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☑ Journal of Ethnopharmacology

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0378-8741	JOURNAL OF ETHNOPHARMACOLOGY	ADMINISTRAÇÃO PÚBLICA E DE EMPRESAS, CIÊNCIAS CONTÁBEIS E TURISMO	A1
0378-8741	JOURNAL OF ETHNOPHARMACOLOGY	ANTROPOLOGIA / ARQUEOLOGIA	A1
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15 September 2019

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Reviews

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Ethnobotanical uses, biological activities and chemical properties of Kei-apple [*Dovyalis caffra* (Hook.f. & Harv.) Sim]: An indigenous fruit tree of southern Africa

Adeyemi Oladapo Aremu, Khayelihle Ncama, Abiodun Olusola Omotayo

Article 111963

Research Papers

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The antithrombotic activity of the active fractions from the fruits of *Celastrus orbiculatus* Thunb through the anti-coagulation, anti-platelet activation and anti-fibrinolysis pathways

Jingjing Zhou, JianXiu Zhai, Wenlin Zheng, Na Han, ... Jun Yin

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Dalspinin isolated from *Spermacoce hispida* (Linn.) protects H9c2 cardiomyocytes from hypoxic injury by modulating oxidative stress and apoptosis

R. Lakshmi Sundaram, Hannah R. Vasanthi

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Bioactivities of serotonin transporter mediate antidepressant effects of *Acorus tatarinowii* Schott

Feng-Hong Zhang, Zhi-Mei Wang, Yan-Ting Liu, Ji-Sheng Huang, ... Yan-Tong Xu

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Mass spectrometry-based urinary metabolomics for the investigation on the mechanism of action of *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim. leaves against ischemic stroke in rats

Rongjin Wang, Liqiang Shi, Shu Liu, Zhiqiang Liu, ... Zhongying Liu

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Carica papaya seed extract slows human sperm

V. Ghaffarilaleh, D. Fisher, R. Henkel

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Ethnopharmacology in the Upper Guadiana River area (Castile-La Mancha, Spain)

Diego Rivera, Alonso Verde, José Fajardo, Concepción Obón, ... Emilio Laguna

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Protective effect of Shaoyao Decoction against colorectal cancer via the Keap1–Nrf2–ARE signaling pathway

Xiaoyan Wang, Shakir M. Saud, Xiwen Zhang, Weidong Li, Baojin Hua

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Toxicological findings about an anticancer fraction with casearins described by traditional and alternative techniques as support to the Brazilian Unified Health System (SUS)

Paulo Michel Pinheiro Ferreira, Denise Barbosa Santos, Jurandy do Nascimento Silva, Amanda Freitas Goudinho, ... Claudia Pessoa

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Abstract | Graphical abstract

Abstract

Ethnopharmacological relevance

Extracts, essential oils and molecules from *Casearia sylvestris* have popularly shown pharmacological actions against chronic diseases, as anxiety, inflammation, cancer and dyslipidemia. In the context of antitumoral therapy, we investigated *in vitro*, *ex vivo* and *in vivo* toxicological changes induced by a Fraction with Casearins (FC) and its

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Xiaopeng Ai, Ya Hou, Xiaobo Wang, Xiaoyan Wang, ... Qi'en Li
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The effect of isoflavaspidic acid PB extracted from *Dryopteris fragrans* (L.) Schott on planktonic and biofilm growth of dermatophytes and the possible mechanism of antibiofilm

Haoqi Lin, Xueping Liu, Zhibin Shen, Wanqiu Cheng, ... Tao Jiang
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Hazard assessment of *Maerua subcordata* (Gilg) DeWolf. for selected endpoints using a battery of *in vitro* tests

Mebrahtom Gebrelibanos Hiben, Lenny Kamelia, Laura de Haan, Bert Spenkelink, ... Ivonne M.C.M. Rietjens
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Anxiolytic- and anxiogenic-like effects of *Montanoa tomentosa* (Asteraceae): Dependence on the endocrine condition

Erika Estrada-Camarena, Isabel Sollozo-Dupont, Dannia Islas-Preciado, María Eva González-Trujano, ... Carolina López-Rubalcava
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Maitham A. Khajah, Khaled Y. Orabi, Sana Hawai, Hanan G. Sary, Ahmed Z. EL-Hashim
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Traditional detoxification of *Jatropha curcas* L. seeds

Rayane C. Albino, Rosemar Antoniassi, Adelia F. de Faria-Machado, Fausto K. Ferraris, ... Danilo R. Oliveira
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UPLC-QTOF/MS-based metabolomics analysis of plasma reveals an effect of Xue-Fu-Zhu-Yu capsules on blood-stasis syndrome in CHD rats

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Rhamnus prinoides (gesho): A source of diverse anti-biofilm activity

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Binding of the polysaccharide from *Acanthopanax giraldii* Harms to toll-like receptor 4 activates macrophages

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A lipid-soluble extract of Pinellia pedatisecta Schott enhances antitumor T cell responses by restoring tumor-associated dendritic cell activation and maturation

Yumeng Wang, Haixia Huang, Sheng Yao, Guiling Li, ... Suiqi Gui

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Ethnobotanical survey of medicinal plants in central Abyan governorate, Yemen

Mohamed Al-Fatimi

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The contribution of beta-2 adrenergic, muscarinic and histamine (H_1) receptors, calcium and potassium channels and cyclooxygenase pathway in the relaxant effect of *Allium cepa* L. on the tracheal smooth muscle

Arghavan Memarzia, Fatemeh Amin, Saeideh Saadat, Mehrdad Jalali, ... Mohammad Hossein Boskabady

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waterborne enterobacter *Cloacae* in rodents: *in-vitro, Ex-vivo and in-vivo* evidences

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Anti-aggregant effect of butanolic extract of Rubia tinctorum L on platelets *in vitro* and *ex vivo*

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Genotoxicity evaluation of a *Phragmitis rhizoma* extract using a standard battery of *in vitro* and *in vivo* assays

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Therapeutic effect of Dongbai-Tonglin-Fang, a Chinese herbal formula, on urinary tract infection in rat model

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Gardenia jasminoides extract without crocin improved atopic dermatitis-like skin lesions via suppression of Th2-related cytokines in Dfe-induced NC/Nga mice

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Rhodiola crenulata attenuates apoptosis and mitochondrial energy metabolism disorder in rats with hypobaric hypoxia-induced brain injury by regulating the HIF-1α/microRNA 210/ISCU1/2(COX10) signaling pathway

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Preclinical development of gastro-protective botanical candidate from *Woodfordia fruticosa* ***(Linn.) Kurz:*** Chemical standardization, efficacy, pharmacokinetics and safety pharmacology

Inshad A. Khan, Amarinder Singh, Durga P. Mindala, Samdarshi Meena, ... Ram A. Vishwakarma
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Anti-inflammatory activity of *Protium spruceanum* (Benth.) Engler is associated to immunomodulation and enzymes inhibition

Tatiane Roquete Amparo, Janaína Brandão Seibert, Fernando Augusto Siqueira Mathias, João Filipe Pereira Vieira, ... Paula Melo de Abreu Vieira

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Active compounds isolated from *Plantago subulata* L. via wound healing and antiinflammatory activity guided studies

Yasin Genc, U.Sebnem Harput, Iclal Saracoglu

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Effects of *Agriophyllum squarrosum* extracts on glucose metabolism in KKAy mice and the associated underlying mechanisms

Saqier, Shuyin Bao, Shuying Han, Wuliji Ao

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An herbal-compound-based combination therapy that relieves cirrhotic ascites by affecting the L-arginine/nitric oxide pathway: A metabolomics-based systematic study

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Patagonian berries as native food and medicine

Guillermo Schmeda-Hirschmann, Felipe Jiménez-Aspee, Cristina Theoduloz, Ana Ladio
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Neoline is the active ingredient of processed aconite root against murine peripheral neuropathic pain model, and its pharmacokinetics in rats

Yohei Tanimura, Masato Yoshida, Kan'ichiro Ishiuchi, Masahiro Ohsawa, Toshiaki Makino
Article 111859

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Volume 241, 15 September 2019, 112004

Toxicological findings about an anticancer fraction with casearins described by traditional and alternative techniques as support to the Brazilian Unified Health System (SUS)

Paulo Michel Pinheiro Ferreira [a b], Denise Barbosa Santos [a b], Jurandy do Nascimento Silva [a b], Amanda Freitas Goudinho [a], Carla Lorena Silva Ramos [a b], Patrícia Canteri de Souza [c], Ricardo Sérgio Couto de Almeida [c], Diego Sousa Moura [d], Rhaul de Oliveira [d], Cesar Koppe Grisolia [d], Alberto José Cavalheiro [e], Ana Amélia de Carvalho Melo-Cavalcante [b], José Roberto de Oliveira Ferreira [f], Manoel Odorico de Moraes Filho [g], Claudia Pessoa [g]

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[e] Chemistry Institute, State University Júlio de Mesquita Filho, Araraquara, Brazil
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Abstract

Ethnopharmacological relevance

Extracts, essential oils and molecules from Casearia *sylvestris* have popularly shown pharmacological actions against chronic diseases, as anxiety, inflammation, cancer and dyslipidemia. In the context of antitumoral therapy, we investigated *in vitro*, *ex vivo* and *in vivo* toxicological changes induced by a Fraction with Casearins (FC) and its component Casearin X isolated from *C. sylvestris* on animal and vegetal cells, and upon invertebrates and mammals.

Material and methods

Cytotoxicity was carried out using normal lines and absorbance and flow cytometry techniques, Artemia salina nauplii, *Danio rerio* embryos and meristematic cells from *Allium cepa* roots. Acute and 30 days-mice analysis were done by behavioral, hematological and histological investigations and DNA/chromosomal damages detected by alkaline Cometa and micronucleus assays.

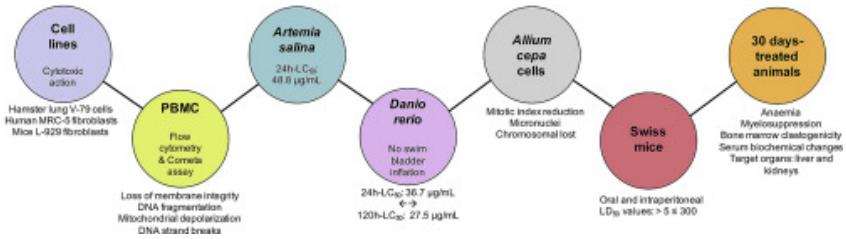
Results

FC was cytotoxic against lung and fibroblasts cells and caused DNA breaks, loss of integrity and mitochondrial depolarization on *ex vivo* human leukocytes. It revealed 24 h-LC_{50} values of 48.8 and 36.7 μg/mL on *A. salina* nauplii and *D. rerio* embryos, reduced mitotic index of *A. cepa* roots, leading to cell cycle arrest at metaphase and anaphase and micronuclei. FC showed i.p. and oral LD_{50} values of 80.9 and 267.1 mg/kg body weight. Subacute i.p. injections induced loss of weight, swelling of hepatocytes and tubules, tubular and glomerular hemorrhage, microvesicular steatosis, lung inflammatory infiltration, augment of GPT, decrease of albumin, alkaline phosphatase, glucose, erythrocytes, and lymphocytes, and neutrophilia ($p > 0.05$). FC-treated animals at 10 mg/kg/day i.p. caused micronuclei in bone marrow and DNA strand breaks in peripheral leukocytes.

Conclusions

This research postulated suggestive side effects after use of FC-related drugs, demonstrating FC as antiproliferative and genotoxic on mammal and meristematic cells, including human leukocytes, teratogenicity upon zebrafish embryos, myelosuppression, clastogenicity, and morphological and biochemical markers indicating liver as main target for FC-induced systemic toxicity.

Graphical abstract



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Introduction

In Brazil, the history of phytotherapy as primary health care has been motivated by social movements, national guidelines and recommendations from the World Health Organization in order to increase

therapeutic resources, recover traditional knowledge, preserve biodiversity, stimulate organic agriculture and environmental protection (Damian et al., 2014).

Phytoterapy received more attention after publication of the Resolution 971/2006, which regulated the *Política Nacional de Práticas Integrativas e Complementares* (National Policy on Integrative and Complimentary Practices) and the *Política Nacional de Plantas Medicinais e Fitoterápicos* (National Policy of Medicinal Plants and Phytotherapeutics). This document introduced medicinal plants into the Brazilian Unified Health System (SUS) and assembled data regarding ethnopharmacological evidences (specially about safety and efficacy) to generate the RENISUS (*Relação Nominal de Plantas Medicinais de Interesse ao Sistema Único de Saúde*/National Relation of Medicinal Plants for Brazilian Health System) to create a guideline of medicinal plants – a list with 71 species. This list can be accessed by scientists for the development of new and folk drugs (Brasil, 2006; 2009).

RENISUS mentions *Casearia sylvestris* Sw. (Salicaceae), known as "guaçatonga", a well distributed shrub in South America (Brasil Ministério da Saúde, 2009). Extracts, essential oils and molecules from *C. sylvestris* have shown analgesic (Piovezan et al., 2017), anti-*Helicobacter pylori* (Spósito et al., 2019), antioxidant (Araújo et al., 2015), anxiolytic (Araújo et al., 2017), hypolipidemic (Schoenfelder et al., 2008; Espinosa et al., 2015), and antiulcerogenic (Basile et al., 1990; Esteves et al., 2005) actions and cardiovascular protection (Brant et al., 2014). Moreover, Brazilian Karajá Indian tribe and natives from the Shipibo-Conibo tribe (Peru) have historical habits of preparing leaf extracts to treat snake bites, wounds, diarrhea, flu and chest colds, probably due to their antifungal and antibacterial activity (Da Silva et al., 2008a; Espinosa et al., 2015; Pereira et al., 2017).

Cytotoxicity against cancer lines is one of the most important biological activity of clerodane diterpenes from *Casearia* and it has been demonstrated they induce DNA fragmentation, phosphatidylserine externalization, cell cycle arrest and reduction of ERK phosphorylation and cyclin D1 expression (Santos et al., 2010; Ferreira et al., 2010, 2014; 2016; Ferreira-Silva et al., 2017). We also displayed a promising potentiality of a fraction from *C. sylvestris* leaves whose tumor inhibition rates ranged from 33 to 67% for human carcinomas and glioblastomas and from 35 to 90% for Sarcoma 180 murine cells (Ferreira et al., 2016).

In the antineoplastic therapy, the determination of toxicity requires attention especially for patients in advanced cancer stages, when side effects overcome benefits and survival increasing is not accompanied by improvement of quality of life. In this context, about 72% of symptoms/signs of drug toxicity in humans can be defined in laboratory animals. Man-laboratory animal correlations are higher for cardiovascular, gastrointestinal and hematological alterations. Hypersensitivity, cutaneous reactions and hepatotoxicity present lower parallelism (Olson et al., 2000; Williams et al., 2002). Therefore, it remains essential to study molecular, cellular and tissue events involved in the progression/retraction of cancer and pharmacological details of cytotoxic agents, especially those that affect integrity of key organs. Herein, we investigated *in vitro*, *ex vivo* and *in vivo* toxicological changes induced by a Fraction with Casearins (FC) and its main component Casearin X isolated from *C. sylvestris* leaves on animal and vegetal cells, and upon invertebrates and mammals, providing a complete profile of possible side effects following use of FC-related drugs.

Section snippets

Chemicals, isolation and structure identification

Fetal calf serum, RPMI 1640 medium, trypsin-EDTA, Ficoll-Hypaque, penicillin and streptomycin were purchased from Cultilab (Campinas, Brazil). Doxorubicin, resazurin, EDTA, Tris-HCl, Triton X-100, propidium

iodide (PI), dimethylsulfoxide (DMSO), rhodamine 123, acetic carmine, and Schiff's reagent were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). NaCl, $MgSO_4$, HCl, $MgCl_2$, $CaCl_2$, KCl, $NaHCO_3$ and citrate were obtained from Vetec Química (Rio de Janeiro, Brazil).

Leaves of *C. sylvestris*…

Cytotoxic activity and DNA damages

In vitro antiproliferative activity of FC was investigated on 3 adherent lines by the Alamar Blue assay. As previously demonstrated for human PBMC (Ferreira et al., 2014), FC was also cytotoxic against hamster lung V-79 cells, human MRC-5 fibroblasts and mice L-929 fibroblasts cells (Table 1).

Outcomes by flow cytometry revealed that FC reduced membrane integrity (0.8 μg/mL: 65.2 ± 1.8%) and caused DNA fragmentation (0.4 and 0.8 μg/mL: 13.1 ± 1.6 and 31.8 ± 1.1%) and mitochondrial depolarization …

Discussion

It remains problematical developing target medicines to affect the course of diseases since most of them have been removed from the market (Harvey, 2014; Newman and Cragg, 2016) because failure in phase I, II and/or III clinical trials due to lack of efficacy (~30–56% of failures), toxicity concerns (~30%), and commercial issues (~20–28%) (Arrowsmith and Miller, 2013), demonstrating approval about 10% (Hay et al., 2014). In general, the main objective of toxicity studies is to identify the…

Conclusions

A fraction with casearins from *C. sylvestris* leaves revealed cytotoxicity on normal human, mouse and hamster cells, teratogenicity upon zebrafish embryos, larval toxicity on *A. salina* nauplii, antiproliferative and clastogenic action on meristematic *A. cepa* cells, caused cell death suggestive of apoptosis in leukocytes. Its main component - Casearin X - induced DNA damages on *ex vivo* and *in vivo* leukocytes. FC was considered acutely toxic if orally or intraperitoneally inoculated. Additionally, …

Conflicts of interest

The authors declare there are no conflicts of interest.…

Author's contributions

PMPF coordinated the research, managed scientific and funding supports, wrote and revised the manuscript. PMPF and DBS performed *in vivo* acute and subacute studies with mice, including histological, genotoxic and hematological techniques; JNS, AFG and CLSR carried out investigations about genotoxic and clastogenic action on meristematic cells from *Allium cepa* roots and toxicity on *Artemia salina* nauplii; PCS, RSCA, DSM, RO and CKG performed, acquired and analyzed all data about embryotoxicity…

Acknowledgments

We thank to the Brazilian agencies “Fundação de Amparo à Pesquisa do Estado do Piauí” [FAPEPI (Public Call 004/2011, grant number 034/2012) and “Conselho Nacional de Desenvolvimento Científico e Tecnológico” [CNPq (#484286/2011-0)] for financial support. Dr. Paulo Michel Pinheiro Ferreira is also grateful to the CNPq (#305086/2016-2) for the personal scholarship.…

Recommended articles

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IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans
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P.M.P. Ferreira *et al.*
Morphological and biochemical alterations activated by antitumor clerodane diterpenes

Chem. Biol. Interact. (2014)

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2023, Fitoterapia

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In vitro and in vivo acute toxicity of an artificial butter flavoring

2023, Journal of Toxicology and Environmental Health - Part A: Current Issues

Antimitotic and toxicogenetic action of Stevia urticifolia aerial parts on proliferating vegetal and mammalian cells: in vitro and in vivo traditional and replacement methods

2022, Journal of Toxicology and Environmental Health - Part A: Current Issues

Systematic approach to identify novel antimicrobial and antibiofilm molecules from plants' extracts and fractions to prevent dental caries

2021, Journal of Visualized Experiments

Chemotherapeutic and Safety Profile of a Fraction from Mimosa caesalpiniifolia Stem Bark

2021, Journal of Oncology

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Abstract

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cytotoxic evaluation using the MTT test (3- [4,5-dimethyl-thiazol-2-yl] -2,5-diphenyl tetrazolium bromide), serial [6]-G dilutions (1.56–100 μg mL-1) were performed, and S-180, HL-60 and peripheral blood mononuclear cells (PBMC) were treated for 72 h. The IC50 of [6]-G were 1.14, 5.73 and 11.18 μg mL-1 for HL-60, S-180 and PBMC, respectively

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Toxic, cytogenetic and antitumor evaluations of [6]-gingerol in non-clinical *in vitro* studies

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ABSTRACT

Gingerol - [6]-gingerol ((S)-5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-3-decanone; [6]-G) - is a phenolic compound with several pharmacological properties. Herein, the aim of the study was to evaluate the toxicogenic effects of [6]-G on Artemia salina nauplii, Allium cepa, HL-60 cell line and Sarcoma 180 (S-180) ascitic fluid cells.For toxic and genotoxic analysis, it was used [6]-G concentrations of 5, 10, 20 and 40 μg mL-1. For cytotoxic evaluation using the MTT test (3- [4,5-dimethyl-thiazol-2-yl] -2,5-diphenyl tetrazolium bromide), serial [6]-G dilutions (1.56–100 μg mL-1) were performed, and S-180, HL-60 and peripheral blood mononuclear cells (PBMC) were treated for 72 h. The IC50 of [6]-G were 1.14, 5.73 and 11.18 μg mL-1 for HL-60, S-180 and PBMC, respectively, indicating a possible selectivity against tumor cell lines. At higher concentrations ($> 10\,\mu g\,mL^{-1}$), toxicity and genotoxicity were observed in the *A. cepa* test, especially at $40\,\mu g\,mL^{-1}$. Mechanisms indicating apoptosis, such as toxicity, cytotoxicity and nuclear abnormalities (bridges, fragments, delays, loose chromosomes and micronuclei) suggest that [6]-G has potential for antitumor pharmaceutical formulations.

1. Introduction

Plants and their bioactive compounds have been found in medicinal practices since antiquity [1]. Approximately 80% of the worldwide population depend on traditional medicine and more than 60% of clinically approved anticancer drugs are derived from medicinal plants [2].

Phytochemicals integrate a heterogeneous set of bioactive compounds classified according to the structural variants, including the polyphenols, which are characterized by the presence of aromatic rings, containing one or more hydroxyl groups [3]. Polyphenols are characterized by diverse biological properties, such as antioxidant activity [4], anti-inflammatory [5] and antitumoral effects [6]. They have demonstrated anticancer activity [7] and apoptotic induction in several tumor cell lines due to pro-oxidant effects at high concentrations or in the presence of metal ions, they can behave as pro-oxidants, leading to DNA degradation and free radical sequestration, providing protective and preventive cellular effects [8–11].

The ginger rhizome - *Zingiber officinale* - belongs to the Family Zingiberaceae, is commonly used as a spice in foods and beverages, and

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has abundant bioactive compounds, including the aromatic polyphenols [4]-, [6]-, [8]-, [10]- and [12]-gingerol. Gingerols are a series of phenolic compounds that differ in the length of their unbranched alkyl side chains, with [6]-G as the most identified form in the fresh rhizome [12,13]. Ginger extract and its pungent components, such as [6]-gingerol, is known to exhibit many biological effects including anti-inflammation, antioxidation and anticancer activity. With respect to anticancer activity, ginger and its constituents have been shown to inhibit the proliferation of and induce apoptosis of a variety of cancer cell types *in vitro* [14–16]. In addition, the use of ginger for the chemoprevention of colorectal cancer has attracted attention in the past years [17–18].

In this context, the assessment of genetic toxicity is of great importance for identifying and characterizing the role of mutagens and carcinogens in human health, including measuring primary damage, gene mutations and chromosomal damages [19,20]. In this context, it is important to apply non-clinical *in vitro* toxicological tests to identify antitumor activities of anticancer candidates, as a strategy for novel drug formulation. Therefore, the aim of the study was to evaluate the toxicogenetic and antioxidant effects of [6]-G in non-clinical models of *A. salina* and *A. cepa*, as well as its cytotoxic effects against PBMCs and tumor cell lines.

2. Materials and methods

2.1. Reagents and materials

Gingerol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium dichromate ($K_2Cr_2O_7$) and copper sulphate ($CuSO_4$•$5H_2O$) obtained from Dynamics Química Contemporânea LTDA (São Paulo, Brazil) were used as standard for the tests of *A. salina* and *A. cepa*. Hydrogen peroxide (H_2O_2) was purchased from Dynamics Química Contemporânea LTDA (São Paulo, Brazil). For the MTT test, the RPMI 1640 medium, penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) was used. Doxorubicin (Dox) was obtained from Eurofarma Laboratories S.A. (São Paulo, Brazil). [6]-G was solubilized at four different concentrations (5, 10, 20 and 40 μg mL^{-1}) using dH_2O and dimethylsulfoxide (DMSO) solvents obtained from Merck Millipore (São Paulo, Brazil).

2.2. Chromatographic analysis of the sigma standard [6]-G

The standard compound [6]-G was analyzed by coupled technique HPLC-DAD, LC–MS. For the analysis, 10 μg mL^{-1} in 95% methanol of the standard was prepared. Analysis by HPLC-DAD was performed on a high-performance chromatograph (CLAE), SHIMADZU®, model LC-20 A. CBM-20 A controller, UV–vis detector with diode arrangement (DAD), model SPD-M20, column C18 Phenomenex Luna, 5 μm (250 × 460 mm) in gradient MeOH: H_2O (5–100% MeOH).

LC-HRESIMS spectra were obtained using a Thermo Instruments MS system (LTQ XL/LTQ Orbitrap Discovery) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler, and Accela pump). The following conditions were used: capillary voltage 45 V, capillary temperature 250 °C, auxiliary gas flow rate 10–20 arbitrary units, sheath gas flow rate 40–50 arbitrary units, spray voltage 4.5 kV, mass range 100–2000 amu (maximum resolution 30,000). The HPLC system was run using SunFire analytical C_{18} column (5 μm, 100 Å, 4.6 × 150 mm), with a gradient of 0.1% formic acid in H_2O: 0.1% formic acid in MeOH 0–100% in 30 min, at a flow rate of 1 $mL.min^{-1}$.

2.3. Test samples

The compound [6]-G was diluted in dH_2O to reach the final concentrations of 5, 10, 20 and 40 μg mL^{-1} for the *A. salina* and *A. cepa*. For the MTT test, serial dilution was employed from the initial concentration of 5 $mg.mL^{-1}$ of [6]-G, diluted in sterile DMSO.

2.4. Brine shrimp lethality bioassay (BSLB)

Initially, for general toxicity evaluation, it was used the lethality bioassay with *A. salina* [21], which is an excellent preliminary bioassay used to test the toxicity of a potential anticancer compound. For that, the cysts were incubated in artificial saline water (39.35 mM NaCl, 18.66 mM $MgCl_2$•6 H_2O, 28.16 mM Na_2SO_4, 8.84 mM $CaCl_2$•$2H_2O$ and 9.38 mM of KCl in 1000 mL of water) at 25–30 °C, with pH adjusted (9.0) using Sodium bicarbonate (Na_2CO_3). After 48 h of incubation, ten live and active nauplii were transferred to test tubes containing [6]-G at the concentrations of 5, 10, 20 and 40 μg mL^{-1}.

The final volume of each sample was adjusted to 5 mL with artificial saline and chlorine-free water (1:1, $v.v^{-1}$). Samples were maintained under the same incubation condition for an additional 24–48 h. Live nauplii were counted macroscopically to determine the number of deaths per concentration. Salt water and $K_2Cr_2O_7$ (5 μg mL^{-1}) were used as negative (NC) and positive (PC) controls, respectively. The treatments were performed in triplicate and the results expressed as a percentage of mortality. The toxicity definition was based on the toxicity scales of McLaughlin et al. (1993) according to the scale, lethal concentration of 50% (LC50) > 1000 μg / ml was considered non-toxic; 500–1000 μg / ml was considered low toxicity; 100–500 μg / ml was considered moderate toxicity ; < 100 μg / ml was considered very toxic or high toxicity.

2.5. Allium cepa test

The *Allium cepa* test was performed to better characterize [6]-gingerol as a potent cytotoxic agent, and evaluate its genotoxicity, since it is a compound commonly found in food and beverages [6]-G toxic/cytotoxic activity was evaluated using roots and meristematic cells of white onion bulbs (*A. cepa*) purchased from a local market of Teresina-PI (Brazil). The bulbs were washed in chlorine-free water and each experimental group (five bulbs) was exposed to [6]-G concentrations (5, 10, 20, 40 μg mL^{-1}), including negative (chlorine-free water) and positive (2.40 ± 10^{-3} mM copper sulfate) control groups. It was counted the number and length of roots (toxic effect), mitotic index (cytotoxic effect) and frequencies of chromosomal abnormalities in root meristematic cells (genotoxic and mutagenic effect) after 24–48 h exposure time (ET) [22]. The roots were then sectioned, fixed in Carnoy solution (ethanol: glacial acetic acid, 3:1, $v.v^{-1}$) for 24 h at 4 °C and stored in 70% ethanol. Thereafter, the roots were hydrolyzed with 1 N hydrochloric acid (HCl), exposed to Schiff's solution and stained with 2% acetic Carmine, and subsequent observation and photomicroscopic capture with 400x magnification to analyze genotoxicity / mutagenicity. A total of 1000 cells/bulb were analyzed, totaling 5000 cells per treatment group. The following parameters were observed for toxic and cytotoxic activity: (a) mean number and root growth (CR); (b) Mitotic index (MI), (c) phase index (FI), (d) frequency of mutagenicity (FM) and (e) frequency of mitotic chromosomal aberrations (CA), calculated by the following equations:

$$MI = Nm/Nt \times 100 \quad (1)$$

$$FI = Nf/Nt \times 100 \quad (2)$$

$$FM = Nmi/Nt \times 100 \quad (3)$$

$$CA = Nac/Nm \times 100 \quad (4)$$

where, IM (%) as the percentage of mitotic index; Nm is the number of cells in mitosis; Nt the total number of cells; IF (%) the percentage rate of phase; and Nf the number of cells in mitosis in the phase; FM (%) the frequency of mutagenicity, Nmi the number of micronuclei; AC (%) the index of mitotic chromosomal aberrations; and Nac the number of chromosomal aberrations.

2.6. Cell viability

The MTT assay [23] was used to evaluate the [6]-G cytotoxic capacity and cancer cell selectivity. Three different cell cultures established in the NTF laboratory from the University of Piauí (Brazil) were used, including HL-60, a human hematopoetic leukemia cell line, S180, a murine solid tumor (sarcoma) cell line and PBMCs. The cells were distributed in 96-well plates, at a density of 1.0×10^6 cells. mL^{-1}. The experimental groups (triplicates) were subdivided into negative control (culture medium), positive control (doxorubicin, 0.3–2 $\mu g\, mL^{-1}$), and [6]-G at different concentrations (1.56, 3.12, 6.25, 12.25, 25.50 and 100 $\mu g\, mL^{-1}$). The samples were incubated for 68 h at 37 °C and 5% CO_2. Thereafter, 20 µl of the MTT solution (5 mg.mL^{-1}) was added to the cultures and reincubated for 4 h. After this time, the culture medium was discarded and the plates were carefully stored and protected from the light and left overnight for complete drying. Subsequently, the precipitate was resuspended in 100 µl of isopropyl alcohol. For the quantification of the reduced salt by the viable cells, the absorbances were measured in microplate reader at 550 nm [24]. Absorbance data were normalized to control (Treatment Absorbance / Control Absorbance × 100).

2.7. Statistical analysis

The results were presented as mean ± standard error (SE). All analyzes were performed using the GraphPad Prism 6 software and using the Shaphiro-Wilk test. The data were previously normalized. The comparison between the groups was performed by the ANOVA test with Tukey hoc post. Values of $p < 0.05$ were considered statistically significant. Assay data for *A. salina* and MTT were normalized, log-transformed and subjected to a non-linear regression analysis in order to find the IC50 for both assays.

3. Results

3.1. [6]-G HPLC-UV and HPLC-ES-MS

The chromatogame HPLC-UV and the HRESIMS mass spectrum are shown in Supplementary Figure. The peak at 25.22 min presented UV characteristic of gingerol compounds, having maximum UV absorption at 279 nm and smallpeak at 230 nm. The protonated $[M+H]^+$ molecule presented ion in *m/z* 295.1919, $[M+Na]^+$ adduct ion in 317.1734 and a sodium dimer $[2M+Na]^+$ ion in *m/z* 611.3531.

3.2. Toxic effects of [6]-G in A. salina

Toxic effects were not detected ($p > 0.05$) in the lowest concentration (5 $\mu g\, mL^{-1}$). However, it was evident a reduction of survival rate followed by increasing [6]-G concentrations, with survival rate ranging from 93.33 ± 3.33 to 16.66 ± 3.33% at 24 h ET and from 83.33 ± 3.33 to 0.00 ± 0.00% at 48 h ET (Fig. 1). Higher concentrations (80 and 100 $\mu g\, mL^{-1}$) showed higher toxicity in both exposure time, with no statistical differences from PC. The LC_{50} values of [6]-G were 40.57 and 24.08 $\mu g\, mL^{-1}$ for 24 and 48 h ET, respectively. According to McLaughlin et al. (1993), the toxicity scale showed that [6] -G has high toxicity for both exposure times analyzed (LC50 < 100 µg mL -1).

3.3. Effect of [6]-G on cell viability in PBMC, S-180 primary culture and HL-60 lineage

The [6]-G concentrations reduced cell viability of peripheral blood mononuclear cells (PBMC), S-180 and HL-60 when compared to negative control group (NC), especially at the highest concentration ($p < 0.05$). After treatment with [6]-G, PBMC presented survival rate of 76.03 ± 2.33% to 21.67 ± 2.33% between the concentrations of 1.56 to 100 $\mu g\, mL^{-1}$, respectively, while Dox-treated cells (0.3 $\mu g\, mL^{-1}$) showed survival rate of 18.68 ± 2.90%, indicating that [6]-G was less toxic than Dox at concentrations from 1.56 to 25 $\mu g\, mL^{-1}$. Conversely, cytotoxic effects in PBMC at higher [6]-G concentrations (50 and 100 $\mu g\, mL^{-1}$) did not differ from the PC. In relation to tumor cell lines S-180 and HL-60, all [6]-G tested concentrations were less toxic than Dox (2.0 $\mu g\, mL^{-1}$). For both cell lines, at lower concentrations (1,5625 −6.25 $\mu g\, mL^{-1}$ for S180 and 1,5625 −3.12 $\mu g\, mL^{-1}$ for HL-60) there were no statistical differences among each other. Additionally, no differences were observed between the concentrations of 50 and 100 $\mu g\, mL^{-1}$ for S180 and HL-60 (Fig. 2).

Lower LC_{50} values indicates better efficiency of [6]-G on HL-60 (1.14 $\mu g\, mL^{-1}$) and S-180 (5.73 $\mu g\, mL^{-1}$) lines, demonstrating its capacity to reduce tumor cell viability. On the other hand, for PBMC cells it was required higher [6]-G amounts to achieve 50% inhibition (11.18 $\mu g\, mL^{-1}$), suggesting a possible selectivity of [6]-G.

3.4. Toxic effects of [6]-G in A. cepa test

The toxic effects of [6]-G on *A. cepa* roots were evaluated by macroscopic parameters (number and root growth). Root size reduction was observed during treatment with [6]-G in a concentration-dependent manner. All concentrations of [6]-G caused significant reduction of root sizes ($p < 0.05$) when compared to the NC (presented root length of 18.11 ± 0.22 and 19.17 ± 0.22 mm after 24 and 48 h, respectively), whose the smallest root sizes were found at the concentration of 40 $\mu g\, mL^{-1}$ (5.93 ± 0.15 mm in 24 h and 4.68 ± 0.17 mm in 48 h exposure).

A. cepa bulbs treated with [6]-G presented reduction of roots at 20 and 40 $\mu g\, mL^{-1}$ when compared to the NC ($p < 0.05$, 24 h ET). Groups treated with [6]-G in both ET (24 and 48 h) were statistically different from the PC, showing less toxic effect on root number, except in the

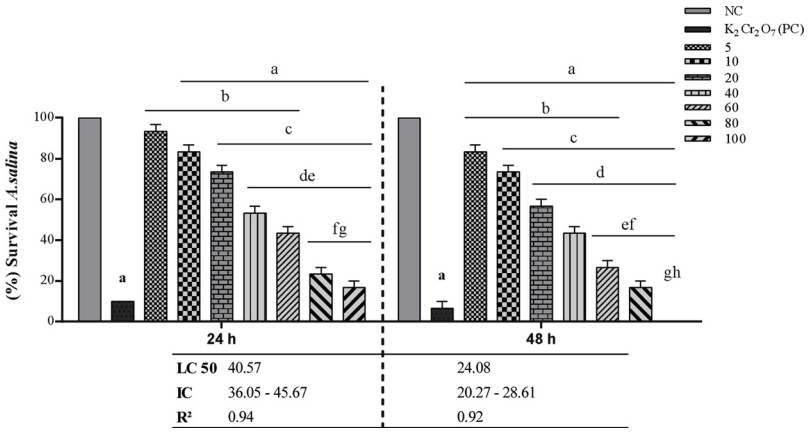

Fig. 1. Toxic effects of [6]-G in A. *salina*. Values are presented as the mean ± SE (n = 3, triplicate, 10 live nauplii/tube); $p < 0.05$ when compared to [a]NC, [b]$K_2Cr_2O_7$ and concentrations [c]5; [d]10; [e]20; [f]40; [g]60; [h]80 and [i]100 $\mu g\, mL^{-1}$ of the [6]-G in the same time period. ANOVA one-way followed by Tukey's test. NC: negative control; PC ($K_2Cr_2O_7$): potassium dichromate (5 $\mu g\, mL^{-1}$); LC_{50}: concentration indicating 50% lethality; CI: confidence interval; R2: coefficient of determination.

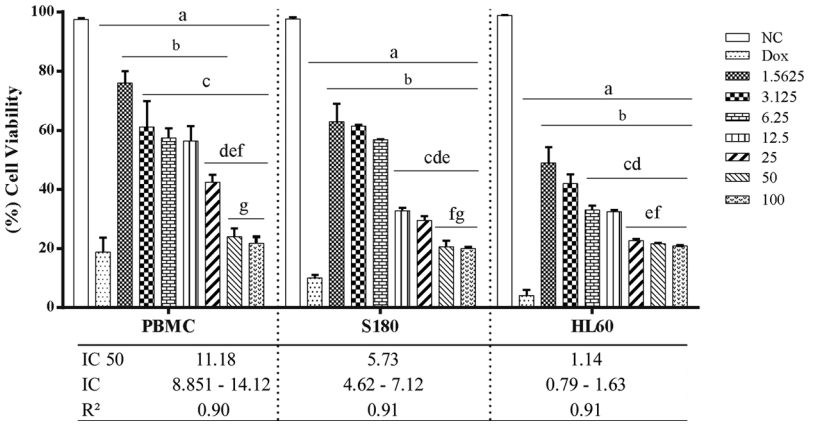

Fig. 2. Cell viability assessed by the MTT assay in PBMC, S-180 and HL-60 cells after 72 h exposure. Values represent the mean ± SE of three independent experiments (n = 3). $p < 0.05$ when compared to [a]NC, [b]Dox and [6]-G at [c]1.56; [d]3.12; [e]6.25; [f]12.5; [g]25; [h]50 and [i]100 μg mL^{-1}. NC: untreated cells. LC_{50}: lethal concentration indicating 50% inhibition; IC: confidence interval; R2: coefficient of determination. ANOVA one-way followed by Tukey's test.

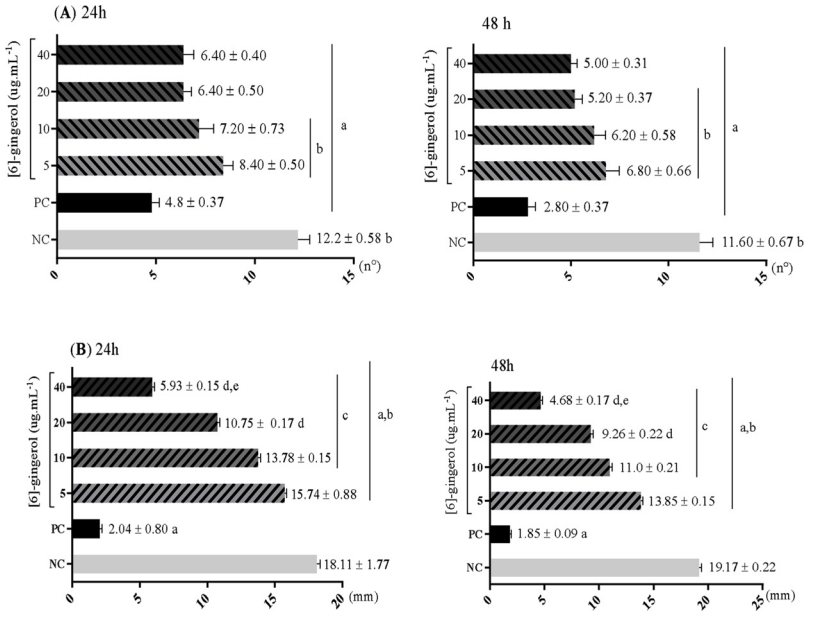

Fig. 3. (A) Mean number of roots *A. cepa* in 24 and 48 h. (B) Mean length (mm) of roots *A. cepa* in 24 and 48 h. Values are means ± SE (n = 5); $p < 0.05$ significantly different between the concentrations and controls in the same period of time, when compared to the [a]NC; [b]PC ($CuSO_4$); [c]5 μg mL^{-1}; [d]10 μg mL^{-1}; [e]20 μg mL^{-1}; [f]40 μg mL^{-1} with exposure of 24 h and 48 h. ANOVA one way followed by Tukey's test.

highest concentration 40 μg mL^{-1} of 48 h exposure (Fig. 3).

3.5. [6]-G Cytotoxicity in A. cepa meristematic cells

The exposure of *A. cepa* meristematic region to [6]-G increased the number of cells in interphase and reduction of dividing cells (prophase, metaphase, anaphase and telophase) measured by MI ($p < 0.05$). Significant MI reduction was observed in a concentration-dependent manner, but [6]-G presented less cytotoxic effects in comparison with PC (Table 1).

3.6. [6]-G Mutagenicity in A. cepa

Chromossomal aberrations (fragments, loose chromosomes and anaphase delay) increased significantly at 40 μg mL^{-1} in both ET when compared to NC, suggesting that [6]-G concentrations above 40 μg mL^{-1} are able to induce genotoxic effects. Mutagenicity was not as found at 5, 10 and 20 μg mL^{-1} (Table 2).

The Fig. 4 shows the profile of *A. cepa* meristematic cells treated with [6]-G in interphase (Fig. 4A), normal cellular division (prophase, metaphase, anaphase and telophase) (Fig. 4B–E) and defective mitosis, showing different chromosomal aberrations (Fig. 4F–M), such as chromosome bridge in anaphase, loose chromosomes, fragments, chromosomal delays, and micronuclei formation (Fig. 4J).

4. Discussion

Bioactive compounds are often toxic at high concentrations, especially in relation to DNA damage [25]. In the *A. salina* assay, low [6]-G concentrations (5 μg mL^{-1}) of [6]-G showed no toxic effect against nauplii, but toxicity effects were observed at concentrations above 10 μg mL^{-1}. Essays with *A salina* lethality can be used as a preliminary, rapid and simple bioassay to test extracts of plants and organic compounds. In most cases, the results are well correlated with cytotoxic and antitumor properties [26]. Such toxicity may also be associated with oxidative mechanisms, where studies indicate that [6]-G therapeutic potential can be attributed to its chemical structure [27,28], since quinones are often designed from phenolic precursors and participate in enzymatic and/or non-enzymatic redox cycles, which can generate ROS, cellular damage and induce apoptosis similar to antitumor agents [29].

The cytotoxic effects of numerous natural compounds as well as cell survival of healthy and tumor cell lines can be evaluated by the MTT method, which is based on the ability of the dehydrogenase enzyme, present in metabolically active cells, to cleave the MTT tetrazolium rings and form violet-colored formazan crystals insoluble in aqueous solutions. Consequently, the number of metabolically active cells is proportional to the amount of formazan crystals produced [23,24]. Herein, [6]-G exhibited cytotoxicity on PBMC and tumor cells (S-180 and HL-60). In fact, previous outcomes have reported that different [6]-

Table 1
Phase (IF) and mitotic index (MI) profile of meristematic cells of *Allium cepa* roots (n = 5 bulbs/group) after 24 and 48 h of exposure to [6]-G.

Treatments	ÍF (%)					IM (%)
	Interphase	Prophase	Metaphase	Anaphase	Telophase	
24h						
Negative control	44.20 ± 0,6	44.32 ± 0.47	5.18 ± 0.32	3.48 ± 0.22	2.82 ± 0.16	55.80 ± 1.36
$CuSO_4$	83.58 ± 0,69[a]	9.30 ± 0.96[a]	3.32 ± 0.31[a]	2.30 ± 0.15[a]	1.50 ± 0.10[a]	16.42 ± 0.69[a]
[6]-G (μg mL^{-1})						
5	49.44 ± 1.07[ab]	40.02 ± 1.27[ab]	4.52 ± 0.31	3.50 ± 0.30	2.40 ± 0.12	50.44 ± 0.69[ab]
10	52.34 ± 1.33[abc]	38.60 ± 0.65[ab]	3.92 ± 0.27	2.94 ± 0.34	1.94 ± 0.11	47.40 ± 0.98[ab]
20	56.92 ± 1.22[abcd]	35.42 ± 1.04[abcd]	3.48 ± 0.35	2.32 ± 0.27	1.86 ± 0.23	43.08 ± 1.22[abcd]
40	60.32 ± 3.89[abcde]	32.66 ± 3.87[abcde]	3.26 ± 0.31[b]	2.14 ± 0.20	1.62 ± 0.16	39.68 ± 3.9[abcd]
48 h						
Negative control	40.34 ± 0.76	47.36 ± 0.46	5.34 ± 0.42	3.78 ± 0.21	3.18 ± 0.21	59.66 ± 1.70
$CuSO_4$	87.68 ± 0.70[a]	6.26 ± 0.65[a]	2.90 ± 0.24[a]	1.88 ± 0.14[a]	1.36 ± 0.10[a]	12.40 ± 1.47[a]
[6]-G (μg mL^{-1})						
5	50.98 ± 1.12[ab]	39.76 ± 1.35[ab]	4.02 ± 0.16	3.08 ± 0.13	2.16 ± 0.11	49.02 ± 1.12[ab]
10	53.94 ± 0.90[abc]	37.64 ± 0.76[ab]	3.78 ± 0.19	2.74 ± 0.28	1.90 ± 0.14	46.26 ± 0.82[ab]
20	58.04 ± 0.84[abcd]	34.76 ± 0.77[abcd]	3.32 ± 0.47	2.14 ± 0.23	1.74 ± 0.11	41.96 ± 0.84[abcd]
40	61.86 ± 4.27[abcde]	32.06 ± 4.24[abcde]	2.72 ± 0.37[b]	1.86 ± 0.19	1.50 ± 0.15	38.14 ± 4.27[abcd]

The values are presented as the mean ± SE calculated from the percentage of phase index IF = Nf / Nt x 100 and mitotic IM = Nm / Nt x 100, of 5000 cells/concentration; $p < 0.05$ significantly different (in the same cell division phase) when compared to [a]NC (dechlorinated water); [b]PC (copper sulfate); [c]5 μg mL^{-1}; [d]10 μg mL^{-1}; [e]20 μg mL^{-1}; [f]40 μg mL^{-1} in the 24 and 48 h treatment period. ANOVA one way followed by Tukey's test.

G concentrations can induce apoptosis and reduce cell viability in HeLa cervical carcinoma (25–175 μg mL^{-1}), LoVo (5–15 μg mL^{-1}), pancreas PANC 1 (5–20 μM), glioblastoma U87 (10–100 μM) and leukemia K562 and U937 (10–200 μM) cell lines [30–34]. Additionally, S-180 cell line is one of the most used cell lines for investigations related to *in vivo*, *in vitro* and *ex-vivo* antitumor activity [35–37]. Sarcoma models are fundamental for understanding the molecular biology of cancer, since sarcomas are distinguished by molecular aberrations such as mutations, deletion, intergenes, gene amplifications, and translocations [38].

[6]-G toxic effects were also evaluated in *A. cepa* roots, a test commonly used indicated for cytotoxicity, genotoxicity and mutagenicity evaluation of synthetic and natural compounds. [6]-G toxicity was firstly observed by macroscopic parameters (number and root growth) and revelaed concentration-dependent results after 24 and 48 h exposure. Delays in root growth, as well as root growth inhibition and appearance of wilting roots represent a response to cytotoxic agents [39]. Inhibition of root growth generally correlates with apical meristematic activity, inhibition of protein synthesis and cell elongation during differentiation [40,41].

Cell cycle analysis allows the determination of cytotoxicity by the frequency of cell division. [6]-G induced caused reduction of cell division (prophase, metaphase, anaphase and telophase) and in all groups tested (5, 10, 20 and 40 μg mL^{-1}), there was MI reduction, especially in higher concentrations (20 and 40 μg mL^{-1}). Although evaluated in plant cells, our study corroborates with [6]-G cytotoxicity observed in human colon tumor cell lines (LoVo) [33].

Mitotic Index reduction may be due to blockage of one or more mitotic phases or delay in cell progression [22]. Our data indicate that [6]-G-treated meristematic cells presented accumulation of cells in prophase, which may be an indication of prophase blockage. A 50% reduction in MI when compared to NC is a limiting value, this is the so-called cytotoxic limit value. The reduction < 50% induces a sublethal effect and < 22% causes a lethal effect [38]. Exposure to [6]-G demonstrated sublethal effect at concentrations between 10 and 40 μg mL^{-1} in 24 and 48 h ET. The reduction of mitotic activity may be due to the inhibition of DNA synthesis or G2 blockade, preventing the cell from entering mitosis [39]. It may also be due to impaired nucleoprotein synthesis and reduced ATP levels that provide energy for spindle elongation, microtubule dynamics and chromosomal movement [40].

Table 2
Chromosomal aberrations (CA) and frequency of mutagenicity (FM) in *Allium cepa* meristematic cells.

Treatments	AC (%)				FM (%)
	Anaphase bridges	Chromosome fragments	Loose chromosomes	Anaphase delay	
24h					
Negative control	0.00 ± 0.00	0.07 ± 0.04	0.03 ± 0.03	0.07 ± 0.04	0.00 ± 0.00
$CuSO_4$	2.68 ± 0.17[a]	2.69 ± 0.27[a]	5.85 ± 0.15[a]	5.71 ± 0.13[a]	0.38 ± 0.02[a]
[6]-G (μg mL^{-1})					
5	0.00 ± 0.00[b]	0.08 ± 0.04[b]	0.04 ± 0.04[b]	0.15 ± 0.07[b]	0.00 ± 0.00[b]
10	0.00 ± 0.00[b]	0.08 ± 0.05[b]	0.08 ± 0.05[b]	0.33 ± 0.05[b]	0.00 ± 0.00[b]
20	0.04 ± 0.04[b]	0.36 ± 0.05[b]	0.27 ± 0.08[b]	0.46 ± 0.07[ab]	0.00 ± 0.00[b]
40	0.20 ± 0.05[b]	0.51 ± 0.12[abcd]	0.40 ± 0.06[abc]	1.46 ± 0.06[abcd]	0.10 ± 0.04[a]
48 h					
Negative control	0.00 ± 0.00	0.66 ± 0.40	0.03 ± 0.03	0.10 ± 0.04	0.00 ± 0.00
$CuSO_4$	4.04 ± 0.19[a]	3.94 ± 0.45[a]	8.32 ± 0.56[a]	8.13 ± 0.42[a]	0.82 ± 0.04[a]
[6]-G (μg mL^{-1})					
5	0.00 ± 0.00[b]	0.20 ± 0.06[b]	0.12 ± 0.05[b]	0.24 ± 0.04[b]	0.00 ± 0.00[b]
10	0.00 ± 0.00[b]	0.34 ± 0.11[b]	0.17 ± 0.04[b]	0.47 ± 0.04[b]	0.00 ± 0.00[b]
20	0.04 ± 0.04[b]	0.57 ± 0.06[b]	0.28 ± 0.08[b]	0.57 ± 0.05[b]	0.00 ± 0.00[b]
40	0.31 ± 0.03[b]	1.47 ± 0.05 [abcde]	0.84 ± 0.03[ab]	1.68 ± 0.09[abcde]	0.29 ± 0.03[a]

The values are presented as the mean ± SE calculated from the frequency of chromosomal aberrations in mitosis CA = N_{CA}/Nm × 100 and mutagenicity FM = Nmi / Nt × 100, of 5000 cells/concentration); $p < 0.05$ significantly different (between tested concentrations/same column) when compared to [a]NC (dechlorinated water); [b]PC (copper sulfate); [c]5 μg mL^{-1}; [d]10 μg mL^{-1}; [e]20 μg mL^{-1}; [f]40 μg mL^{-1} after 24 and 48 h. ANOVA one way followed by Tukey's test.

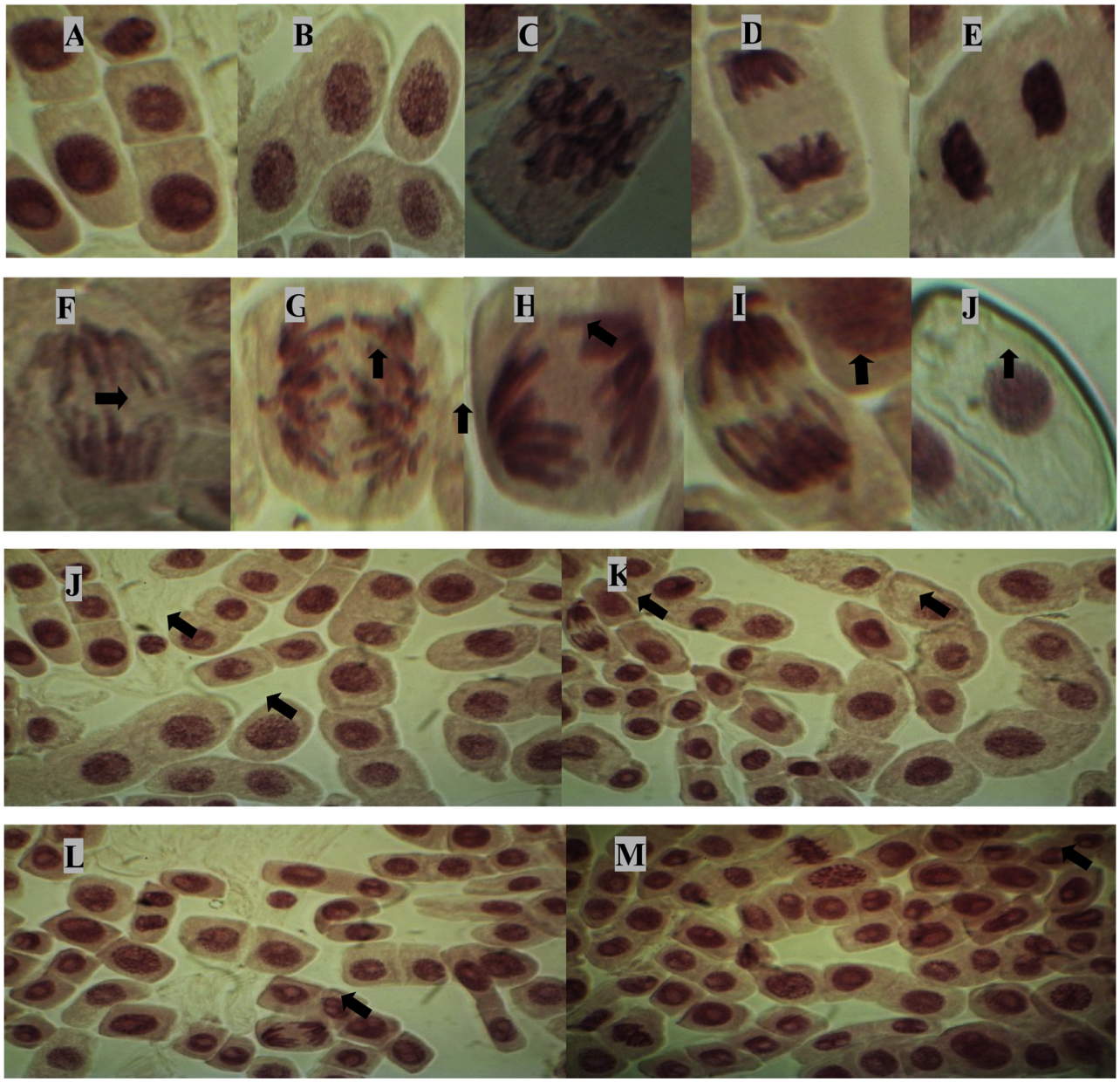

Fig. 4. Photomicrographic profile of meristematic cells of *A. cepa* observed in different phases of cell division: interphase (A), prophase (B), metaphase (C), anaphase (D), telophase (E). Chromosomal abnormalities: chromosome bridge in anaphase (F), chromosomal fragments (G), chromosome delays (H), loose chromosomes and (I) micronucleus (J). Treatment [6]-G: 5 μg mL^{-1} (J); 10 μg mL^{-1} (K); 20 μg mL^{-1} (L); 40 μg mL^{-1} (M). Increase of 40×.

In addition to the mito-depressive effects, the [6]-G treatment presented a series of chromosomal abnormalities during mitosis, with a gradual increase in chromosomal abnormalities (CA) associated with growing concentrations. Such genotoxic effects were found at the anaphase-telophase stage, and the most common abnormalities were anaphasic anomalies > chromosome fragments > loose chromosomes > anaphasic bridges, and CA amplified significantly at 40 μg mL^{-1}.

Chromosomal aberrations can be induced by changes in chromosome structures or number of chromosomes, naturally or as a consequence of exposure to physical or chemical agents. The bridges and chromosomal breaks observed by [6]-G exposure may be due to chromosome adhesion [41], since they tend to remain united and, if separated, cause chromosomal breaks. The occurrence of nuclear damage may be due to the inhibitory activity of phytochemicals on DNA biosynthesis [42]. The chromosomal delay also observed in groups treated with [6]-G are probably asssociated to result from abnormal spindle activity and organizational faults [43].

Mutagenic effects are observed by DNA damage when the cell is exposed to mutagenic agents, resulting in a chromosomal fragmentation (clastogenic origin) or loss of whole chromosomes (aneugenic). When the damage is not repaired, it can be fixed and expressed as micronucleus (MN) after a cycle of cell division [44]. [6]-G also showed mutagenicity due to increased micronucleus frequency as evidenced at 40 μg mL^{-1}. In a previous study [30], morphological changes were detected in HeLa (human cervical carcinoma) cells exposed to [6]-G, including altered nuclear morphology, chromatin condensation and cell fragmentation, which became more evident with increased [6]-G concentration.

In addition to [6]-G cytogenetic damages, there are also *in vitro* and *in vivo* reports of a number of molecular targets including reduction of oxidative damage in cell structures (MDA, ROS, $ONOO^-$, NO_2/NO_3), induction of antioxidant enzymes (SOD, GSH, GSHP, CAT, GPx), inhibition of proinflammatory cytokines and mediators (TNFα, IL-1β, IL6,

IL8, SAA1, NO, iNOS, COX1/2), induction of pro-apoptotic activity in tumor cells (increase and reactivation of p53, activation of caspases -3, 7, 8, 9, cytochrome *c* release, increase Bax:Bcl2 ratio, cell cycle regulation (cycle interruption in G0/G1, S, G2/M), reduction of cyclins (A, B1, CDK1, Cdc25B and Cdc25C), suppression of vascular endothelial growth factor (VEGF) and decreased expression of matrix metalloproteinases (MMPs) [17,45–48].

Gingerol, as well as their derivatives, demonstrate antitumor activity against several cancer cell lines, and the semi-synthetic analog SSi6, a chemical modification of [6]-G molecule, increased selective cytotoxic effects on MDA-MB-231 breast cancer cells. Unlike [6]-G, SSi6 allowed autophagy followed by caspase-independent apoptosis and ROS formation. This data suggest that structural modifications of natural compounds may be an interesting strategy to develop antitumor drugs [49].

Yao and colleagues (2018) have shown that [6]-G selectively suppressed M2 macrophages (promote tumor progression) and elevated M1 macrophages (antitumor) percentage in a mice model of urethane induced lung carcinoma. So, [6] -G reconfigures M2 macrophages into M1 due to reduction of arginase-1 and ROS formation [50]. Moreover, [6]-G analysis in neuroblast human cell line (SHSY5Y) exposed to β-amyloid peptide ($A\beta_{25\text{-}35}$) [33], a neuropathological marker for Alzheimer's disease, showed that the pre-treatment with [6]-G (10 μM) significantly reduced the $A\beta_{25\text{-}35}$ cytotoxicity, including reduction of malondialdehyde levels and ROS formation.

Reports also showed that [6]-G can induce ROS generation in tumor cell lines of chronic (K562) and acute myeloid leukemia (U937) cell lines, leading to disruption of the G2/M cell cycle, decreased expression of cell cycle proteins (cyclin B1, Cdk1, Cdc25B and Cdc25C), and alteration of the cellular oxidant status, inducing generation of mitochondrial ROS [47,48].

5. Conclusion

The [6]-G induced toxicity and cytotoxicity in *A. salina* and *A. cepa* in concentrations > 10 μg mL^{-1}. Its cytotoxicity was also indicated in S-180, HL-60, as well as in PBMC, although presenting selectivity against the tumor cells (S-180 and HL-60). The genotoxicity of the compound was observed in *A. cepa*, especially at 40 μg mL^{-1}, by the formation of micronuclei, bridges, loose chromosomes and delays. The data indicate that cytogenetic mechanisms may be related to [6]-G antitumor activity, as a result of cellular oxidative effects.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2019.108873.

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Pharmacological and physicochemical profile of arylacetamides as tools against human cancers

Paulo Michel Pinheiro Ferreira, Kátia da Conceição Machado, Stefânia Neiva Lavorato, Fátima de Cássia Evangelista de Oliveira, ... Ricardo José Alves

Article 114692

Abstract

Arylacetamides are widely used as synthetic intermediates to obtain medicinal substances. This work evaluated *in vitro* antiproliferative activity of ten 2-Chloro-*N*-arylacetamides on human normal and cancer cells and detailed *in vivo* toxicological and anticancer investigations. Initially, cytotoxic colorimetric assays were performed using tumor lines, peripheral blood mononuclear cells (PBMC) and erythrocytes. Compounds **2**, **3** and **4** were tested for acute toxicity (50, 150 and 300 mg/kg) and for subacute antitumoral capacity in HCT-116 colon carcinoma-bearing xenograft mice for 15 days at 25 mg/kg/day. Most compounds revealed cytotoxic action on tumor lines and PBMC, but activity on human erythrocytes were not detected. Molecular dipole moment

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Pharmacological and physicochemical profile of arylacetamides as tools against human cancers

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ABSTRACT

Arylacetamides are widely used as synthetic intermediates to obtain medicinal substances. This work evaluated *in vitro* antiproliferative activity of ten 2-Chloro-*N*-arylacetamides on human normal and cancer cells and detailed *in vivo* toxicological and anticancer investigations. Initially, cytotoxic colorimetric assays were performed using tumor lines, peripheral blood mononuclear cells (PBMC) and erythrocytes. Compounds **2**, **3** and **4** were tested for acute toxicity (50, 150 and 300 mg/kg) and for subacute antitumoral capacity in HCT-116 colon carcinoma-bearing xenograft mice for 15 days at 25 mg/kg/day. Most compounds revealed cytotoxic action on tumor lines and PBMC, but activity on human erythrocytes were not detected. Molecular dipole moment, lipophilicity and electronic constant of aryl substituents had effects upon *in vitro* antiproliferative capacity. More common *in vivo* acute behavioral signals with compounds **2**, **3** and **4** were muscle relaxation, reduction of spontaneous locomotor activity and number of entries in closed arms and increased number of falls andtime spent in open arms, suggesting diazepam-like anxiolytic properties. Decrease of grabbing strength and overall activity were common, but palpebral ptosis and deaths occurred at 300 mg/kg only. Compounds **2** and **3** reduced colon carcinoma growth (21.2 and 27.5%, respectively, $p < 0.05$) without causing apparent signals of organ-specific toxicity after subacute exposure. The structural chemical simplicity of arylacetamides make them cost-effective alternatives and justifies further improvements to enhance activity, selectivity and the development of pharmaceutical formulations.

1. Introduction

Cancer is a leading cause of death worldwide and the number of new cases is expected to increase considerably over the next decades, according to World Health Organization (WHO, 2017). There are many types of cancer treatment and the best selection depend on the cancer type and stage (Pazdur et al., 2002). Among them, chemotherapy is one of the most important and recommended treatments. However, several drawbacks, like drug resistance and adverse side effects, habitually limit adherence and treatment success (Carlotto et al., 2013; Willyard, 2016; Rapoport, 2017). On the other hand, these drawbacks have stimulated the search for new and secure anticancer drugs.

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In this context, 2-Chloro-*N*-arylacetamidesbelongs to a chemical class widely used as synthetic intermediates to obtain bioactive substances for medicinal and chemical purposes. Chloroacetamides produce pharmaceutical intermediates during organic synthesis and are used as preservative of shampoos, cutting oils, plastics, coatings slab and shower gel (Amrutkar et al., 2012; Jain et al., 2013; Harkov et al., 2013). Reports have also demonstrated their potential as herbicidal, antimicrobial action against filamentous fungi (*Aspergillus niger*), yeasts (*Candida albicans*), bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*), and on drug-sensitive strain of *Mycobacterium tuberculosis* (Hamm and Speziale, 1956; Marco-Contelles and Gomez-Sanchez, 2005; Katke et al., 2011; Aschale, 2012).

However, to the best of our knowledge, their antiproliferative activity has not been evaluated so far. Studies on the mechanism of action of chloroacetanilides have indicated that the biological activity of this class could be attributed to its ability to alkylate important bionucleophiles (Jablonkai, 2003; Helleday et al., 2008; Singh et al., 2011; Swift and Golsteyn, 2014). These features make chloroacetanilides an interesting subject of study as potential anticancer agents. Thus, in this work, we evaluated the *in vitro* antiproliferative activity of ten 2-Chloro-*N*-arylacetamides, differing in aromatic substituents, against human normal and cancer cells, Subsequently, *in vivo* toxicological investigations and analysis of anticancer action using a xenograft model of human carcinoma were performed to assess chemotherapeutic applications and toxic profile.

2. Material and methods

2.1. Chemistry

Compounds **1–10** were synthesized (Table 1) and fully characterized by their melting points and IR, ^{1}H and ^{13}C NMR spectra as described by Lavorato et al. (2017). All compounds were solubilized in sterile dimethylsulfoxide (DMSO, Vetec, Brazil).

2.2. Cell culture and animals' facilities

Human leukemia (HL-60), ovarian (OVCAR-8), glioblastoma (SF-295), colon (HCT-116), and liver (HEPG-2) tumor lines andperipheral blood mononuclear cells (PBMC) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin, at 37 °C in a 5% CO_2 atmosphere (Shel Lab CO_2 Incubator, USA).

For PBMC isolation, heparinized human blood samples (from healthy, non-smoker donors who had not taken any drug for at least 15 days prior to sampling, aged 18–35 years old) were collected. Then, PBMC were isolated by the standard method of density-gradient centrifugation over Ficoll-Hypaque (Cultilab, Campinas, Brazil). After some days, extra blood collection was performed and a suspension of red blood cells (2%) was prepared. All studies were executed in accordance with Brazilian guidelines (Law 466/2012, National Council of Health), the Declaration of Helsinki and with the Universal Declaration on Bioethics and Human Rights of UNESCO.

For *in vivo* studies, Swiss (*Mus musculus*) and CB17 severe combined immunodeficiency (SCID) female mice were obtained from the animal facilities at Universidade Federal do Piauí (UFPI, Teresina, Brazil) and at Fundação Oswaldo Cruz (FIOCRUZ, Salvador, Brazil), respectively. Swiss mice were kept in ventilated racks (Alesco™, Brazil), while CB17-SCID animals were maintained in well-ventilated sterile cages (Tecniplast™, Germain), according to international standards for production and maintaining of germ-free animals. All animals were housed under standard conditions of light (12:12 h light/dark cycle) and temperature (22 ± 1 °C), with access to sterile commercial rodent stock diet (Nutrilabor, Campinas, Brazil) and water *ad libitum*. All procedures were approved by the Committee on Animal Research at FIOCRUZ (#006/2015) and UFPI (#202/2016) and followed Brazilian (*Colégio Brasileiro de Experimentação Animal* - COBEA) and International rules on the care and use of experimental animals (Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes).

2.3. Cytotoxicity analysis

The cytotoxic action was assessed by colorimetric assays after 72 h exposure. Cell proliferation was determined spectrophotometrically using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter). Control groups (negative and positive) received the same amount of solvent (DMSO 0.1%). Doxorubicin (0.005–5 μg/mL) was used as positive control.

2.3.1. Antiproliferative assays with human tumor cells

The cytotoxicity on HL-60, OVCAR-8, SF-295, HCT-116 and HEPG-2 was determined by the MTT assay (Mosman, 1983), which determines the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide (MTT) to a purple formazan product. Line cells were plated in 96-well plates ($0.3–0.7 \times 10^5$ cells/well) and incubated to allow cell adhesion or equilibration (suspension cultures). Twenty-four hours later, compoundswere added to each well (0.2 - 10 μg/mL). After 69 h of incubation, the supernatant was replaced with fresh medium containing 10% MTT, and the cells incubated for an additional 3 h. The plates were centrifuged, formazan product was dissolved in DMSO and absorbance was read at 595 nm.

2.3.2. Antiproliferative study with human normal peripheral blood mononuclear cells

All compounds were also investigated on human PBMC using the Alamar Blue™ assay. PBMC were washed and resuspended (3×10^5

Table 1
Acute toxic effects of the synthetic arylacetamides **2**, **3** and **4** after intraperitoneal injection in Swiss mice.

Group	Dose (mg/kg)	Signs of toxicity		
		Survival	1 h	24 h
Negative control	–	5/5	–	–
Compound **2**	150	5/5	–	Decreased grabbing strength and overall activity
	300	3/5	Increased defecation	Increased defecation, decreased general activity and grabbing strength, and presence of palpebral ptosis
Compound **3**	150	5/5	–	Decreased overall activity
	300	0/5	Decreased grabbing strength and overall activity	Decreased overall activity, presence of palpebral ptosis, increased urination and defecation
Compound **4**	150	5/5	Decreased overall activity	Decreased grabbing strength and overall activity
	300	4/5	Decreased overall activity and body tonus	Reduction of general activity, corneal reflex, and grabbing strength, and increased urination and defecation

Data from n = 5 animals/group. Negative control was treated with the vehicle used to dilute the drug (DMSO 5%).

cells/mL) in supplemented RPMI-1640 medium plus 4% phytohemagglutinin for growth stimulation. PBMC were then plated in 96-well plates (3×10^5 cells/well in 100 μL of medium). After 24 h, compounds dissolved in DMSO were added to each well (0.2 - 25 μg/mL) and cells were incubated for 72 h. Twenty-four hours before the end of the incubation, 20 μL of resazurin (Alamar Blue™) stock solution (0.156 mg/mL) (Sigma Aldrich Co., USA) were added to each well. The absorbance was read at 570 and 595 nm and the drug effect was expressed as the percentage of the control (Ferreira et al., 2015).

2.3.3. Hemolytic assay

Molecules were tested for hemolytic activity according to Santos et al. (2010) at 250 μg/mL in 96-well plates during 60 min at room temperature (25 °C) using suspension of human erythrocytes (2% in 0.85% NaCl containing 10 mM $CaCl_2$). After centrifugation, hemoglobin levels in the supernatants were determined at 540 nm.

2.4. Acute toxicity and behavior analysis

Taking into consideration to minimize pain and suffering as well as ensuring the robustness and reproducibility of the experiments, it was adopted a methodology recommended by the acute toxic class method - Guideline 423 – described in the "Guideline for Testing of Chemicals" from OECD to evaluate the acute toxicity (OECD, 2001). It described that testing in one sex (usually females) is now considered sufficient because, although there is little difference in sensitivity between the sexes, in those cases where differences are observed females are generally slightly more sensitive. Since the compounds **2**, **3** and **4** were not tested previously, and their toxicity was not described yet, the initial dose administered to animals was 300 mg/kg. It is important to note that before administrations, all animals were acclimatized for 5 days. Administrations were performed and mice were observed for 14 days. Negative groups received DMSO 5% in distilled water since compounds were dissolved in pure DMSO.

Following the administration, the animals were fed restricted for 2 h and observed after 60 min and 24 h. Thereafter, animals were observed daily until the 14th day. According to the daily Hippocratic screening, the following signs were assessed: general activity, irritability, touch response, response to tail clamping, writhing, righting reflex, grip strength, auricular reflex, corneal reflex, tremors, convulsions, ptosis, piloerection, cyanosis, and death. It was also evaluated the variation of body weight, food and water consumption, and production of excretions (urination and defecation) using metabolic cages (Lucio et al., 2000). Due to the occurrence of deaths, it was proceeded a new administration at lower doses according to OECD 423. Doses observed the limit of 0.1 mL/10 g of body weight. Range of the LD_{50} was estimated according to the Globally Harmonized System (GHS) (Brazil, 2013).

For behavior investigations, experiments were performed as described below. Diazepam (2 mg/kg, oral by gavage) was administered as standard drug. Doses were chosen base on the toxicity studies and OECD guidelines. After each animal, the cleaning of the field was performed with a paper towel humidified with alcohol 70% to remove excreta left by prior animals. All analyzes were conducted with each animal singly.

2.4.1. Open field test

The exploratory activity was verified using an open field made of acrylic (transparent walls and black floor, with dimensions of 30 × 30 × 15 cm) divided into 9 quadrants and based on the model described by Archer (1973) and Araújo et al. (2017). Thirty minutes after the treatment, animals were placed in the center of the open field. Afterwards, the number of intersections or crossings with four legs (spontaneous locomotor activity - SLA), number of self-cleaning behavior (grooming) and number of lifting without lean against the walls (rearing) were accounted for 5 min.

2.4.2. Rota rod test

The test route rod assesses the degree of muscle relaxation or motor incoordination induced by bioactive substances (Araújo et al., 2017). Each mouse was placed with all four feet onto a bar of 2.5 cm diameter, 25 cm high from the floor, in a rotation of 17 rpm for a period of 3 min. The duration of permanence in the swivel bar, in seconds (s), and the number of falls, with three renewals at maximum, was recorded.

2.4.3. Elevated plus maze test

The elevated plus maze apparatus consists of two open arms (30 × 5 cm) and two closed arms (30 × 25 × 5 cm) crossed perpendicularly. In this frame, the animal is placed 60 cm above the ground exactly on the intersection of the arms (central platform, 5 × 5 cm) with its head turned to the entry of closed arms (Lister, 1987). The animals were placed on the intersection of the arms 30 min after treatment and observed for 5 min. The parameters quantified in this test were number of entries into the open arms (NEOA) and time spent in the open arms (TSOA).

2.5. In vivo xenograft assay with human colon carcinoma

HCT-116 cells were maintained in supplemented RPMI-1640 medium, counted in Neubauer chamber and subcutaneously implanted into the left hind axillary of CB-17mice (2×10^7 cells/mL/animal). On the next day, animals were randomly divided into five groups ($n = 12$ each) and the substances (**2**, **3** and **4**) dissolved in DMSO 5% were intraperitoneally administered for 15 days at 25 mg/kg/day. Negative and positive controls received DMSO 5% (i.p.) and 5FU (15 mg/kg/day, i.p.), respectively.

On the 16thday, animals were anaesthetized with ketamine (90 mg/kg) + xylazine (4.5 mg/kg) for blood collection from each animal *via* retrorbital plexus (Waynforth, 1980) using sterile tubes and heparinize pipettes to determine the profile of circulating peripheral leukocytes and analyzed at 400× magnification in May-Grünwald-Giemsa-stained blood smears (two per animal) to obtain differential amount of white blood cells (WBC). The absolute count of a leukocyte subtype was calculated as the product of its respective differential percentage and total leukocyte count (Biermann et al., 1999).

Afterwards, all mice were sacrificed by cervical dislocation and tumors, livers, kidneys, hearts and lungs were dissected out, weighed and fixed in 10% formaldehyde for examination of size, color changes and hemorrhages. The inhibition ratio of tumor growth (%) was calculated as follows: inhibition ratio (%) = [(A - B)/A] x 100, where A is the average tumor weight in the negative control, and B is the average in each separately treated group.

2.6. Evaluation of intestinal motility

Female Swiss mice were randomly divided into 6 groups ($n = 8$ animals/group): negative control (DMSO 5%, i.p.), positive control for increased intestinal transit (Bisacodil, 10 mg/kg, oral), positive control for reduced intestinal transit (Atropine sulfate, 5 mg/kg, i.p.) and the substances (**2**, **3** and **4**) (50 mg/kg, i.p.). Thirty minutes later, animals received 0.3 mL of activated charcoal 10% in carboxymethylcellulose 1.5% by gavage. After additional 30 min, mice were euthanized by dislocation cervical and the small intestines were withdrawn (from the pylorus to the beginning of the cecum). Outcomes were expressed as a percentage of the total length of the small intestine (Harrison et al., 2004) as follows: Intestinal Transit (%) = Distance journeyed by activated carbon/Total length of small intestine x 100.

2.7. Statistical analysis

Values of IC_{50} and their 95% confidence intervals were obtained by nonlinear regression using the GraphPad program (Intuitive Software for Science, San Diego, CA). Differences were evaluated by comparing

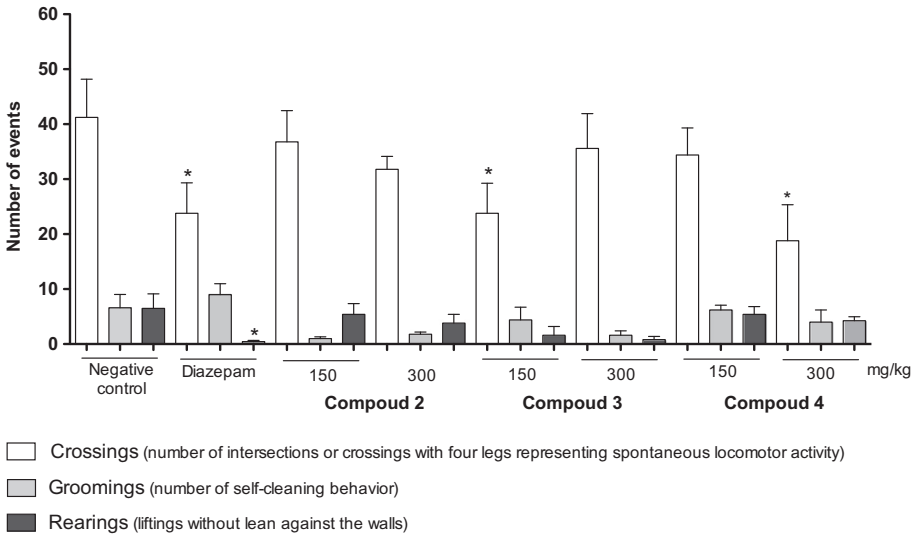


Fig. 1. Behavioral assessment of mice treated with synthetic arylacetamides. Results were expressed as mean ± S.E.M. ($n = 5$ animals/group). Negative control received DMSO 5%. Positive control was treated with Diazepam (2 mg/kg/day). *$p < 0.05$ compared to the negative control by ANOVA followed by Student-Newman-Keuls.

data using one-way analysis of variance (ANOVA) followed by the Newman-Keuls test ($p < 0.05$). All *in vitro* studies were carried out in duplicate and represent independent biological evaluations.

3. Results

3.1. Acute toxicity and behavioral changes

The compound **2** reduced grabbing strength and overall activity at 150 mg/kg and increased defecation, decreased general activity and grabbing strength, and presence of palpebral ptosis at the highest dose (300 mg/kg) (Table 1). Two animals died at 300 mg/kg after 24 h. Compound **3**, which has bromine in its chemical structure, decreased overall activity (apathy and absence of body tonus), and all animals died at 300 mg/kg after 24 h exposure. At 150 mg/kg, death was not detected but decreasing of general activity was obvious. Compound **4** diminished general activity, corneal reflex and grabbing strength, and increased urination and defecation at 300 mg/kg, and one death was noticed. At 150 mg/kg, death was not detected but decreasing of overall activity and grabbing strength were apparent.

The open field test (Fig. 1) revealed reduction in the number of crossings after administration of compound **3** at 150 mg/kg (23.8 ± 6.8) and compound **4** at 300 mg/kg (18.8 ± 4.3) when compared to the negative group (41.3 ± 6.9 crossings) ($p < 0.05$).

The elevated plus maze, an experimental model used to investigate the modulation of anxiety *status* and exploratory activity of animals, showed that compounds **2** at 300 mg/kg and **4** at 150 and 300 mg/kg increased TSOA (179.0 ± 16.7, 196.2 ± 15.3 and 269.0 ± 10.4 s) and reduced TSCA (121.0 ± 17.0, 103.8 ± 15.3 and 38.8 ± 9.0 s) in a similar way displayed with diazepam (199.6 ± 27.3 s and 125.5 ± 14.0 s), if compared to the negative control (100.2 ± 8.4 e 199.8 ± 8.4 s), respectively ($p < 0.05$, Table 2). Moreover, the compound **4** changed NEOA for values (1.0 ± 0.3 entries) lower than those seen in the negative control group (3.6 ± 0.4 entries, $p < 0.05$). Meanwhile, the total number of entries was reduced by compounds **3** (150 mg/kg: 3.2 ± 1.5) and **4** (2.2 ± 0.5 entries, $p < 0.05$).

Compounds **2** (300 mg/kg: 2.8 ± 0.2) and **4** (150 mg/kg: 2.4 ± 0.2; 300 mg/kg: 2.8 ± 0.2 falls) increased number of falls in the rota rod device (Table 3) when compared to the negative control (1.0 ± 0.4) ($p < 0.05$), but only compound **2** at 300 mg/kg diminished permanence time in the swivel bar for 46.8 ± 10.6 s, a value nearly 4-fold lower than that found for negative group (173.8 ± 2.9 s).

Deaths and/or behavior changes were noted with doses of 150 and 300 mg/kg, we also analyzed single doses at 50 mg/kg as suggested by OECD (2001). At this dose, significant outcomes and deaths were not found when compared to negative control (details not showed).

3.2. In vitro cytotoxicity on tumor and normal cells and toxicity

A series of 2-chloro-*N*-arylacetamides (**1**−**10**) was evaluated for their cytotoxicity against five tumor cell lines. These compounds were obtained by the reaction of ten *para*-substituted aniline derivatives with 2-chloroacetic anhydride at room temperature (Lavorato et al., 2017). With exception of compounds **6** and **9**, all compounds displayed IC_{50} values ranging from 4.9 to 50.1 μM (Table 3). Compound **2** (R = Cl) was the most active against three of the five tumor cell lines studied in this work (HL-60, SF295 and HCT-116 cell lines). Additionally, compounds **3** (R = Br) and **4** (R = NO_2) were the only active substances against the most resistant cell line SF-295.

Interestingly, the compounds did not cause significant *in vitro* cytolytic action against human erythrocytes until the concentration tested (250 μg/mL). On the other hand, most molecules were also cytotoxic on proliferating human PBMC, and IC_{50} values ranged from 3.4 to 7.6 μg/mL (Table 3).

Physicochemical data, including the influence of van der Waals volume (vdWV), Hammet's electronic constant (σ), lipophilicity, and dipole moment and their relationship are described in Table 4 and Figs. 2, 3, 4 and 5. They are examined in the Discussion section.

3.3. In vivo antitumor action and subacute effects

Compounds **2**, **3** and **4** were tested for *in vivo* antitumor activity using a xenograft model of colon carcinoma. Only compounds **2** and **3** significantly reduced tumor growth in 21.2% (0.57 ± 0.02 g) and 27.5% (0.53 ± 0.04 g) at 25 mg/kg/day (Table 5) when compared to the negative control (0.73 ± 0.04 g respectively, $p < 0.05$). Comparably, 5-FU, as positive control, also cause tumor reduction.

Deaths or changes in relative weight of organs and in hematological parameters were not detected in arylacetamides-treated animals ($p > 0.05$) but livers increased in 5-FU-treated mice (6.18 ± 0.39 g, $p < 0.05$) in comparison with negative control animals (4.95 ± 0.14 g) (Table 6). Similarly, only animals receiving 5-FU revealed decreasing of erythrocytes, leukocytes, hemoglobin content and hematocrit levels ($p < 0.05$, Table 6).

The compounds 2, 3 and 4 were not able to cause diarrhea. Moreover, they did not alter the intestinal transit (71.1 ± 2.3, 57.9 ± 4.6 e 72.2 ± 4.9%, respectively) when compared to the negative group (67.3 ± 4.0%). On the other hand, Bisacodil increased

Table 2
Effect of synthetic arylacetamides **2**, **3** and **4** on the number of entries in open arms (NEOA), number of entries in closed arms (NECA), time spent in open arms (TSOA), time spent in closed arms (TSCA), and total number of entries into open and closed arms determined by the elevated plus maze test, and on motor coordination analyzed by the rota rod test.

Group	Dose (mg/kg)	NEOA	NECA	TSOA (s)	TSCA (s)	Total number of entries	Number of falls	Time of permanence in revolving bar (s)
Negative control	–	3.6 ± 0.4	4.6 ± 0.4	100.2 ± 8.4	199.8 ± 8.4	8.2 ± 0.8	1.0 ± 0.4	173.8 ± 2.9
Diazepam	2	3.4 ± 0.9	3.2 ± 0.2*	199.6 ± 27.3*	125.5 ± 14.0*	6.6 ± 1.7	3.0 ± 0.5*	71.0 ± 3.5*
2	150	2.2 ± 0.4	1.8 ± 0.4*	137.0 ± 12.4	163.0 ± 12.4	4.0 ± 0.6	1.8 ± 0.4	160.6 ± 8.3
	300	2.8 ± 0.4	1.8 ± 0.4*	179.0 ± 16.7*	121.0 ± 17.0*	4.6 ± 0.7	2.8 ± 0.2*	46.8 ± 10.6*
3	150	2.0 ± 0.8	1.4 ± 0.7*	153.8 ± 57.8	182.8 ± 14.6	3.2 ± 1.5*	1.2 ± 0.7	157.8 ± 15.3
	300	2.8 ± 0.6	2.4 ± 0.7	162.2 ± 36.3	172.3 ± 14.6	5.2 ± 1.2	1.6 ± 0.7	143.8 ± 16.4
4	150	1.8 ± 0.4	2.6 ± 0.5	196.2 ± 15.3*	103.8 ± 15.3*	4.4 ± 0.9	2.8 ± 0.2*	144.5 ± 17.3
	300	1.0 ± 0.3*	1.2 ± 0.2*	269.0 ± 10.4*	38.8 ± 9.0*	2.2 ± 0.5*	2.4 ± 0.2*	139.3 ± 10.4

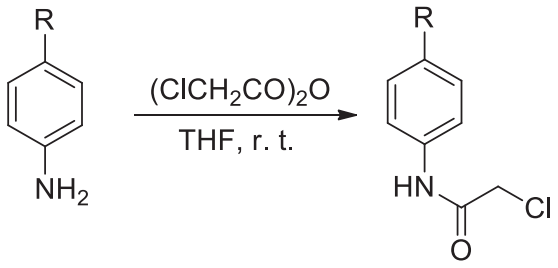
Values are means ± S.E.M. n = 5 animals/group. Negative control was treated with the vehicle used to dilute the drug (DMSO 5%).
* $p < 0.05$ compared with the negative control by ANOVA followed by Newman-Keuls test.

the distance travelled by activated charcoal for 85.1 ± 3.7% and atropine (muscarinic blocker) reduced the intestinal transit for 39.8 ± 4.7% ($p < 0.05$).

4. Discussion

The interest in molecular modeling, combinatorial chemistry and other techniques of chemical synthesis in medicinal chemistry is responsible for the newest therapeutic agents against parasites and cancers, and to treatinflammatory, neurodegenerative and sensory disorders (Soares et al., 2009; Ferreira et al., 2015; Lopes et al., 2015; Araújo et al., 2016; Almeida et al., 2017). Despite some studies relating bioactivity of synthetic arylacetamides, this is the first work focusing on the antiproliferative properties of 2-chloro-*N*-arylacetamides. Subsequently, we also performed *in vivo* preclinical evaluation about their toxicological and antitumoral capacity using xenograft model of colon

Table 3
Antiproliferative potentiality of arylacetamides **1–10** on human tumor lines and primary culture of peripheral blood mononuclear cells (PBMC) and analysis of hemolytic capacity.



1: R = H
2: R = Cl
3: R = Br
4: R = NO_2
5: R = CH_3
6: R = OCH_3
7: R = $COCH_3$
8: R = SO_2NH_2
9: R = COOH
10: R = $COOCH_2CH_3$

Compound	R	IC_{50} [μg/mL (μM)]						Hemolysis (%)*
		HL-60	OVCAR-8	SF-295	HCT-116	HepG-2	PBMC	
1	H	6.0 (35.38)	8.5 (50.1)	> 10 (> 59.0)	8.4 (49.53)	4.5 (26.7)	7.6 (44.6)	0.06 ± 0.16
2-chloro-*N*-phenylacetamide		5.4–6.8	7.5–9.7		7.5–9.4	3.8–5.4	6.1–9.4	
2	Cl	1.0 (4.9)	> 10 (> 49.0)	5.6 (27.44)	3.4 (16.66)	2.4 (11.8)	3.4 (11.8)	5.22 ± 6.97
2-chloro-*N*-(4-chlorophenyl)acetamide		0.6–1.5		4.1–7.7	2.5–4.5	2.0–2.9	3.0–3.8	
3	Br	8.4 (33.8)	7.4 (29.78)	9.8 (39.44)	7.0 (28.17)	4.2 (16.7)	5.7 (22.9)	0.45 ± 0.32
N-(4-bromophenyl)-2-chloroacetamide		6.7–10.5	6.4–8.6	8.2–11.8	5.5–8.9	3.2–5.4	4.7–6.8	
4	NO_2	4.8 (22.37)	4.4 (20.5)	8.9 (41.47)	4.7 (21.9)	2.4 (11.2)	3.0 (14.1)	0.11 ± 0.05
2-chloro-*N*-(4-nitrophenyl)acetamide		4.2–5.9	4.1–4.8	7.7–10.3	3.8–5.8	1.8–3.2	2.3–4.0	
5	CH_3	5.9 (32.13)	6.9 (37.58)	> 10 (> 54.5)	10.0 (54.46)	2.8 (15.3)	> 25 (> 136.1)	0.07 ± 0.01
2-chloro-*N*-(*p*-tolyl)acetamide		4.6–7.7	6.0–8.1		8.6–11.5	2.3–3.5		
6	OCH_3	> 10 (> 50.1)	> 10 (> 50.1)	> 10 (> 50.1)	> 10 (> 50.1)	nd	nd	nd
2-chloro-*N*-(4-methoxyphenyl)acetamide								
7	$COCH_3$	8.3 (39.23)	> 10 (> 47.3)	> 10 (> 47.3)	9.6 (45.36)	1.9 (9.0)	5.2 (24.4)	0.01 ± 0.01
N-(4-acetylphenyl)-2-chloroacetamide		7.3–9.5			7.8–11.9	1.5–2.4	4.1–6.6	
8	SO_2NH_2	5.6 (22.52)	9.8 (39.41)	> 10 (> 40.2)	6.5 (26.14)	2.4 (9.6)	6.1 (24.5)	0.01 ± 0.02
2-chloro-*N*-(4-sulfamoylphenyl)acetamide		4.6–7.0	6.8–14.2		4.5–9.1	1.8–3.1	4.9–7.7	
9	COOH	> 10 (> 46.8)	> 10 (> 46.8)	> 10 (> 46.8)	> 10 (> 46.8)	nd	nd	nd
4-(2-chloroacetamido)benzoic acid								
10	$COOCH_2C_3$	> 10 (> 41.4)	7.5 (31.03)	> 10 (> 41.4)	> 10 (> 41.4)	2.9 (12.0)	4.8 (19.8)	0.11 ± 0.26
Ethyl 4-(2-chloroacetamido)benzoate			6.3–8.9			2.3–3.7	3.6–6.3	
Doxorubicin	–	0.02 (0.04)	1.3 (2.4)	0.2 (0.4)	0.01 (0.02)	0.11 (0.21)	1.9 (3.2)	–
		0.01–0.02	1.0–1.9	0.2–0.3	0.01–0.02	0.17–0.25	1.4–2.4	

Data are presented as IC_{50} values and 95% confidence intervals for leukemia (HL-60), ovarian (OVCAR-8-1), glioblastoma (SF-295), colon (HCT-116), and liver (HEPG-2) tumor lines determined by MTT assay, and for primary culture of human peripheral blood mononuclear cells (PBMC) performed by Alamar Blue assay. Doxorubicin was used as positive control. All experiments were performed in duplicate and represented independent biological evaluations. Nd: not determined.
* Values are presented as percentage of hemolysis ± S.E.M. at 250 μg/mL.

Table 4
Physicochemical properties of compounds (**1–10**).

Compound	R	σ_R[a]	ClogP[b]	vdWV[c] (Å^3)	Dipole moment[d] (D)
1	H	0	1.75	143.68	0.957
2	Cl	0.23	2.35	157.61	2.580
3	Br	0.23	2.52	161.93	2.287
4	NO_2	0.78	1.69	166.65	5.880
5	CH_3	−0.17	2.26	160.45	0.977
6	OCH_3	−0.27	1.59	169.78	2.148
7	$COCH_3$	0.50	1.31	179.88	4.063
8	SO_2NH_2	0.57	0.35	191.47	6.352
9	COOH	0.45	−1.66	171.45	2.484
10	$COOCH_2CH_3$	0.45	2.11	205.83	3.078

[a] σ_R: Hammet's substituent electronic constant; obtained from Hansch and Leo (1979).
[b] ClogP: calculated partition coefficient; calculated using MarvinSketch 6.2.0 [ChemAxon, 2012, (https://www.chemaxon.com/marvin/sketch/index.php)].
[c] vdWV: van der Waals volume; calculated using MarvinSketch 6.2.0.
[d] calculated using HyperChem [HyperChem(TM) Professional 8.0.8, Hypercube, Inc., 1115 NW 4th Street, Gainesville, Florida 32,601, USA].

carcinoma.

Herein, synthetic arylacetamides presented different antiproliferative activity according to the tumor cell line tested and the nature of the aryl substituent at the *para* position. Compound **6** (R = OCH_3) and **9** (R = COOH) were considered inactive against all cell lines they were evaluated whereas only compounds **3** (R = Br) and **4** (R = NO_2) were active against all tumor cells. We also investigated the role of several physicochemical properties of the evaluated compounds, like lipophilicity, dipole moment and van der Waals volume, as well as electronic effects of the aryl substituent in antitumor potential of this chemical class. To this analysis, IC_{50} values in micromolar (μM) were used.

Although we could not clearly establish the effect of aryl substituent to antiproliferative activity of 2-chloro-*N*-arylacetamides, some aspects should be highlighted. The unsubstituted compound **1** was one of the least active compounds, indicating that the presence of a substituent at *para* position is a contributing factor to activity. Among *para*-substituted compounds, only **2** (R = Cl), **3** (R = Br) and **4** (R = NO_2) were active against glioblastoma SF-295 cells. These compounds present van der Waals volumes ranging from 157.61 to 166.65 Å^3. This may indicate that volume occupied by such compounds can be an important feature to the cytotoxic activity. Nevertheless, based only in this parameter, we would expect compound **5** (R = CH_3), whose van der Waals volume is 160.45 Å^3, it would be also active on SF-295 line. So, besides steric aspects, it is likely that the electronic effect of the aryl substituent may also interfere on the activity against this cell line. The electronic effect of a substituent can be measure by its Hammet's electronic

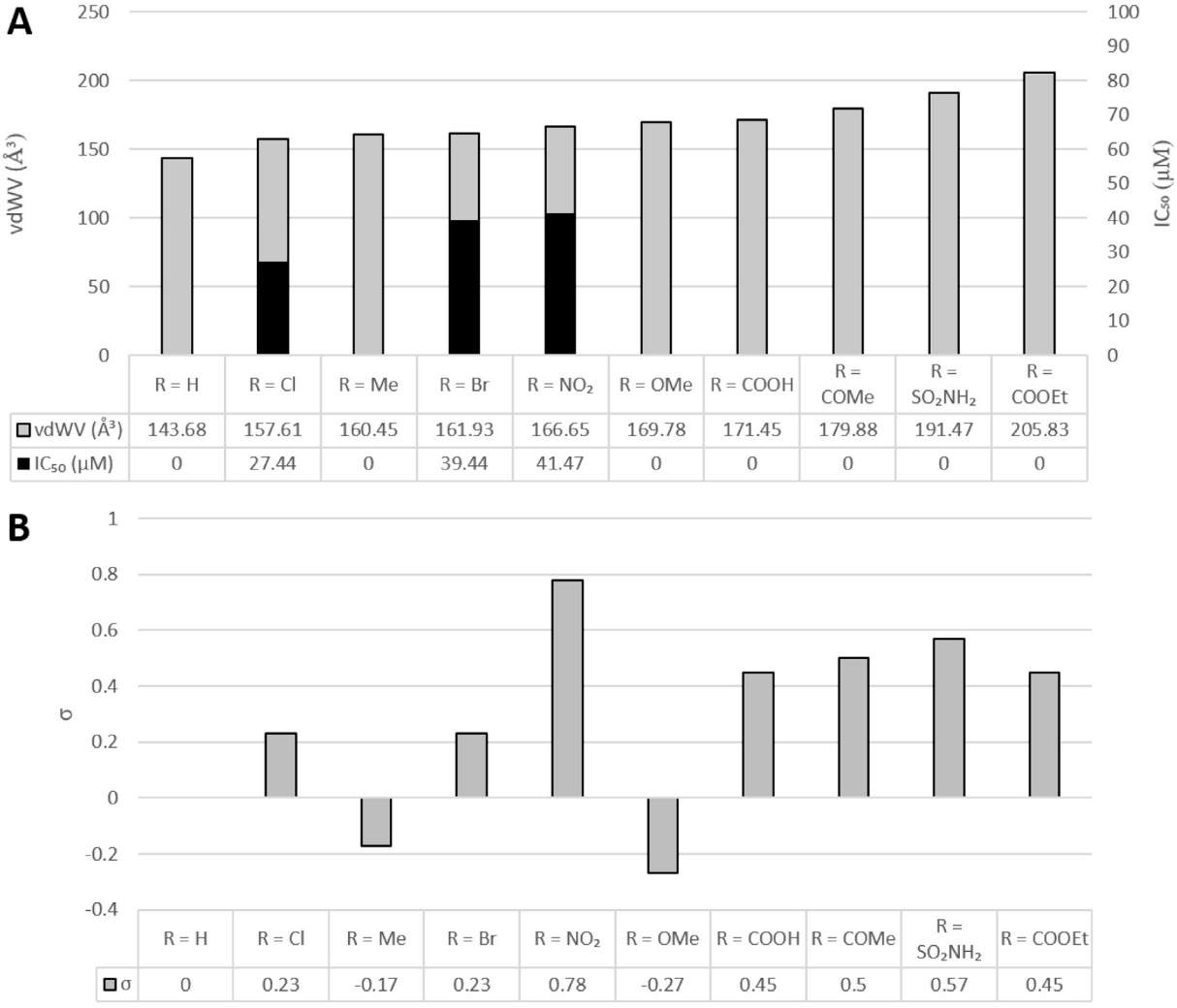


Fig. 2. The influence of van der Waals volume (vdWV) and Hammet's electronic constant (σ) on the antiproliferative activity of 2-chloro-*N*-arylacetamides against SF-295 cell line. A - vdWV (grey columns) and IC_{50} (black columns) values of active compounds against SF-295 arranging in ascending order of vdWV values B - σ values (grey columns) of active compounds against SF-295.

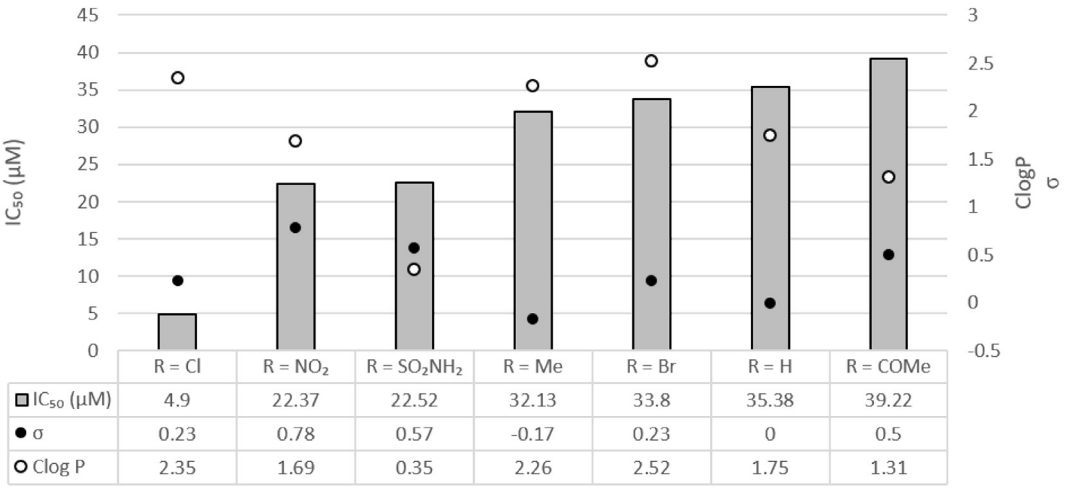


Fig. 3. The influence of Hammet's electronic constant (σ) and lipophilicity (ClogP) on the antiproliferative activity of 2-chloro-N-arylacetamides against HCT-116 cell line. A) IC_{50} (grey columns) and σ (black balls) values of active compounds against HCT-116 arranging in ascending order of IC_{50} values. B) ClogP values (grey columns) of active compounds against HCT-116.

constant (σ). A positive σ indicates that the substituent is an electron-withdrawing group, whereas a negative σ is related to an electron-donating group (Tavares, 2004). According to their σ values, chloro, bromo and nitro play an electron-withdrawing effect, whereas methyl group plays an electron-donating effect, which can contribute to explain the bioactivity differences of these compounds. Thus, this information indicates that the compounds with an electron-withdrawing substituent present a potential antiproliferative activity against glioblastoma SF-295 cells.

The antiproliferative activity of 2-chloro-*N*-arylacetamides against OVCAR-8 cell line can also be partially explained by Hammet's electronic constant of ring substituents (σ). We observed that the most potent compound against OVCAR-8 cell line, compound **4** (R = NO_2), presents a substituent with the highest and positive σ of the series, indicating its high electron-withdrawing character. On the other hand, the least active compound **1** (R = H) have no substituent, so the σ value

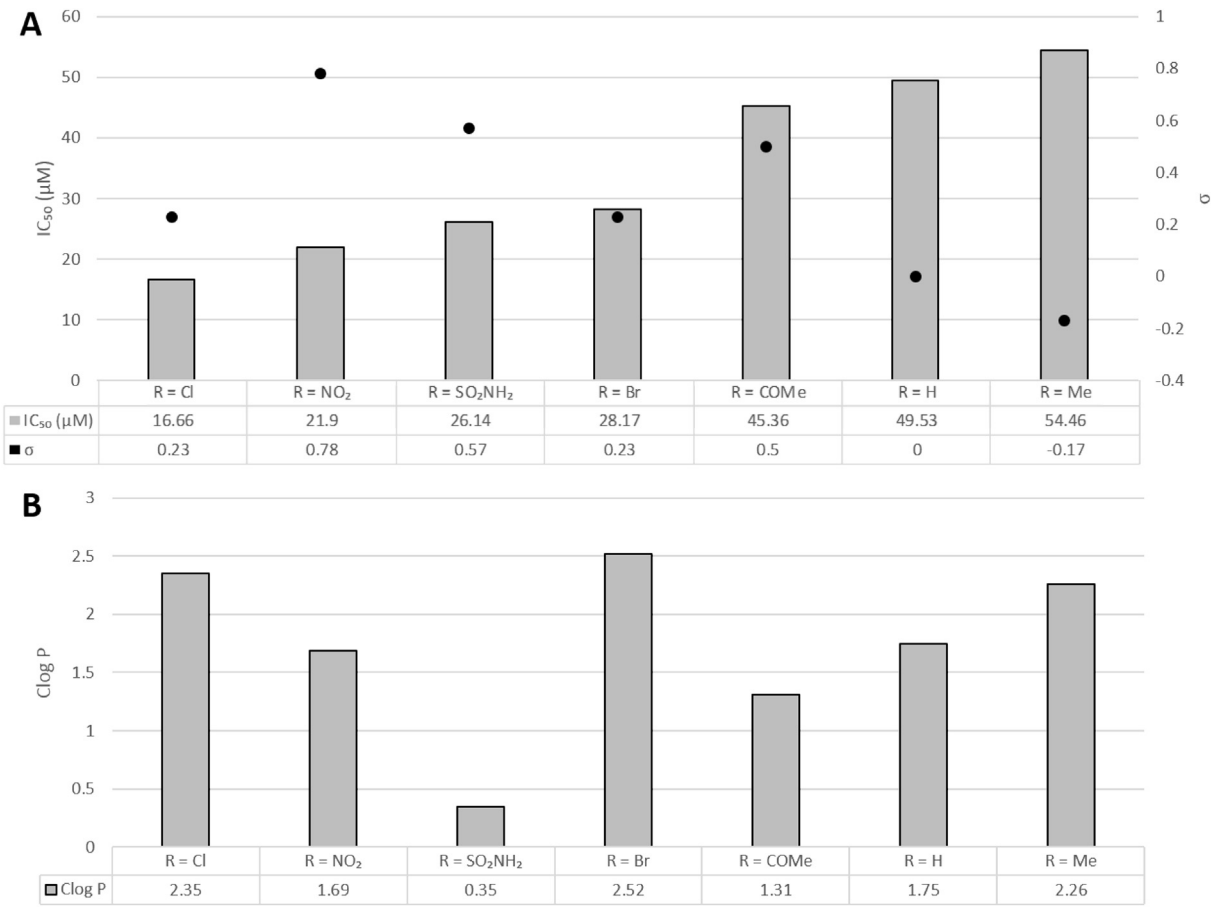
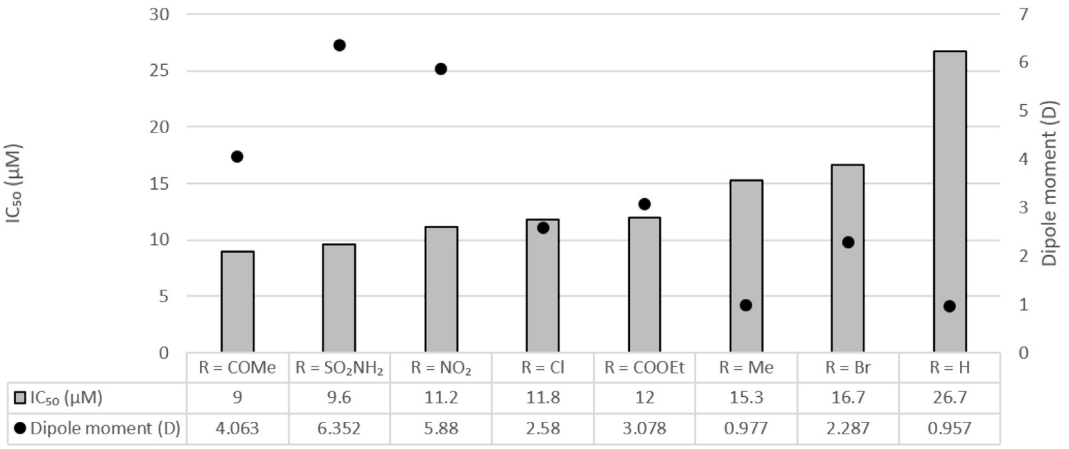


Fig. 4. The relationship among IC50 values (columns in grey) and Hammet's electronic constant (σ) (black balls) and lipophilicity (ClogP) (white balls) of active 2-chloro-N-arylacetamides against HL-60 cell line. The data is arranged in ascending order of IC_{50} values.



	R = COMe	R = SO_2NH_2	R = NO_2	R = Cl	R = COOEt	R = Me	R = Br	R = H
IC_{50} (μM)	9	9.6	11.2	11.8	12	15.3	16.7	26.7
Dipole moment (D)	4.063	6.352	5.88	2.58	3.078	0.977	2.287	0.957

Fig. 5. The relationship between IC50 (columns in grey) and dipole moment (balls in black) values of active 2-chloro-N-arylacetamides against HepG2 cell line. The data is arranged in ascending order of IC_{50} values.

attributed to hydrogen is zero. Accordingly, we would expect compound **8** (R = SO_2NH_2), with the second highest σ value (0.57), to be more active against tumor lines. However, outcomes frustrate this possibility. Probably, this occurred because this compound has the lowest lipophilicity among active compounds (ClogP = 0.35). A compound with low lipophilicity is less able to cross cell membranes, which reduces its ability to act into tumor cells (Rutkowska et al., 2013). Although possessing a lower value of σ for bromine, compound **3** (R = Br) present higher cytotoxic potential than **8** (R = SO_2NH_2), probably due to its higher ClogP. Indeed, we found a negative correlation among IC_{50} values and σ e ClogP, as described in the following equation:

$IC_{50} = -\ 9.2985\ ClogP - 27.7010\ \sigma + 59.8736$ ($n = 6$; $R = 0.901$).

The correlation model indicates that both physicochemical properties can influence the antiproliferative activity of these compounds against OVCAR-8 cells, increasing the activity or reducing IC_{50} values as the values of these two parameters increase. Although it is not a predictive model, it is in accordance with our qualitative analysis of active compounds in the present study. The high electron-donating character of methoxy group in compound **6**and the low lipophilicity of **9** (R = COOH) could explain the absence of activity of these compounds. However, based only on the physiochemical characteristics, we could not find a logical reason for the unexpected inactivity of compounds **2** (R = Cl) and **7** (R = $COCH_3$) against this cell line.

On the other hand, compound **2** (R = Cl) presented a potent antiproliferative effect against HL-60 cells, with an IC_{50} significantly lower than the IC_{50} of the other compounds. We were not able to propose any structure-activity relationship regarding this cell line with the data available thus far. In this case, we cannot establish a correlation among the antiproliferative profile of active compounds against HL-60 cells and their electronic and lipophilicity features. Compounds **4** (R = NO_2), **7** (R = $COCH_3$) and **8** (R = SO_2NH_2), with electron-deficient aromatic rings, present different activity profiles, since **4** and **8** are almost twice active than **7**. Compounds **4** and **8** are also more active than **3** (R = Br), which is the one whose physicochemical properties presented in Table 4 most resemble the properties of compound **2**, the most active compound against this cell line. Although the lipophilicity of **2** can justify its better activity compared to **4** and **8**, the same property cannot explain why **3** has a higher IC_{50} value than **5** (R = CH_3), even though it has an electron-deficient aromatic ring.

The electronic constant of the ring substituent (σ) seems also to affect the antiproliferative activity of these compounds against HCT-116 cells. We observe a tendency towards the increase of the activity as σ increases, *i.e.*, as the ring electronic density decreases. However, compounds **2** (R = Cl) and **3** (R = Br) do not follow this trend. We believe that the high lipophilicity of **2** and **3**, represented by their high ClogP values, can be a positive contribution to their antitumor activity.

Regarding the activity against HepG2 cell line, the compound's dipole moment seems to influence the antiproliferative action of 2-chloro-*N*-arylacetamides, increasing their activity as its value increases. Compounds **4** (R = NO_2), **7** (R = $COCH_3$) and **8** (R = SO_2NH_2) present the highest values of dipole moment among the evaluated compounds and were also the most active against HepG2 cells, whereas compound **1** (R = H), the least active against HepG2, displayed the lowest dipole moment.

In MTT assays, compound **9** (R = COOH) was inactive against all tumor cell lines. Interestingly, compound **10** (R = $COOCH_2CH_3$), the ethyl ester of **9**, was active against two lines only. Since bioassays were run at pH~7.4, compound **9** was in its ionized form. Then, its antiproliferative potential may be reduced since it cannot permeate membranes and reach inside tumor cells (Lima, 2007). If this is true, the low antiproliferative activity of **10** may be explained by its *in-situ* hydrolysis and conversion to **9**.

Table 5
Effects of synthetic arylacetamides on relative weight of key organs and on tumor growth in **CB-17 mice** bearing HCT-116 colon carcinoma after 15 days of intraperitoneal treatment.

Group	Dose (mg/kg/day)	Mice weight (g)	Survival	Liver	Kidney	Lungs	Heart	Tumor (g)	Tumor inhibition (%)
				(g/100 g)					
Negative control	–	20.03 ± 0.43	12/12	4.95 ± 0.14	1.46 ± 0.04	0.67 ± 0.04	0.51 ± 0.02	0.73 ± 0.04	–
5-Fluorouracil	15	18.53 ± 0.88	7/12	6.18 ± 0.39*	1.59 ± 0.10	0.82 ± 0.05	0.59 ± 0.05	0.32 ± 0.06*	56.6*
Compound 2	25	19.00 ± 0.31	12/12	4.99 ± 0.23	1.45 ± 0.06	0.72 ± 0.04	0.61 ± 0.04	0.57 ± 0.02*	21.2*
Compound 3	25	18.60 ± 0.44	12/12	5.08 ± 0.26	1.54 ± 0.06	0.78 ± 0.04	0.56 ± 0.02	0.53 ± 0.04*	27.5*
Compound 4	25	19.98 ± 0.70	12/12	5.02 ± 0.31	1.33 ± 0.08	0.71 ± 0.04	0.51 ± 0.03	0.67 ± 0.05	8.2

Values are means ± S.E.M. $n = 12$ animals/group. Negative control was treated with the vehicle used to dilute the drug (DMSO 5%). 5-Fluorouracil was used as positive control.

* $p < 0.05$ compared with the negative control by ANOVA followed by Newman-Keuls test.

Table 6
Hematological profile of **CB17 mice** bearing HCT-116 colon carcinoma after 15 days of intraperitoneal treatment with synthetic arylacetamides.

Group	Dose (mg/kg/day)	Erythrocytes (10^6 /mm^3)	Hemoglobin (g/dL)	Hematocrit (%)	Platelets (10^3 /mm^3)	Total leukocytes (10^3 /mm^3)	Differential counting of leukocytes (%)		
							Lymphocytes	Monocytes	Granulocytes
Negative control	–	9.88 ± 0.32	12.59 ± 0.47	46.31 ± 1.58	633.6 ± 56.20	2.62 ± 0.41	42.17 ± 3.65	29.72 ± 3.65	28.20 ± 4.31
5-Fluorouracil	25	8.27 ± 0.20*	10.81 ± 0.41*	41.03 ± 1.81*	653.3 ± 102.5	1.53 ± 0.15*	41.08 ± 6.36	31.83 ± 3.28	27.08 ± 3.57
Compound 2	25	9.81 ± 0.34	13.19 ± 0.29	50.14 ± 1.10	549.6 ± 51.20	3.23 ± 0.11	27.16 ± 1.66	40.72 ± 1.01	31.04 ± 1.97
Compound 3	25	9.77 ± 0.20	12.77 ± 0.09	48.46 ± 0.61	453.6 ± 71.46	3.33 ± 0.61	37.90 ± 4.61	36.70 ± 2.96	25.34 ± 3.56
Compound 4	25	8.98 ± 0.07	12.26 ± 0.23	46.40 ± 1.11	473.3 ± 56.21	1.86 ± 0.23	40.30 ± 4.42	31.72 ± 3.14	27.96 ± 2.16

Values are means ± S.E.M. $n = 12$ animals/group. Negative control was treated with the vehicle used to dilute the drug (DMSO 5%). 5-Fluorouracil was used as positive control.

* $p < 0.05$ compared with the negative control by ANOVA followed by Newman-Keuls test.

The initial analysis of structure-activity relationship indicates that physicochemical features work jointly to affect antitumor effects of this series. By the way, the compounds that stood out in the studies – **2** (R = Cl), **3** (R = Br) and **4** (R = NO_2) – have lipophilic character and lower van der Waals volumes. These qualities contribute to the ability to cross cell membranes and to accumulate inside tumor cells. Moreover, these three compounds have electron-withdrawing aromatic substituents and a dipole moment that contribute to accentuate electrophilic properties of chloroacetamide derivatives. Since biological activities of this chemical class have been attributed to the ability to alkylate nucleophiles, like protein and nucleic acids (Jablonkai, 2003; Helleday et al., 2008; Singh et al., 2011; Swift and Golsteyn, 2014), these findings suggest that the antiproliferative activity described here may also be attributed to the same mechanism of action.

In the present work, only a small set of compounds was tested in order to assess whether this chemical class has a potential use as antitumor agents. So, the small dataset limits the performance of conclusive QSAR analysis. Moreover, the antiproliferative activity was determined against tumor cells, not against an isolated specific target. This could explain why we have not found a linear correlation between the activity and a specific physicochemical property (Scior et al., 2009). In this case, the activity-correlated properties lipophilicity and van der Waals volume are likely to influence the antitumor action by affecting the ability of compounds to permeate cell membranes to reach this target. On the other hand, the properties dipole moment and electronic effect of aromatic substituent may have a greater influence on the compound recognition by macromolecules inside cell, its interaction with its molecular target and its alkylating profile, directly affecting the biological activity.

When assayed on human erythrocytes, none of the compounds showed critical lytic action, suggesting cytotoxicity is not related to the direct action on cellular membrane disruption. On the other hand, only compound **5** (R = CH_3) was non-toxic towards PBMC cells ($IC_{50} > 25$ μg/mL). So, most compounds did not reveal selective cytotoxicity only upon tumor cells. Interestingly, **5** is the only evaluated compound with an electron-donating aromatic substituent (Table 4), which contributes to reduce the electrophilicity of the compound and consequently its ability to alkylate bionucleophiles. This suggests that the cytotoxicity may also be modulate by the electrophilic character of the compound in the same way as the antiproliferative activity previously discussed.

Given that the toxic profile of a probable drug must be part of early steps for the development of new medications (Brazil, 2013; Ferreira et al., 2015; Araújo et al., 2018), we also analyzed the acute toxicity of the compounds that stood out to determine toxic effects and to establish safe dosages for subsequent pharmacological studies.

According to the guideline OECD 423 and based on the Globally Harmonized System, compounds **2**, **3** and **4** presented intermediate toxicity, since all of them are classified in the Category 3 (LD_{50} value: > 50 mg/kg < 300 mg/kg) (OECD, 2001). Indeed, studies have suggested that some 2-chloroacetamides present LD_{50} values ranging from about 30 to 300 mg/kg of body weight, and that such toxicity apparently depends neither on routes of administration nor species-specific results (CDC, 2018; National Library of Medicine, 2018).

In the hippocratic screening study, the most common events were reduction of general activity and loss of grapping strength. These effects suggest central nervous system depressant activity despite loss of the righting reflex had not been observed. Such findings indicate these compounds may have central depressant action or selective sedative activity. The loss of grapping strength is an indication for skeletal muscle relaxant activity, which may be peripherally (at the neuromuscular junction) or centrally located (Carlini and Burgos, 1979; Kanjanapothi et al., 2004; Araújo et al., 2017). With this in mind, motor effects using a revolving bar were evaluated, and it was detected that compounds **2** (by reducing time on the revolving bars and increasing falls at 300 mg/kg) and **4** (by increasing falls at 150 and 300 mg/kg) have a myorelaxant activity and cause light but significant psychomotor retardation. It is likely that motor effects are associated with ataxia caused by some 2-Chloroacetamides (CDC, 2018).

Reduction ofcrossingswas noticed for compounds **3** and **4** suggesting modifications in SLA of treated animals, but none of the three compounds altered the number of groomings and rearings, indicating such compounds do not interfere in the exploratory activity of mice subjected to open spaces nor affect the motor coordination. On the other hand, compounds **2**, **3** and **4** reduced the NECA and compounds **2** and **4** increased TSOA (and reduced TSCA) in a very similar way seen for diazepam (standard drug).These conclusions were found using the plus maze test, which consists of a more specific anxiety model for the assessment of anxiolytic drugs with capacity to reduce the rejection animals present to walk to the open arms, since this behavior is conditioned by the fear or stress in aversive environments (Walf and Frye, 2007; Neumann et al., 2011).

In an overview, the acute behavioral signals more commonly associated with compounds **2**, **3** and **4** with were muscle relaxation and reduction of locomotor activity in the revolving bar with consequent increase in the number of falls, decrease of NECA and increase of TSOA, suggesting sedative actions. Such actions surprisingly exhibit characteristics of anxiolytic properties related to the diazepam, mainly for compound **2** (R = Cl) and **4** (R = Br). These behavioral data confirm the reduction of general activity, coordination of the motor system (grabbing strength) and muscle tonus from Hippocratic screening tests (Walf and Frye, 2007; Almeida et al., 2012).

Ash (2004) demonstrated that the LD_{50} of some 2-chloroacetamides on mice is around 100 mg/kg body weight, which could justify, at least in part, the presence of toxicity signs at 150 mg/kg. Behavioral effects analyzed in glyphosate-based herbicide-treated mice showed impairment effects upon the central nervous system probably due to alterations in neurotransmission pathways that participate or regulate locomotor activity, anxiety and memory involving GABAergic, dopaminergic, serotonergic and/or cholinergic systems (Baier et al., 2017).

Benzodiazepines, as diazepam and alprazolam, present a range of

actions – sedative/hypnotic, anxiolytic, anticonvulsant and muscle relaxant – and most of them were found in arylacetamides-treated animals. It is likely such synthetic compounds do not activate $GABA_A$ receptors directly but, instead, are positive allosteric modulators of the effects of GABA and allow lower concentrations of this neurotransmitter to open the Cl^- channels, like most benzodiazepines. As a consequence of the enhancement of GABA's inhibitory activity caused by benzodiazepines, the brain's output of excitatory neurotransmitters including norepinephrine, serotonin, dopamine and acetylcholine is reduced (Sigel and Steinmann, 2012; Miller and Aricescu, 2014). Further pharmacological investigations are in progress to confirm the mechanism(s) of action because this was not the main focus of this research.

Based on the absence of toxicity with doses of 150 mg/kg and considering that a drug has a good safety profile if its therapeutic index exceeds the value of 8–10 (Tamargo et al., 2015), we elected the dose of 25 mg/kg/day for efficacy assays. As *in vivo* models, xenograft animals are technically represented by athymic nude mice, severely compromised immunodeficient (SCID) mice. They have been extensively used to monitor tumorigenicity and tumor growth, can simulate the complexity of genetic and epigenetic abnormalities in human tumors and aid in the development of individualized molecular approaches (Morton and Houghton, 2007; Jung, 2014).

In the present study, we chose colon carcinoma as preclinical cancer model due to its epidemiological importance: a) colorectal neoplasm is the third mostly common occurring cancer in the world; b) causes about 694,000 deaths a year (10% of all cancer deaths); c) presents wide geographical variation in incidence with rates varying ten-fold in both sexes, and d) 95% of them are adenocarcinomas, especially in countries characterized by high or very high indices of development and/or income (two thirds of cases), which strongly demonstrates how certain lifestyle factors affect the risk of developing colorectal carcinomas (Ferlay et al., 2015; Mármol et al., 2017).

We showed that compounds **2** and **3** significantly reduced colon carcinoma tumor growth. We believe that their higher lipophilicities, represented by high ClogP values, has positive (but partial) role in this antitumor activity. Furthermore, the electronic constant of the ring substituent (σ) seems also to affect the antiproliferative activity of the series against *in vitro* HCT-116 cells, being observed a tendency towards the increase of bioactivity as σ increases, *i.e.*, as the ring electronic density decreases. However, compounds **2** (R = Cl) and **3** (R = Br) do not follow this trend, and *in vivo* tumor inhibition rates were equivalent ($p > 0.05$).

Some 2-chloro-*N*-arylacetamides behave as alkylating agents, especially those molecules containing sulfhydryl groups (Jablonkai and Hatzios, 1991; Jablonkai, 2003). It is also worth to note that classical classes of antitumor molecules, like nitrogen mustards, nitrosoureas and quinones causes cell death clearly associated with electronic and lipophilic properties (Hansch et al., 1972; Denny and Wilson, 1986; Gourdie et al., 1990; Driebergen et al., 1986).

Compounds tested *in vivo* neither caused macroscopical/morphological damage on key organs, affected intestinal transit nor altered figurative elements of the peripheral blood, which suggest that subacute exposure for 15 days at 25 mg/kg/day neither promoted direct action on blood cells/hematopoiesis nor showed target organ toxicity, a great advantage if we take into consideration that most antineoplasic drugs clinically available cause myelosuppression (anemia, leucopenia with neutropenia), hepatotoxicity, diarrhea, and alopecia, besides cardiotoxicity, opportunistic infections, peripheral neuropatia, nausea, vomiting, anorexia, fatigue and tiredness (Carlotto et al., 2013; Ferreira and Pessoa, 2017; Rapoport, 2017; Nurgali et al., 2018).

In summary, most of 2-chloro-*N*-arylacetamide derivatives showed moderate to high antiproliferative action on tumor lines, cell toxicity on normal dividing leukocytes, and *in vivo* investigations demonstrated diazepam-like anxiolytic properties of compounds **2** and **4**, including muscle relaxation and reduction ofspontaneous locomotor activity, general mobility and muscle tonus. Physicochemical characters as van der Waals volume, molecular dipole moment, lipophilicity and electronic constant may work together to alter the anticancer capacity, but the evaluation of more compounds of this chemical class is imperative to better understanding their structure-activity relationship. Compounds **2** and **3** reduced tumor growth in xenograft colon carcinoma-bearing mice without causing apparent signals of organ-specific toxicity after subacute exposure. The structural chemical simplicity of these arylacetamides make them cost-effective alternatives and justifies further improvements to enhance activity and selectivity and the development of pharmaceutical formulations.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Abstract

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role. Thus, in the present work, we decided to evaluate the genotoxic and mutagenic effects of ketamine on human peripheral blood leukocytes (PBLs) and *Salmonella typhimurium* (TA98, TA97a, TA100, and TA102) through several well-established experimental protocols based on different parameters in the presence or not of exogenous metabolizing S9 fraction. Our data revealed that ketamine induces a weak

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Abstract

Ketamine is a potent uncompetitive NMDA receptor antagonist that provides amnesia, analgesia, environmental dissociation and immobility, where it has its cytotoxic effect well described in the literature. However, the work on its genotoxic/mutagenic potentials are scarce and insufficient and does not allow a reasonable evaluation of its role. Thus, in the present work, we decided to evaluate the genotoxic and mutagenic effects of ketamine on human peripheral blood leukocytes (PBLs) and Salmonella typhimurium (TA98, TA97a, TA100, and TA102) through several well-established experimental protocols based on different parameters in the presence or not of exogenous metabolizing S9 fraction. Our data revealed that ketamine induces a weak cytotoxic effect on human PBLs after 24 h and is devoided of hemolytic effects. A small amount of DNA strand breaks levels were detected in the modified comet assay (employment of FPG enzyme) only at highest concentrations (500 and 700 μg/mL) of ketamine, highlighting our pro-oxidant data regarding ketamine. However, the oxidative DNA lesions were almost completely repaired which reflects in the lack of mutagenesis (micronuclei and chromosomal aberrations) on human PBLs and no increases in revertants numbers on *S. typhimurium*/microsome test (500 to 5000 μg/plate). In summary, ketamine is a weak oxidative DNA damaging agent and is devoid of mutagenic properties on eukaryotic and prokaryotic models.

Introduction

In general, anesthetics are able to modify structurally the DNA molecules interfering in their functions, being considered by many authors as exogenous genotoxic agents (Alleva et al., 2003). In the last decades,

there has been an increase in the number of agents used in anesthesiology; however, most of them do not have a complete understanding of their potential effects on the health of patients (Nogueira et al., 2016). Therefore, according to (Schifilliti et al., 2011), the choice of a more appropriate technique for the patient is guaranteed from the knowledge of the possible toxic effects of anesthetics.

Ketamine is a noncompetitive antagonist of cyclohexylamine *N*-methyl-D-aspartate (NMDA) receptor which provides amnesia, analgesia, dissociation of the environment and immobility. In addition, this drug has favorable effects on the cardiovascular and pulmonary systems, where this characteristic is particularly valuable for the induction of anesthesia in hypovolemic patients (BEGEC et al., 2013; Leffa et al., 2016).

The toxicity of ketamine to various cell types and tissues is well described in the literature (Leffa et al., 2016; Ozturk et al., 2014; Liu et al., 2013). According to Shan et al., 2018) and Ito et al. (2015) the most likely mechanism of toxicity would be through the generation of reactive oxygen species (ROS). In a study proposed by Liu et al. (2013), mice were shown to be exposed for a long period of time to ketamine, with elevated levels of ROS as well as neuronal cell death.

However, in general, few studies have evaluated DNA damage induced by ketamine (Leffa et al., 2016). It is also worth noting that the European Monitoring Center for Drugs and Drug Addiction (EMCDDA) published a report in 2002, underlining the absence of relevant mutagenic studies *in vivo* with ketamine (EMCDDA, 2002). In addition, >15 years after the publication of the EMCDDA document, Committee of Experts on Drug Dependence at the World Health Organization (WHO) in 2015, during the 37th Meeting, they also revealed a lack of conclusive studies on the mutagenic potential in of ketamine (WHO, 2015). Recently, Braz and Karahalil (2015) published a review study on the *in vivo* potential of genotoxicity of anesthetics already used and those currently used in anesthesia, and authors conclude that many efforts should be addressed due to the conflicting results and few found in the scientific literature. In addition, available genotoxic / mutagenic studies with ketamine are scarce and insufficient and do not allow a reasonable evaluation of their genotoxic potential (EMCDDA, 2002).

Therefore, due to the wide use of the drug for several purposes, it is necessary to evaluate the safety of its use, where the knowledge about this drug will allow a conscious and rational use, avoiding errors (Leffa et al., 2016). Therefore, this study was designed to evaluate *in vitro* the genotoxic and mutagenic effects of ketamine hydrochloride in human PBLs stimulated by phytohemagglutinin and *Salmonella typhimurium* (a prokaryotic model) through several well-established experimental protocols based on different parameters.

Section snippets

Chemicals

The ketamine hydrochloride was purchased from Sigma Chemical (St. Louis, MO). Fetal bovine serum, phytohemagglutinin, RPMI 1640 medium, trypsin-EDTA, glutamine, penicillin and streptomycin were purchased from GIBCO® (Invitrogen, Carlsbad, CA, USA). Low-melting point agarose and agarose were obtained from Invitrogen (Carlsbad, CA, USA). Formamidopyrimidine DNA-glycosylase (FPG, also known as MutM) was obtained from BioLabs (New England, USA). Cyclophosphamine was from Asta Medica. The S9…

Ketamine has a weak cytotoxic potential and no hemolyitic property

After 24 h exposure, the colorimetric assay for assessing cell metabolic activity showed ketamine has a perfil of low cytotoxicity on phytohemagglutinin-stimulated leukocytes cultures at concentrations up to 700 μg/mL. Using negative control cultures as a reference, tested substance did not provoke cytotoxicity as

observed on ketamine-treated cells in all almost concentrations evaluated (Fig. 1A). A weak cytotoxicity was mainly observed just when cells cultures were exposed to high…

Discussion

Ketamine is a widely used anesthetic in clinical practice and have demonstrated it induces neurotoxicity and has a modulatory effect on the cells of the immune system (Pavlovic et al., 2018). However, the precise mechanism of ketamine toxicity still remains unclear. These observations raised the concern whether similar toxicity occurs in the human leukocytes as a biosensor to monitoring of systemic ketamine genetic toxicity of patients under clinical treatments such as neuropathologies.

In…

Conclusion

In conclusion, ketamine was shown to have a low cytotoxic effect on PBLs after 24 h of exposure. Oxidative lesions on the DNA were detected but repaired significantly, which reflected the absence of clastogenic / aneugenic effects on PBLs and absence of mutagenesis in the *Salmonella* / Microsome test.…

Statement of author contributions

Bruno Coêlho Cavalcanti contributed to the conception of this project, data acquisition and analyses, and drafting, revising and the final approval of the manuscript. **João Batista de Andrade Neto** contributed to the conception of this project and drafting, revising and the final approval of the manuscript. **Antônio Adailson de Sousa Silva** contributed to data acquisition and analyses and drafting, revising and the final approval of the manuscript. **Francisco Stefânio Barreto** contributed to the…

Declaration of Competing Interest

The authors declare no conflicts of interest concerning to this article.…

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> of oxidative stress associated with a diminished pool of intracellular antioxidant defense has been reported in patients recovering from surgery, and many anesthetics also interfere in the metabolism of ROS (Rao et al., 1997; Lases et al., 2000; Durak et al., 1999).…

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Antitumoral effects of [6]-gingerol [(S)-5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-3-decanone] in sarcoma 180 cells through cytogenetic mechanisms

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ABSTRACT

Background: [6]-Gingerol [(S)-5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-3-decanone] is a phenolic substance reported for several ethnopharmacological usage by virtue of its antioxidant, antiemetic, anti-inflammatory and anticancer properties. This study assessed the antitumoral effects of [6]-Gingerol in primary cells of Sarcoma 180 as well as in peripheral blood lymphocytes of mice.

Methods: The effect of [6]-Gingerol was assessed by applying cytogenetic biomarkers as indicative of genotoxicity, mutagenicity and apoptosis. Ascitic liquid cells were treated with [6]-Gingerol at concentrations of 21.33, 42.66 and 85.33 μM and subjected to the cytotoxicity assays using Trypan blue test and the comet assay, as well as the cytokinesis-block micronucleus assay. Doxorubicin (6 μM) and hydrogen peroxide (85.33 μM) were used as positive controls.

Results: [6]-Gingerol, especially at concentrations of 42.66 and 85.33 μM, showed notable cytotoxicity in Sarcoma 180 cells by reducing cell viability and cell division rates via induction of apoptosis. Genotoxicity at the concentrations used was punctuated by the increase in the index and frequency of DNA damage in tested groups. [6]-Gingerol, at all concentrations tested, did not induce significant aneugenic and/or clastogenic effects. It did, however, induced other nuclear abnormalities, such as nucleoplasmic bridges, nuclear buds and apoptosis. The genotoxic effects observed in the cotreatment with H_2O_2 (challenge assay) employing neoplastic and healthy cells, indicated that [6]-Gingerol may induce oxidative stress.

Conclusions: Observations suggest that [6]-Gingerol may be a candidate for pharmaceutical antitumoral for-

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mulations due to its cytotoxicity and to mechanisms associated with genetic instability generated by nuclear alterations especially by apoptosis.

1. Introduction

The World Health Organization [102] predicts that about 75 million people will fall victim to some type of cancer until 2030, with an estimate of 17 million deaths throughout the world. The estimated occurrence of new cases of cancer is 600,000 in Brazil for each year of the biennium 2018–2019 [1]. Several therapeutic advances were achieved involving the most common types of cancer (breast, prostate, colon and lung); however, rare and heterogeneous tumors of mesenchymal origins, such as sarcomas, progress further in comparison to our understanding of cancer mechanisms [2,3,79].

Sarcomas are relatively more common in children and young adults and are classified into two main subcategories (soft and bone tissue sarcomas).These are broadly distributed in the organism [3,103] with more than 70 subtypes [80,104], occurring in cartilage, muscle, blood vessels, nerves and fat [97]. In medical oncology, each patient suffering from a sarcoma is an *outlier* [80], and these constitute < 1% of all existent neoplasms in treatment centers, which normally results in a delay in their diagnosis [81]. Sarcoma models are fundamental for understanding cancer's molecular biology [4,5]. Sarcoma 180 being the most used in experimental studies due to presenting high dissemination and proliferation ratios, allows comparative studies for the use of natural and/or synthetic substances with toxic potentials [6–8].

Based on the three distinct stages of carcinogenesis (initiation, promotion and progression), chemopreventive agents must have the ability to inhibit, retard, or even reverse the tumor growth process [9,82]. The development of antitumoral therapies such as surgery, radiotherapy, cytotoxic chemotherapy and selective treatment methods have considerably raised cancer survival [10,83]. Although conventional chemotherapy offers many therapeutic advances, it still contains severe limitations, considering the development of resistance, limited efficacy against a series of solid tumors, side effects, intolerance and non-selectivity [11,84].

Administration of chemopreventive phytochemicals in small doses tends to mainly affect cancer cells and cancer stem cells (CSCs), while further increase in cellular stress may lead to irreversible damage [12–14,85]. This may especially be achieved by therapeutic generation of oxygen reactive species (ROS) that causes apoptosis in cancer cells [14,15]. On the other hand, alterations caused by phytochemicals in regular cells can be tolerated without affecting their physiological response [85], thus indicating the importance of studies with other candidate substances for antitumoral agents. Natural products generally present multi-directed actions with minimum side effects, which makes them ideal candidates for cancer therapy [16,86].

Chemotherapy can be prescribed to control, minimize or delay the development of cancer by the use of cytotoxic drugs, since the cytotoxic effect when it reaches adequate selectivity is able to eliminate cancerous cells [17,18]. [6]-Gingerol has shown various protective effects (antioxidant, anti-inflammatory and anti-tumor) in cellular systems [19]. [6]-Gingerol, found in ginger rhizome, it is recognized as the main active component, available in significant amounts in the fresh rhizome, responsible for most of the pharmacological activities [20,21], showed anti-proliferative effects in *in vitro* tests against cervical (HeLa, CaSki, SiHa) [22]; leukemic (K562, LAMA84, JURLMK1, U937, HL60, NB4) [15]; hepatic (HepG2) [23] and glioblastoma (U87) tumor cell lines [24]. Such carcinogenic strains demonstrated sensitivity to increased ROS during treatment with [6]-Gingerol, leading to suppression of growth and apoptotic induction.

The employment of cytogenetic biomarkers to assess the mutagenic/genotoxic activity of natural and synthetic compounds makes it possible to analyze genetic instability [25,26,87] in healthy and in tumor tissues/cells. The micronucleus test (MN) being widely used is to detect cytogenetic alterations such as clastogenicity, aneugenicity and other nuclear anomalies indicative of cell death [14,26,27,88]. The comet assay also contributes, initially in the assessment of genotoxicity by DNA chain rupture [89], as well as by oxidative damage [105]. Thus, this study aimed to assess the possible antitumoral actions of [6]-Gingerol in a non-clinical model, and explored the cytogenetic mechanisms indicative of genotoxicity, mutagenicity and apoptosis in S-180 primary cell.

2. Materials and methods

2.1. Reagents and chemicals

The isolated compound [6]-Gingerol ($C_{17}H_{26}O_4$) (PubChem CID: 442793), molecular weight 294.39 g/mol, of ≥ 98 % (HPLC) purity was procured from Sigma-Aldrich St. Louis, MO, USA. Culture medium RPMI 1640, penicillin and streptomycin were obtained from GIBCO® (Invitrogen, Carlsbad, CA, USA). Doxorubicin (Dox) was obtained from Eurofarma Laboratórios S.A. (São Paulo, Brazil); while hydrogen peroxide (H_2O_2) was obtained from Dinâmica Química Contemporânea LTDA (São Paulo, Brazil). Dox was used in a single concentration (6 μM, solubilized in dH_2O). H_2O_2 was prepared at a final concentration of 10 mM (diluted in dH_2O).

2.2. Analysis of the sigma standard [6]-Gingerol

The standard compound [6]-Gingerol was confirmed through coupled techniques such as HPLC-DAD, LC–MS [28].

2.3. Test sample preparation

The isolated compound [6]-Gingerol was diluted in sterile dH_2O until it reached the final concentrations of 21.33, 42.66 and 85.33 μM, previously chosen in cell viability assays in non-neoplastic cells with an IC_{50} of 47.7 μM and in Sarcoma 180 cells with IC of 24.44 μM, posteriorly used in CBMN, comet and cotreatment with H_2O_2 assays. It is worth emphasizing that such concentrations were also reported in antitumoral activity studies [29,30].

2.4. Primary cell culture of Sarcoma 180

Ascitic liquid cells of mice with S-180 were obtained from the peritoneal cavity (UFPI, #167/16). After 10 days of inoculation, the ascitic fluid containing tumor cells was removed by puncture in the abdominal cavity of the animal. Then, the S-180 cells were counted in Neubauer's chamber (4×10^6 cells/mL) and incubated in RPMI 1640 culture medium supplemented with 1 mM L-glutamine GIBCO® (Invitrogen, Carlsbad, CA, USA), 10 % (v/v) fetal bovine serum (FBS; Sigma-Aldrich, USA) and 1% (p/v) penicillin/streptomycin (Sigma-Aldrich, USA). The cells were cultured at 37 °C for 48–72 h concomitantly with the test substance and the controls as per the experimental requirement [31].

2.5. Cell viability via Trypan blue assay in S-180 cells

Cell viability was assessed through the application of the Trypan blue exclusion test according to Strober [32]. The cells were cultured at 37 °C for 48 h followed by treatment with test substance for further 24

h. After 72 h of total incubation, 90 μL of the cell suspension (0.5×10^6 cells/mL) were taken from the cultures and 10 μL of Trypan blue was added. Non-viable cells were counted with the aid of the Neubauer chamber in phase contrast microscopy with a magnification of 400×, based on their blue coloration, being considered as dead cells. In contrast, viable cells do not exhibit this staining because of their ability to expel Trypan blue.

2.6. Cytokinesis-blocking micronucleus assay (CBMN)

The CBMN assay was conducted according to Fenech [107] with some adaptations. Cells were cultured in flasks containing 2 mL of culture medium RPMI 1640 supplemented with phytohemagglutinin A GIBCO® (Invitrogen, USA), 1 mM/L L-glutamine GIBCO®, 10 % (v/v) FBS and 1% (p/v) penicillin/streptomycin. Total 20 μL of S-180 cell suspension (0.5×10^6 cells/mL) were added in culture flask, followed by addition of the test and control solutions. Cells were incubated for 48 h at 37 °C. After this period, 14.29 μM of Cytochalasin B (Sigma, USA) was added to the cells, and the flasks were incubated further for more 24 h. At the end of the total 72 h incubation, the cells were transferred to falcon tubes and centrifuged at 800 rpm for 5 min. Then, the supernatant was removed, and the cell pellet was gently agitated for further centrifugation after addition of 5 mL of fixative (methanol: acetic acid, 5:1) and 3 drops of formaldehyde in each tube. The procedure was repeated twice using 3:1 fixative and without formaldehyde. Finally, the supernatant was discarded, and 2–4 drops of the cell suspension were dripped onto slides, which were stained with 5% Giemsa solution for 7 min. Slides, previously codified, were analyzed in a blind test with the aid of the optical microscope (1000x), considering the cytogenetic damage present in 1000 cells per slide in duplicate. The nuclear division index (NDI) was calculated using the following formula: NDI = (M1 + 2M2 + 3M3 + 4M4)/N, where M1-M4 represents the number of viable cells with 1–4 nuclei, and N* the total number of viable cells. In addition, the nuclear division index considering cytotoxicity (NDIC) was calculated according to the following equation: NDIC = (Ap + Nec + M1 + 2(M2) + 3(M3) + 4(M4))/N* where Ap represents the number of cells in apoptosis; Nec, the number of cells in necrosis; M1-M4, the number of viable cells with 1–4 nuclei; and N*, the total number of viable and non-viable cells according to Fenech [108].

2.7. Comet assay

The alkaline version of the comet assay was conducted as described by Speit and Rothfuss [109]. Samples of 10 μL of the S-180 cell suspension (0.5×10^6 cells/mL) were mixed with a thin layer of 0.75 % low melting point agarose (90 μL) and placed on pre-coated slides with 1.5 % regular agarose. The slides were then dipped in lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10 with addition of 1% Triton X-100 and 10 % DMSO at the time of use) for up to 72 h at 4 °C. After this period, slides were incubated in alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min; then, they were exposed to an electric current of 300 mA and 25 V (0.90 V/cm) for 15 min in an electrophoresis chamber. Finally, slides were neutralized with Tris buffer (0.4 M and pH 7.5) and stained with silver nitrate solution. The slides were analyzed according to their photomicrograph profile of the cells (magnification of 400× under optical microscope) and results were expressed as damage index (DI) and damage frequency (DF) of 100 cells in duplicate. The DI was calculated using the formula: DI = Σ (number of cells in a given damage class X damage class), which ranged from 0 to 400 and DF by the following formula: DF = 100 - number of class 0 cells.

2.8. Assessment of genotoxicity mechanism by cotreatment with H_2O_2 (challenge assay)

We assessed in the experiment the involvement of oxidative stress as a mechanism for the induction of genotoxicity according to Luzhna et al. [78]. To that effect, 10 μL samples of S-180/peripheral blood cell suspensions (0.5×10^6 cells/mL) were mixed with a thin layer of 0.75 % low melting point agarose (90 μL) and deposited on slides pre-covered with 1.5 % regular agarose. Then, slides were exposed to [6]-Gingerol (21.33, 42.66, 85.33 μM), both isolated and in cotreatment with H_2O_2 (10 mM), for 5 min. The slides were then dipped in lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10 with addition of 1% Triton X-100 and 10 % DMSO at the time of use) for 5 min, at 4 °C. After this step, we proceeded with the continuation of the comet assay, according to the previous topic.

2.9. Statistical analysis

With the goal of determining divergence between treatments, data were expressed as the mean ± standard deviation (SD). Statistical significance was determined using one way-ANOVA followed by post hoc Tukey's test, considering values of $p < 0.05$ as significant. The program *Graphpad prism* (intuitive software for Science, San Diego, CA, USA) was applied. All studies were performed in duplicate, from independent biological evaluations.

3. Results

3.1. Assessment of purity of [6]-Gingerol by HPLC–UV and HPLC–ES-MS

Confirmation of the substance [6]-Gingerol employing HPLC-UV and HPLC-ES-MS was previously performed and published by [28].

3.2. Assessment of cytotoxicity of [6]-Gingerol by Trypan blue exclusion test

[6]-Gingerol, at concentrations of 21, 33, 42, 66, 85, 33 μM, as well as Dox (6 μM) interfered significantly the cell viability of the S-180 primary cells. It showed IC_{50} of 18.09 μM, IC: 14.03–23.38 and R2 =

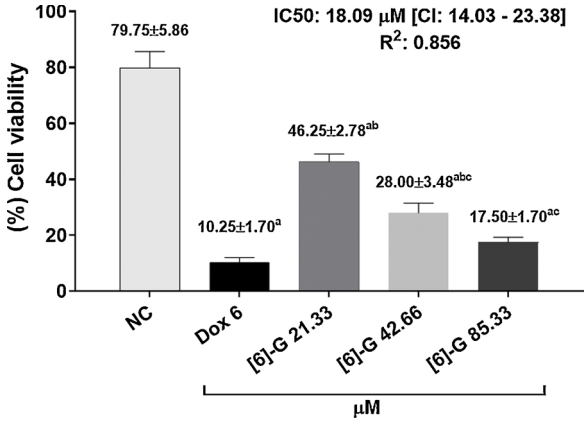

Fig. 1. Cytotoxicity assessment for [6]-Gingerol and IC_{50} determination. S-180 cells were treated with NC, Dox (6 μM), and [6]-Gingerol (21.33, 42.66 and 85.33 μM) at indicated concentrations for 24 h. Cell viability was assessed by Trypan blue exclusion test. Values represent the mean ± standard error of four independent experiments. One-way ANOVA followed by Tukey's post-test was applied for statistical analysis. [a] $p < 0.05$ compared to the NC group, [b] $p < 0.05$ compared to the Dox group and [c] $p < 0.05$ compared to the 21.33 μM group. Dox, doxorubicin; NC, negative control.

0.856 (Fig. 1), indicating cytotoxic effects by reducing viable cells when compared to NC (negative control). We did not observe statistical differences between the higher concentrations (42.66, 85.33 μM), which showed a significant reduction of their viability when compared to the smaller concentration (21.33 μM). We also verified that the concentration of 85.33 μM was as cytotoxic as Dox, being possible to observe a significant increase of cytotoxicity with the increase of [6]-Gingerol concentration.

3.3. Genotoxic effects of [6]-Gingerol in S-180 primary cells by comet assay

The genotoxicity assessment results demonstrate that [6]-Gingerol treatment induced an increase in the ratio of damage to tumor cells (S-180) independent of test concentration. Even if it differed statistically from NC, it showed a lower genotoxic effect when compared with Dox (Fig. 2A). Concerning damage frequency, we also verified the significant increase in the genotoxicity at all the concentrations of [6]-Gingerol, when compared to NC. While only the higher concentration of [6]-Gingerol induced an effect similar to Dox (6 μM). We also observed a significant difference between the lower (21.33 μM) and the higher (85.33 μM) concentrations of the groups treated with [6]-Gingerol regarding damage frequency (Fig. 2B).

The photomicrographic profile (optical microscopy 400×) exposes the outlines of S-180 primary cells treated with [6]-Gingerol, making it possible to record the distinctions between the groups in relation to the types (classes) of DNA damage (Fig. 3). S-180 tumor cells exposed to [6]-Gingerol presented increasing damage according to the increase in concentration. Thus, we verified in the distinct treatments damage levels 1 and 2 (21.33 μM); 2 and 3 (42.66 μM); 3 and 4 (85.33 μM), and [6]-Gingerol presented damage similarity with the Dox-treated group, especially at the highest concentration (85.33 μM), due to the frequency of damage levels 3 and 4.

3.4. Assessment of genotoxic mechanism of [6]-Gingerol in a primary S-180 cells

The Damage Index (DI) analysis verified that the tested concentrations for [6]-Gingerol induced genotoxicity in S-180 and non-tumor cells of healthy mice. Genotoxic damage caused by [6]-Gingerol in S-180 at the two highest concentrations (42.66 and 85.33 μM) was similar to the one observed in group H_2O_2; these also differed when compared to NC. However, when in cotreatment with H_2O_2 in S-180 cells, [6]-Gingerol significantly increased the damage induced by H_2O_2; while this effect was observed at the highest concentration in peripheral blood (PB) cells.

In healthy cells (PB), [6]-Gingerol induced toxicity at all tested concentrations as observed in the DI analysis. Although in cotreatment, the lower concentration showed modulatory effects on oxidative damage by H_2O_2 and widened H_2O_2 damage at the two highest concentrations (42.66 and 85.33 μM). Differences between PB and S-180 concerning genotoxic damage were observed in the NC for H_2O_2, as well as in the cotreatment for all concentrations.

When assessing damage frequency (DF), [6]-Gingerol demonstrated genotoxic action similar to H_2O_2 for S-180 only at the highest concentration. During cotreatment, however, none of the concentrations differed from data obtained for H_2O_2; in PB cells there was also genotoxic induction observed by statistical difference with NC, although in a non-similar way compared to H_2O_2. Differences in response to oxidative damage in S-180 and non-neoplastic cells were observed for the NC and for [6]-Gingerol only at the lowest concentration.

3.5. Antitumoral effects of [6]-Gingerol in S-180 cells by assessing the cytokinesis-blocking micronucleus assay

Assessing the antitumoral effects of [6]-Gingerol (Table 1), data indicate that the substance did not induce mutagenic effects via formation of micronuclei in S-180 cells when compared to NC. Although it was verified by a significant increase in the number of nucleoplasmic bridges at the highest tested concentration (85.33 μM). The test also showed nuclear buds at concentrations 42.66 and 85.33 μM, yet not differing from the results obtained with Dox. [6]-Gingerol induced cytotoxicity in S-180 tumor cells by reducing the cell division index (CDI), overall at its highest tested concentration (85.33 μM) and by the nuclear division index considering apoptosis and necrosis (NDINC). Concentrations of 42.66 and 85.33 μM, when compared to Dox, did not diverge statistically.

The Fig. 5 represents the photomicrographic profile (optic microscopy 1000×) of the cytogenetic alterations observed in S-180 cells treated with [6]-Gingerol. Photomicrographic analysis reiterates the obtained results for cytogenetic damage and cytotoxic action of [6]-Gingerol against S-180 primary cells, making it possible to highlight in increase of nuclear buds and nucleoplasmic bridges., Besides the cytotoxic potential, these results provided the evidence by showing apoptotic and necrotic cells. These data indicate that [6]-Gingerol induced nuclear alterations besides antitumoral activity by induction of cytotoxicity especially by apoptosis induction. It is worth mentioning that alterations generated by [6]-Gingerol were similar to those caused by Dox, mainly at the highest tested concentration.

The increase in concentration of [6]-Gingerol proportionately induced an increase in apoptosis in tumor cells (S-180). We could also verify that the higher concentrations (42.66, 85.33 μM) did not differ between each other, besides showing cytotoxic effects similar to Dox in S-180. However, data concerning necrosis indicated a significant absence of necrotic lesions when compared to NC (Fig. 6).

4. Discussion

[6]-Gingerol is a phenolic substance and a potential candidate for

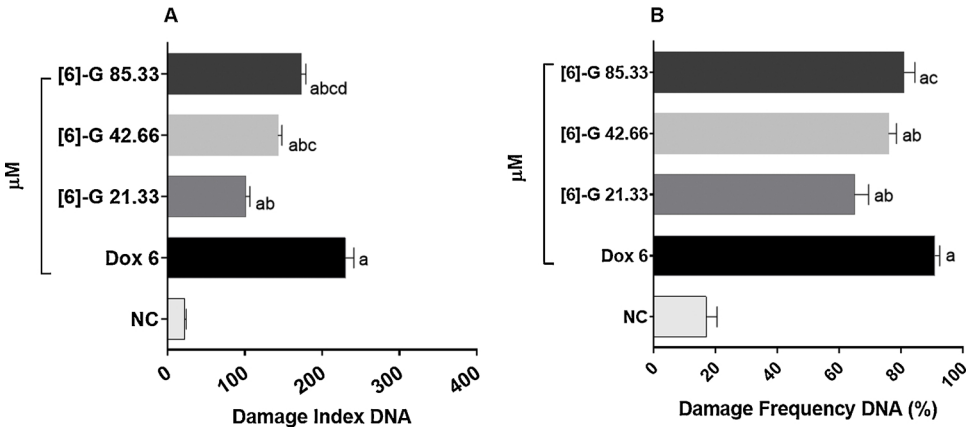

Fig. 2. Genotoxicity of [6]-Gingerol in primary culture of S-180 cells. Cells were treated with NC, Dox (6 μM), and [6]-Gingerol (21.33, 42.66 and 85.33 μM) at indicated concentrations for 24 h. **A**: Damage Index. **B**: Damage Frequency. Values represent the mean ± standard error of four independent experiments. One-way ANOVA followed by Tukey's post-test was applied for statistical analysis. [a] $p < 0.05$ compared to the NC group, [b] $p < 0.05$ compared to the Dox group, [c] $p < 0.05$ compared to the 21.33 μM group and e [d] $p < 0.05$ as compared to the 42.66 μM group. Dox, doxorubicin; NC, negative control.

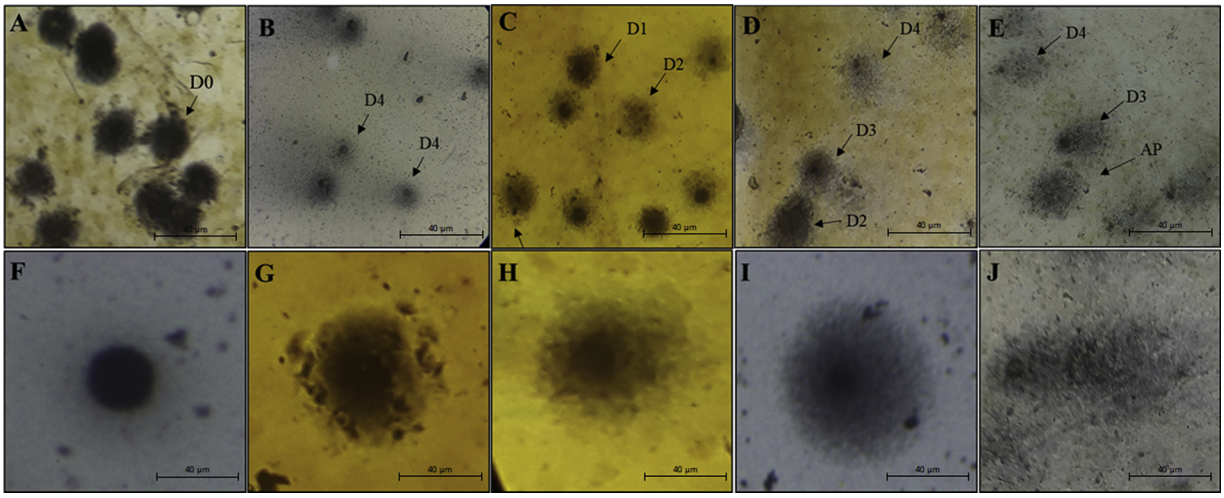

Fig. 3. Photomicrographic profile genotoxicity in a S-180 primary cells. Cells were treated with NC, Dox (6 μM), and [6]-Gingerol (21.33, 42.66 and 85.33 μM) at indicated concentrations for 24 h. Cells were analyzed by comet assay. **A**: untreated cells. **B**: dox (6 μM). **C**: [6]-Gingerol 21.33 μM. **D**: [6]-Gingerol 42.66 μM. **E**: [6]-Gingerol 85.33 μM. **F**: Damage 0 (D0). **G**: Damage 1 (D1). **H**: Damage 2 (D2). **I**: Damage 3 (D3). J: Damage 4 (D4). Staining with silver nitrate.

Table 1
Cytogenetic mechanisms of [6]-gingerol in S-180 cells.

Treatment	Cytogenetic damage			Cytotoxicity	
S – 180					
	Micronucleus	Brigdes	Nuclear Buds	IDN	IDNC
NC	1.75 ± 1.02	2.80 ± 0.41	1.70 ± 0.12	1.58 ± 0.20	1.62 ± 0.04
Dox (6 μM)	36.50 ± 0.35[a]	35.50 ± 3.53[a]	17.00 ± 1.41[a]	1.14 ± 0.03[a]	1.12 ± 0.02[a]
[6]-gingerol					
21.33 μM	2.00 ± 0.41[b]	4.00 ± 2.80[b]	5.50 ± 0.70[b]	1.49 ± 0.07[b]	1.19 ± 0.03[a b]
42.66 μM	4.50 ± 0.70[b]	18.50 ± 0.70[abc]	15.50 ± 2.12[ac]	1.41 ± 0.02[ab]	1.16 ± 0.005[a]
85.33 μM	4.50 ± 0.70[b]	31.50 ± 2.12[acd]	17.00 ± 2.82[ac]	1.18 ± 0,06[a]	1.12 ± 0.03[a]

Cells were treated with NC, Dox and [6]-gingerol at indicated concentrations for 24 h followed by assessment of indicative of antitumoral effects through cytokinesis-blocking micronucleus assay. Values represent the mean ± SD of four independent experiments. One-way ANOVA followed by Tukey's post-hoc test was applied for statistical analysis. [a] $p < 0.05$ compared to the NC group, [b] $p < 0.05$ compared to the Dox group, [c] $p < 0.05$ compared to the 21.33 μM group and [d] $p < 0.05$ as compared to the 42.66 μM group. IDN = M1 + 2 (M2) +3 (M3) +4 (M4)) / N, where M1-M4 represents the number of viable cells with 1–4 nuclei and N*, the total number of viable cells. IDNC = (Ap + Nec + M1 + 2 (M2) + 3 (M3) + 4 (M4)) / N *. Ap, represents the number of cells in apoptosis; Nec, the number of cells in necrosis. M1-M4, the number of viable cells with 1–4 nuclei; and N*, the total number of viable and non-viable cells. NC, negative control (untreated cells); Dox, doxorubicin.

antitumoral agent due to the mechanisms associated to toxicity. The Trypan blue exclusion test with a S-180 primary cell (Fig. 1) supported the observations which was further verified with a significant reduction in cell viability (IC_{50} of 18.09 μM), especially at the higher concentration (85.33 μM) and as compared to the antineoplastic agent doxorubicin. These data corroborate other studies on the antitumoral effects of [6]-Gingerol [23,30,33] and the systematic literature survey by De Lima et al. [19]. [6]-Gingerol was reported to cause reduction in viability in the treatment of tumor cells [22,34] as well as caused the apoptotic induction in cell cultures of cancers of cervical (10–60 μM), colon (5–15 μM), pancreas (5–20 μM), breast (2–10 μM), glioblastoma (10–100 μM) and leukemia (10–200 μM) [15,24,29,30,35–37].

Amongst the proposed mechanisms for Dox-mediated cell death, we highlight the increase in oxidative stress, possibly by the quinone group of Dox which can be oxidized to a semiquinone radical by the addition of an electron [38,39]. While the semiquinone radicals react rapidly with oxygen to generate hydrogen superoxide and peroxide, causing DNA damage and cell death. Dox also acts as an iron chelator, where the Dox-iron complex catalyzes the conversion of hydrogen peroxide into highly reactive hydroxyl radicals [6,40]. Therefore, studying cytotoxicity stands out as a screening method with high yield, strategic in the evaluation of healthy and tumor cell lines exposed to anticancer agents, allowing the study of numerous substances in a brief period of time [90]. The cytotoxic action of [6]-Gingerol in tumor cell lines was also evidenced by the inhibitory effect of viability on human colorectal adenocarcinoma (HCT-15) with IC_{50} of 29 μM [41]. In addition, [6]-Gingerol (150 and 299 μM) promoted antitumoral and pro-apoptotic characteristics in human colon cancer (HCT-116), leading to a reduction of the cell growth rate by 22 % and 28 % at respective tested concentrations. [6]-Gingerol also inhibited the cell viability in SW480 (17 %), HT-29 (28 %), LoVo (13 %) and Caco-2 (8%) at concentrations above 200 μM. Studies also pointed out that [6]-Gingerol induces cell cycle arrest in colon cancer cell lines with an increase in phase G1 and reduction of phase S of mitosis [35]. The anticancer activity of the majority of natural products generally acts through regulation of the immunologic function, inducing apoptosis or inhibiting cell proliferation [91]. However, isolated substances of food origin, such as [6]-Gingerol, should be submitted to toxicogenic potential analysis [92] in tumor and non-tumor tissues in order to verify possible cytogenetic alterations [88]. In addition, it also requires analyzing DNA chain ruptures [89] and oxidative damage [105].

Treatment of tumor cells (S-180) with [6]-Gingerol (Fig. 2) showed an induction of genotoxicity by an increase of the DI independent of the concentration. It also increased DF, especially at 85.33 μM, with a significant similarity to Dox. It is worth emphasizing that in the alkaline version of the comet assay the detected damage may be associated to

single and/or double-strand breaks, alkali-labile sites and oxidative damage [93]. In a study conducted by Lin et al. [30] using human colon tumor cell lines (LoVo), [6]-Gingerol was able to induce cell cycle arrest at the phase G2/M along with elevation of the levels of ROS and p53 phosphorylation. Thus, the generation of ROS induced by [6]-Gingerol is known for increasing DNA damage in cancer cells [94], as observed for chronic (K562) and acute (U937) myeloid leukemia cells [15]. DNA damage caused by both external agents and endogenous processes may overload the functional DNA repair mechanism and may cause genetic instability with the formation of mutations [42–45].

[6]-Gingerol induced genotoxic damage in S-180 cells and in non-tumor cells of the peripheral blood (PB) of healthy mice (Fig. 4), especially at the two higher concentrations which was equivalent to the effects of H_2O_2. These data point to the possible similarities in the generation of oxidative stress. In cotreatment with H_2O_2 in S-180 cells, [6]-Gingerol increased oxidative damage induced by the peroxide, indicating oxidative and/or pro-oxidative effects. However, in PB, these effects were observed only at the higher concentration. Despite H_2O_2 not being a free radical, it is a species with a high reactive potential, since it participates in the generation of the hydroxyl radical ($OH^{\cdot}$). The later has a potentially deleterious actions and constitutes in the most reactive radicals. Since it can alter the nearby cellular structures, H_2O_2 still differs from the free radicals by the greater stability and ability to cross the cell membranes. Suppression of ROS using phytochemicals is crucial for cancer chemoprevention and at the same time, they have also been recognized as ROS-inducing agents in a wide variety of cancer cells [98]. These showed elevated ROS levels (particularly H_2O_2) and are adapted to survive under these conditions. Some phytochemicals further disrupt this "balance", raising the amount of ROS to lethal levels, and lead to the activation of various pathways including apoptosis in neoplastic cells. Some phenolic antioxidants act as pro-oxidants in certain circumstances (high pH with high concentrations of transition metal ions and O_2) which favor the onset of their auto-oxidation process [95]. In the study with H_2O_2, it was evident with the statistical differences of genotoxicity by the increase of the damage index between S-180 and PB cells of healthy mice. This effect was observed using damage frequency only on the negative control and at the lowest [6]-Gingerol concentration. When compared to non-tumor cells, tumor cells showed increased levels of ROS [98], which acts as a detrimental condition to tumor cells. Since they rely on a robust endogenous antioxidant system, it attenuates oxidative stress to proliferate [106].

Still on the oxidative mechanisms, [6]-Gingerol is able to induce lysosomal destabilization, as well as mitochondrial perturbances, causing damage to the electron transport chain. This leads to the production of ROS which induces even more DNA damage [46]. Lysosomal and mitochondrial destabilization has been recognized as a characteristic of cell damage induced by oxidative stress [47–50]. Several studies have suggested that the accumulation of EROs derived from lysosomes and mitochondria is a crucial factor for mechanisms of cell death by apoptosis and autophagy [51–53]. There are also reports that the exposure of cancer cells to phytochemicals induces a sustained response to DNA damage that can lead to cell death by apoptosis [96,99].Cytogenetic mechanisms can lead to cell death by apoptosis and/or necrosis, as observed in cells submitted to the CBMN assay [54,55]. In this study, [6]-Gingerol did not induce clastogenic and aneugenic effects by formation of micronuclei in S-180 cells (Table 1). However, there are contrasting reports showing that [6]-Gingerol (at concentrations of 5–40 μM) induced an increase in the frequency of micronuclei in HepG2 cells [46]. Mutagenic effects depend on the model organism or the concentration/dose used [56–58]. However, mutagenic effects and genetic instability of [6]-Gingerol were observed by the increase of nucleoplasmic bridges at the highest concentration (85.33 μM), as well as the formation of nuclear buds, with a significant increase at 42.66 and 85.33 μM. Nucleoplasmic bridges can occur through rearrangements (dicentric chromosomes, ring chromosomes and chromosomes involved in telomeric associations), where the chromosomes are pulled to the opposite poles during anaphase [59]. Presence of nuclear buds is linked to chromosomic fragments [60], as a mechanism to eliminate the excess of chromosomes in a response process to aneuploidy [61], as well as after nucleoplasmic bridges breaks [62].

It is worth mentioning that lesions on the genetic material can lead to the formation of chromosomal rearrangements (deletions, duplications, inversions and translocations) [63,64]. Thus, genomic instability plays a critical role in the initiation and progression of cancer, with the presence of aneuploidy, tetraploidy, lose or fragmented chromosomes [65,66], facilitating the formation of micronuclei [67,68]. We have extensively analyzed the genomic instabilities, chromosomal rearrangements, and other cytogenetic aberrations in different chronic models. The cytogenetic based analysis of the effect of diets, familial history, and alternative therapies on genomic instability in breast cancer patients was reported [69,70], as well as correlations between risk factors for breast cancer and genetic instability in cancer patients was drawn [70]. Furthermore, the toxicogenomic profiling of omeprazole and the modulatory effects of retinol palmitate and ascorbic acid was reported using chromosomal rearrangements studies [14].

The use of S-180 as an experimental model in the evaluation of the cytotoxic effect of natural substances [71] as [6] -G, is fundamental for understanding the molecular biology of cancer, since sarcomas are distinguished by molecular aberrations such as mutations, intergenic deletions, gene amplifications and translocations [4], murine tumor cells present as an important preclinical tool in the study of antitumor properties [72].

The cytogenetic mechanisms observed in S-180 tumor cells exposed to [6]-Gingerol may be related to the level of cytotoxicity by the reduction of NDI and NDIC, especially in 42.66 and 85.33 μM, similar to that observed for Dox (Table 1). This showed that increased

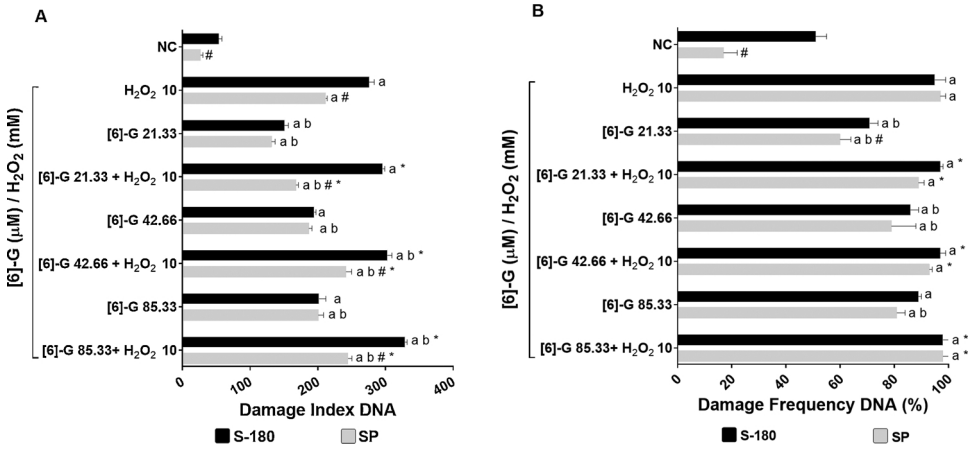

Fig. 4. Genotoxicity of [6]-Gingerol in S-180 primary cell culture and peripheral blood (PB) of mice without neoplastic changes in cotreatment with hydrogen peroxide. Cells were treated with NC, H_2O_2 (10 mM), and [6]-Gingerol (21.33, 42.66 and 85.33 μM) at indicated concentrations for 24 h. **A**: Damage Index. **B**: Damage Frequency. Values represent the mean ± SD of four independent experiments. One-way ANOVA followed by Tukey's post-test was applied for statistical analysis. [a] $p < 0.05$ compared to the NC group, [b] $p < 0.05$ compared to the H_2O_2 group, [#] $p < 0.05$ compared to the S-180 group at the same concentration, * $p < 0.05$ compared to the [6]-Gingerol without H_2O_2 in the same concentration. PB, peripheral blood; NC, untreated cells.

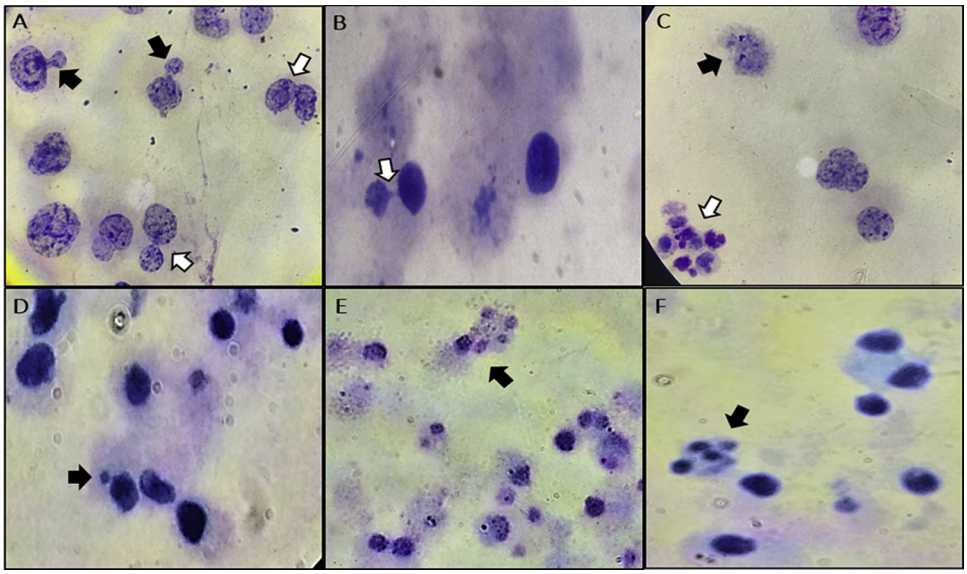

Fig. 5. Photomicrographic profile of S-180 cells treated with [6]-Gingerol. Cells were treated with [6]-Gingerol (21.33, 42.66 and 85.33 μM) and other substances at indicated concentrations for 24 h and CBMN assay was performed at magnification 1000×. Black arrow in **A**: nuclear buds. White arrow in **A**: nucleoplasmic bridges. Arrow in **B**: nucleoplasmic bridges. Black arrow in **C**: necrosis. White arrow in **C** apoptosis. Arrow in **D**: micronucleus. Arrow in **E**: apoptosis. Arrow in **F**: apoptosis.

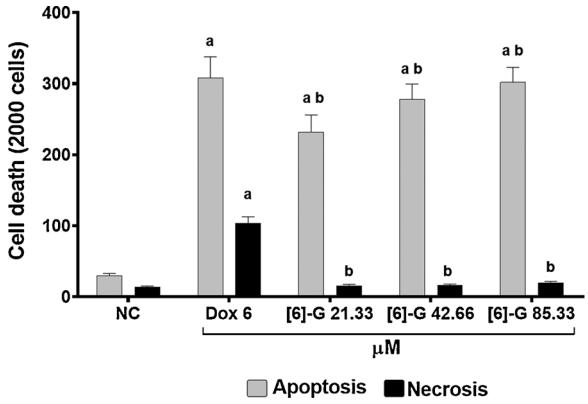

Fig. 6. Antitumoral effects of [6]-Gingerol by apoptosis and necrosis in S-180 primary culture. Cells were treated with [6]-Gingerol for 72 h followed by assessment of apoptotic and necrotic cell population. Values represent the mean ± SD of four independent experiments. S-180. NC: untreated cells. Dox: 6 μM. [6]-Gingerol (21.33, 42.66 and 85.33 μM). One-way ANOVA followed by Tukey's post-test was applied for statistical analysis. [a] $p < 0.05$ compared to the NC group, [b] $p < 0.05$ compared to the Dox group in the same treatment week.

concentration of [6]-Gingerol influenced the increase in the number of apoptotic cells in tumor cells (S-180). While at higher concentrations, these demonstrated the similar Dox-like cytotoxic effects in S-180. There have been reports that [6]-Gingerol induces cell death by autophagy and caspase 3-mediated apoptosis and decreased expression of the NF-κB, AKT and Bcl2 genes in HeLa cell lines. On the other hand, there has been an increase in the expression of pro-apoptotic TNFα, Bax and cytochrome C [29]. According to Wang et al. [73], [6]-Gingerol may also inhibit Bcl-2 expression with subsequent induction of apoptosis in HL-60 cells. Apoptotic effects of [6]-Gingerol have also been related to the release of cathepsin D into the cell cytoplasm, preceding the release of mitochondrial cytochrome C with subsequent induction of apoptosis in HepG2 [74].

As previously reported, [6]-Gingerol showed antitumoral effects by induction of apoptosis (Fig. 6). Programmed cell death is a key process in the mechanism of action of chemotherapeutic agents against tumor cells [75–77]. This process can be mediated by caspases through the activation of death receptors at the cell surface, resulting in the activation of caspase-3 and 7. As for the mitochondrial pathway, Bcl-2 was the first apoptosis inhibitor to be discovered, acting through the control of mitochondrial membrane permeabilization procedures, regulating the release of cytochrome C [100,101]. There are reports of association between cell viability and apoptosis with ROS, as previously described. Exposure of the human glioblastoma cell line (U87) to [6]-Gingerol showed a reduced viability and increased apoptosis at concentrations of 10 − 100 μM. This was achieved both through the generation of ROS and increased expression of pro-apoptotic proteins (p53, Bax) and Bid cleavage, whereas the reduction of the level of anti-apoptotic proteins (cFLIP, Bcl-2, XIAP) [24]. In SW-480 (colon cancer) tumor cells treated with [6]-Gingerol, we observe a significant cleavage of pro-caspases -8 and -9 and effector caspases -3 and -7 in a concentration-dependent manner. Cleavage of the PARP protein, which is a caspase-3 substrate, has also been observed. In the treatment of cells with 200 and 300 μM [6]-Gingerol, the 116-kDa form of PARP was fragmented at 89-kDa, confirming a caspase-mediated apoptosis [37].

5. Conclusions

Results for this study demonstrate the antitumoral abilities of [6]-Gingerol in S-180. It induced cytotoxic response through apoptosis and limited DNA damage, as well as by it increased the genotoxicity. Genotoxic effects were mainly associated to the generation of ROS which further associates with the indicatives of genetic instability. [6]-Gingerol induced nuclear alterations of the types of nucleoplasmic bridges and nuclear buds, which culminated in to apoptosis, as observed by the significantly increased number of apoptoses. [6]-Gingerol demonstrated antitumoral effects through cytogenetic mechanisms with an induction of apoptosis, suggesting that the substance possesses antitumoral potential and can be used in formulations for cancer therapy alone or in combination of other modulators of the antitumoral biomarkers.

Authors contribution

AMTDL and ACDR performed primary experiments; JVDOS and JRDOF performed secondary experiments; ACSD, APMM, AMOFDM and MVOBDA performed data analysis and literature survey for manuscript preparation; TDJADSA, MFCJP, DCDNR, PMPF and JMDCS provided secondary experimental support, analyzed data, and assisted in manuscript preparation; MTI and SKM analyzed data, wrote manuscript and communicated the research paper; AADCMV overall supervised the research work, performed data analysis, and assisted in manuscript preparation.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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ORIGINAL ARTICLE

Non-clinical toxicity of (+)-limonene epoxide and its physio-pharmacological properties on neurological disorders

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Abstract

The compound (+)-limonene epoxide has antioxidant, anxiolytic, and antihelminthic properties. However, investigations to determine its long-term exposure were not performed. We investigated the systemic toxicological profile after chronic exposure as well as the antidepressant and antiepileptic potentialities of (+)-limonene epoxide on mice. Initially, we evaluated acute toxicity on *Artemia salina* nauplii and cytotoxicity on mice erythrocytes and peripheral blood mononuclear cells (PBMC). Aftterwards, mice were chronically treated for 120 days by gavage with (+)-limonene epoxide (25, 50, and 75 mg/kg/day) and this exposure was assessed by pathophysiological measurements. For antidepressant and anticonvulsivant analysis, we performed the forced swimming and tail suspension protocols and pentylenetetrazol- and picrotoxin-induced seizures, respectively. (+)-Limonene epoxide showed a LC_{50} value of 318.7 μg/mL on *A. salina* shrimps, caused lysis of red blood cells at higher concentrations only but did not show cytotoxicity on PMBC, which suggests pharmacological safety if plasma concentrations do not exceed 100 μg/mL. Macroscopic, hematological, clinical chemistry, and nutritional changes were not detected, though focal areas of hepatic necrosis, inflammatory infiltrate, and karyolysis have been detected at 75 mg/kg/day. The compound inhibited the developing of pentylenetetrazol- and picrotoxin-induced seizures, decreased deaths, and reduced immobility times, mainly at 75 mg/kg. So, it reversed reserpine effects, suggesting antidepressant effects should be linked to serotonergic and/or adrenergic transmission. It is feasible that (+)-limonene epoxide plays a benzodiazepine-like anticonvulsive action and may be also recommended as an antidote for poisonings caused by central depressants.

Keywords Antidepressant action · Antiepileptic properties · Biochemical tissue markers · Chronic effects · Terpenoid derivative

Introduction

Systemic/complex illnesses such as neurological/psychosocial diseases, cancers, and cardiovascular and respiratory disorders are the biggest health problems without a perspective of cure (Emdin et al. 2015; Moshé et al. 2015; Araújo et al. 2017; Ferreira and Pessoa 2017). In this context, disorders involving the central nervous system, including anxiety, depression, phobias, schizophrenia, Alzheimer's, Parkinson's, and convulsion, present a high prevalence and require treatments with psychotropic drugs for controlling and reestablishing behavior and humor. Depression is one of the most disabling and prevalent neurological/psychosocial disorders, affecting about 15 to 25% of the population, and studies predict that until 2020, it will be the second most diagnosed disease worldwide, which illustrates its severity and social impacts (Kubacka et al. 2016; Pytka et al. 2016; Szczechowiak et al. 2019).

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From the symptomatological point of view, depression is characterized by sadness, loss of interest or pleasure, sleep and appetite disorders, tiredness, low self-esteem, or low concentration, which strongly impairs the individual's ability to deal with daily problems, and sometimes even induce suicide. Moreover, there is a strong association between depression and other comorbidities, such as epilepsy (Rojas et al. 2011; Mosińska et al. 2016). The treatment with antiepileptic drugs as well as social adaptive capacity was also recognized as risk factors for epilepsy in patients with depression. On the other hand, depression is often seen as a reaction to the stigma of epilepsy and associated with poor quality of life. Well-established patients with epilepsy exhibit rates of depression and suicide up to 4–5-fold greater when compared with the healthy population (Moshé et al. 2015; Yan et al. 2015; Blaszczyk and Czuczwar 2016).

To overcome such a situation, aromatic plants and their active compounds have been investigated to treat neurological chronic diseases. Additionally, most of them have revealed low toxicity (Silva et al. 2012; Dutra et al. 2016). The (+)-limonene epoxide, a semi-synthetic terpenoid derivative from limonene, is considered a promising molecule due to its antioxidant, anxiolytic, and antihelminthic properties (Almeida et al. 2012, 2014a, b). However, investigations to determine its toxicity after long-term administration were not performed. For behavioral and toxicological studies of natural and synthetic compounds, non-clinical toxicological evaluations, generally performed in rats and mice, are considered essential for developing and validation of new drugs and to understand their applicability for humans and pharmaceutical innovation (Houck and Kavlock 2008; Lima et al. 2012; Ferreira et al. 2015, 2019). So, we investigated the systemic toxicological profile after chronic exposure, as well as the antidepressant and antiepileptic potentialities of (+)-limonene epoxide on mice.

Methods

Chemicals

Fetal calf serum, RPMI 1640 medium, trypsin-EDTA, Ficoll-Hypaque, penicillin, phytohemagglutinin, and streptomycin were purchased from Cultilab (Campinas, Brazil). Doxorubicin, (+)-limonene epoxide, ketamine, flumazenil, reserpine, paroxetine, diazepam, imipramine, xylazine, and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). NaCl, $MgSO_4$, HCl, $MgCl_2$, $CaCl_2$, KCl, EDTA, $NaHCO_3$, Tween 80, triton X-100, hematoxylin, eosin, dimethylsulfoxide (DMSO), and citrate were obtained from Vetec Química (Rio de Janeiro, Brazil).

Cell and animal facilities

Artemia salina L. (Artemiidae) eggs were obtained from Maramar Pet™ from the local market (Teresina, Brazil). Eggs of *A. salina* were hatched (10 mg/L) at 25 °C in artificial seawater (NaCl 77.2%, $MgSO_4$ 9.6%, $MgCl_2$ 7.1%, $CaCl_2$ 3.3%, KCl 2.1%, and $NaHCO_3$ 0.6%) prepared with non-chlorinated mineral water. Incubation was performed at 25 °C with constant aeration for 24 h to allow nauplius hatching.

Adult male and female Swiss mice (*Mus musculus* Linnaeus, 1758) were obtained from the Universidade Federal do Piauí (UFPI), Teresina, Brazil. They were kept in well-ventilated cages under standard conditions of light (12 h with alternative day and night cycles) and temperature (22 ± 1 °C) and were housed with access to commercial rodent stock diet (Nutrilabor, Campinas, Brazil) and water ad libitum. All procedures were approved by the Committee on Animal Research at UFPI (#091/2014) and followed Brazilian (*Colégio Brasileiro de Experimentação Animal* - COBEA) and International rules on the care and use of experimental animals (Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes).

Heparinized blood samples from mice were collected from the retrorbital plexus using sterile tubes and heparinized pipettes. Peripheral blood mononuclear cells (PBMC) were isolated by the standard method of density-gradient centrifugation over Ficoll-Hypaque.

Acute toxicity assay on brine shrimps

To perform the acute toxicity test, dormant eggs of *Artemia salina* L. (Artemiidae) were hatched (10 mg/L) at 25 °C in artificial seawater (McLaughlin and Rogers 1998). After 48 h, hatched larvae were collected ($n = 10$) and added to diluted concentrations of (+)-limonene epoxide with seawater (50–1000 μg/mL). After 24 h of incubation, living/dead larvae were counted. The negative control group was represented only by seawater and potassium dichromate (10–100 μg/mL) was used as a positive control. Then, the LC_{50} value was determined (Hamilton et al. 1977).

Hemolytic assay

The compound was solubilized in DMSO 10 mg/mL and tested for hemolytic action (15.7–1000 μg/mL) in 96-well plates during 60 min at environmental temperature (25 °C) using a suspension of 2% erythrocytes from mice prediluted in 0.85% NaCl with 10 mM $CaCl_2$ (Carvalho et al. 2013). After centrifugation, free hemoglobin contents were assessed using a multiplate reader (PG Instruments Ltd.®, T80+ UV/VIS

Spectrometer, UK) at 450 nm. Triton X-100 2% was used as a positive control.

MTT assay

The cytotoxicity on PBMC was determined by the MTT assay (Mosmann 1983). Cells were washed, resuspended in supplemented RPMI-1640 medium plus 4% phytohemagglutinin, and plated in 96-well plates (3×10^5 cells/mL). After 24 h, the compound was added to each well at a final concentration of 100 μg/mL. Doxorubicin (0.3 μg/mL) was used as a positive control. Cells of negative control and positive control and those treated with (+)-limonene epoxide were exposed to the same DMSO percentage (0.1%). Following 68 h of incubation, a fresh medium containing 10% MTT was added and cells were incubated for 4 h. Formazan product was dissolved in DMSO and absorbance was read using a multiple reader (PG Instruments Ltd™, T80+ UV/VIS Spectrometer, UK) at 595 nm.

Assessment of systemic chronic toxicity: physiological and tissue markers

Analyses were performed according to the Organization for Economic Co-Operation and Development (OECD 2018), with minor modifications in order to include more data. OECD (2018) suggests an exposure period of 12 months and measurements (weighing) and regular detailed observations (hematological examination, urinalysis, clinical chemistry), as well as necropsy procedures and histopathology. Male ($n = 5$/group) and female ($n = 5$/group) mice weighing between 25 and 30 g were divided into four groups. All animals ($n = 10$/dose) were orally treated for 120 days with 25, 50, and 75 mg/kg/day of (+)-limonene epoxide (0.1 mL/10 g). The negative control group received only the vehicle (Tween 80 0.05% and dissolved in saline 0.9%).

Behavioral observations During the study, systematic behavioral observations were performed to evaluate the Hippocratic screening, which provides a general estimate of toxicity, state of consciousness, mood, motor system activity, motor coordination, and reflexes. Some parameters (general activity, vocal finesse, irritability, touch response, writhing, righting reflex, muscle tone, grasping strength, ataxia, atrial reflex, corneal reflex, tremors, convulsions, straubysis, anesthesia, lacrimation, palpebral ptosis, urination, defecation, piloerection, respiration, and death) were evaluated during the 120-day period on interspersed days. Signs of toxicity, as well as the time of its appearance, intensity, duration, and progression, were also observed. Body mass and feed pattern of animals were also quantified: ingested volume of water, weight of feed consumed, and amount of excreta produced (Lucio et al. 2000; Cunha et al. 2013).

Blood parameters and tissue analysis On the 121st day, all animals were anesthetized with ketamine (90 mg/kg)-xylazine (4.5 mg/kg) for blood collection from the retrorbital plexus using sterile tubes. Blood was conditioned in two types of tubes: (i) with anticoagulant ethylenediamine tetraacetic acid (EDTA) (HB, Laborlab™, Brazil) for determination of hematological parameters and (ii) without anticoagulant to obtain serum for evaluation of biochemical parameters.

Hematological parameters (red and white cells and platelets) were performed in total blood samples using an automatic analyzer (Advia120/Hematology Siemens™, Germany). The differential leukocyte count was performed in hematological smears stained with May-Grünwald-Giemsa. For biochemical analysis, blood samples were centrifuged at 2000 rpm for 5 min. Then, we examined physiological markers of the liver (aspartate aminotransferase (AST), alanine aminotransferase (AST), alkaline phosphatase (ALP), total and direct bilirubin, total protein), kidneys (blood urea nitrogen (BUN), creatinine), pancreas (glucose), triglycerides, and total cholesterol using Labmax 240 automated apparatus (Labtest™, Brazil) and Labtest® kits and following manufacturer's recommendations.

Afterwards, animals were euthanized to dissect out the liver, kidneys, and hearts to obtain wet relative weights and for macroscopic analysis. Next, livers were fixed with 10% buffered formalin, processed, cut into small pieces to prepare histological sections (4–7 μm), and stained with hematoxylin and eosin (H&E). Morphological analyses were performed by a pathologist under light microscopy (Olympus™, Tokyo, Japan).

Neuropharmacological evaluations

Mice were divided into experimental groups ($n = 6$ animals/group) and orally treated (0.1 mL/10 g) with 25, 50, and/or 75 mg/kg/day of (+)-limonene epoxide. The vehicle (negative control group) received 0.05% Tween 80 dissolved in 0.9% saline. For each protocol, appropriate positive controls were used.

Antidepressant evaluation Animals received the tested compound for 30 days. As positive controls, we used reserpine, paroxetine, and imipramine (0.25, 20, and 50 mg/kg i.p., respectively) (Steru et al. 1985; Porsolt et al. 1987).

Forced swimming test To assess the swimming performance, a 22-cm-diameter and 40-cm-high tank containing fresh water at 26 ± 1 °C was used (filled up to half) (Porsolt et al. 1987). Thirty minutes after the treatments, the animals were placed one by one in the tank to note immobility time (in seconds) for 5 min. The animal was considered immobile when it remained floating in the water, making only gentle movements necessary to keep its head above water.

Tail suspension test The animals were suspended and maintained for 5 min with adhesive tapes about 1 cm from the tip of the tail on a platform 58 cm above the bench (Steru et al. 1985). The time spent immobile was the dependent measure. Mice that climbed up their tails for more than 20% of the total trial time were removed from the analysis. All mice were carefully monitored for any adverse effects during the test and were quickly removed if they display signs of unusual distress (i.e. constant vocalization or damage to their tails) (Can et al. 2012).

Antiepileptic evaluation After 30 min of treatments, all groups received pentylenetetrazol (60 mg/kg, i.p.) to induce clonic seizures (Smith et al. 2007) or picrotoxin (8 mg/kg), a specific blocker of the $GABA_A$-type chloride channels (Calcaterra and Barrow 2014). The latency and percentage reduction of clonic convulsions and the incidence of deaths were recorded up to 24 h. Then, immediately after administration of the seizure agent, mice were individually placed in plastic boxes and observed to determine the latency of the first epilepsy, percentage of animals with seizures, and mortality rate (Lehmann et al. 1988; Bum et al. 2001). As pharmacological tools, animals from control groups were pretreated with oral doses of diazepam (2 mg/kg, a benzodiazepine positive allosteric modulator of $GABA_A$ receptors) (Calcaterra and Barrow 2014) or flumazenil (5 mg/kg, a benzodiazepine antagonist) (Saxona et al. 2010) combined or not with (+)-limonene epoxide at 75 mg/kg after 30 min.

Statistical analysis

Nonlinear regression was used to calculate average values of IC_{50} (50% growth inhibition of cell proliferation), EC_{50} (50% effective concentration of hemolysis), and LC_{50} (50% death population of animals) (Intuitive Software for Science, San Diego, CA). All in vitro experiments were performed twice, representing independent biological evaluations in alternated days/weeks, in which each concentration sample was tested in triplicate. Differences were evaluated by comparing data (mean ± standard error of mean (S.E.M.)) using one-way analysis of variance (ANOVA) followed by the Newman-Keuls test ($p < 0.05$).

Results and discussion

Toxicity effects

Several methods are useful to predict toxicology (and side effects), such as animal cells, vegetal molds, microalgae, invertebrate animals, and mammals (ANVISA 2013; Carvalho et al. 2016; Ferreira et al. 2019; Tsang et al. 2019). The microcrustacean *Artemia salina* Leach (1819), a cosmopolitan invertebrate, is an easy-to-maintain animal under laboratory conditions, sensitive, and of low cost, which has been widely used by the scientific community as (eco)toxicological biosensor since it determines preliminary toxicity of natural and synthetic products with potential biological activity (Amenya et al. 2011; Silva et al. 2016). Herein, we used phase II nauplii (48 h after hatching eggs) determining a concentration-effect curve (Gad 2014).

The compound (+)-limonene epoxide showed a LC_{50} of 318.7 ± 45.9 μg/mL ($R^2 = 0.8243$) while the positive control potassium dichromate caused 100% mortality (Fig. 1a). According to Meyer et al. (1982), molecules with $LC_{50} <$ 1000 μg/mL are considered to be toxic. However, David et al. (2001) classify substances with $LC_{50} < 100$ μg/mL as very toxic. On the other hand, it suggests that this molecule possesses pharmacological activity, since this bioassay has demonstrated a good correlation with other biological properties such as antioxidant, antimicrobial (Martins et al. 2014; Islam et al. 2016), and antitumor activities (Ferreira et al. 2016, 2019).

On the other hand, (+)-limonene epoxide caused increased percentage of damage to red blood cells only at the two highest concentrations tested (500 μg/mL, 69.5 ± 0.02%; 1000 μg/mL, 77.9 ± 0.1%) when compared with the negative control (Fig. 1b). Erythrocyte membranes contain a high concentration of polyunsaturated fatty acids, which makes them very vulnerable to reactions involving chemical direct aggressions, resulting in pores or disruption. So, the evaluation of cytotoxic potential in erythrocytes is a reliable in vitro model to investigate toxic effects of natural and synthetic samples (Brandão et al. 2005).

Knowing the lytic potential occurs at higher concentrations (> 500 μg/mL) and that white blood cells are potential undesirable targets of cytotoxic drugs (Gomes-Júnior et al. 2015), the compound was also evaluated in PBMCs. However, (+)-limonene epoxide did not show cytotoxicity on primary culture of normal mice leukocytes, which suggests pharmacological safety if plasma concentrations do not exceed 100 μg/mL, especially if we take into consideration continuous therapeutic uses, as seen in chronic pathologies like anxiety, depression, phobias, schizophrenia (Matson and Mahan 2010; Nunes and Hallak 2014; Araújo et al. 2018), Alzheimer's (Szczechowiak et al. 2019), Parkinson's, and convulsions (Moshé et al. 2015).

Then, based on these results and the absence of acute toxicity on mice (Almeida et al. 2014a, b), the oral toxicity of (+)-limonene epoxide was assessed after chronic exposure (120 days). Repeated doses protocols allow the verification of systemic toxicological effects on biochemical, hematological, functional, and morphological parameters (Lucio et al. 2000; Bakoma et al. 2013; Harvey 2014; Bampi et al. 2019). First of all, we performed Hippocratic screenings on the 121st day. Afterwards, physiological, behavioral, and symptomatic parameters were analyzed. Although behavioral changes were

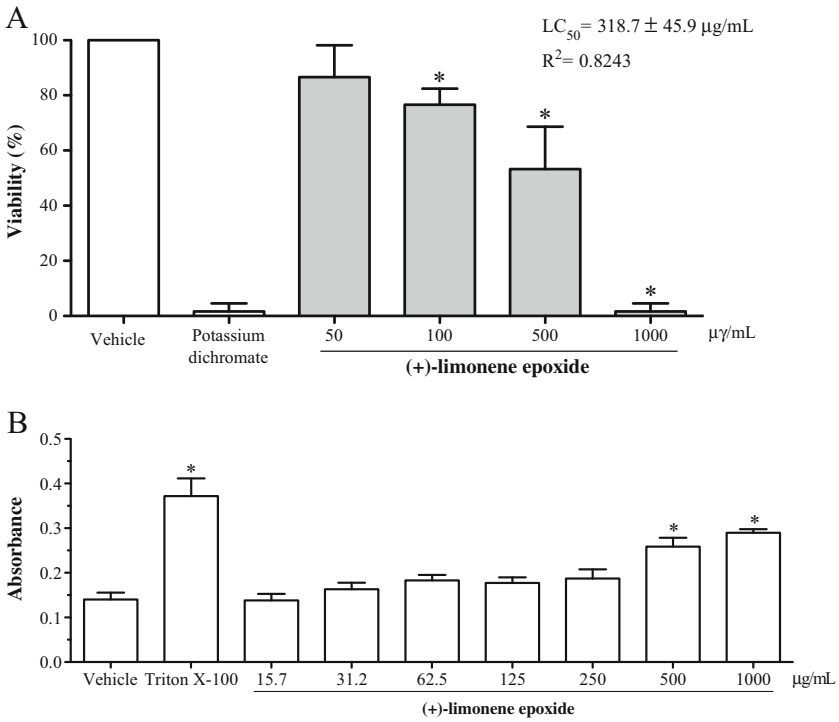

Fig. 1 **a** Viability of *Artemia salina* nauplii after 24 h of exposure to (+)-limonene epoxide ($n = 10$ nauplii/concentration). **b** Evaluation of hemolysis on Swiss mice erythrocytes after 1 h exposure. *$p < 0.05$ compared with the negative control (vehicle) followed by ANOVA followed by Student-Neuman-Keuls. Values are mean ± S.E.M. Positive controls were represented by potassium dichromate (50 µg/mL) and triton X-100 (2%)

not detected (Table 1), four deaths were noticed (two at 50 mg/kg/day and two at 75 mg/kg/day). However, mortality was also observed in the vehicle group, a finding that can be attributed to chronic treatments. General alterations in the body and organ relative weights and feed and water consumptions or excreta production were not detected ($p > 0.05$). In repeated toxicity studies, dietary and behavioral changes are commonly evaluated, as well as feed and water consumption. Moreover, from a toxicological point of view, investigations consider loss of body weight as really important if it changes at least 10% (Lu et al. 2014; Morais et al. 2016; Silva et al. 2016).

Although macroscopic and physiological changes were not detected, it was also decided to evaluate hematological and biochemical parameters, since they have the capacity to detect early and more subtle toxicity of xenobiotics, and serum enzymes may be used, individually or in combination, to indicate hepatocellular, bone, intestinal, heart, renal, and pancreatic damages (Ramaiah 2007; Morais et al. 2016; Ferreira et al. 2016, 2019). Similarly, no significant changes were observed in the hematological and biochemical profile of the animals treated with (+)-limonene epoxide (Table 2; Table 3, $p > 0.05$) (Branco et al. 2011). Once again, the present study corroborates previous outcomes that demonstrated the absence or low toxicity of some monoterpenes (Hariri et al. 2011; Costa et al. 2012; Nogueira Neto et al. 2012).

Exposure to xenobiotics, such as drugs, carcinogens, and secondary metabolites, can cause liver injuries since it is the main organ responsible for the biotransformation of such compounds and, in association with the kidneys, carry out

Table 1 Macroscopic, physiological and behavioral findings of Swiss mice treated with (+)-limonene epoxide for 120 days by gavage

Group/dose		N	Sex	Death	Mortality latency (days)	Feed consumption (g)	Water consumption (mL)	Excreta production (g)	Body weight (g)	Heart g/100 g body weight	Kidneys	Lung
(+)-Limonene epoxide	Vehicle	5	Male	1	48	77.6 ± 1.7	128.0 ± 1.7	60.6 ± 2.1	28.6 ± 0.9	0.4 ± 0.01	1.1 ± 0.02	0.8 ± 0.01
		5	Female	1	69	76.4 ± 1.7	127.2 ± 1.6	59.2 ± 1.9	29.6 ± 0.2	0.5 ± 0.03	1.0 ± 0.10	0.8 ± 0.03
	25 mg/kg/day	5	Male	0	–	75.2 ± 2.6	127.2 ± 1.5	60.6 ± 1.9	28.6 ± 0.7	0.4 ± 0.01	1.1 ± 0.10	0.8 ± 0.10
		5	Female	0	–	77.0 ± 2.1	128.0 ± 2.1	60.4 ± 1.5	29.2 ± 0.4	0.5 ± 0.01	1.1 ± 0.04	0.8 ± 0.10
	50 mg/kg/day	5	Male	1	41	78.6 ± 2.7	130.8 ± 1.4	63.2 ± 2.1	28.2 ± 0.7	0.4 ± 0.01	1.1 ± 0.02	0.8 ± 0.03
		5	Female	1	38	77.0 ± 3.1	129.8 ± 1.6	60.8 ± 1.4	28.4 ± 0.5	0.4 ± 0.03	1.0 ± 0.04	0.8 ± 0.10
	75 mg/kg/day	5	Male	2	56	80.2 ± 2.9	130.6 ± 2.1	63.4 ± 2.2	27.8 ± 0.9	0.4 ± 0.01	1.3 ± 0.02	0.8 ± 0.01
		5	Female	1	88	78.0 ± 2.4	128.8 ± 1.7	62.2 ± 1.7	28.4 ± 0.7	0.5 ± 0.01	1.1 ± 0.10	0.8 ± 0.02

Results are expressed as mean ± standard error of mean (S.E.M.) ($n = 5$ animals/group). *$p < 0.05$ compared with control by ANOVA followed by the Student-Newman-Keuls test

Table 2 Hematological parameters of Swiss mice orally treated with (+)-limonene epoxide for 120 days

Parameters	Sex	Vehicle	(+)-Limonene epoxide		
			25 mg/kg	50 mg/kg	75 mg/kg
Erythrocytes (mm^3)	Females	7.2 ± 0.5	7.6 ± 0.4	6.6 ± 0.7	8.4 ± 0.8
	Males	8.4 ± 0.5	6.8 ± 0.6	8.2 ± 0.6	7.4 ± 0.9
Hemoglobin (g /L)	Females	12.6 ± 0.9	12.6 ± 0.7	12.0 ± 1.6	11.4 ± 1.2
	Males	13.8 ± 0.2	11.8 ± 0.9	12.4 ± 6.2	11.0 ± 1.1
Hematocrit (%)	Females	41.8 ± 1.7	43.0 ± 0.9	41.2 ± 2.1	39.0 ± 2.8
	Males	43.4 ± 0.5	41.0 ± 1.7	42.4 ± 7.7	41.0 ± 1.7
VCM (fL)	Females	48.0 ± 2.2	47.2 ± 0.7	46.8 ± 2.1	47.2 ± 2.1
	Males	44.0 ± 0.2	43.2 ± 5.3	44.6 ± 1.8	43.2 ± 5.3
HCM (pg)	Females	14.6 ± 0.7	15.2 ± 1.0	16.0 ± 0.9	15.6 ± 0.9
	Males	16.0 ± 0.2	14.2 ± 1.2	14.6 ± 1.6	13.2 ± 1.4
CHCM (g/dL)	Females	33.4 ± 1.3	33.2 ± 0.9	32.2 ± 0.6	33.2 ± 0.9
	Males	36.0 ± 0.4	31.8 ± 2.8	30.2 ± 2.1	31.8 ± 2.8
RDW (fL)	Females	11.8 ± 0.9	12.6 ± 0.7	12.4 ± 1.1	12.6 ± 0.7
	Males	11.8 ± 0.2	10.6 ± 1.1	11.0 ± 1.3	10.6 ± 1.1
Platelets (mm^3)	Females	270.2 ± 10.7	269.6 ± 8.1	269.4 ± 7.3	269.6 ± 8.0
	Males	269.2 ± 7.6	264.4 ± 1.3	266.2 ± 9.9	268.4 ± 9.9
Total leukocytes (mm^3)	Females	7.6 ± 0.9	7.0 ± 1.0	7.20 ± 0.5	7.0 ± 0.7
	Males	6.6 ± 0.2	6.2 ± 1.0	5.80 ± 1.2	6.2 ± 1.0
Neutrophils (%)	Females	16.0 ± 1.4	15.6 ± 1.8	17.8 ± 0.4	17.2 ± 1.3
	Males	18.4 ± 0.5	15.6 ± 1.5	16.2 ± 2.3	14.4 ± 1.7
Eosinophils (%)	Females	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
	Males	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.04
Lymphocytes (%)	Females	73.4 ± 3.8	70.8 ± 4.0	72.6 ± 4.3	70.8 ± 4.0
	Males	78.0 ± 0.5	71.8 ± 5.6	68.8 ± 5.9	71.8 ± 5.6
Monocytes (%)	Females	2.2 ± 0.6	1.7 ± 0.5	2.2 ± 0.6	1.8 ± 0.5
	Males	1.8 ± 0.2	2.4 ± 1.0	2.0 ± 0.2	2.2 ± 0.9

Results are expressed as mean ± standard error of mean (S.E.M.) ($n = 5$ animals/group). *$p < 0.05$ compared with control by ANOVA followed by the Student-Newman-Keuls test

metabolization of exogen and endogen molecules, including plant-derived hepatotoxic components. Therefore, the liver is susceptible to chemical and oxidative lesions, especially in chronic conditions (Alvarado-Rico and Castro 2010; Ferreira et al. 2016; Shalan et al. 2017; Ferreira et al. 2019).

Herein, livers from vehicle-treated animals showed the presence of mild passive congestion of venules and venous branches, and hemorrhage focal areas with fibrinoid exudate (Fig. 2a and b). Medium and periterminal portions of hepatocytes have increased due to the presence of cytoplasmic vacuolar degeneration. Moreover, hyperplasia of Kupffer cells and focal areas of necrosis with mild inflammatory cellular infiltrate, predominantly mononuclear cells and some neutrophils, were also found. Animals treated with (+)-limonene epoxide at 25, 50, and 75 mg/kg/day also exhibited similar morphological characteristics to the animals from the control group, though additional some aspects can be emphasized: (i) sinusoidal congestion adjacent to areas of confluent necrosis was observed in some granular-looking hepatocytes (25 mg/kg/day, Fig. 2c and d); (ii) hepatocytes with microvacuolar degeneration and hepatonecrosis (50 mg/kg/day, Fig. 2e and f); iii) necrosis and mixed inflammatory infiltrate, including macrophages, vacuolar degeneration, and karyolysis (75 mg/kg/day, Fig. 2g and h).

Acute and chronic experimental studies with D-limonene, the precursor of (+)-limonene epoxide, have shown fairly low toxicity, although the treatment with D-limonene has caused nephrotoxicity in male rats and hepatoxicity in male and female rats (Shimada et al. 2002; Kim et al. 2013; Ramos et al. 2015).

Generally, liver histological changes are corroborated by serum hepatic functional analytes, such as AST, ALT, alkaline phosphatase, gamma-glutamyl transferase, albumin, bilirubin, urea, and cholesterol (Ramaiah 2007). Nevertheless, indications of liver damages were not corroborated by biochemical analysis after chronic administration of (+)-limonene epoxide. Furthermore, the presence of different levels of injuries (from a cellular swelling to necrosis with the presence of

Table 3 Serum biochemical parameters of Swiss mice orally treated with (+)-limonene epoxide for 120 days

Parameters	Sex	Vehicle	(+)-Limonene epoxide		
			25 mg/kg	50 mg/kg	75 mg/kg
Glucose (mg/dL)	Females	77.4 ± 4.7	78.0 ± 3.7	78.0 ± 4.0	77.0 ± 6.5
	Males	78.2 ± 3.8	77.8 ± 6.5	78.0 ± 4.0	78.0 ± 4.2
Urea (mg/dL)	Females	49.2 ± 2.5	48.2 ± 2.2	48.6 ± 2.2	49.0 ± 2.6
	Males	48.8 ± 0.7	48.4 ± 0.5	49.8 ± 1.1	49.2 ± 0.9
Creatinine (mg/dL)	Females	0.5 ± 0.1	0.46 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
	Males	0.3 ± 0.02	0.3 ± 0.03	0.3 ± 0.1	0.3 ± 0.1
Uric acid (mg/dL)	Females	2.2 ± 0.2	2.1 ± 0.2	2.2 ± 0.1	2.3 ± 0.3
	Males	1.9 ± 0.1	2.1 ± 0.1	2.0 ± 0.1	1.9 ± 0.1
Triglycerides (mg/dL)	Females	104.4 ± 2.7	102.0 ± 1.6	103.2 ± 2.2	104.8 ± 4.1
	Males	97.8 ± 3.9	98.6 ± 2.1	99.8 ± 1.1	99.0 ± 2.5
Total cholesterol (mg/dL)	Females	83.4 ± 1.9	79.0 ± 1.3	78.8 ± 1.2	82.8 ± 4.6
	Males	75.6 ± 3.8	74.6 ± 3.9	72.8 ± 6.7	73.0 ± 6.4
Total proteins (g/dL)	Females	0.8 ± 0.6	0.6 ± 0.5	0.5 ± 0.2	0.6 ± 0.2
	Males	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.6	0.7 ± 0.6
AST (U/mL)	Females	82.8 ± 6.1	76.0 ± 5.2	87.0 ± 2.4	87.2 ± 1.0
	Males	83.8 ± 3.5	82.8 ± 4.2	77.4 ± 6.3	78.0 ± 6.9
ALT (U/mL)	Females	51.0 ± 2.1	49.8 ± 2.2	49.8 ± 1.2	53.0 ± 1.0
	Males	51.2 ± 1.6	52.4 ± 0.2	52.0 ± 0.4	49.8 ± 1.7
Alkaline phosphatase (U/L)	Females	150.4 ± 8.0	149.6 ± 2.6	148.8 ± 2.3	150.0 ± 4.8
	Males	150.6 ± 1.9	144.2 ± 5.9	143.4 ± 7.6	139.6 ± 9.5
Total bilirubin (mg/dL)	Females	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
	Males	0.2 ± 0.1	0.1 ± 0.03	0.2 ± 0.1	0.2 ± 0.1
Direct bilirubin (mg/dL)	Females	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
	Males	0.1 ± 0.02	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1

Results are expressed as mean ± standard error of mean (S.E.M.) ($n = 5$ animals/group). *$p < 0.05$ compared with control by ANOVA followed by the Student-Newman-Keuls test

polymorphonuclear neutrophil granulocytes, sometimes associated with the disorganization of hepatocyte cords), indicates cell death followed by tissue regeneration, but serum hepatic markers did not change, suggesting moderate damage since augmentation of hepatic markers (transaminases, mainly) frequently requires more severe conditions of tissue injuries, such as intense necrosis (Ramaiah 2007; Ferreira et al. 2019).

Neuropharmacological properties

The World Health Organization (WHO) has stimulated the development of effective strategies to reduce episodes and prevent recurrences of depression and it classifies depression as a priority area for health since medications are frequently related to adverse effects and negative impacts on the quality of life (Hall et al. 2015; Wang et al. 2016). Therefore, animal models have fundamental importance for a better understanding of pathophysiology of neural disorders and for the elaboration of new therapeutic alternatives. Despite that there is no animal standard that corresponds exactly to the human depression *status*, there are procedures that induce behavioral situations with similar conditions to the human behavior pathologies (Nunes and Hallak 2014; Moreno et al. 2015; Araújo et al. 2017, 2018).

Antidepressant action

Herein, antidepressant activities of (+)-limonene epoxide were demonstrated using the forced swimming and tail suspension protocols. Both of them are routinely applied to search for compounds with antidepressant action (Guzmán-Gutiérrez et al. 2015; Yan et al. 2015). The behavior of immobility exhibited by mice under subjected inevitable and/or inescapable stress reflects behavioral despair, which in turn may mimic behavioral disorders in humans, such as depression (Zhen et al. 2012). The forced swim test is the most indicated to distinguish between selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (Antkiewicz-Michaluk et al. 2015).

The compound reduced the immobility relative time (53.6, 72.7 and 88.6%) at all doses tested [25 mg/kg (91.2 ± 4.8 s), 50 mg/kg (53.7 ± 2.6 s) and 75 mg/kg (22.3 ± 2.0 s)],

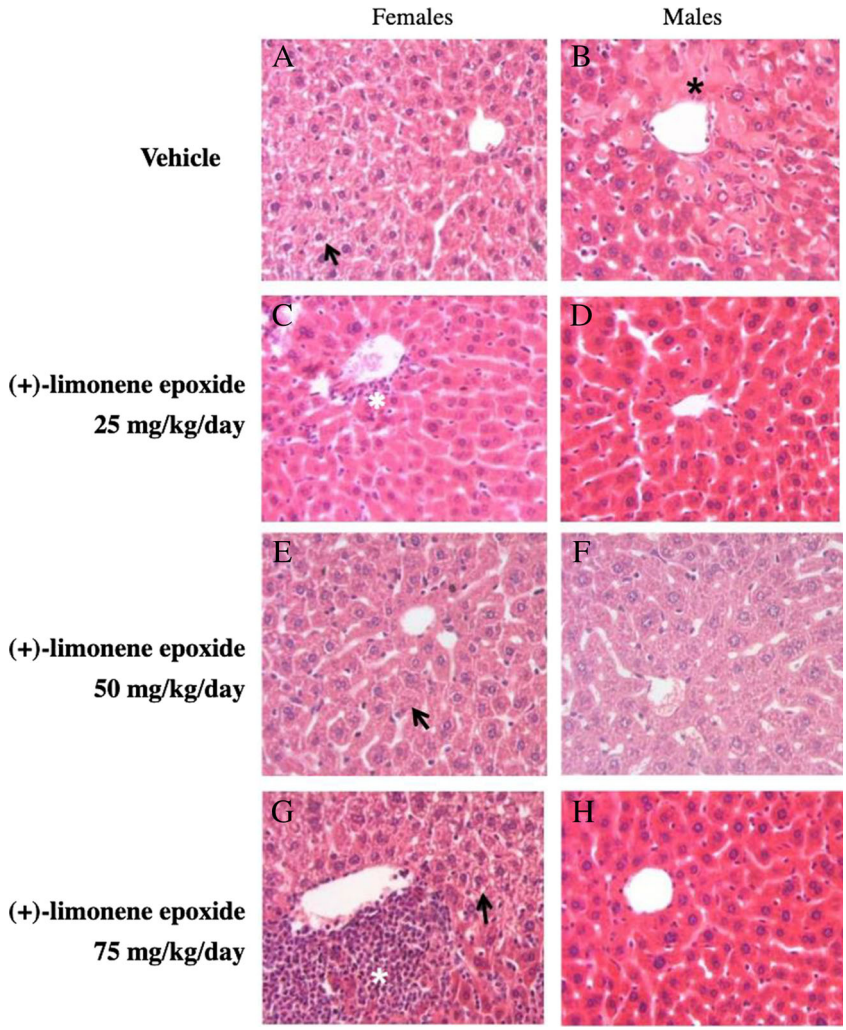

Fig. 2 Histological analysis in livers of Swiss mice treated with (+)-limonene epoxide by gavage for 120 days. Negative controls (**a**, **b**) received vehicle (Tween 80 0.05 % dissolved in saline 0.9 %). Mice were treated with 25 mg/kg/day (**c**, **d**), 50 mg/kg/day (**e**, **f**) and 75 mg/kg/day (**g**, **h**) of (+)-limonene epoxide. Black arrows: cytoplasmic vacuolar degeneration; Black asterisks: fibrinoid exudate; White asterisks: inflammatory infiltrate. Hematoxylin-eosin staining. Light microscopy magnification, 200x. Scale: 10 μm

respectively ($p < 0.05$), when compared with the vehicle group (196.5 ± 4.9 s) (Fig. 3a). However, only the dose 75 mg/kg reduced immobility time when compared with imipramine (48.7 ± 3.6 s) and paroxetine (73.0 ± 6.0 s) ($p < 0.05$). Reserpine promoted a significant increase (34.8%) in immobility time (264.8 ± 5.1 s). Meanwhile, imipramine (48.7 ± 3.6 s) and paroxetine (73.0 ± 6.0 s) reduced it by 75.2 and 62.8%, ($p < 0.05$). Afterwards, the highest dose of (+)-limonene epoxide (75 mg/kg) was administered 30 min after reserpine, paroxetine, or imipramine. Such association caused a reduction in immobility time (89.8 ± 7.8, 35.5 ± 5.2, and 13.7 ± 1.7 s) when compared with the groups receiving only reserpine, paroxetine, or imipramine, respectively ($p < 0.05$).

In relation to the tail suspension investigations, the antidepressants used as positive controls (paroxetine, 46.3 ± 3.1 s; imipramine, 35.2 ± 1.6 s) and (+)-limonene epoxide (25 mg/kg, 66.1 ± 2.4 s; 50 mg/kg, 43.5 ± 3.5 s; 75 mg/kg, 21.0 ± 3.0 s) significantly reduced immobility times (76.2, 81.9, 74.0, 77.7, and 89.2%, respectively) when compared with the vehicle group (195.3 ± 5.1 s) (Fig. 3b). On the contrary, reserpine increased the immobility time to 254.3 ± 5.3 s ($p < 0.05$), as expected for an antiadrenergic standard drug. Similarly, (+)-limonene epoxide 75 mg/kg administered 30 min before reserpine, imipramine, or paroxetine caused a reduction of immobility (49.0 ± 3.0, 15.0 ± 1.4 and 18.8 ± 2.2 s) when compared with the groups only with reserpine, paroxetine, or imipramine, respectively ($p < 0.05$).

(+)-limonene epoxide 75 mg/kg revealed promising outcomes when compared with the standard paroxetine and imipramine ($p < 0.05$), though doses used have not been similar. Previously, we had already demonstrated that (+)-limonene epoxide, in similar doses and conditions, showed anxiolytic activity and did not cause muscle relaxation (rotated rod test) or sedative effect (open field test) (Almeida et al. 2012).

Reserpine is a historically important psychopharmacology drug that postulates the role of monoamines on mood and behavior. Lots of patients under reserpine treatment display severe hypertension and develop advanced symptoms of depression (Antkiewicz-Michaluk et al. 2015). Herein, we showed that (+)-limonene epoxide is able to reverse

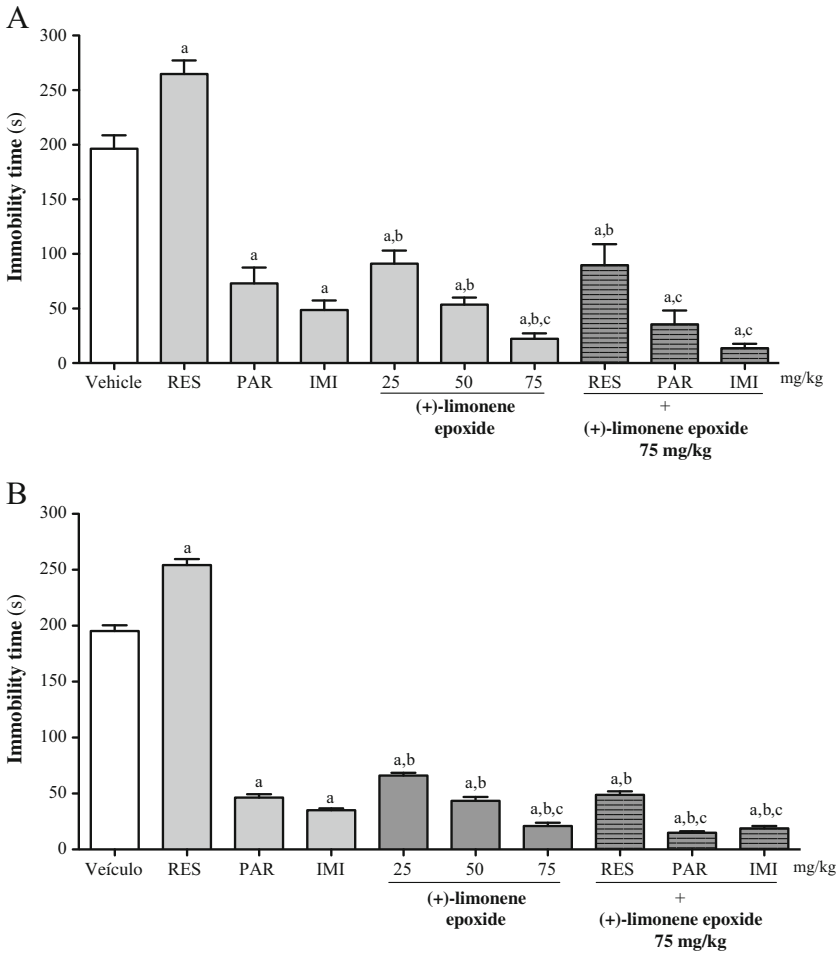

Fig. 3 Effects of (+)-limonene epoxide (25, 50 or 75 mg/kg) on the mice immobility time after a single oral dose by gavage. **a** Determined by forced swimming test. **b** Determined by tail suspension test. Reserpine (RES), Paroxetine (PAR) or Imipramine (IMI) were used as control drugs (0.25, 20 and 50 mg/kg i.p., respectively). Results are expressed as mean ± standard error of mean (S.E.M.). $^{a}p < 0.05$ compared with the negative control (vehicle) by ANOVA followed by the Student Newman-Keuls test; $^{b}p < 0.05$ compared with reserpine by ANOVA followed by Student-Neuman-Keuls; $^{c}p < 0.05$ compared with paroxetine or imipramine by ANOVA followed by Student-Neuman-Keuls

immobility reserpine effects. Since reserpine is an inhibitor of amine uptake and storage within synaptic vesicles and causes depletion of noradrenaline and serotonin in central and peripheral neurons, it was used to reproduce a pharmacological model of depression (Gao et al. 2013). In such a way, the antidepressant effects of (+)-limonene epoxide are likely associated with serotonergic and/or adrenergic neurochemical transmission. Therefore, we also used imipramine, a tertiary amine, as tricyclic antidepressant that blocks serotonin and noradrenaline uptakes from the synaptic cleft (Wasik et al. 2013; Jaworska and Malek 2014).

The association of paroxetine plus (+)-limonene epoxide 75 mg/kg reduced the immobility of the animals when compared with paroxetine alone in both protocols (forced swim test, 51.4%; tail suspension test, 57.4%), indicating that such association was beneficial and synergistic. Paroxetine is a stronger serotonin uptake inhibitor than fluoxetine, fluvoxamine, and sertraline and has poorer action on noradrenaline reuptake. In addition, it has little affinity for alpha-adrenergic, histaminergic type 1 (H_1), 5-hydroxytryptamine type 2 (5-HT_2), and dopaminergic D_2 receptors, avoiding or reducing side effects on the central and autonomic nervous systems, although it presents mild anticholinergic activity. It elevates brain levels of serotonin (5-HT) via adenosine triphosphatase (ATPase) inhibition from presynaptic neurons, resulting in a sudden increase in serotonin concentration in the synapse, predominantly in the synaptic cleft around the neuronal cell body. This rise in serotonin availability would be responsible for side effects (Carhart-Harris and Nutt 2017).

Antiepileptic action

Studies have shown that patients with persistent epileptic seizures tend to develop depression (Mosińska et al. 2016). Epilepsy is a common disorder with a high negative impact on the quality of life, but treatment is mainly directed to the blockade of symptoms rather than treating or curing the primary cause (Blumenfeld 2011). Thus, in this present study, we also evaluated the antiepileptic action of (+)-limonene epoxide.

Outcomes of (+)-limonene epoxide effects on picrotoxin-induced convulsions are described in Table 4. Only the highest dose (75 mg/kg) increased the latency for the first epileptic seizure (66.7%: 1125.7 ± 18.3 s) compared with the vehicle group (675.5 ± 12.8) ($p < 0.05$). This same dose

Table 4 Effects of (+)-limonene epoxide administered by gavage on picrotoxin-induced seizures in mice

Treatment	Dose (mg/kg)	Latency for the first seizure (s)	Epileptic seizure inhibition (%)	Inhibition of death (%)
Vehicle	–	675.5 ± 12.8	0	0
Diazepam	2	1221.1 ± 12.1*	100*	100*
(+)-Limonene epoxide	25	693.6 ± 11.0	0	0
	50	728.7 ± 15.3	0	00
	75	1125.7 ± 18.3*	75*	75*

All animals received a single dose of picrotoxin (8 mg/kg, i.p.) before diazepam or (+)-limonene epoxide. Results are expressed as mean ± standard error of mean (S.E.M.) ($n = 6$ animals/group). *$p < 0.05$ compared with control by ANOVA followed by the Student-Newman-Keuls test

causes a significant reduction of epileptic seizures (25%) and death (25%) when compared with the vehicle group ($p < 0.05$). On the other hand, all doses of (+)-limonene epoxide (25 mg/kg: 281.1 ± 21.6 s; 50 mg/kg: 375.8 ± 33.0 s and 75 mg/kg: 772.5 ± 31.5 s) increased latency for the first epileptic seizure (59.4, 113.1, and 338%, respectively) after a single dose of pentylenetetrazol (60 mg/kg) in comparison with the vehicle (176.4 ± 14.0 s) ($p < 0.05$, Table 5). Nevertheless, only higher doses of (+)-limonene epoxide (50 and 75 mg/kg) inhibited the development of epileptic seizures (20 and 90%) and reduced pentylenetetrazol-induced deaths (20 and 80%), respectively ($p < 0.05$). Diazepam (2 mg/kg) also reduced the number of epileptic seizures and deaths induced by pentylenetetrazol and picrotoxin and increased latency for the first seizure (850.9 ± 19.0 and 1221.1 ± 12.1 s) ($p < 0.05$). Flumazenil reversed the antiepileptic action of diazepam (flumazenil + diazepam, 173.2 + 25.2 s) as well as effects of (+)-limonene epoxide at 75 mg/kg (flumazenil plus (+)-limonene epoxide, 173.8 + 9.9 s) after pentylenetetrazol administration ($p < 0.05$).

Epileptic seizures have been linked to increased release of excitatory neurotransmitters, such as glutamate, and/or reduction of inhibitory neurotransmitters, such as gamma-aminobutyric acid (GABA) or other types of glutamatergic and/or GABAergic pathways (Kesim et al. 2012; Calcaterra and Barrow 2014).

As shown above, (+)-limonene epoxide increased time to the onset of epileptic seizures and 75 mg/kg also inhibited chemically induced convulsions and epilepsy-induced and diminished deaths. Since pentylenetetrazol and picrotoxin are antagonists of $GABA_A$ receptors, interacting with barbituric binding site (Zhu et al. 2012), it is feasible that (+)-limonene epoxide plays a similar anticonvulsive action to the benzodiazepines and acts on the GABAergic system. This hypothesis was confirmed with flumazenil, since its use prior to diazepam blocks benzodiazepine antiepileptic effects after pentylenetetrazol injection (Saxona et al. 2010). So, it very possible (+)-limonene epoxide triggers chloride (Cl^-) channels associated with type A receptors for GABA ($GABA_A$) (Löscher and Schmidt 2006; Calcaterra and Barrow 2014) and it can also be used as an antidote in poisonings caused by central depressants, especially barbiturates.

The antiepileptic action described here corroborates previous studies claiming anxiolytic effects of (+)-limonene epoxide, since such action also occurs, at least in part, via benzodiazepine receptors (Almeida et al. 2012). Moreover, drugs that inhibit pentylenetetrazol-induced seizures and raise the threshold against the development thereof are effective against

Table 5 Effects of (+)-limonene epoxide administered by gavage on pentylenetetrazol-induced seizures in mice

Treatment	Dose (mg/kg)	Latency for the first seizure (s)	Epileptic seizure inhibition (%)	Inhibition of death (%)
Vehicle	–	176.3 ± 14.0	0	0
(+)-Limonene epoxide	25	281.1 ± 21.6[a]	0	0
	50	375.8 ± 33.0[a]	20[a]	20[a]
	75	772.5 ± 31.5[a]	90[a]	80[a]
Diazepam	2	850.9 ± 19.0[a]	100[a]	100[a]
Flumazenil + diazepam	5 + 2	173.2 + 25.2[b]	0[b]	0[b]
Flumazenil + (+)-limonene epoxide	5 + 75	173.8 + 9.9[c]	0[c]	0[c]

All animals received a single dose of pentylenetetrazol (60 mg/kg, i.p.) before diazepam or (+)-limonene epoxide. Results are expressed as mean ± standard error of mean (S.E.M.) ($n = 6$ animals/group). [a] $p < 0.05$ compared with vehicle by ANOVA followed by Student-Neuman-Keuls; [b] $p < 0.05$ compared with diazepam by ANOVA followed by Student-Neuman-Keuls; [c] $p < 0.05$ compared with (+)-limonene epoxide 75 mg/kg by ANOVA followed by Student-Neuman-Keuls

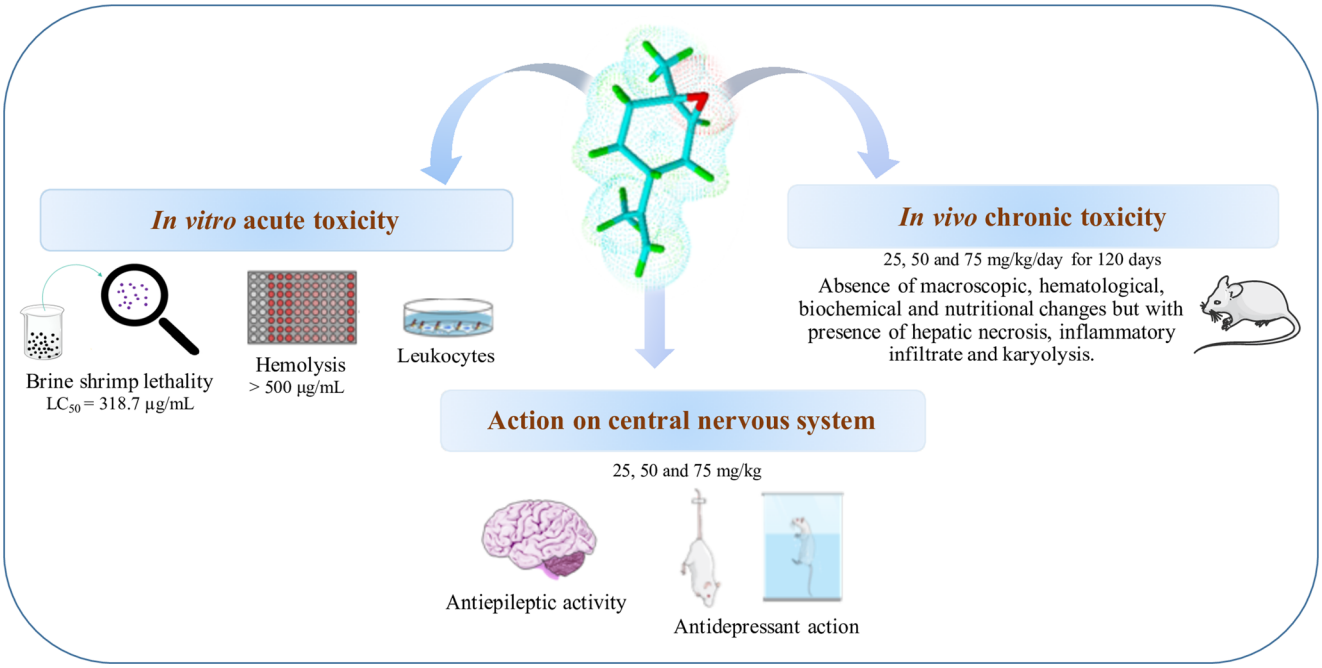

Fig. 4 Summary of in vitro and in vivo biological actions of (+)-limonene epoxide

seizures of absence. Meanwhile, compounds that reduce the spread and duration of seizures are active against focal seizures (e.g., tonic-clonic convulsions) (Rang and Dale 2007).

Taking into consideration that anticonvulsivant and antidepressant drugs are chronically used, liver damages are generally found (Voican et al. 2014). Between 1975 and 1999, 16 out of a total of 45 approved drugs were withdrawn from the market for safety reasons and 22% (10 of 45) were hepatotoxic (Lasser et al. 2002), making hepatoxicity the most common type of lesion induced by bioactive compounds (Lee 2003), despite the fact that about 72% of the symptoms and/or signs of toxicity of such compounds in humans are delineated, mimicked, and visualized in laboratory animals (Olson et al. 2000). In this context, (+)-limonene epoxide has presented additional benefits since it caused slight hepatic changes and liver biochemical changes were not detected.

Figure 4 briefly illustrates our toxicological and neuropharmacological outcomes with (+)-limonene epoxide using in vitro and in vivo techniques.

Conclusions

In conclusion, (+)-limonene epoxide showed low toxic action against *A. salina* nauplii, hemolytic potential only at higher concentrations and absence of cytotoxicity on peripheral blood mononuclear cells. Subchronic toxicity studies revealed very low oral toxic potential and only slight and reversible hepatic morphological changes were seen in the absence of biochemical, hematological and nutrition profile changes. (+)-limonene epoxide inhibited the development of pentylenetetrazol- and picrotoxin-induced seizures, reduced deaths, and decreased the immobility relative time, suggesting its antidepressant effects are linked to serotonergic and/or adrenergic neurochemical transmission. It is feasible that (+)-limonene epoxide triggers Cl^- channels associated with $GABA_A$ receptors and plays a like-benzodiazepine anticonvulsive action. Exhibiting such properties, (+)-limonene epoxide may be also recommended as antidote in poisonings caused by central depressants, especially barbiturates. Its structural chemical simplicity makes it a cost-effective alternative and justifies further improvements to enhance activity and selectivity and to develop pharmaceutical formulations.

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Authors' contribution All authors participated in the study. AACA and RBFC performed the chronic toxicity studies and neuropharmacological/behavioral evaluations. JROF and DD carried out hemolytic, MTT, and *Artemia salina* assays. LSL supervised and supported neuropharmacological experiments. MSR executed histopathological analysis. JMCS provided financial and scientific supports and PMPF planned the research, managed scientific and financial supports, supervised all steps, and wrote the article. The authors also declare that all data were generated in-house and that no paper mill was used.

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Compliance with ethical standards

All procedures were approved by the Committee on Animal Research at UFPI (#091/2014) and followed Brazilian (*Colégio Brasileiro de*

Experimentação Animal - COBEA) and International rules on the care and use of experimental animals (Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes).

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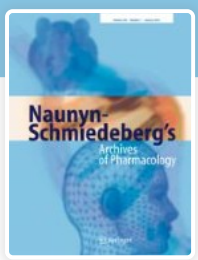

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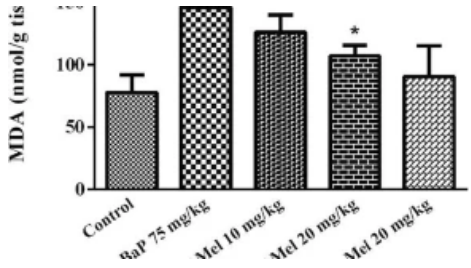

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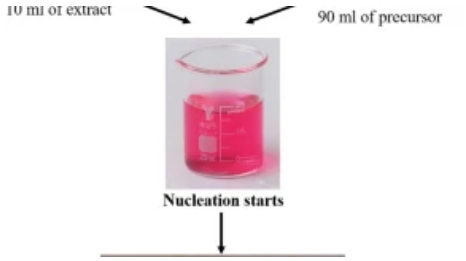

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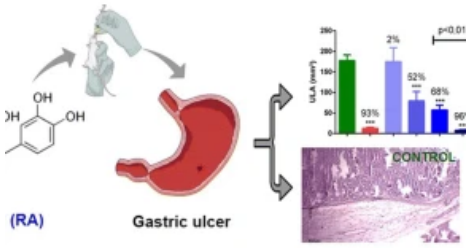

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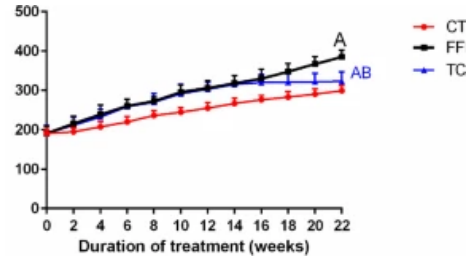

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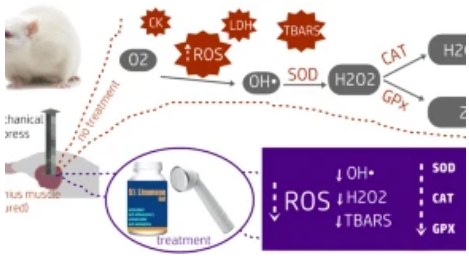

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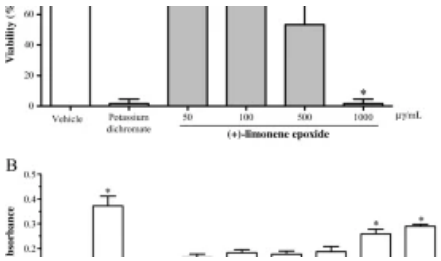

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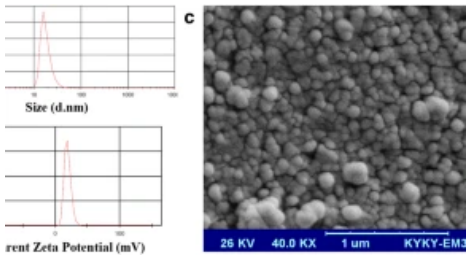

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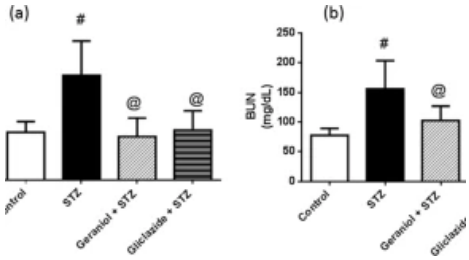

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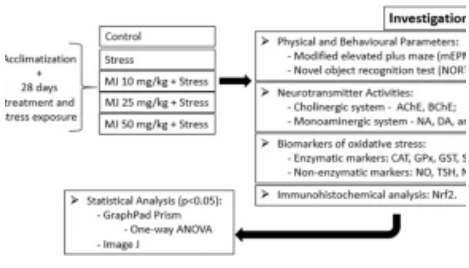

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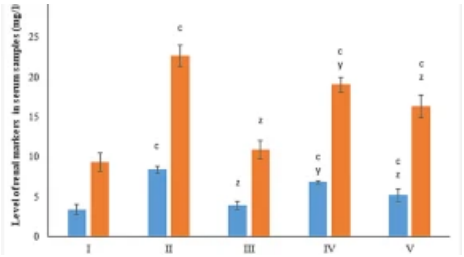

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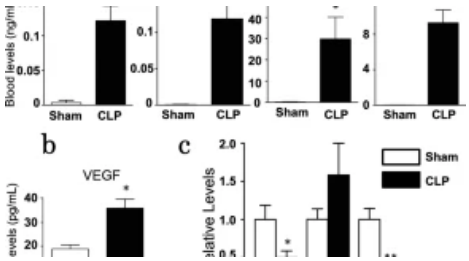

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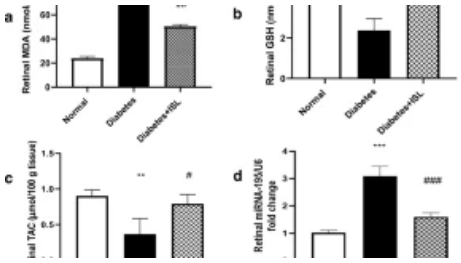

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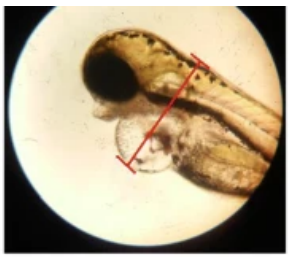

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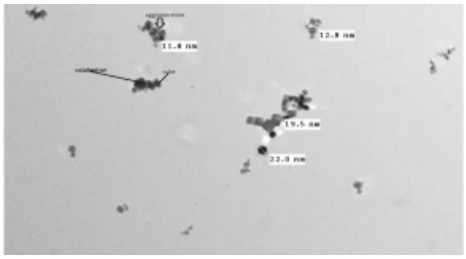

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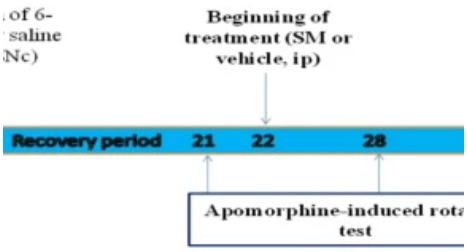

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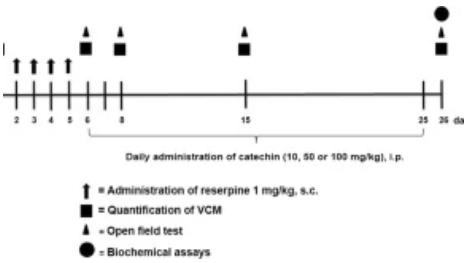

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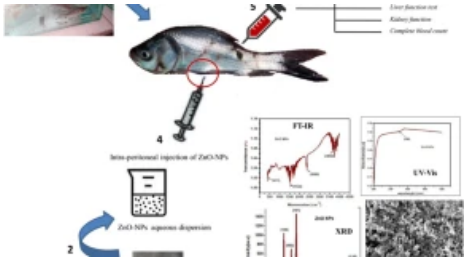

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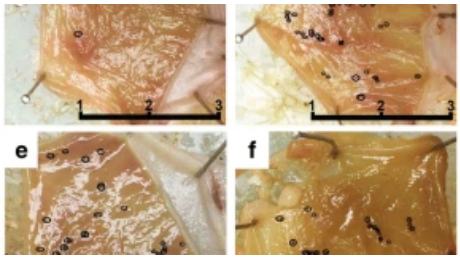

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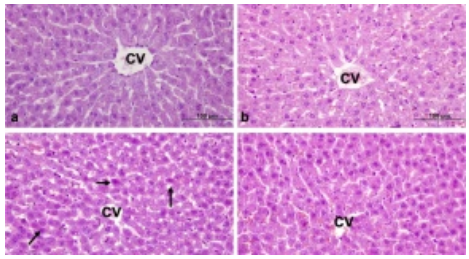

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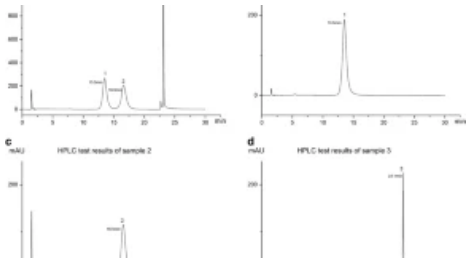

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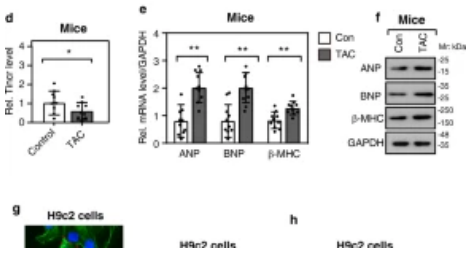

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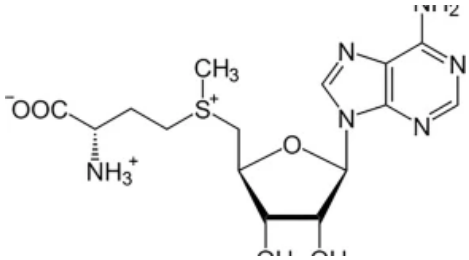

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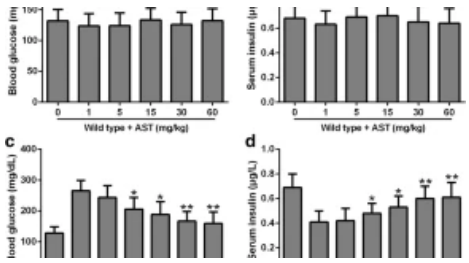

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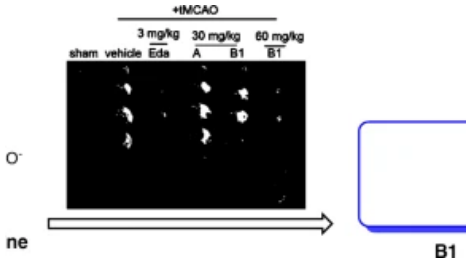

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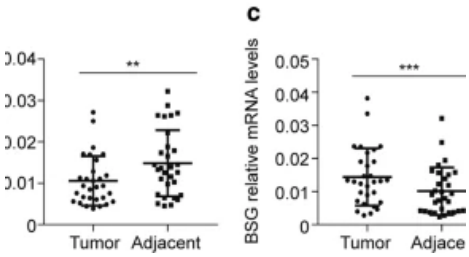

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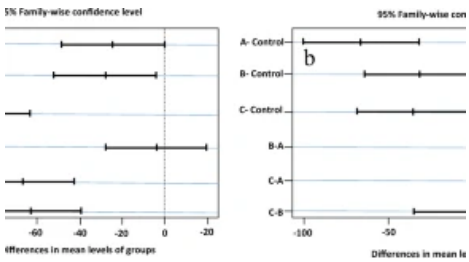

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Chemopreventive effect of troxerutin against hydrogen peroxide-induced oxidative stress in human leukocytes through modulation of glutathione-dependent enzymes

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Chemopreventive effect of troxerutin against hydrogen peroxide-induced oxidative stress in human leukocytes through modulation of glutathione-dependent enzymes

Bruno Coêlho Cavalcanti[a,b], João Batista de Andrade Neto[a,c,d], Antônio Adailson de Sousa Silva[a], Francisco Stefânio Barreto[a], José Roberto de Oliveira Ferreira[e], Hemerson Iury Ferreira Magalhães[f], Cecília Rocha da Silva[a,c], Ícaro Gusmão Pinto Vieira[g], Nágila Maria Pontes Silva Ricardo[g], Hélio Vitoriano Nobre Júnior[a,c], and Manoel Odorico Moraes[a,b]

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ABSTRACT

Troxerutin is a natural flavonoid present abundantly in tea, coffee, olives, wheat, and a variety of fruits and vegetables. Due to its diverse pharmacological properties, this flavonoid has aroused interest for treatment of various diseases, and consequently prompted investigation into its toxicological characteristics. The aim of this study was to evaluate the genotoxic and mutagenic effects and chemoprotective activity attributed to troxerutin using human peripheral blood leukocytes (PBLs) through several well-established experimental protocols based upon different parameters. Data demonstrated that troxerutin (100 to 1000 µM) induced no marked cytotoxic effect on PBLs after 24 hr, and did not produce strand breaks and mutagenicity. Regarding chemoprevention, this flavonoid attenuated cytotoxicity, genotoxicity, and mutagenicity initiated by hydrogen peroxide (H_2O_2) in human PBLs. Further, troxerutin demonstrated no marked cytotoxic effect on PBLs and exerted a protective effect against oxidative stress induced by H_2O_2 through modulation of GSH-dependent enzymes.

KEYWORDS

Chemoprevention; cytotoxicity; flavonoid; genotoxicity; mutagenicity

Introduction

The use of natural or synthetic substances to prevent the development of carcinogenesis is known as chemoprevention (Arroo, Wang, and Bhambra 2020; Gandini et al. 2009; González-Vallinas et al. 2013, Silva et al. 2020; Tai and Toh 2013). This strategy might occur through different mechanisms, such as interference in angiogenesis, elimination of reactive oxygen species (ROS), interaction with specific enzymes, or DNA repair mechanisms (Bilecová-Rabajdová et al. 2013; Estrella-Parra et al. 2019; Gontijo et al. 2018; Gontijo, Dos Santos, and Viegas 2017). Chemoprevention is also associated with enzymes of the glutathione (GSH) system involved in the detoxification process and maintaining the redox balance in the body, enabling cells to overcome conditions such as oxidative stress (Narayanankutty, Job, and Narayanankutty 2019), which is known to signal pro-oncogenic factors.

Several natural products demonstrated potential chemoprotective effects against development of cancer with minimal side effects (Majolo et al. 2020; Tuttis et al. 2018). Among these compounds are flavonoids (Marnewick et al. 2009; Tuttis et al. 2018). Troxerutin, a flavonoid (more accurately a hydroxyethylrutoside) derived from rutin (Panat et al. 2016), is found abundantly present in tea, coffee, olives, wheat, and a variety of fruits and vegetables. In comparison with rutin, it is more readily absorbed from the human digestive tract (Shan et al. 2017; Wang et al. 2017). Due to its diverse pharmacological properties, such as nephro- and hepato-protective, antioxidant, and anti-inflammatory properties, troxerutin has attracted investigation to determine potential toxicological

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characteristics (Baluchnejadmojarad et al. 2017; Marzin et al. 1987; Subastri et al. 2015).

Lu et al. (2011) showed that troxerutin reversed the cognitive deficit induced by high cholesterol levels by activating the PI3K/Akt/CREB pathway and inhibiting the stress pathway of the endoplasmic reticulum in the hippocampus of mice. Zhang et al. (2015b) demonstrated that troxerutin also displayed beneficial properties for the treatment of 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47)-induced hepatotoxicity by lowering depletion of NAD+, with consequent reduction of oxidative stress and suppression of nuclear translocation of NF-kB p65, thus attenuating the transcription of inflammatory genes in mouse liver.

However, a wide variety of studies indicate that flavonoids promote DNA damage in eukaryotic cells (Duthie, Johnson, and Dobson 1997; George and Vasantha Rupasinghe 2017). Consequently it is essential to conduct genotoxicity tests of natural molecules to determine ways to prevent or ameliorate those effects (Cinthura, Gayathri, and Priya 2017; Saks et al. 2017). Subastri et al. (2015) noted that troxerutin-DNA interaction mechanisms involve groove bonds with formation of hydrogen bonds as well as hydrophobic bonds. In another study, Marzin et al. (1987) reported that troxerutin did not markedly damage DNA and exhibited a protective effect against injury induced by oxidative stress.

Maurya et al. (2005) demonstrated that intraperitoneal (ip) administration of troxerutin in mice before and after exposure to total body radiation significantly blocked formation of micronuclei (MN) in blood reticulocytes. Based upon these data, the objective of this study was to examine the cytotoxic, genotoxic, and mutagenic effects of troxerutin using human peripheral blood leukocytes (PBLs), as well as to assess potential chemoprotective activity.

Materials and methods

Chemicals

The rutin used in troxerutin semi-synthesis was provided by the Technological Development Park (PADETEC). The synthesis of troxerutin was performed and characterized according to a previously described method by Miranda et al. (2020).

Fetal bovine serum (FBS) and Dulbecco's modified eagle medium (DMEM) were purchased from Cultilab (Campinas, SP, Brazil). RPMI 1640, trypsin-EDTA, glutamine, penicillin, streptomycin, phytohemagglutinin (PHA), low-melting-point agarose, and agarose were purchased from Invitrogen (Carlsbad, CA, USA). Cytochalasin-B, reduced glutathione (GSH), oxidized glutathione (GSSG), 5,5′-dithiobis (2-nitrobenzoic acid) (Ellman's Reagent, DTNB), dihydronicotinamide adenine dinucleotide phosphate tetrasodium salt (NADPH), methyl methanesulfonate (MMS), and Alamar Blue cell viability reagent were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). 8-oxoguanine DNA glycosylase (hOGG1) was obtained from New England BioLabs (USA). *N*-(1-naphthyl)-ethylenediamine dihydrochloride was purchased from Merck (USA). Hydrogen peroxide (H_2O_2) was obtained from Vetec (Brazil). Doxorubicin (Doxolem®) was purchased from Zodiac Produtos Farmacêuticos S/A (Brazil). All other chemicals and reagents used were of analytical grade.

Isolation of peripheral blood leukocytes (PBLs)

Blood was collected from three healthy donors at the Hematology and Hemotherapy Center of Ceara State (Brazil) with an average age of 24 ± 3.05 years. The procedure was approved by the Human Research Ethics Committee of Federal University of Ceará (COMEPE-UFC – protocol number 281/09). Lymphocytes were isolated by the standard method of density-gradient centrifugation over Histopaque-1077. Cells were washed and resuspended in RPMI 1640 medium supplemented with 20% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C under 5% CO2. Phytohemagglutinin (2.5%) was added at the beginning of culture. After 48 hr incubation, cells were treated with the test substances (Cavalcanti et al. 2020b, 2020a)

Vitro assessment of cytotoxicity

The Alamar Blue test was performed with PBLs (1.5 x 10^6 cells/ml) after incubation for 24 hr with test substances. Troxerutin (100, 500, or 1000 μM) and methyl methanesulfonate (MMS) at 4 × 10^{-5}

M dissolved in DMSO were added to each well, and control groups received the same amount of vehicle. After incubation, 10 µl stock solution (0.312 mg/ml) Alamar Blue (Resazurin, Sigma–Aldrich Co) was added to each well. The absorbance was measured using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter®) and the drug effect was quantified as % of control absorbance at 570 nm and 595 nm. The absorbance of Alamar Blue in culture medium was measured at a higher wavelength and a lower wavelength. The absorbance of the medium was also determined at the higher and lower wavelengths. The absorbance of the medium alone was subtracted from the absorbance of the medium plus Alamar Blue at the higher wavelength. This value is called AO_{HW}. The absorbance of the medium alone was subtracted from the absorbance of medium plus Alamar Blue at the lower wavelength. This value is called AO_{LW}. A correction factor, R_0, was calculated from AO_{HW} and AO_{LW}, where $R_0 = AO_{LW}/AO_{HW}$. The % by which Alamar Blue reduced cell viability was expressed as % reduction = ALW – (AHW x R0) x 100) (Cavalcanti et al. 2020b, 2020a; Lograd o et al. 2010)

Treatment of PBL cultures with troxirutin

For conventional genotoxic and mutagenic experiments, PBLs (5 x 10^5 cells/ml) were treated with troxerutin (100, 500, or 1000 µM) or MMS (4 x 10^{-5} M) dissolved in DMSO (0.1%), without PBS, for 24 hr at 37°C. In addition, troxerutin was tested for its antioxidant potential to exert an antimutagenic effect. The cytokinesis-block MN test as well as the alkaline and modified versions of the comet assay were used after pre-, co-, and post-treatment for 1 hr with 150 µM H_2O_2-exposed PBLs (0.5 x 10^5 cells/ml), with troxerutin at concentrations ranging from 100 to 1000 µM (without PBS) for 3 hr in order to correlate possible mechanisms of modulation (intra- and extracellular reactions) and effect on DNA repair. In the pretreatment protocol, treated cells were washed with PBS (pH 7.4) and submitted to the mutagen experimental protocol, and at the end of H_2O_2 exposure. PBLs were washed with PBS before being treated with the tested samples (post-treatment protocol)(Cavalcanti et al. 2012; Milošević-Dordević et al. 2015). For these sets of experiments, cell viability and apoptosis induction were also monitored, along with analysis of oxidative stress of troxerutin-treated cells.

Cell viability and morphological characterization of apoptotic PBLs

After treatment, trypan blue-excluding cells were counted in a Neubauer chamber in cell aliquots retrieved from the cultures. The number of apoptotic cells was also determined at the end of each treatment by use of the acridine orange (AO)/ethidium bromide (EB) staining assay: 25 µl the cell suspension was mixed with 1 µl dye solution (AO 100 µg/ml + EB 100 µg/ml in PBS) and spread on a slide, where 300 cells were counted per data point. The % of apoptotic cells was then calculated (Cavalcanti et al. 2010, 2008).

Cytokinesis-block MN assay

Treatments took place 48 hr after culture initiation and lasted for 3 hr (pre- and post-treatments) or 1 hr (co-treatment), subsequently inhibitor of cytokinesis cytochalasin B at 3 µg/ml (final concentration in medium) was added 2 hr before harvest, which took place 72 hr after start of culture. Cells were harvested, re-suspended in a 75 mM KCl solution, maintained at 4 °C for 3 min (mild hypotonic treatment), and fixed with cold methanol/acetic acid (3:1). This fixation step was repeated twice, after which the cells were re-suspended in a small volume of methanol/acetic acid and placed in droplets onto clean slides. These were stained with 10% Giemsa (pH 6.8) for 6 min, mounted, and coded prior to microscopic analysis. Micronuclei (MN) were counted in 2,000 binucleated cells (BNC) with well-preserved cytoplasm. The identification of MN was carried out according to Fenech (2000).

Vitro alkaline comet assay

The alkaline comet assay was performed as described by Singh et al. (1988) with minor modifications (Hartmann and Speit 1997), following the recommendations of the

International Workshop on Genotoxicity Test Procedures (Tice et al. 2000). At the end of the treatment (1, 3, or 24 hr), cells were washed with ice-cold PBS, detached with 100 µl trypsin (0.15%), and re-suspended in complete RPMI medium. Next, 20 µl cell suspension (approximately 10^6 cells/ml) was mixed with 0.75% low-melting-point agarose and immediately spread onto a glass slide precoated with a layer of 1% normal-melting-point agarose. The agarose was allowed to set at 4 °C for 5 min. The slides were then incubated in ice-cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100 and 10% DMSO, pH 10) at 4 °C for a minimum of 1 hr to remove cell proteins, leaving the DNA as "nucleoids." After the lysis procedure, the slides were placed on a horizontal electrophoresis unit, which was filled with fresh buffer (300 mM NaOH and 1 mM EDTA, pH>13) to cover the slides for 20 min at 4 °C to enable DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted for 20 min at 25 V and 300 mA (0.86 V/cm). After electrophoresis, the slides were neutralized (0.4 M Tris, pH 7.5), stained with ethidium bromide (20 µg/ml), and analyzed using a fluorescence microscope. All the above steps were conducted under yellow light or in the dark to prevent additional DNA damage. Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed for each test substance concentration. Cells were scored visually and assigned to one of the five classes, according to tail size (from undamaged-0, to maximally damaged-4), and a damage index (DI) value was calculated for each sample of cells. The DI thus ranged from 0 (completely undamaged: 100 cells x 0) to 400 (with maximum damage: 100 cells x 4) {Formatting Citation}. The protective effect of troxerutin on H_2O_2-induced genotoxicity (150 µM per 1 hr incubation) was calculated according to Rao and Tiwari (2006):

% Reduction = (A-B/A-C) x 100

where **A** corresponds to the DI induced by H_2O_2, **B** corresponds to the DI induced by anti-genotoxic treatment (H_2O_2 + troxerutin) and **C** corresponds to the DI assigned to the negative control (distilled water).

Measurement of oxidized guanine and intracellular reactive oxygen species (ROS)

Oxidized guanine and ROS production were estimated by the modified alkaline comet assay and by using 2′,7′-dichlorofluorescein diacetate (H_2 DCFDA) as fluorescence probe, respectively, after H_2O_2 (150 µM for 1 hr) challenge (pre-, co-, and post-treatment protocols described above). For experiments conducted only with troxerutin, the exposure time was 3 hr (Cavalcanti et al. 2020b, 2020a, 2012).

The comet assay was performed as described above with minor modifications. Briefly, slides were removed from the lysis solution and washed three times in enzyme buffer (40 mM HEPES, 100 mM KCl, 0.5 mM Na_2EDTA, 0.2 mg/mL BSA, pH 8), drained, and incubated with 50 µl hOGG1 (human 8-hydroxyguanine DNA-glycosylase) for 30 min at 37°C. Images of 100 randomly selected cells (50 cells from each of two replicate slides) were visually analyzed per group. The quantity of oxidized guanine (hOGG1-sensitive sites) was then determined by subtracting the number of strand breaks (samples incubated with buffer alone) from the total number of breaks obtained after incubation with hOGG1. For ROS detection, at the end of the treatments cells were incubated with 20 µM H_2-DCFDA for 30 min in the dark at 37°C. Cells were then harvested, washed, re-suspended in PBS, and analyzed immediately via flow cytometry (Guava EasyCyte Mini; Guava Technologies, Inc., Hayward, CA, USA) (Cavalcanti et al. 2020b, 2020a, 2012).

Measurement of intracellular reduced glutathione (GSH) content and activities of GSH-dependent enzymes

After H_2O_2 (150 µM for 1 hr) challenge (pre-, co-, and post-treatment protocols described above), GSH content was determined by a spectrophotometric assay based upon formation of 5-thio-2-nitrobenzoate (TNB) from DTNB, according to Akerboom and Sies (1981) with minor modifications. Briefly, treated (100, 500, or 1000 µM) and untreated PBLs (1.5 x 10^6 cells/ml) were washed with ice-cold PBS, re-suspended in 0.1 M sodium phosphate-5 mM EDTA, pH 8, and

sonicated to obtain the cell homogenate. An equal volume of 2 M $HClO_4$-4 mM EDTA was added to the cell extract, and the precipitated proteins were pelleted by centrifugation at 8,000 g for 15 min at 4° C. The supernatant was neutralized with 2 M KOH, and the insoluble residue was removed by centrifugation under the same conditions. For spectrophotometric determination, 910 µl cell extract supernatant or of a standard GSH solution, in the same phosphate–EDTA buffer, was mixed with 50 µl 4 mg/ml NADPH in 0.5% (w/v) $NaHCO_3$, 20 µl 6 U/ml glutathione reductase (GR) in phosphate-EDTA buffer, and 20 µl 1.5 mg/mL DTNB in 0.5% $NaHCO_3$. The increase in absorbance was measured at 412 nm. The results were normalized by protein content (Lowry et al. 1951), and expressed as µg/mg protein.

To estimate glutathione peroxidase (GPx, EC 1.11.1.9) activity, 0.2 ml cell lysate was added to 0.2 ml 0.4 M phosphate buffer (pH 7.4), 50 µl 4 mM GSH, 15 µl 2.5 mM H_2O_2 and 0.1 ml 10 mM sodium azide and then incubated in a water bath at 37°C for 7 min. Subsequently, 0.5 ml 10% TCA was added and each tube was centrifuged at 4500 g for 5 min. Then 1 ml supernatant was added to 2 ml 0.4 M Tris buffer (pH 8) and 50 µl DTNB. The OD was read at 412 nm. The enzyme activity was expressed as µmol of GSH oxidized/min/mg protein (Kaur et al. 2008).

Glutathione reductase (GR, EC.1.6.4.2) activity was measured following the method of Goldberg (1983) with minor modifications. Glutathione reductase is required for the NADPH-dependent conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH). Briefly, the reaction mixture contained 1.5 ml 0.1 M PBS (pH 7.3), 0.1 ml 15 mM EDTA, and 0.1 ml 70 mM GSSG. Cell lysate was added and incubated at room temperature for 15 min, after which 0.05 ml 9.6 mM NADPH was added. The exponential decrease of NADPH (absorbance/min) was detected spectrophotometrically at 340 for 3 min. The control was run without GSSG. The activity of GR was expressed as µmoles NADPH oxidized/min/mg of protein.

Statistical analysis

All experiments were performed in triplicate in three independent experiments. All statistical analyses were carried out using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA). For oxidative stress experiments, hemolytic, comet, CAs, and micronucleus assays, data are presented as means ± SD and were compared by analysis of variance (ANOVA) followed by the Tukey's test.

Results

Troxerutin effects on PBLs cytotoxicity, genotoxicity or mutagenicity

After 24 hr incubation, Alamar Blue assay and trypan blue dye exclusion test showed troxerutin did not produce any significant toxic effects on PBL cultures at concentrations up to 1000 µM (Figure 1). The lack of cytotoxicity was correlated well with our data regarding genomic DNA damage (comet assay) and mutagenesis (MN test). Figure 2 indicates that troxerutin is devoid of genotoxic/mutagenic potential after 24 h treatment at concentrations ranging from 100 to 1000 µM.

Troxerutin attenuation of H_2O_2–initiated cytotoxicity, genotoxicity and mutagenicity

The ability of nontoxic concentrations of troxerutin to reduce deleterious effects of H_2O_2 was measured by the trypan blue assay and AO/EB DNA-binding fluorescent dye-based approach (Avelar-Freitas et al. 2014; Liegler et al. 1995). Figure 3 shows that when compared to cell cultures treated with H_2O_2 alone, troxerutin significantly prevented reduction in cell viability (Figure 3a) in both pre- and post-treatment protocols, as well as decreased the frequency of cells which shared characteristics linked to apoptotic cell death, such as peripheral chromatin condensation, nuclear disassembly, and presence of apoptotic bodies (Figure 3b). In the co-treatment experiments, cell viabilities (greater than 90%) and frequencies of cells with apoptotic morphology were similar to those of untreated cells at all three concentrations examined (Figure 3).

Troxerutin chemopreventive effects on H_2O_2 challenge were also assessed at the DNA level. Data related to number of DNA strand breaks and frequencies of micronucleated PBLs are depicted in Figure 4. Troxerutin did not alter the numbers of

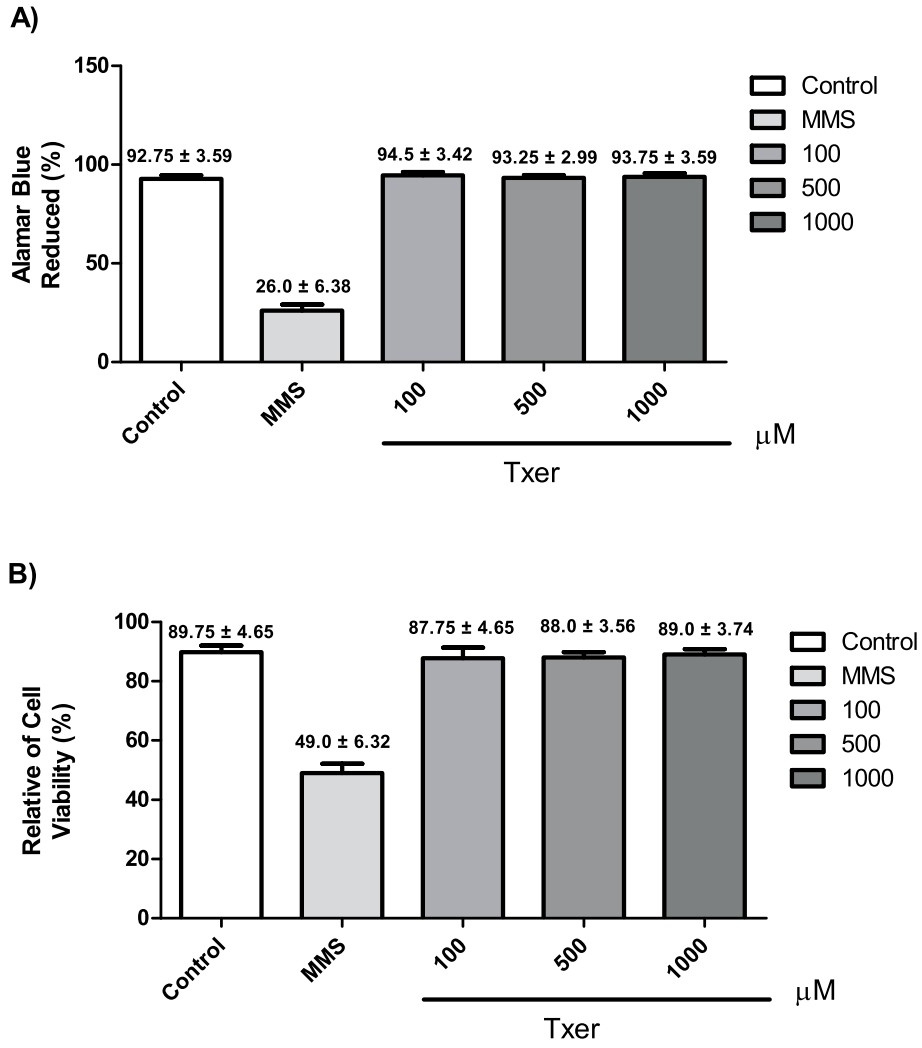

Figure 1. Cytotoxic effects of troxerutin (Txer) on PBLs by Alamar Blue test (panel A), and on cell viability using trypan blue dye exclusion after 24 h of exposure (panel B). Negative control (C) was treated with the vehicle (DMSO) used to dilute the test substances, and 4×10^{-5} M methylmethanesulfonate (MMS) was used as the positive control. Bars represent the mean ± S.D. of three independent experiments. *p < .05 vs. control – ANOVA followed by Tukey's test.

DNA strand breaks after 1 hr exposure, but significantly inhibited oxidative lesions of DNA mediated by H_2O_2 at all three studied concentrations (Figure 4a). Further, this flavonoid exhibited a significant antigenotoxic potential (more than 94% protection) against oxidative DNA damaging properties attributed to H_2O_2 (Figure 5).

The cytokinesis-block MN test demonstrated that H_2O_2, after a short exposure period (3 hr), increased the occurrence of MN cells, which is in agreement with its deleterious action on DNA. In contrast, co-exposure experiments noted that troxerutin displayed marked protection against the ability of H_2O_2 to induce cytogenetic abnormalities such as MN (Figure 4b). Both experimental protocols (pre- and post-treatments) showed troxerutin displayed potential to diminish the mutagenic effects of H_2O_2 (Table 1). Pre-exposure of cultured PBLs to troxerutin before H_2O_2 challenge produced a significant reduction in the frequency of micronucleated PBLs in a concentration-dependent manner. Troxerutin at concentrations equal to 100 and 500 μM induced decreases of MN cells of at approximately 24% and 35%, respectively, in relation to H_2O_2-exposed PBLs, peaking at almost 64% at 1000 μM. A similar scenario was observed in cultures treated with troxerutin after exposure to H_2O_2. In post-treatment experiments, troxerutin induced a significant protective effect, especially at the highest concentrations. Incubation of cells with 100 μM of troxerutin diminished MN formation by approximately 33%, whereas at the highest two

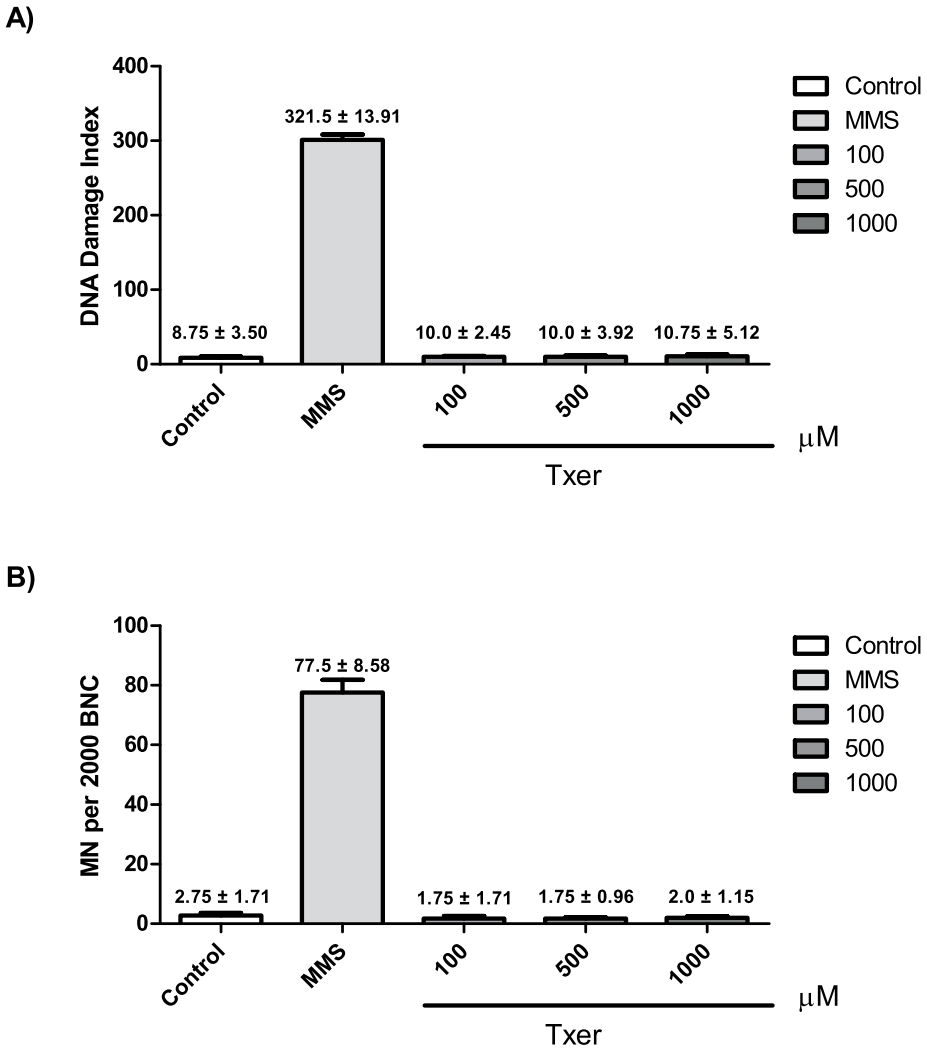

Figure 2. Effects of troxerutin (Txer) on PBL DNA damage index (panel A) and micronucleated cell frequency (panel B) in the cytokinesis-block micronucleus (MN) test only in binucleated cells (BCN) after 24 h exposure. Negative control (C) was treated with the vehicle (DMSO) used to dilute the test substances, and 4×10^{-5} M methylmethanesulfonate (MMS) was used as the positive control. Bars represent the mean ± S.D. of three independent experiments. *$p < .05$ vs. control – ANOVA followed by Tukey's test.

concentrations the levels of protection were greater: 81.76% and 90.24% for 500 and 1000 μM, respectively.

Troxerutin protected PBLs against oxidative stress induced by H_2O_2

Troxerutin at the tested concentrations did not significantly alter the oxidative parameters used as biomarkers (Table 2). After treatments, the levels of intracellular ROS and GSH were similar to negative control values, as were the levels of 8-hydroxy-2-deoxyguanosine (8-OH-dG), which is a major factor associated with oxidative DNA damage and an important marker of cell oxidative stress. Pretreatment with troxerutin significantly reduced intracellular levels of ROS, accompanied by an increase in the pool of GSH (Table 2). In this case, the lowest concentration of troxerutin was able to neutralize almost 41% of free radicals generated by H_2O_2 treatment, and at higher concentrations, preexposure to troxerutin diminished by approximately 58% (500 μM) and approximately 65% (1000 μM) the intracellular ROS induced by H_2O_2. The previous exposure of PBLs to troxerutin stimulated the formation of cellular GSH twofold higher at 100 μM; 2.5-fold higher at 500 μM and 2.8 greater at 1000 μM in relation to PBLs exposed to H_2O_2. These results

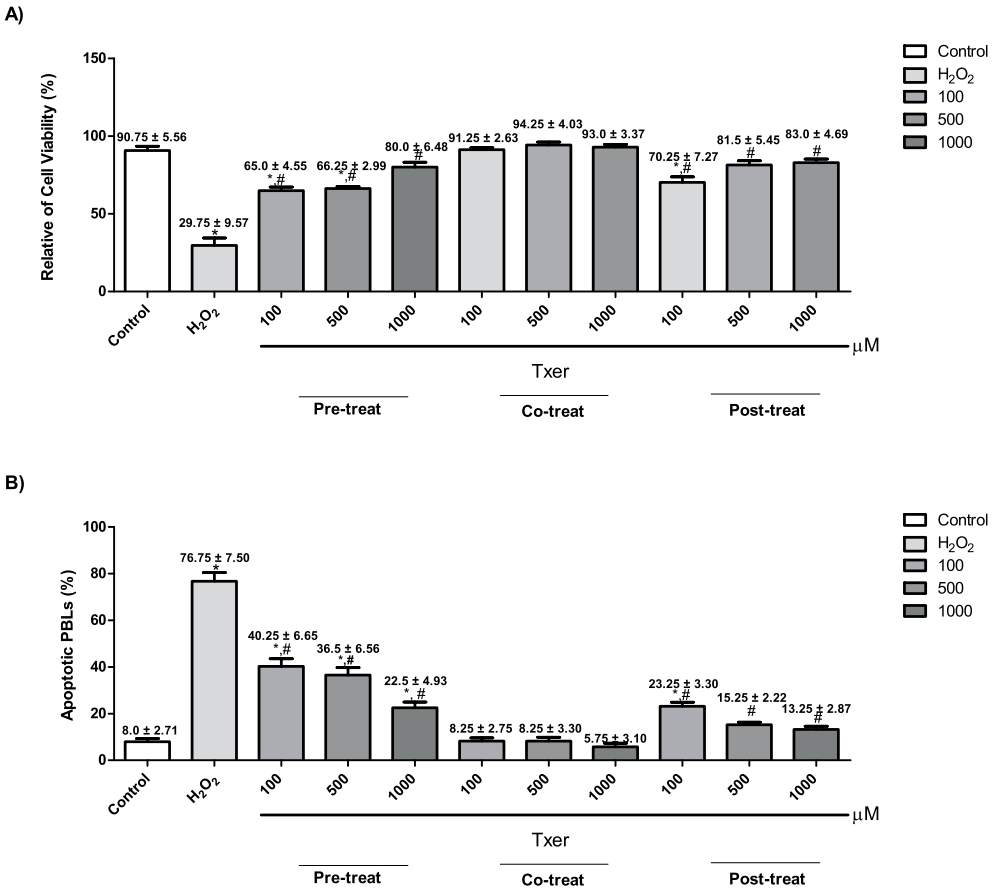

Figure 3. Effects of pre-, co-, and post-treatments with troxerutin (Txer) on H_2O_2 (150 μM)-induced cytotoxicity. After treatments, PBL viability and the induction of apoptotic cells were measured by the trypan blue dye exclusion test (panel A) and AO/EB staining (panel B), respectively. Negative control (C) was treated with the vehicle (DMSO) used to dilute the troxerutin. Bars represent the mean ± SD of three independent experiments. *$p < .05$; vs. negative control; #$p < .05$; vs. H_2O_2 – ANOVA followed by Tukey's test.

are associated with the protective property of this flavonoid against free radicals. Further, ROS scavenging characteristics of troxerutin were important for genome protection against deleterious effects of free radicals. Data displayed in Table 2 indicate that pre-exposure to troxerutin blocked the formation of 8-OH-dG in DNA molecules initiated by H_2O_2. The degree of inhibition against 8-OH-dG genome formation varied in a concentration-dependent manner: 31%, 48.56%, and 61.24% for 100, 500, and 1000 μM, respectively. A different result was observed when PBLs were treated at same time with troxerutin and H_2O_2, as presented in Table 2. Co-exposure protocols were more effective regarding chemopreventive action, and the treatments did not alter ROS levels or consume intracellular content of GSH in relation to negative control. In addition, co-incubation protected against DNA oxidative damage (hOGG1-sensitive sites) by more than 90% compared to cell cultures exposed only to H_2O_2 at all troxerutin concentrations. Finally, when troxerutin was added to cell cultures after H_2O_2 challenge, the levels of ROS were similar to negative control values (Table 2). However, GSH content was lower than in untreated PBLs, but 1.5 to twofold greater than GSH content of H_2O_2-treated cells, suggesting that troxerutin contributes to regeneration of the GSH pool. Regarding the levels of 8-OH-dG formation, post-treatments noted that troxerutin significantly lowered the occurrence of 8-OH-dG by 52.68% (100 μM), 68.2% (500 μM) and 82.52% (1000 μM) after exposure for 3 hr.

Troxerutin stimulated effects on GSH-dependent enzymes

Figure 6 illustrates the activities of GPx and GR of control and experimental groups. The activities of GSH-dependent enzymes (GPx and GR) of PBLs incubated with troxerutin (100, 500, or 1000 uM)

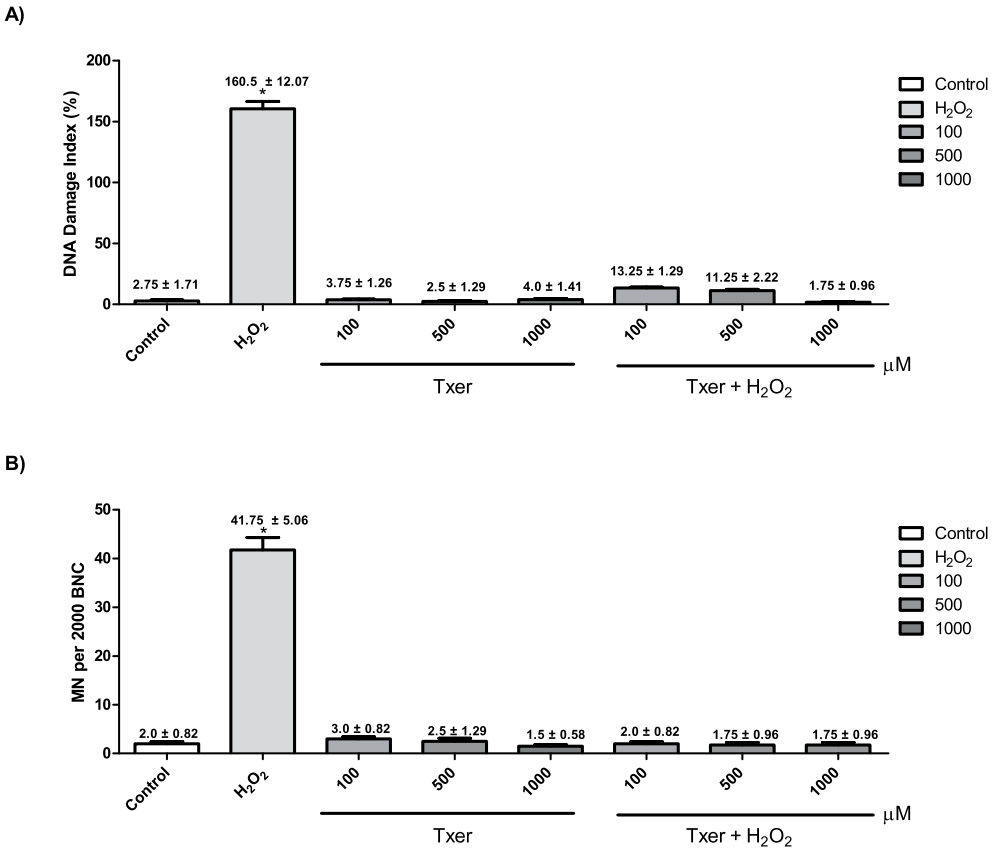

Figure 4. Effects of troxerutin (Txer) on DNA damage index and its ability to protect DNA from oxidative damage induced by H_2O_2 (150 μM) after 1 h exposure using the alkaline version of the comet assay (panel A), and on PBL micronucleated generation after 3 h exposure and its chemopreventive effects (co-treatment, 1 h) on 150 μM H_2O_2-induced micronuclei in PBLs (panel B). * $p < .05$ compared to negative control; #p < .05 compared to H_2O_2 by ANOVA followed by Tukey's test. Data are presented as means ± SD of three independent experiments in triplicate.

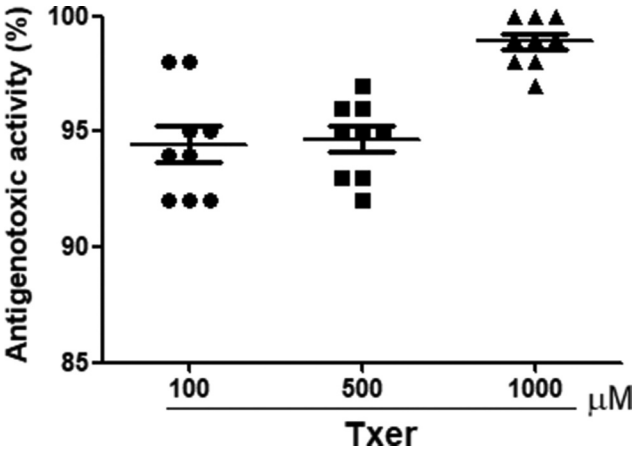

Figure 5. Antigenotoxic effects of troxerutin (Txer) on clastogenicity induced by H_2O_2 (150 μM) after 1 h exposure using the alkaline version of the comet assay. Data are presented as means ± SD of three independent experiments in triplicate.

Table 1. Effects of pre-, and post-treatments of troxerutin on H_2O_2-induced mutagenesis on PBLs.

	Substances	Treatments	MN per 2000 BNC
	Vehicle[a]	-	2.33 ± 1.50
	H_2O_2[b]	150 μM	43.16 ± 8.28*
H_2O_2 challenge	Pre-exposure	100 μM	32.58 ± 0.17*
		500 μM	28.16 ± 1.56*,#
		1000 μM	15.52 ± 0.11*,#
	Post-exposure	100 μM	28.74 ± 1.15*,#
		500 μM	7.87 ± 1.20*,#
		1000 μM	4.21 ± 0.13*,#

[a]DMSO used for diluting the troxerutin; [b]Positive control; *p < 0.05 compared to vehicle group; # $p < 0.05$ Compared to positive control (H_2O_2) by ANOVA followed by Tukey test. Data are presented as means ± S.D. for three independent experiments in triplicate.

alone were similar to those of control. In contrast, the activities of GPx and GR in H_2O_2-treated PBLs were significantly lower than control. PBL cultures exposed to troxerutin before H_2O_2 challenge displayed enhanced activities of GPx and GR in relation to cell cultures exposed only to H_2O_2. In addition, post-treatment experimental groups showed troxerutin also induced a significant

Table 2. Effects of troxerutin on the intracellular ROS generation, modulation of GSH pool, as well as detection of 8-hydroxy-2-deoxyguanosine (8-OH-dG) after H_2O_2 challenge.

	Substances	Treatments	% ROS	GSH (µg/mg protein)	hOGG1-sensitive sites (damage index)
	Vehicle[a]	-	3.38 ± 0.75	4.03 ± 0.27	4.56 ± 0.81
	H_2O_2[b]	150 µM	71.43 ± 4.28**	1.12 ± 0.13**	249.20 ± 14.45*
	Troxerutin	100 µM	2.64 ± 0.21*	3.92 ± 0.15*	3.14 ± 0.25$^{\#}$
		500 µM	3.11 ± 0.10*	4.01 ± 0.21*	5.37 ± 0.11$^{\#}$
		1000 µM	2.87 ± 0.10*	3.96 ± 0.10*	3.16 ± 0.21$^{\#}$
H_2O_2 challenge	Pre-exposure	100 µM	42.34 ± 0.80*,**	2.37 ± 0.22*,**	171.82 ± 8.23$^{*,\#}$
		500 µM	29.35 ± 1.12*,**	2.85 ± 0.10*,**	128.17 ± 1.52$^{*,\#}$
		1000 µM	24.78 ± 0.21*,**	3.21 ± 0.25*,**	96.58 ± 2.06$^{*,\#}$
	Co-exposure	100 µM	4.11 ± 0.10*	3.78 ± 0.33*	16.94 ± 2.38$^{*,\#}$
		500 µM	3.57 ± 1.28*	4.07 ± 0.25*	10.10 ± 1.05$^{*,\#}$
		1000 µM	2.52 ± 0.55*	3.93 ± 0.21*	6.81 ± 0.57$^{\#}$
	Post-exposure	100 µM	4.16 ± 0.30*	1.68 ± 0.16*,**	117.92 ± 8.13$^{*,\#}$
		500 µM	4.55 ± 0.11*	2.11 ± 0.10*,**	79.24 ± 2.49$^{*,\#}$
		1000 µM	4.49 ± 0.05*	2.29 ± 0.22*,**	43.56 ± 1.17$^{*,\#}$

[a]DMSO used for diluting the troxerutin; [b]Positive control; *p < 0.05 compared to vehicle group; $^{\#}$ $p < 0.05$ Compared to positive control (H_2O_2) by ANOVA followed by Tukey test. Data are presented as means ± S.D. for three independent experiments in triplicate.

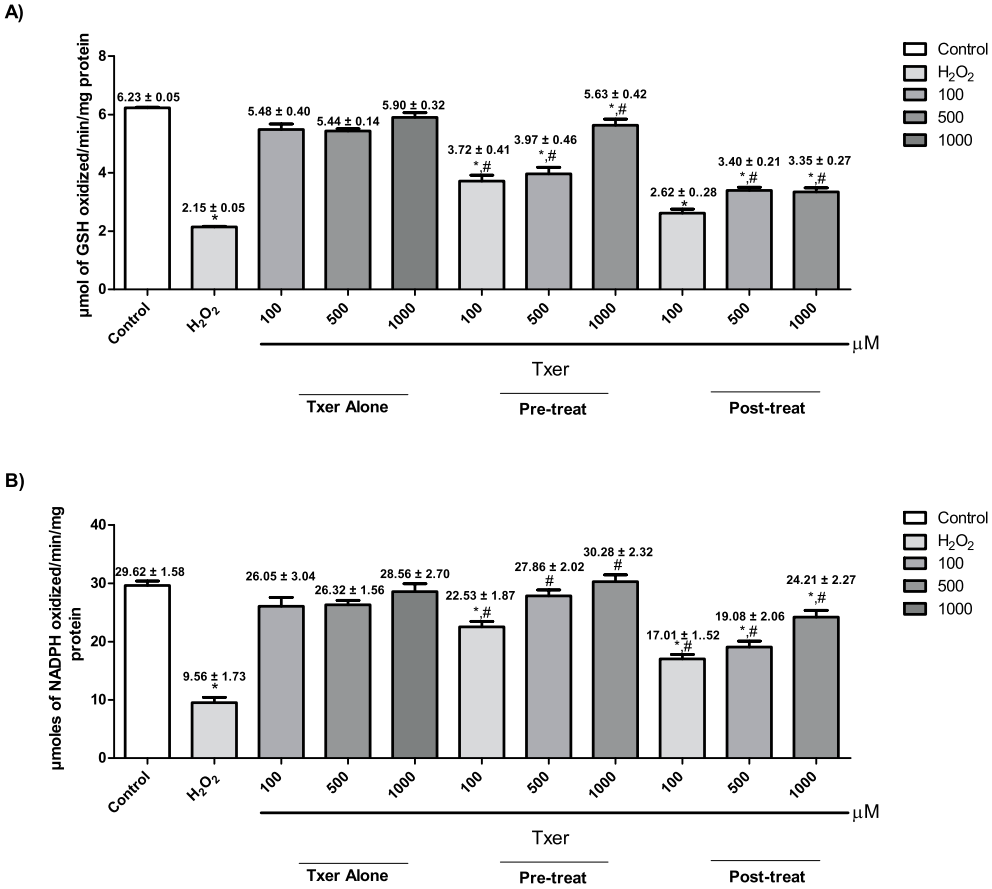

Figure 6. Effects of pre- and post-treatments with troxerutin (Txer) challenged with 150 µM H_2O_2 on the activity of glutathione peroxidase (GPx) and glutathione reductase (GR). Negative control (C) was treated with the vehicle (DMSO) used to dilute the troxerutin. Bars represent the mean ± SD of three independent experiments. *p < .05; vs. negative control; $^{\#}$p < .05; vs. H_2O_2 – ANOVA followed by Tukey's test.

elevation in GPx and GR activities, indicating that troxerutin is an inducer of GSH-dependent enzymes.

Discussion

Several human clinical trials with troxerutin alone or in combination with other drugs such as carbazochrome found that treatments are safe and well-tolerated even at elevated doses (i.e., 7 g per day orally for up to 6 months), with no apparent contraindications (Basile et al. 2001; Glacet-Bernard et al. 1994; Squadrito, Altavilla, and Oliaro Bosso 2000). Our evaluation of the cytotoxic effects of troxerutin revealed that this flavonoid did not exhibit any cytotoxicity against cultured PBLs after exposure for 24 h, even at 1000 μM (Figure 1).

Regarding the use of new drugs during the therapeutic treatment of diseases, the search for new chemical molecules (natural or synthetic) devoid of substantial toxic effects is an important feature in the process of developing new drugs. In fact, flavonoids do not tend to have cytotoxic effects on cells or tissues, presenting a good selectivity index, for example, between cancerous cells and non-cancerous ones, which stimulates the great interest in the development of new anti-cancer drugs based on of flavonoids (Plochmann et al. 2007; Sghaier et al. 2011). As with flavonoids, in general, troxerrutin also has a reliable index of selectivity between tumor and non-tumor cells (Thomas, George, and Selvam 2019).

DNA molecules are among the biological targets of many synthetic and natural bioactive compounds, and as well different drugs including anticancer, antibiotic, and antiviral that are in clinical use or undergoing clinical trials. One class of DNA-binding chemical which has undergone study is small ligand molecules. These molecules, when binding with DNA, interfere in cell physiology and functions by modulating gene expression in DNA (Gibson 2002; Rauf et al. 2005; Sharma et al. 2014; Zhang et al. 2015b).

In general, flavonoids have often been evaluated for their interaction with DNA (Nafisi et al. 2009; Nunes et al. 2020; Sharma et al. 2014). Panat et al. (2016) and Subastri et al. (2015) characterized the mode of interaction of troxerutin with calf thymus DNA, which occurred through DNA groove binding, probably in minor grooves of DNA. Subastri et al. (2015) reported that troxerutin did not produce any modifications of the native B-form of DNA and that this flavonoid preserved the native conformation of the DNA molecule. Subastri et al. (2015) also found that troxerutin was unable to initiate DNA strand breaks but was able to protect DNA against oxidative stress (hydroxyl radical generated by Fenton's reactions).

The natural flavonoid troxerutin and other semi-synthetic flavonoids (i.e. 7-Mono-O- (b-hydroxyethyl) -rutoside) have significant antioxidative effects (Lemmens et al. 2015, 2014; Panat et al. 2016). One of the most striking characteristics related to the mechanism of action of flavonoids is the mitigation power of free radicals. This is reflected in the high and persistent consumption of foods rich in these compounds and in the correlation with the reduction in the development of inflammatory diseases

(Diplock et al. 1998; Middleton and Kandaswami 1994).

Considering the previous information regarding DNA-binding capacity of troxerutin, one might expect to observe some type of genotoxic effect on PBLs following exposure. However, our data from the standard comet and MN tests noted that troxerutin is devoid of genotoxic/mutagenic properties after long (24 hr) exposure (Figure 2) or short (1 hr) treatment (Figure 4). In agreement with our findings, Marzin et al. (1987) reported that troxerutin on human lymphocytes did not significantly induce chromosome or chromatid breaks after 24 hr treatment in the presence or absence of exogenous metabolizing S9 fraction at concentrations up to 10 mg/ml, and failed to alter MN frequency in mouse erythrocytes treated with 5 or 10 g/kg flavonoid. In another set of mutagenic experiments, Marzin et al. (1987) showed troxerutin exerted no mutagenic effect on hamster lung fibroblasts (V79 cells) at concentrations ranging from 0.3 to 10 mg/ml, as well was no mutagenic effect in the *Salmonella typhimurium*/microsome assay at concentrations up to 5 mg/ml in the absence or presence of S9 mix. These earlier results of the absence of damage to DNA by troxerutin in human PBLs strengthen our cytotoxic data (Figure 1), corroborating published studies reporting its safety in clinical uses.

Various investigators described the potent *in vivo* and *In Vitro* antioxidant effects of troxerutin (Elangovan et al. 2016; Panat et al. 2016; Vinothkumar et al. 2014a, 2014b). Our data demonstrated that troxerutin exerted significant chemopreventive effects when PBLs were challenged with H_2O_2, as evidenced by monitoring cell viability and cell morphological changes (Figure 3) as well as other oxidative stress parameters summarized in Table 2. The greatest chemoprotective effect of troxerutin was observed during co-exposure with H_2O_2, which might be attributed to its scavenging properties on free radicals. Chemopreventive effects were also found when PBLs were treated before and after H_2O_2 challenge. Indeed, pre-, and post-exposure to troxerutin stimulated the activities of GSH-dependent enzymes (Figure 6), which helped cells reduce cytotoxicity mediated by H_2O_2 (Figure 3). Further, treatment with troxerutin (pre- or post-exposure) was accompanied by a gradual increase of the GSH intracellular pool, as well as significant fall in levels of cellular ROS (Table 2), accompanied by elevation in activities of GPx and GR (Figure 6). Several animal studies reported that troxerutin reduced cell apoptosis by decreasing production of ROS and enhancing the activities of antioxidative enzymes, such as superoxide dismutase, catalase, GPx, GR, and glutathione-S-transferase (Farajdokht et al. 2017; Raja and Saranya 2019; Shan et al. 2017; Vinothkumar et al. 2014a, 2014b).

Conclusions

In conclusion, troxerutin demonstrated low cytotoxic effect on PBLs after exposure for 24 hr. In addition, this flavonoid exerted a protective effect on PBLs against oxidative stress induced by H_2O_2. Although the mechanisms underlying chemopreventive action of troxerutin are not fully understood, our findings demonstrated that this flavonoid appears to act by stimulating the production of GSH-dependent enzymes.

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Disclosure statement

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Abstract

No carcinogenesis or mutagenesis studies have been carried out with etomidate. The current study showed that etomidate has weak cytotoxic potential after 48h exposure in human lymphocytes and has no hemolytic activity. The weak cytotoxicity seems to be related with redox imbalance of etomidate (40.9 and 81.9μM) treated lymphocytes. At both etomidate concentrations, a slight decrease of the levels of GSH intracellular content and a significant increase in the amount of carbonylated proteins were observed after 48h. The contribution of oxidative stress to genetic toxicity was only perceived when the enzyme Fpg was applied in the comet assay. Etomidate (40.9 and 81.9μM) is a weak generator of oxidative DNA damage in lymphocytes. These damages

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Etomidate is devoid of genotoxicty and mutagenicity in human lymphocytes and in the *Salmonella typhimurium*/microsomal activation test

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ABSTRACT

No carcinogenesis or mutagenesis studies have been carried out with etomidate. The current study showed that etomidate has weak cytotoxic potential after 48 h exposure in human lymphocytes and has no hemolytic activity. The weak cytotoxicity seems to be related with redox imbalance of etomidate (40.9 and 81.9 μM) treated lymphocytes. At both etomidate concentrations, a slight decrease of the levels of GSH intracellular content and a significant increase in the amount of carbonylated proteins were observed after 48 h. The contribution of oxidative stress to genetic toxicity was only perceived when the enzyme Fpg was applied in the comet assay. Etomidate (40.9 and 81.9 μM) is a weak generator of oxidative DNA damage in lymphocytes. These damages to DNA probably were repaired, since no DNA strand breaks were detected in the standard alkaline comet assay (in the presence or absence of hepatic S9 microsomal fraction) without Fpg. Also, no micronucleated lymphocytes or carrying chromosomal aberrations were observed. Finally, etomidate (2046.8 and 4093.5 μM) was not mutagenic in the *Salmonella*/microsome mutagenicity assay, which used four *Salmonella typhimurium* strains (TA97a, TA98, TA100, and TA102) to detect frameshift and base-substitution mutations. In summary, etomidate is a weak oxidative DNA damaging anesthetic and is devoid of mutagenic properties in eukaryotic and prokaryotic models.

1. Introduction

In recent decades there have been several improvements in the management and administration of anesthetics, but the use of these substances can have a significant impact on the health of patients (Braz and Karahalil, 2015). Recent studies have shown the genotoxicity of anesthetics in patients undergoing surgery and in health professionals exposed occupationally, but these findings are controversial (Braz et al., 2011). Although the safety of anesthesia has improved considerably, there are still some adverse effects and unexpected results, so it is important to investigate the genotoxic and mutagenic potential of these compounds (Karahalil et al., 2005). Etomidate is an imidazole derivative that was first synthesized in 1964, initially developed as an antifungal agent, but its anesthetic activity was discovered during the animal testing phase (Liu et al., 2015). Its anesthetic effect is due to the subunit-dependent bond with γ-aminobutyric acid type A (GABAARs) receptors, which produces fast and potent action (Liu et al., 2019).

Etomidate has become important to induce analgesia in critically ill patients, as it has the characteristic of maintaining respiratory and hemodynamic stability (Nyman et al., 2016). Despite having a good hemodynamic profile, in 1983 Ledingham and Watt showed that intensive care patients receiving prolonged etomidate infusions had increased mortality in relation to those receiving benzodiazepines (Hulsman and Hollmann, 2018).

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Since then, several studies have confirmed that etomidate acts by suppressing endogenous glucocorticoid biosynthesis in the adrenal glands due to inhibition of the enzyme 11β-hydroxylase (CYP11B1), which catalyzes cortisol production from deoxycortisol (Wang et al., 2014; Forman, 2011). Studies have shown that at the concentration of 200 ng/mL (1 μM) etomidate has analgesic effects, but at a concentration of 10 ng/mL it interferes in cortisol production, withCI_{50} for cortisol biosynthesis of 1 nM (Zolle et al., 2008).

Regarding the cellular cytotoxicity, Wu et al. (RS et al., 2011) showed that the exposure of raw leukemia cells 264.7 to etomidate leads to morphological changes and reduction of cell viability, with consequent increases in apoptosis-inducing factor levels, cytochrome *c*, endonuclease G, caspase-9 and greater expression of 17 genes that may be involved in the apoptosis-inducing effect.

In one study using the neuroblastoma N2a cell line, etomidate also induced concentration-dependent apoptosis. In addition, etomidate led to loss of mitochondrial membrane potential, resulting in the generation of reactive oxygen species (ROS) in N2a cells and also showed significant modulation of pro-apoptotic proteins, including ADP-ribose polymerase (PARP), cleaved PARP, caspase-9 and proaspase-3 (Wang et al., 2014; Chen et al., 2018).

However, according to Braz, and Karahalil (Braz and Karahalil, 2015), there are no studies of the genotoxic and mutagenic potential of etomidate, so knowing the effects of this agent in genetic material can be a valuable support to better understand its possible mechanisms of action. Thus, the aim of this study was to evaluate the cytotoxic effect of etomidate in lymphocytes from human peripheral blood, as well as its genotoxic and mutagenic potential in human lymphocytes and prokaryotic cells of *Salmonella typhimurium*.

2. Materials and methods

2.1. Chemicals

The etomidate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum, phytohemagglutinin, RPMI 1640 medium, trypsin-EDTA, glutamine, penicillin and streptomycin the Gibco brand were purchased from Invitrogen (Carlsbad, CA, USA). Low-melting point agarose and agarose were also obtained from Invitrogen. Formamidopyrimidine DNA-glycosylase (Fpg, also known as MutM) was obtained from New England BioLabs (Ipswich, MA, USA). Cyclophosphamine was acquired from Asta Medica. The S9 fraction, prepared from the livers of Sprague–Dawley rats pretreated with the polychlorinated biphenyl mixture Aroclor 1254, was purchased from Moltox Inc. (Boone, NC, USA). Colchicine, cytochalasin-B (Cyt−B), L-histidine, biotin, aflatoxin B1, sodium azide, 4-nitroquinoline-oxide (4-NQO), methylmethanesulfonate (MMS), reduced glutathione (GSH), NADPH, glutathione reductase, 5,50-dithionitrobenzoic acid (DTNB) and doxorubicin (DXR) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade.

2.2. Peripheral blood lymphocyte isolation

Blood was collected from 3 healthy at the Hematology and Hemotherapy Center of Ceara State (Brazil) with an average age of 24 ± 3.05 years. The procedure was approved by the Human Research Ethics Committee of Federal University of Ceará (COMEPE-UFC – protocol number 281/09). Lymphocytes were isolated by the standard method of density-gradient centrifugation over Histopaque-1077. Cells were washed and resuspended in RPMI 1640 medium supplemented with 20% fetal bovine serum, 2 mM glutamine, 100 U/mL of penicillin and 171.9 μM of streptomycin, at 37 °C under 5% CO_2. Phytohemagglutinin (2.5%) was added at the beginning of culture. After 48 h of culture, cells were treated with the test substances.

2.3. Assessment of cell viability

The Alamar Blue test was performed with lymphocytes (1×10^6 cells/mL) after 24 and 48 h exposure to the test substance. Etomidate (1.3 to 81.9 μM in water) was added to each well and incubated for appropriate times. Control groups received the same amount of vehicle. Twenty-four hours before the end of incubation, 10 μL of stock solution (0.312 mg/mL) of Alamar Blue (Resazurin, Sigma-Aldrich) was added to each well. The absorbance was measured using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter®) and the drug cytotoxicity was quantified as the percentage of control absorbance at 570 nm and 595 nm. In this test, the absorbance of Alamar Blue in the culture medium is measured at a higher wavelength and lower wavelength. The absorbance of the medium itself is also measured at the same higher and lower wavelengths. The absorbance of the medium alone is then subtracted from the absorbance of medium plus Alamar Blue at the higher wavelength. This value is called AO_{HW}. The absorbance of the medium alone is also subtracted from the absorbance of medium plus Alamar Blue at the lower wavelength to obtain the AO_{LW}. A correction factor, R_0, can be calculated from AO_{HW} and AO_{LW}, where $R_0 = AO_{LW}/AO_{HW}$. The percentage by which Alamar Blue is reduced by viable cells was expressed as follows: % reduced = A_{LW} - (A_{HW} x R_0) x 100 (Lograd o et al., 2010).

2.4. Hemolytic assay

The hemolytic test was performed in 96-well plates following the method described by (Berlinck et al., 1996). Each well received 50 μl of 0.85% NaCl solution containing 10 mM $CaCl_2$. The first well represented the negative control (1% DMSO). In the second well, 50 μL of test substance (1 mg/mL) and 50 μL of 1% DMSO were added. Etomidate was tested at concentrations ranging from 40.9 to 4093.5 μM. The compound was serially diluted with 0.85% NaCl until the 11th well in a row. The last well received 50 μL of 0.2% Triton X-100 (in saline) to obtain 100% hemolysis (positive control). Each well then received 100 μL of a 2% suspension of mouse erythrocytes in saline containing 10 mM $CaCl_2$. After incubation at room temperature for 4 h and centrifugation, the supernatant was transferred to a new plate, and the hemoglobin released was measured using a multiplate reader (Spectra Count, Packard, Ontario, Canada) at 540 nm. Experiments were performed in triplicate in three independent trials.

2.5. Measurements of intracellular GSH content

The reduced glutathione (GSH) content was determined 24 and 48 h after etomidate treatment by a spectrophotometric assay based on the formation of 5-thio-2-nitrobenzoate (TNB) from DTNB, according Akerboom, and Sies to (Akerboom and Sies, 1981) with minor modification. The positive control was H_2O_2 at 150 μM for 1 h exposure. Briefly, treated (40.9 and 81.9 μM etomidate) and untreated cells (1.5×10^6 cells/mL) were washed with ice-cold PBS, resuspended in 0.1 M sodium phosphate-5 mM EDTA, pH 8.0, and sonicated to obtain the cell homogenate. An equal volume of 2 M $HClO_4$–4 mM EDTA was added to the cell extract, and the precipitated proteins were pelleted by centrifugation at 8000 *g* for 15 min at 4 °C. The supernatant was neutralized with 2 M KOH, and the insoluble residue was removed by centrifugation under the same conditions. For spectrophotometric determination, 900 μL of the cell extract supernatant or of a standard GSH solution, in the same phosphate-EDTA buffer, was mixed with 50 μL of 4 mg/mL NADPH in 0.5% (*w/v*) $NaHCO_3$, 20 μL of 6 U/mL glutathione reductase in phosphate-EDTA buffer, and 20 μL of 2523 μM DTNB in 0.5% $NaHCO_3$. The increase in absorbance was measured at 412 nm. The results were normalized by protein content (Lowry et al., 1951), and were expressed as μM protein.

2.6. Quantification of protein carbonylation

The intracellular oxidation of proteins in cells was determined by measuring the carbonyl groups generated in some amino acid side chains using the dinitrophenylhydrazine (DNPH) derivatization method (Buss et al., 1997). The level of protein carbonyls in etomidate-treated cells was measured for 48 h. H_2O_2 at 150 μM during 1 h exposure served as positive control. At the end of the treatments, lymphocytes were collected and the cell lysates were prepared by ultrasonication. The protein present in the cell lysates was quantified using Lowry's method (Lowry et al., 1951). Protein samples (900 mL; 5 mg/mL) were incubated with or without DNPH solution for 60 min at 37 °C in the dark. The reaction mixture was added to 1 mL of 10% trichloroacetic acid (4 °C) for 25 min. The suspension was then centrifuged at 5000 rpm for 30 min (4 °C) and the supernatant fraction was discarded. The pellet was washed three times with ethanol/ethyl acetate (1:1, *v/v*), resuspended in 45 mM Tris-HCl (pH 7.4) buffer and incubated for 10 min at 37 °C. The solubilized protein in the buffer was normalized to the protein content (Lowry et al., 1951) and the absorbance was measured at 375 nm using a spectrophotometer (Spectra Count, Packard, Ontario, Canada). Total protein carbonylation content was determined as nmol/mg protein.

2.7. Comet assay

The standard alkaline comet assay (single cell gel electrophoresis) was performed as previously described (Collins, 2004) in the presence or not of a rat liver exogenous metabolic activation system (S9 fraction). After treatment (6 h), cells were washed with ice-cold PBS, trypsinized, and resuspended in complete medium. Then, 20 μL of cell suspension (0.7×10^5 cells/mL) was dissolved in 0.75% low-melting point agarose, and immediately spread onto a glass microscope slide pre-coated with a layer of 1% normal melting point agarose. Agarose was allowed to set at 4 °C for 5 min. Slides were then incubated in ice-cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100, and 10% DMSO, pH 10.0) at 4 °C for at least 1 h in order to remove cell membranes, leaving DNA as "nucleoids". In the modified comet assay, slides were removed from the lysis solution, washed three times in enzyme buffer (40 mM Hepes, 100 mM KCl, 0.5 Mm Na_2EDTA, 0.2 mg/mL BSA, pH 8.0), and incubated with Fpg (30 min 37 °C). Slides were placed in a horizontal electrophoresis unit and incubated with fresh buffer solution (300 mM NaOH, 1 mM EDTA, pH 13.0) at 4 °C for 20 min in order to allow DNA unwinding and the expression of alkali-labile sites. Electrophoresis was conducted for 20 min at 25 V (94 V/cm). All the above steps were performed under yellow light or in the dark in order to prevent additional DNA damage. Slides were then neutralized (0.4 M Tris, pH 7.5) and stained using the silver staining protocol described by (Nadin et al., 2001). After the staining step, gels were left to dry at room temperature overnight and analyzed under a light microscope. One hundred cells (50 cells from each of two replicate slides for each treatment) were selected and analyzed for DNA migration. These cells were visually scored according to tail length into five classes: (1) class 0: undamaged, without a tail; (2) class 1: with a tail shorter than the diameter of the head nucleus; (3) class 2: with a tail length 1-2× the diameter of the head; (4) class 3: with a tail longer than 2× the diameter of the head; and (5) class 4: comets with no heads. The value of damage index (DI) was assigned to each sample. DI is an arbitrary score based on the number of cells in the different damage classes, which are visually scored by measuring DNA migration length and the amount of DNA in the tail. DI ranges from 0 (no tail: 100 cells × 0) to 400 (with maximum migration: 100 cells × 4) (Burlinson et al., 2007). MMS (4 × 10–5 M), cyclophosphamide (179.1 μM) and H_2O_2 (250 μM) were used as positive controls for all comet assay protocols.

2.8. Cytokinesis-block MN assay

After treatment (24 h) in the presence or not of a rat liver exogenous metabolic activation system (S9 fraction), cells were washed twice with medium, and cytochalasin-B (6.3 μM) was added to the cultures 48 h after initiation, as described by (Fenech, 2000a). Cells were harvested 72 h after the treatment starting point, resuspended in a 75 mM KCl solution, maintained at 4 °C for 3 min (mild hypotonic treatment), and fixed with a cold methanol/acetic acid (3:1) solution. This fixation step was repeated twice, and finally the cells were resuspended in a small volume of methanol/acetic acid and dropped onto clean slides. These were stained with 10% Giemsa (pH 6.8) for 6 min, mounted and coded prior to microscopic analysis. Micronuclei were counted in 2000 binucleated cells (BNC) with well-preserved cytoplasm. The identification of micronuclei was carried out according to Fenech et al.(Fenech, 2000b). MMS (4×10^{-5} M), and cyclophosphamide (179.1 μM) were used as positive controls.

2.9. Chromosomal aberrations (CAs) test

After the end of treatment (24 h), cells were washed with ice-cold PBS and re-cultivated in complete RPMI medium for 48 h. Colchicine (0.0016%) was added 2 h before fixation (72 h). Chromosomes were prepared according to standard procedures (Moorhead et al., 1960). Hypotonic treatment with KCl (0.75 M, 37 °C) was applied for 15 min. The cells were fixed with methanol and acetic acid (3:1), and the fixative solution was changed twice. Air-dried slides were stained with Giemsa (5%, pH 6.8) for 7 min and scored for CAs according to Savage (Savage, 1976). MMS (4×10^{-5} M), and cyclophosphamide (179.1 μM) were used as positive controls. Only well-spread metaphases were examined. Three hundred metaphases per culture were analyzed for the presence of CAs. The mitotic index was determined for 2000 cells and given as the number of mitoses per 100 cells (%) (Arni and Hertner, 1997).

2.10. Salmonella/microsome mutagenicity assay

Salmonella typhimurium TA98, TA97a, TA100, and TA102 were kindly provided by B. M. Ames (University of California, Berkeley, CA, USA). Mutagenicity was tested by the pre-incubation procedure. The S9 metabolic activation mixture (S9 mix) was prepared according to Maron (Maron and Ames, 1983). Briefly, 100 μL of each test bacterial culture ($1–2 \times 10^9$ cells/mL) was incubated at 37 °C with different concentrations of etomidate dissolved in water in the presence or absence of S9 mix for 20 min, without shaking. Subsequently, 2 mL of soft agar (0.6% agar, 0.5% NaCl, 50 M histidine, 50 M biotin, pH 7.4, 42 °C) was added to the test tube and poured immediately onto a plate of minimal agar (1.5% agar, Vogel-Bonner E medium, containing 2% glucose). Aflatoxin B1 (3.2 μM/plate) was used as positive control for all strains (in the presence of metabolic activation with S9 mix), 4-nitroquinoline-oxide (4-NQO, 5.3 μM/plate) for TA97a, TA98, and TA102 and sodium azide (15.4 μM/plate) for TA100 (absence of S9 mix). Plates were incubated in the dark at 37 °C for 48 h before counting the revertant colonies.

2.11. Statistical analysis

All experiments were performed independently three times. All statistical analyses were carried out using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA). For oxidative stress experiments, hemolytic, comet, CAs, and micronucleus assays, data are presented as means ± SD and were compared by analysis of variance (ANOVA) followed by the Tukey test. *Salmonella*/microsome mutagenicity data were analyzed using the Salmonel software. A compound was considered positive for mutagenicity only when: (a) the number of revertants was at least twice the spontaneous yield (MI ≥ 2;

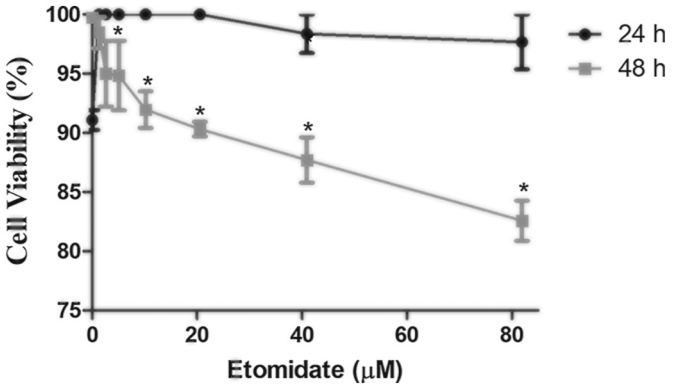

Fig. 1. Cellular viability, assayed by Almar blue test in humans PBLs (Peripheral blood leukocytes) treated for 24 h (black) and 48 h (gray) with etomidate (1.3-81.9 μM). Data presentded as mean values ± SDfrom three independent experiments in triplicate.* Analysis of variance (ANOVA) followed by the Turkey test ($p < 0.05$).

MI = mutagenic index: number of induced colonies in the sample/number of spontaneous colonies in the negative control samples); (b) a significant response was obtained in the analysis of variance ($p \leq .05$); and (c) a reproducible positive dose response ($p \leq .01$) was present, as evaluated by the Salmonel software.

3. Results and discussion

Some studies have been demonstrated that intravenous anesthetic agents cause transient depression of the immune system in humans (Walton, 1979; Käbisch et al., 1986; Liu and FL, 2012). Overall, a considerable number of studies have reported that anesthetics used in clinical practice induce significant but transient immunosuppression in subjects undergoing surgery under general anesthesia, especially regarding of the absolute counts of blood peripheral lymphocytes early in the postsurgical period (Hansbrough et al., 1984; Decker et al., 1996; Kim et al., 2017). In the current study, after 24 h exposure, the Alamar Blue assay showed etomidate did not elicit any significant cytotoxic effects on cell cultures at concentrations up to 81.9 μM. However, cultures exposed to etomidate for 48 h showed a slight decrease in the viability of lymphocytes (Fig. 1), whereas the fraction of non-viable cells increased from 10.30 to 17.74% in cultures exposed to 40.9 and 81.9 μM of etomidate, respectively (Fig. 1). In this respect, (Spiers and EM, 1983) showed that etomidate is devoid of any depressant effect on the immune system. In contrast, (Devlin et al., 1994) reported that at plasma concentrations used clinically for anesthesia obtained after induction doses, etomidate at 40.9 μM caused depression of total T-lymphocyte proliferation *in vitro* after exposure in the presence of different concentrations of phytohemagglutinin, but lymphocytes that did not show mitosis after etomidate treatment were still viable, as detected by the trypan blue exclusion test.

Moreover, etomidate is devoid of hemolytic properties since no hemolysis was observed after anesthetic treatment at concentrations ranging from 40.9 to 4093.5 μM. Our data are relevant, since in clinical practice the commercially available formulation of etomidate contains the organic solvent propylene glycol, which is associated with intravascular hemolysis caused by the high osmolality of etomidate dissolved in propylene glycol (Nebauer et al., 1992). Additionally, hemolysis is not associated with a more physiological solvent (*i.e.*, lipid emulsion). In a lipid emulsion, etomidate has an osmolality close to the physiological one, so the hemolytic effect is significantly reduced (Doenicke et al., 1997).

Interaction between reactive oxygen species (ROS) and cell macromolecules can result in oxidative damage and cell dysfunction. Regarding anesthetics, some studies have shown that different intravenous anesthetic agents can induce an increase intracellular ROS production and cause tissue damages (Murphy et al., 1993; Cavalcanti et al., 2020). A high level of oxidative stress associated with a diminished pool of intracellular antioxidant defense has been reported in patients recovering from surgery, and many anesthetics also interfere in the metabolism of ROS (Rao et al., 1997; Lases et al., 2000; Durak et al., 1999). Here, after 24 h of etomidate incubation, no evidence regarding ROS formation was noted (Table 1). On the other hand, lymphocytes exposed to etomidate during 48 h presented a significant ($p < .05$) reduction of GSH content and increase of the levels of oxidized proteins (Table 1), indicating that etomidate somehow induces the generation of intracellular ROS. In treated cell cultures, the decreases of cell viability were well correlated with ROS generation ($r = 0.925$, $p < .05$). Furthermore, ROS formation with the accompanying intracellular depletion of antioxidants, in particular glutathione, invariably precedes and is associated with apoptosis (Cavalcanti et al., 2011; Da Silva et al., 2011).

As far we know, there are no data available regarding the *in vivo* and *in vitro* genotoxicity and mutagenicity potential of etomidate. In the present study, etomidate was not able to induce *in vitro* DNA strand breaks and cytogenetic abnormalities in lymphocytes, as evaluated by the standard alkaline comet assay (Table 2), cytokinesis-block micronucleus (Table 3) and chromosomal aberration tests (Table 4), even when treatments occurred in the presence of the exogenous metabolizing S9 mix. The modified comet assay with the employment of Fpg showed that oxidized nucleotides occurred after short exposure time (6 h) with etomidate (Table 2). Fpg recognizes oxidized purines, principally 8-oxoguanine, and also ring-opened purines or formamidopyrimidines, as well as some alkylated DNA bases (Azqueta et al., 2011). However, the increment of DNA fragmentation (Fpg-sensitive sites) as measured by the damage index (DI; Table 2) after electrophoretic race indicated that DNA oxidative lesions could be repaired, since no DNA damage induced by etomidate was observed by the standard version of the alkaline comet assay (Table 2).

Interestingly, the cell proliferation indicators of cytogenetic procedures such as the percentage of binucleated cells (Table 3) and the mitotic index (Table 4) revealed that etomidate did not induce

Table 1
Effects of etomidate on peripheral blood leukocytes intracelular glutathione and protein oxidation after 24 h and 48 h exposure.

Compounds	Exposure time	Treatments	Glutathione (μg/mg protein)	Protein carbonylation (nmol/mg protein)
Vehicle[a]	24 h	0.1%	4.37 ± 0.10	1.04 ± 0.22
Hydrogen Peroxide[b]		150 μM	2.56 ± 0.21*	11.60 ± 0.55*
Etomidate		40.9 μM	4.24 ± 0.33	1.11 ± 0.10
		80.1 μM	4.31 ± 0.25	1.09 ± 0.10
Vehicle[a]	48 h	0.1%	4.82 ± 0.55	1.38 ± 0.10
Hydrogen Peroxide[b]		150 μM	0.77 ± 0.11*	17.84 ± 0.81*
Etomidate		40.9 μM	3.71 ± 0.21*	2.63 ± 0.10*
		80.1 μM	3.45 ± 0.10*	3.01 ± 0.10*

[a]Dimethyl sulfoxide; [b] Hydrogen Peroxide (Positive control); *$p < .05$ compared to vehicle group by ANOVA followed by Tukey test. Data are presented as means ± SD for three independent experiments in triplicate.

Table 2
Effects of etomidate on human peripheral blood leukocytes DNA damage index after 6 h exposure with and without metabolic activation (S9 mix) and Formamidopyrimidine DNA-glycosylase (Fpg).

Compounds	Treatments	Damage Index (−S9)	Damage Index (+S9)	Damage Index (Fpg enzyme)
Vehicle[a]	0.1%	9.69 ± 0.11	7.21 ± 0.10	8.44 ± 0.10
Hydrogen Peroxide[b]	250 μM	139.25 ± 4.15*	123.31 ± 2.30*	263.74 ± 5.10*,#
Methylmethanesulfonate[b]	4×10^{-2} μM	327.15 ± 4.85*	312.20 ± 7.10*	305.11 ± 3.70*
Cyclophosphamide[b]	179.1 μM	13.48 ± 0.10	139.21 ± 4.07*,‴	11.14 ± 0.10
Etomidate	40.9 μM	7.61 ± 0.75	8.30 ± 0.11	23.85 ± 0.21*,#
	81.9 μM	13.35 ± 0.25	11.13 ± 0.10	27.45 ± 1.15*,#

[a]Dimethyl sulfoxide; [b] Positive control; *$p < .05$ compared to saline group by ANOVA followed by Tukey test. Data are presented as means ± SD for three independent experiments in triplicate; ‴p < .05 compared to experiments conducted in the absence of S9 mix by ANOVA followed by Tukey test. Data are presented as means ± SD for three independent experiments in triplicate. #p < .05 compared to experiments conducted in the absence of S9 mix and Fpg enzyme by ANOVA followed by Tukey test. Data are presented as means ± SD for three independent experiments in triplicate.

Table 3
Effects of etomidate on human peripheral blood leukocytes micronucleated cell frequency in the micronucleus test with and without metabolic activation (S9 mix).

		-S9		+S9	
Compounds	Treatments	% Binucleated cells	Micronucleus per 2000 binucleated cells [c]	% Binucleated cells	Micronucleus per 2000 binucleated cells [c]
Vehicle[a]	0.1%	85.93 ± 1.10	2.52 ± 0.15	87.51 ± 0.20	2.13 ± 0.05
Methylmethanesulfonate [b]	4×10^{-2} μM	31.42 ± 5.19*	69.28 ± 4.10*	62.85 ± 2.17*	61.47 ± 0.11*
Cyclophosphamide[b]	179.1 μM	84.08 ± 0.10	3.21 ± 1.10	67.21 ± 4.16*,#	44.28 ± 6.10*,#
Etomidate	40.9 μM	80.39 ± 1.11	2.64 ± 0.50	84.94 ± 3.85	2.11 ± 0.55
	81.9 μM	81.63 ± 1.25	2.39 ± 0.10	83.01 ± 0.05	2.47 ± 0.10

[a]Dimethyl sulfoxide; [b] Positive control; [c] Micronucleus frequency is expressed per 2000 binucleated cells; *$p < .05$ compared to vehicle group by ANOVA followed by Tukey test. Data are presented as means ± SD for three independent experiments in triplicate; #p < .05 compared to experiments conducted in the absence of S9 mix by ANOVA followed by Tukey test. Data are presented as means ± SD for three independent experiments in triplicate.

Table 4
Mitotic index, frequency of chromosomal aberrations, and numeric changes in cultured human peripheral blood leukocytes after etomidate exposure with and without metabolic activation (S9 fraction).

Compounds	S9 mix	Treatments	Mitotic index (%) [c]	Number of aberrations[d]			Aberant cells (%)[e]
				R	P	E	
Vehicle[a]	−	0.1%	7.92 ± 0.50	1	1	0	0.66 ± 0.57
Methylmethanesulfonate [b]	−	4×10^{-2} μM	2.36 ± 0.11*	36	0	0	12.00 ± 1.73*
Cyclophosphamide[b]	−	179.1 μM	7.64 ± 0.10*	1	0	0	0.46 ± 0.40
Etomidate	−	40.9 μM	7.73 ± 0.22	2	0	0	0.66 ± 0.57
	−	81.9 μM	7.64 ± 0.10	0	0	0	0.0
Vehicle[a]	+	0.1%	7.61 ± 0.11	4	0	0	1.33 ± 0.57
Methylmethanesulfonate [b]	+	4×10^{-2} μM	2.25 ± 0.10*	34	3	2	13.00 ± 5.19*
Cyclophosphamide[b]	+	179.1 μM	4.62 ± 1.10*	19	3	4	8.66 ± 1.52 *,#
Etomidate	+	40.9 μM	7.50 ± 0.11	0	0	0	0.0
	+	81.9 μM	7.55 ± 0.20	1	0	0	0.46 ± 0.40

[a] Dimethyl sulfoxide; [b] Positive control; [c] determined for 2000 cells (means ± SD); [d]number of aberrations per 150 metaphases analyzed: R, ruptures (chromosome and chromatid); P, polyploid cells; E, endoreduplication); [e]percentage of cells with at least one aberration; *p < .05 compared to vehicle group by ANOVA followed by Tukey test. Data are presented as means ± SD for three independent experiments in triplicate; #$p < .05$ compared to experiments conducted in the absence of S9 mix by ANOVA followed by Tukey test. Data are presented as means ± SD for three independent experiments in triplicate.

impairment of cell cycle progression. These data can be explained by the fact that the cell cultures were not exposed continuously to etomidate during protocol execution. In fact, lymphocytes were exposed during 24 h to the anesthetic, after which the cells were re-incubated for 48 h (without drug) until harvesting to detect cytogenetic abnormalities such as micronuclei and chromosomal aberrations. So, during recovery time (48 h without etomidate) the DNA lesions were probably repaired, which did not allow us to detect the cytogenetic endpoints. Also, etomidate (2046.8 and 4093.5 μM /plate) did not shown any mutagenic effects (in the presence or not of metabolizing S9 fraction) in all four strains of *S. typhimurium* (TA98, TA97a, TA100, and TA102) (Tables 5 and 6). These concentrations were not toxic to TA100 strain and therefore did not interfere in the growth of *S. typhimurium*. However, we observed signs of toxicity at concentrations above 4093.5 μM for the TA100 strain.

In conclusion, etomidate showed a weak cytotoxic effect in human peripheral blood lymphocytes and was unable to cause hemolysis at a concentration similar to that used in clinical practice. The observed cell toxicity can be linked to oxidative stress induced by the anesthetic. The weak and reversible genotoxic effect of etomidate contributed to the non-formation of cytogenetic abnormalities such as the induction of micronucleated cells and chromosomal aberrations (numerical and structural) in human lymphocytes. Furthermore, etomidate at a concentration of 4093.5 μM was found to be devoid of mutagenic effects in prokaryotes (*S. typhimurium*), according to data from the *Salmonella*/microsome mutagenicity assay.

Table 5
Induction of *his* + revertants in TA98 and 97a *S.typhimurium* frameshift strains by etomidate with and without metabolic activation (S9 fraction).

S. typhimurium		TA98				TA97a			
		-S9		+S9		-S9		+S9	
Compounds	Treatments	Rev/plate[c]	Mutagenic Index [d]	Rev/plate[c]	Mutagenic Index [d]	Rev/plate[c]	Mutagenic Index [d]	Rev/plate[c]	Mutagenic Index [d]
Vehicle[a]	–	24.07 ± 2.83	–	30.20 ± 1.08	–	43.21 ± 1.10	–	63.52 ± 3.81	–
Positive control[b]		412.95 ± 26.47*	17.15	773.11 ± 32.28*	25.59	884.23 ± 78.39*	20.46	1781.55 ± 59.61*	28.04
Etomidate	2046.8 μM	11.46 ± 0.15	0.47	41.10 ± 0.50	1.36	37.52 ± 1.10	0.86	64.37 ± 0.11	1.01
	4093.5 μM	20.81 ± 0.10	0.86	36.43 ± 1.03	1.20	28.27 ± 0.56	0.65	62.16 ± 2.20	0.97

[a] Water.
[b] Positive control: (−S9) 4-nitroquinoline 1-oxide (2.6 μM/plate); (+S9) aflatoxin B1 (3.2 μM/plate).
[c] Number of revertants/plate presented as means ± SD for three independent experiments in triplicate.
[d] Mutagenic Index (number of *his* + induced colonies in the sample/number of spontaneous *his* + colonies in the negative control).
* $p < .05$ compared to vehicle group.

Table 6
Induction of *his* + revertants in TA98 and 97a *S. typhimurium* base-substitution strains by etomidate with and without metabolic activation (S9 fraction).

S. typhimurium		TA100				TA102			
		-S9		+S9		-S9		+S9	
Compounds	Treatments	Rev/plate[c]	Mutagenic Index [d]	Rev/plate[c]	Mutagenic Index [d]	Rev/plate[c]	Mutagenic Index [d]	Rev/plate[c]	Mutagenic Index [d]
Vehicle[a]	–	104.78 ± 13.51	–	131.16 ± 15.24	–	342.71 ± 46.38	–	468.90 ± 22.41	–
Positive control[b]		1849.84 ± 37.55*	17.65	1637.82 ± 51.49*	12.48	1961.59 ± 72.36*	5.72	1955.11 ± 68.57*	4.16
Etomidate	2046.8 μM	110.91 ± 10.84	1.05	132.90 ± 1.18	1.01	366.27 ± 21.48	1.06	463.53 ± 36.49	0.98
	4093.5 μM	103.75 ± 2.88	0.99	139.81 ± 19.47	1.06	342.21 ± 15.60	0.99	460.27 ± 24.26	0.98

[a] Water.
[b] Positive control: (−S9) 4-nitroquinoline 1-oxide (5.3 μM /plate); (+S9) aflatoxin B1 (3.2 μg/plate).
[c] Number of revertants/plate presented as means ± SD for three independent experiments in triplicate.
[d] Mutagenic Index (number of *his* + induced colonies in the sample/number of spontaneous *his* + colonies in the negative control).
* $p < .05$ compared to vehicle group.

Declaration of Competing Interest

None.

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