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ORIGINAL ARTICLE

# Phytochemical study and evaluation of cytotoxicity, mutagenicity, cell cycle kinetics and gene expression of *Bauhinia holophylla* (Bong.) Steud. in HepG2 cells in vitro

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**Abstract** *Bauhinia holophylla* (Bong.) Steud. (Fabaceae) is a plant used in Brazilian folk medicine to treat diabetes and inflammation. This study evaluated the phytochemical properties, cytotoxic, apoptotic, mutagenic/antimutagenic effects and alterations in gene expression (RNAm) in HepG2 cells treated with the *B. holophylla* extract. The phytochemical profile highlight the presence of flavonoids isorhamentin and quercetin derivates. The MTT assay was used to evaluate the cytotoxicity of different concentrations for different treatment times. Three concentrations (7.5, 15, 30 μg/mL) were chosen for assessment of apoptosis (AO/EB), mutagenicity (micronucleus), and cell cycle kinetics (flow cytometry). Thereafter, the concentration of 7.5 μg/mL was chosen to evaluate the protective effects against DNA damage induced by benzo[*a*]pyrene (B[*a*]P). At concentrations higher than 7.5 μg/mL (between 10 and 50 μg/mL), the extract was cytotoxic, induced apoptosis, and caused antiproliferative effects. However, it did not induce micronucleus and a reduction of apoptotic and micronucleated cells was observed in treatments that included the extract and B[*a*]P. The protective effect is attributable to the presence of flavonoids, described as antioxidants, inhibitors of DNA adduct and activators of detoxifying enzymes.

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The results of the present study such as absence of cytotoxic and mutagenic effects and protective effects against known carcinogens suggest that *B. holophylla* has potential for use soon as herbal medicine.

**Keywords** Antimutagenic · Micronucleus · Flavonoids · Flow cytometry · RT-qPCR

## Introduction

Herbal medicines are substances of natural origin that became universally popular in primary care health, especially in emerging countries like Brazil (Santos et al. 2012; David et al. 2015). Medicines and herbal compounds are popularly presumed as safe for human consumption without any adverse effect on health and, therefore, they are used in self-medication without medical prescription (Obici et al. 2008). However, literature data demonstrate that substances in medicinal preparations may have toxic effects, including DNA damage (Verschaeve et al. 2004; Sisenando et al. 2009; Rodeiro et al. 2014). The detection and evaluation of potential damages caused by plant compounds are of fundamental importance for minimizing adverse effects of plant extracts, specially when they are used in long-term treatments (Cardozo et al. 2006).

The *Bauhinia* L. genus (Fabaceae) comprises about 300 species, popularly known as "paw of cow's" or "cow's hoof" due to the shape of its leaves. These species are widely distributed in tropical countries, including countries in Africa, Asia and South America (Lewis et al. 2005). The leaves and stem bark have often been used in folk medicine to treat different diseases, particularly diabetes, infections and inflammatory processes (Da Silva and Cechinel Filho 2002; Cechinel Filho 2009).

In the last decade, the interest in these plants increased considerably since studies about biological properties of different species of *Bauhinia* and their isolated compounds have been investigated in vivo and in vitro experimental models. Reports on their biological activities have generated controversies, such as the ethanolic extract of *Bauhinia platypetala* Burch. ex Benth., showed no cytotoxic effects in *S. cerevisiae*, but increased DNA damage in mammalian V79 cells (Santos et al. 2012). Components of the leaves of *Bauhinia monandra* Kurz did not affect the frequency of reverse mutations in strains of *Salmonella typhimurium* (TA97, TA98, TA100 and TA102) with and without metabolic activation (Sisenando et al. 2009). The extract of barks of *Bauhinia variegata* L. showed chemopreventive effects both in vitro (HEp2 and HBL-100 cells lines) and in vivo (Swiss albine mice) (Rajkapoor et al. 2006; Agrawal and Pandey 2009; Pandey and Agrawal 2010).

According to Cechinel Filho (2009), several studies with species of this genus, in general, confirm their therapeutic properties, such as a high antidiabetic potential. These effects were associated with the presence of high contents of flavonoids, such as quercetin-3-o-α-(2″-galloyl)-rhamnoside and kaempferol-3-o-α-(2″-galloyl)-rhamnoside (Estrada et al. 2005; Gonzalez-Mujica et al. 2005). Gonzalez-Mujica et al. (2003) suggested that aqueous leaf extract of *Bauhinia megalandra* Griseb. inhibited intestinal glucose absorption, as well as glucose-6-phosphatase and hepatic gluconeo-genesis, suggesting that this plant might help control hyperglycemia in diabetic patients.

Given the importance of investigating medicinal plants and due to the fact that there are no reports in the literature about the phytochemical composition and the effects of the *B. holophylla* extract in mammalian cells in vitro, this study investigated the ethanolic extract obtained from the leaves of this plant, through the phytochemical characterization; analysis of their cytotoxic, mutagenic, antimutagenic, apoptotic properties and also its effects on gene expression (mRNA) and cell cycle kinetics in human HepG2 (human hepatocellular carcinoma) cells.

## Materials and methods

### Chemicals

Dulbecco's Modified Eagle Medium (DMEM) (Cat No.: 11885084) fetal bovine serum (FBS) (Cat No.: 12657-029), 1% antibiotic–antimycotic solution (Cat No.: 15240-062) were purchased from Gibco (Grand Island, NY, USA). Sodium bicarbonate (CAS: 144-55-8), Dimethylsulfoxide (DMSO; CAS: 67-68-5), benzo[*a*]pyrene (B[*a*]P; CAS: 50-32-8), Cytochalasin B (CAS: 14930-96-2), acridine orange (AO; CAS: 10127-02-3), ethidium bromide (EB; CAS: 1239-45-8), trypan blue (CAS: 72-57-1), ethanol (EtOH; CAS:

64-17-5), methanol (MeOH; CAS: 67-56-1), RNAse solution (Cat. No.: 10109142001), propidium iodide (PI; CAS: 25535-16-4) and KiCqStart® SYBR Green Primers (CAS: KSPQ 12012) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The MTT salt [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; CAS: 298-93-1], agarose (UltraPure™—Cat. No.: 16500500), DNase I (1 U/μL—Cat. No.: EN0521), Oligo-DT$^{12–18}$ (Cat. No.: 18418012); Random Primers (Cat. No.: 48190011) and SuperScript III RT enzyme (Cat. No.: 18080-044) were purchease from Invitrogen (Eugene, OR, USA). PureLink™ RNA Mini Kit Ambion (Cat No.: 12183020) and Platinum® SYBR® Green qPCR SuperMix UGD (Cat. No.: 11733-038) were obtained in Life Technologies (Carlsbad, CA, USA).

## Plant material, extraction and phytochemical study

Leaves (1.6 kg) of *Bauhinia holophylla* were collected in the Botanical Garden of Bauru, located on the banks of Highway Commander João R. de Barros—SP 225 (22°20′30″S e 49°00′30″W) in January of 2011. Identification of the specie was performed by Professor Vaz, A.M.S.F. of the Herbarium of the Botanical Garden of Rio de Janeiro, where the exsiccate have been deposited with record number RB 507.043.

*Bauhinia holophylla* leaves were hot air dried in a kiln with air circulation at 45 °C for 72 h under reduced light exposure and sprayed in a knife mill. The extract was prepared by exhaustive percolation of the powder (220 g) using EtOH:$H_2O$ (7:3 *v*/*v*) and the extractors were concentrated under reduced pressure at temperature below 40 °C. The extract was transferred to tared glass and 65 g of hydroalcoholic extract (yield of 29.5%) were obtained.

The HPLC-PAD system consisted of a PU-2089S Plus (Jasco, Hachioji, Tokyo, Japan) pump equipped with a MD-2015 Plus Photodiode Array Detector (PAD, Jasco) and AS-2055 automatic injector (Jasco, Japan). The column used was a Luna C18 (250 × 4.6 mm, i.d.) with a particle size of 5 μm (Phenomenex, Torrance, CA, USA) maintained at 35 °C. The eluents were: $H_2O$ + 0.1% formic acid (eluent A) and MeOH + 0.1% formic acid (eluent B). Gradient system: 25–100% of B in A in 70 min. Injected volume: 20 μL. The chromatogram was monitored at 200–600 nm.

FIA-ESI-IT-MS spectral data were obtained using an Accela (Thermo Scientific, San Jose, CA, USA) LCQ Fleet with Ion Trap (IT) 3D and ionization by eletrospray (ESI). 70% EtOH extract was dissolved in MeOH-$H_2O$ (8:2) and injected in the ESI source by flow injection analysis (FIA) using a syringe pump at a final concentration of 10 μg; flow rate was 33 μL/min. Capillary voltage was − 20 V, spray voltage was 4 kV, and tube lens offset was − 55 V. Capillary temperature was 275 °C. Nitrogen was used both as drying gas at a flow rate of 60 (arbitrary units) and as nebulising gas. Nebulizer temperature was set at 280 °C, and a potential of − 4 V was used on the capillary. Negative ion mass spectra were recorded in range *m*/*z* 100–1000. The collision energy for MS/MS was adjusted to 10–25%.

The extract was diluted in ethanol 70% (EtOH 70%; EtOH:$H_2O$ distilled—7:3 *v*/*v*) to give a stock solution at a concentration of 5000 μg/mL, which was conditioned under − 20 °C in the absence of light, filtered in sterile membrane. This stock solution was used to prepare the working solutions 100× concentrated (500–5000 μg/mL) also using EtOH 70% as solvent. After this, an aliquot of each working solution was used to treat cells at 1× (5–50 μg/mL), resulting in a final ethanol concentration in culture of 0.7%. All procedures followed the *OECD Guideline No. 129* (guidance document to estimate starting doses for acute oral systemic toxicity tests) that recommended the solvents (i.e. ethanol) should not exceed 1% (*v*/*v*) in the final treatment medium and also included as controls in all assays to demonstrate absence of adverse effects.

## Cell line and culture conditions

HepG2 cells were kindly provided by the Laboratory of Nutrigenomics of FCFRP of University of São Paulo, Brazil. This cell line was chosen because it is a good experimental model in vitro by possessing some inducible enzymes metabolizing phase I and II, which are able to activate and detoxify xenobiotics, thus predicting in vitro hepatotoxic effects of drugs in humans (Atienzar et al. 2014; Lee et al. 2015). HepG2 cells were cultured in 10 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% FBS, 1% antibiotic–antimycotic solution and 0.024% sodium bicarbonate at 37 °C in 5% $CO_2$. All experiments were conducted between the third and eighth

cell passage, and HepG2 cells under these conditions have a cell cycle of approximately 24 h. All experiments were performed in independent biological triplicate with positive (B[*a*]P—20 µM—504 µg/mL) and negative (NC; phosphate saline buffer, PBS) controls.

### MTT assay

The MTT assay was used to determine the cytotoxicity of *B. holophylla* extract in HepG2 cells using the protocol described by Mosmann (1983). Approximately $1.0 \times 10^4$ cells were plated in each well of 96-well plates in 200 µL of complete culture medium. After 24 h of stabilization, 10 different concentrations of the extract (final concentrations in culture: 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 µg/mL) and control solvent (EtOH 70%) in minimal medium (without FBS) were added to HepG2 cells (seven wells per concentration) in a time of 3, 24, 48 and 72 h in incubator of $CO_2$ at 5% and 37 °C. After the appropriate time, the treatments were removed and the MTT solution (5 mg/mL) dissolved in complete medium was added to each well and incubated for 4 h. Finally, the MTT solution was removed and DMSO was added to each well, and the absorbance was measured with a spectrophotometer (Uniscience, São Paulo, SP, Brazil) at 550 nm. The percentage of viable cells was calculated according to the formula:

$$\text{Cell viability } (\%) = \frac{\text{Absorbance treated cells}}{\text{Absorbance control cells}} \times 100$$

### Cytokinesis blocking micronucleus assay (CBMN)

CBMN was performed according to the method described by Fenech (2007). First, $0.5 \times 10^6$ HepG2 cells were placed in each culture flask containing 5 mL of complete medium and stabilized for 24 h; firstly, the mutagenic potential of the extract was assessed and for this the culture medium was replaced by solutions containing three concentrations of the *B. holophylla* extract (final concentration in culture: 7.5, 15 and 30 µg/mL) for further 24 h. Positive (B[*a*]P—504 µg/mL—PC), negative (phosphate-buffered saline, PBS—NC) and solvent (EtOH—0.7%—SC) controls were also included in all experiments. After this, a concentration of the extract that showed no mutagenic effect (7.5 µg/mL) was chosen to assess possible antimutagenic effect using three different associated treatments: (1) simultaneous (SIM: treatment with both, extract and B[*a*]P, for 24 h), (2) pre-treatment (PRE: treatment with the extract for 24 h followed by B[*a*]P for 24 h), and (3) post-treatment (POST: treatment with B[*a*]P for 24 h followed by the extract for 24 h). Positive (B[*a*]P—504 µg/mL—PC), negative (phosphate-buffered saline, PBS—NC) and solvent (EtOH 0.7%—SC) controls were also included in these experiments. After all protocols of treatments (no associated and associated), the cells were incubated for 30 h with cytochalasin B (3 µg/mL). The method used in this technique to slide preparation followed the same method described by our research group (Ribeiro et al. 2015; Serpeloni et al. 2015). Cytological analysis was performed on a Nikon Eclipse E200 light microscope (Tokyo, Japan). To determine the frequency of cells with MN, 3000 binucleated cells with preserved cytoplasm (1000 cells per replicate) were analyzed according to the criteria described by Kirsch-Volders et al. (2000). The percentage of DNA damage reduction (%R) was obtained from the formula proposed by Waters et al. (1990) as described below:

$$\%\text{R} = \frac{[\%\text{MN[PC]} - [\%\text{MN Treatment}]]}{[\%\text{MN[PC]} - [\%\text{MN NC}]]} \times 100$$

The Nuclear Division Index (NDI) was also determined analyzing 500 cells/slide (1500 cells per treatment). To calculate the NDI, the following formula as proposed by Eastmond and Tucker (1989) was applied:

$$\text{NDI} = \frac{\text{M1} + 2(\text{M2}) + 3(\text{M3}) + 4(\text{M4})}{\text{N}}$$

where M1, M2, M3 and M4 = number of cells with one, two, three or four nuclei, respectively, N = total number of viable cells analyzed.

### Apoptosis/necrosis assay

The induction of apoptosis as well as the possible protective effects of *B. holophylla* against B[*a*]P-induced cell death were assessed according to the protocol described by McGahon et al. (1995). In total, $1.0 \times 10^5$ cells were placed in 24-well plates and were treated as described in the CBMN assay. The methods were performed using the same procedure described

by Ribeiro et al. (2015). The cells were mixed with 1 µL of staining solution (100 µg/mL of AO and 100 µg/mL of EB) and 600 cells per treatment (200 per replicate) were analyzed with a Nikon fluorescence microscope, with an excitation wavelength of 515–560 nm and a 590 nm barrier filter. To validate these experiments, a Trypan blue exclusion assay was performed counting the number of blue staining cells (unviables) in Newbauer chamber, considering the cell viability at least 90%. The results are presented as the percentage (%) of viable, apoptotic and necrotic cells; the sum of the cells in each treatment was considered 100%. The percentage of reduced numbers (%R) of apoptotic cells (AC) was calculated as for CBMN.

$$\%\mathrm{R} = \frac{[\%\mathrm{AC[B[a]P]} - [\%\mathrm{AC\ Treatment}]]}{[\%\mathrm{AC[B[a]P]} - [\%\mathrm{AC\ NC}]]}.$$

## Cell cycle assay

Flow cytometry was performed according to the protocol of Ormerod (2000). A total of $1.0 \times 10^5$ cells was plated in 24-well plates and treated as described in the CBMN assay. The methods were performed using the same procedure described by Ribeiro et al. (2015). The cells were fixed in EtOH 70% solution, treated with 50 µL of RNase solution (1 mg/mL in water), 50 µL of PI (400 µg/mL in water) and incubated at 37 °C for 30 min. The samples were then subjected to fluorescence activated cell sorting (FACS, FACSVantage, Becton–Dickinson, Franklin Lakes, NJ, USA). The data were analyzed using the Cell Quest software (Becton–Dickinson), and cell cycle profiles were examined using the Flow Jo software (Tree Star Incorporation, Ashland, OR, USA).

## Gene expression (RT-qPCR)

In experiments of gene expression, initially a total of $0.5 \times 10^6$ HepG2 cells were plated in 25 $cm^3$ culture flasks with 10 mL of complete DMEM medium and stabilized for 24 h. Then, the culture medium in the culture flasks was replaced by 5 mL of DMEM medium and their respective treatments in no associated and associated protocols. However, based on the results of previous experiments (MTT, CBMN, cell cycle and apoptosis/necrosis), for this experiment only the *B. holophylla* extract (7.5 µg/mL) was evaluated in the no associated protocol (only the extract without B[*a*]P addition). In the associated protocol (extract + B[*a*]P) the protective effect of *B. holophylla* extract (7.5 µg/mL) was evaluated only in post-treatment (POST: treatment with B[*a*]P for 24 h followed by the extract for 24 h).

Total RNA extraction was performed with the PureLink™ RNA Mini Kit and the RNA was quantified and checked for quality (ratio of absorbance 260/280 between 1.7 and 2.0) by spectrophotometry (NanoDrop 2000C, Thermo Scientific, San Jose, CA, USA). RNA integrity was also assessed by electrophoresis on a denaturing agarose gel (1%), as described by Aranda et al. (2012). The RNA samples were treated with amplification grade DNase I (1 U/µL), according to the manufacturer's instructions. The cDNAs were synthesized from 500 ng of total RNA using Oligo-$DT^{12-18}$, random primers and SuperScript III enzyme according to the manufacturer's protocol.

For the gene of interest *TP53* (*tumor protein p53*; F: 5'CCATCCACTACAACTACAT'3; R: 5'GCACAAACACGCACCTC'3) and reference genes *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*; F: 5'GGGCATCCTGGGCTACACT'3; R: 5'GGTCCAGGGGTCTTACTC'3) and *HPRT1* (*hypoxanthine guanine phosphoribosyl transferase 1*; F: 5'TCCTCTGCTCCGCCACCG'3; R: 5'TCATCAC TAATCACGACGCC'3) the primers were designed using *Gene Runner* Software, version 3.05 (1994). The primers for the *CAT* (*catalase*; F: 5'CTCTTCTGGA CAAGTACAATG'3; R: 5'AGGAGAATCTTCATC CAGTG'3), *GSR* (*glutathione reductase*; F: 5'GACC TATTCAACGAGCTTTAC'3; R: 5'CAACCACCTT TTCTTCCTTG'3), *GPx1* (*glutathione peroxidase 1*; F: 5'CTACTTATCGAGAATGTGGC'3; R: 5'CA GAATCTCTTCGTTCTTGG'3), *NFE2L2* (*nuclear factor erythroid 2-like 2*; F: 5'CGTTTGTAGATGA CAATGAGG'3; R: 5'AGAAGTTTCAGGTGACT GAG'3), *XPC* (*xeroderma pigmentosum complementation group C*; F: 5'ACTGATGGATACATCGTC TG'3; R: 5'TTCTCCTTCTCCTTCCTTTC'3) and *CYP1A1* (*cytochrome P450 family 1, subfamily A, polipeptide 1*; F: 5'CATTAACATCGTCTTGGAC C'3; R: 5'TCTTGGATCTTTCTCTGTACC'3) genes were obtained from KiCqStart® SYBR Green Primers. The efficiency of each primer was determined by testing serial dilutions of five cDNA concentrations until the best annealing temperature to be obtained.

The PCR analyses were performed using the *Techne Quantum*$^{TM}$ *Real-Time PCR Cycler System* with the Platinum® SYBR® Green qPCR SuperMix UGD in a final volume of 10 µL, using 20 pmol of each oligonucleotide primer and 50 ng of the cDNA template. The reaction mixture was subjected to the following amplification program: 95 °C for 5 min; 40 cycles at 95 °C for 15 s, 60 °C for 15 s and 72 °C for 15 s. A melting curve was also performed, ranging from 50 °C to 95 °C. All experiments were performed in two independent biological experiments and the reactions were performed in technical triplicates.

### Statistical analysis

The data obtained from the MTT, CBMN (including NDI) and apoptosis/necrosis assays were evaluated by ANOVA followed by Tukey's test. The test for linear trend was used to assess effects of concentration-dependent in MTT test. The data obtained from flow cytometry were analyzed by ANOVA followed by Dunnett's test. Statistical analysis was performed with GraphPad Prism 5 software (La Jolla, CA, USA). All data are presented as mean ± standard deviation (X ± SD), with a significance level of $p \leq 0.05$.

Data for gene expression were analyzed by *Quantsoft V.1.1.30*$^{TM}$ Software (Techne). The REST program uses the statistical model *Pair Wise Fixed Reallocation Randomization Test* and was used to evaluate the data obtained from qPCR to determine the target genes that showed differential expression (Pfaffl 2001; Pfaffl et al. 2002). The calculations were normalized from the reference genes *GAPDH* and *HPRT1* and were considered differentially expressed compared to the negative control (PBS) in the "no associated" protocol and to the positive control (PBS + B[*a*]P) in the "associated protocol" when the relative expression was greater than 2 (up-regulated) or less than 0.5 (down-regulated) and $p \leq 0.05$.

## Results

### Chemical profile obtained by HPLC-PAD and FIA-ESI-IT-MS

The constituents identified in the EtOH 70% extract of *B. holophylla* were characterized on the basis of UV and MS espectral data. The spectroscopic data obtained in this study are in concordance with data previously published by our group with the same extract (Rozza et al. 2015).

The HPLC-PAD analysis of the chromatogram peaks with bands at $\lambda_{max}$ = 330–380 and 240–280 nm were related to flavonoid derivatives of flavonols (Andersen and Markhan 2006). The direct flow injection analysis (FIA-ESI–MS) highlight the presence of five main precursor ions present in the EtOH 70% extract of *B. holophylla* (Fig. 1). Fragmentation of the main precursor ions at, *m/z* 433 $[M–H]^-$, *m/z* 447 $[M–H]^-$, *m/z* 463 $[M–H]^-$ confirmed the presence of quercetin-o-pentoside, quercetin-o-deoxyhexoside and quercetin-o-hexoside, respectively (Fig. S1; Supplementary Material). Diagnostics mass fragments at *m/z* 301 characterized quercetin and the precursor ion at *m/z* 315 $[M–H]^-$, isorhamnetin. The neutral losses of 132, 162 and 146 mass units allowed the identification of pentosides, hexosides, deoxyhexosides. These data are compatible with those reported for the identification of flavonoid derivatives (Cuyckens and Claeys 2004).

### Cytotoxicity

Figure 2 shows the percentages of viable HepG2 cells after treatment with different concentrations of the *B. holophylla* extract at different times of treatment in the MTT assay. It is possible to note that at the treatment times of 3 and 48 h, no concentration of the extract changed the viability of HepG2 cells. However, after 24 and 72 h, the highest concentration and concentrations equal or higher than 10 µg/mL showed, respectively, a significant reduction in cell viability. Therefore, the concentrations chosen to other tests were 7.5; 15 and 30 µg/mL.

### Mutagenic and apoptotic effects

In the micronucleus test no concentration of *B. holophylla* extract showed mutagenic potential in HepG2 cells (Table 1). The data obtained of NDI showed that the three concentrations of the extract significantly reduced the cell proliferation. The test for linear trend ($F = 37.73$ and $p > 0.0002$) indicated that this reduction was concentration-dependent. However, this effect was also observed for cells treated with EtOH 0.7% that showed a significant reduction of NDI, which may have contributed to

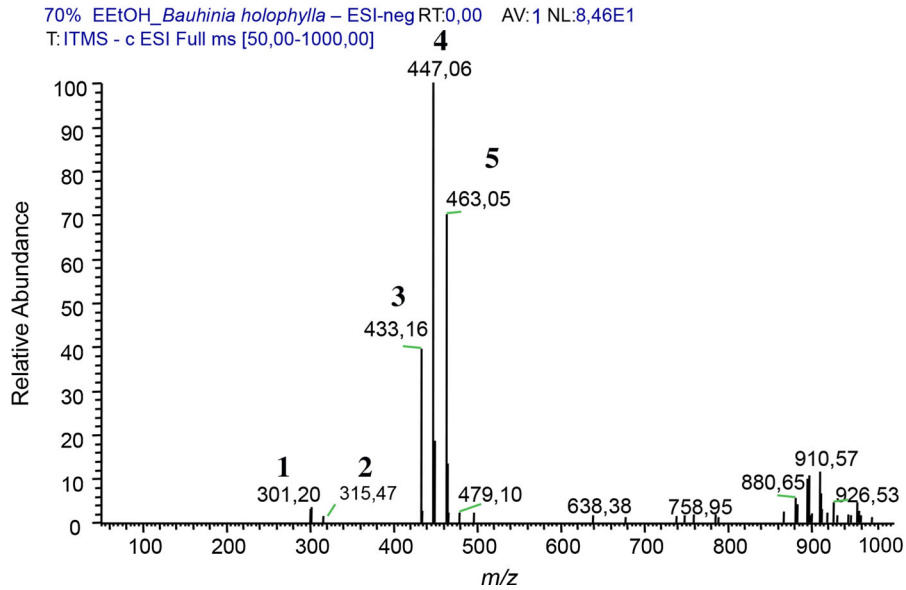


**Fig. 1** Representative FIA-ESI–MS spectra of the EtOH 70% leaves extract of *Bauhinia holophylla* obtained in negative mode confirming the presence of flavonoids derivatives. *1*—quercetin; *2*—isorhamentin; *3*—quercetin-o-pentoside; *4*—quercetin-o-deoxyhexoside; *5*—quercetin-o-hexoside. * For fragmentations, see Figure S1 in the Supplementary Material

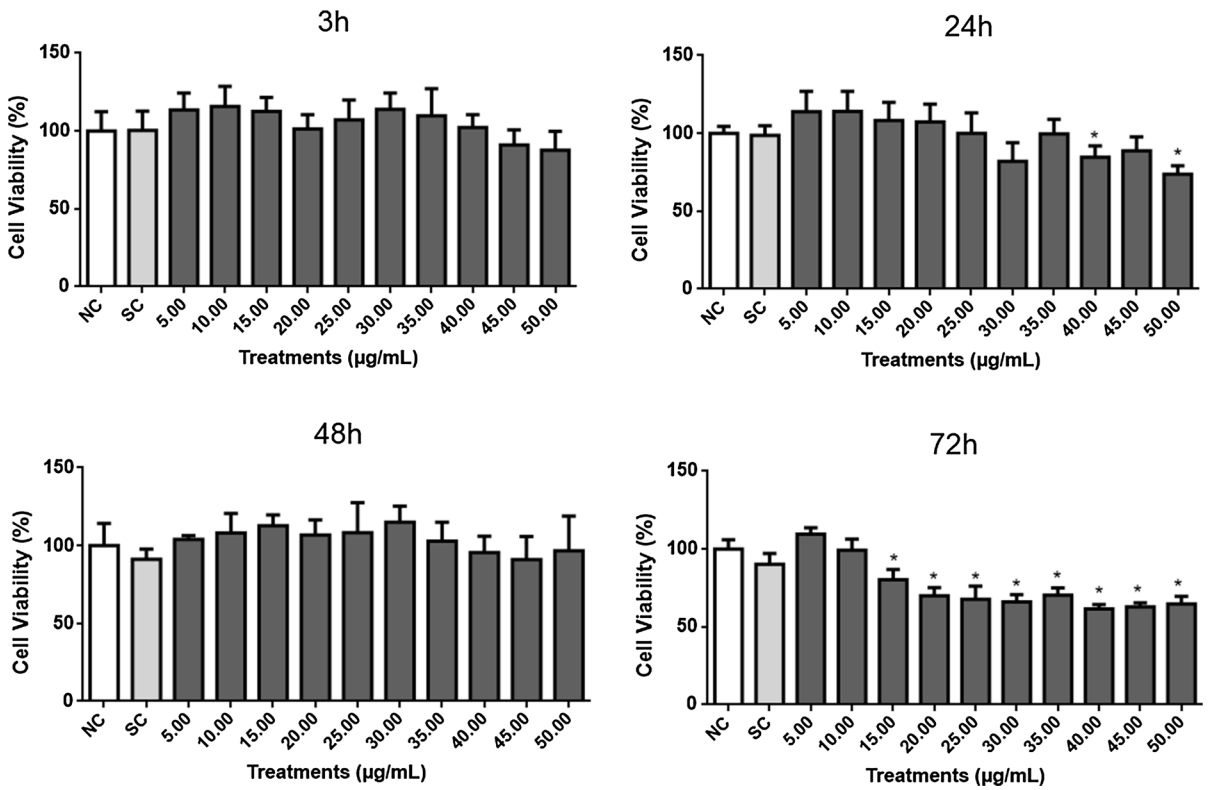


**Fig. 2** Percentage of viable HepG2 cells observed after treatment with different concentrations of *Bauhinia holophylla* in the MTT assay. Asterisk values significantly different from NC by ANOVA followed by Tukey's test ($p \leq 0.05$). The values represent the mean ± standard deviation (X ± SD) and the results are expressed as the percentage relative to the NC (100%). NC: Negative Control (phosphate buffered saline; PBS); SC: Solvent Control (EtOH 0.7%)

**Table 1** Frequency of micronucleated (MN) HepG2 cells, nuclear division index (NDI) and percentage of micronucleated cells (% MNs cells) obtained from three independent biological replicates after 24 h of treatment with three different concentrations of *B. holophylla* extract and their respective controls

| | Micronucleated cells | | | | % MNs cells | NDI | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Repetitions | | | X ± SD | | Repetitions | | | X ± SD |
| | I | II | III | | | I | II | III | |
| Treatments (μg/mL) | | | | | | | | | |
| NC | 16 | 22 | 17 | 18.33 ± 3.22[a] | 1.83 | 1.77 | 1.71 | 1.69 | 1.72 ± 0.04[a] |
| PC | 28 | 30 | 30 | 29.33 ± 1.16[b] | 2.93 | 1.53 | 1.51 | 1.56 | 1.53 ± 0.03[b] |
| SC | 14 | 13 | 17 | 14.67 ± 2.08[a] | 1.47 | 1.52 | 1.53 | 1.56 | 1.54 ± 0.02[b] |
| Extract (μg/mL) | | | | | | | | | |
| 7.5 | 18 | 20 | 13 | 17.00 ± 3.61[a] | 1.70 | 1.54 | 1.57 | 1.59 | 1.57 ± 0.02[b] |
| 15 | 17 | 16 | 18 | 17.00 ± 1.00[a] | 1.70 | 1.41 | 1.41 | 1.40 | 1.41 ± 0.01[c] |
| 30 | 14 | 15 | 11 | 13.33 ± 2.08[a] | 1.33 | 1.39 | 1.34 | 1.42 | 1.38 ± 0.04[c] |

A total of 3000 binucleated cells per treatment (1000 cells per replicate) was counted to score the MN cells and 1500 cells (500 cells per replicate) were counted for the nuclear division index (NDI)

NC: Negative Control (phosphate buffered saline; PBS), PC: Positive Control (B[*a*]P—504 μg/mL—20 μM), SC: Solvent Control (EtOH 0.7%); X ± SD = mean ± standard deviation

*Values with the same letter ([a,b,c]) did not differ from each other by ANOVA followed by Tukey's test ($p \leq 0.05$)

similar data obtained with cells treated only with the extract.

The increase of apoptotic cells compared to the negative control was concentration-dependent, according to the linear trend test ($F = 11.85$ and $p < 0.0005$) (Fig. 3). Spite of this increase have been occurred in a dose-dependent manner, only the result obtained with the highest concentration (30 μg/mL) was statistically different from the control. Since the concentration of 7.5 μg/mL displayed the lowest frequency of micronucleated cells compared to normal control group (Table 1) and had not induced apoptotic cells (Fig. 3), it was chosen to evaluate the protective effect of this extract against B[*a*]P in HepG2 cells.

Cell viability after the Trypan blue test was always above 92% in all experiments. The solvent used for dilution of the extract did not affect viability at any treatment time evaluated (data not shown).

## Protective effects (antimutagenicity assays)

As shown in Table 2, all associated treatments of the *B. holophylla* extract with B[*a*]P showed a protective effect on DNA damage caused by B[*a*]P in the CBMN test. The %R of MN was 76% for simultaneous, 75% for pre-treatment and 83% for post-treatment, demonstrating the antimutagenic potential of this extract. In the post-treatment protocol the extract also inhibited cell proliferation. The extract at concentration of 7.5 μg/mL in all treatment protocols reduced the NDI in comparison to NC.

As showed in Fig. 4, the *B. holophylla* extract also presented a protective effect on apoptosis assay in treatments when compared to the positive control. The significative results were obtained after 3 h where the percentage of reduction of apoptotic cells were 75, 60 and 55%, for, respectively, SIM, PRE and POST. After 24 h of treatment the percentage of apoptotic cells was reduced to 51 (SIM), 24 (PRE) and 50% (POST).

## DNA content analysis

The results of cell cycle analysis performed by flow cytometry using differential staining with propidium iodide are shown in Fig. 5a, b. It was observed that *B. holophylla* extract, in the three concentrations assessed, significantly decreased the population of cells in G1 and increased the population of cells in the S phase, compared to the NC (Fig. 5a). This demonstrates a possible arrest of the cell cycle and decreasing of proliferation of HepG2 cells.

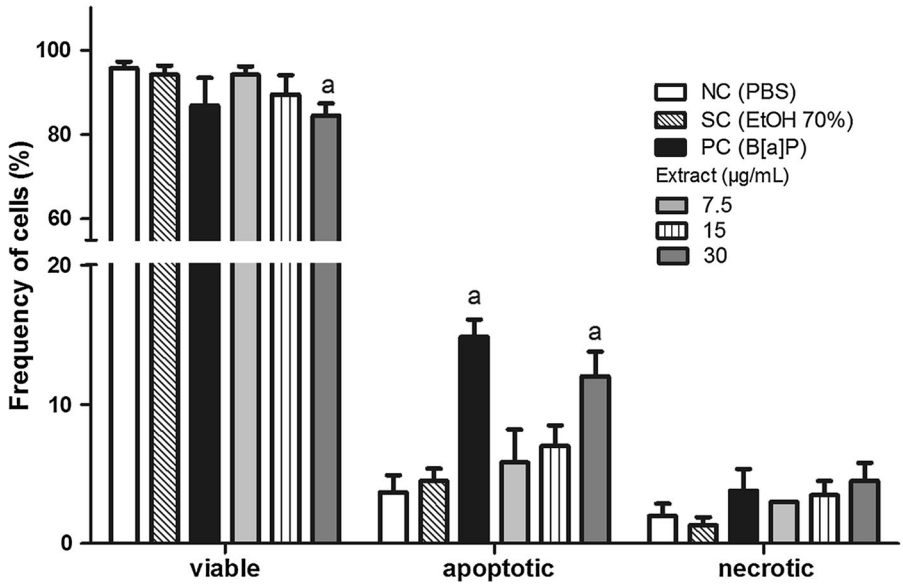


**Fig. 3** Percentage (%) of viable, apoptotic and necrotic HepG2 cells after 24 h of treatment with different concentrations of the *Bauhinia holophylla* extract. Values with letter differ from negative control in ANOVA followed by Tukey's test ($p \leq 0.05$). The values represent the mean ± standard deviation (X ± SD) of experiments performed in triplicate; a total of 600 cells was analyzed for each treatment. NC: Negative Control (phosphate buffered saline; PBS); SC: Solvent Control (EtOH 0.7%). PC: Positive Control (B[*a*]P—504 µg/mL—20 µM)

The evaluation of the protective effect (Fig. 5b), indicated that the associated treatments with B[*a*]P in the protocols of SIM and POST did not altered the population of cells in G1 phase, but increased the population of cells in S phase compared to B[*a*]P. In PRE, there was an increase in the population of cells in G1 phase and a decreasing of the population G2/M.

### Gene expression

The results obtained after the RT-qPCR experiments to evaluate the effects of *B. holophylla* extract (7.5 µg/mL) in gene expression are shown in Table 3. The extract showed no change in the mRNA levels of genes involved in responses to oxidative stress or DNA repair; however, it was able to increase significantly the *CYP1A1* gene. The positive control in both experiments (alone or associated) showed a significant increase in gene expression of *CYP1A1*, involved in the metabolism of B[*a*]P. PC/Post (PBS + B[*a*]P) also showed a significant increase in *XPC* transcripts, involved in DNA repair. When the extract was associated to B[*a*]P in POST, it also did not change mRNA levels of genes related to oxidative stress/DNA repair and only *CYP1A1* gene was upregulated.

## Discussion

Many medicinal plants can provide a relief or cure the symptoms of a disease as well as traditional medicaments (FDA 2006). The number of natural products consumed by the Brazilian population, such as teas and phytotherapics, constantly increase, especially in places where folk medicine is traditionally used in the treatment of chronic diseases (Geller et al. 2015; Da Justa Neves and Caldas 2015). However, for the use of phytotherapies and medicinal plants in chemoprevention it is also important to assess their possible hazards and risks to the human health.

The mutagenicity is a critical and decisive toxicological endpoint in the evaluation of compounds such as drugs, pesticides, food additives and vegetal extracts and that need to be properly investigated during a product's safety assessment process (FDA 2006). Nowadays, some regulatory agencies as "European Food Safety Authority—EFSA" require the execution of at least three different tests for mutagenicity in vitro in the initial identification of hazard potential (EFSA 2008; Llana-Ruiz-Cabello et al. 2015). In this context, it is common practice to make short-term trials for detection of mutagenic and

**Table 2** Frequency of micronucleated (MN) HepG2 cells, nuclear division index (NDI) and percentage of micronucleated cells (% MNs cells) obtained from three independent biological replicates after 24 h of treatment with the *B. holophylla* extract and B[*a*]P in simultaneous, pre- and post-treatment protocols with their respective controls

| Treatments (μg/mL) | Micronucleated cells | | | | % MN cells | %R | NDI | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Repetitions | | | X ± SD | | | Repetitions | | | X ± SD |
| | I | II | III | | | | I | II | III | |
| Simultaneous | | | | | | | | | | |
| NC | 16 | 11 | 11 | 12.67 ± 2.89[a] | 1.27 | | 1.71 | 1.70 | 1.71 | 1.71 ± 0.01[a] |
| PC | 24 | 20 | 28 | 24.00 ± 4.00[b] | 2.40 | | 1.39 | 1.45 | 1.40 | 1.42 ± 0.03[b] |
| SC | 14 | 13 | 16 | 14.33 ± 1.53[a] | 1.43 | | 1.55 | 1.50 | 1.52 | 1.52 ± 0.02[c] |
| SIM | 15 | 15 | 16 | 15.33 ± 0.58[a] | 1.53 | 76 | 1.61 | 1.65 | 1.59 | 1.62 ± 0.03[d] |
| Pre-treatment | | | | | | | | | | |
| NC | 11 | 14 | 14 | 13.00 ± 1.73[a] | 1.30 | | 1.68 | 1.65 | 1.62 | 1.65 ± 0.03[a] |
| PC | 32 | 31 | 29 | 30.67 ± 1.53[b] | 3.07 | | 1.45 | 1.42 | 1.47 | 1.45 ± 0.02[b] |
| SC | 15 | 15 | 16 | 15.33 ± 0.58[a,c] | 1.53 | | 1.62 | 1.55 | 1.56 | 1.58 ± 0.04[a] |
| PRE | 16 | 17 | 19 | 17.33 ± 1.53[c] | 1.73 | 75 | 1.42 | 1.52 | 1.45 | 1.46 ± 0.05[b] |
| Post-treatment | | | | | | | | | | |
| NC | 11 | 14 | 14 | 13.00 ± 1.73[a] | 1.30 | | 1.68 | 1.65 | 1.620 | 1.65 ± 0.03[a] |
| PC | 24 | 27 | 30 | 27.00 ± 3.00[b] | 2.70 | | 1.42 | 1.33 | 1.342 | 1.36 ± 0.05[b] |
| SC | 15 | 15 | 16 | 15.33 ± 0.57[a] | 1.53 | | 1.62 | 1.55 | 1.562 | 1.58 ± 0.04[a] |
| POST | 15 | 15 | 16 | 15.33 ± 0.58[a] | 1.53 | 83 | 1.44 | 1.38 | 1.456 | 1.43 ± 0.04[b] |

A total of 3000 binucleated cells per treatment (1000 cells per replicate) was counted for the MN frequency and a 1500 cells (500 cells per replicate) for the nuclear division index (NDI)

NC: Negative Control (phosphate buffered saline; PBS), PC: Positive Control (B[*a*]P—504 μg/mL—20 μM), SC: Solvent Control (EtOH 0.7%); X ± SD = mean ± standard deviation

*Values with the same letter ([a,b,c]) did not differ from each other by ANOVA followed by Tukey's test ($p \leq 0.05$)

antimutagenic substances using in vitro model, as HepG2 cells. In addition to the initial descriptions reported in introduction, this cell line is widely used for the pharmaceutical and chemical industries as an initial screening to determine the hazard of new chemical compounds eliciting drug-induced liver injury (DILI) in humans (Sison-Young et al. 2015).

There are some reports about biological properties of species of *Bauhinia* genus, but these data are still controversial. The study of Bresolin and Vargas (1993) showed that the *Bauhinia forficata* L. aqueous extract was able to induce mutagenicity in vitro in TA102 strain of *S. typhimurium* after metabolization with S9 fraction (*Salmonella*/microsome assay—Ames test), but the data of the present study with human metabolizing cells (HepG2) showed that *B. holophylla* extract did not induce mutagenicity in three concentrations tested. Although in both studies the cells have been able to metabolize extracts, it should be noted that these were from different species of *Bauhinia* and in addition the assay used by Bresolin and Vargas (1993) asseseds gene mutation in bacteria (Ames test), whereas in the present study with HepG2 cells, the parameter assessed was chromosomal mutation (CBMN).

Santos et al. (2012) when performing experiments using the alkaline comet assay, observed genotoxicity effects of *B. platypetala* ethanolic extract in V79 cells in vitro at concentrations above 50 μg/mL. In the present study, the concentrations tested (7.5, 15 and 30 μg/mL) were not mutagenic and concentrations over 30 μg/mL have not been evaluated in mutagenicity testing because they were cytotoxic in MTT test.

These divergent results obtained with different extracts of species of the genus *Bauhinia* can be explained by the review published by Cechinel Filho (2009) on the chemistry and biological potential of different plants of this genus. He showed that the majority of compounds present in these plants are flavonoids, which have enormous potential for

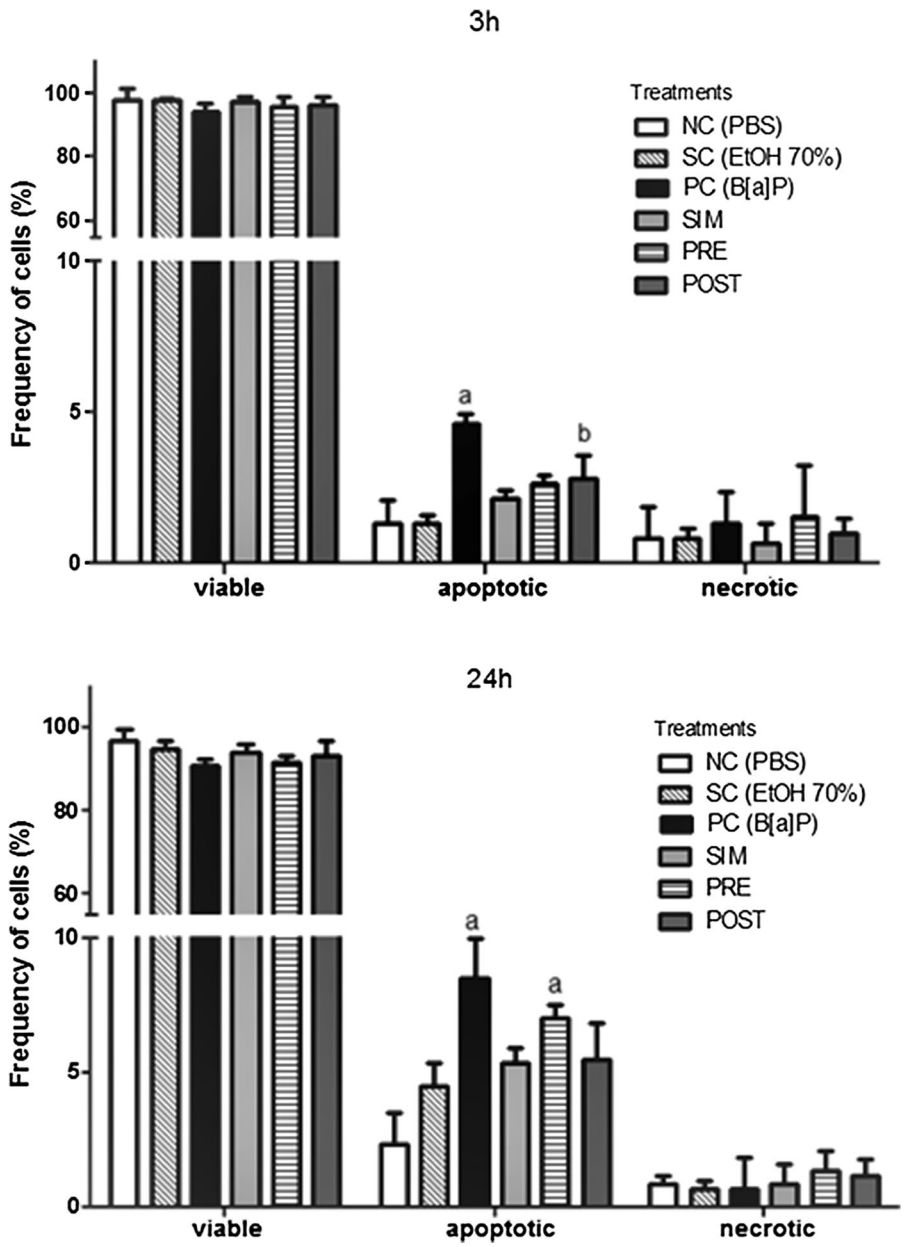


**Fig. 4** Percentage (%) of viable, apoptotic and necrotic HepG2 cells after 3 or 24 h of treatment with the *Bauhinia holophylla* extract and B[*a*]P using pre-treatment (PRE), simultaneous treatment (SIM) and post-treatment (POS) protocols. [a]Value significantly different from negative control by ANOVA followed by Tukey's test ($p \leq 0.05$). [b]Value significantly different from positive control by ANOVA followed by Tukey's test ($p \leq 0.05$). The values represent the mean ± standard deviation (X ± SD) of experiments performed in triplicate; a total of 600 cells was analyzed per treatment. NC: Negative Control (phosphate buffered saline; PBS); SC: Solvent Control (EtOH 0.7%); PC: Positive Control (B[*a*]P—504 µg/mL—20 µM)

treatment of diabetes; however, the quality and amount of these flavonoids vary from species to species and produce different biological results. The chemical profile of the extract used in the present study was determined by HPLC-PAD and FIA-ESI-IT-MS confirming the presence mainly of flavonol-o-glycosides derivatives of quercetin and isorhamnetin as previously described by Rozza et al. (2015).

The chemical constituents present in the *B. holophylla* extract could be also responsible by the protector effect observed against DNA damage induced by B[*a*]P. This substance is a known pre-carcinogen found in cigarette smoke, cooked food and fuel. B[*a*]P needs to be activated by the metabolism enzyme cytochrome P4501A1 (CYP1A1) during the metabolic process (also occurring in HepG2 cells) to then become an intermediate DNA-reactive metabolite with mutagenic action (Burczynski and Penning 2000; Caiment et al. 2015). This metabolite can cause oxidative damage to DNA by the production of

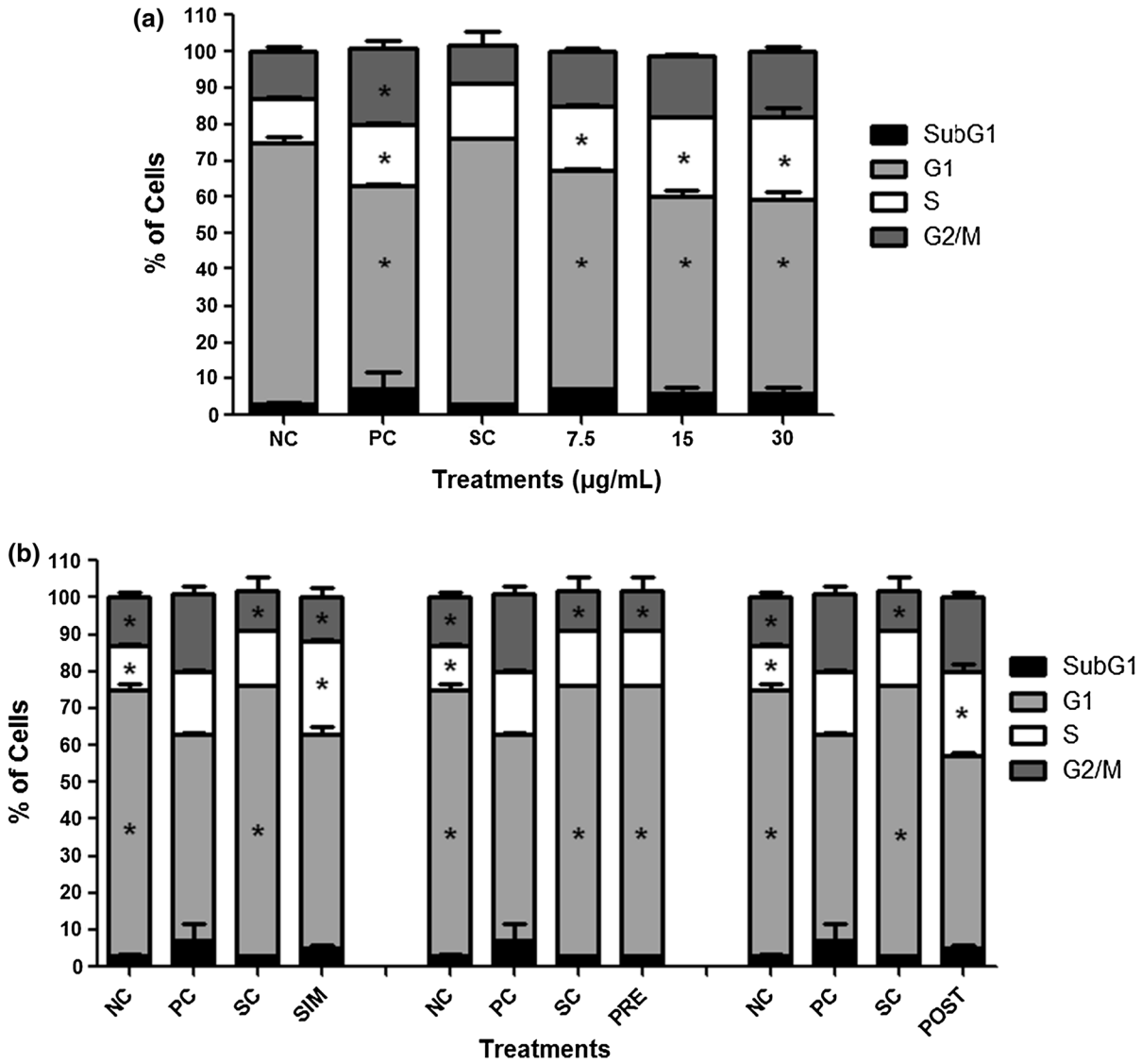


**Fig. 5** Frequency of HepG2 cells observed in each phase of the cell cycle by flow cytometry after treatment with **a** different concentrations of the *B. holophylla* extract and **b** *Bauhinia holophylla* extract and benzo[*a*]pyrene (B[*a*]P) in simultaneous (SIM), pre- (PRE) and post-treatment (POST) protocols. **a** * values were significantly different from the NC as analyzed by ANOVA followed by Dunnett's test ($p \leq 0.05$). **b** * values were significantly different from the PC as analyzed by ANOVA followed by Dunnett's test ($p \leq 0.05$). NC: Negative Control (phosphate buffered saline; PBS); SC: Solvent Control (EtOH 0.7%); PC: Positive Control (B[*a*]P—504 µg/mL—20 µM). X ± SD = mean ± standard deviation. SIM: simultaneous treatment, PRE: pre-treatment, POST: post-treatment

reactive species (RS), form adducts in the DNA molecule and cause damage to other cellular structures (Shiizaki et al. 2013). Due the fact of needs activation by cellular metabolism and by occasional detoxification by phase II enzymes (GSTs, UGTs), it is expected that the frequency of MNs after treatments with B[*a*]P will be lower compared to other traditional chemotherapeutics (Wu et al. 2003).

Several biological properties have been attributed to flavonoids and polyphenols such as antioxidants and anti-inflammatory (Kim et al. 2012; Hatia et al. 2014; Chen et al. 2014; Rozza et al. 2015). The antioxidant potential of these electron donors has been confirmed in vitro cell culture and cell-free systems that eliminated ROS (Roleira et al. 2015). Delgado et al. (2008) have shown that dietary phenolic compounds could

**Table 3** Gene expression analyzed by RT-qPCR in HepG2 cells after 24 h of treatment with *B. holophylla* (7.5 μg/mL) alone or in combination with benzo[*a*]pyrene (B[*a*]P) in post-treatment protocol and their respective control groups

| Gene symbol[c] | B[*a*]P | | *B. holophylla* | | EtOH 0.7% | | PBS + B[*a*]P | | B[*a*]P + *B. holophylla* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | FC[a] | $p^{(a)}$ | FC[a] | $p^{(a)}$ | FC[a] | $p^{(a)}$ | FC[b] | $p^{(b)}$ | FC[b] | $p^{(b)}$ |
| *CAT* | 1.384 | **0.006** | 1.395 | **0.009** | 1.306 | 0.161 | 0.948 | 0.546 | 1.275 | **0.045** |
| *NFE2L2* | 0.852 | 0.302 | 1.246 | 0.136 | 0.659 | 0.131 | 1.061 | 0.737 | 0.810 | 0.447 |
| *GPX1* | 0.951 | 0.673 | 1.024 | 0.916 | 0.938 | 0.805 | 1.050 | 0.878 | 0.938 | 0.869 |
| *GSR* | 0.677 | **0.008** | 0.813 | 0.073 | 1.190 | 0.411 | 0.883 | 0.572 | 1.138 | 0.212 |
| *XPC* | 0.916 | 0.576 | 0.916 | 0.394 | **1.491** | **0.133** | **2.362** | **0.017** | 0.919 | 0.740 |
| *TP53* | 0.698 | 0.099 | 1.046 | 0.832 | 1.052 | 0.853 | 1.580 | **0.009** | 0.745 | **0.024** |
| *CYP1A1* | **8.792** | **0.000** | **3.236** | **0.003** | **0.439** | **0.004** | **16.366** | **0.003** | 1.050 | 0.814 |

*Values in bold were considered significantly different compared to its respective control group ($p \leq 0.05$ + fold change (FC) higher than 2)

[a]Differences in gene expression were obtained comparing treatments with extract in relation to the negative control group (PBS)

[b]Differences in gene expression were obtained comparing the associated treatments in relation to the positive control group (PBS + B[*a*]P—PC/POST). All reactions were performed in technical triplicates. EtOH 0.7%: Solvent Control; B[a]P: 504 μg/mL—20 μM

[c]Gene list: *CAT*, *catalase*; *NFE2L2*, *nuclear factor erythroid 2-like-2*; *GPX1*, *glutathione peroxidase* 1; *GSR*, *glutathione reductase*; *XPC*, *xeroderma pigmentosum complementation group C*; *TP53*, *Tumor Protein p53*; *CYP1A1*, *cytochrome P450, family 1, subfamily A, polypeptide 1*

protect the DNA of HepG2 cells from pyrimidine breaks and DNA oxidation induced by B[*a*]P.

Data published in other studies with flavonoids such as quercetin and isorhamnetin, the main flavonoids found in the chemical analysis, showed that they possess antimutagenic effect and stimulate DNA repair mechanisms after oxidative damage in in vitro culture systems (Ramos et al. 2008; Rupasinghe et al. 2014; Roleira et al. 2015; Waizenegger et al. 2015). However, analyzing the data obtained from gene expression by RT-qPCR assays with *B. holophylla* extract and treatments associated with B[*a*]P, no alteration in the levels of mRNA of antioxidant genes (*CAT, GSR, GPX1, NFE2L2*), tumor suppressor (*TP53*) or DNA repair (*XPC*) pathways was found. The fact that may have contributed to protective effects of *B. holophylla* is that flavonoids may also induce phase metabolizing II enzymes, such as Glutathione-S-Transferases (GSTs), NAD(P)H: Quinone Oxidoreductases (NQOs) and UDP-glucuronosyltransferases (UGTs), who possibly detoxified mutagenic intermediates that become less reactive metabolites and reduce DNA damage (Rupasinghe et al. 2014; Chen et al. 2014). This can be explained by considering that in the post-treatment in CBMN and apoptosis assays (3 and 24 h) there was a significant reduction in the frequency of MNs and apoptotic cells.

Finally, it was found that the *B. holophylla* extract showed no cytotoxic effects, but showed high antiproliferative potential on HepG2 cells when tested alone or in treatments associated with B[*a*]P, as observed in the Nuclear Division Index (NDI) and in flow cytometry, especially at the higher tested concentrations. The significant increase in the population of cells in S phase in all treatment protocols (associated or not) may be due to an arrest of DNA synthesis and of the cell replication, induced by flavonoids constituents of the extract. Our data corroborate studies of other authors who have shown that flavonoids as quercetin (Zhang et al. 2012), disrupted cell cycle in S phase decreasing the proliferation in cell cultures. These events resulted in a decrease in the number cells that have completed the division, as demonstrated in the present study by the NDI, and in this case, it is possible that B. holophylla extract when at high concentrations may present a possible hazard to human cells due the antiproliferative potential.

Considering the parameters and conditions used in this study, it is clear that the data obtained are of great importance, since they indicate that the use of *B. holophylla* extract at low concentrations ($\leq$ 7.5 μg/mL) possibly does not cause adverse effects to human cells. Moreover, the extract showed protective effects that can be associated with a possible

chemopreventive activity against carcinogenic agents, such as B[*a*]P. However, despite of the significant results obtained in human metabolizing cells, an analysis of the molecular mechanism of action of this extract in the same cell type, such as evaluation of the expression of phase II metabolizing genes and in vivo testing should be performed to prove such effects and ensure accurately the absence of hazard from the use of this medicinal plant as well as its possible chemoprotective effect.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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