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Foreword

This research was developed to provide the native food industry with reliable information on specific potential health benefits of native herbs and fruits, as evaluated using cell culture models. These evaluations are cheaper and faster to perform than clinical studies conducted with humans, are used to screen large number of samples and to select the most valuable sources for further nutritional/ human studies. This report represents the first systematic evaluation of commercially grown native Australian herbs and fruits with respect to: i) cell-protective and genome-protective capacity; ii) anti-proliferative activities against human cancer cells and equivalent normal cells; iii) bioavailability of plant components to human cells; and iv) inhibitory activities towards two key enzymes relevant to metabolic syndrome: α-glucosidase, responsible for the digestion of sugars; and pancreatic lipase, responsible for the digestion of fats.

This original research for the first time presents exceptionally high antioxidant capacities of native Australian herb extracts, exhibited within a live cell. This finding demonstrates that the addition of Australian native herbs to food products may deliver antioxidants capable of scavenging free radicals and protecting the cells and their components from the damaging effects of oxidative stress. All evaluated polyphenolic-rich extracts obtained from native herbs and fruits evaluated within this study displayed differential killing ability; specifically, an ability to suppress the proliferation of cancer cells of the digestion system while sparing the normal (non-transformed) cells. Extracts from anise myrtle in particular exhibited superior activity. All polyphenolic-rich extracts successfully inhibited the activities of α-glucosidase and pancreatic lipase enzymes, which, respectively, might be useful towards the management of type two diabetes and obesity. Moreover, the presented research has demonstrated an uptake of phenolic compounds isolated from herbs and fruits by human cells using the Caco-2 cell monolayer model, which indicates that the physiologically active compounds originating from the native foods evaluated within this study are bioavailable to human cells.

This research can be used to inform consumers about food products containing Australian native herbs and fruits. Australian native food industry managers and marketing teams aiming at the development of new markets for native food products domestically and overseas will benefit from this information.

This report, an addition to RIRDC's diverse range of over 2000 research publications, forms part of our New Plant Products R&D program, which aims to facilitate the development of new industries based on plants or plant products that have commercial potential for Australia.

Most of RIRDC's publications are available for viewing, free downloading or purchasing online at www.rirdc.gov.au. Purchases can also be made by phoning 1300 634 313.

Craig Burns
Managing Director
Rural Industries Research and Development Corporation

Abbreviations

AM	Anise myrtle
BL	Bay leaf
BN	Binuclear cell
C3G Eq	Cyanidin 3-glucoside equivalent
CAA	Cellular antioxidant activity
CBMN	Cytokinesis-block micronucleus
CH Eq	Catechin hydrate equivalent
CHA	Chlorogenic acid
CHA Eq	Chlorogenic acid equivalent
CQA	Caffeoylquinic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DP	Davidson's plum
DW	Dry weight
EA Eq	Ellagic acid equivalent
EMEM	Eagle's minimum essential medium
FRAP	Ferric reducing antioxidant power
GA Eq	Gallic acid equivalent
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
HPLC-DAD	High performance liquid chromatography-diode array detector
IC_{50}	Inhibitor concentration needed to reduce cell response by 50 per cent
LM	Lemon myrtle
MN	Micronuclei
NBud	Nuclear bud
NDI	Nuclear division index
NPB	Nucleoplasmic bridge
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate buffered saline
Q3G	Quercetin 3-glucoside
Q3R	Quercetin 3-rutinoside
Q3R Eq	Quercetin 3-rutinoside equivalent
Q Eq	Quercetin equivalent
QD	Quandong
R Eq	Rutin equivalent
REB	Rabbit eye blueberry
RNA	Ribonucleic acid
SD	Standard deviation
SHB	Southern highbush blueberry
TP	Tasmannia pepper leaf
TRC	Total reducing capacity

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Executive Summary

What the report is about

This report presents potential health-enhancing properties of polyphenolic-rich extracts obtained from selected commercially grown native Australian herbs and fruits, as evaluated in an array of *in vitro* assays. The following specific health properties were targeted:

- cellular and genome-protective activities
- anti-proliferative activities against human cancer cells
- differential killing ability for human colon and stomach cell models
- bioavailability of plant components to human cells
- inhibitory activities towards key enzymes relevant to diabetes (α-glucosidase) and obesity (pancreatic lipase).

Who is the report targeted at?

This report is targeted at:

- the Australian native food industry
- the general food, pharmaceutical and cosmeceutical industries
- the health-conscious consumer.

Where are the relevant industries located in Australia?

As recorded in 2005, the Australian native food industry comprises over 90 active individual producers, harvesters, wholesalers and restaurant owners, delivering to the Australian and international market approximately 200 products made of native Australian edible plants. The industry is represented in all states of Australia.

The number of native plants growers is continuously increasing and research is needed to utilise their products in the development of unique Australian value-added foods, to understand their value to human health and to educate consumers about these products.

Background

Recently, native Australian edible plants have been described as being exceptionally rich sources of antioxidants, superior to blueberry which is renowned for its health properties. Antioxidants scavenge the free radicals that are constantly generated in the human body, preventing or reducing oxidative stress and the development of chronic conditions in which oxidative stress is indicated.

Phenolic compounds were identified as the main source of antioxidant capacity in the native foods examined. Phenolic compounds are not only powerful antioxidants but are frequently coupled with a number of health benefits. Phenolic compounds act as modulators of metabolic processes, and as such are essential food components that facilitate normal functions of human cells. Native Australian plants developed locally are a rich source of phenolic compounds with unique compositions that may complement commonly consumed western fruits and vegetables. Therefore, an opportunity exists for the Australian native food industry to research and identify health properties of native edible plants and utilise this information to create unique health-promoting Australian foods.

The most convincing way to evaluate the health-promoting properties of foods is through human studies. However, these studies are usually limited to very few samples, are expensive and take a long time to complete. Additionally, the complexity of the human body and the involvement of other factors may lead to non-conclusive results. Cell-culture-based systems, however, allow screening of a larger number of samples in a short time, are much cheaper and are more reproducible. Previously used exclusively for drug development, this system that 'mirrors' the behaviour of cells within a body is here applied successfully to evaluate large numbers of plant-derived bioactives for their health effects. This project has been designed to utilise an array of *in vitro* assays, based on human cell cultures and isolated enzymes, to obtain information about potential health benefits of polyphenolic-rich extracts from leading commercially grown native Australian herbs and fruits. Substantiation studies in animals and humans are required to confirm findings obtained through cell culture studies.

Aims/objectives

The aim of this project was to investigate the potential health benefits arising from consumption of selected commercially grown Australian native species of primary importance for the industry: Tasmannia pepper leaf (*Tasmannia lanceolata* R. Br., Winteraceae), anise myrtle (*Syzygium anisatum* Vickery, Craven & Biffen, Myrtaceae), lemon myrtle (*Backhousia citriodora* F.Muell, Myrtaceae), quandong (*Santalum acuminatum* A.D.C., Santalaceae) and Davidson's plum (*Davidsonia pruriens* F. Muell., Cunoniaceae). 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.

Methods used

Aqueous polyphenolic-rich extracts were assessed in an array of human cell culture-based and isolated enzyme-based assays (recognised as cell culture/enzyme models for phytochemical research and suitable for the screening of multiple samples to identify potential health benefits), as follows:

- preparation of lyophilised polyphenolic-rich extracts from herbs and fruits and purification of extracts using an open column chromatography method
- analysis of phenolic compounds using high performance liquid chromatography
- antioxidant activity in reagent-based assays (ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC))
- cellular antioxidant activity (CAA) assay
- cellular protection against hydrogen peroxide (H_2O_2)-induced cell death
- cytokinesis-block micronucleus (CBMN) cytome assay
- MTT colorimetric cell viability assay
- flow cytometry analysis of cancer cells exposed to plant extracts
- Caco-2 bioavailability assay
- suppression of α-glucosidase enzyme activity
- suppression of pancreatic lipase enzyme activity.

Results/key findings

Evaluation of polyphenolic-rich extracts obtained from native Australian herbs (Tasmannia pepper leaf, anise myrtle, lemon myrtle) and fruits (Davidson's plum and quandong) in an array of *in vitro* assays revealed for the first time the following unique potential health-enhancing qualities:

- Davidson's plum and Tasmannia pepper leaf extracts were identified as the richest sources of phenolic compounds of the fruits and herbs tested, as quantified per gram dry weight (gDW), followed by anise myrtle and lemon myrtle extracts. The extracts comprised predominantly phenolic acids and flavonoids. Ellagic acid was identified in anise myrtle, lemon myrtle and Davidson's plum.

- Davidson's plum extract among the fruit extracts and anise myrtle extract among the herb extracts exhibited the highest total reducing capacity (using the FRAP assay), while Tasmannia pepper leaf compounds exhibited the highest oxygen radical scavenging ability (using the ORAC assay).

- Phenolic compounds originating from all evaluated plant sources appeared to be bioavailable to human cells based on results using the Caco-2 cell monolayer model. Anise myrtle and quandong phenolic compounds showed the highest bioavailability which was 2-fold that of Tasmannia pepper leaf. An intensive uptake of anise myrtle phenolic compounds occurred within the first hour from their delivery to cell culture.

- Phenolic compounds of all the extracts tested were able to enter a live cell and act as antioxidants within the cell as determined by the cellular antioxidant activity (CAA) assay. Tasmannia pepper leaf extract exhibited superior CAA, followed by anise myrtle extract. These results suggest potential protective effect on cell components (DNA, RNA, lipids, enzymes, etc.) from the damaging action of endogenous free radicals. All extracts exhibited a protective effect on HepG2 (human hepatocellular liver carcinoma) cells from H_2O_2-induced cell death, with extracts from Davidson's plum and quandong showing activities superior to blueberries. This indicates cytoprotective ability of plant extracts against exogenous reactive oxygen species.

- None of the extracts applied at high concentrations of 0.5 mg/mL and 1.0 mg/mL were toxic to HT-29 (human colorectal adenocarcinoma) cells. With the exception of quandong extract applied at a concentration of 1.0 mg/mL, none caused direct DNA damage in HT-29 cells.

- All extracts displayed anti-proliferative activity against isolated human cancer cells, with the highest activity displayed by anise myrtle extract. None of the extracts affected the proliferation or induced the death of equivalent normal cells.

- Induction of apoptosis (programmed cell death), which is the preferred way to remove cancer cells from the human body without inflammation, was identified as the mechanism of anti-proliferative activities against human cancer cells, based on flow cytometric analysis and the CBMN cytome assay. Anise myrtle extract among herb extracts, and Davidson's plum extract among fruit extracts displayed the highest pro-apoptotic activities.

- Anise myrtle emerged as an important source of bioavailable phytochemicals that most effectively suppressed the proliferation of, and induced apoptosis of, cancer cells without affecting the growth of normal cells. The presence of ellagic acid at high levels in the anise myrtle extract may be responsible for its enhanced physiological activities. Further laboratory testing would be needed to confirm the active ingredients.

- All extracts effectively inhibited isolated α-glucosidase and pancreatic lipase enzymes, both of which are involved in metabolic syndrome. Anise myrtle, lemon myrtle and Davidson's plum extracts exhibited strong inhibitory activity against α-glucosidase and Tasmannia pepper leaf and quandong extracts exhibited superior activity against pancreatic lipase enzymes. These results

suggest a potential for the application of the evaluated herbs and fruits in the suppression of metabolic syndrome. Human nutritional / clinical trials would be needed to confirm these effects.

Implications for relevant stakeholders

These are the first results describing potential health modulating activities of polyphenolic-rich extracts obtained from commercially grown native Australian herbs and fruits, through their antioxidant and cell-protective capacities, potential chemo-preventative properties and their abilities to inhibit α-glucosidase and pancreatic lipase enzymes. The results of this study should be used to develop further *in vivo* testing priorities and, if confirmed through nutritional/human studies, they can serve the industry to popularise and educate consumers about their products. Subsequently, inclusion of native Australian herbs and fruits in a wide variety of food products would provide new sources of health-promoting compounds. The wider community would benefit from consumption of novel original Australian food products delivering important bioavailable health-promoting compounds.

Introduction

It has been estimated that the human diet contains approximately 20 000 unique compounds, although far fewer than 100 of these are classified as essential for life. Epidemiological, laboratory and clinical studies have consistently revealed that many non-essential components of our diet are capable of modulating health. Moreover, there is a rapidly increasing body of literature demonstrating that many tissues, as well as the resident bacteria colonising the large intestine, convert numerous ingested compounds to metabolites that affect the replication, differentiation and function of various cell types. The identification of the bioactive compounds present in foods and the elucidation of their molecular mechanisms of action attract considerable interest in the biomedical sciences and are prerequisites for the development of new foods that promote health and that might be used to treat diseases.

Phenolic compounds, a large group of phytochemicals accounting for several thousands of molecules, are abundant micronutrients in our diet that play a role in the prevention of degenerative diseases such as cancer, diabetes and cardiovascular diseases. The health effects of polyphenols depend on the amount and variability of consumed compounds. Therefore, identifying new sources of phenolic compounds and their incorporation into a daily diet will serve to maintain good health and is an ongoing task for nutritionists. Recently, native Australian plants have been identified as exceptionally rich sources of phenolic compounds and there exists the opportunity for industry to utilise these untapped resources for the development of uniquely Australian premium health products.

Compositions of phytochemicals in plants differ significantly and this is reflected by the range of health benefits arising from their consumption. Each plant may exert specific physiological activities depending on the type of phytochemicals and their concentration levels. Information about these activities, which can later be utilised in the development of food products, can be obtained via screening plant extracts using a range of assays.

This project was developed to provide the Australian native food industry with reliable information on potential specific health benefits of commercially grown native Australian herbs: Tasmannia pepper leaf (*Tasmannia lanceolata*), anise myrtle (*Syzygium anisatum*), lemon myrtle (*Backhousia citriodora*), and fruits: quandong (*Santalum acuminatum*) and Davidson's plum (*Davidsonia pruriens*), which are among the most common commercially cultivated native Australian edible plants. The results of this study should be used to develop further *in vivo* testing priorities and to confirm the potential health-enhancing properties of the evaluated species through nutritional/human studies.

Objectives

The purpose of the project was to obtain reliable information about the potential health benefits, as identified through *in vitro* studies, arising from consumption of three of the most important commercially grown native Australian herbs: Tasmannia pepper leaf (*Tasmannia lanceolata* R. Br., Winteraceae), anise myrtle (*Syzygium anisatum* Vickery, Craven & Biffen, Myrtaceae), lemon myrtle (*Backhousia citriodora* F.Muell, Myrtaceae); and two native Australian fruits: quandong (*Santalum acuminatum* A.D.C., Santalaceae) and Davidson's plum (*Davidsonia pruriens* F. Muell., Cunoniaceae). This was addressed by the determination of:

- yield, compositional analysis and antioxidant capacities of a polyphenolic-rich extract obtained from each plant source
- cellular- and genome-protective properties of extracts
- anti-proliferative activities of extracts against human cancer cells
- differential killing ability of extracts in human colon and stomach cell models
- bioavailability of plant components to human cells
- inhibitory activities of plant extracts towards key enzymes relevant to diabetes: α-glucosidase; and obesity: pancreatic lipase.

Information obtained within this project complements recently published studies on health-beneficial properties of polyphenolic-rich extracts from Illawarra plum (*Podocarpus elatus* Endl., Podocarpaceae), Kakadu plum (*Terminalia ferdinandiana* Exell, Combretaceae), muntries (*Kunzea pomifera* F. Muell., Myrtaceae) and native currant (*Acrotriche depressa* R.Br., Epacridaceae) (Tan et al. 2011a, 2011b, 2011c).

Methodology

Plant material

Samples were selected and provided by the Australian Native Food Industry Ltd. Dry samples of Tasmannia pepper leaf (*Tasmannia lanceolata* R. Br., Winteraceae) were supplied by the company Diemen Pepper (Birchs Bay, Tasmania, Australia); anise myrtle (*Backhousia citriodora* F.Muell, Myrtaceae) and lemon myrtle (*Syzygium anisatum* Vickery, Craven & Biffen, Myrtaceae) were obtained from Australian Rainforest Products (Lismore, NSW, Australia). Davidson's plum (*Davidsonia pruriens* F. Muell., Cunoniaceae) was supplied by the Australian Rainforest Products (Lismore, NSW, Australia) and samples of quandong (*Santaluam acuminatum* A.D.C. Santalaceae) were supplied by Outback Pride (Reedy Creek, South Australia, Australia). Rabbit eye and highbush blueberries (*Vaccinum* spp., Ericaceae) used as reference samples for fruits were obtained from the Blueberry Farm of Australia (Corrindi, NSW, Australia). The berries were obtained from a very late harvest after most harvesting for fresh-market sales had been completed. Commercially available bay leaf (*Laurus nobilis* L., Lauraceae) from Hoyts Food Industries Pty Ltd (Moorabbin, Victoria, Australia) was included as a reference sample for herbs.

Chemicals

AAPH (2,2-azobis (2-methylpropionamide) dihydrochloride), chlorogenic acid, dimethyl sulfoxide (DMSO), fluorescein, gallic acid, quercetin, Trolox, XAD-16, Eagle's minimum essential medium (EMEM), Folin-Ciocalteau reagent, acetonitrile, ethanol, TPTZ (2,4,6-tripyridyl-*s*-triazine), ABAP (2,2′-azobis (2-amidinopropane) dihydrochloride), DCFH-DA (2′,7′-dichlorofluorescin diacetate) and Hank's balanced salt solution (HBSS) were purchased from Sigma-Aldrich, Inc (Sydney, NSW, Australia). Standards of phenolic compounds were also purchased from Sigma-Aldrich Inc (Sydney, NSW, Australia), with the exception of cyanidin 3-glucoside, which was purchased from Bicolour (Portugal).

Cell cultures

All cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA), except BL13 (human bladder transitional cell carcinoma) cells which were obtained from Dr D Brookes (Brookes et al. 1998), and cultured at 37°C in a humidified 5% CO_2 atmosphere in media containing 10% 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; HT-29 (colorectal adenocarcinoma) was cultured in McCoy's 5a medium; HL-60 (acute promyelocytic leukaemia) was cultured in Iscove's modified Dulbecco's medium (IMDM) containing 20% FBS; CCD-18Co (colon non-transformed) was cultured in EMEM; and Hs 738.St/Int (mixed stomach and intestine non-transformed) was cultured in Dulbecco's modified Eagle's medium (DMEM). Caco-2 monolayers were also cultured on DMEM medium, with the exception that phenol red was excluded. BL13 cells were cultured in RPMI medium (Invitrogen Australia Pty Ltd, Mulgrave, Victoria, Australia) and HepG2 (human hepatocellular liver carcinoma) in EMEM. Experiments were conducted at passages less than 40.

Preparation of lyophilised polyphenolic-rich extracts from plant sources

Herbs and fruits were weighed and ground. A 5-fold volume of acidified methanol (80% methanol, 19% H_2O and 1% acetic acid, v/v/v) was added, stirred for 2 hours at 4°C and centrifuged for 20 minutes at 10 000 rpm at 4°C (Sorvall RC-5B; DuPont, Wilmington, DE, USA). The supernatant was collected and the extraction was repeated twice. The third extraction was carried out overnight. The supernatants from 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 extracts were 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/v). The eluate was collected and evaporated under reduced pressure at 37°C using a rotary evaporator. The purification was repeated and the resulting fraction was dissolved in a minimum volume of 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:

$$\text{Yield (\%)} = (\text{LF} \times 100) / \text{ES}$$

where LF is the weight of lyophilised fraction (g) and ES is the weight of the extracted sample (g).

Analysis of phenolic compounds by a high performance liquid chromatography-diode array detector (HPLC-DAD)

Analysis of phenolic compounds in extracts was carried out using a high performance liquid chromatography (HPLC) system that consisted of two LC-10AD pumps, SPD-M10A diode array detector (DAD), CTO-10AS column oven, DGU-12A degasser, SIL-10AD auto-injector and SCL-10A system controller (Shimadzu Co, Kyoto, Japan) equipped with a 250 x 4.6 mm i.d., 5 μ Luna C18(2) column (Phenomenex, Lane Cove, NSW, Australia). The following solvents in water with a flow rate of 1.0 mL/minute were used: A) 0.5% triflouroacetic acid in water; and B) 95% acetonitrile and 0.5% triflouroacetic acid in water. The elution profile was a linear gradient elution for B of 10% over 10 minutes followed by an increase to 50% over 45 minutes, and then to 80% over 15 minutes. The column was washed with 100% solvent B for 10 minutes. 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 (anthocyanins). The major phenolic compounds were identified through co-chromatography with authentic standards. Hydroxybenzoic acids and flavanols were quantified as gallic acid equivalents (GA Eq), cinnamic acids were quantified as chlorogenic acid equivalents (CHA Eq), flavonols and stilbenes were quantified as rutin equivalents (R Eq) and anthocyanin compounds were quantified as cyanidin 3-glucoside equivalents (C3G Eq). The results are presented as milligram of compound per gram of extract dry weight (for example, mg C3G Eq/g DW).

Total phenolic content (Folin-Ciocalteu assay)

The total phenolic content was determined using the Folin-Ciocalteu method (Singleton and Rossi 1965). Diluted extracts were directly assayed at 600 nm with gallic acid as a standard. Measurements were done in microplates using a microplate reader model Multiscan RC, version 4 (Labsystems, Finland) operated by the DeltaSoft3 program (Elisa Analysis for the Macintosh with interference for the Multiscan Microplate Readers, BioMetallics Inc, 1995). The analysis was conducted in triplicate. Results were expressed as milligrams of gallic acid (gallic acid equivalent, GA Eq) per gram dry weight of the lyophilised powder (mg GA Eq/g DW).

Total flavonoid content

The total flavonoid content assay was performed as described by Michalska and coworkers (2007). Briefly, lyophilised extracts were solubilised in 80% methanol in water (1 mg/mL), followed by an addition of 50 µL of 5% $NaNO_2$ and incubation at room temperature for 6 minutes. Following this, 300 µL of 10% $AlCl_3 \bullet 6H_2O$ solution was added, and followed by a further incubation at room temperature for another 5 minutes. One millilitre of 1M NaOH was then added, mixed using a vortex and absorbance was read at 510 nm using a spectrophotometer. The total flavonoid content of the extracts was calculated based on a +(-) catechin hydrate calibration curve and expressed as +(-) catechin hydrate equivalents per gram of dry weight (mg CH Eq/g DW).

Ferric reducing antioxidant power (FRAP) assay

The assay was conducted according to Benzie and Strain (1996) with minor modifications. Thirty µL of water and 10 µL of fruit extracts (diluted, as needed to obtain a clear reading) were mixed with 200 µL FRAP reagent consisting of ferric chloride and 2,4,6-tripyridyl-*s*-triazine (TPTZ). The absorbance was measured after 4 minutes at 600 nm. The reducing capacity was calculated using the absorbance difference between sample and blank and a further parallel Fe(II) standard solution. Results were expressed as micromoles of Fe^{2+} per 100 mg dry weight of the lyophilised powder (µmol Fe^{2+}/100 mg DW). Measurements (in triplicate) were done in microplates as described for total phenolics.

Oxygen radical absorbance capacity (ORAC) for hydrophilic compounds assay

The ORAC for hydrophilic compounds assay for oxygen radical scavenging capacity was conducted according to Prior et al. (2005) and Ou et al. (2001). The samples (in triplicate) were mixed with a fluorescein (15 nM) solution and a solution of 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH, 360 mM) both in phosphate-buffered saline (PBS, 75 mM, pH 7.0). Both AAPH and PBS buffer were warmed to 37°C prior to use. The fluorescence was recorded until it reached zero (excitation wavelength 495 nm, emission wavelength 515 nm) in a Varian Cary Eclipse Fluorescence Spectrophotometer (Varian Australia Pty Ltd) equipped with an automatic thermostatic autocell holder at 37°C. A calibration curve was constructed daily by plotting the calculated differences of area under the fluorescein decay curve between the blank and the sample for a series of standards of Trolox solutions in the range of 6.25–75 µg/L. The results were expressed as µmol Trolox equivalents per gram dry weight of the lyophilised powder (µmol Trolox Eq/g DW).

Cellular antioxidant activity (CAA) assay

The assessment of CAA was determined according to Tan et al. (2011c) and Wolfe and Liu (2007). Briefly, $1x10^5$/mL of HepG2 (human hepatocellular liver carcinoma) cells were plated in 96-well microplates and incubated for 24 hours. Next, the media were removed and the cells were washed using PBS. The cells were then treated with different concentrations of plant extracts (10 µL in PBS) added to 80 µL of PBS, followed by addition of 10 µL 2',7'-dichlorfluorescein-diacetate (Sigma-Aldrich Inc, Sydney,NSW, Australia) (250 µM in PBS), and incubated for 1 hour. Subsequently, the cells were washed using 100 µL of PBS, and 100 µL of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) in Hank's Balanced Salt Solution (600 µM) was added. The fluorescence was measured at 485 nm excitation and 538 nm emission wavelengths every 5 minutes over 1 hour (POLARstar Omega - Fluorescence polarization microplate reader, BMG Labtech, Germany). The final fluorescence values were corrected for the blank sample readings, and a time versus fluorescence graph was plotted. A quercetin standard was used to express the results as quercetin equivalents per gram of dry weight of extracts.

Cellular protection against H_2O_2-induced cell death

Cellular protection against hydrogen peroxide (H_2O_2)-induced death of HepG2 cells was determined via a cell viability assay using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Invitrogen Australia Pty Ltd, Mulgrave, Victoria, Australia) assay. Initially, cells ($5x10^5$/mL) were incubated for 24 hours at 37°C in 96-well clear-walled microplates (Thermo Fisher Scientific), before treatment with a range of concentrations of purified polyphenolic extracts for 23 hours, followed by the addition of H_2O_2 (20 mM) for a further 1 hour. Wells were then washed with PBS, 5 mg/mL MTT solution added and the microplate further incubated for 4 hours. The microplate was then drained and the resulting MTT formazan product was dissolved in DMSO. The plate was shaken and absorbance measured at 600 nm using a spectrophotometer (Labsystems Multiskan MS; Thermo Fisher Scientific).

Cytokinesis-block micronucleus (CBMN) cytome assay

The CBMN cytome 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 colorectal adenocarcinoma cells ($5x10^5$/mL) were incubated for 24 hours at 37°C in 24-well plates (Thermo Fisher Scientific). The medium was replaced and cells treated with 0.5 and 1 mg/mL concentrations of purified polyphenolic extract for 24 hours. The treatment was removed and medium replaced with 4.5 µg/mL cytochalasin B (Sigma-Aldrich Inc, Sydney, New South Wales) in medium for 24 hours to block the cells from entering cytokinesis. Cells were then harvested using Tryple Express (Sigma-Aldrich Inc, Sydney, NSW, Australia) and applied to a microscope slide (3 inch x 1 inch) using a cytospin centrifuge (Cytospin 3; Shandon Scientific Limited, Cheshire, United Kingdom). Cells were fixed and stained with Diff-Quik stains (Lab Aids, Narrabeen, NSW, Australia), air-dried and cover-slipped with Depex medium (Merck, Whitehouse Station, NJ, 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 1000x magnification using an oil immersion lens, with sufficient light and precise focus to ensure clear vision of each cell observed.

Scoring was based on the previously described CBMN Cyt scoring criteria (Fenech 2007). The biomarkers scored included frequency of binucleated (BN) cells with micronuclei (MNed BN), with nucleoplasmic bridges (NPB), with nuclear buds (NBud), frequency of necrotic (Necro) and apoptotic (Apop) cells. The nuclear division index was calculated from the ratio of mono-, bi- and multinucleated cells (Eastmond and 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 micronuclei (MN), binuclear cells (BN), nucleoplasmic bridges (NPB) and nuclear buds (BNud). Each treatment concentration and control was assessed six times.

MTT colorimetric cell viability assay

Cell sensitivity of AGS, HT-29, CCD-18Co and Hs 738.St/Int to native Australian plants extracts was determined via cell viability using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Invitrogen Australia Pty Ltd, Mulgrave, Victoria, Australia). Initially, cells (5x105/ml) were incubated for 24 h at 37 °C in 96-well clear-walled microplates (Thermo Fisher Scientific), before treatment with a range of concentrations of purified polyphenolic extracts for 23 h. Subsequently, the medium (containing sample) was removed and wells were washed twice with warm (37°C) PBS to remove any traces of samples; 5 mg/ml MTT solution was then added and the microplate further incubated for 4 h. The microplate was then drained and the resulting MTT formazan product dissolved in DMSO. The plate was shaken and absorbance measured at 600 nm using a spectrophotometer (Labsystems Multiskan MS; Thermo Fisher Scientific). At least four independent measurements were performed for each treatment and the experiments were conducted twice.

Flow cytometry analysis

The analysis has been conducted according to Tan et al. (2011c). For the time-course experiment, HL-60 cells ($5x10^5$/mL) were plated in $25cm^2$ culture flasks and treated with 0.40 mg/mL purified polyphenolic extract for 3, 12 and 24 hours, 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 Australia Pty Ltd, Mulgrave, Victoria, Australia) for 15 minutes 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 10 000 events were acquired for each measurement and the cells were properly gated for analysis. Based on the time-course data, a single time point (6 hours) was chosen and the induction of apoptosis was analysed with a range of concentrations. HL-60 cells ($5x10^5$/mL) were plated in $25cm^2$ culture flasks and treated with purified

polyphenolic extract at concentrations of 0.4, 0.8 and 1.6 mg/mL for 6 hours, with untreated cells as a control. Following the required incubation time, cells were harvested, stained and analysed, as previously stated.

Bioavailability assay

Caco-2 cells were prepared according to Ferruzzi and coworkers (2002) and were grown and differentiated in 6-well plastic dishes until full confluency. In order to starve the cells, 48 hours prior to the experiment they were exposed to basal DMEM medium (without addition of bovine serum and phenol red). Prior to initiating experiments, monolayers were washed twice with 1mL basal DMEM medium and fresh DMEM phenol-red-free medium, enriched with plant extract at a concentration of 1 mg/mL in each well. The cells were incubated for 4 hours in a humidified atmosphere of air/CO_2 (95:5%) at 37°C. After 4-hours incubation the medium was removed and monolayers were washed twice with ice-cold PBS. The cells were collected in 1mL fresh PBS and centrifuged (5 minutes, 2000 rpm at 4°C). The supernatant was removed and the cells were extracted by adding 200 µL of acidified methanol (5% acetic acid/95% methanol), followed by vortexing and sonication for 1 minute. The cells were left on ice for 30 minutes to facilitate the extraction process. Subsequently, samples were centrifuged (10 minutes at 10 000 rpm; Eppendorf 5415D Centrifuge; Eppendorf-Netheler-Hinz, Hamburg, Germany) with the supernatant collected. The extraction was repeated one more time resulting in 400 µL of the total extract volume. The extract was placed in HPLC vials and immediately injected into the HPLC for analysis as described above.

Alpha-glucosidase inhibition assay

The α-glucosidase inhibition was determined as described by Ikarashi and coworkers (2010) using sucrose as a substrate with slight modifications. An α-glucosidase enzyme solution was prepared by dissolving 100 mg of intestinal acetone powders from rat (Sigma-Aldrich Inc, Sydney, NSW, Australia) in 1 mL of 0.1 M maleate buffer (pH 6) and homogenised using an ultrasonicator for 6 minutes on a 30 second rest cycle. The enzyme solution was centrifuged at 3000 g for 30 minutes and the supernatant was diluted to 1:2 (v/v) using the buffer solution. Sample solutions of 20 µL were mixed with 20 µL of 2% sucrose (w/v) in maleate buffer. The enzymatic reaction was initiated by adding 20 µL of enzyme solution to the mixture and incubated at 37°C for 60 minutes. The enzymatic reaction was terminated by heating at 100°C for 10 minutes. Then, 20 µL of sample mixture was mixed with 3mL of colour reagent (Glucose CII-Test Wako; Wako Pure Chemical Industries, Osaka, Japan) at 37°C for 5 minutes and the absorbance was measured at 505 nm. Negative controls were prepared as described by replacing the sample solution with the buffer solution. Control and sample blanks were prepared by replacing the enzyme and sucrose by the buffer solution, respectively. The relative α-glucosidase inhibition was calculated using the following formula:

$$\%\text{Inhibition} = \{(A_{CB}–A_C) – (A_{SB}–A_S)\} / (A_{CB} –A_C) \text{ x } 100$$

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

Lipase inhibition assay

The lipase inhibitory activity was assayed as described by Shimura and coworkers (1992) using 4-methylumbelliferyl oleate as substrate, except for the porcine pancreatic lipase (Sigma type II) which was prepared using a concentration of 0.085g/mL. The relative lipase inhibition activity was calculated using the following formula:

$$\%\text{Inhibition} = (1–(F_S–F_{SB}) / (F_C–F_{CB})) \text{ x}100$$

where F_S and F_C are the values of samples and negative control, respectively, measured fluorometrically at an emission wavelength of 450 nm and excitation of 320 nm, whereas F_{SB} and F_{CB} were the fluorescence readings of sample and control blank, respectively.

Statistical analysis

The mean of results and standard deviations (SD) were calculated based on at least three independent evaluations (n=3). One way ANOVA followed by Tukey's post-hoc test were performed to assess differences between the samples at the level of $p<0.05$. All IC_{50} values were calculated from the corresponding dose inhibition curve according to their best fit shapes based on at least four reaction points using Microsoft Excel. Statistical correlation analyses were performed using Graphpad Prism 5 (Graphpad Software, CA, USA).

Results

Characteristics of purified phenolic-rich extracts obtained from native Australian fruits and herbs

Yield, total phenolic and total flavonoid content

Recent reports described a high antioxidant capacity of selected edible native Australian plants and phenolic compounds were identified as the major source of this capacity (Konczak et al. 2010a, 2010b; Konczak et al. 2009). Pure phenolic compounds evaluated individually, as well as crude phenolic-rich extracts obtained from plants are powerful antioxidants, responsible for a number of health properties of plant-originating foods (Noroozi et al. 1998; Liu 2004; Bagchi et al. 1999). Polyphenolic-rich extracts of four native Australian fruits: Kakadu plum, Illawarra plum, muntries and native currant exhibited enhanced antioxidant and cell-protective activities, induced apoptosis of cancer cells and enhanced immune responses (Tan et al. 2011a, 2011b, 2011c), suggesting strong capabilities of these native plants to modulate health. Therefore this study was designed to evaluate health attributes of hydrophilic extracts obtained from commercially grown native Australian herbs and fruits of particular importance to the Australian native food industry. Hydrophilic (water soluble) compounds, extracted from three herbs, anise myrtle, lemon myrtle and Tasmannia pepper leaf, and two fruits, quandong and Davidson's plum, were purified using open column chromatography in order to capture and concentrate phenolic compounds. In order to compare the properties of native Australian herbs and fruits to other well-known edible plants, reference samples were also incorporated: bay leaf as a reference sample for herbs and blueberries (rabbit eye blueberry and/or southern highbush blueberry) as reference sample/s for fruits.

The yields of alcohol-based hydrophilic extracts are presented in Table 1. Among herbs, the highest extraction yield was generated from Tasmannia pepper leaf (162.6 per cent that of the reference sample bay leaf). The extraction yields of anise myrtle and lemon myrtle were comparable to bay leaf.

The level of total phenolics in Tasmannia pepper leaf extract was 3-fold that of a bay leaf and levels in anise myrtle and lemon myrtle extracts were respectively, 2.3- and 2.0-fold that of bay leaf extract. The level of total phenolic compounds in concentrated polyphenolic-rich extract produced from quandong was comparable to that in blueberry extracts. Davidson's plum extract contained the highest level of phenolics, superior to all evaluated extracts (Table 1). In all herb and fruit extracts generated within this study unknown compounds originating from the respective plants were also present at various levels, especially in quandong (45.6 per cent), lemon myrtle (33.9 per cent), anise myrtle (27.1 per cent). These additional compounds could affect the potential physiological activities of phenolics in either a synergistic, additive or antagonistic manner.

Flavonoids, a group of polyphenolic compounds that are ubiquitous in plants and present in the human diet, are most commonly known for their enhanced antioxidant properties, which are significantly higher than the antioxidant capacity of vitamins C and E and display an array of health-promoting properties (Noroozi et al. 1998; Bagchi et al. 1999). Among the herbs tested in this study, the highest level of flavonoids were found in Tasmannia pepper leaf extract (1.6-fold that of bay leaf), followed by anise myrtle extract (1.3-fold that of bay leaf). Lemon myrtle extract contained less flavonoids then bay leaf (Table 1). Davidson's plum extract contained the highest level of flavonoids among all samples evaluated, which was 2-fold that of blueberry extracts. The level of flavonoids in quandong was also higher than that in blueberry extract.

Table 1 **Extraction yield, total phenolics, total flavonoids and antioxidant capacities of extracts obtained from native Australian herbs and fruits and reference samples: bay leaf (reference sample for herbs) and blueberries (reference samples for fruits).**

Source of polyphenolic-rich extract	Yield (%)	Total phenolics (mg GA Eq/g DW)	Total flavonoids (mg CH Eq/g DW)	TRC (μmol Fe^{+2}/g DW)	ORAC (μmol Trolox E/g DW)
Herbs					
Anise myrtle	4.93	728.9±25.8[b]	213.96±13.4[b]	8054±15.2[a]	7564±1272[a]
Lemon myrtle	5.74	660.5±58.8[b]	134.25±21.2[c]	5025±10.9[b]	4136±594[b]
Tasmannia pepper leaf	8.52	911.9±57.8[a]	255.96±3.3[a]	4444±12.2[c]	12789±996[a]
Bay leaf	5.24	319.3±5.80[c]	161.54±3.6[c]	3040±17.5[d]	4945±715[b]
Fruits					
Quandong	10.15	543.3±17.5[b]	246.58±4.5[b]	3225±13.3[d]	6028.4±953[a]
Davidson's plum	10.10	949.1±50.5[a]	352.53±3.1[a]	9258±16.1[a]	8791.5±370[a]
Rabbit eye blueberry	N/A	504.3±29.0[b]	187.43±1.8[c]	6098± 5.3[c]	3931.5±196[b]
Southern highbush blueberry	N/A	550.5±19.0[b]	172.49±7.3[d]	4811±26.7[b]	3266.5±233[b]

GA Eq: gallic acid equivalent; CH Eq: catechin hydrate equivalent; Q Eq: quercetin equivalent; TRC: total reducing capacity; ORAC: oxygen radical absorbance capacity; N/A: data not available.

Antioxidant capacity of purified polyphenolic-rich extracts

The levels of antioxidant capacity of plant extracts were evaluated in two reagent-based assays (FRAP and ORAC) and one cell-based assay (CAA). The total reducing capacity (TRC) was evaluated with the help of the ferric reducing antioxidant power (FRAP) assay, which measures the total redox-activity of plant extract (Halvorsen et al. 2006). The radical scavenging capacity was evaluated in the oxygen radical absorbance capacity (ORAC) assay that measures an ability to scavenge oxygen free radicals, which are the main type of free radicals generated in the human body during metabolic processes (Prior et al. 2005).

Among the herbs examined in this study, anise myrtle extract exhibited the highest TRC, and was followed by lemon myrtle and Tasmannia pepper leaf extracts. All native herb extracts had higher TRC than the reference sample bay leaf (Table 1). ORAC results showed that Tasmannia pepper leaf extract was nearly twice as efficient in scavenging oxygen free radicals as anise myrtle and three times more efficient than lemon myrtle (Table 1). Generally, in both reagent-based assays native Australian herbs displayed superior antioxidant capacities to the bay leaf extract. Similar differences among these three plant sources were reported previously for their crude alcoholic extracts (Konczak et al. 2010a; Konczak et al. 2009). The present result indicates that concentrated phenolic-rich extracts generated within this study represent the qualities of crude extracts from the respective plants.

Among fruits, Davidson's plum extract exhibited superior antioxidant capacity, with the oxygen radical scavenging ability 1.5-fold that of quandong and 2.5-fold that of the blueberry references and the TRC 3-fold that of quandong and 2-fold that of the southern highbush blueberry reference. A different result has been reported earlier for crude extracts from the same fruits, where the oxygen radical scavenging capacity of Davidson's plum was only 50 per cent of that of quandong and the TRC was 1.8-fold that of quandong (Konczak et al. 2010b; Konczak et al. 2009).

The superior antioxidant activity of Davidson's plum purified phenolic-rich extract obtained within this study clearly indicates that crude extract of Davidson's plum contained a vast amount of compounds that significantly contributed to the dry weight, but did not contribute to antioxidant capacity (such as polysaccharides), and these compounds have been removed during the purification process.

Compositional analysis of purified polyphenolic-rich extracts

The major compounds detected in polyphenolic-rich extracts obtained from herbs and fruits were phenolic acids (ellagic acid, chlorogenic acid, *p*-coumaric acid) and flavonoids (quercetin, myricetin, rutin, hesperedin, anthocyanins) (Table 2). Ellagic acid has been identified in two Australian herbs: anise myrtle and lemon myrtle; and one Australian fruit: Davidson's plum (Table 2). The HPLC-DAD chromatogram of extracts from these plants monitored at 280 nm wavelength had a characteristic 'hemp' or elevated baseline (Appendix Figure A1A). Following acid hydrolysis of these extracts, elevated levels of ellagic acid were detected (250 nm wavelength; Appendix Figure A1B). Before the hydrolysis experiment, the large peak representing ellagic acid was not present. Therefore, these results indicate the presence of ellagitannins in the evaluated sources.

Anise myrtle and lemon myrtle belong to the Myrtaceae family. The presence of ellagic acid and polymeric compounds in the leaves of anise myrtle, and, at a lower level, in lemon myrtle (Table 2) is consistent with the Myrtales, an order especially characterised by ellagitannins (Bate-Smith 1962).

In agreement, polymeric phenolic compounds were detected in a number of spices and condiments belonging to the Myrtaceae family, such as clove (*Syzygium aromaticum* (L.) Merrill & Perry) (Shan et al. 2005), *Syzyguim glomeratum, S. venosum* and *S. mauritanum* (Neergheen et al. 2006) and Jamaica pepper berries (*Pimenta dioica* (L.) Merr) (Nakatani 2000). In addition to the polymeric phenolic compounds found in anise and lemon myrtle, the following flavonoids were also detected: quercetin, myricetin and hesperetin (Table 2). Gornall and coworkers (1979) reported that beside polymeric phenolics, flavonols (especially myricetin) and their O-methylderivatives are the typical

feature of the Myrtaceae family. Similarly, the leaves of *Marlierea grandiflora* Berg, collected at Espirito Santo state of southeast Brazil, contained quercetin, quercitrin, myricetin 3-rhamnoside, ellagic acid and 3-O-mythylellagic acid (Amaral et al. 2001).

Table 2 Major phenolic compounds identified in extracts of herbs and fruits (mg/g DW).

Compound	Anise myrtle	Lemon myrtle	Tasmannia pepper leaf	Davidson's plum	Quandong
Ellagic acid*	666.9±109.1	461.9±21.1		181.6±2.2	
p-coumaric acid			51.5±7.4		
Chlorogenic acid	8.05±1.4		289.3±14.6		258.7±7.2
Quercetin	29.1±4.9	31.7±4.4	152.9±2.2	6.08±0.4	9.9±0.6
Myricetin	17.6±3.6	35.3±6.8		9.87±0.6	
Hesperetin		160.6±35.8			
Rutin			119.4±8.6	5.91±0.4	
Cyanidin 3-glucoside			0.05±0.01		1.73±0.02
Cyanidin 3-rutinoside			0.02±0.001		
Delphinidin sambubioside				9.17±0.05	
Cyanidin sambubioside				4.3±0.2	
Pelargonidin sambubioside				12.7±0.6	
Peonidin sambubioside				13.0±0.6	
Malvidin sambubioside				7.22±0.4	

*Ellagic acid was identified and calculated as ellagic acid equivalent (mg EA Eq/g DW) following hydrolysis.

We have identified ellagic acid in Davidson's plum fruit. Bate-Smith (1977) has evaluated dry herbarium specimens (leaves) of 46 species of the Cunoniaceae family from Australia and South Africa, including Davidson's plum dry leaf. Myricetin has been reported as the major flavonol of Davidson's plum dry leaf and was followed by quercetin. However, ellagic acid was not detected. In the alcoholic extract of Davidson's plum fruit polymeric compounds (tannins) were detected. Following acid hydrolysis, a high level of ellagic acid was identified (Table 2). In a more recent study, Fogliani and coworkers (2005) evaluated antimicrobial properties and scavengers of superoxide anions of fifty species belonging to the family Cunoniaceae from New Caledonia. *Cunonia macrophylla* leaf extract demonstrated an ability to inhibit the growth of bacteria and fungi. Six components, including ellagic acid, were identified as the biologically active compounds obtained after hydrolysis.

In addition to ellagic acid, quercetin and myricetin, Davidson's plum extract contained five anthocyanins (cyanidin, peonidin, delphinidin, pelargonidin and malvidin, Table 2) to which the fruit owes the crimson colour of the flesh and skin. Due to the presence of anthocyanins, Davidson's plum juice/extract can successfully be used as a natural food colour (Busch Jensen et al. 2011). The presence of ellagic acid, the antimicrobial and antifungal properties of which have been reported (Fogliani et al.

2005), suggests potential for future studies towards the application of Davidson's plum as a source of natural food ingredients with both antimicrobial and colouring properties.

Phenolic compounds detected in native Australian plant extracts in this study are highly bioactive molecules, with ellagic acid and ellagitannins reported as strong anti-inflammatory and chemo-preventative agents (Heber 2008; Mandal and Stoner 1990; Daniel and Stoner 1991; Lesca 1983). Ellagitannins are large molecules and after consumption are not absorbed intact into the blood stream but are hydrolysed to ellagic acid. They are also metabolised by gut flora into urolithins which are conjugated in the liver and excreted in the urine. The urolithins are also bioactive and are reported to inhibit prostate cancer cell growth (Heber 2008). Ellagitannins and ellagic acid are responsible for the health properties of pomegranates, raspberries, blackberries, strawberries, walnuts and other plant foods. They display antimicrobial and antiviral properties. Phenolic compounds, including phenolic and hydroxicinnamic acids and flavonoids are efficient antioxidants. The flavonoids: quercetin, catechin and kaempferol are known powerful antioxidants (Noroozi et al. 1998) and these were also detected in the phenolic-rich extracts evaluated within this study. The composition of plant extracts indicates potential physiological activities and these are subsequently examined within the present study.

Cellular and DNA protection by polyphenolic-rich extracts

Antioxidant activities of plant extracts within a live cell

Both reagent-based assays (FRAP and ORAC) employed in this study provided information about the antioxidant capacities of compounds as determined through a chemical reaction. The cellular antioxidant activity (CAA) assay provided complementary information about the efficiency of phytochemicals as antioxidants at the cellular level, and as such is more relevant when considering the effect on humans. The final result of this assay depends on uptake, distribution and metabolism of the antioxidant compounds in a live cell. This information cannot be obtained through reagent-based antioxidant activity assays. According to Wolfe and Liu (2007), in comparison to animal models, the CAA is a cost-effective and fast way to obtain important information on the efficiency of antioxidants within a live cell.

The primary source of antioxidants entering a live cell and consequently scavenging free radicals, is food. Antioxidants are required in order to reduce the concentration of constantly produced free radicals generated in normal metabolic processes. High concentrations of free radicals induce oxidative stress within a cell, followed by oxidative damage to biomolecules such as deoxyribonucleic acid (DNA), ribonucleic acids (RNAs), proteins and lipids (Halliwell 2007). These damages initiate chronic inflammation and are the major factors in the development of chronic diseases such as cancer, diabetes, Alzheimer's diesease, cataracts, cardiovascular diseases, and age-related functional decline.

Table 3 Cellular antioxidant activity (CAA) of polyphenolic-rich extracts obtained from Australian native herbs and fruits and reference samples.

Plant source	**CAA** (µmol Q Eq/g DW)
Herbs	
Anise myrtle	106.6±2.6[b]
Lemon myrtle	88.0±2.0[c]
Tasmannia pepper leaf	154.6±1.2[a]
Bay leaf	44.6±2.1[d]
Fruits	
Quandong	95.8±9.4[b]
Davidson's plum	88.4±1.5[b]
Rabbit eye blueberry	412.9±1.1[a]
Southern highbush blueberry	348.7±0.4[a]

Note: bay leaf (reference sample for herbs) and blueberries (reference samples for fruits); evaluated in human hepatocellular liver carcinoma cells (HepG2 cells).

Among herb extracts, superior CAA was displayed by Tasmannia pepper leaf extract, followed by anise myrtle extract (Table 3). The CAA of lemon myrtle, quandong and Davidson's plum was significantly lower than that of reference samples of blueberries, higher than that of bay leaf and similar to that of Kakadu plum (71.5±11.3 µmol Q Eq/g DW) (Tan et al. 2011c). The prerequisite of cellular antioxidant activity of phytochemicals is their uptake by a cell. Similar to blueberries, Tasmannia pepper leaf extract is comprised of monomers of flavonoids and hydroxycinnamic acids; relatively small molecules which cross the cell wall more efficiently than large polymeric compounds present in other extracts. Polyphenolic-rich extracts of anise myrtle, lemon myrtle and Davidson's plum comprise of mixtures of monomeric and polymeric phenolic compounds. With regards to the polymeric compounds, after consumption their uptake by human cells will depend on a hydrolysis in human digestion system and/or their degradation by gut flora (Heber 2008). Therefore it can be expected that the CAA of plant extracts comprising of polymeric compounds within a live body may be higher than suggested by this study.

In conclusion, the results of the CAA assay are indicative of the function of unique compounds found within native Australian herbs and fruits in entering and consequently acting as antioxidants within a live cell.

Cellular protection from H_2O_2-induced cell death

Hydrogen peroxide (H_2O_2) is an important reactive oxygen species generated within the human body. In the experimental setting evaluated below, H_2O_2 induces cell death. HepG2 (hepatocellular carcinoma human cells) were treated with plant extracts, applied at low concentration, which did not affect the proliferation of HepG2 cells over 23 hours. Subsequently, the cells were challenged by an addition of 20 mmol H_2O_2 to the culture medium. In the negative control (no plant extract added) the addition of H_2O_2 to the culture medium reduced the percentage of live cells by approximately 40 per cent (Figure 1, black bar). The positive control (Figure 1, white bar) represents cell growth without any treatment.

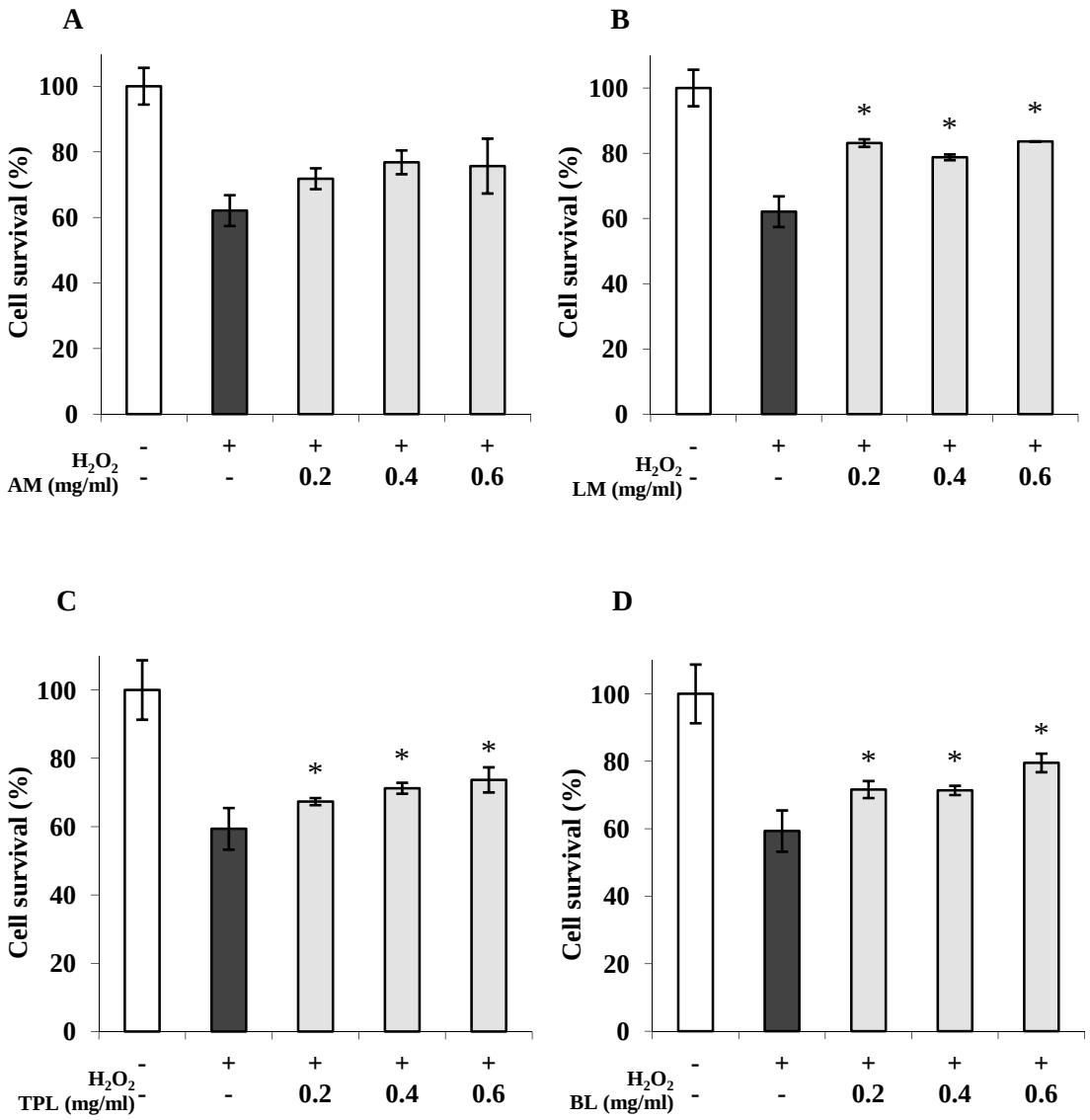


Figure 1 Cellular protection of HepG2 cells by polyphenolic-rich extracts obtained from Australian native herbs (AM: anise myrtle; LM: lemon myrtle; TP: Tasmannia pepper leaf) and reference sample, bay leaf (BL).

First column: no H_2O_2; second column: 20 mmol H_2O_2; all other columns represent treatments with H_2O_2 and plant extracts. The data represents the mean ± standard deviation of six independent events. An asterisk indicates significant difference with H_2O_2 only treated cell ($p<0.05$).

Pre-treatment of cells with plant extracts, applied at concentrations from 0.2 to 0.6 mg/mL prior to H_2O_2 challenge, significantly reduced the cytotoxic effect of H_2O_2, exhibited by higher cell survival rate (Figure 1). At the lowest concentration of 0.2 mg/mL lemon myrtle extract was the most effective with a 50 per cent reduction of cell death caused by H_2O_2. Increasing the concentrations of anise myrtle, lemon myrtle and bay leaf extracts to 0.6 mg/mL didn't enhance their cell-protective effects. Only in the case of Tasmannia pepper leaf extract was a dose-dependent effect observed.

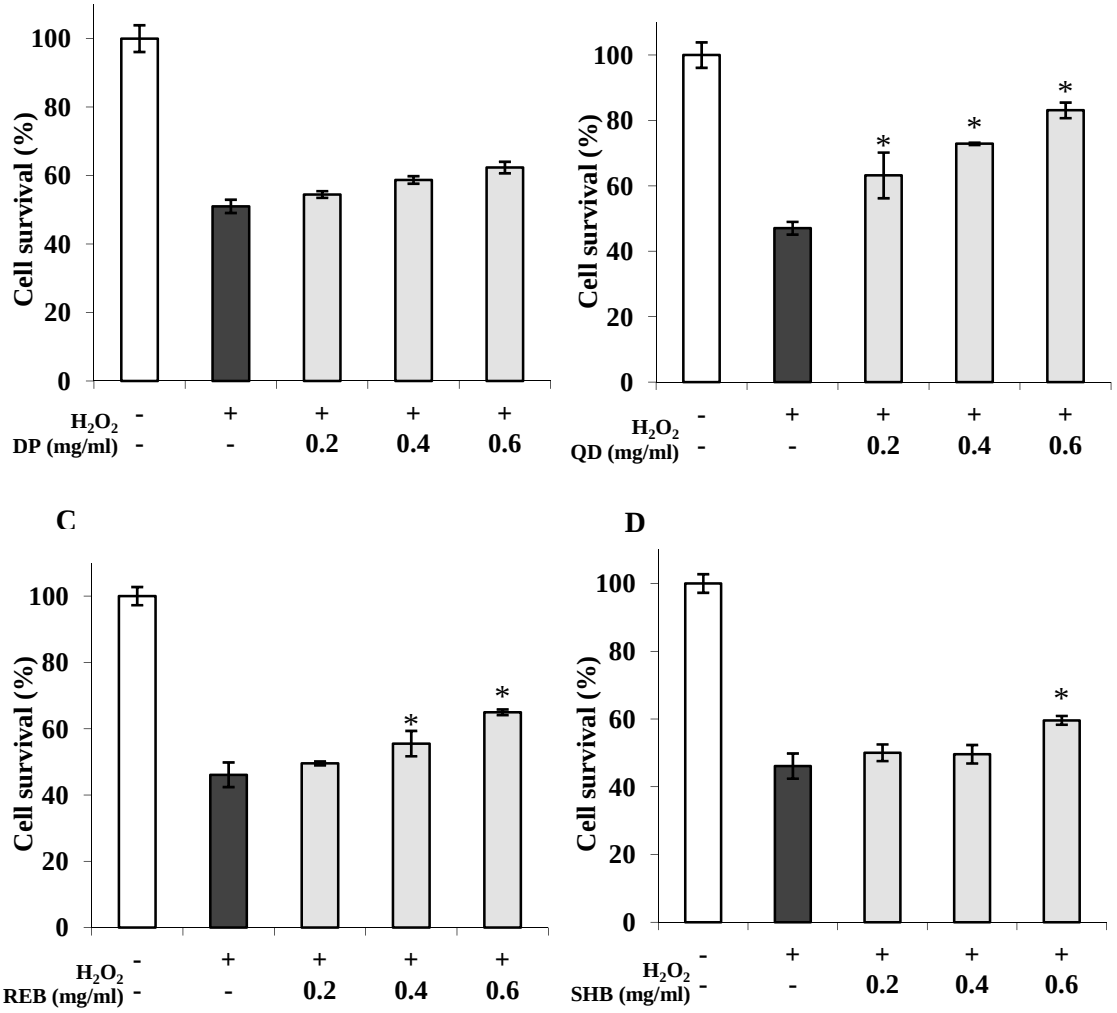


Figure 2 Cellular protection of HepG2 cells by polyphenolic-rich extracts obtained from Australian native fruits (DP: Davidson's plum; QD: quandong) and reference samples of blueberry (REB: rabbit eye blueberry; SHB: southern highbush blueberry).

First column: no H_2O_2; second column: 20 mmol H_2O_2; all other columns represent treatments with plant extracts at concentrations given for 23 hr followed by 1hr of H_2O_2 (20 mol) treatment. The data represents the mean ± standard deviation of six independent events. An asterisk indicates significant difference with H_2O_2 only treated cell ($p<0.05$).

Among the fruits tested, quandong's extract most efficiently reduced the H_2O_2 damage in a dose-dependent manner, while the effect of Davidson's plum extract was lower. The presence of polymeric compounds in Davidson's plum extract might be responsible for this effect. Cellular protection by native fruits' extracts was superior (quandong) or similar (Davidson's plum) to that of the reference samples of blueberries (Figure 2).

This experimental data suggests that phenolic-rich extracts from both Australian native herbs and fruits effectively reduce cell damage caused by reactive oxygen species.

Evaluation of direct DNA damage by polyphenolic-rich extracts

The Cytokinesis block micronucleus (CBMN) cytome assay is a well-established and comprehensive approach for measuring DNA damage, cytostasis and cytotoxicity (Fenech 2007). DNA damage events are scored specifically in once-divided binucleated (BN) cells and 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. Cytostatic effects are measured via the proportion of mono-, bi- and multinucleated cells and cytotoxicity via necrotic and/or apoptotic cell ratios. MNi originate from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division. The cytokinesis-block micronucleus (CBMN) assay is the preferred method for measuring MNi in cultured human and/or mammalian cells because scoring is specifically restricted to once-divided BN cells, which are the cells that can express MNi. In this study, HT-29 colorectal adenocarcinoma cells were exposed to various concentrations of purified polyphenolic extracts (0.5 and 1.0 mg/mL) and the frequency of the various cytome biomarkers was determined (Table 4).

Following the treatment with all evaluated polyphenolic-rich plant extracts, the number of apoptotic HT-29 cells increased 5 to 7-fold in comparison to the control group. Importantly, the level of necrotic cells did not increase indicating that the extracts at the applied concentrations were not cytotoxic to HT-29 cell. In the case of the Australian herbs, induction of apoptosis was concomitant with the reduction of binucleate cell frequency. Therefore, it can be speculated that cell damage leading to apoptosis (form of cell death in which a programmed sequence of events leads to the elimination of cells without releasing harmful substances into the surrounding area) may potentially occur during cytokinesis or there may be an inhibition of cell cycle progression. Due to the reduction of binucleate cells, the nuclear division index decreased, which suggests potential cytostatic effect of herbs on HT-29 cells. This effect was not visible after the treatment with fruit extracts. Davidson's plum extract at concentration of 1.0 mg/mL, induced an increase in BN cell numbers, which indicates an impact on the mitotic status of HT-29 cells.

In determining the frequency of the various biomarkers of DNA damage (MNi, NPBs and NBUD), with the exception of quandong extract, which applied at the concentration of 1.0 mg/mL increased the number of binucleated cells with micronuclei, no significant differences were obtained when compared to the control group which is suggestive that, with the exception of quandong (1.0 mg/mL), none of the applied treatments induced increased rates of DNA damage within HT-29 cells.

The results obtained in this set of experiments exhibited cellular- and DNA-protective capacities of Australian native herb and fruit polyphenolic extracts. The compounds present in native herbs and fruits successfully enter a live cell and exhibit antioxidant activity within a cell, protecting cell components from endogenous free radicals. They also exhibit cellular-protective activities against cell death from externally applied (exogenous) oxygen free radicals. With the exception of quandong applied at concentration of 1.0 mg/mL, plant extracts did not cause any direct damage to the DNA of cells. None of the plant extract at the applied concentrations was toxic to the HT-29 cells. All extracts applied at concentrations of 0.5 and 1.0 mg/mL induced apoptosis of HT-29 cells, which suggests their potential chemo-preventative properties.

Table 4 Frequency of HT-29 CBMN Cytome biomarkers following treatment with various concentrations of purified polyphenolic-rich Australian native plant extracts.

	Frequency of cell type[a]								
	Mononuclear	**Binuclear**	**Multi**	**Apoptotic**	**Necrotic**	**NDI**	**BN with MN**	**BN with NPB**	**BN with 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	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	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	4.9 ± 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	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	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	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	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	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	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	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*	9.6 ± 1.5	11.3 ± 0.9

[a] The results represent the mean ± SD per 500 cells of at least two counted slides. An asterisk (*) represents significant difference ($p<0.05$) in particular cell type between sample-treated and control slides.

CBMN: cytokinesis-block micronucleus; AM: anise myrtle; LM: lemon myrtle; TP: Tasmannia pepper leaf; DP: Davidson's plum; QD: Quandong; NDI: nuclear division index; MN: micronuclei; BN: binuclear cell; NPB: nucleoplasmic bridges; NBud: nuclear buds.

Potential chemo-preventative properties of polyphenolic-rich extracts

Anti-proliferative activities against human cancer cells and differential killing ability of extracts

The ability of plant extracts to suppress the proliferation of human cancer cells may be indicative of their potential chemo-preventative properties. Within this study, we have evaluated cell sensitivity of the cancer cell lines: HT-29 (colorectal adenocarcinoma) and AGS (gastric adenocarcinoma); and the respective normal cells: CCD-18Co (colon non-transformed) and Hs 738.St/Int (mixed stomach and intestine non-transformed) as representatives of the digestive system, where cells are directly exposed to food components and their metabolites. Additionally, the sensitivity of BL13 (human bladder transitional cell carcinoma) and HepG2 (human hepatocellular liver carcinoma) cells to plant extracts were evaluated. The cells were exposed to a range of concentrations of plant extracts from 0.0 to 2.0 mg/mL over 24 hours and the effect on cell proliferation was monitored.

Each of the polyphenolic-rich extracts demonstrated a reduction of cancer cell proliferation (HT-29, AGS, BL 13 and and HepG2) in a dose-dependent manner. The effects of anise myrtle, lemon myrtle and Tasmannia pepper leaf extracts were more pronounced than the effects of quandong and Davidson's plum extracts. Anise myrtle exhibited the lowest IC_{50} values (concentration of plant extract to reduce cell proliferation in 50 per cent) for all the cancer cell lines tested, with IC_{50} values from 0.38±0.02 for HepG2 cells to 0.76±0.03 for HT-29 cells (Table 5), while Tasmannia pepper leaf extract also showed strong anti-proliferative activity against BL13 cells (0.56±0.10). It is highly possible that the most efficient anti-proliferative effect of anise myrtle polyphenolic-rich extract against all cancer cell lines occurs due to the presence of high levels of ellagitannins and ellagic acid (Table 2). The chemo-preventative effect of ellagic acid has been well known with the suppression of esophagal tumorigenesis in rats (Heber 2008; Mandal and Stoner 1990) and reduction of neoplasia in mice (Daniel and Stoner 1991) being well demonstrated. Interestingly, the anti-proliferative activity of bay leaf extract used within this study as a reference sample was similar to that of anise myrtle (Table 5). This result is in support of the recently demonstrated and pronounced anti-proliferative activity of *L. nobilis* alcoholic extracts against human breast adenocarcinoma (MCF7) cell line (Al-Kalaldeh et al. 2010).

The extracts of both fruits (quandong and Davidson's plum) exhibited lower anti-proliferative activity against AGS than against HT-29, BL13 and HepG2 cell lines (Table 5), which indicates cell line–plant extract specificity. A dose-dependent reduction of AGS cell proliferation has been observed, however, at a lower level than that of other cell lines. In the case of Davidson's plum extract, at the highest concentration of 2.0 mg/mL, the cell proliferation rate was reduced from 100 per cent to 66.5 per cent and in the case of quandong the reduction was from 100 per cent to 78.3 per cent. In general, the anti-proliferative activities of Davidson's plum and quandong extracts against cancer cell lines were lower than those of polyphenolic-rich extracts obtained from other native Australian fruits (Illawarra plum, Kakadu plum, muntries and native currant) (Tan et al. 2011a). Davidson's plum and quandong also exhibited lower anti-proliferative activities than the reference samples of blueberry (Table 5).
The experiments presented clearly demonstrate an increased sensitivity of cancer cells to plant extracts in comparison to the equivalent normal cells. This is documented by the significantly lower IC_{50} values for HT-29 and AGS than for CCD-18Co and Hs 738.St/Int. In the case of normal cell lines, no significant reduction in cell viability after 24 hours exposure to the extracts was observed. The most pronounced differential killing ability was exhibited by anise myrtle extract due to the high value of extrapolated IC_{50} for the Hs738.St/Int cell line. The differences in sensitivities of cancer and normal cells to anise myrtle and lemon myrtle extracts are presented in Figure 3.

All extracts evaluated within this study demonstrated differential killing ability, or an ability to reduce the proliferation of cancer cells without a damaging effect on normal cells. The degree of anti-proliferative activity of plant extracts against different cancer cells varied, which clearly suggests cell

line–plant extract specificity. Polyphenolic-rich extracts from herbs exhibited more pronounced anti-proliferative activities than the extracts obtained from fruits. The cell line–plant extract specificity can be explained with compositional differences of the extracts with regards to the identity of phytochemicals and their levels. Moreover, each of the extracts evaluated within this study, may contain other phytochemicals, which naturally occur in the evaluated herbs and fruits. These compounds may also exert physiological activities enhancing the anti-proliferative effect through additive or synergistic interactions or reducing the anti-proliferative effect through antagonistic interaction.

Table 5 Concentration of purified polyphenolic-rich extracts from native Australian plants and reference samples resulting in 50% cell viability (IC_{50}) of human cancer and non-transformed cells.

Plant extract	IC_{50} (mg/mL) ± SD[a]					
	HT-29*	CCD-18Co	AGS	Hs738.St/Int	BL13	HepG2
Quandong	1.88±0.07	>2.0	>2.0	>2.0	1.35±0.08	2.30±0.5
Davidson's plum	1.35±0.23	>2.0	>2.0	>2.0	1.35±0.03	0.78±0.02
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
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

The IC_{50} and SD were obtained via nonlinear regression and are expressed as the mean ± SD, determined from the results of the MTT assay of three independent experiments with four replicates each. The IC_{50} values are presented as the amount of fruit extract per mL of culture [IC_{50} (mg/mL) ± SD]. * cell types: HT-29: colorectal adenocarcinoma; CCD-18Co: colon non-transformed; AGS: gastric adenocarcinoma; Hs 738.St/Int: mixed stomach and intestine non-transformed; BL-13: bladder cancer; HepG2: liver hepatocellular carcinoma.

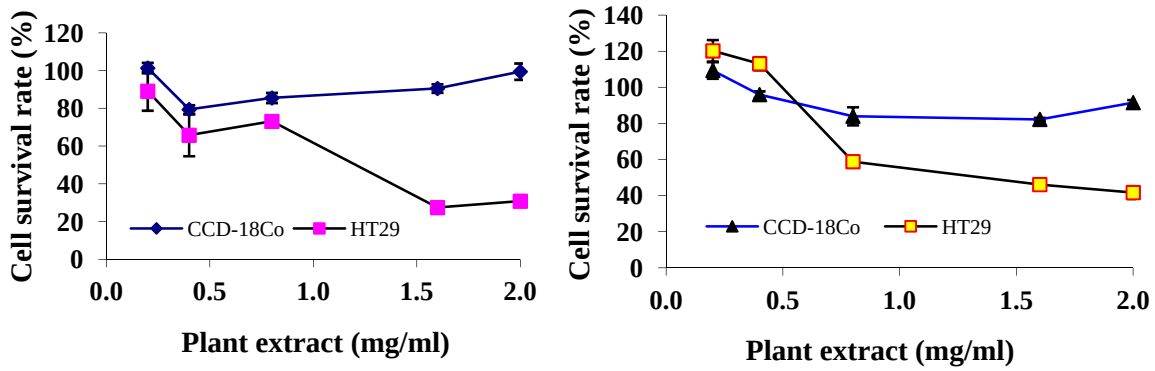


Figure 3 Effect of anise myrtle (left) and lemon myrtle (right) extracts on the proliferation of normal colon cells (CCD-18Co) and colon cancer cells (HT-29).

Mechanism of anti-proliferative activities of polyphenolic-rich extracts against cancer cells: pro-apoptotic capacity of plant extracts

In order to understand the mechanism of anti-proliferative activity of plant extracts against cancer cell lines, flow cytometry analysis of HL-60 (human promyelocytic leukaemia) was conducted. Contrary to the cell lines associated with the digestive tract, which are adhesive and grow on the surface of a culture vessel, the HL-60 cells grow in suspension as single cells. Flow cytometry analysis requires each cell to be assessed individually, and therefore, the HL-60 cell line is the most suitable for such analysis. The HL-60 cells were treated with plant extracts for 3, 12 and 24 hours and subsequently the cell populations were assessed for the presence of apoptotic cells. As presented in Figure 4, each of the plant extracts induced apoptosis of HL-60 cells and the percentage of apoptotic cells (the sum of ‘apoptotic’ (red bar) and ‘late apoptotic’ (grey bar) cells) increased with the treatment time, indicating that apoptosis induction is a time-dependent process.

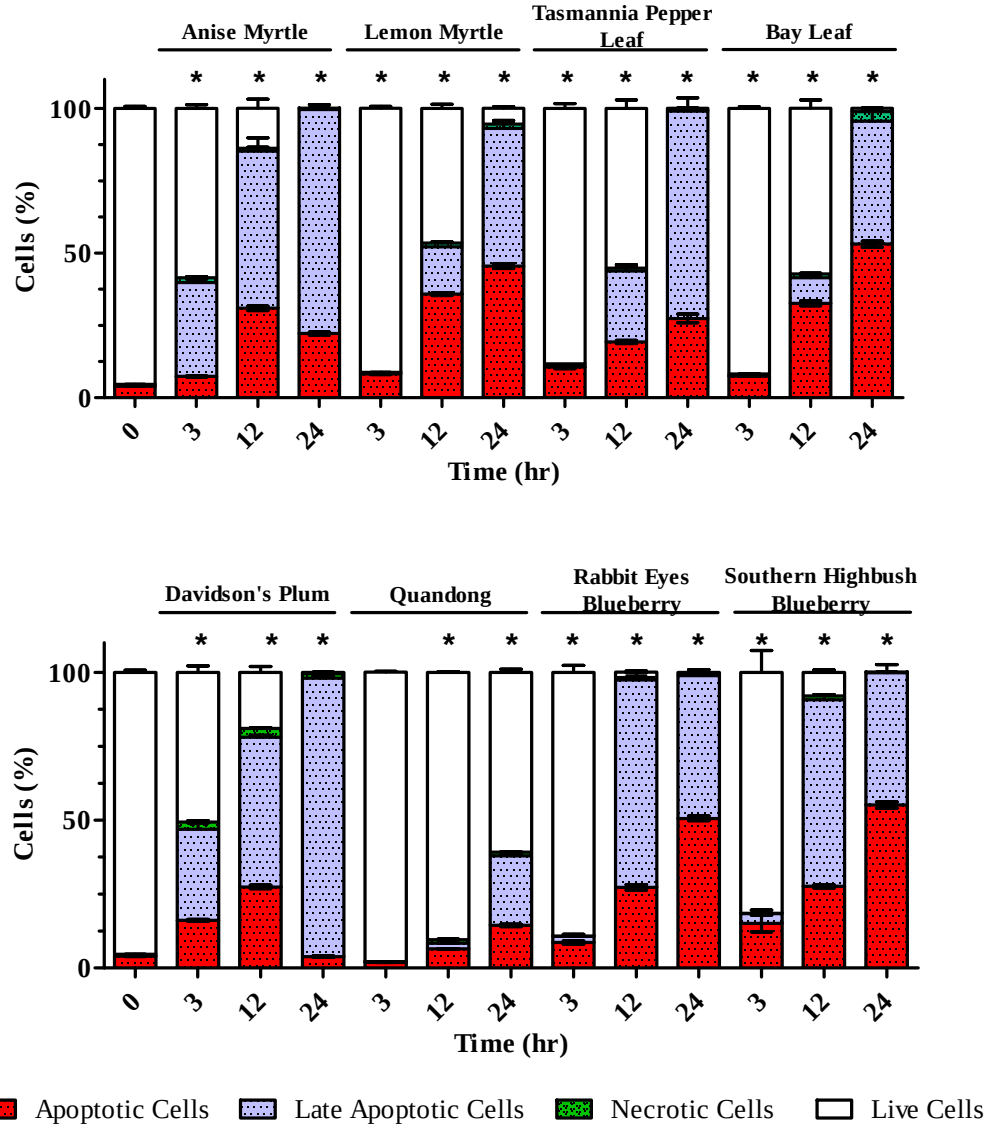


Figure 4 Flow cytometric analysis of HL-60 cells treated with purified polyphenolic-rich extracts (0.4 mg/ml) obtained from Australian native herbs (top chart) and fruits (bottom chart) and reference samples for 3, 12 and 24 hours.

Percentage of live (white), apoptotic (red), late apoptotic (grey) and necrotic cells (green) are presented as a mean value ± SD of the percentage of cells in each population obtained from three independent experiments. An asterisk (*) represents a significant difference ($P<0.05$) between the percentage of live cells and the combined percentages of apoptotic and late apoptotic cells.

Among the herbs studied, anise myrtle extract exhibited the largest immediate effect and induced the highest level of apoptotic cells after 3 and 12 hours of treatment. After 24 hours of treatment with anise myrtle extract, nearly all cells become apoptotic. Lemon myrtle, Tasmannia pepper leaf and bay leaf extracts, produced a similar percentage of apoptotic cells after 24 hours. With regards to fruit extracts, Davidson's plum effectively induced cell apoptosis and the pattern of apoptosis induction was similar to both that of anise myrtle and the reference samples of blueberries. To the contrary, the efficiency of quandong extract to induce apoptosis of cancer cells was low, with approximately 10 per cent of apoptotic cells after 12 hours of treatment and 40 per cent after 24 hours of treatment (Figure 4). Anise myrtle and Davidson's plum extracts exhibited the largest immediate effect on cancer cells with the highest level of apoptosis within the first 3 hours of treatment.

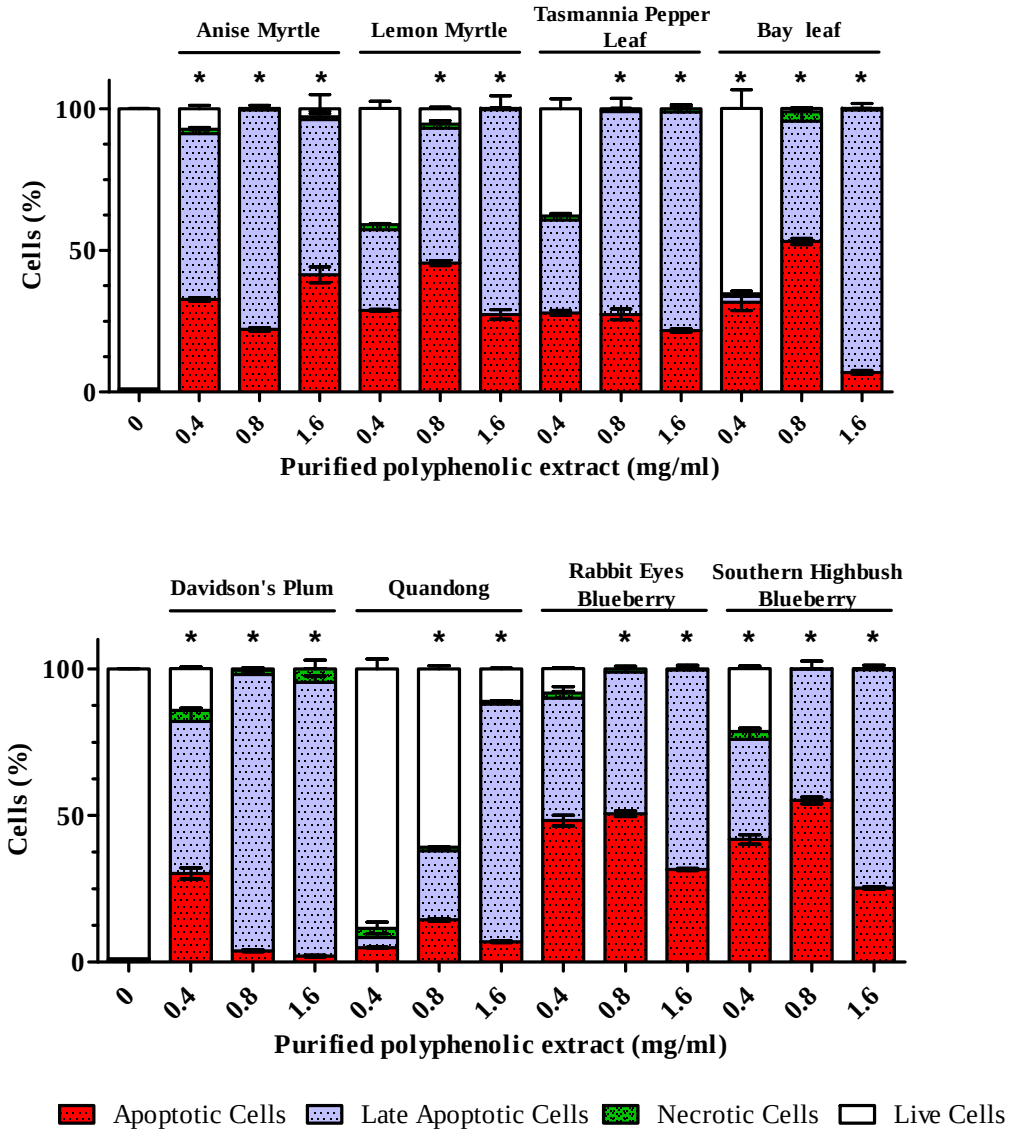


Figure 5 Flow cytometric analysis of HL-60 cells treated with purified polyphenolic-rich extracts obtained from Australian native herbs (top chart) and fruits (bottom chart) and reference samples at doses of 0.4, 0.8 and 1.6 mg/mL over 24 hours.

Percentage of live (white), apoptotic (red), late apoptotic (grey) and necrotic cells (green) are presented as a mean value ± SD of the percentage of cells in each population obtained from three independent experiments. An asterisk (*) represents significant difference ($P<0.05$) between the percentage of live cells and the combined percentages of apoptotic and late apoptotic cells.

Examination of the pro-apoptotic activity at a single time point (24 hours) with various doses of plant extracts revealed a clear dose-dependent response only for quandong extract (Figure 5). Lemon myrtle, Tasmannia pepper leaf and bay leaf exhibited a similar pattern with 50 per cent (±10 per cent) of apoptosis induction at the lowest concentration of 0.4 mg/mL. In case of anise myrtle and Davidson's plum, an application of 0.4 mg/mL of plant extract induced apoptosis of >80 per cent (Figure 5). This shows the remarkable pro-apoptotic activity of extracts from the Australian native plants evaluated within this study.

The results of this study confirm the ability of polyphenolic-rich extracts obtained from Australian native herbs and fruits to induce apoptosis of cancer cells, as observed in the CBMN assay. The induction of apoptosis has been confirmed in two varied human cancer cell lines: HT-29 (human colon

adenocarcinoma) used in the CBMN assay; and HL-60 (human promyelocytic leukaemia) used in the flow cytometric analysis.

In conclusion, the above presented results indicate that Australian native herbs and fruits exhibit anti-proliferative and pro-apoptotic activities in cancer cell lines without significant damage to non-transformed (normal) cells. Further research is needed to understand the exact mechanism, with characterisation of the use of plant extracts as signalling molecules in modulation of molecular pathways.

Bioavailability of phytochemicals from Australian native plant species to human cells

Consumption of a diet rich in fruits and vegetables reduces significantly the risk of chronic diseases, such as Alzheimer's and cardiovascular disease, diabetes or cancer (Noroozi et al. 1998; Liu 2004; Bagchi et al. 1999; Halliwell 2007; Block et al. 1992). The protective effect of fruits and vegetables has been attributed to phytochemicals acquired through the diet. Phenolic compounds, particularly flavonoids, are one of the major groups of plant components essential to maintain good health, vitality and well-being. Flavonoid compounds have been identified in phenolic-rich extracts obtained from all Australian native plants evaluated within this study. Some fruits and/or vegetables and their extracts are more efficient in health prevention and curing than others (Beattie et al. 2005). These effects arise from the composition of phytochemicals accumulated by the plant cell, their bioaccessibility from the food matrix, their uptake by human cells and their following metabolism.

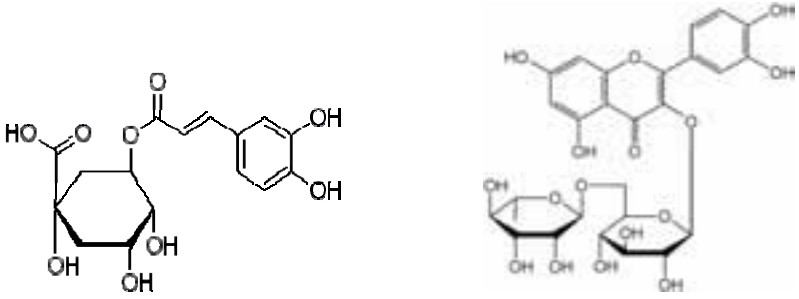
The literature concerning bioavailability of phytochemicals from whole foods is still limited. The use of humans and animals in clinical studies to understand the uptake and bioavailability of compounds is difficult, complex and expensive. Alternatively, an attractive option is the use of an *in vitro* model, where the uptake of an isolated compound or mixture by a human cell nurtured in cell culture medium can be captured and monitored over time. A well-established model to study bioavailability of compounds is the Caco-2 cell culture model. Caco-2 cells are derived from human colon adenocarcinoma and differentiate into polarised enthrocyte-like monolayers, acting similarly to intestinal epithelial cells. Permeability coefficients determined for the human Caco-2 monolayer have been shown to correlate highly with human absorption *in vivo* (Lennernas 1997). The model has effectively been used to study the uptake of drugs, and later, food components, and has been proposed as a useful system to study bioavailability of whole food phytochemicals (Boyer et al. 2004; Liu and Finley 2005).

Understanding the bioavailability of a pure component helps to understand the bioavailability of the same compound when present at a complex mixture of phytochemicals. The uptake of compounds from a mixture is complicated and many other factors need to be considered such as concentrations and forms of individual phytochemicals, interactions with other food components as well as the food matrix which affects the kinetics of the release of compounds to the environment. It is possible that the bioavailability of the same compounds may differ among foods, depending on the food matrix, and will differ from the bioavailability of a pure compound.

Uptake of pure compounds: chlorogenic acid and quercetin 3-rutinoside by Caco-2 monolayers

Chlorogenic acid (CHA) and quercetin are among the most common phenolic compounds present in extracts of native herbs and fruits evaluated in this study. Chlorogenic acid (Figure 6), also known as caffeoylquinic acid (CQA), is an ester of caffeic acid and cyclitol quinic acid and represents the hydroxycinnamic acids subgroup of phenolic acids. Depending on the substitution pattern, three isomers of chlorogenic acid exist in nature: 3-CQA (chlorogenic acid), 4-CQA and 5-CQA (neochlorogenic acid) with 3-CQA and 5-CQA being the most common. Among our samples, chlorogenic acid is the main phenolic compound of Tasmannia pepper leaf.

Quercetin belongs to the flavonoids subgroup of phenolic compounds and is among the most common flavonoids found in fruits and vegetables. It is present at high levels in onions and apples. In plant cells, the aglycon of quercetin may have various sugar moieties, with the most common being glucoside or rutinoside (Figure 6). Both, quercetin glucosides and quercetin rutinosides, are present in extracts of Australian native plants.



Chlorogenic acid Quercetin 3-rutinoside

Figure 6 Molecular structure of chlorogenic acid (left) and quercetin 3-rutinoside (right).

Chlorogenic acid (CHA) and quercetin 3-rutinoside (Q3R) were selected as model molecules to understand the bioavilability of pure compounds within this study. Caco-2 monolayers were starved for 24 hours and subsequently chlorogenic acid and quercetin 3-rutinoside individually were added to basic culture media for 4 hours. The amounts of both compounds extracted from Caco-2 cells after 4 hours of feeding are presented in Figure 7. Examples of HPLC chromatograms representing peaks of the detected compounds are presented in Appendix Figure A2.

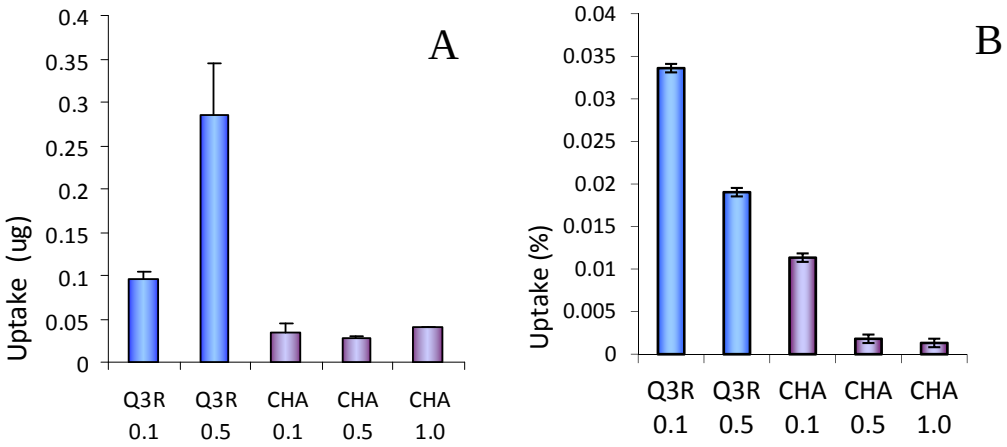


Figure 7 Uptake of pure compounds: chlorogenic acid (CHA) and quercetin 3-rutinoside (Q3R) by Caco-2 monolayers, presented as: A) micrograms (µg) of compound extracted from cells/well; B) percentage of compound added to the culture medium.

At each concentration tested, a larger uptake of Q3R than CHA was observed. CHA is the smaller molecule with the exact molecular mass of 354.1, while the molecular mass of Q3R is 610.1. However, at the concentration of phytochemicals in the culture medium of 0.1 mg/mL the uptake of Q3R was 2-fold that of CHA. A further increase of phytochemical concentration in the medium to 0.5 mg/mL (a 5-fold increase) resulted in a 3-fold increase of Q3R uptake. This result is in agreement with Boyer and coworkers (2004), who monitored the uptake of quercetin 3-glucoside (Q3G) from shallot extract by Caco-2 monolayers and reported that an increase of Q3G concentration in the culture medium from 1 to 9 nmol brought an increase in uptake from approximately 0.1 to 0.16 nmol.

Contrary to the Q3R uptake, a higher concentration of CHA in the culture medium did not result in an increase of CHA uptake by Caco-2 cells. Earlier, Hollman and coworkers (1995) proposed that quercetin glucosides may be actively absorbed and the presence of a sugar moiety may play a role in this process. The mechanism of uptake of various phytochemicals is not fully understood. It is not clear if active transport plays a major role in this process or the permeability of Q3R is higher than that of CHA. In-depth studies need to be conducted to understand this process. However, this experiment clearly demonstrated that both pure compounds, CHA and Q3R, were successfully absorbed by Caco-2 cell monolayers.

Various phytochemicals, when applied in a pure form, are absorbed in different manners by human cells. The process might be related to molecular structure and size and also to mechanisms of uptake. When phytochemicals are presented as components of food, their uptake will also depend on their bioaccessibility from the food matrix and release into the digestive system as well as their digestive stability.

Uptake of phenolic compounds from plant extracts

The uptake of phytochemicals from polyphenolic-rich extracts of Australian native herbs and fruits by Caco-2 cell monolayers over a period of 4 hours is presented in Figure 8. Total uptake of phenolic compounds varied from 0.17 µg/well (Tasmannia pepper leaf) to 0.36–0.38 µg/well (anise myrtle and quandong). The amount of extract applied to each culture well was 3 mg and the level of uptake was from 0.014 to 0.040 per cent. The previous experiment revealed that uptake of pure CHA by Caco-2 monolayers is lower than the uptake of pure Q3R over the same time (Figure 7); the lowest level of uptake of Tasmannia pepper leaf phytochemicals, where CHA is the main compound of the extract, corresponds with these results. In the case of other plant extracts, their composition was more complex, which may positively affect the uptake of phytochemicals.

This experiment clearly showed that phenolic compounds obtained from Australian native plants are absorbed by human cells and the percentage of absorbed compounds is very low (0.015 to 0.040 per cent). It needs to be remembered, however, that within the cell culture experiment the surface area of the Caco-2 monolayer is only 3 cm^2. This area represents only a small fraction of the human digestive system that is estimated to be 900 cm long.

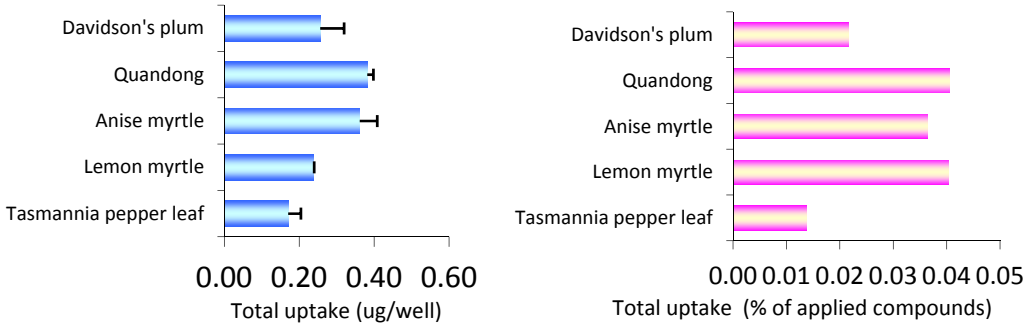


Figure 8 Total uptake of phenolic compounds from polyphenolic-rich extracts of Australian native herbs and fruits by Caco-2 monolayers.

Compounds detected at 280 nm were quantified as gallic acid equivalent (GA Eq), compounds detected at 320 nm were quantified as chlorogenic acid equivalent (CHA Eq) and compounds detected at 370 nm were quantified as quercetin 3-rutinoside equivalent (Q3R Eq). Total uptake represents a sum of compounds detected at 280, 320 and 370 nm. Each value represents the mean ±SD of two independent observations within the same experiment.

The Caco-2 cell monolayer extracts obtained after 4 hours of cell feeding with plant compounds (at concentration of 1.0 mg/mL) were analysed for the presence of selected major individual plant compounds: chlorogenic acid representing hydroxycinnamic acids and quercetin, myricetin, hesperetin, and rutin representing flavonols (Figure 9), as peaks representing these compounds were clearly visible on the HPLC chromatogram and were identified through co-chromatography with

standards. Beside these compounds, a number of minor peaks were detected at various wavelengths, depending on the plant extract, as follows: Tasmannia pepper leaf: 320 and 370 nm; lemon myrtle: 370 nm; anise myrtle: 280 and 370 nm; quandong: 320 nm; and Davidson's plum: 280 and 370 nm. The peaks detected at 280 nm represent benzoic acids and flavanols (Davidson's plum and anise myrtle), peaks at 320 nm represent hydroxycinnamic acids (Tasmannia pepper leaf and quandong) and peaks at 370 nm represent flavonols (Tasmannia pepper leaf, Davidson's plum, anise myrtle and lemon myrtle). It needs to be noted, that the level of uptake of individual compounds from a mixture depends on a number of factors: initial composition of the mixture, degradation rate of phenolic compounds in culture medium (or in a human digestion system), and accumulation of degradation products as well as the mechanism of uptake of individual compounds. In the case of Tasmannia pepper leaf, CHA is the main compound of the plant extract. However, the total uptake of flavonols (quercetin and derivatives detected at 370 nm wavelength) exceeded that of CHA (Figure 9A).

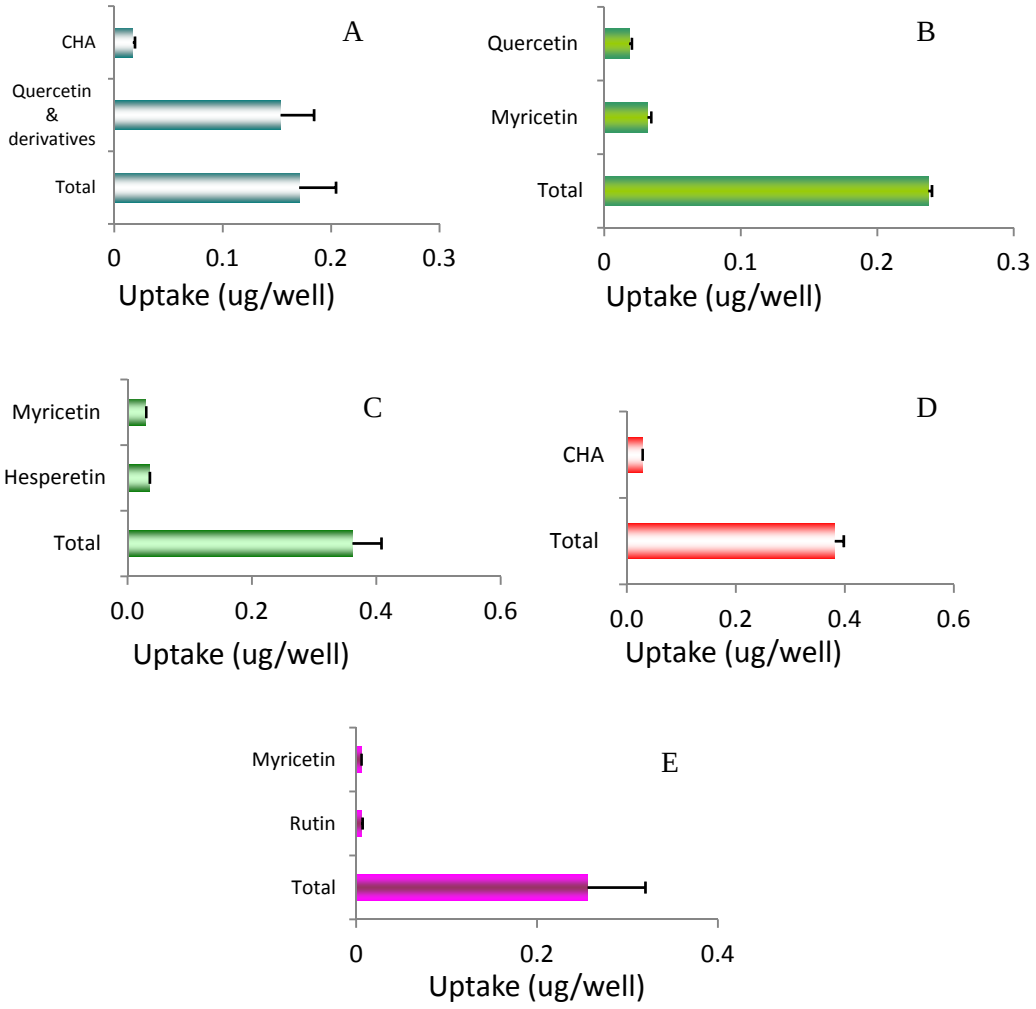


Figure 9 Total uptake of phenolics and major compounds of polyphenolic-rich extracts from Australian native herbs and fruits by Caco-2 monolayers.

A: Tasmannia pepper leaf; B: Lemon myrtle; C: Anise myrtle; D: Quandong; E: Davidson's plum. Compounds detected at 280 nm were quantified as gallic acid equivalent (GA Eq), compounds detected at 320 nm were quantified as chlorogenic acid equivalent (CHA Eq) and compounds detected at 370 nm were quantified as quercetin 3-rutinoside equivalent (Q3R Eq). Total uptake represents a sum of compounds detected at 280, 320 and 370 nm. Each value represents the mean ±SD of two independent observations within the same experiment.

Flavonols are present in foods predominantly in glycosylated form and the type of sugar influences uptake. For example, absorption of quercetin is more rapid and efficient after ingestion of onions, which are rich in glucosides, than after ingestion of apples containing both glucosides and various other glycosides (Manach et al. 2004). These observations are in agreement with our results presented above.

The kinetics of uptake of phenolic compounds from the plant extracts was monitored using anise myrtle phytochemicals as an example, applied at a concentration of 1 mg/mL. At selected time points Caco-2 monolayers were collected and plant-originating compounds were extracted from the cells (Figure 10). The Caco-2 cell extract contained two groups of phenolic compounds: detected at 280 nm (phenolic acids/catechins) and at 370 nm (flavonols). Myricetin is one of the major compounds present in anise myrtle extract and it was also detected in the Caco-2 cell extract. The highest uptake by human cells of myricetin as well as other phenolic compounds was observed after 1 hour of feeding the plant extract to Caco-2 cells. This result is in agreement with other studies reporting the maximum absorption of food components from 15 minutes to 2 hours after ingestion (Hollman et al. 1995).

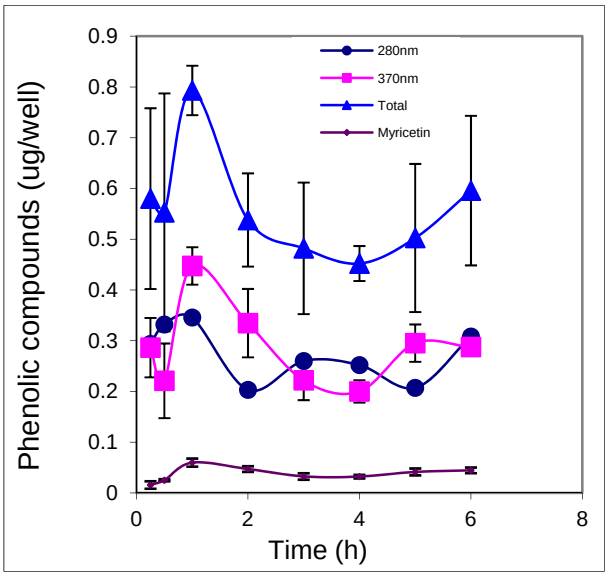


Figure 10 Time course of phenolic compound uptake from anise myrtle extract by Caco-2 monolayers.

In a human cell or human plasma, the level of food components is affected by their metabolites and/or degradation products that will appear over time. Human studies have shown that in the case of some phenolic compounds, such as hesperetin from orange juice, a second plasma peak was observed, indicating an uptake of this compound from the digestion system 10 to 12 hours after ingestion. This secondary uptake was presumably due to the activities of intestinal bacteria that can release flavonoid aglycones from their metabolites (glucoronides) (Manach et al. 2003).

Results from our study demonstrate an uptake of pure phenolic compounds (CHA and Q3R) as well as phenolic compounds from a plant-originating mixture by Caco-2 cell monolayers. Q3R was absorbed much more efficiently than CHA. Accordingly, a lower total uptake of CHA-rich Tasmannia pepper leaf phenolic compounds was observed in comparison to other extracts. The maximum absorption was observed within 30 to 60 minutes of incubation time.

Inhibitory potential of Australian native herbs against key enzymes relevant to metabolic syndrome: α-glucosidase and pancreatic lipase

Metabolic syndrome, characterised by glycemic index imbalance, glucose intolerance, hypertension, dyslipidemia and/or obesity, is an early sign of potential future development of a chronic condition, frequently type 2 diabetes. Type 2 diabetes is characterised by a rapid increase of blood glucose level after food consumption, a condition also known as postprandial hyperglycemia. This rapid increase can be reduced through the 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, which catalyses the cleavage of glucose from disaccharides and oligosaccharides, facilitating an uptake of glucose into the blood stream. Hence, inhibition of α-glucosidase activity reduces glucose uptake. Pancreatic lipase is another enzyme relevant to metabolic syndrome; it is the key enzyme which hydrolyses triglycerides into glycerol and fatty acids, facilitating an uptake of fat (triglycerides) in the intestine. Inhibition of lipase activity reduces dislipidemia (high levels of blood cholesterol and triglycerides).

Phytochemicals have been reported to suppress the activity of various human enzymes. An endemic Sri Lankan plant *Cassia auriculata* (Leguminosae) exerts a strong inhibitory effect against α-glucosidase, comparable to that of the therapeutic drug acarbose (Abesundara et al. 2004). The inhibitory activities of plant extracts vary among the plants depending on their phytochemical composition. Matsui and coworkers (2001a) reported significant differences in α-glucosidase inhibitory activity among 16 anthocyanin-containing plant extracts. Molecular structure and substitution pattern of anthocyanin molecules strongly affected their inhibitory activities (Matsui et al. 2001b). Forty-seven plant species, belonging to 29 families were described as sources of α-glucosidase inhibitors, with the active components being: alkaloids, stilbenoids (polyphenol), triterpene, acids (chlorogenic acid, betulinic acid, syringic acid, vanillic acid, bartogenic acid, oleanolic acid, dehydrotrametenolic acid, corosolic acid, ellagic acid, ursolic acid, gallic acid), phytosterol, myoinositol, flavonoids, flavonolignans, anthraquinones, anthrones, xanthones, feruloylglucosides, flavanone glucosides, acetophenone glucosides, glucopyranoside derivatives, genine derivatives, flavonol, anthocyanin and others (Benalla et al. 2010).

The objective of the present study was to evaluate for the first time, the functional potential of three of the most commonly used Australian native herbs: anise myrtle, lemon myrtle and Tasmannia pepper leaf and two Australian native fruits: Davidson's plum and quandong against the principal components of the metabolic syndrome: hyperglycemia and dislipidemia. Anise myrtle and lemon myrtle leaves, rich in essential oils and with a distinct flavour (Southwell et al. 2000), superficially resemble bay leaf and are used in a similar manner: in seasoning and in preparation of main meals. Additionally, due to their unique flavour, they are also frequent components of herbal infusions. Tasmannia pepper leaf, due to its pungency (Drager et al. 1998), is used in cooking and as an additive in processed foods, e.g. cheese. Both Davidson's plum and quandong are used as constituents of sauces and chutneys. During food preparation, food consumption and digestion, their constituents are released and may interact with a number of digestive enzymes. It is important to mention that interaction between food components and the above mentioned enzymes occurs in the digestion system, therefore bioavailability of the active compounds is not necessary for these activities to occur.

Inhibitory activities against α-glucosidase

All of the herb and fruit extracts evaluated in this study inhibited the activity of α-glucosidase enzyme in a dose-dependent manner (Figure 11). The inhibitory activity of Australian native herbs extracts exceeded that of the reference sample, bay leaf extract. Anise myrtle and lemon myrtle exhibited more pronounced activity than Tasmannia pepper leaf. Chlorogenic acid and ellagic acid are among the major compounds in, respectively, Tasmannia pepper leaf and anise myrtle/lemon myrtle extracts. Chlorogenic acid was identified as the major anti-diabetic compound of *Nerium indicum*, an India-

Pakistan-originated shrub belonging to the oleander family which is used as a folk remedy for type 2 diabetes in some regions of Pakistan. Ingestion of the leaves of *N. indicum* before a meal is known to lower the postprandial glucose levels in type 2 diabetic patients (Ishikawa et al. 2007). Recently, ellagic acid was identified as the main antidiabetic agent in strawberries (da Silva et al. 2010).

A strong positive correlation was identified between the level of phenolic compounds and IC_{50} of *α*-glucosidase inhibitory activity ($R^2 = 0.999$). Various degrees of correlation between phenolic content and α-glucosidase inhibitory activity were reported for various plant extracts. Ranilla and coworkers (2010) observed a moderate positive correlation between total phenolic level and *α*-glucosidase inhibitory activity for commonly used medicinal plants in Latin America. Phenolic-rich extracts of Virginia raspberries efficiently inhibited α-glucosidase, however, the extracts were not effective against lipase (Zhang et al. 2010).

Davidson's plum extract was more effective in reducing α-glucosidase enzyme activity than quandong and blueberry, with the exception of the lowest concentration of 0.1 mg/mL (Figure 11). In Davidson's plum extract we have detected polymeric compounds that under hydrolysis break down to form ellagic acid (Table 2). According to Artz and coworkers (1987), generally, proanthocyanidins or condensed tannins (polymeric compounds) are prone to complexation with proteins, while dimers are less effective and some simple flavonols do not precipitate proteins. The conformation of proteins also plays an important role: tightly coiled globular proteins exhibit low affinity for tannins; whereas conformationally open proteins are prompt to form complexes with tannins (Hagerman and Butler 1981). Accordingly, the composition of the extracts evaluated in this study affects their protein-binding ability. Polymeric compounds dominate in anise myrtle and at lower levels are present in lemon myrtle and Davidson's plum extracts, which might be the reason for enhanced inhibition of α-glucosidase enzyme activity. Tasmannia pepper leaf and quandong extracts comprise monomeric compounds and displayed relatively lower inhibitory activity towards α-glucosidase.

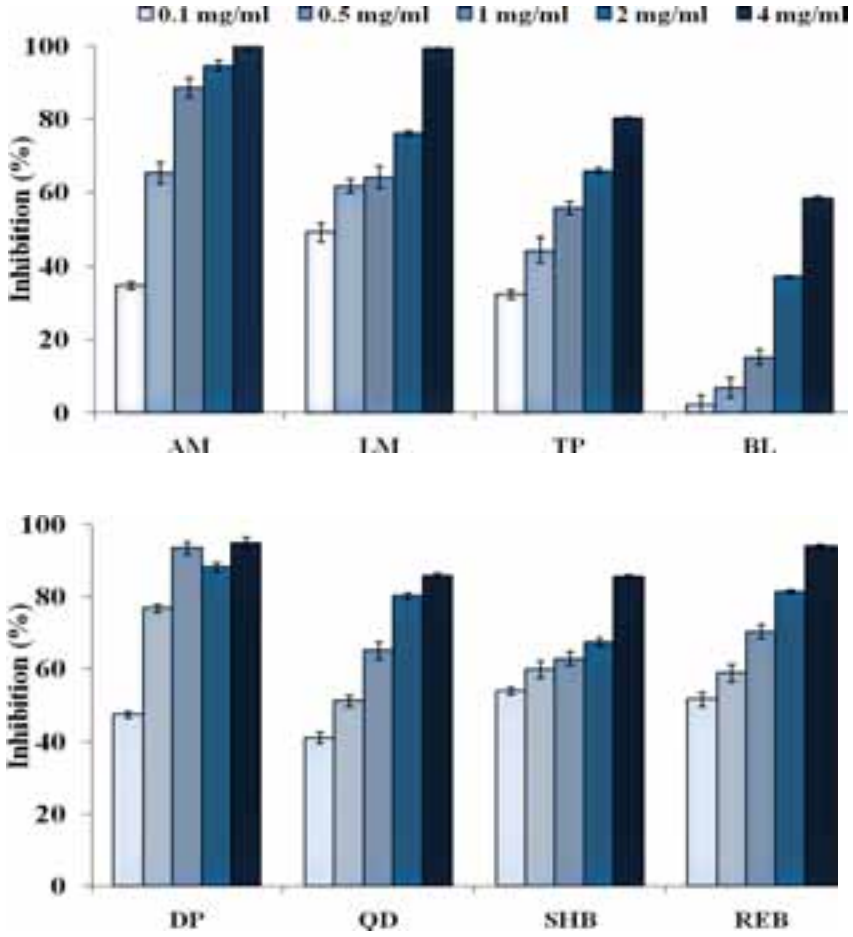


Figure 11 Relative α-glucosidase inhibitory activity of polyphenolic-rich extracts obtained from Australian native herbs and fruits and relevant reference samples.

AM: anise myrtle; LM: lemon myrtle; TP: Tasmannia pepper leaf; BL: bay leaf (reference sample); DP: Davidson's plum; QD: quandong; SHB: southern highbush blueberry (reference sample); REB: rabbit eye blueberry (reference sample).

Inhibitory activities against pancreatic lipase

With regards to lipase, inhibitory activity of Tasmannia pepper leaf extract dominated over the anise myrtle and lemon myrtle extracts (Figure 12). The activity of Davidson's plum was lower than other fruits included in this study. The dominance of cinnamic acids (chlorogenic acid and *p*-coumaric acid) in Tasmannia pepper leaf might be responsible for this activity. Sugiyama and coworkers (2007) reported that lipase inhibitory activity by various phenolics depended on their degree of polymerisation: at least pentameric procyanidins were required for a sufficient inhibition of a pancreatic lipase. Dimers as well as flavan-3-ols were not active. Chalcones and cinnamic acids exhibited moderate activities, lower than that of pentameric proanthocyanidins. Detailed knowledge on the composition of polyphenolic-rich extracts is required in order to understand the degree of suppression of both enzymes by plant extracts.

Arai and coworkers (1999) evaluated an alcoholic extract (70 per cent ethanol) of the South American medicinal herb *Eugenia uniflora* (Myrtaceae), closely related to anise myrtle and lemon myrtle, for an ability to inhibit increases in glucose and triglyceride levels in the plasma of ICR strain mice after administration of sugars and corn oil, respectively. The extract was fractionated into six fractions

depending on polarity and molecular size. Fractions 1–4 were most efficient in inhibiting the increase of plasma glucose level and fractions 3 and 4 inhibited triglyceride levels. Further, the authors found that inhibition of postprandial hypertriglyceridemia by fractions 3 and 4 occurred due to inhibition of pancreatic lipase. These results are in support of the findings by Sugiyama and coworkers (2007) showing that the enzyme–phenolic compound specificity affects the inhibitory action of phenolic-rich extracts, and is responsible for a different degree of α-glucosidase and pancreatic lipase inhibition by the extracts evaluated in this study.

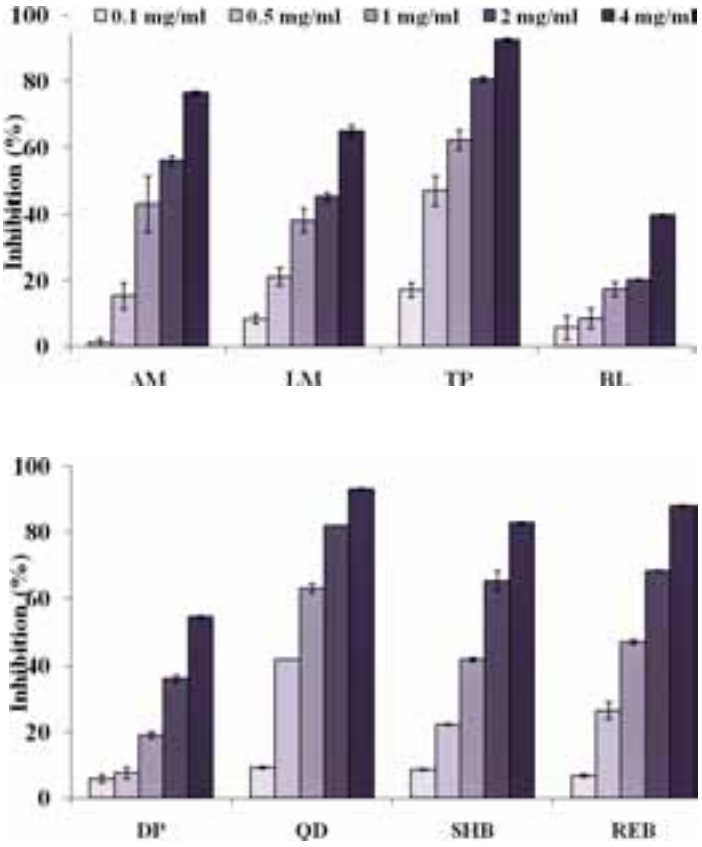


Figure 12 Relative lipase inhibitory activity of polyphenolic-rich extracts obtained from Australian herbs and fruits and relevant reference samples.

AM: anise myrtle; LM: lemon myrtle; TP: Tasmannia pepper leaf; BL: bay leaf (reference sample); DP: Davidson's plum; QD: quandong; SHB: southern highbush blueberry (reference sample); REB: rabbit eye blueberry (reference sample).

A number of plants representing the Myrtaceae family are known ethnopharamceuticals. *Syzygium cumini* L., (also known as *Eugenia jambolana* Lam.) and *Eucalyptus globulus* Labill are two Indian medicinal plants with a recognised hypoglycaemic potential. Multiple studies summarised by Mukherjee et al. (2006) describe the blood glucose lowering effect of both alcoholic and water extracts of *Syzygium cumini* seeds and dry leaves, as identified through animal studies. An enhancement of insulin release and an increase in insulin serum activity were suggested as the potential mechanisms of action. An alcoholic extract of *Syzygium cumini* bark exhibited superior anti-diabetic properties among seven herbs from the Philippines, administered to mice together with glucose (Villasenor and Lamadrid 2006). Gray and Flatt (1998) identified a reduction of hyperglycaemia by a water extract of *Eucalyptus globulus* in mice. An insulin-releasing effect, as identified and based on *in vitro* studies with a pancreatic beta-cell line, was suggested as the mechanism of action. *Eugenia uniflora* L., Myrtaceae, is among the most common remedies in Argentina, Brazil, Uruguay and Paraguay. Leaf infusion is used in the treatment of fever, rheumatism, stomach diseases, disorders of the digestive

tract, hypertension, yellow fever and gout, and has a role in reducing weight and diminishing blood pressure, as well as acting as a diuretic (Consolini et al. 1999). In agreement, according to Arai and co-workers (1999), five out of six ethanolic (70 per cent) fractions of *Eugenia uniflora* leaves inhibited an increase of plasma glucose level in mice in an oral glucose tolerance test. The effect was due to the inhibition of glucose absorption from the intestine. Two of the fractions inhibited an increase of plasma triglycerides in an oral corn oil tolerance test due to the inhibition of fat decomposition in the intestine (Arai et al. 1999). In north-eastern Argentina the same plant is used as an antihypertensive agent. *In vivo* studies with rats confirmed a hypotensive effect of a crude aqueous extract of dry leaves through direct vasodilation (Consolini et al. 1999). Australian native plants evaluated in this study are closely related to the ethnopharmaceuticals from Southern America.

This investigation for the first time revealed inhibitory activities of Australian herb and fruit extracts against two key enzymes involved in metabolic syndrome: α-glucosidase and pancreatic lipase. This study indicates further health benefits arising from consumption of the edible native plants evaluated in this study.

Conclusions

This study revealed for the first time a number of potential health-modulating activities of polyphenolic-rich extracts obtained from three selected Australian native herbs: Tasmannia pepper leaf, anise myrtle and lemon myrtle; and two Australian native fruits: Davidson's plum and quandong, as assessed using an array of *in vitro* assays. Polyphenolic-rich extracts obtained from these plants comprised predominantly phenolic acids (ellagic acid, chlorogenic acid, *p*-coumaric acid), flavonoids (quercetin, myricetin, hesperedin, anthocyanins) and polymeric phenolic compounds (ellagitannins).

Extracts of Davidson's plum and lemon myrtle exhibited superior total reducing capacities, higher than reference samples of blueberry and bay leaf, respectively. Tasmannia pepper leaf extract displayed an oxygen radical absorbance capacity that was superior to all plants studied. All extracts displayed cytoprotective activities, as demonstrated, respectively, by effective protection of HepG2 cells from H_2O_2-induced cell death and cellular antioxidant activities. With the exception of quandong extract applied at 1.0 mg/mL, none of the evaluated extracts caused DNA damage in HT-29 cells. All extracts evaluated within this study exhibited differential killing ability, or an ability to reduce the proliferation of cancer cells without a damaging effect on normal cells with the extract from anise myrtle displaying superior activity. The degree of anti-proliferative activities of plant extracts against different cancer cells varied, which suggests cell line–plant extract specificity. Polyphenolic-rich extracts from herbs exhibited more pronounced activities than extracts obtained from fruits. Induction of cancer cell apoptosis was established as the mechanism of anti-proliferative activities. Pro-apoptotic activities were confirmed in two human cancer cell lines using two different approaches: HT-29 (human colorectal adenocarcinoma) cells were used in the CBMN assay; and HL-60 (human promyelocytic leukaemia) cells were used for flow cytometric analysis. As indicated by the CBMN assay, in the case of herbal extracts, induction of apoptosis occurred through cell damage during cytokinesis or an inhibition of cell cycle progression. These results suggest potential chemo-preventative properties of the evaluated extracts.

The activity of plant extracts in the cellular antioxidant activity (CAA) assay indicates their uptake and metabolism by a live cell. The study with Caco-2 cell monolayer model confirms that compounds originating from the native herbs and fruits evaluated in this study were bioavailable to human cells.

All extracts actively inhibited the activity of isolated α-glucosidase, with extracts from anise myrtle, lemon myrtle and Davidson's plum being the strongest inhibitors. Extracts from Tasmannia pepper leaf and quandong were the most efficient inhibitors of pancreatic lipase. The inhibitory activities against isolated enzymes: α-glucosidase and pancreatic lipase suggest potential anti-diabetic and anti-obesity properties, respectively.

The results obtained within this study suggest a number of potential health-enhancing properties of the evaluated native Australian herbs and fruits. These results have been generated in an array of cell culture based assays. Further nutritional/clinical studies with humans are required to confirm the identified potential health benefits.

Appendix

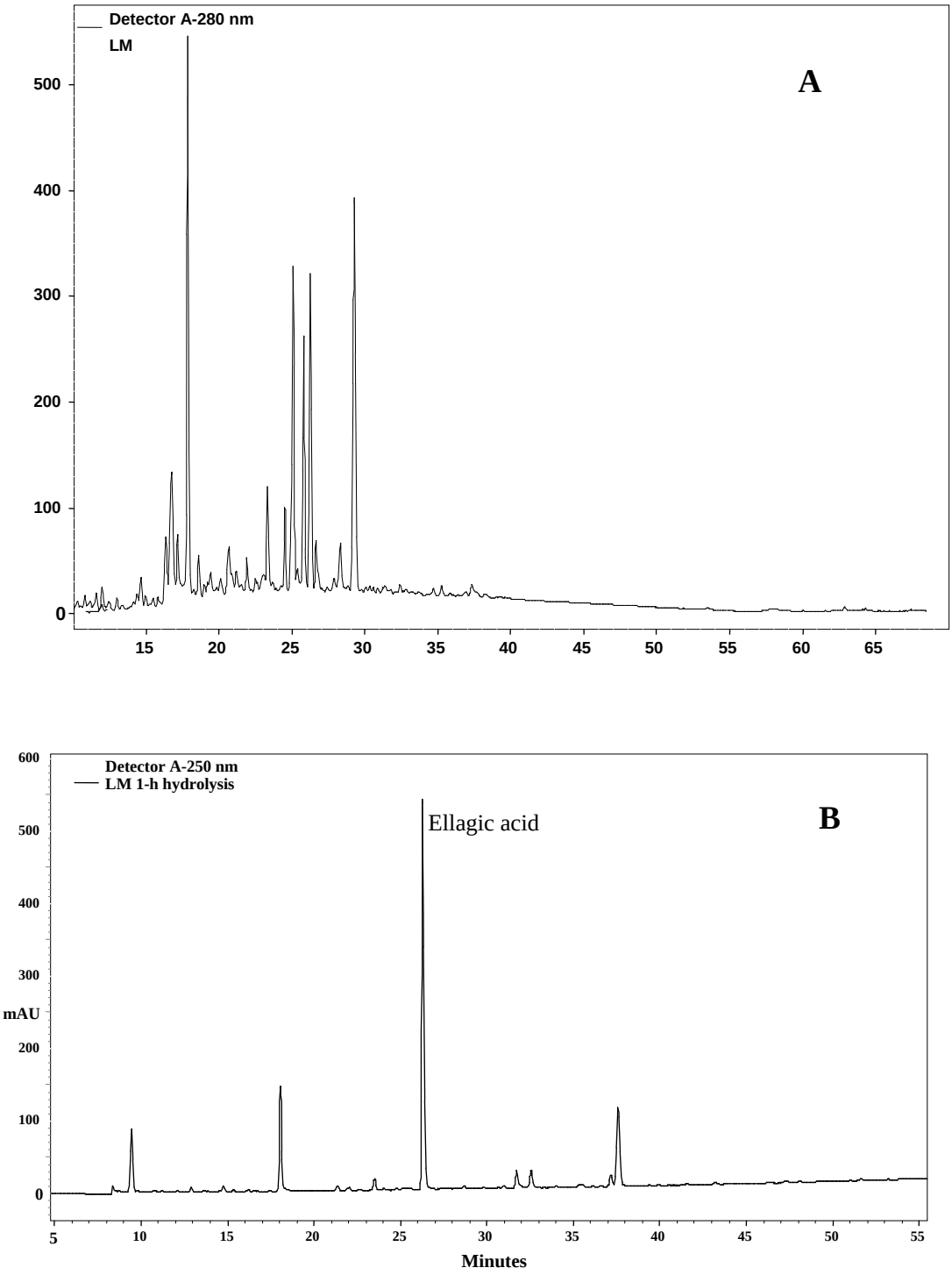


Figure A1 HPLC chromatogram of lemon myrtle extract.

A) Crude methanolic extract (λ=280 nm) showing the presence of an elevated baseline representing polymeric compounds. B) Extract after hydrolysis of tannins (λ = 250 nm) with the major peak representing ellagic acid.

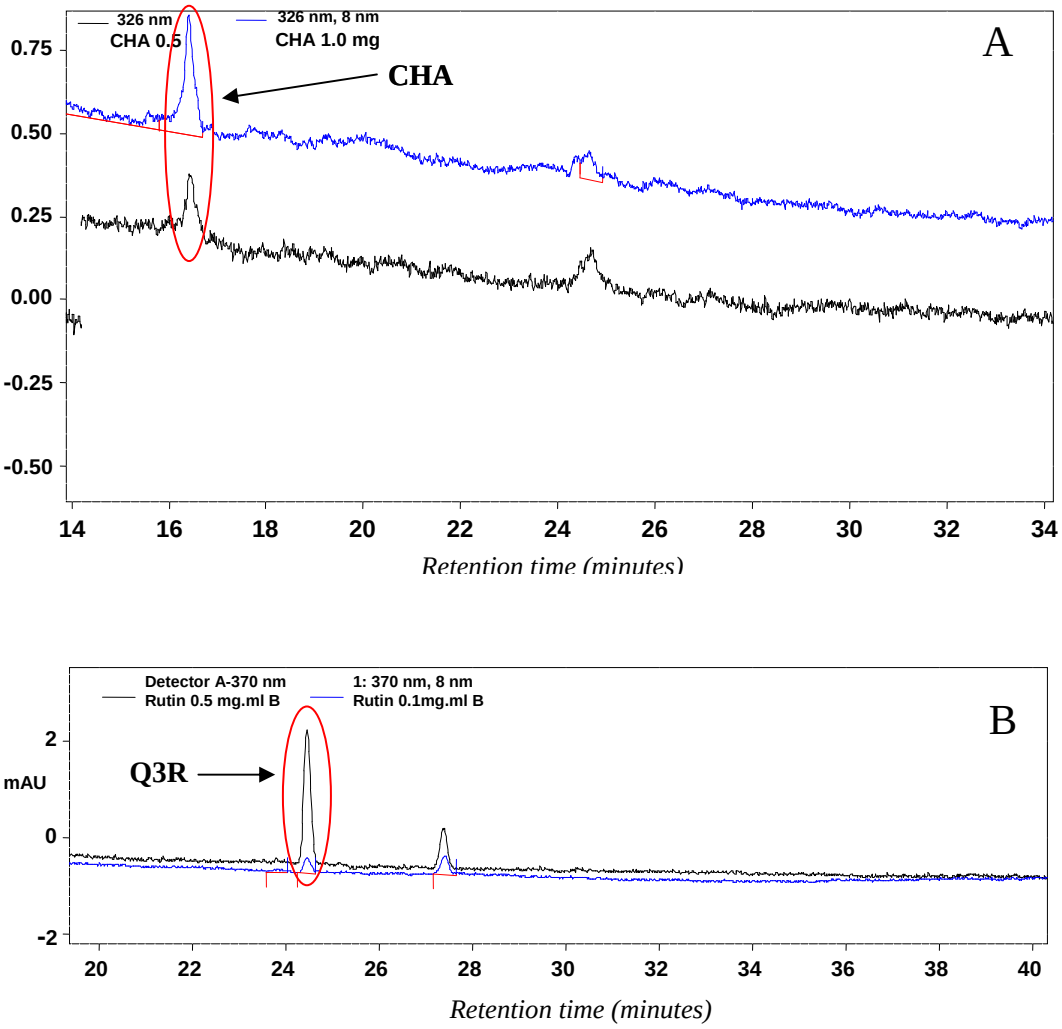


Figure A2 **High performance liquid chromatography of Caco-2 cell monolayer extract after cell exposure of 4 hours to: A) chlorogenic acid at concentrations 0.5 mg/mL and 1.0 mg/mL, and B) quercetin 3-rutinoside at concentrations of 0.1 and 0.5 mg/mL.**

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Potential Physiological Activities of Selected Australian Herbs and Fruits

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The aim of this project was to investigate the potential health benefits arising from consumption of selected commercially grown Australian native species of primary importance for the industry: Tasmannia pepper leaf, anise myrtle, lemon myrtle, quandong and Davidson's plum. This work complements recent studies on health attributes of Kakadu plum, Illawarra plum, muntries and native currant. Jointly these studies represent the first evaluation of specific health-enhancing properties of commercially grown native Australian herbs and fruits.

This report is targeted at the Australian native food industry, the general food, pharmaceutical and cosmeceutical industries and the health-conscious consumer.

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