Original Research

Maytenus ilicifolia dry extract protects normal cells, induces apoptosis and regulates Bcl-2 in human cancer cells

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Abstract

Maytenus is the largest genus of the family Celastraceae and the species *Maytenus ilicifolia* (popularly known as 'Espinheira Santa'). It is widely used in traditional Brazilian medicine to treat stomach conditions including nausea, gastritis, and ulcers. In this study, the apoptotic effects of a spray-dried extract of *M. ilicifolia* (SDEMI) was evaluated using human hepatocellular cells (HepG2), colorectal carcinoma cells (HT-29), and normal keratinocytes (HaCaT). Cells were treated with SDEMI for 4 and 24 h, then were assayed for levels of apoptosis, caspase-3, and Bcl-2 by flow cytometry, immunostaining, and Western blot, respectively. Significant differences between groups were determined using analysis of variance ($P < 0.05$). For HepG2 and HT-29 cells treated with SDEMI, various cytotoxic effects were observed compared with control cells at all timepoints assayed ($P < 0.001$). Furthermore, positive caspase-3 staining and down-regulation of Bcl-2 were observed, consistent with the induction of cell death detected in these cell lines. In contrast, treatment of HaCaT cells with SDEMI was associated with a protective effect compared with control cells at both timepoints ($P < 0.001$). For example, increased expression of Bcl-2 and negative caspase-3 staining were detected. Taken together, these results suggest that SDEMI protects normal cells, while SDEMI mediates induction of apoptosis via down-regulation of Bcl-2 and involvement of caspase-3 in human carcinoma cells.

Keywords: plants, human cancer cells, caspase 3, Bcl-2, apoptosis

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Introduction

Currently, the mechanism of action of many anticancer compounds or drugs involves their capacity to promote apoptosis. Moreover, there are many mechanisms by which apoptosis can be induced.[1] Compounds that suppress the proliferation of malignant cells by inducing apoptosis represent a useful mechanistic approach for both cancer chemoprevention and chemotherapy. However, unfavourable side effects and the development of resistance associated with many anticancer agents that are widely used represent significant challenges.

Drug resistance is a major problem in the treatment of many diseases, including cancer. Certain cancer cells have been well-characterized for their resistance to chemotherapy in the clinic. In addition, increasing evidence strongly suggests that drug-resistant cancer cells that survive chemotherapy are responsible for the reappearance of tumours and a poor prognosis. Accordingly, drug resistance leads to the failure of tumour treatment.[2] Therefore, new methods are needed to overcome cancer resistance to conventional treatments. Since natural compounds can modulate apoptosis pathways that are frequently blocked in human cancers, these compounds may

provide novel opportunities for cancer drug development.[3] In particular, extracts of plants of the family Celastraceae are used as insecticides in traditional agriculture, as well as for the treatment of stomach problems, fever, rheumatism, arthritis, and cancer.[4] Maytenus is the largest genus of this family,[5] and the species, Maytenus ilicifolia, is referred to as Espinheira Santa in Brazilian herbal medicine. Espinheira Santa is well-known for its ability to alleviate stomach pain and nausea, as well as for its treatment of ulcers, gastritis,[6,7] and inflammation.[8] Moreover, in vitro, the leaves of M. ilicifolia have been shown to relax the endothelium of rat aortic rings.[9]

Analyses of the active compounds in M. ilicifolia have identified flavonoids and tannins.[10,11] Correspondingly, an anticancer effect of the genus M. ilicifolia has previously been reported,[12] with its plant extracts affecting signalling pathways affecting apoptosis.[13,14] In particular, two major pathways have been well-characterized. One involves a mitochondrial pathway, where Bcl-2 family members, including the anti-apoptotic Bcl-2 protein,[15] are responsible for regulating apoptosis in response to various stimuli. Accordingly, mitochondria are key mediators of cell apoptosis. In the present study, the objective was to evaluate whether M. ilicifolia can selectively induce apoptosis and down-regulation of Bcl-2 in human carcinoma cells versus normal cells.

Materials and methods

Plants

Aerial parts of M. ilicifolia were supplied by CPQBA-UNICAMP (Centro Pluridisciplinar de Pesquisas Clínicas, Biológicas e Agrícolas-Universidade Estadual de Campinas-Campinas, SP, Brazil). A voucher specimen was deposited at the UFRGS herbarium under number ICN111772.

Spray-dried extract of *M. ilicifolia* (SDEMI)

Dried and ground leaves of M. ilicifolia were extracted by infusion with distilled water (1 : 10, w/v) for 15 min. Colloidal silicon dioxide (Aerosil 200, Degussa, São Paulo, Brazil) was added to the miscella in a 2 : 8 ratio of adjuvant to dry residue.[16] The dispersion was dried using a Production Minor spray-dryer (GEA, Copenhagen, Denmark) provided with a rotating disk. The operational conditions were 9500 rpm rotational disk speed, 149°C inlet temperature, 99°C outlet temperature, and 140 mL/min feed ratio. Quantitative analyses of SDEMI were performed using liquid chromatography according to Soares et al.[17] Catechin and epicatechin content in SDEMI was calculated to be 10.8 and 25.7 μg/mg, respectively.

Cell culture

Non-cancerous keratinocytes (HaCaT), and the cancer cell lines, HT-29 a colorectal adenocarcinoma and HepG2 a hepatocarcinoma were purchased from the Culture Collection of the Faculty of Medicine (University of São Paulo, São Paulo, Brazil). All cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (CULTILAB LTDA/Brazil). Three concentrations (0.5, 1.0, or 2.0 mg/mL) of SDEMI were used in culture medium. SDEMI solution was filtered using a 0.22 mm minipore membrane, aliquoted, and stored at −20°C. SDEMI was thawed just prior dilution in culture medium.

Cell viability

Due to the different sensitivity of cancer cells to the SDEMI, the optimal exposure time for each cell line was determined in a pilot study in order to obtain dose-dependent effect. Viability was determined at 24 h for HT-29 and HepG2. Cell viability was determined by trypan blue exclusion assay. Briefly, cell aliquots were mixed with same volume of 0.5% (w/v) trypan blue and incubated at room temperature for 5 min. The number of viable cells was calculated using a haemocytometer.

Flow cytometry

To verify and quantify cells treated with SDEMI, and to identify cell cycle stage and cell viability, cells were stained with propidium iodide (PI). PI emits different fluorescence intensities depending on the phase of the cell cycle, and these differences can be analysed by photomultiplier detectors in a flow cytometer (BD Facscanto II). For these assays, cells were plated in six-well plates (5×10^5 cells/well) in 2 mL medium/well. After 24 h, cells were treated with varying concentrations of SDEMI for 4 or 24 h. Control cells were maintained in culture medium without SDEMI. When cells reached approximately 80% confluence, they were harvested, added to 70% ethanol and centrifuged (5 min at 300 × g). Cell pellets were resuspended in 200 μL PI solution (20 mL phosphate-buffered saline (PBS), 20 μL 0.1% Triton X-100, 200 mg/mL RNase, 20 μg/mL PI), and incubated for 30 min at room temperature (RT) in flow cytometry tubes protected from light. Samples were analysed using a FACScalibur cytometer (BD Bioscience, Franklin Lakes, NJ, USA) and CELLQuest™ software (BD Biosciences). The percentage of cells in each phase of the cell cycle was quantified and data are presented in histograms.

Caspase-3 activity

Cultured cells were plated on glass coverslips in 24-well plates (5×10^4 cells/well) and grown for 3–7 days. After 24 h, they were treated with the SDEMI (0.5, 1.0 or 2.0 mg/mL) for 4 or 24 h. The cells were then washed, fixed with paraformaldehyde, permeabilized by Triton-X, and incubated with rabbit polyclonal anti-caspase-3 (Abcam, San Francisco, CA, USA) diluted 1 : 500 in PBS containing bovine serum albumin (5%; Life Technologies do Brasil LTDA, São Paulo, Brazil) for 1 h at RT in a humid atmosphere. The primary antibody was detected with Alexa Fluor 488 goat anti-rabbit secondary antibody (Abcam), and 4,6-diamidino-2-phenylindole (Life Technologies do Brasil LTDA) was used for nuclear staining. The immunostained coverslips were examined under a LSM 510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany). Images were obtained with laser excitation at 442 nm. Images selected were representative of the majority of the cells.

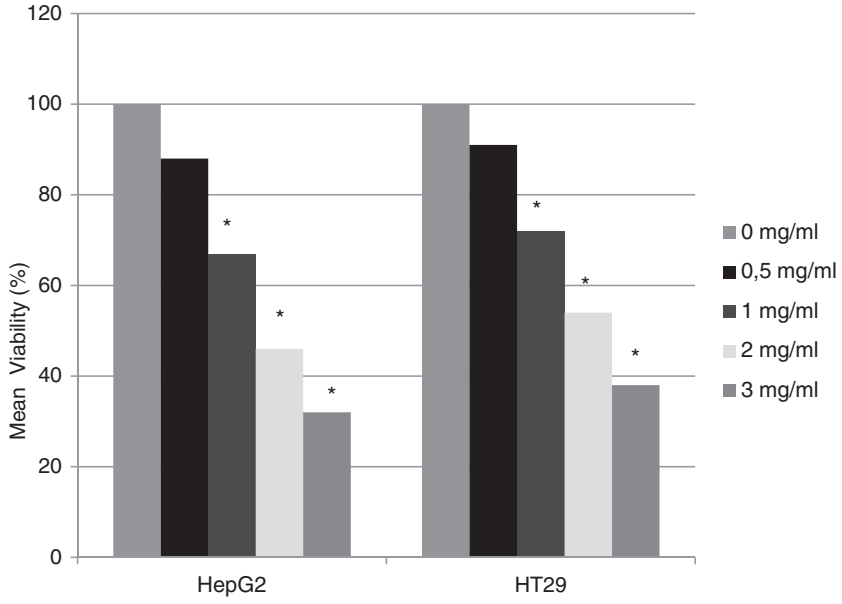

Figure 1 Effect of *Maytenus ilicifolia* dry extract (SDEMI) on the viability of various cancer cells derived from different origins. Cells were treated with different concentrations of SDEPN as indicated. The exposure time for HepG2 and HT-29 24 h. Cell viability was calculated by trypan blue exclusion assay (mean % viability). The asterisk (*) represented significantly different value from control ($P < 0.05$)

Western blotting

Equal amounts of protein (50 µg/lane) from treated and untreated cells were loaded and electrophoresed on 12% sodium dodecyl sulphate-polyacrylamide gels. Proteins were then blotted onto polyvinyl-difluoride membranes (PolyScreen, NEN Life Sciences, USA). The membranes were subsequently dried and blocked in 5% non-fat milk in PBS/0.1% Tween-20. Membranes were then incubated with a mouse Anti-Bcl-2 (Abcam) diluted 1 : 2000, followed by an appropriate secondary antibody conjugated to horseradish peroxidase (1 : 1000). Bcl-2 bound antibodies were detected using an enhanced chemiluminescence system (Amersham, USA) and the signals recorded on x-ray film. Membranes were then incubated with β-actin antibody (Sigma Chemical Co., St Louis, MO, USA) and an appropriate secondary antibody as a loading control.

Statistical analysis

All experiments were performed at least in triplicate, and significant differences between groups were calculated using analysis of variance and Bonferroni's test, as indicated. A P-value less than 0.05 was considered statistically significant.

Results

Viability of different cancer cells after SDEMI treatment

To fully characterize the anticancer potential of the SDEMI, we analysed its effect on the viability of various cancer cells derived from different origins including colorectal adenocarcinoma and hepatocarcinoma. The viability of these different cancer cells was significantly decreased by SDEMI treatment in a dose-dependent manner as shown in Figure 1. However, different cancer cells responded to SDEMI with different sensitivity. SDEMI exerted a potent effect on reducing the viability of hepatocarcinoma and adenocarcinoma cells. The doses that were near the half maximal inhibitory concentration (IC_{50}) were 1.0 and 2.0 mg mL. The viability of these different cancer cells was significantly decreased by SDEMI treatment in a dose-dependent manner shown in Figure 1, which show that the IC_{50} doses of SDEMI are 1.0 and 2.0 mg/mL, respectively. The dose of 0.5 mg/mL was included in the assay, because smaller doses have lower toxicity, mainly in normal cells. These concentrations were chosen for the following steps.

Detection of apoptosis

Flow cytometric analysis was performed to detect hypodiploid cells, counted as cells with less DNA content than that of cells in the G1 phase of the cell cycle. Figure 2 shows the hypodiploid population present in normal HaCaT cells treated with 2 mg/mL SDEMI. The hypodiploid populations of HepG2 and HT-29 cancer cells, each treated with 1.0 and 2 mg/mL SDEMI for 4 and 24 h, are also shown. To fully characterize the anticancer potential of SDEMI, cell viability was also assayed. For HaCaT, HepG2, and HT-29 cancer cells treated with SDEMI for 4 h, significant effects on cell viability were detected at 1.0 and 2 mg/ml SDEMI ($P < 0.05$). After 24 h, effects on cell viability were detected for all doses of SDEMI (0.5, 1, and 2 mg/mL SDEMI; $P < 0.05$). For HepG2 and HT-29 cells, viability decreased following SDEMI treatment at both 4 and 24 h timepoints in a dose-dependent manner ($P < 0.001$), although the sensitivity of the two cell lines differed (Figure 2). In contrast, SDEMI increased the cell viability of HaCaT cells (Figure 3).

Caspase-3 activity

To study mechanism(s) mediating SDEMI-induced apoptosis, caspase-3 activation in SDEMI-treated cells was detected

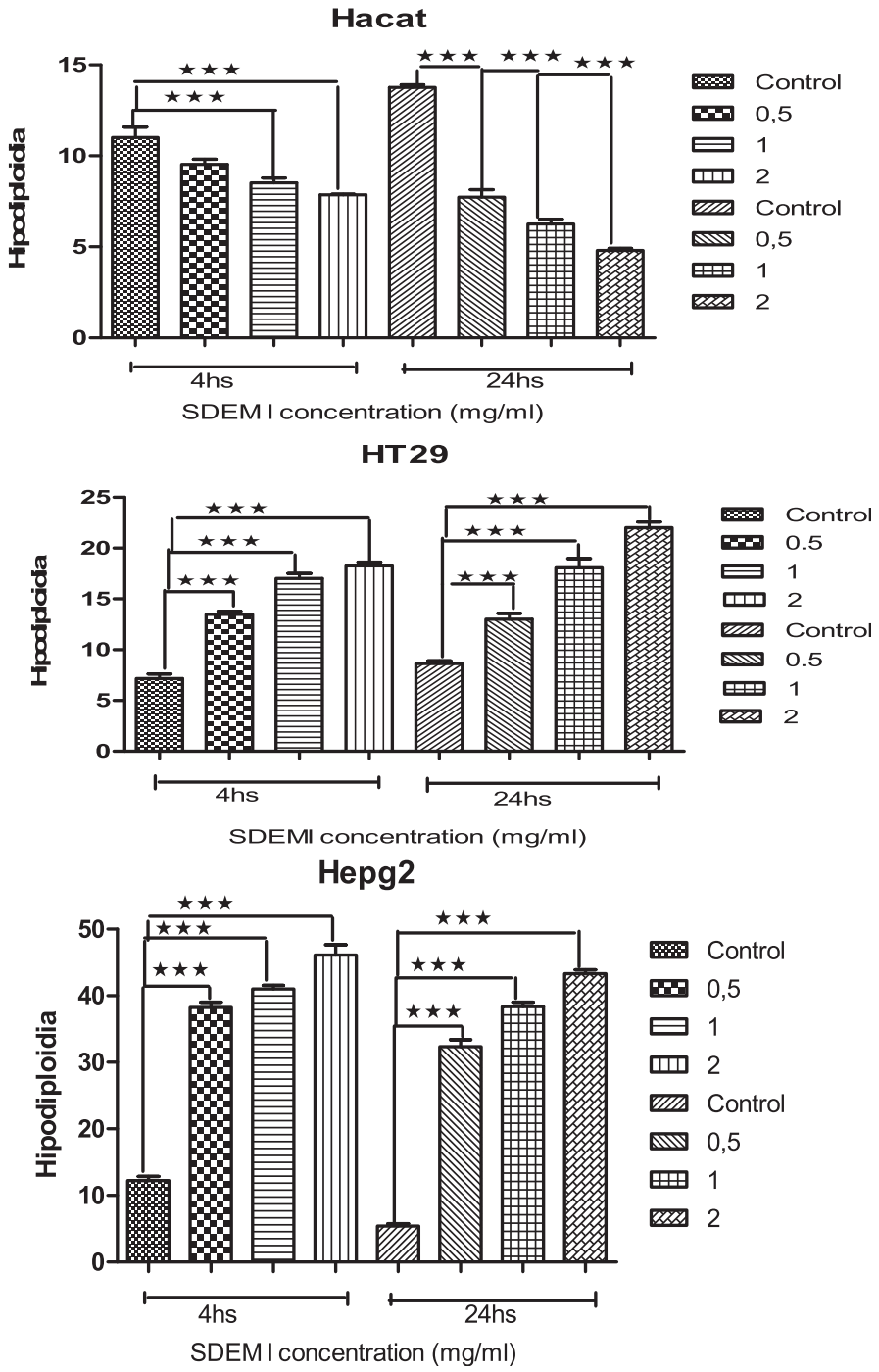

Figure 2 Effect of spray-dried extract of *Maytenus ilicifolia* (SDEMI) on HaCaT; HepG2, and HT-29 by flow cytometry. HaCaT (SDEMI 2.0 mg/mL for 4 or 24 h); HepG2 (SDEMI 1 or 2 mg/mL for 4 or 24 h); HT-29 (SDEMI 1 or 2 mg/mL for 4 or 24 h)

using immunofluorescent microscopy. Representative images of HaCaT, HepG2, and HT-29 cells following SDEMI treatment are shown in Figure 3 (a–c, respectively). While negative staining for caspase-3 indicated that HaCaT cell death following SDEMI treatment was not mediated by an apoptotic process (Figure 4a), positive staining indicated that HepG2 and HT-29 cell death were mediated by an apoptotic process (Figure 4b,c).

Expression of Bcl-2

In Figure 5, Western blotting performed to detect the levels of Bcl-2 in HaCaT, HepG2, and HT-29 cells is shown. Increased expression of Bcl-2 in HaCaT cells treated with SDEMI was observed. This corresponds with a protective effect of SDEMI that prevents induction of apoptosis. No expression of Bcl-2 in HepG2 cells and weak expression for Bcl-2 in HT-29 cells treated with SDEMI was observed.

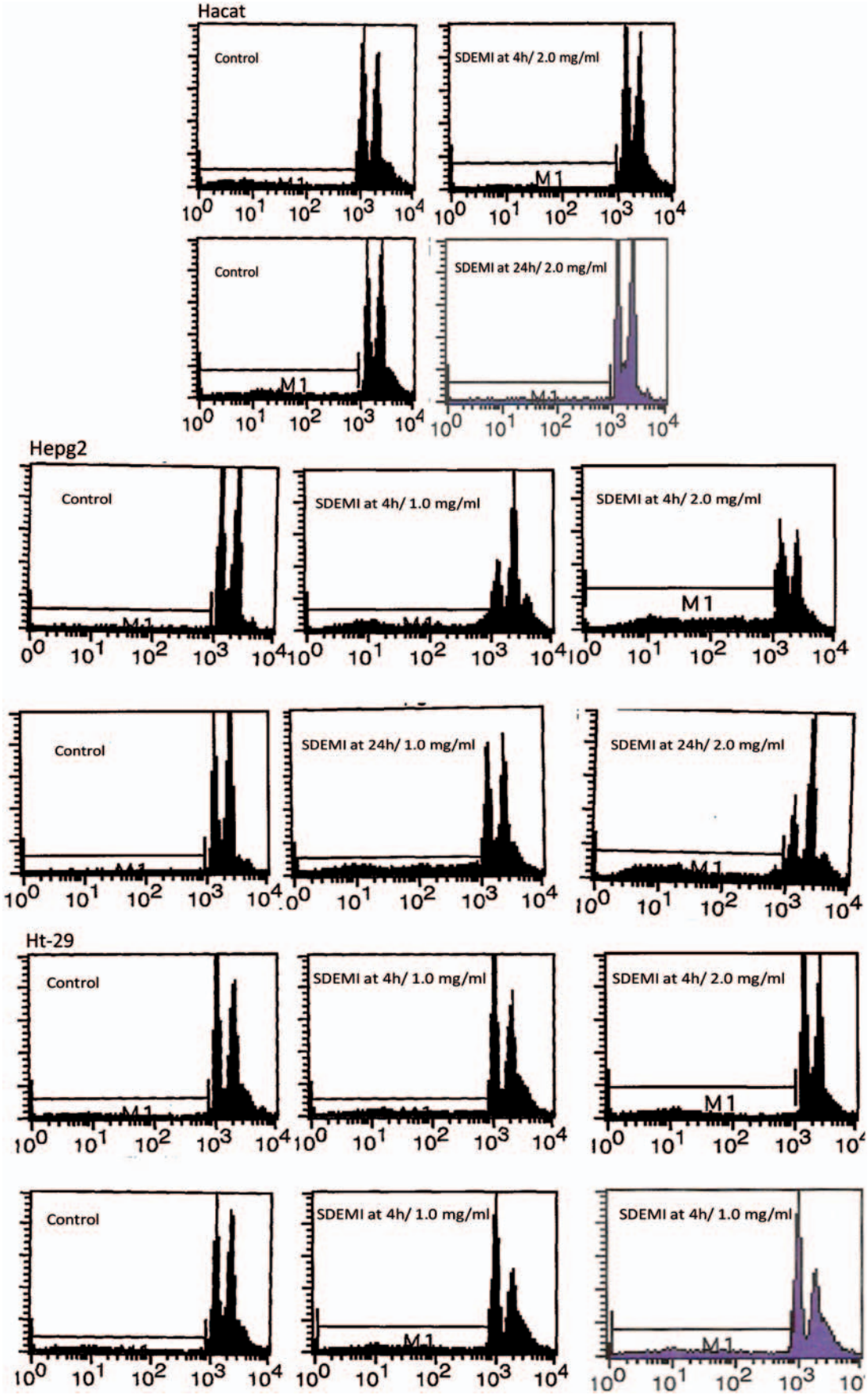

Figure 3 Effect of SDEPN on the viability of various cancer cells derived from different origins. HaCaT, HT-29, and HepG2 cells were treated for 4 or 24 h with SDEMI. HaCat: 1 and 2 mg/kg (4 h) and 0.5; 1 and 2 mg/kg (24 h); HT-29: 0.5; 1 and 2 mg/kg (4 and 24 h). HepG2: 0.5; 1 and 2 mg/kg (4 and 24 h) versus control group (***$P < 0.05$)
(A color version of this figure is available in the online journal)

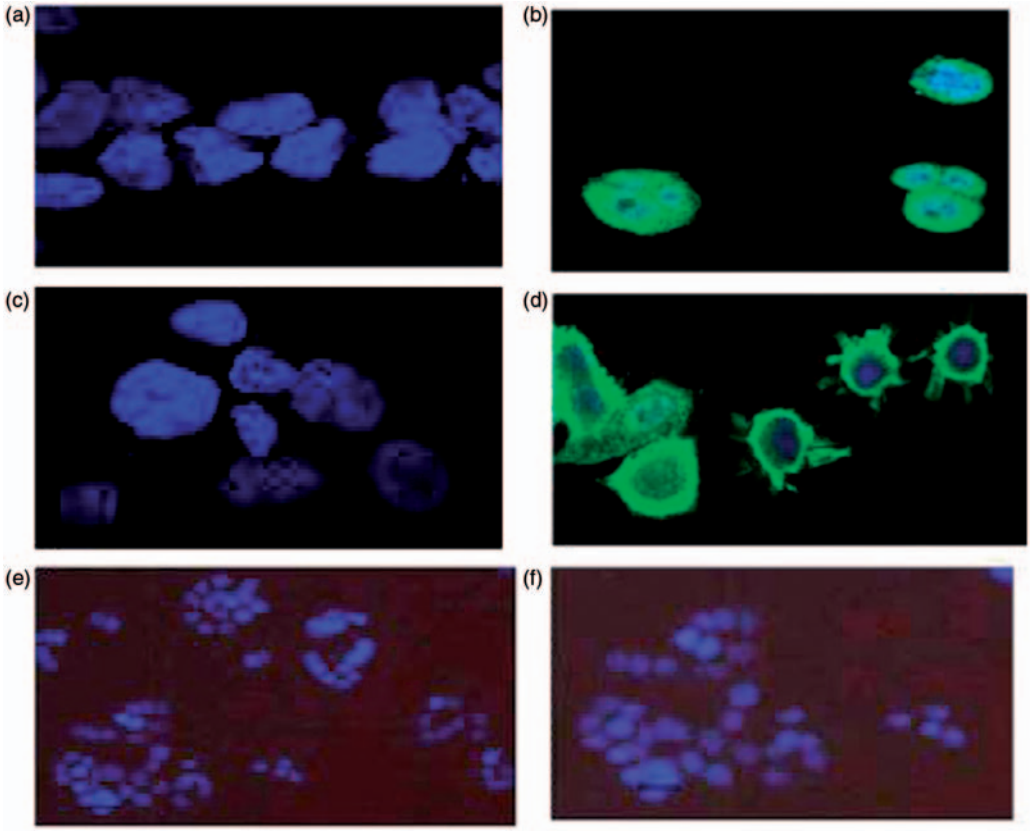

Figure 4 Caspase-3 detection confirms apoptosis induction in HepG2 and HT-29 cells. Cells were stained with 4,6-diamidino-2-phenylindole (blue) and an anticaspase-3 antibody (green) to detect caspase-3 activation as described in Materials and methods section. Control untreated cells are shown in (a) HepG2, (c) HT-29 and (e) HaCaT. After exposure to 2 mg/mL spray-dried extract of *Mytenus ilicifolia* (SDEMI) for 24 h, both HepG2 (b) and HT-29 (d) cells were positive for active caspase-3. Exposure to the same dose for 24 h did not activate caspase-3 in the HaCaT cells (f) (A color version of this figure is available in the online journal).

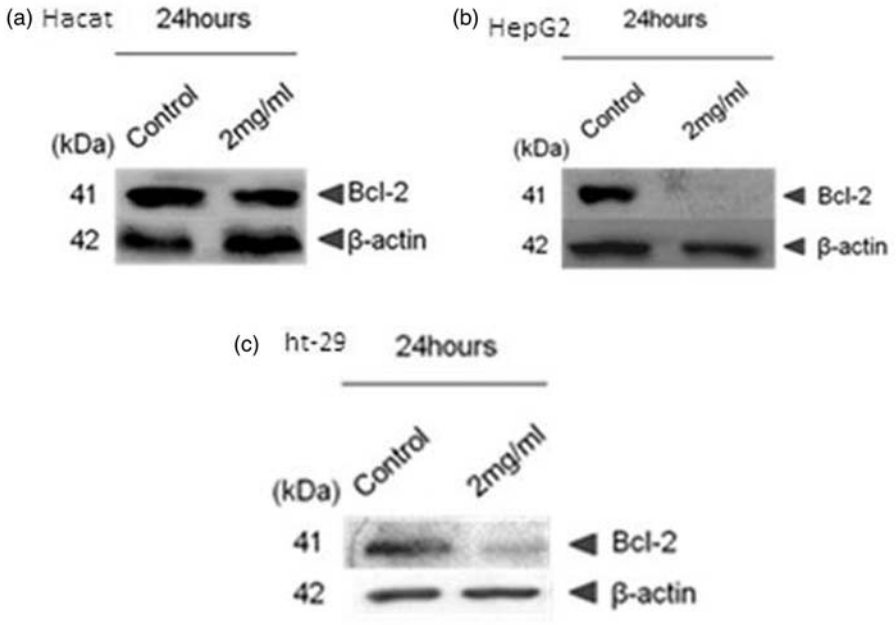

Figure 5 Expression of Bcl-2 was detected in total protein extracts determined by Western blotting (WB), with detection of β-actin used as a loading control. (a) Up-regulation of Bcl-2 detected in HaCaT cells treated with SDEMI for 24 h. (b) Absence-binding observed in HepG2 cells treated with SDEMI for 24 h. (c) Weak-binding detected in HT-29 cells subjected to SDEMI for 24 h

Discussion

Herbal medicines are widely used in Brazil, and currently constitute a market that is expanding. In Brazil, the agency primarily responsible for regulating medicinal plants and their derivatives is the National Health Surveillance Agency (ANVISA). The Ministry of Health also plays a role in protecting and promoting public health by ensuring the safety of products and services.[18] Currently, there are 162 plant species that have derivatives registered at ANVISA.[19] Among the species with the highest number of registration entries is M. ilicifolia, a plant species native to Brazil that has a high medicinal value due to its ability to treat gastritis and gastric ulcers.[20]

In vitro, M. ilicifolia has been shown potently inhibit the proliferation of cytotoxic leukaemia cells (HL-60).[12] However, it has not previously been investigated whether M. ilicifolia modulates an apoptotic pathway in colorectal and hepatocellular cancer cells.

Induction of apoptosis can be recognized by characteristic biochemical and morphological alterations of the plasma membrane, mitochondria, and nucleus. The associated phenotypes include cell shrinkage, membrane blebbing, chromatin condensation, and formation of a DNA ladder with multiple fragments.[21] Apoptosis is regulated and executed by the interplay of many genes responsive to various stimuli. In carcinoma cells, a variety of chemical reagents have been found to induce apoptosis via different pathways, including the Bcl-2-related pathway.[22,23] Bcl-2 proteins predominantly localize on the outer mitochondrial membrane, and mediate anti-apoptotic effects by stabilizing the mitochondrial membrane and inhibiting the permeability of transition pores. As a result, the release of cytochrome c[24] is affected, which is responsible for the activation of pro-caspase-9 which promotes downstream caspase activation, including that of caspase-3.[25] Furthermore, caspase-3 activation is essential for mediating nuclear changes associated with apoptosis.[26] In the present study, expression of Bcl-2 and caspase-3 were detected by Western blot and immunofluorescence, respectively, in order to elucidate the molecular details of SDEMI-induced apoptosis.

When HT-29 and HepG2 cells were treated with SDEMI for 4 and 24 h, cell growth of both cell lines was significantly inhibited in a concentration-dependent manner. Moreover, the inhibition of growth was associated with increased rates of apoptosis. Induction of apoptosis was confirmed with the detection of reduced levels of Bcl-2 proteins and activation of caspase-3 in HT-29 and HepG2 cells. Both Bcl-2 and caspase-3 have been shown to be apoptosis-related regulators. Correspondingly, spray-dried extracts of Phyllanthus niruri (SDEPN) were also observed to be selectively toxic against the HepG2 cancer cell line. However, no toxic effects were observed in HT-29 cells in response to SDEPN.[27] In addition, SDEPN induced apoptotic effects via caspase-3 in the HepG2 cancer cell line.[28]

Expression of Bcl2 decreased in HepG2 and HT-29 cells 24 h after the application of 2 mg/mL SDEMI. These results indicate that SDEMI induces apoptosis by down-regulating expression of Bcl-2. It was also observed that HepG2 cells were more sensitive to SDEMI treatment in terms of Bcl-2 inactivation compared with HT-29 cells.

In 2004, Soares et al.[17] published a validation assay and demonstrated its capacity to separate and quantify catechin and epicatechin present in aqueous extracts of leaves from M. ilicifolia. Catechin and epicatechin are procyanidins, which are types of flavonoids. Many natural products, including polyphenolic compounds, exhibit chemopreventive effects in various human cancer cell lines, as well as in carcinogen-induced tumour models in experimental animals.[29] However, information on the potential role of procyandins in cancer prevention, particularly in vivo, is limited.

Previously, catechin hydrate was observed to induce apoptosis and the expression of caspase-3 in a time- and dose-dependent manner in a cervical cancer cell line (SiHa).[30] Cathechin hydrate was also shown to induce apoptosis in MCF-7 human breast cancer cells concomitant with enhanced expression of pro-apoptotic genes such as caspase-3.[31]

In the present study, SDEMI was observed to protect keratinocytes, as detected by the statistically significant reduction in the percentage of hypodiploid cells present in SDEMI-treated HaCaT cells (Figure 2a) compared with control cells (Figure 2e). These results were further confirmed by an absence of caspase-3 labelling in SDEMI-treated HaCaT cells (Figure 3a). These results are consistent with those of Araújo Júnior et al.,[28] where P. niruri was found to protect keratinocytes from the effects of apoptosis in vitro. Taken together, these results support a growing body of evidence that plant medicines can be cytotoxic towards cancer cells, yet mediate a protective effect for normal cells.

In conclusion, the results of the present study demonstrate that SDEMI can induce apoptosis in human HT-29 and HepG2 cancer cells. SDEMI-induced apoptosis was associated with down-regulation of Bcl-2 and activation of a caspase-3-dependent signalling pathway. Conversely, the protective effect of SDEMI on normal keratinocytes was confirmed with detection of caspase-3 inactivation and up-regulation of Bcl-2. Taken together, these results suggest that SDEMI represents a potential therapeutic candidate for the treatment of cancer, and also mediates a protective effect for normal cells.

Authors contribution

RFAJ and GCBG participated in the design of the studies; RFAJ, ALSLO, JBP and VBG did the experimental study of cancer cells; LALS, PRP and TPS were responsible for the preparation of extracts; RFAJ analysed the statistical data; RFAJ and AAA wrote and corrected the paper

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