Original Research

A dry extract of *Phyllanthus niruri* protects normal cells and induces apoptosis in human liver carcinoma cells

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Abstract

The ability to induce apoptosis is an important marker for cytotoxic antitumor agents. Some natural compounds have been shown to modulate apoptosis pathways that are frequently blocked in human cancers, and therefore, these compounds provide novel opportunities for cancer drug development. *Phyllanthus,* a plant genus of the family Euphorbiaceae, exhibits multiple pharmacological actions. Of these, *Phyllanthus niruri* extracts exhibit significant antitumor activity, which is consistent with the traditional medicinal use of this plant. To examine the apoptotic effects of a spray-dried extract of *P. niruri* (SDEPN), human hepatocellular carcinoma cells (HepG2, Huh-7), colorectal carcinoma cells (Ht29) and keratinocytes (HaCaT) were exposed to the extract for 4, 8 and 24 h. Flow cytometry and caspase-3 immunostaining were used to detect apoptosis, while analysis of variance was applied to identify significant differences between groups ($P < 0.05$). At all timepoints, the SDEPN induced significantly different cytotoxic effects for HepG2 and Huh-7 cells compared with control cells ($P < 0.001$). In contrast, the SDEPN had a protective effect on HaCaT cells compared with control cells at all timepoints ($P < 0.001$). In caspase-3 assays, activation was detected after cell death was induced in Huh-7 and HepG2 cancer cells by the SDEPN. In combination, these results indicate that the SDEPN is selectively toxic towards cancer cell lines, yet is protective towards normal cells.

Keywords: apoptosis, caspase-3, hepatocellular carcinoma, *Phyllanthus niruri*

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Introduction

Tremendous progress has been made in the prevention, detection and treatment of various cancers over the last 50 years. However, late stage diagnoses, inadequate strategies for addressing aggressive metastasis and a lack of clinical procedures for overcoming multidrug resistance continue to challenge the successful treatment of cancers.[1,2] Correspondingly, there is a great need for the development of novel and effective cancer therapies.

Apoptosis is an important cellular process for a variety of biological systems in which cell death occurs without inflammation. Once induced, apoptosis involves a sequential process which causes: (i) chromatin condensation, characterized by intense basophillic staining; (ii) nucleolar disintegration; and (iii) reduced cell volume with a concomitant increase in cell density. In the latter case, although compaction of cytoplasmic organelles occurs, mitochondria remain morphologically normal. Subsequent steps in apoptosis include the division of ribosomes, organelles

and nuclear material into membrane-bound apoptotic bodies. These apoptotic bodies are then shed from epithelial surfaces, or are removed by phagocytic cells (such as macrophages) which surround epithelial cells. In solid tumors, apoptotic bodies are removed by adjacent tumor cells.[3]

To identify cytotoxic antitumor agents, induction of apoptosis is a key marker. Moreover, cancers that do not undergo apoptosis in response to appropriate stimuli represent a key cause of treatment failure. 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.[4] The anticancer effect of the genus, *Phyllanthus,* a plant genus of the family Euphorbiaceae, has been reported to have multiple known pharmacological actions. In Brazilian herbal medicine, *Phyllanthus niruri* is called Quebra Pedra (stone breaker), and has been shown to inhibit the formation of calcium oxalate in the kidney,[1,5] to mediate antimutagenic, anticarcinogenic and antitumor actions,[6,7] and antioxidant, hepatoprotective[8,9] and antihyperuricemic properties.[9]

Due to a paucity of evidence regarding the possible cytotoxicity of a spray-dried extract of *P. niruri* (SDEPN), the aim of this study was to determine the cytotoxicity of this extract for human hepatocellular carcinoma cells (HepG2, Huh-7), colorectal carcinoma cells (Ht29) and keratinocytes (HaCaT). The capacity for the SDEPN to preferentially induce apoptosis in cancer cells versus normal cells was also examined.

Materials and methods

Plants

The aerial parts of *P. niruri L.* were supplied by the 'Centro Pluridisciplinar de Pesquisas Químicas e Biológicas' (CPQBA-UNICAMP) from the Campinas State University, Brazil. The plant was dried at 40°C for a week in an air oven. Separated leaves and branches were reduced in a knife mill (Retsch basic sieve, Haan, Germany). A voucher specimen was deposited at the Herbarium of the Federal University of Rio Grande do Sul under number ICN 111765.

Dry extract preparation

The dried and ground aerial parts from *P. niruri* were used to prepare aqueous extracts by decoction, using raw material in distilled water (7.5%; w/v). The aqueous extract was dried using a Niro Atomizer Production Minor spray drier (Geo Niro, Søborg, Denmark) to obtain the dried product (SDEPN). Colloidal silicon dioxide (Aerosil®200, Degussa, São Paulo, Brazil) was used as a drying agent at 30% (w/w) based on the dry residue of the extracted solution. The SDEPN was standardized as 12.33 mg/g of gallic acid by high-pressure liquid chromatography, according to De Souza *et al.*[10] and as 99.4 mg/g total flavonoids by visible and ultraviolet spectroscopy according to Soares *et al.*[11]

Cell culture

Non-cancerous cells (HaCaT; keratinocytes) and cancer cells (HT29 [colorectal adenocarcinoma], HepG2 [hepatocarcinoma] and Huh-7 [hepatocarcinoma]) were purchased from the Culture Collection of the Faculty of Medicine (University of São Paulo [USP], Sao Paulo, Brazil). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Cultilab LTDA, São Paulo, SP, Brazil).

Cell viability

Due to the different sensitivity of cancer cells to the SDEPN, the optimal exposure time for each cell line was determined in a pilot study in order to obtain a dose-dependent effect. Viability was determined at 4, 8 and 24 h for HT29, Huh-7 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 five minutes. The number of viable cells was calculated using a hemocytometer.

Cytotoxicity assay

Three concentrations (0.5, 1.0 or 2.0 mg/mL) of SDEPN diluted in DMEM culture medium were used and filtered via a 0.22 mm minipore membrane. The solutions were aliquoted and stored at −20°C, thawed just before testing, and diluted with the culture medium. The final concentration of DMSO for all treatments was less than 0.1%. Culture medium was used in the control group. Cisplatine 2.0 mmol/L was used in the positive control. For this study, cells were incubated in either the absence or presence of different concentrations of the SDEPN for 4, 8 and 24 h and exposed to different concentrations of the SDEPN for different time periods.

Morphology

HaCaT, HT29, HepG2 and Huh-7 cells were plated (5×10^5 cells/well in 2 mL medium) in six-well culture plates, and allowed to attach for 24 h. Then the cells were treated with the SDEPN (0.5, 1.0 or 2.0 mg/mL) for 4, 8 or 24 h. After treatment with the SDEPN, cells were observed under a phase-contrast microscope and morphological changes associated with apoptosis, such as membrane blebbing and formation of apoptotic bodies, were recorded.

Hypodiploid cell detection

Flow cytometric analysis was performed to detect hypodiploid cells and to analyze cell cycle progression. Cancer cells were plated in six-well plates (5×10^5 cells/well in 2 mL medium). After 24 h, they were treated with the SDEPN (0.5, 1.0 or 2.0 mg/mL) for 4, 8 or 24 h. The cells were harvested by trypsinization when they reached approximately 80% confluence. The cells were stored in 70% ethanol and centrifuged for five minutes at $300 \times g$. They were then re-suspended in 200 μL propidium iodide

(PI) buffer (20 mL phosphate-buffered saline [PBS], 20 μL 0.1% Triton X-100, 200 mg/mL RNase, 20 μg/mL PI), placed in FACS tubes (Cultilab LTDA), and incubated for 30 min at room temperature (RT) protected from light. The labeled cells were captured with a FACSCaliber cytometer (BD Bisocience, Franklin Lakes, NJ, USA), and analyzed with CellQuest software (BD Biosciences). The percentage of cells in different cell cycle phases was quantified, and the data were expressed as 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 SDEPN (0.5, 1.0 or 2.0 mg/mL) for 4, 8 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 (BSA 5%; Life Technologies do Brasil LTDA, São Paulo, Brazil) for one hour 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.

Statistical analysis

All experiments were performed in triplicate at a minimum. The significance of differences between groups was calculated by applying analysis of variance and Bonferroni's test, as indicated. A P value of less than 0.05 was considered statistically significant.

Results

Viability of different cancer cells after SDEPN treatment

To fully characterize the anticancer potential of the SDEPN, we analyzed 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 SDEPN treatment in a dose-dependent manner as shown in Figure 1. However, different cancer cells responded to the SDEPN with different sensitivity. The SDEPN exerted a potent effect on reducing the viability of hepatocarcinoma cells including HepG2 and Huh-7, within a few hours. HT29 cells were more tolerable to the SDEPN. The doses that were near the IC_{50} (The half maximal inhibitory concentration) were 0.5, 1.0 and 2.0 mg mL; these concentrations were chosen for the following steps.

Morphological observation

Morphological changes were observed with light microscopy to determine the potential mechanism underlying the cytotoxic effect of the SDEPN. In the control cells, nuclei were round in shape and appeared homogeneous, whereas in SDEPN-treated cells, chromatin condensation at the nuclear periphery and nuclear fragmentation were observed. Treatment with the SDEPN resulted in different degrees of morphological changes. The most obvious change was rounding of the cells (Figure 2). The changes appeared after the cells were treated for four hours and became obvious after 24 h. Comparing the control HepG2 and Huh-7 cells (Figures 2a, c) to those treated with 1 mg/mL SDEPN for

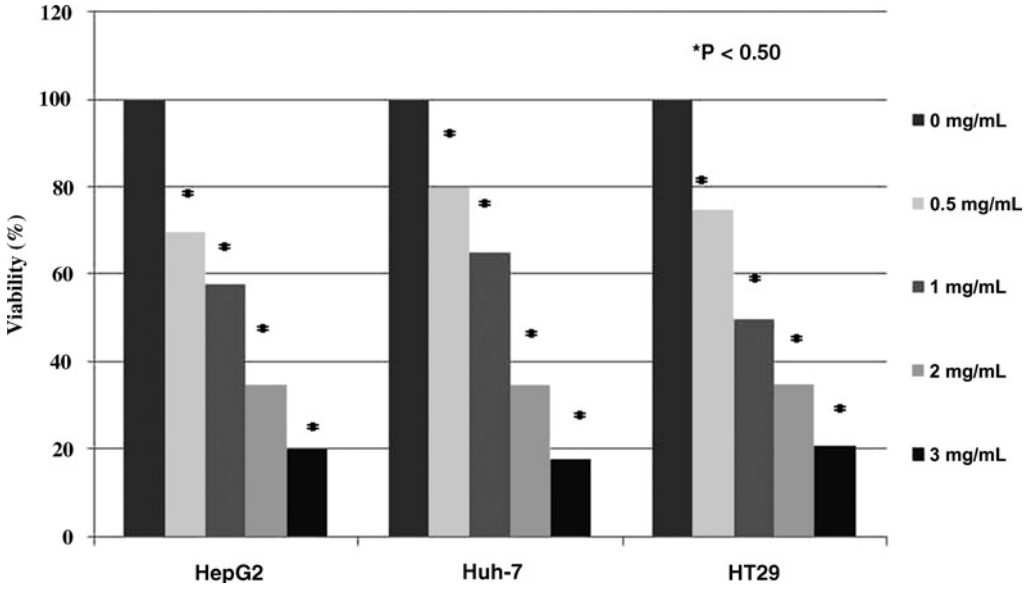

Figure 1 Effect of the spray-dried extract of *Phyllanthus niruri* (SDEPN) on the viability of various cancer cells derived from different origins. Cells were treated with different concentrations of the SDEPN as indicated. The exposure time for HepG2, HT29 and Huh-7 was 4, 8 and 24 h. Cell viability was calculated by trypan blue exclusion assay. The asterisk (*) represented significantly different value from control, $P < 0.05$

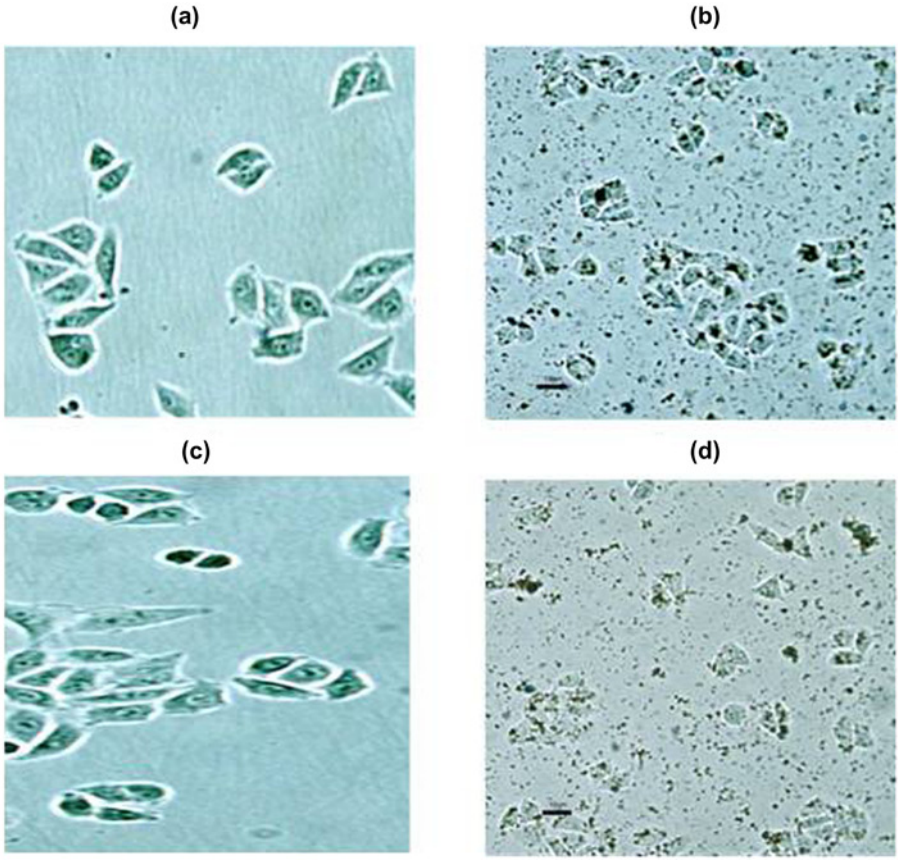

Figure 2 Effect of the spray-dried extract of *Phyllanthus niruri* (SDEPN) on HepG2 and Huh-7 cell morphology. Phase-contrast images of control HepG2 (a) and Huh-7 (c) control cells, and HepG2 (b) and Huh-7 (d) cells exposed to 1 mg/mL SDEPN were taken after eight hours. Magnification: ×100. (A color version of this figure is available in the online journal)

eight hours revealed typical morphological characteristics for cell apoptosis, such as cell condensation, plasma membrane blebbing and formation of apoptotic bodies. These changes implied that the growth inhibitory effect of the SDEPN might result from induction of apoptosis.

Apoptosis detection

One of the specific characteristics of apoptosis is DNA fragmentation, which leads to the appearance of hypodiploid cells.[12] Flow cytometric analysis was performed to detect hypodiploid cells, counted as cells with less DNA content than those in G1 of the cell cycle. Figure 3 shows the hypodiploid population of HepG2 and Huh-7 cells after exposure to 2 mg/mL SDEPN at four hours and 24 h. To fully characterize the anticancer potential of the SDEPN, we analyzed its effect on the viability of various cancer cells derived from different origins, as well as on normal cells (HaCaT). The viability of the cancer cells was decreased significantly by SDEPN treatment in a dose-dependent manner. However, the SDEPN had the opposite effect on HaCaT cells (Figure 4a). Cancer cells responded to the SDEPN with different sensitivities. The SDEPN potently reduced the viability of HepG2 and Huh-7 liver cancer cells ($P < 0{,}001$) within a few hours (Figures. 4c, d). Colorectal (HT29) cancer cells were more tolerant of the SDEPN ($P > 0.01$; Figure 4).

Caspase-3 activity

To study the mechanism mediating SDEPN-induced apoptosis, caspase-3 activation in SDEPN-treated cells was detected by confocal immunofluorescent microscopy. Representative microphotographs of cells after SDEPN treatment, including HaCaT, HepG2 and Huh-7 cells, are shown in Figure 5. Negative staining for caspase-3 indicated that HaCaT cell death after SDEPN treatment was not mediated by an apoptotic process (Figure 5f). Positive staining for caspase-3 indicated that Huh-7 and HepG2 cell death after SDEPN treatment was mediated by an apoptotic process (Figure 5).

Discussion

Induction of apoptosis in cancer cells represents an efficient strategy for cancer therapy. Moreover, there are a variety of stimuli that trigger apoptosis in cancer cells, including irradiation and chemotherapeutic agents. However, most of these stimuli have poor specificity, and therefore, normal cells are also damaged. Correspondingly, the focus of cancer research currently includes the identification of apoptosis-inducing drugs that have high specificity for cancer cells.[13] As a result, plant-derived agents have attracted attention due to their anticancer properties. For example, the studies of cancer therapy have involved the

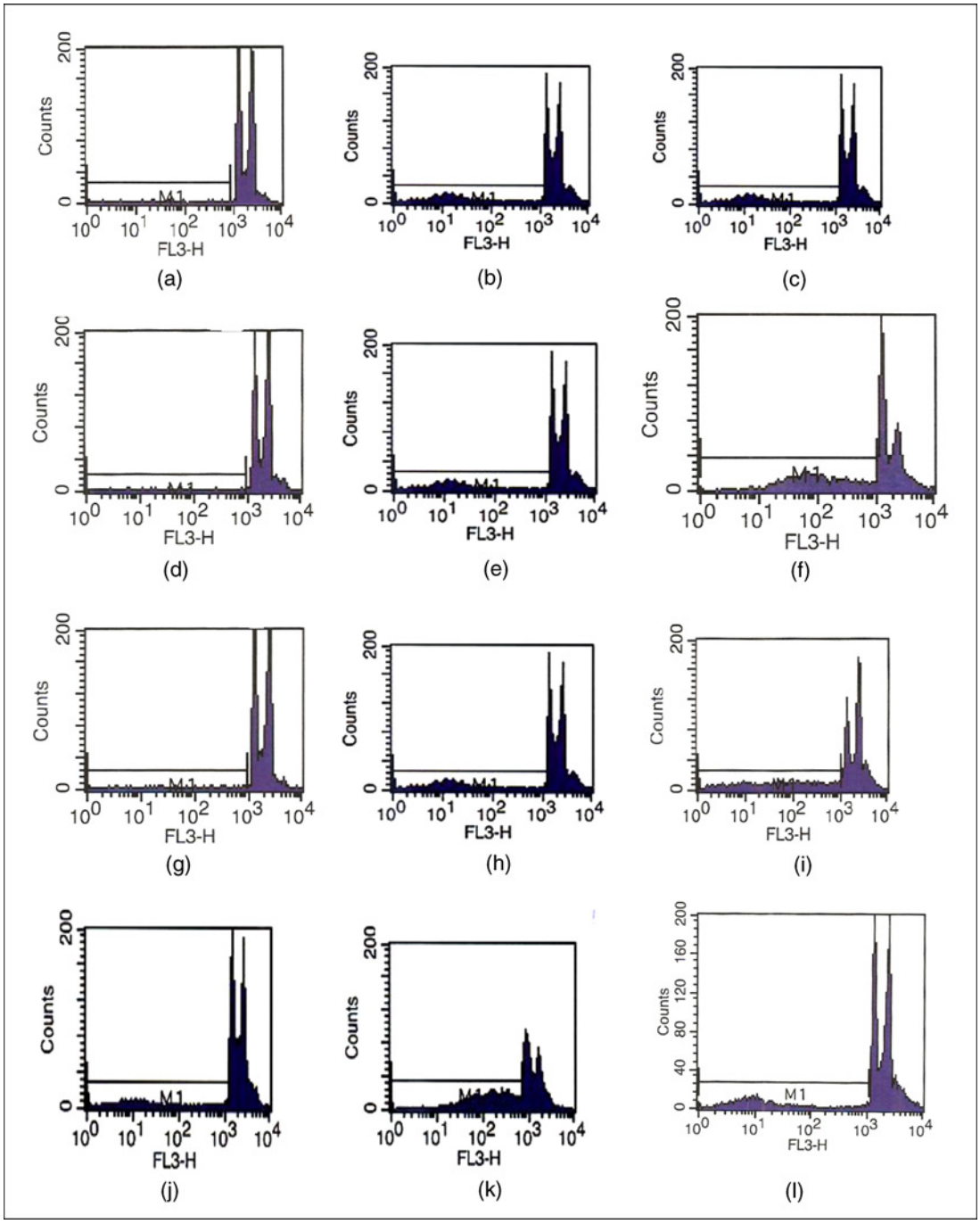

Figure 3 DNA damage in liver cancer cells exposed to the spray-dried extract of *Phyllanthus niruri* (SDEPN). Hypodiploid Huh-7 (a–f) and HepG2 (g–m) cells were detected by flow cytometry as described in the Materials and methods section. Cells were left untreated for 4 (a, g) or 24 h (d, j), treated with 2.5 mmol/L cisplatine for 4 (b, h) or 24 h (e, l) or treated with 2 mg/mL SDEPN for 4 (c, i) or 24 h (f, m)

antitumor effects of plant-derived agents where several phenomena characteristic of apoptosis have been observed. These phenomena included cell cycle arrest, the generation of DNA damage and the activation of caspases.[14,15]

In the present study, the SDEPN did not induce cell death in colorectal cancer cells (HT29). Correspondingly, immunofluorescence studies of treated HT29 cells did not detect expression of caspase-3. These results are consistent with clinical evidence that colorectal cancer cells (including HT29) are highly resistant to chemotherapeutic drugs that induce apoptosis, possibly due to the tumor stem cell activity associated with this type of cancer.[16] In addition, loss of caspase-3 expression has also been associated with resistance to apoptosis and resistance to therapy.[17]

On the other hand, the SDEPN was able to induce cell death in liver cancer cells (HepG2 and Huh-7) within a few hours. For example, at each timepoint assayed, DNA fragmentation was detected in Huh-7 cells treated with the SDEPN (at 1 mg/mL and 2 mg/mL). For HepG2 cells, the most extensive DNA fragmentation was observed at

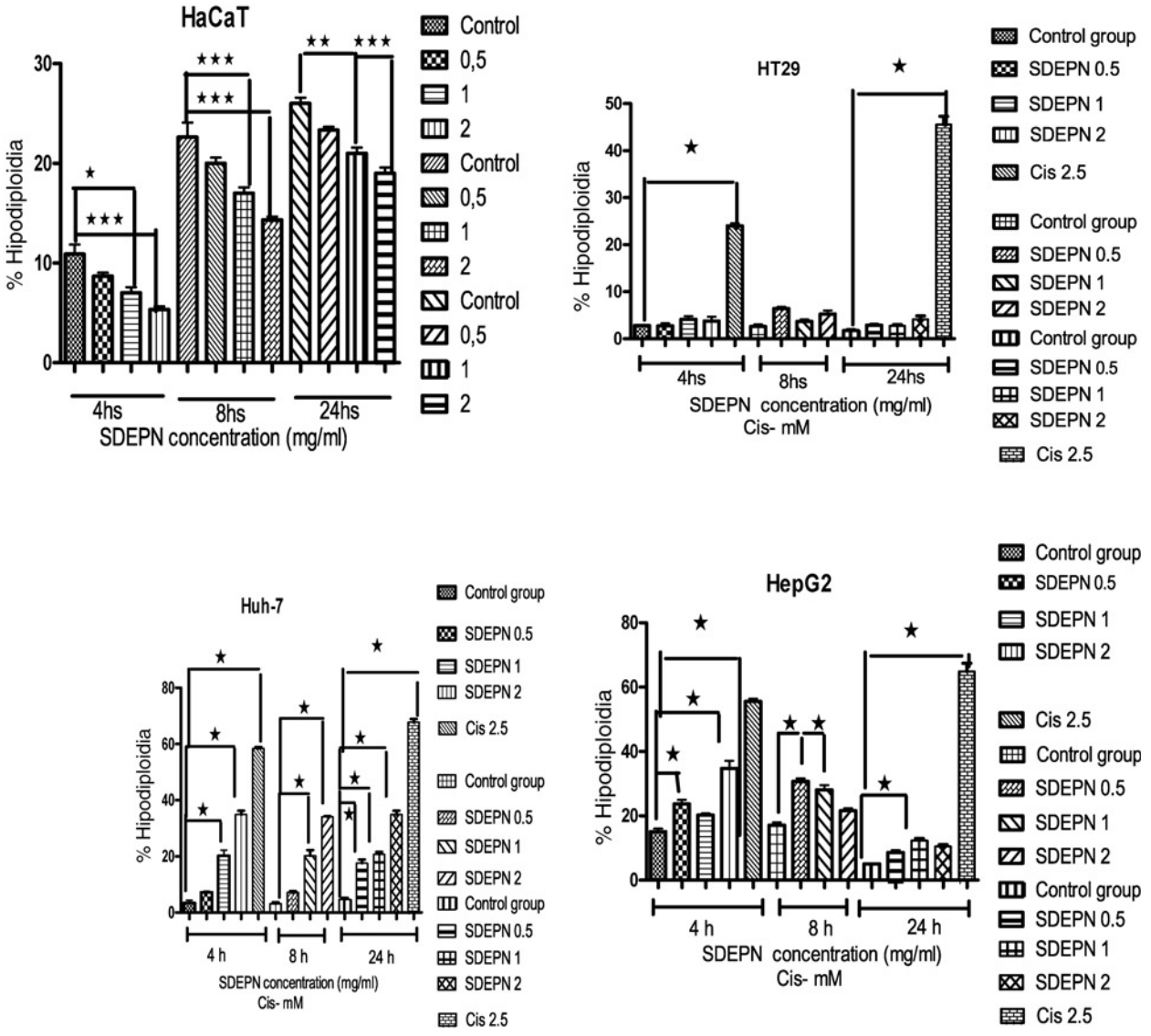

Figure 4 Effect of the spray-dried extract of *Phyllanthus niruri* (SDEPN) on the viability of various cancer cells derived from different origins. HaCaT (a), HT29 (b), Huh-7 (c) and HepG2 (d) cells were treated with different concentrations of the SDEPN as indicated for 4, 8 or 24 h. Cell death was assessed by flow cytometry as described in the Materials and methods section. Data represent the means ± the standard error of the mean (SEM) calculated from three individual experiments

four hours with treatments of 0.5 and 2.0 mg/mL SDEPN. However, after eight hours, DNA fragmentation was the highest for cells treated with SDEPN ranging from 0.5 to 1 mg/mL. Accordingly, high levels of caspase-3 expression were detected in both HepG2 and Huh-7 hepatocellular carcinoma cell lines. Apoptotic activity has been detected in other species of *Phyllanthus*, and has also been accompanied by increased expression of caspase-3. These species have included Lewis lung carcinoma cells,[18] skin melanoma and prostate cancer cells.[19] It was also proposed by Persad *et al.*[20] that overexpression of caspase-3 may serve as a diagnostic and therapeutic marker for hepatocellular carcinomas.

The SDEPN was observed to protect keratinocytes, as detected by the statistically significant reduction in the percentage of hypodiploid cells present (Figure 3a) compared with the control group (Figure 4e). These results were further confirmed by an absence of caspase-3 labeling (Figure 4f), consistent with the results of Sarkar and Sil.[9] In the latter study, *P. niruri* was found to inhibit apoptosis via a caspase-3-dependent mitochondrial death pathway in hepatocytes *in vitro*.

The SDEPN protected the keratinocytes, as seen by a statistically significant reduction in the percentage of hypodiploid cells (Figure 3a) compared with the control group (Figure 4e), and further confirmed by a lack of caspase-3 labeling (Figure 4f), results similar to those of Sarkar and Sil.[9] These researchers showed that *P. niruri* inhibited apoptosis via a caspase-3-dependent mitochondrial death pathway in hepatocytes *in vitro*.

Plant-derived polyphenols, including flavonoids and gallic acid, are the main components of *P. niruri* extracts.[21,22]

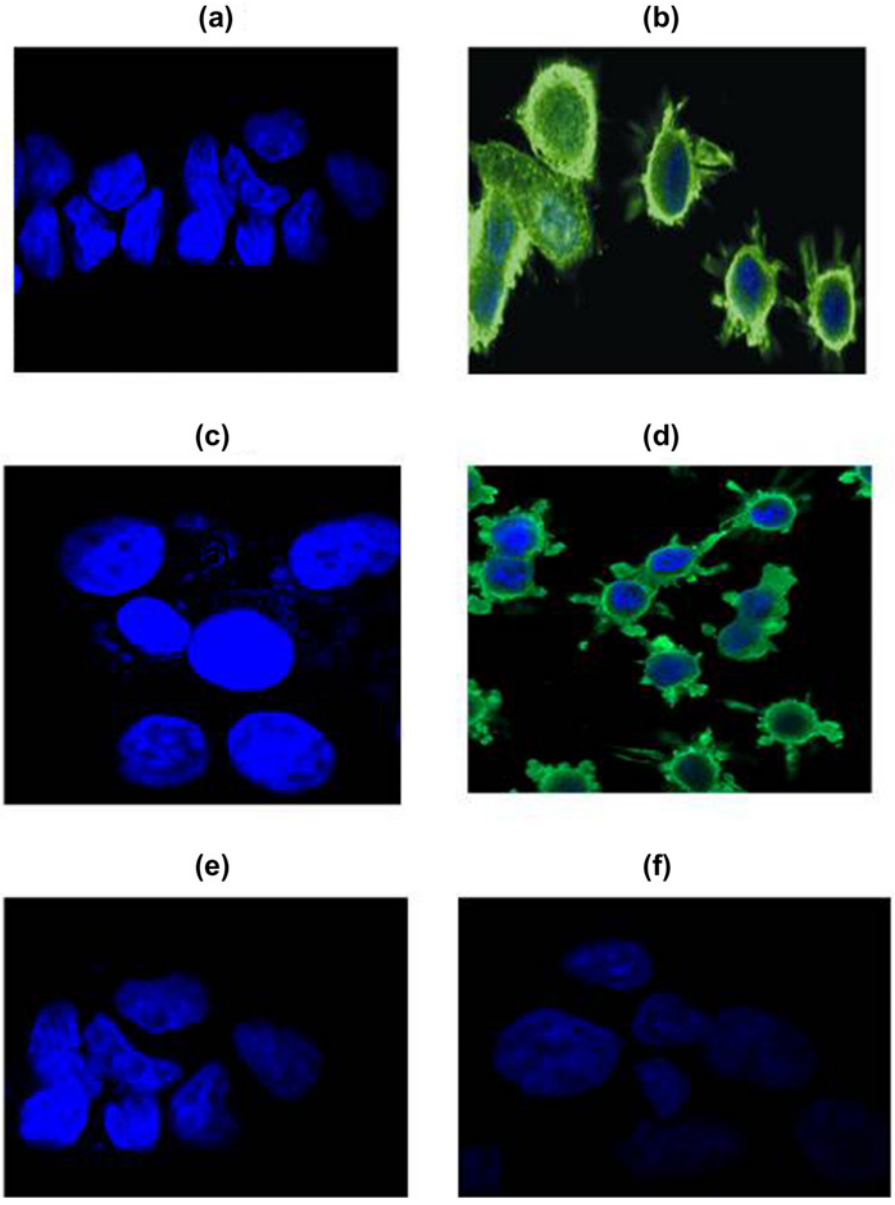

Figure 5 Caspase-3 detection confirms apoptosis induction in HepG2 and Huh-7 cells. Cells were stained with 4,6-diamidino-2-phenylindole (blue) and an anticaspase-3 antibody (green) to detect caspase-3 activation as described in the Materials and Methods section. Control untreated cells are shown in (a) HepG2, (c) Huh-7 and (e) HaCaT. After exposure to 2 mg/mL spray-dried extract of *Phyllanthus niruri* (SDEPN) for eight hours, both HepG2 (b) and Huh-7 (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)

Furthermore, dried extracts are often employed as the therapeutically active material in the manufacture of phytopharmaceuticals. There are several advantages attributed to this extraction method, including chemical and physical stability.[10,11] In the present study, flavonoids and gallic acid were also detected in the SDEPN extracts used, consistent with Soares *et al.*[11] and De Souza *et al.*[10] In a study by Ji *et al.*,[23] NCI-H460 cells treated with gallic acid activated caspase-3 in a time-dependent manner, thereby supporting the role of caspase-3 in gallic acid-induced apoptosis. Additional studies have demonstrated the anticancer activity of *P. niruri* extracts for skin and lung cells.[24]

In summary, the SDEPN was found to significantly inhibit the proliferation of two human cancer cell lines *in vitro*. The cytotoxic effect of the SDEPN involved the induction of apoptosis, combined with increased expression of the apoptosis signaling pathway by caspase-3. Taken together, these results demonstrate that the SDEPN can mediate a significant protective effect on keratinocytes, which involves an inhibition of apoptosis and signaling by caspase-3. Furthermore, these results provide *in vitro* evidence to support the use of the SDEPN for the chemoprevention and treatment of cancer cells.

Author contributions: RFdAJ and GCBG participated in the design of the studies. RFdAJ, JGLP, HDOM and ALCdSLO did the experimental study of cancer cells. LALS, PRP and TPdS were responsible for the preparation of extracts.

RFdAJ analyzed the statistical datas. RFdAJ and AAdA wrote and corrected the paper.

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