LDH, proliferation curves and cell cycle analysis are the most suitable assays to identify and characterize new phytotherapeutic compounds

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Abstract Brazilian flora biodiversity has been widely investigated to identify effective and safe phytotherapeutic compounds. Among the investigated plant species, the *Byrsonima* genus exhibits promising biological activities. This study aimed at evaluating the cytotoxicity of *B. correifolia*, *B. verbascifolia*, *B. fagifolia* and *B. intermedia* extracts using different assays in two cell lines (primary gastric and HepG2 cells). The different extract concentrations effects on cell viability were assayed using the MTT, aquabluer, neutral red and LDH assays. Non-cytotoxic concentrations were selected to generate cell proliferation curves and to assess cell cycle kinetics by flow cytometry. *Byrsonima* extracts differentially affected cell viability depending on the metabolic cellular state and the biological parameter evaluated. *B. fagifolia* and *B. intermedia*

extracts exhibited lower cytotoxic effects than *B. correifolia* and *B. verbascifolia* in all assays. The results obtained with LDH and flow cytometry assays were more reliable, suggesting that they can be useful in the screening for herbal medicine and to further characterize these extracts as phytotherapeutic compounds.

Keywords Antioxidant · *Byrsonima* sp. · Cytotoxicity · Cytostatic · Flow cytometry · Herbal medicine

Introduction

Plants are an important source of natural active compounds, many of which can be used as models

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for the new drugs synthesis, because they exhibit an almost inexhaustible range of diversity structure and physical–chemical and biological properties. Approximately 25 % of drugs prescribed worldwide are estimated to be derived from plants (Rates 2001). More than 50 % of the drugs that were approved over the last 30 years come directly or indirectly from natural products. In the cancer area, out of the 175 molecular drugs introduced between 1940s and 2010, 85 are natural products or derived from natural products (Newman and Cragg 2012).

Brazilian flora biodiversity has been widely investigated with respect to its therapeutic potential and has led to the identification of new phytotherapeutic compounds with demonstrated efficacy (Brasil 2006). Among the promising plant species, those belonging to the *Byrsonima* genus are noteworthy. This genus belongs to the family Malpighiaceae and contains approximately 150 species widely distributed throughout tropical America. Brazil is home to approximately 50 % of the *Byrsonima* species, which are found primarily in the North, Northeast and Central regions but may also be found in the Southeast region of the country, in “cerrado” areas (Brazilian savanna). In Brazil, these species are particularly known as “muricis” and, depending on the color of their flowers and fruits or place of occurrence, are differentiated as “murici da várzea”, “murici da mata”, or “murici-amarelo”, among others (revised by Guilhon-Simplicio and Pereira 2011).

Some *Byrsonima* species had already been characterized phytochemically such as *Byrsonima verbascifolia* (Dosseh et al. 1980), *B. intermedia* (Sannomiya et al. 2007) and *B. fagifolia* (Lima et al. 2008; Sannomiya et al. 2007a, b). All of them contain several constituents in common, like amentoflavone, (+)-catechin, (–)-epicatechin, and quercetin derivatives, but the amount present of these compounds differs (Sannomiya et al. 2005a, b, c; Rinaldo et al. 2010).

Some species exhibit high nutritional value and can be consumed as raw fruits or in the form of jams, sweets or ice cream. Furthermore, ethnopharmacological studies have demonstrated the popular use of *Byrsonima* plants for the treatment of diarrhea, fever, asthma, and skin infections (Guilhon-Simplicio and Pereira 2011). Research has confirmed the biological activities of these plants, especially with respect to topical and systemic anti-inflammatory activity (Maldini et al. 2009; Moreira et al. 2011), antioxidant (Pereira et al. 2015) and antimicrobial activities (Michelin et al. 2008).

Lima et al. (2008) and Santos et al. (2012), studying, respectively, *B. fagifolia* and *B. intermedia* extracts, observed gastroprotective and antidiarrheal activities, supporting the traditional use of this genus to treat gastrointestinal disorders. *B. verbascifolia* was identified as an antiviral agent (Cecilio et al. 2012). Data compiled from biological activities of *Byrsonima* species showed an incipient number of studies about *B. correifolia*.

Espanha et al. (2014) demonstrated that *B. correifolia*, *B. verbascifolia*, *B. fagifolia* and *B. intermedia* exhibit no mutagenic properties using the Ames test. Moreover, the authors have observed antimutagenic activity of all standardized extracts from these species. However, there are no data concerning the influence of these extracts on cell viability or any determination of their non-cytotoxic concentrations in human cell lines for the treatment of different diseases.

According to Weyermann et al. (2005) it is not advisable to conclude about cytotoxicity of a compound on the results obtained from only one method. Therefore, the aim of this study was to evaluate, using several assays, cell viability and non-cytotoxic concentrations of four extracts from different species of *Byrsonima*, *B. correifolia*, *B. verbascifolia*, *B. fagifolia* and *B. intermedia* and choose the most appropriate test to determine the cytotoxicity, measure the non-cytotoxic concentrations of extracts as a precondition for the use of these species as phytotherapeutic compounds and to evaluate their new biological activities.

Materials and methods

Plant material and extraction

The leaves of each species were collected in different locations in Brazil: *B. correifolia* A. Juss. (Voucher specimen number: 27151, deposited in Graziella Barroso Herbarium, at Federal University of Piauí) in Piauí (Northern region); *B. verbascifolia* (L.) DC. (Voucher specimen number: 481, deposited in Herbarium of the Federal University of Tocantins), *B. fagifolia* Nied. (Voucher specimen number: 743, deposited in Herbarium of the Federal University of Tocantins) in Tocantins (Midwest region) and *B. intermedia* A. Juss. (Voucher specimen number: 1426, deposited in UNICAMP Herbarium, in São Paulo (Southeast region)). The extraction process and

preparation was the same as that described by Espanha et al. (2014).

Cell lines and cell culture

Two human cell lines were employed: primary normal gastric epithelium (GAS) and hepatocellular carcinoma (HepG2). The first cell line was provided by Dr. Rommel Rodríguez Burbano from the Federal University of Pará (UFPA) and was cultivated in Dulbecco's Modified Eagle's Medium (DMEM—Gibco, Grand Island, NY, USA) high glucose, supplemented with 10 % fetal bovine serum (FBS—CAS: 12657-029, Gibco) and with 0.06 g/L penicillin (CAS: 113-98-4, Sigma-Aldrich, St. Louis, MO, USA), 0.10 g/L streptomycin (CAS: 3810-74-0, Sigma-Aldrich) and 0.024 % sodium bicarbonate (CAS: 1444-55-8, Sigma-Aldrich). The second cell line was provided by Dra. Lusânia Maria Gregg Antunes from the Faculdade de Ciências Farmacêuticas—Universidade de São Paulo and was cultivated in DMEM supplemented with 15 % FBS, antibiotic–antimycotic solution (CAS: 15240-062, Gibco) and sodium bicarbonate. Cultures were maintained at 37 °C in a 5 % CO₂ atmosphere and 95 % relative humidity.

Chemical agents

Benzo[a]pyrene—B[a]P (3,4-Benzo[a]pyrene—CAS: 50-32-8, Sigma-Aldrich), is an environmental pollutant carcinogen used in experimental models for cancer research (SIGMA-ALDRICH 2012). B[a]P is a polycyclic aromatic hydrocarbon (PAH) and was used as a DNA damage inducer at a concentration of 5.04 µg/mL. The B[a]P metabolites generated by the CYP1A1 enzyme acts as DNA intercalating agents and causes bases dissociation leading to the apurinic sites generation in the nucleic acid molecule (Uno et al. 2006).

Doxorubicin (DXR) (Adriamycin—CAS: 23214-92-8, Sigma-Aldrich) acts as a DNA intercalating agent in mammalian cells. DXR blocks DNA and RNA synthesis, inhibits DNA topoisomerase II and has clastogenic and aneugenic effects, causing single-strand breaks and membranes damage (Dhawan et al. 2003). This drug was used at a final concentration of 0.2 µg/mL.

Cytotoxicity assays

The four cytotoxicity assays used in this study were as follows: (1) MTT; (2) neutral red; (3) lactate dehydrogenase and (4) aquabluer.

Before treatments, the cells were sub-cultivated, washed with phosphate buffered saline (PBS) (pH 7.4) and trypsinized (trypsin 0.05 %—CAS: 25300-054—Gibco).

For all cytotoxicity assays, 1.0×10^4 HepG2 or GAS cells were seeded in 96-well culture plates 24 h prior to the treatments. After this period, the culture medium was removed and the cells were washed with PBS. Subsequently, in each well 200 µL of serum-free medium was added containing 1 of 10 different concentrations of plant extracts based on the maximal dilution obtained in the solvent (SV—7 H₂O: 3 MeOH). The final concentrations of the extracts in the cell cultures were 0.08; 0.15; 0.31; 0.61; 1.2; 2.4; 4.8; 9.75; 19.5 and 39 µg/mL for *B. correifolia*, and 0.15; 0.31; 0.61; 1.2; 2.4; 4.8; 9.75; 19.5; 39 and 78 µg/mL for *B. verbascifolia*, *B. fagifolia* and *B. intermedia*. These concentrations were obtained diluting the extracts in a stock solution of solvent concentrated 100×. From this, an aliquot was used to treat cells in a final methanol concentration in culture of 0.3 %. Cells treated only with PBS were used as negative controls, and another group was exposed to extract solvent (SV) for 24 h in order to prove that the solvent used is not cytotoxic. The EC₅₀ was calculated using the MTT and neutral red assays to determine the dose-dependent effect on cell lethality using the software CalcuSyn (Biosoft 1996–2005). Three non-cytotoxic concentrations were chosen for the cell proliferation and flow cytometry analyses. All experiments were performed in triplicate (three cultures/treatment).

MTT assay

The MTT assay described by Mosmann (1983) has been widely used as a measure of cytotoxicity and cell viability after the exposure of living cells to toxic substances. The mitochondrial capacity and integrity are related to cell viability (Bernhard et al. 2003). In this assay, the tetrazolium salt (-3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide—CAS: 298-93-1, Sigma-Aldrich) is yellow in color and is easily incorporated by viable cells and reduced by the

mitochondrial enzyme succinate dehydrogenase to the formazan compound, which is purple in color.

The technique was performed as described by Serpeloni et al. (2015) and Ribeiro et al. (2016). The results are expressed as the percentage of viable cells concerning the negative control group (PBS), which was considered to represent 100 % viability. Finally, the viability was obtained using the following formula:

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

AquaBluer

The cell viability was also assessed using the aquabluer assay (also known as Alamar Blue). This assay monitors the reducing environment of the living cell. Viable cells with active metabolism can reduce resazurin into the resorufin product that is pink and fluorescent (Rampersad 2012). The assay was carried out according to the manufacturer's recommendations (MultiTarget Pharmaceuticals LLC, Salt Lake City, UT, USA). The first step was to seed 1.0×10^3 of HepG2 cells in 96-well culture plates 24 h prior of the treatments. After this period, the culture medium was removed and the cells were washed with phosphate buffer saline (PBS). Subsequently, in each well 200 μ L of complete medium was added containing the same concentrations of plant extracts described in cytotoxicity assays section. Cells treated only with PBS were used as negative controls, and another group was exposed to extract solvent (SV) for 24 h. The plates were read in a fluorescence plate reader with intensity of 540ex/590em. The results are expressed as the percentage of viable cells with respect to the negative control group (PBS), which was considered to represent 100 % of viability; the cell viability was obtained using the same formula shown in “[MTT assay](#)” section.

Neutral red

The cell viability assessment using the neutral red assay was performed according to Repetto et al. (2008) with some modifications. The neutral red assay is based on the ability of viable cells to bind and incorporate the supravital dye neutral red (CAS: 553-24-2—Sigma-Aldrich).

This dye is weakly cationic and penetrates cell membranes by passive diffusion. The dye accumulates intracellularly in lysosomes, where it binds to anionic and/or phosphate groups of the lysosomal matrix. The quantity of dye incorporated into cells is measured by spectrometry and is directly proportional to the cell number with intact membranes.

After treatment, 100 μ L of neutral red solution (33 μ g/mL of neutral red powder diluted in PBS) was added per well, and the cells were incubated for 3 h at 37 °C. Subsequently, 0.2 mL of 1 % acetic acid in 50 % ethanol was added to each well, and the absorbance was read at 540 nm in a spectrophotometer.

The results are expressed as a percentage of viable cells concerning the negative control group (PBS), which was considered to exhibit 100 % viability; the cell viability was obtained using the same formula as shown in “[MTT assay](#)” section.

Lactate dehydrogenase assay

Cytosolic lactate dehydrogenase (LDH—CAS: 86-2-30—Labtest, Lagoa Santa, MG, BR) catalyzes the conversion of pyruvate to lactate in the presence of NADH. Cells with damaged membranes release the LDH enzyme into the extracellular medium. The increase in absorbance at 340 nm due to the oxidation of NADH is proportional to LDH activity in the sample. To determine total LDH activity, cells from the positive control group were treated with 1 % Triton 100 $\times$. After treatment, samples were analyzed using a commercial LDH kit following the manufacturer's recommendations, and LDH activity was expressed as U/mL.

Cell proliferation curves

Cell growth curves were generated by seeding 2.5×10^4 HepG2 or GAS cells per well in 24-well culture plate in a final volume of 2 mL of complete culture medium. Three concentrations of each extract were tested, and positive controls (B[a]P—5.04 μ g/mL for HepG2 cells and DXR—0.2 μ g/mL for GAS cells), a negative control (PBS) and a solvent control (SV—0.3 % of methanol in culture) were included. All treatments were performed with independent cultures in triplicate. The different concentrations of each extract were evaluated at 24, 48, 72, 96 and 120 h of exposure. After each exposure time, cells were trypsinized, and 20 μ L was collected to count the number of cells.

A proliferation curve based on the determination of the total protein content was also performed using the technique proposed by Da Costa Lopes et al. (2000), where the proteins were dyed with 1 % bromophenol blue. The steps were the same as performed by studies of Cilião et al. (2015) and Serpeloni et al. (2015).

Flow cytometry

The cell cycle analysis was performed as described by Ribeiro et al. (2016) and Serpeloni et al. (2015), 3.0×10^5 cells were seeded in complete culture medium in a final volume of 2.5 mL in 12-well plates and were treated according to the protocol described before. Control groups were also included (positive DXR 0.2 µg/mL for GAS cells and B[a]P 5.04 µg/mL for HepG2 cells), negative (PBS) and solvent (SV).

At the time of harvest, the medium was removed and reserved, and the cells were washed with PBS, trypsinized and resuspended with the medium previously reserved. Cell suspensions were centrifuged at 700g for 5 min. At this time, the supernatant was removed, and the pellet was washed with PBS, following further centrifugation. Subsequently, PBS was removed, and the pellet was resuspended in 2 mL of cold 70 % ethanol and kept at -20°C until analysis.

Before reading, the sample was stored at -20°C and it was centrifuged (700g, 4°C for 5 min), and 70 % ethanol was replaced by 1 mL of ice cold PBS, followed by additional centrifugation. After centrifugation the PBS was removed, and the cells were resuspended in 200 µL of propidium iodide solution (10 mL PBS: 10 µL Triton: RNase 100 µL: 40 µL propidium iodide) and kept at room temperature for 30 min. 5000 cells were analyzed using a FACS Vantage (Becton–Dickinson, Franklin Lakes, NJ, USA) flow cytometer. Data were analyzed using Cell Quest software (Becton–Dickinson), and cell cycle profiles were designed using the Flow Jo software (Tree Star Incorporation, Ashland, OR, USA). The results are expressed as the percentage of cells in different phases of the cell cycle: subG1 (cell death), G1, S and G2/M.

Data analysis

All results were evaluated using the GraphPad Prism 6[®] software system (La Jolla, CA, USA). Statistical analyses were conducted using one-way variance analysis (ANOVA). The samples homogeneity was

tested using Bartlett's test, followed by Tukey's test. All data are presented as the mean $\pm$ SD, with a significance level of $p \leq 0.05$.

Results

In the MTT assay, the *B. correifolia*, *B. verbascifolia* and *B. intermedia* species exhibited similar characteristics. The lowest concentrations of these extracts resulted in an increase in the viability rate to both cell lines analyzed (Fig. 1). This result prompted us to conduct the following experiments: (1) extracts incubation with tetrazolium salt in the cells absence; (2) cells incubation with extracts for 23 h followed by washing and lysis for 1 h and (3) use of the aqua bluer assay in HepG2 cells to assess if the redox state of the cells was responsible for the apparent cell viability increase (Fig. 2).

Concentrations equal to or greater than 1.2, 4.8 and 9.75 µg/mL of *B. correifolia*, *B. verbascifolia* and *B. intermedia*, respectively, reacted with the tetrazolium salt in absence of cells increasing the absorbance (data not showed) and the cellular lysates demonstrated that MTT reacted with *B. correifolia*, *B. verbascifolia* and *B. intermedia* at concentrations equal to and greater than 4.8; 19.5 and 78 µg/mL in HepG2 cells and 9.75; 39 and 78 µg/mL in GAS cells, respectively, also increasing the cell viability (data not showed). However, this increase in the cell viability at lower concentrations was not observed when the aquabluer assay was used (Fig. 2). Likewise, when the neutral red (Fig. 3) and LDH (Fig. 4) assays were used in both cell lines, no increase in cell viability was observed. These data show that the MTT assay is not recommended, at least not alone, to evaluate the cytotoxicity of plant extracts.

Byrsonima correifolia

The aquabluer assay results showed that the *B. correifolia* concentrations equal or greater than 1.2 µg/mL in HepG2 were cytotoxic (Fig. 2a). In the neutral red assay, concentrations equal to or greater than 2.4 or 0.61 µg/mL in HepG2 and GAS cells, respectively, resulted in cytotoxicity (Fig. 3a1, a2). In the LDH assay (Fig. 4) the cytotoxic concentrations were equal or higher than 4.8 µg/mL for both cell lines (Fig. 4a2). It is possible to note that regardless of the assay performed, the highest concentrations of the extracts were always cytotoxic.

Fig. 1 Percentage of viable HepG2 (1) and GAS (2) cells after 24 h treatment with different concentrations of extracts (MTT assay): **a** *B. correifolia*; **b** *B. verbascifolia*; **c** *B. fagifolia*; and **d** *B. intermedia*; negative control—NC (PBS) and solvent (SV) groups. The results represent mean $\pm$ SD of independent cultures in triplicate. NC represents 100 % viability. PBS phosphate buffered saline. Values significantly differing from the negative control group by ANOVA and Tukey test: hash symbol increase and asterisk decrease

The EC_{50} values calculated using neutral red assay data were 11.44 $\mu\text{g/mL}$ ($r = 0.721$) and 16.11 $\mu\text{g/mL}$ ($r = 0.952$) to HepG2 and GAS cells, respectively.

Concentrations of 0.08, 0.31 and 1.2 $\mu\text{g/mL}$ were selected based on the results of the three cell viability assays described above for the evaluation of cell proliferation curves and flow cytometry analysis.

Fig. 2 Evaluation of viability of HepG2 cells treated with **a** *B. correifolia*; **b** *B. verbascifolia*; **c** *B. fagifolia*; and **d** *B. intermedia*; negative control—NC (PBS) and solvent (SV) in aquabluer assay. The results represent mean $\pm$ SD of independent cultures. NC represents 100 % viability. Asterisk values differ significantly from the negative control group by the ANOVA and Tukey test ($p \leq 0.05$)

The proliferation curve results demonstrated that the three concentrations did not affect the cell viability or cell proliferation of either cell line even after 120 h of treatment (Fig. 5a).

The cell cycle analysis in HepG2 cells (Fig. 6a1) revealed a decrease in the cell number in the G_1 phase of the cell cycle at concentrations of 0.08 and 1.2 $\mu\text{g/mL}$ relative to the negative control group. An increase in the cells number in the sub G_1 phase was also observed in the 0.31 and 1.2 $\mu\text{g/mL}$ treatments. In GAS cells, treatment with 0.08 $\mu\text{g/mL}$ resulted in no change in cell cycle distribution relative to the negative control (Fig. 6a2), whereas the concentrations of 0.31 and 1.2 $\mu\text{g/mL}$ induced an increase in the cell number in sub G_1 and decrease in the cell number in G_1 . Furthermore, the 1.2 $\mu\text{g/mL}$ concentration increased the cell number in G_2 (Fig. 6a2).

Byrsonima verbascifolia

HepG2 and GAS cells treated with different concentrations of the *B. verbascifolia* extract exhibited different responses to the tests performed.

In aquabluer assay it is possible to note that the concentrations of *B. verbascifolia* equal or greater than 1.2 $\mu\text{g/mL}$ presented cytotoxicity to HepG2 cells

(Fig. 2b). In the neutral red assay (Fig. 3), a decrease in cell viability was observed at all concentrations, except for 0.15 and 0.31 $\mu\text{g/mL}$ in HepG2 cells (Fig. 3b1) and 0.15 $\mu\text{g/mL}$ in GAS cells (Fig. 3b2). However, in the LDH assay (Fig. 4), concentrations between 19.5 and 78 $\mu\text{g/mL}$ (Fig. 4b1, b2) were cytotoxic for both cell lines, and 9.75 was also cytotoxic to GAS cells.

The EC_{50} values were calculated for HepG2 cells (5.41 $\mu\text{g/mL}$; $r = 0.918$) and for GAS cells (342.12 $\mu\text{g/mL}$; $r = 0.73$) using the neutral red assay.

Based on aquabluer, neutral red and LDH tests, the concentrations of 0.15; 0.61 and 2.4 $\mu\text{g/mL}$ were selected for the evaluation of cell proliferation curves and flow cytometry. Through both counting cell numbers and measuring protein levels (Fig. 5b1, b2), we determined that HepG2 and GAS exhibited the same profile of proliferation in response to treatment.

The flow cytometry data of the HepG2 cells demonstrated that none of the concentrations interfered with the cell cycle distribution relative to the negative control (Fig. 6b1). Nevertheless, in GAS cells (Fig. 6b2), all concentrations reduced the cell number in the G_1 phase, whereas the concentrations 0.15 and 0.61 $\mu\text{g/mL}$ increased the cell number in the S and G_2/M phases.

Fig. 3 Cell viability of HepG2 (1) and GAS (2) cells 24 h after treatment with different extracts concentrations (Neutral Red assay): **a** *B. correifolia*; **b** *B. verbascifolia*; **c** *B. fagifolia*; and **d** *B. intermedia*; negative control—NC (PBS) and solvent (SV). The results represent mean $\pm$ SD of independent cultures in triplicate. NC represents 100 % viability. Asterisk values differ significantly from the negative control group by the ANOVA and Tukey test ($p \leq 0.05$)

Byrsonima fagifolia

The aquabluer results showed that the *B. fagifolia* concentrations equal or greater than 9.75 µg/mL decreased HepG2 cells viability (Fig. 2c).

In the neutral red assay (Fig. 3c) and LDH assay (Fig. 4c), only the highest concentration tested (78 µg/mL) was cytotoxic for both cell lines. This extract exhibited low cytotoxicity in both cell lines, with EC₅₀ values higher than the

Fig. 4 Lactate dehydrogenase enzyme activity of HepG2 (1) and GAS (2) cells after 24 h treatment with different concentrations of extracts (LDH assay): **a** *B. correifolia*; **b** *B. verbascifolia*; **c** *B. fagifolia*; **d** *B. intermedia*; negative control—NC (PBS); positive control—PC (1 % Triton 100×) and solvent (SV) groups. The results represent mean values $\pm$ SD of independent cultures in triplicate. Asterisk values differ significantly from the negative control group by ANOVA and Tukey test ($p \leq 0.05$)

Fig. 5 Proliferation curves of HepG2 (1) and GAS (2) cells by total proteins quantification ($\log_2$ mean of absorbance) after treatment with extracts of **a** *B. correifolia*; **b** *B. verbascifolia*; **c** *B. fagifolia*; **d** *B. intermedia*; negative control (PBS); positive controls (B[a]P—5.04 $\mu\text{g}/\text{mL}$ and DXR—0.2 $\mu\text{g}/\text{mL}$) and solvent (SV). The results represent mean $\pm$ SD of independent cultures in triplicate

concentrations tested in neutral red assay (data not shown).

The concentrations of 0.15, 0.61 and 2.4 $\mu\text{g}/\text{mL}$ were selected for the evaluation of cell proliferation curves and flow cytometry analysis. As with other extracts, the three concentrations of *B. fagifolia* evaluated using cell growth curves had no effect on the proliferation rate of either cell line tested (Fig. 5c).

The flow cytometry data (Fig. 6) revealed that the lowest concentration (0.15 $\mu\text{g}/\text{mL}$) decreased the number of HepG2 cells in the G₁ phase, and the concentration of 0.61 $\mu\text{g}/\text{mL}$ increased the cell number in the G₁ and S phases relative to the negative control group (Fig. 6c1). With respect to GAS cell, at lower (0.15 $\mu\text{g}/\text{mL}$) and higher (2.4 $\mu\text{g}/\text{mL}$) concentrations, a decrease in the cell number in the G₁ phase was observed, and an increase in the cell number in S

Fig. 6 Percentage of HepG2 (1) and GAS (2) cells in different phases of the cell cycle after treatment with extracts of **a** *B. correifolia*—Bc; **b** *B. verbascifolia*—Bv; **c** *B. fagifolia*—Bf; **d** *B. intermedia*—Bi; negative (PBS); positive (B[a]P—5.04 µg/mL or DXR 0.2 µg/mL) and solvent (SV) control groups. The

cultures were exposed to the compounds for 24 h. The results represent mean values $\pm$ SD of independent cultures in triplicate. Asterisk values differ significantly from the negative control group as analyzed by the ANOVA and Tukey test

phase was observed at higher concentrations (Fig. 6c2).

Byrsonima intermedia

The results of aquabluer assay demonstrated that the concentrations of *B. intermedia* equal or greater than 2.4 µg/mL reduced the HepG2 cell viability (Fig. 2d).

In the neutral red assay (Fig. 3), concentrations between 9.75 and 78 µg/mL were cytotoxic to HepG2 cells, and concentrations between 2.4 and 78 µg/mL were cytotoxic to GAS cells (Fig. 3d1, d2). In the LDH assay (Fig. 4), concentrations between 9.75 and 78 µg/mL were cytotoxic in HepG2 cells (Fig. 4d1), and none of the concentrations were cytotoxic in GAS cells (Fig. 4d2). The EC₅₀ values in the neutral red assay were 44.27 µg/mL ($r = 0.913$) for HepG2 cells and for GAS cells, the EC₅₀ value fell outside the range of concentrations tested (data not shown). These data demonstrate different responses between the cell lines evaluated.

Concentrations of 0.15, 0.61 and 2.4 µg/mL were selected for cell proliferation curves evaluation (Fig. 5) and flow cytometry (Fig. 6). The proliferation curves data indicated treatment of HepG2 and GAS cells with the extract had no effect on the proliferation rate relative to untreated cells (Fig. 5d1, d2).

However, the cell cycle analysis for HepG2 cells (Fig. 6d1) revealed that 0.15 µg/mL extract reduced the cells number in the G₁ phase and increased the cell number in the G₂/M phase. The concentration of 0.61 µg/mL decreased the cell number in the G₁ phase, and the highest concentration increased the cell number in the G₁ phase and decreased the cell number in S phase (Fig. 6d1).

The three concentrations similarly interfered in the cell distribution of GAS cells by reducing the cell number in the G₁ phase and increasing the cell number in the S and G₂/M phases (Fig. 6d2).

Discussion

In Brazil, the *Byrsonima* genus is widely used in folk medicine for the treatment of many diseases (Guilhon-Simplicio and Pereira 2011). Species of this genus possess different pharmacological activities, which may be related to the presence of chemical compounds such as catechins and derivatives, gallic acid and its

derivatives, and flavonoids (Sannomiya et al. 2005a, b, c, 2007a, b; Rinaldo et al. 2010; Guilhon-Simplicio and Pereira 2011).

To analyze the cell viability and cytotoxic properties of potential new phytotherapeutics, which are drugs with different chemical compounds, the best approach is to use several methods, thus analyzing different cellular parameters to reduce confounding factors and obtain reliable results (Weyermann et al. 2005; Abdullah et al. 2014). In the present study, different assays were employed to evaluate cytotoxicity to different cellular targets: respiratory chain activity (MTT), reducing cellular environment (Aqua-blue), membrane integrity (LDH) and lysosomal activity (Neutral Red). The MTT assay is used in many areas, especially in the identification of new drugs; however, this assay has limitations. The disadvantages of this assay have been demonstrated since it was proposed by Mosmann (1983) and the limitations of this assay that interfere in the results are: presence of polyphenolic hydroxyl groups, pH and components of the medium, metabolism and cell concentrations (Scudiero et al. 1988; Plumb et al. 1989; Mei Han et al. 2010; Wang et al. 2011; Van Tonder et al. 2015).

The four *Byrsonima* extracts exhibited differential effects on cell viability depending on the methodology applied, and on the qualitative and quantitative differences in the chemical compounds, as related by Sannomiya et al. (2005a, b, c) and Rinaldo et al. (2010). The extracts assessed in the present study contain considerable levels of polyphenols, mainly quercetin (Guilhon-Simplicio and Pereira 2011).

The phytochemical studies reported in the literature with polar extracts of leaves of *Byrsonima* species such as *B. crassa*, *B. intermedia* and *B. basiloba* showed the occurrence of (+)-catequin, (–)-epicatechin, proanthocyanidins, glycosylated flavones, amantoflavone, gallic acid, methyl gallate and galloyl quinic acid derivatives (Sannomiya et al. 2005a, b, c, 2007a, b).

B. fagifolia differs from the other studied species from the point of chemical view. Four glycosylated flavones were isolated from the polar extract while twenty-one galloyl quinic acid derivatives and five flavonoids were identified from the infusion of the leaves (Sannomiya et al. 2007a, b; Lima et al. 2008). This fact can explain the low cytotoxicity of *B. fagifolia* relative to the others species (*B. coriifolia*

and *B. verbascifolia*) in the MTT, aquabluer, neutral red and LDH assays (Figs. 1c, 2c, 3c, 4c). As *B. fagifolia*, the extract of *B. intermedia* also exhibited a low cytotoxic effect (Figs. 1d, 2d, 3d, 4d) and amentoflavone is present in high concentrations in *B. intermedia*.

The in vitro cytotoxicity assays are the first parameters to evaluate the toxicity of a compound and determine non-cytotoxic concentrations of new drugs (OECD—Genetic Toxicology Test Guidelines 2015). The toxicity is the major cause for the high failure rate (40–50 %) of pharmaceutical drug (Sumantran 2011) but also false the positive results in mutagenicity tests (Kirkland et al. 2007). So, taking into account this and according to the importance of medicinal plants as a source of new drugs (Newman and Cragg 2012), the low cytotoxicity of *B. fagifolia* and *B. intermedia*, combined with the ethnopharmacological knowledge of these species (Guilhon-Simplicio and Pereira 2011) encourage future studies to investigate the therapeutic potential and action mechanism of *B. fagifolia* and *B. intermedia* species.

Contrary to what was obtained for *B. fagifolia* and *B. intermedia*, many cytotoxic concentrations were detected when HepG2 and GAS cells were treated with extracts of *B. correifolia* and *B. verbascifolia*; the EC₅₀ values calculated for both extracts were among the 10 concentrations evaluated in the neutral red assays.

Given the evidence that molecules that have cytotoxic effect may also have anticancer activity, detecting compounds with cytostatic effect is extremely important for the identification of new anticancer agents (Sumantran 2011; Di Giorgio et al. 2011). Therefore, the population should carefully use *B. correifolia* and *B. verbascifolia* extracts, and investigations of these extracts should be restricted to therapeutic use in cancer treatment.

The assay based on the ability of viable cells to bind and incorporate the supravital dye neutral red exhibited a good sensitivity. However, some chemicals can induce irreversible precipitation of neutral red dye in fine crystals, resulting in an overestimation of toxic effects (Repetto et al. 2008). This effect may explain the apparent increase on toxicity observed in the present study for all concentrations (except 0.15 µg/mL) of *B. verbascifolia* in both cell lines (Fig. 2b). The data obtained with flow cytometry and proliferation curves confirmed that the concentrations of 0.61

and 2.4 µg/mL (Fig. 2b) did not increase the cell number in subG₁ phase and did not change the cell cycle kinetics.

The loss of integrity or damage of cellular membranes due to cell death results in the release of cytosolic enzymes as LDH. Enzyme activity level correlates with the level of cell death/membrane damage and provides a precise measurement of cytotoxicity induced by chemical compounds (Korzeniewski and Callewaert 1983).

Ulukaya et al. (2008) suggested caution in comparing cell viability assays because the MTT assay, depending on the drugs of choice, seem to give rise to higher viability levels than the ATP assay. Using plant extracts of *Mangifera indica*, Abdullah et al. (2014) detected a high correlation among the MTT, LDH and neutral red assays.

In the present study, after exposure to *Byrsonima* extracts, cytotoxicity was measured with the MTT, aquabluer, LDH, neutral red assays, and the different sensitivities of each assay were analyzed. The LDH assay demonstrated to be the most reliable once and no false positive or negative results were observed.

After performing the cytotoxicity assay, we selected three non-cytotoxic concentrations of each extract to identify possible therapeutic concentrations for the production of phytotherapeutic compounds. It is extremely important to evaluate how the concentrations of these extracts influence cell proliferation because it is possible to observe cytostatic effects independent of cytotoxic effects. To evaluate the cell proliferation, the best choice is to measure total cellular protein content, which is not dependent of cell metabolism (Skehan et al. 1990; Van Tonder et al. 2015). For this, sulforhodamine B (SRB) or bromophenol blue (Skehan et al. 1990; Cilião et al. 2015; Serpeloni et al. 2015) can be used as dye of cellular total protein content. The results obtained using the cell proliferation curves with bromophenol blue suggest that the three concentrations of all extracts evaluated did not alter the proliferative rate of either cell line in long-term treatment (Fig. 5).

Flow cytometry is a sensitive technique for cell cycle analysis. It evaluates individual cells by morphology, DNA content and cell viability. Concentrations of 0.31 and 1.2 mg/mL of *B. correifolia* extract induced cell death in flow cytometry analysis in both cell lines tested (Fig. 6) while the neutral red assay also detected decrease in cell viability but it was not

significant (Fig. 3a1, a2). The cell death was not observed in the LDH assay because this assay detects the release of the enzyme lactate dehydrogenase by cells with damage, which does not occur during the apoptosis (Fig. 4a1, a2).

Regarding *B. verbascifolia*, it is clear that all concentrations of this extract did not affect the cell cycle of HepG2 cells, whereas in GAS cells the lowest concentrations decreased the cell number in G₁ phase but increased the cell number in G₂/M, which could indicate that the cell cycle arrest was due to DNA repair. These data are consistent with those of Zhang et al. (2009), who demonstrated that flavonols like quercetin were able to induce cell cycle arrest in the G₂/M phase in the human esophageal squamous cell carcinoma cell line (KYSE-510). The cells accumulation in G₂ may be due to DNA damage checkpoint (that may sense some of the persistent DNA lesions from the previous S phase as being inappropriately or not fully replicated DNA) (Kastan and Bartek 2004) and prevents the mitosis onset. Therefore, flow cytometry analysis was very useful to indicate how low and moderate cytotoxic concentrations of these extracts influence the cell cycle.

A comprehensive cytotoxic effect determination of plant extract requires methods that evaluate cell proliferation rate (growth curve) of cell lines exposed to short and long term treatments; and the use of various methodologies with different cellular targets, directed for detecting dead/damaged cells (LDH assay), and to detect a decrease in viable cell metabolism (MTT test, neutral red and aquabluer), avoiding false negative/positive and ensuring more reliable results (Weyermann et al. 2005; Abdullah et al. 2014).

Conclusion

The present study demonstrated that the most appropriate tests to determine the cytotoxicity and/or measure the non-cytotoxic concentrations of new phytotherapeutic compounds are the LDH assay, proliferation curve and cell cycle analysis by flow cytometry. *B. correifolia* and *B. verbascifolia* should be used carefully in light of their cytotoxic effects, whereas *B. fagifolia* and *B. intermedia* exhibit low cytotoxicity and should be further investigated for their use as chemopreventive agents. Although the

Byrsonima genus is extensively used in Brazilian folk medicine, this study is the first to assess the effects of these extracts on human cell lines and contribute to characterize these species as herbal medicines.

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Compliance with ethical standards

Conflict of interest The authors have declared no conflicts of interest.

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