



Antimutagenic and antirecombinagenic activities of noni fruit juice in somatic cells of *Drosophila melanogaster*

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ABSTRACT

Noni, a Hawaiian name for the fruit of *Morinda citrifolia* L., is a traditional medicinal plant from Polynesia widely used for the treatment of many diseases including arthritis, diabetes, asthma, hypertension and cancer. Here, a commercial noni juice (TNJ) was evaluated for its protective activities against the lesions induced by mitomycin C (MMC) and doxorubicin (DXR) using the Somatic Mutation and Recombination Test (SMART) in *Drosophila melanogaster*. Three-day-old larvae, trans-heterozygous for two genetic markers (*mwh* and *flr*³), were co-treated with TNJ plus MMC or DXR. We have observed a reduction in genotoxic effects of MMC and DXR caused by the juice. TNJ provoked a marked decrease in all kinds of MMC- and DXR-induced mutant spots, mainly due to its antirecombinagenic activity. The TNJ protective effects were concentration-dependent, indicating a dose-response correlation, that can be attributed to a powerful antioxidant and/or free radical scavenger ability of TNJ.

Key words: antigenotoxicity, doxorubicin, mitomycin C, SMART, noni juice.

INTRODUCTION

Morinda citrifolia L. (Rubiaceae) - popularly known in Hawaii and Brazil as noni, is also called “indian mulberry”, “ba ji tian”, “nono”, “nonu”, “cheese fruit”, and “nhau”. It is one of the most traditional and popular medicinal plants in Polynesia, and its use has been recorded for over 2000 years (Earle 2001). Noni is native to Southeastern Asia (Indonesia) and Australia, this small evergreen tree

or shrub is noted for its extremely wide range of environmental tolerances and now has a pantropical distribution, found even at Central and South America (from Mexico to Panama, Venezuela and Surinam) (Nelson 2006). All parts of the plant, especially the fruit, have been utilized as a food source or for its medicinal properties (Cardon 2003, Wang et al. 2002). As a medicinal plant, noni has been used to prevent and cure several diseases. Its therapeutic effects include antimicrobial, analgesic, hypotensive, anti-inflammatory, anticancer and

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immunological system stimulation effects (Chan-Blanco et al. 2006, Wang et al. 2002, Yu et al. 2008). Currently, the use of *Morinda citrifolia* has become widespread, and its products are commercially available in health food stores, chain grocery stores specializing in natural foods, and on the internet. Both leaves and fruits are processed and sold as capsules, tea, and juice forms, although the fruit juice is the predominant formulation.

The anti-inflammatory, analgesic, hypotensive and cardiovascular activities of *Morinda citrifolia* were reviewed in Chan-Blanco et al. (2006). Antimicrobial effects of ethanol and hexane extracts of noni have been described, including their antitubercular activity, with the extracts inhibiting the growth of *Mycobacterium tuberculosis* by 89-95% (Saludes et al. 2002). Immunomodulatory effects were demonstrated for commercially available juice (Tahitian Noni® Juice - TNJ); polysaccharide-rich substance from noni juice (noni-ppt) and fruit juice concentrates, both *in vivo* and *in vitro* (Hirazumi and Furusawa 1999, Hirazumi et al. 1996, Palu et al. 2008), and recently Harada et al. (2009) detected neuroprotective effect of noni juice against the development of ischemic neuronal damage in mice.

Furthermore, noni-ppt showed antitumor activity in the Lewis lung peritoneal carcinomatosis model (Hirazumi and Furusawa 1999) and prophylactic and therapeutic potential against the immunomodulator-sensitive sarcoma 180 tumor system (Furusawa et al. 2003). The anticarcinogenic properties of the TNJ have been observed at the initiation stage of chemical carcinogenesis, by preventing the carcinogen-DNA adduct formation and/or by acting as an antioxidant (Wang and Su 2001).

Over 150 phytochemical compounds have already been identified in the noni plant (for a review see Chan-Blanco et al. 2006), and the major micronutrients are phenolic compounds, organic acids and alkaloids. The fruit is described to have flavonoids, lignans and coumarins (Potterat

and Hamburger 2007). Although anthraquinones occur nearly exclusively in the roots (Deng et al. 2007, Ohsawa and Ohba 1993), a potent quinone reductase (QR) inducer, 2-methoxy-1,3,6-trihydroxyanthraquinone, has been reported to be present in the fruit, which could account for the cancer chemopreventive activity exerted by noni (Pawlus et al. 2005).

Noni has been tested in various bioassays, *in vitro* and *in vivo*, to indicate the absence and/or evaluate its genotoxic potential, including gene mutation (HPRT), unscheduled DNA synthesis (UDS), Comet assay, Ames test (Westendorf et al. 2007), micronucleus in mouse, chromosomal aberration in human lymphocytes (Edwards 2002, 2003) and somatic mutation and homologous recombination in *Drosophila melanogaster* (Franchi et al. 2008).

The purpose of this study was to directly evaluate antimutagenic and/or antirecombinagenic effects of TNJ using the Somatic Mutation and Recombination Test (SMART). This assay is based on the loss of heterozygosity (LOH) induction that may occur through various mechanisms, such as point and chromosomal mutations, as well as mitotic recombination. This versatile short-term *in vivo* assay detects simultaneously mutational and mitotic recombination, being able to quantify the recombinagenic activity of a compound in a genotoxicity screening (Franchi et al. 2009, Téllez et al. 2007, Toledo et al. 2008).

MATERIALS AND METHODS

CHEMICAL COMPOUNDS

In this study we used a commercial noni fruit juice produced by Morinda Inc (Tahitian Noni® Juice – manufactured by Tahitian Noni International Inc. 333 West River Park Drive Provo, UT 84604, USA; and imported by Morinda International Brazil Ltda. – São Paulo – Brazil), that was introduced in the global market in 1996 and became commercialized

in Brazil in 2002. Mitomycin C (Mitocin[®] – MMC – CAS N. 50-07-7, manufactured by Kyowa Hakko Kirin Co. Ltd. and imported by Bristol-Myers Squibb Farmacêutica S.A. – Santo Amaro – SP – Brazil) and doxorubicin (Oncodox[®] – DXR – CAS N. 23214-92-8 manufactured by Cipla Ltd. – Goa – India and imported by Meizler Biopharma S.A.) were used as positive controls. The compounds were dissolved in distilled water immediately before use, and the solvent was used as negative control.

THE SOMATIC MUTATION AND RECOMBINATION TEST (SMART)

The wing SMART is based on the identification of wing hairs with mutant phenotypes that represent the occurrence of injuries at DNA level. Such alterations are primordially induced in cells of the imaginal discs, which mitotically divide to originate the adults' wings. Thereby, to obtain more detailed data about the antigenotoxic profile of TNJ, we employed the standard version of the wing SMART in *Drosophila melanogaster* (Andrade et al. 2003, Graf et al. 1984).

FLY STOCKS

The following stocks of *D. melanogaster*, with genetic markers on the left arm of chromosome 3, were used: (i) *mwh/mwh*, carrying the wing cell marker 'multiple wing hairs' (*mwh* abbreviated) and (ii) *ftr³/In(3LR)TM3, ri p^p sep bx^{34e} es Bd^S* (*ftr³* abbreviated).

The wing cell marker 'flare' (*ftr³*) is a zygotic recessive lethal gene, which is maintained in the strain over the balancer chromosome TM3.

STANDARD (ST) CROSSING

The crossings were carried out *en masse* for 3 days, in glass vials containing standard culture medium, using 80 *ftr³* virgin females and 30 *mwh* males (Graf and van Schaik 1992). The following progeny was produced from this cross: marker-heterozygous

flies (*mwh +/+ ftr³*) with phenotypically wild-type wings; and balancer-heterozygous flies (*mwh +/+ TM3, Bd^S*) with phenotypically serrate wings.

TREATMENTS

Eggs derived from ST crossing were collected for 8 h on standard medium enriched with baker's yeast supplemented with sucrose. After 72 ± 4 h, third-instar larvae were collected by flotation in running water and placed in bottles containing 0.9 g of *Drosophila* instant medium (Carolina Biological Supply Company, Burlington, NC, USA) rehydrated with 3 mL of the treatment solutions. The co-treatment was carried out by mixing the mutagens, MMC (0.05 mM) or DXR (0.2 mM), with three concentrations of TNJ (25%, 50%, and 75% v/v). Larvae were fed on instant medium until pupation (about 48 h). After emergence, adult flies were collected from the treatment vials and stored in 70% ethanol. Their wings were mounted in Faure's solution on slides and wing hair mutations were analyzed under a 400 \times magnification.

SCORING OF WINGS

The induction of LOH in the marker-heterozygous flies produce two mutant clones types: (i) single spots, either *mwh* or *ftr³*, resulting from point or chromosome mutations as well as mitotic recombination, and (ii) twin spots, consisting of both *mwh* and *ftr³* subclones, originating exclusively from mitotic recombination (Graf et al. 1984). In the balancer-heterozygous genotype, *mwh* spots reflect predominantly somatic point mutation and chromosome mutation, since mitotic recombination involving the balancer chromosome and its structurally normal homologue is a lethal event (Vogel et al. 1999).

DATA COLLECTION AND STATISTICAL ANALYSIS

Two complete independent experimental procedures were performed. As the data of each individual

experiment were not heterogeneous (p clearly < 0.05 in the Kruskal-Wallis Test), all data were pooled for statistical testing. The adults from each experimental group were collected and both flies' wings were mounted on slides. Both dorsal and ventral sides of the wings were analyzed; altogether approximately 48,800 cells were examined per fly.

The relative frequencies of each type of mutant clone per fly in a treatment series were compared pair-wise (i.e., genotoxin versus genotoxin + TNJ) using the conditional binomial test according to Kastenbaum and Bowman (1970). The data was evaluated according to the multiple decision procedure proposed by Frei and Würzler (1988, 1995) resulting in four possible diagnostic: positive, negative, inconclusive or weak positive antigenotoxicity.

RESULTS

Prior to the antigenotoxicity assessment, TNJ was submitted to a dose range test, demonstrating that concentrations from 25 to 100% v/v do not exert toxic effects (Franchi et al. 2008). The concentrations used to assess TNJ antigenotoxic effects ranged from 25 to 75% v/v and were co-administered with 0.05 mM of MMC or 0.2 mM of DXR. These concentrations induce genotoxic effects without affecting fly survival, as also observed in previously published results (Rezende et al. 2011, Santos 1999).

The antigenotoxic effects of TNJ, measured by the wing SMART, after chronic co-exposure to MMC and DXR are summarized in Table I. TNJ showed a statistically significant ($p < 0.05$) inhibitory effect against MMC and DXR in the frequencies of total spots for both genotypes (*mwh/flr³* and *mwh/TM3,Bd^S*), although only a weak positive effect was observed in flies treated with the lowest dose of TNJ + MMC.

For the *mwh/flr³* flies, the total spot frequency was reduced by 41, 68 and 85% in MMC co-treatments, and 66, 79 and 90% in DXR co-

treatment, for concentrations of 25, 50 and 75% of TNJ, respectively. TNJ also had a significant ($p < 0.05$) positive antimutagenic action against MMC and DXR in the *mwh/TM3,Bd^S* flies, considering the total spot category. Mutant clones induced by recombination are not recovered in the balancer-heterozygous flies, indicating that the spots detected in this genotype are all of mutational origin (point and/or chromosomal). The inhibition observed was of 63, 71 and 97% for MMC-treated flies; and of 83, 92 and 96% for DXR-treated flies, for concentrations of 25, 50 and 75% of TNJ, respectively.

The effect on mutation and recombination induced by both genotoxins, and its modulation by TNJ is shown in Figure 1. MMC (0.05 mM) induced a total of 24.17 spots per fly through a combination of mutation and recombination, while 25, 50 and 75% concentrations of TNJ decreased this frequency to 14.60, 8.25 and 4.40, respectively. MMC alone induced 5.31 spots per fly due exclusively to mutation, and 18.86 due to mitotic recombination. TNJ administered in the culture media reduced the MMC mutational frequency to 2.27, 1.86 and 0.60, while the MMC-recombinational frequency was decreased to 12.33, 6.39 and 3.80 respectively, demonstrating a dose-response effect for both MMC-genotoxic events.

DXR (0.2 mM) induced a total frequency of 10.37 spots, but when TNJ (25, 50 and 75%) were co-administered this frequency dropped to 3.80, 2.43 and 1.35 (Table I). DXR induced 0.85 spots per fly due exclusively to mutation and all TNJ concentrations reduced DXR mutation to a similar value of ~0.30 spots (Figure 1). We observed a clear dose response behavior to DXR recombinational frequency that changed from 9.49 to 3.41, 2.10 and 1.05, in TNJ concentrations of 25, 50 and 75%, respectively. In terms of TNJ antigenotoxic potential these frequencies represent a drop of 56-66% and 63-87% in the frequency of mutation and recombination induced by DXR.

DISCUSSION

In a previous report we have demonstrated that TNJ (25, 50, 75 and 100% v/v) has no genetic toxicity effects in somatic cells of *Drosophila melanogaster* (Franchi et al. 2008), indicating the absence of TNJ mutational and recombinational actions. Previous studies have also shown that TNJ did not induce gene mutations at the HPRT locus (in 0.003 to 3 $\mu\text{L}/\text{mL}$ dose range), in presence and absence of S9 mix. In the same study, no mutagenic activity of ethyl-acetate extract from noni juice (100-fold concentrated) was observed in Chinese hamster V79-cell line (Westendorf et al. 2007). There was no increase in micronuclei nor any evidence of

systemic toxicity in mice ascribed to dehydrated TNJ (10 g/kg body weight) when administered via oral gavage (Edwards 2002). Also, no significant increases were noted in the frequency of chromosome aberrations in cultured cells from human lymphocytes (625, 1250, 2500, and 5000 $\mu\text{g}/\text{mL}$) in presence or absence of S9 mix (Edwards 2003, Ratanavalachai et al. 2008, West et al. 2006).

Considering these findings, we decided to evaluate the antimutagenic and/or antirecombinogenic action of TNJ. In our study, the co-treatment with different concentrations of TNJ plus DXR or MMC induced a statistically significant dose-response reduction in the frequencies of spots in flies.

TABLE I
Effect of the co-treatment (TNJ + MMC and TNJ + DXR) in somatic cells of *Drosophila melanogaster* using the wing SMART standard cross.

Genotype and compounds	N. of flies (N)	Spots per fly (n° of spots)		statistical diagnosis ^a		Total spots <i>m=2</i>	Total <i>mwh</i> clones ^c (n)	Clone induction frequencies (per 10 ⁵ cells per cell division) ^d (n/NC)ef	Inhibition ^g (%)	
		Small single spots (1-2 céls) ^b <i>m=2</i>	Large single spots (>2 céls) ^b <i>m=5</i>	Twin spots <i>m=5</i>						
<i>mwh/flr</i> ³										
Neg. Control	40	0.73 (29)	0.13 (5)	0.10 (4)	0.95 (38)	38	1.95			
MMC (0.05mM)	40	12.05 (482)	7.90 (316)	4.23 (169)	24.18 (967)	925	47.39 [45.44]			
MMC+ TNJ25%	40	6.78 (271)	5.08 (203)	2.75 (110)	14.60 (584)	562	28.79 [26.84]	40.93		
MMC+ TNJ50%	40	3.70 (148)	2.85 (114)	1.70 (68)	8.25 (330)	319	16.34 [14.40]	68.31		
MMC+ TNJ75%	40	1.90 (76)	1.50 (60)	1.00 (40)	4.40 (176)	171	8.76 [6.81]	85.01		
<i>mwh/ TM3, Bd</i> ⁵										
Neg. Control	40	0.40 (16)	0.05 (2)	h	0.45 (18)	18	0.92			
MMC	40	3.78 (151)	1.30 (52)		5.08 (203)	203	10.40 [9.48]			
MMC + TNJ25%	40	1.70 (68)	0.48 (19)		2.18 (87)	87	4.46 [3.53]	62.76		
MMC + TNJ50%	40	1.38 (55)	0.43 (17)		1.80 (72)	72	3.69 [2.77]	70.78		
MMC + TNJ75%	40	0.50 (20)	0.08 (3)		0.58 (23)	23	1.18 [0.26]	97.26		

TABLE I (CONTINUATION)

Genotype and compounds	N. of flies (N)	Spots per fly (n° of spots)		statistical diagnosis ^a				Total spots <i>m</i> =2	Total <i>mwh</i> clones ^c (n)	Clone induction frequencies (per 10 ⁵ cells per cell division) ^d (n/NC)ef	Inhibition ^g (%)		
		Small single spots (1-2 céls) ^b <i>m</i> =2	Large single spots (>2 céls) ^b <i>m</i> =5	Twin spots <i>m</i> =5		Total spots <i>m</i> =2							
<i>mwh/flr³</i>													
Neg. Control	40	0.35	(14)	0.03	(1)	0.03	(1)	0.40	(16)	16	0.82		
DXR (0.2mM)	40	1.43	(57)	7.03	(281)	1.93	(77)	10.38	(415)	400	20.49	[19.67]	
DXR + TNJ25%	40	1.28	(51)	-	1.28 (51)	+	1.25 (50)	+	3.80 (152)	+	147	7.53 [6.71]	65.89
DXR + TNJ50%	40	1.05	(42)	-	0.60 (24)	+	0.78 (31)	+	2.43 (97)	+	97	4.97 [4.15]	78.90
DXR + TNJ75%	40	0.90	(36)	+	0.30 (12)	+	0.15 (6)	+	1.35 (54)	+	54	2.77 [1.95]	90.09
<i>mwh/TM3,Bd^S</i>													
Neg. Control	40	0.28	(11)	0.00	(0)			0.28	(11)	11	0.56		
DXR	40	0.73	(29)	0.13	(5)			0.85	(34)	34	1.74	[1.18]	
DXR + TNJ25%	40	0.35	(14)	+	0.03 (1)	i	h	0.38	(15)	+	15	0.77 [0.20]	83.05
DXR + TNJ50%	40	0.30	(12)	+	0.03 (1)	i		0.33	(13)	+	13	0.67 [0.10]	91.53
DXR + TNJ75%	40	0.30	(12)	+	0.00 (0)	+		0.30	(12)	+	12	0.61 [0.05]	95.76

^aStatistical diagnosis according to Frei and Würzler (1988): +, positive; -, negative; i, inconclusive. *m*, factor of multiplication for evaluation of results significantly negatives. Levels of significance $\alpha=0,05$. ^bIncluding rare single spots *flr³*. ^cConsidering clones *mwh* for single spots *mwh* and for twin spots. ^dCalculated accordance with Frei et al. (1992). ^eC = 48,800, i. e., approach number of cells examined for individual. ^fNumbers between keys are the corrected frequencies of induction in relation incidence estimate of the negative control. ^gCalculated according to Abraham (1994): (genotoxin alone-genotoxin plus TNJ/genotoxin alone)×100. ^hJust single spots *mwh* can be observed in heterozygous individual *mwh/TM3,Bd^S*, since TM3 balancer chromosome does not contain the mutant gene *flr³*.

DXR and MMC are two well known antineoplastic agents used in the treatment of solid tumors (Begleiter 2000, Minotti et al. 2004). DXR inhibits the activity of the enzyme topoisomerase II, inducing DNA strand breaks (Islaih et al. 2005, Resende et al. 2006), while MMC acts primarily by promoting DNA crosslinkage (Efimov and Fedyunin 2010, Riley and Workman 1992, Tomasz et al. 1987). Through these mechanisms, MMC and DXR are able to induce mutations and

chromosomal aberrations in both tumor and non-tumor cells. Moreover, cellular enzymes are capable of converting DXR and MMC into free radical metabolites (Benchekroun et al. 1993, Dusre et al. 1989, Menegola et al. 2001), which in turn induce damage to several molecules, such as DNA.

Many studies have suggested the co-administration of antineoplastic agents and free radical scavengers, such as antioxidants, to reduce the genotoxicity of such treatments in non-tumor

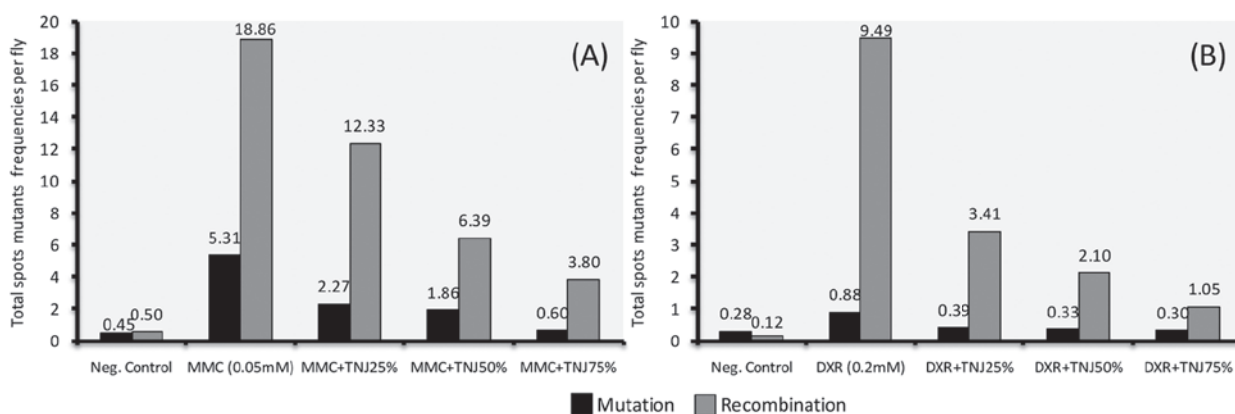


Figure 1 - Contribution of mutation and recombination to total mutant spots frequencies per fly in trans-heterozygous flies treated with MMC (A) or DXR (B) in combination with TNJ (25%, 50%, 75%). The recombinagenic action was calculated according to Graf et al. (1992) and Sinigaglia et al. (2006) as follows: Frequencies (F) = total *mwh* clones (n)/number of flies (N); Mutation Frequencies (FM) = frequencies of *mwh* clones in balancer-heterozygous/frequencies of *mwh* clones in marker trans-heterozygous; Recombination Frequencies (FR) = $1-FM$; Frequencies of Total Spots (FT) = total spots in *mwh/flr³* flies (considering *mwh* and *flr³* spots)/number of flies; Mutation = $FT \times FM$; Recombination = $FT \times FR$.

cells (Amara-Mokrane et al. 1996, Antunes et al. 2007, Antunes and Takahashi 1998, Costa and Nepomuceno 2006, Fragiorge et al. 2007, Gentile et al. 1998, Tavares et al. 2006). So, the noni juice may be promising in this scenario.

In this study, TNJ produced a marked decrease in all kinds of MMC- and DXR-induced mutant spots. However, the largest effect observed in the co-administration of TNJ with MMC and DXR was antirecombinagenic, resulting in 82 and 87% reductions, respectively.

The mechanisms by which TNJ exerts its antigenotoxic activity are not clear at the present. However, antioxidant and/or free radical scavenger activities could be suggested. The radical scavenging activity has been measured *in vitro* using the terazolium nitroblue (TNB) assay, by assessing the juice's potential capacity to protect cells or lipids from oxidative alteration promoted by a superoxide anion radical (SAR). The SAR scavenging activity of TNJ was 2.8 times higher than that of vitamin C, and 1.4 times higher than that of pycnogenol and grape seed powder (Wang and Su 2001). TNJ's protective effects were proportional to the concentrations applied, indicating a dose-response correlation.

Iridoids and potent antioxidant phenolic compounds, such as deacetylasperulosidic acid, scopoletin, isoscapoletin, aesculetin, 7-hydroxycoumarin (7-HC), quercetin, americanin A have been identified in noni fruits (Dussossoy et al. 2011, Ikeda et al. 2009, Liu et al. 2007, Su et al. 2005, West et al. 2011). These findings suggest that several compounds, in particular iridois and phenolic compounds, could contribute separately or synergistically to an antioxidative and antigenotoxic activity in TNJ.

Alternatively, the antigenotoxic activity detected for TNJ could be attributed to the presence of QR inducers. These compounds, such as 2-methoxy-1,3,6-trihydroxyanthraquinone (Pawlus et al. 2005), scopoletin and quercetin (Nitteranon et al. 2011), have been described in noni fruit. QR is a phase II metabolizing enzyme that is induced in conjunction with other protective phase II enzymes. This induction of phase II enzymes, such as QR, is considered as a cancer chemopreventive - since potential oxidative and electrophilic molecules can be more readily metabolized and excreted before its interaction with cellular macromolecules, such as DNA. QR is also responsible for maintaining the reduced states of antioxidants such as α -tocopherol

and coenzyme Q10. QR inducers are sometimes referred to as “indirect antioxidants”, and this activity is considered protective at the initiation stage of carcinogenesis (Dinkova-Kostova and Talalay 2000, Kensler 1997).

This study has successfully used the SMART to demonstrate the protective effects of TNJ on the genotoxicity of DXR and MMC. We conclude that TNJ provides greater protection against these drugs, and that antirecombinagenic activity was the predominant effect. A dose-response relationship was also observed and might be attributed mainly to their powerful scavenger ability. Nevertheless, further experiments should be carried out to gain a better understanding of the mechanism of action of noni phytochemicals and to ensure their safe clinical use.

RESUMO

Noni, nome Havaiano da fruta de *Morinda citrifolia* L., é uma planta medicinal e tradicional da Polinésia amplamente usada para o tratamento de muitas doenças entre as quais artrite, diabetes, asma, hipertensão e câncer. Neste trabalho, um suco comercial de noni (TNJ) foi avaliado para suas atividades protetoras contra as lesões induzidas por mitomicina C (MMC) e doxorubicina (DXR) usando o teste de mutação e recombinação somática em *Drosophila melanogaster*. Larvas de terceiro estágio, trans-heterozigotas para dois genes marcadores (*mwh* e *flr³*), foram cotratadas com TNJ mais MMC ou DXR. Observamos uma redução dos efeitos genotóxicos de MMC e DXR pelo suco. TNJ induziu uma forte redução em todos os tipos de manchas mutantes induzidas por MMC e DXR principalmente devido a uma atividade antirecombinante. Os efeitos protetores de TNJ foram dependentes da concentração, indicando uma correlação dose-resposta, os quais podem ser atribuídos a uma potente ação antioxidante e/ou habilidade de sequestrar radicais livres pelo TNJ.

Palavras-chave: antígeno toxicidade, doxorubicina, mitomicina C, SMART, suco de noni.

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