Title

Antioxidant and cytoprotective activities of polyphenols from native Australian fruits

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Abbreviations: ARE, antioxidant response element; **Keap1,** Kelch-like ECH-associated protein 1; **Nrf2,** nuclear factor-erythroid 2p45 (NF-E2)-related factor 2

Keywords:

Antioxidant activity / Kakadu Plum / Native Australian fruits / Nrf2 / Polyphenols

Abstract

Antioxidant and cellular protective functions were studied in 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*). In several reagent-based assays (total phenolics, ferric ion reducing antioxidant power (FRAP) assay and oxygen radical absorbance capacity (ORAC) assay), each fruit showed significant antioxidant activity against a blueberry (*Vaccinum sp.*, cv. Biloxi) standard. Kakadu Plum exhibited the greatest antioxidant capacity, 1936%, 1333% and 236% greater than the blueberry standard in each assay respectively. Protection of RAW 264.7 cells against hydrogen peroxide induced apoptosis, and levels of nuclear factor-erythroid 2p45 (NF-E2)-related factor 2 (Nrf2) and Kelch-like ECH-associated protein 1 (Keap1) proteins was also examined. Of the selected fruits, Kakadu Plum and Muntries were found to protect against hydrogen peroxide induced cell death, whilst Kakadu Plum and Native Currant exerted a dose-dependent increase in the Nrf2/Keap1 ratio. The results suggest Kakadu Plum demonstrates the greatest potential for antioxidant activity, yielding 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/Keap 1 ratio.

1 Introduction

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 potential damage, mutations and ultimately the formation of cancer [1]. Defence against oxidative stress is therefore an important factor in preventing the development of many diseases. Dietary polyphenols are strong antioxidants, able to scavenge and intercept free radicals, preventing damage to cellular molecules [2]. 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 [3]. 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 be extremely important, through promoting many antioxidant and drug-metabolising enzymes [4]. Accordingly, polyphenols which are able to interact with these pathways and mechanisms are therefore more likely to exhibit beneficial physiological activities.

Native Australian fruits provide significant potential for the discovery of novel antioxidant compounds for use as pharmaceuticals or functional foods. The significant and extensive history of the local indigenous population allowed for a constant refinement of knowledge on the properties and effectiveness of various plant and food sources [5]. Therefore, fruits which were consumed traditionally may have increased potential for positive health benefits. As a result, four native Australian fruits were selected, representing a diverse range of botanical environments and among the most popular fruits traditionally consumed in the regions in which they are found. 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*). As an insight into their therapeutic potential, Kakadu Plum was even considered by certain tribes more as a medicine than a food [6]. These fruits, except Native Currant, have been previously reported to contain high levels of polyphenols and strong antioxidant activity [7]. Additionally Illawarra Plum has exhibited anti-proliferative and apoptotic activity in various cancer cell lines [8].

The present study aims to provide a definitive characterisation of the antioxidant activity in selected native Australian fruits through an array of antioxidant assays. More importantly, we have examined the level of Nrf2 and Kelch-like ECH-associated protein 1 (Keap1) – two downstream transcription factors, for the impact of polyphenolic compounds on the antioxidant enzymatic pathways.

2 Materials and methods

2.1 Collection of fruit material

Illawarra Plum (*Podocarpus elatus* Endl., *Podocarpaceae*), Kakadu Plum (*Terminalia ferdinandiana* Exell, *Combretaceae*) and Muntries (*Kunzea pomifera* F. Muell., *Myrtaceae*) were purchased from Tanamera Native Produce Pty Ltd., Obum Obum, Qld, Australia. Native currant (*Acrotriche depressa* R.Br., *Epacridaceae*) 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

Samples were prepared according to Velioglu et al. [9] with minor modifications. Randomly selected fresh fruits were snap-frozen in liquid nitrogen and freeze-dried under vacuum (Benchtop 2K; Virtis, Gardiner, NY, USA). Freeze-dried samples were finely ground into a powder using a grinder (Multigrinder; Sunbeam, Botany, NSW, Australia), and a 200 mg portion extracted with 3 ml of acidified methanol (80% methanol, 19.9% H_2O and 0.1% HCl, v/v/v) and sonicated for 15 minutes at room temperature. The extracts were centrifuged for 10 minutes at 10,000 x *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 [9] as described previously [7]. 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 [10], as described previously [11]. 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) assay

Oxygen radical scavenging capacity was determined using the ORAC assay as described previously [8]. 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 raw fruit material of the four native fruits was initially weighed and ground into a pulp using a heavy duty blender (Waring Laboratory Science, Torrington, CT, USA). A 2-fold volume of acidified methanol (80% methanol, 19.9% H_2O and 0.1% HCl, v/v/v) was then added, stirred for 15 minutes and centrifuged for 25 minutes at 11,000 x *g* at 4°C (Sorvall RC-5B; DuPont, Wilmington, DE, USA) 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 in triplicate. The supernatant from the consecutive extractions were combined and the solvent evaporated under reduced pressure at 37°C using a rotary evaporator (Rotavapor R-205; Buchi, Flawil, Switzerland) to produce a methanolic extract.

The methanolic extract was further purified using an XAD-7HP (Sigma-Aldrich, St. Louis, MO, USA) resin column (300 x 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 using a rotary evaporator. 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 representing a polyphenolic-rich fraction of the fruit.

2.7 Quantification of phenolic compounds (HPLC-DAD)

Quantification of phenolic acids and anthocyanins in the purified polyphenolic extracts was conducted according to Kammerer et al. [12] and Terahara et al. [13] 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 autoinjector, and SCL-10A system controller (Shimadzu Corporation, Kyoto, Japan) equipped with a 250 x 4.6 mm i.d., 5 µm Luna C18(2) column (Phenomenex, Torrance, CA, USA). Analytical HPLC was run at 25°C and monitored at 280 nm, 320 nm and 370 nm for phenolic acids and at 520 nm for anthocyanins.

For phenolic compounds, including phenolic acids, flavonols, stilbenes and flavanols, 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 minutes in solvent A, followed by 30% B in 10 minutes, 50% B in 15 minutes and then to 100% B in 1 minute. Phenolic acids were calculated as µmol of gallic acid equivalents (GAEs) per mg of dry weight (µmol GAEs/mg DW).

For 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 minutes in solvent A, followed by 100% B in 4 minutes. Anthocyanins were calculated as µg of cyanidin 3-glucoside equivalents (CEs) per mg of dry weight (µg CEs/mg DW).

2.8 Cell culture

Cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) 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,

USA). 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 [14]. Initially, Hep G2 cells ($1x10^5$/ml) were incubated for 24 hours at 37°C in 96-well clear-walled microplates (Thermo Fisher Scientific), before growth medium was removed and wells gently washed with PBS. Cells 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 a further 1 hour. 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 applied and fluorescence measured every 5 minutes for 1 hour, with excitation at 485 nm and emission at 535 nm (Wallac 1420 Multilabel Counter; PerkinElmer, Waltham, MA, USA). Negative control wells contained cells treated with DCFH-DA and oxidant (ABAP) in the absence of samples. Blank wells contained cells treated without oxidant. 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 (MTT colourimetric cell viability assay)

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 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 hydrogen peroxide (H_2O_2; 100 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 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

The preparation of cell lysates from Hep G2 cells was conducted according to Tanigawa et al. [15] with minor modifications. Initially, Hep G2 cells ($1x10^6$ per flask) were incubated for 24 hours at 37°C in 25 cm^2 flasks. Fresh medium was then added and a range of concentrations of purified polyphenolic extracts added for 9 hours. Cells were harvested by scraping in ice-cold PBS and collected by centrifugation at 500 x *g* for 5 minutes. Cells were then lysed in modified RIPA buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% deoxycholate, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and protein inhibitor cocktail (Sigma-Aldrich). The lysates were homogenised in an ultrasonicator for 12.5 minutes on a 10 second sonication and 20 second rest cycle, and centrifuged for 15 minutes at 15,000 x *g* at 4°C. The supernatants were collected and the protein concentration evaluated using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Equivalent amounts of protein were boiled at 100°C in SDS buffer for 10 minutes 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, USA), 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, USA). 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, USA).

2.13 Statistical analyses

Results are expressed as mean ± standard deviation of at least three independent experiments. Differences between samples or treatment and control values was determined using one-way ANOVA with Tukey's post-hoc test (GraphPad, San Diego, CA, USA). Results were considered to be significant when p value < 0.05.

3 Results

3.1 Total phenolics and antioxidant capacity of Australian native fruits

The Folin-Ciocalteau assay was used to determine the total phenolic content of the four selected native Australian fruits in comparison with a blueberry control (Table 1). Each of the four native Australian fruits demonstrated a greater level of phenolic content compared to that of the blueberry control. Kakadu Plum was found to contain the highest level of phenolics (1936% of that of blueberry), followed by Native Currant (380%), Illawarra Plum (241%) and Muntries (128%).

The antioxidant capacity of the native Australian fruits was determined using the FRAP assay and the ORAC assay (Table 1). As expected, the results of the FRAP assay demonstrated a high correlation with the Folin-Ciocalteau assay (Table 2), with Kakadu Plum exhibiting the greatest antioxidant capacity (1333% of that of blueberry), followed by Native Currant (369%), Illawarra Plum (254%) and Muntries (153%). The ORAC assay revealed larger oxygen radical scavenging capacities for all four native fruits compared to the blueberry control. The variances 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%). Positive correlations between each of the three assays were observed (Table 2).

3.2 Extraction and quantification of phenolic compounds from Australian native fruits

The phenolic compounds in fruits have been found to display increased bioactivity [16]. The identification of these bioactive compounds is important in elucidating the mechanism of action of any demonstrated bioactivity. Subsequently, a phenolic-rich fraction was extracted from each of the selected native Australian fruits, analysed and subjected to evaluation of potential physiological activities.

The greatest yield of phenolic compounds from the initial fresh weight was from the extraction of Kakadu Plum (1.30%), followed by Native Currant (0.73%), Muntries (0.54%) and Illawarra Plum (0.31%) (Table 3). The Kakadu Plum extract also contained the highest level of phenolic compounds (4.796 µmol GAEs/mg DW), followed by Illawarra Plum (2.525 µmol GAEs/mg DW), Muntries (1.613 µmol GAEs/mg DW) and Native Currant (0.393 µmol GAEs/mg DW). The extraction yields and quantification of phenolic compounds in the extracts suggests Kakadu Plum is a rich source of phenolic compounds. With regards to the anthocyanin content, Illawarra Plum contained significant levels (0.996 µmol CEs/mg DW), followed by Native Currant (0.074 µmol CEs/mg DW) and Muntries (0.002 µmol CEs/mg DW). No anthocyanins were detected in the Kakadu Plum extract.

3.3 Cellular antioxidant activity of Australian native fruits

The results of the CAA assay revealed that Kakadu Plum exhibited the greatest cellular antioxidant activity with an EC_{50} value (µg/ml) of 153.0 ± 24.5 (Table 4), 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. Comparing the relative antioxidant activity using the chemical and cell culture methods, it has been shown that Kakadu Plum and to a lesser extent Illawarra Plum has consistently demonstrated significant antioxidant activity. In contrast, the order of the antioxidant activity for Native Currant and Muntries are different using the two methods.

3.4 Cellular protection of RAW 264.7 cells from hydrogen peroxide induced apoptosis

Increased levels of hydrogen peroxide, an important ROS, result s in oxidative stress, cellular damage and apoptosis. Ultimately, it leads to the development of various diseases including cancer [17]. To characterise the potential protective effects of native Australian fruits, the cell viability of H_2O_2-exposed RAW 264.7 cells was determined through MTT assay. Cells were exposed to the purified polyphenolic extracts (at levels which did not induce cytotoxicity) for 23 hours before the addition of H_2O_2 for 1 hour.

Of the four native fruits, only Kakadu Plum and Muntries showed a protective effect, with a significant difference between H_2O_2-only treated cells (Figure 1). Illawarra Plum and Native Currant did not attenuate the H_2O_2-induced cytotoxicity as assessed by the MTT assay. The results indicate the cytoprotective activity of Kakadu Plum and Muntries in protecting RAW 264.7 cells from H_2O_2-induced cytotoxicity.

3.5 Western blot for antioxidant enzymes

The upregulation of cellular antioxidant and detoxification enzymes represents a significant biological mechanism by which cells exert antioxidant activity. 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 [18]. 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 Figure 2, 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.

4 Discussion

The four selected native Australian fruits have each been found to exhibit varying levels of antioxidant activity in a number of assay systems. Using reagent based antioxidant assays, each fruit was found to contain high levels of total phenolics and significantly greater antioxidant capacity than the blueberry standard. Blueberries are highly recognised for their antioxidant activity and potential health benefits [19]. Positive correlations were notably observed between the total phenolics and FRAP assays, which could be due to similar experimental mechanisms, of using electron transfer reactions [20]. It is therefore recommended that a comparison with an alternative antioxidant system, such as the ORAC assay that involves hydrogen atom transfer reactions and is most relevant to human biology among all

reagent-based assays, be conducted [21]. In this study, we have employed the ORAC assay and found that all native Australian fruits exhibited superior oxygen radical scavenging activity to blueberry, with outstanding activity recorded for Kakadu Plum.

The results of reagent-based antioxidant testing indicate Kakadu Plum contains the greatest level of phenolic compounds and the highest antioxidant capacity, followed in order by Native Currant, Illawarra Plum and Muntries. Additionally, all four selected native Australian fruits were found to compare favourably to the blueberry control, suggesting their potential as novel sources of antioxidants for beneficial health impact. The results obtained for Illawarra Plum, Kakadu Plum and Muntries, are in agreement with previously reported antioxidant capacities of these fruits [7-8]. Moreover, these results also indicate that phenolic complexes of the evaluated four native Australian fruits represent mixtures of phenolic compounds that should effectively neutralise oxidants by direct reduction with electron transfer and by radical quenching with hydrogen atom transfer mechanisms.

The reagent based assays are useful in screening samples efficiently and in a cost-effective manner. However, they also have significant limitations with an inability to accurately predict activity *in vivo*. The 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 remaining cost-effective and relatively fast [14]. The CAA assay was conducted to provide a more appropriate evaluation of antioxidant activity in a biological system. The assay measures the sample's ability to prevent the formation and action of peroxyl radicals within a cell. The DCFH compound which diffuses across the cellular membrane oxidises into the fluorescent DCF in the presence of added peroxyl radicals. The sample's antioxidant activity is then calculated by the decrease in fluorescence in comparison to control cells and is expressed as an EC_{50} value. Accordingly, the results of the CAA assay, utilised to partially address several of these limitations, confirms the significant activity of the Kakadu Plum sample.

The results of the four native Australian fruits compare favourably with previous screenings of fruits and vegetables using these antioxidant assays. The cellular antioxidant activity of the Kakadu Plum in particular is much greater than a range of commonly consumed fruits, although the method of extraction differed slightly [22]. Similarly, the total phenolics, FRAP and ORAC values for each native Australian fruit ranked highly against a comparable study by Proteggente et al. [23] on the

antioxidant activity of regularly consumed fruits and vegetables. Other previous studies including screenings of red wines and medicinal plants, highlight the significant antioxidant activity displayed by the selected native Australian fruits, and Kakadu Plum especially [24-25].

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 defences, such as the ARE and antioxidant and detoxification enzymes. Subsequently, by examining the effect of the polyphenolic extracts on the Nrf2/Keap1 ratio, it will provide important insight into the potential upregulation of antioxidant enzymes. Of the four extracts, only Kakadu Plum and Native Currant increased the Nrf2/Keap1 ratio, suggesting that these two fruits may provide useful cellular antioxidant protection. Equally important, the cytoprotective effects of the extracts with hydrogen peroxide exposure were also investigated using RAW 264.7 cells. This represents an important mechanism for antioxidant activity [26-27]. Kakadu Plum and Muntries were found to protect against hydrogen peroxide induced cell death, but not Illawarra Plum and Native Currant. Interestingly, the cytoprotective abilities of Kakadu Plum and Muntries, may be explained by a pro-oxidant effect. Polyphenols have been found to exhibit a pro-oxidant effect, both in cell culture and *in vivo* [28], and this is particularly relevant for Kakadu Plum, which contains an extremely rich mixture of polyphenols. The polyphenols, either directly or through the formation of a radical of the polyphenol itself after free radical scavenging, may induce a mild repeated cellular oxidative stress [2]. This in turn may boost cellular antioxidant defence systems, as may occur with an increased Nrf2/Keap1 ratio. This effect has been previously documented in common flavonoids such as quercetin [26].

The experiments presented above, examine the antioxidant activity of rich mixtures of polyphenols extracted from selected native Australian fruits. These rich mixtures facilitate highly complex synergistic or antagonistic actions between compounds, which must be considered in the assessment of antioxidant activity. This is particularly true of the Kakadu Plum extract, which contains over 100 compounds. Although a complete chemical characterisation of the active constituents has not been conducted, our laboratory has previously identified quercetin/hesperitin glucosides and kaempferol/luteolin glycosides, with catechin-based hexose-containing glycosides being tentatively identified [29]. The Illawarra Plum extract predominantly contains cyanidin 3-glucoside with smaller

amounts of pelargonidin 3-glucoside, with the Muntries extract containing minor levels of delphinidin 3-glucoside and cyanidin 3-glucoside [11]. Finally, the Native Currant extract contains significant levels of several anthocyanins, including cyanidin 3-glucoside, cyanidin 3-rutinoside, delphinidin 3-glucoside and delphinidin 3-rutinoside, with minor levels of rutin.

In conclusion, these results suggest that of the four selected native Australian fruits, only Kakadu Plum exhibits both strong antioxidant and cytoprotective properties. Kakadu Plum demonstrates the greatest potential for antioxidant activity, yielding consistently potent results in all reagent-based 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. Polyphenols from native Australian fruits, and particularly Kakadu Plum, may have significant ability to protect against oxidative stress, and subsequently represent promising potential for further beneficial health applications.

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Tables

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

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

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

Table 2. Relationship between the levels of phenolic compounds and antioxidant capacity for four native Australian fruits and blueberry (cv. Biloxi).

	r^2
Total phenolics vs. FRAP[a]	0.996
Total phenolics vs. ORAC	0.795
ORAC vs. FRAP	0.842

[a] n=5 for each assay.

Table 3. Yields of purified polyphenolic extracts from four native Australian fruits and quantification (HPLC-DAD) of phenolic compounds.

Purified polyphenolic extract	Yield (%)	Phenolic compounds (µmol GAEs/mg DW)	Anthocyanins (µmol CEs/mg DW)
Illawarra Plum	0.31	0.301 (2.53)[a]	0.996
Kakadu Plum	1.30	4.80	-
Muntries	0.54	1.61	0.002
Native Currant	0.73	0.369 (0.393)[a]	0.074

[a] Phenolic compounds including anthocyanins

Table 4. Cellular antioxidant activity (CAA) of purified polyphenolic native Australian fruit extracts

	CAA EC_{50} (µg/ml) [a]	**CAA (µmol of QE/g purified extract)**
Illawarra Plum	233.2 ± 20.2 a	46.3 ± 3.9
Kakadu Plum	153.0 ± 24.5 b	71.5 ± 11.3
Muntries	758.0 ± 103.8 c	14.4 ± 2.1
Native Currant	537.9 ± 12.0 c	20.0 ± 0.5

[a] Data represents the mean ± standard deviation of at least three independent experiments. Values in each column with no letters in common are significantly different ($p<0.05$) using ANOVA and post-hoc analysis.

Figure Legends

Figure 1. Effect of purified polyphenolic extracts on H_2O_2-induced cell death in RAW 264.7 macrophages using the MTT assay. Cells were treated with different concentrations of purified polyphenolic extracts for 23 hours followed by the addition of H_2O_2 (100 mM) for a further 1 hour, and the viability of the cells determined by MTT assay. 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.

Figure 2. 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 hours. The expression of the Nrf2, Keap1 and α-tubulin proteins was detected by Western blot analysis. 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.

Figure 1

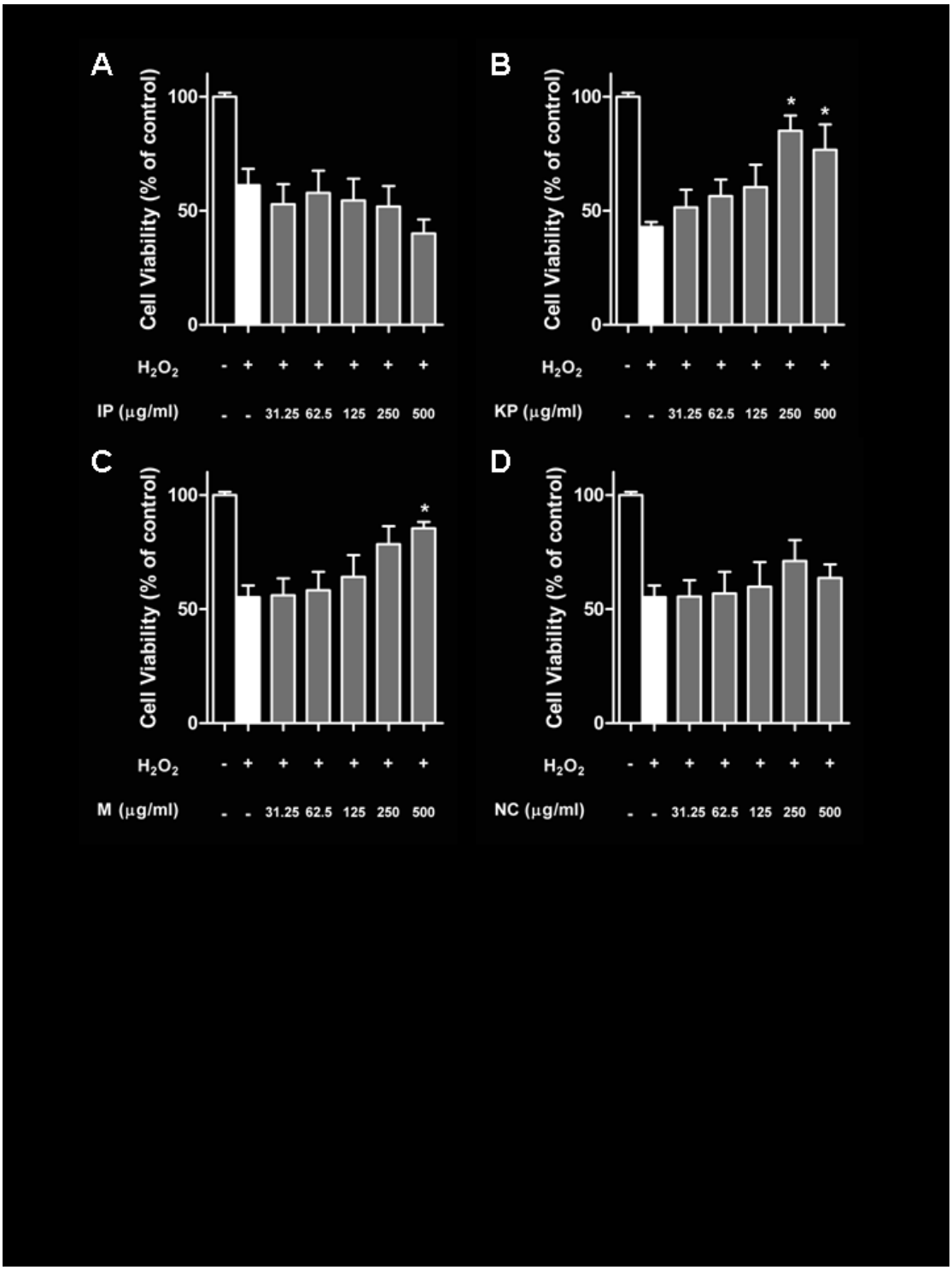
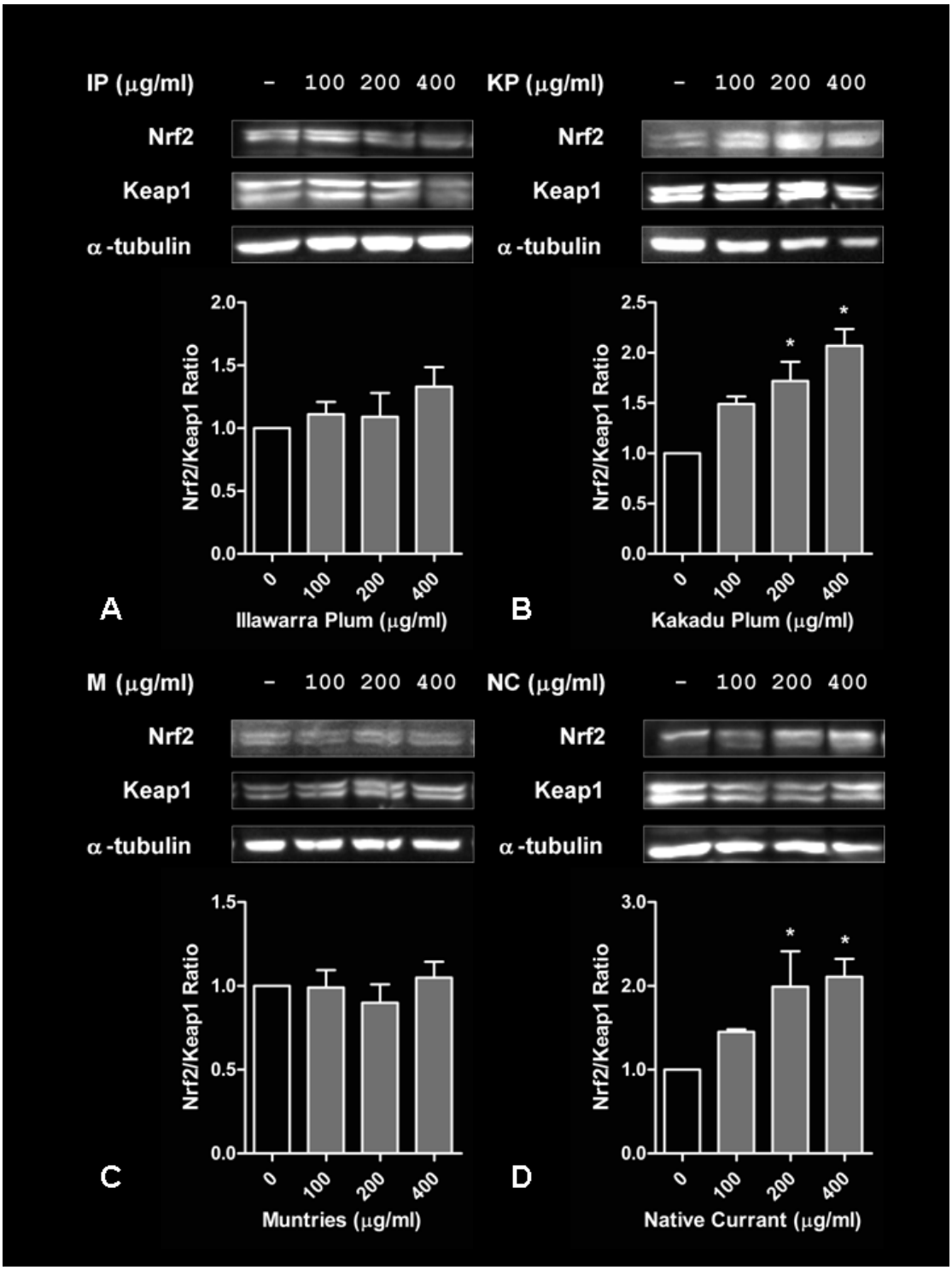

A
B
C
D
Cell Viability (% of control)
100
50
0
H_2O_2 - + + + + + +
IP (μg/ml) - - 31.25 62.5 125 250 500
KP (μg/ml) - - 31.25 62.5 125 250 500
M (μg/ml) - - 31.25 62.5 125 250 500
NC (μg/ml) - - 31.25 62.5 125 250 500
*

Figure 2


IP (μg/ml) – 100 200 400
Nrf2
Keap1
α-tubulin
Nrf2/Keap1 Ratio
Illawarra Plum (μg/ml)
A
KP (μg/ml) – 100 200 400
Nrf2
Keap1
α-tubulin
Nrf2/Keap1 Ratio
Kakadu Plum (μg/ml)
B
M (μg/ml) – 100 200 400
Nrf2
Keap1
α-tubulin
Nrf2/Keap1 Ratio
Muntries (μg/ml)
C
NC (μg/ml) – 100 200 400
Nrf2
Keap1
α-tubulin
Nrf2/Keap1 Ratio
Native Currant (μg/ml)
D