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Effect of Hecogenin on DNA instability

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ABSTRACT

Hecogenin is a sapogenin found in Agave species in high quantities and is responsible for the many therapeutic effects of these medicinal plants. In addition, this compound is also widely used in the pharmaceutical industry as a precursor for the synthesis of steroidal hormones and anti-inflammatory drugs. Despite Hecogenin being widely used, little is known about its toxicological properties. Therefore, the present study aimed to investigate the cytotoxic, genotoxic and mutagenic effects of Hecogenin on HepG2 cells. Cytotoxicity was analyzed using the MTT test. Then, genotoxic and mutagenic potentials were assessed by comet assay and cytokinesis-block micronucleus assay, respectively. Cytotoxic effect was observed only when cells were exposed to concentrations of Hecogenin equal or higher than 100 μM. Although a lower concentration of Hecogenin caused DNA damage, a reduction on nuclear mutagenic markers in HepG2 cells was observed. The results indicated that Hecogenin treatment generated DNA damage, but in fact it would be repaired, avoiding dissemination of the damage throughout the cell division. Further studies need to be performed to confirm the observed protective effect of Hecogenin against genomic instability.

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1. Introduction

Sapogenins are the non-polar residue of amphipathic glycosides named Saponins [1]. The therapeutic properties of different classes of saponins such as analgesic, anti-inflammatory and anti-tumoral effects have already been demonstrated [2]. Furthermore, sapogenins are of great interest for the pharmaceutical industry as a source for the development of new drugs [3].

Hecogenin is a sapogenin found in *Agave sisalana* species (commonly known as "sisal"), which are extensively spread throughout tropical and subtropical regions [4]. Brazil is one of the largest producers of sisal, representing 69% of worldwide production [5]. Plants of the *Agave* genus are used as therapeutic agents by Chinese Traditional Medicine in scabiosis, in reducing pain, treating different inflammatory conditions and even against cancer [6].

Hecogenin has been indicated as the responsible agent for sisal therapeutic effect due to its beneficial properties involving anti-inflammatory, antioxidant, antifungal, hypotensive, anti-hyperalgesic and anti-nociceptive effects [7–9]. Moreover, Hecogenin is used in the pharmaceutical industry as a precursor of steroidal anti-inflammatory and steroidal hormone drugs [10]. Despite Hecogenin being widely used and its beneficial effects, information about its toxicity is still very scarce. Currently, the information available about Hecogenin toxicological effects has been tested using a high concentration of Hecogenin-rich extracts on animal models [11]. Unlike animal models, in vitro cell-based models provide mechanistic understanding and present some advantages such as simplicity, low cost, and reproducibility. They are commonly used in basic science for pharmaceutical research along with toxicological investigation [12].

In the literature, studies aimed to investigate Hecogenin mechanisms of cytotoxicity have been reported using different cell lines such as A549 human lung cancer cell, human rheumatoid arthritis synovial cell and 1547 osteosarcoma cell lines [13–15]. These cell lines are interesting models to investigate cytotoxic and anti-

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tumoral properties of a diversity of substances, but since Hecogenin is used as a medicinal molecule, it is important to take into account toxicology in environments that allow metabolization, simulating what happens in live organisms [16]. For instance, the Human hepatoma cell line (HepG2) that contains endogenous metabolizing enzymes is an in vitro model that helps to assess xenobiotics that need a previous bioactivation [17]. Until now, there have been no reports of Hecogenin toxicity using this cell line. In addition, genetic toxicology helps understand the deleterious effect in a subclinical stage caused by exposure to different toxic substances [18]. Using genetic biomarkers on this cell model would broaden the scope of the toxicological assessment. Therefore, this work aimed to assess cytotoxic, genotoxic and mutagenic potentials of Hecogenin on HepG2 cells.

2. Materials and methods

2.1. Reagents and compounds

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), cytochalasin B, cyclophosphamide, Giemsa dye, silver nitrate and SYBR Green were purchased from Sigma-Aldrich (St. Louis, MO, EUA). Normal-melting point (NMP) agarose, low-melting point (LMP) agarose, Dulbecco's modified Eagle's medium (DMEM), streptomycin/penicillin, fetal bovine serum and trypsin were acquired from Life Technologies (Carlsbad, CA, EUA).

2.2. Hecogenin extraction from A. sisalana

Hecogenin was kindly provided by Prof. Dr. José Maria Barbosa-Filho, from the Pharmaceutical Sciences Department, UFPB. Protocol for the isolation, purification and identification performed for obtaining Hecogenin from *A. sisalana* is detailed in Cerqueira et al. [7]. First, 5 kg of *A. sisalana* leaves were extracted with ethanol using a soxhlet dispositive for 24 h. Afterward, solvent was removed under reduced pressure and the residue was hydrolyzed by refluxing with ethanolic hydrochloric acid for 4 h. After cooling and filtering, the acid-insoluble residue was extracted with hexane in a soxhlet apparatus for 12 h and the extract was recrystallized with acetone and analyzed by MNR for identification. The Hecogenin was extracted by acetylation with an acetic anhydride/pyridine mixture, then the isolated Hecogenin was analyzed by HPLC and the purity reached 98%.

Hecogenin dry powder was suspended in acetone before use. The final acetone concentration in culture was less than 1%, as indicated by Burgess et al. [19].

2.3. HepG2 cell culture

Human hepatoma cell lines (HepG2) were purchased from an American Culture Collection (ATCC, Rockville, MD). First, cells were cultivated in DMEM medium, supplemented with 10% fetal bovine serum, 1% streptomycin/penicillin and 2% l-glutamine at 37 °C and 5% CO_2.

2.4. Cell viability assessment (MTT test)

The MTT assay was performed to determine cell viability through the energetic cell metabolism. First, the MTT salt is reduced by succinic dehydrogenase in mitochondria to formazan, an insoluble violet crystal [20]. The MTT assay was performed following the protocol previously described by Mosmann [21] with minor modifications. Then, 10^4 cells per well were seeded in 96-well flat bottom culture plates and incubated overnight at 37 °C and 5% CO_2. After incubation, cells were exposed to concentrations of Hecogenin between 0 μM (negative control: medium + 1% vehicle), 10 μM, 50 μM, 100 μM, 150 μM and 200 μM for 24 h. MTT was added at a final concentration of 1 mg/mL and incubated for 4 h. Then, the medium was removed and 100% ethanol was added to dissolve the formed formazan crystals. Absorbance was determined at 570 nm in a spectrophotometer. The experiment was performed three times independently.

2.5. Genotoxic assessment (Comet assay)

Comet assay was performed to investigate the genotoxic effect of Hecogenin. This assay is based on the fact that damaged DNA loses its association with nuclear proteins while undamaged DNA does not. The resulting DNA fragments as consequence of the genetic damage can be observed microscopically after electrophoresis as "comets", and the degree of DNA damage is estimated considering the tail size, the integrity of the nucleoid and the relationship of both [22].

Comet assay was performed as previously described [22] with minor modifications. First, cells were seeded in 6-well plates and treated with four different concentrations of hecogenin, 0 μM (negative control: medium + 1% vehicle), 10 μM, 25 μM and 50 μM, and 30 μM of H_2O_2 (positive control) for 24 h. Cell suspension was mixed with 1% (w/v) low-melting agarose and loaded onto slides pre-coated with 1.5% (w/v) normal melting agarose. After agarose solidification, the slides were submerged in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl with 10% DMSO and 1% Triton X – 100 freshly added; pH 10.0; 3 h). Then, the nucleoids were submerged in electrophoresis buffer (10 M NaOH and 200 mM EDTA; pH 13.0, 4 °C) for 20 min for DNA denaturation. Electrophoresis was conducted for 30 min at 30 V and 400 mA. Afterward, the slides were washed with neutralization buffer (0.4 M of Tris-HCl buffer; pH 7.5) and absolute ethanol. Slides were stained with 0.02% silver nitrate solution, according to Nadin et al. [23].

Fifty nucleoids per experiment were visually scored in an optical microscope (Olympus, Japan), totalizing 150 nucleoids per treatment, as described by Collins et al. [24]. Each comet was given an arbitrary unit of 0–4 (0–undamaged; 4–maximum damage). Damage score was thus assigned to each sample ranging from 0 (no damage: 50 cells x 0) to 200 (maximum damage: 50 cells x 4). Then, a mean was calculated for each treatment.

2.6. Mutagenicity assessment (CBMN assay)

DNA damage that overcame the repairing process can progress to chromosome abnormalities causing a mutagenic effect. Micronuclei (MN) characterized by lost genetic material from DNA double strand breaks acquiring the morphology of a small nucleus is one of resulting genetic consequence that can be seen in cells affected by mutagen substances and has been extensively used as a biomarker of mutagenicity. Moreover, other cytological characteristics help us understand the resulting genetic instability. For instance, amplified genes could be excluded from the nucleus, leaving a nucleoplasmic connection between it and the main nucleus, or could also be created from dicentric chromosomes that can form a continuous link between the nuclei in a binucleated cell. These clastogenic or aneugenic effects are characterized by the occurrence of nuclear buds (NBUDs), and nucleoplasmic bridges (NBRDs) [18,25].

The CBMN assay was performed according to the protocol described by Vasquez et al. [26], with minor modifications. First, HepG2 cells (5×10^5) were seeded in 6-well plates and allowed to attach overnight. Then, the cells were treated with 0 μM (negative control: medium + 1% vehicle), 10 μM, 25 μM and 50 μM of Hecogenin, and positive control (cyclophosphamide 0.2 mg/mL) for 24 h. Afterward, cytochalasin B was added (final concentration, 3.5 μg/mL) following 24 h of incubation. Cells were washed with hypotonic solution (0.075 M KCl, 4 °C, 3 min) and fixed with

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