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A comparative study of the modulatory effects of (−)-cubebin on the mutagenicity/recombinogenicity induced by different chemical agents

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ABSTRACT

(−)-Cubebin (CUB) is a lignan isolated from dry seeds of *Piper cubeba*. We aimed to assess its genotoxic potential and influence on chromosomal damage (frequency of micronuclei – MN) induced by doxorubicin (DXR) in V79 cells and by urethane (URE) in somatic *Drosophila melanogaster* cells. Our findings indicate an absence of a CUB-mediated genotoxic effect at the concentrations tested. The results also revealed that CUB significantly reduced the frequency of MN induced by DXR, with a mean reduction of 63.88%. In a previous study, our research group demonstrated an absence of CUB-mediated mutagenic effects through the wing Somatic Mutation and Recombination Test (SMART) in *Drosophila*. In the present study, we used the standard and high bioactivation versions of the SMART to estimate the antigenotoxic effects of CUB associated with URE. At lower concentrations, the recombination level decreased, but at the highest concentration, the recombination level increased. Our data and previous studies suggest that CUB may act as a free radical scavenger at low concentrations, a pro-oxidant at higher concentrations when it interacts with the enzymatic system that catalyzes the metabolic detoxification of DXR or URE, and/or an inducer of recombinational DNA repair.

1. Introduction

Plants have been used throughout history as a primary source of food, fuel, and medicine (van der Kooy et al., 2009). In the phytochemistry field, medicinal plants are biochemically characterized by their possible bioactive compounds, which can be identified, isolated and subjected to detailed structural analysis (Petronilho et al., 2012). Evidenced by their traditional use and through scientific studies, natural products have been important participants in drug discovery, providing novel structures that can be used as potential drugs (Newman and Cragg, 2012). Many compounds isolated from plants have shown therapeutic promise. Among the plants investigated to date, those of the Family Piperaceae exhibit the greatest therapeutic potential (Reshmi et al., 2010).

The genus *Piper* has over 700 species distributed in both hemispheres, and some species have been used in Ayurvedic medicine because of their medicinal properties (Prasad et al., 2005). Phytochemical studies of Piperaceae extracts have revealed the presence of several bioactive molecules, such as amides, benzoic acids, chromenes, phenylpropanoids, terpenes, alkaloids and lignans (Vanin et al., 2008).

Piper cubeba Linn. (Piperales, Piperaceae), popularly known as *pimenta de Java* (in Brazil), *kemukus* (in Indonesia), cubeb or tailed pepper, thrives worldwide in tropical and subtropical regions (De Rezende et al., 2011). Phytochemical studies of *P. cubeba* extracts have identified the presence of terpenes, alkaloids and lignans (Elfahmi et al., 2007). In comparison to other species of this genus, *P. cubeba* has received less attention, but the essential oils and lignans have been more intensively investigated as *P. cubeba* accumulates both groups of compounds in relatively high amounts (Elfahmi et al., 2007).

Recent *P. cubeba* studies have aimed at investigating the biological activities of crude extracts (Silva et al., 2007; Pandey and Singh, 2009), essential oils (Magalhães et al., 2012), as well as isolated compounds, including (−)-cubebin (CUB), a dibenzylbutyrolactolic lignan (Silva et al., 2005) isolated from dry *P. cubeba* seeds that possess a broad range of biological activities (Aboul-Enein et al., 2011; Bastos et al., 2001; De Rezende et al., 2011; Maistro et al., 2011; Silva et al., 2005, 2007, 2009) and its semi-synthetic derivative (−)-hinokinin (HK) (Medola et al., 2007; Resende et al., 2010; Silva et al., 2007). The interest in lignans, such as CUB and HK, has grown due to such biological effects (Saleem et al., 2005).

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Recently, our research group demonstrated that CUB does not exhibit mutagenic effects, but it instead protects against Doxorubicin-induced mutations assessed by the wing Somatic Mutation and Recombination Test (SMART) in *Drosophila melanogaster* (De Rezende et al., 2011). The absence of mutagenicity and the antimutagenic potentials of HK were also evaluated in micronucleated polychromatic erythrocytes (MNPCE) from the peripheral blood of Wistar rats (Medola et al., 2007). Additionally, an *in vitro* micronucleus test (MNT) using Chinese hamster lung fibroblast V79 cells was performed (Resende et al., 2010).

Micronuclei (MN) are derived from chromosomal fragments and whole chromosomes that were retained in anaphase. Therefore, the MNT can be used to demonstrate both clastogenic and aneugenic effects (Fenech, 2007).

The Somatic Mutation and Recombination Test (SMART) in *D. melanogaster* is a short-term *in vivo* assay for chemical mutagenic or recombinagenic activity (Graf et al., 1984, 1989; Graf and van Schaik, 1992). The test measures loss of heterozygosity (LOH) induction, which may occur through point mutations, chromosomal aberrations, and mitotic recombination in two sections of *D. melanogaster* chromosome 3. The SMART has been successfully used to assess the mutagenic/antimutagenic effects of isolated compounds (De Rezende et al., 2009, 2011; Sotibran et al., 2011; Vázquez-Gómez et al., 2010).

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method is a standard, easily executable assay for assessing the free radical scavenging potential of an antioxidant molecule. DPPH is a stable radical in solution, appears purple and absorbs 515–517 nm in methanol. The acceptance of a hydrogen (H) atom from the scavenger molecule, i.e., the antioxidant, results in the reduction of DPPH to $DPPH_2$, visualized by a color change from purple to yellow (Mishra et al., 2012).

In the present study, the *in vitro* MNT in binucleated Chinese hamster lung fibroblast cells (V79) was used to assess CUB's mutagenic and the anti-mutagenic potentials against DXR-induced mutagenicity. We also investigated CUB's protective effects against Urethane-induced mutation and recombination through SMART.

2. Materials and methods

2.1. (−)-Cubebin

The CUB used in this study was kindly provided by Márcio Luis Andrade e Silva, PhD, of the Research Group on Natural Products, University of Franca (UNIFRAN), Franca, SP, Brazil. Additional information about CUB isolation and purification can be found in Silva et al. (2005).

2.2. Cells and culture conditions

Chinese hamster lung fibroblast (V79) cells were kindly supplied by Ilce Mara de Syllos Cólus, PhD (Universidade Estadual de Londrina, Londrina (PR), Brazil). Cells were maintained as a monolayer in plastic culture flasks (25 cm^2) in HAM-F10 (Sigma–Aldrich Co., St. Louis, MO, USA) and DMEM (Sigma–Aldrich Co., St. Louis, MO, USA) (1:1) medium supplemented with 10% fetal bovine serum (Nutricell, Campinas, SP, Brazil), antibiotics (0.01 mg/mL streptomycin, CAS:3810-74-0, and 0.005 mg/mL penicillin, CAS:113-98-4; Sigma–Aldrich Co., St. Louis, MO, USA), and 2.38 mg/mL Hepes (CAS:7365-45-8; Sigma–Aldrich Co., St. Louis, MO, USA) at 37 °C in a BOD-type chamber (Model: 347CD, FANEM Ltda., São Paulo, SP, Brazil). The average cell cycle time was 12 h under these conditions. The micronucleus assay was performed using cells grown in 5.0 mL of culture medium.

2.3. Maintenance conditions of D. melanogaster strains

Originally, the *D. melanogaster* stocks were kindly donated by Dr. Ulrich Graf of the Physiology and Animal Husbandry Institute of Animal Sciences (University of Zurich, Schwerzenbach, Switzerland). Three *D. melanogaster* strains, (1) the *multiple wing hairs*: *y*; *mwh j*; (2) the *flare-3*: ***flr^3/In(3LR)TM3, ri p^p sep l(3)89Aa bx^{34e} e Bd^S*** and (3) the ***ORR/ORR; flr^3/In(3LR)TM3, ri p^p sep l(3)89Aa bx^{34e} e Bd^S***, were maintained in glass vials filled with a maintenance medium (i.e., banana, sucrose, yeast and methylparaben) under light/dark cycles (12:12), at 25 ± 1 °C and approximately 60% humidity in a BOD-type chamber (Model: SL224, SOLAB – Equipamentos para Laboratórios Ltda., São Paulo, SP, Brazil).

2.4. Mutagenicity tests

2.4.1. Cytokinesis-block micronucleus test in V79 cells

To determine CUB concentrations, the clonogenic efficiency assay was performed. The concentrations tested ranged from 2 to 2048 μM, and we chose the final concentrations of 2.0, 4.0, 8.0 and 16.0 μM, using cytotoxicity as a criterion (Franken et al., 2006).

For the experiments, 10^6 cells were seeded into tissue culture flasks, incubated for two cycles (24 h) in complete HAM-F10/DMEM medium, washed with phosphate-buffered saline (PBS, pH 7.4), and treated with each concentration of (−)-cubebin alone or in combination with the mutagen DXR (0.5 μg/mL) for 3 h in serum-free medium. At the end of this period, cells were washed twice with PBS, fresh serum-supplemented medium containing 3 μg/mL cytochalasin-B (CAS: 14930-96-2; Sigma–Aldrich Co., St. Louis, MO, USA) was added, and the cells were incubated for an additional 17 h. At harvest time, cells were rinsed twice with 5 mL PBS, trypsinized using 0.025% trypsin–EDTA and centrifuged for 5 min at 900 rpm. The pellet was hypotonized in 1% sodium citrate plus one drop of 1% formaldehyde and carefully homogenized with a Pasteur pipette. This cell suspension was centrifuged under the same conditions. The pellet was resuspended in methanol:acetic acid (3:1) and again homogenized with a Pasteur pipette. Fixed cells were transferred to slides and stained with 5% Giemsa.

Binucleated cells (6000) were scored per treatment, yielding 2000/treatment/repetition. We employed the criteria established by Fenech (2000) to analyze the micronucleus and binucleated cells.

The nuclear division index (NDI) was determined for 3000 cells analyzed per treatment, yielding 1000 cells per repetition. Cells with a well-preserved cytoplasm containing 1–4 nuclei were scored. The NDI was calculated according to Eastmond and Tucker (1989) using the following formula:

$$\mathrm{NDI} = \frac{(\mathrm{M1} \pm 2(\mathrm{M2}) \pm 3(\mathrm{M3}) \pm 4(\mathrm{M4}))}{N},$$

where M1–M4 represent the number of cells with 1, 2, 3 or 4 nuclei, respectively, and *N* is the total number of viable cells.

DNA damage was induced using the chemotherapeutic agent doxorubicin (DXR; CAS 23214-92-8, Pharmacia Brasil Ltda., Brazil), which was dissolved in distilled water immediately before treatment to a final concentration of 0.5 μg/mL. This dose was determined in pilot experiments. Positive (DXR) and negative controls were also analyzed. All assays described here were repeated independently three times to ensure reproducibility.

2.4.1.1. Calculation of the percent reduction in DNA damage. The percent reduction of DXR-induced damage by CUB was calculated as the number of damaged cells after treatment with the DNA damage-inducing agent DXR minus the number of damaged cells after antigenotoxicity treatment × 100, divided by the number of damaged cells after treatment with the DNA damage-inducing agent minus the number of damaged control cells (Waters et al., 1990).

2.4.1.2. Statistical analysis. The results were evaluated by analysis of variance (ANOVA) and the Tukey test at $p < 0.05$, where the significance metric represented the comparison between the responses in the experimental condition with the negative control in the genotoxicity assay and with the positive control when the antigenotoxicity of (−)-cubebin was determined based on its capacity to reduce DXR-induced DNA damage.

2.4.2. Somatic Mutation and Recombination Test – SMART

2.4.2.1. Crosses. The absence of CUB-mediated mutagenic effects was previously observed by our research group using the *Drosophila* wing SMART (De Rezende et al., 2011).

Based on these data, the antigenotoxic effects of (−)-cubebin on urethane-induced somatic mutations in *D. melanogaster* were also assessed by SMART. Two crosses were performed to produce the experimental larval progeny: (1) Standard (ST) cross (virgin females of strain *flr^3/In(3LR)TM3, ri p^p sep l(3)89Aa bx^{34e} e Bd^S* crossed with *mwh/mwh* males) and (2) High bioactivation (HB) cross (virgin females of strain *ORR/ORR*; *flr^3/In(3LR)TM3, ri p^p sep l(3)89Aa bx^{34e} e Bd^S* crossed with *mwh/mwh* males). The latter cross is highly sensitive to promutagens and procarcinogens because of the increased level of cytochrome P450 present in the *ORR/ORR*; *flr^3* strain (Hällström and Blanck, 1985; Saner et al., 1996). Both crosses produced two types of progeny, which were distinguished phenotypically by the *Bd^S* marker: (i) marker-heterozygous (MH) flies (*mwh+/+flr^3*) with phenotypically wild-type wings and (ii) balancer-heterozygous (BH) flies (*mwh/TM3, Bd^S*) with phenotypically serrate wings. *Additional information* on *these* and other *strains* can be found *elsewhere* (Dapkus and Merrell, 1977; Graf et al., 1989; Graf and van Schaik, 1992; Hällström and Blanck, 1985; Saner et al., 1996).