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

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

Key words: Central composite rotatable design, ellagic acid, enzyme assisted extraction, Kakadu plum fruit, *Terminalia ferdinandiana.*

INTRODUCTION

A species of *Terminalia* endemic to Australia – *Terminalia ferdinandiana* Exell, Combretaceae, commonly known as Kakadu plum, has a significant history as a food source and traditional medicine by the Australian aboriginal

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population for centuries. Fruits are consumed to cure headaches and to alleviate the symptoms of colds and flu while the pounded fruit is used as an antiseptic and a soothing balm for aching limbs (Konczak et al., 2010). Kakadu plum grows across large areas of Australia: Western Australia, to the south of Broome and Northern Territory, near the Arnhem Land and the Gulf area in the east, covering a range of environmental conditions (Wrigley, 1988).

Kakadu plum is a rich source of polyphenolic compounds for example ellagic acid (EA) and its hydrolysable tannin, ellagitannin (Konczak et al., 2014; Williams et al., 2014). EA and its derivatives are present at high levels in many *Terminalia* plants (Pfundstein et al., 2010) and are reported to be responsible for their perceived health promoting and biological activities (Dhanani et al., 2015). Konczak et al. (2014) reported free EA (fEA) levels of the Kakadu plum fruit are in the range of 3050 to 14020 mg/100 g DW of while a recent study by Williams et al. (2014) reported the lower level of 626 to 980 mg/100 g DW of Kakadu plum fruit. The polyphenol EA has been reported to exert anti-carcinogenic, anti-bacterial, anti-fungal, antiviral, and anti-inflammatory activities (Ahad et al., 2014; Hseu et al., 2012; Sarabhai et al., 2013).

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

To date not one of the published studies on Kakadu plum has reported a water based extraction method as EA is not only insoluble in water but is also difficult to solubilize in commonly used organic solvents in sufficient quantities for formulation (Bala et al., 2006). This characteristic is the main contributor to the low oral bioavailability of EA (Seeram et al., 2004). Use of organic solvents in these extraction processes has several disadvantages. Most notably safety hazards, high energy consumption, risk to the environment and toxicological effects (Teo et al., 2010). As most of these solvents are not suitable for food applications, there is a need for an EA-rich aqueous extract of Kakadu plum as a functional ingredient for use in the food and beverage industry. At present, Kakadu plum is incorporated as a puree or powder into food without the associated benefits of any enrichment procedures (Smyth et al., 2012).

Incorporating the powdered form of Kakadu plum can be challenging as experiments conducted in our laboratories have shown that Kakadu plum freeze-dried powder when mixed with water forms a jelly like matrix that makes incorporation of this fruit problematic. Initial metabolomic profiling of Kakadu plum fruit conducted by our research group indicated the presence of sugars that are commonly associated with the polysaccharide pectin (Müller-Maatsch et al., 2016). The presence of pectin is a well-known obstacle to the efficient extraction of bioactive compounds from plant matrices as this highly resistant complex of the plant cell wall entraps bioactive compounds (Padayachee et al., 2012; Phan et al., 2015). In fact many of the naturally existing phenolic compounds in fruits and vegetables are usually covalently bound to insoluble polymers (Peleg et al., 1991). Therefore the presence of pectic polysaccharides in Kakadu plum matrix may have the potential to bind EA and other bioactives. Therefore, there is a growing need to develop a food grade compatible extract of Kakadu plum with high bioactive levels, into an easily incorporable format for use as a functional ingredient.

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

In order to overcome issues regarding the solubility of EA, a water co-solvent system was used which is an attractive alternative to expedite the release of EA from Kakadu plum. In these co-solvent systems, the solubility of organic compounds can be improved by altering the composition of the co-solvent and has been reported useful for other plant-based phytochemicals e.g. curcumin (John et al., 2013). In order to facilitate enhanced release of EA from pectin in Kakadu plum cell matrix, an enzyme assisted extraction (EAE) method was developed and evaluated. EAE techniques have been reported to facilitate extraction of bioactive compounds from complex plant polysaccharides like pectin (Lenucci et al., 2015; Miron et al., 2013). EAE has several benefits with higher extraction yield, faster extraction, higher recovery, reduced solvent use and lower energy consumption with the added advantage of being environmentally friendly (Puri et al., 2012).

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

MATERIALS AND METHODS

Plant material

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

Chemicals

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

Determination of moisture content

The moisture content of the freeze-dried Kakadu plum powder was determined according to AOAC official method 964.22 (Association of Official Analytical Chemists, 1995). Briefly, in triplicate, each sample (1 g) was dried for approximately 16 h to a constant weight at 70°C in a vacuum oven (W. C. Heraeus GmbH, Hanau, Germany). The difference between initial weight and constant weight after drying was taken as moisture lost and hence moisture content of the sample.

Metabolomic analysis of Kakadu plum fruit

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

Extraction of polar metabolites from freeze-dried Kakadu plum fruit

Sub-samples (0.1 g) of the lyophilized and milled samples of Kakadu plum fruit were weighed into pre-chilled tared 2 mL microcentrifuge vials. To each vial, 600 µL of extraction solution containing 2:1 (v/v) of 100% methanol and chloroform, including 10 µL of the internal standards (1 mM sorbitol; 10 mM valine; 5 mM myristic acid) was added. The vials were vortexed for 30 s. This was followed by addition of 200 µL chloroform and vortexing for 30 s. The samples were incubated at 70˚C for 15 min in a shaking water bath at 850 rpm. After incubation, 400 µL of ultrapure water added and mixed by vortexing. All the samples were centrifuged at 12054 g at room temperature for 15 min. After centrifugation the upper polar and non-polar lower phases were carefully transferred to new vials. The polar phase was washed using 300 µL chloroform followed by centrifugation at 12054 g at room temperature for 10 min. The washed polar supernatant was transferred to a new vial. The lower non-polar phase left after this centrifugation was combined with the previous non-polar phase. The resulting extracts were stored at -80˚C till further analysis.

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

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

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

Central composite rotatable design (CCRD) for enzyme treatment

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

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

The total number of 20 statistically designed batch experiments (detailed in Table 2) were performed for different combinations of the variables X_1 to X_3 on the response variables Y_1 to Y_3. The 20 experiments were conducted in a randomized order. The experimental design and data analysis were carried out using the Minitab 16 software (Minitab Pty Ltd, Sydney, Australia).

The analysis of variance (ANOVA) for each of the three response variables given in the Tables 3 to 4 indicated significance $p<0.05$ for regression analysis using the second order polynomial model in Equation 1 for all regression models including the linear, square and interaction terms.

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_1^2 + \beta_5X^2 + \beta_6X_3^2 + \beta_7X_1X_2 + \beta_8X_1X_3 + \beta_9X_2X_3 \quad (1)$$

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

Model fitting from response surface methodology

The thick matrix of plant polysaccharide like pectin hinders effective extraction of bioactive compounds from plant cells as these polysaccharides protects the bioactive compounds by trapping them inside (Padayachee et al., 2012). Phytochemicals that are retained by polysaccharide- lignin network of the plant cell are not accessible to solvents in routine extraction processes. Use of pectolytic enzymes allows hydrolysis of plant cell wall components resulting in an increase in cell wall permeability, with subsequent higher extraction yields.

In order to devise an efficient extraction technique that will allow enhanced extraction of bioactive compounds from Kakadu plum

tissue within an easily incorporable liquid format pectinase enzyme was used. Poor solubility and hence reduced bioavailability are limiting factors for the clinical application of EA. To overcome the challenges due to the insolubility of EA in water, propylene glycol was used as a solvent. Propylene glycol is generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA), with aqueous systems of propylene glycol as high as 40% being regarded as nontoxic for human consumption (Liu et al., 2005). Therefore, the Kakadu plum extracts reported here can be considered safe for use in food industry applications.

For our study, we selected three independent variables - solvent volume (%, w/w) (X_1), concentration of enzyme (mg/L) (X_2) and time of hydrolysis (h) (X_3). The response variables measured were concentration of EA (Y_1), concentration of AA (Y_2) and total polyphenolic content (Y_3). The total number of experiments for this study was 20 (Table 2).

Determination of EA content in Kakadu plum extracts

Due to solubility concerns of EA within the enzymatic digestion samples, the pH of each of the extracts was adjusted to 7.5 from their initial pH of ~3.5 (Williams et al., 2016). EA solubility is pH dependent and preliminary analysis showed that levels of EA significantly increased at a higher pH. For the extraction of fEA from the liquids after enzymatic digestion, 1.0 mL of each liquid extract were filtered through a 0.45 µm syringe filter into a HPLC vial, N_2 was introduced, capped and stored at -80°C prior to analysis.

The quantification of EA content in different extracts of Kakadu plum were conducted by HPLC-DAD analysis, as described by Williams et al. (2014). For analysis, an aliquot of 20 µL of sample was injected. Spectra for all wavelengths between 220 and 600 nm were recorded by the photodiode array detector. The fEA was identified by the chromatographic behaviour in comparison with EA commercial standards and UV spectra. fEA content was expressed as mg/100 g DW.

Determination of ascorbic acid content in Kakadu plum extracts

Experiments conducted to determine the effect of pH on AA concentration showed that concentration of AA is higher at lower pH (supplementary table S1). Therefore, the extracts at their original pH of ~3.5 were directly subjected to AA determination. For extraction of AA from the liquid extracts obtained from the enzymatic digestion 0.1 mL of each of the samples was extracted with the AA extraction solution consisting of 1% (m/v) citric acid containing 0.05% (m/v) ethylenediamine tetra-acetic acid (EDTA) as the disodium salt in 50% (v/v) methanol. An aliquot of the diluted samples were filtered through a 0.45 µm filter syringe prior to HPLC analysis. Concentration of AA in the extracts was determined as per Williams et al. (2014).

Determination of total phenolic content (TPC)

The total phenolic content of the liquid extracts was determined using the Folin Ciocalteau (FC) method (Singleton and Rossi, 1965). Diluted extracts were analysed at 750 nm with Gallic acid as a standard using a microplate reader (Infinite M200, Tecan Australia Pty Ltd, Melbourne, Australia). The analysis was carried out in triplicate and expressed as micromoles of total phenolics Gallic acid equivalents, (GAE) per gram of sample. TPC was calculated by the following formula:

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

Statistical analysis

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

RESULTS AND DISCUSSION

Sugar analysis from the polar extracts of Kakadu plum fruit

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

The presence of mannose, rhamnose, galacturonic acid, arabinose glucose and fructose in Kakadu plum fruit suggests the existence of the polysaccharide pectin in the fruit tissue as these sugars are often incorporated into pectin (Müller-Maatsch et al., 2016). The possible presence of pectin in Kakadu plum tissue was further provided by the observations that when dissolved in water (1:4 w/w), Kakadu plum powder formed a gel like consistency. Similar gel formations have been reported by pectin compounds in different fruit materials e.g. prickly pear fruit (*Opuntia albicarpa*) (Lira-Ortiz et al., 2014) and pomelo (*Citrus maxima*) (Methacanon et al., 2014).

Several of the sugars reported in the current study have also been identified in the gum or crude extracts of other *Terminalia* members. In a study analysing the gum of two *Terminalia* species *T. sericea* and *T. superba,* presence galactose, arabinose, rhamnose, mannose and xylose along with highly branched polysaccharides consisting of galacturonic, glucuronic and 4-*o*-methylglucuronic acids were described (Anderson and Bell, 1974). In another traditional medicinal *Terminalia* member - *T macroptera*, Zou et al. (2014) reported the presence of the monosaccharides - rhamnose, mannose, galactose, galacturonic acid, arabinose and glucose in the crude extracts of root, stem and leaves. The galactose sugar was present in the root, stem and

Table 1. Chemical characterisation (free and total EA; AA and moisture content) of the initial starting material, that is, freeze dried kakadu plum puree.

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

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

Model fitting from response surface methodology

The analysis of variance (ANOVA) for each of the three response variables (Table 3) indicated significance $p<0.05$ for regression analysis using the second order polynomial model in Equation 1 for all regression models including the linear, square and interaction terms. This model is good for determining EA, AA and TPC as the lack of fit is not significant ($p>0.05$) (Table 4). The response models for each response variables showing only the terms with significance ($p<0.05$) estimated regression coefficients (Table 3).

The mean values of the level of EA, AA and TPC of the extracts are shown in Table 2. The level of EA in the extracts ranged from 53.6 to 266.6 mg/100 g DW. The highest level of EA was detected when 1.5% solvent volume with 300 mg/L enzyme concentration and time of reaction 15 h was employed. The multiple regression analysis for the response variable EA indicated that the regression model for EA was significant ($p<0.05$) and did not present lack of fit. Results indicated the regression coefficient of the linear (β_3), square (β_6) and interaction (β_8) terms had significant effects on EA levels. The predicted model can be described by Equation 2 in terms of coded values. The co-efficient of determination (R^2) of the regression EA was 81.18% (Table 3).

$$Y_1 = 90.7 + 105\ X_1 + 0.213\ X_2 - 21.13\ X_3 - 48.0\ X_1X_1 - 0.000089\ X_2X_2 + 0.710\ X_3X_3 - 0.165\ X_1X_2 + 10.76\ X_1X_3 + 0.0080\ X_2X_3 \quad (2)$$

Significance levels β_3 = ***$p<0.001$, β_6 = **$p<0.05$ and β_8 **$p<0.05$.

ANOVA testing for the response variable AA indicated that the regression model for AA was not significant ($p>0.05$). Furthermore the results also suggested that the regression coefficient of any of the linear, square and interaction terms had any significant effects on AA levels with the co-efficient of determination (R^2) of the regression AA being only 33.60% (Table 3). Consequently the obtained Equation 3 cannot adequately describe the extraction of AA with the current parameters.

Therefore, the presented model cannot be adequately applied to the preparation of AA rich extracts without modifying the tested parameters. Thus the enzymatic digestion and subsequent breakdown of the plant cell wall material did not have a significant impact on the release of AA. This can possibly be attributed to the fact that AA is not trapped within the plant cell wall. AA is synthesized in the plant cytosol (Loewus, 1980) and is distributed into the chloroplasts, vacuole, and apoplast of the plant cell (Rautenkranz et al., 1994).

$$Y_2 = 138.3 - 70.7\ X_1 - 0.104\ X_2 + 2.30\ X_3 + 24.1\ X_1X_1 - 0.000017\ X_2X_2 + 0.005\ X_3X_3 + 0.0374\ X_1X_2 - 1.69\ X_1X_3 + 0.00565\ X_2X_3 \quad (3)$$

TPC values of the extracts ranged from 48.46 to 105.78 mg GAE/g with the highest values observed with the experimental design when 1.5% solvent volume with 300 mg/L enzyme concentration and 15 h reaction time is applied. ANOVA for the response variable TPC (Table 4) indicated that the regression model for TPC was significant ($p<0.05$) and did not exhibit any lack of fit. Results indicated the regression coefficient of the linear (β_3) and interaction (β_8) terms had significant effects on TPC. The predicted model for TPC can be described by Equation 4 in terms of coded values. The co-efficient of determination (R^2) of the regression TPC was 78.12% (Table 3).

$$Y_3 = 75.0 + 33.6X_1 + 0.0383X_2 - 7.58X_3 - 21.2\ X_1X_1 + 0.000012\ X_2X_2 + 0.170\ X_3X_3 - 0.0406\ X_1X_2 + 4.56\ X_1X_3 + 0.00160X_2X_3 \quad (4)$$

Significance levels β_3 = ***$p<0.05$ and β_8 = **$p<0.05$.

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

Verification of predictive model

Parameters for each of the three independent variables, that is, solvent volume (%), enzyme concentration (mg/L)

Table 2. The coded levels of the variables used in the CCRD and responses obtained from the study.

No.	Variables X_1 to X_3						Responses Y_1 to Y_3			Extraction yield	
	X_1	True values X_1	X_2	True values X_2	X_3	True values X_3	Y_1	Y_2	Y_3	Y_1	Y_2
1	1	2	1	100	1	12	218.9	70.6	104.74	42.1	0.37
2	0	1.5	0	300	0	7	103.9	66.3	59.89	20.0	0.35
3	-1	1.5	-1	300	-1	-1.40	108.8	83.1	67.52	20.9	0.43
4	1	2	1	500	1	2	53.6	68.0	48.46	10.3	0.35
5	-1	2.34	-1	300	-1	7	98.4	110.5	52.12	18.9	0.58
6	1	1	1	100	1	12	127.5	99.7	79.05	24.5	0.52
7	0	1.5	0	300	0	7	142.6	64.6	85.70	27.4	0.35
8	-1	1.5	-1	300	-1	15.40	266.6	92.8	105.78	51.3	0.48
9	0	1.5	0	300	0	7	150.5	69.4	86.38	28.9	0.36
10	1	2	1	100	1	2	100.0	77.9	58.73	19.2	0.41
11	1	1	1	100	1	2	160.5	71.0	90.86	30.9	0.37
12	0	1.5	0	300	0	7	108.2	63.7	66.98	20.8	0.33
13	0	1.5	0	300	0	7	130.1	112.1	82.04	25.0	0.58
14	1	1	1	500	1	12	178.9	97.4	91.38	34.4	0.51
15	-1	0.66	-1	300	-1	7	108.8	98.6	67.04	20.9	0.51
16	1	1	1	500	1	2	135.7	65.2	84.60	26.1	0.34
17	1	2	1	500	1	12	160.0	102.3	88.65	30.8	0.53
18	-1	1.5	-1	636.35	-1	7	124.7	64.4	78.61	24.0	0.35
19	0	1.5	0	300	0	7	198.0	104.9	91.82	38.1	0.55
20	-1	1.5	-1	-36.35	-1	7	124.4	106.3	73.29	23.9	0.55

[1]The coded levels of 3 variables in the CCRD are shown with the actual quantities. The low settings in the experiment are identified by -1 in coded units and the high settings are identified by 1 in coded units. X_1, solvent volume in % (w/w); X_2, enzyme concentration in mg/L and X_3, time of reaction in h. [2]Y_1 = concentration of fEA in mg/100 g DW; Y_2 = concentration of AA in mg/100 g DW; Y_3 = Total phenolic content in mg Gallic acid equivalent/g (mg GAE/g); Extraction yield for Y_1 and Y_2 are expressed as %. Extraction yield (%) = (weight of the extract x 100) / (weight of the original sample).

and time (h) were selected to obtain the highest levels of the response variables. As EA was the parameter that fitted the statistical model most appropriately, it was this response that we aimed to verify. In addition EA has aslo exhibited considerable antimicrobial activity and therefore would be a likely candidate in developing a natural antimicrobial agent from Kakadu plum. The suitability of the model equation for predicting the optimum response values was tested using 7 treatments described in Table 5. Three additional treatments were included to confirm the critical role that the pectinase enzyme plays in enabling the release of the bioactive compounds from the pectin (refer to supplementary data Table S2). These include treatment S1 with 3.5% solvent concentration, 0 mg/L enzyme concentration and 24 h of reaction time; treatment S2 with 3.5% solvent concentration, 1000 mg/L enzyme concentration and 24 h reaction time and treatment S3 with 3.0% solvent concentration, 800 mg/L enzyme concentration and 15 h of time. Expected values of EA in each of treatments were calculated with the help of of Equation 2.

These new extractions were submitted to the same experimental analytical procedures as those applied initially. The observed and predicted values, along with the computed absolute errors (AE) are presented in Table 5. The predicted EA values for 7 treatments (selected as they

Table 3. Response surface regression with coefficients for responses obtained from the study.

Model	Y_1				Y_2				Y_3			
	Coef	SE Coef	T	P	Coef	SE Coef	T	P	Coef	SE Coef	T	P
Constant	138.8	11.7	11.86	0.000	80.79	8.20	9.85	0.000	78.57	4.24	18.53	0.000
X_1	-6.41	7.76	-0.83	0.428	0.40	5.44	0.07	0.943	-5.15	2.81	-1.83	0.097
X_2	-6.42	7.76	-0.83	0.427	-3.75	5.44	-0.69	0.506	-0.83	2.81	-0.30	0.774
X_3	36.68	7.76	4.72	0.001	7.63	5.44	1.40	0.191	10.66	2.81	3.79	0.004
X_1X_1	-11.99	7.56	-1.59	0.144	6.01	5.30	1.13	0.283	-5.31	2.74	-1.94	0.081
X_2X_2	-3.57	7.56	-0.47	0.647	-0.67	5.30	-0.13	0.902	0.48	2.74	0.17	0.865
X_3X_3	17.75	7.56	2.35	0.041	0.13	5.30	0.03	0.980	4.26	2.74	1.56	0.151
X_1X_2	-16.5	10.1	-1.62	0.136	3.74	7.11	0.53	0.61	-4.06	3.68	-1.10	0.296
X_1X_3	26.9	10.1	2.65	0.024	-4.24	7.11	-0.60	0.565	11.41	3.68	3.10	0.011
X_2X_3	8	10.1	0.79	0.45	5.65	7.11	0.79	0.446	1.60	3.68	0.43	0.673
	R^2 = 81.18%				R^2 = 33.60%				R^2 = 78.12%			
	R^2 (adj) = 64.23%				R^2 (adj) = 0.00%				R^2 (adj) = 58.44%			
	R^2 (pred) = 36.40%				R^2 (pred) = 0.00%				R^2 (pred) = 29.08%			

[1]X_1, is solvent concentration (% w/w); X_2, enzyme concentration (mg/L); X_3, time (h); [2]Y_1, fEA (mg/100 g DW); Y_2, AA (mg/100 g DW); Y_3, TPC (mg GAE/g).

possessed values that fell within the statistical model for the three variables), were consistent with the predicted values. The strong correlation between the actual and predicted results confirmed that the response model was adequate to reflect the expected optimization. Because of the low absolute error values obtained by the comparison between observed and predicted values, the proposed model could be used to predict the response value.

By increasing values of the three variables, it is possible to obtain extracts with higher concentration of EA as demonstrated by extract S1 with 173.2 mg/100 g DW, treatment S2 with 392.1 mg/100 g DW and treatment S3 with 322.2 mg/100 g DW of EA (Table S2). Treatment S1 and S2 had identical values for solvent concentration and time of reaction and differed only in terms of the enzyme concentration.

Treatment S1 contained no enzyme whereas treatment S2 utilised 1000 mg/L (Table S3).

Extraction yield

For the 20 extracts the EA extraction yield ranged from 10.3 to 51.3% (Table 2), with the highest yield observed for the solvent concentration of 1.5%, enzyme concentration of 1.5 mg/L and 15 h for time of reaction. The extraction yield observed for AA was significantly lower and ranged from 0.33 to 0.58% (Table 2) which could again be attributed to the fact that unlike EA, AA is not trapped by the plant cell wall materials with enzyme digestion not contributing towards its release. The extraction yield for treatment S2 was 75.4% and was significantly higher than treatment S1 with no enzyme digestion with extraction of 33.3%, followed by treatment S3 with 800 mg/L of enzyme giving an extraction yield of 62.0%. This amply demonstrated that digesting the plant cell walls indeed facilitated the release of EA.

The use of enzymes in extraction processes has been previously reported to enhance recovery of bioactives. In ginger, pre-treatment with enzymes prior to solvent extraction resulted in higher yields of oleoresin and gingerol than the controls. Acetone extraction after enzyme treatment yielded 20% oleoresin and 12.2% gingerol compared to the control (15% oleoresin and 6.4% gingerol) (Nagendra et al., 2013). EAE also resulted in significantly higher yield for kaempferol-glucosides from cauliflower (kaempferol-3-feruloyldiglucoside 37.8 and kaempferol-3-glucoside 58.4 mg rutin equivalent /100 g dry weight) (Huynh et al., 2014). In citrus peels, EAE also resulted in increased yield of phenolic compounds (25.90 to 39.72%)

Table 4. Analysis of variance for lack of fit of models obtained from the CCRD study.

Model	Y_1					Y_2					Y_3				
	DF	Adj SS	Adj MS	F	P	DF	Adj SS	Adj MS	F	P	DF	Adj SS	Adj MS	F	P
Regression	9	35498.7	3944.3	4.79	0.011	9	2046.64	227.404	0.56	0.800	9	3860.15	428.91	3.97	0.021
Linear	3	19495	6498.3	7.89	0.005	3	989.71	329.902	0.82	0.514	3	1922.93	640.98	5.93	0.014
X_1	1	561.7	561.7	0.68	0.428	1	2.15	2.148	0.01	0.943	1	362.84	362.84	3.36	0.097
X_2	1	563.7	563.7	0.68	0.427	1	192.18	192.178	0.48	0.506	1	9.42	9.42	0.09	0.774
X_3	1	18369.6	18369.6	22.31	0.001	1	795.38	795.38	1.97	0.191	1	1550.67	1550.67	14.35	0.004
Square	3	7541.5	2513.8	3.05	0.079	3	546.24	182.081	0.45	0.723	3	744.59	248.2	2.30	0.140
X_1X_1	1	2073.1	2073.1	2.52	0.144	1	521.1	521.104	1.29	0.283	1	406.28	406.28	3.76	0.081
X_2X_2	1	183.7	183.7	0.22	0.647	1	6.46	6.458	0.02	0.902	1	3.28	3.28	0.03	0.865
X_3X_3	1	4541.7	4541.7	5.52	0.041	1	0.26	0.255	0.00	0.980	1	261.49	261.49	2.42	0.151
Interaction	3	8462.2	2820.7	3.43	0.06	3	510.69	170.229	0.42	0.742	3	1192.63	397.54	3.68	0.051
X_1X_2	1	2170.4	2170.4	2.64	0.136	1	112.07	112.066	0.28	0.610	1	131.58	131.58	1.22	0.296
X_1X_3	1	5784.1	5784.1	7.03	0.024	1	143.57	143.569	0.35	0.565	1	1040.65	1040.65	9.63	0.011
X_2X_3	1	507.7	507.7	0.62	0.45	1	255.05	255.05	0.63	0.446	1	20.39	20.39	0.19	0.673
Residual error	10	8232.4	823.2			10	4045.29	404.529			10	1080.89	108.09		
Lack-of-Fit	5	2342.2	468.4	0.4	0.833	5	1523.02	304.604	0.60	0.703	5	298.72	59.74	0.38	0.843
Pure error	5	5890.2	1178			5	2522.27	504.455			5	782.17	156.43		
Total	19	43731.1				19	6091.93				19	4941.04			

[1]X_1 is solvent concentration (% w/w), X_2 enzyme concentration (mg/L) and X_3 is time (h); [2]Y_1 is fEA (µg/ml); Y_2 is AA (µg/ml); Y_3 is TPC (mg GAE/g).

Table 5. Treatments selected for verification.

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

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

(Li et al., 2006).

Conclusion

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

Supporting information

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

Conflicts of Interests

The authors have not declared any conflict of interests.

Abbreviations

EAE, enzyme assisted extraction; **CCRD,** central composite rotatable design; **EA,** ellagic acid; **AA,** ascorbic acid; **TPC,** total phenolic content; **DW,** dry weight; **GAE,** gallic acid equivalent; **GCMS,** gas chromatography mass spectrometry; **HPLC-DAD,** high-performance liquid chromatography with photodiode array detection.

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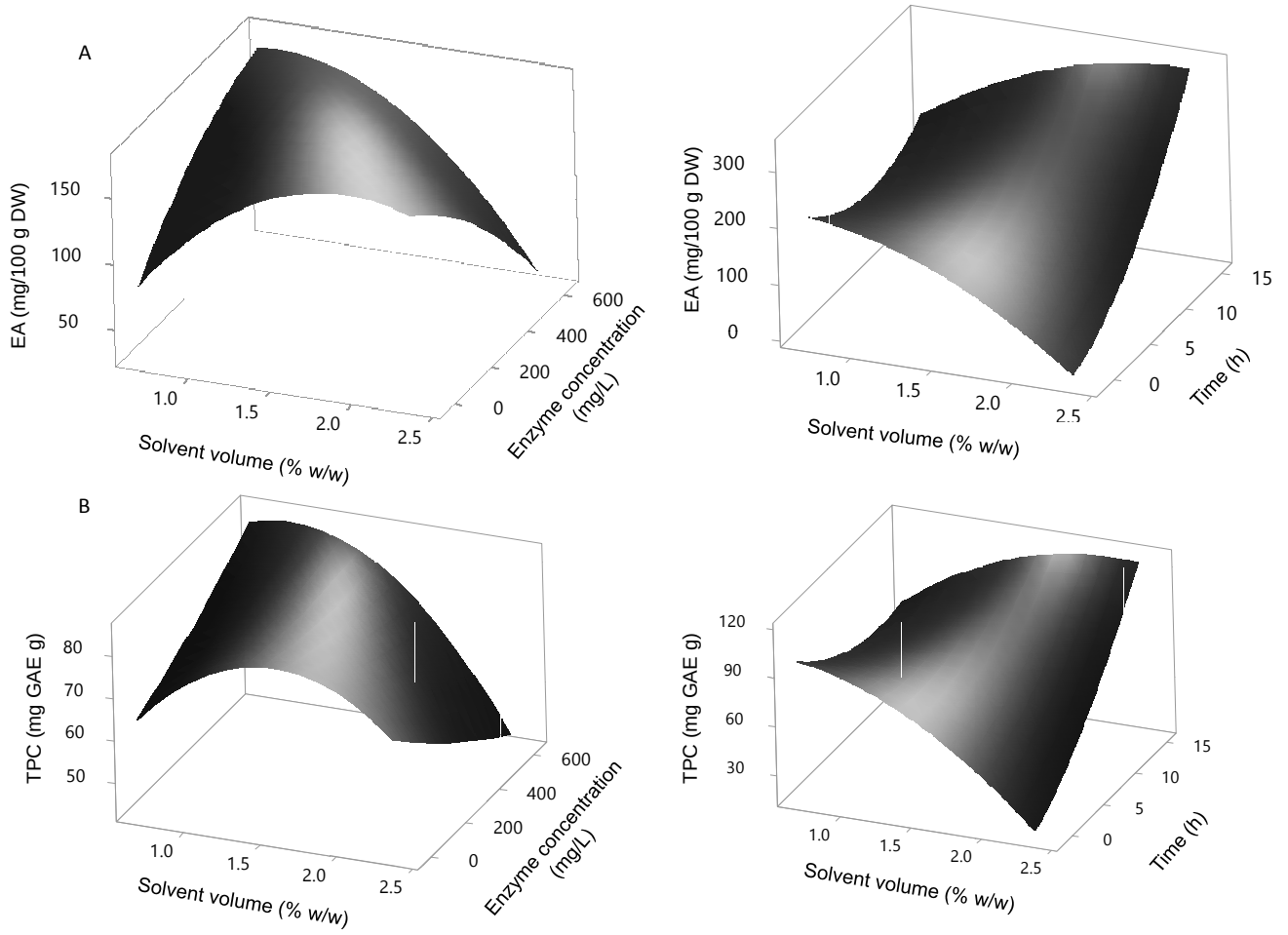

Figure S1. Effect of solvent volume (% w/w), enzyme concentration (mg/L) and time (h) on [A] EA concentration (mg/100 g DW) and [B] TPC (mg GAE/g) in the extracts is shown as surface plots.

Table S1. Effect of pH on concentration of AA in Kakadu plum extracts from initial experiments.

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

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

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

[1]Different letters (that is, a, b, c) across rows denote significant differences between mean EA concentrations in each treatments according to a Tukey−Kramer HSD.