



Characterization of alkyl phenols in cashew (*Anacardium occidentale*) products and assay of their antioxidant capacity

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Abstract

In this study the content of anacardic acids, cardanols and cardols in cashew apple, nut (raw and roasted) and cashew nut shell liquid (CNSL) were analysed. The higher amounts (353.6 g/kg) of the major alkyl phenols, anacardic acids were detected in CNSL followed by cashew fibre (6.1 g/kg) while the lowest (0.65 g/kg) amounts were detected in roasted cashew nut. Cashew apple and fibre contained anacardic acids exclusively, whereas CNSL also contained an abundance of cardanols and cardols. Cashew nut (raw and roasted) also contained low amounts of hydroxy alkyl phenols. Cashew nut shell liquid was used for a basic fractionation of the alkyl phenol classes and the individual anacardic acids, major cardanols and cardols were purified to homogeneity from these fractions by semi-preparative HPLC and definitively identified by nano-ESI-MS-MS, GC-MS and NMR analyses. The hexane extracts (10 mg/ml) of all cashew products tested plus CNSL, displayed significant antioxidant capacity. Cashew nut shell liquid was the more efficient (inhibition = 100%) followed by the hexane extract of cashew fibre (94%) and apple (53%). The antioxidant capacity correlated significantly ($P < 0.05$) with the concentration of alkyl phenols in the extracts. A mixture of anacardic acids (10.0 mg/ml) showed the higher antioxidant capacity ($IC_{50} = 0.60$ mM) compared to cardols and cardanols ($IC_{50} > 4.0$ mM). The data shows that of these substances, anacardic-1 was by far the more potent antioxidant ($IC_{50} = 0.27$ mM) compared to cardol-1 ($IC_{50} = 1.71$ mM) and cardanol-1 ($IC_{50} > 4.0$ mM). The antioxidant capacity of anacardic acid-1 is more related to inhibition of superoxide generation ($IC_{50} = 0.04$ mM) and xanthine oxidase ($IC_{50} = 0.30$ mM) than to scavenging of hydroxyl radicals. At present a substantial amount of cashew fibre is mostly used in formulations of animal or poultry feeds. The data presented in this study, indicates that this waste product along with CNSL, both of which contain high contents of anacardic acids, could be better utilized in functional food formulations and may represent a cheap source of cancer chemopreventive agents.

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Keywords: Antioxidants; Anacardic acids; Cardols; Cardanols; HPLC; Mass spectrometry; Reactive oxygen species; Cashew apple; Cashew fibre; Cashew nuts; GC-MS; Xanthine oxidase

Abbreviations: DHBA, dihydroxybenzoic acids; DMSO, dimethyl sulphoxide; GC-MS, gas chromatography-mass spectrometry; HP, Hewlett-Packard; HPLC, high performance liquid chromatography; UV, ultraviolet; ROS, reactive oxygen species.

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1. Introduction

Anacardium occidentale Linn (cashew) a member of the family Anacardiaceae is a tropical tree indigenous to Brazil, which is now extensively cultivated in India and east Africa. India is the largest producer of cashew

nut, accounting for almost 50% of world exports. The tree also yields the so-called cashew apple to which the nut is attached. This is very juicy, fibrous and also edible.

Compared with the nut however, cashew apple (and the fibre it contains) is almost completely neglected in commercial terms. There is considerable potential for its exploitation. A number of processes have been developed for converting cashew apple into various products, such as juice, jams, syrups and various beverages. The peduncle (pseudofruit) contributes to human nutrition by supplying vitamin C, averaging 200 mg/100 g of juice, four times higher than that of orange juice (Menezes and Alves, 1995).

Cashew nut shell liquid (CNSL) is an important agricultural by-product of cashew nut production. The potential annual availability of this material, which accounts for about 32% of the shell, is enormous. Industrial application of CNSL-based products are numerous, including brake linings, paints and primers, foundry chemicals, lacquers, cements, speciality coatings (Menon et al., 1985) and transformed cardanol for gasoline stabilization (Castro Dantas et al., 2003).

The biologic activities of CNSL components have attracted considerable attention, in the areas of, for example molluscicidal activity (Kubo et al., 1986), anti-tumour activity (Itokawa et al., 1987; Itokawa et al., 1989; Kubo et al., 1993a,b), antimicrobial activity (Himejima and Kubo, 1991; Kubo et al., 1993a,b; Muroi and Kubo, 1993), inhibition of α -glucosidase, invertase and aldose reductase (Toyomizu et al., 1993), inhibition of tyrosinase (Kubo et al., 1994), uncoupling effects on liver mitochondria (Toyomizu et al., 2000), antioxidant activity (Amorati et al., 2001) and xanthine oxidase inhibition (Masuoka and Kubo, 2004). Cashew apple and cajuina juice are also reported to possess mutagenic, antioxidant potential and antimutagenic activity (Cavalcante et al., 2003).

The class of compounds present in CNSL are also present in *Ginkgo* extracts; in the former they are called anacardic acids and in the latter ginkgolic acids but both classes are 6-alkylsalicylic acids or 2-hydroxy-6-alkylbenzoic acids. The former are reported to be anti-tumour agents (Itokawa et al., 1987; Itokawa et al., 1989; Kubo et al., 1993a,b) and non-carcinogens but the latter are reported to be cytotoxic agents (Siegers, 1999; Westendorf and Regan, 2000; Hecker et al., 2002). However it should be noted that there is no solid proof of a strong allergic reaction to these alkylphenols when taken orally. For instance, reports have not been filed on adverse health effects of *Ginkgo* homeopathic mother tinctures (Woerdenbag and van Beek, 1997; Woerdenbag and de Smet, 2000). despite the fact that such extracts contain 2.2% (22,000 ppm) of ginkgolic acids (Siegers, 1999) and they have been consumed by many people over decades. Nevertheless for some reason

these compounds are regarded as suspect, and the larger manufacturers limit the total alkylphenol concentration in the final standardised commercial extract to between 5 and 10 ppm.

In this study the content of anacardic acids, cardanols and cardols in cashew apple, fibre, nut (raw and roasted) and CNSL were analysed. The anacardic acids, cardols and cardanols were fractionated as classes, and the individual alkyl phenols purified to homogeneity, for measurement of their antioxidant capacity and ability to inhibit xanthine oxidase in comparison to other known antioxidants such as 1-(+)-acetoxypinoresinol, caffeic acid, dimethyl sulphoxide, hydroxytyrosol, salicylic acid, trolox, and tyrosol.

2. Materials and methods

2.1. Reagents

Acetic acid, 2-deoxyguanosine, dimethyl sulfoxide, ethylenediaminetetraacetic acid, hypoxanthine, methanol, xanthine, and xanthine oxidase were obtained from Merck (Darmstadt, Germany). K_2HPO_4 and KH_2PO_4 were obtained from Serva (Heidelberg, Germany). 2, 3-Dihydroxybenzoic acid (2,3-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA), salicylic acid, trolox, and $FeCl_3 \cdot 6H_2O$ were obtained from Aldrich Chemie (Steinheim, Germany). Cinnamic acid, *o*-, *m*- and *p*-coumaric acids were obtained from Fluka Chemie (Buchs, Switzerland). Hydroxytyrosol was synthesized and purified as described by Owen et al. (2000c) whereas tyrosol and 1-(+)-acetoxypinoresinol were isolated and purified to homogeneity from extravirgin olive oil (Owen et al., 2000b). All solutions were made up in double distilled water.

2.2. Cashew products studied (sources)

Cashew apple, cashew nuts (raw and roasted), cashew fiber and CNSL [kind gifts from Dr. Debora Garrutti (Embrapa, Brasilia, Brasil) and Jaime Thomas de Aquino (Cione, Fortaleza, Brasil)] were studied to quantitate the phenolic substances by analytical and spectroscopic analyses and for assessment of antioxidant capacity.

2.3. Cashew product extraction

Cashew nuts, cashew apple and cashew fiber (10 g) were extracted twice (3 h) with hexane in a Soxhlet apparatus to remove lipid. After drying, they were further extracted (3 \times 3 h) with methanol. Cashew apples were lyophilised prior to extraction. Cashew nut shell liquid (30 g) was obtained by heating (175 °C) the fruit (100 g) in an oven for 45 min.

2.4. Isolation of phenolic antioxidants from CNSL

The purification of anacardic acids, cardanols and cardols from CNSL (10 g) was conducted as described by Paramashivappa et al. (2001). Briefly anacardic acids were isolated as their calcium salts, the acid-free CNSL was treated with ammonia and extracted with hexane/ethyl acetate (98:2) to separate the mono-phenolic cardanol components, followed by ethyl acetate/hexane (80:20) to yield the cardols.

2.5. High performance liquid chromatography (HPLC)

Analytical HPLC was conducted on a Hewlett-Packard (HP) 1090 liquid chromatograph fitted with a C-18, reversed phase (5 μm) column (25 cm \times 4 mm ID; Latak, Eppelheim, Germany). The mobile phase used was 2% acetic acid in double distilled water (solvent A) and methanol (solvent B), utilizing the following gradient over a total run time of 40 min: initially 50:50 solvents A and B followed by an increase in solvent B to 100% in 20 min, held isocratically for a further 20 min at a flow rate of 1.0 ml/min. Phenolic compounds in the eluent were detected with a UV-diode-array detector (HP 1040 M) set at 250, 278 and 315 nm. The amount of phenolic compounds in the extracts and alkyl phenol fractions was determined using calibration curves, generated with authentic standards in duplicate, by measuring the UV absorption at λ_{max} as a function of concentration in the range 0.025–4.0 mM. When authentic standards were unavailable, sufficient quantities of the compounds were isolated and purified to allow the preparation of standard curves in the same range as above.

For detection of the products of ROS interaction with salicylic and cinnamic acid in the hypoxanthine/xanthine oxidase assay the mobile phase described by Owen et al. (2000b) was used with the DAD detector set at 278 and 325 nm to detect the products, 2,5-dihydroxybenzoic acid and 2,3-dihydroxybenzoic acid and *o*-, *m*- and *p*-coumaric acids of reactive oxygen species (ROS) attack on both substrates, respectively. After incubation 20 μl of the reaction mixtures were injected into the HPLC. The flow rate of the mobile phase was 1 ml/min with a total run time of 45 min. The amount of phenolic compounds in the assay mixtures was again determined using calibration curves, generated with authentic standards in duplicate, by measuring the UV absorption at λ_{max} as a function of concentration in the range 0.025–4.0 mM. Instrument control and data handling were performed with the HP Chemstation software on a PC.

2.6. Semi-preparative HPLC and fraction collection

Semi-preparative HPLC was conducted on an Agilent 1100 liquid chromatograph fitted with a similar

C18 column (10 mm ID). For separation of individual compounds in extracts of cashew products plus the anacardic acid, cardanol and cardol fractions, the mobile phase consisted of acetonitrile–water–acetic acid (80:20:1) run in the isocratic mode. (Shobha and Ravindranath (1991)) at a flow rate of 3.0 ml/min. The alkyl phenols in the eluate were detected with a UV diode-array detector (HP 1040 M) set at 278 and 315 nm. Peaks eluting from the column were collected on an Agilent 220 Microplate Sampler and subsequently freeze-dried.

2.7. Gas chromatography–mass spectrometry (GC–MS)

Analyses were performed exactly as described by Owen et al. (2000a,b). Prior to GC–MS, dried methanolic extracts (40 $\mu\text{g/ml}$) were derivatized by addition of 100 μl of BSTFA (bis(trimethylsilyl)trifluoroacetamide) at 30 $^{\circ}\text{C}$ for 30 min.

2.8. Electrospray ionisation mass spectrometry (ESI–MS)

Samples purified by semi-preparative HPLC were dissolved in methanol, and spectra were recorded on a Finnigan MAT TSQ 7000 triple-quadrupole mass spectrometer (Finnigan, San Jose, California, USA) equipped with a nanoelectrospray source (EMBL, Heidelberg, Germany), using both the positive- and negative-ion modes. Argon was used as collision gas at a nominal pressure of 2.5 mTorr (1 Torr = 133.3 Pa). Gold-plated glass capillaries for sample spraying were prepared in-house using a microcapillary puller (Type-87-B, Sutter Instruments). The applied voltage was 400–700 V, and the mass scan range was 20–2600 D.

2.9. Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance (NMR) spectra were recorded in CD_3OD solvent on a Bruker AC-250 (Bruker Analytik, Rheinstetten, Germany) at a ^1H frequency of 250.133 MHz and ^{13}C frequency of 62.896 MHz, using conventional 1D Fourier transform techniques ($^1\text{D } ^1\text{H}$, ^{13}C with broadband ^1H decoupling, ^{13}C DEPT) for all compounds. Compounds (ca. 10–50 mg) were dissolved in 0.4 ml CD_3OD (99.96% D) in 5-mm diameter sample tubes. The following conventional measurements were conducted, namely 1D ^1H and ^{13}C (broadband decoupled and DEPT-135).

2.10. Hypoxanthine/xanthine oxidase assay

2.10.1. Salicylic acid model

The assay used to determine the antioxidant potential of cashew extracts and the individual purified phenolic

compounds was based on the methods of Owen et al. (2000b, 2003). Phenolics I–VIII were dissolved in the assay buffer (1.0 ml) and tested in the concentration range of 0–4 mM. A 10- μ l aliquot of xanthine oxidase (18 mu) in 3.2 M NH_4SO_4 was added to initiate the reactions. The sample tubes were incubated for 3 h at 37 °C, at which time the reaction was complete. A 20 μ l portion of the reaction mixture was analysed by HPLC using the mobile phase and conditions described by Owen et al. (2000b, 2003).

Samples for determination of the antioxidant capacity of alkyl phenol classes were prepared by placing 0–500 μ l of methanol solutions (10.0 mg/ml) in 15 ml centrifuge tubes (in duplicate) and removing the solvent under a stream of nitrogen. Dried sample material was suspended in the assay buffer (1.0 ml) prior to addition of xanthine oxidase.

The extent of dihydroxyphenol (2,5-dihydroxybenzoic acid and 2,3-dihydroxybenzoic acid) produced by hydroxyl radical (HO^\bullet) attack on salicylic acid was determined from standard curves of the respective phenols.

2.10.2. Cinnamic acid model

To assess the antioxidant capacity of salicylic acid, experiments were conducted exactly as described above with cinnamic acid (2 mM) as aromatic probe. The products of hydroxyl radical attack (*o*-, *m*-, and *p*-coumaric acid) on this phenolic acid were detected at 278 nm and quantitated against their respective standard curves at the same wavelength.

2.11. Assay of superoxide anion generated by xanthine oxidase

Superoxide anion was generated enzymatically in a xanthine oxidase system. The reaction mixture consisted of 2.7 ml of sodium carbonate buffer (40 mM) containing 0.1 mM EDTA (pH 10.0), 0.06 ml of 10 mM xanthine, 0.06 ml of sample solution (dissolved in DMSO), 0.03 ml of 0.5% bovine serum albumin and 0.03 ml of nitroblue tetrazolium. To the mixture at 25 °C, 0.12 ml of xanthine oxidase (0.04 units) was added, and the absorbance at 560 nm recorded for 90 s (formation of blue formazan). Control experiments were carried out by replacing sample solutions with the same amount of DMSO. The rate of superoxide anion generation was calculated from the proportional increase in absorbance.

2.12. Assay of uric acid generated by xanthine oxidase

The reaction mixture consisted of 2.76 ml of 40 mM sodium carbonate buffer containing 0.1 mM EDTA (pH, 10.0), 0.06 ml of 10 mM xanthine and 0.06 ml of sample dissolved in DMSO. The reaction was started by addition of 0.12 ml xanthine oxidase (0.04 units)

and the absorbance at 293 nm was recorded for 90 s. Rate of uric acid formation was calculated from the proportional increase in absorbance.

2.13. 2-Deoxyguanosine assay

The buffer system described for the HX/XO system was used with omission of hypoxanthine and replacement of salicylic acid by 2-deoxyguanosine (2 mM). The generation of ROS was initiated by the addition of ascorbic acid (500 μ M). The reaction tubes were incubated for 24 h at 37 °C, and 20 μ l of the reaction mixture was analyzed by HPLC as described above. The amount of 8-oxo-2-deoxyguanosine produced by the reaction of ROS with 2-deoxyguanosine was determined from a standard curve of the reaction product (Owen et al., 2003).

2.14. Statistics

For the five antioxidant assays described, the volume of phenolic extracts or the concentration of purified phenolic compounds, producing a 50% inhibition of oxidation (IC_{50}) were determined using the table curve program (Jandel Scientific, Chicago, IL).

3. Results

3.1. Content of alkyl phenols in cashew products

The hexane and methanol Soxhlet extracts of the various cashew products differed considerably in the content of alkyl phenols as judged by comparison with external standard curves. By far the higher amount (353.6 g/kg) of the major alkyl phenols, anacardic acids were detected in CNSL, while the lower amount (0.65 mg/kg) was detected in roasted cashew nuts (Table 1). Cashew apple and fibre contained anacardic acids exclusively, whereas cashew nuts (raw and roasted) contained both anacardic acids, and cardols. Cashew nut shell liquid contained an abundance of anacardic acids, cardols and cardanols and was therefore used for a basic fractionation of the alkyl phenol classes as described in Section 2. Analytical HPLC of these classes is shown in Fig. 1. The individual anacardic acids, major cardanols and cardols (Fig. 2) were purified to homogeneity from these fractions by semi-preparative HPLC and definitively identified by nano-ESI-MS, (Table 2), GC-MS (Table 3). NMR analyses (data not shown) were entirely consistent with the data of Van Beek and Wintermans (2001).

3.2. Antioxidant capacity of cashew product extracts

Cashew nut shell liquid, and hexane extracts of the other cashew products tested, displayed significant

Table 1
The amount (g/kg) of alkyl phenols detected in the various cashew products

Alkyl phenol	g/kg			
	CNSL	Fibre	Nut	Apple
Anacardic acid-1	153.50	1.81	(0.58), (0.35) ^a	0.22
Anacardic acid-2	107.96	1.81	(0.20), (0.12) ^a	0.32
Anacardic acid-3	92.12	2.49	(0.28), (0.18) ^a	0.56
Cardanol-1	97.61	n.d.	n.d.	n.d.
Cardanol-2	65.37	n.d.	n.d.	n.d.
Cardanol-3	55.31	n.d.	n.d.	n.d.
Cardol-1	98.18	n.d.	(0.22), (0.11) ^a	n.d.
Cardol-2	46.65	n.d.	(0.07), (0.05) ^a	n.d.
Total	716.70	6.10	(1.06), (0.64) ^a	1.10
Cashew fruits ^b	215.01			

n.d. = not detected.

^a Cashew nut (roasted).

^b Calculated from the CNSL yield (30.0 ml) after extraction of raw cashew fruits (100 g).

antioxidant capacity (Table 4). The more efficient being CNSL (100%) followed closely by the hexane extract of

cashew fibre which caused 94% inhibition. The antioxidant capacity correlated significantly ($P < 0.05$) with the concentration of alkyl phenols in the extracts.

To establish which class of alkyl phenols were responsible, selective isolation of the anacardic acids, cardanol and cardol fractions from CNSL was conducted as described in Section 2.

As depicted in Fig. 3 a mixture of anacardic acids (10.0 mg/ml) showed by far the higher (Table 5) antioxidant capacity ($IC_{50} = 0.6$ mM) compared to cardols and cardanols ($IC_{50} > 4.0$ mM) in terms of inhibition of ROS attack on salicylic acid and also with respect to inhibition of xanthine oxidase (anacardic acids, $IC_{50} = 0.35$ mM; cardols and cardanols, $IC_{50} > 4.0$ mM). The inhibition profile of xanthine oxidase by anacardic acids is depicted in Fig. 4.

The purified major anacardic acids (1–3) along with cardanol-1 and cardol-1 were subjected to similar antioxidant assays. The data shows (Table 6) that of the anacardic acids, anacardic acid-1 ($IC_{50} = 0.27$ mM) displayed (Fig. 5) a much stronger antioxidant capacity

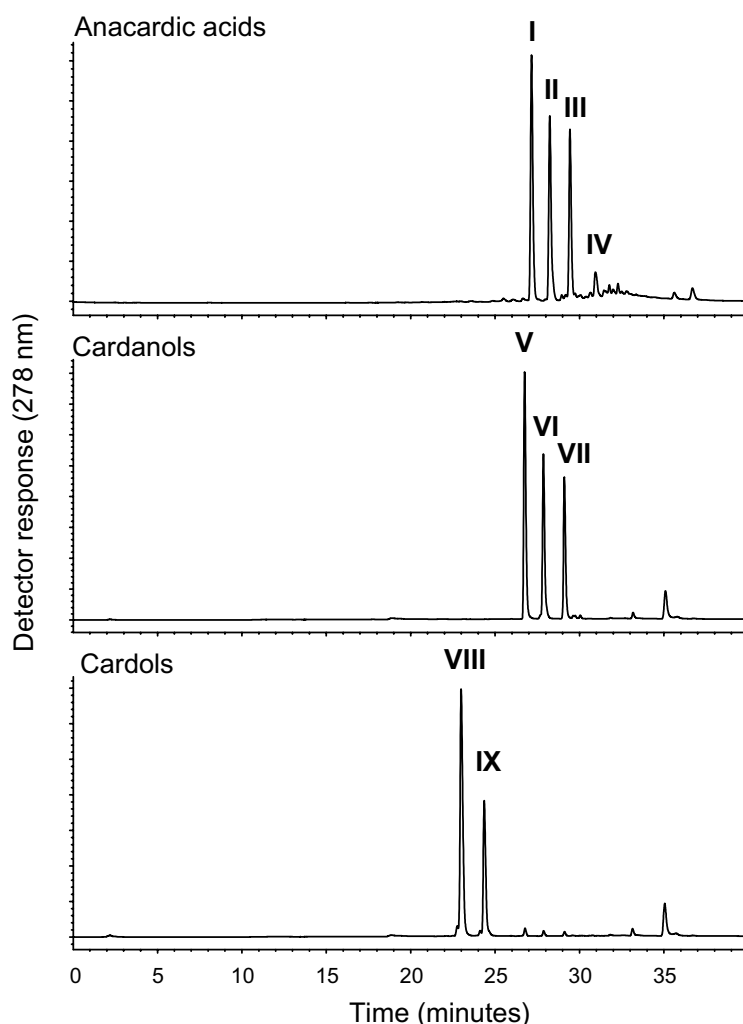
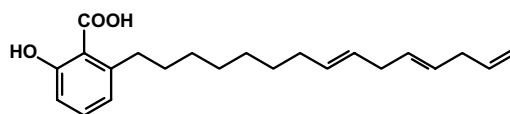
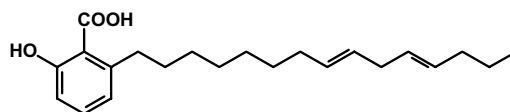


Fig. 1. Analytical HPLC chromatograms at 278 nm of the anacardic acid, cardanol and cardol fractions isolated from CNSL.

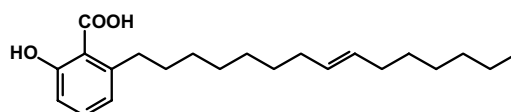
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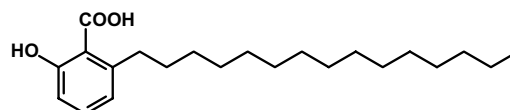
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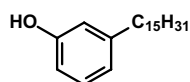
Anacardic acid-3 (III)



Anacardic acid-4 (IV)



Cardanols (V-VII)



Cardols (VIII-IX)

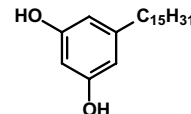


Fig. 2. Structures of the anacardic acids, cardanols and cardols isolated from CNSL.

Table 2

Nano-ESI-MS data for individual alkyl phenols, purified to homogeneity from cashew nut shell liquid by semi-preparative HPLC

Alkyl phenol	<i>m/z</i>				
	Exact mass (calc.)	$[M - H]^-$	$[2M - H]^-$	$[M + H]^+$	$[M + Na]^+$
Anacardic acid-1	342.219	341.1	683.4	343.0	365.0
Anacardic acid-2	344.235	343.1	687.4	345.0	367.0
Anacardic acid-3	346.251	345.1	691.5	347.1	369.0
Anacardic acid-4	348.266	347.1	695.5	n.d.	371.0
Cardanol-1	298.230	297.1	595.5	n.d.	321.2
Cardanol-2	300.245	299.4	599.5	301.3	323.3
Cardanol-3	302.261	301.3	603.5	n.d.	325.3
Cardanol-4	304.277	303.3	607.5	305.1	n.d.
Cardol-1 ^a	314.225	313.1	627.4	315.0	337.0
Cardol-2 ^b	316.240	315.3	631.4	317.3	339.3

n.d. = not detected.

^a $[3M - H]^- = 941.5$.^b $[3M - H]^- = 947.5$.

compared to anacardic acids-2 ($IC_{50} = 0.56$ mM) and -3 ($IC_{50} = 0.77$ mM) and while not such a clear relation existed, anacardic acid-1 ($IC_{50} = 0.19$ mM) inhibited xanthine oxidase to a greater extent than anacardic

acids-2 and -3 (Fig. 6). The antioxidant capacity of anacardic acids 1–3 was far greater than a range of phenolic compounds tested for comparison (Table 7). Furthermore anacardic acid-1 was by far the more

Table 3
GC–MS data for individual alkyl phenols purified to homogeneity, from cashew nut shell liquid by semi-preparative HPLC

	Alkyl phenol	TMS groups	M ⁺⁺ (calc.)	m/z (rel. intensity, %)	
				M ⁺⁺	Major fragment ions ^a
I	Anacardic acid-1	2	486	486 (3)	471 (100), 396 (46), 219 (68), 147 (44)
II	Anacardic acid-2	2	488	488 (8)	473 (100), 398 (52), 219 (58), 147 (39)
III	Anacardic acid-3	2	490	490 (7)	475 (100), 400 (9), 219 (35), 147 (16)
IV	Anacardic acid-4	2	492	492 (1)	477 (100), 402 (1), 219 (19), 147 (6)
V	Cardanol-1	1	370	370 (26)	355 (4), 180 (100), 179 (65)
VI	Cardanol-2	1	372	372 (34)	357 (4), 180 (100), 179 (39)
VII	Cardanol-3	1	374	374 (42)	359 (2), 180 (100), 179 (22)
VIII	Cardanol-4	1	376	376 (77)	361 (5), 180 (100), 179 (25)
IX	Cardol-1	2	458	458 (12)	443 (4), 281 (15), 268 (100)
X	Cardol-2	2	460	460 (19)	445 (5), 281 (16), 268 (100)

^a Base peak in bold face.

Table 4
Antioxidant capacity of hexane extracts (10 g) of cashew apple, fiber, nut (raw and roasted), and cashew nut shell liquid (CNSL) as determined in the hypoxanthine/xanthine oxidase assay

Extract (10 mg/ml in assay buffer)	Inhibition ^a (%)
Cashew nut shell liquid (CNSL)	100
Cashew fiber	94
Cashew apple	53
Cashew nut (raw)	41
Cashew nut (roasted)	37

^a Inhibition of ROS attack on salicylic acid.

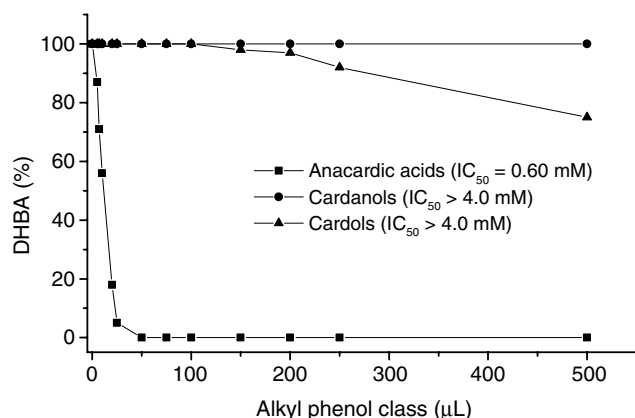


Fig. 3. Inhibition of reactive oxygen species attack on salicylic acid by the anacardic acid, cardanol and cardol fractions isolated from CNSL, in the hypoxanthine/xanthine oxidase HPLC-based assay.

Table 5
Antioxidant capacity of anacardic acid, cardanol and cardol mixtures in the hypoxanthine/xanthine oxidase assay

Alkyl phenols	IC ₅₀ (mM)	
	DHBA (4 mM _{inh} %)	Xanthine oxidase (4 mM _{inh} %)
Anacardic acids	0.60 (100)	0.35 (100)
Cardols	>4.0 (22)	>4.0 (26)
Cardanols	>4.0 (0)	>4.0 (0)

Inh % = at alkyl phenol concentration (4 mM).

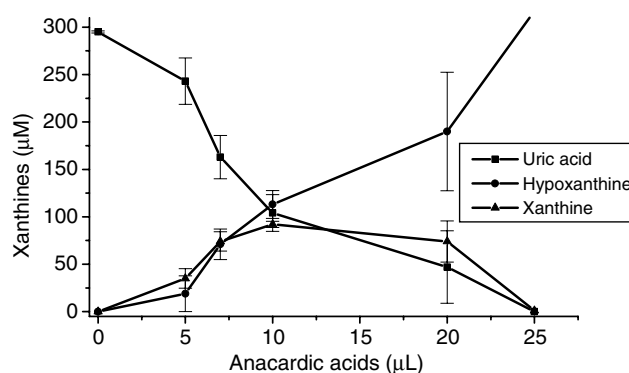


Fig. 4. Inhibition of xanthine oxidase by the anacardic acid, cardanol and cardol fractions isolated from CNSL, in the hypoxanthine/xanthine oxidase HPLC-based assay.

Table 6
Antioxidant capacity of alkyl phenols, purified to homogeneity by semi-preparative HPLC in the hypoxanthine/xanthine oxidase assay

Alkyl phenol	IC ₅₀ (mM)	
	DHBA (4 mM _{inh} %)	Xanthine oxidase (4 mM _{inh} %)
Anacardic acid-1	0.27 (100)	0.19 (100)
Anacardic acid-2	0.56 (95)	0.38 (100)
Anacardic acid-3	0.77 (93)	0.36 (99)
Cardanol-1	>4.0	>4.0
Cardol-1	1.71 (83)	0.97 (95)

Inh % = at alkyl phenol concentration (4 mM).

potent antioxidant (IC₅₀ = 0.27 mM) compared to both cardol-1 (IC₅₀ = 1.71 mM) and cardanol-1 (IC₅₀ > 4.0 mM). The antioxidant capacity of anacardic acid-1, appeared to be much more related to inhibition of xanthine oxidase (IC₅₀ = 0.19 mM) than to scavenging of hydroxyl radicals which was confirmed by the 2-deoxyguanosine assay (IC₅₀ > 4.0 mM). Therefore identical experiments to those described by Masuoka and Kubo (2004) were conducted. The data (Fig. 7) shows that anacardic acid-1, inhibits both generation of superoxide (IC₅₀ = 36 μM) and xanthine oxidase (IC₅₀ = 301 μM) in these assay systems. Inhibition of superoxide and

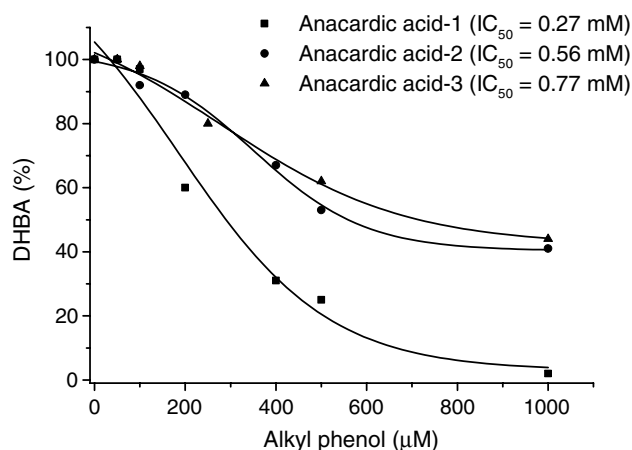


Fig. 5. Inhibition of reactive oxygen species attack on salicylic acid by anacardic acid-1, cardanol-1 and cardol-1 isolated from CNSL, in the hypoxanthine/xanthine oxidase HPLC-based assay.

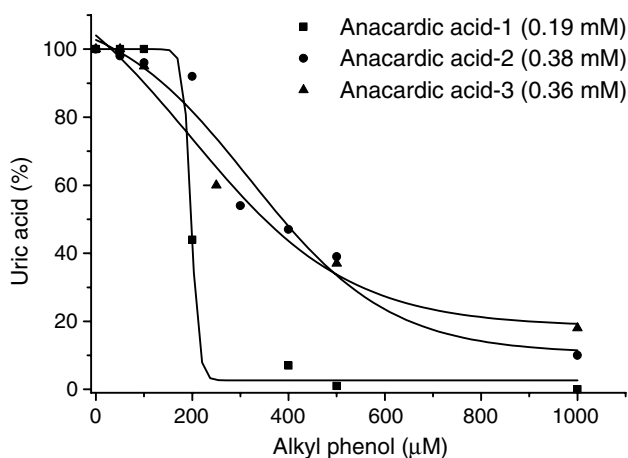


Fig. 6. Inhibition of xanthine oxidase by anacardic acid-1, cardanol-1 and cardol-1 isolated from CNSL, in the hypoxanthine/xanthine oxidase HPLC-based assay.

Table 7

Antioxidant capacity of anacardic acids in the hypoxanthine/xanthine oxidase assay compared to other known antioxidants

Compound	IC ₅₀ (mM)
Anacardic acid-1	0.27
Anacardic acid-2	0.56
Anacardic acid-3	0.77
1-(+)-Acetoxypinoresinol	0.90
Hydroxytyrosol	1.34
Dimethylsulphoxide	2.30
Tyrosol	2.51
Salicylic acid ^a	4.07
Caffeic acid	6.05
Trolox	12.24

^a Evaluated in the hypoxanthine/xanthine oxidase cinnamic acid model.

xanthine oxidase was hyperbolic and sigmoidal, respectively indicating that the former is more efficient than the latter.

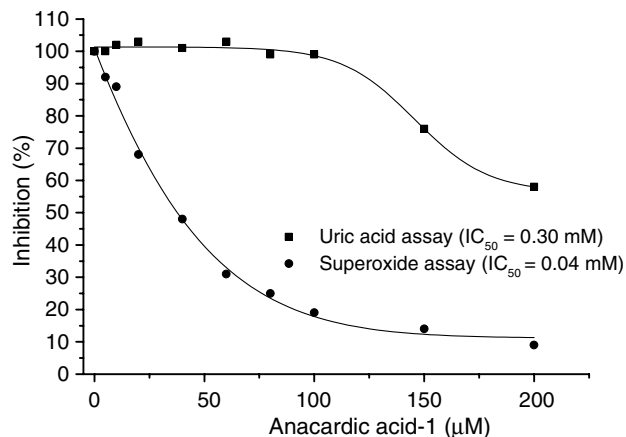


Fig. 7. Inhibition of superoxide generation and xanthine oxidase activity by anacardic acid-1 in the superoxide and uric acid assays.

4. Discussion

The results of this study show that CNSL extracted from cashew nuts by heating contains a range of alkyl phenols. These consist of anacardic acids, cardanols and cardols. Cashew nut shell liquid, as a crude mixture appeared to be a potent scavenger of reactive oxygen species, and, inhibitor of xanthine oxidase. Selective fractionation of the major alkyl phenols within CNSL revealed that the active principles are anacardic acids and to a much lesser extent cardols. Of the former, anacardic acid-1 containing three double bonds in the alkyl side-chain, confers greater antioxidant and enzyme inhibition capacity than the other acids possessing 1–2 double bonds. Amelioration of ROS attack on salicylic acid by anacardic acids in the hypoxanthine/xanthine oxidase assay is predominantly a function of suppression of superoxide generation and xanthine oxidase inhibition, rather than scavenging of reactive oxygen species as confirmed by the 2-deoxyguanosine assay and supports the data of Masuoka and Kubo (2004). Although anacardic acids share the same phenolic ring system as salicylic acid, the presence of a phytyl side-chain confers far greater antioxidant capacity on the former than the latter. The reason for this is that, salicylic acid acts only as a scavenger of ROS, whereas anacardic acids potentially inhibit generation of superoxide anion and uric acid by xanthine oxidase as described by Masuoka and Kubo (2004). This also appears to be the reason for their greater antioxidant capacity compared to a range of other known antioxidants (Table 7). Because anacardic acids possess a phytyl side-chain, it would be of interest to evaluate whether or not these compounds can be incorporated into cellular membranes in a similar manner to Vitamin E. Such a phenomenon is currently under study in our laboratories.

Comparison of the effects of CNSL with hexane extracts of other cashew products revealed that all show

antioxidant capacity to various extents. This correlated significantly with the absolute amount of alkyl phenols, in particular, the concentration of anacardic acids. In this regard, extracts of cashew fibre, which is a waste product (from cashew apple) in the commercial production of cashew nuts, containing a high content of anacardic acids, shows impressive potential cancer chemopreventive activities.

Support for this comes from the study of Cavalcante et al. (2003) who demonstrated that fresh (CAJ) compared to processed cashew apple juice (cajuina) has higher antioxidant capacities, as measured by protection of DNA damage induced by ROS, and this correlates with the anacardic content of CAJ (17.9 mg/100 g) and cajuina (0.41 mg/100 g) which is in agreement with other reports (Itokawa et al., 1987; Kubo et al., 1993a,b).

Anacardic acids are also known to inhibit enzymes such as prostaglandin synthase (Grazzini et al., 1991), tyrosinase (Kubo et al., 1994) and lipoxygenase (Shobha et al., 1994). They also strongly inhibit digestive enzymes such as α -glucosidase, invertase and aldose reductase, whereas the structurally related salicylic acid lacking an alkyl phenyl side-chain, is a very weak inhibitor (Toyomizu et al., 1993). Furthermore salicylic acid and cardanols at a much higher concentration than anacardic acids are required for mitochondrial uncoupling activity (Toyomizu et al., 2000).

Cancer epidemiologic data for the colorectum throughout Brazil shows an increase of 33.1% between 1979 (3.44/100,000) and 2000 (4.59/100,000) but currently the rate is far lower in the northeast (2.7–3.4/100,000) compared to the south (13.3–21/100,000) and a probable reason for this is the far higher consumption of fruits and vegetables, especially cashew in the northeast, compared to the south, where a high-fat western type diet is consumed.

A substantial amount of cashew apple and heat damaged and broken kernel (after processing) are mostly used in formulations of animal or poultry feeds. The data presented in this study indicate that these waste products which contain high contents of anacardic acids with high antioxidant capacity, could be better utilized in functional food formulations (Cione, 2000) and may represent a cheap source of cancer chemopreventive agents.

Because there is no available data on the variability of alkyl phenol content in cashew products with regard to season, climate, cultivar and age of fruit the effect of these are currently being studied in our laboratory.

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