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Title: 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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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(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(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(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

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Full title
Phenolic content and potential health benefits of Australian grown faba beans

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Concise title
Health benefits of Australian grown faba beans

Key words:
Faba bean, phenolic compound, antioxidant, health benefit
Abstract:
The impact of dry roasting on phenolic constituents and functional properties of three faba bean cultivars (Nura, Rossa and TF) were investigated. These functional properties included antioxidant and chemopreventative capacities, as well as the inhibitory effects on angiotensin converting enzyme (ACE), α-glucosidase and lipase. Chromatograms of on-line post column derivatisation assays coupled with high performance liquid chromatography revealed the existence of active phenolics eluted at the relatively less polar region in the coloured cultivars, which was lacking in the white-coloured cultivar, TF. Roasting reduced the total phenolic and flavonoid contents in all faba bean cultivars and was found to diminish antioxidant activity by 10-40% as measured by the reagent-based assays [diphenylpicrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS), and Oxygen Radical Absorption Capacity (ORAC)]. Results from the cell culture-based assay [Cellular Antioxidant Activity (CAA)] showed an increase tendency in CAA after roasting in coloured cultivars. Cellular protection ability of the faba bean phenolics against hydrogen peroxide-induced DNA damage was further assessed using RAW264.7 cells. Both of the raw and cooked bean extracts were shown to inhibit proliferation of all human cancer cell lines evaluated, despite the cooked phenolic extracts having no effect on the non-transformed human cells. To clarify the mechanism of antiproliferative activity, flow cytometric analyses were performed and the results showed that faba bean extracts successfully induced cell apoptosis of HL-60 (acute promyeloctic leukaemia) cells. The ACE, α-glucosidase and lipase inhibitory activities of bean extracts demonstrated in vitro suggested that the phenolic extracts from faba beans were able to inhibit all enzymes tested. Overall, extracts from the buff-coloured cultivar (Nura) exhibited comparable bioactivities as the red-coloured cultivar (Rossa), while the white-coloured cultivar (TF) contained the lowest phenolic content, antioxidant activity and enzyme inhibition activities. These results are important to promote the utilisation of faba beans in human diets for various health benefits.
1. Introduction

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 cultivars 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 which was 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 content including phenolic compounds, oligosaccharides\(^3\), enzyme inhibitors, phytosterols and saponins\(^4,5\). Intake of legumes is reported to potentially contribute to lowering the risk of cancer\(^6\), cardiovascular disease\(^7\), antihypertension 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 a significant decrease in polyphenols, enzyme inhibitors, phytic acid, some minerals and vitamins, and increase the protein digestibility of faba beans\(^12,13\). Interestingly, Acar *et al.*\(^14\) reported an increase in the antioxidant capacity of different types of pulses including black bean, borlotti bean, kidney bean, red soybean, yellow bean, giant lentils and chickpea after roasting at 150°C for 60 minutes, with an initial fall in antioxidant capacity in the yellow and red soybeans after roasting for 10 minutes. Comparatively, in faba beans, the tannin content increased after roasting at 149°C/20 min and 177°C/18 min, but decreased after roasting for 204°C/14 min and 232°C/12 min\(^14\).

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\(^\text{(16-18)}\), catechins\(^\text{(19)}\), flavonols\(^\text{(20)}\), isoflavones\(^\text{(21)}\), phenolic acids\(^\text{(22)}\) and tannins\(^\text{(23-25)}\) are natural antioxidants\(^\text{(26)}\). 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\(^\text{(27, 28)}\).

Phenolic extracts of different types of beans were found to have antioxidant activities\(^\text{(26)}\), protective effects against radical-induced DNA damage\(^\text{(29)}\), antimutagenic\(^\text{(30)}\) and anticancer\(^\text{(31)}\) properties. Many have reported substantial amounts of phenolic compounds in raw\(^\text{(32)}\) and cooked faba beans\(^\text{(33)}\), but limited reports focus on the health benefits of faba bean phenolic compounds nor the impact of food preparation heat processes on the retention and activities of phenolic compounds. The present study aims to investigate the potential health benefits of extracts of raw and cooked faba beans extracts in the prevention of chronic diseases including hypertension, diabetic, obesity and different types of cancers in vitro. 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.

2. Materials and Methods

Plant Materials

Three faba beans cultivars including Nura (buff), Rossa (red) and TF (white) 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, NSW, Australia) for one hour (approximately 50g per batch in a single layer on a foil tray and agitated gently after 30 minutes for uniform heating). Roasted beans were cooled at room temperature and ground into flour using an IKA-Universalmühle M20 Grinder (Janke and Kunkel, Staufen, Germany).
Preparation of phenolic extracts

Extraction of phenolic compounds was carried out by dispersing flour in aqueous acetone (acetone:water, 70:30, v/v)\(^{16}\) at a solid to solvent ratio of 1:10 and shaking for two hours at room temperature. Supernatant was collected after centrifugation at 4000g using an Eppendorf 5415D Centrifuge (Eppendorf-Netheler-Hinz, Hamburg, Germany) for five minutes 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, Switzerland) and then freeze-dried using a Christ-Alpha 1–4 freeze dryer (Biotech International, Germany). 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 0.45µm Millipore filters 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 antioxidant capacity (ORAC) was carried out as described by Prior et al.\(^{36}\).

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

The ABTS\(^{–+}\) radical cation was prepared by dissolving ABTS (Sigma Aldrich, Madrid, Spain) in deionised water (7mM) and mixed with 2.45mM of potassium persulfate overnight to allow complete reaction. The solution was diluted using water to obtain an absorbance of 0.70±0.02 at 734nm and filtered through a polypropylene membrane (0.45µm)\(^{37}\).

On-line PCD with High Performance Liquid Chromatography (HPLC)

Analysis of the antioxidant activity was carried out on-line using ABTS cation radical. The HPLC system (ProStar model 410) consisted of Phenomenex Luna 5U C18 column (100A pore size; 150x 3mm), preceded by a guard column (Phenomenex,
4×3.0 mm), 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 (8µL) of extract sample (50mg/mL) dissolved in solvent A was injected in a gradient of 0-48% phase B in 40 minutes at a flow-rate of 0.4mL/min. UV spectra were recorded at 280nm. Post column on-line antioxidant activity 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 0.4mL/min. The absorbance of the reaction products was measured by a UV-Vis detector at 414nm.

Cell cultures

All cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD) except 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 (RPMI; Invitrogen Corporation, Australia); AGS (gastric adenocarcinoma) in F-12K Ham’s medium (Invitrogen Corporation, Australia); Hep G2 (hepatocellular) in Eagle’s minimum essential medium (EMEM; Sigma-Aldrich, Australia); HT-29 (colorectal adenocarcinoma) in McCoy’s 5a modified medium (Invitrogen Corporation, Australia); RAW264.7 (macrophage; Abelson murine leukemia virus-induced tumor) in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, Australia); HL-60 (acute promyelocytic leukaemia) in Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen Corporation, Australia) and CCD-18Co (colon normal) cells were cultured in EMEM. Each medium was supplemented with 100µg/mL streptomycin and 1000units/mL penicillin (Invitrogen Corporation, Carlsbad, CA) and 10% foetal calf serum, with the exception of IMDM for HL-60 which required 20% serum.

Cellular antioxidant activity (CAA) assay

The assessment of CAA was determined according to Tan et al. (2010)(39) and Wolfe & Liu (2007)(40). Briefly, 1x10⁵/mL of Hep G2 cells were plated in 96-wells microplates and incubated for 24 hours. Next, the media was removed and the cells were washed using phosphate buffered saline (PBS). The cells were then treated with different concentration of faba bean extracts (10µL in PBS) added to 80µL of PBS, followed by addition of 10µL 2',7'-dichlorofluorescein-diacetate (Sigma-Aldrich)
(250µM in PBS), and incubated for one hour. After that, the cells were washed using 100µL of PBS and added to 100µL of 2,2’-azobis (2-amidinopropane) dihydrochloride in Hank’s Balanced Salt Solution (600µM). Subsequently, the fluorescence was measured at 485nm excitation and 538nm emission wavelength every five minutes over one hour. The final fluorescence values were corrected from the blank sample readings, and a time versus fluorescence graph was plotted. A quercetin standard was used to express the results as quercetin equivalents per gram of dry weight of beans.

Antiproliferation assay

The assessment of antiproliferation activity of the faba bean lyophilized extracts were carried out on adhesive cells BL13, AGS, Hep G2 and HT-29 using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay, as outlined by Tan et al.\textsuperscript{39}. PBS was used to dissolve the sample extract.

Assessment of apoptosis and cytolysis by flow cytometry

Suspensions (4.5mL) of HL60 cells (2.5-5×10\textsuperscript{5}/mL) were treated with 225µL (0.8mg/mL) of extracts for 3, 12 and 24 hours in 25cm\textsuperscript{2} 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 1.6mg/mL in PBS) for 24 hours. Cells were stained with Annexin V-Alexa Fluor 488 annexin V/Dead cell apoptosis kit with Alexa Flour 488 annexin V and PI for flow cytometry (Invitrogen Corporation, Australia) according to the manufacturer’s directions. After the set incubation time, cells were harvested, washed with cold phosphate buffered saline (PBS) and resuspended in Annexin-binding buffer. After that, 100µL of cells were stained by adding 5µL of Annexin V and 1µL of Propidium iodine and incubated for 10 minutes at room temperature. Next, cells were mixed with 400µL of Annexin-binding buffer and immediately placed on ice. Analysis was performed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and FlowJo software (TreeStar Inc., Ashland, OR, USA) to determine the extent of cell apoptosis and lysis. From 3000 to 10,000 events were acquired for each measurement and cell populations were gated for analysis.

Cellular protection against hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})
The cellular protection against H$_2$O$_2$ assay was carried out using RAW264.7 cells according to Tan et al.\textsuperscript{(39)} except the concentration of H$_2$O$_2$ used was 40mM.

**Angiotensin converting enzyme (ACE) inhibition assay**

The ACE inhibition assay was carried out as described in Shalaby, Zakora & Otte\textsuperscript{(41)} using furanacroloyl-Phe-Glu-Glu (FAPGG) as a substrate. Results were expressed as µg of captopril equivalents per gram of dry weight of beans.

**Alpha-Glucosidase inhibition assay**

The α-Glucosidase inhibition was determined as described by Ikarashi et al.\textsuperscript{(27)} using sucrose as a substrate with slight modifications. An α-glucosidase enzyme solution was prepared by dissolving 100mg of intestinal acetone powders from rat (Sigma Aldrich, Australia) in 1mL of 0.1M maleate buffer (pH 6) and homogenised using an ultrasonicator for six minutes on a 30 second rest cycle. The enzyme solution was centrifuged at 3000g for 30 minutes and the supernatant was diluted to 1:2 (v/v) using the buffer solution. Sample solutions (20µL) were mixed with 2% sucrose (w/v) in maleate buffer (20µL). The enzymatic reaction was initiated by adding enzyme solution (20µL) to the mixture and incubated at 37°C for 60 min. The enzymatic reaction was terminated by heating at 100°C for 10 minutes. Sample mixture (20µL) was then used to react with the colour reagent (Glucose CII-Test Wako, Wako Pure Chemical Industries, Osaka, Japan) (3mL) at 37°C for 5 minutes and the absorbance was measured at 505nm. 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 by the buffer solution. The relative α-glucosidase inhibition was calculated using the following formula:

\[
\text{%Inhibition} = \left\{ \frac{(A_{CB} - A_{C}) - (A_{SB} - A_{S})}{(A_{CB} - A_{C})} \right\} \times 100
\]

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.\textsuperscript{(28)} using 4-methylumbelliferyl oleate as the substrate, except the porcine pancreatic lipase (Sigma type II) was prepared using a concentration of 0.085g/mL. The relative lipase inhibition activity was calculated using the following formula:
%\text{Inhibition} = \left(1 - \frac{(F_S - F_{SB})}{(F_C - F_{CB})}\right) \times 100

Where $F_S$ and $F_C$ were the values of samples and negative control measured fluorometrically at an emission wavelength of 450nm and excitation of 320nm by a Varian Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies), and $F_{SB}$ and $F_{CB}$ were the fluorescence readings of sample and control blank.

\textit{Statistical analysis}

The significant differences between mean values were calculated based on at least three independent evaluations ($n=3$) and the standard deviations (SD) were also calculated. Student’s $t$ test was conducted to assess differences between the samples at the level of $p<0.05$. All 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. Statistical correlation analyses were performed using Graphpad Prism 5 (Graphpad Software, CA, USA). Results for correlation analysis were considered statistically significant when the $p<0.05$.

\textbf{3. Results and Discussion}

\textit{3.1 Total phenolic content (TPC), total flavonoid content (TFC) and antioxidant capacities}

The effects of roasting on TPC, TFC and antioxidant capacities of the extracts from three faba bean cultivars are presented in Table 1. Compared to the raw sample, a higher extraction yield was obtained for the roasted samples with an approximately 10% increase. The differences between the cultivars with regards to the extraction yield were negligible. The levels of TPC and TFC in the Rossa cultivar were not significantly different from Nura cultivar in raw and roasted beans. The TPC in the coloured cultivars was about four times of that in the TF cultivar while the TFC was about three times higher. After roasting, TPC and TFC contents in the Nura and Rossa cultivars were about two times the value of the TF cultivar. The white-coloured TF cultivar (termed “tannin free”), was developed for its low tannin content thus explaining the low TPC and TFC detected in this study.

In the case of Nura and Rossa cultivars, roasting led to a significant decrease in the total phenolic and flavonoid contents with an approximately 50% and 30% reduction respectively. These results support earlier findings that heating applied through
various cooking methods decreased the phenolic contents in different types of legumes\(^{(42)}\). However, after roasting, the TFC of the TF cultivar was reduced by half.

The antioxidant capacities of faba beans were evaluated using three assays: DPPH, TEAC and ORAC (Table 1). The antioxidant capacity of the Nura and Rossa cultivars was comparable in all assays. However, the antioxidant capacity of the TF cultivar was about 2- to 3-times (ORAC and TEAC assay, respectively) and 6-times (DPPH assay) lower than the antioxidant capacity of Rossa and Nura. Significant decreases in antioxidant capacities of the Rossa and Nura cultivars occurred after roasting. However, roasting did not affect the antioxidant activity in TF cultivar significantly as tested by the DPPH and ORAC assays.

A high correlation was observed between the TPC, TFC (0.950, \(p<0.01\)) and the antioxidant capacities, as evaluated using DPPH (0.929, \(p<0.01\)), TEAC (0.963, \(p<0.01\)) and ORAC (0.872, \(p<0.05\)) assays. The HPLC-post column derivatisation (PCD) profiles of phenolic compounds (280nm) extracts from the three raw faba bean cultivars (Figure 1) show that the phenolic compounds eluted in two separate regions, which can be arbitrarily classified as relatively polar (0-15 minutes) and less polar (15-40 minutes) regions. The HPLC-PCD results also showed that in the case of Rossa and Nura cultivars, most of the antioxidant activities were contributed by the less polar region. HPLC chromatograms of the Nura (Fig. 1a) and Rossa (Fig.1b) extracts presented distinct active peaks in the less polar region. Moreover, in the case of Rossa cultivar extract, traces of anthocyanins were also detected (520nm, data not presented), which may contribute towards the antioxidant capacity. However, HPLC chromatogram of the TF extract (Fig. 1c) lacked active compounds in the relatively less polar region. All the three cultivars appeared to have similar HPLC-PCD 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\(^{(18)}\). These compounds appeared at the relatively less polar region in HPLC-PCD profiles (Figure 1) and are likely to be responsible for antioxidant capacity of the extracts. High molecular weight phenolic compounds were reported previously to be very active hydrogen donors thereby radical quenchers\(^{(43)}\). The white-coloured cultivar TF...
contained noticeably lower TPC and TFC, and subsequently exhibited significantly lower antioxidant capacity. The HPLC chromatogram of the TF extract (Figure 1c) shows a lack of active phenolic compounds at the less polar region, which might be the polymeric compounds. Our results confirm earlier findings by Madhujith, Amarowicz & Shahidi(29), who also were not able to detect anthocyanidin and procyanidin in the white bean varieties.

3.2 Cellular protection by faba bean extracts

3.2.1 Cellular antioxidant activity (CAA) 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 can not 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). The extracts of the two cultivars: Nura and Rossa were evaluated in this study. In contrast to the results obtained in the reagent-based assays, where both cultivars exhibited similar antioxidant capacity, in the CAA assay, extract from the Rossa cultivar exhibited superior antioxidant activity in both raw and roasted beans (Table 2). No significant differences were found between the CAA of extracts obtained from the raw and roasted beans as expressed in µM quercetin equivalent/g of bean. However, the IC$_{50}$ of Nura shows a tendency for a higher uptake of compounds present in the extracts of roasted bean. The HPLC chromatogram presented in Figure 1 clearly demonstrates compositional differences between the compounds detected at 280nm in the extracts of Nura and Rossa cultivars. While the “hump” at the less polar region dominated in the extracts of Nura and Rossa cultivars could be the polymeric compounds, monomeric anthocyanins were also detected in the extract of the Rossa cultivar (data not presented). A variety of phenolic compounds are likely to contribute to the different cell uptake rates within an hour. It can also be speculated, that heat application during roasting or cooking of faba beans will cause a partial oxidation of polymeric compounds which will affect the uptake and reflect on the antioxidant capacity within a cell. The IC$_{50}$ values of CAA of the faba beans are slightly lower than those of lentil (670µg/mL), yellow pea (780µg/mL) and green peas (1280µg/mL) as reported in Xu & Chang(44) assayed using AGS cells.
3.2.2 Cellular protection against H$_2$O$_2$

Hydrogen peroxide is a reactive oxygen species which is present in live cells and used in experimental models. In this experimental model we have evaluated the protective effect of the faba bean extracts against H$_2$O$_2$ induced apoptosis in RAW264.7 cells. Extracts obtained from the raw faba beans of Nura and Rossa cultivars, applied at concentrations of 0.0-0.4mg/mL, exhibited cellular protection against H$_2$O$_2$ in a dose-dependent manner (Figure 2a and 2c). At concentrations higher than 0.4mg/mL the protection diminished due to the commencement of antiproliferative effects, or possibly pro-oxidative effects of phenolic compounds at high concentrations. The pro-oxidant effect might be caused by the interaction of the added phenolic compounds with undefined components of the culture media resulting in generation of H$_2$O$_2$\(^{(45)}\). The same tendency was observed for extracts obtained from the roasted Nura cultivar (Figure 2b). However, in the case of extracts obtained from the roasted Rossa cultivar, the protective effect was observed only at concentrations higher than 0.4mg/mL (Figure 1d). In comparison with other results on the protection of RAW264.7 cells from H$_2$O$_2$ induced injury\(^{(39)}\), the faba bean extracts appeared less efficient than that of Kakadu plum extract. However, the crude faba bean extracts were used for this assay, whereas in the case of Kakadu plum, purified (using XAD-7 resin column) and concentrated phenolic extract was used. Chow et al.\(^{(46)}\) found that 25µM and 50µM of quercetin (but not rutin and quercitrin) posed potent protection of RAW264.7 cells against H$_2$O$_2$ induced injury.

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

The effects of extracts obtained from the raw and roasted faba bean cultivars: 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, are presented in Table 3. The extracts, applied at a concentration range of 0.2–2.0mg/mL, exhibited a dose dependent suppression of the proliferation of human cancer cells. Both raw and roasted faba bean extracts of the Nura and Rossa cultivars, suppressed the proliferation of cancer cells in a similar manner (Figure 3, Figure 4). On the other hand, the extracts of roasted Nura and Rossa cultivars did not affect the proliferation of non-transformed colon human cells (CCD-18Co), while effectively suppressing the proliferation of the counter part, human colon cancer cells (HT-29) (Figure 4a).
Heating appeared to cause a decrease in phenolic contents and antioxidant capacities which were in line with some but not all of the antiproliferative results. This suggests different types of phenolic compounds in the faba bean extracts might exert a diverse degree of activity on specific target sites of cells. The IC_{50} value represents the concentration required to inhibit 50% of cell proliferation, therefore, a lower IC_{50} indicates a greater antiproliferation ability. The IC_{50} values of the raw Nura extract (<0.2mg/mL) was lower than that of green pea (3.25mg/mL), chickpea (3.23mg/mL) and lentil (1.27mg/mL), but the IC_{50} value of the raw Rossa extract was higher than the lentil extract tested on AGS cells\cite{44}. Seeram et al.\cite{47} found different cancer cell lines had diverse levels of sensitivity to phenolic compounds extracted from cranberries using different cell viability testing assays. The raw Nura extracts poses exceptionally high antiproliferative effects on AGS and Hep G2 cells in comparison to the raw Rossa extract. The reason is not known and the results are under further investigation. However, in support of our findings, the IC_{50} values for six types of berry extracts tested on six different human tumor cell lines were all reported to be less than 0.2mg/mL\cite{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, an approach used in chemotherapy treatments\cite{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 leukemia cells) to the lyophilised extracts obtained from raw and roasted faba beans induced cell apoptosis. The percentage of apoptotic cells increased with a greater extract concentration (Figure 5a). After the first three hours of incubation, early apoptotic events were detected. The number of apoptotic cells increased with the treatment time (Figure 5b), with the greatest percentage of apoptotic cells induced by both of the raw and roasted faba bean extracts over 24 hours. 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 faba bean extracts.

3.4 Inhibition of ACE, 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 that are responsible for sugar and lipid digestion. Inhibition of α-glucosidase activities could potentially reduce starch digestion and sugar absorption, therefore contributing to a lower postprandial hyperglycaemic response, whereas inhibition of lipase activity could reduce fat uptake contributing towards weight maintenance.

The phenolic extracts of both of the raw and roasted faba beans inhibited the activity of all the investigated enzymes. Condensed tannins (proanthocyanidins) in faba beans are prone to complexation with proteins\(^{50}\). Therefore the observed inhibition of the various investigated enzymes investigated in this study is likely due to the formation of proanthocyanidin-enzyme complexes\(^{51}\).

The Nura cultivar extract exhibited the greatest ACE inhibition activity in both raw and roasted forms, followed by the extracts of the Rossa and TF cultivars (Figure 6). Unfortunately, roasting significantly reduced the ACE inhibition activity in faba beans, except for the TF cultivar.

Roasting was found to decrease the level of α-glucosidase inhibitory activities of all the investigated cultivars (Table 4). Among the extracts of roasted beans, extracts from Rossa exhibited the highest α-glucosidase inhibition activities, followed by Nura and TF. A similar decrease of α-glucosidase inhibitory activity after thermal processing of the most coloured bean cultivars has been reported by Ranilla \textit{et al.}\(^{8}\).

The Rossa cultivar extract exhibited the strongest lipase inhibitory activity followed by the Nura and TF cultivars. Contrary to the results of roasting effect on the other types of enzyme, heating was found to cause an increase in lipase inhibitory activity
in all faba bean cultivars (Table 5). Similarly to our results, Zadernowski et al.\(^{(51)}\) also reported lipase inhibition activity in both faba bean and pea varieties.

The Pearson’s correlation disclosed that ACE results were positively correlated with TPC (0.875), TFC (0.917), DPPH radical scavenging activity (0.805) and TEAC (0.810) results \((p<0.05)\), but not significantly correlated with ORAC results (0.546, \(p=0.26\)). The data suggests that ACE inhibition of faba bean extracts might be due to the phenolic compounds. On the other hand, the results of \(\alpha\)-glucosidase and lipase inhibitory activity did not correlate with the results of other assays. Zhang et al.\(^{(52)}\) also found no correlation between the TPC and \(\alpha\)-glucosidase inhibitory activity among extracts of seven raspberry cultivars. This suggests that total phenolic contents were not directly related to the \(\alpha\)-glucosidase and lipase inhibition activities in faba beans. Ranilla et al.\(^{(8)}\) reported positive and negative correlations of the TPC with the \(\alpha\)-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 \(\alpha\)-glucosidase inhibitory activity respectively. This suggests that the relationship between phenolic compounds and \(\alpha\)-glucosidase activity depends on the composition of phytochemical 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 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 interactions between those constituents.

The phenolic extracts of three faba bean cultivars exhibited significant differences in activities as evaluated using a range of \textit{in vitro} assays. Therefore, this study suggests a possible role for plant breeders to select cultivars 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\textsuperscript{[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 with humans, before drawing a final conclusion on the faba bean polyphenols in relation to human health.

4. Conclusions
Crude extracts of faba beans evaluated in this 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 (hydrogen peroxide). In addition, these extracts showed inhibitory effects on angiotensin converting enzyme, $\alpha$-glucosidase and lipase as studied using \textit{in vitro} methods. Faba bean extracts suppressed proliferation of different types of cancer cells in a dose depending manner on different types of cancer cells, but posed negligible effects on the CCD-18Co (normal colon) cells particularly after roasting. Roasting decreased antioxidant activities of faba bean extracts based on chemical analysis, however, the effects of roasting on cellular and enzymatic assays varied depending on the assays. Nura, as the representative of the most common faba bean colour (buff) was shown to exhibit comparable potential health benefits to the dark red-colour cultivar (Rossa). The white-coloured cultivar (TF) contained, overall, the lowest level of tested health benefits. 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.

5. Acknowledgements
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O’Connell for growing the faba bean samples, Kah Yaw Ee, Karunrat Sakulnarmrat, Micheal Loughlin, Paul Burton and Marie Rose for technical assistance.
6. References


42. Ranilla LGl, Genovese MIs, Lajolo FM (2009) Effect of different cooking conditions on phenolic compounds and antioxidant capacity of some selected Brazilian bean (Phaseolus vulgaris L.) cultivars. J Agric Food Chem 57, 5734-5742.


Table 1. Extraction yield, total phenolic content, total flavonoid content, DPPH radical scavenging activity, Trolox equivalent antioxidant capacity (TEAC) and oxygen radical antioxidant capacity (ORAC) of raw and roasted faba bean cultivars*.

<table>
<thead>
<tr>
<th></th>
<th>Raw bean</th>
<th></th>
<th>Roasted bean</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td><strong>Extraction yield (mg of extracts/gDW)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>77.06</td>
<td>0.60</td>
<td>83.92 b</td>
<td>3.19</td>
</tr>
<tr>
<td>Rossa</td>
<td>83.19 a,b</td>
<td>0.34</td>
<td>90.41 b</td>
<td>4.83</td>
</tr>
<tr>
<td>TF</td>
<td>78.70 a</td>
<td>0.42</td>
<td>88.51 b</td>
<td>1.41</td>
</tr>
<tr>
<td><strong>Total phenolic content (mg GAEq/gDW)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>3.56 a</td>
<td>0.1</td>
<td>1.75 c</td>
<td>0.21</td>
</tr>
<tr>
<td>Rossa</td>
<td>3.75 a</td>
<td>0.1</td>
<td>1.86 c</td>
<td>0.13</td>
</tr>
<tr>
<td>TF</td>
<td>0.99 b</td>
<td>0.15</td>
<td>1.11 b</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Total flavonoid content (mg CEq/gDW)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>2.85 a</td>
<td>0.13</td>
<td>0.88 b</td>
<td>0.07</td>
</tr>
<tr>
<td>Rossa</td>
<td>2.95 a</td>
<td>0.15</td>
<td>1.04 b</td>
<td>0.05</td>
</tr>
<tr>
<td>TF</td>
<td>1.01 b</td>
<td>0.01</td>
<td>0.48 c</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>DPPH radical scavenging activity (µmol TEq/gDW)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>53.37 a</td>
<td>1.21</td>
<td>34.34 d</td>
<td>1.69</td>
</tr>
<tr>
<td>Rossa</td>
<td>48.10 b</td>
<td>3.16</td>
<td>37.34 d</td>
<td>2.45</td>
</tr>
<tr>
<td>TF</td>
<td>7.46 c</td>
<td>0.26</td>
<td>9.59 e</td>
<td>0.99</td>
</tr>
<tr>
<td><strong>Trolox equivalent antioxidant capacity (µmol TEq/gDW)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>72.69 a</td>
<td>2.86</td>
<td>49.75 c</td>
<td>1.79</td>
</tr>
<tr>
<td>Rossa</td>
<td>71.28 a</td>
<td>7.73</td>
<td>55.95 d</td>
<td>2.79</td>
</tr>
<tr>
<td>TF</td>
<td>25.63 b</td>
<td>0.56</td>
<td>35.81 e</td>
<td>3.46</td>
</tr>
<tr>
<td><strong>Oxygen radical antioxidant capacity (µmol TEq/gDW)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>109.10 a,b</td>
<td>11.05</td>
<td>94.43 b,c</td>
<td>11.02</td>
</tr>
<tr>
<td>Rossa</td>
<td>142.80 e</td>
<td>11.19</td>
<td>116.90 d</td>
<td>9.61</td>
</tr>
<tr>
<td>TF</td>
<td>78.21 c,d</td>
<td>4.17</td>
<td>73.91 d</td>
<td>4.92</td>
</tr>
</tbody>
</table>

*Results are based on at least three independent measurements (n=3). The data marked by the same letters were not significantly different (p<0.05) in the respective assay.

† mmol GAEq/gDW - mmol of gallic acid equivalents per g of dry bean
‡ mmol CEq/gDW - mmol of catechin equivalents per g of dry bean
§ mmol TEq/gDW - mmol Trolox equivalents per g of dry bean
Table 2. Cellular antioxidant activity of the lyophilised extracts obtained from two raw and roasted faba bean cultivars: Nura and Rossa.

<table>
<thead>
<tr>
<th>Faba bean cultivar</th>
<th>Cellular Antioxidant Activity</th>
<th>µmol QE/g of DW†</th>
<th>EC\textsubscript{50} (µg/mL)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean SD</td>
<td>Mean SD</td>
<td></td>
</tr>
<tr>
<td><strong>Raw</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>7.8 0.5</td>
<td>402.27 27.1</td>
<td></td>
</tr>
<tr>
<td>Rossa</td>
<td>15.8 4.2</td>
<td>208.0 60.4</td>
<td></td>
</tr>
<tr>
<td><strong>Roasted</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>8.9 6.5</td>
<td>277.8 69.7</td>
<td></td>
</tr>
<tr>
<td>Rossa</td>
<td>13.9 4.2</td>
<td>241.3 73.1</td>
<td></td>
</tr>
</tbody>
</table>

†µmol QEq/g DW- µmol quercetin equivalents per gram of dry bean

‡Results are based on three independent experiments (n=3).

‡EC\textsubscript{50}- concentration of lyophilised extract in culture medium (µg/mL) able to scavenge effectively 50% of free radicals within a cell.
<table>
<thead>
<tr>
<th>Faba bean cultivar</th>
<th>AGS (gastric)</th>
<th>BL13 (bladder)</th>
<th>Hep G2 (liver)</th>
<th>HT-29 (colon)</th>
<th>CCD-18 Co (colon)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>SD</td>
<td>SD</td>
<td>SD</td>
<td>SD</td>
</tr>
<tr>
<td>Raw bean</td>
<td>Nura</td>
<td>&lt; 0.2</td>
<td>-</td>
<td>&lt;0.2</td>
<td>1.46</td>
</tr>
<tr>
<td></td>
<td>Rossa</td>
<td>1.04</td>
<td>0.06</td>
<td>2.33</td>
<td>0.18</td>
</tr>
<tr>
<td>Roasted bean</td>
<td>Nura</td>
<td>1.64</td>
<td>0.05</td>
<td>1.74</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Rossa</td>
<td>2.03</td>
<td>0.05</td>
<td>1.74</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Results are presented as concentration (mg/mL) of freeze dried extract in culture medium needed to achieve growth suppression of 50% (IC$_{50}$). Sample concentration ranged from 0.2-2.0mg/mL. The results were obtained via nonlinear regression and are based on at least four replicates.
Table 4. Effects of roasting on the α-glucosidase inhibitory activity of faba bean extracts.

<table>
<thead>
<tr>
<th>Faba bean cultivar</th>
<th>α-Glucosidase inhibition</th>
<th>Relative percentage (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Raw</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>63.10</td>
<td>0.49</td>
</tr>
<tr>
<td>Rossa</td>
<td>68.28</td>
<td>1.95</td>
</tr>
<tr>
<td>TF</td>
<td>59.66</td>
<td>0.49</td>
</tr>
<tr>
<td>Roasted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>30.00</td>
<td>0.49</td>
</tr>
<tr>
<td>Rossa</td>
<td>44.14</td>
<td>0.98</td>
</tr>
<tr>
<td>TF</td>
<td>19.31</td>
<td>2.93</td>
</tr>
<tr>
<td>Reference sample</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acarbose</td>
<td>85.96</td>
<td>3.79</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Faba bean cultivar</th>
<th>α-Glucosidase inhibition</th>
<th>Relative percentage (%)</th>
<th>μmol Acarbose equivalent/g of dry bean*</th>
<th>IC_{50} (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Raw</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>63.10</td>
<td>0.49</td>
<td>259.39</td>
<td>13.61</td>
</tr>
<tr>
<td>Rossa</td>
<td>68.28</td>
<td>1.95</td>
<td>493.82</td>
<td>102.96</td>
</tr>
<tr>
<td>TF</td>
<td>59.66</td>
<td>0.49</td>
<td>182.78</td>
<td>9.59</td>
</tr>
<tr>
<td>Roasted</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>30.00</td>
<td>0.49</td>
<td>7.35</td>
<td>0.38</td>
</tr>
<tr>
<td>Rossa</td>
<td>44.14</td>
<td>0.98</td>
<td>36.42</td>
<td>3.82</td>
</tr>
<tr>
<td>TF</td>
<td>19.31</td>
<td>2.93</td>
<td>2.43</td>
<td>0.75</td>
</tr>
</tbody>
</table>

*Relative α-glucosidase inhibition of raw and roasted faba bean cultivars. Results are based on an average of three determinations (mean±SD, n=3). The concentration of the samples used was set at 2mg/mL.
Table. 5. IC\textsubscript{50} and relative lipase inhibitory activity (mean±SD, n=3) of the raw and roasted faba bean cultivars.

<table>
<thead>
<tr>
<th>Faba bean cultivar</th>
<th>Lipase inhibition</th>
<th>IC\textsubscript{50} (mg/mL)\textsuperscript{†}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Relative percentage (%)\textsuperscript{*}</td>
<td>Mean</td>
</tr>
<tr>
<td>Raw</td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Nura</td>
<td>21.28</td>
<td>1.61</td>
</tr>
<tr>
<td>Rossa</td>
<td>48.15</td>
<td>1.87</td>
</tr>
<tr>
<td>TF</td>
<td>18.55</td>
<td>2.67</td>
</tr>
<tr>
<td>Roasted</td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Nura</td>
<td>34.00</td>
<td>0.78</td>
</tr>
<tr>
<td>Rossa</td>
<td>55.24</td>
<td>2.56</td>
</tr>
<tr>
<td>TF</td>
<td>30.08</td>
<td>2.96</td>
</tr>
</tbody>
</table>

\textsuperscript{*}Relative lipase inhibitory of raw and roasted faba bean cultivars. Result was based on an average of three determinations (mean±SD, n=3). The sample concentration was set at 40mg/mL.

\textsuperscript{†}The final concentration of polyphenol extract required for 50% inhibition of the enzyme activity under assay conditions.
Fig. 1. Chromatograms of high performance liquid chromatography (HPLC) and on-line post column derivatisation assay (PCD) of extracts obtained from a raw plant material of faba bean cultivars: Nura (a), Rossa (b) and TF (c).
Fig 2. Cellular protection against H$_2$O$_2$ (40mM) of RAW264.7 cells by extracts obtained from raw (a) and roasted Nura (b), raw (c) and roasted Rossa (d). Results are mean value±SD of four replicates. The data marked by different letters were significantly different (p<0.05).
Fig. 3. The effect of extracts obtained from the raw Nura and Rossa cultivars of faba beans on proliferation of non-transformed human colon cells, CCD-18Co (a) and human colon cancer cells, HT 29 (b). Results are based on at least three independent experiments with four replicates (mean±SD, n=4).
Fig. 4. The effect of extracts obtained from roasted Nura and Rossa cultivars of faba beans on proliferation of non transformed human colon cells, CCD-18Co (a) human colon cancer cells, HT-29 (b). Results are based on at least three independent experiments with four replicates (mean±SD, n=4).
Fig. 5. Apoptosis of human leukaemia cells, HL60 induced by raw and roasted faba bean polyphenolic extracts as determined by flow cytometric analysis based on different dose-response (a) and time course (extract concentration: 0.8mg/mL) (b). Results were based on three independent flow cytometric analysis (mean±SD, n=3) for 3000 to 10000 cells in each population and are presented as a percentage of each cell population.
Fig. 6. Angiotensin converting enzyme inhibition of raw and roasted faba bean cultivars. Results were expressed as µg captopril equivalents per gram of dry weight of beans (mean±SD, n=3). The data marked by different letters were significantly different ($p<0.05$).