Food and Chemical Toxicology 49 (2011) 1235–1241

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

The effect of the dibenzylbutyrolactolic lignan (−)-cubebin on doxorubicin mutagenicity and recombinogenicity in wing somatic cells of *Drosophila melanogaster*

A.A.A. de Rezende [a], M.L.A. e Silva [b], D.C. Tavares [b], W.R. Cunha [b], K.C.S. Rezende [b], J.K. Bastos [c], M. Lehmann [d], H.H.R. de Andrade [e], Z.R. Guterres [f], L.P. Silva [g], M.A. Spanó [a,*]

[a] *Universidade Federal de Uberlândia, Uberlândia, MG, Brazil*
[b] *Universidade de Franca, Franca, SP, Brazil*
[c] *Universidade de São Paulo, Ribeirão Preto, SP, Brazil*
[d] *Universidade Luterana do Brasil, Canoas, RS, Brazil*
[e] *Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[f] *Universidade Estadual do Mato Grosso do Sul, Mundo Novo, MS, Brazil*
[g] *Universidade Estadual Paulista Júlio de Mesquita Filho, Assis, SP, Brazil*

ARTICLE INFO

Article history:
Received 6 January 2011
Accepted 2 March 2011
Available online 6 March 2011

Keywords:
Somatic Mutation And Recombination Test
SMART
Toxicity
Cyp6A2

ABSTRACT

The dibenzylbutyrolactolic lignan (−)-cubebin was isolated from dry seeds of *Piper cubeba* L. (Piperaceae). (−)-Cubebin possesses anti-inflammatory, analgesic and antimicrobial activities. Doxorubicin (DXR) is a topoisomerase-interactive agent that may induce single- and double-strand breaks, intercalate into the DNA and generate oxygen free radicals. Here, we examine the mutagenicity and recombinogenicity of different concentrations of (−)-cubebin alone or in combination with DXR using standard (ST) and high bioactivation (HB) crosses of the wing Somatic Mutation And Recombination Test in *Drosophila melanogaster*. The results from both crosses were rather similar. (−)-Cubebin alone did not induce mutation or recombination. At lower concentrations, (−)-cubebin statistically reduced the frequencies of DXR-induced mutant spots. At higher concentrations, however, (−)-cubebin was found to potentiate the effects of DXR, leading to either an increase in the production of mutant spots or a reduction, due to toxicity. These results suggest that depending on the concentration, (−)-cubebin may interact with the enzymatic system that catalyzes the metabolic detoxification of DXR, inhibiting the activity of mitochondrial complex I and thereby scavenging free radicals. Recombination was found to be the major effect of the treatments with DXR alone. The combined treatments reduced DXR mutagenicity but did not affect DXR recombinogenicity.

1. Introduction

Piper cubeba Linn. (Piperaceae) is a pepper plant that is widely distributed in tropical and subtropical regions and is popularly known as *pimenta de Java* (in Brazil), *kemukus* (in Indonesia) or cubeb pepper. It is known that extracts from *P. cubeba* have anti-inflammatory (Bastos et al., 2001; Choi and Hwang, 2003; Yam et al., 2008a), anti-type IV allergic (Choi and Hwang, 2003), antileishmanial (Bodiwala et al., 2007), genotoxic (Junqueira et al., 2007), antineoplasic (Yam et al., 2008b), and molluscicidal activities (Pandey and Singh, 2009).

Lignans are a class of secondary plant metabolites that are produced by the oxidative dimerization of two phenylpropanoid units. Interest in lignans and their synthetic derivatives has grown due to their applications for cancer chemotherapy and their various other pharmacological effects (Saleem et al., 2005). Here, (−)-cubebin, a dibenzylbutyrolactolic lignan, was isolated from the dry seeds of *P. cubeba* L. This lignan is known to possess anti-inflammatory (Bastos et al., 2001; da Silva et al., 2005), analgesic (da Silva et al., 2005), and antimicrobial activities (Silva et al., 2007, 2009). Although some (−)-cubebin derivative compounds ((−)-hinokinin; (−)-*O*-benzyl cubebin; (−)-*O*-(*N*,*N*-dimethylamino-ethyl)-cubebin) inhibit the free amastigote forms of *Trypanosoma cruzi*, the natural (−)-cubebin molecule itself, which is used as the starting compound to obtain the evaluated dibenzylbutyrolactolic derivatives, does not affect the growth of trypomastigote forms of *T. cruzi* (de Souza et al., 2005). (−)-Cubebin and derivatives isolated from *P. cubeba* were found to significantly inhibit cytochrome P450 (CYP3A4) (Usia et al., 2005, 2006) and the NADH oxidase activity of mitochondrial complex I (Saraiva et al., 2009).

* Corresponding author. Tel.: +55 34 32182505; fax: +55 34 32182203.
E-mail address: maspano@ufu.br (M.A. Spanó).

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doi:10.1016/j.fct.2011.03.001

The anthracycline antibiotic Doxorubicin (DXR), a drug that targets topoisomerase II (Top2) (Islaih et al., 2005), is one of the most effective anticancer drugs used in the clinic (Lyu et al., 2007). This drug may induce mutations by intercalating formaldehyde adducts in the DNA (Spencer et al., 2008) or by inducing the formation of oxygen free radicals (Navarro et al., 2006), single- and double-strand DNA breaks (Lyu et al., 2007) and somatic recombination (Lehmann et al., 2003; Valadares et al., 2008; de Rezende et al., 2009; Sousa et al., 2009).

The wing Somatic Mutation And Recombination Test (SMART) using *Drosophila melanogaster* was developed to detect the loss of heterozygosity of suitable gene markers that have detectable phenotypes that are expressed on the wings. This assay is an efficient and quick method for quantitating the recombinogenic and mutagenic potential of chemical and physical agents (Graf et al., 1996; Vogel et al., 1999; Spanó et al., 2001). For this purpose, two crosses are typically used: the standard (ST) cross (Graf et al., 1989) and a high bioactivation (HB) cross (Graf and van Schaik, 1992). The ST cross uses strains carrying basal levels of the metabolizing cytochrome P450 enzyme (Cyp6A2) and is used to detect direct-acting genotoxins. The HB cross uses strains with high levels of Cyp6A2 and is used to detect indirect-acting genotoxins that exert their genotoxic activity only when metabolized (Frölich and Würgler, 1989; Graf and van Schaik, 1992; Saner et al., 1996).

Several reports have demonstrated the probability of pharmacokinetic interaction between natural compounds and herbal products with conventional drugs when they are administered simultaneously (Usia et al., 2006). Here, we examine the mutagenicity and recombinogenicity of the dibenzylbutyrolactolic lignan (−)-cubebin when administered alone or simultaneously with DXR.

2. Materials and methods

2.1. Chemical compounds and media

(−)-Cubebin was isolated and purified from the seeds of *P. cubeba* L. as previously described (Silva et al., 2007). Doxorubicin (DXR) (Rubidox® – Laboratório Químico Farmacêutico Bergamo Ltda., Taboão da Serra, SP, Brazil) (CAS 23214–92-8) was obtained from the Hospital de Clínicas da Universidade Federal de Uberlândia, MG, Brazil. Ultrapure water (18.2 ΩX) was obtained from a MilliQ system (Millipore, Vimodrone, Milan, Italy). (−)-Cubebin was dissolved in a mixture of 1% Tween 80 (Fluka, Buchs, Switzerland) and 3% ethanol (Neon, São Paulo, Brazil) in ultrapure water. DXR was dissolved in ultrapure water. The solutions were always prepared immediately before use. As an alternative medium, instant mashed potato flakes (Yoki® Alimentos S. A., São Bernardo do Campo, SP, Brazil) were used. The structural formula of (−)-cubebin is shown in Fig. 1.

2.2. The Somatic Mutation And Recombination Test (SMART)

Two crosses were carried out to produce the experimental larval progeny. The first was the standard (ST) cross, in which virgin females of strain *flr*^{3}/*In(3LR)TM3, ri p*^{p} *sep l(3)89Aa bx*^{34e} *e Bd*^{S} were crossed with *mwh/mwh* males (Graf et al., 1984, 1989). The second was the high bioactivation (HB) cross, in which virgin females of strain *ORR/ORR*; *flr*^{3}/*In(3LR)TM3, ri p*^{p} *sep l(3)89Aa bx*^{34e} *e Bd*^{S} were crossed with *mwh/mwh* males (Graf and van Schaik, 1992). Each cross produced marker-heterozygous (MH) flies (*mwh flr*$^+$/*mwh*$^+$ *flr*^{3}) with normal wings and balancer-heterozygous (BH) flies (*mwh flr*$^+$/*mwh*$^+$ *TM3*, *Bd*^{S}) with serrated wings. From both crosses, eggs were collected for 8 h in culture glass bottles with an agar-agar base (4% w/v) topped with a thick layer of live baker's yeast supplemented with sucrose. After 72 h (± 4 h), third instar larvae were washed out of the bottles with ultrapure water (MilliQ system) and collected in a stainless steel strainer. For chronic feeding, four sets of vials for each cross were prepared with 1.5 g of mashed potato flakes (Yoki® Alimentos S.A., Brazil) and 5 ml of a solution containing (−)-cubebin alone (at a final concentration of 0.25, 0.5, 1.0, 2.0 or 4.0 mM) or with DXR (0.2 mM). Negative (1% Tween-80 and 3% ethanol in distilled water) and positive (DXR 0.2 mM) controls were included in both experiments. The experiments were conducted at 25 ± 1 °C and approximately 60% humidity. The larvae were counted before the distribution in two series of these vials. The number of hatched flies was used to calculate the survival rates upon exposure. From the other two sets of vials, the hatched flies were stored in 70% (v/v) ethanol. The wings were removed and mounted on slides with Faure's solution (gum arabic 30 g, glycerol 20 ml, chloral hydrate 50 g and distilled water 50 ml) and analyzed for spots under a compound microscope at 40x magnification. Frequency and size of single and twin spots were recorded. Single spots (*mwh* or *flr*^{3}) can result from mutational events, chromosome aberrations or mitotic recombination (crossing over between the two marker genes). Twin spots (*mwh* and *flr*^{3}) are produced exclusively by mitotic recombination (crossing over between the marker *flr*^{3} and the centromere of chromosome 3). The wings of BH flies were mounted and analyzed after verifying that positive responses were obtained in the MH progeny. In the wings of BH flies, only *mwh* single spots can be recovered. These spots are due to only mutational events because recombination is suppressed in inversion-heterozygous cells with the multiply inverted TM3 balancer chromosome (Graf et al., 1984; Guzmán-Rincón and Graf, 1995).

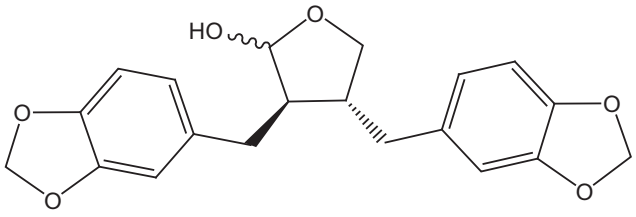

Fig. 1. Structural formula of (−)-cubebin.

2.3. Statistical analysis

For each treated series, 50 flies of both sexes were scored. The multiple-decision procedure (Frei and Wurgler, 1988) was used to analyze the data, resulting in three different diagnoses: negative, positive or inconclusive. The frequency of each type of spot (small single, large single or twin) and the total frequency of spots per fly for each treatment were compared pair-wise (i.e., negative control versus (−)-cubebin; positive control (DXR) alone versus DXR plus (−)-cubebin) according to Kastenbaum and Bowman (1970) with $p = 0.05$ (Frei and Wurgler, 1988, 1995). All inconclusive and weak results were analyzed with the non-parametric U-test of Mann, Whitney and Wilcoxon (a = b = 0.05, one sided) (Frei and Wurgler, 1995). Based on the clone induction frequency per 10^5 cells, the recombinogenic activity was calculated as follows. Frequency of mutation (FM) = frequency of clones in BH flies/frequency of clones in MH flies. Frequency of recombination (FR) = 1 − frequency of mutation (FM). Frequencies of total spots (FT) = total spots observed in MH flies (considering *mwh* and *flr*^{3} spots)/number of flies (Santos et al., 1999; Sinigaglia et al., 2004, 2006). Based on the control-corrected spot frequencies per 10^5 cells, the percentage of (−)-cubebin inhibition was calculated as: (DXR alone − (−)-cubebin plus DXR/DXR alone) − 100 (Abraham, 1994). Statistical comparisons of survival rates were made with the Chi-squared test for ratios of independent samples.

3. Results

Third instar larvae of *D. melanogaster* obtained from both (ST and HB) crosses were fed chronically (approximately 48 h) with (−)-cubebin (0.25, 0.5, 1.0, 2.0 or 4.0 mM) alone or in combination with DXR (0.2 mM). Each treatment was done in duplicate. The data were pooled after verifying that there were no significant differences between repetitions. The concentrations were chosen based on a dose response test, for which the survival rates of flies are given in Table 1. Although 4.0 mM (−)-cubebin alone significantly decreases the survival rates, this concentration was also tested in association with DXR.

At concentrations below 2.0 mM, (−)-cubebin was not found to be toxic. Thus, we found it particularly important to evaluate the modulatory effects of low concentrations on DNA damage induced by DXR in somatic cells of *D. melanogaster*. The highest concentration (4.0 mM) of (−)-cubebin was found to be toxic in the ST cross, when administered alone or combination with DXR. A significant decrease in survival rates relative to the negative control group (ultrapure water) was observed. This concentration was also found to be toxic in the HB cross when administered with DXR.

The results of the ST cross of the SMART assays using *D. melanogaster* are depicted in Table 2. In the MH individuals, (−)-cubebin alone did not show any genotoxicity at the doses used. The DXR treatment, as expected, induced positive results for all categories of spots when compared to the negative

Table 1
Survival rates upon exposure to different concentrations of (−)-cubebin alone or in combination with doxorubicin relative to control groups (ultrapure water and doxorubicin) in the wing Somatic Mutation And Recombination Test in *D. melanogaster*.

Compounds		Standard cross		High bioactivation cross	
DXR (mM)	Cubebin (mM)	Survival (%)	*p*-value	Survival (%)	*p*-value
0	0	95		95	
0	0.25	90	>0.05	90	>0.05
0	0.5	100	>0.05	90	>0.05
0	1.0	95	>0.05	90	>0.05
0	2.0	85	>0.05	90	>0.05
0	4.0	60	<0.05	90	>0.05
0.2	0	95	>0.05	95	>0.05
0.2	0.25	85	>0.05	80	>0.05
0.2	0.5	85	>0.05	80	>0.05
0.2	1.0	85	>0.05	80	>0.05
0.2	2.0	75	>0.05	70	>0.05
0.2	4.0	50	<0.05	65	<0.05

Statistical comparisons of survival rates were made with the Chi-squared test for ratios of independent samples.

control. Lower concentrations of (−)-cubebin (0.25, 0.5 or 1.0 mM) when administered with DXR (0.2 mM) were found to inhibit DXR-induced DNA damage (54.66, 26.21 and 26.84%, respectively). The simultaneous administration of DXR with (−)-cubebin (2.0 mM), however, was found to significantly increase the number of DXR-induced wing spots (by 94.30%).

The wings of the BH flies resulting from the simultaneous application of both drugs were also mounted and scored. This procedure enabled us to quantify the contribution of mutagenic and recombinogenic events to the final genotoxicity observed (Frei et al., 1992; Graf et al., 1992).

In the BH individuals of the ST cross, DXR (0.2 mM) induced a significant increase in the mutant spot frequency relative to the negative control. In the combined treatments, only 0.5 mM (−)-cubebin significantly reduced the total number of spots induced by DXR. The other concentrations of (−)-cubebin (0.25, 1.0, 2.0 and 4.0 mM) did not affect DXR mutagenicity.

Based on the clone induction frequency per 10^5 cells, we compared the number of observed spots in the MH and BH flies and quantified the contribution (%) of mutation and recombination to the total number of observed spots (Frei et al., 1992; Graf et al., 1992; Abraham, 1994). We found that the induced spots were mainly due to recombination. We also found that (−)-cubebin does not affect the recombinogenic activity of DXR (Fig. 2).

The results of the HB cross of the SMART assays with *D. melanogaster* are summarized in Table 3. The results obtained with the MH individuals of the HB cross treated with (−)-cubebin alone were negative at all tested concentrations. DXR statistically increased all categories of spots when compared to the negative control. When administered with DXR, all concentrations of (−)-cubebin were found to statistically inhibit DXR-induced DNA damage, although the inhibition have not been dose-related.

In the BH individuals, DXR induced a significant increase in mutant spots, relative to the negative control. Compared to DXR alone, the treatments of 0.25, 1.0 or 4.0 mM (−)-cubebin administered with DXR produced significantly fewer mutant spots, while 0.5 and 2.0 mM (−)-cubebin had no effect on the production of mutant spots.

Table 2
Summary of results obtained with the *Drosophila* wing spot test (SMART) in the marker-heterozygous (MH) and balancer-heterozygous (BH) progeny of the standard cross (ST) after chronic treatment of larvae with (−)-cubebin and Doxorubicin (DXR).

Genotypes and treatments		Number of flies	Spots per fly (number of spots) statistical diagnosis[a]				Spots with *mwh* clone[c]	Frequency of clone formation/10^5 cells per cell division[d]		Recombination (%)	Inhibition[e] (↓) or induction[e] (↑)(%)
DXR (mM)	(−)-Cubebin (mM)		Small single spots (1–2 cells)[b]	Large single spots (>2 cells)[b]	Twin spots	Total spots		Observed	Control Corrected		
mwh/flr3											
0	0	50	0.32 (16)	0.06 (03)	0.02 (01)	0.40 (20)	20	0.82			
0	0.25	50	0.16 (08)−	0.00 (00)−	0.00 (00)−	0.16 (08)−	08	0.33	−0.49		
0	0.50	50	0.16 (08)−	0.00 (00)−	0.00 (00)−	0.16 (08)−	08	0.33	−0.49		
0	1.00	50	0.14 (07)−	0.04 (02)−	0.00 (00)−	0.18 (09)−	09	0.37	−0.45		
0	2.00	50	0.16 (08)−	0.08 (04)−	0.02 (01)−	0.26 (13)−	13	0.53	−0.29		
0	4.00	50	0.22 (11)−	0.02 (01)−	0.00 (00)−	0.24 (12)−	12	0.49	−0.33		
0.20	0	50	2.78 (139)+	2.38 (119)+	2.66 (133)+	7.72 (386)+	371	15.20	14.38	91.90	
0.20	0.25	50	1.20 (60)*	0.92 (46)*	1.56 (78)*	3.68 (184)*	179	7.34	6.52	86.02	54.66 ↓
0.20	0.50	50	1.52 (76)*	1.58 (79)*	2.56 (128)	5.66 (283)*	279	11.43	10.61	94.27	26.21 ↓
0.20	1.00	50	1.40 (70)*	1.46 (73)*	2.80 (140)	5.66 (283)*	277	11.34	10.52	88.10	26.84 ↓
0.20	2.00	50	3.42 (171)*	4.08 (204)*	6.92 (346)*	14.42 (721)*	702	28.76	27.94	94.59	94.30 ↑
0.20	4.00	50	2.62 (131)	2.04 (102)	2.72 (136)	7.38 (369)	354	14.20	13.38	94.10	06.94
mwh/TM3											
0	0	50	0.26 (13)	0.00 (00)	[f]	0.26 (13)	13	0.53			
0.20	0	50	0.50 (25)+	0.10 (05)+		0.60 (30)+	30	1.23	0.70		
0.20	0.25	50	0.40 (20)	0.10 (05)		0.50 (25)	25	1.01	0.48		
0.20	0.50	50	0.08 (04)*	0.24 (12)		0.32 (16)*	16	0.64	0.11		
0.20	1.00	50	0.40 (20)	0.26 (13)*		0.66 (33)	33	1.34	0.81		
0.20	2.00	50	0.66 (33)	0.10 (05)		0.76 (38)	38	1.56	1.03		
0.20	4.00	50	0.42 (21)	0.00 (00)*		0.42 (21)	21	0.85	0.32		

Marker-trans-heterozygous flies (*mwh/flr*^{3}) and balancer-heterozygous flies (*mwh/TM3*) were evaluated.

* $P \leqslant 0.05$ vs. DXR only.

[a] Statistical diagnoses according to Frei and Wurgler (1988, 1995). *U*-test, two sided; probability levels: −, negative; +, positive; i, inconclusive; $P \leqslant 0.05$ vs. untreated control;

[b] Including rare *flr*^{3} single spots.

[c] Considering *mwh* clones from *mwh* single and twin spots.

[d] Frequency of clone formation: clones/flies/48,800 cells (without size correction).

[e] Calculated as{[DXR alone − DXR + (−)-cubebin] /DXR} × 100, according to Abraham (1994).

[f] Balancer chromosome *TM3* does not carry the *flr*^{3} mutation and recombination is suppressed, due to the multiple inverted regions in these chromosomes.

Table 3
Summary of results obtained with the *Drosophila* wing spot test (SMART) in the marker-heterozygous (MH) and balancer-heterozygous (BH) progeny of the high bioactivation cross (HB) after chronic treatment of larvae with (−)-cubebin and Doxorubicin (DXR).

Genotypes and treatments		Number of flies	Spots per fly (number of spots) statistical diagnosis[a]				Spots with *mwh* clone[c]	Frequency of clone formation/10^5 cells per cell division[d]		Recombination (%)	Inhibition[e] (%)
DXR (mM)	(−)-Cubebin (mM)		Small single spots (1–2 cells)[b]	Large single spots (>2 cells)[b]	Twin spots	Total spots		Observed	Control Corrected		
mwh/flr3											
0	0	50	0.38 (19)	0.08 (04)	0.08 (04)	0.54 (27)	27	1.11			
0	0.25	50	0.28 (14)–	0.08 (04)–	0.02 (01)–	0.38 (19)–	19	0.78	−0.33		
0	0.50	50	0.26 (13)–	0.02 (01)–	0.04 (02)–	0.32 (16)–	16	0.66	−0.45		
0	1.00	50	0.40 (20)–	0.08 (04)–	0.00 (00)–	0.48 (24)–	24	0.98	−0.13		
0	2.00	50	0.40 (20)–	0.04 (02)–	0.00 (00)–	0.44 (22)–	22	0.90	−0.21		
0	4.00	50	0.44 (22)–	0.16 (08)–	0.02 (01)–	0.62 (31)–	30	1.22	0.10		
0.20	0	50	2.80 (140)+	2.78 (139)+	3.20 (160)+	8.78 (439)+	420	17.21	16.10	90.89	
0.20	0.25	50	1.74 (87)*	1.50 (75)*	1.66 (83)*	4.90 (245)*	245	10.04	8.93	92.62	44.54
0.20	0.50	50	2.32 (116)	1.46 (73)*	2.30 (115)*	6.08 (304)*	304	12.46	11.35	87.38	29.50
0.20	1.00	50	2.54 (127)	2.18 (109)*	2.64 (132)	7.36 (368)*	363	14.88	13.77	93.11	14.47
0.20	2.00	50	2.28 (114)	1.64 (82)*	2.16 (108)*	6.08 (304)*	301	12.34	11.23	88.48	30.25
0.20	4.00	50	2.20 (110)*	1.20 (60)*	2.34 (117)*	5.74 (287)*	285	11.67	10.56	89.79	34.41
mwh/TM3											
0	0	50	0.26 (13)	0.00 (00)	[f]	0.26 (13)	13	0.53			
0.20	0	50	0.50 (25)+	0.30 (15)+		0.80 (40)+	40	1.64	1.11		
0.20	0.25	50	0.50 (25)	0.00 (05)		0.50 (25)*	25	1.02	0.49		
0.20	0.50	50	0.70 (35)	0.00 (00)		0.70 (35)	35	1.42	0.89		
0.20	1.00	50	0.50 (25)	0.00 (00)*		0.66 (25)*	25	1.02	0.49		
0.20	2.00	50	0.66 (33)	0.10 (05)*		0.76 (38)	38	1.56	1.03		
0.20	4.00	50	0.42 (21)	0.00 (00)*		0.42 (21)*	21	0.85	0.32		

Marker-trans-heterozygous flies (*mwh/flr*^{3}) and balancer-heterozygous flies (*mwh/TM3*) were evaluated.
* $P \leqslant 0.05$ vs. DXR only.
[a] Statistical diagnoses according to Frei and Wurgler (1988, 1995). *U*-test, two sided; probability levels: −, negative; +, positive; i, inconclusive; $P \leqslant 0.05$ vs. untreated control;
[b] Including rare *flr*^{3} single spots.
[c] Considering *mwh* clones from *mwh* single and twin spots.
[d] Frequency of clone formation: clones/flies/48,800 cells (without size correction).
[e] Calculated as{[DXR alone − DXR + (−)-cubebin] /DXR} × 100, according to Abraham (1994).
[f] Balancer chromosome *TM3* does not carry the *flr*^{3} mutation and recombination is suppressed, due to the multiple inverted regions in these chromosomes.

By comparing the number of observed spots in the MH flies and BH flies, we once again found that the induced spots were mainly due to recombination. We found that (−)-cubebin displays only anti-mutagenic activity and does not interfere with DXR recombinogenicity (Fig. 2).

4. Discussion

The mutagenic and/or recombinogenic potential of (−)-cubebin, alone and in combination with the chemotherapeutic agent DXR, was assayed using two crosses (ST and HB) of the wing Somatic Mutation And Recombination Test (SMART) in *D. melanogaster*. We obtained similar data in both crosses and found that (−)-cubebin alone does not modify the frequency of spontaneous mutant spots in this test system.

The reference mutagen DXR significantly increased all categories of spots. Previous studies using the SMART assay have shown that the major mutational contribution of DXR is its ability to induce recombination (Lehmann et al., 2003; Valadares et al., 2008; de Rezende et al., 2009; Sousa et al., 2009). DXR is a chemotherapeutic agent that induces single- and double-stranded DNA breaks, which are processed by recombinational DNA repair pathways. DNA double-strand breaks (DSBs) are a serious threat to the cell and can lead to chromosomal aberration, mutation and cancer. DSBs in human cells are repaired via non-homologous DNA end joining and homologous recombination repair pathways (Poplawski et al., 2010). Homologous recombination can result in a loss of heterozygosity or genetic rearrangements. Some of these genetic alterations can be correlated with the manifestation of recessive heritable diseases and may play a primary role in carcinogenesis. More likely, however, they are probably involved in secondary and subsequent steps of carcinogenesis that reveal recessive oncogenic mutations (Bishop and Schiestl, 2002, 2003).

There is an increasing amount of evidence to suggest that cancer and other mutation-related diseases can be prevented not only by limiting exposure to recognized risk factors but also with the intake of protective factors and by modulating the defense mechanisms of the host organism. This strategy, referred to as chemoprevention, can be pursued either with suitable pharmacological agents and/or by dietary factors (Ferguson et al., 2005). Here, we examined the mutagenicity and recombinogenicity of the dibenzylbutyrolactolic lignan (−)-cubebin extracted from seeds of *P. cubeba* L. when administered alone or simultaneously with the pharmacological agent DXR.

The association of (−)-cubebin with DXR in the ST cross was found to produce different results depending on the concentration used. At lower concentrations (0.25, 0.5 or 1.0 mM) (−)-cubebin significantly reduces the frequency of DXR-induced mutant spots. At 2.0 mM, (−)-cubebin strongly increases DXR-induced mutagenicity and recombinogenicity, affecting all types of spots (small single, large single and twin spots). The magnitude of the comutagenicity was found to be considerable, leading to an enhancement of 94.30%. At 4.0 mM, (−)-cubebin slightly reduces the frequency of DXR-induced DNA damage. Given the significant decrease in survival rates that was observed in flies treated with 4.0 mM (−)-cubebin (Table 1), we think that the tendency of reduction in DXR-induced DNA damage can be attributed to cytotoxicity, rather than a protective effect of (−)-cubebin.

In the HB cross, all concentrations of (−)-cubebin were found to significantly reduce the frequency of DXR-induced mutant spots.

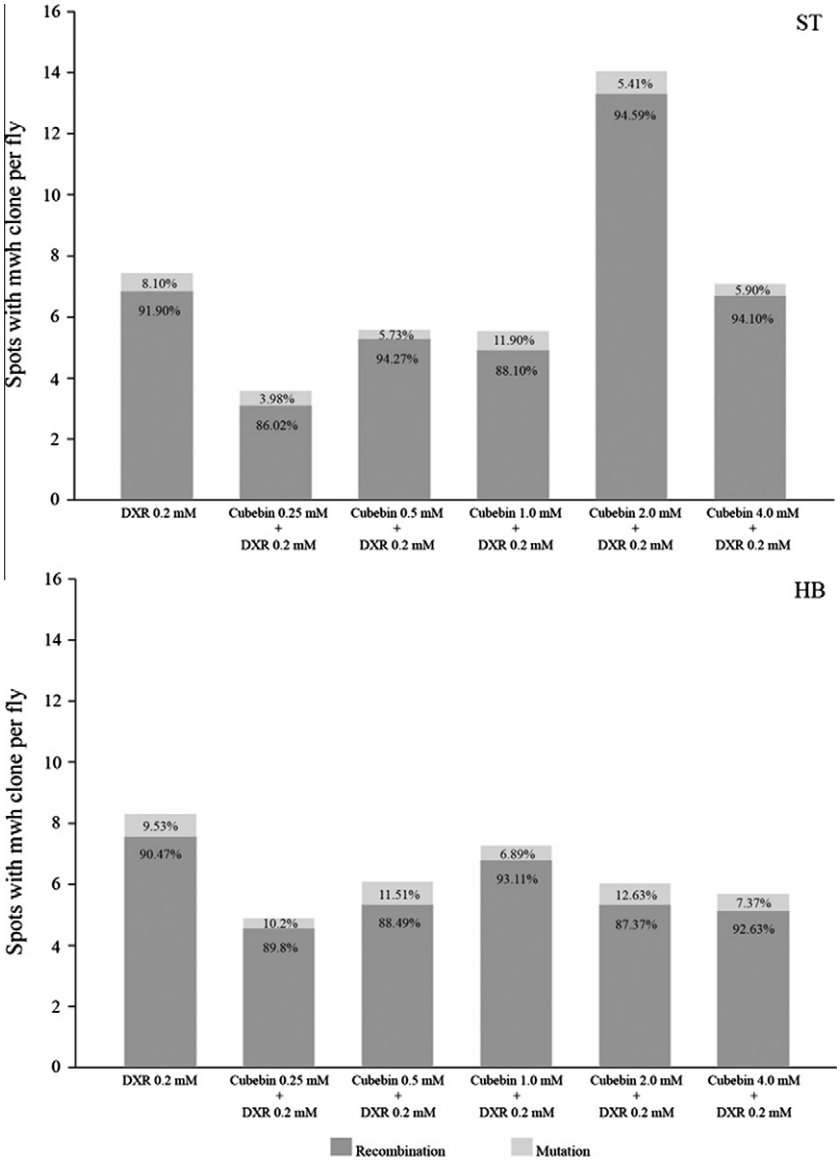

Fig. 2. Contribution of recombination and mutation (in percentage) to total mwh wing spot induction observed in MH individuals from the ST and HB crosses treated with DXR alone and in combination with different concentrations of (−)-cubebin.

However, given the significant decrease in survival rates observed in the ST cross (Table 1), we conclude that the reduction in DXR-induced mutant spots that can be observed upon treatment with 0.2 or 0.4 mM (−)-cubebin in the HB cross could be due to cytotoxicity.

Similar data have been generated for (−)-hinokinin (HK), a dibenzylbutyrolactolic lignan that is obtained by from (−)-cubebin, using the micronucleus (MN) test in V79 Chinese hamster lung fibroblasts to assay the effect on DXR clastogenicity. At lower concentrations, HK significantly protects against DXR-induced MN formation. This effect is thought to be due to of the ability of HK to quench reactive oxygen species. At higher concentrations, HK potentiates DXR-induced clastogenicity, suggesting that higher doses of HK may increase the oxidative stress generated by DXR (Resende et al., 2010).

The cytochrome P450 (CYP) superfamily is the most important drug-metabolizing enzyme group that activates promutagens (Takiguchi et al., 2010). Previous studies have shown that an absence of mutagenicity may result from the metabolic deactivation of a compound. For example, nor-nitrogen mustard (NNM) was found to be non-mutagenic when fed to adult flies in a sex-linked recessive lethal test. In combination with 1-phenylimidazole (PhI), an inhibitor of cytochrome P-450, sufficient quantities of the mutagen was able to reach the gonads and produce significant genetic damage. These data suggest that NNM is deactivated by this family of cytochrome P-450 (Zijlstra and Vogel, 1988). It has been proposed that a similar mechanism can explain the significant potentiating action of tannic acid when administered simultaneously with either of the alkylating agents methylmethanesulfonate or nitrogen mustard in a SMART assay in *D. melanogaster* (Lehmann et al., 2000).

Previous studies have shown that MeOH- and EtOAc-soluble fractions of *P. cubeba* significantly inhibit the human CYP3A4-mediated metabolism of (*N*-methyl-^{14}C)erythromycin (Usia et al., 2006). Thus, it is possible that (−)-cubebin acts by interacting with the enzyme systems that catalyze the metabolic detoxification of DXR in *D. melanogaster*. Such an interaction could explain the high frequency of mutant spots observed at 2.0 mM (ST cross) and the

cytotoxic effect observed at 2.0 mM (HB cross) and 4.0 mM (in both ST and HB crosses).

Mitochondria have also been often implicated in cell death. (−)-Cubebin and its derivatives typically inhibit the mitochondrial complex I, as demonstrated by the inhibition of glutamate/malate-supported state 3 respiration of mitochondria and the NADH oxidase activity of submitochondrial particles (Saraiva et al., 2009). The inhibition of mitochondrial complex I is another possible mechanism to explain the toxicity observed at high (−)-cubebin concentrations.

In the *Drosophila* SMART assay (−)-cubebin significantly alters the generation of DXR-induced wing spots, which are the result of somatic mutation and mitotic recombination. Our data, in combination with the data from previous studies using different compounds, allow us to suggest that (−)-cubebin may act as a free radical scavenger at low concentrations and a pro-oxidant at higher concentrations when it interacts with the enzymatic system that catalyzes the metabolic detoxification of DXR. Alternatively, (−)-cubebin may inhibit mitochondrial complex I, leading to cell death.

In this study, we showed that (−)-cubebin can positively or negatively influence DXR-induced mutagenicity, depending on the concentration. With the long-term goal of developing chemoprevention strategies, future work will focus on investigating the conditions under which (−)-cubebin can prevent genome damage, its mechanisms of action and its pharmacokinetic interaction with conventional drugs.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Universidade de Franca (UNIFRAN) and Universidade Federal de Uberlândia (UFU). Alexandre Azenha Alves de Rezende was supported by a fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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