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Isoflavones as Apoptosis Inducers in Human Hepatoma HuH-7 Cells

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Nine flavonoids isolated from the ethyl acetate extract of *Pycnanthus angolensis* were assayed for their potential apoptosis induction activities in human hepatoma HuH-7 cells. These flavonoids include eight isoflavones, namely irilone (1), tectorigenine (2), formononetin (3), genistein (4), 2′-hydroxybiochanin A (5), mixture of biochanin A (6) and prunetin (7), and 4′,7-dihydroxy-2′-methoxyisoflavan (8), and the flavanone liguiritigentin (9). Their chemical structures were characterized by spectroscopic methods including 2D NMR experiments. Methodology for cell death detection included the LDH assay, Hoechst staining, TUNEL staining and general caspase-3-like activity assay. The compounds tested showed higher apoptosis induction profiles in HuH-7 cells compared with the control. Caspase activity assays confirmed the apoptosis inducing activity of these flavonoids.

Keywords: Pycnanthus angolensis; Myristicaceae; apoptosis induction; HuH-7 cells; isoflavones; flavonoids.

INTRODUCTION

Flavonoids are important bioactive plant-synthesized natural products. They exhibit a wide range of biological activities, such as antioxidant, antiviral, antiinflammatory, antibacterial, antiallergic, hepatoprotective, cytotoxic, estrogenic, antiestrogenic and pro-apoptotic (Li *et al.*, 1993; Nkengfack *et al.*, 2001; Hodek *et al.*, 2002; Li *et al.*, 2008; Martín *et al.*, 2010). There are numerous reports in the literature of the past two decades, suggesting that the dietary intake of certain flavonoids is inversely related to the outbreak of breast, liver and prostate cancer (Lagiou *et al.*, 2008; Banerjee *et al.*, 2008; Sarkar and Li, 2002; Seufi *et al.*, 2009). Several *in vitro* and *in vivo* studies support the chemoprotective effects of flavonoids.

Apoptosis, also known as 'programmed cell death', is a phenomenon of the body to eliminate the unwanted and cancerous cells. The ability of internal or external stimuli to induce cell death is therefore recognized for its immense therapeutic potential (Elmore, 2007). Many natural products, such as several polyphenols, alkaloids, terpenoids and saponins can induce apoptosis by interacting with important molecular targets such as DNA, microtubules, biomembranes and receptors. This process is carried out by the involvement of various enzymes (Taraphdar *et al.*, 2001).

In our ongoing search for bioactive compounds from medicinal plants (Duarte *et al.*, 2007, 2009; Mansoor *et al.*, 2009; Ramalhete *et al.*, 2009; Lage *et al.*, 2010), we have been studying the stem bark of the African tree *Pycnanthus angolensis* (Welw.) Warb. (Myristicaceae). *Pycnanthus angolensis* has been reported to contain interesting compounds, such as terpenoid-quinones (Wabo *et al.*, 2007) and cyclolignane derivatives (Nono *et al.*, 2010). In our study, several lignans from the CH_2Cl_2 extract were isolated. Some of them showed antimalarial (Abrantes *et al.*, 2008) and/or antitumor-promoting activities (Pusztai *et al.*, 2010). Further phytochemical studies of the ethyl acetate extract of this plant yielded eight isoflavones **1–8** and a flavanone (**9**). This is a report on the isolation and apoptosis induction activity of these flavonoids.

MATERIAL AND METHODS

General experimental procedures. NMR spectra were recorded on a Bruker ARX-400 NMR spectrometer (^{1}H-NMR 400 MHz; ^{13}C-NMR 100.61 MHz), using $CDCl_3$ as solvent. Column chromatography was carried out on SiO_2 (Merck 9385). TLC were performed on precoated SiO_2 F_{254} plates (Merck 5554 and 5744), visualized under UV light and by spraying with a solution of H_2SO_4–MeOH (1:1) followed by heating. HPLC was carried out on a Merck-Hitachi instrument, with UV detection, using a Merck LiChrospher 100 RP-18 (10 μm, 250 × 10 mm) column.

Plant material. The stem bark of *Pycnanthus angolensis* was collected in São Tomé and Príncipe islands and identified by Professor Jorge Paiva (plant taxonomist), of the Instituto Botânico, University of Coimbra. A voucher specimen (no MM426) has been deposited at the Instituto Botânico, University of Coimbra, Coimbra, Portugal.

Extraction and isolation. The air-dried powdered stem bark of *P. angolensis* (3.25 kg) was extracted with CH_2Cl_2 at room temperature as described previously

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(Abrantes *et al.*, 2008). The residue was further extracted with EtOAc (3 × 5 L) yielding a residue (56 g) that was suspended in MeOH: H_2O, 1:1 (0.4 L) and partitioned with CH_2Cl_2 (3 × 0.4 L) to obtain 6.54 g of CH_2Cl_2 extract. The CH_2Cl_2 extract was subjected to silica gel column chromatography (5 × 80, 230 g SiO_2), eluted with gradient solvents *n*-hexane–EtOAc (1:0 to 6:4, 10% gradient, 0.4 L each eluent; 6:4 to 4:6, 5% gradient, 0.8 L each eluent; 4:6 to 0:1, 5% gradient, 0.4 L each eluent), and EtOAc–MeOH (1:0 to 1:1, 5% gradient, 0.4 L each eluent) to obtain eight major fractions (A–H), which were then combined on the basis of the TLC profile. Fraction D (398 mg) was purified on silica gel column (2 × 40, 27 g SiO_2) with *n*-hexane–EtOAc of increasing polarity (1:0 to 8:2, 2% gradient, 0.25 L each solvent; 8:2 to 0:1, 10% gradient, 0.25 L each eluent) to give a mixture of biochanin A (**6**) and prunetin (**7**) (122 mg) in a 3:1 ratio. Fraction E (477 mg) was submitted to silica gel column (2 × 50, 50 g SiO_2) eluted with *n*-hexane–EtOAc (1:0 to 7:3, 2% gradient, 0.6 L each eluent; 7:3 to 3:7, 10% gradient, 0.2 L each eluent; 0:1, 0.2 L) and EtOAc–MeOH (19:1, 0.2 L) to afford ten subfractions (E1–E10). Irilone (**1**) (10 mg) was purified from subfraction E5 by crystallization (EtOAc:MeOH). Subfraction E6 was further purified by semi-preparative RP-HPLC, eluted with MeOH–H_2O (3:2, 254 nm, 3.0 mL/min, Rt 24 min) to give compound **5** (2′-hydroxybiochanin A, 10 mg). Subfraction E7 was purified by semi-preparative RP-HPLC, eluted with MeOH–H_2O (3:2, 254 nm, 3.0 mL/min, R_t 16 min) to give a mixture, which was further purified by preparative TLC ($CHCl_3$:MeOH, 24:1, 3×) to give pure compounds **2** (tectorigenine, 2 mg) and **8** (4′,7-dihydroxy-2′-methoxyisoflavan, 5 mg), respectively. Subfractions E8 and E9 were purified by preparative TLC ($CHCl_3$–MeOH 19:1, and 24:1, 2×, respectively), yielding compounds **4** (genistein, 5 mg), **9** (liquiritigentin, 5 mg) and **3** (formononetin, 3 mg).

Compounds tested. All the isolated flavonoids, irilone (**1**), tectorigenine (**2**), formononetin (**3**), genistein (**4**), 2′-hydroxybiochanin A (**5**), mixture of biochanin A (**6**) and prunetin (**7**), and 4′,7-dihydroxy-2′-methoxyisoflavan (**8**), and the flavanone liguiritigentin (**9**) were tested for apoptosis induction activity. Based on HPLC and NMR data, the purity of the isolated compounds was more than 95%. Compounds were dissolved in DMSO to prepare a stock solution of 50 μM. For apoptosis assays, 20 μM concentration of each compound was used. Controls containing only DMSO solvent were included in all experiments. Recombinant human TGF-β1 (R&D Systems Inc., Minneapolis, MN, USA) was also used as a positive control (origin: Chinese Hamster Ovary cell line; purity: >97%, by SDS-PAGE under reducing conditions and visualized by silver stain).

Cell culture. Human hepatoma HuH-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Grand Island, NY, USA) supplemented with 100 U/mL penicillin, 100 U/mL streptomycin, 0.25 U/mL amphotericin B and 10% fetal bovine serum (Invitrogen). Cells were plated at either 1×10^5 cells/cm^2 for morphological assessment of apoptosis and viability assays, or 2×10^5 cells/cm^2 for protein extraction. HuH-7 cells were then incubated in medium supplemented with 20 μM of flavonoids for 24 h. Finally, the cells were either fixed for microscopic assessment of apoptosis or processed for cell viability assays. Cytosolic proteins were extracted for caspase activity assays, after treating the cells with 20 μM of flavonoids for 12 h.

Cell viability. Cell viability was measured by lactate dehydrogenase (LDH) assay (Sigma-Aldrich, St Louis, MO, USA). The cells were centrifuged at 250 × **g** for 4 min and aliquots were transferred to a clean flat-bottom plate. LDH assay mixture was added to each sample in a volume equal to twice the volume of medium removed for testing in 96-multiwell plates. Plates were incubated at room temperature for 20–30 min and the absorbance was measured spectrophotometrically at a wavelength of 490 nm.

Evaluation of apoptosis. Hoechst labeling of cells was used to detect apoptotic nuclei. In brief, the cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4, for 10 min at 25 °C, incubated with Hoechst dye 33258 (Sigma-Aldrich) at 5 μg/mL in PBS for 5 min, and then washed with PBS and mounted using PBS: glycerol (3:1, v/v). Fluorescent nuclei were scored and categorized according to the condensation and staining characteristics of chromatin. Normal nuclei showed non-condensed chromatin dispersed over the entire nucleus. Apoptotic nuclei were identified by condensed chromatin, contiguous to the nuclear membrane, as well as nuclear fragmentation of condensed chromatin. Three random microscopic fields per sample of approximately 150 nuclei were counted, and mean values expressed as the percentage of apoptotic nuclei. In addition, DNA fragmentation was evaluated using an ApopTag® peroxidase *in situ* apoptosis detection kit (Serologicals Corp, Norcross, GA, USA) for transferase mediated dUTP-digoxigenin nick-end labelling (TUNEL) staining. Briefly, the cells were fixed in 4% paraformaldehyde in PBS, pH 7.4, for 10 min at room temperature, and post-fixed in precooled ethanol:acetic acid (2:1, v/v) for 5 min at −20 °C. The cells were treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. After adding the equilibration buffer, the cells were treated with terminal deoxynucleotidyltransferase (TdT) and digoxigenin-dNTPs for 60 min at 37 °C. Cells were then treated with antidigoxigenin- peroxidase for 30 min at 37 °C, colorized with 3,3′-diaminobenzidine (DAB) substrate, and counterstained with 0.5% methyl green. Finally, the cells were coverslipped with mounting medium before analysis by phase-contrast microscopy. The number of TUNEL-positive cells was counted on a computer screen grid from at least three random fields. Microscopy assessments were performed with a Zeiss AX10 microscope (Carl Zeiss, Jena, Germany) equipped with a Leica DFC490 camera (Leica Wetzlar, Germany).

Caspase-3-like activity assay. General caspase-3-like activity was determined in cytosolic protein extracts after harvesting and homogenization of cells in isolation buffer containing 10 mM Tris–HCl buffer, pH 7.6, 5 mM magnesium chloride, 1.5 mM potassium acetate, 2 mM dithiothreitol and protease inhibitor cocktail tablets (Complete; Roche Applied Science, Mannheim, Germany). General caspase-3-like activity was evaluated by enzymatic cleavage of chromophore p-nitroanilide (pNA) from the substrates DEVD-pNA (Sigma-Aldrich). The proteolytic

reaction was carried out in isolation buffer containing 50 μg cytosolic proteins and 50 μM substrate. The reaction mixtures were incubated at 37 °C for 1 h, and the formation of pNA was measured at 405 nm using a 96-well plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis. All data were expressed as mean ± SEM from at least three separate experiments. Statistical analysis was performed using GraphPad Instat version 3.00 (GraphPad Software, San Diego, CA, USA) for the Student's *t*-test. Values of $p < 0.05$ were considered significant.

RESULTS

Phytochemical studies of the CH_2Cl_2 soluble fraction of the EtOAc extract of *Pycnanthus angolensis* have led to the isolation of nine flavonoids including eight isoflavones, irilone (**1**), tectorigenine (**2**), formononetin (**3**), genistein (**4**), 2′-hydroxybiochanin A (**5**), biochanin A (**6**), prunetin (**7**) and 4′,7-dihydroxy-2′-methoxyisoflavan (**8**), and a flavanone liguiritigentin (**9**). Compounds **6** and **7** were isolated in mixture (3:1). The structures of these compounds were characterized by the comparison of their spectroscopic data with those reported in the literature (Dhar and Kalla, 1973; Agrawal *et al.*, 1984; Chang *et al.*, 1994; Chun-qing *et al.*, 1997).

The ability of compounds **1–9** to induce cell death was evaluated by a general screening of cell viability, using the LDH assay, after exposure of human hepatoma HuH-7 cells to 20 μM of each compound for 24 h. Compound **4** was the most toxic, increasing cell death by ~50%, compared with the control ($p < 0.01$) (Fig. 1). Exposure to either compounds **1**, **2**, **5** or a mixture of **6** and **7** resulted in increases in cell death ranging from ~40% to 20% ($p < 0.05$).

Next, the ability of the compounds to induce apoptosis was evaluated by assessing the nuclear morphology of HuH-7 cells after Hoechst staining, following incubations with flavonoids **1–9**, for 24 h (Fig. 2a). Apoptotic nuclei were characterized by condensed chromatin contiguous to the nuclear membrane, as well as nuclear fragmentation. Incubation with the isoflavone mixture (**6** and **7**) showed almost 30% of apoptotic cells ($p < 0.01$) (Fig. 2b). Compounds **1**, **2**, **5** and **8** also induced increased levels of apoptosis up to ~20%, compared with the control ($p < 0.01$). Genistein (**4**), showed the lowest activity among all the flavonoids, inducing only 16% of apoptosis ($p < 0.01$).

Apoptosis induction by flavonoids **1–9** was also analysed by caspase-3-like activity assays. The results confirmed the morphological data after Hoechst staining. All the tested compounds proved to be caspase-3 inducers. In fact, caspase-3-like activity increased by 80% after incubation with isoflavone **1** compared with the controls ($p < 0.01$) (Fig. 3). In addition, isoflavones **2**, **3**, **4**, a mixture of **6** and **7**, and **8** also induced caspase-3-like activity by ~50% ($p < 0.05$).

To further confirm the apoptosis induction activity, isoflavone **1** was selected and the TUNEL assay was performed (Fig. 4). The results corroborated the apoptosis induction nature of isoflavone **1**, showing increased numbers of nuclei with DNA fragmentation.

DISCUSSION

In the present study, all tested compounds (**1–9**) (Fig. 5) showed higher levels of apoptosis induction in human hepatoma HuH-7 cells compared with the non-exposed control cells. In cytotoxicity assays, compounds showed varying levels of LDH activity (Fig. 1). LDH is a soluble enzyme located in the cytosol. The enzyme is released into the surrounding culture medium, upon cell damage or lysis that occurs during both apoptosis and necrosis. LDH activity in the culture medium is therefore used as an indicator of cell membrane integrity and a marker of cytotoxicity. Isoflavone **4**, genistein, increased cytotoxicity by 50%, and was followed by **2** and **5**, and **1** and a mixture of **6** and **7** in cytotoxic activity. The higher value of cytotoxicity of isoflavone **4** in the LDH assay, but lowest apoptosis induction profile in Hoechst staining (Fig. 2a and 2b) might be due to its necrosis-based mode of action, rather than apoptosis.

In the general caspase-3-like assay, all compounds exhibited increased enzymatic activity (Fig. 3), confirming the Hoechst staining apoptosis induction profiles. Caspases are a family of proteins that are one of the main executors of the apoptotic process. They belong to a group of enzymes known as cysteine proteases

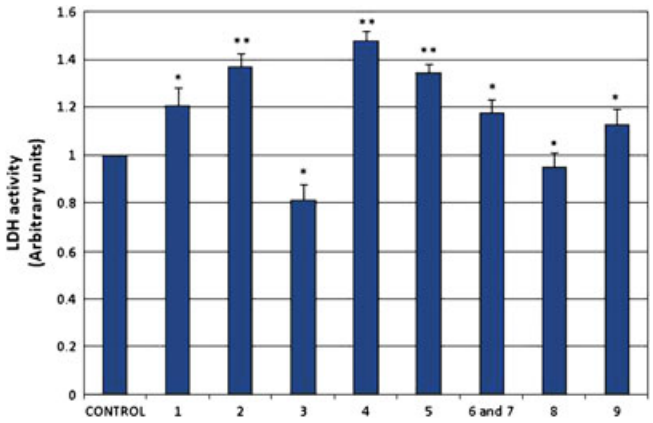

Figure 1. General cell death as assessed by the LDH assay after exposure of human hepatoma HuH-7 cells to either DMSO solvent (control) or 20 μM flavonoids (**1–9**) for 24 h. Results are expressed as the mean numbers ± SEM of at least three different experiments. *$p < 0.05$ and **$p < 0.01$ from control.

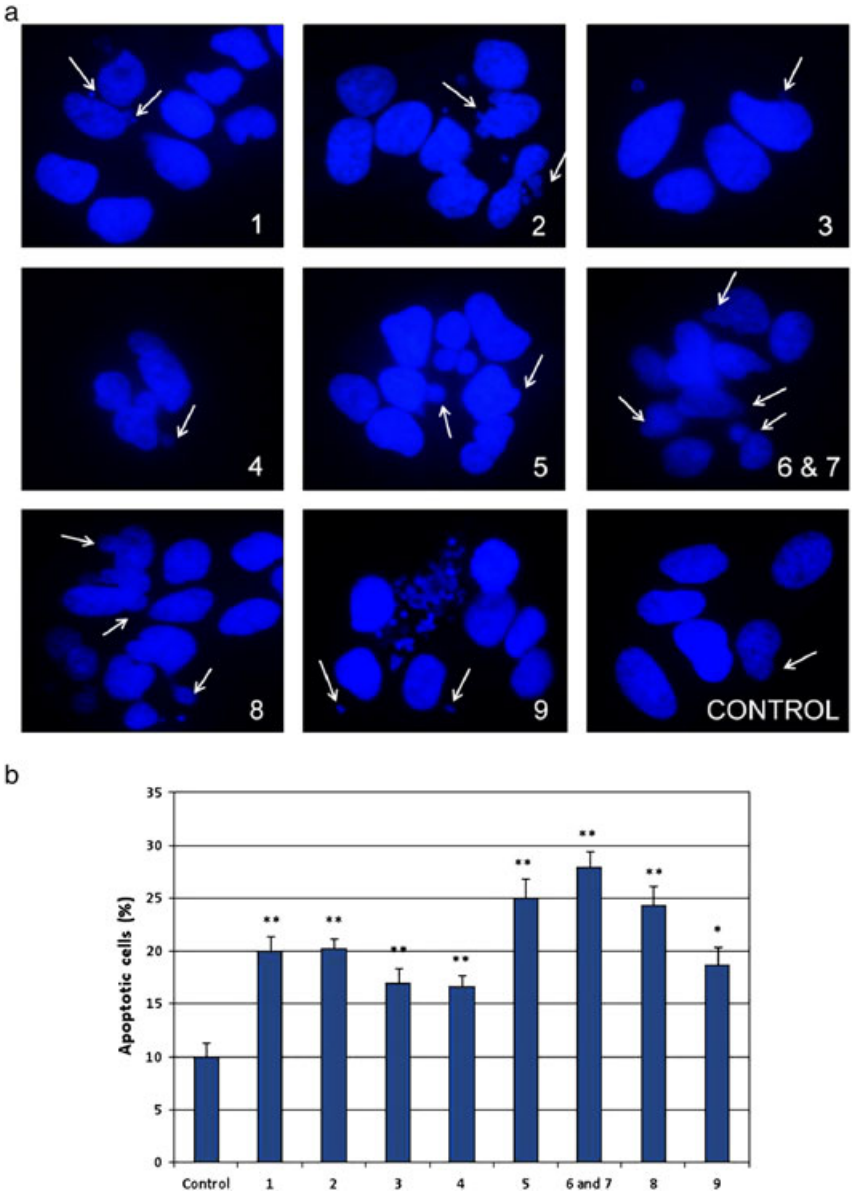

Figure 2. (a) Fluorescence microscopy of Hoechst staining after incubation of human hepatoma HuH-7 cells with either DMSO solvent (control) or 20 μM flavonoids **1–9** for 24 h. Arrows indicate apoptotic bodies. (b) Percentage of apoptotic cells as assessed by nuclear morphology changes after Hoechst staining in human hepatoma HuH-7 cells exposed to either DMSO solvent (control) or 20 μM flavonoids (**1–9**) for 24 h. Results are expressed as the mean numbers ± SEM of at least three different experiments. $*p < 0.05$ and $**p < 0.01$ from control. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr

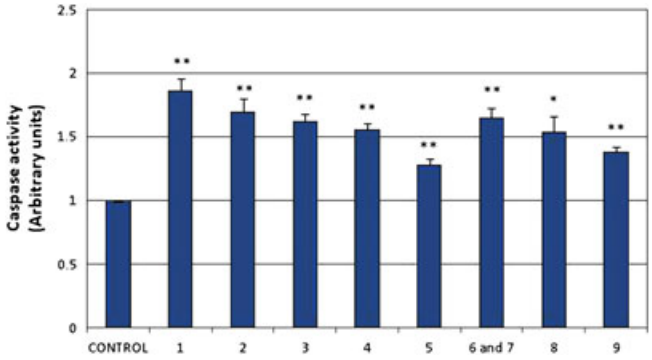

Figure 3. Caspase-3-like activity after exposure of human hepatoma HuH-7 cells to either DMSO solvent (control) or 20 μM flavonoids (**1–9**) for 12 h. Results are expressed as the mean numbers ± SEM of at least three different experiments. $*p < 0.05$ and $**p < 0.01$ from control.

and exist within the cell as inactive pro-forms or zymogens. These zymogens can be cleaved to form active enzymes following the induction of apoptosis. Induction of apoptosis via death receptors typically results in the activation of initiator caspases, such as caspase-8 or -10. These can then activate downstream

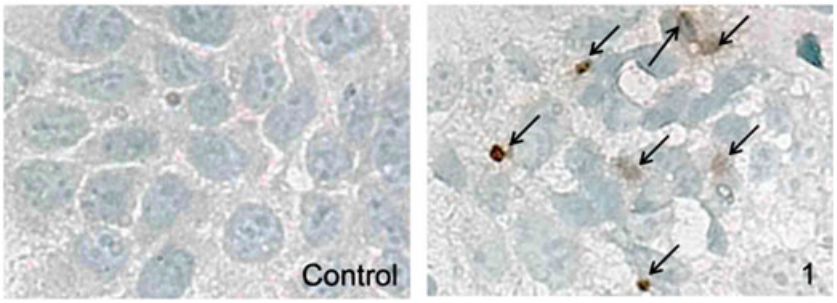

Figure 4. TUNEL staining after incubation of human hepatoma HuH-7 cells with either DMSO solvent (control) or 20 μM isoflavone **1** for 24 h. Arrows indicate TUNEL-positive nuclei. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr

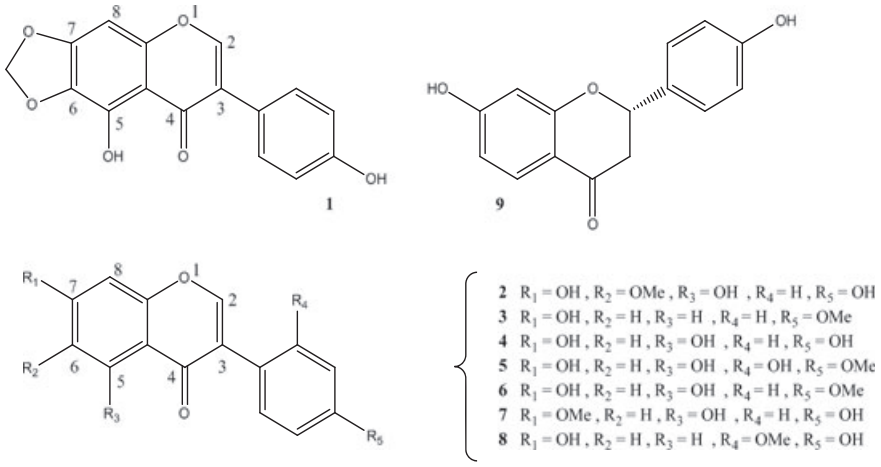

Figure 5. Chemical structures of flavonoids **1–9**.

effector caspases, such as caspase 3, -6 and -7. Effector caspases are responsible for the cleavage of key cellular proteins, such as cytoskeletal proteins, which lead to the typical morphological changes observed in cells undergoing apoptosis. Irilone (**1**) exhibited the highest caspase activity, reflecting an increase of ~80% in caspase-3-like activity compared with the control. Isoflavones **2–4**, a mixture of **6** and **7**, and **8** showed at least a 50% increase in caspase activity. These studies corroborated the ability of these flavonoids to induce apoptosis in HuH-7 cells.

Irilone (**1**), which showed the highest level of caspase-3 like activity, was further studied for its pro-apoptotic activity using the TUNEL assay. DNA fragmentation represents a characteristic hallmark of apoptosis, and the TUNEL assay is an established method for detecting DNA fragments. The presence of TUNEL positive nuclei in cells treated with compound **1** corroborated its apoptosis induction profile (Fig. 4).

To summarize, all flavonoids exhibited higher levels of apoptosis induction than those of the control in human hepatoma HuH-7 cells. Flavonoids, because of their antioxidant behavior and ability to interact with different enzymes are considered as important apoptosis inducers (Rice-Evans, 2001). This study supports the notion that intake of certain flavonoids such as genistein rich foods (Sarkar and Li, 2002) have a chemoprotective effect against various types of cancer. Further studies to dissect mechanisms of action of these compounds are essential clearly to define their effects.

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Conflict of interest

None of the authors have any conflict of interest related to this study. There is no ethical issue with this study.

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