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Antioxidant and cytoprotective activities of native Australian fruit polyphenols

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ABSTRACT

Four native Australian fruits, 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*) were examined for antioxidant and cellular protective activities. Each fruit showed significantly greater antioxidant activity than a blueberry (*Vaccinum sp.*, cv. Biloxi) reference with Kakadu Plum exhibiting 13.3-fold and 2.4-fold activity of blueberry in the ferric ion reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC-H) assays, respectively. A lyophilised polyphenolic-rich extract of Kakadu Plum exhibited the greatest cellular antioxidant activity (CAA assay) of 71.5 μmol QE/g, and was followed by Illawarra Plum, Native Currant and Muntries (46.3, 20.0 and 14.4 μmol QE/g, respectively). Polyphenolic-rich extracts of Kakadu Plum and Muntries (but not Illawarra Plum and Native Currant) extracts efficiently protected RAW 264.7 cells against hydrogen peroxide induced apoptosis in a dose-dependent manner. Kakadu Plum and Native Currant polyphenolic-rich extracts increased the Nrf2/Keap1 ratio, suggesting activation of the antioxidant response element (ARE) through the Nrf2/Keap1 complex. The results suggest Kakadu Plum exhibits the greatest antioxidant potential, exerting antioxidant activity through free radical scavenging and affecting two (Nrf2/Keap1) downstream transcription factors.

1. Introduction

Native Australian fruits provide significant potential sources of novel antioxidant compounds for use as pharmaceuticals or functional foods. The extensive history of the local indigenous population has allowed a constant refinement of knowledge on the properties and effectiveness of various plant and food sources (Lassak & McCarthy, 2001). Therefore, traditionally consumed fruits may have potential for positive health benefits.

Four native Australian fruits, representing a diverse range of botanical environments and among the most popular fruits consumed traditionally in their native regions were selected for this study. The fruits were 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*). Kakadu Plum was considered by certain tribes more as a medicine than a food (Isaacs, 1996). These fruits, except Native Currant, have been selected based on our preliminary antioxidant testing of a range of native Australian fruits, as they contain high levels of polyphenols and exhibit strong antioxidant activity in a range of reagent-based antioxidant activity assays (Konczak, Zabaras, Xiao, Shapira, & Lee, 2008; Netzel, Netzel, Tian, Schwartz, & Konczak, 2006, 2007). Additionally Illawarra Plum has exhibited anti-proliferative and apoptotic activity in various cancer cell lines (Konczak et al., 2008).

Oxidative stress is an imbalance in the redox status of a cell, between the production of reactive oxygen species (ROS) and antioxidant defence mechanisms, leading to damage, potential mutations and ultimately the formation of cancer (Halliwell, 2007). Defence against oxidative stress is therefore an important factor in preventing the development of many diseases. Dietary polyphenols are potent antioxidants, able to scavenge and intercept free radicals, preventing damage to cellular molecules (Moskaug et al., 2005). Antioxidant action however, is not limited to ROS scavenging, and includes the upregulation of antioxidant and detoxification enzymes, modulation of cell signalling and gene expression and other cellular effects (Eberhardt & Jeffery, 2006). One particular transcription factor, nuclear factor-erythroid 2p45 (NF-E2)-related factor 2 (Nrf2), and its interaction with the antioxidant response element (ARE), has been shown to promote many antioxidant and drug-metabolising enzymes (Yu & Kensler, 2005). Accordingly, polyphenols which are able to interact with these pathways are likely to exhibit beneficial physiological activities.

The present study aims to evaluate the cytoprotective potential of a few selected native Australian fruits by examining the most

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significant aspect of antioxidant activity through a comprehensive array of antioxidant assays with an objective to utilise these sources for the development of functional foods for the protection against antioxidant stress. In particular, this study provides a detailed in-depth investigation of the related downstream mechanisms including the expression of the antioxidant enzymatic pathway with two (Nrf2 and Kelch-like ECH-associated protein 1 (Keap1)) downstream transcription factors. The major polyphenols extracted from the selected native Australian fruits were also characterised by HPLC-DAD and LC-PDA-MS/MS.

2. Materials and methods

2.1. Collection of fruit material

Illawarra Plum, Kakadu Plum and Muntries were purchased from Tanamera Native Produce Pty Ltd., Obum Obum, Qld, Australia. Native Currant was provided by Dr Maarten Ryder from CSIRO Sustainable Ecosystems, Urrbrae, SA, Australia. Blueberry (*Vaccinum* spp. cv. Biloxi, *Ericaceae*) was obtained from Blueberry Farms of Australia, Corrindi, NSW, Australia.

2.2. Preparation of crude methanolic extracts

Freeze-dried fruits for the Folin–Ciocalteau, FRAP and ORAC assays were finely ground and extracted using acidified methanol as described previously (Konczak et al., 2008). Briefly, a 200 mg portion was extracted with 3 ml of acidified methanol (80% methanol, 19.9% H_2O and 0.1% HCl, v/v/v) and sonicated for 15 min at room temperature. The extracts were centrifuged for 10 min at 10,000×*g* and the supernatant collected. The pellet was re-extracted another two times. The combined supernatants (9 ml) were immediately utilised in the Folin–Ciocalteau, FRAP and ORAC antioxidant assays.

2.3. Folin–Ciocalteau assay

The total phenolic content of the native fruits was determined using the Folin–Ciocalteau assay as described previously (Netzel et al., 2007). The total phenolic content of the samples was expressed as gallic acid equivalents per g of dry weight (μmol GAE/g DW) based on a gallic acid standard curve, and standardised against a blank control in sextuplicate wells.

2.4. Ferric ion reducing antioxidant power (FRAP) assay

Total reducing capacity was determined using the FRAP assay (Benzie & Strain, 1996), as described previously (Netzel et al., 2006). The reducing capacity of the samples was expressed as μmol of Iron (II) per g of dry weight (μmol Fe^{2+}/g DW) based on an Iron (II) sulphate standard curve, and standardised against a blank control in sextuplicate wells.

2.5. Oxygen radical absorbance capacity (ORAC-H) assay for hydrophilic compounds

Oxygen radical scavenging capacity was determined using the ORAC assay as described previously (Konczak et al., 2008). The antioxidant capacity of the samples was expressed as μmol of Trolox per g of dry weight (μmol Trolox/g DW) based on a Trolox standard curve.

2.6. Preparation of purified polyphenolic extracts

The weighed raw fruit material of the four native fruits was ground into a pulp using a heavy duty blender. A 2-fold volume of acidified methanol (80% methanol, 19.9% H_2O and 0.1% HCl, v/v/v) was then added, stirred for 15 min and centrifuged for 25 min at 11,000×*g* at 4 °C with the supernatant collected. An equivalent volume of acidified methanol was re-added to the fruit material and left overnight at 4 °C. The process of addition of acidified methanol, centrifugation and collection of supernatant was conducted three times. The supernatant from the consecutive extractions were combined and the solvent evaporated under reduced pressure at 37 °C to produce a methanolic extract.

The methanolic extract was further purified using an XAD-7HP (Sigma-Aldrich, St. Louis, MO) resin column (300×60 mm i.d.). The extract was dissolved with acidified water (99% H_2O, 1% acetic acid, v/v), applied to the column, washed with acidified water and eluted with 70% ethanol (70% ethanol, 29% H_2O, 1% acetic acid, v/v/v). The eluate was collected and evaporated under reduced pressure at 37 °C. The purification was repeated, with 80% methanol (80% methanol, 19.9% H_2O, 0.1% trifluoroacetic acid, v/v/v), and the eluate was collected and evaporated as previously described. The resulting fraction was dissolved in purified water and freeze-dried under vacuum to obtain a fine lyophilised powder rich in polyphenols.

2.7. Quantification (HPLC-DAD) and identification (LC-PDA-MS/MS) of phenolic compounds

Quantification of phenolic compounds and anthocyanins in the purified polyphenolic extracts was conducted according to Kammerer, Claus, Carle, and Schieber (2004) with minor modifications. The HPLC system consisted of two LC-10AD pumps, SPD-M10A diode array detector, CTO-10AS column oven, DGU-12A degasser, SIL-10AD auto-injector, and SCL-10A system controller (Shimadzu Corporation, Kyoto, Japan) equipped with a 250×4.6 mm i.d., 5 μm Luna C18(2) column (Phenomenex, Torrance, CA). Analytical HPLC was run at 25 °C and monitored at 280 nm, 320 nm and 370 nm for phenolic compounds and specifically at 520 nm for anthocyanins.

For phenolic compounds the following solvents in purified water with a flow rate of 1.0 ml/min were used: A, 2% acetic acid and B, 1% acetic acid and 50% acetonitrile. The elution profile was a linear gradient elution for B of 10% to 24% over 30 min in solvent A, to 30% B in 10 min, 50% B in 15 min and then to 100% B in 1 min. Total phenolic compounds, including anthocyanins were calculated as μg of gallic acid equivalents (GAEs) per mg of dry weight (μg GAEs/mg DW).

For the specific quantification of anthocyanins, the following solvents in purified water with a flow rate of 1.0 ml/min were used: A, 1.5% phosphoric acid and B, 1.5% phosphoric acid, 20% acetic acid and 25 % acetonitrile. The elution profile was a linear gradient elution for B of 25% to 40% over 30 min in solvent A, to 100% B in 4 min. Anthocyanins were calculated as μg of cyanidin 3-glucoside equivalents (CEs) per mg of dry weight (μg CEs/mg DW).

LC-PDA-MS/MS analysis was conducted on a Quantum triple stage quadrupole (TSQ) mass spectrometer (ThermoFinnigan, NSW, Australia) as described previously (Konczak et al., 2008).

2.8. Cell culture

Cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were cultured at 37 °C in a humidified 5% CO_2 atmosphere in media containing 10% foetal bovine serum (FBS), 100 μg/ml streptomycin and 100 units/ml penicillin (Invitrogen Corporation, Carlsbad, CA). Hep G2 (hepatocellular carcinoma) was cultured in Eagle's minimum essential medium (EMEM; Invitrogen Corporation); and RAW 264.7 (murine macrophage) was cultured in Dulbecco's Modified Eagle's medium (DMEM; Invitrogen Corporation).

2.9. Cellular antioxidant activity (CAA) assay

Antioxidant activity was determined using the CAA assay with Hep G2 cells as described previously (Kelly L. Wolfe & Liu, 2007). Briefly, Hep

G2 cells ($1x10^5$/ml) were treated with a range of concentrations of purified polyphenolic extracts and 25 μM 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Sigma-Aldrich) in PBS and incubated for 1 h. Next, 600 μM of 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP; Sigma-Aldrich) in 100 μl of Hanks' Balanced Salt Solution (HBSS; Sigma-Aldrich) was added and fluorescence measured every 5 min for 1 h, with excitation at 485 nm and emission at 535 nm (Wallac 1420 Multilabel Counter; PerkinElmer, Waltham, MA). The antioxidant capacity of the samples was expressed as μmol of quercetin (Sigma-Aldrich) equivalents per g of dry weight (μmol QE/g DW) based on a quercetin control.

2.10. Cellular protection against H_2O_2 induced cell death

Cell sensitivity of RAW 264.7 cells to native Australian fruit extracts was determined via cell viability using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Invitrogen Corporation). Initially, cells ($5x10^5$/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, followed by the addition of hydrogen peroxide (H_2O_2; final concentration 100 μM) for a further 1 h. Wells were then washed with PBS; 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).

2.11. Preparation of cell lysates

Preparation of cell lysates from Hep G2 cells was conducted according to Tanigawa, Fujii, and Hou (2007) with minor modifications. Briefly Hep G2 cells were treated with a range of concentrations of purified polyphenolic extracts for 9 h. Cells were then lysed in modified RIPA buffer. The lysates were homogenised in an ultrasonicator and centrifuged for 15 min at 15,000×*g* at 4 °C. The supernatants were collected and the protein concentration evaluated using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Equivalent amounts of protein were boiled at 100 °C in SDS buffer for 10 min to produce whole-cell lysates.

2.12. Western blot analysis

The whole-cell lysates containing bromophenol blue were run on a 4–12% Bis-Tris gel (NuPAGE; Invitrogen Corporation) and transferred to PVDF membrane using the iBlot Gel Transfer System (Invitrogen Corporation). Following blotting, using the SNAP i.d. Protein Detection System (Millipore Corp., Bedford, MA), the membrane was blocked, incubated with primary antibody, washed, incubated with secondary antibody, washed and incubated with chemiluminescent alkaline phosphate (AP) substrate (Immobilon; Millipore Corp.). Blocking buffer consisted of Tris buffered saline containing 0.1% Tween-20 (TBS/T) and 1% BSA, and washing buffer consisted of TBS/T. Primary antibodies against Nrf2, Keap1 and α-tubulin, and AP-conjugated anti-goat, anti-mouse and anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Bound antibodies were then detected and the relative amounts of proteins associated with specific antibody were quantified using a chemiluminescent imager (FluorChem SP; Alpha Innotech, San Leandro, CA).

2.13. Statistical analyses

The results are expressed as mean ± SD of at least three independent experiments. Differences between different treatment groups and the control counterparts were determined using one-way ANOVA with Tukey's post-hoc test (GraphPad, San Diego, CA), with $p<0.05$ as statistically significant.

3. Results and discussion

3.1. Antioxidant capacity of Australian native fruits

In this study, to bioprospect four selected native Australian fruits for their antioxidant potential, a range of assays were used which represent two different mechanisms of quantifying antioxidant capacity. The FRAP and Folin–Ciocalteu assays are based on electron transfer mechanisms and measure the total concentration of redox-active compounds (Prior, Wu, & Schaich, 2005). The FRAP assay does not measure thiol antioxidants such as glutathione and is preferred by some researchers as a measure of antioxidant capacity (Halvorsen et al., 2006). The ORAC assay, based on hydrogen atom transfer reaction, indicates the ability of fruit extracts to scavenge specifically the oxygen free radicals, and as such is most relevant to human biology among all reagent-based assays (Magalhaes, Segundo, Reis, & Lima, 2008).

Antioxidant capacities of the four selected native Australian fruits are presented in Table 1. Commercially available Australian-grown blueberry has been included as a reference sample, due to the recognition for a high antioxidant potential and related health benefits of blueberries (Faria et al., 2005). The results of the FRAP assay revealed Kakadu Plum exhibited the greatest antioxidant capacity (1333% of that of blueberry), followed by Native Currant (369%), Illawarra Plum (254%) and Muntries (153%). Similarly, the ORAC assay revealed larger oxygen radical scavenging capacities for all four native fruits compared to the blueberry reference. The differences between samples however were not as significant. Kakadu Plum again demonstrated the greatest antioxidant capacity (236% of that of blueberry), followed by Native Currant (182%), Illawarra Plum (144%) and Muntries (113%). These results are in agreement with earlier findings (Konczak et al., 2008; Netzel et al., 2007) and suggest that with regards to antioxidant capacity, independent fruit samples obtained in different years display similar properties.

The Folin–Ciocalteau assay, recognised as one of the standard antioxidant testing procedures (Prior et al., 2005), measures the level of total phenolics in natural products based on oxidation/reduction mechanisms. Similar to the results obtained in FRAP and ORAC assays, each of the four native Australian fruits demonstrated greater Folin–Ciocalteu values than blueberry (Table 1). The Folin–Ciocalteu values of Kakadu Plum were the highest (1936% of that of blueberry), followed by Native Currant (380%), Illawarra Plum (241%) and Muntries (128%).

We compared our results with those from a comparable study by Proteggente et al. (2002) on the antioxidant activity of regularly consumed fruits and vegetables, including strawberry, raspberry and apple. The FRAP and ORAC values for each native Australian fruit are significantly greater than all fruits and vegetables tested, when adjusted to equivalent units. The results also compared favourably with studies on grape lines, and Chinese herbal preparations (Jacob, Hakimuddin, Paliyath, & Fisher, 2008; Liu, Qiu, Ding, & Yao, 2008). Although an interference of other compounds (sugars, vitamin C, organic acids, etc.) needs to be considered, the results described above may suggest that phenolic compounds present in the fruits may contribute towards their antioxidant capacities. In order to confirm this hypothesis, phenolic compounds were isolated, quantified and their identity was investigated.

3.2. Extraction and quantification of phenolic compounds from Australian native fruits

The hydrophilic extraction of native Australian fruits, followed by purification using an open column chromatography, has yielded five water soluble fractions, which were further evaluated for the presence of phenolic compounds. The HPLC-DAD analysis revealed that the Kakadu Plum extract contained the highest level of total

Table 1
Total phenolics and antioxidant capacity (FRAP, ORAC and CAA) of four native Australian fruits and blueberry (cv. Biloxi).

	FRAP[a] (μmol Fe^{2+}/g DW)	ORAC[b] (μmol Trolox/g DW)	Total phenolics (μmol GAE/g DW)	CAA[c] (μmol of QE/g purified extract)
Blueberry	340.4 ± 18.1 (100) a	770.0 ± 126.5 (100) a	186.1 ± 10.8 (100) a	–
Illawarra Plum	864.2 ± 156.8 (254) b	1111.1 ± 138.4 (144) ab	449.2 ± 90.0 (241) bc	46.3 ± 3.9 a
Kakadu Plum	4538.4 ± 105.8 (1333) d	1816.6 ± 49.6 (236) c	3602.4 ± 109.1 (1936) d	71.5 ± 11.3 b
Muntries	520.0 ± 16.9 (153) ab	866.7 ± 137.9 (113) a	237.9 ± 7.4 (128) ab	14.4 ± 2.1 c
Native Currant	1255.6 ± 24.4 (369) c	1402.1 ± 78.1 (182) b	706.8 ± 35.1 (380) c	20.0 ± 0.5 c

[a] Data represents the mean ± standard deviation of at least three independent experiments. The data in parentheses (%) represent values normalised to blueberry (cv. Biloxi). Values in each column were separately analysed using ANOVA and post-hoc analysis. Values with no letters in common are significantly different from other values in the same column ($p<0.05$). FRAP, ferric ion reducing antioxidant power.
[b] ORAC, oxygen radical absorbance capacity.
[c] CAA, cellular antioxidant activity.

phenolic compounds (815.8 ± 184.4 μg GAEs/mg DW), followed by Illawarra Plum (429.6 ± 23.9), Muntries (274.5 ± 0.1) and Native Currant (66.9 ± 0.1).

Anthocyanins were identified in the Illawarra Plum, Native Currant and Muntries extracts. The highest level was recorded for Illawarra Plum (447.3 ± 19.6 μg CEs/mg DW), followed by Native Currant (33.4 ± 1.3) and Muntries (1.1 ± 0.04). The LC/MS evaluation (Fig. 1) of the extract revealed that the main anthocyanin of Illawarra Plum is cyanidin 3-glucoside (422.0 ± 4.4). Traces of delphinidin 3-glucoside (4.6 ± 0.05) and pelargonidin 3-glucoside (20.7 ± 24.1) were also present. This result is in agreement with earlier reports which identified cyanidin 3-glucoside in Illawarra

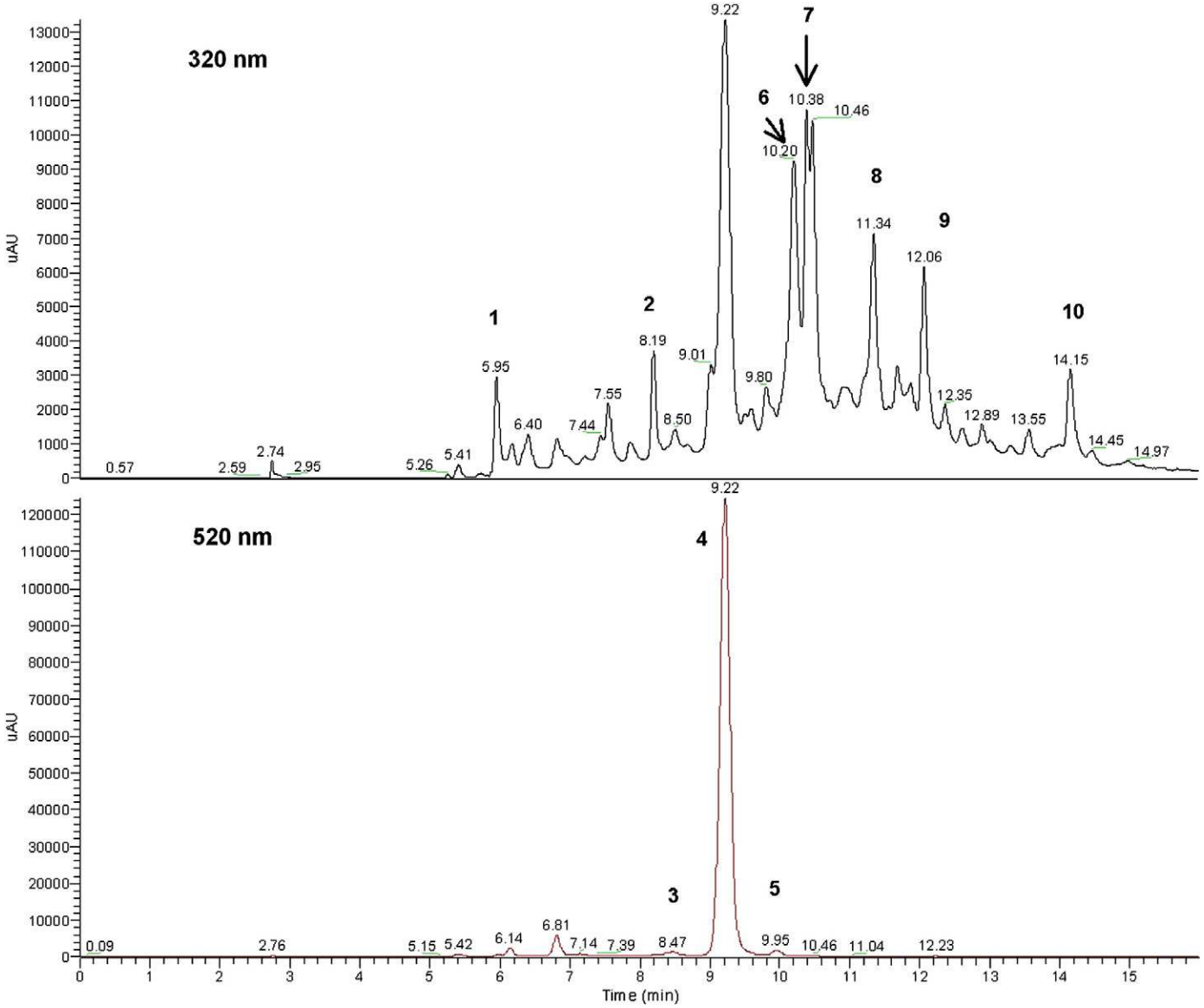

Fig. 1. Identification and quantification of phenolic compounds detected in the lyophilised Illawarra extract. 1: unknown, MS fragmentation (precursor, product) $[463]^-$, 301; concentration: 3.3 μg/g; 2: unknown, $[465]^-$, -; 3.3 μg/g; 3: delphinidin 3-glucoside, $[465]^+$, 303, 1.8 μg/g; 4: cyanidin 3-glucoside, $[449]^+$, 287, 330 μg/g; 5: pelargonidin 3-glucoside, $[431]^+$, 271, 6.0 μg/g; 6: unknown, $[593]^-$, -, 9.9 μg/g; 7: luteolin/kaempferol glucoside, $[447]^-$, 285, 22.5 μg/g; 8: unknown, $[463]^-$, 9.3 μg/g; 9: quercetin glucoside, $[463]^-$, 301, 5.8 μg/g and 10: unknown, 3.6 μg/g.

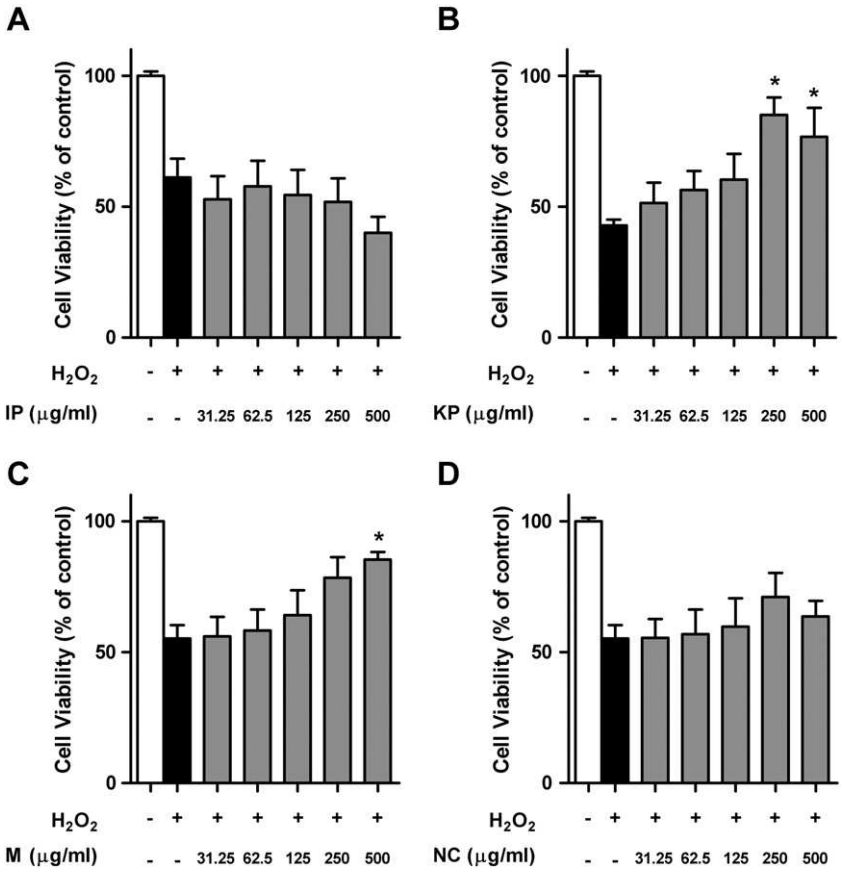

Fig. 2. Effect of purified polyphenolic extracts on H_2O_2-induced cell death in RAW 264.7 macrophages using the MTT assay. The cells were treated with different concentrations of purified polyphenolic extracts for 23 h followed by the addition of H_2O_2 (100 μM) for a further 1 h, and the viability of the cells determined by MTT assay. The data represents the mean ± standard deviation of at least three independent experiments. An asterisk indicates significant difference with H_2O_2 only treated cells ($p<0.05$). (A) IP, Illawarra Plum. (B) KP, Kakadu Plum. (C) M, Muntries. (D) NC, Native Currant.

Plum (Netzel et al., 2006). The UV trace at 320 nm highlighted the presence of several other minor components such as a quercetin glucoside, a luteolin or kaempferol (negative product m/z 285) glucoside, as well as rutin (negative parent m/z 609, product mz 301; 0.11 μg/g).

The main anthocyanins detected in the Native Currant extract, were cyanidin 3-glucoside (15.3 ± 0.5), cyanidin 3-rutinoside (6.1 ± 0.3), delphinidin 3-glucoside (4.1 ± 0.2) and delphinidin 3-rutinoside (8.0 ± 0.3). The Muntries extract contained minor levels of cyanidin 3-glucoside (0.8 ± 0.05) and delphinidin 3-glucoside (0.3 ± 0.01). The Kakadu Plum extract contained no anthocyanins, however the HPLC-DAD chromatogram of this extract revealed the presence of a very rich mixture of compounds detected mainly at 280 nm. This indicates the presence of compounds such as benzoic acids, catechins and proanthocyanidins. Earlier studies of Kakadu Plum with LC/MS tentatively identified quercetin (or hesperitin) glucosides as wells as kaempferol (or luteolin) glycosides with catechin-based hexose-containing glycosides also in the mixture (Konczak, Zabaras, Dunstan, & Aguas, 2010).

3.3. Cellular antioxidant activity of Australian native fruits

The reagent-based antioxidant assays are useful in screening samples efficiently. However, they also have significant limitations with an inability to accurately predict activity *in vivo*. Use of cell culture models to assess antioxidant activity helps to address some of the limitations of chemical based antioxidant assays. Cell culture models consider issues of uptake, distribution and metabolism, whilst being cost-effective and relatively rapid (Kelly L. Wolfe & Liu, 2007). The CAA assay was conducted to provide a more appropriate evaluation of antioxidant activity in a biological system. The results of the CAA assay (Table 1) revealed that the purified polyphenolic extract of Kakadu Plum exhibited the greatest cellular antioxidant activity with an EC_{50} value (μg/ml) of 153.0 ± 24.5, which was significantly lower than all the other samples. Illawarra Plum had the next lowest EC_{50} value (233.2 ± 20.2) followed by Native Currant (537.9 ± 12.0) and Muntries (758.0 ± 103.8). These results indicate that Kakadu Plum may have the greatest antioxidant activity within this biological system, followed by Illawarra Plum.

The cellular antioxidant activity of Kakadu Plum in particular is significantly greater than extracts from a range of commonly consumed fruits including wild blueberry, strawberry, raspberry, cranberry and apple (K.L. Wolfe et al., 2008) and extracts from various legumes such as green pea, yellow pea, chickpea and lentil (Xu & Chang, 2009; Xu & Chang, 2010), although the method of extraction or cell line differed slightly. Pomegranate and blackberry however, were found to have higher CAA values than Kakadu Plum (K.L. Wolfe et al., 2008). Specific polyphenols, such as luteolin, myricetin and quercetin-3-β-D-glucoside, have also been demonstrated to have significant cellular antioxidant activity (K.L. Wolfe & Liu, 2008).

3.4. Cellular protection of RAW 264.7 cells from hydrogen peroxide induced apoptosis and upregulation of antioxidant enzymes (Nrf2/Keap1 ratio)

Hydrogen peroxide is an important ROS in oxidative stress. To characterise the potential cytoprotective effects of native Australian fruits, the cell viability of H_2O_2-exposed RAW 264.7 cells was

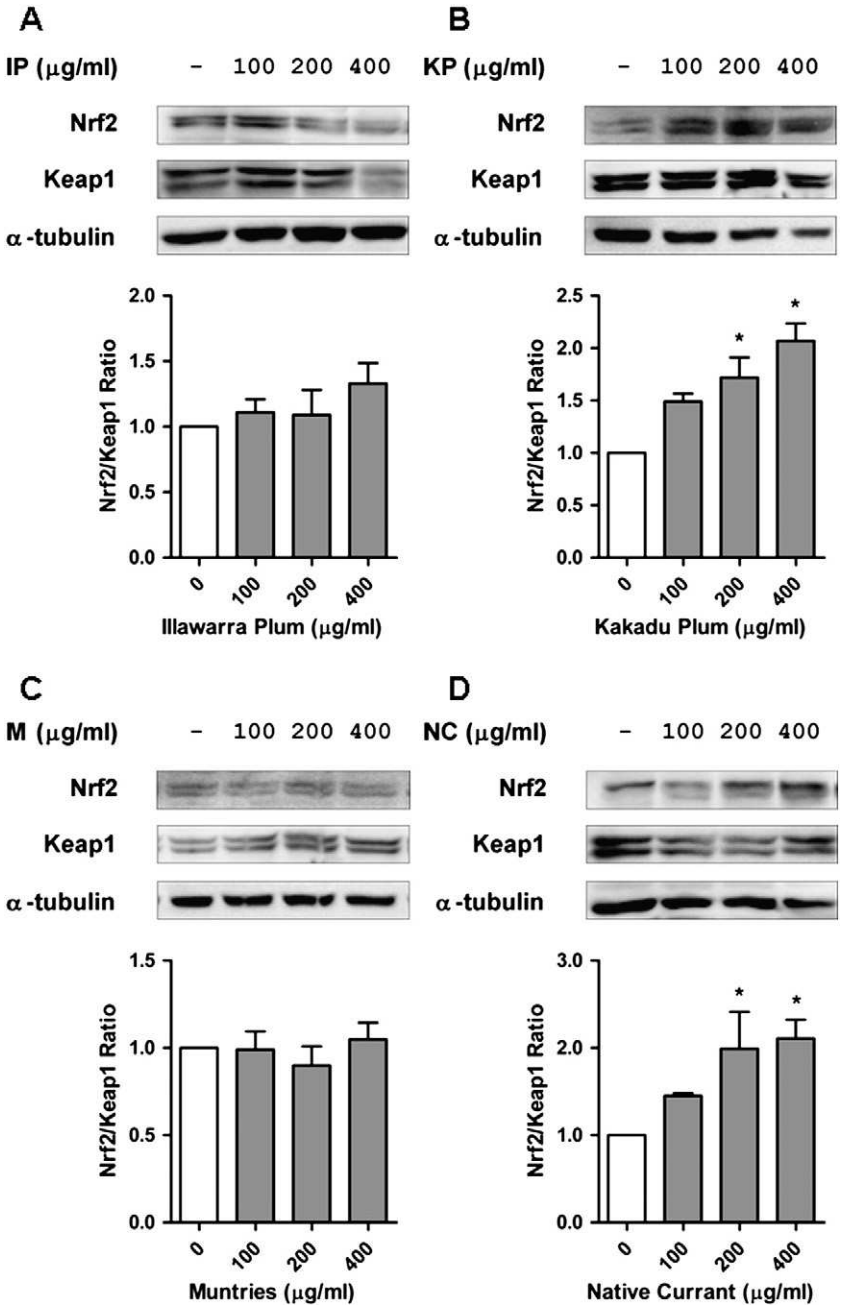

Fig. 3. Effect of purified polyphenolic extracts on steady-state Nrf2 and Keap1. HepG2 cell lysates were prepared after exposure to different concentrations of purified polyphenolic extracts for 9 h. The expression of the Nrf2, Keap1 and α-tubulin proteins was detected by Western blot analysis. The data represents the mean ± standard deviation of at least three independent experiments. An asterisk indicates significant difference with negative control ($p<0.05$). (A) IP, Illawarra Plum. (B) KP, Kakadu Plum. (C) M, Muntries. (D) NC, Native Currant.

determined through MTT assay. This represents an important mechanism for antioxidant activity (Chow, Shen, Huan, Lin, & Chen, 2005; Lin, Shen, Lin, Yang, & Chen, 2007). Of the four native fruits, at concentrations which did not affect cell viability, only Kakadu Plum and Muntries showed a protective effect, with a significant difference between H_2O_2-only treated cells (Fig. 2). Illawarra Plum and Native Currant did not attenuate the H_2O_2-induced cytotoxicity. The results indicated the cytoprotective activity of Kakadu Plum and Muntries in protecting RAW 264.7 cells from H_2O_2-induced cytotoxicity. Common polyphenols such as quercetin, but not its glycosides quercetrin or rutin (Chow et al., 2005), and baicelein, but not its glycoside baicalin (Lin et al., 2007), and a polyphenolic leaf extract from *Agastache rugosa*, a common plant in traditional Chinese medicine (Oh et al., 2006), have also been demonstrated to display cytoprotective properties in RAW 264.7 cells.

While the above mentioned assays examine the ability of the selected native Australian fruits to significantly scavenge radical species; it is known however, that antioxidant activity involves more intricate cellular defence, such as the ARE and antioxidant and detoxification enzymes. The ARE is a gene sequence which promotes many antioxidant and detoxification enzymes, and the ARE itself is largely regulated by the Nrf2-Keap1 complex (Eggler, Gay, & Mesecar, 2008). Therefore, to determine the activation of the ARE by the selected native Australian fruits, we measured the levels of Nrf2 and Keap1 proteins in Hep G2 cells upon exposure to varying concentrations of the purified polyphenolic

extracts using Western blot analysis. As shown in Fig. 3, the Kakadu Plum and Native Currant purified polyphenolic extracts exerted a dose-dependent increase in the Nrf2/Keap1 ratio, indicating an increase in the relative levels of Nrf2 and subsequent activation of the ARE. The Illawarra Plum and the Muntries extracts did not display significant differences in the Nrf2/Keap1 ratio at the concentrations tested, suggesting no activation of the ARE occurred. Important chemopreventive polyphenols such as quercetin, EGCG and green tea extracts, resveratrol and various anthocyanins have all been reported to activate Nrf2 and induce expression of phase II antioxidant enzymes (Chen, Jang, Li, & Surh, 2005; Shih, Yeh, & Yen, 2007; Tanigawa et al., 2007).

4. Conclusion

Of the four selected native Australian fruits, only Kakadu Plum displays both strong antioxidant and cytoprotective properties. Kakadu Plum demonstrates the greatest potential for antioxidant activity, displaying consistently potent results in all reagent and cell culture-based assays. It exerts antioxidant activity through several mechanisms, including free radical scavenging and an increased Nrf2/Keap1 ratio with activation of the ARE. The potential synergy between the various antioxidant pathways may enhance the potential antioxidant protection, but further studies are required to completely elucidate the antioxidant mechanisms. Polyphenols from native Australian fruits, and particularly Kakadu Plum, may represent significant potential to be further developed as a functional food for the protection against oxidative stress and additional beneficial health applications.

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