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Original Research Article

Effects of (−)-cubebin (*Piper cubeba*) on cytotoxicity, mutagenicity and expression of *p38 MAP kinase* and *GSTa2* in a hepatoma cell line

Andressa Megumi Niwa [a,*], Juliana Cristina Marcarini [b], Daniele Sartori [a], Edson Luis Maistro [c], Mário Sérgio Mantovani [a]

[a] Departamento de Biologia Geral, Universidade Estadual de Londrina (UEL), Londrina (PR), Brazil
[b] Instituto de Biociências, Universidade Estadual Paulista – UNESP, Rio Claro, São Paulo, Brazil
[c] Departamento de Fonoaudiologia, Universidade Estadual Paulista Julio de Mesquita Filho (UNESP), Marília, São Paulo, Brazil

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ABSTRACT

(−)-Cubebin is a lignan extracted from the seeds of the pepper *Piper cubeba*, a commonly eaten spice with beneficial properties, including trypanocidal, anti-inflammatory, analgesic, anti-proliferative and leishmanicidal activities. Because of its therapeutic potential, we investigated the effects of (−)-cubebin on the cytotoxicity, cell proliferation kinetics, mutagenicity and expression of *p38 MAP kinase* and *glutathione S-transferase a2 (GSTa2)* using real-time RT-PCR in *Rattus norvegicus* hepatoma cells. We found that 280 μM (−)-cubebin was cytotoxic after 24, 48 and 72 h of exposure, but not mutagenic at 0.28 μM, 2.8 μM and 28 μM after 26 h. Similarly, exposure to 0.28 μM, 2.8 μM and 28 μM (−)-cubebin for 24, 48, 72 and 96 h did not alter the cell proliferation kinetics. Cells exposed to 28 μM (−)-cubebin for 24 h did not exhibit changes in *p38 MAP kinase* and *GSTa2* expression, indicating that cellular changes were not induced by extracellular stimuli and that (−)-cubebin is likely not metabolized via this pathway. Our results suggest that high levels of (−)-cubebin should be consumed with caution due to the cytotoxic effect observed at the highest concentration. However, at lower concentrations, no cytotoxic, mutagenic or proliferative effects were observed, providing further evidence of the safety of consuming (−)-cubebin.

1. Introduction

The pepper *Piper cubeba*, known as the cubeb pepper, tailed pepper or Java pepper, is a popular spice consumed throughout Europe as well as in many other countries, including Arabia, India, Indonesia and Morocco (Junqueira et al., 2007). This pepper has been used since the Middle Ages as both a spice and in the practice of traditional medicine for the treatment of various diseases. The genus *Piper* includes more than 1000 species of plants, which grow as weeds, brush or, less frequently, as standing or sustained trees (Junqueira et al., 2007), and they are primarily found in Asia and the New World (Nunes et al., 2007).

Recent studies have shown that the lignan dibenzylbutyrolactone (−)-cubebin can be extracted from the seeds of *P. cubeba*. This lignan has several beneficial properties, including trypanocidal (Bastos et al., 1999; de Souza et al., 2005), analgesic, anti-inflammatory (Bastos et al., 2001), leishmanicidal (Bodiwala et al., 2007) and anti-proliferative (Yam et al., 2008) activities. Lignans (from the Latin *lignum*, meaning wood) are phytoestrogens found in a wide variety of plants (Krajčová et al., 2009). They consist of dimers formed via the oxidative coupling of cinnamyl alcohols with each other or with cinnamic acids (Barbosa Filho, 2004).

Despite the substantial consumption of *P. cubeba* in various countries, few studies have evaluated the risks associated with the consumption of this species. Given the promising therapeutic potential of this lignan, the objective of this study was to assess the cytotoxicity, mutagenicity and cell proliferation kinetics in HTC cells (a *Rattus norvegicus* hepatoma cell line) treated with (−)-cubebin. We also examined cells exposed to (−)-cubebin to determine the expression of *p38 MAP kinase*, which is involved in several cellular functions, such as inflammation, cell growth, differentiation, cell cycle regulation and cell death (Zarubin and Han, 2005), and of *glutathione S-transferase* (*GST*), which is involved in the metabolism of xenobiotics. Collectively, these experiments provide data that contribute to the safe therapeutic usage of (−)-cubebin.

2. Materials and methods

2.1. (−)-Cubebin extraction and preparation

(−)-Cubebin was obtained from powdered seeds of *P. cubeba* imported from India via maceration with 96% ethanol. Details

* Corresponding author at: Departamento de Biologia Geral – CCB, Universidade Estadual de Londrina – Campus Universitário, Rodovia Celso Garcia Cid, Pr 445 Km 380, Campus Universitário, P.O. Box 6001, Londrina, Paraná, CEP: 86051-980, Brazil. Tel.: +55 43 3371 4977.
E-mail address: andressamn@yahoo.com.br (A.M. Niwa).

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regarding the isolation of (−)-cubebin were provided by Maistro et al. (2011). The (−)-cubebin, which was estimated to have a purity of 99% by HPLC and spectral data analyses, with a yield of 2.24 mg/g dry seed. The (−)-cubebin was diluted in dimethyl sulfoxide (DMSO) (Mallinckrodt Chemicals, USA). The concentration of DMSO did not exceed 0.25% of the culture medium.

2.2. Induction of DNA damage

Doxorubicin (18.4 μM; Adriblastina®, Pharmacia, Italy) was dissolved in culture medium and used to induce DNA damage for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity and cell proliferation kinetics assays. For the cytokinesis-block micronucleus assay, the mutagen benzo[a]pyrene (Fluka, Switzerland), which indirectly induces DNA damage, was used at a concentration of 79 μM and dissolved in 0.8% DMSO.

The use of doxorubicin was based on preliminary studies from our group just to select which the cytotoxic concentration. The use of benzo[a]pyrene as positive control in micronucleus assay was due to the use of HTC cells (from rat hepatoma) to verify their functional metabolic activity. Benzo[a]pyrene is an indirect-acting mutagen, i.e., must be metabolized by cells to cause DNA damage (Verma et al., 2012), providing evidence that HTC cells during the micronucleus assay were metabolically active.

2.3. HTC cells

A *R. norvegicus* hepatoma cell line (HTC cells) was acquired from the Rio de Janeiro Cell Bank (RJBC/UFRJ). HTC cells were grown at 37 °C in culture flasks (25 cm^2) containing a 1:1 mixture of Dulbecco's modified Eagle's medium:nutrient mixture F-12 (Ham) (Gibco, USA) and supplemented with an antibiotic–antimycotic solution (Gibco, USA) and 10% fetal bovine serum (FBS) (Gibco, USA).

2.4. MTT cytotoxicity assay

The MTT assay (Invitrogen, Life Technologies, USA) was performed according to the protocol described by Mosmann (1983). Seeded 96-well culture plates (2.5×10^4 cells/well) were incubated at 37 °C for 24 h. After this period, the following treatments were administered for 24, 48 and 72 h: control (0.25% DMSO); cytotoxicity inducer (18.4 μM doxorubicin); and (−)-cubebin at 0.028 μM, 0.28 μM, 2.8 μM, 28 μM and 280 μM. The selection of concentrations used in MTT assay was based on in vivo study of Maistro et al. (2011). DMSO at a concentration of 0.25% was also administered to wells containing culture medium alone (no cells) for use as a "blank" control. Three independent experiments were performed, and each experiment included twelve replicates.

2.5. Cell proliferation kinetics

Changes in cell proliferation were quantified in HTC cells exposed to (−)-cubebin for 24, 48, 72 and 96 h by manually counting cells. Approximately 10^5 cells per culture tube (10 cm^2) were incubated under the following treatment conditions: control (0.025% DMSO); DNA damage inducer (18.4 μM doxorubicin); and (−)-cubebin (0.28 μM, 2.8 μM and 28 μM). The same treatment conditions were tested in the remaining experiments. Three independent repetitions were performed in each treatment.

2.6. Cell viability assay

Cell viability was measured by manually counting cells stained with 0.2% trypan blue reagent (Gibco, USA) in a Neubauer chamber, according to Mauro et al. (2011). Every 24 h, 100 cells from each treatment were counted over a total period of 96 h. The number of viable versus nonviable cells was expressed as a percentage of the total cell viability. Three independent experiments were performed for each treatment condition.

2.7. Cytokinesis-block micronucleus assay and determination of the nuclear division index

HTC cells (10^6) were grown in culture flasks (25 cm^2) for 24 h prior to the administration of the different treatments. Binucleated cells were obtained via incubation with 3 μg/mL cytochalasin-b (Sigma–Aldrich, Germany) for 27 h. After this period, the cells were harvested according to a previously published protocol (Martins de Oliveira et al., 2002). The slides were analyzed according to criteria determined by Fenech (2000). Three independent experiments were performed.

The nuclear division index (NDI) was also determined from these slides and verified according to Eastmond and Tucker (1989). Three independent experiments were performed.

2.8. Real-time RT-PCR

To evaluate *p38 MAP kinase* and *GSTa2* gene expression, 10^6 HTC cells were grown in culture flasks (25 cm^2) for 24 h and then exposed to 28 μM (−)-cubebin for a period of 12 h. After this period, the cells were trypsinized, and total RNA was extracted using TRIzol LS (Invitrogen, Life Technologies, USA). The extracted RNA was treated with 1 U of DNase I Amplification Grade (Invitrogen, Life Technologies, USA). The purity of the sample was confirmed using a spectrophotometer (Eppendorf Biophotometer, Germany), and sample integrity was verified via electrophoresis on a 0.8% agarose gel.

cDNA was synthesized from 1 μg of total RNA in a reaction containing 0.25 mM dNTPs (Invitrogen, Life Technologies, USA), 10 pM oligo-dT (Invitrogen, Life Technologies, USA), 4 U of RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, Life Technologies, USA) and 200 U of M-MLV reverse transcriptase (Invitrogen, Life Technologies, USA) in a total reaction volume of 20 μL. cDNA synthesis was carried out in a thermocycler at 37 °C for 50 min, followed by 70 °C for 15 min.

Real-time PCR was performed in a PTC 200 DNA Engine Cycler thermocycler using the Chromo4 detection system (MJ Research BIO-RAD). PCR amplification of each sample was performed in a total reaction volume of 20 μL, which included 2 μL of cDNA and 0.25 μM of each forward and reverse primer, to amplify partial regions of the *p38 MAP kinase* and *GSTa2* genes (Table 1). cDNA amplification was measured according to the fluorescence emitted by the SYBR Green fluorophore (10 μL; contained in the Platinum SYBR Green qPCR Supermix-UDG kit, Invitrogen, Life Technologies, USA). The PCR cycling conditions were as follows: an initial denaturation step at 50 °C for 2 min followed by 95 °C for 3 min

Table 1
Oligonucleotide primers used for real-time RT-PCR.

Gene	Primer sequence	Reference
GAPDH	F: 5′ ACAAGATGGTGAAGGTCGGTGTCA 3′ R: 5′ AGCTTCCCATTCTCAGCCTTGACT 3′	Tso et al. (1985)
p38	F: 5′ AAGTCATTAGCTTTGTGCCACCGC 3′ R: 5′ AGTGGGATGGACAGAACAGAAGCA 3′	In this study[a]
GSTa2	F: 5′ AGCCATGGCCAAGACTACCTTGTA 3′ R: 5′ AGAGGTCAGAAGGCTGGCATCAAA 3′	Pickett et al. (1986)

[a] The sequence of the forward and reverse primers was designed using the IDT software (http://www.idtdna.com/Scitools/Applications/Primerquest).

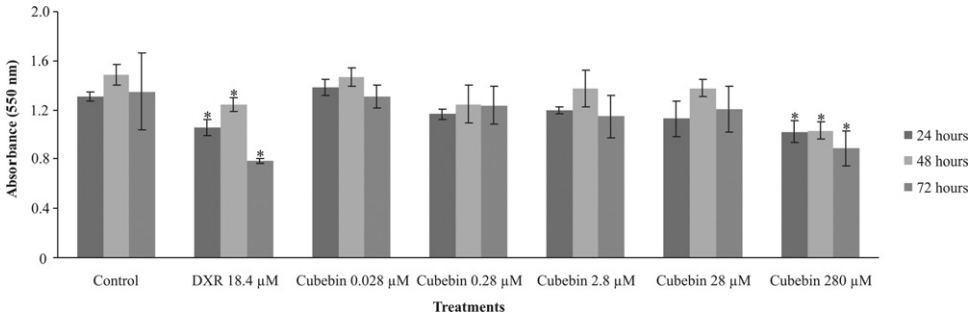

Fig. 1. Average absorbance observed in MTT cytotoxicity assay after 24, 48 and 72 h of incubation with (−)-cubebin. *Control*, culture medium 0.5% DMSO; *DXR*, 18.4 μM doxorubicin (DNA damage inducer). *Significant difference compared with the control ($p < 0.05$).

and 39 cycles of 95 °C for 20 s, 60 °C for 30 s and 72 °C for 20 s, with a final step at 95 °C for 10 s followed by 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 in steps of 0.5 °C every 5 s. Each PCR amplification was performed in two biological experiments and with three mechanical replicates. The data were normalized to the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene.

2.9. Statistical analysis

The data obtained from the MTT, cell proliferation kinetics and micronucleus assays were assessed using analysis of variance (ANOVA) followed by Dunnett's test. Comparisons were performed between the (−)-cubebin treatments and the control group using GraphPad Instat, where $p < 0.05$ was considered statistically significant.

The expression levels of *p38 MAP Kinase* and *GSTa2* were determined using a method previously published by Pfaffl (2001). The statistical analyses were performed using REST-384 software (Pfaffl et al., 2002).

3. Results

The absorbance readings for HTC cells exposed to 0.028, 0.28, 2.8 and 28 μM (−)-cubebin for 24, 48 and 72 h were not significantly different from the control group, suggesting that (−)-cubebin is not cytotoxic when administered at these concentrations. However, exposure to 280 μM (−)-cubebin resulted in a significant difference from the control group, suggesting that this substance is cytotoxic at high concentrations (Fig. 1). We omitted the 280 μM (−)-cubebin treatment condition from further testing because cell death may skew the results of the following experiments. Next, we evaluated whether the proliferation of HTC cells exposed to 0.28, 2.8 and 28 μM (−)-cubebin was altered after 24, 48, 72 and 96 h of exposure. At these three concentrations of (−)-cubebin, the cell proliferation kinetics of the HTC cells were not altered at any of the time points examined, and there were no significant differences detected compared to the control group (Fig. 2). We confirmed that there was no cytotoxicity at these concentrations using a cell viability assay, in which no significant difference was observed in the cells exposed to (−)-cubebin compared to the control group at any of the time points examined (Fig. 3).

To determine whether (−)-cubebin is able to induce DNA damage, a cytokinesis-block micronucleus assay was performed. No significant difference was found in the number of micronuclei detected in the groups exposed to (−)-cubebin for 26 h compared to the control group, demonstrating that (−)-cubebin is not mutagenic at concentrations of 0.28, 2.8 and 28 μM (Fig. 4). There was also no significant difference in the NDI compared to the control group under these conditions (Fig. 4).

In the real-time PCR reactions we considered the average values of efficiency 80%. Next, we assessed whether HTC cells exposed to 28 μM (−)-cubebin for 24 h exhibited altered levels of *p38 MAP kinase* and *GSTa2* gene expression. p38 MAP kinase and GSTa2 are

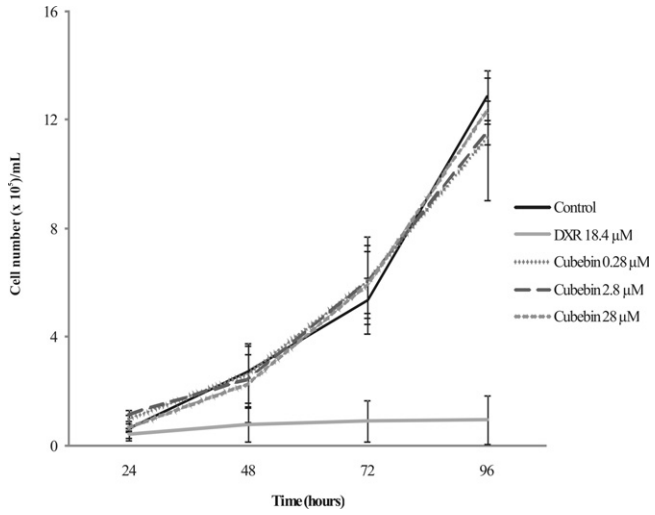

Fig. 2. Growth curve of HTC cells exposed to (−)-cubebin for 24, 48, 72 and 96 h. *Control*, culture medium with 0.025% DMSO; *DXR*, 18.4 μM doxorubicin (DNA damage inducer).

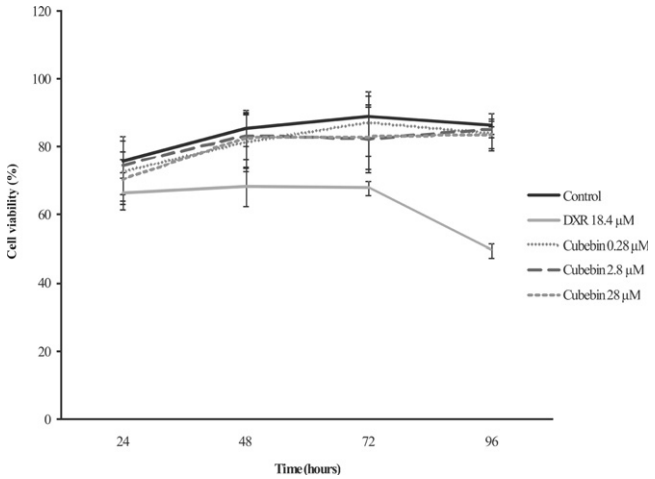

Fig. 3. Percentage HTC cells viability after exposure to (−)-cubebin for 24, 48 and 72 h. *Control*, culture medium with 0.025% DMSO; DXR, 18.4 μM doxorubicin (DNA damage inducer).

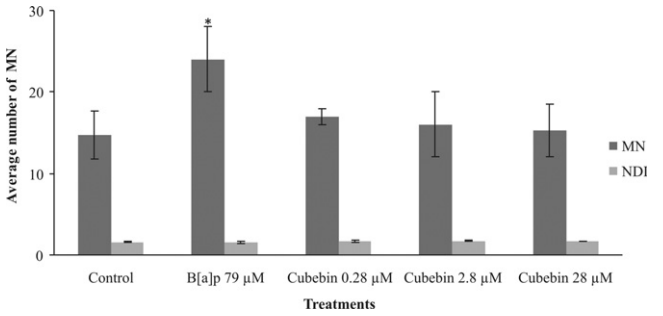

Fig. 4. Mean and standard deviation of the number of micronuclei (MN) and NDI in HTC cells exposed to (−)-cubebin for 26 h. *Control*, culture medium with 0.04% DMSO; B[a]p, 79 μM benzo[a]pyrene (DNA damage inducer); *MN*, micronucleus; *NDI*, nuclear division index. *Significant difference compared with the control ($p < 0.05$).

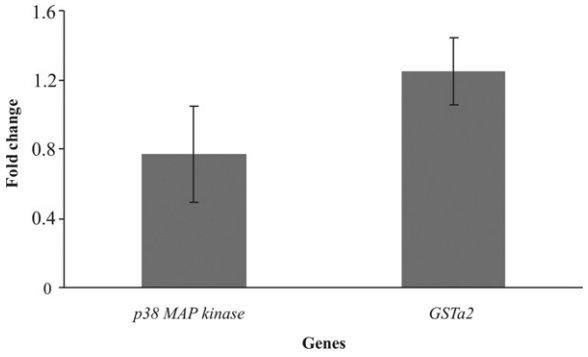

Fig. 5. Evaluation of the relative expression levels of *p38 MAP kinase* and *GSTa2* by real-time RT-PCR in HTC cells exposed to 28 μM (−)-cubebin for 12 h. The data were normalized to *GPDH*. No significant difference was found in gene expression levels according to the REST-384 software (Pfaffl et al., 2002). The comparison was performed with the control group.

involved in cell signaling and the metabolism of xenobiotics, respectively. The analysis of their expression could provide insight into the cellular alterations resulting from exposure to (−)-cubebin. However, we did not observe significant differences in *p38 MAP kinase* and *GSTa2* expression in cells exposed to 28 μM (−)-cubebin compared to control cells (Fig. 5).

4. Discussion

The use of medicinal plants by the general population dates back quite a long time and continues to be a common practice, highlighting the importance of performing studies to assess their genotoxicity (Teixeira et al., 2003). (−)-Cubebin is a lignan of great interest due to its trypanocidal (Bastos et al., 1999; Esperandim et al., 2013; de Souza et al., 2005), analgesic, anti-inflammatory (Bastos et al., 2001), leishmanicidal (Bodiwala et al., 2007) and anti-proliferative activities (Yam et al., 2008). Due to the promising therapeutic effects of (−)-cubebin and considering the substantial consumption of *P. cubeba* in some countries, we examined a number of factors related to its safe consumption. The *R. norvegicus* hepatoma cells (HTC cells) used in this study are proficient at metabolizing xenobiotics. Thus, HTC cells represent an ideal model system for use in toxicity studies because they mimic the role of the liver in metabolizing xenobiotics, primarily from food sources in humans.

Published reports on the mutagenicity of (−)-cubebin are scarce. Maistro et al. (2011) observed a clastogenic effect in murine bone marrow cells treated with 500 mg/kg and 2000 mg/kg (−)-cubebin. In the same study, no genotoxic effect was observed in the peripheral blood cells of mice treated with 250 mg/kg (−)-cubebin for 24 h and at concentrations of 500 mg/kg and 2000 mg/kg for 4 h.

The mutagenicity and recombinogenicity effects of (−)-cubebin alone or in combination with doxorubicin were investigated in *Drosophila melanogaster* by de Rezende et al. (2011). These authors found that treatment with 0.25, 0.5 or 1.0 mM (−)-cubebin reduced the DNA damage induced by 0.2 mM doxorubicin. However, administration of doxorubicin in combination with 2 mM (−)-cubebin significantly increased DNA damage, suggesting that this lignan can positively or negatively influence doxorubicin-induced mutagenicity, depending on the concentration applied (de Rezende et al., 2011).

In addition to the work performed examining (−)-cubebin directly, some researchers have studied plant extracts containing (−)-cubebin (Junqueira et al., 2007; Medola et al., 2007; Resende et al., 2010). In a study by Junqueira et al. (2007), mice treated with seed extracts from *P. cubeba* at concentrations of 1.0 g/kg and 1.5 g/kg for 48 and 72 h were examined for clastogenicity. This analysis demonstrated a significant increase in micronucleated polychromatic erythrocytes in the blood when compared with a control group. Similarly, treatment with these concentrations of the seed extract for 24 h significantly increased the number of micronuclei in mouse bone marrow cells. Our data suggest that (−)-cubebin is not the component of *P. cubeba* seeds that is responsible for the genotoxicity observed in vivo. The concentration of the compound used and the exposure time could also contribute to the variance observed between these studies.

In addition to performing morphological analyses, we evaluated whether treatment with 28 μM (−)-cubebin alters the expression of genes involved in cell signaling (*p38 MAP kinase*) and the metabolism of xenobiotics (*GSTa2*). MAP kinases are key members of a signaling pathway that plays a role in response to a variety of extracellular stimuli (Zarubin and Han, 2005). The MAP kinase family has been divided into the following four distinct subgroups: (1) ERKs, which are kinases that are regulated by extracellular stimuli; (2) JNK/SAPKA-c-Jun N-terminal kinase, which is activated by stress; (3) BMK1-ERK5/large MAP kinase 1; and (4) the protein kinase p38 group (Ono and Han, 2000). The p38 kinases have been shown to be involved in inflammation, cell growth, differentiation, cell cycle regulation and cell death (New and Han, 1998). The *glutathione S-transferases* (*GSTs*) are members of a multigene family involved in the detoxification and, in some cases, activation of a wide variety of chemicals (Eaton and Bammler, 1999). GST proteins are the major detoxifying enzymes involved in phase II detoxification and are primarily found in the cytosol (Sheehan et al., 2001). We found that the exposure of HTC cells to 28 μM (−)-cubebin for 24 h did not result in an alteration of the expression of *p38 MAP kinase*, suggesting that (−)-cubebin does not induce any significant cellular changes. This finding confirms the results obtained in the cytotoxicity and proliferation assays, in which 28 μM (−)-cubebin was not found to be cytotoxic and did not affect cell growth. Additionally, HTC cells exposed to 28 μM (−)-cubebin for 24 h did not show altered expression of *GSTa2*, suggesting that (−)-cubebin is not metabolized via this pathway. The gene expression data further imply that (−)-cubebin is safe and does not alter important cellular events.

5. Conclusion

Our data suggest that (−)-cubebin, which is obtained from *P. cubeba*, should be consumed with caution, as the highest concentration tested (280 μM) was found to be cytotoxic. The other three concentrations used here (0.28 μM, 2.8 μM and 28 μM) were not mutagenic, and the results of these treatments provide insight into the safe use of this lignan. Consistent with this finding and in support of (−)-cubebin's therapeutic potential,

(−)-cubebin does not affect the proliferation of rat liver tumor cells. Finally, HTC cells exposed to 28 μM (−)-cubebin for 24 h did not show altered expression of *p38 MAP kinase* and *GSTa2*, again indicating that (−)-cubebin can be consumed safely, as it does not alter important cellular pathways.

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