Marchantin A, a cyclic bis(bibenzyl ether), isolated from the liverwort Marchantia emarginata subsp. tosana induces apoptosis in human MCF-7 breast cancer cells

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A B S T R A C T

Liverwort constituents have been reported to exert a broad spectrum of biological activities. In this study, we used a bioactivity-guided separation of an extract from the liverwort species Marchantia emarginata subsp. tosana to determine its anticancer activity. A high level of the active ingredient was isolated from this liverwort and its chemical structure was identified and characterized by various spectra. It was found to be identical to a well-known compound, marchantin A, a cyclic bisbibenzyl ether. However, no anticancer activities of this compound have previously been reported. We found that marchantin A efficiently induced cell growth inhibition in human MCF-7 breast cancer cells, with an IC50 of 4.0 µg/mL. Fluorescence microscopy and a Western blot analysis indicated that marchantin A actively induced apoptosis of MCF-7 cells. The levels of cleaved caspase-8, cleaved caspase-3, cleaved caspase-9, and cleaved poly (ADP ribose) polymerase (PARP) increased. However, the level of Bid markedly decreased in a dose- and time-dependent manner. We also evaluated the anticancer activities of marchantin A on the regulation of cell cycle regulators such as p21, p27, cyclin B1, and cyclin D1. The p21 and p27 gene expressions increased markedly while cyclin B1 and D1 gene expression decreased markedly by treatment with marchantin A. Many report demonstrated that liverwort was suggested to possess potent antioxidant activity. Our results indicate that marchantin A possesses free radical-scavenging activity (EC50 = 20 µg/mL). Taken together, for the first time, the compound marchantin A from liverworts demonstrated to be a potent inducer of apoptosis in MCF-7 cells.

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1. Introduction

There are about 6000 species of liverwort identified in the world. Liverworts contain oil bodies in their cells and elaborate mainly sesquiterpenes, diterpenes and lipophilic aromatics as their major chemical constituents. Several biological activities of liverworts are triggered from these active ingredients. Plants of Marchantia species had been used as traditional medicine in ancient China. Many bioactive components, particularly the bis(bibenzyl) ethers, such as marchantin A–C, have been isolated from various Marchantia species [1]. Marchantia emarginata subsp. tosana (Marchantiaceae) is a common thallus liverwort species. Humulane-type sesquiterpenes and bis(bibenzyl)
compounds had been reported from the Japanese liverwort _M. tosana_ [1,2]. In Taiwan, this liverwort is widely distributed in moist and endolithic areas at low elevations, however, the chemical components as well as their bioactivities have not been seriously investigated. In the present study, we found that the Taiwanese species _M. tosana_ contains a fairly high amount of marchantin A, which had been isolated from _M. tosana_ previously, yet never in such rich scale. Marchantin A has been reported to possess diverse biological activities, such as antifungal [3], antimicrobial [4], anti-inflammatory [5], antioxidative, and skeletal muscle relaxing [6] activities. Askawa demonstrated that marchantin A was efficient against fungi of several species [7] and inhibited LPS-induced NO in RAW264.7 cells [5]. As far as we know, no anticancer activities of this compound have yet been reported. In the present study, we are interested in the anticancer activities of marchantin A against human breast cancer MCF-7 cells.

Apoptosis, or programmed cell death, plays an essential role in controlling cell numbers in many developmental and physiological settings. Its morphological characteristics include cell shrinkage, nuclear condensation, plasma membrane blebbing, chromosomal DNA fragmentation, and the formation of apoptotic bodies [8]. Recent reports have shown that many anticancer drugs or chemoprevention agents act by inducing apoptosis to inhibit tumor promotion and progression. Mitochondria are currently regarded playing a central role in mediating “intrinsic death signals” and can serve as a novel target for chemotherapies [9,10]. Cytochrome c (Cyt c) is a mitochondrial protein that can activate caspase-9. However, the release of Cyt c is regulated by anti- and proapoptotic members of the Bcl-2 family (such as Bcl-2, Bcl-xL, Bax, Bak, Bad, and Bid). Once in the cytoplasm, it binds Apaf-1 to procaspase-9, leading to activation of caspase-9 and triggering of the caspase cascade [11]. Many parameters of mitochondrial physiology have been shown to be hallmarks of apoptosis. These include loss of the mitochondrial membrane potential, generation of reactive oxygen species (ROS), termination of oxygen consumption, and the release of Cyt c [12]. In the present study, we investigated the effect of marchantin A on the induction of apoptosis in MCF-7 cells.

Breast cancer is the second most common type of cancer and the fifth most common cause of cancer death in the world. The incidence of breast cancer had increased 4-fold in the past 25 years (1980–2005) according to the statistics of Department of Health in Taiwan. In Taiwan, incidence of female breast cancer was from 12.7 per 100,000 women in 1980 to 49 per 100,000 women in 2005. In 2008, calculated 182,460 new cases of invasive breast cancer will be diagnosed in women in the United States and breast cancer is expected to cause 40,480 deaths in the US. In North America, women have the highest incidence of breast cancer. Although no one method can predict the development of breast cancer, several risk factors have been found and allow clinicians to identify women at highest risk [13]. Recent research focused on exploring options such as chemoprevention to reduce the rate of developing breast cancer among high risk women. Conventional therapeutic strategies include surgery, radiation, and chemotherapy. Due to their side effects and limited effectiveness, there is still a pressing need for the development of anti-breast cancer drugs. Natural products exerting diverse bioactivities and possessing unique structural properties are an important source for the development of novel anticancer drugs [14]. In this study, the anticancer effects of marchantin A, a class of secondary metabolites produced exclusively in liverworts (_M. tosana_), like the dimer of stilbenoids, were evaluated in human MCF-7 cells.

2. Materials and methods

2.1. Plant material

_M. tosana_ (Fig. 1a) was collected from Nankang, Taipei, and the species was identified by Prof. Shan-Hsiung Lin (Department of Life Science, Tunghai University, Taiwan). The specimen was stored at NatureWise Biotech and Medicals Corp. Samples were collected in the summer 2007.

2.2. Extraction and isolation of marchantin A

The air-dried and powdered material of _M. tosana_ (500 g) was extracted with 5 L of MeOH, sonicated for 1 h, then left to stand for 2 weeks at room temperature (24–25 °C). The filtered methanol extract was evaporated to dryness under reduced pressure to obtain a crude extract (11.7 g), which was suspended in water (500 mL) and partitioned with n-hexane and EtOAc (3 × 500 mL). A bioassay test showed that the EtOAc (ethyl acetate) extract exhibited significant anticancer activity than n-hexane or water extract against MCF-7 cells. The EtOAc extract was evaporated under reduced pressure (1.8 g). A portion (1.0 g) of the EtOAc extract was chromatographed over a silica gel column and eluted with an n-hexane:EtOAc gradient system (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10, then pure MeOH) to obtain ten (F1–F10) fractions. These fractions were assayed for their effects on MCF-7 cell proliferation. The most active fraction (F4) was purified again by silica gel column and eluted with an n-hexane:EtOAc gradient system (from 10% to 60%). The inhibitory activities of these eluted fractions against MCF-7 cells were screened in a proliferation platform assay. The active fractions were purified on a reversed-phase preparative high-performance liquid chromatographic/ultraviolet (HPLC/UV) unit. Conditions included a Phenomenex Luna C18 silica gel column (10.0 × 250 mm, USA), a solvent system of methanol:water (80:20), a flow rate of 3.0 mL/min, detection at UV 210 nm, the temperature of column at 25 ± 1 °C, and the elution conditions are used the isocratic. Fractions with a retention time of 7.3 min containing the active ingredient were collected. The active ingredient was identified as a known compound, marchantin A (Fig. 1b). Marchantin A, a white amorphous solid, has a molecular formula of C_{28}H_{24}O_{5}, as deduced from 13C NMR (CPD and DEPT) data and HREIMS (440.1632). By means of 1H, 13C NMR analysis (Table 1) and 2D NMR (including HMOC, HMBC, COSY, and NOESY methods), the structure was elucidated as marchantin A [15].
Marchantin A and inhibition of cell growth. (a) Morphology of the liverwort, *M. tosana*, which was collected in summer 2007. (b) The structure of the active ingredient, marchantin A. (c) Cell growth inhibition activity of marchantin A after treatment with a fixed concentration of 10 \( \mu \text{g/mL} \) for 24 and 72 h or (d) treatment with various concentrations of 2.5–10.0 \( \mu \text{g/mL} \) for 48 h on human MCF-7 breast cancer cells. (e) MCF-7 cells were untreated or treated with marchantin A at a fixed concentration of 7.5 \( \mu \text{g/mL} \) for 24, 48, and 72 h. Cells were counted and determined by a trypan blue exclusion assay.

**Fig. 1.** Structure of marchantin A and inhibition of cell growth. (a) Morphology of the liverwort, *M. tosana*, which was collected in summer 2007. (b) The structure of the active ingredient, marchantin A. (c) Cell growth inhibition activity of marchantin A after treatment with a fixed concentration of 10 \( \mu \text{g/mL} \) for 24 and 72 h or (d) treatment with various concentrations of 2.5–10.0 \( \mu \text{g/mL} \) for 48 h on human MCF-7 breast cancer cells. (e) MCF-7 cells were untreated or treated with marchantin A at a fixed concentration of 7.5 \( \mu \text{g/mL} \) for 24, 48, and 72 h. Cells were counted and determined by a trypan blue exclusion assay.

Fig. 1a–c, data presented were from three independent experiments with similar results. Fig. 1d and e, data represent means ± SD (n = 3).
humidified atmosphere of 95% air and 5% CO2. Marchantin
vine serum (FBS), a 1% dilution of penicillin–streptomycin,
Taiwan). MCF-7 cells were cultured in Dulbecco’s modified
Industry Research and Development Institute (Hsinchu,
2.4. Cell culture
pared at a fixed concentration of 10 mg/mL. Cells
A was dissolved in dimethyl sulfoxide (DMSO) and pre-
various concentrations (2.5, 5.0, 7.5, and 10.0
incubated for 14 h before being treated with DMSO or with

2.3. Analytical conditions for marchantin A
The M. tosana profile in the total methanol extract and
marchantin A were analyzed with a reversed-phase
HPLC/UV unit. The conditions included a Luna Phenome-
marchantin A were analyzed with a reversed-phase
UV 210 nm, the temperature of the column at 25 ± 1
elution conditions are isocratic, and the volume of injec-
profile in the total methanol extract and
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2.5. Morphological analysis of apoptotic cells
MCF-7 cells were cultured on six-well culture plates
(3 × 10^6/well) and treated with marchantin A at a fixed
concentration of 10.0 µg/mL for 24 h. After treatment, cells
were fixed with methanol for 30 min, washed with phos-
phate-buffered saline (PBS), and then stained with 50 µg/
ml of propidium iodide (PI) in the presence of 50 µg/mL
RNase A. The morphology of nuclear chromatin was de-
formed. MCF-7 cells were plated at a density of 3
C. The cell pellets were collected by centrifugation,
resuspended in 1 mL of iced 100% ethanol, then incubated overnight at
−20 °C. The cell pellets were collected by centrifugation,
resuspended in 1 mL of hypotonic buffer (0.5% Triton X-
100 in PBS and 1 µg/mL RNase A), and incubated at 37 °C
for 30 min. Then, 1 mL of the PI solution (50 µg/mL) was
added, and the mixture was allowed to stand at 4 °C for
30 min. The cellular DNA content was then analyzed by
FACScan cytometry (Becton Dickinson).

2.6. Analysis of the cell cycle
MCF-7 cells (1 × 10^6) in a 100-mm dish were treated
with various concentrations (2.5–10.0 µg/mL) of marchan-
tin A for 48 h or cells were treated with a fixed concentra-
tion of 10 µg/mL marchantin A for 2, 4, 6, and 8 h. Cells
were trypsinized and collected with ice-cold PBS. Cells
were resuspended in 200 µL PBS and fixed by adding
800 µL of iced 100% ethanol, then incubated overnight at
−20 °C. The cell pellets were collected by centrifugation,
resuspended in 1 mL of hypotonic buffer (0.5% Triton X-
100 in PBS and 1 µg/mL RNase A), and incubated at 37 °C
for 30 min. Then, 1 mL of the PI solution (50 µg/mL) was
added, and the mixture was allowed to stand at 4 °C for
30 min. The cellular DNA content was then analyzed by
FACScan cytometry (Becton Dickinson).

2.7. Annexin V-staining
Double staining for Annexin-V-fluorescein isothioocy-
nate (FITC) binding and for cellular DNA using PI was per-
formed. MCF-7 cells were plated at a density of 3 × 10^5
cells/well on 6-well plates. After treatment with or without
the marchantin A (10 µg/mL) for 2, 4, and 6 h, or treatment
with 2.5–10.0 µg/mL of marchantin A for 48 h, cells were
trypsinized and collected in ice-cold PBS. The cells were
washed twice with cold PBS and resuspended in 1 mL
binding buffer (BD PharMingen, San Diego, CA). They were
then stained with Annexin-V-FITC and PI according to the
protocol from BD PharMingen. Briefly, 2 µL of Annexin-V-
FITC (50 µg/mL) and 1 µL of PI (500 µg/mL) were added
to a 100 µL solution of cells and the solution was incubated for
15 min at room temperature in the dark. Binding buffer
was then added, and early and late apoptosis was visual-
ized by constructing a dot-plot using a Becton Dickinson
FACScan. Green fluorescence from the Annexin-V-FITC
was determined using an FL1 detector having a bandpass
filter with specifications 530 ± 15 nm. Red fluorescence
from PI was determined using an FL2 detector having a
bandpass filter with specifications 585 ± 21 nm. A total of
10,000 events were recorded for each sample.

2.8. Western blot assay
MCF-7 cells (1 × 10^6) on 100-mm dishes were treated
with various concentrations (0, 2.5, 5, 7.5, and 10 µg/mL)

Table 1
<table>
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<th>δC (mult.)b</th>
<th>HMBC (H → C)</th>
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<td>121.2 (d)</td>
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<td>6.92 d (8)</td>
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<td>139.1 (s)</td>
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<td>30.3 (t)</td>
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<td>35.5 (t)</td>
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<td>11'</td>
<td>156.7 (s)</td>
<td></td>
<td></td>
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<td>128.9 (d)</td>
<td>10', 11', 14'</td>
</tr>
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<td>13'</td>
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<td>112.0 (d)</td>
<td>10', 11'</td>
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<td>14'</td>
<td>6.39 d (7.8)</td>
<td>123.2 (d)</td>
<td>8', 10', 13'</td>
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</table>

a Data without multiplicities were obtained using the COSY-45 and
HMBC pulse sequences.
b Multiplicities were obtained from DEPT experiments.

2.4. Cell culture
Human MCF-7 cells were purchased from the Food
Industry Research and Development Institute (Hsinchu,
Taiwan). MCF-7 cells were cultured in Dulbecco’s modified
Eagle’s medium (DMEM, Gibco) containing 10% fetal bo-
vine serum (FBS), a 1% dilution of penicillin–streptomyacin,
and 2 mM glutamine. Cells were maintained at 37 °C in a
humidified atmosphere of 95% air and 5% CO2. Marchantin
A was dissolved in dimethyl sulfoxide (DMSO) and pre-
pared at a fixed concentration of 10 mg/mL. Cells
(1 × 10^6 per dish) were cultured in a 100-mm dish and incubated for 14 h before being treated with DMSO or with
various concentrations (2.5, 5.0, 7.5, and 10.0 µg/mL) of
marchantin A at different time points. Marchantin A is a
small compound (MW 440.16 Da). When these values
were converted to molarities, they were 5.7, 11.4, 17.0,
and 22.7 µM, respectively. All of these treatments with
various doses of marchantin A. The vehicle (DMSO) in
the cell culture medium was fixed concentration at 2 µL/mL.
Cells were counted and determined by a trypan blue exclu-
sion assay.

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of marchantin A for 48 h, or cells were treated with a fixed concentration of 10 μg/mL of marchantin A for 24 and 72 h. After treatment, cells were collected and resuspended in 100 μL lysis buffer. Equal amounts of proteins (30 μg) were mixed with 2× sample buffer and resolved by 12.5% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) for β-actin, poly (ADP ribose) polymerase (PARP), Bid, caspase-3, caspase-8, and caspase-9 detection. Proteins were electrotransferred to an Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), and equivalent protein loading was verified by staining the membrane with the reversible dye, amido black (Sigma Chemical, St. Louis, MO, USA). This was followed by overnight blocking with a solution composed of 20 mM Tris–HCl (pH 7.4), 125 mM NaCl, 0.2% Tween 20, and 3% bovine serum albumin (BSA). Specific antibodies used were anti-human PARP and Bid (1:500 of a rabbit polyclonal antibody; Cell Signaling Technology, Danvers, MA, USA), anti-caspase-3, anti-caspase-8, and caspase-9 antibodies (1:800 of mouse monoclonal antibody; Cell Signaling Technology). These proteins were detected by chemiluminescence (ECL, Amersham, GE Healthcare, Buckinghamshire, UK).

2.9. Real-time quantitative PCR analysis

Total RNA from MCF-7 cell was treated with marchantin A (0, 2.5, 5.0, 7.5, and 10 μg/mL) for 24 h. Cells were lysed, and the total RNA was extracted with a Qiagen RNeasy Mini kit (Qiagen, Valencia, CA, USA). All real-time quantitative PCR primers were designed with Primer Express Software (Applied Biosystems, Foster City, CA) based on GenBank sequence data. Each real-time quantitative PCR reaction contained 30 ng of cDNA, each primer at 100 nM, and 7.5 μL of SYBR Green PCR Master Mix (1-step kit, Applied Biosystems) in a total volume of 15 μL. Each sample was performed in triplicate on an Applied Biosystems Prism 7500 Fast Sequence Detection System and the relative mRNA levels were calculated by the comparative threshold cycle method with GAPDH as an internal control. Multiple PCR was performed using the following primers for humans: p21 forward 5'-CAGACCGAGCATGACAGA-3' and reverse 5'-TTAGGGCTTCCTGAGGAG-3'; cyclin B1 forward 5'-AGCTTGGCTGCTGTAAGAG-3' and reverse 5'-GCCATGGATCCCTTGCTGAGA-3'; cyclin D1 forward 5'-TTAGGGCTTCCTGAGGAG-3' and reverse 5'-ATGCTGGCGCTGAGTACGT-3'; p53 forward 5'-ATGCGGCGCTGCTGAGGAG-3' and reverse 5'-GCCATGGATCCCTTGAGGAG-3'; p27 forward 5'-CTGCAACCGAGCATGACAGA-3' and reverse 5'-GCCATGGATCCCTTGAGGAG-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5'-ATGCTGGCGCTGCTGAGGAG-3' and reverse 5'-GCCATGGATCCCTTGAGGAG-3'. After reverse transcriptase step at 48 °C for 30 min, then enzyme activation reaction at 95 °C for 10 min. Initial denaturation at 95 °C for 15 s, anneal/extend at 60 °C for 1 min, 40 cycles were performed in the duration.

2.10. DPPH free radical-scavenging activity

The free radical-scavenging capacities of marchantin A (5–30 μg/mL) and caffeic acid phenyl ester (CAPE) (1–16 μg/mL), a well-known antioxidant used as a positive control at various concentrations were measured with 1.0 mL of 0.3 mM 1,2-diphenyl-2-picrylhydrazyl (DPPH) in methanol. The DPPH radical has a deep violet color due to its unpaired electron, and its radical-scavenging capability can be followed spectrophotometrically by an absorbance loss at 517 nm when the pale-yellow non-radical form is produced. The mixtures were vigorously shaken and left to stand at room temperature for 30 min in the dark. The absorbance at 517 nm was measured against methanol as a blank. The ability to scavenge DPPH radicals was then calculated by the following equation:

\[
\text{Scavenging effect (\%)} = \frac{1 - (A_{517} \text{ of sample}/A_{517} \text{ of control}) \times 100%}{1}
\]

The EC_{50} value was defined the concentration of a test compound which scavenge 50% of the free radicals.

2.11. Densitometric analyses

Bands were scanned with Adobe Photoshop 6.0 (Adobe System Inc., San Jose, CA, USA). The mean density of each band was analyzed by the Scion image program. In each case, blots were subjected to multiple exposures on the film to ensure that the band density was in the linear range. The data are presented in terms of fold change over internal control (β-actin) for each treatment. All data presented were from three independent experiments with similar results.

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3. Results and discussion

3.1. Purification and identification of marchantin A

The active ingredient, called marchantin A (Fig. 1b), was isolated by repeated passages of the total methanol extract of the liverwort through chromatography. Final purification of the active ingredient was achieved by HPLC/UV on a reversed-phase column. The total content marchantin A was roughly 6.6% of the methanol extract of liverwort (M. tosana). Fig. 2 shows the HPLC profile of the total methanol extracts of liverwort, indicating liverwort is rich in marchantin A. Marchantin A was previously isolated from several different species of liverworts, and was first identified from Marchantia species. It exhibits antibacterial and antifungal activities against several species [3]. However, no anticancer activities of this compound (marchantin A) had previously been reported. In this report, we investigated the mechanism of apoptosis of human MCF-7 breast cancer cells induced by marchantin A.

3.2. Inhibition of cell growth by marchantin A

MCF-7 cells were treated with marchantin A at a fixed concentration of 10 μg/mL for 24 and 72 h (Fig. 1c) or treated with various concentrations (2.5, 5.0, 7.5, and 10 μg/mL) of marchantin A for 48 h (Fig. 1d). Treatment with marchantin A at a fixed concentration of 7.5 μg/mL for 24, 48,

![Fig. 3. Marchantin A induced apoptosis in MCF-7 cells. (a) Effect of marchantin A on the morphology of nuclear chromatin. MCF-7 cells were untreated or treated with marchantin A at a concentration of 10 μg/mL for 24 h and stained as described in Section 2. Morphological changes of nuclear chromatin were then viewed under a confocal microscope. (b) Flow cytometric analysis of marchantin A-treated MCF-7 cells after fluorescinated anti-Annexin V monoclonal antibody binding (FL1-H) and propidium iodide uptake (FL2-H). Cells were treated with or without 10 μg/mL of marchantin A for 2, 4, and 6 h, or treated with 2.5–10.0 μg/mL of marchantin A for 48 h, and subsequently measured by flow cytometry. The lower right quadrant represents early apoptosis; the upper right quadrant represents late apoptosis. (c) Flow cytometric analysis of MCF-7 cells treated with a fixed concentration of marchantin A at 10 μg/mL for 2, 4, 6, and 8 h or (d) treated with marchantin A at various concentrations of 2.5–10.0 μg/mL for 48 h and stained with propidium iodide as described in Section 2. Following the flow cytometric analysis, the cellular DNA profile was analyzed by Cell Quest software.

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and 72 h significantly inhibited cell proliferation as shown in Fig. 1e. All these results demonstrated that marchantin A possesses the ability to inhibit cell growth, with an IC50 of 4.0 \( \mu \text{g/mL} \). We also evaluated marchantin A on several different cancer cell lines such as Rat c6 glioma cells, human Hs683 glioma cells, human HT-29 colon cancer cells, and human A549 lung cancer cells. All of these cell lines exhibited similar results to those of MCF-7 cells (data not shown). A related compound, marchantin C was reported to induce microtubule depolymerization and suppress tumor cell growth [16]. Our data demonstrated that suppression of tumor cell growth capacity by marchantin A was similar to that of marchantin C.

### 3.3. Marchantin A induces apoptotic cell death in MCF-7 cells

Through a cell growth inhibition assay (Fig. 1c and d), we showed that marchantin A induced significant cell death in MCF-7 cells. Inhibition of cell proliferation was clearly present in marchantin A-treated cells. The effect of cell death appeared to occur through the induction of apoptosis. The cytotoxic effect of marchantin A was further evidenced by nuclear condensation under a confocal microscope as shown in Fig. 3a. The morphological features of apoptosis, such as chromatin condensation and nuclear fragmentation, were seen in marchantin A-treated cells. We next questioned whether marchantin A induced apoptosis in MCF-7 cells. Here, we used annexin V-staining and flow cytometry to evaluate whether marchantin A caused cell death is via apoptosis (Fig. 3b). Cells were treated with marchantin A (10 \( \mu \text{g/mL} \)) in 0, 2, 4, and 6 h for the apoptosis analysis. Our result indicates no significantly increased in a time-course dependent in apoptotic cell population following marchantin A treatment (data not shown). Furthermore, MCF-7 cells were treated with 2.5–10.0 \( \mu \text{g/mL} \) of marchantin A for 48 h. The result indicated a dose-dependent increase (1.58–10.91%) in apoptotic cell population following marchantin A treatment. To investigate the induction of a sub-G1 cell population, the DNA content of MCF-7 cells was treated with marchantin A at a fixed concentration of 10 \( \mu \text{g/mL} \) for 2, 4, 6, and 8 h (Fig. 3c) or treated with various concentrations of marchantin A for 48 h and then analyzed by flow cytometry as shown in Fig. 3d. Marchantin A-treated cells for 8 h, markedly increased the G2/M cell population from 20.5% to 33.3% and the G0/G1 cell population dropped markedly from 60.0% to 42.7% as shown in Fig. 3c. The results of flow cytometric analysis indicated that marchantin A increased the sub-G1 cell population from 0.7% to 6.8%, the G0/G1 cell population dropped significantly from 62.3% to 36.1%, and the G2/M cell population increased markedly from 17.1% to 48.0% in a dose-dependent manner as shown in Fig. 3d. These results suggested that marchantin A efficiently influenced cell cycle progression in a period times (2–8 h) and induced apoptosis in MCF-7 cells may be needed incubation over 24 h. Marchantin C was reported to significantly induce cell cycle arrest at the G2/M phase in A172 and HeLa cells [16]. Our data also demonstrated that marchantin A suppressed tumor cell growth similarly to that of marchantin C.

### 3.4. Marchantin A induces apoptosis through the activation of caspases

Apoptosis is a programmed cell suicide in which caspases are the central components of this process. In mammalian cells, caspases involved in apoptosis are classified into two groups according to their function and structure [17]. In this present study, we examined on how marchantin A induced apoptosis by activating caspases-3, -8, and -9 in MCF-7 cells. First, we evaluated whether caspase-dependent signal pathways were involved. Treatment of cells with a fixed concentration (10 \( \mu \text{g/mL} \)) of marchantin A for 24 and 72 h or with various concentrations (2.5, 5.0, 7.5, and 10.0 \( \mu \text{g/mL} \)) for 48 h resulted in a significant increase in the cleaved form of PARP (85 kDa) as shown in Fig. 4a. Furthermore, our results also demonstrated that protein levels of the active forms of caspases-3 and -9 were markedly increased in dose- and time-dependent manners as shown in Fig. 4b and c. Activation of caspase-9 is a very...
important pathway, because it can induce apoptosis through the mito-
chondrion-dependent pathway. Therefore, evaluation of Bid was an
important issue. Our findings suggested that marchantin A decreased
Bid protein levels (Fig. 4d) in dose- and time-dependent manners. Fur-
thermore, we were interested in evaluating caspase-8 levels following
marchantin A treatment. Because caspase-8 plays an important role in
both the mitochondrion-dependent and -independent pathways in apop-
tosis [18], we further evaluated whether caspase-8 might be activated
during the induction of apoptosis by marchantin A, as shown in Fig. 4e.
Our data demonstrated that marchantin A-caused a marked decrease in
the procaspase-8 protein level and a slight increase in the cleaved cas-
pase-8 protein level in a dose-dependent manner. These results suggested
that marchantin A increased protein levels of the cleaved forms of PARP,
and caspases-3, -8, and -9. In this study, we assessed the apoptotic effect
induced by marchantin A on MCF-7 cells and found that this effect might
occur through a mitochondrion-dependent pathway.

3.5. Effects of marchantin A on cell cycle regulators

Recently, a study demonstrated that cyclins D1 and E and the cyclin-
dependent kinase inhibitor, p21(CDKN1A), are important regulators of cell
cycle in breast cancer [19]. Many reports also demonstrated that cell
Fig. 4 (continued)
Fig. 5. Regulation of cell cycle regulator genes in marchantin A-treated MCF-7 cells. Cell cycle regulator genes expression was used real-time Q-PCR analysis. MCF-7 cells were treated marchantin A with various concentrations at 0, 2.5, 5.0, 7.5, and 10.0 µg/mL for 24 h. (a) p21, (b) p27, (c) cyclin B1, and (d) cyclin D1 cell cycle regulator genes were determined by Q-PCR as described in Section 2. We have used the t-test for statistical analysis. The data represent means ± SD (n = 3); * p < 0.05; ** p < 0.02.
growth inhibition can be regulated by cell cycle regulators, including the cyclin-dependent kinase inhibitor, p21waf1 [20], and cyclins B1 [21], D1 [22], and E [23]. We investigated the effect of inhibiting MCF-7 cell growth by marchantin A. Using a real-time quantitative PCR (Q-PCR), we studied gene expressions of those cell cycle regulators following treatment of MCF-7 cells with marchantin A at various concentrations (0, 2.5, 5.0, 7.5, and 10.0 μg/mL) for 24 h. The results are shown in Fig. 5. In each case, multiple gene analyses with GAPDH were used to standardize mRNA levels over the course of the experiment. The p21 and p27 gene expressions increased markedly by treated with marchantin A at concentrations from 5.0 to 10.0 μg/mL as shown in Fig. 5a and b; while cyclin B1 and D1 gene expression decreased markedly after treatment with marchantin A at concentrations from 2.5 to 7.5 μg/mL as shown in Fig. 5c and d. Many reports have revealed an association of cancer and cell cycle regulation. Cell cycle regulators are also frequently found to be mutated in many human cancers [24]. Therefore, cell cycle regulation has been a target for controlling cancers. In this report, we evaluated the anticancer activities of marchantin A not only by evaluating its apoptotic mechanism but also by investigating its effects on cell cycle regulators. P21 is a very important cell cycle regulator and tumor suppress gene [25]. P21 gene overexpression can increase the cell population at the G0/G1 phase after treatment with marchantin A (0–5.0 μg/mL) as shown in Fig. 3c. Many histone deacetylase (HDAC) inhibitors such as SAHA [26], trichostatin A [27], so- dium butyrate [28], LAQ-824 [29], and MS-275 [30], were reported to cause p21 gene overexpression. Cyclin B1 is another important cell cycle regulator. Our data demonstrated that cyclin B1 gene expression was markedly suppressed after cells were treated with marchantin A. How- ever, marchantin C treated human A172 glioma cells markedly increased cyclin B1 levels [16]. These results implied that the anticancer activities of marchantin A and C may go through different regulatory pathways.

3.6. Free radical-scavenging activity

The liverwort has been reported to exert a broad spectrum of biological activities such as cytotoxic, antifungal, antimicrobial, and antioxidant properties. We were interested in evaluating the antioxidant property of marchantin A. The free radical-scavenging activities of various concentrates of marchantin A and CAPE (a potent antioxidant agent used as a positive control [31]) were evaluated with DPPH, and the results are shown in Fig. 6. The respective EC_{50} values of marchantin A and CAPE were 20.0 and 5.5 μg/mL indicating that marchantin A was not as strong an antioxidant as CAPE. Previous studies had been reported that the neighboring hydroxyl groups on the aromatic ring are essential for the antioxidant activity of flavonoids [32]. The chemical structure of marchantin A clearly demonstrates that two hydroxyl groups attached at C1' and C6' are very important parameters for scavenging free radicals of DPPH. For the same reason we believe that the antioxidant activity of marchantin A is stronger than that of marchantin C, because marchantin C has no hydroxyl group at the 6' position. Similarly, two adjacent hydro- xyl groups at C1' and C6' are also very important for inducing cytotoxicity. Many reports suggested that phenolic compounds in DMEM cell culture medium could produce hydrogen peroxide (H_{2}O_{2}) [33,34]. This free radical producing agent may induce cytotoxicity or apoptosis in cultured cells. This leads us to think that marchantin A induces apoptosis in MCF-7 cells may partly due to generating of H_{2}O_{2}.

In summary, we think that the marchantin A inhibited proliferation of MCF-7 cells via apoptosis. Our data demonstrated that marchantin A pos- sibly induced apoptosis through a caspase-dependent pathway. Cyclin B1, cyclin D1, P27, and p21 were significantly changed by treating the cells with marchantin A. These results clearly show that marchantin A has strong capability to influence the cell cycle process. Flow cytometric data demonstrated that low doses (<5 μg/mL) of marchantin A arrested cell growth at the G0/G1 phase, while high doses (7.5–10.0 μg/mL) arrested cell growth at the G2/M phase. The antioxidant and cytotoxic effects may be related to the chemical structure of marchantin A. The neighbor- ing hydroxyl groups attached at C1' and C6' on the aromatic ring are important for the antioxidant and cytotoxic activities of marchantin A. Both marchantin A and marchantin C have similar chemical structures and anticancer activities and may have potential to be developed into candidate drugs for chemotherapy.

Conflict of interest

None of the authors have financial relationship with a commercial entity that has an interest in the content of this study.

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