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Potential Antioxidant, Antiinflammatory, and Proapoptotic Anticancer Activities of Kakadu Plum and Illawarra Plum Polyphenolic Fractions

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Kakadu plum (Terminalia ferdinandiana Exell, Combretaceae) and Illawarra plum (Podocarpus elatus Endl., Podocarpaceae) extracts were fractionated, using a bioassay-guided approach and screened for antioxidant activity [oxygen radical absorbance capacity (ORAC) and cellular antioxidant activity (CAA) assays] and antiinflammatory activity (nitrite concentration and prostaglandin E_2 release in lipopolysaccharide (LPS)-activated murine macrophages). Among 8 fractions obtained from KP and 5 fractions obtained from IP, fraction KPF5 from KP exhibited superior activity in all assays, with an ORAC value of 3,776 ± 603 μmol Trolox/g DW and a CAA value of 52.2 ± 8.6 μmol quercetin equivalents/g DW. In addition, KPF5 further demonstrated an upregulation of the Nrf2/Keap1 ratio in Hep G2 cells. KPF5 also inhibited the expression of COX-2 and iNOS in LPS-activated murine macrophages, potentially through the NF-κB, p44/42 mitogen activated protein kinase and Akt pathways. KPF5 also induced apoptosis and DNA damage in HT-29 cells, as determined by the cytokinesis block micronucleus cytome assay.

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INTRODUCTION

Novel preventative strategies for cancer are essential. Despite being a largely preventable disease (1), cancer still contributes significantly (19%) to the total burden of disease in Australia (2). Epidemiological investigations have revealed that a high intake of fruits and vegetables reduces cancer incidence (3), and it has been estimated that up to 25% of cancer deaths are due to inappropriate diet (4). Dietary phytochemicals are believed to have a protective effect against the development of cancer (5), and, consequently, interest in their use as chemopreventive agents has increased substantially. Carcinogenesis, however, is a complex process, encompassing a multitude of pathological and biochemical events (6). A systematic and wide-ranging approach to the discovery of dietary compounds with chemopreventive activity is necessary. Oxidative stress, inflammation, and the evasion of apoptosis are 3 key biological mechanisms in the development of the characteristic hallmarks of cancer (7–9). Consequently, antioxidant, antiinflammatory, and proapoptotic anticancer activities represent several broad biological mechanisms, which collectively are important in preventing, suppressing, or reversing the development of carcinogenesis. Phytochemicals, which exhibit biological activities through either of these mechanisms, may prove to have the greatest potential as chemopreventive agents (10).

Native Australian fruits offer enormous opportunities for the discovery of chemopreventive phytochemicals (11). The sheer diversity in the flora is illustrated with 2,440 species of fruiting rainforest plants found in the tropical regions of Australia alone (12). This diversity, however, necessitates an appropriate method of selection for the most promising fruits for research. An ethnobotanical analysis combined with previous screening studies of native Australian fruits for the above mentioned 3 broad biological mechanisms resulted in the selection of Kakadu plum (Terminalia ferdinandiana Exell, Combretaceae) and Illawarra plum (Podocarpus elatus Endl., Podocarpaceae) for further studies (13–15). Kakadu plum and Illawarra plum are amongst the most traditionally popular fruits for the Aboriginal populations in the areas in which they are found (16,17). The Kakadu plum was even considered by certain tribes more as medicine than food (18) and is among the world's highest known sources of vitamin C (19). Furthermore, previous studies have revealed the significant in vitro antioxidant activity of Kakadu plum and Illawarra plum, much greater than other native Australian fruits and a blueberry reference (13,20). Both fruits have also been found to exhibit in vitro antiinflammatory activity in lipopolysaccharide (LPS)-activated murine macrophages (15) and in vitro antiproliferative and apoptotic activity in various cancer cell lines (14,21).

In the present study, purified polyphenolic fractions of Kakadu plum and Illawarra plum were first screened for antioxidant and antiinflammatory activity using in vitro cell culture-based assays. The best-performing fraction was then further examined in a range of experimental systems to elucidate the mechanism of these activities. These experimental systems have all been previously used in the literature and in studies with the whole extracts of native Australian fruits and provide a reference point to the activities displayed by the Kakadu plum and Illawarra plum fractions (13–15). Specifically, antioxidant activity was determined by levels of nuclear factor-erythroid 2p45 (NF-E2)-related factor 2 (Nrf2) and Kelch-like ECH-associated protein 1 (Keap1) in Hep G2 cells; antiinflammatory activity was determined by cyclooxygenase (COX)-1, COX-2 and inducible nitric oxide synthase (iNOS) levels in LPS-activated murine macrophages with possible molecular pathways of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), p44/42 mitogen activated protein kinase (MAPK), and Akt also examined; and finally, proapoptotic anticancer activity was determined by the CBMN Cyt assay in HT-29 cells.

MATERIALS AND METHODS

Preparation of Polyphenolic Extracts and Fractions From Native Australian Fruits

The native Australian fruits Illawarra plum (Podocarpus elatus Endl., Podocarpaceae) and Kakadu plum (Terminalia ferdinandiana Exell, Combretaceae) were purchased from Tanamera Native Produce Pty., Ltd., Obum Obum, Australia. The raw fruit material of Illawarra plum and Kakadu plum was initially weighed and ground into a pulp using a heavy-duty blender (Waring Laboratory Science, Torrington, CT). A 2-fold volume of acidified ethanol (80% ethanol, 19.9% H_2O, and 0.5% TFA, vol/vol/vol) was then added, stirred for 15 min, and centrifuged for 25 min at 11,000 $\times$ g at 4°C (Sorvall RC-5B; DuPont, Wilmington, DE) with the supernatant collected. An equivalent volume of acidified ethanol was re-added to the fruit material and left overnight at 4°C. The addition of acidified ethanol, centrifugation, and collection of supernatant was conducted in triplicate. The supernatant from 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 an ethanolic extract.

The ethanolic extract was further purified and fractionated using an XAD-7HP (Sigma-Aldrich, St. Louis, MO) resin column (300 $\times$ 60 mm i.d.). The extract was dissolved with acidified water (99.5% H_2O, 0.5% TFA, vol/vol), applied to the column, washed with acidified water, and eluted with a stepwise gradient of various concentrations of 500 ml acidified ethanol (0.5% TFA) ranging from 20% to 100% ethanol in 10% increments. The eluate was collected every 100 ml and analyzed by high-performance liquid chromatography (HPLC). Eluates with similar HPLC profiles were combined to create different fractions. Subsequently the fractions were evaporated under reduced pressure at 37°C using a rotary evaporator. The resulting dry fractions were dissolved in purified water and freeze-dried under vacuum to obtain a fine lyophilized powder representing phenolic-rich fractions of the fruit.

Quantification of Phenolic Compounds by HPLC-Diode-Array Detection

Quantification of phenolic compounds and anthocyanins in the fractions was conducted according to Kammerer et al. (22) and Terahara et al. (23) with minor modifications. The HPLC system consisted of 2 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 $\times$ 4.6 mm i.d., 5 μm Luna $C_{18(2)}$ column (Phenomenex, Torrance, CA). Analytical HPLC was run at 25°C and monitored at 280 nm, 326 nm, and 370 nm for phenolic acids and at 520 nm for anthocyanins. The following solvents in purified water with a flow rate of 1.0 ml/min were used: solvent A 0.5% TFA and solvent B 95% acetonitrile and 0.5% TFA. The elution profile was a linear gradient elution for B of 0% to 10% over 8 min in solvent A, followed by 30% B in 20 min and then to 100% B in 1 min. Phenolic compounds were quantified as μmol of gallic acid equivalents (GAEs) per mg of dry weight (μmol GAEs/mg DW) at 280 nm, μmol of chlorogenic acid equivalents (μmol CAEs/mg DW) at 326 nm, and μg of cyanidin 3-glucoside equivalents (μg CEs/mg DW) at 520 nm.

LC-PDA-MS/MS

LC-PDA-MS/MS analysis was conducted on a Quantum triple stage quadrapole (TSQ) mass spectrometer (Thermo Fisher Scientific, Waltham, MA), equipped with a quaternary solvent delivery system, a column oven, a photo-diode array detector, and an autosampler. An aliquot (5 μl) of an aqueous methanolic (80%) solution of the fraction of interest was chromatographed on a 150 × 2.1 mm i.d., 5 μm Luna $C_{18(2)}$ column (Phenomenex), which was heated to 30°C. The following solvents with a flow rate of 200 μl/min were used: solvent A, 0.5% formic acid in purified water and solvent B, 0.5% formic acid in acetonitrile. The elution profile was a linear gradient for B of 10% to 100% over 20 min in solvent A. Ions were generated using an electrospray source in the positive mode under conditions set following optimization using chlorogenic acid. MS experiments in the full scan (parent and product-specific) and the selected reaction monitoring (SRM) mode were conducted.

Cell Culture

All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD) 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, Carlsbad, CA). Hep G2 (hepatocellular carcinoma) was cultured in Eagle's minimum essential medium (EMEM; Invitrogen Corporation); HT-29 (colorectal adenocarcinoma) was cultured in McCoy's 5a medium (Invitrogen); and RAW 264.7 (murine macrophage) was cultured in Dulbecco's Modified Eagle's medium (DMEM; Invitrogen).

Oxygen Radical Absorbance Capacity (ORAC) Assay

Oxygen radical scavenging capacity was determined using the ORAC assay as described previously (21). Briefly, fluorescein (120 nM) and diluted sample were inserted into a fluorescence spectrophotometer and equilibrated at 37°C. AAPH was added and fluorescence ($\lambda_{ex} = 495$ nm; $\lambda_{em} = 515$ nm) recorded every 5 s and a kinetic curve generated. 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.

Cellular Antioxidant Activity (CAA) Assay

Antioxidant activity was determined using the CAA assay (24) as described previously (13). Briefly, Hep G2 cells were treated with the purified polyphenolic fractions and 25 μM DCFH-DA and incubated for 1 h. After the addition of 600 μM ABAP, fluorescence ($\lambda_{ex} = 485$ nm; $\lambda_{em} = 535$ nm) was recorded every 5 min for 1 h. 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.

Measurement of Nitrite Concentration

Nitrite concentration in culture supernatant was determined by Griess reaction. RAW 264.7 cells (3×10^5 per well) were incubated for 24 h at 37°C in 48-well plates (Iwaki; Asahi Techno Glass, Chiba, Japan). The medium was removed and fresh serum-free medium was then added for 2.5 hours to eliminate the influence of FBS. The cells were treated for 1 h with a range of concentrations of purified polyphenolic fractions before exposure to 40 ng/ml LPS for 12 h. Equal volumes of the culture supernatant were mixed with modified Griess reagent (Sigma-Aldrich) for 15 min at room temperature in the absence of light. Nitrite concentration was measured by absorbance levels at 540 nm using a spectrophotometer, against a sodium nitrite standard curve. Nitrite, an end product of nitric oxide (NO) metabolism, was used as a measure of NO production.

Measurement of Prostaglandin E_2 (PGE_2) Production

PGE_2 concentration in culture supernatant was determined with a PGE_2 enzyme immunoassay kit (Sapphire Biosciences, Redfern, Australia) according to the manufacturer's instructions. RAW 264.7 cells (5×10^5 per well) were incubated for 24 h at 37°C in 6-well plates. The medium was removed and fresh serum-free medium was then added for 2.5 h to eliminate the influence of FBS. The cells were treated for 1 h with a range of concentrations of purified polyphenolic fractions before exposure to 40 ng/ml LPS for 12 h. The level of PGE_2 released into the culture medium was determined by measuring absorbance levels at 412 nm using a spectrophotometer against a PGE_2 standard curve.

Western Blot Analysis

The preparation of Hep G2 cell lysates was according to Tanigawa et al. (25) with minor modifications as described previously (15). The preparation of RAW 264.7 cell lysates was conducted according to Hou et al. (26) with minor modifications as described previously (15). The whole-cell lysates containing bromophenol blue were analyzed by Western blot analysis as described previously (13). Primary antibodies against Nrf2, Keap1, COX-1, COX-2, iNOS, and α-tubulin, and AP-conjugated antigoat, antimouse, and antirabbit secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies against IκBα, phospho-IκBα, IKKα, IKKβ, phospho-IKKα/β, p44/42 MAP kinase, phospho-p44/42, and phospho-Akt were obtained from Cell Signaling Technology (Beverly, MA). Bound antibodies were detected and the relative amounts of proteins associated with specific antibody were quantified using a chemiluminescent imager (FluorChem SP; Alpha Innotech, San Leandro, CA). The mean and SD of the relative densities and statistical differences were determined using triplicate data. Images of Western blots are representative of the triplicate data.

Cytokinesis-Block Micronucleus Cytome (CBMN Cyt) Assay

The CBMN Cyt assay was conducted using the cytochalasin B technique according to Fenech (27) with minor modifications, as described previously (14). The assay was conducted to measure the different endpoints in untreated and purified polyphenolic fraction treated cells. Scoring was based on the previously

TABLE 1
Quantification (HPLC-DAD) of phenolic compounds from Kakadu plum (KPFs) and Illawarra plum purified polyphenolic fractions (IPFs)

Purified Polyphenolic Fraction	Phenolic Compounds (μmol GAE/mg DW) Absorbance (280 nm)	Phenolic Compounds (μmol CAE/mg DW) Absorbance (326 nm)	Anthocyanins (μmol CE/mg DW) Absorbance (520 nm)
KPF1			
KPF2	0.041	0.002	
KPF3	0.187		
KPF4	0.294		
KPF5	0.313		
KPF6	0.343		
KPF7	0.075		
KPF8	0.260		
IPF1	0.003		
IPF2	0.035		
IPF3	0.026		0.053
IPF4		0.001	0.010
IPF5			0.002

μmol GAE/mg DW = μmol of gallic acid equivalents per mg of dry weight; μmol CAE/mg DW = μmol of chlorogenic acid equivalents per mg of dry weight; μmol CE/mg DW = μmol of cyanidin 3-glucoside equivalents per mg of dry weight.

described CBMN Cyt scoring criteria (27). The biomarkers scored include frequency of binucleated (BN) cells with micronuclei (MN-BN), with nucleoplasmic bridges (NPB), with nuclear buds (NBud), and frequency of necrotic and apoptotic cells. The nuclear division index (NDI) was calculated from the ratio of MN, BN, and multinucleated cells (28). A total of 500 cells were scored per slide to determine ratios of MN cells, BN cells, multinucleated cells, necrotic and apoptotic cells. A total of 500 BN cells were scored per slide to determine frequency of MN, MN-BN, NPB, and NBud. Each treatment concentration and control was assessed in sextuplicate.

Statistical Analyses

All results are expressed as mean ± SD of at least 3 independent experiments. Differences between treatment and control values or between samples were determined using 1-way analysis of variance (ANOVA) with Tukey's post hoc test (GraphPad, San Diego, CA), with $P < 0.05$ as statistically significant.

RESULTS

Characterization of Kakadu Plum and Illawarra Plum Fractions

Among the various native Australian fruits screened for potential physiological activities, Kakadu plum and Illawarra plum exhibited significant in vitro antioxidant, antiinflammatory, and proapoptotic anticancer activity (13–15). Subsequently, these 2 fruits were subjected to bioassay-guided fractionation, a well-established paradigm to yield a fraction that would assist in determining any novel, bioactive compounds (29). The fractionation of the Kakadu plum extract yielded 8 distinct fractions (termed KPF1–KPF8), whereas the fractionation of Illawarra plum yielded 5 distinct fractions (termed IPF1–IPF5). As shown in Table 1, the Kakadu plum fractions comprised mainly constituents with a maximum UV absorbance at 280 nm, which suggests the presence of components such as hydroxybenzoic acids and flavan-3-ols. The greatest amounts of these compounds were contained within KPF5 and KPF6, although KPF1 contained no detectable compounds. The major components of the Illawarra plum fractions were anthocyanins, detected with a maximum UV absorbance at 520 nm. IPF3 contained the greatest amounts of anthocyanins.

Assessment of Potential Physiological Activity of Kakadu Plum and Illawarra Plum Purified Fractions

Antioxidant Capacity (ORAC Assay) and Cellular Antioxidant Activity (CAA Assay)

Each Kakadu plum and Illawarra plum fraction was screened for antioxidant activity using the ORAC and CAA assays (Table 2). Among the Kakadu plum fractions, KPF8 exhibited the greatest oxygen radical scavenging ability, followed by KPF5 and KPF4. Of the Illawarra plum fractions, IPF3 contained the greatest scavenging ability, followed by IPF2. Each Kakadu plum and Illawarra plum fraction, however, displayed a lower ORAC value than whole extracts of Kakadu plum and Illawarra plum, respectively. The CAA assay revealed KPF1 contained the greatest cellular antioxidant activity, followed by KPF5 and KPF4. The anthocyanin-rich IPF3 again contained the greatest

TABLE 2
Antioxidant capacity (ORAC assay) and cellular antioxidant activity (CAA assay) of Kakadu plum (KPFs) and Illawarra plum purified polyphenolic fractions (IPFs)

Purified Polyphenolic Fraction	ORAC (μmol Trolox/g DW)	CAA EC_{50} (μg/ml)	CAA (μmol QE/g DW)
Quercetin	—	10.8 ± 1.1	—
KPF1	$1886.9 \pm 15.3\,a$	162.9 ± 28.1	$67.2 \pm 10.9\,a$
KPF2	$3484.4 \pm 38.7\,bc$	276.6 ± 29.2	$39.1 \pm 3.9\,bc$
KPF3	$2090.1 \pm 470.3\,ac$	303.7 ± 69.4	$36.6 \pm 7.6\,bc$
KPF4	$3583.2 \pm 377.0\,b$	277.1 ± 48.6	$39.6 \pm 7.3\,bc$
KPF5	$3776.1 \pm 602.8\,bd$	210.0 ± 35.9	$52.2 \pm 8.6\,ab$
KPF6	$2966.2 \pm 326.8\,abc$	349.0 ± 13.6	$30.8 \pm 1.2\,cd$
KPF7	$2650.6 \pm 603.4\,abc$	440.8 ± 73.4	$24.9 \pm 4.5\,cd$
KPF8	$5109.3 \pm 321.7\,d$	714.2 ± 152.5	$15.6 \pm 3.5\,d$
Kakadu plum whole extract	5996.6 ± 18.6	153.0 ± 24.5	71.5 ± 11.3
IPF1	$903.9 \pm 340.7\,A$	5766.3 ± 1101.2	$1.9 \pm 0.33\,A$
IPF2	$2441.9 \pm 362.9\,AB$	809.9 ± 90.7	$13.4 \pm 1.5\,AB$
IPF3	$2937.7 \pm 369.1\,A$	299.0 ± 70.0	$37.5 \pm 10.0\,C$
IPF4	$2019.5 \pm 367.7\,AB$	1012.1 ± 35.2	$10.6 \pm 0.37\,AB$
IPF5	$1694.2 \pm 806.1\,AB$	482.8 ± 99.0	$22.7 \pm 4.7\,BC$
Illawarra plum whole extract	5050.0 ± 817.5	233.2 ± 20.2	46.3 ± 3.9

Values in each column with no letters in common are significantly different ($P < 0.05$) using analysis of variance and post hoc analysis. μmol Trolox/g DW = μmol of Trolox per g of dry weight; μmol QE/g DW = μmol of quercetin equivalents per g of dry weight.

cellular antioxidant activity amongst the Illawarra plum fractions. The CAA values for the whole Kakadu plum and Illawarra plum extracts were also greater than the individual fractions of each fruit.

Assessment of Antiinflammatory Activity

The Kakadu plum and Illawarra plum purified polyphenolic fractions were also screened for antiinflammatory activity in LPS-activated murine macrophages (RAW 264.7), by measuring nitrite concentration using Griess reaction and PGE_2 release using an enzyme immunoassay (Fig. 1). Each Kakadu plum fraction induced a dose-dependent decrease in nitrite concentration, with the greatest reductions by KPF6 and KPF5 (Fig. 1A). Fractions KPF2 to KPF8 all displayed significant nitrite concentration reductions in a dose-dependent manner over the concentration range 25-400 μg/ml. Of the Illawarra plum fractions, however, the greatest decreases were seen with IPF5 and IPF3, but only at the higher concentration range of 100–400 μg/ml (Fig. 1A). The results of the PGE_2 enzyme immunoassay revealed similar trends, with each Kakadu plum fraction inhibiting PGE_2 in a dose-dependent manner, except KPF6 (Fig. 1B). The greatest inhibition was displayed by KPF7 and KPF5. Of the Illawarra plum fractions, there was a similar pattern of dose-dependent inhibition of PGE_2 enzyme activity, except for IPF1 and IPF4. IPF5 again displayed the greatest antiinflammatory activity of the Illawarra plum fractions in inhibiting PGE_2 release (Fig. 1B).

Screening for the antioxidant and antiinflammatory capacity of the Kakadu plum and Illawarra plum purified polyphenolic fractions, illustrated the enhanced activity in various assays of the KPF5 fraction over the other Kakadu plum and Illawarra plum fractions. Although other fractions displayed greater activity in individual assays, KPF5 showed the greatest activity overall. There are several key biological mechanisms through which a chemopreventive agent may exert its activity (10) and, consequently, KPF5 was chosen for detailed biological mechanistic investigation into its potential antioxidant, antiinflammatory and proapoptotic anticancer activity and secondly, chemical fingerprinting as determined by LC-PDA-MS/MS.

Molecular Mechanism of the Potential Biological Activity of KPF5

Potential Activation of the ARE (Nrf2/Keap1 ratio) by KPF5

The effect of KPF5 on the potential activation of the antioxidant response element (ARE), through the levels of Nrf2 and Keap1 in Hep G2 cells is presented in Fig. 2. At concentrations equal or higher than 200 μg/ml, KPF5 significantly increased the Nrf2/Keap1 ratio in a dose-dependent manner. This indicates an increase in the relative levels of Nrf2, potentially resulting in the activation of the ARE.

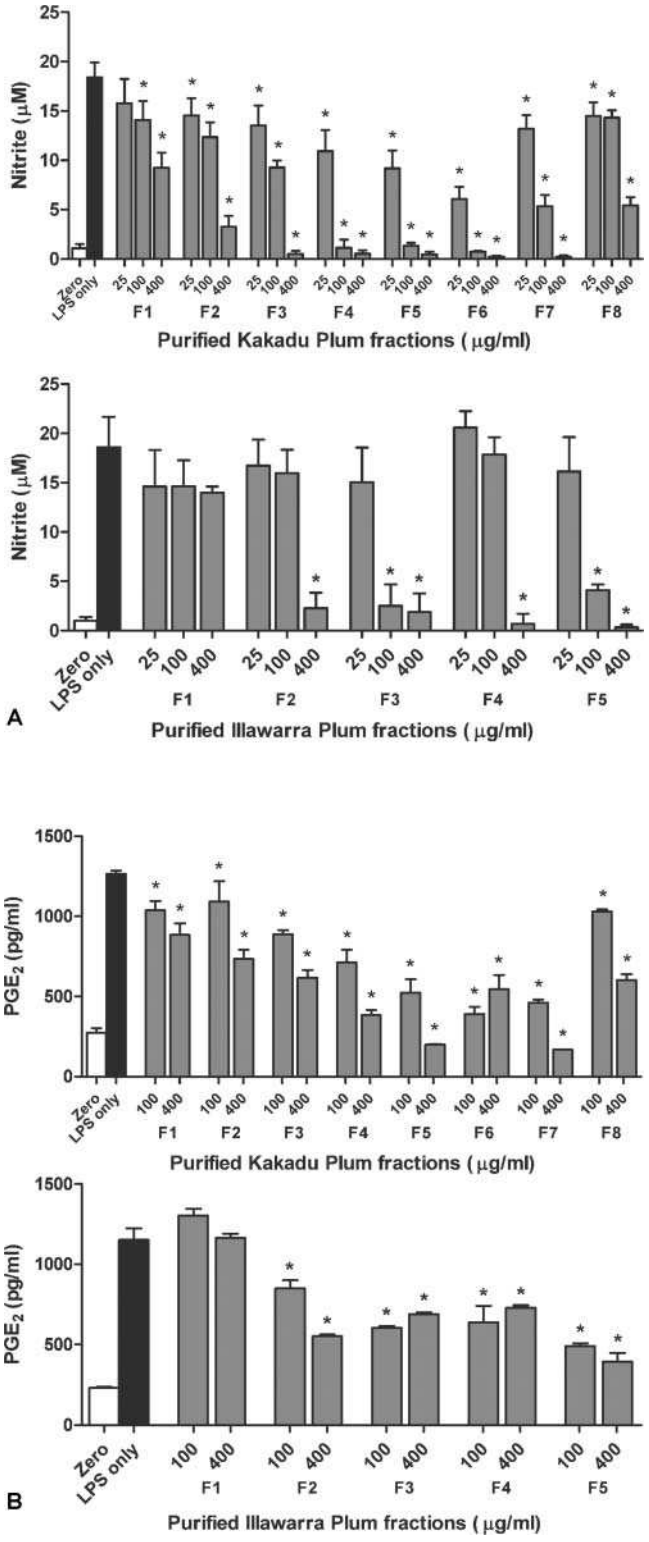

FIG. 1. Dose-dependent effect of Kakadu plum and Illawarra plum purified polyphenolic fractions on nitrite concentration (A) and prostaglandin E_2 (PGE_2) release (B). RAW 264.7 cells were exposed to serum-free medium for 2.5 h, different concentrations of purified polyphenolic fractions for 1 h and LPS for a further 12 h. The nitrite concentration or level of PGE_2 release was then measured. An asterisk indicates significant difference with lipopolysaccharide control ($P < 0.05$) of triplicate experiments.

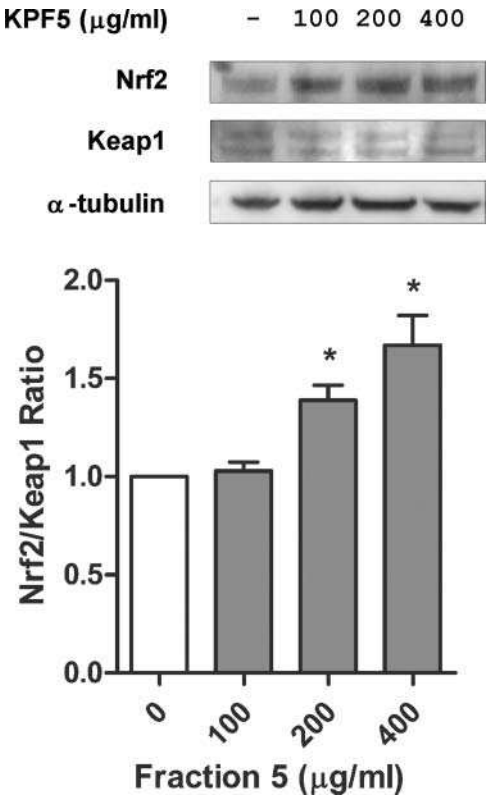

FIG. 2. Effect of the selected Kakadu plum purified fraction (KPF5) on steady-state Nrf2 and Keap1. Hep G2 cell lysates were prepared after exposure to different concentrations of KPF5 for 9 h. The expression of the Nrf2, Keap1, and α-tubulin proteins was detected by Western blot analysis. An asterisk indicates significant difference with negative control ($P < 0.05$) of triplicate experiments. Keap1 = Kelch-like ECH-associated protein 1; Nrf2 = nuclear factor-erythroid 2p45 (NF-E2)-related factor 2.

Effect of KPF5 on Key Inflammatory Enzymes and Pathways in LPS-Activated Murine Macrophages

Figure 3 illustrates the effect of KPF5 on the expression of the COX-1, COX-2, and iNOS enzymes, monitored in RAW 264.7 cells. Treatment with KPF5 induced a dose-dependent decrease in levels of COX-2, with no effect on the levels of COX-1 at the concentrations tested. Furthermore, KPF5 induced a significant reduction of iNOS across the concentrations (100–400 μg/ml) tested. These results indicate that KPF5 components modulated the activity of key inflammatory enzymes.

In order to further examine KPF5 against several molecular pathways that are important in the regulation of COX-2 and iNOS mediated inflammation, the effects of KPF5 on the NF-κB, p44/42 MAPK, and Akt pathways were characterized by exposure of RAW 264.7 cells to KPF5 (Fig. 4). The results indicated KPF5 inhibited the phosphorylation and degradation of IκBα. KPF5 also inhibited the phosphorylation of p44/42 MAPK and Akt, suggesting that KPF5 may exert antiinflammatory activity through the NF-κB, p44/42 MAPK, and Akt pathways.

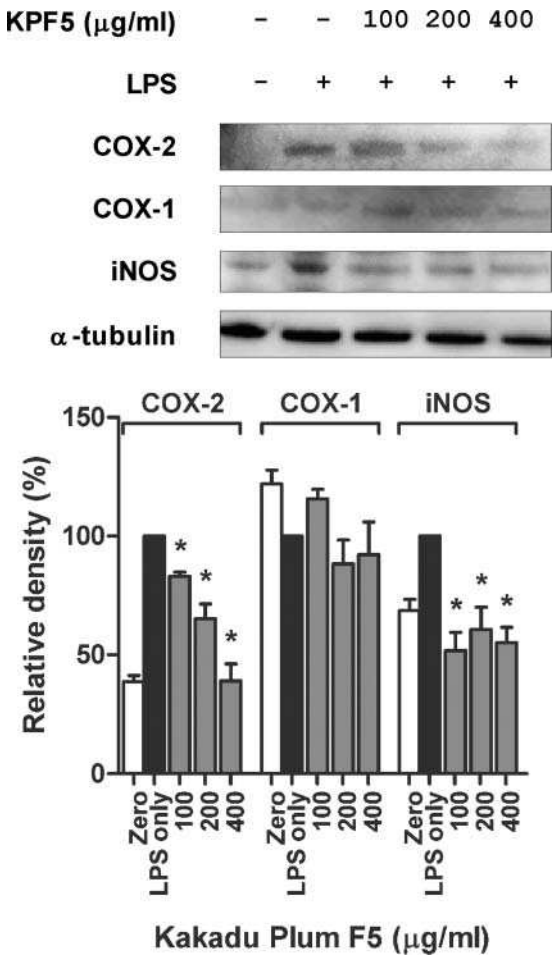

FIG. 3. Expression of COX-2, COX-1, and iNOS in RAW 264.7 cells treated with the selected Kakadu plum purified fraction (KPF5). RAW 264.7 cell lysates were prepared after exposure to serum-free medium for 2.5 h, different concentrations of KPF5 for 1 h, and LPS for a further 12 h. The expression of the COX-2, COX-1, iNOS, and α-tubulin proteins was detected by Western blot analysis. An asterisk indicates significant difference with lipopolysaccharide (LPS) control ($P < 0.05$) of triplicate experiments. COX = cyclooxygenase; iNOS = inducible nitric oxide synthase; LPS = lipopolysaccharide.

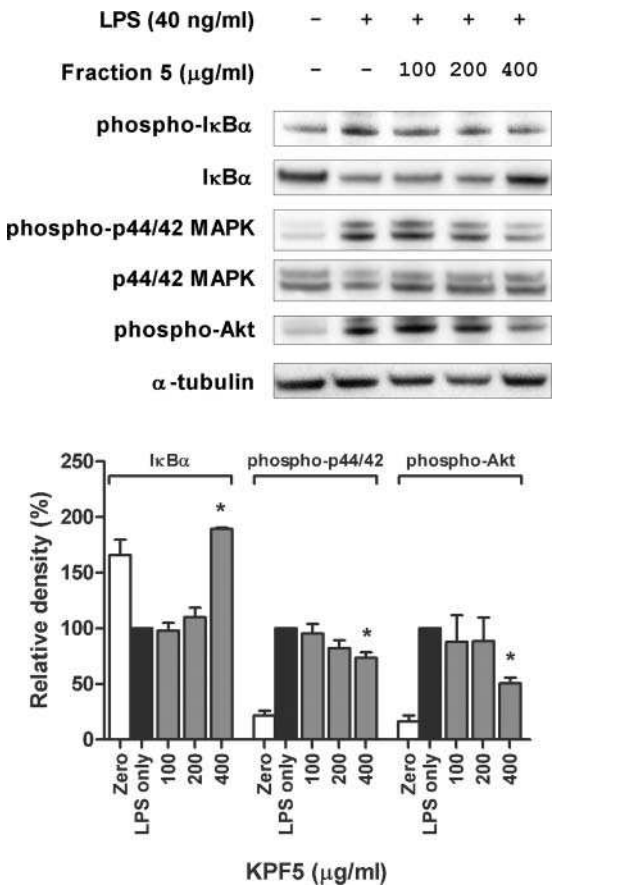

FIG. 4. Effect of the selected Kakadu plum purified fraction (KPF5) on NF-κB, p44/42 mitogen-activated protein kinase (MAPK), and Akt pathways. RAW 264.7 cell lysates were prepared after exposure to serum-free medium for 2.5 h, different concentrations of KPF5 for 1 h, and lipopolysaccharide (LPS) for a further 30 min. Data represent the mean ± SD of at least 3 independent experiments. An asterisk indicates significant difference with LPS control ($P < 0.05$). The expression of the IκBα, phospho-IκBα, IKKα, IKKβ, phospho-IKKα/β, p44/42 MAP kinase, phospho-p44/42, phospho-Akt, and α-tubulin proteins was detected by Western blot analysis.

Genotoxic, Proapoptotic and Cytostatic Effects of KPF5 in HT-29 Cancer Cells in the CBMN Cyt Assay

HT-29 cells were exposed to KPF5 and the frequency of various biomarkers was scored (Table 3). The results revealed that KPF5 induced apoptosis in HT-29 cells, with increased numbers of apoptotic cells at both KPF5 0.5 mg/ml and 1.0 mg/ml, whereas there were no changes in the level of necrotic cells. KPF5 also induced a cytostatic effect on HT-29 cells, with a decrease in NDI for KPF5 1.0 mg/ml. This treatment induced a substantial decrease in the numbers of BN cells, indicating the various markers of DNA damage could not be quantified. For the KPF5 0.5 mg/ml treatment, however, there was a statistically significant increase in the number of MN, further suggesting KPF5 induced DNA damage in HT-29 cells.

Chemical Fingerprinting of KPF5 Determined by LC-PDA-MS/MS

LC-PDA-MS/MS was conducted to gain a better understanding of the nature of active components in fraction KPF5. The presence of 4 major compounds with mass-to-charge ratio (m/z) values of 433, 449 (2 compounds), and 785 were confirmed. The product mass spectra of these parent ions were then obtained (Fig. 5). The mass spectra revealed fragmentation patterns that are atypical of common phenolic compounds, suggesting these compounds may not be common polyphenols. The identity of these compounds could not be established from the common neutral-loss values. The mass spectra, however, revealed the two 449 m/z compounds are structurally similar and are likely to be isomers, as both exhibited common neutral loss values of 150, 124, and 110 m/z.

TABLE 3
Frequency of various cell types of HT-29 cells in cytokinesis block micronucleus cytome cultures treated with various doses of the selected Kakadu plum purified fraction (KPF5)

	Frequency of Cell Type									
Cell Type	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
KPF5 0.5 mg/ml	323.9 ± 21.5*	94.1 ± 14.5	3.2 ± 2.2	70.3 ± 30.8*	8.4 ± 5.7	1.24 ± 0.02	13.3 ± 3.7	22.9 ± 5.5*	2.5 ± 2.4	22.6 ± 25.9
KPF5 1.0 mg/ml	343.5 ± 23.0*	5.0 ± 2.7*	0.5 ± 0.6*	144.8 ± 20.0*	6.2 ± 4.0	1.02 ± 0.01*				

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. NDI = nuclear division index; MN = micronuclei; BN = binuclear; NPB = nucleoplasmic bridges; NBud = nuclear buds.

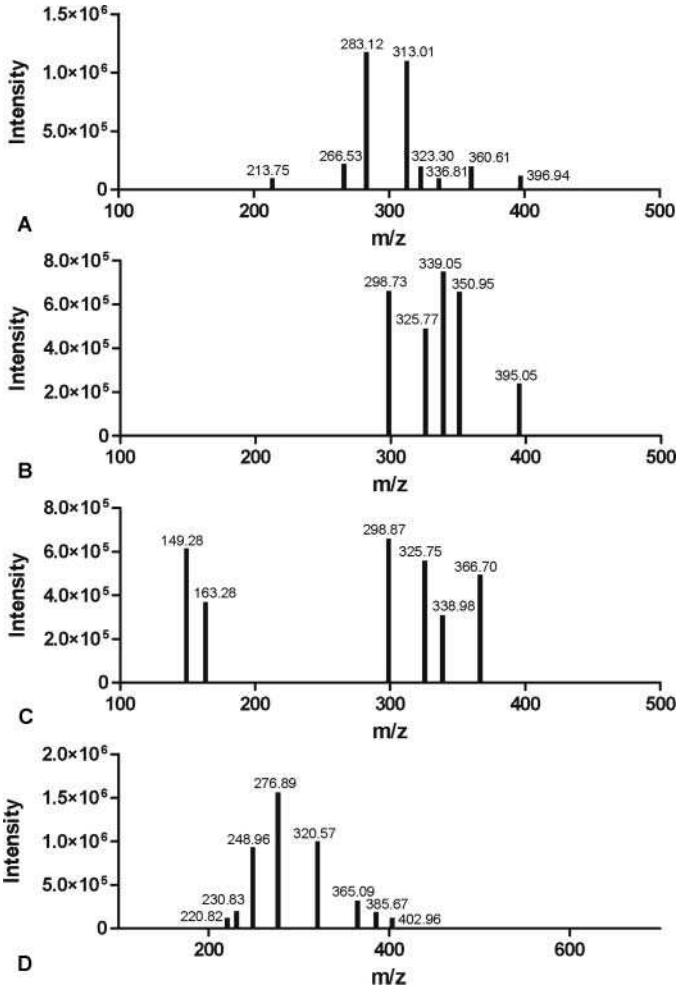

FIG. 5. LC-PDA-MS/MS of major parent compounds identified in Kakadu plum purified fraction 5 following positive mode electrospray ionization mass spectrometry. A: 433 m/z parent compound. B: 449 m/z parent compound 1. C: 449 m/z parent compound 2. D: 785 m/z parent compound.

DISCUSSION

The results demonstrate the utility of bioassay-guided fractionation of 2 native Australian fruits, Kakadu plum and Illawarra plum. This may aid in the identification of biologically active compounds in native Australian fruits used traditionally by the indigenous population. The Kakadu plum fractions mostly contained compounds with a maximum absorbance at 280 nm, consistent with previous results for the whole Kakadu plum purified polyphenolic extract (13). Similarly, the Illawarra plum fractions contained significant levels of anthocyanins, with IPF3 predominantly containing cyanidin 3-glucoside (99%) and minor pelargonidin 3-glucoside (1%) levels. The anthocyanins within IPF4 and IPF5, were found to consist only of cyanidin 3-glucoside. This is again consistent with previous results that showed that of the total anthocyanin content within a whole extract of Illawarra plum, cyanidin 3-glucoside comprised 99% (30).

The screening results of the Kakadu plum and Illawarra plum fractions for antioxidant and antiinflammatory activity revealed KPF5 to possess a range of significant biological activities that demands subsequent detailed chemical fingerprinting as well as molecular mechanistic analysis. The results for KPF5 compared with the Kakadu plum purified polyphenolic whole extract, showed slightly decreased antioxidant activity, with lower ORAC and CAA values, suggesting the whole extract components may provide an additive or synergistic effect or may affect the uptake of the active polyphenolic compounds into cells. The effect of both KPF5 and the whole extract on nitrite concentration and PGE_2 release in LPS-activated murine macrophages was similar (15). IPF3 was the highest-performing Illawarra plum fraction and, similar to Kakadu plum, IPF3 displayed lower ORAC and CAA values compared to the whole extract. The extent of nitrite inhibition between IPF3 and the whole extract were also comparable; however, the whole extract had a greater inhibitory effect on PGE_2 release (15).

The Kakadu plum whole extract has previously been assayed for antioxidant, antiinflammatory, and proapoptotic anticancer activity using the same assays used in the present study (13–15). A comparison of the results reveals KPF5 showed similar activities to the Kakadu plum whole extract, including an upregulation of the Nrf2/Keap1 ratio, indicating potential activation of the ARE. The ARE is a base sequence found in the promoter regions of a wide spectrum of genes, promoting many antioxidant and drug-metabolizing enzymes (31). The inhibition of COX-2 and iNOS by KPF5, although having no effect on levels of COX-1 in LPS-activated murine macrophages, was again comparable to previous results for the whole extract (15). This inhibition of inflammatory processes indicates the potential for protection against cancers occurring from chronic inflammation (32). Interestingly however, while KPF5 demonstrated potential inhibition of the NF-κB pathway similar to the whole extract, KPF5 also inhibited the phosphorylation of p44/42 MAPK and Akt, which was not noted with the whole extract (15). This indicates KPF5 acts via alternative mechanisms than the whole extract, suggesting either compounds within the whole extract may be exerting an antagonistic effect that hinders its ability to act via these pathways, or the specific active compounds are at a much lower concentration in the whole extract. These pathways are important in inflammatory processes. The CBMN Cyt assay results are similar for both KPF5 and the whole extract in inducing DNA damage, a cytostatic effect, and a proapoptotic effect in HT-29 cells (14). Apoptosis represents a potent form of defense against cancer, and mutations with the apoptosis machinery are common to virtually all cancers (33).

Many other well-characterized polyphenols and fruit extracts also display similar antioxidant, antiinflammatory, and proapoptotic anticancer activities to KPF5. They include various green tea extracts and (−)-epigallocatechin gallate (EGCG), a major component of green tea, which reduce H_2O_2-induced cytotoxicity, increase Nrf2 expression, inhibit COX-2 and iNOS expression, and induce apoptosis in cancer cell lines (25,34–36).

Quercetin and genistein are other common flavonoids that display similar activities (37–40).

The LC/MS analysis revealed the presence of at least 4 major compounds in the KPF5 fraction. Each compound displayed a complex fragmentation pattern. For example, the compounds with 449 m/z contained common neutral loss values of 150, 124, and 110 m/z. These values do not correlate with characteristic sugar moieties (e.g., pentoses, hexoses, and others), typically seen in compounds such as glycosides. Ultimately, an extensive study involving isolation of individual compounds from the KPF5 fraction and subsequent elucidation of their molecular structure with the help of 2D or 3D nuclear magnetic resonance (NMR) is needed. The results obtained within this study clearly suggest the presence of compounds in KPF5 that may ultimately contribute to its enhanced bioactivity.

Our study has shown that Kakadu plum contains an extremely rich mixture of phytochemicals. The presence of polyphenols, such as quercetin/hesperitin glucosides, kaempferol/luteolin glycosides, and catechin-based hexose-containing glycosides, have already been reported (41). However, the HPLC chromatogram of the Kakadu plum extract is extremely rich, with over 60 individual compounds represented by separate peaks. Moreover, our LC/MS chemical fingerprinting analysis has revealed that peaks with slightly different molecular masses are overlapping, clearly indicating that the number of individual compounds in the fruit extracts is likely to be significantly larger.

In summary, KPF5 exhibited a range of significant biological activities in vitro, including antioxidant activity, the inhibition of COX-2 and iNOS in LPS-activated murine macrophages and proapoptotic activity in HT-29 cells. KPF5 may potentially target multiple pathways and have multiple mechanisms of action to exert its chemopreventive activity, highlighting its significant chemopreventive potential. Further research is warranted to obtain a more complete understanding of the complex synergistic, additive, or antagonistic interactions between the different compounds found within this fraction and to completely elucidate the potential of KPF5 as a chemopreventive agent.

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