Potential health properties of selected commercially grown native Australian herbs and fruits

Author:
Sakulnarmrat, Karunrat

Publication Date:
2012

DOI:
https://doi.org/10.26190/unsworks/15870

POTENTIAL HEALTH PROPERTIES OF SELECTED COMMERCIALLY GROWN NATIVE AUSTRALIAN HERBS AND FRUITS

A thesis submitted for the degree of

Doctor of Philosophy

By

Karunrat Sakulnarmrat

Food Science & Technology

UNSW, Australia

UNSW

THE UNIVERSITY OF NEW SOUTH WALES

Food Science and Technology

School of Chemical Engineering

Faculty of Engineering

The University of New South Wales

August 2012

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ACKNOWLEDGEMENTS

First of all, my great appreciation and admiration is directed to Dr. Izabela Konczak, my main supervisor at Food and Nutritional Science Australia, CSIRO for her indescribable guidance, endless support and encouragement for my work and her patience during my learning process as well as excellent facilities and good opportunity provided by CSIRO for producing all the results.

Thank you to Dr. George Srzednicki, my supervisor at UNSW for his advice, support and his generous assistance. My thanks to UNSW for the conference grant.

Thank you Dr. Dimitrios Zabaras for LCMS data, Dr. Michael Bull for flow cytometry analysis, Food and Nutritional Science, North Ryde as well as Dr. Thomas Phillip and Dr. Michael Fenech at Food and Nutritional Science in Adelaide for giving a chance to learn CBMNCyts experiment from his laboratory.

For their support, I would like to extend my deepest gratitude to the following organisations and people: The Royal Thai Government for providing funds to support "Strategic scholarships for food science department for nine Rajamangala Universities", which offered the opportunity for myself to study abroad.

Rajamangala University of Technology Isan (RMUTI) is gratefully acknowledged, especially sincere thanks to Dr. Viroj Limkaisang, vice president of planning and developing department and Dr. Surasak Ratree, assistant president of planning and developing department who gave me support to obtain the grants.

All my friends for the wonderful time that we have shared together during my period of studying, especially Aaron C. Tan who was wellship in setting up the experiments and who provided excellent guidance. Miss Siem Doo Siah, Miss Pimpinan Somsong, Miss Onanong Kaisoon, Miss Yu (Sabrina) Guo, Mr. Abdullah Dalar and all royal Thai students at UNSW for their friendship, kindness and support.

Thanks to friends from analytical laboratory Mahshid and Udayshika for their support and kind advice.

I would like to extend my final thanks to my beloved family for their love, support, understanding and encouragement. Thank you very much for always beside me and listen to me during the good and bad time.

LIST OF PUBLICATIONS

1. Sakulnarmrat, K. & Konczak, I. (2012). Composition of native Australian herbs polyphenolic-rich fractions and *in vitro* inhibitory activities against key enzymes relevant to metabolic syndrome, *Food Chemistry*, 134(2): 1011-1019.

2. Sakulnarmrat, K., Fenech, M., Thomas, P. & Konczak, I. (2013). Cytoprotective and pro-apoptotic activities of native Australian herbs polyphenolic-rich extracts. *Food Chemistry*, 136: 9-17.

3. Sakulnarmrat, K. & Konczak, I. (2012). Composition and inhibitory activities towards digestive enzymes of polyphenolic-rich fractions obtained from Davidson's plum and quandong. *LWT- Food Science and Technology* (submitted).

Conference presentations

1. Sakulnarmrat, K., Konczak, I., Zabaras, D. & Srzednicki, G. (2010). Anti-proliferative effect of extracts from Australian indigenous herbs against human cancer cells, *Food Innovation Asia Conference 2010: Indigenous Food Research and Development to Global Market*, June 17-18, 2010, BITEC, Bangkok, THAILAND. (Poster presentation)

2. Sakulnarmrat, K., Konczak, I. & Srzednicki, G. Inhibitory potential of native Australian herbs against key enzymes relevant to metabolic syndrome. *5th International Conference on Polyphenols and Health*. Sitges Barcelona, SPAIN, October 17-20, 2011. (Poster presentation)

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ABREVIATIONS

AA	Arachidonic acid
AAPH	2,2′-azobis (2-methylpropionamide) dihydrochloride
ABAP	2,2′-azobis (2-amidinopropane) dihydrochloride
A_C	Absorbance of control
A_{CB}	Absorbance of control blank
A_C-DEVD-pNA	Acetyl-Asp-Glu-Val-Asp p-nitroanilide
ACE	Angiotensin converting enzyme
AGS	Human gastric adenocarcinoma
AM	Anise myrtle
A_S	Absorbance of sample
A_{SB}	Absorbance of sample blank
ATCC	American Type Culture Collection
BL13	Human bladder transitional cell carcinoma
ANOVA	Analysis of variance
AUC	Area under the curve
BL	Bay leaf
BN	Binucleated cell
BSA	Bovine serum albumin
CAA	Cellular antioxidant activity
CAT	Catechin hydrate
CAT E	Catechin hydrate equivalent
CBMNCyt	Cytokinesis-block micronucleus cytome
CCD-18Co	Human colon normal
CHA	Chlorogenic acid
CHA E	Chlorogenic acid equivalent
C3-G E	Cyanidin 3-glucoside equivalent
COX	Cyclooxygenase
DAD	Diode array detector
DAG	Diacylglycerol
DCFH-DA	2′,7′-dichlorofluorescin diacetate

DL	the weight of the extracted sample
DMAC	4-Dimethylaminocinnamaldehyde
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DP	Davidson's plum
DTT	1,4-Dithiothreitol
DW	Dry weight
EA	Ellagic acid
EA E	Ellagic acid equivalent
EC_{50}	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
EG	Ellagic acid glycosides
EGCG	(-)-epigallocatechin gallate
EMEM	Eagle's minimum essential medium
ET	Ellagitannins
FBS	Foetal bovine serum
F-C	Folin-Ciocalteu
Fe^{2+} E	Fe^{2+} equivalents
FRAP	Ferric reducing antioxidant power
FS	Fluorescence reading of sample
F12-K	Ham's nutrient mixture medium
GA E	Gallic acid equivalents
GRAS	Generally recognised as safe
HBSS	Hanks' Balanced Salt Solution
HepG2	Hepatacellular carcinoma
HL-60	Human acute promyolocytic leukaemia
HPLC	High performance liquid chromatography
HPLC-DAD	High performance liquid chromatography-diode array detector
HRP	Horse radish peroxidise
HT-29	Human colorectal adenocarcinoma
IAP	Inhibition of apoptosis protein
IC_{50}	Half maximal inhibitory concentration

i.d.	Inside diameter
IMDM	Iscove's modified Dulbecco's medium
iNOS	Inducible nitric oxide synthase
Keap1	Kelch-like ECH-associated protein 1
LC	Liquid chromatography
LF	the weight of lyophilised fraction
LM	Lemon myrtle
LPS	Lipopolysaccharide
M	Myricetin
MAPK	Mitogen activated protein kinase
McCoy's 5a	McCoy's 5a modified medium
MNi	Micronuclei
4-MUO	4-methylumbelliferyl oleate
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
m/z	Mass-to-charge ratio
NBud	Nuclear bud
NDI	Nuclear division index
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NPB	Nucleoplasmic bridge
ORAC	Oxygen radical absorbance capacity
P38	Protein 38
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PGE_2	Prostaglandin E_2
Pl3K	Phophatidylinositol 3-kinase
PKC	Protein kinase C
pNA	p-nitroaniline
Q	Quercetin
QE	Quercetin equivalent
Q3G	Quercetin 3-glucoside
Q3R	Quercetin 3-rutinoside
Q3R E	Quercetin 3-rutinoside equivalent

QD	Quandong
REB	Rabbit eye blueberry
R E	Rutin equivalents
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
RONS	Reactive oxygen nitrogen species
ROS	Reactive oxygen species
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SHB	Southern highbush blueberry
TBS/T	Tris buffered saline containing 0.1% Tween-20
TFA	Trifluoreoacetic acid
TP	Total phenolic
TPL	Tasmannia pepper leaf
TPTZ	2,4,6-Tripyridyl-s-triazin
TRC	Total reducing capacity
Vis	Visible

Symbols

-	Anion
+	Cation
•	Radical
M	Molarity
N	Normality
n	Nano
p	Critical p-value or significance level
r	Correlation coefficient
λ_{ex}	Fluorescein excitation wavelength
λ_{em}	Fluorescein emission wavelength

ABSTRACT

The population of overweight and obese people, including children and adults, has increased markedly during the past several decades. Obesity has been recognised as a prerequisite condition associated with increased risk of cancers such as colon and rectum, kidney, pancreas, thyroid, gallbladder, and possibly other cancer types. Population suffering from obesity have also a higher risk of coronary heart disease, stroke, high blood pressure, diabetes, and a number of other chronic diseases. A high intake of fruits, herbs and vegetables is associated with a reduction of cardiovascular incidences.

Numerous studies have confirmed that phytochemicals within plant foods are proposed are responsible for much of their health-protective effects. An ethnobotanical approach, with a systematic evaluation of traditional uses of indigenous plant foods, represents an effective method of selecting candidates for research. Indigenous plants have long history of use and recently they are promoted to commercial production nationally and internationally. Native Australian plants provide a possible source of novel health-promoting phytochemicals due to an extensive history of the uses of locally available plants by the Australian Aboriginal population, the unique characteristics of the Australian environment and, finally, the recent scientific evidences of their biological activities.

The aim of the present research was to evaluate potential health-promoting properties of three selected commercially grown native Australian herbs: Tasmannia pepper leaf (*Tasmannia lanceolata,* Winteraceae), anise myrtle (*Syzygium anisatum,* Myrtaceae) and lemon myrtle (*Backhousia citriodora*, Myrtaceae) and two fruits: Davidson's plum (*Davidsonia pruriens* F. Muell*.,* Cunoniaceae) and quandong (*Santalum acuminatum*, A.D.C., Santalaceae). Bay leaf (*Laurus Nobilis*, Lauraceae) as well as rabbit eye (*Vaccinium ashei*) and southern highbush (*V.darrowii* x *V. corymbosum*) blueberries were also included as reference samples.

Polyphenolic-rich extracts obtained from these plant sources were characterised with regards to their composition and antioxidant capacitates using an array of both, reagent-

based and cellular-based assays. Total phenolics (TP), ferric ion reducing antioxidant power (FRAP) assay and oxygen radical absorbance capacity (ORAC) assay showed that Tasmannia pepper leaf extract had 3-times higher level of total phenolics (911.9 ± 58 mg GA E/gDW) and was followed by anise myrtle and lemon myrtle (2.3- and 2.0-times, respectively greater than that of bay leaf). Tasmannia pepper leaf extract exhibited an outstanding ORAC values (12789 ± 996 µMol Trolox E/gDW), and also displayed the greatest antioxidant activity in the cellular antioxidant activity (CAA) assay (154.6 ± 1.17 µmol Q E/gDW). Tasmannia pepper leaf extract contained the highest level of total flavonoids (255.9 ± 3.3 mg Cat E/gDW). Ellagic acid and derivatives were the dominant compounds of anise myrtle (67.0%) and lemon myrtle (46.2%) fractions, accompanied by flavonoids (catechin, myricetin, hesperetin, quercetin). Tasmannia pepper leaf extract comprised of chlorogenic acid (28.8%) coupled with quercetin (11.39%). Tasmannia pepper leaf extract, inhibited efficiently the activity of α-glucosidase (IC_{50}: 0.83 mg/mL) and pancreatic lipase (IC_{50}: 0.60 mg/mL). Anise myrtle and lemon myrtle extracts had a pronounced α-glucosidase inhibitory activities (IC_{50}: 0.30 and 0.13 mg/mL, respectively) and were less effective against lipase. Tasmannia pepper leaf applied at a concentration of 1.0 mg/mL inhibited the activity of angiotensin converting enzyme (ACE) in 29.6%, and was closely followed by anise myrtle extract (25.6%).

Davidson's plum extract contained predominantly ellagic acid and ellagitannins (18.1%) accompanied by flavonoids (myricetin, quercetin, rutin, and anthocyanins) and had the highest level of total phenolic (949 ± 239 mg GA E/gDW). Davidson's plum extract also exhibited superior antioxidant capacity (FRAP: 9258 ± 926 µmol Fe^{+2}/gDWand ORAC: 8791.5 ± 370 µMol Trolox E/gDW) to quandong, rabbit eye and southern highbush blueberry. It had similar inhibitory activities against isolated α-glucosidase enzyme (IC_{50} of 0.13 mg/mL) to rabbit eye and southern highbush blueberry (IC_{50} = 0.097 and 0.091 mg/mL, respectively) and was the most efficient inhibitor of angiotensin converting-enzyme (ACE 91.2% inhibition at extract concentration of 1 mg/mL). Quandong extract comprised hydroxycinnamic acids, quercetin and cyanidin 3-glucoside, and was the most effective against pancreatic lipase (IC_{50}: 0.60 mg/mL). Various levels of correlation between the level of isolated digestive enzyme-inhibitory activities and total phenolics and antioxidant capacities were identified, indicating a specificity of individual phenolic compounds present in the isolated fractions to complex with proteins.

Purified polyphenolic-rich extracts obtained from herbs and fruits were further evaluated for potential chemopreventive effects using a wide range of assays to assess cytoprotective, anti-proliferative, pro-apoptotic and anti-inflammatory activities. The evaluation of cytoprotective and anti-proliferative activities was conducted using a variety of cancer (HT-29; colorectal adenocarcinoma, AGS: gastric adenocarcinoma, HepG2: hepatocellular carcinoma and BL-13; bladder) and equivalent normal (CCD-18Co; colon normal and Hs738.St/Int; mixed stomach and intestine normal) cell lines. All herb and fruit extracts reduced the proliferation of cancer cell, with anise myrtle exhibiting an outstanding anti-proliferative effect. No significant reduction of cell viability of non-transformed cells (CCD-18Co and Hs 738.St/Int) was observed. The suppression of the proliferation of cancer cells was due to induction of apoptosis, as identified based on flow cytometry analysis of acute promyelocytic leukaemia cancer (HL-60) cells. This was confirmed by identified induction of caspase-3 activity. The results of the CBMNCyt assay suggested no direct DNA damage in HT-29 cells as a result of treatment with all extracts, applied at final concentrations of 0.5 and 1.0 mg/mL. The results of this study revealed a number of potential health-promoting properties of phytochemicals obtained from native Australian herbs and fruits.

Finally, anti-inflammatory activity of polyphenolic-rich extracts has been assessed by measuring their effect on the production of nitric oxide (NO•) concentration and prostaglandins E_2 (PGE_2) in LPS activated hepatocellular carcinoma (HepG2) model. With the exception of lemon myrtle, all evaluated extracts inhibited production of nitric oxide (NO•). All extracts inhibited the release of prostaglandin E_2 (PGE_2). Tasmannia pepper leaf was the most effective in inhibiting nitric oxide (NO•) release at 400 µg/mL and Davidson's plum showed the greatest effect in inhibition of prostaglandin E_2 (PGE_2) production. These results suggested that herbs and fruits extracts down-regulated the key enzymes vJ involved inflammatory process: inducible nitric synthase (iNOS) and cyclooxygenase (COX-2).

In summary, this is the first study which revealed valuable health attributes of the selected commercially grown native Australian herbs and fruits. Future studies should be conducted to confirm these results at *in vivo* levels. If confirmed, these results would

suggest potential application of these plants as a novel source for nutraceutical/food industry.

Chapter 1

Introduction

Epidemiological studies conducted over the last decade reported a clear link between the quality of consumed food and the risk of life style-related diseases, such as type 2 diabetes, high blood pressure, heart attack and colorectal cancer. For example, only 2 to 5 percent of all colon cancers are hereditary, with the remaining 95 to 98% being preventable (Jasperson *et al.*, 2010). Individual behaviour such as choice of consumed food and physical activities are indicated as the main approaches that could help to maintain good health and to prevent chronic diseases. Currently, application of natural products for prevention of chronic conditions received a significant attention of health-conscious consumers. Especially this is valid for physiologically active phytochemicals present in common foods that contribute to their various health benefits and are characterised with a noticeable lack of side effects, which frequently is the limiting factor of chemotherapeutic agents (Manson *et al*., 2005). As a result, World Cancer Research Fund has promoted a series of recommendations presented in its Second Expert Report, suggesting an increase in daily intake of various fruits and vegetables to at least five servings per day (World Cancer Research Fund, 2007). It is estimated that individual who consume daily several serving of fruits and vegetable would receive approximately 1 gram per day of total polyphenol (Manach *et al.*, 2004).

Over the last decade obesity has emerged as one of the major problems in public health that leads towards the development and promoting of an array of diseases such as metabolic syndrome, inflammation and cancers (Lavie *et al.*, 2009). Obesity is also considered as a prerequisite condition of other chronic diseases such as cancers, which in 2008 were found to cause death of 12.4 million worldwide. Currently, in Australia, cancer is ranked as the first cause of mortality with more than 43,000 death estimated in 2010. In addition, it has been estimated that the number of new patients suffering from this condition in 2010 was about 114,000 cases (Cancer Council Australia, 2012).

Multiple epidemiological studies reported that sufficient consumption of fruits and vegetables is associated with improved health and decreased risk of cancer incidents (Surh, 2003; Kraft *et al.*, 2008). Phenolic compounds naturally abundant in plants possess antioxidant activity and play a significant role in preserving intracellular oxidative balance (Babich *et al.*, 2011). They also act as antioxidant, anti-inflammatory, anti-diabetic and cancer-preventative agents (Tsuda, 2008; Barbosa *et al.*, 2011). The evidences suggested that multifunctional polyphenols could be considered as an appropriate target for the development of nutraceuticals/food supplements for the prevention/suppression of metabolic syndrome related chronic diseases.

The rich Australian flora comprises approximately 24,000 species of native plants with about 5000 edible and 5000 medicinal species (Cooper, 2004), and represents a vast and untapped natural resource that has a potential to be intensively utilised by the food and pharmaceutical industries. The edible fruits, herbs and spices served as a sole source of food and medicine to the indigenous population for thousands of years (Roberts *et al.*, 1990). In 1990, selected edible native Australian plants were introduced to commercial production (Ahmed & Johnson, 2000). Currently they deliver a number of products with a total value of about $AUD 14 million annually (excluding macadamia nut, with production value of about $AUD 150 million annually), as estimated by the Australian Native Foods Industry Ltd. (ANFIL, 2012).

Native Australian fruits, herbs and spices have already been introduced into supermarkets and speciality shops in Australia, and are becoming available to the wider community and are used in restaurants that promote a unique Australian cuisine. Some studies have reported that edible native plants possess unique sensory properties (Hodgson & Wahlqvist, 1993), potentially indicating presence of a rich mixture of phytochemicals. Other studies have reported that native Australian plants are rich sources of polyphenols that contribute to a high antioxidant capacity (Konczak *et al.*, 2008; Konczak *et al.,* 2010a; Konczak *et al.*, 2010b; Tan *et al.,* 2011a). It can be speculated that incorporation of native fruits and herbs into daily diet could have a positive impact on human health.

To date information on the potential health promoting properties of native Australian plants is insufficient. Therefore, more studies need to be conducted to provide systematic

information on their composition and health enhancing properties. The mechanisms of their activities need to be elucidated through physiological studies, which subsequently may help to bring forward their utilisation as alternative health promoting foods and pharmaceutical constituents, stimulating national and international economy.

Polyphenols are plant secondary metabolites ubiquitously found in commonly consumed fruits and vegetables, comprising more than 10,000 compounds (Haslam, 1998). These non-essential compounds are known to modulate biological processes, and provide beneficial health effects. They have been recognised to be responsible for the protective properties of fruits, vegetables, herbs and spices in age-related chronic diseases (e.g. cancer and cardiovascular disease), (Kroon & Williamson, 2005). Polyphenols are primarily recognised for their excellent antioxidant properties. A number of other biological activities of polyphenols include anti-inflammatory, antibacterial, antiviral activities and cancer prevention (Scalbert *et al.*, 2005). Phenolic compounds represent a particularly rich family of phytochemicals with physiological activities these are widely investigated. It has been reported that phenolic compounds play an important role as antioxidants which effectively reduce oxidative stress, linked to their chemopreventive activity (Scalbert *et al.*, 2005). Accordingly, polyphenol-rich diet arising from consumption of fruits and vegetables has been associated with the decrease of colon cancer incidences (Yang *et al.*, 2001).

Based on the available literature on the role of phenolic compounds in maintaining health, and available information on native Australian edible plants, it can be hypothesised that phenolic compounds present in the native Australian fruits and herbs may exert physiological activities. To prove/disprove this hypothesis, five plant sources selected by the industry: Tasmannia pepper leaf (*Tasmannia lanceolata*: TPL), anise myrtle (*Syzygium anisatum*: AM), lemon myrtle (*Backhousia citriodora*: LM), quandong (*Santalum acuminatum*: QD) and Davidson's plum (*Davidsonia pruriens*: DP) were evaluated in an array of reagent-based and cell culture based assays. Elucidating the potential effects behind polyphenols mechanism is an essential step towards understanding their health benefit. The assessment tool used in this study was an *in vitro* model procedure, which provided fast results, is cost efficient and allows screening of a large number of samples in a short time, which is much cheaper than human studies and

is reproducible. The cell culture based system that "mirrors" behaviour of cells within a body, previously used exclusively for drug development, is at present successfully applied to evaluate large numbers of plant-derived bioactives for their health effects.

Due to the fact that edible plant sources were selected for the current study, cell models relevant to digestive system were mostly used, including human cancer cells isolated from colon (HT-29), stomach (AGS) and bladder (BL-13). In addition, human liver hepatoma cells (HepG2) and human promyelocytic leukemia cells (HL-60) were also employed as they represent standard cell models for selected procedures. Liver is known as the central organ that plays an essential part in metabolism and detoxification process (Bleibel *et al.*, 2007). Equivalent normal cells were included in order to compare and evaluate cytotoxic effects. Further, the mechanism of apoptosis induction and anti-inflammatory properties were researched. Inhibitory activities of plant extracts against key enzymes relevant to metabolic syndrome: α-glucosidase, pancreatic lipase, angiotensin converting enzyme (ACE) were also evaluated.

The aim of this study was to investigate the potential health-promoting properties of selected commercially grown native Australian herbs and fruits. This work complements recent studies on health attributes of Kakadu plum, Illawarra plum, muntries and native currant (Tan *et al.* 2011a, 2011b, 2011c). Jointly these studies represent the first evaluation of specific health-enhancing properties of commercially grown native Australian herbs and fruits.

Chapter 2

Literature Review

2.1 Native Australian plants – source for food industry

Australian native flora represents a vast resource of attractive edible plants. Cooper (Cooper, 2004) described 2440 species of fruiting rainforest plants in tropical Queensland. Of these, 500 species extend into New South Wales, 500 into Northern Territory and up to 300 occurring also in Western Australia. Cherikoff and Isaacs (1990) identified 245 native edible species of plants from rainforest habitats (rainforest bush foods) and 231 from dryland (dryland bush foods). For the Sydney region alone the same authors have reported 208 edible species. To the indigenous people of Australia, the Aboriginals, edible native Australian fruits have served as a source of food and medicine for thousands of years (Roberts *et al.*, 1990). These fruits were reported to possess unique nutritious and organoleptic characteristics (Hodgson & Wahlqvist, 1992).

Over the last 20 years multiple projects have been undertaken to generate data on the composition of Australian Aboriginal foods and to evaluate their nutritional values. Selected foods have been evaluated predominantly for the presence of protein, fat, carbohydrate, fiber, ash, energy, minerals and vitamins (Konczak *et al.*, 2009). In recent years, native edible plants (bushfood plants) have increased in popularity. A number of commercially significant crops have been identified and research on their propagation, breeding and cultivation has been undertaken (Ahmed & Johnson, 2000). Selected native Australian fruits have already entered commercial production and are available from local growers, in supermarkets, restaurants and are sold overseas.

Among the commercially grown native Australian edible plants, two fruits: Davidson's plum (*Davidsonia pruriens* F. Muell., Cunoniaceae) and quandong (*Santalum acuminatum*, A.D.C., Santalaceae) as well as three herbs: Tasmannia pepper leaf

(*Tasmannia lanceolata,* Winteraceae), anise myrtle (*Syzygium anisatum,* Myrtaceae) and lemon myrtle (*Backhousia citriodora*, Myrtaceae) are of economical importance due to their use in commercially available products (Table 2.1). After consultations with the Australian Native Food Industries Ltd. (ANFIL) representing the native food industry, these sources have been selected for present study.

Table 2.1 Major native Australian plants and usages

Common name	Main use
Tasmannia pepper leaf	Leaf for spice
Lemon myrtle	Fresh or dried herb, tea blend and beverage, oil, dairy, biscuits, breads, confectionery, pasta, syrups, liqueurs, flavoured oils, packaged fish/salmon. Dipping sauces, simmer sauces. For use in sweet and savoury dishes.
Anise myrtle	Leaf for spice. Sweet and savoury, teas, drinks, syrups, glazes, cakes, biscuits, dressings, sauces and ice-creams
Quandong	Fruit used mainly in dried halved form, stored up to 8 years. Used in products such as jams, preserves, sauces, relishes, juices, deserts and ice creams
Davidson's plum	Used in jams, sauces, drinks, wine bakery products, mixed into yoghurt.

Source: ANFIL (2012a)

2.2 Biology and ecology of native Australian herbs and fruits selected for the study

2.2.1 Tasmannia pepper leaf

Seven species of Tasmannia pepper leaf (*Tasmannia lanceolata*, Winteraceae) were identified in Australia, all of which have pepper tasting leaves (Southwell & Brophy,

1992). *Tasmannia lanceolata* (Poir.) Baill., known as mountain pepper, is the main commercial species. The plant represents a medium shrub, up to 5 m high, with dark green leaves and distinctive crimson stems (Figure 2.1) (Dragar *et al.*, 1998). The leaves and berries have a pepper flavour, and are used in savoury dishes. Research on Tasmannia pepper leaf has focused on preparation of herbal remedies and extraction of oils (Bryant, 2005). Southwell and Brophy (1992) identified the constituents of essential oils of the seven Australian Tasmannia species, and found that the characteristic pepper flavour of the species is due to the presence of sesquiterpen polygodial which elicits a warm and pungent taste (Read, 1996). Recently, in Australia, selected restaurants specialising in native foods use the leaves and berries in preparation of dishes called the 'Australian cuisine'. Tasmannia pepper leaf and berries, from both natural and cultivated sources, are being more widely used as a flavouring agent by food industry, for example, in speciality cheese (Agboola & Radovanovic-Tesic, 2002). Leaf is used as spice blends and in baking products, cosmetic applications, flavour extract, sauces, chutneys, flavoured cheeses, olive oils and confectionary (ANFIL, 2012a).

Figure 2.1 Tasmannia pepper leaf
Source: Konczak *et al.* (2009)

2.2.2 Anise myrtle

Anise myrtle (*Syzygium anisatum*, Myrtaceae) also known as *Backhousia anisata* and *Anetholea anisata*, ringwood or aniseed tree, is a rare Australian rainforest tree that has a

dense crown and grows up to 45 m. The leaves are 6 – 12 cm long with prominently wavy margins and aniseed aroma (Figure 2.2). Flowers are white and sweetly scented, borne in panicles. The fruit are dry papery capsules 5 mm long (ANFIL, 2012). Coloured cream flowers are eucalyptus like and appear in late spring to early summer. The leaf from cultivated plantations, also known in trade as aniseed myrtle or anise myrtle, is used as a bushfood spice and is distilled for the essential oil.

The essential oil of anise myrtle contains predominately (E)–anethole (trans–anethole) and methyl chavicol with minor amounts of alpha-pinene, cineole, (Z)–anethole, alphe farnesene and anisaldehyde (Southwell *et al*., 1996). With regard to the composition of essential oils, two distinct chemotypes of anise myrtle have been identified: one rich in (e)–anethole (more than 90%) which is preferred; the other rich in methyl chavicol (60 – 75%), which is a known carcinogen (Wilkinson & Cavanagh, 2005). The leaf, used as a herb, provides an aniseed flavour to sweet and savoury dishes as well as to cosmetics (Konczak *et al.,* 2010a). In the food industry, the leaves are used ether fresh or dry ground and the price is approximately 38 Australian dollars per kilogram (Robin, 2004).

Figure 2.2 Anise myrtle
Source: Konczak *et al.* (2009)

The therapeutic properties of one of the constituents of anise myrtle, anethole, made it being used as expectorant sedative and stimulant in anti-cough medicines. Further applications are in food and beverages and fragrance industry as raw material for

cosmetics (Fenaroli, 1975). Generally, anethole is regarded as safe (GRAS: generally recognised as safe) for internal consumption as a flavouring agent. Plants containing anethole have traditionally been used to assist with weight loss, lactation and stomach complaints.

2.2.3 Lemon myrtle

Lemon myrtle (*Backhousia citriodora*, Myrtaceae) is native to the subtropical rainforests of Queensland. The leaves have a high content of citral that gives thema distinctive lemone-lime fragrance. Citral accounts for over 90% of the plants essential oil, compared to about 3% in lemon oil (Southwell *et al*., 2000). The leaves are dried or processed to extract an essential oil. The dried leaves are used as a tea, potpouri or spice (Figure 2.3); the essential oil is used as a food and beverage flavouring, air freshener, disinfectant and in a range of body care products. It flowers prolifically with large bunches of small white flowers on the ends of the branches. It is a common garden plant in Brisbane. Since the early 1990s, around 1.4 million lemon myrtle trees have been established in plantations in Australia, mainly in Queensland but also in northern New South Wales. The trees are formed into hedges that can be mechanically harvested.

Figure 2.3 Lemon myrtle
Source: Konczak *et al.* (2009)

Australian Lemon Myrtle Ltd. is known to be the leading producer which manages 1.2 million trees alone in an organically certified production system. In 2007, the plantation of lemon myrtle trees established in Australia could produce 2,100 tonnes of lemon myrtle (Foster, 2009). The farm gate price of whole fresh leaf is $36 AUD dollars per kilogram, (ANFIL, 2012b).

Australian native herbs from both natural and cultivated sources are being more widely used as a flavouring agent by food industry (Ahmed & Johnson, 2000), for example, have been used in speciality cheese (Agboola & Radovanovic-Tesic, 2002). Lemon myrtle is particularly expected to become an important commercial product due to the high contents predominantly of citral compounds in essential oil (Hayes & Markovic, 2000). The antimicrobial activity of the essential oil and other extracts of lemon myrtle have been evaluated, with respect to their potential application as topical pharmaceutical products (Burke *et al.*, 2004; Hayes & Markovic, 2002).

2.2.4 Quandong

Quandong, known by the scientific name as *Santalum acuminatum*, A.D.C., Santalaceae, occurs naturally in Western Australia and South Australia, and can also be found some area of Queensland, Victoria and the Northern Territory. Native peach, desert peach and wild peach are the common names of this fruit. The quandong tree is parasitic and growing on other trees, plantings in orchards require companion plantings. The companion plantings may also be productive, such as acacias producing wattle seeds. The quandong fruit is generally bright red in colour (Figure 2.4) and contains a large seed that accounts for around 50 percent of the total weight of the fruit in the wild but less than this in irrigated orchard plantings.

The fruit is a rich source of vitamin C and is a traditional food of indigenous Australians. It has been a staple food and exceptionally valuable commodity of some desert Aboriginal tribes (Zola & Gott, 1992). Surplus fruit was collected and dried for up to 8 years for later consumption; dried fruit was reconstituted in water when needed. Quandong was also an important food source for early European settlers (Clarke, 2007). It was eaten fresh or made into a range of food products, particularly preserves. Currently

quandong is used in jams, preserves, sauces, relishes, juices, deserts and icecream. The fruit is rather acidic and contains appreciable amounts of carbohydrate. It is high in protein compared with most fruits (ANFIL, 2012a).

Figure 2.4 Quandong fruits
Source: Konczak *et al.* (2009)

The seed is used predominantly for medicinal purposes. The distinctive, textured seeds are strung and worn as body ornaments or necklaces. The fruit of some trees have a tasty kernel that is extracted when it can be heard knocking inside the shell. These oily kernels are either eaten raw or pounded so the oil can be removed and used as a cosmetic to smooth the skin of face and body comparable to almond and apricot oils used in European cosmetics (Rivett *et al.*, 1989). The wood is used for fire (Zola & Gott, 1992). The production of quandong is sourced from both: wild harvest, particularly by indigenous Australians, and from orchards. In 2001, there were around 26,000 quandong trees in orchards. The plantings were largely irrigated (Lethbridge, 2004). The expectation was that each irrigated quandong tree would annually produce about 3 to 5 kg of mature fruit, equivalent to 0.75 – 1.2 kg of dried flesh (Loveys & Justias, 1994). It is not economically viable to supply quandongs to fresh market and instead the fruit is processed in orchards. In 2006, an estimated weight of 10 tonnes of quandongs were gathered from the wild and a further 4 tonnes were harvested from orchards (Hele *et al.*, 2006). Amongst Australian aborigines quandongs were much valued for their medicinal properties. Specialised uses of quandong included a form of tea which was drunk as a purgative. Quandong tree roots were also ground down and used as an infusion for the treatment of rheumatism.

Typically quandong leaves were crushed and mixed with saliva to produce a topical ointment for skin sores and boils. Oil rich kernels were also processed in a similar fashion to treat skin disorders. Quandong kernels could also be eaten and some tribal groups were known to employ crushed kernels as a form of hair conditioning oil. Ingeniously, Australia's aborigines appeared to be aware that Quandongs were a preferred food source of emus, and that a ready supply of quandong seeds could befound in their droppings (Exploroz, 2012).

2.2.5 Davidson's plum

Davidson's Plum (*Davidsonia pruriens*, F. Muell., Cunoniaceae), also known as *Ooray*, is found in tropical rainforests in Queensland and northern New South Wales of Australia. This rainforest tree grows to 12 m and has pinnate leaves with slightly irritant hairs and small flowers. Leaves are compound, alternate or opposite. The fruits are purple (Figure 2.5) about 5 cm long, and are found to be strongly acidic. Due to it intensive crimson colour on the outside and scarlet inside Davidson's plum is highly prized for jam (Jensen *et al*., 2011). There are two seeds though usually only one is fertile. The fruit is eaten by cassowaries, sulphur-crested cocatoos and double-eyed fig-parrots (Cooper, 2004).

Figure 2.5 Davidson's plum
Source: Konczak *et al.* (2009)

2.3 Phytochemicals from Australian native plants

Biologically active, non-nutritive components in the plant-based diet, other than traditional nutrients, that have a beneficial effect on human health have been defined as phytochemicals (Johnson & Williamson, 2003). More than thousand compounds that have a physiological effect have been identified (Boyer & Liu, 2004). Generally, plant produces these compounds to protect itself from environmental challenges such as ultraviolet radiation and pathogens and the same compounds were found to be beneficial for the protection of human health. A large number of recent studies demonstratedthat phytochemicals play an important role in protecting against major chronic diseases as well as health problems associated with ageing such as cancer, cardiovascular disease, inflammation and lipid oxidation (Surh, 2003; Liu, 2003; Lee & Lee, 2006). Their antimicrobial activity has also been scientifically validated (Khan *et al*., 2009).

According to Liu (2004) physiologically active phytochemicals have been classified into 5 groups: carotenoids, phenolic compounds, alkaloids, nitrogen containing compounds and organosulfur compounds (Figure 2.6). Some of the well-known phytochemicals are lycopene in tomatoes representing the group of carotenoids, allyl sulphides found in onions, leeks and garlic and isoflavones in soy and flavanoids in fruits representing the group of phenolic compounds (Figure 2.7) (Ayoola *et al.,* 2008). Flavonoids and phenolic acids play an important role in plant colour, taste and smell and make a major contribution to total dietary polyphenols, with 60% and 30%, respectively (Nichenametla *et al*., 2006). Flavonoids and phenolic acids were identified as powerful antioxidants (Zheng & Wang, 2001) and their ability to protect cell against oxidative damage was reported as the mechanism of the protective effects of fruits and spices (Babich *et al.*, 2011).

Recent studies identified modulatory activities of selected phytochemicals on multiple metabolic pathways. Many studies have reported that phytochemicals inhibit carcinogenesis by inhibiting phase I enzymes, and induction of phase II enzymes, scavenging reactive agents, such as reactive oxygen species (ROS), suppressing the abnormal proliferation of early, preneoplastic lesions, and inhibit the growth of various cancer cells (Manson, 2003; Wolfe *et al.*, 2003; Eberhardt *et al.*, 2000; Tan *et al.*, 2011a).

Studies on native Australian plants polyphenolic-rich extracts reported potential chemopreventative activities of Kakadu plum, muntries, Illawarra plum and native current, associated with a high level of antioxidant capacities (Tan *et al.*, 2011b). Additionally, epidemiological and clinical studies with humans have highlighted that a low risk of cancer is closely related to a diet rich in multiple antioxidants contributed by whole food rather than to a one supplement with an individual antioxidant (Liu, 2003; Lee & Lee, 2006).

2.4 Dietary polyphenols

Polyphenols are plant secondary metabolites which are moderately water-soluble, with molecular weight of 500 to 4,000 Da. More than 8,000 individual molecules of phenolic compounds have been identified (Dai & Mumper, 2010) and it is expected that currently this number is significantly higher. Generally, phenolic compounds are classified into 4 groups, based on: i) the number of phenol rings that they contain and ii) the structural elements binding the rings. As such, the compounds are differentiated between phenolic acids, flavonoids, stilbenes and lignans (Dai & Mumper, 2010).

2.4.1 Phenolic acids

Phenolic acids are the organic compounds, which contain a phenolic ring and an organic carboxylic acid function (C6–C1 skeleton). Phenolic acids can be divided into two groups: hydroxybenzoic acids and hydroxycinnamic acids. Gallic acid is the common derivatives of benzoic acid, beside *p*-hydroxybenzoic acid, protocatechuic acid, vanillic acid. Hydroxycinnamic acids are derivatives of benzoic acid and cinnamic acid with hydroxyl groups and methoxy groups substituted at various points on the aromatic ring (Marinova & Yanishlieva, 2003) (Figure 2.8) and are a major class within the phenolic compounds, widely distributed in plants kingdom (Yang *et al.*, 2001). The most common hydroxycinnamic acid derivatives are *p*-coumaric acid, caffeic acid and ferulic acid (Figure 2.9). These acids are frequently formed as simple esters and join with quinic acid or glucose in foods. In contrast, hydroxybenzoic acid is predominantly found in form of glucosides. Chlorogenic acid is the most common hydroxycinnamic acid found in most plants (Manach *et al.,* 2004).

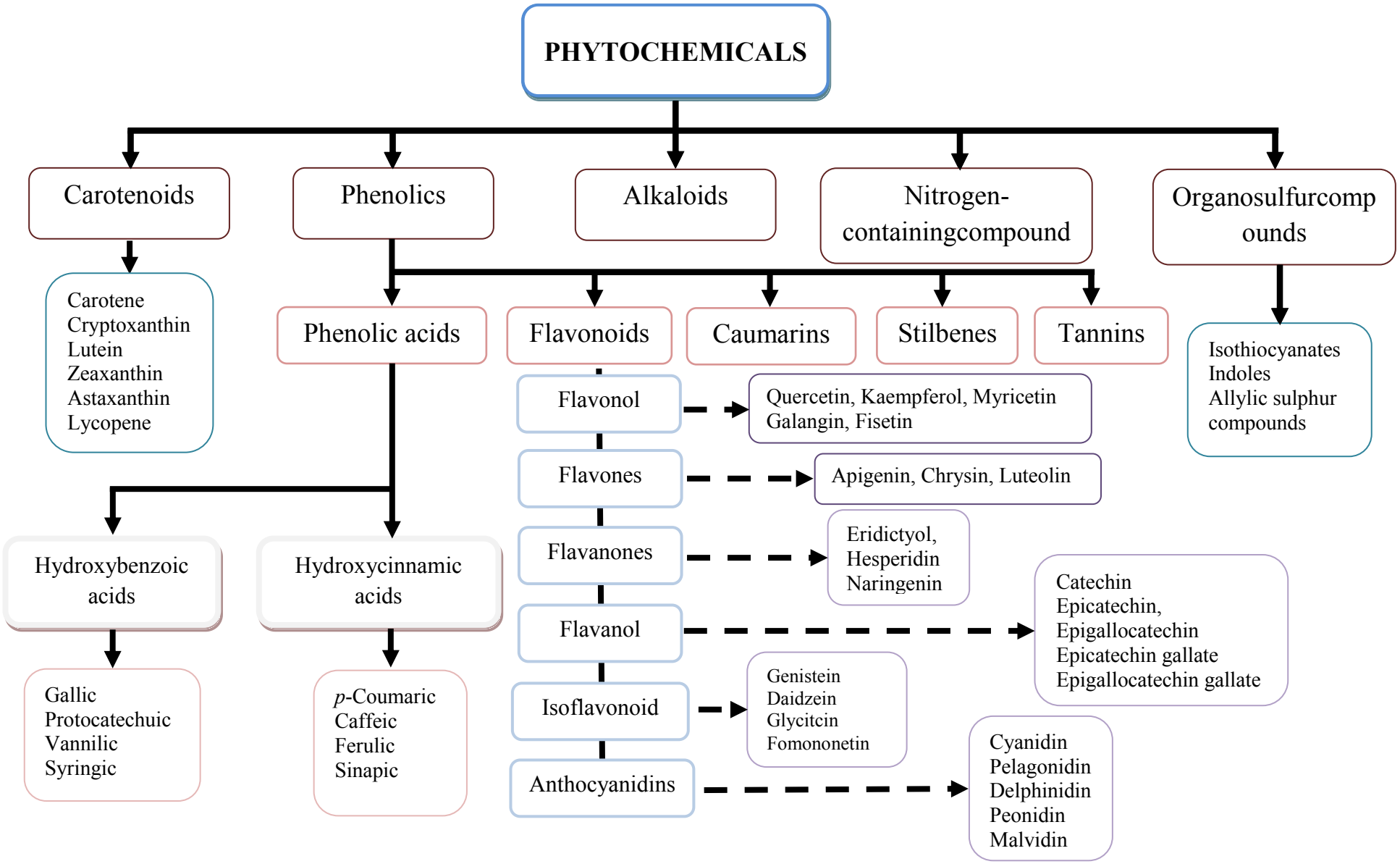


Figure 2.6 Classification of dietary phytochemicals (adapted from Liu, 2004)

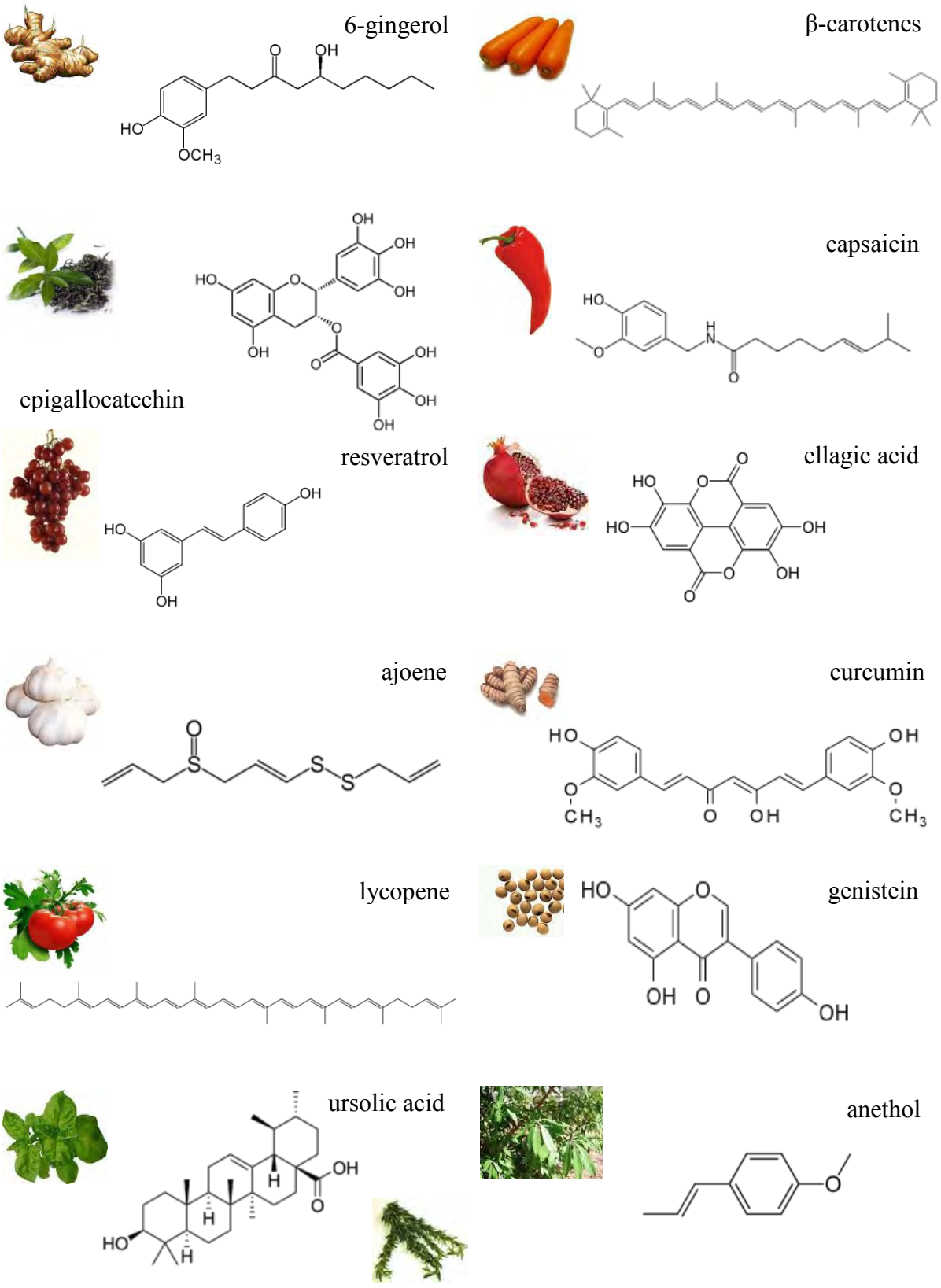
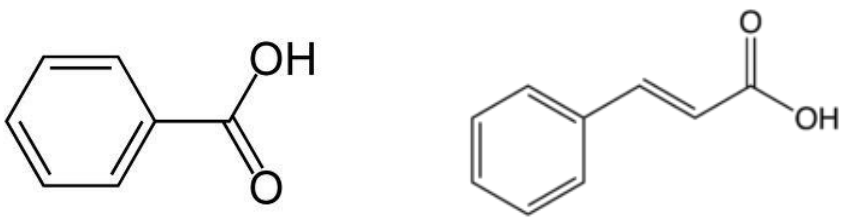


Figure 2.7 Sample of representative phytochemicals and their dietary sources (adapted from Surh, 2003)

A highly potent phenolic acid is the ellagic acid, a dilactone of hexahydroxydiphenic acid, which in turn is a dimeric condensation product of gallic acid (Tomás-Barberán & Clifford, 2000). Ellagic acid is present in many red fruits and berries, including raspberries, strawberries, blackberries, cranberries, pomegranate and some nuts (e.g. pecans and walnuts). The highest levels of ellagic acid are found in raspberries (Vattem & Shetty, 2005). In plants, ellagic acid is present in the form of ellagitannins, comprising ellagic acid bound to a sugar molecule. Ellagic acid has been reported to be a strong antioxidant, which could play an effective role in antiviral, antibacterial, anti-mutagen and anti-cancer properties of plant foods (Seeram *et al.*, 2005; Mertens-Talcott *et al.*, 2003; Sharma *et al.*, 2010; Landete, 2011).

Studies have also shown potent anti-cancer activity *in vitro* against cancer cells of breast, oesophagus, skin, colon, prostate and pancreas. More specifically, ellagic acid was documented to prevent the destruction of P53 gene, decrease in total hepatic mucosal cytochromes and an increase in some hepatic phase II enzyme activities, thereby enhancing the ability of the target tissues to detoxify the reactive intermediates. Phenolic acids have been identified as strong inhibitors of carcinogenesis at the initiation and promotion stages of tumour induced by different carcinogenic compounds (Yang *et al.*, 2001).



Benzoic acid Cinnamic acid

Figure 2.8 Benzoic acid and cinnamic acid structure

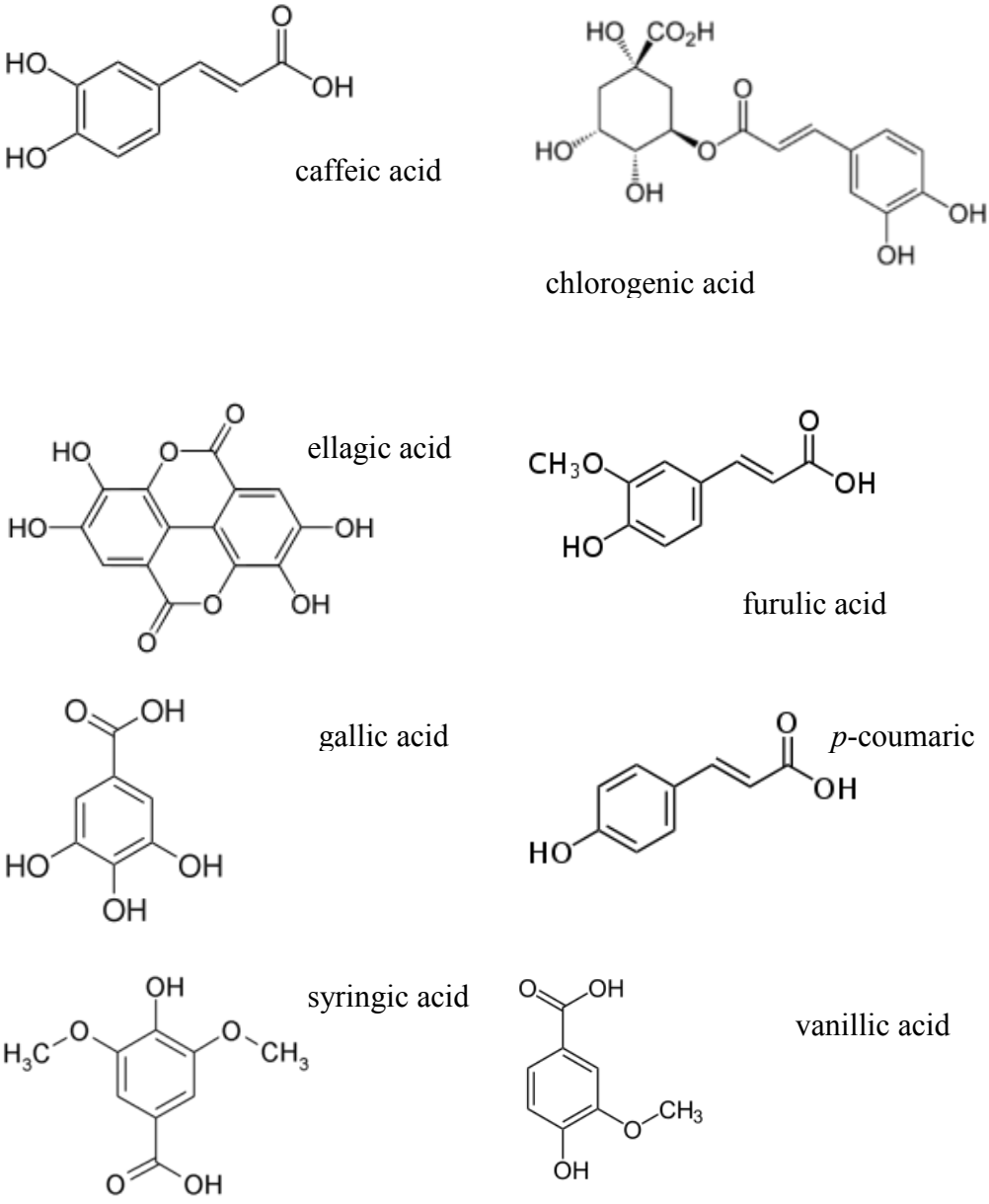


Figure 2.9 Chemical structure of main phenolic acids

2.4.2 Flavonoids

Flavonoids are water soluble phenolic molecules with more than 4,000 identified compounds (Ren *et al.*, 2003). The compounds have basic skeleton structure of phenyl benzopyrone structure (C6–C3–C6), consisting of 15 carbon atoms and can be visualized as two benzene rings (A and B rings) which are joined together with a short three carbon

chain (C ring). One of the carbons of the short chain is always connected to a carbon of one of the benzene rings, either directly or through an oxygen bridge, thereby forming a third middle ring, which can be six membered (Huang *et al.*, 2011) (Figure 2.10).

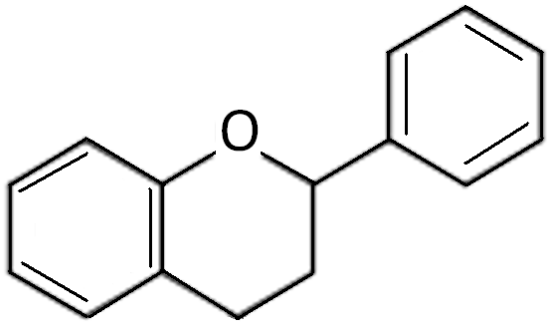


Figure 2.10 Flavonoid structure

The flavonoids comprise 6 major subgroups including chalcone, flavone, flavonol, flavanone, anthocyanins and isoflavonoids (Figure 2.11). Flavonoids are found in most plants with the most important dietary sources being fruits, vegetables, tea and soybeans. Flavonoids (beside carotenoids) are also responsible for the colouring of plants (Anderson, 2007). Green tea and black tea were recorded to contain of about 25% flavonoids (Harold & Graham, 1992). Other important sources of flavonoids are apples and onion which contain predominantly quercetin, and citrus fruits which contain rutin and hesperidin (Boyer & Liu, 2004). Daily intake of flavonoids can vary between 50 to 500 mg (Thomasset *et al*., 2007).

Flavonoids have received attention due to potent antioxidant capacities and a wide array of biochemical functions contributing towards many health promoting effects (Kroon & Williamson, 2005). They are involved in immune function including gene expression, enzyme activity and cholesterol and histamine metabolism. The beneficial health effects associated with these compounds are anti-allergic activities, reduction of coronary heart disease risk, anti-cancer, anti-inflammatory and anti-viral activities (Khan *et al.*, 2009; Kim *et al.*, 2010; Lee *et al.*, 2010; Tan *et al.*, 2011b).

Epidemiological studies have shown that heart diseases are inversely related to flavonoid intake (Hsieh & Ofori, 2007). Tea flavonoids reduce the oxidation of low-density lipoprotein, lower the blood levels of cholesterol and triglycerides. Through prevention of the oxidation of low-density lipoprotein flavonoids reduce the risk of the development of atherosclerosis (Doyon & Labrecque, 2008). Soy flavonoids known as isoflavones can reduce blood cholesterol and help to prevent osteoporosis. Soy flavonoids are also used to ease menopausal symptoms.

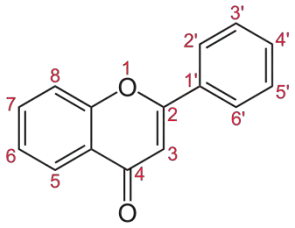
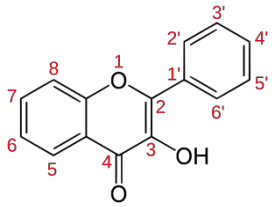
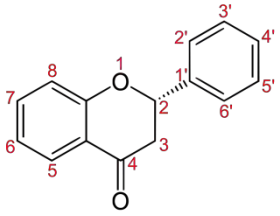
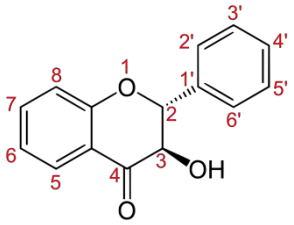
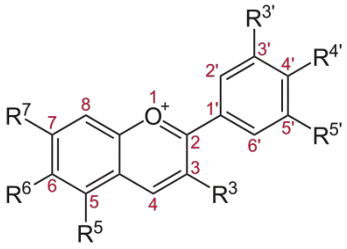
Flavone		Luteolin Apigenin Tangeritin
Flavonol		Quercetin , Kaempferol Myricetin, Fisetin Isorhamnetin, Pachypodol Rhamnazin
Flavanone		Hesperetin, Naringenin Eriodictyol, Homoeriodictyol
Flavanonol		Taxifolin
Anthocyanin		

Figure 2.11 Structure of main class of dietary flavonoids

Additionally, flavonoids have been reported to exert anti-inflammatory actions and to modulate immune function (Boots *et al.*, 2008; Tan *et al*., 2011c). For example, the well-known flavonoid quercetin possesses an ability to relieve hay fever, eczema, sinusitis and asthma (Johnson & Williamson, 2003). Quercetin in combination with other flavonoids, inhibits a number of enzymes including bradykinin (Bamard *et al*., 1993) and tyrosine kinase (Hur *et al.*, 1994). Rutin and quercetin have been shown to regulate the activity of hormones, including thyroid hormones, such as transport and metabolism (Manach *et al.*, 2003).

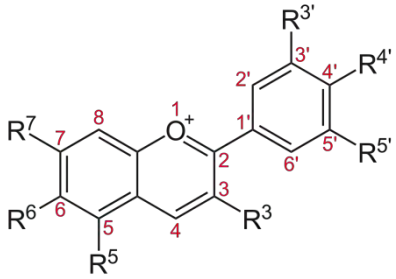
Mullen *et al.* (2008) fed rats with radiolabeled [2–^{14}C]quecetin–4′–glucoside and analysed the products of its metabolism over 24 h. On entering of cecum and colon, in particular 3–hydroxyphenylacetic acid (also detected in faces and urine) and 3, 4–dihydroxyphenylacetic acid were detected, indicating the convertion to phenolic acid by colonic microflora. Hippuric acid was also detected in urine and it decreased markedly over 24–48 h and 48–72 h period. The result shows that most of [2–^{14}C]quecetin–4′–glucosidehas been converted to phenolic acids in gastrointestinal tract (Mullen *et al.*, 2008). A high physiological activity of polyphenols found in green tea have been documented, such as their ability to inhibit a variety of processes associated with cancer cell growth, survival, and metastasis (Boyer & Liu, 2004).

2.4.3 Anthocyanins

Anthocyanins commonly exist as glycosides and acylglycosides and are responsible for the blue and red colours of fruits, for example berries, cherries, and plums, and vegetables such as red cabbage and radishes, and some grains and roots (Kris-Etherton & Keen, 2002). More than 700 molecules of various anthocyanins have been found in nature represented by 10 different anthocyanidins (Table 2.2) (Anderson, 2006). Anthocyanins are strong antioxidants and contribute to some of the beneficial effects of fruits and vegetables on human health. Many studies reported that anthocyanins have anti-inflammatory, anti-cancer and chemo-protective properties (Middleton *et al.*, 2000: Tan *et al*., 2011b; Tan *et al*., 2011c; Ravoori *et al.*, 2012).

Blueberries are a well know source of anthocyanins. Blueberry extract possess a high antioxidant capacity (Faria *et al*., 2005), suppresses the proliferation of cancer cells (Martin *et al*., 2003), angiogenesis (Bagchi *et al.,* 2004) and induces the apoptosis of cancer cell (Lazze *et al*., 2004). Tan *et al.* (2011a) reported that anthocyanins found in native Australian fruit Illawarra plum and native currant inhibited the COX2 and iNOS enzymes, which indicates their potential anti-inflammatory activities.

Table 2.2 Structure of some selected anthocyanidins and their substitutions



Anthocyanidin	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Aurantinidin	−H	−OH	−H	−OH	−OH	−OH	−OH
Cyanidin	−OH	−OH	−H	−OH	−OH	−H	−OH
Delphinidin		−OH	−OH	−OH	−OH	−H	−OH
Europinidin		−OH	−OH	−OH	$-OCH_3$	−H	−OH
Luteolinidin	−OH	−OH	−H	−H	−OH	−H	−OH
Pelargonidin	−H	−OH	−H	−OH	−OH	−H	−OH
Malvidin	$-OCH_3$	−OH	$-OCH_3$	−OH	−OH	−H	−OH
Peonidin	$-OCH_3$	−OH	−H	−OH	−OH	−H	−OH
Petunidin	−OH	−OH	$-OCH_3$	−OH	−OH	−H	−OH
Rosinidin	$-OCH_3$	−OH	−H	−OH	−OH	−H	$-OCH_3$

2.4.4 Tannins

Tannins are high molecular weight compounds ranging from 500 to over 3,000 Da in case of gallic acid ester and up to 20,000 Da in case of proanthocyanidins (Khanbabaee & Van Ree, 2001; Han *et al.*, 2007). Tannins constitute the third important group of phenolics,

and they are subdivided into hydrolysable and condensed tannins (Okuda, 2005). The hydrolysable tannins are esters of gallic acid (gallo- and ellagi-tannins), while the condensed tannins are polymers of polyhydroxyflavan- 3-ol monomers (Okuda, 2005). A third subdivision, the phlorotannins consist entirely of phloroglucinol and have been isolated from several genera of brown algae (Kang *et al.*, 2011). Tannins are polyphenols, which in a solution bind with protein, basic compounds, such as alkaloids or heavy metallic ions, which makes them insoluble and induce precipitation (Okuda & Ito, 2011).

Proanthocyanidins have a wide variety of biological activities such as antimutagenicity, inhibition of low-density lipoprotein oxidation, anti-inflammatory, antiviral and antihypertention capacities (Joshi *et al.*, 2001). They were reported to inhibit the growth of breast cancer cells both *in vitro* (Agarwal *et al.*, 2000) and *in vivo* (Kim *et al.*, 2004; Mantena *et al.*, 2006). The study of Meeran and Katiyar (2007) also suggested that proanthocyanidins induce apoptotic cell death in a dose-dependent manner by 26 – 58%, which was associated with an increased protein expression of proapoptotic Bax, decreased expression of antiapoptotic Bcl-2 and Bcl-xl, loss of mitochondrial membrane potential, and cleavage of caspase-9, caspase-3 and PARP. Cytotoxicity of proanthocyanins associated with the induction of apoptosis without affecting the growth and viability of the normal cells has also been shown in human prostate cancer cells (Bagchi *et al.*, 2002; Agarwal *et al.*, 2002).

Proanthocyanidins from grape seed extract were shown to inhibit epidermal growth factor-induced and constitutively active mitogen-activated protein kinase (MAPK) signalling in human prostate cancer (DU145) cells, which may have a possible role in antiproliferation and apoptosis of cancer cells (Tyagi *et al.*, 2003). Grape seed extract also inhibited the proliferation of colorectal carcinoma cell lines. The inhibition was associated with a pro-apoptotic activity involving a loss of mitochondrial membrane potential and activation of caspase-3 in these cells (Hsu *et al*., 2009).

Ellagitannins is the largest group of hydrolysable tannins characterised by the presence of one or more hexahydroxydiphenoyl (HHDP) unit(s) on a glucopyranose core. The HHDP group is biosynthetically formed through intramolecular, oxidative C-C bond formation between neighbouringgalloyl groups in galloylglucoses (Aaby *et al.*, 2005). Koponen *et*

al. (2007) found that ellagitannins and ellagic acid occur in high concentrations in raspberries about 263 – 330 mg/100 gFW (Koponen *et al.*, 2007). Ellagitannins found in berries were reported to inhibit proliferation of various cancer celllines. Mullen *et al.* (2002) reported that ellagitannins found in raspberries contribute significantly to the antioxidant activity and vasodilation properties. The same author also observed that Sanguiin H-6, the most abundant ellagitannin found in raspberries, was a major contributor to antioxidant capacity, beside vitamin C and anthocyanin compounds (Mullen *et al*., 2002). Similarly, Ross *et al.* (2007) suggested that the antiproliferative activity of raspberries is predominantly associated with ellagitannins.

Pomegranate juice and its ellagitannins were also reported to inhibit proliferation, induce apoptosis and suppress inflammatory cell signalling in colon cancer cell lines (Seeram *et al*., 2005; Larrosa *et al*., 2006). The same authors highlighted that the efficacy of pomegranate juice is higher than its purified ellagitannins. This indicates synergistic action of ellagitannins and other bioactive compounds, such as anthocyanins and flavonols abundant in pomegranate juice. In agreement, polyphenols in muscadine grape skin inhibit the growth of colon cancer cells and induce apoptosis (Yi *et al*., 2005). Fraction isolated from red muscadine grapes and rich in ellagic acid, ellagic acid glycosides and ellagitannins induce apoptosis, decrease cell number and cause alterations in cell cycle kinetics in colon carcinoma cells (Mertens-Talcott *et al*., 2006).

The same authors underlined that the efficiency of ellagitannins observed in these studies is probably due to ellagic acid produced by hydrolysis. In agreement, the study has demonstrated that pomegranate and raspberry ellagitannins produce ellagic acid in the cell culture media (Larrosa *et al*., 2006; Ross *et al.*, 2007), and ellagic acid reduces cell proliferation and induces cell cycle arrest and apoptosis (Losso *et al*., 2004, Larrosa *et al*., 2006). Cerda *et al.* (2005) reported that ellagitannins are not absorbed by human. In contrast, ellagitannins will hydrolyse to yield ellagic acid, which is then further metabolised by colonic microflora, increasing the levels of bioavailable derivatives (Cerda *et al*., 2005).

2.5 Health benefits of foods

Phytochemicals may be able to play an essential role in many chronic disease preventive approaches. There is a wide range of potential downstream applications including the use of phytochemicals to produce functional foods, pharmaceutical applications, in chemoprevention and other alternative therapies.

2.5.1 Functional foods

Functional foods are considered to be any foods or food components consumed as part of the usual diet that may provide demonstrated physiological benefits and/or the ability to help reduce the risk of chronic diseases beyond basic nutritional functions (Doyon & Labrecque, 2008). During the last decade, efforts have been made to investigate the effects of plant foods and natural antioxidants on the prevention of chronic diseases. As a result of many completed studies and ongoing research, it is strongly believed that the dietary consumption of antioxidant-rich fruit, vegetables, herbs, or their phytochemicals constituents, plays an essential role in protecting the body's cells from damage including reduction of the risk factors associated with many common diseases. These can encourage the health- conscious consumers to increasingly consider consumption of functional food that may be perceived as a food, addressing age-related health conditions, tapping to its pharmacological significance. As a result the quality of life will be improved and life expectancy would continue to lengthen, which will also reduce healthcare expenses (Arai *et al.*, 2008).

The San Diego-based Nutrition Business Journal (NBJ) estimated that the value of global functional foods and supplement markets in 2001 was about US$62 billion and US$50 billion, respectively (CSIRO, 2012a). Japan ranked first in functional food consumption is also included in this estimation. Australia, the seventh among these markets leaders, had an estimated value of about US$700 and US$560 million, respectively for functional foods and supplements. Functional foods provide an alternative pathway for individuals to conveniently obtain dietary nutrients to address significant health problems (Hsieh & Ofori, 2007) either through prevention or treatment. Preventing or managing specific conditions and ensuring overall health and well-being through this convenient method is

the best approach to maintain a good health and provides economic benefits to consumers (Siro *et al.,* 2008).

The upward trend of functionality of foods is amplified by the relatively new and growing area of nutrigenomics, albeit their health benefits may show considerable variability, partly due to genetic variations (Furguson, 2009). Generally, nutrigenomics examines the impact of diets and dietary compounds on gene expression and protein expression and modification, metabolism and overall health (Brown & van der Ouderaa, 2007). Functional foods from Australian plants do not only address public health concerns. The increasing demand for functional foods will be an avenue for more sustainable forms of agriculture increasing standards of food supply chains (Smith, 2008), which promises potential solutions to some of the challenges facing the world's food system such as rising prices, food recalls and a global food shortage (Wallinga & Maizes, 2008). In brief, this has the potential for significant commercial benefits for the food, biotechnological and agricultural industries.

2.5.2 Pharmaceutically active food components

Pharmacologically active substances in functional quantities may be potentially present in food in the form of nutritional bioactives (Tulp *et al.*, 2006) which significantly overlap with the commercial production of pharmaceutically prepared supplements. Nutritional bioactives implicated in preventing diseases or slowing disease progression will often target the same enzymes, receptors and transcription factors, as those targeted by the pharmaceutical industry (Schwager *et al.,* 2008). An evidence of this is the growing interest by the pharmaceutical industry in exploring nutritional components and functional foods (Siro *et al.,* 2008).

Nutrient-based agents are gaining increasing exposure as chemopreventive agents and many are undergoing clinical trials. These may focus on specific cancers or population groups, or provide benefit for the population as a whole (Greenwal & Dunn, 2009). However, the properties of these agents which act on specific molecular and cellular targets in cell culture and animal models should be identified (Siro *et al.,* 2008). Nutrient-based agents are generally regarded as being safe, nontoxic, and effective and

with an ability for long-term usage (Siro *et al.,* 2008). Chemopreventive agents, on the other hand, which work through different mechanisms and modes of action, may produce a synergistic effect which increases efficacy and minimises toxicity (Johnson & Williamson, 2003).

Moreover, chemotherapeutic agents are largely costly and less effective as the disease progresses, highlighting the benefits and potential of chemoprevention as an approach for cancer control. The use of dietary chemopreventive substances, however, may also have further applications in combination with traditional chemotherapeutic agents. This may be in the prevention of the occurrence of cancer, prevention of metastatic spread or treatment of cancer (Russo, 2007). Chemopreventive agents have been ideally considered to have the ability to kill cancerous cells without toxicity to non-transformed cell. A high efficacy in multiple sites is also stated including the capability for oral consumption, a known mechanism of action, low cost, and acceptance by population (Rajamanickam & Agarwal, 2008). Currently natural products have obtained great attention for cancer prevention especially the antioxidants presented in common food and beverages, owing to their various health benefits, noticeable lack of side effects and the limitations of other chemotherapeutic agents (Manson *et al*., 2005). Several studies have shown that natural products derived from edible plants interfere with a specific stage of the carcinogenic process (Nishino *et al.*, 2007; Tan *et al*., 2011b). Green tea is the most widely consumed beverage in the world and is especially popular in the eastern countries. Because of its abundant, scientifically proven, beneficial effects on human health, green tea has received considerable attention (Ho *et al.*, 2007). Tea has been shown to inhibit tumorigenesis *in vivo*, involving in different organs such as the stomach, liver, small intestine and colon.

The inhibitors of tumorigenesis contributed by polyphenols are classified into three categories according to the sequence in the carcinogenic process in which the protective properties are exerted. The first group consists of compounds that interfere with the formation of carcinogens from precursor substances. The second group represents compounds that prevent complete carcinogens from reaching the critical sites in target cells, known as blocking agents, which primarily act by retarding the activation or facilitating the detoxification and removal of xenobiotics. Finally, the third group of

inhibitors play a role to impair, delay or reverse the expression of malignancy after exposure to carcinogens, and are called suppressing agents (Figure 2.12).

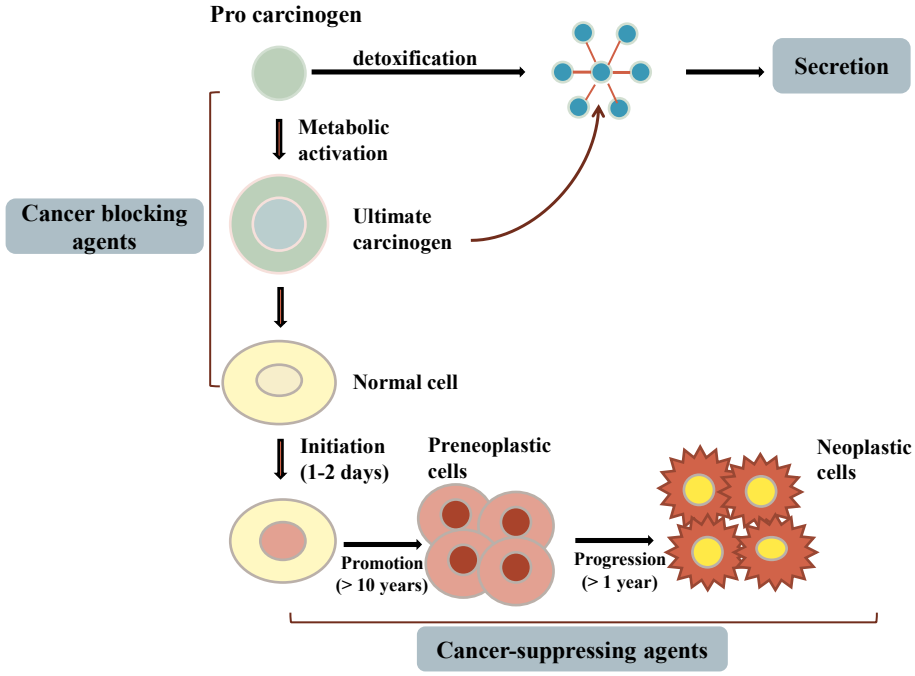


Figure 2.12 Dietary phytochemicals that block or suppress multistage carcinogenesis (adapted from Surh, 2003)

2.6 Potential mechanisms of cancer chemoprevention by phytochemicals

Cancer is an uncontrolled cell growth showing a naturally lengthy multistage development of pathogenesis. A large number of studies over the last decade have investigated a variety of anticancer drugs for prevention and treatment. Dietary polyphenols now become well-known physiologically active molecules that are considered to be alternative agents, providing the ultimately potential properties preventing malignant cell growth. Recently, the mechanistic information of phytochemicals acting as chemopreventive agents has been established, presenting the potential properties to interfere with various stages of tumour development, such as promotion and progressions. These include the modulation of mitogenic signalling, cell-cycle regulatory molecules, survival/apoptotic signalling, angiogenic and metastatic

events in cancer cells, blocking metabolic activation and/or DNA binding of carcinogens, stimulation of detoxification, repair of DNA damage, suppression of cell proliferation and metastasis or angiogenesis and induction of differentiation or apoptosis of pre-cancerous cells (de Flora & Ferguson, 2005). Phytochemicals evaluated in these studies have been shown to possess efficient anti-proliferative properties against various cancerous cells *in vitro*, as well as tumour growth *in vivo*. Primary prevention is achieved by the prevention of occurrence of the disease by early detection. Secondary prevention looks at the reversion of tumours at a malignant stage, while tertiary prevention attempts to prevent local recurrences as well as invasion and metastasis (Figure 2.13).

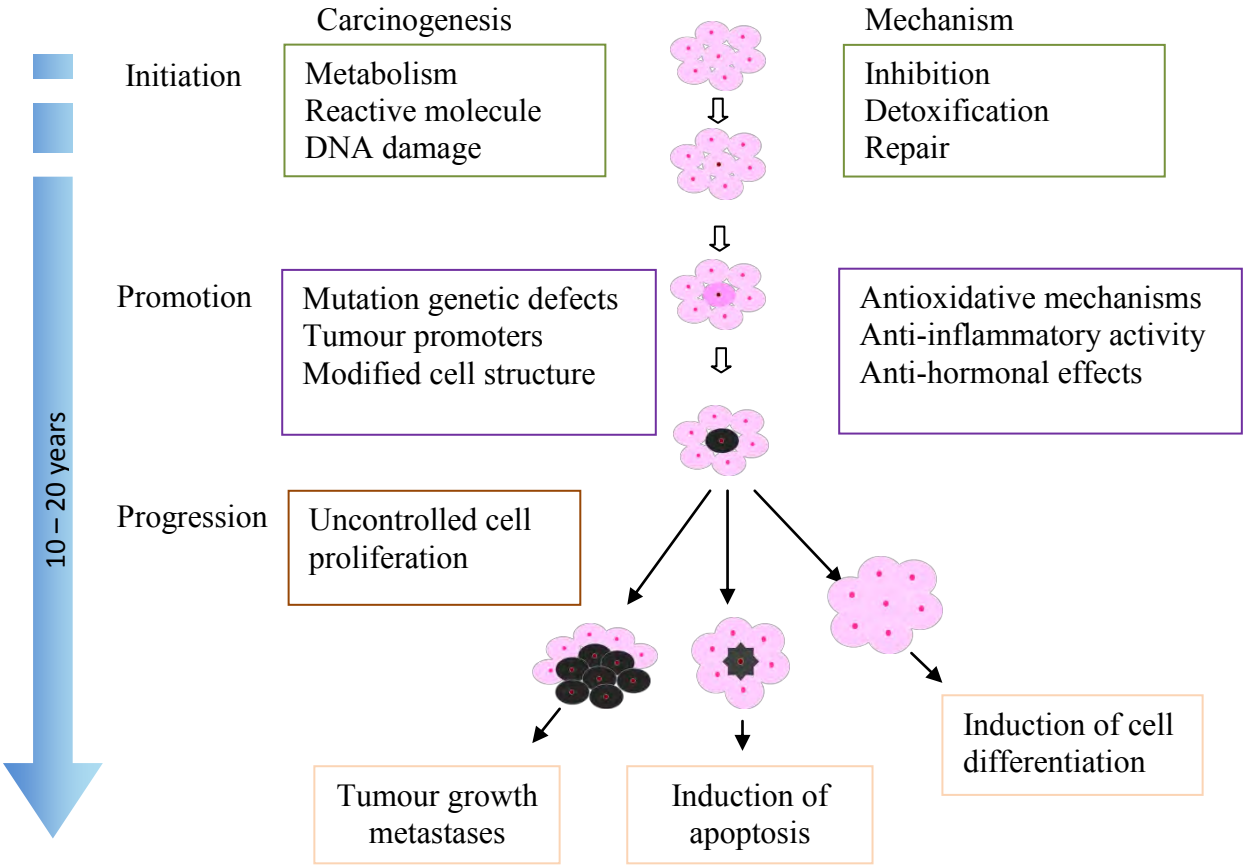


Figure 2.13 Carcinogenesis and mechanism of chemoprevention (adapted from de Flora & Ferguson, 2005)

2.6.1 Inhibition of the tumour cells growth

Two major mechanisms of cell death have been described which are apoptosis and necrosis. Necrosis is a form of traumatic cell death that results from acute cellular injury, and involves the external damage of cell, mediated by destruction of the plasma membrane. Apoptosis is different from necrosis as the processes associated with apoptosis in disposal of cellular debris do not damage the organism.

Apoptosis is the programmed cell death (PCD) which occurs in multicellular organisms (Rajesh *et al.*, 2009). In fact, apoptosis is one of the main types of programmed cell death which involves a series of biochemical events leading to specific changes in cell morphology and physiology and ultimately death of cell. Characteristic cell morphology of cells undergoing apoptosis includes blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (Hengartner, 2000). Two major mechanisms of cell death have been described which are apoptosis and necrosis.

Necrosis is a form of traumatic cell death that results from acute cellular injury, and involves in the external damage of cell, mediated by destruction of the plasma membrane. Apoptosis is different from necrosis as the processes associated with apoptosis in disposal of cellular debris do not damage the organism. Apoptosis plays a potent role as the most efficient form of defence against cancer (Ghavami *et al*., 2009). The ability of a neoplastic cell to evade apoptosis represents a significant characteristic which translates into its malignant ability and chemotherapeutic resistance. Apoptosis is a major mechanism by which many current synthetic anticancer and chemotherapeutic agents achieve their target. As such, apoptosis emerges as an important mechanism by which dietary compounds may exhibit chemopreventive potential. Apoptosis may remove cells undergoing neoplastic transformation when other cellular defence systems have failed to block the carcinogenesis process upstream. Removing the genetically damaged, pre-initiated or neoplastic cells by induction of apoptosis or cell cycle arrest represents a cornerstone of the chemopreventive paradiam (D′Agostini *et al*., 2005).

Apoptosis represent a complex molecular process involving more than a hundred proteins actively participating in various actions from signal transduction to execution of key cytoskeletal structures and command centre DNA (Khan *et al.*, 2007). Particularly, apoptosis progresses through one of three pathways which are the extrinsic pathway, the intrinsic pathway or the granzyme B (GrB) pathway (Boivin *et al.*, 2009). The extrinsic pathway involves induction via the activation of death receptors on the cell surface. The intrinsic pathway relies on an increase in mitochondrial permeability and cytochrome c release. The GrB pathway involves the exposure of sensitive target cells to the cytotoxic cell protease GrB (Ghavami *et al.*, 2009).

2.6.1.1 Extrinsic Pathway

The extrinsic pathway begins outside the cell and is associated with the activation of specific pro-apoptotic receptors on the cell surface or death receptors (DR). The specific molecules known as pro-apoptotic ligands (Figure 2.14), act as the activator of this process. These include Apo2L/TRAIL and CD95L/FasL and bind their cognate receptors DR4/DR5 and CD95/Fas, respectively (Fulda & Debatin, 2006; Rowinsky, 2005). The DISC recruits procaspase-8, activating caspase-8 which in turn directly cleaves and activates caspase-3. The extrinsic pathway, in contrast to intrinsic pathway, triggers individually the apoptosis of the p53 protein (Elmore, 2007).

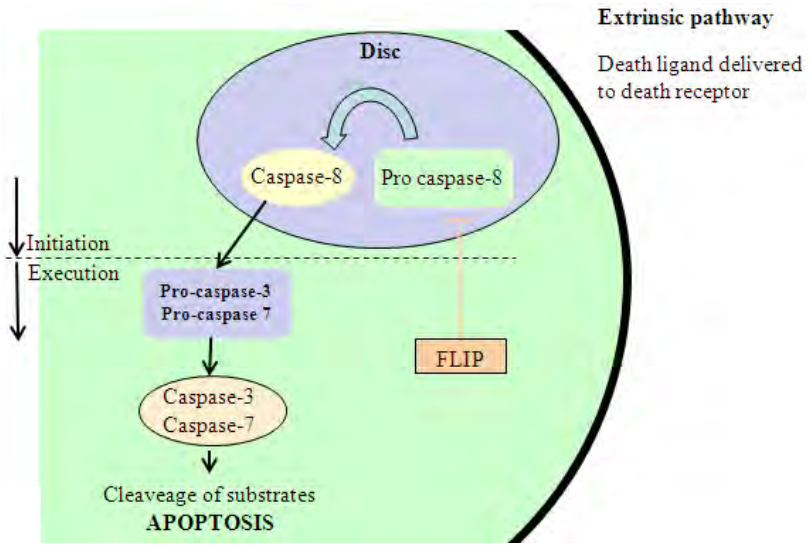


Figure 2.14 Elements of the extrinsic apoptotic pathway (adapted from Riedl & Salvesen, 2007)

Ligand binding induces receptor clustering and forming a death-inducing signalling complex (DISC) as the enrolment of the adaptor protein Fas-associated death domain (FADD) and the initiator caspases 8 or 10 as procaspases (Pan *et al.*, 2008). The DISC formation can induces procaspase molecules into close proximity of one another, facilitating their autocatalytic processing and release into the cytoplasm where they activate effector caspases 3, 6, and/or 7, thereby converging on the intrinsic pathway (Figure 2.15) (Ghobrial *et al.*, 2005; Lavrik *et al.*, 2005).

Dimerisation may play a crucial role to activate caspase 8, and clustering of the receptors and the associated DISC may enhance this activation (Bao & Shi, 2007). DISC formation is modulated by several inhibitory mechanisms, including c-FLICE inhibitory protein (c-FLIP), which exerts its effects on the DISC by interacting with FADD to block initiator caspase activation and decoy receptors, which can block ligand binding or directly abrogate pro-apoptotic receptor stimulation (Ashkenazi, 2008). The extrinsic pathway considered on the DISC activation can also adopts the effector caspase machinery as the intrinsic pathway.

2.6.1.2 Intrinsic Pathway

The intrinsic pathway is initiated inside the cell (Figure 2.15) which occurs in response to cellular signals resulting from DNA damage, a defective cell cycle, detachment from the extracellular matrix, hypoxia, loss of cell survival factors, or other types of cell stress (Coultas & Strasser, 2003). This pathway involves the release of pro-apoptotic proteins that activate caspase enzymes from the mitochondria. This process ultimately triggers apoptosis (Coultas & Strasser, 2003). The mitochondrial pathway is regulated largely by the Bcl-2 family of proteins, including various proapoptotic proteins such as Bcl-2 antagonist of cell death (Bad), Bcl-2-interacting domain death agonist (Bid), Bcl-2 antagonist/killer (Bak), and various anti-apoptotic proteins such as Bcl-2 and Bcl-C.

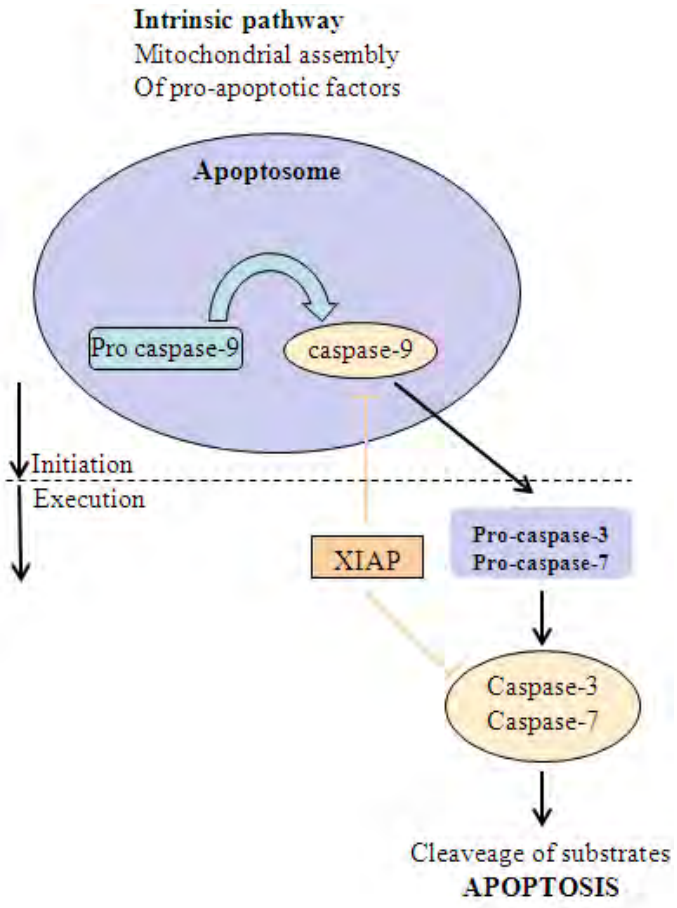


Figure 2.15 The role of intrinsic pathway *(*adapted from Riedl & Salvesen, 2007)

As the ratio of pro apoptotic family members to anti-apoptotic family members becomes grater, pores form in the outer mitochondrial membrane (Hail & Lotan, 2009). Subsequently cytochrome c is released into the cytoplasm from the mitochondrial intermembranous space. Other proapoptotic factors released from the mitochondria include various procaspases, apoptotic protease-activating factor 1 (Apaf-1), endonuclease G and apoptosis-inducing factor. An apoptosome is formed from the binding of cytochrome c, Apaf-1, adenosine triphosphate and procaspase–9 via catalysis. However, it has become clear that cancer cells are often reliant on these aberrancies for continued survival. Perhaps, counter-intuitively, cancer cells can in fact be more prone to apoptosis than normal cells. The apoptosis-prone phenotype of cancer cells is masked and counterbalanced by up-regulation of one or more anti-apoptotic mechanisms. Therefore, it is of enormous therapeutic interest to selectively tip the balance of the cellular fate of

cancer cells towards apoptosis. Indeed, the rational design of novel agents that can selectively induce apoptosis in cancer cells is a rapidly developing field, as exemplified by the plethora of such agents reported in contemporary literature.

The main pathways of apoptotic signalling are extrinsic and intrinsic as well as perforin/granzyme pathway (Figure 2.16). Each requires specific triggering signals to begin an energy–dependent cascade of molecular events. Each pathway activates its own initiator caspase (8, 9, 10), which in turn will activate the executioner caspase–3. However, granzyme A works in a caspase-independent fashion. The execution pathway results in characteristic cytomorphological features including cell shrinkage, chromatin condensation, membrane blebbing, nuclear fragmentation and apoptotic bodies and finally phagocytosis of the apoptotic bodies by adjacent parenchymal cells, neoplastic cells or macrophages.

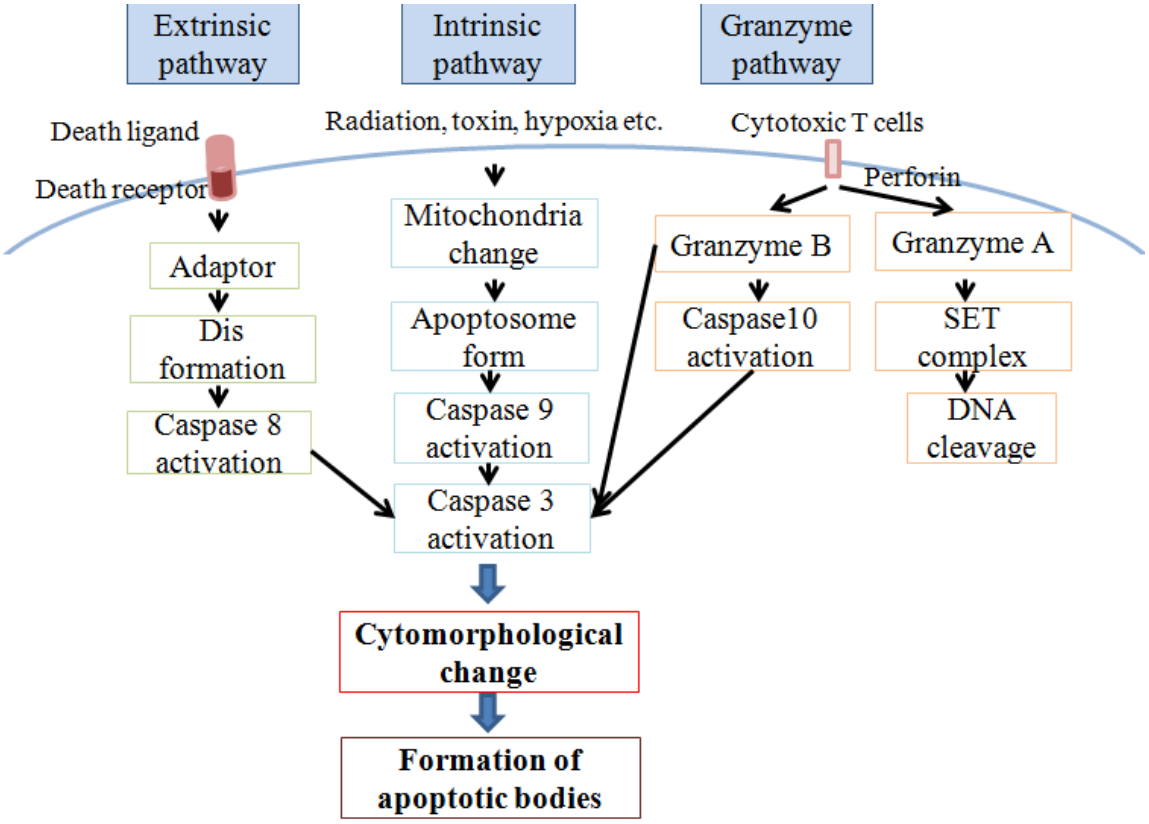


Figure 2.16 Schematic of apoptosis pathways (adapted from Elmore, 2007)

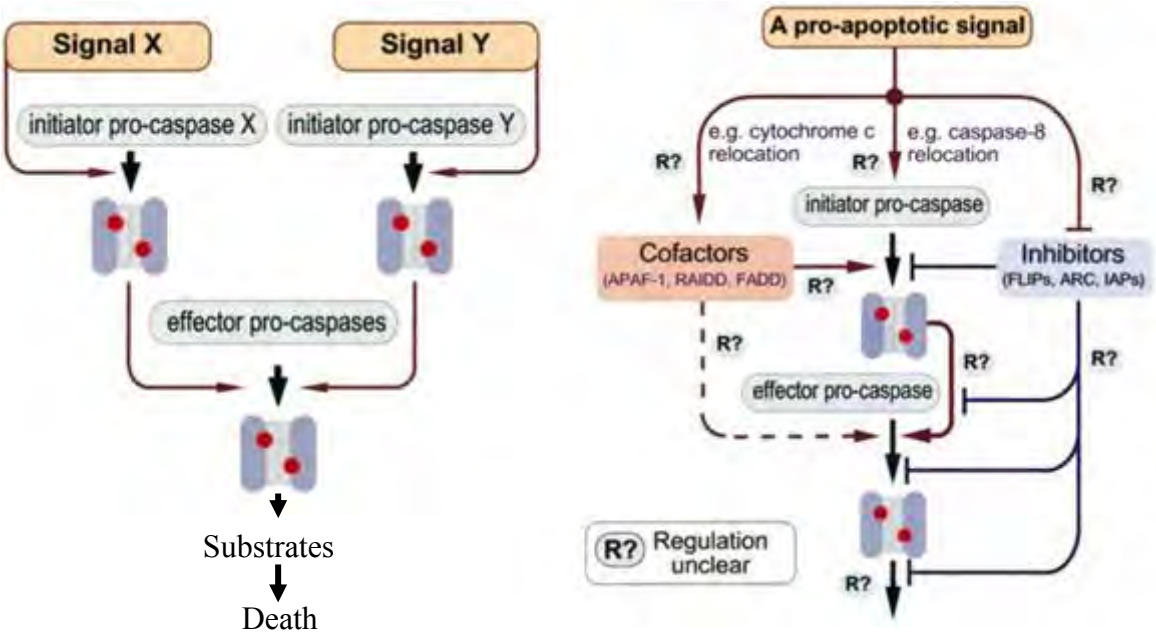
2.6.1.3 Granzyme B pathway

GrB is a serine protease primarily stored in the cytotoxic granules of cytotoxic lymphocytes, and implicated as a major mechanism of cytotoxic T cell and natural killer in cell-mediated elimination of cells. It may also be expressed by various other immunological cell types under pro-inflammatory condition (Boivin *et al.,* 2009). The GrB pathway typically involves GrB release from a cytotoxic lymphocyte and subsequent uptake by the target cell. Internalisation of GrB is facilitated by perforin, a molecule capable of forming pores in intracellular membranes. Once inside the cells, GrB is able to initiate apoptosis through numerous pathways (Lawen, 2003). The primary pathway of GrB is the cleavage of Bid and the consequent mitochondrial release of cytochrome c and apoptosome formation. GrB however, may also directly activate caspases such as caspase-3 or cleave caspase substrates such as the inhibitor of caspase-activated deoxyribonuclease (ICAD), (Lord *et al.,* 2003).

2.6.1.4 Caspase cascade

The caspase cascade plays a vital role in the induction, transduction, amplification, and execution of apoptotic signals within the cell (Figure 2.17) (Kurokawa & Kornbluth, 2009). The caspases are a group of intracellular cysteine enzymes that are activated through both the intrinsic and extrinsic pathway of apoptosis. These destroy the essential cellular proteins which lead to controlled cell death. There are generally two subgroups of caspase activation during apoptosis. The initiator caspases, consisting of caspase-2, -8, -9 and -10, are activated through the apoptosis-signalling pathways and the effector caspases, consisting of caspase-3, -6 and -7, which in an expanding cascade, are carrying out apoptosis (Hengartner, 2000; Lavrik *et al.*, 2005; Pop & Salvesen, 2009).

Caspase cascades are initiated through assembly of multiprotein complexes that trigger activation of the initiator caspases, which are then released and able to activate the downstream effector caspases. Caspase activity is normally held in check by c-FLIP and the IAP protein family, of which at least 10 have been identified including XIAP, cIAP1, cIAP2, ILP2, MLIAP, SURVIVIN, and BRUCE (Lavrik *et al.*,2005).



A. Caspase cascade B. Regulation of caspase activity

Figure 2.17 Caspase cascade in apoptotic cells and a model for caspase regulation (adapted from Thornberry *et al.*, 1997)

Referencing to Figure 2.17A, early observations that, during apoptosis, common morphological changes occur in tissues and species led to the suggestion that this process is governed by a conserved biochemical system. It is now clear that these changes are due to the activities of a common set of effector caspases. The observation that distinct death signals result in the same manifestations of apoptosis is explained by the binding that effector caspases are activated by different initiator caspases, each of which is activated by a set of proapoptotic signals. For figure 2.17B, available evidence suggests that caspases are regulated by opposing effects of activators and inhibitors. A signal apparently initiates three pathways involving cofactors, initiator caspases, and inhibitors. Activation of co-factors (for example, cytochrome c relocation from mitochondria to cytoplasm), modification of the caspase (for example, relocation of caspase-8 to a receptor complex), and inactivation of inhibitors together result in activation of the initiator caspase. The dashed line from cofactors to effector caspases reflects the possibility that effector caspases may be activated by an autocatalytic mechanism.

Regulation is likely to be even more complicated; for example, active caspases may be involved in feedback mechanisms.

The inhibition of apoptosis (IAP) proteins are characterized by the presence of between 1 and 3 specific domains called baculoviral repeats (BIRs), which are directly involved in their caspase-inhibitory activity. While not directly involved in apoptotic signalling per se, some of these proteins prevent cell death by suppressing endogenous initiator and effector caspase activity (see Figure 2.15). Emerging evidence also suggests that IAPs may play a role in modulating cell division (Schimmer, 2004). The IAPs SURVIVIN and c-IAP1 are overexpressed in several malignancies (Schimmer, 2004).

2.6.2 Antioxidant activity and oxidative stress

Epidemiological studies have shown that large intakes of fruit and vegetables protect against a range of chronic diseases and problems associated with ageing. This is often attributed to a high intake of phytochemicals with antioxidant activity, as this is thought to be the mechanism underpinning many of these protective effects. Antioxidants are phytochemicals, vitamins and other nutrients that protect cells from damage caused by free radicals (Babich *et al.*, 2011). *In vitro* and *in vivo* studies have shown that antioxidants can prevent the free radical damage that is associated with cancer and heart disease (Lazze *et al.,* 2004; Chen *et al.,* 2003). Antioxidants can be found in fruits and vegetables, culinary herbs and medicinal herbs, grains, bark of trees and others. The study on several culinary and medicinal herbs reported that the antioxidant level of herbs can be as high as 465 mmol Fe^{2+} per 100 gDW (Dragland *et al.,* 2003).

The consumption of smaller quantities of multiple phytochemicals may result in more health benefits than the consumption of larger quantities of fewer phytochemicals. Numerous studies with plant phytochemicals showed that phytochemicals with antioxidant activities may reduce risk of cancer and improve heart health. Antioxidants deactivate free radicals by donation of electrons and converting them into harmless molecules. Antioxidants can play a significant role as agents that prevent or inhibit oxidation. They are both naturally occurring or are synthetic substances that can protect cells from the damaging effects of oxygen free radicals (Bagchi *et al.,* 2000). A number

of nutrients have antioxidant properties, for example vitamin E, manganese, glutathione, Co Q, vitamin C, selenium, carotenoid compounds and phenolic compounds (Bagchi *et al.,* 2000). These antioxidants all appear to be involved in the elimination of carbon-centered radicals and peroxyl radicals (Gropper *et al*., 2005).

Free radicals, highly unstable molecules, are formed as part of natural metabolism. They are also formed in the body due to external sources such as environmental factors, smoking, pesticides, pollution and radiation. Free radicals react easily with the essential molecules of body, including DNA, fat and proteins. All organic and inorganic materials consist of atoms, which can be bound together to form molecules. Each atom has a specific number of positively charged protons, and negatively charged electrons. Free radicals are atoms that possess an unpaired electron, and therefore are highly unstable. To regain stability they are prompt to receive electrons from other atoms, thereby forming neutral molecules.

To regain the stability, free radicals try to steal electrons from other molecules, thereby changing their chemical structure. As a result, the molecule becomes a free radical itself, causing a chain reaction which can result in the destruction of a cell. Antioxidants have the property to neutralize free radicals without becoming free radicals themselves because they are stable in both forms. In other words, antioxidants are chemicals offering their own electrons to the free radicals, thus preventing cellular damage. However, when the antioxidant neutralizes a free radical it becomes inactive. Therefore we need to continuously supply our body with antioxidants. This is of importance because the action of free radicals could increase the risk of diseases such as cancer and heart problems and could accelerate ageing.

The existence of free radical in a human body at a low level is a part of the homeostasis. However, accumulation of free radicals at high levels creates an oxidative stress that is responsible for aging and a number of chronic diseases due to the damage to cell organelles such as lipids of the cell membrane, proteins and DNA. The damaged cells may eventually initiate mutation and formation of cancer (Klaunig & Kamendulis, 2004). The studies have shown that oxidative damage to cells and tissues are induced in chronic diseases development such as cancers, aging, cataract, myocardial infraction and

atherosclerotic cardiovascular disease (Liu & Finley, 2005; Eberhardt & Jeffery, 2006; Halliwell, 2007). The authors reported that antioxidants inhibit oxidative stress by acting at different stages in the oxidation reaction and may have multiple mechanisms of action.

In biological systems, an antioxidant can be defined as any substance that, in low concentration compared with the oxidisable substrate, significantly delays or prevents oxidation of that substrate. The substrate such as the oxidisable compound, is usually a lipid, but can be also a protein, DNA, or carbohydrate. In the case of lipid oxidation, the main mechanism of antioxidants is to act as radical chain-breakers. The preventive antioxidant oxygen scavenging or blocking the pro-oxidant effects is the other form of mechanism by binding proteins that contain catalytic metal sites (Frankel & Meyer, 2000). The complexity of antioxidants needs to be taken into account in free radical assays in testing for antioxidant activity.

The complexity of a multi-component oxidative biological material is overlooked compared to oxidation model systems that are models of lipids in their real environment. There are a large number of methods to determine the antioxidant activity of compounds. The antioxidant activity may vary widely depending on the environment of the lipid substrate. For antioxidant evaluation using a radical scavenging test, it should be recalled that this method can evaluate only the radical scavenging activity of the compound, and not the other antioxidant mechanisms, such as metal chelation. In addition, the antioxidant action is more complex in real foods and biological systems where several mechanisms become effective (Frankel & Meyer, 2000).

2.6.2.1 The mechanism of antioxidant activity

Autoxidation is a free radical chain process which is initially generated by light, heat, radiation or metal ions. The mechanism of autoxidation reaction can be described in terms of initiation, propagation and termination reactions illustrated as follows:

Chain initiation

$$ROOH + RH \longrightarrow RO\bullet + \bullet OH + RH \longrightarrow RO\bullet + H_2O + R\bullet$$

$$RO\bullet + RH \xrightarrow{\text{Fast}} R\bullet + ROH$$

Chain propagation

$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$

Chain termination

$2ROO^{\bullet} \longrightarrow 2RO^{\bullet} + O_2 \longrightarrow ROH + QO + O_2$

The reactions can be divided into three stages including chain initiation, propagation and termination, respectively. For the initiation process, some event causes free radicals to be formed. Free radicals, for example, can be produced purposefully by the decomposition of a radical initiator such as benzoyl peroxide. In some cases, initiation stage is induced by a process that is not well understood but is thought to be thespontaneous reaction of oxygen with a material by abstracting a hydrogen atom from their molecules in the propagation step. Destructive autoxidation processes also are initiated by pollutants such as those in smog. Once free radicals are formed, they react in a chain to convert the material to a hydroperoxide. The chain is ended by termination reactions in which free radicals collide and combine their odd electrons to form a new bond (Vertuani *et al.*, 2004).

One of the particular sources of primary catalysts which induce oxidation *in vitro* and *in vivo* systems is oxygen and reactive oxygen species (ROS), as well as reactive nitrogen species (RNS). Both oxygen species occur due to normal cell metabolism and can play a beneficial physiological role at low to moderate concentrations (Valko *et al.,* 2006). ROS includes superoxide (O_2 •-), peroxyl (ROO•), alkoxyl (RO•), hydroxyl (HO•) and nitric (NO•) radicals, which are the oxygen centred free radical (Figure 2.18).

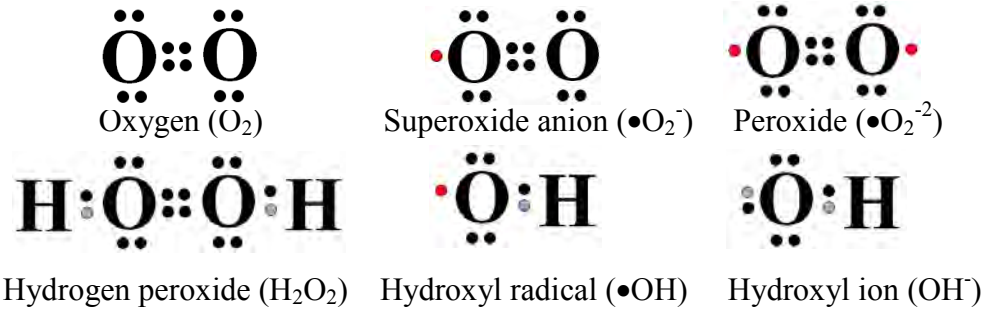


Figure 2.18 Electron structures of common reactive oxygen species

A vast number of flavonoid compounds act as efficient antioxidants in biological systems. For instance, quercetin effectively inhibited ROS and RNS using the U937 monoblastic and CEM lymphocytic cell lines (Cossarizza *et al.,* 2009). According to the study of Tanigawa *et al.* (2007), quercetin also enlarges ARE-mediated binding activity through increased Nrf2 expression. Genistein - a phytoestrogen, belonging to the isoflavones which can be found in soybean, soy drink and related soy food (Szkudelska & Nogowski, 2007) inhibited LPS−induced nitrite production in cultured macrophages and protected against LPS−induce necrosis despite its ability to cause apoptosis. Foti *et al.* (2005) also reported that genistein suppress iNOS activity and iNOS gene expression. It is suggested that the reduction in free radical production should result from the scavenging of ROS and RNS, direct inhibition of iNOS enzyme activity, and inhibition of iNOS gene expression (Vertuani *et al.*, 2004). Therefore, suppression of NO level by phytochemicals may be a new and efficient approach for the treatment of inflammation and cancer.

2.6.2.2 Cellular antioxidant assay

The cellular antioxidant assay (CAA) utilizes 2',7'-dichlorofluorescin diacetate (DCFH-DA) as a probe incorporated into cultured human HepG2 liver cancer cells (Wolfe & Liu, 2007). HepG2 cells absorb non-polar DCFH-DA by passive diffusion and aredeacetylated by cellular esterases. As a result, polar 2',7'-dichlorofluorescin (DCFH) is formed, trapped within the cells. Peroxyl radicals ($ROO^{\bullet}$) produced from 2, 2'-azobis (2-amidinopropane) (ABAP) lead to the oxidation of DCFH to form a fluorescent compound dichlorofluorescein (DCF). These DCF are scavenged peroxyl radicals by reacting withthe antioxidant compounds or phytochemicals obtained from plant extracts (Wolfe *et al.*, 2008). The level of fluorescence generated in the system is proportional to the level of oxidation. The decrease in cellular fluorescence compared to the control cells indicates the antioxidant capacity of the compounds and is expressed as an EC_{50} value.

2.6.2.3 Antioxidant response element (ARE)

The induction of many cytoprotective enzymes in response to reactive chemical stress is regulated primarily at the transcriptional level, mediating by a *cis*-acting transcriptional

enhancer sequence named antioxidant response element (ARE). This element initially found in the promoters of genes encoding phase II detoxification enzymes, antioxidant and drug-metabolising enzymes, providing co-factors and reducing equivalents and molecula chaperone/stress response gene (Yu & Kensler, 2005; Nguyen *et al.*, 2009).

The core ARE sequence was defined as 5'-TGACnnnGCA-3' based on mutational analysis of the rat GST A1 promoter sequence (Erickson *et al.*, 2002). The ARE possesses structural and biological features, indicating its unique properties responsive to oxidative stress. It is activated not only in response to H_2O_2 but specifically by chemical compounds with the capacity to either undergo redox cycling or be metabolically transformed to a reactive or electrophilic intermediate (Nguyen *et al.*, 2009). Moreover, compounds that have the propensity to react with sulfhydryl groups such as diethyl maleate, the isothiocyanates, and dithiothiones are also potent inducers of ARE activity. Therefore, alteration of the cellular redox status due to elevated levels of ROS andelectrophilic species and/or a reduced antioxidant capacity (e.g. glutathione) appears to be an important signal for triggering the transcriptional response mediated by this enhancer. Therefore, ARE-mediated gene expression and the resulting induction of the antioxidant enzymes have been considered as an essential protection mechanism of cells against endogenous and/or exogenous carcinogenic intermediates.

Induced expression of genes by ARE inducers is highly dependent on the basic region-leucine zipper (bZIP) transcription factor. In particular, transcription factor nuclear factor E2-related factor 2 (Nrf2) has been known to be an important driven ARE-mediated gene expression, more efficient than Nrf1 (Chen & Kong, 2005). Chan and Kwong (2000) have found the reduction of basal and the induction of expression of antioxidant genes by Nrf2-null mice. The study also reported Nrf2-null mice influence in the increase of oxidative stress and decreased reducing activity and antioxidant capacity (Chan & Kwong, 2000). This evidence indicated that Nrf2/ARE pathway represents a critical role in regulating the intracellular redox status (Figure 2.19).

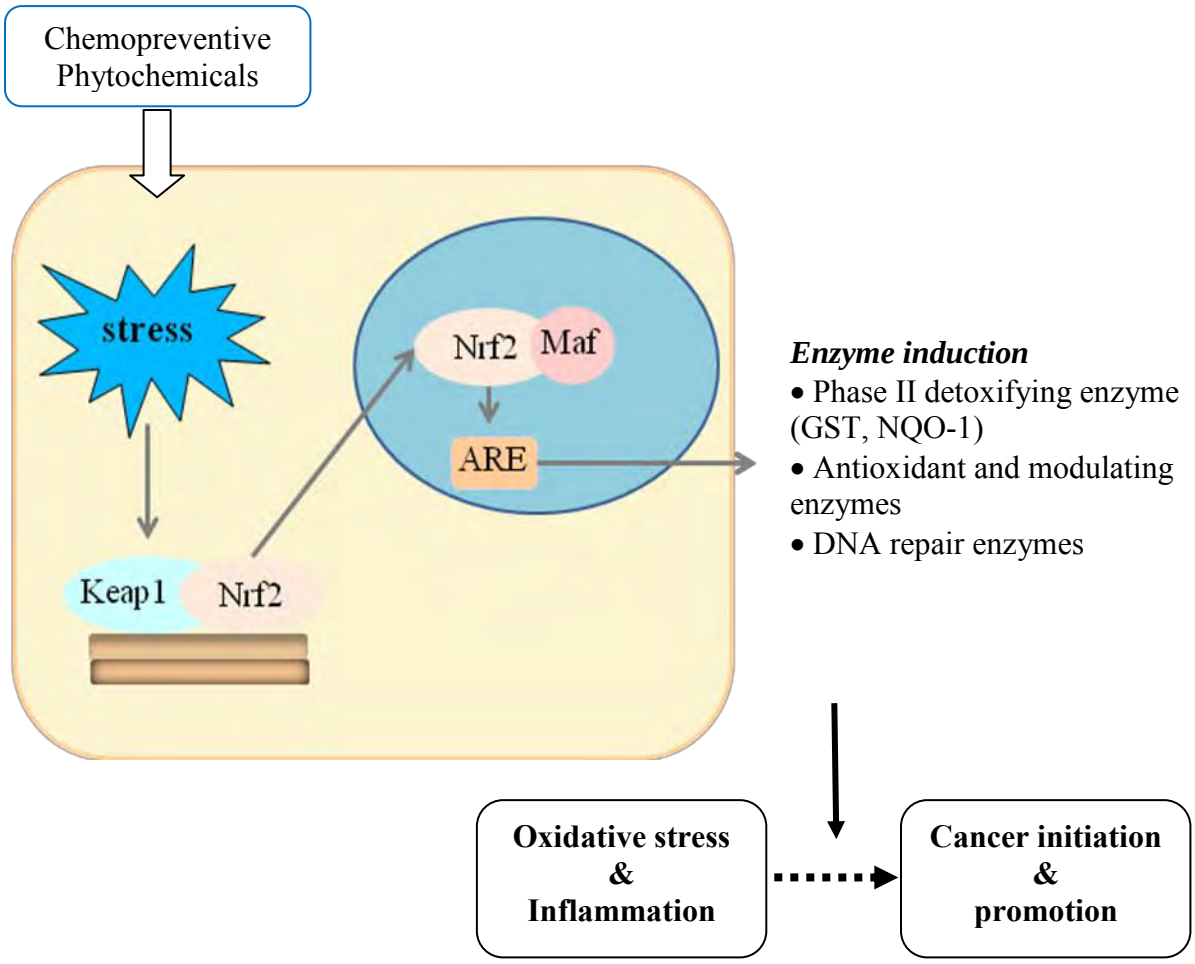


Figure 2.19 Role of the Keap1-Nrf2-ARE system in the regulation of the antioxidant response (adapted from Surh, 2003)

Nrf2 has been further confirmed in the strong response of the expression of antioxidant genes. The Nrf2-knockout mice have been found to have not only reduced levels of antioxidant enzymes but also more susceptibility to xenobiotics and environmental poisons compared to wild-type mice (Chen & Kong, 2005). The same response has been observed which Nrf2 knockout mice, which also demonstrated greater susceptibility to carcinogenesis and loss of inductive response to chemoprotective agents, intenselyrelative to regulation of the inflammatory process and the proteasome system (Yu & Kensler, 2005).

2.6.3 Anti-inflammatory activity of plant phenolics

Inflammation is the basic mechanism of body responding to injury. Pain is considered to be the most frequent symptom of the inflammatory process. Chronic inflammation may also involve a causative factor in a variety of cancers such as bladder, colon, pancreas, stomach and other cancers and may similarly be the final stage of years of inflammation (Balkwill & Mantovani, 2001) (Figure 2.20). The longer the inflammation persists, the more risk of cancer increases. Chronic inflammation is reported to be a predominant driver of alimentary tract cancer (Johnson, 2007).

The chronic inflammatory states may be triggered by microbial infections, autoimmune disease or inflammation of unknown origin, and 15 – 20% deaths from cancer are estimated to be linked with underlying infection or inflammatory responses. Nonsteroidal anti-inflammatory drugs (NSAIDs) are also known to reduce the risk of developing certain cancers and the associated mortality (Johnson, 2007). Chronic use of aspirin is reported to reduced rates of colorectal and oesophageal cancer (Corley *et al.*, 2003). Hence, acute inflammation, such as occurs in response to a transient infection, is not regarded as a risk factor for the development of neoplasia, although many of the same molecular mediators are generated in both acute and chronic inflammation. In general, inflammatory leukocytes such as neutrophils, monocytes, macrophages, and eosinophils provide the soluble factors that are thought to mediate the development of inflammation-associated cancer, although other cells, including the cancer cells themselves also participate. Variuos sources and cellular responses of reactive oxygen species are shown in Figure 2.20.

Inflammatory mediators include metabolites of arachidonic acid, cytokines, chemokines, and free radicals. Chronic exposure to these mediators leads to increased cell proliferation, mutagenesis, oncogene activation, and angiogenesis. The ultimate result is the proliferation of cells that have lost normal growth control. Animal models provide experimental evidence that chronic inflammation can promote cancer and further insights into possible mechanisms (Figure 2.21). Inflammation is a normal physiological process in the innate immune responses which generally occur in response to tissue injury.

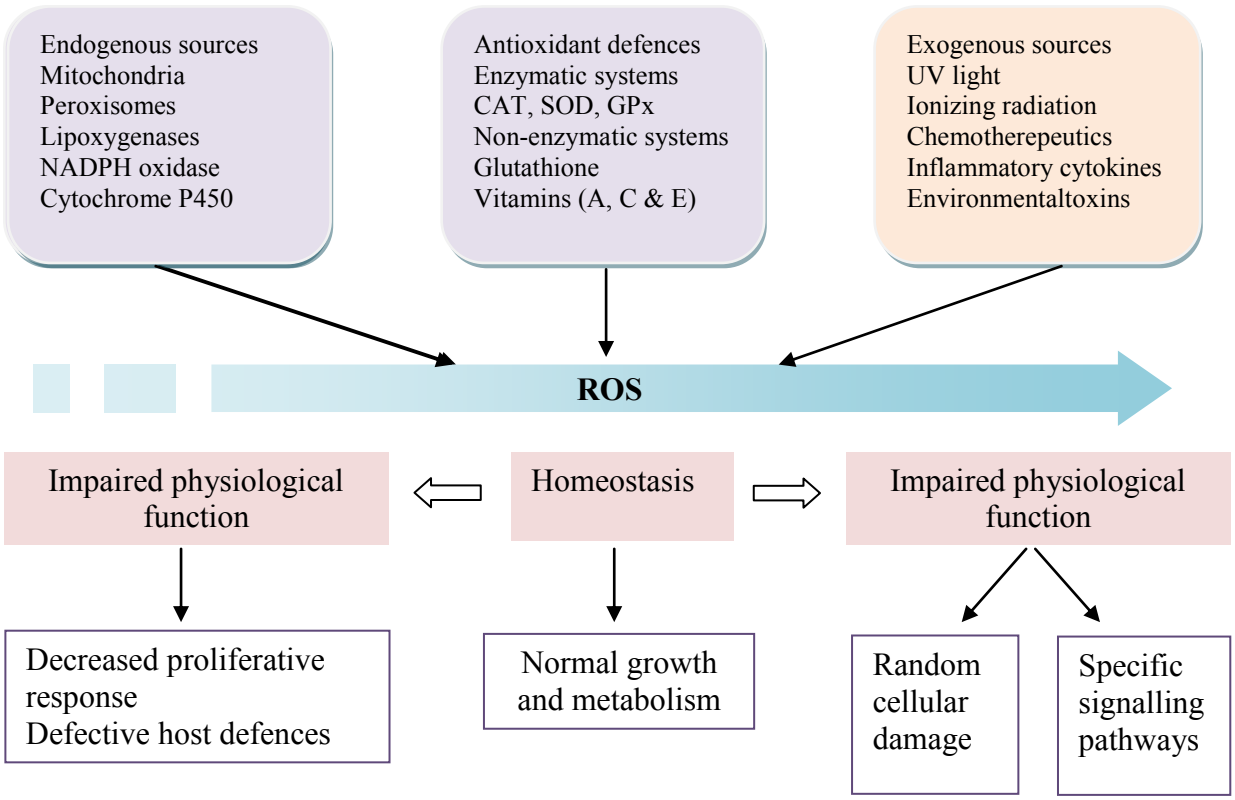


Figure 2.20 Sources and cellular responses of reactive oxygen species (adapted from Finkel & Holbrook, 2000)

In acute inflammation at the site of injury, it results in an increased supply of blood, greater vascular permeability and migration of white blood cells. Inflammatory cells, such as neutrophils, eosinophils and mononuclear phagocytes become stimulated and induce oxidant-generating enzyme, while soluble mediators such as acute-phase proteins, eicosanoids and cytokines are also produced.

High levels of oxidants and free radicals are produced in an effort to combat invading pathogens and foreign bodies and eliminate infected host tissue (Ohshima *et al*., 2005). A significant increase in non-mitochondrial oxygen consumption, or respiratory burst occurs, as an electron is reduced from oxygen generating the reactive species precursor superoxide anion (O_2^-) such as myeloperoxidase and eosiniphil peroxidise, which can ultimately result in the generation of nitrogen oxide (NO_2) or various non-radical oxygen species such as HOCl or HOBr (Zhang *et al.*, 2002). During acute inflammation, this

innate immune response represents the first line of immune defence, and modulates the activation of the adaptive immune response. In chronic inflammation however when the cause of injury in not resolved rapidly, the roles are often reversed, whereby the adaptive immune response causes excessive and ongoing activation of innate immune cells (de Visser & Eichten, 2006).

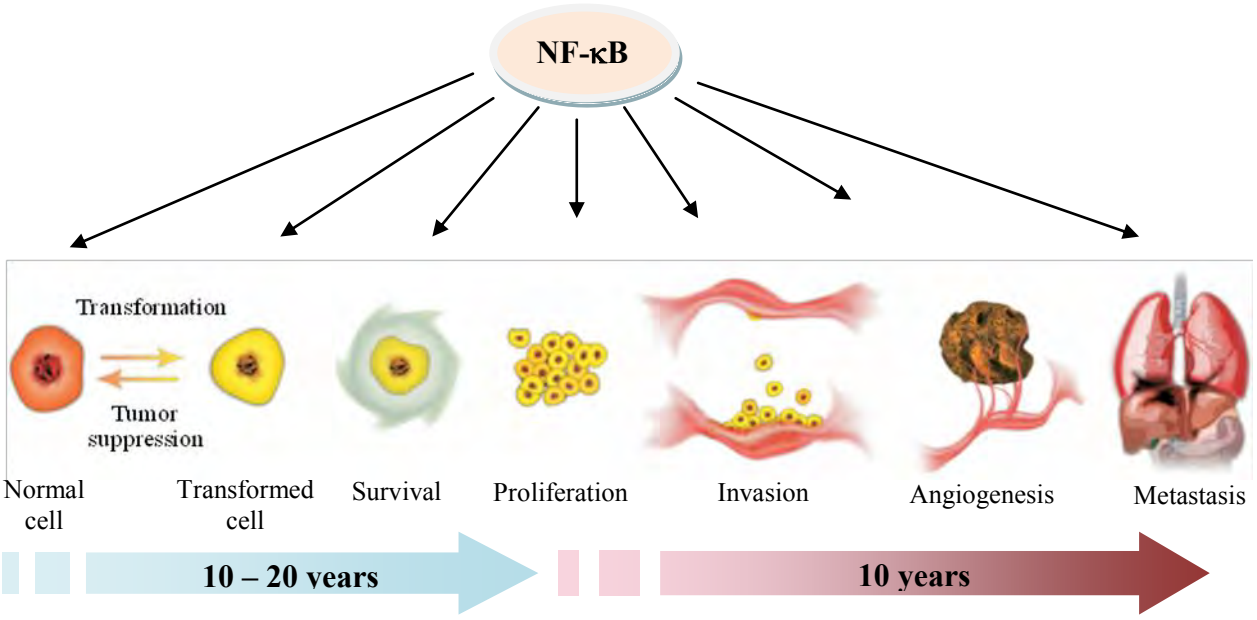


Figure 2.21 Roles of NF-κB-mediated inflammatory pathway in cellular transformation, cancer survival, proliferation, invasion, angiogenesis and metastasis (adapted from Aggarwal *et al.*, 2009)

These chronically activated innate immune cells can exacerbate chronic tissue damage by further producing damaging reactive species, reactive aldehyde, cotokines, chemokines and growth factors to the microenvironment which enables the disruption and alteration of normal biological processes. This leads to genomic disability, not only predisposing the development of cancer, but causing primary organ dysfunction and systemic complications. Excessive tissue remodelling and loss of tissue function due to tissue destruction, protein and DNA alterations cased by either the innate or adaptive immune response also leads to an increased risk of cancer development (Perwez Hussain & Harris, 2007).

2.6.4 Links with the immune response (Cancer-related inflammation and cancer immunoediting)

The immune system is a system of biological structures and processes within an organism that protects against disease from outside invader. To function properly, an immune system must detect a wide variety of agents from viruses to parasitic worms and distinguish them from the organism's own health tissue. Inflammation, generally known as one of the protective properties of the immune system is a broad and complex physiological process (Medzhitov, 2008). Epidemiological studies have confirmed that the inflammatory together with immune systems may inhibit the development of cancer. The primary roles in the prevention of tumours contributed by immune system include host protection from virus-induced tumours by elimination or suppression, eliminating pathogens and prompt resolution of inflammation andcollectively identifying and eliminating tumour cells in certain tissues which rely on their tumour-specific antigens (TSAs) expression (Vesely *et al.*, 2011). The last role is known as cancer immunosurveillance.

Adaptive immune response plays an indispensable part in performing tumour surveillance and elimination, based on the innate immune systems for the initial activation, representing a paradoxical mean by which inflammation promotes antitumour activity. Dendritic cells, the key initiators of adaptive immune response are suppressed by the induction of IL-10 signalling. However, multiple pathways have also been marked to be an efficiently inhibitory pathway against tumour immunity (Mantovani *et al.*, 2008). Immunosupressive mediators released from tumour cells employ mechanism to incapacitate the host-mediated antitumour responses as well as tumour progressive facilitating (Allavena *et al.*, 2008). Adaptive immune response of tumor-associated and specific antigens is susceptible to be able to play a role in essentiallypotential machinery to control cancer development (Vesely *et al.*, 2011).

2.6.4.1 Targets for chemoprevention by anti-inflammatory actions

A number of studies in cancerous mouse models as well as humans with cancer have confirmed that particular innate and adaptive immune cell types, effector molecules and

pathway play a key role as collectively function via extrinsic tumour-suppressor mechanisms (Vesely *et al.*, 2011). Chemoprevention has been recognised as a feasible approach for cancer prevention. One approach is devoted to modulation of inflammatory mechanisms, which occurs through various pathways including the generation of reactive species with oxidant generating enzymes (e.g. inducible nitric oxide synthase (iNOS)), various cytokines, mediators of inflammation (e.g. cyclooxygenase (COX)-2) and specific molecular signalling (e.g. nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), Figure 2.21). These are thought to be a potential approach to develop new strategies as molecular targets in the prevention, early detection and inflammation related cancers treatment (Ohshima *et al.*, 2005; Perwez Hussain &Harris, 2007). Non-steroidal anti-inflammatory drugs (NSAIDs) have been reported to reduce the incidence of certain malignancies such as colorectal, oesophageal, breast, lung, and bladder cancers, by which cyclooxygenase (COX) enzymes converted arachidonic acid to prostaglandins (PGs) and thromboxane. The inflammatory molecule is a target enzyme including inducible nitric oxide synthase (iNOS), mediators of inflammation such as cyclooxygenase (COX)-2 and specific molecular pathways such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Kim *et al.*, 2009).

2.6.4.2 iNOS and NO•

NO• is generated by NO synthase (NOS), the enzyme involved in the various immune-pathological and physiological processes and play a vital part in inflammation associated cancer. For normal condition, NO• plays an essential role as a key signalling molecule, related in vasodilation, neurotransmission and host defence. Overwhelming production of NO• free radical results in an imbalance inredox status and inflammatory microenvironment, producing proneoplastic functions (Keibel *et al.*, 2009).

There are three major isoforms of NO (1) NOS1 (neuronal NOS, nNOS), (2) NOS2 (inducible NOS, iNOS) and (3) NOS3 (endothelial NOS, eNOS). NOS1 and NOS3 are largely constitutive isoforms, which by the co-operating of calcium regulatory protein-calmodulin and present similar NO release kinetics. NOS3 or eNOS is known as a constitutive enzyme initially found in the endothelium. NOS2 or iNOS is an inducible nitric oxide synthase (iNOS) present in various cell types depending on inflammatory

stimulation such as macrophages. This produces a large number of NO• in a range of micromolar concentration and can be sustained for a prolonged period of time. However, only a range of picomolar and nanomolar levels are observed from NOS1 and NOS3 (Gharavi & El-Kadi, 2003).

The expression of iNOS is regulated by a variety of stimuli such as inflammatory cytokines, NF-κB, hypoxia and Wnt-pathway involve in iNOS induction (Perwez Hussain &Harris, 2007; Kundu & Surh, 2008). A low level of NO affects iNOS transcription which by activate NF-κB and up-regulate iNOS expression, while down-regulating transcription at the high levels (Bogdan, 2001). NO• can also bind with other cellular components to form various RNS such as oxygen and generate N_2O_3 as a product, which is able to remove amine groups from various DNA bases. A highly reactive nitrating and oxidising species is controlled by coupling with O_2 •- forms peroxynitrite anion (ONOO-) by diffusion (Ohsima *et al.*, 2005).

The tumour suppressor gene p53 frequently interweave with NO•. The p53 is known as a key molecular node for regulating the expression of a specific set of genes relevant to inflammatory stimulus. The co-operation between NO• and p53 can produce pro-tumorigenic and anti-tumourigenic effects. For normal unstressed cells, NO• can activate the p53 tumour suppressive pathway. The induction of oncogenic mutations in the p53 gene by NO• has been observed athigher levels viainflammatory conditions, resulting from the different pathway that NO• can react. NO• predominantly acts through cGMP-dependent pathways at the lower concentration. When the concentration increases, NO• acts directly by co-operation with biological macromolecules or the formation of other RNS (Perwez Hussain, & Harris, 2007).

p53 is mutated or part of its regulatory circuit is functionally inactivated in most type of cancers, which highlights its effects in tumorigenesis prevention (Jin & Levine, 2001). The p53 protein is a sequence-specific DNA-binding, resulting in the transcriptional regulation of genes, which are involved in mediating key cellular processes such as DNA repair, cell-cycle arrest, senescence and apoptosis (Riley *et al.*, 2008). For unstressed cells, p53 protein is down-regulated by protein binding (e.g. MDM2, COP1 or JNK) which leads to p53 degradation through the ubiquitin/proteasome signalling. According to

the p53 up-regulated of most of these genes, p53 level is very low in normal cells. It occurs is involved in regulating the cellular antioxidant defence network and preventing or repairing mutations (Perwez Hussain& Harris, 2007). Increasing p53 activity caused by reactive species and extended oxidative stress affects the enhancing of reactive species production, thereby contributing to the responses such as cytostatic and pro-apoptotic effect of p53 to eliminate cells with mutations. Both pro-oxidant and antioxidant properties, relying on the level of reactive species or oxidative stress are thought to play important roles (Bensaad & Vousden, 2005). Inducible nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (COX-2) are responsible for the formation of nitric oxide (NO•), various reactive nitrogen species (RNS) and prostaglandin E_2 (PGE_2). These products are involved in different paths of cellular processes which lead to the development and progression of cancer (Figure 2.22).

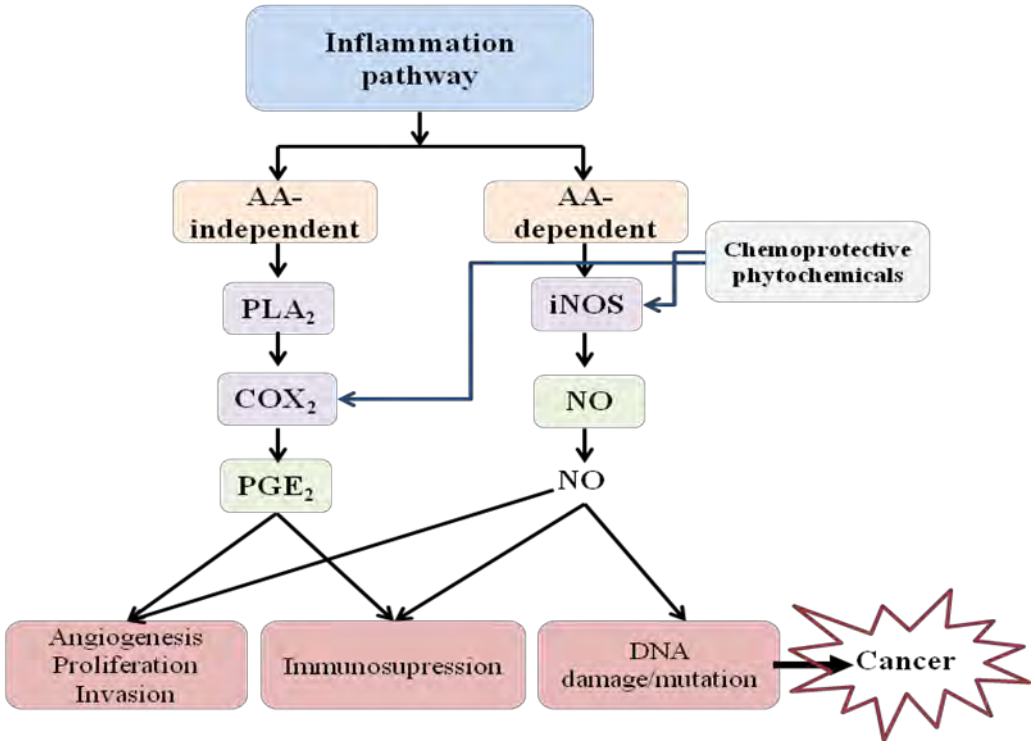


Figure 2.22 Signalling pathways involved in inflammation-induced cancer (adapted from Ohshima *et al*., 2005 and Philpott & Ferguson, 2004)

2.6.4.3 COX-2 and PGE_2

Arachidonic acid (AA) is an essential polyunsaturated fatty acid present in the phospholipids in particular phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositides of a number of physiological and pathophysiological processes. It is abundant in the brain muscles and liver membrane. The AA production, known as AA cascade derives from the phospholipid-bound form, by phospholipase A_2 (PLA_2) enzyme, cleaving the fatty acid off and can also be catalysed by diacylglycerol lipase and produce DAG as a product. AA can be catalysed by one of the key enzymes, cyclooxygenese (COX), lipooxygenase (LOX) or CYP450 to produce various metabolites (Hyde & Missailidis, 2009).

For instance, COX converts AA to produce prostaglandin H_2 (PGH_2) which is known as the precursor of the series-2 prostanoids. This reaction occurs through two active sites including a heme with peroxidise activity and a cyclooxygenase site. The first site is responsible for the changing from unstable PGG_2 to PGH_2 rapidly, while the second site involves in the conversion from AA to the hydroperoxy endoperoxide prostaglandin G_2 (PGG_2). Both pathways react via H atom abstraction from AA by a tyrosine radical generated by the peroxidise active site, which by the reaction of two oxygen (O_2) molecules and AA, produces PGG_2. Subsequently, PGH_2 then acts as the intermediate agentto produce all further substrates, catalysed by a number of cell-specific isomerises. These products include various prostaglandins such as PGE_2, prostaglandin D_2 (PGD_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin I_2 (PGI_2) and thromboxane A_2 (TXA_2) (Hyde & Missailidis, 2009).

Cyclooxygenase (COX) is an enzyme responsible to the formation of important biological mediators known as prostanoids (e.g. prostaglandins, prostacyclin and thromboxane). COX comprises two major isoforms including COX-1 mostly found in many tissues such as kidney, lung, stomach small intestine and colon, whereas COX-2 is undetectable in normal cells. COX-2 is known as an inducible key enzyme in inflammation, hypoxia and Wnt-signalling. COX-2 is catalyse conversion of arachidonic acid to prostaglandin E_2 (PGE_2), results in the increase of PGE_2 production during the inflammatory process (Dinarello, 2010). The epidemiological studies have reported the role of COX-2 as

amolecular target of cancers. The study has confirmed the protective effects of nonsteroidal anti-inflammatory drugs (NSAIDs), acted as the inhibitors of COX activity for colorectal cancers. COX-2 is overexpressed in early and advanced colorectal cancer tissues, which result in a poorer clinical outcome.

2.6.4.4 Prostaglandins

Prostaglandins are like hormones which are formed by most cells in the body. They act as autocrine and paracrine lipid mediators (e.g. they work at or immediately adjacent to their site of synthesis). Without storing, prostaglandins are synthesized *de novo* from membrane-released arachidonic acid (Figure 2.23) when cells are activated by mechanical trauma or by specific cytokine, growth factor, and other stimuli such as collagen and adenosine diphosphate (ADP) in platelets, bradykinin and thrombin in endothelium (Funk, 2001).

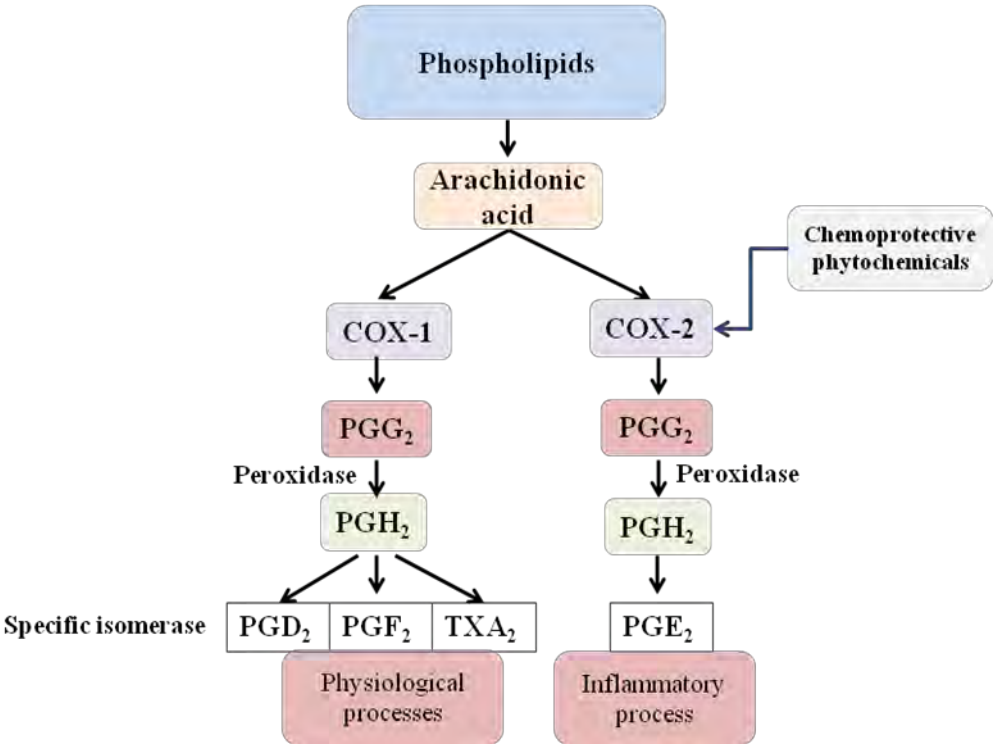


Figure 2.23 Arachidonic acid (AA) metabolism cascade via the cyclooxygenase (COX) pathway (adapted from Kwon *et al.*, 2007 and Hyde & Missailidis, 2009)

The isomer of COX, COX-1 and COX-2 enzymes is derived from arachidonic acid (AA), producing various specific isomerases of prostaglandins (PG) and thromboxanes (TXA). The constitutive COX-1 produces prostaglandin D_2 (PGD_2), prostaglandin F_2 (PGF_2) and thromboxane A_2 (TXA_2), which are involved in physiological processes. Prostaglandin E2 (PGE2), the main product released from inducible COX-2 is known as a key component that strongly relates to inflammatory pathway. Prostaglandins, in particular PGE_2 have also played as a key mediator in carcinogenesis, activating several downstream signalling pathways such as the epidermal growth factor receptor pathway (Han & Wu, 2005) and also include the modulation of immune responses, protection of the gastrointestinal mucosa, regulation of blood clotting and maintenance of renal homeostasis. These pathological processes have been identified on a basis of prostaglandins as well as inflammation, pain, fever and swelling (Greenhough *et al.*, 2009).

PGE_2 is produced by all cell types of the body, with epithelia, fibroblasts, and infiltrating inflammatory cells, representing the major sources of PGE_2 in an immune response (Kalinski, 2012). The study reported that the genetic/pharmacological disruption of PGE_2 receptors decreases tumour formation in colon carcinoma mouse models (Mutoh *et al.*, 2002). PGE_2 has also been reported to play a significant role in the protumourigenic effects of COX-2 in particular of colorectal cancerby increasing the levels of PGE_2 observed in both colorectal adenomas and carcinomas. The excentric COX-2 expression has occurred in the majority of colorectal cancers, accounting for more than 80%. NSADI known as the selective COX-2 inhibitor has been recognised to reduce the numbers of sporadic colorectal adenomas found in the study using a randomised double-blind placebo-controlled trial model (Greenhough *et al.*, 2009).

The alterations to cyclooxygenase-2 (COX-2) expression and subsequently the overexpression of its enzymatic product prostaglandin E_2 (PGE_2) has been accepted as a key role linked to the development of colorectal cancer. The mechanism exerts various number of pathways including promoting the proliferation, survival, angiogenesis, migration and invasion of tumours (Greenhough *et al.*, 2009). The interaction between PGE_2 and specific cell-surface G-protein-coupled receptors (EP1-EP4) and subsequent downstream signalling pathways is considered to be involved in the pro-survival,

proliferation and cell growth effects. PGE_2 has also been found to be involved in several proposed pathways such as the increase in levels of B-cell leukaemia/lymphoma 2 (Bcl-2) expression via the Ras-MAPK/ERK pathway, action through the PI3K/Akt pathway, cyclic adenosine monophasphate (cAMP)/proteinkinase A signalling and epidermal growth factor receptor (EGFR) signalling (Greenhough *et al.*, 2009; Vivanco & Sawyers, 2002). Wnt-signalling is now clearly thought to be able to contribute to human tumour progression (Polakis, 2012), which activates β-catenin/T-cell factor (TCF) signalling. The Wnt-pathway is believed as an important pathway, in particular for colorectal cancer in promoting the acquisition of a progenitor or stem cell-like phenotype contributing to the limitless replicative potential in cancer (Greenhough *et al.*, 2009).

2.6.4.5 Links between iNOS and COX-2

The co-operation between iNOS and COX-2 has been reported, which by iNOS specific bind with COX-2, producing COX-2 post-translationally by *S*-nitrosylation. As a result, the catalytic activity of COX-2 is increased, representing the potential synergistic molecular interactions between both key enzymes in inflammatory process (Khanapure *et al.*, 2007). The study using animal model has reported the interlink-effects between all three enzymes, contributingjointly to the development of cancer (Perwez Hussain, Hofseth & Harris, 2003).

2.7 Inhibitory activity against key enzyme relevant to metabolic syndrome

2.7.1 Metabolic syndrome

Metabolic syndrome is a condition of the biochemical processes involved in the normal functional condition of human body that increases the opportunity of developing a cardiovascular disease and diabetes. There are five conditions concerning metabolic risk factors, which occur individually or tend to occur together. At least three metabolic risk factors occurring together would result in metabolic syndrome diagnosis. These factors include abdominal obesity, high blood pressure, low blood levels of the good cholesterol (HDL), high blood levels of the bad cholesterol (LDL), high blood levels of triglycerides and insulin.

An exceptional metabolic syndrome is characterised by insulin resistance that occurs due to interaction of genetic and environmental factors. Each component of metabolic syndrome could play an important part in significantly increasing the risk of developing one or more diseases. For examples, the increasing risk of type 2-diabetes and heart disease were found to relate to the obesity. Hypertension is also the most important risk factor causing stroke, as well as heart disease and insulin resistance and can be the first step to type 2-diabetes, associated with the risk of developing heart disease, kidney disease and blindness.

A rapid growth of metabolic syndrome was reported in both children and adults with almost one-fourth of the developed world's population (International Diabetes Foundation). Australia has one of the highest prevalences of overweight and obesityamong developed countries, contributing to metabolic syndrome (IOTF, 2010). Approximately 29 percent of adults (age 25 and over) were classed to have a metabolic syndrome (Zimmet *et al.*, 2005). The body mass index (BMI) has commonly been used to measure the obesity condition, defined as weight-to-height ratio, and is considered to be a reasonable reflection of body fat. BMI is calculated by dividing body weight in kilograms by the square of height in metres (kg/m^2). Among adults, a person with a BMI greater than 25 kg/m^2 is considered overweight, while a BMI greater than 30 kg/m^2 is considered obese, which relates to a high risk of adverse health outcomes (Parliament of Australia, 2012).

Obesity is a central component of the metabolic syndrome, which encompasses factors such as abdominal adiposity, insulin resistance, hypertension and atherogenic lipid profiles (Bray & Champagene, 2004). There is also evidence that psychosocial health of overweight and obese individuals is affected (Wellman & Friedberg, 2002). Epidemiological studies show that increasing BMI is associated with greater risk of mortality and morbidity from conditions including non-insulin dependent diabetes mellitus, coronary heart disease, hypertension, hyperlipidemia, reproductive abnormalities, osteroarthritis, back pain and certain cancers (NHMRC, 2003; Wellman & Friedberg, 2002).

Obesity has emerged as a crisis in public health leading to hypertension, high blood pressure and cardiovascular diseases. In 2005, the cost of being overweight and obesity in Australia has been estimated at $21 billion annually (Colagiuri *et al*., 2010). It is important to understand the mechanism of suppression of obesity related to metabolic syndrome, which could positively impact on the future public health. Polyphenols, efficient antioxidant, were found to have the potential to suppress obesity by decreasing the level of lipid and protein oxidation (Figure 2.24).

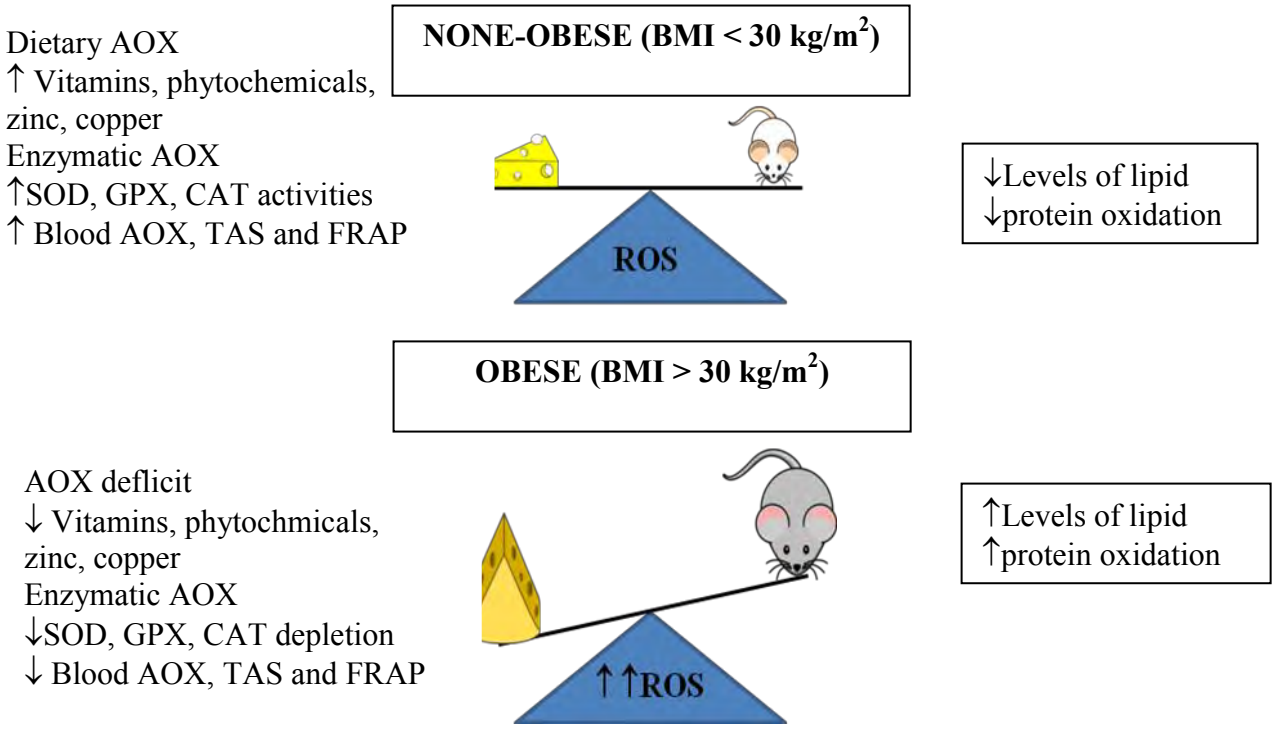


Figure 2.24 Prooxidant–antioxidant balance with non-obese status (top) and obesity (bottom) (adapted from Vincent & Taylor, 2006)

Increased dietary intake of antioxidants and tissue enzymatic and nonenzymatic antioxidants match the pro-oxidant processes with non-obese status. In obesity, an antioxidant deficit exists. Available antioxidants are overpowered by excessive ROS formation, shifting the system toward oxidative stress. ROS, reactive oxygen species; AOX, antioxidant; SOD, superoxide dismutase; GPX, glutathione peroxidase; CAT, catalase; TAS, total antioxidant status; FRAP, ferric acid reducing potential

2.7.2. Regulatory effect of phytochemicals on carbohydrates metabolism

Dietary carbohydrates involving starch and sugar are a staple of the human diet, and the oxidation of carbohydrates is the central energy-yielding pathway in most nonphotosynthetic cells. The studies on dietary polyphenols and their metabolites reported that these could influence and regulate the digestion, absorption and metabolism of carbohydrates (Hanhineva *et al.*, 2010). Most carbohydrates must be hydrolysed to small molecules, monosaccharides, in the upper gastrointestinal tract prior the occurrence of circulated absorption. The insulin will be secreted from the β-cells of the islets of Langerhans, the regions of pancreas containing its endocrine, system due to the excessive glucose concentration in blood stream. Insulin also plays a role in the uptake of glucose in peripheral tissue, muscle, adipose tissue and kidney. It elevates storage of glucose in liver in form of glycogen, accompanied with lipolysis inhibition occurring in adipose tissue. Glucagon, an essential hormone to maintain the glucose homeostasis that is secreted from the pancreatic α-cells, also plays a part in regulating the blood glucose level, when the glucose level is below normal.

Hanhineva *et al.*, (2010) presented possible mechanisms of regulation of glucose metabolism by dietary polyphenols. The mechanism involves multiple steps as follows: inhibition of carbohydrate digestion and glucose absorption in the intestine, stimulation of insulin secretion from the pancreatic β-cells, modulation of glucose release from liver, activation of insulin receptors and glucose uptake in the insulin-sensitive tissues, and modulation of hepatic glucose output (Figure 2.25). As a result, the glucose level in blood could be regulated and maintained.

The study of Thom (2007) has confirmed the ability of chlorogenic acid enriched instant coffee to induce a reduction in body fat and mass, resulting from decreasing of glucose absorption. The reduction of glucose absorption would ultimately lead to an increase in the consumption of fat reserves, due to the reduced availability of glucose as an energy source (Thom, 2007). Since coffee drinking and obesity appear co-existing in most developed societies the efficacy of these products in those already regularly exposed to caffeine remains to be demonstrated. However, it is important to note that a major consequence of blocking digestion of carbohydrates in the proximal gut is colonic

fermentation which leads to increased microbial production of gas in the bowel; this effect can limit its use (Thom, 2007). In addition, Ishikawa and co-workers (2007) reported that ingestion of leaves of an Indian plant *Nerium indicum* Mill., used as a folk remedy for type II diabetes, reduced postprandial blood glucose level in humans. In endemic Sri Lankan plant *Cassia auriculata* (Leguminosae), the α-glucosidase was also reported to be comparable to that of a therapeutic drug acarbose (Abesundara *et al.*, 2004).

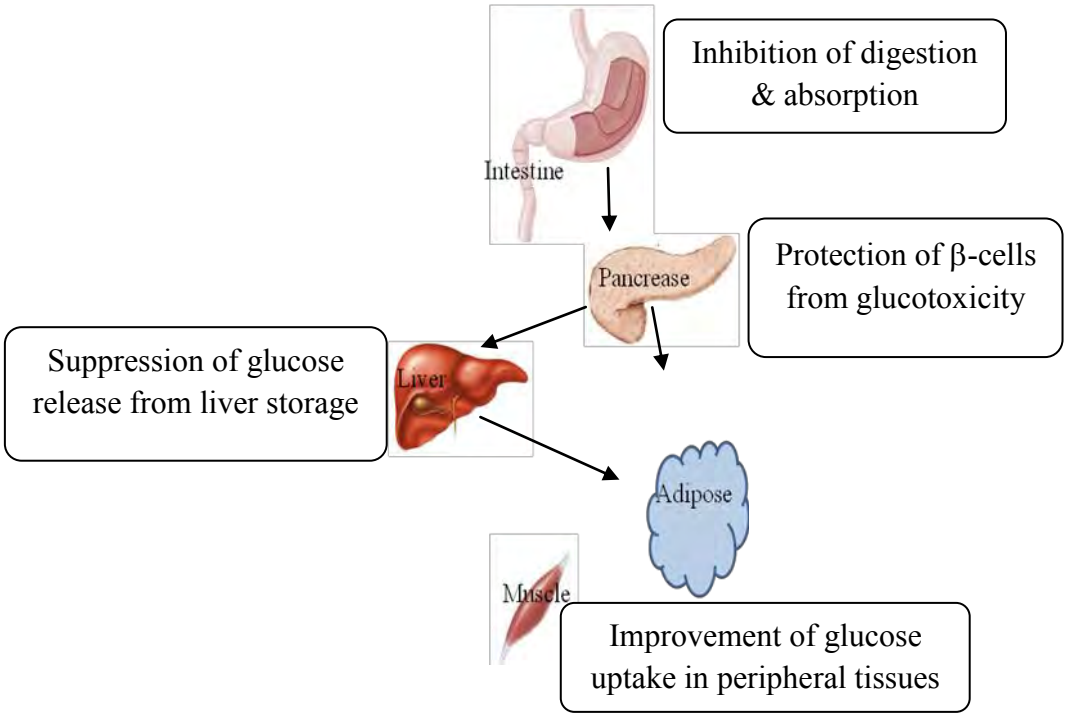


Figure 2.25 Potential sites of action of dietary polyphenols on carbohydrate metabolism and glucose homeostasis (adapted from Hanhineva *et al.*, 2010)

2.7.3. Regulatory effect of phytochemicals on lipids metabolism

Energy metabolism is originating from triacylglycerols hydrolysis, generating free fatty acids and glycerol, assisted by lipases enzymes. Glycerol will be absorbed and eventually rejoin fatty acids in the intestinal cells. Bile salts or bile acids, created by liver will interact with hydrophobic molecules to digest less soluble compounds such as fatty acids.

These components have been known to play an important role for fatty acid and fat-soluble vitamins (e.g. A, D, E, and K) absorption. Pancreatic lipase which is responsible for the majority of digestive action, uses as cofactor a small protein called co-lipase, which binds both to lipase and to the micelle surface. As a result, free fatty acids are produced and absorbed in the small intestine. Soluble fiber has been considered to bind with bile acids, which itself cannot be absorbed. Therefore, fiber-bound bile acids can eliminate cholesterol store in body leading to a reduction in serum cholesterol.

Grove and Lambert (2010) described a regulatory effect of tea on the metabolism of lipids. According to these authors, tea polyphenols play a role in inhibition of pancreatic lipase, phospholipase and SGLT1 in small intestine. They are also associated with decreasing *de novo* lipogenesis, resulting in increasing β-oxidation, GLUT1 and GLUT4 expression (Figure 2.26). Therefore, carbohydrate uptake will be reduced and modulate body weight and energy balance. *Cissus quadrangularis*, folk Indian plant, has also been reported to reduce serum triglyceride levels (Oben *et al.*, 2007).

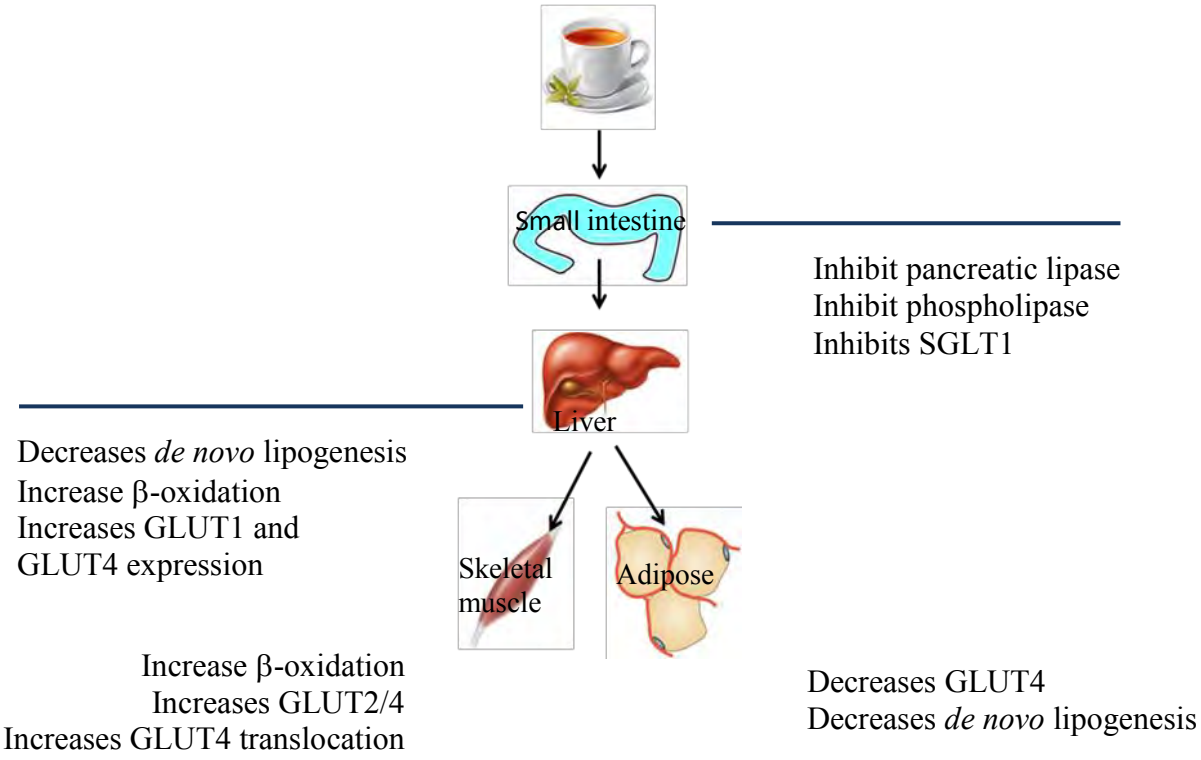


Figure 2.26 Potential sites of action of tea polyphenols on energy metabolism (adapted from Grove & Lambert, 2010)

The animal studies have suggested that the consumption of blueberries or its bioactive polyphenolic contents may provide several health benefits including protection against inflammation and modulation of obesity and adiposity (Lau *et al.*, 2007). In addition, the study of purified anthocyanins extracts from blueberries also highlighted the reduction of body and adipose tissue weight observed in high fat diet of C57BL/6J mice model compared to high fat-fed controls, supplemented with 2.9 mg/g purified anthocyanins extract (Cinti *et al.*, 2005). A similar result was also reported by Lumeng *et al.* (2007) where purified anthocyanins from blueberries lowered serum triglycerides, cholesterol and leptin levels excluding liver lipids and triglycerides levels.

2.7.4. Regulatory effect of phytochemicals on hypertension

Angiotensin converting enzyme (ACE) is an important enzyme for controlling blood pressure. Angiotensin I converting enzyme is a glycoprotein peptidyldipeptide hydrolase, which in its somatic form, has two active binding sites; N- and C-terminal (Actis-Goretta *et al.*, 2006). ACE catalyzes angiotensin I hydrolysis leading to the formation of vasoconstrictor, angiotensin II, resulting in high blood pressure (Shalaby *et al.*, 2006). Therefore, ACE inhibition is attributable to inducing antihypertensive effect.

Bioactive compounds from edible plants have been suggested to possess ACE inhibitory activity. Several studies haveinvestigated ACE inhibitory compounds from various sources (e.g. tea, berries and soybean) and have reported ACE inhibition activity. Zibadi *et al.* (2008) reported that pycnogenol, a proanthocyanin oligomer isolated from French maritime pine (*Pinus maritime* L.) was an effective mediator of blood pressure regulation in humans, possibly due to the inhibition of ACE. Kozuma *et al.* (2005) demonstrated blood pressure lowering effect in humans with mild hypertension of aqueous extracts of green coffee beans, containing chlorogenic acid as the main compound. The aqueous or alcoholic extracts from *Magnolia liliflora* and *Magnolia officinalis* herbs (Black *et al.*, 1996) as well as aqueous extracts of ginger (Ranilla *et al.*, 2010) have been reported to reduce blood pressure. Polyphenolics-rich extracts obtained from herbs were reported to modulate the activity of angiotensin I-converting enzyme (ACE) (Balasuriya & Rupasinghe, 2011).

The animal studies also reported the efficacy of polyphenol rich diet-induced antihypertension. Suzuki *et al.* (2002) demonstrated ACE inhibitory activity of pure chlorogenic acid in hypertensive rats. Potenza *et al.* (2007) studied the effect ofadministration of 200 mg/kg body weight EGCG for 3 weeks in spontaneously hypertensive rats, a rodent model of the metabolic syndrome. The result showed significant decrease in blood pressure, associated with insulin sensitivity and adiponectin levels increasing.

2.7.5 The role of inflammation in the metabolic syndrome

An increasing number of research papers reported that the development of obesity is accompanied by chronic, low-grade inflammation. This evidence is characterised by an accumulation of macrophages in the adipose tissue and the liver. The other evidences include an increasing release of inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) from these macrophages and increasing the levels of these cytokines in the blood stream.

The studies on obese humans and animal obesity models also reported similar changes. It is now widely accepted that metabolic disorders such as insulin resistance and cardiovascular disease are influenced by the presence of local and systemic inflammation. In addition, the metabolic syndrome is characterized by inflammation. Inflammatory markers, such as C-reactive protein (CRP), are predictors of metabolic syndrome and related events in humans. Several studies suggest that inflammation is the link between obesity and these related pathologies. Therefore, the strategy to reduce the risk of obesity-induced metabolic syndrome may be considered by reduction of inflammation.

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Equipment, chemicals and reagents

A list of chemicals and reagents used and their suppliers is shown in Table 3.1. All chemicals were of analytical, HPLC or molecular biology grade, where required. The list of equipment used in the experiments is shown in Table 3.2.

Table 3.1 List of chemicals, reagents and their suppliers

Chemicals	Supplier
Chemicals:	
AAPH [(2,2′-azobis (2-methylpropionamide) dihydrochloride]	Sigma-Aldrich, St. Louis, MO, USA
ABAP [(2,2′-azobis (2-amidinopropane) dihydrochloride]	Sigma-Aldrich, St. Louis, MO, USA
Acarbose glucobay	BayerAG, Leverkusen, Gemany
Acetic acid	Ajax Finechem, Taren Point, NSW, Australia
Acetonitrile	Honeywell International, Burdick & Jackson, Morristown, NJ, USA
Albumin from bovine serum (minimum 96%) electrophoresis	Sigma-Aldrich, St. Louis, MO, USA
Alexa Fluor 488 annexin V	Invitrogen Co., Carlsbad, CA, USA

Table 3.1 (continued)

Chemicals	Supplier
Aluminium chloride hexahydride ($AlCl_3 \bullet 6H_2O$)	Sigma-Aldrich, St. Louis, MO, USA
Angiotensin converting enzyme	Sigma-Aldrich, St. Louis, MO, USA
Annexin V- kit	InvitrogenCo., Carlsbad, CA, USA
Fetal bovine serum albumin (FBS)	Sigma-Aldrich, St. Louis, MO, USA
5-Bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP)	Sigma-Aldrich, St. Louis, MO, USA
Bromophenol blue	Sigma-Aldrich, St. Louis, MO, USA
Camptothecin	Sigma-Aldrich, St. Louis, MO, USA
Captopril	Sigma-Aldrich, St. Louis, MO, USA
Caspase 3 Assay Kit, Colorimetric	Sigma-Aldrich, St. Louis, MO, USA
(+)- Catechin hydrate	Sigma-Aldrich, St. Louis, MO, USA
Chlorogenic acid	Sigma-Aldrich, St. Louis, MO, USA
Cyanidin 3-glucoside	Sigma-Aldrich, St. Louis, MO, USA
Cytochalasin B	Sigma-Aldrich, St. Louis, MO, USA
DCF(2′,7′-dichlorofluorescin diacetate)	Sigma-Aldrich, St. Louis, MO, USA
D-(+) glucose solution (45%)	Sigma-Aldrich, St. Louis, MO, USA
Depex mouting medium	Sigma-Aldrich, St. Louis, MO, USA
Diethy pyrocarbonate (DEPC)	Sigma-Aldrich, St. Louis, MO, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, St. Louis, MO, USA
4-Dimethylaminocinnamaldehyde	Sigma-Aldrich, St. Louis, MO, USA
Ellagic acid	Sigma-Aldrich, St. Louis, MO, USA
Ethanol	Honeywell International
Ethidium bromide	Sigma-Aldrich, St. Louis, MO, USA
Fluorescein	Sigma-Aldrich, St. Louis, MO, USA
Folin-Ciocalteu reagent	Merck, Pty Ltd., Kilsyth, Vic, Australia
Formaldehyde	Sigma-Aldrich, St. Louis, MO, USA
Furanacroloyl-Phe-Glu-Glu (FAPGG)	Sigma-Aldrich, St. Louis, MO, USA
Gallic acid	Sigma-Aldrich, St. Louis, MO, USA

Table 3.1 (continued)

Chemicals	Supplier
Glucose CII-Test Wako	Wako Pure Chemical Industries, Osaka, Japan
Glycerol	Sigma-Aldrich, St. Louis, MO, USA
Griess reagent	Sigma-Aldrich, St. Louis, MO, USA
Hanks' balance Salt Solution (HBSS)	Sigma-Aldrich, St. Louis, MO, USA
Hemacolour Rapid staining	Merck, Pty Ltd., Kilsyth, Vic, Australia
Hesperidin	Sigma-Aldrich, St. Louis, MO, USA
Hydrochloric acid (HCl)	Ajax Finechem, Taren Point, NSW, Australia
Hydrogen peroxide (H_2O_2)	Sigma-Aldrich, St. Louis, MO, USA
Iron (III) chloride ($FeCl_3$)	Merck, Pty Ltd., Kilsyth, Vic, Australia
Iron (II) sulphate ($FeSO_4$)	Merck, Pty Ltd., Kilsyth, Vic, Australia
Lipopolysaccharide (LPS, E. Coli 055:B5)	Sigma-Aldrich, St. Louis, MO, USA
Luteolin	Sigma-Aldrich, St. Louis, MO, USA
Maleic acid buffer	Nacalia tesque, Kyoto, Japan
McIlvaine's buffer	Sigma-Aldrich, St. Louis, MO, USA
4-methylumbelliferyl oleate (4-MUO)	Sigma-Aldrich, St. Louis, MO, USA
Myricetin	Sigma-Aldrich, St. Louis, MO, USA
Methanol	Honeywell International
MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)	InvitrogenCo., Carlsbad, CA, USA
Nitro blue tetrazolium (NBT: tablet)	Sigma-Aldrich, St. Louis, MO, USA
N-[3-(2-Furyl)acryloyl]-Phe-Gly-Gly	Sigma-Aldrich, St. Louis, MO, USA
Orlistat	Sigma-Aldrich, St. Louis, MO, USA
Penstrep	InvitrogenCo., Carlsbad, CA, USA
PGE_2 enzyme immunoassay kit	Sapphire Biosciences, Redfern, NSW, Australia
Porcine pancreas	Sigma-Aldrich, St. Louis, MO, USA
Propidium iodide	InvitrogenCo., Carlsbad, CA, USA

Table 3.1 (continued)

Chemicals	Supplier
Protease inhibitor cocktail	Sigma-Aldrich, St. Louis, MO, USA
Quercetin	Sigma-Aldrich, St. Louis, MO, USA
Quercetin dihydrate	Sigma-Aldrich, St. Louis, MO, USA
Rat intestinal acetone powder	Sigma-Aldrich, St. Louis, MO, USA
Rutin	Sigma-Aldrich, St. Louis, MO, USA
Sodium carbonate (Na_2CO_3)	Ajax Finechem, Taren Point, NSW, Australia
Sodium chloride (NaCl)	Sigma-Aldrich, St. Louis, MO, USA
Sodium hydroxide (NaOH)	Sigma-Aldrich, St. Louis, MO, USA
Sodium nitrite ($NaNO_2$)	Sigma-Aldrich, St. Louis, MO, USA
S-9 mix	Wako Pure Chemical Industries, Japan
TPTZ (2,4,6-Tripyridyl-s-triazin)	Merck, Pty Ltd., Kilsyth, Vic, Australia
Trifluoroacetic acid (TFA)	Sigma-Aldrich, St. Louis, MO, USA
Trolox	Sigma-Aldrich, St. Louis, MO, USA
Tryple Express for trypsinization	Sigma-Aldrich, St. Louis, MO, USA
Trysin EDTA (10x)	Sigma-Aldrich, St. Louis, MO, USA
WST-1 for proliferation	Sigma-Aldrich, St. Louis, MO, USA
XAD-16 resin for purification	Sigma-Aldrich, St. Louis, MO, USA

Table 3.2 List of equipments and consumables

Item	Supplier
Autoclave bags	Sterrilope Australia
Blender	Waring Laboratory Science, Torrington, CT, USA
Cell culture flask (25 mL, 75 mL)	Corning Incorporated, NY, USA
Centrifuge	Sorvall RC-5B; DuPont, Wilmington, DE, USA

Table 3.2 (continued)

Item	Supplier
Centrifuge 5424	Eppendorf, AG, Hamburg, Germany
Disposable pasteur pipette	Chase Scientific glass Inc. Australia
Eppendorf (1.5 mL, 2.0 mL)	Eppendorf, AG, Hamburg, Germany
Falcon tube (15 mL, 50 mL)	Corning incorporated, NY, USA
FACSCalibur flow cytometer	Becton Dickinson, Franklin Lakes, NJ, USA
FlowJo software	TreeStar Inc., Ashland, OR, USA
Fluorescence spectrophotometer	Cary Eclipe; Varian, Inc., Palo Alto,
Glove	Nitratex, Australia
Graph Pad Prism (5.04)	GraphPad Software, Inc., CA, USA
Heating plate for tubes	Reacti-Therm Heating/stirring module, Thermoscientific, Rockford, USA
Hemocytometer	Reichert, Buttalo, NY, USA
HPLC: LC-10AD pumps, SPD-M10A diode array detector (DAD), CTo-10AS column oven, DGu-12A degasser, SIL-10AD autoinjector, and SCL-10A system controller	Shimadzu Corporation, Kyoto, Japan
Incubator	Sanyo, Japan
Individual pipette (5 mL, 10 mL, 25 mL)	Socorex, Swiss, Switzerland
Luna C18column (250 x 4.6 mm i.d., 5 µm)	Phenomenex, Torrance, CA, USA
Magnetic stirrer	John Morris Scientific Australia
Microscope slide (7.6 cm x 2.54 cm)	Hawksley, UK
Micropipette	Interpath, Australia
Microplate reader	Wallac 1420 Multilabel Counter;
Olympus BH-2	Olympus, Tokyo, Japan
Pipettes (2 µL, 5 µL, 200 µL, 1 mL)	Eppendorf, AG, Hamburg, Germany
pH meter	Sentron integrated Sensor technology
Quartz cuvettes	Starna Pty. Ltd., Baulkhan Hills, NSW,

Table 3.2 (continued)

Item	Supplier
	Australia
Rotary evaporator rotavapor R-205	Buchi, Switzerland
Rotor JA14 14,000 rpm serial no. 02U8152	Beckman, USA
Shaker	Titertek, Huntsville, AL, USA
Sonicator	Unisonic, Australia
Spectrophotometer	Labsystems Multiskan MS; Thermo Fisher Scientific, Waltham, MA, USA
Synergy UV	Millipore, Australia
Syringe filter hydrophilic (0.2, 0.4 μm)	Sartorius stedium biotech, Germany
Syringe single use	Terumo, Australia
XAD-16 resin column (300 x 60 mm i.d.)	Selby, Australia
Vortex	Selby, Australia
48-well microplate	Thermo Fisher Scientific, Australia
96-well microplate	Sarstedt Australia, Technology Park,
Slide	Living stone, Australia
Rack	Edwards, Australia
Counter	Upgreen, Taiwan
Tips	Quality Scientific plastic, USA

3.1.2 Collection of plant material

Commercial samples of Tasmannia Pepper Leaf (*Tasmannia lanceolata,* R. Br.), anise myrtle (*Syzygium anisatum*, Vickery, Craven & Biffen), lemon myrtle (*Backhousia citriodora,* F. Muell), quandong (*Santalum acuminatum)* and Davidson's plum (*Davidsonia pruriens*) were obtained from Australian Native Food Industry Ltd. Additionally, rabbit eye and southern highbush blueberries (*Vaccinum spp.*) used as reference samples for fruits were obtained from the Costa Exchange Ltd., Corrindi (NSW, Australia). Commercially available bay leaf (*Laurus nobilis* L., Lauraceae) (Hoyts Food

Industries Pty Ltd., Moorabbin, Victoria, Australia) was included as a reference sample for herbs.

3.1.3 Mammalian cell lines and culture media

All cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured at 37°C in a humidified 5% carbon dioxide (CO_2) atmosphere in media containing 10% foetal bovine serum (FBS), 100 µg/mL streptomycin and 100 units/mL penicillin (Invitrogen Corporation, Carlsbad, CA, USA) unless otherwise stated. AGS (gastric adenocarcinoma) was cultured in F12-K Ham's medium; BL13 (bladder cancer) was grown cultured in RPMI; CCD-18Co (colon normal) was cultured in Eagle's minimum essential medium (EMEM; Sigma-Aldrich); HepG2 (hepatocellular carcinoma) was cultured in EMEM; Hs 738.St/Int (mixed stomach and intestine normal) was cultured in Dulbecco's Modified Eagle's medium (DMEM); and RAW 264.7 (murine macrophage) was also cultured in Dulbecco's Modified Eagle's medium (DMEM); HT-29 (colorectal adenocarcinoma) was cultured in McCoy's 5a medium; HL60 (acute promyelocytic leukaemia) in Iscove's modified Dulbecco's medium (IMDM). Experiments were conducted at passages less than 40.

3.2 Preparation of polyphenolic-rich extracts

3.2.1 Preparation of lyophilised mixtures of bioactive compounds isolated from plant sources

The lyophilized extracts were prepares as described earlier (Konczak *et al.*, 2008). Briefly, the raw plant material of the native herbs and fruits was initially weighed and ground into a pulp using a heavy duty blender (Waring Laboratory Science, Torrington, CT, USA). A 2-fold volume of acidified ethanol (80% ethanol, 19% H_2O and 1% Acetic acid, v/v) was then added, stirred for 2 h at low temperature (4°C) and centrifuged for 20 min at 10,000 rpm at 4°C (Sorvall RC-5B; DuPont, Wilmington, DE, USA; rotor Beckman JA14 14,000 rpm serial no. 02U8152 USA. The supernatant was collected and the extraction was repeated twice. The third extraction was carried out overnight. The supernatants from the consecutive extractions were combined and the solvent evaporated

under reduced pressure at 40°C using a rotary evaporator (Rotavapor R-205; Buchi, Switzerland).

The concentrated alcoholic extract was further purified using an XAD-16 resin column (300 x 60 mm i.d.). The extracts were dissolved with acidified water (99% H_2O, 1% acetic acid, v/v), applied to the column, washed with acidified water and eluted with 80% ethanol (80% ethanol, 19.9% H_2O, 0.1% trifluoroacetic acid, v/v). The eluate was collected and evaporated under reduced pressure at 37°C using a rotary evaporator. The purification was repeated. The resulting fraction was dissolved in purified water and freeze-dried under vacuum to obtain a fine lyophilized powder representing a polyphenolic-rich fraction. The extraction yield was calculated as a percentage of the original raw plant material according to the formula: Yield (%) = (LF x 100)/DL, where LF was the weight of lyophilised fraction (g) and DL was the weight of the extracted sample (g).

3.3 Determination and quantification of purified polyphenolic-rich extracts

3.3.1 Total phenolic content (Folin-Ciocalteu assay)

The total phenolic content of the native plants was investigated using the Folin-Ciocalteu assay as describe by Konczak *et al.* (2010a). The plant extracts diluted (1:50) in distilled water, were added to Folin-Ciocalteu reagent diluted (1:10) in distilled water in a 96-well microplate (Sarstedt Australia, Technology Park, SA, Australia) and shaken for 3 min. Absorbance was measured at 600 nm using a spectrophotometer (Labsystems Multiskan MS; Thermo Fisher Scientific, Waltham, MA, USA), to account for an ascorbic acid correction. Following this, 6% Na_2CO_3 was added, and the microplate shaken for a further 15 min. The absorbance was then measured at 600 nm. The total phenolic content of the samples was expressed as gallic acid equivalents per gram dry weight of the lyophilised powder (µmol GAE/gDW), based on gallic acid standard curve, and standardised against a blank control in triplicate wells.

3.3.2 Determination of total flavonoid content

The total flavonoids content was performed as described by Michalska *et al.* (2007). Briefly, extracted samples were diluted (1:5) in 80% methanol water followed by adding 50 µL of 5% $NaNO_2$ and incubated at room temperature for 6 min. Following this, 300 µL of 10% $AlCl_3 \bullet 6H_2O$ solution was added and further incubate at room temperature for another 5 min. One mL of 1M NaOH was then added, mixed with vortex and absorbance read at 510 nm using spectrophotometer (Labsystems Multiskan MS; Thermo Fisher Scientific, Waltham, MA, USA). The total flavonoid content of the extracts was calculated and expressed as +(-) cathechin hydrate equivalents per gram of dry weight (mg Cat E/gDW) based on catechin standard curve.

3.3.3 Determination of proanthocyanidin content

Anthocyanidin formation in a hydrochloric medium with ferric ammonium sulphate as catalyst was performed as explained by Li *et al.* (1996). The proanthocyanidin content was expressed as milligrams of +(-) cathechin hydrate equivalents per gram of dry weight (mg Cat E/gDW) based on catechin standard curve.

3.3.4 Determination of ellagitannins and quantification of ellagic acid level

Acid hydrolysis of the extracts and quantification of ellagic acid was performed according to da Silva Pinto *et al.* (2008). Briefly, ten milligrams of polyphenol-rich extract were dissolved in 2 mL of 2N trifluoroacetic acid (TFA) in a pyrex glass tube. The tube was placed into the heating instrument (Reacti-Therm Heating/stirring module, Thermoscientific, Rockford, USA) and incubated at 120°C for 2 h. Subsequently the solution was transferred into 5 mL volumetric flask and 80% methanol was used to adjust the volume. The sample was mixed well and analysed using HPLC. The levels of ellagic acid and derivatives were quantified as ellagic acid equivalent (mg EA E/gDW) based on ellagic acid calibration curve at 250 nm.

3.3.5 Quantification of phenolic compounds (HPLC-DAD)

Quantification of phenolic compounds and anthocyanins in the purified polyphenolic extracts was conducted according to Kammerer *et al.* (2004) and Terahara *et al.* (2000) with minor modifications. The HPLC system consisted of two LC-10AD pumps, SPD-M10A diode array detector (DAD), CTo-10AS column oven, DGu-12A degasser, SIL-10AD autoinjector, and SCL-10A system controller (Shimadzu Corporation, Kyoto, Japan) equipped with a 250 x 4.6 mm i.d., 5 µm Luna C18(2) column (Phenomenex, Torrance, CA, USA). The following solvents in water with a flow rate of 1.0 mL/minwere used: A, 0.5% triflouroacetic acid (TFA) in water and B, 95% acetonitrile and 0.5% TFA in water. The elution profile was a linear gradient elution for B of 10% over 10 min followed by an increase to 50% over 45 min, and to 80% over 5 min. The column was washed with 100% solvent B for 15 min. Analytical HPLC was run at 25°C and monitored at 280 nm (hydroxybenzoic acids and flavanols), 326 nm (hydroxycinnamic acids, stilbenes), 370 nm (flavonols) and 520 nm. Hydroxybenzoicacids and flavanols were quantified as gallic acid equivalents (GA E), cinnamic acids were quantified as chlorogenic acid equivalents (CHA E), flavonols and stilbenes were quantified as rutin equivalents (R E) and anthocyanin compounds were quantified as cyanidin 3-glucoside equivalents (C3G E). The results are presented per gram of dry weight (e.g. mg C3G E/gDW) of the lyophilized polyphenolic-rich extract.

3.4 Antioxidant activity studies

3.4.1 Ferric Ion Reducing Antioxidant Power (FRAP) assay

Total reducing capacity was determined using the FRAP assay conducted according to Konczak *et al.*, 2010a. The FRAP reagent was initially prepared consisting of 10 mL of 300 mmol/L acetate buffer, 10 mL of 20 mmol/L $FeCl_3$ and 1 mL of 10 mmol/L TPTZ solution. The acetate buffer (pH 3.6) consisted of 3.1 g of sodium acetate and 16 mL acetic acid per litre of water. The TPTZ solution consisted of 31.2 mg of TPTZ in 10 mL HCl. Initially, the samples diluted in water, were added to FRAP reagent in a 96-well microplate (Sarstedt Australia) and shaken for 15 sec. After incubation for 8 min, the absorbance was measured at 600 nm using a spectrophotometer (Labsystems Multiskan

MS; Thermo Fisher Scientific). The reducing capacity of the samples was expressed as micromoles of Iron (II) per gram dry weight of the lyophilised powder (μmol Fe^{2+}/gDW) based on an Iron (II) sulphate standard curve, and standardised against a blank control in triplicate wells.

3.4.2 Oxygen Radical Absorbance Capacity (ORAC) assay

Oxygen radical scavenging capacity was determined using the ORAC assay according to Konczak *et al.* (2010a). Initially, preparations of fluorescein (120 nM) and AAPH (360 mM) were formulated in phosphate buffered saline (PBS; 75 mM, pH 7.0). Fluorescein and diluted sample was added to quartz cuvettes (Starna Pty. Ltd., Baulkhan Hills, NSW, Australia) and inserted into a fluorescence spectrophotometer (Cary Eclipe; Varian, Inc., Palo Alto, CA, USA) and allowed to equilibrate at 37°C under rigorous stirring. Following this, AAPH was added to each cuvette and fluorescence measurements initiated immediately. Fluorescence (λ_{ex} = 495 nm; λ_{em} = 515 nm) was recorded every 5 seconds until the fluorescence reached zero and a kinetic curve generated. The area under the curve (AUC) was integrated and standardised against a blank control. The measurements were carried out in triplicate. The antioxidant capacity of the samples was expressed as μmol Trolox equivalents per gram dry weight of the lyophilised powder (μmol Trolox E/gDW) based on a Trolox standard curve.

3.4.3 Cellular Antioxidant Activity (CAA) assay

The cellular antioxidant activity (CAA) was conducted according to Tan *et al.* (2011a) and Wolfe & Liu (2007). Initially, HepG2 cells ($1x10^5$/mL) were incubated for 24 h at 37°C in 96-well clear-walled microplates (Thermo Fisher Scientific, Sydney, Australia). Subsequently, the medium was then removed and the wells were gently washed with PBS. A range of concentrations of purified extracts were treated in four replicates. Following this, 80 μL of PBS and 10 μL of 250 μM DCFH-DA solutions were then added to each well and incubated for 1 h. The plate was then drained and washed with PBS. The 100 μL of 600 μM ABAP was added to the cells in 100 μL of HBSS. The plate was placed into fluorescence (excitation wavelength 495 nm, emission wavelength 515

nm, 37°C) and was measured every 5 min for 1 h. After blank subtraction, the area under curve for fluorescence versus time was integrated to calculate the CAA value at each concentration of plants (Wolfe & Liu, 2007); see equation 3.1

$$CAA\ unit = 100 - (\int SA / \int CA) x 100 \quad (3.1)$$

where $\int SA$ is the integrated area under the sample fluorescence versus time curve and $\int CA$ is the integrated area from the control curve. The median effective dose (EC_{50}) was then determined for the plants from the graph pad prism 5 and/or Microsoft Excel. The EC_{50} values were expressed as mean ±SD for triplicate set of data obtained from the same experiment. EC_{50} values were converted to CAA values, expressed as micromoles of quercetin equivalents (Q E) per gram of dry weight.

3.4.4 Cellular protection against H_2O_2 induced cell death (MTT assay)

According to anti-proliferative activity against cancer cell lines, the different concentrations of phenolic compounds considered as having no effect on cell growth (0 – 0.6 mg/mL) were selected (García-Alonso *et al.*, 2006). Cell sensitivity of RAW 264.7 and HepG2 cells to native Australian plant extracts was determined via cell viability using the colourimetric MTT assay. Initially, cells (5 x 10^5/mL) were incubated for 24 h at 37°C in 96-well clear-walled microplates (Thermo Fisher Scientific, Sydney, Australia), before treatment with a range of concentrations of purified polyphenolic-rich extracts for 23 h, followed by the adding of H_2O_2 (20 mM) for a further 1 h. Wells were then gently washed with 200 µL PBS, 5 µL with MTT (HT-29 & CCD-18Co) and/or premix WST-1 solution (AGS & BL-13) added and the plate was then further incubated for 4 h and 2 h, respectively. The microplate was then shaken for 10 min and absorbance measured at 450 nm using a spectrophotometer.

3.5 Pro-apoptotic anticancer activity studies

3.5.1 Cell viability using MTT assay

The MTT assay was conducted as described previously (Tan *et al.*, 2011b). Initially, cells (5 x 10^5/mL) were incubated for 24 h at 37°C in 96–well clear–walled microplates (Thermo Fisher Scientific, Sydney, Australia). Subsequently a range of concentrations of purified polyphenolic-rich extracts were applied over 24 h. Next, the medium and samples were removed from each cell and the wells were gently washed with PBS. A 100 μL of PBS and 10 μL of 5 mg/mL MTT solution were then added to each well and the cultures were further incubated for 4 h. The MTT formazan product was dissolved with dimethyl sulfoxide (DMSO). The plate was shaken for 10 min and the absorbance was measured at 595 nm using a spectrophotometer. The results were expressed as the optical density ratio of the treatment to control. At least 6 measurements were conducted for each treatment.

3.5.2 Measurement of apoptosis by flow cytometry

The analysis has been conducted according to Tan *et al.* (2011b). For the dose response, HL–60 cells (5 x 10^5/mL) were plated in 25cm^2 culture flasks and treated with purified polyphenolic-rich extract at concentrations of 0.4, 0.8 and 1.6 mg/mL for 6 h, with untreated cells as a control. Following the required incubation time, cells were harvested, stained and analysed, as previously stated.

For the time response experiment HL–60 cells (5 x 10^5/mL) were plated in 25 cm^2 culture flasks and treated with 0.4 mg/mL purified polyphenolic extract for 3, 12 and 24 h, with untreated cells as a control. Cells were then harvested, washed with cold PBS and resuspended in annexin-binding buffer before staining with Alexa Fluor 488 annexin V and propidium iodide (Invitrogen Corporation) for 15 min at room temperature. The samples were then analysed immediately after staining using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and FlowJo software (TreeStar Inc., Ashland, OR, USA). A total of 20,000 events were acquired for each measurement and the cells were properly gated for analysis.

3.5.3 Determination of caspase-3 activity

Caspase-3 activity was measured using a colorimetric caspase-3 assay kit (Sigma-Aldrich), according to the manufacturer's instructions. Apoptosis was induced in HL−60 cells with treatment of 0.8 mg/mL purified polyphenolic-rich extract for 24 h, with untreated cells as a control. As per the assay kit protocol, the cells were harvested and washed with PBS, resuspended in lysis buffer, and incubated on ice for 20 min. Cell lysates were collected by centrifugation and analysed immediately. The lysates were incubated at 37°C with the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) in the presence and absence of the inhibitor Ac-DECF-CHO in a 96−well microplate (Thermo Fisher Scientific). Hydrolysis of the substrate Ac-DEVD-pNA was induced by caspase-3, causing the release of the p-nitroaniline (pNA) moiety. Caspase activity was directly proportional to the level of pNA released and quantified spectrophotometrically at 405 nm using a microplate reader (Wallac 1420 Multilabel Counter; PerkinElmer).

3.5.4 Cytokinesis-block micronucleus cytome (CBMN Cyt) assay

The cytokinesis-block micronucleus cytome (CBMN Cyt) assay was conducted using the cytochalasin B technique as described by Fenech (2007) with minor modifications, to measure the different endpoints in untreated and purified polyphenolic extract treated cells. HT-29 (5 x 10^5/mL) treated with 0.5 and 1.0 mg/mL concentrations of purified polyphenolic-rich extract were cultured at 37°C in 48-well plates (Thermo Fisher Scientific) for 24 h. The medium was changed and extract was applied into wells including control. Following this, the treatment was removed and replaced with fresh medium containing 4.5 µg/mL cytochalasin B for exactly 24 h to inhibit the cell division after mitosis stage. Cells were then harvested using Tryple Express (or 0.02% v/v Trypsin, Sigma Aldrich), and deactivated with 500 µL medium without additive. Following this, cells were resuspended, centrifuged and removed medium. Twenty microlitre of fixative (methanol) were then used to resuspend before applied cells to a microscope slide (7.5 cm x 2.5 cm) by dropping method. Cells were fixed and stained with Diff-Quik stains (Sigma, NSW, Australia), air-dried and coverslipped with Depex medium (Sigma, USA). All slides were coded to avoid bias in slide-scoring, and were

analysed by a trained single scorer to ensure consistency in scoring. An Olympus BH-2 (Olympus, Tokyo, Japan) light microscope was used at 100x magnification using an oil immersion lens, with sufficient light and precise focus to ensure clear vision of each cell observed.

Scoring was performed according to Fenech (2007). The biomarkers scored included frequency of binucleated (BN) cells with micromuclei (MN-BN), with nucleoplasmic bridges (NPB), with nuclear buds (NBud) and frequency of necrotic and apoptotic cells. The nuclear division index (NDI) was calculated from the ratio of mono-, bi- and multinucleated cells (Eastmond & Tucker, 1989). A total of 500 cells were scored per slide to determine ratios of mononucleated cells, binucleated cells, multinucleated cells, necrotic and apoptotic cells. A total of 500 binucleated cells were scored per slide to determine frequency of MNi, MN-BN, NPB and NBud. Each treatment concentration and control was assessed in duplicate.

3.6 Anti-inflammatory activity studies

3.6.1 Measurement of nitrite concentration

Nitrite concentration in culture supernatant was determined by Griess reaction according to Uto *et al.* (2005) with minor modifications. Initially, HepG2 cells (3×10^5 per well) were incubated for 24 h at 37°C in 48 well plates (Thermo Fisher Scientific, Australia). Fresh serum-free medium was then added for 2.5 h to eliminate the influence of FBS. The cells were treated for 1 h with a range of concentrations of purified polyphenolic-rich extracts before exposure to 40 ng/mL LPS for 12 h. Equal volumes of the culture supernatant were mixed with modified Griess reagent for 15 min at room temperature in the absence of light. Nitrite concentration was measured by absorbance levels at 540 nm against a sodium nitrite standard curve using a spectrophotometer.

3.6.2 Measurement of PGE_2 production

Prostaglandin E_2 (PGE_2) concentration in culture supernatant was determined with a PGE_2 enzyme immunoassay kit (Sapphire Biosciences, Redfern, NSW, Australia)

according to the manufacturer's instructions. Initially, HepG2 cells (5 x 10^5 per well) were incubated for 24 h at 37°C in 6 well plates. Fresh serum-free medium was then added for 2.5 h to eliminate the influence of FBS. The cells were treated for 1 hwith a range of concentrations of purified polyphenolic-rich extracts before exposure to 40 ng/mL LPS for 12 h. The level of PGE_2 released into the culture medium was determined by measuring absorbance levels at 412 nm using a spectrophotometer against a PGE_2 standard curve.

3.7 Metabolic syndrome

3.7.1 α-glucosidase inhibitory assay

The α-glucosidase inhibitory activity was calculated by measuring the amount of glucose hydrolysed from sucrose according to Matsui *et al.* (2001). Initially, rough enzyme solution was prepared using rat intestinal acetone powder (Sigma Aldrich, St. Louis, Mo., USA) as the source of α-glucosidase. One hundred milligrams of rat intestinal acetone powder added to 1 mL of 0.1M maleic acid buffer (pH 6.0; Nacalia tesque, Kyoto, Japan). The mixture was then homogenised using ultrasonicator for 6 min (30 sec sonication and 30 sec rest cycle), followed by centrifugation at 3000 rpm for 30 min. The supernatant was collected and diluted two times with 0.1 M maleate buffer by, and was used as the enzyme solution for the maltase reaction. The 2% sucrose (Sigma-Aldrich) solution was prepared in maleic acid buffer.

The stock solution of the native Australian plant extracts (12 mg/mL) was prepared in maleic acid buffer. The solution was sonicated for 20 min and then filtered using 0.45 µm filter. From these stock solutions, a gradient of concentrations was prepared via serial dilution in maleic acid buffer (final concentration: 0.1, 0.5, 1.0, 2.0 and 4.0 mg/mL). The varying concentrations of native Australian fruit extracts were then combined with equal volumes of rough enzyme liquid and 2% maltose. Blank controls, negative controls and maltose controls were also included. Blank controls consisted of maleic acid buffer, sample concentration and rough enzyme liquid. Negative controls consisted of 2% maltose or 2% sucrose, maleic acid buffer and rough enzyme liquid. Maltose or sucrose

controls consisted of 2% maltose or 2% sucrose and maleic acid buffer only. Acarbose (final concentration: 0.01, 0.1, 0.5, 1.0, 2.0 and 20.0 mg/mL), which was used as a positive control, were diluted with 0.1 M maleate buffer. Next, 20 µL of sample solution and 20 µL of maltose solution were mixed.

The enzyme reaction was initiated by adding 20 µL of the enzyme solution and then vortexed briefly, followed by heating at 37 °C for 1 h in a water bath. Following this, the mixtures were boiled 100°C for 10 min. Then 20 µL of mixture were added with colour reagent (Glucose CII-Test Wako, Wako Pure Chemical Industries, Osaka, Japan). The colour reagent when mixed with a sample, determines the level of transformation of glucose from α-form to β-form through the formation of a red pigment. The mixtures were then incubated at 37°C for a further 5 min and the absorbance read at 505 nm using a spectrophotometer (Labsystems Multiskan MS; Thermo Fisher Scientific, Waltham, MA, USA). The α-glucosidase inhibitory activities of the samples were expressed as glucose equivalents based on a glucose standard curve (Wako Pure Chemical Industries). The rate of α-glucosidase inhibition was calculated as a percentage of the control using equation 3.2:

$$\% \quad inhibition = (A_C - A_S)A_C x100 \qquad 3.2$$

Where A_C is the absorbance of the control and A_S is the absorbance of the sample after subtracting the absorbance of the blank.

3.7.2 Pancreatic lipase inhibitory assay

The inhibition of lipase activity was determined by measuring the amount of 4-methylumbelliferone released from 4-methylumbelliferyl oleate (4-MUO) by pancreatic lipase (type II) from porcine pancreas (Sigma, Australia) according to Shimura *et al.* (1992). Initially, stock solutions of the sample extracts were prepared at a concentration of 12 mg/mL. A gradient of concentrations was then prepared via serial dilution in McIlvaine's buffer. The porcine lipase enzyme was dissolves in McIlvaine's buffer, pH = 7.4, at concentration 0.085 g/mL. The solution was centrifuged (10min/10,000g) and the enzyme containing supernatant was collected. Fifty µL of the supernatant were used for each reaction mixture. The varying concentrations of sample extracts in McIlvaine's

buffer were combined with 0.1 mM 4-methylumbelliferyl oleate (4-MUO; Sigma-Aldrich, Australia) dissolved in DMSO. Blank controls, positive control and an inhibitor control were also included. Blank controls consisted of Mcllvaine's buffer and 4-MUO. The control consisted of buffer, 4-MUO and lipase. The inhibitor control consisted of sample, DMSO and lipase. Each mixture was added to the tubes, vortexed and incubated at 37°C for a further 20 min. Following incubation, 1 mL of 0.1N HCl was added to terminate the reaction. Subsequently the pH of the mixture was adjusted to 4.3 with a help of 2 mL of 0.1 M sodium citrate. The amount of 4-methylumbelliferone released by the lipase was measured fluorometrically (excitation wavelength 495 nm, emission wavelength 515 nm, 37°C) using the Cary Eclipse (Varian, Inc., Palo Alto, CA, USA) fluorescence spectrophotometer. The lipase inhibition levels were determined by fluorescence values as a percentage of the control (see equation 3.3).

$$\% \quad inhibition = \left(\frac{(A_{cb} - A_c) - (A_{sb} - A_s)}{(A_{cb} - A_c)}\right) x100 \qquad 3.3$$

3.7.3 Angiotensin converting enzyme (ACE) assay

Angiotensin converting enzyme (ACE) plays an important part in regulation of blood pressure and normal cardiovascular function. It catalyses the conversion of angiotensin I to angiotensin II, leading to an increase of blood pressure. ACE-inhibition also prevents formation of angiotensin II, thus lowering blood pressure, which was carried out as described in Shalaby *et al.* (2006). Moreover, it is a glycoprotein peptidyldipeptide hydrolase, with main known functions being to cleave histidyl-leucine from antiogensin I and forming the potent vasoconstrictor angiotensin II, and degrading bradykinin to inactive peptides (Dzau, 2001). The ACE inhibition assay was carried out as described by Shalaby *et al.* (2006) using furanacroloyl-Phe-Glu-Glu (FAPGG) as a substrate and the results were expressed as a percentage of ACE inhibition.

3.8 Statistical analysis

The mean of results were calculated based on at least three replicates in three independent experiments (n = 3) with corresponding standard deviations (SD). ANOVA and Tukey's

post hoc analysis were conducted to assess differences between the samples at the level of $p < 0.05$. All IC_{50} values were calculated from the corresponding dose inhibition curve according to their best fit shapes based on at least four reaction points using Microsoft Excel. Statistical correlation analyses were performed using Graphpad Prism 5.04 (Graphpad Software, CA, USA). Results for correlation analysis were considered statistically significant when the $p < 0.05$.

Chapter 4

Composition of purified polyphenolic-rich extracts obtained from native Australian herbs and fruits

4.1 Introduction

Preparation of purified polyphenolic-rich extracts, extraction of bioactive compounds from plant material is the first step in analysis of their identity and their characterisation. To facilitate efficient extraction, at first plant materials in various forms, such as dried, frozen or fresh, are homogenised by blender, to increase the surface area and subsequently, the contact between extracting solvent and sample (Wang & Weller, 2006). Ethanol is frequently used as it is safe for human consumption, efficient and widely applied solvent (Prior *et al.*, 2001; Shi *et al.*, 2005). In this study, an acidified ethanolic solvent has been used. Addition of acid into solvent is an optional step, and it is recommended when anthocyanins are present in order to prevent their degradation. For this purpose both, weak acids and strong acids are suggested to be used. Revilla *et al.* (1998) and Nicoue *et al.* (2007) stated that trifluoroacetic acid (TFA) at a level of 0.5 - 3.0% or hydrochloric acid (HCl) at concentration of less than 1% should be used for anthocyanin extraction. In order to remove some other unwanted components such as sugar and pectin, purification using an open-column chromatography (XAD-16HP particles) is recommended (Figure 4.1) (Naczk & Shahidi, 2004). After purification, the sample is condensed and freeze-dried under vacuum to obtain a fine lyophilized powder representing polyphenolic-rich fraction, which subsequently is subjected to analysis.

Hydrolysis is frequently used to simplify the chromatographic analysis of ellagitannins with acid hydrolysis. Strong inorganic acids such as hydrochloric acid (HCl) and trifluoroacetic acid (TFA) are normally chosen for this treatment, with acid concentration between 1 to 2 N and 30 min to 2 h reaction time, cleaved glycosidic bound between phenolic compounds and sugar molecules linked to structure (da Silva Pinto *et al.*, 2008).

Raw material

Pulp

Blend (Waring laboratory Science), USA

Extraction

Centrifuging

Sorvall RC-5B; Dupont, USA

Purification

Condensation

Rotavapor R-205; Buchi, Flawil, Switzerland

Freeze-dring

Phenolic rich-fraction

Figure 4.1 Extraction process of purified phenolic-rich extract obtained from Davidson's plum

Total phenolic content (TPC) assay, which utilises the Folin-Ciocalteu (F-C) reagent, is a well-known method that provides a convenient, rapid, cost efficient and reproducible estimation of total phenolic content in food products and biological samples (Huang *et*

al., 2005). This method relies on the transfer of electron in alkaline medium from phenolic compounds to phosphormolybdic/ phosphotungstic acid complexes. As a result, the colour of reaction mixture changes from yellow to blue, which can be monitored spectrophotometrically. While the Folin-Ciocalteu method has been considered useful to determine total content of phenolics from plant food, it is non-specific, it detects simultaneously all groups of phenolic compound in extracts, and beside phenolic compounds, other phytochemicals may also participate in the reaction, such as vitamin C or sugars, which may affect the final result (Naczk & Shahidi, 2004). The high performance liquid chromatography (HPLC) method allows to separate, tentatively identify and quantify individual phenolic compounds and therefore has been recognised as the standard practice for quantification of phenolics (Becker *et al.*, 2004). HPLC coupled with diode array detector (HPLC-DAD) is the most accurate and prevalent approach for analysis of phenolic compounds (Naczk & Shahidi, 2004; Robbins, 2003).

Application of liquid chromatography mass spectrometry (LCMS) allows compounds identification. LC/MS has become a powerful technique providing useful information of molecular formula and structural elucidation of the constituents of extracts. LC/MS has been recognised as the best technique for identification and characterisation of phenolic compounds from plant extracts (Flamini, 2003). Identification and quantification of phenolic compounds obtained from native Australian herbs and fruits used in this study have been performed using LC/MS in the preliminary research (Konczak *et al.*, 2010a; Konczak *et al.*, 2010b).

4.2 Results

4.2.1 Extraction yields

The extraction yields of purified polyphenolic-rich extracts obtained from herbs and fruits evaluated in this study, presented as a percentage of the original raw plant material, are shown in Table 4.1. Among herbs, TPL produced the highest yield, which was approximately 162% that of bay leaf used as a reference sample for herbs. The extraction yields obtained from LM and AM were similar to that of BL. Both native Australian

fruits: DP and QD produced similar amounts of purified polyphenolic-rich extracts, which were double of these of the reference samples, blueberries.

4.2.2 Phenolic content

4.2.2.1 Total phenolic content

The levels of phenolic compounds in plant samples (Table 4.1) were quantified using reagent-based assay and HPLC. The results obtained from the reagent-based F-C method indicate that TPL originated extract had the highest total phenolics that was 3-fold higher than that of a reference sample for herbs, BL extract. AM and LM had respectively 2.3-fold and 2-fold higher level of total phenolics than BL. DP extract contained the highest level of phenolics, superior to all evaluated extracts. QD had similar level of total phenolic content as blueberries. The result suggests that among the evaluated plants sources TPL among herbs and DP among fruits are the richest sources of phenolic compounds.

The quantification of phenolic compounds conducted using HPLC shows lower values than identified using the F-C assay. The HPLC quantification revealed that DP extract contained approximately 4-times less phenolics than indicated by the F-C values, and REB and SHB had respectively, 30 and 37% less phenolics than indicated by the F-C values. QD had 42% less phenolics than indicated by the F-C value. Similarly, the extracts of LM, AM and TPL contained respectively 65%, 55% and 60% less phenolics than indicated by the F-C values.

4.2.2.2 Total flavonoids

The total flavonoids contents in the evaluated extracts ranged from 134.3 ± 21.2 to 352.5 ± 3.1 mg catechin hydrate equivalent per gram dry weight (Table 4.1). Among herbs, the highest level of flavonoids was found in TPL extract (1.6-fold that of BL), followed by AM extract (1.3-fold that of BL). LM extract contained less total flavonoids than BL and had the lowest total flavonoids among all extracts. In contrast, the highest total flavonoids

Table 4.1 Yield and total phenolics (TP) content in extracts of native Australian herbs and fruits

Sample	Yield (%)	Total Phenolics (F-C) (mg G E[1]/gDW)	Phenolic compounds (HPLC) (mg/gDW)				Total	Total flavonoids (mg Cat E[5]/gDW)	Total proanthocyanidins (mg Cat E/gDW)
			280 nm (GA E[1])	326 nm (CHA E[2])	370 nm (R E[3])	520 nm (C3-G E[4])			
Herbs									
Anise myrtle	4.93	729 ± 26[b]	74.2 ± 5.2	25.7 ± 1.5	230 ± 19	ND	329.9	213.5 ± 13.4[b]	31.0 ± 0.0[b]
Lemon myrtle	5.74	661 ± 59[b]	15.7 ± 2.0	35.3 ± 3.4	181 ± 35	ND	232	134.3 ± 21.2[c]	16.3 ± 3.9[c]
Tasmannia pepper leaf	8.52	912 ± 58[a]	ND[6]	359 ± 18	ND	0.43 ± 0.05	359.43	255.9 ± 3.3[a]	16.1 ± 2.5[c]
Bay leaf	5.24	319 ± 5.8[c]	N/A[7]	N/A	N/A	N/A		161.5 ± 3.6[c]	48.5 ± 3.0[a]
Fruits									
Davidson's plum	10.1	949 ± 199[a]	156 ± 31	ND	21.9 ± 0.7	46.4 ± 2.1	224	352.5 ± 3.1[a]	382.5 ± 10.0[a]
Quandong	10.2	543 ± 18[b]	ND	313 ± 18	ND	2.02 ± 0.2	315.02	246.6 ± 4.5[b]	10.0 ± 2.5[b]
Rabbit eyes blueberry	4.47	504 ± 29[b]	ND	160 ± 4.9	ND	233.1 ± 5.2	393.1	187.4 ± 1.8[c]	23.3 ± 6.3[b]
Southern highbush blueberry	5.66	551 ± 19[b]	ND	161 ± 14	ND	169 ± 20	330	172.5 ± 2.2[d]	15.8 ± 3.8[b]

All data represent the mean ± standard deviation of at least three independent experiments. Values in each column with different superscripts are significantly different ($p < 0.05$), calculated using ANOVA and Tukey's post hoc.[1]GA E: Gallic acid equivalent. [2]CHA E: Chlorogenic acid equivalent. [3] RE: Rutin hydrate equivalent. [4]C3-G E: Cyanidin 3-glucoside equivalent. [5]Cat E: Catechin hydrate equivalent. [6]ND: not detected.[7]N/A: not available.

level was observed in DP extract, which was 2-fold that of blueberry extracts. The level of flavonoids in QD was also higher than that in blueberry extracts.

4.2.2.3 Total proanthocyanidins

The data clearly outline the richest source of proanthocyanidin - DP (382.5 ± 10.0 mg Cat E/gDW), superior to all other extracts, while quandong had the lowestlevel of proanthocyanidin (10.0 ± 2.5 mg Cat E/gDW) (Table 4.1). In case of herbs, all extracts had lower level of total proanthocyanidin than BL.

4.2.2.4 Quantification and identification of phenolic compounds by high performance liquid chromatography

4.2.2.4.1 Phenolic compounds in purified polyphenolic-rich extracts of herbs

The major compounds detected in purified polyphenolic-rich extracts obtained from herbs and fruits are presented in Table 4.2. Identification by liquid chromatography mass spectrometry and quantification of phenolic compounds present in native Australian herbs and fruits evaluated in this study have been descried earlier (Konczak *et al.*, 2010a; Konczak *et al.*, 2010b); and this study is based on earliear results and represents its continuation. The major compounds identified in the purified polyphenolic-rich extracts included phenolic acids (ellagic acid, chlorogenic acid) and flavonoids (quercetin, myricetin, rutin, hesperetin, anthocyanins). The HPLC analysis revealed that the major phenolic compounds of Tasmannia pepper leaf extracts are phenolic acid and flavonoids (Figure 4.2A).

Table 4.2 Phenolic compounds identified in Tasmannia pepper leaf, anise myrtle and lemon myrtle (mg/gDW)

Compound	Anise Myrtle	Lemon Myrtle	Tasmannia pepper leaf
Ellagic acid	152.9 ± 0.7	102.0 ± 5.8	ND
Ellagic acid derivatives*	514.0 ± 10	359.9 ± 27	ND
Chlorogenic acid	ND	ND	288 ± 10
Catechin	17.3 ± 4.5	ND	ND
Quercetin**,♦	29.1 ± 4.9	31.3 ± 6.2	45.6 ± 4.4
Quercetin 3-rutinoside♦	ND	ND	68.3 ± 9.4
Myricetin♦	1.04 ± 0.2	1.20 ± 0.2	ND
Hesperetin♦	4.10 ± 0.6	5.37 ± 1.1	ND
Rutin	ND	ND	ND
Cyanidin 3-glucoside◊	ND	ND	0.37 ± 0.01
Cyanidin 3-rutinoside◊	ND	ND	0.02 ± 0.001

*Ellagitannins and ellagic acid glycosides were quantified as ellagic acid equivalent following hydrolysis based on the peak area at 250 nm; **Includes quercetin glycosides with the exception of quercetin 3-rutinoside; ♦Myricetin, hesperetin, quercetin and derivatives were quantified as quercetin 3-rutinoside equivalent based on the peak area at 370 nm; ◊Cyanidins were quantified as cyanidin 3-glucoside equivalent.

Chlorogenic acid was the main component of TPL extract present at the level of 288.2 ± 10.2 mg/gDW. TPL contained the highest level of quercetin and quercetin derivatives (113.9 ± 13.8 mg/gDW) among all evaluated extracts. Trace of anthocyanins, cyanidin 3-glucoside and cyanidin 3-rutinoside were also identified. Quercetin was the dominating compound in AM and LM extracts, with 29.1 ± 4.9 and 31.3 ± 6.2 mg/gDW, respectively (Figure 4.2). Catechin was presented only in AM (17.3 ± 4.5 mg/gDW). Another HPLC chromatogram of AM extract is shown in Figure 4.3.

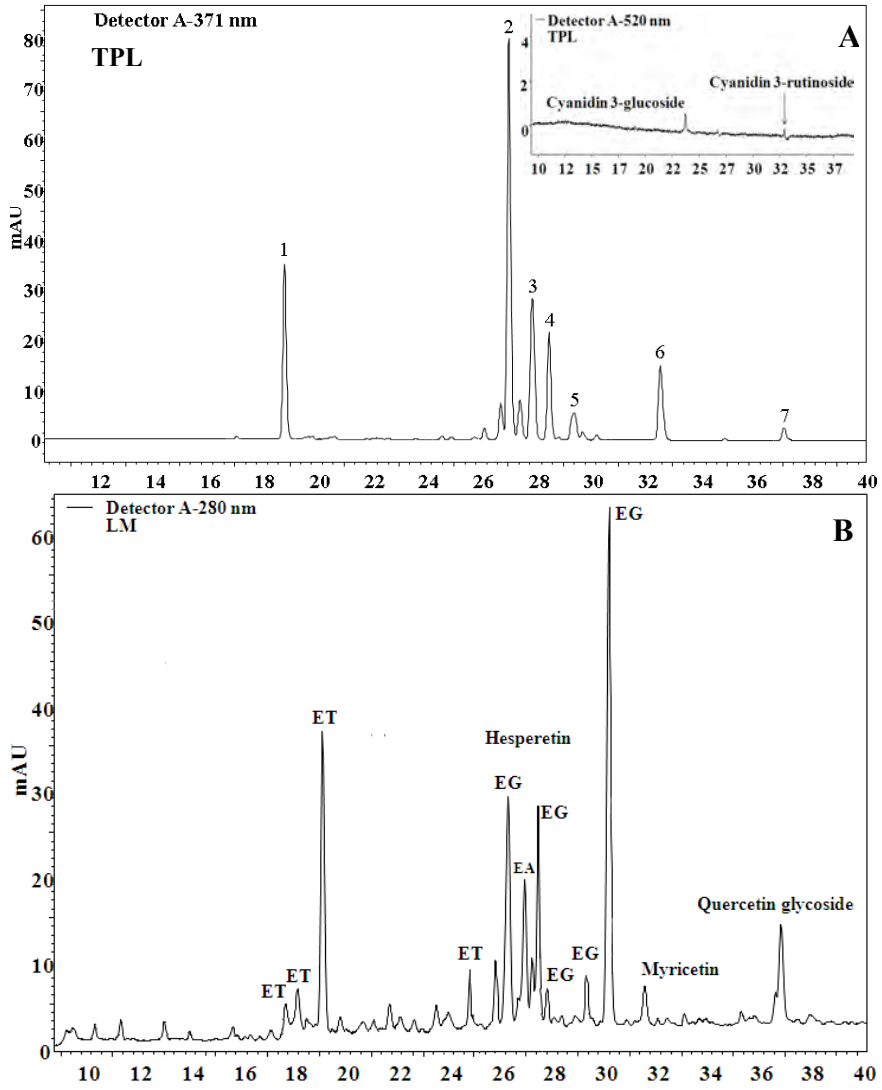


Figure 4.2 HPLC chromatography of purified polyphenolic-rich extracts obtained from herbs: Tasmannia pepper leaf (A) and lemon myrtle (B). TPL chromatogram: 1 and 3 – chlorogenic acid, 2 – quercetin 3-rutinoside, 4, 5, 6, 7 – quercetin glycosides; insert represents the HPLC chromatogram of anthocyanins. ET: ellagitannin. EG: ellagic acid glycoside.

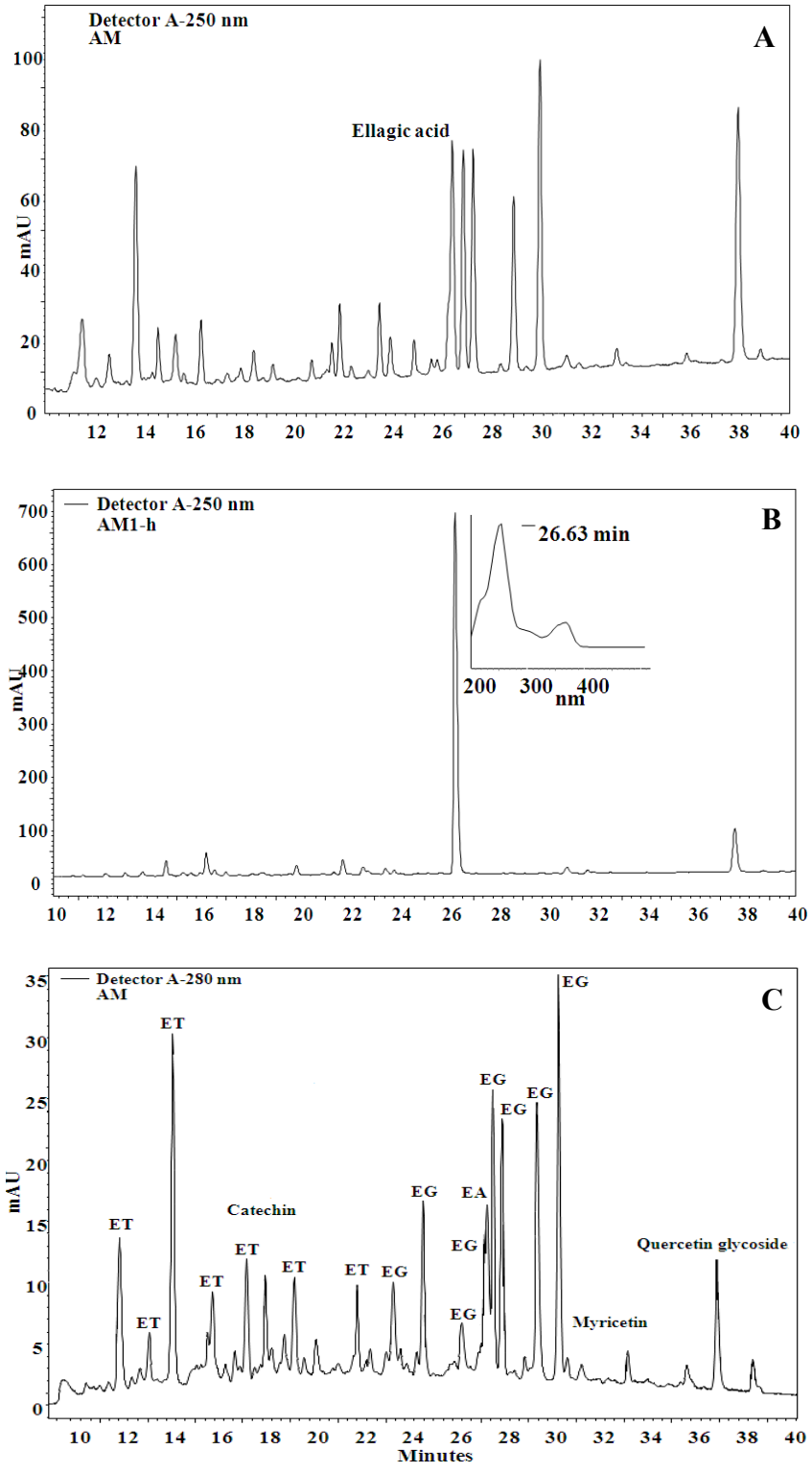


Figure 4.3 HPLC chromatogram at 250 nm of AM extract before hydrolysis (A) and after hydrolysis (B). Insert represents a spectrum of ellagic acid. At 280 nm (C); ET: ellagitannin. EG: ellagic acid glycoside.

Acid hydrolysis performed on the purified polyphenolic-rich AM, LM and DP extracts resulted in the disappearance of multiple peaks and release of an extremely high level of ellagic acid (Figure 4.3B), indicating the presence of ellagic acid derivatives. These derivatives were tentatively identified based on their UV spectral properties (Aaby *et al.*, 2005; Zafrilla *et al.*, 2001; Lee & Talcott, 2004 and Mullen *et al.*, 2002). The UV spectral data suggested presence of two groups of ellagic acid derivatives. The first group was characterised by a spectrum with maximum absorption only below 280 nm (Figure 4.4). These spectral properties characterise true ellagitannins, hydrolysable conjugates containing one or more hexahydroxydiphenoyl (HHDP) groups, which are highly polar and therefore elute on the reverse phase HPLC chromatogram at earlier retention times (Figure 4.4).

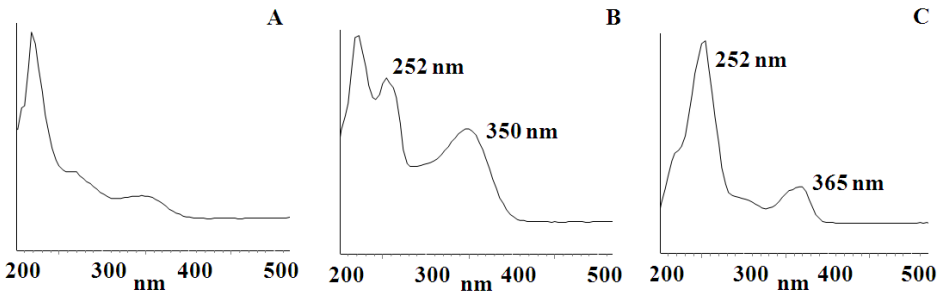


Figure 4.4 Typical UV spectra of (A) ellagitannin, ET; (B) ellagic acid glycoside, EG and (C) ellagic acid found in anise myrtle

The second group of peaks had UV spectra with maximum absorption at 252 nm and 350 nm (Figure 4.4B), which is similar to that of free ellagic acid (252 nm and 365 nm) (Figure 4.4C) and is a typical feature of ellagic acid glycosides (Aaby *et al.*, 2005; Lee and Talkott, 2004; Zafrilla *et al.*, 2001) (Figure 4.3B & 4.3C). These compounds are less polar than ellagitannins and therefore, elute on the reverse phase HPLC chromatogram at later retention times (Aaby *et al.*, 2005). Their various retention time and similar but not identical UV spectra suggest differences in the substitution of the phenolic hydroxyl groups of the ellagic acid nucleous, most probably various sugar moieties. Ellagic acid and its derivatives were also identified in lemon myrtle (Table 4.2).

4.2.2.4.2 Phenolic compounds in purified polyphenolic-rich extracts of fruits

Anthocyanins, 3-sambubiosides of delphinidin, cyanidin, peonidin, pelargonidin, petunidin and malvidin coupled with flavonoids (myricetin, quercetin rutinoside and quercetin hexoside) were identified in DP extract (Figure 4.6A & B). Antocyanins represented significant part of the DP extract, contributing approximately 5% of the polyphenolic-rich mixture (Table 4.3). Flavonoids: rutin (5.91 ± 0.4 mg/gDW) and myricetin (9.87 ± 0.6 mg/gDW) were the other main constituents of DP extract. Similar to AM and LM, DP chromatogram showed a significant 'hump' at 280 nm, indicating presence of polymeric compounds (Figure 4.5). Acid hydrolysis of the extract resulted in the disappearance of the hump and multiple peaks and an appearance of a large peak visible at 250 nm (Figure 4.5B), which, based on spectral data and co-chromatography with ellagic acid, was identified as ellagic acid (Figure 4.5).

Based on spectral characteristics: the maximum absorption only below 280 nm, the peaks that appeared before 26 min retention time and disappeared after acid hydrolysis, were tentatively identified as true ellagitannins (ET, Figure 4.5B), hydrolysable conjugates containing one or more hexahydroxydiphenoyl (HHDP) groups (Aaby *et al.*,2005). Only 2 peaks (26.8 min and 27.2 min retention time) had an additional absorption peak at 365 nm, which suggests that these peaks represent ellagic acid glycosides (Aaby *et al.*, 2005; Lee and Talkott, 2004; Zafrilla *et al.*,2001). Ellagic acid (36.4 ± 5.0 mg/gDW) and ellagic acid derivatives (145 ± 7.2 mg/gDW) identified in DP extract were the major constituents and accounted for 18% of the total phenolics (Table 4.3).

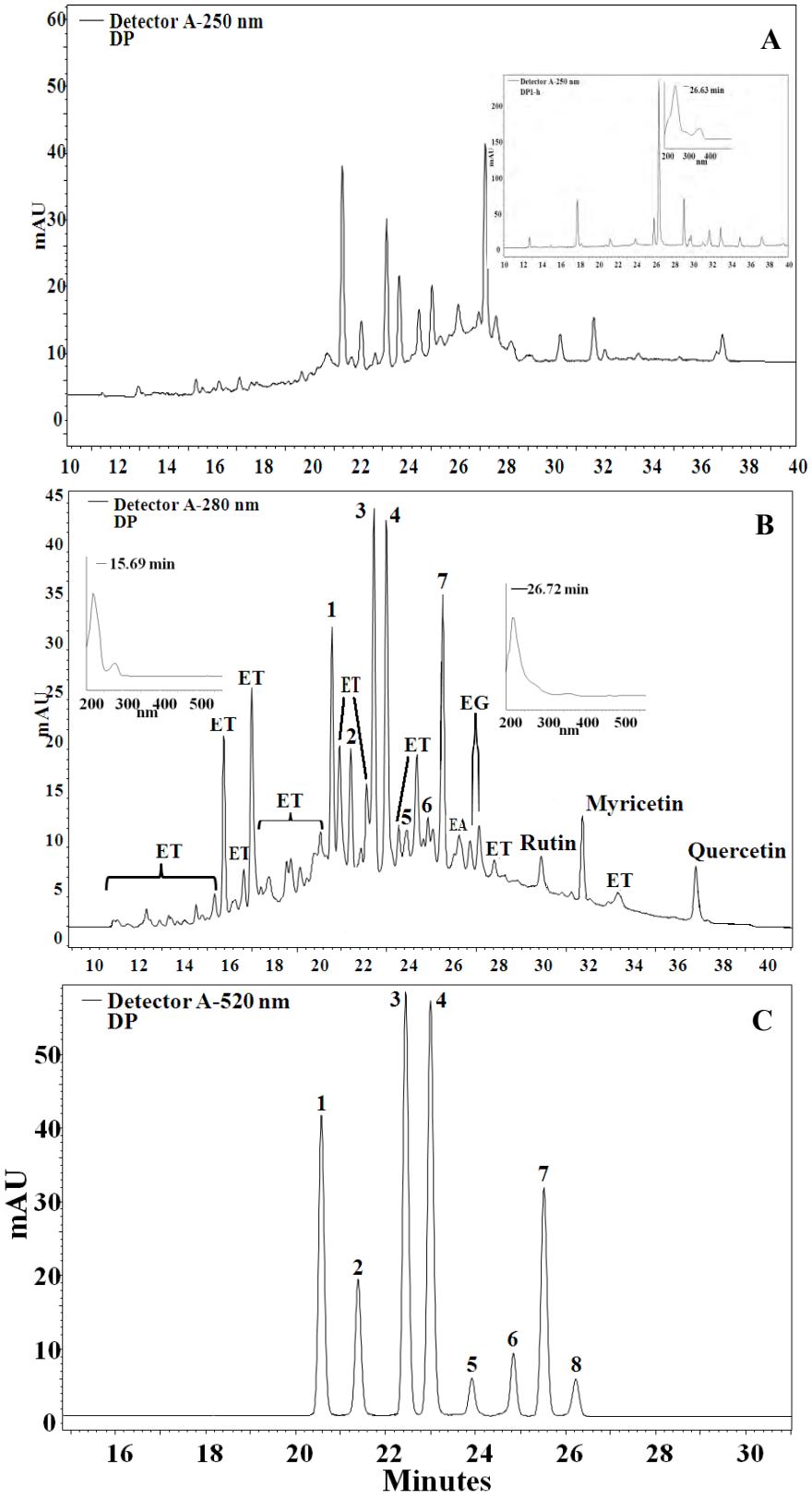


Figure 4.5 Chromatogram of purified polyphenolic-rich extract obtained from Davidson's plum. A) before hydrolysis; insert after hydrolysis at 250 nm; B) at 280 nm;ET: ellagitannin; EA: ellagic acid; EG: ellagic acid glycoside; R: rutin; M: myricetin; Q: quercetin.; C) 1: delphinidin 3-sambubioside; 2: cyanidin 3-sambubioside; 3: pelargonidin 3-sambubioside; 4: peonidin 3-sambubioside; 5 and 6: unknown anthocyanins; 7: malvidin 3-sambubioside, 8: unknown anthocyanin.

Table 4.3 Phenolic compounds identified in Davidson's plum and quandong (mg/gDW)

Compound	Davidson's plum	Quandong
Ellagic acid	36.4 ± 5.0	ND
Ellagic acid derivatives*	145 ± 7.2	ND
Chlorogenic acid	ND	259 ± 7.2
Catechin	ND	ND
Quercetin**,♦	6.08 ± 0.4	9.9 ± 0.6
Quercetin 3-rutinoside♦	ND	ND
Myricetin♦	9.87 ± 0.6	ND
Hesperetin♦	ND	ND
Rutin	5.91 ± 0.4	ND
Cyanidin 3-glucoside◊	ND	1.73 ± 0.02
Cyanidin 3-rutinoside◊	ND	ND
Delphinidin sambubioside	9.17 ± 0.05	ND
Cyanidin sambubioside	4.3 ± 0.2	ND
Pelargonidin sambubioside	12.7 ± 0.6	ND
Peonidin sambubioside	13.0 ± 0.6	ND
Malvidin sambubioside	7.22 ± 0.4	ND

*Ellagitannins and ellagic acid glycosides were quantified as ellagic acid equivalent following hydrolysis based on the peak area at 250 nm; **Includes quercetin glycosides with the exception of quercetin 3-rutinoside; ♦Myricetin, hesperetin, quercetin and derivatives were quantified as quercetin 3-rutinoside equivalent based on the peak area at 370 nm; ◊Cyanidins were quantified as cyanidin 3-glucoside equivalent.

In contrary to DP extracts, monomeric compounds dominated in QD extract, with hydroxycinnamic acids as the major constituents. Chlorogenic acid was the major compound and contributed to 13.3% dry weight of the mixture and was followed by an isomer of coumaric acid, which contributed 12.6% (Figure 4.6, Table 4.3). With regards to the anthocyanin content, only cyanidin-3 rutinoside identified as cyanidin has been detected in the quandong extract (Table 4.3). Two hydroxycinnamic acids were detected at 14.84 and 17.09 min as the major constituents in QD extract (Figure 4.6). Chlorogenic

acid was the major compound and contributed 13.3% to the dry weight of the mixture and was followed by an isomer of coumaric acid, which contributed 12.6%

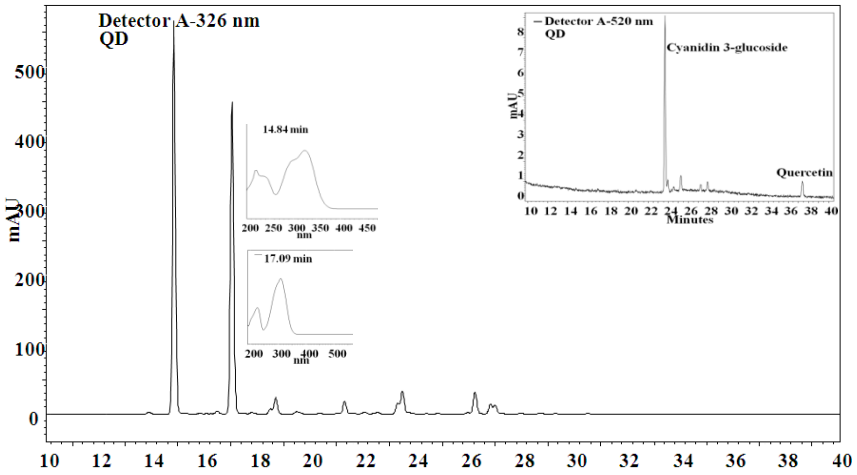


Figure 4.6 HPLC chromatogram of purified polyphenolic-rich extract obtained from quandong, QD. Chromatogram: chlorogenic acid; inserts represent cyaniding 3- glucoside and quercetin.

4.3 Discussion

TPL among herbs as well as DP and QD among fruits had higher extraction yield than the yield of respective reference samples. This result suggests that these selected native Australian herbs and fruits may serve as a good source of phenolic compounds.The evaluation of total phenolic content by F-C method showed higher level of total phenolic than their evaluation by HPLC. This is probably due to the presence of other interfering compounds such as sugars, ascorbic acid, aromatic amines and unanticipated phenols (Singleton & Rosi, 1965). This result shows that despite purification of the extracts, other compounds that could interfere in the reaction were still present in the polyphenolic-rich extracts prepared in this study. The composition of purified polyphenolic-rich extract of TPL and DP obtained within this study resembled composition of crude extract of Tasmannia pepper berry and leaf reported earlier (Konczak *et al.*, 2010a). Similarly, DP had the highest level of total phenolics content and exhibit superior to all evaluated extracts in this study, which shows in the results obtained by Konczak *et al.* (2010b). The

results suggested that the extraction and purification method applied in the present study did not induce major changes in the composition of phenolic compounds.

This study identified for the first time presence of ellagic acid and ellagitannins in AM and LM. Both plants belong to the Myrtaceae family and the presence of ellagic acid and polymeric compounds in the leaves of these extracts is consistent with the order Myrtales (Bate-Smitth, 1962). The same compounds were detected in other plants extracts of Myrtaceae family such as clove (*Syzygium aromaticum* (L.) Merrill & Perr) (Shan *et al.*, 2005) and *Syzygium glomeratum*, S. *venosum* and S. *Mauritanum* (Neergheen *et al.*, 2006). Ellagitannins are usually present in small berry fruits such as raspberries (sanguiin H-6 and lambertianin C) (Borges *et al.*, 2010), strawberry (Seeram *et al.*, 2006) and grape (McDougall *et al.*, 2005). Ellagitannins identified in raspberry and cloudberry account for 77 – 88% of the total phenolic compounds (Hakkinen *et al.*, 1999). Ellagitannins and derivatives were also detected as major components in Davidson's plum.

Beside ellagitannins, the presence of flavonoids was reported for the leaves of *Marlierea grandiflora* Berg, Myrtaceae, of southeast Brazil (myricetin 3-rhamnoside, quercetin, quercitrin, ellagic acid and 3-*O*-mythylellagic acid; Amaral *et al.,* 2001). The presence of myricetin is the typical feature of the Myrtaceae family (Gornall *et al.,* 1979). Anthocyanins were identified in DP with small amount in QDand TPL. Anthocyanins are the compounds responsible to various colours of plant organs (fruits, flowers, tubers, leaves, grains) that vary from pink to red to purple and blue. For example they are the predominant phenolic compounds of raspberry and blueberry (Borges *et al.*, 2010). DP is a large, intensely pigmented, crimson fruit. Recently, it has been evaluated as a novel source of natural food colour for food industry (Jensen *et al.*, 2011).

Chorogenic acid was the major compound indentified in both TPL and QD. Chlorogenic acid is one of the most abundant phenolic compounds found in fruits, vegetable and coffee and contributes toward their antioxidant capacity. In addition, ellagic acid and derivatives are gaining popularity as highly bioactive compounds (Landete, 2011). These compounds have been extensively studied for their health benefit. Consequenly, identification of phenolic compounds in particular ellagitannins and ellagic acid in native

Australian herbs and fruits may provide valuable information for food/nutraceutical industry with regards to their utilisation in health-enhancing products.

4.4 Conclusion

The results presented in this chapter can be summarised as the follows:

(i) Native Australian herbs (AM and LM) and fruit (DP) have been identified as new sources of ellagic acid and ellagitannins.

(ii) TPL has been identified as exceptionally rich source of chlorogenic acid.

(iii) Native Australian herbs and fruits evaluated in this study represent superior sources of phenolic compounds to the respective reference samples: BL, REB and SHB.

Chapter 5

Antioxidant capacity of purified polyphenolic-rich extracts obtained from native Australian herbs and fruits

5.1 Introduction

Application of more than one antioxidant testing method has been suggested to provide a comprehensive prediction of antioxidant efficacy of food and biological systems (Frankel & Meyer, 2000: Prior *et al.*, 2005). This is because both, foods and biological samples represent complex matrices, where various factors affect the antioxidant activity of compounds, including the colloidal properties of the substrates, the conditions and stages of oxidation and the localisation of antioxidants in different phases (Frankel & Myer, 2000). To evaluate the antioxidant capacity of polyphenolic-rich extracts, Ferric Reducing Antioxidant Power (FRAP) assay and Oxygen Radical Absorbance Capacity (ORAC) assay were selected. These two reagent-based *in vitro* assays are widely used as they are fast, efficient, inexpensive and simple. FRAP assay assesses antioxidant power of phytochemicals through reduction of ferric to ferrous ion at low pH, which results in development of a purple colour of ferrous-tripyridyltriazine complex (Benzie & Strain, 1996). The Oxygen Radical Absorbance Capacity (ORAC) assay measures the ability of phytochemicals to scavenge oxygen free radicals, which are the dominating type of free radicals present in a human body. Because of this, ORAC is considered as imitating antioxidant activity of phenols in a biological system and, subsequently, is one of the assays that have received considerable attention. Moreover, ORAC integrates both, time and degree of activity of antioxidants. In particular, when analysing samples using ORAC assay, the lag phases of their antioxidant capacities are taken into account (Prior *et al.*, 2003). This is especially beneficial when measuring foods and supplements that contain complex ingredients with various slow and fast acting antioxidants, as well as ingredients which combine effects that cannot be pre-calculated (Magalhaes *et al.*, 2008).

Both reagent-based assays (FRAP and ORAC) employed in this study provide information about antioxidant capacities of compounds as determined through a chemical reaction. The cellular antioxidant activity (CAA) assay has been developed to satisfy the requirement for more biologically relevant cell culture-based model assessing antioxidant activity (Liu & Finley, 2005). The CAA assay provides complementary information about the efficiency of phytochemicals as antioxidants at a cellular level, and as such is more relevant when biologic objects are concerned (humans). The final result of this assay depends on uptake, distribution and metabolism of antioxidant compounds in a live cell. This information cannot be obtained through reagent-based antioxidant activity assays. In comparison to animal model, the CAA is a cost-effective and fast way to obtain animportant information on the efficiency of antioxidants within life cells (Wolfe & Liu, 2007). Excessive hydrogen peroxide (H_2O_2), an important ROS have been implicated in the rate of cellular injuries that subsequently developed to various deseases such as cancer (Klaunig & Kamendulis, 2004). Therefore, evaluation the ability of plant extracts in cellular protective effect against induced H_2O_2 is also investigated as an essential mechanism for antioxidant activity (Chow *et al.*, 2005; Lin *et al.*, 2007).

5.2 Results

5.2.1 Antioxidant capacity

5.2.1.1 Antioxidant capacity as evaluated using reagent-based assays

Table 5.1 presents the results of antioxidant tests. The result of the FRAP assay demonstrated that all purified polyphenolic-rich native herbs extracts had higher total reducing capacities than the reference sample BL. Among them, AM exhibited the highest antioxidant capacity (2.5-fold that of bay leaf) and was followed by LM and TPL extract. The ORAC assay revealed that, with the exception of LM, all extracts had superior oxygen radical scavenging capacities, than the respective reference samples. In case of herbs, TPL extract was nearly twice as efficient in scavenging oxygen free radicals as BL extract. Similar differences among these three herbs were reported previously for their crude alcoholic extracts (Konczak *et al.*, 2010a).

DP extract had the greatest total reducing capacities (ferric reducing antioxidant power, FRAP assay), superior to that of all extracts. The FRAP values were respectively, 1.5-fold and 1.92-fold these of REB and SHB. DP extract also exhibited the highest oxygen radical absorbance capacity (ORAC assay), which was 2.2-fold and 2.7-fold these of rabbit eye and southern highbush blueberries (Table 5.1). QD had lower FRAP value comparable to that of blueberry extracts. However, quandong extract had 1.5 times higher ORAC value than the ORAC value of REB and 1.8 times higher than that of SHB.

Table 5.1 Antioxidant capacity (FRAP and ORAC assay) of purified polyphenolic-rich extracts obtained from native Australian herbs and fruits

Samples	FRAP[a] (µmol Fe^{+2}/gDW)	ORAC[b] (µMol Trolox E/gDW)
Herbs		
Anise myrtle	8054 ± 15.2[a]	7564 ± 1272[a]
Lemon myrtle	5025 ± 10.9[b]	4136 ± 594[b]
Tasmannia pepper leaf	4444 ± 12.2[c]	12789 ± 996[a]
Bay leaf	3040 ± 17.5[d]	4945 ± 715[b]
Fruits		
Davidson's plum	9258 ± 16.1[a]	8791.5 ± 370[a]
Quandong	3225 ± 13.3[d]	6028.4 ± 953[a]
Rabbit eye blueberry	6098 ± 5.3[c]	3931.5 ± 196[b]
Southern highbush blueberry	4811 ± 26.7[b]	3266.5 ± 233[b]

All data represent the mean ± standard deviation of at least three independent experiments. Values in each column with different superscripts are significantly different ($p < 0.05$) as calculated using ANOVA and Tukey's multiple comparison test. [a]FRAP, Ferric Reducing Antioxidant Power. µmol Fe^{+2}/gDW, µmol of Iron (II) per g of dry weight.[b]ORAC: Oxygen Radical Absorbance Capacity. µM Trolox E/gDW, micromole Trolox equivalent per g of dry weight.

5.2.1.2 Antioxidant capacity as evaluated within a life cell

The results of the CAA assay revealed that all purified polyphenolic-rich extracts obtained from herbs showed higher value in comparison to BL extract. TPL exhibited the

greatest cellular antioxidant activity among herbs with an $EC_{50}$of 192.2 ± 52.5 µg/mL (Table 5.2), which was significantly lower than EC_{50} of all the other herbs. AM and LM were less efficient, with EC_{50} value 278.7 ± 26.5 µg/mL and 337.7 ± 29.9 µg/mL, respectively.

Table 5.2 Cellular antioxidant activities of purified polyphenolic-rich extracts obtained from native Australian herbs and fruits expressed as EC_{50} and CAA values (Mean ± SD, *n* = 3)

Samples	CAA units	
	CAA (µmol Q E/gDW)[a]	**CAA EC_{50}(µg/mL)[b]**
Herbs		
Anise myrtle	106.6 ± 2.64[b]	278.7 ±26.5
Lemon myrtle	88.0 ± 2.03[c]	337.7 ± 29.9
Tasmannia pepper leaf	154.6 ± 1.17[a]	192.2 ± 52.5
Bay leaf	44.6 ± 2.10[d]	666.9 ± 21.1
Fruits		
Davidson's plum	88.4 ± 1.48[b]	336.1 ± 14.9
Quandong	95.8 ± 9.40[b]	310.3 ± 14.0
Rabbit eye blueberry	412.9 ± 1.05[a]	72.0 ± 10.6
Southern highbush blueberry	348.7 ± 0.4[a]	85.1 ± 9.2

[a]Data represent the mean ± standard deviation of at least three independent experiments. Values in each column with different superscripts are significantly different ($p < 0.05$) as calculated using ANOVA and Tukey's multiple comparison test. [a]µmol Q E/gDW, µmol of quercetin equivalents per g of dry weight; [b]EC_{50}: half maximal effective concentration.

Earlier presented data showed that TPL had the highest total phenolics contents at 912 ± 58 mg GA E/gDW (Table 4.1) and oxygen radical scavenging capacity (ORAC values) of 12789 ± 996 µmol Trolox E/gDW. Yet, it does not have the highest FRAP values (Table 5.1). TPL extracts comprised predominantly chlorogenic acid and quercetin (Table 4.2). The CAA assay indicates that phenolic compounds of the TPL extract could efficiently enter a life cell and consequently act as antioxidant within a biological system. DP and

QD had approximately 5-fold higher EC_{50} CAA values than these of reference samples of blueberries. This indicates that the extract of blueberries would be a more efficient scavenger of free radicals in a life cell than the extracts of DP and QD. The CAA values of these two fruits were similar to those of kakadu plum (71.5 ± 11.3 μmol Q E/gDW) (Tan *et al.*, 2011a).

5.2.2 Correlation analysis

Various relationships were observed between the total phenolics (TP), total flavonoids, total proanthocyanidins and antioxidant capacities of herbs and fruits extracts (excluding references) evaluated in this study (Table 5.3). In case of herbs, significant positive correlation was found between total phenolics and ORAC values (r = 0.981), while total phenolics and FRAP showed a lack of correlation (r = 0.157). No significant correlation was found between FRAP and ORAC values. A high correlation has been found between TP and ORAC values of TPL extract. However no correlation was found between TP and FRAP values. In summary, the molecular antioxidant response of phenolic compounds varies remarkably, depending on their chemical structure. Similar relationship has been reported by Satue-Gracia *et al.* (1997). Thus, the antioxidant activity of an extract cannot be predicted on the basis of its total phenolic content. A strong correlation has been identified between total flavonoids and ORAC (r = 0.917), while there was no correlation between total flavonoids and FRAP (r = -0.003). Total phenolics and total flavonoids also exhibited moderately positive correlation (r = 0.826).

In case of fruits, a significant correlation was observed between total phenolics content of the fruit extracts and their ORAC values (r = 0.816) and total phenolic & FRAP values (r = 0.724). This indicates that phenolic compounds are the major constituents contributing to the oxygen radicals scavenging activity. The result revealed that ORAC values significantly correlated with total flavonoids (r = 0.991) and total proantocyanidin (r = 0.759). A strong influence of proanthocyanidins and flavonoids on antioxidant capacities was also found: TP, FRAP and ORAC values highly correlated with total proantocyanidins (r = 0.991, 0.804 and 0.759, respectively) while TP and ORAC showed high correlation with total flavonoids (r = 0.876 and 0.991, respectively) (Table 5.3).

Table 5.3 Relationship between the levels of phenolic compounds and antioxidant capacity for native Australian herbs and fruits

	TP	FRAP	ORAC	CAA	Total Flavonoids	Total Proanthocyanidins
Herbs						
TP	1	0.157	0.981	0.999	0.826	-0.069
FRAP		1	0.072	-0.213	0.003	0.925
ORAC			1	0.983	0.917	-0.016
Fruits						
TP	1	0.724	0.816	0.356	0.876	0.991
FRAP			-0.326	-0.054	0.416	0.804
ORAC			1	-0.748	0.991	0.759

5.2.3 Cellular protection from hydrogen peroxide (H_2O_2) induced cell death in HepG2 cells

Within this study, MTT assay was employed to evaluate the potential protective activity of purified polyphenol-rich extracts against H_2O_2-induced cell death. This study has been conducted using HepG2 cell line as its represent frequently applied model systems. The assay requires the use plant extracts at low levels, at which there is not suppression of cell proliferation (García-Alonso *et al.*, 2006). Through a preliminary study these concentrations were established to be from 0.2 – 0.6 mg/mL (results presented in the Appendix C). Median cytotoxic concentration of hydrogen peroxide was 20 mM at 50% of cell survival. With increasing incubation time from 3 to 23 h, twenty-three h proved to be a sufficient time to suppress the activity of H_2O_2 –induced cell death.

5.2.3.1 Cellular protection against H_2O_2 –induced cell death by purified polyphenolic-rich herbs extracts

To evaluate the potential cellular protection of purified polyphenolic-rich obtained from herb extracts from H_2O_2induced cell death, HepG2 (hepatocellular human carcinoma cells) were pre-treated over 23 h with 0.2 – 0.6 mg/mL (a range of concentration that does

not affect the proliferation of HepG2 (result presented in Appendix C) and then challenged with 20 mM H_2O_2 over 1 h. Incubation of HepG2 cells with different doses of purified polyphenol-rich extracts and H_2O_2 (20 mM) resulted in a significant protection of cells from H_2O_2-induced injury (Figure 5.1).

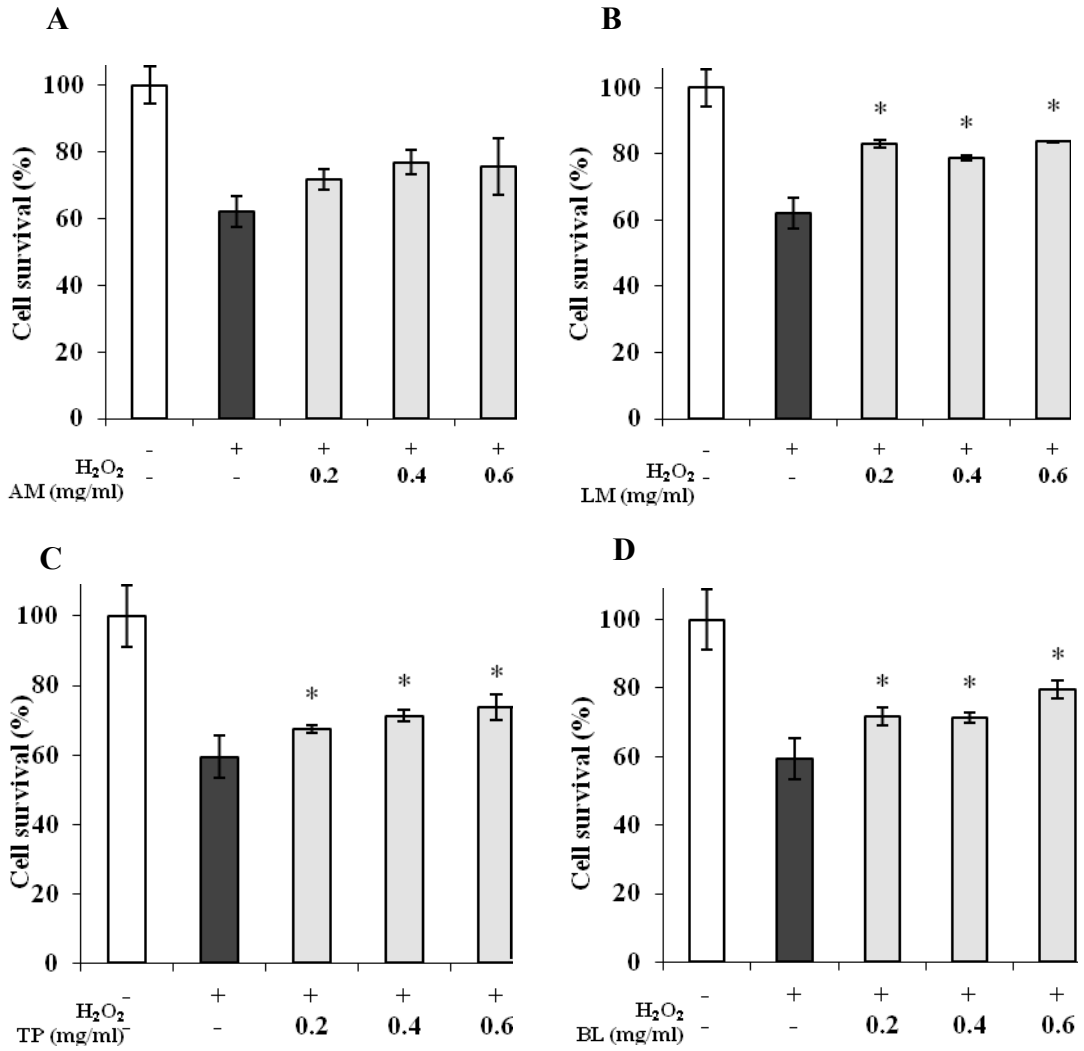


Figure 5.1 Effect of purified polyphenolic-rich extract of AM, LM, TPL and BL on H_2O_2 induced cell death in HepG2 cells using the MTT assay

Cells were treated with different concentrations (0.2, 0.4 and 0.6 mg/mL) of purified polyphenolic-rich extracts for 23 h in the presence of H_2O_2 (20 mM) for 1 h. The cellular viability was detected by MTT assay. Data are expresses as the mean standard deviation of at least three independent experiments. Treatments: (-) no H_2O_2 (cell only); (+) H_2O_2 (cells) without or with addition of plant extracts.*$p < 0.05$ indicates a significant difference between indicated groups, as analysed by Tukey's post hoc.

The addition of H_2O_2 to the culture medium reduced the viability of HepG2 cells (control) by approximately 40% (Figure 5.1). Pre-treatment with all herbal extracts reduced the

adverse effect of H_2O_2 by up to 50%. Only TPL extract showed a clear dose-dependent effect. At the lower concentration of 0.2 mg/mL, LM extract was the most effective with a 50% reduction in H_2O_2 induced cell death. The results revealed that polyphenolics derived from native Australian herbs may possess cytoprotective properties toward HepG2 cells when subjected to H_2O_2 induced cellular death.

5.2.3.2 Cellular protection against H_2O_2 –induced cell death by purified polyphenolic-rich fruits extracts

QD extract exhibited protection of HepG2 cell against H_2O_2 –mediated cellular death in a dose-dependent manner after pre-incubation for 23 h. At the lowest concentration of 0.2 mg/mLQD extract significantly reduced H_2O_2- induced cell death by 40% (Figure 5.2), compared with the control. DP extract was comparable to both blueberry species, with about 10% and 15% cell survival observed at 0.4 and 0.6 mg/mL, respectively (Figure 5.2).

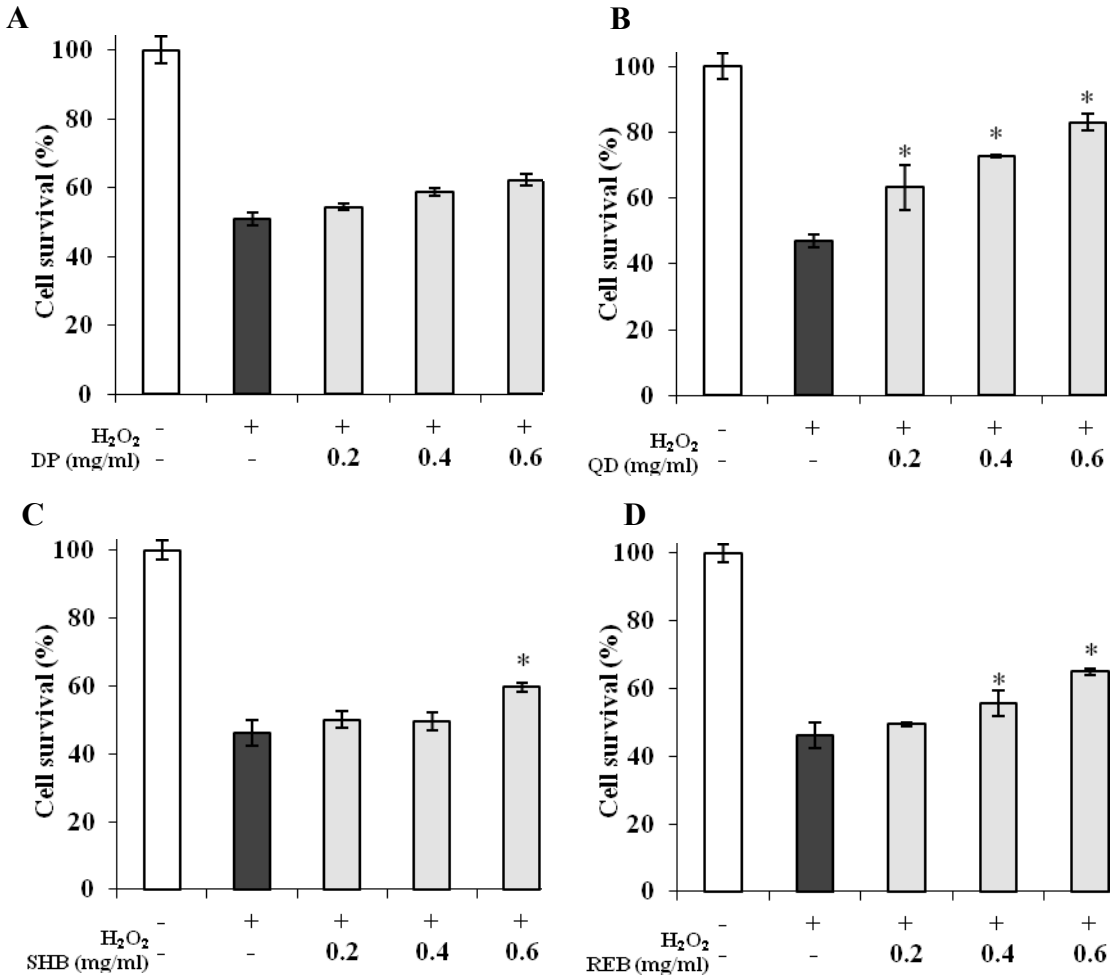


Figure 5.2 Effect of purified polyphenolic-rich extract of DP, QD, REB and SHB on H_2O_2 induced cell death in HepG2 cells using the MTT assay

Cells were treated with different concentrations (0.2, 0.4 and 0.6 mg/mL) of purified polyphenolic-rich extracts for 23 h in the presence of H_2O_2 (20 mM) for 1 h. The cellular viability was detected by MTT assay. Data are expresses as the mean standard deviation of at least three dependent experiments. Treatments: (-) no H_2O_2 (cell only); (+) H_2O_2 (cells) without or with addition of plant extracts. *$p < 0.05$ indicates a significant difference between indicated groups, as analysed by Tukey's post hoc.

5.3 Discussion

Each extracts exhibited varying levels of antioxidant activity in a number of assays. The results from reagent-based antioxidant activity *in vitro* demonstrated that all purified polyphenolic-rich extracts obtained from native Australian herbs and fruits comprised greater level of phenolic compounds than the respective reference samples. AM extract had superior total reducing capacity (TRC: FRAP value) and TPL extract had an outstanding oxygen radical absorbance capacity (ORAC value), which correlated with CAA value. DP exhibited the highest level of antioxidant activity in both, FRAP and ORAC assays. The results clearly showed that native Australian herbs and fruits can deliver more phenolic compounds than the respective reference samples used in this study and therefore can be considered as a novel source of phenolic compounds for application in health promoting food.

A high positive correlation was identified between TP and ORAC values for herbs (r = 0.981) and fruits (r = 0.816) extracts. Such high value indicated that phenolic compounds are responsible for the detected radical scavenging ability. Various levels of correlation between total phenolics and antioxidant capacities were reported in the literature. Giovanelli and Buratti (2009) studied wild blueberries (*Vaccinium myrtillus*) and four highbush blueberry (*V.corymbosum*) cultivars; they reported a significant correlation between total phenolic content and antioxidant activity as detected using DPPH assay (Giovanelli & Buratti, 2009). A strong positive correlation between total phenolic content and FRAP (r = 0.7929) and TEAC (r = 0.8043) was also reported for tea infusion (Fu *et al.*, 2011). Similarly, Song *et al.* (2010) found a positive correlation between total phenolic content and antioxidant activity of Chinese medicinal plants, as measured by FRAP (r = 0.8998) and TEAC (r = 0.8844). Katalinic *et al.*, 2006 reported strong correlation (r = 0.9825) between total phenolic contents and FRAP obtained from

medicinal plant crude extracts. However, some authors reported a weak correlation between total phenolic contents and antioxidant capacity, such as in extracts obtained from crude strawberry (r = 0.6599 for FRAP and (r = 0.4438 for TEAC) (Rekika *et al.*, 2005). A strong correlation has been found between total flavonoid and ORAC (r = 0.917), while there was no correlation between total flavonoid and FRAP (r = -0.003). This can be explained with a fact that flavonoid compounds readily deliver hydrogen cation and therefore are predominantly active in ORAC assay (Davalos *et al.*, 2004).

Similarly, Tan *et al.* (2011a) reported larger antioxidant capacities of polyphenolic-rich extracts obtained from other native Australian fruits (Kakadu plum, Illawarra plum, muntries and native currant) than those of blueberry reference. In contrast, different results have been reported earlier for crude extracts from the same fruits, where the oxygen radical absorbance capacity of DP was only 50% of that of quandong and the TRC was 1.8-fold that of quandong (Konczak *et al.*, 2009). The superior antioxidant activity of DP purified polyphenolic-rich extract obtained within this study clearly indicates that crude extract of DP contained a vast amount of compounds that significantly contributed to the dry weight, without significant contribution to antioxidant capacity (such as polysaccharides), and these compounds have been removed during the purification process.

There are many different mechanisms through which polyphenol molecules can react with other compounds (e.g. donating an electron or hydrogen atom), while being involve in multiple reaction mechanisms (Prior *et al.*, 2005). As such, multiple assays are necessary and caution must be taken when antioxidant activities are evaluated (Prior *et al.*, 2005). Pro-oxidant activity is questioned to determine whether or not the reaction will occur *in vivo* and cause harm to human cells according to antioxidant mechanisms (Halliwell, 2008). Depending on their molecular structure, different phenolic compounds may give different responses in reagent-based antioxidant testing methods, based on various molecular mechanisms of the chemical reaction. As mentioned above, (5.2.1.1) the FRAP and ORAC assays represent two different approaches to evaluate antioxidant capacity. Accordingly, phenolic compounds that readily donate hydrogen cation will exert antioxidant activity in ORAC assay, but not in FRAP. TPL extract, comprises

predominantly chlorogenic acid and quercetin that have been shown to exhibit high ORAC activity (Davalos *et al.*, 2004; Ou *et al.*, 2001).

The results of the CAA assay demonstrated that the purified polyphenolic-rich extracts from herbs exhibited significant cellular antioxidant activity, with an outstanding result obtained for TPL. All fruit extracts, however, have shown lower CAA value than that of blueberry extracts. In comparison, all extracts obtained from herbs showed significantly greater CAA values than extracts from a range of commonly consumed fruits such as wild blueberry, strawberry, raspberry, cranberry and apple (Wolfe & Liu, 2008). The EC_{50} of all the evaluated herbs, including the reference sample, were significantly lower than those of sow thistle (*Sonchus oleraceus* L. 238 Asteraceae) extract, a native to Europe and central Asia medicinal plant, which exhibited EC_{50} of 3.21 mg/mL (or 3210 μg/mL) (McDowell *et al.*, 2011).

Similar CAA values were observed for both, TPL and QD extracts. As described earlier, TPL and QD contained monomeric compounds such as chlorogenic acid, *p*-coumaric acid and quercetin/derivatives. According to Manach *et al.* (2004), these monomeric compounds are relatively well absorbed by life cell phytochemicals, which can explain the superior cellular antioxidant activity of the TPL extract. An essential aspect related to CAA assay includes cellular uptake, metabolism and distribution of bioactive compounds, which are important modulators of bioactivity (Wolfe *et al.*, 2008). In accordance, the CAA assay may provide a better prediction of antioxidant capacity in biological systems. According to the result, it can be speculated that after consumption of AM and LM predominant in polymeric compounds, gut flora in digestive system will degrade these compounds. Consequently, this may possibly reflect that CAA level detected in biological systems such a human body is higher than this study.

Similarly to AM and LM, the extract of DP comprises a mixture of monomeric and polymeric phenolic compounds. In the setting of the CAA assay it is possible that these polymeric compounds were not able to enter the life cells. However, the polymeric compounds are expected to be digested or degraded by gut flora depending on hydrolysis in biological digestion system (Heber, 2008). Therefore, it may be expected that such

compounds will exert antioxidant activity at various stages of food digestion in a life organism.

The cytoprotective effects of the extracts with H_2O_2 exposure were investigated using HepG2 cells (Aherne & O´Brien, 1999), representing a relevant mechanism for antioxidant capacity in biological system. TPL and QD extract showed a clear dose-dependent effect in protecting cells from a cellular damage induced by H_2O_2. The results revealed that TPL and QD extracts have a potent cytoprotective activity in protecting cells from H_2O_2-induced death.QD extract, however, was less efficient than kakadu plum extract (85% cell survival at 0.6 mg/mL QD and 0.2 mg/mL Kakadu plum) (Tan *et al.*, 2011a). Literatures have also reported various efficiently cellular protective effects of other compounds. Myricetin, quercetin (Aherne & O'Brien, 1999) and rutin (Alia *et al.*, 2006), green tea polyphenols, quercetin (Jiao *et al.*, 2003; Alia *et al.*, 2006) as well as esculetin (6,7-dihydroxycoumarin) (Subramaniam & Ellis, 2011) exhibited similar cellular protective effect against H_2O_2 induced oxidative damage in HepG2 cells.

Chang and Lin (2012) have observed that 95% ethanolic extract of air-dried fruit *Terminalia chebula* Retzinhibited H_2O_2- induced PC12 cell death. This traditional medicinal fruit used for its homeostatic and/or cardiotonic activities showed more pronounced, 40-time higher activity than QD extract (at 5 µg/mL: 64.2 ± 6.0 % cell survival), although the pre-incubation time was different (Chang & Lin, 2012). Various fruits phenolic extracts (cranberry, apple, red grape, red plum) also reduced tBHP-induced oxidative stress in human lung fibroblast cells (CCD-25LC) (Boateng & Verghese, 2012).

Miccadei *et al.* (2008) reported a similar ability to that of chlorogenic acid of polyphenolic extracts obtained from edible part of artichoke in preventing the loss of total intracellular glutathione (GSH) and the accumulation of malondialdehyde (MDA) by HepG2 cells. Chow *et al*. (2005) reported a significant protection by quercetin not quercetin derivatives against H_2O_2-induced cytotoxicity in RAW264.7 (400 µM and 24 hpre-treatment). Pre-incubation of individual compounds (ellagic acid, chlorogenic acid, caffeic acid and ferulic acid) with the rat pheochromocytoma (PC12) cells reduced the

cytotoxicity and loss of GSH induced by H_2O_2 challange. Ellagic acid was more efficient in cellular death prevention than other phenolic acids (Pavlica & Gebhardt, 2005).

Bioactive compounds originating from red raspberry (hydroxycinnamic acids, ellagic acid derivatives, quercetin derivatives, chlorogenic acid, caffeic acid) exhibited cytotoxic, cytoprotective properties, antioxidative/prooxidative effect, and effect on total glutathione concentration in human laryngeal carcinoma (HEp2) and colon adenocarcinoma (SW 480) cell lines with selectively SW 480 cells being more efficient than HEp2 cells. The antioxidative effect of raspberry leaf extract was observed in HEp2 cells treated with H_2O_2, as opposed to SW 480 cells, where raspberry leaf extract induced reactive oxygen species formation. Raspberry leaf extracts increased total glutathione level in HEp2 cells (24 h), which was suggesting the influence of by-products generated from cellular metabolism (Durgo *et al.*, 2012).

5.4 Conclusions

This chapter demonstrated that the polyphenols from native Australian herbs and fruits may have significant potential to protect cellular system against oxidative stress. The results can be summarised as follows:

(i) AM exhibited the highest FRAP value and TPL showed an outstanding ORAC activity.

(ii) Among fruits, DP showed the greatest both FRAP and ORAC activity.

(iii) Among herbs, TPL and QD among fruits exhibited the greatest cellular antioxidant activity in the CAA assay.

(iv) Purified polyphenolic-rich extracts obtained from herbs and fruits exhibited cytoprotective properties against H_2O_2-induced cellular death in HepG2.

Chapter 6

Potential chemopreventive properties of purified polyphenolic-rich extracts obtained from native Australian herbs and fruits

6.1 Introduction

The assessment of cell viability, including cell growth and proliferation has become an essential technique for cell-based studies. These assays are widely used as a routine method for screening a various samples depending on different objective of each study. Cell viability assessment is defined as the measurement of proliferation of a life cells in the presence or absence of a particular substance or samples, for a specified period of time (Houghton *et al.*, 2007). In contrast to cell viability, cell proliferation is the measurement of actively dividing cells in a sample, expressed as the actual number or proportion of proliferating cells in cell culture, or as relative number of cell population in assays, without detection of inert non-growing healthy cells (Houghton *et al.*, 2007).

Most viability assays depend on one of two characteristic factors, which is metabolic activity or cell membrane integrity of healthy cells. Practically, the metabolic activity is evaluated in cell populations via incubation with a tetrazolium salt such as MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)) or WST-1 (water soluble tetrazolium salts). The first two reagents represent the same mechanisms: tetrazolium salt is cleaved into a coloured formazan product by an enzyme present in mitochondria of active cells, which is measured by spectrophotometry (Houghton *et al.*, 2007).

To evaluate the growth inhibitory and pro-apoptotic activity of phytochemicals, a number of assays have been developed depending on the specific kinetics and synchrony occurred in a system. Flow cytometric technique is known as a well-established method to characterise morphological and functional features of cells undergoing apoptosis. This

conventional technique is used routinely for apoptosis assessment. Principally, scattered light detect changes in the plasma membrane of apoptotic cells, which allows to identify apoptotic, early apoptotic, necrotic and life cells. However, the limitation of this method is that it does not provide direct morphologic evidences of cell death. Beside the changes described above, non-specific changes to apoptosis may also appears, which may also be present in cells undergoing necrosis. In this situation it may be difficult to distinguish between late apoptotic and necrotic cells (Steensma *et al.*, 2003). Therefore, in order to characterise apoptosis, a combination of various analysing techniques is suggested in order to deliver a reliable result (Lovborg *et al.*, 2005).

The cytokinesis block micronucleus (CBMN) cytome assay is a well-established and comprehensive approach for measuring three outcomes: DNA damage, cytostasis and cytotoxicity (Fenech 2007). DNA damage events are scored specifically in once-divided binucleated (BN) cells using cytochalasin B, an inhibitor of microfilament ring assembly required for the completion of cytokinesis. These cells include (a) micronuclei (MNi), a biomarker of chromosome breakage and/or whole chromosome loss which has been shown to be predictive of increased cancer risk, cardiovascular mortality and are significantly elevated in both Alzheimer's and Parkinson's disease, (b) nucleoplasmic bridges (NPBs), a biomarker of DNA misrepair and/or telomere end-fusions, and (c) nuclear buds (NBUDs), a biomarker of elimination of amplified DNA and/or DNA repair complexes. MNi, NPBs and NBuds are nuclear anomalies commonly found in cancer, which represent a common phenotype of chromosomally unstable cells (Brassesco *et al.*, 2009).

6.2 Anti-proliferative activity of purified polyphenolic-rich extracts

The cell sensitivity of the transformed cell lines: AGS, HT-29, BL13, HepG2, and the equivalent (or normal): CCD-18Co and Hs738.St/Int cell lines to purified polyphenolic-rich extracts obtained from native Australian plants was determined using the colourimetric MTT assay. Gastric and colorectal cell lines were selected as representatives of the digestive system, and therefore directly exposed to food compounds

and their metabolites. The cell sensitivity of the HL-60 was also evaluated in order to assess potential effects on immunological cells.

Each of the purified polyphenolic-rich extracts demonstrated a reduction in cell viability of the cancer cell lines AGS, BL13 and HT-29 in a dose-dependent manner. The cytotoxic activities (IC_{50}; 50% inhibition concentrations, mg/mL) of extracts against various cancer cell lines are presented in Table 6.1. AM exhibited the lowest IC_{50} values for HepG2 cells (0.38 ± 0.02 mg/mL) (Table 6.1), whilst TPL also showed strong anti-proliferative activity against BL-13 (IC_{50} = 0.56 ± 0.1 mg/mL). In comparison to the equivalent normal cell lines, the gastric and colorectal cancer cell lines showed an increased sensitivity to each extract, with the exception of DP and QD against gastric cancer cell lines.

AM showed similar growth inhibitory activity for BL13 (0.56 ± 0.05 mg/mL) and AGS (0.59 ± 0.05 mg/mL), followed by HT-29 (0.76 ± 0.03). LM showed similar anti-proliferative activity against all cancer cell lines with IC_{50} of HT-29 1.35 ± 0.14 mg/mL, AGS 1.25 ± 0.53, BL13 1.12 ± 0.35, and HepG2 1.36 ± 0.08. In view of these results, LM was less potent than BL (Table 6.1). The above results demonstrate that anise myrtle had the most efficient antiproliferative effect, similar to that of a reference sample BL. In addition, there was a difference in anti-proliferative activity of the extracts against different cancer cells, which suggested a cell line-phytochemicals specificity.

In case of fruits, both, DP and QD, showed moderate antiproliferative activity against cancer cell lines, but were less potent than blueberries. The effects of the varios concentration of herb and fruit extracts on cell viability are shown in Figure 6.1 and 6.2, respectively. Cells were treated with varying concentrations for 24 h and viability was determined using the MTT assay. Sample concentration (mg/mL) versus cell viability data (% of control) was graphed, and the area under this viability-dose curve was integrated. Differences between values were determined by one-way ANOVA with Tukey's post hoc test, with an asterisk representing $p < 0.05$ (Figure 6.1).

Table 6.1 Concentration of purified polyphenolic-rich extracts obtained from native Australian herbs and fruits in 50% cell viability (IC50) of human cancer and non-transformed cells

Plant extract	IC_{50} (mg/mL)[a] ± SD					
	HT-29*	**CCD-18Co**	**AGS**	**Hs738.St/Int**	**BL13**	**HepG2**
Herbs						
Anise myrtle	0.76 ± 0.03	>2.0	0.59 ± 0.05	>2.0[4.1±0.63]	0.56 ± 0.05	0.38 ± 0.02
Lemon myrtle	1.35 ± 0.14	>2.0	1.25 ± 0.53	>2.0[3.0±0.23]	1.12 ± 0.35	1.36 ± 0.08
Tasmannia pepper leaf	1.39 ± 0.09	>2.0	1.88 ± 0.25	>2.0[2.25±0.18]	0.56 ± 0.10	1.13 ± 0.19
Bay leaf	0.75 ± 0.08	>2.0	0.36 ± 0.03	>2.0	0.51 ± 0.08	0.72 ± 0.06
Fruits						
Davidson's plum	1.35 ± 0.23	>2.0	>2.0	>2.0	1.35 ± 0.03	0.78 ± 0.02
Quandong	1.88 ± 0.07	>2.0	>2.0	>2.0	1.35 ± 0.08	2.30 ± 0.5
Rabbit eye blueberry	1.51 ± 0.15	>2.0	0.78 ± 0.07	>2.0	0.56 ± 0.13	0.36 ± 0.02
Southern highbush blueberry	0.93 ± 0.17	>2.0	1.04 ± 0.32	>2.0	0.72 ± 0.07	0.69 ± 0.04

[a]The $IC_{50}$was obtained via nonlinear regression and are expressed as the mean ± SD, determined from the results of the MTT assay of 3 independent experiments with 4 replicates each. The IC_{50} values are presented as the amount of fruit extract per mL of culture [IC_{50} (mg/mL) ± SD]. HT-29: colorectal adenocarcinoma, CCD-18Co: colon non-transformed, AGS: gastric adenocarcinoma, Hs738.St/Int: mixed stomach and intestine non-transformed, BL-13: bladder cancer, HepG2: liver hepatocellular carcinoma.

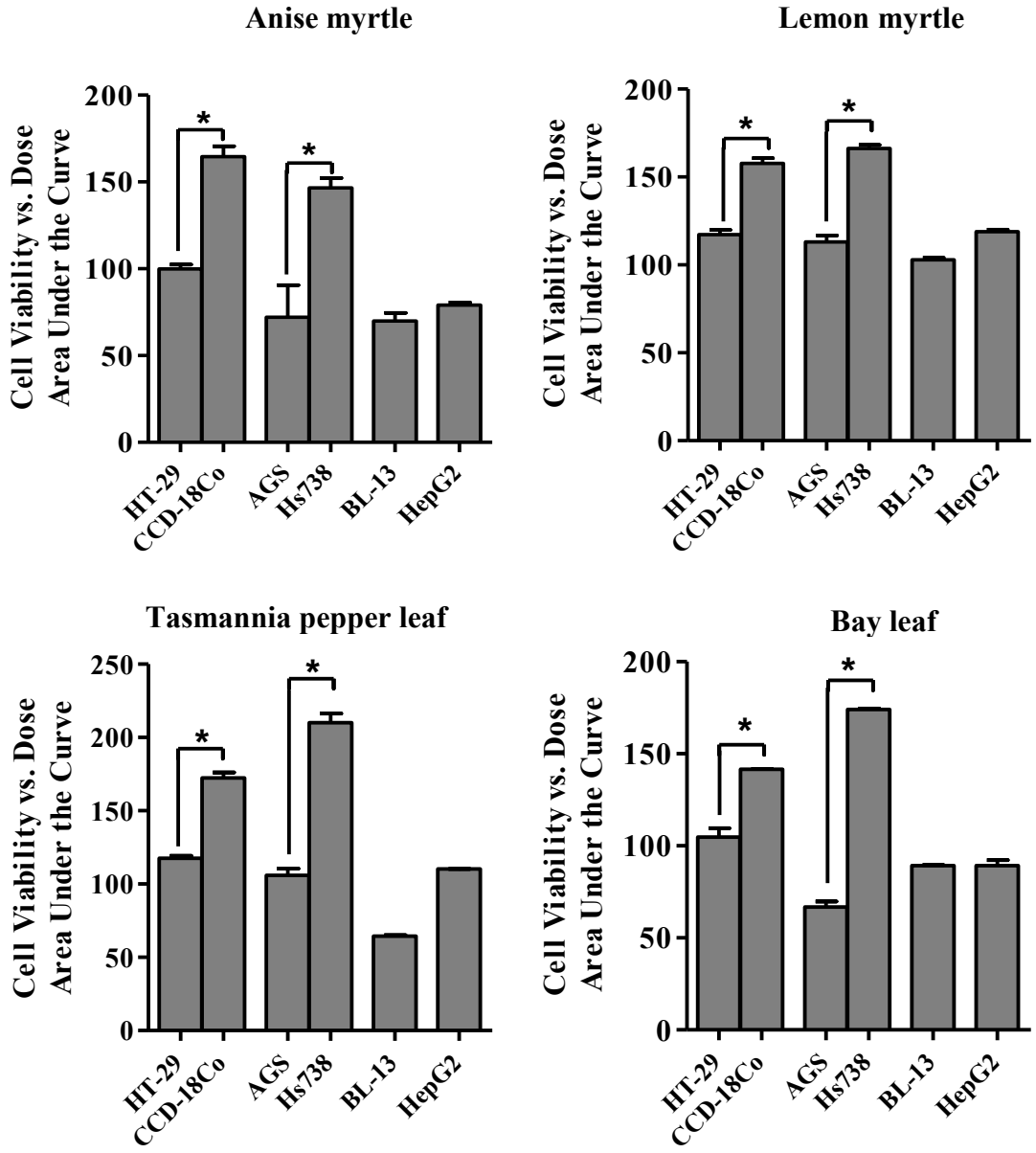


Figure 6.1 Dose-dependent effect of purified polyphenolic-rich extracts obtained from native Australian herbs on cancer cells (AGS, HT-29, BL13, HepG2) and non-transformed cells (CCD-18Co, Hs738.St/Int)

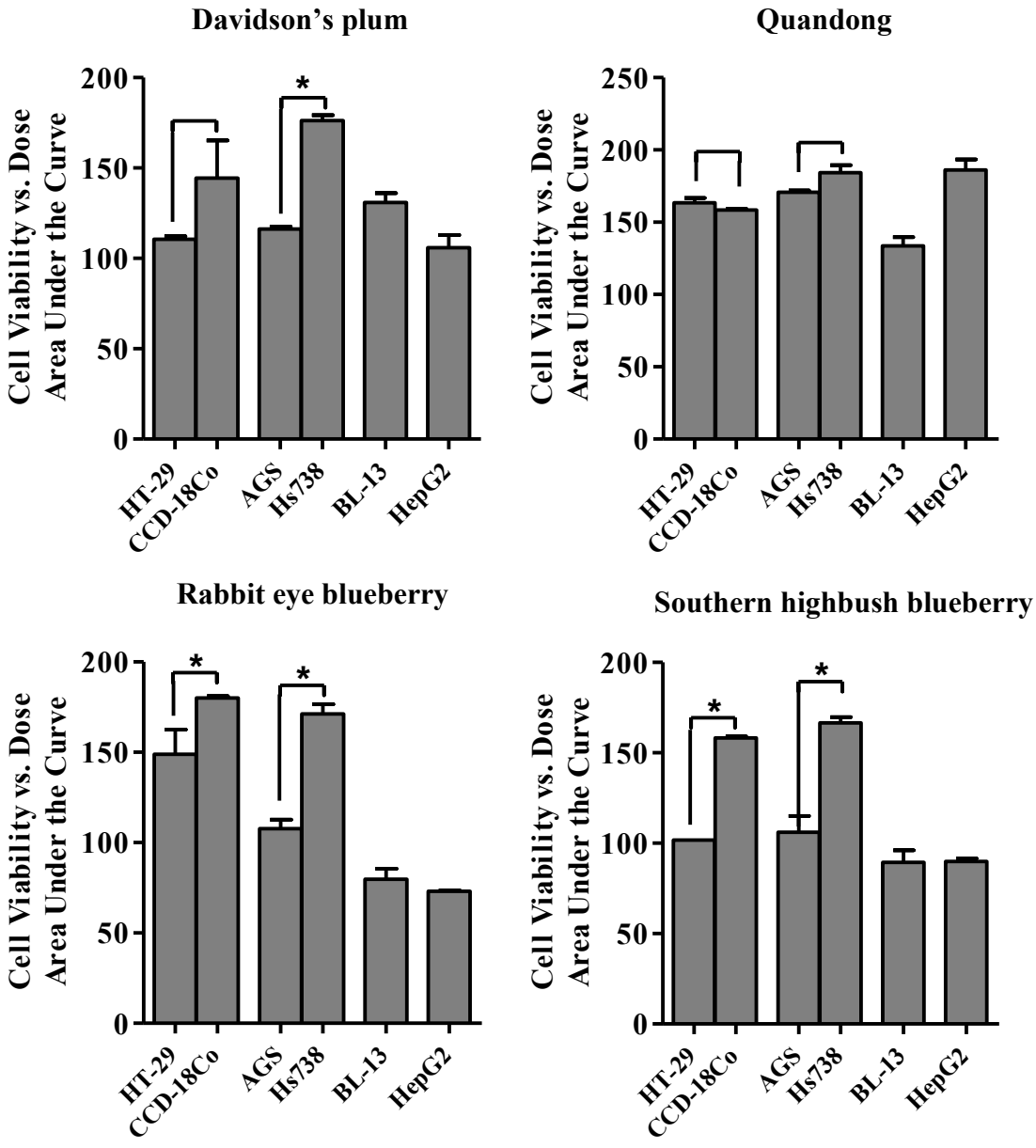


Figure 6.2 Dose-dependent effect of purified polyphenolic-rich extracts obtained from native Australian fruits on cancer cells (AGS, HT-29, BL13, HepG2) and non-transformed cells (CCD-18Co, Hs738.St/Int)

Cells were treated with varying concentrations for 24 h and viability was determined using the MTT assay. Sample concentration (mg/mL) versus cell viability data (% of control) was graphed, and the area under this viability-dose curve was integrated. Differences between values were determined by one-way ANOVA with Tukey's post hoc test, with an asterisk representing $p < 0.05$ (Figure 6.2). The cell viability data for each

cell line were also expressed graphically as area under the curve between cell viability and concentration. In case of herbs significant differences were observed between AGS and Hs738.St/Int and HT-29 and CCD-18Co, the cancerous cells and equivalent non-transformed cell. Among fruits, DP, REB and SHB showed significant difference between the gastric cancer cells AGS and non-transformed equivalent Hs738.St/Int. There was no obvious relationship between total phenolic content and inhibition of cell proliferation for herbs with *r* ranged between 0.0224 and 0.071. A strong negative correlation was found between total phenolic content and antiproliferative activity of HT-29 for fruits (r = -0.9387). The anti-proliferative effect of the native Australian herbs and fruits extracts against various cancer cell lines indicate the potential of plant extracts to exert anticancer activity, without affecting the proliferation of their equivalent normal cells. Purified polyphenolic-rich extracts obtained from herbs exhibited more pronounced anti-proliferative activities than the fruit extracts as indicated by the lower IC_{50} value (Table 6.1).

6.3 Determination of apoptosis in cancer cells treated with purified polyphenolic-rich extracts

To identify induction of apoptotic cells by purified polyphenolic-rich extracts, two experiments were conducted: evaluation of dose response and time response of human promyelocytic leukemia (HL-60) cells. Evaluation of cells treated with various concentrations of plant extract at a single time point (6 h) showed that native Australian herbs (Figure 6.3A) and fruits (Figure 6.4A) extracts induced a high number of apoptotic cells.

In case of herbs, the percentage of total apoptotic cells increased with increasing concentration showing dose dependent effect. At a concentration of 0.4 mg/mL, the highest percentage of total apoptotic cells was found in AM (91.1 ± 1.4), followed by LM (57.2 ± 2.0) similarly in TPL (60.6 ± 2.4). They were all higher than inBL (33.8 ± 0.2). The percentage of total apoptotic cells reached more than 95% after increase concentration to 0.8 mg/mL and 1.6 mg/mL. At the highest concentration (1.6 mg/mL), AM showed 96.1 ± 2.9, LM 99.6 ± 5.0, TPL 98.7 ± 2.0 of total apoptotic cells. For all herbs, the percentage of necrotic cells was also significantly lower, indicating cell death

induced by apoptotic pathway. At 1.6 mg/mL, the percentage of necrotic cells observed in AM was 0.98 ± 0.1%, in LM 0.37 ± 0.02%, and TPL 1.20 ± 0.1%.

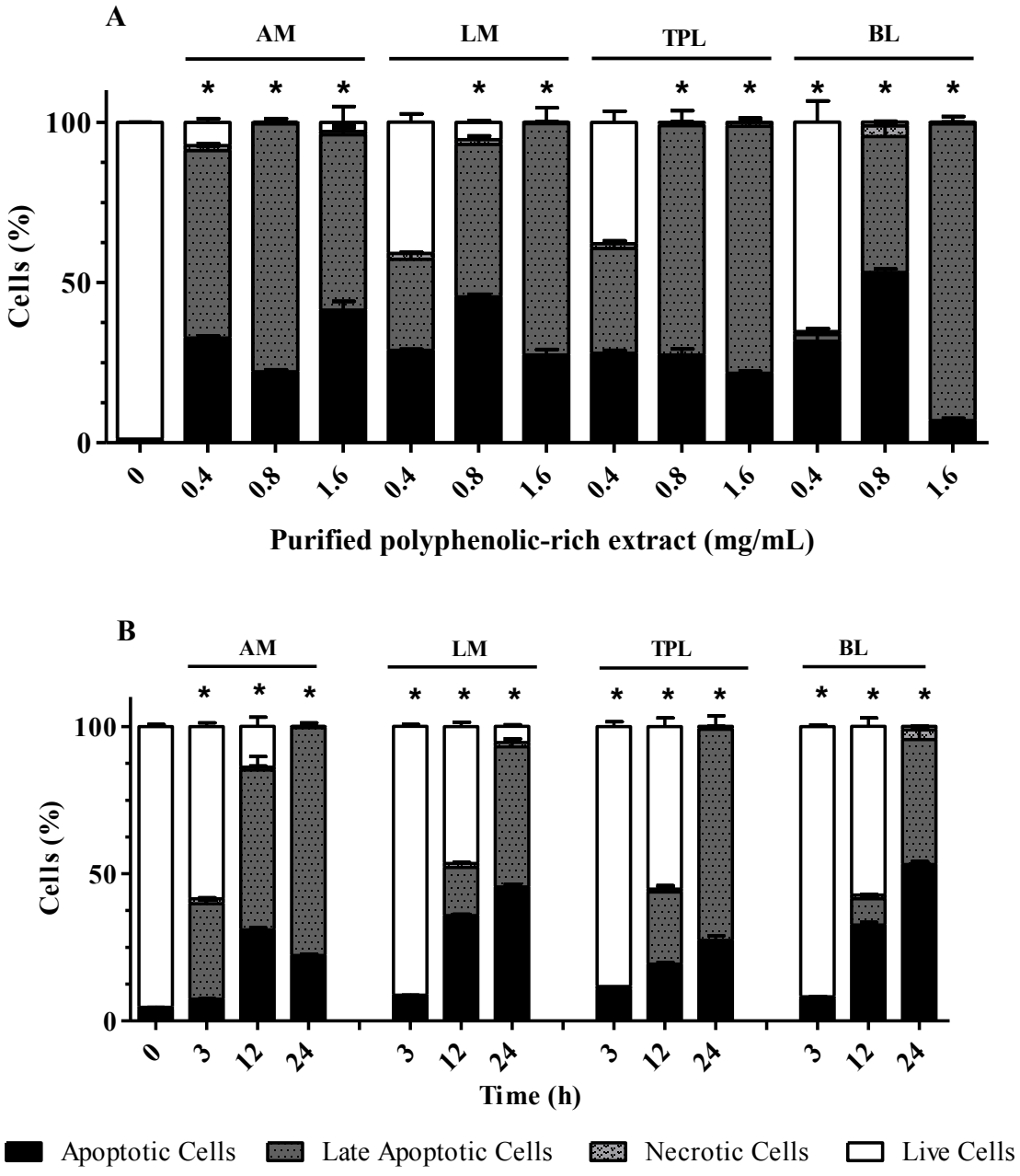


Figure 6.3 Flow cytometric analysis of HL-60 cells treated with purified polyphenolic-rich extracts obtained fromnative Australian herbs

Percentage of live (white), apoptotic (black), late apoptotic (dark grey) and necrotic (light grey) cells were measured in cells stained with annexin V and propidium iodide. (A) Dose-response experiment. Cells were treated with 0.4, 0.8 and 1.6 mg/mL of purified

polyphenolic-rich extracts for 6 h. (B) Time-response experiment. Cells were treated with 0.4 mg/mL of purified polyphenolic extracts for 3, 12 and 24 h. Data represent the mean ± standard deviation of the percentage of cells in each population obtained from three independent experiments. An asterisk represents significant difference ($p < 0.5$) between percentage of live cells and percentage of apoptotic and late apoptotic cells added.

The time-response experiment was conducted using a single concentration of plant extracts of 0.4 mg/mL, as at this concentration approximately 50% of life cells were observed. The percentage of apoptotic cells increased with an increase of the treatment time, with the greatest number of total apoptotic cells being found at 24 h (Figure 6.3B). Over the time of experiment a steady increase of late apoptosis events was observed. This phenomenon favoured the progression of apoptotic cells from early to late stages of apoptosis. The percentages of late apoptotic plus apoptotic cells at 3, 12 and 24 h for AM were 39.8 ± 1.8 %, 85.2 ± 7.0 and 99.6 ± 3.1 respectively; for LM 8.62 ± 0.8, 52.1 ± 1.2 and 93.1 ± 4.7, for TPL 11.5 ± 1.6, 43.8 ± 3.8 and 99.0 ± 9.2, and for BL 8.04 ± 0.5, 41.5 ± 3.1 and 95.6 ± 6.3.A time-dependent response was found for all herbs, with the most efficient response observed for anise myrtle at early stage. At 3 h, AM showed the greatest potential to induce apoptosis. The percentage of necrotic cells was low over time, indicating the mechanism of cell death as apoptosis rather than necrosis.

Similar trend has been observed in case of fruits, with DP showing a greater potential to induce apoptosis at the lowest concentration (0.4 mg/mL) than QD. The activity of DP extract was close to that of a reference sample, REB. At a concentration of 0.4 mg/mL, the percentage of total apoptotic cells of DP was 82.1 ± 4.3, QD 8.4 ± 0.9, REB 90.0 ± 4.3, and SHB 76.0 ± 3.8. After increase in concentration to 0.8 mg/mL, the percentage of total apoptotic cells reached 98.0 ± 0.7 for DP, 37.9 ± 0.8 for QD, 98.9 ± 2.2 for REB, and 99.9 ± 2.7 for SHB. At a concentration of 1.6 mg/mL, the percentage of apoptotic cells after the treatment with DP was 95.4 ± 1.5, QD 88.0 ± 0.8, REB 99.6 ± 1.4, and SHB 99.7 ± 1.4.

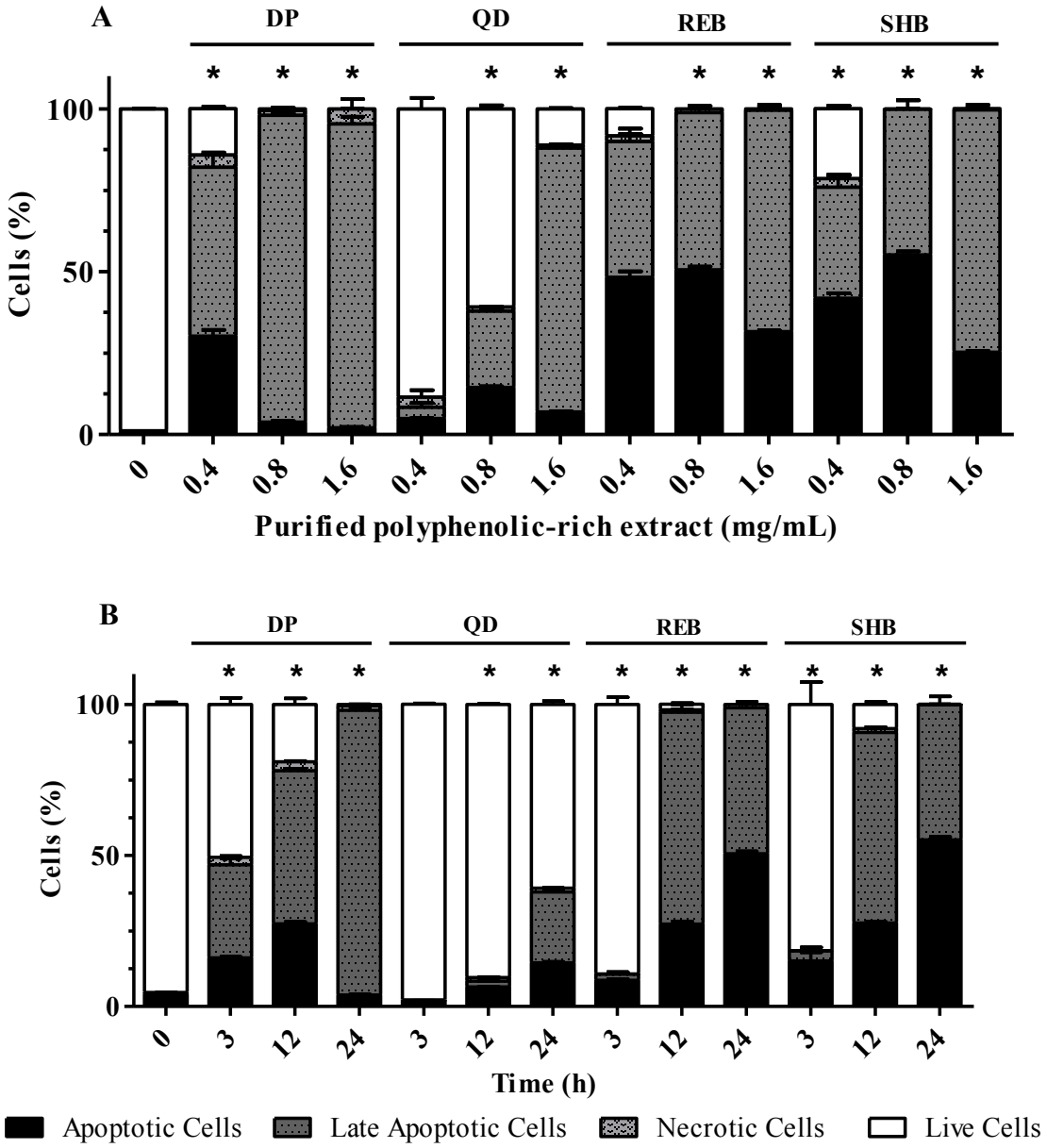


Figure 6.4 Flow cytometric analysis of HL-60 cells treated with purified polyphenolic-rich extracts obtained from native Australian fruits

Percentage of live (white), apoptotic (black), late apoptotic (dark grey) and necrotic (light grey) cells were measured in cells stained with annexin V and propidium iodide. (A) Dose-response experiment. Cells were treated with 0.4, 0.8 and 1.6 mg/mL of purified polyphenolic-rich extracts for 6 h. (B) Time-response experiment. Cells were treated with 0.4 mg/mL of purified polyphenolic-rich extracts for 3, 12 and 24 h. Data represents the mean ± standard deviation of the percentage of cells in each population obtained from

three independent experiments. An asterisk represents significant difference (*$p < 0.5$) between percentage of live cells and percentage of apoptotic and late apoptotic cells added.

Among fruits, DP extract showed the highest ability to induce apoptotic pathway within 3 h, than other fruit extracts evaluated in this study. The percentage of total apoptotic cells increased in a time dependent manner. The efficiency of quandong extract was comparable to those of reference samples. The percentages of total apoptotic cells at 3, 12 and 24 h treatment with DP were 46.9 ± 1.0, 78.1 ± 1.7 and 98.0 ± 6.3, for QD extract 2.07 ± 1.2, 8.4 ± 0.3 and 37.9 ± 6.4, for REB 10.7 ± 4.6, 97.4 ± 3.0 and 98.9 ± 1.6, and for SHB 18.4 ± 8.3, 90.7 ± 2.5 and 99.9 ± 7.2. These results indicate that DP is superior to other samples with regards to the induction of apoptosis at early state. Similarly to herbs, the percentage of necrotic cells was almost stable over time, which confirms the ability of fruits extracts to induce apoptosis.

6.4 Determination of caspase-3 activity

Following the induction of apoptosis by the extracts, further study was conducted to assess the involvement of caspase-3 protease activity-induced apoptosis using human promyelocytic leukemia (HL-60) cells. The sequential activation of caspase family plays an essential role in denaturing the cellular infrastructure during intrinsic apoptosis pathway. To observe caspase-3 activity the cells were treated with plant extracts at a concentration of 0.80 mg/mL, and the presence of caspase-3 assay was monitored spectrophotometrically.

The induction of caspase-3 activity was observed after cells treatment with each of the native Australian herb and fruit extracts (Figure 6.5A & B). For each of the samples investigated, caspase-3 activity was detected as early as at 3 h time point, with the highest level of caspase-3 at the 6 h time point. The caspase-3 activity then decreased at 12 h. This evidence supports the induction of caspase-3 as an earlyevent in the induction of apoptosis. A comparison of the four native herbs extracts at the 6 h time point revealed that the TPL and LM induced the greatest level of caspase-3 activity. All native

Australian herbs extracts showed significantly higher activity than that of a reference sample BL.

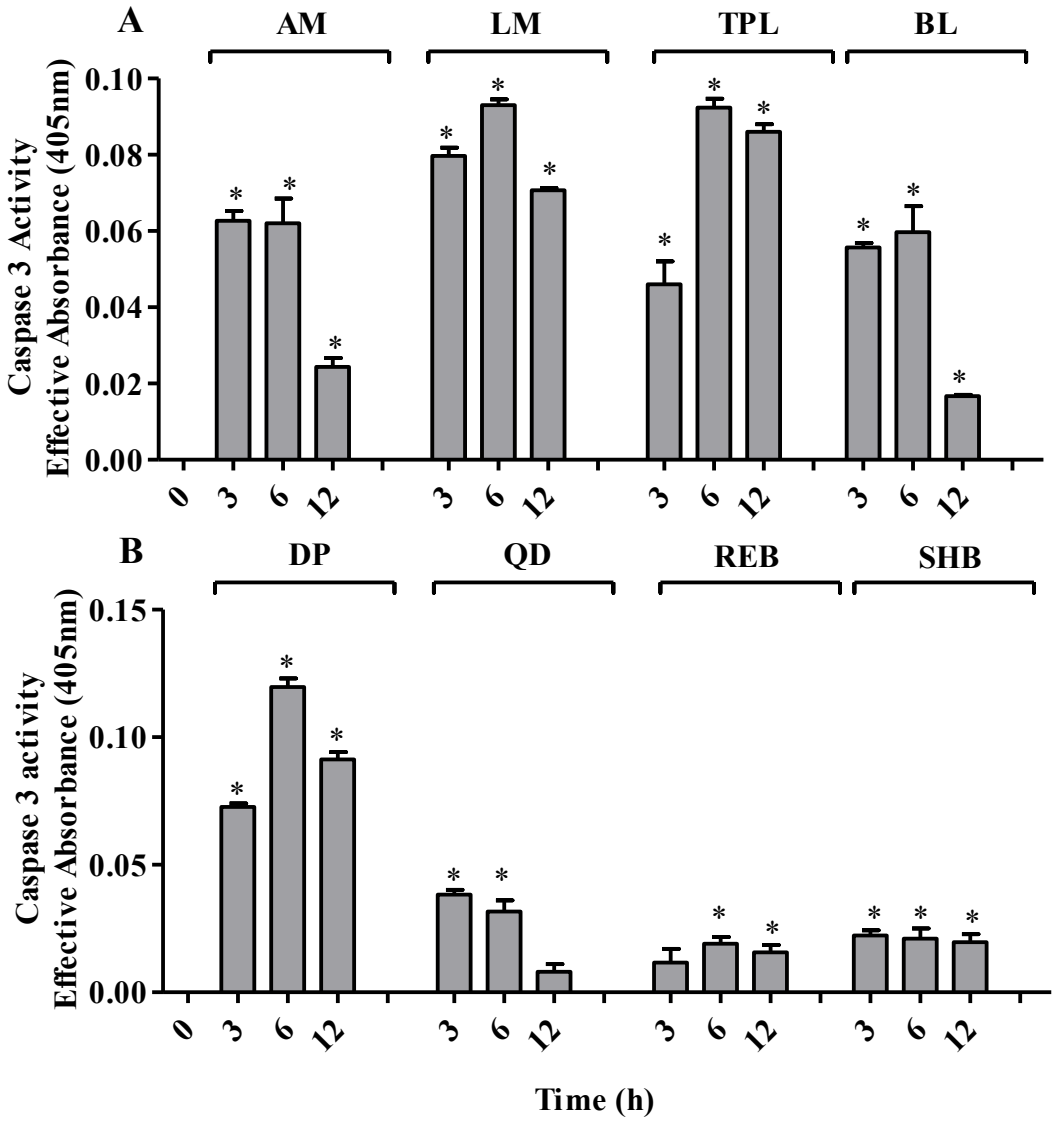


Figure 6.5 Level of caspase-3 activation of HL-60 cells treated with purified polyphenolic-rich extracts obtained from native Australian herbs (A) and fruits (B)

At 6 h, LM treated HL-60 cells showed the highest overall level of caspase-3 activation (0.093 ± 0.002 effective absorbance), which was similar to that induced by TPL (0.092 ± 0.007). The induction of caspase-3 activity by AM (0.062 ± 0.01) was the same as by BL (0.060±0.01). At 12 h time point, a small decrease of caspase-3 activity was observed after the treatment with TPL extract (10%), followed by LM (20%), and AM (0.02 ± 0.004).

In case of fruits, DP induced the greatest level of caspase-3 activity. QD exhibited lower level efficiency to induce caspase-3, which was slightly higher than that of blueberries. Similarly to the treatment with herbs extracts, the highest efficacy of the extract to induce caspase-3 activity was detected at 6 h time point. These results suggest that the evaluated purified olyphenolic-rich extracts induce the process of apoptosis via caspase pathway.

6.5 Genotoxic and pro-apoptotic effects of purified polyphenolic-rich native Australian herb and fruit extracts

Dietary compounds may cause damage to cancer cells through numerous pathways. To determine the mechanistic basis for the cytotoxic effects of the native Australian plants to cancer cells, the CBMB Cyt assay was conducted as a result of the presence of genotoxicity (Fenech, 2007) and provides information on potential cytotoxic and cytostatic effects. In this study, HT-29 colorectal adenocarcinoma cells were exposed to various concentrations of purified polyphenolic-rich extracts (0.5 and 1.0 mg/mL) and the frequency of the various cytome biomarkers was determined (Table 6.2). All purified polyphenol-rich extracts significantly increased the frequency of apoptotic cells in comparison to the control treatment group.

Presence of herbs and fruit extracts in the culture medium resulted in an increase of theapoptotic cells level in comparison to control. The level of apoptotic cells increased from control to 70.4 ± 6.7% after treatment with AM (0.5 mg/mL) and 74.0 ± 7.1% (1.0 mg/mL), to 52.0 ± 17.0% after treatment with LM (0.5 mg/mL) and 69.5 ± 2.1% (1.0 mg/mL), and63.4 ± 6.3% after treatment with TPL (0.5 mg/mL) and 62.8 ± 19.1% (1.0 mg/mL), DP induced apoptosis in 51.0 ± 1.4% (0.5 mg/mL) and 70.0 ± 11.6% (1.0 mg/mL) and QD – in 52.0 ± 10% (0.5 mg/ml) and 58.1 ± 5.1% (1.0 mg/ml). There were no significant differencesin the number of necrotic cell for each extract. A significant decrease in NDI was observed for each herb compared to control cells with the exception of AM 0.5 mg/mL (1.30 ± 0.03) and TPL 1.0 mg/mL (1.24 ± 0.02). The lower number of NDI arose from a substantial decrease in the number of BN cells. In contrast, DP and QD treatment resulted in an increase of NDI, especially at concentration of 1.0

Table 6.2 Frequency of various cell types of HT-29 cells in CBMN cultures treated with various doses of purified polyphenolic-rich extracts obtained from native Australian herbs and fruits

	Frequency of cell type[a]									
	Mononuclear	Binuclear	Multi	Apoptotic	Necrotic	NDI	MN in BN	Total MN in BN	NPB	NBud
Control	339.3 ± 22.3	136.3 ± 21	5.3 ± 2.1	9.0 ± 1.7	10.0 ± 1.0	1.31 ± 0.05	1.8 ± 0.3	1.8 ± 0.3	9.7 ± 3.8	9.9 ± 5.5
Herbs										
AM 0.5 mg/mL	307.1 ± 4.2*	98.8 ± 7.7*	12.7 ± 2.7	70.4 ± 6.7*	11.1 ± 0.5	1.30 ± 0.03	3.3 ± 0.8	3.3 ± 0.8	9.8 ± 4.3	20.9 ± 6.8*
AM 1.0 mg/mL	346.5 ± 3.5	83.5 ± 12.0*	1.0 ± 0.4	74.0 ± 7.1*	10.5 ± 0.7	1.20 ± 0.01*	4.1 ± 3.3	4.1 ± 3.3	4.9 ± 3.4	7.9 ± 0.8
LM 0.5 mg/mL	350.5 ± 34.6*	84.0 ± 22.6*	4.0 ± 4.2	52.0 ± 17.0*	9.5 ± 0.7	1.21 ± 0.04*	2.3 ± 2.0	2.3 ± 2.0	3.5 ± 1.0	5.7 ± 1.5
LM 1.0 mg/mL	356.5 ± 10.6*	63.0 ± 4.2*	3.0 ± 1.4	69.5 ± 2.1*	8.0 ± 2.8	1.16 ± 0.02*	2.0 ± 1.3	2.0 ± 1.3	3.1 ± 1.2	6.7 ± 0.2
TP 0.5 mg/mL	344.2 ± 1.1	80.1 ± 4.1*	4.1 ± 1.6	63.4 ± 6.3*	8.1 ± 2.6	1.21 ± 0.0*	1.8 ± 0.7	1.8 ± 0.7	4.2 ± 0.4	9.6 ± 2.4
TP 1.0 mg/mL	330.2 ± 21.1	96.0 ± 0.5*	4.0 ± 2.9	62.8 ± 19.1*	7.0 ± 1.3	1.24 ± 0.02	3.2 ± 0.9	3.2 ± 0.9	9.7 ± 4.5	20.8 ± 7.2*
Fruits										
DP 0.5 mg/mL	285.5 ± 19.1	140.5 ± 4.9	12.0± 11.3	51.0 ± 1.4*	11 ± 4.2	1.38 ± 0.07	4.5 ± 2.1	4.5 ± 2.1	3.0 ± 1.4	8.0 ± 2.8
DP 1.0 mg/mL	237.1 ± 13.3*	172.0 ± 3.3*	12.4 ± 2.2	70.0 ± 11.6*	8.4 ± 0.7	1.47 ± 0.01*	2.7 ± 0.8	2.7 ± 0.8	8.1 ± 3.9	17.4 ± 6.2*
QD 0.5 mg/mL	278.8 ± 11.5	155.5 ± 2.7	7.6 ± 3.2	52.0 ± 10*	6.1 ± 1.1	1.39 ± 0.02	2.8 ± 0.7	2.8 ± 0.7	8.5 ± 3.8	13.7 ± 0.3
QD 1.0 mg/mL	262.7 ± 6.4	161.9 ± 3.8	9.0 ± 1.8	58.1 ± 5.1*	8.1 ± 4.2	1.42 ± 0.02*	7.1 ± 1.7*	7.1 ± 1.7*	9.6 ± 1.5	11.3 ± 0.9

[a] The results represent the mean ± standard deviation per 500 cells of at least 2 counted slides. An asterisk represents significant difference ($p < 0.05$) in particular cell type between treated sample and control slides. CBMN, cytokinesis-block micronucleus. NDI, nuclear division index. MNi, micronuclei.BN, binuclear. NPB, nucleoplasmic bridges. NBud, nuclear buds.

mg/mL for both fruits. The frequency of the various biomarkers of DNA damage (MNi, NPB and NBud) was scored. No significant differences of MNi were observed after treatment with each extract. Only AM (0.5 mg/mL) and TPL (1.0 mg/mL) treatments resulted in a high number of NBud compared to control cells. The results suggested no induction of DNA damage in HT-29 cells by the extracts.

6.6 Anti-inflammatory activity

Inflammation and chronic inflammatory mechanisms represent a complex process involved in the development of major chronic conditions including cancer and atherosclerosis (Finch & Crimmins, 2004). The emerging evidence from epidemiological studies has confirmed significant correlation between inflammation and cancer. Various literature sources also reported that chronic inflammatory disease increases the risk of numerous types of cancer including bladder, cervical, gastric, intestinal, oesophageal,ovarian, prostate and thyroid cancers, accounting for 15 – 20% of all cancer malignancy worldwide arrising from infections and inflammatory responses (Balkwill & Mantovani, 2001; Mantovani *et al.*, 2008). It is now becoming clear that an association between inflammatory response and cancer impacts every single tumorigenesis at various steps of development process such as initiation, promotion, malignant conversion, invasion and metastasis (Grivennikov *et al*., 2010).

Phytochemicals have long been used to treat inflammatory disorders and related diseases (Krishnaswamy, 2008; El Beyrouthy *et al.*, 2008). Several plant extracts and isolated compounds have been reported to exert anti-inflammatory activity. Tan *et al.* (2011c) reported anti-inflammaroty activity of purified polyphenolic-rich native Australian fruits as well as turmeric (*Curcuma longa*), fruits, herbs and spices (Muller*et al.*, 2010). Quercetin has also been found to inhibit NF-κB, iNOS and COX-2 activity (Davis *et al.*, 2009) and inhibits iNOS, COX-2 and C-reactive protein (CRP), and down-regulates NF-κB and TNF-a secretion (Garcia-Mediavilla *et al.*, 2008).

Commercially available ellagic acid exhibited anti-inflammatory properties by down-regulating iNOS, COX-2, TNF-a and IL-6 via inhibition of NF-κB. Ellagic acid also

exerted chemopreventive effect on colon carcinogenesis after administration, as investigated in male Wistar albino rats [treatment with ellagic acid 60 mg/kg bodyweight/every day p.o. for 30 weeks] (Umesalma, & Sudhandiran, 2010). Anthocyanins from berries have also shown anti-inflammatory effects in both *in vitro* and *in vivo* models (Seeram *et al.*, 2001; Jean-Gilles *et al.*, 2012). The anti-inflammatory activity of polyphenolic rich extracts obtained from selected native Australian fruits; e.g. kakadu plum has been reported (Tan *et al.*, 2011c).

This chapter describes potential anti-inflammatory activities of selected native Australian herbs: TPL, AM and LM and fruits: DP and QD. Purified polyphenolic-rich extracts were evaluated in LPS-activated murine macrophages for their anti-inflammatory effect, and the release of $NO^{\bullet}$ and PGE_2, which are the products of the inducible nitric synthase (iNOS) and cyclooxygenase (COX- 2) enzymes, were monitored. Nitric oxide (NO•) is an important free radical molecule generated by L-arginine in metabolic reaction catalysed by nitric oxide synthases. NO• is an important cellular signalling molecule, having a vital role in many biological processes, especially it plays an essential part in the regulation of various physiological functions in cardiovascular and immune systems.

6.6.1 Effect of native Australian herbs and fruits on nitric oxide (NO•) concentration

The inhibitory activities of the purified polyphenolic-rich extracts against nitric oxide (NO•), as evaluated in HepG2 cells, are presented in Figure 6.6. Each purified polyphenolic-rich extract reduced the concentration of nitric oxide in a concentration-dependent manner.

All herbs extracts, applied in the concentration range between 25 - 400 µg/mL, caused a reduction in nitric oxide levels. At the lowest concentration of 25 µg/mL, TPL exhibited similar efficiency as LM, and both extracts were more potent than AM extract. Extracts obtained from the native herbs were more effective inhibitors of $NO^{\bullet}$ production than BL extract. Among fruits extracts, DP and QD were significantly stronger inhibitors of nitric oxide production than REB and SHB extracts (Figure 6.6B). At the lowest and the highest concentrationsquandong was more potent inhibitor of nitric acid than DP. This result

suggests that native Australian herbs and fruits polyphenolics may suppress the production of $NO^{\bullet}$, which is involved in inflammatory processes.

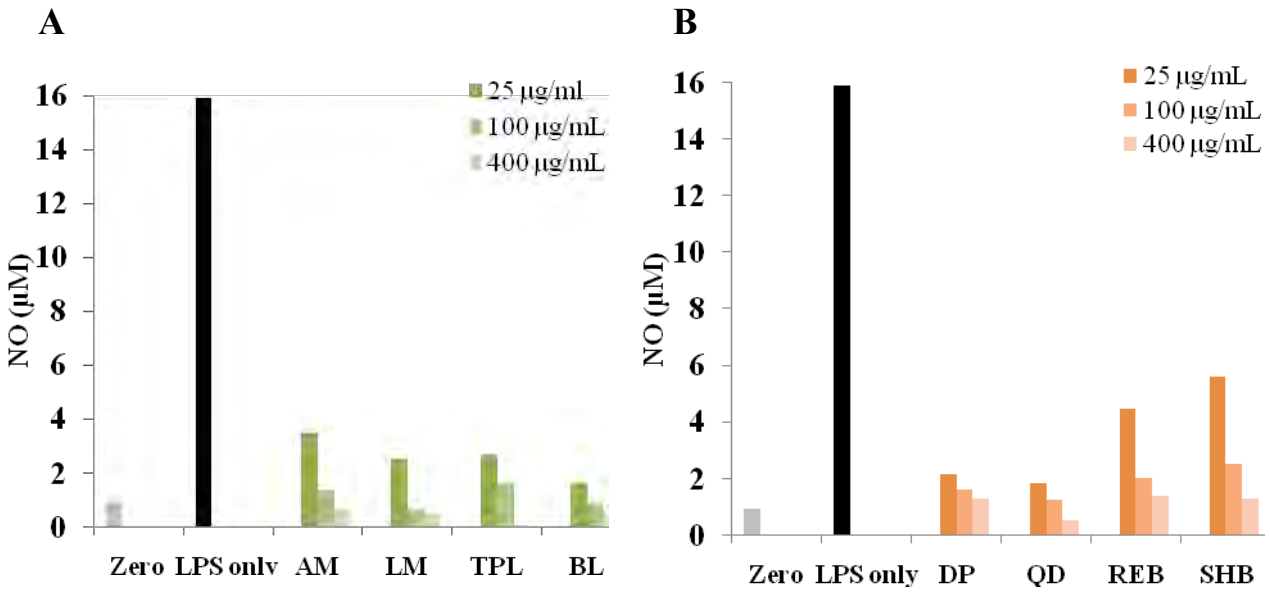


Figure 6.6 Dose-dependent effects of purified polyphenolic-rich extract obtained from native Australian herbs (A) and fruits (B) on nitrite concentration

HepG2 cells were exposed to serum-free medium for 2.5 h, different concentrations of purified polyphenolic-rich extracts for 1 h and LPS for further 12 h. The nitrite concentration (A) or level of PGE_2 release (B) was then measured. Data represent the mean ± standard deviation of at least three independent experiments. An asterisk indicates significant difference with LPS control ($p < 0.05$).

6.6.2 Effect of native Australian herbs and fruits on prostaglandin (PGE_2) production

Anti-inflammatory activity of the extracts was further evaluated by measuring their potency on the pro-inflammatory mediator PGE_2 production in activated hepatocellular carcinoma (HepG2) cells. PGE_2 has been recognised to be the principal constituent of the COX-2 enzymes. Each purified polyphenolic-rich extract effectively inhibited the generation of PGE_2 (Figure 6.7A & B). Among herbs extracts applied at a low concentration (100 µg/mL), TPL was the most efficient inhibitor, and was followed by LM and BL extracts. AM showed significantly lower efficiency than the other extracts. At higher concentration (400 µg/mL), TPL completely inhibited PGE_2 production, and

was followed by AM and BL extracts (Figure 6.7A). The increase in concentration of lemon myrtle extract didn't affect the PGE_2 level. In case of fruits, each purified polyphenolic-rich extract inhibited PGE_2 production in a dose-dependent manner (Figure 6.7B). At the lower concentration of 100 µg/mL, QD activity was comparable to these of two blueberry extracts, whereas DP was less efficient. However, at the higher concentration of 400 µg/mL, DP extract was superior in reducing PGE_2 level.

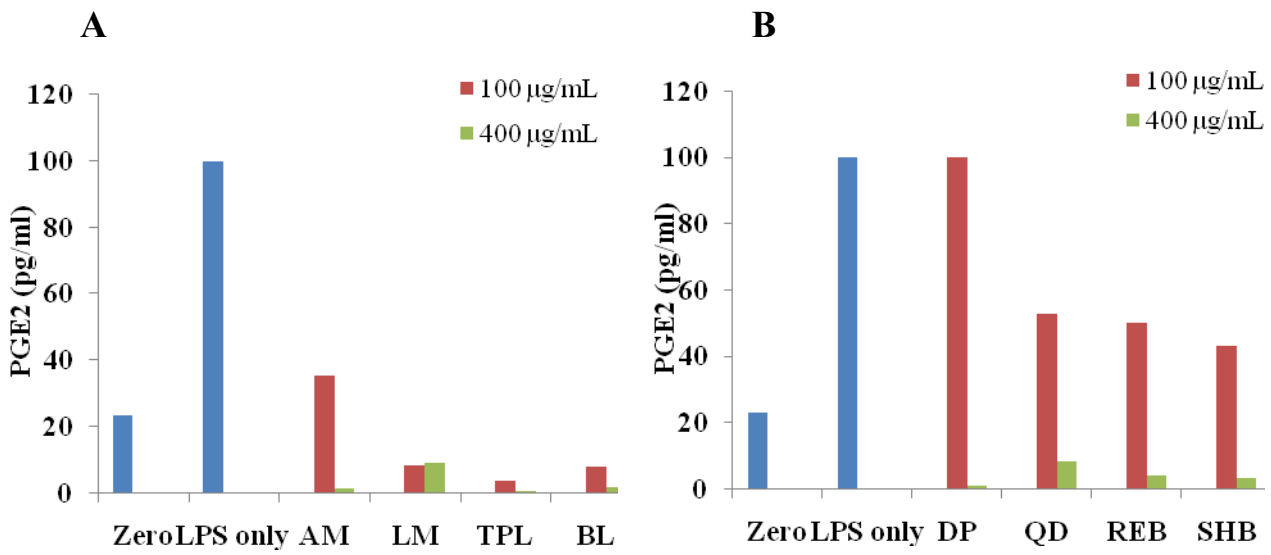


Figure 6.7 Dose-dependent effects of purified polyphenolic-rich extracts obtained from native Australian herbs (A) and fruits (B) on prostaglandin E_2 (PGE_2) release

HepG2 cells were exposed to serum-free medium for 2.5 h, different concentrations of purified polyphenolic extracts for 1 h and LPS for further 12 h. The nitrite concentration (A) or level of PGE_2 release (B) was then measured. Data represents the mean ± standard deviation of at least three independent experiments. An asterisk indicates significant difference with LPS control ($p < 0.05$).

6.7 Discussion

This study has revealed the ability of purified polyphenolic-rich extracts obtained from native Australian herbs and fruits to inhibit proliferation of cancer cells without negative effect on their equivalent non-transformed cells. The extracts demonstrated pro-apoptotic

activity against various cancer cell lines. The results are in agreement with those of Tan *et al.* (2011a), who reported antiproliferative activity and cytotoxic effects of purified polyphenolic-rich extracts of native Australian fruits (kakadu plum, muntries, Illawarra plum and native current) against various cancer cell lines.

The mechanisms of inhibition of proliferation or induction of cell death as an effect of treatment with plant extracts were investigated. Flow cytometry analysis of HL-60 cells revealed different proportions of apoptotic and necrotic cells at different concentrations of the extracts and over time. It should be noted that the apoptotic cells eventually are degraded into necrotic cells. The annexin-staining used allows detection of the cells at an early stage of apoptosis (Van Engeland *et al.*, 1996) and in a rather narrow time-window, which is then followed by a longer period of DNA fragmentation and lysis detected as 'necrotic' cells. In this study, various techniques were used to assess apoptosis, including flow cytometry with annexin V and propidium iodide staining, caspase-3 activity and CBMN assay. The results showed that AM is the most efficient inducer of apoptosis, as documented by the highest levels of apoptotic and late apoptotic cells at 24 h of treatment. LM and TPL were comparable to BL. DP among fruits effectively induced cell apoptosis. The pattern of apoptosis induction was similar to both that of AM and the reference samples of blueberries, with the highest level of apoptosis detected at the first 3 h. The efficiency of QD extract to induce apoptosis was low.

Induction of apoptosis by polyphenols or polyphenolic-rich extracts in HL-60 cells, in a combination with morphological assessment, DNA fragmentation and flow cytometry, were reported for epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), resveratrol, deguelin and xanthone, isolated from traditional Chinese herb, *Gentianopsis paludosa* (Han & Kim, 2009; Surh *et al.*, 1999; Lambert *et al.*, 2005).

Further method was also employed to identify apoptosis through monitoring the activity of caspase-3. The induction of proteases of the caspase family is characterised as the most important biochemical event occurring during apoptosis process (Kurokawa & Kornbluth, 2009). Each extract was found to induce the expression of caspase-3, with the greatest levels of enzyme activity at 6 h with the exception of QD. Herbs were superior in the induction of caspase 3 activity. Similarly, commercial green tea polyphenols showed

inhibitory activity of HL-60 cells proliferation (IC_{50} 49.5 µg/mL) with incubation time double of that applied in this study (48 h). Green tea polyphenols also induced apoptosis in HL-60 cells through down-regulation of Bcl-2 expression level and caspase-3 activity (Han *et al.*, 2009).

The CBMN Cyt assay determines DNA damage, cytoxicity and cytostasis caused by the purified polyphenolic-rich extracts. HT-29 cell lines were selected as the most suitable for this assay that they provid large cells and easy to count. The concentrations applied in this assay were chosen to reveal potential genotoxicity without causing significant cell death. Only QD at 1.0 mg/mL showed significantly different and higher number of micronuclei (MNi) compared to the control cells. The result suggested chromosome breakage/loss, DNA misrepair, telomere end-fusions and gene amplification may all be the predominant mechanism by which cytotoxicity occurs. The other extracts did not induce DNA damage. The result also revealed that the induction of apoptosis in HT-29 cells was accompanied by a decrease in BN cell numbers. This evidence was only observed in all herbs, indicating that damage may occur during cytokinesis or an inhibition of cell division.

The results also showed that purified polyphenolic-rich extracts obtained from native Australian herbs and fruits inhibited accumulation of nitric oxide (NO•) and release of PGE_2, the primary products of iNOS and COX-2 inflammatory enzymes. In particular herbs exhibited more pronounced inhibitory activity against nitric oxide and PGE_2 production in LPS-activated hepatocellular carcinoma (HepG2) cells. At the highest concentration (400 µg/mL), TPL and QD were more efficient inhibitors of NO• concentration. TPL and AM extracts among herbs and DP among fruits exhibited the greatest potential to suppress the release of PGE_2.

Each herb and fruit extracts showed different potency in various assays, which is believed to depend on the phytochemical composition. AM, LM and DP extracts contain ellagic acid and derivatives as major constituents, whereas TPL and QD contain predominantly monomeric compounds (chlorogenic acid: Table 4.2 & 4.3). Over the past decade, anti-inflammatory properties of natural products obtained from traditional medicines and fruits were extensively investigated *(*Middleton *et al.*, 2000). Khan and Mukhtar (2008) reported that epigallocatechin gallate (EGCG) exhibited anti-inflammatory effect, through

inhibition of NF-κB signalling pathway, increased IκB levels while inhibiting NF-κB nuclear translocation. EGCG also inhibited the expression of iNOS and COX-2 and subsequent $NO^{\bullet}$ and PGE_2 production (Chen & Zhang, 2007), without effects on COX-1 expression observed by Shankar *et al.* (2007).

Ellagic acid has been reported to exert inhibitory activity against proliferation of human osteogenic sarcoma (HOS) cell line (IC_{50} 6.5 μg/mL) (Han, Lee & Kim, 2006). The authors also reported its potential to induce apoptosis via activation of caspase 3 Specifically, the study reported that ellagic acid interacts with other polyphenolic compounds in a synergistic manner to induce the caspase-3 activity, increase Bax, a pro-apoptotic protein, resulting in a decrease in the ratio of Bcl-2/Bas, one of the major events involved in apoptotic regulation (Mertens-Talcott & Percival, 2005).

Tasaki *et al.* (2008) highlighted a high tumour selectivity of ellagic acid, with no mortality or treatment-related negative clinical signs in rats treated with about 120 mM daily over 90 days (Tasaki *et al.*, 2008). The extremely low concentration of ellagic acid and its high effectiveness suggested that ellagic acid may be applicable for clinical use. In addition, the study reported that ellagic acid inhibited proliferation and induced accumulation of the S-phase cells in the cell cycle of HL60, activated apoptotic pathway by regulating caspase-3 activity and enhanced ATRA-induced differentiation (Hagiwara *et al.*, 2010).

Anthocyanins, in particular cyanidin 3-glucoside found in fruits, have been previously reported to inhibit cell growth, induce apoptosis in a human breast carcinoma cell line (HS578T) through caspase-3 and PARP regulation and down-regulated the expression of DKs and cyclins (Chen *et al.*, 2005). In addition, the agycones of cyanidin and delphinidin have also showed anti-proliferative activity against HL-60 and colorectal adenocarcinoma (HCT116) cells (Katsube *et al.*, 2003).

Polyphenols from native Australian fruits may have significant pro-apoptotic anticancer activity. The combination of antioxidant, pro-apoptotic and anti-inflammatory potential may provide a multi-targeted approach to the inhibition of the initiation, progression and promotion of carcinogenesis. Native Australian herbs and fruits have marked potential

anti-proliferative and pro-apoptotic effects, however a more complete chemical profile of these materials need to be elucidated to further characterise the molecular pathways and understand potential mechanisms of action.

6.8 Conclusions

This chapter demonstrated chemopreventive effects of purified polyphenolic-rich extracts obtained from native Australian herbs and fruits. The results can be summarised as follows:

(i) Purified polyphenolic-rich extracts from each native Australian herbs and fruit, exhibited selectively anti-proliferative activity against a panel of cancer cell lines (HT-29, AGS, BL13 and HepG2) in comparison to normal cell lines (CCD-18Co, Hs 738.St/Int) with an outstanding result by AM.

(ii) Purified polyphenolic-rich extracts from each native Australian herbs and fruits induced apoptosis in a human promyelocytic leukaemia cell line (HL-60).

(iii) The induction of apoptosis by purified polyphenolic-rich extracts from native Australian herbs and fruits involved caspase-3.

(iv) Purified polyphenolic-rich extracts obtained from herbs and fruits induced apoptosis in HT-29 cells (CBMN Cyt assay).

(v) Purified polyphenolic-rich extracts obtained from TPL and QD inhibited most efficiently expression of nitric oxide (NO•), the key product of inflammatory process at 400 μg/mL.

(vi) Purified polyphenolic-rich extracts obtained from TPL, AM and DP exhibited the greatest potency to inhibit the expression of PGE_2, principal product of COX-2 at 400 μg/mL.

Chapter 7

Inhibitory activity against digestive enzyme relevant to metabolic syndrome

7.1 Introduction

Metabolic syndrome is a leading health disorder worldwide (International Diabetes Foundation, 2012) and a predisposing factor in the development of diabetes and cardiovascular diseases. Diabetes, high blood pressure, high cholesterol, high triglycerides and obesity are the symptoms of metabolic syndrome. The linkage between metabolic syndrome and chronic inflammation in white adipose tissue is also an important biological feature and becomes systemic (Emanuela *et al.*, 2012).

Polyphenols possesse multifactorial properties associated with their antioxidant, anti-inflammatory and neuroprotective effects and they also enhance insulin function (Broadhurst *et al.*, 2000). Compounds suitable for metabolic syndrome treatment should possess hypoglycaemic, hypolipidemic and antioxidant properties (Fraga, 2005; Manach *et al*. 2005; Scalbert *et al*. 2005). Polyphenols could possibly lead to the development of new sources for safer agents on metabolic syndrome management.

Metabolic syndrome, characterised by glycemic index imbalance, glucose intolerance, hypertension, dyslipidemia and/or obesity, is an early sign of potential future development of chronic conditions, such as type 2 diabetes, which is characterised by postprandial hyperglycemia - a rapid increase of blood glucose level after food consumption. The rapid increase of blood glucose can be reduced through inhibition of enzymes involved in the release of glucose from foods and this approach is used in the management of type 2 diabetes, with the main target being α-glucosidase enzyme.

α-Glucosidase is a membrane-bound enzyme located in the epithelium of the small intestine, and catalyses the cleavage of glucose from disaccharides and oligosaccharides, which facilitates an uptake of glucose into the blood stream. Hence, inhibition of α-glucosidase activity reduces glucose release and subsequently the uptake. Consumption of α-glucosidase inhibitors naturally occurring in food is an important supporting factor in management of postprandial hyperglycemia. Two other enzymes that impact on metabolic syndrome are pancreatic lipase (Grove *et al.*, 2011) and angiotensin I-converting enzyme (ACE) (Balasuriya & Rupasinghe, 2011). Lipase, primarily produced in the pancreas, hydrolyses lipids to form fatty acids so they can be absorbed in the human digestive system. Pancreatic lipase is the key enzyme which hydrolyses triglyceride into glycerol and fatty acids, facilitating an uptake of fat (triglycerides). Angiotensin converting enzyme (ACE) plays an important part in regulation of blood pressure and normal cardiovascular function. It catalyses the conversion of angiotensin I to angiotensin II, which increases blood pressure, therefore inhibition of ACE may help to reduce hypertension (Shalaby *et al.*, 2006).

To understand the mechanisms that polyphenols act as mediator to balance the system is essential in order to develop effective strategies to prevent chronic inflammatory signalling from white adipose tissue and metabolic syndrome management. Therefore, this study tries to assess the ability of native Australian herbs and fruits to regulate enzyme relevant to metabolic syndrome. The various methods were performed include α-glucosidase, pancreatic lipase and angiotensin-converting enzyme inhibitory activities.

7.2 Inhibitory activities against α-glucosidase

A dose-dependent inhibition of α-glucosidase was observed for all polyphenolic-rich extracts evaluated in this study (Figure 7.1). Among the herb extracts, at each concentration tested, the inhibitory activities of TPL, AM and LM polyphenolic-rich extracts were higher than that of BL extract. AM and LM exhibited more pronounced inhibitory activities against α-glucosidase (IC_{50} = 0.30 mg/mL and 0.13 mg/mL, respectively) than TPL (IC_{50}= 0.83 mg/mL) and BL (IC_{50} = 3.21 mg/mL). At the concentration of 1.0 mg/mL the α-glucosidase inhibition rate of AM extract was 88.9 ± 2.7%, LM 64.1 ± 3.1%, and TPL 55.7 ± 1.7% (Figure 7.1A).

Table 7.1 α-Glucosidase inhibitory activity of purified polyphenolic-rich extracts obtained from native Australian herbs and fruits, compared to reference samples

Inhibiting agent	α-glucosidase	
	IC_{50} [a] (mg/mL)	Acarbose E[b] (μmol/gDW)
Herbs		
Anise myrtle	0.30±0.02[b]	164.1±3.9[b]
Lemon myrtle	0.13±0.04[a]	359.0±18.8[a]
Tasmannia peppep leaf	0.83±0.36[c]	54.9±4.8[c]
Bay leaf	3.21±0.14[c]	15.2±0.2[c]
Fruits		
Davidson's plum	0.13±0.001[b]	375±3.9[d]
Quandong	0.39±0.01[a]	124±2.7[c]
Rabbit eye blueberry	0.097±0.002[c]	502±11[b]
Southern highbush blueberry	0.091±0.001[c]	532±8.3[a]

[a]IC_{50} – half maximul inhibitory concentration.Means with different superscripts in the same column are significantly different at the ($p < 0.05$) level; $n = 3$. [b]Acarbose E: acarbose equivalent.

Similarly, purified polyphenolic-rich extracts obtained from fruits also exhibited a dose-dependent inhibition of isolated α-glucosidase (Figure 7.1B). At the concentration of 1.0 mg/mL DP extract had the greatest inhibitory activity of 93.5 ± 1.8%. At the same concentration the α-glucosidase inhibitory activity of QD extract was significantly lower (65.1 ± 2.4%) and comparable to that of REB (62.8 ± 1.9%) and SHB (70.3 ± 2.0%). However, when applied at the lowest concentration of 0.1 mg/mL, blueberries exhibited more pronounced inhibitory activities than DP and QD. This resulted in the lowest IC_{50} values of 0.097 and 0.091, respectively, for REB and SHB (Table 7.1), closely followed by DP (IC_{50} of 0.13).

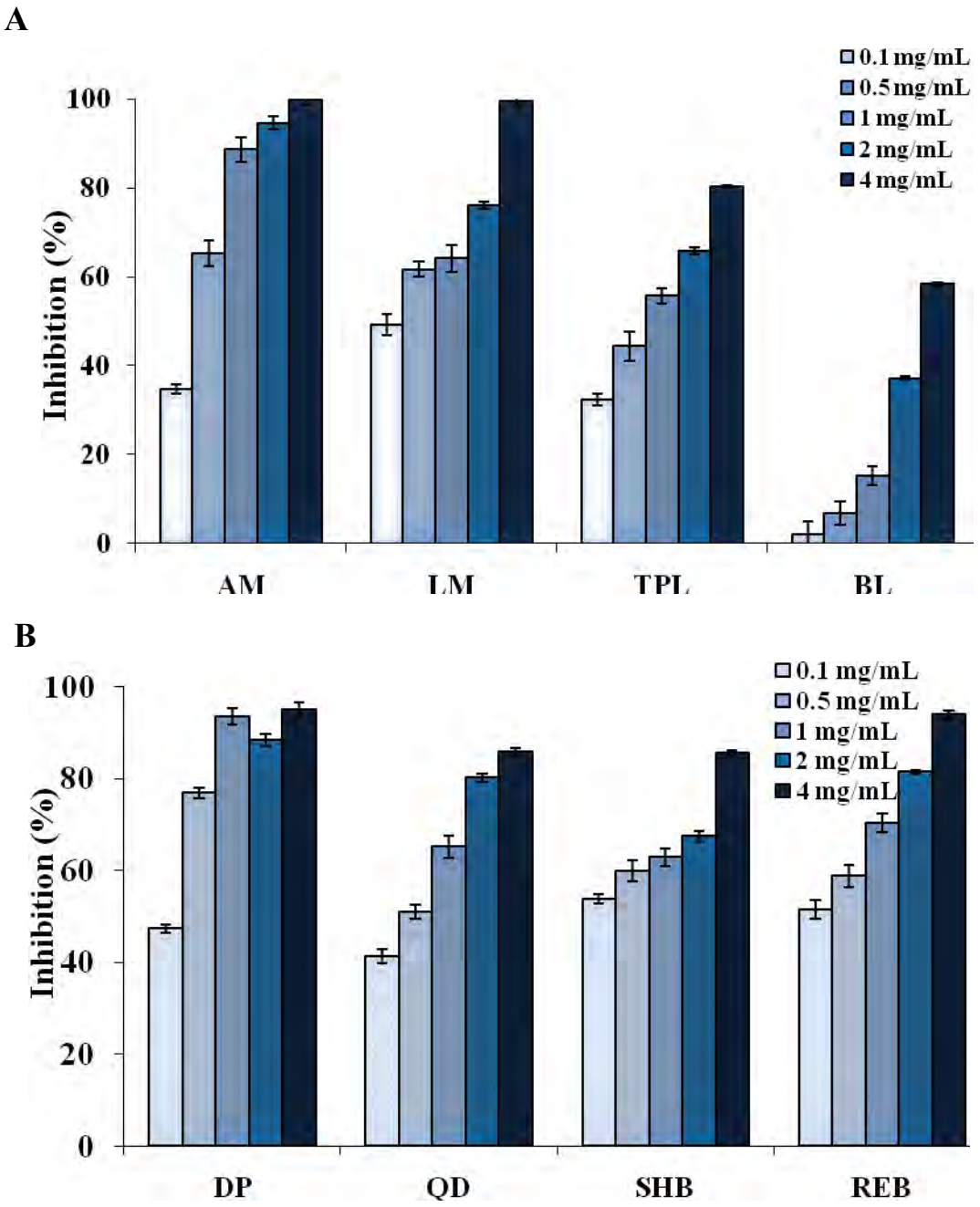


Figure 7.1 Dose-dependent inhibition of α-glucosidase activity of purified polyphenolic-rich extracts obtained from native Australian herbs (A) and fruits (B)

7.3 Regulatory effect of phytochemicals on lipids metabolism

Evaluation of the purified polyphenolic-rich extracts for lipase inhibitory activities revealed that TPL extract was the strongest inhibitor of isolated pancreatic lipase at each concentration tested (Figure 7.2A). When applied at a concentration of 1.0 mg/mL, the

inhibition rate by the TPL extract was 62.2 ± 2.9%, by AM 42.9 ± 8.4% and LM 38.2 ± 3.5%. Accordingly, the IC_{50} of TPL extract had the lowest value of 0.60 mg/mL (Table 7.2).

Table 7.2 Lipase inhibitory activity of purified polyphenolic – rich extracts obtained from native Australian herbs and fruits, compared to reference samples

Inhibiting agent	Pancreatic lipase	
	IC_{50}[a] (mg/mL)	Orlistat E[b] (μmol/gDW)
Herbs		
Anise myrtle	1.55 ± 0.25[b]	2.6 ± 0.1[b]
Lemon myrtle	2.51 ± 0.3[b]	1.7 ± 0.0[bc]
Tasmannia pepper leaf	0.60 ± 0.03[a]	7.3 ± 0.8[a]
Bay leaf	6.3 ± 0.27[c]	0.7 ± 0.0[c]
Fruits		
Davidson's plum	1.74 ± 0.02[c]	2.16 ± 0.03[c]
Quandong	0.60 ± 0.01[a]	6.30 ± 0.07[a]
Rabbit eye blueberry	0.94 ± 0.01[b]	4.00 ± 0.06[b]
Southern highbush blueberry	1.02 ± 0.08[b]	3.69 ± 0.31[b]

[a]IC_{50} – half maximum inhibitory concentration.Means with different superscripts in the same column were significantly different at the level ($p < 0.05$); n = 3. [b]Orlistat E: orlistat equivalent.

Among fruits, QD extract was the most efficient inhibitor at each concentration tested and was followed by the blueberries (Figure 7.2B). When applied at a concentration of 1.0 mg/ml, the inhibition rate by the QD extract was 63.0 ± 1.5%, by DP 19.0 ± 1.0% and by REB and SHB 46.8 ± 0.7% and 41.8 ± 0.6%, respectively. Consequently, QD had the lowest IC_{50} of 0.6 mg/mL (Table 7.2). In contrast, DP extract exhibited approximatelythree times higher, represented by IC_{50} of 1.74 mg/mL (Table 7.2).

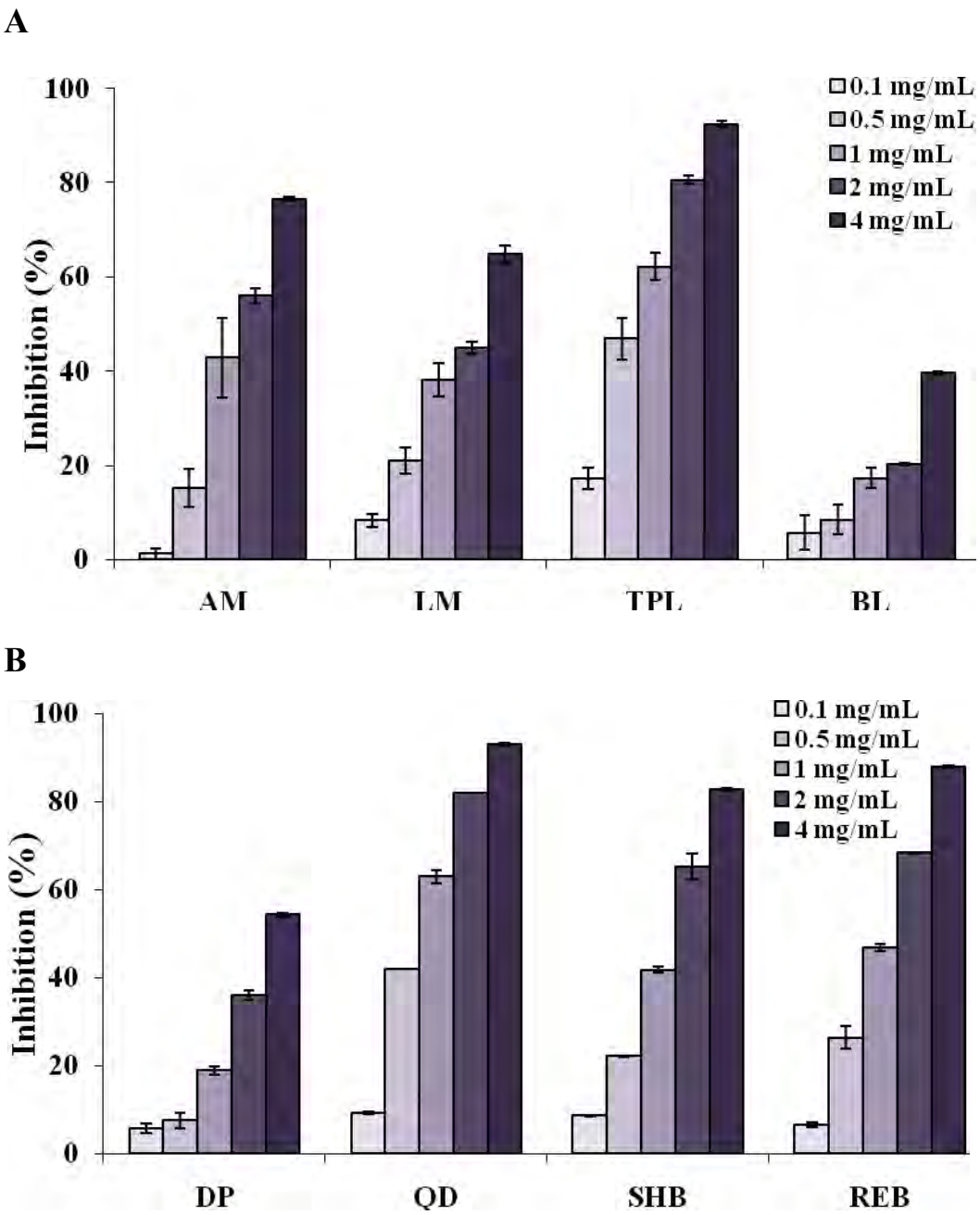


Figure 7.2 Dose-dependent inhibition of pancreatic lipase activity of purified polyphenolic-rich extracts obtained from native Australian herbs (A) and fruits (B)

7.4 Angiotensin converting enzyme (ACE) assay

All purified polyphenolic-rich extracts of the native Australian herbs applied at concentration of 1 mg/mL inhibited the activity of ACE (Table 7.3), with TPL and AM extracts showing comparable and higher activity than LM extract. Comparable ACE

inhibitory activities (approximately 30%) have been reported for aqueous extracts of ginger (Ranilla *et al.*, 2010) and red currant (da Silva Pinto *et al.*, 2008).

Both purified polyphenolic-rich extracts obtained from the native Australian fruits, applied at the concentration of 1.0 mg/mL inhibited the activity of isolated ACE (Table 7.3) with an outstanding inhibitory activity of DP extract (91.3% inhibition). It can be speculated that polymeric compounds present in DP extract could produce a similar effect to pycnogenol. Similarly, purified ellagitannin from strawberry was reported to inhibit ACE, however it was less potent than DP extract evaluated in this study (22% inhibitory activity observed at a concentration of 50 mg/mL) (da Silva Pinto *et al.*, 2010a).

Table 7.3 Angiotensin converting enzyme inhibitory activities of purified polyphenolic – rich extracts obtained from native Australian herbs and fruits, comparable to reference samples.

Inhibiting agent	**Angiotensin converting enzyme[a]**	
	Inhibition (%)	Captopril E[b] (μmol/g DW)
Herbs		
Anise myrtle	25.9 ± 4.2[a]	50.0 ± 8.8[a]
Lemon myrtle	13.0 ± 4.2[b]	29.6 ± 4.9[b]
Tasmannia pepper leaf	29.6 ± 4.2[a]	58.1 ± 9.7[a]
Bay leaf	13.9 ± 2.8[b]	30.5 ± 3.4[b]
Fruits		
Davidson's plum	91.3 ± 1.4[a]	487.2 ± 28.0[a]
Quandong	22.2 ± 1.4[b]	28.3 ± 1.6[b]
Rabbit eye blueberries	ND	6.8 ± 1.2[b]
Southern highbush blueberries	ND	5.8 ± 1.0[b]

[a]Angiotensin converting enzyme inhibition was evaluated using extracts at concentration of 1.0 mg/mL. Means with different letters in the same column were significantly different at the level ($p < 0.05$); $n = 3$. [b]Captopril E: catopril equivalent. ND = not detected.

7.5 Discussion

The results reveal specificity of phenolic compounds-enzyme interaction. Artz *et al.* (1987) reported that proanthocyanidins or condensed tannins are prone to complexation with proteins, and dimers are less effective. The same authors reported that the protein binding activity of simple flavonols varies, with some not precipitating proteins. According to Hagerman and Butler (1981) the conformation of proteins plays an important role in protein-phenolic compound interaction: tightly coiled globular proteins exhibit low affinity for tannins whereas proteins with an open conformation form complexes with tannins rapidly. Therefore the formation of protein – phenolic compound complexes, resulting in inhibition of an enzyme activity, depends on both: the molecular structure of a phenolic compound and the protein structure.

A number of plant species representing the Myrtaceae family are traditionally used as anti-hyperglycemic agents. For example, *Syzygium cumini* is the traditional folk remedy for type II diabetes in the Philippines and *in vivo* study with male Swiss Webster mice with alcoholic bark extract has confirmed the anti-diabetic properties (Villasenor & Lamadrid, 2006). Effective inhibition of hyperglycemia in mice by aqueous extract of *Eugenia uniflora* (Myrtaceae) found in South America, Southern Asia and Africa has been reported by Arai *et al.* (1999). The same study also demonstrated inhibition of hypertriglyceridemia and reduction of fats decomposition in the mice intestine. Suppression of pancreatic lipase activity was reported as the mechanism of action. Consolini*et al.* (1999) reported a hypotensive effect of aqueous extract of *Eugenia uniflora* (Myrtaceae) dry leaves exhibited through direct vasodilatation, supporting the traditional use of this plant as antihypertensive agent. In another study, among a number of phenolic fractions obtained from red raspberry, only fraction containing ellagitannins (lambertianin C and sanguiin H-6) exhibited vasolidation activity on aorta rings isolated from male New Zealand rabbit (Mullen *et al.*, 2002). Based on these results and datafound in this study, it can be speculated that ellagitannins identified in AM, LM and DP extracts play a role in inhibiting various digestive enzymes.

These levels of α-glucosidase enzyme inhibition are higher or similar to the inhibition by aqueous extracts of the majority of herbs and spices from Latin America, which, applied

at a concentration of 1.25 mg/mL, suppressed the enzyme activity by 20 to 40% (Ranilla *et al.*, 2010). The IC_{50} values of α-glucosidase inhibition (Table 7.1) for LM, AM and TPL polyphenolic-rich extracts are lower (AM and LM) or comparable (TPL) to the reported IC_{50} of aqueous extracts of an anti-diabetic folk medicine cinnamon bark (0.42 to 2.96 mg/mL), with the exception of the Saigon cinnamon with an IC_{50} value higher than 4 mg/mL (Adisakwattana *et al.*, 2011).

AM and LM extracts exhibited slightly higher *α*-glucosidase inhibitory activities than TPL. The purified polyphenol-rich extracts evaluated in this study were more effective inhibitors of α-glucosidase than methanolic extract of muscadine grape from south eastern United States (IC_{50} of 1.92 mg/mL) (You *et al.*, 2012). *Terminalia chebula*Retz.or black myrobalan is a fruit native to Pakistan and India that is used as carminative, deobstruent astringent and expectorant reagent in folk medicine. Three ellagitannins have been identified as the active components of this fruit: chebulanin, chebulagic acid and chebulinic acid. Two of them: chebulanin and chebulagic acid, evaluated individually, with IC_{50} of 690 μM (0.45 mg/mL) and 97 μM (0.093 mg/mL), respectively, were comparable inhibitors of α-glucosidase to DP, REB and SHB polyphenolic extracts obtained in this study (Gao *et al.*, 2007). Crude ethanolic extract of 'Ovation' strawberry cultivar [comprising chlorogenic acid (57.9 ± 3.6 μg/gFW), ellagic acid (28.2 ± 2.2 μg/gFW), and quercetin (3.3 ± 0.4 μg/gFW)] was the most effective inhibitor of α-glucosidase of 14 strawberry cultivars extracts with 80% suppression rate at the concentration of 0.1 mg/mL (IC_{50} of 0.05 mg/mL) (Cheplick *et al.*, 2010). This inhibitory effect was 2- to 8-times stronger than that of any polyphenolic extract evaluated in our study.

Strawberry ellagitannins were reported to be effective natural agents against hyperglycaemia and hypertension (da Silva Pinto *et al.*, 2010a). Similarly, Zhang *et al.* reported that ellagic acid exhibited 2-times greater *α*-glucosidase inhibitory effect than chlorogenic, gallic, gentisic, benzoic, vanillic, caffeic, coumaric and ferulic acid as well as quercetin and rutin. The same authors highlighted that cyanidin-diglucoside, pelargonidin-3-rutinoside, and catechin are also the active compounds of raspberries that inhibit α-glucosidase activity. Red raspberry, arctic bramble and cloudberry are rich sources of ellagitannins which account for 77 - 88% of total phenolic compounds

(Hakkinen *et al.*, 1999). Extracts of various cultivars of raspberry effectively inhibited hyperglycaemia *in vitro* (Zhang *et al.*, 2010). These results are in support toobservations made in this study of the α-glucosidase inhibitory activity of AM, LM and DP extracts and suggest that ellagic acid, ellagitannins and anthocyanins might be the active components responsible for the suppression of α-glucosidase enzyme activity.

Obesity, one of the physiological abnormalities representing metabolic syndrome, is becoming a common condition worldwide: in year 2008 about 1.46 billion adults (21.8%) were estimated to be overweight (Wang *et al.*, 2011). Obesity frequently leads to adverse chronic condition, especially diabetes mellitus, coronary heart disease, certain forms of cancer, and sleep-breathing disorders (Kopelman, 2000). Pancreatic lipase is the key enzyme which hydrolyses triglyceride into glycerol and fatty acids, facilitating an uptake of fat (triglycerides). According to Grove *et al.* (2011) inhibition of pancreatic lipase was the possible mechanism of modulatory activity of epigallocatechin-3-gallate isolated from green tea in fat absorption in male C57bl/6 mice on a high fat diet, which resulted in increased fecal lipid content (by 29.4%) and reduced final body weight.

Hsu and Yen (2008) described inhibitory effects of phenolic compounds on obesity and their underlying molecular signalling mechanisms. Among fifteen phenolic acids and 6 flavonoids they identified *o*-coumaric acid and rutin as the most potent inhibitors (61.3 and 83.0%, respectively) of adipogenesis in 3T3-L1 adipocytes (Hsu & Yen, 2007). In a follow up *in vivo* study with rats, the same authors found that feeding *o*-coumaric acid and rutin resulted in significant decrease of serum lipid profiles, insulin, and leptin. Moreover, the levels of hepatic triacylglycerol and cholesterol also significantly decreased. The authors concluded that intake of rutin and *o*-coumaric acid can be beneficial for the suppression of a high-fat-diet-induced dyslipidemia (Hsu *et al.*, 2009). Two isomers of chlorogenic acid: 3-*O*-caffeoylquinic acid (chlorogenic acid) and its structural isomer, 5-*O*-caffeoylquinic acid, were reported as the major antihyperglycemic principles present in the leaves of *Nerium indicum*, an Indian folk remedy for type 2 diabetes (Ishikawa *et al.*, 2007). Chlorogenic acid is present at high levels in the TPL and QD extracts. It can be suspected that a high level of hydroxycinnamic acids in these two extracts might have contributed towards the suppression of pancreatic lipase activity.

Similarly, chlorogenic acid-rich polyphenolic extracts obtained from a herb TPL was more efficient inhibitor of pancreatic lipase (IC_{50} = 0.6 mg/mL) than the ellagitannins-rich extracts obtained from AM and LM (IC_{50} = 1.5 and 2.5 mg/mL, respectively) (Sakulnarmrat & Konczak, 2012). Hawthorn fruits of the *Crataegus* species are used in China and Europe as traditional medicinal plants to strengthen heart function, lower blood lipids levels, and dilate blood vessels to promote blood circulation. The fruit rich in epicatechin and chlorogenic acid, exhibited pronounced hypolipidemic properties, when evaluated *in vivo* with New Zealand white rabbits (Zhang *et al.*, 2002).

Hypertension, elevated cholesterol levels, and dyslipidemia are the classic risk factors of cardiovascular disease (Fernandez, 2007). According to Mittal and Singh (2010) hypertension is becoming another common condition with about 25% of the world's adult population suffering, and this is estimated to increase to 29% by 2025. Over-expression of the ACE enzyme leads to an increase of blood pressure. Identification and incorporation of natural ACE inhibitors into foods may help to control this burden. For example, pycnogenol, a proanthocyanin oligomer isolated from French maritime pine (*Pinus maritima* L.) was identified as an effective modulator of blood pressure in humans, possibly due to the inhibition of ACE (Zibadi *et al.*, 2008).

All purified polyphenolic-rich extracts of the native Australian herbs and fruits, applied at the concentration of 1.0 mg/mL inhibited the activity of isolated ACE (Table 7.3) with an outstanding inhibitory activity of DP (91.3% inhibition). However, AM and LM extracts were found to be less efficient than DP extracts (26% and 13.0%, respectively). TPL showed more efficient in ACE inhibitory activity than quandong (30% and 22%, respectively). It can be speculated that polymeric compounds present in DP extract could produce a similar effect to pycnogenol. Similarly, purified ellagitannin from strawberry was reported to inhibit ACE, however it was less potent than DP extract evaluated in this study (22% inhibitory activity observed at a concentration of 50 mg/mL) (da Silva Pinto *et al.*, 2010a). The ACE inhibitory activity of quandong extract (approximately 30%) was comparable to that of aqueous extracts of red currant (da Silva Pinto *et al.*, 2010b), but was much lower than inhibitory activities of purified anthocyanins: delphinidin-3-*O*-sambubioside (IC_{50} = 84.5 µg/mL) and cyanidin-3-*O*-sambubioside (IC_{50} = 68.4 µg/mL) isolated from *Hibiscus sabdariffa* (Ojeda *et al.*, 2010).

7.6 Conclusions

The results suggest that all purified polyphenolic-rich extracts obtained from native Australian herbs and fruits possess inhibitory activities against α-glucosidase, pancreatic lipase and angiotensis I-converting enzyme. The results indicate a high affinity to bind to these enzymes *in vitro*, reducing their activities.

(i) AM and LM extracts had a pronounced effect against α-glucosidase and TPL was equally effective against both α-glucosidase and pancreatic lipase.

(ii) DP extract effectively suppressed the activities of isolated α-glucosidase; QD extract was most efficient inhibitor of pancreatic lipase.

(iii) DP was a superior to all evaluated samples inhibitor of angiotensin I-converting enzyme activity.

Chapter 8

Conclusions and recommendations

The objective of the current studies was to provide systematic information on potential health-beneficial properties of purified polyphenolic-rich extracts obtained from selected commercially grown native Australian herbs and fruits. TPL and DP extracts displayed the highest extraction yields, superior to these of reference samples, which indicates that native Australian herbs and fruits may serve as a good source of phenolic compounds.

The evaluated extracts comprised predominantly of phenolic acids (ellagic acid, chlorogenic acid, *p*-coumaric acid), flavonoids (quercetin, myricetin, hesperetin, anthocyanins) and polymeric phenolic compounds (ellagitannins).Ellagitannins and derivatives were detected as the major compounds of AM, LM and DP. The main compound of TPL and QD extracts was chlorogenic acid. All identified constituents have been known as excellent antioxidant compounds. Some of these constituents have previously been reported in crude extracts and some compounds were firstly detected in this study (ellagitannins and derivatives). This result suggested that purification method applied in this study did not affect the composition of purified extract in comparison to crude extracts.

TPL extracts had an outstanding oxygen radical absorbance capacity to all evaluated extracts. TPL and QD exhibited similar CAA value and showed a clear dose-dependent protection from cellular damage induced by H_2O_2. All extracts exhibited various anti-proliferative activities against cancer cells without damaging effect on normal cells. Purified polyphenolic-rich extracts obtained from herbs had a higher anti-proliferative activity than fruit extracts with AM displaying a superior activity.

AM and DP exhibited the greatest pro-apoptotic activities as demonstrated by flow cytometry analysis using human promyelocytic leukaemia (HL-60) and CBMN assay

using human colorectal adenocarcinoma (HT-29) cells. Each extract induced capase-3 activity, suggesting induction of apoptosis occurs via caspase-3. These results demonstrate potential chemo-preventative properties of the evaluated extracts.Total phenolic and antioxidant capacities showed various selectivity and sensitivity against key enzyme relevant to digestive enzymes such as α-glucosidase, pancreatic lipase and angiotensin converting enzyme, resulting from different levels of phytochemicals and their composition. No significant correlation has been found between antioxidant capacity and anti-proliferative, pro-apoptotic and anti-inflammatory activities. Proliferation of cancer cells is driven through various signalling pathways, among which caspase 3-expression is a key executioner enzyme induced apoptotic signalling.

The identification and quantification of compounds present in purified polyphenolic-rich extracts obtained from native Australian herbs and fruits have led to identification of ellagitannins, ellagic acid and derivatives, potentially active compounds naturally found in small berries such as strawberry and raspberry. These provide a new source of effective bioactive compounds. The investigations of the antioxidant capacity, cytoprotective, pro-apoptotic, anti-inflammatory as well as inhibitory activities relevant to digestive enzymes and blood-pressure lowering effects of those plants provided valuable evidence of potential health benefits. The evaluated plants represent novel sources of chemopreventative phytochemicals. These findings were possible due to vast botanical diversity of the Australian environment and flora, extensive history of traditional Aboriginalknowledge and recent scientific discoveries. A simple and repetitive approach such as reagent–based and cell culture–based assays is very useful tool for screening studies. The studies providean initialevidence that polyphenol-rich extracts obtained from native Australian herbs and fruits represent a potential new candidate for treatment of metabolic syndrome; they possess anti-proliferative, pro-apoptotic and anti-inflammatory effects, thus raising the possibility of a new application of these compounds for the development in nutraceuticals/food industry.

Future research directions

Base on reagent and cell culture–based assay, the present study has provided valuable systematic informationwhich can be further extended towards understanding of *in vivo*

chemopreventative mechanisms. Biological systems, such as human body, are complex and the effect of consumed phytochemicals depends on their absorption, metabolism, distribution and concentration in blood stream and targeted tissues. The concentration of polyphenols is known to be rather low in a blood stream and targeted targets, where these compounds exert their antioxidant action in sufficient amount (Manach *et al.*, 2004: Manach *et al.*, 2005).

Several epidemiological studies have revealed correlation between health-beneficial effects and an intake and bioavailability of polyphenols (Manach *et al.*, 2005; Han, Shen & Lou, 2007). Therefore, the future study could be extended to the concept of bioavailability by which integrative approaches should be considered. These include intestinal absorption, metabolism by the microflora, intestinal and hepatic metabolism, nature of circulating metabolites, binding to albumin, cellular uptake, accumulation in tissues, and biliary and urinary excretion (Manach *et al.*, 2004). Interaction between polyphenols and food matrix is definitely complex, resulting in affecting their absorption and metabolism. To understand these interactions, extensive future studies are required, in particularon metabolites as the initial material. After food consumption, metabolite is released and bind with protein and lipid. Consequently, metabolites produced *in vivo* such as methyl ethers, glucuronides, and sulphates reach in biological fluids andtissues only in nanomolar range. Further studies of their fate may provide a knowledge about biological mechanism of their delivery to target tissues. In addition, clinical and animal *in vivo*studies are considered as an essential source of knowledge that facilitates the progress in understanding the mechanism of physiological and pathological events related to bioavailability. Complete understanding of the bioavailability would subsequently provide information that will allow to translatethe potential *in vitro* results to human. Novel mechanistic pathways by which polyphenols inhibit the key enzymes of the digestive system relevant to metabolic syndrome need to be researched in order to generate a mechanistic understanding, which further may provide promising drugs to reduce the metabolic syndrome burden.

Chapter 9

References

Aaby, K., Skrede, G. & Wrolstad, R.W. (2005). Phenolic composition and antioxidant activities in flesh and achenes of strawberries (*Fragaria ananasa*). *Journal of Agricultural and Food Chemistry*, 53: 4032-4040.

Abesundara, K.J.M., Matsui, T. & Matsumoto, K. (2004). α-Glucosidase inhibitory activity of some Sri Lanka plant extracts, one of which, *Cassia auriculata,* exerts a strong antihyperglycemic effect in rats comparable to the therapeutic drug acarbose. *Journal of Agricultural and Food Chemistry,* 52: 2541-2545.

Actis-Goretta, L., Ottaviani, J.I. & Fraga, C.G. (2006). Inhibition of Angiotensin Converting Enzyme Activity by Flavanol-Rich Foods. *Journal of Agricultural and Food Chemistry*, 54: 229-234.

Adisakwattana, S., Lerdsuwankij, O., Poputtachai, U., Minipun, A. & Suparpprom, C. (2011). Inhibitory activity of cinnamon bark species and their combination effect with acarbose against intestinal α-glucosidase and pancreatic α-amylase. *Plant Foods for Human Nutrition*, 66: 143-148.

Agarwal, C., Sharma, Y., Zhao, J. & Agarwal, R. (2000). Polyphenolic fraction from grape seeds causes irreversible growth inhibition of breast carcinoma MDA-MB468 cells by inhibiting mitogen-activated protein kinases and inducing G1 arrest and differentiation. *Clinical Cancer Research*, 2000: 2921-2930.

Agarwal, C., Singh, R.P. & Agarwal, R. (2002). Grape seed extract induces apoptotic death of human prostate carcinoma DU145 cells via caspases activation accompanied by

dissipation of mitochondrial membrane potential and cytochrome c release. *Carcinogenesis*, 23: 1869-1876.

Agboola, S. O., & Radovanovic-Tesic, M. (2002). Influence of Australian native herbs on the maturation of vacuum-packed cheese. *LWT-Food Science and Technology*, 35: 575–583.

Aggarwal, B.B., Kuiken, M.E.V., Iyer, L.H., Harikumar, K.B. & Sung, B. (2009). Molecular Targets of Nutraceuticals Derived from Dietary Spices: Potential Role in Suppression of Inflammation and Tumorigenesis. *Experimental Biology and Medicine*, 234: 825-849.

Aherne, S.A. & O'Brien, N.M. (1999). Protection by the flavonoids myricetin, quercetin and rutin against hydrogen peroxide-induced DNA damage in Caco-2 and Hep G2 cells, *Nutrition and Cancer*, 34 (2): 160-166.

Ahmed, A.K. & Johnson, K.A. (2000). Horticultural development of Australian native edible plants. *Australian Journal of Botany*, 48: 417–426.

Alía, M. Ramos, S., Mateos, R., Granado-Serrano, A.B., Bravo, L. & Goya, L. (2006). Quercetin protects human hepatoma HepG2 against oxidative stress induced by *tert*-butyl hydroperoxide. *Toxicology and Applied Phamacology*, 212: 110-118.

Allavena, P., Garlanda, C., Borrello, M. G., Sica, A. & Mantovani, A. (2008). Pathways connecting inflammation and cancer. *Current Opinion in Genetics and Development*, 18: 3-10.

Amaral, A.C.F., Kuster, R.M., de Santana Bessa, W., Barnes, R.A., Kaplan, M.A.C. & Wessjohann, L.A. (2001). Flavonoids and other phenolics from leaves of two *Marlierea* species (*Myrtaceae*). *Biochemical Systematics and Ecology*, 29: 653-654.

Anderson, O.M. (2006). Flavonoids: Chemistry, Biochemistry and Applications. CRC Press, Boca Raton FL.
Anderson, O.M. (2007). Flavonoids, Chemistry, Biochemistry and Applications. *Journal of Natural Products*, 70(1): 140.

ANFIL (2012). http://www.cse.csiro.au/research/nativefoods/nativefoods_website.pdf accessed on August 2012.

ANFIL (2012a). http://www.anfil.org.au/index.php/flavour-of-the-month/ accessed on19th July, 2012.

ANFIL (2012b). http://www.anfil.org.au/idex.php/industry-profile/spices/Lemon-Myrtle/ accessed on 19th July, 2012.

Arai, I., Amagaya, S., Komatsu, Y., Okada, M., Hayashi, T., Kasai, M., Aerisawa, M. & Momose, Y. (1999). Improving effects of the extracts from *Eugenia uniflora* on hyperglycemia and hypertriglyceridemia in mice. *Journal of Ethnopharmacology*, 68: 307-314.

Arai, S., Yasuoka, A. & Abe, K. (2008). Functional food science and food for specified health use policy in Japan: state of the art. *Current Opinion in Lipidology*, 19(1): 69-73.

Artz, W.E., Bishop, P.D., Dunker, A.K., Schanus, G. & Swanson, B.G. (1987). Interaction of synthetic proanthocyanidin dimer and trimer with bovine serum albumin and purified bean globulin fraction G-1. *Journal of Agricultural and Food Chemistry*, 35: 417-421.

Ashkenazi, A. (2008). Directing cancer cells to self-destruct with pro-apoptotic receptor agonists. *Nature Reviews Drug Discovery*, 7: 1001-1012.

Ayoola, G. A., Folawewo A. D., Adesegun S. A., Abioro O. O., Adepoju-Bello A.A. & Coker, H.A.B. (2008). Phytochemical and antioxidant screening of some plants of apocynaceae from South West Nigeria. *African Journal of Plant Science*, 2(9): 124-128.

Babich, H., Schuck, A.G., Weisburg, J.H., & Zuckerbraun, H.L. (2011). Research strategies in the study of the pro-oxidant nature of polyphenol nutraceuticals. *Journal of Toxicology,* doi:10.1155/2011/467305.

Bagchi, D., Bagchi, M., Stohs, S.J., Das, D.K., Ray, S.D., Kuszynski, C.A., Joshi, S.S. & Pruess, H.G. (2000). Free radicals and grape seed proanthocyanidin extract: Importance in human health and disease prevention. *Toxicology*, 148: 187-197.

Bagchi, D., Bagchi, M., Stohs, S.J., Ray, S.D., Sen, C.K. & Preuss, H.G. (2002). Cellular protection with proanthocyanidins derived from grape seeds. *Annals of the New York Academy of Sciences*, 957: 260-270.

Bagchi, D., Sen, C.K., Bagchi, M. & Atalay, M. (2004). Anti-angiogenic, antioxidant, and anti-carcinogenic properties of a novel anthocyanin-rich berry extract formula, *Biochemistry (Mosc)*, 69: 75-80.

Balasuriya, B.W.N. & Rupasinghe, H.P.V. (2011). Plant flavonoids as angitensin converting enzyme inhibitors in regulation of hypertension. *Functional Foods in Health and Disease*, 5: 172-188.

Balkwill, F. & Mantovani, A. (2001). Inflammation and cancer: back to Virchow? *The Lancet*, 357: 539-545.

Bamard, D.L., Smee, D.F., Huffman, J.H., Meyerson, C.R. & Sidwell, R.W. (1993). Review of quercetin and related bioflavonoids. *Chemotherapy*, 39: 203-211.

Bao, Q. & Shi, Y. (2007). Apoptosome: a platform for the activation of initiator caspases. *Cell Death and Diffentiation*, 14: 56-65.

Barbosa, A. C. L., da Silva Pinto, M., Sarkar, D., Ankolekar, C., Greene, D. & Shetty, K. (2011). Influence of varietal and pH variation of antihyperglycemia and antihypertension properties of long-term stored apples using *in vitro* assay models. *Journal of Food Biochemistry*, doi: 10.1111/j.1745-4514.2011.00554.

Bate-Smith, E.C. (1962). The phenolic constituents of plants and their taxonomic significance. *The Botanical Journal of the Linnean Society,* 58: 95-173.

Becker, E.M., Nissen, L.R. & Skibsted, L.H. (2004). Antioxidant evaluation protocols: food quality or health effects. *European Food Research and Technology*, 219: 561-571.

Bensaad, K. & Vousden, K.H. (2005). Savior and slayer: the two faces of p53. *Nature Medicine*, 11: 1278-1279.

Benzie, I.F.F. & Strain, J.J. (1996). The ferric reducing ability of plasma (FRAP) as a measurement of "antioxidant power": the FRAP assay. *Analytical Biochemistry*, 239: 70-76.

Black, H.R., Ming, S., Poll, D.S., Wen, Y.F., Zhou, H.Y., Zhang, Z.Q., Chung, Y.K. & Wu, Y.S. (1996). A comparison of the treatment of hypertension with Chinese herbal and western medication. *The Journal of Clinical Hypertension*, 24: 371–378.

Bleibel, W., Kim, S., D'Silva, K. & Lemmer, E.R. (2007). Drug-induced liver injury: Review article. *Digestive Diseases and Sciences*, 52: 2463–2471.

Boateng, J., & Verghese, M. (2012).Protective effects of the phenolic extracts of fruits against oxidative stress in human lung cells. *International Journal of Pharmacology*, 8(3): 152-160.

Bogdan, C. (2001). Nitric oxide and the regulation of gene expression. *Trends in Cell Biology*, 11: 66–75.

Boivin, W.A., Cooper, D.M., Hiebert, P.R. & Granville, D.J. (2009). Intracellular versus extracellular granzyme B in immunity and disease: challenging the dogma. *Laboratory Investigation,* 89: 1195–1220.

Boots, A.W., Wilms, L.C., Swennen, E.L., Kleinjans, J.C., Bast, A. & Haenen, G.R. (2008). *In vitro* and ex vivo anti-inflammatory activity of quercetin in healthy volunteers. *Nutrition*, 24: 703–710.

Borges, G., Degeneve, A., Mullen, W. & Crozier, A. (2010). Identification of flavonoid and phenolic antioxidants in black currants, blueberries, raspberries, red currants, and cranberries. *Journal of Agricultural and Food Chemistry*, 58: 3901-3909.

Boyer, J. & Liu, R.H. (2004). Review: Apple phytochemicals and their health benefits *Nutrition Journal*, 3: 5-20.

Brassesco, M.S., Valera, E.T., Neder, L., Castro-Gamero, A.M., Arruda, D., Machado, H.R., Sakamoto-Hojo, E.T. & Tone, L.G. (2009). Polyploidy in atypical grade II choroid plexus papilloma of the posterior fossa. *Neuropathology*, 29: 293–298.

Bray, G. & Champagene, C. (2004). Obesity and the metabolic syndrome: Implications for dietetics practitioners. *Journal of the American Dietatic Association*, 104: 86-89.

Broadhurst, C.L., Polansky, M.M. & Anderson, R.A. (2000). Insulin-like biological activity of culinary and medicinal plant aqueous extracts in vitro. *Journal of Agricultural and Food Chemistry*, 48: 849-852.

Brown, L. & van der Ouderaa, F. (2007). Nutritional genomics: food industry applications from farm to fork. *British Journal of Nutrition*, 97: 1027-1035.

Bryant, G. (2005). The Random House Encyclopedia of Australian Native Plants. Random House, Sydney, Australia.

Burke, B.E., Baillie, J. E. & Olson, R.D. (2004). Essential oil of Australian lemon myrtle (*Backhousia citriodora*) in the treatment of molluscum contagiosum in children. *Biomedicine and Pharmacotherapy*, 58: 245–247.

Cancer Council of Australia (2012).
http://www.cancer.org.au/aboutcancer/FactsFigures.htm, accessed on 21st February, 2012

Cerda, B., Tomás-Barberán, F.A. & Espín, J.C. (2005). Metabolism of antioxidant and chemopreventive ellagitannins from strawberries, raspberries, walnuts, and oak-aged wine in humans: identification of biomarkers and individual variability. *Journal of Agricultural and Food Chemistry*, 53: 227-235.

Chan, J.Y. & Kwong, M. (2000).Impaired expression of glutathione synthetic enzyme genes in mice with targeted deletion of the Nrf2 basic-leucine zipper protein. *Biochemica Biophysica Acta*, 1517: 19-26.

Chang, C.L., & Lin, C.S. (2012). Phytochemical composition, antioxidant activity and neuroprotective effect of*Terminalia chebula retzius* extracts. *Evidence-Based Complementary and Alternative Medicine*, doi:10.1155/2012/125247.

Chen, C. & Kong, A.-N.T. (2005). Dietary cancer-chemopreventive compounds: from signaling and gene expression to pharmacological effects. *Trends in Pharmacological Sciences*, 26: 318-326.

Chen, C.C., Hsu, J.D., Wang, S.F., Chiang, H.C., Yang, M.Y., Kao, E.S., Ho, Y-C. & Wang, C-J. (2003). *Hibiscus sabdariffa* extract inhibits the development of atherosclerosis in cholesterol-fed rabbits. *Journal of Agricultural and Food Chemistry,* 51: 5472-5477.

Chen, P.-N., Chu, S.-C., Chiou, H.-L., Chiang, C.-L., Yang, S.-F.& Hsieh, Y.-S. (2005). Cyanidin 3-glucoside and peonidin 3-glucoside inhibit tumor cell growth and induce apoptosis *in vitro* and suppress tumor growth *in vivo*. *Nutrition and Cancer*, 53: 232-243.

Chen, L. & Zhang, H.-Y.(2007). Cancer preventive mechanisms of the green tea polyphenol (-)-epigallocatechin-3-gallate. *Molecules*, 12: 946-957.

Cheplick, S., Kwon, Y. I., Bhowmik, P. & Shetty, K. (2010).Phenolic-linked variation in strawberry cultivars for potential dietary management of hyperglycemia and related complications of hypertension. *Bioresource Technology*, 101: 404-413.

Cherikoff, V. & Isaacs, J. (1990). The bushfood handbook. How to gather, grow, process and cook Australian wild foodf. Ti Tree Press, Sydney, Australia, p.82.

Chow, J-M., Shen, S-C., Huan, S.K., Lin, H-Y. & Chen, Y-C. (2005). Quercetin, but not rutin and quercitrin, prevention of H_2O_2-induced apoptosis via anti-oxidant activity and heme oxygenase 1 gene expression in macrophages. *Biochemical Pharmacology*, 69: 1839-1851.

Cinti, S., Mitchell, G., Barbatelli, G., Murano, I., Ceresi, E., Faloia, E., Wang, S., Fortier, M., Greenberg, A.S. & Obin, M.S. (2005). Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *The Journal of Lipid Research*, 46: 2347-2355.

Clarke, P. A. (2007). *Aboriginal people and their plants*, Rosenberg Publishing, Dural, NSW, Australia.

Colagiuri, S., Lee, C.M.Y., Colagiuri, R., Magliano, D., Shaw, J.E., Zimmet, P.Z. & Caterson, I.D. (2010). The cost of overweight and obesity in Australia. *The Medical Journal of Australia*, 192 (5): 260-264.

Consolini, A.E., Baldini, O.A.N. & Amat, A.G. (1999). Pharmacological basis for the empirical use of *Eugenia uniflora* L. (Myrtaceae) as antihypertensive. *Journal of Ethnopharmacology*, 66: 33-39.

Cooper, W. (2004). *Fruits of the Australian Tropical Rainforest*, Nokomis Edition Pty Ltd., Melbourne, Australia.

Corley, D.A., Kerlikowske, K., Verma, R., & Buffler, P. (2003). Protective association of aspirin/NSAIDs and esophageal cancer: a systematic review and metanalysis.

Gastroenterology, 124: 47-56.

Cossarizza, A., Ferraresi, R., Troiano, L., Roat, E., Gibellini, L., Bertoncelli, L., Nasi, M. & Pinti, M. (2009). Simultaneous analysis of reactive oxygen species and reduced glutathione content in living cells by polychromatic flow cytometry. *Natural Protocols*, 4: 1790–1797.

Coultas, L. & Strasser, A. (2003). The role of the Bcl-2 protein family in cancer. *Seminars in Cancer Biology*, 13: 115-123.

CSIRO (2012a). http://www.csiro.au/proprietaryDocuments/FunctionalFood112004.pdf accessed on October, 2012.

CSIRO (2012b). http://www.cse.csiro.au/research/nativefoods/nativefoods_website.pdf, accessed on 21st February, 2012.

D′Agostini, F., Izzotti, A., Balansky, R.M., Bennicelli, C. & de Flora, S. (2005). Modulation of apoptosis by cancer chemopreventive agents. *Mutation Research*, 591: 173-186.

Dai, J. & Mumper, R.J. (2010). Review Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules*, 15: 7313-7352.

da SilvaPinto, M**.,** Lajolo, F.M.,& Genoves, M.I. (2008). Bioactive compounds and quantification of total ellagic acid in strawberries (*Fragaria x ananassa* Duch.) *Food Chemistry*, 107(4): 1629-1635.

da Silva Pinto, M., de Carvalho, J.E., Lajolo, F.M., Genovese, M.I. & Shetty, K. (2010a). Evaluation of antiproliferative, anti-type 2 diabetes, and antihypertension potentials of ellagitannins from strawberries (*Fragaria x ananassa* Duch.) using *in vitro* models. *Journal of Medicinal Food*, 13: 1027–1035.

da Silva Pinto, M., Kwon, Y-I., Apostolidis, E., Lajolo, F.M., Genovese, M.I. & Shetty, K. (2010b). Evaluation of red currants (*Ribes rubrum* L.), black currants (*Ribes nigrum* L.), red and green gooseberries (*Ribes UVA-CRISPA*) for potential management of type 2 diabetes and hypertension using *in vitro* models. *Journal of Food Biochemistry*, 34: 639-660.

Davalos, A., Gomez-Cordoves, C. & Bartolome, B. (2004). Extending applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. *Journal of Agricultural and Food Chemistry*, 52: 48–54.

Davis, J.M., Murphy, E.A. & Carmichael, M.D. (2009). Effects of the dietary flavonoid quercetin upon performance and health. *Curr. Sports Med. Rep.*, 8: 206-213.

De Flora, S. & Ferguson, L.R. (2005). Overview of mechanism of cancer chemopreventive agents. *Mutation Research*, 591: 8-15.

De Visser, K.E. & Eichten, A. (2006). Paradoxical roles of the immune system during cancer development. *Nature Reviews Cancer*, 6: 24-37.

Dinarello, C.A. (2010). Anti-inflammatory agents: Present and future. *Cell*, 140: 935–950.

Doyon, M. & Labrecque, J.A. (2008). Functional foods: a conceptual definition. *British Food Journal*, 110 (11): 1133-1149.

Dragar, V.A., Garland, S.M. & Menary, R.C. (1998). Investigation of the variation in chemicals composition of *Tasmannia lanceolata* solvent extract. *Journal of Agricultural and Food Chemistry*, 46: 3210-3213.

Dragland, S., Senoo, H., Wake, K., Holte, K. & Blomhoff, R. (2003). Several culinary and medicinal herbs are important sources of dietary antioxidants. *American Society for Nutritional Sciences*, 133: 1286-1290.

Durgo, K., Belscak-Cvitanovic, A., Stancic, A., Franekic, J. & Komes, D. (2012). The bioactivepotential of red raspberry (Rubus idaeus L.) leaves in exhibiting cytotoxic and cytoprotective activity on human laryngeal carcinoma and colon adenocarcinoma. *Journal of Medicinal Food*, 15(3): 258-268.

Dzau, V.J. (2001). Tissue angiotensin and phthobiology of vascular disease: A unifying hypothesis, *Hypertension*, 37: 1047-1052.

Eastmond, D. & Tucker, J. (1989) Identification of aneuploidy-inducing agents using cytokinesis-blocked human lymphocytes and an antikinetochore antibody. *Environmental and Molecular Mutagenesis*, 13: 34-43.

Eberhardt, M.V. & Jeffery, E.H. (2006). When dietary antioxidants perturb the thiol redox. *Journal of the Science of Food and Agriculture*, 86: 1996-1998.

Eberhardt, M., Lee, C. & Liu, R.H. (2000). Antioxidant activity of fresh apples. *Nature* 2000, 405: 903-904.

El Beyrouthy, M., Arnold, M. Delelis-Dusollier, A. & Dupont, F. (2008). Plants used as remedies antirheumatic and antineuralgic in the traditional medicine of Lebanon. *Journal of Ethnopharmacology*, 120(3): 315-334.

Elmore, S. (2007). Apoptosis: A review of programme cell death. *Toxicologic Pathology*, 35(4): 495-516.

Erickson, A.M., Nevarea, Z., Gipp, J.J. & Mulcahy, R.T. (2002). Identification of a variant antioxidant response element in the promoter of the human glutamate-cysteine ligase modifier subunit gene. *The Journal of Biological Chemistry*, 277(34): 30730-30737.

Exploroz (2012).
http://www.exploroz.com/Forum/Topic/84294/Wildflower_PhotosThe_Quandong.aspx, accessed on 11th February, 2012

Fenaroli, G. (1975). Handbook Flavor Ingred. 2nd Ed. vol. 2 Cleveland: OH CRC Press.

Fenech, M. (2007). Cytokinesis-block micronucleus cytome assay. *Nature Protocols*, 2 (5): 1084-1104.

Fernandez, M.L. (2007). The metabolic syndrome. *Nutrition Reviews*, 64: S30-S34.

Finch, C.E. & Crimmins, E.M. (2004). Inflammatory exposure and historical changes in human life-spans. *Science*, 305: 1736-1739.

Finkel, T. & Holbrook, N.J. (2000). Oxidants, oxidative stress and the biology of ageing.*Nature*, 408: 239-247.

Flamini, R. (2003). Mass spectrometry in grape and wine chemistry. Part I: polyphenols. *Mass Spetrometry Reviews*, 22(4): 218-250.

Foster, M. (2009). *Emerging Animal and Plant Industries-their value to Australia*, Rural Industries Research and Development Corporation, 2 Edition, (RIRDC Pub. No. 09/004), p. 50.

Foti, P., Erba, D., Riso, P., Spadafranca, A., Criscuoli, F. & Testolin, G. (2005). Comparison between daidzein and genistein antioxidant activity in primary and cancer lymphocytes. *Archives of Biochemistry and Biophysics*, 433: 421-427.

Frankel, E.N. & Meyer, A.S. (2000). The problem of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *Journal of the Science of Food and Agriculture*, 80: 1925-1941.

Fu, L., Xu, B-T., Gan, R-Y., Zhang, Y., Xu, X-R., Xia, E-Q.& Li, H-B. (2011). Total phenolic contents and antioxidant capacities of hurbal and tea infusions. *International Journal of Molecular Sciences*, 12: 2112-2124.

Fulda, S. & Debatin, K.M. (2006). Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene*, 25: 4798-4811.

Funk, C.D. (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science*, 294: 1871-1875.

Furguson, L, R. (2009). Nutrigenolics approaches to functional foods. *Journal of the American Dietetic Association,* 109: 452-458.

Gao, H., Huang, Y-N., Xu, P-Y., & Kawabata, J. (2007). Inhibitory effect on α-glucosidase by the fruits of *Terminalia chebula* Retz. *Food Chemistry*, 105: 628-634.

Garcia-Mediavilla, V., Crespo, I., Collado, P. S., Esteller, A., Sánchez-Campos, S., Tunón, M. J., *et al.* (2007). The anti-inflammatory flavones quercetin and kaempferol cause inhibition of inducible nitric oxide synthase, cyclooxygenase- 2 and reactive C-protein, and down-regulation of the nuclear factor κB pathway in Chang liver cells. *European Journal of Pharmacology*, 557(2–3): 221–229.

Garcia-Alonso, J., Ros, G. & Jesus Periago, M. (2006). Antiproliferative and cytoprotective activities of a phenolic-rich juice in HepG2 cells. *Food research international*, 39: 982-991.

Gharavi, N. & El-kadi, A.O.S. (2003). Measurement of nitric oxide in murine hepatoma hepa1c1c7 cells by revesed phase HPLC with fluorescene detection. *The Journal of Pharmaceutical Science*, 6(2): 302-307.

Ghavami, S., Hashemi, M., Ande, S.R., Yeganeh, B., Xiao, W., Eshraghi, M. Bus, C.J., Kadkhoda, K., Wiechec, E. & Halayko, A.J. (2009). Apoptosis and cancer: mutations within caspase genes. *British Medical Journal*, 46: 495-510.

Ghobrial, I.M., Witzig, T.E. & Adjei, A.A. (2005). Targeting apoptosis pathways in cancer therapy. *Cancer Journal for Clinicians*, 55: 178-194.

Giovanelli, G. & Buratti, S. (2009). Comparison of polyphenolic composition and antioxidant activity of wild Italian blueberries and some cultivated varieties. *Food Chemistry*, 112: 903–908.

Gornall, R.J., Bohm, B.A. & Dahlgren, R. (1979). The distribution of flavonoids in the angiosperms. *Bot. Not*., 132: 1-30.

Greenhough, A., Smartt, H.J.M., Moore, A.E., Roberts, H.R., Williams, A.C., Paraskeva, C., & Kaidi, A. (2009). REVIEW: The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. *Carcinogenesis*, 30(3): 377–386.

Greenwal, P. & Dunn, B.K. (2009). Do we make optimal use of the potential of cancer prevention? *Recent Results Cancer Reseach*, 181: 3-17.

Grivennikov, S., Greten, F.R., & Karin, M. (2010). Review: Immunity, Inflammation, and cancer. *Cell*, 140: 883-899.

Gropper, D.R., Smith J.L. & Groff, J.L. (2005). Advanced nutrition and human metabolism.4th edition Belmont, USA.Thomson Wadsworth. pp. 259-367.

Grove, K.A. & Lambert, J.D. (2010). Laboratory, Epidemiological, and human intervention studies show that tea (*Camellia sinensis*) may be useful in the prevention of obesity. *Journal of Nutrition*, 140: 446–453.

Grove, K.A., Sae-tan, S., Kennett, M.J. & Lambert, J.D. (2011). (-)-Epigallocatechin-3-gallate inhibits pancreatic lipase and reduces body weight gain in high fat-fed obese mice. *Obesity*. E.J.C.N. doi:10.1038/oby.2011.139

Hagerman, A.E. & Butler, L.G. (1981). The specificity of proanthocyanidin-protein interactions. *The Journal of Biological Chemistry*, 256 (9): 4444-4497.

Hagiwara, Y., Kasukabe, T., Kaneko, Y., Niitsu, N. & Okabe-Kado, J. (2010). Ellagic acid, a natural polyphenolic compound, induces apoptosis and potentiates retinoic acid-induced differentiation of human leukemia HL-60 cells. *International Journal of Hematology*, 92: 136–143.

Hail, N.Jr. & Lotan, R. (2009). Cancer chemoprevention and mitochondria: targeting apoptosis in transformed cells via the disruption of mitochondrial bioenergetics/redox state. *Molecular Nutrition and Food Research*, 53: 49-67.

Hakkinen, S., Heinonen, M., Karenlampi, S., Mykkanen, H. Suuskanen, J., & Torronen, R. (1999).Screening of selected flavonoids and phenolic acids in 19 berries. *Food Research International*, 32: 345-353.

Halliwell, B. (2007). Oxidative stress and cancer: have we moved forward?. *Biochemical Journal,* 401(1): 1-11.

Halliwell, B. (2008). Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and *in vivo* studies? *Archives Biochemistry and Biophysics*, 476: 107-112.

Han, D.H., Lee, M.J. & Kim, J.H. (2006). Antioxidant and apoptosis-inducing activities of ellagic acid. *Anticancer Research*, 26: 3601-3606.

Han, D.H. & Kim, J.H. (2009).Difference in growth suppression and apoptosis induction of EGCG and EGC on human promyelocytic leukemia HL-60 cells. *Archives of Pharmacal Research*, 32: 543-547.

Han, D.H., Jeong, J.H. & Kim, J.H. (2009). Anti-proliferative and apoptosis induction activity of green tea polyphenols on human promyelocytic leukemia HL-60 cells.*Anticancer Research*, 29: 1417-1422.

Han, X., Shen, T. & Lou, H. (2007). Review: Dietary polyphenols and their biological significance. *International Journal of Molecular Sciences*, 8: 950-988.

Han, C. & Wu, T. (2005). Cyclooxygenase-2-derived prostaglandin E2 promotes human cholangiocarcinoma cell growth and invasion through EP1 receptor-mediated activation of the epidermal growth factor receptor and Akt. *The Journal of Biological Chemistry*, 280: 24053–24063.

Hanhineva, K., Törrönen, R., Bondia-Pons, I., Pekkinen, J., Kolehmainen, M., Mykkänen, H. & Poutanen, K. (2010). Impact of dietary polyphenols on carbohydrate metabolism. *International Journal of Molecular Sciences*, 11: 1365-1402.

Harold, N. & Graham, P.D. (1992). Green tea composition, consumption and polyphenol chemistry. *Preventive Medicine*, 21: 334-350.

Haslam, E. (1998). *Practical polyphenolics: from structure to molecular recognition and physiological action*, Cambridge University Press, Cambridge, UK.

Hayes, A. J., & Markovic, B. (2000). Toxicity of Australian essential oil *Backhousiacitriodora* (Lemon myrtle). Part 1. Antimicrobial activity and in vitro cytotoxicity. *Food and Chemical Toxicology*, 40: 535–543.

Heber, D. (2008). Multitargeted therapy of cancer by ellagitannins, *Cancer Letters*, 269 (2): 262-268.

Hele, A., Latham, Y., Ryder, M., O'Hanlon, M. & Lethbridge, B. (2006). Quandong production. Government of South Australia Primary Industries and Resources SA Factsheet No: 17/03.

Hengartner, M.O. (2000). Insight review articles: The biochemistry of apoptosis. *Nature*, 207: 770-776.

Ho, Y.C., Yang, S.F., Peng, C.Y., Chou, M.Y. & Chang, Y.C. (2007). Epigallocatechin-3-gallate inhibits the invasion of human oral cancer cells and decreases the productions of matrix metalloproteinases and urokinase-plasminogen activator, *Journal of Oral Pathology and Medicine*, 36 (10): 588–593.

Hodgson, J.M. & Wahlqvist, M.L. (1993). Nutrition and health of Victorian Aborigines (Kooris). *Asia Pacific Journal of Clinical Nutrition*, 2: 43-57.

Houghton, P., Fang, R., Techatanawat, I., Steventon, G., Hylands, P. J. & Lee, C.C. (2007). The sulphorhodamine (SRB) assay and other approaches to testing plant extracts and derived compounds for activities related to reputed anticancer activity. *Methods*, 42: 377-387.

Hsieh, Y.-H.P. & Ofori, J.A. (2007). Innovations in food technology for health. *Asia Pacific Journal of Clinical Nutrition*, 16(supppl): 65-73.

Hsu, C-P., Lin, Y-H., Chou, C-C., Zhou, S-P, Hsu, Y-C., Liu, C-L., Ku, F-M. & Chung, Y-C. (2009). Mechanisms of Grape Seed Procyanidin-induced Apoptosis in Colorectal Carcinoma Cells. *Anticancer Research*, 29: 283-290.

Hsu, C-L.& Yen, G-C. (2007). Effects of flavonoids and phenolic acids on the inhibition of adipogenesis in 3T3-L1 adipocytes. *Journal of Agricultural and Food Chemisty*, 55: 8404–8410.

Hsu, C-L.& Yen, G-C. (2008). Phenolic compounds: Evidence for inhibitory effects against obesity and their underlying molecular signaling mechanisms. *Molecular Nutrition and Food Research*, 52: 53-61.

Hsu, C-L, Wu, C-H., Huang, S-L., & Yen, G-C. (2009). Phenolic compounds rutin and *o*-coumaric acid ameliorate obesity induced by high-fat diet in rats. *Journal of Agricultural and Food Chemistry*, 57: 425–431.

Huang, D., Ou, B. & Prior, R.L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*, 53: 1841-1856.

Huang, W-Y., Cai, Y-Z. & Zhang, Y. (2011). Natural phenolic compounds from medicinal herbs and dietary plants: Potential use for cancer prevention. *Nutrition and Cancer*, 62(1): 1-20.

Hur, C.Q., Chen, K., Shi, Q., Kikushkie, R.E., Cheng, Y.C. & Lee, K.H. (1994). *Journal of Natral Products*, 57: 42-50.

Hyde, C.A.C. & Missailidis, S. (2009). Inhibition of arachidonic acid metabolism and its implication on cell proliferation and tumour-angiogenesis. *International Immunopharmacology*, 9: 701-715.

IOTF (2011). International Obesity Task Force.Global prevalence of adult obesity. http://www.iotf.org/database/documents/GlobalPrevalenceofAdultObesityJanuary2010.pdf, accessed on 11th July 2011.

Ishikawa, A., Yamashita, H., Hiemori, M., Inagaki, E., Kimoto, M., Okamoto, M., Tsuji, H., Nemon, A.N., Mohammadi, A. & Natori, Y. (2007). Characterization of inhibitors of postprandial hyperglycemia from the leaves of *Nerium indicum*. *Journal of Nutritional Science and Vitaminology*, 53: 166-173.

Jasperson, K.W., Tuohy, T.M., Meklason, D.W. & Burt, R.W. (2010). Hereditary and familial colon cancers. *Gastroenterology*, 138: 2044-2058.

Jean-Gilles, D., Li, L., Ma, H., Yuan, T., Chichester, C.O. & Seeram, N.P. (2012). Anti-inflmmatory effects of polyphenolic-enriched red raspberry extract in an antigen-induced arthritis rat model. *Journal of Agricultural and Food Chemistry*, 60: 5755-5762.

Jensen, M.B., Bergamo, C.A. L-D., Payet, R.M., Liu, X., & Konczak, I. (2011). Influence of copigment derived from Tasmannia pepper leaf on Davidson's plum anthocyanins. *Journal of Food Science*, 76: C447-C453.

Jin, S. & Levine, A.J. (2001). The p53 functional circuit. *Journal of Cell Science*, 114: 4139-4140.

Johnson, I.T. (2007). Phytochemicals and cancer. *Proceeding of the Nutrition Society*, 66: 207-215.

Johnson, I. & Williamson, G. (2003). Phytochemical functional foods, Woodhead Publishing, TJ international, Padstow, Cornwall, England.

Joshi, S.S., Kuszynski, C.A. & Bagchi, D. (2001). The cellular and molecular basis of health benefits of grape seed proanthocyanidin extract. *Current Pharmaceutical Biotechnology*, 2: 187-200.

Kalinski, P. (2012). Regulation of immune responses by prostaglandin E_2. *The Journal of Immunology*, 188: 21-28.

Kammerer, D., Claus, A., Carle, R. & Schieber, A. (2004). Polyphenol screening of pomace from red and white grape varieties (Vitis Vinifera L.) by HPLC-DAD-MS/MS. *Journal of Agricultural and Food Chemistry*. 52: 4360-4367.

Kang, S-M., Lee, S-H., Heo, S-J, Kim, K-N. & Jeon, Y-J. (2011). Evaluation of antioxidant properties of a new compound, pyrogallol-phloroglucinol-6,6'-bieckol isolated from brown algae, *Ecklonia cava*. *Nutrition Research Practice*, 5(6): 495-502.

Katalinic, V., Milos, M., Kulisic, T. & Jukic, M. (2006). Screening of 70 medicinal plant extracts for antioxidant capacity and total phenols. *Food Chemistry*, 94: 550-557.

Katsube, N., Iwashita, K., Tsushida, T., Yamaki, K. & Kobori, M. (2003). Induction of apoptosis in cancer cells by Bilberry (Vaccinium myrtillus) and the anthocyanins. *Journal of Agricultural and Food Chemistry*, 51: 68-75.

Keibel, A., Singh, V. & Sharma, M.C. (2009). Inflammation, microenvironment, and the immune system in cancer progression. *Current Pharmaceutical Design*, 15: 1949-1955.

Khan, N., Afaq, F. & Mukhtar, H. (2007). Apoptosis by dietary factors: the suicide solution for delaying cancer growth. *Carcinogenesis*, 28: 233-239.

Khan, N. & Mukhtar, H. (2008). Multitargeted therapy of cancer by green tea polyphenols. *Cancer Letters*, 269: 269-280.

Khan, R., Isalam, B., Akram, M., Shakil, S., Ahmad, A., Ali, S.M., Siddiqui, M. & Khan, A.U. (2009). Antimicrobial activity of five herbal extracts against multi drug resistant (MDR) strains of bacteria and fungus of clinical origin. *Molecules*, 14(2): 586-597.

Khanapure, S.P., Garvey, D.S., Janero, D.R. & Letts, L.G. (2007). Eicosanoids in inflammation: biosynthesis, pharmacology, and therapeutic frontiers. *Current Topics in Medicinal Chemistry*, 7: 311-340.

Khanbabaee, K. & Van Ree, T. (2001). Tannins classification and definition. *Natural Product Reports*, 18: 641-649.

Kim, H., Hall, P., Smith, M., Kirk, M., Prasain, J.K., Barnes, S. & Grubbs, C. (2004). Chemoprevention by grape seed extract and genistein in carcinogeninducedmammary cancer in rats is diet dependent. *Journal of Nutrition*, 134: 3445S–3452S.

Kim, Y.S., Lee, Y.M., Kim, H., Kim, J., Jang, D.S., Kim, J.H. & Kim, J.S. (2010a). Anti-obesity effect of *Morus bombycis* root extract: Anti-lipase activity and lipolytic effect. *Journal of Ethnopharmacology*, 130: 621–624.

Kim, H-J., Kim, S-K., Kim, B-S., Lee, S-H., Park, Y-S., Park, B-K., Kim, S-J., Kim, J., Choi, C., Kim, J.S., Cho, S.D., Jung, J.W., Roh, K.H., Kang, K.S. & Jing J.Y. (2010b). Apoptotic effect of quercetin on HT-29 colon409 cancer cells via the AMPK signaling pathway. *Journal of Agricultural and Food Chemistry*, 58: 8643–8650.

Kim, Y.S., Young, M.R., Bobe, G., Colburn, N.H. & Milner, J. A. (2009). Bioactive food components, inflammatory targets, and cancer prevention. *Cancer Prevention Research*, 2(3): 200−208.

Klaunig, J.E. & Kamendulis, L.M. (2004).The role of oxidative stress in carcinogenesis. *Annual Review of Pharmacology and Toxicology*, 44: 239-267.

Konczak, I., Zabaras, D., Xiao, D., Shapira, D. & Lee, G. (2008). Screening native Australian fruits for health-promoting properties. Anti-proliferative and pro-apoptotic

activity of Illawarra Plum. *Journal of Clinical Biochemistry and Nutrition*, 43 (1): 543-547.

Konczak, I., Zabaras, D., Dunstan, M., Aguas, P., Roulle, P. & Pavan, A. (2009). Health benefits of Australian native foods. An evaluation of health-enhancing compounds.Commercial report for the RIRDC. (RIRDC Pub. No. 09/133), p. 41.

Konczak, I., Zabaras, D., Dunstan, M., & Aguas, P. (2010a). Antioxidant capacity and phenolic compounds in commercially grown native Australian herbs and spices. *Food Chemistry*, 122: 260-266.

Konczak, I., Zabaras, D., Dunstan, M. & Aguas, P. (2010b). Antioxidant capacity and phenolic compounds in commercially grown native Australian fruits. *Food Chemistry,* 123: 1048-1054.

Kopelman, P.G. (2000). Obesity as a medical problem. *Nature,* 404: 635-643.

Koponen, J.M., Happonen, A.M., Mattila, P.H. & Torronen, A.R. (2007). Contents of anthocyanins and ellagitannins in selected foods consumed in Finland. *Journal of Agricultural and Food Chemistry*, 55: 1612–1619.

Kozuma, K., Tsuchiya, S., Kohori, J., Hase, T. & Tomokitsu, I. (2005). Antihypertensive effect of green coffee bean extract on mildly hypertensive subjects. *Hypertension Research*, 28: 711-718.

Kraft, T.F.B., Dey, M., Rogers, R.B., Ribnicky, D.M., Gipp, D.M., Cefalu, W.T., Raskin, I. & Lila, M.A. (2008). Phytochemical composition and metabolic performance-enhancing activity of dietary berries traditionally used by native North Americans. *Journal of Agricultural and Food Chemistry*, 56: 654-660.

Kris-Etherton, P.M. & Keen, C.L. (2002). Evidence that the antioxidant flavonoids in tea and cocoa are beneficial for cardiovascular health. *Current Opinion in Lipidology*, 13: 41–49.

Krishnaswamy, K. (2008). Traditional Indian spices and their health significance. *Asia Pacific Journal of Clinical Nutrition*, 17(S1): 265–268.

Kroon, P. & Williamson, G. (2005). Polyphenols: dietary components with established benefits to health? *Journal of the Science of Food and Agriculture*, 85: 1239-1240.

Kundu, J.K. & Surh, Y.-J. (2008). Inflammation: gearing the journey to cancer. *Mutatation Research*, 659: 15-30.

Kurokawa, M. & Kornbluth, S. (2009). Caspases and kinases in a death grip. *Cell,* 138: 838-854.

Kwon, K.H., Barve, A., Yu, S., Huang, M.-T. & Kong, A.-N.T. (2007). Cancer chemoprevention by phytochemicals: potential molecular targets, biomarkers and animal models. *Acta Pharmacologica Sinica*, 28: 1409-1421.

Lambert, J.D., Hong, J., Yang, G.-Y., Liao, J. & Yang, C.S. (2005). Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations. *American Journal of Clinical Nutrition*, 81: 284S-291S.

Landete, J.M. (2011). Ellagitannins, ellagic acid and their derived metabolites: A review about source, metabolism, functions and health. *Food Research International*, 44: 150-1160.

Larrosa, M., Tomas-Barberan, F.A. & Espin, J.C. (2006). The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway. *The Journal of Nutritional Biochemistry*, 17: 611–625.

Lau, F.C., Bielinski, D.F. & Joseph, J.A. (2007). Inhibitory effects of blueberry extract on the production of inflammatory mediators in lipopolysaccharide-activated BV2 microglia. *Journal of Neuroscience Reseach*, 85: 1010-1017.

Lavie, C.J., Milani, R.V. & Ventura, H.O. (2009). Obesity and cardiovascular disease: risk factor, paradox, and impact of weight loss. *Journal of the American College of Cardiology*, 53: 1925-1932.

Lavrik, I.N., Golks, A. & Krammer, P.H. (2005). Caspases: pharmacological manipulation of cell death. *The Journal of Clinical Investigation*, 115: 2665-2672.

Lawen, A. (2003). Apoptosis-an introduction. *Bio essays*, 25: 888-896.

Lazze, M.C., Savio, M., Pizzala, R., Cazzalini, O., Perucca, P., Scovassi, A.I., Stivala, L.A. & Bianchi, L. (2004). Anthocyanins induce cell cycle perturbations and apoptosis in different human cell lines. *Carcinogenesis*, 25: 1427-1433.

Lee, J-H., Jeong, Y-J., Lee, S-W., Kim, D., Oh, S-J., Lim, H-S., Oh, H-K., Kim , S-H, Kim, W-J. & Jung, J-Y. (2010). EGCG induces apoptosis in human laryngealepidermoid carcinoma Hep2 cells via mitochondria with the release of apoptosis-inducing factor and endonuclease G. *CancerLetters*, 290: 68–75.

Lee, J.-H. & Talcott, S.T. (2004). Fruit maturity and juice extraction influences ellagic acid derivatives and other antioxidant polyphenolics in muscadine grapes. *Journal of Agricultural and Food Chemistry*, 52: 361-366.

Lee, K.W. & Lee, H.J. (2006). The roles of polyphenols in cancer chemoprevention. *Biofactor*, 26: 105-121.

Lethbridge, B. (2004). “The new crop industries handbook”, Edited by S. Salvin, M.Bourke and T. Byrne, RIRDC, 373 – 376.

Li, Y-G., Tanner, G. & Larkin, P. (1996). The DMACA-HCL Protocol and the Threshold Proanthocyanidin Content for Bloat Safety in Forage Legumes. *Journal of Scientific Food Agriculture*, 70: 89-101.

Lin, H.Y., Shen, S.C., Lin, C.W., Yang, L.Y. & Chen, Y.C. (2007). Baicalein inhibition of hydrogen peroxide-induced apoptosis via ROS-dependent heme oxygenase 1 gene expression. *Biochimica et Biophysica Acta*, 1773: 1073-1086.

Liu, R.H. (2003). Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *American Journal of Clinical Nutrition,* 78: 517S-520S.

Liu, R.H. (2004). Potential synergy of phytochemicals in cancer prevention: mechanism of action. *Journal of Nutrition*, 134(12S): 3479S-3485S.

Liu, R.H. & Finley, J. (2005). Potential cell culture models for antioxidant research. *Journal of Agricultural and Food Chemistry*, 53: 4311-4314.

Lord, S.J., Rajotte, R.V., Korbutt, G.S. & Bleackley, R.C. (2003). Granzyme B: a natural born killer. *Immunological Reviews*, 193: 31-38.

Losso, J.N., Bansode, R.R., Trappey, A., Bawadi, H.A. & Truax, R. (2004). In vitro anti-proliferative activities of ellagic acid. *The Journal of Nutritional Biochemistry*, 15(11): 672-678.

Lovborg, H., Gullbo, J. & Larsson, R. (2005). Screening for apoptosis - classical and emerging techniques.*Anticancer Drugs*, 16: 593-599.

Loveys, B.R. & Justias, M. (1994). Stimulation of germination of Quandong (*Santalum acuminatum*) and other Australian native plant seeds. *Australian Journal of Botany,* 42: 565-574.

Lumeng, C.N., Bodzin, J.L. & Saltiel, A.R. (2007). Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *The Journal of Clinical Investigation*, 117: 175-184.

Magalhaes, L.M., Segundo, M.A., Reis, S. & Lima, J.L.F.C. (2008). Review article Methodological aspects about in vitro evaluation of antioxidant properties. *Analytica Chimica Acta*, 613: 1-19.

Manach, C., Morand, C., Gil-Izquierdo, A., Bouteloup-Demange, C. & Remesy, C. (2003).Bioavailability in humans of the flavanones hesperidin and narirutin after the ingestion of two doses of orange juice. *European Journal of Clinical Nutrition*, 57: 235-242.

Manach, C., Scalbert, A., Morand, C., Rémésy, C. &Jimenez, L. (2004). Polyphenols: food sources and bioavailability. *American Journal of Clinical Nutrition*, 79: 727-747.

Manach, C., Williamson, G., Morand, C., Scalbert, A. & Rémésy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans I. Review of 97 bioavailability studies. *American Journal of Clinical Nutrition*, 81: 230S-242S.

Manson, M.M. (2003). Cancer prevention - the potential for diet to modulate molecular signalling. *Trends in Molecular Medicine,* 9: 11-18.

Manson, M.M., Farmer, P.B., Gescher, A. & Steward, W.P. (2005). Innovative agents in cancer prevention. *Recent Results in Cancer Research*, 166: 257–275.

Mantena, S.K., Baliga, M.S. & Katiyar, S.K. (2006). Grape seed proanthocyanidins induce apoptosis and inhibit metastasis of highly metastatic breast carcinoma cells. *Carcinogenesis*, 27: 1682–1691.

Mantovani, A., Allavena, P., Sica, A., Balkwill, F. (2008). Cancer-related inflammation. *Nature*, 454: 436-444.

Marinova, E.M. & Yanishlieva, N.V. (2003).Antioxidant activity and mechanism of action of some phenolic acids at ambient and high temperatures. *Food Chemistry*, 81(2): 189-197.

Martin, S., Favot, L., Matz, R. & Lugnier, C., Anriantsitohaina, R. (2003). Delphinidin inhibits endothelial cell proliferation and cell cycle progression through a transient activation of ERK-1/-2. *Biochemical Pharmacology,* 65(4): 669-675.

Mateus, N. (2005). Antioxidant properties of prepared blueberry (Vaccinium myrtillus) extracts. *Journal of Agricultural and Food Chemistry*, 53: 6896-6902.

Matsui, T., Ueda, T., Oki, T., Sugita, K., Terahara, N. & Matsumoto, K. (2001).α-Glucosidase inhibitory action of natural acylated anthocyanins I. Survey of natural pigments with potent inhibitory activity. *Journal of Agricultural and Food Chemistry,* 49: 1948-1951.

McDougall, G.J., Shpiro, F., Dobson, P., Smith, P., Blake, A. & Stewart, D. (2005). Different polyphenolic components of soft fruits inhibits alpha-amylase and alpha-glucosidase. *Journal of Agricultural and Food Chemistry*, 53: 2760-2766.

McDowell, A., Thompson, S., Stark, M., Ou, Z-Q. & Gould, K.S. (2011). Antioxidant activity of puha (*Sonchus oleraceus* L.) asassessed by the Cellular Antioxidant Activity (CAA) Assay. *Phytotherapy Research*, 25: 1876–1882.

Medzhitov, R. (2008). Origin and physiological roles of inflammation. *Nature*, 454: 428-435.

Meeran, S.M. & Katiyar, S.K. (2007). Grape seed proanthocyanidins promote apoptosis in human epidermoid carcinoma A431 cells through alterations in Cdki-Cdk-cyclin cascade, and caspase-3 activation via loss of mitochondrial membrane potential. *Experimental Dermatology*, 16: 405–415.

Mertens-Talcott, S.U., Talcott, S.T. & Percival, S.S. (2003). Low concentrations of quercetin and ellagic acid synergistically influence proliferation, cytotoxicity and apoptosis in MOLT-4 human leukemia cells. *Journal of Nutrition*, 133: 2669–2674.

Mertens-Talcott, S.U. & Percival, S.S. (2005). Ellagic acid and quercetin interact synergistically with resveratrol in the induction of apoptosis and cause transient cell cycle arrest in human leukemia cells. *Cancer Letters*, 218: 141-151.

Mertens-Talcott, S.U., Lee, J-H, Percival, S. & Talcott, S.T. (2006). Induction of cell death in Caco-2 human colon carcinoma cells by ellagic acid rich frations from muscadine grapes (*Vitis rotundifolia*). *Journal of Agricultural and Food Chemistry*, 54: 5336-5343.

Miccadei, S., Venere, D.D., Cardinali, A., Romano, F., Durazzo, A., Foddai, M.S., Fraioli, R., Mobarhan, S., &Maiani, G. (2008). Antioxidative and apoptotic properties of polyphenolic extracts from edible part of artichoke (*Cynara scolymus* L.) on cultured rat hepatocytes and on human hepatoma cells. *Nutrition and Cancer*, 60(2): 276-283.

Michalska, A., Ceglinska, A., Amarowicz, R., Piskula, M.K. Szawara-Nowak, D. &Zielinski, H. (2007).Antioxidant contents and antioxidative properties of Traditional Rye Breads. *Journal of Agricultural and Food Chemistry*, 55: 734-740.

Middleton, E. Jr., Kandaswami, C. & Tehoharides, T.C. (2000). The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer, *Pharmacology Reviews,* 52: 673–751.

Mittal, B.V. & Singh, A.K. (2010). Hypertension in the developing world: challenges and opportunities. *American Journal of Kidney Diseases,* 55(3): 590-598.

Mueller, M., Hobiger, S. & Jungbauer, A. (2010). Anti-inflammatory activity of extracts from fruits, herbs and spices. *Food Chemistry*, 122: 987-996.

Mullen, W., McGinn, J., Lean, M.E.J., MacLean, M.R., Gardner, P., Duthis, G.G., Yokota, T. & Crozier, A. (2002). Ellagitannins, Flavonoinds and other phenolics in red raspberries and their contribution to antioxidant capacity and vasorelaxation properties. *Journal of Agricultural and Food Chemistry,* 50: 5191-5196.

Mullen, W., Rouanet, J-M., Auger, C., Teiss` Edre, P-L., Caldwell, S.T., Hartley, R.C., Lean, M.E.J., Edwards, C.A. & Crozier, A. (2008). Bioavailability of [2-^{14}C] quercetin-4′-glucoside in rats. *Journal of Agricultural and Food Chemistry*, 56: 12127–12137.

Mutoh, M., Watanabe, K., Kitamura, T., Shoji, Y., Takahashi, M., Kawamori, T., Tani, K., Kobayashi, M., Maruyama, T., Kobayashi, K., Ohuchida, S., Sugimoto, Y., Narumiya, S., Sugimura, T. & Wakabayashi, K. (2002). Involvement of prostaglandin E receptor subtype EP(4) in colon carcinogenesis. *Cancer Reseach*, 62: 28–32.

Naczk, M. &Shahidi, F. (2004). Extraction and analysis of phenolics in food. *Journal of Chromatography A*, 1054(1-2): 95-111.

Neergheen, V.S., Soobrattee M.A., Bahorun, T. & Aruoma, O.I. (2006). Characterization of the phenolic constituents in Mauritian endemic plants as determinants of their antioxidant activities *in vitro*. *Journal of Plant Physiology*, 163: 787-799.

Nguyen, T., Nioi, P. &Pickett, C.B. (2009). The Nrf2-antioxidant response element signalling pathway and its activation by oxidative stress. *The Journal of Biological Chemistry*, 284: 13291-13295.

NHMRC, Clinical Practice Guidelines for the management of overweight and obesity in adults. 2003, NHMRC: Canberra.

NHMRC (2012).
http://www.nhmrc.gov.au/grants/research-funding-statistics-and-data/cardiovascular-disease, accessed on 21st February, 2012.

Nichenametla, S.N., Taruscio, T.G., Barney, D.L. & Exon, J.H. (2006). A review of the effects and mechanisms of polyphenolics in cancer. *Critical Review of Food Science and Nutrition*, 46: 161-183.

Nishino, H., Satomi, Y., Tokuda, H. & Masuda, M. (2007). Cancer control by phytochemicals. *Current Pharmaceutical Design*, 13(33): 3394–3399.

Oben, J.E., Enyegue, D.M., Fomekong, G.I., Soukontoua, Y.B. & Agbor, G.A. (2007).The effect of Cissus quadrangularis (CQR-300) and a Cissus formulation (CORE) on obesity and obesity-induced oxidative stress. *Lipids in Health and Disease*, 6: 4-12.

Ohshima, H., Tazawa, H., Sylla, B.S. & Sawa, T. (2005). Prevention of human cancer by modulation of chronic inflammatory processes. *Mutatation Reseach,* 591: 110-122.

Ojeda, D., Jiménez-Ferrer, E., Zamilpa, A., Herrera-Arellano, A., Tortoriello, J. & Alvarez, L. (2010). Inhibition of angiotensin convertin enzyme (ACE) activity by the anthocyanins delphinidin- and cyanidin-3-*O*-sambubiosides from *Hibiscus sabdariffa*. *Journal of Ethnopharmacology*, 127: 7–10.

Okuda, T. (2005). Systematics and health effects of chemically distinct tannins in medicinal plants. *Phytochemistry*, 66: 2012-2031.

Okuda, T. & Ito, H. (2011). Tannins of constant structure in medicinal and food plants-hydrolyzable tannins and polyphenols related to tannins. *Molecules*, 16: 2191-2217.

Ou, B., Hampsch-Woodill, M. & Prior, R. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescin as the fluorescent. *Journal of Agricultural and Food Chemistry,* 49: 4619-4626.

Pan, M.-H., Ghai, G. & Ho, C.-T.(2008). Food bioactives, apoptosis, and cancer. *Molecular Nutrition and Food Reseach*, 52: 43-52.

Parliament of Australia (2012).
http://www.aph.gov.au/library/intguide/sp/obesity.htm, accessed on 11th February 2012.

Pavlica, S., & Gebhardt, R. (2005). Protective effects of ellagic and chlorogenic acids against oxidative stress in PC12 cells. *Free Radical Research*, 39(12): 1377-1390.

Perwez Hussain, S., Hofseth, L.J. & Harris, C.C. (2003). Radical causes of cancer. *Nature Reviews Cancer*, 3: 276-285.

Perwez Hussain, S. & Harris, C.C. (2007). Inflammation and cancer: an ancient link with novel potentials. *International Journal of Cancer*, 121: 2373-2380.

Philpott, M. & Ferguson, L.R. (2004). Immunonutrition and cancer. *Mutation Reseach*, 551: 29-42.

Polakis, P. (2012). Wnt signalling in cancer. *Cold Spring Harbor Perspectives in Biology*, 4: a008052.

Pop, C. & Salvesen, G.S. (2009) Human caspases: activation, Specificity, and regulation. *The Journal of Biological Chemistry*, 284: 21777-21781.

Potenza, M.A., Marasciulo,F.L., Tarquinio,M., Tiravanti, E., Colantuono,G., Federici, A., Kim, J-A. Quon, M.J. & Montagnani, M. (2007). EGCG, a green teapolyphenol, improves endothelial function and insulin sensitivity, reduces blood pressure, and protects against myocardial I/R injury in SHR. *American Journal of Physiology Endocrinology and Metabolism*, 292(5): 1378-1387.

Prior, R.L., Lazarus, S.A., Cao, G., Muccitelli, H. & Hammerstone, J.F. (2001). Identification of procyanidins and anthocyanins in blueberries and cranberries (Vaccinium spp.) using high performance liquid chromatography/mass spectrometry. *Journal of Agricultural and Food Chemistry*, 49: 1270-1276.

Prior, R. L., Hoang, H., Gu, L., Wu, X., Bacchiocca, M., Howard, L., Hampsch-Woodill, M., Huang, D., Ou, B. & Jacob, R. (2003). Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORAC)) of plasma and other biological and food samples. *Journal of Agricultural and Food Chemistry*, 51: 3273–3279.

Prior, R.L., Wu, X. & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, 53: 4290-4302.

Rajamanickam, S. & Agarwal, A. (2008). Natural products and colon cancer: current status and future prospects. *Drug Development Research*, 69 (7): 460–471.

Rajesh, Pl., Rastogi, R. & Rajeshwar, P.S. (2009). Apoptosis: molecularmechanisms and pathogenicity. *EXCLI Journal*, 8: 155-181.

Ranilla, L.G., Kwon, Y-I, Apostolidis, E. & Shetty, K. (2010). Phenolic componds, antioxidant activity and *in vitro* inhibitory potential against key enzymes relevant for hyperglycemia and hypertension of commonly used medicinal plants, herbs and spices in Latin America. *Bioresource Technology*, 101: 4676-4689.

Ravoori, S., Vadhanam, M.V., Aqil, F., & Gupta, R. (2012). Inhibition of estrogen-mediated mammary tumorigenesis by blueberry and black raspberry. *Journal of Agricultural and Food Chemistry*, 60: 5547−5555.

Ren, W.Y., Qiao, Z.H., Wang, H.W., Zhu, L. & Zhang, L.(2003). Flavonoids: promising anticancer agents. *Medical Care Research and Review*, 23: 519–534.

Revilla, E., Ryan, J.-M. & Martin-Ortega, G. (1998). Comparison of Several Procedures Used for the Extraction of Anthocyanins from Red Grapes. *Journal of Agricultural and Food Chemistry*, 46: 4592-4597.

Rhode, J., Fogoros, S., Zick, S., Wahl, H., Griffith, K.A., Huang, J. & Liu, J.R. (2007). Ginger inhibits cell growth and modulates angiogenic factors in ovarian cancer cells. *BMC Complementary and Alternative Medicine,* 7: 44-48.

Rice-Evans, C.A., Miller, N.J., Bolwell, P.G., Bramley, P.M. & Pridham, J.B. (1995). The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Reseach*, 22: 375–383.

Riedl, S.J. & Salvesen G.S. (2007). The apoptosome: signalling platform of cell death. *Nature Reviews Molecular Cell Biology,* 8: 405-413.

Riley, T., Sontag, E., Chen, P. & Levine, A. (2008). Transcriptional control of human p53-regulated genes. *Nature Reviews Molecular Cell Biology*, 9: 402–412.

Rivett, D.E., Jones, G.P. & Tucker, D.J. (1989). *Santalum acuminatum* fruit: a prospect for horticultural development. In: Wickens, G.E., Haq, N. and Day, P. (Eds.), pp. 208-221.

Roberts, R.G., Jone, R., & Smith, M.A. (1990). Thermoluminescence dating of a 50,000-year-old human occupation site in northern Australia. *Nature*, 345(6271): 153-156.

Robin, J. (2004). Native foods-overview. In Salvin, S., Bourke, M. & Byrne, T., The new crop industry handbook, vol. 04/125: 338-345. RIRDC Publication.

Robbins, R. (2003). Phenolic acids in foods: an overview of analytical methodology. *Journal of Agricultural and Food Chemistry*, 51: 2866-2887.

Ross, H.A., McDougall, G.J. & Stewart, D. (2007). Antiproliferative activity is predominantly associated with ellagitannins in raspberry extracts. *Phytochemistry*, 68: 218-228.

Rowinsky, E.K. (2005). Targeted induction of apoptosis in cancer management: the emerging role of tumor necrosis factor-related apoptosis-inducing ligand receptor activating agents. *Journal of Clinical Oncology*, 23: 9394-9407.

Russo, G.L. (2007). Ins and outs of dietary phytochemicals in cancer chemoprevention. *Biochemical Pharmacology,* 74: 533-544.

Sakulnarmrat, K. & Konczak, I. (2012). Composition of native Australian herbs polyphenolic-rich fractions and *in vitro* inhibitory activities against key enzymes relevant to metabolic syndrome. *Food Chemistry*, 34: 1011-1019.

Scalbert, A., Manach, C., Morand, C. & Rémésy, C. (2005). Dietary polyphenols and the prevention of diseases. *Critical Reviews in Food Science and Nutrition*, 45: 287–306.

Schimmer, A.D. (2004). Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice. *Cancer Research*, 64: 7183-7190.

Schwager, J., Mohajeri, M.H., Fowler, A. & Weber, Pl. (2008).Challenges in discovering bioactives for the food industry. *Current Opinion in Biotechnology*, 19: 66-72.

Seeram, N.P., Momin, R.A., Nair, M.G. & Bourquin, L.D. (2001). Cyclooxygenase inhibitory and antioxidant cyanidin glycosides in cherries and berries. *Phytomedicine*, 8: 362-369.

Seeram, N.P., Adams, L.S., Henning, S.M., Niu, Y., Zhang, Y., Nair, M.G. & Heber, D. (2005). *In vitro* antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. *The Journal of Nutritional Biochemistry,* 16: 360–367.

Seeram, N.P., Lee, R., Scheuller, S. & Heber, D. (2006). Identification of phenolic compounds in strawberries by liquid chromatography electrospray ionization mass spectroscopy. *Food Chemistry*, 97: 1–11.

Shalaby, S.M. Zakora, M. & Otte, J. (2006). Performance of two commonly used angiotensis-converting enzyme inhibition assays using FA-PGG and HL as substrates, *Journal of Dairy Research*, 73: 178-186.

Shan, B., Cai, Y.Z., Sun, M. & Corke, H. (2005). Antioxidant capacity of 26 spice extracts and characterisation of their phenolic constituents. *Journal of Agricultural and Food Chemistry*, 53: 7749-7759.

Shankar, S., Ganapathy, S. & Srivastava, R.K. (2007). Green tea polyphenols: biology and therapeutic implications in cancer. *Frontiers in Bioscience*, 12: 4881-4899.

Sharma, M., Li, L., Celver, J., Killian, C., Kovoor, A. & Seeram, N.P. (2010). Effects of fruit Ellagitannin extracts, ellagic acid, and their colonic metabolite, urolithin A, on Wnt signalling. *Journal of Agricultural and Food Chemistry*, 58(7): 3965–3969.

Shi, J., Nawaz, H., Pohorly, J., Mittal, G., Kakuda, Y. & Jiang, Y. (2005). Extraction of polyphenolics from plant material for functional foods-engineering and technology. *Food Reviews International,* 21: 139-166.

Shimura, S., Tsuzuki, W., Kobayashi, S. & Suzuki, T. (1992). Inhibitory effect on lipase activity of extracts from medicinal herbs. *Bioscience, Biotechnology and Biochemistry*, 56: 1478-1479.

Siro, I., Kapolna, E., Kapolna, B. & Lugasi, A. (2008). Functional food: Product development, marketing and consumer acceptance-a review. *Appetite*, 51: 456-467.

Smith, B.G. (2008). Developing sustainable food supply chains. *Philosophical Transactions of the Royal Society Biological Science,* 363: 849-861.

Song, F-L., Gan, R-Y., Zhang, Y., Xiao, Q., Kuang, L. & Li, H-B. (2010). Total phenolic contents and antioxidant capacities of selected Chinese medicinal plants. *International Journal of Molecular Sciences*, 11: 2362-2372.

Southwell, I.A. & Brophy, J.J. (1992). Differentiation within the Australian *Tasmannia* by essential oil comparison. *Phytochemistry,* 31: 3073-3081.

Southwell, I.A., Russell, M., Smith, R.L.&Archer, D.W. (2000). *Backhousia citriodora* F. Muell.(Myrtaceae), a superior source of citral. *Journal of Essential Oil Research*, 12(6): 735-741.

Steensma, D.P., Timm, M. & Witzig, T.E. (2003). Flow cytometric methods for detection and quantification of apoptosis. *Methods in Molecular Medicine*, 85: 323-332.

Subramaniam, S.R., & Ellis, E.M. (2011). Esculetin-induced protection of human hepatoma HepG2 cells against hydrogen peroxide is associated with the Nrf2-dependent induction of the NAD(P)H: Quinone oxidoreductase 1 gene. *Toxicology and Applied Pharmacology*, 250: 130–136.

Surh, Y.-J., Hurh, Y.J., Kang, J.Y., Lee, E., Kong, G. & Lee, S.J. (1999). Resveratrol, an antioxidant present in red wine, induces apoptosis in human promyelocytic leukemia (HL-60) cells. *Cancer Letters*, 140: 1-10.

Surh, Y-S.(2003). Cancer chemoprevention with dietary phytochemicals. *Nature Review Cancer*, 3: 768-780.

Suzuki, A., Kagawa, D., Fuji, A., Ochiai, R., Tokimitsu, I. & Saito, I. (2002). Short and long-term effects of ferulic acid on blood pressure in spontaneously hypertensive rats. *American Journal of Hypertension*, 15: 351-357.

Szkudelska, K. & Nogowski, L. (2007). Genistein-a dietary compound inducing hormonal and metabolic changes. *The Journal of Steroid Biochemistry and Molecular Biology*, 105: 37-45.

Tan, A.C., Konczak, I., Ramzan, I. & Sze, D.M-Y. (2011a). Antioxidant and cytoprotective activities of native Australian fruit polyphenols. *Food Research International*, 44: 2034-2040.

Tan, A.C., Konczak, I., Ramzan, I. & Sze, D.M-Y. (2011b). Native Australian fruit polyphenols inhibit cell growth and induce apoptosis in human cancer cell lines. *Nutrition and Cancer*, 63(3): 444-455.

Tan, A.C., Konczak, I., Ramzan, I. & Sze, D.M-Y. (2011c). Native Australian fruit polyphenols inhibit COX-2 and iNOS expression inLPS-activated murine macrophages. *Food Research International*, 44: 2362-2367.

Tanigawa, S., Fujii, M. & Hou, D.-X.(2007). Action of Nrf2 and Keap1 in ARE-mediated NQO1 expression by quercetin. *Free Radical Biology and Medicine*, 42: 1690-1703.

Tasaki, M., Uemura, T., Maeda, M., Ishii, Y., Okamura, T., Inoue, T., Kuroiwa, Y., Hirose, M. & Nishikawa, A. (2008). Safety assessment of ellagic acid, a food additive, in a subchronic toxicity study using F344 rats. *Food and Chemical Toxicology*, 46(3): 1119–1124.

Terahara, N., Konczak-Islam, I., Nakatani, M., Yamakawa, O., Goda, Y. & Honda, T. (2000). Anthocyanins in callus induced from purple storate root of lpomoea batatas L. *Phytochemistry*, 54: 191-922.

Thom, E. (2007). The effect of chlorogenic acid enriched coffee on glucose absorption in healthy volunteers and its effect on body mass when used long-term in overweight and obese people. *The Journal of International Medical Research*, 35: 900–908.

Thomasset, S.C., Berry, D.P., Garcea, G., Marczylo, T., Steward, W.P. & Gescher, A.J. (2007). Dietary polyphenolic phytochemicals-promising cancer chemopreventive agents in humans? A review of their clinical properties. *International Journal of Cancer*, 120: 451-458.

Thornberry, N.A., Rano, T.A., Peterson, E.P., Rasper, D.M., Timkey, T., Garcia-Calvo, M., Houtzager, V.M., Nordstrom, P.A., Roy, S., Vaillancourt, J.P., Chapman, K.T. & Nicholson, D.W. (1997). A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *The Journal of Biological Chemistry*, 272(29): 17907-17911.

Tomás-Barberán, F. A. & Clifford, M.N. (2000). Dietary hydroxybenzoic acid derivatives – Nature, occurrence and dietary burden. *Journal of the Science of Food and Agriculture*, 80: 1024-1032.

Tsuda, T. (2008). Regulation of adipocyte function by anthocyanins; Possibility of preventing the metabolic syndrome. *Journal of Agricultural and Food Chemistry*, 56: 642-646.

Tulp, M., Bruhn, J.G. & Bohlin, L. (2006). Food for thought. *Drug Discovery Today*, 11: 1115-1121.

Tyagi, A., Agarwal, R. & Agarwal, C. (2003). Grape seed extract inhibits EGF-induced and constitutively active mitogenic signaling but activates JNK in human prostate carcinoma DU145 cells: possible role in antiproliferation and apoptosis. *Oncogene*, 22: 1302-1316.

Umesalma, S. & Sudhandiran, G. (2010). Differential inhibitory effects of the polyphenol ellagic acid on inflammatory mediators NF-κB, iNOS, COX-2, TNF-α, and IL-6 in 1,2-Dimethylhydrazine-induced rat colon carcinogenesis. *Basic & Clinical Pharmacology & Toxicology*, 107: 650-655.

Valko, M., Rhodes, C.J., Moncol, J., Izakovic, M. & Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico Biological Interactions*, 160: 1-40.

Vattem, D.A. & Shetty, K. (2005). Biological functionality of ellagic acid: A Review. *Journal of Food Biochemistry*, 29: 234–266.

Vertuani, S., Angusti, A. & Manfredini, S. (2004). The antioxidants and pro-antioxidants network: An overview. *Current Pharmaceutical Design*, 10: 1677-1694.

Vesely, M.D., Kershaw, M.H. Schreiber, R.D. & Smyth, M.J. (2011). Natural innate and adaptive immunity to cancer. *Annual Review of Immunology*, 29: 235–271.

Villasenor, I.M. & Lamadrid, M.R.A. (2006). Comparative anti-hyperglycemic potential of medicinal plants. *Journal of Ethnopharmacology*, 104: 129-131.

Vincent, H.K. & Taylor, A.G. (2006). Biomarkers and potential mechanisms of obesity-induced oxidant stress in humans. *International Journal of Obesity*, 30: 400–418.

Vivanco, I. & Sawyers, C.L. (2002). The phosphatidylinositol 3-kinase/AKT pathway in human cancer. *Nature Reviews Cancer*, 2: 489-501.

Wallinga, D. & Maizes, V. (2008). Foraging for healthy food in the global economy; ten steps we can all take. *Explore (NY)*, 4: 385-388.

Wang, Y.C., McPherson, K., Marsh, T., Gortmaker, S.L. & Brown, M. (2011). Health and economic burden of the projected obesity trends in the USA and UK. *The Lancet,* 378: 815-25.

Wang, L. & Weller, C.L. (2006). Recent advances in extraction of nutraceuticals from plants. *Trends in Food Science & Technology*, 17: 300-312.

Wellman, N.S. & Friedberg, B. (2002). Causes and consequences of adult obesity: health, social and economic impacts in the United States. *Asia Pacific Journal Clinical Nutrition*, 11(suppl 8): S705-S709.

Wolfe, K.L. & Liu, R.H. (2007). Cellular antioxidant activity (CAA) assay for assessing antioxidants foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, 55: 8896-8907.

Wolfe, K.L. & Liu, R.H. (2008). Structure-Activity Relationships of flavonoids in the cellular antioxidant activity assay. *Journal of Agricultural and Food Chemistry,* 56: 8404-8411.

Wolfe, K.L., Kang, Xinmei, He, X., Dong, M., Zhang, Q. & Liu, R.H. (2008). Cellular antioxidant activity of common fruits. *Journal of Agricultural and Food Chemistry*, 56: 8418-8426.

Wolfe, K., Wu, X. & Liu, R.H. (2003). Antioxidant activity of apple peels. *Journal of Agricultural and Food Chemistry*, 51: 609-614.

World Cancer Research Fund (2007). *Food, nutrition, physical activity, and the prevention of cancer: a global perspective*, American Institute for Cancer Research, Washington D.C., USA.

Yang, C.S., Landau, J.M., Huang, M.T. & Newmark, H.L. (2001). Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annual Review of Nutrition*, 21: 381-406.

Yi, W., Fischer, J. & Akoh, C.C. (2005). Study of anticancer activities of muscadine grape phenolics in vitro. *Journal of Agricultural and Food Chemistry*, 53: 8804-8812.

You, Q., Chen, F., Wang, X., Jiang, Y. & Lin, S. (2012). Anti-diabatic activities of phenolic compounds in muscadine against alpha-glucosidase and pancreatic lipase. LWT-*Food Science and Technology*, 46: 164-168.

Yu, X. &Kensler, T. (2005). Nrf2 as a target for cancer chemoprevention. *Mutation Research,* 591(1-2): 93-102.

Zafrilla, P., Ferreres, F. & Tomas-Barberan, F.A. (2001). Effect of processing and storage on the antioxidant ellagic acid derivatives and flavonoids of red raspberry (*Rubus idaeus*) jams. *Journal of Agricultural and Food Chemistry,* 49: 3651-3655.

Zhang, L., Li, J., Hogan, S., Chung, H., Welbaum, G.E. & Zhou, K. (2010). Inhibitory effect of raspberries on starch digestive enzyme and their antioxidant properties and phenolic composition. *Food Chemistry*, 119: 592–599.

Zhang, Z., Ho, W.K.K., Huang, Y., James, A.E., Lam, L.W. & Chen, Z-Y. (2002). Hawthorn fruit is hypolipidemic in rabbits fed a high cholesterol diet. *Journal of Nutrition*, 132: 5–10.

Zheng, W., & Wang, S. Y. (2001). Antioxidant activity and phenolic compounds in selected herbs. *Journal of Agricultural and Food Chemistry*, 49: 5165–5170.

Zibadi, S., Rohdewald, P.J., Park, D. & Watson, R.R. (2008). Reduction of cardiovascular risk factors in subjects with type 2 diabetes by Pycongenol supplementation. *Nutrition Research*, 28: 315-320.

Zimmet, P.Z., Alberti, K.G.M.M. & Shaw, J.E. (2005). Editorials: Mainstreaming the metabolic syndrome: a definitive definition. *The Medical journal of Australia*, 183 (4): 175-176.

Zola, N. & Gott, B. (1992). *Koorie Plants Koori People - Traditional Aboriginal Food, Fibre and Healing Plants of Victoria*, (1996 reprint), Koorie Heritage Trust, Melbourne, 29.

Chapter 10
Appendices

APPENDIX A: Chromatograms of standard

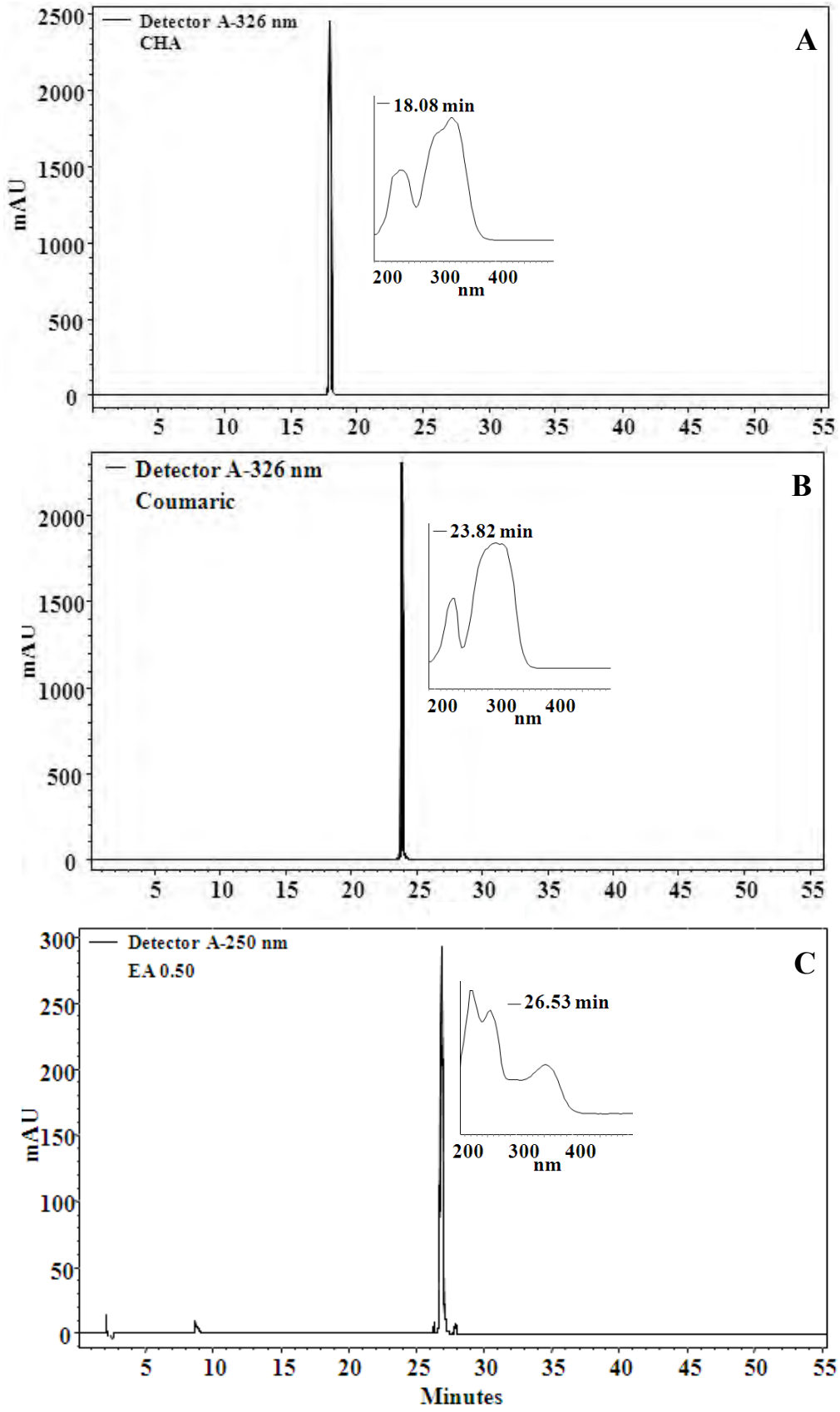


Figure A.1 Chromatogram of phenolic acid standard (A) chlorogenic acid (B) coumaric acid and (C) ellagic acid

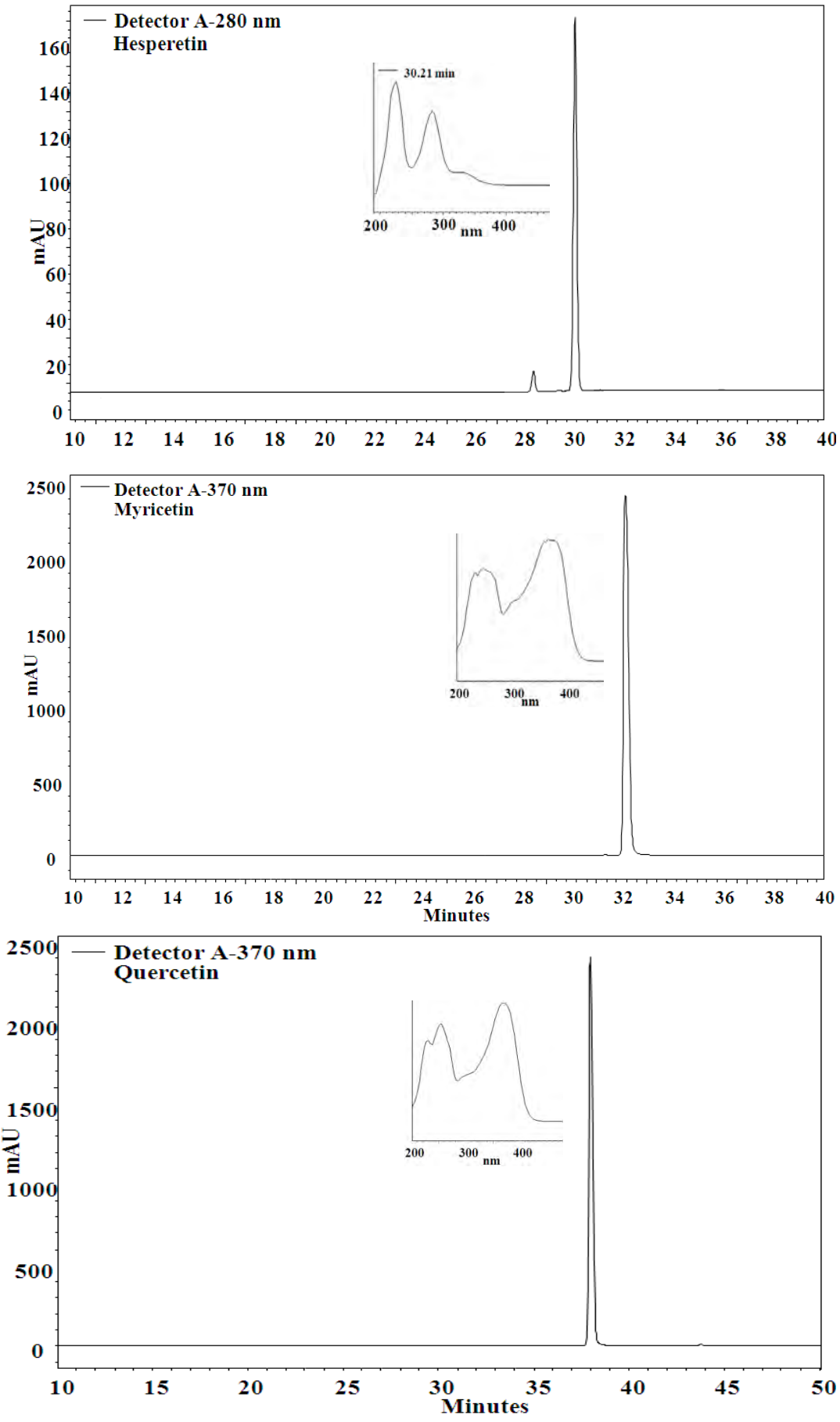
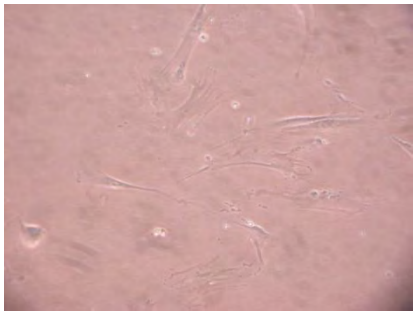


Figure A.2 Chromatogram of some flavonoids (hesperetin, myricetin, quecetin)

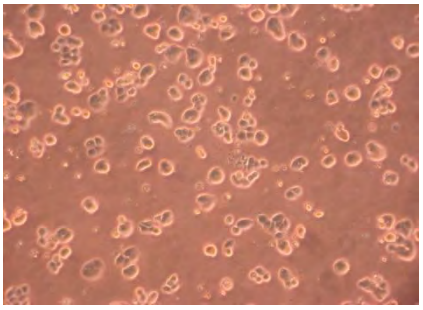
APPENDIX B: Cell lines and media

Table B.1 List of cell lines and media

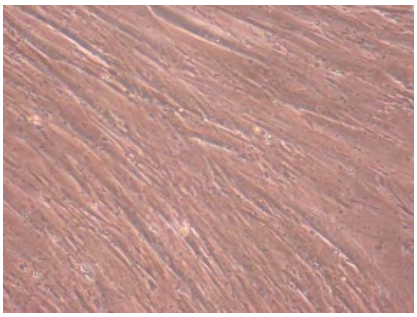
Cell line	Medium
AGS (gastric adenocarcinoma)	F12-K Ham's medium
HT-29 (colorectal adenocarcinoma)	McCoy's 5a medium
HL-60 (acute promyelocytic leukaemia)	Iscove's modified Dulbecco's medium (IMDM) containing 20% FBS
CCD-18Co (colon normal)	Eagle's minimum essential medium (EMEM)
Bladder cells (BL13)	RPMI medium
RAW 264.7	Dulbecco's Modified Eagle's medium (DMEM)/F-12)
HepG2	Eagle's minimum essential medium (EMEM)
Hs738.St/Int	Dulbecco's Modified Eagle's medium (DMEM/F-12)



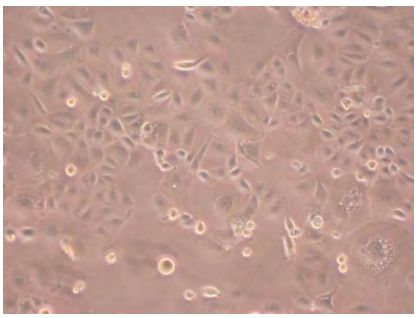
Colon normal (CCD-18Co)



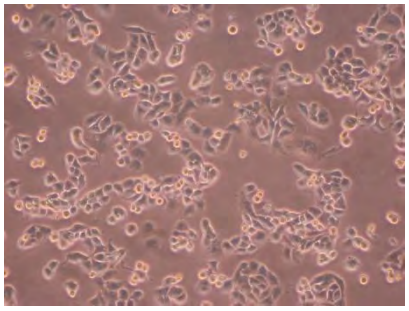
Colorectal adenocarcinoma (HT-29)



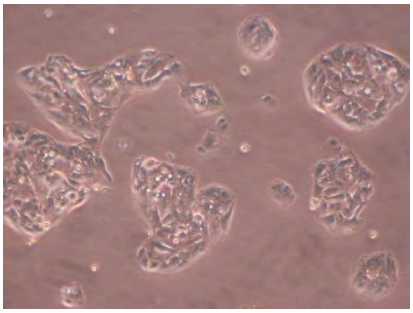
Hs738.St/Int



Gastric adenocarcinoma (AGS)



Bladder cells (BL13)



Hepatocellular carcinoma (HepG2)

Figure B.1 Cancerous cells and non-transform cells

APPENDIX C: Cellular protection from hydrogen peroxide (H_2O_2) induced cell death

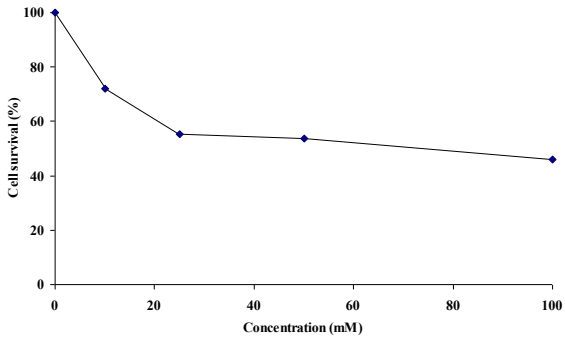


Figure C.1 Effect of H_2O_2 on cell proliferation of HepG2 cells

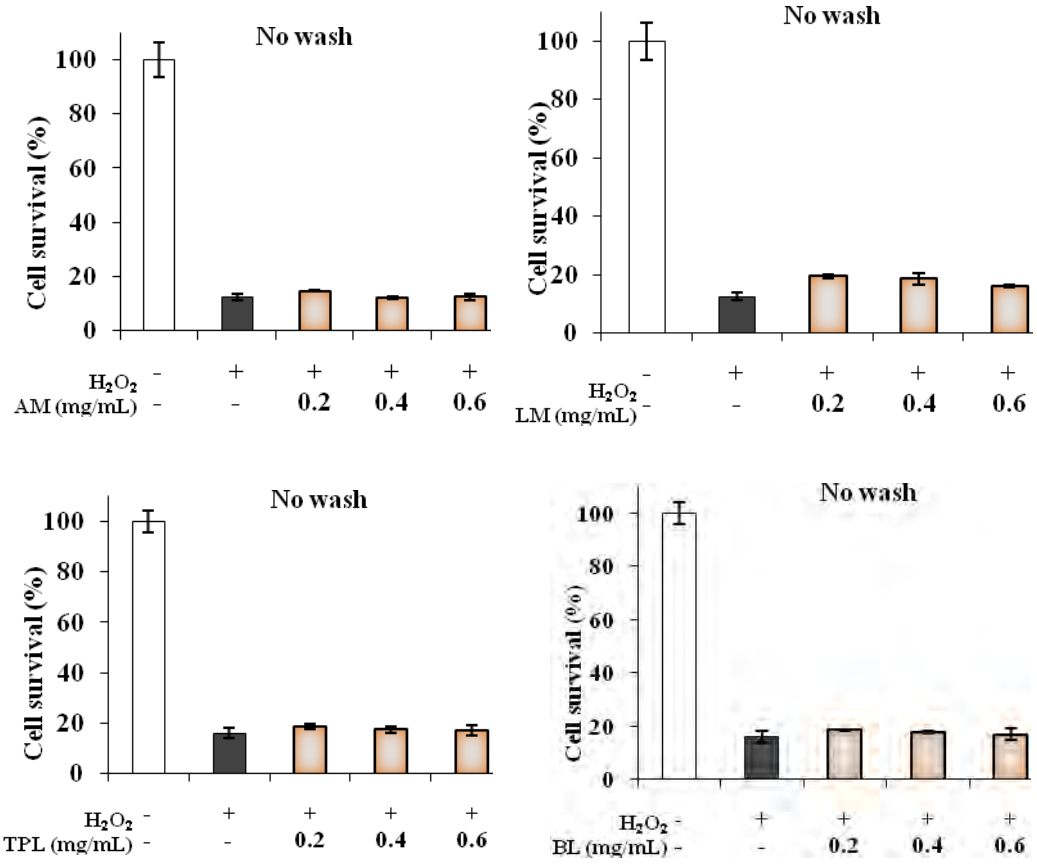


Figure C.2 Effect of purified polyphenolic-rich herbs extracts on H_2O_2 –induced cell death in RAW 264.7 cells using the MTT assay without washing step

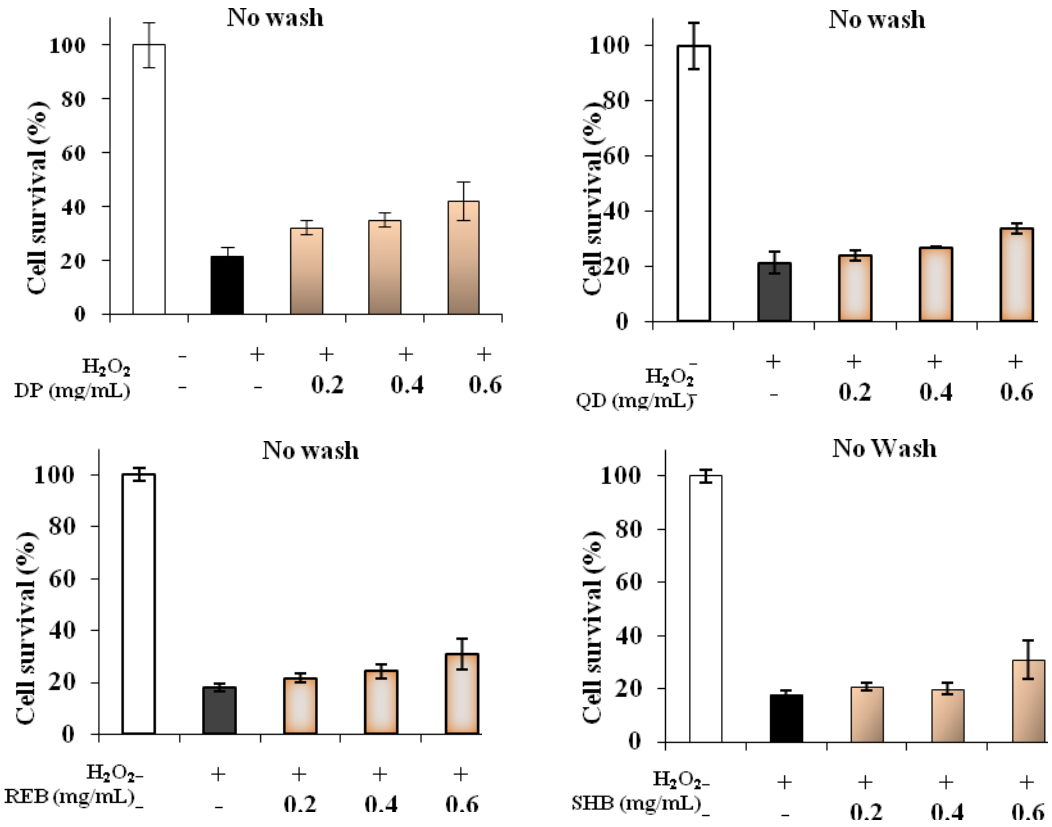


Figure C.3 Effect of purified polyphenolic-rich fruits extracts on H_2O_2 –induced cell death in RAW 264.7 cells using the MTT assay without washing step

APPENDIX D: Anti-proliferative activity

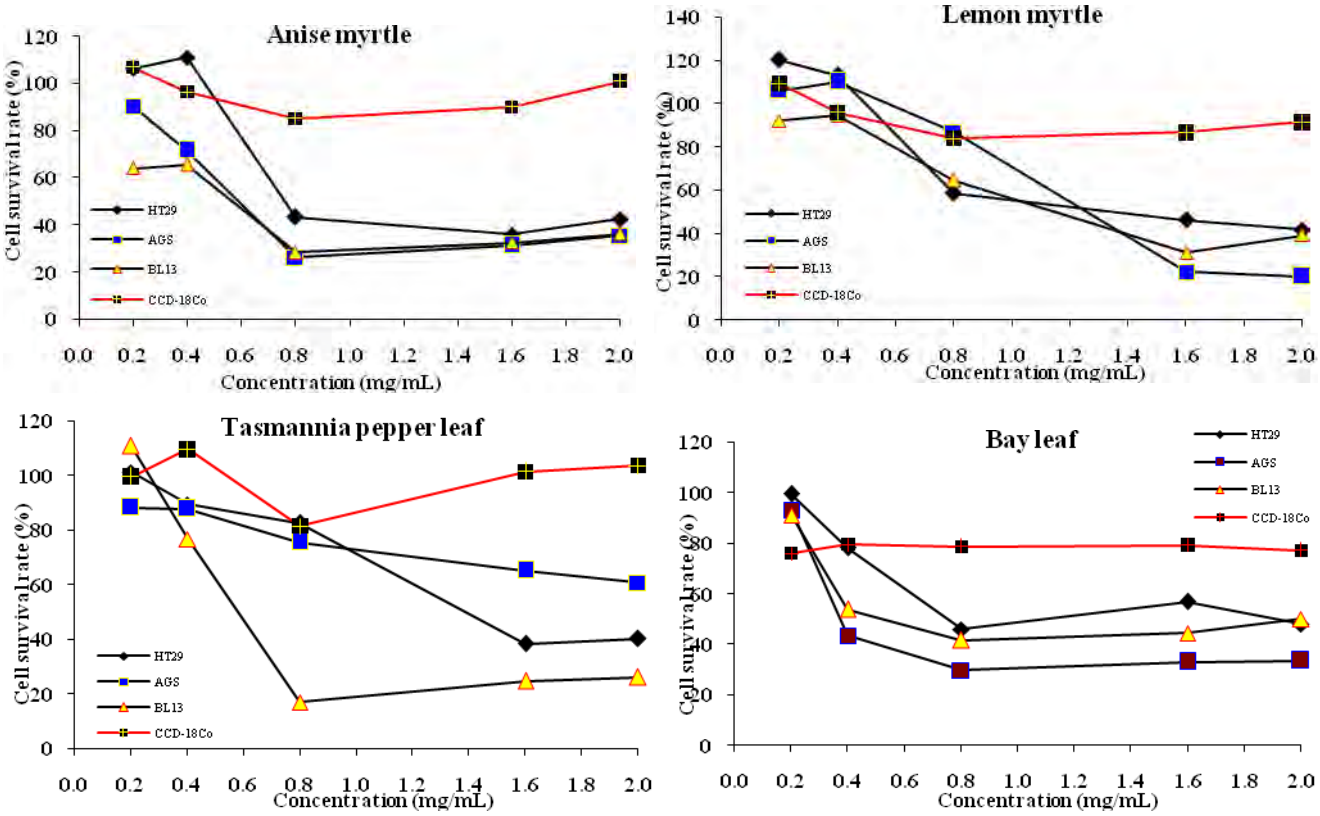


Figure D.1 Anti-proliferative activity of purified polyphenolic-rich herbs extracts against cancerous cell lines and normal cell lines

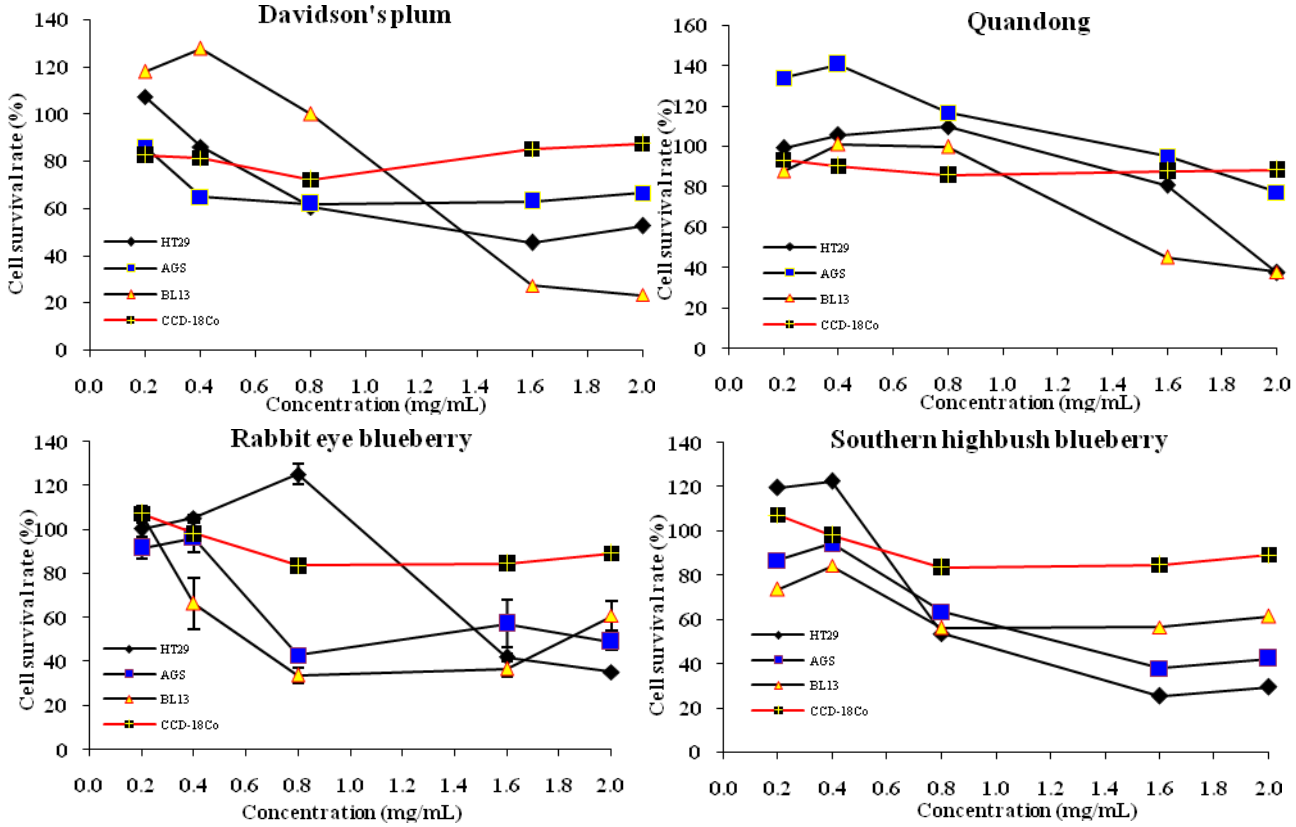


Figure D.2 Anti-proliferative activity of purified polyphenolic-rich fruits extracts against cancerous cell lines and normal cell lines

APPENDIX E: Biography

Miss Karunrat Sakulnarmrat obtained her bachelor degree (Chemical Engineering) from Suranaree University of Technology (SUT), Thailand in 2000 and she continued her master degree (Chemical Engineering) at Chulalongkorn University, Thailand in 2001 (Chemical Engineering). She is a teaching permanent staff of Food Science Department, Faculty of Agricultural and Technology at Rajamangala University of Technology Isan (RMUTI), Surin campus, Thailand. She has awarded a scholarship by her university for PhD program at University of New South Wales (UNSW), Sydney Australia, and collaborated with CSIRO Food and Nutritional Science Australia for 3 years.