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Original Research

Antitumor effects of citrinin in an animal model of Sarcoma 180 *via* cytogenetic mechanisms

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Abstract: Citrinin (CIT) is a cytotoxic, hepatotoxic, nephrotoxic and cardiotoxic metabolite obtained from *Penicillium citrinum*, that has been increasingly searched as an anticancer drug candidate. In this study, we assessed the antitumor effects of citrinin, using cytogenetic biomarkers for genotoxicity in Sarcoma 180 (S-180) ascitic fluid cells of mice. Citrinin, extracted from *P. citrinum* acetonitrile extract, was characterized by LC-MS. Cytotoxic assessment was done through using comet (alkaline version) and micronucleus assays. In S-180 cells, CI_{50} of CIT was 3.77 µg/mL, while at 12.5 and 100 µg/mL, CIT was as cytotoxic as doxorubicin (2 µg/mL). At 0.5, 1.0 and 2.0 µg/mL, it induced genotoxicity and mutagenicity in S-180 cells, especially at 2 µg/mL, triggering oxidative damage similar to hydrogen peroxide (10 mM). The antitumor effects were evidenced by a marked increase in S-180 cells apoptosis and necrosis due to clastogenic and/or aneugenic cytogenetic effects (micronucleus formation), as well as by induction of nucleoplasm bridges and nuclear buds, culminating in S-180 apoptosis and necrosis. CIT has potential as drug candidate for antitumor purposesbyinvolving cytogenetic mechanisms.

Key words: Sarcoma 180; Citrinin; Cytogenetic; Apoptosis.

Introduction

Cancer consists of the pathological process that promotes abnormal, without control and progressive cell growth of the tissue, through cell proliferation (1). In the United States, in 2016, 1,685,210 new cases of distinct types of cancer were detected; among them, 595,690 cancer deaths (2), and in less developed countries, they account for approximately 57% of cases and 65% of deaths (3).

Sarcomas are malignant neoplasms that affect the body's supporting tissues, characterizing a mesenchymal origin (4). The assessment of molecular mechanisms behind the disease through using reliable models, markedly contributes to the advancement of therapies to these neoplasms (5). Often complex, sarcomas cause

structural and numerical chromosomal alterations, which prompts searches to elucidate cytogenetic damages (6).

Multidisciplinary approaches, like surgery, cytotoxic therapy and immunotherapy are necessary and should be carefully combined for cancer treatment (7). However, it is still necessary to improve our knowledge about both intrinsic and extrinsic cell mechanisms implicit in tumor development, as well as the capacity for therapeutic response (8). In the search for most efficient treatments, given the distinct types of cancers, science deposits hope in non-clinical research, where in animal models and in vitro approaches are pivotal (9).

Endophytic fungi of marine origin and their metabolites gather a lot of attention in drug discovery and exploration, as they have probable therapeutic potential (10) as candidates for anticancer drugs (11), since several studies report their antitumor activities by apoptosis induction and cell proliferation inhibition (12,13). The genus Penicillium shows cytotoxic and anticancer activity (14-17), as observed in citrinin (CIT) obtained from *Penicillium citrinum*, which presents cytotoxic, antitumor (12,13,18-21), as well as neuroprotective (22) effects. On the other hand, antagonistic substances, such as those with antioxidant and cytotoxic effects, as reported for CIT, point to a good option for cancer therapy (23).

Many antitumor agents are known to be genotoxic besides presenting an immense potential for inducing genetic changes in target tissues (24), such as cytogenetic changes involving aneuploidies, deletions, insertions, breaks and loss of chromosomes (25). Thus, cytogenetic biomarkers able to detect DNA damages are relevant and useful in genetic risk analysis, providing essential data on oncological biological effects (26), as in the example of comet assay, that can detect the levels of DNA damage, such as breaks in single and double strands (27), as well as oxidative damages (28), and,on the other side, cytokinesis-block micronucleus assay (CBMN), that allows to assess cytogenetic damages from various cytotoxic agents in binucleated cells (29). In this way, the assessment of cytogenetic markers in Sarcoma 180 (S-180) ascitic fluid cells of mice is of utmost importance, because it works as a control of health therapies effectiveness, as for example, in incorporating new substances into chemotherapeutic protocols (30,31). Thus, in this study we assessed the antitumor effects of CIT throughusing cytogenetic biomarkers suggestive of genotoxic and mutagenic potential, apoptosis and necrosis in cultures of S-180 ascitic fluid of mice.

Materials and Methods

General experimental procedure for the isolation of CIT

We used standard analytic solvents from companies Synth, Dinâmica, Merck: methanol, ethyl, n-hexane, acetonitrile. These solvents were used during extraction and partitioning. For the open column chromatographic separation, we used the stationary phase Sephadex LH-20 Sigma-Aldrich. For isolating and growth of microorganisms, we used solid media: Parboiled rice (Marcon®) and PDA (Potato Dextrose Agar - Sigma®). Experiments for ^{1}H RMN (500 MHz), ^{13}C RMN (126 MHz), HMBC, HMQC were obtained in a Bruker Avance DRX-500 spectrometer using solvents CDC_{l3} and DMSO-d_6 (CIL and Isotec-INC), with non-deuterated residual solvent as an internal reference.

Mass spectrum electrospray ionization operated in positive mode using aLTQ Orbitrap XL Hybrid with Fourier transform (Thermo Scientific Instruments) coupled to a CLAE Thermo Instruments system (PDA Accela Detector, Accela automatic display and Accela pump, Thermo Scientific Instruments). The following conditions were used: capillary voltage, 4.5 kv; dry temperature, 260 °C; gas flow ratio 10-20 arbitrary units, desolvation gas stream 40-50 arbitrary units, pulverization tension 4.5 kV; mass range 100-1.000 m/z (maximum resolution 30.000).

Red macroalgae *D. marginata* was collected in December 2009, at the northern region of São Paulo, at the Fortaleza Beach, city of Ubatuba, Brazil (23º24'93 ' and 45º03'41"W), during low tide, and was identified by Nair Yakoya (Instituto de Botânica, São Paulo, Brazil). The exsicatum was deposited at the Herbarium of the Instituto de Botânica, São Paulo, Brazil (N° SP400960).

Identification of Penicillium citrinumand CITcharacterization

The endophytic fungus *P. citrinum* was isolated through a previously described standard procedure (32), from the internal tissue of red macroalgae *D. marginata*. After isolation, the pure culture of *P. citrinum* was sent to and classified by Anil Sazak (Ondokuz Mayıs University/ Samsun Turkey).

P. citrinum was grown in 5 Erlenmeyer flasks, each containing 90 g rice. After the incubation period, cultures were combined, ground and extracted using CH_3OH (5 x 250 mL). The solvent was evaporated, producing a raw extract of CH_3OH, then dissolved in CH_3CN and partitioned with hexane, generating extract acetonitrile (EACNPc). A portion (900 mg) of the *P. citrinum* acetonitrile extract (EACNPc) was fractioned using chromatography in a Sephadex LH-20 column (70 cm x 3.0 cm),eluted with MeOH:DCM (1:1), yielding 45 fractions of approximately 80 mL each. Analysis by CCD and CLAE-DAD/EM-IES [Column C18; MeOH:H_2O (5-100% MeOH), 254 nm], allowed for another sorting between 16 new groups of fractions, by chromatogram similarity (FDm1Se-FDm16Se). Fraction FDm9Se (79 mg), after washing with methanol, supplied us with substance CIT (30 mg).

CIT was solubilized in 10% DMSO and distilled water. In this study, CIT was administered at 0.5, 1.0 and 2 µg/mL. These concentrations were chosen from preliminary tests of cell viability with MTT assay in a macrophage culture of mice cell line RAW 264.7, as suggested by Andrade (33).

Sarcoma 180 primary culture

Sarcoma 180 (S-180) tumor maintenance procedures in *Swiss* mice were approved by the ethics committee on animal experimentation of UFPI (nº 167/16, ANNEX B). Tumor cells were maintained in mice through weekly intraperitoneal transfers. Ten days after inoculation, tumor cells-containingascitic fluid was removed by abdominal cavity puncture and centrifuged (500 g,

5 min, 4 °C) to obtain the cellular pellet. Then, S-180 cells were counted in Neubauer's chamber (4 x 10^6 cells/mL) and incubated in RPMI 1640 culture medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum and 1% (p/v) penicillin/streptomycin), kept in a stove at 37 °C for 72 h, as were test substance and controls.

Cell viability through MTT assay in a S-180 primary culture

The MTT {[3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium] bromide} assay was conducted following Mosmann (34). Cells were distributed in 96-wells plates, with 0.8x10^6 cells/mL. Both test substance and controls were incubated for 68 h along with S-180 cells. After 72 h of incubation period, we added to the cultures 20 µL of the MTT solution (5 mg/mL) and re-incubated them for 4 h in a stove at 37 ºC and 5% CO_2. Passed this period, the culture medium was discarded, and plates were cautiously stored away from light and left overnight for complete drying. Then, 100 µL of isopropyl alcohol was used to resuspend the precipitate. To quantify the reduced formazan salt in living cells, the absorbances were analyzed with the aid of the plate spectrophotometer, at the wavelength of 550 nm.

Study of genotoxicity mechanism through cotreatment with H_2O_2

Genotoxicity induction through measuring the oxidative stress performance was done according to the method described by Luz et al. (35). Briefly, S-180 cell suspension aliquots (10 µL; 0.5 x 10^6 cells/mL) were mixed with 0.75% low melting point agarose (90 µL) and placed on pre-coated slides with 1.5% regular agarose. Then, slides were exposed to CIT (2 µg/mL),by itself and with hydrogen peroxide (H_2O_2, 10 mM) for 5 min. Finally, slides were immersed in the lysis solution for 5 min, at 4 °C. Finally, thecomet and CBMN assays were performed according to the methods described by Speit and Rothfuss (36) and Fenech (37), respectively, with some modifications. Doxorubicin was used as positive control (PC) and olive oil as negative control (NC).

Statistical analysis

Absorbance data obtained from the MTT test were normalized and transformed into a logarithm and subjected to a non-linear regression to be found at LC_{50}. To define the differences between treatments, data expressed as mean±SD (standard deviation) were compared by analysis of variance (ANOVA), two-way and Bonferroni post-test, withvalues $p<0.05$ considered statistically significant. Graph Pad Prism software, version 6.0 for Windows (Graph Pad Software, La Jolla California USA). All studies were performed in duplicate.

Results and Discussion

Characterization of isolated CIT from P.citrinumacetonitrile extract

Analyses of RMN ^1H and ^{13}C led us to CIT identification, HRESIMS *m/z* $[M+H]^+$ 251.0917 (calculated for $C_{13}H_{14}O_5$) and *m/z* 273.0735 $[M+Na]^+$ (Figure 1). The substance structure was decoded when comparing with MS and ^1H and ^{13}C RMN databases, as also with data

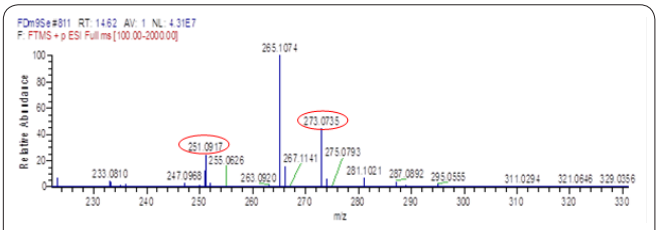

Figure 1. Mass spectrum (EM-IES (+)) of Citrinin.

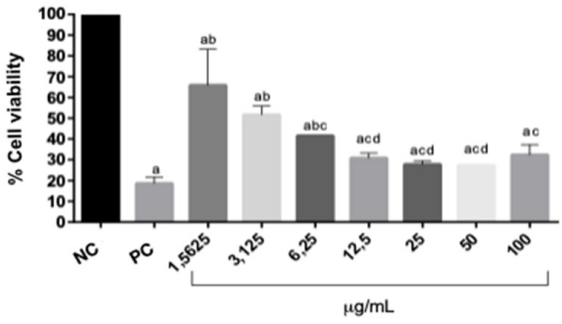

Figure 2. Citrinin (CIT) action on cell viability in S-180 ascitic fluid cells of mice. NC: negative control, untreated cells; PC: positive control, doxorubicin (2 µg/mL). CIT: citrinin. Log: logarithm. Different letters mean significant differences between groups: [a] $p<0.05$ compared to the NC group, [b] $p<0.05$ compared to the PC group, and [c] $p<0.05$ compared to the 1.56 group, [d] $p<0.05$ compared to a concentration of 3.12 µg/mL.

from the literature (38,39). CIT was obtained in the form of pointed crystals of orange color, UV λmax (MeOH) 318 nm. ^1H RMN ($CDCl_3$, 500 MHz) 8.23 (*s*, H-1), 4.76 (*qd*, *J*=6.8, H-3), 2.97 (*qd*, H-4), 1.23 (*d*, *J*=6.8, H-9), 1.35 (*d*, *J*=7.2, H-10), 2.01 (*s*, H-11).

Cytotoxic effects of CIT in S-180 cell viability

Natural products extracted from endophytic fungi have revealed to be promising sources of therapeutic agents (10). CIT obtained from *P. citrinum* acetonitrile extract presented a CI_{50} of 3.77 µg/mL (95% CI: 2.57-5.54 µg/mL, R^2=0.9066). Polyketides are among the main classes of natural cytotoxic products for tumor cells and, in this context, can be used as chemotherapeutics (40). Studies have point to CIT cytotoxicity through oxidative stress and apoptosis induction, via the ERK1 and ERK2 pathways, in addition to cell cycle arrest by acting on p53 and p21 signaling pathways (41-43), and triggering modifications in antioxidant enzymatic defenses (44).

S-180 cell viability was analyzed by the MTT assay 72 h after exposure. The PC, doxorubicin, and the other CIT concentrations significantly reduced cell viability when compared to NC. CIT concentrations between 12.5 and 100 µg/mL were as cytotoxic as PC (Figure 2), as already evidenced by other studies (45,46). CIT cytotoxic and antitumoral activities have been already assessed in other cancer cell lines, such as cervical cancer (HeLa), oral carcinoma (KB), prostate cancer (LNCap), liver (HepG2) and lung cancer (LU-1), and breast adenocarcinoma (MCF-7) cell lines (39,42,47).

Genotoxic effects of CIT in S-180 ascitic fluid cells of mice through comet assay

Investigations on genotoxicity can be done through the comet assay *in vitro,* and *ex vivo* by DNA damage assessment and repairing capacity (48), which in its al-

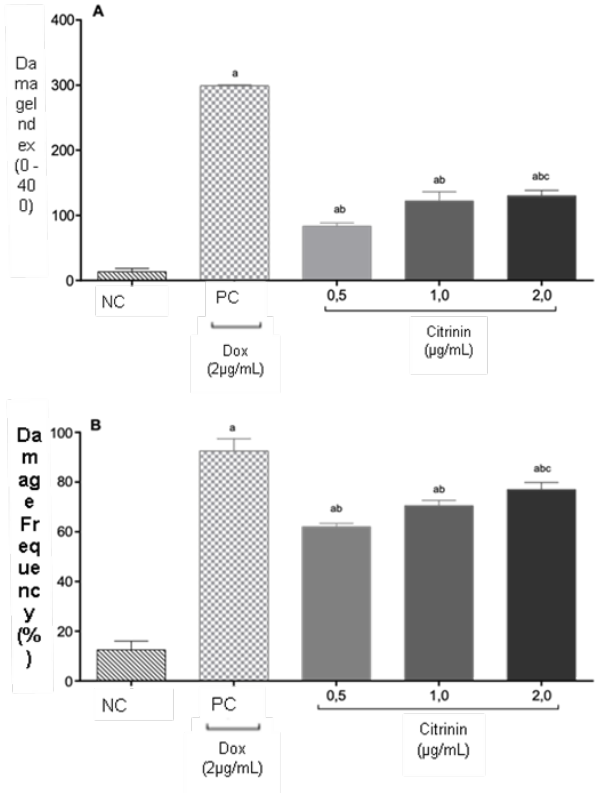

Figure 3. Genotoxicity in S-180 ascitic fluid cells of mice, after 72 h of exposure. **A)**Damage Index. **B)** Damage Frequency. NC: negative control, untreated cells; PC: positive control, doxorubicin (Dox) 2 µg/mL. CIT: Citrinin (0.5, 1.0 and 2 µg/mL). Different letters mean significant differences between groups: [a] $p<0.05$ compared to the NC group; [b] $p<0.05$ compared to the PC group; [c] $p<0.05$ compared to a concentration of 0.5 µg/mL.

kaline version can detect breaks in single and/or double strands of DNA and oxidative damage (49). CIT, in all tested concentrations, and specially in 2 µg/mL, induced genotoxic effects in S-180 cells, through increasing DNA damage index and damage frequency, when compared to NC. The higher concentration tested led to significant genotoxic damage in relation to the lower concentration **(Figure 3)**. Studies have shown that CIT can induce ROS-mediated DNA damage (50,51). Genotoxic damage can trigger genomic instability and provoke DNA alterations (52).

Assessment of oxidative damage induction as a CIT genotoxicity mechanism in S-180 cells in mice

In the genotoxic effects' analysis via comet assay, CIT induced, at the concentrations tested, increased

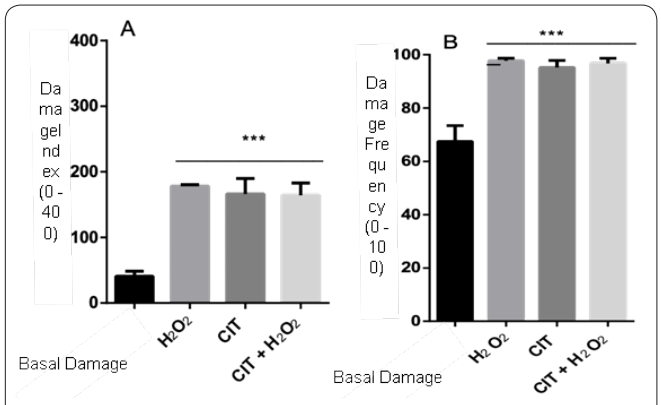

Figure 4. CIT genotoxicity by mechanisms associated with oxidative damage, in S-180 ascitic fluid cells co-treated with H_2O_2. In **A.** DNA damage index and in **B.** Damage frequency. Basal damage: negative control, untreated cells. H_2O_2 (10 mM): CIT: citrinin (2 µg/mL). Different letters mean significant differences between groups: [a] $p<0.05$ compared to the NC group, [b] $p<0.05$ compared to the H_2O_2 group 10 and [c] $p<0.05$ compared to the CIT group.

damage index and DNA damage frequency in S-180 cells. Moreover, in the searchforgenotoxicity mechanisms in S-180 ascitic fluid cells, in the challenge assay with H_2O_2, no statistical differences were observed between CIT and H_2O_2, as well as between CIT and CIT combined with H_2O_2. However, statistical differences were observed in relation to baseline damage of S-180 ascitic fluid tumoral cells (**Figure 4**). Indeed, the stressor agent (H_2O_2) is a marker of H_2O_2-induced damage (53). Nonetheless, it is worth mentioning that CIT has a free OH group in its carbons (C8 and C12) and is capable of inducing DNA damage through OH radical formation, thus leading to oxidative damage (51), as so happens in the H_2O_2 mechanism of oxidative stress induction (54).

Antitumoral effects of CIT through cytogenetic damage to S180 ascitic fluid cells of mice assessed by the CBMN assay

CITgenerated mutagenic effects in S180 cells at all tested concentrations by inducing nuclear changes,namely increasing micronuclei (MN), the number of nucleoplasmic bridges and nuclear buds, especially at higher concentrations, when compared with untreated (NC) cells (Table 1). As observed in other studies, CIT induced MNformation in HepG2 cell lines (55). These alterations characterize mutations resulting from the installation of chromosome lesions, non-disjunction during anaphase, gene amplification, necrosis

Table 1. CIT-induced mutagenicity inof S-180 cells at different concentrations (0.5; 1.0 and 2.0 µg/mL) evaluated through the cytokinesis-block micronucleus assay (CBMN).

	Mutagenic Damage and Cytogenetic Alterations		
Treatments	**Micronuclei**	**Nucleoplasmic Bridges**	**Nuclear Buds**
NC	6.00 ± 2.10	4.67 ± 2.52	7.00 ± 2.00
PC	27.67 ± 1.15^{***a}	39.67 ± 2.08 ^{***a}	26.67 ± 1.15 ^{***a}
CIT 0.5 µg/ml	11.00 ± 1.10^{***b}	9.00 ± 1.73^{***b}	11.00 ± 5.29^{***b}
CIT 1.0 µg/ml	15.33 ± 5.50^{**a***b}	11.33 ± 1.15^{***b}	28.67 ± 1.53^{***a}
CIT 2.0 µg/ml	24.67 ± 3.06^{***a}	14.00 ± 4.10^{*a***b}	27.33 ± 1.15^{***a}

*p < 0.05; ** p < 0.01; *** p<0.001; differences statistically different a to the negative control (NC), b to the positive control (PC).

Table 2. Nuclear division index (NDI), nuclear damage index indicative of cytotoxicity (NDIC), apoptosis and cell necrosis frequency in S-180 cells, induced by CI, assessed through the cytokinesis-block micronucleus assay (CBMN).

			ToxicogeneticDamage	
Treatments	**NDI**	**NDIC**	**Apoptosis**	**Necrosis**
NC	1.65 ± 0.02	1.60 ± 0.01	29.33 ± 2.31	16.33 ± 2.52
PC	1.19 ± 0.02^{***a}	1.18 ± 0.02^{***a}	302 ± 17.44	109 ± 11.53
CIT 0.5 µg/mL	1.55 ± 0.06^{***b}	1.49 ± 0.02^{***b}	29.67 ± 8.1^{***b}	17.6 ± 2.08^{***b}
CIT 1.0 µg/mL	1.34 ± 0.06^{**a**b}	1.31 ± 0.05^{**a**b}	60.67 ± 4.04^{**a***b}	37 ± 2.65^{*a***b}
CIT 2.0 µg/mL	1.27 ± 0.02^{**a*b}	1.22 ± 0.07^{***a}	67.33 ± 11.3^{***a***b}	45.67 ± 3.21^{**a***b}

*p<0.05; ** p<0.01; *** p<0.001; differences statistically different a to the negative control (NC), b to the positive control (PC).

and apoptosis, which results in MN formation (mainly from chromosomal fragments or whole chromosomes breaks, unsegregated in themitotic spindle during cell division) (56). It is worth noting that chromosomal alterations contribute to MN formation, also viewed as a biological marker of cancer (57).

MNs are present in cells that divide that have chromosome breaks without centromeres (acentric fragments) and/or whole chromosomes, unable to move to the cell poles over the cell cycle and become encapsulated by the nuclear envelope during telophase (58); thus, increased MN frequency is associated with deficiencies in responses to DNA lesions. Nucleoplasmic bridges and nuclear buds are important markers to assess cytogenetic alterations. In this way, nucleoplasmic bridges are derived from dicentric chromosomes (providing an additional measure of chromosome rearrangement), whereas nuclear buds represent a mechanism in which cells remove amplified DNA, functioning as an important marker of gene amplification (59,60). Several gene amplification manifestations are associated with a malignant phenotype and may appear during the cell cycle (61). Chromosomal instability contributes to human neoplasms through poor chromosome disjunction and aneuploidy, in addition to DNA damage, mutations and chromosome translocations (1), which may culminate in apoptosis. Briefly, apoptosis is a mechanism of programmed cell death, composed of nucleus fragmentation, chromatin condensation, and chromosomal DNA disintegration (62). CIT was also able to induce cytotoxicity at concentrations of 1 and 2 µg/ mL, through reducingthe nuclear division index (NDI) and nuclear damage index indicative of cytotoxicity (DNIC), when compared to NC. These data indicate that CIT cytotoxic effects are related to cytogenetic damagesthat occur by induction of mutagenic nuclear alterations, which can lead to genetic instability and cell death. In addition, it should be emphasized that CIT induced similar effects for DNIC, MN and nuclear buds on cells treated with doxorubicin, especially at the highest concentration (**Table 2**). Several studies have indicated that tumor cell growth inhibition by apoptosis is an important phenomenon for antitumor therapy, as it stimulates cell death by reducing more complex inflammatory events and consequently limiting cytotoxic effects in healthy tissues (13,63).

CIT, at high doses (140 and 420 mg/kg), induces oxidative stress through reactive oxygen species (ROS) overproduction and mitochondrial loss through the liberation of the cytochrome-*c* complex, leading to apoptosis, as well as lipid peroxidation (64) and may be related to increased *Bax-Bcl2* ratio, membrane potential loss, cytochrome-*c*induction and caspases activation (18). Studies have related CIT cytotoxicity with apoptosis induction (18,65) and necrosis induction in hepatic cells (66,67), while others have also pointed that the CIT-induced apoptotic signaling is convergent to the mitochondrial-dependent pathway (20,21,50,68,69), through ROS increase, as the main inducer of apoptotic signaling (12).

The intrinsic apoptotic pathway (mitochondria-dependent) is mediated by intracellular signals, such as cytogenetic damage, high concentrations of cytosolic calcium and oxidative stress, that converge at mitochondrial level in response to different stress conditions and function as triggers for mitochondrial pathway initiation (70). The immediate activation of pro-apoptotic members of the Blc-2 family neutralizes the anti-apoptotic proteins Bcl-2 and Bcl-xL (both responsible for controlling mitochondrial membrane permeability), thus allowing the rupture of mitochondrial external membrane permeability, and therefore proteins from the intermembranous space spread in the cytosol (62). These proteins include what we call apoptogenic factors, such as cytochrome-*c*, which play a key role in mitochondrial-dependent apoptosis activation (70).

CIT, at all tested concentrations, and specially at 2.0 µg/mL, exerted antitumor and cytotoxic effects in primary cultures of S-180. The antitumor effect was observed through several cytogenetic mechanisms, which lead to: (i) cytotoxicity induction by reducing cell viability; (ii) genotoxicity induction by triggering oxidative stress; (iii) clastogenic and/or aneugenic events due to MN induction, as well as nucleoplasmic bridges and nuclear buds formation; (iv) cytotoxicity by means of nuclear abnormalities when observing NDI and NDIC reduction, which led to apoptosis and necrosis. This study also points to the fact that oxidative mechanisms may be implicated in cytogenetic alterations involved in CIT antitumor activity.

Conflict of interest

None declared.

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