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Evaluation of lignan (–)-cubebin extracted from *Piper cubeba* on human colon adenocarcinoma cells (HT29)

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ABSTRACT

The dibenzylbutyrolactone lignan (–)-cubebin, which is extracted from the seeds of the pepper *Piper cubeba*, has shown promise as an anti-inflammatory, analgesic, leishmanicidal, antiproliferative, and trypanocidal compound. Given the therapeutic potential of (–)-cubebin, this study aimed to investigate its safety profile by analyzing cytotoxicity, mutagenicity, cell proliferation kinetics, induction of apoptosis, and expression of pro-apoptotic genes in human colon adenocarcinoma cells (HT29) exposed to (–)-cubebin. MTT cytotoxicity assays demonstrated that (–)-cubebin was cytotoxic only at 280 μ M, whereas it was not cytotoxic at 2.8, 14, or 28 μ M. Data demonstrated that (–)-cubebin was not mutagenic as evidenced by a micronucleus (MN) assay, did not alter cell-growth kinetics over 4 d, and showed absence of induced apoptosis after 24 h. Further, *CASP8* and *CASP9* gene expression was not markedly changed in HT29 cells exposed to 28 μ M or 70 μ M (–)-cubebin for 12 h. Based on our observations, (–)-cubebin was cytotoxic at a concentration of 280 μ M, suggesting that the use of this concentration should be avoided. However, lower concentrations exerted no apparent damaging effects, indicating that this lignan is safe to use for pharmacological purposes at certain concentrations.

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The use of natural products with therapeutic properties is as old as human civilization, and for a long time, minerals, plants, and animal products were the main sources of drug production (Rates, 2001; Santos et al., 2014; Maistro et al., 2015). Despite recent strides in modern medicine, plants still provide an important contribution to health (Calixto, 2000; Ryu et al., 2014). However, even though natural products are used extensively by the general population, many medicinal plants have not been subjected to rigorous scientific testing, and their properties are extremely variable (Capasso et al., 2000). The indiscriminate use of natural products by the general population demonstrates the need for studies that evaluate both cytotoxicity and mutagenicity of these compounds to generate data regarding the safety of their consumption.

In this context, the pepper *Piper cubeba*, known as Java pepper or, in English, as “cubeb pepper” is a medicinal plant that has been widely used

throughout Europe since the Middle Ages, as well as in Saudi Arabia, India, Indonesia, and Morocco (Junqueira et al., 2007). Investigation of the dibenzylbutyrolactone lignan (–)-cubebin, which is extracted from the seeds of *Piper cubeba*, led to the isolation of several compounds with interesting biological activities (Souza et al., 2004). Lignans are a class of secondary plant metabolites that are produced by the oxidative dimerization of two phenylpropanoid units (Rezende et al., 2011). Various lignans exert anticancer, antioxidant, antimicrobial, anti-inflammatory, immunosuppressive, antiviral, and hepatoprotective effects (Kernan et al., 1997; Saleem et al., 2005; Zhang et al., 2007).

Several beneficial properties of (–)-cubebin were reported, including trypanocidal (Bastos et al., 1999; Souza et al., 2005), analgesic, anti-inflammatory (Bastos et al., 2001), leishmanicidal (Bodiwala et al., 2007), and antiproliferative (Yam et al., 2008b) activities. To enhance these

therapeutic effects, a number of (–)-cubebin derivatives were synthesized, including (–)-*O*-acetyl cubebin, (–)-*O*-benzyl cubebin, (–)-*O*-(*N,N*-dimethylaminoethyl)-cubebin, (–)-hinokinin, and (–)-6,6′-dinitrohinokinin. (–)-Hinokinin, an oxidized derivative of (–)-cubebin, displaying increased analgesic, anti-inflammatory (Souza et al., 2004), and more efficacious trypanocidal activity than benznidazole, which is the standard drug used to treat Chagas disease (Souza et al., 2005).

(–)-Cubebin and its derivatives are therefore of interest to the pharmaceutical industry. Because (–)-cubebin displays therapeutic promise and serves as the starting point for the production of more effective synthetic derivatives, studies that seek to evaluate possible harmful effects on cells are important to determine the safety profile of (–)-cubebin. Maistro et al. (2011) found that (–)-cubebin produced genotoxic effects in Swiss mice by performing micronucleus (MN) assays on bone marrow cells and comet assays on peripheral blood lymphocytes (PBL). These data showed that caution is necessary and that more studies are needed to assess the risks of (–)-cubebin ingestion before it is prescribed to humans. Importantly, there have been no apparent reports on the influence of (–)-cubebin on cultured human cells. Because data regarding the safe use of (–)-cubebin are still lacking, the objective of this study was to assess cytotoxicity, mutagenicity, cell proliferation kinetics, apoptosis induction, and expression of pro-apoptotic genes in human colon adenocarcinoma cells (HT29) treated with (–)-cubebin.

Materials and methods

(–)-cubebin

(–)-Cubebin was obtained from ethanolic extracts of *Piper cubeba* seeds as described by Maistro et al. (2011) and was provided by Dr. Edson Luis Maistro of UNESP (Universidade Estadual Paulista), Marília-SP. (–)-Cubebin was diluted in dimethyl sulfoxide (DMSO) (Mallinckrodt Chemicals). The concentration of DMSO in culture medium did not exceed 0.25%.

DNA-damaging agents

For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) cytotoxicity and cell proliferation kinetics assays, 1.84 μ M doxorubicin (Adriblastina, Pharmacia) was used to induce DNA damage. In the cytokinesis-block micronucleus (CBMN) assay, DNA damage was induced by the direct-acting mutagen methyl methanesulfonate (MMS, Acros Organics) at 200 μ M in 0.2% DMSO. To evaluate the induction of apoptosis, the apoptosis-inducing chemotherapeutic drug camptothecin (Acros Organics) was used at 43 μ M in 1% DMSO.

HT29 cells

The HT29 cells used in this study were generously provided by Prof. João Ernesto de Carvalho of CPQBA (Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas), UNICAMP (Universidade de Campinas). Cells were grown in 25-cm² culture flasks containing Dulbecco's modified Eagle's medium (DMEM, Gibco, BRL) supplemented with 100× antibiotic–antimycotic solution (Gibco, BRL) and 10% fetal bovine serum (FBS; Gibco, BRL) and incubated at 37°C in a 5% CO₂ atmosphere (UV UltraSafe HF 212). The experiments were conducted using 1.6×10^4 cells/cm²/250 ml.

Cytotoxicity assay

The MTT assay (Invitrogen, Life Technologies) was based on the protocol described by Mosmann (1983). Briefly, 24-well culture plates (3×10^4 cells/well) were incubated at 5% CO₂ and 37°C for 24 h. Afterward, HT29 cells were exposed to 2.8, 28, or 280 μ M (–)-cubebin for 24, 48, or 72 h. Then, 0.167 mg/ml MTT dissolved in serum-free culture medium was added to each well and the precipitates were dissolved in DMSO after 4 h. Three independent experiments were performed in quadruplicate. The percent cell viability was calculated according to Huang et al. (2005).

Cytokinesis-block micronucleus assay (CBMN) and nuclear division index

HT29 cells (10^6) were grown in 25-cm² culture flasks for 24 h and then exposed to 2.8, 14, or 28 μ M (–)-cubebin for 27 h. The same concentrations were tested in other experiments. The concentrations used in the CBMN assay were based on data from the MTT assay protocol. As only 2.8 μ M and 28 μ M (–)-cubebin were not cytotoxic, these concentrations were selected for other evaluations in accordance with the guidelines of the CBMN assay, which recommend the evaluation of noncytotoxic concentrations. Binucleated cells (BNC) were obtained by adding 3 μ g/ml cytochalasin B (Sigma-Aldrich) along with the treatments. After 27 h, cells were collected according to Martins de Oliveira et al. (2002), with modifications. Cells were washed with PBS, detached with 0.1% trypsin–ethylenediamine tetraacetic acid (EDTA; Gibco, BRL) at 37°C, inactivated with culture medium, and centrifuged at $90 \times g$ for 5 min. Next, cells were homogenized with 1% sodium citrate hypotonic solution. Formaldehyde (15 μ l) was subsequently added to the cell suspension, which was immediately homogenized. Cells were again centrifuged, the supernatant was discarded, and the cells were fixed with methanol:acetic acid 3:1 v/v for 10 min. The solution was then centrifuged for 5 min at $90 \times g$, and the cell pellet was used for the confection of blades, which were stained with 5% Giemsa. One thousand BNC were analyzed per treatment per repetition. The slides were analyzed according to Fenech (2000). Each experiment was repeated three times. The same slides prepared for BNC with MN analyses were utilized to assess the nuclear division index (NDI). The NDI was verified according to the method of Eastmond and Tucker (1989).

Cell proliferation kinetics

Changes in cell proliferation were evaluated in HT29 cells exposed to 2.8, 14, or 28 μ M (–)-cubebin for 24, 48, 72, and 96 h (1.6×10^5 cells per 10-cm² tube). After each treatment period, cells were harvested with 0.1% trypsin-EDTA, stained with trypan blue, and counted in a Neubauer chamber. Data are presented as the

total number of cells (viable + unviable) with greater than 95% viable cells/treatment. Analyses were performed in triplicate.

Assessment of apoptosis induction in situ

HT29 cells (1.4×10^4) were cultured on coverslips placed in a 6-well plate, and treatments (control, 2.8, 14, or 28 μ M (–)-cubebin) were added after 24 h of stabilization. Cells were incubated for 24 h, washed with phosphate-buffered saline (PBS), fixed with 3:1 v/v methanol:acetic acid for 5 min, and successively submerged in 95, 75, 50, and 25% ethanol. Next, the coverslip was immersed in McIlvaine buffer (pH 3.8) for 5 min and stained with 0.01% acridine orange for 5 min. Analysis was performed on a fluorescence microscope (excitation filter 420–490 nm and barrier filter 520 nm; Nikon Phase Contrast 1.25). Normal cells were distinguished from apoptotic cells by counting 500 cells per treatment according to the method described by Rovozzo and Burke (1973). Three independent experiments were performed.

Real-time PCR

To evaluate pro-apoptotic CASP8 and CASP9 gene expression, 5×10^5 HT29 cells were grown in 25-cm² culture flasks. Two concentrations of (–)-cubebin (28 and 70 μ M) were evaluated for a period of 12 h. After this period, cells were trypsinized, and their total RNA was extracted with Trizol LS (Invitrogen, Life Technologies). The extracted RNA was treated with 0.1 U of Amplification Grade DNase I (Invitrogen, Life Technologies). The purity of the sample was determined using a spectrophotometer (Eppendorf Biophotometer), and sample integrity was verified by 0.8% agarose gel electrophoresis.

Next, cDNA was synthesized from 1 μ g total RNA using 0.25 mM dNTPs (Invitrogen, Life Technologies), 10 pM Oligo-dT (Invitrogen, Life Technologies), 4 U RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen, Life Technologies), and 200 U of M-MLV reverse transcriptase (Invitrogen, Life Technologies) in a final reaction volume of 20 μ l. Samples were incubated in a thermocycler at 37°C for 50 min followed by 70°C for 15 min.

Table 1. Primers Used for Real-Time Reverse-Transcription Qualitative PCR.

Gene	Primers	Reference
<i>GPDH</i>	F:5' GAAGGTGAAGGTCGGAGTC 3' R:5' GAAAGATGGTGATGGGATT 3'	Sugaya et al. (2005) with modifications
<i>CASP8</i>	F:5' GCAAAAGCACGGGAGAAAGT 3' R:5' TGCATCCAAGTGTGTTCCATTC 3'	Castaneda and Rosin-Steiner (2006)
<i>CASP9</i>	F:5' GCTCTTCCTTTGTCATCTCC 3' R:5' GTTTTCTAGGGTTGGCTTCG 3'	Chen et al. (2009)

Real-time polymerase chain reaction (PCR) using 2 μ l cDNA was performed on a PTC 200 DNA Engine Cycler thermocycler using the Chromo 4 detection system (MJ Research BIO-RAD) and 0.375 μ M of each forward and reverse primer, which amplified partial regions of the *CASP8* and *CASP9* genes (Table 1) in a final reaction volume of 20 μ l. Fragment amplification was detected by the fluorescence emitted by the SYBR Green fluorophore (10 μ l) contained in the Platinum SYBR Green qPCR Supermix-UDG kit (Invitrogen, Life Technologies). The PCR conditions were as follows: an initial denaturation step at 50°C for 2 min; 95°C for 3 min; 39 cycles of 95°C for 20 s, 60°C for 30 s, and 72°C for 20 s; and a final step consisting of 95°C for 10 s and 40°C for 1 min. Melting-curve analysis was performed at the end of the reaction by increasing the temperature from 50°C to 95°C at 0.5°C intervals every 5 s. The reactions were performed in two biological and three mechanical replicates. In this study, data were normalized to the glyceraldehyde-3-phosphate dehydrogenase (*GPDH*) gene.

Statistical analysis

Data obtained from the MTT cytotoxicity assay, cell proliferation kinetic assay, MN assay, and induction of apoptosis were evaluated by analysis of variance (ANOVA) followed by Dunnett's test using GraphPad Instat, with the significance threshold set at $p < .05$ for comparing different concentrations of (–)-cubebin with the control group. Gene expression levels were determined by the method of Pfaffl (2001), with statistical analyses performed using REST-384 software (Pfaffl et al., 2002).

Results

The viability of HT29 cells exposed to 2.8 or 28 μ M (–)-cubebin for 24, 48, and 72 h did not differ significantly from controls, indicating that (–)-cubebin was not cytotoxic at these concentrations (Figure 1). However, there was a significant reduction in viability of HT29 cells exposed to 280 μ M (–)-cubebin in relation to controls at 24, 48, and 72 h (Figure 1). Therefore, only 2.8–28 μ M (–)-cubebin levels were used for subsequent experiments.

CBMN assay were performed to verify whether (–)-cubebin is able to induce DNA damage, and no significant differences in number of MN was detected in cells exposed to (–)-cubebin for 27 h compared with controls, indicating that 2.8, 14, or 28 μ M (–)-cubebin were not mutagenic (Figure 2). Similarly, no significant difference was observed with regard to the NDI (Figure 2).

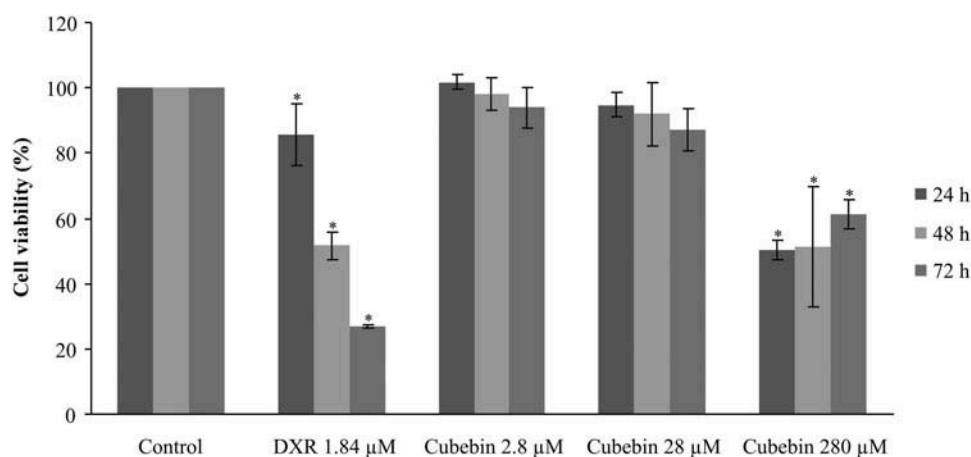


Figure 1. Percentage of viability of HT29 cells exposed to (–)-cubebin for 24, 48 and 72 h. Control (DMSO 0.025%); DXR 1.84 μ M (inductor of DNA damage). *Significant difference ($p < 0.05$) in relation to control cells HT29.

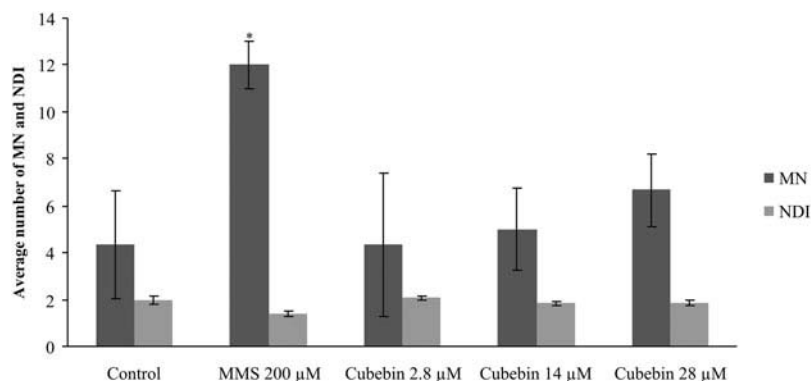


Figure 2. Mean and standard deviation of the number of micronuclei (MN) and NDI in HT29 cells exposed to (–)-cubebin for 27 h. (Control 0.025%); MMS 200µM - Methyl methanesulfonate 200µM (inductor of DNA damage). *Significant difference ($p < 0.05$) in relation to control cells HT29.

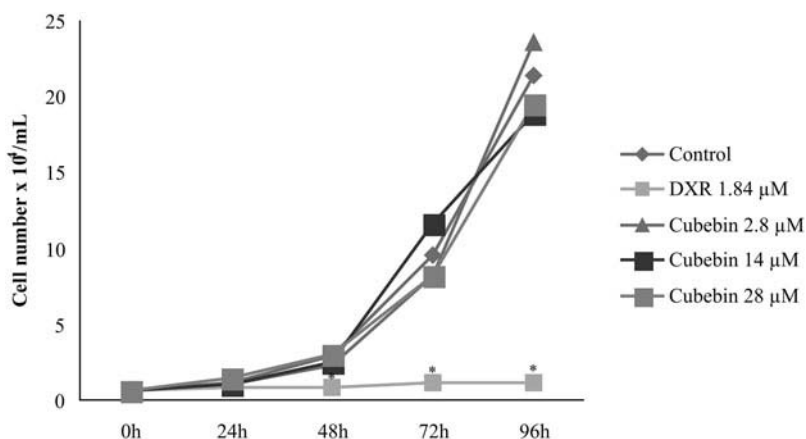


Figure 3. Growth curve of HT29 cells exposed to (–)-cubebin for 24, 48, 72 and 96 h. Control (DMSO 0.025%); DXR 1.84µM - Doxorubicin (inductor of DNA damage). *Significant difference ($p < 0.05$) in relation to control cells HT29.

Whether (–)-cubebin affected the growth of HT29 cells was also determined as increased tumor cell proliferation may be harmful for patients with cancer. However, cell proliferation kinetics analysis revealed no significant change in the growth of HT29 cells exposed to (–)-cubebin for 24, 48, 72 and 96 hr (Figure 3).

The induction of apoptotic cell death by (–)-cubebin was also examined because this property may be exploited to prevent or inhibit tumor growth. (–)-Cubebin did not induce cell death by apoptosis following treatment for 24 h, as no marked differences in number of apoptotic cells were found between (–)-cubebin-treated cells and controls (Figure 4).

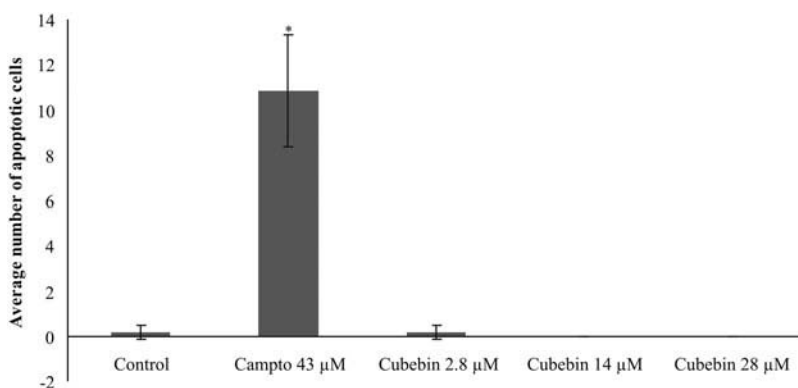


Figure 4. Mean and standard deviation of the number of apoptotic cells found in 1500 HT29 cells exposed to (–)-cubebin for 24 h. Control (DMSO 0.025%); Campto 43 µM - Camptothecin 43µM (inductor of DNA damage). *Significant difference ($p < 0.05$) in relation to control cells HT29.

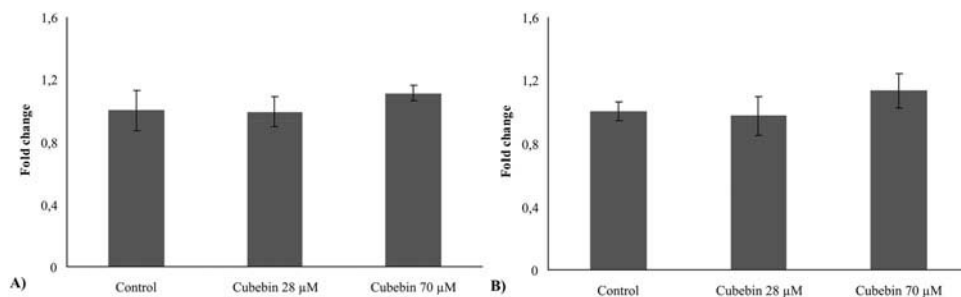


Figure 5. Evaluation of relative expression of CAS8 (a) and CASP9 (b) genes by RT-qPCR in HT29 cells exposed to (–)-cubebin (28 and 70 µM) for 12 h. Data were normalized with the GPDH gene. Control (DMSO 0.0625%). No significant difference.

As apoptotic morphological features were not observed in cells treated with (–)-cubebin, early apoptotic events induced by both extrinsic and intrinsic pathways were determined by examining CASP8 and CASP9 gene expression. Compared with control cells, the relative expression of CASP8 and CASP9 was not markedly altered by treatment with 28 or 70 µM (–)-cubebin (Figure 5). Therefore, our data confirm that treatment of HT29 cells with 28 µM or 70 µM (–)-cubebin did not induce apoptosis by either intrinsic or extrinsic pathways.

Discussion

The use of medicinal plants by the general population remains a common practice, which makes studies of their genotoxicity essential (Teixeira et al., 2003). In this context, (–)-cubebin is a lignan of great interest because it has trypanocidal (Bastos et al., 1999; Souza et al., 2005), anti-inflammatory, analgesic (Bastos et al., 2001) leishmanicidal (Bodiwala et al., 2007), and antiproliferative activities (Yam et al., 2008b). Exposure of HT29 cells to (–)-cubebin at 2.8 or 28 µM for 24, 48, 72, and 96 h did not markedly alter cell proliferation kinetics, confirming the absence of cytotoxicity. Only the highest concentration of (–)-cubebin tested (280 µM) was cytotoxic at 24, 48, and 72 h, with an approximate 50% reduction in HT29 cell viability (Figure 1). There are no other apparent published data regarding cytotoxicity and growth of intestinal cells exposed to (–)-cubebin.

Niwa et al. (2013) studied the effects of (–)-cubebin in an *R. norvegicus* hepatoma cell line (HTC cells) to verify its safe use in a metabolizing cell. Evidence indicated that 28 mM

(–)-cubebin treatment for 24 h did not markedly alter p38 MAP kinase and GSTa2 expression and was not mutagenic in HTC cells, but cytotoxicity was observed. Some studies of *Piper cubeba* plant extracts have been performed in other cell lines. Treatment with an ethanol extract of *Piper cubeba* (P9605) inhibited the proliferation of LNCaP human prostate adenocarcinoma cells at concentrations above 40 µg/ml (Yam et al., 2008a), and 30 µg/ml P9605 extract reduced the growth of MCF-7 human breast carcinoma cells (Yam et al., 2008b). Yam et al. (2008a) also found that treating LNCaP cells with 30 µg/ml *Piper cubeba* extract and 84 µM (–)-cubebin produced five- and twofold increases, respectively, in apoptotic cell death after 48 h. In contrast, in the present study, exposure to 2.8, 14, or 28 µM (–)-cubebin did not markedly alter apoptotic cell death after treatment for 24 h. (–)-Cubebin also did not significantly alter expression of CASP8 and CASP9 genes, which are involved in the extrinsic and intrinsic apoptosis pathways, respectively, indicating that there is no induction of early events of apoptotic cell death in (–)-cubebin-treated cells. Evaluating the expression of these genes may provide general information regarding induction of apoptosis in HT29 cells treated with (–)-cubebin. Multiple factors such as time of exposure to the drug and concentrations utilized may account for these differences. In addition, the HT29 cells used in this investigation do not possess hormone receptors, which may explain lack of any changes in growth and cell death in this cell line.

Information regarding the safe use of (–)-cubebin was obtained using the CBMN assay. (–)-Cubebin displayed no mutagenic activity and did not markedly alter the NDI in HT29

cells after 27 h at all concentrations evaluated. The conservation of NDI indicates that cells displayed the same pattern of nuclear division regardless of treatment with (–)-cubebin, reducing possible errors associated with counting of MN in BNC. Taken together, these data suggest that at concentrations tested, (–)-cubebin did not produce clastogenic or aneugenic damage in HT29 cells. Our data are contradictory to those of Maistro et al. (2011), who observed a clastogenic effect of (–)-cubebin at 500 and 2000 mg/kg in murine bone-marrow cells. In the same study, no genotoxic effect was observed in peripheral blood cells of mice treated with (–)-cubebin at a concentration of 250 mg/kg for 24 h and at concentrations of 500 mg/kg and 2000 mg/kg for 4 h.

The mutagenicity and recombinogenicity of (–)-cubebin alone or in combination with doxorubicin were investigated by Rezende et al. (2011) in *Drosophila melanogaster*. Data demonstrated that 0.25, 0.5, or 1 mM (–)-cubebin reduced DNA damage mediated by 0.2 mM doxorubicin. However, administration of doxorubicin with 2 mM (–)-cubebin significantly increased DNA damage, suggesting that this lignan might positively or negatively influence doxorubicin-induced mutagenicity, depending on the concentration (Rezende et al., 2011). Junqueira et al. (2007) reported a significant rise of number of MN in peripheral blood cells of mice and in bone-marrow cells of rats treated with *Piper cubeba* extract (1 or 1.5 g/kg body weight). However, in our study, no genotoxicity was found in cells exposed to (–)-cubebin, suggesting that the genotoxicity observed for this compound may be dependent upon the cell/tissue type.

Many investigators have been interested in analyzing various (–)-cubebin derivatives (Silva et al., 2005; Souza et al., 2004; 2005, Medola et al., 2007; Saraiva et al., 2007) in order to enhance their biological activities. Medola et al. (2007) studied the compound (–)-hinokinin, which is an oxidized derivative of (–)-cubebin, and found no genotoxic effect but a reduction of chromosome damage in mice peripheral blood cells, as well as an antioxidant effect. Resende et al. (2010) noted that (–)-hinokinin did not affect number of MN in Chinese hamster lung fibroblast cells (V79) at concentrations up to

128 μ M. In addition, at low concentrations, (–)-hinokinin was effective in reducing DNA damage induced by methyl methanesulfonate or doxorubicin treatment, but at high concentrations (–)-hinokinin potentiated DNA damage induced by these agents (Resende et al., 2010). Similar results were obtained by Rezende et al. (2011) in studies with (–)-cubebin. Saraiva et al. (2007) showed that (–)-hinokinin exerted greater activity against epimastigotes than the drug benznidazole, which is the standard drug used for the treatment of Chagas disease. These results demonstrate that (–)-cubebin, a lignan extracted from a natural product, serves as a starting point for production of potentially more active compounds, highlighting the importance of natural products in the search for new medicines.

The data obtained in the present study suggest that (–)-cubebin exerts low toxicity at concentrations up to 28 μ M, as cytotoxicity, mutagenicity, apoptotic cell death, or enhanced growth was not observed in HT29 cells. It is possible that cytotoxic concentrations may induce apoptosis in HT29 cells, but this was not tested. (–)-Cubebin was cytotoxic at 280 μ M, decreasing cell viability by approximately 50% after treatment for 24 h. Therefore, caution is needed when using (–)-cubebin at high concentrations.

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