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Organic acids in Kakadu plum (*Terminalia ferdinandiana*): The good (ellagic), the bad (oxalic) and the uncertain (ascorbic)

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ABSTRACT

The phenolic ellagic acid (EA) is receiving increasing attention for its nutritional and pharmacological potential as an antioxidant and antimicrobial agent. The Australian native Kakadu plum (*Terminalia ferdinandiana*) fruit is an abundant source of this phytochemical. The fruit also contains large amounts of vitamin C (mainly as ascorbic acid, AA) and possibly the undesirable oxalic acid (OA). Regular consumption of high oxalate foods poses a variety of health risks in humans including interference with calcium absorption and kidney stone formation. Oxalate is also the end-product of AA metabolism so that consumption of fruit with heightened AA content has the potential to elevate urinary oxalate levels. The aims of this study were to investigate the distribution of EA and the presence of other bioactives in other Kakadu plum tissues. Chemical analysis of Kakadu plum fruit and leaves for EA (free and total), OA (water-soluble and total), calcium (Ca) and AA indicated that EA and AA concentrations were high in the fruit while the leaves had significantly higher EA levels but little or no detectable AA. OA content in fruit and leaves was substantial with the fruit being placed in the high-Oxalate category. These findings suggest that there is potential to elevate oxalate levels in the urine of susceptible people and intake of fruit-derived products should be closely monitored. By measuring tissues collected from specific trees, high EA-producing or low OA-containing individuals were identified.

1. Introduction

Ellagic acid (EA) and its derivatives are of interest primarily for their nutritional and pharmacological potential as antioxidants and antimicrobial agents (reviewed by Landete, 2011). Until recently this phytochemical was believed to be limited to only a few fruit such as strawberries (da Silva Pinto, Lajolo, & Genovese, 2008), *Rubus* berries including raspberries and boysenberries (Kähkönen et al., 1999) and muscadine grapes (Lee & Talcott, 2004). There is now a rapidly expanding search for plant sources of this compound (Netzel, Netzel, Tian, Schwartz, & Konczak, 2007; Pfundstein et al., 2010) with the fruit of the Australian native plant Kakadu plum (*Terminalia ferdinandiana*) being found to possess elevated concentrations (Konczak, Maillot, & Dalar, 2014; Williams, Edwards, Pun, Chaliha, & Sultanbawa, 2014). Taking advantage of this abundance of bioactives with functional properties, the fruit is already being employed in such diverse products as dietary supplements, skin care and pharmaceutical products as well as an ingredient in the increasingly popular gourmet bush-food market (Konczak et al., 2014; Mohanty & Cock, 2012). Complicating any comprehensive and accurate assessment of EA levels in plants is the fact that EA occurs in several forms with vastly dissimilar solubilities, reactivities and antioxidant efficiencies. These forms are free EA (Fig. 1a), EA glycosides and the polymeric ellagitannins (ET) (da Silva Pinto et al., 2008; Williams, Edwards, Chaliha, & Sultanbawa, 2016). To accurately evaluate any EA-containing products' bioactive potential it is necessary to quantify the different forms present.

Although the commercial use of Kakadu plum fruit has increased, little information is available about the distribution of EA or the presence of other bioactives in the various Kakadu plum tissues. This is surprising as leaves often possess similar or even higher phytochemical levels when compared to the more commonly consumed plant tissues; documented examples being sweet-potato (Williams et al., 2013) and Tasmanian pepper (*Tasmannia lanceolata*) (Konczak et al., 2009). Awareness of this has recently occurred when Shami, Philip, and Muniandy (2013) described substantial antibacterial and antioxidant

Abbreviations: AA, ascorbic acid; Ca, calcium; EA, ellagic acid; ET, ellagitannins; OA, oxalic acid; Ox, oxalate.

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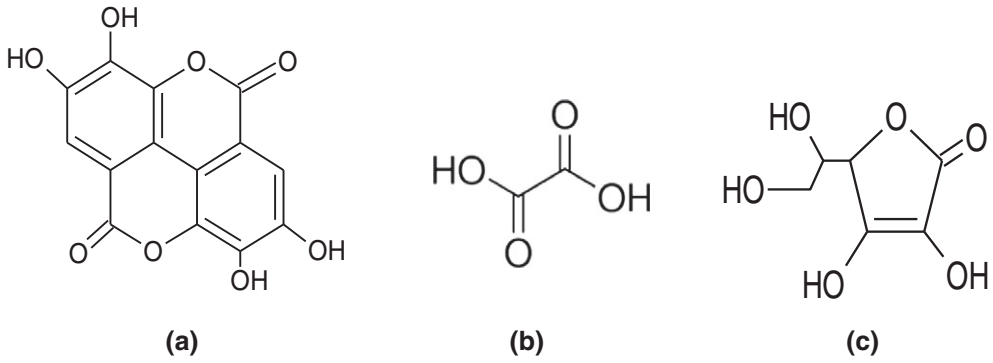

Fig. 1. Structure of (a) ellagic acid, (b) oxalic and (c) ascorbic acid.

activities of an Australian plant mixture that included the leaves of the Kakadu plum. Although these activities were directly related to the amount of phenolic compounds the active constituents within the plants were not identified. Similarly, Courtney, Sirdaarta, Mathews, and Cock (2015) reported that leaf extracts from Kakadu plum exhibited potent inhibitory activity against the bacterial triggers of several auto-immune inflammatory diseases, which these authors believed was initiated by the high tannin content.

Oxalic acid (OA) (Fig. 1b) or more commonly its' conjugated base oxalate (Ox) is present at elevated levels in the leaves and stalks of many plants most notably, rhubarb, spinach and bamboo shoots (reviewed in Noonan & Savage, 1999). Foods high in oxalate can bind with dietary calcium (Ca) and render it unavailable for absorption. Ingestion of such foods may also play a central role in enhancing urinary Ox levels, occasionally leading to the formation of calcium oxalate kidney stones in the urinary tract of susceptible people (Nouvenne et al., 2008).

Since Ox interferes with Ca absorption, several research groups maintain that any studies into its adverse effects must be considered in terms of the foods' Ox:Ca ratio (Fassett, 1973; Noonan & Savage, 1999). Noonan and Savage (1999) suggested that foods with an Ox:Ca ratio greater than two would contain little utilizable Ca allowing Ox to bind with the Ca from other foods eaten at the same time.

Additionally, the intestinal absorption rate of Ox ingested depends on its solubility (Holmes, Assimos, & Goodman, 1998) with most authors agreeing that greater nutritional problems are triggered by the soluble component (potassium or sodium salt) as it is more readily absorbed than its insoluble counterpart (calcium Ox) (Holmes & Kennedy, 2000; Hönow & Hesse, 2002).

Oxalates excreted by the kidneys come from the metabolism of Ox-precursors such as ascorbic acid as well as Ox-containing foods (Kohlmeier, 2003). Earlier reports estimated that dietary Ox accounts for only 10% of the excreted Ox with the remaining 90% derived from the metabolism of Ox-precursors. Of the latter 30–50% is contributed by ascorbic acid (AA) (Noonan & Savage, 1999; Williams & Smith, 1983). A later study revised the dietary contribution upwards to a much higher 45% (Holmes & Kennedy, 2000). Regardless, most investigators still recommend that susceptible people refrain from additional AA intake to limit the risk associated with kidney stone formation (Ferraro, Curhan, Gambaro, & Taylor, 2015; Nouvenne et al., 2008).

Given the developing interest in the utilisation of Kakadu plum tissues as a source of bioactives, it is essential to obtain the relevant data for assessment of their importance as either health-beneficial or anti-nutritional compounds. Thus, the aims of this study were to investigate the distribution and concentration of these compounds in various plum tissues as well as examining possible impediments to the development of any derived products. To achieve this, the distribution of EA forms in composite puree and whole fruit obtained from the 2014 harvest as well as ripe fruit and leaves of varying maturity collected in 2015 from individual Kakadu plum trees growing in different locations in the Wadeye district of the Northern Territory, Australia were evaluated. In addition AA, total and water-soluble Ox and Ca contents were also measured and the Ox:Ca ratio calculated. By focussing on bioactives present in fruit and leaves from individual trees we have obtained an estimate of the variability. This may assist with future selection and cultivation of generally more EA rich or conversely reduced Ox content individuals of this Australian native plant.

2. Materials and methods

2.1. Reagents

EA and AA were purchased from Sigma-Aldrich Inc. (Sydney, NSW, Australia). The HPLC-grade methanol, formic acid and acetonitrile were purchased from Thermo Fisher Scientific (Melbourne, Victoria, Australia). All other chemicals including OA were of analytical grade and purchased from Thermo Fisher Scientific.

2.2. Plant materials and preparation

Approximately 2000 kg of whole Kakadu plum fruit were acquired from the April 2014 harvest at Wadeye, Northern Territory, Australia. The fruit were cleaned and sanitised with 200 ppm chlorine solution before grinding in an Apex mill (Fallsdell Machinery Pty. Ltd., NSW, Australia) and a puree was formed by passing the milled fruit through a paddle finisher (Fallsdell Machinery Pty. Ltd.) containing a 2 mm screen where most of the seeds were collected. These operations were performed on site at Wadeye. The puree was frozen in 4 kg batches and transported frozen to the Brisbane laboratories. On arrival 500 g samples were stored in air-tight, sealable plastic bags at −20 °C.

A selection of fruit from the 2014 harvest not used in puree manufacture was also transported frozen to Brisbane. On arrival batches of 1 kg of whole fruit were stored in air-tight, sealable plastic bags at −20 °C.

Whole Kakadu plum fruit and leaves were gathered in April 2015 from specified individual trees located around Wadeye (see Table 1); From each tree 20 whole ripe fruit (based on colour and fullness of the fruit) and 10 leaves (of varying maturity) were batched in air-tight, sealable plastic bags and transported fresh, under refrigerated

Table 1
Location within the Wadeye district of the Northern Territory, Australia of six individual Kakadu plum trees whose leaves and fruit were collected for organic acid and Ca content determination.

Description	Location
Tree #5	Air Force Hill
Tree #10	Kuy Crossing, Yederr Rd
Tree #15	Before Double Crossing
Tree #18	Between Yederr Rd & Double Crossing
Tree #19	Yelcher Beach
Tree #27	Yelcher Beach

conditions to Brisbane. On arrival they were immediately freeze-dried, finely ground in a Retsch MM301 cyromill (Retsch GmbH, Haan, Germany) and stored at −20 °C.

Approximately 350 g of the 2014 puree and whole fruit were freeze-dried and finely-ground using the above equipment immediately and after 3 months and stored at −20 °C.

2.3. Moisture content of the freeze-dried powders

The moisture content of the freeze-dried powders and original puree was determined according to Association of Official Analytical Chemists (1995), official method 964.22. Triplicate 1 g samples were dried for approximately 16 h to a constant weight at 75 °C in a vacuum oven (W. C. Heraeus GmbH, Hanau, Germany). The difference between initial weight and constant weight after drying was taken as moisture lost and hence moisture content of the sample.

2.4. Extraction of free EA

Free EA was extracted with 100% methanol according to Williams et al. (2016).

Triplicate samples (≈0.1 g) of the freeze-dried powders were accurately weighed into a 15 mL centrifuge tube and 5 mL (3 × extractions) of methanol was added and sonicated for 10 min. After centrifugation (≈3220*g*, 5 min at 20 °C) the clear supernatant was transferred to a 25 mL volumetric flask and made to volume with methanol. About 1.5 mL of the diluted supernatant was passed through a 0.45 μm syringe filter into a HPLC vial, N_2 was introduced and the vial capped and stored at −80 °C.

The concentration of the extracted EA was monitored by HPLC-DAD as detailed below (Section 2.6).

2.5. Extraction and hydrolysis of EA glycosides and ETs

Acid hydrolysis of the methanol extracts was performed according to Williams et al. (2014). A 2 mL aliquot of the methanol extract was pipetted into a 5 mL Reacti-Therm (Thermo Fisher Scientific, Bellefonte, PA, USA) vial containing a stirring slug. The methanol was evaporated under nitrogen and 2 mL of 2 N trifluoroacetic acid (TFA) was added to the vial which was then capped and mixed to dissolve the residue. The vial was placed into the Reacti-Therm heater/stirrer unit (Thermo Fisher Scientific, Bellefonte, PA, USA) where the contents were hydrolysed at 120 °C for 120 min. After hydrolysis the vial was cooled and the contents transferred into a 5 mL volumetric flask with methanol. About 1.5 mL of this solution was passed through a 0.45 μm syringe filter into a HPLC vial, N_2 was introduced, and the vial capped and stored at −80 °C.

2.6. Determination of free and total EA content

Reverse-phase HPLC was the method chosen for identifying and quantifying EA in the samples (Williams et al., 2016).

An aliquot (10 μL) was analysed using a Shimadzu (Shimadzu Co., Kyoto, Japan) HPLC system consisting of a system controller (SCL-10Avp), degasser (DCU-12A), pump A (LC-10AD), pump B (LC-10ADvp), auto-sampler (SIL-20C), column oven (CTO-10AC) and a photo-diode array detector (SPD-M10Avp) linked to Labsolutions software. Optimal separation of the EA was achieved on a reversed-phase C_{18} Acclaim Polar Advantage II, 3 μm, 4.6 × 150 mm column (Thermo Fisher Scientific) with matching guard column. Both columns were maintained at 30 °C. Optimal separation required gradient elution. The solvents consisted of (A) 0.1% formic acid (v/v) in water and (B) 0.1% formic acid (v/v) in acetonitrile. The gradient began isocratically with 15% solvent B for 2 min, followed by a linear gradient from 15 to 25% B for 10 min, from 25 to 30% B for 10 min, from 30 to 90% B for 3 min and then isocratic for 4 min. Re-equilibration steps over 8 min returned the system to initial conditions. A flow rate of 1.5 mL/min was maintained for each step.

Spectra for all wavelengths between 220 and 600 nm were recorded by the photodiode array detector. Quantification (before and after acid hydrolysis) was performed by comparison to a six-point calibration curve (0–200 μg/mL) prepared by dissolving EA commercial standard in 100% methanol. Linearity was assessed by calculating slope, y – intercepts and correlation coefficients using a least squares regression equation. The calibration curve so produced gave rise to the acceptable correlation coefficient of 0.9997 (ICH Guidelines, 1995).

The limits of detection (LOD) and quantification (LOQ) were calculated in accordance with 3.3× (ϭ/s) and 10× (ϭ/s) criteria where ϭ = residual standard deviation of the response factors and s = slope of the calibration curve (Shrivastava & Gupta, 2011). The LOD and LOQ for the method was 0.26 and 0.80 μg/mL respectively. A good accuracy for the method was confirmed with the recovery values of 100% as previously shown in Williams et al. (2016).

EA content for all forms was expressed as mg/g DW (dry weight) after moisture determinations.

2.7. Extraction of water-soluble and total Ox

Extraction of water-soluble and total oxalates and quantification were performed according to Ohkawa (1985).

Triplicate samples (0.4 g) of the freeze-dried powders were accurately weighed into a small beaker and 10 mL of water was added. The mixture was allowed to stand at room temperature (≈22 °C) with occasional stirring before being filtered through Whatman No. 41 filter paper with the clear filtrate being transferred to a 100 mL glass beaker. The water was evaporated overnight in an oven (Thermoline Scientific, Wetherill Park, NSW, Australia) set at 80 °C. On removal from the oven the beaker was allowed to cool and the residue dissolved in 20 mM phosphate buffer pH 2.4. The dissolved residue was transferred to a 100 mL volumetric flask making sure to wash the beaker several times with buffer. After transferring all the washings to the volumetric flask it was made to volume with the phosphate buffer pH 2.4 solution. About 1.5 mL of this solution was passed through a 0.45 μm syringe filter into a HPLC vial and the vial capped and stored at −80 °C.

To extract the total Ox, ≈0.4 g of the freeze-dried powders were accurately weighed into a 50 mL glass tube. 5 mL of 25% hydrochloric acid was added to the tube which was then capped and mixed before being placed into an oven set at 80 °C for 120 min. After this the vial was cooled and the contents were filtered (Whatman No. 41 filter paper) into a 100 mL volumetric flask followed by several washes of phosphate buffer pH 2.4. The flask contents were transferred to a beaker and the pH adjusted to 2.4 with 50% potassium hydroxide solution utilising a pH meter (TPS labCHEM, Australia) to monitor. After transferring back to the 100 mL volumetric flask, it was made to volume with phosphate buffer pH 2.4. About 1.5 mL of this solution was passed through a 0.45 μm syringe filter into a HPLC vial.

2.8. Determination of water-soluble and total Ox content

An aliquot (10 μL) was analysed on a Shimadzu HPLC system with an attached SDD-M10A VP diode array detector. The operating conditions were similar to those outlined by Libert (1981) i.e.: reversed phase column Synergi Hydro RP 80A, 4 μm, 250 × 4.5 mm (Phenomenex, NSW, Australia); mobile phase of 20 mM phosphate buffer pH 2.4 at a flow rate 1 mL/min; column temperature maintained at 35 °C and the OA peak was monitored at 214 nm.

The OA was quantified from a standard curve prepared by dissolving standard OA in the pH 2.4 phosphate buffers. The LOD was calculated to be 33 mg/100 g with a LOQ of 100 mg/100 g (as per Section 2.6 above).

2.9. Determination of Ca

The detailed procedures for sample digestion using a microwave digestion system (MarsXpress, CEM, Matthews, NC, USA) and analysis that employed inductively coupled plasma optical emission spectrometry (ICP-OES, Varian Australia, VIC, Australia) are those outlined previously in Carter, Tinggi, Yang, and Fry (2014); Schoendorfer et al. (2011). In brief, the dried samples were accurately weighed (0.3 g) into Teflon vessels and concentrated nitric acid (4 mL) was added. The digested samples were suitably diluted prior to ICP-OES analysis. Appropriate reference materials (polar leaves DC73350, bush branches DC73348, Spinach SRM 1570) were used for quality control and assurance. The references materials were treated similarly throughout the analysis. The recoveries of Ca from these reference materials ranged from 99 to 108% while the LOD was calculated at 0.03 mg/kg with a LOQ of 1.0 mg/kg (as per Section 2.6 above).

2.10. Extraction of AA

The methods for sample preparation and HPLC analysis adopted in this study were based on those outlined by Dennison, Brawley, and Hunter (1981) and Gökmen, Kahraman, Demir, and Acar (2000).

AA was determined by weighing 100 mg of the freeze-dried sample into a 15 mL centrifuge tube followed by 10 mL of extracting solution consisting of 1% (m/v) citric acid containing 0.05% (m/v) ethylenediamine tetra-acetic acid (EDTA) as the disodium salt in 50% (v/v) methanol. After being vortexed for 20 s the tubes were centrifuged at ≈3220*g* for 5 min and 1 mL of clear supernatant was added to a 10 mL volumetric flask and made to volume with extracting solution. An aliquot of this solution was filtered through a 0.45 μm syringe filter prior to immediate HPLC analysis.

2.11. Determination of AA content

The content of AA was determined by HPLC with separation being achieved with a Waters (Waters Associates, Rydalmere, NSW, Australia) HPLC system consisting of a pump (LC-515), auto-sampler (Plus 717), UV–visible detector (model 481) linked to Varian Star software (Version 6.41). A 5 μm Supelcosil LC-NH_2, 4.6 × 250 mm column (Supelco, Sigma Aldrich, Sydney, NSW, Australia) efficiently separated ascorbic acid isocratically by using a solution of 40:60 (v/v) methanol: 0.25% K_2HPO_4 (m/v) buffer (adjusted to pH 3.5 with phosphoric acid) as mobile phase. The flow rate was 1.0 mL/min. An aliquot of 10 μL of sample was injected and the AA peak was detected at 245 nm and identified and quantified by comparison to a commercial standard.

A stock AA solution was prepared by dissolving reference grade AA in extracting solution to achieve a concentration of 1000 mg/L. Due to stability concerns the AA solutions (stock and calibration) were prepared fresh on each day of testing. A calibration curve was acquired by plotting the peak area against AA concentration of four calibration standards (5, 10, 20 and 50 mg/100 mL) diluted from the stock solution with extracting solution, tested in triplicate by HPLC. The concentration range selected was higher than that suggested for most fruit due to the higher levels reported for Kakadu plum fruit (Williams et al., 2014). Linearity was calculated as outlined above in Section 2.6. The UV – visible detector employed gave a linear response over the concentration range 5–50 mg/100 mL. Furthermore the calibration curve gave rise to an acceptable correlation coefficient of >0.999 as specified by the ICH Guidelines (1995). The LOD and LOQ (calculated as given per Section 2.6) for the AA method was 2.9 and 8.1 mg/100 mL respectively. A good accuracy for the method was confirmed with recoveries of 95–100%.

The AA concentrations of the samples were expressed as mg/100 g DW.

2.12. Statistical analysis

All analyses were run in triplicate and were expressed as means ± standard deviation (SD). Statistical analysis was performed by using the XLSTAT-Pro software package version 7.0 (XLSTAT Addinsoft, Paris, France). One-way ANOVA followed by the least significant differences (LSD) test was used to compare the means of the free/total EA and total Ox and Ca levels of the leaves and whole fruit (with and without frozen storage). A p value < 0.05 was regarded as indicating significant difference.

3. Results and discussion

3.1. The good – ellagic acid

The current measurements confirmed that Kakadu plum fruit is a very rich source of EA (Table 2; Fig. 2), comparable to the previous result for a similar whole fruit combination (980 mg/100 g DW) collected from the 2013 Wadeye harvest (Williams et al., 2014). Previous assessment of free EA levels in Kakadu plum fruit collected from sites across northern Australia yielded a range of 3050 to 14,020 mg/100 g DW (Konczak et al., 2014) much higher than those exhibited by the present extracts. However in a survey of related *Terminalia* species, Pfundstein et al. (2010) presented the lower distribution of 228–410 mg/100 g DW for the free EA content, comparable with the values from the current study for fruit collected from individual trees i.e. 381 to 957 mg/100 g DW (Fig.2). Discrepancies between research groups in measuring EA are not unexpected as most have noted that EA values (even for the same plant material) vary markedly, the disparities being attributed to different extracting conditions (da Silva Pinto et al., 2008; Williams et al., 2014, 2016) as well as the often cited genetic diversity, climate and soil conditions, ripening stage and storage conditions (Konczak et al., 2014).

In the current study the free and total EA levels in the leaves of this plant were significantly higher ($p < 0.05$) than those of the fruit collected from the same trees (Fig. 2). In fact the mean concentration of the leaves was of the order of 2.8 times higher for the free form while the total content was approximately 6 times higher than the comparable fruit (Table 2). Furthermore Fig. 2 highlighted that leaves collected from Trees 15, 18 and 27 exhibited the highest total EA content. Conversely the percentage of free to total EA exhibited by the leaves was a low 29% when compared with the 64% for fruit; this low result together with our previous findings outlined in Williams et al. (2016) indicated a greater presence of the complex ETs in the leaves (Fig.2). The higher overall concentrations for the leaves together with this low ratio may offer enhanced antioxidant efficiencies as activity is directly correlated with the number of hydroxyl groups present per molecule giving rise to the following sequence of decreasing activity: - ETs; free EA and EA glycosides (Pfundstein et al., 2010; Zafrilla, Ferreres, & Tomas-Barberan, 2001).

Table 2
Free and total EA content in composite (purees and whole flesh) samples of Kakadu plum fruit stored at −20 °C together with average values of whole flesh and leaves collected from six individual Kakadu plum trees.

Description	Free EA (mg/100 g DW)	Total EA (mg/100 g DW)
Puree–2014 (month 0)	520 ± 11	1496 ± 76
Puree–2014 (month 3)	710 ± 10	1165 ± 24
Whole fruit–2014 (month 0)	795 ± 9	1726 ± 334
Whole fruit–2014 (month 3)	857 ± 56	1214 ± 192
Average whole fruit (indiv.)	622[a] ± 205	976[c] ± 223
Average leaves (indiv.)	1724[b] ± 159	5848[d] ± 1046

For the two columns different letters (a–d) showed a significant difference ($p < 0.05$) for the mean of the free and total EA as analysed by the ANOVA test and then least significant differences (LSD) test. Values used are means of triplicate analyses.

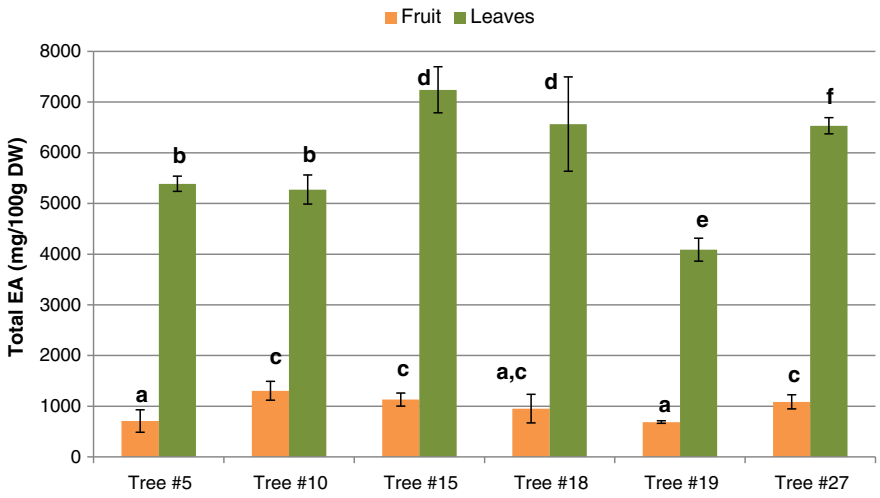

Fig. 2. Total EA content of tissues (whole fruit and leaves) collected from six selected individual Kakadu plum trees. Different letters (a–f) showed a significant difference ($p < 0.05$) as analysed by the ANOVA test and then least significant differences (LSD) test. Values used are means of triplicate analyses.

It was perceived that free EA content increased markedly with frozen storage (Table 2) agreeing with previous storage trials that noted a rapid increase due to the hydrolysis of the fruits' ETs to the simpler form (Aaby, Wrolstad, Ekeberg, & Skrede, 2007; Zafrilla et al., 2001). This may become an issue when adopting harvesting and storage practices as well as formulating decisions on the eventual use of the plum flesh.

3.2. The bad - oxalate

High concentrations of total OA were found in all the frozen Kakadu plum fruit (Table 3; Figs. 3 and 4). To place in context any Ox problem, more commonly consumed vegetables and fruit that are recognised Ox – rich foods have been added to Table 3. Note to facilitate this comparison the fresh weight of some recorded results were converted to dry weight on the application of water content obtained from the USDA National Nutrient Database (2013), for bamboo shoots from Judprasong, Charoenkiatkul, Sungpuang, Vasanachitt, and Nakamanong (2006) and star fruit (*Averrhoa carambola*) from Morton (1987).

Table 3
Total oxalate (mg/100 g DW), calcium (mg/100 g DW) and oxalate/calcium (mEq) ratio of composite (whole flesh and purees) samples of Kakadu plum fruit together with average values of whole flesh and leaves collected from six individual Kakadu plum trees stored at −20 °C.

Description	Total oxalate (mg/100 g DW)	Calcium (mg/100 g DW)	Oxalate/Ca (mEq)
Puree–2014 (month 0)	2466 ± 252	287 ± 20	3.8
Puree–2014 (month 3)	2573 ± 125	273 ± 17	4.2
Whole fruit–2014 (month 0)	2638 ± 37	249 ± 8	4.7
Whole fruit–2014 (month 3)	2633 ± 115	298 ± 3	3.9
Average whole fruit (indiv.)	2717[a] ± 601	243[c] ± 48	5.2 ± 1.6
Average whole leaves (indiv.)	1636[b] ± 657	752[d] ± 329	1.1 ± 0.1
[1]Star fruit (*A. carambola*)	2220	60	16.4
[2]Rhubarb (raw)	4296 − 20,875	625 − 1906	7.9
[2]Spinach	3720 − 14,651	930 − 1418	4.3
[3]Bamboo shoots	2740	224	5.4

For the two columns different letters (a–d) showed a significant difference ($p < 0.05$) for the mean of the free and total EA as analysed by the ANOVA test and then least significant differences (LSD) test. Values used are means of triplicate analyses.

[1] From Hönow and Hesse (2002) with the Ca content obtained from Morton (1987).

[2] From Noonan and Savage (1999) with moisture content from USDA National Nutrient Database (2013).

[3] From Judprasong et al. (2006) with the Ca content obtained from Feleke (2013).

A 1996 conference whose outcomes were summarised by Graham and Hart (1997) recorded concerns about inadequate evidence being available on the safe use of commercially viable Australian native plants that included Kakadu plum fruit. A two-tier investigation (Hegarty, Hegarty, & Wills, 2001) provided scientific information about possible adverse effects. The first stage comprising a review of scientific, industry and popular literature revealed no evidence that the edible flesh from Kakadu plum fruit possessed any undesirable properties although they did note that the leaves from a related *Terminalia* species (*T. oblongata*) were believed to be poisonous to cattle and sheep. The second stage involved conducting a range of chemical tests on selected plant species. OA was found in the frozen Kakadu plum fruit extracts at the 524 mg/100 g DW level (re-calculated using a moisture content of 54.2 g/100 g), much lower than the values found in the current study (Table 3). Hönow and Hesse (2002) measured the total Ox content in star fruit (*Averrhoa carambola*) which contained 2220 mg/100 g DW and according to these and later authors (Ruan et al., 2013) placed it in the high – Ox food category (Table 2). Bearing in mind that the values for all the fruit examined in the current study are consistently higher than this, care needs to be exercised when consuming Kakadu plum products not to exceed the daily Ox intake of 50 mg per day (recommended by the American Dietetic Association, 2005). In general the Ox concentrations of the leaves were significantly lower ($p < 0.05$) than their fruit counterparts and much lower than the leafy vegetables known to contain high Ox levels such as spinach, rhubarb and bamboo shoots (Table 3; Fig. 3). In general the leaves collected from individual trees showed a similar trend except for those collected from Yelcher Beach (Fig. 3) where the total OA was on a par with the fruit taken from the same tree (Tree# 19) or even more unexpected significantly higher ($p < 0.05$) when compared to its fruit counterpart for Tree# 27 (Fig. 3).

As the presence of Ox in foods has been linked to the reduction of the bioavailability of essential minerals such as Ca, several authors have advocated that any discussion of the adverse effects of Ox must include the Ox: Ca ratio (Noonan & Savage, 1999). The subsequent measurement of Ca of all the tested extracts allowed the calculation of this ratio (Table 2). The authors (Fassett, 1973; Noonan & Savage, 1999) classified foods into three groups based on this ratio: 1) plants with ratio >2;2) ratio of approximately 1 and 3) plants with a ratio <1. Foods with a ratio >2 are seen to have a high Ox content and in conjunction with little or no utilizable Ca, possess the ability to bind Ca from other foods eaten at the same time. All the Kakadu plum fruit displayed Ox:Ca ratios >2 indicating an ability to demineralize other foods (Table 3). On the other hand the leaves had Ox:Ca ratios that placed them with plants such as

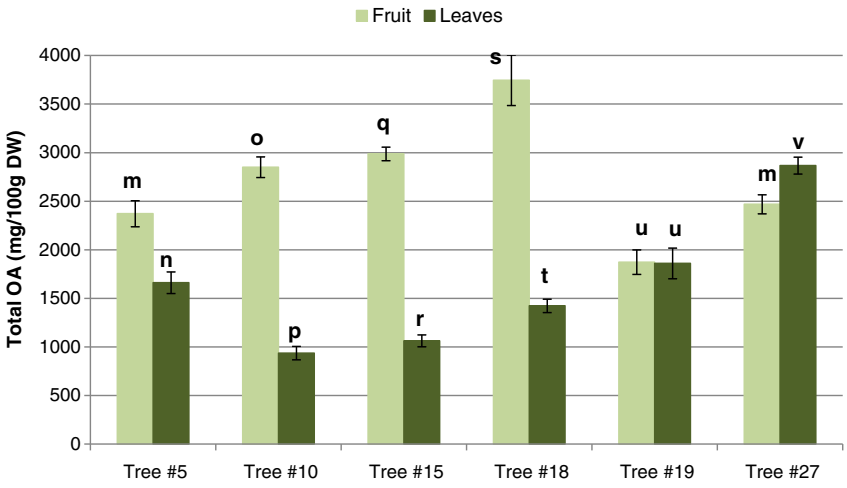

Fig. 3. Total OA content of tissues (whole fruit and leaves) collected from individual Kakadu plum trees. Different letters (m–v) showed a significant difference ($p < 0.05$) as analysed by the ANOVA test and then least significant differences (LSD) test. Values used are means of triplicate analyses.

potato, amaranth and green tea (Noonan & Savage, 1999) suggesting a limited capacity to bind available Ca from other sources (Table 3).

One of the main factors influencing the intestinal absorption rate of Ox and hence its bioavailability is the water-soluble characteristics of the predominant form (Hönow & Hesse, 2002; Siener, López-Mesas, Valiente, & Blanco, 2016). Recognising its limitations most research groups (Israr, Frazier, & Gordon, 2013; Massey, 2007) believe the calculation of the soluble to total Ox ratio is the most appropriate indicator of bioavailability of this compound. A representation of this ratio calculated for whole fruit and the leaves is given in Fig. 4. Again for comparison the ratio for the star fruit was calculated. It's been recognised that the soluble Ox present has greater intestinal absorption than its insoluble counterpart conceivably imparting greater nutritional problems (Savage, Vanhanen, Mason, & Ross, 2000). Applying these criteria to the current results, the fruit extracts all possess substantial quantities of water-soluble Ox (Fig. 4), in fact with higher percentages than the star fruit, a plant tissue with well-documented anti-nutritional problems associated with its level of Ox (Fang et al., 2007).

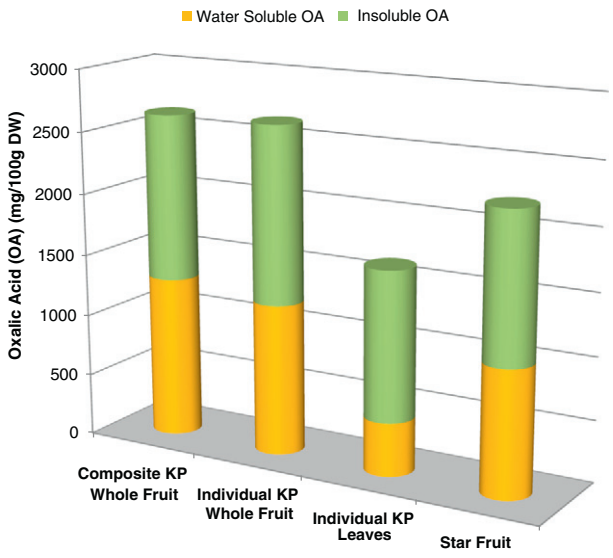

Fig. 4. Total OA (water-soluble + insoluble) content in composites of whole Kakadu plum fruit as well as tissues (whole fruit and leaves) collected from individual Kakadu plum trees Values used are means of triplicate analyses.

3.3. The uncertain – ascorbic acid

Besides dietary intake, there is a metabolic link between AA and OA and evidence that a high intake of AA can result in an increase in urinary Ox (Linster & Van Schaftingen, 2007). There is, however, continuing debate about the contribution these two sources make to the body's level of Ox in the urine. Hughes and Norman (1992) suggested that the contribution of diet extends to only 10–20% while Holmes and Kennedy (2000) put this figure at around 45% and in some individual cases as high as 72%. In a recent review Nouvenne et al. (2008) concluded that high intakes of AA, up to 1500 mg/d, produced only modest increases in urinary Ox levels. Intake below this rate they suggested had no effect on kidney stone formation. A recent study into dietary and supplementary vitamin C intake and the associated risk of kidney stone development found a substantial increase only for men and when the AA intake exceeded 1000 mg/d (Ferraro et al., 2015). This study confirms the high AA levels in Kakadu plum fruit extracts previously found (Konczak et al., 2014; Williams et al., 2014) and compare them with values reported for star fruit (Table 4).

AA levels in the composite fruit extracts were very close to those found previously, 14,038 mg/100 g DW in Williams et al. (2014) and comparable with the average value of 15,190 mg/100 g DW reported by Konczak et al. (2014).

The data obtained from the individual fruit is consistent with the composite results with an average value of 11,133 mg/100 g DW

Table 4
Concentration of ascorbic acid (AA) (mg/100 g DW) and the corresponding amount (g) of Kakadu plum fruit extracts that can be added without exceeding 1000 and 1500 mg intake of AA. Where specified the fruit composites were stored at −20 °C for 0 and 3 months.

Description	AA (mg/100 g DW)	Amount Freeze-dried powder (g) to exceed 1000 mg/d AA/d	Amount Freeze-dried powder (g) to exceed 1500 mg/d
Puree–2014 (month 0)	19,183 ± 32	5.2	7.8
Puree–2014 (month 3)	17,645 ± 428	5.7	8.6
Whole fruit–2014 (month 0)	18,157 ± 135	5.5	8.3
Whole fruit–2014 (month 3)	16,366 ± 855	6.1	9.2
Average whole fruit (indiv.)	11,133 ± 901	9.0	13.5
Star fruit	344	290	436

Values are means ± SD of triplicate analyses.

although a very wide range of AA concentrations was noted fluctuating from 85 to 25,667 mg/100 g DW. Konczak et al. (2014) also observed this high variability in AA content suggesting genetic differences and environmental conditions were largely responsible. No AA was detected in the leaves.

While the underlying metabolic link between AA and OA remains unclear, there is enough evidence to infer that a high intake of AA can result in a moderate increase in urinary Ox (Ferraro et al., 2015; Taylor, Stampfer, & Curhan, 2004; Thomas, Elinder, Tiselius, Wolk, & Akesson, 2013). As the fruit is already being used as a natural source of vitamin C in dietary health supplements (Mohanty & Cock, 2012) it was thought beneficial to calculate the dose of the tested Kakadu plum fruit powders that can be added without exceeding 1000 and 1500 mg intake of AA as specified in Ferraro et al. (2015) and Nouvenne et al. (2008) respectively (Table 4). The two AA intake thresholds presented represent current thinking and are not meant in any way to be definitive.

4. Conclusions

Our study focussed on three organic acids with two being much sought-after constituents for functional food and nutraceutical applications. Kakadu plum fruit contains high levels of all three: the potent antioxidant and antimicrobial EA, the anti-nutritional OA and the equally active antioxidant, AA. We found that leaves most notably those collected from trees 15, 18 and 27 were an even better source of EA, particularly of the complex ETs which may possess greater antioxidant capacity than the free form. When the role EA plays in these plants' enhanced antimicrobial properties becomes more evident, further incentive for greater utilisation of the leaves as an alternative source of this important phytochemical may eventuate.

Kakadu Plum fruit also contains very high levels of OA particularly those from trees 10, 15 and 18 in conjunction with little utilizable Ca as indicated by a ratio of OA to Ca much higher than 2. This places them in a category with star fruit (*A. carambola*), rhubarb and spinach, all with well documented Ox problems. Exacerbating this problem is the fact that the fruit has a high percentage of soluble oxalates which are more easily absorbed in the intestinal tract. In general the leaves possessed lower OA concentrations and in conjunction with higher Ca content would seem to indicate a reduced Ox problem.

The third organic acid present in high concentrations in Kakadu Plum fruit is ascorbic acid. Since AA metabolism generates more OA it adds to the problem that high OA content presents already.

It is becoming clear that the presence of high Ox levels together with the potential to create even higher amounts through metabolism of elevated AA content could limit some nutraceutical applications. For applications requiring smaller amounts, e.g. as a food additive, the Ox and AA levels should pose little or no health risks. The much lower Ox content and the significantly higher EA concentrations make the Kakadu plum leaves a less problematic source of bioactives, although the lack of AA may reduce its attractiveness for these applications. Furthermore an obstacle to this tissues utilisation is a total lack of information regarding the active constituent that provoked reports of poisoning in sheep and cattle when fed the leaves of a related Terminalia species (Hegarty et al., 2001).

Future studies should attempt to clarify the association between dietary/supplementary AA and OA intake, Ox metabolism and kidney stone formation. Kakadu plum tissues provide a rich source of bioactive compounds, so a next step in any commercialisation for nutraceutical applications should entail the experimental testing on animal models in various combinations and formulations.

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