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Native Australian fruit polyphenols inhibit cell growth and induce apoptosis in human cancer cell lines

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Abstract

Apoptosis is one of the most critical forms of defence against cancer, and the induction of apoptosis by dietary polyphenols represents significant potential for cancer preventive activity. The present study examined polyphenols extracted from selected 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*) for anti-proliferative activity against a panel of cancer and normal cell lines. Each fruit selectively inhibited the growth of cancer cell lines in a dose-dependent manner. The mechanism of growth inhibition of the human promyelocytic leukaemia cells (HL-60) was determined to be apoptosis by morphological assessment, DNA fragmentation, flow cytometry and caspase-3 induction. Furthermore, Kakadu Plum was found to activate caspase-7, -9 and poly (ADP-ribose) polymerase (PARP) suggesting it acts via the intrinsic apoptosis pathway. The same fruit also caused direct DNA damage in colon adenocarcinoma cells (HT-29) as detected using the cytokinesis-block micronucleus cytome (CBMN Cyt) assay.

Introduction

Apoptosis is arguably one of the most potent forms of defence against cancer, and a mechanism by which many current anticancer and chemopreventive agents induce their effect. It is a form of programmed cell death, characterised by cell shrinkage, chromatin condensation, inter-nucleosomal DNA fragmentation and the formation of apoptotic bodies [1]. Apoptosis typically progresses through the extrinsic pathway, with the activation of death receptors on the cell surface, or the intrinsic pathway, with an increase in mitochondrial permeability and cytochrome *c* release [2]. The caspase cascade is a central component of the apoptosis process and important in both pathways. Caspase-3 is particularly vital, an executioner caspase activated by both pathways, it cleaves numerous cellular proteins including structural, nuclear and cytoskeletal proteins and signalling molecules, activates other caspases and activates the caspase-activated DNase [3]. Mutations in the apoptosis machinery are common to virtually all cancers [4]. Dietary compounds have been demonstrated to modulate the pathways which lead towards apoptosis of mutated cells [5]. This activity is consistent with the epidemiological evidence that a phytochemical-rich diet, with a high intake of fruits and vegetables, leads to a reduction in cancer incidence [6]. Polyphenols are a particular class of phytochemicals known for having a range of potent biological activities [7].Total dietary intake of polyphenols has been estimated as reaching as high as 1 g per day in Western diets [8]. They have been found to exhibit strong antioxidant, anti-inflammatory, anti-carcinogenic and pro-apoptotic activity [9-10].

The Australian flora offers enormous opportunities for the selection of novel plants that in addition to being nutritious possess health-enhancing properties. At least 2440 species of fruiting rainforest plants were identified in the tropical regions of Australia alone [11]. The indigenous people of Australia, the Aboriginals, have consumed native plants for thousands of years [12]. To date only limited general compositional data of around 500 indigenous foods has been collated [13]. This included information on content of water, protein, fat, carbohydrates, vitamins and minerals for around 100 native fruits. This research indicated that native Australian foods do not differ in their nutritional composition to the fruits and vegetables consumed by western societies [14]. Additionally, native Australian plants have been reported to possess unique sensory characteristics [15], potentially indicating a rich matrix of phytochemicals and flavour compounds.

Intensive antioxidant testing of native Australian fruits and herbs has previously been undertaken in our laboratory, revealing high levels and an exceptionally rich composition of phytochemicals [16-18]. Among these phytochemicals are a number of compounds known for health-enhancing properties. A purified anthocyanin-rich extract obtained from Illawarra Plum effectively induced apoptosis of colon adenocarcinoma cells *in vitro* [19]. Consequently, four traditionally consumed native Australian fruits were selected, 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*). These fruits were among the most popularly consumed fruits traditionally, in the areas in which they are found [20-21]. They also represent a diverse range of botanical environments across the Australian continent, from the tropical northern regions to the rainforest eastern regions, and are among the most commercially viable fruits, as identified by local industry [16].

In order to further identify potential anticancer or chemopreventive capacity in the selected native Australian fruits and to understand the underlying mechanisms, purified polyphenolic extracts were assayed for anti-proliferative activity against a panel of cancer cell lines and their equivalent normal cells. The mechanism of cytotoxicity was further characterised with investigations into the induction of apoptosis, including caspase-3 activation, and potential genotoxic damage with the cytokinesis-block micronucleus cytome (CBMN Cyt) assay.

Materials and methods

Native Australian fruit purified polyphenolic extract preparations

The native Australian fruits, 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. Purified polyphenolic extracts were prepared as described previously [19]. Briefly, fruit material was extracted using acidified methanol (80% methanol, 19.9% H_2O and 0.1% HCl, v/v/v), purified twice on an XAD-7HP

column (Sigma-Aldrich, St. Louis, MO, USA) and freeze-dried to produce a lyophilised powder representing a purified polyphenolic fruit fraction.

Quantification of phenolic compounds (HPLC-DAD)

Quantification of phenolic acids and anthocyanins in the purified polyphenolic extracts was conducted according to Kammerer et al. [22] and Terahara et al. [23] with minor modifications as described previously [16].

Cell culture

All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) 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 normal) was cultured in Eagle's minimum essential medium (EMEM); and Hs 738.St/Int (mixed stomach and intestine normal) was cultured in Dulbecco's Modified Eagle's medium (DMEM). Experiments were conducted at passages less than 40.

Isolation of human peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors' buffy coat with informed consent from the Australian Red Cross Blood Service. PBMCs were isolated from the buffy coat via Ficoll-Hypaque (Pharmacia-Amersham, Uppsala, Sweden) density-gradient centrifugation according to the manufacturer's instructions.

MTT colorimetric cell viability assay

Cell sensitivity of AGS, HT-29, CCD-18Co and Hs 738.St/Int 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) as described previously [19].

ATP luminescence cell viability assay

Cell sensitivity of HL-60 and PBMCs to native Australian fruit extracts was determined via cell viability using the luminescent CellTiter-Glo ATP assay (Promega, Madison, WI, USA) as described previously [24].

Morphological assessment of apoptosis

The characteristic morphological changes of apoptosis, including chromatin condensation and nuclear blebbing, were assessed by fluorescent microscopy after Hoechst 33342 staining. HL-60 cells were treated with 0.75 mg/ml purified polyphenolic extract for 24 hours with untreated cells as a control. After washing with PBS, cells were resuspended in 4% PBS-buffered formaldehyde. Cells were applied to a cell culture cover slip and fixed at 4°C for 20 minutes. After further PBS washing, cells were stained with 2 µg/ml Hoechst 33342 (Sigma-Aldrich) for 20 minutes at room temperature. Cells were again washed with PBS, cover slips mounted onto microscope slides and examined and photographed with a DAPI filter on a Nikon Eclipse 90i (Nikon Corporation, Tokyo, Japan) fluorescence microscope.

Analysis of DNA fragmentation

The analysis of inter-nucleosomal DNA fragmentation was conducted via agarose gel electrophoresis. Initially, HL-60 cells ($5x10^5$/ml) were plated in 25cm^2 culture flasks and treated with 0.75 mg/ml purified polyphenolic extract for 24 hours, with untreated cells as a control. Genomic DNA was then extracted using the NucleoSpin Tissue Kit (Macherey–Nagel GmbH, Düren, Germany) according to the manufacturer's instructions. The DNA samples were electrophoresed on 2% agarose gel (Sigma-Aldrich), and the DNA in the gel visualised using a Typhoon 8600 variable mode imager (Pharmacia-Amersham) following ethidium bromide (Sigma-Aldrich) staining. A 100-bp marker (N3231S, New England Biolabs Inc., Beverly, MA, USA) was used as a reference marker.

Measurement of apoptosis by flow cytometry

Time Course. HL-60 cells ($5x10^5$/ml) were plated in 25cm^2 culture flasks and treated with 0.75 mg/ml purified polyphenolic extract for 3, 6, 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 Corporation) 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 20,000 events were acquired for each measurement and the cells were properly gated for analysis.

Dose Response. 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 0.375, 0.75 and 1.5 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.

Determination of caspase-3 activity

Caspase-3 activity was measured using a colorimetric caspase-3 assay kit (Sigma-Aldrich), according to the manufacturer's instructions. Apoptosis was induced in HL-60 cells with treatment of 0.75 mg/ml purified polyphenolic extract for 24 hours, with untreated cells as a control. Caspase activity was quantified specrophotometrically at 405 nm using a microplate reader (Wallac 1420 Multilabel Counter, PerkinElmer, Waltham, MA, USA).

Preparation of cell lysates

The preparation of cell lysates was conducted according to Hou et al. [25] with minor modifications. Initially, HL-60 cells ($2.5x10^6$ per flask) were incubated for 3 hours at 37°C in 25 cm^2 flasks. At various time points (1, 2, 4 and 6 hours), 0.75 mg/ml of Kakadu Plum purified polyphenolic extracts was added. Harvested pellets were lysed and the protein concentration evaluated using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equivalent amounts of protein were boiled at 100°C in SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT and 0.1% bromophenol blue) for 10 minutes to produce whole-cell lysates.

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 horseradish peroxidase (HRP) 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 α-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies against caspase-3, cleaved caspase-3, caspase-7, cleaved caspase-7, caspase-9, cleaved caspase-9, poly (ADP-ribose) polymerase (PARP) and cleaved PARP and HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA, 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).

Cytokinesis-block micronucleus cytome (CBMN Cyt) assay

The CBMN Cyt assay was conducted using the cytochalasin B technique as described by Fenech [26] 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) in medium for 24 hours to block the cells from entering cytokinesis. Cells were then harvested using Tryple Express (Sigma-Aldrich) and applied to a microscope slide (3 in. x 1 in.) 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 coverslipped 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 [26]. The biomarkers scored include 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 [27]. 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 MN, BN, NPB and BNud. Each treatment concentration and control was assessed in sextuplicate.

Statistical analyses

Results are expressed as mean ± standard deviation of at least three independent experiments. IC_{50} values were calculated through nonlinear regression analysis, and statistical significance calculated in all experimental sets using the unpaired two-tailed Student's t-test or one-way ANOVA with Tukey's post-hoc test where appropriate, with $p<0.05$ as statistically significant (GraphPad, San Diego, CA, USA).

Results

Characterisation of purified polyphenolic extracts

The purified polyphenolic extracts were analysed by HPLC for total phenolic content (Table I). The Kakadu Plum (4.80 µmol GAEs/mg DW) extract was found to contain the highest level of overall phenolic compounds, followed by Illawarra Plum (2.53 µmol GAEs/mg DW), Muntries (1.61 µmol GAEs/mg DW) and Native Currant (0.393 µmol GAEs/mg DW). Illawarra Plum (0.996 µmol CEs/mg DW) and Native Currant (0.074 µmol CEs/mg DW) extracts were found to contain significant levels of anthocyanins, whilst no anthocyanins were detected in the Kakadu Plum extract; and only minute levels of anthocyanins were found in the Muntries (0.002 µmol CEs/mg DW) extract.

The Illawarra Plum extract predominantly contained cyanidin 3-glucoside with trace amounts of pelargonidin 3-glucoside, while the Muntries extract contained minor levels of delphinidin 3-glucoside and cyanidin 3-glucoside [17]. The Native Currant extract contained significant levels of several anthocyanins, including cyanidin 3-glucoside, cyanidin 3-rutinoside, delphinidin 3-glucoside and delphinidin 3-rutinoside, with minor levels of rutin. The Kakadu Plum extract was found to be a complex mixture of over 100 compounds. However, quercetin/hesperitin glucosides and kaempferol/luteolin glycosides have been identified in the extract, with catechin-based hexose-containing glycosides also tentatively identified [18].

Anti-proliferative activity of purified polyphenolic extracts

The cell sensitivity of the AGS, HT-29, CCD-18Co and Hs 738.St/Int (gastric and colorectal) cell lines were selected as representatives of the digestive system, and therefore directly exposed to food compounds and their metabolites. The cell sensitivity of the HL-60 cell line and PBMCs were selected to assess potential effects on immunological cells. Each of the purified polyphenolic extracts demonstrated a reduction in cell viability of the cancer cell lines AGS and HT-29 in a dose-dependent manner. Native Currant exhibited the lowest IC_{50} values (mg/ml) for both AGS (0.238 ± 0.027) and HT-29 (0.271 ± 0.076) (Table I), whilst Kakadu Plum also showed strong anti-proliferative activity against HT-29 (0.385 ± 0.036). Interestingly, the gastric and colorectal cancer cell lines showed an increased sensitivity to each extract in comparison to the equivalent normal cell lines, with lower IC_{50} values for AGS and HT-29 than CCD-18Co and Hs 738.St/Int. The cell viability data for each cell line was also plotted graphically, and the areas under the curve were calculated and compared. Similarly significant differences were seen between gastric and colorectal cancer cell lines to their equivalent normal cell lines (Figure 1).

The extracts also demonstrated anti-proliferative activity against HL-60 and PBMCs in a dose-dependent manner. The Illawarra Plum extract showed greater growth inhibition to HL-60 (0.337 ± 0.068 mg/ml) than PBMCs (0.506 ± 0.044), along with the Muntries extract to HL-60 (1.34 ± 0.019) versus PBMCs (1.83 ± 0.007) (Table I). The Kakadu Plum extract however, although exhibiting the greatest potency to HL-60 cells (0.257 ± 0.074), produced a similar IC_{50} value for PBMCs (0.239 ± 0.031). Similarly, Native Currant demonstrated an equivalent anti-proliferative effect to PBMCs (0.567 ± 0.010) and HL-60 (0.590 ± 0.087).

The IC_{50} values were also standardised against total phenolic content to offer a more appropriate comparison between the different fruit extracts (Table I). From the standardised values in each of the cancer cell lines, it can be seen that Native Currant continued to show significant inhibition of proliferation. With regards to the effectiveness of polyphenolic complexes against transformed (cancer) and non-transformed (normal) cell lines, the Kakadu Plum extract exhibited the greatest differences; 5.2 times more extract was required to suppress the growth of the CCD-18Co (colon normal) cell line than the HT-29 (colorectal adenocarcinoma) cell line. With regards to the Hs 738.St/Int (mixed stomach and intestine normal) versus AGS (gastric adenocarcinoma) cell

lines, the difference was 2.3. The results suggest lower toxicity of Kakadu Plum polyphenolics to the non-transformed cell lines in comparison to other fruit extracts used in this study.

The anti-proliferative activity of the native fruit extracts on cancer cell lines, suggest potential for the polyphenolics from native fruits to exert anticancer activity. Furthermore, the greater inhibition of cell viability in cancer cells in comparison with their normal cells indicates the potential for selectivity to cancer cells of the polyphenolic compounds.

Determination of apoptosis in cancer cells by purified polyphenolic native Australian fruit extracts

The morphological changes of cancer cells after exposure to the purified polyphenolic extracts were assessed. Treatment with each extract (0.75 mg/ml over 24 hours) demonstrated the morphological changes in the nucleus characteristic of apoptosis; chromatin condensation, nuclear blebbing and formation of apoptotic bodies in the native extracts treated HL-60 cells compared to the untreated control (Figure 2A). The negative control (non-treated cells) exhibited normal nuclear morphology with a diffuse chromatin structure represented by light staining. The results demonstrated that each native fruit extract may induce apoptosis in HL-60 cells. DNA fragmentation is a significant feature of apoptosis. Figure 2B revealed the time-dependent DNA laddering at approximately 180-200 base pairs by each native fruit extract. After treatment with each of the purified polyphenolic extracts, DNA fragmentation was visible after 3 hours of treatment, with the most pronounced effect after 6 hours. The negative control however, showed no significant DNA ladder. These results provided further evidence to the induction of apoptosis in HL-60 cells by native Australian fruit extracts.

Flow cytometric data revealed that over the various time points for each of the native fruit extracts, the percentage of apoptotic HL-60 cells increased over time with the greatest number of total apoptotic cells at 24 hours (Figure 3). The percentage of late apoptotic cells also increased steadily over time, indicating the progression of apoptotic cells from early to late stages of apoptosis. The percentage of late apoptotic plus apoptotic cells at 3, 6, 12 and 24 hours for Illawarra Plum were 44.6 ± 1.6%, 47.0 ± 7.0, 50.1 ± 7.6 and 57.6 ± 7.7 respectively, for Kakadu Plum were 18.0 ± 5.3, 36.4 ± 2.4, 50.4 ± 7.7 and 66.6 ± 3.2, for Muntries were 20.4 ± 6.5, 32.2 ± 3.5, 48.2 ± 6.8 and 61.5 ± 6.2, and for Native Currant were 36.1 ± 6.8, 56.5 ± 3.8, 76.7 ± 4.9 and 88.7 ± 4.9. The induction of apoptosis in HL-60 occurred in a time-dependent manner. Illawarra Plum exhibited a

level of apoptosis which remained constant after 3 hours. This could be the effects of specific Illawarra Plum extract compounds, especially cyanidin 3-glucoside, or lower stability of the compounds over time. The percentage of necrotic cells was low in comparison with the apoptotic cells and remained stable over the various time points, demonstrating the mechanism of cell death as apoptosis rather than necrosis.

Examination of the induction of apoptosis at a single time point and with various doses showed that for the Illawarra Plum and Muntries extracts, the percentage of total apoptotic cells increased with increasing concentrations indicating a dose-dependent response (Figure 3B). The percentage of necrotic cells was also significantly lower for both Illawarra Plum and Muntries. For Kakadu Plum and Native Currant however, the percentage of necrotic cells increased at the highest concentration (1.5 mg/ml). The percentage of apoptotic and late apoptotic cells also showed no significant differences between the various concentrations.

Determination of caspase-3 activity

As shown in Figure 3, each native fruit extract induced the activation of caspase-3. For each of the samples investigated, caspase-3 activity was detected as early as 3 hours, with the greatest level of caspase-3 at the 6 hour time point. The caspase-3 activity then decreased at 12 and 24 hours. This supports the induction of caspase-3 as an early event in the induction of apoptosis. A comparison of the four native fruit extracts at the 6 hour time point revealed that the Native Currant extract (0.075 ± 0.005 effective absorbance) induced the greatest level of caspase-3 activity, followed by Kakadu Plum (0.038 ± 0.014) and Muntries (0.039 ± 0.008), with Illawarra Plum (0.015 ± 0.003) exhibiting the lowest level of caspase-3 activity.

Taken as a whole, the data presented above; evidence of the morphological changes, DNA fragmentation, flow cytometry and caspase-3 activation, demonstrated that the native Australian fruit extracts inhibited the proliferation of HL-60 cells via the induction of apoptosis through a caspase-3 dependent pathway.

Activation of caspases and degradation of PARP by Kakadu Plum-induced apoptosis

In this study, Western blot was used to determine the involvement of caspases in the apoptotic response of HL-60 cells induced by Kakadu Plum. The latter was chosen based on the significant apoptotic response in previous

experiments in conjunction with its considerable potential in additional physiological activities, including antioxidant and anti-inflammatory properties (data not shown).

As shown in Figure 4, the treatment with Kakadu Plum caused the activation of caspase-3, -7 and -9 in a time-dependent manner. The appearance of cleaved forms at 4 and 6 hours was consistent with data from flow cytometric and caspase-3 colorimetric assay analysis. Kakadu Plum also caused the cleavage of PARP in a time-dependent manner. PARP, cleaved by caspase-3, is widely utilised in the detection of apoptosis and blocks access of DNA repair enzymes to fragmented chromatin and prevents DNA damaging signalling [28]. The results suggested that Kakadu Plum induced apoptosis through pathways dependent on caspase-3, -7 and -9.

CBMN Cyt assay

The CBMN Cyt assay is a well established approach to determine the presence of genotoxicity [26], and measures three outcomes: DNA damage, cytostasis and cytotoxicity. DNA damage is evaluated by scoring for three biomarkers; micronuclei, nucleoplasmic bridges and nuclear buds. Cytotoxicity is determined by apoptotic and necrotic cell ratios, while cytostatic effects are measured by the nuclear division index (NDI).

In this study, HT-29 colorectal adenocarcinoma cells were exposed to various concentrations of purified polyphenolic extracts and the frequency of various biomarkers was scored (Table II). Of the various cell treatments, only Kakadu Plum 0.5 mg/ml (103.3 ± 29.7 per 500 cells) and 1.0 mg/ml (139.1 ± 9.0) and Native Currant 1.0 mg/ml (33.1 ± 15.3) displayed an increased level of apoptosis compared with the control cells. There was also an increased level of necrotic cells with Kakadu Plum 0.5 mg/ml (12.4 ± 4.6) and 1.0 mg/ml (11.1 ± 5.8), although the ratio of apoptotic cells was still significantly greater. In Illawarra Plum and Muntries, there were no significant increases in apoptotic or necrotic cell ratios. The NDI was significantly affected by Illawarra Plum 0.5 mg/ml (1.36 ± 0.07) and 1.0 mg/ml (1.29 ± 0.01) with an increase compared to control cells. Kakadu Plum 1.0 mg/ml (1.04 ± 0.01) however showed a decrease in NDI. This was largely due to a substantial decrease in the numbers of BN cells. As a result, the extremely low numbers of BN cells for this cell treatment were insufficient to obtain counts for the various biomarkers of DNA damage. Native Currant 0.5 mg/ml and 1.0 mg/ml also showed a decreased number of BN cells, although both Native Currant and Muntries did not exhibit significant differences in NDI. In measuring the various biomarkers of DNA damage (MNi, NPB and NBud) only

one cell treatment, Kakadu Plum 0.5 mg/ml, displayed greater levels compared to control cells. The number of MNi-BN cells (22.8 ± 6.6 per 500 BN cells), number of MNi (35.0 ± 5.3), NPBs (4.2 ± 3.2) and NBuds (25.0 ± 16.6) were all significantly greater. None of the other cell treatments showed signs of DNA damage in HT-29 cells.

Discussion

The present results demonstrate that polyphenolic-rich extracts from native Australian fruits possess anti-proliferative and pro-apoptotic activity against various cancer cell lines. The data suggests that all four fruits exhibit differential activity, being more selective for gastric and colorectal cancer cell lines over their normal cell equivalents, with a lower IC_{50} in each case. Analysis on normal cells is often overlooked, despite the fact its importance may actually relate to potential usefulness as chemotherapeutic agents in the clinical setting [29]. Screening for differential activity using PBMCs is particularly important to identify samples which kill cancer cells whilst sparing or even proliferating normal immunological cells [24]. This potential immunomodulatory action may stimulate T-cell and natural killer cell proliferation and increase tumour-specific immunosurveillance [30]. The Muntries extract showed a significant difference between HL-60 cells and PBMCs, indicating its potential immunomodulatory actions, whilst the Illawarra Plum, Kakadu Plum and Native Currant extracts displayed no difference. It must be noted however, that most of the PBMCs are in G_0 phase when collected, and therefore the data is related to PBMC that are not dividing [31]. Furthermore, it is important to note that the PBMC comprises a range of immunological distinct subsets such that proliferation may be restricted to a particular subset.

To understand the mechanisms of the suppression of cancer cell growth, detailed investigations into the induction of apoptosis were conducted. Cell death largely occurs through apoptosis, necrosis or accidental cell death, and it has become evident now that apoptosis and necrosis represent a continuum of cell death and features of both may be evident in the same cell [32]. A multitude of cellular events occur in apoptosis, and many biochemical events may not be specific to apoptosis. Additionally, not all events may occur in every apoptotic cell, during every stage of apoptosis or in response to all apoptosis-inducing stimuli [33]. Consequently it is necessary to conduct combination of morphological and biochemical techniques to yield the most reliable data on the determination of apoptosis [32]. Accordingly, in this study we have employed a range

of techniques, including a morphological assessment via fluorescence microscopy, visualisation of DNA fragmentation by agarose gel electrophoresis and flow cytometric analysis with annexin V and PI staining, and revealed all four native Australian fruit extracts induced apoptosis in HL-60 cells. The data also indicated that Native Currant is the most potent inducer of apoptosis with significant levels of apoptotic and late apoptotic cells at 24 hours seen in flow cytometric analysis. However, it can also be seen that at 3 hours, Illawarra Plum induces the greatest level of apoptosis, which remained constant at 24 hours.

Following the determination of apoptosis, the extracts were also assayed for caspase-3 induction. Each extract was found to induce the expression of caspase-3 induction, with the greatest levels at 6 hours. Native Currant induced the greatest levels of caspase-3 followed by Kakadu Plum, Muntries and Illawarra Plum. This indicates that apoptosis occurs via caspase-3 induction. The Kakadu Plum extract was also assayed for caspase-3, -7 and -9 induction and PARP cleavage by Western blot. The activation of caspase-9 suggests the involvement of the mitochondrial mediated (intrinsic) pathway of apoptosis. Caspase-9 subsequently cleaves caspase-3 and -7, which are both effector caspases. The activation of caspase-3 and -7 was also seen in a time-dependent manner. These effector caspases then proteolytically cleave a broad spectrum of cellular targets for apoptosis such as PARP [3]. The cleavage of PARP by Kakadu Plum indicates the induction of apoptosis occurs through this caspase cascade.

Lastly, the CBMN Cyt assay was conducted to provide information on potential genotoxic damage caused by the purified polyphenolic extracts. The results however, showed only the Kakadu Plum extract induced DNA damage at the concentrations tested, with numbers of MNi, NPB and NBud, all significantly different to the control suggesting chromosome breakage/loss, DNA misrepair, telomere end-fusions and gene amplification may all be a predominant mechanism by which cytotoxicity occurs. The concentrations were chosen at levels which would reveal potential genotoxicity without causing significant cell death. The other three fruit extracts did not induce DNA damage. Overall, it suggests cytotoxicity in these other fruits and the induction of apoptosis is likely to occur through an alternative mechanism. Furthermore, the CBMN Cyt assay also provides cytostatic and cytotoxic information. This revealed that Kakadu Plum and Native Currant induced apoptosis in HT-29 cells, coupled with a decrease in BN cell numbers, potentially indicating damage may occur during cytokinesis or an

inhibition of cell cycle progression. Illawarra Plum, which has previously been shown to induce apoptosis in HT-29 cells [19], also exhibited impacts on the mitotic status of HT-29 cells with an increase in BN cell numbers.

The results for the selected native Australian fruits demonstrate their remarkable pro-apoptotic activity. Kakadu Plum in particular, exhibited strong activity against human promyelocytic leukaemia HL-60 cells. Previous studies have shown similar effects on HL-60 cells with constituents of green tea and red wine, such as (-)-epigallocatechin 3-gallate (EGCG) and resveratrol [34-36].

Overall, the results indicate that native Australian fruits exhibit anti-proliferative and pro-apoptotic activity in cancer cell lines greater than in normal cells. The induction of apoptosis involved an increase in caspase-3 expression, however it did not include direct DNA damage with the exception of Kakadu Plum. Kakadu Plum-induced apoptosis in particular involves the activation of caspases-3, -7 and -9. The fruits have marked potential as anticancer or chemopreventive agents, however more work is needed to elucidate a complete chemical profile of the fruits, further characterise modulation of molecular pathways, and understand potential bioavailability and toxicity impacts.

Acknowledgements

We thank Dr Erin Symonds and Professor Michael Fenech from CSIRO Food and Nutritional Sciences, Adelaide, Australia for their guidance with the CBMN Cyt assay. Funding of this research and PhD scholarship to A.T. by the CSIRO Food Futures Flagship, Australia is gratefully acknowledged.

Conflict of Interest Statement: None declared.

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Tables

Table I: Total phenolic and anthocyanin content and concentration producing 50% cell viability (IC_{50}) for purified polyphenolic native Australian fruit extracts on AGS, HT-29, CCD-18Co, Hs738, HL-60 cells or PBMCs.

	Phenolics (µmol GAE/mg DW)[c]	**Anthocyanins (µmol CE/mg DW)**[d]	**IC_{50} (mg/ml) ± SD**[a] **[IC_{50} (µmol GAE/ml) ± SD]**[b]					
			HT-29	**CCD-18Co**	**AGS**	**Hs 738.St/Int**	**HL-60**	**PBMC**
Illawarra Plum	0.301 [2.53][e]	0.996	0.896 ± 0.049 [2.26 ± 0.124]	1.39 ± 0.036 [3.50 ± 0.091]	0.939 ± 0.100 [2.37 ± 0.252]	>2 [>5.05]	0.337 ± 0.068 [0.851 ± 0.171]	0.506 ± 0.044 [1.28 ± 0.110]
Kakadu Plum	4.80	-	0.385 ± 0.036 [1.85 ± 0.171]	>2 [>9.59]	0.872 ± 0.067 [4.18 ± 0.320]	>2 [>9.59]	0.257 ± 0.074 [1.24 ± 0.353]	0.239 ± 0.031 [1.15 ± 0.146]
Muntries	1.61[c]	0.002	0.927 ± 0.081 [1.50 ± 0.130]	>2 [>3.23]	0.978 ± 0.133 [1.58 ± 0.215]	>2 [>3.23]	1.34 ± 0.019 [2.16 ± 0.031]	1.83 ± 0.010 [2.95 ± 0.012]
Native Currant	0.369 [0.393][c]	0.074	0.27 ± 0.076 [0.107 ± 0.030]	0.70 ± 0.113 [0.274 ± 0.045]	0.238 ± 0.027 [0.094 ± 0.011]	1.26 ± 0.132 [0.494 ± 0.052]	0.590 ± 0.087 [0.232 ± 0.034]	0.567 ± 0.010 [0.167 ± 0.111]

[a] The IC_{50} and SD were obtained via nonlinear regression and are expressed as the mean ± SD, determined from the results of the MTT or ATP assay of three independent experiments. [b] The IC_{50} values are presented as the amount of fruit extract per ml of culture [IC_{50} (mg/ml) ± SD] and the amount of polyphenolic compounds per ml of culture [IC_{50} (µmol GAE/ml) ± SD]. [c] µmol of gallic acid equivalents per mg of dry weight (µmol GAE/mg DW). [d] µmol of cyanidin 3-glucoside equivalents per mg of dry weight (µmol CE/mg DW). [e] Phenolic compounds including anthocyanins.

Table II: Frequency of various cell types of HT-29 cells in CBMN cultures treated with various doses of purified polyphenolic native Australian fruit extracts.

	Frequency of Cell Type[a]									
	Mononuclear	**Binuclear**	**Multi**	**Apoptotic**	**Necrotic**	**NDI**	**MN-BN**	**#MN**	**NPB**	**NBud**
Control	388.1 ± 17.2	99.9 ± 17.4	3.2 ± 0.8	5.6 ± 1.7	3.2 ± 0.8	1.22 ± 0.03	8.4 ± 3.7	11.7 ± 4.0	0.4 ± 0.9	11.2 ± 4.7
IP 0.5 mg/ml	316.7 ± 30.0*	170.5 ± 33.7*	4.2 ± 0.9	6.2 ± 5.3	2.5 ± 2.3	1.36 ± 0.07*	4.0 ± 2.2	4.9 ± 2.0	1.6 ± 1.8	4.6 ± 2.9
IP 1.0 mg/ml	353.5 ± 4.0	136.1 ± 7.1*	4.1 ± 0.8	3.5 ± 2.5	2.8 ± 1.6	1.29 ± 0.01*	8.1 ± 3.3	10.8 ± 5.5	1.7 ± 1.2	10.5 ± 8.8
KP 0.5 mg/ml	312.9 ± 27.5*	63.8 ± 9.0*	7.7 ± 9.6	103.3 ± 29.7*	12.4 ± 4.6*	1.21 ± 0.06	22.8 ± 6.6*	35.0 ± 5.3*	4.2 ± 3.2*	25.0 ± 16.6*
KP 1.0 mg/ml	338.2 ± 13.9*	9.9 ± 1.5*	1.7 ± 1.7	139.1 ± 9.0*	11.1 ± 5.8*	1.04 ± 0.01*	-	-	-	-
M 0.5mg/ml	373.0 ± 20.6	101.4 ± 14.2	3.9 ± 1.4	13.8 ± 6.2	7.9 ± 6.4	1.23 ± 0.03	3.3 ± 1.2	5.0 ± 2.5	1.1 ± 0.7	4.0 ± 3.3
M 1.0 mg/ml	350.9 ± 9.5	116.0 ± 4.4	4.2 ± 0.5	21.7 ± 9.1	7.1 ± 2.8	1.26 ± 0.01	5.1 ± 2.0	6.7 ± 3.2	1.2 ± 0.9	9.0 ± 4.8
NC 0.5 mg/ml	398.8 ± 6.6	71.5 ± 6.6*	2.0 ± 2.7	23.4 ± 12.2	4.2 ± 1.5	1.16 ± 0.02	5.6 ± 4.4	7.8 ± 5.9	0.6 ± 0.8	3.2 ± 3.6
NC 1.0 mg/ml	394.5 ± 15.0	65.0 ± 5.9*	3.7 ± 3.6	33.1 ± 15.3*	3.7 ± 1.0	1.16 ± 0.01	6.9 ± 3.7	10 ± 7.4	0.6 ± 0.8	3.3 ± 1.8

[a] The results represent the mean ± SD per 500 cells of at least 4 counted slides. An asterisk represents significant difference ($p<0.05$) in particular cell type between sample-treated and control slides. CBMN, cytokinesis-block micronucleus. IP, Illawarra Plum. KP, Kakadu Plum. M, Muntries. NC, Native Currant. NDI, nuclear division index. MN, micronuclei. BN, binuclear. NPB, nucleoplasmic bridges. NBud, nuclear buds.

Legends to Figures

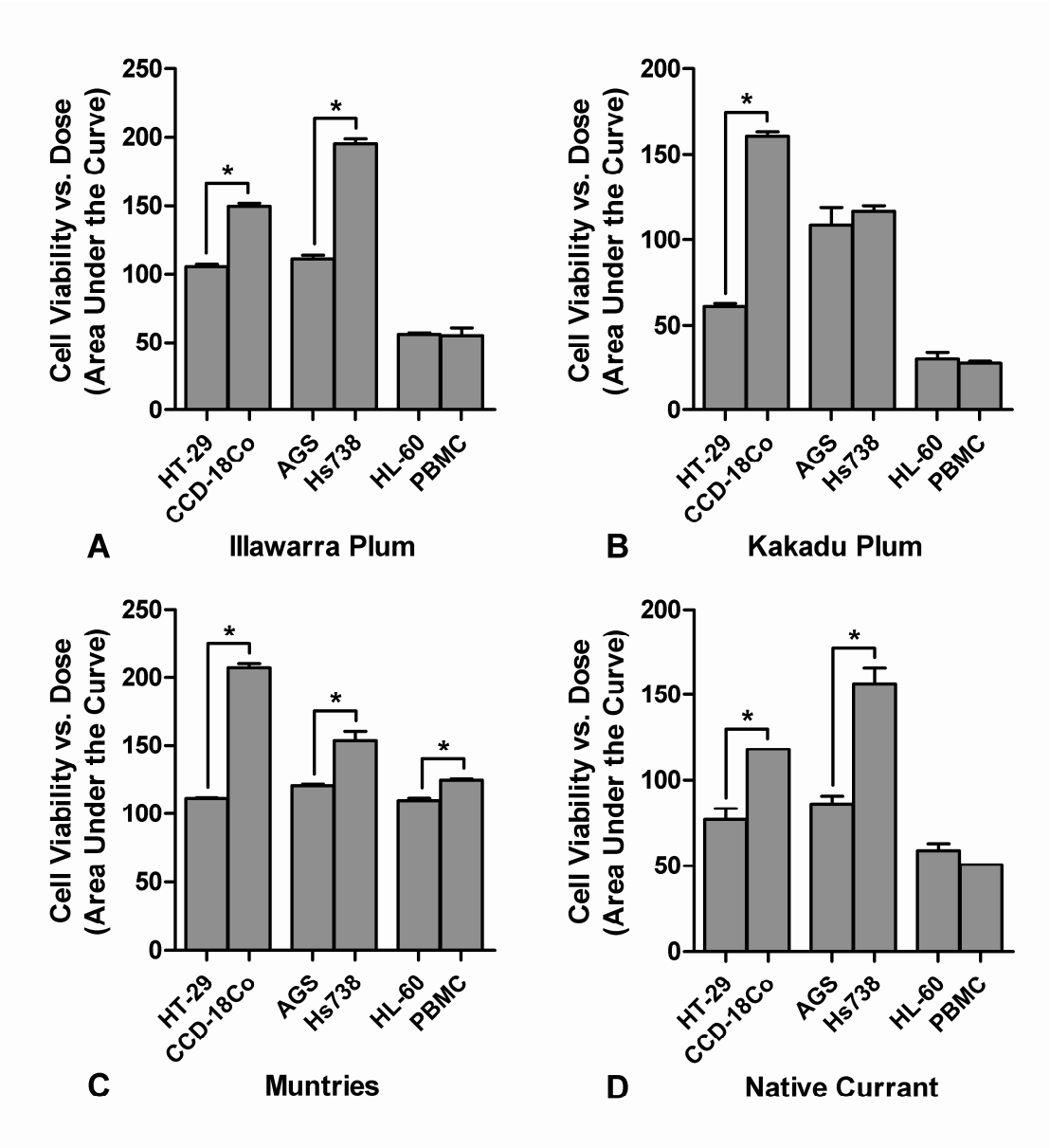

Fig. 1: Dose-dependent effect of purified polyphenolic native Australian fruit extracts on AGS, HT-29, CCD-18Co, Hs738, HL- 60 cells or PBMCs. Cells were treated with varying concentrations for 24 hours and viability was determined using the MTT or ATP assay. Data was graphed for sample concentration (mg/ml) versus cell viability (% of control), and the area under this viability-dose curve was integrated. Differences between values were determined by one-way ANOVA with Tukey's post-hoc test, with an asterisk representing $p<0.05$. (A) Illawarra Plum. (B) Kakadu Plum. (C) Muntries. (D) Native Currant.

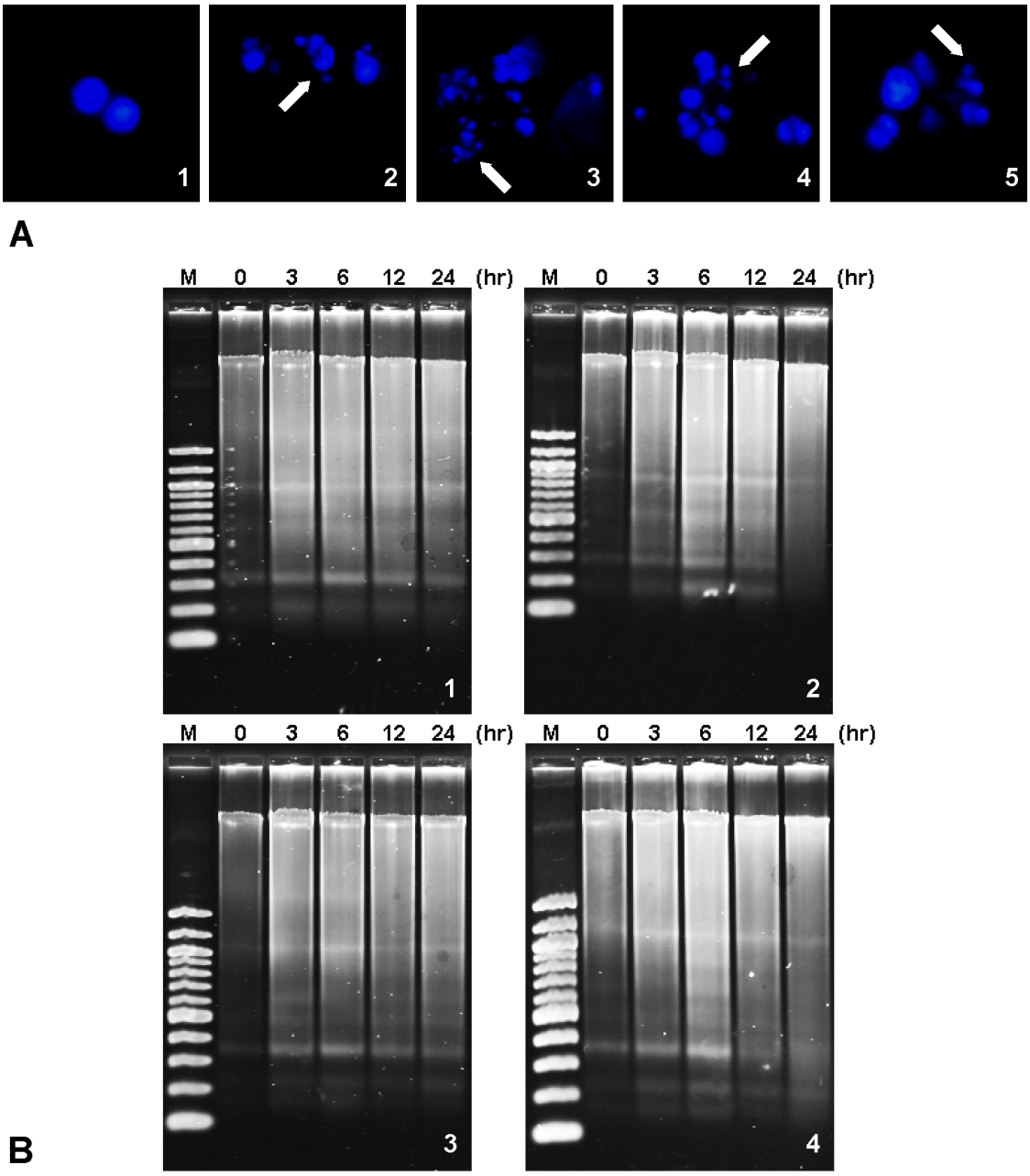

Fig. 2: Purified polyphenolic native Australian fruit extract induced apoptosis in HL-60 cells. (A) Nuclear morphology of cells stained with Hoechst 33342. Cells were treated with 0.75 mg/ml of native Australian fruit extract for 24 hours. (1) Control. (2) Illawarra Plum. (3) Kakadu Plum. (4) Muntries. (5) Native Currant. (B) Time-dependent DNA fragmentation of HL-60 cells treated with 0.75 mg/ml of native Australian fruit extracts. Following treatment, DNA was extracted, separated by 2% agarose gel electrophoresis and visualised after ethidium bromide staining. M, DNA 100bp marker. (1) Illawarra Plum. (2) Kakadu Plum. (3) Muntries. (4) Native Currant.

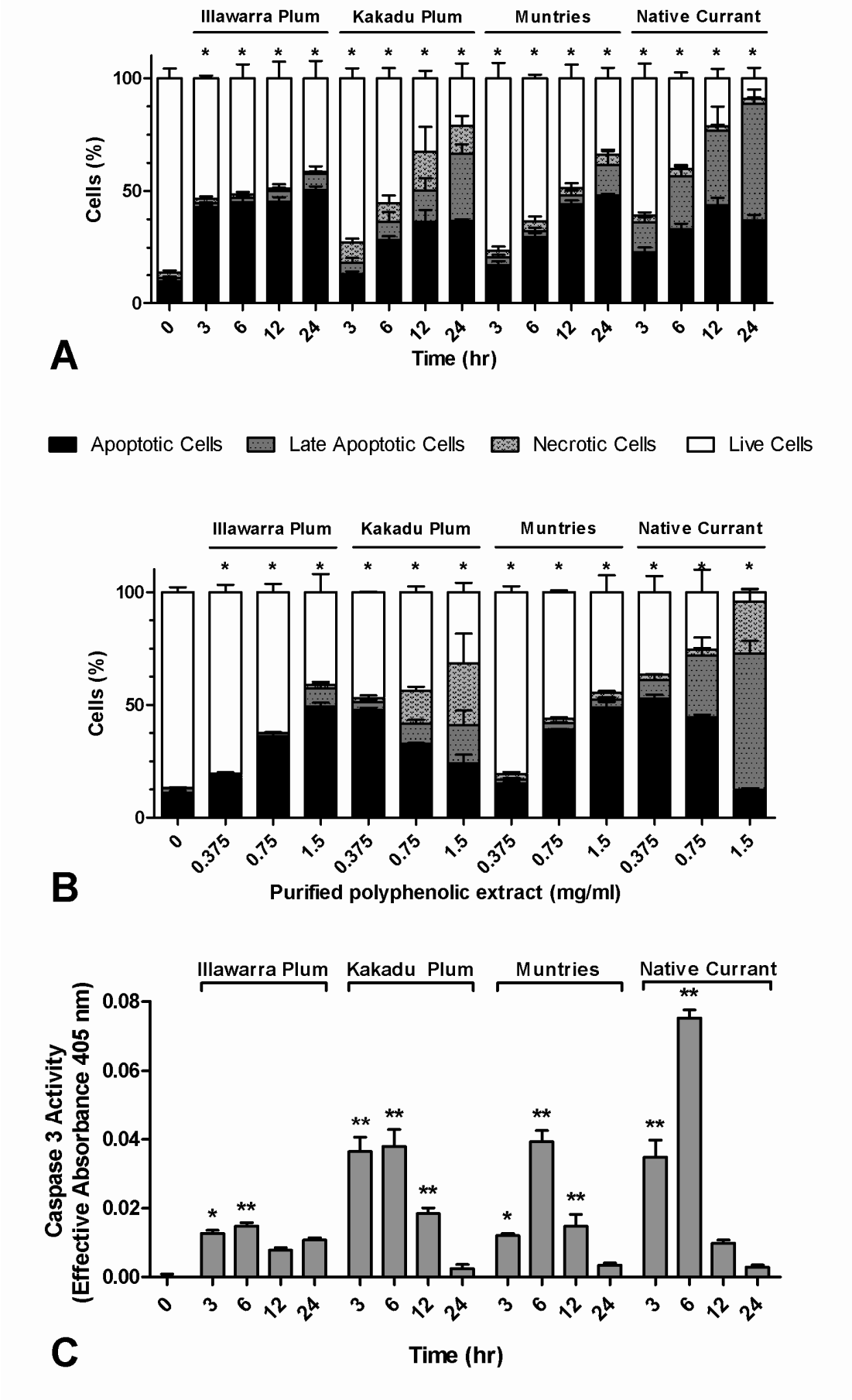
Illawarra Plum
Kakadu Plum
Muntries
Native Currant
Cells (%)
100
50
0
0 3 6 12 24 3 6 12 24 3 6 12 24 3 6 12 24
Time (hr)
A
Apoptotic Cells
Late Apoptotic Cells
Necrotic Cells
Live Cells
Illawarra Plum
Kakadu Plum
Muntries
Native Currant
Cells (%)
100
50
0
0 0.375 0.75 1.5 0.375 0.75 1.5 0.375 0.75 1.5 0.375 0.75 1.5
Purified polyphenolic extract (mg/ml)
B
Illawarra Plum
Kakadu Plum
Muntries
Native Currant
Caspase 3 Activity
(Effective Absorbance 405 nm)
0.08
0.06
0.04
0.02
0.00
0 3 6 12 24 3 6 12 24 3 6 12 24 3 6 12 24
Time (hr)
C

Fig. 3: Flow cytometric analysis and level of caspase-3 activation of HL-60 cells treated with purified polyphenolic native Australian fruit extracts. (A, B) Flow cytometric analysis. Percentage of live (white), apoptotic (black), late apoptotic (dark grey) and necrotic cells (light grey) were measured in cells stained with annexin V and propidium iodide. Data represents the mean value ± standard deviation of the percentage of cells in each population obtained from three independent experiments. An asterisk represents significant difference ($p<0.05$) between percentage of live cells and percentages of apoptotic and late apoptotic cells added. (A) Time-course experiment. Cells were treated with 0.75 mg/ml of purified polyphenolic extracts for 3, 6, 12 and 24 hours. (B) Dose response experiment. Cells were treated with 0.375, 0.75 and 1.5 mg/ml of purified polyphenolic extracts for 6 hours. (C) Level of caspase-3 activation. Cells were treated with 0.75 mg/ml of native Australian fruit extracts and cellular extracts were analysed using a colorimetric caspase-3 assay kit. Data represents the mean value ± standard deviation of the effective absorbance value at 405 nm compared to control cells of three independent experiments. An asterisk represents significant difference (* $p<0.05$; ** $p<0.01$) between sample treated and negative control absorbances.

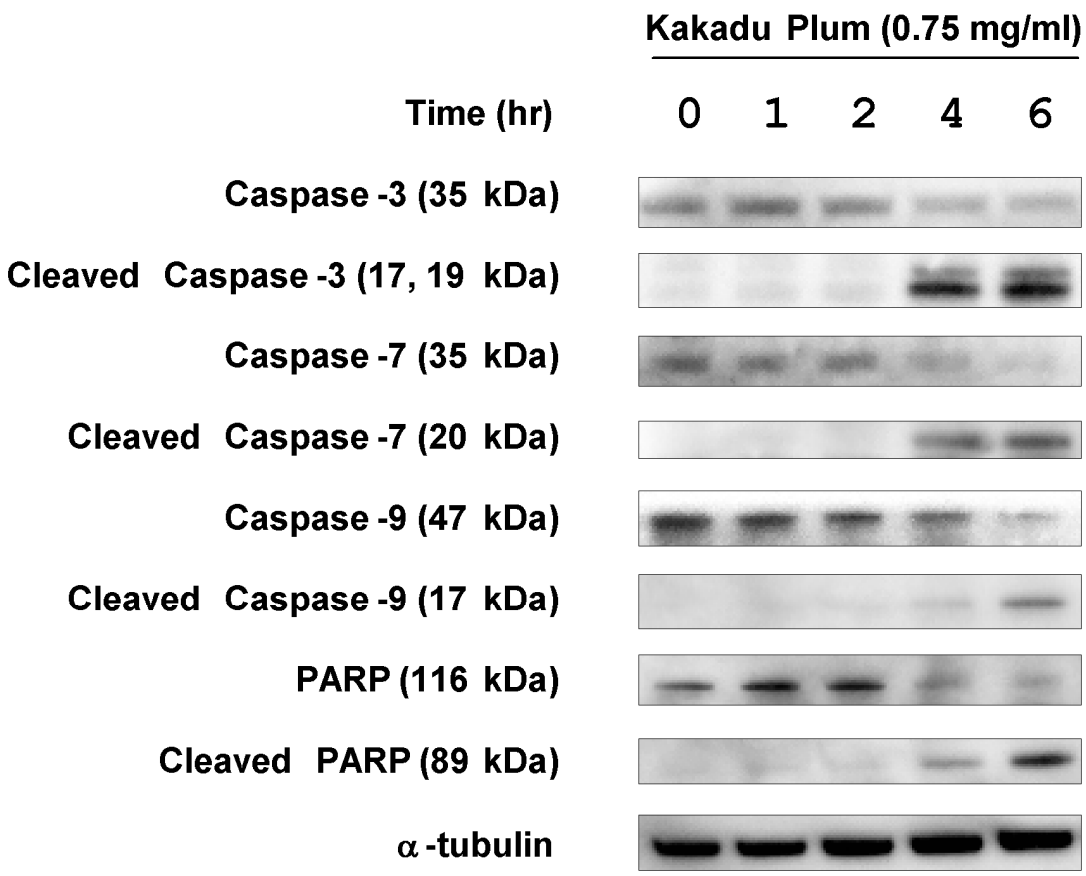

Fig. 4: Activation of caspases and PARP cleavage in HL-60 cells treated with Kakadu Plum purified polyphenolic extract. Cells were treated with 0.75 mg/ml of Kakadu Plum for 1, 2, 4 and 6 hours. Cleavage of PARP and activation of caspases were determined by Western blot analysis. Experiments were conducted in triplicate and results shown are representative.