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Contributed Paper

Antiproliferative Activity of Berberine from *Coscinium fenestratum* on NCI-H838 Cell Line

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ABSTRACT

Berberine is the main cytotoxic compound in *Coscinium fenestratum*. Although, cytotoxic activity of berberine on many cancer cell lines have been studied but effect of berberine on NCI-H838 cell line is still not reported. In the present study, antiproliferative activity and mechanism of berberine isolated from *C. fenestratum* against NCI-H838 were investigated. NCI-H838 cells were treated with various concentrations of berberine and the cytotoxic activity was evaluated by MTT method. Berberine exhibited cytotoxicity by dose and time dependent manner with the IC_{50} values at 24, 48 and 72 h were 111.9$\pm$7.0 μM, 92.4$\pm$1.2 μM and 68.4$\pm$7.9 μM, respectively. Additionally, apoptotic in berberine treated cell was detected by DAPI staining. The associated proteins for apoptosis were examined by western blotting and it was found that berberine downregulated Bcl-2, procaspase 3, 6, 7 and 8 but upregulated caspase 7 by dose dependent manner. Moreover, berberine induced G2/M arrest was determined by the flow cytometric method. Taken together indicated that the potential of antiperiferal activity of berberine mediated through the Bcl-2/caspase-dependent pathway and G2/M cell cycle arrest.

Keywords: berberine, *Coscinium fenestratum*, NCI-H838, apoptosis, caspase, G2/M arrest

1. INTRODUCTION

Coscinium fenestratum (Gaertn.) Colebr., is a plant in the family Menispermaceae. It is a woody climbing shrub with cylindrical stem, externally yellowish brown and internally yellow in colour. Its stem has often been used as a substitute for berberis, but it can be readily distinguished by the presence of large vessels in the wood, absence of annual rings and the crenate ring of sclerenchyma beneath the cortex. The stem yields a yellow dye, which is used either alone or in combination with turmeric and other coloring materials [1]. In Thailand, this plant is commonly known as "Hamm", it is distributed mainly in the northeast of Thailand especially in Nong Khai and Nakorn Panom provinces. Decoction of

the stem of *C. fenestratum* has been used in Thai traditional medicine of rural people in the northeast of Thailand for a long time. Its stem has been claimed to possess hypoglycaemic, hypotensive, laxative and anti-diabetic activities [2]. In phytochemical studies, *C. fenestratum* has afforded berberine as the main alkaloidal constituent and a smaller amount of protoberberine [3-5].

Berberine (5,6-dihydro-9,10-dimethoxy-benzo[g]-1,3-benzodioxole[5,6-d-a] quinolizinium), isoquinoline alkaloid, has been extensively studied and known to exhibit multiple pharmacological activities such as antimicrobial activity towards bacteria, fungi and viruses [6,7], antimalarials [8], antiinflammatory [9], antihypertension [10], lower blood glucose level [11] reduce blood lipid [12] and antiproliferative [13] activity. It was also shown to inhibit the *in vitro* growth of a number of human cancer cell lines.

From our previous study, it was found that the main cytotoxic compound from *C. fenestratum* was berberine (Figure 1) and it showed cytotoxic and apoptosis effects against HL-60 cell line [14]. However, cytotoxic effect of berberine on each cancer cell line is significantly different and the effect of berberine against non-small cell lung adenocarcinoma has still not been reported yet. Therefore, the aim of the present study is to find out the antiproliferative effect and apoptotic pathway of berberine underlying human non-small cell lung adenocarcinoma, NCI-H838.

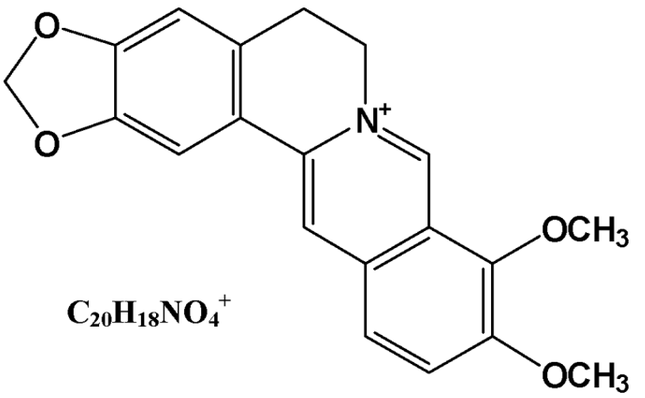

Figure 1. Chemical structure of berberine isolated from *C. fenestratum*.

2. MATERIALS AND METHODS

2.1 Berberine Extraction and Purification from *Coscinium fenestratum*

Dried stem of *C. fenestratum* was purchased from a local district in Chiang Mai, Thailand. Berberine was isolated by the method described previously [14]. First, the stem powder was extracted by methanol. Then the methanol extract was separated with C18 resins (Polygoprep 100-20 C_{18}, Macherey-Nagel) open column using water follow by water/acetonitrile (7:3), acetonitrile and methanol as mobile phase. Berberine was extracted in the methanol fraction. This fraction was further purified by RP-HPLC using C18 preparative column (10×250 mm, 5 μm spherical, VYDAC) and two mobile phases, A (5% acetonitrile, 0.1% trifluoroacetic

acid) and B (95% acetonitrile, 0.1% trifluoroacetic acid). The RP-HPLC condition used was the gradient elution profile of 0-5-15-20-30 min/0-35-60-100-100%B at a flow rate of 2 mL/min. The eluent was monitored by measuring UV absorption at 214 nm and the major peak was collected, which was berberine.

2.2 Antiproliferative Activity of Berberine on NCI-H838 Cell Line

Non-small cell lung adenocarcinoma (NCI-H838) cell line was cultured in RPMI medium and supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B. The cell was maintained in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air. Antiproliferative activity of berberine was studied by methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. Cells were trypsinized and seeded in 96 well plates (2×10^4 cells/well) in 100 µL of RPMI medium and allowed to adhere for 24 h. Berberine was dissolved in dimethyl sulfoxide (DMSO). Then, cells were treated with 100 µL of RPMI medium containing various concentrations of berberine and 0.25% DMSO as control. The cells were subsequently incubated for 24, 48 and 72 h, the medium was aspirated, and 100 µL fresh medium containing 0.5 mg/mL MTT was added to assess cell viability. After incubation for 4 h at 37°C, the media was aspirated, and 100 µL of DMSO was added to each well. The plates were shaken to dissolve the reduced MTT, and the absorbance was measured at 570 nm and 630 nm by microtiter plate reader (MRX II, DYNEX). The percentage of cell survival in each well was determined by dividing the absorbance of the test well by the average absorbance in wells treated only with DMSO. All samples were tested in triplicate and repeated at least three times.

2.3 Apoptosis Detection by DAPI Staining

NCI-H838 1×10^6 cells were seeded in 6 cm dish (3 mL/well), and incubated at 37°C for 24 h. The cells were treated with 80 and 100 µM berberine and 0.25% DMSO as control for 24 h, then cells were detached by trypsinization and washed with ice cold phosphate buffer saline (PBS). The cells were incubated with 1 µg/mL 4′-6-diamidino-2-phenylindole (DAPI) in PBS for 15 min in dark at room temperature then washed with PBS twice, and the suspension (5 µL) was placed on a glass slide and covered with a cover slip. The morphology of the cell's nuclei was observed using a fluorescence microscope (Olympus BX51 Series). Apoptosis rate of NCI-H838 cell was determined by random counting from 200 to 300 cells/sample.

2.4 Effect of Berberine on Apoptosis by Western Blot Analysis

NCI-H838 1×10^6 cells were plated onto 6 cm dish (3 mL/well), and incubated at 37°C for 24 h. The cells were treated with 80, 100, 120 and 140 µM berberine and 0.25% DMSO as control for 24 h. Cells were collected and lysed by the addition of ice-cold lysis buffer (150 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 2 mM ethylenediaminetetra acetic acid, 0.2% sodium dodecyl sulfate, 1% protease inhibitor cocktail). After leaving on ice for 20 min, the mixtures were centrifuged (12,000 rpm) at 4°C for 20 min, and the supernatants were collected as the whole-cell extracts. The protein concentrations were determined with Bio-Rad protein assay reagent using bovine serum albumin (BSA) as a standard. Equivalent amounts of 10 µg protein were added to SDS-PAGE Laemmli buffer (250 mM Tris base, pH 6.8, 40%

glycerol, 8% SDS, 20% β-mercaptoethanol, 0.01% bromophenol blue) and then proteins were separated by SDS– PAGE (12.5% gel). The PVDF Immobilon-P membrane and blotter paper were cut to the same size as the gel. The gel, membrane and blotter paper were equilibrated in transfer buffer (5.8 g/l Tris-HCl pH 8.8, 2.9 g/l glycine and 20% (v/v) methanol) for 10 min. Then, the gel and membrane were assembled. The separated proteins were transferred to the PVDF membrane using semi-dry transfer blot cell at 250 mA for 1.30 h. The membranes were incubated with 5% (w/v) skim milk in PBST (PBS containing 0.1% (v/v) Tween-20) for 2 h to block the nonspecific binding protein sites. The membrane was incubated with procaspase 3, 6, 7, 8, Bcl-2 antibody (Abcam, diluted 1:2,000 in PBST) at room temperature for 2 h. The membrane was washed 3 times with PBST for 5 min, and were incubated with appropriate horseradish peroxidase-conjugated secondary antibody (Abcam, diluted up to 1:2,000 in PBST) for 2 h at room temperature, then washed 3 times with PBST for 5 min. Immunoreactive bands were visualized by the enhanced chemiluminescence Western blotting detection system (ECL™ kit; GE Healthcare). The same membranes were used sequentially to detect several proteins by stripping and reblotting. To verify equal protein loading, membranes were re-blotted with an anti-β-actin monoclonal antibody.

2.5 Determination cell cycle by PI staining

NCI-H838 1×10^6 cells were seeded in 6 cm dish (3 mL/well), and incubated at 37°C for 24 h. The cells were treated with 40, 60 and 80 μM berberine and 0.25% DMSO as control for 24 h. All cells were collected by trypsinization and washed with cold PBS. Then, 70% of cold ethanol was slowly added to the cell pellet while vortexing. Cells were fixed with 70% ethanol for at least 30 minutes at 4°C. Before analysis, the 70% ethanol was removed. The cell pellet was washed with PBS twice and incubated with 100•μg/ml RNAse in PBS at 37°C for 30 min. Cells were stained with 50•μg/ml propidium iodide (PI) at 37°C in the dark for 15 min. Samples were immediately analysed by flow cytometry (BD FACScan). Cell cycle distribution was determined using Cell Quest software.

3. RESULTS AND DISCUSSIONS

3.1 Antiproliferative Activity of Berberine on NCI-H838 Cell Line

Berberine was extracted and purified from *C. fenestratum* to yield 3.68% (w/w). This compound was shown cytotoxic activity against many cancer cell lines [13-17]. In this study, berberine from *C. fenetratum* also could inhibit human non-small cell lung adenocarcinoma, NCI-H838, cell proliferation by dose and time dependent manner as shown in Figure 2. In addition, IC_{50} values (the concentration required that inhibited cell growth by 50% relative to untreated cancer growth) were calculated. It was found that, the IC_{50} values at 24, 48 and 72 h were 111.9 ± 7.0 μM, 92.4 ± 1.2 μM and 68.4 ± 7.9 μM, respectively.

3.2 Apoptosis Detection by DAPI Staining

The cells were died about 50% and 20% when treated with 100 and 80 μM berberine, respectively. Thus these concentrations were chosen for study effect of berberine on cell apoptosis. NCI-H838 cells were treated with 80 and 100 μM berberine for 24 h, then the nuclei was stained with DAPI and nuclear bodies were observed by fluorescence microscope as shown in Figure 3. Berberine could induce apoptosis in NCI-H838 cells. The cells that treated with 80 μM berrberine contained apoptotic cells 2.65 fold while 100 μM berberine treated cells consisted apoptotic cells 5.27 fold when compared with

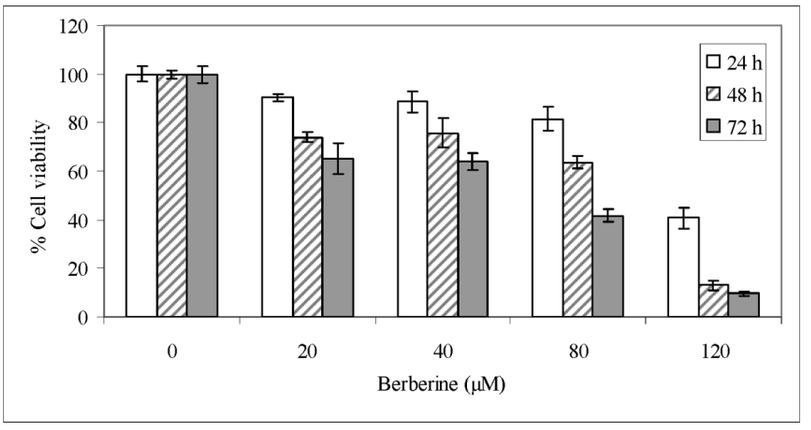

Figure 2. Antiproliferative activity of berberine from *C. fenestratum* on NCI-H838. NCI-H838 cells were treated with or without 20, 40, 80 and 120 µM berberine for 24, 48 and 72 h, then cell viability was determined by MTT assay.

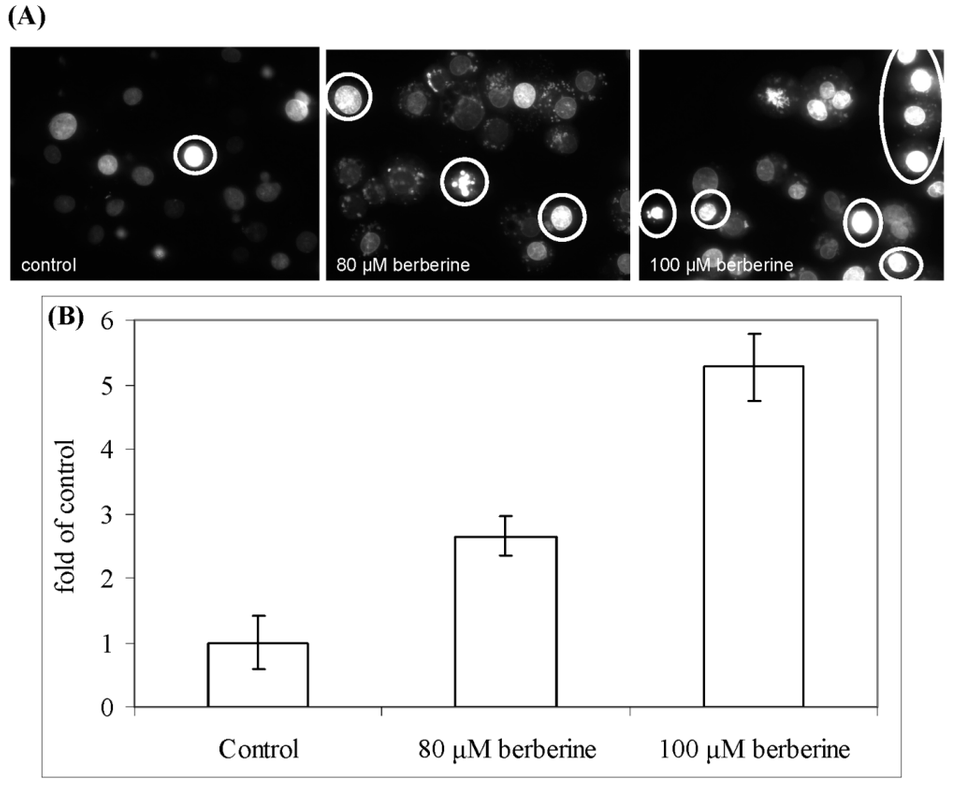

Figure 3. Effect of berberine on NCI-H838 cell apoptosis detected by DAPI staining. NCI-H838 cells were treated with and without 80 and 100 µM berberine for 24 h, then stained with DAPI and observed under fluorescence microscope (**A**). Apoptotic nuclei are shown in blue circle. Apoptosis rate was determined by random counting from 200 to 300 cells/sample and the apoptosis cells were calculated when compared with control (**B**).

the control. From this result it was found that berberine could induce apoptosis in NCI-H838 cell the same as in the other cell lines such as U937 [13], HL-60 [14-16] and SNU-5 [17] cell line.

3.3 Effect of berberine on apoptosis by western blot analysis

NCI-H838 cells were treated with 80, 100, 120 and 140 μM berberine for 24 h, then collected total cell lysate and observed protein in apoptosis pathway including procaspase 3, 6, 7, 8 and Bcl-2 and β-actin by western blotting. The results are shown in Figure 4. It was found that NCI-H838 cells treated with higher concentrations of berberine, procaspase 3, 6, 7 and 8 and Bcl-2 were significantly decreased in order, but the cleavage caspase 7 was increased while the level of β-actin, protein loading control, was stable.

Bcl-2 is an integral membrane protein located mainly on the outer membrane of mitochondria and a member of protein in BCL-2 family. The proteins of this family have an important role in the regulation of programmed cell death associated with the mitochondrial membrane. Bcl-2 acts to inhibit apoptosis by prevent cytochrome c release from mitochondria [18]. Our result showed that berberine reduced the antiapoptotic Bcl-2 protein level as a result of cytochrome c was released from mitochondria and then induce apoptosis.

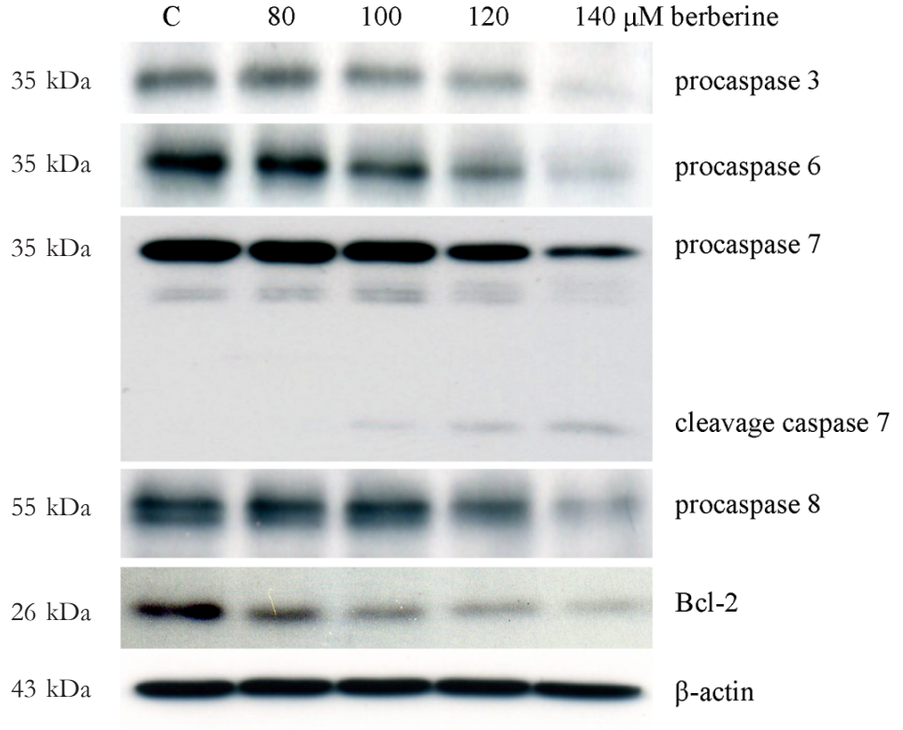

Figure 4. Western blot analysis of some proteins in apoptosis pathway of control, 80, 100, 120 and 140 μM berberine treated NCI-H838 cell at 24 h. β-actin was used as loading protein control.

The caspase-cascade system plays essential roles in the apoptosis. Caspases are cysteine-aspartic protease in a family of cysteine proteases. Caspases exist as inactive proenzymes known as procaspases that undergo proteolytic processing at conserved aspartic residues to produce two subunits, large and small subunits, that dimerize to form the active enzyme. Sequential activation of caspases played a central role in the execution-phase of cell apoptosis [19, 20]. Generally, there are two pathways through which the caspase family proteases can be activated: one is the death signal-induced, death receptor-mediated pathway; the other is the stress-induced, mitochondrion-mediated pathway (i.e. a caspase-9-dependent pathway) [20]. In the cell treated berberine, the amount of procaspase 7 was decreased while cleavage caspase 7 (active form) was increased. This was confirmed that procaspase 3, 6, 7 and 8 were activated to be active enzymes. Therefore, the active caspase 3, 6, 7, 8 were increased and induced apoptotic substrates in down stream pathway.

3.4 Determination cell cycle by PI staining

Berberine could induce cell cycle arrest in some cell lines [16, 17, 21] therefore effect of berberine on NCI-H838 cell cycle arrest would be studied. The concentrations that lower than IC_{50} (40, 60 and 80 μM of berberine) were used for study effect of cell cycle arrest because high concentration of berberine (>80 μM) could induce more apoptosis in this cell line. The cells were treated with 40, 60 and 80 μM of berberine for 24 h and then before stained with PI to observe cell cycle. The results are shown in Figure 5. When cell was treated with higher concentrations of berberine, percentage of cells in G2/M phase was increased in dose dependent manner. It had been reported that berberine could induce G2/M phase cell cycle arrest in HL-60 and SNU-5 cell lines [16, 17], while G0/G1 arrest was observed in murine leukemia L1210 cell line [21]. On the other hand, berberine has no effect on the cell cycle profile of the U937 and B16 cells was detected [13], suggesting that berberine induced different cell cycle arrest depending on cell types. Therefore, the mechanism of berberine is not unclear in all cell types. From our result, it was found that berberine could induce G2/M phase arrest in NCI-H838 cell. It had been reported that, berberine could bind with DNA and induced apoptosis [15], therefore, berberine might be able to arrest cell cycle by this reason.

The possible signaling pathway of apoptosis in NCI-H838 cell inducing by berberine is shown in Figure 6. In berberine treated cells, the cells were blocked at G2/M phase and undergo apoptosis if the damage was severely to be repaired [22]. An alternative effect of berberine was to activate caspase 8, and then the downstream pathways of caspase 8 were activated. Caspase 8 activated procaspase 3 to be caspase 3, then this active caspase 3 activated procaspase 6 and 7 to be caspase 6 and 7 [20]. Additionally, berberine could reduce antiapoptotic Bcl-2 level causing cytochrome c released from mitochondria to the cytosol and activated caspase 9 [18]. Then this caspase 9 activated procaspase 3 and 7. Next, active caspase 3, 6 and 7 activated downstream apoptosis substrates. Finally, apoptosis would be induced [18, 20].

4. CONCLUSION

Berberine isolated from *C. fenestratum* exhibited cytotoxic effect on NCI-H838 cell line in dose and time dependent manner. Apoptotic effect of berberine on this cell line was observed. In addition, berberine could reduce the protein levels of Bcl-2, procaspase 3, 6, 7 and 8 but it could increase the protein

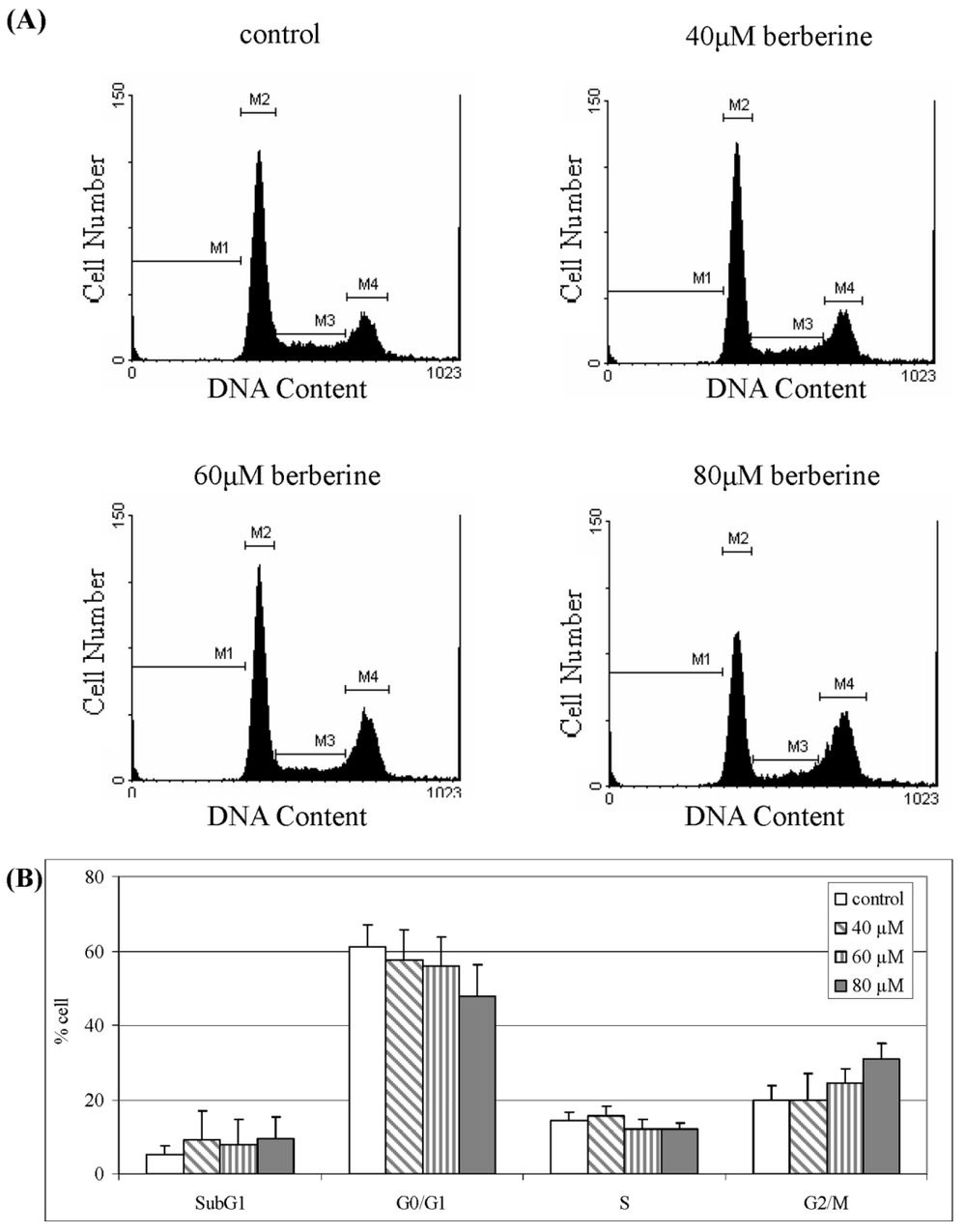

Figure 5. Cell cycle analysis of control, 40, 60 and 80 μM berberine treated NCI-H838 cell at 24 h measure by flow cytometry (**A**), and percentage cell in early and late apoptosis were calculated in histogram (**B**).

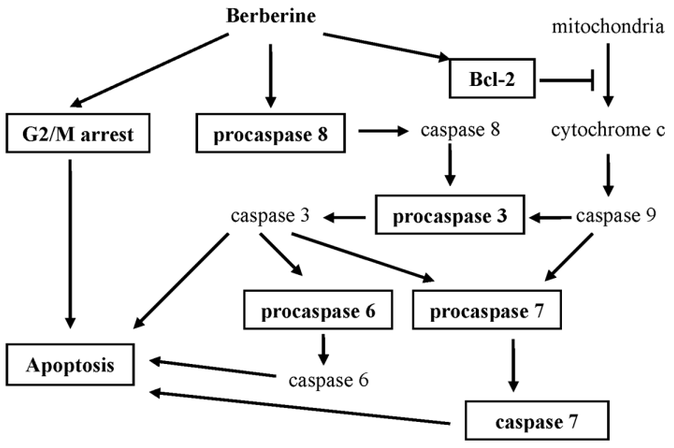

Figure 6. Proposed signaling pathway of apoptosis in NCI-H838 cell line which was induced by berberine. Berberine induced G2/M arrest, decrease procaspase 3, 6, 7, 8 and Bcl-2, but increase caspase 7, causing apoptosis in NCI-H838 cells. The signals in rectangle were from this study.

level of caspase 7 in NCI-H838 cells. Moreover, berberine could induce G2/M arrest in this cell line. Taken together, the results clearly demonstrate that berberine induces apoptosis of NCI-H838 cell line through the Bcl-2/caspase-dependent pathway and G2/M arrest and the possible mechanism has been proposed.

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REFERENCES

[1] Shirwaikar A., Rajendran K. and Punitha I.S.R., Antidiabetic activity of alcoholic stem extract of *Coscinium fenestratum* in streptozotocin-nicotinamide induced type 2 diabetic rats, *J. Ethnopharmacol.*, 2005; **97**: 369-374.

[2] Wattanathorn J., Uabundit N., Itarat W., Mucimapura S., Laopatarakasem P. and Sripanidkulchai B., Neurotoxicity of *Coscinium fenestratum* stem, a medicinal plant used in traditional medicine, *Food Chem. Toxicol.*, 2006; **44**: 1327-1333.

[3] Malhotra S., Taneja S.C. and Dhar K.L., Minor alkaloid from *Coscinium fenestratum*. *Phytochem.*, 1989; **28**, 1998-1999.

[4] Pinho M.M.P., Pinto M.M.M., Kijjoa A., Pharadia K., Diaz J.G. and Herz W., Protoberberine alkaloids from *Coscinium fenestratum*, *Phytochem.*, 1992; **31**: 1403-1407.

[5] Rojsanga P., Gritsanapan W. and Suntornsuk L., Determination of berberine content in the stem extracts of *Coscinium fenestratum* by TLC densitometry, *Medical Principles and Practice*, 2006; **15**: 373-378.

[6] Freile M.L., Giannini F., Pucci G., Sturniolo A., Rodero L., Pucci O., Balzareti V. and Enriz R.D., Antimicrobial activity of aqueous extracts and of berberine isolated from *Berberis heterophylla*, *Fitoterapia*, 2003; **74**: 702-705.

[7] Hayashi K., Minoda K., Nagaoka Y., Hayashi T. and Uesato S., Antiviral activity of berberine and related compounds against human cytomegalovirus, *Bioorg. Med. Chem. Lett.*, 2007; **17**: 1562-1564.

[8] Iwasa K., Nishiyama Y., Ichimaru M., Moriyasu M., Kim H.S., Wataya Y., Yomori T., Takashi T. and Lee D.U., Structure-activity relationships of quaternary protoberberine alkaloids having an antimalarial activity, *Eur. J. Med. Chem.*, 1999; **34**: 1077-1083.

[9] Lee C.H., Chen J.C., Hsiang C.Y., Wu S.L., Wu H.C. and Ho T.Y., Berberine suppresses inflammatory agents-induced interleukin-1β and tumor necrosis factor-α productions via the inbibition of IκB degradation in human lung cells, *Pharmacol. Res.*, 2007; **56**: 193-201.

[10] Liu J.C., Chan P., Chen Y.J., Tomlinson B., Hong S.F. and Cheng J.T., The antihypertensive effect of the berberine derivative 6-protoberberine in spontaneously hypertensive rats, *Pharmacology*, 1999; **59**: 283-289.

[11] Zhang H., Wei J., Xue R., Wu J.D., Zhao W., Wang Z.Z., Wang S.K., Zhou Z.X., Song D.Q., Wang Y.M., Pan H.N., Kong W.J. and Jiang J.D., Berberine lowers blood glucose in type 2 diabetes mellitus patients through increasing insulin receptor expression, *Metabolism*, 2010; **59**: 285-292.

[12] Zhao W., Xue R., Zhou Z.X., Kong W.J. and Jiang J.D., Reduction of blood lipid by berberine in hyperlipidemic patients with chronic hepatitis or liver cirrhosis, *Biomed. Pharmacother.*, 2008; **62**: 730-731.

[13] Letašiová S., Jantová S., Čipák L., and Múčková M., Berberine-antipoliferative activity in vitro and induction of apoptosis/necrosis of the U937 and B16 cells, *Cancer Lett.*, 2006; **239**: 254-262.

[14] Tungpradit R., Sinchaikul S., Phutrakul S., Wongkham W. and Chen S.T., Anti-Cancer Compound Screening and Isolation: *Coscinium fenestratum*, *Tinospora crispa* and *Tinospora cordifolia*, *Chiang Mai J. Sci.*, 2010; **37(3)**: 476-488.

[15] Kuo C.L., Chou C.C., and Yung B.Kuo C.L., Chou C.C. and Yung B.Y.M., Berberine complexes with DNA in the berberine-induced apoptosis in human leukemic HL-60 cells, *Cancer Lett.*, 1995; **93**: 193-200.

[16] Lin C.C., Lin S.Y., Chung J.G., Lin J.P., Chen G.W. and Kao S.T., Down-regulation of cyclin B1 and up-regulation of weeI by berberine promotes entry of leukemia cells into the G2/M-phase of the cell cycle, *Anticancer res.*, 2006; **26**: 1097-1104.

[17] Lin J.P., Yang J.S., Lee J.H., Hsieh W.T. and Chung J.G., Berberine induces cell cycle arrest and apoptosis in human gastric carcinoma SNU-5 cell line, *World J. Gastroenterol*, 2006; **12**: 21-28.

[18] Yang J., Liu X., Bhalla K., Kim C.N., Ibrado A.M., Cai J., Peng T.I., Jones D.P. and Wang X., Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked, *Science*, 1997; **275**: 1129-1132.

[19] Launay S., Hermine O., Fontenay M., Kroemer G., Solary E. and Garrido C., Vital functions for lethal caspases, *Oncogene*, 2005; **24**: 5137-5148**.**

[20] Fan T.J., Han L.H., Cong R.S. and Liang J., Caspase family proteases and apoptosis, *Acta Biochimica et Biophysica Sinica*, 2005; **37**: 719-727.

[21] Jantová S., Čipák L., Čerňáková M., and Košťáloválová D., Effect of berberine on proliferation, cell cycle and apoptosis in HeLa and L1210 cells, *J. Pharmacy and Pharmaco.*, 2003; **55**: 1143-1149.

[22] King K.L. and Cidlowski J.A., Cell cycle regulation and apoptosis, *Annu. Rev. Physiol.*, 1998; **60**: 601-617.