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In Vitro and In Vivo Apoptosis-Inducing Antileukemic Effects of *Mucuna macrocarpa* Stem Extract on HL-60 Human Leukemia Cells

Kuan-Hung Lu, MS[1], Yuh-Fang Chang, BS[1], Pen-Hui Yin, PhD[2], Ting-Ting Chen, MS[3], Yu-Ling Ho, PhD[4], Yuan-Shiun Chang, PhD[1], and Chin-Wen Chi, PhD[2,5]

Abstract

Mucuna macrocarpa Wallich (Leguminosae) is believed to hold blood circulation activating effects, and has been used as a folk remedy in Southeast Asia for the treatment of various hematologic and circulatory-related ailments. The objective of this study was to investigate whether crude methanolic extract of *M macrocarpa* (CMEMM) possessed antileukemic effects on HL-60, human leukemia cells. CMEMM was prepared from dried stems of this plant, and its apoptosis-inducing effects were investigated using HL-60 cells in vitro and in vivo. With treatment of 25 to 75 μg/mL CMEMM, the in vitro antiproliferative effect on HL-60 cells increased in a dose- and time-dependent manner during the 72-hour treatment period. The concentration of CMEMM that exhibited a 50% growth inhibition (IC_{50}) for 72-hour exposure was 36.4 μg/mL. Apoptosis triggered by CMEMM in HL-60 cells was confirmed by the following observations: (*a*) characteristic apoptotic nuclear fragmentation, (*b*) dose-dependent accumulation of sub-G_1 phase in cell cycle analyses, (*c*) increased percentages of annexin V-positive apoptotic cells, and (*d*) dose-dependent elevation of active caspase-3. Furthermore, an in vivo tumor growth suppression effect by CMEMM (500 mg/kg/d intraperitoneally) was observed in mouse xenografts. The results suggest that CMEMM exerts antileukemic effects via an apoptotic pathway in HL-60 cells, and could be a candidate for developing antileukemic agents in the future.

Keywords

Mucuna macrocarpa, antileukemia, apoptosis, HL-60, xenograft, caspase-3

Introduction

Mucuna macrocarpa Wallich belongs to the Leguminosae family. It is a large woody climber found in forests at altitudes of 100 to 1500 meters, and is distributed throughout Taiwan and Southeast Asia.[1] Dried stems of this plant have been used in folk medicine to activate blood circulation for various hematologic and circulatory-related ailments, such as anemia, hemoptysis, dysmenorrheal, pain in waist and knees, numbness of hands and feet.[2] Additionally, crude drug of this medicinal plant has been used as a remedy for the treatment of diabetes mellitus.[3] In phytochemical investigations, amino acids, lipids, and triterpenoids have been isolated.[4,5] Moreover, it has also been demonstrated to possess diuretic and spasmolytic activities in rat models;[6] however, there has been no studies on the anticancer effect of this plant.

Leukemia, a cancer of the blood or bone marrow, is clinically and pathologically subdivided into 2 different forms, myeloid and lymphoid. Acute lymphoblastic leukemia (ALL) more frequently occurs in children, whereas the risk of acute myeloid leukemia (AML) is higher in adults. Acute promyelocytic leukemia (APL) is a distinct subtype of AML characterized by a specific genetic alteration, affecting the retinoic acid receptor-α, and leading to a blockage in the differentiation of granulocytic cells.[7] APL is unique from other forms of AML in its striking response to anthracyclines and

[1]China Medical University, Taichung, Taiwan
[2]Taipei Veterans General Hospital, Taipei, Taiwan
[3]National Taiwan Sport University, Taoyuan, Taiwan
[4]Huangkuang University, Taichung, Taiwan
[5]National Yang-Ming University, Taipei, Taiwan

Corresponding Author:
Chin-Wen Chi, Department of Medical Research and Education, Taipei Veterans General Hospital, No. 201, Sec. 2, Shih-Pai Road, Taipei 112, Taiwan
Email: cwchi@vghtpe.gov.tw

differentiating agents such as all-trans retinoic acid (ATRA) or arsenic trioxide; however, the relapse rate has been reported to be about 30% to 40%.[8] Despite significant progress in the treatment of these diseases, therapy is still unsuccessful in many patients. Prognosis is particularly poor in adult AML;[9] moreover, ATRA is known to have more frequent adverse effects in pediatric patients.[10] Therefore, developing new therapeutic drugs for refractory or recurrent leukemia is a worthwhile task.

Apoptosis, a programmed cell death, plays an important role in the development of various diseases, including cancer.[11] In recent years, interest has been focused on the manipulation of apoptotic processes in the treatment and prevention of cancers.[12] Much effort has been directed toward the search for herbs that influence apoptosis as alternative cancer therapies because of their low toxicity and costs.[13,14] In addition, recent reports have demonstrated that extracts from herbal medicines exert antileukemic effects by inducing apoptosis in human leukemia cells.[15,16]

In the present study, the in vitro apoptosis-inducing ability of crude methanolic extract of *M macrocarpa* (CMEMM) was investigated on human leukemia cell line, HL-60. Antiproliferative activity was examined by trypan blue exclusion assay. CMEMM-induced apoptosis was determined by typical apoptotic morphologic changes, cell cycle analysis, annexin V binding assay, and caspase-3 activation analysis. Furthermore, the in vivo antitumor effect was examined using human leukemia xenograft model.

Materials and Methods

Reagents

RPMI-1640 medium, fetal bovine serum (FBS), gentamycin, trypan blue dye solution, and phosphate-buffered saline (PBS; pH 7.4) were purchased from Gibco (Grand Island, NY). Dimethyl sulfoxide (DMSO) and ethidium bromide were purchased from Sigma (St. Louis, MO). Acetic acid and methanol were purchased from Merck (Darmstadt, Germany). Cycle TEST PLUS DNA Reagent Kit was purchased from Becton Dickinson (San Jose, CA). ANNEX100F Kit (annexin V: FITC assay kit) was purchased from AbD Serotec (Kidlington, UK). Active Caspase-3 FITC Mab Apoptosis Kit was purchased from BD Pharmingen (San Diego, CA).

Plant Material and Extraction

Mucuna macrocarpa Wallich (Leguminosae) was collected in Nantou County, Taiwan in July 2006. The material was identified by Professor Yuan-Shiun Chang in the Graduate Institute of Chinese Pharmaceutical Sciences, China Medical University; a voucher specimen (MM 950729) has been deposited in the same institute. Crude methanolic extract of the stems of *M macrocarpa* (CMEMM, yield = 13.9% of dry weight) was prepared as described below. The stems were cut into small pieces, air-dried for 1 week, and extracted with methanol for 48 hours at room temperature 3 times. All extracts were mixed, and the mixture was filtered and concentrated using a rotary evaporator until dry.

Cell Culture and CMEMM Treatment

Human promyelocytic leukemia HL-60 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). HL-60 cells were cultured in RPMI-1640 medium containing 10% FBS and 0.01 mg/mL gentamycin and incubated in a humidified atmosphere of 5% CO_2 at 37°C. CMEMM was dissolved in DMSO and diluted in culture medium to obtain final concentrations of 25, 50, and 75 μg/mL. Cells (1×10^5 cells/mL) were seeded into 60-mm dishes and exposed to vehicle or various concentrations of CMEMM for 24 to 72 hours. The concentration of DMSO (0.1%) did not affect the cell viability. Cells were harvested and counted by hemocytometer at 24, 48, and 72 hours after treatment with CMEMM and stored for further examinations.

Cell Proliferation Assay

Cell growth was determined by trypan blue exclusion assay. Cells were collected at indicated times following CMEMM exposure. After centrifugation, cells were resuspended in culture medium and stained with 0.4% trypan blue solution. Viable cells were counted using a hemocytometer. The percentages of growth inhibition were calculated by comparing the cell numbers of CMEMM group with that of the control.

Cytological Examination

Ethidium bromide fluorescence staining method was used to observe apoptotic morphology of individual cells. Briefly, cells (1×10^6 cells/mL) were washed twice with PBS and collected on microscope slides by cytospin (Shandon Cytospin Cytocentrifuge). This was followed by fixation with 25% (v/v) acetic acid in methanol for 10 minutes and staining with ethidium bromide solution (20 μg/mL) at room temperature. Photographs were taken under an inverted fluorescence microscopy (Olympus IX-70). Fragmented nuclei were suggestive of apoptosis.

Cell Cycle Analysis

DNA staining was carried out using Cycle TEST PLUS DNA Reagent Kit. Briefly, cells (1×10^6 cells/mL) were

washed and stained for DNA content according to the kit protocol. Fluorescence intensity of propidium iodide (PI) was determined using a FACScan flow cytometer and analyzed by CellQuest software (Becton Dickinson, San Jose, CA).

Annexin V-FITC/PI Analysis

Annexin V-FITC/PI staining method was used to identify and quantify apoptotic cells. Cells (2×10^5 to 5×10^5 cells/mL) were treated according to the manufacturer's instruction of ANNEX100F Kit. FITC/PI fluorescence intensity was measured using a FACScan flow cytometer to differentiate between viable (annexin V-negative and PI-negative), early apoptotic (annexin V-positive, PI-negative), and necrotic cells (annexin V-positive and PI-positive). The extent of apoptosis was quantified according to the percentage of annexin V-positive cells.

Active Caspase-3 Analysis

The amount of active caspase-3, a marker for cells undergoing apoptosis, was measured using Active Caspase-3 FITC Mab Apoptosis Kit. The FITC-conjugated monoclonal rabbit anti-active caspase-3 antibody specifically recognizes the active form of caspase-3 in human or mouse cells. Briefly, cells (1×10^6 cells/0.5 mL) were permeabilized, fixed, and stained for active caspase-3 according to the Active Caspase-3 FITC Staining Protocol. The cells were then analyzed by flow cytometry to determine FITC fluorescence intensity.

Human Leukemia Xenograft Model

In vivo testing of antileukemic effect of CMEMM was performed in xenograft models and carried out with ethics committee approval (No. 97-148) from Taipei Veterans General Hospital. A total of 22 male athymic nude mice (BALB/c nude, with body weight of 20 to 24 grams and aged 5 weeks) were obtained from the National Laboratory Animal Center (Taipei, Taiwan) and kept in an autoclaved cage with polyester fiber filters to avoid contact with pathogens. The mice were housed under sterilized conditions with a constant temperature of 25 ± 2°C and 12-hour light:12-hour dark cycles. All animal diet and water were autoclaved before feeding to the mice ad libitum. The mice were acclimatized to the housing condition at least 1 week before the experiment. Both sides of lateral thighs of the nude mice were injected subcutaneously with 1×10^6 HL-60 cells. When palpable tumors (4 to 6 mm in diameter) arose 3 weeks after injection, the mice were randomly divided into 3 groups (7 to 8 mice per group). The experimental groups were treated with CMEMM extracts (100 or 500 mg/kg/d in autoclaved water), and the control was treated with autoclaved water by daily intraperitoneal (i.p.) injection. Tumor size and body weight were monitored daily throughout the experiment. Tumor volumes were measured by a digital caliper and calculated according to the following formula: tumor volume (mm^3) = $0.4 \times L \times W^2$, where L and W were the major and minor dimensions of the tumor, respectively.[17] After 21 days of treatment, the mice were euthanized and tumors were removed. The final body and tumor weights were recorded for the calculation of percentage ratio final tumor-to-body weight. Tumors, livers, kidneys, and spleens were collected, fixed, embedded, and stained with hematoxylin and eosin (H&E) for pathological analyses.

Phytochemical Analysis

Phytochemical components of CMEMM were analyzed by chromatographic methods. Briefly, CMEMM was suspended in distilled water and partitioned successively with different poloarities of organic solvents such as *n*-hexane, chloroform, ethyl acetate, and *n*-butanol. Chemical components were isolated from these partitioned fractions using column chromatography. By means of infrared (Nicolet Impact 400 FT-IR spectrophotometer), EI-MS (VG Platform II Mass Spectrometer), and ^{1}H NMR and ^{13}C NMR (Bruker DPX-200 FT-NMR), the structures of chemical components were identified on the basis of spectral evidence. The major isolated components from CMEMM were further determined by the application of HPLC (Waters 2695 separation module). HPLC chromatogram was carried out on XBridge RP-18 endcapped column (5 μm pore size, 250 × 4.6 mm, inner diameter) by an isocratic elution of 2% aqueous acetic acid and acetonitrile (63:37, v/v) solvent. The peaks were recorded with a photodiode array detector (Waters 996 PDA detector) at 254 nm, and the solvent flow rate was kept at 0.8 mL/min.

Statistical Analysis

To assess statistical significant differences between the control and CMEMM-treated groups, the data were analyzed by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test using the SPSS for Windows 10.0 version software. Differences were considered significant when $P < .05$.

Results

Antiproliferative Effect of CMEMM on HL-60 cells

As illustrated in Figure 1, CMEMM reduced the proliferation of HL-60 cells in a dose- and time-dependent manner. Significant growth inhibitions ($P < .05$) were observed for cells treated with 50 and 75 μg/mL of CMEMM. Similar response profiles were observed in the 48- and 72-hour

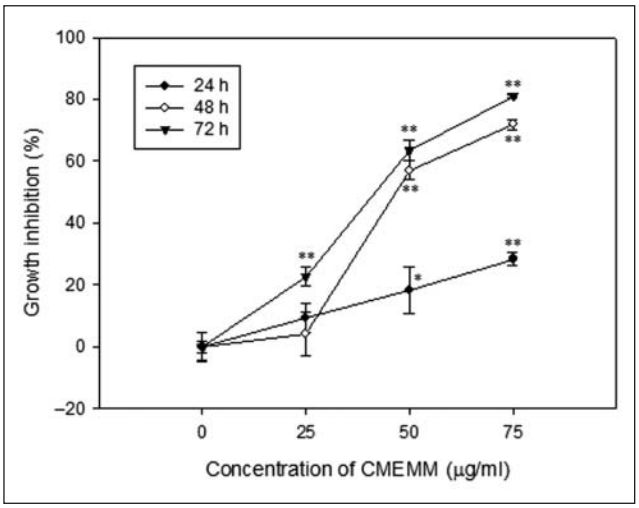

Figure 1. A ntiproliferative effects of crude methanolic extract of *Mucuna macrocarpa* (CMEMM) on HL-60 cells
Cells (1×10^5cells/mL) were seeded onto 60-mm dishes and exposed to dosages of CMEMM ranging from 25 to 75 μg/mL for up to 72 hours. Control cells were treated with 0.1% dimethyl sulfoxide (DMSO) in medium. The percentages of growth inhibition were measured by trypan blue exclusion assay and calculated by comparing the cell numbers with that of the controls. Each value represents the mean ± standard error (mean ± SE) of 6 replicate cultures from 3 independent experiments. *P < .05 and **P < .01 indicate significant difference from the control value.

treatment groups. Growth inhibitions were most obvious after 72 hours of treatment; the percentage of growth inhibition increased from 22.7% to 81.0% as the dosage of CMEMM increased from 25 to 75 μg/mL. Time response data demonstrated that 50% growth inhibition (IC_{50}) was observed at 36.4 μg/mL for 72 hours.

Nuclear Morphological Changes Induced by CMEMM

After 72 hours incubation with CMEMM, nuclear morphological alterations in HL-60 cells were determined by ethidium bromide staining. As shown in Figure 2, 25 μg/mL CMEMM-treated cells had similar nuclear morphology as that of the control. However, the nuclei of 50 and 75 μg/mL CMEMM-treated cells presented with condensed, fragmented chromatin and formation of apoptotic bodies, which were in clear contrast to the spherical and intact nuclei of the control.

Cell Cycle Progression in CMEMM-treated cells

After exposure to 25, 50, or 75 μg/mL of CMEMM for 24 to 72 hours, the cell cycle of HL-60 cells were analyzed using flow cytometry. As shown in Figure 3, CMEMM-treated HL-60 cells at sub-G_1 phase (or hypodiploid apoptotic cells) increased in a dose-dependent manner. After 72 hours of treatment, the percentage of 75 μg/mL CMEMM-treated

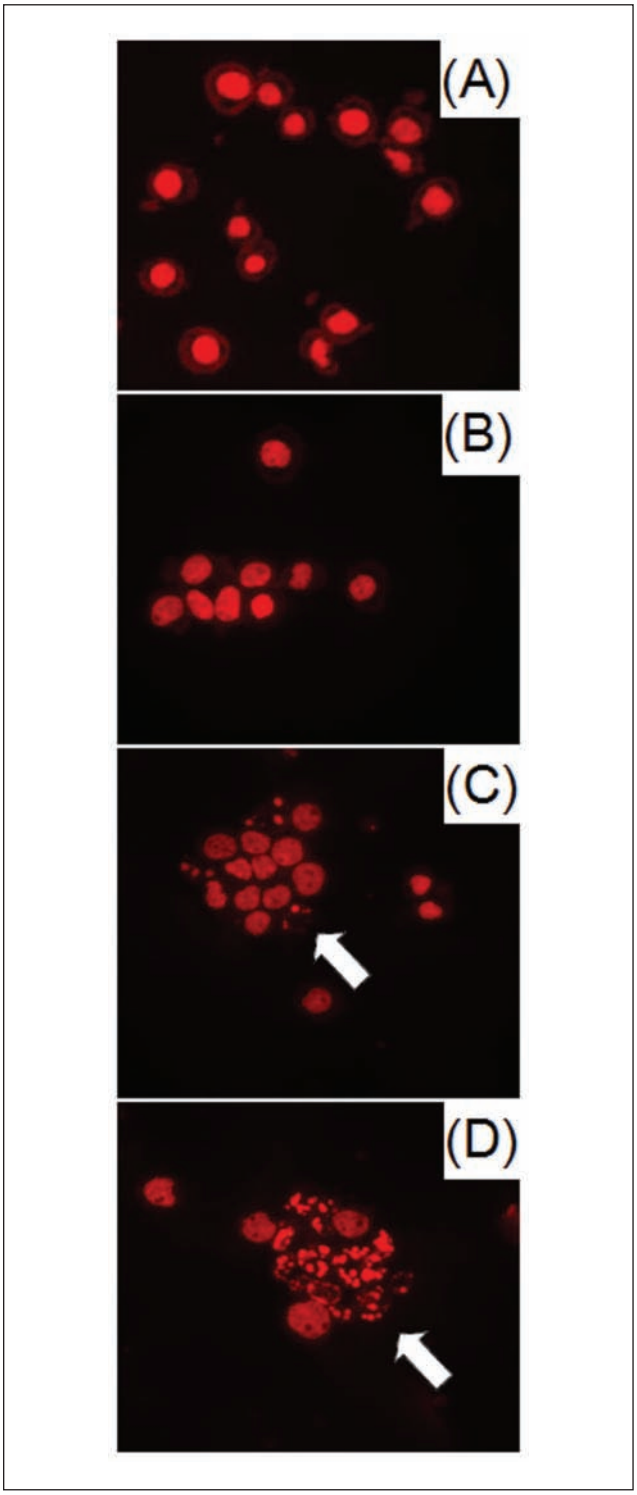

Figure 2. Nuclear morphological changes induced by crude methanolic extract of *Mucuna macrocarpa* (CMEMM)
Nuclear morphology of HL-60 cells treated with (A) 0.1% dimethyl sulfoxide (DMSO; control), (B) 25 μg/mL CMEMM, (C) 50 μg/mL CMEMM, and (D) 75 μg/mL CMEMM for 72 hours. The nuclei were stained with ethidium bromide. Arrows indicate apoptotic bodies of nuclear fragmentation. Magnification ×200.

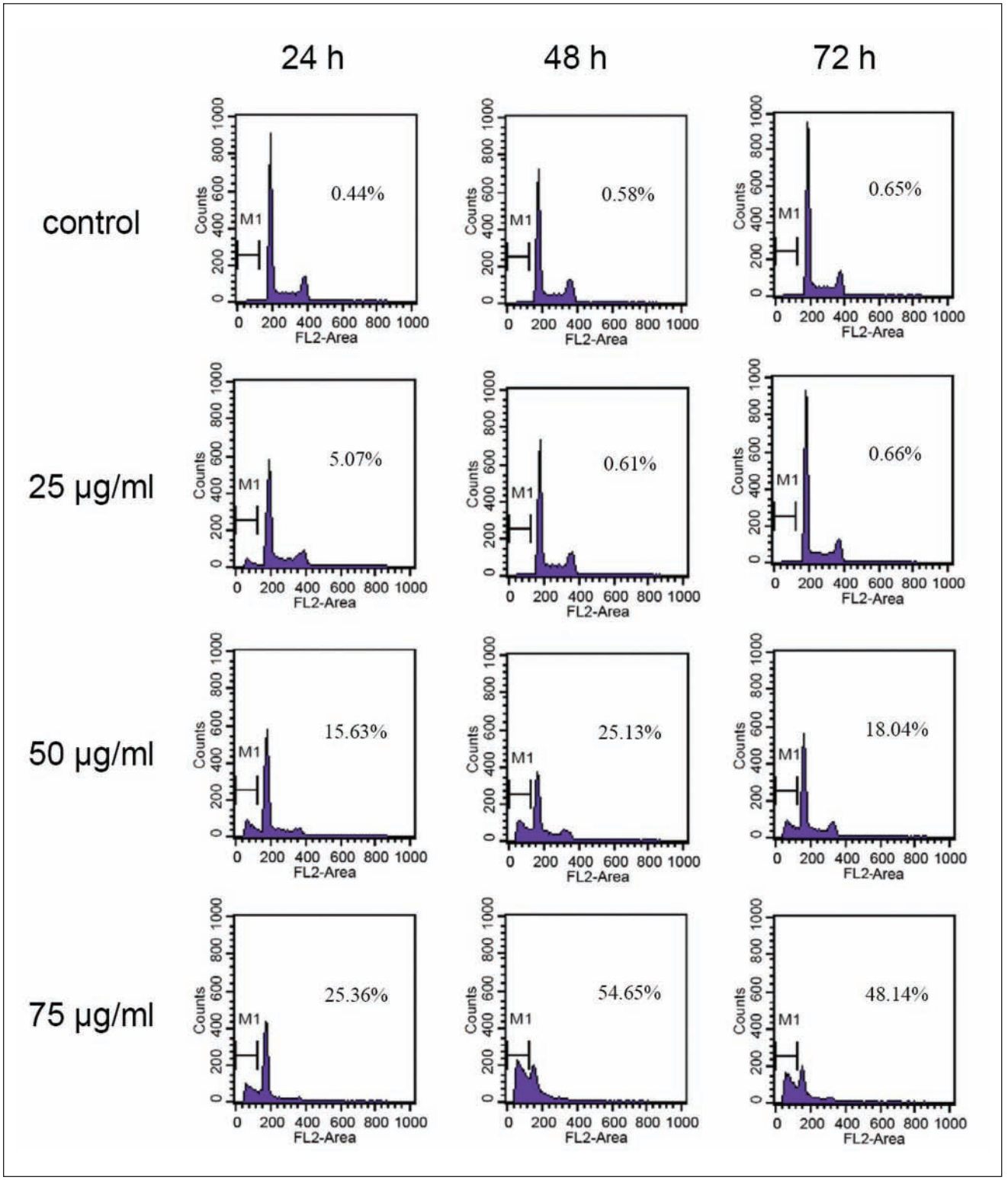

Figure 3. Cell cycle progression in crude methanolic extract of *Mucuna macrocarpa* (CMEMM)-treated cells
Cell cycle analyses of HL-60 cells treated with 0.1% dimethyl sulfoxide (DMSO; control), 25 µg/mL CMEMM, 50 µg/mL CMEMM, and 75 µg/mL CMEMM for 24 to 72 hours. After treatment with CMEMM, cells were collected and stained with propidium iodide, and analyzed for DNA content by flow cytometry. The percentages of sub-G1 or hypodiploid cells were analyzed by CellQuest software. The representative cell cycle progressions in CMEMM-treated or vehicle control cells were from 1 of 3 independent experiments.

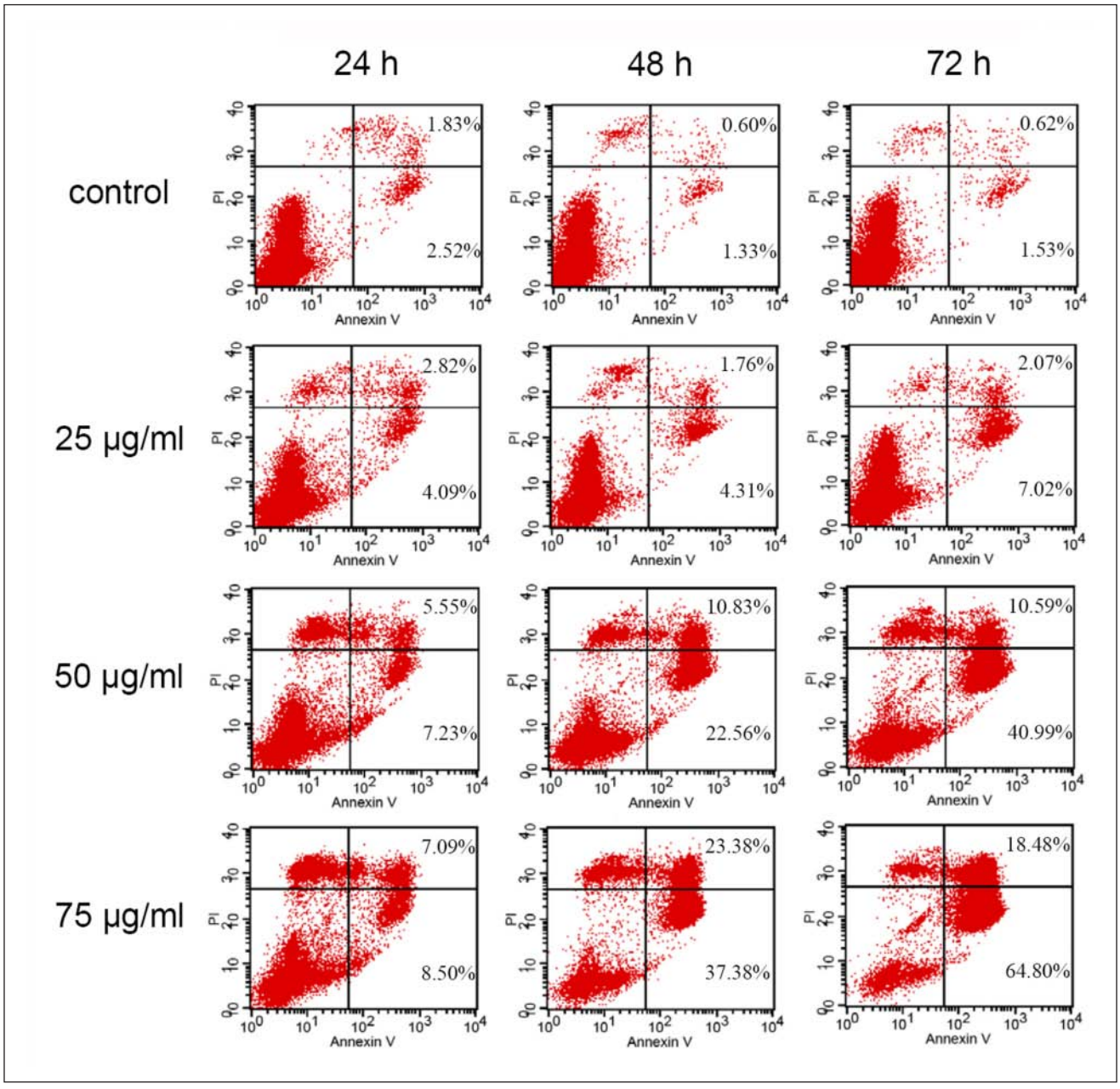

Figure 4. Annexin V-FITC/PI analyses of crude methanolic extract of *Mucuna macrocarpa* (CMEMM)-treated cells
HL-60 cells were treated with 0.1% dimethyl sulfoxide (DMSO; control), 25 µg/mL CMEMM, 50 µg/mL CMEMM, and 75 µg/mL CMEMM for 24 to 72 hours. Quantitative percentages of apoptotic and necrotic cells of CMEMM-treated cells were measured using flow cytometry. Data represent the result from 1 of 3 independent experiments.

cells at sub-G_1 phase increased from 25.4% to 54.7%. As for the control, no significant changes were observed (0.4% to 0.7%).

Annexin V Binding in CMEMM-Treated Cells

Annexin V staining can identify apoptosis at an early stage. To further confirm and quantify apoptosis in HL-60 cells triggered by CMEMM, cells were stained with annexin V-FITC/PI, and then analyzed by flow cytometry. After treatment with 25, 50, or 75 µg/mL CMEMM for 24 to 72 hours, the percentage of annexin V-positive apoptotic cells increased in a dose- and time-dependent manner (Figure 4). During the 72-hour treatment period, the percentage of early apoptotic cells in the 75 µg/mL CMEMM-treatment group increased from 8.5% to 64.8%. As for the control group, no significant changes were observed (1.3% to 2.5%). Furthermore, the percentage of necrotic cells increased from

0.6% to 1.8% and from 7.1% to 23.4% for the control group and 75 μg/mL CMEMM treatment group, respectively.

Involvement of Active Caspase-3 in CMEMM-Treated Cells

Because caspase-3 is a key member in the caspase family of cysteine proteases that cleave protein substrates and give rise to characteristic apoptotic morphology, the activation of caspase-3 has been used as an index of apoptosis.[18] To investigate whether CMEMM-induced apoptosis in HL-60 cells involved the activation of caspase-3, cells were analyzed using flow cytometry with active caspase-3-specific antibody. As shown in Figure 5, caspase-3 activity in HL-60 increased after treatment with 25, 50, or 75 μg/mL of CMEMM for 24 hours in a dose-dependent manner. The percentage of cells containing active caspase-3 increased from 2.8% as in the control to 42.5% in cells treated with 75 μg/mL of CMEMM.

In Vivo Antiproliferation Effect of CMEMM on HL-60 in Mouse Xenograft Models

To further examine whether CMEMM can effectively suppress growth in vivo, HL-60 cells were transplanted into athymic nude mice. After tumor formation, the mice were treated either with CMEMM (100 or 500 mg/kg/d, i.p.) or with vehicle PBS as control. CMEMM at dosage of 500 mg/kg/d significantly inhibited the growth of subcutaneously transplanted HL-60 cells in athymic mice after 21 days of treatment, as supported by both the tumor volume (Figure 6A) and the final tumor weight measurements (Figure 6B). The relative tumor volume (ratio of tumor volume on each test day to that of day 1) of 500 mg/kg/d CMEMM-treated animals were significantly ($P < .05$) reduced when compared with the control from days 5 to 21. At the end of the study, the relative tumor volume of 100 mg/kg/d CMEMM-treated and control animals had average relative tumor volumes of 16.8 and 16.6, respectively. In contrast, the 500 mg/kg/d CMEMM-treated animals had an average relative tumor volume of 9.4. Moreover, the final tumor-to-body weight ratio for the 500 mg/kg/d CMEMM-treated animals was significantly ($P < .05$) reduced when compared with the controls. No obvious acute toxicity of CMEMM was observed during the treatment period, and there was no significant difference between the body weight of the treated and control animals. Also, no tissue damage was observed in the livers, kidneys, and spleens following examination of the tissue slices stained with H&E (data not shown).

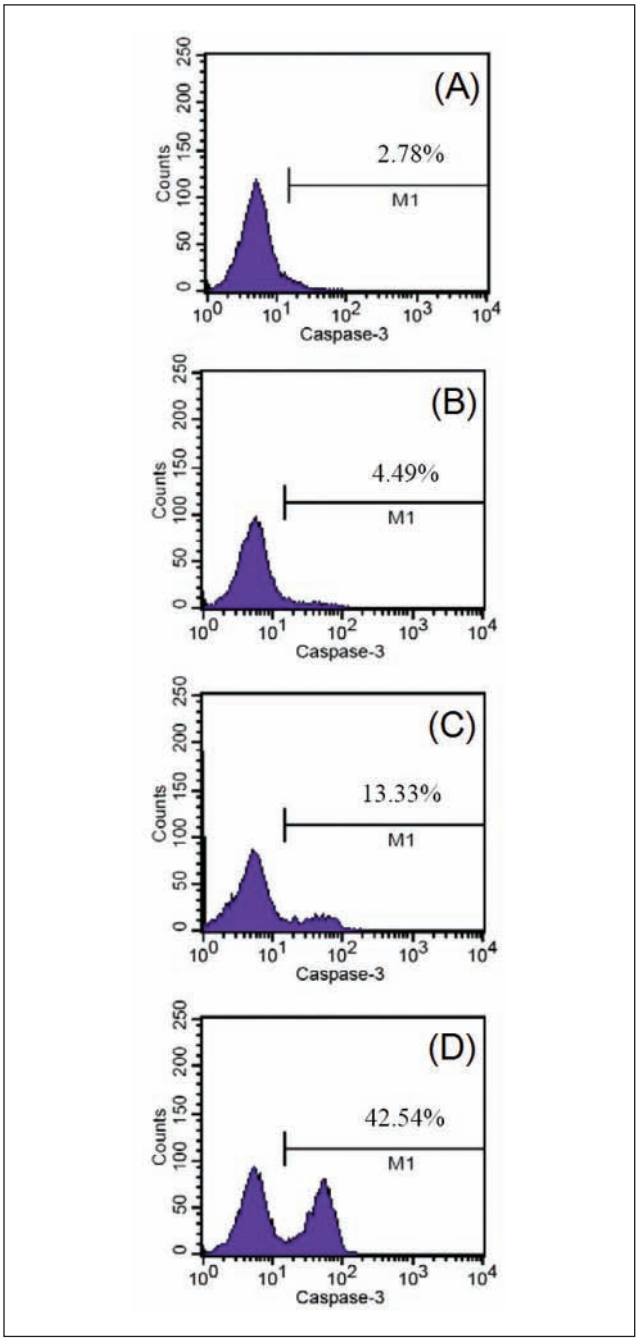

Figure 5. Activation of caspase-3 in crude methanolic extract of *Mucuna macrocarpa* (CMEMM)-treated cells
Active caspase-3 analyses were performed on HL-60 cells treated with (A) 0.1% dimethyl sulfoxide (DMSO; control), (B) 25 μg/mL CMEMM, (C) 50 μg/mL CMEMM, and (D) 75 μg/mL CMEMM for 24 hours. After treatment with CMEMM, cells were permeabilized, fixed and stained, and analyzed for active caspase-3 by flow cytometry. Data represent the result from 1 of 3 independent experiments.

Chemical Components and HPLC Chromatogram of CMEMM

As shown in Figure 7A, nine chemical components were isolated from CMEMM. Based on the NMR, IR, and MS spectroscopic data, these compounds were identified by comparison of spectroscopic data with literature reports. These included 1 lipid (tetracosanoic acid[19]), 1 sterol

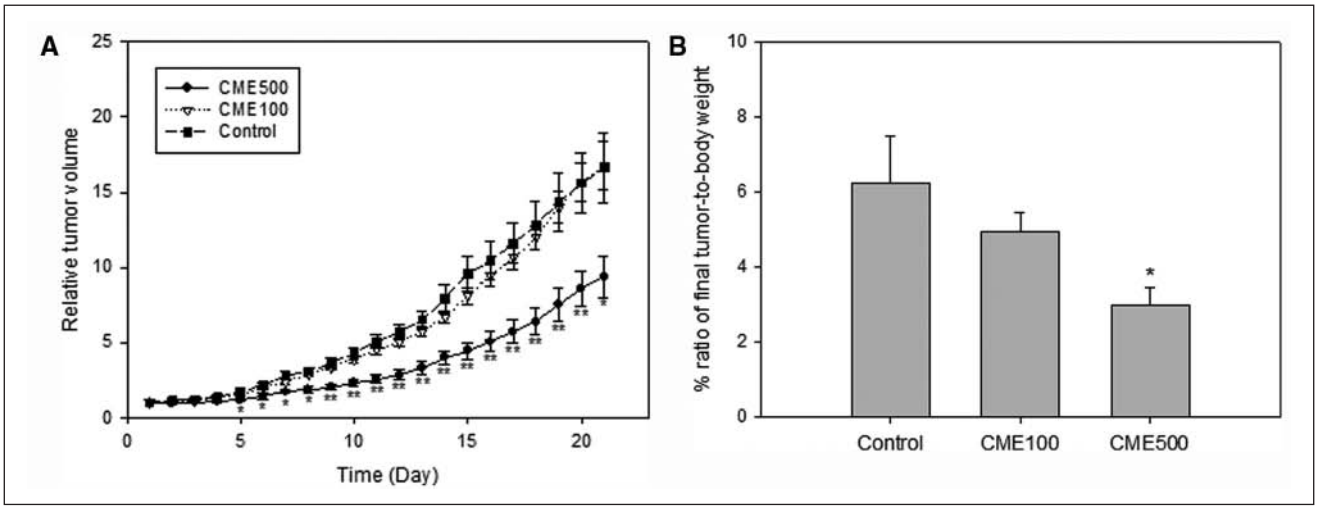

Figure 6. In vivo antiproliferative effect of crude methanolic extract of *Mucuna macrocarpa* (CMEMM) on human leukemia HL-60 xenografts

When the tumors were palpable (4 to 6 mm), BALB/c nude mice were treated either with vehicle control or with CMEMM by daily intraperitoneal injection. The animals (7 to 8 mice per group) were treated with 100 mg/kg/day CMEMM (CME100), 500 mg/kg/day CMEMM (CME500), or with phosphate-buffered saline (PBS) as vehicle control for 21 days. A, For relative tumor volume, results were expressed as mean ± standard error (mean phosphate-buffered saline (PBS) as vehicle control for 21 days. A, For relative tumor volume, results were expressed as mean ± standard error (mean ± SE) calculated from the ratio of tumor volume on each test day to that of day 1. B, For percentage ratio of final tumor-to-body weight, results were expressed as the mean ± SE. **P* < .05 and ***P* < .01 indicate significant difference from the control value.

(mixture of β-sitosterol and stigmasterol[20]), 2 carbohydrates (sucrose[21] and D-pinitol[22]), 1 triterpenoid (friedelin[23]), and 4 isoflavones (medicarpin[24], calycosin[25], afrormosin,[26] and genistein[27]). As shown in Figures 7B and 7C, calycosin, afrormosin, and genistein were used as marker components for the standardization of flavonoid ingredients of CMEMM by high-performance liquid chromatography (HPLC) chromatograms.

Discussion and Conclusions

In the theory of traditional Chinese medicine, blood-activating and stasis-resolving herbs have been used for the treatment of various hematologic and circulatory related ailments since ancient times. Since these medicinal herbs can relieve symptoms related to leukemia, they have been employed as clinical dialectical therapy for patients with myeloproliferative disorders by the Chinese in recent years.[28] Additionally, recent reports have demonstrated that antileukemic effects of these herbs were exerted by induction of apoptosis in human leukemia cells.[29,30] In the present study, we have for the first time examined the antileukemic activity of *M macrocarpa*, seeking scientific support for its traditional use.

The current study demonstrated that CMEMM was effective against the proliferation of human promyelocytic leukemia cells, HL-60 (Figure 1). However, 2 lymphoid leukemia cell lines, Jurkat T and Molt-3 cells, showed similar responses but were less sensitive to CMEMM-induced growth inhibition in comparison to HL-60 in our preliminary study (data not shown). Similarly, Chang et al [31] had also reported differences in sensitivity between promyelocytic and lymphoid leukemia cells when exposed to the same agents in cell proliferation assays. APL cells (HL-60) were more sensitive to drug-induced growth inhibition than ALL cells (Molt-3). An important finding is that apoptosis in HL-60 cells triggered by CMEMM was confirmed not only by the characteristic apoptotic morphology (Figure 2) and accumulation at sub-G_1 phase (Figure 3) but also by an increase of annexin V-FITC/PI staining (Figure 4). Moreover, active caspase-3 elevation was found to be a key mediator for CMEMM-induced apoptosis (Figure 5). These results together suggest that CMEMM exerts antileukemic effects via an apoptotic pathway in HL-60 cells.

Furthermore, the present in vivo results confirmed the growth-inhibitory effect of CMEMM on human leukemia xenografts in athymic nude mice. Throughout the 21 days of treatment by i.p. injection, CMEMM at 500 mg/kg/d was efficacious in tumor suppression, and the ratio of final tumor-to-body weight was significantly reduced in comparison to that of the control (Figure 6). However, in our pilot study, treating mice with 500 mg/kg/d of CMEMM in their daily gavage did not result in observable tumor suppressing effect on HL-60 xenografts. This implied that the extent of bioavailability in CMEMM-treated animals through gavage was not as high as i.p. injection probably because of pharmacokinetic behavior of the different administration routes. To improve bioavailability of CMEMM, pharmacological

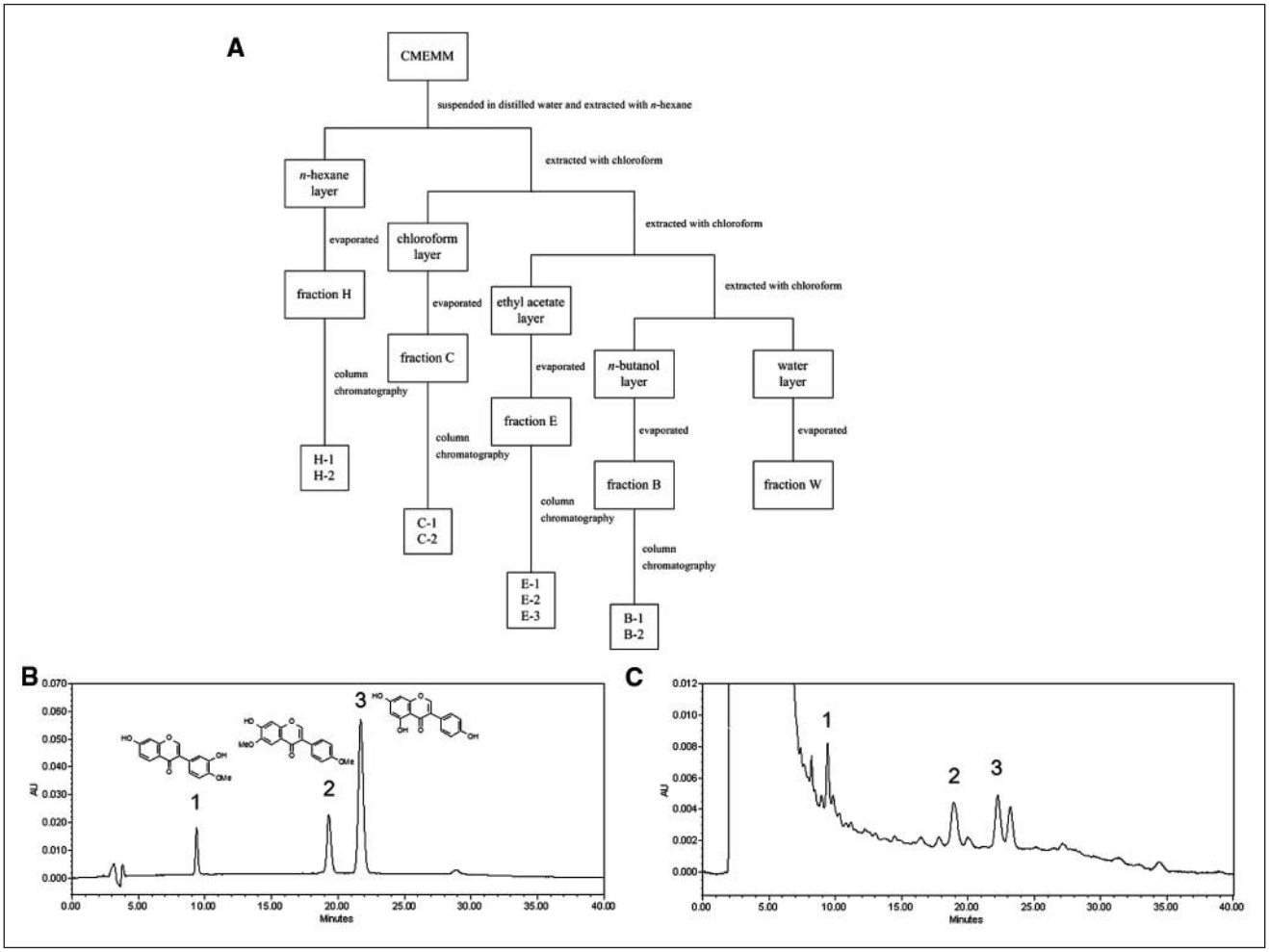

Figure 7. Phytochemical analyses of crude methanolic extract of *Mucuna macrocarpa* (CMEMM)
A, Chemical components were isolated from CMEMM using chromatographic methods: tetracosanoic acid (H-1), friedelin (H-2), mixture of β-sitosterol and stigmasterol (C-1), medicarpin (C-2), calycosin (E-1), afrormosin (E-2), genistein (E-3), sucrose (B-1), and D-pinitol (B-2). B and C, high-performance liquid chromatography (HPLC) chromatograms show a standard mixture and CMEMM: (1) calycosin; (2) afrormosin; (3) genistein. (For HPLC chromatographic conditions, please see the Materials and Methods section.)

studies such as dosage form design and formulation development will be worthwhile tasks in the future.

Using column chromatography and spectroscopy, 9 components from the stem of *M macrocarpa* were isolated and identified as 1 lipid, 1 sterol, 2 carbohydrates, 1 triterpenoid, and 4 isoflavones (Figure 7A). In previous studies of the bioactive isoflavones isolated in this experiment, medicarpin and genistein had been reported to exert antileukemic effects by inducing apoptosis in HL-60 cells;[32,33] calycosin had been reported to exert antiproliferative effects in U937 lymphoma cells;[34] and afrormosin had been reported to exhibit inhibitory effects on TPA-induced tumor promotion in mouse.[35] Since medicarpin was absent from the HPLC chromatogram because of its relatively low content, the other bioactive isoflavones were identified in CMEMM with pure marker components (Figure 7B). Although not shown in the present study, the HPLC quantitative analysis showed the content of genistein was 0.001% in air-dry stems. It has been reported recently that IC_{50} value of genistein for 48-hour exposure was 18.2 μM in HL-60 cells.[36] It was reasonable to assume that genistein was not the main component in CMEMM that induced growth inhibition on HL-60 cells because of the low content of genistein in air-dried stems of this plant. This implied that the antileukemic effect of *M macrocarpa* may have resulted from the interaction of different flavonoids and other constituents.

In conclusion, we presented evidence that treating human leukemia HL-60 cells with CMEMM led to the induction of apoptosis. Therefore, our results suggested that CMEMM could be a candidate for developing antileukemic

agents. For future therapeutic application, further studies of CMEMM in comedications with synthetic drugs are now in progress in our laboratory.

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Authors' Note

Dr Yuan-Shiun Chang and Dr Chin-Wen Chi contributed equally to this work. Correspondence may also be addressed to Dr Yuan-Shiun Chang at Graduate Institute of Chinese Pharmaceutical Sciences, China Medical University, No. 91, Hsueh-Shih Road, Taichung 404, Taiwan. *E-mail address*: yschang@mail.cmu.edu.tw

Declaration of Conflicting Interests

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