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Cytotoxic and genotoxic effects of ethanolic extract of *Euphorbia hyssopifolia* L. on HepG2 cells

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ABSTRACT

Ethnopharmacological relevance: *Euphorbia hyssopifolia* L. is a weed with recognized antimicrobial potential employed in Indian, Asian and Latin–American popular medicine. However, little is known with regard to its toxic potential. The present study aimed to investigate the cytotoxic and genotoxic effects of ethanolic extract of *E. hyssopifolia* in HepG2 cell culture.
Materials and methods: Phytochemical screening of ethanolic extract was carried out to determine the presence of active secondary plant metabolites. Six concentrations (0.00001, 0.0001, 0.001, 0.01, 0.1 and 1.0 mg/mL) of ethanolic extract were tested by the MTT assay to verify cytotoxicity. Then, genotoxic evaluations (alkaline comet assay and cytokinesis-block micronucleus assay – CBMN) were carried out in HepG2 cells with extract concentrations of 0.01, 0.1 and 1.0 mg/mL.
Results: Mono and sesquiterpenes, triterpenes and steroids, and flavonoids were the main classes found in the phytochemical screening. Extract concentrations used in the MTT assay showed no cytotoxic activity. On the other hand, genotoxic activity was verified at 0.1 and 1.0 mg/mL in the alkaline comet assay. Additionally, the 1.0 mg/mL concentration induced severe cell damage leading to death in the CBMN assay, indicating a cytotoxic effect for this concentration in the latter method.
Conclusion: The use of *E. hyssopifolia* extract for medicinal purposes should be avoided, because concentrations above 0.01 mg/mL may pose risk to human health due to cytotoxic and/or genotoxic effects.

Abbreviations: HepG2, human hepatoma cell culture; MTT assay, colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay; CBMN assay, cytokinesis-block micronucleus assay; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; MMS, methyl methanesulfonate; LMP, low-melting-point agarose; MN, micronucleus; NB, nuclear bud; NPB, nucleoplasmatic Bridge

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1. Introduction

Euphorbia hyssopifolia L. (Euphorbiaceae), a weed with recognized antimicrobial potential, has been employed in Indian, Asian and Latin–American popular medicine (Kane et al., 2009; Yang et al., 2005). The plant is empirically used as tea (aerial part) for colds, indigestion and back pains, being sometimes used as a tonic (Abo, 1994). In scientific assays, leaf extracts showed cicatrizing and antiviral activity against HIV (water extract; above 6–8 mg/mL), and antibacterial activity against a wide spectrum of both gram-positive and gram-negative bacteria (ethanolic extract; above 0.05–2 mg/mL), with potential use in gonorrhea treatment (Matsuse et al., 1999; Alisi and Abanobi, 2012). However, there are no reports for its root usage in popular medicine.

On the other hand, leaf extracts of some *Euphorbia* species (*E. balsamifera*, *E. heterophylla* L., *E. hirta* L., *E. lateriflora* Schumach. & Thonn. and *E. helioscopia* L.) presented toxic potential, being able to reduce leukocyte and lymphocyte number and to cause the emergence of hepatic lesions in rats (Adedapo et al., 2004; Kheyrodin and Ghazvinian, 2012). For *E. hyssopifolia* leaf extract, for instance, doses above 100 mg/kg were hepatotoxic in rats (Igwenyi et al., 2011). However, despite their toxic potential, several *Euphorbia* species are used in popular medicine, but doses and respective effectiveness are not clear. Therefore, toxicity tests using several model organisms, such as cell culture and animals are necessary (Mwine and Van Damme, 2011). Thus, the present study aimed to investigate the cytotoxic and genotoxic effects of *E. hyssopifolia* ethanolic extract on human hepatoma cell culture (HepG2).

2. Material and methods

2.1. Plant material

Plants of *E. hyssopifolia* were collected at Federal University of Pernambuco (Recife – Pernambuco, Brazil). A voucher specimen of the plant has been deposited at the herbarium of Federal University of Pernambuco, under reference number UFPE1793.

2.2. Extract preparation

Aerial parts of the plants (270 g), including stem, leaves and inflorescences, were dried for seven days at 50 °C. The dried material (100 g) was mixed in 1 L of absolute ethanol and kept for a period of 24 h accompanying occasional shaking and stirring. The filtrate (ethanolic extract) obtained was evaporated using rotary evaporator (45 °C) until obtaining a semisolid product. For preparation of the initial concentration, 200 mg of the semisolid extract were mixed to 2 mL of ethanol and dissolved into distilled water adjusted to 200 mL.

2.3. Phytochemical screening of extract

The plant extracts were screened for the presence of alkaloids, monoterpenes and sesquiterpenes, triterpenes and steroids, saponnins, flavonoids, phenylpropane glycosides, cynnamic derivatives, hydrolysable tannins, condensed tannins and coumarins according to Randau et al. (2004).

2.4. Cell lines

Culture of HepG2 (human hepatoma cell line from the Cell Bank of Rio de Janeiro, Brazil) cells were cultivated in 25 cm^2 cell culture flasks containing MEM medium (EARLE, Cultilab), 10 mL of sodium pyruvate 100 mM (Gibco) and 1.0 mL of antibiotic–antimycotic solution (penicillin 10,000 IU/mL/streptomycin 10 mg/mL, Cultilab), supplemented with 10% fetal bovine serum (FBS; Gibco) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.5. MTT assay

The cytotoxic effects of plant extracts were assessed by the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, as described by Mosmann (1983) with some modifications. Cells were seeded in 96-well plates and incubated overnight at 37 °C in a humidified atmosphere containing 5% CO_2. A range of six concentrations of *E. hyssopifolia* extract (0.00001, 0.0001, 0.001, 0.01, 0.1 and 1.0 mg/mL) and cells were incubated for 4 h. Negative and positive control treatments were carried out with culture medium MEM and 1% Triton X-100, respectively. After exposure, the treatments were removed from the wells, to which MTT (Thiazolyl Blue Tetrazolium Bromide, CAS No. 298-93-1, Sigma) was then added. At the end of the incubation period, the MTT was discarded and 100 μL of DMSO (dimethyl sulfoxide) was added. The plate was read at a spectrophotometer, using a 540 nm filter.

2.6. Genotoxicity assays

The cells were exposed to concentrations 0.01, 0.1 and 1.0 mg/mL of *E. hyssopifolia* extract for 4 h. Negative and positive control treatments were carried out with culture medium MEM and methyl methanesulfonate (MMS, Sigma-Aldrich, CAS 66-27-3), respectively. After the exposure period, samples were used in the alkaline comet assay and micronucleus assay.

2.6.1. Alkaline comet assay

The alkaline comet assay was performed according to Singh et al. (1988) with some modifications. The slides were prepared by adding 20 μL of cell suspension with 120 μL of low-melting-point agarose (LMP) in pre-gelatinized slides, being kept at 4 °C for 20 min. The slides were exposed to the lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris and 8.0 g of NaOH, pH 10) at 4 °C for 1 h. Subsequently, the slides were immersed into a cold electrophoresis solution (300 mM NaOH and 1 mM EDTA solution, pH > 13) for 20 min, for DNA denaturation. Electrophoresis was carried out at 40 V and 300 mA for 20 min. The slides were stained with a 50 μL of GelRed® solution. The analysis of the slides was done immediately after staining by using a fluorescence microscope with excitation filter of 510–560 nm and barrier filter of 590 nm. One hundred nucleoids were analyzed per slide, totalizing 600 nucleoids per treatment. The score of each treatment was verified by multiplying the number of nucleoids observed in each damage class by the value of the class (0, 1, 2 or 3).

2.6.2. Cytokinesis-block micronucleus assay (CBMN assay)

After the application of treatments, the cells were washed twice with 5 mL of phosphate buffered saline (PBS). To avoid cytokinesis, 50 μL of cytochalasin B (300 μg/mL – Sigma) were added into the culture medium after 28 h of incubation, according to Malini et al. (2010) with modifications. 1.5 mL of hypotonic solution (1% sodium citrate) was added, and the cells were fixed in Carnoy (methanol:acetic acid 3:1, v-v). The slides were stained with 5% Giemsa and analyzed afterwards under light microscopy. For MN quantification, 9000 binucleated cells were counted per treatment. Besides micronuclei (MN), also nuclear buds (NB) and nucleoplasmic bridges (NPB) were quantified.

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