

***In vitro* investigations of the potential health benefits of Australian-grown faba beans (*Vicia faba* L.): chemopreventative capacity and inhibitory effects on the angiotensin-converting enzyme, α -glucosidase and lipase**

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(Submitted 6 September 2011 – Accepted 26 October 2011)

Abstract

The functional properties, including antioxidant and chemopreventative capacities as well as the inhibitory effects on angiotensin-converting enzyme (ACE), α -glucosidase and pancreatic lipase, of three Australian-grown faba bean genotypes (*Nura*, *Rossa* and *TF(Ic*As)*483/13*) were investigated using an array of *in vitro* assays. Chromatograms of on-line post column derivatisation assay coupled with HPLC revealed the existence of active phenolics (hump) in the coloured genotypes, which was lacking in the white-coloured breeding line, *TF(Ic*As)*483/13*. Roasting reduced the phenolic content, and diminished antioxidant activity by 10–40% as measured by the reagent-based assays (diphenylpicrylhydrazyl, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) and oxygen radical absorbance capacity) in all genotypes. Cell culture-based antioxidant activity assay (cellular antioxidant activity) showed an increase of activity in the coloured genotypes after roasting. Faba bean extracts demonstrated cellular protection ability against H₂O₂-induced DNA damage (assessed using RAW264.7 cells), and inhibited the proliferation of all human cancer cell lines (BL13, AGS, Hep G2 and HT-29) evaluated. However, the effect of faba bean extracts on the non-transformed human cells (CCD-18Co) was negligible. Flow cytometric analyses showed that faba bean extracts successfully induced apoptosis of HL-60 (acute promyelocytic leukaemia) cells. The faba bean extracts also exhibited ACE, α -glucosidase and pancreatic lipase inhibitory activities. Overall, extracts from *Nura* (buff-coloured) and *Rossa* (red-coloured) were comparable, while *TF(Ic*As)*483/13* (white-coloured) contained the lowest phenolic content and exhibited the least antioxidant and enzyme inhibition activities. These results are important to promote the utilisation of faba beans in human diets for various health benefits.

Key words: Faba beans: Phenolic compounds: Antioxidants: Health benefits

Faba beans (*Vicia faba* L.), also commonly known as fava, horse and broad beans, are widely consumed in different parts of the world including Egypt, Sudan, The Netherlands, Spain, Saudi Arabia, India and China. Their seed coat colours range from white, buff (or beige), purple, green to red, with the buff-coloured beans being the most commonly accepted for human consumption. In 2008, the worldwide production of dry faba beans was approximately 4.1 million tonnes, and the export trade was valued at approximately US\$291 million⁽¹⁾.

Faba beans are grown in Australia as a break crop and exported to the Middle East and Asia⁽²⁾, with export trade valued at approximately US\$58 million in 2008⁽¹⁾.

Pulses are well known to be an economical source of protein, carbohydrate and fibre, and are low in fat. Pulses are also incorporated in human diets for their additional nutritional benefits, especially their microconstituents including phenolic compounds, oligosaccharides⁽³⁾, enzyme inhibitors, phytosterols and saponins^(4,5). Intake of legumes is reported to potentially

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); ACE, angiotensin-converting enzyme; CAA, cellular antioxidant activity; DPPH, diphenylpicrylhydrazyl; EC₅₀, half maximal effective concentration; EMEM, Eagle's minimum essential medium; IC₅₀, half maximal inhibitory concentration; IMDM, Iscove's modified Dulbecco's medium; ORAC, oxygen radical absorbance capacity; TEAC, Trolox equivalent antioxidant capacity; TFC, total flavonoid content; TPC, total phenolic content.

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lower the risk of cancer⁽⁶⁾, CVD⁽⁷⁾, hypertension and diabetes⁽⁸⁾. Some of the microconstituents are currently marketed as functional foods and nutraceutical ingredients⁽⁹⁾. Also, there have been many attempts to incorporate pulses into food products for enrichment of product quality and additional health benefits^(10,11).

A wide range of methods are used to prepare faba beans including soaking, boiling and roasting. Heating was reported to result in significant decreases in polyphenols, enzyme inhibitors, phytic acid, some minerals and vitamins, but increase protein digestibility of faba beans^(12,13). Interestingly, Acar *et al.*⁽¹⁴⁾ reported that roasting at 150°C for 60 min increased the antioxidant capacity of different types of pulses including black bean, borlotti bean, kidney bean, red soybean, yellow bean, giant lentils and chickpea, with an initial fall observed in the yellow and red soybeans after roasting for 10 min. Comparatively, in faba beans, the tannin content increased after roasting at 149°C/20 min and 177°C/18 min, but decreased after roasting at 204°C/14 min and 232°C/12 min⁽¹⁵⁾.

Phenolic compounds are one of the microconstituents which have been gaining an increasing interest for their health-promoting properties, largely defined by their antioxidant activity. Previous research has reported different types of phenolic compounds found in faba beans, such as procyanidins^(16–18), catechins⁽¹⁹⁾, flavonols⁽²⁰⁾, isoflavones⁽²¹⁾, phenolic acids⁽²²⁾ and tannins^(23–25), which are natural antioxidants⁽²⁶⁾. Phenolic compounds extracted from a variety of plant materials have been reported to have an ability to inhibit carbohydrate and lipid digestion, therefore preventing them from absorption. These could potentially lower the postprandial hyperglycaemic response and contribute towards weight maintenance^(27,28).

Phenolic extracts of different types of beans were found to have antioxidant activities⁽²⁶⁾, protective effects against radical-induced DNA damage⁽²⁹⁾, as well as antimutagenic⁽³⁰⁾ and anticancer⁽³¹⁾ properties. Many have reported substantial amounts of phenolic compounds in raw⁽³²⁾ and cooked faba beans⁽³³⁾, but limited reports focus on the health benefits of faba bean phenolic compounds and also on the impact of food preparation heat processes on the retention and activities of phenolic compounds. The present study hence aims to investigate *in vitro* the potential health benefits of crude extracts from raw and cooked faba beans in the prevention of chronic diseases including hypertension, diabetes, obesity and different types of cancers. The results could support increased consumption of faba beans and the development of new food products using faba beans, enhancing the exploitation of the crop and providing better returns to growers.

Materials and methods

Plant materials

For the purpose of the study, three faba bean genotypes including cv. *Nura* (buff-coloured), cv. *Rossa* (red-coloured) and breeding line *TF(Ic*As)*483/13* (white-coloured) were grown at the Wagga Wagga Agricultural Institute experimental field in NSW, Australia in 2008. Harvested beans were air-dried and then stored at –20°C until analysis.

Dry roasting

Roasting was performed at 150°C using dry heat in an oven (Premium Laboratory Oven, Thermoline Scientific) for 1 h (approximately 50 g per batch in a single layer on a foil tray and agitated gently after 30 min for uniform heating). Roasted beans were cooled at room temperature and ground into flour using an IKA-Universalmühle M20 Grinder (Janke and Kunkel).

Preparation of phenolic extracts

Extraction of phenolic compounds was carried out by dispersing the flour in aqueous acetone (acetone–water, 70:30, v/v)⁽¹⁶⁾ in a solid:solvent ratio of 1:10 and shaking for 2 h at room temperature. The supernatant was collected after centrifugation at 4000 g using an Eppendorf 5415D Centrifuge (Eppendorf-Netheler-Hinz) for 5 min at 5°C. A second extraction was performed on the residue and the extracts were pooled, concentrated under reduced pressure at 40°C using a rotary evaporator (Rotavapor R-205; Buchi) and then freeze-dried using a Christ-Alpha 1–4 freeze dryer (Biotech International). The extracts were stored at –20°C until used. Distilled water was used to dissolve the dried extract and the reconstituted extracts were filtered through 0.45 µm Millipore filters (Millipore Australia Pty Ltd, Australia) before analysis. All extractions and measurements were performed at least in triplicate, except extractions from the raw beans which were performed in duplicate.

Total phenolic content, total flavonoid content and antioxidant capacity assays

The total phenolic content (TPC) assay was conducted according to Konczak *et al.*⁽³⁴⁾. The total flavonoid content (TFC), diphenylpicrylhydrazyl (DPPH) radical scavenging capacity and Trolox equivalent antioxidant capacity (TEAC) assays were performed as described by Michalska *et al.*⁽³⁵⁾. The oxygen radical absorbance capacity (ORAC) assay was carried out as described by Prior *et al.*⁽³⁶⁾.

Preparation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) free radical for post column derivatisation assay

The 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS)⁺ radical cation was prepared by dissolving ABTS (Sigma Aldrich) in deionised water (7 mM) and mixed with 2.45 mM of potassium persulphate overnight to allow complete reaction. The solution was diluted using distilled water to obtain an absorbance of 0.70 (SD 0.02) at 734 nm and filtered through a polypropylene membrane (0.45 µm)⁽³⁷⁾.

On-line post column derivatisation with HPLC

Analysis of the antioxidant activity was carried out on-line using ABTS cation radical. The HPLC system (ProStar model 410, Varian, Inc., Australia) consisted of a Phenomenex Luna 5U C18 column (100A pore size; 150 × 3 mm), preceded by a guard column (Phenomenex, 4 × 3 mm), a Varian 240I pump



and a Varian 335 PDA Detector. The mobile phase A was water–acetic acid (99:1; v/v) and phase B was methanol–acetonitrile (50:50; v/v). An aliquot (8 μ l) of the extract sample (50 mg/ml) dissolved in solvent A was injected and eluted in a gradient of 0–48% phase B for 40 min at a flow-rate of 0.4 ml/min. UV spectra were recorded at 280 nm. Post column antioxidant activity on-line was determined on the HPLC eluent from the system which arrived at a 'T' piece and reacted with ABTS⁺ that was added at a flow rate of 0.4 ml/min. The absorbance of the reaction products was measured by a UV–Vis detector (Model 9050, Varian, Inc., Australia) at 414 nm.

Cell cultures

All cells lines were purchased from the American Type Culture Collection except the BL13 (human bladder transitional cell carcinoma) cells which were obtained from Dr D. Brookes⁽³⁸⁾. All cells were cultured at 37°C in a humidified 5% CO₂–95% air atmosphere. BL13 cells were cultured in Roswell Park Memorial Institute medium (Invitrogen Corporation); AGS (gastric adenocarcinoma) in F-12K Ham's medium (Invitrogen Corporation); Hep G2 (hepatocellular) in Eagle's minimum essential medium (EMEM; Sigma-Aldrich); HT-29 (colorectal adenocarcinoma) in McCoy's 5a modified medium (Invitrogen Corporation); RAW264.7 (macrophage; Abelson murine leukaemia virus-induced tumour) in Dulbecco's modified Eagle's medium (Sigma-Aldrich); HL-60 (acute promyelocytic leukaemia) in Iscove's modified Dulbecco's medium (IMDM; Invitrogen Corporation) and CCD-18Co (colon normal) cells were cultured in EMEM. Each medium was supplemented with 100 μ g/ml streptomycin and 1000 units/ml penicillin (Invitrogen Corporation) and 10% fetal calf serum, with the exception of IMDM for HL-60 which required 20% serum.

Cellular antioxidant activity assay

The assessment of cellular antioxidant activity (CAA) was determined according to Tan *et al.*⁽³⁹⁾ and Wolfe & Liu⁽⁴⁰⁾. Briefly, 1×10^5 /ml of Hep G2 cells were plated in a ninety-six-well microplate and incubated for 24 h. Next, the medium was removed and the cells were washed using PBS. The cells were then treated with different concentrations of faba bean extracts (10 μ l in PBS), added to 80 μ l of PBS, followed by the addition of 2',7'-dichlorofluorescein-diacetate (Sigma-Aldrich) (10 μ l) (250 μ M in PBS), and incubated for 1 h. Subsequently, the cells were washed using PBS (100 μ l) and added to 100 μ l of 2,2'-azobis (2-amidinopropane) dihydrochloride in Hank's balanced salt solution (600 μ M). The fluorescence was measured at 485 nm excitation and 538 nm emission wavelengths for every 5 min in 1 h. The final fluorescence values were corrected for the blank sample readings, and a time *v.* fluorescence graph was plotted. A quercetin standard was used to express the results as quercetin equivalents per g of dry weight of beans.

Antiproliferation assay

The assessments of antiproliferation activity of the faba bean crude extracts were carried out on the adhesive cells, BL13,

AGS, Hep G2 and HT-29 using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay, as outlined by Tan *et al.*⁽³⁹⁾. PBS was used to dissolve the sample extracts.

Assessment of apoptosis and cytolysis by flow cytometry

Suspensions (4.5 ml) of HL-60 cells ($2.5\text{--}5 \times 10^5$ /ml) were treated with 225 μ l (0.8 mg/ml) of extracts for 3, 12 and 24 h in 25 cm² culture flasks in triplicate. Untreated cells were used as a control. For the dose–response evaluation, the cells were treated with three different extract concentrations (0.4, 0.8 and 1.6 mg/ml in PBS) for 24 h. Cells were stained with Annexin V-Alexa Fluor 488 annexin V/Dead cell apoptosis kit with Alexa Fluor 488 annexin V and PI for flow cytometry (Invitrogen Corporation) according to the manufacturer's directions. After the set incubation time, the cells were harvested, washed with cold PBS and resuspended in Annexin-binding buffer. Following this, 100 μ l of cells were stained by adding 5 μ l of Annexin V and 1 μ l of propidium iodine and incubated for 10 min at room temperature. Next, the cells were mixed with 400 μ l of Annexin-binding buffer and immediately placed on ice. Analysis was performed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson) and FlowJo software (TreeStar Inc.) to determine the extent of cell apoptosis and lysis. From 3000 to 10 000 events were acquired for each measurement and the cell populations were gated for analysis.

Cellular protection against H₂O₂

The cellular protection against H₂O₂ assay was carried out using RAW264.7 cells according to Tan *et al.*⁽³⁹⁾, except that the concentration of H₂O₂ used was 40 mM.

Angiotensin-converting enzyme inhibition assay

The angiotensin-converting enzyme (ACE) inhibition assay was carried out as described in Shalaby *et al.*⁽⁴¹⁾ using furanacroyl-Phe-Glu-Glu as substrates. Results were expressed as μ g of captopril equivalents/g of dry weight of beans.

α -Glucosidase inhibition assay

The α -glucosidase inhibition was determined as described by Ikarashi *et al.*⁽²⁷⁾ using sucrose as substrates, with slight modifications. An α -glucosidase enzyme solution was prepared by dissolving 100 mg of intestinal acetone powders from rat (Sigma-Aldrich) in 1 ml of 0.1 M maleate buffer (pH 6) and homogenised using an ultrasonicator for 6 min on a 30 s rest cycle. The enzyme solution was centrifuged at 3000 g for 30 min and the supernatant was diluted to 1:2 (v/v) using the buffer solution. Sample solutions (20 μ l) were mixed with 2% sucrose (w/v) in maleate buffer (20 μ l). The enzymatic reaction was initiated by adding enzyme solution (20 μ l) to the mixture and incubated at 37°C for 60 min. The enzymatic reaction was terminated by heating at 100°C for 10 min. Sample mixture (20 μ l) was then used to react with the colour reagent (Glucose CII-Test Wako, Wako Pure Chemical Industries) (3 ml) at 37°C

for 5 min and the absorbance was measured at 505 nm. Negative controls were prepared as described by replacing the sample solution with the buffer solution, whereas control and sample blanks were prepared by replacing the enzyme and sucrose solutions with the buffer solutions. The relative α -glucosidase inhibition was calculated using the following formula:

$$\text{Percentage of inhibition} = \frac{(A_{CB} - A_C) - (A_{SB} - A_S)}{(A_{CB} - A_C)} \times 100$$

where A_S and A_C were the absorbance of sample and negative control, and A_{SB} and A_{CB} were the absorbance of sample and control blanks.

Lipase inhibition assay

The lipase inhibitory activity was assayed as described by Shimura *et al.*⁽²⁸⁾ using 4-methylumbelliferyl oleate as the substrate, except that the porcine pancreatic lipase (Sigma type II) was prepared using a concentration of 0.085 g/ml. The relative lipase inhibition activity was calculated using the following formula:

$$\text{Percentage of inhibition} = (1 - (F_S - F_{SB}) / (F_C - F_{CB})) \times 100$$

where F_S and F_C were the values of samples and negative control measured fluorometrically at an emission wavelength of 450 nm and excitation of 320 nm by a Varian Cary Eclipse

Fluorescence Spectrophotometer (Agilent Technologies), and F_{SB} and F_{CB} were the fluorescence readings of sample and control blanks.

Statistical analysis

The significant differences between mean values were calculated based on at least three independent evaluations ($n = 3$) and the standard deviations were also calculated. Student's t test was conducted to assess differences between the samples at the level of $P < 0.05$. All half maximal inhibitory concentration (IC_{50}) values were calculated from the corresponding dose inhibition curve according to their best-fit shapes based on at least four reaction points using Microsoft Excel (Microsoft Corp, USA). Statistical correlation analyses were performed using Graphpad Prism 5 (Graphpad Software). Results for correlation analysis were considered statistically significant when $P < 0.05$.

Results and discussion

Total phenolic content, total flavonoid content and antioxidant capacities

The effects of roasting on TPC, TFC and antioxidant capacity of the extracts from three faba bean genotypes are presented in Table 1. In comparison to the raw sample, a higher extraction yield was obtained for the roasted samples (approximately 10% higher). The difference between the genotypes with

Table 1. Extraction yield, total phenolic content, total flavonoid content, diphenylpicrylhydrazyl (DPPH) radical scavenging activity, Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) of crude extracts from the raw and roasted faba bean genotypes

(Mean values and standard deviations of at least three independent measurements, $n = 3$)

	Raw bean		Roasted bean	
	Mean	SD	Mean	SD
Extraction yield (mg of extracts/DW)				
<i>Nura</i>	77.06 ^a	0.60	83.92 ^{a,b}	3.19
<i>Rossa</i>	83.19 ^{a,b}	0.34	90.41 ^b	4.83
<i>TF(Ic*As)*483/13</i>	78.70 ^a	0.42	88.51 ^b	1.41
Total phenolic content (mg GAEq/g DW)				
<i>Nura</i>	10.68 ^a	0.32	5.24 ^c	0.62
<i>Rossa</i>	11.26 ^a	0.19	6.38 ^c	1.44
<i>TF(Ic*As)*483/13</i>	2.97 ^b	0.44	3.35 ^b	0.20
Total flavonoid content (mg CEq/g DW)				
<i>Nura</i>	2.85 ^a	0.13	1.77 ^c	0.14
<i>Rossa</i>	2.95 ^a	0.15	2.09 ^d	0.09
<i>TF(Ic*As)*483/13</i>	1.01 ^b	0.01	0.95 ^b	0.11
DPPH radical scavenging activity ($\mu\text{mol TE/g DW}$)				
<i>Nura</i>	53.37 ^a	1.22	34.34 ^d	1.69
<i>Rossa</i>	48.11 ^b	3.16	37.34 ^d	2.45
<i>TF(Ic*As)*483/13</i>	7.47 ^c	0.26	9.59 ^c	0.99
TEAC ($\mu\text{mol TE/g DW}$)				
<i>Nura</i>	72.69 ^a	2.86	49.75 ^c	1.79
<i>Rossa</i>	71.28 ^a	7.72	55.95 ^d	2.79
<i>TF(Ic*As)*483/13</i>	25.63 ^b	0.09	35.81 ^e	3.46
ORAC ($\mu\text{mol TE/g DW}$)				
<i>Nura</i>	109.10 ^{a,b}	11.05	94.43 ^{b,c}	11.02
<i>Rossa</i>	142.80 ^e	11.19	116.90 ^a	9.61
<i>TF(Ic*As)*483/13</i>	78.21 ^{c,d}	4.17	73.91 ^d	4.92

DW, dry weight; GAEq, gallic acid equivalents; CEq, catechin equivalents; TE, Trolox equivalents.

^{a,b,c,d,e} Mean values with unlike superscript letters were significantly different in the respective assays ($P < 0.05$).

regards to the extraction yield was negligible. The level of TPC and TFC of *Rossa* was not significantly different from *Nura* in both of their raw and roasted beans, except that the roasted *Rossa* had slightly higher TFC than the roasted *Nura*. The TPC in the raw beans of coloured genotypes were about four times, while the TFC was about three times higher than *TF(Ic*As)*483/13*. After roasting, the TPC and TFC contents of *Nura* and *Rossa* were about two times higher than *TF(Ic*As)*483/13*. The white-coloured *TF(Ic*As)*483/13* (termed 'tannin free'), was developed for its low tannin content, thus explaining the low TPC and TFC detected in the present study.

Roasting led to a 40–50% decrease in the TPC, and a 30–40% decrease in the TFC of both genotypes, *Nura* and *Rossa*. On the other hand, roasting only reduced the TPC and TFC of *TF(Ic*As)*483/13* by 13 and 7%, respectively. These results support earlier findings that heating applied through various cooking methods decreased the phenolic contents in different types of legumes⁽⁴²⁾.

The antioxidant capacities of faba beans were evaluated using three assays: DPPH, TEAC and ORAC (Table 1). The antioxidant capacities of *Nura* and *Rossa* were comparable in all assays. However, the antioxidant capacity of *TF(Ic*As)*483/13* was lower than the antioxidant capacities of the other two genotypes; about two to three times (ORAC and TEAC assays, respectively) and six times lower (DPPH assay). A significant decrease in the antioxidant capacity of *Rossa* and *Nura* occurred after roasting. However, roasting did not affect the antioxidant activity of *TF(Ic*As)*483/13* significantly as tested by the DPPH and ORAC assays.

A high correlation was observed for the TPC with TFC (0.98, $P < 0.001$) and the antioxidant capacities, as evaluated using DPPH (0.93, $P < 0.01$), TEAC (0.96, $P < 0.01$) and ORAC (0.87, $P < 0.05$) assays. The HPLC-post column derivatisation profiles of crude extracts detectable at 280 nm from the raw beans of three genotypes (Fig. 1) showed that the phenolic compounds eluted in two separate regions, which can be arbitrarily classified as relatively polar (0–15 min) and less polar (15–40 min) regions. The HPLC-post column derivatisation results also showed that most of the antioxidant activities in *Rossa* and *Nura* were contributed by the less polar region. Distinct active peaks in the less polar region were observed in the HPLC chromatograms of *Nura* (Fig. 1(A)) and *Rossa* (Fig. 1(B)). Moreover, traces of anthocyanins were also detected (520 nm, data not presented) in the extract from *Rossa*, which may contribute to the antioxidant capacity. In contrary, the HPLC chromatogram of the extract from *TF(Ic*As)*483/13* (Fig. 1(C)) lacked active compounds in the relatively less polar region. However, the three genotypes appeared to have similar HPLC-post column derivatisation profiles in the relatively polar region.

Faba beans with coloured seed coat were reported to contain low and high molecular weight phenolic compounds such as flavanols and proanthocyanidins⁽¹⁸⁾. The high molecular weight compounds were likely to appear at the relatively less polar region in the HPLC-post column derivatisation profiles (Fig. 1(A) and (B)) and contribute to the antioxidant capacity. High molecular weight phenolic compounds were reported

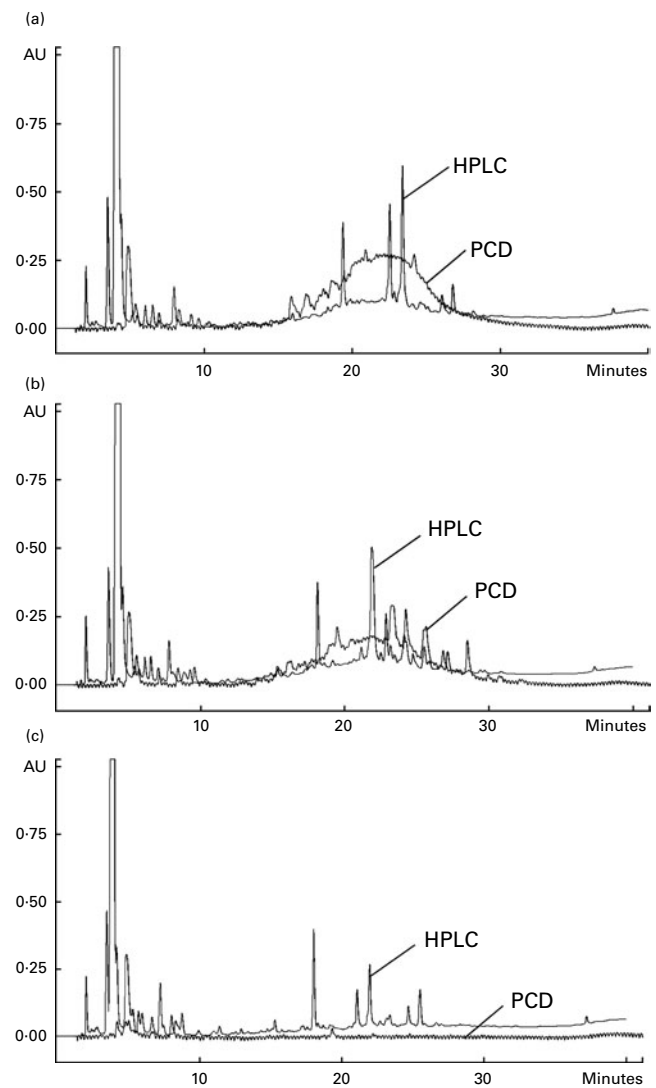


Fig. 1. Chromatograms of HPLC and on-line post column derivatisation (PCD) assay of crude extracts obtained from the raw faba bean genotypes: (A) *Nura*, (B) *Rossa* and (C) *TF(Ic*As)*483/13*.

previously to be very active hydrogen donors, and thereby radical quenchers⁽⁴³⁾. The white-coloured *TF(Ic*As)*483/13* contained noticeably lower TPC and TFC, and subsequently exhibited significantly lower antioxidant capacity. The HPLC chromatogram of extract from *TF(Ic*As)*483/13* (Fig. 1(C)) showed a lack of active phenolic compounds at the less polar region, which might be the polymeric compounds. The present results confirm earlier findings by Madhujith *et al.*⁽²⁹⁾, who also were not able to detect anthocyanidin and procyanidin in the white bean varieties.

Cellular protection by faba bean extracts

Cellular antioxidant activity assay. The CAA assay evaluates antioxidant activity at the cellular level. The final result of this assay depends on uptake, distribution and metabolism of the antioxidant compounds in a live cell. This information cannot

Table 2. Cellular antioxidant activity of the crude extracts obtained from the raw and roasted faba bean genotypes, *Nura* and *Rossa* (Mean values and standard deviations of three independent experiments, *n* 3)

Faba bean genotype	Cellular antioxidant activity			
	$\mu\text{mol QE/g DW}$		EC_{50} ($\mu\text{g/ml}$)*	
	Mean	SD	Mean	SD
Raw bean				
<i>Nura</i>	7.8 ^a	0.5	402.3	27.1
<i>Rossa</i>	15.8 ^a	4.2	208.0	60.4
Roasted bean				
<i>Nura</i>	8.9 ^a	6.5	277.8	69.7
<i>Rossa</i>	13.9 ^a	4.2	241.3	73.1

$\mu\text{mol QE/g DW}$, μmol quercetin equivalents per g of bean (dry weight basis); EC_{50} , half maximal effective concentration.

^a Mean values with same superscript letters were not significantly different.

* Concentration of the lyophilised extract in the culture medium ($\mu\text{g/ml}$) able to scavenge 50% of free radicals effectively within a cell.

be obtained in a reagent-based antioxidant testing. In comparison to animal models, the CAA is a cost-effective and fast way to obtain important information on the efficiency of antioxidants within a cell⁽⁴⁰⁾. Extracts from *Nura* and *Rossa* were evaluated in this study. No significant difference was found between the CAA of extracts obtained from both genotypes, regardless of heat treatment, as expressed in μmol quercetin equivalent/g dry weight of bean (Table 2). However, half maximal effective concentration (EC_{50}) of the roasted *Nura* showed a tendency

for a higher uptake of extracts than its raw beans. The HPLC chromatogram (Fig. 1) demonstrated compositional differences between the compounds detected at 280 nm in extracts from *Nura* and *Rossa*. The 'hump' at the less polar region dominated in extracts from both *Nura* and *Rossa* could be the polymeric compounds, while phenolic acids and flavonols were greater in the extract from *Rossa* (data not presented). A variety of phenolic compositions were likely to contribute to the different cell uptake rates and efficiency of protection against peroxy radicals, within an hour. It can also be speculated that heat application during roasting of faba beans caused a partial oxidation of polymeric compounds which would affect the uptake and reflect on the antioxidant capacity within a cell. The CAA EC_{50} values of the faba beans are slightly lower than those of lentil (670 $\mu\text{g/ml}$), yellow pea (780 $\mu\text{g/ml}$) and green peas (1280 $\mu\text{g/ml}$) as reported in Xu & Chang⁽⁴⁴⁾ assayed using AGS cells.

Cellular protection against H_2O_2 . H_2O_2 is a reactive oxygen species which is present in live cells and is used in experimental models. In this experimental model, we evaluated the protective effect of the faba bean extracts against H_2O_2 -induced apoptosis in RAW264.7 cells (Fig. 2). Extracts obtained from the raw faba bean genotypes, *Nura* and *Rossa*, were applied at concentrations of 0.1–0.4 mg/ml, and exhibited cellular protection against H_2O_2 in a dose-dependent manner (Fig. 2(A) and (C)). However, the protection diminished at concentrations higher than 0.4 mg/ml due to the commencement of

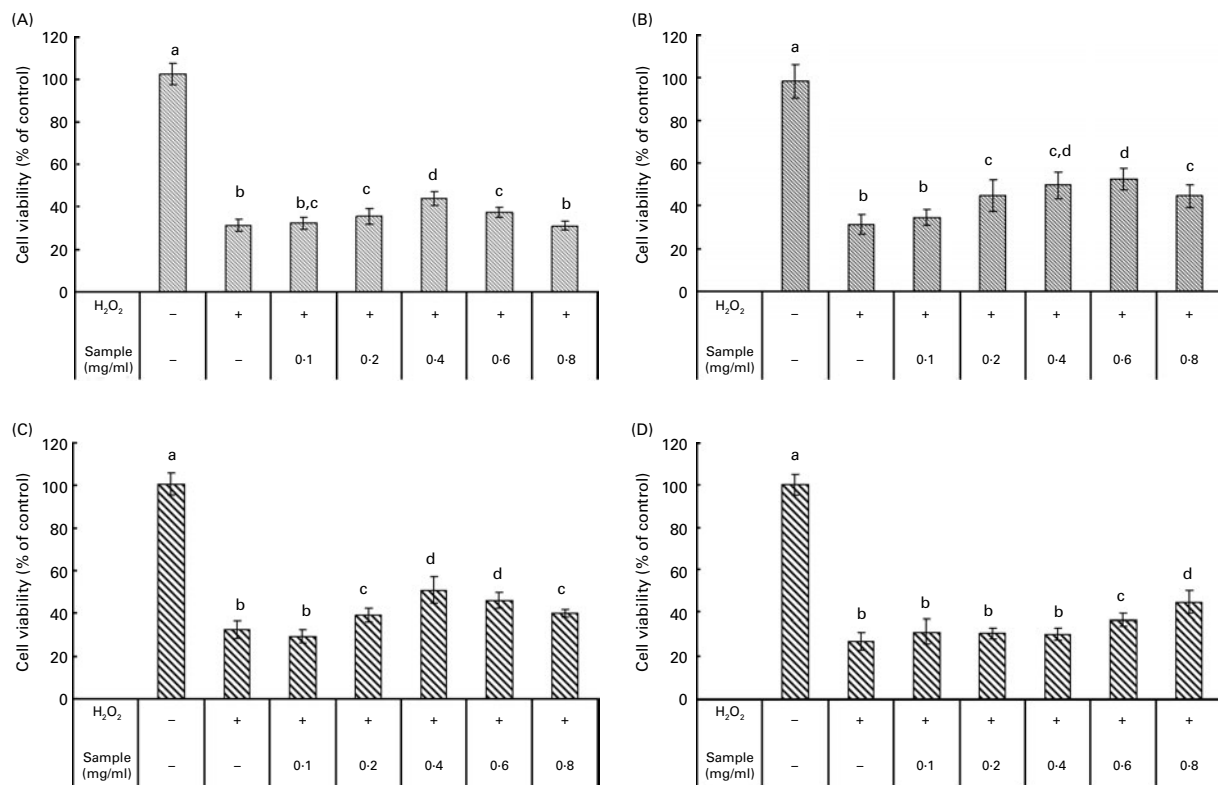


Fig. 2. Cellular protection against H_2O_2 (40 mM) on RAW264.7 cells by crude extracts obtained from the (A) raw and (B) roasted *Nura*; (C) raw and (D) roasted *Rossa*. Values are means of four replicates, with standard deviations represented by vertical bars. ^{a,b,c,d} Mean values with unlike letters were significantly different ($P < 0.05$).

Table 3. Effects of faba bean crude extracts on the proliferation of human cancer cells: AGS, BL13, Hep G2 and non-transformed cells: CCD-18 Co

(Mean values and standard deviations)

Faba bean genotype	IC ₅₀ * (mg/ml)									
	AGS (gastric)		BL13 (bladder)		Hep G2 (liver)		HT-29 (colon)		CCD-18Co (colon)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Raw bean										
<i>Nura</i>	<0.2	–	1.90	0.15	<0.2	–	1.46	0.06	>2	–
<i>Rossa</i>	1.04	0.06	2.33	0.18	1.59	0.06	1.37	0.07	>2	–
Roasted bean										
<i>Nura</i>	1.64	0.05	1.74	0.06	1.55	0.34	1.45	0.06	>2	–
<i>Rossa</i>	2.03	0.05	1.74	0.09	1.60	0.07	1.87	0.04	>2	–

IC₅₀, half maximal inhibitory concentration.

* Results were presented as concentration (mg/ml) of crude extracts in the culture medium needed to achieve suppression of cell growth by 50% (IC₅₀). Sample concentration ranged from 0.2 to 2.0 mg/ml. The results were obtained via nonlinear regression and based on at least four replicates.

antiproliferative effects, or possibly due to the pro-oxidative effects of phenolic compounds at high concentrations. The pro-oxidative effect might be caused by the interaction of the added phenolic compounds with undefined components from the culture media, resulting in the generation of H₂O₂⁽⁴⁵⁾. The same tendency was observed for extracts obtained from the roasted *Nura* (Fig. 2(B)). On the other hand, the protective effects by extracts obtained from the roasted *Nura* and *Rossa* were observed at concentrations at 0.2 and 0.6 mg/ml, respectively (Fig. 2(B) and (D)). In comparison to other results on the protection of RAW264.7 cells from H₂O₂-induced injury, the faba bean extracts appeared less efficient than that of Kakadu plum extract⁽³⁹⁾. However, the crude faba bean extracts were used for this study, whereas purified and concentrated Kakadu plum extracts (using XAD-7 resin column) were used. Chow *et al.*⁽⁴⁶⁾ found that 25 and 50 μM of quercetin (but not rutin and quercitrin) posed potent protection of RAW264.7 cells against H₂O₂-induced injury.

Effects of faba bean extracts on proliferation and apoptosis of cancer cells

The effect of extracts obtained from the raw and roasted faba bean genotypes, *Nura* and *Rossa*, on the proliferation of different types of cancer cells including AGS, HT-29, BL13, Hep G2 and one non-transformed cell line, CCD-18Co, is presented in Table 3. The crude faba bean extracts, applied at a concentration range of 0.2–2.0 mg/ml, exhibited a dose-dependent suppression of all of the tested human cancer cell proliferations, while exhibiting negligible proliferation effect on the non-transformed human colon CCD-18Co cells. Extracts from the raw *Nura* suppressed some proliferation of non-transformed colon cells, CCD-18Co, while the raw *Rossa* did not (Fig. 3(A)). Extracts from raw beans of both genotypes effectively suppressed proliferation of the human colon cancer cells, HT-29 (Fig. 3(B)). On the other hand, extracts from the roasted *Nura* and *Rossa* did not affect the proliferation of the non-transformed CCD-18Co cells (Fig. 4(A)), but effectively suppressed the proliferation of the human colon cancer cells, HT-29 (Fig. 4(B)).

Heating appeared to cause decreases in phenolic contents and antioxidant capacities, which were in line with some but not all of the antiproliferation results. This suggests that different types of phenolic compounds in the faba bean extracts might exert a diverse degree of activities on specific target sites of cells. The IC₅₀ value represents the concentration required to inhibit 50% of cell proliferation, and therefore, a lower IC₅₀ indicates a greater antiproliferation ability. The IC₅₀ values of extract from the raw *Nura* (<0.2 mg/ml) and

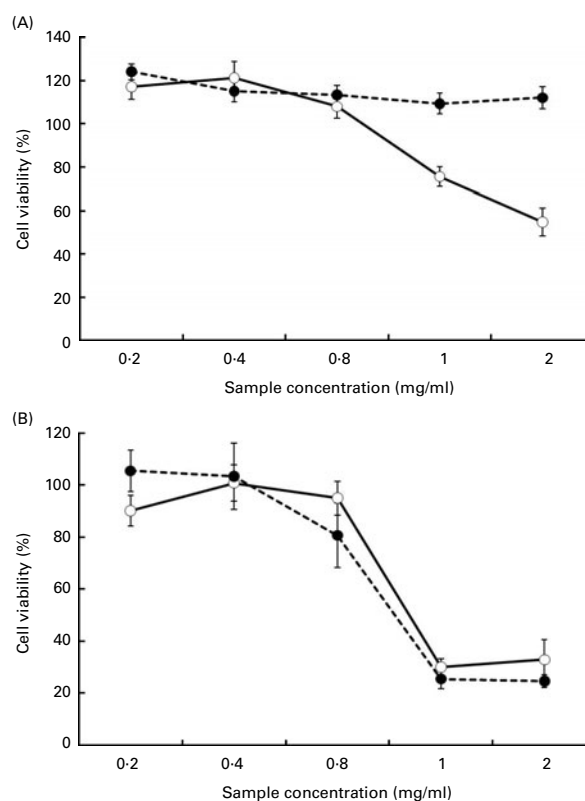


Fig. 3. The effect of crude extracts obtained from the raw faba bean genotypes, *Nura* (○) and *Rossa* (●) on proliferation of (A) non-transformed human colon cells, CCD-18Co and (B) human colon cancer cells, HT-29. Values are means of at least three independent experiments with four replicates, with standard deviations represented by vertical bars, *n* 4.

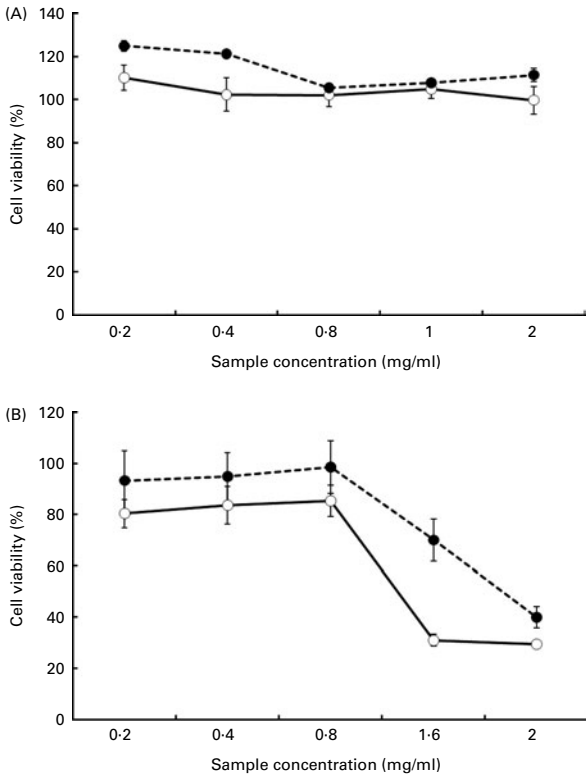


Fig. 4. The effect of crude extracts obtained from the roasted faba bean genotypes, *Nura* (—○—) and *Rossa* (---●---) on proliferation of (A) non-transformed human colon cells, CCD-18Co and (B) human colon cancer cells, HT-29. Values are means of at least three independent experiments with four replicates, with standard deviations represented by vertical bars, *n* 4.

Rossa (1.04 mg/ml) were lower than green pea (3.25 mg/ml), chickpea (3.23 mg/ml) and lentil (1.27 mg/ml) as tested on AGS cells⁽⁴⁴⁾. Seeram *et al.*⁽⁴⁷⁾ found that different cancer cell lines had diverse levels of sensitivity to phenolic compounds extracted from cranberries using different cell viability testing assays. Extracts from the raw *Nura* posed exceptionally high antiproliferative effects on AGS and Hep G2 cells in comparison to the raw *Rossa*. The reason is not known and the results are under further investigation. However, in support of our findings, the IC₅₀ values of six types of berry extracts tested on six different human tumour cell lines were all reported to be less than 0.2 mg/ml⁽⁴⁸⁾.

In order to understand the mechanism behind the suppression of cancer cell proliferation, an investigation to identify apoptotic and necrotic cells within the populations treated by the faba bean extracts was carried out. Apoptosis is a natural cell death process which cancer cells evade. Induction of apoptosis in cancer cells is the preferred way to remove them from the human body, which is an approach used in chemotherapy treatments⁽⁴⁹⁾. Food compounds that are able to induce apoptosis of cancer cells might contribute to cancer prevention.

The flow cytometric analysis revealed that exposure of HL-60 (human promyelocytic leukaemia cells) to crude extracts obtained from the raw and roasted faba bean genotypes, *Nura* and *Rossa*, induced cell apoptosis (Fig. 5). The percentage of apoptotic cells increased with greater extract concentrations

(Fig. 5(A)). After the first 3 h of incubation, early apoptotic events were detected. The number of apoptotic cells increased over the treatment time (Fig. 5(B)), with the greatest percentage of apoptotic cells induced by both, the raw and roasted faba bean extracts, over 24 h. In addition, the percentage of necrotic cells remained very low. This result suggests that the suppression of cancer cell proliferation was due to induction of apoptosis by the applied faba bean extracts.

Inhibition of angiotensin-converting enzyme, α-glucosidase and lipase

ACE is a key blood pressure regulator which is responsible for vasoconstriction that leads to an increase in blood pressure. Inhibition of ACE activity can potentially prevent ACE from elevating blood pressure, reducing the incidence of hypertension. The enzymes, α-glucosidase and lipase are important enzymes in the digestive tract and are responsible for sugar and lipid digestion, respectively. Inhibition of α-glucosidase activity could potentially reduce starch digestion and sugar absorption, therefore contributing to a lower postprandial hyperglycaemic response, whereas the inhibition of lipase activity could reduce fat uptake contributing to weight maintenance.

The raw and roasted faba bean extracts inhibited the activity of all the investigated enzymes. Condensed tannins

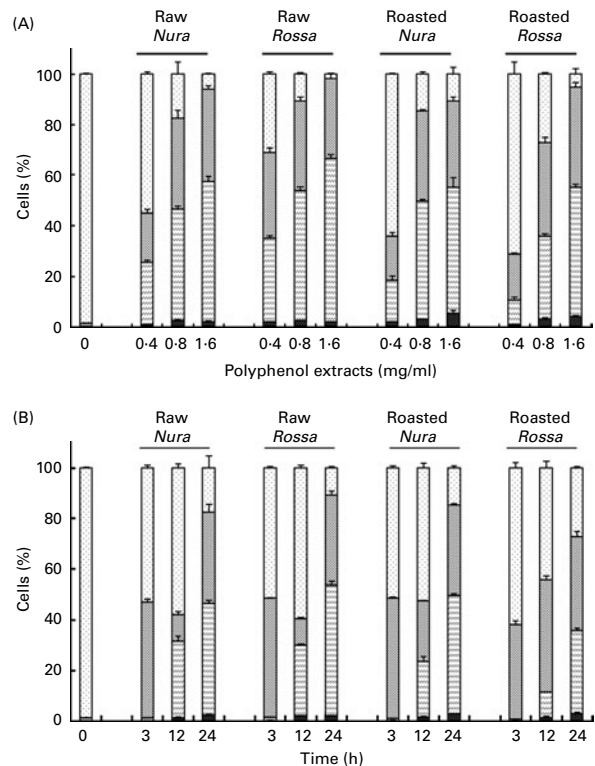


Fig. 5. Apoptosis of human leukaemia cells, HL-60, induced by crude extracts obtained from the raw and roasted faba beans as determined by flow cytometric analysis based on (A) different dose–response and (B) time course (extract concentration: 0.8 mg/mL). Values are means of three independent flow cytometric analysis for 3000–10 000 cells in each population and presented as a percentage of each cell population, with standard deviations represented by vertical bars, *n* 3. ■, Necrotic cells; □, late apoptotic cells; ▨, early apoptotic cells; □, viable cells.

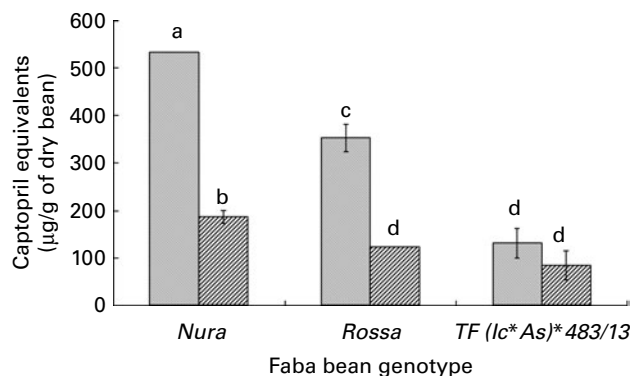


Fig. 6. Angiotensin-converting enzyme inhibition by crude extracts obtained from the raw (▨) and roasted (▨) faba bean genotypes. Results were expressed as µg of captopril equivalents/g of dry weight of beans. Values are means, with standard deviations represented by vertical bars, *n* 3. a,b,c,d Mean values with unlike letters were significantly different (*P* < 0.05).

(proanthocyanidins) in faba beans are prone to forming complexes with proteins⁽⁵⁰⁾. Therefore, the observed inhibition of the various enzymes investigated in the present study is probably due to the formation of proanthocyanidin–enzyme complexes⁽⁵¹⁾.

Extracts from *Nura* exhibited the greatest ACE inhibition activity in both of the raw and roasted beans, followed by extracts from *Rossa* and *TF(Ic*As)*483/13* (Fig. 6). Unfortunately, roasting reduced ACE inhibition activity in the faba beans significantly, except for *TF(Ic*As)*483/13*.

Roasting was found to decrease the level of α-glucosidase inhibitory activity of all the investigated genotypes (Table 4). Among extracts obtained from both of the raw and roasted beans, *Rossa* exhibited the highest α-glucosidase inhibition activity, followed by *Nura* and *TF(Ic*As)*483/13*. Similar decreases of α-glucosidase inhibitory activities after thermal processing in most of the coloured bean genotypes were also reported by Ranilla *et al.*⁽⁸⁾.

Extracts from *Rossa* exhibited the strongest lipase inhibitory activity followed by *Nura* and *TF(Ic*As)*483/13* (Table 5).

Table 4. Effects of roasting on the α-glucosidase inhibitory activity of faba bean crude extracts

(Mean values and standard deviations, *n* 3)

Faba bean genotype	α-Glucosidase inhibition (%)*	
	Mean	SD
Raw bean		
<i>Nura</i>	63.10	0.49
<i>Rossa</i>	68.28	1.95
<i>TF(Ic*As)*483/13</i>	59.66	0.49
Roasted bean		
<i>Nura</i>	30.00	0.49
<i>Rossa</i>	44.14	0.98
<i>TF(Ic*As)*483/13</i>	19.31	2.93
Reference sample		
Acarbose	85.96	3.79

* Relative α-glucosidase inhibition activities of crude extracts from the raw and roasted faba bean genotypes. Results were based on the averages of three determinations. The concentrations of the samples (including the reference sample) used were set at 2 mg/ml.

Table 5. Half maximal inhibitory concentration (IC₅₀) and relative lipase inhibitory activity of crude extracts from the raw and roasted faba bean genotypes

(Mean values and standard deviations, *n* 3)

Faba bean genotype	Lipase inhibition		IC ₅₀ (mg/ml)†
	Relative percentage*		
	Mean	SD	
Raw			
<i>Nura</i>	21.28	1.61	81.43
<i>Rossa</i>	48.15	1.87	43.28
<i>TF(Ic*As)*483/13</i>	18.55	2.67	105.60
Roasted			
<i>Nura</i>	34.00	0.78	70.26
<i>Rossa</i>	55.24	2.56	40.72
<i>TF(Ic*As)*483/13</i>	30.08	2.96	72.17

* Relative lipase inhibitory activity of crude extracts from the raw and roasted faba bean genotypes. Results were based on an average of three determinations. The sample concentrations were set at 40 mg/ml.

† The final concentration of faba bean crude extracts required to achieve the inhibition of enzymatic activity by 50% under assay condition.

In contrary to the results of roasting effect on the other types of enzymes, heating was found to cause an increase in lipase inhibitory activity in all faba bean genotypes. Similar to the present results, Zadernowski *et al.*⁽⁵¹⁾ also reported lipase inhibitory activity in both faba bean and pea varieties.

The Pearson's correlation disclosed that ACE results were positively correlated with the TPC (0.88), TFC (0.83), DPPH radical scavenging activity (0.81) and TEAC (0.81) results (*P* < 0.05), but not significantly correlated with the ORAC results (0.55, *P* = 0.26). The data suggest that ACE inhibition of faba bean extracts might be caused by the phenolic compounds. In contrary, the results of α-glucosidase and lipase inhibitory activities did not correlate with the results of other assays. Zhang *et al.*⁽⁵²⁾ also found no correlation between the TPC and α-glucosidase inhibitory activity among the extracts of seven raspberry cultivars. This suggests that TPC were not directly related to the

α-glucosidase and lipase inhibition activities in faba beans. Ranilla *et al.*⁽⁸⁾ reported positive and negative correlations of the TPC with the α-glucosidase and ACE inhibitions as being 0.24 and −0.42, respectively, in the different dry bean cultivars. On the other hand, Silva Pinto *et al.*⁽⁵³⁾ and Mai *et al.*⁽⁵⁴⁾ who tested Brazilian strawberries and Vietnamese edible plants found positive relationships between the TPC and the α-glucosidase inhibitory activity, respectively. This suggests that the relationship between phenolic compounds and α-glucosidase activity depends on the phenolic compositions and subsequently could be plant-specific.

In fact, the solvent used in this study possibly extracted constituents other than phenolic compounds in faba beans, such as trypsin inhibitors⁽⁵⁵⁾, oligosaccharides⁽⁵⁶⁾, vicine and convicine⁽⁵⁷⁾, lipase⁽⁵⁸⁾, saponins⁽⁵⁹⁾ and particularly phytate which has been reported to have the ability to bind with proteins⁽⁶⁰⁾, and these microconstituents might affect the results of enzyme inhibition assays. The strong bioactivities of faba bean could be a result of synergistic interaction between those constituents.

The phenolic extracts of three faba bean genotypes exhibited significant differences in activities as evaluated using a range of *in vitro* assays. Therefore, the present study suggests a possible role for plant breeders to select genotypes for specific health functionality. Faba bean flour or fractionated components can be potentially incorporated into new food products as ingredients to impart desirable health benefits. It would be useful to determine the actual faba bean (or ingredient) intake required to have significant desirable health-promoting effects. However, the actual bioavailability of the phenolic compounds (especially proanthocyanidins) to humans remains unclear^(61,62). Therefore, further research is required to (1) identify these native compounds and their metabolic products and (2) examine their effects on the human digestive system (e.g. metabolised by the colonic microflora) through human clinical studies, before drawing a final conclusion on the faba bean polyphenols in relation to human health.

Conclusions

The crude phenolic extracts obtained from the raw and roasted faba beans evaluated in the present study exhibited potential health-benefiting properties, including potent antioxidant activities (based on both reagent- and cell-based assays), chemopreventative effects (through induction of cancer cell apoptosis) and protection against reactive oxygen species, H₂O₂. In addition, these extracts showed inhibitory effect on ACE, α -glucosidase and lipase as studied using *in vitro* methods. Faba bean extracts suppressed proliferation of different types of cancer cells in a dose-dependent manner, but posed negligible effect on the CCD-18Co (colon normal) cells particularly after roasting. According to the reagent-based assays, roasting decreased the antioxidant activities of faba bean extracts; however, the roasting effect on cellular and enzymatic assays varied. Overall, *Nura* (buff-coloured) and *Rossa* (red-coloured) exhibited comparable functional properties, while *TF(Ic*As)*483/13* (white-coloured) contained the lowest level of all tested functional properties. The present study encourages a wider utilisation of faba bean in human diets for its potential health benefits due to their known microconstituents, such as phenolics.

Acknowledgements

Funding for the present study was provided by Food Futures Flagship, CSIRO Food and Nutritional Sciences; Grains Research & Development Corporation (GRDC Research Code GRS166) and EH Graham Centre for Agricultural Innovation (an alliance between Charles Sturt University and NSW Department of Primary Industries). The support received from the Charles Sturt University Postgraduate Writing-Up Award Scheme is gratefully acknowledged. The authors thank Jeffrey Paull, Peter Matthews, Eric Armstrong and Gerard O'Connor for providing samples, and Dr Michelle Bull for acquiring sample data using the flow cytometer. The order of authors reflects their relative contributions. The authors declare no conflict of interest.

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