grown under eCO_2 relative to their respective controls. Moreover, significant increases (about 1.5–2.1 fold) were reported for total flavonoids and phenols in both plants as affected by eCO_2.

3.3.2. Ionome and vitamins are influenced by CO_2 enrichment

The results presented in Table 5 shows that basil and peppermint contained comparable amounts of the detected minerals, except for Cu, which was 3-fold higher in basil. Moreover, among the detected vitamins, the levels of α and γ-tocopherol (vitamin E) and α-carotene (vitamin A) were significantly higher in basil. For the two plants, the levels of P, Na, Mn and Cd were not significantly affected by eCO_2, while that for K, Ca, Mg, Cu and Zn were significantly improved. On the other hand, phylloquinone (vitamin K1) was significantly accumulated in both plants as affected by eCO_2, while the level of α-carotene was improved in basil and that of α- tocopherol was increased in peppermint only.

3.4. CO_2 enrichment improves the biological activities of basil ad peppermint

Under aCO_2, basil and peppermint showed similar FRAP scavenging activity, while that for DPPH was higher in basil than in peppermint (Table 6). Similarly, basil showed significantly higher anti-protozoal (*Trypanosoma cruzi*) and anti-bacterial (*Escherichia coli* and *Streptococcus* sp.) activities compared with peppermint. However, similar anti-cancer activities were recorded for both plants. Total antioxidant, anti-protozoal and antibacterial activities were improved in both plants when grown under eCO_2 as compared with their values in the respective aCO_2 plants (Table 6). However, these eCO_2-induced improvements were mainly more evident in peppermint than in basil. On the other hand, the influence of eCO_2 on the anticancer activities of basil and peppermint was dependent on the tested cell line. Whereas, eCO_2 treatment improved the activity of both plants against T24P cell line, but had no significant effect on anti-Colo205 activity. Moreover, eCO_2 enhanced the activity of basil against HepG2 and that for peppermint against embryonic kidney adenocarcinoma (cell line 293).

3.5. Global change in metabolomes of basil and peppermint as affected by eCO_2

To obtain a global metabolic view of the similarities and differences among the two plant species and CO_2 levels, the full dataset was subjected to a Principal Component Analysis (PCA). The first two principal components (PC1 and PC2) accounted for 96.94% of the variance (Fig. 2). A clear separation was observed for the two CO_2 scenarios along PC2 (42.6%) but not for the two plant species. PC2 was mainly loaded on parameters related to biological activities, antioxidants and fatty acids. PCA analysis also showed that measured parameters related to biological activities and phenolics and vitamins were positively correlated to each other. Moreover, Hierarchical Clustering Analyses showed quantitative differences in the metabolic profiles of the two plants and in their responsiveness to eCO_2 (Fig. 1). The metabolic changes provoked by eCO_2 showed some variability depending on the type of metabolite and the tested plant species. Out of 94 metabolites analyzed by HPLC and GCMS, 38 and 31were significantly improved in basil and peppermint, respectively. In fact, the aforementioned metabolic changes could be attributed to the direct and/or indirect influence of eCO_2 on the processes of photosynthesis, photorespiration, glycolysis, TCA cycle, mitochondrial electron transport and/or C allocation to various primary and secondary metabolites.

4. Discussion

Plant growth and development are greatly dependent on photosynthetic carbon assimilation, whereas the products of photosynthesis serve as sources of energy and as building blocks for a variety of cell compartments and C based metabolites. It is well documented that

Table 5
Contents of inorganic nutrients, vitamins (μg g^{-1} dry weight) total glutathione, and ascorbate (μmol g^{-1} fresh weight) in basil and peppermint grown under ambient (aCO_2) and elevated (eCO_2). Values are mean ± standard error of three independent replicates. Means followed by the same lower-case letter in a row do not differ significantly at the 0.05 probability level.

	Basil		*Peppermint*	
	aCO_2	eCO_2	aCO_2	eCO_2
Minerals				
K	2.63 ± 0.14a	8.95 ± 1.87b	2.16 ± 0.08a	9.05 ± 1.09b
Ca	0.31 ± 0.07a	1.83 ± 0.29b	0.48 ± 0.09a	2.13 ± 0.34b
Mg	0.37 ± 0.01a	1.44 ± 0.38b	0.25 ± 0.01a	1.61 ± 0.31b
P	5.98 ± 0.23a	4.84 ± 2.40a	6.86 ± 0.27a	5.41 ± 2.69a
Na	0.03 ± 0.00a	0.11 ± 0.08a	0.03 ± 0.00a	0.05 ± 0.01a
Cu	0.21 ± 0.01b	2.00 ± 0.73c	0.07 ± 0.00a	3.27 ± 0.18c
Fe	0.02 ± 0.00a	0.18 ± 0.09a	0.03 ± 0.00a	0.08 ± 0.06a
Mn	0.02 ± 0.00a	0.08 ± 0.05a	0.02 ± 0.00a	0.03 ± 0.00a
Cd	0.05 ± 0.03a	0.13 ± 0.13a	0.03 ± 0.02a	0.18 ± 0.10a
Zn	0.04 ± 0.00a	0.30 ± 0.03b	0.03 ± 0.00a	0.41 ± 0.20b
Glutathione	0.51 ± 0.07b	0.76 ± 0.11c	0.31 ± 0.02a	0.59 ± 0.09b
Ascorbate	3.70 ± 0.40a	4.83 ± 0.50b	2.69 ± 0.12a	5.20 ± 0.34b
α-Carotene	0.29 ± 0.01b	0.35 ± 0.01c	0.09 ± 0.00a	0.12 ± 0.00a
β-Carotene	0.06 ± 0.00a	0.07 ± 0.00a	0.002 ± 0.00a	0.003 ± 0.00a
β-Cryptoxanthin	0.03 ± 0.00a	0.04 ± 0.01a	0.03 ± 0.00a	0.04 ± 0.00a
α-tocopherol	3.52 ± 0.19b	3.67 ± 0.46b	2.89 ± 0.11a	3.57 ± 0.11b
B-tocopherol	0.005 ± 0.00a	0.007 ± 0.00a	0.004 ± 0.00a	0.005 ± 0.00a
γ-tocopherol	0.50 ± 0.02bc	0.56 ± 0.06c	0.34 ± 0.01a	0.40 ± 0.02ab
δ-tocopherol	0.04 ± 0.00a	0.08 ± 0.04a	0.04 ± 0.00a	0.04 ± 0.00a
Total tocopherol	8.01 ± 0.31a	8.47 ± 1.23a	8.19 ± 0.36a	8.89 ± 0.07a
Phylloquinone	0.006 ± 0.00a	0.009 ± 0.00b	0.006 ± 0.00a	0.009 ± 0.00b

eCO_2 could improve the photosynthetic rate by suppressing the oxygenation reaction of rubisco, leading to improved C gain (Pérez-López et al., 2009; Watanabe et al., 2014). In the current study, regardless of the plant species, the eCO_2-induced plant growth was consequent with the improved rate of photosynthesis and accumulation of NSC (sucrose, fructose, glucose and starch). Such accumulation of NSC could explain the fertilization effect of eCO_2 because sugars are known to have pleiotropic roles in regulation of plant growth and metabolism. Similarly, improvement in the rate of photosynthesis and accumulation of NSC as well as enhanced growth in response to eCO_2 have been reported in several plant species, such as Arabidopsis, tobacco, tomato, wheat and *Isatis indigotica* (Leakey et al., 2009; Aranjuelo et al., 2011; Li et al., 2013, 2017; Noguchi et al., 2015). CO_2 enrichment not only improved the accumulation of NSC in basil and peppermint, but also enhanced their breakdown via dark respiration and, consequently, affected the accumulation of TCA cycle intermediates. In this regard, up-regulation of carbohydrate metabolism and dark respiration has been observed in plants grown under eCO_2 (Wang and Curtis 2002; Leakey et al., 2009; Li et al., 2013). Indeed, the response of TCA cycle intermediates to eCO_2 is variable depending on the tested plant species and the experimental conditions, especially N nutrition. For example, under sufficient N, fumarate, malate and total content of TCA organic acids had increased in Arabidopsis grown in high CO_2 (Watanabe et al., 2014). While, under low N, the levels of all TCA cycle organic acids were reported to be reduced in leaves of wheat exposed to eCO_2 (Aranjuelo et al., 2013).

The close coordination between N and C metabolisms is well recognized at both the biochemical and transcriptional levels, whereas the synthesis of organic N requires C skeleton and energy being provided by C metabolism (Nunes-Nesi et al., 2010). Therefore, increasing CO_2 may promote the synthesis of amino acids by improving the availability of the required intermediates and energy through up-regulation of C metabolism. However, the inhibition of the oxygenation reaction of rubisco as affected by eCO_2 will reduce the photorespiratory N recycling and affect the glutamine synthetase–glutamate synthase pathway (Lea et al., 2006). Hence, impact of eCO_2 on the biosynthesis

Table 6
Total antioxidant (FRAP, μmole Trolox g^{-1} dry weight; DPPH, % inhibition) antiprotozoal (% reduction in parasite), antibacterial (diameter of inhibition zone, mm) and anticancer (% dead cells) activities of basil and peppermint grown under ambient (aCO_2) and elevated (eCO_2). Values are mean ± standard error of three independent replicates. Means followed by the same lower-case letter in a row do not differ significantly at the 0.05 probability level.

	Basil		*Peppermint*	
	aCO_2	eCO_2	aCO_2	eCO_2
Total antioxidant activity				
FRAP	36.90 ± 0.88a	66.41 ± 2.41b	39.84 ± 1.16a	71.21 ± 1.71b
DPPH	49.57 ± 1.46b	81.27 ± 2.09c	33.69 ± 0.65a	88.65 ± 2.47d
Antiprotozoal				
Trypanosoma cruzi	4.01 ± 0.11b	6.57 ± 0.28c	2.84 ± 0.09a	6.620.27c
Anti-bacterial				
Streptococcus sp.	19.52 ± 0.47b	31.43 ± 1.21c	15.56 ± 0.45a	30.93 ± 2.04c
Escherichia coli	20.63 ± 0.66b	34.30 ± 1.58c	12.84 ± 0.42a	35.25 ± 0.87c
Anti-cancer				
HepG2	62.38 ± 2.09a	79.75 ± 5.05b	70.82 ± 2.48ab	82.02 ± 2.51b
Colo205	63.65 ± 2.39a	72.67 ± 2.49ab	71.97 ± 1.75ab	95.81 ± 12.39b
293	74.34 ± 1.67ab	83.99 ± 3.37bc	72.72 ± 2.00a	87.51 ± 2.19c
T24P	70.54 ± 1.19a	90.32 ± 3.83b	68.36 ± 2.09a	90.92 ± 3.64b

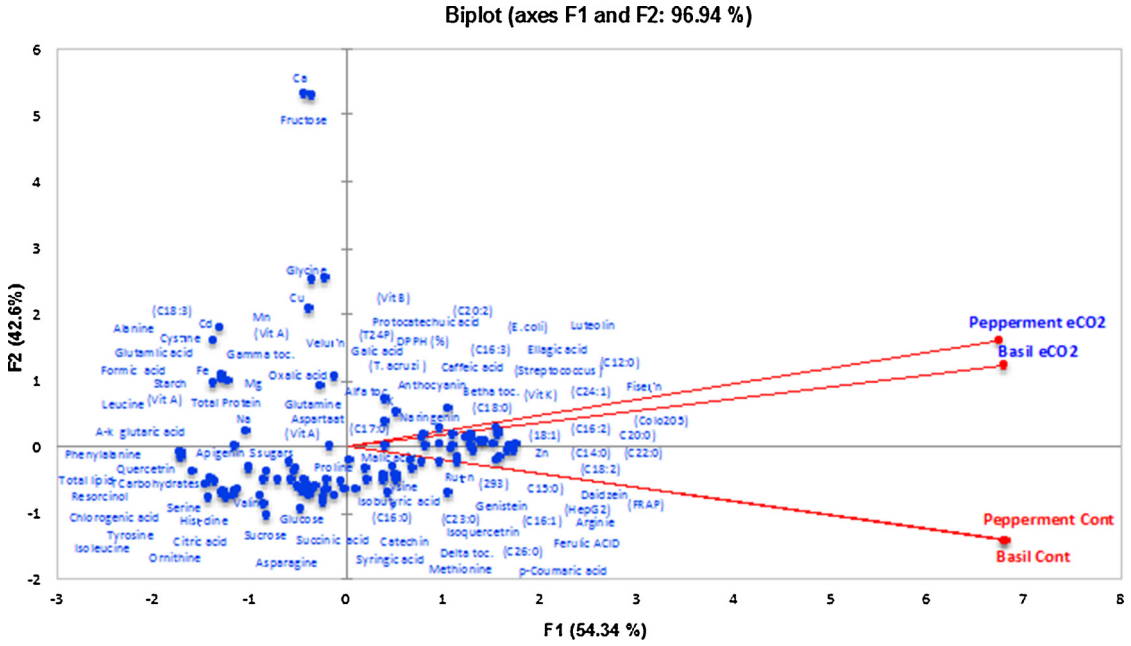

Fig. 2. Principal component analysis of the levels of 94 metabolite in basil and peppermint grown under aCO_2 or eCO_2. Variances explained by the first two components (PC1 and PC2) appear in parentheses.

of amino acids is expected to show some kind of complexity. In the present study, the individual amino acids in basil and peppermint responded differently to eCO_2, but none was reduced in any plant. Maintenance of the level and/or significant accumulation of some amino acids reported in the current study under eCO_2 may be ascribed to the enhanced availability of C skeleton together with the sufficient supply and uptake of inorganic N and/or enhanced nitrate reduction. Supporting this explanation, the levels of some amino acids, such as glutamine, asparagine, glutamate, aspartate, and alanine have been reported to accumulate under eCO_2 and sufficient N nutrition (Geiger et al., 1998; Yu et al., 2012; Watanabe et al., 2014). Moreover, there is some evidence for the positive impact of CO_2 enrichment on nitrate uptake and nitrate reductase activity in several plant species such as *Plantago major*, cucumber and tobacco (Fonseca et al., 1997; Larios et al., 2001; Matt et al., 2001).

Fatty acid biosynthesis is also dependent on carbohydrate metabolism, where *de novo* biosynthesis is initiated by the ATP-dependent carboxylation of acetyl-CoA, mainly derived from the glycolytic pathway, to produce malonyl-CoA that provides the carbon atoms required for fatty acid elongation (Brown et al., 2010). Compared with sugars and amino acids, few studies have dealt with the effect of eCO_2 on fatty acids biosynthesis in plants. Regarding the present results, although basil and peppermint have comparable levels of most of the detected fatty acids, however there was an apparent difference in their responsiveness to eCO_2 (Table 3). Such disparity may be attributed to the degree of availability of substrates and/or metabolic energy required for fatty acid biosynthesis. Supporting this explanation, in basil, that has greater levels of NSC and higher respiration rate than peppermint, the total content of lipids and about one-half of the individual fatty acids were significantly increased as affected by eCO_2, which is not the case for peppermint. Huang et al. (1999) reported that eCO_2 had no obvious effect on the *n*-fatty acid chain length, but decreased the ratio of unsaturated fatty acids in birch seedlings (*Betula pendula*). Moreover, reduction in the levels of the majority of fatty acids in roots of radish, carrot and turnip has been reported in response to eCO_2 (Azam et al., 2013).

Because reactive oxygen species and free radicals are known as major causes of chronic diseases, an important factor that determines the medicinal value of plants is their content of antioxidant metabolites such as phenolic compounds, vitamins and some minerals like Se, Zn, Cu and Mn (Idso et al., 2002; Ghasemzadeh et al., 2016; Fedor et al., 2017). It is well documented that basil and peppermint are characterized by high antioxidant potentials (Lv et al., 2012; Figueroa-Pérez et al., 2014; Riachi and De Maria 2015; Szymanowska et al., 2015). The biosynthesis of antioxidant metabolites and assimilation of essential elements is dependent on intermediates of C and N metabolism (Bryant et al., 1983; Herms and Mattson 1992; Asensi-Fabado and Munné-Bosch 2010). Therefore, by its impact on primary metabolism and/or nutrient uptake, eCO_2 is expected to affect the levels of phenolics, vitamins and minerals (Watanabe et al., 2014; Noguchi et al., 2015; Asif et al., 2017). In this regard, eCO_2 has been reported to improve the antioxidant activities of the medicinal plants ginger, strawberry and *Labisia pumila* (Wang et al., 2003; Ghasemzadeh et al., 2010; Jaafar et al., 2012). In the current study, the stimulatory effect of eCO_2 on antioxidant metabolites and minerals, which was more obvious on total ascorbate, glutathione, anthocyanins, flavonoids, and minerals than vitamins, other than ascorbate, and phenolic acids, was accompanied by a significant increase in the total antioxidant capacity in both basil and peppermint. Similarly, eCO_2-induced improvement in the antioxidant capacity of some medicinal plants was attributed to the enhanced levels of the non-enzymatic antioxidants ascorbate, glutathione, anthocyanins and flavonoids, such as quercetin and kaempferol (Wang et al., 2003; Ghasemzadeh et al., 2010; Ghasemzadeh and Jaafar 2011; Ibrahim and Jaafar 2011; Jaafar et al., 2012). In fact, the impact of eCO_2 on the levels of vitamins in plants is poorly undertaken, but ascorbic acid (vitamin C) was reported to be improved in fruits of sour orange and strawberry grown under CO_2 enrichments (Idso et al., 2002; Wang et al., 2003). Moreover, in accordance with our results, Azam et al. (2013) reported that eCO_2 treatment had resulted in accumulation of Ca, Mn and Cu in roots of carrot, turnip and radish. However, CO_2 enrichment has been reported to reduce the levels of foliar minerals in several plant species (Roberntz and Linder 1999; Luomala et al., 2005; Teng et al., 2006). This influence was mainly attributed to dilution effect rather than reduced nutrient uptake.

In addition to their antioxidant capacities, basil and peppermint are known for their antimicrobial and anticancer properties (Lv et al., 2012; Riachi and De Maria 2015; Szymanowska et al., 2015). In the current study, hierarchical clustering of biological activities that were

assayed in basil and peppermint showed that they were all grouped into a single cluster (Fig. 1d). This suggests that the different biological activities detected may share common underlying mechanisms and/or causative metabolic compounds. The present results also revealed that the relatively higher antiprotozoal, antibacterial and DPPH radical scavenging activities recorded in basil than in peppermint was paralleled to its higher levels of most of the detected phenolics and flavonoids. In addition, eCO_2, that caused significant accumulation of flavonoids, greatly improved the antioxidant, antiprotozoal and antibacterial activities of the two medicinal herbs. In agreement, Ghasemzadeh and Jaafar (2011) reported that CO_2 enrichment had improved the anticancer activity of two ginger varieties against some human cancer cell lines (MDA-MB-231 and MCF-7). Such eCO_2-induced improvement in biological activities was attributed to the paralleled increase in phenolics and flavonoids content. Our findings also suggested a positive correlation between antibacterial, antiprotozoal and anticancer activities of basil and peppermint and their contents of phenolic compounds, especially flavonoids. Supporting this explanation, phenolic compounds have been recognized for their anticancer (Roleira et al., 2015), antiprotozoal (Sun et al., 2016) and antimicrobial (Kumar et al., 2013; Mohamed et al., 2017) properties. Moreover, several investigations have ascribed the biological activities of basil and peppermint to their secondary metabolites such as terpenoids, phenolics, flavonoids and steroids (Figueroa Pérez et al., 2014; Ghasemzadeh et al., 2016).

5. Conclusion

In conclusion, basil and peppermint have qualitatively similar metabolic profiles. However, quantitative variations were recorded in the relative levels of many of the detected compounds. Their metabolites can be classified into three main groups depending on their responsiveness to eCO_2. The first group was enhanced by eCO_2 in both plants; it includes NSC, fumarate, glutamine, glutathione, ascorbate, phylloquinone (vitamin K1), anthocyanins and majority of flavonoids and minerals. The second group of metabolites was enhanced in one plant but not in the other as affected by eCO_2; involving those were improved in basil (ornithine, leucine, some fatty acids, total lipid content, resorcinol and α-carotene) and those were increased in peppermint (asparagine, proline, cystine, total proteins and α-tocopherol). The third group of metabolites was not significantly affected by eCO_2. Interestingly, the recorded enhancement in biological activities in both plants was consequent with improvement in the levels of anthocyanins, flavonoids and vitamin K1. Therefore, this study suggests that the metabolic changes provoked by eCO_2 show some kind of variability depending on the plant species, metabolite under study and the cellular activity involved.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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