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Effect of *Derris scandens* extract on a human hepatocellular carcinoma cell line

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Abstract. The incidence rate of hepatocellular carcinoma (HCC) remains high in numerous countries, including Thailand. There are numerous different lines of HCC treatment; however, various side effects and the resistance of cancer cells during treatment remain issues. At present, traditionally used herb plants have been widely used as alternatives to cancer therapy. *Derris scandens* is a Thai traditional herb which is commonly found in Thailand and widely used as a traditional medicine for numerous different diseases. The cytotoxicity of *D. scandens* ethanolic extract on a HCC cell line (HCC-S102) was determined using an MTT assay. Following treatment with *D. scandens* ethanolic extract, the induction of apoptosis was determined by Annexin V and dead cell assays, and then confirmed by the upregulation of cleaved poly(ADP-ribose) polymerase. Furthermore, a proteomic approach was used in order to study protein alteration upon treatment with *D. scandens* ethanolic extract coupled with liquid chromatography-tandem mass spectrometry analysis for protein identification. The results suggested that *D. scandens* ethanolic extract resulted in cytotoxicity against HCC-S102 cells, as the half-maximal inhibitory concentration values were 36.0±1.0, 29.6±0.6, and 22.6±1.5 μg/ml at 24, 48 and 72 h, respectively. Apoptotic cells were induced following treatment with *D. scandens*. The comparative proteomic profiles of *D. scandens* ethanolic extract-treated and untreated cells revealed various protein targets for anticancer activity including heterogeneous nuclear ribonucleoprotein (hnRNP) K, hnRNP A2/B1, stomatin-like 2 and GAPDH. In the present study, the anticancer activity of *D. scandens* ethanolic extract was demonstrated to affect the cell proliferation of HCC-S102 via an apoptotic pathway. The alteration in these proteins provides a better understanding of the mechanism of action of *D. scandens*, which may be a promising anticancer agent for the treatment of patients with HCC in the future.

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Introduction

Hepatocellular carcinoma (HCC), a primary liver cancer, is one of the most prevalent causes of cancer-associated mortality globally and originates from hepatocyte cells in the liver (1). Various risk factors, including chronic viral infection (including with hepatitis B and C viruses), aflatoxin exposure, alcohol ingestion and iron overload, contribute to the development of HCC (2). The incidence rate of HCC is increasing in numerous Asian countries, including Thailand (1). A majority of patients with HCC have a poor prognosis due to the first detection of disease at an advanced stage. Numerous different therapies have been devised in order to improve the prognosis and long-term survival of patients; however, limitations remain, including severe side effects such as fatigue, pain, diarrhea, nausea, vomiting, hair loss and the resistance of cancer cells to treatment (3). At present, there is a great deal of interest in traditional plant medicines, which may be used for cancer therapy or in combination with other cancer treatments.

Derris scandens, locally known as 'Thao-wan-Priang' in Thailand, belongs to the family of Leguminosae or Fabaceae. It is well-known as an Asian medicinal plant, which grows as a woody vine throughout Southeast Asia, including in Thailand. Dried stems of *D. scandens* have been used as an expectorant, antitussive, diuretic and anti-dysentery agent (4), and additionally for the treatment of several diseases including osteoarthritis, inflammation and muscle pains (4,5). A previous study revealed that *D. scandens* ethanolic extract has potential anti-metastatic activity in cholangiocarcinoma and hepatoma cell lines equal to paclitaxel (Taxol; 10^{-9} M), which was used as positive control (6). Furthermore, extracts from *D. scandens* have been revealed to exert anti-proliferative effects against colon cancer by upregulating B-cell lymphoma 2-associated X protein (Bax), which is pro-apoptotic, and downregulating B-cell lymphoma 2 (Bcl-2) anti-apoptotic proteins (7).

Proteomic analysis is used extensively in the field of cancer research. The main principle of this technique is to separate

proteins in two dimensions according to their isoelectric point and molecular mass. Mass spectrometry is used for protein identification following two-dimensional (2D) electrophoresis (8). These processes of proteomic analysis have been used as crucial tools in order to comprehensively monitor, identify and characterize the variations of proteins for numerous different diseases (9,10). Thus, proteomic analysis is a useful tool to examine and identify the changes in protein expression in a HCC cell line in response to traditional plant treatment.

In the present study, the cytotoxicity of *D. scandens* ethanolic extract on a human HCC cell line, HCC-S102, was examined. Induction of cytotoxicity via apoptosis was additionally studied. As the mechanisms underlying the anti-proliferative properties of *D. scandens* on this cell line are yet to be reported, 2D electrophoresis was performed to identify protein alterations which will improve understanding of the mode of action.

Materials and methods

Plant preparation. D. scandens was purchased from a Thai medicinal herb shop in Bangkok, Thailand. Stem parts were selected and prepared using ethanol extraction. Briefly, the stem parts of dry plants were chopped and ground into small pieces. Dried ground plant materials (50 g) were percolated with absolute ethanol and then shaken with an orbital shaker at 60 rev/min for 22 h at room temperature. The plant materials in absolute ethanol solution were then filtered using Whatman filter paper no. 4 (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), followed by drying under decreased pressure to yield 62.93 mg. Ethanolic plant extracts were dissolved in dimethylsulfoxide (DMSO) at 200 mg/ml and stored as a stock solution at -20˚C.

Cell culture. The HCC-S102 cell line, established from a Thai patient (11), was kindly provided by Dr Sumalee Tungpradabkul (Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand). Cells were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences), 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and 125 ng/ml amphotericin B (Gibco; Thermo Fisher Scientific, Inc.). All cultures were incubated in a CO_2 incubator at 37˚C in a humidified atmosphere of 5% CO_2. Culture medium was replenished three times per week.

Cytotoxic activity. HCC-S102 cells were used to examine the cytotoxic effect of *D. scandens* using an MTT assay (12). Cells in culture medium were plated in 96-well plates at a density of $5x10^3$ cells/well and incubated at 37˚C overnight. The cells were then treated with various concentrations of *D. scandens* ethanolic extract (0-50 μg/ml) for 24, 48 and 72 h in addition to DMSO (negative control) and doxorubicin (positive control) at various concentrations (0-2.7 μg/ml) for 24, 48 and 72 h. Subsequently, medium was discarded and replaced with 100 μl fresh medium containing tetrazolium dye (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) solution (0.5 mg/ml). All plates were incubated for 2 h at 37˚C. Then, the medium was removed and formazan product was dissolved by the addition of 100 μl DMSO to each well. Absorbance was measured at 550 nm with subtracted background at 650 nm using a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Protein extraction. Cells treated with 20 and 30 μg/ml *D. scandens* ethanolic extract for 72 h and control cells (treated with 0.5% DMSO) were separately washed with 0.25 M sucrose to remove salt. Cells were then scraped into 450 μl 0.25 M sucrose containing cOmplete™, Mini Protease Inhibitor Cocktail Tablets (ratio of 1:10, Sigma-Aldrich; Merck KGaA) and centrifuged at 778 x g for 15 min at 4˚C. The pellets were resuspended in lysis buffer containing 9 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 2% dithiothreitol (DTT), 2% ampholine [pH 3-10, the same range as that of the immobilized pH gradient (IPG) strip] and 1% protease inhibitor cocktail, followed by sonication on ice and centrifugation at 13,800 x g for 10 min at 4˚C. Protein concentrations were determined using a Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) as previously described (13).

2D gel electrophoresis and image analysis. Amounts of 200 μg of each sample were applied onto Immobiline™ Drystrips (7 cm, non-linear, pH 3-10 gradient; Bio-Rad Laboratories). IPG strips were incubated at room temperature overnight. First-dimension isoelectric focusing was performed at 6,500 V/h, 55 μA per gel strip using an Ettan IPGphor 3 (GE Healthcare, Little Chalfont, UK). For second-dimension SDS-PAGE, the IPG strips were equilibrated in an equilibration buffer at room temperature for 15 min for each step. Electrophoresis was performed in a SE 600 Ruby apparatus (GE Healthcare Life Sciences) at 10 mA/gel, followed by Coomassie blue R-250 staining as previously described (14). Gels were scanned using Labscan software (version 5.0; GE Healthcare Life Sciences) and analyzed using ImageMaster 2D Platinum (version 7.0; GE Healthcare Life Sciences). All experiments were performed independently in triplicate. Protein spots revealing a significant difference in volume ratio ($P<0.05$) were selected for protein identification by mass spectrometry analysis.

In-gel digestion. Protein spots were subjected to in-gel tryptic digestion as described previously (14). Briefly, the gels were cut into small pieces and destained using 0.1 M NH_4HCO_3 in 50% acetonitrile at 30˚C for 20 min, followed by reduction with 10 mM DTT at 60˚C for 45 min and alkylation with 100 mM iodoacetamide at 25˚C for 30 min, respectively. Following the removal of reagents, gel pieces were completely dried and digested using 0.3 μg trypsin (Promega Corporation, Madison, WI, USA) in 30 μl digestion buffer at 37˚C overnight. The digestion buffer was collected for protein identification.

Protein identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Identification of protein spots were performed by nanoflow liquid chromatography coupled with amaZon speed ion trap mass spectrometry (Bruker Corporation, Billerica, MA, USA). All trypsinized peptides were concentrated and desalted on a 75 μm internal diameter x100 mm C_{18} EASY-nLC™ column (Thermo Fisher Scientific, Inc.). Subsequently, the peptides were eluted by

gradient separation using eluents A and B, which were 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. In total, 6 μl sample was injected into the nano-LC system and separated at a flow rate of 0.5 μl/min for 30 min using the following gradient: 0 min 95% A, 20 min 60% A, 20.5 min 5% A, 29 min 5% A and 29.5 min 95% A, followed by MS/MS equipped with the CaptiveSpray™ source using 1.0 sec automatic scan rate with 0.1 sec interscan delay. For MS/MS analysis, parent mass peaks with a range between 50 and 3,000 *m/z* were selected. Collision energy was fixed at 1,300 V. MS/MS data were processed and converted into mgf files using Compass software (version 1.4; Bruker Corporation) and proteins were identified using the MASCOT search engine (www.matrixscience.com). Search parameters were set as follows: Database, Swiss-Prot and NCBInr; taxonomy, *Homo sapiens*; enzyme, trypsin; two missed cleavages allowed. Peptide and MS/MS fragment ion mass tolerance were set at 1.2 and 0.6 Da, respectively. Proteins with a consistent molecular mass and isoelectric point gel spot with a MASCOT score of >25 using a P-value of ≤0.05 were considered to be positively identified.

Western blot analysis. Protein samples were extracted using radioimmunoprecipitation assay buffer that contained cOmplete™, Mini Protease Inhibitor Cocktail Tablets. A total of 10 μM NaF (New England BioLabs, Inc., Ipswich, MA, USA), 1 mM of Na_3VO_4 (Sigma-Aldrich; Merck KGaA) and 20 mM of β-glycerophosphate (Merck KGaA) were used as phosphatase inhibitors. A total of 15 μg protein was loaded into each lane and was separated by SDS-PAGE (12.5% gel) and electrophoretically transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 3% bovine serum albumin for 1 h at room temperature and subsequently incubated at 4˚C overnight with primary antibodies as follows: Rabbit polyclonal anti-poly(ADP-ribose) polymerase (PARP; 1:1,000; cat. no. 9542; Cell Signaling Technology, Inc., Danvers, MA, USA), mouse monoclonal anti-α-tubulin (1:3,000; cat. no. 3873; Cell Signaling Technology, Inc.), rabbit polyclonal anti-heterogeneous ribonucleoprotein K (hnRNP K, 1:1,000; cat. no. 4675; Cell Signaling Technology), rabbit monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1,000; cat. no. ab75834; Abcam, Cambridge, UK), mouse monoclonal anti-hnRNP A2/B1 (1:1,500; cat. no. ab6102; Abcam), rabbit polyclonal anti-peroxiredoxin-4 (Prx4; 1:10,000; cat. no. ab15574; Abcam), rabbit monoclonal anti-oxygen-regulated protein 150 (ORP150, also known as HYOU1 or hypoxia-upregulated protein 1; 1:1,000; cat. no. ab134944; Abcam) and rabbit monoclonal anti-stomatin-like protein 2 (STOML2; 1:2,000; cat. no. ab191884; Abcam). The membranes were then washed 3 times with Tris-buffered saline with 0.1% Tween 20 and was incubated with a horseradish peroxidase (HRP)-conjugated polyclonal rabbit anti-mouse immunoglobulin (cat no. P0260; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) or polyclonal swine anti-rabbit immunoglobulin (cat no. P0217; Dako; Agilent Technologies, Inc.) (dilution, 1:2,000) at room temperature for 1 h. Bands were detected using electrochemiluminescence reagent (GE Healthcare Life Sciences) and images were captured using the ImageQuant™ LAS 4000 digital imaging system (GE Healthcare Life Sciences).

Annexin V and dead cell assay (flow cytometry). A total of $2x10^5$ cells (2 ml/well) was seeded in 6-well plates at 37˚C overnight. The control condition group contained 0.5% DMSO as a vehicle control. For treated cells, cells were treated with *D. scandens* ethanolic extract at various concentrations (20 and 30 μg/ml) and further incubated at 37˚C in a humidified atmosphere of 5% CO_2 for 24, 48 and 72 h. Following incubation, cells were trypsinized and resuspended at $1x10^6$ cells/ml prior to staining with a 1:1 ratio of Muse® Annexin V and Dead Cell Assay kit (EMD Millipore, Billerica, MA, USA) at room temperature in the dark for 20 min. Apoptotic analysis was performed using Muse™ Cell Analyzer (EMD Millipore), with Muse™ Analysis Software version 1.3.0.0.

Statistical analysis. All data are presented as the mean ± standard deviation. Statistical analysis was performed by two-way analysis of variance with Tukey's Honest Significant Difference post-hoc analysis), using GraphPad Prism (version 7; GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Cytotoxic activity of D. scandens ethanolic extract on HCC-S102 cells. To estimate the cytotoxic effect of *D. scandens* ethanolic extract on HCC-S102 cells, an MTT assay was performed. Half-maximal inhibitory concentration (IC_{50}) values were calculated, and the IC_{50} values were 36.0±1.0, 29.6±0.6, and 22.6±1.5 μg/ml at 24, 48 and 72 h, respectively (Fig. 1A). Additionally, doxorubicin was used as a positive control, and the IC_{50} values for the doxorubicin-treated cells were 1.1±0.03, 0.6±0.01 and 0.3±0.02 μg/ml at 24, 48 and 72 h, respectively (Fig. 1B).

These results reveal that *D. scandens* ethanolic extract exerts a cytotoxic effect on HCC-S102 cells in dose- and time-dependent manner compared with the control. On the basis of the inhibitory concentration of *D. scandens* ethanolic extract, the IC_{50} and IC_{80} were 20 and 30 μg/ml, respectively, and so these doses were selected for further studies.

Detection of apoptosis of HCC-S102 cells induced by D. scandens ethanolic extract. The apoptosis-inducing effect of *D. scandens* ethanolic extract on HCC-S102 cells was quantified using Annexin V and dead cell assays. HCC-S102 cells were exposed to *D. scandens* ethanolic extract at concentrations of 20 and 30 μg/ml for 24, 48 and 72 h. Following treatment, a gradual increase in Annexin V-positive-staining was detected in cells treated with 20 and 30 μg/ml *D. scandens* ethanolic extract compared with the control (Fig. 2A).

The proportions of total apoptotic cells demonstrated that the treatment of *D. scandens* ethanolic extract on HCC-S102 induced apoptosis in a dose- and time-dependent manner. Thus, total apoptotic cells were significantly increased (P<0.01) in cell groups treated with 20 and 30 μg/ml *D. scandens* ethanolic extract for 48 and 72 h compared with the control, and significantly increased (P<0.01) in the group treated with 30 μg/ml *D. scandens* ethanolic extract for 24 h compared with the control (Fig. 2B).

To confirm whether *D. scandens* ethanolic extract was associated with an induction of apoptosis in HCC-S102 cells,

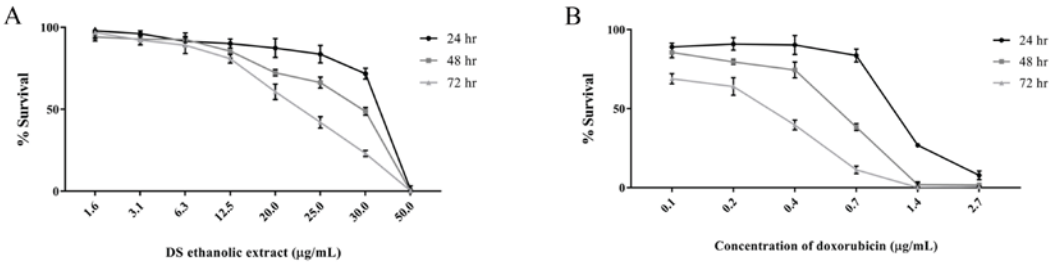


Figure 1. Cytotoxic effect of *D. scandens* ethanolic extract and doxorubicin on the HCC-S102 cell line. The cells were exposed to (A) *D. scandens* ethanolic extract or (B) doxorubicin (positive control) at the indicated dosages for 24, 48 and 72 h, and cell viability was determined using an MTT assay. Results are expressed as the mean ± standard deviation of three independent experiments. DS, *Derris scandens*.

the expression levels of cleaved PARP were determined using western blot analysis. The results revealed that treatment of HCC-S102 cells with *D. scandens* ethanolic extract increased the band intensity of cleaved PARP, particularly at 30 μg/ml (at 89 kDa; Fig. 3A). Therefore, 30 μg/ml *D. scandens* ethanolic extract resulted in significantly (P<0.01) increased expression levels of cleaved PARP after 24, 48, and 72 h of treatment (Fig. 3B). An increase in cleaved PARP expression levels confirmed the hypothesis that *D. scandens* ethanolic extract induced the death of HCC-S102 cells via the apoptotic pathway.

Effects of D. scandens ethanolic extract treatment by proteomic analysis. In order to identify any differences in protein expression, proteins were extracted from HCC-S102 cells treated with 20 and 30 μg/ml *D. scandens* ethanolic extract for 72 h. The 2D patterns of control and *D. scandens* ethanolic extract-treated cells (Fig. 4A-C) were then analyzed using ImageMaster 2D Platinum program. As a result, ~466 protein spots were detected in *D. scandens* ethanolic extract-treated (either 20 or 30 μg/ml) cells and, the majority of proteins were revealed to have statistically significant differences in their expression levels >1.5-fold change (P<0.05; data not shown), compared with the equivalent spots in control cells. Among these, 18 protein spots that were identified to have significantly different expression levels were selected for further analysis by LC-MS/MS and subsequently identified using the MASCOT search engine and SwissProt database.

In total, 18 proteins were identified, as presented in Table I. In total, 6 upregulated proteins and 12 downregulated proteins were identified in *D. scandens* ethanolic extract-treated (20 and 30 μg/ml) HCC-S102 cells compared with the control. The upregulated proteins included endoplasmin (Hsp90B1), heat-shock cognate 71 kDa protein (Hsc70), cytoplasmic dynein 1 intermediate chain 2 (DC1I2), UDP-N-acetylhexosamine pyrophosphorylase-like protein 1, ORP150 and type I cytoskeletal keratin 9.

Downregulated proteins included chromobox protein homolog 3 (CBX3), 60S acidic ribosomal protein P0, filamentous actin-capping protein subunit α-1, retinal dehydrogenase 1 (RALDH1), aldo-keto reductase family 1 member C1 (AKR1C1), GAPDH, neurocalcin-δ, cytochrome bc_1 complex subunit 1 (UQCRC1), glutaredoxin-3 (TXNL2), peroxiredoxin-4 (Prx4), hnRNP K, hnRNP A2/B1 and STOML2.

Validation of differential 2D electrophoresis protein expression levels by western blot analysis. To validate the difference of the expression levels of the proteins of interest, which have been reported to be involved in the survival of cancer cells (15-20), western blot analysis was used to confirm the differences of protein expression levels between *D. scandens* ethanolic extract-treated cells and untreated cells. ORP150 was revealed to be significantly increased (P<0.0001) in *D. scandens* ethanolic extract-treated cells compared with the control, which is consistent with the 2D electrophoresis result. Similarly, STOML2 was revealed to be significantly decreased in 20 μg/ml *D. scandens* ethanolic extract-treated cells (P<0.05), and Prx4, STOML2, hnRNP K and hnRNP A2/B1 were all significantly decreased in 30 μg/ml treated cells (P<0.05) compared with the control (Fig. 5A and B).

Discussion

Cancer is a major health problem, thus developments of novel cancer treatments are required. It has been reported that certain plant extracts have the potential to be used as anticancer drugs by enhancing the immune system, inducing cell differentiation, inhibiting telomerase activities and inducing apoptosis *in vitro* and *in vivo* (21). In Thailand, certain herbs have been widely used for a long time. However, the underlying molecular mechanisms of action of the Thai herbs (e.g., *Bridelia ovata* Decne or *Dioscorea membranacea* Pierre) for cancer treatment are still being investigated. In the present study, the effect of *D. scandens* stem ethanolic extract on the HCC-S102 cell line were determined.

According to the criteria of cytotoxicity activity for crude extracts from the American National Cancer Institute (NCI), a concentration of plant extract ≤30 μg/ml with an incubation time of between 48 and 72 h is considered to be 'active' due to the calculation of the IC_{50} value (22). The *D. scandens* ethanolic extract was investigated for cytotoxic activity in HCC-S102 cells. The IC_{50} value following treatment for 72 h was 22.6±1.5 μg/ml, which is of potential interest in terms of the NCI criteria. One previous study indicated that ethanolic crude extract of *D. scandens* exhibited potent cytotoxic activity against the lung adenocarcinoma cell line (CORL-23) with an IC_{50} value of 21.04±0.57 μg/ml following treatment for 72 h (23). Furthermore, the cytotoxic activity of *D. scandens* ethanolic extract has been investigated using other lung cancer cell lines (namely, A549 and NCI-H226) compared with lung normal cells, with the results indicating that *D. scandens* ethanolic extract exhibited an increased cytotoxic activity in lung cancer cell lines compared with normal lung cells (IC_{50}, 65.04 μg/ml) (24).

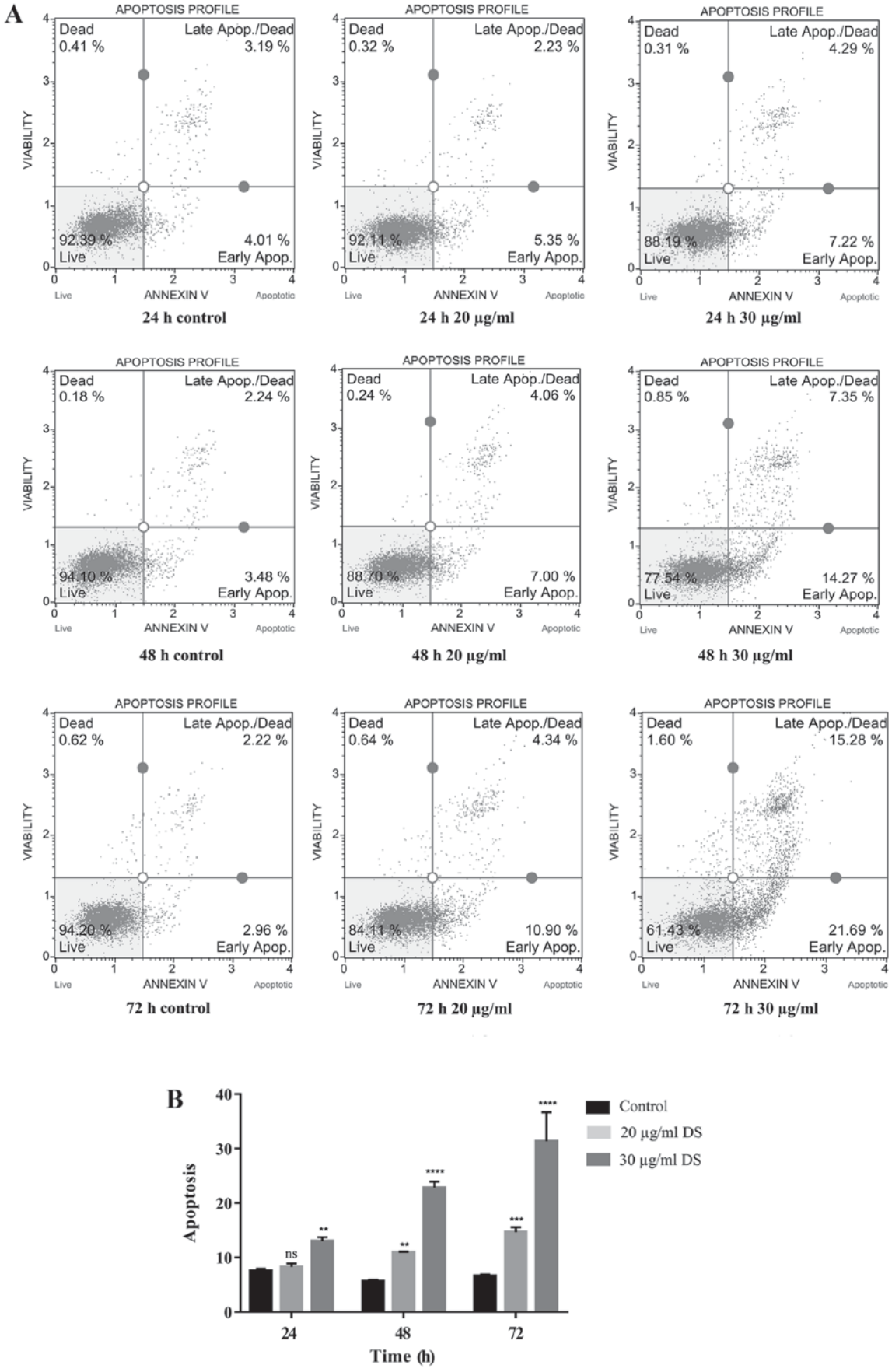


Figure 2. Effect of *D. scandens* ethanolic extract on the apoptosis of the HCC-S102 cell line. (A) Flow cytometry with Muse analysis demonstrated that the proportion of total apoptotic cells increased in the HCC-S102 cell line with increasing doses and durations of treatment with *D. scandens* ethanolic extract. (B) Percentage of total apoptotic cells observed following treatment with 20 and 30 μg/ml *D. scandens* ethanolic extract for 24, 48 and 72 h compared with the control. Results are expressed as the mean ± standard deviation. **P<0.01 and ***P<0.001 vs. the control. NS, non-significant; DS, *Derris scandens* ethanolic extract.

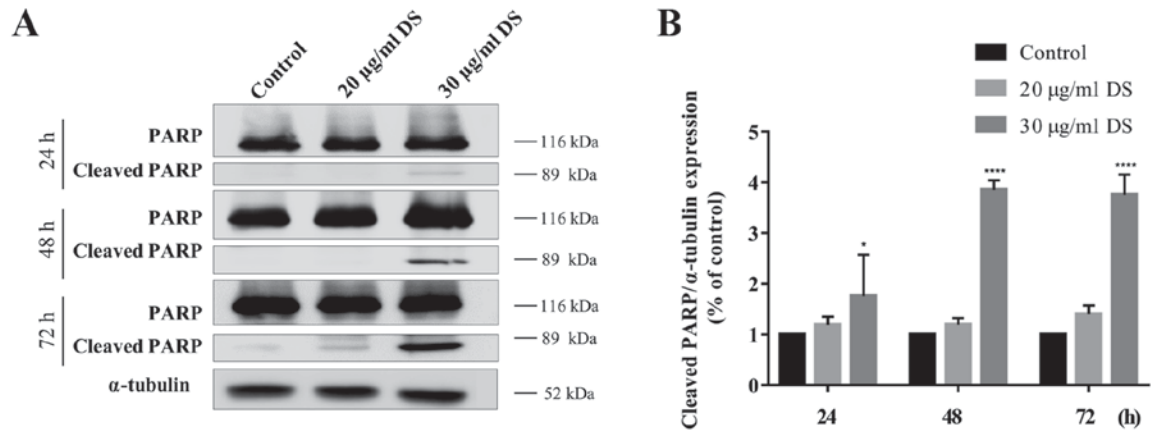


Figure 3. Effect of *D. scandens* ethanolic extract on the expression levels of cleaved PARP in the HCC-S102 cell line. (A) Western blot analysis results revealed an upregulation of the expression of cleaved PARP (89 kDa) following treatment with *D. scandens* ethanolic extract for 24, 48 and 72 h. (B) Fold changes of cleaved PARP expression levels compared with the control at different treatment doses (20 and 30 μg/ml) and times (24, 48 and 72 h). α-tubulin (52 kDa) was used as a loading control. Results are expressed as the mean ± standard deviation. *P<0.05 and ***P<0.001 vs. the control. DS, *Derris scandens* ethanolic extract; PARP, poly(ADP-ribose) polymerase.

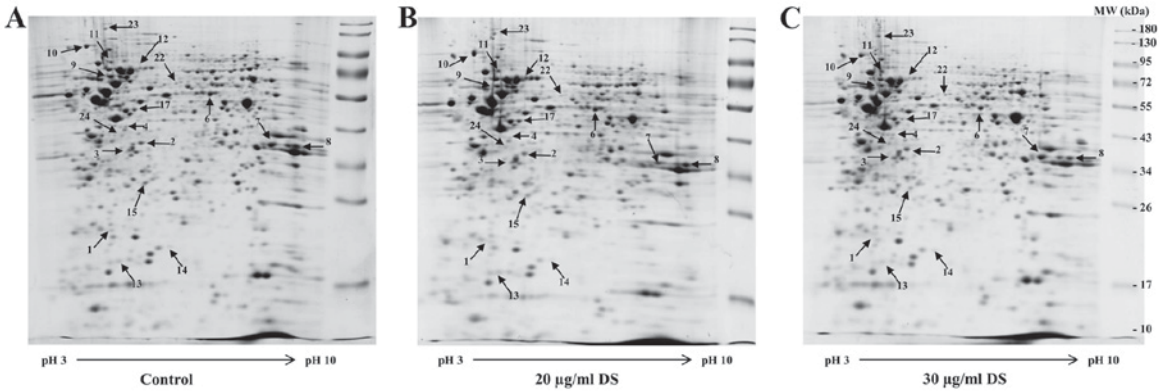


Figure 4. 2D electrophoresis map of proteins from the HCC-S102 cell line treated with *D. scandens* ethanolic extract. 2D electrophoresis was performed on a pH 3-10 non-linear immobilized pH gradient strip (7 cm), followed by SDS-PAGE (12.5% gel). Proteins were isolated after exposure of the cells to (A) control and (B) 20 and (C) 30 μg/ml *D. scandens* ethanolic extract for 72 h. The gels were stained with Coomassie blue R-250. 2D, two-dimensional; DS, *Derris scandens* ethanolic extract; MW, molecular mass.

D. scandens ethanolic extract has been revealed to have anti-proliferative effects against colon cancer cell lines, mediating cell death through necrosis in the SW480 colon cancer cell line (7). Hematulin *et al* (5) reported that colon cancer cells (HT-29) treated with *D. scandens* ethanolic extract exhibited increased apoptosis induction compared with cells treated with a combination of radiation and *D. scandens* ethanolic extract. However, cell death from *D. scandens* ethanolic extract in combination with radiation was increased compared with in *D. scandens* ethanolic extract alone, suggesting that other modes of cell death were involved in colon cancer cell lines.

To determine how *D. scandens* ethanolic extract exerts cytotoxicity on the HCC-S102 cell line, a cell death mechanism, apoptosis, was studied using an Annexin V and dead cell assay, which detects the translocation of phosphatidylserine from the inner to the outer cell-surface membrane, which occurs during apoptosis. Using a double stain of Annexin V-fluorescein isothiocyanate (FITC) and 7-aminoactinomycin D (7-AAD), cells were classified as living cells or cells at early or late stages of apoptosis. HCC-S102 cells treated with *D. scandens* ethanolic extract were stained with Annexin V-FITC and 7-AAD, and then quantified using flow cytometric analysis. Results revealed that cell apoptosis from *D. scandens* ethanolic treatment was dose- and time-dependent. At higher doses, treated cells were killed via the apoptosis pathway following incubation with *D. scandens* extract for 48 and 72 h.

PARP is a nuclear DNA-binding protein, which is typically cleaved by caspase-3 during apoptosis, thus the detection of PARP cleavage is used as an indicator of apoptosis (25). The results of the present study revealed that there was a significantly increased intensity of cleaved PARP protein in treated cells at different doses and times compared with the control. This corresponds with the results of the flow cytometric experiments, and confirms that *D. scandens* ethanolic extract treatment induced apoptosis in HCC-S102 cells.

Proteomic analysis was used to investigate differentially expressed proteins between HCC-S102 cells treated with *D. scandens* ethanolic extract for 72 h and untreated control cells. A total of 18 significantly different protein spots were analyzed using Image Master and identified by LC-MS/MS. Cells treated with *D. scandens* extract possessed 6 proteins

Table I. Differentially expressed proteins in *D. scandens* ethanolic extract treated HCC-S102 cells identified by liquid chromatography-tandem mass spectrometry.

Spot no.	SwissProt entry name	Protein name	Molecular mass, kDa kDa/ isoelectric point	Protein score	Peptides matched	Sequence coverage, %	Fold change of protein volume	
							Control and 20 μg/ml *D. scandens* ethanolic extract	Control and 30 μg/ml *D. scandens* ethanolic extract
Upregulated proteins								
10	ENPL_HUMAN	Endoplasmin	92.4/4.8	609	13	15	(+) 1.68	(+) 1.17
11	DC1I2_HUMAN	Cytoplasmic dynein 1 intermediate chain 2	71.4/5.1	226	3	7	(+) 1.90	(+) 1.37
12	HSP7C_HUMAN	Heat-shock cognate 71 kDa protein	73.6/5.9	60	1	2	(+) 2.03	(+) 1.76
14	K1C9_HUMAN	Type I cytoskeletal keratin 9	62.0/5.1	90	1	3	(+) 0.77	(+) 1.54
22	UAP1L_HUMAN	UDP-N-acetylhexosamine pyrophosphorylase-like protein 1	56.9/5.9	61	1	3	(+) 1.23	(+) 1.63
23	HYOU1_HUMAN	Hypoxia-upregulated protein 1	111.3/5.2	435	7	9	(+) 1.34	(+) 1.81
Downregulated proteins								
1	CBX3_HUMAN	Chromobox protein homolog 3	20.8/5.2	292	4	22	(-) 1.42	(-) 1.46
2	RLA0_HUMAN	60S acidic ribosomal protein P0	34.3/5.7	66	2	9	(-) 1.53	(-) 1.20
3	CAZA1_HUMAN	Filamentous actin-capping protein subunit α-1	32.9/5.5	186	3	15	(-) 1.62	(-) 1.05
4	GLRX3_HUMAN	Glutaredoxin-3	37.4/5.3	119	2	8	(-) 1.50	(-) 1.07
6	AL1A1_HUMAN	Retinal dehydrogenase 1	54.8/6.3	654	11	29	(-) 1.56	(-) 1.55
7	AK1C1_HUMAN	Aldo-keto reductase family 1 member C1	36.8/8.0	133	3	9	(-) 1.80	(-) 1.15
7	ROA2_HUMAN	Heterogeneous nuclear ribonucleoprotein A2/B1	37.4/8.9	131	2	8	(-) 1.80	(-) 1.15
8	G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase	36.0/8.6	146	3	13	(-) 1.09	(-) 1.56
9	HNRPK_HUMAN	Heterogeneous nuclear ribonucleoprotein K	50.9/5.4	311	6	19	(-) 1.76	(-) 1.57
13	NCALD_HUMAN	Neurocalcin-δ	22.2/5.2	58	1	6	(-) 1.05	(-) 1.98
15	PRDX4_HUMAN	Peroxiredoxin-4	30.5/5.8	104	2	12	(-) 1.28	(-) 1.93
17	QCR1_HUMAN	Cytochrome bc_1 complex subunit 1	52.6/5.9	160	4	10	(-) 1.03	(-) 1.57
24	STML2_HUMAN	Stomatin-like protein 2	38.5/6.8	143	3	13	(-) 1.12	(-) 1.83

(+) and (-) represent the upregulation or downregulation, respectively, of the protein fold change compared with the control. Spot numbers refer to Fig. 4.

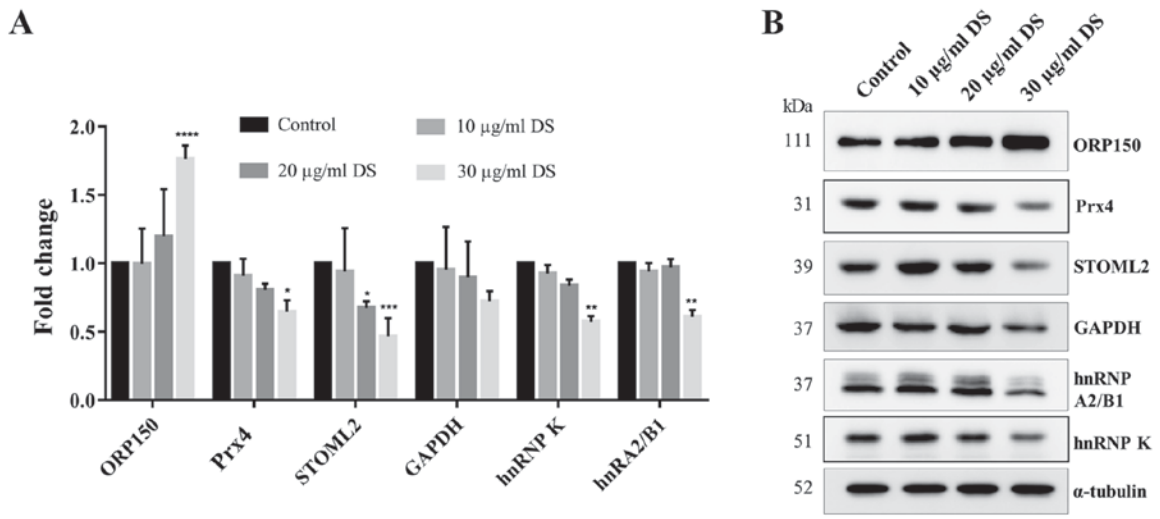


Figure 5. Verification of the expression of representative proteins from two-dimensional electrophoresis on the HCC-S102 cell line treated with *D. scandens* ethanolic extract. Cells were treated with different concentrations of *D. scandens* ethanolic extract (10, 20 and 30 μg/ml) for 72 h and subjected to SDS-PAGE (12.5% gel), followed by western blot analysis. (A) Fold change of and (B) western blot analysis results for ORP150, Prx4, GAPDH, STOML2, hnRNP A2/B1 and hnRNP K. α-tubulin was used as loading control. Results are expressed as the mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. the control. DS, *Derris scandens* ethanolic extract; ORP150, oxygen-regulated protein 150; Prx4, peroxiredoxin-4; STOML2, stomatin-like protein 2; hnRNP, heterogeneous nuclear ribonucleoprotein.

with higher expression and 13 proteins with lower expression compared with untreated controls. A majority of proteins involved in stress response including Hsp90B1, Hsc70 and ORP150 were upregulated. Downregulated proteins involved in cell metabolism included RALDH1, AKR1C, GAPDH, UQCRC1 and STOML2. In addition, TXNL2 and Prx4 which serve functions as antioxidants were downregulated. Proteins participating in transcriptional regulation including CBX3 and hnRNP K were downregulated, as were proteins involved in signal transduction including hnRNP A2/B1. Among these proteins, ORP150, Prx4, GAPDH, STOML2, hnRNP A2/B1 and hnRNP K proteins were selected to validate the differential expression of these proteins between treated and untreated cells using western blot analysis. In agreement with the proteomic results, western blot analysis revealed that ORP150 was upregulated whereas Prx4, GAPDH, STOML2, hnRNP A2/B1 and hnRNP K proteins were downregulated.

Prx4 is a member of the thiol-specific antioxidant group of proteins, and is normally expressed in the endoplasmic reticulum (ER) of cells (15). Its general function is to serve as an antioxidant by decreasing the production of reactive oxygen species (ROS). In addition, a previous study revealed that Prx4 additionally participated in oxidative protein folding as a molecular chaperone in order to facilitate protein folding in the ER (26). High expression of Prx4 has been reported in numerous different tumor types, including lung cancer, colorectal cancer and high-grade glioma (primary brain malignancy) (15,27,28). For example, Prx4 is highly expressed in and associated with the development of human lung cancer, and has been suggested to be useful as a good prognostic marker of cancer progression in early-stage squamous cell carcinoma of lung cancer (15). Additionally, a previous study used immunohistochemistry and quantitative polymerase chain reaction to demonstrate that the expression of the Prx4 gene and protein were higher in colorectal cancer tissues compared with normal tissues (27). Notably, Kim *et al* (28) have previously revealed that Piperlongumine, a natural plant product, suppressed the expression of Prx4 and subsequently increased ROS and ER stress levels in glioma cells. Excess misfolded or unfolded proteins in ER may induce the ER stress response, which may be detected by the expression of ORP150.

ORP150 is a notable molecular chaperone in the ER, which belongs to the heat-shock protein 70 family (29). The ORP150 system is a part of the ER machinery associated with the folding and assembly of secretory and membrane proteins in the ER (16). ORP150 expression was induced by numerous stress conditions including the accumulation of protein unfolding and the depletion of glucose or oxygen (16). Under stress conditions, ORP150 may accumulate in the ER, serving a cytoprotective function for cells. The upregulation of ORP150 has been reported in numerous types of cancer including breast cancer (28), bladder cancer (30) and nasopharyngeal carcinoma (31). Prx4 downregulation concomitantly with the upregulation of ORP150 indicates an increase in ER stress and ROS levels in the HCC-S102 cell line. The evidence suggests that, together, prolonged unfolded protein accumulation and persistent ER stress in cancer cells may initiate pro-apoptotic signaling (32). Thus, it may be deduced that these consequences will occur in HCC-S102 cells when treated with *D. scandens* ethanolic extract.

GAPDH and STOML2 are involved in cell metabolism. GAPDH is a key regulatory enzyme of glycolysis, and serves a crucial function in several biological processes including the control of gene expression, DNA replication and repair and apoptosis (33). There is evidence that GAPDH expression is increased in various human cancer types, including ovarian, lung and colon cancer types (17,34,35). Furthermore, previous studies have revealed that GAPDH knockdown by small interfering RNA (siRNA) results in a significant decrease in the proliferative, migratory and invasive abilities of lung squamous carcinoma cells *in vitro* (36). STOML2 is a protein on the mitochondrial inner membrane; however,

its functions remain unknown (37). STOML2 has been implicated in serving an important function in sustaining the mitochondria membrane potential and ATP production, and additionally affects cell activity including cell motility and cell growth in tumor cells (18). Furthermore, it has been reported that the downregulation of STOML2 increases the sensitivity of cancer cells to chemotherapeutic treatments by inducing cancer cell apoptosis and cell cycle arrest (18). The results of the present study revealed the downregulation of GAPDH and STOML2, which may be one of the mechanisms of *D. scandens* ethanolic extract resulting in cytotoxicity in HCC-S102 cells.

The hnRNP family are considered to be an important factor in mRNA processing and biogenesis (38). The present study revealed a decrease in the expression levels of hnRNP K and hnRNP A2/B1 in the HCC-S102 cell line treated with *D. scandens* ethanolic extract. hnRNP K is a member of the hnRNP family. It exerts specific binding for the c-myc promoter and functions as a transcription factor (39). Association of hnRNP K with tumor development in various types of cancer have been reported. A previous study revealed that hnRNP K may be involved in hepatocarcinogenesis by enhancing hepatitis B and hepatitis C replication and cell proliferation (19). In lung cancer, the downregulation of hnRNP K by siRNA resulted in the inhibition of cell growth and increased apoptosis in a lung cancer cell line (A549) (40). In addition, there is evidence that hnRNP K suppresses apoptosis in HCC and nasopharyngeal carcinoma (41,42).

hnRNP A2/B1 is another member of the hnRNP family that was revealed to be downregulated in the present study. hnRNP A2/B1 has several cellular functions including regulating gene expression at the transcriptional and translational level. hnRNP A2/B1 has been reported to be overexpressed in tumorigenesis, and may be a potential biomarker for early detection, particularly in lung cancer (43). To assess the risk of human liver cancer, the increased expression and cytoplasmic localization of hnRNP A2/B1 may be used as a diagnostic biomarker (20). Furthermore, the inhibition of hnRNP A2/B1 expression has been demonstrated to increase the sensitivity of cancer cells to chemotherapy, stimulate apoptosis and additionally modulate the promotion of pancreatic cancer (44). hnRNP K and hnRNP A2/B1 expression levels were decreased with the treatment with *D. scandens* ethanolic extract in the present study. Therefore, this may be one of the mechanisms involved in the cytotoxic activity against HCC-S102 cells.

In conclusion, the present study demonstrated that the effect of *D. scandens* ethanolic extract on HCC-S102 cell line occurs through the apoptotic pathway. The proteomic profiling of cells treated with *D. scandens* ethanolic extract identified numerous protein targets for anticancer activity including hnRNP K, hnRNP A2/B1, STOML2 and GAPDH. It may be suggested that the decrease in the survival of HCC-S102 cells by alterations in these proteins may potentiate the lethal effect of *D. scandens*-induced Prx4 downregulation and excess ER stress as indicated by ORP150 upregulation. Consequently, the present results indicate the association of the apoptosis mechanism with cell cytotoxicity upon treatment of *D. scandens* ethanolic extract. However, further studies on the function of each protein are necessary in order to elucidate the mode of action of *D. scandens* ethanolic extract.

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