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Original article

The effect of maturity and season on health-related bioactive compounds in wild harvested fruit of *Terminalia ferdinandiana* (Exell)

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Summary This study reports changes in the concentrations of important health-related bioactive compounds (vitamin C and ellagic acid) and morphology of wild harvested Kakadu plum fruits that were collected during three harvest seasons and four maturities. The results showed that fruit weight increased, whereas fruit length and width changed slightly with the advance in maturity. Vitamin C increased up to 20 folds (from 1.2 to 21.2% dry weight [DW]) from immature to mature stage, whereas ellagic acid decreased approximately three times (from 6.5 to 2.1% DW). Similar trends were observed over the three harvest seasons studied. A positive correlation between fruit weight and vitamin C, whereas a negative correlation with ellagic acid was observed, indicating that maturity plays an important role in contributing to the variation of ellagic acid and vitamin C. Season also had an effect and showed the influences of rainfall, temperature and solar exposure on the biosynthesis of vitamin C and ellagic acid.

Keywords Ellagic acid, Kakadu plum, maturity, rain fall, seasonal effect, solar exposure, temperature, *Terminalia ferdinandiana* Exell, vitamin C, wild harvest.

Introduction

Terminalia ferdinandiana (Exell), commonly known as Kakadu plum (KP), belongs to the family Combretaceae, a native tree of Australia (Brock, 2005; Gorman *et al*., 2020). Fruits of this tree play an important role in the Australian Aboriginal communities as food or traditional medicinal ingredient (Lim, 2012). Recently, KP fruits have been reported to exhibit a wide range of health-related benefits measured *in vitro*, including antimicrobial, antioxidant, anti-inflammatory and anticancer properties (Tan *et al*., 2011; Akter *et al*., 2019; Chaliha & Sultanbawa, 2019). The functional properties of KP fruits are associated with the exceptionally high levels of vitamin C of up to 32 g/100g dry weight (DW) or 5.3 g/100g fresh weight (FW), ellagic acid (3.1–14.0 g/100g DW), and ellagic acid–related compounds such as glycosides and polymeric ellagitannins (Clifford & Scalbert, 2000; Konczak *et al*., 2014). Vitamin C, ellagic acid and its derivatives have been associated with health-promoting biological functions, including antioxidant properties, protecting against free radicals, improvement of the renal activity and inhibiting cancer cell proliferation (Losso *et al*., 2004; Ahad *et al*., 2014; Mortensen & Lykkesfeldt, 2014).

The Australian Indigenous people have a long history of harvesting KP fruits from the wild, where wild harvest might explain potential inconsistencies in fruit quality from season to season. Seasonal effects associated with fluctuations in environmental conditions (e.g., temperature and rainfall) during fruit growth have been also reported to affect fruit growth and the nutritional composition of a wide range of crops such as grapes and berries (Lee & Kader, 2000; Cozzolino *et al*., 2010; Teixeira *et al*., 2013; Hykkerud *et al*., 2018).

KP fruit season normally starts from November to December, ending around June (Gorman *et al*., 2019b), where the fruits usually grow from the dry season through the wet season, particularly in the Northern Territory of Australia (Gorman *et al*., 2019a).

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Differences in climatic conditions among harvest seasons might cause insecurity to the KP Industry and Indigenous enterprises, as they might be the cause of variations in the composition and consistency of this fruit and subsequently its quality. Anecdotally, a large fluctuation in the concentration of both vitamin C and ellagic acid has been reported in wild harvested KP fruits collected from different growing sites, accessions and even between individual trees at the same location (Konczak *et al*., 2014). However, available information between different seasons and maturities in wild harvested fruits of KP is incomplete.

In addition to the variations in the concentration of bioactive compounds and fruit quality parameters caused by seasonal effects, fruit maturity also plays an important role in determining the quality, sensory attributes and biological functions of plant-based food products (Kader, 1999; Pinillos *et al*., 2016; Vieira *et al*., 2018; Aubert *et al*., 2021). Therefore, harvesting KP fruit at the appropriate maturity is a key factor to ensure that the fruit fulfils the requirements for optimum compositional levels and to maximise the accumulation of bioactive compounds. This information could be subsequently used to monitor and predict postharvest storage and shelf-life to meet the growing demand for this type of products. Unlike other fruits that are traditionally collected at the ripe stage to optimise the harvesting conditions and quality, there is a potential to harvest KP fruits at the time when fruits reach the highest levels of some of the bioactive compounds to target different markets or food applications. Therefore, it will be important to better understand the relationships between fruit maturity and the accumulation of bioactive compounds during fruit growth to enable the KP industry and Indigenous enterprises to develop a standard harvest protocol for the KP fruit grown in the wild.

The current study aimed to investigate the effects of both maturity and season on the concentrations of two health-related bioactive compounds, vitamin C and ellagic acid, as well as on the morphology (length, weight and width) of wild harvested KP fruits from the Northern Territory of Australia.

Materials and methods

Reagents

Commercial chemical standards (HPLC grade) of L-ascorbic acid and ellagic acid were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Other reagents, including 1,4-dithiothreitol, meta-phosphoric acid, formic acid, acetic acid, hydrochloric acid and organic solvents used throughout the study, were of analytical grade and sourced from Sigma-Aldrich or Merck (Castle Hill, NSW, Australia).

Sample collection

KP fruits were collected from Thamarrurr region, Northern Territory (Australia), after obtaining a permit from the Northern Territory Parks and Wildlife Commission and permission from the Traditional Owners. The season normally starts from December to June in Northern Territory (Australia), and the KP fruit samples used in the current study were randomly harvested from three individual trees in April 2016 and 2017, and from six individual trees in April 2019. After harvesting, the fruits (ca. 5 kg per tree) were manually allocated into four different maturity groups from immature to mature (approximately 500 g per maturity stage; Fig. 1), using a visual method based on differences in the fruit shape and the degree of fruit fullness between maturity stages as previously described by Sultanbawa *et al*. (2018). A maturity index guideline describing exterior differences between the four maturity stages was included in Fig. 1. After sample allocation, whole fruit samples were kept at refrigerated conditions (4 °C) during transportation to the laboratory. The weight, length and width of the refrigerated fresh fruit samples were measured. After that, the whole intact fruit samples were frozen and lyophilised at −50 °C for 48 h (CSK Climateck, CSK Scientific, Brisbane, QLD, Australia). Lyophilised fruits were processed, where the flesh and seeds were separated using a laboratory blender (Waring®, Australian Scientific, Sydney, NSW, Australia). The freeze-dried flesh was retained and milled into a fine powder using a Mixer Mill (MM 400 Retsch, Thermo Fisher Scientific, Brisbane, QLD, Australia). The KP powdered samples were stored at −35 °C for further chemical analyses.

Climate data, such as temperature, rainfall and sun exposure, were obtained in a monthly format (from December to April) for each harvest season from the Australian Government's Bureau of Meteorology website (http://www.bom.gov.au/climate/data/, accessed on 15th September 2020).

Fruit morphology

To measure fruit morphology, ten fresh KP fruit samples were randomly selected from each maturity stage and from individual trees. The morphological parameters (width and length) were measured in the randomly selected samples using a 150-mm Digital Calliper (Craftright Engineering Works, Jiangsu, China). The fruit weight was also recorded using a laboratory scale (g ± 0.01 g) (Sartorius CP224S, Gottingen, Germany).

Extraction and analysis of ellagic acid

Analysis of total ellagic acid (free and conjugated forms) was performed following the method previously

Degree of fullness

0-25%	25-50%	50-75%	75-100%
Fruit has a long and skinny shape with a very sharp pointy concave tip at fruit's top end. At the stem end, there is a small slightly concave bulge. There are two obvious veins from the bottom to the fruit's top end which exist on each side of the fruit. Fruit colour is green to light brown.	Fruit has a long shape, like Stage 1, with a slightly pointy concave tip at fruit's top end. At the stem end, there is a slightly convex bulge. The fruit is more inflated compared to Stage 1. Two veins still exist on each side of the fruit but are only visible from the middle to the fruit's top end. Fruit colour is green to light brown.	Fruit is an egg-like shape with a pointy convex tip at the fruit's top end. At the stem end, there is a very light, but mostly flat small bulge. Fruit is approx. double the inflated degree of stage 1 fruit. The veins alongside the fruit are less visible. Fruit colour is light green to yellow.	Fruit is round with a fully inflated shape and plump, approximately 3 times the inflated degree of Stage 1 fruit. The veins alongside the fruit are invisible. The fruit has a bulging, convex blunted tip at the fruit's top end. At the stem end, the fruit no longer has a distinguishable mound. Fruit colour is light green to light yellow.

Figure 1 KP fruits harvested at four different maturity stages based on percentage fullness of the fruit: 0–25% (Stage 1), 25–50% (Stage 2), 50–75% (Stage 3), and 75–100% (Stage 4).

reported by Williams *et al*. (2016) with slight modifications. In brief, approximately 100 mg KP fruit powdered samples was extracted with 80% (*v*/*v*) methanol containing 0.01N HCl using a vortex, followed by sonication for 10 min at room temperature. Both the free and conjugated ellagic acids released in the extract (supernatant) were collected after centrifugation at 3900 g for 5 min at 20 °C (Eppendorf Centrifuge 5810 R, Hamburg Germany). The pellets were re-extracted twice with absolute methanol, followed by centrifugation as described above. The supernatants were combined and subjected to a Waters™ UHPLC-PDA system (Waters, Milford, MA, USA) for analysis and quantification of free ellagic acid released in the extract (Fig. 2b). To quantify ellagic acid existing as esterified form, 2 mL of the extract was transferred into a 5-mL Reacti-Therm vial (Thermo Fisher Scientific) and evaporated until dryness under nitrogen, using a Thermo Scientific™ Reacti-Vap Evaporator system. After evaporation, 2 mL 2N HCl was added into the Reacti-Therm vials for overnight hydrolysis at 90 °C following the method of Williams *et al*. (2016). Ellagic acid released after the hydrolysis was dissolved into absolute methanol and injected into an UPLC-PDA system for quantification of total ellagic acid (both free and conjugated forms) (Fig. 2c). The extraction and hydrolysis were conducted in triplicate.

Liquid chromatography analysis was performed using a Waters™ UHPLC-PDA system and a Waters BEH Shield RP C18 column (100 × 2.1 mm *i.d*; 1.7 μm) maintained at 40 °C. Mobile phases consisted of 0.1% formic acid in milli-Q water (eluent A) and 0.1% formic acid in acetonitrile (eluent B). Gradient elution programmed for B at a flow rate of 0.3 mL min^{-1} was as follows: 5% B for 1 min, 20% B for 4 min, 40% B for 10 min, 100% B for 2 min and re-equilibration for 5 min before the next injection. Total ellagic acid was quantified using an external calibration curve of ellagic acid monitored at 254 nm (Fig. 2a). The percentage coefficient of variation (% CV) of ellagic analysis in all the samples was in the range of 0.14–11.8%.

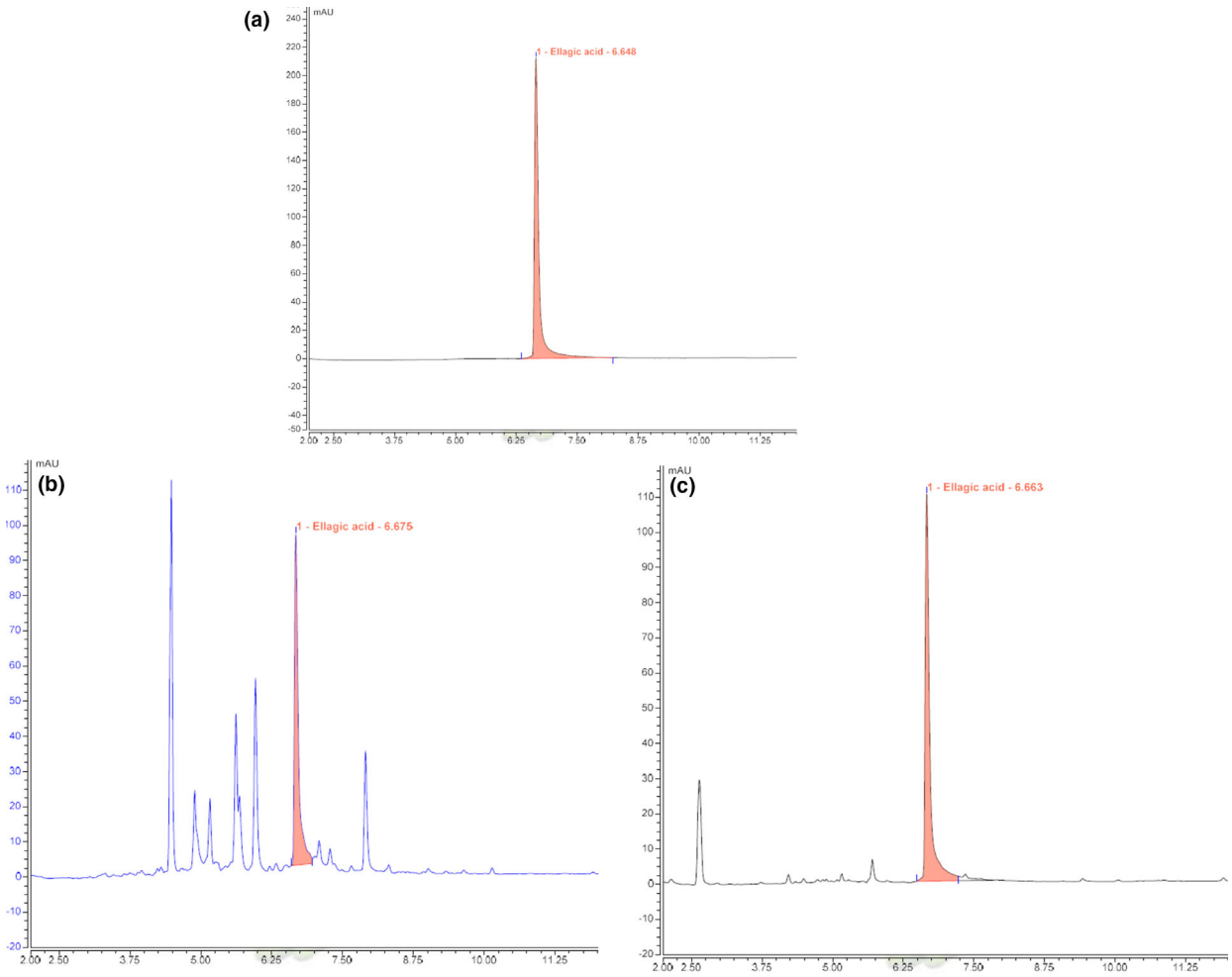

Figure 2 Representative UHPLC-PDA chromatograms @ 254 nm of (a) ellagic acid standard at 100 ppm and the sample extracts (b) before and (c) after acid hydrolysis.

Extraction and analysis of vitamin C

Analysis of vitamin C in KP fruit powdered samples (in triplicate) followed the method described by Phan *et al*. (2019) using a Waters Acquity UPLC-PDA system and a Waters HSS-T3 column (150 × 2.1 mm *i.d*; 1.8 μm) maintained at 25 °C with an isocratic elution (0.2 mL min^{-1} of 0.1% aqueous formic acid). Vitamin C was quantified using an external calibration curve of L-ascorbic acid monitored at 245 nm. The percentage coefficient of variation (%CV) of vitamin C analysis in all the samples was in the range of 0.7–5.7%.

Statistical analysis

A general linear model (GLM) was selected as an appropriate statistical approach which enables to specify the degree of interaction between multiple independent variables and to perform analysis of variance (ANOVA) for both balanced and unbalanced data (Shaw & Mitchell-Olds, 1993; Cnaan *et al*., 1997; SAS Institute, 2015). In the current study, data with unbalanced repeated measurements (three individual trees for seasons 2016 and 2017 and six individual trees for season 2019) were analysed using a GLM procedure, followed by Tukey's method of multiple comparison (Minitab 17® for Windows, Minitab Inc., State College, PA, USA).

Linear correlation was also used to show the relationships between the bioactive compounds analysed, fruit morphological and abiotic parameters (temperature, rainfall, solar exposure) using Graphpad Prism version 8.3 (GraphPad Software, San Diego, CA, USA). A P value of ≤0.05 was used to determine significant differences between treatments (e.g., maturity and season).

Results and discussion

Effect of maturity and season on fruit morphology

Table 1 shows the differences in fruit morphology associated with maturity in the KP fruit samples collected from immature (M1) to mature (M4) stages. Fruit samples collected over the three seasons showed a steady increase in fruit weight from 1.8 to 3.2 g from M1 to M4, although no statistically significant differences were observed. Both fruit length and width slightly decreased with maturity ($P > 0.05$; Table 1), ranging from 23.6 to 35.3 mm and 15.3 to 20.1 mm, respectively. Fruit weight results are in agreement with those reported in the literature, where the average weight of KP fruit samples collected from the Northern Territory of Australia ranged from 1.5 to 3.4 g (Konczak *et al*., 2014). However, both fruit length and width were higher compared with those reported in the literature where values averaged between 20 mm in length and 10 mm in width (Lim, 2012). A significant increase in fruit weight, followed by minor changes in fruit length as fruit mature, has been also reported in other edible indigenous fruit samples such as Natal plum (*Carissa macrocarpa*) (Ndou *et al*., 2019). No statistically significant differences in fruit morphological parameters between the three seasons analysed were observed, implying that the changes in environmental conditions (assumably temperature, rainfall and sunlight) over the three harvest seasons might not have an effect on these parameters measured in the wild harvested KP fruit samples.

Effect of maturity and season on vitamin C

Figure 3 summarises the vitamin C results of the KP fruit samples (four maturities and three seasons). The results showed an increase in vitamin C concentration associated with the advance of maturity (from M1 to M4) over the three seasons evaluated. The concentration of vitamin C in the KP fruit samples significantly increased from 1.2 to 21.2% DW from M1 to M4 (Fig. 3). These results suggested a direct effect of fruit maturity on the biosynthesis and accumulation of vitamin C in the KP samples during fruit growth. Overall, our findings are in agreement with those reported by others, where fruits have been sourced from domesticated crops such as strawberry, pomegranate and white guava. These authors described an increase in vitamin C synthesis associated with fruit maturity and/or ripening (Olsson *et al*., 2004; Soares *et al*., 2007; Fawole & Opara, 2013). For instance, the highest concentration of vitamin C in strawberry fruit could be observed in samples harvested at the late ripening stages or full maturity (e.g., dark colour) (Olsson *et al*., 2004). Similarly, the highest concentration of vitamin C in pomegranate (Fawole & Opara, 2013) and white guava (Soares *et al*., 2007) was observed at late maturity stages. It has been proposed that vitamin C synthesis and metabolism in fruit tissues followed the D-galacturonate pathway, which in short converts L-galactono-1,4-lactone to ascorbate by the activity of the enzyme L-galactono-1,4-lactone dehydrogenase (Fenech *et al*., 2018). In addition, the biosynthesis of ascorbic acid becomes more active in fruits with full ripening or fruits that reached the late stages of maturity (Fenech *et al*., 2018). Badejo

Table 1 Morphological parameters of KP fruits and climate conditions at the collection site over three seasons

		Morphology *			Climatic condition **				
Season	Maturity stages	Weight (g)	Length (mm)	Width (mm)	Min. air temp. (°C)	Max. air temp. (°C)	Aver. Temp (°C)	Rainfall (mL)	Solar exposure (MJ m^{-2})
2016	1	1.8 ± 0.4	35.3 ± 6.3	20.1 ± 2.3	24.9 ± 1.2	34.3 ± 1.2 a	29.6 ± 0.5 a	223.4 ± 200.2 ab	18.9 ± 1.8 b
	2	2.3 ± 0.5	27.5 ± 0.6	16.7 ± 2.7					
	3	3.1 ± 1.3	27.9 ± 3.6	17.1 ± 2.9					
	4	3.2 ± 1.4	26.6 ± 5.1	17.2 ± 3.1					
2017	1	1.8 ± 0.5	26.2 ± 3.2	17.5 ± 1.4	24.4 ± 0.8	32.5 ± 0.9 b	28.5 ± 0.4 b	381.3 ± 176.2 a	19.3 ± 1.8 b
	2	2.7 ± 1.5	27.2 ± 9.6	16.3 ± 2.3					
	3	2.7 ± 1.9	25.0 ± 9.7	15.8 ± 3.4					
	4	3.2 ± 1.8	23.8 ± 8.6	16.0 ± 3.2					
2019	1	2.0 ± 0.5	25.2 ± 4.0	15.6 ± 1.5	25.1 ± 1.3	34.0 ± 0.6 a	30.0 ± 0.8 a	157.3 ± 75.9 b	22.5 ± 1.4 a
	2	2.0 ± 0.5	23.8 ± 2.3	15.3 ± 1.8					
	3	2.5 ± 0.4	24.6 ± 1.7	15.4 ± 1.0					
	4	2.8 ± 0.5	23.6 ± 2.0	15.9 ± 1.9					

(*) Morphological data present mean ± SD ($n = 3$ for 2016 and 2017; $n = 6$ for 2019). (**) Average data of climatic condition collected monthly during KP fruit growth and ripening from December to April at the Port Keats Airport station (Bureau of Meteorology Station number: 014948; Northern Territory, Australia; http://www.bom.gov.au/climate/data/). Different letters at the same column indicate significant differences at $P \leq 0.05$.

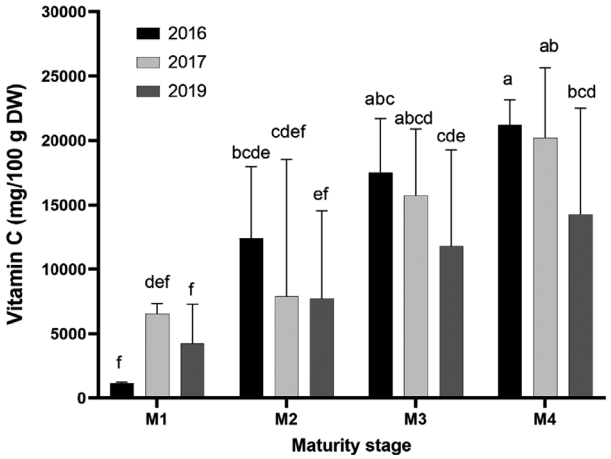

Figure 3 Changes in the concentration of vitamin C associated with fruit maturity over three seasons. Data present mean ± standard deviation (n = 3 for 2016 and 2017, n = 6 for 2019); Different letters indicate significant differences at $P \leq 0.05$.

et al. (2012) also reported that photosynthesis during immature or green fruit development stages of tomato could reduce ascorbate production. This association between maturity and ascorbic acid might explain the observation of low concentration of vitamin C in the immature KP fruit samples analysed.

Regarding the seasonal effect (year of harvest) on the concentration of vitamin C, the results of this study showed that fruit samples collected during the 2016 season had a higher vitamin C content than those samples harvested in 2017 and 2019. However, no statistical differences were observed between the seasons ($P > 0.05$, Fig. 3). It was observed that the 2016 season had the highest temperature and a moderate rainfall compared with the other two years, whereas the 2019 season had the lowest rainfall (see Table 1). These results suggested that temperature and moderate rainfall during fruit growth might be the crucial factors in explaining the exceptionally high concentration of vitamin C in the 2016 KP fruit samples.

Domesticated fruit crops have environmental requirements to optimise yield, where the pool of vitamin C might be modulated by abiotic factors such as sunlight exposure or temperature (Gautier *et al*., 2008; Zechmann *et al*., 2011; Suzuki *et al*., 2014; Fenech *et al*., 2018). These factors are well known to have a specific role in the antioxidant cellular system of higher plants (Jimenez *et al*., 2002; Massot *et al*., 2013). It has been also reported that a simultaneous increase in vitamin C level in plant tissues that underwent oxidative stress (e.g., photosynthetic tissues) might have an additional beneficial effect on plant tolerance (Fenech *et al*., 2018). It has been proposed by different authors that oxidative stress might occur during both fruit development and ripening (Brennan & Frenkel, 1977; Rogiers *et al*., 1998; Jimenez *et al*., 2002; Huan *et al*., 2016; Fenech *et al*., 2018). An early study in 1981 compared the content of vitamin C between wild tomato species grown naturally in Peru and Mexico (e.g., both coastal areas and river valleys, less than 1000 m above sea level with abundant rainfalls) (Esquinas-Alcazar, 1981). In this study, the authors suggested that high sun radiation and warm temperatures could explain why individual tomato plants tended to have higher concentrations of vitamin C (Esquinas-Alcazar, 1981), which are similar to those encountered in the Northern Territory of Australia. It is also important to note that KP fruit grows naturally in the wild, where the combination of sunlight, rainfall and temperature might influence the biosynthesis of vitamin C. These factors are not controlled by anthropogenic interventions as it is happening in domesticated crops (Lee & Kader, 2000; Koyama *et al*., 2012; Teixeira *et al*., 2013; Sun *et al*., 2017; Fenech *et al*., 2018). In addition, large standard deviations observed in Fig. 3 could be due to tree-to-tree variation, clearly reflecting a considerable effect of wild harvest condition to the content of vitamin C of KP fruits. Konczak *et al*. (2014) has reported a large fluctuation in the concentration of both vitamin C and ellagic acid between individual KP trees collected at the same geographical location, which supported the obtained results of the current study.

Limited research reported the biosynthesis of vitamin C in wild harvested fruits. However, based on the current knowledge in ascorbate biosynthesis in cultivated plants, it seems that both temperature (high) and rainfall (moderate) might be the main driving factors that contribute to the exceptionally high concentrations of vitamin C found in the analysed KP fruit samples. These factors are closely associated with the commonly harsh conditions found in North Australia.

Effect of maturity and season on ellagic acid

Figure 4 shows the content of ellagic acid in the KP fruit samples analysed at different maturity stages and seasons. Statistically significant differences were observed in the concentration of ellagic acid in the wild harvested KP fruits. However, no statistically significant differences could be found in the interactions between maturity and season. Similar to the result of vitamin C, large standard deviations were also observed for the results of ellagic acid. This could be due to tree-to-tree variation (Fig. 4), indicating the effect of the wild harvest and requiring more samples for further studies to substantiate the observation of the current study.

Contrary to the results reported for vitamin C, the total content of free and conjugated ellagic acid in the KP fruit samples decreased with the advance of fruit

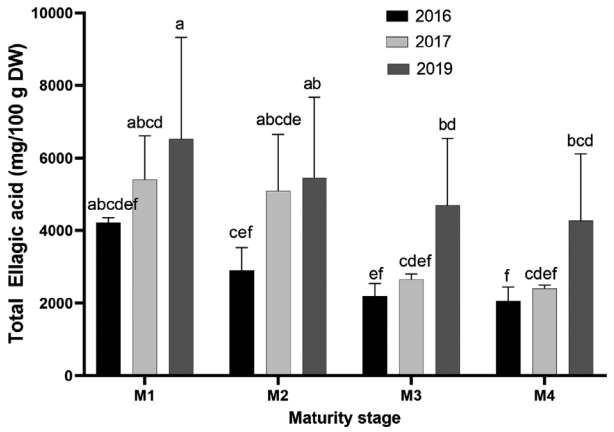

Figure 4 Changes in the concentration of ellagic acid associated with fruit maturity over three seasons. Data present mean ± standard deviation (n = 3 for 2016 and 2017, n = 6 for 2019); Different letters indicate significant differences at $P \leq 0.05$.

maturity ($P < 0.05$; Fig. 4). The same trend was observed in all three seasons analysed. The concentration of ellagic acid found in this study was in a similar range as reported in the literature, where total ellagic acids in different *Terminalia ferdinandiana* accessions varied from 3,050 to 14,020 mg/100 g DW (Konczak *et al*., 2014). A higher concentration of ellagic acid was reported in different strawberry varieties harvested from green to pink maturity compared with the fully mature samples, which have a characteristic red colour (Kosar *et al*., 2004). The effect of maturity on the concentration of ellagic acid was also reported in cultivated red raspberries (cv. Caroline), where the concentration of ellagic acid steadily decreased from 295 μg/100 g fresh weight (FW) at 5% maturity to 45.3 μg/100 g FW at 100% maturity (Wang *et al*., 2009). These results support the findings of the current study that the decrease of ellagic acid might be associated with fruit maturity.

Statistically significant differences were observed in the concentration of ellagic acid related with changes in temperature, rainfall and sunlight exposure between the seasons 2016 and 2019 (Fig. 4). However, no differences were observed between the harvest years 2017 and 2019 ($P > 0.05$). Fruit samples harvested in 2019 had the highest concentration in total ellagic acid (4.28–6.52 g/100 g DW), followed by the fruits collected in 2017 (2.41–5.41 g/100 g DW) and 2016 (2.07–4.21 g/100 g DW). It has been reported that different abiotic conditions such as temperature, solar exposure intensity and rainfall can affect the biosynthesis of ellagic acid and other phenolic compounds (Anttonen & Karjalainen, 2005; Koyama *et al*., 2012; Sun *et al*., 2017; Hykkerud *et al*., 2018). Several studies have reported the effect of 'season' on the biosynthesis and concentration of ellagic acid; for example, in cloudberry fruits harvested from different locations (Hykkerud *et al*., 2018) and walnut fruit (*Juglans regia* L.) (Solar *et al*., 2006).

In this study, differences were observed in temperature and rainfall between the three harvest seasons (Table 1; $P < 0.05$). The 2019 season had the lowest rainfall compared with the other two seasons, but the fruits had the highest ellagic acid content; whereas fruits harvested in 2016 and 2017 (high rainfall) had significantly lower ellagic acid concentrations (Table 1). Overall, these results might indicate that high rainfall during the season determines a decrease in the biosynthesis and accumulation of ellagic acid. Our findings are in agreement with those reported in the literature, suggesting that water stress can modulate the synthesis and accumulation of phenolic compounds in domesticated fruits including grapes (Quiroga *et al*., 2012; Cáceres-Mella *et al*., 2017), cherry tomatoes (Sánchez-Rodríguez *et al*., 2012) and medicinal plants (Albergaria *et al*., 2020).

Relationships between fruit morphology, bioactive compounds and climate parameters

Linear regression was used to assess the relationships between fruit maturity, climate data and bioactive compounds determined in the KP fruit samples. Figure 5a shows a positive correlation (r = 0.43, P = 0.01) between the concentration of vitamin C and fruit weight. This correlation indicated that as fruit increases its weight (dry matter) during maturity, the concentration of vitamin C increases too. This is in agreement with a previous report by others, suggesting that fruit dry matter might be associated with the increase in vitamin C (e.g., demonstrated with sweet pepper fruit (Niklis *et al*., 2002)).

In contrast, the concentration of total ellagic acid decreased with an increase in fruit weight ($P > 0.05$, Fig. 5b), resulting in a negative correlation between vitamin C and total ellagic acid ($r = -0.52$, $P < 0.001$; Fig. 5c). No statistically significant correlations were observed between the other fruit morphological parameters (e.g., fruit width and length) and the analysed bioactive compounds. A possible explanation could be that both parameters, fruit width and length, did not significantly change during fruit development (Table 1).

Figure 6 mostly shows weak to moderate linear regressions between the climate data (average of minimum and maximum air temperatures, rainfall and solar exposure) and the concentration of vitamin C and ellagic acid, except for a significant positive correlation between total ellagic acid and solar exposure (Fig. 6a; P = 0.01). Similar results have been reported in the literature, suggesting that light quality and intensity can have a significant impact on the biosynthesis and

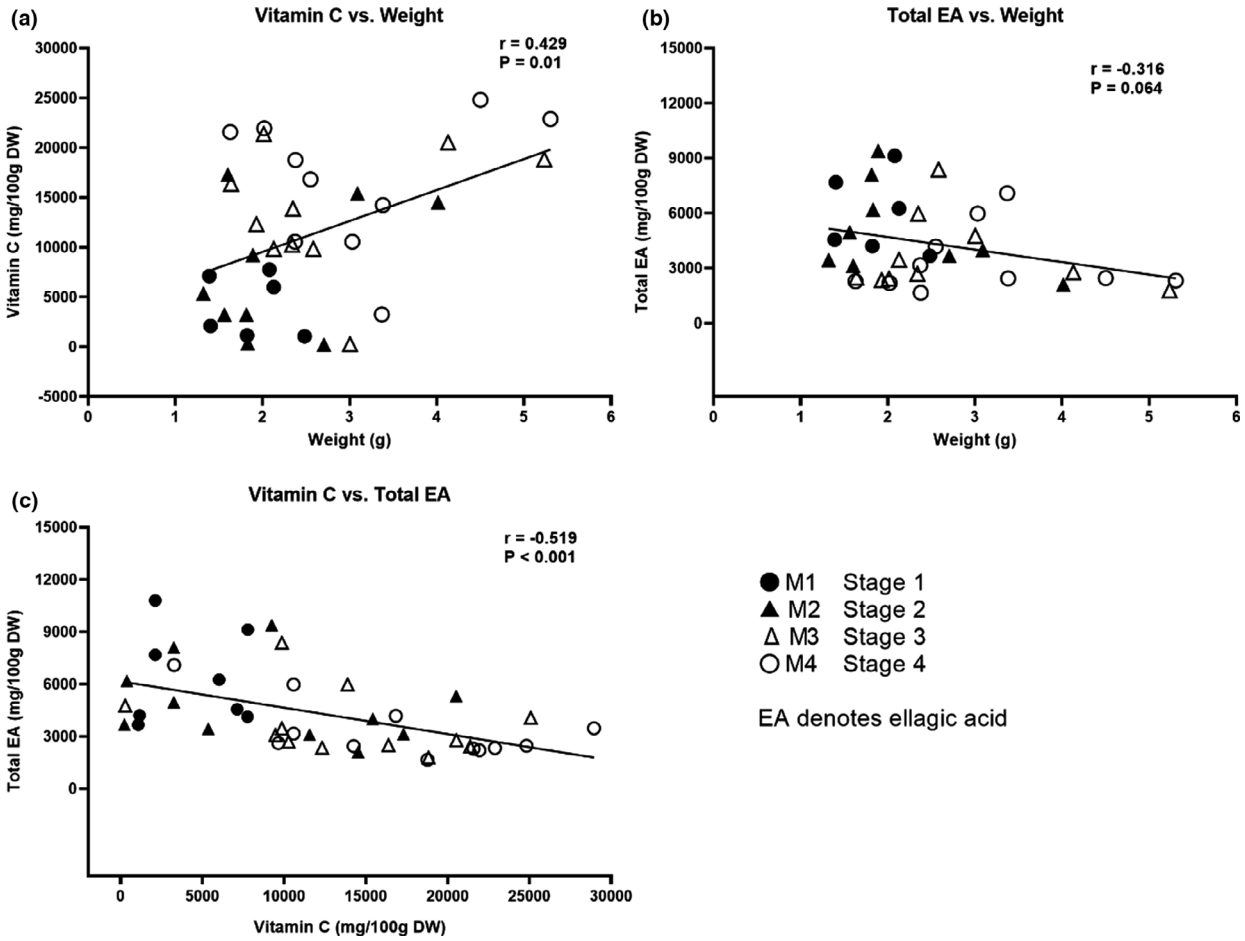

Figure 5 Linear correlations between fruit weight and (a) vitamin C and (b) total ellagic acid, and (c) between vitamin C and total ellagic acid.

accumulation of polyphenols during fruit growth (Wang *et al*., 2009; Koyama *et al*., 2012; Sun *et al*., 2017). Jaakkola *et al*. (2012) reported a "poor" correlation between the biosynthesis of phenolic compounds in wild harvested cloudberry fruits and the average maximum daily temperature, and suggested that the formation of polyphenols was highly related to sunlight exposure. It has been reported in viticulture that hot climate regions could produce grapes with a higher total anthocyanin content compared with grapes that were cultivated in a cooler climate (Cozzolino *et al*., 2010). No statistically significant correlations were found between ellagic acid, average temperature and rainfall. The same trend was observed for the relationships between vitamin C and the climate data (Fig. 6d,e and f).

Conclusions

The current study provided insight into the effects of fruit maturity and season on the variability of health-related bioactive compounds in wild harvested KP fruits. Our results indicated that fruit maturity plays a crucial role in determining the concentration of vitamin C in KP fruits at harvest, whereas total ellagic acid was significantly reduced during ripening. Seasonal effects seem to be a minor factor in terms of accumulation of vitamin C, but had a significant effect on ellagic acid, with solar exposure and intensity as key factors. The obtained results could be important and beneficial for the KP industry and Indigenous enterprises to better understand the variability of vitamin C and ellagic acid with changes in fruit maturity and season. This information is also helpful for further development of a standard harvest protocol for the KP fruit grown in the wild. Even though the study had a limited sample size due to management of wild harvested botanical material and permits for collection with approval from Traditional Owners, a total of twelve trees with four maturity stages per tree, collected over three seasons, gives a significant amount

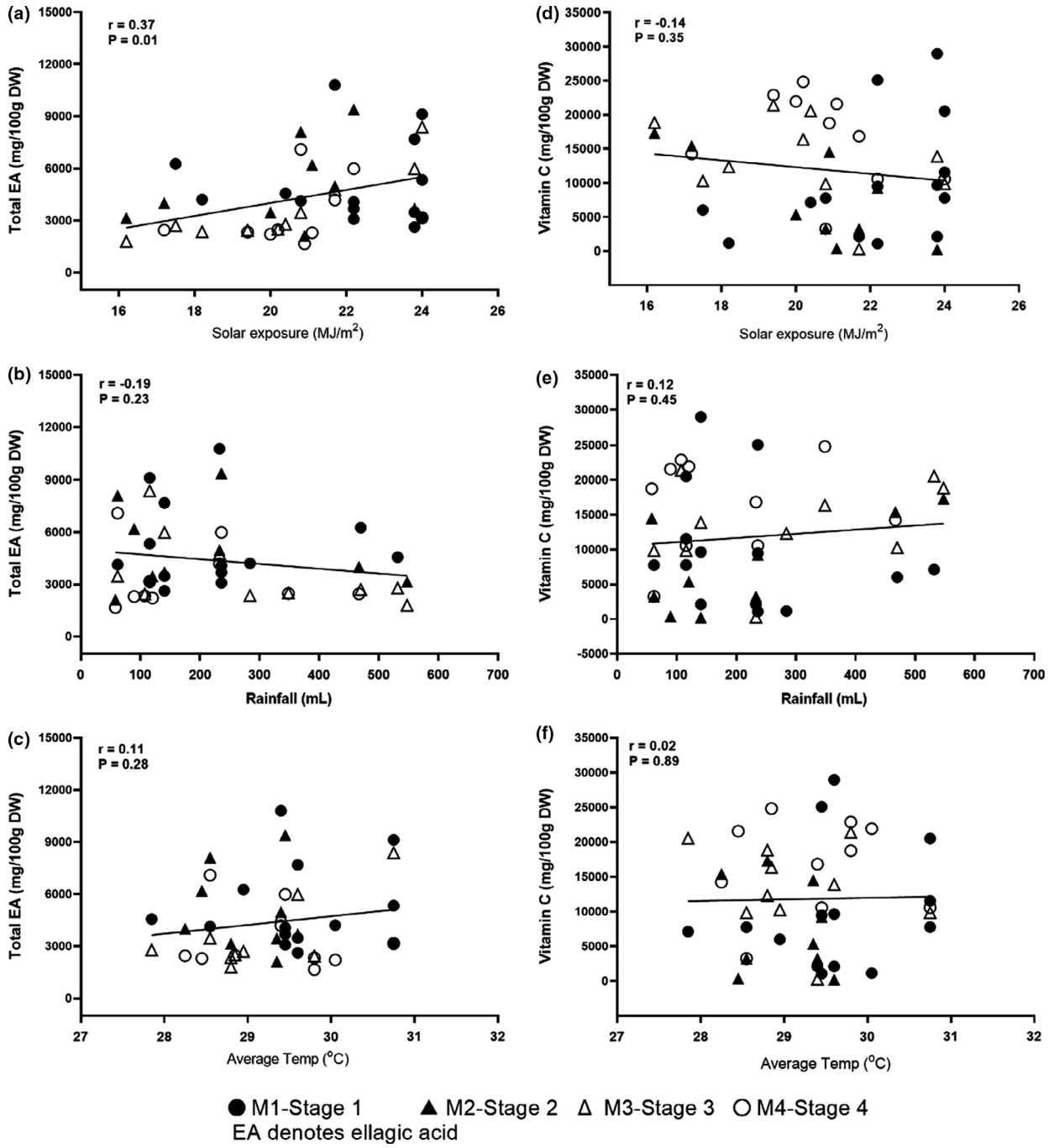

Figure 6 Linear correlations between climate data and total ellagic acid (a, b, c) as well as vitamin C (d, e, f).

of information on the trends of changes in bioactive compounds. Further studies are warranted having a larger samples size, more sampling locations and additional harvest seasons to substantiate the current results. In addition, the physiological processes during ripening affecting the biosynthesis and accumulation of vitamin C and ellagitannins in wild harvested KP fruits should be another focus in future studies. Investigations on health-related properties (e.g., antioxidants, anti-diabetes and anti-inflammatory) of KP fruits harvested at different maturity stages are also recommended.

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Author contribution

Anh Dao Thi Phan: Conceptualization (equal); Data curation (equal); Formal analysis (lead); Investigation (lead); Methodology (equal); Resources (equal); Software (equal); Validation (equal); Writing-original draft (lead); Writing-review & editing (lead). **Maral Seidi Damyeh:** Formal analysis (supporting); Investigation (supporting); Writing-review & editing (supporting). **Mridusmita Chaliha:** Formal analysis (equal); Methodology (equal); Resources (equal); Writing-review & editing (supporting). **Saleha Akter:** Formal analysis (supporting); Investigation (supporting); Methodology (supporting); Writing-review & editing (supporting). **Selina Fyfe:** Formal analysis (supporting); Software (supporting); Writing-review & editing (supporting). **M Netzel:** Conceptualization (supporting); Investigation (supporting); Supervision (equal); Validation (equal); Writing-review & editing (equal). **Daniel Cozzolino:** Conceptualization (equal); Data curation (equal); Methodology (supporting); Software (equal); Supervision (equal); Validation (equal); Visualization (equal); Writing-review & editing (equal). **Yasmina Sultanbawa:** Conceptualization (lead); Funding acquisition (lead); Investigation (lead); Resources (lead); Validation (equal); Visualization (equal); Writing-review & editing (equal).

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Conflict of interest

The authors declare no conflict of interest.

Ethical approval

Ethics approval was not required for this research and the research data are not shared.

Peer review

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Antimicrobial Activity, Total Phenolic and Ascorbic Acid Content of *Terminalia ferdinandiana* Leaves at Various Stages Of Maturity

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Abstract

This work investigated the effect of leaf development (maturity) on morphology, antimicrobial activity, total phenolic (TPC) and ascorbic acid content in leaves of *Terminalia ferdinandiana,* an endemic plant of Australia. The results of this study indicated that total ascorbic acid was in the range of 23.0 to 35.5 mg/100 g dry weight (DW), showing an increase with advance of maturity. TPCin water and methanolic extracts were in the range of 237.3 - 598.6 and 210.3 - 319.6 mg Gallic acid equivalent (GAE)/ g DW, respectively. Leaf extracts exhibited pronounced inhibitory activity towards *Staphylococcus* aureus where total ascorbic acid and TPC were positively correlated with the observed antimicrobial activity. These results indicated that leaves extracts might be used asan alternative to synthetic antimicrobial agents, with a great potential for application as an environmentally friendly sanitizer in the hospitality and healthcare industries.

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Leaf Maturity;
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Staphylococcus Aureus;
Terminalia ferdinandiana.

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Doi:

Introduction

Terminalia ferdinandiana (Exell, Combretaceae), is an endemic plant of Australia, with edible fruits that are extremely rich in antioxidant compounds.[1-6] Like its counter parts from the genus *Terminalia*, this plant has a rich history of being utilised as a traditional medicine by the Australian aboriginal communities. Fruits of this plant have been used to prepare various ailments for the cure of headaches, to alleviating symptoms of colds and fluand as an antiseptic.[1-8] This planthas been also used for its medicinal properties in the same way as *T. carpentariae*, another Australia native *Terminalia* species.[9]

Recent studies have reported on a number of bioactive properties of *T. ferdinandiana* which support many of the traditional medicinal claims of this plant by the Australian Aboriginal communities.[10-12] Some of thesereports also indicated that polar solvent extracts from *T. ferdinandiana* fruit were effectivein inhibiting both Gram-positive and Gram-negative bacteria.[10-12]

Protective effects of *T. ferdinandiana* fruit extracts on oxidative stress and inflammatory pathways have been also reported by other authors.[13-14] The potent biological activity observed in *T. ferdinandiana*is attributed to the presence of enhanced levels of antioxidant bioactive compounds.[15-16] Some of the bioactive phytochemicals detected in *T. ferdinandiana* fruit and leaves include ascorbic acid, ellagic acid, gallic acid, α-tocopherol, ethyl gallate, chebulic acid, corilagin, hydroxycinnamic acid, lutein, tannins, chebulagic acid, exifone, punicalin, castalagin, appanone A-7 methyl ether, xanthotoxin, phthalane, saponins, flavonoids, and terpenes.[1-19] Elevated antioxidant activitywas confirmed by early studies by Netzel and collaborators(2007)who measured the antiradical properties by the TROLOX Equivalent Antioxidant Capacity (TEAC) assay.[15] The antioxidant capacity of 567 T ferdinandiana fruit from 45 geographic sites was also reported demonstrating the important antioxidant activity of this plant.[2, 20-21] Recently, TPC of methanolic extracts of *T. ferdinandiana* fruits and leaf (obtained by accelerated solvent extraction) have been reported to be 12.2 and 11.7 g GAE/100 g DW.[22]

One of the most prominent antioxidant phytochemicals present in *T. ferdinandiana* is ascorbic acid or vitamin C, which is essential for human health. Due to its strong antioxidant properties, ascorbic acid neutralizes reactive oxygen species, prevents the generation of new free radicals by suppressing relevant molecular pathway and assists in the recycling of other antioxidants.[20-21] Vitamin C also plays an important immunomodulatory functions such as regulation of macrophage activity, reduction of inflammatory mediators, and imparting direct bacteriostatic effect at high concentrations.[22]

High levels of ascorbic acid(>14% DW)have been reported in *T. ferdinandiana* fruits.[5-6] Other studies have also reported a wide range of ascorbic acid levels in *T. ferdinandiana* fruits (3.5 - 5.9% FW) and in the range of0.1 - 5.3% FW.[1-4, 5,23] Levels of ascorbic acid in the *T. ferdinandiana* fruit were observed at significantly higher concentrations than other well-known natural sources of ascorbic acid like citrus fruit (0.5% FW ascorbic acid),[24] Acerola fruit (1.0 and 1.4% FW)[24-25] and Camu-Camu (1.8% FW).[24-25]

As the consumer awareness on the health promoting activities of the *T. ferdinandiana* fruit and its products, increasing demands for new applications are driving food industry to find novel applications of *T. ferdinandiana* as a functional food ingredient. Currently, only frozen puree and freeze-dried powder of *T. ferdinandiana* fruit are commercially available; however, anecdotal evidences and recent studies have indicated that other tissues such as leaves, seed coats and kernels could also be used for food and other applications.[22,26] The leaves of *T. ferdinandiana* could be a great candidate for such novel applications. Anecdotally, there are accounts of using broken *T. ferdinandiana* leaves for cleaning by scrubbing hands within the Indigenous Australians indicating potential antimicrobial efficacy of the leaves. A recent study, evaluated the antimicrobial activity of extracts were prepared from different *T. ferdinandiana* tissues including fruits, leaves, seedcoats, and barks. Itwas observed that both fruit and leaf extract exhibited superior antimicrobial activity, against common foodborne bacteria.[22, 26]

As *T. ferdinandiana* is a semi-deciduous tree, it drops its leaves in the dry season, and spouts new leaves at the beginning of the wet season. The characteristic morphology of the leaves should make them easier

to harvest than fruit. Leaves of *Terminalia* plants are usually spirally organised (often crowded) at the ends of the branches, sometimes on short shoots. In fact, the genus name '*Terminalia*' comes from the Latin word 'Terminus' relating to the factthat the leaves are located at the very tips of the shoots. Mature *T. ferdinandiana* leaves areusually large in size, making it possible to collect larger volume of raw material. Although several studies have shown the bioactive potentials of *T. ferdinandiana* leaves, information on the effect of maturity of leaves on their bioactive compounds or bioactivity is not available.

The objective of this work was to provide with information on morphology, antimicrobial activity, total phenolic and ascorbic acid content in *T. ferdinandiana* leaves over different stages of maturity.

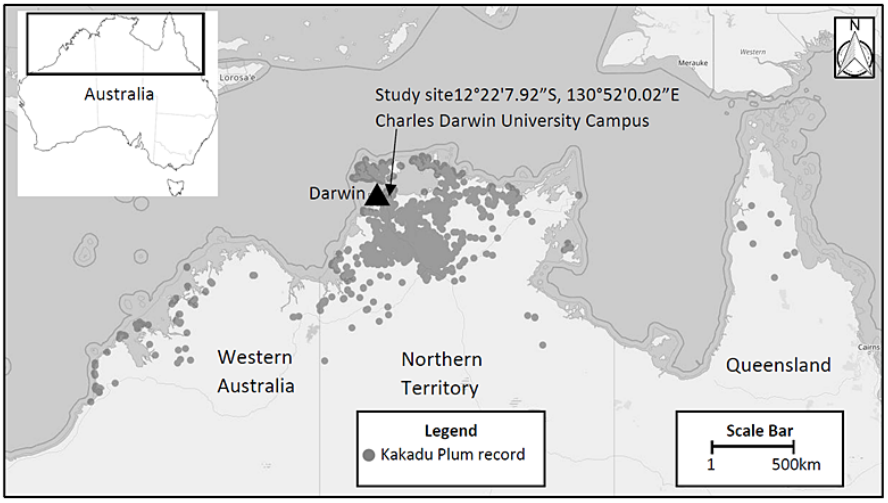


Fig.1: Map showing distribution of *T. ferdinandiana* (as plotted using tree records from Atlas of Living Australia) and approximate locate of study site at Charles Darwin University, Darwin, Northern Territory, Australia

Materials and Methods

Plant Material

Fieldwork for this study was conducted in undeveloped bushland in the northwestern corner of the Charles Darwin University, Casuarina campus (Darwin, Northern Territory, Australia) (see Figure 1). The study site is within one kilometre of the coastal shoreline, comprising open savanna woodland with incipient monsoon forest along old streamlines and is representative of *T. ferdinandiana* habitat.[23] The mean annual rainfall from 1995 to 2020 was 1768 mm and mean daily temperatures ranged from a minimum of 23.2 to a maximum of 32.1°C (Bureau of Meteorology 2020).

Leaf samples were harvested from November 2017 to June 2018. A total of 15 different maturity stages of leaves (10 leaves per stage) were collected from 10 individual trees (AT1 to AT10). Stage 1 to 4 contain immature leaves; stage 5 to 10 contain mature leaves and stage 11 to 15 contain senescing leaves. Leaves of stages 12 to 15 were collected from fewer than 10 individual trees as some of the trees had dropped their leaves ahead of others. Details of the harvesting timeare provided in Table 1. For the current study, 5 maturity stages, were selected from 3 individual trees (10 leaves per stage per tree) for analysis. The selected maturity stages for the current study allowed us to look at leaves at immature stage (e.g. stages 2 and 3), mature stage (e.g. stage 6 and 10) and senescing stage (e.g. stage 15) (see Figure 2).

Table 1: Time of collection of *Terminalia ferdinandiana* leaf samples

	Maturity stages	Time of collection
Immature leaves*	1	14 Nov 2017
	2	14 Nov 2017
	3	14 Nov 2017
	4	14 Nov 2017
Mature leaves	5	9 Jan 2018
	6	14 Feb 2018
	7	12 Mar 2018
	8	14 Apr 2018
	9	14 May 2018
	10	21 May 2018
Senescing leaves	11	30 May 2018
	12**	6 Jun 2018
	13***	13 Jun 2018
	14***	21 Jun 2018
	15***	26 Jun 2018

*These samples were collected on the same time point as the newly sprouted leaves of stage 1. Leaves were divided into stage 2, 3 and 4 on the increase in sizes.
**Leaves from stage 12 were collected from 6 individual trees (10 leaves per tree).
***Leaves from stage 13, 14 and 15 were collected from 4 individual trees (10 leaves per tree).

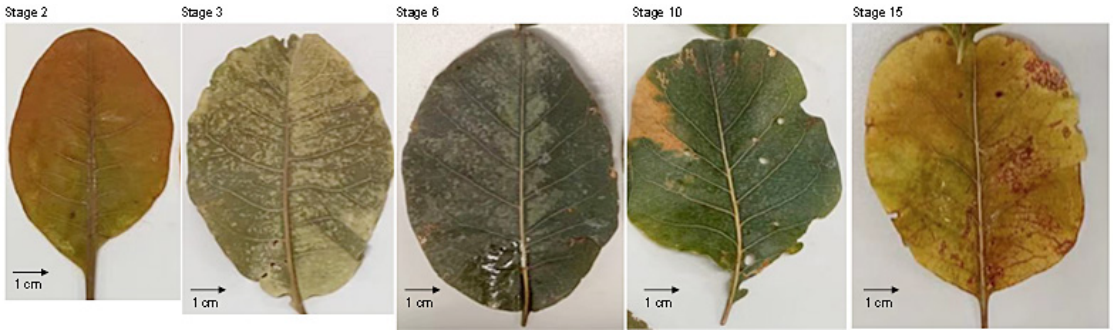


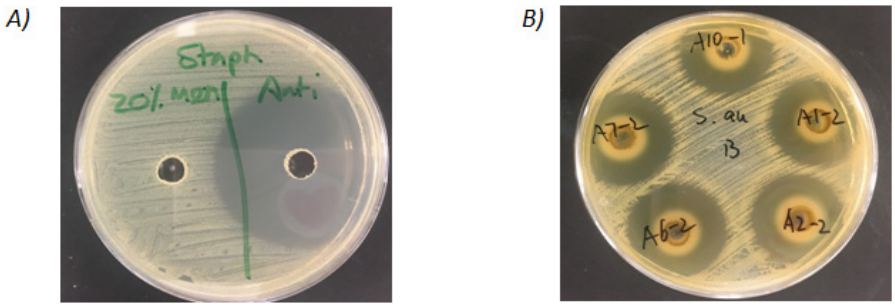
Fig. 2: The selected maturity stages of the current study. Stages 2 and 3 represent the immature leaves, stages 6 and 10 represent the mature leaves and stage 15 represent senescing leaves

Morphological Parameters

Length and width of 10 individual leaves of each maturity stage from three individual trees were measured using adigital calliper (Craftright Engineering Works, Jiangsu, China) followed by measuring the weight on laboratory scales (Sartorius CP224 Sanalytical balance with readability precision 0.01g, Gottingen, Germany). The leaves were frozen at -80°C and then freeze dried at -50°C for 48h (CSK Climatek, Darra, QLD, Australia). After freeze drying, around 2 g of dry leaves were ground using a Retsch MM400 ball mill (Retsch GmbH, Haan, Germany) at a speed of 30 Hz for 1 min.

Extraction of Bioactive Constituents

Bioactive constituents from *T. ferdinandiana* leaves were extracted by either methanol or deionized water. Triplicate samples (approx. 0.5 g) of the freeze-dried powders were accurately weighed into separate centrifuge tubes and individually blended with 5 ml of each solvent. After 30 seconds of vortex-mixing, the tubes were sonicated in an ultrasonic bath (Elma Schmidbauer GmbH, Ruiselede, Belgium) for 5 min at room temperature, followed by another 5 min of gentle agitation. The slurry was subsequently centrifuged at 3900 rpm for 5 min using an Eppendorf 5180 centrifuge (Eppendorf, Hamburg, Germany). The supernatant was carefully transferred and collected while the residues extracted twice again. After 3 times of extractions, a total volume of 15 ml of the crude extracts were combined and evaporated in a rotatory evaporator (Genevac Ltd, Ipswich, Suffolk, England) at 40°C. The dried extracts were freshly reconstituted in 5 mL of aqueous 20% v/v methanol (for methanol extracts) or deionized water (for water extracts) prior to analysis of antimicrobial activities. The reconstitute extract was stored at biomedical freezer (MDF U5312, PHCbi, Panasonic) and daily monitored using a digital thermometer.



Supplementary 2: Representative photos show (B) the inhibition of methanol extracts and (A) antibiotic solution (10 µl of Penicillin and streptomycin at 1g each/10 mL methanol) against *Staphyloccocus areus* and negative control 20% methanol.

Antimicrobial screening

A total of three pathogenic microbial strains were tested in this study to evaluate the antimicrobial activity of *T. ferdinandiana* leaf extracts: *Staphylococcus aureus* NTCC 6571, a Gram-positive bacteria; Escherichia coli NTCC 9001, a Gram-negative bacteria and Candida albicans ATCC 90028 a fungi. The bacterial strains were purchased from American Type Culture Collection, USA or National Collection of Type Cultures (NCTC), UK. Well diffusion assay was used to evaluate the antimicrobial activity of the leaf extracts followed the method published by Phan and collaborators(2019).[27] The inhibition zone was measured using a 150 mm Digital Calliper (Craftright Engineering Works, Jiangsu, China). MIC will be suggested for further investigations uing a widen ranges of microorganisms. All plates were incubated overnight in triplicate (see Supplementary material S2).

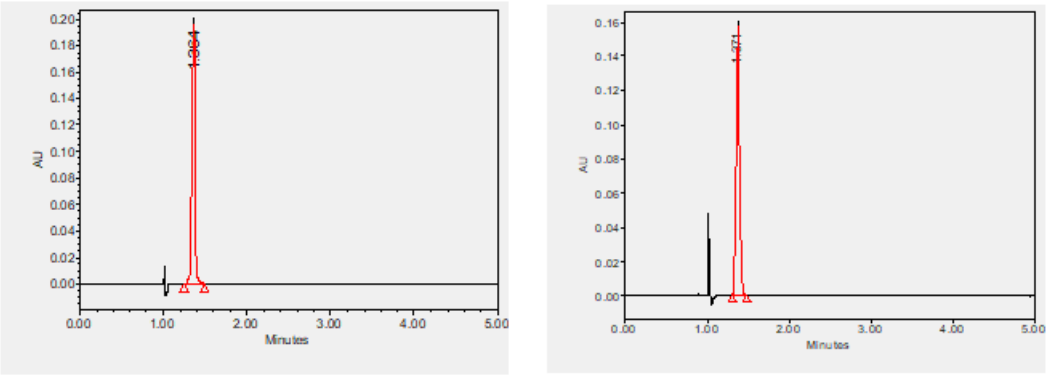
Total Phenolic Content (TPC)

The total phenolic content (TPC) of the samples was determined using the Folin Ciocalteau (FC) method using a micro-plate reader (Sunrise Tecan, Maennedorf, Switzerland).[28] Gallic acid standards ranging from 21 to 105 mg/L were prepared to establish the standard curve for quantification of TPC in the extract. TPC was expressed as mg gallic acid equivalents per gram of sample in dry weight (mg GAE/g DW).

Extraction and Analysis of Ascorbic Acid

Measurement of vitamin C content in *T. ferdinandiana* leaf extracts was conducted by utilizing ultra-high-performance liquid chromatographic–photodiode array (UHPLC-PDA) methodology.[29-30] (See chromatogram S1).

A B



Supplementary 1: UHPLC-PDA acquired at 245 nm of (A) ascorbic acid standard and (B) the leaf extract

Table 2: Morphological data for *Terminalia ferdinandiana* leaves at various stages of maturity

Tree ID	Maturity stage	Length (mm)	Width (mm)	Weight (g)
AT2	2	58.7± 6.5	36.8± 5.1	0.4 ± 0.1
	3	112.5± 8.6	95.5± 10.9	1.9 ± 0.3
	6	159.6± 16.5	123.4± 9.6	4.0 ± 0.9
	10	149.5± 18.0	122.6± 15.8	3.4 ± 0.6
	15	124.8± 30.0	106.3± 16.4	2.4 ± 0.9
AT5	2	80.5± 7.7	50.9± 7.1	0.8 ± 0.2
	3	133.4± 20.4	98.9± 18.1	2.5 ± 0.8
	6	184.6± 17.1	126.3± 16.1	5.1 ± 0.8
	10	162.4± 24.1	118.1± 27.7	3.6 ± 1.1
	15	189.4± 28.4	123.9± 17.1	4.1 ± 1.1
AT9	2	73± 19.9	46.2± 12.2	1.2 ± 0.8
	3	138.1± 25.5	91.4± 15.8	2.5 ± 0.9
	6	187± 23.8	136.1± 14.8	5.0 ± 1.0
	10	168.9± 48.4	119.7± 21.5	3.6 ± 1.7
	15	135.3± 17.7	109.9± 19.0	3.6 ± 1.3

Results are mean ± SD (n = 10)
AT indicate the tree ID of the individual trees collected from the study site at Charles Darwin University, Darwin, Northern Territory, Australia.

Data analysis

Descriptive statistics (average, minimum and maximum values, and standard deviation), principal component analysis (PCA) and multiple linear regression (MLR) wereapplied to inspectthe relevant and interpretable structure in the data set associated with the variables measured in the leaf samples at the different maturities. The optimum number of components in both PCA and MLR analysis was determined internal cross validation (leave one

out).[31] The Unscrambler software (version 10.5, CAMO, Norway) was used to develop the PCA and MLR regression models. Samples were standardised using the pre-processing 1/SD.[31]

Results and Discussion

The current study analysed *T. ferdinandiana* leaves at 5 maturity stages namely immature leaves at stages 2 and 3; mature leaves at stages 6 and 10, and senescing leaves at stage 15. Leaves from stages1 to 4 were collected at the same time as the freshly sprouted leaves (e.g.stage 1). These immature leaves were then divided into 4 stages according to their sizes. From stages 5 to 15, leaves were collected at different time points and grouped according to their stage of development/maturity (Table 1).

Morphological analysis showed that at maturity stage 2, irrespective of the tree, leaves had the smallest length and width, both of which showed a gradual increase in the leaves of stage 3. However, leaves at maturity stages 6, 10 and 15 did not have a clear trend and showed high variability in length, width and weight (Table 2). Tree-to-tree variability has been also observed in *T. ferdinandiana* fruits. The high degree of variability observed in the leaves and fruits could be attributed to the fact *T. ferdinandiana* is a wild harvested plant and often undergoes wide cross-pollination. Interestingly, the variability of the morphological parameters seemed to decrease with the maturity stage of the leaves which is indicated by a lowercoefficient of variation (% CV) in the morphological parameters measured (Table 5).

Table 3: Total ascorbic acid and total polyphenol content *Terminalia ferdinandiana* leaves at various stages of maturity

Tree ID	Maturity stage	Total ascorbic acid (mg/100 g DW)	Total polyphenol content (mg GAE/g DW)	
			Methanolic extracts	Water extracts
AT2	2	ND	533.4 ± 1.5	263.1 ± 0.8
	3	ND	598.6 ± 1.4	301.9 ± 1.3
	6	23.0 ± 0.1	279.8± 2.6	253.9 ± 2.2
	10	29.6 ± 0.5	384.9± 2.4	274.9± 0.5
	15	34.8 ± 0.3	407.5± 1.9	295.9± 0.6
AT5	2	ND	294.8± 1.5	222.2 ± 0.5
	3	ND	330.3± 0.8	231.8 ± 0.6
	6	32.3 ± 0.8	237.3± 1.6	210.3 ± 2.1
	10	34.9 ± 0.3	283.9± 0.9	220.3 ± 2.5
	15	35.5 ± 0.1	247.5± 2.1	215.6 ± 0.4
AT9	2	ND	388.7± 2.3	276.2 ± 0.3
	3	ND	579.6± 0.3	319.6 ± 0.2
	6	22.5 ± 0.4	359.8±1.0	258.4 ± 0.2
	10	32.6 ± 0.5	323.8±0.9	259.0 ± 0.4
	15	34.7 ± 0.7	379.9± 0.7	283.2 ± 0.5

Results are mean± SD (n = 3)

The ascorbic acid levels determined in leaves at maturity stage 6, 10 and 15 were between the ranges of 22.5 to 34.8 mg/100 gDW (Table 3) whereas no peak of ascorbic acid was observedin the UHPLC-PDA chromatogram for leaves sourced from stages 2 and 3, respectively.This data suggested that levels of ascorbic acid in these immature leaves might be present either at a relatively low concentration or lower than the limit of detection (LOD = 0.1 parts per million) of the method used for the analysis.

The lower levels of ascorbic acid in *T. ferdinandiana* leaves compared to fruits have been previously reported by other authors.[5-6] It is well known that ascorbic acid is important bioactive compound having important roles in the plant (e.g. redox reactions, cofactor of enzymes, photosynthesis, hormone biosynthesis, antioxidant function).[32-35] Variation in the ascorbic acid content has been reportedamong different tissues and organs in the same tree by other authors.[32-35] High concentration of ascorbic acid was found in tissues such as leaves and flowers compared with those photosynthetically active such as stems and roots where higher concentrations are present in the meristematic tissues and reproductive organs (e.g. flowers, young fruits).[32-35] It has been also reported that the concentration of ascorbic acid might be affected by the environment and developmental stages of the organ (i.e. mature vs immature fruits).[36] These factors might explain the observed variations in ascorbic acid in the leaf samples analysed.

The total phenolic content (TPC) of the leaf extracts ranged from 210.3 to 598.6 mg/g DW. The TPC values varied from 237.3 to 598.6 mg GAE/g DW in the methanolic extracts and from 210.3 to 319.6 mg GAE/g DW in the water extracts. TPC was higher in the leaf methanolic extracts compared to the water extracts. Similar results were reported by Akter and collaborators (2019). TPC showed a gradual decrease with the advance of maturity, with the highest levels observed in leaves at stage 3 irrespective of the tree in both the methanolic and water extracts (Table 3).

Table 4: Inhibition zones (mm) of methanol and water extracts against *S. aureus.*

Tree ID	Maturity stage	Inhibition zones (mm)	
		Methanolic extracts	**Water extracts**
AT2	2	23.9 ± 0.8	21.8 ± 0.3
3	27.5 ± 0.5	24.9 ± 0.9	
6	24.9 ± 0.9	23.1 ± 0.9	
10	26.3 ± 0.3	24.2 ± 0.6	
15	27.2 ± 0.6	25.5 ± 0.4	
AT5	2	23.6 ± 1.5	21.8 ± 1.3
3	29.1 ± 0.4	23.3 ± 0.6	
6	24.4 ± 0.4	22.6 ± 0.7	
10	26.1 ± 0.1	23.8 ± 0.4	
15	27.7 ± 0.2	25.3 ± 0.4	
AT9	2	24.7 ± 0.4	21.2 ± 0.7
3	28.3 ± 1.0	24.9 ± 0.8	
6	25.7 ± 0.4	23.6 ± 0.7	
10	26.6 ± 0.9	24.2 ± 0.7	
15	27.2 ± 0.7	26.7 ± 0.9	
Antibacterial control*	53.3 ± 0.2	52.8 ± 0.31	
Solvent control**		-	-
Results are mean ± SD (n = 3)			

*1 µg penicillin and streptomycin was used as antibacterial control.
**Solvent controls included 20% (v/v) aqueous methanol for methanolic extracts and water for water extracts

The antimicrobial analysis showed that the methanolic and water extracts of *T.ferdinandiana* leaves have strong inhibitory efficacy (e.g. inhibition zone > 13 mm)[37] against the Gram-positive *S. aureus*, but

no inhibition against the Gram-negative E. coliand thefungi *C. albicans* (Table 4). The antimicrobial efficacy in terms of inhibitory zones were in the range of 23.6 to 29.1 mm and 21.2 to 26.7 mm for methanolic and water extracts, respectively. A recent study has also reported that methanolic and aqueous leaf extracts of *T. ferdinandiana* were good inhibitors of the Gram positive Bacillus anthracis, the etiological agent of anthrax.[19] Akter and collaborators (2019) observed that the methanolic and water extracts of *T. ferdinandiana* leaves inhibited both Gram positive and negative bacteria.[26] The observed difference in the antimicrobial efficacy of *T. ferdinandiana* leaf extracts might be associatedwith the difference in the type of bioactive compounds extracted and their extent of release from the sample matrix as result of extraction method used and geographical location of the studied plant material.[38-39]

Pearson correlation showed strong positive correlation between levels of ascorbic acid and antimicrobial inhibition exhibited by methanolic (Pearson r = 0.60) and water (Pearson r = 0.55) extracts (Table 5). This relationship indicated that ascorbic acid might be a contributing factor, in the observed antimicrobial activity of *T. ferdinandiana* leaf extracts. In fact, a number of studies have reported the antimicrobial efficacy of ascorbic acid against a number of pathogenic bacteria including *S. aureus*, *E coli*, *Helicobacter pylori*, *Campylobacterjejuni* and *Mycobacterium tuberculosis*.[40-42]

Table 5: Descriptive statistics of the variables measured in the leaf samples at different maturity stages

	Mean	SD	CV (%)
Length (mm)	137.2	45.8	33.3
Width (mm)	100.4	34.1	34
Weight (g)	2.9	1.6	55
Total ascorbic (mg/100 g dry weight)	31.1	4.8	15.5
TPC methanolic extracts (mg GAE/g DW)	375	11	2.9
TPC water extracts (mg GAE/g DW)	259	3.2	1.2
Inhibition methanolic extracts	26.2	1.6	6
Inhibition water extracts	23.7	1.4	6

CV Coefficient of Variation)

The TPC of methanolic and water extracts were negatively correlated to total ascorbic acid content (Pearson r = -0.047 and -0.11 respectively). This could be explained by the fact that ascorbic acid may not be the primary contributor to TPC of *T. ferdinandiana*.[43] As mentioned before, *T. ferdinandiana* fruits and leaves are also rich sources of bioactive antioxidant compounds other than ascorbic acid, such as ellagic acid, gallic acid and α-tocopherol.[1-6] Significant positive correlation was observed between the morphological parameters, TPC and antimicrobial inhibition, indicating that with maturity the level of phenolics and antimicrobial efficacy increases (Table 5).

The data was also analysed by principal component analysis (PCA). This method transforms a group of highly correlated variables into new data sets called principal components (PC). Then, data was interpreted using the scores and loadings. This algorithm of PCA reduces the dimensionality of the data but retains most of the variation in the data set, which increases interpretability simultaneously minimizing loss of information.[31] It was observed a variation between samples obtained from different trees (Figure3A) and maturity stages (Figure3B), indicating the existence ofnatural variability between the trees.The PCA loadings allowed to interpret which variables might influence the separation

between leaves (Figure 4). Most of the differences observed between the samples were found to be explained by the morphological parameters, level of ascorbic acid and antimicrobial efficacy. Further more, loadings in PC1 explained the differences in maturity stages where high and positive loadings corresponded well with all chemical variables such as ascorbic acid and TPC.

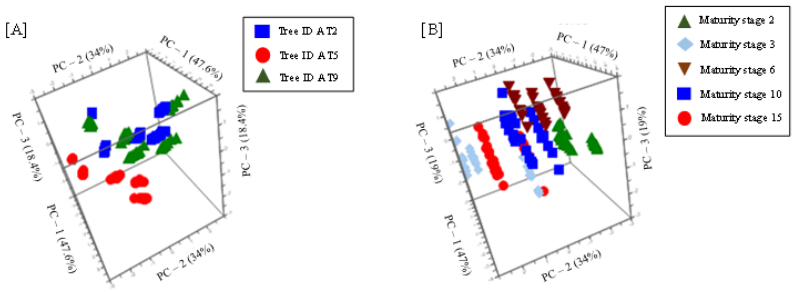


Fig. 3: Principal component analysis (PCA) (A) individual trees and (B) maturity stages of the leaves. The symbols indicate individual subjects

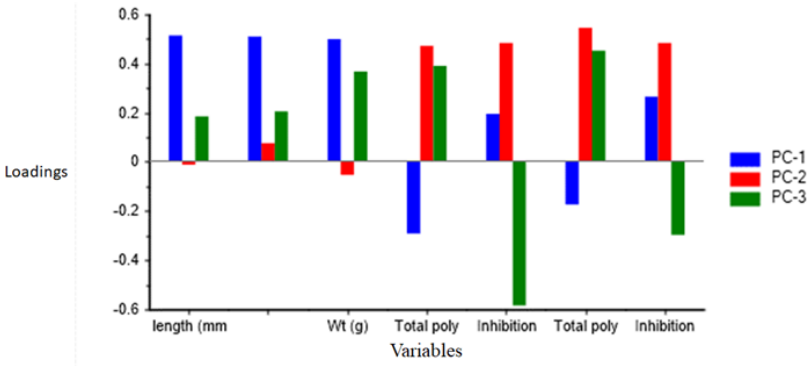


Fig. 4: Loading plots of principal components. Blue, red and green indicate PC-1, 2 and 3, respectively. Variables include: length, width and weight of leaves, TPC and antimicrobial activity of methanolic (MeOH) extracts, TPC and antimicrobial activity of water extracts

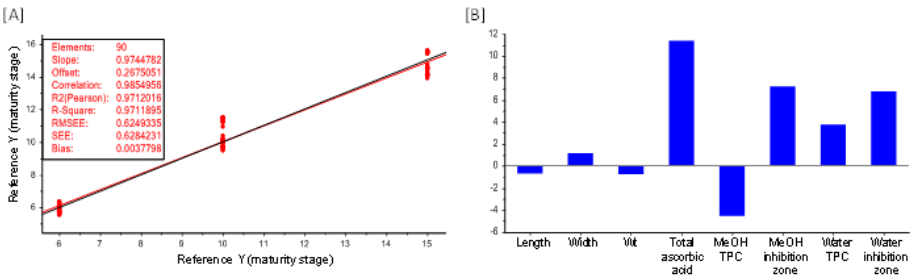


Fig. 5: Multiple linear regression results. Coefficients of regression derived from the multiple linear regression model for length, width and weight of leaves, total ascorbic acid content, TPC and antimicrobial activity of methanolic (MeOH) extracts, TPC and antimicrobial activity of water extracts

Multiple linear regression (MLR) analysis indicated that 97% of the variance in maturity of *T. ferdinandiana* leaves could be explained by the model reported in Figure 5A. The regression coefficients showed that ascorbic acid had a large influence in explaining maturity stages of the leaves, followed by both antimicrobial efficacy and TPC of the water extracts (Figure 5B). This indicates that in addition to the presence of bioactive compounds (e.g. ascorbic acid) other parameters such as antimicrobial activity and TPC might also vary with leaf maturity. Therefore, the concentration of ascorbic acid and TPC might be used as biomarkers to monitor leaf maturity in *T. ferdinandiana*.

Conclusion

For the first time, information on morphology, antimicrobial activity, total phenolic and ascorbic acid content in *Terminalia ferdinandiana* leaves was reported. Variation in the morphological parameters, TPC and ascorbic acid content with advancement of maturity was observed in the samples analysed. Both PCA and MLR analyses indicated that effect of individual trees and maturity stages, where the concentration of ascorbic acid explains the variability in maturity among leaves. However, the data in this study was not based on a large number of biological samples, and hence, is not sufficient to describe the effect of maturity on nutritional composition of *T. ferdinandiana* leaves. The use of statistical techniques such as PCA and MLR regression, allowed us to obtain additional information from the data set allowing for a better interpretation of the differences associated with maturity. Results from this study also indicated the pronounced inhibitory effect of *T. ferdinandiana* against *S. aureus*. Currently only the *T. ferdinandiana* fruit (as a freeze-dried powder and puree) is commercially available as a functional food ingredient, whereas, leaves or any other tissues, are not used for any industry applications. Leaf extracts showed promise as antimicrobial agent, suggesting that might be used as alternative to synthetic antimicrobial agents. Further studies will be recommended on *T. ferdinandiana* leaves, adding a large number of biological samples, trees and maturities, bioactive compounds, and anti-nutritional compounds.

Acknowledgments

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Conflict of Interest

The authors do not have any conflict of interest.

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Hydrolysable tannins, physico-chemical properties, and antioxidant property of wild-harvested *Terminalia ferdinandiana* (Exell) fruit at different maturity stages

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Keywords: Kakadu plum, indigenous fruit, undomesticated harvest, phytochemicals, fruit developmental stages, antioxidant properties.

Abstract

Terminalia ferdinandiana Exell. (Common name: Kakadu plum), is a wild-harvested fruit with limited information regarding the effects of fruit maturity on the phytonutritional properties and bioactivities of the fruit. The present study investigated the changes in hydrolysable tannin compounds, sugar components, physico-chemical parameters, and antioxidant property of wild-harvested Kakadu plum fruits at four different maturity stages, from immature to fully mature. Chebulagic acid, geraniin, chebulinic acid, castalagin, gallic acid, and punicalagin concentrations decreased steadily with advancing fruit maturity. In contrast, the concentrations of elaeocarpusin, helioscopin B, corilagin, 3,4,6-tri-*O*-galloyl-*S*-glucose and ellagic acid increased at the beginning of fruit growth and decreased when the fruits reached full maturity level. In addition, total (sum) amount of the studied hydrolysable tannins was significantly ($p \leq 0.05$) lower in the fully mature samples than the immature one. Total phenolic content (TPC) and DPPH antioxidant radical scavenging activity did not vary significantly between different maturity stages. Pearson's correlation coefficient test indicated that TPC and DPPH positively ($p \leq 0.05$) correlate with most of the studied tannin compounds. Sugars (glucose, fructose, and sucrose), total soluble solid content and titratable acid content increased during the fruit development. Principle component analysis (PCA) enabled us to differentiate the immature and mature samples, based on fruit phytonutritional quality and antioxidant properties. The PCA results also suggested a considerable variability between the individual trees, which is probably because of wild harvest practice.

1 Introduction

Terminalia ferdinandiana Exell., an endemic Australian plant, belongs to the family *Combretaceae* and is one of the 250 species of Terminalia genus (1). It is commonly known as Kakadu plum, billy goat plum, and gubinge, and is mainly distributed in Kimberley region of Western Australia, Northern Territory, and Northern Queensland (2). Kakadu plum is a small to moderate-sized semi-deciduous tree with smooth-skinned, fleshy ovoid drupes, a short beak and yellow-green colored fruits (3). The fruit of this plant has been widely used as traditional food or folk medicine by the Australian Indigenous communities (4). Since Kakadu plum possesses anti-inflammatory, antimicrobial, antioxidant, and anti-cancer properties (5-7), this has led to increased scientific interest in the characterization of phytochemicals, biofunctional properties, and applications. The fruit's functional properties have boosted its popularity in a number of markets, including functional food ingredients, health, cosmetics, and nutraceuticals (2, 8).

Hydrolysable tannin is a group of polyphenolic compounds possessing high molecular weight, complex molecular structure, and relatively strong polarity (9). Terminalia species is well known as a rich source of hydrolysable tannins, of which ellagitannins and gallotannins are dominant and mainly contribute to the reported health-related benefits of this plant species (10, 11). Studies on characterization of hydrolysable tannin compounds in Kakadu plum and elucidation of their molecular structures are limited due to the unavailability of commercial reference materials as well as the diversity and complexity of their molecular structures (11). In most of the recent studies, hydrolysable tannins in Kakadu plum are estimated indirectly through the semi-quantification of their corresponding hydrolyzed metabolites. For instance, quantification of ellagitannins is based on the release of hexahydroxy-diphenic (HHDP) acid, which undergoes spontaneous lactonization to ellagic acid under acidic condition at high temperatures (12-14). The hydrolysable tannin and phenolic acid compounds, such as ellagic acid and corilagin, were identified and quantified as predominant constituents in Kakadu plum fruit powder, using a high-resolution accurate mass (HRAM) spectrometry (15). Williams et al. (13) quantified the hydrolysable tannin compounds in Kakadu plum fruit as total ellagic acid equivalent and reported that the fruit possesses a higher level of ellagic acid (259.1 mg/100g DW) than that of other common ellagic acid-rich fruits such as strawberry (4.8 mg/100g DW) and boysenberry (5.5 mg/100g DW). Furthermore, the total phenolic content (TPC) of Kakadu plum fruit has been reported as 6-fold higher than that of blueberry, a bench-mark antioxidant-rich fruit (5, 13).

Since Kakadu plum is a traditional wild-harvested fruit, a large fluctuation in the bioactive compounds (vitamin C and ellagic acid) has been reported in fruits collected from three seasons and four different maturity stages (14). Phan and co-workers (14) reported a positive correlation between vitamin C and an increase of fruit maturity in wild-harvested Kakadu plum. However, ellagic acid content decreased with fruit maturation. Despite this, little is known about the effects of fruit maturation on the biosynthesis of individual hydrolysable tannin compounds, the accumulation of soluble sugars, and the antioxidant activity in this fruit. In addition, there is insufficient information about the effects of wild-harvest conditions on the variation of bioactive compounds and bioactivities. Konzack *et al.* (2014) (16) reported a significant variation in the levels of bioactive compounds in Kakadu plum fruits harvested from different accessions, locations, and different individual trees at the same location. Additionally, other potential factors including changes in climatic conditions (e.g., rainfall level, solar exposure intensity, and air temperature) can affect the fruit morphology and the biosynthesis of bioactive compounds during fruit growth (14). Therefore, understanding the effect of wild-harvest conditions on the fruit quality at harvest is crucial for researchers, the Australian Indigenous community, Indigenous enterprises, and the Australian bush food industry.

Considering the above, the present study aimed to investigate the effects of fruit maturity to the hydrolysable tannins and phenolic acids, physicochemical properties, sugar components, and antioxidant scavenging activity of wild-harvested Kakadu plum fruits at four different maturity stages as described in Figure 1.

2 Materials and Methods

2.1 Chemical reagents

Polyphenol and sugar standards (HPLC grade, ≥ 95% purity) including gallic acid, ellagic acid, corilagin, 3,4,6-tri-*O*-galloyl-*S*-glucose, castalagin, punicalagin, fructose, glucose and sucrose were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). All the other chemicals and solvents (HPLC/analytical grade) used throughout the study were supplied from Merck (Bayswater, VIC, Australia) or Sigma-Aldrich.

2.2 Plant materials

Kakadu plum fruits were harvested randomly from six individual trees (ca. 5 kg/tree) in Thamarrurr region (Darwin, Northern Territory, Australia). The required permissions were obtained from the Northern Territory Government Parks and Wildlife Commission, and the Traditional Owners. Fruits from each individual tree were sorted into four different maturity stages from immature to fully mature levels (approx. 200 g fruit/each maturity stage), based on differences in the degree of fruit fullness between the maturity stages as reported in previous studies (8, 17). The fruits, reaching less than 25, 25-50, 50-75, and 75-100% degree of fruit fullness, were allocated for the samples at maturity stage 1 (S1), S2, S3, and S4, respectively (Figure 1). Total 24 observations (4 maturity stages x 6 individual trees) were performed for each variable measured in the present study, followed a completely randomized block design.

Next, the fruit samples were kept at 4 °C during transportation from the collection site to the laboratory. After arrival samples were immediately frozen at -35 °C and then freeze-dried at -48 ± 2 °C for 72 h (CSK Climatek, Darra, Queensland, Australia). After separating the freeze-dried pulp and seeds using a laboratory blender (Waring, Australian Scientific, Sydney, NSW, Australia), the freeze-dried fruit pulp were ground into a fine powder using a Mixer Mill (MM400 Retsch, Thermo Fisher Scientific, Brisbane, QLD, Australia). The fruit powdered samples were stored at -35 °C for further analysis.

2.3 Determination of total soluble solid content and titratable acidity

Approximately 1 g of freeze-dried powder samples was mixed with Milli-Q water (1:20; *w/v*) and vortexed for 1 min. The homogeneous mixture was centrifuged at 3900 rpm (25 °C, 10 min) (Eppendorf 5180, Hamburg, Germany). The supernatant was collected for determination of the total soluble solid content (TSS, %) using a digital refractometer (Hanna Instruments Ltd., Leighton Buzzard, UK), and measurements of pH and titratable acidity (TA, expressed as percentage of citric acid equivalent) using an automatic titration unit (Metrohm Dosimat 765, Karl Fischer, Metrohm, Herisau, Switzerland). The analysis was conducted in triplicate.

2.4 Extraction of bioactive compounds

Hydrolysable tannins and other phenolic compounds were extracted by mixing approximately 0.5 g of powdered samples with aqueous methanol (80%, *v/v*) containing 0.01N HCl according to Bobasa *et al.* (2021) (15), with few modifications. The mixtures were sonicated in an ultrasonication bath (Elma

Schmidbauer GmbH, Ruiselede, Belgium) for 15 min, followed by 15-min shaking at room temperature (25 °C) in a reciprocating mixer (RP1812, Paton Scientific, Adelaide, SA, Australia). Next, the mixtures were centrifuged at 3900 rpm (10 min, 25 °C) (Eppendorf 5180 centrifuge). After collecting the supernatant, the residues were re-extracted twice with aqueous methanol as described above. The supernatants were combined and filtered through a 0.2 μm hydrophilic PTFE syringe filter membrane into HPLC vials for subsequent hydrolysable tannin analysis. The extraction was conducted in triplicate.

2.5 UHPLC-HRAM MS/MS analysis

Bioactive compounds in Kakadu plum fruit extract (obtained in section 2.4) were identified and quantified using a Thermo high resolution accurate Q Exactive mass spectrometer (Thermo Fisher Scientific Australia Pty Ltd., Melbourne, VIC, Australia), equipped with a DIONEX Ultimate 3000 UHPLC system. The instrumental method was followed according to Bobasa *et al.* (2021) (15) with some modifications. The compounds were separated in a Waters HSS T3 column (150 × 2.1 mm *i.d*; 1.8 μm) maintained at 40 ºC, with 0.1% formic acid as mobile phase A and acetonitrile containing 0.1% formic acid as mobile phase B. The flow rate was 0.3 mL/min and gradient elution for mobile phase B was as follows: 0-1 min, 5% B; 1-8 min, 5-20% B; 8-15 min, 20-45% B; 15-22 min, 45-100% B; isocratic elution at 100% B for 2 min and recondition to 5% B for 5 min before the next injection. The mass spectrometer was operated in parallel reaction monitoring (PRM) in a negative electrospray ionization (ESI) mode at 35,000 FWHM resolution, AGC target value of 2e5, maximum injection time of 200 ms, and optimized normalized collision energy (NCE) from 25 to 35eV. The inclusion list of 13 interested/targeted hydrolysable tannins and phenolic acids with detail information about the mass features presenting in Table S1. A mix-standard solution (including gallic acid, ellagic acid, corilagin, 3,4,6-tri-*O*-galloyl-*S*-glucose, castalagin, and punicalagin) was prepared in MeOH and was also included in the LCMS analysis to facilitate the compound identification and development of external standard calibration curves for quantification. Thermo Trace Finder™ v.5.1 software (Thermo Scientific, Brisbane, QLD, Australia) was employed for data processing.

2.6 Determination of total phenolic content and DPPH free radical scavenging activity

The TPC and DPPH free radical scavenging activity were employed to determine the antioxidant activity of Kakadu plum fruit extract (obtained from section 2.4). For TPC, Folin-Ciocalteu assay was applied followed the procedure previously reported (18). Gallic acid standard was used to equivalently quantify the TPC. Results are expressed as g of gallic acid equivalent per 100 g sample on dry weight basis (g GAE/100 g DW).

DPPH radical scavenging assay was conducted according to the method previously described (18). Ascorbic acid standard was used to equivalent quantify DPPH scavenging capacity of Kakadu plum fruit extract. Results are expressed as g of ascorbic acid equivalent per 100 g sample on dry weight basis (g AAE/100 g DW).

2.7 Analysis of sugar components

Extraction and analysis of individual soluble sugars in Kakadu plum fruit samples were conducted followed the method previously reported by Hong *et al.* (2021) (19), with minor modifications. Briefly, about 0.5 g of samples (in triplicate) were homogenized with aqueous methanol (62%, *v/v*) using a vortex mixer, followed by incubation in a shaking water bath at 50 ºC for 30 min (LSB18, Grant Instruments, Amsterdam, Netherlands). The supernatant was collected after centrifuging (3900 rpm, 10 min, 25 °C) and the pellet was re-extracted with 62% MeOH for another 2 times. The supernatants were combined, filtered through 0.2 μm hydrophilic PTFE syringe filter membrane into HPLC vials. A Shimadzu Nexera X2 UHPLC system coupled with a Shimadzu MS8045 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) was employed for sugar analysis. The multiple reaction monitoring (MRM) scanned at negative mode was applied for identification and quantification of targeted soluble sugars, including fructose (m/z 179.2 → 113.1), glucose (m/z 179.2 → 89.0), and sucrose (m/z 341.2 → 179.2). Compound separation was performed in a Waters UPLC BEH Amide column (100 × 2.1 mm *i.d.*, 1.7 um; Waters, Dublin, Ireland) maintained at 40 °C, with mobile phase A (80% acetonitrile containing 0.1% NH_4OH) and mobile phase B (0.1% NH_4OH). The gradient program for mobile phase B at a flow rate of 0.2 mL/min was as follows: 0-1 min, 0% B; 1-7 min, 0-40% B; and recondition to the initial condition for 5 min before the next injection. A mix standard solution including glucose, fructose, and sucrose dissolved in Milli-Q water was prepared for establishment of external standard calibration curves for quantification of the soluble sugars detected in the fruit extract. The concentration of sugars is expressed as g per 100 g sample on dry weight basis.

205

206 **2.8 Statistical analysis**

207 Data were calculated and present as the mean and standard error (SE). A general linear model (GLM)
208 procedure was applied to perform the analysis of variance (ANOVA) between the fruit samples
209 collected at different fruit maturity stages, followed by Tukey's multiple comparison post hoc tests
210 using Minitab 17® for Windows (Minitab Inc., State College, PA, USA). A p value of ≤ 0.05 was used
211 to determine significant differences. A Pearson's correlation coefficient test was also applied to test
212 the correlation between antioxidant activity and bioactive compounds studied. A principal component
213 analysis (PCA), including 20 measured variables with 6 replications and full-crossed validation, was
214 performed to visualize the variability in the dataset, using GraphPad Prism software ver. 9.3 (GraphPad
215 Software, San Diego, CA, USA).

216

217 **3 Results and Discussion**

218 **3.1 Changes in hydrolysable tannins and phenolic acids in Kakadu plum fruits with the**
219 **advance of fruit maturity**

220 Figures 2 and 3 show representative mass spectrum and MS^2 fragmentations of 13 individual
221 hydrolysable tannins and phenolic acids detected in Kakadu plum fruit extract. The detected and
222 identified compounds included gallic acid, ellagic acid, corilagin, 3,4,6-tri-O-galloyl-S-glucose,
223 castalagin, geraniin, chebulagic acid, chebulinic acid, punicalalgin and its isomer, elaeocarpusin,
224 chebulic acid, and helioscopin B. Identification of the detected compounds was based on matching the
225 mass features and the MS^2 characteristic fragment ions with that of the commercial standards included
226 in the UHLPC-HRAM-MS/MS analysis (section 2.1) and those reported in literature (15, 20, 21). The
227 results of the present study were consistent with previous studies reported the presence of the
228 corresponding compounds in Kakadu plum fruit (3, 15, 22) as well as in other *Terminalia* species (23,
229 24). The results showed that corilagin has the highest concentration among the studied compounds,
230 ranging from approximately 1600 to 1800 mg/100g DW, followed by 3,4,6-tri-*O*-galloyl-*S*-glucose
231 (1045.7 – 1380 mg/100g DW), ellagic acid (647.9 – 730.1 mg/100g DW), geraniin (111 – 363 mg/100g
232 DW), elaeocarpusin (216.1 – 244.2 mg/100g DW), chebulagic acid (143.1 – 246.3 mg/100g DW), and
233 punicalagin and its isomer (145.1 – 172.4 mg/100g DW). All other compounds studied were present at

234 levels below 100 mg/100g DW (Figure 4). The present study also confirms previous findings that
235 corilagin is one of the major tannin compounds in *Kakadu plum* fruit (3, 15).

236 Figure 4 clearly shows two opposite trends in the biosynthesis of hydrolysable tannins and phenolic
237 acids during the fruit growth. A steady decrease was observed in the concentrations of chebulagic acid,
238 geraniin, chebulinic acid, castalagin, gallic acid and punicalagin with the advance of fruit maturity. In
239 contrast, the levels of elaeocarpusin, helioscopin B, corilagin, 3,4,6-tri-*O*-galloyl-*S*-glucose and ellagic
240 acid were slightly increased from immature stage S1 to S3, and then decreased from S3 to the fully
241 mature stage S4. Similarly, in pomegranate, as the fruit matures, the level of hydrolysable tannins in
242 aril juice declines along with gallic acid and ellagic acid concentrations (25). In addition, several
243 studies have shown a relatively higher level of hydrolysable tannins at the early maturity stage of
244 several tannin-rich fruits such as java plum (*Syzygium Cumini* Lam.), carob (*Ceratonia Siliqua* L.) and
245 different persimmon (*Diospyros kaki* Thunb.) cultivars (26-28).

246 The biosynthesis of the individual hydrolysable tannins and phenolic acids during Kakadu plum fruit
247 development was not significantly different ($p > 0.05$), but the total (sum) levels of all the studied
248 compounds measured at the fully mature stage S4 were significantly lower than those measured at the
249 immature stages (S1 and S2) (Figure 4). This suggests that hydrolyzed tannins in Kakadu plum fruit
250 decreased significantly as the fruit ripens. A decreasing trend in the total ellagic acid content (after acid
251 hydrolysis of ellagitannins) during the ripening process of wild-harvested Kakadu plum fruits was
252 recently reported (14, 17). The reduction in hydrolysable tannins, which is associated with the fruit
253 maturation process, could be attributed to the polymerization of tannin compounds that can potentially
254 bind to other macromolecules such as proteins, polysaccharides, and fibers to form the complex
255 structures (29). According to previous research, this could have limited the extractability of tannin
256 compounds (30). Furthermore, it has been reported that plants produce tannins as secondary
257 metabolites to protect the plants against virus and microbe attacks during fruit development (31). The
258 plant defense mechanism has been reported to contribute to the decrease in the content of bioactive
259 compounds (e.g., persin, epicatechin and catechin) with fruit maturation as reported previously in New
260 Zealand-grown 'Hass' avocado fruit (32).

261

262 **3.2 An increase in total soluble solid content and titratable acidity during fruit development**

263 Table 1 shows the results of total soluble solid content (TSS), pH values, titratable acidity (TA) of
264 Kakadu plum fruits harvested at different stages of maturity. TSS increased from 2.0% during the
265 immature stage (S1) to 4.1% at the mature stage (S4), whereas TA increased rapidly from 2.8% (S1)
266 to 4.8% when the fruit matured (S4). Furthermore, pH, that is inversely correlated with TA, showed a
267 significant decrease ($p \leq 0.05$) at S4. TSS and TA did not differ significantly among the maturity stages,
268 possibly due to a large variation among individual trees due to wild harvesting. The increase in TSS
269 was probably derived from the accumulation of sugars during the fruit development (discussed later in
270 section 3.3) as reported previously (33, 34). The higher TA content in the ripe fruit could be attributed
271 to the accumulation of the exceptional amount of ascorbic acid in Kakadu plum fruits (up to 20% DW)
272 at the full-maturity stage (12, 14). The observed increasing trend in TA is in contrast with the
273 decreasing trend reported for other common domestical fruits such as mango (cv. Cogshall) (35) and
274 pomegranate (*Punica granatum* L.) (36).

275

276 **3.3 Sugar accumulation with the advance of fruit maturity**

277 The changes in the amount of main sugar components (glucose, fructose, and sucrose) and total sugar
278 content during fruit growth are present in Figure 5. Fructose was found as a major sugar component
279 (2.0 – 4.1 g/100g DW), followed by glucose (1.7 – 2.8 g/100g DW), and sucrose (0.7 – 2.8 g/100g
280 DW). The gradual accumulation of sugars during fruit maturation led to an increase in total sugar
281 content from 4.9 to 9.7 g/100g DW from immature to fully mature stage, and consequence the observed
282 increase in TSS (previous section 3.2). However, like TA and TSS results, no significant difference
283 was observed among the four maturity stages. The low levels of glucose, fructose, and sucrose in the
284 immature fruits could be due to their rapid usage by the cells. The early stages of fruit development
285 require sugars to provide energy and intermediates needed for cell division and growth (37). Fruit
286 sugars and total sugars tended to increase at late stages of development probably due to starch
287 breakdown and continuous accumulation of sugars (37); particularly fructose, which increased the total
288 concentration of soluble sugars, and sweetness, to a maximum level at maturity. Recently, changes in
289 the degree of fruit fullness is considered as maturity index for harvesting Kakadu plum fruits (17).
290 Therefore, the obtained results in this study on the increase of sugar content during fruit ripening may
291 be considered as a secondary fruit maturity index, which together with the fruit fullness can facilitate
292 the development of a standard harvest protocol for this wild-harvested fruit.

The sugar content observed in the present study was higher than that was previously reported in Kakadu plum (2.3 g/100g DW) (16) and other *Terminalia* species, including *Terminalia citrina* at 2.6 g/100g DW (38) and *Terminalia chebula* (2.3 g/100g DW, (39)) which could be due to the effects of wild harvest practice depending on multiple environmental factors (14). The large variation in total sugars have been reported among the individual Kakadu plum trees that were harvested at the same location in Northern Territory (16). Individual fruits may exhibit significant variations in their external and internal qualities depending on their location in a tree canopy (40). The differences observed between the same species could be attributed due to the effect of location, sunshine hours, photosynthetically active radiation (40).

3.4 Changes in antioxidant capacity during the fruit growth

Figure 6 shows DPPH free radical scavenging activity and TPC of Kakadu plum fruit harvested at different maturity stages. DPPH scavenging activity varied from 51.4 to 55 g AAE/100 g DW during maturation, with S2 exhibiting slightly higher DPPH scavenging activity than the other maturity stages, although no statistical difference ($p > 0.05$; Fig. 6A). Similarly, there was no significant ($p > 0.05$) difference in TPC values among the studied maturity stages, which was ca. 15 g GAE/100 g DW (Figure 6B). The obtained results are in agreement with the reported TPC values of forty-five accessions of Kakadu plum fruits ranging from 12.2 to 50.5 g GAE/100 g DW (16). The results suggested that fruit maturity is unlikely affecting the antioxidant capacity of wild-harvested Kakadu plum fruits as the biosynthesis of plant secondary metabolites such as phenolics is considerably depended on numerous environmental factors including sunshine hours, soil condition, temperature, air quality and water availability (41-44).

To obtain a better understanding on the relationship between antioxidant capacity and the main bioactive compounds of Kakadu plum fruits, a Pearson's correlation coefficient test was conducted (Table S2). A positive correlation ($r = 0.653$, $p < 0.01$) between DPPH scavenging capacity and TPC was observed. In addition, TPC positively correlated with most of the studied hydrolysable tannins, including 3,4,6-tri-*O*-galloyl-*S*-glucose ($r = 0.55$, $p < 0.01$), chebulinic acid ($r = 0.51$, $p < 0.05$), elaeocarpusin ($r = 0.62$, $p < 0.01$) and helioscopin B ($r = 0.55$, $p < 0.01$); except for castalagin ($r = -0.41$, $p < 0.05$) and punicalagin ($r = -0.39$, $p > 0.05$), which were present in the samples at relatively low amounts (Figure 4). Furthermore, there was a positive correlation between DPPH scavenging

capacity and chebulinic acid, elaeocarpusin and helioscopin B (r = 0.44, 0.51, and 0.45, respectively; $p < 0.01$). The results suggested that phenolic compounds including hydrolysable tannins could contribute to the antioxidant capacity of Kakadu plum fruit, which are consistent with previous research in Kakadu plum (16) as well as other Terminalia species (15, 18, 45).

3.6 Principal components analysis (PCA) indicates the effect of fruit maturity and high variability between the individual trees

PCA was applied to visualize the effects of fruit maturity on the changes of bioactive compounds, sugars, antioxidant capacity and other fruit quality parameters. Generally, the PCA scores plot (Figure 7A), explaining 61.22% of the total variability (PC1 44.13% and PC2 17.09%) in the dataset, demonstrates a clear separation between the studied trees along the PC1. As can be seen in Figure 7A, trees 1, 3, and 5 were grouped together, while trees 2, 4, and 6 clustered in another group. The PC2, however, divides the samples into two distinguished groups according to fruit maturity levels, including the immature group (S1 and S2) and the mature one (S3 and S4). The PCA results highlighted the higher variability in the dataset influenced by variations among the studied trees. This confirms the considerable effect of wild harvest on the quality of Kakadu plum fruit.

PCA loadings plot (Figure 7B) reveals the extent of the contribution of variables measured to the variations in the dataset. In PC1, hydrolysable tannin compounds that found at high concentrations such as helioscopin B (r = 0.9), 3,4,6-Tri-O-galloyl-S-glucose (r = 0.92), elaeocarpusin (r = 0.88), chebulinic acid (r = 0.69) and corilagin (r = 0.77), together with TPC (r = 0.73), glucose (r = -0.77), fructose (r = -0.8), and TA (r = 0.74) contributed to the separation among the trees. In contrast, punicalagin (r = 0.6), chebulinic acid (r = 0.61), chebulic acid (r = 0.55), geraniin (r = 0.72), total sugars (r = -0.46), sucrose (r = -0.52) and TSS (r = -0.710) were mainly responsible for the differences between the fruit maturity stages observed along the PC2. Furthermore, the PCA loadings plot showed that hydrolysable tannins and antioxidant capacity positively corelated with the immature fruit samples (S1, S2), whereas sugar components, TA and TSS were positively correlated with the mature fruits (S3, S4). The obtained results highlight an opposite trend in the dataset that as the fruit matures, more sugars and acids are accumulated, and more hydrolysable tannins are reduced.

4. Conclusions

The obtained results provide a better understanding of the relationship between the fruit maturity and the accumulation of different bioactive compounds and the associated bioactivities during Kakadu plum fruit growth. This can assist the Australian Indigenous enterprises as well as the food and other industries in selecting the appropriate maturity stages to harvest Kakadu plum fruit for further development for functional food ingredients, pharmaceutical and/or nutraceutical products. Generally, the results showed that fruit maturity plays an important role in determining the quality of Kakadu plum fruit at harvest. TSS, TA and sugars increased during the fruit development, whereas phenolic compounds including major hydrolysable tannins exhibited a decreasing trend in the result as fruit ripens. A positive correlation between antioxidant capacity and hydrolysable tannins was observed, suggesting that tannins might mainly contribute to the bioactivity of Kakadu plum fruit. PCA results enabled us to differentiate between the immature and mature fruit samples and highlighted the high variability in the dataset, suggesting the considerable influence of wild harvest condition. Further studies using a larger sample size from different locations and harvest seasons/years are required to substantiate the current results.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Conceptualization, A.P., M.N., D.S. and Y.S.; methodology, A.P., J.Z, M.S., S.S. and D.S.; software, A.P. and J.Z.; validation, A.P., M.N., D.S., and Y.S.; formal analysis, A.P., J.Z, M.S., S.S; data curation, A.P. and J.Z.; writing—original draft preparation, A.P; review and editing, J.Z, M.S., S.S. M.N., D.S. and Y.S.; supervision, D.S. and Y.S.; funding acquisition, Y.S. All authors have read and agreed to the published version of the manuscript.

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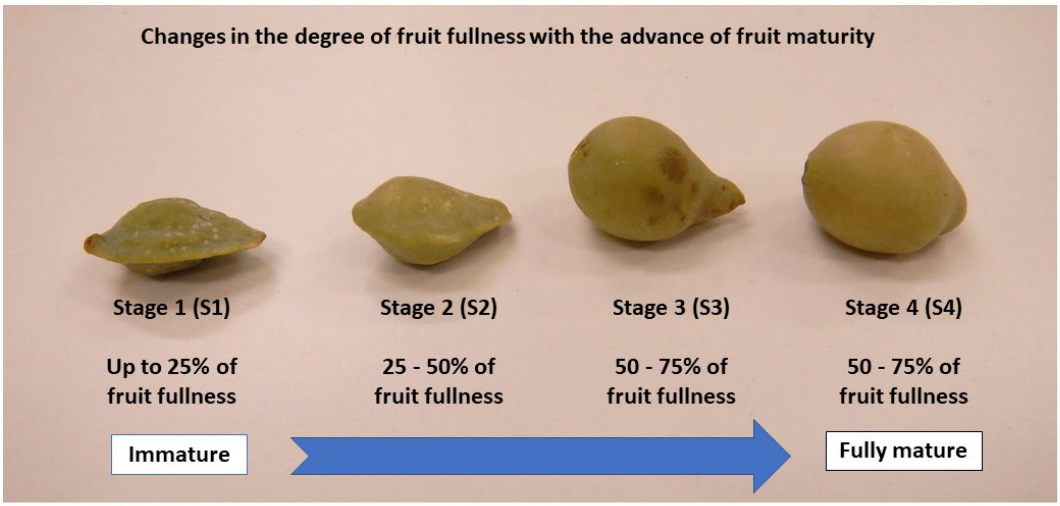
501 **List of Figures**



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503 Figure 1. illustrates changes in the degree of fruit fullness with an increase in fruit maturity

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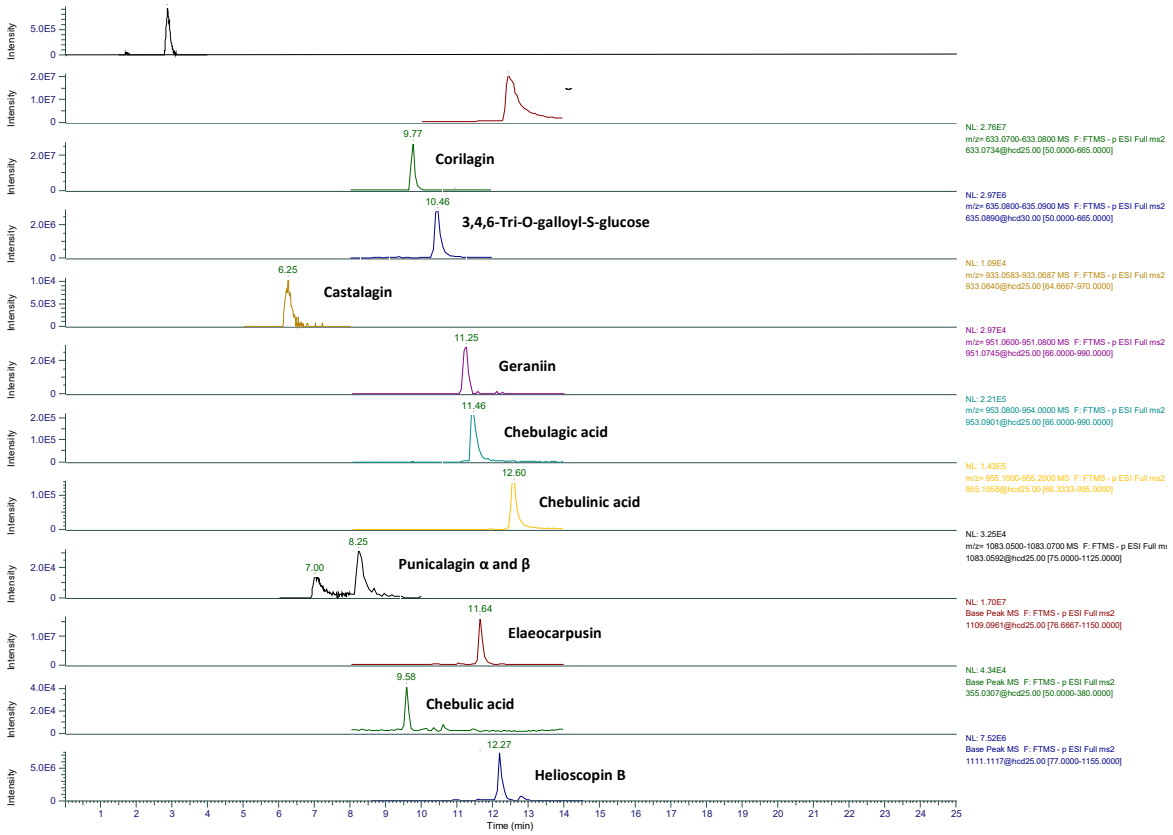

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507 Figure 2. Representative ion chromatograms of 13 main hydrolysable tannins and phenolic acids in

508 Kakadu plum fruit using a HRAM orbitrap mass spectrometer.

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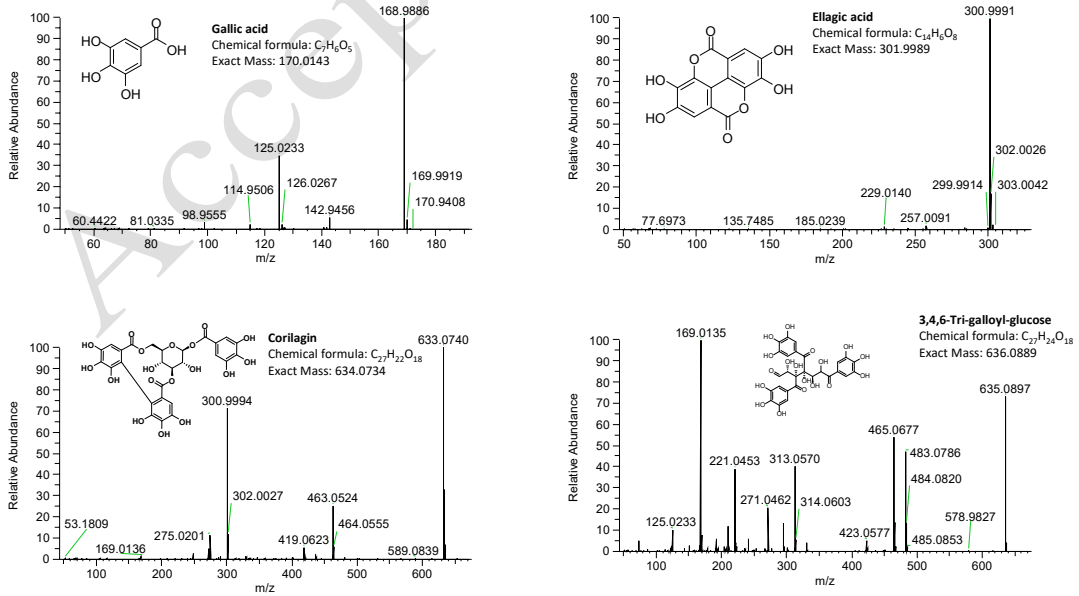

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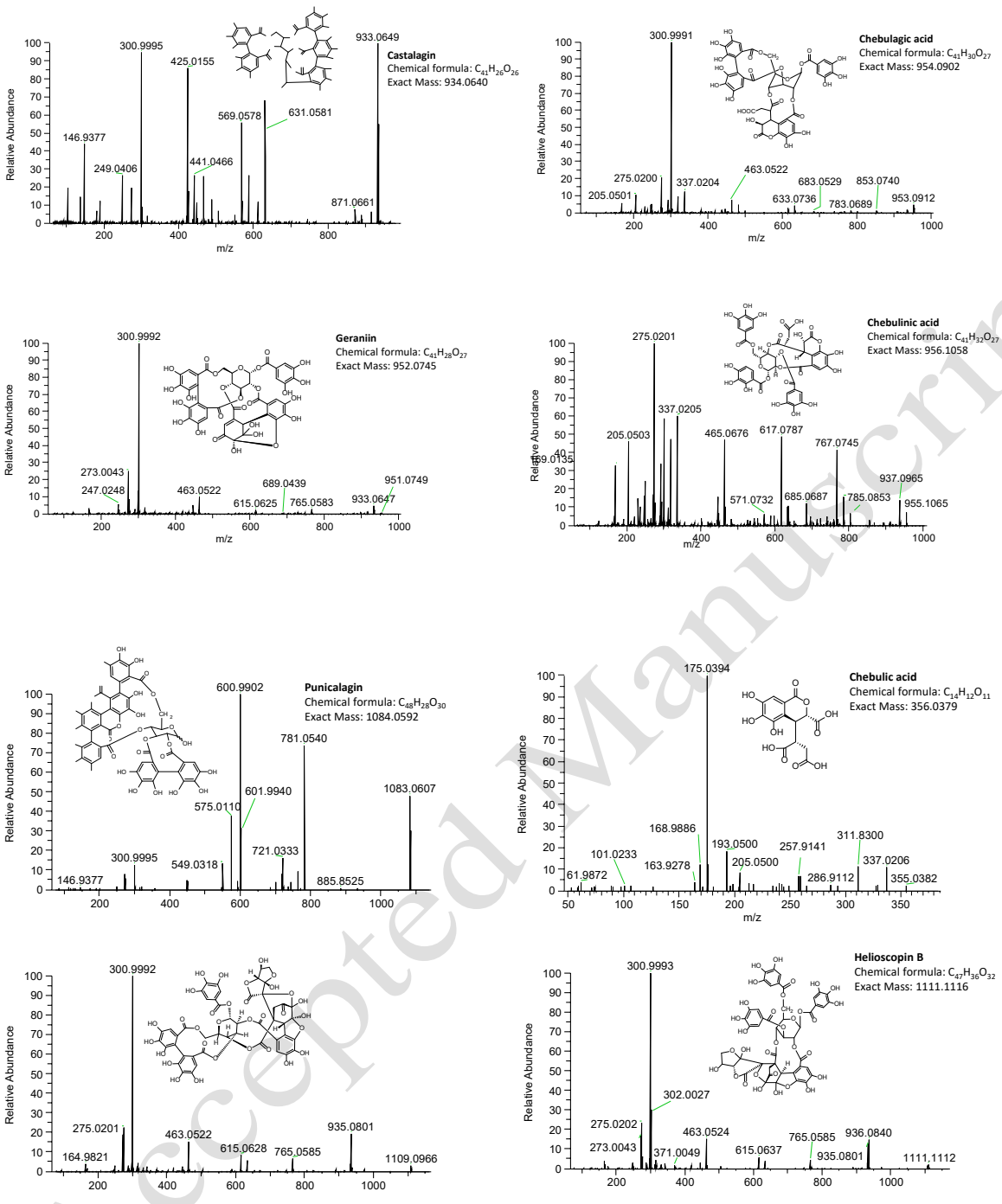
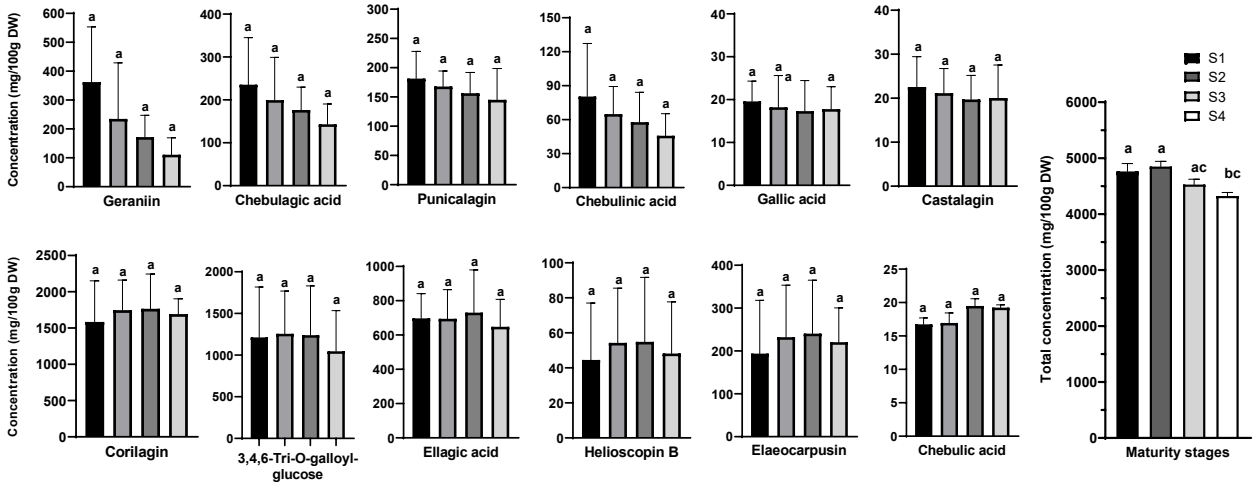
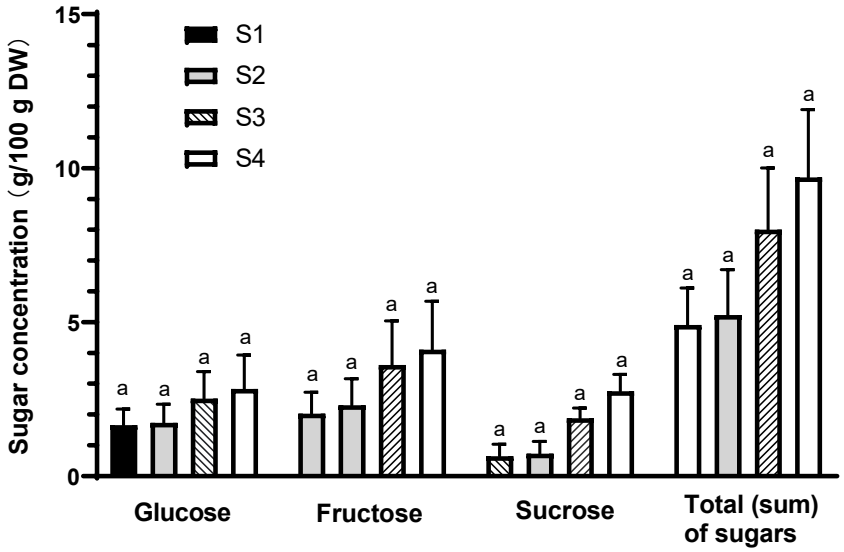


Figure 3. Molecular structures and mass features of the studied compounds using a HRAM orbitrap mass spectrometer.



519

520 Figure 4. Changes in the concentrations of the studied bioactive compounds in Kakadu plum fruit
521 harvested at different maturity stages. Data presents mean ± standard error (SE), n = 6. S1-4 denotes
522 different fruit maturity stages classified from immature stage (S1) to fully-mature stage (S4).
523 Different letters indicate the significant differences at $p \leq 0.05$.



524

525 Figure 5. Individual sugar components and total (sum) sugars of Kakadu plum fruit harvested at
526 different maturity stages. Results are mean ± standard error (SE), n = 6. S1-4 denotes the different
527 maturity stages from immature (S1) to mature (S4). Different letters indicate the significant
528 differences at $p \leq 0.05$.

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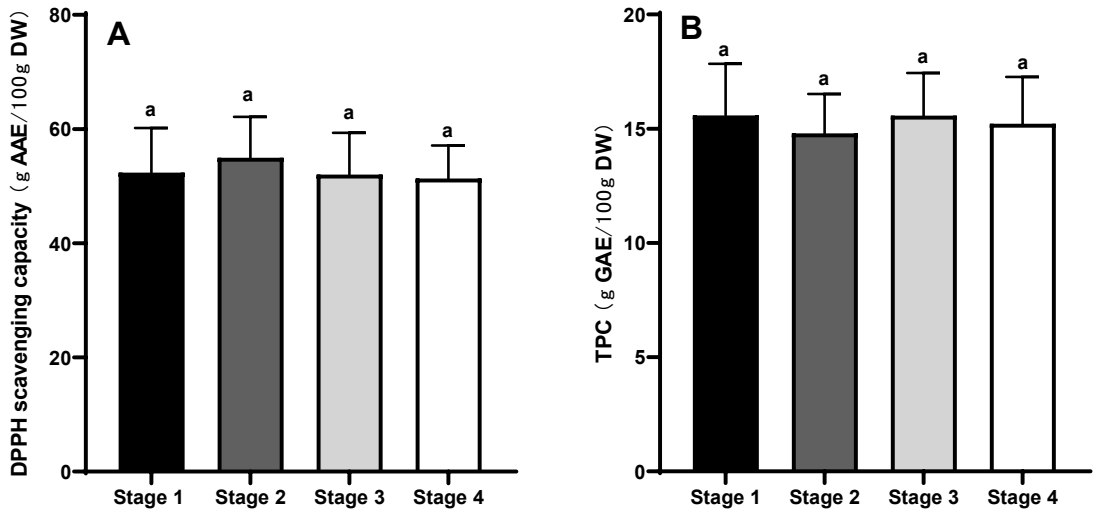

531

532 Figure 6. (A) DPPH free radical scavenging activity and (B) total phenolic content of Kakadu plum
533 fruit at different maturity stages. Data are mean ± SE (n = 6). DPPH scavenging activity and TPC are
534 expressed as ascorbic acid equivalent (g AAE/100 g DW) and gallic acid equivalent per 100 g dry
535 sample (g GAE/100 g DW), respectively. Different letters indicate the significant differences at $p \leq$
536 0.05.

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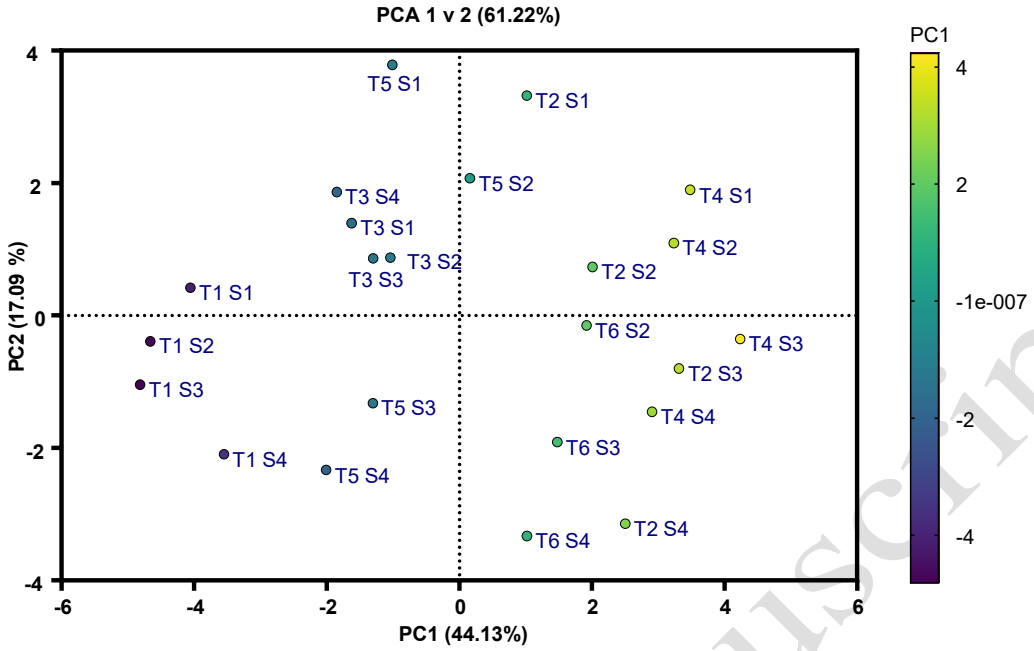

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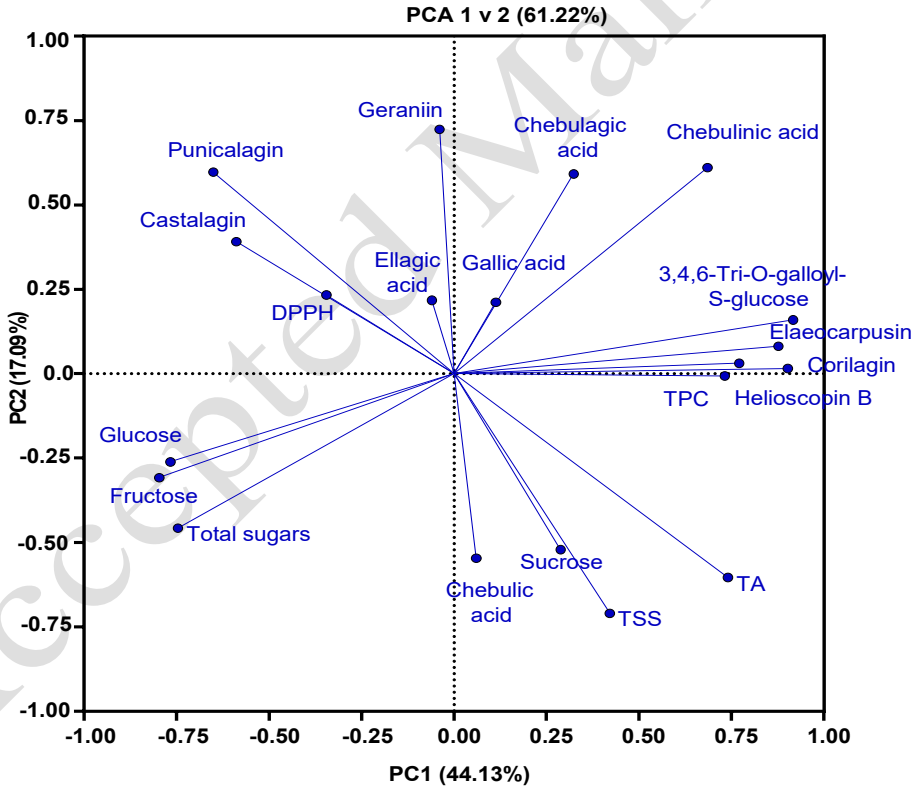

545

546 Figure 7. (A) PCA scores plot and (B) loadings plot.

547 T1-T6, Trees; S1-S4, maturity stages; TPC, Total phenolic content; DPPH, antioxidant capacity; TA,
548 titratable acidity; TSS, total soluble solid content.

549 **List of Tables**

550

551 Table 1. Total soluble solid, titratable acidity, pH value of Kakadu plum fruit at different maturity stages.

Maturity stage	TSS (%)	TA (%)	pH
S1	2.0 ± 0.1 a	2.8 ± 0.8 a	4.03 ± 0.01 a
S2	3.3 ± 0.1 a	3.6 ± 0.6 a	3.87 ± 0.01 ab
S3	3.7 ± 0.1 a	4.5 ± 0.8 a	3.82 ± 0.01 ab
S4	4.1 ± 0.1 a	4.8 ± 0.2 a	3.80 ± 0.01 b

552 Results present mean ± SE, n = 6. S1-4 denotes the different maturity stages from immature (S1) to
553 mature (S4). Different letters indicate the significant differences at $p \leq 0.05$.

554 **Supplementary Materials**

555 **Table S1**. Mass spectrometric data operated in negative mode of targeted hydrolysable tannins and phenolic acids present in Kakadu plum
556 fruit extract at different maturity stages.

Compounds	Molecular formula	Retention time (min)	$[M-H]^-$	Collision energy (eV)	Target transition ion for quantification	Transition ions for confirmation
Gallic acid	$C_7H_6O_5$	2.88	169.0142	25	168.9886	125.0233
Castalagin	$C_{41}H_{22}O_{18}$	6.25	933.0639	25	933.0649	631.0581, 425.0155 300.9995
Punicalagin and its isomer	$C_{48}H_{28}O_{30}$	7.0 8.25	1083.0592	25	600.9902	1083.0607, 781.0540 300.9995
Chebulic acid	$C_{14}H_{12}O_{11}$	9.58	355.0306	25	175.0394	355.0382, 168.9886
Corilagin	$C_{27}H_{22}O_{18}$	9.77	633.0733	25	633.0740	463.0524, 300.9994
3,4,6-Tri-*O*-galloyl-*S*-glucose	$C_{27}H_{24}O_{18}$	10.46	635.0889	30	635.0897	483.0786, 465.0677 169.0135
Geraniin	$C_{41}H_{28}O_{27}$	11.25	951.0745	25	300.9992	933.0647, 463.0522
Chebulagic acid	$C_{41}H_{30}O_{27}$	11.46	953.0901	25	300.9991	953.0912, 463.0522 275.0200
Elaeocarpusin	$C_{47}H_{34}O_{32}$	11.64	1109.0960	25	300.9992	1109.0966, 935.0801 463.0522
Helioscopin B	$C_{47}H_{36}O_{32}$	12.27	1111.1116	25	300.9992	1111.1112, 463.0524 275.0202
Chebulinic acid	$C_{41}H_{32}O_{27}$	12.60	955.1058	25	275.0201	955.1065, 785.0853 465.0676
Ellagic acid	$C_{14}H_6O_8$	12.43	300.9989	35	300.9992	257.0091

557

558 Table S2. Pearson's correlation coefficients between ellagitannins and the values of TPC/DPPH of the wild-harvested Kakadu plum fruits.

	Castalagin	Corilagin	3,4,6-Tri-O-galloyl-S-glucose	Punicalagin	Gallic acid	Ellagic acid	Chebulagic acid	Chebulinic acid	Elaeocarpusin	Chebulic acid	Helioscopin B	Geraniin	TPC	DPPH
Castalagin	1.000													
Corilagin	-0.610**	1.000												
3,4,6-Tri-O-galloyl-S-glucose	-0.506*	0.836**	1.000											
Punicalagin	0.691**	-0.410	-0.531**	1.000										
Gallic acid	0.162	0.044	0.037	0.183	1.000									
Ellagic acid	0.324	-0.192	-0.174	0.358	0.226	1.000								
Chebulagic acid	-0.279	0.594**	0.461*	0.190	-0.019	-0.035	1.000							
Chebulinic acid	-0.246	0.557**	0.748**	-0.084	0.120	0.084	0.624**	1.000						
Elaeocarpusin	-0.359	0.752**	0.822**	-0.461*	0.303	0.104	0.293	0.680**	1.000					
Chebulic acid	-0.620**	0.281	0.132	-0.338	-0.498*	-0.319	0.027	-0.109	-0.140	1.000				
Helioscopin B	-0.509*	0.738**	0.892**	-0.601**	0.190	-0.043	0.168	0.633**	0.874**	0.038	1.000			
Geraniin	-0.022	0.199	0.137	0.449*	-0.145	-0.045	0.882**	0.475*	-0.118	-0.021	-0.175	1.000		
TPC	-0.414*	0.391	0.554**	-0.389	0.120	0.188	0.229	0.506*	0.616**	-0.034	0.554**	-0.015	1.000	
DPPH	-0.201	0.338	0.380	-0.240	0.200	0.347	0.142	0.441*	0.505*	-0.048	0.451*	-0.040	0.653**	1.000

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