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Clarification of the Phenotypic Characteristics and Anti-Tumor Activity of *Hedyotis diffusa*

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Abstract: *Hedyotis diffusa* Willd. (Rubiaceae) is an important folk herb used to prevent and cure hepatitis and liver cancer in Taiwan. For differentiation of *H. diffusa* from counterfeits, macroscopic and microscopic characters of *H. diffusa, H. corymbosa* and *H. tenelliflora* were examined in this study. According to Trypan blue exclusion assay and Western blot analysis, *H. diffusa* had a significant inhibition of cell growth and induction of cell apoptosis in COLO 205 (colon cancer), Hep 3B (hepatocellular carcinoma) and H460 (lung cancer) cell lines. This study also used high-performance liquid chromatography (HPLC) to determine the quality control of *H. diffusa*. The HPLC data showed that ursolic and oleanolic acid are the components of the *H. diffusa*, consisting of approximately 4.66–4.80% and 1.86–1.96%, respectively. Our study also demonstrated that ursolic acid has significant anti-tumor activity in COLO 205, Hep 3B and H460 cancer cells.

Keywords: *Hedyotis diffusa*; COLO 205 Cells; Hep 3B Cells; H460 Cells; Apoptosis; High-Performance Liquid Chromatography; Ursolic Acid.

Introduction

Hedyotis diffusa (Rubiaceae) has been used in Chinese medicine for a long time. *H. diffusa* was found to have neuroprotective, antioxidant and anti-inflammatory activity (Kim *et al.*,

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2001; Lin *et al.*, 2002, 2004). However, some plant species, such as *Hedyotis corymbosa* and *Hedyotis tenelliflora*, have substituted *H. diffusa* under the general name "Peh-hue-juwa-chi-cao" in traditional Chinese and folk medicine. To give the right prescription for an illness, the botanical origin of herb must be demanded critically. In this study, macroscopic and microscopic characters of three original plants of "Peh-hue-juwa-chi-cao" were examined to differentiate *H. diffusa* from counterfeits.

Apoptosis is a major form of cell death and is associated with characteristic morphological changes including the formation of membrane blebs and apoptotic bodies, nuclear condensation and DNA fragmentation. It is widely accepted that there are two principal pathways of apoptosis; namely, caspase-dependent and -independent. Caspases, a family of cysteine proteases, play a critical role during apoptosis. There are at least two major mechanisms by which a caspase cascade resulting in the activation of effector caspase-3 may be initiated by the most apical caspase, one involving caspase-8 and the other involving caspase-9 (Zou *et al.*, 1997; Srinivasula *et al.*, 1998). Therefore, the activation of caspase-3 is required of apoptosis whether the caspase-8 or caspase-9 pathway. Apoptosis-inducing factor (AIF) is a 57 kDa protein that resides mainly within the space between the inner and outer mitochondrial membrane. Upon loss of mitochondrial membrane integrity, AIF is released from the mitochondria to induce nuclear condensation and large-scale DNA fragmentation (Liou *et al.*, 2003). This leads cell death without the participation of caspases; hence, AIF is a key player in eliciting caspase-independent apoptosis in the cells.

Ursolic acid and oleanolic acid are pentacyclic triterpenoic acids and have the same molecular formula and a similar chemical structure. They are the major components of some oriental and traditional wild medicine herbs distributed all over the world. There is a growing interest in the elucidation of the biological roles of both these triterpenoid compounds. Since ursolic acid and oleanolic acid are relatively non-toxic, many investigations have focused on their anti-tumor activity. Both compounds have been shown to act at various stages of tumor development, including inhibition of tumor promotion and induction of tumor cell differentiation. Harmand *et al.* (2003, 2005) have demonstrated that ursolic acid induces apoptosis through mitochondrial intrinsic pathway, caspase-3 activation and cell cycle arrest in tumor cells.

Since plant extracts are composed of complex phytochemical constituents, a proper method is desired for the quality control of the extract of the title plant. High performance liquid chromatography (HPLC) was used to identify the complex phytochemical constituents isolated and quantify a specific constituent in the plant extracts. It is well known that ursolic and oleanolic acid were isolated from many kinds of medicinal plants and are present in the human diet. This study also investigated whether ursolic and oleanolic acid are contained in *H. diffusa*. We attempted to develop a HPLC method for simultaneous determination of oleanolic acid and ursolic acid in alcohol extract of *H. diffusa*. It is expected that oleanolic acid and ursolic acid might be used as a quality control of alcohol extracts of *H. diffusa* by HPLC analysis.

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Materials and Methods

Materials

The voucher specimens (*Hedyotis diffusa*: CMU HD 0411; *Hedyotis corymbosa*: CMU HC 0412; *Hedyotis tenelliflora*: CMU HT 0522) were deposited in School of Chinese Medicine Resources, China Medical University, Taichung, Taiwan. Antipain, aprotinin, dithiothreitol, EGTA, leupeptin, oleanolic acid, pepstatin, phenylmethylsulfonyl fluoride, Tris and ursolic acid were purchased from Sigma Chemical Company (St. Louis, MO); acetonitrile and methanol were HPLC grade and purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA); anti-mouse and anti-rabbit IgG peroxidase-conjugated secondary antibodies were purchased from Jackson Immunoresearch (Hamburg, Germany). Antibodies to various proteins were obtained from the following sources: caspase-3 was obtained from BD Biosciences (San Diego, CA, USA); Apoptosis-inducing factor and β-actin were from Sigma Chemical Company. Enhanced chemiluminescent (Renaissance) detection reagents were obtained from NEN Life Science Products (Boston, MA, USA).

Staining of Transverse Section of the Stem of H. diffusa

A transverse section was obtained at a 1/2 portion of an internode from a stem of *H. diffusa*. The transparent reagent of chloral hydrate was added in the transverse section of stem. After drying near fire, the transverse section of stem was stained with 5% phloroglucinol and 12 N hydrochloric acid. The slides were then mounted by using glycerin/H_2O solution (1:1) and observed by microscopy.

Preparation of H. diffusa

The air-dried plants of *H. diffusa* (170 g) were soaked thrice with 1 ml of 95% ethanol at room temperature for three days. The extracts were filtered. The filtrates were collected and then concentrated under reduced pressure at 40°C. The yield of dry extract of *H. diffusa* was 6.9%.

Cell Cultures

The human hepatocellular carcinoma cell line Hep 3B and lung non-small carcinoma H460 cells were grown in monolayer culture in Dulbecco's modified Eagle's medium (Life Technologies, Rockville, MD, USA) containing 5% fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Rockville, MD, USA) and 2 mM glutamine (Merck, Darmstadt, Germany) at 37°C a humidified atmosphere comprised of 95% air and 5% CO_2. The human colorectal carcinoma cell line COLO 205 were grown in monolayer culture in RPMI containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin and 2 mM glutamine. When cells were treated with

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the alcohol extract of *H. diffusa*, the culture medium containing 1% fetal bovine serum was used. All data presented in this report are from at least three independent experiments showing the same pattern of expression.

Trypan Blue Dye Exclusion Assay

COLO 205, Hep 3B and H460 cells were seeded at a density of $1.5 \times 10^5, 5 \times 10^4$ and 5×10^4 cells/well, respectively, onto a 12-well plate (Falcon, Franklin Lakes, NJ) 48 h before treatment. Drugs were added to the medium at various indicated times and concentrations. The control cultures were treated with 0.1% ethanol. After incubation, cells were washed with PBS (phosphate-buffered saline). The number of viable cells was determined by staining cell population with Trypan blue (Sigma). One part of 0.2% Trypan blue dissolved in PBS was added to one part of the cell suspension and the number of unstained (viable) cells was counted.

Protein Preparation

Adherent and floating cells were collected at the indicated times and washed twice in ice-cold PBS. Cell pellets were resuspended in cell lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin and 5 μg/ml antipain) for 30 min at 4°C. Lysates were clarified by centrifugation at 100,000 g for 30 min at 4°C and the resulting supernatant was collected, aliquoted (50 μg/tube) and stored at −80°C until assay. The protein concentrations were estimated with the Bradford method.

4′,6-Diamidino-2-Phenylindole Dihydrochloride (DAPI) Staining

COLO 205, Hep 3B and H460 cells were seeded onto a 12-well plate 48 h before drug treatment. Cells were cultured for 48 h in 1% serum medium with 0.1% ethanol or various indicated concentrations of alcohol extracts of *H. diffusa.* After treatment, cells were fixed with 3.7% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 and stained with 1 μg/ml DAPI for 5 min at 37°C the cells were then washed with PBS and examined by a fluorescent microscope (Olympus IX 70).

Western Blot Analysis

Samples were separated by various appropriate concentrations (11 and 13%) of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad Life Science Products, Hercules, CA, USA). The SDS-separated proteins were equilibrated in transfer buffer (50 mM Tris, pH 9.0-9.4, 40 mM glycine, 0.375% SDS and 20% methanol) and electrotransferred to Immobilon-P Transfer Membranes (Millipore Corporation, Bedford, MA, USA). The blot was blocked with a solution containing 5% nonfat dry milk in Tris-buffered

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saline (10 mM Tris and 150 mM NaCl) with 0.05% Tween 20 for 1 h, washed and incubated with antibodies to β-actin (1:5000, the detection of β-actin was used as an internal control in all of the data of Western blotting analysis), AIF (1:1000) and caspase-3 (1:1000). Secondary antibody consists of a 1:20,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG. The enhanced chemiluminescent detection system was used for immunoblot protein detection.

HPLC Assay for H. diffusa

HPLC was performed by an Inertsil ODS-2 column (5 μm, 4.6 × 250 mm, GL Science Inc., Tokyo) eluted at a rate of 1.0 ml/min with a mobile phase (pH 8.67) of potassium phosphate buffer solution (pH 7.8) and acetonitrile (54/46, v/v) and UV detector with the detection wavelength set at 216 nm. The sample (1.6 mg) was dissolved in methanol with sonication. To prepare standard solutions, oleanolic and ursolic acid were accurately weighed and dissolved in methanol to give a series of concentrations in the ranges of 10.0–400.0 and $15.0-600.0\ \mu g/ml$, respectively. Calibration graphs were plotted by linear regression analysis of the peak area with concentrations. To quantify oleanolic and ursolic acid in *H. diffusa*, an appropriate amount of the alcohol extract of *H. diffusa* was dissolved in methanol. The afforded sample solution was filtered through a 0.45 μm millipore membrane prior to HPLC analysis.

Results

Clarification of the Phenotypic Characteristics of H. diffusa, H. corymbosa and H. tenelliflora

Some plant species such as *H. corymbosa* and *H. tenelliflora* have substituted *H. diffusa,* which resulted in confusion in the medical effects of *H. diffusa* in the traditional Chinese and folk medicine. In this study, macroscopic and microscopic characters of *H. diffusa*, *H. corymbosa* and *H. tenelliflora* were examined. The external and internal morphology of *H. diffusa*, *H. corymbosa* and *H. tenelliflora* are very similar. This study suggested three significant differences in morphology among them. The flowers are pairs in axillary racemes with pedicels of *H. diffusa*, terminal cymes with pedicels and peduncles of *H. corymbosa* and 1–5 clustered in axillary spike without pedicels and peduncles of *H. tenelliflora* (Fig. 1A). As shown in Fig. 1B, the morphology of stem is nearly circular, circular with 4 angles and nearly elliptical with 4 angles of *H. diffusa*, *H. corymbosa* and *H. tenelliflora*, respectively. The third difference in their morphology is that the structure of papilla was observed in the transverse section of the stem of *H. diffusa* (Fig. 1B). Papilla did not appear on the transverse section of the stem of *H. corymbosa* and *H. tenelliflora*, but they have the significant collenchyma structure (Fig. 1B).

H. diffusa Induced Tumor Cell Death in a Dose-Dependent Manner

This study served to evaluate the effects of alcohol extract of *H. diffusa* on cell death of various human tumor cell lines, such as COLO 205 (colon cancer), Hep 3B (hepatocellular

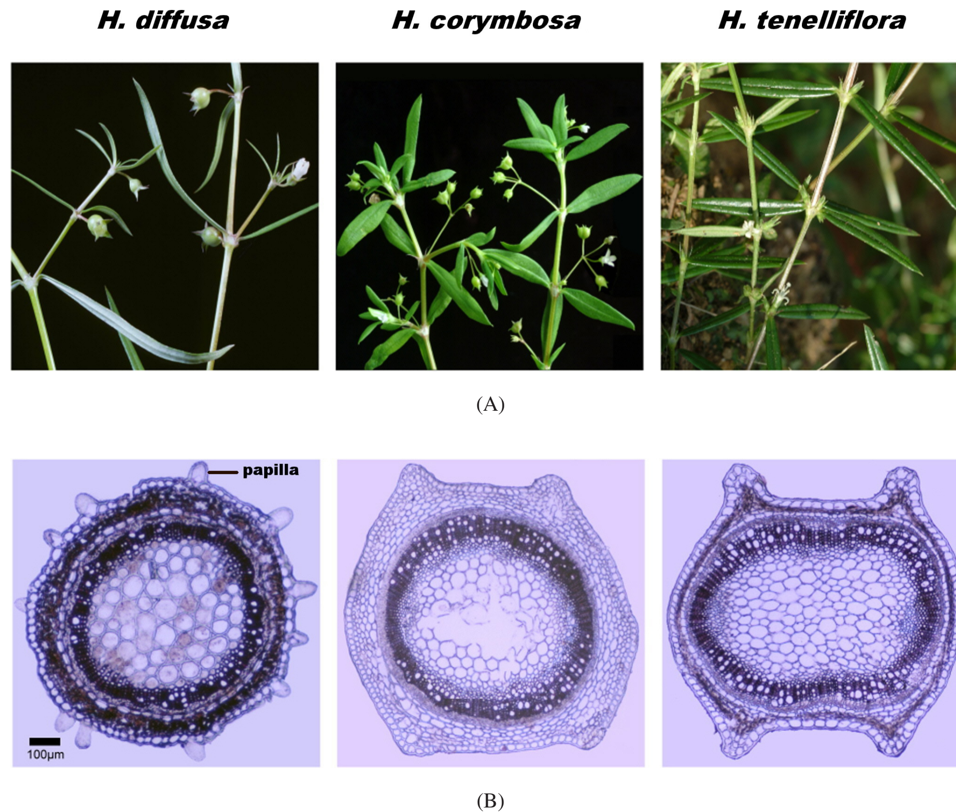

Figure 1. (A) The configuration of the flower of *H. diffusa, H. corymbosa* and *H. tenelliflora*. (B) The outline of transverse section of the stem of *H. diffusa, H. corymbosa* and *H. tenelliflora*.

carcinoma cells) and H460 cells (lung cancer). We determined the effect of *H. diffusa* on cell viability by Trypan blue dye exclusion assay. The data are presented as proportional viability (%) by comparing the treated group with the untreated cells, the viability of which was assumed to be 100%. As shown in Fig. 2, 48 h of continuous exposure to various concentrations of *H. diffusa* (25, 75 and 150 μg/ml) on COLO 205, Hep 3B and H460 cells resulted in dose-dependent decreases in cell number relative to control cultures. The IC_{50}, the half maximal inhibitory concentration, values of *H. diffusa*-induced COLO 205, Hep 3B and H460 cell death were about 151.7, 115.3 and 127.8 μg/ml, respectively, after 48 h treatment. Therefore, 150 μg/ml of *H. diffusa* was chosen for further experiments.

H. diffusa Induces Apoptosis in COLO 205, Hep 3B and H460 Cells

The phenotypic characteristics of *H. diffusa*-treated tumor cells were evaluated by microscopic inspection of overall morphology. COLO 205, Hep 3B and H460 cells formed apoptotic bodies, and then floated after treatment with 150 μg/ml alcohol extracts of *H. diffusa* for 48 h (data not shown). Treatment of COLO 205, Hep 3B and H460 cells with

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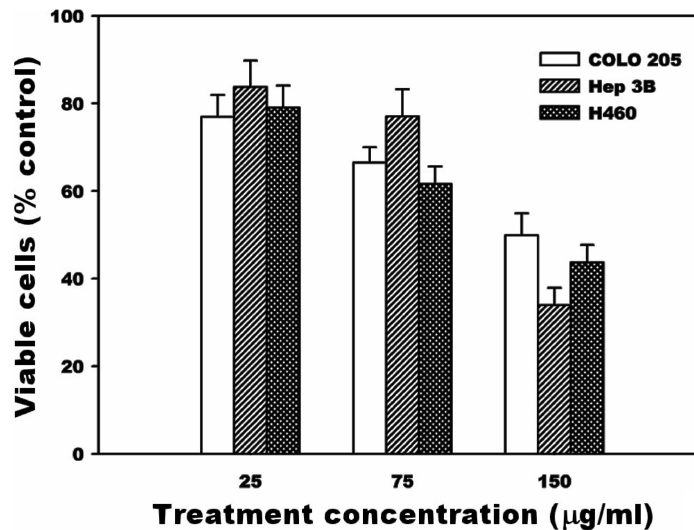

Figure 2. Alcohol extracts of *H. diffusa* induced COLO 205, Hep 3B and H460 cancer cell death. Cells were cultured for 48 h before drug treatment in 12-well plates. Cells were treated with 0.1% ethanol or *H. diffusa* (25, 75 and 150 μg/ml) in the presence of 1% serum at 37°C for 48 h and cells were washed and counted for Trypan blue exclusion with a hemocytometer. All results are expressed as the mean percentage of control ± S.D. of triplicate determinations from three independent experiments.

H. diffusa resulted in changes in nuclear morphology, evidenced by the DAPI staining, a DNA binding dye. There was an increase in the number of nuclear condensations after treatment with *H. diffusa* (150 μg/ml) for 48 h in COLO 205, Hep 3B and H460 cells (Fig. 3). It is noteworthy that the nuclear morphology of Hep 3B is irregular after treatment with H. diffusa (Fig. 3). In this study, the *H. diffusa*-induced tumor cell nuclear morphological change and DNA condensation were observed. Therefore, *H. diffusa*-induced tumor cell death was indicative of a typical apoptosis.

The Effects of H. diffusa on AIF and Caspase-3 Expressions in COLO 205, Hep 3B and H460 Cells

To obtain further support for the induction of apoptosis by *H. diffusa* in tumor cells, the expression of AIF (release from mitochondria to cytosol) and caspase-3 proteins (cleavage of proform), which are the key indicators of intracellular signaling of caspase-independent and -dependent apoptosis, were performed. In this study, the expression of proform of caspase-3 was significantly decreased after treatment with *H. diffusa* for 16 h (Fig. 4). However, AIF protein levels decrease after 16 h treatment with *H. diffusa* (Fig. 4). Based on the above results, *H. diffusa*-induced COLO 205, Hep 3B and H460 cell death provides apoptotic characteristics and triggers changes in the proteins, which are intimately associated with apoptosis. These data suggest that *H. diffusa* induced COLO 205, Hep 3B and H460 cell death by caspase-dependent apoptosis pathway.

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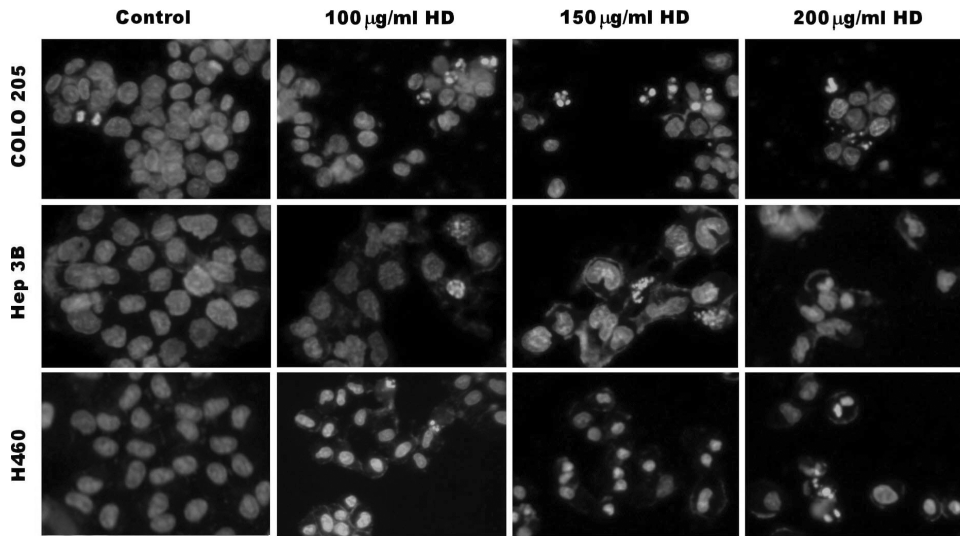

Figure 3. Alcohol extracts of *H. diffusa* induced COLO 205, Hep 3B and H460 cancer cell apoptosis. *H. diffusa* induced phenotypic changes in cell nucleus. COLO 205, Hep 3B and H460 cells were cultured for 16 h in 1% serum medium with 0.1% ethanol (control) or with 100, 150 or 200 μg/ml *H. diffusa* (HD). After treatment, cells were fixed with 3.7% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 and stained with 1 μg/ml DAPI for 5 min at 37°C. Cells were then washed with PBS and examined by fluorescence microscopy. Results are representative of three independent experiments.

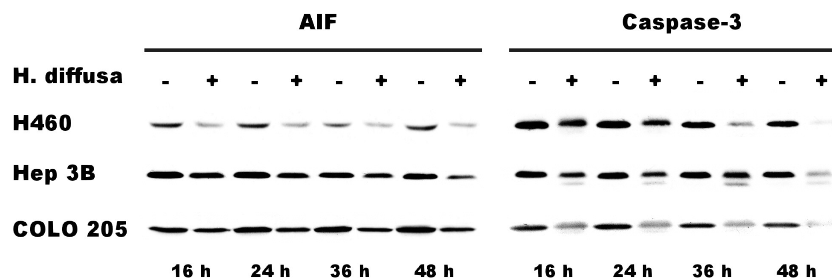

Figure 4. Effects of alcohol extracts of *H. diffusa* on the expression of apoptosis-inducing factor and caspase-3 proteins. The effects of *H. diffusa* (150 μg/ml) on apoptosis-inducing factor (AIF) and caspase-3 proteins was detected by Western blot analysis in COLO 205, Hep 3B and H460 cells. Cells were incubated with 0.1% ethanol or 150 μg/ml *H. diffusa* in the presence of 1% serum for 16, 24, 36 and 48 h. Cell lysates were analyzed by 11% (AIF) and 13% (caspase-3) SDS-PAGE and probed with primary antibodies as described in Materials and Methods. Results are representative of three independent experiments.

Ursolic and Oleanolic Acid were the Constituents of H. diffusa

To demonstrate the amount of ursolic and oleanolic acid contained in the *H. diffusa*, high performance liquid chromatography was used. The HPLC system used a Spherisob octadecylsilyl silica column with acetonitrile and potassium phosphate buffer solution as the mobile phase and detection at 216 nm. Pure oleanolic and ursolic acid showed a retention

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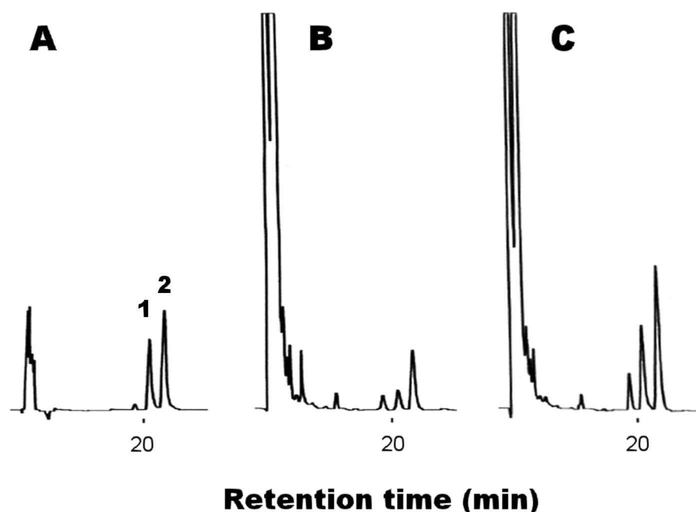

Figure 5. The contents of oleanolic and ursolic acid in *H. diffusa*were analyzed by HPLC. HPLC was performed by an Inertsil ODS-2 column eluted at a rate of 1.0 ml/min with a mobile phase (pH8.67) of potassium phosphate buffer solution and acetonitrile (54/46, v/v) and UV detector with the detection wavelength set at 216 nm. (A) Pure oleanolic (peak 1) and ursolic acid (peak 2) showed a retention time of 21.2 and 23.5 min, respectively. (B) Alcohol extract of *H. diffusa* exhibited two peaks at 21.4 and 23.8 min. (C) Appropriate amounts of oleanolic and ursolic acid were added to alcohol extracts of *H. diffusa* and then analyzed by HPLC.

time of 21.2 and 23.5 min, respectively (Fig. 5A). HPLC analysis of *H. diffusa* exhibited two peaks at 21.4 and 23.8 min (Fig. 5B), which was merged with that of oleanolic and ursolic acid standard (Fig. 5C). The data also showed that ursolic and oleanolic acid are the components of *H. diffusa*, consisting of approximately 4.66–4.80% and 1.86–1.96%, respectively, in each analysis of HPLC.

The Effects of Ursolic Acid and Oleanolic Acid on Tumor Cell Death

This study also evaluated the effects of ursolic and oleanolic acid on cell death of various tumor cell lines, COLO 205, Hep 3B and H460. We determined the effects of ursolic and oleanolic acid on cell viability by Trypan blue dye exclusion assay. The data are presented as proportional viability (%) by comparing the treated group with the untreated cells, the viability of which was assumed to be 100%. As shown in Fig. 6, 24 h of continuous exposure to various concentrations of ursolic acid (3, 10 and 30 μM) on COLO 205, Hep 3B and H460 cells resulted in dose-dependent decreases in cell number relative to control cultures. The IC_{50} values of ursolic acid were 16.3, 13.7 and 7.4 μM for COLO 205, Hep 3B and H460 cancer cells, respectively. The effects of oleanolic acid on COLO 205, Hep 3B and H460 cell death were also investigated. However, treatment with 80 μM oleanolic acid for 72 h had no significant effect on the COLO 205, Hep 3B and H460 cell death (data not shown).

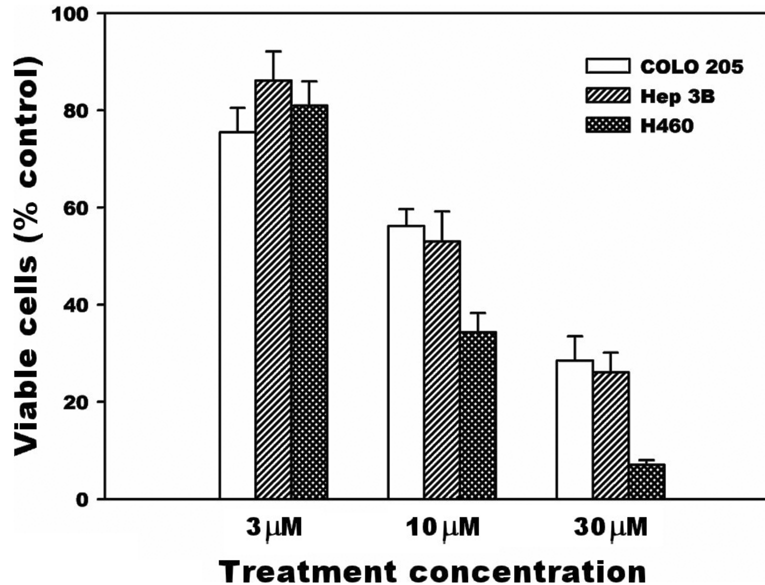

Figure 6. Ursolic acid induced COLO 205, Hep 3B and H460 cancer cell death. Cells were cultured for 48 h before drug treatment in 12-well plates. Cells were treated with 0.1% DMSO or ursolic acid (3, 10 and 30 μM) in the presence of 1% serum at 37°C for 24 h and cells were washed and counted for Trypan blue exclusion with a hemocytometer. All results are expressed as the mean percentage of control±S.D. of triplicate determinations from three independent experiments.

Discussion

H. diffusa is a traditional Chinese medicine with functions of antihepatitis, anticancer, antioxidant and anti-inflammatory activity (Kim *et al.*, 2001; Lin *et al.*, 2002, 2004). Previous treatments of diseases with herbs were more empirical than theoretical. Therefore, clarifying the mechanisms of action of the components of herbs may be important for developing their application. *H. diffusa* is an annual diffuse glabrous herb. Rain sprouts the seed in spring. *H. diffusa* grow luxuriantly, blossom and bear fruit from July to September and then dry up after the tenth month each year. Since a luxuriant growth of *H. diffusa* is only from July to September each year, *H. corymbosa* and *H. tenelliflora*, which are luxuriant throughout the year, were usually used as ersatz plants of *H. diffusa* on the market. Therefore, the ersatz plants of *H. diffusa* are everywhere. In this study, macroscopic and microscopic characters of three original plants of "Peh-hue-juwa-chi-cao" were examined to differentiate *H. diffusa* from counterfeits. This study suggested 3 significant differences in morphology among *H. diffusa, H. corymbosa* and *H. tenelliflora*. The external morphology, such as the shape of flowers and stem, and the tissue section of stem like papilla of *H. diffusa* showed the significant difference between *H. diffusa* and counterfeits.

Apoptosis is a major form of cell death and essential for normal development and for the maintenance of homeostasis. In addition, current anti-neoplastic therapies, chemotherapy and radiation-therapy are likely to be affected by the apoptotic tendencies of cells; thus, this

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process has obvious therapeutic implications (Green *et al.*, 1994). During apoptosis, certain characteristic morphologic events such as nuclear condensation, nuclear fragmentation and cell shrinkage occur (Lee, 2001; Leung *et al.*, 2005). The *H. diffusa*-induced apoptosis was characterized by chromatin condensation in this study. Many investigations have suggested that the apoptotic effect of cells is mediated by a well-characterized transduction process of apoptotic signals, such as caspase-3 (caspase-dependent) and apoptosis-inducing factor (caspase- independent) (Zou *et al.*, 1997; Srinivasula *et al.*, 1998; Liou *et al.*, 2003). Caspases are a family of cysteine proteases, and many of them are implicated as important initiators or effectors of the apoptosis process. To date, at least 14 members of this family have been identified. Among them, the caspase-3 is required for many of the nuclear changes associated with apoptosis, including DNA fragmentation and chromatin condensation (Kim *et al.*, 2001; Kwon *et al.*, 2005). During apoptosis, the proteolytic cleavage of the proform of caspase-3, which resulted in the activation of caspase-3, is requisite (Kim *et al.*, 2001; Kwon *et al.*, 2005). This study has demonstrated that the activation of caspase-3 is involved in *H. diffusa*-induced COLO 205, Hep 3B and H460 cell death. *H. diffusa* has been shown to trigger cell death due to its capability to induce chromatin condensation, directly or indirectly, via activation of caspase-3 in this study. These above data suggested that *H. diffusa* induces apoptotic cell death in COLO 205, Hep 3B and H460 cancer cells.

Oleanolic and ursolic acid have numerous pharmacological activities including anti-inflammatory, anticancer and hepatoprotective effects (Liu, 2005; Yu *et al.*, 2009). Andersson *et al.* (2003) have demonstrated that ursolic acid inhibits proliferation and stimulates apoptosis in colon cancer HT29 cells by activation of alkaline sphingomyelinase. Li *et al.* (2002) also suggested that oleanolic and ursolic acid have an inhibitory effect on tumor cell proliferation through cell cycle arrest in human colon carcinoma cell line HCT15. Therefore, the possible mechanism of anti-tumor activity is that both drugs have the effects on the inhibition of cell proliferation and stimulation of cell apoptosis. Since ursolic and oleanolic acid have been isolated from many kinds of medicinal plants and are present in the human diet, this study also investigated whether ursolic and oleanolic acid are contained in *H. diffusa*. Furthermore, we also hypothesized that ursolic and oleanolic acid might be important constituents in *H. diffusa*-induced tumor cell death in this study. The HPLC data showed that ursolic and oleanolic acid are the components of the *H. diffusa*, consisting of approximately 4.66–4.80% and 1.86–1.96%, respectively. Since plant extracts are composed of complex phytochemical constituents, proper components are desired for a quality control of the extract of the title plant. Therefore, oleanolic acid and ursolic acid might be used as a quality control of alcohol extracts of *H. diffusa* in HPLC analysis. Our study has demonstrated that ursolic acid has significant anti-tumor activity in COLO 205, Hep 3B and H460 cancer cells and the IC_{50} values are 16.3, 13.7 and 7.4 μM, respectively, after 24 h treatment. However, the IC_{50} value of *H. diffusa*-induced COLO 205, Hep 3B and H460 cell death is about 131.6 μg/ml (the mean of 115.3, 127.8 and 151.7 μg/ml), which contains about 13.6 μM of ursolic acid. Based on the above data, ursolic acid may be an important active component and contributes to the anti-tumor activity of alcohol extracts of *H. diffusa*.

In conclusion, *H. diffusa* has been used as a remedy for a long time in Taiwan. *H. diffusa* was found to have a major effect on anti-hepatoma. In this study, *H. diffusa* had a significant effect on cytotoxicity not only in liver cancer cell line, but also in lung and colon cancer cell lines. We have also demonstrated that the *H. diffusa*-induced COLO 205, Hep 3B and H460 cancer cell death is a typical apoptosis that was accompanied by a significant DNA condensation. In general, it is difficult to grasp the concept of herbal dosage. This is mainly owing to the limitations in our knowledge of the herbs; it is also the result of variations in our conception of how herbs may contribute to disease. In order to maintain a safe and stable dose of *H. diffusa*, we identified the phenotypic characteristics and quantified the main active constituent of *H. diffusa*. In this study, we developed a HPLC method and found that oleanolic acid and ursolic acid are the constituents of alcohol extracts of *H. diffusa*. Our study has also demonstrated that ursolic acid may be an active component of *H. diffusa*. We hope that the quality control of *H. diffusa* will be developed to indicate the safety of the drug by quantifying the amount of ursolic acid in *H. diffusa* when it is used in the field of anticancer agents in the future.

Acknowledgments

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