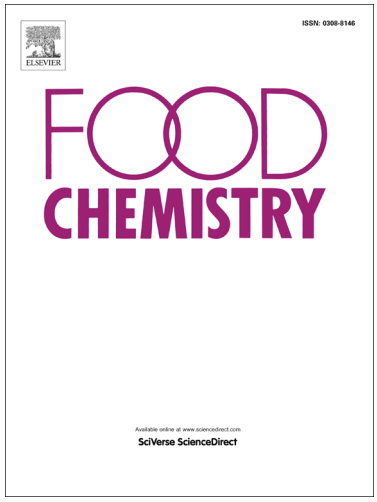
CO_2 treatment improves the hypocholesterolemic and antioxidant properties of fenugreek seeds

Reference:
Hozzein Wael N., Saleh Ahmed M., Habeeb Talaat H., Wadaan Mohammed A. M., Abd Elgaw ad Hamada.- CO_2 treatment improves the hypocholesterolemic and antioxidant properties of fenugreek seeds
Food chemistry - ISSN 0308-8146 - 308(2020), UNSP 125661
Full text (Publisher's DOI): https://doi.org/10.1016/J.FOODCHEM.2019.125661

Journal Pre-proofs



CO_2 treatment improves the hypocholesterolemic and antioxidant properties of fenugreek seeds

Wael N. Hozzein, Ahmed M. Saleh, Talaat H. Habeeb, Mohammed A.M. Wadaan, Hamada AbdElgawad

PII: S0308-8146(19)31787-X
DOI: https://doi.org/10.1016/j.foodchem.2019.125661
Reference: FOCH 125661

To appear in: *Food Chemistry*

Received Date: 30 January 2019
Revised Date: 5 October 2019
Accepted Date: 6 October 2019

Please cite this article as: Hozzein, W.N., Saleh, A.M., Habeeb, T.H., Wadaan, M.A.M., AbdElgawad, H., CO_2 treatment improves the hypocholesterolemic and antioxidant properties of fenugreek seeds, *Food Chemistry* (2019), doi: https://doi.org/10.1016/j.foodchem.2019.125661

CO_2 treatment improves the hypocholesterolemic and antioxidant properties of fenugreek seeds

Wael N. Hozzein[1,2,a,*], Ahmed M. Saleh[3,4,a,*], Talaat H. Habeeb[4], Mohammed A.M. Wadaan[1], Hamada AbdElgawad[2,5]

[1]Bioproducts Research Chair, Zoology Department, College of Science, KingSaud University, Riyadh 11451, Saudi Arabia.

[2]Botany and Microbiology Department, Faculty of Science, Beni-Suef University, Beni-Suef 62521, Egypt.

[3]Department of Botany and Microbiology, Faculty of Science, Cairo University, Giza 12613, Egypt.

[4]Biology Department, Faculty of Science at Yanbu, Taibah University, King Khalid Rd., Al Amoedi, 46423 Yanbu El-Bahr, Saudi Arabia.

[5]Laboratory for Molecular Plant Physiology and Biotechnology, Department of Biology, University of Antwerp, Groenenborgerlaan 171, B-2020, Antwerp 2020, Belgium.

[a]These authors contributed equally to this manuscript and share first authorship.

[*]Corresponding Authors: whozzein@ksu.edu.sa (W.N. Hozzein); asaleh@sci.cu.edu.eg (A.M. Saleh)

Running title:

eCO_2 improves the hypocholesterolaemic activity of fenugreek

Keywords:

CO_2, Fenugreek, hypocholesterolaemic potential, cholesterol micellar solubility, pancreatic lipase activity, bioactive metabolites

Abstract

A high level of serum cholesterol is a major cause of atherosclerosis. Fenugreek is a well-known hypocholesterolaemic agent with amazing phytochemical composition. Due to its impact on plant metabolism, CO_2 enrichment was tested as a strategy to support functional values in fenugreek seeds. Phytochemical composition and biological activities of three fenugreek cultivars (G2, G6 and G30) grown under ambient (aCO_2, 400 μmol mol^{-1}) and elevated CO_2 (eCO_2, 620 μmol mol^{-1}) were assessed. Applying eCO_2 improved physical parameters of fenugreek seeds, and enhanced their biological activities. A significant increase in hypocholesterolaemic potential, as indicated by inhibition of cholesterol micellar solubility and pancreatic lipase activity, was recorded. In addition, antioxidant, anti-lipid peroxidation and antibacterial activities were improved. These enhanced biological activities were accompanied by improved seed chemical composition at the primary and secondary metabolic levels. Therefore ,eCO_2 treatment represents an efficient strategy to increase the hypocholesterolaemic, antioxidant and antibacterial activities of fenugreek seeds.

1. Introduction

Atherosclerosis, a component of cardiovascular diseases, results from the buildup of fatty plaque on artery walls and thus affecting the blood flow (Hansson, 2005). A major cause for atherosclerosis is high levels of serum cholesterol (hypercholesterolemia) due to oxidation by free radicals producing low-density lipoproteins (LDL) leading to the formation of fatty plaque (Suciu et al., 2018). Therefore, keeping the levels of both serum cholesterol and free radicals under control is of immense importance to decrease the risk factors of heart disease. In this sense, the utilization of plant-based foods rich in both hypocholesterolaemic and antioxidant phytochemicals is helpful. Plants produce a vast array of health-promoting phytochemicals. Among these, phenolics, saponins and alkaloids represent the most effective groups, as they have been characterized for their anti-inflammatory, antiallergic, antimicrobial, antidiabetic, anticarcinogenic and/or antioxidant activities (Di Carlo, Mascolo, Izzo, & Capasso, 1999; Francis, Kerem, Makkar, & Becker, 2002; Roberts, 2013). These three groups of plant secondary metabolites have been reported to have hypocholesterolaemic potential mainly through modulating the activity of lipid metabolizing enzymes, interacting with bile acids and/or affecting the excretion of cholesterol in the feces (Birari & Bhutani, 2007; Sosnowska, Podsędek, Redzynia, & Kucharska, 2018). In addition, plant-derived vitamins could provide significant benefits to human health (Asensi-Fabado & Munné-Bosch, 2010).

Fenugreek (*Trigonellafoenumgraecum* L.) is a leguminous plant originally native to Eastern Europe and the Middle East, but now is cultivated worldwide. Seeds and leaves of fenugreek, either in intact or powdered form, and their extracts are widely utilized for both nutritional and medicinal purposes (Acharya, Thomas, &Basu, 2006). Together with its hypocholesterolaemic potential, fenugreek has been characterized for its antioxidant, anti-

inflammatory, antimicrobial, antifungal, anticancer and antidiabetic properties (Yadav & Baquer, 2014). Regarding its hypocholesterolaemic effect, fenugreek seeds treatment decreased the levels of LDL, triglycerides, total cholesterol and/or blood lipids in hypercholesterolemic rats (Belguith-Hadriche et al., 2013; Knott et al., 2017) and coronary artery disease patients (Bordia, Verma, & Srivastava, 1997; Mathern, Raatz, Thomas, & Slavin, 2009). This hypocholesterolaemic capacity of fenugreek seeds has been attributed to its polyphenolic, saponin and alkaloid ingredients (Herrera, Hierro, Fornari, Reglero, & Martin, 2018).

Currently, research in the area of preventive medicine is focused on supporting the functional value of plant based-foods by improving the endogenous levels of their health-promoting primary and secondary compounds (Al Jaouni et al., 2018; Saleh et al., 2018). In this regard, the up-regulation of photosynthesis is an effective strategy to enhance the synthesis of the bioactive phytochemicals by improving the availability of the required precursors and metabolic energy (Al Jaouni et al., 2018; Saleh et al., 2018). Elevated CO_2 (eCO_2), as a substrate for photosynthesis, has been reported to improve the photosynthetic C assimilation, and consequently triggers a set of metabolic events in the target plant, which lead to induced levels of both primary and secondary metabolites (Watanabe et al., 2014). Such improved levels of secondary metabolites, e.g. polyphenol, saponins and alkaloids, have been found to improve the health-promoting properties of several herbal and medicinal plants (Ghasemzadeh & Jaafar, 2011; Jaafar, Ibrahim, & Karimi, 2012; Saleh et al., 2018). Previous research had addressed the impact of extraction and/or administration protocols and conditions on the antioxidant and hypocholesterolaemic potentials of fenugreek seeds (Belguith-Hadriche et al., 2013; Bordia et al., 1997; Herrera et al., 2018; Knott et al., 2017; Mathern et al., 2009). However, an effort to increase these properties in the target seeds is still missing. The aim of the present investigation

was to improve the antioxidant and hypocholesterolaemic activities of fenugreek seeds by supporting the endogenous levels of the underlying phytochemicals using a CO_2 enriched environment as a strategy. To test our hypothesis, fenugreek was grown under two different levels of CO_2, ambient (aCO_2, 400 μmol mol^{-1}) and elevated (eCO_2, 620 μmol mol^{-1}). The yield parameters and the levels of phenolic compounds, saponins, alkaloids and vitamins as well as fatty acids composition of the produced seeds were assessed. The associated changes in the antioxidant and hypocholesterolaemic, as indicated by reduction in micellar cholesterol solubilization and inhibition of pancreatic lipase activity, potentials were investigated. To test the interactive behaviors between biological activities with similar phytochemical basis, the antibacterial activities of the treated seeds were evaluated.

2. Material and methods

2.1. Biological materials, reagents and chemicals

Seeds of fenugreek cultivars were obtained from Agricultural Research Centre – Giza, Egypt. Cholesterol, sodium taurocholate, oleic acid, methyl umbelliferyl oleate (4-MUO), pancreatic lipase, 2,2-diphenyl-1-picryhydrazyl (DPPH), 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ), Iron (III) chloride (FeCl3), trichloroacetic acid (TCA), thiobarbituric acid (TBA), Folin-Ciocalteu reagent, aluminum chloride (AlCl3), Mueller Hinton agar and standards for phenolic acids and flavonoids (caffeic acid, ferulic acid, catechin, gallic acid, p-coumaric acid, chlorogenic acid, kaempferol, quercetin, luteolin, apigenin, naringenin, rutin, tricin, vitexin) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cholesterol analysis kit was purchased from Pointe Scientific Inc. (Canton, MI, USA).

2.2. Experimental set up, growth conditions, and plant harvests

Seeds of three fenugreek cultivars i.e., G2, G6 and G30 were gown in potting mix (Tref EGO substrates, Moerdijk, The Netherlands, 25×20cm pots). Forty pots (20 pots per treatment) were filled with 2kg of loamy soil and organic compost (50:50%) at a humidity of 0.27 g water /g dry soil. These pots were transferred to controlled-growth chambers under two climate conditions, viz: 1) ambient CO_2 (aCO_2, 400±27μmol CO_2 mol^{-1}air); 2) elevated CO_2 (e CO_2, 620±42 μmol CO_2 mol^{-1}air ppm). IPCC-SRES B2-scenario prediction of elevated CO_2 of the year 2100 was the basis for selecting the applied elevated CO_2 concentration (Murray & Ebi, 2012). CO_2 concentration was continuously monitored with a CO_2analyser (WMA-4, PP Systems, Hitchin, UK). Other growth conditions were adjusted to 150 μmol PAR m^{-2} s^{-1}, 16/8 h day/night photoperiod, 21/18^{o}C air temperature and 65% humidity. Soils in the pots were daily watered to stabilize soil water content to 65% of water-holding capacity (SWC). After 2 weeks of transferring pots to climate chambers, germinated plants were ultimately thinned to one per pot. At later stages of the experiment, pots were watered to 75% of the SWC. To avoid chamber-specific bias in this experiment, pots and their corresponding CO_2 treatment were relocated between the two climate chambers every two weeks. At the full maturation stage (around 118 days after sowing), seeds were collected to measure their physical and chemical properties and biological activities.

2.3. Seed preparations for biological activity assay

Seeds from each plant were separately ground and about 4g powder was extracted with ethanol at room temperature for 24h. The extract was centrifuged at 8,000*g* for 25 min and the supernatant was filtered using Whatmann No.1 filter paper. After concentrating the extract using a rotary evaporator, the samples were stored at -20°C until used.

2.4. Hypocholesterolaemic activity

2.4.1. Inhibition of micellar solubility of cholesterol

For measuring the impact of fenugreek seeds on the micellar solubility of cholesterol the method described by Lin, Tsai, Hung, & Pan(2010) was followed. The concentrated seed extract was added to 7 ml of micellar solution (2 mM cholesterol, 10 mM sodium taurocholate, 132 mM NaCl, 5 mM oleic acid, 15 mM sodium phosphate (pH 7.4)) at the rate of 10 mg/ml. The mixture was sonicated for 2 min and incubated in a water bath at 37 °C for 24 h. The micellar solution was then ultracentrifuged at 40,000 rpm for 60 min at 20 °C and 10 µl of the supernatant was used for spectrophotometric determination of cholesterol content at 500 nm by an enzymatic method using a cholesterol analysis kit (Pointe Scientific, C7510). Inhibition activity of micellar solubility of cholesterol for each sample was calculated as follow:

Inhibition activity (%) = [(C-S)/C]*100

Where, C is the cholesterol concentration in control micellar solution and S is cholesterol concentration in micellar mixture containing seed powder.

2.4.2. Pancreatic lipase inhibition assay

The inhibitory activity of seed extract against pancreatic lipase was measured by using 4-MUO as a substrate(Sugiyama et al., 2007). 0.5 mL of the seed extract (different concentrations), was mixed with 0.5 mL of the freshly preparedlipase (1 mg/mL; lipase from porcine pancreas, Sigma-Aldrich). After stirring for 10 min, the mixtures were centrifuged (4000 rpm, 10 min), and then 2 mL of the 4-MUO (0.1mM) solutions was added. The reaction mixture without seed extract was used as a blank. The mixture was incubated at 37 °C. Aliquots of 0.2 mL were taken at different time points, and 4-MUO hydrolysis by lipase was measured at an excitation wavelength of 350 nm and an emission wavelength of 450 nm. A logarithmic regression curve was established to calculate IC_{50} values (mg/mL), defined as the concentration of the extract that

inhibited 50% the activity of the pancreatic lipase.

2.5. Antioxidant capacity

In vitro antioxidant capacity was measured by DPPH and ferric reducing antioxidant power (FRAP) methods (Al Jaouni et al., 2018). About 0.2g of the seed powder were extracted with ethanol (80%) and centrifuged at 14,000 rpm for 20 min. The antioxidant capacity was measured by mixing 0.1 mL of the diluted seed extracts with 0.25 mL of the DPPH solution or FRAP reagent (mixing TPTZ (10mM) and $FeCl_3$ (20mM) in acetate buffer (0.25M, pH 3.6). After incubation at room temperature, the absorbance was measured at 517 nm and 600 nm using spectrometric method, respectively.

2.6. Anti-lipid peroxidation

The degree of lipid peroxidation was measured by the thiobarbituric acid reactive substances (TBARS) method, using an egg yolk homogenate as the lipid rich medium (Ohkawa, Ohishi, & Yagi, 1979). The seed extract and egg homogenate (0.5ml of 10% v/v) were mixed with 15 mM ferrous sulphate (to induce lipid peroxidation), and after 30 min 1.5 ml of 10% TCA was added. The mixture was then transferred to a tube contains 1.5 ml of 0.67% TBA and boiled for 30 min. The chromogen formed was measured at 535 nm.

2.7. Antibacterial activities

The antibacterial activities of the seed ethanolic extracts were tested by the disc diffusion method (bacterial suspension containing 10^6 CFU/ml of the bacterial test strain spread on Muller Hinton agar). The extracts were loaded on sterilized filter paper discs (5 µg/disc). Ethanol was used as a negative control. These discs were placed on the agar plates and incubated for 24 h at 37°C. The inhibition zones were measured by Vernier caliper.

2.8. Metabolites analyses

The concentrations of total soluble sugars in the seed extracts were determined following Nelson's method described by Clark and Switzer (Clark & Switzer, 1977). Quantification of soluble proteins was conducted according to the Folin-Lowry method adopted by Hartree (1972). Total phenolic and flavonoid contents were determined using the Folin–Ciocalteu and aluminum chloride colorimetric assays, respectively (Madany & Saleh, 2015). The contents of alkaloids and saponins in fenugreek seeds were measured according to the methods described by Sreevidya & Mehrotra (2003) and Lai, Hsieh, Huang, & Chou (2013), respectively.

The fatty acids profile was quantified according to Hassan, Saleh, & AbdElgawad (2018). To obtain the lipophilic fraction, powdered seeds were extracted in chloroform/methanol (2:1, v/v) at 25°C. The lipophilic fractions were centrifuged (16,000 rpm, 30 min). Filtered supernatants were quantified (GC-MS analysis, Hewlett Packard 6890, MSD 5975 mass spectrometer, equipped with an HP-5 MS column). Fatty acids were identified using the NIST 05 and Golm Metabolome Databases, http://gmd.mpimp-golm.mpg.de.

Identification and quantification of phenolic compounds were performed by UHPLC-MS/MS analysis following the protocol described by Xavier et al. (2017). A known weight of the dried powdered seeds was extracted with 80% (v/v) ethanol in a water bath at 70 °C for 30 min. After centrifugation (12,000 rpm for 30 min), the supernatant was concentrated using a rotary evaporator (IKA-WERKE-RV06ML; Staufen, Germany). The obtained residue was dissolved in HPLC grade methanol (final concentration = 1000 ppm). Chromatographic analysis of the extract was performed in an Acquity UPLC System (Waters, Milford, CT, USA). An Acquity BEH C18 column (100 mm × 2.1 mm), with a 1.7-µm particle size was used for separation. The mobile phase components were eluent A: ultrapure water containing 0.1% formic acid and eluent B: acetonitrile. The flow rate was 0.2 ml/min and 2 µl of samples were injected,

with a linear gradient starting at 3% B, increased to 100% B in 10 min. 3,5-dichloro-4-hydroxybenzoic acid was used as an internal standard.

Vitamins, i.e., tocopherol, carotene, thiamine, β-cryptoxanthin and phylloquinone, were measured by HPLC (Shimadzu, Hertogenbosch, Netherlands). Tocopherols were extracted in hexane, then separated in Particil Pac column material and measured by a normal phase HPLC (Hamad et al., 2015). Carotene and β-cryptoxanthin contents were extracted in acetone and analyzed by a reversed phase HPLC conducted with a diode array detector (Al Jaouni et al., 2018). Phylloquinone was extracted in methanol and the supernatant was injected in to RP18 column. Phylloquinone was detected by using a reversed phase HPLC methods (Jakob & Elmadfa, 1996).

For further details about the extraction and determination procedures of the measured metabolites please refer to supplementary materials (S1).

2.9. Statistical analyses

Experiments were carried out following a randomized complete block design. Normality and homogeneity of variances were checked using the Kolmogorov–Smirnoff and Levene's tests, respectively. The analyses were performed using the SPSS statistics software package, version 20.0 (IBM Corporation, USA). Differences between eCO_2 and aCO_2 seeds within each cultivar were analyzed using Student's *t*-test at probability levels of 0.05, 0.01 or 0.001.

3. Results

3.1. Elevated CO_2 improves yield and quality of fenugreek seeds

The results presented in Table 1 revealed that under aCO_2 conditions (400 μmol mol^{-1}), the three cultivars showed comparable values for the seed physical parameters, however; there

were significant variations in the levels of the chemical ingredients. Seeds of the G2 cultivar showed the highest values of total phenolics and saponins, however those of G30 contained the highest content of alkaloids. eCO_2 (620 μmol mol^{-1}) treatment increased the seed yield of G2, G6 and G30 fenugreek cultivars by about 70, 53 and 57%, respectively as compared with their respective aCO_2 controls. Moreover, all the associated physical parameters, including seed length, width, mass, thickness and pod length had been improved as a result of eCO_2 treatment, except for the seed width in the case of G6. Not only the physical parameters, but also the total contents of sugars, proteins, phenols, flavonoids, alkaloids and saponins were significantly increased in almost all cultivars in response to eCO_2 treatment. The positive effect of eCO_2 was more pronounced on the accumulation of phenolics than on the saponins and alkaloids.

3.2. CO_2 enrichment enhances the hypocholesterolaemicpotential of fenugreek seeds

The hypocholesterolaemic potential of fenugreek seeds was assessed *in vitro* by measuring the reduction in micellar solubility of cholesterol and inhibition of lipase activity (Figure 1). Under aCO_2 conditions, the fenugreek cultivars studied showed considerable reductions in the micellar solubility of cholesterol, ranged from 50 to 55% (Figure 1a). Such inhibition of micellar solubility was significantly supported by eCO_2 treatment, whereas the inhibition capacity improved by about 28, 36 and 27% in G2, G6 and G30 seeds, respectively, as affected by eCO_2 treatment. Seeds of G6 showed the highest inhibition of micellar solubility under both aCO_2 and eCO_2 conditions, about 55 and 75 % respectively. Regarding the lipase inhibition assay results, the IC_{50} of the fenugreek cultivars tested ranged from 3.4 to 3.9 mg/ml under aCO_2 conditions (Figure 1b). However, these IC_{50} values were significantly reduced in response to eCO_2 treatment by about 25% in all cultivars. Seeds of the three cultivars showed comparable IC_{50} values, 2.6 to 2.7 mg/ml, under eCO_2 conditions.

3.3. Seeds of eCO_2treated-plants possess higher antioxidantcapacity.

Under aCO_2 conditions, the seed extracts of the three fenugreek cultivars showed comparable DPPH radical scavenging capacity, about 50 %, however the highest value in the FRAP assay was obtained by the G6 cultivar (Figure 2a,b). The eCO_2 treatment significantly improved the total antioxidant capacity (TAC) of the three cultivars relative to their respective aCO_2 controls. Similar improvements in the DPPH radical scavenging capacities were observed for seeds of G6 and G30, while the seeds of G2 showed the highest improvement (94%) in the value of FRAP assay as affected by eCO_2. Moreover, for all the tested cultivars, a significant improvement in the anti-lipid peroxidation capacities were recorded in fenugreek seeds produced in eCO_2 conditions (Figure 2c). The highest inhibition of lipid peroxidation was reported in seeds of the G30 plants grown under eCO_2.

3.4. CO_2 enrichment improves the antibacterial activities of fenugreek seeds

The antibacterial activities of the ethanolic seed extracts were tested against four Gram-positive and two Gram-negative bacterial species (Table 3). Regarding the Gram-positive bacteria, eCO_2 treatment resulted in significant improvements in the antibacterial activities of the three fenugreek cultivars against *Bacillus subtilis*, *Streptococcus* sp. and *Staphylococcus aureus* relative to the aCO_2 control. However, the antibacterial activity against *Sarcina lutea* was improved in the seeds of G2 only, in response to eCO_2 treatment. On the other hand, among the Gram-negative bacteria the activity against *Escherichia coli* was significantly improved in the three cultivars, but that for *Pseudomonas aeruginosa* was enhanced in G2 only as affected by eCO_2 treatment.

3.5. Elevated CO_2affected the nutritive value of fenugreek seeds

The levels of individual saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids were assessed in the seeds of three fenugreek cultivars under both aCO_2 and eCO_2conditions (Table 2). The three cultivars showed similar fatty acid profiles, however some variations in the concentrations of the individual fatty acids were recorded. Palmitic (C16:0) and stearic (C18:0) acids were the dominant SFA, meanwhile linoleic acid (C18:2) was the dominant UFA followed by linolenic (C18:3) and oleic acid (18:1) acids. The seeds of the different cultivars showed low SFA/UFA ratio. The eCO_2 treatment significantly increased the levels of some SFA, but did not induce a significant change in the total content of SFA in any cultivar. On the other hand, eCO_2 increased the accumulation of the majority of UFA and their total content in the seeds of the three cultivars. The content of the predominant fatty acid, octadecadienoic (C18:2), was increased by 42, 38 and 40% in the seeds of G2, G6 and G30 plants, respectively, grown at eCO_2. For all cultivars, the plant seeds produced under aCO_2conditions showed low SFA/UFA ratio (about 0.2), while this ratio was further decreased to about 80% of its value in response to eCO_2.

Of the vitamins present in the fenugreek seeds, vitamin C (ascorbic acid) was the predominant vitamin followed by vitamin E (tocopherol). They account for about 50 and 22% of the total vitamins content, respectively (Table 2). The contents of vitamin C, vitamin E, vitamin K (Phylloquinone) and two forms of vitamin A were significantly improved in the seeds of the thee cultivars in response to eCO_2 treatment.

The concentrations of the five phenolic acids and nine flavonoids were quantified in the seeds of G2, G6 and G30 fenugreek cultivars (Table 2). Among these, gallic and caffeic acids were the predominant phenolic acids, while kaempferol, quercetin and rutin were the major flavonoids. Although the fenugreek cultivars in this study had similar phenolic profiles, the

quantitative differences in the levels of individual phenolic acids and flavonoids were detected. The level of gallic acid was significantly increased in G2 and G6, while caffeic acid and catechin were significantly increased in G30 as affected by the eCO_2 treatment. Ferulic acid was significantly increased in all cultivars when grown in the eCO_2 environment. On the other hand, the contents of almost all the detected flavonoids, except for rutin, were significantly increased in response to the eCO_2 treatment in all cultivars.

4. Discussion

Functional foods are those that contribute not only to supplying human nutritional needs, but also contribute to the prevention of chronic diseases or in reducing their risk factors (Roberfroid, 1999). Fenugreek is well recognized as a potent hypocholesterolaemic agent (Belguith-Hadriche et al., 2013; Bordia et al., 1997; Knott et al., 2017; Mathern et al., 2009). The hypocholesterolaemic potential of fenugreek seeds is attributed to the content of secondary metabolites such as phenolic acids, flavonoids, saponins and alkaloids (Herrera et al., 2018). In this sense, increasing the accumulation of these bioactive phytochemicals in fenugreek seeds represents a strategy to support its hypocholesterolaemic activity. In the current study, we have employed CO_2 rich environment (eCO_2) as a tool to achieve the above-mentioned purpose.

Regarding the phytochemical composition of fenugreek seeds, the results obtained herein are consistent with the previous studies (Mansour & El-Adawy, 1994; Naidu, Shyamala, Naik, Sulochanamma, & Srinivas, 2011). They had reported that sugars and proteins are the major chemical constituents of fenugreek seeds, while lower amounts of secondary metabolites were recorded. In accordance with our results, Belguith-Hadriche et al. (2013) and Kenny et al.(2013) had reported that the phenolic profile of fenugreek seeds is dominated with gallic, caffeic,

syringic, p-coumaric, chlorogenic acids, kaempferol, apigenin, naringenin, myricetin and luteolin. The present results revealed that fenugreek seeds is a rich source of UFA, where linolenic (C18:3), linoleic (C18:2) and oleic (C18:1) acids were the most dominant fatty acids. This result is in agreement with Chatterjee et al. (2010) who reported that linoleic, linolenic and oleic acids accounted for 67 % of the total fatty acids content of fenugreek seeds. In line with the literature, the current results showed that fenugreek seeds represent a valuable source of vitamins A, B, C and E (Ahmad, Alghamdi, Mahmood, & Afzal, 2016).

Elevated CO_2 is a well-known bio-fertilizer with a prominent effect on both plant growth and metabolism (Watanabe et al., 2014). The primary action of eCO_2 is to improve the photosynthetic C assimilation through activating the carboxylation reaction of rubisco, the key enzyme in photosynthesis, in the expense of the oxygenation reaction (Pérez-López et al., 2009). The up-regulation of photosynthesis not only enhances plant growth, but also provides the energy and precursors required for the synthesis of various secondary metabolites (Al Jaouni et al., 2018; Saleh et al., 2018). In this context, the current results revealed that eCO_2 treatment had a positive impact on both seed yield and quality of the fenugreek cultivars tested (Table 1). Whereas, the seeds of eCO_2-treated plants showed significantly higher values of the measured physical parameters and improved contents of total sugars, proteins, phenolics, flavonoids, saponins and alkaloids, as compared with aCO_2-grown plants. The eCO_2 treatment affected the levels of individual phenolic acids, flavonoids and vitamins. Previously, eCO_2 (600 ± 50 µmol mol^{-1}) has been reported to improve the net photosynthetic rate and influence the nutrient composition of fenugreek plant (Jain et al., 2007). The accumulation of secondary metabolites in several medicinal plants in response to eCO_2 treatment has been reported. We have recently recorded improved contents of total phenolics and flavonoids in several herbal plants grown

under CO_2 enriched environment(Al Jaouni et al., 2018; Saleh et al., 2018). The promoting action of eCO_2 on the accumulation of saponins and alkaloids was also investigated in several plant species (Agrell, Anderson, Oleszek, Stochmal, & Agrell, 2004; Jia, Zhang, Zhao, Liu, & He, 2018).

The inhibition of pancreatic lipase is a way to reduce the hyperlipidemia, whereas dietary triacylglycerols, the main lipid constituent in the human diet, must be hydrolyzed by the action of pancreatic lipase before it can be absorbed (Birari & Bhutani, 2007). Besides, reduction of micellar solubilization is helpful to retard the absorption of cholesterol by the small intestine(Furune et al., 2014). Proper scavenging of free radicals is also essential to prevent the oxidation of cholesterol into low-density lipoproteins (LDL) (Suciu et al., 2018). In this regard, plant phenolics, saponins and alkaloids have been reported to inhibit lipid metabolizing enzymes and to reduce micellar solubility of cholesterol (Birari & Bhutani, 2007; Sosnowska et al., 2018). In addition, plant vitamins and polyphenols are well characterized for their free radicals scavenging potential(Asensi-Fabado & Munné-Bosch, 2010; Kenny et al., 2013).The eCO_2-induced improvements in the levels of phenolics, saponins, alkaloids and vitamins reported herein could enhance the antioxidant and hypocholesterolaemic activities of the produced seeds. Supporting this hypothesis, regardless of the cultivar, seeds of eCO_2-treated plants possessed elevated TAC (DPPH and FRAP) and showed higher inhibition on lipid peroxidation, micellar solubility of cholesterol and lipase activity. Belguith-Hadriche et al (2013) have attributed the hypocholesterolaemic potential exerted by fenugreek seeds in hypercholesterolemic rats, as indicated by decreased levels of total cholesterol, triglycerides and LDL and increased concentration of the high-density lipoprotein, to the flavonoids such as naringenin kaempferol, apigenin and luteolin. The higher pancreatic lipase inhibitory activity of fenugreek seeds extract

than that of quinoa extract was linked to the higher content of total phenolics and saponins in fenugreek seeds (Herrera et al., 2018). The hypocholesterolaemic potential of purified plant polyphenols, saponins and alkaloids has been reported, both *in vitro* and *in vivo* (Birari & Bhutani, 2007; Sosnowska et al., 2018). Although the impact of eCO_2 treatment on the hypocholesterolaemic activity of medicinal plants has not been discussed in the literature, the positive effect of eCO_2 on other biological activities such as antioxidant, antifungal, antiprotozoal and anticancer has been investigated (Al Jaouni et al., 2018; Ghasemzadeh & Jaafar, 2011; Idso et al., 2002; Jaafar et al., 2012; Saleh et al., 2018; Wang, Bunce, & Maas, 2003). In all of these studies, the researchers have attributed the eCO_2-induced enhancement in biological activity to the elevated levels of biologically active secondary metabolites. Similarly, the present results revealed that eCO_2 treatment exerted a positive impact on the antibacterial activities of fenugreek seeds, which was consistent with the improved levels of secondary metabolites (phenolics, flavonoids, saponins and alkaloids). These results suggest that the hypocholesterolaemic and antibacterial activities seem to be complementary because they share the same underlying phytochemicals.

Fenugreek seeds possess low SFA/UFA ratios which could favor their use in the prevention and/or treatment of hypercholesterolemia, as the intake of saturated fats is linked to cardiovascular diseases (Livingstone, Lovegrove, & Givens, 2012). The eCO_2 treatment increased this property by increasing the levels of the majority of UFA, but not the SFA. Similar to our results, Chatterjee et al.(2010) reported that the fatty acid composition of fenugreek seeds is dominated by UFA. Although the impact of eCO_2 on the levels of individual fatty acids was not investigated, it was concluded that by its up-regulatory role on the process of dark respiration, eCO_2 could enhance the availability of precursors required for fatty acids

biosynthesis (Brown, Slabas, & Rafferty, 2010). Similar to the present results, we have recently reported that eCO_2 treatment improved the ratio of unsaturated fatty acids in four medicinal herbs (Al Jaouni et al., 2018; Saleh et al., 2018).

Conclusion

For the first time, we reported the capacity of eCO_2 to increase the hypocholesterolaemic potential of fenugreek seeds. Such promoting action of eCO_2on the functionality of fenugreek seeds is attributed to its up-regulatory effect on the biosynthesis of biologically active phytochemicals such as phenolics, flavonoids, saponins and alkaloids. The accumulation of these groups of bioactive phytochemicals reinforced the capacity of fenugreek seed extract to inhibit both micellar solubility of cholesterol and pancreatic lipase activity, therefore this could be helpful in reducing the absorption of cholesterol in the small intestine. The eCO_2 treatment was efficient in supporting total antioxidant capacity and the proportion of unsaturated fatty acids in fenugreek seeds.

Acknowledgment

The authors acknowledge funding from the Research and Development (R&D) Program (Research Pooling Initiative), Ministry of Education, Riyadh, Saudi Arabia, (RPI-KSU).

References

Agrell, J., Anderson, P., Oleszek, W., Stochmal, A., & Agrell, C. (2004). Combined effects of elevated CO_2 and herbivore damage on alfalfa and cotton. *Journal of Chemical Ecology*, *30*(11), 2309–2324.

Ahmad, A., Alghamdi, S. S., Mahmood, K., & Afzal, M. (2016). Fenugreek a multipurpose crop: Potentialities and improvements. *Saudi Journal of Biological Sciences*, *23*(2), 300–310.

Al Jaouni, S., Saleh, A. M., Wadaan, M. A. M., Hozzein, W. N., Selim, S., & AbdElgawad, H. (2018). Elevated CO_2 induces a global metabolic change in basil (*Ocimum basilicum* L.) and peppermint (*Mentha piperita* L.) and improves their biological activity. *Journal of*

Plant Physiology, *224–225*(March), 121–131.

Asensi-Fabado, M. A., & Munné-Bosch, S. (2010). Vitamins in plants: occurrence, biosynthesis and antioxidant function. *Trends in Plant Science*, *15*(10), 582–592.

Belguith-Hadriche, O., Bouaziz, M., Jamoussi, K., Simmonds, M. S. J., El Feki, A., & Makni-Ayedi, F. (2013). Comparative study on hypocholesterolemic and antioxidant activities of various extracts of fenugreek seeds. *Food Chemistry*, *138*(2–3), 1448–1453.

Birari, R. B., & Bhutani, K. K. (2007). Pancreatic lipase inhibitors from natural sources: unexplored potential. *Drug Discovery Today*, *12*(19–20), 879–889.

Bordia, A., Verma, S. K., & Srivastava, K. C. (1997). Effect of ginger (*Zingiber officinale* Rosc.) and fenugreek (*Trigonella foenumgraecum* L.) on blood lipids, blood sugar and platelet aggregation in patients with coronary artery disease. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *56*(5), 379–384.

Brown, A., Slabas, A., & Rafferty, J. (2010). Fatty Acid Biosynthesis in Plants — Metabolic Pathways, Structure and Organization. *Lipids in Photosynthesis SE - 2*, *30*, 11–34.

Chatterjee, S., Variyar, P. S., & Sharma, A. (2010). Bioactive lipid constituents of fenugreek. *Food Chemistry*, *119*(1), 349–353.

Clark, J. M., & Switzer, R. L. (1977). *Experimental Biochemistry* (2nd editio). W.H. Freeman & Company, San Francisco.

Di Carlo, G., Mascolo, N., Izzo, A. A., & Capasso, F. (1999). Flavonoids: old and new aspects of a class of natural therapeutic drugs. *Life Sciences*, *65*(4), 337–353.

Francis, G., Kerem, Z., Makkar, H. P. S., & Becker, K. (2002). The biological action of saponins in animal systems: a review. *British Journal of Nutrition*, *88*(6), 587–605.

Furune, T., Ikuta, N., Ishida, Y., Okamoto, H., Nakata, D., Terao, K., & Sakamoto, N. (2014). A study on the inhibitory mechanism for cholesterol absorption by α-cyclodextrin administration. *Beilstein Journal of Organic Chemistry*, *10*, 2827.

Ghasemzadeh, A., & Jaafar, H. Z. E. (2011). Antioxidant potential and anticancer activity of young ginger (*Zingiber officinale* Roscoe) grown under different CO_2 concentration. *Journal of Medicinal Plants Researc*, *5*(14), 3247–3255.

Hamad, I., Abdelgawad, H., Al Jaouni, S., Zinta, G., Asard, H., Hassan, S., … Selim, S. (2015). Metabolic analysis of various date palm fruit (*Phoenix dactylifera* L.) cultivars from Saudi Arabia to assess their nutritional quality. *Molecules*, *20*(8), 13620–13641.

Hansson, G. K. (2005). Inflammation, atherosclerosis, and coronary artery disease. *New England Journal of Medicine*, *352*(16), 1685–1695.

Hartree, E. F. (1972). Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Analytical Biochemistry*, *48*(2), 422–427.

Hassan, M. O., Saleh, A. M., & AbdElgawad, H. (2018). Sonchus oleraceus Residue Improves

Nutritive and Health-Promoting Value of Common Bean (*Phaseolus vulgaris* L.): A Metabolic Study. *Journal of Agricultural and Food Chemistry*, *66*, 2092–2100.

Herrera, T., Hierro, J. N. del, Fornari, T., Reglero, G., & Martin, D. (2018). Inhibitory effect of quinoa and fenugreek extracts on pancreatic lipase and α-amylase under in vitro traditional conditions or intestinal simulated conditions. *Food Chemistry*, *270*(July 2018), 509–517.

Idso, S. B., Kimball, B. A., Shaw, P. E., Widmer, W., Vanderslice, J. T., Higgs, D. J., … Clark, W. D. (2002). The effect of elevated atmospheric CO_2 on the vitamin C concentration of (sour) orange juice. *Agriculture, Ecosystems & Environment*, *90*(1), 1–7.

Jaafar, H. Z. E., Ibrahim, M. H., & Karimi, E. (2012). Phenolics and flavonoids compounds, phenylanine ammonia lyase and antioxidant activity responses to elevated CO_2 in *Labisia pumila* (Myrisinaceae). *Molecules (Basel, Switzerland)*, *17*(6), 6331–6347.

Jain, V., Pal, M., Raj, A., Khetarpal, S., Physiology, P., Agricultural, I., & Delhi-, N. (2007). Photosynthesis and nutrient composition of spinach and fenugreek grown under elevated carbon dioxide concentration. *Biologia Plantarum*, *51*(3), 559–562.

Jakob, E., & Elmadfa, I. (1996). Application of a simplified HPLC assay for the determination of phylloquinone (vitamin K1) in animal and plant food items. *Food Chemistry*, *56*(1), 87–91.

Jia, X., Zhang, C., Zhao, Y., Liu, T., & He, Y. (2018). Three years of exposure to lead and elevated CO_2 affects lead accumulation and leaf defenses in *Robinia pseudoacacia* L. seedlings. *Journal of Hazardous Materials*, *349*(126), 215–223.

Kenny, O., Smyth, T. J., Hewage, C. M., & Brunton, N. P. (2013). Antioxidant properties and quantitative UPLC-MS analysis of phenolic compounds from extracts of fenugreek (*Trigonella foenum-graecum*) seeds and bitter melon (*Momordica charantia*) fruit. *Food Chemistry*, *141*(4), 4295–4302.

Knott, E. J., Richard, A. J., Mynatt, R. L., Ribnicky, D., Stephens, J. M., & Bruce-Keller, A. (2017). Fenugreek supplementation during high-fat feeding improves specific markers of metabolic health. *Scientific Reports*, *7*(1), 12770.

Lai, L.-R., Hsieh, S.-C., Huang, H.-Y., & Chou, C.-C. (2013). Effect of lactic fermentation on the total phenolic, saponin and phytic acid contents as well as anti-colon cancer cell proliferation activity of soymilk. *Journal of Bioscience and Bioengineering*, *115*(5), 552–556.

Lin, Y. H., Tsai, J. S., Hung, L. B., & Pan, B. S. (2010). Hypocholesterolemic effect of compounded freshwater clam protein hydrolysate and *Gracilaria*. *Food Chemistry*, *123*(2), 395–399.

Livingstone, K. M., Lovegrove, J. A., & Givens, D. I. (2012). The impact of substituting SFA in dairy products with MUFA or PUFA on CVD risk: evidence from human intervention studies. *Nutrition Research Reviews*, *25*(2), 193–206.

Madany, M. M. Y., & Saleh, A. M. (2015). Phytotoxicity of Euphorbia helioscopia L. on *Triticum aestivum* L. and *Pisum sativum* L.. Annals of Agricultural Sciences, 60(1), 141–151.

Mansour, E. H., & El-Adawy, T. A. (1994). Nutritional potential and functional properties of heat-treated and germinated fenugreek seeds. *LWT-Food Science and Technology*, *27*(6), 568–572.

Mathern, J. R., Raatz, S. K., Thomas, W., & Slavin, J. L. (2009). Effect of fenugreek fiber on satiety, blood glucose and insulin response and energy intake in obese subjects. *Phytotherapy Research*, *23*(11), 1543–1548.

Murray, V., & Ebi, K. L. (2012). IPCC special report on managing the risks of extreme events and disasters to advance climate change adaptation (SREX). *Journal of Epidemiology and Community Health*, *66*, 759–760.

Naidu, M. M., Shyamala, B. N., Naik, J. P., Sulochanamma, G., & Srinivas, P. (2011). Chemical composition and antioxidant activity of the husk and endosperm of fenugreek seeds. *LWT-Food Science and Technology*, *44*(2), 451–456.

Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, *95*(2), 351–358.

Pérez-López, U., Robredo, A., Lacuesta, M., Sgherri, C., Muñoz-Rueda, A., Navari-Izzo, F., & Mena-Petite, A. (2009). The oxidative stress caused by salinity in two barley cultivars is mitigated by elevated CO_2. *Physiologia Plantarum*, *135*(1), 29–42.

Roberfroid, M. B. (1999). What is beneficial for health? The concept of functional food. *Food and Chemical Toxicology*, *37*(9–10), 1039–1041.

Roberts, M. F. (2013). *Alkaloids: biochemistry, ecology, and medicinal applications*. Springer Science & Business Media.

Saleh, A. M., Selim, S., Jaouni, S. Al, & AbdElgawad, H. (2018). CO_2 enrichment can enhance the nutritional and health benefits of parsley (*Petroselinum crispum* L.) and dill (*Anethum graveolens* L.). *Food Chemistry*, *269*(February), 519–526.

Sosnowska, D., Podsędek, A., Redzynia, M., & Kucharska, A. Z. (2018). Inhibitory effect of black chokeberry fruit polyphenols on pancreatic lipase – Searching for most active inhibitors. *Journal of Functional Foods*, *49*(June), 196–204.

Sreevidya, N., & Mehrotra, S. (2003). Spectrophotometric method for estimation of alkaloids precipitable with Dragendorff's reagent in plant materials. *Journal of AOAC International*, *86*(6), 1124–1127.

Suciu, C. F., Prete, M., Ruscitti, P., Favoino, E., Giacomelli, R., & Perosa, F. (2018). Oxidized low density lipoproteins: the bridge between atherosclerosis and autoimmunity. Possible implications in accelerated atherosclerosis and for immune intervention in autoimmune rheumatic disorders. *Autoimmunity Reviews*.

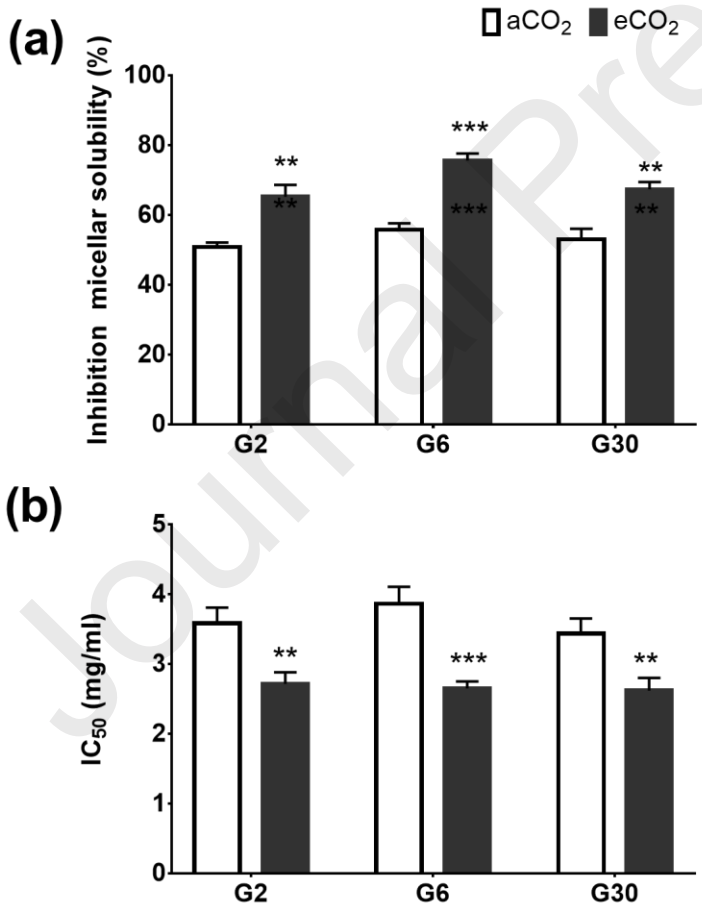
Sugiyama, H., Akazome, Y., Shoji, T., Yamaguchi, A., Yasue, M., Kanda, T., & Ohtake, Y. (2007). Oligomeric procyanidins in apple polyphenol are main active components for inhibition of pancreatic lipase and triglyceride absorption. *Journal of Agricultural and Food Chemistry*, *55*(11), 4604–4609.

Wang, S. Y., Bunce, J. A., & Maas, J. L. (2003). Elevated carbon dioxide increases contents of antioxidant compounds in field-grown strawberries. *Journal of Agricultural and Food Chemistry*, *51*(15), 4315–4320.

Watanabe, C. K., Sato, S., Yanagisawa, S., Uesono, Y., Terashima, I., & Noguchi, K. (2014). Effects of elevated CO_2 on levels of primary metabolites and transcripts of genes encoding respiratory enzymes and their diurnal patterns in arabidopsis thaliana: Possible relationships with respiratory rates. *Plant and Cell Physiology*, *55*(2), 341–357.

Xavier, J. de A., Valentim, I. B., Camatari, F. O. S., de Almeida, A. M. M., Goulart, H. F., Ferro, J. N. de S., … Goulart, M. O. F. (2017). Polyphenol profile by UHPLC-MS/MS, anti-glycation, antioxidant and cytotoxic activities of several samples of propolis from the northeastern semi-arid region of Brazil. *Pharmaceutical Biology*, *55*(1), 1884–1893.

Yadav, U. C. S., & Baquer, N. Z. (2014). Pharmacological effects of *Trigonella foenum-graecum* L. in health and disease. *Pharmaceutical Biology*, *52*(2), 243–254. https://doi.org/10.3109/13880209.2013.826247



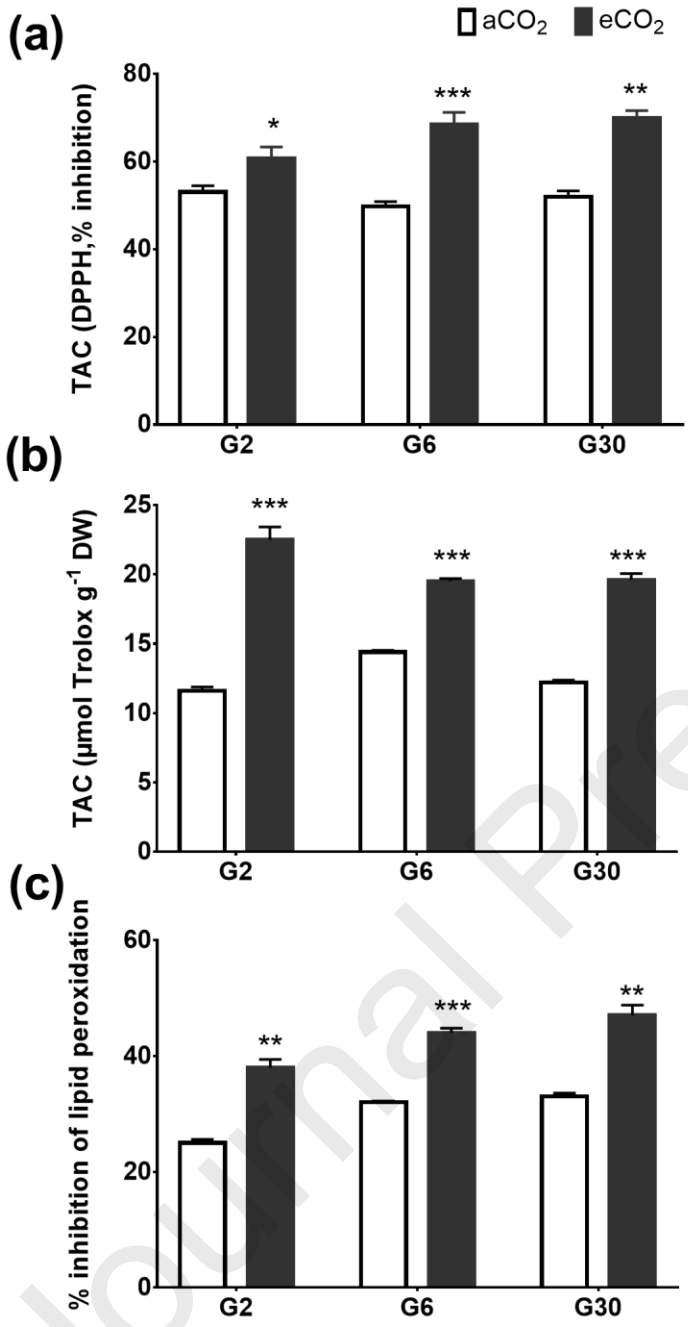


Figure captions

Figure 1. Inhibition of micellar solubility of cholesterol (a) and IC_{50} values against pancreatic lipase activity (b) of fenugreek seeds produced under two levels of CO_2, ambient (400 µmol CO_2 mol^{-1} air, aCO_2) and elevated (620 µmol CO_2 mol^{-1} air, eCO_2). Values are mean ± standard error

of three independent replicates. Asterisks indicate significant differences from the respective aCO_2 control as revealed by Student *t* test, $^{**}P < 0.01$, $^{***}P < 0.001$.

Figure 2. DPPH radical scavenging capacity (a), FRAP (b) and anti-lipid peroxidation activity (c) of fenugreek seeds produced under two levels of CO_2, ambient (400 µmol CO_2 mol^{-1} air, aCO_2) and elevated (620 µmol CO_2 mol^{-1} air, eCO_2). Values are mean ± standard error of three independent replicates. Asterisks indicate significant differences from the respective aCO_2 control as revealed by Student *t* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 1. Physical and chemical parameters of fenugreek seeds produced under two levels of CO_2, ambient (400 µmol CO_2 mol^{-1} air, aCO_2) and elevated (620 µmol CO_2 mol^{-1} air, eCO_2).

Parameter	**G2**		**G6**		**G30**	
	aCO_2	**eCO_2**	**aCO_2**	**eCO_2**	**aCO_2**	**eCO_2**
Physical parameters						
Seed length (mm)	2.40±0.14	3.13±0.26*	2.07±0.02	2.94±0.12*	2.77±0.14	3.58±0.10*
Seed width (mm)	1.81±0.06	2.14±0.04*	2.23±0.10	2.66±0.10	1.63±0.08	2.49±0.10*
Seed thickness (mm)	0.72±0.04	0.97±0.02*	0.89±0.02	1.08±0.02*	0.92±0.02	1.06±0.02*
Seed mass (mg/seed)	6.25±0.14	7.93±0.14*	5.86±0.26	6.96±0.30*	5.76±0.16	7.94±0.26*
Pod length (cm)	6.84±0.12	9.93±0.48*	6.81±0.12	10.99±0.4**	8.03±0.14	11.19±0.20**
Seed yield (mg/pod)	77.2±2.45	131.1±.8.03*	60.1±2.12	92.3±6.60*	76.0±3.01	119.6±5.30*
Chemical parameters (mg/g)						
Total sugar	302.4±10.80	398.8±11.2*	333.5±14.62	501±23.40**	297.1±9.80	423.1±18.80**
Total protein	227.8±11.20	217.9±15*	210.2±10.2	213.8±9.80*	251.7±6.20	248.1±12.20**
Total phenols	12.90±0.44	21.20±1.10*	9.80±0.32	18.91±1.00**	10.63±0.20	17.32±0.60**
Total flavonoids	0.22±0.02	0.53±0.02*	0.43±0.06	0.75±0.00*	0.36±0.01	0.81±0.06**
Total alkaloid	21.9±0.62a	32.1±0.96**	22.9±0.68	42.1±1.2**	31±1.02	37.5±1.1*
Total saponins	14.8±0.44a	15.7±1.00	9.8±0.24	17.7±0.6**	12.6±0.40	18.6±0.56*

Values are mean ± standard error of three independent replicates. Asterisks indicate significant differences from the respective aCO_2 control as revealed by Student *t* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 2. Concentrations of individual fatty acids, vitamins (µg g^{-1} dry weight), phenolic acids, flavonoids (mg g^{-1} dry weight) in seeds of fenugreek plants grown under two levels of CO_2, ambient (400 µmol CO_2 mol^{-1} air, aCO_2) and elevated (620 µmol CO_2 mol^{-1} air, eCO_2).

	G2		**G6**		**G30**	
	aCO_2	**eCO_2**	**aCO_2**	**eCO_2**	**aCO_2**	**eCO_2**
Fatty acids						
C12:0	0.02±0.00	0.05±0	0.03±0a	0.04±0	0.03±0	0.09±0**
C14:0	0.43±0.06	0.37±0.02	0.36±0.02	0.38±0.04	0.37±0.02	0.35±0.02
C15:0	0.18±0.02	0.38±0.04*	0.21±0.02	0.36±0.02*	0.23±0	0.22±0.04
C16:0	5.30±0.32	5±0.2	6.9±0.32	6.4±0.22	7.1±0.26	6.5±0.24
C17:0	0.58±0.04	0.6±0.02	0.63±0.04	1.45±0.08**	0.56±0.02	0.58±0.02
C18:0	3.20±0.20	4±0.22	3.6±0.14	3.5±0.22	3.2±0.12	4.7±0.28*
C20:0	1.30±0.06	1.9±0.1*	1.7±0.16	3.5±0.26*	1.4±0.1	2.4±0.22
C22:0	0.41±0.02	0.55±0.04	0.7±0.02	1.09±0.1	0.64±0	0.83±0.04*
C24:0	0.15±0.02	0.37±0.04*	0.25±0	0.33±0.06	0.23±0	0.47±0.04*
Total SFAs	11.6±0.46	13.2±0.14	14.5±0.32	17.1±0.5	13.7±0.3	16.1±0.54
C12:1	0.07±0.02	0.07±0.00	0.07±0	0.12±0*	0.07±0	0.07±0
C16:1	0.17±0.00	0.37±0.02***	0.17±0	0.38±0.02**	0.2±0	0.43±0.02**
C17:1	0.13±0.02	0.32±0.02*	0.22±0.02	0.32±0.02	0.16±0	0.19±0.02
C18:1	8.21±0.26	12.29±0.36**	11.81±0.48	18.82±0.3***	11.51±0.32	18.47±0.5***
C20:1	0.04±0.00	0.03±0.00	0.03±0	0.04±0	0.04±0	0.07±0*
Total MUFAs	8.60±0.26	13.1±0.36**	12.3±0.48	19.7±0.32***	12.2±0.52	19.1±0.52***
C18:2	35.16±1.82	49.92±2.54*	39.13±1.56	54±1.7*	34.71±1.4	48.56±1.04**
C18:3	15.92±0.62	20.42±0.98	13.24±0.64	20.82±0.74**	13.63±0.34	19.27±0.72*
C20:2	0.15±0.02	0.15±0.00	0.14±0	0.15±0	0.14±0	0.14±0
Total PUFAs	51.2±2.10	70.5±1.86*	52.5±1.52	75±2.04**	48.5±1.28	68±1.12***
SFA/UFA	0.194	0.158	0.224	0.181	0.226	0.185
Vitamins						

Vitamin A						
α-Carotene	0.58±0.04	0.79±0.08	0.33±0.04	1.15±0.1**	0.49±0.04	1.03±0.1*
β-Carotene	0.15±0.01	0.73±0.1*	0.31±0.06	0.24±0.02	0.21±0.02	0.48±0.06*
β-Cryptoxanthin	0.08±0.01	0.29±0.06*	0.10±0.01	0.24±0.02*	0.07±0.01	0.16±0.02
Vitamin B (Thiamine)	0.06±0	0.1±0.02	0.05±0	0.07±0	0.06±0	0.36±0.06*
Vitamin C (Ascorbic Acid)	1.57±0.04	2.87±0.18**	1.71±0.08	2.85±0.06***	1.86±0.04	2.83±0.12***
Vitamin E (Tocopherol)	0.72±0.04	1.2±0.08*	0.78±0.06	1.21±0.06*	0.82±0.06	1.36±0.08*
Vitamin K (Phylloquinone)	0.21±0.02	0.46±0.04***	0.16±0.02	0.25±0.02**	0.13±0.02	0.21±0.02**
Phenolic acids						
Galic acid	5.32±0.31	6.87±0.29*	6.75±0.18	9.05±0.68**	8.16±0.28	8.52±0.23
Ferulic acid	0.04±0.00	0.05±0.00**	0.03±0.00	0.04±0.00**	0.02±0.00	0.11±0.00**
p-Coumaric acid	1.04±0.15	1.09±0.05	1.65±0.11	2.1±0.15*	1.67±0.15	1.79±0.10
Caffeic acid	3.84±0.18	4.15±0.25	3.21±0.18	3.3±0.20	2.56±0.11	2.93±0.10*
Catechin	1.53±0.13	1.49±0.15	0.57±0.10	0.85±0.03	0.72±0.05	0.96±0.03**
Flavonoids						
kaempferol	0.82±0.01	1.33±0.05*	1.43±0.05	1.41±0.08	1.41±0.08	1.46±0.08
Quercetin	1.85±0.13	2.52±0.05**	1.41±0.08	1.98±0.08**	0.98±0.10	1.26±0.06*
Luteolin	0.06±0.00	0.08±0.00**	0.05±0.00	0.08±0.00*	0.08±0.00	0.10±0.00
Rutin	1.29±0.08	1.31±0.05	0.99±0.05	1.00±0.05	0.71±0.03	0.75±0.03
Tricin	1.04±0.10	2.37±0.28*	0.71±0.05	1.25±0.05**	0.63±0.00	0.83±0.03***
vitexin	0.55±0.03	0.74±0.00**	0.42±0.03	0.63±0.03**	0.37±0.03	0.52±0.03**
Myricetin	0.64±0.05	1.25±0.10*	0.46±0.03	0.76±0.03**	0.40±0.02	0.58±0.03**
Apigenin	0.19±0.03	0.26±0.00**	0.15±0.01	0.20±0.00**	0.08±0.01	0.12±0.00*
Naringenin	0.93±0.20	1.19±0.13	0.56±0.05	1.55±0.08***	0.62±0.05	0.83±0.08*

Values are mean ± standard error of three independent replicates Asterisks indicate significant differences from the respective aCO_2 control as revealed by Student *t* test, **P* < 0.05, ***P* < 0 .01, ****P* < 0 .001.

Table 3. Antibacterial activity (diameter of inhibition zone, mm) of fenugreek seeds produced under two levels of CO_2, ambient (400 µmol CO_2 mol^{-1} air, aCO_2) and elevated (620 µmol CO_2 mol^{-1} air, eCO_2).

Bacterial species	**G2**		**G6**		**G30**	
	aCO_2	**eCO_2**	**aCO_2**	**eCO_2**	**aCO_2**	**eCO_2**
Gram-positive						
Bacillus subtilis	15.63±1.23	29.67±4.61**	18.97±1.03	23.63±1.05*	25.77±1.79	31.53±0.28*
Streptococcus sp.	20.67±1.55	35.20±2.01**	14.80±0.62	32.67±2.37**	20.60±0.4	31.77±2.41*
Sarcina lutea	18.60±2.01	31.67±1.05**	20.71±0.92	23.11±2.05	26.73±2.37	27.10±3.11
Staphylococcus aureus	18.13±1.00	24.23±0.86**	21.93±2.06	31.17±1.72*	19.57±1.67	34.17±2.20*
Gram-negative						
Escherichia coli	17.50±1.25	31.53±0.93***	14.33±1.01	21.43±0.88**	28.91±1.62	34.43±1.59*
Pseudomonas aeruginosa	16.83±1.41	26.10±2.58*	16.30±1.65	18.83±1.82	22.12±3.29	24.07±2.28

Values are mean ± standard error of three independent replicates. Asterisks indicate significant differences from the respective aCO_2 control as revealed by Student *t* test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- eCO_2 treatment supports the hypocholesterolaemic potential of fenugreek seeds.
- eCO_2-treated seeds are more potent in inhibiting cholesterol micellar solubility.
- Inhibitory action of fenugreek seeds against pancreatic lipase is promoted by eCO_2.
- eCO_2 induces the accumulation of phenolics, flavonoids, saponins and alkaloids.