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Impact of Growing Location on Kakadu Plum Fruit Composition and In Vitro Bioactivity as Determinants of Its Nutraceutical Potential

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Abstract: Growing location is known to affect the metabolite content and functionality of wild harvested fruits. *Terminalia ferdinandiana*, commonly known as Kakadu plum (KP), is among the most commercially important native Australian bush foods. Therefore, we evaluated the composition and in vitro bioactivity of aqueous acidified ethanol (AAE) and water extracts prepared from KP fruit wild harvested in the Northern Territory (NT) and Western Australia (WA). Compositional analysis included vitamin C, total ellagic acid (TEA), and total phenolic content (TPC), while in vitro bioactivity was assessed through anti-inflammatory (RAW 264.7 macrophages) activity and cell viability (Hep G2) assay. The IC_{50} of the extracts ranged from 33.3 to 166.3 µg/mL for NO inhibition and CC_{50} from 1676 to 7337 µg/mL for Hep G2 cell viability inhibition. The AAE KP fruit extracts from the NT exhibited potent anti-inflammatory activity and impacted Hep G2 cell viability more than other extracts, most likely due to TEA (3189 mg/100 g dry weight (DW)), vitamin C (180.5 mg/g DW) and TPC (196 mg GAE/g DW) being higher than in any other extract. Overall, the findings of the present study are promising for using KP fruit and derived products in functional foods, nutraceuticals, or dietary supplements.

Keywords: *Terminalia ferdinandiana*; kakadu plum; Australian grown; anti-inflammatory; antiproliferative; vitamin C; ellagic acid; ellagitannins; bioactive compounds

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1. Introduction

Edible plants are common targets for alternative therapies as these sources are more likely to be recognised as safe, and approved by regulaory bodies. In addition, many epidemiological and experimental studies have showed that polyphenol-rich plants and derived products can modulate non-communicable diseases (NCDs), making these plant sources important targets for the development of functional foods and nutraceuticals [1–4]. *Terminalia ferdinandiana*, commonly known as Kakadu Plum (KP), is one such target that has been investigated for more than 10 years. Kakadu plum is a native Australian bush food, wild harvested for its high vitamin C content [5]. This fruit is also rich in polyphenols such as ellagic acid and ellagitannins, including hydrolysable tannins [6], which play important roles in its in vitro antioxidant [7] and antimicrobial [8] activities. However, studies exploring other biological effects of KP and derived extracts are very limited.

Inflammation is an important biological defense to injury, infection, and "foreign materials" [9] that contributes to uncontrolled inflammation causing chronic non-communicable diseases (NCDs), including cancer and inflammatory bowel disease (IBD), cardiovascular, and neurodegenerative diseases [10,11]. Aggravated by industrialization, lifestyle, genetics, and infections [12], NCDs contributed to approximately 80% of the global disease burden

in 2020 and are still increasing at an alarming rate [13]. NCDs not only severely impact patient quality of life, but the associated psychological disturbances also impact families and communities [14,15].

Macrophages play a central role in inflammation by producing cytokines, reactive oxygen species, and nitric oxide (NO)—a signaling molecule synthesized from arginine by the enzyme nitric oxide synthase (NOS) [16,17]. In normal physiological conditions, NO exhibits a range of important functions such as vasodilation and modulation of cell-mediated immunity [18]. However, excess NO associated with chronic inflammation can break DNA double strands [19], form peroxynitrite, which damages lipids [20] and proteins, and cause oxidative injury, necrosis, and apoptosis [21]. These biochemical processes promote growth of cancer cells that can infiltrate and destroy normal body tissues [22].

The treatment of NCDs involves lifestyle changes and therapeutic treatments. For example, inflammation is treated with non-steroidal anti-inflammatory drugs (NSAIDs), and chemotherapy is administered for the treatment of cancer [23,24]. However, NSAIDs can produce adverse gastrointestinal and cardiovascular effects [24] and chemotherapy drugs cause severe cytotoxicity [25]. These serious side effects have prompted researchers to identify alternative therapies with less side effects, leading to extensive research into plant-based therapies.

Anti-inflammatory activity is commonly examined in vitro using lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells to produce NO [16,26]. The direct measurement of NO is extremely challenging due its short half-life and rapid oxidation to nitrite and nitrate; however, nitrite is a stable metabolite that can be used to estimate total NO production in cells [27] via the Griess [16] and 2,3-Diaminonaphthalene (DAN) [26] assays. In the DAN assay, non-fluorescent DAN reacts with NO_2^- to yield 2,3-naphthotriazole offering the most sensitive measurement for NO produced from NOS [28]. This assay directly correlates NO production with nitric oxide synthase activity [29].

Chemotherapy involves the administration of cytotoxic agents to control the rapid proliferation of cancer cells [30]. In addition to in vivo animal studies and human clinical trials, substantial in vitro research has been dedicated to identifying safer cytotoxic compounds with less side effects that can impact cancer cell viability and reduce cancer cell proliferation [31]. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), or MTT assay, is the most used technique [30,32] to measure cell viability. However, it has been reported that phenolics, proteins, and carbohydrates can reduce MTT-based reagents and interfere with cell viability measurements [33,34]. In this regard, Akter and co-workers [34] compared different assays to investigate if antioxidant-rich KP fruits and derived extracts influence cell viability measurements. The authors reported that the CyQUANT® (Thermo Fisher Scientific Corporation, Waltham, MA, USA) NF Cell Proliferation Assay reagent was not impacted by KP extracts. Furthermore, the CyQUANT® NF Cell Proliferation Assay has been applied in several studies investigating cancer cell viability in response to different treatments [35–37].

Therefore, the aims of the present study were to explore the composition and in vitro bioactivity of KP fruit wild harvested from two main growing regions in Australia, the Northern Territory and Western Australia, to evaluate the impact of growing location on fruit composition and resultant bioactivity. Vitamin C, total ellagic acid, and total phenolic content were determined in the wild harvested KP fruit samples, while in vitro bioactivity was assessed through anti-inflammatory activity and cell viability. Anti-inflammatory activity was measured through the DAN assay and the CyQUANT® NF Cell Proliferation assay was used to observe the impact of KP fruit extracts on cell viability.

2. Materials and Methods

2.1. Reagents and Mammalian Cell Lines

Growth media (RPMI 1640), Dulbecco's modified eagle medium (DMEM), Dulbecco's phosphate buffered saline without calcium and magnesium (PBS), Hank's Balanced Salt Solution (HBSS), Penicillin and Streptomycin, glutamax, trypsin-EDTA, and fetal bovine

serum (FBS) were sourced from Gibco (Invitrogen/Life Technologies Pty Ltd., Mt. Waverley, VIC, Australia). Nunc™ F96 MicroWell™ Black polystyrene plates were purchased from Invitrogen (Thermo Fisher Scientific Corporation, Waltham, MA, USA). CyQUANT® NF Cell Proliferation Assay reagent was purchased from Invitrogen (Thermo Fisher Scientific Corporation). Lipopolysaccharide (LPS) (from *Escherichia coli* 0111: B4) (LPS), 2,3-diaminonaphthalene (DAN), quercetin, and Hep G2 cell lines were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). The murine macrophage cell line, RAW264.7 was from the American Type Culture Collection (Rockville, MA, USA).

2.2. Plant Material Collection and Extract Preparation

The mature KP fruit samples were wild harvested in Karajarri country and Yawuru conservation in the Kimberley's, WA, as well as Darwin, the Northern Territory, in January 2020 (Figure S1). Ten KP trees from each site or location were randomly selected and approximately 50–100 fresh fruits were collected from each tree. The samples were put in plastic bags and transported under refrigerated conditions to the laboratory for analysis. After manually deseeding the fruits, the remaining pulp and peels were blended to a puree using mortar and pestle first, and then a Waring 8010S Laboratory Blender (Waring® Laboratory Science, Torrington, CT, USA).

This was conducted for each site and location to generate composite sample batches. The fruit puree was freeze-dried under vacuum (Lindner & May Ltd., Brisbane, QLD, Australia), milled to provide a homogenous powder and stored at −80 °C for further analysis.

Extracts were prepared using water and aqueous acidified ethanol (80% ethanol, 19% water, 1% *w*/*v* citric acid) as described previously [6]. Briefly, 0.1 g of sample material from each site and location was mixed with 5 mL of extraction solvent (either water or acidified ethanol). After that, the mixture was vortexed (30 s), sonicated (15 min) and centrifuged (4000 rpm, 15 min). The obtained supernatant was collected and the procedure repeated two more times. An aliquot of the collected supernatant was kept at −80 °C for total phenolic and ellagic acid analysis while the remaining supernatant was freeze-dried under vacuum (Lindner & May Ltd.) for the anti-inflammatory and cell viability assays. The whole procedure was performed in triplicate.

Lyophilized acidified ethanol extracts were reconstituted in dimethyl sulfoxide (DMSO) and water extract concentrations were selected based on findings reported by Akter and colleagues [34,38]. Samples for anti-inflammatory experiments were diluted in RPMI-1640 growth media to achieve 500, 250, 125, 63, 31 & 15.5 µg/mL. For Hep G2 cell viability assays, samples were diluted in HBSS to achieve 70,000, 60,000, 50,000, 30,000, 10,000, 5000, 2000, 500, and 50 µg/mL. Quercetin solutions were diluted in growth media to achieve 15,000, 11,000, 8000, 6000 & 3000 µg/mL.

2.3. Total Phenolic Content (TPC), Total Ellagic Acid (TEA), and Vitamin C

TPC, TEA, and vitamin C were determined according to Bobasa et al. [6]. However, as already described in Section 2.2, KP fruit samples from the NT and WA were extracted with both water and aqueous acidified ethanol (AAE) and analysed separately. Briefly, the TPC was determined using the Folin-Ciocalteu reagent a micro-plate absorbance reader (Infinite M200, Tecan Austria GmbH, Grodig, Austria) at 700 nm. Two mL of the supernatant (Section 2.2) were added into a 5 mL Reacti-Therm vial (Fisher Scientific, Bellefonte, PA, USA) containing a stirring bar. For the TEA analysis, the solvents were evaporated under nitrogen and subjected to an overnight hydrolysis at 90 °C using 2 M HCl. After hydrolysis, the vial was cooled, and the content extracted with 5 mL 100% methanol to determine the total ellagic acid content. Vitamin C, L-ascorbic acid (L-AA) and dehydroascorbic acid (DHAA), was extracted by 3% meta-phosphoric acid containing 8% acetic acid and 1 mL ethylenediaminetetraacetic acid (EDTA). DHAA, which was also present in the extracts/samples, was reduced to L-AA prior to analysis. Both ellagic acid and total vitamin C (TVC, sum of L-AA and reduced DHAA) were analysed using a Waters AcquityTM UPLC-PDA system.

2.4. Nitric Oxide Production in RAW 264.7 Cells Using the Diaminonaphthalene Assay

2.4.1. RAW 264.7 Cell Culture

RAW 264.7 macrophages were cultured in RPMI 1640 with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% FBS. Cells were grown in vented 175 cm^2 flasks (Nunc™ EasYFlask™ Cell Culture Flasks, Thermo Fisher Scientific Corporation) at 37 °C and 5% CO_2 in humidified air with media being replaced every 2–3 days. The cells were detached at 90% confluence using 0.25% (*v*/*v*) trypsin-EDTA, resuspended in fresh growth media, counted, and diluted to obtain 8×10^5 cells/well in 96-well plates. Cell plating, sample administration, and media changes were performed with an epMotion® 5075t liquid handling system (Eppendorf, Hamburg, Germany) [38].

2.4.2. Nitric Oxide Production by RAW264.7 Cells

Nitric oxide production was measured using the DAN assay according to Breger et al. [26] and Suleria et al. [16]. In 96-well plates, 8×10^5 RAW 264.7 cells resuspended in 100 µL growth media, were added to each well with 20 µL 300 ng/mL LPS and either 20 µL of extract, quercetin (15,000–3000 µg/mL), or growth media. Cells were incubated at 37 °C and 5% CO_2 for 48 h before 20 µL cell media was transferred from each well into a new 96-well plate. To prepare a standard curve, 20 µL sodium nitrite (3.13–200 µM) and media were also added to the new 96-well plate. Next, 80 µL RO water was added to each well followed by 10 µL DAN reagent. The plate was then mixed in the dark at 25 °C for 10 min before 20 µL 2.8 N NaOH was added to each well. The plate was mixed gently and incubated at room temperature in the dark for one minute before fluorescence was measured (360 nm excitation and 430 nm emission) using a Spectramax M3 multi-mode microplate reader (Molecular Devices, San Jose, CA, USA). Nitrite concentration was extrapolated from the sodium nitrite standard curve and expressed as a percentage decrease in NO production compared to the LPS treatment control. The concentration required to reduce 50% NO production is defined as half maximal inhibitory concentration (IC_{50}) [16,26].

Cell viability was also assessed in the remaining cells using the CyQUANT® NF Cell Proliferation Assay. CyQUANT® NF DNA binding dye (74 µL) was added to each well using a manual multichannel pipette. The plate was then covered and incubated at 37 °C for 1 h before fluorescence was measured (485 nm excitation and 530 nm emission) using a Spectramax M3 multi-mode microplate reader (Molecular Devices). Cell viability was calculated as a percentage of viable cells compared to the HBSS control [34].

2.5. Hep G2 Cell Viability Assay

In this study, the Hep G2 hepatic tumor cell line was selected to explore the impact of KP extracts on cell viability as Hep G2 cells provide an acceptable model for studying anticancer drugs [39].

2.5.1. Cell Culture

All cell numbers and growth conditions were adapted from Akter et al. [38]. Hep G2 cells were maintained in DMEM supplemented with 10% FBS (*v*/*v*), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM Glutamax. Cells were grown in vented culture flasks at 37 °C and 5% CO_2. To investigate the impact of KP extracts on Hep G2 cell viability, 5×10^4 Hep G2 cells/well were grown in 96-well plates for 24 h prior to cell treatment.

2.5.2. Cell Viability Assay

Hep G2 cell viability was examined by the CyQUANT® NF Cell Proliferation Assay as described previously [38]. The concentration required to reduce 50% of viable cell was represented as 50% cytotoxic concentration (CC_{50}).

2.6. Statistical Analysis

Results are reported as mean ± SEM (n = 6). The mean difference between extracts was compared by one-way ANOVA followed by Tukey's and Dunnett's T3 post hoc tests using

the SPSS statistical software package 20.0 (SPSS Inc., Chicago, IL, USA). One sample *t*-test and Wilcoxon test, as well as unpaired *t*-test were also performed to determine the differences between two groups. The IC_{50} and CC_{50} were calculated using normalized response variable curves fitted by GraphPad Prism version 9 (San Diego, CA, USA). *p*-values < 0.05 were considered significant.

3. Results and Discussion

3.1. TPC, TEA, and Vitamin C

Extract composition is presented in Table 1. To the best of our knowledge, this is the first study to report free ellagic acid (FEA) and TEA, obtained after acid hydrolysis, in KP fruit wild harvested from different growing regions. Overall, TEA and FEA were significantly higher in matched extracts from NT compared to WA. For example, TEA in the NT AAE KP fruit extracts was 1.3-fold higher than that in the WA AAE extracts, similarly TEA in NT water extracts was 1.6-fold higher than TEA in WA water extracts. The highest FEA was measured in the NT AAE extracts (1228 ± 23 mg/100 g DW) while the lowest FEA was determined in the WA water extracts (43 ± 1.3 mg/100 g DW). These results also showed that AAE extracts contained significantly more TEA and FEA compared to water extracts.

Table 1. Total phenolic content (TPC), free ellagic acid (FEA), ellagitannins (ETs), total ellagic acid (TEA) and vitamin C of KP fruits wild harvested from the Northern Territory (NT) and Western Australia (WA).

	Samples	FEA (mg/100 g DW)	ETs [&] (mg EAE/100 g DW)	TEA (mg/100 g DW)	TPC (mg GAE/g DW)	Vitamin C (mg/g DW)		
						L-AA	DHAA	TVC
NT	NT AAE	1228 ± 23.1 [d]	1961.6 ± 24.4 [d]	3189.6 ± 25.7 [d]	196 ± 3.6 [d]	171 ± 0.7 [b]	9.6 ± 1.2 [a]	180.5 ± 1.0 [b]
	NT water	148 ± 3.8 [b]	373.1 ± 4.0 [b]	521.1 ± 3.5 [b]	88.8 ± 5.0 [b]			
WA	WA AAE	1045 ± 9.4 [c]	1449.7 ± 10.1 [c]	2494.7 ± 11.1 [c]	174 ± 5.0 [c]	116.3 ± 1.3 [a]	8.7 ± 1.0 [a]	125 ± 3.0 [a]
	WA water	43 ± 1.3 [a]	276 ± 5.2 [a]	318.8 ± 9.1 [a]	64.3 ± 5.0 [a]			

Values are expressed as mean ± SEM of triplicate measurements, mean comparison using One-Way ANOVA followed by Tukey's Post Hoc test for ellagic acid and unpaired *t*-test for vitamin C. Different letters in the same column indicate significant difference ($p < 0.05$). mg EAE: milligram ellagic acid equivalent; mg GAE/g DW: milligram gallic acid equivalent per gram dry weight. L-AA: L-ascorbic acid, DHAA: dehydroascorbic acid, TVC: total vitamin C (sum of L-AA and reduced DHAA). [&] calculation adapted from Williams et al. [40].

Compared to previous studies, the levels of FEA found in NT and WA KP fruit extracts were considerably higher than the FEA content reported in other fruits, such as strawberry (4.8 ± 0.1 mg/100 g DW) and boysenberry (5.5 ± 0.6 mg/100 g DW) methanol extracts [40]. Williams and co-workers [41] also determined TEA in 80% aqueous acidified methanol extracts prepared from boysenberry and strawberry, again TEA concentrations were much lower than those in the present study (96 and 32 mg/100 g DW for boysenberry and strawberry versus 2494 and 3189 mg/100 g DW for WA and NT KP). Furthermore, the TEA content in KP fruit was also higher than that reported in several other species of economic importance, such as guava, blackberry, and walnuts [42] that place KP fruit as a valuable source of EA.

Table 1 shows significant ($p < 0.05$) differences in TPC. Like the EA trends, the highest TPC was measured in NT AAE extracts, followed by WA AAE, NT water, and WA water (with the lowest TPC of 64.3 mg GAE/g DW). The TPC findings (considering 85% moisture content in fresh KP fruits) were considerably higher than that found in many common fruits and vegetables, such as watermelon (0.10 ± 0.01 mg GAE/g FW), orange (1.3 ± 0.01 mg GAE/g FW), chives (6.78 ± 0.90 mg GAE/g FW), and pakchoi (7.11 ± 0.37 mg GAE/g FW) [43,44]. It should be also noted that the TPC assay employed in this study used the Folin-Ciocalteu (F-C) reagent creating an electron transfer-based (antioxidant capacity) assay that measures the reducing capacity of a sample. This means that the F-C reagent also reacts with non-phenolic compounds such as ascorbic acid (vitamin C) that might contribute to the high TPC results shown in Table 1 [45,46].

Like EA and TPC, vitamin C content also differed significantly ($p < 0.05$) between NT and WA KP fruit (18% versus 12.5% vitamin C per DW) (Table 1). However, these results are still within the vitamin C concentration range reported previously for KP fruit and derived powder (14–19% per DW) [40,47]. Notably, the 12.5% vitamin C content in the WA KP fruit samples was still considerably higher than that found in common vitamin C rich fruits, such as oranges, apples, and grapes [48]. These findings further highlight KP fruit and derived products as a natural and abundant source of vitamin C.

The significant variations observed in total phenolic, FEA, TEA, and vitamin C contents between fruit harvested from the NT and WA are most likely due to environmental differences, such as micro-climate, soil composition, rainfall, and sun exposure, rather than differences in maturity, as all fruits were ripe and collected at a similar time [7,40].

3.2. Effect of KP Fruit Extracts on Inflammation Induced by LPS In Vitro

3.2.1. Cell Viability

RAW 264.7 cell viability was assessed to ensure that all cell treatments, including KP extracts and quercetin, did not significantly decrease cell viability. Statistically non-significant ($p > 0.05$) differences were observed between the cells treated with the five concentrations of the AAE and water extracts in both region (500, 250, 125, 63, 31 &/15.5 µg/mL) and quercetin (15,000, 11,000, 8000, 6000 & 3000 µg/mL) relative to LPS treated control (Figure 1).

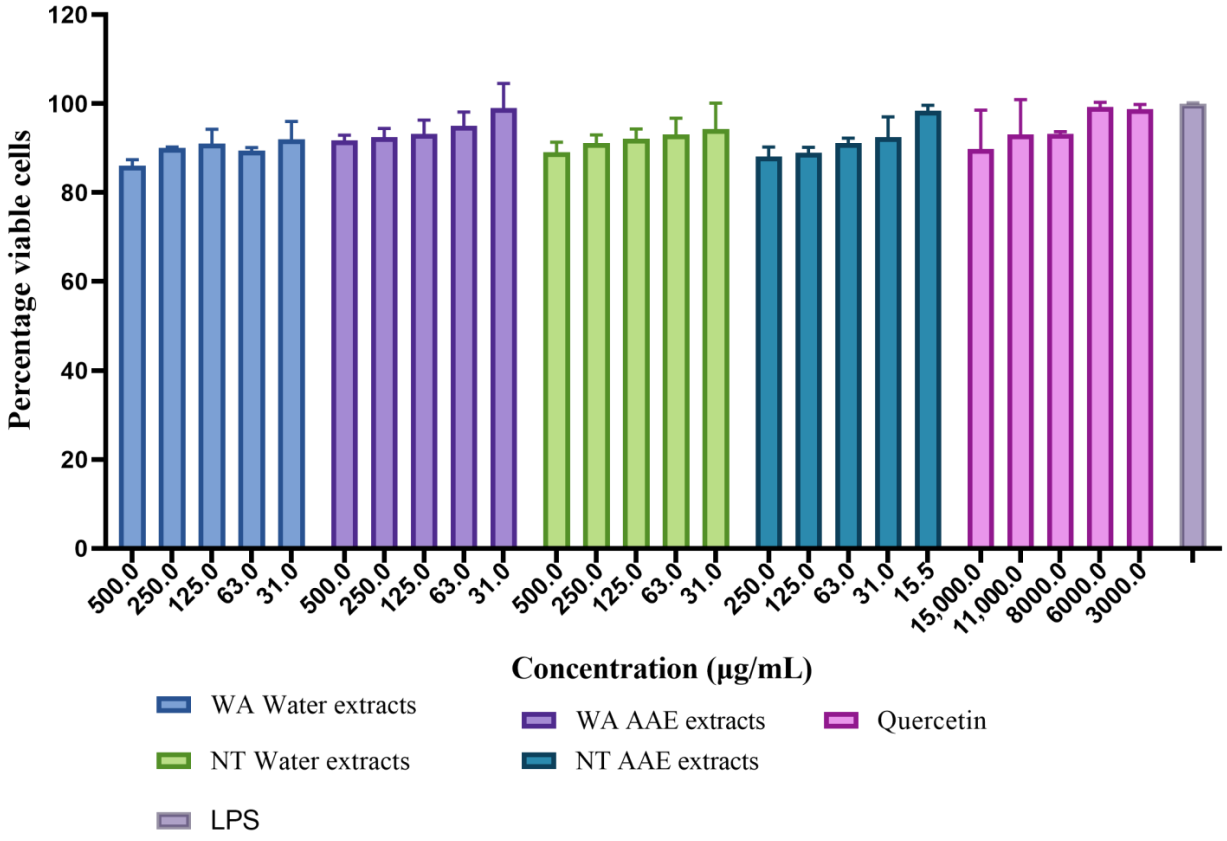

Figure 1. Determination of cell viability in RAW 264.7 macrophages treated with LPS and Kakadu Plum extracts, prepared from fruits wild harvested in Western Australia (WA) and the Northern Territory (NT). Values are expressed as mean ± SEM of triplicate measurements of three independent experiments. AAE: aqueous acidified ethanol, LPS: Lipopolysaccharide (300 ng/mL).

3.2.2. Inhibition of NO Production

Figure 2 and Table 2 show that samples treated with either KP extracts, or quercetin significantly ($p < 0.05$) decreased NO production. The NT AAE, NT water, WA AAE and WA water extracts inhibited NO production by 50% at concentrations of 33.3 ± 1.3, 52.4 ± 2.1, 157.0 ± 1.5 and 163.3 ± 1.3 µg/mL, respectively (Table 2). The IC_{50} demonstrated in the NT fruits, both AAE and water extracts, are comparable to *Eucalyptus eximia*, *E. acmenoides*, and *E. notabilis* leaf ethanol extracts that demonstrated 50% NO inhibition in LPS-stimulated RAW 264.7 cells at 34.14 ± 7.1, 56.93 ± 11.8 and 53.84 ± 7.7 µg/mL, respectively [49]. The highest concentration of NT water extracts (500 µg/mL) inhibited ≥90% of NO production like *F. suspense* aqueous extracts (2000 µg/mL, >90%) [50], whereas the highest dose of WA water extracts (500 µg/mL) reduced NO production (61%) greater than that of *C. militaris* fruit water extracts (1250 µg/mL, 51%) [51].

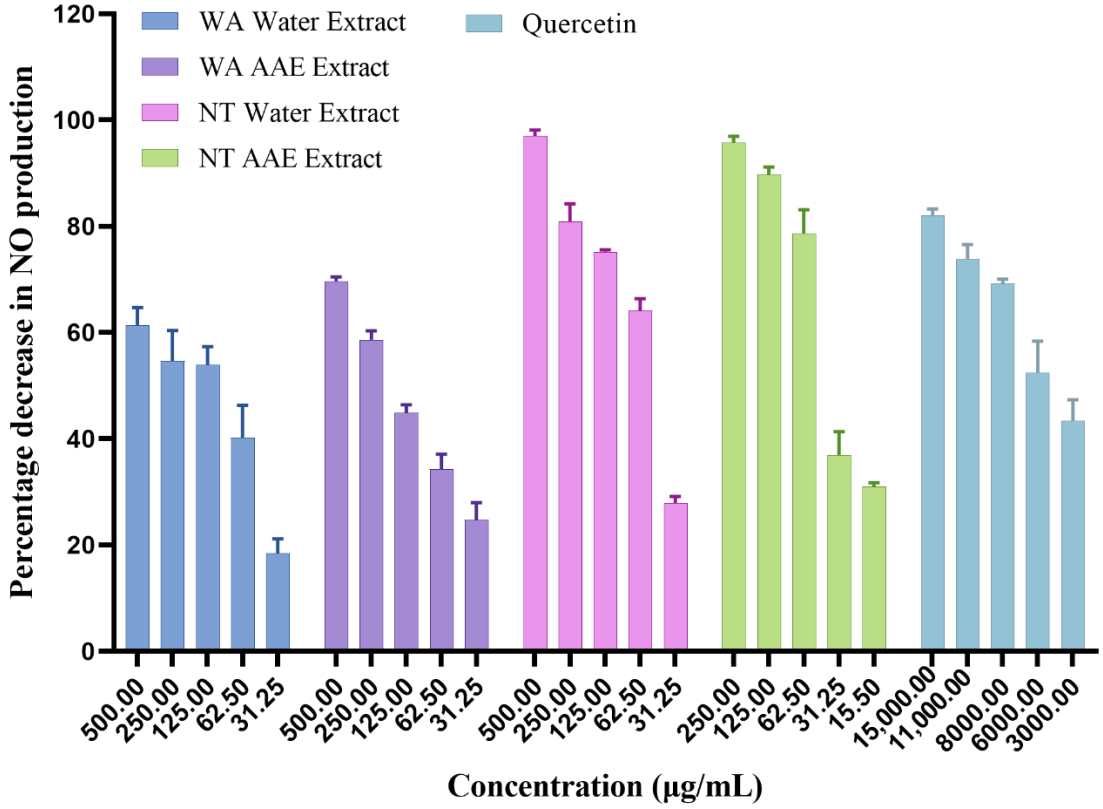

Figure 2. Anti-inflammatory activities, expressed as percentage inhibition of nitric oxide (NO) production, of KP fruits wild harvested in Western Australia (WA) and the Northern Territory (NT), in RAW 264.7 macrophages. Values are expressed as mean ± SEM of triplicate measurements of three independent experiments.

Table 2. IC_{50} values for anti-inflammatory activity determined in RAW264.7 macrophages.

Extracts	Inhibition of NO Production (IC_{50} µg/mL)
WA Water	166.3 ± 1.3 ^{c}
WA AAE	157.0 ± 1.5 ^{c}
NT Water	52.4 ± 2.1 ^{b}
NT AAE	33.3 ± 1.3 ^{a}
Quercetin	4269.3 ± 3.1 ^{d}

Values are expressed as mean ± SEM of triplicate measurements of three independent experiments. IC_{50}: half-maximal inhibitory concentration, anti-inflammatory activity is compared using unpaired *t*-test, different letters in the same column indicate significant difference ($p < 0.05$). NT: Northern Territory, WA: Western Australia, AAE: Aqueous acidified ethanol.

Our results, presented in Figure 2, show that the NT AAE extract was the most potent inhibitor of NO in LPS-stimulated RAW264.7 cells (Table 2). Solvent extracts prepared from other wild edible plants, such as *A. horridus* [52], *S. officinalis*, *R. officinalis* and *M. piperita* [53], also have strong anti-inflammatory activities in vitro. In addition, hydrolysable tannins in a KP fruit AAE extract [6] such as corilagin [54], 2,3,6-tri-O-galloyl-β-D-glucose, chebulinic acid [55], ellagic acid, gallic acid and punicalagin A&B [56], have also been found to inhibit production of pro-inflammatory cytokines and mediators in LPS-stimulated RAW 264.7 cells. Interestingly, the NT fruit AAE extract contained the highest TEA and may explain the potent anti-inflammatory activity associated with this extract (Figure 2 and Table 2).

Furthermore, our study found strong anti-inflammatory properties (Table 2) in the water extracts (NT water IC_{50} = 52.4 ± 2.1 µg/mL and WA water IC_{50} = 166.3 ± 1.3 µg/mL). Similarly, water extracts prepared from *T. arjuna* fruits inhibited NO production in RAW 264.7 cells more than acetone and methanol extracts [57]. Water extracts in *T. chebula* fruit also retained the highest level of gallic acid (553.79 ± 3.76 nmol/mg), almost 50-fold higher, compared to punicalagin, chebulagic acid, and chebulinic acid [58]. Gallic acid, 3,4,6-tri-O-galloyl-β-D-glucose, corilagin and ellagic acid [59–62] were also identified in aqueous extracts of *T. chebula* and *T. bellirica* fruits. Similarly, Diop et al. [63] and Markom et al. [64] reported corilagin, ellagic acid and punicalagin in the aqueous extracts of other plant species such as *P. niruri* and *C. aculeatum*. Subsequently, the compounds that are present in aqueous extracts of related *Terminalia* species could also be present in our samples and may be responsible for the potent anti-inflammatory activities observed in vitro.

However, the NT water extract exhibited potent (IC_{50} 52.4 ± 2.1 µg/mL) anti-inflammatory activities accompanied by WA water and AAE extracts, respectively (Table 2). These differences could be justified by the level of vitamin C (Table 1) which provide different degree of protection to the oxidation of phenolic compounds [65].

3.3. Hep G2 Cell Viability Results

The impact of KP extracts on Hep G2 cell viability was measured using the CyQUANT® NF Cell Proliferation assay. Hep G2 cell viability in response to 50–70,000 µg/mL KP extracts is shown in Figure 3, while CC_{50} values are displayed in Table 3. Hep G2 cell viability decreased with increasing KP extract concentrations producing more than 90% cell death at 70,000 µg/mL of all the extracts. The NT AAE impacted Hep G2 cell viability significantly ($p < 0.05$) more than other extracts producing the lowest CC_{50} value (1676 ± 1.1 µg/L), followed by WA AAE (2456 ± 1.1 µg/mL), WA water (5440 ± 1.0 µg/mL), and NT water (7337 ± 1.5 µg/mL) water extracts.

Overall, NT water extracts impacted cell viability the least with a CC_{50} value of 7337 ± 1.5 µg/mL; however, this extract is 6, 8, 11, 14, and 16-fold more potent than the half maximal cytotoxic concentration reported by Chu et al. [66] in Hep G2 cells in response to 80% acetone extracts of spinach, cabbage, red pepper, yellow onion, and broccoli, respectively. Liu and co-worker [67] also evaluated cytotoxic activities of four fresh raspberry varieties (Heritage, Kiwigold, Goldie, and Anne) using Hep G2 cells. The potent inhibitor of cell proliferation was Goldie raspberry (EC_{50} = 11.7 ± 0.6 mg/mL), almost seven times the NT AAE dose required to kill 50% of Hep G2 cells.

Extracts prepared from plants rich in hydrolysable tannins (HTs) produced antiproliferative effects in various cancer cell lines [68,69]. For instance, pomegranate fruit husk water [70] and *S. cumini* pulp 75% aqueous acidified ethanol [71] extracts contained punicalagin and ellagic acid that contributed to their cytotoxic activities in cancer cells. Pellati et al. [72] determined chemoprotective activities of HTs isolated from *T. chebula* fruits decoction where punicalagin > chebulinic acid > chebulagic acid > 3,4,6-tri-O-galloyl-β-D-glucose > corilagin produced more than 80% cancer cell death. The ellagitannins (ETs) isolated from *P. emblica*, including ellagic acid, corilagin, chebulagic acid, and elaeocarpusin, also exhibited an anti-proliferative effect [68,69]. The findings from the present study also suggest that the observed impact on Hep G2 cell viability could be due to high EA or HT

contents in the NT fruits. For example, NT AAE extracts contained the highest EA and ET contents and exhibited the strongest anti-inflammatory activity and impacted Hep G2 cell viability more than any other extract. The physicochemical properties of EA and HTs, like being soluble in polar solvents, most likely explain the significant cellular responses to AAE extracts. Furthermore, the differences between NT and WA fruit composition where NT AAE exhibited the highest TPC, FEA, and ETs (as displayed in Table 1), suggest regional differences influenced by growing location and climate.

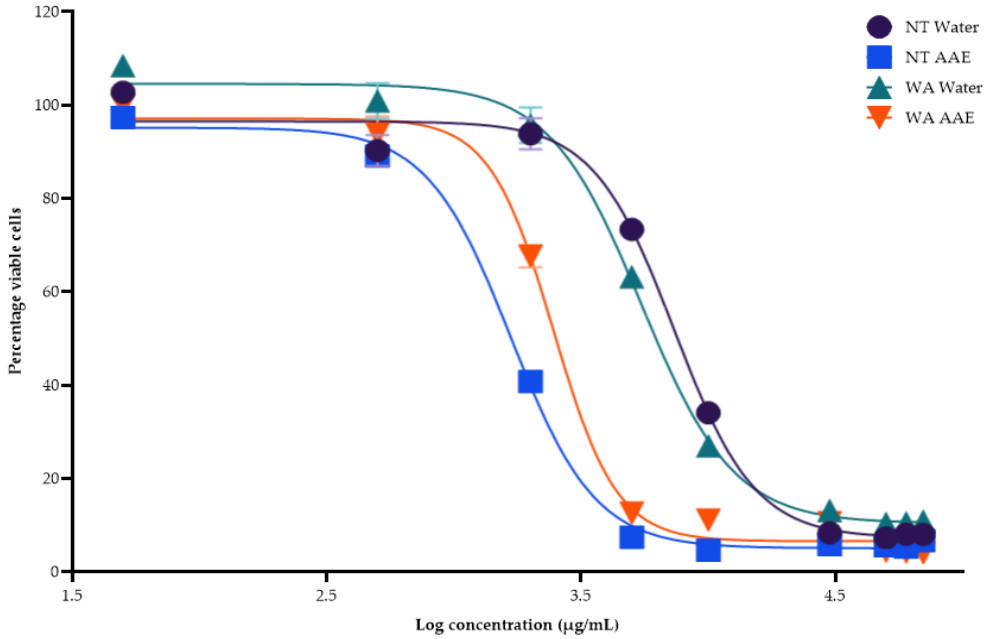

Figure 3. Comparison of the Northern Territory (NT) and Western Australia (WA) KP fruits aqueous acidified ethanol (AAE) and water extracts cell cytotoxicity as percentage of viable Hep G2 cells using the CyQUANT® NF Cell Proliferation Assay.

Table 3. CC_{50} values for Hep G2 cells using the CyQUANT® NF Cell Proliferation Assay.

Extracts	Cytotoxicity (CC_{50} µg/mL)
WA Water	5440 ± 1.0 ^{c}
WA AAE	2456 ± 1.1 ^{b}
NT Water	7337 ± 1.5 ^{d}
NT AAE	1676 ± 1.1 ^{a}

Values are expressed as mean ± SEM of triplicate measurements of three independent experiments. CC_{50}: half-maximal cytotoxic concentration, cytotoxicity is compared using one sample t-test and Wilcoxon test, different letters in the same column indicate significant difference ($p < 0.05$). NT: Northern Territory, WA: Western Australia, AAE: Aqueous acidified ethanol.

4. Conclusions

To the best of our knowledge, this is the first study reporting the in vitro anti-inflammatory and cytotoxic activities of KP fruits wild harvested from two Australian growing regions: NT and WA. Fruit extracts, prepared with water or AAE, exhibited strong anti-inflammatory and cytotoxic activities in vitro. Samples from the NT showed potent inhibition of NO production in LPS-stimulated RAW264.7 cells and impacted Hep G2 cell viability more than the other extracts. In particular, AAE extracts prepared from KP fruit wild harvested from the NT exhibited greater bioactivity, most likely due to high TEA, vitamin C, and TPC. Overall, the results from this in vitro study clearly demonstrate the potential of KP fruit and derived products to be used in nutraceuticals or dietary supplements. However,

further research is warranted to determine the full range of biological activities and to substantiate the in vitro results in vivo.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nutraceuticals3010002/s1, Figure S1: *Terminalia ferdinandiana* (Kakadu plum).

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