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Non-protein amino acids in Australian acacia seed: Implications for food security and recommended processing methods to reduce djenkolic acid

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ABSTRACT

Seed of Australian acacia species, *Acacia colei*, *Acacia elecantha*, *Acacia torulosa*, *Acacia turmida* and *Acacia saligna*, were analysed for the presence of toxic non-protein amino acids and the levels of essential amino acids. Amines were derivatised with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate before analysis using liquid chromatography electrospray ionisation triple quadrupole mass spectrometry (LC-ESI-QQQ-MS). Multiple reaction monitoring (MRM) with optimised transitions and collision energies for each analyte were employed. The known nephrotoxic compound djenkolic acid was found to be present at elevated levels in all species tested. The lowest levels were in *A. colei* (0.49% w/w) and the highest in *A. saligna* (1.85% w/w). Observed levels of djenkolic acid are comparable to measured and reported levels found in the djenkol bean. Subsequent testing of seed processing methods showed djenkolic acid levels can be significantly reduced by over 90% by dry roasting at 180 °C rendering the seed safe for human consumption.

1. Introduction

The seed of certain species of Australian acacia have been shown to have significant potential to improve rural livelihoods and reduce malnutrition in semi-arid regions of Africa. Acacias provide a range of benefits including environmental services, such as nitrogen fixation and wind speed reduction; they produce valuable fuelwood and building materials and they produce edible seed (Adewusi, Falade, Oyedapo, Rinaudo, & Harwood, 2006; Cunningham et al., 2008; Harwood, Rinaudo, & Adewusi, 1999; Rinaudo, 2001; Rinaudo & Cunningham, 2007; Rinaudo, Patel, & Thomson, 2002; Thompson, Harwood, & Rinaudo, 1996; Yates, 2010).

The use of acacia seed as a food source is not new, with up to forty species known to have been eaten regularly by Aboriginal people in Australia for thousands of years (Devitt, 1992; Latz & Green, 1995; Lister, Holford, Haigh, & Morrison, 1996; Midgley,

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Brand, Harwood, Annison, & Richardson, 1991; O'Connell et al., 1983; Orr & Hiddens, 1987). At the present time, acacia seed is produced and marketed as 'wattleseed' by a small, but growing industry in Australia. Australian acacia seed of a number of species have been analysed by several groups, and shown to contain crude protein at between 22% and 27%, carbohydrate between 21% and 57%, and fats between 7% and 15% (Yates, 2014). Acacia seed is rich in the amino acid lysine, making it an excellent complement to the predominantly cereal based diets of semi-arid Africa (Adewusi, Falade, & Harwood, 2003; Yates, 2010). The seed of *Acacia colei* was the subject of a human volunteer trial in Niger in 1995 (Adewusi et al., 2006), and has been consumed regularly in the Maradi district since that time.

Acacia seed is known to contain several antinutritional factors, which if not removed through appropriate processing or cooking could reduce absorption of nutrients. The presence of trypsin inhibitors, oxalate, phytate, saponins and tannins are reported by Adewusi, Falade, Harwood (2011), Adewusi et al. (2003) and Ee and Yates (2013), though not in concentrations likely to be injurious to human health. Non-protein amino acids (NPAAs) also occur in acacia seed and may have potentially antinutritional or toxic effects (Bell, 2003). For example, Falade, Adewusi, and Harwood (2012) show that (S)-carboxyethyl cysteine in acacia seed interferes with the absorption of methionine in rats.

This study screened and quantified the levels of a variety of NPAAs in five species of acacia seed in order to assess the health risk that these compounds may pose if the seeds become a regular part of the human diet. Amino acids, NPAAs, biogenic amines and many other amines can be selectively derivatised and quantified using 6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate (Aqc) (Boughton et al., 2011). The initial screen comprised the NPAAs: (S)-carboxymethylcysteine (CMC), lanthionine, djenkolic acid (DJK), mimosine and canavanine.

In the first part of the study NPAAs were measured in the seeds of five species: *A. colei*, *Acacia elacantha*, *Acacia torulosa*, *Acacia tumida* and *Acacia saligna*. The *A. saligna* seed was tested in two preparations, as a raw seed and as a roasted seed. A sample of soybean was also tested as a negative control for comparative purposes. When concerning levels of djenkolic acid were found, commercially sourced Djenkol bean was also tested as a positive control, then a second part of the study sought to determine the most effective means of processing for reduction of this compound in three of the five species of acacia and the effect of processing methods on essential amino acids content.

The species selected for testing are all under investigation for their potential contribution to agricultural systems in semi-arid regions of Africa. *A. colei* has been used as a human food in Niger for over 15 years. *A. torulosa*, *A. elacantha* and *A. tumida* have performed well in agronomy trials and show potential for good seed yields in Niger and other parts of the Sahel (Cunningham & Abasse, 2005). *A. saligna* has been planted widely in the dry highlands of Tigray, in northern Ethiopia, with seed production representing a significant potential food resource (Hagazi, 2011; Yates, 2010). The species trialled in the Sahel could be expected to perform similarly in the Ethiopian lowlands, where many of the livelihood and land degradation problems seen in Niger are also evident.

2. Materials and methods

2.1. General

All chemicals and solvents used for analysis were purchased from Sigma Aldrich (Australia) and were either of analytical or mass spectrometric grades. The Aqc reagent was synthesized accordingly (Cohen & Michaud, 1993) and deionised water (18.2 MΩ) was produced using a Synergy UV Millipore System (Millipore) and was used throughout. An Eppendorf 5415R refrigerated mircrocentrifuge (Eppendorf AG, Hamburg, Germany) cooled to 0 °C was used for centrifugation. External calibration curves within the concentration range of 100 nM to 100 μM were prepared from a combined solution containing all standards.

Acacia seed lots from *A. colei*, *A. elacantha*, *A. torulosa*, *A. tumida* were sourced from trial plots growing at Danja, Niger. *A. saligna* seed lots were source from naturalised stands growing in Tigray, Ethiopia. Roasted *A. saligna* seeds were prepared by the Tigray Agricultural Research Institute, Ethiopia and were subject to 10 min pan-roasting over a charcoal fire. Djenkol beans and soybean were sourced from a commercial retail supplier.

2.2. Acacia seed pre-treatments

2.2.1. Dehusking

Raw seed was deshuked by cracking the seed coat then manually peeling the seed coat away, hardened seed coat from roasted seeds was removed by crushing the seed then removing as much seed coat as possible. Dehusking and separation of raw meal from *A. colei* seed proved difficult due to the small size of the seed.

2.2.2. Roasting of raw seeds

For initial roasting samples of *A. colei*, *A. saligna* and *A. torulosa* seed were pan roasted for 10 min at 180 °C prior to homogenisation. For time course roasting experiments lots of ten seeds from *A. colei*, *A. saligna* and *A. torulosa* were roasted in aluminium foil boats for 2, 4, 6, 8 and 10 min at 180 °C in a conventional fan forced oven. Each roasted seed lot was then split into two groups where one group was homogenized whole and the other dehusked then homogenized.

2.2.3. Germination of seeds

Seeds from *A. colei*, *A. saligna* and *A. torulosa* were germinated by pouring enough boiling water to submerge seeds placed on sterilised large filter paper in a petri dish. Seeds were soaked overnight and excess water removed. Samples were germinated on the laboratory bench and a separate lot in the dark for three days. Seedlings were snap frozen in liquid nitrogen and freeze dried.

2.2.4. Soaking of seed

Lots of ten seeds from *A. colei*, *A. saligna* and *A. torulosa* were placed in 15 ml Falcon tubes then soaked in deionised water (500 μl) for 24 h at 23 °C, the water was removed then frozen and stored at −20 °C. Seeds were snap frozen in liquid nitrogen then freeze dried.

2.3. Homogenisation of acacia seeds, djenkol bean and soybean

Frozen djenkol beans were hand crushed using a mortar and pestle to a course powder prior to homogenisation.

Raw or pre-treated acacia seeds (500 mg), raw soybean (500 mg) and course djenkol bean powder (500 mg) were homogenised in an IKA Yellowline A-10 water cooled mill grinder (IKA-Werke GmbH & Co. KG, Stuafen, Germany) for 30–60 s prior to extraction.

Freeze dried germinated acacia seedlings were homogenized at −20 °C in cryo-tubes (Precellys, Bertin Technologies, Montigny-le-Bretonneux, France) containing 1.4 mm ceramic beads and methanol (500 μl) containing internal standard ($^{13}C_5$,^{15}N-valine, 25 μM) using a Cryomill (Precellys 24, Bertin Technologies, Montigny-le-Bretonneux, France) with cryo attachment using 3 × 30 s program at 6000 rpm with 45 s delay intervals.

2.4. General extraction procedure

To 20 mg of homogenised material was added methanol (500 µl) containing internal standard ($^{13}C_5$,^{15}N-valine, 25 µM). The mixture was vortexed and centrifuged at 13,200 rpm for 1 min at 0 °C, the supernatant transferred to a new vial then 500 µl of deionised water added to the remaining pellet. The mixture was vortexed, centrifuged at 13,200 rpm for 5 min at 0 °C and the supernatant combined with the methanol extract. The combined phases were washed with 200 µl of dichloromethane, the extracts transferred to a new vial and diluted 10-fold in methanol:water (1:1) and stored at −20 °C prior to derivatisation.

2.5. Measurement of amino acids and NPAA's

2.5.1. Generation of standard curves

Amino acid standard curves were generated following the procedure of (Boughton et al., 2011). Authentic NPAA standards of (*S*)-carboxymethylcysteine (CMC), lanthionine, djenkolic acid (DJK), mimosine and canavanine were dissolved in methanol:water (1:1) containing tris(2-carboxyethyl)phosphine (TCEP, 10 mM) and ascorbic acid (1 mM) to a concentration of 10 mM. Individual standards were diluted individually or mixed to create a combined solution then diluted to a concentration of 2.5 mM using volumetric glassware. A standard curve was generated by serially diluting individual standards or combinations of standards to 150, 100, 50, 25, 10, 5, 1, 0.5 and 0.1 µM using methanol:water (1:1) containing tris(2-carboxyethyl)phosphine (TCEP, 10 mM) and ascorbic acid (1 mM).

2.5.2. Derivatisation procedure

Derivatisation by 6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate (Aqc) was performed by following the procedure of (Boughton et al., 2011). A 10 µl aliquot of each sample was added to 70 µl of borate buffer (200 mM, pH = 8.8 at 25 °C) containing tris(2-carboxyethyl)phosphine (TCEP, 10 mM), ascorbic acid (1 mM) and 2-aminobutyric acid (25 µM) as internal standard, used for instrumental error correction. The resulting solution was vortexed and 20 µl of Aqc reagent (10 mM dissolved in 100% ACN) added then immediately vortexed. The samples were heated with shaking at 55 °C for 10 min, centrifuged and transferred to HPLC vials containing inserts.

2.5.3. Instrumentation

An Agilent 1200 LC-system coupled to a 6410 Electrospray Ionization-Triple Quadrupole Mass Spectrometer (MS) (Agilent Technologies, Santa Clara, CA, USA) was used for quantification. Injection volumes of 1 µl of samples or standards were used. Ions were monitored in the positive ion mode using a dynamic MRM (DMRM) method optimized for each analyte (see Supplementary Table 1). The source, collision energies and fragmentor voltages were optimized for each analyte by infusing a derivatised standard with LC eluent. The following source conditions were used for the Agilent 6410 QQQ-MS: sheath gas temperature 315 °C, gas flow 10 l min^{-1}, nebulizer pressure 45 psi and capillary voltage 3800 V.

2.5.4. Liquid chromatography

Aqueous mobile phase of 0.1% (v/v) formic acid in water (solvent A) and organic phase 0.1% (v/v) formic acid in acetonitrile (solvent B) were used. For LC experiments run on a 1200 LC-system (Agilent Technologies, Santa Clara, USA). A Zorbax Eclipse XDB-C18 Rapid Resolution HT 2.1 × 50 mm, 1.8 µm (Agilent Technologies, Santa Clara, USA) column was used with a flow rate of 300 µl min^{-1}, maintained at 30 °C, resulting in operating pressures below 400 bar with a 19 min runtime. A gradient was run from 0 to 2 min using 1% solvent B, linearly raised to 15% solvent B over 7 min, then raised to 30% solvent B over 5 min, followed by re-equilibration at 1% solvent B for 5 min.

2.6. Data analysis

Amino acids and NPAAs were quantified and corrected for instrumental error using MassHunter Quantitative Analysis Software (Version: 6.0, Agilent Technologies, Santa Clara, USA). Quantified results were exported into Microsoft Excel 2010 then manually corrected for extraction efficiency by determination of the median response for $^{13}C_5$,^{15}N-valine labelled internal standard then calculation of individual correction factors.

3. Results

3.1. Derivatisation of non-protein amino acids and detection by LC-QQQ-MS

The NPAAs canavanine, (S)-carboxymethylcysteine (CMC), djenkolic acid (DJK), lanthionine and mimosine each possess at least one reactive amine group that can be selectively derivatised using 6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate (Aqc) under standard conditions (Boughton et al., 2011). The NPAAs canavanine, CMC and mimosine contain only a single reactive amine group and were successfully derivatised and when analysed by LC-QQQ-MS in the positive ion mode the corresponding Aqc derivatives were observed as the singly charged $[M + Aqc + H]^+$ ion (see Supplementary Table 1 and Fig. 1). Precursor ion for canavanine was found at *m*/*z* 374.2 with retention time 10.4 min, CMC *m*/*z* 350.2 at 10.3 min and mimosine *m*/*z* 369.2 at 9.8 min. Both DJK and lanthinone contain two reactive amines and each NPAA was doubly derivatised once at each amine group under standard conditions. Analysis by LC-QQQ-MS observed precursor ions to be present as the doubly charged $[M + (2 \times Aqc) + (2 \times H)]^{2+}$ ion; DJK *m*/*z* 298.2 at 6.3 min and lanthionine *m*/*z* 275.1 at 9.6 min. Amines derivatised with Aqc can undergo gas phase fragmentation by using collision induced dissociation leading to generation of a characteristic 171 Amq fragment ion (Boughton et al., 2011). For each NPAA derivative a collision energy of 20V was employed with cell accelerator voltages optimised to produce an optimal Amq 171 response with voltages ranging from 5 to 7 V (see Supplementary Table 1). Once optimised a multiple reaction monitor was employed to monitor each NPAA and other essential amino acids using LC-QQQ-MS. The linear calibration range was found for most derivatives to lie between 0.5 and 100 µm with lower limits of detection of 0.05–5 µm (see Supplementary Table 1). Quantitation of NPPAs and amino acids in acacia was achieved by comparison against an external calibration curve containing all analytes. Raw data was corrected for instrumental error and extraction efficiency by using two internal standards 2-aminobutyric acid and $^{13}C_5$,^{15}N-valine. For each internal standard percent relative standard deviations were found to be <10%RSD (data not shown).

3.2. Screen of five acacia species, soybean and djenkol bean for NPAA's

Samples from *A. colei*, *A. elacantha*, *A. torulosa*, *A. tumida* and *A. saligna* seed lots were homogenised, extracted then screened for measureable amounts of the NPAAs: canavanine, (S)-carboxymethylcysteine (CMC), djenkolic acid (DJK), mimosine and lanthionine. Very little or no response was observed for CMC, canavanine, lanthionine and mimosine with responses below the lowest standard employed (0.5 µM). However, results indicated that large amounts of DJK was present in each of the acacia varieties tested (Table 1) where concentrations ranged from 0.5% to 1.9% of the whole seed mass, with *A. torulosa* and *A. saligna* displaying the highest

Table 1
Calculated concentration of Djenkolic acid (g/100 g) occurring in a variety of sample types including: the seeds of five Acacia species, an authentic imported from sample of commercially lightly roasted *A. saligna* from Ethiopia, with soybean and a commercially supplied djenkol bean used as negative and positive controls respectively; compared to literature reported concentrations of djenkolic acid in the djenkol bean (Areekul et al., 1976; Lucas et al., 1988).

Sample	Average djenkolic acid detected g/100 g (±standard error, $n = 3$)
A. colei	0.49 (±0.02)
A. elacantha	0.54 (±0.01)
A. saligna (raw)	1.90 (±0.06)
A. saligna (commercially roasted)	1.63 (±0.1)
A. torulosa	1.74 (±0.02)
A. tumida	0.94 (±0.05)
Soybean	<0.001
Djenkol bean (measured)	5 (±0.75)
Djenkol bean (literature reported)	1–2

Table 2
Djenkolic acid content expressed as a relative percentage of raw whole seed of *A. colei*, *A. saligna* and *A. torulosa* whole seed, pan roasted seed, soaked seed, germinated seed (light and dark), seed husk and seed meal.

Sample	*A. colei*	*A. saligna* (%)	*A. torulosa* (%)
Raw whole seed	100%	100	100%
Roast whole seed	2%	34	3
Soaked seed	126%	90	50
Light germination	83%	154	48
Dark germination	33%	89	27
Raw meal (dehusked)	N/A	140	93
Roast meal	6%	78	16
Husk-roast	8%	2	0

Table 3
Djenkolic acid content (g/100 g ± standard error, $n = 3$) in the whole seed of roasted *A. colei*, *A. saligna* and *A. torulosa*.

Time (min) roasted at 180 °C	*A. colei*	*A. saligna*	*A. torulosa*
0 (Raw)	0.237 ± 0.010	1.189 ± 0.041	0.721 ± 0.023
2	0.113 ± 0.000	0.901 ± 0.030	0.448 ± 0.016
4	0.053 ± 0.001	0.811 ± 0.012	0.506 ± 0.001
6	0.017 ± 0.000	0.396 ± 0.009	0.050 ± 0.001
8	0.005 ± 0.000	0.144 ± 0.004	0.064 ± 0.000
10	0.002 ± 0.000	0.039 ± 0.000	0.003 ± 0.000

concentrations 1.74% and 1.90% respectively. Comparative testing of soybean as negative control showed DJK content to be negligible at less than 0.001%. Measurement of DJK content in djenkol bean as a positive control returned a much higher DJK content of 5% (Table 1), greater than literature reported values in the range of 0.3–2% (Areekul, Kirdudom, & Chaovanapricha, 1976; Lucas, Guerrero, Sigales, & Sotelo, 1988).

3.3. Djenkolic acid content present in whole seed, meal or husk and the effect of different processing methods

The quantity of DJK present in whole seed, meal and the husk was determined after homogenisation of seed. For raw meal, seeds were carefully dehusked by cracking and peeling the seed coat from the seed or after homogenisation seed coat fragments were removed prior to analysis. Results indicate that in all cases the seed meal was observed to contain the highest concentrations of DJK (Table 2). The effect of roasting, soaking and germination of acacia seed was then explored to determine an appropriate method for processing seed using rudimentary village accessible conditions. Pan roasting led to large reductions in the DJK content of whole seed decreasing by 66–98% and for roasted meal decreasing by 22–94%. Germination of the seed in the light leading to reductions of 17–52% or gain in reported response 154%, germination in the dark led to reductions of between 11% and 73.

With pan roasting of seeds providing the greatest reduction of DJK content in the whole seed; the effect of oven roasting 3 acacia species under more controlled conditions was tested over a period of 10 min in a fan forced oven at 180 °C (Table 3) sampling every 2 min. Results showed a significant drop in DJK content over a 10 min roast by >95% and after 8 min of roasting DJK levels were reduced by over 90% for all species. *A. saligna* seeds showed the highest concentrations and slowest DJK reduction over time requiring at least 8 min to reach 90% reduction of DJK content in comparison to *A. colei* and *A torulosa* which showed DJK reductions of >90% after 6 min.

It is possible that the degradation of DJK could result in the production of toxic amines such as cysteine-S-sulphonic acid. Accurate mass profiling of the derivatised roasted extracts when compared to the derivatised raw seed meal extracts did not show the presence of any new amine products forming (data not shown). The Aqc derivatisation agent is specific for amines and the lack of any new peaks suggests DJK is degraded via another pathway such as the Maillard reaction or pyrolysis at higher temperatures.

3.4. Effect of roasting on essential amino acid content of acacia seed meal

The concentration of essential amino acids including Lysine (Lys), Methionine (Met), Histidine (His), Threonine (Thr), Leucine (Leu), Isoleucine (Ile), Phenylalanine (Phe) and Tryptophan (Trp) were determined in seed meal over the course of roasting to determine any effect upon nutritional content (Table 4). Results indicate that *A. torulosa* showed the highest amount of amino acid degradation after 10 min of roasting with six of the nine essential amino acids degraded by over 90%. In contrast *A. colei* and *A. saligna* only saw His degrade >90% with other amino acids ranging from 81% through to only 17% reduction. There were two exceptions for *A. colei* which saw a slight increase in the levels of Ile to 103% and Val to 111%. Within these same samples DJK was observed to significantly decrease after 10 min of roasting.

4. Discussion

4.1. The risks of djenkolic acid

Djenkolic acid was first identified as the cause of acute kidney disease in people who ate djenkol bean (*Archidendron jirringa*), which is considered a delicacy in south-east Asia (van Veen & Hyman, 1933). Patients afflicted by 'djenkolism' complain of nausea, acute pain in the loins and kidney region and haematuria. The toxicity is due to the tendency of DJK to precipitate in the acidic environment of the kidney, forming needle-like crystals that cause mechanical damage to the kidney (Barceloux, 2008; van Veen & Hyman, 1933). The DJK exposure levels associated with episodes of djenkolism seem to vary widely. Vigneaud and Patterson (1936) found that some people became ill after eating as little as half of a 15 g bean, whilst others were able to eat up to ten beans without illness. Studies have identified risks in chronic exposure to DJK. Areekul, Muangman, Bohkerd, and Saenghirun (1978) suggested that extended exposure to DJK could be implicated in the formation of kidney stones and Wiwanitkit (1995) found that long term consumption of djenkol beans was related to haematuria in children in Thailand. Shukri, Mohamed, Mustapha, and Hamid (2011) conducted a 90 day study that

Table 4
Concentration of essential amino acids (pmoles mg^{-1} ± S.E.) in acacia seeds after roasting for between 0 and 10 min.

Species	Time (min)	His	Thr	Lys	Met	Val	Ile	Leu	Phe	Trp
A. colei	0	4890 ± 321	425 ± 6.7	1778 ± 12.2	49.6 ± 0.7	316 ± 3.8	162 ± 1.8	152 ± 2.1	258 ± 1.8	328 ± 7.7
	2	3350 ± 103	366 ± 3.9	1374 ± 10.1	31 ± 0.2	248 ± 1.5	114 ± 0.5	92.3 ± 1	183 ± 2.5	235 ± 7.8
	4	1899 ± 53	278 ± 1.1	940 ± 13.7	31.4 ± 0.1	217 ± 1.4	96.6 ± 1.3	73.9 ± 2.8	143 ± 4.3	272 ± 11.8
	6	2365 ± 261	265 ± 1.6	1280 ± 20.1	31.1 ± 0.6	393 ± 2.3	193 ± 1.1	155 ± 0.7	150 ± 2.8	176 ± 6.6
	8	1149 ± 78.3	205 ± 4.9	586 ± 13.3	15 ± 0.9	158 ± 2.7	63.7 ± 1.3	46.5 ± 0.9	50.2 ± 1.1	86 ± 3.3
	10	496 ± 41.5	137 ± 0.7	1543 ± 37.3	21.4 ± 0.6	350 ± 1.4	167 ± 6.6	111 ± 4.3	53.7 ± 2.5	77.5 ± 4.1
A. saligna	0	2820 ± 325	646 ± 34.5	1519 ± 77.8	31.3 ± 0.7	287 ± 1.4	93 ± 2.1	129 ± 3.9	181 ± 12.8	496 ± 27.9
	2	1252 ± 117	1013 ± 29.5	956 ± 67.4	17.3 ± 0.3	226 ± 0.9	76.8 ± 0.3	57.5 ± 1.2	136 ± 1.8	923 ± 24.3
	4	643 ± 47	592 ± 4.6	1262 ± 51	18.8 ± 0.8	326 ± 1.9	73.2 ± 0.4	71.4 ± 0.4	218 ± 2.3	365 ± 7.5
	6	551 ± 44.4	532 ± 20.1	1153 ± 36.5	17.5 ± 0.5	266 ± 4.5	64.6 ± 0.4	62.8 ± 0.8	131 ± 2.8	347 ± 7.1
	8	190 ± 7	468 ± 5.1	654 ± 0.8	29.8 ± 0.4	188 ± 0.9	44.3 ± 0.9	40.8 ± 0.7	62.7 ± 0.2	118 ± 0.4
	10	220 ± 9.1	270 ± 2.5	762 ± 2.8	10.2 ± 1.5	218 ± 2.1	86.8 ± 0.1	86.4 ± 3.1	79.4 ± 0.2	94.6 ± 0.3
A. torulosa	0	946 ± 72.3	548 ± 12.1	1137 ± 46.5	111 ± 0.9	341 ± 0.5	116 ± 1.9	129 ± 3.8	197 ± 5	960 ± 36.5
	2	727 ± 23.3	494 ± 16.3	1123 ± 10.8	16.2 ± 0.6	374 ± 1	87.1 ± 0.9	74.9 ± 1.3	144 ± 2.9	769 ± 17.1
	4	665 ± 15.1	411 ± 2.4	986 ± 13.7	16 ± 0.3	252 ± 1.6	57.2 ± 0.6	50.2 ± 0.4	90.7 ± 1.1	1313 ± 10
	6	836 ± 70.5	281 ± 5.1	1377 ± 29.4	15.5 ± 0.5	225 ± 1.8	55.3 ± 0.7	33 ± 0.2	46 ± 0.5	490 ± 2.8
	8	122 ± 20.8	328 ± 0.8	414 ± 10.1	38.4 ± 0.2	292 ± 1.7	77.3 ± 0.6	53.9 ± 1.3	52.5 ± 0.8	298 ± 2.3
	10	20.4 ± 0.9	86.3 ± 1	64.4 ± 2.4	9.2 ± 0.3	94.3 ± 1.1	17 ± 0.3	11.8 ± 0.3	9.4 ± 0.2	22 ± 0.5

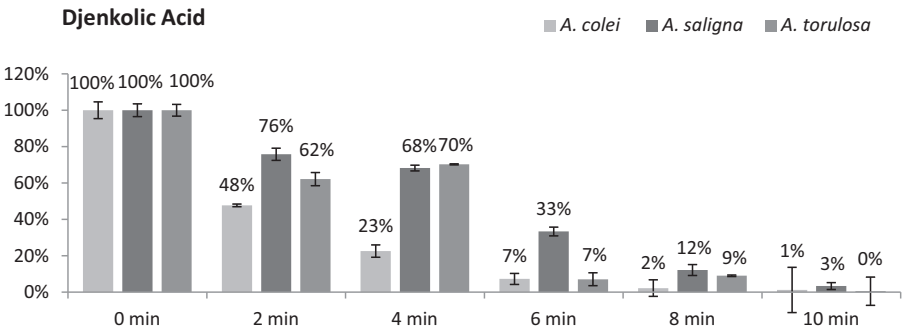

Fig. 1. Relative percentage of djenkolic acid present in acacia seeds after oven roasting at 180 °C for periods of 0–10 min (bars indicate standard error, $n = 3$).

showed that relatively low levels of DJK exposure could result in lesions to the heart, liver, pancreas and kidney of rats.

4.2. Comparing the DJK levels of djenkol bean and acacia seed

Djenkol beans are reported to contain DJK at levels between 1 and 2% (Areekul et al., 1976; Lucas et al., 1988). Under the methodology used in this study, a much higher DJK content of 5% was identified within the sample of djenkol beans tested (Table 1). Acacia seed DJK content in the species tested was found to range from 0.49% for *A. colei*, to 1.9% for *A. saligna*. If eaten raw and without removing the seed husk, 100 g of *A. colei* would provide 490 mg of DJK, whilst the same quantity of *A. saligna* would provide 1900 mg of DJK. Djenkol bean has been reported to cause illness at ingestion rates that would deliver between 150 and 3000 mg of DJK. On the basis of these figures, the levels of djenkolic acid are similar to those found in djenkol bean and suggests that unprocessed acacia seed is unsafe to eat. If partial processing occurs, wherein the seed husk is removed but no roasting occurs, then DJK levels may rise by a further 20–30%, since very little DJK is found in the husk (Table 2).

4.3. Village accessible methods – effects of germination, fermentation and roasting upon DJK levels

Acacia seed could be expected to make the greatest nutritional contribution amongst poor communities in semi-arid regions, yet levels of DJK in acacia seed need to be significantly reduced if it is to be a useful and safe source of food for humans. In poor rural communities, limited technologies are available for the processing of foodstuffs, these being separation of fractions (typically by grinding and sieving), soaking (leeching), roasting, boiling, germinating (sprouting) and fermenting. To be realistic and relevant to people's lives, any recommended processing method would need to reflect one or more of these limited and very traditional methods. A number of other toxic amino acid including β-*N*-oxalyl-α,β-diaminopropionic acid (β-ODAP), the causative agent of neurolathryism in humans, have previously been shown to be readily reduced using village accessible technologies (Lambein, Kuo, Kusama-Eguchi, & Ikegami, 2007). β-ODAP is present in grass pea (*Lathryus sativus*) at similar levels to DJK in acacia and upon roasting ground seed material for up to 60 min at temperatures of 150 °C the content of β-ODAP can be reduced by up to 88%, rendering the roasted seed safe for human consumption (Akalu, Johansson, & Nair, 1998; Grela, Studzinski, & Matras, 2001).

The processing methods tested in this study compared the DJK content of raw whole seed, roast whole seed, soaked seed, seed germinated in light and in darkness, raw meal, roast meal and roast husk. Though all methods had some effect in reducing DJK, the roasting of acacia seed was most effective, reducing DJK content by more than 90% over 10 min of roasting for the three species tested (Table 3, Fig. 1). Optimal roasting time for *A. colei* and *A. torulosa* was 6 min at 180 °C. Inexplicably, given that it is a relatively small seed with a thin husk, *A. saligna* required a further 4 min of roasting to achieve the same 90% reduction in DJK. The kinetics of DJK degradation relative to published β-ODAP results are most likely due to the hotter temperatures employed during oven roasting in our study. Given the widespread use of wood fire and charcoal for cooking, roasting in a pan is recommended as the best method for processing of acacia seed.

4.4. Implications for the use of acacia seed as a food in semi-arid and famine prone areas

Due to a high protein content acacia seed has significant potential as a food in famine prone areas. The results of this study indicate that DJK is present in unprocessed acacia seed at levels that may raise health concerns. The results show that if appropriate processing methods are employed, DJK levels can be reduced by more than 90%. Roasting is the most effective method for removal of DJK, but the effects of roasting differ between species of seed. *A. saligna* needs 20% more time for roasting than either *A. colei* or *A. torulosa* to achieve the same levels of DJK reduction.

Roasting affects all amines in acacia seed to some degree, including amino acids bound within protein, by degradation via the Maillard reaction from which a complex series of poorly characterised products are formed or pyrolysis at higher temperatures (Hodge, 1953).

In the case of *A. saligna*, it is possible that the degree of roasting required to reduce DJK to levels safe for human consumption will also reduce the levels of important free amino acids. Our results show that the free amino acids Lys and Met were reduced by 13–94% and 57–92%, respectively, branch chain amino acids Val, Leu and Ile are reduced 7–91%, aromatic amino acids Phe and Trp reduced 65–98%, Thr 58–84% and basic amino acid His 76–98%. The significance of this amino acid reduction would need to be assessed in light of the overall diet available to a person.

In the real-world of poor communities facing food shortage, toxins in food pose serious risks as people can tend to discount their safety in order to maximise scarce resources (Baro & Deubel, 2006; Getahun, Lambein, Vanhoorne, & van der Stuyft, 2003). In the case of acacia seed, discounting may take the form of people choosing to roast less or not at all. A number of plausible scenarios under resource scarce conditions may be envisaged where the seed is prepared without adequate roasting and incorporated into the diet thereby exposing individuals to DJK at possibly unsafe levels. Such DJK exposure would in all likelihood be harmless if it were occasional or short term. However, if the pattern were to continue over weeks or months, illness may result, an outcome made more likely by general poor health brought about by malnutrition. Such a scenario is not an argument against the use of acacia seed as a food. Rather, it is an argument for a thorough education effort explaining how acacia seed needs to be processed in order to ensure safety.

5. Conclusion

Results from screening five species of Australian acacia for toxic non-protein amino acids in seed provided surprisingly high concentrations of the nephrotoxin djenkolic acid. The high levels of djenkolic acid observed raise serious concerns for human health if raw acacia seed meal is consumed or the levels are not reduced by food processing methods. Under resource poor (famine) or extended exposure a significant risk of the development of djenkolism exists. Exploration of appropriate food processing methodology demonstrated djenkolic acid content could be easily reduced to safe levels for human consumption by roasting the whole seed or seed meal for short periods of time, thereby significantly reducing any potential risks associated with consumption. The wider implications of reduction of essential free and protein bound amino acid content will need to be assessed in conjunction with the potential overall diet of individuals under famine conditions but are beyond the scope of the present study. Further, appropriate public education into safe processing methods of acacia seed could further ameliorate potential risks. Results from this study also indicate that there are no negative implications for acacia seed use in the Australian Bushfoods industry. Roasting levels employed in Australia, coupled with use in relatively low concentrations (i.e., <10%) means there is no health risk in contemporary Australian usage of acacia seed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.01.072.

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