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ORIGINAL ARTICLE

Toxicity and mutagenic activity of benzophenone isolated from *Garcinia brasiliensis* fruit in healthy and *Schistosoma mansoni* infected mice

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ABSTRACT

7-epiclusianone (7-epi), a benzophenone isolated from fruits of *Garcinia brasiliensis*, presents remarkable biological activities as the schistosomicidal. However, there is a need to evaluate the toxic effects of 7-epi by analyzing liver function markers (aspartate-aminotransferase, alanine-aminotransferase, albumin, total proteins, and glucose) and a renal marker (creatinine), and beside that also comet (COM), micronucleus (MN), apoptosis (APOP), and histopathological in vivo analyzes. Although 7-epi at doses of 100.0 and 300.0 mg/kg (for five consecutive days) did not cause biochemical alterations to the male swiss mice (8 weeks old), it was not able to mitigate the damage caused by *S. mansoni* eggs. In the COM, MN, and APOP assays with marrow and the colon, 7-epi at doses of 10.0, 50.0, and 100.0 mg/kg (for fourteen consecutive days) caused no damage to the DNA and was able to protect the cell from damage caused by doxorubicin (16.0 mg/kg) and *N*, *N*'-dimethylhydrazine (30.0 mg/kg) both by peritoneal route for fourteen days. In the histopathological exams, the liver showed no changes in the dose of 100.0 mg/kg of 7-epi, however, some changes were observed in the 300.0 mg/kg dose. The results obtained in these experiments reveal that 7-epi can be used safely for the treatment of murine experimental schistosomiasis.

KEY WORDS: *Garcinia brasiliensis*; 7-epiclusianone; *Schistosoma mansoni*; biochemical analysis; cytotoxicity; mutagenicity; genotoxicity

ABBREVIATIONS

7-epi: 7-epiclusianone; **COM:** comet; **MN:** micronucleus; **APOP:** apoptosis; **AST:** aspartate-aminotransferase; **ALT:** alanine-aminotransferase; **EEE:** ethanolic extract; **TLC:** Thin-layer chromatography; **LC-MS/MS:** spectra and high performance liquid chromatography coupled to sequential mass spectrometry; **H&E:** haematoxylin and eosin; **DXO:** doxorubicin chloridate; **DMH:** N'-dimethylhydrazine; **PS:** physiological solution; **ANOVA:** Analysis of Variance; **CRD:** completely randomised design; **PCEs:** polychromatic erythrocytes; **MNPCE:** micronucleated polychromatic erythrocytes; **NaCl 0.9%:** sodium chloride; **TM:** tail moment.

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Introduction

Brazilian biodiversity offers many examples of common and rare plant life that deserve to be better explored. *Garcinia brasiliensis* (commonly known as a bacupari) is a plant native to the Amazon region, typically in tropical distribution, and widespread in the Brazilian territory. The fruit of this species has been used in alternative medicine for the treatment of peptic ulcers and urinary and tumour diseases. *Garcinia* species are a rich source of oxygenated and prenylated phenolic derivatives and some have been shown to exhibit antifungal, anti-inflammatory, antitumour, antioxidant, and anti-lipidemic properties (Veloso *et al.,* 2018). Among the variety of plant extracts described so far, 7-epi (Figure 1), a tetraprenylated benzophenone isolated from the epicarp of the native plant *G. brasiliensis*, has traditionally been used in folk medicine for the treatment of different diseases (Sales *et al.,* 2015).

Recently, studies conducted by our research group have shown that tetraprenylated benzophenone 7-epi demonstrated a considerable *in vitro* effect against cercariae, schistosomulos, and adult worms of *S. mansoni* indicating that this compound should be evaluated in animal trials as an alternative medicine for schistosomiasis (Castro *et al.,* 2015).

Schistosomiasis is a parasitic illness caused by trematodes of the genus *Schistosoma* and it is a neglected disease linked to poverty due to its association with a lack of sanitation and the domestic use of contaminated water (Silva *et al.,* 2017). There are approximately 258 million people infected with schistosomiasis in 78 countries, accounting for 200 million deaths annually. Moreover, 800 million people are estimated to be at risk of this infection (Neves *et al.,* 2016). There is no vaccine for schistosomiasis and chemotherapy relies heavily on a single drug (praziquantel) raising concerns about drug resistance, which highlights the need of more research in new vaccine and drug targets (Samoil *et al.,* 2018).

Toxicological genetics is one of the areas of science that has been dedicated to research on the genotoxic and mutagenic properties of agents, to which organisms are exposed (Costa *et al.,* 2008). To assess the safety of using these substances, genotoxicity tests such as the comet assay and mutagenicity tests such as the micronucleus (MN) test, *in vivo* mammalian cells, usually mice, are used. In this way, genotoxicity studies help to evaluate the safety and effectiveness of natural products (Bast *et al.,* 2002) considering that the mutagens are directly or indirectly linked to carcinogenesis (Papamokos & Silins, 2016). Histopathological tests and biochemical parameters used to evaluate liver and renal function are also of great importance since the toxic action of a substance can often compromise multiple organs (Abdel-Daim *et al.,* 2016).

Despite the many beneficial actions of plants, it is important to determine whether any of their constituents can have toxic effects and to evaluate whether the plants' metabolism can also generate metabolites with this same activity (Sousa & Viccini 2011). Thus, the indiscriminate application of medicinal plants and their constituents, without cytotoxic knowledge and knowledge of the genotoxic and mutagenic potential of their compounds, might represent a risk to human health (Sousa *et al.,* 2010). The present work was carried out to evaluate the toxic effects of 7-epi using biochemical, histopathological, and mutagenic approaches, since previous studies conducted by our research group have demonstrated the significant *in vivo* schistosomicidal activity of 7-epi, revealing that it as a promising schistosomicidal compound (Castro *et al.,* 2018).

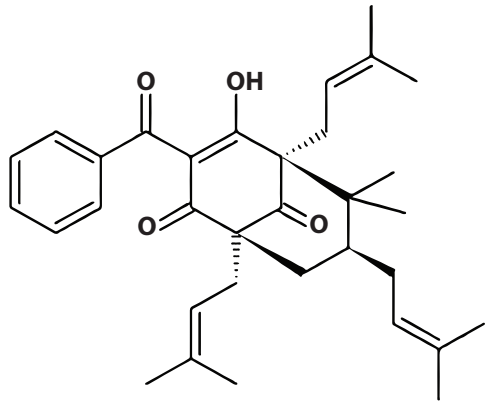

Figure 1. Chemical structure of 7-epiclusianone ($C_{33}H_{42}O_4$, MW: 502.68 g/mol).

Materials and methods

Collection of plant material

G. brasiliensis plants were obtained from the herbarium of the Federal University of Viçosa (MG, Brazil) after identification using the specimen voucher number VIC2604. 7-epi (molecular weight (Mw): 502.695 g/mol) was purified from *G. brasiliensis* pericarp following the methodology described by Castro *et al.* (2018), using only the hexane-soluble fraction of the ethanol extract as described below.

Preparation of extracts

Epicarps from *G. brasiliensis* fruits were air-dried at 40 °C for 8 days with continuous moisture monitoring. After the material was completely dry, it was pulverized in a knife grinder (Tecnal, Piracicaba, SP, Brazil), producing 1 kg of ground sample. The dried grounded epicarps were extracted by maceration with 3.0 l of ethanol 99.6% (MERCK, Germany) at room temperature (15 to 25 °C). The extracts were then filtered and concentrated using a rotary evaporator (IKA, Campinas, SP, Brazil) under reduced pressure at 45 °C. This procedure was repeated five times yielding a total of 80.6 g of fruit pericarp from the ethanolic extract (EEE).

Purification and isolation of 7-epiclusianone

To isolate the bioactive compound (7-epi), EEE was subjected to column chromatography (8×100 cm) on silica gel (230–400 mesh). Samples were eluted with increasingly polar mixtures of hexane/ethyl acetate and ethyl acetate/ethanol to give 50 fractions of 200 ml each. These fractions were concentrated on a rotary evaporator at a reduced pressure and were analyzed by Thin-layer chromatography (TLC) (eluent: hexane/ethyl acetate in different proportions) and vanillin solution in sulphuric acid and under ultraviolet light (λ= 254 and 365 nm) as developers. These fractions were pooled into four groups according to their similarities and compared with the standard 7-epi previously isolated from EEE. The 4–10 fractions were then rechromatographed on a silica gel (230–400 mesh) column (8×100 cm) and eluted with crescent polarity mixtures of n-hexane/ethyl-acetate and ethyl-acetate/ethanol to purify prenylated benzophenone 7-epi. The purity of 7-epi was 99.0% at 254 nm on the chromatogram, which presented the highest number of eaks, depicted in Figure S1 in supplementary material.

The structure of the isolated compound was determined by ^{1}H NMR and ^{13}C NMR spectra and high-performance liquid chromatography coupled to sequential mass

spectrometry (LC-MS/MS). The data were compared to those verified in a previous study investigating the chemical structure of this compound (Derogis *et al.,* 2008; Dos Santos *et al.,* 1999; Santos *et al.,* 1998). Data from ^{1}H NMR and ^{13}C NMR spectra and LC-MS/MS are available as Supplementary Figures S2, S3, S4 and Table S1.

Tests for the evaluation of biochemical parameters and histopathological analyses

Experimental design for the in vivo study

Male swiss mice (8 weeks old) weighing approximately 20 g were infected subcutaneously (Holanda *et al.,* 1974) with 100±10 cercariae of *S. mansoni* (LE strain) obtained from Laboratory of Schistosomiasis, Rene Rachou Research Center-Fiocruz, Brazil. All the experiments and procedures were approved by the Ethical Committee for Animal Research (Protocol #408/2012). The animals were separated into 2 groups, one group from infected animals (45 days after infection) and another from uninfected animals. Both groups were subdivided into groups of (i) untreated animals; (ii) animals treated with corn oil; (iii) animals treated with 100.0 mg/kg of 7-epi and (iv) treated with 300.0 mg/kg of 7-epi. Each group containing 10 animals (n=10) that were treated with 7-epi orally by gavage for 5 consecutive days and euthanized on the sixth day after treatment. The 30 and 90 mg of 7-epi was suspended in 1 ml of corn oil and administered orally (one dose per day for five consecutive days) at 100.0 mg/kg and 300.0 mg/kg, respectively. The animals were euthanized one day after treatment and perfusion was performed to collect worms in the mesentery and liver. Serum was collected from the infected and uninfected animals for analysis of the biochemical parameters and the liver of the uninfected animals removed for histopathological analyses All these tests were performed in triplicate.

Biochemical parameters in serum of mice

The serum of sacrificed mice was collected for the spectrophotometrical evaluation of glucose, total protein, albumin, and the activities of transaminases (AST and ALT); creatinine levels were measured using commercial Labtest® kits (Lagoa Santa, MG, Brazil) following the protocol described by the manufacturer. The analyses were performed in an Automatic Analyzer (Labmax 240®, Lagoa Santa, MG, Brazil).

Histological examination of uninfected mice liver

For histological analysis, livers were fixed in 10% neutral-buffered formalin and processed by routine methods. Sections of 5.00 µm were stained with haematoxylin and eosin (H&E) and analysed using a Zeiss Axio Scope A1 light microscope (Carl Zeiss, Germany) equipped with a camera (Axio Cam CC3, Zeiss) by a pathologist.

In vivo toxicological analyses of 7-epiclusianone

Experimental design for the in vivo study

The toxicological analyses of 7-epi were verified in uninfected and infected Swiss mice (8 weeks old) obtained from the Laboratory of Schistosomiasis, Rene Rachou Research Center-Fiocruz, Brazil. All the experiments and procedures were approved by the Ethical Committee for Animal Research (Protocol #408/2012). The mice were fed *ad libitum* with a commercial pelleted diet (Nuvilab Produtos Agropecuarios Ltda, Curitiba, PA, Brazil) and water and divided into 16 treatment groups; groups 2 to 12 contained uninfected animals and groups 13 to 16 contained *S. mansoni* infected animals (45 days of infection), with 8 animals in each group. The experiment was carried out with the following groups: group 1, positive control, animals received water by gavage plus doxorubicin chloridate via intraperitoneal injection (DXR, 16.0 mg/kg; Rubidox®, Bergamo Laboratory, São Paulo, SP, Brazil); group 2, positive control, animals received water by gavage plus N,N'- dimethylhydrazine via intraperitoneal injection (DMH, 30.0 mg/kg, Sigma-Aldrich, St. Louis, MO, USA); group 3, negative control, animals received water by gavage plus intraperitoneal physiological saline solution (PS, NaCl 0.9% w/v); groups 4, 5, and 6 received DMH (30.0 mg/kg) plus three doses of 7-epi, (10.0, 50.0, and 100.0 mg/kg body weight, respectively), which were provided orally at 10.0 ml/kg plus PS; groups 7, 8, and 9 received DXR (16.0 mg/kg) plus 7-epi (10.0, 50.0, and 100.0 mg/kg of body weight (b.w.), respectively); groups 10, 11, and 12 received NaCl 0.9% (10.0 ml/kg) plus 7-epi (10.0, 50.0, and 100 mg/kg b.w., respectively); groups 13, 14, and 15 received only 7-epi (10.0, 50.0, and 100.0 mg/kg b.w., respectively); and group 16 received only NaCl 0.9% (10.0 ml/kg) and was used to test for an interaction between this anti-cancer drug and 7-epi. Either DXR, DMH, or PS was provided 24 h before the termination of the experiment (day 14). At the end of the study, all animals were anesthetized with ketamine (1.0 mg/kg b.w.) and xylazine (0.05 mg/kg b.w.) and then euthanized. At necropsy, blood, bone marrow cells, and liver were collected from all animals. All animals were weighed every two days to track the growth and nutrient utilization performance of uninfected and infected mice fed commercial diet and treated with 7-epi.

In vivo bone marrow MN test, in vivo gut MN test, and apoptosis and comet assays

For the mutagenicity and antimutagenicity analysis of 7-epi, the bone marrow micronucleus (MN) test was used according to the protocol described by Silva *et al.* (2014). Two thousand polychromatic erythrocytes (PCE) were analyzed per mouse in slides blindly scored using a light microscope at ×1000 magnification. For the gut micronucleus test and apoptosis analyses, the colons were excised, flushed with NaCl 0.9% to remove fecal debris, cut open longitudinally, and rolled from caecum to anus, as described by Moolenbeek & Ruitenberg (1981). These "swiss rolls" were fixed in 10% (v/v) neutral formalin, embedded in paraffin, and sectioned thorough the roll (5.0 µm). For the *in vivo* gut micronucleus test, we followed the methods described by Vanhauwaert *et al.* (2001), using adjusted coloring conditions to obtain a better contrast of the nucleus against the cellular material. For this purpose, the slides were subjected to hydrolysis by being dipped for 15 min at room temperature in HCl 5 N, rinsed in distilled water three times, immersed in the dark in Schiff's reagent (Merck, Germany) for 90 min and then washed for 5 min in flowing water. After being rinsed in water, the slides

were counterstained with fast-green (Vetec Química Fina LTDA, Brazil) (0.5% w/v) for 4 min and rinsed in absolute ethanol. For gut MN count, 1000 colonic epithelial cells and their respective total number of crypts were scored manually from each animal, using a light microscope at ×1000 magnification. For the apoptosis analyses, "swiss roll" slides were stained with hematoxylin-eosin (HE) and assayed under light microscopy at ×400 magnification (Silva *et al.,* 2014). With regard to identification of apoptotic cells, a total of 20 perpendicular well-oriented crypts were examined in each animal, counting the total number of epithelial cells in each one. The apoptotic cells were identified as previously described by Silva *et al.,* (2014).

Comet (single-cell gel electrophoresis assay)

The peripheral blood comet assay was executed, as described in Azevedo *et al.* (2016) with some modifications. For this test, 7.0 µl of blood were mixed with 73.0 µl of low-melting-point agarose (5.0%) to form a microgel and spread on 1.5% agarose pretreated microscope slides. For the liver, sample approximately 0.4 g liver were placed in 2000.0 µl of cold Hanks' solution in a Petri dish and sliced into fragments to obtain a cell suspension. After that, liver cell suspensions (80.0 µl) were mixed with 240.0 µl 0.5% low-melting-point agarose and spread onto microscope slides, precoated with 1.5% normal melting agarose (Martins *et al.,* 2017).

The slides of liver and blood were then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl pH 10.0, 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA).), and 10% DMSO) for 24 h at 4 °C. After 24 h, the slides were placed into a horizontal electrophoresis unit and the DNA was allowed to rest for 20 min in alkaline solution containing 300 mM NaOH and 1 mM EDTA (pH≥13.0). Electrophoresis was conducted at 0.7 V/cm, 300 mA, 80 W (LPS-300V, Loccus Biotecnologia, Cotia, Brazil). The slides were neutralised for 20 min in neutralisation buffer (0.4 M Tris, pH 7.5) and stained with Red Gel 1:10,000 v/v (Thermo Fisher Scientific, São Paulo, Brazil) for fluorescence analysis. The fluorescent labelled DNA was visualised using a Nikon Eclipse 80i fluorescence microscope (400×) (Nikon, Minato, Tóquio, Japão). We used the parameters tail moment, tail length and percentage of DNA in the comet tail for analyzing the DNA damage. Fifty nucleoids were randomly counted from each slide for a total of one hundred cells per animal. The resulting images were captured and processed with image analysis software (TriTek CometScore™ v1.5, Sumerduck, VA, USA).

Statistical analysis

For the tests evaluating the biochemical parameters and histopathological results, the statistical analyses were performed using GraphPad Prism (version 5.0); the differences between the groups were determined using Analysis of Variance (ANOVA) followed by a Tukey's multiple comparison test. Values were considered significant when $p<0.05$.

The analyses of the 7-epi toxicological experiment were conducted according to a completely randomized design (CRD) with three replications. Analysis of variance (ANOVA) and the Tukey average comparison test were applied to the chemical analysis data and the *in vivo* test data (growth performance and nutrient utilization, oxidative stress, and comet assay). The Chi square test; test was used to compare the micronucleus numbers.

The percentage of reduction in micronucleated and apoptotic cells, was calculated according to Azevedo *et al.,* (2003), using the following formula: reduction (%) = (mean frequency of damage in A – mean frequency of damage in B/mean frequency of damage in A – mean frequency of damage in C) × 100; where A = positive control group treated with DMH or DXR; B = group treated with 7-epi + DMH or DXR; C = negative control group treated NaCl 0.9%. The statistical software used was SAS (Statistical Analysis System), version 9.2, licensed for UFV (Federal University of Viçosa, Brazil) in 2008. The results were considered statistically significant when the *p*-values were 0.05 or less.

Results and discussion

Tests for the evaluation of biochemical toxicity markers and histopathological analyses

Renal problems due to schistosomiasis have been studied by several authors (Duarte *et al.,* 2014; Eid *et al.,* 2017; Neves *et al.,* 2016b). To support possible renal failure, creatinine concentration was evaluated in animals treated with 100.0 mg/kg and 300.0 mg/kg 7-epi (Figure 2). At these doses, no differences were observed between the infected and uninfected groups, showing that neither the infection nor the 7-epi treatment caused kidney damage to rats.

The levels of transaminases, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), among others, increase as a result of metabolic changes in the liver, such as drug administration, schistosomiasis, cirrhosis, hepatitis, liver cancer, and jaundice (Robinson *et al.* 2014). The identification of mild, moderate, and severe elevations in the levels of ALT and AST in the liver can be used to determine the extent of liver damage (Wang *et al.,* 2015). Both of these enzymes in the body carry out processes of the intermolecular transfer of an amino group from donor gamma-amino acids to the acceptor alpha-ketonic acid without the intermediate formation of ammonium. Transamination plays a key role in the intermediate metabolism as it provides the synthesis and the destruction of some amino acids in the body. These enzymes are released into the blood when there is cell damage (Kvan *et al.,* 2019).

In this study, the uninfected mice that were treated with the two concentrations of 7-epi, presented no changes in the enzymes AST and ALT. Therefore, it can be assumed that 7-epi have no toxic effect on the liver (Figure 2). However, the infected mice have shown an increase of these two enzymes ($p>0.0001$) in the serum. This was observed in the control groups (control and oil control) and in the groups treated with the two 7-epi

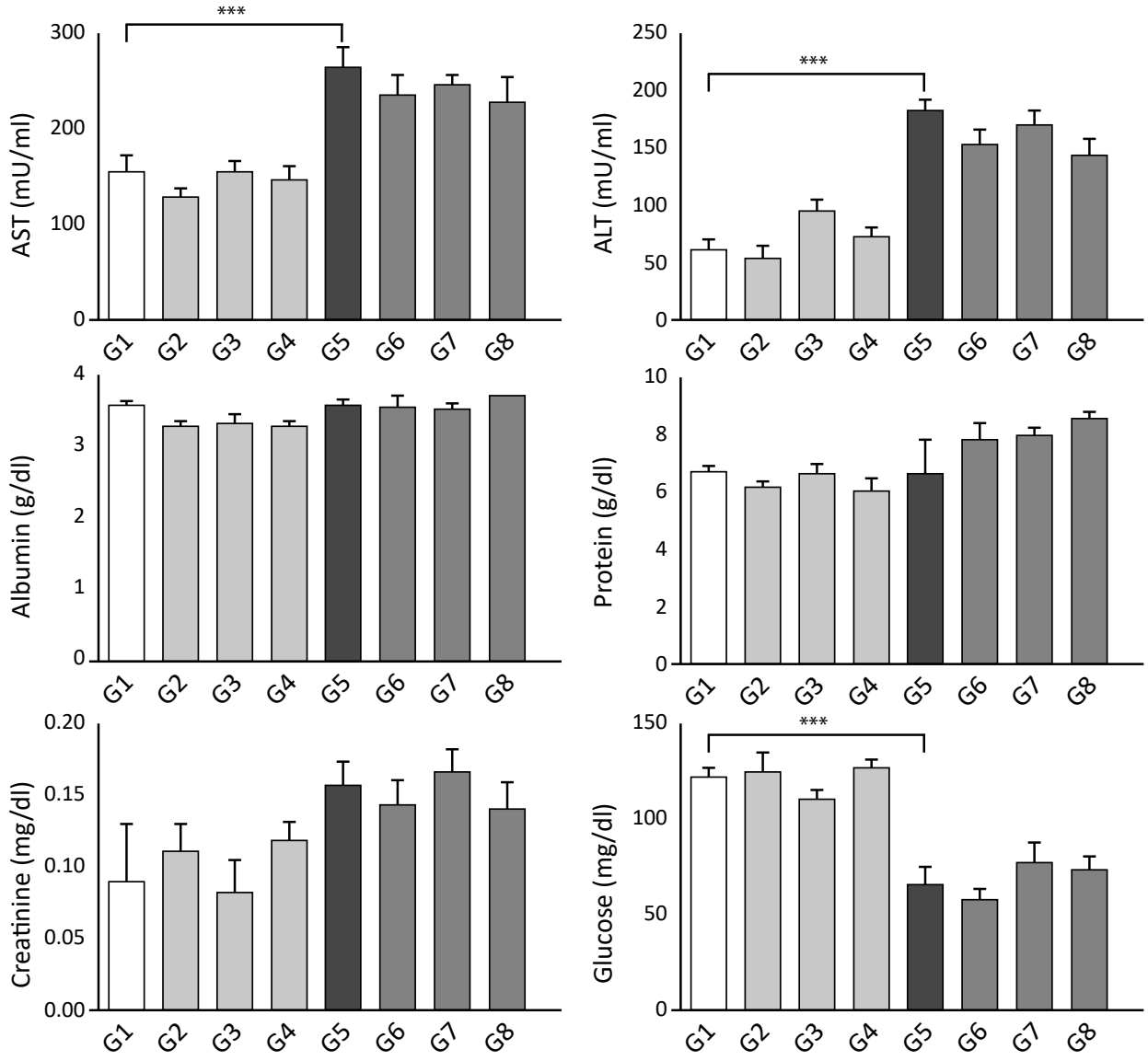

Figure 2. Evaluation of biochemical markers of toxicity in animals from the uninfected and infected groups. Animals (after 45 days of infection) were treated over 5 consecutive days with 7-epiclusianone. G1= uninfected control; G2= uninfected control treated with oil; G3= uninfected and treated with 7-epi 100.0 mg/kg, G4= uninfected and treated with 7-epi 300.0 mg/kg; G5= infected control; G6= infected control with oil; G7= infected and treated with 7-epi 100.0 mg/kg; G8= infected and treated with 7-epi 300.0 mg/kg. Statistically significant difference ***$p<0.0001$ when comparing the G1 with G5.

concentrations (100.0 and 300.0 mg/kg). This suggests that 7-epi was not able to ameliorate the altered liver function, which was the induced by the schistosomiasis.

To evaluate the organic involvement of the infected mice, the serum albumin concentration and total protein concentration were measured 45 days after infection. The liver is the only site of albumin synthesis and can be affected by liver disease, nutritional status, hormonal balance, and osmotic pressure (Allan *et al.*, 2014). To assess possible hepatic impairment, serum albumin and total protein levels (Figure 2) were measured in infected and uninfected mice. There was no change in the albumin or total protein levels in the infected animals compared to the uninfected animals, indicating that 7-epi did not induce hepatic damage and there was a normal functioning of the liver in albumin synthesis. The results presented here are in agreement with the hypothesis proposed by Smithers and Walker (1961), in addition to Atta *et al.* (2006), that schistosomal hypoalbuminemia is transient and there is a decrease in the concentration of this protein in the sera of the infected animals in the 5th, 6th, and 7th week of infection before the levels return to normal during the 8th week of infection.

S. mansoni are facultative anaerobes and use the glucose of their host to obtain energy (Ali *et al.*, 2015). According to Figure 2, 7-epi did not alter serum glucose levels in uninfected mice; therefore, in infected mice, there was a significant reduction ($p>0.0001$) in serum glucose levels; however, 7-epi was unable to increase these levels. According Ali *et al.* (2015), an important consequence of hepatic injury in schistosomiasis is the stimulation of glycolysis, which is manifested by a markable reduction

in levels of plasma glucose, hepatic glucose, and glycogen, and this is seen when comparing infected mice to control mice (Aly & Aly, 2006). This probably occurs due to the active manipulation and adaptation of the parasites to the host, in addition to the destruction of liver tissue, which results in the liver's inability to metabolism proteins and fats or to use glucose and store glycogen.

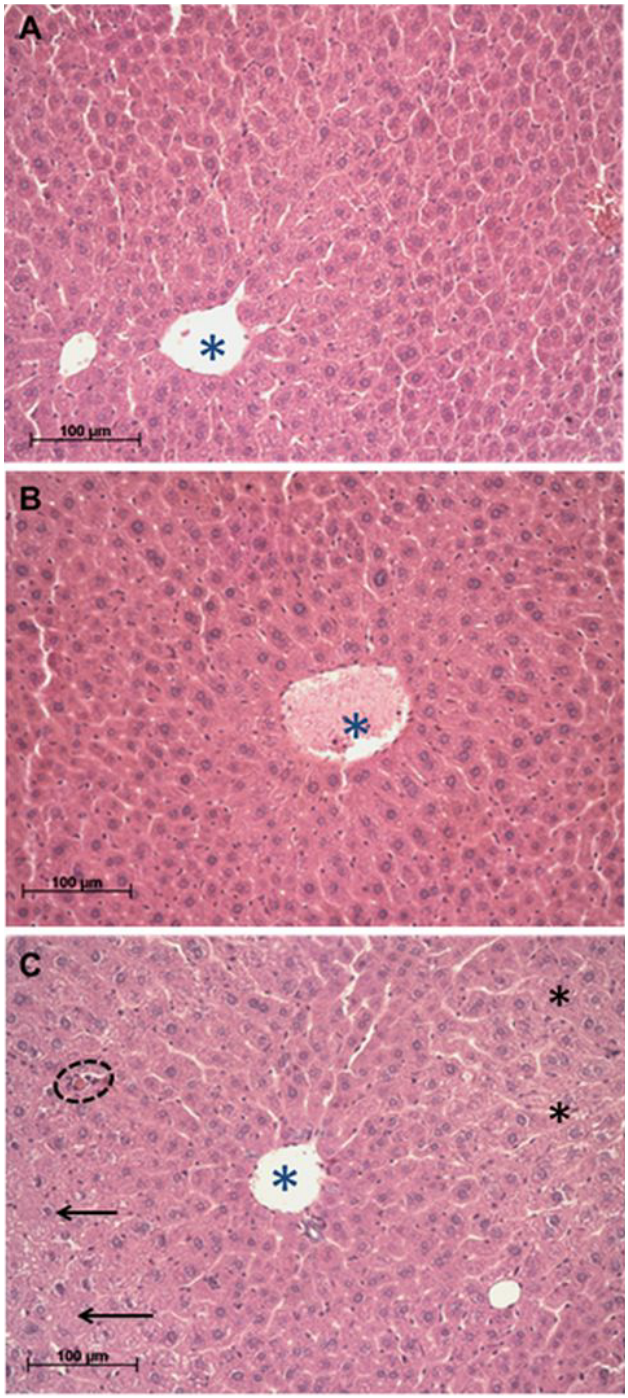

Figure 3. Histological examination of non-infected mouse liver after 7-epi treatment. Photomicrographs of livers after administration of 7-epi at (A) 0, (B) 100 and (C) 300 mg/kg, respectively. Mice injected with 100 mg/kg of epi-7 did not show any significant histological injury. The livers of mice injected with 300 mg/kg of 7-epi show inflammation (circle), necrotic cells with karyolytic or picnotic nuclei (arrows), and disorganization of hepatic cords (asterisk) at proximity to the centrilobular vein (blue asterisk). Original magnification 200× (bar= 100 µm).

In this study, it was possible to conclude that 7-epi is a substance that does not influence the biochemical parameters of uninfected mice; however, it was not able to revert the toxic effects caused by the disease. Similarly, the liver from mice administered with 100.0 mg/kg of 7-epi exhibited no significant microscopic alterations compared to the control group. Conversely, the histological alterations in the liver tissue of the 300.0 mg/kg 7-epi-treated mice were inflammation, necrosis, and disorganization of the hepatic parenchyma in some areas adjacent to centrilobular vein. These histological changes can be observed in mice treated with 300.0 mg/kg of 7-epi compared to control animals. The liver of mice from this group did not show significant histological changes that would indicate major liver toxicity, in agreement with the maintenance of baseline levels of ALT and AST (Figure 3).

7-epiclusianone mutagenic effects *in vivo*

In the present work, *in vivo* analyses were performed to evaluate the mutagenic effects of 7-epi compared to spontaneously occurring damage. The possible induction of apoptosis was studied in the intestinal epithelium since it is an important process in the eradication of cells that have suffered DNA damage due to the presence of mutagenic and/or genotoxic compounds (Mukhopadhyay *et al.,* 2014). The regulation of apoptotic has also been associated with different processes of oncogenesis, such as initiation, progression, and metastasis. In addition, it has also been associated with autoimmune diseases, Parkinson's, and Alzheimer's (Weber *et al.,* 2013).

The sensitivity of this *in vivo* assay was demonstrated by the positive/DMH response, which exhibited significant increases in the frequency of apoptosis (4.04% apoptotic cells) compared to the negative control/NaCl 0.9% group (1.79% apoptotic cells) (Table 1). The three concentrations of 7-epi (10.0, 50.0, and 100.0 mg/kg) showed a protective effect against mutagenic lesions compared to the positive group, presenting a reduction of 41.92, 78.89, and 107.79% apoptotic cells, respectively. The reduction in the number of apoptotic cells indicates that 7-epi protected the cells from DNA damage. In addition, 7-epi does not cause an imbalance in cellular homeostasis compared to the positive control group treated with the inducing substance DMH. Consequently, the animals treated with 7-epi did not activate mechanisms of cell death. The protective effect was observed by a similar level of apoptosis to the negative control group, which evidences the spontaneous levels of apoptosis in the animals (Table 1). In the animals infected with *S. mansoni*, the negative control group (G16: infected + NaCl 0.9%) presented no statistical difference compared to the negative control group of uninfected animals (G3: NaCl 0.9%), demonstrating that the parasite does not induce apoptosis.

The MN test is an *in vivo* assay widely used for the detection of clastogenic agents (which break down chromosomes) and aneugenic agents, which induce aneuploidy

Table 1. Effect of 7-epi on apoptosis frequency.

Groups/Treatments (number of animals)	Total crypts [a]	Total cells [b]	Cell/ crypt	Number of apoptosis/ group (average ± SD)	% Apoptotic cells (average ± SD)	Reduction (%)
G2: DMH Positive control (n=5)	100	2991	29.91	21.20±3.2	4.04±0.92	
G4: Epi 1 + DMH (n=5)	100	2961	29.61	16.40±4.40	2.79±0.72*	41.92
G5: Epi 2 + DMH (n=6)	120	3356	27.97	12.17±2.8	2.18±0.53*	78.89
G6: Epi 3 + DMH (n=7)	140	4095	29.25	8.86±1.6	1.54±0.31*	107.79
G3: Negative control NaCl 0.9% (n=4)	8	2202	27.53	9.75±1.7	1.79±0.44	
G10: Epi 1 + NaCl 0.9% (n=4)	80	2149	26.87	10.50±3.1	1.98±0.65	
G11: Epi 2 + NaCl 0.9% (n=6)	120	3675	30.63	11.17±2.6	1.95±0.77	
G12: Epi 3 + NaCl 0.9% (n=4)	8	2143	26.79	11.25±2.2	2.13±0.53	
G16: Infected + NaCl 0.9% (n=5)	100	3396	33.96	14.80±4.4	2.30±1.04	
G13: Infected + Epi 1 (n=5)	100	3256	32.56	11.00±3.8	1.87±1.05	
G14: Infected + Epi 2 (n=5)	100	3183	31.83	18.00±8.3	3.00±1.74	
G15: Infected + Epi 3 (n=3)	60	2097	34.95	22.33±6.4	2.96±1.08	

DMH: N,N-dimethylhydrazine 30.0 mg/kg; NaCl solution 0.9%. Epiclusianone: Epi 1: 10 mg/kg, Epi 2: 50 mg/kg, Epi 3: 100 mg/kg. *Statistically significant difference when comparing the positive control DMH (G2) to the other groups (Chi square test; $p<0.05$). Reduction (%) = (mean frequency of damage in A - mean frequency of damage in B/ mean frequency of damage in A - mean frequency of damage in C) × 100, where A= positive control group treated with DMH; B= group treated with 7-epi + DMH; C= negative control group treated with NaCl 0.9%. [a] The value means 20 crypts/animal. [b] Number of cells resulting from 20 crypts/animal

or abnormal chromosomal segregation. A MN can be formed from an entire chromosome when damage occurs to the mitotic apparatus of the cell or in the chromosome itself (Araldi *et al.,* 2015).

A significant increase in the frequency of *micronucleated polychromatic erythrocytes* (MNPCE) in mice bone marrow was observed in the positive/DXR group (3.41% MNPCE) compared to the negative control group NaCl 0.9% (0.81% MNPCE), which validates this *in vivo* assay. Moreover, there were no cytotoxic effects in any of the experimental groups according to their polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) ratios. This result suggests a normal proliferation of bone marrow cells (PCE) compared to the number of mature erythrocytes, NCE (Aquino *et al.,* 2011). In addition, there were no mutagenic effects above the level of spontaneous damage demonstrated by no difference between the negative control (G5/NaCl 0.9%) of the uninfected animals and the animals treated with 7-epi with NaCl 0.9% (m/v).

To analyze the antimutagenic effect of 7-epi, the groups G2, G3, and G4, which received concentrations of 7-epi at 10.0, 50.0, and 100.0 mg/kg, respectively, were compared to uninfected animals treated with DXR (30.0 mg/kg) (G1/positive control group). It was possible to observe a remarkable reduction in MN frequency (Table 2), corresponding to 81.21%, 43.71% and 58.65%, at doses of 10.0 mg/kg; 50.0 mg/kg, and 100.0 mg/kg, respectively. Therefore, this demonstrates that there is an important protective effect of 7-epi on the bone marrow cells against DXR (30.0 mg/kg).

Our results are in agreement with Carvalho-Silva *et al.* (2012), who demonstrated that 7-epi did not cause mutagenic effects since it was not able to induce damage (micronuclei) in the DNA of bone marrow cells of mice. Studies have demonstrated that some natural products, such as *Copaifera lansdorffii* (Alves *et al.* 2013), *Styrax camporum* (Francielli de Oliveira *et al.* 2012), and *Solanum lycocarpum* (Munari *et al.* 2014), in addition to not presenting cytotoxic effects, were able to reduce the cytotoxic action of a mutagenic agent. A study by Santa-Cecília *et al.* (2012) demonstrated that 7-epi was able to regulate inflammatory phagocyte superoxide anion release through a mechanism controlled by protein tyrosine phosphorylation and by the direct stimulation of protein kinase C. Possibly, the antimutagenic and antioxidant activities shown by 7-epi might contribute to an anticarcinogenic effect and have antitumor activity, thus promoting the study of new drugs of natural origin as prophylactic agents in cancer prevention.

The MN colon test has been widely used since intestinal epithelial cells are the first to have contact with the food eaten. In addition, these cells have a high capacity of turnover (renewal) facilitating the detection of clastogenic and aneugenic effects of chemical compounds not detected by the micronucleus test of marrow (Uno *et al.,* 2015).

Similarly to the bone marrow micronuclei, all the groups that received the 7-epi + NaCl 0.9% did not present a difference compared to the negative control of infected and not infected animals, demonstrating that this substance does not present gut mutagenic effects above the level of spontaneous mutations.

It was observed that exposure of the uninfected animals to the concentrations of 7-epi (G4, G5, and G6) + DMH also decreased the MN frequency in the colonocytes (Table 3), with a reduction in micronuclei of 74.38, 99.17, and 96.22% with the three doses of 10.0, 50.0, and 100.0 mg/kg, respectively, compared to the positive control (G2/DMH). These results also confirm the protective effect of 7-epi at the three doses assessed against the DMH-inducing substance on intestinal epithelial cells at all concentrations analyzed.

Table 2. The effect of 7-epiclusianone on the frequency of micronucleated polychromatic erythrocytes (MNPCE) in the bone marrow of mice.

Groups/Treatments (number of animals)	Total cells	PCE	NCE	PCE/NCE	MNPCE	%MN**	Reduction (%)
G1: DXR Positive control (n=6)	12000	454.5	295.6	1.5	15.5	3.41	
G7: Epi 1 + DXR (n=8)	16000	645.9	354.1	1.8	8.4	1.30*	81.21
G8: Epi 2 + DXR (n=7)	14000	495.0	379.4	1.3	11.3	2.27*	43.71
G9: Epi 3 + DXR (n=7)	14000	665.2	491.3	1.4	15.0	2.26*	44.39
G3: Negative control NaCl 0.9% (n=9)	18000	681.0	434.4	1.6	5.5	0.81*	58.65
G10: Epi 1 + NaCl 0.9% (n=6)	12000	486.1	263.3	1.8	2.3	0.46	
G11: Epi 2 + NaCl 0.9% (n=7)	14000	593.0	407.0	1.5	6.1	1.04	
G12: Epi 3 + NaCl 06.9% (n=7)	14000	509.8	365.3	1.4	4.6	0.91	
G: Infected + NaCl 0.9% (n=7)	14000	529.1	345.9	1.5	6.6	1.25	
G13: Infected + Epi 1 (n=6)	12000	461.4	288.6	1.6	5.0	1.08	
G14: Infected + Epi 2 (n=8)	16000	591.9	405.1	1.5	8.1	1.37	
G15: Infected + Epi 3 (n=5)	10000	399.3	225.8	1.8	3.0	0.75	

DXR: doxorubicin 30.0 mg/kg b.w.; NaCl solution 0.9%; NCE: normochromatic erythrocytes; PCE: polychromatic erythrocytes; MNPCE: micronucleated polychromatic erythrocytes; MN: micronucleus; n= 2000 analyzed cells/animal, Epiclusianone: Epi 1: 10 mg/kg, Epi 2: 50 mg/kg, Epi 3: 100 mg/kg. *Statistically significant difference when comparing the positive control DXR (G1) to the other groups (Chi square test; $p<0.05$). Reduction (%) = (mean frequency of damage in A - mean frequency of damage in B/ mean frequency of damage in A - mean frequency of damage in C) × 100, where A= positive control group treated with DXR; B= group treated with 7-epi + DXR; C= negative control group treated with NaCl 0.9%. **The values correspond to the total micronucleted cell in relation of total counted cell from each group.

Table 3. Results of the *in vivo* gut epithelial cell micronuclei test.

Groups/Treatments (number of animals)	Total cells	Total crypts [a]	Cells/crypt [b]	Total micronucleated cells	%MN**	MN Reduction (%)
G2: DMH Positive control (n=6)	6000	151	39.74	99	1.65	
G4: Epi 1 + DMH (n=6)	6000	151	39.74	45	0.75*	74.38
G5: Epi 2 + DMH (n=6)	6000	186	32.26	27	0.45*	99.17
G6: Epi 3 + DMH (n=7)	7000	188	37.23	34	0.49*	96.22
G3: Negative control NaCl 0.9% (n=5)	5000	130	38.46	22	0.44	
G10: Epi 1 + NaCl 0.9% (n=5)	5000	132	37.88	27	0.54	
G11: Epi 2 + NaCl 0.9% (n=6)	6000	194	30.93	37	0.62	
G12: Epi 3 + NaCl 0.9% (n=5)	5000	130	38.46	18	0.36	
G16: Infected NaCl 0.9% (n=5)	5000	132	37.88	26	0.52	
G13: Infected + Epi 1 (n=7)	7000	166	42.17	45	0.64	
G14: Infected + Epi 2 (n=6)	6000	143	41.96	39	0.65	
G15: Infected + Epi 3 (n=)	5000	120	41.67	31	0.62	

n: number of animals; DMH: N,N-dimethylhydrazine 30.0 mg/kg; NaCl solution (0.9%) 1.00 ml/kg; Epiclusianone: Epi 1: 10 mg/kg, Epi 2: 50 mg/kg, Epi 3: 100 mg/kg. MN: micronucleated cell; *Statistically significant difference when comparing the positive control DMH (G2) to the other groups (Chi square test; $p<0.05$). Reduction (%) = (mean frequency of damage in A - mean frequency of damage in B/ mean frequency of damage in A - mean frequency of damage in C) × 100, where A= positive control group treated with DMH; B= group treated with 7-epi + DMH; C= negative control group treated with NaCl 0.9%. **The values correspond to the total micronuclated cell in relation of counted cell from each group; [a] Number of crypts resulting from 2000 cell/animal. [b] Ratio between number of cells and crypts per animal.

The decrease in the number of MN observed in colonocytes and polychromatic marrow erythrocytes of animals treated with 7-epi indicates that there was protection against DXR damage to the bone marrow and DMH damage to the colon, which demonstrates that the damage that was not perpetuated and transmitted to the daughter cells during cell multiplication.

Therefore, 7-epi can be considered antimutagenic according to Felicidade *et al.* (2014), who consider any substance capable of reducing the frequency of spontaneous or induced mutations, regardless of the mechanism of action, as antimutagenic.

The results presented in this study are consistent with those of Rocha-Sales *et al.* (2016), who confirmed the structure-activity relationship and the potential utility of prenylated benzophenone, 7-epi, as a substance with antitumor activities.

For the comet test, peripheral blood cells and liver cells of the studied animals were used. This test assesses damage before it is repaired, which nucleoids with the

highest DNA damage presenting the highest migration of DNA, resembling the image of a comet, indicating the amount of DNA breakage in the cell (Azevedo *et al.* 2019). For both, the liver and blood cells, the parameters tail length, % DNA in comet tail, and tail moment were used. The tail moment reflects measures of the smallest detectable size of DNA migration (reflected in tail length size) and the comet tail length is directly proportional to the amount of DNA damage and the number of DNA breaks (represented by the % DNA in comet tail), demonstrating the relationship between the breaks and the drag of the genetic material (Jain *et al.* 2015; Rawat *et al.* 2018).

It was possible to observe that 7-epi did not induce peripheral blood leucocyte or liver cell DNA damage according to any of the three parameters evaluated in infected and uninfected animals. Therefore, neither the schistosomiasis nor the 7-epi treatment were able to induce injury in cells liver and blood DNA above the level of spontaneous damage. In this study, it was possible to observe that 7-epi did not induce DNA damage the liver cells of infected and uninfected animals according to the three parameters evaluated. Analyzing such parameters in the liver cells, it was observed that in some doses 7-epi was able to protect cells from damage caused by DXR

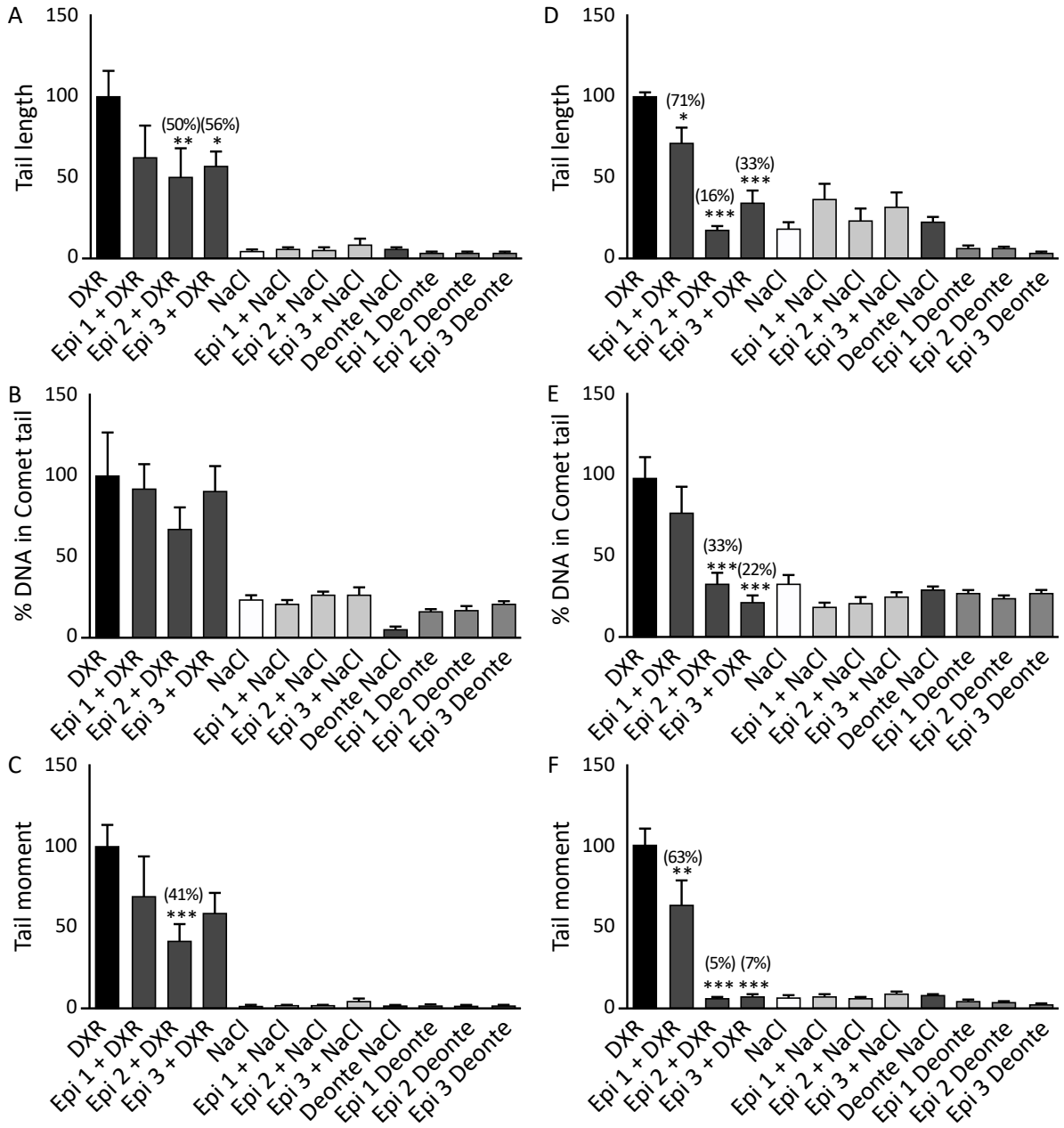

Figure 4. Comet liver cells and peripheral blood leucocytes. A, B and C liver cells: tail length, % DNA in comet tail and tail moment; D, E and F peripheral blood leucocyte: tail length, % DNA in comet tail and tail moment. DXR: doxorubicin 30mg/kg b.w.; NaCl: physiological solution (0.9% NaCl). Epi: epiclusianone; Epi 1: 10.0 mg/kg; Epi 2: 50.0 mg/kg; Epi 3: 100.0 mg/kg. Statistically significant difference in relation to the control group (DXR): *0.0.5; **0.001, ***0.0001. Values in parentheses represent the % protection of 7-epi against DXR in liver ad blood cells.

and this can be observed by the levels of protection of 7-epi, which was by 56.0% for the tail length and 41.0% for the tail moment, as shown in Figure 4 (A and C).

Analyzing the parameters of the comet test using peripheral blood leucocytes revealed that the 3 tested doses of 7-epi were able to protect cells from damage caused by DXR. This was highlighted by the protection levels of 7-epi, which range from 16.0% to 71.0% for tail length, from 5.0% to 63.0% for tail moment and 22.0% to 33.0% for % DNA in comet tail, according presented in Figure 4 (D, E and F).

The present study demonstrated that 7-epi, in addition to not presenting a genotoxic/mutagenic effect, was able to reduce the chromosomal damage induced by DXR, which demonstrates its potential to be used as a potent and promising chemoprotective substance. Although schistosomiasis is a disease that causes many types of damage to the host, in our work, it was not possible to observe damage according to the evaluated parameters.

Conclusion

Based on the results of this study it can be concluded that 7-epi does not alter the biochemical parameters evaluated, demonstrating that this substance does not present hepatic or renal toxicity; however, this substance was not able to protect these organs from the damage caused by *S. mansoni* infection. In histopathological analyses, 7-epi does not promote significant changes in the liver of uninfected animals.

The results obtained in the comet test, the micronucleus tests of bone marrow and the colon, and the apoptosis tests revealed that 7-epi did not cause damage to the DNA and was able to protect DNA from the damage caused by DXR and DMH.

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Author Contributions

Laila Santos Vieira Giannini, Tatieska de Paula Maia, Lellis Henrique Costa, Flávia Fernanda Bubula Couto, Raquel Lopes Martins Souza, Luciana Azevedo, Marcos José Marques.performed *in vivo* experimental analyses, André Luiz Machado Viana and Maria Rita Rodrigues performed biochemical analysis, Marcelo Henrique dos Santos carried out all chemical analyses, Carine Ervolino de Oliveira, performed histopathological analyses.

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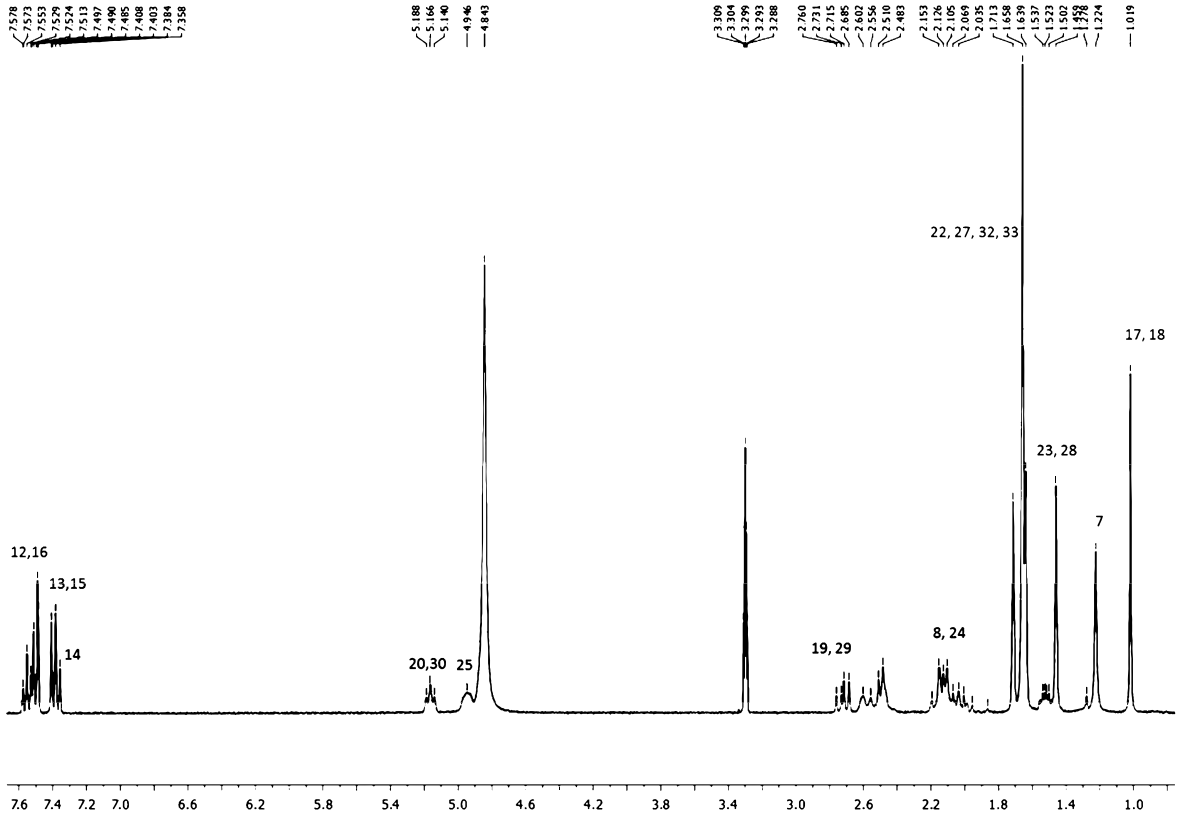

Figure S1. 1H-NMR spectrum of 7-epiclusianone (CD3OD, 300MHz).

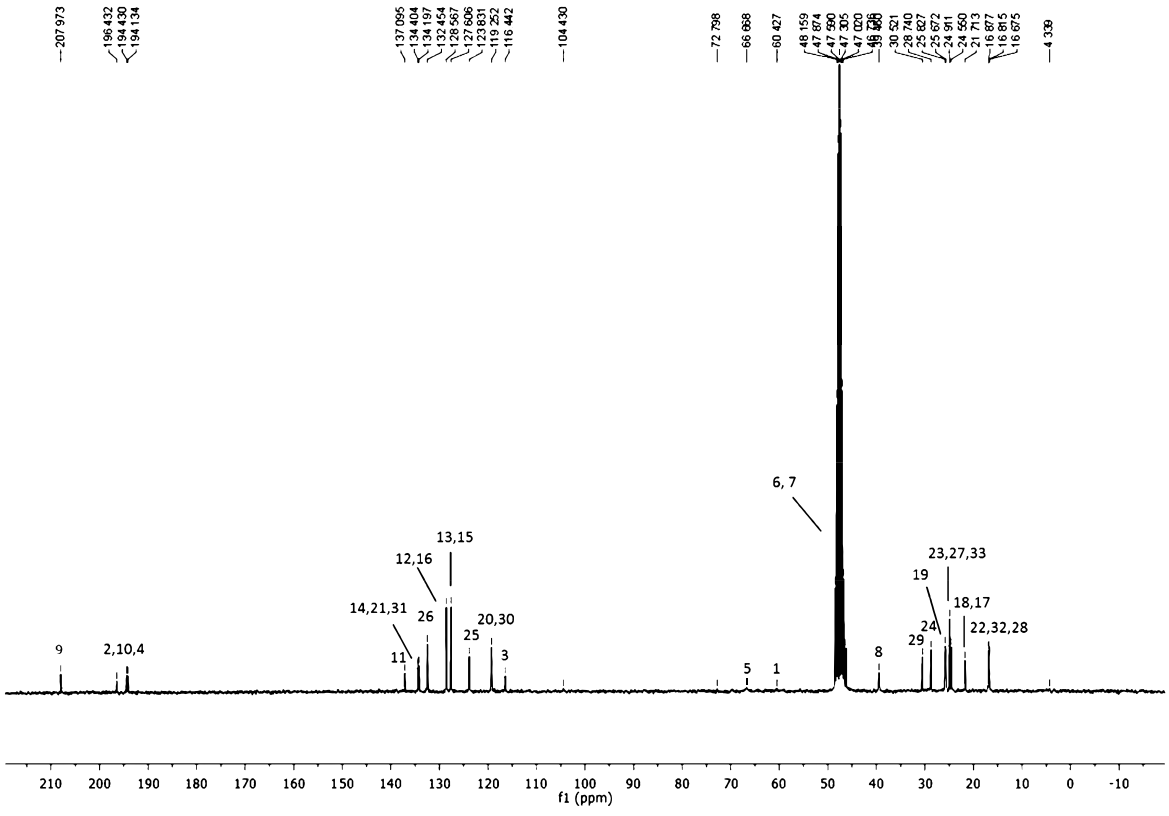

Figure S2. ^{13}C-NMR spectrum of 7-epiclusianone (CD_3OD, 75 MHz).

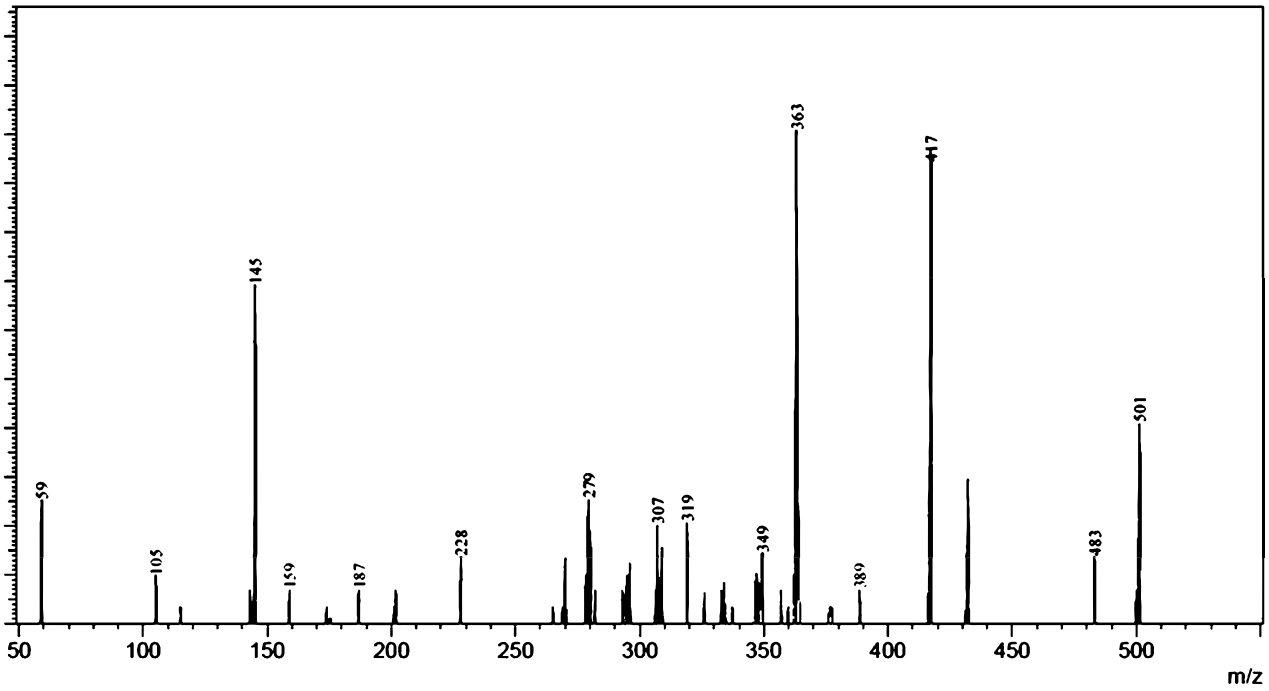

Figure S3. MS spectrum of 7-epiclusianone.

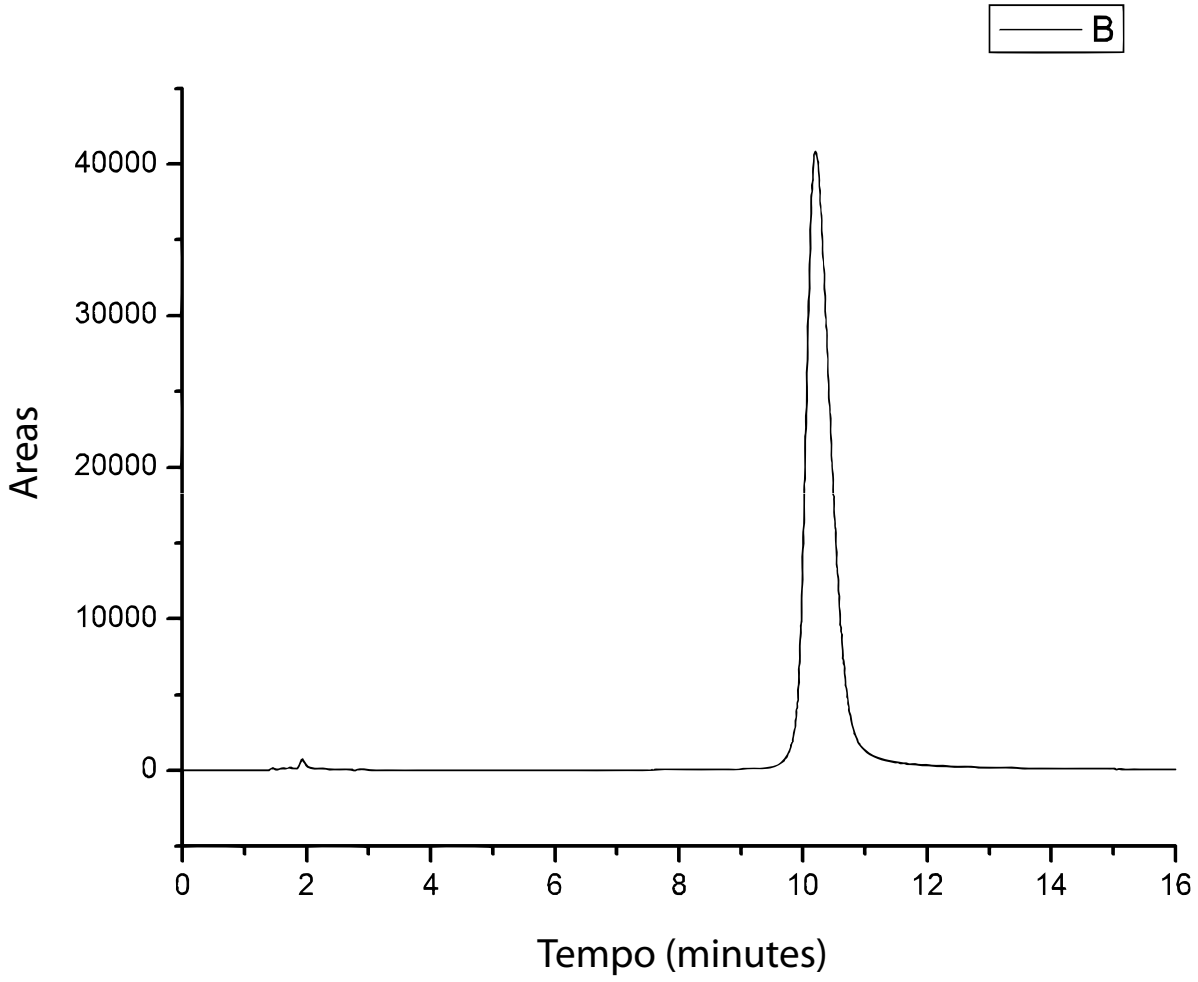

Figure S4. Chromatogram of the isolated 7-epiclusianone sample at 245 nm.

Table S1. Assignment of chemical shifts of carbon and hydrogen atoms of 7-epiclusianone based on NMR data.

	δ_C		δ_H
C			
1	60.42		
2	196.43		
3	116.42		
4	194.13		
5	66.66		
6	48.16		
9	207.97		
10	194.43		
11	137.05		
21	134.40		
26	132.45		
31	134.19		
CH		**CH**	
7	46.23	7	1.22
12,16	128. 56	12, 16	7.57
13,15	127.60	13, 15	7.35
14	132.45	14	7.52
20	119.25	20	5.18
25	123.83	25	4.94
30	119.25	30	5.16
CH_2		**CH_2**	
8	39.46	8	2.12
19	25.82	19	2.76
24	28.74	24	2.15
29	30.52	29	2.68
CH_3		**CH_3**	
17	21.71	17	1.01
18	25.82	18	1.01
22	16.87	22	1.65
23	24.91	23	1.52
27	24.56	27	1.63
28	16.67	28	1.50
32	16.81	32	1.71
33	25.67	33	1.71

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