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Research Article

Cashew (*Anacardium occidentale)* apple juice lowers mutagenicity of aflatoxin B1 in *S. typhimurium* TA102

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Abstract

Cashew (*Anacardium occidentale*) is a medicinal plant native to Brazil and also yields a nutritious fruit juice. Its large pulpy pseudo-fruit, referred to as the cashew apple, contains high concentrations of vitamin C, carotenoids, phenolic compounds and minerals. Natural and processed cashew apple juice (CAJ/cajuina) are amongst the most popular juices in Brazil, especially in the north-east. Both juices have antioxidant potential and suppress mutagenicity of hydrogen peroxide. In the present study we evaluated the inhibitory effects of CAJ/cajuina on Aflatoxin B1(AFB_1)–induced mutation, using the *Salmonella/*microsome assay with the experimental approaches of pre-, co- and post-treatments. Both CAJ/cajuina suppress AFB_1-induced mutagenesis in strain TA102 when applied in co- and in post-treatment. Possible mechanisms for anti-mutagenicity in co-treatment are (a) interaction with S9 enzymes, (b) metabolization to non-mutagenic compounds of AFB_1 or (c) inactivation of S9 potential. Total suppression of AFB_1 mutagenicity was observed in co-treatment with both CAJ and cajuina. Post-treatment anti-mutagenicity of both juices suggests a modulation of activity of error-prone DNA repair. CAJ/cajuina may be considered promising candidates for control of genotoxicity of AFB_1 and may thus be considered as health foods with anti-carcinogenic potential. This promising characteristic warrants further evaluation with *in vivo* studies.

Key words: cashew apple juice, cajuina, anti-mutagenicity.

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Introduction

Cashew apple, the pseudofruit of the cashew tree (*Anacardium occidentale*), is widely consumed in the northeast of Brazil. It is regularly drunk as fresh cashew apple juice (CAJ) or as processed juice (cajuina). Studies have shown CAJ to have anti-bacterial, anti-fungal and anti-tumor activities (Kubo *et al.*, 1993a; 1993b; Kozubek *et al.*, 2001) as well as anti-oxidant effects (Melo Cavalcante *et al.*, 2003) and anti-mutagenic activity (Santos *et al.*, 2002; Melo-Cavalcante *et al.*, 2003). As fruit juices CAJ/cajuina are complex mixtures, containing high concentrations of vitamin C, various carotenoids, phenolics (quercetin, anacardic acid, tannin) and metals as biologically active compounds (Melo-Cavalcante *et al.*, 2003). A large number of epidemiological studies have shown the protective effects of vegetables and fruits against cancer; this is attributed to the fact that they contain anti-mutagens as well as anti-carcinogens (Ames, 2001; Paolini and Nestle, 2003; Edenharder *et al.*, 2003).

Chemoprevention is a promising additional method to environmental control for reducing human exposure to environmental and dietary carcinogens (Ames, 2001; De Flora *et al.*, 2003; Park *et al.*, 2003). Anti-mutagens and anti-carcinogens are common components in many traditional herbal remedies and dietary therapies (Zeiger, 2003; Aruoma, 2003; Surch and Ferguson, 2003). Aflatoxin B1 (AFB1) is a secondary metabolite of the fungus *Aspergillus flavus* (Groopman *et al.*, 1991). Epidemiological studies have shown strong correlation between hepatocarcinoma and exposure to AFB_1 (Sotomayor *et al.*, 1999; Karekar *et al.*, 2000). AFB_1 is activated to AFB1-8,9-epoxide by the cytochrome P450 mono-oxygenase system. This metabolite binds covalently to DNA, RNA, and proteins (Groopman *et al.*, 1991; Sotomayor *et al.*, 1999).

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In our present study we report the inhibitory effects of CAJ/cajuina on the mutagenic activity of AFB1 in *Salmonella/*microsome assay, using different pre-, co- and post-treatment approaches.

Materials and Methods

Preparation of juice from *Anacardium occidentale*

To produce fresh CAJ, cashew fruits, obtained from the State of Piauí, Brazil, were washed and sterilized by soaking them for about 5 s in 70% ethanol and subsequent flaming. The cashew apples were then macerated and the juice sieved using sterile equipment. An aliquot was tested for absence of microorganisms and the juice samples were frozen at -20 °C. Cajuina was derived from CAJ by centrifugation of the macerated fruits, clarification with gelatin, filtration and thermal treatment (1 h at 100 °C), according to the manufacturer`s information (Lili Doces, Teresina, PI, Brazil). The chemical compounds identified in CAJ/cajuina (Melo Cavalcante *et al.*, 2003) are given in Table 1.

Chemicals

Aflatoxin B_1 (AFB_1) was dissolved in dimethylsulfoxide (DMSO), both of which were purchased from Sigma (St. Louis, MO, USA).

Strain

Salmonella typhimurium strain TA102 (*his G428*, *rfa*, pKM101, PAQI), as described by Maron and Ames (1983) and Mortelmans and Zeiger (2000), was used for mutagenicity assay. The test strain was kindly supplied by Dr. B.N. Ames, University of California, Berkeley, U.S.A.

Microsomal fraction

The post-microsomal S9 fraction, prepared from livers of Sprague-Dawley rats treated with the polychlorinated biphenyl mixture Aroclor 1254, was purchased from Molecular Toxicology Inc. (Maltox^{TM}, Annapolis, Maryland, USA). The S9 metabolic activation mixture was prepared according to Maron and Ames (1983) and Mortelmans and Zeiger (2000).

Anti-mutagenicity analysis

Anti-mutagenicity of CAJ/cajuina against AFB1 was assessed using the standard plate incorporation assay as described by Maron and Ames (1983) and Mortelmans and Zeiger (2000), with the methodological variations described by Melo-Cavalcante *et al.* (2003). An overnight culture of TA102 was washed with 5 mL of 0.2 M phosphate buffered saline (PBS, pH 7.4). The dose of AFB1 was 10 μL/plate, a concentration that does not show toxicity when mixed with juices, while the doses of CAJ (10, 25 and 50 μL/plate) and cajuina (100, 500 and 2000 μL/plate) were selected in preliminary dose range-finding assays. The final criterion to select juice doses was their non-toxicity. We used the following controls: a) for AFB1; H_2O + AFB1+ bacteria + S9mix; b) for juice; H_2O + juice + bacteria ± S9mix; c) for S9mix; juice + bacteria + AFB1, with omission of S9 fractions and d) for bacteria; H_2O + bacteria + S9mix. Incubation was at 37 °C with continuous gentle shaking, followed by centrifugation at 3,000 rpm for 20 min (RT6000, Sorvall Instruments, DUPONT, USA). The anti-mutagenic evaluation was done by the following treatments: **pre-** (juice + bacteria in fresh nutrient broth (4 h), wash bacteria and add AFB1 + S9mix (20 min), wash bacteria and plate), **co-** (**A**- Bacteria + juice and AFB1 + S9mix (20 min), wash bacteria and plate. **B**- Juice + AFB1 (20 min) + S9mix (20 min), add to the bacteria and plate. **C**- AFB1 + S9mix (20 min), add juice (20 min), add bacteria and plate) and **post**- (**A**- Bacteria + AFB1 + S9mix (20 min), wash bacteria, add the juice and plate. **B-** Bacteria + AFB1 + S9mix (20 min), wash and incubate with juice in fresh broth (30 min), wash bacteria and plate. **C**- Bacteria + AFB1 + S9mix (20 min), wash and further incubate in fresh broth (3 0min), add juice and plate). Each sample was assayed in triplicate and data are presented as means ± SD of two independent assays. Anti-mutagenicity for each dose of CAJ/cajuina against AFB1 was calculated according to Melo-Cavalcante *et al.* (2003) as follows: percentage of inhibition (I%) = [1-(B/A)] x 100, where A represents the number of revertants/plate containing AFB1 and B represents the number of revertants/plate containing AFB1 and juices. The number of spontaneous revertants was subtracted from all plate counts. The anti-mutagenic effect of CAJ/cajuina, at non-toxic doses, was given as ID_{50}, the dose causing a 50% reduction of mutagenicity in the test system. Toxicity is indicated when a decrease > 70% in the number of *his*+ revertant colonies on plate with juice and AFB_1 in relation to the number of spontaneous revertants is observed, as well as in the absence of background lawn

Table 1 - Chemical components of CAJ and of cajuina.

Juices[a]	Total carotenoids mean ± SD[b]	Total phenols mean ± SD	Condensed tannin mean ± SD	Quercetin mean ± SD	Anacardic acid mean ± SD	Ascorbic acid mean ± SD
CAJ	0.32 ± 0.0**	11.9 ± 0.3**	61.1 ± 0.5**	0.23 ± 0.03	17.9 ± 0.4**	120.80 ± 4.1**
Cajuina	0.01 ± 0.0**	8.6 ± 0.4**	13.0 ± 4.0**	0.28 ± 0.03	0.41 ± 0.0**	1.56 ± 0.4**

[a]Concentrations expressed in mg/100g. [b]Mean value of at least three independent experiments ± SD (Melo- Cavalcante *et al.*, 2003). Statistical significance, one-way ANOVA Dunnett's Multiple Comparison Test. ** $p < 0.01$.

and/or complete absence of growth of pinpoint non-revertants, according to Mortelmans and Zeiger (2000) and Melo-Cavalcante *et al.* (2003). Co-mutagenic activities were considered to have occurred when the number of revertants on the plates with juices and AFB1 were significantly higher than those containing AFB_1 only.

Statistical analysis

Statistical significance was determined by One-Way Analysis of Variance (ANOVA) using the Statistical Package for Social Science (SPSS, Chicago, 1993). Dunnett's test was used to determine whether the means of the treatments differed significantly from the positive mutagenic control. The mean difference is significant at the level of 0.01 (**).

Results and Discussion

In preliminary studies using *S. typhimurium* strain TA102 we ascertained that neither CAJ nor cajuina, at a dose of 100 µL/plate, were non-mutagenic either with or without metabolic activation (Melo-Cavalcante *et al.*, 2003). As shown in Table 2, AFB1-induced mutagenesis was suppressed by CAJ/cajuina. Protective effects against AFB1-induced mutagenesis have already been described for juices of apricots, oranges, Brussels sprouts, carrots, yellow/red peppers, tomatoes (Rauscher *et al.*, 1998) and *doesang* (Korean fermented soypaste) extracts (Park *et al.*, 2003).

In pre-treatment, CAJ increased the mutagenicity of AFB1, suggesting a co-mutagenic effect. However, cajuina did not show any statistically significance for co-mutagenicity, but had a significant indication of toxicity at 2000 µL/plate. The lack of anti-mutagenic effect in pre-treatments with both juices (Table 2) could be attributed to the loss of anti-mutagenic substances of CAJ/cajuina during washing of the juice-treated bacteria with phosphate buffer (pH 7.4) and/or to the alteration of pH due the auto-oxidation of polyphenols that occurs mainly at pH values above neutrality (Rueff *et al.*, 1989). This could cause

Table 2 - Anti-mutagenic activity of CAJ/cajuina against AFB_1-induced mutagenesis in TA102 with metabolic activation.

Procedure	Dose[a]	CAJ *His*+ revertants/plate[c]	Inhibition %[d]	Dose[b]	Cajuina *his*+ revertants/plate[c]	Inhibition %[d]
Positive control[e]	-	714 ± 92	NT	-	752 ± 49	NT
Spont. Revert.	-	300 ± 23	NT	100	298 ± 27	NT
S9 mix control	10	358 ± 12	NT	100	366 ± 25	NT
	25	364 ± 17	NT	500	327 ± 25	NT
	50	406 ± 44	NT	2000	377 ± 17	NT
Pre-treatment	10	1420 ± 125[f] **	NT	100	875 ± 144	NT
	25	1108 ± 29 [f] **	NT	500	822 ± 119	NT
	50	1464 ± 130 [f] **	NT	2000	207 ± 17[g] **	NT
Co-treatment A	10	200 ± 20 [g] **	NT	100	120 ± 27[g] **	NT
	25	208 ± 12 [g] **	NT	500	115 ± 10[g] **	NT
	50	203 ± 10 [g] **	NT	2000	102 ± 21[g] **	NT
Co-treatment B	10	668 ± 26**	11	100	336 ± 28**	92
	25	506 ± 53**	50	500	478 ± 54**	60
	50	406 ± 13**	74	2000	500 ± 47**	56
Co-treatment C	10	CAG[h]	NT	100	243 ± 36	112
	25	322 ± 35**	95	500	354 ± 34**	88
	50	346 ± 15**	89	2000	344 ± 22**	90
Post-treatment A	10	342 ± 44**	90	100	154 ± 24 [g] **	NT
	25	341 ± 22**	90	500	258 ± 35 **	108
	50	CAG[h]	NT	2000	394 ± 9**	79
Post-treatment B	10	172 ± 8 [g] **	NT	100	143 ± 25[g]	NT
	25	350 ± 18**	88	500	180 ± 2 [f] **	NT
	50	330 ±12**	93	2000	411 ± 78**	75
Post-treatment C	10	218 ± 20 **	119	100	238 ± 8 **	113
	25	306 ± 30 **	99	500	343 ± 34**	90
	50	351 ± 21**	88	2000	377 ± 26**	83

Each experiment was repeated 2 times. [a]Dose of CAJ in µL/plate. [b]Dose of cajuina in µL/plate. [c]Mean of three plates. After 48 h of incubation the number of revertants was counted and percentage of inhibition was calculated according to Melo-Cavalcante *et al.* (2003). I% = [1-(B/A)] X 100, where A represents plates containing AFB_1 and B represents the plate containing AFB_1 and juice. [d]Percentage of inhibition. I% ≥ 50% was considered to show antimutagenicity. [e]Plates containing only AFB_1 (10 µL/plate). [f]Co-mutagenic activities were considered to have occurred when the number of revertants on the plates with juices and AFB1 were higher that than those containing AFB1only. [g]Samples of juice in presence of AFB1 with values of revertants colonies > 70% as compared with spontaneous revertants are considered indicative of toxicity (Mortelmans and Zeiger, 2000). [h]Complete absence of growth. NT (inhibition not detected). Statistical significance, one–way ANOVA followed by Dunnett's Multiple Comparison Test. ** p ≤ 0.01.

destruction of anti-mutagenic substances in CAJ/cajuina, *e.g.* condensed tannins, quercetin and other phenolic compounds (Melo-Cavalcante *et al.*, 2003).

However, many known anti-mutagenic chemicals of juices may also act as co-mutagens, *e.g.* vanillin and tannic acid. In many cases polyphenols are anti-mutagenic, depending on whether they are present before, during or after exposure to the relevant mutagen (Ferguson, 2001; Surch and Ferguson, 2003; Zeiger, 2003).

When CAJ/cajuina and strain TA102 were incubated with AFB_1 for 20 min at 37 °C with washing (co-treatment A), we observed a decrease in the number of his^+ revertants/plate below the number of spontaneous mutants of the negative control for both juices, indicating the toxic effects of this treatment (Table 2). However, CAJ/cajuina in preliminary tests did not indicate toxicity at the dose used and neither was this observed for the dose of AFB_1. Cashew apple juice has been shown to be cytotoxic and a potent anti-bacterial agent due the presence of anacardic acid (Kubo *et al.*, 1993b) and resorcinolic acid (Kozubek *et al.*, 2001). This could explain the toxicity observed in co-treatment A caused by the adverse effects of chemopreventive agents (Lee and Park, 2003). Although the toxicological effect of anacardic acid and resorcinolic acid has been investigated, the mechanisms of cytotoxic action are not yet clear.

Is known that under certain experimental conditions, many anti-oxidants can induce adverse effects, depending on their redox potential; accepting or donating electrons may render them either protective or toxic (De Flora, 1998; De Flora *et al.*, 2001). One proposed mechanism of action for the toxicity of anacardic acid and resorcinolic acid is their strong interaction with biological membranes. This interaction may be responsible for their anti-bacterial, fungicidic and cytotoxic activity (Kozubek *et al.*, 2001).

However, when CAJ/cajuina were co-incubated with AFB_1 for 20 min at 37 °C without washing before adding strain TA102 and plating (co-treatment B), anti-mutagenic activity was observed (Tables 2 and 3). Therefore, inhibition or competition for S9 enzymes seems to be the main anti-mutagenic mechanism of CAJ/cajuina, as already observed in studies on the anti-mutagenesis of *Phyllanthus orbicularis* extracts against aromatic amines (Ferrer *et al.*, 2001).

One possible mechanism of anti-mutagenesis is juice-AFB_1 metabolite interaction. This was suggested by the results of adding juices and strain TA102 and plating after co-incubation of AFB_1 with S9mix for 20 min at 37 °C (co-treatment C). A high anti-mutagenic effect was found, with about 95% inhibition of AFB_1-induced mutagenesis (Table 2). This suggests a possible anti-mutagenic mechanism of CAJ/cajuina whose function would be to interact with the mutagenic metabolites of AFB_I and transform them to non-mutagenic compounds. This anti-mutagenicity could be attributed to a large number of natural juice compounds (Table 1 and Table 3), *i.e.* carotenoids, phenols (quercetin and tannin), anacardic acid and ascorbic acid, all with anti-oxidant and anti-mutagenic properties (Melo-

Table 3 - Effects and possible active compounds of CAJ/cajuina against Aflatoxin B_1 in *Salmonella typhimurium* TA102.

Procedure	Effects of CAJ	Active compounds of CAJ	Effects of Cajuina	Active compounds of Cajuina
Pretreatment	Co-mutagenic	Ascorbic acid Phenols Condensed Tannins	Indicates toxicity	Phenols Condensed Tannins Anacardic acid
Co-treatment A	Indicates toxicity	Anacardic acid	Indicates toxicity	Anacardic acid
Co-treatment B	Anti-mutagenic	Carotenoids Ascorbic acid Phenols Condensed Tannins Quercetin	Anti-mutagenic	Phenols Condensed Tannins Quercetin
Co-treatment C	Anti-mutagenic	Carotenoids Ascorbic acid Phenols Condensed Tannins Quercetin	Anti-mutagenic	Phenols Condensed Tannins Quercetin
Post-treatment A	Anti-mutagenic Indicates toxicity	Phenols Condensed Tannins Quercetin	Anti-mutagenic Indicates toxicity	Phenols Condensed Tannins Quercetin
Post-treatment B	Anti-mutagenic Indicates toxicity	Phenols Condensed Tannins Quercetin	Anti-mutagenic Indicates toxicity	Phenols Condensed Tannins Quercetin
Post-treatment C	Anti-mutagenic	Phenols Condensed Tannins Quercetin	Anti-mutagenic	Phenols Condensed Tannins Quercetin

Cavalcante *et al.*, 2003). Carotenoids and vitamin C, which are widely distributed in fruits, play a role in genomic stability (Fenech, 2001) and were shown to inhibit metabolic activation of AFB1, benzo[a]pyrene and cyclophosphamide *in vitro* and *in vivo* (Odin *et al.*, 1997; Rauscher *et al.*, 1998). The phenolic compounds of the juices do not react covalently with AFB1; however inhibition of enzyme activation could lead to the formation of a chemical complex (Loarca-Pina *et al.*, 1996; Cardador-Matinez *et al.*, 2002) or to the transformation of AFB_1 to non-toxic products (Premalatha and Sachdanandam, 2000). Polyphenols may reduce production of the active metabolites through down-regulation of the relevant phase I enzymes, and/or may directly interfere with DNA adduct formation (Ferguson, 2001).

In addition CA/cajuina showed excellent anti-oxidant potential based on their capacity to scavenge free peroxyl radicals as measured in the Total Radical-trapping Antioxidant Potential (TRAP) assay that showed lowered oxidative damage-induced mutagenesis by co- and post-treatments (Melo-Cavalcante *et al.*, 2003).

Anti-mutagenicity of various anti-oxidants, *e.g.* flavones and flavanols, against AFB1 has also been observed (Francis *et al.*, 1989; Kusamram *et al.*, 1998) and antocyanins (Tedesco *et al.*, 2001) and galangin (Heo *et al.*, 2001) show similar activity.

We observed some anti-mutagenic effect in post-treatment A, for both juices, at 10 and 25 μL/plate for CAJ and 500 and 2000 μL/plate for cajuina. In post-treatments B and C, CAJ showed high anti-mutagenic potential at 25 and 50 μL/plate, inhibiting up to 99% of the mutagenicity of AFB1 (Table 2). However, cajuina in post-treatment B showed this inhibitory effect only at 2000 μL/plate and in post-treatment C at 500 and 2000 μL/plate (Table 2). This high anti-mutagenic potential at some doses of the post-treatment suggests protection by phenolic compounds, *i.e.* by quercetin, antocyanins and tannic acid, from error-prone DNA repair mechanisms (Melo-Cavalcante *et al.*, 2003; Ferguson, 2001; De Flora *et al.*, 2001).

In conclusion, the present study demonstrates that CAJ/cajuina may protect *S. typhimurium* strain TA102 against AFB1-induced DNA damage (Table 2) by various mechanisms, including the possible interaction with S9 enzymes and transformation of AFB_1 and its mutagenic metabolites to non-mutagenic compounds. The stimulation of repair and/or reversion of DNA damage as observed in post-treatment could be another anti-mutagenic mechanism of CAJ/cajuina. This protection can be attributed to the presence of chemically active components in both juices (Table 3), which have already been shown to be involved in the protection of DNA (Melo-Cavalcante *et al.*, 2003).

Our results indicate that CAJ/cajuina could be useful in protecting against a variety of compounds with mutagenic potential, that, once activated by the host, can produce mutagenic DNA adducts.

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