Chiang Mai J. Sci. 2010; 37(3) : 476-488
www.science.cmu.ac.th/journal-science/josci.html
Contributed Paper

Anti-Cancer Compound Screening and Isolation: *Coscinium fenestratum*, *Tinospora crispa* and *Tinospora cordifolia*

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Received: 8 October 2009
Accepted: 24 January 2010

ABSTRACT

The isolations of active anti-cancer compounds from *Coscinium fenestratum, Tinospora crispa* and *Tinospora cordifolia* are rarely reported. In this study, the isolation of anti-cancer compound from these herbs was performed. Cytotoxic activity of the water and methanol extracts of these herbs were evaluated. The methanol extract of *C. fenestratum* showed the most cytotoxic activity, and contained a major compound of berberine with 3.68%. Cytotoxic screening of berberine against ten cancer cell lines and one normal cell (PMBC) showed the most cytotoxic activity against HL-60 leukemia cells with an IC_{50} of 1.41 ± 0.7 μg/mL. The selective index (SI) value of berberine against HL-60 cells and PBMC cells were 0.142, indicating the selective cytotoxicity to cancer cells. Interestingly, berberine uptake by cancer cell correlated with cytotoxic activity. In addition, the apoptosis of HL-60 cells against berberine were detected by DAPI staining, and quantitated by Annexin V-FITC staining. Berberine, the effective compound from *C. fenestratum*, appears to possess anticancer potential.

Keywords: *coscinium fenestratum*, *tinospora crispa*, *tinospora cordifolia*, cancer, cytotoxicity.

1. INTRODUCTION

C. fenestratum, *T. crispa* and *T. cordifolia* are herbaceous vines of the family *Menispermaceae* and are mostly used in Thai traditional medicine to treat a wide variety of ailments [1]. These herbs have extensive pharmacological activities. *C. fenestratum* exhibits antioxidant [2,3], antiplasmodial [2], antibacterial [4], antidiabetic [2,3,5], antiproliferative [6,7], hypotensive [2] and neurotoxic activities [1,8]. *T. crispa* exhibits antihyperglycaemic [9], antiproliferative [7] and antimalarial [10,11] activities and also has a mild cardiotonic effect [12]. *T. cordifolia* has immunomodulating [13,14], antidiabetic [15] and antineoplastic [16,17] activities. It can also reduce the metastatic potential of melanoma cell in mice [18], decrease symptoms of allergic rhinitis [19] and protect mice against

lipopolysaccharide induced endotoxic shock [20,21]. Even though several pharmacological studies of *C. fenestratum, T. crispa* and *T. cordifolia* have been widely investigated, there are a few prosperities in effort to isolate anti-cancer compounds by using crude extracts from them. Moreover, the cytotoxic activities of these herbs against cancer cells are not completely elucidated. In this study, we aimed to evaluate the anti-cancer effect of water and methanol extracts of these Menispermaceae herbs (*C. fenestratum*, *T. crispa* and *T. cordifolia*) on cancer cells and selectively isolate the potent anti-cancer compound. Then the effect of active compound on cancer cell death or apoptosis was examined.

2. MATERIALS AND METHODS

2.1 Preparation of Herb Extracts

Dried stems of *C. fenestratum*, *T. crispa* and *T. cordifolia* were purchased from a local district in Chiang Mai, Thailand. Stem powders of these herbs were extracted with methanol and water. For methanol extraction, the powders (200 g) were soaked in 1.0 L of methanol with stirring for 24 h at room temperature. After centrifugation and filtration, the residue was extracted twice with methanol using the same method. The pooled supernatants of methanol extracts were evaporated to dryness under vacuum using a rotary evaporator. For water extraction, the powders (200 g) were boiled in 1.5 L of water at 100°C for 6 h. After centrifugation and filtration, the supernatants were lyophilized. Methanol and water extracts were subjected to test the cytotoxic activity prior to further isolation.

2.2 Isolation of Active Compound by Column Chromatography

Methanol extract (100 mg) was dissolved in 10 mL of methanol and loaded into an open column (1x20 cm) which contained C18 resins (Polygoprep 100-20 C_{18}, Macherey-Nagel) as a stationary phase. The elution began with water, water/acetonitrile (7:3), acetonitrile and methanol, respectively. The eluted samples were collected in four fractions, dried under evaporator, and then the cytotoxic activity was tested. The sample with high cytotoxic activity was re-dissolved in a small volume of methanol and underwent column chromatography on a RP-HPLC by using C18 preparative column (10x250 mm, 5 µm spherical, VYDAC). Two mobile phases, A (5% acetonitrile, 0.1% trifluoroacetic acid) and B (95% acetonitrile, 0.1% trifluoro-acetic acid), were filtered through a 0.22 mm Millipore filter and degassed before use. 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. The major peak of eluted sample was collected. For determination of active compound's purity, the C18 analytical column (4.6x250 mm, 5 µm spherical, ASTEC) was used. The RP-HPLC condition used was the gradient elution profile of 0-30-40 min/0-100-100%B at a flow rate of 1 mL/min. The eluent was also monitored by measuring UV absorption at 214 nm.

2.3 Mass Spectrometry and NMR Spectroscopy

The active compound was analyzed by a high resolution ESI-TOF mass spectrometer (BioTOF III; Bruker Daltonics, Inc., Billerica, MA, USA). NMR spectra (H^1 and C^{13} NMR) of this compound in MeOD were recorded on the Bruker Avance 500 spectrometer at 500 MHz, with standard pulse sequences provided by Bruker.

2.4 Cancer Cell Culture

For cancer cell culture, twelve cancer cell lines were cultured. Acute promyelocytic leukemia (HL-60), non-small cell lung

adenocarcinoma (NCI-H838) and large cell lung carcinoma (NCI-H661) cell lines were cultured in RPMI medium. Nasopharyngeal carcinoma (NPC-TW01), breast adenocarcinoma (MCF-7) and gastric carcinoma (MKN-45) cell lines were cultured in DMEM medium. Uterine sarcoma (MEA-SA) and colon carcinoma (HCT-116) cell lines were cultured in McCoy's 5A medium. Classic small cell lung carcinoma (NCI-H1876) cell line was cultured in DMEM/Ham's F12 (1:1) medium. Renal carcinoma (A498) and hepatocellular carcinoma (HepG2) cell lines were cultured in MEM medium. Squamous cell lung carcinoma (SW900) cell line was cultured in Leibovitz's L-15 medium. All cultured media were 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. Cell cultures were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air.

2.5 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

In this study, PBMCs were used as normal cells compared with cancer cells. PBMCs were isolated from healthy volunteers by the density gradient centrifugation method using Ficoll–Paque™ PLUS (GE Healthcare). In brief, the blood samples with an equal volume of Ficoll–Paque™ PLUS were added into the centrifuge tube. After the PBMC layer was harvested by centrifugation at 1,900 rpm for 30 min, cells were washed twice with PBS buffer. Cells were seeded at 10^6 cells/mL in RPMI medium and cultured at 37°C in an atmosphere of 5% CO_2 and 95% air for 1 day prior to cell treatment.

2.6 Cytotoxic Screening of Active Compound using WST and MTT Assays

Inhibition of cell proliferation was measured to evaluate the cytotoxic effect of each sample on cancer and normal cells. In order to determine the cytotoxicities of all samples, cells ($5x10^4$ cells/well) were treated with tested samples at various concentrations of 5, 10, 50, 100, 200, 300, 400 and 500 μg/mL for crude extracts, and 1, 5, 10, 50 and 100 μg/mL for isolated fraction or pure compound, for 24 h. DMSO (0.25%) was used for dissolving samples and as a control in all treated times. Determination the cytotoxic activity by WST or MTT assay was depended on cell type, adherent or suspension cell. Cell proliferation of suspension cell (HL-60 and PBMC) was measured using WST-8 assay. In a few words, 10 μL WST-8 solution was added to each well. After incubation for 4 h at 37°C, the cell viability was measured at wavelengths of 450 nm and 630 nm with microtiter plate reader (MRX II, DYNEX). Cell proliferation of adherent cell (NCI-H838, NPC-TW01, NCI-H1876, NCI-H661, MES-SA, SW900, MKN-45, HCT-116 and A498) was measured using MTT assay. In short, the medium was removed and replaced with 100 μL fresh medium containing 10% MTT solution (5 mg/mL). After incubation for 4 h at 37°C, the solution was aspirated and 100 μL DMSO was added to dissolve the dye precipitated cells. The absorbance was measured at wavelengths of 570 nm and 630 nm. The percentage of viable cells at different concentrations of samples was plotted relative to control values, and the IC_{50} values were computed. The IC_{50} value was defined as the concentration required that inhibited cell growth by 50% relative to untreated cancer growth. The selectivity index (SI) was the ratio of IC_{50} of cancer cell to the IC_{50} of normal cell. Each experiment was performed in triplicate and repeated at least three times. Results were expressed as the mean ± SD.

2.7 Berberine Uptake by Cell

Cancer cells were seeded in 6 cm dish ($1x10^6$ cells/dish) for 24 h. Fresh medium with 50 μM berberine was replaced and incubated for 15 min. Cells were washed with cold PBS (3 times) and collected by trypsinization. The cell pellet was washed with cold PBS and lyophilized. Berberine form cell pellet was extracted by 100 μL methanol and determined absorbance at 340 nm by spectrophotometer (NanoDrop, ND-1000). Berberine concentration was calculated by compare with berberine standard curve.

2.8 Detection of Apoptosis by DAPI staining

HL-60 cells were seeded at $2x10^6$ cells/mL in 6-well plate (5 mL/well), and incubated at 37°C for 24 h. The cells were treated with 5, 10 and 20 μM berberine and 0.25% DMSO (control) for 24 h. After centrifugation, the cells were washed with PBS buffer and re-suspended in PBS containing 1 μg/mL DAPI solution. After incubation at room temperature for 15 min in dark condition, cells were washed twice with PBS then the suspension (5 μL) was placed on a glass slide and covered with a cover slip. The morphology of the cells' nuclei was observed using a fluorescence microscope (Olympus BX51 Series).

2.9 Quantification of Apoptosis by Flow Cytometry

The Annexin V-FITC apoptosis detection kit was used to detect apoptosis by flow cytometry. To be brief, HL-60 cells were seeded at $2x10^4$ cells/mL in a 6 well plate (5 mL/well), and incubated at 37°C for 24 h. The cells were treated with 5, 10 and 20 μM berberine for 24, 48 and 72 h. After centrifugation, the cells were washed with cold PBS buffer and re-suspended in 1X binding buffer at a concentration of $1x10^6$ cells/mL. The suspensions (100 μL) were transferred to a flow cytometry tube then added with 5 μL of Annexin V-FITC and 5 μL of PI, respectively. After incubation at room temperature for 15 min in dark condition, the suspensions were added with 400 μL of PBS buffer and analyzed using BD FACScan and Cell Quest software.

3. RESULTS AND DISCUSSIONS

3.1 Herb Extraction and Preliminary Cytotoxic Screening

Three herbs of *C. fenestratum*, *T. crispa* and *T. cordifolia* were extracted with water and methanol from 200 g of dried stem powders. The water extracts were prepared by boiling and the supernatants were then freeze-dried to yield 25.64 g (12.82%), 18.84 g (9.42%) and 21.56 g (10.78%), respectively (Table 1).

Table 1. Extraction of three Menispermaceae herbs with water and methanol and their cytotoxic activities against cancer cells at 24 h.

	IC_{50} (μg/mL)					
Subject	***C. fenestratum***		***T. crispa***		***T. cordifolia***	
	water	**methanol**	**water**	**methanol**	**water**	**methanol**
1. Yield (%)	12.82	13.80	9.42	5.40	10.78	11.76
2. Cancer cells						
- HL-60	384±13.5	120±10.0	>500	>500	>500	>500
- HepG2	>500	>500	>500	>500	>500	>500
- MCF-7	>500	>500	>500	>500	>500	>500

IC_{50} > 500 μg/mL is undetectable for excess concentrations used.

Meanwhile, the methanol extracts were prepared by thrice extraction with methanol and the supernatants were then evaporated to yield 27.60 g (13.80%), 10.80 g (5.40%) and 23.52 g (11.76%), respectively. The effect of crude extracts on growth inhibition of HL-60, HepG2 and MCF-7 cancer cells was determined by WST or MTT assay. Only the HL-60 cell growth was inhibited by *C. fenestratum* water and methanol extracts with IC_{50} values of 384±13.5 μg/mL and 120±10.0 μg/mL, respectively (Table 1). In contrast, *T. crispa* and *T. cordifolia* extracts had no significant cytotoxicity to these three cancer cells with the IC_{50}>500 μg/mL, which surplused all tested concentrations. From IC_{50} values, *C. fenestratum* methanol extract was more effective than the water extract along 72 h of treatment time. This result was similar to other reports, in which the methanol extract of *C. fenestratum* showed higher antiproliferative activities against lung carcinoma cells, murine lewis lung carcinoma (LLC) cells, A549 human lung adenocarcinoma cells and L929 mouse fibrosarcoma cells than that of water extract [6,8]. Therefore, the *C. fenestratum* methanol extract was further used to isolate the active compound.

3.2 Isolation of Active Compound from *C. fenestratum* Methanol Extract

The procedure for fractionation of the extract from *C. fenestratum* herb is shown in Figure 1. According to the methanol extract exhibited effective cytotoxic activity against HL-60 cells, the methanol extract was further used to isolate the active compound, and HL-60 cell line was chosen in the screening experiment for selecting the active fraction during the isolation processes. The methanol extract was first subjected to the C-18 open column chromatography and four fractions were collected with the elution solvents of water, water/acetonitrile (7:3), water/acetonitrile (1:1) and methanol, respectively. The yields of four fractions were 1.14%,

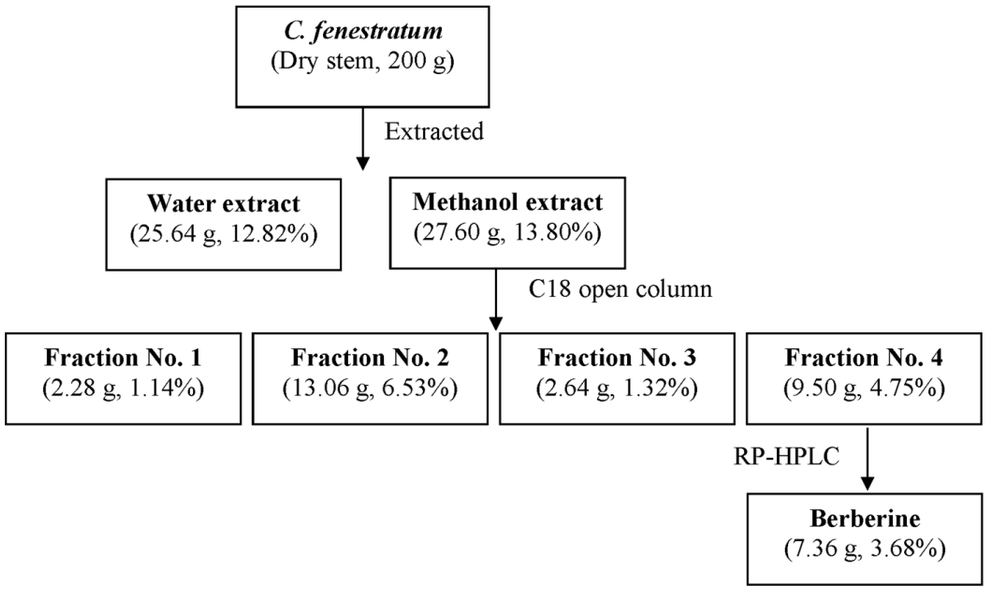

Figure 1. Procedure for fractionation of the extracts from *C. fenestratum* herb. Step and percentage yield (w/w) of cytotoxic active compound purification from *C. fenestratum*.

6.53%, 1.32% and 4.75%, respectively. After examining the cytotoxic activity of these fractions against HL-60 cells for 24, 48 and 72 h, fraction No. 4 showed the most effective cytotoxic activity at 72 h among the fractions examined and the IC_{50} from fraction No. 4 was 4.4±0.7 μg/mL (Table 2). The cytotoxic activity was proportional to the time of treatment. To isolate of active compound in fraction No.4, fraction No.4 was further purified by RP-HPLC using semi-preparative C18 column (Figure 2). HPLC chromatogram of fraction No.4 showed two major peaks, but only the second peak with a higher peak area showed the cytotoxic activity. To obtain the high purity of active compound and

Table 2. Cytotoxic activity of *C. fenestratum* crude extracts and 4 fractions isolated by C18 open column chromatography from methanol extract against HL-60 cells.

Samples	IC_{50} (μg/mL) ± SD		
	24 h	48 h	72 h
Water	384 ± 13.5	34.6 ± 1.5	9.8 ± 1.7
Methanol	120 ± 10.0	6.3 ± 0.4	5.3 ± 0.4
Fraction No. 1	>100	>100	>100
Fraction No. 2	>100	>100	>100
Fraction No. 3	47.0 ± 3.0	27.4 ± 1.0	28.0 ± 0.8
Fraction No. 4	51.0 ± 5.0	26.5 ± 0.7	4.4 ± 0.7

IC_{50} > 100 μg/mL is undetectable for excess concentrations used.

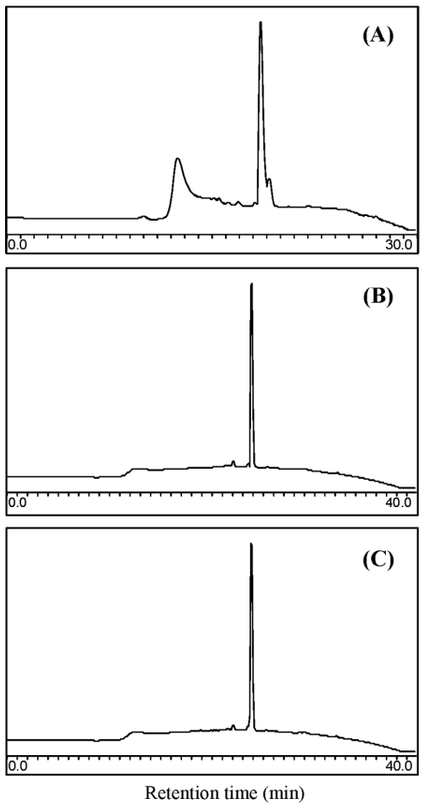

Figure 2. HPLC chromatograms of *C. fenestratum* methanol extract fractionated from fraction No.4 and the isolated major component from fraction No.4. Fraction No.4 fractionated from C-18 open column chromatography was analyzed by RP-HPLC using semi-preparative C-18 column (**A**) and the major component from this fraction was further analyzed by RP-HPLC using analytical C-18 column (**B**). The HPLC chromatogram of commercial available berberine chloride from Sigma was used for comparison (**C**).

determine its structure, this fraction was re-purified by RP-HPLC. The active compound was analyzed by using a high-resolution ESI-TOF mass spectrometer, and the monoisotopic mass of active compound was 336.1 [M^+: $C_{20}H_{18}NO_4^+$]. The structure of this compound was then analyzed by H^1 and C^{13} NMR. H^1 NMR (500 MHz, MeOD): δ 9.77 (1H, s), 8.69 (1H, s), 8.10 (1H, J = 9.1 Hz), 8.00 (1H, J = 9.1 Hz), 7.65 (1H, s), 6.96 (1H, s), 6.10 (2H, s), 4.94 (2H, J= 6.4 Hz), 4.21 (3H, s), 4.11 (3H, s), 3.31 (2H, J = 6.4 Hz). C^{13} NMR (500 MHz, MeOD): δ 152.3, 152.2, 150.1, 146.5, 145.9, 139.8, 135.3, 132.0, 128.2, 124.6, 123.5, 122.0, 121.6, 109.5, 106.7, 103.8, 62.7, 57.7, 57.3, 28.3. On the basis of the above data, this active compound was identified as berberine by direct comparison with an authentic sample using MS, NMR and RP-HPLC. The chemical structure of berberine is shown in Figure 3. Quantitative analysis of berberine content by RP-HPLC using C18 analytical column compared with commercial available authentic sample of berberine revealed the presence of 30.0% berberine in methanol extract and 96.9% berberine in isolated fraction No.4. So, the relative content of berberine in *C. fenestratum* stem derived from a part of methanol extraction was calculated to be 4.14%. From our results, three isolation steps of berberine from *C. fenestratum* methanol extract gave 3.68% yield, which was less than calculation only 0.46% but more than the yield of ethanol extracts by maceration extraction (3.37%), percolation extraction (3.08%) and Soxlet extraction (2,67%) [22]. Therefore, this isolation of berberine from *C. fenestratum* stem was not only a simple procedure, but also was able to provide higher percentage yield of berberine, compared to previous studies.

3.3 Cytotoxicity Evaluation and cell uptake of berberine

Although the cytotoxic effects of commercial berberine chloride toward many cancer and non-cancer cells have been reported that the cell sensitivity to berberine increased in order B16 < EAC < V79 < U937 < L1210 < NIH-3T3 < HeLa cells [23-25],

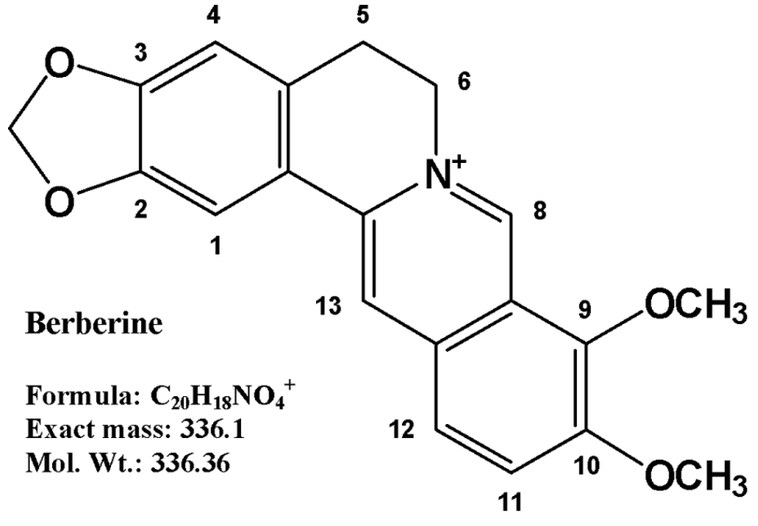

Figure 3. Chemical structure of berberine isolated from *C. fenestratum* methanol extract.

other cancer types have not yet been elucidated. In this study, the cytotoxic activity of isolated berberine was examined in 10 cancer cell lines (HL-60, NCI-H838, NCI-H1876, NCI-H661, SW900, NPC-TW01, MES-SA, MKN-45, HCT-116 and A498) and 1 normal cells (PBMC). The cytotoxic activity expressed in terms of relative absorbance of drug-treated cells, in comparison to control cells, and IC_{50} values (for 72 h) of berberine are presented in Table 3. The cancer cell sensitivity to berberine increased in order HL-60 < NCI-H838, NPC-TW01 < NCI-H1876 < NCI-H661 < MES-SA < SW900 < MKN-45 < HCT-116 < A498 cells. IC_{50} value of normal cells, PBMC, was higher than 7 cancer cell lines, equal with MKN-45 cells but lower than in HCT-116 and A498 cells. Therefore, berberine has selective toxicity to cancer and normal cells. Berberine was more effective to inhibit cell growth of HL-60 and lung cancer cells (NCI-H838, NCI-H1876, NCI-H661 and SW900) than other cancer cells. On the other hand, the selective index (SI) value of berberine was relatively lower in HL-60 cells than in other cancer cells, with same IC_{50} values. This indicated that berberine has selective toxicity to HL-60 cells and less toxic to normal cells, compared with other cancer cells. Because berberine shows height absorbance at 340 nm, so berberine uptake by cells were determined by measuring A_{340} of berberine extracted from cells and comparison with berberine standard curve. The results are shown in Table 1. The IC_{50} value of the cell uptake with more berberine

Table 3. Cytotoxic activity and drug uptake of berberine against 10 cancer and normal cells.

Cells	Cell type	IC_{50} (μg/mL) ± SD[a]	SI[b]	Berberine uptake (μM)[c]
PBMC	Peripheral blood mononuclear cell[d]	9.93 ± 5.4	-	nd
HL-60	Acute promyelocytic leukemia	1.41 ± 0.7	0.142	9.476±1.053
NCI-H838	Non-small cell lung adenocarcinoma	1.86 ± 1.3	0.187	9.284±1.270
NPC-TW01	Nasopharyngeal carcinoma	1.86 ± 0.1	0.187	9.196±0.703
NCI-H1876	Classic small cell lung carcinoma	2.16 ± 1.8	0.217	5.507±0.522
NCI-H661	Large cell lung carcinoma	2.49 ± 1.0	0.251	5.132±0.492
MES-SA	Uterine sarcoma	4.72 ± 0.2	0.476	3.202±0.548
SW900	Squamous cell lung carcinoma	5.47 ± 1.7	0.551	2.792±0.230
MKN-45	Gastric carcinoma	9.78 ± 0.5	0.985	2.851±0.411
HCT-116	Colon carcinoma	11.15 ± 3.6	1.124	2.734±0.438
A498	Renal carcinoma	32.35 ± 5.8	3.258	nd

[a] Cells were treated with berberine for 72 h.
[b] SI; The selectivity index is the ratio of IC_{50} of cancer cell to the IC_{50} of normal cell.
[c] Cells were treated with berberine for 15 min.
[d] PBMC(s) was obtained from normal healthy volunteer and used to compared with several cancer cells.
nd; not determine

was lower than the cell uptake with less berberine. Indicating that, cytotoxic activity of berberine was correlated with drug uptake.

3.4 Determination of Apoptosis in HL-60 Cells

After cell treatment with 5, 10 and 20 μM berberine for 24 h, the morphological change in the nuclei of HL-60 apoptotic was visualized by fluorescence microscopy using DAPI staining (Figure 4). Nuclei are considered to have the normal phenotype when glowing bright and homogenously. While apoptotic nuclei can be identified by the condensed chromatin gathering at the periphery of the nuclear membrane or a total fragmented morphology of nuclear bodies. The characteristic condensed nuclei of apoptotic cells were distinctly visible at 10 and 20 μM berberine. The quantitative analysis of HL-60 apoptotic cells was also examined by flow cytometry using Annexin V and PI. Early apoptosis of HL-60 cells could be induced with 10 and 20 μM berberine, but 5 μM berberine could not induce apoptosis when compared to control at any time period (Figure 5). Moreover, 20 μM berberine could induce late apoptosis at 72 h. This result was similar to the study of Kuo et al., in which berberine chloride (25 μg/mL, ~14.87 μM) could induce morphological changes and internucleosomal DNA fragmentation, characteristic of apoptosis in HL-60 cells [26].

4. CONCLUSIONS

In this study, the anti-cancer activity of water and methanol extracts from *C. fenestratum*, *T. crispa* and *T. cordifolia* stems were determined by the cell-based screening assay, and to selectively isolate the anti-cancer compound from the active extract. Results showed methanol extract of *C. fenestratum* as having more potent cytotoxic activity against HL-60 cells than that of *T. crispa* and *T. cordifolia*, even compared with water extracts and other cancer cell lines. Berberine is the major active compound from *C. fenestratum* methanol extract and was simply isolated by C-18 chromatography coupled with cell-based screening assay. The cytotoxic evaluation of berberine against cancer and normal cells showed the most selective toxicity in HL-60 cells, as compared to normal cells and other cancer cells. It also exhibited the ability to induce apoptosis in HL-60 cells. Although berberine is the major active compound that was isolated from *C. fenestratum* methanol extract, there are many components that are specific to a variety of cancer cells and still require further identification and study of their biological activities. Therefore, the cell-based screening assay is useful for evaluation of anti-cancer activity in many medicinal herbs and also selective isolation of other anti-cancer compounds.

ACKNOWLEDGEMENTS

This work is supported by the Royal Golden Jubilee Ph.D. Program (Grant No. PHD0050/2547) under The Thailand Research Fund (TRF; Bangkok, Thailand), the Graduate School, Chiang Mai University (Chiang Mai, Thailand), and Academia Sinica (Taipei, Taiwan).

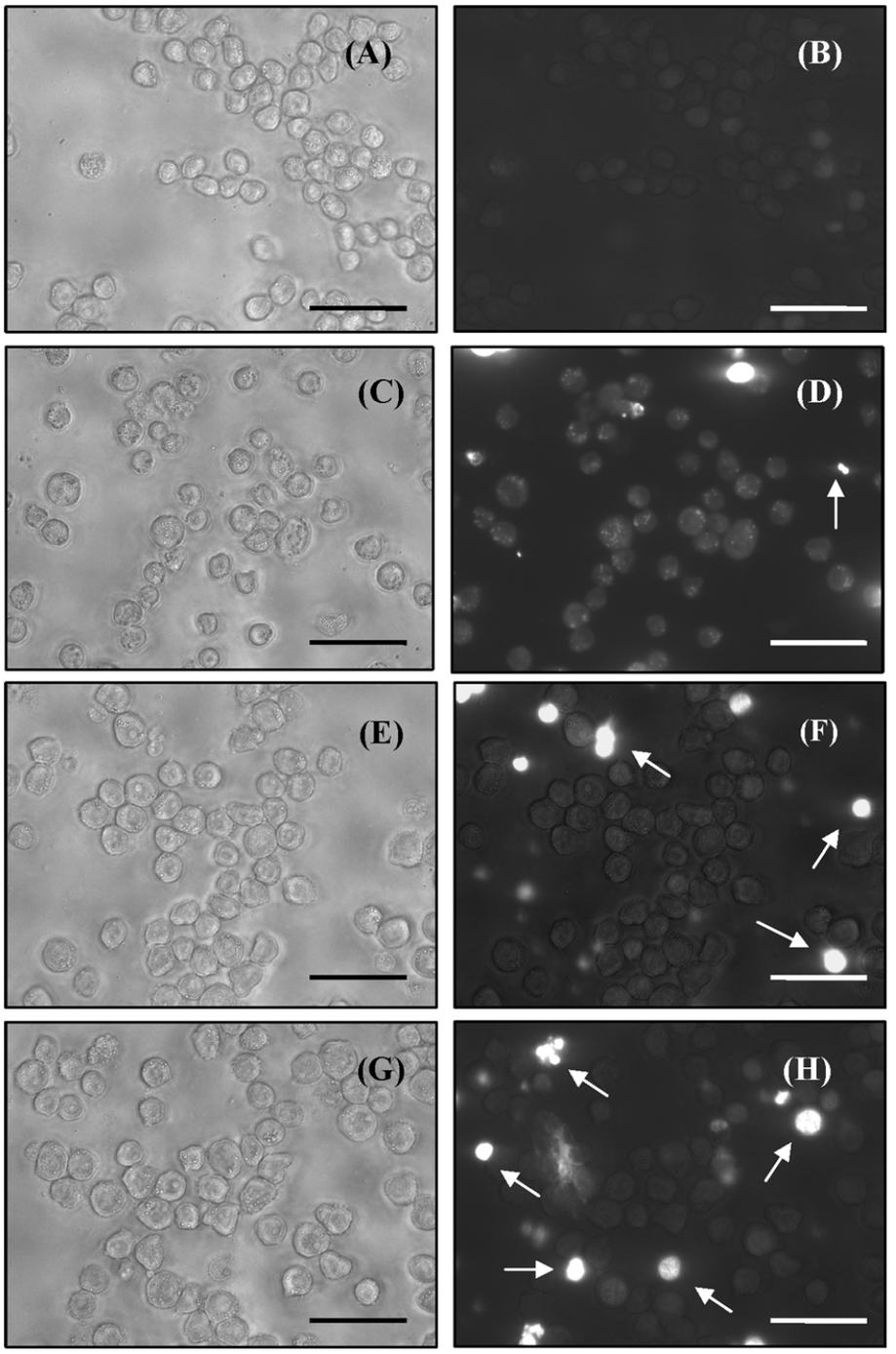

Figure 4. Fluorescent microscopic analysis of berberine-induced apoptotic HL-60 cells stained with DAPI. Cells were incubated without (**A**,**B**) or with 5 (**C**,**D**), 10 (**E**, **F**) and 20 (**G**, **H**) μM berberine for 24 h before stained with DAPI and observed with under white light and fluorescence. White arrows represent the apoptotic DAPI stained nucleus (scale bar = 50μm).

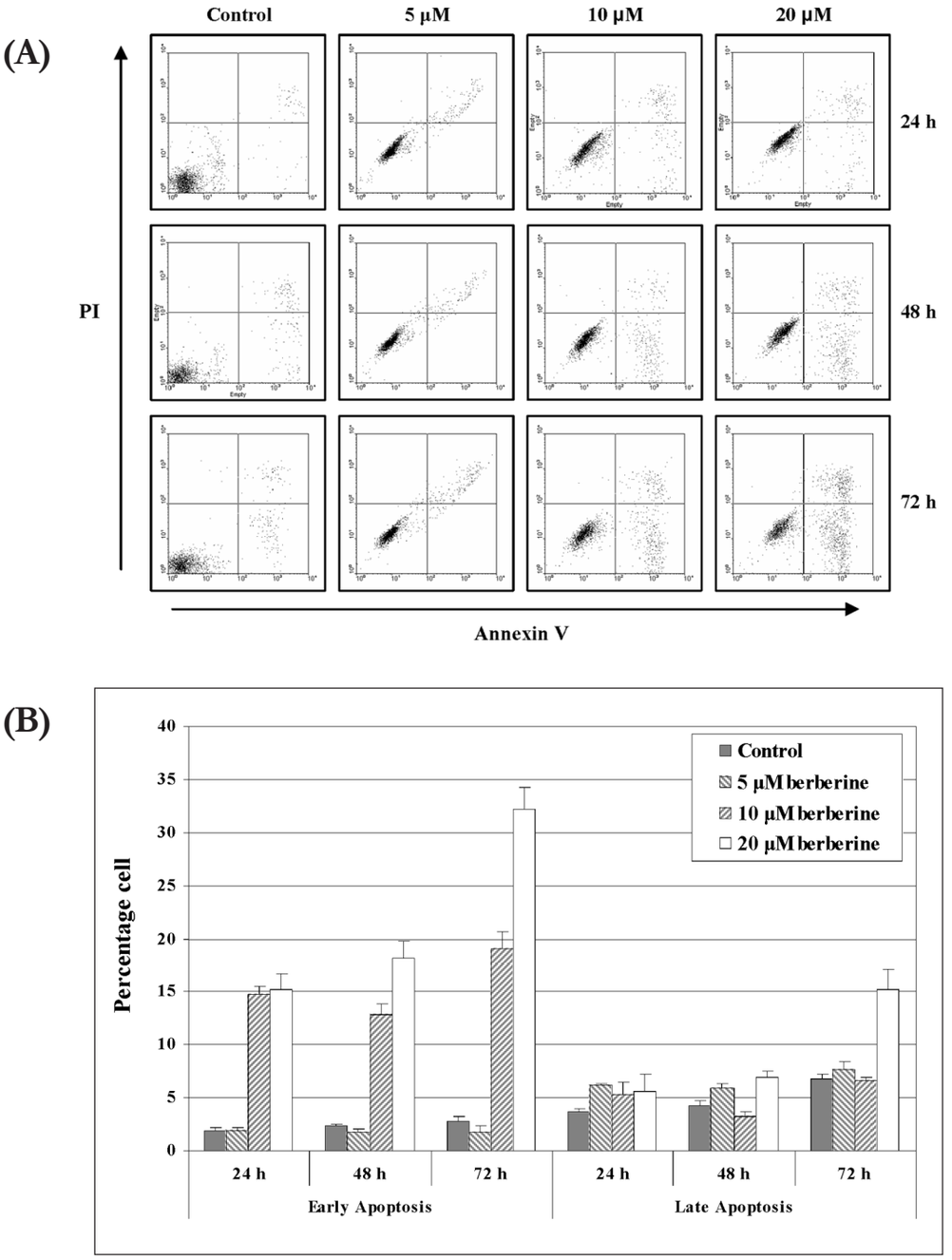

Figure 5. Annexin V-FITC staining of the apoptosis in HL-60 cells against berberine. Apoptosis of HL-60 cells was induced by berberine (5, 10 and 20 µM) for 24, 48 and 72 h. Cells were stained with Annexin V-FITC then examined by flow cytometry analysis (**A**), and percentage cell in early and late apoptosis were calculated in histogram (**B**).

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