Health benefits of Australian grown faba beans (*Vicia faba* L.): effects of food processing

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by Siem Doo Siah

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Certificate of Authorship

I hereby declare that this submission is my own work and to the best of my knowledge and belief, understand that it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma at Charles Sturt University or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by colleagues with whom I have worked at Charles Sturt University or elsewhere during my candidature is fully acknowledged. I agree that this thesis be accessible for the purpose of study and research in accordance with normal conditions established by the Executive Director, Library Services, Charles Sturt University or nominee, for the care, loan and reproduction of thesis, subject to confidentiality provisions as approved by the University.

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List of Publications

Peer reviewed papers:


Conference presentations:

Abstract
This *in vitro* study evaluated the food processing effects on the health benefits of Australian grown faba beans (*Vicia faba* L.). The extractability of phenolic compounds from 12 faba bean genotypes using 80% methanol (v/v) and 70% acetone (v/v) were compared. The faba bean acetone extracts exhibited a higher total phenolic content (TPC), total flavonoid content (TFC) and total proanthocyanidins (TPro) than those of methanol extracts by two times. The acetone extracts also exhibited a higher level of di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) radical scavenging activity, total equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant power (FRAP) than those of methanol extracts by 6-, 6- and 2 times, respectively. Evaluation on phenolic compounds in the acetone extracts using a high performance liquid chromatography-post column derivatisation (HPLC-PCD) system showed a HPLC ‘hump’ at the less-polar region of chromatograms which gave a substantial antioxidant response. The active ‘hump’ was not detected in the methanol extracts from all genotypes and acetone extracts from the white-coloured genotype.

Quantification and characterization of phenolic compounds in the acetone extracts of processed beans were carried out in comparison to those of the unprocessed beans. Soaking did not have a dramatic effect on the phenolic content and antioxidant capacity of faba beans. Soaking and heat processing decreased the phenolic content and antioxidant capacity in faba beans as shown by an array of chemical-based analyses. Autoclaving treatment exerted a greater level of destruction on phenolic content and antioxidant capacity of faba beans than that of boiling treatment. The HPLC-PCD profiles of boiled versus autoclaved coloured-beans further supported these findings, where the active HPLC ‘hump’ was only detected in the chromatograms of boiled bean extracts but not in those of autoclaved bean extracts.

The effect of dry roasting on phenolic content and antioxidant capacity of buff-coloured genotype, *Nura* was investigated. Roasting for up to 120 min retained a higher phenolic content and antioxidant capacity in faba beans than those of other heat treatments. Roasting was shown to cause generations of new compounds as demonstrated by the chromatograms of acetone extracts from roasted beans in comparison to that of unprocessed beans. The acid- and alkaline-hydrolysed extracts exhibited entirely different HPLC profiles. Eight types of phenolic acids, catechin and epicatechin in the bean extracts were tentatively quantified.
The effect of dry roasting on cellular functional properties of faba beans was studied. Cell culture-based antioxidant activity assay [Cellular Antioxidant Activity (CAA)] showed that roasting faba beans from the coloured-genotypes resulted in a higher antioxidant capacity, which was contrary to the results obtained from the chemical-based analyses. The faba bean extracts exhibited cellular protection against hydrogen peroxide-induced DNA damage, assessed using Abelson murine leukemia virus-induced tumor (macrophage) (RAW264.7) cells, and inhibited proliferation of all human cancer cell lines [bladder transitional cell carcinoma (BL13), gastric adenocarcinoma (AGS), hepatocellular carcinoma (Hep G2) and human colorectal adenocarcinoma HT-29)] evaluated. However, the effect of extracts on the non-transformed human cells (CCD-18Co) was negligible. Flow cytometric analyses showed that the extracts successfully induced apoptosis of acute promyelocytic leukaemia (HL-60) cells. The faba bean extracts also exhibited ACE, α-glucosidase and pancreatic lipase inhibitory activities.

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<th>Description</th>
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<tbody>
<tr>
<td>AAPH</td>
<td>2,2'-Azobis(2-amidino-propane) dihydrochloride</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid</td>
</tr>
<tr>
<td>A_c</td>
<td>Absorbance of control</td>
</tr>
<tr>
<td>A_cB</td>
<td>Absorbance of control blank</td>
</tr>
<tr>
<td>Ac-DEVD-pNA</td>
<td>Acetyl-Asp-Glu-Val-Asp p-nitroanilide</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>AGS</td>
<td>Human gastric adenocarcinoma</td>
</tr>
<tr>
<td>AlCl_3.6H_2O</td>
<td>Aluminium chloride hexahydrate</td>
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<tr>
<td>CF_3COOH</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>CHAeq</td>
<td>Chlorogenic acid equivalents</td>
</tr>
<tr>
<td>CO_2</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>Cu^+</td>
<td>Cuprous</td>
</tr>
<tr>
<td>Cu^{2+}</td>
<td>Cupric</td>
</tr>
<tr>
<td>DCFH-DA</td>
<td>2',7'-Dichlorofluorescin-diacetate</td>
</tr>
<tr>
<td>DMAC</td>
<td>4-Dimethylaminocinnamaldehyde</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPPH</td>
<td>1,1-Diphenyl-2-picrylhydrazyl radicals</td>
</tr>
<tr>
<td>DW</td>
<td>Dry weight basis</td>
</tr>
<tr>
<td>EC_{50}</td>
<td>Half maximal effective concentration</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle’s minimum essential medium</td>
</tr>
<tr>
<td>Eqn</td>
<td>Equation</td>
</tr>
<tr>
<td>FAPGG</td>
<td>Furanacroyl-Phe-Glu-Glu</td>
</tr>
<tr>
<td>F_c</td>
<td>Fluorescence reading of control</td>
</tr>
<tr>
<td>F_{SB}</td>
<td>Fluorescence reading of sample blank</td>
</tr>
<tr>
<td>Fe^{2+}</td>
<td>Ferrous</td>
</tr>
<tr>
<td>Fe^{3+}</td>
<td>Ferric</td>
</tr>
<tr>
<td>FeCl_3.6H_2O</td>
<td>Iron (III) chloride</td>
</tr>
<tr>
<td>FeSO_4</td>
<td>Iron(II)sulphate</td>
</tr>
<tr>
<td>FRAP</td>
<td>Ferric reducing antioxidant power</td>
</tr>
<tr>
<td>F_s</td>
<td>Fluorescence reading of sample</td>
</tr>
<tr>
<td>F_{SS}</td>
<td>Fluorescence reading of sample blank</td>
</tr>
<tr>
<td>FW</td>
<td>Fresh weight</td>
</tr>
<tr>
<td>GAE</td>
<td>Gallic acid equivalents</td>
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<tr>
<td>H_2O_2</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>Hep G2</td>
<td>Human hepatocellular</td>
</tr>
<tr>
<td>HL-60</td>
<td>Human acute promyelocytic leukaemia)</td>
</tr>
<tr>
<td>HO^·</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>HOO</td>
<td>Hydroperoxyl</td>
</tr>
<tr>
<td>HOCI</td>
<td>Hypochorous acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HT-29</td>
<td>Human colorectal adenocarcinoma</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td>IC_{50}</td>
<td>Half maximal inhibition concentration</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s modified Dulbecco’s medium</td>
</tr>
<tr>
<td>K_2SO_4</td>
<td>Potassium persulfate</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>Acronym</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>---------</td>
<td>--------------</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>Sodium carbonate</td>
</tr>
<tr>
<td>NaNO₂</td>
<td>Sodium nitrite</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NO</td>
<td>Nitrogen oxide, Nitric oxide</td>
</tr>
<tr>
<td>O₂</td>
<td>Diatomic oxygen molecule</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>Superoxide</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>Peroxynitrite anion</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCD</td>
<td>Post column derivatisation</td>
</tr>
<tr>
<td>RAW264.7</td>
<td>Macrophage; Abelson murine leukemia virus-induced tumor</td>
</tr>
<tr>
<td>RE</td>
<td>Rutin equivalents</td>
</tr>
<tr>
<td>RO</td>
<td>Alkoxy</td>
</tr>
<tr>
<td>ROO</td>
<td>Peroxy</td>
</tr>
<tr>
<td>ROOH</td>
<td>Hydroperoxide</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>Sc</td>
<td>Slope for control blank</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviations</td>
</tr>
<tr>
<td>Ss</td>
<td>Slope for sample</td>
</tr>
<tr>
<td>TEAC</td>
<td>Trolox equivalent antioxidant capacity</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TFC</td>
<td>Total flavonoid content</td>
</tr>
<tr>
<td>TPC</td>
<td>Total phenolic content</td>
</tr>
<tr>
<td>Trolox</td>
<td>Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>QE</td>
<td>Quercetin equivalents</td>
</tr>
<tr>
<td>vis</td>
<td>Visible</td>
</tr>
</tbody>
</table>

**Symbols**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>·</td>
<td>Anion</td>
</tr>
<tr>
<td>+</td>
<td>Cation</td>
</tr>
<tr>
<td>·</td>
<td>Radical</td>
</tr>
<tr>
<td>%</td>
<td>Percent</td>
</tr>
<tr>
<td>a*</td>
<td>Redness and greenness on the tristimulus system (CIELAB)</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>b*</td>
<td>Yellowness and blueness on the tristimulus system (CIELAB)</td>
</tr>
<tr>
<td>°C</td>
<td>Degree celcius</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>g</td>
<td>Gravity force</td>
</tr>
<tr>
<td>m</td>
<td>Milli-</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>L*</td>
<td>Lightness on the tristimulus system (CIELAB)</td>
</tr>
<tr>
<td>M</td>
<td>Molarity</td>
</tr>
<tr>
<td>μ</td>
<td>Micro</td>
</tr>
<tr>
<td>n</td>
<td>Nano</td>
</tr>
<tr>
<td>p</td>
<td>Critical p-value or significance level</td>
</tr>
<tr>
<td>r²</td>
<td>Coefficient of determination</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
</tbody>
</table>
Chapter 1 Literature Review

1.1 Faba bean (*Vicia faba* L.)

Faba beans are cultivated in different parts of the world as human foods and animal feeds. Faba beans belong to the family of Leguminosae (Fabacear), and may be known as field bean, broad bean, horse bean, tick bean, windsor bean, hava, feve, *baakla* and *bakla shim* (Adsule & Akpapunam, 1996). Pictures of fresh faba beans in pods are shown in Figure 1-1, while mature buff-coloured faba beans are shown in Figure 1-2.

![Figure 1-1 Fresh faba bean pods and seeds](image1)

Faba beans are widely cultivated in the temperate and subtropical regions, and ranked the fourth most important legume crop in the world behind dry beans, dry peas and chickpea (Alghamdi, 2009). The total world production of faba beans in 2009 was 4.2 million tonnes, worth a total export value of US$266 million (FAOSTAT, 2010).
Faba beans can be harvested at vegetative stage when the pods and seeds are fresh and green, and used as a vegetable. Alternatively, they can be harvested at maturity stage after the pods and beans dry out, in which the resulting dry seeds are termed pulses (Schneider, 2002). Faba bean plants can grow up to two meters at maturity stage and each pod carries 2-4 seeds. The seed coat colours can be diverse from white, buff (beige), green, red, purple or violet, spotted to black. Faba bean 100-seed weights and sizes can vary also depending on the genotypes (Cabrera & Martin, 1986; Nozzolillo, Ricciardi & Lattanzio, 1989), water supply, genetic and environmental conditions (Alghamdi, 2009).

1.2 Faba beans in Australia
In Australia, fava beans are a winter-grown crop and are usually grown as a break crop. The seeds are harvested mechanically when they reach full maturity and dry (Figure 1-3), where their stems, leaves and pods turned brittle and darken in colour (Figure 1-4).

Figure 1-3 Faba bean plants at maturity stage and ready to be harvested
It is reported that Australia exported an average of 126 kilo tonnes of faba beans annually from 2005 to 2009, valued at approximately US$48 millions and comprised around 22% of the total world exports for human consumption market (FAOSTAT). Some faba beans are used domestically as animal feeds, especially if lower in seed quality. Faba beans are cultivated in Victoria, New South Wales and Western Australia. Faba bean plants in Australia usually have a relatively shorter height at about one meter, but it depends on seasonal conditions. The buff-coloured faba beans are prefered by the international food markets.

1.3 Major chemical constituent in faba bean
Comparisons of major chemical constituents, mineral, amino acid and vitamin contents in six colour- and white-flowering faba bean cultivars cultivated in Germany are presented in Table 1-1, 1-2 and 1-3 (Makkar et al., 1997).
Table 1-1 Major chemical constituent and energy contents of faba bean from colour- and white-flowering faba bean cultivars

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>Colour-flowering</th>
<th>White-flowering</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein (g kg⁻¹)</td>
<td>257 - 289</td>
<td>259 - 304</td>
</tr>
<tr>
<td>Soluble protein (g kg⁻¹)</td>
<td>21.6 - 26.4</td>
<td>18.7 - 21.4</td>
</tr>
<tr>
<td>Lipid (g kg⁻¹)</td>
<td>13.9 - 18.9</td>
<td>15.0 - 25.4</td>
</tr>
<tr>
<td>Ash (g kg⁻¹)</td>
<td>31.6 - 41.8</td>
<td>33.1 - 40.9</td>
</tr>
<tr>
<td>Crude fibre (g kg⁻¹)</td>
<td>99.3 - 142.5</td>
<td>87.8 - 128.9</td>
</tr>
<tr>
<td>Starch (g kg⁻¹)</td>
<td>406.6 - 485.2</td>
<td>417.0 - 476.7</td>
</tr>
<tr>
<td>Reducing sugar (g kg⁻¹)</td>
<td>40.9 - 80.6</td>
<td>28.1 - 89.6</td>
</tr>
<tr>
<td>Energy (MJ kg⁻¹)</td>
<td>18.4 - 18.7</td>
<td>18.0 - 18.8</td>
</tr>
</tbody>
</table>

Sources: Makkar et al. (1997)

Table 1-2 Mineral content in faba beans grown at Germany

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>Colour-flowering</th>
<th>White-flowering</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (g kg⁻¹)</td>
<td>1.0-1.55</td>
<td>1.33-2.10</td>
</tr>
<tr>
<td>Phosphorus (g kg⁻¹)</td>
<td>5.00-7.10</td>
<td>4.88-7.10</td>
</tr>
<tr>
<td>Magnesium (g kg⁻¹)</td>
<td>1.33-1.70</td>
<td>0.22-1.66</td>
</tr>
<tr>
<td>Sodium (g kg⁻¹)</td>
<td>0.033-0.09</td>
<td>0.044-0.33</td>
</tr>
<tr>
<td>Potassium (g kg⁻¹)</td>
<td>11.53-15.30</td>
<td>12.10-14.90</td>
</tr>
<tr>
<td>Copper (mg kg⁻¹)</td>
<td>9.96-18.93</td>
<td>8.89-21.07</td>
</tr>
<tr>
<td>Manganese (mg kg⁻¹)</td>
<td>12.20-23.25</td>
<td>13.34-17.73</td>
</tr>
<tr>
<td>Zinc (mg kg⁻¹)</td>
<td>32.42-94.11</td>
<td>41.00-63.21</td>
</tr>
<tr>
<td>Iron (mg kg⁻¹)</td>
<td>52.21-91.90</td>
<td>49.90-68.92</td>
</tr>
<tr>
<td>Aluminium (mg kg⁻¹)</td>
<td>4.43-27.73</td>
<td>2.22-16.59</td>
</tr>
<tr>
<td>Sulphur (mg kg⁻¹)</td>
<td>6.71-13.29</td>
<td>7.76-14.40</td>
</tr>
</tbody>
</table>

Source: Makkar et al. (1997)

Table 1-3 Amino acids contents (g/100g protein) in faba beans grown at Germany

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>Colour-flowering</th>
<th>White-flowering</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>6.28-8.56</td>
<td>5.80-7.23</td>
</tr>
<tr>
<td>Leucine</td>
<td>7.35-8.27</td>
<td>6.60-7.77</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.29-4.64</td>
<td>3.76-4.24</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.90-1.01</td>
<td>0.79-1.10</td>
</tr>
<tr>
<td>Cystine</td>
<td>1.19-1.42</td>
<td>1.10-1.41</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.13-5.25</td>
<td>3.58-4.86</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.67-3.97</td>
<td>3.76-4.27</td>
</tr>
<tr>
<td>Valine</td>
<td>3.75-5.64</td>
<td>4.20-5.14</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.89-3.46</td>
<td>2.70-4.15</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.88-4.39</td>
<td>3.76-4.07</td>
</tr>
<tr>
<td>Serine</td>
<td>4.89-5.53</td>
<td>4.68-5.35</td>
</tr>
<tr>
<td>Glutamic acid (includes glutamine)</td>
<td>15.86-17.29</td>
<td>14.20-15.89</td>
</tr>
<tr>
<td>Asaotic acid (includes asparagines)</td>
<td>10.12-10.98</td>
<td>9.67-10.40</td>
</tr>
<tr>
<td>Proline</td>
<td>4.42-6.29</td>
<td>4.68-5.75</td>
</tr>
<tr>
<td>Glycerine</td>
<td>4.47-4.93</td>
<td>4.15-4.71</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.53-4.60</td>
<td>3.43-4.41</td>
</tr>
<tr>
<td>Arginine</td>
<td>9.76-12.10</td>
<td>8.80-10.94</td>
</tr>
</tbody>
</table>

Source: Makkar et al. (1997)

Faba beans, similarly to other types of pulses, are a good source of carbohydrate, protein and fibre regardless of flowering types, and are low in fat content. The starch
and fat contents in the white- and colour-flowering faba bean cultivars are comparable. Faba beans are limiting in ascorbic acid and sulphur containing amino acids (methionine and cystine) in general. Conversely, the lysine content in faba bean is much higher than cereal grains (Alghamdi, 2009).

1.4 Minor constituents in faba bean and their health effects

Faba bean are reported to contain a variety of minor constituents, such as phenolic compounds, protease inhibitors, phytates, α-amylase inhibitors (Borowska, Giczewska & Zadernowski, 2003; Oomah et al., 2011), lectins, saponins (Sharma & Sehgal, 1992), α-galactosidase (Vidal-Valverde et al., 1998), oligosaccharides (Pridham, 1958; Sosulski, Elkowicz & Reichert, 1982), tocopherols, phytosterols, squalenes, triterpenic acids (Kalogeropoulos et al., 2010), L-3,4-dihydrophenylalanine (L-DOPA), vicine and convicine (Goyoaga et al., 2008). Some of these minor constituents are considered to have antinutritional properties. Nevertheless, many of the minor constituents are associated with potential health benefits, and they are progressively being recognised and introduced to modern diets (Schneider, 2002; Patterson, Maskus & Dupasquier, 2009; Carbonaro, 2011). Although the beneficial properties of these minor constituents can sometimes be controversial (Harland & Morris, 1995; Madar & Stark, 2002; Campos-Vega, Loarca-Pina & Oomah, 2010). The controversial issues are largely related to concerns on intake of the minor constituents in order to prevent diseases effectively (Bouayed & Bohn, 2010; Gelati & O'Brien, 2004). Table 1-4 summarises minor constituents in faba beans and their health effects. Also, a review on the important minor consituents in faba beans is given below.
<table>
<thead>
<tr>
<th>Minor constituents</th>
<th>Contents</th>
<th>Chemical characteristic</th>
<th>Reported health related effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vicine and convicine</td>
<td>Vicine 1.3% and convicine 0.5%</td>
<td>Pyrimidine derivatives</td>
<td>Favism (susceptible to patients who is genetically defect in glucose-6-phosphate dehydrogenase)</td>
<td>1</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>2%</td>
<td>L-3,4-dihydrophenylalanine</td>
<td>Treat Parkinson disease patients; lowering blood pressure</td>
<td>2-4</td>
</tr>
<tr>
<td>Enzymatic inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-amylase Protease (trypsin-chymotrypsin inhibitor)</td>
<td>4171U/g</td>
<td>Protein</td>
<td>Anti-HIV reverse transcriptase activities; anticancer; antifungal; antimitogenic; inhibit digestion of nutrients</td>
<td>5-7</td>
</tr>
<tr>
<td>Lectin</td>
<td>27.1-27.2 mg/mL</td>
<td>Protein</td>
<td>Cause agglutination of blood cells; anticancer</td>
<td>8-9</td>
</tr>
<tr>
<td>Phytic acid</td>
<td>2.17%</td>
<td>Myo-inositol hexaphosphoric acid</td>
<td>Inhibits colon carcinogenesis (from unknown plant origin); react with protein therefore affecting protein availability; chelate metals (eg. iron and zinc)</td>
<td>10-14</td>
</tr>
<tr>
<td>0.71-1.15%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saponin</td>
<td>1.8-3.17%</td>
<td>Amphiphatic in nature, with one hydrophobic triterpenoid or steroidal aglycone and one or more oligosaccharide moieties</td>
<td>Anticarcinogenesis (from Wistaria brachybotrys); antimicrobial and anti-inflammatory (from tea-leaf); haemolytic activity and toxicity; lower plasma cholesterol (from soya bean, soapwort and Quillaia);</td>
<td>8, 15-18</td>
</tr>
<tr>
<td>Oligosaccharides</td>
<td>Raffinose (0.2%), stachyose (0.5-0.8%) and verbascose (1.85-3.05%)</td>
<td>Linkage of sugar molecules, fiber like material that can retain water, cannot be digested in small intestine but fermentable in large intestine</td>
<td>Act as prebiotic; cause flatulence</td>
<td>19-20</td>
</tr>
<tr>
<td>Phenolic compounds</td>
<td>0.5-2%</td>
<td>Ubiquitous in plants, at least a ring structure attached with one or more hydroxyl groups</td>
<td>Antimutagenic (from common beans); antioxidant; inhibit digestive enzymatic activities;</td>
<td>8, 21-23</td>
</tr>
</tbody>
</table>


‡U/gDW= one unit of inhibitor required to reduce the activity of enzyme by one unit
§minimum amount per mL assay medium to produce haemagglutination
1.4.1 Vicine and convicine

One of the most well-known antinutrients in faba beans are their undesired pyrimidine derivatives, vicine and convicine that can cause favism (Lagoja, 2005). Favism is an X-chromosome-linked inherited enzymatic defect which more commonly affects male children (Kattamis, Kyriazakou & Chaidas, 1969). It is a haemolytic anaemia and occurs in patients who are deficient in enzyme glucose-6-phosphate dehydrogenase in their red blood cells. Favism can be fatal particularly in infants, however, the risks and incidences associate with favism decline with age (Belsey M., 1973; Kattamis, Kyriazakou & Chaidas, 1969; Mehta, 1994). Faba beans contain about 1.3% and 0.5% of vicine and convicine respectively (Hussien et al., 1986). It is possible to apply gamma-radiation for diminishing of vicine and convicine in faba beans (Jaddou, 1998). It is also possible to lower vicine and convicine through breeding (Hussien et al., 1986; Gutierrez et al., 2006).

1.4.2 L-DOPA

The L-DOPA is one of most important components in faba beans. The L-DOPA is a precursor of neurotransmitter dopamine, which is known to help manage Parkinson disease (Rabey et al., 1992; Rabey et al., 1993). The effect of L-DOPA in lowering blood pressure in animals (Hornykiewicz, 1958) and humans (Saito et al., 1991) has also been reported. There is about 20 mg/gDW of L-DOPA in faba beans, and the content spontaneously increases throughout nine days of seedling or sprouting process (Goyoaga et al., 2008), which could be amplified by application of natural elicitors such as fish protein hydrolysates (Randhir, Shetty & Shetty, 2002).

1.4.3 Lectin

Lectin is a type of protein that binds specifically to sugar moieties. Lectin also has abilities to agglutinate cells and it binds specifically to cell surface receptors (Sela, Wang & Edelman, 1975; Sharon & Lis, 2004). Although faba beans contain lectin (haemagglutinin activity (Alonso, Aguirre & Marzo, 2000; Makkar et al., 1997), the haemagglutinin activity in faba beans is considered very low and probably has no physiological effect (Grant et al, 1983). The majority of hemagglutinin activity is contributed from faba bean seed cotyledon, and heat processing using autoclave is
shown to diminish all haemagglutinating activity in faba beans (Marquardt et al., 1975).

1.4.4 Enzyme inhibitors

Enzymes inhibitors such as trypsin, α-amylase, α-galactosidase are regarded as antinutrients due to their effects on digestion and absorption of macronutrients. Enzyme inhibitors in faba beans become a concern particularly when they are used as animal feeds (Wynne G., 1981). There are 4171 U/g of amylase inhibition (Shekib, El-Iraqi & Abo-Bakr, 1988) and 0.1% protease inhibition activities (Ye & Ng, 2002) determined in faba beans. White-coloured faba bean genotypes have a significantly lower enzymatic inhibition activity (Moneam, 1990). Faba bean hulls contribute to most of the trypsin inhibitor activity (Marquardt et al., 1975), while soaking and dehulling are shown to be effective in lowering trypsin inhibitor contents in faba beans (Bakr, 1996).

1.4.5 Phytic acids

Phytic acid is a myo-inositol hexaphosphoric acid, which is a major form of phosphorus storage in many seeds (Williams, 1970). Phytic acid is also a natural antioxidant (Graf, Empson & Eaton, 1987) that can be found in many cereals and legumes at a level ranging between 1-5% (Cheryan & Rackis, 1980). Phytic acid in faba bean has raised concern due to their ability to bind with proteins thus affecting protein digestibility (Carnovale, Lugaro & Lombardi-Boccia, 1988), particularly when faba beans are used as animal feeds. Phytic acid has been reported to chelate metals (eg. iron and zinc) and restricts absorption of minerals physiologically (Griffiths, 1982; Wynne G., 1982; Glahn & Wortley, 2002; Luo, Xie & Cui, 2010). There is about 0.71-1.15% (Carnovale, Lugaro & Lombardi-Boccia, 1988) or up to 2% (Alonso, Aguirre & Marzo, 2000) of phytic acids in faba beans. Also, phytic acid is thought to inhibit carcinogenesis (Hirose et al., 1991; Shamsuddin, 1992; Liao et al., 2007). Soaking and germination of faba beans followed by dehulling are reported to lower the level of phytic acid in faba beans (Bakr, 1996).
1.4.6 Saponins

Saponins have an amphipathic property, with its aglycone (triterpenoid or steroidal) and one or more oligosaccharides being hydrophobic and hydrophilic, respectively. Therefore, saponins are a strong emulsifying agent (Price, Johnson & Fenwick, 1987; Shi et al., 2004). Saponins are recognised as antinutrients due to their toxicity when administrated at a high dosage to animals (Oleszek, Junkuszew & Stockmal, 1999), which can result in haemolytic activities (Khalil & El-Adawy, 1994). Nevertheless, saponins from various plant sources exhibit adjuvant activities, which are largely defined by their chemical structures rather than their haemolytic activity (Oda et al., 2000). Faba beans contain two types of saponins similar to soya saponins group B (Amarowicz et al., 1997). There are about 3 and 1.8% of saponins in colour- and white-flowering faba bean cultivars, respectively (Makkar et al., 1997). Saponins can lower plasma cholesterol level (Kwawano-Takahashi et al., 1986; Sidhu & Oakenfull, 1986), and exhibit anticancer properties (Wu et al., 1990; Konoshima et al., 1992). Saponins from tea-leafs has also been reported to contain antimicrobial and anti-inflammatory abilities as assessed using animal models (Sagesaka et al., 1996).

1.4.7 Oligossacharides

Oligossacharides in faba beans include α-galactoside raffinose (0.2%), stachyose (0.5-0.8%) and verbascose (1.85-3.05%) (Dini et al., 1989). Oligossacharides are soluble in aqueous ethanol (Lattanzio et al., 1986). Ingestion of legumes containing oligosaccharides has gained concern for causing flatulence (Calloway, Hickey & Murphy, 1971). Oligosaccharides are indigestible in small intestines, thereby being passed to the large intestines and fermentable by the microbial flora which results in generation of volatile fatty acids and gases (Rivero-Urgell & Santamaria-Orleans, 2001). However, this is becoming known as one of the functional properties of oligosaccharides that acts as prebiotics by increasing the growth of specific bifidobacteria in the guts and decreasing pathogenic microorganism populations (Mitsuoka, Hidaka & Eida, 1987; Juskiewicz et al., 2006; Kapiki et al., 2007). In addition, oligosaccharides are often linked to anti-carcinogenicity (Burns & Rowland, 2000; Niba & Niba, 2003). However, a human study concluded that trans-galacto-oligosaccharides are completely digestible and do not bring negative nor positive
effects to the intestinal microflora (Alles et al., 1999), which is contrary to that of fructo-oligosaccharides (Sakai et al., 2001).

1.4.8 Phenolic compounds

There are different types of phenolic compounds in faba beans (see section 1.6) including proanthocyanidins (tannins), phenolic acids, hydroxycinnamic acids, and flavonoids. Phenolic compounds are thought to be an antinutrient mainly due to presence of tannins that may complex with some digestive enzymes such as α-amylases, trypsins (Griffiths & Moseley, 1980) and lipases, particularly at high concentrations (Longstaff & McNab, 1991). This could restrict availability of some macronutrients for absorption following consumption of faba beans (Helsper et al., 1993). The health effects of phenolic compounds from a variety of plant materials have been extensively studied. The health benefits associated with phenolic compounds are antioxidant (Chaieb et al., 2011), antimitagenic (De Mejia, Castano-Tostado & Loarca-Pina, 1999), antibacterial (Rauha et al., 2000) and anticancer (Pan et al., 2010) activities. Faba bean phenolic extracts and their fractions are widely reported to have potent antioxidant potentials based on reagent based assays (Amarowicz et al., 1996; Amarowicz et al., 2004). In addition, phenolic compounds of faba beans can inhibit radical-induced DNA damage (Madhujith, Amarowicz & Shahidi, 2004). Conversely, tannins (proanthocyanidin) in faba beans are reported to exhibit inhibition activity on lipase and lipoxygenase (Karamac et al., 2007; Zadernowski et al., 2001); have a strong tendency to complex and precipitate proteins (Naczk et al., 2001); inhibit α-amylase and lipase activities (Longstaff & McNab, 1991).

1.4.9 Other substances in faba bean with potential health effects

Apart from the known minor constituents in faba beans, some substances that are not clearly identified in faba beans can also exert a significant effect on human healths. For instance, an unknown substance in faba beans have potent protective effects against strychnine-induced but not picrotoxin-induced convulsions that can cause deaths in mice where the reported administration of 0.01 mL/g of water extract from cooked beans outperformed Diazepam (20 mg/kg) (an anti-convulsant drug) by increasing the survival rate in mice prior to exposure to highly toxic strychnine (0.112
mg/kg) in a dose-dependent manner (Salih & Mustafa, 2008). Consumption of faba beans is also proven to lower the serum glucose, insulin, triacylglycerol, total cholesterol, lower-density-lipid (LDL) cholesterol, and very-low-density-lipoprotein (VLDL) cholesterol, while significantly increasing the glucagon and high-density-lipoprotein (HDL) cholesterol in human subjects (Fruhbeck, Monreal & Santidrian, 1997). This effect could be attributed to phenolics, saponin or polyunsaturated lecithin that might be presented in the protein concentrate and its ethanol soluble factors or other unknown constituents from faba beans (Zadernowski et al., 2001; Mengheri et al., 1984). Feeding rats with whole faba bean extract has been reported to be more effective than that of protein isolates in lowering the plasma LDL-, VLDL-cholesterol, hepatic cholesterol and triacylglycerol, although HDL is not altered (Mararulla et al., 2001). The hypocholesterolemic effect caused by the faba bean extracts could be due to an increase in glucagon and a decrease in insulin level, or, by inducing conversion of cholesterol to bile acid in liver which increases fecal bile acid and steroid fecal excretions (Fruhbeck et al., 1997; Jaya et al., 1981 Macarulla et al., 2001). Conversely, it is speculated by Yang et al. (1984) that nitrosations may be caused by reaction of organic compounds in faba bean extracts and nitroso derivatives in human gastric producing carcinogenic N-nitroso compounds.

1.5 Effect of faba bean seed coat colour on minor constituents

Table 1-5 compares contents of minor constituents in white- and coloured-flowering faba bean cultivars. Coloured-flowering faba bean cultivars have significantly higher contents of total phenols, tannins, condensed tannins and saponins than that of white-flowering cultivars; it is an inverse for their trypsin inhibitor (Makkar et al., 1997). The lectin activity and phytate contents are similar in both of the white- and coloured-flowering cultivars. Unlike the coloured-flowering cultivars, condensed tannins can not be detected in the white-flowering faba bean cultivars.
Table 1-5 Comparison of minor constituents in coloured- and white-flowering faba bean cultivars grown in Germany

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>Coloured-flowering</th>
<th>White-flowering</th>
<th>Significance difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total phenols a (g kg⁻¹)</td>
<td>20.1±5.7</td>
<td>4.5±0.4</td>
<td>---</td>
</tr>
<tr>
<td>Tannins a (g kg⁻¹)</td>
<td>14.1±4.4</td>
<td>0.14±0.06</td>
<td>---</td>
</tr>
<tr>
<td>Condensed tannins b (g kg⁻¹)</td>
<td>26.2±7.1</td>
<td>nd</td>
<td>---</td>
</tr>
<tr>
<td>Trypsin inhibitor activity c</td>
<td>1.85±0.09</td>
<td>3.05±0.34</td>
<td>---</td>
</tr>
<tr>
<td>Lectin activity d (mg mL⁻¹)</td>
<td>27.1±5.1</td>
<td>27.2±9.4</td>
<td>-</td>
</tr>
<tr>
<td>Saponin e (g kg⁻¹)</td>
<td>31.7±5.4</td>
<td>18.3±1.2</td>
<td>---</td>
</tr>
<tr>
<td>Phytate f (g kg⁻¹)</td>
<td>16.6±2.3</td>
<td>15.0±2.7</td>
<td>-</td>
</tr>
</tbody>
</table>

Source: Makkar et al. (1997)

a As tannin acid equivalent
b As leucocyanidin equivalent
c As mg trypsin inhibited g⁻¹ dry matter
d Minimum amount per mL assay medium which produced haemagglutination
e As diosgenin equivalent
f Significant difference between coloured- and white-flowering groups, p < 0.001; -, not significant; nd, not detected

1.6 Phenolic compounds in faba bean

Faba beans are rich in phenolic compounds and exhibit potent antioxidant activities (Amarowicz et al., 2004). There are various contents and types of flavonoids including flavonols and flavones present in hydrolysed hot methanol extracts from faba bean seed coats differing in colours (Nozzolillo et al., 1989). However, none of the mentioned flavonoids can be found in extracts from the white-coloured, while anthocyanins can only be detected in extracts from the violet-coloured seeds. These results are in agreement with other reports that phenolic contents in faba beans vary among genotypes (Bekkara et al., 1998; Amarowicz et al., 2004; Vincenzi et al., 2006), which can be influenced by their genetic variance (Cabrera & Martin, 1989) and growing conditions (Oomah et al., 2011; Vincenzi et al., 2006). Tannins make up 72-82% of the total phenolic content in seed coats of coloured-faba beans (Nasar-Abbas et al., 2008), where the seed coats comprise about 13% of total seed weight (Griffiths, 1981). The condensed proanthocyanidins among white and brown-coloured faba beans can vary immensely between 0 and 6% (Cansfield, Marquardt & Campbell, 1980). Table 1-6 summarises different phenolic compounds in faba beans as analysed using chromatography as reported by a collection of studies. The phenolic compounds in faba beans include flavones, flavanones, anthocyanins, flavan-3-ols, isoflavones, hydroxybenzoic acids and hydroxycinnamic acids.
Table 1-6 Phenolic compounds in faba bean as analysed using chromatography

<table>
<thead>
<tr>
<th>Group</th>
<th>Class</th>
<th>Phenolic compounds</th>
<th>Amounts/Remarks</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td>Flavones</td>
<td>Quercetin</td>
<td>20mg/kgFW (acid hydrolysis)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kaempferol</td>
<td>&lt;2mg/kgFW (acid hydrolysis)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24.2 µg/100gFW</td>
<td>7*</td>
</tr>
<tr>
<td>Flavanones</td>
<td></td>
<td>Luteolin</td>
<td>&lt;1mg/kgFW (acid hydrolysis)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myricetin</td>
<td>26mg/kgFW (acid hydrolysis)</td>
<td>3</td>
</tr>
<tr>
<td>Anthocyanins</td>
<td></td>
<td>Cyanidin</td>
<td>Presence (alkaline hydrolysis)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Delphinidin</td>
<td>Presence (alkaline hydrolysis)</td>
<td></td>
</tr>
<tr>
<td>Flavan-3-ols</td>
<td></td>
<td>Catechin</td>
<td>128.3±160.60 mg/kgFW</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16.23 mg/100gFW</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chrysir</td>
<td>76.9 µg/100gFW</td>
<td>7*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24.2 µg/100gFW</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Luteolin</td>
<td>&lt;2mg/kgFW (acid hydrolysis)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myricetin</td>
<td>26mg/kgFW (acid hydrolysis)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24.2 µg/100gFW</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24.2 µg/100gFW</td>
<td>7*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Luteolin</td>
<td>&lt;1mg/kgFW (acid hydrolysis)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myricetin</td>
<td>26mg/kgFW (acid hydrolysis)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24.2 µg/100gFW</td>
<td>7</td>
</tr>
<tr>
<td>Isoflavones</td>
<td></td>
<td>Daidzein</td>
<td>5.0 mg/kgDW</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Genistein</td>
<td>19.9 mg/kgDW</td>
<td>6</td>
</tr>
<tr>
<td>Non-flavonoids</td>
<td>Hydroxybenzoic</td>
<td>p-Hydroxybenzoic acid</td>
<td>0.7 mg/100gDW of hulls (alkaline hydrolysis)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>acids</td>
<td>Gallic</td>
<td>15.8 µg/100gFW</td>
<td>7*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.5 mg/100gDW of hulls</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16.2 µg/100gFW</td>
<td>7*</td>
</tr>
<tr>
<td>Hydroxycinnamic</td>
<td>Caffeic acid</td>
<td>Cholorogenic acid</td>
<td>16.0 µg/100gFW</td>
<td>7*</td>
</tr>
<tr>
<td>acids</td>
<td></td>
<td>Gallic</td>
<td>22.8 µg/100gFW</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cinnamic acid</td>
<td>22.8 µg/100gFW</td>
<td>7*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Coumaric</td>
<td>0.5 mg/100gDW of hulls;</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>o-Coumaric acid</td>
<td>1.6 mg/100gDW of flour (alkaline hydrolysis)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-Coumaric acid</td>
<td>40.0 µg/100gFW</td>
<td>7*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ferulic</td>
<td>0.5 mg/100gDW of flour;</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sinapic</td>
<td>40.0 µg/100gFW</td>
<td>7*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vanillic acid</td>
<td>14.1 µg/100gFW</td>
<td>7*</td>
</tr>
</tbody>
</table>

1.7 Effect of processing on macro- and minor constituents of faba beans and their products

Faba beans, as other types of pulses, are subjected to heat processing before human consumption. A number of reports have described the effect of processing methods on nutrient contents of faba beans including boiling (Abusin, Hassan & Babiker, 2009; Vidal-Valverde et al., 1998), pressure cooking (autoclaving/canning) (Khalil & Mansour, 1995), frying (Hamza, El-Tabey & Stegemann, 1987) and dry heating (Anderson et al., 1994). Heating such as steaming and freezing can also affect amine and phenolic contents of fresh faba bean samples (Wolosiak et al., 2010). The effect of heat processing on macro- and minor-constituents of cooked faba bean products in the form of dishes based on different recipes have also been reported (Hussien et al., 1986; Hamza, El-Tabey & Stegemann, 1987). It is a common practice to soak faba beans followed by heat processing to shorten the cooking time (Youssef et al., 1987), therefore the effect of incorporation of different soaking processes prior to cooking on nutritional quality of faba beans have been investigated (Fernandez et al., 1996; Fernandez et al., 1997; Vidal-Valverde et al., 1998). Effect of dehulling on nutritional quality of processed faba beans has also been reported (Ward, Marquardt & Campbell, 1976; Sharma & Sehgal, 1992). Findings on effects of different processing methods on macro- and minor-constituents in faba beans are summarized in Table 1-7.
### Table 1-7 Effect of soaking processes on chemical constituents in faba bean

<table>
<thead>
<tr>
<th>Soaking methods</th>
<th>Reference</th>
<th>Remarks</th>
<th>Effect on major constituents</th>
<th>Effect on minor constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soaking in water</td>
<td>1</td>
<td>Soaking in distilled water for nine hr (1:3, w/v)</td>
<td>↓: Starch, fructose and sucrose inplace</td>
<td>↓: Oligosaccharide, hemicelluloses, non-diary fibre and calcium ↓: Cellulose and lignin =: Phytic acid</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Soaking in deionised water overnight (1:3, w/v) (dehulled beans)</td>
<td>=: Protein, fibre, fat and carbohydrate</td>
<td>↑: Tannins ↓: Ash</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Soaking in distilled water (1:5, w/v) for 12 hr (dehulled beans)</td>
<td>↓: Protein and fibre =: Phytic acid =: Ash</td>
<td>=: Ash</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Soaking beans in tap water for 12 hr at 37°C (1:10, w/v)</td>
<td>↓: Phytic acid and saponin</td>
<td>=: Phytic acid</td>
</tr>
<tr>
<td>Soaking in citric acid</td>
<td>1</td>
<td>Soaking in 0.1% citric acid (pH 5) for nine hr (1:3, w/v)</td>
<td>↓: Starch, fructose and sucrose ↑: Glucose =: Phytic acid</td>
<td>↓: Oligosaccharide, hemicelluloses, non-diary fibre and calcium ↑: Cellulose and lignin =: Phytic acid</td>
</tr>
<tr>
<td>Soaking in sodium bicarbonate</td>
<td>1</td>
<td>Soaking in 0.07% sodium bicarbonate for nine hr (1:3, w/v)</td>
<td>↓: Starch, fructose and sucrose ↑: Glucose =: Phytic acid</td>
<td>↓: Oligosaccharide, hemicelluloses and non-diary fibre ↑: Cellulose =: Lignin, calcium and phytic acid</td>
</tr>
</tbody>
</table>

1. Vidal-Valverde et al. (1998); 2. Anderson et al. (1994); 3. Youssef et al. (1987); 4 Sharma & Sehgal (1992) (↓, reduction; ↑, increase; =, unchanged)

### 1.7.1 Effect of soaking on the chemical constituents of faba beans

It can be observed from the findings in Table 1-7 that a variety of soaking parameters have been used in those studies. The applied conditions in soaking faba beans vary including bean to soaking medium ratio; duration of soaking time; dehulling or not dehulling prior to soaking and the types of soaking medium. The different conditions applied in soaking faba beans cause dissimilar effects on macro- and minor-constituents in faba beans. Therefore, it is not easy to draw a general conclusion regarding the extent of soaking effect on nutritional quality of faba beans. However, it can be observed that soaking faba beans generally lowers starch, fructose and sucrose contents, while increases glucose contents. Soaking dehulled faba beans either causes increases or did not change protein, fibre, fat, carbohydrate and ash contents. Soaking faba beans reduces oligosaccharide, hemicelluloses and non-diary fibre contents, but increases cellulose content regardless of soaking media choices. Soaking faba beans increases trypsin inhibitor and tannin contents, while lowering phytic acid and saponin contents.
1.7.2 Effect of heat processing on the chemical constituents of faba beans

Table 1-8 summarises effects of boiling, autoclaving and dry heating on chemical constituents of faba beans. A variety of treatment conditions are applied in the respective processing methods, such as choices of soaking and cooking medium; duration of soaking and cooking processes; beans to medium ratios for both soaking and cooking processes; and dehulling or not dehulling prior to cooking. Therefore, it is difficult to draw a concluding remark on how heat processing methods affect macro- and minor-constituents in faba beans. However, it is probably reasonable to conclude that boiling faba beans does not alter protein, carbohydrate, fat, ash and fibre; decreases starch, fructose, sucrose contents; increases fat content occasionally.

Table 1-8 Effect of heat processing methods on chemical constituents in faba bean

<table>
<thead>
<tr>
<th>Processing methods</th>
<th>Reference</th>
<th>Remarks</th>
<th>Effect on major constituents</th>
<th>Effect on minor constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boiling</td>
<td>1</td>
<td>Soaking beans in distilled water for nine hr (1:3, w/v); soaking water was drained off and boiling beans for 35min (1.0: 6.7, w/v)</td>
<td>↓: Starch, fructose and sucrose</td>
<td>↓: Oligosaccharide, hemicelluloses, non-dietary fibre and calcium ↑: Cellulose and lignin =: Phytic acid</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Soaking beans in distilled water (1:20, w/v) for 12 hr; soaking water was drained off and boiled beans in tap water (3mL/g of seed) for about 45 min</td>
<td>=: protein, carbohydrate, reducing sugar and fat</td>
<td>↓: Oligosaccharide, tannins, phytic acid, vicine, trypsin inhibitor and haemagglutinin activity =: Raffinose, ash and convicine</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Boiling beans in deionised water for 40 min (1:4, w/v), the cooking water was discarded (dehulled beans)</td>
<td>↑: Fat =: Protein, fibre and carbohydrate, ↓: Trypsin inhibitor and tannin ↓: Ash</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Soaking beans in tap water for eight hr; soaking water was drained off</td>
<td>↓: Protein and carbohydrate ↑: Moisture and fibre =: fat</td>
<td>↓: Tannin, phytic acid, ash and polyphenol</td>
</tr>
<tr>
<td>1</td>
<td>Soaking beans in 0.1% citric acid (pH 5) for nine hr (1:3, w/v); soaking water was drained off and boiling beans for 35min (1.0: 6.7, w/v)</td>
<td>↓: Fructose and sucrose =: Starch</td>
<td>↓: Oligosaccharide, hemicelluloses, non-dietary fibre, calcium and phytic acid ↑: Cellulose and lignin =: Phytic acid</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Soaking beans in 0.07% sodium bicarbonate for nine hr (1:3, w/v); soaking water was drained off and boiling beans for 35 min (1.0: 6.7, w/v)</td>
<td>↓: Fructose and sucrose =: Starch</td>
<td>↓: Oligosaccharide and hemicelluloses ↑: Cellulose and non-dietary fibre =: Lignin, calcium and phytic acid</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>Conditions</td>
<td>Changes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autoclaving</td>
<td>1. Soaking in distilled water (1:20, w/v) for 12 hr; soaking water was drained off and autoclaving beans at 121°C for 30 min; 2. Soaking beans in tap water for 12 hr at 37°C (1:10, w/v); beans were autoclaved in distilled water (1:2, w/v) at 1.05kg/cm² pressure</td>
<td>↓: Protein, carbohydrate, reducing sugar and fat; ↓: Oligosaccharide, tannin, phytic acid, vicine, convicine, trypsin inhibitor and haemagglutinin activity; =: Ash; ↓: Phytic acid and saponin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry heating</td>
<td>1. 120°C (1 atm) for 15 min; 2. 149°C for 20 min (dehulled beans); 3. 177°C for 18 min (dehulled beans); 4. 204°C for 14 min (dehulled beans); 5. 232°C for 12 min (dehulled beans)</td>
<td>↓: Starch, fructose and sucrose; ↑: Cellulose; ↑: Fat; =: Protein and carbohydrate; ↑: Fat; =: Protein, fibre and carbohydrate; ↑: Fat; =: Protein, fibre and carbohydrate; ↑: Fat; =: Protein, fibre and carbohydrate; ↑: Fat; =: Protein, fibre and carbohydrate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


In general, boiling faba beans decreases oligosaccharide, hemicelluloses, polyphenol, tannins, phytic acid, vicine, non-dietary fibre, trypsin inhibitor, haemagglutinin activity and calcium contents. Nevertheless, soaking faba beans in a 0.07% sodium bicarbonate solution prior to boiling does not alter lignin, calcium and phytic acids in faba beans. Autoclaving also does not alter protein, carbohydrate, reducing sugar, ash and fat contents in faba beans. But, autoclaving faba beans lower oligosaccharide, tannin, phytic acid, vicine, convicine, trypsin inhibitor, haemagglutinin activity, phytic acid and saponin contents. Conversely, dry heating treatment on whole faba beans reduces starch, fructose, sucrose, oligosaccharide, hemicelluloses, calcium and phytic acid contents; increases cellulose; does not alter lignin contents. Dry heating dehulled faba beans increases fat content; does not alter protein, ash, fibre and carbohydrate contents; decreases trypsin inhibitor contents. Interestingly, dry heating faba beans at 149°C/20 min and 177°C/18 min raises tannin contents, while dry heating at 204°C/14 min and 232°C/12 min decreases tannin contents.
1.8 Fortification of food products using faba bean and other pulses

It has been an interest in incorporating faba bean flours to make different types of food products for fortification and nutrient enriching purposes. For instance, tofu made from faba bean flours contains a higher protein but lower lipid contents than that of soybean flour (Zee et al., 1988). Fortification of wheat flour with faba bean flours by 15% in yeast-leavened breads produces satisfactory end products to sensory panels (Finney, Morad & Hubbard, 1980). Also, substitution of wheat flours with faba bean flours by up to 30% in flat breads helps to improve the end product sensory attributes as reported by the same authors. Substitution wheat flours with a different proportions of decorticated cracked broad bean flour (DCBF) by up to 10% in Egyptian ‘Balady’ flat breads produces an overall acceptable end product as that of control ‘Balady’ breads (Abdel-Kader, 2000). Nevertheless, a more recent report on fortification of pasta made of durum wheat semolina by up to 35% of faba bean flours affects sensory quality of the end products negatively with a higher hardness and fracturability, which could be attributed to the non-gluten protein from the faba bean flours (Petitot et al, 2010). Another food product, tempeh, a deep fried potato based snack food is fortified using faba bean flours by up to 50% which improves the taste of end products significantly, as well as prolongs the shelf life of the end products (Grzeskowiak & Berghofer, 1990). The prolonged shelf life of end products is claimed to be caused by antioxidants in the tempeh containing faba bean flours, which is further varified by Berghofer et al. (1998). There are also many reports on fortification of food products using other types of pulses, such as substituting wheat flours with chickpea flours in making of sponge and layer cakes (Gomez et al., 2008); replacement of soybean flours with lupin flour in making tofu products (Jayasena, Khu & Nasar-Abbas, 2008); fortification of spaghetti prepared from durum wheat semolina by roasted and non-roasted navy, pinto or lentil flours (Bahnassey & Khan, 1986), as well as blending of lupin flours to wheat flours in bread baking (Campos & El-Dash, 1976).

Fortification using flours from faba beans and other pulses in making of different food products can potentially improve nutritional values and sensory properties of end products, although it could also affect physicochemical, cooking and sensory qualities of end products (Finney et al., 1980; Bahnassey & Khan, 1986; Grzeskowiak & Berghofer, 1990). Therefore, successful fortification formulas require study on the right proportion of legume flour substitution (Jayasena et al., 2008),
processing parameters (Petitot et al., 2010) and improver enrichment (Campos & El-Dash, 1976).

1.9 Free radicals and reactive oxygen species (ROS)

Generation of reactive oxygen species (ROS) in human bodies as part of the redox signaling process in an aerobic environment is an inevitable normal physiological state. This redox signaling balances generation of ROS, and the activity is modulated by a series of enzymatic and non-enzymatic antioxidant systems (Tan, Schubert & Maher, 2001). However, oxidative stress occurs as a result of redox imbalance which causes an increase in generation of ROS and leads to development of diseases as a result of the deleterious effects on cells and disorders (De Bont & Van Larebeke, 2004). The ROS could be produced from a variety of sources including mitochondria, xanthine oxidase, NADPH oxidases and dysfunctional NO synthases (Shah & Channon, 2004). They can be free radical or non-radical species, and either positively and negatively charged (Yoshikawa, Naito & Kondo, 1997). Some examples of ROS are shown in Table 1-9.

<table>
<thead>
<tr>
<th>Free radicals</th>
<th>Formula</th>
<th>Origin/Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide</td>
<td>O₂⁻</td>
<td>Primary ROS formed in metabolic processes through addition of one electron to O₂ and can initiate secondary radical reaction</td>
</tr>
<tr>
<td>Hydroxyl</td>
<td>HO</td>
<td>Products of O₂⁻ and H₂O₂ reaction or superoxide-driven Fenton reaction. It is highly reactive to compounds close to its site such as DNA strands and bases</td>
</tr>
<tr>
<td>Peroxyl, alkoxyl</td>
<td>ROO⁻, RO⁻</td>
<td>Initial new round of lipid peroxidation</td>
</tr>
<tr>
<td>Hydroperoxyl</td>
<td>HOO⁻</td>
<td>Protonated form of O₂⁻, initiates fatty acid peroxidation</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>NO</td>
<td>Participates in smooth muscle relaxation, neurotransmission, defense mechanisms and blood pressure regulation, it is highly reactive with radicals especially O₂⁻</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Non radicals</th>
<th>Formula</th>
<th>Origin/Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen peroxide</td>
<td>H₂O₂</td>
<td>Generated in vivo by superoxide dismutase and other oxidase reaction</td>
</tr>
<tr>
<td>Singlet oxygen</td>
<td>¹O₂</td>
<td>Could be taking part in phagocytosis and generated by eosinophils peroxidase and polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>Hypochlorous acid</td>
<td>HOCl</td>
<td>Product of H₂O₂ and Cl⁻ and react with O₂⁻ and produce HO₂⁻, involves in important killing of bacterial</td>
</tr>
<tr>
<td>Alkylhydroperoxide</td>
<td>ROOH</td>
<td>Degradation of lipid hydroperoxides at presence of transition metals to form alkoxyl radical</td>
</tr>
</tbody>
</table>

Free radicals are broadly defined as any species that have one or more unpaired electrons and able to take part in one-electron transfer reactions (Halliwell & Gutteridge, 1990). They include an atom of hydrogen (one unpaired electron), most transition metal ions and an oxygen molecule. As free radicals have an unpaired
electron, they are unstable in nature and are often reactive. The unpaired electron of a radical is passed from molecule to molecule and turns the recipient into a free radical and neutralising the donor. A free radical with an unpaired atom is usually represented by a dot at the upper right of the chemical, “X”. It can be negatively or positively charged, X⁻ or X⁺. Generation of some complex free radical products can occur by ionising radiation of biological materials. Whereas, ionizing radiation on water mainly generates H⁺, OH⁻ and eaq⁻, and when the radiation is directed onto aqueous solutions, those hydrated electrons readily interact with neighbouring biomolecules (Niki, 1997). If there is sufficient intrinsic energy from non-ionizing radiation to generate homolysis of covalent bonds, free radicals can be formed (Slater, 1984). Sources of free radicals can be generated from inflammation, strenuous exercise, detoxification, exposure to certain chemicals, radiation, ultraviolet (UV) light, alcohol, cigarette smoke, air pollutants, excess free calcium, pesticides, excess stored or unbound iron and high fat diets (Inserra, Ardestani & Watson, 1997; Jia & Misra, 2007; Mena, Ortega & Estrela, 2009)

The biochemistry of free radicals is described in details by Cheeseman & Slater (1993) and the authors explained that free radicals can be formed by:

- Hemolytic bond fission: X:Y → X⁻ + Y⁺ (Eqn. 1)
- Electron-transfer reaction: A + e⁻ → A⁻ (Eqn. 2)

While ions can be formed by heterolytic fission: (3) X:Y → X⁻ + Y⁺ (Eqn. 3)

1.9.1 Hydrogen peroxide, superoxide and hydroxyl radicals (H₂O₂, O₂⁻, HO⁻)

The diatomic oxygen molecule (O₂) itself is an important free radical, that can be reduced to form superoxide free radical anion (O₂⁻). The reaction of two superoxide molecules can form H₂O₂ and O₂ by superoxide dismutase, where the H₂O₂ are commonly generated in biological systems:

\[ 2 \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \] (Eqn. 4)

Under normal circumstances, the H₂O₂ does not tend to be reactive and it is steadily converted to water by the enzymes catalase and glutathione peroxidase. In fact, the production of H₂O₂ at a high concentration during the ‘respiratory burst’ of phagocytes is necessary to defend pathogen invasion by phagocytosis (Iyer, Islam & Quastel, 1961), which is subjected to antibacterial enzymes (such as myeloperoxidase) and generated by polymorphonuclear leukocytes. The H₂O₂ and O₂⁻ do not exert strong anti-microbial activities, unless they are at a high concentration
(Hyslop et al., 1995; Imlay & Linn, 1986). The addition of myeloperoxide and iodine, bromide and chloride ions can increase production of H$_2$O$_2$ (Klebanoff, 1968). The H$_2$O$_2$ and O$_2^-$ are precursors of more reactive oxidizing radicals particularly at presence of transition metal ions, such as iron and cuprous. The O$_2^-$ converts ferric (Fe$^{3+}$) to ferrous (Fe$^{2+}$) and cupric (Cu$^{2+}$) to cuprous (Cu$^{+}$) respectively, which can then react with H$_2$O$_2$ to produce reactive hydroxyl radicals (·OH) (Halliwell, 1996), this is termed Fenton reaction (Eqn. 5). Other metals including chromium, cobalt, vanadium, cadmium, arsenic and nickel are also thought to take part in the Fenton reaction, which mediates formation of free radicals (Valko et al., 2006). The equation below is an example of the Fenton reaction that results in the production of ·OH (Zepp, 1992):

$$H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^+ + Fe^{3+} \quad \text{(Eqn. 5)}$$

The OH$^-$ is extremely reactive, and has a half-life of $10^{-9}$ sec when reacting with 1M linoleate and it can react immediately with any reactive species close to it (Pryor, 1986). According to Slater (1984) and Pryor (1986), a radical such as OH$^-$ has a relatively smaller radius of diffusion and is less selective to its substrate, thus has a greater reactivity, which is the reverse to a radical such as a peroxyl radical that has a relatively less reactivity.

### 1.9.2 Hypochlorous acid (HOCl)

Myeloperoxidase generates HOCl in the phagosome during phagocytosis at presence of chlorine (Albrich, McCarthy & Hurst, 1981; McKenna & Davies, 1988):

$$H_2O_2 + Cl^- \rightarrow HOCl + OH^- \quad \text{(Eqn. 6)}$$

The HOCl has a strong antibacterial activity (Foote, Goyne & Lehrer, 1983; Harrison & Schultz, 1976). Although HOCl has a high antibacterial activity, the presence of H$_2$O$_2$ would neutralise the effect by converting HOCl to OH$^-$ (Hampton, Kettle & Winterbourn, 1998).

### 1.9.3 Hydroperoxide, hydroperoxyl, peroxyl and alkoxy (ROOH, HOO$^-$, ROO$^-$, RO$^-$):

The HOO$^-$ is the simplest form of peroxyl radical. Peroxyl radicals can be generated from ROOH, a H-abstraction through free radical chain reactions (Chan et al., 1982). The type of organic R group attached to peroxyl radicals does not alter reactivity but physical properties of ROO$^-$ and RO$^-$. The RO$^-$ has a shorter half-live than ROO$^-$. 
hence a greater reactivity (Marnett, 1987). The stable carbon-oxygen bond limits the ROO' from dissociating into O₂ (Marnett, 1987). The propagation step of lipid peroxide chain reaction is initiated when a lipid radical is formed through the hemolytic (Eqn. 1) or thermal cleavage (Porter, Caldwell & Mills, 1995). The lipid radical reacts with O₂ and generates ROO', and then the ROO' react with a RH group to generate a ROOH and a free radical, R' (Gardner, 1989).

\[ \text{R'} + \text{O}_2 \rightarrow \text{ROO'} \quad \text{(Eqn. 7)} \]
\[ \text{ROO'} + \text{RH} \rightarrow \text{R'} + \text{ROOH} \quad \text{(Eqn. 8)} \]

This chain reaction can keep on going, or terminate when two ROO' combine to form non radical products.

\[ 2\text{R'} \rightarrow \text{R-R} \quad \text{(Eqn. 9)} \]
\[ 2\text{ROO'} \rightarrow \text{ROO-OOR} \quad \text{(Eqn. 10)} \]
\[ 2\text{RO'} \rightarrow \text{RO-OR} \quad \text{(Eqn. 11)} \]

The auto lipid peroxidation of polyunsaturated and monosaturated fatty acids cause oxidative deteriorations of fatty foods (Chan et al., 1982).

1.9.4 Singlet oxygen (\( ^1\text{O}_2 \))

Singlet oxygen is not a free radical but it can initiate a chain reaction of lipid peroxidation after reacting with polyunsaturated fatty acids or low-density lipoprotein (LDL) (Bus, Aust & Gibson, 1974; Thomas & Pryor, 1980). The myeloperoxidase-H₂O₂-halide systems generate only a mimima amount of \( ^1\text{O}_2 \) under physiological conditions as measured using infrared spectrophotometers (Kanofsky et al., 1984). However, there is generation of significant amounts of \( ^1\text{O}_2 \) by polymorphonuclear leokocytes as a result of phagocytosing using a new method applying 9,10-diphenylanthracene- and perylene-coated beads (Steinbeck, Khan & Karnovsky, 1992). There is a significant amount of \( ^1\text{O}_2 \) generated by human eosinophils peroxidase at presence of 100 µM bromide at pH 7.0, where negligible amount of \( ^1\text{O}_2 \) is produced by myeloperoxidase and lactoperoxidase under the same condition (Kanofsky et al., 1988).
1.9.5 Nitrogen oxide (NO∙)

The NO∙ participates in a variety of physiological processes, more notably in blood pressure regulation, muscle relaxation and neurotransmission. This radical diffuses freely in the cytoplasm and through plasma membranes.

1.10 Oxidative stress and human diseases

Excessive free radicals or a disorder in the modulation of intracellular molecules and signaling pathways (“redox signaling”) leads to oxidative stress, which generates ROS in human bodies (Shah & Channon, 2004). Enzymes including glutathione peroxidase, glutathione reductase, catalase and superoxide dismutase, intercept and inactivate the ROS to manage damages that could be exerted to proteins, lipids and deoxyribonucleic acid (DNA) as part of defense system against oxidative stress in our bodies. However, the level of oxidative stress varies from time to time and our body often fails to adapt precisely to the fluctuating stresses. This results in the leaking of ROS from the antioxidant defense capacity, and hence this imposed oxidative stress is unavoidable (Davies K., 1995). Aging causes a decrease in the level of antioxidant enzymes and leads to an increased risk of diseases that are linked to oxidative stress, such as cancer development and immune dysfunction incidences (Inserra et al., 1997). The free radicals produced in cells can cause severe cell injuries to membrane, proteins, lipids, carbohydrates and nucleotides, and these damages inside any parts of the human body can cause cancers (Weitzman & Gordon, 1990; Wiseman & Halliwell, 1996). Oxidative damage in human bodies, particularly the elderly, results in a failure of oxidative energy metabolism, which is the basic cause of neurodegenerative diseases, including Alzheimer’s disease (Gonzalez-Lima, Valla & Cada, 1998). Moreover, chronic oxidative stress in human bodies is linked to an increased incidence and severity of various diseases such as cardiovascular diseases, neurodegenerative diseases, chronic inflammation and cancers (Yoshikawa et al., 1997).

1.10.1 Oxygen radical species cause DNA damage and induce mutation

As mentioned earlier, the glutathione peroxidases take part in antioxidant defense capacity by reducing H₂O₂ to water, but this process is disturbed by the presence of transition metals which cause the generation of OH′. When the reactive OH′ are
generated close to the DNA, they can directly attack the DNA bases and deoxyribosyl backbone, which results in alterations in DNA strands. The modification of the genetic material in a cell is served as the initial step of mutagenesis, where misreading at DNA transcription and replication stages can induce point mutation, which results in activation of oncogene or loss of tumor suppressors, and leads to cell proliferation and cancer development (Strand et al., 1993; Prolla, 1998; Jackson & Loeb, 2001; Jones & Thompson, 2009). Double-strand-break of DNA is reported to promote likelihood of DNA synthesis errors (Strathern, Shafer & McGill, 1995). Other types of ROS can also potentially involve in initiation of carcinogenesis. Tumor cells evade apoptosis, a process that cells undergoing phagocytosis without liberating their toxic intracellular contents as part of the antioxidant defense system, while oxidative stress accelerates cancer progression (Farber, 1991; Buttke & Sandstrom, 1994). There are other causes of oxidative stress and their relation to DNA damage leading to carcinogenesis, where some of the causes being tobacco smoke, ultraviolet radiation, fatty acids in food, ethanol, iron and copper ions as summarized by Dreher & Junod (1996).

1.10.2 Reactive oxygen species (ROS) incurs inflammation

Chronic accumulation of leukocytes, lipid, and smooth muscle cells in the inner layer of vascular walls can form an atherosclerotic plaque (Cybulsky & Gimbrone, 1991; Ross, 1995). Adhesion of leukocytes to endothelial lining of vascular walls can cause inflammation and thrombosis (Sluiter et al., 1993). Generation of ROS by neutrophils as a result of an oxygen burst is an inflammatory response (Coxon et al., 1996). This antioxidant defense process damages pathogens, disturbs the apoptosis of neutrophils, and also damages host cells probably due to leakage of ROS (Kasahara et al., 1997; Hampton et al., 1998; Jackson & Loeb, 2001). The Nitric oxide (NO) plays an important role in regulating blood pressure. The ROS inactivates NO leading to a functional NO deficiency, which can result in regional hypertension (Roberts et al., 2000; Vaziri, Ni & Barton, 2000; Barton, Ni & Vaziri, 2001), which is one of the important risk factors that promotes pathogenesis of atherosclerosis (Touyz, 2004). Inflammation processes accelerate generation of $\text{O}_2^-$ and NO$^\cdot$, which encourages formation of peroxynitrite anion (ONOO$^\cdot$). The ONOO$^\cdot$ is not a radical but has a strong oxidation property. It reacts with carbon dioxide (CO$_2$), or can be degraded to become a substrate for myeloperoxidase, which can contribute to modification of
LDL and arthelogenesis (Carr, McCall & Balz, 2000). Some other diseases such as gastritis, ulcerative colitis, pancreatitis and chronic hepatitis are also associated with chronic inflammation and malignancies (Jackson & Loeb, 2001). Chronic inflammation is one of the important risks leading to carcinogenesis (Ames, Shigenaga & Hagen, 1993).

1.10.3 Free radicals damage low density lipoprotein (LDL)

It is demonstrated that oxidation of LDL can lead to atherogenesis and atherosclerotic coronary heart disease (Witztum & Steinberg, 1991; Parthasarathy, Steinberg & Witztum, 1992). Free radicals can cause modification of different cell types that are present at intestinal cell walls, such as endothelial and smooth muscle cells within LDL (Morel, DiCorleto & Chisolm, 1984), which results in cellular LDL receptors failing to recognise the LDL and a decline in macrophage scavenger receptor from uptake of oxidised LDL. Hypertension is a risk factor leading to atherosclerosis and possibly together, they enhance arterial oxidative stress (Alexander, 1995). ROS can also alter the protein structure by modifying amino acid side chains that leads to protein degradation or crosslinking (Wolff, Garner & Dean, 1986; Davies, Delsignore & Lin, 1987). This may also change the enzymatic activity, hence their site-specificity and functionality (Fucci et al., 1983; Rice-Evans & Bruckdorfer, 1992; Shacter, 2000).

1.11 Phenolic compounds

Plants synthesize a large number of different organic compounds, which are classified into primary and secondary metabolites. Primary metabolites including lipids, carbohydrates and proteins and they are essential for plant photosynthesis, respiration, growth and development. Some higher plant species synthesize secondary metabolites or phytochemicals that are diverse in structures. The secondary metabolites do not involve the continuance of fundamental life process, but they have an important role in interacting with the environment (Oksman-Caldentey & Inze, 2004). Phenolic compounds are one of the important secondary metabolites and are synthesized through phenylpropanoid metabolism pathways (Figure 1-5). They are thought to protect plants from herbivores and microbial infections. Conversely, phenolic compounds are also thought to play an important role in attracting pollinators and seed-dispersing animals.
The functionality of phenolic compounds in plants are thought to include responsiveness to biotic and abiotic stresses including UV radiation, wounding, pathogen attack, low temperature and nutrient deficiency (Dixon & Paiva, 1995; Winkel-Shirley, 2002); or, it could be due to the presence of heavy metals in the growing environment (Michalak A., 2006). Therefore, the phenolic compounds can be allelopathic agents, UV protectants and also signal molecules in formation of nitrogen-fixing root nodules in legumes (Crozier, Jaganath & Clifford, 2006). Some phenolic classes such as anthocyanins are responsible for the pigmentation in parts of plants (eg. fruit skins) (Koes, Verweij & Quanttrocchio, 2005), while the oxidised proanthocyanidins also are brown pigments in the seed coat of mature seeds (Lepiniec et al., 2006).

Phenolic compounds are made up of chemical ring structure/s attached to one or multiple hydroxyl groups. There are a wide range of phenolic compounds such as hydrolysable tannins (metabolites of galic or ellagic acids), condensed tannins (proanthocyanidins), hydroxycinnamic and simple hydroxybenzoic acids. Phenolic compounds contribute partially to color and taste of fruits such as orange, red, blue,
violet and purple colours (Shahidi & Naczk, 1995; Belitz, Grosch & Schieberle, 2004). Different plant species or varieties of a same species could exhibit distinct phenolic levels; or, to a lesser extent, phenolic profiles as a result of responses to dissimilar environmental conditions and stresses (Hakkinen & Torronen, 2000; Marles, Balasubramanian & Bett, 2010).

In general, phenolic compounds can be grouped into two types, flavonoids and non-flavonoids. Flavonoids and non-flavonoids are known to associate with many health benefits (see 1.4.8), which is largely attributed to their ability to donate hydrogen or electron and acts as free radical scavengers, hence, antioxidant activity. Many articles have discussed the relationships between structures of phenolic compounds and antioxidant activity (Rice-Evans, Miller & Paganga, 1996; Rice-Evans, Miller & Paganga, 1997; Natella et al., 1999). A number of others have also dealt with effect of extraction solvent systems on extractabilities of phenolic compounds; applications of different methodologies in analysing phenolic compounds (Escribano-Bailon & Santos-Buelga, 2003; Naczk & Shahidi, 2004, 2006; Robards, 2003); metabolism, nutritional significance and the relation between diseases and phenolic compound consumption (Manach et al., 2005; D'Archivio et al., 2010; Del Rio et al., 2010). Therefore, the content below largely concentrates on distributions and health effects of the phenolic compounds.

1.11.1 Flavonoids

Flavonoid is one type of phenolic compounds derived from phenylpropanoid which is based on \( C_6C_3C_6 \) flavan nucleus (Figure 1-6). Subclasses of flavonoids such as flavones, flavonols, flavanones, flavan-3-ols, anthocyanins and isoflavones are classified depending on the degree of saturation and oxidation of the flavan nucleus heterocyclic ring (C-ring) (Figure 1-7).

![Figure 1-6 Basic structure and numbering system of the parent flavonoid. Source: Stalikas (2007)](image-url)
The backbone structure of a flavonoid is consisted of two aromatic rings (A and B rings) and it is usually linked by a six-membered oxygenated heterocyclic ring, or C ring. The structure of isoflavones differs with its B-ring attached to C-3 of the heterocyclic C-ring. Most of the flavonoids in plants are in glycosylated form, which influences its solubility, stability and bioavailability (Kim et al., 2006). Apart from that, hydroxylation and acylation of flavonoids also affect their polarity (Fucci et al., 1983). Flavonoids occur as O-glycosides and not so frequently as C-glycosides (Koh & Mitchell, 2008). The sugars of the O-glycoside are usually attached to hydroxyl group of flavonoid nucleus at C-3 and/or C-7 positions, while the C-glycosides are connected to either C-6 or C-8 positions. The C-glycosides are not cleaved by acid hydrolysis which differs from that of O-glycosides, and the acetyl, malonyl or organic acid residues would substitute the sugars (Bandykova & Yugin, 1979; Koh & Mitchell, 2008).

### 1.11.1.1 Flavan-3-ols
Proanthocyanidins or condensed tannins are formed as a result of oxidative condensation of monomeric flavan-3-ol (eg. catechin, epicatechin and epicatechin
gallate) to dimers, oligomers and then polymers, based on hetero- or homo-monomer units (Xie & Dixon, 2005). Interestingly, catechin with gallate groups (e.g. epicatechingallate and epigallocatechin gallate), as well as a pyrogallol-type structure on B-ring exhibits a relatively higher anti-cancer activity (Achiwa et al., 1997; Saeki et al., 2000). Hydrolysis of proanthocyanidins using acid in alcoholic solutions yields anthocyanidins (Bate-Smith & Lerner, 1954). There have been many efforts in analysing proanthocyanidins and its monomeric units which account for their potential health related benefits (De Bruyne et al., 1999; Arts et al., 2001). Consumption of foods and beverages, including green tea (Demeule et al., 2002; Babu & Dongmin, 2008), red wine (Corder et al., 2006), cocoa or chocolate (Steinberg, Bearden & Keen, 2003), grape seed (Dinicola et al., 2010) and apple (Boyer & Liu, 2004) have been widely promoted for their potential health functional properties against cardiovascular diseases and cancers due to their catechin contents. Nevertheless, many other types of foods including vegetables, spices, fruits and legumes are also found to be rich in catechins and proanthocyanidins (Arts et al., 2000; Gu et al., 2003; Harnly et al., 2006). The degree of condensation or polymerization and monomer types of proanthocyanidins are known to be related to astringency and bitterness (Lea & Arnold, 1978), as well as their their tendency to complex with proteins (Pringent et al., 2009).

1.1.1.2 Flavonones
Hesperidin and naringenin are two types of flavonones that are abundant in citrus fruits (Yusof, Ghazali & King, 1990; Wang, Chuang & Ku, 2007), and they can also be found in grape fruits (Jagetia & Reddy, 2011). Hesperidin has potential chemopreventive activity against colon cancer induced by 1,2-dimethylhydrazine (Aranganathan & Nalini, 2009) as demonstrated by animal model experiments, while naringenin has protective effects against cadmium-induced hepatotoxicity (Renugadevi & Prabu, 2010) and dextran sodium sulphate-induced colitis (Amaro et al., 2009).

1.1.1.3 Flavonols
Myricetin, quercetin and kaempferol and their glycosides are among flavonols that are commonly found in vegetables leaves, fruits, legumes and beverages (Herrmann, 1988; Sampson et al., 2002). Flavonols are shown to have in vitro chemopreventive ability against Medulloblastoma, a malignant brain tumor in children (Labbe et al.,
Moreover, rutin, which is being one of the well known flavonol glycoside, together with other flavonols are reported to have anti-inflammatory activity by animal models (Guardia et al., 2001). In addition, rutin is also reported to have ability to suppress tumor growth in rats (Deschner et al., 1991). However, flavonols are shown to exhibit a greater level of bioavailability than its glycoside, rutin (Manach et al., 1997). Both quercetin and kaempferol are widely distributed in vegetables, especially free standing leaves as a response to light, except for onions (Herrmann, 1988). Quercetin distributes relatively more widely in fruits than kaempferol and myricetin (Hoffmann-Ribani, Huber & Rodriguez-Amaya, 2009). Supplementation of quercetin is shown to be effective in reducing blood pressure of stage 1 hypertensive patients (Edwards et al., 2007). Seven types of flavonol glycosides are quantified in berries, and their contents vary when harvested in different seasons (Yang et al., 2009). The level of flavonol contents can also vary among white grape cultivars (Castillo-Munoz et al., 2010).

1.11.1.4 Flavanones
Flavanone including hesperidin, naringin and taxifolin are mostly found in citrus fruits (Kawai et al., 1999; Medina-Remon et al., 2011). Taxifolin is also detected in heartwood and the bark of some tree species (Hergert & Goldschimid, 1958). Flavanones have been long reported to have anti-inflammatory activities and chemopreventive effects (Gupta et al., 1971; Tanaka et al., 1997; Benavente-Garcia et al., 2007). Only consumption of flavanones but not the other five other classes of flavonoids is reported to cause an inverse relationship with stomach adenocarcinoma as reported by epidemiology studies (Lagiou et al., 2004).

1.11.1.5 Flavones
Apigenin and luteolin and their glycosides are two types of flavones often present in many vegetables especially at their leafy parts (Herrmann, 1988; Miean & Mohamed, 2001; Wang et al., 2003). Flavones are reported to exhibit anti-carcinogenesis potential. For instance, flavones are reported to have cytostatic and cytotoxic effects on some types of cancers in vitro (Kaur et al., 1992; Huang et al., 1999; Yin et al., 2001); luteolin is shown to activate apoptosis (programmed cell death) in transformed colon epithelial cells but not the non-transformed normal cell counterparts through both cell culture and animal models (Wenzel et al., 2000); apigenin exhibits the greatest potency in inducing apoptosis on HL-60 cells comparing to other types of
flavonoids including quercetin, myricetin and kaempferol (Wang, Lin-Shiau & Lin, 1999). Nevertheless, epidemiology studies investigating on relationships between flavone intake and cancer risks give contradictory findings (Hertog et al., 1994; Hertog et al., 1995; Hirvonen et al., 2001). Luteolin is also reported to have inhibitory activity on α-glucosidase and amylase (Kim, Kwon & Son, 2000).

1.11.1.6 Anthocyanins

Anthocyanin is one of the flavonoids that have been gaining a substantial interest, possibly due to the fact that most of them including malvidin, cyanidin and apigenidin are plant pigmentation and contribute to different colours in beverages (Bakker & Timberlake, 1985; Bridle & Timberlake, 1997). The colours of anthocyanins range from red to purple/blue and they are reported to have health benefits (Zafra-Sone et al., 2007). Anthocyanins are present in most of the red- and purple/blue-coloured fruits and vegetables (Goiffon, Brun & Bourrier, 1991; Kahkonen et al., 2003; Sondheimer & Kertesz, 1948), olives (Romani et al., 1999), black beans (Takeoka et al., 1997) and kidney beans (Choung et al., 2003). It has been an ongoing interest of the food industry to use anthocyanins as colouring agents (Francis, 1989). However, stability of pigmentation in food products over time remains a challenge to the food industry because the pigmentation is prone to enzyme degradation and/or interaction with other components in food matrixes (Wrolstad, Durst & Lee, 2005). Structural alternation of anthocyanins cause changes in colour visually (Stintzing et al., 2002; De Freltas & Mateus, 2006). Furthermore, interaction of anthocyanins with tannins is thought to result in colour changes in red wines during the aging process (Liao, Cai & Haslam, 1992; Singleton & Trousdale, 1992). Anthocyanins are reported to have potent antioxidant, anti-inflammatory (Hwang et al., 2011) and chemopreventative activities (Vareed et al., 2006; Zhang et al., 2008). Anthocyanins also have protective effects against DNA damage induced by tert-butyl-hydroperoxide in smooth muscle and hepatoma cells of rats (Lazze et al., 2003). Feeding rodents with purified extracts of blueberries and strawberries exhibits preventative effect against obesity in rats, this is the inverse for that of whole blueberries and strawberries (Prior et al., 2008; Prior et al. 2009). Conversely, ingesting strawberries rich in anthocyanins is thought to be associated with a lower postprandial inflammation and insulin secretion (Edirisinghe et al., 2011).
1.11.1.7 Isoflavones

Legumes are known to contain isoflavones such as genistein and daidzein (Kaufman et al., 1997) which have estrogen-like activity (Zhao et al., 2005). Consumption of soybeans are reported to have a variety of health benefits, such as lowering lipid peroxidation and increasing the ability of LDL to resist oxidation, thereby preventing atherosclerosis as reported in a human study (Wiseman et al., 2000); improving vascular reactivity, thereby having a cardiovascular benefit to post-menopausal women (Goodman-Gruen & Kritz-Silverstein, 2001; Lissin et al., 2004); increasing secretion of interleukin-6 in serum of middle-aged women, which is thought to improve their immune system (Jenkins et al., 2002); lowering cholesterol in human (Zhou, Melby & Watanabe, 2004). Furthermore, isoflavones exhibit potential chemopreventative activities including prevention of photocarcinogenesis (Moore et al., 2006), as well as inducing apoptosis of prostate and pancreas cancer cells as evidenced by many in vitro studies (Kyle et al., 1997; Li et al., 2004). The cancer preventative ability of isoflavones is further supported by epidemiological studies that relates consumption of isoflavones with a lower risk of prostate and breast cancers in Japanese men and women (Iwasaki et al., 2007; Kurahashi et al., 2007). Nevertheless, effect of phytoestrogens as one type of isoflavones in protecting or promoting breast cancers remain contrary and inconclusive (Rice & Whitehead, 2006).

1.11.2 Non-flavonoids

Non-flavonoids include hydroxybenzoic and hydroxycinnamic (phenylpropanoids) acids and they are termed phenolic acids. Hydroxybenzoic and hydroxycinnamic acids are built up of C₆C₁ and C₆C₃ skeletons (Crozier et al., 2006).

1.11.2.1 Phenolic acids

Hydroxybenzoic acids are grouped according to the number of hydroxyl groups being attached to a phenol ring structure. They include monohydroxybenzoic acids (eg. salicylic, 3- and 4-hydroxybenzoic acids), dihydroxybenzoic acids (eg. protocatechuic and gentisic acids) and trihydroxybenzoic acid (eg. gallic and syringic acids). Hydroxycinnamic acids include coumaric, caffeic, ferulic, chlorogenic and sinapic acids. In general, hydroxycinnamic acids exhibit a greater antioxidant activity (H- or electron donating ability) than that of hydroxybenzoic acids (Rice-Evans et al., 1996). However, antioxidant activity among individual group of phenolic acids can
vary depending on the number and position of hydroxyl groups and substitutes. Notably, a higher antioxidant activity is observed with the presence of a \( \text{CH}==\text{CH}-\text{COOH} \) group in cinnamic acids than in benzoic acids (Chimi et al., 1991), as well as methoxy substitutions at the ortho-position of monophenols (Cuvelier, Richard & Berset, 1992). Phenolic acids are widely distributed in plant materials and bind covalently to the cell wall polysaccharides mainly through ester (although some are ether-linked) and glycosidic linkages to hemicellulose arabinose units (Moore & Jung, 2001; Scalbert et al., 1985). They are usually determined simultaneously by hydrolyzing using acid and/or alkaline in order to liberate phenolic acids (Dabrowski & Sosulski, 1984; Mattila & Hellstrom, 2007; Ross, Beta & Arntfield, 2009). Nevertheless, the acid hydrolysing processes may encounter some losses in hydrocinnamic acids in the analysis (Krygier et al., 1982; Robbins R., 2003). Phenolic acids are ubiquitous in many types of vegetables, fruits, grains and legumes (Zhou et al., 2004; Mattila & Hellstrom, 2007; Russell et al., 2009), as well as beverages (Shahrzad & Bitsch, 1996). However, the results of phenolic acid determinations can vary according to the extraction and determination methodologies (Escarpa & Gonzalez, 2001; Nardini & Ghiselli, 2004; Ross et al., 2009).

Phenolic acids have been reported to have potent anti-cancer properties (Kampa et al., 2003) and have abilities to inhibit adipogenesis which is associated with anti-obesity benefits in vitro (Hsu, Huang & Yen, 2006; Hsu & Yen, 2007b). Gallic acid is found to have protective effect against dyslipidaemia and hepatosteatosis induced by high fat diets in rats (Hsu & Yen, 2007a). Interestingly, phenolic acids of the trihydroxylated derivatives are demonstrated to have a potent cytotoxicity and antiproliferation effect on various cancer cell lines but not on that of non-transformed cell line counterparts (Gomes et al., 2003). The derivatives of caffeic and gallic acids with methyl and octyl esters have a significantly lower anti-proliferation effect on cancer cell lines than their propyl ester counterparts (Fiuza et al., 2004). Esters of \( p \)-hydroxybenzoic acids are reported to have an increasing toxicity in proportion to the length of alkyl esters. However, the overall toxicity is low as demonstrated by acute toxicity studies (Mathews et al., 1956). The safety of applying alkyl esters of \( p \)-hydroxybenzoic acids (parabens) in food and pharmaceutical products as preservatives has gained attentions (Soni et al., 2001; Kawaguchi et al., 2009). It is claimed that the parabens might have an estrogenic activity (Hu & Aizawa, 2003; Lemini et al., 2003). Conversely, an increasing antimicrobial activity is found in
proportional to the length of alkyl esters of various phenolic acids (Merkl et al., 2010). Seven out of nine types of phenolic acids are shown to have protective effects against oxidative DNA damage, except for 3-hydroxybenzoic acid that has an inverse effect as revealed by an in vitro study (Lodovici et al., 2001). Moreover, o-coumaric acid is also shown to have antiplatelet activity through both in vitro and in vivo studies, this may be beneficial in preventing vascular diseases (Luceri et al., 2007).

**1.11.2.2 Hydrosable tannins**

Condensation of gallic acids and hexahydroxydiphenolic acids forms gallotannins, while condensation of hexahydroxydiphenic acids derives ellagic tannins, the condensation of the mentioned phenolic acids can lead to forming of high-molecular-weight polymers (Bravo, 1998). Gallotannins and ellagitannins are both hydrosable tannins that are readily hydrolysed to release gallic acid and/or ellagic acid by acid, alkaline, hot water or enzymatic actions. Inversely, condensed tannins are not hydrolysable (Crozier et al., 2006). Nevertheless, it is known that some flavonoids such as catechin gallates are consisted of gallic acid esterified from catechin, which contain both properties as hydrosable and condensed tannins (De Bruyne et al., 1999; Schofield, Mbugua & Pell, 2001). Both types of tannins share similar abilities to complex proteins and carbohydrates (Reed , 1995). A great number of health related functional properties of hydrosable tannins from different plant materials have been reported. Hydrosable and condensed tannins are known to be abundant in many plant species (Tarascou et al., 2010). Condensed tannins have been reported to exhibit different health benefits: the 70% acetone extract from Eugenia jambos L. and isolated hydrosable tannins from the extract exhibit in vitro anti-cancer ability via induction of apoptosis (Yang et al., 2000); pomegranate extract rich in hydrosable tannins demonstrates an anti-inflammatory arthritis ability in rats (Shukla et al., 2008); tannic acid lower hepatic lipogenesis and atherogenesis in mice effectively (Do et al., 2011); and, tannins from Mouriri pusa can potentially reduce inflammatory gastric ulcer (Vasconcelos et al., 2010). Interestingly, hydrosable tannins exhibit a significantly greater suppression activity on tumor cells than that of epigallocatechin-3-o-gallate as shown by Tanimura et al. (2005). Ellagic acid from ellagitannins has recently gained interests for its potential health functional properties such as prevention of atherosclerosis (Yu et al., 2005; Chang et al., 2008) and other types of cancers (Wang, Chen, & Yang, 1999) through in vitro and in vivo models. In particular, ellagic acids have protective effect against colon cancers by
inducing apoptosis in the colon cells but not the non-transformed cells *in vitro* (Larrosa et al., 2006).

### 1.12 Conclusion

Generation of ROS in the human body is part of the normal physiological processes, which is modulated by a series of enzymatic and non-enzymatic antioxidant systems. Nevertheless, the human body often fails to precisely modulate the related redox signaling process, which can lead to oxidative stress. Oxidative stress is a potential source of tissue inflammations, DNA mutations, cardiovascular diseases and cancers. Hypertension has been recognised as one of the important factors that accelerate the cause of diseases.

Some minor constituents from plant sources are thought to provide health benefits, although negative health effects are sometimes reported. Among the minor constituents, phenolic compounds have been widely linked to antioxidant activities and associated with health benefits, including anti-inflammation, anti-cancer, anti-hypertension, anti-atherosclerosis and the prevention of other diseases.

Faba beans are an important ingredient in many traditional dishes in different parts of the world, and are gaining an increasing popularity in the modern world for their health benefits. These benefits are due to their major as well as minor constituents, particularly phenolic compounds, which are thought to be natural antioxidants. Although faba beans have had an important place in the world export market, further research to explore and confirm the beneficial health properties of faba beans can potentially increase the popularity and utilization of faba beans as human foods, thereby bringing better returns to growers. Furthermore, there is a potential to develop faba bean cultivars which have specific health properties.

There have been a limited number of studies on the phenolic composition of faba beans. Also, there is a lack of understanding on the functionalities of the phenolic composition in faba beans; particularly the impact of cooking has on these functionalities.

The current study aims to:

1. Optimise the extraction of functional compounds from faba bean phenolic extracts and chemically characterise the compounds within these extracts.
2. Assess the effect of processing methods including soaking, boiling, autoclaving and dry roasting on phenolic contents and antioxidant properties of a range of faba bean genotypes.
3. Determine the potential health benefits of faba bean extracts (before and after cooking) by measuring their potential: anticancer and antihypertensive properties as well as their ability to inhibit digestive tract enzymes including $\alpha$-glucosidase and lipase.
Chapter 2 Materials and Methods

2.1 Sample preparation and storage

The 12 selected faba genotypes consisted of five different seed coat colours:

(1) white: breeding line $TF(\text{Ic}^*\text{As})^*483/13$ (Figure 2-1);

Figure 2-1 Faba bean group: white ($TF(\text{Ic}^*\text{As})^*483/13$)

(2) green: cv. *Icarus* (Figure 2-2);

Figure 2-2 Faba bean group: green (*Icarus*)

(3) red: cv. *Rossa* (Figure 2-3);

Figure 2-3 Faba bean group: red (*Rossa*)
(4) purple: cv. Deep Purple (Figure 2-4) and

Figure 2-4 Faba bean group: purple (Deep Purple)

(5) buff: breeding line 974*(611*974)/42, breeding line 1269*483/6-1, breeding line 1323/3, cv. Doza, cv. Farah, cv. Fiord, cv. Nura, breeding line S95007/1 (Figure 2-5)

Figure 2-5 Faba bean group: buff (a. 974*(611*974)/42, b. 1269*483/6-1, c. 1323/3, d. Doza, e. Farah, f. Fiord, g. Nura and h. S95007/1)

All bean samples were grown to maturity at Wagga Wagga Agricultural Institute, Australia and mechanically harvested at the same time. Bean samples from the 2008 growing season were cleaned by removing foreign materials, loosely packed and stored in the dark at room temperature for five months to naturally dry, followed by storing at -18°C. Faba beans from the 2009 growing season were dried at 50°C for three days before storing at -18°C and used for the acid and alkaline hydrolysis
(section 2.7), fractionation of extracts (section 2.8.4), cell culture (section 2.9) and enzymatic tests (section 2.10). Bean samples were either used as whole seeds or ground into flours using an IKA-Universalmühle M20 Grinder (Janke and Kunkel, Staufen, Germany) and stored in screw cap plastic containers at -18°C.

2.2 Processing

2.2.1 Dry Roasting

Roasting was conducted at 150°C using dry heat on an open tray in an oven (Premium Laboratory Oven, Thermoline Scientific, NSW, Australia) for 60 min (Acar et al., 2009). The roasted beans were cooled at room temperature and ground into flour using an IKA-Universalmühle M20 Grinder (Janke and Kunkel, Staufen, Germany).

2.2.2 Soaking

A sample of raw faba beans (10 g) was added to 50 mL of distilled water, allowed to soak for 12 hr overnight at room temperature (Nasar-Abbas et al., 2008). After soaking, the water was drained and the soaked beans were blotted dry using paper towels. The softened bean samples were added to 100 mL of aqueous acetone (acetone:water, 70:30, v/v) and the extraction of the phenolic compounds was performed as described in the below section 2.3.

2.2.3 Boiling

A sample of soaked bean (from 10 g of raw faba beans prepared in section 2.2.2) was added into 100 mL of boiling distilled water and allowed to boil at atmospheric pressure for 40 min. The cooking broth was drained, freeze dried, and kept at -18°C for further tests. The boiled beans was centrifuged at 2000 g for 5 min at 4°C to remove excess water and a solution of aqueous acetone (100 mL) was added and the extraction was performed as described in section 2.3.

2.2.4 Autoclaving

A sample of raw faba bean was added with 50 mL of low calcium content distilled water (80 mg of calcium chloride/L), allowed to soak for 12 hr at room temperature.
After draining the soaking solution, a solution of 2% sodium chloride (100 mL) was added to the soaked beans and autoclaved at 115°C for 20 min (Revilla & Vivar-Quintana, 2008). The cooking broth was drained, freeze dried and kept at -18°C for further analysis. The autoclaved beans were centrifuged to remove excess water, and a solution of aqueous acetone (100 mL) was added to the beans to perform extraction as described in section 2.3.

2.3 Preparation of crude phenolic extract
Extraction of phenolic compounds was carried out on all the raw and processed faba bean samples as outlined in section 2.2 above by dispersing bean or ground flour in aqueous acetone (Merghem et al., 2004) or aqueous methanol (methanol:water, 80:20, v/v) (Michalska et al., 2007) at a solid to solvent ratio of 1:10 and shaking (150 rpm, Orbital Mixer; Ratek OM11, Australia) for 2 hr at room temperature. Similarly for the soaked, boiled and autoclaved bean samples, the same solid (bean weight dry basis) to aqueous acetone ratio were applied for extraction, blended using a hand held blender (Multiquick/Minipimer Professional MR 5550, Braun Kronberg, Germany) for 35 s and shaken for 2 hr. Supernatant was collected after centrifugation at 4000 g for 5 min at 5°C. A second extraction was performed on the residue while maintaining the same sample to extraction solvent ratio 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 to obtain a fine lyophilised powder using a Christ-Alpha 1–4 freeze dryer (Biotech International, Germany). The extracts were stored at -20°C until required. Distilled water was used to dissolve the dried extract and these reconstituted extracts were filtered through 0.45 µm Millipore filters before analysis. All extractions and measurements were performed at least in triplicate, except extraction from the raw beans which was performed in duplicate.

2.4 Preparation of purified phenolic extract
Purification was carried out on the crude phenolic extracts from raw Nura and Rossa beans as described by Terahara et al. (2000) and Netzel et al. (2007). Open chromatography column filled with 1 kg of Amberlite XAD7 mesh 20-60 acrylic resin (Sigma-Aldrich) was used for purification of crude phenolic extracts. New resin (1 kg for each column) was washed three times using distilled water, tightly packed into the open columns with dimension 690 cm² and 560 cm² for purification of Nura and Rossa extracts respectively due to the varying column sizes. The resin was filled
with distilled water while loading the crude extracts from the top of the column. A solution of 1% acetic acid (v/v) (2 L) was used to elute the impurities and was discarded. Next, a pure acetone (2 L) was used to elute the purified extract gradually. The eluted extract in acetone was concentrated using a rotary evaporator and freeze-dried.

2.5 Determination of physical properties of faba bean

2.5.1 Colour determination

Colour determination was conducted using a Minolta Chroma Meter CR-310 (Minolta Co., Osaka, Japan) with a CR-A33E class light projection tube and a white calibration tile. A white container was used to hold bean samples and at least two layers of the bean samples were placed in containers for each measurement to minimise the background interference. Colour was defined by the L*, a* and b* tristimulus system (CIELAB), where L* was a measure of lightness (0= black; 100=white); a* was a measure of red/green hue (+60= red; -60= green); and b* was a measure of blue/yellow hue (+60= yellow; -60= blue).

2.5.2 Hydration and swelling coefficients

Hydration coefficient was determined by soaking the raw beans for 12 hr at room temperature in deionised water. The hydration coefficient was expressed as a percentage of increase in bean weight (Nasar-Abbas et al., 2008):

\[
\text{Hydration coefficient} = \frac{\text{Weight of bean seeds after soaking}}{\text{Weight of bean seeds before soaking}} \times 100
\]

The raw bean samples were placed in a graduated cylinder and soaked in deionised water for 12 hr to determine swelling coefficient as described above. After soaking, the water volume displaced was estimated and the swelling coefficient expressed in percentage was calculated as below (Nasar-Abbas et al., 2008):

\[
\text{Swelling coefficient} = \frac{\text{Volume of bean seeds after soaking}}{\text{Volume of bean seeds before soaking}} \times 100
\]
2.6 Chemical assays

2.6.1 Total phenolic content (TPC) assay

The total phenolic content (TPC) was determined according to Konczak et al. (2010). A sample of extract solution (25 µL) was inserted into a 96-well microplate. A solution of Folin-Ciocalteu reagent (Sigma-Aldrich) (diluted 10-fold with distilled water) (125 µL) was added to the microplate and the microplate was shaken for 3 min at room temperature. The absorbance of ascorbic acid was read at 600 nm. After that, a sample solution of 6% sodium carbonate (Na$_2$CO$_3$, Ajax Chemicals) in distilled water (w/v) (125 µL) was added to the mixture and shaken for another 15 min at room temperature. The absorbance was then measured at 600 nm again. The final absorbance of sample was obtained after subtraction from the absorbance of sample blank and the absorbance of ascorbic acid. A gallic acid standard (Sigma) (seven different concentrations from 0.01-0.10 mg/mL) was included in the experiment and the TPC was expressed as mg of gallic acid equivalent per gram of dry bean (GAE/gDW).

2.6.2 Total flavonoid content (TFC) assay

The total flavonoid content (TFC) assay was determined according to Michalska et al. (2007), except that the total volume was reduced to fit in a microcuvette. Sample extracts (125 µL) were diluted with 600 µL of distilled water, followed by addition of 5% sodium nitrite (NaNO$_2$, Ajax Finechem) in distilled water (w/v) (37.5 µL). Mixtures were allowed to stand at room temperature for 6 min. After that, a sample of 10% aluminium chloride hexahydrate (AlCl$_3$.6H$_2$O, Sigma-Aldrich) in distilled water (w/v) (75 µL) was added to the mixtures and allowed to stand for another 5 min at room temperature. A solution of 1 M sodium hydroxide (NaOH) (250 µL) was added to the mixtures, and the absorbance was read at 520 nm after mixing. A catechin standard curve made up of at least six data points prepared from different concentrations (0.01-0.20 mg/mL) of (+)-catechin gallate (Sigma) was generated under the same conditions. The flavonoid content was expressed as mg of catechin equivalent per gram of dry bean (CE/gDW).
2.6.3 Total proanthocyanidin content (TPro) assay

The proanthocyanidin content (TPro) of the extracts were determined according to the hydrochloric acidified 4-dimethylaminocinnamaldehyde (DMAC-HCL) protocol as described in Li, Tanner & Larkin (1995) with a reduced volume to fit in a 96-well microplate. The 2% 4-dimethylaminocinnaldehyde (DMAC, Sigma) (w/v) was prepared in a mixture of cold methanol and 6 N HCl (1:1, v/v). The 9 mL of DMAC mixture was diluted with 25 mL of pure methanol to make up a diluted DMAC solution. Sample extract (160 µL) was inserted into a 96-well microplate, followed by addition of 50% methanol (v/v) (80 µL). After that, the diluted DMAC solution (40 µL) was added into the wells to begin the reaction. The mixture was incubated at room temperature (about 25°C) for 20 min and the absorbance was measured at 640 nm. A catechin standard curve (0.02-0.10 mg/mL) was prepared from (±)-catechin hydrate (Sigma) which was assayed under the same conditions. The results were expressed as mg of catechin equivalent per gram of dry bean (CE/gDW).

2.6.4 Trolox equivalent antioxidant capacity (TEAC) assay

The Trolox equivalent antioxidant capacity (TEAC) assay measures the relative ability of extracts to react with the radical cation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS·+) (Arts et al., 2004). A solution of blue/green colour ABTS·+ chromophore was produced by mixing 2.45 mM potassium persulfate (K₂S₂O₈, Sigma) and 7 mM of ABTS (Sigma) in deionized water (1:1, v/v), stored overnight in the dark for at least 16 hr. The ABTS·+ solution was then diluted using deionized water to achieve an absorbance of 0.7±0.010 measured at 734 nm. Sample extract (100 µL) was added to the diluted ABTS·+ solution (1 mL), well mixed and allowed to stand at room temperature for 5 min before measuring the absorbance at 734 nm. At least seven different concentrations (5-500 µM) of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid standard (Trolox, Sigma-Aldrich) in water (0.02-0.5 mM) were prepared under the same conditions to establish a Trolox equivalent antioxidant capacity (TEAC) standard curve. The results were expressed as µM of Trolox equivalent per gram of dry bean (TE/gDW).
2.6.5 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay determines the relative ability of compounds to scavenge 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH) (Michalska et al., 2007), except that the total volume was reduced to fit in microcuvettes. A solution of DPPH was prepared by dissolving 10 mg of DPPH in 25 mL of 80% methanol (v/v). Sample extract (50 µL) was mixed with the DPPH solution (125 µL), followed by dilution with 1 mL of deionised water. The solution mixture was allowed to stand at room temperature in the dark for 20 min and the absorbance was measured at 517 nm. A Trolox standard curve was established from at least seven data points (25-1000 µM), which was prepared at the same condition. The results were expressed as µM of Trolox equivalent per gram of dry bean (TE/gDW).

2.6.6 Oxygen radical absorbance capacity (ORAC) assay

The antioxidant activity was determined using an oxygen radical absorbance capacity (ORAC) assay as outlined by Prior et al. (2003) using a FLUOstar Omega UV-Vis microplate reader (BMG Labtechnologies, Offenburg, Germany) with slight modifications. The fluorescein stock solution was prepared by dissolving 0.0255 g of fluorescein (Sigma-Aldrich) in 50 mL of phosphate buffer (pH 7, 0.075 M). The fluorescein stock solution (50 µL) was diluted with 10 mL of buffer solution. The fluorescein working solution was freshly prepared everyday by adding a solution of the fluorescein stock solution (800 µL) with 50 mL of buffer solution, incubated in a water bath at 37°C. Similarly, 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) solution (79.6 µM) was freshly prepared every day. To begin the ORAC assay, a solution of sample extract (20 µL) was pipetted into a 96-well microplate, containing the fluorescein working solution (150 µL) and incubated at 37°C for 30 min in the chamber of the microplate reader. After that, the AAPH solution (25 µL) was auto-injected by the liquid handling system and the mixture was mixed at 500 rpm for 5 s. The fluorescein readings were measured at an excitation wavelength at 485 nm and an emission wavelength at 520 nm (60 cycles, 90 s/cycle and 10 flashes/well) every min for 90 min. The ORAC results were calculated using a MARS Data Analysis Software (Program version: 2.10R3) (BMG Labtechnologies).
The net area under the fluorescein decay curve (AUC) of sample was obtained by subtracting the AUC from the control blank. A Trolox standard curve with seven data points (0.5-50 µM) was included in every microplate and a linear regression equation was established using the net AUC obtained from different Trolox concentrations ($r^2=0.98$). The gain was adjusted before addition of AAPH using the well containing the highest concentration of Trolox. The final results were expressed as µM Trolox equivalent per gram of dry bean (TE/gDW).

2.6.7 Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power (FRAP) assay was used to measure the ferric reducing ability of faba bean extracts (Benzie & Strain, 1996). A solution of 2,4,6-tripyridyl-s-triazin (TPTZ) (10 mM) was made by dissolving 31.2 mg of TPTZ in 10 mL of HCl solution (40 mM). A 10 mL aliquot of iron (III) chloride (FeCl$_3$.6H$_2$O) solution (20 mM) in water (w/v) was added to 1 mL of TPTZ solution (10 mM) and 10 mL of acetate buffer (300mM, pH 3.6) to make up an FRAP-reagent solution. Sample extract solution (10 µL) was inserted into a 96-well microplate, followed by an addition of a solution of distilled water (30 µL). The FRAP-reagent solution (200 µL) was added to the sample mixtures and incubated for 8 min after shaking the microplate for 10-15 s. After that, the absorbance was measured at 595 nm. A standard curve made up from five different concentrations of iron(II)sulphate (FeSO$_4$) (0.5-2.5 mmol/L) was included in each microplate, and the results were expressed as mmol of Fe$^{2+}$ equivalents per gram of dry bean (Fe$^{2+}$eq/gDW).

2.7 Hydrolysis

2.7.1 Acid hydrolysis

The acid hydrolysis of the purified phenolic extract was performed as described in Daniel et al. (1989) and Arnous & Meyer (2008). Dried sample extract (10 mg) was hydrolysed using 2 mL of trifluoroacetic acid (2 M CF$_3$COOH, Sigma-Aldrich) solution for 2 hr at 120ºC in a heating block (Reacti-Therm III Heating/Stirring Module, Thermo Scientific, USA). After that, the acid solution was made up to 5 mL in a volumetric flask with 80% methanol (v/v) and stored at -18ºC. The solution was filtered using a 0.45 µm polypropylene membrane (Millipore) before analysis.
2.7.2 Alkaline hydrolysis

The alkaline hydrolysis method was adapted from Luthria & Pastor-Corrales (2006). The purified phenolic extract (10 mg) was hydrolysed using 2 M NaOH (5 mL) for 30 min at 45°C (laboratory oven, Memmert, Germany). After the alkaline hydrolysis, the mixture was acidified by adding 7.2 M HCl (1.4 mL). The free conjugated phenolic acids were then released by adding ethyl acetate (6.4 mL) and vortexed for 20 s. The extraction of free conjugated phenolic acids using ethyl acetate was repeated for three times, and the ethyl acetate was pooled from each extraction. The combined ethyl acetate was evaporated under vacuum by a rotary evaporator to a complete dryness. The dried remains were dissolved in 80% methanol (v/v) (5 mL) and vortexed for 30 s (three times). The solution was filtered using a 0.45 μm polypropylene membrane (Millipore) before analysis.

2.8 Separation using column chromatography

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

The 7 mM ABTS⁺ solution was prepared by dissolving the ABTS in deionised water and mixed with 2.45 mM of K₂S₂O₈ (1:1, v/v) overnight to allow a complete reaction as what described in section 2.6.4. The solution was diluted using distilled water to obtain an absorbance of 0.70±0.02 at 734 nm and filtered through a 0.45 μm polypropylene membrane (Millipore).

2.8.2 On-line PCD with high performance liquid chromatography (PCD-HPLC) for phenolic compound profiling

Analysis of the antioxidant activity was carried out on-line using ABTS⁺ cation radicals (Ee et al., 2011), where the HPLC eluent was allowed to react with the ABTS solution directly. The HPLC system (ProStar model 410) consisted of 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 μL) of extract sample (50 mg/mL) was dissolved in the solvent A and injected to the HPLC system. Separation of compounds was performed by gradient elution: 0-5% phase B (6 min), 5-30% phase B (12 min), 30-40% phase B (6 min), 40-45% phase B (6 min), 45-52% phase B (3 min), 52-100% phase B (3 min) and 100% phase B for 4 min,
using a Phenomenex Luna 5U C18 column (100A pore size; 150 × 3mm) and preceded by a guard column (Phenomenex, 4 × 3mm). The UV spectra were recorded at 280 nm. The PCD antioxidant activity of the HPLC eluent was determined on-line when 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 at 414 nm.

### 2.8.3 Separation using HPLC for characterisation and quantifying phenolic compounds

The HPLC system (Shimadzu, Japan) for characterisation and quantifying phenolic compounds consisted of an auto injector (SIL-10AD VP), a degasser (DGU-20A), pumps (LC-10AD), a diode array detector (SPD-M10A), a column oven (CTO-10AS) and a system controller (SCL-10A). The phases A and B were 0.5% trifluoroacetic acid (TFA, Sigma-Aldrich) (v/v) in distilled water and acetonitrile:TFA:water (95:0.5:4.5, v/v/v) respectively. The column oven was maintained at 25ºC and the solvent elution rate was set at 1 mL/min. The gradient elution method used to separate phenolic compounds was outlined by Netzel et al. (2007) with modification, where phase B was eluted from 0-10% over first 10 min, 10-50% for 45 min, 50-80% for 15 min, 80-100% for 15 min and lastly an isocratic elution with 100% phase B over 10 min, for an overall 95 min HPLC run. The absorbances of HPLC eluents were monitored at four different wavelengths: 260 nm for determination of hydroxybenzoic acids/flavanols; 326 nm for determination of hydroxycinnamic acids; 370 nm for determination of flavonols and 520 nm for determination of anthocyanins. The amounts of respective phenolic classes were expressed as GAE for hydroxybenzoic acids/flavanols; chlorogenic acid equivalents (CHAeq) for hydroxycinnamic acids; rutin equivalents (RE) for flavonols and cyanidin 3-glucoside equivalents (C3Geq) for anthocyanins.

### 2.8.4 Fractionation of phenolic extracts using Preparative HPLC

Fractionation of crude phenolic extracts was carried out using a preparative scale HPLC system (ProStar Prep HPLC system, Varian) consisting of a Varian ProStar ultraviolet-visible (UV-Vis) detector (Model 320), Varian PrepStar Solvent Delivery Module pumps (Model SD-1) and a Varian ProStar fraction collector (Model 701).
An aliquot (2 mL) of crude extract from the dry roasted *Nura* for 60 min at 150°C (0.25 g/mL) in phase A was injected for separation using a Varian Dynamax Microsorb 100-5 C18 reverse phase column (250 mm × 21.4 mm). The gradient elution profile was the same as the HPLC-PCD system described in 2.8.2 with a flow rate of 15 mL/min. Two fractions: fraction A and B, were collected which represented the elution from 1-10 min and 10-30 min respectively from the analytical HPLC system. The collected fractions were concentrated by evaporation under vacuum and lyophilised.

2.9 Analysis using cell culture

2.9.1 Cell cultures

All cells lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD) except for the human bladder transitional cell carcinoma (BL13) cells which were obtained from Dr. D. Brookes (Brookes et al., 1998). All cells were cultured at 37°C in a humidified 5% CO₂/95% air atmosphere. The BL13 cells were cultured in the Roswell Park Memorial Institute medium (RPMI; Invitrogen Corporation, Australia); human gastric adenocarcinoma (AGS) cells in the F-12K Ham’s medium (Invitrogen Corporation, Australia); human hepatocellular carcinoma (Hep G2) cells in the Eagle’s minimum essential medium (EMEM; Sigma-Aldrich, Australia); human colorectal adenocarcinoma (HT-29) cells in the McCoy’s 5a modified medium (Invitrogen Corporation, Australia); Abelson murine leukemia virus-induced tumor (macrophage) (RAW264.7) cells in the Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, Australia); human acute promyelocytic leukaemia (HL-60) cells in the Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen Corporation, Australia) and human colon normal (CCD-18Co) cells were cultured in the EMEM. Each medium was supplemented with 100 µg/mL of streptomycin, 1000 units/mL of penicillin (Invitrogen Corporation, Carlsbad, CA) and 10% foetal calf serum, except that the IMDM for cultivating the HL-60 cells required 20% foetal calf serum.

2.9.2 Cellular antioxidant activity (CAA) assay

The assessment of cellular antioxidant activity (CAA) was achieved using an antioxidant assay described by Tan et al. (2010) and Wolfe & Liu (2007). Briefly, 1 × 10⁵/ mL of Hep G2 cells were plated in 96-wells microplates and incubated for 24 hr.
Next, the media was removed and the cells were washed using phosphate buffered saline (PBS). The cells were then treated with faba bean crude extract (10 µL in PBS) and addition of a solution of PBS (80 µL). After that, a solution of 250 µM 2',7'-dichlorofluorescein-diacetate (DCFH-DA, Sigma-Aldrich) in PBS (10 µL) was added to the microplate and the microplate was incubated for 1 hr. Subsequently, the cells were washed using 100 µL of PBS and added with 100 µL of 2,2'-azobis (2-amidinopropane) dihydrochloride in Hank’s Balanced Salt Solution (600 µM). The fluorescence was measured at 485 nm excitation and 538 nm emission wavelengths for every 5 min continuously in 1 hr. The final fluorescence values were corrected for 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 (QE/gDW).

2.9.3 Antiproliferation assay

The antiproliferation activity assessment of the crude faba bean extract was carried out on adhesive cells (BL13, AGS, Hep G2 and HT-29) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) colorimetric assay (Tan et al., 2010). Briefly, 2.7 × 10^5/ mL of Hep G2 and 1 × 10^5/ mL of other cells in 100 µL medium were seeded into each well of a 96-well microplate. After incubating for 24 hr, the cells were treated with the sample extracts (0.2-2.0 mg/mL) and allowed to incubate for another 24 hr. The medium was removed, the cells were washed using 100 µL of PBS, and the wells were refilled with 100 µL of PBS. Subsequently, 10 µL of MTT (5 mg/mL) was added and the cells were allowed to incubate for 4 hr. After that, the excess MTT was removed, and the MTT-formazan was dissolved using 100 µL of dimethyl sulphoxide (DMSO, Sigma). The microplate was shaken for 10 min and the absorbance was measured at 595 nm.

2.9.4 Cellular protection against hydrogen peroxide (H_2O_2)

The cellular protection against hydrogen peroxide (H_2O_2) assay was carried out using RAW264.7 cells (Tan et al., 2010). A range of crude extract concentrations were chosen to perform this experiment which did not markedly suppress the cell growth as observed in the above antiproliferation assays (section 2.9.3). A solution of medium (100 µL) containing 5 × 10^5/ mL of cells were plated into a 96-well
microplate and the cells were incubated for 24 hr. After that, a sample extract (10µL) was added to the cells and allowed an incubation of another 23 hr. The media was removed and cells were washed using PBS. After that, 100 µL of H₂O₂ (40 mM) was added into the wells and incubated for 1 hr. One group of wells was reserved as a control, where no sample and H₂O₂ were added to the cells. Another group of wells was reserved as a negative control, where only H₂O₂ was added to the cells. After 1 hr of incubation, the H₂O₂ was removed followed by washing using PBS. An aliquot of PBS (100 µL) and a 10 µL of MTT solution (5 mg/mL) were added to each well to perform the MTT assay as described in section 2.9.3.

2.9.5 Assessment of apoptosis and cytolysis by flow cytometry

An aliquot of (4.5 mL) of HL60 cell suspensions (5 × 10⁵/mL) were treated with 225 µL (0.8 mg/mL) of crude phenolic extracts from faba beans for 3, 12 and 24 hr in a 25 cm² culture flask. The 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). The cells were then stained using an 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 instructions. After the set incubation time, cells were harvested, washed with PBS and resuspended in an Annexin-binding buffer. After that, 100 µL of cells were stained by adding 5 µL of Annexin V and 1 µL of propidium iodine and incubated for 10 min at room temperature. Next, cells were mixed with 400 µL of Annexin-binding buffer and immediately transferred onto ice. Analysis was performed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and a 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 the cell populations were gated for analysis.

2.9.6 Determination of caspase-3 activity

The caspase-3 activity was conducted on HL-60 cells using a caspase-3 assay kit (Sigma-Aldrich) according to the manufacturer’s instruction. This assay determines the caspase-3 activity where the ability of cell lysates to cleave the peptide substrate
acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) and release p-nitroanline (pNA).

A solution containing 1x10⁶/ mL of cells (7.5 mL) was seeded in a 25cm² flask. A sample of crude extracts (0.8 mg/mL) (375 µL) was added and the cells were incubated at the designated time points (3, 6, 12 and 24 hr). After the incubation time, the cells were harvested, centrifuged at 600 g for 5 min to remove to the media. The cell pellets were washed in 1 mL of PBS. The cell pellets were resuspended in 500 µL of a 1 × Lysis Buffer, transferred onto ice and allowed to incubate for 20 min. After that, the cell pellets were centrifuged at 15,000 g for 15 min at 4ºC. The supernatants containing cell lysates were collected for analysis. Untreated cells were used as a control for comparison.

On a 96-well microplate, a sample (25 µL) of cell lysates from the treated cells and a non-treated control were plated and added with 65 µL of 1×Assay Buffer, followed by addition of the Caspase-3 substrate (10 µL) (2 mM). The microplate was covered and the mixtures were incubated overnight at 37ºC. The absorbance was then measured at 405 nm. The results were expressed as folds of caspase-3 activity versus control.

2.10 Enzymatic assays

2.10.1 Angiotensin converting enzyme inhibitory activity

The angiotensin converting enzyme (ACE) inhibition assay was carried out as described in Shalaby, Zakora & Otte (2006) using furanacroloyl-Phe-Glu-Glu (FAPGG) as a substrate. The ACE enzyme solution from rabbit lung (Aldrich Chemicals) (0.25 unit/mL) was freshly prepared everyday by adding 1 mL of 50 mM tris-HCl buffer containing 0.3 M sodium chloride (pH 7.5) into a vial of 0.25 unit of ACE enzyme. A sample of crude extract or buffer (control) (10 µL) and ACE solution (10 µL) were added to the wells of a 96-well microplate at room temperature. The microplate was transferred to the incubator of a FLUOstar Omega UV-Vis spectrophotometer (BMG Labtech, Germany), and 150 µL of the FAPGG solution (0.1 M) was injected into the wells through the liquid handling system. The mixture was mixed thoroughly for 5 s at 500 rpm and the absorbance was recorded every 30 s for 30 min at 340 nm. The ACE activity was calculated using the following formulation:

\[
ACE \text{ inhibition} \% = (1 - \frac{S_S}{S_C}) \times 100
\]
Where $S_S$ is the slope for the sample, and $S_C$ is the slope for the control blank.

A catopril standard curve ranging from 40-400 µM captopril (Sigma Chemicals) was used and the results were expressed as µg of captopril equivalents per gram of dry bean.

### 2.10.2 Alpha-Glucosidase inhibition assay

The α-glucosidase inhibition was determined as described by Ikarashi et al. (2010) using sucrose as a substrate with slight modifications. An α-glucosidase enzyme solution was prepared by dissolving 100 mg of intestinal acetone powders from rat (Sigma Aldrich, Australia) 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. Crude extract (20 µL) was mixed with 2% sucrose (w/v) in maleate buffer (20 µL). The enzymatic reaction was initiated by adding enzyme solution (20 µL) 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 µL) was then used to react with the colour reagent (Glucose CII-Test Wako, Wako Pure Chemical Industries, Osaka, Japan) (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 with the buffer solutions. 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.

### 2.10.3 Lipase inhibition assay

The lipase inhibitory activity was assayed as described by Shimura et al. (1992) using 4-methylumbelliferyl oleate as substrate. The porcine pancreatic lipase (Sigma type II) solution was prepared by dissolving 0.085 g of lipase in 1 mL of a Mcilvaine’s buffer (pH 7.4) and underwent centrifugation for 10 min at 10000 g. The supernatant was collected for the experiment. Crude extract (50 µL) was added with 100 µL of 4-methylumbelliferyl oleate solution (0.1 mM). The lipase solution (50 µL) was then
added to initiate the reaction. The mixture was allowed to incubate in a 37°C water bath for 20 min. After the incubation period, 0.1 N HCL (1 mL) followed by 0.1 M sodium citrate (2 mL) were added into the mixture and mixed well to stop the enzymatic reaction. The mixture was measured at 450 nm emission and 320 nm excitation wavelengths.

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 a negative control measured fluorometrically at an emission wavelength at 450 nm and excitation wavelength at 320 nm, and \(F_{SB}\) and \(F_{CB}\) were the fluorescence readings of the sample and control blank.

2.11 Statistical analysis

All figures containing line and bar charts were plotted from the averages of samples as described in the above Materials and Methods section. Vertical error bars with caps were included where appropriate to show the standard deviations that were calculated using Microsoft Excel 2010 (Microsoft Inc, USA). Alphatical superscripts were included in the charts to show the statistical differences between data points where appropriate. The statistical differences were calculated using Prism 5 (Graphpad Software, CA, USA).

2.11.1 Chapter 3

The analysis of variance (ANOVA) on data was carried out using GenStat 13th edition [http://www.vsn-intl.com](http://www.vsn-intl.com) (VSN International Ltd, UK). There were two independent extractions performed for both the acetone and methanol extraction systems. Depending on the variable, there were one (extraction yield, TPC, TPro and FRAP) or three (TFC, DPPH and TEAC) measurements per extraction. This data was analysed by ANOVA with genotype, extraction and their interaction as terms in the model. Where there were three measurements per extraction, extraction was included in the model as a blocking factor. Examination of the residuals from the models indicated that data for some variables needed to be log or square root transformed so errors had common variance. Means for each genotype by solvent extraction system combination are shown in Tables 3-1, 3-2 and 3-3 together with LSD calculated at the
5% level. Means for each genotype by solvent extraction system combination were also used to calculate the Pearson correlations in Tables 3-4 and 3-5.

For colour (L*, a* and b* values), six independent measurements were made and this data was analysed by ANOVA with genotype as the only term in the model. Student t-test were performed to generate the significant differences ($p<0.05$) in Table 3-6 using Prism 5 (Graphpad Software, CA, USA).

2.11.2 Chapter 4

The data was analysed using GenStat 13th edition [http://www.vsn-intl.com](http://www.vsn-intl.com) (VSN International Ltd, UK. There were two independent extractions performed for the acetone extraction system on unprocessed beans; three independent extractions performed for the acetone extraction system on processed beans after undergoing soaking, boiling and autoclaving treatments, respectively; three independent cooking broths were collected from boiling and autoclaving treatments respectively for assessments. Depending on the variable, there were one (extraction yield, TPC, TPro and FRAP) or three (TFC, DPPH and TEAC) measurements per extraction/cooking broth. This data was analysed by ANOVA. Where there were three measurements per extraction, treatment was included in the model as a blocking factor. Examination of the residuals from the models indicated that data for some variables needed to be square root transformed so errors had common variance. Means for each genotype are shown in Tables 4-1, 4-2 and 4-3 together with LSD calculated at the 5% level. Means for each genotype were also used to calculate the Pearson correlations in Tables 4-4.

2.11.3 Chapter 5

The data was analysed using GenStat 13th edition [http://www.vsn-intl.com](http://www.vsn-intl.com) (VSN International Ltd, UK. There were two independent extractions performed for the acetone extraction system on unprocessed beans; three independent extractions performed for the acetone extraction system on processed beans after undergoing dry roasting treatments for individual time points. Depending on the variable, there were one (extraction yield, TPC, TPro and FRAP) or three (TFC, DPPH and TEAC) measurements per extraction. This data was analysed by ANOVA. Where there were three measurements per extraction, treatment was included in the model as a blocking...
factor. Means for each genotype are shown in Tables 5-1 and 5-2 together with LSD calculated at the 5% level.

There were two independent extractions performed for the acetone extraction system on unprocessed beans (whole beans). Three separate runs to collect the Fraction A and B respectively. There was one (extraction yield, TPC, TPro, FRAP and ORAC) measurement per extraction/fraction. This data was analysed by ANOVA using Prism 5 (Graphpad Software, CA, USA) and the means for each measurement are shown in Table 5-3.

There were two independent extractions performed for the acetone extraction system on unprocessed beans (none-hydrolysed sample). Two hydrolysis treatments were performed on the alkaline- and acid-hydrolysed samples, respectively. There were two measurements per none-hydrolysed and hydrolysed samples. This data was analysed by ANOVA. The means for each genotype are shown in Table 5-4. The data from alkaline-hydrolysed samples was analysed by student’s t-test (two tailed) using Prism 5 (Graphpad Software, CA, USA) and the means for each measurement are shown in Tables 5-5 and 5-6.

2.11.4 Chapter 6

The data was analysed using GenStat 13th edition [http://www.vsn-intl.com](http://www.vsn-intl.com) (VSN International Ltd, UK). There were two independent extractions performed for the acetone extraction system on unprocessed beans; three independent extractions performed for the acetone extraction system on processed beans after undergoing dry roasting treatments. Depending on the variable, there were one (extraction yield, TPC and ORAC) or three (TFC, DPPH and TEAC) measurements per extraction. This data was analysed by ANOVA. Means for each genotype are shown in Tables 6-1 and 6-4 together with LSD calculated at the 5% level.

There were four concentrations per single extract applied in the CAA, proliferation and lipase inhibition studies. There were at least three measurements performed per selected concentration of extract. The half maximal effective concentration (EC\textsubscript{50}) or half maximal inhibition concentration (IC\textsubscript{50}) in Tables 6-2, 6-3 and Table 6-5 was obtained via nonlinear regression using Prism 5 (Graphpad Software, CA, USA).

There was one measurement per extraction and the data was analysed by ANOVA. Means for each genotype are shown in Table 6-4 together with LSD calculated at the 5% level.
Chapter 3 Phenolic content and antioxidant capacity of Australian grown faba bean genotypes as affected by solvent extraction systems

3.1 Introduction
Vegetables, fruits and pulses are important sources of different types of phenolic compounds, which are natural antioxidants in human diets (Amarowicz & Pegg, 2008). Consumption of pulses has been associated with potential health benefits in lowering risks of different chronic diseases (Geil & Anderson, 1994). Recent investigations have provided more evidences that bean consumption is potentially beneficial in reducing cardiovascular disease (Finley, Burrell & Reeves, 2007), inflammatory activity (Oomah, Corbe & Balasubramanian, 2010), mutagenic activity (De Mejia, Castano-Tostado & Loarca-Pina, 1999) and cancer (Hughes, Ganthanvorn & Wilson-Sanders, 1997; Thompson et al., 2008).

Faba beans are usually buff (light tan) in colour but genotypes existing with seed coat colours ranging from white, buff, green, red to purple. The buff-coloured faba beans are preferred by the major international food markets. The relationships between seed coat colours, phenolic contents and antioxidant activities of pulses are controversial. Darker-coloured bean cultivars are thought to contain a higher phenolic content and antioxidant capacity as reported by Elias, De Fernandez & Bressani (1979). However, conflicting findings exist as to wheat was reported by Beninger & Hosfield (2003) and Deshpande & Cheryan (1987). It is commonly known that beans with varying seed coat colours contain different types of dominant phenolic compounds (Ranilla, Genovese & Lajolo, 2007). For instance, beans with black, red and brown-coloured seed coats contain cyanidin, while delphinidin can only be found in extracts from red- and brown-coloured beans (Madhujith, Amarowicz & Shahidi, 2004). The levels of phenolic acids and radical-induced DNA damage inhibition can also vary among beans with different seed coat colours (Madhujith, Amarowicz & Shahidi, 2004). Condensed tannins, exist in the coloured-beans but not in the white-coloured ones, are known to contribute to potent antioxidant activities (Troszynska & Ciska, 2002).

In general, phenolic compounds are grouped into simple phenolic acids including hydroxybenzoic acids and hydrocinnamic acids, flavonoids, stilbenes, lignans and tannins depending on their chemical structures with one or more aromatic rings attached to one or more hydroxyl groups (Dai & Mumper, 2010; Rice-Evans, Miller
& Paganga, 1996). Quantification and identification of phenolic compounds in crude plant extracts can be time consuming and the analyses require sophisticated equipment. Simple and quick reagent-based assays and chromatography profiling can help with the evaluations.

Different solvent extraction systems are applied to extract phenolic compounds in many plant materials, where methanol- and acetone-water mixtures being two of the most commonly used. It has been shown that different combination of solvent types and choices of acid addition in the extraction solvent have significant impacts on the extractable phenolic compounds from a variety of legume seeds (Xu & Chang, 2007). For instance, it is advisable to extract anthocyanins using slightly acidified methanol; while acetone is more efficient in extracting proacyanidins from foods (Escribano-Bailon & Santos-Buelga, 2003). A variety of solvent systems have been applied to extract phenolic compounds from faba beans, which include water (Almeida, Greiner et al., 2008; Wolosiak et al., 2010), pure methanol (Tomas-Barberan, Garcia-Grau & Tomas-Lorente, 1991), acetone-methanol-water (7:7:6; v/v/v), 70% ethanol (v/v) (Chaieb et al., 2011), 70% acetone (v/v) (Merghem et al., 2004) and 80% acetone (v/v) (Amarowicz et al., 1996). Among them, the combination of alcohols with water are more efficient than using a single pure solvent in extraction of phenolic compounds from faba beans, possibly due to the fact that combinations of solvents are capable of extracting a range of less polar aglycones and sugar attached glycosides (Escribano-Bailon & Santos-Buelga, 2003).

In the present study, two solvent extraction systems: methanol-water (80:20; v/v) and acetone-water (70:30; v/v) are selected to extract phenolic compounds from 12 Australian grown faba bean genotypes. The faba beans consisted of five different seed coat colour groups including white, buff, green, red and purple. The investigation aims to estimate the dominant groups and types of phenolic compounds in faba bean genotypes, and their antioxidant capacity. The application of a variety of reagent based assays also aims to reveal the specific antioxidant mechanisms of phenolic compounds in faba beans. In addition, comparison between the phenolic content and antioxidant capacity of faba bean genotypes can potentially assist in selection of genotypes for future breeding of antioxidant rich faba bean genotypes.
3.2 Results

3.2.1 Colour determination

Seed coat colour, seed weight and description of 12 faba bean genotypes are shown in Table 3-1. In a CIELAB colour system, L* value indicates lightness/darkness, where 0 is black through the grey scale to 100 that is white. Therefore, the violet-coloured Deep Purple and red-coloured Rossa had the lowest L* value, followed by green-coloured Icarus and other buff-coloured genotypes.

Table 3-1 Seed coat colours (L*, a* and b* values based on the CIELAB colour system), seed weight and description of the 12 faba bean genotypes†‡§∫

<table>
<thead>
<tr>
<th>Faba bean genotype</th>
<th>Seed coat colour</th>
<th>Seed weight (g/100 seeds)</th>
<th>Description</th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TF(Ic*As)*483/13</td>
<td>White</td>
<td>60</td>
<td>Tannin free, white hilum breeding line (combination of white flower, white testa and white hilum)</td>
<td>56.0a</td>
<td>1.4a</td>
<td>17.3a</td>
</tr>
<tr>
<td>974*(611*974)/42</td>
<td>Buff§</td>
<td>85</td>
<td>Breeding line, low discolouration† at Tamworth</td>
<td>49.0c</td>
<td>7.1b</td>
<td>17.7def</td>
</tr>
<tr>
<td>1269*483/6-1</td>
<td>Buff§</td>
<td>83</td>
<td>Breeding line, good disease resistance, white hilum, low discolouration∫ at Tamworth</td>
<td>48.7cd</td>
<td>7.9a</td>
<td>18.8a</td>
</tr>
<tr>
<td>1323/3</td>
<td>Buff</td>
<td>60</td>
<td>Germplasm from ICARDA</td>
<td>46.5a</td>
<td>3.6a</td>
<td>17.9cde</td>
</tr>
<tr>
<td>Doza</td>
<td>Buff</td>
<td>45</td>
<td>Variety, from Northern New South Wales</td>
<td>48.0de</td>
<td>4.5d</td>
<td>17.4b</td>
</tr>
<tr>
<td>Farah</td>
<td>Buff</td>
<td>67</td>
<td>Variety, selected from Fiesta, based on germplasm from Spain</td>
<td>47.7cd</td>
<td>7.0a</td>
<td>18.4abc</td>
</tr>
<tr>
<td>Fiod</td>
<td>Buff</td>
<td>46</td>
<td>Variety, based on germplasm from Greece</td>
<td>47.0a</td>
<td>5.9e</td>
<td>17.6defg</td>
</tr>
<tr>
<td>Nura</td>
<td>Buff</td>
<td>53</td>
<td>Variety, progeny of Icarus*Ascot</td>
<td>49.1e</td>
<td>5.9e</td>
<td>18.5a</td>
</tr>
<tr>
<td>S95007/1</td>
<td>Buff</td>
<td>67</td>
<td>Germplasm from ICARDA</td>
<td>50.0b</td>
<td>2.3f</td>
<td>18.0bcd</td>
</tr>
<tr>
<td>Icarus</td>
<td>Green</td>
<td>80</td>
<td>Variety, based on germplasm from Ecuador</td>
<td>45.3h</td>
<td>-2.2h</td>
<td>17.5fgh</td>
</tr>
<tr>
<td>Rossa</td>
<td>Red</td>
<td>53</td>
<td>Variety, based on germplasm from Ecuador</td>
<td>28.3i</td>
<td>5.0i</td>
<td>0.9h</td>
</tr>
<tr>
<td>Deep Purple</td>
<td>Dark Purple</td>
<td>41</td>
<td>Single plant selection from Fiod, made in Western Australia</td>
<td>29.0i</td>
<td>4.4i</td>
<td>-1.1i</td>
</tr>
</tbody>
</table>

†ICARDA- Internation Center for Agricultural Research in the Dry Areas. The data marked by the same superscripts in each column were not significantly different (p<0.05). ‡L* - lightness (0= black; 100=white); a* - red/green (+60= red; -60= green); b* - blue/yellow (+60= yellow; -60= blue). §Seed coat colour was between buff and green, buff in colour but with modifier genes that result in a green tinge. ∫Discolouration refers to the darkening rate of the seed coat after harvest.

As expected, TF(Ic*As)*483/13 had the highest L* value, due to its white-coloured seed coat. Some of the buff-coloured genotypes, such as 1323/3, Fiod and Farah were darker than the other buff-coloured genotypes including 974*(611*974)/42,
1269*483/6-1 and S95007/1. The a* value refers to the redness/greenness, where a positive a* value indicates a redder hue, while a negative a* value indicates a greener hue. Only green-coloured *Icarus* showed a negative a* value, while the rest of the genotypes showed positive a* values. The b* value signifies the yellowness/blueness, where only *Deep Purple* had a negative b* value that appeared in the blue hue region. On the other hand, *Rossa* had a very low but positive b* value. The rest of the white-, buff- and green-coloured genotypes shared similar b* values.

### 3.2.2 Extraction yields

The extraction yields of crude phenolic extracts from all of the genotypes comprised about 7-9% of the total bean weight (dry basis) regardless of choices of solvent system (Table 3-2). The extraction yields from *TF(Ic*/*As)*483/13 was higher when extracted using 80% methanol (v/v) that that of extracted using 70% acetone (v/v), however, the inverse was true for *974*(611*974)/42 and *Fiord*. For all the other faba bean genotypes, the extraction yields from methanolic and acetone extract pairs for each genotype were not significantly different.

### 3.2.3 Effect of solvent systems on the phenolic content of 12 faba bean genotypes

The effect of two different solvent extraction systems on the total phenolic content (TPC), total flavonoid content (TFC) and total proanthocyanidins (TPro) of the 12 faba bean genotypes are shown in Table 3-2. The comparisons between methanol and acetone extracts were completed using the original data instead of the transformed data. The TPC of acetone extracts from all genotypes were 2-3 times greater than that of their methanol extracts counterparts, except for *TF(Ic*/*As)*483/13. Among the acetone extracts, *TF(Ic*/*As)*483/13 exhibited the lowest TPC, which was 2-4 times lower than that of coloured-genotypes. The highest TPC was found in *Fiord, Nura, Icarus, Rossa* and *Deep Purple*.
Table 3-2 Comparison of extraction yield, total phenolic content, total flavonoid content of 12 faba bean genotypes extracted using 80\% methanol (v/v) and 70\% acetone (v/v) respectively.

<table>
<thead>
<tr>
<th>Faba bean genotype</th>
<th>Extraction yield (g/100gDW)†</th>
<th>TPC (mgGAE/gDW)‡</th>
<th>TFC (mgCE/gDW)§</th>
<th>TPro (mgCE/gDW)∫</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>80% Methanol</td>
<td>70% Acetone</td>
<td>80% Methanol</td>
<td>70% Acetone</td>
</tr>
<tr>
<td>TF (lc*As)483/13</td>
<td>8.8ahi</td>
<td>7.9ghi</td>
<td>1.5ghi (2.3)</td>
<td>1.7a (2.8)</td>
</tr>
<tr>
<td>974*(611*974)/42</td>
<td>7.8ikj</td>
<td>8.6abcde</td>
<td>1.3i (1.6)</td>
<td>2.8c (8.0)</td>
</tr>
<tr>
<td>1269*483/6-1</td>
<td>7.5k</td>
<td>7.3k</td>
<td>1.3i (1.7)</td>
<td>2.3i (5.4)</td>
</tr>
<tr>
<td>1323/3</td>
<td>8.5abcde</td>
<td>8.8abc</td>
<td>1.5kghi (2.3)</td>
<td>3.0i (8.9)</td>
</tr>
<tr>
<td>Doza</td>
<td>8.2defgh</td>
<td>8.5abcde</td>
<td>1.4ghi (2.0)</td>
<td>2.9ghi (8.6)</td>
</tr>
<tr>
<td>Farah</td>
<td>8.2defgh</td>
<td>8.6abcde</td>
<td>1.4ghi (1.8)</td>
<td>2.9ghi (8.5)</td>
</tr>
<tr>
<td>Fiord</td>
<td>8.4bcdefg</td>
<td>9.2a</td>
<td>1.5kghi (2.3)</td>
<td>3.5i (12.0)</td>
</tr>
<tr>
<td>Nura</td>
<td>7.5jk</td>
<td>7.7ijk</td>
<td>1.4ghi (2.0)</td>
<td>3.3i (10.9)</td>
</tr>
<tr>
<td>S95007/1</td>
<td>8.7jk</td>
<td>8.1defgh</td>
<td>1.6ghi (2.5)</td>
<td>2.7i (7.5)</td>
</tr>
<tr>
<td>Icarus</td>
<td>8.6abcde</td>
<td>8.7abcd</td>
<td>1.5kghi (2.3)</td>
<td>3.3i (10.8)</td>
</tr>
<tr>
<td>Rossa</td>
<td>8.7abcd</td>
<td>8.3bcdefgh</td>
<td>1.6i (2.6)</td>
<td>3.4i (11.2)</td>
</tr>
<tr>
<td>Deep Purple</td>
<td>8.3bcdefgh</td>
<td>8.9ab</td>
<td>1.5kghi (2.1)</td>
<td>3.3i (10.8)</td>
</tr>
<tr>
<td>5% LSD (genotype × extraction)</td>
<td>0.7</td>
<td>0.2</td>
<td>0.08</td>
<td>0.05</td>
</tr>
</tbody>
</table>

The data marked by the same superscripts within two types of solvent extract systems in the respective assay were not significantly different (p<0.05)

†g/100gDW- gram of extract per 100 gram of dry bean
‡TPC- Total phenolic content; mgGAE/gDW- mg gallic acid equivalents per gram of dry bean; Square root transformed to achieve equal variances in the population for obtaining 5% LSD (the original data were in brackets)
TFC- Total flavonoid content; mgCE/gDW- mg of catechin equivalents per gram of dry bean; Log(2.7) transformed to achieve equal variances in the population for obtaining 5% LSD (the original data were in brackets)

TPro- Total proanthocyanidins; mgCE/gDW- mg of catechin equivalents per gram of dry bean
Similarly, the TFC of acetone extracts from all genotypes was 2-3 times higher than their methanol extract counterparts, except for \( TF(Ic*As)*483/13 \). The acetone extracts from \( Nura, Icarus, 974*(611*974)/42 \) and \( Rossa \) similarly exhibited the highest TFC among all genotypes.

The acetone extracts of all faba bean genotypes contained a significantly higher TPro content than their methanol extract counterparts. Among the methanol extracts, \( TF(Ic*As)*483/13 \) and \( S96007/1 \) contained the highest TPro. The highest TPro contents of the acetone extracts were found in \( Icarus, Fiord, Nura \) and \( Deep Purple \).

### 3.2.4 Effect of solvent systems on the antioxidant activities of 12 faba bean genotypes

The effect of two different solvent extraction systems on the antioxidant activities of 12 faba bean genotypes is shown in Table 3-3. The comparisons between methanol and acetone extracts were completed using the original data instead of the transformed data. The acetone extracts exhibited a 4-8 times greater level of DPPH radical scavenging activity than the methanol extracts for all tested genotypes, except for \( TF(Ic*As)*483/13 \). Conversely, the methanolic extract of \( TF(Ic*As)*483/13 \) had a similar level of DPPH radical scavenging activity to other genotypes. Nevertheless, the acetone extract of \( TF(Ic*As)*483/13 \) had a much lower DPPH radical scavenging activity compared to other genotypes. The highest level of DPPH radical scavenging activity was found in the acetone extracts from \( Fiord, Icarus \) and \( Deep Purple \).
Table 3-3 Effect of different solvent systems, 80% methanol (v/v) and 70% acetone (v/v) on the level of antioxidant activities of 12 Australian grown faba bean genotypes with different seed coat colours

<table>
<thead>
<tr>
<th>Faba bean genotype</th>
<th>DPPH (µmolTE/gDW)</th>
<th>TEAC (µmolTE/gDW)</th>
<th>FRAP (µmolFe²⁺eq/gDW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>80% Methanol</td>
<td>70% Acetone</td>
<td>80% Methanol</td>
</tr>
<tr>
<td>TF(Ic*As)*483/13</td>
<td>2.2^m (8.9)</td>
<td>2.0^m (7.3)</td>
<td>2.5 (12.0)</td>
</tr>
<tr>
<td>974<em>611</em>974/42</td>
<td>2.1^h (8.1)</td>
<td>3.9^h (48.1)</td>
<td>2.6 (13.2)</td>
</tr>
<tr>
<td>1269*483/6-1</td>
<td>2.0^km (7.3)</td>
<td>3.4^km (29.3)</td>
<td>2.5^km (12.0)</td>
</tr>
<tr>
<td>1323/3</td>
<td>1.9^if (6.6)</td>
<td>3.7^if (39.4)</td>
<td>2.4^if (10.8)</td>
</tr>
<tr>
<td>Doza</td>
<td>2.0^h (7.3)</td>
<td>3.8^h (43.6)</td>
<td>2.4^h (10.8)</td>
</tr>
<tr>
<td>Farah</td>
<td>1.9^f (6.6)</td>
<td>3.8^f (43.6)</td>
<td>2.4^f (10.8)</td>
</tr>
<tr>
<td>Fiord</td>
<td>2.1^hl (8.1)</td>
<td>4.2^hl (64.8)</td>
<td>2.4^hl (10.8)</td>
</tr>
<tr>
<td>Nura</td>
<td>2.1^h (8.1)</td>
<td>4.0^h (53.1)</td>
<td>2.6^h (13.2)</td>
</tr>
<tr>
<td>S95007/1</td>
<td>2.1^h (8.1)</td>
<td>3.7^h (39.4)</td>
<td>2.6^h (13.2)</td>
</tr>
<tr>
<td>Icarus</td>
<td>2.2^hn (8.9)</td>
<td>4.1^hn (58.7)</td>
<td>2.5^hn (12.0)</td>
</tr>
<tr>
<td>Rossa</td>
<td>2.3^h (9.8)</td>
<td>3.9^h (48.1)</td>
<td>2.7^h (14.6)</td>
</tr>
<tr>
<td>Deep Purple</td>
<td>2.2^h (8.9)</td>
<td>4.1^h (58.7)</td>
<td>2.5^h (12.0)</td>
</tr>
<tr>
<td>5% LSD (genotype x extraction)</td>
<td>0.1</td>
<td>0.1</td>
<td>1.62</td>
</tr>
</tbody>
</table>

The data marked by the same superscripts within two types of extraction solvent systems in the respective assay were not significantly different (p<0.05)

1DPPH-2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity; µmolTE/gDW- µmol Trolox equivalents per gram of dry bean; Log_{2.7} transformed to achieve equal variances in the population for obtaining 5% LSD (the original data were in brackets)

2TEAC- Trolox equivalent antioxidant capacity; µmolTE/gDW- µmol Trolox equivalents per gram of dry bean; Log_{2.7} transformed to achieve equal variances in the population for obtaining 5% LSD (the original data were in brackets)

3FRAP- Ferric Reducing Antioxidant Power; µmolFe²⁺eq/gDW- µmol Fe²⁺ equivalents per gram of dry bean

The TEAC of the acetone extracts from all genotypes were about 2-7 times greater than that of methanol extracts. Rossa exhibited the highest TEAC among the methanol extracts. Conversely, Fiord and Icarus exhibited the highest TEAC among the acetone extracts. The acetone extract of TF(Ic*As)*483/13 had the lowest TEAC, while its methanolic extract had a TEAC that close to the median of all the faba bean genotypes.

The ferric reducing antioxidant power (FRAP) of the acetone extracts from faba bean genotypes were about 2-3 times greater than those of the methanol extracts, except that the acetone extract of TF(Ic*As)*483/13 was only 1.3 times higher than its methanol extracts. The TF(Ic*As)*483/13 exhibited the lowest FRAP among the acetone extracts, which was the inverse to those of Fiord and Deep Purple.
The correlations between TPC, TFC, TPro, DPPH, TEAC and FRAP for faba bean methanol and acetone extracts from 12 faba bean genotypes are shown in Table 3-4 and Table 3-5, respectively. The TFC of methanol extracts was positively correlated with DPPH ($r^2=0.92$), while a positive ($0.59<r^2<0.74, p<0.05$) or no correlation was shown between all other assays. Furthermore, TPro and TPC did not show correlation with all other assays, except that a positive correlation between TPC and FRAP was established ($r^2=0.66, p<0.05$). The correlations between all assays were high ($r^2>0.88, p<0.001$) for acetone extracts, except for TPro. The TPro of acetone extracts were not correlated with all other assays.

Table 3-4 Correlations of total phenolic content (TPC), total flavonoid content (TFC), total proanthocyanidins (TPro), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, total equivalents antioxidant capacity (TEAC) and ferric reducing antioxidant power (FRAP) of methanol extracts from 12 faba bean genotypes

<table>
<thead>
<tr>
<th></th>
<th>TPC</th>
<th>TFC</th>
<th>TPro</th>
<th>DPPH</th>
<th>TEAC</th>
<th>FRAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPC</td>
<td>1.00</td>
<td>0.48</td>
<td>0.16</td>
<td>0.51</td>
<td>0.48</td>
<td>0.66*</td>
</tr>
<tr>
<td>TFC</td>
<td>1.00</td>
<td>-0.04</td>
<td>0.92***</td>
<td>0.74*</td>
<td>0.59*</td>
<td></td>
</tr>
<tr>
<td>TPro</td>
<td>1.00</td>
<td>-0.03</td>
<td>0.06</td>
<td>0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPPH</td>
<td>1.00</td>
<td>0.75*</td>
<td></td>
<td>0.62*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEAC</td>
<td></td>
<td>1.00</td>
<td>0.68*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRAP</td>
<td></td>
<td></td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The results were analysed using the Pearson’s correlation (***, $p<0.001$; *, $p<0.05$)
Table 3-5 Correlations of total phenolic content (TPC), total flavonoid content (TFC), total proanthocyanidins (TPro), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, total equivalents antioxidant capacity (TEAC) and ferric reducing antioxidant power (FRAP) of acetone extracts from 12 faba bean genotypes.

<table>
<thead>
<tr>
<th></th>
<th>TPC</th>
<th>TFC</th>
<th>TPro</th>
<th>DPPH</th>
<th>TEAC</th>
<th>FRAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPC</td>
<td>1.00</td>
<td>0.93***</td>
<td>0.33</td>
<td>0.94***</td>
<td>0.92***</td>
<td>0.92***</td>
</tr>
<tr>
<td>TFC</td>
<td>1.00</td>
<td>0.33</td>
<td>0.97***</td>
<td>0.94***</td>
<td>0.88***</td>
<td></td>
</tr>
<tr>
<td>TPro</td>
<td>1.00</td>
<td>0.37</td>
<td>0.27</td>
<td>0.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPPH</td>
<td>1.00</td>
<td>0.98***</td>
<td>0.94***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEAC</td>
<td>1.00</td>
<td>0.91***</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRAP</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The results were analysed using the Pearson's correlation (***, p<0.001).

3.2.6 High performance liquid chromatography-post column derivatisation (HPLC-PCD) profiles

The HPLC-post column derivatisation (PCD) profiles of the crude phenolic extracts (in 70% acetone versus 80% methanol) from four faba bean genotypes representing different seed coat colour groups: white (TF(Ic*As)*483/13), buff (Nura), green (Icarus) and red (Rossa) are illustrated in Figures 3-1, 3-2, 3-3 and 3-4, respectively. The HPLC-PCD profiles of faba bean genotypes other than those mentioned were included in Appendix 1. The faba bean phenolic compounds from both solvent extraction systems eluted in two separate regions of the HPLC chromatogram: retention time 0-15 min and retention time 15-40 min, which were arbitrarily classified as polar and less-polar regions.
Figure 3-1 HPLC-PCD profiles (280 nm) of white-coloured faba bean genotype, \(TF(Ic^*As)^{483/13}\) extracted using: (a) 70% acetone (v/v) and (b) 80% methanol (v/v)

Figure 3-2 HPLC-PCD profiles (280 nm) of buff- or beige-coloured faba bean genotype, \(Nura\) extracted using: (a) 70% acetone (v/v) and (b) 80% methanol (v/v)
Figure 3-3 HPLC-PCD profiles (280 nm) of green-coloured faba bean genotype, *Icarus* extracted using: (a) 70% acetone (v/v) and (b) 80% methanol (v/v)

Figure 3-4 HPLC-PCD profiles (280 nm) of red-coloured faba bean genotype, *Rossa* extracted using: (a) 70% acetone (v/v) and (b) 80% methanol (v/v)
The HPLC profiles of both acetone and methanol extracts from \( TF(1c^*As)^*483/13 \) (Figure 1A) appeared to be similar, where only the polar region of their chromatograms had an active PCD response at about retention time 5 min, while the less-polar region lacked active compounds. The HPLC-PCD profiles of both methanol and acetone extracts from all of the faba bean genotypes contained similar major peaks eluted at retention times: 4, 18, 21 and 22 min. Nevertheless, there were a few additional small active peaks appearing at the polar regions of chromatograms of acetone extracts from all genotypes, which were absent in that of methanol extracts. A noticeable low level of HPLC ‘hump’ appeared in the chromatogram of the three coloured genotypes at the less-polar region (Figure 3-2, 3-3 and 3-4; retention time: 15-25 min). Moreover, these HPLC ‘humps’ directly corresponded to a substantive PCD response indicating a potent antioxidant activity, which were not prominent in the methanol extracts.

3.2.7 Analysis of phenolic classes in faba bean extract

The HPLC results in Table 3-6 showed that both of the crude acetone extracts from the buff-coloured \( Nura \) and red-coloured \( Rossa \) contained three major phenolic classes including the hydroxybenzoic acids/flavanols, hydroxycinnamic acids and flavonols, which were detectable at 280 nm, 326 nm and 370 nm, respectively. However, none of the anthocyanins was detectable at 520 nm in both of the genotypes.

Table 3-6 Quantification of phenolic classes in faba bean genotypes crude acetone extracts using HPLC

<table>
<thead>
<tr>
<th>Detection wavelength (nm)</th>
<th>Phenolic class</th>
<th>Faba bean genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( Nura )</td>
</tr>
<tr>
<td>260</td>
<td>Hydroxybenzoic acids/ flavanols</td>
<td>2.94±0.04\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>(mgGAE/gDW)\textsuperscript{†}</td>
<td></td>
</tr>
<tr>
<td>326</td>
<td>Hydroxycinnamic acids</td>
<td>0.16±0.00\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>(mgCHAeq/gDW)\textsuperscript{‡}</td>
<td></td>
</tr>
<tr>
<td>370</td>
<td>Flavonols (mgRE/gDW)\textsuperscript{§}</td>
<td>0.18±0.01\textsuperscript{a}</td>
</tr>
<tr>
<td>520</td>
<td>Anthocyanins</td>
<td>-</td>
</tr>
</tbody>
</table>

\textsuperscript{†}mgGAE/gDW – mg of gallic acid equivalents per gram of dry bean  
\textsuperscript{‡}mgCHAeq/gDW – mg of chlorogenic acid equivalents per gram of dry bean  
\textsuperscript{§}mgRE/gDW – mg of rutin equivalents per gram of dry bean  

\textsuperscript{a,b}The data marked by different superscripts across columns in the respective measurement wavelengths were significantly different (p<0.05)
The results showed that the extract of Rossa contained a significantly higher amount of all the three different classes of phenolic compounds including hydroxybenzoic acids/ flavanols, hydroxcinnamic acids and flavonols than those of Nura. There were 2.94±0.04 mgGAE/gDW of hydroxybenzoic acids/flavanols, 0.16±0.00 mgCHAEq/gDW of hydroxcinnamic acids and 0.18±0.01 mgRE/gDW of flavonols in the extract of Nura, respectively. There were 3.18±0.09 mgGAE/gDW of hydroxybenzoic acids, 2.42±0.09 mgRE/gDW of flavonols and 1.86±0.11 mgCHAEq/gDW of hydroxcinnamic acids in the extract of Rossa, respectively.

3.3 Discussion

3.3.1 Extraction × genotype interaction

The ANOVA analysis indicates that the main factors: solvent extraction systems (extraction) and genotypes, on the variables (extractopm yields, TPC, TFC, TPot, DPPH radical scavenging activity, TEAC and FRAP) are respectively significant, except for the extraction of extraction yields. Furthermore, the extraction × genotype interaction is significant for all of the mentioned variables. There is a much greater range in means for the acetone extraction than methanol extraction of all variables except for the extraction yields. In addition, there is little correlation between rankings for acetone extracts and rankings for methanol extracts of all variables, which also means the ranking orders for methanol and acetone extracts of 12 faba bean genotypes are completely different (Appendix 2).

The comparison of means at 5% LSD shows that the extraction yields from both solvent extraction systems are similar, except that the extraction yields from acetone extracts of 974*(611*974)/42 and Fiord are higher than that of their methanol extracts. Furthermore, the extraction yields of acetone extract is lower than that of methanol extract for TF(Ic*As)*483/13. These inconsistent effects from both factors reflect on the significant extraction × genotype interaction.

Although the mean for the acetone extraction is equally significantly higher than the mean for the methanol extraction from all genotypes, a significant extraction × genotype interaction is shown for the variables TPC, TEAC and FRAP. This interaction is termed ordinal interaction where the lines formed by means of acetone and methanol extracts are parallel. The interaction is significant because the slope of lines is not parallel in the ordinal interaction, which is reflected from the much greater range in means for the acetone extracts than that of methanol extracts, as well
as the little correlation between rankings for acetone and rankings for methanol extraction as mentioned earlier.

The extraction × genotype interaction is shown to be significant for the variables TFC and DPPH. Similarly, the mean for acetone extracts are significantly higher than that of methanol extracts, which contributes to ordinal interaction. However, the mean for acetone extracts of \( TF(Ic*As)*483/13 \) is lower than that of methanol extracts together with the non-parallel slope are the sources of the significant interaction.

The genotype × extraction interaction for the variable TPro is also significant. This is attributed to the fact that the mean of acetone extracts from \( 1269*483/6-1 \) and \( S95007/1 \) are lower than the mean of methanol extracts which is the inverse to those of other genotypes, together with the non-parallel slope resulting in the significant interaction.

### 3.3.2 Efficiency of extraction solvents

This study demonstrates that the 70% acetone (v/v) method is generally more efficient than the 80% methanol (v/v) in extracting phenolic compounds in faba beans with a higher level of antioxidant activity. These results are in agreement with other reports from Xu & Chang (2007), Rocha-Guzman, Herzog, Gonzalez-Laredo, Ibarra-Perez, Zambrono-Galvan & Gallegos-Infante (2007) and Chavan, Shahidi & Naczk (2001), where aqueous acetone is demonstrated to be able to extract phenolic compounds from legume seeds more efficiently in comparison to aqueous methanol. The aqueous acetone is more effective than methanol in extracting phenolic compounds in faba beans which might be attributed to the ability of aqueous acetone to extract the active HPLC ‘humps’ at the relatively less-polar regions of chromatograms.

### 3.3.3 Role of genotype difference (and colour) in the nature of phenolic compound

The structural diversity of phenolic compounds have a major effect on their antioxidant properties based on scavenging radicals, chelating redox-active metals and ability to inhibit certain enzymes such as xanthine oxidase (Cotelle, 2001). The phenolic contents and antioxidant capacity among faba bean genotypes are significant different which are likely due to different phenolic compounds existing in their dissimilar seed coat colours. Although extraction of phenolic compounds from the
coloured-faba beans using aqueous acetone do not result in a greater amount of extraction yield than that of using methanol generally, the acetone extracts exhibit a noticeably higher level of TPC, TFC and antioxidant capacity. In addition, HPLC-PCD provides an insight that the majority of antioxidant activities of faba bean extracts are coming from the HPLC ‘humps’ as demonstrated in the chromatograms of extracts from coloured-genotypes. These active peaks cannot be separated through varying polarities and elution flow rates, continuing to elute jointly as a ‘hump’, hence they appear to be highly associated. Similar HPLC ‘humps’ in the chromatographic profiles of cider apples and faba beans at a wavelength of 280 nm have also been reported by Guyot, Doco, Souquet, Moutounet & Drilleau (1997) and Merghem et al. (2004) respectively. The ‘humps’ are later being characterised to be highly polymerised and oligomeric procyanidins and reportedly too complex to be easily separated for analysis. Different classes of phenolic compounds exist in the faba bean extracts as quantified using HPLC as demonstrated in our study, this also suggests that most of the compounds in the faba bean extracts are visible at 280 nm, where phenolic acids, flavan-3-ols or catechins are detectable.

The darker-coloured beans have been reported to exhibit a higher phenolic content and antioxidant capacity (Elias, De Fernandez, & Bressani, 1979). However, some lighter-coloured genotypes such as the buff-coloured genotypes 974*(611*974)/42, Fiord and Nura, have been demonstrated to exhibit a comparable level of antioxidant activity to those of darker-coloured genotypes including Rossa, Deep Purple and Icarus as shown in the current study. These findings will be received with interest by the food industry since buff-coloured faba beans are the most commonly commercialised for international food markets. Variation in phenolic contents might be related more greatly to flower colour than testa colour (Cabrera & Martin, 1986). This is particularly true for the tannin-free genotypes, which are known to have white-flowers with no spots (Crofts, Evans & McVetty, 1980). There are possibilities of breeding low tannin faba beans by selection of varieties that have white flowers with yellow-spots, while regular faba bean varieties have white flowers with black spots as proposed by Cabrera & Martin (1989).

The variation in the phenolic contents among faba bean genotypes may be caused by unintentional cross breeding between genotypes. For instance, cross breeding soybean hybrids containing low flavonoids content results in offsprings with a low polyphenol content and antioxidant capacity and vice versa (Malencic, Maksimovic,
Popovic & Miladinovic, 2008). A similar outcome for eggplant hybrids has also been reported (Prohens, Rodriguez-Burruel, Raigon & Nuez, 2007).

Genotype contributes to significant differences in the phenolic contents and antioxidant capacity of faba beans as shown in this study is in agreement with other studies (Chaieb et al., 2011; Oomah, Luc, Leprelle, Drover, Harrison & Olson, 2011). The TEAC and FRAP levels of faba bean methanolic samples obtained from the present findings are comparable to what reported by Pellegrini, Serafini, Salvatore, Del Rio, Bianchi & Brighenti (2006). The TPC, TFC, DPPH radical scavenging activity and TEAC of the acetone extracts in the present study are also comparable or higher than those of the eight different types of coloured peas and beans extracted using 80% acetone (v/v) (Xu & Chang, 2007). However, in comparison to the faba bean methanolic extracts in the current study, the crude ethanolic extracts of 13 faba beans grown in Tunisia contain higher TPC, TFC and FRAP approximately 15-, 5- and 50-times (Chaieb et al., 2011). Moreover, the ethanolic extracts also exhibit higher TPC, TFC and FRAP than that of acetone extracts from samples in this study by about 4-, 3- and 20-times. The polarity ranking order for the three types of solvent extraction systems is acetone > methanol > ethanol. As acetone extracts from faba beans exhibited a higher phenolic content and antioxidant capacity than those of methanol extracts, it is be expected that ethanol extracts would contain the lowest phenolic content and antioxidant activity. However, the markedly higher levels of phenolic content. However, the markedly higher levels of phenolic content and antioxidant capacities in faba beans cultivated at Tunisia than those of methanol and acetone extracts in the present study might be attributed to the genetic, agronomic and environment variabilities in comparison to the present study (Oomah et al., 2011).

In general, the white-coloured TF(Ic*As)*483/13 exhibits a lower level of phenolic content and antioxidant activity in comparison to that of coloured-genotypes. Unlike the coloured-genotypes, TF(Ic*As)*483/13 appears to have similar levels of TPC and DPPH radical scavenging activity in both of its methanol and acetone extracts. The TF(Ic*As)*483/13 also has a lower extraction yield when extracted using aqueous acetone than that of the methanol extract, which is the inverse to all the other genotypes. The acetone extract of TF(Ic*As)*483/13 also contains a significant lower level of TFC than its methanol extract. This is likely due to TF(Ic*As)*483/13 is the only tannin free genotype with a white hilum (combination of white flower, white testa and white hilum) among the faba bean genotypes studied in the present study.
Tannins or proanthocyanidins in the coloured-genotypes is likely to contribute to the majority of antioxidant activity as proposed earlier. Conversely, the antioxidant activity in TF(Ic*As)*483/13 is likely contributed from non-tannin components, such as monomeric phenolic compounds detectable at 280 nm as observed in the HPLC-PCD chromatographs of the TF(Ic*As)*483/13 extract. This is because proanthocyanidins are known to be not detectable in faba beans with white-coloured seed coats (Nozzolillo, Ricciardi & Lattanzio, 1989).

Efforts have been ongoing in breeding faba bean cultivars with low tannin contents for the purpose of feeding monogastric animals (Duc, 1991; Van Der Poel, Dellaert, Van Norel & Helsper, 1992). This is because tannins that are present in the diet of monogastric animals could result in protein complexation and lower animal weights (Jansman, Verstegen, Huisman & Van Den Berg, 1995). However, the tannin-free beans tend to have a lower proportion of seed coats (Cabrera & Martin, 1986). The tannin-free beans also are more susceptible to seed coat cracking and plant diseases (Kantar, Hebblethwaite & Pilbeam, 1996; Ondrej & Hunady, 2007), which makes their adaptation to farming situations difficult.

3.3.4 Mechanism of antioxidant activity in faba bean

The averaged FRAP values of the acetone extracts are 2-times higher than that of methanol extracts. In comparison, the averaged values of DPPH radical scavenging activity and TEAC of acetone extracts are higher than their methanol counterparts by 6- and 5-times respectively. This suggests that the antioxidant activity of faba bean acetone extracts are largely based on free radical scavenging activities rather than reducing powers. In support of this, it has been reported that the antioxidant activity of faba beans is mainly based on chain-breaking ability rather than chelating activity with transition metals (Carbonaro, Virgili & Carnovale, 1996).

3.3.5 Correlation between phenolic contents and antioxidant activity

As pointed out by Prior, Wu & Schaich (2005), no single assay can reflect the true total antioxidant capacity of one sample. Therefore, it is important to use assays involving different reaction mechanisms to justify the antioxidant mechanism of a test sample. The TPC has been recognised as one of the best assays to determine total phenols, although the chemistry involved can be complex (Schofield, Mbugua & Pell,
In addition, the substances responsible for the reaction of antioxidant activity possibly include compounds other than phenolics, such as sugars (Singleton, Orthofer & Lamuela-Raventos, 1999). The TPC, DPPH radical scavenging activity, TEAC and FRAP assays are based on an electron transfer mechanism (Huang, Ou & Prior, 2005). However, they are conducted under different working conditions and reaction time lengths. The TPC, FRAP and TEAC assays measure the hydrophilic antioxidants, where the TPC assay is conducted in an alkaline condition and it is the inverse for that of FRAP. Conversely, the DPPH radical scavenging activity measures the antioxidants that can be dissolved in aqueous methanol.

The correlation between data from various variables (assays) for acetone extracts is much higher than that of methanol extracts. This might suggest that aqueous acetone is more efficient than aqueous methanol in extracting relevant phenolic compounds that exhibit a high level of antioxidant activity. The antioxidant activity is likely to be contributed from the HPLC ‘hump’ in the chromatograms of acetone extracts. Interestingly, when the correlation analysis is carried out on both of the methanol and acetone extracts together instead of separately as what presented, it generates a higher correlation relationship. This is because the data points collected from the two solvent extraction systems form two distinct populations that scatter toward opposite ends of the correlation line (Appendix 3). This results in a straight correlation line and thus, a higher correlation. This observation indicates the importance of carrying out correlation analysis on scatter plots, not only on numerical outcomes.

The correlation of TPro with all other assays is relatively low regardless of solvent system choices. In fact, the level of TPro content is very low in comparison to TFC and TPC. This might be suggesting that the antioxidant activity of crude faba bean extracts is mainly due to the flavonoids rather than proanthocyanidins. However, this contradicts to what proposed earlier. Therefore, it is a more reasonable suggestion that the TPro values are possibly underestimated. This could be due to the limited accessibility of polymeric proanthocyanidins in the reaction, especially given the fact that 4-dimethylaminocinnamaldehyde (DMACA) reacts exclusively with the end units of proanthocyanidins (Prior & Gu, 2005).
Chapter 4 Effect of soaking, boiling and autoclaving on the phenolic contents and antioxidant capacity of faba beans with different seed coat colours

4.1 Introduction

Many staple dishes are prepared from pulses. For example, faba beans are served as Ful Medames and Taamia in Egypt and Sudan; lentils and/or chickpeas are stewed to form a thick paste, Dal (or Dahl), and served with rice or roti in India and Southern Asia; adzuki beans are regularly utilised in patisserie desserts and bakery fillings in Japan; black beans or other types of beans are served as Feijoada in some African countries. Some pulses including cowpeas or black eye peas, black beans and mung beans are used as ingredients for soups and desserts in some of the Chinese and South East Asian cuisines. Among many types of pulses, faba beans are reported to contain a considerable phenolic content and antioxidant activity (Amarowicz et al., 1996). Since pulses are not commonly eaten raw, there have been a variety of thermal processing methods applied to pulses to achieve desirable sensory and, sometimes, increased nutritional properties. Soaking pulses prior to thermal processing is a common practice to shorten the cooking time. It has been reported that different processing methods including soaking, boiling, steaming, roasting and pressure cooking have a significant effect on phenolic content and antioxidant capacity of various types of pulses (Acar et al., 2009; Baoteng et al., 2008; Xu & Chang, 2008). The phenolic content and antioxidant capacity of faba beans are also affected by cooking as reported by Amarowicz et al. (2004) and Chaieb et al. (2011). However, the study on phenolic content and antioxidant capacity of dry faba beans as affected by thermal processes is limited, particularly on faba bean grown in Australia which contributes to about 20% of the world export market (FAOSTAT, 2010). Furthermore, previous reports have not considered cooking broth, but focused on processed beans only (Khalil & Mansour, 1995; Vidal-Valverde et al., 1998).

This chapter aims to study the effect of soaking, boiling and autoclaving (pressure cooking) on phenolic contents and antioxidant capacity of Australian grown faba bean genotypes with different seed coat colours. Acetone extracts of faba bean contained a higher phenolic content and antioxidant capacity than that of methanol
extracts as demonstrated in Chapter 3. Therefore, the 70% acetone (v/v) extraction system is chosen to extract phenolic compounds of faba beans in this study. Phenolic content and antioxidant capacity of both cooking broth and cooked beans were assessed.

4.2 Results

4.2.1 Hydration and swelling coefficients of faba beans

Table 4-1 details the hydration and swelling coefficients of five faba bean genotypes with different seed coat colours. The soaking process caused whole beans to hydrate and swell to about 2-folds in size compared to their unprocessed beans. The results showed that the hydration coefficients of *Icarus* and *Rossa* were the highest among the tested genotypes. The swelling coefficient of *Icarus*, *Rossa* and *Doza* were also the greatest among the genotypes. Conversely, swelling coefficient of the white-coloured *TF(Ic*×*As)*483/13 was the lowest among all genotypes.

<table>
<thead>
<tr>
<th>Faba bean genotype</th>
<th>Seed coat colour</th>
<th>Hydration coefficient (%)</th>
<th>Swelling coefficient (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>TF(Ic</em>×*As)*483/13</td>
<td>White</td>
<td>192.9b</td>
<td>192.6c</td>
</tr>
<tr>
<td>Doza</td>
<td>Buff</td>
<td>190.4b</td>
<td>216.7ab</td>
</tr>
<tr>
<td>Nura</td>
<td>Buff</td>
<td>189.9b</td>
<td>210.2b</td>
</tr>
<tr>
<td><em>Icarus</em></td>
<td>Green</td>
<td>212.5a</td>
<td>222.2a</td>
</tr>
<tr>
<td><em>Rossa</em></td>
<td>Red</td>
<td>210.8a</td>
<td>222.2a</td>
</tr>
<tr>
<td>5% LSD</td>
<td></td>
<td>4.4</td>
<td>9.9</td>
</tr>
</tbody>
</table>

*The data marked by the same letters were not significantly different (p<0.05) in the respective column.*

4.2.2 Effect of soaking, boiling and autoclaving on phenolic content of faba bean

Soaking and boiling were conducted on five faba bean genotypes: *TF(Ic*×*As)*483/13, *Doza*, *Nura*, *Icarus* and *Rossa*. Three faba bean genotypes including *TF(Ic*×*As)*483/13, *Nura* and *Rossa* were selected to undergo autoclaving processes. The effect of soaking, boiling and autoclaving on extraction yields, total phenolic (TPC), total flavonoid (TFC) and total proanthocyanidins (TPro) contents of faba bean genotypes is tabulated in Table 4-2.
Table 4-2 Effect of soaking, boiling and autoclaving on the phenolic content of faba bean*

<table>
<thead>
<tr>
<th>Faba bean genotype</th>
<th>Unprocessed</th>
<th>Soaked</th>
<th>Boiled</th>
<th>Cooking broth</th>
<th>Autoclaved</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bean</td>
<td>Bean</td>
<td>Bean</td>
<td>Cooking broth</td>
<td>Bean</td>
</tr>
<tr>
<td>TF(Ic*As)*483/13</td>
<td>78.7abc</td>
<td>79.5a</td>
<td>24.8a</td>
<td>106.2a</td>
<td>85.3bc</td>
</tr>
<tr>
<td>Doza</td>
<td>86.1abc</td>
<td>76.3a</td>
<td>24.8a</td>
<td>103.4a</td>
<td>n/a</td>
</tr>
<tr>
<td>Nura</td>
<td>77.1abc</td>
<td>78.2a</td>
<td>28.2a</td>
<td>86.7c</td>
<td>80.2a</td>
</tr>
<tr>
<td>Icarus</td>
<td>86.9abc</td>
<td>79.9a</td>
<td>29.6a</td>
<td>91.8bc</td>
<td>n/a</td>
</tr>
<tr>
<td>Rossa</td>
<td>83.2abc</td>
<td>85.3a</td>
<td>29.6a</td>
<td>101.6ab</td>
<td>87.0a</td>
</tr>
<tr>
<td>5% LSD</td>
<td>6.7</td>
<td>10.0</td>
<td>5.0</td>
<td>11.2</td>
<td>17.1</td>
</tr>
<tr>
<td>TPC (mgGAE/gDW)†</td>
<td>2.8c</td>
<td>2.8f</td>
<td>0.7b</td>
<td>1.8a</td>
<td>0.7b</td>
</tr>
<tr>
<td>Doza</td>
<td>8.6b</td>
<td>4.6c</td>
<td>2.3a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Nura</td>
<td>10.9a</td>
<td>6.5ab</td>
<td>2.4a</td>
<td>1.7a</td>
<td>1.8a</td>
</tr>
<tr>
<td>Icarus</td>
<td>10.8a</td>
<td>5.6bc</td>
<td>2.2a</td>
<td>1.9a</td>
<td>n/a</td>
</tr>
<tr>
<td>Rossa</td>
<td>11.2a</td>
<td>7.7a</td>
<td>2.4a</td>
<td>1.9a</td>
<td>1.9a</td>
</tr>
<tr>
<td>5% LSD</td>
<td>0.5</td>
<td>1.6</td>
<td>0.4</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>TFC (mgCE/gDW)§</td>
<td>1.01d (1.02)</td>
<td>0.71d</td>
<td>0.23d</td>
<td>0.26bc</td>
<td>0.21b</td>
</tr>
<tr>
<td>Doza</td>
<td>1.53c (2.34)</td>
<td>1.01c</td>
<td>0.54c</td>
<td>0.21c</td>
<td>n/a</td>
</tr>
<tr>
<td>Nura</td>
<td>1.69a (2.86)</td>
<td>1.81a</td>
<td>0.90a</td>
<td>0.34a</td>
<td>0.43a</td>
</tr>
<tr>
<td>Icarus</td>
<td>1.80a (3.24)</td>
<td>1.47b</td>
<td>0.88a</td>
<td>0.29ab</td>
<td>n/a</td>
</tr>
<tr>
<td>Rossa</td>
<td>1.72a (2.96)</td>
<td>1.77a</td>
<td>0.80b</td>
<td>0.31ab</td>
<td>0.44a</td>
</tr>
<tr>
<td>5% LSD</td>
<td>0.10</td>
<td>0.13</td>
<td>0.05</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>TPro (mgCE/gDW)∫</td>
<td>0.27ab</td>
<td>0.20a</td>
<td>0.08a</td>
<td>0.06ab</td>
<td>0.14b</td>
</tr>
<tr>
<td>Doza</td>
<td>0.19bc</td>
<td>0.08c</td>
<td>0.04c</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Nura</td>
<td>0.28ab</td>
<td>0.14b</td>
<td>0.06b</td>
<td>0.05b</td>
<td>0.27a</td>
</tr>
<tr>
<td>Icarus</td>
<td>0.32ab</td>
<td>0.11bc</td>
<td>0.06b</td>
<td>0.07a</td>
<td>n/a</td>
</tr>
<tr>
<td>Rossa</td>
<td>0.13ab</td>
<td>0.08c</td>
<td>0.04c</td>
<td>0.04b</td>
<td>0.17b</td>
</tr>
</tbody>
</table>
| 5% LSD             | 0.11        | 0.04   | 0.01   | 0.02         | 0.07       | 0.14         

*The data marked by the same letters were not significantly different (p<0.05) in respective assay. The n/a indicated data was not analysed.
†g/100gDW- gram of extract per 100 gram of dry bean
‡TPC- Total phenolic content; mgGAE/gDW- mg gallic acid equivalents per gram of dry bean
§TFC- Total flavonoid content; mgCE/gDW- mg of catechin equivalents per gram of dry bean; data of uprocessed beans were transformed by square roots to achieve equal variances in the population for statistical analyses, the non-transformed data (actual results) are shown brackets
∫TPro- Total proanthocyanidins; mgCE/gDW- mg of catechin equivalents per gram of dry bean

The three processing methods were briefly described below (details are in the Chapter 3, section 3.2):

(1) soaking: beans were soaked in distilled water for 12 hr at room temperature;
(2) boiling: beans were soaked in distilled water for 12 hr at room temperature, and boiled in an open container for 40 min.
(3) autoclaving: beans were soaked in calcium chloride (CaCl₂) for 12 hr at room temperature, and autoclaved for 20 min at 115°C.
4.2.2.1 Effect of soaking on the phenolic content of faba bean

Soaking caused slight changes in yields of TPC in the faba beans in comparison to that of unprocessed beans. The extraction yield of phenolic extracts from soaked beans of all tested genotypes were not significantly different from each other. There were more than 50% of TPC retained in the soaked beans of coloured-genotypes after undergoing the soaking process in comparison to that of unprocessed beans. The soaked beans of Rossa and Nura retained the highest TPC among all genotypes. Oppositely, the soaked beans of TF(Ic*As)*483/13 exhibited the lowest TPC after soaking.

In comparison to the unprocessed beans, there were about 40-70% of TFC retained in the soaked beans of all genotypes after soaking. Among the genotypes, Nura and Rossa retained the highest TFC after soaking. Conversely, the TFC in the soaked bean of TF(Ic*As)*483/13 was the lowest.

In comparison to the unprocessed beans, there were about 30-70% of TPro retained in the soaked beans of all genotypes following the soaking treatment. The white-coloured genotype TF(Ic*As)*483/13 exhibited the highest level of TPro in its soaked beans among the tested genotypes. Inversely, the soaked beans of Rossa, Icarus and Doza contained the lowest levels of TPro among all genotypes.

4.2.2.2 Effect of boiling on phenolic contents of pre-soaked faba beans

The extraction yields of phenolic extracts from the boiled beans were about 30-40% of that of unprocessed beans. On other hand, boiling faba beans generated increased extraction yields in the cooking broths, which were greater than that of unprocessed beans. The extraction yields of cooking broths from the coloured-genotypes were about 10-20% greater than that of unprocessed beans. Whereas, the extraction yields of cooking broth from TF(Ic*As)*483/13 were about 35% greater than that of unprocessed beans. Therefore, the combination of extraction yields from the boiled beans and cooking broths of all genotypes was about 1.4-1.7 times greater than those of unprocessed and pre-soaked beans. The extraction yields from boiled beans of all genotypes were not significantly different from each other. However, the extraction yields of cooking broths from Nura and Icarus were the lowest among the tested genotypes. Comparatively, TF(Ic*As)*483/13, Doza and Rossa generated the highest amounts of extraction yields in the cooking broths among the genotypes.

In comparison to the unprocessed beans, there was about 20-30% of TPC retained in the boiled beans of coloured-genotypes after boiling, while there was another 20%
TPC leached into the cooking broths. The remaining 50-60% of TPC was likely destroyed during the heating process. Conversely, there was about 25% of TPC retained in the boiled beans of \(TF(Ic*As)^{483/13}\) in comparison to that of unprocessed beans, and there was about 65% of TPC leached to its cooking broths. The boiled beans of coloured-genotypes exhibited a similar TPC, which was significantly higher than that of \(TF(Ic*As)^{483/13}\). All genotypes produced a similar TPC in their cooking broths after boiling regardless of seed coat colours.

There was a greater TFC retained in the boiled beans than was leached into the cooking broths of coloured-genotypes: 20-30% was retained in the boiled beans and 10% was leached to the cooking broths. The remaining 60-70% of TFC was likely diminished during the heating process. There were about 25% of TFC retained in the boiled beans of \(TF(Ic*As)^{483/13}\) compared to the unprocessed beans, while a similar level of TFC was leached to the cooking broths. The boiled beans of Nura and Icarus contained the highest TFC among all genotypes. Conversely, \(TF(Ic*As)^{483/13}\) contained the least TFC. The cooking broths of Nura, Icarus and Rossa contained the highest TFC.

In comparison to the unprocessed beans, there was about 20-30% of TPro retained in the boiled beans of all tested genotypes, and a similar level of TPro was leached into their cooking broths. The remaining 40-60% of TPro was likely destroyed by the heat treatment. The boiled beans of \(TF(Ic*As)^{483/13}\) contained the highest level of TPro among all tested genotypes, while soaked beans of Rossa and Doza had the lowest TPro content. Among all genotypes, the cooking broths from Icarus and \(TF(Ic*As)^{483/13}\) exhibited the highest level of TPro.

4.2.2.3 Effect of autoclaving on phenolic content of pre-soaked faba bean

There was a small increase in the extraction yields from autoclaved beans in comparison to that of unprocessed beans. The extraction yields of cooking broths were about four times greater (by weight) than that of unprocessed beans. After autoclaving the coloured-genotypes, a higher TPC leached to the cooking broths than what was retained in the autoclaved beans: approximately 20% was leached and 17% was retained. Conversely, a 40% of TPC was leached to the cooking broths and a 25% retained in the autoclaved beans for \(TF(Ic*As)^{483/13}\). The autoclaved beans and cooking broths of Nura and Rossa exhibited a similar level of TPC, and they were significantly higher than those of \(TF(Ic*As)^{483/13}\).
A similar relationship of leaching and retainment of TPC was found for TFC of faba beans after autoclaving. After autoclaving the coloured-beans, there was about 20% of TFC being leached to their cooking broth and 25% of TFC being retained in the autoclaved beans in comparison to the unprocessed beans. Conversely, there was a higher TFC leached to the cooking broths than being retained in the autoclaved beans of \(TF(Ic*As)*483/13\) comparing to the unprocessed beans: 40% in the cooking broth and 25% in the autoclaved beans. The coloured-genotypes exhibited a significantly higher TFC than that of \(TF(Ic*As)*483/13\) in both of the autoclaved beans and cooking broths.

The autoclaving process caused different changes to TPro content of three faba bean genotypes: 52% was retained in the autoclaved beans of \(TF(Ic*As)*483/13\); almost 100% retained in the autoclaved beans of \(Nura\) and an increase by 130% in the autoclaved beans of \(Rossa\). The level of TPro in cooking broths of \(TF(Ic*As)*483/13\), \(Nura\) and \(Rossa\) was higher than that of unprocessed beans by 1.0-, 1.5- and 2.4 folds, respectively.

4.2.3 Effect of soaking, boiling and autoclaving on antioxidant activities of faba bean

The effect of processing on the levels of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, total equivalents antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) of faba bean genotypes is shown in Table 4-3. The processing methods applied on faba beans are soaking, boiling and autoclaving.
Table 4-3 Effect of soaking, boiling and autoclaving on the antioxidant activities of faba bean*

<table>
<thead>
<tr>
<th>Faba bean genotype</th>
<th>Unprocessed Bean</th>
<th>Soaked Bean</th>
<th>Boiled Bean</th>
<th>Cooking broth</th>
<th>Autoclaved Bean</th>
<th>Cooking broth</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH (µmolTE/gDW) </td>
<td>TF(Ic*As)*483/13</td>
<td>2.7± (7.5)</td>
<td>23.3c</td>
<td>7.7c</td>
<td>4.3d</td>
<td>4.6c</td>
</tr>
<tr>
<td>Doza  </td>
<td>6.7± (44.2)</td>
<td>33.8c</td>
<td>17.1b</td>
<td>4.1c</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Nura  </td>
<td>7.3± (53.3)</td>
<td>49.9c</td>
<td>23.4a</td>
<td>5.6c</td>
<td>8.7b</td>
<td>20.0a</td>
</tr>
<tr>
<td>Icarus  </td>
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<td>39.9c</td>
<td>22.6a</td>
<td>4.8c</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Rossa  </td>
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<td>50.0c</td>
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<td>5.4c</td>
<td>9.9c</td>
<td>9.9c</td>
</tr>
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<td>n/a</td>
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<td>0.6</td>
<td>1.0</td>
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<td>15.98c</td>
<td>7.27c</td>
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<td>8.35b</td>
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<td>14.38c</td>
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<td>n/a</td>
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<td>37.57a</td>
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</tr>
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<td>8.14</td>
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</tr>
<tr>
<td>FRAP (µmolFe²⁺ eq/gDW)</td>
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<td>9.04a</td>
<td>1.92d</td>
<td>4.54c</td>
<td>3.24c</td>
</tr>
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<td>5.29c</td>
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<td>n/a</td>
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</tr>
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</tr>
<tr>
<td>Rossa  </td>
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<td>6.97a</td>
<td>6.61a</td>
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<tr>
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<td>0.20</td>
<td>0.47</td>
<td>0.63</td>
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</tbody>
</table>

*The data marked by the same letters were not significantly different (p<0.05) in respective assay. The n/a signified data was not analysed.

†DPPH- 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity; µmol TE/gDW - µmol Trolox equivalents per gram of dry bean; data of unprocessed bean were transformed by square roots to achieve equal variances in the population, the non-transformed data were in brackets

‡TEAC- Trolox equivalent antioxidant capacity; µmolTE/gDW- µmol Trolox equivalents per gram of dry bean

§ORAC- Oxygen radical absorbance capacity; µmolTE/gDW- µmol Trolox equivalents per gram of dry bean

¶FRAP- Ferric reducing antioxidant power; µmolFe²⁺eq/gDW- µmol Fe²⁺ equivalents per gram of dry bean

4.2.2.1 Effect of soaking on antioxidant activities of faba bean

The soaking treatment resulted in no change or a reduction in the level of DPPH radical scavenging activity of coloured-genotypes when compared to their unprocessed beans, where there was about 70-100% being retained in the soaked beans. Inversely, an increase in the level of DPPH radical scavenging activity by 3-times was observed in the soaked beans of TF(Ic*As)*483/13 comparing to its unprocessed beans. Among the tested genotypes, the soaked beans of Nura, Rossa and Icarus contained the highest levels of DPPH radical scavenging activity, while TF(Ic*As)*483/13 and Doza exhibited the lowest activity.

After soaking, there was about 30-40% of TEAC retained in the soaked beans of coloured-genotypes comparing to the unprocessed beans. There was a relatively
higher TEAC retained in the soaked beans of \(TF(Ic*As)^{483/13}\) than those of coloured-genotypes, where about 60% of TEAC was retained in the soaked beans of \(TF(Ic*As)^{483/13}\). The soaked beans of \(Nura\) and \(Rossa\) exhibited the highest TEAC among the genotypes, while \(TF(Ic*As)^{483/13}\) was the inverse. The soaking process increased the ORAC in the soaked beans of \(TF(Ic*As)^{483/13}\) by about 7% when compared to the unprocessed beans. Conversely, soaking caused decreases in ORAC of \(Nura\) and \(Rossa\), where 80-90% of ORAC was retained in their soaked beans. The soaked beans of \(Rossa\) and \(Nura\) contained the highest ORAC. The ORAC in the soaked beans of \(TF(Ic*As)^{483/13}\) was the lowest among the tested genotypes. The soaking treatment caused a slight decrease in FRAP of all faba bean genotypes comparing to unprocessed beans regardless of seed coat colours, where at least 80% of FRAP was retained in the soaked beans. Among the tested genotypes, \(Doza\), \(Ross\) and \(Nura\) exhibited the highest FRAP in the soaked beans. Inversely, the soaked beans of \(TF(Ic*As)^{483/13}\) contained the lowest FRAP.

4.2.2.2 The effect of boiling on antioxidant capacity of pre-soaked faba bean

After boiling, a higher level of DPPH radical scavenging activity was measured in the boiled beans of all genotypes than their cooking broths: about 40% and 100% of DPPH were measured in the coloured- and white-genotype boiled beans respectively, while the cooking broths exhibited 60% and 10% of DPPH radical scavenging activity that of unprocessed white- and coloured-genotypes. The level of DPPH radical scavenging activity in the boiled beans of \(Nura\), \(Icarus\) and \(Rossa\) was not significantly different from each other, but it was significantly higher than those of \(Doza\) and \(TF(Ic*As)^{483/13}\). The cooking broths of \(Nura\) and \(Rossa\) exhibited the highest level of DPPH radical scavenging activity among the tested genotypes. Conversely, the cooking broths from \(Doza\) and \(TF(Ic*As)^{483/13}\) contained the lowest level of DPPH radical scavenging activity. After boiling the coloured-beans, a higher TEAC was measured in boiled beans than cooking broths: 20% in the boiled bean and 15-20% in the cooking broths. It was the lowest for the white-coloured \(TF(Ic*As)^{483/13}\), where 60% and 30% of TEAC was measured in the cooking broths and boiled beans, respectively. The TEAC in the boiled beans of \(Nura\), \(Icarus\) and \(Rossa\) was similar, and they were significantly higher than those of \(Doza\) and \(TF(Ic*As)^{483/13}\). The cooking broths from \(Rossa\)
and TF(Ic*As)*483/13 contained the highest level of TEAC among the tested genotypes, while the other three genotypes exhibited a similar TEAC.

Boiling Nura and Rossa caused a higher ORAC in boiled beans than that of cooking broths: 30-40% in the boiled beans versus 30% in the cooking broths. This was the reverse of the TF(Ic*As)*483/13, where 30% ORAC was measured in its boiled beans comparing to 50% ORAC in its cooking broths. The boiled beans of Nura and Rossa had a similar ORAC, which were significantly higher than that of TF(Ic*As)*483/13. The ORAC of the cooking broths from all three genotypes was similar.

After boiling Nura, Icarus and Rossa, a higher level of FRAP was exhibited in boiled beans than that of cooking broths, which was the inverse for that of TF(Ic*As)*483/13. After boiling the coloured-beans, about 30-40% and 30-35% of FRAP was measured in the boiled beans and cooking broths. There was about 20% and 40% of FRAP measured in the boiled beans and cooking broths of TF(Ic*As)*483/13, respectively. The boiled beans of Rossa contained the highest FRAP among the tested genotypes, which was the inverse for that of TF(Ic*As)*483/13. The cooking broths of Rossa and Nura exhibited the highest FRAP, which was the opposite for that of TF(Ic*As)*483/13.

4.2.2.3 The effect of autoclaving on antioxidant capacity of pre-soaked faba bean

After autoclaving faba beans, the cooking broths exhibited a higher level of DPPH radical scavenging activity than that of autoclaved beans. In comparison to the unprocessed beans of Nura and Rossa, there was about 16% and 20-40% of DPPH radical scavenging activity measured in the autoclaved beans and cooking broths respectively. In comparison to unprocessed beans, 60% of DPPH radical scavenging activity was measured in the autoclaved TF(Ic*As)*483/13, while its cooking broth demonstrated a 1.2 times higher DPPH radical scavenging activity. The autoclaved beans of Rossa exhibited the highest level of DPPH radical scavenging activity among the tested genotypes, followed by that of Nura and TF(Ic*As)*483/13. The cooking broths of Nura contained the highest level of DPPH radical scavenging activity among the tested genotypes, while the cooking broths of Rossa and TF(Ic*As)*483/13 contained a similar level of DPPH radical scavenging activity.

Following the autoclaving processes on faba beans, a higher TEAC was measured in the cooking broths than that of autoclaved beans. In comparison to the unprocessed beans, about 15% of TEAC was measured in the autoclaved beans of Nura and Rossa.
while a 50-55% of TEAC was measured in their cooking broths. Conversely, 30% of TEAC was measured in autoclaved beans of TF(Ic*As)*483/13, while the TEAC of its cooking broth was 1.4 times higher than that of unprocessed beans. The autoclaved beans of Nura and Rossa contained a similar level of TEAC, and they were higher than that of TF(Ic*As)*483/13. The cooking broths from Rossa exhibited the highest TEAC, while Nura and TF(Ic*As)*483/13 were the inverse. After autoclaving the faba beans, the cooking broths exhibited a higher ORAC than that of autoclaved beans. In comparison to the unprocessed beans, there was about 20-30% of ORAC measured in the autoclaved beans of TF(Ic*As)*483/13, Nura and Rossa, while about 40-60% of ORAC measured in its cooking broths. Both of the autoclaved beans and cooking broths from Nura and Rossa contained a comparable level of ORAC and they were significantly higher than that of TF(Ic*As)*483/13. After autoclaving the faba beans, the cooking broths was shown to contain a higher FRAP than that of the autoclaved beans. For the coloured-genotypes, there was about 45-50% of FRAP measured in their autoclaved beans, while a 50-60% of FRAP was measured in their cooking broth following the autoclaving process. Conversely, about 30% and 40% of FRAP retained in the autoclaved beans of TF(Ic*As)*483/13 versus its cooking broth. In both of the autoclaved beans and cooking broths of all tested genotypes, Rossa exhibited the highest FRAP, followed by Nura and TF(Ic*As)*483/13.

4.2.4 Correlation between phenolic content and antioxidant activity

The Pearson’s correlation of data from the variables: TPC, TFC, TPro, DPPH radical scavenging activity, TEAC, ORAC and FRAP assays are summarised in Table 4-4. The data points from that of unprocessed beans were excluded. The statistical analysis revealed a high correlation between all assays, except for TPro. The correlations between TPro with other assays were insignificant, while exhibiting a relatively low but significant positive correlation with TEAC (0.52, p<0.01).
Table 4-4 Pearson’s correlation of the effect of soaking, boiling and autoclaving on total phenolic content (TPC), total flavonoid content (TFC), total proanthocyanidins (TPro), 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, total equivalent antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC) and ferric radical antioxidant power (FRAP) of faba bean genotypes

<table>
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<tr>
<th></th>
<th>TPC</th>
<th>TFC</th>
<th>TPro</th>
<th>DPPH</th>
<th>TEAC</th>
<th>ORAC</th>
<th>FRAP</th>
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<td>0.19</td>
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<td>0.86***</td>
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<td>0.90***</td>
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<td>0.90***</td>
<td>0.93***</td>
<td>0.89***</td>
</tr>
<tr>
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<td>0.18</td>
<td>0.52**</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
<td>0.27</td>
</tr>
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<td>0.86***</td>
<td>0.86***</td>
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<td>0.74***</td>
<td>0.74***</td>
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</tr>
</tbody>
</table>

***significant correlation at p<0.001
**significant correlation at p<0.01

4.2.5 High performance liquid chromatography-Post column derivatisation (HPLC-PCD) of faba bean processed by soaking, boiling and autoclaving

The HPLC-PCD profiles of phenolic extracts from the soaked beans of Nura (Figure 4-1b) appeared to be very similar to that of unsoaked beans (Figure 4-1a), with an exception of having a lower HPLC peaks and PCD responses. Similar to what was observed in section 3.2.6, there were two major groups of phenolic compounds eluted at two different regions of chromatograms: (i) the first 10 min and (ii) 10-40 min, respectively. These two phenolic groups appeared to be relatively hydrophilic and less hydrophilic in nature, which were arbitrary named as the polar and less-polar regions, respectively. The phenolic compounds eluted at the less-polar region as a HPLC ‘hump’ with inseparable peaks which gave a substantial PCD response after reacting with ABTS⁺.

Figure 4-1 High performance liquid chromatography-post column derivatisation (HPLC-PCD) profiles of phenolic extracts from (a) raw and (b) soaked beans of faba bean genotype, Nura
Figure 4-2 shows the HPLC-PCD profiles of phenolic extracts from the boiled beans (Figure 4-2a) and the cooking broths (Figure 4-2b) of *Nura* following the boiling process, as well as the autoclaved beans (Figure 4-2c) and cooking broths of *Nura* following the autoclaving process (Figure 4-2d). The HPLC-PCD profiles of the boiled beans (Figure 4-2a) appeared to be similar to that of soaked beans (Figure 4-1b), with the two described phenolic groups separating by polarity, but at a lower level. In contrast, there was no obvious active HPLC ‘hump’ observed for the phenolic compounds eluted at the less-polar region for the cooking broth from the boiling process (Figure 4-2b).

Conversely, the HPLC profiles of the autoclaved beans (Figure 4-2c) and its cooking broths (Figure 4-2d) were similar. The phenolic compounds eluted at the less-polar regions of the chromatograms of both autoclaved beans and cooking broths had no active HPLC ‘hump’, although phenolic compounds eluted at the polar region of their chromatograms were similar to that of boiled beans.
4.3 Discussion

4.3.1 Effect of processing on the extraction yield, hydration and swelling coefficients of faba bean

It is a common household practice to pre-soaked dry pulses for a period of time, which serves as an initial hydration stage in order to facilitate the following cooking process. Pre-soaked beans are hydrated and softened, therefore taking a shorter time for thermal processing the beans to achieve acceptable palatabilities. Our results agree with what were reported by El-Refai et al. (1988) and Nasar-Abbas et al. (2008) that soaking faba bean for more than 12 hr causes an increase in bean sizes by about 2-folds in comparison to unprocessed beans. Among the tested genotypes, the white-coloured breeding line, $TF(1c^{*}A_{s})^{*}483/13$ has a noticeably lower hydration and swelling capabilities, which is the inverse of the darker-coloured genotypes, Icarus and Rossa. This observation also agrees with the possibility that genotypes with darker seed coat colours contain a higher fibre content and hull proportion (Crofts, Evans & McVetty, 1980; Shirzadegan, 1985).

Many factors could affect water uptake of faba beans, which include seed coat to cotyledon ratios, initial moisture and mineral contents. These factors are associated with bean maturity stages, genotypes, storage conditions and growing conditions (Chang et al., 1994; Shehata, Bakr & Shimi, 1983; Youssef & Bushuk, 1984; Noaman et al., 1988), where the hulls or seed coat components of faba bean increase over storage time but water uptake ability is lowered (El-Refai et al., 1988). Other factors involving processing conditions such as length of soaking time and the temperature of soaking medium also have a significant effect on the water uptake of faba beans (Henderson & Ankrah, 1985). Although moisture uptake and cookability of dry faba bean correlate with an increased soaking time and temperature, the effect comes to a plateau when the maximum water absorption is achieved (Henderson & Ankrah, 1985). This study design has tried to eliminate some of the variabilities in the mentioned factors by standarding the processing methods, storage environment, growing duration and condition.

The current study shows that soaking faba beans did not have as great an effect on the extraction yields of soaked beans comparing to that of unprocessed beans. However, heat treatments performed on the pre-soaked faba beans resulted in increased extraction yields in the cooking broth and as well as increased extraction
yields from the cooking broth and processed beans for unprocessed beans. This finding suggests that, although soaking has successfully hydrated the bean tissues and plant cells, it probably does not cause a drastic physicochemical change in beans. Conversely, the heat treatments has probably caused a severe fracture of seed physical structure and facilitated the components such as soluble sugars (eg. α-galactosides) (Sanchez-Mata, Hurtado & Diez-Marques, 1999) and protein (Hamza et al., 1987) to leach into the cooking broths, thereby generating extraction yields in the cooking broths. In addition, although the cooking broth is collected from supernatant of the cooked beans, it is possible that some residues of soluble fibres or to a lesser extent, insoluble fibres could also be present (Kutos et al., 2003). In support of this, a loss of total solids by 30% after stewing faba beans in comparison to that of unprocessed beans has been reported by Noaman et al. (1988). It is also reasonable to expect that the heating process may cause degradations of large molecules including polysaccharides, proteins and tannins, as well as generations of new compounds in the cooked beans or cooking broths. For instance, soaking followed by boiling faba beans is reported to reduce starch, fructose and sucrose in cooked beans but generations of glucose which is not present in unprocessed beans (Vidal-Valverde et al., 1998). Also, boiling and autoclaving faba beans similarly lower other constituents including stachyose, tannins, B-group vitamins, phytic acid, vicine, convicine, trypsin inhibitor and mineral contents, possibly due to denaturation or degradation of those chemical constituents or otherwise the molecules are leached to the soaking and cooking broths (Hamza et al., 1987; Khalil & Mansour, 1995). Interestingly, thermal processes conducted on pinto beans cause decreases in insoluble fibre contents but increases in resistant starch contents (Kutos et al., 2003a). On the other hand, it is reported that soaking and heating processes cause degradation of saponins in navy beans (Shi et al., 2009).

Our results show that the extraction yield of cooking broths obtained from the autoclaving process was greater than that of boiling process. This might be signifying that soaking in a low calcium solution followed by autoclaving enhanced the permeability of seed membrane, thereby permitting a greater amount of soluble sugars, fibres and other constituents to leach into the cooking broths. This is supported by findings on autoclaving faba and navy beans cause a greater reduction in saponin, tannins and other constituents compared to ordinary boiling as reported by Khalil & Mansour (1995) and Shi et al. (2009). Autoclaving faba beans results in
disappearances of the HPLC ‘hump’ elutes at the relatively less-polar region in the chromatograms of autoclaved bean extracts as shown in our findings. Conversely, the HPLC ‘hump’ is present in the chromatograms of boiled bean extracts. This observation also agrees that a greater level of destruction occurs to faba beans by performing autoclaving than boiling. Nevertheless, the faba beans appear to be more intact after being autoclaved than that of being boiled as observed visually in this study. This might be due to CaCl₂ solutions that are being applied in both of the soaking and autoclaving medium. Conversely, deionised water is used as soaking and boiling medium. It has been known that addition of CaCl₂ in the soaking or cooking medium results in firmer and more intact beans (McCurdy et al., 1983). This is possibly due to the diffusion of calcium ions into the beans, where the calcium ions combine with the pectin in the cell walls that causes a firmer texture (Luh, Wang & Daoud, 1975). The autoclaved beans are darker in colour in comparison to that of boiled beans, which might be indicating formations of new compounds as a result of Maillard reactions, which could also partly contribute to the antioxidant activity as suggested by Xu & Chang (2008).

During soaking, dry beans absorb water through a simple diffusion process over time to aid in softening of the beans, and facilitate the cooking process. The chemical composition in pulses affects water uptake abilities and food processing properties as discussed. The differences in pulse genotypes could have a significant effect on the variation in physiochemical properties of beans including seed sizes, seed coat thickness, seed coat colours, bean shapes and hardness (Giczewska & Borowska, 2003; Kader, 1995; Rowland, 1977). Although the protein contents of faba beans solely do not have a significant impact on their water uptake ability as reported by Kader (1995), it is rational to expect that water absorption by a plant material made up of protein and starch primarily, as well as their synergistic interactions with other minor constituents in beans during the thermal processing will impact on the level of protein denaturation, starch gelatinisation and pasting properties. This could influence the final antioxidant activity of processed beans (Xu & Chang, 2008). It is reported that losses of phenolic contents during cooking is not entirely due to destruction of phenolic compounds, but it can be possibly attributed to the chemical properties of phenolic compounds such as formation of new insoluble components with other organic substances (Satwadhar, Kadam & Salunkhe, 1981). Therefore, it is possible that cooking faba beans that are known to contain a substantial amount of tannins
would lead to a strong tannin-protein interaction (Carbonaro, Virgili & Carnovale, 1996). However, the substantial variations in chemical composition among bean genotypes sometimes are not sufficient to cause significant affects on the overall sensory properties of cooked beans (Casanas et al., 2006).

4.3.2 Effect of processing on the phenolic compound and antioxidant capacities of faba bean

The TPC, TFC, TPro levels and DPPH, TEAC, ORAC and FRAP activities measured in the boiled coloured-beans were generally higher than those of their cooking broths, although the TPro of autoclaved beans and cooking broth from Icarus and Rossa were comparable. Conversely, the autoclaved coloured-beans exhibited lower TPC, TFC, TPro levels, as well as lower levels of DPPH, TEAC, ORAC and FRAP activities than their cooking broths (except that the DPPH radical scavenging activity of autoclaved beans from Rossa was similar to its cooking broth). This observation further suggests that soaking coloured-faba beans followed by autoclaving using low calcium medium incurs a greater level of destruction on the seed coat and tissue which permits leaching of active phenolic compounds into the cooking broths in comparison to that of boiling treatment carried out on coloured-faba beans. This result agrees with other reports that carried out evaluations on other types of beans (Rocha-Guzaman et al., 2007; Xu & Chang, 2008). Nevertheless, boiling and autoclaving chickpeas have been reported to cause a comparable level of destruction on their tannin contents (El-Adawy, 2002). Conversely, soaking processes probably would not alter the physicochemical properties of faba beans extensively as demonstrated by the similar HPLC-PCD profiles for the extracts of soaked and unprocessed beans.

In general, findings from this study show that soaking, boiling and autoclaving lower phenolic contents and antioxidant capacity in faba beans, which agrees with what was reported by Abusin, Hassan & Babiker (2009). It has been reported that different processing methods cause reductions in phenolic contents and antioxidant capacity in various types of pulses: green pea, yellow pea, chickpea, lentil (Xu & Chang, 2008), mung bean (Barroga, Laurena & Mendoza, 1985), common bean (Elias, De Fernandez & Bressani, 1979) and some Indian wild beans (Vadivel, Kunyanga & Biesalski, 2011). Although soaking has no significant effect on water soluble polyphenol content of faba beans compared to that of unprocessed beans as
determined using the Folin-Denis reagent (Alonso, Auirre & Marzo, 2000). Furthermore, inconsistent effects on phenolic contents and antioxidant capacities in Brazilian bean cultivars by thermal treatments are also reported, where the solvent extraction solvent used in the mentioned study is aqueous methanol (Ranilla, Genovese & Lajolo, 2009). This is very likely because of different solvents including water and aqueous methanol are applied in the mentioned studies to extract phenolic compounds, and the solvent extraction systems are not able to extract the less-polar compounds as represented by the HPLC ‘hump’ in the chromatograms of faba bean extracts which is high in antioxidant activity as discussed in Chapter 3. The processing methods are sometimes shown to cause an increase in phenolic content and antioxidant capacity of faba beans, this might be due to heating processes breakdown the HPLC ‘hump’, which is likely to be polymeric compounds, and facilitate extraction of phenolic compounds thereby resulting in an increase in phenolic content and antioxidant capacity.

The potent antioxidant activity in faba beans is thought to be contributed from the highly polymerised proanthocyanidins or tannins as discussed in Chapter 3 and by Carbonaro, Virgili & Carnovale (1996). The proanthocyanidins could be compounds elute at the less-polar region of chromatograms, or HPLC ‘hump’ as observed in the chromatograms of acetone extracts from coloured-faba beans. The HPLC ‘humps’ are not detected in the chromatograms of extracts from autoclaved beans and that of cooking broths. However, both of the mentioned bean extracts and cooking broths exhibit a considerable phenolic content and antioxidant capacity. These findings suggest that the antioxidant activity of those extracts could be contributed from compounds that are not detectable by UV-detector of the HPLC system, for example, carbohydrates and proteins. Also, these compounds could be generated as a result of degradation of macromolecules or forming of new compounds during heating processes. This is especially true for \( TF(Ic*As)^{483/13} \): the level of DPPH radical scavenging activity and TEAC of the respective combined bean extracts and cooking broth from \( TF(Ic*As)^{483/13} \) after the boiling and autoclaving treatments are higher than their unprocessed beans. In support of this, antioxidant activity of processed beans is thought to be caused by formation of aglycones from degradation of the glucosides of flavonoids (Xu & Chang, 2008).

Our results show that, although the applied processing methods significantly reduce the phenolic content and antioxidant capacity of various faba bean genotypes, there is
a considerable amount of phenolic compounds with potent antioxidant activity being retained in the boiled and autoclaved beans, as well as being leached to their cooking broths. Therefore, it is desirable to consume cooked faba beans together with their cooking broths for a maximum potential health benefit. Although it has been reported that diets with a high phenolic content, particularly tannins, reduces protein digestibility thereby preventing absorption of some nutrients using animal models (Zdunczyk et al., 2003). However, food preparation processes might be playing an important role in neutralising intake of phenolic compounds from foods, as well as governing bioavailability of nutrients, such as minerals (ElMaki et al., 2007). In support of this, it is reported that supplementing cooked common beans together with cooking broths cause weight gains in rats, while consumption of raw common beans result in weight loss in the rodents (Elias, De Fernandez & Bressani, 1979). Consequently, it would be desirable to find out the right dose for faba bean consumption in order to obtain the maximum health benefits.

In general, the white-coloured breeding line faba beans, TF(Ic*As)*483/13 has a lower phenolic content and antioxidant capacity than the coloured-genotypes, which agrees with what reported by Elias, De Fernandez, & Bressani (1979). The buff-coloured Nura exhibits a remarkably comparable phenolic content and antioxidant capacity to the red-coloured Rossa and green-coloured Icarus as demonstrated from this study. Seeds with a relatively smaller size are likely to have a greater seed coat area, thereby higher tannin contents because most of the tannins are located at the seed coats (De Mejia et al., 2003; Osman, Elaziz & ElHassan, 2010). Nevertheless, the seed sizes of Nura and Rossa are the same and they are smaller than that of Icarus, where the seed size is expressed in g of seeds per 100 seeds as shown in Chapter 3.

4.3.3 Correlations between various variables

The high positive correlations between all variables except for that involving TPro assay suggest that antioxidant activity in faba beans is likely to be contributed from phenolic compounds, but not proanthocyanidins. This contradicts to what was proposed earlier that antioxidant activity in the coloured-genotypes is contributed from tannins. Therefore, the insignificant correlations of TPro with other assays are likely due to the limited assayable proanthocyanidins in those extracts. This is because the DMACA applied in the TPro assay reacts solely with the end units of
proanthocyanidins (Prior & Gu, 2005) and this has been discussed in Chapter 3. In support of this, although the autoclaving process is found to lower TPC and TFC in all tested faba bean genotypes as shown in the current study, there is a substantial increase in the TPro level in the autoclaved beans comparing to the unprocessed beans. This could be because of the autoclaving process has successfully degraded or dissociated the highly polymerised proanthocyanidins which therefore increasing the amount of assayable proanthocyanidins. Conversely, a decrease in TPro of faba beans is observed after undergoing the boiling processes, which may suggest that soaking followed by boiling treatments are not sufficient to degrade proanthocyanidins in faba beans. This assumption is confirmed by our chromatography findings, where the HPLC ‘hump’ eluting at the less-polar region can only be observed in the chromatograms of extracts from the boiled beans, but not that of autoclaved beans, nor their cooking broths. This observation is also in line with what shown in the current study that a greater extraction yield is generated from faba beans undergoing the autoclaving in comparison to that of boiling processes, which indicates a greater extent of destruction is exerted on faba beans during the autoclaving treatment than boiling.

It is interesting to note that data points obtained from the evaluations on extracts from the unprocessed beans are likely to scatter at the top end of correlation lines regardless of choice of assays (Appendix 3), this would influence the correlation analysis significantly. Therefore, the data points from that of unprocessed beans are not considered in the correlation analyses in the present study. Nevertheless, the correlations between the TPro assays and all other assays are poor both with and without inclusion of data points from unprocessed beans. The correlations between the FRAP with TPC and TFC assays, as well as between that of DPPH with TFC appears to be in an exponential relationship. This exponential relationship is likely to cause a low correlation. These observations propose the importance of carrying out correlation analysis with care to avoid misleading interpretations.

The chemical mechanism of ORAC assay involves hydrogen atom transfer reactions, while that of TPC, DPPH radical scavenging activity, TEAC and FRAP assay are based on the electron-transfer reaction (Huang, Ou & Prior, 2005). In view of the appreciable antioxidant activity in both of the raw and processed faba beans as tested using an array of assays in our study, it is apparent that the antioxidant mechanism of the phenolic compounds in faba beans are complex and involving hydrogen atom
transfer and electron transfer capabilities. Different thermal processing treatments that are carried out on faba beans further complex the assessment on antioxidant mechanism of faba bean phenolic compounds, which is attributed to different classes and types of phenolic compounds present in faba beans as shown in our study. Although this is only an in vitro study on the phenolic compounds and antioxidant capacity of faba beans, findings obtained from this study support that phenolic compounds could be acquired from consuming faba beans for potential antioxidant capacities, thereby encouraging wider utilisations of faba beans.
Chapter 5 Effect of roasting on phenolic composition and antioxidant properties of Australian grown faba bean (*Vicia faba* L.)

5.1 Introduction

It is often necessary to heat treat pulses to increase their palatability for human consumption. Roasting is one way to cook pulses which is a method that uses dry heat. There have been many studies on the impact of dry heat treatments on phenolic content and antioxidant capacity of various plant materials including coffee beans (Del Castillo, Ames & Gordon, 2002), cocoa beans (Payne et al., 2010), citrus peels (Jeong et al., 2004), wheat brans (Kim et al., 2006) and wattle seeds (Ee et al., 2011). Also, there are some reports that discuss the effect of roasting on various types of pulses (Baoteng et al., 2008; Siddhuraju, 2006) and nuts (Chandrasekara & Shahidi, 2011; Chukwumah et al., 2007). However, there is a limited number of studies which focus on effect of dry heating on phenolic content and antioxidant activity of faba beans, and some of the findings are contradictory. For instance, roasting faba beans at a low temperature for a long time (LTLT; 149°C/20 min) causes an increase in the tannin content, which is the inverse to that of roasting at high temperature for a short time (HTST; 232°C/12 min) as reported by Anderson et al. (1994). Furthermore, the effects of dry heating on phenolic content and antioxidant capacity of other types of beans are inconsistent (Acar et al., 2009; Baoteng et al., 2008).

This chapter aims to evaluate the effect of dry roasting on phenolic content and antioxidant capacity of Australian grown faba beans. The phenolic extracts from roasted faba beans are fractionated to aid in investigation of phenolic compounds. Acid and alkaline hydrolyses are carried out on the phenolic extracts of faba beans to simplify chromatography (Antolovich et al., 2000; Rehwald, Meier & Sticher, 1994) and liberate the bound phenolic acids (Moore & Jung, 2001; Scalbert et al., 1985) aiding in determination of flavan-3-ols, hydroxybenzoic and hydroxycinnamic acids.

5.2 Results

5.2.1 Effect of roasting on phenolic content of faba bean

The effect of dry roasting on extraction yields, total phenolic content (TPC), total flavonoid content (TFC) and total proanthocyanidins (TPro) of faba bean cultivar, *Nura* for different time points is shown in Table 5-1. Roasting the faba beans for 10
to 120 min resulted in a significantly higher extraction yield in comparison to that of unprocessed beans by about 10%. The extraction yields of phenolic extracts from the roasted beans were similar regardless of the roasting duration.

### Table 5-1 Effect of dry roasting on extraction yields and phenolic contents of faba beans

<table>
<thead>
<tr>
<th>Roasting time (minute)</th>
<th>Extraction yield (mg/gDW)(^a)</th>
<th>TPC (mgGAE/gDW)(^b)</th>
<th>TFC (mgCE/gDW; %)(^c)</th>
<th>TPro (mgCE/gDW)(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>77.1(^e)</td>
<td>10.7(^e)</td>
<td>2.8(^e)</td>
<td>0.23(^e)</td>
</tr>
<tr>
<td>10</td>
<td>86.2(^a)</td>
<td>8.3(^b)</td>
<td>2.3(^b)</td>
<td>0.16(^b)</td>
</tr>
<tr>
<td>30</td>
<td>86.3(^a)</td>
<td>5.5(^c)</td>
<td>1.6(^c)</td>
<td>0.14(^c)</td>
</tr>
<tr>
<td>60</td>
<td>83.9(^a)</td>
<td>5.2(^c)</td>
<td>1.8(^c)</td>
<td>0.13(^c)</td>
</tr>
<tr>
<td>120</td>
<td>87.7(^a)</td>
<td>6.2(^c)</td>
<td>1.6(^c)</td>
<td>0.16(^b)</td>
</tr>
<tr>
<td>5% LSD</td>
<td>5.0</td>
<td>1.2</td>
<td>0.3</td>
<td>0.03</td>
</tr>
</tbody>
</table>

\(^a\)The data marked with same superscripts in the respective analysis/assay (column) was not significant different.  
\(^b\)g/100gDW- gram of extract per 100 gram of dry bean  
\(^c\)TPC- Total phenolic content; mgGAE/gDW- mg gallic acid equivalents per gram of dry bean  
\(^d\)TFC- Total flavonoid content; mgCE/gDW- mg of catechin equivalents per gram of dry bean  
\(^e\)TPro- Total proanthocyanidins; mgCE/gDW- mg of catechin equivalents per gram of dry bean

The TPC in the unprocessed beans was significantly higher than those of roasted beans regardless of roasting duration. Roasting the beans for 10 min retained 80% of TPC comparing to that of unprocessed beans. The TPC of roasted beans was about 50-60% of that of unprocessed beans. Roasting the beans for 10 min retained the highest TPC in faba beans comparing to that of beans being roasted for longer periods of time. Roasting for 30, 60 and 120 min retained a similar TPC in faba beans.

After roasting the beans for 10 min, there was about 80% of TFC retained in the roasted beans comparing to that of unprocessed beans. Moreover, prolong roasting the faba beans for up to 120 min retained TFC in faba beans by about 60%. Roasting the beans for 30, 60 and 120 min retained a similar TFC in faba beans, and they were significantly lower than that of beans being roasted for 10 min.

Roasting the beans for 10 min and onwards retained 60-70% of TPro in faba beans when comparing to that of unprocessed beans. Beans roasted for 30 and 60 min had lower TPro levels than beans roasted for 10 and 120 min.

#### 5.2.2 Effect of roasting on faba bean antioxidant activity

The effect of dry roasting on antioxidant activities of faba bean extracts was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, total equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant power (FRAP) assays, and the results are presented in Table 5-2. In comparison to the
unprocessed beans, roasting the faba beans for up to 120 min retained the DPPH radical scavenging activity in the beans by 50-60%. Roasting for 10 and 60 min retained a similar level of DPPH radical scavenging activity in faba beans, and they were significantly higher than that of beans being roasted for 30 and 120 min.

Table 5-2 Effect of dry roasting on the antioxidant activities of faba bean at designated time points

<table>
<thead>
<tr>
<th>Roasting time (minute)</th>
<th>DPPH (µmolTE/gDW)*</th>
<th>TEAC (µmolTE/gDW)†</th>
<th>FRAP (µmolFe²⁺eq/gDW)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>53.4a</td>
<td>72.7a</td>
<td>18.2b</td>
</tr>
<tr>
<td>10</td>
<td>34.0b</td>
<td>66.2b</td>
<td>19.3ab</td>
</tr>
<tr>
<td>30</td>
<td>24.1c</td>
<td>57.4c</td>
<td>19.2ab</td>
</tr>
<tr>
<td>60</td>
<td>34.3b</td>
<td>49.8d</td>
<td>19.5b</td>
</tr>
<tr>
<td>120</td>
<td>27.8c</td>
<td>61.8ace</td>
<td>19.7a</td>
</tr>
<tr>
<td>5% LSD</td>
<td>4.7</td>
<td>4.6</td>
<td>1.5</td>
</tr>
</tbody>
</table>

*The data marked with same superscripts in the respective assay (column) were not significantly different (p<0.05).
†DPPH- 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity; µmolTE/gDW- µmol Trolox equivalents per gram of dry bean
‡TEAC- Trolox equivalent antioxidant capacity; µmolTE/gDW- µmol Trolox equivalents per gram of dry bean
§FRAP- Ferric reducing antioxidant power; µmolFe²⁺eq/gDW- µmol Fe²⁺ equivalents per gram of dry bean

There were about 70-90% of TEAC retained in the roasted faba beans compared to the unprocessed beans. Roasting the beans for 10 min and 120 min retained the highest TEAC, which was the inverse of that of beans being roasted for 60 min. In comparison to the unprocessed faba beans, roasting for 120 min increased FRAP in faba beans significantly. Roasting faba beans for 10, 30 and 60 min also similarly increased the FRAP in faba beans but the changes were not significantly different from that of unprocessed beans.

5.2.3 Effect of roasting on HPLC-PCD profiles of faba bean phenolic extracts

Figure 5-1 shows the high performance liquid chromatography-post column derivitisation (HPLC-PCD) profiles of extracts from the raw and roasted beans. The chromatogram of phenolic extracts from the unprocessed faba bean is shown in Figure 5-1a. The chromatograms of phenolic extracts from the faba beans being roasted for 60 min and 120 min are shown in Figures 5-1b and 5-1c. The chromatograms of faba bean extracts eluted in two separate regions: retention time 0-10 min and retention time 10-30 min, and they are arbitrarily grouped as polar and less-polar regions, respectively.
Figure 5-1 High performance liquid chromatography-post column derivatisation (HPLC-PCD) profiles of crude phenolic extracts from (a) uncooked faba bean, (b) roasted faba bean for 60 min and (c) 120 min at 150°C (*, new/altered peaks)

In the chromatograms of the extracts from unprocessed and roasted faba beans, there was a large PCD response or antioxidant activity corresponding to the compounds eluted at retention time 4 min. However, the antioxidant responses of phenolic compounds from the extracts of beans being roasted for 120 min was lower than that of beans being roasted for 60 min. Some new or altered HPLC peaks were observed in the chromatograms of extracts from the roasted beans at retention time 10-30 min in comparison to that of unprocessed beans (marked by *), and the peaks were more noticeable in the extract obtained from the beans being roasted for 120 min.
5.2.4 Phenolic content and antioxidant activity of fractions from roasted faba bean

The crude acetone extracts of the roasted faba beans (Nura) were fractionated from the phenolic compounds eluted at the polar (0-10 min) and less-polar regions (10-30 min) respectively, termed Fraction A and B (Table 5-3). The extraction yields of phenolic extracts from the whole beans was the greatest (83.92±3.19 mg/gDW), followed by that of Fraction A (24.29±0.74 mg/gDW) and B (4.95±0.12 mg/gDW). However, sum of the extraction yields of Fraction A and B only accounted for about 35% of that of whole beans.

### Table 5-3 Phenolic content and antioxidant activities of Fraction A and B from the crude extract from roasted faba bean (roasting at 150°C for 60 minutes)

<table>
<thead>
<tr>
<th></th>
<th>Extraction yield (mg/gDW)</th>
<th>TPC (mgGAE/gDW)</th>
<th>TPro (mgCE/gDW)</th>
<th>FRAP (µmolFe2+ eq/gDW)</th>
<th>ORAC (µmolTE/gDW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole bean</td>
<td>83.92±3.19</td>
<td>5.24±0.63</td>
<td>0.13±0.01</td>
<td>19.46±0.68</td>
<td>94.43±16.30</td>
</tr>
<tr>
<td>Fraction A</td>
<td>24.29±0.74</td>
<td>0.34±0.04</td>
<td>0.001±0.000</td>
<td>1.09±0.17</td>
<td>18.36±1.60</td>
</tr>
<tr>
<td>Fraction B</td>
<td>4.95±0.12</td>
<td>2.31±0.09</td>
<td>0.09±0.01</td>
<td>8.14±0.35</td>
<td>19.81±5.60</td>
</tr>
</tbody>
</table>

The results marked with different superscripts in the individual column were significantly different from each other (p<0.05)

*1g/100gDW- gram of extract per 100 gram of dry bean

*TPC- Total phenolic content; mgGAE/gDW- mg of gallic acid equivalents per gram of dry bean

*TPro- Total proanthocyanidins; mgCE/gDW- mg of (+)-catechin equivalents per gram of dry bean

*FRAP- Ferric reducing antioxidant power; µmolFe2+eq/gDW- µmol ferric ions equivalents per gram of dry bean

*ORAC- Oxygen radical absorbance capacity; µmolTE/gDW- µmolTrolox equivalents per gram of dry bean

The whole bean extract also exhibited the greatest TPC, TPro, FRAP and oxygen radical absorbance capacity (ORAC) comparing to that of fractions. Fraction B exhibited a significantly higher TPC, TPro, FRAP than that of Fraction A, but it had a similar ORAC to Fraction A. The sum of Fraction A and B comprised about 40% (TPC), 70% (TPro), 47% (FRAP) and 40% (ORAC) of that of whole bean extracts, respectively.

5.2.5 Effect of acid and alkaline hydrolysis on phenolic composition of faba bean extracts

Table 5-4 compares the phenolic compositions of extracts from Nura and Rossa after undergoing alkaline and acid hydrolysis, respectively. The phenolic compound was
grouped into four different classes based on the respective detectable wavelengths at 280, 326, 370 and 520 nm.

Table 5-4 Effect of acid and alkaline hydrolysis on phenolic composition of faba bean extracts

<table>
<thead>
<tr>
<th>Detection wavelength</th>
<th>Detectable phenolic classes</th>
<th>Nura</th>
<th>Rossa</th>
<th>5% LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hydrolysis treatment</td>
<td>None</td>
<td>Alkaline</td>
<td>Acid</td>
</tr>
<tr>
<td>280 nm</td>
<td>Hydroxybenzoic acids/flavanols (mgGAE/gDW)†</td>
<td>2.95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.92&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>326 nm</td>
<td>Hydroxycinnamic acids (mgCHAeq/gDW)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.03&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.45&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td>360 nm</td>
<td>Flavonols (mgRE/gDW)&lt;sup&gt;ξ&lt;/sup&gt;</td>
<td>0.18&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.39&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.45&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>520 nm</td>
<td>Anthocyanidins (mgC3G eq/gDW)&lt;sup&gt;∫&lt;/sup&gt;</td>
<td>n.d.</td>
<td>0.12&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.19&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

† The data marked by different superscripts measured at the respective wavelength were significantly different (p<0.05). n.d. = not detectable.
‡ mgGAE/gDW- mg of gallic acid equivalents per gram of dry bean
§ mgCHAeq/gDW- mg of chlorogenic acid equivalents per gram of dry bean
ξ mgRE/gDW- mg of rutin equivalents per gram of dry bean
∫ mgC3Geq/gDW- mg of cyanidin 3-glucoside equivalents per gram of dry bean

The hydroxybenzoic acids/flavanols were detected at 280 nm and quantified as gallic acid equivalents (GAE/gDW); hydroxycinnamic acids were measured at 326 nm and expressed as chlorogenic acid equivalents (CHAeq/gDW); flavonols were quantified at 370 nm and expressed as rutin equivalents (RE/gDW), and lastly the anthocyanidins were assessed at 520 nm and expressed as cyanidin 3-glucoside equivalents (C3G/gDW). Both Nura and Rossa extracts contained mostly hydroxybenzoic acids/flavanols regardless of the hydrolysis treatments. The non-hydrolysed extracts from both Nura and Rossa contained primarily hydroxybenzoic acids/flavanols, followed by flavonols, hydroxycinnamic acids and had no anthocyanidin. The non-hydrolysed Rossa extract exhibited a significantly higher level of all three detectable phenolic classes than that of non-hydrolysed Nura.

The alkaline-hydrolysed Nura extracts contained lower amounts of hydroxybenzoic acids/flavanols and hydroxycinnamic acids than, while exhibiting a similar amount of flavonols to that of non-hydrolysed extracts. Conversely, the contents of hydroxybenzoic acids/flavanols, hydroxycinnamic acids and flavonols in the alkaline-hydrolysed Rossa extracts were lower than that of non-hydrolysed extracts.
Interestingly, the alkaline hydrolysis carried out on both *Nura* and *Rossa* extracts resulted in generations of anthocyanidins in their hydrolysed extracts, where the anthocyanidins were not detected in their non-hydrolysed samples. The contents of hydroxybenzoic acids/flavanols in the acid-hydrolysed *Nura* extract were lower than that of non-hydrolysed *Nura* extract. The level of hydroxycinnamic acids and flavonols in the acid-hydrolysed *Nura* extract were similar to that of non-hydrolysed *Nura* extract. Conversely, the contents of hydroxybenzoic acids/flavanols, hydroxycinnamic acids and flavonols in the acid-hydrolysed *Rossa* extracts were lower than that of non-hydrolysed *Rossa* extract. A similar level of anthocyanidins was detected in both of the acid-hydrolysed *Nura* and *Rossa* extracts, which were not detected in their respective non-hydrolysed extracts.

The alkaline-hydrolysed *Nura* extract contained a significantly higher amount of hydroxybenzoic acids/flavanols; a similar level of hydroxycinnamic acids and anthocyanidins; and, a lower level of flavonols in comparison to that of alkaline-hydrolysed *Rossa* extract. Conversely, a significant higher level of flavonols was observed in the acid-hydrolysed *Rossa* extracts than that of acid-hydrolysed *Nura* extracts, whilst the levels of hydrobenzoic acids/flavanols, hydroxycinnamic acids and anthocyanins were similar in both of the mentioned extracts.

### 5.2.6 Determination of phenolic acids and flavan-3-ols in faba bean

The retention times of standard mixtures of phenolic acids (Figure 5-2a) and flavan-3-ol (Figure 5-2b) were measured simultaneously at 280 nm. The retention time was used as a reference to identify phenolic acids and flavan-3-ols in faba bean extracts. After that, the standard was used to spike the identified HPLC peaks to further confirm the corresponding phenolic acids.
Figure 5-2 HPLC chromatogram of mixed (a) phenolic and (b) flavan-3-ol standards

There was an appearance of HPLC ‘hump’ eluting at retention time 10-30 min in the chromatogram of the purified non-hydrolysed *Nura* extract (Figure 5-3a). The chromatograms of acid- (Figure 5-3b) and alkaline-hydrolysed (Figure 5-3c) extracts were entirely different from each other, and they were also different from that of non-hydrolysed extract. The baseline of chromatogram of the acid-hydrolysed extract was flatter than that of alkaline-hydrolysed extract.

Figure 5-3 HPLC profiles of faba bean extracts after (b) alkaline hydrolysis and (c) trifluoroacetic acid hydrolysis of the (a) purified non-hydrolysed extract

The HPLC peaks of both alkali- and acid-hydrolysed extracts spread across the entire time scales of the chromatograms. Furthermore, there was an appearance of a small ‘hump’ observed at 58-76 min in the chromatogram of acid-hydrolysed extract. Eight types of phenolic acids were tentatively identified in both alkaline-hydrolysed *Nura* and *Rossa* extracts (Table 5-5) by comparing the retention times as well as co-eluting (spiking) with the purchased standards (Figure 5-2).
The phenolic acids found in the alkaline-hydrolysed samples tentatively included gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, ellagic acid and o-coumaric acid. The levels of all types of phenolic acids were similar in both of the Nura and Rossa extracts, except that the level of p-coumaric and o-coumaric acids were significantly higher in the former. Table 5-6 shows the content of flavan-3-ol in the alkaline-hydrolysed Nura and Rossa extracts. There were similar amounts of (+)-catechin in both of the alkaline-hydrolysed Nura and Rossa extracts. However, the level of (-)-epicatechin in the alkaline-hydrolysed Rossa extract was 2-folds of that of alkaline-hydrolysed Nura extracts.

Table 5-6 Quantification of flavan-3-ols in alkaline-hydrolysed faba bean extracts using HPLC*

<table>
<thead>
<tr>
<th>Flavan-3-ol (mg/100gDW)</th>
<th>(+)-Catechin</th>
<th>(-)-Epicatechin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nura</td>
<td>11.91±2.28a</td>
<td>9.10±0.95a</td>
</tr>
<tr>
<td>Rossa</td>
<td>9.66±0.84a</td>
<td>18.95±1.25b</td>
</tr>
</tbody>
</table>

*Results were analysed using student unpaired t-test (two tailed) based on two independent experiments and presented as mg phenolic acid per gram of dry bean (mean±SD; n=2). Results marked with different superscripts in individual column were significantly different from each other (p<0.05).

5.3 Discussion

5.3.1 Effect of dry roasting on extraction yield and phenolic compounds of faba bean

The dry heating process causes a significant reduction in the level of phenolic content and antioxidant activity of faba beans. The finding is in agreement with other studies conducted on sicklepod seeds (Vadivel, Kunyanga & Biesalski, 2011) and moth beans (Siddhuraju, 2006), where extract solvents used are methanol:water and acetone:water mixtures respectively. The extract yields of roasted beans are higher...
than that of unprocessed beans as reported in the current study, which contradicts a study on the wattle seeds similarly applying aqueous acetone to extract phenolic compounds (Ee et al., 2011). Also, roasting wattle seeds results in a dramatic increase in TPC (10-folds) and TFC (4-folds) as reported by the same author. The dissimilar finding is likely attributed to different types of phenolic compounds in wattle seeds, where they are primarily succinic and gallic acids. However, in view of a much greater number of HPLC peaks and inseparable HPLC ‘humps’ as shown in the chromatograms of faba bean extracts in the current study, it is evident that the phenolic classes in faba bean extracts are much more varied than that of wattle seeds and thereby responding differently to the dry heat treatment. In support of this, it has been reported that dry toasting a variety of pulses causes dissimilar changes in TPC of the pulses (Baoteng et al., 2008). Therefore, changes in the phenolic contents as a respond to heat treatment can be plant specific, which relies greatly on the nature and types of phenolic compounds in the particular plant materials. Furthermore, dry heating conditions can also have significant effect on the changes in phenolic contents of beans. For instance, dry heat treatment at 149°C for 20 min is reported to double the amount of tannins, while heat treatment at 232°C for 12 min reduces almost 40% of tannins in dehulled faba beans (Anderson et al., 1994).

5.3.2 Generation and changes in phenolic profile of faba bean undergoing roasting processes

It can be observed that dry roasting causes degradations of phenolic compounds in faba beans as evident by chromatograms of extracts from the roasted faba beans with a flatter HPLC ‘hump’ than that of unprocessed beans. Similarly, new phenolic compounds have been reported to generate in extracts of roasted peanuts comparing to that of unprocessed peanuts (Chukwumah et al., 2007). Roasting faba beans causes generations of new phenolic compounds and alteration of the level of phenolic compounds as demonstrated in the current study. This is supported by a finding on catechin content of cocoa beans is gradually reduced with an increasing roasting temperature, but the reverse is true for its epicatechin content (Payne et al., 2010). The appearance of a HPLC ‘hump’ eluted at the less-polar regions of the chromatograms of faba bean crude and purified extracts is likely to be proanthocyanidins as discussed earlier in Chapters 3 and 4. This is evident by generation of anthocyanins following hydrolysis of faba bean extracts in the current
study, and this observation is supported by Bate-Smith & Lerner (1954). Furthermore, appearances of proanthocyanidins eluted as a HPLC ‘hump’ in chromatograms have also been reported in acetone extracts of faba beans (Merghem et al., 2004) and cider apples (Guyot et al., 1998). It is known that roasting can alter procyanidins in the food materials containing proanthocyanidins, such as peanut skins (Yu et al., 2006; Lou et al., 1999). Nevertheless, roasting tends to alter the minor (small) than those of major (big) HPLC peaks in the chromatograms of faba bean extracts as demonstrated in our study. This indicates that the major peaks are more heat-labile, and possibly larger in molecular weights.

5.3.3 Antioxidant activity and its mechanism in faba bean undergoing dry roasting

The mechanism of TPC, FRAP, DPPH radical scavenging activity and TEAC assays are based on electron-transfer reaction. The FRAP assay measures reducing capacity, while the other assays measure inhibition ability on the free radical species or radical scavenging ability (Dai & Mumper, 2010). The ORAC assay measures antioxidant capacity based on the hydrogen transfer mechanism (Huang, Ou & Prior, 2005). Among the mentioned assays, only FRAP is conducted in an acidic medium, while TPC is measured under an alkaline condition. The HPLC-PCD result reveals that most of the phenolic compounds in faba bean extracts by less-polar nature are based on free radical scavenging activity (on-line reaction with ABTS\(^+\)). The phenolic compounds in faba bean with reducing capability are heat stable, because there is no substantial change in FRAP of extracts from roasted beans in comparison to that of unprocessed beans.

It is no doubt that choices of solvent extraction systems can have a significant effect on the extractability of phenolic compounds in various plant materials as evident in Chapter 3. Furthermore, heat treatments also can exert significant effects in phenolic compounds and antioxidant activity of faba beans as shown in Chapter 4. For example, roasting small black soybeans increases the level of DPPH radical scavenging activity, TEAC, Maillard reaction products, as well as improving protective effect against \( \text{H}_2\text{O}_2 \) of roasted small black soybeans, where distilled water was used as solvent extraction system (Kim et al., 2011); roasting chickpea, giant lentil, black bean, borlotti bean, red and yellow soybeans increases TEAC where the TEAC was measured by reacting the bean powder directly with the ABTS\(^+\) solution prepared in an ethanol/water (50:50, v/v) mixture (Acar et al., 2009). In view of
findings, roasting mainly reduce the antioxidant capacity mainly in faba beans which is likely because 70% acetone (v/v) is applied in the current study. It might be possible that the applied extraction solvent systems in the mentioned studies using water and aqueous ethanol are not efficient in extracting phenolic compounds with less-polar nature, as agreed by what reported in Chapter 3. Nevertheless, the applied heat treatments possibly increase extractability of phenolic compounds in the roasted beans in comparison to that of unprocessed beans, and in turn increasing the antioxidant activity and functionality. For instance, dry heat treatment releases the bound phenolic compounds from citrus peels and increases its antioxidant activity when extracted using water and 70% ethanol extracts (Jeong et al., 2004). Conversely, it is likely that the solvent system applied in the current study (aqueous acetone) has successfully extracted the majority, if not all, of the extractable phenolic compounds in the faba beans. In fact, dry heating has been reported to reduce proanthocyanidins or tannins in various nuts and legumes (Chandrasekara & Shahidi, 2011; Tan, Wong & de Lumen, 1984), which is thought to be caused by alterations of tannin binding sites with proteins as a result of heat treatments (Siebert, Troukhanova & Lynn, 1996) and lead to decreases in the antioxidant activity of plant materials (Arts et al., 2002). Dry toasting lowers phenolic, flavonoids and proanthocyanidin contents in soybeans, although the same treatment increases those of black-eyed peas, kidney and pinto beans as extracted using aqueous methanol (Baoteng et al., 2008). This further supports that effect of heat processing on phenolic compounds in plant materials relies largely on the nature of the plant materials.

The roasting process is known to increase non-enzymatic browning Maillard reaction products in plant materials and the Maillard reaction products are reported to contribute to antioxidant activity, which is particularly true for roasted coffee beans (Delgado-Andrade, Rufian-Henares & Morales, 2005; Yanagimoto et al., 2002). Although DPPH radical scavenging activity and TEAC in the roasted faba beans are lower than that of unprocessed beans, there is an increase in the antioxidant activities of faba beans being roasted for longer than 30 min, which is probably because of formation of Maillard reaction products.

5.3.4 Phenolic content and antioxidant activity of fractions from roasted faba bean

Although the extraction yields of Fraction A are five times greater than that of Fraction B, Fraction B exhibits a higher level of TPC, TPro and FRAP. Furthermore,
HPLC-PCD profiles of the roasted faba bean extracts also demonstrate that most of the antioxidant activities are contributed from the Fraction B. This suggests that majority of potent antioxidants in faba bean are less-polar in nature, although they are low in amount. Conversely, both fractions A and B contain an equal ORAC. This suggests that antioxidant activity in Fraction A is likely to base on hydrogen transfer mechanism, which is not reflected in the HPLC-PCD analyses. There is a great loss in TPC, TPro, FRAP and ORAC in faba bean extracts after being fractionated, as shown by comparing those of the whole bean extract to the sum of both fractions. The loss in phenolic content and antioxidant activity of fractionated extracts in comparison to that of whole bean extracts might be attributed to process handling errors including the collection and freeze drying processes and sample overloading relative to the column capacity, or it could be retention of some matrix compounds in the preparative column (Obied, Prenzler & Robards, 2008).

5.3.5 Effect of acid and alkaline hydrolysis on phenolic compositions in faba bean

Various types of phenolic components are observed in the extracts of Nura and Rossa including hydroxybenzoic acids, hydroxycinnamic acids and flavonols. The findings from current study confirm that there is a relatively low but a significant amount of proanthocyanidins and anthocyanins (detectable after hydrolysis) in both the buff- and red- coloured-faba bean cultivars, where hydrolysis of synthetic proanthocyanidins is known to generate anthocyanidins (Porter, Hrstich & Chan, 1986). The anthocyanins in faba beans could be cyanidin and delphinidin as reported earlier (Leung et al., 1979; Troszynska et al., 2006). The appearance of a small HPLC ‘hump’ in the chromatogram of acid-hydrolysed Nura extract is possibly due to presence of less common flavan-3-ols with an interflavan bond of 4→6 bonds, rather than an 4→8 bond, because the former is more resistant to cleavage (Schofield, Mbugua, & Pell, 2001). Also, the appearance of an extremely non-polar small HPLC ‘hump’ at retention time 48-76 min in the chromatograms of acid-hydrolysed extracts indicates presence of large molecule non-hydrolysable tannins which were not hydrolysable by the applied acidic condition (Merghem et al., 2004). The clearly dissimilar HPLC profiles obtained from the acid- versus alkaline-hydrolysed extracts of Nura indicates that the choices of hydrolysis methodology greatly influences the types of detectable phenolic acids in faba bean extracts. The detectable phenolic acids in the alkaline-hydrolysed extract are 2-folds that of...
alkaline-hydrolysed extract. This observation agrees with other reports studying on various types of dry beans (Ross, Beta & Arntfield, 2009) and wheat bran (Verma, Hucl & Chibbar, 2009). This is likely because the acid hydrolysis treatment carried on extracts at a higher temperature and for a longer period of time than that of alkaline hydrolysis treatment results in a greater loss of esters (Krygier, Sosulski & Hogge, 1982).

5.3.6 Determination of phenolic acids and flavan-3-ols in faba bean after acid and alkaline hydrolysis

The detection wavelength at 280 nm has been applied widely for the detection of benzoic acid and hydroxycinnamic acid derived phenolic compounds simultaneously. Acid hydrolysis on plant extracts for determination of phenolic acids is more commonly practiced due to it being more applicable in the human digestive system. Moreover, alkaline hydrolysis has been reported to degrade phenolic compounds especially hydroxycinnamic acids (Robbins, 2003). However, the identification and quantification of phenolic acids of faba beans in the current study is carried out on the alkaline-hydrolysed extracts because a higher phenolic content is obtained compared to that of acid-hydrolysed extract. There are eight types of phenolic acids and two types of flavan-3-ols detected in the alkaline-hydrolysed extract of faba beans. It must be emphasized that the mentioned identified compounds in the alkaline-hydrolysed extracts in this study are only tentative due to the spectral analysis on individual HPLC peaks can not be used to confirm their identity. The identified compounds have similar retention time but unmatched spectrum data to the authentic phenolic standards, this might caused by poor resolution of compounds based on retention time resulting in overlapping peaks. Although both hydrolysis procedures successfully diminish the HPLC ‘humps’, the both hydrolysis treatments on the extracts causes generation of many small HPLC peaks that spread across almost the entire baseline of their chromatograms. This signifies that phenolic acids and flavonoids in faba bean extracts have a wide range of molecular weights and structures. Moreover, the faba bean extracts also contain extremely non-polar compounds which elute beyond retention time 70 min (with approximately 80-100% of solvent B). There are many unidentified phenolic compounds in the hydrolysed-extract of faba beans other than those are identified in this study. The poorly resolved compounds in the alkaline-hydrolysed extract generally have an optimal absorbance at 200-250 nm (Appendix
The reason could be the strong disturbance from sample matrix that contains other UV active organic molecules absorbing UV at 200-250 nm (Robbins & Bean, 2004); or, it could also be the trifluoroacetic acid used in the hydrolysis process that causes acetylation of hydroxyl groups of phenolic compounds and thereby a hypochromic shift (Hussein & Bruggeman, 1999).
Chapter 6 *In vitro* investigation of potential health benefits of

Australian grown faba bean: antioxidant activities, chemopreventative capacity and inhibitory effects on the angiotensin converting enzyme, \( \alpha \)-glucosidase and lipase\(^1\)

6.1 Introduction

Pulses are well known to be an economical source of protein, carbohydrate and fibre. Pulses are also low in fat content and containing minor constituents such as phenolic compounds, oligosaccharides (Bouhnik et al., 1997), enzyme inhibitors, phytosterols and saponins (Campos-Vega, Loarca-Pina & Oomah, 2010; Mathers, 2002). Intake of legumes is reported to potentially lower the risk of cancers (Aune et al., 2009), cardiovascular diseases (Anderson, Smith & Washnock, 1999), hypertensions and diabetes (Ranilla et al., 2010). Some of the micro-constituents are currently marketed as functional foods and nutraceutical ingredients (Ferguson, 2001). Also, there have been many attempts to incorporate pulses into food products for enrichment of product quality and additional health benefits (Gomez et al., 2008; Patterson, Maskus & Bassett, 2010).

Phenolic compounds are one of the minor constituents in plants that have been gaining an increasing interest for their health-promoting properties, largely defined by their antioxidant activity (Agboola et al., 2010; Aviram, Rosenblat & Fuhrman, 2008). Previous research has reported that different types of phenolic compounds are found in faba beans such as procyanidins (Merghem et al., 2004), catechins (Arts, Van De Putte & Hollman, 2000), flavonols (Hertog, Hollman & Katan, 1992), isoflavones (Kaufman et al., 1997), phenolic acids (Sosulski & Dabrowski, 1984) and tannins (Bekkara et al., 1998; Karamac et al., 2005; Moneam, 1990), and they are natural antioxidants (Amarowicz et al., 2004). 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 (Ikarashi et al., 2010; Shimura et al., 1992).

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\(^1\) The content in this chapter was accepted for publication in the British Journal of Nutrition (in press)
Phenolic extracts of different types of beans are reported to have antioxidant activities (Amarowicz et al., 2004), protective effects against radical-induced DNA damage (Madhujith, Amarowicz & Shahidi, 2004), antimutagenic (Meijia, Castano-Tostado & Loarca-Pina, 1999) and anticancer (Itoh, Umekawa & Furuichi, 2005) properties. There are also numerous of reports on the substantial amounts of phenolic compounds in raw (Amarowicz et al., 1996) and cooked faba beans (Kalogeropoulos et al., 2010). Conversely, there is a limited number of reports which focus on the health benefits of faba bean phenolic compounds or the impact of food preparation using heat treatments on retention of the health functional properties of phenolic compounds.

Base on the results in Chapter 4 and 5, roasting treatment is shown to be relatively more effective in retaining phenolic compounds and antioxidant activities of faba beans comparing to that of boiling and autoclaving treatments. An investigation on the effect of roasting on phenolic content and antioxidant activity, as well as the potential health benefits of faba beans in preventing chronic diseases including hypertension, diabetic, obesity and different types of cancers in vitro is carried out in the current study. Three faba bean genotypes with different seed coat colours including Nura (buff), Rossa (red) and TF(Ic*As)*483/13 (white) are selected for a more comprehensive comparison purpose.

6.2 Results and Discussion

6.2.1 Total phenolic content, total flavonoid content and antioxidant capacities

The effect of roasting on Total Phenolic Content (TPC), Total Flavonoid Content (TFC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, total equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) of the extracts from three faba bean genotypes are presented in Table 6-1.
Table 6-1 Extraction yield, total phenolic content (TPC), total flavonoid content (TFC), di(phenyl)-(2,4,6-trinitrophenyl)miminoazanii (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 genotype

<table>
<thead>
<tr>
<th></th>
<th>Raw bean</th>
<th>Roasted bean</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Extraction yield (mg of extracts/gDW)</strong>†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>75.1c</td>
<td>83.9b</td>
</tr>
<tr>
<td>Rossa</td>
<td>86.8ab</td>
<td>89.6a</td>
</tr>
<tr>
<td>TF(İc*As)*483/13</td>
<td>88.1ab</td>
<td>88.5ab</td>
</tr>
<tr>
<td>5% LSD</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td><strong>Total phenolic content (mgGAEq/gDW)</strong>‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>10.9a</td>
<td>5.2b</td>
</tr>
<tr>
<td>Rossa</td>
<td>11.2a</td>
<td>6.4b</td>
</tr>
<tr>
<td>TF(İc*As)*483/13</td>
<td>2.8c</td>
<td>3.4c</td>
</tr>
<tr>
<td>5% LSD</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td><strong>Total flavonoid content (mgCEq/gDW)</strong>§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>2.8b</td>
<td>1.8d</td>
</tr>
<tr>
<td>Rossa</td>
<td>3.0b</td>
<td>2.1c</td>
</tr>
<tr>
<td>TF(İc*As)*483/13</td>
<td>1.0c</td>
<td>1.0e</td>
</tr>
<tr>
<td>5% LSD</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td><strong>DPPH radical scavenging activity (µmolTE/gDW)</strong>∫</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>53.4a</td>
<td>34.3c</td>
</tr>
<tr>
<td>Rossa</td>
<td>48.1b</td>
<td>37.3c</td>
</tr>
<tr>
<td>TF(İc*As)*483/13</td>
<td>7.5d</td>
<td>9.6e</td>
</tr>
<tr>
<td>5% LSD</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td><strong>Trolox equivalent antioxidant capacity (µmolTE/gDW)</strong>∫</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>72.7a</td>
<td>49.8c</td>
</tr>
<tr>
<td>Rossa</td>
<td>71.3a</td>
<td>56.0b</td>
</tr>
<tr>
<td>TF(İc*As)*483/13</td>
<td>25.6a</td>
<td>35.8d</td>
</tr>
<tr>
<td>5% LSD</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td><strong>Oxygen radical absorbance capacity (µmolTE/gDW)</strong>∫</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>111.9b</td>
<td>94.4c</td>
</tr>
<tr>
<td>Rossa</td>
<td>136.8a</td>
<td>116.9b</td>
</tr>
<tr>
<td>TF(İc*As)*483/13</td>
<td>69.9d</td>
<td>73.9d</td>
</tr>
<tr>
<td>5% LSD</td>
<td>15.5</td>
<td></td>
</tr>
</tbody>
</table>

*The data marked by the same superscripts were not significantly different (p<0.05) in the respective assay.
†g/100gDW- gram of extract per 100 gram of dry bean
‡mgGAEq/gDW- mg of gallic acid equivalents per gram of dry bean
§mgCEq/gDW- mg of catechin equivalents per gram of dry bean
∫µmolTEq/gDW- µmol Trolox equivalents per gram of dry bean

Roasting caused an increase in extraction yields in Nura, but insignificant change in Rossa and TF(İc*As)*483/13. The extraction yields from roasted Nura was significantly lower than that of roasted Rossa, but not significantly different from that of roasted TF(İc*As)*483/13. The raw and roasted Nura and Rossa exhibited a similar TPC and TFC, except that the TFC of roasted Rossa was slightly higher than that of roasted Nura. The TPC and TFC in the roasted beans of Nura and Rossa were about two times higher than that of TF(İc*As)*483/13.

The TPC and TFC of roasted Nura and Rossa were reduced by 40-50% and 30-40% respectively. Nevertheless, roasting did not effect TPC and TFC in TF(İc*As)*483/13. These results was supported by findings earlier that heating
applied through various cooking methods decreased the phenolic contents in different types of legumes (Ranilla et al., 2010).

The antioxidant capacities of faba bean were evaluated using three assays: DPPH radical scavenging activity, TEAC and ORAC (Table 6-1). A significant decrease in the antioxidant activities of Rossa and Nura occurred after roasting. However, roasting did not affect the levels of antioxidant activities in TF(1c*As)*483/13 significantly as tested using the DPPH and ORAC assays.

6.2.3 Cellular protection by faba bean extracts

6.2.3.1 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 cannot 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 (Wolfe & Liu, 2007). Extracts from Nura and Rossa were evaluated in this study. No significant difference was found between the CAA of extracts obtained from either genotype, regardless of heat treatment (Table 6-2). However, extract from the roasted Nura exhibited a lower EC₅₀ value than its raw bean counterparts, which suggested a tendency for a higher uptake of compounds.

Table 6-2 Cellular antioxidant activity of the crude extracts obtained from the raw and roasted faba bean genotypes, Nura and Rossa

<table>
<thead>
<tr>
<th>Faba bean genotype</th>
<th>Cellular Antioxidant Activity†</th>
<th>EC₅₀ (µg/mL)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw bean</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>7.8b</td>
<td>402a</td>
</tr>
<tr>
<td>Rossa</td>
<td>15.8a</td>
<td>208b</td>
</tr>
<tr>
<td>Roasted bean</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>11.8ab</td>
<td>278b</td>
</tr>
<tr>
<td>Rossa</td>
<td>12.7ab</td>
<td>262b</td>
</tr>
<tr>
<td>5% LSD</td>
<td>6.4</td>
<td>114</td>
</tr>
</tbody>
</table>

*The data marked by the same superscripts were not significantly different.
†µmolQE/gDW: µmol quercetin equivalents per gram of dry bean
‡EC₅₀: concentration of the lyophilised extract in the culture medium (µg/mL) able to scavenge 50% of free radicals effectively within a cell.

The HPLC chromatogram as shown in section 5.2.6 demonstrated compositional differences between the compounds detected at 280 nm in extracts from Nura and Rossa. The HPLC ‘hump’ at the less polar region dominated in extracts from both
*Nura* and *Rossa* could be polyphenolic compounds (Chapter 2, 3 and 4). A variety of phenolic compounds were likely to contribute to the different cell uptake rates and efficiency of protection against peroxyl radicals, within an hour. It can also be speculated that heat application during roasting of faba bean caused a partial oxidation of polymeric compounds, which subsequently would affect the uptake and reflect on the antioxidant capacity within a cell. The CAA EC$_{50}$ values of the faba bean are slightly lower than those of lentil (670 µg/mL), yellow pea (780 µg/mL) and green peas (1280 µg/mL) as reported in Xu & Chang (2009) assayed using AGS cells.

### 6.2.3.2 Cellular protection against H$_2$O$_2$

Hydrogen peroxide (H$_2$O$_2$) is a reactive oxygen species which presents 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$_2$O$_2$ induced apoptosis in RAW264.7 cells (Figure 6-1) was evaluated. 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$_2$O$_2$ in a dose-dependent manner (Figure 6-1a and 6-1c). However, the protection diminished at concentrations higher than 0.4 mg/mL due to the commencement of 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 generation of $\text{H}_2\text{O}_2$ (Lapidot, Walker & Kanner, 2002). The same tendency was observed for extracts obtained from the roasted *Nura* (Figure 6-1b). The protective effect by extracts obtained from the roasted *Nura* and *Rossa* were observed at concentrations at 0.2 and 0.6 mg/mL respectively (Figure 6-1b and 6-1d). In comparison to other results on the protection of RAW264.7 cells from $\text{H}_2\text{O}_2$ induced injury, the faba bean extracts appeared less efficient than that of Kakadu plum extract (Tan et al., 2010). 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. (2005) also found that 25 µM and 50 µM of quercetin (but not rutin and quercitrin) posed potent protection of RAW264.7 cells against $\text{H}_2\text{O}_2$ induced injury.

### 6.2.4 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 human cancer cells including gastric adenocarcinoma (AGS), colorectal adenocarcinoma (HT-29), bladder transitional cell carcinoma (BL13), hepatocellular (Hep G2) and one human non-transformed colon normal cell line (CCD-18Co), is presented in Table 6-3. It is shown that the raw *Nura* extracts possessed superior anti-proliferation activities on AGS and Hep G2 cells with IC$_{50}$ values lower than 0.2 mg/mL in comparison to that of raw *Rossa* extract. Inversely, the raw *Rossa* extract is more efficient in suppressing the proliferation of HT-29 cells than that of raw *Nura* extract. The roasted *Nura* extracts also demonstrated a greater anti-proliferation ability on all tested cancer cell types, except for that of BL13. The raw and roasted bean extracts from both coloured-genotypes are shown to have a negligible effect on the non-transformed CCP-18Co cells, with an IC$_{50}$ value greater than 2 mg/mL which was the highest sample concentration applied in the present study.
Table 6-3 Effects of faba bean crude extracts on the proliferation on human cancer cells: gastric adenocarcinoma (AGS), colorectal adenocarcinoma (HT-29), bladder transitional cell carcinoma (BL13), hepatocellular (Hep G2); and human non-transformed: normal colon cells (CCD-18Co)

<table>
<thead>
<tr>
<th>Faba bean genotype</th>
<th>IC₅₀ (mg/mL)</th>
<th>AGS (gastric)</th>
<th>BL13 (bladder)</th>
<th>Hep G2 (liver)</th>
<th>HT-29 (colon)</th>
<th>CCD-18Co (colon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nura</td>
<td></td>
<td>&lt; 0.2</td>
<td>1.90±0.15</td>
<td>&lt;0.2</td>
<td>1.46±0.06</td>
<td>&gt;2</td>
</tr>
<tr>
<td>Rossa</td>
<td></td>
<td>1.04±0.06</td>
<td>2.33±0.18</td>
<td>1.59±0.06</td>
<td>1.37±0.07</td>
<td>&gt;2</td>
</tr>
<tr>
<td>Roasted bean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td></td>
<td>1.64±0.05</td>
<td>1.74±0.06</td>
<td>1.55±0.34</td>
<td>1.45±0.06</td>
<td>&gt;2</td>
</tr>
<tr>
<td>Rossa</td>
<td></td>
<td>2.03±0.05</td>
<td>1.74±0.09</td>
<td>1.60±0.07</td>
<td>1.87±0.04</td>
<td>&gt;2</td>
</tr>
</tbody>
</table>

*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 0.2–2.0 mg/mL. The results were obtained via nonlinear regression and based on at least four replicate.

The crude faba bean extracts, applied at a concentration range of 0.2–2.0 mg/mL, exhibited a dose dependent suppression of the proliferations of all tested human cancer cells, while exhibited negligible effect on the proliferation of human normal colon cells. Extracts from the raw Nura slightly suppressed proliferation of non-transformed colon cells, while the raw Rossa did not (Figure 6-2a). Extracts from raw beans of both genotypes effectively suppressed proliferation of the HT-29 cells (Figure 6-2b).
Figure 6-2 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\(^*\).

\(^*\)Results were based on at least three independent experiments (mean±SD, n=3)

Extracts from the roasted *Nura* and *Rossa* did not affect the proliferation of the non-transformed CCD-18Co cells (Figure 6-3a), but effectively suppressed the proliferation of the human colon cancer cells, HT-29 (Figure 6-3b).

Figure 6-3 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\(^*\).

\(^*\)Results were based on at least three independent experiments with four replicates (mean±SD, n=4)

Heating appeared to cause decreases in phenolic content and antioxidant activity, which were in line with some but not all of the antiproliferation results. This suggests...
different types of phenolic compounds in the faba bean extracts might exert a diverse degree of activities 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 extract from the raw Nura (<0.2 mg/mL) and 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 (Xu & Chang, 2009). Seeram et al. (2004) 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 and the reason is not known. However, in support of our findings, the IC$_{50}$ values of six types of berry extracts tested on six different human tumor cell lines were all reported to be less than 0.2 mg/mL (Seeram et al., 2006).

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. The flow cytometric analysis revealed that exposure of HL-60 (human promyelocytic leukemia cells) to crude extracts obtained from the raw and roasted faba bean genotypes, Nura and Rossa induced cell apoptosis (Figure 6-4). The percentage of apoptotic cells increased with greater extract concentrations (Figure 6-4a). After the first three hr of incubation, early apoptotic events were detected. The number of apoptotic cells increased over the treatment time (Figure 6-4b), with the greatest percentage of apoptotic cells induced by both of the raw and roasted faba bean extracts over 24 hr. 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.
Figure 6-4 Apoptosis of human leukaemia cells, HL60 induced by crude extracts obtained from the raw and roasted faba bean as determined by flow cytometric analysis based on (a) different dose-response and (b) time course (extract concentration: 0.8 mg/mL)\(^*\).

\(^*\)Results were based on three independent flow cytometric analysis (mean±SD, n=3) for 3000 to 10000 cells in each population and presented as a percentage of each cell population.

The faba bean extracts were further incubated with cancer cell for different incubation periods to investigate the activation of caspase-3 activity. Activation of caspase-3 is often involved in the mediation of apoptosis that causes apoptotic
chromatin condensation and DNA fragmentation in cells and lead to loss of cell viability (Porter & Janicke, 1999; Woo et al., 1998). However, cancer cells often evade the natural cell death process, apoptosis, while the 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 (Fulda & Debatin, 2006). Food compounds that are able to induce apoptosis of cancer cells might contribute to cancer prevention. The results in Figure 6-5 showed that the caspase-3 activity was detected throughout the 3, 6, 12 and 24 hr after incubation of faba bean extracts (0.8 mg/L) on the HL-60 cells.

![Figure 6-5 Level of caspase-3 activation after treating human leukaemia cells (HL60) with faba bean extracts (0.8 mg/mL) for 3, 6, 12 and 24 hr in comparison to the non-treated control.](image)

*An asterisk represents significant difference (*p<0.05; **p<0.01; ***p<0.001) between sample and control.*

The trend of caspase-3 activity induced by the raw bean extracts from both Nura and Rossa was similar with a decrease of caspase-3 activity was observed after incubation for six hr, although the extracts from raw Nura appeared to exhibit a greater level of caspase-3 activity in comparison to extracts from the raw Rossa. After roasting, extract from the roasted Nura was shown to exhibit an increasing level of caspase-3 activity with the highest activity at the 24 hr was observed. Conversely, the highest caspase-3 activity was detected after incubating extracts from the roasted Rossa for six hr. The caspase-3 activity as early as three hr incubation time was also reported
after incubating various Australian native fruit purified extracts (0.75 mg/mL) similarly on HL-60 cells, where the maximum caspase-3 activity was detected at incubation time for six hr (Tan et al., 2011). However, Huang et al. (2007) reported a continuous increasing trend of caspase-3 activity after incubating the trypsin inhibitor from sweet potato storage roots on NB4 promyelocytic leukemia cells throughout 0-72 hr.

6.2.5 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 (Danser et al., 1995; Hansson et al., 1999). 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 activity could potentially reduce starch digestion and sugar absorption, therefore contributing to a lower postprandial hyperglycaemic response (Bischoff, 1994; Keup et al., 1977), whereas inhibition of lipase activity could reduce fat uptake contributing towards weight maintenance (Drent & Van Der Veen, 1995; Goldberg, 1996).

The raw and roasted faba bean extracts inhibited the activity of all of the investigated enzymes. Condensed tannins (proanthocyanidins) in faba bean are prone to forming complexes with proteins (Naczk et al., 2001). Therefore the observed inhibition of the various enzymes investigated in this study is likely due to the formation of proanthocyanidin-enzyme complexes (Zadernowski et al., 2001). Extracts from both of the raw and roasted Nura exhibited the greatest ACE inhibition activity, followed by extracts from Rossa and TF(Ic*As)*483/13 (Figure 6-6).
Unfortunately, roasting reduced ACE inhibition activity in the faba bean significantly, except for TF(Ic*As)*483/13. This could be attributed to changes in phenolic-protein interactions that occurred following the heat treatment (Kosinska et al., 2011; Siebert, Troukhanova & Lynn, 1996).

Roasting was found to decrease the level of α-glucosidase inhibitory activity of all investigated genotypes (Table 6-4). Among extracts obtained from both of the raw and roasted beans, Rossa exhibited the highest level of α-glucosidase inhibition activity, followed by Nura and TF(Ic*As)*483/13. Similar decreases in α-glucosidase inhibitory activities after thermal processing most of the coloured-genotypes were also reported by Ranilla et al. (2010).

Table 6-4 Effects of roasting on the α-glucosidase inhibitory activity of faba bean crude extracts

<table>
<thead>
<tr>
<th>Faba bean genotype</th>
<th>α-Glucosidase inhibition (%)</th>
<th>Raw bean</th>
<th>Roasted bean</th>
<th>Reference sample (Acarbose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nura</td>
<td>66.7a</td>
<td>33.6c</td>
<td>99.8</td>
<td></td>
</tr>
<tr>
<td>Rossa</td>
<td>69.4a</td>
<td>50.7b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TF(Ic*As)*483/13</td>
<td>61.8bc</td>
<td>26.0f</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% LSD</td>
<td>13.9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

Extracts from Rossa exhibited the strongest lipase inhibitory activity followed by Nura and TF(Ic*As)*483/13 (Table 6-5). In contrary to the results of roasting effect on the other types of enzymes, heating was found to cause an increase in level of lipase inhibitory activity in all faba bean genotypes. Similar to our results,
Zadernowski et al. (2001) also reported lipase inhibition activity in both faba bean and pea varieties.

Table 6-5 IC$_{50}$ and relative lipase inhibitory activity (mean±SD, n=3) of crude extracts from the raw and roasted faba bean genotypes

<table>
<thead>
<tr>
<th>Faba bean genotype</th>
<th>Lipase inhibition IC$_{50}$ (mg/mL)$^\dagger$</th>
<th>Relative percentage (%)$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Raw</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>23.9$^d$</td>
<td>81.4 (SE= 3.5)</td>
</tr>
<tr>
<td>Rossa</td>
<td>44.9$^b$</td>
<td>43.3 (SE= 2.0)</td>
</tr>
<tr>
<td>TF(lc*As)*483/13</td>
<td>17.6$^e$</td>
<td>105.6 (SE= 8.7)</td>
</tr>
<tr>
<td><strong>Roasted</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nura</td>
<td>28.3$^c$</td>
<td>70.3 (SE= 2.1)</td>
</tr>
<tr>
<td>Rossa</td>
<td>48.9$^a$</td>
<td>40.7 (SE= 0.9)</td>
</tr>
<tr>
<td>TF(lc*As)*483/13</td>
<td>26.6$^{cd}$</td>
<td>72.2 (SE= 2.7)</td>
</tr>
<tr>
<td>5% LSD</td>
<td></td>
<td>3.2</td>
</tr>
</tbody>
</table>

$^*$Relative lipase inhibitory activity of crude extracts from the raw and roasted faba bean genotypes. The sample concentrations were set at 40 mg/mL.

$^\dagger$The final concentration of faba bean crude extracts required to achieve inhibition of enzymatic activity by 50% under assay condition, the results were obtained using linear regression with application of sample concentrations ranged from 10-40 mg/mL.

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 suggests that ACE inhibition of faba bean extracts might be caused by the phenolic compounds. In contrary, the results of $\alpha$-glucosidase and lipase inhibitory activities did not correlate with the results of other assays. Zhang et al. (2010) also found no correlation between the TPC and $\alpha$-glucosidase inhibitory activity among extracts of seven raspberry cultivars. This suggests that phenolic content was not directly related to the $\alpha$-glucosidase and lipase inhibition activities in faba bean. Ranilla et al. (2010) 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, Pinto et al. (2008) and Mai et al. (2006) who tested Brazilian strawberries and Vietnamese edible plants and found positive relationships between the TPC and the $\alpha$-glucosidase inhibitory activity. This suggests that the relationship between phenolic compound and $\alpha$-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 bean, such as trypsin inhibitors (Helsper et al., 1993), oligosaccharides (Lattanzio et al., 1986), vicine and convicine (Brown & Roberts, 1972), lipase (Dundas, Herderson & Eskin, 1978), saponins (Amarowicz et al., 1997)
and particularly phytate which has been reported to have the ability to bind with proteins (Sharma & Sehgal, 1992), and these minor constituents might affect the results of enzyme inhibition assays. The strong bioactivities of faba bean could be a result of synergistic interaction between those constituents.
Chapter 7 Conclusions and recommendation for future studies

7.1 Conclusions
An extensive study of faba bean phenolic extracts is carried out in this study. The effect of different food processing methods including soaking, boiling, autoclaving and roasting on the functional properties of faba bean are investigated. Furthermore, the bioactivities of faba bean extracts such as anti-cancer, anti-hypertension and inhibition of α-glucosidase and lipase are characterised in vitro.

Choices of solvent extraction system have a significant effect on the measured level of extractable phenolic compounds and antioxidant activity of faba beans as demonstrated in the current study. It is shown that 70% acetone (v/v) is a more suitable solvent than 80% methanol (v/v) for the purpose of extracting the maximum amount of phenolic compounds with a high antioxidant activity in faba beans. This finding may be useful if faba bean extracts are used in the pharmaceutical or natural medicine disciplines, as yield of bioactive compounds is an important factor in the economic viability of producing such products. Faba bean genotypes with different seed coat colours are shown to exhibit inconsistent responses to the treatments using two different solvent extraction systems. In general, buff- and darker-coloured genotypes are demonstrated to have a comparable phenolic contents and antioxidant capacity, and they are higher than those of white-coloured genotype. The findings suggest that faba bean genotypes with a high phenolic content and antioxidant capacity could be considered as one of the important traits in the future breeding programs for the purpose of enhancing bioactivities. The 70% acetone (v/v) extracts are used to perform subsequent investigations on the phenolic content and antioxidant capacity of faba beans as affected by different processing methods.

Soaking, boiling and autoclaving are shown to cause losses in phenolic content and antioxidant activity of faba beans which are likely due to leaching of active compounds into the soaking and cooking medium. However, a substantial amount of phenolic compounds are also being retained in the cooked beans. Boiling is shown to be a better method than autoclaving in retaining phenolic contents of faba beans. This information suggests home cooked faba beans may contain a higher level of phenolic compounds than that of industrially processed faba beans. The results also demonstrate the importance of utilising soaking solutions and cooking broths in our diets to ensure the maximum consumption of bioactive compounds. The fact that
antioxidant capacity does not vary greatly between the highly- and buff-coloured beans as a result of processing suggests that consumption of commercially cultivated genotypes such as *Nura* provides an equivalent health benefit to consumption of the exotic coloured genotypes.

In comparison to boiling and autoclaving, roasting is the most effective cooking method in retaining antioxidant activity in faba beans. The results propose that cooking methods are available to retain the maximum health benefits in faba beans. Although the phenolic compounds in faba bean extracts are altered and the phenolic content is generally lowered as a result of dry heat treatment on faba beans, there is generation of new compounds contributing to potent antioxidant activity. This is valuable information because roasting has not just improved the desirable flavours and aroma of roasted faba beans, the evidence of an increased antioxidant activity in faba beans after roasting for a prolonged period further encourages a wider utilisation of faba beans in baked/roasted products. Consumers and industrial processors should be encouraged to adopt dry heat treatments in processing faba beans and their products. The buff-coloured and red-coloured faba beans are shown to contain different phenolic profiles suggesting different genotypes may potentially offer different health benefits.

Extracts from the raw and roasted faba beans are demonstrated to exhibit a range of bioactivities. These bioactivities include anti-oxidant, anti-cancer, and anti-hypertension activities, as well as potential anti-obesity properties through the ability to inhibit digestion of glucose and lipids through inhibition of digestive enzymes α-glucosidase and lipase. Although the roasted faba beans exhibit a lower level of antioxidant activity than those of unprocessed beans, extracts from the roasted beans retain a significant amount of biological activities. This demonstrates that cooked faba bean can be a useful source of bioactive compounds. The newly discovered biological activities of faba bean have the potential to increase the consumption of this commodity both domestically and internationally.

Consumers are becoming increasingly health conscious, knowledge on the wide ranging potential health properties of faba beans is likely to result in this pulse becoming more popular with consumers. It is hoped that this increase in demand will assist in boosting the prices that farmers receive for their crops, and result in more farmers including this crop in their farming system. This would provide an additional
benefit to farmers through an increased level of nitrogen fixation in their soil resulting in a more profitable and sustainable farming system.

7.2 Recommendation for future studies

The findings in the current study demonstrate the ability of faba beans to deliver a high level of biologically active compounds in our diet. The level of biologically active molecules has not been actively selected in breeding programs, suggesting that there may be a potential to increase the level of biologically active molecules through selection of genotypes with high phenolic levels. The variation in phenolic levels between the genotypes studied in this project demonstrates that there is a genetic basis to phenolic levels. Exploiting this genetic variation may result in genotypes with even greater health benefits. Further studies are also required to assess the impact of environment and agronomic practices on phenolic levels. It may be possible to boost the potential health benefits of faba beans by growing them in different environments or using different agronomic practices.

The current study reveals the potential health benefits of faba beans, however, their potential detrimental effects also deserves considerations. It is possible that faba beans may have anti-nutritional effects, for instance, tannins in faba bean are known to have antinutritional properties, because of their ability to reduce digestibility of nutrients, particularly protein (Jansman et al., 1995). Further research is also needed to demonstrate the bioavailability of phenolic compounds, because the health benefits of these compounds can only be realized if they are available to our body (D'Archivio et al., 2010; Kushi, Meyer & Jacobs, 1999; Rice & Whitehead, 2006). The limitations that need to be addressed include the quantification of doses required to have a physiological effect, as well as confirming that the in vitro results can predict in vivo activity. This task could be achieved by (1) identifying the phenolic compounds in faba beans which are providing the health benefits and their metabolic products and (2) examine the effects of these phenolic compounds on human systems through clinical studies. In addition, the role of food matrix influences, food processing and storage effects, as well as interaction of polyphenols with gut microbiota need to be assessed (Porrini & Riso, 2008; Scalbert et al., 2002).

The anti-cancer potential of faba bean extracts revealed by this study warrants further studies in determination of cancer cell pro-apoptotic mechanism induced by faba bean extracts. Future studies could concentrate on the effect of faba bean extracts in modulating detoxification of endogenous reactive oxygen species, particularly in
relation to phase II enzymes that are strongly linked to regulation of inflammatory processes. This would provide more direct and convincing evidence on faba bean pro-apoptotic activity.

This study demonstrates that faba bean extracts exhibit inhibition activities on ACE, α-glucosidase and pancreatic lipase in vitro, which suggests consumption of faba beans or faba bean extracts could potentially contribute to prevention of hypertension and obesity. To confirm this hypothesis, in vivo clinical studies using animal and human models are needed to fully understand the biological processes that result from faba bean consumption. If these trials provide positive results, further work would be needed to understand dose requirements and may also lead to the development of new therapeutic drugs.

The attempt to characterize the individual phenolic compounds is not successful due to the complex nature of the faba bean extracts. The study designs could be improved by utilizing normal phase-HPLC (Rigaud et al., 1993), HCl-butanol (Takahata et al., 2001), thiolysis-HPLC (Guyot, Marnet & Drilleau, 2001) and electrospray ionization mass spectrometry (Guyot et al., 1997) to determine the identity of bioactive compounds in faba beans.

This research suggests the potential value in supplementing faba bean flours in food products, particularly those are subjected to dry heating such as baking or roasting. It is, however, important to study the end product quality and sensory attributes as affected by incorporating faba beans or their extracts in the products.

It needs to be emphasized that the health benefits of faba beans demonstrated in the current study may not be solely due to phenolic compounds present in the extracts. It is possible that other minor constituents in the extract such as phytic acids, oligosaccharides and enzyme inhibitors may contribute to the observed bioactivities.

The overall health benefits of faba bean observed in this study could be due to synergistic effects of phenolic compounds and other minor constituents. Further study may include purification and isolation of selected minor constituents and phenolic compounds. This approach could elucidate their individual and combined effect in contributing to biological activity of the extract. However, as the purification process often modifies the true nature and bioactivity of the individual compound, we may never be able to develop a clear understanding of what compounds in the faba bean extracts are providing the biological activities.
References


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coronary heart disease and cancer in the seven countries study. *Archives of Internal Medicine, 155*, 381-386.


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Appendix 1 HPLC-PCD profiles of methanol versus acetone extracts from faba bean genotypes differing in seed coat colours

Figure 1 Methanol (bottom) versus acetone (top) extracts from buff-coloured faba bean genotype, 974*(611*974)/42
Figure 2 Methanol (bottom) versus acetone (top) extracts from buff-coloured faba bean genotype, 1269*483/6-1
Figure 3 Methanol (bottom) versus acetone (top) extracts from buff-coloured faba bean genotype, 1323/3
Figure 4 Methanol (bottom) versus acetone (top) extracts from buff-coloured faba bean genotype, *Doza*
Figure 5 Methanol (bottom) versus acetone (top) extracts from buff-coloured faba bean genotype, *Farah*.
Figure 6 Methanol (bottom) versus acetone (top) extracts from buff-coloured faba bean genotype, *Fiord*
Figure 7 Methanol (bottom) versus acetone (top) extracts from buff-coloured faba bean genotype, *S95007/1*
Figure 8 Methanol (bottom) versus acetone (top) extracts from purple-coloured faba bean genotype, *Deep Purple*
Appendix 2 Ranking orders of material (extraction) yields, TPC, TFC, DPPH radical scavenging activity, TEAC and FRAP of methanol versus acetone extracts of 12 faba bean genotypes

Figure 1. Ranking of methanol (blue-coloured bars) and acetone (orange-coloured bars) of material (extraction) yields (MYLD), total phenolic content (TPC), total flavonoid content (TFC), total proanthocyanidin content (Tpro), ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH) and total antioxidant capacity (TEAC) of 12 Australian grown faba bean genotypes
Figure 2. Ranking of methanol (blue-coloured bars) and acetone (orange-coloured bars) of material (extraction) yields (MYLD), total phenolic content (TPC), total flavonoid content (TFC), total proanthocyanidin content (Tpro), ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH) and total antioxidant capacity (TEAC) of 12 Australian grown faba bean genotypes.
Appendix 3 Scatterplots of correlations between various assays in Chapter 3
Figure 1 Scatter plots of correlations between TPC, TFC, TPro, DPPH radical scavenging activity, TEAC and FRAP assays (A= acetone extracts; M= Methanol extracts) (Faba bean genotype: 1-1269*483/6-1; 2-1323/3; 3-974*(611*974)/42; 4-Deep Purple; 5-Doza; 6-Farah; 7-Fiord; 8-Icarus; 9-Nura; 10-Rossa; 11-S95007/1; 12-TF(Ic*As)*483/13)
Appendix 4 HPLC-PCD profiles of extracts and cooking broths from soaked, boiled and autoclaved faba bean genotypes differing in seed coat colours

Figure 1 HPLC-PCD profiles of acetone extracts from soaked beans of (A) buff-coloured Doza; (B) green-coloured Icarus; (C) red-coloured Rossa and (D) white-coloured TF(Ic*As)*483/13
Figure 2 HPLC-PCD profiles of acetone extracts from boiled beans of (A) buff-coloured Doza; (B) green-coloured Icarus; (C) red-coloured Rossa and (D) white-coloured TF(Ic*As)*483/13
Figure 3 HPLC-PCD profiles of cooking broths following boiling processes on (A) buff-coloured Doza; (B) green-coloured Icarus; (C) red-coloured Rossa and (D) white-coloured TF(Ic*As)*483/13
Figure 4 HPLC-PCD profiles of acetone extracts from autoclaved beans of (A) buff-coloured Doza; (B) green-coloured Icarus; (C) red-coloured Rossa and (D) white-coloured TF(Ic*As)*483/13

Figure 5 HPLC-PCD profiles of cooking broths following autoclaving processes on (A) buff-coloured Doza; (B) green-coloured Icarus; (C) red-coloured Rossa and (D) white-coloured TF(Ic*As)*483/13
Appendix 5 Scatterplots of correlations between various assays in Chapter 4
Figure 1 Scatterplots of correlations between TPC, TFC, TPro, DPPH, TEAC, ORAC and FRAP assays in Chapter 4 (U=unprocessed, S=soaked, B=boiled, A=autoclaving) (D= Doza, I= Icarus, N=Nura; R= Rossa; T= TF(Le*As)*483/13)
Figure 2 Scatterplots of correlations between TPC, TFC, TPro, DPPH, TEAC, ORAC and FRAP assays in Chapter 4 with exclusion of unprocessed beans. (S=soaked, B=boiled, A=autoclaving) (N=Nura; R=Rossa; T=TF(Ic^*As)^*483/13)
Appendix 6 HPLC and spectra diagrams for identification of phenolic acids and flavonoids in faba bean extracts

Figure 1 HPLC profile of alkaline hydrolysed Nura extract
Figure 1 HPLC diagram of alkaline hydrolysed *Nura* extract spiked with phenolic acid standards
Figure 2 HPLC diagram of alkaline hydrolysed *Nura* extract spiked with flavonoid standards
Table 1 Comparison of spectra obtained from HPLC standards versus hydrolysed *Nura* extract

<table>
<thead>
<tr>
<th>Standard</th>
<th>Spectrum of standard</th>
<th>Spectrum of hydrolysed <em>Nura</em> extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td><img src="image1" alt="Spectrum at 9.76 min." /></td>
<td><img src="image2" alt="Spectrum at 9.71 min." /></td>
</tr>
<tr>
<td></td>
<td><img src="image3" alt="Spectrum at 8.99 min." /></td>
<td><img src="image4" alt="Spectrum at 12.90 min." /></td>
</tr>
<tr>
<td>Protochatechuic acid</td>
<td><img src="image5" alt="Spectrum at 9.76 min." /></td>
<td><img src="image6" alt="Spectrum at 9.71 min." /></td>
</tr>
<tr>
<td></td>
<td><img src="image7" alt="Spectrum at 8.99 min." /></td>
<td><img src="image8" alt="Spectrum at 12.90 min." /></td>
</tr>
</tbody>
</table>
Chlorogenic acid

Caffeic acid
**p-Coumaric acid**

- Spectrum at time 18.74 min.
- Spectrum at time 23.57 min.
- Spectrum at time 21.01 min.
- Spectrum at time 25.17 min.

**Ferulic acid**

- Spectrum at time 21.01 min.
- Spectrum at time 25.17 min.
Ellagic acid

Spectrum at time 26.63 min.

Spectrum at time 26.54 min.

Spectrum at time 22.79 min.

Spectrum at time 28.97 min.

o-Coumaric acid

Spectrum at time 22.70 min.

Spectrum at time 26.97 min.

Spectrum at time 28.87 min.
(+-)catechin

(-)epicatechin
Appendix 7 Anti-proliferation activity of faba bean extracts on cancer cell lines

Figure 1 Antiproliferation activity of acetone extracts from raw (top) and roasted (bottom) faba beans on human gastric adenocarcinoma (AGS) cells

Figure 2 Antiproliferation activity of acetone extracts from raw (top) and roasted (bottom) faba beans on human bladder transitional cell carcinoma (BL13) cells
Figure 3 Antiproliferation activity of acetone extracts from raw (top) and roasted (bottom) faba beans on human hepatocellular carcinoma (Hep G2) cells

Figure 4 Antiproliferation activity of acetone extracts from raw (top) and roasted (bottom) faba beans on human colorectal adenocarcinoma (HT-29) cells

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Figure 5 Antiproliferation activity of acetone extracts raw (top) and roasted (bottom) faba beans on Abelson murine leukemia virus-induced tumor (macrophage) (RAW264.7) cells