E X D 1 2 9 0 B
Journal Name Manuscript No.
Dispatch: 25.4.11 Journal: EXD CE: Samuel Chelliah
Author Received: No. of pages: 6 PE: Nagappan

DOI:10.1111/j.1600-0625.2011.01290.x
www.blackwellpublishing.com/EXD

Original Article

# Proteomics displays cytoskeletal proteins and chaperones involvement in *Hedyotis corymbosa*-induced photokilling in skin cancer cells

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**Abstract:** Photodynamic therapy was found to be an effective therapy for local malignant tumors. This study demonstrated that 80 $\mu g$/ml *Hedyotis corymbosa* extracts with 0.8 J/cm$^2$ fluence dose caused M21 skin cancer cell death. Photoactivated *H. corymbosa*-induced M21 cell death is a typical apoptosis that is accompanied by nuclear condensation, externalization of phosphatidylserine and the changes in protein expression of apoptosis-related proteins, such as Bcl-2 and caspase family members. This study applied 2D electrophoresis to analyse the proteins involved in the photoactivated *H. corymbosa*-induced M21 cell apoptosis. We found 12 proteins to be markedly changed. According to the results of protein sequence analysis of these altered protein spots, we identified that the expression of cytoskeletal proteins and chaperones were involved in the photoactivated *H. corymbosa*-induced M21 cell apoptosis. We further demonstrated that photoactivated *H. corymbosa* caused a significant effect on the cytoskeleton distribution and mitochondrial activity in M21 cells. Based on the above findings, this study characterized the effects and mechanisms of the photoactivated *H. corymbosa*-induced apoptosis in M21 skin cancer cells.

**Key words:** apoptosis – cytoskeleton – *Hedyotis corymbosa* – M21 skin cancer cells – mitochondria – photodynamic therapy

*Accepted for publication 21 March 2011*

## Introduction

Photodynamic therapy (PDT) of cancer is based on the administration of a tumor-localizing photosensitizing agent, which is activated by light of appropriate wavelength. PDT has been partly limited by side effects, such as prolonged cutaneous phototoxicity. PDT has recently been approved by the FDA for the treatment of precancerous lesions, namely actinic keratoses. However, PDT of skin cancers has been limited to the treatment of non-hyperkeratotic lesions because of ineffective penetration of photosensitizer into the thicker lesions. It is important to enhance tissue penetration of photosensitizers and reduce side effects (1,2). *Hedyotis corymbosa* (Rubiaceae) is extensively used in traditional Chinese medicine for the treatment of skin disease, hepatitis and cancer. We now found that *H. corymbosa* extracts exhibited significant phototoxicity in M21 cells in a series of tests. Therefore, we attempted to develop *H. corymbosa* as a photosensitizer for PDT for skin malignancies.

Apoptosis is a major form of cell death, which involves many factors and signal transduction molecules. It plays a key role in pathogenesis and therapy for many diseases and as a mechanism of PDT-induced cell death (3,4). Proteomics is a method to elucidate cellular processes at the molecular level (5,6). This study used 2D gel electrophoresis to analyse the proteins involved in the photoactivated *H. corymbosa*-induced apoptosis in M21 cells. Apoptosis is associated with changes in cell morphology that suggest prominent involvement of the cytoskeleton. Actin appears to play an important role in the early stage of apoptosis (4,7). The cytoskeleton also may facilitate the mitochondrial recruitment of pro-apoptotic proteins to the cytosol, e.g. cytochrome *c* or apoptosis-inducing factor (AIF) (7,8).

Molecular chaperones ensure correct protein folding and the continued maintenance of protein structure. Many molecular chaperones were initially identified as heat-shock proteins (HSPs) and as they are synthesized in response to stress, such as heat, hypoxia and ATP depletion. HSP have a complex, but they primarily have anti-apoptotic role in apoptosis. HSP70 inhibits apoptosis by preventing recruitment of procaspase-9 from the apaf-1 apoptosome (9). HSP27 inhibits cytochrome *c*-dependent activation of procaspase-9 (10). Overexpression of HSP60 prevents apoptosis by protecting mitochondrial function in cardiac myocytes (11). It has also been demonstrated that HSP60 accelerated activation of caspase-3 during apoptosis (12). Therefore, the HSP have a significant and complex role in apoptosis.

The major purpose of this study was to characterize the effects and mechanisms of the *H. corymbosa* extracts-induced phototoxicity in M21 skin cancer cells. This study demonstrated that *H. corymbosa* extracts and a small light dose caused apoptosis. We identified multiple cytoskeletal proteins and chaperones that were involved in M21 cell apoptosis induced by photoactivated *H. corymbosa*.

## Methods

### Materials

The voucher specimen (*Hedyotis corymbosa*: CMU HC 08002) was deposited in School of Chinese Medicine Resources, China

Medical University, Taichung, Taiwan. Tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin were purchased from Sigma Chemical Company (St. Louis, MO, USA). Antibodies to various proteins were obtained from the following sources: AIF, β-actin, Bcl-2, Bax, caspase-3, caspase-8, cytochrome *c*, protein disulphide isomerase (PDI), HSP27 and HSP70 antibodies were from Sigma Chemical Company; 150-kDa oxygen-regulated protein (ORP150) was from IBL (Japan); caspase-4 and caspase-9 were from Calbiochem (Cambridge, MA, USA); HSP60 was from Calbiochem (Germany). Horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit IgG and rabbit anti-sheep IgG were from Abcam.

**Source and preparation of *Hedyotis corymbosa***

*Hedyotis corymbosa* is an annual diffuse glabrous herb. *H. corymbosa* grow luxuriantly, blossom and bear fruit from July to September and then dried up after the tenth month each year. *H. corymbosa* used in this study was harvested in August in Taiwan Taichung city. The botanical origin of *H. corymbosa* was identified by Dr Chao-Lin Kuo (School of Chinese Medicine Resources, China Medical University). The air-dried whole plant, including roots, stems, leaves, flowers and fruits, of *H. corymbosa* (570 g) was soaked thrice with 5 l of 95% ethanol at room temperature for 3 days. The extracts were filtered. The filtrate was collected and then concentrated under reduced pressure at 40°C. The yield of dry extract of *H. corymbosa* was about 9%. *H. corymbosa* extracts were dissolved in dimethylsulphoxide (DMSO) and total concentration of DMSO in the medium was <0.2%. The stock of *H. corymbosa* extracts (100 μg/ml) was aliquoted (20 μl/tube) and stored at −20°C until assay. Absorption spectrum of *H. corymbosa* extracts was recorded in the range of 400–700 nm on a μQuant™ microplate spectrophotometer (BioTek, Winooski, VT, USA). The absorption spectrum of *H. corymbosa* extracts is shown in Figure S1.

**Cell culture**

The human skin cancer cell line M21 was kindly provided by Dr Feng-Yao Tang (China Medical University). M21 cells were grown in monolayer culture in ~~RPMI~~ medium 1640 (Invitrogen Corporation, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Rockville, MD, USA) at 37°C in a humidified atmosphere comprised of 95% air and 5% $CO_2$. When M21 cells were treated with *H. corymbosa* extracts, the culture medium containing 1% FBS was used. All data presented in this study are from at least three independent experiments.

**Light source**

The irradiation source was a set of fluorescent lamp (20 W; China Electric MFG Corporation, Taiwan) located at a made-to-measure box. The wavelength of fluorescent lamp is in the range of 400–700 nm. The intensity of light was measured as Lux, a system of international illumination measure. Lux was inverted to light dose (J/cm$^2$). The cells were irradiated at 20, 40 and 60 W for 30 min that correspond to 0.4, 0.8 and 1.2 J/cm$^2$ light dose. The light dose in the sample was constant for all the experiments.

**Morphological investigation and mitochondrial reductase activity**

M21 cells were seeded onto 12-well plate 48 h before being treated with drugs. The cells were incubated with 0.1% DMSO or various indicated concentrations of *H. corymbosa* extracts for various indicated times and then irradiated with 0.4, 0.8 and 1.2 J/cm$^2$ light dose. Morphological investigation and mitochondrial reductase activity were performed as previously described (13).

**Assay of lactate dehydrogenase (LDH) release**

M21 cells were incubated with 0.1% DMSO or with 40 or 80 μg/ml *H. corymbosa* extracts for various indicated times (in phenol-free RPMI medium 1640 with 0.1% bovine serum albumin) and then irradiated with the light dose of 0.8 J/cm$^2$. After irradiation, the supernatant was removed and centrifuged at ~~13 000 rpm~~ for 10 min. LDH activity in the supernatant was determined with the LDH detection kit (Roche Applied Science, Germany).

**Apoptosis detection**

Apoptotic characteristics were detected by 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining and annexin V-FITC/PI double-staining assay as previously described (13).

**Protein preparation and Western blot analysis**

Protein preparation and Western blot analysis were performed as previously described (13). The protein concentrations were estimated with the Bradford method. Equal amounts of protein (50 μg) were separated by various indicated concentrations of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the SDS-separated proteins were electrotransferred to Immobilon-P Transfer Membranes ~~(Millipore)~~. Membranes were probed with antibodies to β-actin (1:5000), AIF (1:1000), Bax (1:500), Bcl-2 (1:1000), caspase-3 (1:1000), caspase-4 (1:12000), caspase-8 (1:8000), caspase-9 (1:200), cytochrome *c* (1:10000), HSP27 (1:8000), HSP60 (1:1000), HSP70 (1:1000), ORP150 (1:1000) and PDI (1:1000). Secondary antibody consisted of a 1:20 000 dilution of HRP-conjugated goat anti-mouse IgG (for β-actin, Bcl-2, Bax, caspase-3, caspase-8, HSP60, HSP70, ORP150 and PDI) or HRP-conjugated-rabbit IgG (for AIF, caspase-4, caspase-9 and HSP27) and rabbit anti-sheep IgG (for cytochrome *c*).

**Two-dimensional gel electrophoresis**

Two-dimensional gel electrophoresis, NanoLC-MS/MS analysis and database searches were performed as previously described (14). Gels were stained with standard silver-staining protocol.

**Localization of F-actin and microtubules**

Cells grown on glass plates were incubated with vehicle alone or 40 μg/ml *H. corymbosa* extracts for 4 h and then irradiated with 0.8 J/cm$^2$ light dose. To visualize F-actin, cells were fixed in 3.7% formaldehyde, permeabilized with 1% Triton X-100 and incubated with $1.9 \times 10^{-7}$ M TRITC-phalloidin for 40 min. To detect tubulin, cells were incubated for 30 min with 250 nM Tubulin Tracker™ Green reagent. After washing with PBS, the cells were observed using fluorescent microscope ~~(H600L; Nikon)~~.

**Measurement of mitochondrial function**

M21 cells were incubated with vehicle alone or 40 μg/ml of *H. corymbosa* extracts for 4 h and then irradiated with 0.8 J/cm$^2$ light dose. Mitochondrial activity, the opening of mitochondrial permeability transition (MPT) pore and mitochondrial membrane potential (MMP) were measured as previously described (13,15). To detect mitochondrial activity, cells were incubated for 30 min at 37°C with 100 nM MitoTracker Red CMXRos and observed by fluorescent microscope (H600L; Nikon). To measure the opening of MPT pore, the fluorescence intensity of calcein was measured with FACSCanto flow cytometer (excitation, 488 nm; emission,




 

530 nm) and analysed using ModFit LT 3.0 Software (Verity Software House, Topsham, ME, USA). MMP was determined by flow cytometry analysis of JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide)-stained cells.

### Statistical analysis

Statistically significant difference from the control group was identified by Student's *t*-test for paired data.

## Results

### *Hedyotis corymbosa* extracts induced phototoxicity of M21 cells

This study served to evaluate the effects of alcohol extract of *H. corymbosa* on cell death of M21 cells in irradiation condition. Figure 1 shows the results of MTT cytotoxic assay on M21 cells after *H. corymbosa* extracts with irradiation. The cell death is drug dose- and irradiation dose-dependent. As shown in Fig. 1b, pretreatment with 80 $\mu$g/ml *H. corymbosa* extracts for 4 h and then irradiation with 0.8 J/cm$^2$ light dose appeared to have a 50% cell death of M21 cells. Light alone did not affect cell survival in this study. *H. corymbosa* extracts did not show any dark toxicity at the evaluated concentrations (40, 80 and 120 $\mu$g/ml for 48 h) following MTT assay (Fig. 1d). These results suggested that *H. corymbosa* extracts may be a photosensitizer.

### Photoactivated *Hedyotis corymbosa* extracts induced M21 cell apoptosis

To further investigate whether the photoactivated *H. corymbosa* extracts-induced cell death is a typical apoptosis of M21 cells, the characteristics of apoptosis, such as the integrity of plasma membrane, nuclear morphology and externalization of phosphatidylserine, were evaluated in this study. LDH is a cytoplasmic enzyme that is released from the cell following plasma membrane damage. Photoactivated *H. corymbosa* extracts had no significant effect on the release of LDH (Table S1). It indicated that the cell membrane was not disrupted by photosensitized *H. corymbosa*, even though cells were incubated with 80 $\mu$g/ml *H. corymbosa* extracts for 6 h and then irradiated with 0.8 J/cm$^2$ fluence dose. Treatment of M21 cells with *H. corymbosa* extracts and irradiation resulted in the changes in nuclear morphology, evidenced by DAPI staining (Fig. 2A). After 30-min irradiation of *H. corymbosa*-sensitized cells, there was a gradual increase in the amount of nuclear condensation (Fig. 2A). Staining cells simultaneously with annexin V/propidium iodide allows the discrimination of intact cells (FITC−/PI−), early apoptotic (FITC+/PI−) and late apoptotic cells (FITC+/PI+). As shown in Figure S2, treatment of cells with *H. corymbosa* extracts and irradiation resulted in a dose-dependent increase in late apoptotic cells.

LOW RESOLUTION FIG

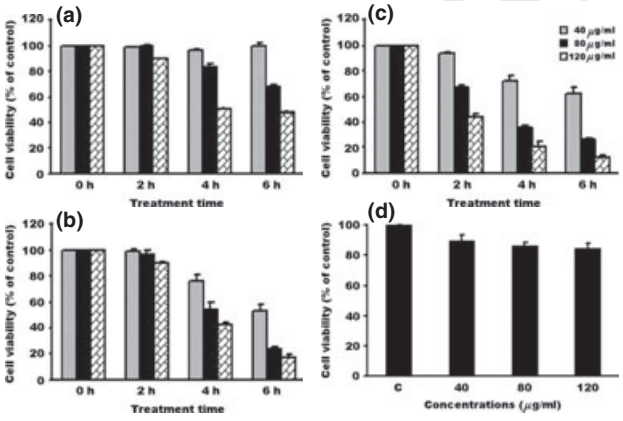


**Figure 1.** Evaluation of phototoxicity after incubation of M21 cells with *Hedyotis corymbosa* extracts and irradiation with fluence dose. Cells were incubated with 0.1% dimethylsulphoxide or with 40, 80 or 120 $\mu$g/ml *H. corymbosa* extracts for 2, 4 or 6 h and then irradiated with 0.4 (a), 0.8 (b) and 1.2 (c) J/cm$^2$ fluence dose. The viable cells were measured by MTT assay, and fraction of viable cells was calculated by defining the absorption of cells without treatment of *H. corymbosa* extracts as 100%. In light-shield condition (d), *H. corymbosa* extracts (40, 80 or 120 $\mu$g/ml; 48 h) did not exhibit significant cell toxicity of M21 cells. All results are expressed as the mean % control ± SD of triplicate determinations from four independent experiments.

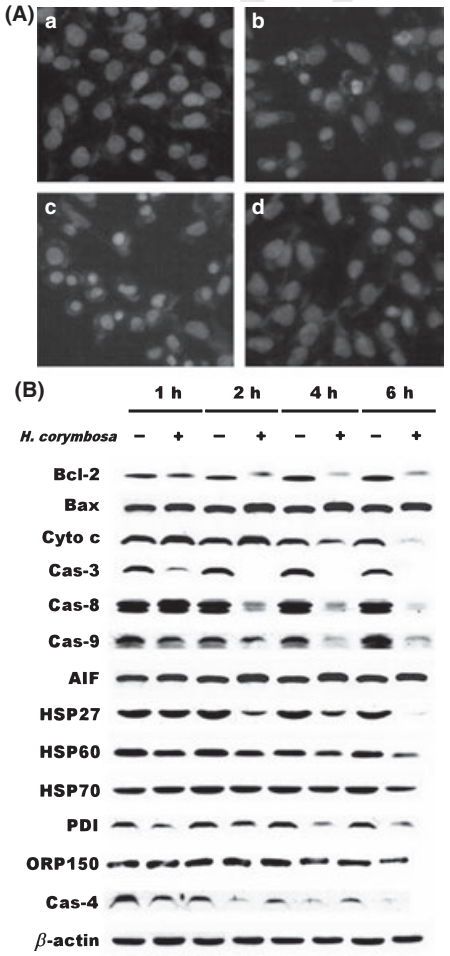


**Figure 2.** Photoactivated *Hedyotis corymbosa* extracts induced M21 cell apoptosis. (A) The photodynamic effect of *H. corymbosa* on M21 cell nuclear morphology. Cells were incubated with 0.1% dimethylsulphoxide (a) or with 40 (b) or 80 (c) $\mu$g/ml *H. corymbosa* extracts for 4 h and then irradiated with 0.8 J/cm$^2$ fluence dose. After irradiation, cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100 and stained with 1 $\mu$g/ml 4′,6-diamidino-2-phenylindole dihydrochloride. In light-shield condition (d), cells were incubated with 80 $\mu$g/ml *H. corymbosa* extracts for 4 h. The cells were then examined by fluorescent microscope (300×). (B) Effects of photoactivated *H. corymbosa* on the expression of apoptosis-related proteins and chaperones in M21 cells. Cells were incubated with 0.1% dimethylsulphoxide or 80 $\mu$g/ml *H. corymbosa* extracts for 1, 2, 4 or 6 h and then irradiated with 0.8 J/cm$^2$ fluence dose. Cell lysates were analysed by 5% (ORP150), 9% [heat-shock proteins (HSP) 60 and HSP70], 10% (apoptosis-inducing factor, caspase-8 and protein disulphide isomerase), 12% ($\beta$-actin), 13% (Bcl-2, caspase-3, caspase-9 and HSP27) and 14% (Bax, caspase-4 and cytochrome *c*) SDS-PAGE and then probed with primary antibodies as described in Methods. The detection of $\beta$-actin was used as an internal control in all of the data of Western blotting analysis. −: control cells; +: *H. corymbosa* extracts-treated cells; Cas: caspase; Cyto *c*: cytochrome *c*. All results are representative of three independent experiments.

**Photodynamic effects of *Hedyotis corymbosa* extracts on the expression of apoptosis-related proteins of M21 cells**

To obtain further support for the induction of apoptosis by photoactivated *H. corymbosa* extracts of M21 cells, the expression of apoptosis-related proteins, such as caspase family members, AIF, cytochrome *c*, Bcl-2 and Bax, were evaluated. Exposure of M21 cells to 80 $\mu$g/ml *H. corymbosa* extracts for 1 h and 0.8 J/cm$^2$ fluence dose resulted in decreases in the protein levels of Bcl-2 and pro-form caspase-3, pro-form caspase-8 and pro-form caspase-9 (Fig. 2B). The protein expression of cytochrome *c* was increased during treatment with 80 $\mu$g/ml *H. corymbosa* extracts for 2 h and 0.8 J/cm$^2$ fluence dose, but cytochrome *c* level was decreased after 2 h (Fig. 2B). Photoactivated *H. corymbosa* extracts induced a significant increase, however, in the protein expression of AIF and Bax (Fig. 2B).

**Identification of differentially expressed proteins by 2D gel**

This study further investigated the proteins involved in the photoactivated *H. corymbosa*-induced M21 cell apoptosis, protein lysate of control and *H. corymbosa*-photosensitized cells were analysed by 2D gel, and the induced protein spots were detected by the PDQuest software. After the treatment of cells with 80 $\mu$g/ml *H. corymbosa* extracts for 4 h and 0.8 J/cm$^2$ fluence dose, we found 12 protein spots to be markedly changed compared to the control cells (Fig. 3). Photoactivated *H. corymbosa* triggered significant decrease in the protein expression of spots 1–6 (Fig. 3). In *H. corymbosa*-photosensitized cells, the protein expression of spots 7–12 is more intense than those in the control cells (Fig. 3). These altered proteins were identified as MTHSP75, PDI, HSP27, vimentin, tropomyosin 4, chaperonin and cathepsin D preproprotein (Table S2) and were classified according to functional category, including chaperones (MTHSP75, PDI, HSP27 and chaperonin) and cytoskeletal proteins (vimentin and tropomyosin 4). It is interesting to note that the chaperonin revealed spot families in the horizontal direction of the 2D gel. These variants of chaperonin are identical in molecular weight but different in pI values (Fig. 3). Spots 4 and 5 were identified as vimentin, and that are identical in pI values but different in molecular weight (Fig. 3).

**Effect of photoactivated *Hedyotis corymbosa* on the protein expression of chaperones in M21 cells**

Because proteomic analysis reveals chaperones involvement in the photoactivated *H. corymbosa*-induced M21 cell apoptosis, the protein expression of mitochondrial or endoplasmic reticulum (ER) chaperones, such as HSP27, HSP60, HSP70, ORP150, PDI and caspase-4, was examined in this study. As shown in Fig. 2B, exposure of M21 cells to 80 $\mu$g/ml of *H. corymbosa* extracts and 0.8 J/cm$^2$ fluence dose resulted in decreases in HSP27, HSP60, HSP70, ORP150, PDI and caspase-4 protein level.

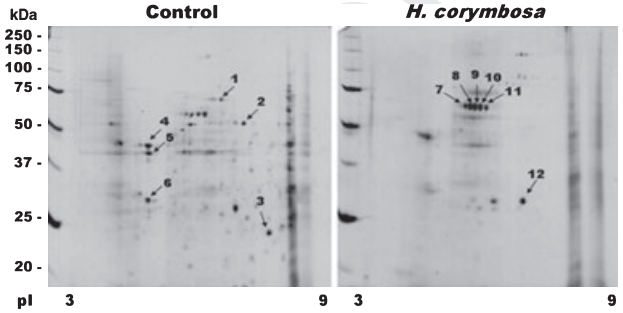


**Figure 3.** Two-dimensional electrophoresis maps of control or *Hedyotis corymbosa*-photosensitized M21 cells. Cells were incubated with 0.1% dimethylsulphoxide or 80 $\mu$g/ml *H. corymbosa* extracts for 4 h and then irradiated with 0.8 J/cm$^2$ fluence dose. Proteins were separated on a pH 3–10 IPG-strip (7 cm) in the first dimension and on a 12% SDS-polyacrylamide gel in the second dimension. Staining of the protein spots was accomplished by silver nitrate. Results are representative of three independent experiments.

**Photodynamic effect of *Hedyotis corymbosa* extracts on cytoskeleton in M21 cells**

In the following experiment, the effect of photoactivated *H. corymbosa* on M21 cell actin microfilaments was examined. In the control cells, actin microfilaments were diffusely present as short microfilaments throughout the M21 cells (Fig. 4A). After the treatment of cells with *H. corymbosa* extracts (80 $\mu$g/ml, 4 h) and fluence dose (0.8 J/cm$^2$), actin microfilaments were significantly reduced and concentrated prominently on the cell membrane margins in many cells (Fig. 4A). In the absence of light, *H. corymbosa* extracts had no significant effect on the distribution of F-actin (data not shown). In addition, photoactivated *H. corymbosa* caused a significant decrease in the fluorescence intensity of F-actin in M21 cells (Fig. 4A). To further examine whether microtubules was injured by photoactivated *H. corymbosa* in M21 cells, TubulinTracker Green reagent was used to detect the $\beta$-tubulin of microtubules in this study. As shown in Fig. 4A, microtubules were present as short fragments throughout the cells after the treatment of M21 cells with 80 $\mu$g/ml *H. corymbosa* extracts and 0.8 J/cm$^2$ fluence dose. However, microtubules are extensive fibrillar in the control cells (Fig. 4A).

**The effect of phalloidin on the photoactivated *Hedyotis corymbosa*-induced cell death and disruption of actin microfilaments of M21 cells**

In this experiment, we determined the effect of phalloidin, an actin-stabilizing agent, on the photoactivated *H. corymbosa* extracts-induced M21 cell death by MTT assay. The photoactivated *H. corymbosa* extracts-induced M21 cell death was partly blocked by pretreatment with 1 $\mu$M phalloidin for 1 h (Fig. 4B). The phenotypic characteristics of *H. corymbosa*-sensitized/irradiated cells were also evaluated. When cells were incubated with *H. corymbosa* extracts (40 and 80 $\mu$g/ml, 4 h) prior to irradiation, a greater percentage of the cells displayed a rounded morphology and eventually detached from the substratum (Fig. 4C). Pretreatment of M21 cells with 1 $\mu$M phalloidin prevented the photoactivated *H. corymbosa*-induced changes in cell morphology (Fig. 4C) and disruption of actin microfilaments (Fig. 4D).

**Effects of photoactivated *Hedyotis corymbosa* on mitochondrial function of M21 cells**

The activity of mitochondria was evaluated in this study. As shown in Figure S3A, the dot-like structures and bright red fluorescence was observed in control cells' cytoplasm using the MitoTracker Red CMXRos. In *H. corymbosa*-photosensitized cells, the red fluorescence was faint (Figure S3A). This result suggested that the capability for MitoTracker Red CMXRos uptake by mitochondria in control cells is higher compared to those in *H. corymbosa*-photosensitized cells. To confirm the possible role of MPT pore in the process of photoactivated *H. corymbosa*-induced apoptosis, we measured the opening of MPT pore in intact cells by flow cytometry. As shown in Figure S3B, treatment with 40 $\mu$g/ml *H. corymbosa* extracts for 4 h and then 0.8 J/cm$^2$ fluence dose resulted in a decrease in calcein fluorescent intensity because of the opening of




 

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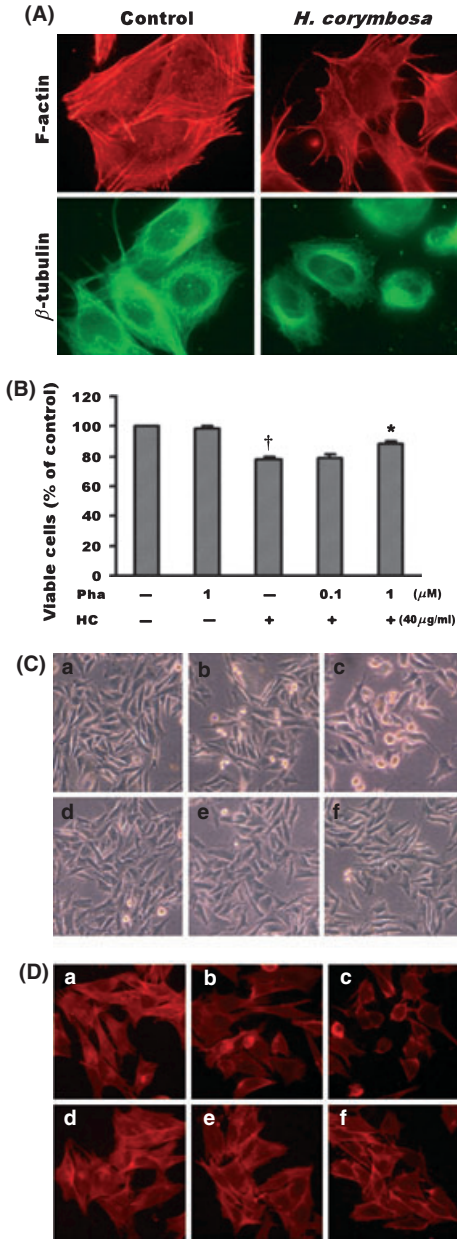


**Figure 4.** The effect of photoactivated *Hedyotis corymbosa* on actin microfilaments and microtubules of M21 cells. Cells were incubated with 0.1% dimethylsulphoxide (DMSO) (control) or 80 μg/ml *Hedyotis corymbosa* extracts for 4 h and then irradiated with 0.8 J/cm$^2$ fluence dose. After irradiation, the localization of F-actin and β-tubulin was examined. The specimens were observed by fluorescent microscope (1000×). (B) Effects of phalloidin on the photoactivated *H. corymbosa* extracts-induced cell death of M21 cells. Cells were incubated with 0.1% DMSO or *H. corymbosa* extracts (HC) for 4 h and then irradiated with 0.8 J/cm$^2$ fluence dose. In phalloidin (Pha) treatment, cells were pretreated with phalloidin for 1 h. The viable cells were measured by MTT assay. Results are expressed as the mean percentage of control ± SD. $^{\dagger}P < 0.01$ compared to the control values. $^{*}P < 0.01$ compared to the photoactivated *H. corymbosa* extracts-treated cells. (C) Effects of phalloidin on the photoactivated *H. corymbosa* extracts-induced changes in cell morphology (150×). (D) Effects of phalloidin on the photoactivated *H. corymbosa* extracts-induced disruption of actin microfilaments (300×). a: control cells; b: 40 μg/ml *H. corymbosa*-treated cells; c: 80 μg/ml *H. corymbosa*-treated cells; d: 1 μM phalloidin-treated cells; e: 1 μM phalloidin- and 40 μg/ml *H. corymbosa*-treated cells; f: 1 μM phalloidin- and 80 μg/ml *H. corymbosa*-treated cells. All results are representative of three independent experiments.

MPT pores. However, treatment with 40 μg/ml *H. corymbosa* extracts for 4 h had no effect on the calcein fluorescent intensity of M21 cells in the dark (Figure S3B). Because the opening of MPT pore is accompanied by decrease in MMP, this study examined the photodynamic effect of *H. corymbosa* extracts on MMP in M21 cells. After cells were treated with *H. corymbosa* extracts and irradiation, a remarkable attenuation of MMP occurred compared to the control cells or the cells treated with 40 μg/ml *H. corymbosa* extracts in the dark (Figure S3C). Based on the above data, mitochondrial function is severely impaired by photoactivated *H. corymbosa* extracts in M21 cells.

## Discussion

This study demonstrated that 80 μg/ml *H. corymbosa* extracts and 0.8 J/cm$^2$ light dose caused cell death of M21 cells. *H. corymbosa* extracts did not show any dark toxicity under the same concentrations for evaluation M21 cell death. Therefore, *H. corymbosa* extracts may be a photosensitizer, which leads to induce M21 cancer cell death. As shown in Figure S1, the absorption spectrum of *H. corymbosa* extracts in ethanol shows the typical Soret band at 420 nm and Q band at 670 nm. It suggests a tetrapyrrolic structure, likely present in a mixture of substances, such as chlorins and porphyrin (16,17). The photosensitizing activity of napthodianthrones and tetrapyrrolic derivatives found in plants has been discovered. Hypericin, a component of *Hypericum* plants, has extensively been used in PDT. The green plant pigment chlorophyll containing the porphyrin-related chlorin ring is partially responsible for this activity (18,19). The purification of photosensitizer from *H. corymbosa* extracts will be further developed in our study. Photodynamic effect occurs when light-activated photosensitizers transfer the energy to oxygen nearby to produce singlet oxygen. Singlet oxygen can then react with biological substrates, resulting in cellular damage (16,17). Therefore, reactive oxygen species (ROS) may play an important role in the *H. corymbosa* extracts-induced photokilling in M21 cells.

In this study, apoptosis was virtually the unique type of cell death in response to *H. corymbosa* extracts and irradiation in M21 cells. During the photoactivated *H. corymbosa*-induced apoptosis, nuclear condensation and externalization of phosphatidylserine were observed in M21 cells. In the experiment of annexin V/PI double staining, exposing the M21 cells to *H. corymbosa* extracts and light resulted in an increase in the late apoptotic cells. According to the results of LDH assay, we also demonstrated that photoactivated *H. corymbosa*-induced M21 cell death was a typical apoptosis, not necrosis. Photosensitized *H. corymbosa* also induced significant changes in pro-form caspase-3, AIF and cytochrome *c* protein levels, which are important indicators of apoptosis.

Proteomic data showed that chaperones, such as MTHSP75, PDI, HSP27 and chaperonin, were involved in the photoactivated *H. corymbosa*-induced M21 cell apoptosis. It is well known that chaperones are found in the ER or mitochondria. Therefore, we focused our attention on the protein expression of mitochondrial and ER molecular chaperones. This study demonstrated that the molecular chaperones in the ER, such as PDI, caspase-4 and ORP150, are involved in the photoactivated *H. corymbosa*-induced M21 cell apoptosis by decreasing protein levels. The ORP150, a new member of HSP family, functions as a molecular chaperone in the ER (20). Caspase-4 has been shown to be localized on the

ER and as an ER stress–specific caspase in human (21,22). Photoactivated *H. corymbosa* also induced a significant decrease in the protein expression of HSP60 and HSP70, which act as mitochondrial molecular chaperone, in this study. These results are consistent with previous observations that have shown an association between the expression of the molecular chaperones and cell death (23,24). The above findings suggest that the protein folding pathway serves as an important apoptotic control point in the photoactivated *H. corymbosa*-induced apoptosis. After treatment with *H. corymbosa* and light, the vimentin and chaperonin revealed spot families in the vertical and horizontal direction, respectively, in the 2D gel. This result seemed to suggest that the single spots of the complex pattern were probably because of post-translational modifications of one particular protein.

Because MTHSP75 is the precursor of HSP70 and located in the matrix of mitochondria, we hypothesized that photoactivated *H. corymbosa* induced M21 cell apoptosis in association with regulation of mitochondrial function. The MPT pore is characterized by opening of the permeability transition pore in the inner mitochondrial membrane, which results in increase in permeability of this membrane to protons, ions and small–molecular weight solutes (25). This increased permeability is also considered to lead to a collapse of the MMP (26,27). We demonstrated that photoactivated *H. corymbosa* extracts induced the opening of MPT pores accompanied by a disruption of MMP in M21 cells. In addition, photoactivated *H. corymbosa* induced a significant reduction in the capability for MitoTracker Red CMXRos uptake by mitochondria. These data suggested that mitochondria may be a target of photoactivated *H. corymbosa* during photoactivated *H. corymbosa*-induced M21 cell death.

It has been indicated that the function of mitochondria is regulated by F-actin (7,8). In the results of 2D gel electrophoresis, we also observed that HSP27, vimentin and tropomyosin significantly decreased after treatment with *H. corymbosa* and fluence. Vimentin and tropomyosin are the important constituents of cytoskeleton. It has been reported that p38-mediated F-actin reorganization was associated with translocation of HSP27 from cytosolic to cytoskeletal fraction (28). The involvement of cytoskeletal actin in apoptosis has been suggested by some morphological studies, which show actin cleavage during morphological apoptosis (29,30). According to the above considerations, this study investigated the role of cytoskeleton during photoactivated *H. corymbosa* extracts-induced M21 cell apoptosis. Photoactivated *H. corymbosa* extracts reduced the length of the stress fibres and amount of F-actin in M21 cells, indicating that the photoactivated *H. corymbosa* extracts-induced M21 cell apoptosis is mediated in part through its effect on actin microfilaments. Furthermore, the photoactivated *H. corymbosa*-induced cell death and disruption of F-actin was partly blocked by pretreatment with 1 $\mu$M phalloidin, an actin-stabilizing agent. Based on the above data, the regulation of cytoskeletal actin is involved in the photoactivated *H. corymbosa* extracts-induced M21 cell apoptosis.

We demonstrated that the photoactivated *H. corymbosa* extracts-induced cell apoptosis is a typical apoptosis that is accompanied by nuclear condensation, externalization of phosphatidylserine and integrated cell membrane. This study applied 2D electrophoresis to analyse the proteins involved in photoactivated *H. corymbosa*-induced M21 cell apoptosis. The cytoskeletal proteins and chaperones were demonstrated to be involved in photoactivated *H. corymbosa*-induced M21 cell apoptosis.

## Acknowledgements

This work was supported by National Science Council Grant NSC 96-2320-B-039-011 and NSC 97-2320-B-039-005-MY3 and the China Medical University Grant CMU97-174 of Republic of China. Chen MY, Chang YH and Lee HZ performed the research, You BJ, Wu CY, Bao BY and Lee HZ analysed the data, Wu YC and Lee HZ contributed essential reagents for the study, Wu YC and Lee HZ designed the research study and You BJ and Lee HZ wrote the paper.

## References


1 Maisch T, Santarelli F, Schreml S *et al.* Exp Dermatol 2010: **19**: e302–e305.
2 Togsverd-Bo K, Lerche C M, Poulsen T *et al.* Exp Dermatol 2010: **19**: e166–e172.
3 Ali S M, Olivo M, Yuen G Y *et al.* Asian J Surg 2002: **25**: 18–26.
4 Leung W C, Hour M J, Chang W T *et al.* Food Chem Toxicol 2008: **46**: 3389–3400.
5 Dallmann K, Junker H, Balabanov S *et al.* Int J Cancer 2004: **108**: 342–347.
6 van den Bogaerdt A J, El Ghalbzouri A, Hensbergen P J *et al.* Biochem Biophys Res Commun 2004: **315**: 428–433.
7 Soldani C, Croce A C, Bottone M G *et al.* Histochem Cell Biol 2007: **128**: 485–495.
8 Bernstein B W, Chen H, Boyle J A *et al.* Am J Physiol Cell Physiol 2006: **291**: C828–C839.
9 Beere H M, Wolf B B, Cain K *et al.* Nat Cell Biol 2005: **2**: 469–475.
10 Garrido C, Bruey J M, Fromentin A *et al.* FASEB J 1999: **13**: 2061–2070.
11 Lin K M, Lin B, Lian I Y *et al.* Circulation 2001: **103**: 1787–1792.
12 Samali A, Cai J, Zhivotovsky B *et al.* EMBO J 1999: **18**: 2040–2048.
13 Lee H Z, Yang W H, Hour M J *et al.* Eur J Pharmacol 2010: **648**: 50–58.
14 Lee H Z, Yang W H, Bao B Y *et al.* Eur J Pharmacol 2010: **642**: 19–27.
15 Lee H Z, Liu W Z, Hsieh W T *et al.* Food Chem Toxicol 2009: **47**: 561–570.
16 Nyman E S, Hynninen P H. J Photochem Photobiol B 2004: **73**: 1–28.
17 Serra A, Pineiro M, Santos C I *et al.* Photochem Photobiol 2010: **86**: 206–212.
18 Stiel H, Marlow I, Roeder B. J Photochem Photobiol B 1993: **17**: 181–186.
19 Ris H B, Altermatt H J, Inderbitzi R *et al.* Br J Cancer 1991: **64**: 1116–1120.
20 Ikeda J, Kaneda S, Kuwabara K *et al.* Biochem Biophys Res Commun 1997: **230**: 94–99.
21 Hitomi J, Katayama T, Eguchi Y *et al.* J Cell Biol 2004: **165**: 347–356.
22 Li J, Xia X, Ke Y *et al.* Biochim Biophys Acta 2007: **1770**: 1169–1180.
23 Lai M Y, Hour M J, Leung W C *et al.* Eur J Pharmacol 2007: **573**: 1–10.
24 Kudo T, Okumura M, Imaizumi K *et al.* Biochem Biophys Res Commun 2006: **344**: 525–530.
25 Zoratti M, Szabo I. Biochim Biophys Acta 1995: **1241**: 139–176.
26 Oliveira K A, Zecchin K G, Alberici L C *et al.* J Bioenerg Biomembr 2008: **40**: 307–314.
27 Crompton M. Biochem J 1999: **341**: 233–249.
28 Nofer J R, Noll C, Feuerborn R *et al.* Biochem Biophys Res Commun 2006: **340**: 751–757.
29 Petrovas C, Mueller Y M, Yang G *et al.* Apoptosis 2007: **12**: 2175–2186.
30 Suria H, Chau L A, Negrou E *et al.* Life Sci 1999: **65**: 2697–2707.


## Supporting Information

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** The absorption spectrum of *Hedyotis corymbosa* extracts in ethanol (1 mg/ml).

**Figure S2.** Annexin V-FITC/PI staining of M21 cells treated with 0.1% dimethylsulphoxide (A) or with 40 (B) or 80 (C) $\mu$g/ml *Hedyotis corymbosa* extracts followed by irradiation with 0.8 J/cm$^2$ fluence dose. Cells were then processed for annexin V-FITC/PI staining and analyzed with flow cytometry. In light-shield condition (D), cells were incubated with 80 $\mu$g/ml *H. corymbosa* extracts for 4 h. Results are representative of three independent experiments.

**Figure S3.** Effects of photoactivated *Hedyotis corymbosa* extracts on mitochondrial function in M21 cells.

**Table S1.** Lactate dehydrogenase release from M21 cells.

**Table S2.** Characteristics of the analyzed spots.

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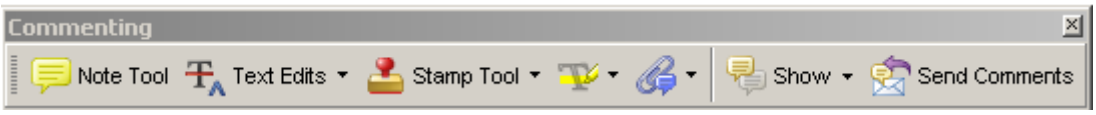


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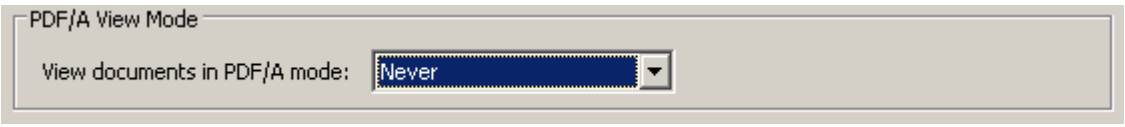


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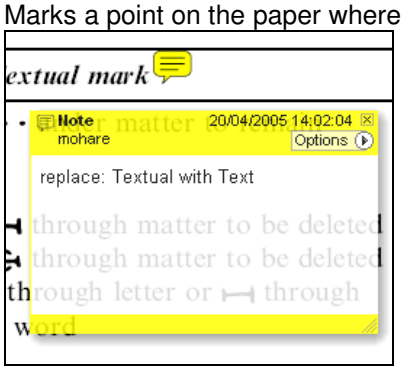


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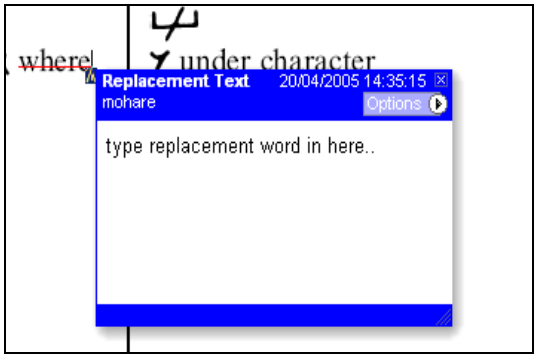


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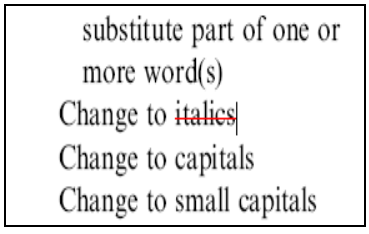


How to use it:

1. Select cursor from toolbar
2. Highlight word or sentence
3. Right click
4. Select Cross Out Text

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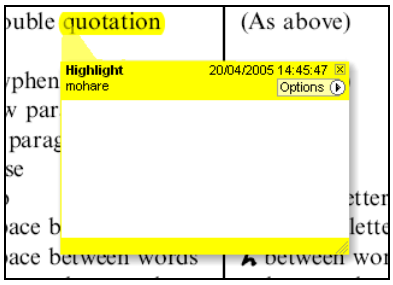


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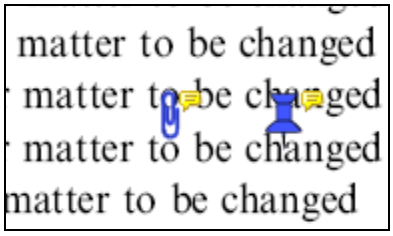


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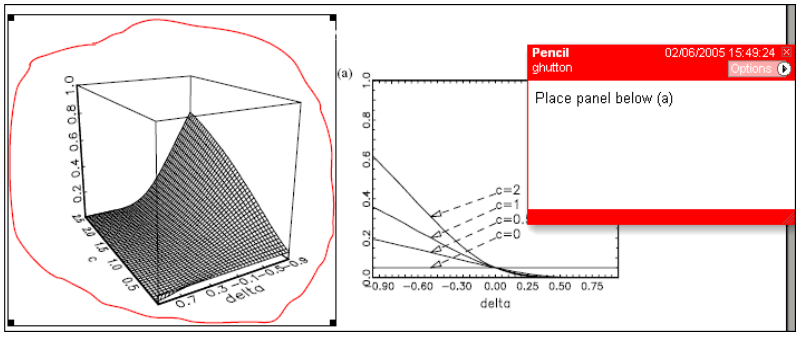


How to use it:

1. Select Tools > Drawing Markups > Pencil Tool
2. Draw with the cursor
3. Multiple pieces of pencil annotation can be grouped together
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