DANIELA REIS JOAQUIM DE FREITAS
(ORGANIZADORA)

AGENDA GLOBAL DE PESQUISA EM CIÊNCIAS BIOLÓGICAS

Ano 2021

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(ORGANIZADORA)

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Atena Editora
Ponta Grossa – Paraná – Brasil
Telefone: +55 (42) 3323-5493
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APRESENTAÇÃO

A pesquisa não pode parar. Isto é um fato. E o livro “Agenda global de Pesquisa em Ciências Biológicas” é a prova de que o Brasil é profícuo quando se trata de pesquisa. Esta obra é composta por trabalhos científicos produzidos em diversas partes do país na forma de artigos originais e de revisão, que abordam desde o cultivo, triagem e citocompatibilidade de células-tronco mesenquimais expostas à nanotubos funcionalizados de carbono multicamadas até o controle de qualidade microbiológica do sururu (*Mytella falcata*) produzido no Rio de Janeiro, ou a análise temporal da disseminação de vegetação exótica em dunas do litoral do Rio Grande do Sul, ou o desenvolvimento do turismo e as mulheres erveiras da Amazônia. Todas estas pesquisas possuem campo dentro das Ciências Biológicas, mas fazem interface com meio Ambiente, Engenharia, Ciências da Saúde, Antropologia, Tecnologia de alimentos, entre outras áreas.

Ao longo de 13 capítulos serão discutidas diferentes temáticas, com embasamento teórico-científico adequado, atualizado e serão revistos conceitos importantes. Este livro é principalmente voltado para os estudantes e profissionais que desejam se aprofundar mais na pesquisa na grande área das Ciências Biológicas, com uma leitura rápida, dinâmica e cheia de possibilidades de aprendizado.

Assim como todas as publicações da Atena Editora, esta obra passou pela revisão de um Comitê de pesquisadores com mestrado e doutorado em programas de pós-graduação renomados no Brasil. Portanto, apresentamos ao leitor um trabalho de qualidade, atualizado e devidamente revisado por pares.

Boa leitura.

Daniela Reis Joaquim de Freitas

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CYTOTOXIC AND GENOTOXIC EFFECTS OF THE GLUTARIMIDE ALKALOID JULOCROTINE

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Regianne Maciel dos Santos Correa
Laboratório de Citogenética Humana, Instituto de Ciências Biológicas, Universidade Federal do Pará
Belém – Pará
https://orcid.org/0000-0002-9837-4304

Plínio Cerqueira dos Santos Cardoso
Laboratório de Citogenética Humana, Instituto de Ciências Biológicas, Universidade Federal do Pará
Belém – Pará
http://lattes.cnpq.br/9371025014746575

Lorena Araújo da Cunha
Laboratório de Citogenética Humana, Instituto de Ciências Biológicas, Universidade Federal do Pará
Belém – Pará
https://orcid.org/0000-0002-1085-3635

Tatiane Cristina Mota
Laboratório de Citogenética Humana, Instituto de Ciências Biológicas, Universidade Federal do Pará.
Belém – Pará
https://orcid.org/0000-0001-8127-8503

Diego Di Felipe Ávila Alcantara
Laboratório de Citogenética Humana, Instituto de Ciências Biológicas, Universidade Federal do Pará.
Belém – Pará
https://orcid.org/0000-0001-5045-2265

Giselle Maria Skelding Pinheiro Guilhon
Laboratório de Química, Instituto de Ciências Exatas e Naturais, Universidade Federal do Pará.
Belém – Pará
https://orcid.org/0000-0002-9257-8998

Rosana de Nazaré Silva Peixoto
Laboratório de Química, Instituto de Ciências Exatas e Naturais, Universidade Federal do Pará.
Belém – Pará
http://lattes.cnpq.br/8910370386641451

Rommel Rodriguez Burbano
Laboratório de Citogenética Humana, Instituto de Ciências Biológicas, Universidade Federal do Pará.
Belém – Pará
https://orcid.org/0000-0002-4872-234x

Marcelo de Oliveira Bahia
Laboratório de Citogenética Humana, Instituto de Ciências Biológicas, Universidade Federal do Pará.
Belém – Pará
https://orcid.org/0000-0001-6820-6521

ABSTRACT: Natural products are potential drugs candidates to treat several pathologies. However, some of these compounds can be toxic to the body and may even have genotoxic properties, causing changes in DNA with consequent increased risk of carcinogenesis. Julocrotine (2-[N-(2-methylbutanolyl)]-N-phenylethylglutarimide) is a natural glutarimide alkaloid isolated mainly from *Croton species* (Euphorbiaceae). *In vitro*

studies have shown that this alkaloid is an antiproliferative agent against the promastigote and amastigote forms of *Leishmania* (L.) *amazonensis.* Thus, the present study aimed to evaluate the cytotoxic and genotoxic effects of julocrotine using the MTT, the comet Assay and the DCFH-DA assay for reactive oxygen species (ROS) assessment in cultured human lymphocytes. The tested alkaloid was isolated from *Croton pullei* Lanj., a species collected in the Amazon Region. Our results showed that julocrotine is not cytotoxic to human lymphocytes at all tested concentrations, including the concentration that it is active against *L. amazonensis*. However, it was genotoxic to human lymphocytes. In addition, an increase in the rate of ROS was also observed. Although julocrotine was not cytotoxic in the assessed conditions, the genotoxic effect observed reinforces the need of caution for its possible use as a drug to treat not only leishmaniasis, but any other pathology.
KEYWORDS: Leishmaniasis; Julocrotine, Comet assay; Cytotoxicity; Reactive oxygen species.

EFEITOS CITOTÓXICOS E GENOTÓXICOS DO ALCALÓIDE GLUTARIMIDA JULOCROTINA

RESUMO: Os produtos naturais são potenciais candidatos a medicamentos para o tratamento de diversas patologias. Porém, alguns desses compostos podem ser tóxicos para o organismo e podem até apresentar propriedades genotóxicas, causando alterações no DNA com consequente aumento do risco de carcinogênese. Julocrotina (2- [N- (2-metilbutanolil)] - N-feniletilglutarimida) é um alcalóide glutarimida natural isolado principalmente de espécies de *Croton* (Euphorbiaceae). Estudos *in vitro* demonstraram que este alcalóide é um agente antiproliferativo contra as formas promastigota e amastigota de *Leishmania* (L.) *amazonensis*. Assim, o presente estudo teve como objetivo avaliar os efeitos citotóxicos e genotóxicos da julocrotina por meio do MTT, ensaio do cometa e ensaio do DCFH-DA para avaliação de espécies reativas de oxigênio (ROS) em cultura de linfócitos humanos. O alcalóide testado foi isolado de *Croton pullei* Lanj., Espécie coletada na região amazônica. Nossos resultados mostraram que a julocrotina não foi citotóxica para os linfócitos humanos em todas as concentrações testadas, incluindo a concentração que é ativa contra *L. amazonensis*. No entanto, foi genotóxico para os linfócitos humanos. Além disso, também foi observado um aumento na taxa de ROS. Embora a julocrotina não tenha sido citotóxica nas condições avaliadas, o efeito genotóxico observado reforça a necessidade de cautela quanto ao seu possível uso como medicamento para tratar não só a leishmaniose, mas qualquer outra patologia.
PALAVRAS - CHAVE: Leishmaniose; Julocrotina; Ensaio do cometa; Citotoxicidade; Espécies reativas de oxigênio.

1 | INTRODUCTION

Brazil has an abundant flora, popularly explored in the prevention, treatment, and cure of different diseases. Among these pathologies, one can mention fungal infections, malaria, diabetes, high cholesterol levels, and certain types of cancers (COELHO-FERREIRA, 2009; CARTAXO *et al.,* 2010). Nevertheless, it is important to note that some constituents of plants

can be toxic to the body. For example, plants used for therapeutic purposes may contain genotoxic properties that can cause changes in DNA (OYEYEMI, 2015). Even though ROS are produced during normal aerobic metabolism in mammalian cells, they are involved in cellular and tissue damage and the free radicals produced by oxidative processes can attack DNA bases or sugars, causing breakage of single strands that can evolve into double-strand breaks and abasic sites in DNA (MARSHALL; BARROWS, 2004; MARSHALL *et al.*, 2009). In that way, it is important to evaluate cytotoxic and genotoxic activities of natural products before their use in any kind of treatment.

Julocrotine (Figure 1) is a natural product included in a small group of alkaloids known as glutarimide alkaloids that have been isolated mostly from Euphorbiaceae species. It was first identified from *Julocroton montevidensis* (NAKANO *et al.,* 1961) and from some *Croton* species, such as *C. membranaceus* (ABOAGYE *et al.,* 2000), *C. cascarilloides* (CUONG *et al.* 2002), *C. cuneatus* (SUAREZ *et al.*, 2004), *C. pullei* var. *glabrior* Lanj. (BARBOSA *et al.*, 2007), among other *Croton* species. More recently, it was isolated from a *Cordia* species (Boraginaceae) (PARKS *et al.*, 2010). Julocrotine is the most common glutarimide alkaloid and therefore the most cited one. Stuart *et al.* (1973), Teng *et al.* (2011), Neves Filho *et al.* (2011), and Silva and Joussef (2011) have synthesized julocrotine and other glutarimide alkaloids.

Figure 1: Chemical structure of julocrotine, a glutarimide alkaloid isolated from Croton pulleivar. *glabrior* (GUIMARÃESet al.,2010).

Guimarães and coworkers (2010) have shown the inhibitory *in vitro* effect of julocrotine on the growth of promastigote and amastigote forms of *Leishmania amazonensis* (L.) with no cytotoxicity against the host cell. Taking into account the observed antileishmanial activity of julocrotine, together with other biological activity that this alkaloid can show, with the possibility to be used in the future for different treatments, this study aimed to evaluate the cytotoxic and genotoxic effects induced by this compound in human lymphocytes using the MTT test, comet assay and DCFH-DA assay for reactive oxygen species (ROS) assessment.

2 I METHODS

2.1 Plant extraction and isolation

Stems of *Croton pullei* Lanj. (sin. *Croton pullei* var. *glabrior*) (SECCO *et al*., 2008) were collected in the municipality of Peixe-Boi, State of Pará, Brazil. A voucher specimen (MG 188,908) was deposited in the herbarium of the Museu Paraense Emílio Goeldi (Belém - PA - Brazil). The botanic material (1,000 g) was extracted with hexane and methanol, successively, at room temperature, yielding the hexane (0.65 g) and methanol (80.00 g) extracts, after concentration under vacuum. From the hexane solution, a white solid precipitated during concentration of the hexane solution. After filtration, the solid was purified by crystallization from a 5% hexane-EtOAc solution, yielding julocrotine (2.50 g). The methanol extract (20.00 g) was suspended in a 3:1 methanol – water solution and successively partitioned with CH_2Cl_2, EtOAc and *n*-BuOH. The concentrated CH_2Cl_2 phase (5.00 g) was fractionated by column chromatography on silica gel using mixtures of hexane, ethyl acetate and methanol in gradients of increasing polarities as mobile phase. The fraction eluted with hexane-EtOAc 27% from the column yielded additional quantity (1.00 g) of julocrotine. The structure of the alkaloid julocrotine (N-(2,6-dioxo-1-phenethyl-piperidin-3-yl)-2-methyl-butyramide), also named as 2-[N-(2-methylbutanoyl)]-N-phenylethylglutarimide, was proposed from NMR spectrometry data. When first isolated, structure confirmation of julocrotine was proposed based on NMR, IR spectrometry and X-ray cristallography data (MOREIRA *et al*., 2008). The drug was dissolved in methanol. For the experiments, julocrotine was diluted in the culture medium (RPMI) until the desired concentrations.

2.2 Culturing Isolated Lymphocytes

Blood samples (20 mL) were obtained by venipuncture from three healthy 20 to 30-year old non-smokers, two females and one male, with no recent history of exposure to mutagens. This study followed the principles of the local ethics committee (Instituto de Ciências da Saúde - Universidade Federal do Pará) and the donors gave written informed consent to participate in the study after a full explanation of the purpose, nature and risk of all procedures. Blood lymphocyte isolation was carried out using the procedure described by Fenech (2000). $1x10^6$ cells/mL were seeded in 5 mL of RPMI 1640 medium (Cultilab, Campinas, SP, Brazil) supplemented with 20% fetal bovine serum (Cultilab, Campinas, SP, Brazil), 4% phytohemagglutinin A (Gibco-Invitrogen, Carlsberg, CA, USA), 0.05 mg/mL gentamicin sulfate (Cultilab, Campinas, SP, Brazil) and 2.16 μM amphotericin B. Cells were cultured at 37°C in an incubator containing 5% CO_2.

2.3 MTT assay

For the MTT assay, isolated lymphocytes were grown in 96-wells culture plates at concentration of 0.5 x 10^6 cells/well and incubated for 24 hours. After the initial period of

incubation, cells were treated with different concentrations of julocrotine for 24 hours. After treatment, 100 µl of MTT (5000 µg/mL) were added to the cells for 3 hours. Then, the MTT was removed and 100 µl of dimethylsulfoxide (DMSO, Sigma®) was added for 1 hour in order to dissolve the formazan obtained during the process. Afterward, DMSO was measured by spectrophotometry (λ= 562 nm). The cell survival was calculated as the absorbance percentage compared to the control absorbance. The julocrotine concentrations used were 19.75; 39.5; 79; 158; 316; 632 and 1264 *µ*M. These concentrations were chosen based on data from the literature (16). *N*-Nitroso-*N*-methylurea (NMU), a known carcinogenic alkylant was used as positive control.

2.4 Comet assay (alkaline version)

For the alkaline version of the comet assay, lymphocytes were grown in sterile 12-wells culture plates (Corning) at a concentration of $1x10^6$ cells/well and treated with different concentrations of julocrotine (79, 158, 316 and 632 *µ*M) for 24 hours. Doxorubicin (5.52 *µ*M) was used as positive control. After treatment, 450 *µ*L of the cell suspension were homogenized with 300 µL of a low-melting-point agarose (0.8%), spread onto microscope slides pre-coated with a normal-melting-point agarose (1.5%) and covered with a coverslip (24x60 mm). After 5 minutes at 4°C, the coverslip was removed and the slides were immersed in cold lysis solution (2.5M NaCl; 100 mM EDTA; 10 mM Tris, 10% DMSO and 1% Triton-X, pH=10). After lysis, the slides were placed in an electrophoresis chamber and covered with freshly made electrophoresis buffer (300 mM NaOH; 1 mM EDTA, pH>13). The electrophoresis was run for 25 minutes (34 V and 300 mA). Afterward, the slides were neutralized by submersion in distilled water (4°C) for 5 minutes and fixed in 100% ethanol for 3 minutes. Staining of the slides was performed immediately before the analyses using ethidium bromide (20 µg/mL). Slides were prepared in duplicate, and 100 cells were screened per sample (50 cells from each slide) using a fluorescent microscope (Olympus BX41) at 40x magnification. The DNA damage index (DI) (i.e. the relative intensity of fluorescence in the comet's tail in function of the frequency of DNA breaks) was visually determined. The following five categories (0-4) were used: class 0 (no damage), class 1 (little damage with a tail length shorter than the diameter of the nucleus), class 2 (medium damage with a tail length one or two times the diameter of the nucleus), class 3 (significant damage with a tail length one or two times the diameter of the nucleus), and class 4 (significant damage with a tail length greater than three times the diameter of the nucleus). Damage index was determined by the following formula:

*DI (au): [(N1*1 + N2*2 + N3*3 + N4*4)] / 100 (total number of analyzed cells)*

where DI = DNA damage index, au = arbitrary unit, N1 - N4 = cells in classes 1, 2, 3 and 4.

2.5 Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay

Intracellular ROS generation was evaluated using the fluorescent probe dichlorofluorescein diacetate (DCFH-DA) (Sigma Chemical Co. / St. Louis, MO, USA). Lymphocytes were grown in sterile 12-wells culture plates (Corning) ($1x10^6$ cells/well) and exposed to julocrotine at different concentrations (316 and 632 μM) for 3 h at 37°C. Thereafter, cells were collected by centrifugation and washed in PBS at 1000 rpm for 5 min. After a new centrifugation, cells were suspended in PBS and DCFH-DA was added to a final concentration of 10 μM. The suspension was incubated in dark for 30 min at 37°C and after a new wash in PBS, the samples were analyzed by spectrophotometry with an emission wavelength of 528 nm and an excitation wavelength of 485 nm. H_20_2 (2mM) was used as positive control.

2.6 Statistical analysis

For parametric data sets, statistical analysis was performed using ANOVA, followed by the Tukey test. For nonparametric data sets, we used Kruskal-Wallis test. The BIOESTAT 5.0 software (AYRES *et al.*, 2007) was used to perform statistical analyses. *P*-values of <0.05 were considered significant.

3 I RESULTS

3.1 MTT assay

The cell viability showed no significant decrease in the lymphocytes survival rates after a 24 hours treatment with julocrotine at all tested concentrations (Figure 2). Using the same experimental conditions, the positive control (NMU) showed a statistically significant decrease in the survival rates. The cell viability values were 69.7, 78.4, 70.6, 64.2, 51.9, 45.7, and 11.7% for the NMU and 121.3, 109.2, 115.4, 105.4, 115.2, 107.2, and 108.2% for julocrotine using concentrations of 19.75, 39.5, 79, 158, 316, 632, and 1264 μM, respectively.

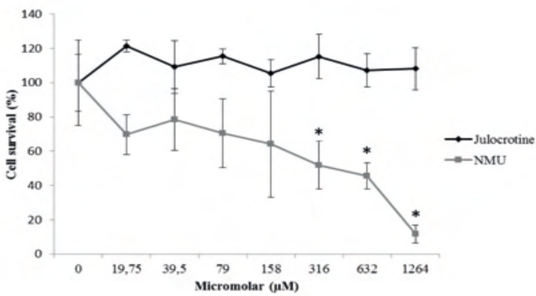

Figure 2: Effects of Julocrotineand NMU (positivecontrol) in human lymphocyteculture analyzed by MTT assay. *p<0.05 (ANOVA/Tukeypost-test) when compared with control. Data are expressed as the mean values obtained from three experiments.

3.2 Comet assay

The comet assay with julocrotine showed that this compound is genotoxic to human lymphocytes only at high concentrations. A dose-response increase of the DNA damage (Damage index = DI) was observed (Figure 3). However, a significant increase ($p<0.05$) of DI was observed only at 632 μM (DI=1.76), when compared to control (DI=0.78).

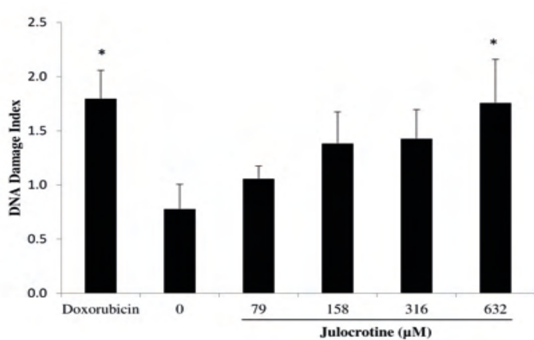

Figure 3: Effects of Julocrotine in human lymphocytes analyzedby comet assay. *$p<0.05$ (ANOVA/Tukey post-test) when compared with control. Data are expressed as the mean values obtained from three experiments; n=3.

3.3 ROS induction

ROS production experiments indicated that there was a significant ($p<0.05$) increase in the rate of such molecules in all tested concentrations (316 μM and 632 μM of julocrotine) in comparison to the negative control (Figure 4).

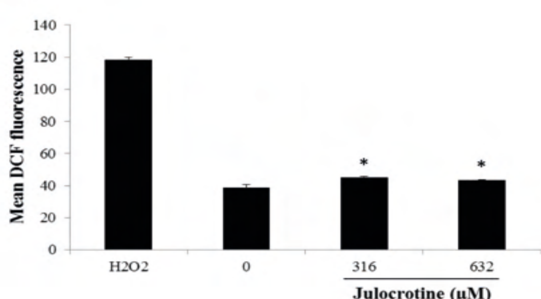

Figure 4: Mean fluorescence intensity ofhuman lymphocytes stained with ROS indicator DCFH-DA after exposition to julocrotine. *$p<0.05$ (ANOVA/Tukey post-test) when compared with control. Data are expressed as the meanvalues obtained from thre

4 I DISCUSSION

The literature shows that alkaloids cytotoxicity is quite variable, as it is expected due to the variety of their structures. In addition to many types of alkaloids, little structures differences can affect most of the biological activities.

There are few cytotoxicity studies of glutarimide alkaloids and no one deals with effects on lymphocytes. Suarez and coworkers have observed that julocrotol and isojulocrotol (two glutarimide alkaloids isolated from *C. cuneatus*, which structures are very close to julocrotine), were reported to exhibit cytotoxic activity against MCF-7 (human breast carcinoma) and Hep-G2 (hepatoma). Julocrotine was also tested in the same study, but the results were considered not significant (SUAREZ *et al*., 2004). A very high activity of julocrotine on the brine shrimp lethality test (*Artemia salina*) was reported (KAPINGU *et al.,* 2005). Antimicrobial effects of julocrotine (against *S. aureus, B. subtilis, E. coli, P. aeruginosa, A. niger, C. albicans*) have been reported, but they were considered insignificant (BAYOR *et al.,* 2009).

The studies of Guimaraes and coworkers have shown that julocrotine (at 79 μM) was not toxic to mouse peritoneal macrophages using MTT assays when treated for periods of 24, 48, and 72 hr (GUIMARÃES *et al*., 2010). According to the same authors, julocrotine showed antileishmanial activity in promastigotes and amastigotes of *L. amazonensis* when tested *in vitro* at concentrations ranging from 79 to 316 μM, and these findings indicated that julocrotine has the ability to diffuse through cell membranes being cytotoxic for intracellular parasites, although not to the host cell. Our results showed that julocrotine did not induce cytotoxic effects in lymphocytes, even at high concentrations (i.e. 1264 μM) and these results do not prevent a possible use of julocrotine on antileishmanial chemotherapy, which has been suggested by Guimarães and coworkers.

In order to compare the cytotoxic effects some alkaloids caused on lymphocytes, using similar experiments, some examples are cited. Cavalcanti *et al*. (2008) showed that the ingenamine G, an alkaloid isolated from the species *Pachychalina alcaloidifera*, induced a moderate cytotoxic effect in human lymphocytes after 24 h of treatment, using the MTT test. Chakraborty *et al*. (2004) showed that the extract of total alkaloids isolated from the root of *Tiliacora racemosa* (a medicinal plant used in India) was not cytotoxic to human lymphocytes; however, the extract proved to be cytotoxic to a panel of four cancer cells, namely, HL-60 (acute leukemia), K-562 (chronic leukemia), MCF-7 (breast adenocarcinoma), and HeLa (cervical carcinoma).

This is the first study dealing with genotoxic effects and ROS production of a glutarimide alkaloid. In the present study, julocrotine induced a genotoxic effect in human lymphocytes only at a high concentration (632 μM). Several authors using the same test have shown genotoxic effects of alkaloids. As an example, Cavalcanti *et al*. (2008) observed that the alkaloid ingenamine G extracted from the *Pachychalina alcaloidifera* significantly

increased the rate of DNA damage in human lymphocytes at concentrations ranging from 15 to 20 μg/mL. The alkaloid vincristine, an anticancer drug with genotoxic effects, induced significant damage to the DNA of human lymphocytes at (WEI *et al*., 2008). Furthermore, Kleinsasser and coworkers found that the alkaloid myosmine induces significant damage in the DNA of tonsil cells and human lymphocytes cells at concentrations ranging from 10 to 50 mM (KLEINSASSER *et al.,* 2003).

Julocrotine also increased the rate of ROS production. Some alkaloids are also known as of oxidative stress inducers. For example, an induction of ROS was observed in human glioblastoma cells (T98G) after treatment with berberine, an alkaloid with antitumor properties (EOM *et al*., 2010). The alkaloid nicotine is also capable of inducing oxidative stress in cells of the central nervous system (NEWMAN *et al*., 2002), in cells of the rat brain (BHAGWAT *et al*., 1998) and in human spermatozoa (ARABI, 2004).

The increased of the rate of ROS caused by julocrotine is in accordance with the studies of Guimarães and coworkers that suggested that one of the probable mechanisms by which julocrotine induces leishmanicidal effects could be the induction of ROS (GUIMARÃES *et al.,* 2010). It is tempting to assume that the genotoxic effects induced by julocrotine in the present study may be due to the ability of alkaloids to induce ROS that can damage DNA, however, further studies are needed in order to confirm such hypothesis, once other mechanisms must also be considered. Some substances, including alkaloids, have the ability to induce aneugenic effects through its interaction with the microtubule protein (GONZÁLEZ-CID *et al.,* 1999). In addition, pyridoacridine alkaloids, besides inducing ROS, have the ability to intercalate with the DNA and change the topology of this molecule, and change the way DNA metabolism enzymes interact with its substrate, which can inhibit many of the processes of metabolism of this molecule, including synthesis and topoisomerization (MARSHALL; BARROWS, 2004). Further studies are needed to define mechanism of the observed genotoxic effects of julocrotine.

The present study has shown no cytotoxic effect of julocrotine at all tested concentrations, but a clear genotoxic effect on lymphocytic cells, although at a very high concentration (632 μM). These observations reinforce the need of caution before the use of this substance as a therapeutic agent, including its possible use as antileishmanial chemotherapy.

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