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Cytoprotective and pro-apoptotic activities of native Australian herbs polyphenolic-rich extracts

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ABSTRACT

Three commercially grown native herbs unique to Australia, Tasmannia pepper leaf (*Tasmannia lanceolata* R. Br., Winteracea; TPL), anise myrtle (*Syzygium anisatum* Vickery, Craven & Biffen, Myrtaceae; AM) and lemon myrtle (*Backhousia citriodora* F. Muell, Myrtaceae; LM) as well as a reference sample bay leaf (*Laurus nobilis* L., Lauraceae; BL) were examined for potential cytoprotective properties. All native herbs exhibited greater cellular antioxidant activity as measured by the cellular antioxidant activity (CAA) assay than bay leaf and reduced the hydrogen peroxide (H_2O_2) induced death of hepatocellular carcinoma (HepG2) cells by 25–50%. All herb extracts reduced the proliferation of colon (HT-29; IC_{50} = 0.75–1.39 mg/ml), stomach (AGS; IC_{50} = 0.59–1.88 mg/ml), bladder (BL13; IC_{50} = 0.56–1.12 mg/ml) and liver (HepG2; IC_{50} = 0.38–1.36 mg/ml) cancer cells. No significant reduction of cell viability of non-transformed colon (CCD-18Co; IC_{50} > 2.0 mg/ml) and mixed stomach and intestine (Hs 738.St/Int; IC_{50} > 2.0 mg/ml) cells was observed. Flow cytometry analysis and the results of the cytokinesis block micronucleus cytome (CBMNCyt) assay conducted with respectively, promyelocytic leukaemia (HL-60) and colon adenocarcinoma (HT-29) cells suggest an increase in apoptosis following treatment with the herb extracts. The occurrence of apoptotic cells coincided with an increase in caspase-3 enzyme activity. The results of the CBMNCyt assay suggested no direct DNA damage in colon adenocarcinoma (HT-29) cells as a result of treatment with all extracts, applied at final concentrations of 0.5 and 1.0 mg/ml.

1. Introduction

Oxidative stress is the result of a homeostatic imbalance between the generation of reactive oxygen and nitrogen species (RONS) and a decrease in either free radical scavenging or the mechanisms used to repair oxidised macromolecules. This may lead to cellular dysfunction and eventual cell death following damage to various cell targets such as DNA, lipids and proteins (Liu & Finley, 2005). Such molecular damage events have been linked to many chronic diseases including cancer (Klaunig & Kamendulis, 2004) cardiovascular disease, diabetes and inflammatory diseases such as inflammatory bowel disease (Eberhardt & Jeffery, 2006; Evans, Dizdaroglu, & Cooke, 2004; Halliwell, 2007). Overwhelming epidemiological evidence strongly correlates the regular consumption of fruits and vegetables with a lower incidence of certain chronic diseases including cardiovascular disease and cancer. This part is thought to be attributed to the cellular protective abilities of naturally occurring antioxidants, which are able to efficiently scavenge RONS, thereby potentially reducing risk of the onset of oxidative stress related diseases (Babich, Schuck, Weisburg, & Zuckerbraun, 2011). Beside the damaging effects on DNA and other cellular molecules, it has been suggested that RONS suppress apoptosis, promote proliferation, invasiveness and metastasis (and possibly angiogenesis), which are the subsequent steps in cancer development and progression (Halliwell, 2007).

Apoptosis, also known as programmed cell death, is a key pathway for normal cellular development and homeostasis. The process can be triggered by a wide variety of physiological and pathological stimuli (Elmore, 2007), associated with morphological changes such as cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation (Hengardner, 2000). Caspases are a family of cysteine proteases that play a key role in the apoptotic process and are highly conserved during development and aging. Caspase-3, the effector caspase in apoptosis, represents a convergence point for two different caspase-dependent apoptotic pathways: the mitochondrial (intrinsic) pathway and the death receptor (extrinsic) pathway. The direct activation of such executioner caspases is thought to be an anticancer strategy which may prove beneficial in treating many cancers in which procaspase-3 concentrations are elevated (Putt et al., 2006).

Selected polyphenols such as quercetin (Kim et al., 2010) and crude extracts of fruits such as strawberry and plum (Ramos, Alia, Bravo, & Goya, 2005) present in the human diet have been shown

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to be effective in the suppression of the proliferation of HT-29 and HepG2 cancer cells, involving the induction of apoptosis. Epigallocatechin-3-gallate (EGCG) isolated from green tea induced-apoptosis in human prostate carcinoma LNCaP cells, shifting the balance between pro- and anti-apoptotic proteins in favour of apoptosis (Hastak et al., 2003). EGCG has also been found to inhibit cellular proliferation of human laryngeal epidermoid carcinoma Hep2 cells through a p53-mediated mitochondrial pathway which is a caspase-independent pathway (Lee et al., 2010).

Australia has been isolated for thousands of years and thus the local plants have developed in isolation to suit the often harsh natural conditions. The native Australian flora is among the most diverse in the world due to the wide range of different environments and plant communities. Commercially grown native Australian herbs and spices contain high levels of phenolic compounds, especially flavonoids, phenolic acids, and tannins (Konczak, Zabaras, Dunstan, & Aguas, 2010; Sakulnarmrat & Konczak, 2012). Commercial production of these herbs has been initiated in 1990's and currently in Australia they are available in supermarkets and specialty shops, are used in seasoning and in preparation of main meals. Additionally, due to their unique flavour they are included into herbal infusions. The endemic Tasmannia pepper, due to its pungency, is used in cooking and as an additive in processed foods, e.g. cheese. As the production and the consumption of these herbs steadily increases there is a need to understand their effect on human health. The present study focused on the evaluation of potential health-enhancing properties of purified polyphenol-rich extracts obtained from three major commercially grown herbs: Tasmannia pepper leaf, anise myrtle and lemon myrtle. Previous studies reported high antioxidant capacities of polyphenolic-rich extracts obtained from these herbs and their dose-dependent inhibitory activities towards the isolated enzymes: α-glucosidase, pancreatic lipase and angiotensin I-converting enzyme (Sakulnarmrat & Konczak, 2012). Within this study potential cellular-protective and pro-apoptotic activities against human cancer cell lines, especially associated with the digestion system, were investigated. DNA damage events, cytostasis and cytotoxicity of these native Australian herbs polyphenols were assessed by the cytokinesis block micronucleus cytome (CBMNCyt) assay (Fenech, 2007).

2. Materials and methods

2.1. Plant material

Commercial samples of dry Tasmannia pepper leaf (*Tasmannia lanceolata* R. Br., Winteracea) were obtained from the Diemen Pepper Company (Tasmania, Australia). Commercial samples of anise myrtle (*Syzygium anisatum* (Vickery, Craven & Biffen, Myrtaceae) and lemon myrtle (*Backhousia citriodora* F. Muell, Myrtaceae) were obtained from Australian Rainforest Products (New South Wales, Australia). Bay leaf (*Laurus nobilis* L., Lauraceae) from Hoyts Food Industries Pty Ltd., Moorabbin, Victoria, Australia) was obtained commercially and included as a reference sample.

2.2. Chemicals

2′,7′-Dichlorofluorescein-diacetate (DCFH), 2,2′-azobis (2-amidinopropane dihydrochloride) (ABAP), dimethyl sulfoxide (DMSO), fluorescein, hydrogen peroxide (H_2O_2), caspase 3 assay kit colorimetric, depex mounting medium, Hanks' balance Salt Solution (HBSS), methanol, ethanol, NaOH, trifluoroacetic acid (TFA), XAD-16 resins, NaH_2PO_4, KH_2PO_4, NaCl, KCl, quercetin and camptothecin were obtained from Sigma–Aldrich, Inc. (Sydney, Australia). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin, streptomycin and alexa fluor 488 annexin V-kit were purchased from Invitrogen (Melbourne, Australia). Hemacolour rapid staining kit was obtained from Merck (USA).

2.3. Preparation of polyphenolic-rich extracts

The polyphenolic-rich extracts were prepared as described previously (Sakulnarmrat & Konczak, 2012).

2.4. Cell lines

All cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured at 37 °C in a humidified 5% carbon dioxide (CO_2) atmosphere in media containing 10% foetal bovine serum (FBS; Invitrogen Corporation, Carlsbad, CA, USA), 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 (Invitrogen Corporation, Carlsbad, CA, USA); BL13 (bladder cancer) was cultured in RPMI 1640 medium (Invitrogen, Australia). CCD-18Co (colon normal) and HepG2 (hepatocellular carcinoma) were cultured in Eagle's minimum essential medium (EMEM; Sigma–Aldrich); Hs 738.St/Int (mixed stomach and intestine normal) and RAW 264.7 (murine macrophage) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corporation, Carlsbad, CA, USA); HT-29 (colorectal adenocarcinoma) was cultured in McCoy's 5a medium (Invitrogen, Australia); HL-60 (acute promyelocytic leukaemia) was cultured in Iscove's modified Dulbecco's medium (Invitrogen, Australia). Experiments were conducted in cell lines with less than 40 passages.

2.5. Cell viability assay

The colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Invitrogen Australia Pty Ltd., Mulgrave, Victoria, Australia) assay was used to assess the sensitivity of AGS, HT-29, CCD-18Co, Hs 738.St/IntBL13 and HepG2 cells to native Australian herbs extracts. The cells (5×10^5/ml) were incubated for 24 h at 37 °C in 96-well clear-walled flat bottomed microplates (Thermo Fisher Scientific) before treatment with a range of concentrations (final concentration 0.0–2.0 mg/ml) of purified polyphenolic-rich extracts over the following 23 h. The polyphenolic-rich extracts applied in this study were water-soluble, and no additional solvents (that could affect cell growth) were required to carry out the experiments. In order to avoid the effect of sample's colour on the final reading, the medium (containing sample) was removed, wells were gently washed with warm (37 °C) PBS to remove any traces of samples; then 100 μl of phosphate buffered saline (PBS) and 5 μl of MTT stock solution (5 mg/ml) were added. The culture was further incubated for 4 h. Next, the microplate was drained and the resulting MTT formazan product dissolved in DMSO. The plate was shaken for 10 min and the absorbance measured (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 separately on two occasions. The results were expressed as the optical density ratio of the treatment to control.

2.6. Cellular antioxidant activity (CAA) assay

The assessment of CAA was conducted using the HepG2 cell line, as previously described (Tan, Konczak, Ramzan, & Sze, 2011a). The results were expressed as quercetin equivalent per gramme of dry weight (mg QE/gDW) of purified polyphenolic-rich lyophilised extracts and EC_{50} (the amount of extract necessary to reduce the oxidative stress in 50%).