



Monitoring quality and bioactivity of Kakadu plum in the Northern Territory

by Y.Sultanbawa, M.Chaliha, A.Cusack, D. Edwards and
D. Williams
May 2018



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Foreword

Australian native foods have unique nutritional, health and functional properties providing considerable potential to contribute to the global market for functional foods. *Terminalia ferdinandiana* (Kakadu plum or various other local names) is a native food, cherished in Indigenous communities as a medicine and food. This small green fruit contains high levels of vitamin C, antimicrobial and antioxidant compounds and grows in abundance across the Top End of Australia.

Innovative commercial applications have been developed based on the unique functional properties of Kakadu plum. The research team at the University of Queensland and the Department of Agriculture and Fisheries, Queensland have developed a natural preservative solution to extend the shelf life of cooked chilled aquacultured prawns, a glazing solution to prevent oxidation and retain colour in frozen cooked prawns and a natural antioxidant and antimicrobial powder to extend the shelf life of pre-prepared frozen meals. These technical innovations transformed the raw Kakadu plum into a functional food ingredient which created diversified markets with novel uses and a higher price for the Kakadu plum.

There was a need to increase the supply of the wild harvested Kakadu plum to meet the demand of the growing national and international markets. The Aboriginal landholders in northern Australia needed to establish a sustainable supply chain by establishing Kakadu plum micro-enterprises within each community interested in harvesting and processing and making a business from this native food.

AgriFutures Australia supported this concept and funded the project on “Monitoring quality and bioactivity of Kakadu plum in the Northern Territory”. Palngun Wurnangat Aboriginal Corporation in Wadeye, in the Northern Territory was the first community that was selected to develop a sustainable wild harvested Kakadu plum supply chain. This community was the most suitable to commence the research work and commercial harvest as they had experience in harvesting Kakadu plums. It was also the community that purchased the processing equipment from Coradji (Pty) Ltd, the largest processor of Kakadu plum products in Australia.

This report outlines research developed to provide the Indigenous communities and native food industry with reliable information on the retention of bioactive compounds during harvesting, processing and storage of Kakadu plum. This will enable the development of product standards which in turn will provide the industry with scientific evidence to expand and explore new market opportunities globally.

Data on the composition of key bioactive compounds will be an important tool to both optimise the quality and promote the benefits of Kakadu plum. Additionally, these parameters are needed for establishing quality assurance processes that can validate quality and bioactivity across different batches of the same plant source and throughout the storage life of the product.

This report represents the first systematic monitoring of quality and bioactivity of wild harvested and processed Kakadu plum products. In addition, it also reports on training of Indigenous communities in best production and quality control practices to ensure a consistent Kakadu plum product, to supply to main stream agriculture, aquaculture and food industries. Finally, the report provides information on processing and packaging improvements which will ensure the quality and safety of the product over extended storage periods. The results from this study can be used in developing product standards for Kakadu plum and open new markets.

This report is an addition to AgriFutures Australia's diverse range of over 2000 research publications and it forms part of our Emerging Industries arena, which aims to establish high potential rural industries.

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John Harvey
Managing Director
AgriFutures Australia

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Abbreviations

AA	Ascorbic acid
CCRD	Central composite rotatable design
DW	Dry weight
EA	Ellagic acid
EAE	Enzyme assisted extraction
FRAP	Ferric reducing ability of plasma
FW	Fresh weight
GA	Gallic acid
GAE	Gallic acid equivalent
GCMS	Gas Chromatography Mass Spectrometry
HPLC-DAD	High-performance liquid chromatography with photodiode array detection.
LCMS	Liquid chromatography mass spectrometry
LOD	Limits of detection
LOQ	Limits of quantification
NLC	Northern Land Council
ROS	Reactive oxygen species
SCFA	Short chain fatty acid
SEM	Scanning electron microscopy
TO	Traditional Owners
TPC	Total phenolic content

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Executive Summary

What the report is about

This report includes the monitoring of changes in quality and bioactivity of Kakadu plum in Northern Australia, based on work with Indigenous communities who are wild harvesters of Kakadu plum. This is the first systematic study that has evaluated key bioactive compounds in Kakadu plum during commercial wild harvests, assessing these compounds in different geographical locations and determining tree to tree and within tree variations during several harvesting seasons. This is the first study to report on changes in key bioactive compounds during different maturity stages in Kakadu plum and the potential to develop value added products from fruit and seed kernels. Work on the biotransformation of Kakadu plum was found to increase the growth of probiotic bacteria and produced metabolites that are beneficial to human health.

This project has trained the Indigenous communities in adopting best production and quality control practices in developing value added Kakadu plum products. The measurement of these bioactive compounds has enabled the development of product specification information that can be used for marketing purposes.

Who is the report targeted at?

- The Indigenous communities interested in making Kakadu plum a business
- The Indigenous communities interested in making Kakadu plum a business
- The Australian native food industry
- The general food and beverage industry
- Extraction companies interested in developing functional food ingredients
- Food standards and regulatory authorities

Where are the relevant industries located in Australia?

- Indigenous communities located in Northern Australia.
- Aqua culture prawn industry located in Queensland.
- Australian Native Food Industry throughout Australia.
- Food and beverage industry throughout Australia.

Background

The University of Queensland and the Department of Agriculture and Fisheries, Queensland were collaborators on an AgriFutures funded project titled "Changes in quality and bioactivity of native foods during storage". Kakadu plum was one of the native fruits assessed in this project. This study confirmed the high level of vitamin C and phytochemicals present in Kakadu plum and its stability during storage. The unique properties of Kakadu plum has enabled the commercial use of this native plum as a functional food ingredient.

The current needs for establishing a processing unit for Kakadu plum in Wadeye is training in good production practices including the training of workers in good hygiene, quality control to ensure a consistent product and monitoring the quality of the product from harvest, through to processing and storage

This project will address the needs for producing Kakadu plum products (frozen, puree and whole) with known quality and safety in Wadeye, Thamarrurr Region, Northern Territory. The processing of Kakadu plum will be done by the Palngun Wurnangat Aboriginal Corporation PWAC (Women's Centre). The women of this association are the new owners of the Kakadu plum processing business, formerly owned by Coradji (Pvt) Ltd, one of the largest producers

of Kakadu plum processed products in Australia. The current production practices will be assessed and best practices will be recommended to minimise losses in quality and bioactivity. This study will enable the PWAC to adopt best production and quality control practices for the processing and storage of Kakadu plum that will maximise product quality and meet shelf life expectations of customers. This project will also empower the women of the Wadeye community with food manufacturing skills, understanding the nutritional value of native foods such as Kakadu plum and importance of processing and retaining quality over long storage periods. These skills can be extended to other native foods which will result in the reduction of post-harvest losses and availability of native food throughout the year for their own consumption and further value addition by other industries. An expected outcome is to establish similar harvesting, processing and storage models in other Indigenous communities interested in making Kakadu plum a business.

Aims/objectives

The objectives of this project are:

- To assess the current harvesting, processing, packaging and storage of Kakadu plums in terms of good production and quality control practices in a remote community setting in Northern Australia.
- To train Indigenous communities and others on good production and quality control practices
- To test the raw materials and processed product for quality and bioactivity
- To assist in the scaling up of production and address the challenges during such operations
- To monitor the product throughout the supply and distribution chain and during storage to determine product consistency
- To develop product standards for Kakadu plum
- To identify other nutritional, bioactive and health properties in Kakadu plum to target new markets.

Methods used

Wild harvested Kakadu plum fruits and leaves from different geographical locations were subjected to the following analysis:

- Analysis of ascorbic acid levels
- Analysis of free and total ellagic acid levels
- Proximate analysis for macro nutrients
- Analysis of minerals
- Microbial quality standard plate count and yeasts & moulds
- Fatty acid composition

Results/key findings

This research has yielded a range of significant findings, suitable for immediate adoption by the Indigenous communities interested in making Kakadu plum a commercial product. The product specification sheets, production protocols and quality control measures gives confidence to mainstream agriculture and food industries to purchase Kakadu plum products from Indigenous communities.

- This study confirms the potential of using Kakadu plum fruit as a functional ingredient with high antioxidant capacity and other bioactivities due to the presence of ascorbic acid and ellagic acid in significant quantities.
- This is the first study reporting on the different stages of maturity classified according to fullness of Kakadu plum fruit and changes in ascorbic acid and ellagic acid levels during ripening.
- The strong correlation between the visual fullness measurements and the total ellagic acid and ascorbic acid content at the four stages of maturity established for Kakadu plum give market opportunities for the functional ingredient industry.
- The leaves of kakadu plum had a higher content of ellagic acid, a key bioactive with known health properties.
- An enzyme assisted aqueous Kakadu plum extract has been developed which has potential to be used as a natural preservative in foods.
- The Kakadu plum kernel has the potential to be utilized as a novel protein source for dietary purposes and non-conventional supply of linoleic, palmitic and oleic acids.
- Kakadu Plum biotransformation led to an increase in the growth of beneficial *Lactobacilli* bacteria and produced metabolites including short chain fatty acids and urolithins that were beneficial to human health.
- Product specification sheets for chemical and microbial quality were developed for wild harvested Kakadu plum puree and freeze dried powder. These are being used by the main stream agriculture and food industries when purchasing these Kakadu plum products.
- Puree and freeze dried powders of Kakadu plum from the Wadeye community has been used by the prawn aquaculture industry in Queensland from 2014 onwards.
- The Kakadu plum business operating as a micro-enterprise has given opportunities for seasonal work especially for PWAC women and an income to the Wadeye Aboriginal community members. This has empowered the Wadeye community members, especially the women.

A Kakadu Plum Indigenous Network (KPIN) was established as an outcome of the Native Trees and Fruit Workshop held in Broome WA in (March 2017). Participants now share and communicate their Kakadu Plum experiences with each other to support the development of an Indigenous-led Kakadu plum industry.

Implications for relevant stakeholders

- If adopted, this research will enable the setting of Kakadu plum micro-enterprises in Indigenous communities throughout northern Australia. It is envisaged these enterprises will be completely owned and controlled by the local Indigenous community.
- The benefits of developing product specification standards for the Kakadu plum gives confidence to the main stream agriculture and food industry in quality of the end product.
- Improved product information, including information on stability of bioactive compounds in Kakadu plum and identification of chemical markers during storage will support quality control and product description, which will improve market confidence in the end product

- Market expansion through product innovation and scientific evidence to support product claims
- Improved manufacturing guidelines for products and accurate information for labelling
- Sustainable growth for the Kakadu plum industry based on an increased understanding of protocols and products.

Recommendations

Information obtained from this project can be used to promote Kakadu plum products as functional ingredients supported by credible scientific data. The economic benefit of getting access to global markets and gaining customer confidence is very significant and could increase sales significantly. Opportunities for cross-industry use of Kakadu plum extracts as natural preservatives and antioxidants in other food systems present innovative applications. Such applications could be considered only the beginning of potential uses of this functional ingredient, not only in food and beverages but also in the complementary health care and cosmetic industries.

Growth of the Kakadu plum industry will increase cultivation, harvesting and processing of native foods in Indigenous communities, which will create much-needed employment in remote areas. In most Indigenous communities, it is the women who cultivate and harvest native foods, and growth of this industry would give economic power to women.

Chapter 1 Physicochemical Properties of Kakadu plum

1.1 Introduction

Australia is a vast, mostly arid country, with a unique flora and an ancient indigenous culture whose knowledge has barely been tapped. One way of ensuring nutrition security is to identify and grow a diversity of foods that provide the nutrients essential for sustaining a healthy lifestyle. The Aboriginal people in Australia have subsisted on indigenous plants for over 40,000 years [1]. There are hundreds of edible indigenous plant species in Australia used by Aboriginal people for their nutritional and medicinal value, yet their full potential as foods remains hidden. Kakadu plum (*Terminalia ferdinandiana*) is one such indigenous food plant.

Kakadu plum is an important traditional food in the diet of Aboriginal people in Northern Australia [2] and has several nutraceutical properties. *T. ferdinandiana* is also known as Gubinge, Billygoat plum, Green plum and Bush plum, and by different aboriginal names such as Arangal, Gabiny (by Yawuru community), Gubinge (Bardi community north of and around Broome), Kabinyn (Nyul Nyul), Madoorr (Bardi community near One Arm Point of Dampier peninsular), Manmohban (in Kune language in Maningrida area), Mi-marl-arl (by Wadeye community in Northern Territory- NT) and Murunga (Eastern Arnhem Land of NT).

For the purpose of this report it will be referred to as Kakadu plum. It is endemic to mainly the coastal (<40 km inland) tropical savannas of northern Australia from Broome in the west to the Gulf of Carpentaria in the east and is most abundant on Indigenous tenured land [3], refer Figure 1A and B for distribution of *Terminalia* sp. globally and *T. ferdinandiana* which is endemic to Australia.

Kakadu plum belongs to the genus *Terminalia* which is in the family Combretaceae.

Terminalia ferdinandiana is a tropical tree growing up to about 10 m with large light green to yellow leaves. The fruit is about 2 cm long and 1 cm in diameter and varies in colour from light green to yellow with some exhibiting a reddish tint (Figure 2). The fruit contains a single large seed [4, 5]. The tree begins to flower at the end of the dry season, from September to November and fruits from the middle of the wet season to the early dry from January to June [6].

The nutritional value of Kakadu plum has been reported previously [4, 7, 8]. However, the exploration of Kakadu plum as a rich source of bioactive phytochemicals rather than its macro- and micro- nutrients appears to offer the most value to the functional food industry.

Oxidative stress in the human body occurs as a result of the accumulation and action of free radicals generated as by-products of essential metabolic reactions and through exposure to environmental factors [9]. The free radicals (or reactive oxygen species, ROS) so generated cause damage to tissue, cells, nucleic acids, proteins and lipids and can result in a general decline in optimum body function [10-12]. This damage has been linked to the development of degenerative diseases such as cancer [13], cardiovascular diseases [14], neural degeneration [15] and diabetes [16]. The major sources of antioxidants, compounds that alleviate oxidative stress in our bodies are fruit and vegetables [17, 18]. In recent decades there has been renewed

interest in Australian native plant foods as rich sources of these antioxidant bioactives, most notably Kakadu plum fruit [19-22].

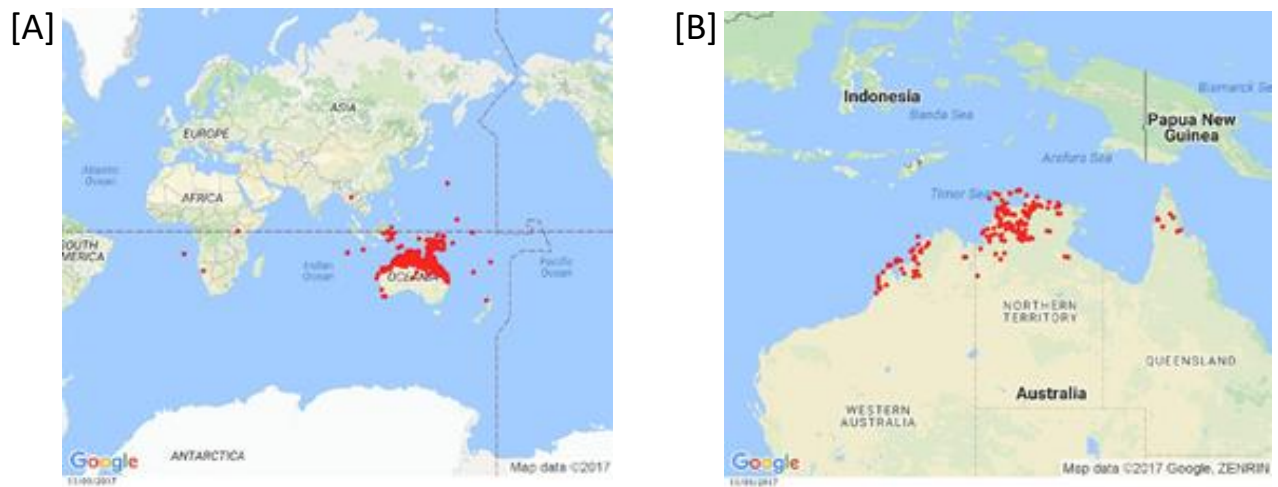


Figure 1 [A] Global distribution of *Terminalia* spp and [B] Distribution of *Terminalia ferdinandiana* in Australia [Source: The Atlas of Living Australia]

It has been known for many years that vitamin C (whose active forms are ascorbic acid (AA) and dehydroascorbic acid (DHAA) reduce oxidative stress effectively [23, 24], refer to Figure 3. To utilise this property, the functional food industry has searched for natural sources of AA [25].

Kakadu plum has exceptionally high AA content and high levels of antioxidants. This is particularly important given that humans and primates cannot synthesize AA due to the lack of the enzyme L-gulonolactone oxidase, therefore AA has to be obtained from the diet. The AA distribution in fruits can vary from 5 mg/100g fresh weight (FW) in apple to 59 mg/100g FW in oranges [26]. AA is essential for collagen synthesis and insufficient amounts can lead to scurvy, it is also advocated in high doses to prevent colds and the role it plays in the treatment of cancer although not yet proven cannot be ignored [27, 28]. Kakadu plum with high levels of AA can be eaten fresh or incorporated into foods and beverages to obtain the recommended daily requirement for adult men 90 mg/day and women at 75 mg/day. The tolerable upper intake level of vitamin C for adults is 2000 mg/day [29].

Natural vitamin C sources are preferred as it is believed they are better able to reduce free radicals (as measured by the ORAC assay) and have greater bioavailability when compared to the synthetic counterpart [25]. Citrus fruit are the best known source of natural vitamin C with an average content of approximately 0.5% FW [30]. A natural substitute for citrus is Acerola (*Malpighia puniceifolia*) fruit with an average AA content of 1.0% FW [31] with a recent study reporting a content of up to 1.4% FW [32]. This latter study measured even higher amounts of AA in the fruit of the camu-camu (*Myrciaria dubia*) plant. Several studies [7, 19, 33] have reported very high AA levels in Kakadu plum fruit between 3.5 – 5.9% FW. Similar value (range 0.1-5.3% FW) have since been measured in Northern Territory grown Kakadu plum fruit, although there was high variability in the AA content between the growth sites and individual samples collected at each site [21].



Figure 2 *T. ferdinandiana* tree with leaves and fruits. A] A deciduous tree with grey flaky bark. B] Light green large oval shaped leaves, approximately 25 cm X 15 cm in size. Leaves are spirally arranged and dense near the branches. C] Oval almond shaped fruits.

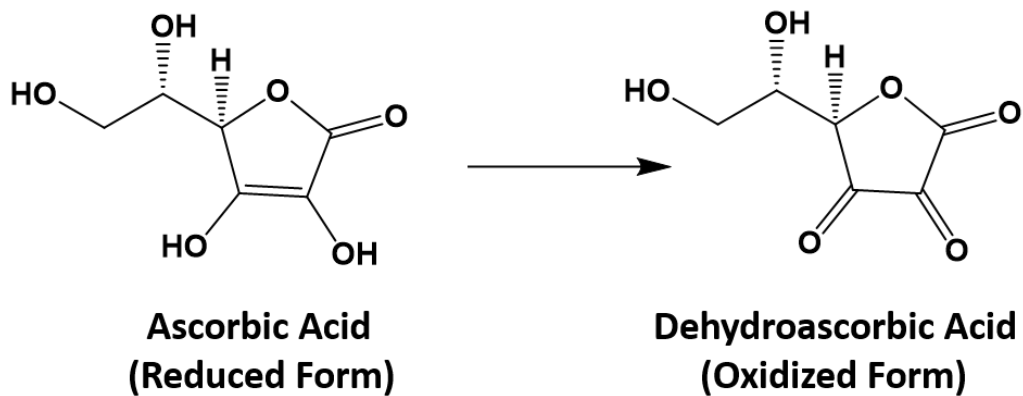
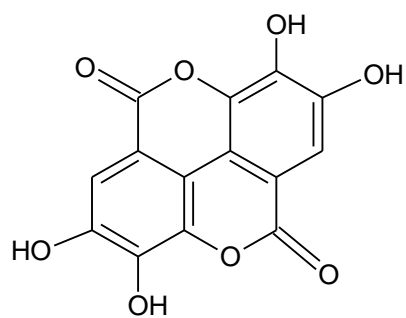


Figure 3 Structure of Ascorbic Acid (AA) and Dehydroascorbic acid (DHAA)



Ellagic acid

Figure 4 Structure of Ellagic acid (EA)

The fruit is an excellent source of the phenolic compounds gallic acid (GA) and ellagic acid (EA), and their related compounds [20, 21] which have well documented *in vitro* antioxidant activity [34, 35]. Very high levels of EA were reported in the Kakadu plum fruit tested by Konczak et al., (2014) [21], far higher than values shown by *Rubus* berries, among the richest sources of EA [36]. This suggests that Kakadu plum fruit is a unique edible source of this phytochemical. EA occurs in plant foods in different forms: free EA, EA glycosides and polymeric ellagitannins [36]. The overall structure of these forms has a pronounced effect on their antioxidant efficiency refer to Figure 4. Although all EA forms are believed to function as antioxidants their efficiency depends on their chemical structure, most notably the number of available hydroxyl groups [36-38].

The aim of this study is to determine the physiochemical parameters and changes of bioactive compounds AA and EA in different geographical locations and harvesting seasons. This information will be useful in determining the quality and bioactivity of the Kakadu plum fruit during processing and developing value added products for commercial applications.

1.2 Material and Methods

1.2.1 Sample Collection

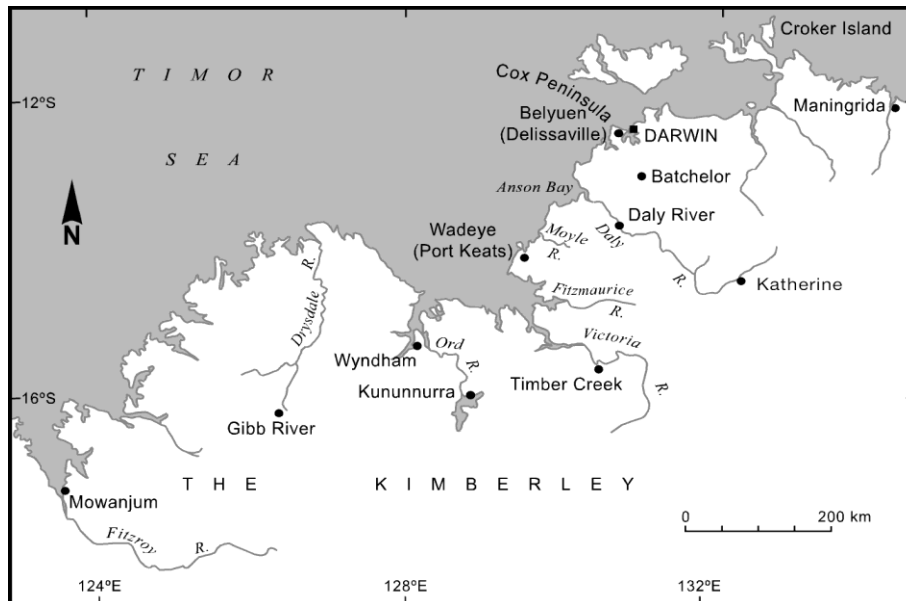


Figure 5 Kakadu plum collection sites in Northern Australia

Kakadu plum samples were collected after obtaining the necessary research permits from the Northern Territory Government (Parks and Wildlife Commission and Department of Primary Industry and Resources) and permission from the Traditional Owners of the particular geographical location where collection occurred. Western Australia (Kimberley region) the samples were collected by the Traditional Owners of the relevant Indigenous Communities and sent to the Health and Food Science laboratories at Coopers Plains, Brisbane for testing. For Kakadu plum collection sites, refer to Figure 5. Samples were collected from natural/wild stands and were given a tree accession number. Botanical specimens collected for identification.

1.2.2 Plant material

Fruit collected from individual Kakadu plum trees in 2015, 2016 and 2017 from different geographical locations in Northern Territory and Western Australia (see Table 1) were treated on arrival at the Health and Food Science Precincts laboratory at Coopers Plains in the following manner: physical measurements weight, width, length were done on the whole fruit and leaves and frozen. The frozen sample of fruit and leaves were freeze-dried, finely ground in a Retsch MM301 cyromill (Retsch GmbH, Haan, Germany) and stored at -80°C . The resulting powders were tested for moisture, EA (free & total) and AA contents.

Table 1 Sample collection locations and tree accession numbers for Kakadu plum

Location	Date	Tree Accession numbers
*Wadeye (Northern Territory)	3-11 April 2015	20151-201528
Kimberley (Western Australia)	20-30 December 2015	Jpp1-Jpp14, 46b,47,48,49a, 46WP,W/P 049,W/P 052(i), W/P 052(ii), W/P 053
Wadeye (Northern Territory)	18-25 April 2016	20161-201673
Milingimbi (Northern Territory)	27-29 April 2016	201682-201697
Darwin (Northern Territory)	26 April 2016	201674-201681
Wadeye (Northern Territory)	16-22 April 2017	20171-201737

*Location codes for Wadeye – A, B, C, D, E, F, G, H, I, J, K

1.2.3 Proximate analysis

Proximate and mineral analysis was performed on the freeze dried powders of composite samples of Kakadu plum tissues from Wadeye harvested in 2015, including flesh, seed coat, kernel and leaves at Symbio Alliance, Eight Mile Plains, Queensland, Australia. Symbio is a National Association of Testing Authorities (NATA) accredited laboratory that complies with ISO/IEC 17025:2005. The analysis were done according to the NATA approved in house methods or AOAC methods: protein by AOAC method 990.03 [39]; fat by AOAC method 991.36 [40]; saturated, mono-unsaturated, poly-unsaturated and trans fat by in house method CFH068.2; moisture by AOAC 934.01 [41], ash by AOAC method 923.03 [42] (AOAC, 2000); total sugar by CFH001.1, total dietary fibre by CF057; available carbohydrate by CF029.1; energy by CF030.1; crude fibre by AOAC 962.09 (AOAC, 1990) and dry matter by in house method CF006.1.

The methods for analysis of minerals and trace elements/metals have previously been described in Carter et al. 2015 [43] and Tinggi et al 2015 [44]. The analysis was carried out using a 7700 Inductively Coupled Plasma (ICP)-MS (Agilent Technologies Australia Pty. Ltd., VIC, Australia) and ICP-OES (Agilent Technologies Australia Pty. Ltd., VIC, Australia) after microwave digestion (MarsXpress, CEM Corporation, Matthews, NC, USA).

1.2.4 Determination of size of whole fruits and leaves

Ten Kakadu Plum fruits and leaves were chosen at random from different geographical locations in the Northern Territory and Western Australia and measured for length and width using 150 mm Digital Caliper (Craftright Engineering Works, Jiangsu, China).

1.2.5 Determination of weight of fruit and seeds

Ten whole fruit and leaves from different geographical locations in the Northern Territory and Western Australia were weighed on laboratory scales (Sartorius CP224S, Gottingen, Germany). The fruit were cut down one side with a knife and the flesh peeled off the seed, then weighed separately and the pulp/seed ratio was calculated.

1.2.6 Determination of moisture content

The moisture content of the freeze-dried material was determined from the weight loss after drying to a constant weight in a vacuum oven at 70°C [45].

1.2.7 Extraction and the determination of free EA content

Free EA was extracted with 100 % methanol as per the methods described previously by Williams et al. [46, 47]. Briefly, duplicate samples (≈ 0.1 g) of the freeze-dried powders were accurately weighed into a 15 mL centrifuge tube and 5 mL (3 x extractions) of aqueous acidified methanol (1L contains 800 mL methanol; 198 mL H₂O and 2 mL conc. HCl) was added and sonicated for 10 min with occasional shaking. After centrifugation ($\approx 3220g$, 5 min at 20°C) the clear supernatant was transferred to a 25 mL volumetric flask and made to volume with aqueous acidified methanol. About 1.5 mL of the diluted supernatant was passed through a 0.45 μ m syringe filter into a HPLC vial, N₂ 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:

1.2.8 Extraction and the determination of total EA content

Acid hydrolysis of the methanol extracts was performed according to Williams et al. (2014) [22]. A 2 mL aliquot of the aqueous acidified methanol extract was pipetted into a 5 mL Reacti-Therm (Thermo Fisher Scientific, Bellefonte, PA, USA) vial containing a stirring slug. The liquid was evaporated under nitrogen and 2 mL of 2N hydrochloric acid (HCl) was added to the vial which were mixed to dissolve the residue. The uncapped vial was placed into the Reacti-Therm heater/stirrer unit (Thermo Fisher Scientific, Bellefonte, PA, USA) where the contents were hydrolyzed at 90°C overnight. After hydrolysis the vial was cooled and the contents transferred into a 5 mL volumetric flask with methanol. About 1.0 mL of 100% methanol (x 4) was added to the tubes, capped, shaken before transferring to 15 mL centrifuge tubes. Final volume was made up to 5 mL with 100% methanol before 1.5 mL of this solution was passed through a 0.45 μ m syringe filter into a HPLC vial, N₂ was introduced, and the vial capped and stored at -80°C.

Reverse-phase HPLC was the method chosen for identifying and quantifying EA in the samples [46]. An aliquot (10 μ L) 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 C₁₈ Acclaim Polar Advantage II, 3 μ m, 4.6 x 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 μ g/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 $3.3 \times (\bar{\sigma}/s)$ and $10 \times (\bar{\sigma}/s)$ criteria where $\bar{\sigma}$ = residual standard deviation of the response factors and s = slope of the calibration curve [48]. The LOD and LOQ for the method was 0.26 and 0.80 $\mu\text{g}/\text{mL}$ respectively. A good accuracy for the method was confirmed with the recovery values of 100 % as previously shown in Williams et al. (2016) [46].

EA content for all forms was expressed as mg/100 g DW (dry weight) after moisture determinations.

1.2.9 Extraction and the determination of AA content

The methods for sample preparation and HPLC analysis adopted in this study were based on those outlined by Dennison et al. (1981) [49] and Gökmen et al. (2000) [50].

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 20s the tubes were centrifuged at $\approx 3220 g$ 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.

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-NH₂, 4.6 x 250 mm column (Supelco, Sigma Aldrich, Sydney, NSW, Australia) efficiently separated AA isocratically by using a solution of 40:60 (v/v) methanol: 0.25% K₂HPO₄ (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 duplicate by HPLC. The concentration range selected was higher than that suggested for most fruit due to the higher levels reported for Kakadu plum fruit [22]. 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 greater than 0.999 as specified by the International Council for Harmonisation (ICH) of Technical Requirements for Pharmaceuticals for Human Use Guidelines [51]. The LOD and LOQ (calculated as given per Section 1.2.8) for the AA method was 2.9 and 8.1 mg/100mL 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.

1.2.10 Statistical Analysis-

Results were expressed as mean \pm standard deviation. The Pearson's correlation coefficients were performed using XLSTAT 2013 program and GraphPad Prism Version 7.03.

1.3 Results and Discussion

Botanical specimens of the *Terminalia ferdinandiana* plant were authenticated by the experts in Queensland Herbarium, Brisbane Botanic Gardens Mt Coot-tha, Queensland Australia, where botanical specimens were retained for future reference (AQ522453).

The size and weight of fresh Kakadu plum fruits including the seed and leaves varied in different locations, in northern Australia, refer to Table 2, Table 3, Table 4 and Table 5. The length and width of fruits from the 2016 harvest ranged from (16.4-37.4 mm & 8.0-20.1 mm) in Wadeye, in Milingimbe (21.3 – 29.0 mm & 13.7-19.0 mm) and in Darwin (21.8-30.2 mm & 14.2-18.6 mm) respectively. The weight of the fruit from the 2016 harvest ranged from (1.0-4.6 g) in Wadeye, (1.7-5.2 g) in Milingimbi and (1.7-3.7 g) in Darwin.

The size and weight of fresh Kakadu plum fruits including the seed in the Kimberley, Western Australia, the size and weight of fresh Kakadu plum fruits from the 2015 harvest ranged from (16.4 – 28.7 mm) in length, (13.1 – 19.5 mm) in width and (1.3 – 4.1 g) in weight.

The length and width of leaves from the 2016 harvest ranged from (12.7-23.9 cm & 8.1-15.2 cm) in Wadeye, in Milingimbe (11.6 – 18.3 cm & 9.2-14.9 cm) and in Darwin (12.4-18.5 cm & 10.9-13.9 cm) respectively. The weight of the fruit from the 2016 harvest ranged from (2.9-8.7 g) in Wadeye, (2.8-8.0 g) in Milingimbi and (3.7-7.4 g) in Darwin.

The weight of a fresh seed based on 100 fruits from Wadeye (after removing flesh) is approximately 0.4 g. Seed contributes to 13% of the total weight of a fresh plum, while the edible flesh was 87% (data not presented). Konczak, et al (2014) reported edible flesh of 73.4% for the Northern Territory and 81.5% for Western Australia [21].

Literature reports, fruit size of about 20 mm length and 10 mm in width and leaf size approximately 25 cm length 15 cm in width [4, 5], which falls within the ranges found in this study. Konczak, et al (2014) reported that the average fresh weight of Kakadu plum varied from 1.5 – 3.4 g in the NT [21].

Table 2 Physical measurement of the fruits & leaves of Kakadu plum from Wadeye, Northern Territory 2016 harvest

Tre e ID	Locat ion	Weight (g)	Length (mm)	Width (mm)	Weight (g)	Length (cm)	Width (cm)
Fruit				Leaves			
1	E	2.2±0.4	24.0±1.6	14.0±1.2	4.0±2.1	16.8±4.8	11.0±2.6
2	E	2.6±0.3	26.5±3.1	14.1±0.8	3.1±0.9	14.4±2.1	10.2±1.6
5	E	2.7±0.5	26.1±3.3	16.0±1.2	5.0±1.6	17.8±4.0	12.9±1.9
7	E	3.2±0.5	29.8±2.6	16.6±0.8	5.4±1.6	20.0±4.0	13.5±2.2
11	E	4.5±0.8	24.3±2.3	17.4±1.4	4.5±0.8	17.3±1.9	12.7±1.2
12	E	4.3±0.5	27.7±1.7	18.8±1.1	5.1±1.7	18.9±4.0	12.6±2.3
13	E	2.2±0.4	26.5±3.7	15.8±1.0	5.3±1.9	17.9±4.3	12.4±1.8
14	E	1.7±0.3	21.5±2.4	13.3±1.6	5.8±1.1	17.8±2.2	15.2±1.2
15	E	2.1±0.3	24.4±1.5	16.6±1.6	6.1±0.8	20.2±1.7	14.4±1.0
16	E	2.9±0.6	21.0±2.8	12.3±1.3	4.1±1.1	17.7±2.7	12.0±1.7
18	E	2.5±0.4	30.2±1.2	19.5±0.7	2.9±0.6	14.0±1.6	11.3±1.3
19	E	2.9±0.6	23.8±2.0	16.1±2.0	4.3±2.0	16.4±3.2	12.7±2.1
20	E	2.4±0.5	24.8±1.7	15.1±1.5	7.7±1.7	20.7±2.6	14.6±1.6
21	F	3.3±0.2	31.3±1.9	15.6±0.9	6.6±3.6	18.2±5.1	12.5±3.4
22	F	2.7±0.4	25.7±2.6	15.6±1.0	5.6±1.8	18.7±3.2	12.4±2.0
23	F	3.8±1.5	37.4±4.2	20.1±1.4	5.2±1.8	16.4±2.8	12.3±1.7
24	F	4.0±0.5	33.0±2.8	16.9±1.6	4.7±1.3	18.2±3.2	11.0±1.4
25	F	3.7±0.4	29.0±2.9	16.8±0.6	6.4±1.4	18.7±1.5	13.5±1.3

Tree ID	Location	Weight (g)	Length (mm)	Width (mm)	Weight (g)	Length (cm)	Width (cm)
Fruit				Leaves			
26	F	1.8±0.4	25.7±1.8	14.5±1.0	7.0±1.3	19.7±2.4	13.7±1.1
28	G	1.4±0.3	18.4±1.6	12.5±0.8	3.8±1.1	14.5±2.4	10.6±1.8
29	G	4.3±0.5	25.6±1.2	17.5±0.9	5.0±1.4	15.4±2.1	11.7±1.5
30	G	1.1±0.3	22.0±2.3	13.4±1.0	4.5±1.1	16.5±2.0	12.1±0.9
31	G	1.5±0.3	20.7±1.9	14.6±2.0	8.7±2.3	23.9±3.0	14.9±1.5
32	G	2.0±0.4	19.6±2.0	14.4±1.1	4.5±1.6	15.2±3.0	12.2±2.5
34	G	1.3±0.2	21.2±1.2	14.8±1.2	6.3±2.4	20.5±4.5	12.4±2.3
35	H	2.5±0.3	23.8±1.1	15.7±0.7	5.1±1.3	16.2±2.5	11.7±1.6
36	H	4.0±0.6	27.5±1.1	17.5±1.2	5.3±1.1	18.2±2.5	13.4±1.4
37	H	2.0±0.4	22.2±1.3	14.1±0.7	5.6±2.4	16.5±2.8	12.1±2.4
38	H	2.1±0.4	23.9±2.1	14.1±1.3	4.3±1.0	17.2±1.7	11.6±1.7

Measurements are based on average of 10 fruits and leaves (mature)

Table 3 Physical measurement of the fruits of Kakadu plum from Wadeye, Northern Territory 2016 harvest

Tree ID	Location	Weight (g)	Length (mm)	Width (mm)	Weight (g)	Length (cm)	Width (cm)
Fruit				Leaves			
39	B	2.5±0.4	20.8±1.4	15.0±1.2	4.6±1.4	15.8±2.1	11.3±1.8
40	B	1.5±0.2	23.3±2.0	13.5±1.3	4.4±1.2	14.5±2.0	11.3±1.4
41	B	2.1±0.7	25.4±2.8	13.8±1.2	4.9±1.0	15.4±1.6	12.5±1.5
42	B	2.1±0.5	22.3±1.9	13.1±0.9	4.4±0.9	13.7±1.2	11.8±1.1
43	B	1.7±0.4	22.9±2.1	14.4±0.8	3.2±0.9	12.7±3.0	10.2±1.4
45	B	1.3±0.2	21.9±2.5	14.5±0.9	2.6±0.7	12.8±2.1	9.1±1.7
46	B	1.3±0.1	22.8±1.8	14.3±0.7	3.0±1.0	12.7±2.0	9.1±1.4
48	D	2.2±0.3	24.8±1.7	15.7±1.2	6.0±2.5	17.1±3.2	13.5±2.7
49	D	1.8±0.3	30.0±2.1	17.6±0.9	5.1±2.4	16.5±4.1	12.2±3.0
50	D	1.5±0.5	21.6±1.8	12.5±1.1	5.6±1.9	18.2±3.2	12.0±1.8
51	D	2.2±0.2	22.9±2.2	14.9±1.4	5.8±1.7	19.2±3.4	12.9±1.4
52	D	1.7±0.3	25.5±2.1	15.9±1.1	4.7±1.7	17.0±3.7	12.2±2.1
53	D	1.4±0.2	23.3±3.0	13.6±1.2	4.7±1.7	15.3±2.3	11.4±1.6
54	D	1.9±0.2	22.2±4.0	14.3±1.7	4.5±0.6	16.9±1.2	11.6±0.9
55	D	1.3±0.1	21.6±1.6	14.1±0.5	4.7±1.3	17.5±2.9	12.4±1.5
59	A	1.5±0.2	23.2±1.8	15.1±1.3	3.0±1.0	12.4±1.9	10.2±1.5
60	A	1.6±0.6	21.5±2.0	14.5±1.0	3.3±0.7	14.0±1.2	9.2±0.7
61	A	2.2±0.5	22.4±2.1	13.9±0.7	3.4±1.1	14.2±2.2	8.1±1.5
62	A	1.6±0.8	16.4±1.7	8.0±1.6	3.6±1.2	14.6±3.0	10.3±1.5
63	I	2.6±0.5	25.4±2.6	15.2±1.2	4.6±1.1	15.9±1.8	11.6±1.6
64	I	1.7±0.3	23.5±1.8	15.1±1.0	5.4±1.5	18.5±2.6	13.6±1.7
65	I	4.6±0.7	27.4±1.6	18.3±1.2	4.7±2.1	15.7±3.6	12.3±2.1
66	J	1.6±0.3	23.7±5.4	13.4±0.7	5.1±2.1	16.9±3.8	12.6±2.9
67	J	1.2±0.2	20.2±1.3	14.2±1.1	6.0±1.4	18.9±3.0	13.7±2.0

Tree ID	Location	Weight (g)	Length (mm)	Width (mm)	Weight (g)	Length (cm)	Width (cm)
		Fruit			Leaves		
68	J	3.0±0.4	22.9±1.7	17.6±1.5	6.8±2.0	18.7±3.2	15.9±2.1
69	J	1.0±0.1	23.2±3.1	15.6±1.0	3.6±0.6	14.2±1.2	10.9±1.3
70	J	1.7±0.3	24.2±1.7	15.2±1.1	5.0±1.5	17.6±3.0	12.3±2.3
71	K	1.9±0.3	23.6±1.3	15.9±0.6	6.5±2.8	19.2±4.3	14.9±2.6
72	K	2.2±0.3	21.8±1.7	14.3±0.6	6.2±2.6	18.4±3.6	13.4±2.2
73	K	2.2±0.4	23.2±2.0	14.4±0.9	4.8±1.4	15.2±2.1	12.5±1.2

Measurements are based on average of 10 fruits and leaves (mature)

Table 4 Physical measurement of the fruits of Kakadu plum from Darwin and Milingimbi Northern Territory 2016 harvest

Tree ID	Weight (g)	Length (mm)	Width (mm)	Weight (g)	Length (cm)	Width (cm)
Fruit			Leaves			
Darwin						
74	3.5±0.3	21.8±1.2	16.7±1.1	4.0±1.0	13.6±1.6	10.9±1.1
75	2.5±0.4	24.5±1.9	15.2±1.5	4.7±1.5	15.0±2.5	11.6±1.3
76	2.2±0.3	23.4±1.4	14.2±0.9	6.2±1.8	18.5±2.3	12.6±1.4
77	3.5±0.6	24.3±2.0	17.3±1.7	3.7±0.6	12.4±1.0	10.6±1.2
78	3.7±0.6	30.3±2.2	18.6±1.7	4.3±1.7	14.6±3.3	10.9±1.6
79	3.1±0.4	27.2±1.3	16.3±1.0	4.1±1.7	14.2±3.0	11.1±2.9
80	1.7±0.4	22.6±1.3	13.3±0.8	4.6±1.2	16.2±2.1	11.1±1.0
81	2.9±0.5	25.4±2.2	15.8±1.3	7.4±2.3	18.5±3.7	13.9±2.4
Milingimbi						
82	3.3±0.3	23.7±1.8	17.2±0.5	5.5±1.2	16.4±1.4	12.2±1.4
83	3.2±0.9	25.6±2.5	17.4±1.4	5.5±1.1	15.7±2.0	14.9±1.6
84	3.9±0.9	25.2±3.2	15.9±1.7	4.8±1.3	15.8±3.0	11.8±1.4
85	4.9±0.9	29.0±2.5	18.5±1.3	8.0±1.7	18.2±1.6	14.3±1.9
86	5.2±1.2	29.0±2.6	19.1±1.8	6.4±1.6	18.3±2.5	13.7±1.6

Tree ID	Weight (g)	Length (mm)	Width (mm)	Weight (g)	Length (cm)	Width (cm)
	Fruit			Leaves		
87	3.1±0.8	24.1±2.8	15.9±1.7	6.6±1.6	17.1±2.6	14.3±1.2
88	3.9±0.7	27.8±2.3	17.3±1.3	5.9±0.7	17.2±2.0	12.6±1.5
90	2.1±0.4	28.8±2.7	14.3±1.1	3.6±1.0	14.4±2.0	10.2±1.3
91	1.7±0.5	23.7±1.6	16.8±1.5	4.2±1.4	15.4±3.0	11.9±1.4
92	2.5±0.3	26.0±1.3	13.7±0.8	5.5±1.3	17.2±2.7	13.1±1.3
93	1.9±0.4	21.6±2.5	13.8±0.9	3.4±0.8	13.8±1.8	10.1±2.3
94	2.3±0.4	22.3±2.7	14.3±1.3	4.3±1.1	14.9±2.3	12.1±1.4
95	2.2±0.2	21.3±3.4	13.9±0.7	2.8±1.6	11.6±4.4	9.2±2.6
97	2.8±0.8	23.6±2.9	13.9±1.6	6.4±1.6	17.5±2.5	12.5±1.1

Measurements are based on average of 10 fruits and leaves (mature)

Table 5 Physical measurement of the fruits of Kakadu plum from the Kimberley Western Australia 2015 harvest

Tree ID	Weight (g)	Length (mm)	Width (mm)
Jpp1	1.9±0.6	20.9±3.0	13.5±3.3
Jpp2	2.8±0.2	23.1±1.5	16.6±0.7
Jpp4	3.2±0.6	24.0±1.5	17.4±0.7
Jpp5	2.6±0.9	22.0±3.9	17.5±1.8
Jpp6	3.1±0.4	24.1±2.8	17.0±1.3
Jpp7	2.4±0.5	26.6±2.9	15.6±1.4
Jpp8	4.1±0.7	27.7±3.0	19.5±1.0
Jpp9	3.1±0.3	23.5±2.7	18.0±0.9
Jpp10	2.9±0.8	25.0±2.8	17.1±1.8
Jpp11	4.0±0.4	26.4±1.3	19.4±1.1
Jpp12	2.7±0.5	20.7±1.5	17.1±0.8
Jpp13	4.1±0.5	28.7±4.1	18.6±0.7
Jpp14	2.7±0.3	24.3±2.2	15.7±1.2
46WP	1.9±0.4	21.0±2.0	14.3±1.1
WP051	1.9±0.3	21.4±1.5	13.7±1.1
47	1.6±0.5	19.9±1.7	15.0±2.0
48	1.6±0.7	20.1±2.2	14.2±1.9
49a	1.9±0.3	19.7±0.8	14.4±0.9
EAC	3.8±0.8	29.5±4.9	18.5±1.9
W/P052(i)	1.5±0.4	16.4±1.2	13.1±1.5
W/P052(ii)	1.3±0.5	18.4±2.4	13.5±1.4
W/P053	1.9±0.4	23.6±3.3	14.2±1.1

Measurements are based on average of 10 fruits and leaves (mature)

Table 6 Proximate composition of Kakadu plum tissues (Dry weight basis)

Units (% w/w)	Kakadu plum tissues			
	Fruits	Leaves	Seedcoats	Kernels
Protein	4.7	7.1	2.3	32.0
Fat	0.9	6.5	0.6	35.1
Saturated Fat	0.3	2.5	0.1	5.8
Mono-unsaturated Fat	<0.1	0.4	0.2	9.8
Poly-unsaturated Fat	0.6	3.7	0.4	19.4
Trans Fat	<0.01	<0.01	<0.01	<0.01
Moisture (air)	6.0	3.0	2.2	4.0
Ash	5.5	5.9	0.6	4.5
Dietary Fibre (Total)	45.9	44.0	90.2	21.2
Dry Matter	94.0	97.0	97.8	96.0
Crude Fibre	13.5	14.6	62.8	11.6
Energy (KJ/100g)	1,110	1283	853	2065
Total Sugar (g/100g)	2.3	2.5	<0.10	0.49
Available Carbohydrate (%)	37.0	33.4	4.0	3.2
Sodium (Na) (mg/100g)	40	12	10	8.6
Vitamin C (mg/100g)	22,000			

Fruit, seed coat and kernels are from a composite sample of Kakadu plum fruit harvested in Wadeye, 2015 and processed into puree and freeze dried powder. A composite sample of leaves also harvested in Wadeye, 2015. Results reported on a dry weight basis.

According to Brand-Miller et al (1998) average composition of Kakadu plum samples tested for macronutrients for g/100g edible portion, converted to dry weight basis revealed a protein, fat, fibre and total carbohydrate percentage of 3.3, 2.1, 29.2 and 70.8 respectively . Except for protein the fat, fibre and total carbohydrates are higher in the Brand-Miller study (Table 6). It must be noted that the Kakadu plum tissue samples for this study were taken from Wadeye, 2015 harvest as a composite sample. The kernels are a good source of protein and fat, seed coat is high in dietary fibre and the fruit is high in AA.

Table 7 Mineral composition of Kakadu plum tissues

	Fruits (mg/100g)	Leaves (mg/100g)	Seedcoats (mg/100g)
Major Elements			
Ca	295.00 ± 0.62	1343.00 ± 13.66	131.10 ± 18.55
K	2717.79 ± 99.42	1179.10 ± 22.08	264.60 ± 0.39
Mg	203.77 ± 0.71	402.80 ± 2.16	24.30 ± 3.75
Na	212.35 ± 43.25	202.10 ± 79.83	95.70 ± 10.87
P	72.80 ± 1.50	63.60 ± 1.72	20.40 ± 3.89
Trace Elements			
Fe	1.70 ± 0.00	3.35 ± .07	3.90 ± 0.70
Zn	2.15 ± 1.63	1.95 ± 0.07	0.28 ± 0.04
Co	0.01 ± 0.00	0.09 ± 0.00	<0.01
Ni	0.49 ± 0.00	0.12 ± 0.00	0.04 ± 0.00
Cu	1.40 ± 0.00	0.64 ± 0.01	0.96 ± 0.06
Cr	0.07 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Mn	5.10 ± 0.00	25.50 ± 0.70	1.30 ± 0.00
Sr	4.00 ± 0.00	13.00 ± 0.00	1.40 ± 1.41
Mo	0.04 ± 0.00	0.01 ± 0.00	<0.01
Se	<0.01	<0.01	<0.01
B	1.95 ± 0.07	3.50 ± 0.00	0.42 ± 0.05
Ba	2.30 ± 0.00	2.80 ± 0.00	0.65 ± 0.00

Heavy metals

As	0.0005	0.0005	0.0005
Cd	0.0005	0.0005	0.0005
Hg	0.0005	0.0005	0.0005
Pb	0.0005	0.0005	0.042 ± 0.00

Data are presented as mean ± SD of duplicate determinations. Means are not significantly different.

Konczak et al (2009) reported the following values for Zn (0.57), Mg (203.8), Ca (282.5), Fe (3.99), Na (10.5), K (1905.5), Mn (3.5), Cu (0.303), Mo (0.019) on dry weight basis for freeze dried deseeded Kakadu plum fruit [52]. These mineral values are comparable to the Ca, Mg and K reported for Kakadu plum fruit in this study. The heavy metal concentrations of Kakadu plum tissues as given in Table 7 are 0.0005 mg/100g DW for all of the metals tested except the Pb content of the seedcoats which is 0.04 mg/100g. Wide variations exist in the mineral contents of all the Kakadu plum tissues tested this could be due geographical variation, mineral uptake efficiency in plants and soil conditions.

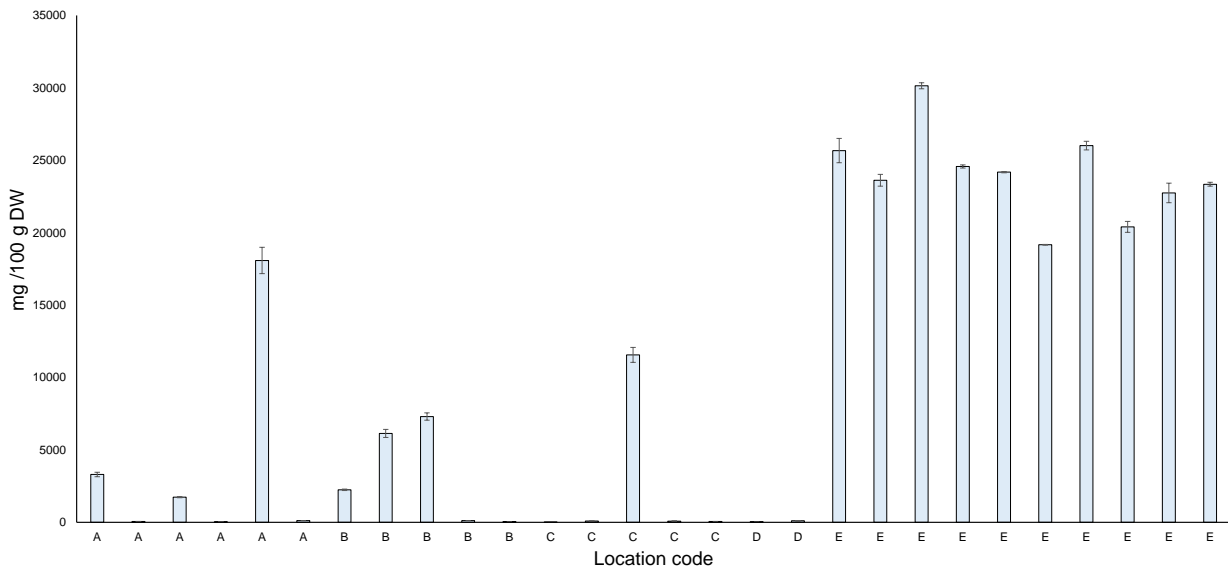


Figure 6 Levels of AA in Kakadu plum fruit samples from Wadeye, Northern Territory 2015

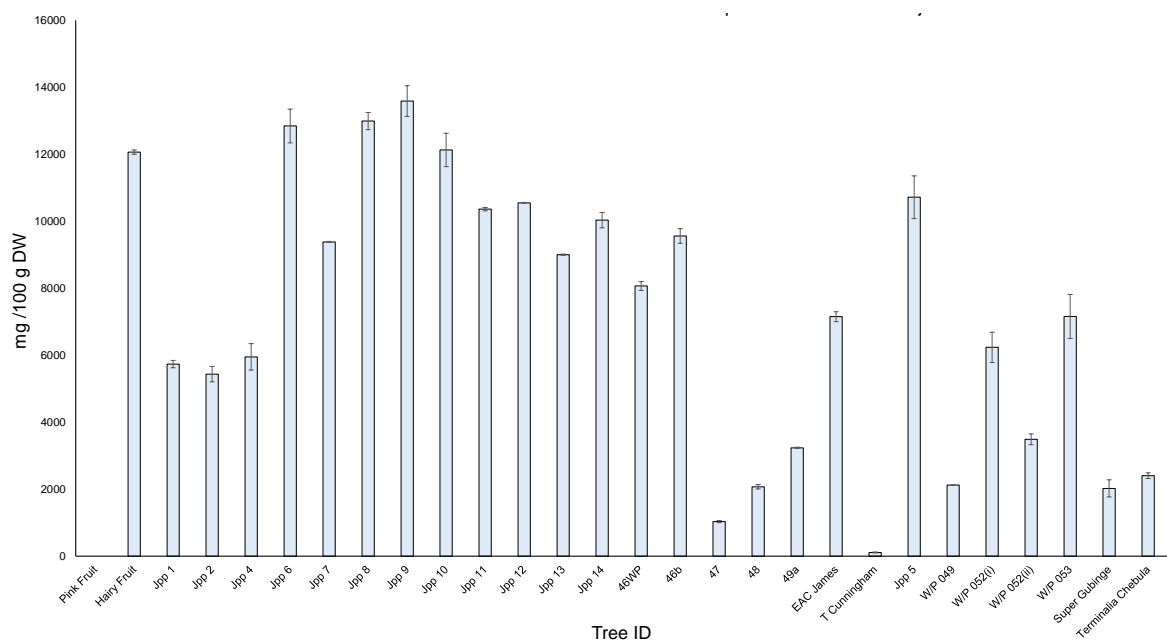


Figure 7 Levels of AA in Kakadu plum fruit samples from Kimberley, Western Australia 2015

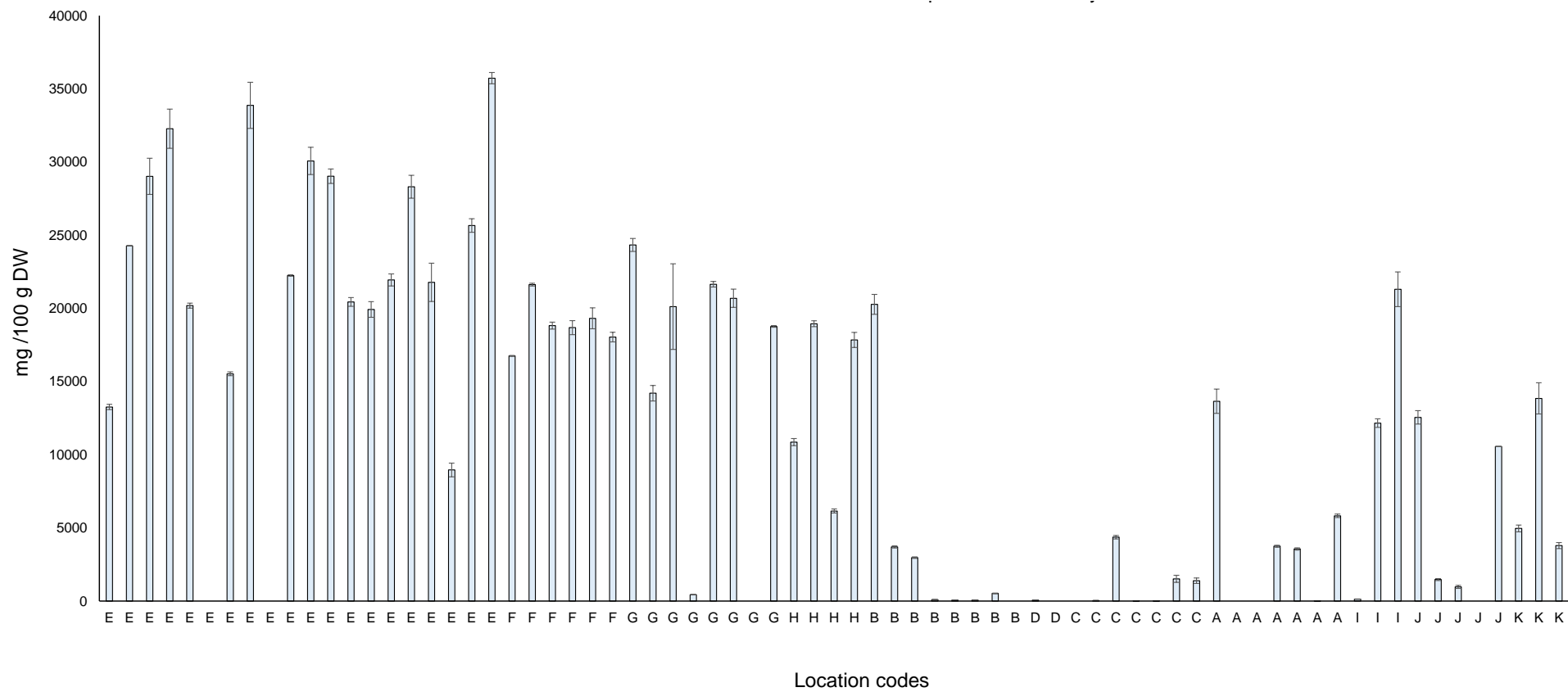


Figure 8 Levels of AA in Kakadu plum fruit samples from Wadeye, Northern Territory, 2016

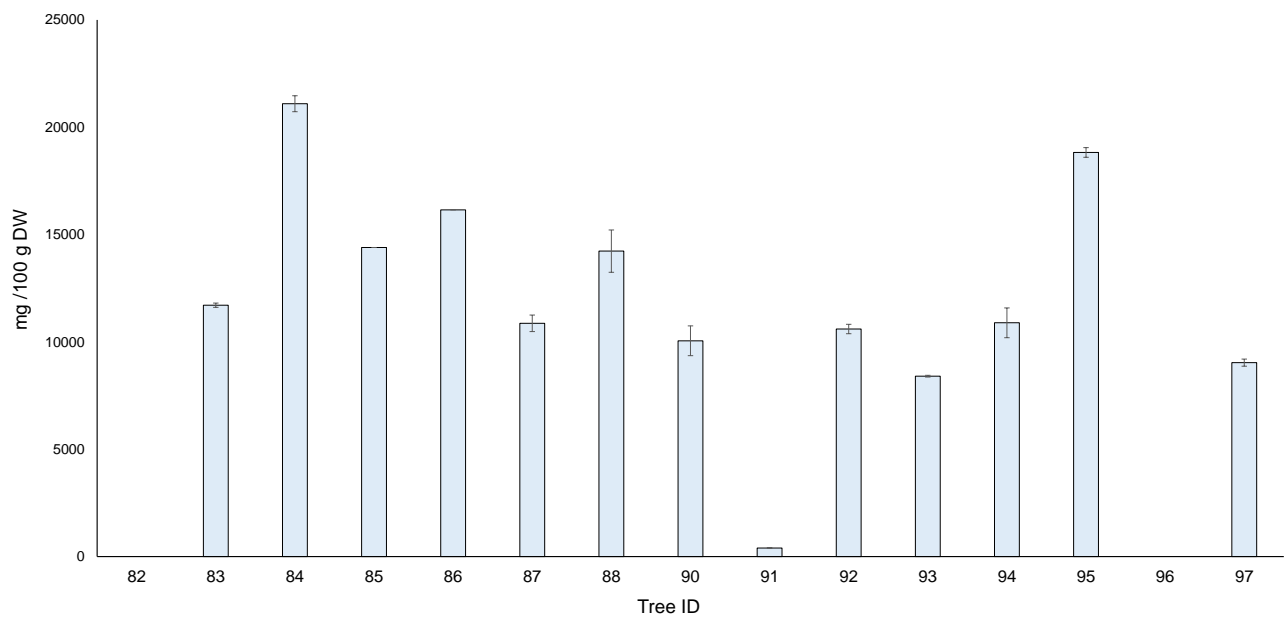


Figure 9 Levels of AA in Kakadu plum fruit samples from Milingimbi, Northern Territory, 2016

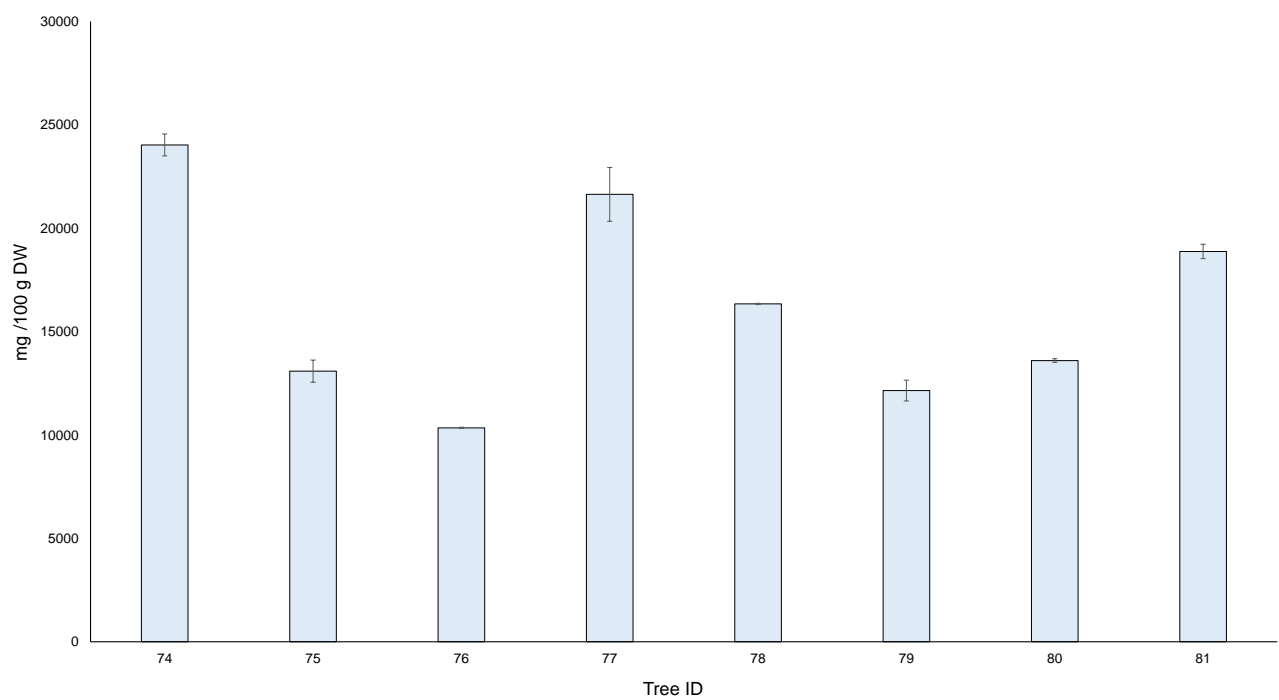


Figure 10 Levels of AA in Kakadu plum fruit samples from Darwin, Northern Territory, 2016

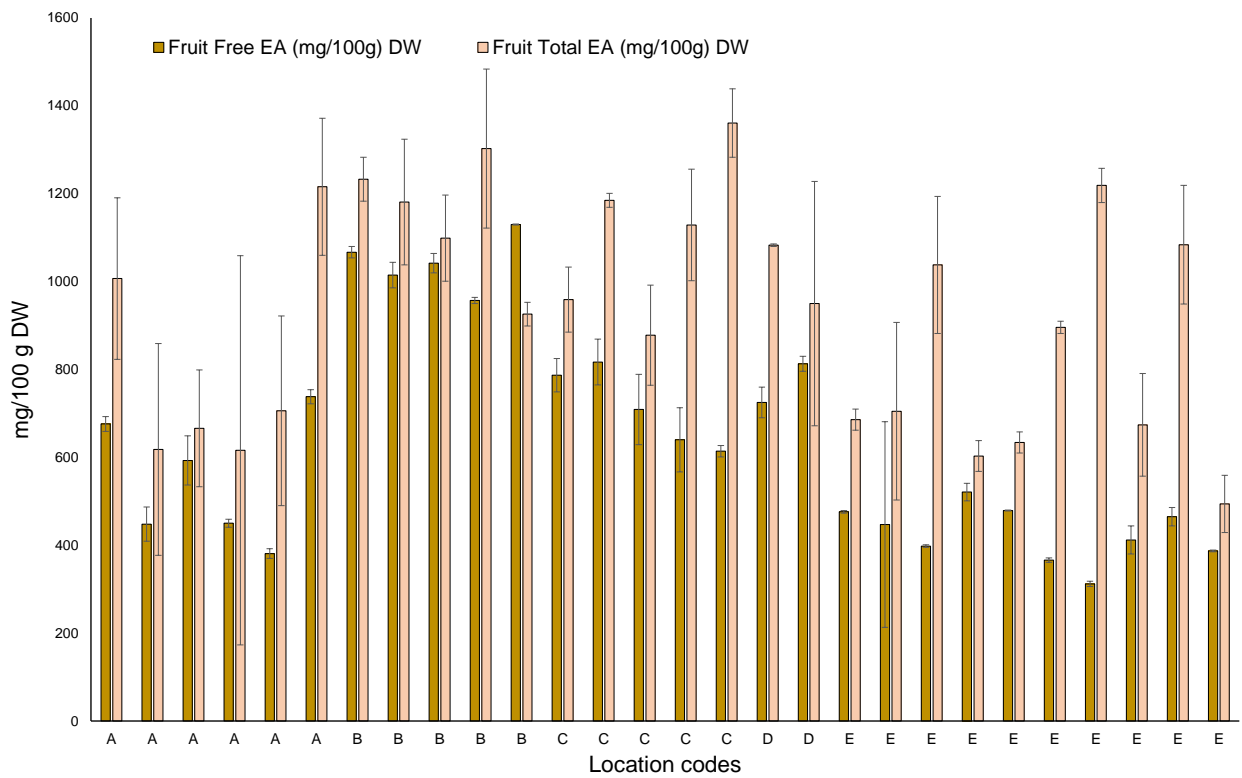


Figure 11 Levels of EA in Kakadu plum fruit samples from Wadeye Northern Territory, 2015

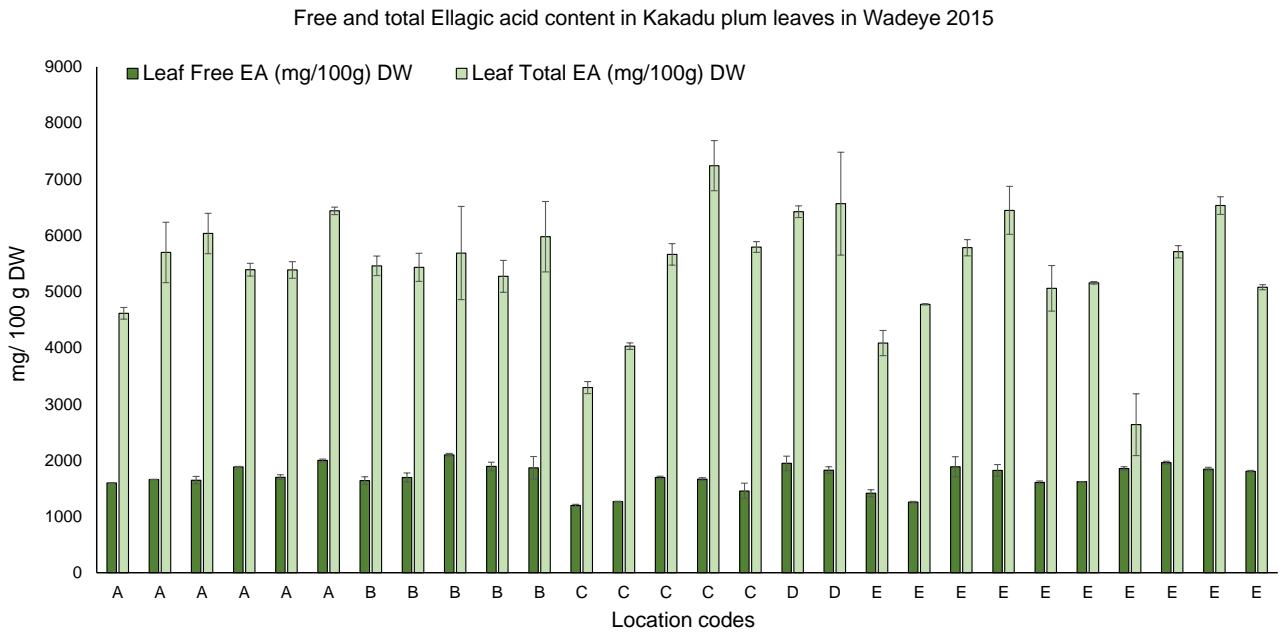


Figure 12 Levels of EA in Kakadu plum leaf samples from Northern Territory 2015

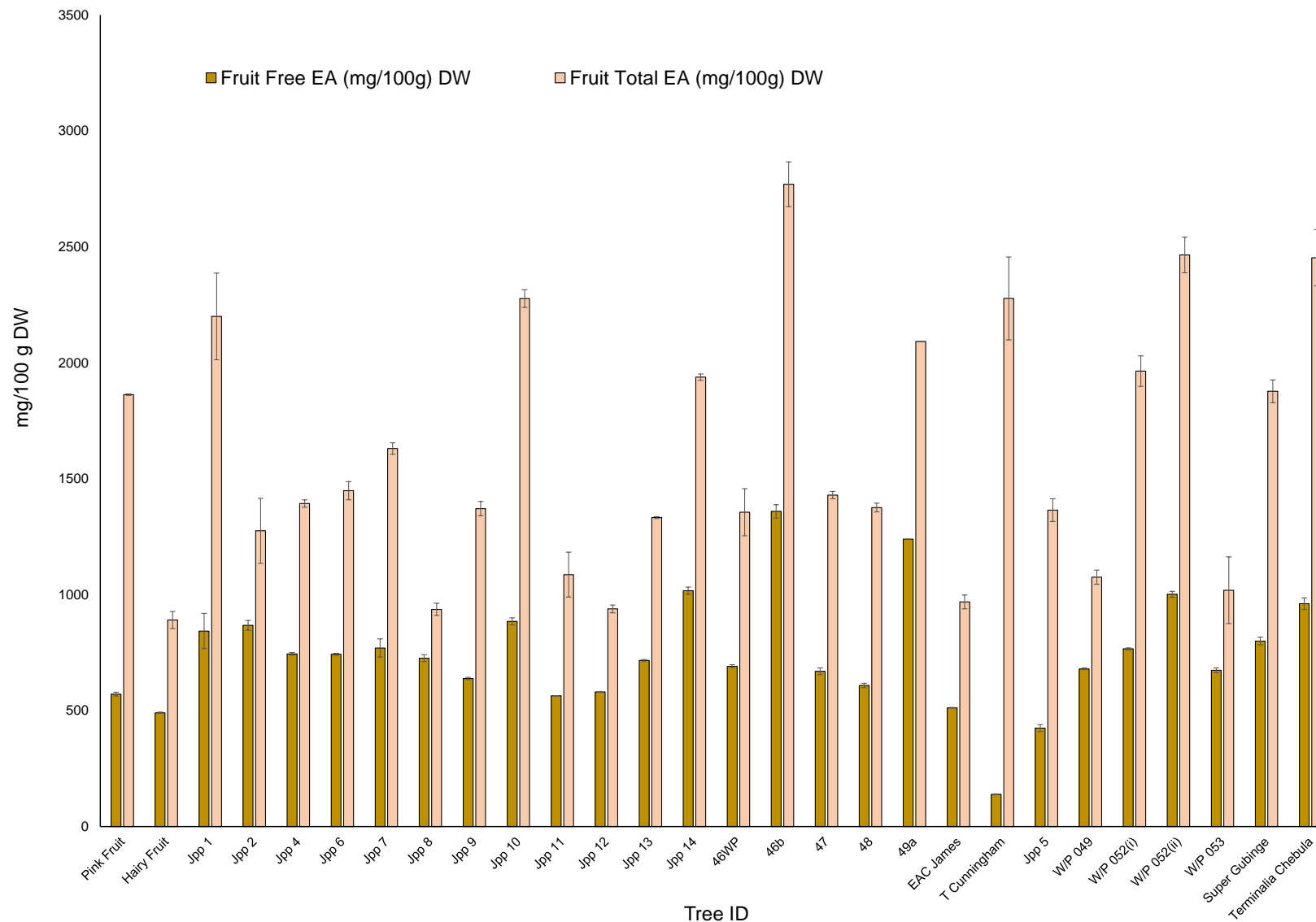


Figure 13 Levels of EA in Kakadu plum fruit samples from Kimberley, Western Australia 2015

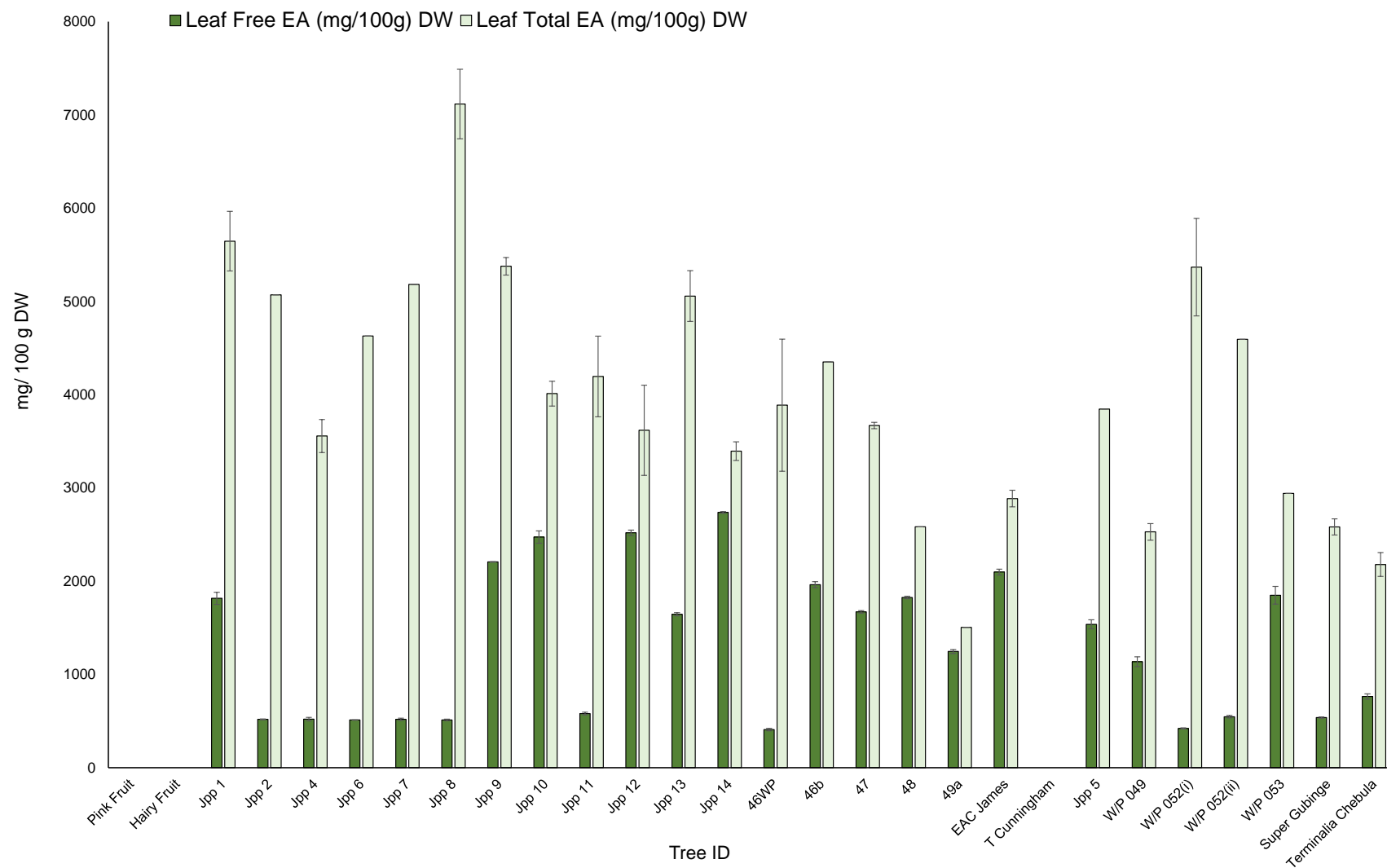


Figure 14 Levels of EA in Kakadu plum leaf samples from Kimberley, Western Australia 2015

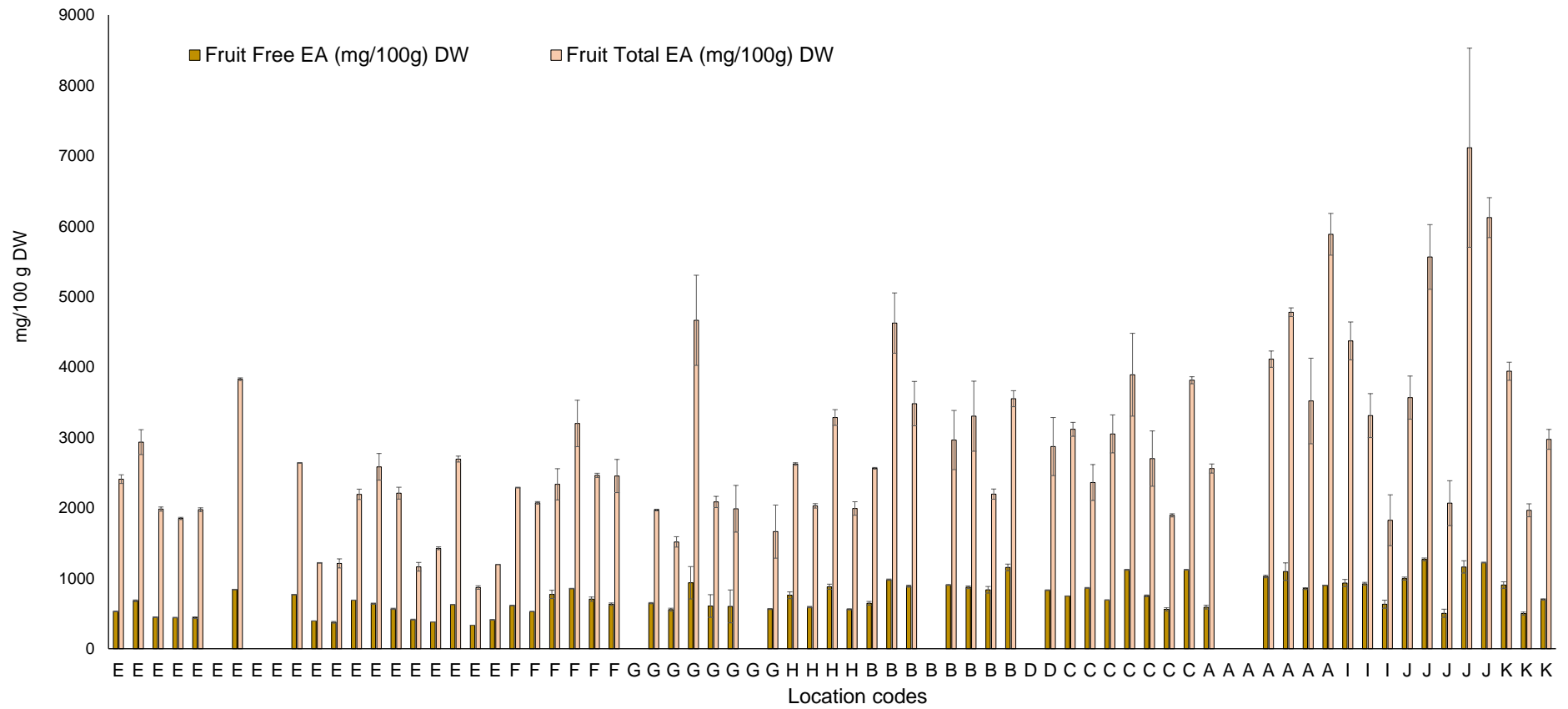


Figure 15 Levels of EA in Kakadu plum fruit samples from Wadeye Northern Territory, 2016

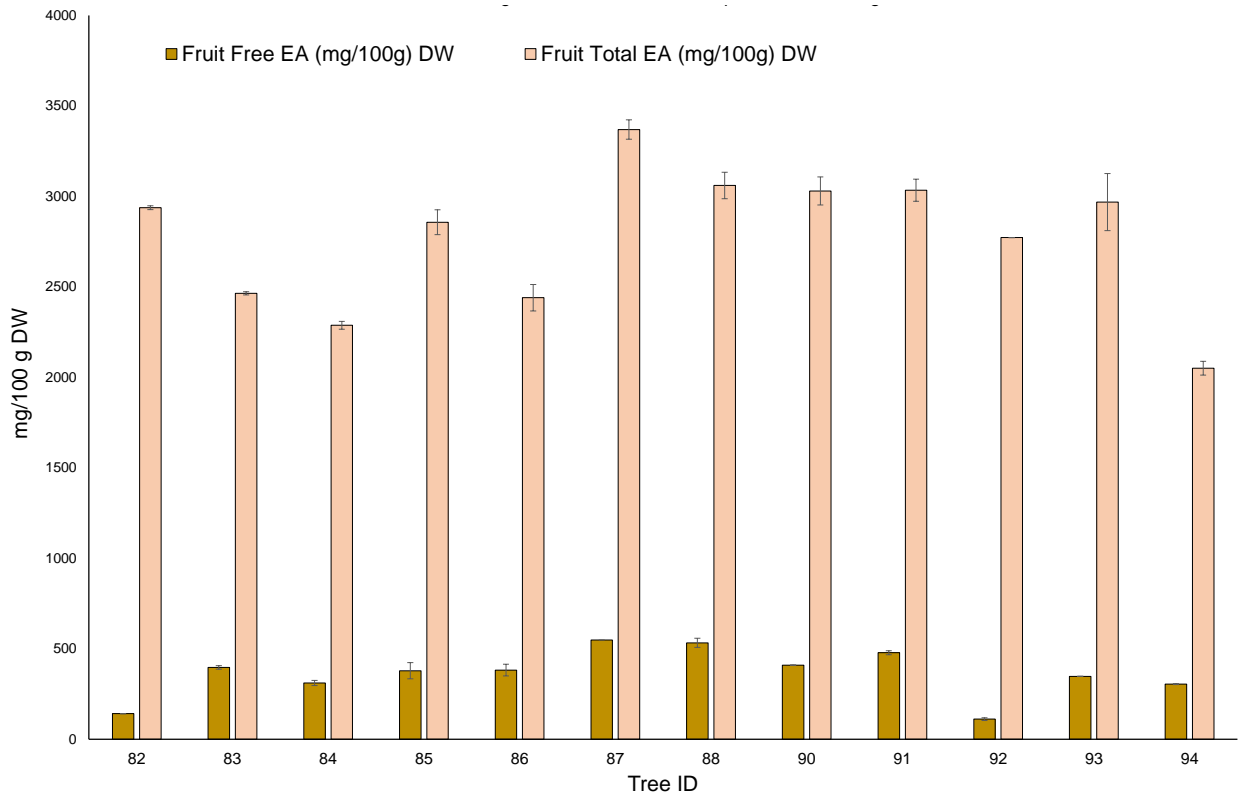


Figure 16 Levels of EA in Kakadu plum fruit samples from Milingimbi, Northern Territory, 2016

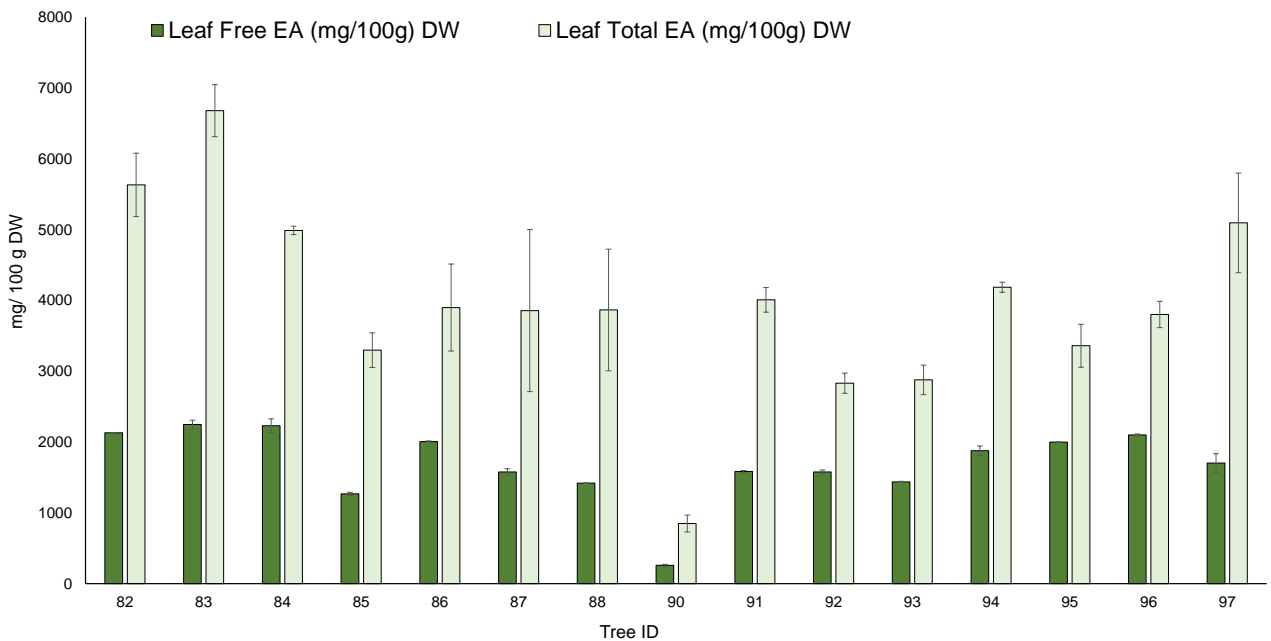


Figure 17 Levels of EA in Kakadu plum leaf samples from Milingimbi, Northern Territory, 2016

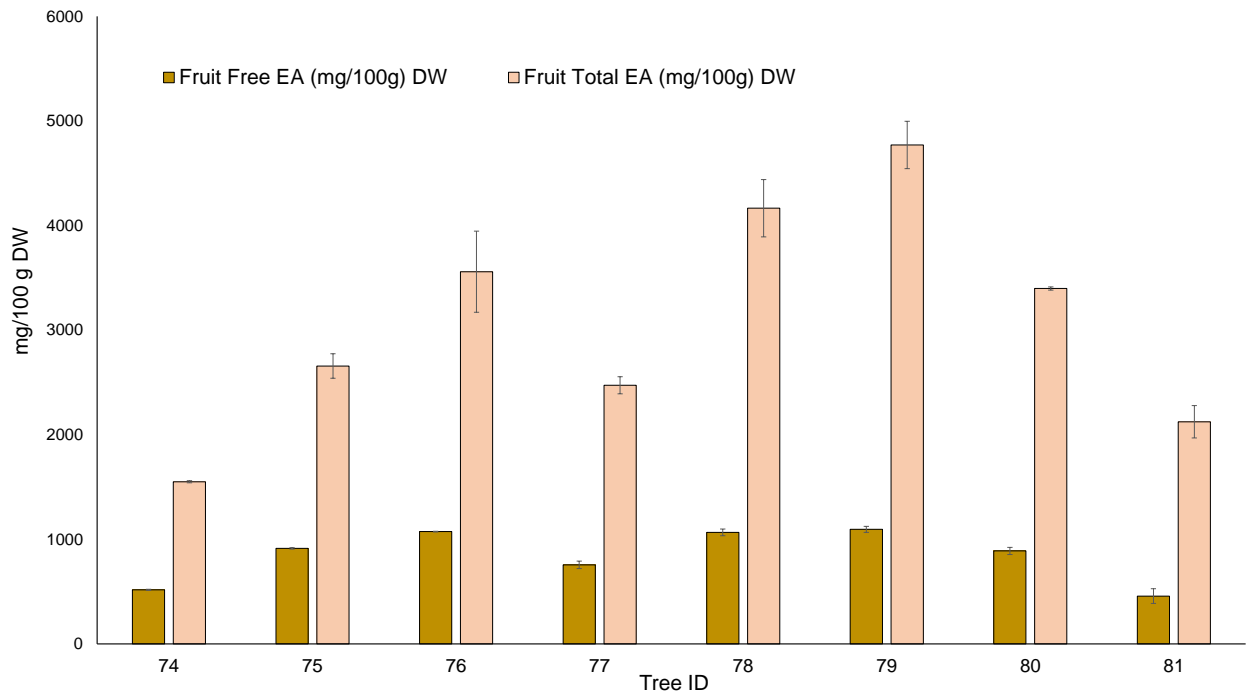


Figure 18 Levels of EA in Kakadu plum fruit samples from Darwin, Northern Territory, 2016

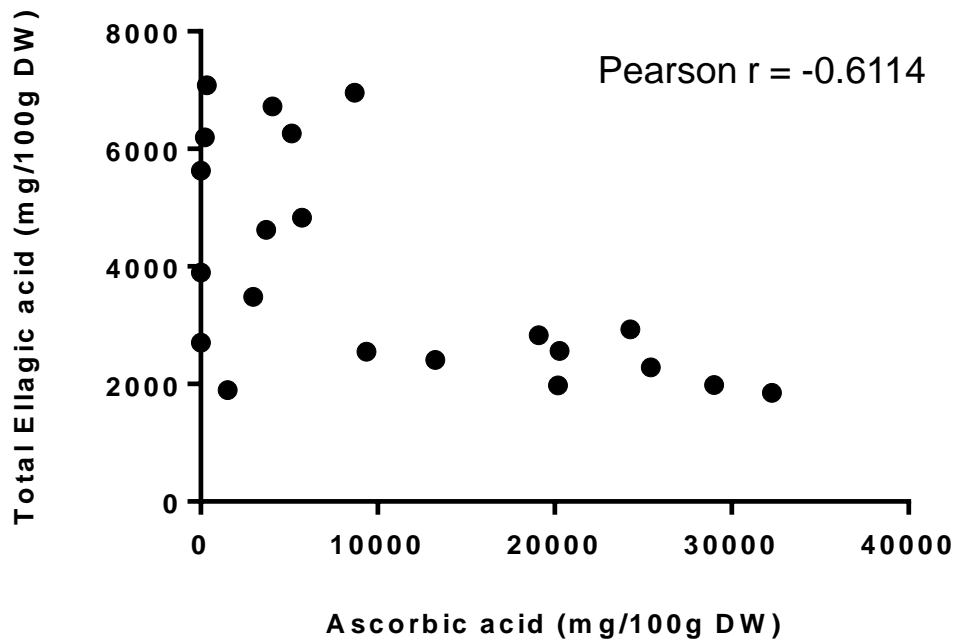


Figure 19 Correlation between Total EA and AA over two harvesting seasons 2016 and 2017 in Wadeye, Northern Territory.

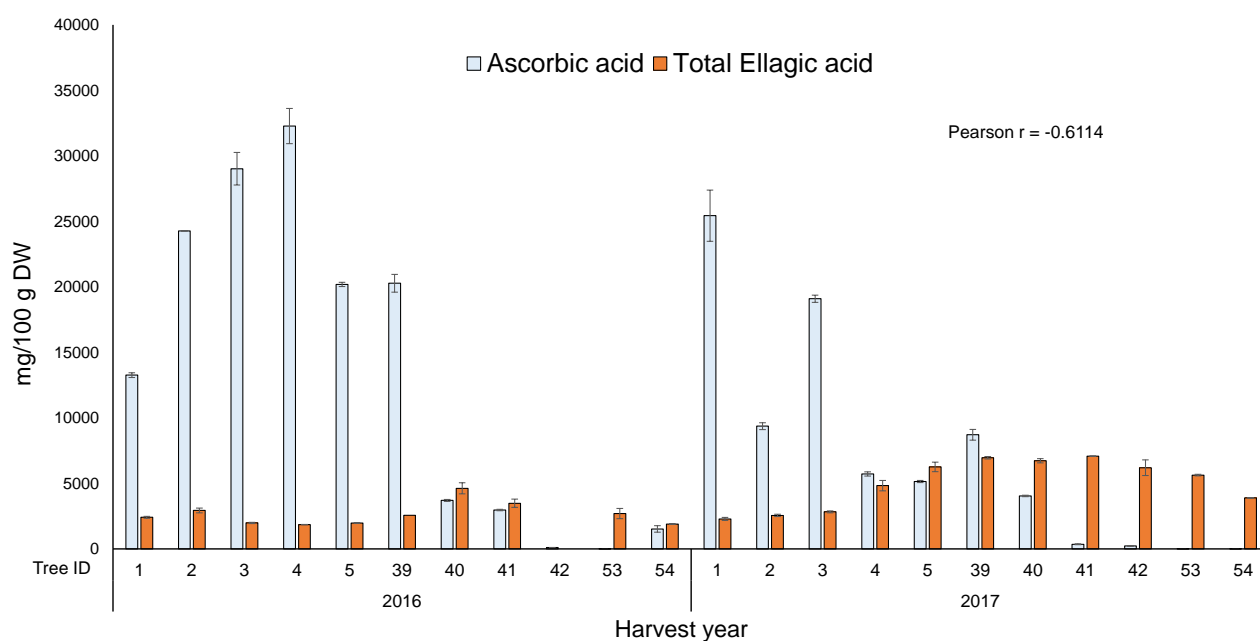


Figure 20 Changes in Total EA and Ascorbic acid over two harvesting seasons 2016 and 2017 in Wadeye, Northern Territory.

As reported by Konczak et al (2014) there is significant tree to tree variability in the EA and AA content in the Kakadu plum fruits from different geographical locations and within each location [21]. However, when composite samples were tested the vitamin C was within 10,000 – 25,000 mg/100g DW [Figure 6, Figure 7, Figure 8, Figure 9 and Figure 10] and the total EA within 1000 – 6000 mg/100g DW [Figure 11, Figure 13, Figure 15, Figure 16 and Figure 18].

The leaves had a higher EA content than the Kakadu plum fruit [Figure 12,

Figure 14 and Figure 17] and this is in agreement to Williams et al (2016), who reported the leaves being a better source of EA, particularly the complex ellagitannins which may possess greater antioxidant than the free form [53].

Within all these variation there was a negative Pearson r (-0.6114 $p < 0.05$) correlation between the AA and EA indicating there is a converse relationship [Figure 19]. For example in 2016 harvest at Wadeye, the AA levels were high and the EA levels were low and the opposite effect was observed in 2017.

A study into *Terminalia* fruit phenolics (not including *T. ferdinandiana*) confirmed that the antioxidant capacity of the identified EA and the polymeric ellagitannins (ETs) depended in part, on the number of hydroxyl groups per molecule [38]. ETs (several containing up to 16 hydroxyl groups per molecule) possessed the highest activities as judged by the ORAC (Oxygen Radical Absorbance Capacity) and FRAP (Ferric Reducing Ability of Plasma) assays but interestingly, EA itself was ranked highly on a molar basis by the DPPH assay. The authors speculated this was due to the comparatively higher density of hydroxyls within the smaller EA molecule. Studies that have measured the different forms in *Rubus* berries (such as blueberries and blackberries) [36] and strawberries [54] have indicated that the content was predominantly in the polymeric ET form. Knowledge of the relative proportions of EA forms will also be important in assessing the effect of processing and storage on these antioxidant compounds.

1.4 Conclusion and recommendations

The rich nutritional and phytochemical profiles reported for Kakadu plum demonstrates the great potential of this traditional Australian fruit as a functional food and ingredient. The significantly high antioxidant capacity due to its much elevated ascorbic acid and polyphenolic content clearly indicates its application in food systems as a natural alternative to enhance quality. If the unique combination of these polyphenolic compounds and ascorbic acid can be maintained successfully on processing new and novel opportunities in developing even more effective antioxidant formulations may be realised.

This study confirms the potential of using Kakadu plum fruit as a functional ingredient with high antioxidant capacity and other bioactivities due to the presence of AA and EA in significant quantities.

The variation observed in the wild harvested Kakadu plum from different geographical locations warrants further study to understand the reason for this diversity. It is important to assess if this variation in AA and phytochemicals such as EA is due to genetics or the environment. Further study is required to understand other bioactive compounds that could be present in Kakadu plum that is of commercial value.

Chapter 2 Kakadu plum (*Terminalia ferdinandiana*) – Fruit maturity, ripening and quality relationships

2.1 Introduction

Quality, the degree of excellence of fresh fruits and their products is a combination of attributes and properties that give each commodity importance in reference to human food. The relative value of each quality component depends on the commodity and its intended use, whether fresh or processed and this will vary among different players in the supply chain such as producers, handlers and consumers [55]. Quality attributes of fresh fruits and vegetables include appearance, texture, flavour, nutritional and safety factors.

Maturity at harvest is a key factor that determines the storage life and final fruit quality. Fruits harvested when it is immature will be of poor quality and lead to uneven ripening. When fruits are harvested when it is over mature due to delayed harvesting, they can be susceptible to decay and results in postharvest losses. Therefore, getting the right maturity stage is important to retain the quality of the fruit and shelf life [56, 57].

There are different methods of determining maturity indices in fruits and vegetables. Most of the indices differ according to the fruit type. An ideal maturity index can be measured non-destructively, is different at distinctive stages of maturity and does not change with time of storage. However, an ideal non-destructive maturity index is difficult to find for most fruit types.

The common methods of determining the maturity of a fruit according to Prasad et al., (2018) [58] are:

Visual methods: depends on the outward appearance of the fruits and would include skin colour, size (mass and volume), fullness of fruit and presence of dry mature leaves.

Physical methods: firmness, specific gravity, ease of separation of abscission.

Chemical methods: total soluble solids, titratable acidity and starch content.

Computational methods: days from flower bloom or bud initiation stage until optimum fruit development.

Physiological methods: respiration and aroma development.

Structure and shape are used to determine maturity indices for fruits, for example the finger angle shape can be used for bananas and in general the maturing fruit becomes less angular and more rounded [59].

Colour is one of the most important characteristics of horticultural produce that is used in agriculture for estimating the maturity of fruits. Colour is closely related to chemical and physical properties for horticultural produce. In fruits, the phenolic state estimation has been studied extensively and can be related to certain stages of maturity and to the antioxidants and other health benefits [60, 61].

Determining visual measurements for different stages of maturity of Kakadu plum is a challenge. From the reported literature, colour was a popular tool to measure maturity in different horticultural produce. However, from the variation in colour of Kakadu plum fruit

refer to Figure 21, it was clear colour measurements would not be a suitable method to assess fruit maturity. The variation in colour could be due to cross breeding between the different *Terminalia* spp. in the wild.

The aim of this study was to assess the most suitable method for determining the different maturity stages for Kakadu plum. The ideal method would be a non-destructive visual method which could be correlated to some of the destructive methods such as chemical assessments.



Figure 21 Colour variation in wild harvested Kakadu plum

2.2 Material and Methods

2.2.1 Collection of Kakadu plum fruit for maturity indices data

Kakadu plum fruit samples to assess the changes in the key chemical markers EA and AA were collected during the harvesting season April 2016 and 2017 for Kakadu plum in Wadeye, Northern Territory. In 2016 and 2017 samples were collected from 3 individual trees, each representing fruits that were at different stages of maturity within the same tree. The fruits were harvested, cooled and immediately frozen and transported to the Health and Food Sciences Precinct laboratories, at Coopers Plains, Queensland for physico-chemical assessments.

2.2.2 Physical measurements of Kakadu plum fruit

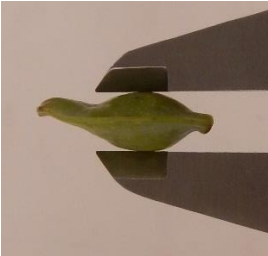
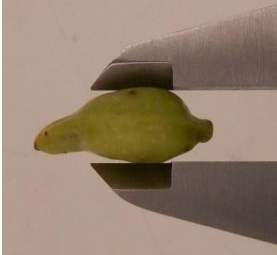

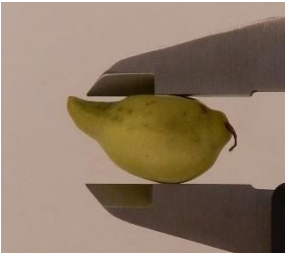
Whole fruit samples from 2016 and 2017 season were weighed on laboratory scales (Sartorius CP224S, Gottingen, Germany) and length, width and fullness was measured using a 150 mm Digital Caliper (Craftright Engineering Works, Jiangsu, China). The samples from different stages of maturity (Table 8) were freeze dried, separated from the seeds, milled and kept at -80°C till further analysis.

2.2.3 Assessing the ellagic acid (EA) and ascorbic acid (AA) content

Refer to Chapter on Physicochemical and nutritional properties of Kakadu plum [page no 22] for in house methods used to determine the EA and AA content of the fruit.

AA analysis were also done at a NATA accredited lab, Symbio Laboratories, Eight Mile Plains, Brisbane, Queensland, accredited for compliance with ISO/IEC 17025 – Testing. AA was analysed by HPLC according to method (CFH061), it is a NATA accredited method, which is developed in house and not according to AOAC. Quality Control of the test follow NATA requirement. QC and duplicate assays performed at the minimum rate of one per batch or 5% whichever the greater.

Table 8 Maturity indices based on fullness of the fruit at different stages of maturity.

Shape of fruit	Percentage fullness (%)	*Maturity Stage from 1-4
	0-25	1
	25-50	2
	50-75	3
	100	4

*Maturity stage from 1-4 would indicate a range from immature to mature.

2.3 Results and Discussion

Table 9 Physico-chemical and nutritional properties of Kakadu plum at different stages of maturity in Wadeye

Tree ***ID	Maturity Stage	Number of fruits	Weight (g)	Length (mm)	Width (mm)	Fullness (mm)	*AA (mg/100g DW)	*Total EA (mg/100g DW)
2017 Harvest								
4	1	9	2.13±0.34	28.46±2.01	18.55±0.92	10.93±0.52	**6017	6262±361
4	2	11	3.09±0.49	26.80±2.02	18.20±0.86	13.77±0.98	**15416	4005±203
4	3	9	4.13±0.57	26.58±1.30	19.18±1.25	16.91±1.19	**20534	2781±272
4	4	12	4.50±0.81	25.33±2.20	19.11±1.44	17.94±1.22	**24819	2469±200
10	2	4	1.83±0.22	28.09±2.07	14.06±0.52	10.85±0.45	**370	6195±602
10	3	27	2.34±0.33	27.63±1.61	14.35±0.70	13.09±0.90	**10267	2699±63
10	4	7	3.38±0.60	27.87±2.26	15.95±0.78	15.58±1.31	**14242	2452±67
13	1	10	1.39±0.18	23.88±2.74	16.50±1.94	8.86±0.49	**7121	4563±208

Tree ***ID	Maturity Stage	Number of fruits	Weight (g)	Length (mm)	Width (mm)	Fullness (mm)	*AA (mg/100g DW)	*Total EA (mg/100g DW)
13	3	23	1.64±0.19	20.91±1.37	13.84±0.68	11.46±1.02	**16377	2498±62
13	4	15	1.63±0.27	18.09±1.46	12.90±0.98	13.10±0.98	**21583	2294±27
2016 Harvest								
15	2	5	1.6±0.29	23.73±1.41	16.21±1.16	9.90±0.53	*17297±39 9	3141±83
15	3	18	2.01±0.32	23.80±1.49	16.12±1.34	11.60±0.78	*21380±37 7	2343±33
15	4	23	2.02±0.42	23.33±2.47	15.56±1.57	12.01±1.08	*21946±41 1	2209±87
23	1	3	1.82±0.42	35.27±6.33	20.05±2.31	10.10±0.54	*1154±1	4212±138
23	2	2	4.02±0.57	38.35±2.98	19.21±0.97	13.95±0.69	*14516±28 9	2114±108
23	3	27	5.23±0.72	38.90±2.67	20.94±1.32	16.98±1.07	*18829±23 7	1794±29
23	4	11	5.30±0.88	36.36±2.15	20.90±1.59	17.78±1.24	*22895±39 9	2335±222
34	2	18	1.32±0.29	20.34±2.01	14.78±2.27	10.82±0.80	*5352±123	3449±83

Tree ***ID	Maturity Stage	Number of fruits	Weight (g)	Length (mm)	Width (mm)	Fullness (mm)	*AA (mg/100g DW)	*Total EA (mg/100g DW)
34	3	6	1.93±0.51	20.89±1.06	14.26±1.72	13.84±1.69	*12318±55 9	2349±338
34	4	46	2.38±0.44	20.12±1.17	15.23±1.10	15.64±1.38	*18767±53	1664±377

*The coefficient of variation (cv) of the HPLC analysis of EA and AA in all the samples were less than 10%

** Vitamin C analysis for samples 4, 10 and 13 at different stages of maturity was done at Symbio laboratories, quality Control and duplicate assays performed at the minimum rate of one per batch or 5% whichever the greater.

*** Tree ID is the accession number,

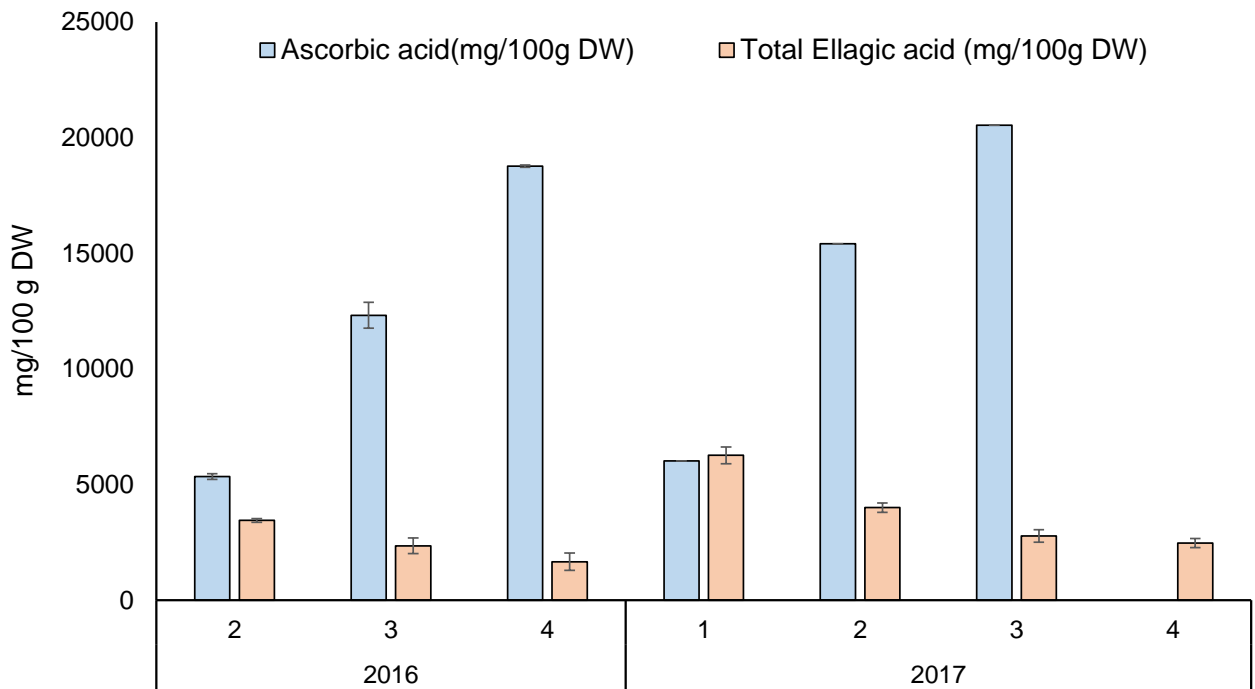


Figure 22 Changes in Total EA and AA at different stages of maturity over two harvesting seasons for Kakadu plum



Figure 23 Maturity Indices Chart for Kakadu plum from Northern Territory at four stages of ripening

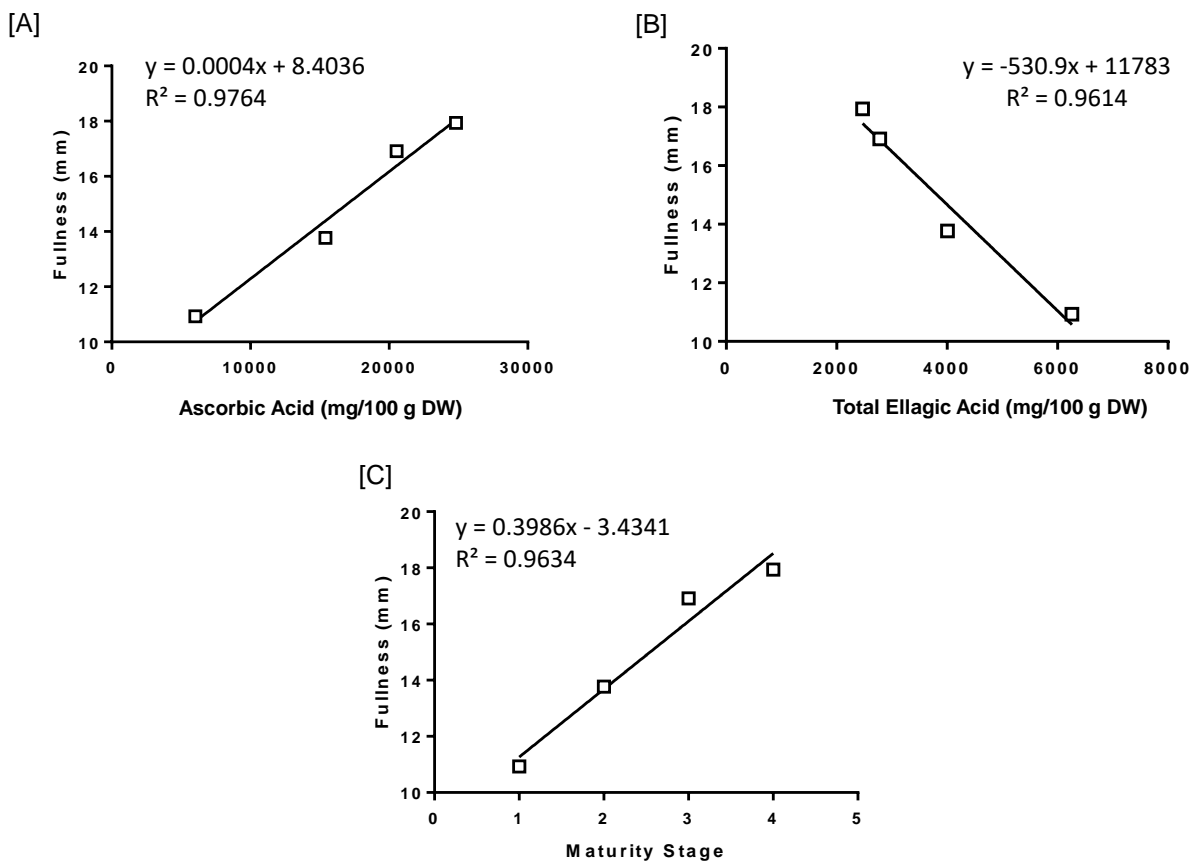


Figure 24 Correlating visual (non-destructive) and chemical (destructive) results

Due to the variation in colour of the Kakadu plum fruit it was decided to develop maturity indices charts based on fullness of the fruit. The maturity stages of Kakadu plum was established as percentage fullness ranging from 0-100% relating to stage 1 (0-25%), stage 2 (25-50%), stage 3 (50-75%) and stage 4 (100%).

Stage 1 had the highest total EA content followed by stages 2, 3 and 4 (Figure 22 and Figure 23). Conversely, stage 4 had the highest AA content followed by 3, 2 and 1 (Figure 22 and Figure 23). As the maturity stage and ripening increased, the total EA content decreased and the total AA content increased (Figure 23).

It is clear from the results there is a strong correlation ($R^2 > 0.95$) between the non-destructive visual measurement of fullness and destructive chemical assessments of total EA and total AA (Figure 24).

Changes in phenolic acids during the ripening stage has been reported by Wang et al., (2016) in the Jujube fruit from *Ziziphus jujuba* growing in Asia, especially China. EA and caffeic acid content of the Jujube fruit decreased with ripening and maturity stage [62]. Pomegranate juice at three different stages of maturity (unripe, mid-ripe and full-ripe) indicated a decline in total phenolics and total tannins as maturity advanced while the total anthocyanin, total flavonoid and AA increased [63].

Cranberry (*Vaccinium macrocarpon*) known for its health attributes (maintenance of urinary tract health and antioxidants status) were assessed for changes in bioactive compounds during different stages of maturity. Fruits were investigated at light green, blush, light red and dark red maturation stages. A converse relationship was found between total phenolics and

anthocyanins as fruits mature. Total phenolic content declined while the anthocyanin content increased from green to dark red stage [64].

With all these studies it is clear that bioactive compounds present in any horticultural product may vary based on genetic and environmental factors. A study on pepper grown under greenhouse conditions assessed the impacts of cultivar, fruit maturity stage (mature versus immature), and growing season on the concentration of bioactive compounds. This study revealed that the mature peppers generally had the highest AA and capsaicinoids compared to the immature peppers. Highly significant interactions among cultivar, growing season and maturity stage were observed for the key bioactive compounds including AA, capsaicinoids, flavonoids and total phenolics. This indicates that the key bioactive compounds contributing to health value of fruits and vegetables is a function of cultivar, maturity stage and production season [65].

2.4 Conclusion and future recommendations

This is the first study reporting on the different stages of maturity classified according to fullness of Kakadu plum fruit and changes in bioactive compounds during ripening. The data obtained from this study suggests there is a strong correlation between the visual fullness measurements and the total EA and AA content at the four stages of maturity established for Kakadu plum. The total EA content decreased with increased maturity and the total AA content increased with maturity. These findings have provided novel information on how best to select the appropriate maturity stage of Kakadu plum fruits that contains the highest amount of EA and AA giving the best health and functional properties. This gives an opportunity to target different markets based on requirements. For markets looking for potent sources of natural AA as health supplements or in the food industry as functional ingredients, harvesting at stage 4 of maturity would be recommended. Those markets looking for high EA, then a less mature stage would be more suited.

Future studies should include developing objective and non-destructive methods (visible and near infrared; Vis/NIR spectroscopy) for determining quality attributes especially the chemical markers related to nutritional and health value. Studies should also be done to determine other bioactive compounds and their changes during the different maturity stages. Ethylene production during the different stages of maturity should be assessed to determine if Kakadu plum is a climacteric or non-climacteric fruit.

Chapter 3 Harvesting, processing and storage of Kakadu plum

3.1 Background

The University of Queensland in collaboration with Department of Agriculture and Fisheries (DAF), Queensland were funded by AgriFutures Australia (formerly known as RIRDC) to work on a project titled "Changes in quality and bioactivity of native foods during storage". Kakadu plum was one of the native fruits assessed in this project. This study confirmed the high level of vitamin C and phytochemicals present in Kakadu plum and its stability during storage. The unique properties of Kakadu plum has enabled the commercial use of this native plum as a functional food ingredient. It has been successfully used in extending the shelf life and improving the quality of chilled and frozen seafood (prawns). This has opened new markets for Kakadu plum at the same time it has also put pressure on the producers of Kakadu plum products to ensure consistent quality and bioactivity and availability for use in commercial applications. Coradji (Pvt) Ltd one of the largest producers of Kakadu plum processed products such as purees and freeze dried powder have sold their business to the Palngun Wurnangat Aboriginal Corporation PWAC (Women's Centre), Wadeye, Tharrurr Region in the Northern Territory. This association is an independently owned indigenous women's organisation and the main shared language spoken is Murrinhpatha. Palngun Wurnangat means "Strong Women Together" PWAC was established in 1990 to provide support to families by delivering social and economic activities which involve and benefit the people of the region. Access to real jobs and continuing cultural heritage are prime goals. This Women's Association runs a cluster of small connected micro-businesses, and are familiar with the procedures involved in running a business.

This study will include the assessing of current production practices of Kakadu plum that are wild harvested in the Tharrurr Region, Wadeye community and training of PWAC women and Tharrurr rangers in Wadeye on good production and quality control practices. Development of product standards will provide producers and buyers with agreed reference point in terms of quality, bioactivity and food safety. This would be an important component in developing new markets for Kakadu plum. Implementation of good production and quality control practices at the processing unit in Wadeye will also give confidence to buyers as they are assured of a quality and safe product. An anticipated outcome is the adoption of this harvesting and processing model for Kakadu plum by other Indigenous communities in Northern Australia.

3.2 Materials and Methods

3.2.1 Harvesting and Processing of Kakadu plum in Wadeye

A group of researchers from the University of Queensland, DAF, Queensland, Charles Darwin University and EcOz Environmental Services visited Wadeye from 2014- 2017 details given in Table 10.

Table 10 Summary of training for Kakadu plum harvesting and processing

Location	Date	Harvesting & processing and training of Indigenous community members
Wadeye (Northern Territory)	18-26 April 2014	Setting of puree processing equipment at Wadeye and harvesting and freezing of Kakadu plum fruit. Training of PWAC members and Thamarrurr Rangers
Wadeye (Northern Territory)	3-11 April 2015	Training in harvesting, processing and storage with the new blast freezer & packaging equipment
Wadeye (Northern Territory)	18-25 April 2016	Monitoring of training in harvesting, processing and storage
Milingimbi (Northern Territory)	27-29 April 2016	Training in harvesting, processing and storage of Crocodile Islands Rangers
Kimberley (Western Australia)	19 – 21 July, 2016	Training in harvesting, processing and storage of Mamabulanjin Aboriginal Corporation
Wadeye (Northern Territory)	16-22 April 2017	Training in Kakadu plum value addition and diet diversification for PWAC members and Thamarrurr Rangers

3.2.2 Commercial harvesting of Kakadu plum fruits

The PWAC members are responsible for getting the necessary permission from Traditional Owners (TO) for harvesting Kakadu plum on their lands. They also require a commercial permit from Northern Land Council (NLC).

3.2.3 Training and Registration for the harvesting of Kakadu plum (Mi Marrarl) in Wadeye

This training was established in the Wadeye community by the PWAC in partnership with Rose Read. The training material has been provided in Appendix 1 (page no 126).

Training and Protocols developed for the harvesting and processing of Kakadu plum in frozen whole fruit and puree partnership with the University of Queensland and DAF, Queensland.

Training of PWAC member and Thamarrurr rangers in operating the blast freezer and packaging equipment.

Thamarrurr rangers trained in collection of Kakadu plum tissue samples for research purposes and GPS tracking of tree locations.

3.3 Results and Discussion

3.3.1 Product specification sheets

Specification for Kakadu plum powder

Botanical name: *Terminalia ferdinandiana*

Source: Wild harvested by indigenous communities in the Northern Territory. As fruits are wild harvested chemical makers are given as a broad range.

Plant tissue used: Fruit flesh

Type of preparation: Dry powder

Characteristics:

General appearance: pale greenish to yellow/amber powder

Moisture content: Less than 8% w/w

Total ash: Less than 6% w/w

Ascorbic acid content: 10,000 – 25,000 mg/100g

Total Ellagic acid content: 1000 – 6000 mg/100g

Heavy metals: Less than 20 ppm

Particle size: More than 90% passed through a 100 micron sieve

Storage: Refrigerated below 4°C

Microbial limits:

Standard Plate Count : Less than 10,000 CFU/g

Yeast and Moulds Count: Less than 1000 CFU/g

Enterobacteriaceae: Less than 10 CFU/g

Coagulase-positive *staphylococci* : Less than 100 CFU/g

Escherichia coli : Not detected in 1 g

Salmonella spp. : Not detected in 25 g

Specification for Kakadu plum puree

Botanical name: *Terminalia ferdinandiana*

Source: Wild harvested by indigenous communities in the Northern Territory. As fruits are wild harvested chemical makers are given as a broad range.

Plant tissue used: Fruit flesh

Type of preparation: Frozen puree

Characteristics:

General appearance: semi-liquid pale greenish to yellow/amber puree

Solids content: 13 – 17% w/w

Moisture content: 83 - 87% w/w

Total ash: Less than 1% w/w

Ascorbic acid content: 1500 – 3750 mg/100g

Total Ellagic acid content: 100 - 600 mg/100g

Heavy metals: Less than 5 ppm

Storage: Frozen storage: $-20 \pm 2^{\circ}\text{C}$

Microbial limits:

Standard Plate Count : Less than 10,000 CFU/g

Yeast and Moulds Count: Less than 1000 CFU/g

Enterobacteriaceae: Less than 10 CFU/g

Coagulase-positive *staphylococci* : Less than 100 CFU/g

Escherichia coli : Not detected in 1 g

Salmonella spp. : Not detected in 25 g

3.3.2 Kakadu plum harvesting seasons

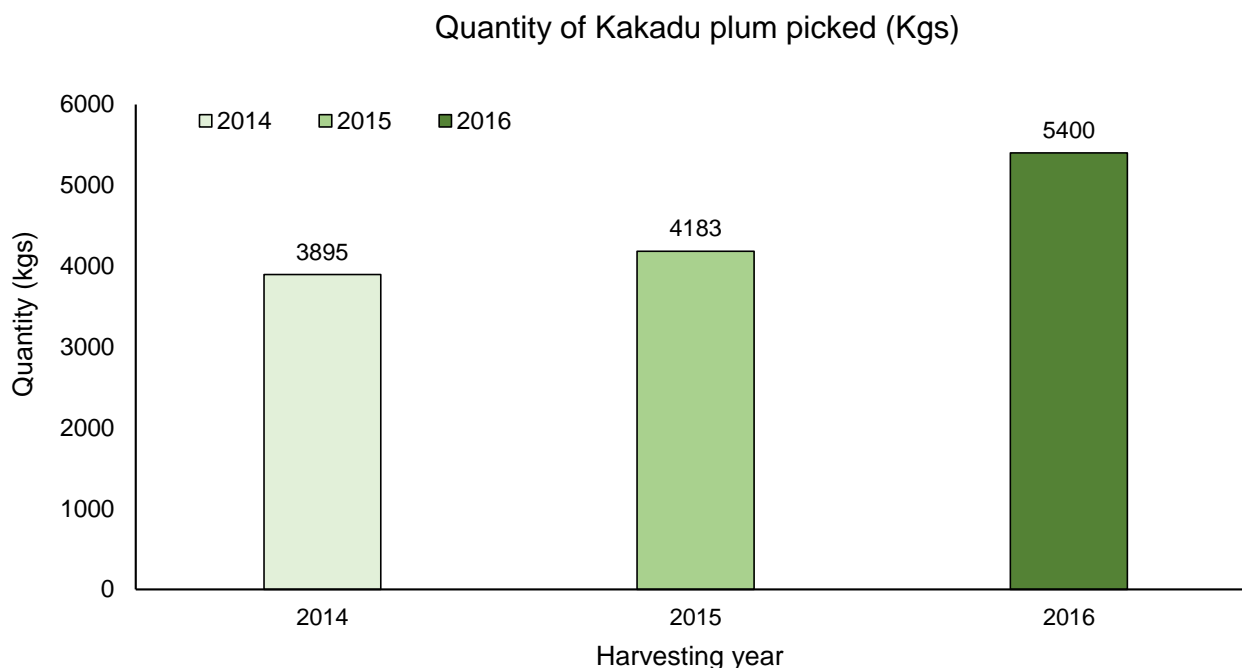


Figure 25 Quantity of Kakadu plum fruit harvested from 2014 - 2016

Harvest of Kakadu plum has been occurring in the Thamarrurr Region since about 2006. Until 2010, most of these harvests were organised through the Indigenous Ranger Program, the Thamarrurr Rangers. Collections were generally in the order of 300-500 kg per season and it was purchased by Coradji. The value chain for this fruit once it had been frozen was by barge to Darwin, by refrigerated truck to Sydney (where it was processed), then to Melbourne to freeze dry and then to the USA. Payment for raw fruit collected took a couple of months and would have gone back to the Ranger program.

A different model was used in 2010 where money was paid to all community members for supply of Kakadu plum fruit and payment was up front. The harvests were much bigger and probably only restricted by the amount of money available to pay up front, the length of the ripening of the fruit and the ability to store the fruit frozen. Amounts of 3000 kg in 2010/11 and up to 5400 kg in 2016 (Figure 25) have been harvested paying pickers up front.

3.3.3 Harvest (2014)

For the 2014 harvesting season, a total of 3895 Kg was harvested. While the harvesting and collection of the fruits were being done by the indigenous women and their families in Wadeye. The researchers with PWAC members and Thamarrurr Rangers set up the equipment for processing of Kakadu plum. About 2 tonnes of fruit was processed into the puree. The yield of fruit to puree was between 70 - 75%. The processed puree was packaged and frozen, for sampling refer to Table 11 for microbial quality. The women and rangers were trained in the hygienic processing of Kakadu plum and a new sanitisation step was introduced. Details for harvesting and processing protocols, and standard operating procedure for sanitisation, processing, packaging and storage are given below.

Harvesting, sorting and storage protocols for Kakadu plum in indigenous communities

Harvesting Kakadu Plum

This is how the Kakadu plum must be picked.

The cooperative will not buy your fruit if it has not been picked to these instructions.

The cooperative will only buy fruit from pickers with relevant Government Permits.

1. Identify Kakadu plum tree – Find the kakadu plum tree (*Terminalia ferdinandiana*), make sure you have the correct tree
2. What to pick

Pick ripe fruit only



Pick the fruit by hand and place in a suitable container given by the cooperation

Do not drop or bruise the fruit through rough handling

3. Do not pick fruit that are not ripe and flat, pick fruit that have a full body.

Do not pick soft fruit

Do not pick fruit that has fallen off the tree and is on the ground

Do not pick in any area that have been sprayed such as old cattle or sheep stations, or council parks and streets

Do not pick fruit that has been eaten by insects or birds

Sorting and Storage of the Kakadu plums

4. Storage of the picked fruit before bringing it to the collection sites

Discard sticks/leaves from fruit and place the fruits in a refrigerator if you cannot bring it on the same day to the collection site.

Fruits must be brought to the collection site within 24 hours of picking and storing in refrigerated conditions.

Do not transport or store fruit in containers that have ever contained chemicals of any type.

5. Delivering the fruit. The fruit must be delivered refrigerated to the collection site within 24 hours of picking. At the collection site the fruits must be weighed and checked for quality. Once this is done the fruits are placed on plastic trays and blast frozen till the core temperature of the fruit reaches -30 - -35°C . The blast frozen tray of fruits is placed on a pallet in a storage freezer at -20°C . This storage freezer could be a freezer container. Once the pallet is full of the trays it is shrink wrapped and kept at -20°C ready for transport.

Standard Operating Procedures for Sanitisation, Processing, Packaging and Storage

1. Preparation of frozen whole Kakadu plums for processing

- Thaw the whole frozen Kakadu plum fruits to room temperature
- Weigh each container of fruit and record the net weight and other delivery details on the batch processing form
- Take a composite sample of fruit for chemical assays from 4 boxes off each pallet
- Prepare 200 parts per million (ppm) of chlorine solution for the sanitisation of the fruits.
- Dip a known quantity of fruit in potable water to remove dust and soil.
- Dip the same batch of fruit in 200 ppm of chlorine solution and keep for 5 minutes
- Final dip in potable water before placing in trays to drain the water

2. Milling of Kakadu plum fruits to separate the seed from the pulp

- Clean and sanitise the milling equipment with 200 ppm of chlorine water.
- Set the mill to run with knives forwards, ie. sharp edge forward.
- Place a screen with 12 mm holes into the mill.
- Assemble the components of the mill.
 - Set the mill to run at 1440 RPM using the small set of pulleys.
 - Place a previously sanitised plastic container under the mill and switch the mill on. Eye protection and hearing protection must be worn when the mill is operating.

- Allow the fruit to slowly slide down the inlet chute into the mill.
- Collect the milled fruit into the plastic container as it falls from the opening on the bottom of the mill.
- Use a plastic scraper to remove any milled fruit caught inside the mill when the production run is finished.

3. Preparation of puree of Kakadu plum fruits after milling

- Set the paddle finisher to rotate at 830 RPM using the small to large set of pulleys.
- Place screens with 2mm holes into the finisher.
- Place a previously sanitised plastic container under the finisher to collect the puréed fruit.
- Place a plastic bag under the outlet on the end of the machine to collect the seeds and material too large or hard to go through the screen.
- Tip the milled fruit slowly into the inlet chute on the machine and using the hand pusher, push the fruit into the machine. Do not overload the capacity of the finisher in order to prevent non-processed fruit from being ejected into the waste container.
- Collect the de-seeded, puréed fruit as it falls from the opening on the bottom of the finisher.
- Use a plastic scraper to remove any puréed fruit caught inside the finisher when the production run is finished.

4. Packaging and Storage of pureed Kakadu plum

- Take the containers of de-seeded puréed fruit and weigh approximately 4kg into a 100 micron 25cm x 40cm polyethylene bag. Squash the bag to remove excess air and ensure the packed puree is spread evenly and is no more than 20 mm thickness.
- Seal the bag using the electric bar heat sealer for 4 seconds at a congealing time of 4 seconds.
- Pack six sealed bags of puree flat in 30cm x 45cm cardboard boxes using the following method:
 - place one cardboard divider on bottom of empty box
 - place 2 sealed bags of puree on top
 - add another cardboard divider
 - place 2 more sealed bags of puree on top of the second divider
 - add another cardboard divider
 - place 2 more sealed bag of puree on top of the third divider

- add another cardboard divider
- seal the boxes with tape.
- Accurately record the weight of each filled bag on the record form.
- Take a 50g sample of the puréed fruit for chemical and microbiological assay from the start, middle and end of the production run.
- Place the sealed boxes into frozen storage at -15°C to -20°C immediately after packing.

5. Cleaning up of processing equipment and processing area

- After completion of the processing run disassemble the mill and the pureeing equipment, hose and wash the parts in potable water and finally in 200 ppm of chlorine solution
- Hose the mill, puree equipment, plastic containers etc with a high pressure water cleaner to remove excess material. This should be done thoroughly to ensure no seeds or skin etc remain on the equipment as moulds could grow with time.
- Spray or wash all equipment with 200 ppm of chlorine solution, dry all plastic containers by draining and place into storage.
- Hose the floors and walls with potable water and finally wash the floors with 200 ppm of chlorine solution.

Table 11 Microbial quality of Kakadu plum samples

Date	Description	CFU/g		
		Standard Plate Count	Yeast	Mould
25/05/2014	Kakadu plum puree made with 2 Year old Plums (Pureed 23.4.14)	8.20E+02	8.50E+01	1.05E+02
25/05/2014	Kakadu plum puree Month 1 composite	2.04E+03	1.20E+02	1.10E+02
25/05/2014	Whole fruit picked from the ground Kuy Yadder	8.15E+02	0.00E+00	1.35E+02
25/05/2014	Kakadu plum puree 2 kg 24.4.14 Top left Corner	1.64E+03	1.10E+02	1.45E+02
25/05/2014	Kakadu plum puree 2 kg 24.4.14 middle of the bag	2.48E+03	2.00E+02	1.75E+02
25/05/2014	Kakadu plum puree 2 kg 24.4.14 Bottom right Corner	2.12E+03	1.10E+02	1.50E+02
30/08/2010	Kakadu plum frozen from 2008 dried (Coradji)	8.10E+03	<100	1.00E+02
30/08/2010	Kakadu plum frozen plum from 2008 minced (Coradji)	1.40E+04	<100	<100

Table 12 Chemical and microbial quality of composite samples of Kakadu plum freeze dried powder

Composite Kakadu plum puree/freeze dried powders	Free EA(mg/100g DW)	Total EA(mg/100g DW)	Vitamin C (mg/100g DW)	Total Plate Count CFU/g	Yeast & mould count CFU/g
16070-403-1- (Wadeye 2014) Pureeing in Brisbane 2015	980		17785	<100	<100
16070-403-2 (Wadeye 2014) Pureeing Wadeye	695		18849	2000	<100
16083-503 (Wadeye 2014) Pureeing Wadeye	820		16807	800	<100
201497 Wadye 2014	520	1496	19,183		
201598 Wadeye 2015	453	1856	21898		
201599 Wadeye 2015			21,000		
201599 Wadeye 2015 (puree) FW			3,400		

All the samples were within the limits given below in the Kakadu plum puree specification sheet. Standard Plate Count – less than 10,000 CFU/g and Yeast and Moulds Count – less than 1000 CFU/g. Vitamin C within 10000 – 25000 mg/100 g DW and total EA 1000 – 6000 mg/100 g DW (Table 11 and Table 12). For frozen puree, Ascorbic acid content was between 1500 to 3750 mg/100 g DW.



Figure 26 Processing of Kakadu plum puree

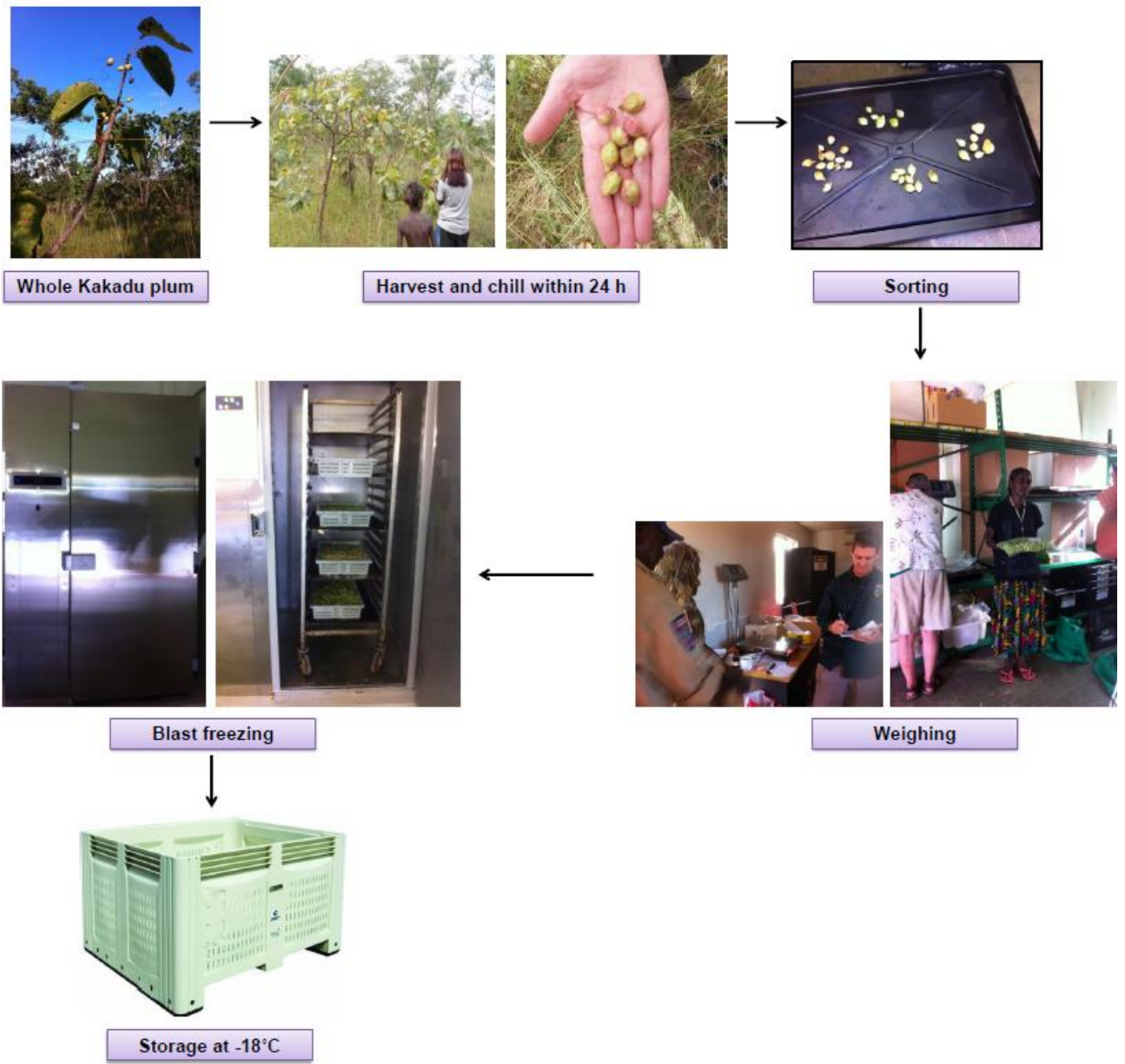


Figure 27 Processing of whole Kakadu plum

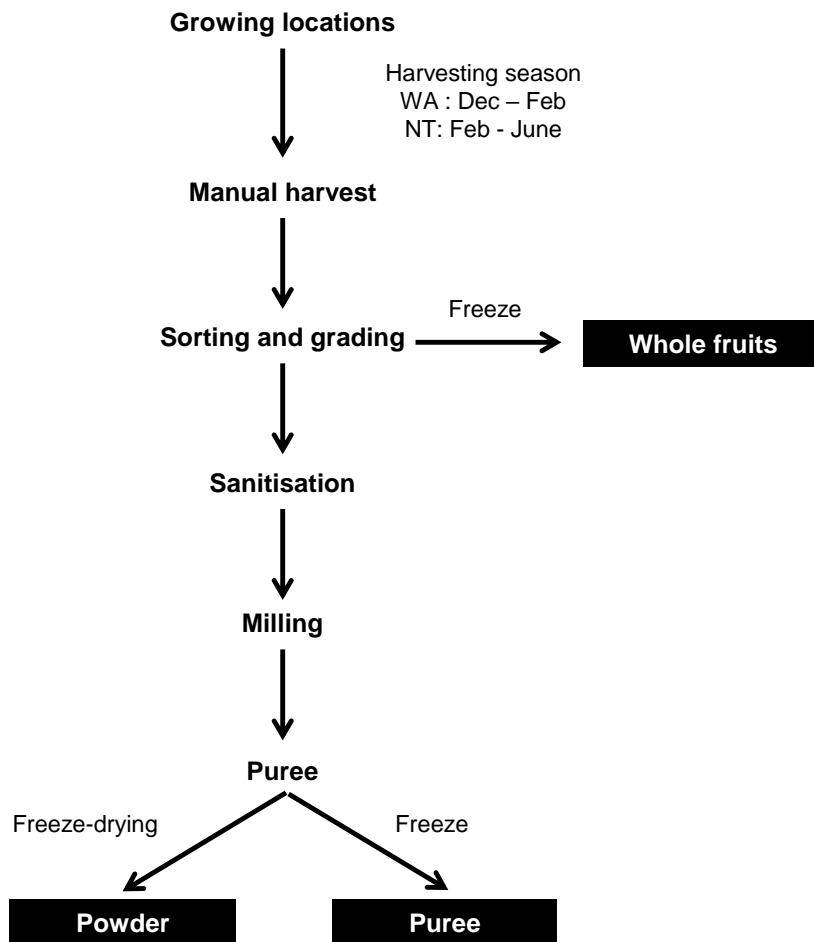


Figure 28 Overview of current processing of *T. ferdinandiana*

After the initial visit in April 2014 and learning from the first harvesting and processing experience, there have been on-going discussions regarding next year's harvest (February – March 2015) with the stakeholders. To improve the quality of the whole fruit and puree it has been recommended to purchase a blast freezer, storage freezers and vacuum sealing packaging machine. There are ongoing discussions on having a central processing plant in Darwin where Kakadu plum fruits from other Indigenous communities can be processed. Processing of Kakadu plum puree and whole fruit are shown in Figure 26 and Figure 27, respectively. Current processing steps for Kakadu plum are shown in Figure 28

3.3.4 Harvest (2015)

For the 2015 harvest the PWAC had purchased a blast freezer, storage freezers and vacuum sealing packaging equipment. Freezing and packaging parameters were determined, 6-8 women from PWAC and Thamarrurr rangers were trained in using the blast freezer and packaging equipment.

Information given by the PWAC for the 2015 season, 4.18 tonnes of Kakadu plum fruit was harvested, there were 148 registered pickers of which 137 were women and 11 men. The harvesting period in Wadeye was 10 weeks from April – July in 2015.

3.3.5 Harvest (2016)

For the 2016 season it was decided by PWAC to stop processing into puree in Wadeye, the puree will be processed in Brisbane and the freeze drying will occur either in Brisbane or Melbourne. Monitoring of the harvesting and processing of Kakadu plum in Wadeye at PWAC from 18 – 25 April 2016. The women are now training other communities in Wadeye, Thamarrurr region who are interested in harvesting kakadu plum. The PWAC have the harvesting and processing protocols implemented in the past two years and have shared the training material and protocols with other Indigenous communities.

PWAC is looking into the possibility of building a cool room receiving area and limiting picking to own country. The Thamarrurr Rangers were trained during the 2016 harvest in collection of samples for research purposes, tagging and GPS tracking of tree locations. Rangers will continue to monitor the harvest to determine who is picking, whether necessary permission from TO's have been obtained and if the wild harvest is done sustainably.

Training programs held in other Aboriginal Communities in Northern Australia

- At the invitation of the Crocodile Islands Rangers in Milimgimbi a presentation on the "Handling and processing of kakadu plums" was done on 27 April 2016 at the Rangers Unit before training the rangers in the harvesting and processing of Kakadu plums.
- At the invitation of the Mamabulungin Aboriginal Corporation the following presentation titled "Why Kakadu plum is so Special? Making a Business from Native Plant products – Gabinge as a case study", which included the training modules made in Wadeye, were made to the following Indigenous communities: Mamabulanjin Aboriginal Corporation, Nyul Nyul Rangers, Twin Lakes Cultural Park, Lombadina Community, Eyes on Country from 19 – 21 July, 2016 Broome, Western Australia.

3.3.6 Harvest (2017)

- For the 2017 season, Wadeye did not harvest Kakadu plum fruits the Indigenous community wanted to rest the trees. In addition, there was a change of management at the Wadeye Palngun Wurnangat women's association now known as Palngun Wurnangat Aboriginal Corporation. There was a need to understand how the Kakadu plum harvesting was going to be managed by the women's centre and discussions are progressing on obtaining the new permits required for commercial harvesting. There is also a need to find new markets and the community is pursuing the signing of contracts with buyers before the 2018 harvest.

- The PWAC members and Thamarrurr Rangers were presented data on maturity indices for Kakadu plum, how to select the fruits based on fullness as a parameter to assess the maturity stage. The same group was trained on using Kakadu plum in a beverage, quantity to use to receive the Required Daily Intake for vitamin C and the possibility of using different native foods including Kakadu plum in diet diversification.
- The Mamabulanjin Aboriginal Corporation in Broome and the Indigenous community at Twin Lakes Cultural Park has adopted the harvesting and processing model currently operating in Wadeye. Broome continued to harvest about 10 tonnes of fruit.
- Puree and freeze dried powders of Kakadu plum from Wadeye continues to be used by the farmed prawn industry in Queensland for shelf life extension of chilled cooked prawns and 75% of the cooked chilled prawn industry in Queensland are using Kakadu plum products.
- Karen Sheldon Catering uses the Freeze dried Kakadu plum powder from Wadeye and other suppliers to extend the shelf life of frozen meal solutions, which also go back to Indigenous communities in addition to the wider community.

A team of four members from UQ, QLD DAF, and the University of McGill, Canada, visited Broome from 11 – 18 March, 2017. This visit to Broome was to present the data on the samples of Kakadu plum that was given for testing by the communities in 2015/2016 and to visit the communities who were interested in harvesting, processing and enrichment planting of Kakadu plum. Presentation of the data and future research directions was done at the Native Fruits and Trees Workshop held on March 14 – 15 at The University of Notre Dame, Australia (Broome campus). This workshop was organised by the Mamabulanjin Aboriginal Corporation and Indigenous Land Corporation, our team of researchers were invited to present on the Kakadu plum work that had been done in Wadeye with the Palngun Wurnangat women's association and Thamarrurr Rangers. A presentation was also done by the Palngun Wurnangat women's association on the processing hub, harvesting protocols and sharing of the business model established in Wadeye. The aim of the workshop was to have an open discussion with the Indigenous community representatives on current harvest activities, aspirations, barriers and opportunities.

3.4 Conclusion and recommendations

The Kakadu plum business operating as a micro-enterprise has given opportunities for seasonal work especially for women and an income to the Wadeye Aboriginal community members. Culturally and socially it has benefited the community as the members harvest in their own country and is a good time for sharing of stories by the elders with the younger generation. The Kakadu plum harvest is a festive season in Wadeye and a period of the year that the entire community look forward to participate. The potential of developing this business model to be completely owned and controlled by the Aboriginal community has made this a worthwhile investment, not just for plums but for other promising native foods as well.

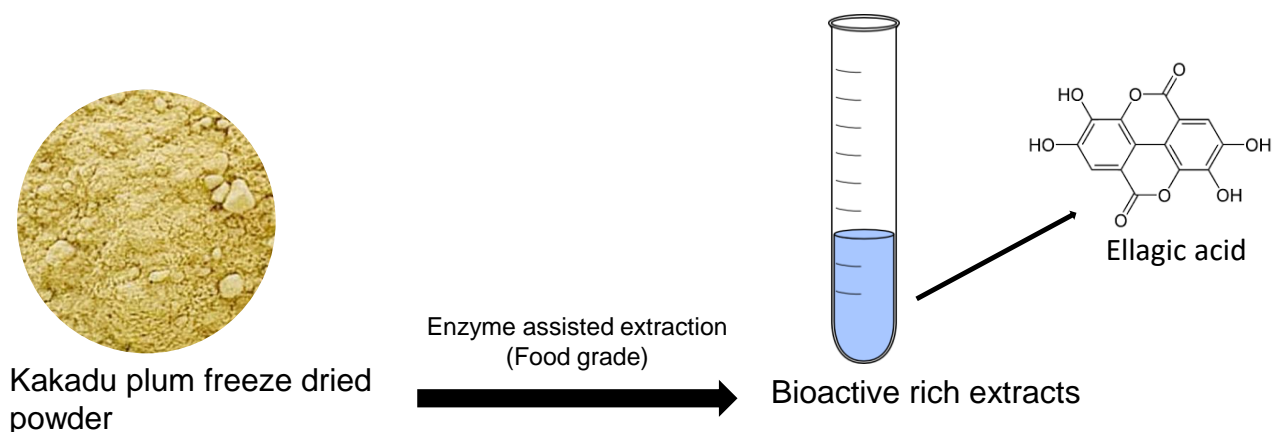
A Kakadu Plum Indigenous Network (KPIN) was established as an outcome of the Native Trees and Fruit Workshop held in Broome WA (March 2017). Participants now share and communicate their Kakadu plum experiences with each other to support the development of an Indigenous-led Kakadu plum industry.

The challenges of running a micro-enterprise from remote regional Australia will continue, however these challenges can be overcome as long as it is something that the community wants and willing to work at.

The Indigenous communities should continue to build partnerships with potential buyers to ensure consistent market for the current supply and expansion to new markets. This will enable building a stronger and sustainable supply and value chain for Kakadu plum.

Chapter 4 Bioactive rich extracts from *Terminalia ferdinandiana* by enzyme-assisted extraction: A simple food safe extraction method

Graphical abstract



Highlights

1. An enzyme assisted method for aqueous KP extracts is reported.
2. Extraction method was optimised using central composite rotatable design.
3. Pectic polysaccharides in KP from metabolomic profiling are reported.
4. These extracts could be applied to a food system as natural preservatives.

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Abstract

A food grade compatible enzyme assisted extraction (EAE) technique for extracting bioactive compounds from freeze-dried Kakadu plum (KP) puree was evaluated. To optimise the extraction, a central composite rotatable design (CCRD) was conducted and effects of solvent concentration, enzyme concentration and time of reaction on extracted levels of free ellagic acid (fEA), ascorbic acid (AA) and total phenolic content (TPC) were determined. In the extracts, concentration of fEA ranged from 53.6 - 266.6 mg/100 g dry weight (DW) of KP puree; AA 63.7 – 112.1 mg/100 g DW and TPC levels of 73.23 -104.74 mg of gallic acid equivalent (GAE)/g. Extraction yield of fEA ranged from 10.3 to 51.3%. The model was found to be suitable for extraction of fEA - an important bioactive compound with documented antimicrobial properties from KP fruit. A solvent (propylene glycol) concentration of 1.5 % (w/w), enzyme (pectolytic enzymes) concentration of 300 mg/L and extraction time of 15 h was ascertained as optimum for the fEA extraction delivering a yield of 51.3%. The extraction method described here facilitates the provision of a simple, cost effective food-grade compatible extract that by-passes the need for organic solvents thereby obtaining an EA-rich aqueous extract with enhanced biological activities. This simple extraction method can also be applied to other EA rich plant material like pomegranate and peel of many common fruits which are generated as food processing by-products and can be easily adopted by numerous industries.

4.1 Introduction

A species of *Terminalia* endemic to Australia – *Terminalia ferdinandiana* Exell, Combretaceae, commonly known as KP, has a significant history as a food source and traditional medicine by the Australian aboriginal population for centuries. Fruits are consumed to cure headaches and to alleviate the symptoms of colds and flu while the pounded fruit is used as an antiseptic and a soothing balm for aching limbs [20]. KP grows across large areas of Australia: Western Australia, to the south of Broome and Northern Territory, near the Arnhem Land and the Gulf area in the east, covering a range of environmental conditions [66].

KP is a rich source of polyphenolic compounds for example ellagic acid (EA) and its hydrolysable tannin, ellagitannin [21, 22]. EA and its derivatives are present at high levels in many *Terminalia* plants [38] and are reported to be responsible for their perceived health promoting and biological activities [67]. Konczak et al. (2014) reported free EA (fEA) levels of the KP fruit are in the range of 3050 to 14020 mg/100 g DW [21] while a recent study by Williams et al. (2014) reported the lower level of 626 - 980 mg/100 g DW of KP fruit [22]. The polyphenol EA has been reported to exert anti-carcinogenic, anti-bacterial, anti-fungal, antiviral, and anti-inflammatory activities [68-70].

As well as EA, KP fruit also contains a high concentration (14038 mg/100g DW) of AA [22] (Williams et al., 2014). Very high values (average of 15190 mg/100g DW) have been measured in Northern Territory grown KP fruit, although there was large variability in the AA content between the growth sites and individual samples collected at each site [21].

To date not one of the published studies on KP has reported a water based extraction method as EA is not only insoluble in water but is also difficult to solubilize in commonly used organic solvents in sufficient quantities for formulation [71]. This characteristic is the main contributor to the low oral bioavailability of EA [72]. Use of organic solvents in these extraction processes has several disadvantages. Most notably safety hazards, high energy consumption, risk to the environment and toxicological effects [73]. As most of these solvents are not suitable for food applications, there is a need for an EA-rich aqueous extract of KP as a functional ingredient for use in the food and beverage industry. At present, KP is incorporated as a puree or powder into food without the associated benefits of any enrichment procedures [74].

Incorporating the powdered form of KP can be challenging as experiments conducted in our laboratories have shown that KP freeze-dried powder when mixed with water forms a jelly like matrix that makes incorporation of this fruit problematic. Initial metabolomic profiling of KP fruit conducted by our research group indicated the presence of sugars that are commonly associated with the polysaccharide pectin [75]. The presence of pectin is a well-known obstacle to the efficient extraction of bioactive compounds from plant matrices as this highly resistant complex of the plant cell wall entraps bioactive compounds [76, 77]. In fact many of the naturally existing phenolic compounds in fruits and vegetables are usually covalently bound to insoluble polymers [78]. Therefore the presence of pectic polysaccharides in KP matrix may have the potential to bind EA and other bioactives. Therefore, there is a growing need to develop a food grade compatible extract of KP with high bioactive levels, into an easily incorporable format for use as a functional ingredient.

This study facilitates the provision of a simple, cost effective food-grade compatible extract that by-passes the need for organic solvents thereby obtaining an EA-rich aqueous extract with enhanced biological activities.

In order to overcome issues regarding the solubility of EA, a water co-solvent system was used which is an attractive alternative to expedite the release of EA from KP. In these co-solvent systems, the solubility of organic compounds can be improved by altering the

composition of the co-solvent and has been reported useful for other plant-based phytochemicals e.g. curcumin [79]. In order to facilitate enhanced release of EA from pectin in KP cell matrix, an enzyme assisted extraction (EAE) method was developed and evaluated. EAE techniques have been reported to facilitate extraction of bioactive compounds from complex plant polysaccharides like pectin [80, 81]. EAE has several benefits with higher extraction yield, faster extraction, higher recovery, reduced solvent use and lower energy consumption with the added advantage of being environmentally friendly [82].

A central composite rotatable design (CCRD) was used to obtain extracts with elevated levels of bioactive compounds from freeze-dried KP puree using EAE and water-propylene glycol as a water co-solvent system. Moreover, a detailed chemical characterization of the initial starting product and subsequent extracts that measured the concentration of EA, AA and total phenolic content (TPC) provided data on the efficiency of the EAE technique.

4.2.1 Materials and methods

4.2.1 Plant material

KP puree (commercial grade), processed in late August 2014 in Wadeye, NT was used for this study. KP puree was freeze-dried (GAMMA 1-15LSC, Crist, Austria) and finely ground in a Retsch MM301 cryomill (Retsch GmbH, Haan, Germany) and subsequently stored at -20°C until further analysis. Chemical composition of this raw material was undertaken to obtain concentrations of free and total EA, AA and moisture content (Table 1). All analyses were conducted in triplicate.

4.2.2 Chemicals

EA and AA (both >95% purity) were purchased from Sigma-Aldrich Inc. (Sydney, NSW, Australia). The HPLC-grade methanol and ethanol were purchased from Thermo Fisher Scientific (Melbourne, Victoria, Australia). Food grade (99.5%) Propylene glycol was purchased from Sigma-Aldrich (Sydney, NSW, Australia). Enzyme Pectinex® Ultra SP-L was procured from Novozymes (KRN05649 Novozymes A/S Krogshoejvej 36, Bagsvaerd, Denmark). The Folin–Ciocalteu phenol reagent (2N) was obtained from Sigma-Aldrich. (Sydney, NSW, Australia). All other chemicals were of analytical grade and purchased from Thermo Fisher Scientific.

4.2.3 Determination of moisture content

The moisture content of the freeze-dried KP powder was determined according to AOAC official method 964.22 (Association of Official Analytical Chemists, 1995). Briefly, in triplicate, each sample (1 g) was dried for approximately 16 h to a constant weight at 70°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.

4.2.4 Metabolomic analysis of KP fruit

Metabolomic analysis of KP fruit was conducted using Gas Chromatography Mass Spectrometry (GCMS).

4.2.4.1 Extraction of polar metabolites from freeze-dried KP fruit

Sub-samples (0.1 g) of the lyophilized and milled samples of KP fruit were weighed into pre-chilled tared 2 mL microcentrifuge vials. To each vial, 600 μL of extraction solution containing 2:1 (v/v) of 100 % methanol and chloroform, including 10 μL of the internal standards (1 mM

sorbitol; 10 mM valine; 5 mM myristic acid) was added. The vials were vortexed for 30s. This was followed by addition of 200 μ L chloroform and vortexing for 30s. The samples were incubated at 70°C for 15 min in a shaking water bath at 850 rpm. After incubation, 400 μ L of ultrapure water added and mixed by vortexing. All the samples were centrifuged at 12054 g at room temperature for 15 min. After centrifugation the upper polar and non-polar lower phases were carefully transferred to new vials. The polar phase was washed using 300 μ L chloroform followed by centrifugation at 12054 g at room temperature for 10 min. The washed polar supernatant was transferred to a new vial. The lower non-polar phase left after this centrifugation was combined with the previous non-polar phase. The resulting extracts were stored at -80°C till further analysis.

4.2.4.2 Gas Chromatography Mass Spectrometry (GCMS) analysis for KP metabolomic profiling

Samples were analysed by a 7890A gas chromatograph (GC) coupled with a 5975C mass spectrometric detector (Agilent, Mulgrave, VIC, Australia). The GC was fitted with a Factor Four™ VF-5ms capillary column (0.25 mm, 0.25 μ m, 30 m length with a 10 m fused guard column) (Varian, Mulgrave, VIC, Australia) with helium (BOC gasses, ultra high purity) used as a carrier gas under a constant flow of 0.718 mL/min. The initial temperature of 70°C was held for 1 min, then increased to 325°C at a rate of 7°C/min and maintained for 3.5 min. The ion source, quadrupole and transfer line were set at 250°C, 150°C and 280°C respectively. For analysis, aliquots of 100 μ L of the polar extracts were freeze dried. Derivatization and introduction of the samples was performed using an automated Multi-Purpose Sampler (MPS-2XL) equipped with a heated agitator (GERSTEL, Germany).

For derivatization, a portion of (100 μ L) of the polar fraction was oximated with methoxylamine hydrochloride in anhydrous pyridine (15 μ L of 40 mg/mL) [Sigma-Aldrich, St Louis, MO., USA] at 37°C for 1 h and then silylated with N,O-Bis (trimethylsilyl) trifluoroacetamide with 1% Trimethylchlorosilane (30 μ L) at 37°C for 2 h.

Subsamples (3 μ L) of the derivatized polar and non-polar extract samples were then injected in split (3:1) mode at 250°C. Metabolites were detected in a total ion count mode and the scanning range was 50-600 m/z at a 2.66 scans/sec rate.

4.2.5 Central composite rotatable design (CCRD) for enzyme treatment

For the experiments, 5 g of the freeze-dried KP puree was weighed into 50 mL pre-labelled Falcon tubes. To each of the tubes, 35 mL of distilled water, and required volumes of solvent [propylene glycol (volume ranged from 1-2% w/w)] and enzyme [pectolytic enzyme (concentration ranged from 100-500 mg/L)] were added. Samples were mixed with the help of a vortex mixer. Treated samples were left on gentle shaking at room temperature for the pre-determined time (ranged from 2-12 h). After completion, samples were centrifuged at 2655 g at 17°C for 30 min in an Eppendorf Centrifuge 5804 R (Eppendorf Austria GmbH, Wein, Austria) until a clear separation can be seen. The supernatants were carefully transferred to new tubes and stored at -20°C until further analysis.

EAE was optimized using a central composite rotatable design (CCRD). The effects variables X_1 solvent volume (1-2% w/w), X_2 enzyme concentration (100-500 mg/L) and X_3 time of reaction (2-1h) on the response variable Y_1 levels of fEA (mg/100 g DW), Y_2 levels of AA (mg/100 g DW) and Y_3 total phenolic content (TPC) (mg GAE/g extract) were investigated.

The total number of 20 statistically designed batch experiments (detailed in Table 14) were performed for different combinations of the variables X_1 to X_3 on the response variables Y_1 to Y_3 . The 20 experiments were conducted in a randomized order. The experimental design and data analysis were carried out using the Minitab 16 software (Minitab Pty Ltd, Sydney, Australia).

The analysis of variance (ANOVA) for each of the three response variables given in the Table 15 and Table 16 indicated significance $p < 0.05$ for regression analysis using the second order polynomial model in Equation 1 for all regression models including the linear, square and interaction terms.

Equation 1

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_1^2 + \beta_5 X_2^2 + \beta_6 X_3^2 + \beta_7 X_1 X_2 + \beta_8 X_1 X_3 + \beta_9 X_2 X_3$$

Where Y = response, X = factors/independent variables and β = regression coefficients

4.2.6 Model fitting from response surface methodology

The thick matrix of plant polysaccharide like pectin hinders effective extraction of bioactive compounds from plant cells as these polysaccharides protect the bioactive compounds by trapping them inside [76]. Phytochemicals that are retained by polysaccharide-lignin network of the plant cell are not accessible to solvents in routine extraction processes. Use of pectolytic enzymes allows hydrolysis of plant cell wall components resulting in an increase in cell wall permeability, with subsequent higher extraction yields.

In order to devise an efficient extraction technique that will allow enhanced extraction of bioactive compounds from KP tissue within an easily incorporable liquid format pectinase enzyme was used. Poor solubility and hence reduced bioavailability are limiting factors for the clinical application of EA. To overcome the challenges due to the insolubility of EA in water, propylene glycol was used as a solvent. Propylene glycol is generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA), with aqueous systems of propylene glycol as high as 40% being regarded as nontoxic for human consumption [83]. Therefore, the KP extracts reported here can be considered safe for use in food industry applications.

For our study, we selected three independent variables - solvent volume (% w/w) (X_1), concentration of enzyme (mg/L) (X_2) and time of hydrolysis (h) (X_3). The response variables measured were concentration of EA (Y_1), concentration of AA (Y_2) and total polyphenolic content (Y_3). The total number of experiments for this study was 20 (Table 14).

4.2.7 Determination of EA content in KP extracts

Due to solubility concerns of EA within the enzymatic digestion samples, the pH of each of the extracts was adjusted to 7.5 from their initial pH of ~3.5 [53]. EA solubility is pH dependent and preliminary analysis showed that levels of EA significantly increased at a higher pH. For the extraction of fEA from the liquids after enzymatic digestion, 1.0 mL of each liquid extract were filtered through a 0.45 μ m syringe filter into a HPLC vial, N_2 was introduced, capped and stored at -80°C prior to analysis.

The quantification of EA content in different extracts of KP were conducted by HPLC-DAD analysis, as described by Williams et al., [22]. For analysis, an aliquot of 20 μ L of sample was injected. Spectra for all wavelengths between 220 and 600 nm were recorded by the photodiode array detector. The fEA was identified by the chromatographic behaviour on comparison with EA commercial standards and UV spectra. fEA content was expressed as mg/100 g DW.

4.2.8 Determination of ascorbic acid content in KP extracts

Experiments conducted to determine the effect of pH on AA concentration showed that concentration of AA is higher at lower pH (supplementary table S1). Therefore, the extracts at their original pH of ~3.5 were directly subjected to AA determination. For extraction of AA

from the liquid extracts obtained from the enzymatic digestion 0.1 mL of each of the samples was extracted with the AA extraction 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. An aliquot of the diluted samples were filtered through a 0.45 µm filter syringe prior to HPLC analysis. Concentration of AA in the extracts was determined as per Williams et al. [84].

4.2.9 Determination of Total Phenolic Content (TPC)

The total phenolic content of the liquid extracts was determined using the Folin Ciocalteu (FC) method [85]. Diluted extracts were analysed at 750 nm with Gallic acid as a standard using a microplate reader (Infinite M200, Tecan Australia Pty Ltd, Melbourne, Australia). The analysis was carried out in triplicate and expressed as micromoles of total phenolics Gallic acid equivalents, (GAE) per gram of sample. TPC was calculated by the following formula:

$$\text{TPC (mg GAE/g)} = \text{GAE (mg/g)} \times [\text{Total volume of extract (mL)} \times 10^{-3} \text{ (L/mL)} \times \text{dilution factor}] / [\text{sample weight (g)} \times 10^{-3} \text{ (mg/g)}]$$

4.2.10 Statistical analysis

All analyses were run in triplicate (n =3) were expressed as means ± standard deviation (SD). The outcomes from CCRD experiment were analysed with the help of Minitab 16 software (Minitab Pty Ltd, Sydney, Australia). All the other statistical analyses were performed by using the XLSTAT-Pro software package version 7.0 (XLSTAT Addinsoft, Paris, France). Differences between means were first analysed by the ANOVA test and then least significant differences (LSD) test (p < 0.05).

4.3 Results and Discussion

4.3.1 Sugar analysis from the polar extracts of KP fruit

Present work, for the first time, reports the results from non-targeted metabolomic profiling of KP fruit. 158 chemically diverse metabolites were identified in the polar extracts including amino acids and their derivatives, fatty acids, organic acids, phenolic acids, steroids, sterols, sugars and sugar alcohol, terpenoids and tocopherol. For the purposes of this study we only report five of the identified sugars namely mannose, rhamnose, galacturonic acid, arabinose glucose and fructose. These sugars have not been previously reported in KP fruit. Perhaps surprisingly, the metabolomic analysis did not show, galactose in the fleshy part of the KP fruit.

The presence of mannose, rhamnose, galacturonic acid, arabinose glucose and fructose in KP fruit suggests the existence of the polysaccharide pectin in the fruit tissue as these sugars are often incorporated into pectin [75]. The possible presence of pectin in KP tissue was further provided by the observations that when dissolved in water (1:4 w/w), KP powder formed a gel like consistency. Similar gel formations have been reported by pectin compounds in different fruit materials e.g. prickly pear fruit (*Opuntia albicarpa*) [86] and pomelo (*Citrus maxima*) [87].

Several of the sugars reported in the current study have also been identified in the gum or crude extracts of other *Terminalia* members. In a study analysing the gum of two *Terminalia* species *T. sericea* and *T. superba*, presence galactose, arabinose, rhamnose, mannose and xylose along with highly branched polysaccharides consisting of galacturonic, glucuronic and 4-*o*-methylglucuronic acids were described [88]. In another traditional medicinal *Terminalia* member - *T macroptera*, Zou et al. (2014) reported the presence of the monosaccharides - rhamnose, mannose, galactose, galacturonic acid, arabinose and glucose in the crude extracts of root, stem and leaves [89]. The galactose sugar was present in the root, stem and

leaves, but the fruit was not examined in this study. The current investigation is the first to report the presence of these pectic polysaccharides in KP.

4.3.2 Model fitting from response surface methodology

The analysis of variance (ANOVA) for each of the three response variables (Table 15) indicated significance $p < 0.05$ for regression analysis using the second order polynomial model in equation 1 for all regression models including the linear, square and interaction terms. This model is good for determining EA, AA and TPC as the lack of fit is not significant ($p > 0.05$) (Table 15). The response models for each response variables showing only the terms with significance ($p < 0.05$) estimated regression coefficients (Table 15).

The mean values of the level of EA, AA and TPC of the extracts are shown Table 15. The level of EA in the extracts ranged from 53.6 to 266.6 mg/100 g DW. The highest level of EA was detected when 1.5% solvent volume with 300 mg/L enzyme concentration and time of reaction 15 h was employed. The multiple regression analysis for the response variable EA indicated that the regression model for EA was significant ($p < 0.05$) and did not present lack of fit. Results indicated the regression coefficient of the linear (β_3), square (β_6) and interaction (β_8) terms had significant effects on EA levels. The predicted model can be described by Equation 2 in terms of coded values. The co-efficient of determination (R^2) of the regression EA was 81.18% (Table 15).

Equation 2

$$Y_1 = 90.7 + 105 X_1 + 0.213 X_2 - 21.13 X_3 - 48.0 X_1 X_1 - 0.000089 X_2 X_2 + 0.710 X_3 X_3 - 0.165 X_1 X_2 + 10.76 X_1 X_3 + 0.0080 X_2 X_3$$

Significance levels $\beta_3 = ***p < 0.001$, $\beta_6 = **p < 0.05$ and $\beta_8 = **p < 0.05$

ANOVA testing for the response variable AA Table 16 indicated that the regression model for AA was not significant ($p > 0.05$). Furthermore the results also suggested that the regression coefficient of any of the linear, square and interaction terms had any significant effects on AA levels with the co-efficient of determination (R^2) of the regression AA being only 33.60% (Table 15). Consequently the obtained equation 3 cannot adequately describe the extraction of AA with the current parameters. Therefore, the presented model cannot be adequately applied to the preparation of AA rich extracts without modifying the tested parameters. Thus the enzymatic digestion and subsequent breakdown of the plant cell wall material did not have a significant impact on the release of AA. This can possibly be attributed to the fact that AA is not trapped within the plant cell wall. AA is synthesized in the plant cytosol [90] and is distributed into the chloroplasts, vacuole, and apoplast of the plant cell [91].

Equation 3

$$Y_2 = 138.3 - 70.7 X_1 - 0.104 X_2 + 2.30 X_3 + 24.1 X_1 X_1 - 0.000017 X_2 X_2 + 0.005 X_3 X_3 + 0.0374 X_1 X_2 - 1.69 X_1 X_3 + 0.00565 X_2 X_3$$

TPC values of the extracts ranged from 48.46 to 105.78 mg GAE/g with the highest values observed with the experimental design when 1.5% solvent volume with 300 mg/L enzyme concentration and 15 h reaction time is applied. ANOVA for the response variable TPC (Table 16) indicated that the regression model for TPC was significant ($p < 0.05$) and did not exhibit any lack of fit. Results indicated the regression coefficient of the linear (β_3) and interaction (β_8) terms had significant effects on TPC. The predicted model for TPC can be described by Equation 4 in terms of coded values. The co-efficient of determination (R^2) of the regression TPC was 78.12% (Table 15).

Equation 4

$$Y_3 = 75.0 + 33.6X_1 + 0.0383X_2 - 7.58X_3 - 21.2 X_1X_1 + 0.000012 X_2X_2 + 0.170 X_3X_3 - 0.0406 X_1X_2 + 4.56 X_1X_3 + 0.00160X_2X_3$$

Significance levels $\beta_3 = ***p < 0.05$ and $\beta_8 = **p < 0.05$

The response variables EA and TPC showed a strong positive correlation (Pearson correlation of EA and TPC = 0.923, $p < 0.05$) indicating that when one variable increases the other variable also increases. Positive linear effects were observed for EA concentration and the independent variable time (Pearson correlation of EA and Time = 0.647, $p < 0.05$). Strong positive correlation was also observed for TPC and time (Pearson correlation of TPC and Time = 0.560, $p < 0.05$). Thus time provided highly significant ($p < 0.05$) effects on EA and TPC levels of the extracts. The square term for time had a significant ($p < 0.05$) effect on the levels of EA. The interaction between solvent volume/time had a significant effect ($p < 0.05$) on the levels of EA and TPC. The interaction of solvent volume and time on the levels of fEA and TPC are shown as surface plots Figure S1.

4.3.3 Verification of predictive model

Parameters for each of the three independent variables i.e. solvent volume (%), enzyme concentration (mg/L) and time (h) were selected to obtain the highest levels of the response variables. As EA was the parameter that fitted the statistical model most appropriately, it was this response that we aimed to verify. In addition EA has also exhibited considerable antimicrobial activity and therefore would be a likely candidate in developing a natural antimicrobial agent from KP. The suitability of the model equation for predicting the optimum response values was tested using 7 treatments described in Table 17. Three additional treatments were included to confirm the critical role that the pectinase enzyme plays in enabling the release of the bioactive compounds from the pectin (refer to supplementary data Table S2). These include treatment S1 with 3.5% solvent concentration, 0 mg/L enzyme concentration and 24 h of reaction time; treatment S2 with 3.5% solvent concentration, 1000 mg/L enzyme concentration and 24 h reaction time and treatment S3 with 3.0% solvent concentration, 800 mg/L enzyme concentration and 15h of time. Expected values of EA in each of treatments were calculated with the help of equation 2.

These new extractions were submitted to the same experimental analytical procedures as those applied initially. The observed and predicted values, along with the computed absolute errors (AE) are presented in Table 17. The predicted EA values for 7 treatments (selected as they possessed values that fell within the statistical model for the three variables), were consistent with the predicted values. The strong correlation between the actual and predicted results confirmed that the response model was adequate to reflect the expected optimization. Because of the low absolute error values obtained by the comparison between observed and predicted values, the proposed model could be used to predict the response value.

By increasing values of the three variables, it is possible to obtain extracts with higher concentration of EA as demonstrated by extract S1 with 173.2 mg/100 g DW, treatment S2 with 392.1 mg/100 g DW and treatment S3 with 322.2 mg/100 g DW of EA (Table S2). Treatment S1 and S2 had identical values for solvent concentration and time of reaction and differed only in terms of the enzyme concentration. Treatment S1 contained no enzyme whereas treatment S2 utilised 1000 mg/L (Table S3).

4.3.4 Extraction yield

For the 20 extracts the EA extraction yield ranged from 10.3 to 51.3% (Table 14), with the highest yield observed for the solvent concentration of 1.5%, enzyme concentration of 1.5 mg/L and 15 h for time of reaction. The extraction yield observed for AA was significantly lower and ranged from 0.33 to 0.58% (Table 14) which could again be attributed to the fact that unlike EA, AA is not trapped by the plant cell wall materials with enzyme digestion not

contributing towards its release. The extraction yield for treatment S2 was 75.4% and was significantly higher than treatment S1 with no enzyme digestion with extraction of 33.3%, followed by treatment S3 with 800 mg/L of enzyme giving an extraction yield of 62.0%. This amply demonstrated that digesting the plant cell walls indeed facilitated the release of EA.

The use of enzymes in extraction processes has been previously reported to enhance recovery of bioactives. In ginger, pre-treatment with enzymes prior to solvent extraction resulted in higher yields of oleoresin and gingerol than the controls. Acetone extraction after enzyme treatment yielded 20% oleoresin and 12.2% gingerol compared to the control (15% oleoresin and 6.4% gingerol) [92]. EAE also resulted in significantly higher yield for kaempferol-glucosides from cauliflower (kaempferol-3-feruloyldiglucoside 37.8 and kaempferol-3-glucoside 58.4 mg rutin equivalent /100 g dry weight) [93]. In citrus peels, EAE also resulted in increased yield of phenolic compounds (25.90–39.72%) [94].

4.4. Conclusion

In this study, EA rich water-based extracts of KP freeze-dried puree, were attained by utilising a food compatible enzyme assisted extraction technique. A CCRD was employed to optimise the extraction process. For concentrate the bioactive EA and increase the level of TPC, solvent concentration % (w/w) and time of reaction (h) were identified as controlling factors. It is possible to employ a mathematical model to obtain KP extracts with elevated EA levels thereby providing a realistic alternative to antimicrobial agents of synthetic origin. The novel extraction method is simple and cost effective and can be adopted by numerous industries. It should dramatically increase the applications for KP in a diverse range of food products where having a water based extract is a preferred option –e.g. as natural preservatives in the food and beverage industry. This method can also be applied to other EA rich plant material like pomegranate and peel of many common fruits which are generated as food processing by-products.

Supporting information

Supporting information associated with this article can be found online. Table S1 - Effect of pH on concentration of AA in KP extracts from initial experiments. Table S2 – Concentration of EA in additional treatment included in the study. Figure S1 Effect of solvent volume (% w/w), enzyme concentration (mg/L) and time (h) on [A] EA concentration (mg/100 g DW) and [B] TPC (mg GAE/g) in the extracts is shown as surface plots.

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Disclosure of conflict of interest

The authors declare no competing financial interest.

Table 13 Chemical characterisation (free and total EA; AA and moisture content) of the initial starting material i.e. freeze dried KP puree

Chemical	Concentration
FEA	520.0 mg/100 g DW
Total EA	1496.0 mg/100 g DW
AA	19183 mg/100 g DW
Moisture	3.9 g/100 g

Table 14 The coded levels of the variables used in the CCRD and responses obtained from the study

o.	Variables X ₁ to X ₃						Responses Y ₁ to Y ₃			Extraction yield	
	X ₁	True values X ₁	X ₂	True values X ₂	X ₃	True values X ₃	Y ₁	Y ₂	Y ₃	Y ₁	Y ₂
1	1	2	1	100	1	12	218.9	70.6	104.74	42.1	0.37
2	0	1.5	0	300	0	7	103.9	66.3	59.89	20.0	0.35
3	-1	1.5	-1	300	-1	-1.40	108.8	83.1	67.52	20.9	0.43
4	1	2	1	500	1	2	53.6	68.0	48.46	10.3	0.35
5	-1	2.34	-1	300	-1	7	98.4	110.5	52.12	18.9	0.58
6	1	1	1	100	1	12	127.5	99.7	79.05	24.5	0.52
7	0	1.5	0	300	0	7	142.6	64.6	85.70	27.4	0.35
8	-1	1.5	-1	300	-1	15.40	266.6	92.8	105.78	51.3	0.48
9	0	1.5	0	300	0	7	150.5	69.4	86.38	28.9	0.36
10	1	2	1	100	1	2	100.0	77.9	58.73	19.2	0.41
11	1	1	1	100	1	2	160.5	71.0	90.86	30.9	0.37
12	0	1.5	0	300	0	7	108.2	63.7	66.98	20.8	0.33
13	0	1.5	0	300	0	7	130.1	112.1	82.04	25.0	0.58
14	1	1	1	500	1	12	178.9	97.4	91.38	34.4	0.51
15	-1	0.66	-1	300	-1	7	108.8	98.6	67.04	20.9	0.51
16	1	1	1	500	1	2	135.7	65.2	84.60	26.1	0.34
17	1	2	1	500	1	12	160.0	102.3	88.65	30.8	0.53
18	-1	1.5	-1	636.35	-1	7	124.7	64.4	78.61	24.0	0.35
19	0	1.5	0	300	0	7	198.0	104.9	91.82	38.1	0.55
20	-1	1.5	-1	-36.35	-1	7	124.4	106.3	73.29	23.9	0.55

¹The coded levels of 3 variables in the CCRD are shown with the actual quantities. The low settings in the experiment are identified by -1 in coded units and the high settings are identified by 1 in coded units. X₁, solvent volume in % (w/w); X₂, enzyme concentration in mg/L and X₃, time of reaction in h.

²Y₁ = concentration of fEA in mg/100 g DW; Y₂ = concentration of AA in mg/100 g DW and Y₃ = Total phenolic content in mg Gallic acid equivalent/g (mg GAE/g)

Extraction yield for Y₁ and Y₂ are expressed as %. Extraction yield (%) = (weight of the extract x 100) / (weight of the original sample).

Table 15 Response surface regression with coefficients for responses obtained from the study

Model	Y ₁				Y ₂				Y ₃			
	Coef	SE Coef	T	P	Coef	SE Coef	T	P	Coef	SE Coef	T	P
Constant	138.8	11.7	11.86	0.000	80.79	8.20	9.85	0.000	78.57	4.24	18.53	0.000
X ₁	-6.41	7.76	-0.83	0.428	0.40	5.44	0.07	0.943	-5.15	2.81	-1.83	0.097
X ₂	-6.42	7.76	-0.83	0.427	-3.75	5.44	-0.69	0.506	-0.83	2.81	-0.30	0.774
X ₃	36.68	7.76	4.72	0.001	7.63	5.44	1.40	0.191	10.66	2.81	3.79	0.004
X ₁ X ₁	-11.99	7.56	-1.59	0.144	6.01	5.30	1.13	0.283	-5.31	2.74	-1.94	0.081
X ₂ X ₂	-3.57	7.56	-0.47	0.647	-0.67	5.30	-0.13	0.902	0.48	2.74	0.17	0.865
X ₃ X ₃	17.75	7.56	2.35	0.041	0.13	5.30	0.03	0.980	4.26	2.74	1.56	0.151
X ₁ X ₂	-16.5	10.1	-1.62	0.136	3.74	7.11	0.53	0.61	-4.06	3.68	-1.10	0.296
X ₁ X ₃	26.9	10.1	2.65	0.024	-4.24	7.11	-0.60	0.565	11.41	3.68	3.10	0.011
X ₂ X ₃	8	10.1	0.79	0.45	5.65	7.11	0.79	0.446	1.60	3.68	0.43	0.673
	R ² = 81.18%				R ² = 33.60%				R ² = 78.12%			
	R ² (adj) = 64.23%				R ² (adj) = 0.00%				R ² (adj) = 58.44%			
	R ² (pred) = 36.40%				R ² (pred) = 0.00%				R ² (pred) = 29.08%			

¹X₁ is solvent concentration (% w/w), X₂ enzyme concentration (mg/L) and X₃ is time (h)

²Y₁ is fEA (mg/100 g DW), Y₂ is AA (mg/100 g DW), and Y₃ is TPC (mg GAE/g)

Table 16 Analysis of variance for lack of fit of models obtained from the CCRD study.

Model	Y ₁					Y ₂					Y ₃				
	DF	Adj SS	Adj MS	F	P	DF	Adj SS	Adj MS	F	P	DF	Adj SS	Adj MS	F	P
Regression	9	35498.7	3944.3	4.79	0.011	9	2046.64	227.404	0.56	0.800	9	3860.15	428.91	3.97	0.021
Linear	3	19495	6498.3	7.89	0.005	3	989.71	329.902	0.82	0.514	3	1922.93	640.98	5.93	0.014
X ₁	1	561.7	561.7	0.68	0.428	1	2.15	2.148	0.01	0.943	1	362.84	362.84	3.36	0.097
X ₂	1	563.7	563.7	0.68	0.427	1	192.18	192.178	0.48	0.506	1	9.42	9.42	0.09	0.774
X ₃	1	18369.6	18369.6	22.31	0.001	1	795.38	795.38	1.97	0.191	1	1550.67	1550.67	14.35	0.004
Square	3	7541.5	2513.8	3.05	0.079	3	546.24	182.081	0.45	0.723	3	744.59	248.2	2.30	0.140
X ₁ X ₁	1	2073.1	2073.1	2.52	0.144	1	521.1	521.104	1.29	0.283	1	406.28	406.28	3.76	0.081
X ₂ X ₂	1	183.7	183.7	0.22	0.647	1	6.46	6.458	0.02	0.902	1	3.28	3.28	0.03	0.865
X ₃ X ₃	1	4541.7	4541.7	5.52	0.041	1	0.26	0.255	0.00	0.980	1	261.49	261.49	2.42	0.151
Interaction	3	8462.2	2820.7	3.43	0.06	3	510.69	170.229	0.42	0.742	3	1192.63	397.54	3.68	0.051
X ₁ X ₂	1	2170.4	2170.4	2.64	0.136	1	112.07	112.066	0.28	0.610	1	131.58	131.58	1.22	0.296
X ₁ X ₃	1	5784.1	5784.1	7.03	0.024	1	143.57	143.569	0.35	0.565	1	1040.65	1040.65	9.63	0.011
X ₂ X ₃	1	507.7	507.7	0.62	0.45	1	255.05	255.05	0.63	0.446	1	20.39	20.39	0.19	0.673
Residual Error	10	8232.4	823.2			10	4045.29	404.529			10	1080.89	108.09		

Lack-of-Fit	5	2342.2	468.4	0.4	0.833	5	1523.02	304.604	0.60	0.703	5	298.72	59.74	0.38	0.843
Pure Error	5	5890.2	1178			5	2522.27	504.455			5	782.17	156.43		
Total	19	43731.1				19	6091.93				19	4941.04			

¹X₁ is solvent concentration (% w/w), X₂ enzyme concentration (mg/L) and X₃ is time (h), ²Y₁ is EA (µg/ml), Y₂ is AA (µg/ml) and Y₃ is TPC (mg GAE/g)

Table 17 Treatments selected for verification

Treatments	Solvent volume (% w/w)	Enzyme conc (mg/L)	Time (h)	Expected EA content (mg/100 g DW)	Actual EA content (mg/100 g DW)	Absolute Error (AE)
1	2.34	636.36	15.41	231.1	204.1 ^a	27.0
2	2.1	550	14.5	170.4	175.7 ^b	5.3
3	1.9	450	15	149.4	151.1 ^c	1.6
4	2	300	14	144.5	130.8 ^d	13.7
5	1.9	300	14	129.4	101.1 ^e	19.3
6	1.8	300	14	114.4	113.6 ^{de}	0.8
7	1.7	300	14	84.3	99.3 ^f	15.1

¹Different letters (i.e., a, b, c, d, e and f) across rows denote significant differences between mean EA concentrations in each Treatments according to a Tukey–Kramer HSD.

Table S1 – Effect of pH on concentration of AA in KP extracts from initial experiments

pH	Concentration of AA (mg/100 g DW)
3.4	521
7.5	313
8.5	361

Table S2 – Concentration of EA in additional treatment included in the study

Treatments	Solvent volume (% w/w)	Enzyme conc (mg/L)	Time (h)	EA content (mg/100 g DW)	Extraction yield (%)
S1	3.5	0	24	208.00 ^c	33.3
S2	3.5	1000	24	471.00 ^a	75.4
S3	3	800	22	387.00 ^b	62.0

¹Different letters (i.e., a, b, c) across rows denote significant differences between mean EA concentrations in each Treatments according to a Tukey–Kramer HSD.

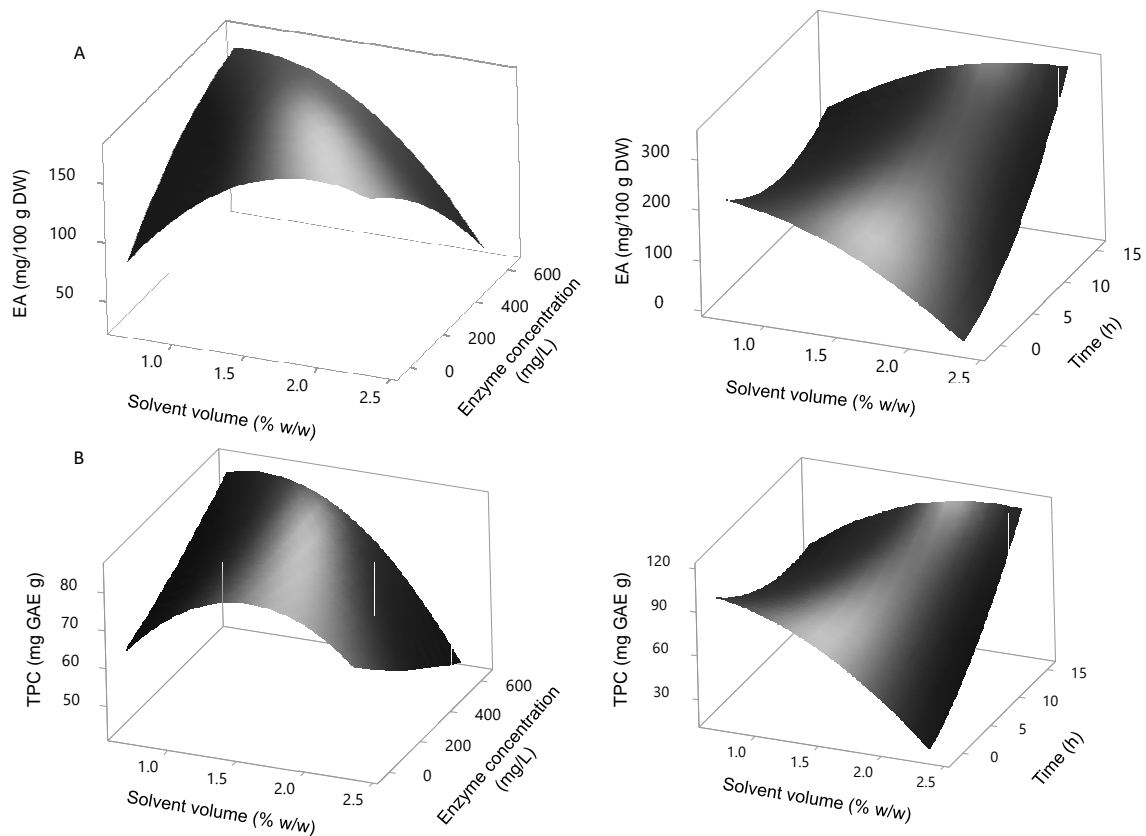


Figure S1 Effect of solvent volume (% w/w), enzyme concentration (mg/L) and time (h) on [A] EA concentration (mg/100 g DW) and [B] TPC (mg GAE/g) in the extracts is shown as surface plots.

Chapter 5 Chemical and Nutritional Composition of *Terminalia ferdinandiana* (Kakadu Plum) Kernels: A Novel Nutrition Source

Abstract:

Terminalia ferdinandiana (Kakadu plum) is a native Australian fruit. Industrial processing of *T. ferdinandiana* fruits into puree generates seeds as a by-product, which are generally discarded. The aim of our present study was to process the seed to separate the kernel and determine its nutritional composition. The proximate, mineral and fatty acid compositions were analysed in this study. Kernels are composed of 35% fat, while proteins account for 32% dry weight (DW). The energy content and fiber were 2065 KJ/100 g and 21.2% DW, respectively. Furthermore, the study showed that kernels were a very rich source of minerals and trace elements, such as potassium (6693 mg/kg), calcium (5385 mg/kg), iron (61 mg/kg) and zinc (60 mg/kg) DW, and had low levels of heavy metals. The fatty acid composition of the kernels consisted of omega-6 fatty acid, linoleic acid (50.2%), monounsaturated oleic acid (29.3%) and two saturated fatty acids namely palmitic acid (12.0%) and stearic acid (7.2%). The results indicate that *T. ferdinandiana* kernels have the potential to be utilized as a novel protein source for dietary purposes and non-conventional supply of linoleic, palmitic and oleic acids.

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5.1 Introduction

Terminalia is the second-largest genus of the combretaceae family, with approximately 250 species growing in tropical and subtropical countries around the world [95]. More than 30 species of *Terminalia* occur in northern regions of Australia [96]. More than 50 species of *Terminalia* have found utility as ingredients in foods and beverages worldwide, as preservatives, raw material for wine and palm sugar, eaten raw and as food supplements [97]. The nutritional and therapeutic properties of *Terminalia* genus can be attributed to the presence of a wide range of phytochemicals, such as phenolic compounds, which encompasses phenolic acids, gallotannins, ellagitannins, proanthocyanidins and other flavonoids [97].

Terminalia ferdinandiana, popularly known as Kakadu plum, is native to Australia. Indigenous Australians (Aboriginal people) have been using this plant as a food and medicine for centuries, for example, refreshing drinks are made from fresh or dried fruits in Western Australia [96]. Fruits are traditionally used as an antiseptic, soothing balm, in colds and flu and in treating a headache [98]. A number of research outcomes have been reported on the antioxidant [19, 20, 99], antibacterial [100, 101], anti-inflammatory [102], anti-apoptotic, cytoprotective and anticancer activities [103] of *T. ferdinandiana* fruits and leaves. Phytochemical analysis has revealed that *T. ferdinandiana* fruit is a rich source of Ellagic acid and its hydrolysable tannins, ellagitannins [22]. Recently, a food safe extraction method of *T. ferdinandiana* fruits for commercial use in the food industry has been suggested [98]. Additionally, a systematic evaluation of the changes in quality and bioactivity of the fruits of *T. ferdinandiana* during processing, packaging and storage has been performed, and key chemical markers have been identified to enable standardized products to be delivered to the consumer [101].

In the last two decades, seeds and kernels from the *Terminalia* genus have been researched and reported for their nutritional properties and health-promoting activities [104-106]. To understand the relationship between the internal quality and genotype of the plant, studies of nut and kernels characteristics and composition are very common. During the industrial processing of *T. ferdinandiana* fruits, the seeds are treated as waste products and have been discarded. Recent studies on many fruit seeds or kernels have shown that they have the potential to be utilized as ingredients for value addition, they are very nutritious and could be used as alternate sources of essential minerals, fatty acids, and proteins [107-110].

To date, no reports have been published on the utilization of the by-products of *T. ferdinandiana* and there is no investigation on the chemical and nutritional composition of *T. ferdinandiana* kernels. The aim of this study was to determine the potential use of the by-product of *T. ferdinandiana* in the industry by determining proximate, mineral and fatty acid compositions to ascertain its nutritional value and potential as a source of food supplement ingredients for the food industry.

5.2 Materials and Methods

5.2.1 Sample Collection and Preparation

Fully ripe and mature fruits of *T. ferdinandiana* were collected from over 600 trees, giving a total harvest of 5000 kg, from native bush land covering an area of 20,000 Km² in Northern Territory, Australia in 2015 and were authenticated by the experts in Queensland Herbarium, Brisbane Botanic Gardens Mt Coot-tha, Queensland, Australia, where botanical specimens were retained for future reference (AQ522453). Seeds were collected as the by-products after pureeing of the fruits, and were stored at -20 °C prior to analysis. *T. ferdinandiana* tissues are illustrated in Figure 29.



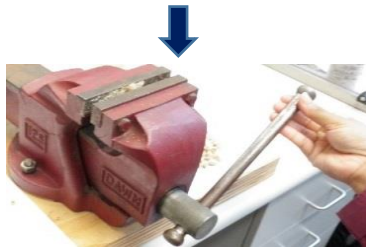
Figure 29 *Terminalia ferdinandiana* tissues. (A) Fresh fruits, (B) Dried seeds, (C) Kernels.

5.2.2 Processing of Seeds

The frozen seeds were thawed, washed and cleaned manually several times to remove the pulp residues with double distilled water. The seeds were then dried in the oven for 48 h at 40 °C. After drying, the seeds were individually cracked using an Engineers' vice size 125 (DAWN, Melbourne, Victoria, Australia) to release the kernels from the seedcoats. The seedcoats and kernels were kept, processed and analyzed separately. The kernels were kept in air-tight containers and placed at -20 °C for further analysis. A flowchart depicting the processing of the seeds is illustrated in Figure 30. During the processing of fruits in the industry in a batch of 100 kg of fruits, 22 kg of seeds can be obtained as by-product. The average weight of a dry seed is 0.5 g and the moisture content is 2.8%. Average weight of a kernel is 0.04 g. The kernel is 8% of the weight of the seed. 1 kg of dry seeds can deliver 80 g of kernels.



Dried seeds of *Terminalia ferdinandiana*



Seeds are cracked in an Engineers' vice



Seedcoats and kernels are separated



Seedcoats



Kernels

Figure 30 Processing of *Terminalia ferdinandiana* seeds to release kernels.

5.2.3 Proximate Composition Analysis

Physicochemical analysis of the kernels of *T. ferdinandiana* was performed at an accredited laboratory (National Association of Testing Authorities (NATA), Symbio Alliance, Eight Mile Plains, Queensland, Australia). The following analyses were done according to AOAC methods: vitamin C, protein (AOAC 990.03, 992.15 & 992.15), fat (AOAC 991.36), saturated, mono-unsaturated, polyunsaturated and trans-fat (AOAC 996.06), moisture (AOAC 925.10), ash (AOAC 923.03), sodium (using ICP-AES), total sugar (AOAC 977.20) and dietary fiber (AOAC 985.29, 991.42 and 993.19). Available carbohydrate and energy were calculated using FSNZ (Food Standards Australia New Zealand) codes.

5.2.4 Fatty Acid Analysis

Dried kernels (ca. 1 g) were finely chopped and extracted with chloroform and methanol (2:1) followed by agitation at room temperature for one hour. The mixture was then centrifuged for 5 min at 3500 rpm and the whole process was repeated twice. The lipid extracts were mixed with boron trifluoride (BF₃)-methanol reagent (20%) and fatty acids were derivatized to fatty acid methyl esters [111]. The methyl esters of the fatty acids were dissolved in heptane and analyzed by GC-MS (Shimadzu QP2010, Shimadzu Corporation, Tokyo, Japan). The GC conditions were as follows: Restek stabilwax capillary column (30 m × 0.25 mm ID × 0.5 µm film thickness) (Restek Corporation, Bellefonte, PA, USA); oven temperature program: the column held initially at 100 °C after injection and the final temperature was increased to 250 °C, total program time was 39:00 min; injector temperature: 250 °C; carrier gas: Helium; linear gas velocity: 42.7 cm/s; column flow: 1.10 mL/min; split ratio: 50.00; injection volume: 1.0 µL. MS conditions were regulated as follows: ion source temperature: 200 °C; interface temperature: 250 °C; mass range: 35–500 atomic mass units. Identification of the compounds was carried out by comparison of their retention times and mass spectra with corresponding data from a standard food industry FAME Mix (Restek Corporation, Bellefonte, PA, USA). A total of 32 individual compounds were analyzed and only the detected ones were recorded along with their quantity compared with the standard.

5.2.5 Mineral and Trace Element Analysis

Accurately weighed 0.3 g of dried *T. ferdinandiana* kernels were taken into teflon vessels of microwave digestion system (MarsXpress, CEM, Matthews, NC, USA) and high-purity nitric acid (70% w/w, 4 mL) was added [44]. The samples were left overnight at room temperature for slow digestion gasses to evolve. The vessels were sealed and microwave-digested at increased temperature with set digestion time [43]. The digested samples were diluted and made up to 40 mL with high-purity water (Milli-Q Element system, Millipore, Bedford, MA, USA). The levels of minerals and trace elements were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES, Vista AX, Varian Australia, Mulgrave, Victoria, Australia), to measure lower levels and for greater sensitivity the analysis was carried out using ICP-MS (7500a, Agilent, Tokyo, Japan). The ICP-MS was equipped with an auto-sampler, integrated sample introduction system and a helium octopole reaction cell to remove polyatomic interferences (⁴⁰Ag ³⁵Cl on ⁷⁵As). The operating conditions were as follows: radio frequency (RF) power 1350W, argon carrier gas 0.8 L/min and helium reaction cell gas flow rate 4.5 mL/min. The standard reference materials were used for the quality control and assurance and treated similarly to the samples throughout the study. The data of quality control and assurance are presented in the Supplementary Material (Table S1).

5.2.6 Statistical Analysis

The data were calculated using Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA). The results are expressed as the mean of triplicate experiments unless otherwise specified.

5. Results and Discussion

5.3.1 Proximate Composition

The proximate composition of *T. ferdinandiana* kernels is summarized in Table 18. Moisture content is an important parameter in terms of the physicochemical properties of plant parts, due to the fact that low moisture content is beneficial for retaining the quality and shelf life of seeds, and this also decreases the susceptibility for microbial growth, premature seed germination, unwarranted fermentation and undesirable biochemical changes. The moisture content is only 4% in the kernels of *T. ferdinandiana*, presenting minimum risk for microbial

growth and undesirable biochemical changes upon storage. A comparable moisture content of 5.5% was reported for *T. catappa* kernels [106]. Furthermore, the results of the present study showed that the kernels were abundant in proteins, with a content of 32% relative to the standard. Protein content of *T. ferdinandiana* is higher than that of *T. catappa* kernels (20.1%) [106]. *T. sericea* kernels contain 46.2% proteins [112], which is higher than *T. ferdinandiana* kernels. Recommended dietary allowances (RDA) for protein are 56 g for a 70 kg man [113]. As the protein content of *T. ferdinandiana* kernels is high, it could be used as an alternative source or dietary supplement for consumers with restricted and compromised protein intake from other sources. Ash content is 4% and dietary fiber 21.2% in *T. ferdinandiana* kernels. Ash content signifies the presence of minerals in the kernel, tissue and the high content of fiber can help in improving the gut health and digestion. The lipid content in *T. ferdinandiana* kernels was found to be 35.1%, with less than 1% in the trans form. The WHO recommends that no more than 1% of our daily energy intake come from trans-fatty acids (TFAs). Based on the present results, it can be concluded that the fat content of *T. ferdinandiana* kernels is devoid of any trans-fat-associated health risk. The fat content in *T. sericea* seed is 32.5% [112], 64.7% in *T. catappa* kernels [106], and in *T. catappa* seed it is 32.7% [105], while in *T. ferdinandiana* kernels it is 35.1%. *T. ferdinandiana* kernels can supply 50% of the RDA of fat with saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fats are in the order of 5.8%, 9.8%, and 19.4%. These proportions are similar to the fatty acid profile determined by GC-MS (Table 19). A diet rich in PUFA is important for the structure and function of proteins, receptors, enzymes and transport molecules whereas the MUFA content may lower blood cholesterol levels, modulate immune function and can improve the fluidity of high-density lipoproteins (HDL) [114]. The results of our present study thus suggest that *T. ferdinandiana* kernels have the potential to be used as an alternative source of MUFA and PUFA.

Table 18. Proximate composition of *Terminalia ferdinandiana* kernels.

<i>T. ferdinandiana</i> Kernels		Nutrition Information (Servings per Package: 1) (Serving Size: 100 g)		
		Quantity per Serving	% Daily Intake */Serving	
Protein	% (w/w)	32.0	32 g	64%
Fat	% (w/w)	35.1	35.1 g	50%
Saturated Fat	% (w/w)	5.8	5.8 g	24%
Mono-unsaturated Fat	% (w/w)	9.8	9.8 g	
Poly-unsaturated Fat	% (w/w)	19.4	19.4 g	
Trans Fat	% (w/w)	<0.01	<0.1 g	
Moisture (air)	% (w/w)	4.0		
Ash	% (w/w)	4.5		
Dietary Fibre (Total)	% (w/w)	21.2	21.2 g	71%

Dry Matter	% (w/w)	96.0		
Crude Fibre	% (w/w)	11.6		
Energy	KJ/100 g	2065	24%	
Total Sugar	g/100 g	0.49	<1 g	<1%
Available Carbohydrate	%	3.2	3.2 g	1%
Sodium (Na)	mg/100 g	8.6	8.6 mg	<1%

* Percentage daily intakes are based on an average adult diet of 8700 KJ. Results are expressed as the mean of triplicate experiments.

Table 19 Fatty acid profile of *Terminalia ferdinandiana* kernels expressed as percentage (\pm SD) of the total fatty acid profile as determined by FAME GC-MS analysis.

Fatty Acid	Percentage (%) \pm SD
<i>Saturated</i>	
C14:0 Methyl myristate	0.1 \pm 0.01
C16:0 Methyl palmitate	12 \pm 0.53
C18:0 Methyl stearate	7.2 \pm 0.13
C20:0 Methyl arachidate	0.7 \pm 0.06
C22:0 Methyl behenate	0.4 \pm 0.13
TSFA	20.4
<i>Monounsaturated</i>	
C16:1 (<i>cis</i> -9) Methyl palmitoleate	0.1 \pm 0.06
C18:1 (<i>cis</i> -9) Methyl oleate	29.2 \pm 0.68
C20:1 (<i>cis</i> -11) Methyl eicosenoate	0.1 \pm 0.04
TMUFA	29.4
<i>Polyunsaturated</i>	
C18:2 (all- <i>cis</i> -9,12) Methyl linoleate	50.2 \pm 1.1
PUFA	50.2

SFA vs. UFA	0.25:1
MUFA vs. PUFA	0.6:1

SFA: saturated fatty acids; UFA: unsaturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; TSFA: total saturated fatty acids; TMUFA: total monounsaturated fatty acids; TPUFA: total polyunsaturated fatty acids; data presented as a mean \pm SD of triplicate experiments.

5.3.2 Mineral and Trace Element Composition

The macro and trace element composition of *T. ferdinandiana* kernels evaluated in this study is presented in Table 20, and the non-essential and heavy metal composition is presented in Table 21. Minerals are essential for proper functioning of the body, and a deviation from the appropriate amounts can cause numerous diseases, clinical syndromes, and illnesses associated with the deficient intake, as well as overuse over time or at a certain time period of life. Hence, reference values are established and reviewed periodically to stipulate the mineral levels that will meet the needs of healthy human individuals. The RDA of the evaluated minerals for a healthy male adult of 70 kg body weight are also presented in Table 20 and Table 21. The high macro-mineral contents were found to be phosphorus 872.8 mg/100 g DW, and calcium at 538.5 mg/100 g DW, while sodium was 120.3 mg/100 g DW and magnesium 421.1 mg/100 g DW (Table 20). These results indicated that the kernels could significantly contribute to the mineral intake in humans. Mineral composition analysis of kernels from *Terminalia* genus is scarce and one report on the mineral composition of *T. catappa* seeds included phosphorus (10), calcium (36.1), magnesium (26.4), iron (375), sodium (5) and potassium (350), in mg/100 g [14]. Kernels from bayberry (*Myrica rubra*) were reported as an abundant source of potassium, containing 780 mg/100 g [115]. The potassium content of white Chinese olive (*Canarium album*) is also high, at 587 mg/100 g [110]. In our study, *T. ferdinandiana* kernels contained 669.3 mg/100 g of potassium, which can be compared to the potassium content of bayberry, Chinese olive, and black Chinese olive. Moreover, the phosphorus levels of *T. ferdinandiana* kernels (872.8 mg/100 g DW) seemed to be much higher compared to the levels of bayberry (32.9 mg/100 g) [107]. Important trace elements found in *T. ferdinandiana* were zinc, manganese, copper and iron at levels of 6, 9.1, 2.5 and 6.1 mg/100 g, respectively, and are within the RDA and AI values. It can be suggested that *T. ferdinandiana* kernels can be a valuable dietary source of these trace elements. These trace elements are important constituents of various proteins and enzymes of our body which are involved in macronutrient metabolism [106]. The levels of molybdenum, arsenic, mercury, cadmium were found at less than 0.1 mg/kg in the kernels, while the lead level was found at 0.13 mg/kg (Table 21). Heavy metal exposure poses significant health risks, which can cause life-threatening diseases, and the toxic effects are influenced by chemical forms, absorption rate, and solubility in body fluids. The toxicity of arsenic depends on the chemical form. The inorganic form of arsenic is more toxic than organic arsenic [44]. Mercury can be readily absorbed and incorporated into tissue proteins and can cause detrimental effects on health. The bioaccessibility and bioavailability of the exposed heavy metals can again vary depending on the chemical forms, time and route of exposure, duration, and concentration of the exposed metals. However, the levels of heavy metals found in *T. ferdinandiana* kernels were within the regulatory limits, suggesting that they may not impose any health risk.

Table 20 Major and trace elements composition of *Terminalia ferdinandiana* kernels (mg/100 g DW).

Mineral Composition															
Major Elements						Micro/Trace Elements									
	Ca	Mg	Na	K	P	Fe	Zn	Mn	Cu	Co	Ni	Mo	Se	Sr	B
Kernels (mg/100 g DW)	538.5	421.1	120.3	669.3	872.8	6.1	6.0	9.1	2.5	0.02	0.17	<0.01	0.02	5.0	0.83
DRI	1200 AI ^a	350 EAR ^a	1.3 AI ^a	4.7 AI ^a	700 RDA ^a	8 RDA ^a	11 RDA ^a	2.3 AI ^a	700 RDA ^a	0.12A I ^a	1.0 UL ^a	34 EAR ^a	45 EAR ^a	1–5 RDA ^a	20 UL ^a
Units	mg	mg	g	g	mg	mg	mg	mg	µg	µg	mg	µg	µg	mg	mg

Results are expressed as the mean of duplicate experiments. RDA—recommended dietary allowance, AI—adequate Intake, UL—tolerable upper intake level, DRI—Dietary reference intakes; EAR—Estimated average requirement. ^aInstitute of Medicine. 2006. *Dietary Reference Intakes: The Essential Guide to Nutrient Requirements*. Washington, DC: The national academic press. ISBN: 978-0-309-15742-1. DOI: <http://doi.org/10.17226/11537>. URL <https://www.nap.edu/read/11537/chapter/1>. Accessed 17 February 2017.

Table 21 Non-essential elements and heavy metal compositions of *Terminalia ferdinandiana* kernels (mg/100 g DW).

	Non-Essential Elements	Heavy Metals			
	Ba	As	Hg	Pb	Cd
Kernels (mg/100 g DW)	0.31	<0.01	<0.01	0.013	<0.01
DRI	0.02 UL ^a	12.5–25 UL ^b	5 UL ^c	25 UL ^c	2.5 UL ^d
Units	mg/kg BW	µg/kg BW/week	µg/kg BW/week	µg/kg BW/week	µg/kg BW/Week

Results are expressed as the mean of duplicate experiments. UL—tolerable upper intake level, DRI—Dietary reference intakes; BW—body weight; ^a Scientific Committee on Health and Environmental Risks (2012). Assessment of the tolerable daily intake of barium. European commission. URL http://ec.europa.eu/health/scientific_committees/environmental_risks/docs/scher_o_161.pdf. Accessed 17 February 2017; ^b Food Safety authority of Ireland (2009). Mercury, Lead, Cadmium, Tin, and Arsenic in Food. Toxicology factsheet series, Issue no. 1. URL www.fsai.ie/WorkArea/DownloadAsset.aspx?id=8412. Accessed 17 February 2017; ^c [116]; ^d Statements on the tolerable weekly intake for cadmium. Panel on contaminants in the food chain. EFSA, 2011, 9(2). URL <https://www.efsa.europa.eu/en/efsajournal/pub/1975>. Accessed 17 February 2017.

5.3.3 Fatty Acid Composition

Fatty acids can be considered the main constituent of all oils and may include saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids [114]. Besides providing high-quality food, vegetable oils can also provide essential nutrients that have a particular clinical significance. PUFA are present in membrane phospholipids in some tissues and can also act as precursors for prostaglandin hormones [117]. On the other hand, SFA are reported to increase cardiovascular disease risk and sometimes can potentiate the risk of cancer and autoimmune disorders [118]. The principal fatty acid components in *T. ferdinandiana* kernels were palmitic (SFA, 12%); oleic (MUFA, 29.3%); and linoleic (PUFA, 50.2%) acids (Table 19).

SFA are reported to impact human health by increasing the plasma low-density lipoprotein (LDL) cholesterol. However, some of the SFA are also reported to increase the high-density lipoprotein (HDL) cholesterol and some of them have little or no significant role in increasing or decreasing the LDL and HDL cholesterol levels [119]. The main SFA found in *T. ferdinandiana* kernels are myristic (0.09%), palmitic (12%), stearic (7.2%), arachidic (0.76%) and behenic (0.4%) acid. The level of myristic acid in *T. ferdinandiana* kernels is only 0.09%.

Unsaturated fatty acids can exist in *cis*- or *trans*-configuration. *Cis*-configuration is found in naturally occurring unsaturated fatty acids, while *trans*-configuration is the result of processing. *Cis*-unsaturated fatty acids are known as potent inducers of adiposomes also referred to as lipid droplets and they have important roles in cell signaling, regulation of lipid metabolism and control of the synthesis and secretion of inflammatory mediators [120]. The MUFA present in *T. ferdinandiana* kernels are palmitoleate (0.2%), oleate (29.3%) and eicosenoate (0.11%). Among the MUFA, oleic acid is the most abundant one found in *T. ferdinandiana* kernels. Oleic acid has been reported to act as an anti-inflammatory and anti-apoptotic agent. The anti-inflammatory mechanism includes down-regulating cyclooxygenase-2 and inducible nitric oxide synthase through the activation of nuclear factor-kappa B [119]. Oleic acid may promote insulin resistance which is contrary to the PUFA which protects from insulin resistance [119]. Oleic acid has also been reported to attenuate blood pressure and risk of developing hypertension [121]. The potential use of *T. ferdinandiana* kernels as a dietary source of oleic acid in reducing the risk and attenuating hypertension requires further investigation. Previous reports on some of the seeds and kernels of the family Combretaceae had reported oleic acid as the most abundant unsaturated fatty acid found in this family [122].

Essential PUFA are α -linolenic (18:3, *n*-3) and linoleic acid (18:2, *n*-6), from which other important PUFA are derived. Recently, essential fatty acids (EFA) have been considered as functional food components and nutraceuticals [120]. Documented roles of EFA include cardioprotective effect (due to their considerable antiatherogenic, antithrombotic, anti-inflammatory, antiarrhythmic, hypolipidemic effects), the fluidity of biological membranes, the function of membrane enzymes and receptors, modulation of eicosanoids production, blood pressure regulation and metabolism of minerals [120]. EFA are also reported to reduce the risk of cardiovascular, cancer, osteoporosis, diabetes and some other serious diseases due to their complex effects on concentrations of lipoproteins [120]. Linoleic acid is an unsaturated omega-6 fatty acid that plays a critical role in the maintenance of the structural and functional integrity of the central nervous system (CNS) and retina [112]. A deficiency can cause skin scaling and hair loss [123]. Linoleic acid (C18:2) is the only PUFA found in *T. ferdinandiana* kernels (50.2%). Therefore, it can be suggested that *T. ferdinandiana* kernels can be used as a potential dietary source of linoleic acid which can increase the systemic pool and subsequently help nourish the CNS and retina.

The WHO recommends that total daily energy intake derived from omega-6 PUFA should be 5–8% and from omega-3 PUFA 2% for an adult male. Studies on the seeds of *T. bellirica*

have reported that 40% of the seed is oil and 35% is protein and major fatty acids were linoleic (31%), palmitic (35%) and oleic (24%) acids and the authors have suggested that kernels could be used as a dietary source of linoleic acid [124]. Reported studies on various plants of *Terminalia* genus included that *T. glucausens* contains palmitic acid (34.9%), myristic acid (0.1%) and stearic acid (4.8%), seed oil of *T. superba* contains behenic acid (C22:0; 1.2%) and the oil of *T. catappa* contains stearic acid (5.8%), myristic acid (1.21%) and arachidic acid (1.3%) [122]. Variations in the fatty acid composition is very common in plants and may be due to a number of reasons including but not limited to soil composition, climate, and specific geographical locations etc.

Nutritionally, the ratio of unsaturated to saturated fatty acids in edible oils and fats is very important. High levels of saturated fatty acids are desirable to increase oil stability. However, SFA become nutritionally undesirable, because high levels of saturated fatty acids are considered to increase the concentration of LDL, affecting the ratio of LDL to HDL and promoting vascular smooth muscle proliferation [125, 126]. The ratio of UFA/SFA for *T. ferdinandiana* kernels is 4, which can be considered favorable for reducing the risk of cardiovascular complications [125]. Again, the relationship between saturated and polyunsaturated FA content is an important parameter for determination of the nutritional value of oils which is expressed as P/S index. Oils and fats with a P/S index > 1 are considered to have nutritional value. Several studies indicate that a higher P/S index means a smaller deposition of lipids in the body. The P/S indexes of *T. ferdinandiana* kernels and some other common oils and fats are shown in Table 5. The P/S index of *T. ferdinandiana* kernels was 2.45, while safflower oil is 10.55 and coconut fat is 0.005. The fatty acid composition of *T. ferdinandiana* kernels is comparable to the composition of soya bean oil (Table 22).

Table 22 Comparison of the fatty acid compositions of *Terminalia ferdinandiana* kernels with commonly consumed oils and fats.

Type of Oil/Fat	Fatty Acid Composition			P/S Index	Reference
	SFA	MUFA	PUFA		
<i>T. ferdinandiana</i> Kernels	20.4	29.6	50.0	2.45	Current study
Coconut	90.5	8.8	0.5	0.005	[36]
Corn	25.1	26.8	48	1.91	
Cottonseed	22.4	35.4	42	1.87	
Soybean	13.5	28.5	57.5	4.26	
Peanut	19.2	58.5	20	1.04	
Safflower	7.2	16.6	76	10.55	
Linseed	9.65	22.1	68	7.05	
Palm kernel	76	22.5	1.25	0.016	
Sunflower seed	8.8	31.5	59.5	6.76	
Canola	9.6	59.5	30.7	3.2	

There are suggestions to reduce the SFA in the diet to suppress the risk of coronary heart diseases (CHD) and cardiovascular diseases (CVD). However, it is important to note that SFA reduction itself cannot suppress the risk. Mostly, reduction of SFA and TFA with their simultaneous replacement by PUFA could lead to a reduction of the risk of CHD. The SFA content of *T. ferdinandiana* kernels was 20.4%, having a very low amount of myristic acid. Based on our results, it can be concluded that the SFA of *T. ferdinandiana* kernels are unlikely to have detrimental health effects by increasing the LDL cholesterol level. Moreover, the kernels were a good source of linoleic acid suggesting *T. ferdinandiana* kernels as a valuable source of EFA that can be used in feed and food.

5.4 Conclusions

To the best of our knowledge, this is the first study on the nutritional composition of *T. ferdinandiana* kernels. The present study indicated that the kernels contain high levels of protein and lipid. The mineral composition of *T. ferdinandiana* kernels reveal a very good source of fundamental minerals and micronutrients. Furthermore, the kernels can be considered as a potential dietary source of linoleic and oleic acid. From a nutritional point of view, our present results suggest that *T. ferdinandiana* kernels have a high nutritional value and may contribute to a healthy diet. The utilization of kernels as a by-product from processing of *T. ferdinandiana* fruit will generate applications as a source of food supplement ingredients for essential fatty acids and can represent an alternate source of protein in the food and feed industry. Ongoing studies on the quality aspects of *T. ferdinandiana* kernels will also include bioaccessibility and bioavailability to substantiate the nutritional value and potential health effects of this unexploited by product.

Supplementary Materials: The following are available online at www.mdpi.com/link, Table S1: Trace element recoveries from reference materials.

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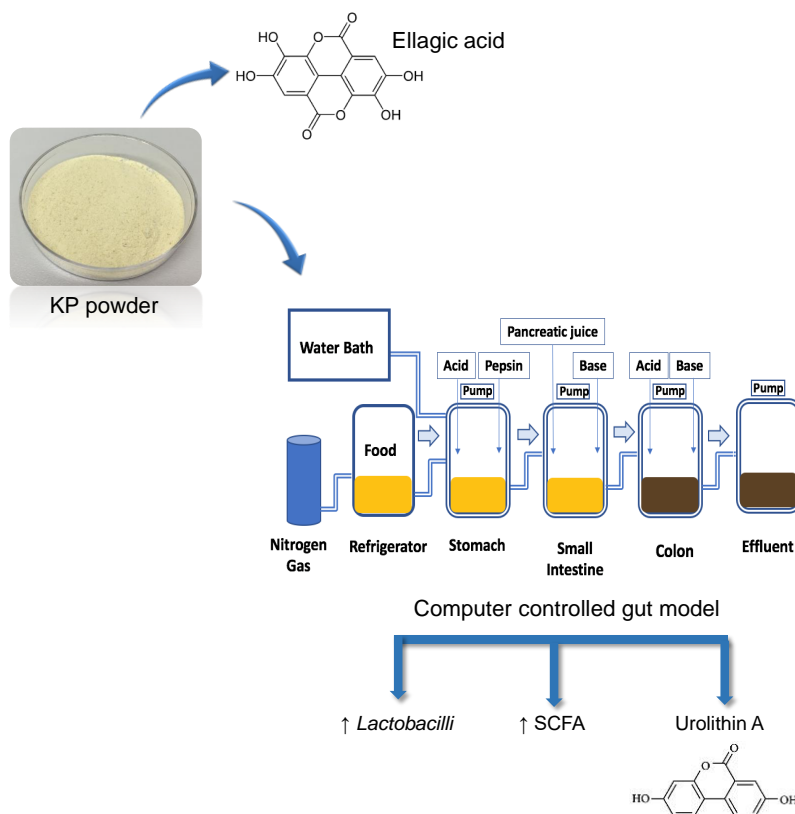
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Chapter 6 Biotransformation of Kakadu plum phytochemicals in a dynamic multistage gastrointestinal model

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Graphical abstract



Highlights

- Kakadu Plum (KP) biotransformation led to an increase in the growth of beneficial *Lactobacilli*.
- KP digested samples significantly increased the concentration of acetic and propionic acid.
- KP treatments maintained the butyric acid concentration at the level as that of the control samples, whereas EA treatment showed significant reduction than the control.
- KP digested samples indicated the presence of Urolithin A.
- Results indicate that consumption of KP may have health promoting properties.

6.1 Introduction

Although consumption of phytochemicals such as polyphenols in fruits and vegetables are associated with health benefits, one major issue with their studies is the limited information about their biotransformation in the gastrointestinal (GI) tract. Polyphenols and other bioactive phytochemicals undergo extensive metabolism by digestive enzymes and gut microflora to generate a variety of smaller molecular weight compounds and hence are poorly absorbed during digestion. Animal and human studies have shown that phytochemicals such as chlorogenic acid, caffeic acid and ferulic acid undergo fermentation by the gut microflora and generate a variety of simpler absorbable phenolic compounds [84, 127].

The human gut microbiota is a complex and dynamic ecosystem that is comprised of thousands of different species of bacteria. The composition of the gut flora varies from individual to individual depending on factors like - region-specific diet, geography, health, host genetics, early microbial exposure and age [128]. These factors are responsible for altering the gut microbiota over time at the individual-level [129]. Despite the variation, the two predominant phyla are - *Bacteroidetes* and *Firmicutes* (>90%) [130, 131]. It is believed that the gut microbial derived phenolic metabolites are responsible for many of the health promoting properties of phytochemicals. Therefore, interaction between phytochemicals and gut microbiota and the subsequent biotransformation of phytochemicals in gut are vital to understand to determine the presumed health promoting properties of plant products. As the healthy intestine is not easily accessible for most research work, a number of *in vitro* models that simulate the intestine are employed for such studies.

To study the effect of digestive processes on polyphenol degradation, *in vitro* gut model studies have typically excluded colonic fermentation. A dynamic multi-reactor gastrointestinal simulator of the human intestinal microbial ecosystem was used in this research work. This model was based on the simulator of the human intestinal microbial ecosystem (SHIME) developed in by Molly et al., 1993 [132]. This model is equipped with multi-compartment simulators of the human gut and was developed due to the awareness that faecal microbiota significantly differ from the *in vivo* colon microbiota in terms of community composition and metabolic activity. Although inoculation of faecal microbiota into single-stage chemostats or static bioreactors was attempted to mimic colon conditions, it was only useful for limited periods of time since environmental parameters such as pH, redox potential, available nutrients and microbial population dynamics constantly change. Thus, static models do not consider the dynamics of transit during digestion or the varying microbial and digestive conditions in different segments of the GI tract [133, 134]. In the dynamic model, the inoculated intestinal microbiota can be maintained over a longer timeframe, and the intermittent supplementation of nutritional medium and the removal of microbial suspension could be simulated [135]. Therefore, a full dynamic gastric model provides a realistic and predictive simulation of the physico-chemical and digestive processing involving the human stomach, small intestine and the colon. The use of this dynamic system allowed monitoring of the biotransformation of the phytochemicals in KP matrix in different segments of the GI tract. Simulated GI model has been applied to study the biotransformation and bioavailability of a number of phytochemicals some of which are summarised in Table 23.

Terminalia ferdinandiana, commonly known as Kakadu plum (KP) is an Australian native plant that was traditionally a part of the the Australian aboriginal diet. This plant has been used as a traditional medicine by the aboriginal community for centuries [136]. KP fruit is known to be one of the richest known sources of vitamin C or ascorbic acid (AA) [4, 20]. KP is also a rich source of phenolic compounds responsible for its high antioxidant activities [20]. *In vitro* studies have indicated that enriched KP extracts possess significant anti-inflammatory and chemopreventive properties [137]. These findings corroborate the traditional medicinal usage of KP. The increase in the awareness of many health promoting properties associated

with KP has led to incorporation of this fruit in diverse products including dietary supplements, skin care and pharmaceutical products as well as an ingredient in the increasingly popular gourmet bush-food market [9, 21]. Many of the health promoting aspects of KP has been attributed to one of its phenolic constituents – Ellagic acid (EA).

EA (2,3,7,8-tetrahydroxy[1]-benzopyrano[5,4,3-cde][1] benzopyran-5,10-dione) is a phenolic compound which until recently was believed to be only present in few fruit such as strawberries [54], *Rubus* berries like raspberries and boysenberries [138] and muscadine grapes [139]. EA has shown significant biological potential including radical scavenging, metal chelating, antibacterial, antiviral, antifibrotic, antiatherogenic, antimutagenic and anticancer activities [140]. Due to these promising potential, search for plant sources for this compound has intensified recently [19]. In plant matrix EA can occur in several forms with significantly different solubilities, reactivities and antioxidant efficiencies and these forms include free EA, EA glycosides and the polymeric tannin – ellagitannin (ET) [46, 54]. Although all the three forms of EA exhibit antioxidative property, the free form has shown the highest antioxidant activity [141]. KP fruit has been reported to contain a high level of EA (in its free form) with one study reporting the levels in the range from 3050 to 14020 mg/100 g DW [21] and 620.6 mg/100 g DW by another study [22]. Despite the difference in the findings both studies clearly showed that KP is a vital source of EA.

It is well known that EA and ET absorption is very low and both undergo gut microbial fermentation to form urolithins [142]. The gut microbial metabolism of ET and EA is shown in Figure 32. The poor bioavailability and the extensive gut catabolism suggest that urolithins rather than ET or EA may be the actual bioactive molecules [143].

6.2 Materials and methods

6.2.1 Materials

KP freeze-dried powder was purchased from Traditional Homeland Enterprise (T.H.E., Victoria, Australia) and milled into a fine consistency using a ball mill (MM301 cryomill, Retsch GmbH, Haan, Germany). The powder was stored at -20°C until further analysis. Ellagic acid (EA), (>95% purity) were purchased from Sigma-Aldrich Inc. (Canada) and included as a reference control.

6.2.2 Computer controlled dynamic human gastrointestinal (GI) model

The GI model used reactors representing the stomach (V1), small intestine (V2; duodenum, jejunum and ileum), and the colon (V3). Batch-culture fermentation was performed based on the method of Tzounis et al. (2008) with modifications [144].

6.2.3 Preparation of faecal slurry

Faecal samples for study were collected from one healthy, non-smoking individual with no history of GI diseases or antibiotic use in the 6 months prior to the study. In order to prepare the faecal solution for the study, faecal samples were freshly collected (on the day of the experiment) and processed within 4 h. In order to prepare the faecal slurry, samples were diluted 1:10 (w/v) with sterile 1X PBS (pH 7.4). The samples were manually filtered through a strainer and stirred before injecting into the GI model (5 ml faecal slurry per vessel).

6.2.4 Batch fermentation

6.2.4.1 Stabilization period

In one water-jacketed fermenter vessel (300 ml), filled with 225 ml of pre-sterilized basal growth medium (composition is summarized in Table 24) was inoculated with 15 ml of faecal

slurry. The content of the vessel was magnetically stirred, pH was adjusted and incubated at 37 °C using a circulating water bath. The pH was maintained at pH 6.8 by Raspberry Pi Model B+ (Raspberry Pi Foundation, Cambridge, UK) connected with a pH circuit (Atlas Scientific, NY, USA) and two miniature peristaltic pumps (Takasago Electric, Inc., Japan) to deliver acid (5 N) and base (5 N), to mimic conditions located in the distal region of the human large intestine (anaerobic; 37°C; pH 6.8). Anaerobic conditions were maintained by purging the vessel with oxygen-free nitrogen gas at 15 ml/min. The temperature of the simulator was kept at 37°C (pH was adjusted to 6.8). The stabilization was carried out for 24 h.

6.2.4.2 Enzymatic digestion

In order to carry out enzymatic digestion, 100 ml autoclaved distilled water (ddH₂O) was placed into the reactor vessels in the GI simulator. The reactor vessels were maintained at 37°C with continuous stirring and nitrogen flushing and the pH of the reactors were adjusted to 6.8. This was followed by addition of KP powder or EA.

The level of EA (free form) in the freeze-dried sample used here is 979 mg/100 g DW. As EA is not considered to be essential for humans, there is no recommended daily intake (RDI) value set for EA. However, KP is also a rich source of AA and the level of AA in the KP sample used here is 20,000 mg/100g. The RDI for AA for healthy 76-kg men is 40 mg and for healthy 62 kg women is 30 mg. It was reported that 200 mg of AA a day is the maximum amount that can be absorbed by human cells, making higher dosing on a daily basis futile. Many currently available KP 'food supplements' contains at least 1 g of KP powder in a capsule form. There are some products that sell KP powder in a ready to drink form which the consumer can take at levels where AA concentration can exceed than the recommended dose. In order to reflect these variation of KP dosage that exists in currently available products, two values of KP were selected. A lower dose of 1g (with a safe limit of AA of 200 mg) and a higher dose of 5 g (with an overdose of AA of 1000 mg). EA (1 g) was selected as a reference control.

The vessels were adjusted to pH 7 and α -amylase (A31176, Sigma, 707.83 mg dissolved in 1.5 ml dH₂O per vessel) was added and incubated for 15 min at 37°C. After 15 min incubation, the pH of the vessels were adjusted to 2 using 1N HCl and pepsin solution (1.67 g dissolved in 2 ml of 0.1 N HCl) was added and incubated for 1 h. At the end of pepsin digestion, the pH was adjusted to 8 using 1N NaOH. Pancreatic juice (prepared by dissolving 485.32 mg of bile extract (8631, Sigma) and 79.416 mg pancreatin (P3292/1750, Sigma) in 1 ml ddH₂O) was added to the vessels followed by incubation for 1.5 h. At the end of pancreatic juice digestion, pH of each vessel was adjusted to 6.8. Samples (5 ml) were collected at time points 0, 8, 12, 36, 24, 48 and 72 h (Figure 33). Samples were immediately used for bacterial enumeration. Remaining samples were stored at -80°C until further use. The experiments were carried out in triplicate.

6.2.5 Ferric reducing ability of plasma (FRAP) assay

To determine the total antioxidant potential in the supernatant of the faecal water obtained from the gut model, the ferric reducing ability of plasma (FRAP) antioxidant capacity assay was employed. In this assay the electron-donating capacity of an antioxidant is measured by the change in absorbance at 593 nm when a blue-coloured Fe²⁺-tripyridyltriazine (Fe²⁺TPTZ) compound is formed from a colourless oxidized Fe³⁺ form [145]. A standard curve was constructed using ferrous sulphate at concentrations ranging from 0.1 to 10 mM. The FRAP reagent was prepared using 10:1:1 ratio of 300 mM acetate buffer (pH 3.6):10 mM TPTZ (2,4,6-tripyridyl-s-triazine): 40 mM HCl at 50°C and 20 mM ferric chloride hexahydrate solution. Once prepared the FRAP solution was immediately incubated for 10 min at 37°C. For the assay, into a 96-well plate, 30 μ L water, 10 μ L standards or samples and 200 μ L

FRAP solution were added, followed by 30 min incubation the absorbance was read at 593 nm in a microplate reader (Infinite PRO 200 series, Tecan Group).

6.2.6 Short chain fatty acid (SCFA) analysis

In order to analyse the SCFA, 3 ml of the samples were centrifuged and filtered using 25 mm syringe filters (0.2 µm, MCE, sterile) (09-719C, Fisher Scientific) and 1 µL of the filtered sample was directly injected into a 6890 series gas chromatograph (GC) system equipped with a flame ionization detector (Agilent Technologies, Santa Clara, CA, USA). Analysis was carried out as described by Sadeghi et al. (2016) [146]. Using a HP-INNOWAS 30 m fused capillary column (Agilent Technologies, Santa Clara, CA, USA), 250 µm internal diameter, with a film thickness of 0.25 µm, SCFA were separated. Helium at flow rate of 1.0 ml/min was used as the carrier gas. The inlet and detector temperatures were 220°C and 230°C, respectively. The oven temperature was initially set at 150°C, held for 10 min and increased by 10°C/min from 150°C to 180°C and then held for 5 min. Identification of the SCFAs were done based on their retention times compared to that of pure standards (Nu-Check Prep, Inc., Waterville, MN). The concentration of SCFA was calculated as mM. Samples were analysed in duplicate from three independent experiments.

6.2.7 Culture of Lactobacilli

Samples removed from the fermentation vessels were subjected to bacterial counts on selective agar plates for enumeration of *Lactobacilli*. *Lactobacilli* spp. were grown anaerobically at 35°C on De Man, Rogosa and Sharpe (MRS) agar (Oxoid, UK) and plates were counted after 3 days of incubation.

6.2.8 Liquid chromatography–mass spectrometry (LCMS) analysis of key metabolites

Digested sample of 5 g KP and 1 g EA were subjected to LCMS analysis. Thawed samples were vortexed and filtered with 25 mm syringe filters (0.2 µm, MCE, sterile; 09-719C, Fisher Scientific) and transferred to vials for liquid chromatography–mass spectrometry (LC–MS) analysis. Metabolites were analysed using a high-performance liquid chromatography (HPLC) equipped with a Phenomenex F5 C18 column (2 x 150 mm, 3 µm). Elution was achieved using solvents A (0.1% formic acid in water) and B (Acetonitrile in 0.1% formic acid). Gradient conditions included – 1 min 5% B, 5 min 30% B, 10 min 70% B, 11 min 95% B and 13 min of 100% B with 0.3 ml/min flow rate. For preparing the samples for injection, 40 µl of sample along with 40 µl 10 µM resveratrol (internal standard) and 120 µL 25% acetonitrile were transferred HPLC vials, volume injected was 15 µl and column temperature was 40°C.

Mass data was obtained with the help of an Agilent 1200 series HPLC system equipped with an Agilent 6210 electrospray ionization, time-of-flight (ESI-TOF) mass spectrometer (Agilent, Santa Clara, CA, USA), with internal mass calibration.

6.2.9 Statistical analysis

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

6.3 Results and discussions

6.3.1 Antioxidant activity

The antioxidant activity plant material are linked to the phenolic acids and flavonoids content. It is important to understand that the antioxidant properties might change due to the biotransformations during the GI digestion. Antioxidant activity was measured in KP (1 g and 5 g) and EA samples after gut model digestion using FRAP (Figure 34). The antioxidant capacity in both 1 g and 5 g KP samples was superior than the EA samples. In 5 g KP, no major changes in the antioxidant capacity was noted indicated by the stable trend through the digestion period. In case of the 1 g KP, the antioxidant capacity was observed to decrease after 12 h. The better antioxidant capacity demonstrated by KP treatment compared to EA could be due to release of other antioxidant phytochemicals such as AA from KP matrix. The drop in antioxidant capacity after 12h in case of the 1 g KP could be due to formation of secondary metabolites due to gut microbial fermentation. Similar observations were made in a study involving simulated 24h GI digestion of peel flour of pomegranate (*Punica granatum*), another fruit rich in EA, a decrease in FRAP values was seen from 'mouth' digestion step to 'intestinal' digestion [147].

6.3.2 Short chain fatty acid (SCFA) analysis

Effect of KP and EA digestion on SCFA levels was analyzed (Figure 35). SCFA analysis showed an increase in total SCFA after gut digestion of the KP samples whereas EA samples showed no significant change in SCFA compared to control.

The significant increase of total SCFA at 8h mark in KP samples indicate the formation of these fermentation products by the colonic microflora. The concentration of acetic acid in KP digested samples was observed to increase after 8 h. The increase was more significant in 5 g KP samples. The peak concentration was observed at 12 h and decreased slightly at 24 h mark. The same observation was observed in the case of propionic acid with respect to both KP treatments. The butyrate concentration across all the treatment groups was lower than acetic and propionic acid concentration. The butyrate concentration was almost identical in both control and KP treated samples and remained constant throughout the digestion. Unlike KP treated samples, in the EA treated samples showed decrease in the concentration all the 3 major SCFAs. Acetic, propionic and butyric acid were also the main SCFA seen after the in vitro simulated digestion of pomegranate peel flour [147]. The higher concentration of SCFA observed in the KP samples compared to EA could be attributed to the presence of dietary fibers like pectin [148] in the KP matrix.

SCFAs produced from microbial fermentation act as important energy sources in the gut and some are transported to other sites for other usage. Acetic acid, the main product of *Bifidobacteria* fermentation plays an important role in mucin secretion [149, 150]. Butyric acid act as an energy source for mucosal cells and may inhibit neoplastic changes in cancer cells [151]. Propionic acid simulates the release of anorectic gut hormones [152]. The reduced levels of propionate and butyrate has been associated with inflammation. For example, butyrate production is low in ulcerative colitis [153] whereas low levels of propionate has been observed in children at risk of asthma [154]. Also, high butyrate has shown to enhance growth of probiotic bacteria and exert anti-inflammatory and anticancer properties. Butyrate also plays important roles in maintaining intestinal health including – regulating trans epithelial fluid transport, ameliorating mucosal inflammation and oxidative stress, repairing epithelial defense barrier and modulating visceral sensitivity and intestinal motility [155]. Therefore, the increase in propionate by KP samples and the ability of KP samples to maintain the butyrate concentration throughout the digestion process indicate potential health benefit.

6.3.3 Effect on Lactobacilli

Effect of KP and EA digestion on growth of the probiotic *Lactobacilli* were analyzed [

Figure 36]. Following gut digestion of KP samples, an increase in *Lactobacilli* count was observed with the highest concentration observed in 5 g KP at 24 h. The EA samples showed an initial increase at 8 h time but dropped after 36 h.

Probiotics such as *Lactobacilli* are considered to be one of the most important bacterial groups for human health by modulating intestinal microbial balance, resisting disease and improving gastrointestinal functions [156]. Present study indicated that KP phytochemicals significantly increased the numbers of *Lactobacilli*. Therefore, the consumption of KP products could be beneficial for the regulation of intestinal micro-ecology, and may improve health.

6.3.4 LC-MS analysis

LC-MS analysis was performed on the 5 g KP samples after gut model digestion. Pure standards of EA, Urolithin A and B were used as reference standard and resveratrol was used as the internal standard. Chromatograms of these compounds are shown in Figure 37.

EA and Urolithin A were detected in the digested 5 g KP samples [Figure 38]. Peak area of EA and Urolithin A are summarised in Table 25 and Table 26. EA was detected over each of the time point with an initial increase at 12 h followed by a gradual decrease at 24 h [Figure 38A]. The concentration of EA in these sample were quantified and provided in Figure 39. Urolithin A was detected in trace amounts at baseline level over time [Figure 38B]. Due to low levels Urolithin A could not be quantified.

Detection of Urolithins in biological samples after consumption of ET or EA rich food is well documented. For example, studies investing the bioavailability of pomegranate a rich source of ET, showed that after the intake of large amounts of pomegranate husk ETs, the main metabolites detected in plasma and urine were urolithins A, B, and C and smaller amounts of Ellagic acid-dimethyl ether glucuronide [157].

Detection of Urolithin A in the digested KP samples is particularly noteworthy, as Urolithin A has been reported to show antioxidant [158, 159], antimalarial [160], anti-inflammatory [161-163] and anticancer [164-166] activity *in vitro*. The biological activity of Urolithin A has also been reported in *in vivo* setting. Urolithin A showed anti-inflammatory and prebiotic effects in a rat model of ulcerative colitis. After administering a diet of either 250 mg/kg/day of an ET-rich pomegranate extract (human equivalent dose HED ~2.5 g in a 70 kg person) or 15 mg/kg/day of synthetic urolithin A (HED ~154mg in a 70 kg person) for 25 days before inducing colon inflammation with dextran sodium sulfate (DSS). Both diets led to decreased expression of inflammatory biomarkers including iNOS and COX-2 in the colonic mucosa. However, the anti-inflammatory activity exerted by Urolithin A was higher than that of the pomegranate extract. Also, only Urolithin A protected the colonic architecture against DSS treatment [167]. Thus, detection of Urolithin A in the KP digested samples indicate that consumption of KP may be beneficial as further health benefits can be achieved via Urolithin A.

6.4 Conclusions

By using the *in vitro* fermentation via a simulated gut model, the biotransformation of KP was analysed. The biotransformation of KP increased the growth of the beneficial prebiotic *Lactobacilli* species. Simulated gut microbial fermentation of KP also increased the

concentration of acetic, propionic and butyric acids increased significantly compared to those of the control. Furthermore, Urolithin A was detected KP samples. The present study provides new insights into how KP phytochemicals may change in the complex gut environment. The results presented here suggest that KP phytochemicals have potential prebiotic-like activity by modulating human intestinal microbiota and generating SCFAs that can contribute health.

Acknowledgements

The authors acknowledge the facilities, and the scientific and technical assistance, of the School of Human Nutrition, McGill University (Montreal, Canada). This project was supported by RIRDC Grant (2014-2018-PRJ 2014000161). M. Chaliha's PhD was supported by the Research Training Program (RTP) Scholarship.

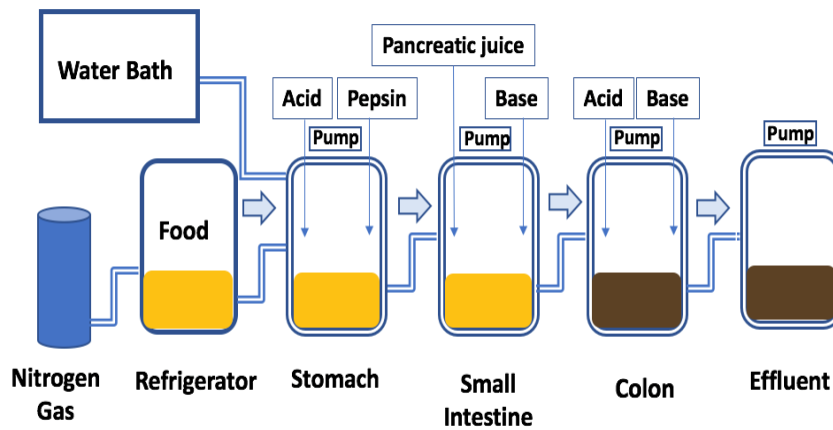


Figure 31 Schematic representation of the simulated gut model (adapted from Ekbatan et al [47])

Table 23. Application of *in vitro* dynamic GI model in investigating biotransformation and bioavailability of phytochemicals

Phytochemical	Major colonic metabolites	Effect on gut flora (if any)	Ref
Herbal formulation Padma Hepaten containing Chebulic ellagitannins	Urolithin	Colonic metabolites and urolithins prevented oxidative injury in cultured rat primary hepatocytes	[168]
Mixture of pure chlorogenic acid, caffeic acid, ferulic acid and rutin	Phenylpropionic, benzoic, phenylacetic and cinnamic acids		[47]
Rye bran and aleurone, wheat bran and aleurone, and oat bran and cell wall concentrate	Phenylpropionic acids	Increased <i>bifidobacteria</i> and <i>lactobacilli</i>	[169]
Sun-dried raisins		increased <i>bifidobacteria</i> and <i>lactobacilli</i>	
Insoluble dietary fibre chitin-glucan	acetate, propionate and butyrate isobutyric, valeric, and isovaleric acid	Decreased ratio <i>Firmicutes/Bacteroidetes</i> and delayed increase in the concentration of <i>Roseburia</i> spp	[170]
Red wine	Gallic acid, protocatechuic acid, 3-O-methylgallic acid, 4-hydroxybenzoic acid, 3,4-dihydroxyphenylpropionic		[171]

Phytochemical	Major colonic metabolites	Effect on gut flora (if any)	Ref
Fresh and pasteurized orange juice	acid, vanillic acid, syringic acid and salicylic acid	<p data-bbox="996 359 1355 582">Fresh orange increased <i>Lactobacillus</i> spp., <i>Enterococcus</i> spp., <i>Bifidobacterium</i> spp., and <i>Clostridium</i> spp. and reduced <i>enterobacteria</i>.</p> <p data-bbox="996 614 1355 758">The pasteurized juice increased <i>Lactobacillus</i> spp. and reduced <i>enterobacteria</i></p>	[172]

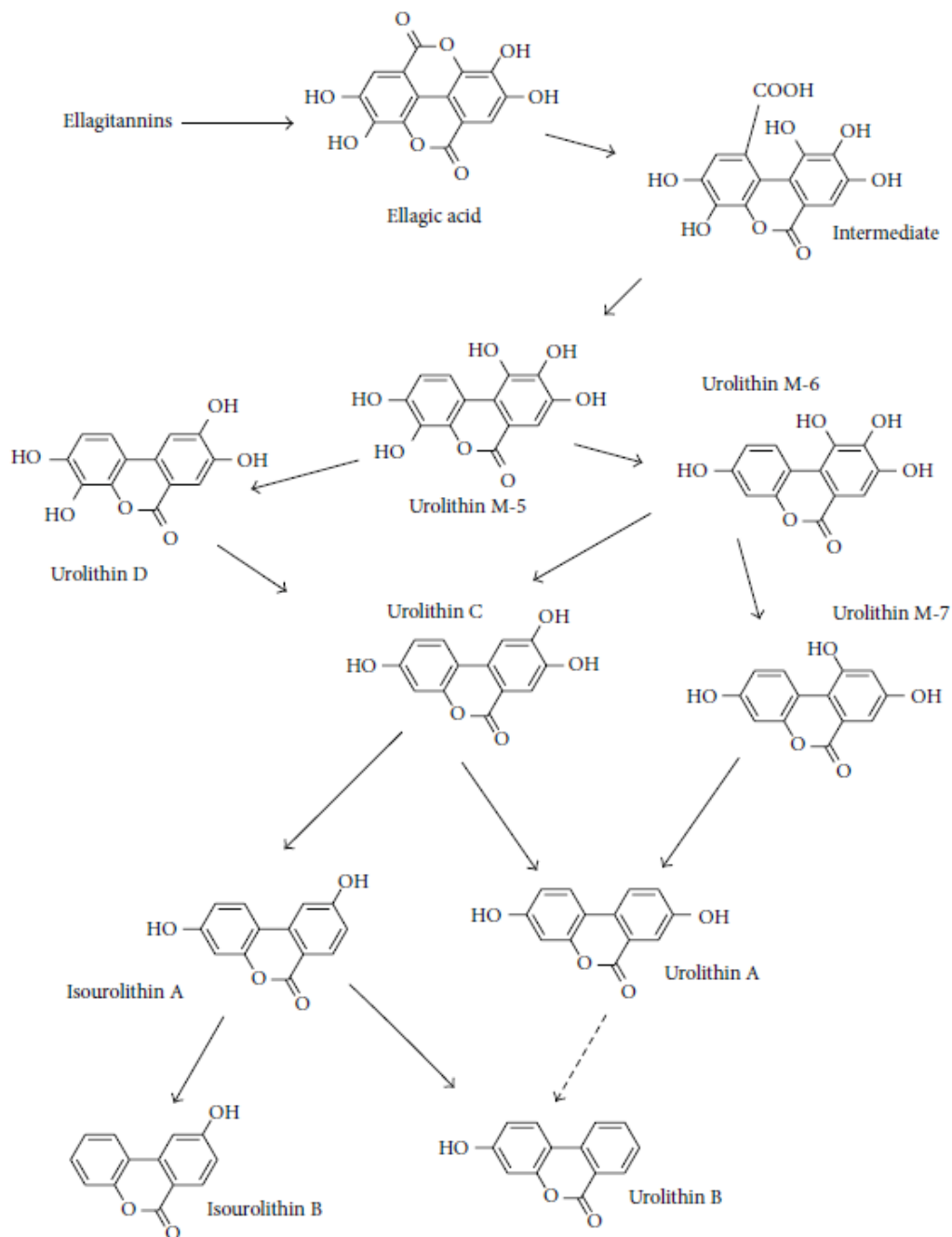


Figure 32 Metabolism of ellagitannins (ET) and Ellagic acid (EA) by gut microbiota (adapted from Espín et al., 2013 [173]).

Table 24 Composition of the basal culture media

Component	Concentration (g/L)
Peptone water	2
Yeast extract	2
Sodium chloride	0.1
Dipotassium hydrogen phosphate	0.04
Potassium dihydrogen phosphate	0.04
Sodium hydrogen carbonate	2
Magnesium sulfate heptahydrate	0.01
Calcium chloride hexahydrate	0.01
Tween 80	2
Vitamin Solution	0.01
L-cysteine	0.5
Bile salts	0.5
Resazurin	0.001

Note : The media was sterilized using a 0.45 µm syringe filter. 45 ml of bacterial media was used per vessel. (9:1 media: fecal slurry).

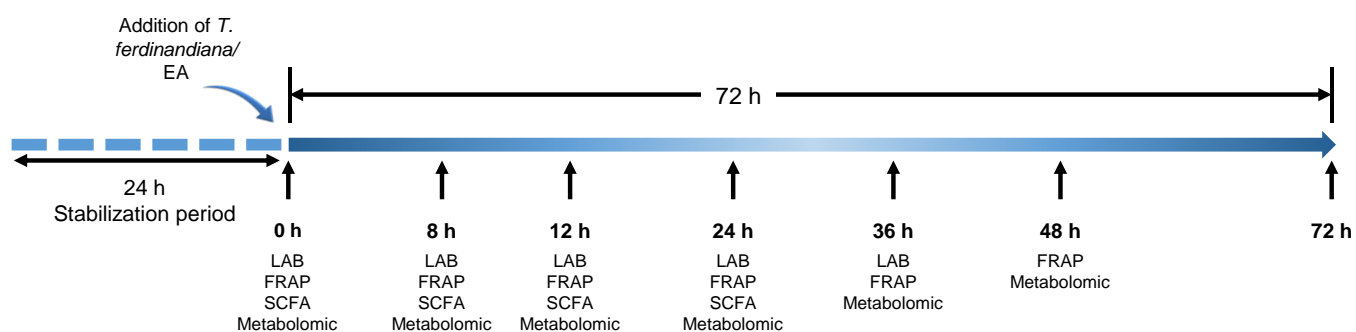


Figure 33 Schematic representation of GI model experiments timeline and sample collection.

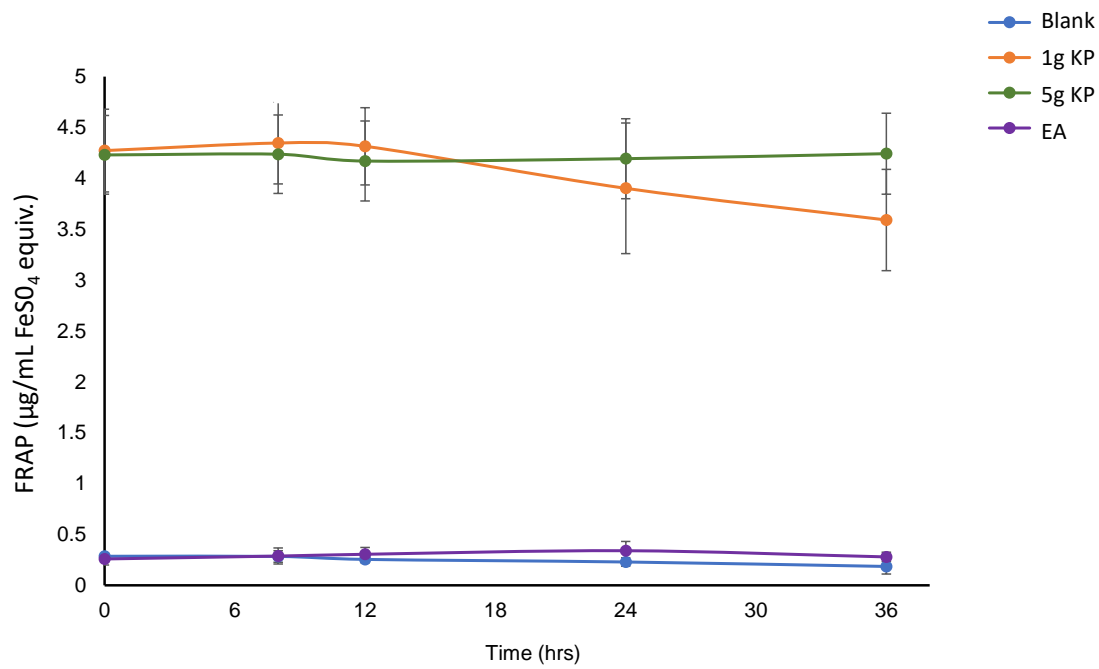


Figure 34 Time course of antioxidant capacity FRAP measures control (blank), KP and EA after gut model digestion. Data are mean \pm SD.

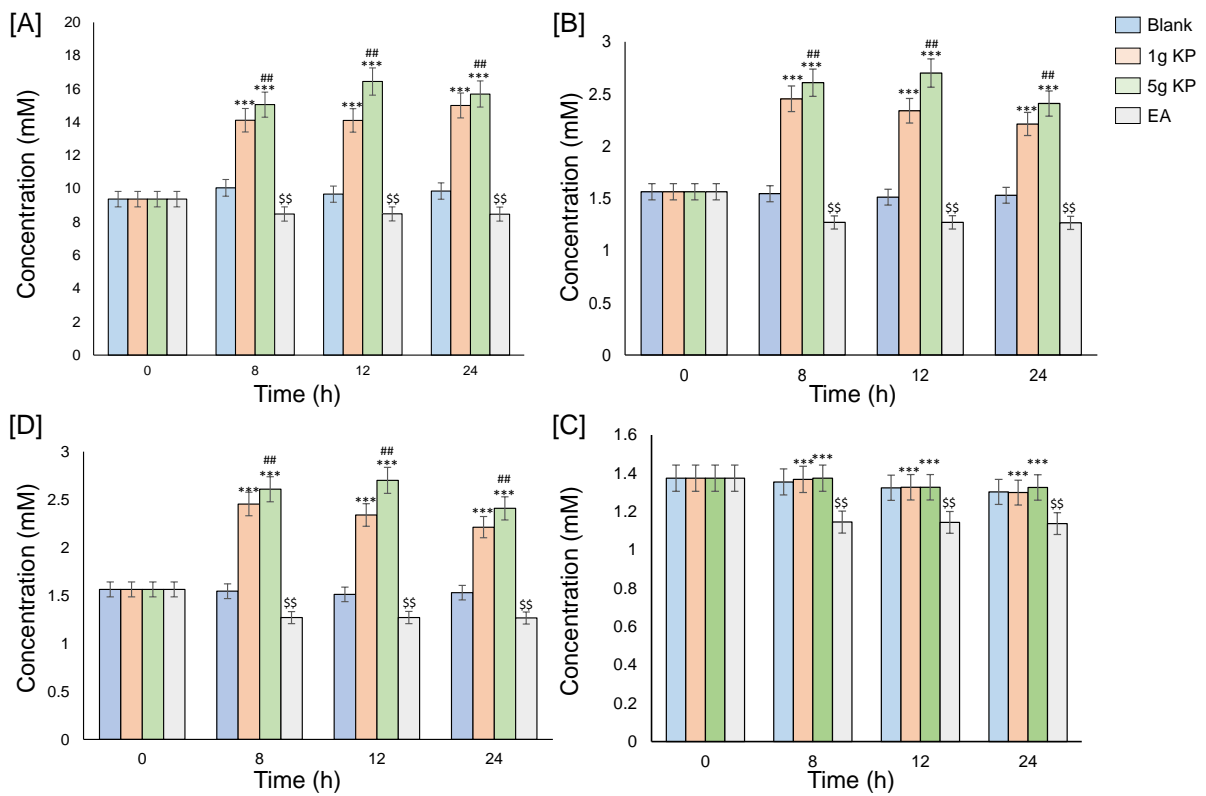


Figure 35 Changes in concentration (mM) of [A] total SCFA, [B] acetic acid, [C] propionic acid and [D] butyric acid. Measurements were carried out after gut model digestion of control (blank), KP and EA. Data are expressed as means \pm SE. Statistics: KP treatment compared to control # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$; EA treatment compared to control \$ $P < 0.05$, \$\$ $P < 0.01$, \$\$\$ $P < 0.001$ and KP vs EA * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

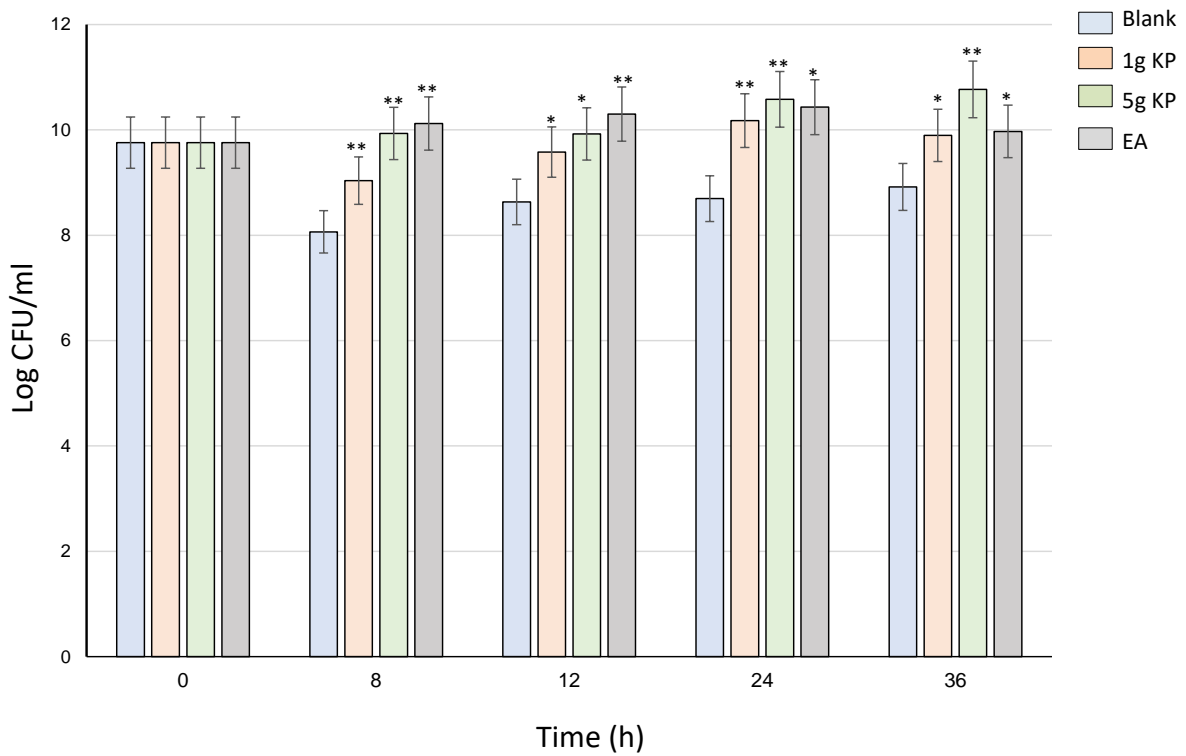


Figure 36 Changes in *Lactobacilli* growth (Log CFU/ml) after gut model digestion of control (blank), KP and EA. Data are expressed as means \pm SE. Statistics: treatment compared to control * $P<0.05$, ** $P<0.01$, *** $P<0.001$, Student's *t*-test.

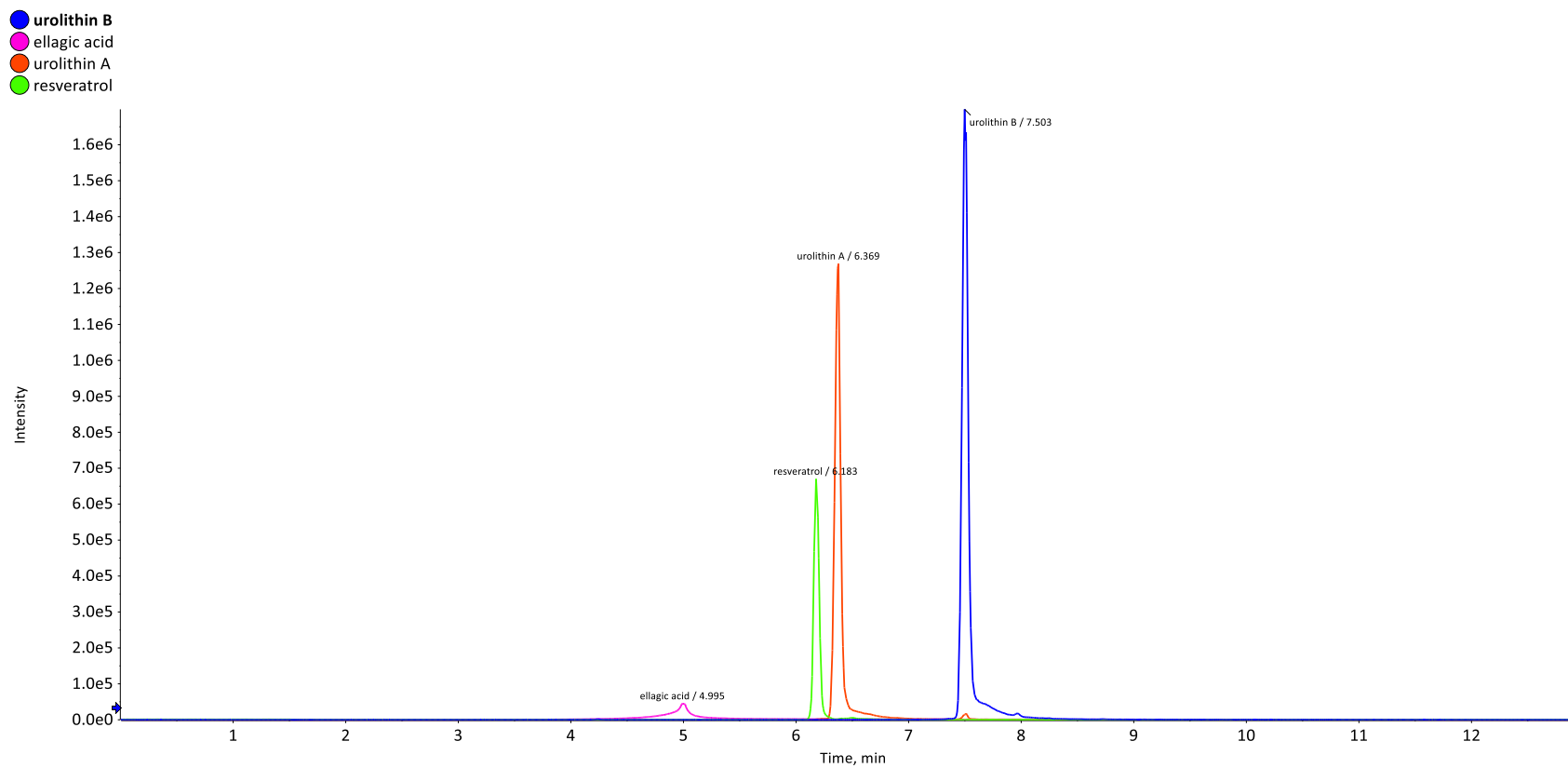


Figure 37 Chromatograms with reference compounds Ellagic acid, resveratrol, Urolithin A and Urolithin B.

Table 25 Peak area of Ellagic acid with respect to IS (resveratrol) in blank control and 5 g KP after gut model digestion

Sample	Time (h)	Ellagic acid	SEM
Blank	0	1.13E+05	34058.84
	8	5.82E+04	15527.63
	12	1.45E+06	152041.7
	24	1.99E+04	3769.836
5 g KP	0	1.13E+05	34058.84
	8	9.08E+06	631156.3
	12	1.05E+07	508975.7
	24	9.19E+06	522515.9

Table 26 Peak area of Urolithin A with respect to IS (resveratrol) in blank control and 5 g KP after gut model digestion

Sample	Time (h)	Urolithin A	SEM
Blank	0	3.62E+04	26975.4
	8	5.30E+04	51525.26
	12	5.54E+04	54320.87
	24	3.42E+04	33271.12
5 g KP	0	3.62E+04	26975.4
	8	1.91E+04	9339.052
	12	1.88E+04	10447.33
	24		

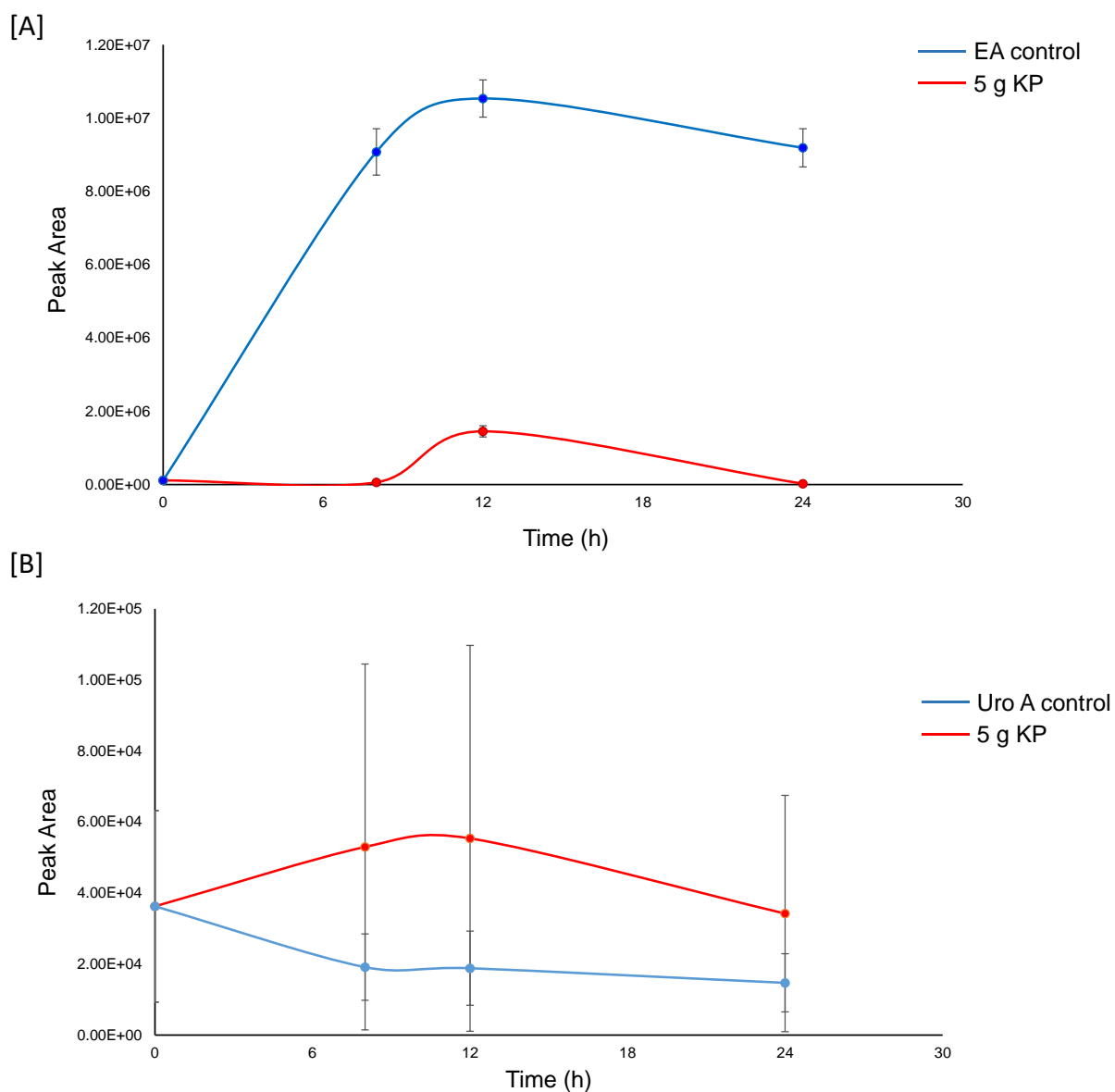


Figure 38 Identification of key metabolites - [A] Ellagic acid and [B] Urolithin A in reference sample and 5 g KP sample (after gut model digestion). [A] Ellagic Acid was detected over each of the time points. Until 12h, EA level showed an increase in 5 g KP sample possibly due to release from the food matrix and then a gradual decrease at time 24h. [B] Urolithin A was detected trace amounts and at baseline concentrations over time and sample type. Data expressed as means \pm SD.

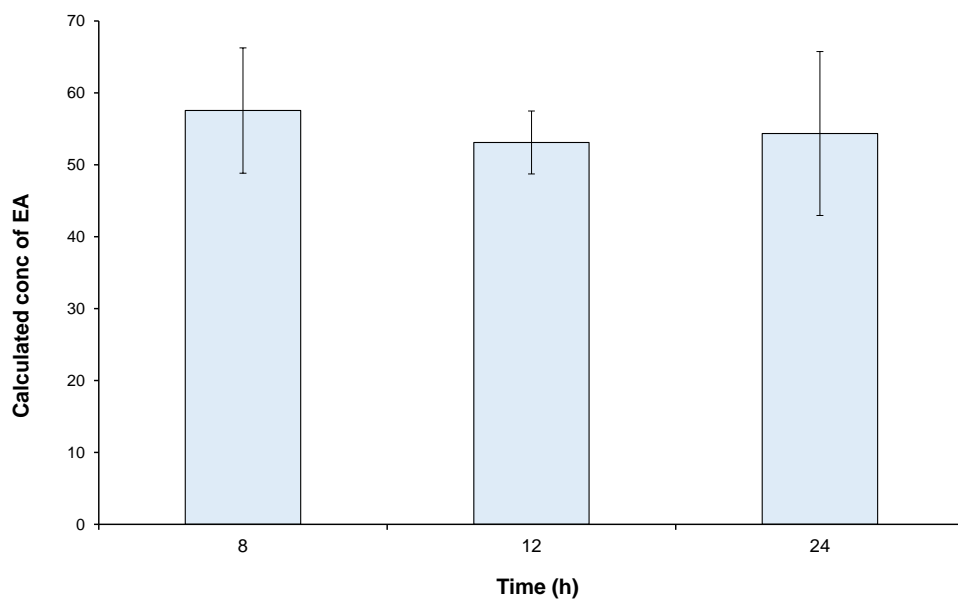


Figure 39 Calculated concentration of EA of 5 g KP samples after gut model digestion.

Appendices

Appendix 1

Training and Registration for the harvesting of Kakadu plum (Mi Marral) in Wadeye. This training program and training of the PWAC members was done by Rose Read in partnership with PWAC and the researchers on this project.

A training video on Kakadu plum harvesting is available for the community to use in Murrinhpatha language.



Bush Plum – Mi Marrarl

Training and Registration
2016

Clean, ripe, fresh plums

- Any size, small or big
- Not flat
- No plums from ground, soft, eaten or big black marks
- No sticks, leaves
- Bring in fresh- same day, next morning



Clean plum – no germs

- Plums make medicine
- Keep plums clean
- Clean hands
- No coughing
- Cover cuts or wound with bandage



- Green bags



Buying plums

- Ripe, full fruit
- Big & small
- Not flat



Care for trees, country

- No broken trees, branches
- Care for country
- Rangers out watching
- Report any damage



Getting to country



- Speak to us or Rangers if you want transport to your country
- We can't promise but we will try and get you there

Hours

Monday – Friday: 9am – 4pm

Saturday: 9am- 12 noon

Closed Sunday: X

Don't pick on Saturday if you can't bring in Saturday morning

Appendix 2

Workshops held to promote the harvesting, processing and storage of Kakadu plum



TERRITORY NATURAL RESOURCE MANAGEMENT 2015 CONFERENCE
10-12 NOVEMBER 2015, DARWIN

get the *dir*ton NRM 

MAKING A BUSINESS FROM NATIVE PLANT PRODUCTS

Kakadu Plum as a case study

TUESDAY
10TH NOVEMBER
12.45PM - 4.30PM
REGISTER FROM 8AM
VIBE HOTEL, DARWIN

Sustainable harvest of natural resources provides an opportunity for economic development in remote areas of northern Australia. Recent growth in market demand and the proposed development of a central processing hub in Darwin may be a 'game changer' for economic opportunity for Indigenous Australians. Harvest of native plant products allows Indigenous Australians to take advantage of their natural resources, whilst utilising and maintaining their intimate knowledge of country. Come along and find out about the growth of the Kakadu Plum industry and what we can learn from what is happening at Wadeye; how the Kakadu Plum model being applied there could be used for sustainable harvest of other native plants; the importance of a central processing hub and regional supply centres; and if you are interested in making a business from Kakadu Plum what you need to do to get started.

Presenters

Angus Melpi and Maureen Simon
Angus and Maureen are Senior Thamarrurr Rangers. They have played key roles in Kakadu plum harvest in Wadeye and will share their perspective on this growing industry.

Julian Gorman
Julian has been working on Indigenous, wildlife – based enterprise through the Charles Darwin University and Northern Land Council since 1999. Much of this work has involved exploring commercial opportunities and Indigenous aspirations in developing enterprise based on Kakadu Plum.

Chris Brady
Chris has worked in natural resource management across northern Australia for almost two decades. Chris was instrumental in expanding the plum harvest in Wadeye and pushing for the development of a central processing hub in Darwin.

Yasmina Sultanbawa
Yasmina is a Senior Research Fellow at the Queensland Alliance for Agriculture and Food Innovation (QAAFI), University of Queensland. She has 18 years' experience in value addition to food and has a track record of working with industry and attracting national and international funds where commercialization has been a key outcome.

\$40 MEMBER | **OR INCLUDED IN FULL CONFERENCE REGISTRATION**
\$50 NON-MEMBER

REGISTER

Please download your registration form at tnrmconference.org.au and submit to events@territorynrm.org.au

WORKSHOP E





For more information contact us: ☎ 08 8942 8300 ✉ events@territorynrm.org.au 🌐 www.tnmconference.org.au

Making a business from Native plant products

Kakadu Plum as a case study

Tuesday 10th November 12.45pm - 4.30pm

Vibe Hotel, Darwin

Vision: An across the north strategy to build a Kakadu plum industry

Purpose of workshop: to let interested parties know what is happening in this space

Four sections to workshop:

- to share the story of the developing Kakadu plum industry (last 15 years)
- to propose a model that can deal with market forces without putting too much pressure on one region/community
- to provide information about costings and participation
- to allow for open discussion

Introductions of presenters: 1250 – 1315

Researchers: Julian Gorman; Chris Brady; Yasmina Sultanbawa

Industry: Kindred Spirits Enterprises: Rhonda Renwich, Ann Shanley, Kathy Havers

Australian Native Food Industry Limited: Amanda Garner

Region Supply Centres: Palngun Wurnangat Association: Margo Northy, Rose Read, Mamabulanjan Aboriginal Corporation: Paul Lane and Neil Gower

Land Managers: Indigenous Thamarru Rangers: Angus Melpi and Maureen Simon

Trainers: Kimberley Training Institute: Kim Courtenay

Section 1: 1315 – 1445 History of development of Kakadu Plum Industry

1315 – 1320 Structure of workshop and introduction to Kakadu Plum -
Julian Gorman

1320-1330 Why is Kakadu Plum special? Chemical properties (ones that have been known for a while such as Vit C through to newer properties such as anti-microbial etc). - Yasmina Sultanbawa

1330-1345 Commercial interest to date - Julian Gorman

1345-1400 Overview of horticultural training and development in Kimberley -
Kim Courtenay

1400-1405 What has limited the growth of this industry to date? Non consistent supply, non-consistent demand, lack of a business structure, support - Julian Gorman

What has changed to overcome these obstacles? New Research and new markets Greater supply

1405 – 1425 Wadeye Supply Hub (Thamarrurr Rangers/Palngun Wurnangat Association) - Angus Melpi, Maureen Simon, Chris Brady, Margo - **Video – transition to a supply hub**

1430-1440 Challenges of building a collaborative regional structure in a regional hub - Paul Lane/Neil Gower – Mamabulanjan Aboriginal Corporation Land tenure, interests of wild harvesters, private indigenous land owners, Outstation Families and Communities.

1440 – 1445: Discussion/Questions

Section 2: 1445-1510 – developing a central processing hub

1445 - 1455: Why is it necessary to have one? Quality control, product specifications, packaging, product development, consistency of supply. -

Yasmina Sultanbawa

1455-1510: The KSE model that is being developed. How it links with regional supply centres and what's its role will be - **Ann Shanley**

1510-1515: Discussion/Questions

Section 3: 1515 – 1600 – costs of setting up regional supply centres/horticulture

1515-1530 What are the costs in setting up a Region supply centre? - Chris Brady, Rose Read, Yasmina Sultanbawa

1530-1540 A brief overview of costs in setting up, training, infrastructure and support in contemplating horticulture - Kim Courtenay

1540 – 1550 Potential for commercialisation of other native plant products - Amanda Garner

1550-1600: Discussion/Questions

Section 4: 1600 – 1630 - Questions and Discussion

Workshop to communicate the importance of gubinge and the potential of making a business from native plant products to the Indigenous communities in the Kimberley Region, Western Australia from 19-21 July 2016.

Monday 18th July

- 6:30 pm – Networking dinner

Tuesday 19th July

- 10:00 am – Meeting with key personnel and/or harvester groups (MAC Conference room)
- 12:00 pm – Lunch at MAC Conference room
- 1:30 pm – Excursion to Balu Buru Gubinge School of Excellence, Skuthorpe Native Tree Nursery, Green Army WWTP North

Wednesday 20th July

- Travel to Beagle Bay Area to meet with:
 - Nyul Nyul Rangers and Mark Rothery
 - Twin Lakes Cultural park – Bruno Dann and Marion Louise Manson
- Information session with Yasmina Sultanbawa
- Information session with Neil Gower
 - T.H.E. Kakadu Plum – Greater Northern Kakadu Plum Co-operative
 - 2016/2017 Harvest Season
 - Broome co-operative
- Look over the gubinge orchard
- 5:30 pm – Dinner at Mangrove – Golden Staircase to the Moon. MAC has invited 20 guests upstairs dining area overlooking Roebuck Bay.

Thursday 21st July

- 7:30 am – Travel to Lombadina (a further 80 kms north from Beagle Bay)
- Meeting with Robert Sibosado and Lombadina Community
- Information session with Yasmina Sultanbawa
- Information session with Neil Gower
 - T.H.E. Kakadu Plum – Greater Northern Kakadu Plum Co-operative
 - 2016/2017 Harvest Season
 - Broome co-operative
- Look over the gubinge orchard

The Mamabulanjin Aboriginal Corporation and Indigenous Land Corporation

Native Fruits and Trees Workshop

Broome March 14th & 15th 2017

A workshop on Native Fruits and Trees was held in Broome from 11-18 March 2017 for indigenous communities, industry and researchers. The aim of the workshop was to have an open discussion with the indigenous community representatives on current harvest activities, aspirations, barriers and opportunities. A Kakadu Plum Indigenous Network (KPIN) was established as an outcome of the Native Trees and Fruit Workshop held in Broome WA (March 2017). Participants now share and communicate their Kakadu Plum experiences with each other to support the development of an Indigenous-led Kakadu plum industry.

Agenda:

Day 1 Tuesday 14th March

<i>Time</i>	<i>Presentation</i>	<i>Presenter</i>
8:30 – 9:00	<i>Registration</i>	
9:00-9:30	<i>Welcome & Workshop Overview</i> <i>Why are we here? Purpose of the workshop.</i>	<i>Paul Lane</i>
9:30-10:30	<i>Introductions – Open discussion from community representatives on current harvest activities, aspirations, barriers and opportunities. What do participants expect to get out of the next two days</i>	<i>Paul Lane</i>
10:30 -11:00	<i>Morning Tea</i>	
11:00 – 12:00	<i>Gubinge/Kakadu Plum Industry Overview –update from Kakadu Plum Working Group.</i> <i>Purpose and Findings of the Collaborative Northern Australia</i>	<i>Ann Shanley (T.H.E)/Jacob Habner (ILC)</i>

Working Group to date (30 mins)

Q&A / Group Discussion facilitated by Paul Lane

12:00-12:30	Local Kimberley Context & Strategy (30 mins)	Neil Gower, Mamabulanjin Aboriginal Corporation
12:30 - 1:30	Lunch	
1:30 – 3:00	Traditional Homeland Enterprise (T.H.E) Q&A / Group Discussion facilitated by Paul Lane	Ann Shanley/ Rhonda Renwick
3:00 – 3:20	Afternoon Tea	
3:20 – 3:30	Engineers without Borders (EWB) (10 mins)	Lizzie Brown
3:30-4:00	Overview of Palngun Wurnangat (Wadeye) (30 mins)	Rose Read and Chris Brady
4:00-4:30	Wrap Up Day One	Neil Gower/Paul Lane

Guests are invited for drinks & canapes at Mangrove Hotel from 6pm to 8pm

Day 2 Wednesday 15th March

Time	Presentation	Presenter
8:30 – 9:30	Summary of Outcomes Day One Discussion	Paul Lane
9:30 – 10:30	National Research Strategy Proposed ARC Linkage Q&A / Group Discussion facilitated by Paul Lane	Paul Lane, Kimberley Institute
10:30 – 10:50	Morning Tea	
10:50 – 11:20	Science behind the Fruit R&D opportunities for Native Fruit Industry Innovative Functional Ingredients from Plant Sources in food applications (20 mins)	Dr Yasmina Sultanbawa, University of Queensland

11:20 – 11:40	<i>Nutritional interventions to prevent metabolic disorders using plant bioactive compounds (20 mins)</i>	<i>A/g Professor Stan Kubow</i>
11:40-12:00	<i>Kimberley Wild Gubinge (Munget Aboriginal Corporation) (20 mins)</i>	<i>Jacinta Monck and Lenny O'Meara</i>
<hr/>		
12:00-12:30	<i>Questions or further discussion from the morning sessions</i> Q&A / Group Discussion facilitated by Paul Lane	<i>Paul Lane</i>
12:30-1:30	<i>Lunch</i>	
1:30-3:00	<i>Potential Way Forward – Challenges & Opportunities</i> Q&A / Group Discussion facilitated by Paul Lane	<i>Paul Lane</i>
3:00-3:30	<i>Closing Remarks</i>	<i>Neil Gower, Mamabulanjin Aboriginal Corporation</i>
Workshop close		
<hr/>		

Stakeholder meetings

Darwin

Stakeholder meeting held on 10 November 2015 with Indigenous communities from Northern Territory and Western Australia including Palngun Wurnangat Aboriginal Corporation, Mamabulanjin Aboriginal Corporation, Kimberley Institute, Kindred Spirits Foundation, EcOz Environmental Services, Charles Darwin University and The University of Queensland.

Brisbane

Stakeholder meeting held on 28th and 29th September 2016 with Indigenous communities from Northern Territory and Western Australia including Palngun Wurnangat Aboriginal Corporation, Mamabulanjin Aboriginal Corporation, The Gundjeihmi Aboriginal Corporation, Kimberley Institute, Kindred Spirits Foundation, EcOz Environmental Services, Charles Darwin University, Indigenous Land Council, Engineers without Borders, Traditional Homeland Enterprises and The University of Queensland.

28th meeting was held at the Health and Food Sciences precinct for the Kakadu plum consortium members to understand the testing and processing of Kakadu plum. One on One meetings were held with the different industry and Indigenous community partners interested in working with Kakadu plum.

29th September meeting was facilitated by Wiley Engineering group and called a “The Discovery Workshop” to brainstorm and determine the markets and strategies that could be adopted for developing markets, value chains and the emerging business model in Wadeye for this new native fruit industry.

Training programs held in other Aboriginal Communities in Northern Australia

- At the invitation of the Crocodile rangers in Milimgimbi a presentation on the “Handling and processing of kakadu plums” was done on 27 April 2016 at the Rangers Unit before training the rangers in the harvesting and processing of Kakadu plums.
- At the invitation of the Mamabulungin Aboriginal Corporation the following presentation titled “Why Kakadu plum is so Special? Making a Business from Native Plant products – Gabinge as a case study”, which included the training modules made in Wadeye, were made to the following indigenous communities: Mamabulanjin Aboriginal Corporation, Nyul Nyul Rangers, Twin Lakes Cultural Park, Lombadina Community, Eyes on Country from 19 – 21 July, 2016 Broome, Western Australia.

Feedback received from a community after my (Dr Yasmina Sultanbawa) visit to Broome from July 18-22, 2016

Thank you so much for your very informative presentation at Mamabulanjin were impressed with the results of your research and would like to stay in contact and help in anyway we can.

Our practices in the wild harvest and aboriginal land & fire management increase yields and help the biodiversity of the country here. We have good examples in our area. Next time you come over to Broome we would like to show you our outstation at Twin Lakes and the healthy bush orchards, if you have the time, or at least give you some samples of other plants with traditional uses. Bruno has a wealth of traditional knowledge for Nyul Nyul-central Dampier Peninsular.

Thanks again from all of us at Twin Lakes and can you please let us know the results of your testing of the seed casing and kernel or any other results you find from gubinge tree or fruits for the Kimberley region.

Warm regards

Bruno & Marion

Co -Founders Twin Lakes Cultural Park

Appendix 3

Conference presentations and publications

The following oral presentation on kakadu plum was made at the **II International Conference on Food Chemistry and Technology**, November 14-16, Las Vegas, USA

Metabolomic profiling and enzyme assisted extraction of bioactive compounds from Kakadu plum (*Terminalia ferdinandiana*) – a native plant of Australia

Mridusmita Chaliha^{1*}, David Williams², Heather. Smyth¹, Sharon Pun², David Edwards² and Yasmina Sultanbawa¹

¹Queensland Alliance for Agriculture and Food Innovation (QAAFI), The University of Queensland, Queensland, Australia

²Department of Agriculture and Fisheries (DAF), Queensland, Australia

Kakadu plum (KP, *Terminalia ferdinandiana*) is a traditional fruit that has been consumed for its nutritional and therapeutic value by indigenous Australians for thousands of years. Presence of high levels of ellagic acid (EA) and ascorbic acid (AA) makes it a rich source of antioxidants.

Non-targeted metabolomic profiling of polar extracts of the KP fruit, seeds and leaves identified 158 metabolites including amino acids, organic acids, phenolic acids, esters and sugars. In addition to detecting AA and gallic acid, previously unreported metabolites such as galacturonic acid, rhamnose, xylitol, xylulose, maltose, myo-inositol and palatinose were tentatively identified. Presence of these sugars indicated the presence of pectin in KP tissue. Therefore chemical extraction and subsequent characterization was carried out on the KP pectin. The extracted pectin (yield 85%) had an equivalent weight of 251 g, 13% methoxyl content and 40% degree of esterification. Non-polar extracts from flesh, seeds and leaf tissues showed the presence of saturated and unsaturated fatty acids, including lauric acid, palmitic acid, steric acid, linolenic acid and oleic acid.

To obtain enhanced release and recovery of bio-active compounds like EA and AA, that are known to get trapped within plant cells, enzymatic assisted extraction of bioactive compounds from KP tissue was carried out with pectinase using a central composite rotatable design (CCRD). The resultant 20 extracts showed high concentration of EA ranging from 51-256 mg/ml and AA ranging from 67-121 mg/ml at alkaline and acidic pH respectively. The total phenolic activity and antimicrobial effects of the extracts were investigated.

The following oral presentations on kakadu plum were made at the **International Conference on Food Safety and Regulatory Measures**, August 17-19 2015, Birmingham, UK

Exploring the metabolomic constituents and bioactive potential of Kakadu plum (*Terminalia ferdinandiana*) – a native plant of Australia

M. Chaliha^{1*}, P. Chrysanthopoulos², M. Hodson², D. Williams³, H. Smyth¹, S. Pun³, D. Edwards³ and Y. Sultanbawa¹

¹Queensland Alliance for Agriculture and Food Innovation (QAAFI), The University of Queensland, Queensland, Australia

²Metabolomic Australia, Australian Institute for Bioengineering and Nanotechnology (AIBN), The University of Queensland, Queensland, Australia

³Department of Agriculture and Fisheries (DAF), Queensland, Australia

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Non-targeted metabolomic profiling of polar extracts of the KP fruit, seeds and leaves identified 158 metabolites including amino acids, organic acids, phenolic acids, esters and sugars. In addition to detecting AA and gallic acid, previously unreported metabolites such as galacturonic acid, rhamnose, xylitol, xylulose, maltose, myo-inositol and palatinose were tentatively identified. Preliminary experiments were carried out to extract and characterize pectin from KP. The extracted pectin (yield 85%) had an equivalent weight of 251 g, 13% methoxyl content and 0.5% degree of esterification. To obtain enhanced release and recovery of active compounds attached to the cells, enzymatic digestion of KP tissue was carried out with pectinase. The resultant extract showed high concentration of EA ranging from 100 – 4400 mg/100 ml and AA ranging from 313 – 521 mg/100 ml at acidic and alkaline pH, respectively. The antioxidant activity and protective effects of this extract containing KP bioactive compounds was investigated with the help of Caco-2 cells where oxidative stress was experimentally induced with hydrogen peroxide. Understanding the KP metabolites and the molecular pathways via which it exerts anti-oxidant activity *in vitro*, will help in identifying new sources of therapeutics that target the debilitating oxidative and inflammatory cascade which is the hallmark of many chronic degenerative disorders.

Innovative functional ingredients from plant sources in food applications

Y. Sultanbawa

Queensland Alliance for Agriculture and Food Innovation (QAAFI), The University of Queensland, Queensland, Australia

New food safety issues emerge for many reasons. It could be due to a microorganism evolving to become a pathogen or a pathogen becoming more virulent. The globalized food supply chain and food production methods are also seen as contributing factors. Other reasons are the change in eating habits of people and their desire to lead a healthy lifestyle. Consequently, there has been an increase in the consumption of fresh/ unprocessed/ additive free food. Food safety and quality and sustainability of production are some of the driving forces that are presently changing the market for fresh food. The challenges of addressing the safety issues have resulted in the development of innovative technologies to improve safety of fresh food. Among, these technologies, the most promising are those based on the search for affordable and environmentally friendly novel technologies. Natural preservation technologies using plant extracts are being increasingly explored to extend the shelf life of fresh food. Plant antimicrobials are phytochemicals which are important for the proper functioning of the plant and used as plant defense agents against microorganisms and other predators. Phenolic compounds are a rich source of antioxidants which can extend the freshness of the product by preventing oxidation. The Australian native food industry with its diverse and rich flora has a huge potential to contribute to the growing natural functional ingredient market. Case studies of natural antimicrobials in extending the storage life of meat, seafood and horticultural products will be discussed with successful commercial applications.

The following poster presentations on kakadu plum were made at the **TropAg2015 International Conference**, November 16-18 2015, Brisbane, Australia

Acids in Kakadu Plum Fruit (*Terminalia ferdinandiana*): The Good (Ellagic), The Bad (Oxalic) and The Uncertain (Ascorbic)

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Recently there has been an increasing interest in the phenolic, ellagic acid (EA) primarily for its nutritional and pharmacological potential as an antioxidant. The occurrence of this important phytochemical is limited to a few fruits e.g. strawberries, *Rubus* berries and the tissues of *Terminalia ferdinandiana* (Kakadu plum). Levels so far reported have varied markedly but all have indicated that Kakadu plum fruit is an abundant source. Investigations have also revealed that this fruit possesses large amounts of the bioactive forms of vitamin C (ascorbic, AA and dehydroascorbic acids, DHAA). However several native Australian plants have been implicated in containing the undesirable oxalic acid (OA). As consumption of oxalate in the diet poses health risks such as kidney stone development, it is important to identify high oxalate containing foods. Related to this is the observation that endogenous oxalate is produced by the breakdown of AA in the liver and is finally excreted in the urine. This study measured the contents of EA; OA and AA in the fruit of the Kakadu plum. The results indicated EA and the bioactive forms of vitamin C were present in very high concentrations especially when compared to well-documented sources such as strawberries and boysenberries. The levels of OA in the fruit admittedly less than that reported for several well known OA-containing vegetables were still considerable. These quantities in conjunction with very high AA levels suggests more detailed investigations are necessary before recommending optimal levels of Kakadu plum fruit that could be included into functional foods.

Exploring the metabolomic constituents and bioactive potential of Kakadu plum (*Terminalia ferdinandiana*) – a native plant of Australia

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Kakadu plum (KP, *Terminalia ferdinandiana*) is a traditional food that has been consumed for its nutritional and therapeutic value by indigenous Australians for thousands of years.

Non-targeted metabolomic profiling of polar extracts of the KP fruit, seeds and leaves identified 158 chemically diverse metabolites including amino acids, organic acids, phenolic acids, esters and sugars. In addition to detecting AA and gallic acid, previously unreported metabolites such as galacturonic acid, rhamnose, xylitol, xylulose, maltose, myo-inositol and palatinose were tentatively identified. Preliminary experiments were carried out to extract and characterize pectin from KP. The extracted KP pectin (yield 85%) had an equivalent weight of 251.17 g, 12.75% methoxyl content and 0.46% degree of esterification.

To obtain enhanced release and recovery of active compounds attached to the cells, enzymatic digestion of KP tissue was carried out with pectinase. The resultant extract showed high concentration of EA ranging from 100 – 4400 mg/100 ml and AA ranging from 313 – 521 mg/ 100 ml at acidic and alkaline pH. The antioxidant activity and protective effects of this extract containing KP bioactive compounds was investigated with the help of Caco-2 cells where oxidative stress was experimentally induced with hydrogen peroxide.

Understanding the KP metabolites and the molecular pathways via which it exerts anti-oxidant activity *in vitro*, will help in identifying new sources of therapeutics that target the debilitating oxidative and inflammatory cascade which is the hallmark of many chronic degenerative disorders.

Oral presentations at conferences/workshops

- Sultanbawa, Y. 2017. Guest speaker at the Women's College 2017 Standing Committee Luncheon. "Australian native plants – Empowering indigenous communities" 29 June, 2017.
- Sultanbawa, Y. 2017. Why Mi-Marral (Kakadu plum) is so special. A workshop held for the Thamarrur Rangers and the Palngun Wurnangat Aboriginal Corporation to introduce value added products from Kakadu plum to the Aboriginal community in Wadeye for diet diversification. April 20-21 April 2017.
- Sultanbawa, Y. 2017. Presentation on 'Unique and Nutritious Queensland Produce' for the Advanced Queensland Innovation Partnerships 2016-2017 Round 2 to the Gugu Badhun community, Greenvale, Townsville, 14 April 2017.
- Sultanbawa, Y. 2017. Innovative Functional Ingredients from Plant Sources in Food Applications. Presentation at the Native Fruits and Trees Workshop, Broome 14-15 March, 2017 organised by Mamabulanjin Aboriginal Corporation and Indigenous Land Council.
- Sultanbawa, Y. 2016. Burdekin Horticulture Forum, North Queensland Dry Tropics. Does the region have the potential to become a food hub? Organised by Growcom and National Landcare Programme, 19 October invited speaker presentation "Kakadu plum case study and how QAAFI can help". October 19, Achacha farm, Bruce Highway Palm Creek, Giru, Townsville.
- Sultanbawa, Y. 2015 Territory Natural Resource Management Conference, 12-13 November, Darwin, Australia, presentation on "Why Kakadu plum is so special at the workshop titled "Making a business from native plant products – Kakadu plum as case study.

Student presentations at conferences/workshops

- Chaliha, M., Williams, D., Smyth, H., Pun, S., Edwards, D. and Sultanbawa, Y. Metabolomic profiling and enzyme assisted extraction of bioactive compounds from Kakadu plum (*Terminalia ferdinandiana*) – native plant of Australia. II International Conference on Food Chemistry and Technology, November 14-16, 2016, Las Vegas, USA (Oral presentation)
- Gorman, J. Brady, C. and Sultanbawa, Y. Looking after country through commercial use of native plant products. Two decades on Conservation through sustainable use of wildlife conference. 30 Aug – 1 Sept. 2016 Brisbane, Australia (Oral presentation)
- Chaliha, M., Shelat, K., Fernando, C., Williams, D., Smyth, H. and Sultanbawa, Y. In vitro antimicrobial effects and mode of action of Kakadu plum (*Terminalia ferdinandiana*) products against pathogenic and spoilage microorganisms. Tropical Agriculture Conference 2017, High impact science to nourish the world, 20-22 November 2017, Brisbane Convention and Exhibition Centre, Brisbane, Australia (Poster presentation).
- Akter, S., Netzel, M., Tinggi, U., Fletcher, M., Williams, D. and Sultanbawa, Y. Nutritional and non-nutritional properties of *Terminalia ferdinandiana* (Kakadu plum) tissues from the Northern Territory. Tropical Agriculture Conference 2017, High impact science to nourish the world, 20-22 November 2017, Brisbane Convention and Exhibition Centre, Brisbane, Australia (Poster presentation).

- Akter, S., Williams, D., Netzel, M., Fletcher, M., Tinggi, U. and Sultanbawa, Y. Kakadu plum (*Terminalia ferdinandiana*) kernels: A novel nutrition source. Australian Institute of Food Science and Technology, 50th Anniversary Convention “The Future Food”, 17-18 July 2017 International Convention Centre, Sydney, Australia (Poster presentation).
- Chaliha, M., Williams, D., Smyth, H., Pun, S., Edwards, D. and Sultanbawa, Y. Metabolomic profiling and enzyme assisted extraction of bioactive compounds from Kakadu plum (*Terminalia ferdinandiana*) – native plant of Australia. II International Conference on Food Chemistry and Technology, November 14-16, 2016, Las Vegas, USA (Oral presentation) accepted
- Williams, D.J., Edwards, D., Chaliha, M., Burren, B and Sultanbawa, Y. Acids in Kakadu Plum Fruit (*Terminalia ferdinandiana*): The Good (Ellagic), The Bad (Oxalic) and The Uncertain (Ascorbic). Meeting the Productivity Challenge in the Tropics, 16-18 November 2015, Brisbane, Australia (POSTER)
- Chaliha, M., Chrysanthopoulos, P., Hodson, M., Williams, D., Smyth, H. and Sultanbawa, Y. Exploring the metabolomic constituents and bioactive potential of Kakadu plum (*Terminalia ferdinandiana*) – a native plant of Australia. Tropical Agriculture Conference 2015, Meeting the Productivity Challenge in the Tropics, 16-18 November 2015, Brisbane, Australia (Poster presentation)

Peer reviewed publications

- Akter, S.; Netzel, M. E.; Fletcher, M. T.; Tinggi, U.; Sultanbawa, Y., Chemical and nutritional composition of *Terminalia ferdinandiana* (kakadu plum) kernels: a novel nutrition source. *Foods* **2018**, *7*, 11.
- Chaliha, M.; Williams, D.; Smyth, H.; Sultanbawa, Y., Extraction and characterization of a novel *Terminalia* pectin. *Food Science and Biotechnology* **2017**.
- Chaliha, M.; Williams, D.; Edwards, D.; Pun, S.; Smyth, H.; Sultanbawa, Y., Bioactive rich extracts from *Terminalia ferdinandiana* by enzyme-assisted extraction: A simple food safe extraction method. *Journal of Medicinal Plants Research* **2017**, *11*, 96-106.
- Williams, D. J.; Edwards, D.; Pun, S.; Chaliha, M.; Burren, B.; Tinggi, U.; Sultanbawa, Y., Organic acids in Kakadu plum (*Terminalia ferdinandiana*): The good (ellagic), the bad (oxalic) and the uncertain (ascorbic). *Food Research International* **2016**, *89*, 237-244.
- Williams, D. J.; Edwards, D.; Chaliha, M.; Sultanbawa, Y., Measuring free ellagic acid: influence of extraction conditions on recovery by studying solubility and UV-Visible spectra. *Chemical Papers* **2016**, *70*, 1078-1086.
- Williams, D. J.; Edwards, D.; Pun, S.; Chaliha, M.; Sultanbawa, Y., Profiling ellagic acid content: The importance of form and ascorbic acid levels. *Food Research International* **2014**, *66*, 100-106.

Appendix 4

Awards won by the members of the Kakadu plum consortium

Plum award for Australian native food industry collaboration

Kakadu plums

27 November 2017

A powder extracted from the Kakadu plum to extend the shelf life of frozen ready-made foods has earned a University of Queensland researcher and her team a national award for community engagement.

Associate Professor Yasmina Sultanbawa from UQ's Queensland Alliance for Agriculture and Food Innovation was recognised for her work with Aboriginal communities and industry in northern Australia to develop the powder.



Her team won the Outstanding Collaboration in Community Engagement award at the annual **Business and Higher Education Round Table (BHERT)** awards held on 21 November 2017.

“Australia’s Indigenous population has long known the health benefits of native bush food, but the mainstream food industry has only been able to harness some of these benefits recently,” Dr Sultanbawa said.

“The Kakadu plum is only the size of an olive but is packed with Vitamin C, antioxidant and antimicrobial properties.”



Associate Professor Yasmina Sultanbawa (centre)

Dr Sultanbawa has worked with government, researchers, industry and Aboriginal communities across the Top End of Australia since 2010, investigating the nutritional and functional value of the plum.

“We developed a puree and a powder that can be added to foods including frozen ready-made meals to extend shelf life up to 18 months, which is very significant advantage,” Dr Sultanbawa said. She said indigenous communities, government, philanthropists and research partners worked collaboratively to develop an innovative business model to harvest, market and distribute the in-demand Kakadu plum fruit puree and powder.



Wild harvesting of Kakadu plums

Twenty tonnes of Kakadu plums are harvested across Northern Australia each year, with plans to increase this wild harvest to more than 100 tonnes to meet commercial demand. The annual BHERT awards aim to reinforce the importance of business-university partnerships in innovation, research and development and teaching.

BHERT Chief Executive Officer Dr Peter Binks praised the Plum Pickings team.

“This an outstanding collaboration, built on good science and interdisciplinary relationships with the objective of creating new business and employment opportunities,” Dr Binks said.

“Our judging panel was impressed by the impact this project has already had, and the long-term prospects for the food preservative industry in Queensland and the Northern Territory.”

The Kakadu plum consortium includes:

Funding partners: AgriFutures Australia, Australian Government Department of Industry, Innovation and Science and The Australian Industry Group

Industry partners: Kindred Spirits Foundation, Karen Sheldon Catering, Australian Native Foods and Botanicals

Research partners: University of Queensland, Department of Agriculture and Fisheries, Charles Darwin University, EcOz Environmental Services

Aboriginal community partners: Palngun Wurnangat Aboriginal Corporation, Gundjeihmi Aboriginal Corporation, Mamabulanjin Aboriginal Corporation, Milingimbi Crocodile Rangers, Thamarrurr Rangers

Captions: top left - Associate Professor Yasmina Sultanbawa (centre); bottom right - wild harvesting of Kakadu plums.

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