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DNA Damage and Cell Death Induced by Crude Extract of the Orchid *Oncidium flexuosum* Sims

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Abstract

Oncidium flexuosum Sims. is an orchid whose leaves are frequently used for therapeutic applications although its action mechanism is not exactly understood. So the aim of this study was investigate its effects in Wistar rat liver, kidney and bone marrow. For this purpose, twenty-five adult male Wistar rats were divided into five groups of 5 animals each: a control group receiving only water, a positive control receiving a mutagen compound and three groups treated for 15 days with 100, 250 or 500 mg/kg/day of a crude hydroalcoholic leaf extract of *O. flexusosum* Sims. No significant alterations were observed in –SH groups, MDA or catalase, showing that the extract does not induce hepatic oxidative damage. There was no evidence of kidney injury and hepatic function alteration (ALT). However, significant mitochondrial swelling was observed in the treated groups and is probably related to cell death induction observed in cells that displayed acidophilia, pyknosis and positive labeling with the TUNEL technique at higher concentrations of the extract. We demonstrated that the hepatotoxicity of the extract resulted from its ability to induce DNA and mitochondrial damage that arrests cells, leading to cell death, probably caused by the flavonoids and tannins present in this extract.

Keywords: Cell death; Micronuclei; Mitochondrial swelling; Oxidative stress

Abbreviations: MMS: Methyl Methanesulfonate; ALT: Alanine Aminotransferase; HEPES: 4-(2-hydroxyethyl)-1-Piperazineethanesulfonic Acid; EGTA: Ethylenediaminetetraacetic Acid; SOD: Superoxide Dismutase; GST: Glutathione S-transferase; FITC: Fluorescein Isothiocyanate; IgG: Immunoglobulin G; DAB: Diaminebenzidyne; Dntp: Deoxyribonucleotide Triphosphates; MN: Micronucleous; FBS: Fetal Bovine Serum; PE: Polychromatic Erythrocytes; MNPCEs: Micronucleated Polychromatic Erythrocytes; SEM: Standard Error of Mean; SDM: Standard Deviation of Mean; RT-PCR: Reverse Transcriptase Polymerase Chain Reaction; HMP: Herbal Medicinal Products; MTP: Mitochondrial Permeability Transition Pore; UTP: Uridine Triphosphate; ATP: Adenosine Triphosphate

Highlights

Orchid leaf extract caused swelling of isolated rat hepatic mitochondria.

Mutagenic activity in erythrocytes was observed.

Hepatic hyperemia, cytoplasmic vacuolization, pseudo-eosinophilia and cell death.

The effects could be attributed to flavonoids and tannins found in the crude extract.

Introduction

Plant extracts and their isolated components can be used for the fabrication of therapeutic products with various purposes [1]. About 80% of the world population uses plants for therapeutic purposes. The herbal product industry has shown an annual growth of 25% in revenue. In addition, the costs of the development of a phytotherapeutic agent are usually lower than those of a synthetic drug [2,3].

Orchids are very versatile plants that are able to adapt to a wide variety of environments. In addition, these plants possess mechanisms for the production, transformation and accumulation of numerous substances, advantageous for their survival and guaranteeing the perpetuation of the species. These natural compounds are responsible for most of the therapeutic activities of orchids, but little is known about the beneficial properties of these plants [4]. A review by Hossain [5] shows the traditional therapeutic uses of orchids with its recent advances in pharmacological investigations that would be a useful reference for plant drug researches, especially in this plant group.

The species *Oncidium flexuosum* Sims., popularly known as the dancing doll orchid, is one of the most important species of the *Oncidium* genus in Brazil that have been used for therapeutic methods [4]. Hydroalcoholic *O. flexuosum* leaf extract was phytochemically analysed using chemical methods showed a predominance of phenolic compounds, such as flavonoids and tannins, and the presence of triterpenes. However, no total alkaloids or saponins were detected [4]. Flavonoids are compounds able to decrease the permeability of capillaries and to increase their resistance. These substances have also

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been reported to reduce dehydroascorbic acid, increasing the use of vitamin C. Additional properties of flavonoids include a scavenger effect by capturing inflammation-derived free radicals An anti-inflammatory activity exerted by inhibiting arachidonic acid peroxidation and as well as the antiallergic, antiviral, hormonal, antimicrobial, and anticancer properties that have been attributed to flavonoids. They have also been accredited an antihyaluronidase and antielastase activity, reducing vascular permeability, an indirect inhibition of platelet aggregation and adhesion, and the activation of enzymes such as cyclooxygenases and lipoxygenases. In addition, flavonoids enhance and improve the quality of collagen fibers [4].

Phenols are not considered to be toxic at normal quantities and under normal conditions, except for polymeric phenols, called tannins, which are able to bind and precipitate proteins from aqueous solutions. In general, the production of high levels of phenols by the plant is associated with its healing potential and the treatment of different diseases such as diarrhea, arterial hypertension, rheumatism, hemorrhage, wounds, burns, intestinal and kidney problems, and urinary tract problems. The ability of phenols to precipitate proteins is responsible for their antimicrobial and antifungal activity [6].

Deleterious effects of a number of chemical and biological toxic compounds on the liver have been investigated looking for their role in genotoxic and oxidative stress [7]. Therefore, the objective of the present study was to investigate the effects of chronic treatment with three different concentrations of a hydroalcoholic extract of *O. flexuosum* Sims. The test was carried out using Wistar rats to evaluate hepatic and renal function, the occurrence of hepatic oxidative damage, histopathological alterations of the kidneys and liver, and liver mitochondrial swelling. In addition, the ability of the extracts to induce micronuclei in bone marrow erythrocytes and cell death in hepatocytes was investigated.

Materials and Methods

Animals

Twenty-five adult male Wistar rats (2 months old) were maintained throughout the experiment at the Center of Animal Experimentation, Centro Universitário Hermínio Ometto (UNIARARAS), on a 12-h light/dark cycle at a temperature of 25°C and air humidity of 60%. The animals received water and Purina ration *ad libitum* and they were randomly divided into five groups: a negative control group (CG) (n=5) receiving only filtered water; a positive control group (PCG) (n=5) receiving water and, on the day of euthanasia, a single dose of methyl methanesulfonate (MMS); and three experimental groups (n=5) treated with the hydroalcoholic extract of *Oncidium flexuosum* Sims. (G100, G250, G500). The study was approved by the Ethics Committee of Centro Universitário Hermínio Ometto, UNIARARAS (protocol 750/2008), and was conducted in accordance with the ethical guidelines of the Brazilian College of Animal Experimentation (COBEA).

Doses of the crude extract used and route of administration

The animals were weighed and received by gavage, for a period of 15 days, predetermined volumes of crude extract from leaves of *O. flexuosum* Sims. The extract was prepared and characterized by Gaspi et al. [4]. Fresh leaves (50 g) were selected, cleaned, and strongly macerated with 300 mL of an aqueous ethanol solution (7:3, v/v) for 4 h at room temperature. This procedure was repeated 3 times with the same powder and the same solvent. After filtration, the solvent was completely evaporated under vacuum at 40°C in a rotary evaporator, and the hydroalcoholic extract was obtained after lyophilization. The yield of the lyophilized extract was 9% [4]. The qualitative identification of the chemical constituents was carried using chemical methods and thin-layer chromatography, and showed a predominance of phenolic compounds, such as flavonoids and tannins, and the presence of triterpenes [4]. Doses of 100, 250 and 500 mg/kg/day were chosen according previous studies of our research group. In addition, the positive control group received 20mg/kg MMS (Sigma, St. Louis, MO, USA) 24 h before euthanasia [8].

Evaluation of renal tubular function by lithium clearance

Fourteen hours before sacrifice, the animals received lithium chloride (Merck, Darmstadt, Germany), 60 µmol/100g body weight, by gavage. The animals were maintained in collective cages, not eating any solid food and ingesting water *ad libitum*. In addition, the animals received by gavage a water overload (5% of body weight) to obtain regular and stable urinary flow during urine collection. One hour after the first overload, a second water overload was applied. After 30 min, the animals were transferred to a metabolic cage and urine was eliminated spontaneously and collected over a period of 2 h in a graded centrifuge tube. The urine sample volumes were calculated and creatinine concentrations were determined in a spectrophotometer (Micronal, 362, São Paulo, Brazil) by the alkaline picrate method at the University Hospital of Universidade Estadual de Campinas (UNICAMP) [9].

Collection of material for analysis

After euthanasia, blood was collected by cardiac puncture of all animals, except for the positive control group. Fresh liver samples were collected for RNA extraction and biochemical analysis, and liver fragments were fixed in buffered 10% formalin. Bone marrow samples were obtained for the micronucleus test and the right kidney was fixed in buffered 10% formalin. Five animals served as positive control (receiving MMS) for the micronucleus test.

The animals were anesthetized with xylazine (20 mg/ml; Rompun®, Bayer S.A., São Paulo, Brazil) and ketamine (50 mg/ml; Ketalar®, Parke-Davis & Co., Wellington, New Zealand).

Evaluation of hepatic oxidative damage

Catalase activity was assayed according to the method of Aebi [10] and expressed as absorbance units/mg protein.

The serum activity of alanine aminotransferase (ALT) was assayed using the Synermed ALT enzymatic kit (Synermed International, Inc., Westfield, IN, USA) according to manufacturer's instructions and absorbance was read at 532 nm.

The levels of reduced groups of total protein (-SH) were measured using liver homogenate in 0.1 M phosphate buffer, pH 7.4, as described by Ellman [11]. Absorbance was measured immediately and after 15 min at 412 nm. The concentration of reduced sulfhydryl groups (-SH) was calculated using the equation (Abs_{final} – $Abs_{initial}$) x 1.57, and is reported as mM [12].

Evaluation of damage to liver mitochondria

Liver fragments from all groups were washed in medium containing 250 mM sucrose, 10 mM HEPES and 1 mM EGTA, pH 7.2-7.3, and homogenized in a Potter-Elvehjem tissue grinder. The homogenate was centrifuged, and the supernatant was removed and again centrifuged. The precipitate was resuspended in medium containing 250 mM sucrose, 10 mM HEPES and 0.3 mM EGTA, pH 7.2-7.3, and again centrifuged. The mitochondrial fraction was resuspended in medium containing 125 mM sucrose and 10 mM HEPES-KOH, pH 7.2-7.3. All procedures were performed at 4°C [13]. Next, 50 µl of the sample was

added to 3 ml biuret reagent. After 10 min, three readings were obtained at 540 nm and the mean was used to calculate protein concentration in the sample using the following equation: absorbance – 0.0026/0.0073 [14]. The pellet containing the mitochondrial fraction (0.5 mg protein) was added to 3 ml medium containing 125 sucrose and 1 mM HEPES, pH 7.2-7.3, and absorbance was monitored kinetically at 540nm over a period of 15 min. After one minute, 40 µl $CaCl_2$ and 40 µl KH_2PO_4 were added and absorbance was read at 540 nm, with mitochondrial swelling being proportional to the decrease in absorbance [13].

Histological preparation

Kidney and liver fragments were dehydrated in an increasing ethanol series and cleared for embedding in liquid paraffin. Histological liver sections of 5 to 7 µm were obtained and stained with hematoxylin-eosin, periodic acid Schiff (PAS), and bromophenol blue to identify glycogen and total protein in the hepatocytes. Kidney sections were submitted to hematoxylin-eosin and PAS. After drying, the sections were mounted on slides with Permount resin.

Immunohistochemistry for the detection of superoxide dismutase (SOD) and glutathione S-transferase (GST)

Sections cut from the same block were processed for indirect immunofluorescence. Liver sections, in which the primary antibody was omitted, were used as the negative control and sections to which human liver membrane lysate (normal fetal tissue ab29880) was added served as the positive control (PC), in order to prove the efficiency of the reaction. All the sections were mounted on silanated slides and antigen retrieval was performed by boiling the slides in citrate buffer at 95°C for 1 h. The sections were incubated with the primary antibody (anti-SOD-1 or anti-GST-1 monoclonal rabbit antibody, EP1727y or EP1938y, respectively; Abcam, Cambridge, MA, USA) (1:250) overnight at 4°C. After washing in PBS, the sections were incubated with the secondary antibody (FITC-conjugated goat anti-rabbit IgG polyclonal antibody; Abcam 6717) (1:250) for 2 h at room temperature in a dark chamber [15]. After successive washes in PBS, the slides were mounted in Permount and fluorescence was detected using an I3 filter at an excitation wavelength of 450 to 490 nm (Leica DM2000).

Evaluation of cell death by the TUNEL method

Paraffin-embedded liver sections were submitted to immunodetection of DNA fragmentation indicative of cell death using the ApopTag® Plus kit (Chemicon Int., Temecula, CA, USA) and *In Situ* Cell Death Detection kit (ISCDDK, Roche, São Paulo, Brazil) according to manufacturer instructions. Both kits use anti-fluorescein peroxidase-conjugated antibody and DAB for development of the reaction. As a positive control, DNAse I was applied before the TUNEL reaction. The negative control (reaction control) consisted of omission of the TUNEL reaction enzyme.

RNA preparation and semiquantitative reverse transcriptase PCR analyses

Total RNA was isolated from approximately 100mg rat liver with the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and digested with DNAse I, amplification grade (Invitrogen). RNA purity and concentration were determined spectrophotometrically. cDNA was synthesized from 2 µg RNA in the presence of dithiothreitol, dNTP, random primers, RNAseOUT, and SuperScript™ II Reverse Transcriptase (Invitrogen) in a final volume of 20 µl. Semiquantitative RT-PCR was used to amplify GSTM1 and Mn-SOD mRNA, and to compare their expression between CE-treated and untreated.

PCR was performed in a final volume of 25 µl containing 1 µl cDNA; 200 µM of each dNTP, 0.2 pmol of each primer (Mn-SOD and β-actin) or 0.4 pmol of each primer (GSTM1); 2 mM $MgCl_2$ (GSTM1), 3 mM $MgCl_2$ (Mn-SOD) or 1.6 mM $MgCl_2$ (β-actin), and 0.04 U *Taq* DNA polymerase (Biotools, Madrid, Spain). The amplification conditions were initial denaturation at 94°C for 3 min for all genes studied, followed by 28 cycles for the β-actin gene, GSTM1, and Mn-SOD. The cycles consisted of denaturation at 94°C for 30 s (β-actin), 1 min (GSTM1 and Mn-SOD); annealing at 57°C for 30 s (β-actin), at 55°C for 1 min (Mn-SOD) or at 60°C for 1 min (GSTM1), and extension at 72°C for 30 s (β-actin) or 1 min (GSTM1 and Mn-SOD).

Primers for amplifications were made by MWG-Biotech AG, and the sequences were as follows: *β-actin*, 5'-AGAGGGAAATCGTGCGTGACA-3' (forward) and 5'-CGATAGTGATGACCTGACCGTCA-3' (reverse); *GSTM1*, 5'- CCAAATTGAGAATTCCACAGCGC-3' (forward) and 5'-TGCCTGCAGGATCCAATGTGGA-3' (reverse); *Mn-SOD*, 5'-GCACATTAACGCGCAGATCA-3' (forward) and 5'-AGCCTCCAGCAACTCTCCTT-3' (reverse).

The amplified products were separated on 1.5% agarose gel stained with ethidium bromide. The gel was photographed using the Kodak Electrophoresis Documentation and Analysis System. The signal intensities of the bands were measured densitometrically using the Scion Image analysis software (Scion Corp., Frederick, MD, USA). Each value was determined as the mean of three densitometric readings. The results are expressed as average ratios of the relative optical densities of GSTM1 and Mn-SOD PCR products in relation to β-actin.

Mutagenicity and micronucleus test

Genotoxic potential is a primary risk factor for long-term effects, such as carcinogenic and reproductive toxicology and degenerative diseases. Several studies employing the micronucleus (MN) test in peripheral lymphocytes or in exfoliated buccal mucosa cells are available in the last decades [16]. For this purpose, the femur was removed from the animals and the epiphysis was cut and medullary canal was washed with fetal bovine serum (FBS) (Cultilab, Campinas, Brazil). The bone marrow sample was centrifuged, the supernatant was discarded and the pellet was homogenized. One drop of the cell suspension was placed on a slide and a smear was prepared. The slides were stained with Wright and Giemsa's solution. A total of 2000 polychromatic erythrocytes (PE) were analyzed per animal for determination of the number of micronuclei, and micronuclei were identified as dark blue staining bodies in the cytoplasm of PEs. The slides were coded and analyzed blindly under a microscope equipped with an immersion objective. The results are reported as the frequency of micronucleated polychromatic erythrocytes (MNPCEs) among 2000 polychromatic erythrocytes per animal. The substance tested is defined as mutagenic when it produces a significant increase ($P<0.05$) in the frequency of MNPCEs in relation to the negative control [17].

Statistical analysis

The results of enzyme quantification, mitochondrial swelling, micronucleus test, and creatinine levels were analyzed by ANOVA using the Prism 3.0 program. The control and treated groups were compared by the Tukey test. The results of mRNA expression are reported as the mean ± SEM and were compared by the Student *t*-test. In all cases, a P value < 0.05 was considered to be statistically significant.

Results

Animals treated with crude extract of *O. flexuosum* Sims. presented no weight loss during the treatment period. Analysis of the biochemical parameters showed no significant alterations in ALT levels in the

treated groups, with a discrete increase in G250 and a small reduction in G100 and G500 being observed (Figure 1A). -SH levels were found to be higher in all groups, especially the G500 group, but the difference was not significant (Figure 1C). MDA levels did not present significant alterations in the treated groups but there was a discrete increase in G100 and G500 and a decrease in G250 when compared with the control animals (Figure 1D). Catalase activity did not show a significant variation in treated animals even though it decreased somewhat in the G250 and G500 groups (Figure 1B). Evaluation of mitochondrial swelling induced by calcium and phosphate showed a significant increase in the variation of absorbance between the extract treated groups and the control group ($P<0.05$ for G100 and $P<0.01$ for G250 and G500) (Figure 1E).

Histological analysis of liver sections showed the presence of hyperemia and hepatocyte alterations such as cytoplasmic vacuolization, pseudo-eosinophilia and nuclear pyknosis in G500 (Figure 2A-2C). PAS and bromophenol blue histochemistry revealed a higher staining for glycogen (Figure 2F) and total proteins (Figure 3A-3D) in hepatocytes of the centrolobular vein region. An increase in the frequency of nuclei positive to the TUNEL method was observed in the liver of animals of G500 (Figure 3F) when compared to the negative control group (Figure 3E), with similar results being obtained with both cell death detection kits.

RT-PCR for the quantification of Mn-SOD and GST-M1 mRNA showed no significant difference in the expression levels of these genes relative to that of β-actin, except for reduced SOD transcripts in G500 (Figure 4A and 4B). These results agree with the immunohistochemical analysis using antibodies against SOD-1 and GST-1, which showed no observable differences in immunostaining between treated and control animals, although an increase of GST levels was detected by the two techniques in G500 (Figure 4C and 4D).

No significant differences in creatinine levels were observed between the treated and control groups (CG=5.117±1.226; G100=5.602±1.181; G250=4.122± 0.563; G500= 3.6875±0.783 - mg/dl) and no renal histopathological alteration of the treated animals were observed. Analysis of mutagenicity by the micronucleus test showed a high frequency of MNPCEs in animals treated with MMS (positive control) and a low frequency in those treated with water (negative control). The extract induced a significant increase ($P<0.01$) in the

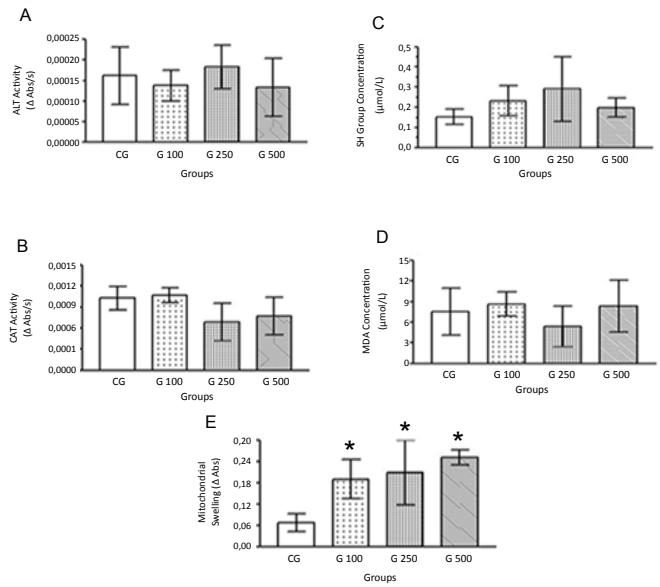

Figure 1: Effect of *in vivo* administration of crude extract of *Oncidium flexuosum* Sims. on the levels of alanine aminotransferase (A) and –SH groups (B), catalase activity (C), lipid peroxidation (D), and mitochondrial swelling (E) in rat livers. Values are expressed as the mean ± SD (n = 5). *Significantly different from the control group (P < 0.05).

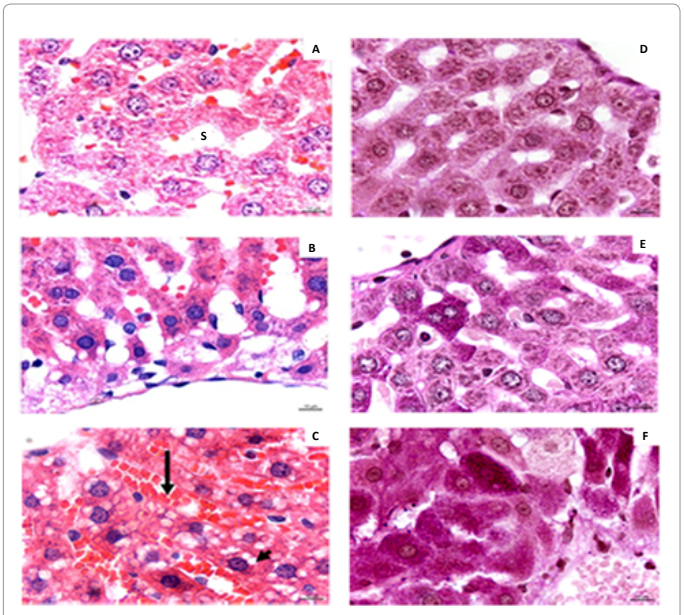

Figure 2: Histological sections of male Wistar rat livers after treatment with Oncidium flexuosum Sims. crude extract. A, B, C) Sections stained with hematoxylin-eosin and D, E, F sections submitted to periodic acid Schiff. A, D) Control group; B, E) group G250; C,F) group G500. Note in B and C, hepatocyte hypertrophy and cytoplasmic vacuolization, and in C, hyperemia (arrow) and nuclear pyknosis. (s) sinusoids.

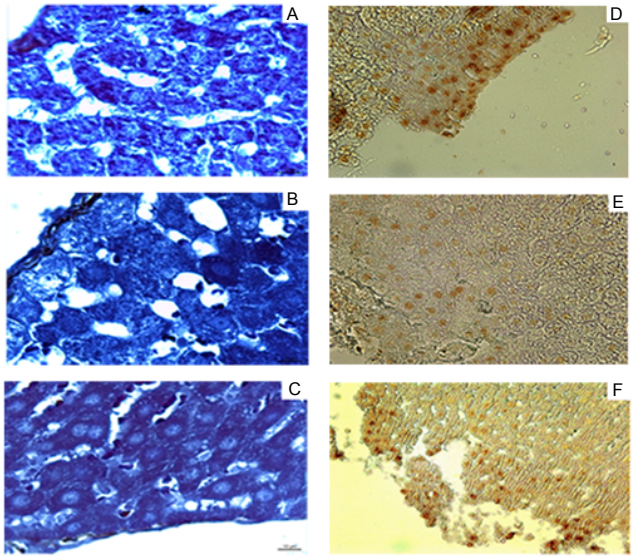

Figure 3: Histological sections of male Wistar rat livers after treatment with Oncidium flexuosum Sims. crude extract. A, B, C) sections stained with bromophenol blue and D, E, F) sections submitted to the TUNEL method. A, E) Control group; B) group G250; C, F) group G500; D) positive control group. TUNEL positive nuclei (brown) was higher in treated animals (F) compared to the negative control (E). Magnification 200X.

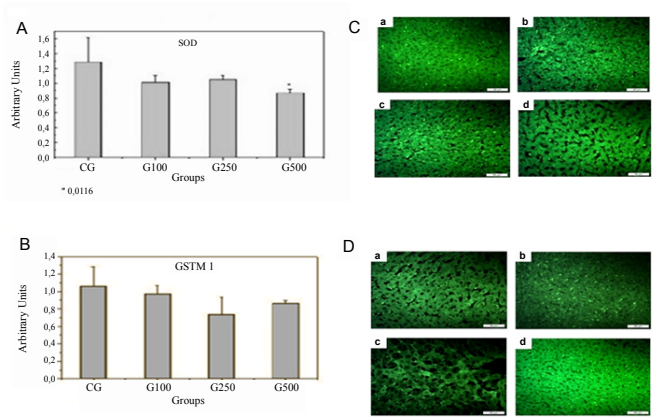

Figure 4: Analysis of Mn-superoxide dismutase (Mn-SOD), glutathione S-transferase (GSTM1) and β-actin mRNA expression by semiquantitative reverse transcriptase-PCR and immunolocalization of anti-SOD1 and anti-GST1 antibody in hepatocytes. Densitometric analysis of liver Mn-SOD (A) and GSTM1 (B) mRNA expression in control and treated animals. Changes in mRNA are expressed as normalized densitometric units relative to β-actin mRNA. Values are the mean ± SEM. $P < 0.05$ indicates statistical significance. Immunohistochemistry for the detection of SOD-1 (C- a CG, b G100, c G250, d G500) and GST-1 (D -a G100, b G250, c G500, d PC).

Chemical	N	MNPCEs/ 10000 PCEs	Mean ± S.D./ Groups
CG	5	0	0.0 ± 0.0
PCG	5	61	48.2 ± 14.114
G100	5	4	0.8 ± 1.3
G250	5	17	3.4 ± 0.55*
G500	5	30	6.0 ± 1.41*

Mean ± Standart desviation; MNPCEs: micronucleated polychromatic erythrocytes; MMS: methyl methanesulfonate; PCEs: polychromatic erythrocytes. (*=0,0000)

Table 1: Effect of the treatment with 100, 250 and 50 mg/kg/day of *Oncidium flexuosum* Sims. crude extract during 15 consecutive days showing the number of micronucleated polychromatic erythrocytes in bone marrow of male rats.

frequency of MNPCEs in the G250 and G500 groups when compared to the negative control (Table 1).

Discussion

At the concentrations of *Oncidium flexuosum* Sims. extract tested, the present study showed hepatic, but not renal toxicity, probably because the extract is metabolized in the liver, thus preventing damage to the kidneys.

Three significant alterations were observed in the treated groups: I) hepatic mitochondrial swelling, II) DNA damage, and III) histopathological changes such as cell death. These data demonstrated that the hydroalcoholic extract was capable of inducing cellular alterations that culminate in cell death and so its intake can be dangerous. So, our study has a great importance because according Jordan et al. [18] although herbal medicinal products (HMP) have been perceived by the public as relatively low risk, there has been more recognition of the potential risks associated with this type of product as the use of HMPs increases.

The significant mitochondrial swelling seen in the liver of rats treated with the three extract concentrations suggests that the extract causes alterations in the hepatic mitochondrial membrane. Probably, the mitochondrial membrane damage resulted in a swelling that is a pathological reaction frequently triggered by the opening of a high-conductance permeability transition pore (MTP) in the inner mitochondrial membrane. This is an efficient method to investigate membrane damage [19]. Investigative methods used in our study do not allow us conclude that the mitochondrial swelling was induced by the opening of MTP and it would be useful to employ other techniques in future studies to monitor changes of mitochondrial Ca^{2+}, $\Delta\Psi m$ and onset of the MTP [20].

Oxidative stress has been pointed out as a major event responsible for mitochondrial swelling [20]. However in the present study, no significant differences in -SH quantification, MDA levels, CAT activity, or cellular expression and quantification of SOD and GST transcripts in treated groups were observed when compared with the control group. These data suggest that no activation of an antioxidant system and transcription of stress associated genes occurred. This observation can be explained by fact that flavonoids have a scavenger effect [4] which could protect cells from oxidative stress. Furthermore, it has been shown that changes in hepatocyte mitochondrial membrane permeability is related to cell death and our results confirmed this event using the TUNEL method [20].

Most often, exposure to low doses of toxicants can result in autophagy or apoptosis, whereas higher levels of the same toxicant might cause necrosis. Studies with arsenic trioxide, for example, revealed that low doses of this agent triggered Bax/Bak-dependent apoptosis, whereas higher doses caused mitochondrial permeability transition (MPT) and apoptotic/necrotic cell death. The latter observation illustrates the frequent *in vivo* findings that distinct cell death modalities may coexist within the same lesion and that interference with certain signaling pathways or critical cell functions might result in cell death by a different mechanism [21]. Liver sections of the treated groups showed the presence of hyperemia, cytoplasmic vacuolization, pseudo-eosinophilia and nuclear pyknosis that characterize a cell death process. However, further studies are needed to elucidate the mechanism of cell death induced by the extract studied here. So far, could be employed the VirtualToxLab method that according Vedani et al. [22] is an in silico technology for estimating the toxic potential of this natural product.

The extract also induced DNA damage as demonstrated by an increase in the frequency of MNPCEs in bone marrow. Errors in mitotic chromosome segregation generate DNA breaks, via the formation of structures called micronuclei [23,24]. Thus, we suggest that some compound present in the extract has the ability to interact with DNA promoting breaks that induce signaling mechanisms, such a Ca^{+2} influx, causing hepatocyte mitochondrial damage that culminate in cell death. All these suppositions could be tested with isolated mitochondria from treated rat liver.

Relationship between DNA breaks and mitochondrial permeability alterations was also observed [25] as side effects of neosergeolide, a quassinoid isolated from *Picrolemma sprucei* Hook. f. (Simaroubaceae). It is a secondary metabolite with important biological properties, including cytotoxic and antitumor properties. A newly identified ginsenoside from *Panax notoginseng* (ginseng) was active against a variety of cancer cells *in vitro* and *in vivo* by induction of apoptosis and G1 phase arrest and inhibited the growth of breast cancer by inhibiting MDM2 (human homologue of the mouse double minute 2) oncogene expression [26].

In a review of the mutagenic activity of medicinal plants, Varanda et al. [27] showed that several of these plants have genotoxic activity. One example is *Strychnos pseudoquina* St. Hil. ("quina do cerrado"), a native plant of the Brazilian savannah used for the treatment of liver and intestinal diseases, which is rich in alkaloids and flavonoids and was able to induce micronuclei formation. The extract of *Byrsonima crassa* ("murici"), a plant used for the treatment of ulcers, was found to be mutagenic in mice and this effect was attributed to the presence

of amentoflavone. *Myrciaria tenella* Berg. (Cambui), *Smilax campestris* (Japecanga), *Tripodanthus acutifolius* (Erva-de-Passarinho), and *Cassia corymbosa* Benth. (Senna) have also been shown to have mutagenic activity [27], probably due to the presence of flavonoids, tannins and anthraquinones in their extracts.

As mentioned earlier, Gaspi et al. [4] phytochemically characterized the *O. flexuosum* Sims. extract and identified total flavonoids and tannins as the main secondary compounds. This composition, which is basically beneficial, depending on the dose and duration of treatment, may cause cell damage, leading to cell death and other deleterious effects. However, there are some evidences that these compounds can have therapeutic applications. Valdameri et al. [28] suggested that flavone (2-phenyl-4H-1-benzopyran-4one) could induce changes in mitochondrial membrane properties. This compound is found in some cereal grains and generates several biological activities, including: apoptosis induction, cell cycle arrest, caspase activation and inhibition of tumor cell proliferation. Similarly, a recent review showed that honey has been considered a "natural cancer vaccine" because it is rich in flavonoids, which presents anticancer properties. The mechanisms suggested are rather diverse such as various signaling pathways, including stimulation of TNF-alpha (tumor necrosis factor-alpha) release, inhibition of cell proliferation, induction of apoptosis, and cell cycle arrest [29].

Williams et al. [30] demonstrated that fifteen stilbenoids, including a new phenanthraquinone and two new dihydrostilbenes, were identified in a commercial *Oncidium* sp. This compound inhibited proliferation of NCI-H460 and M14 cancer cell lines, probably inducing these cells to apoptosis. Susanti et al. [31] for example, demonstrated a presence of arctigenin, a natural plant lignin, in the extract of *Arctium lappa* L. and showed its cytotoxicity attained by inhibiting proliferation of liver cancer cells, leading to the induction of apoptosis. Future *in vitro* analysis for viability and cell proliferation using different doses of *O. flexuosum* Sims. hydroalchoolic extract might demonstrate this potential.

In addition, the treatment with 500mg/kg/day of the extract resulted in increased accumulation of proteins as demonstrated by histochemical staining with bromophenol blue, as well as inducing an accumulation of glycogen in the centrolobular region. These observations demonstrated a cell response in terms of the secretion and accumulation of biomolecules induced by the extract studied. Higher protein quantity in the cytosol could be caused by the ability of phenols (tannins) to precipitate proteins [6].

The accumulation of glycogen seen in the treated animals suggests impaired glycogenolysis, i.e., the occurrence of cell damage impairing the release of glucose into the blood. The liver is the main site of glycogen to contribute to the regulation of blood glucose. Glycogen synthesis, which is located in the cytosol, depends on the UTP supply and hence on ATP supply resulting from both mitochondrial oxidative phosphorylation and cytosolic glycolysis [32]. Mitochondrial damage could cause imbalance between glycolytic ATP generation and ATP loss, inducing cell death after an injury [20].

Our results make it evident that the extract studied can interfere with liver cell function, causing DNA and mitochondrial damage and cell death, which raises many questions about what mechanisms are involved in this events and that future analysis will be important to elucidate these questions.

Conclusion

The present results suggest that flavonoids and tannins found in the crude extract of *O. flexuosum* Sims. leaves, are responsible for the mutagenic potential of the extract and its ability to induce hepatic mitochondrial damage and to trigger cell death mechanisms in rat hepatocytes, demonstrating its toxicity when doses of 250 mg/kg/day or higher are ingested for 15 days. On the other hand, these findings highlight the importance of *in vitro* studies using transformed cells to achieve a better understanding of these effects.

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Authors' contributions

GDCSA, ECMSZ, CAO and AAP were responsible for designing and coordinating the study as well as for data interpretation and writing of the manuscript. MPL, CSO, DKZ, JAO, RB and GDCSA performed the experiments. GLGP were involved on creatinine concentrations analysis. FOGG and JEC prepared and provided the crude extract. All authors read and approved the final manuscript.

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