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The Leaf Extracts of *Toona sinensis* and Fermented Culture Broths of *Antrodia camphorata* Synergistically Cause Apoptotic Cell Death in Promyelocytic Leukemia Cells

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Abstract

Toona sinensis is a common edible vegetable that is used in certain Chinese dishes and has importance in folk medicine. The leaf extracts of *T sinensis* possess and exhibit anticancer efficacy against various cancer cell types. In Taiwanese folklore, *Antrodia camphorata*, also known as "Niu-Cheng-Zi," is used in traditional medicine to treat various illnesses. Its fruit and mycelium possess various potent antiproliferative properties. Two studies from our group have reported that *T sinensis* or *A camphorata* has the ability to cause apoptosis in various cancer cells. Conversely, underlying molecular mechanisms and any beneficial effects remain unknown. This study shows anticancer efficacy for both *T sinensis* and *A camphorata* co-treatments that target HL-60 cells. The combination index values indicate that 40 µg/mL of *T sinensis* and 25 µg/mL of *A camphorata* as a combined treatment shows a synergetic effect, which reduces HL-60 cell proliferation. Alternately, this treatment exhibited no cytotoxic effects for human umbilical vein endothelial cells. Western blot data showed that *T sinensis* and *A camphorata* as a combined treatment result in augmented expression of apoptosis, cytochrome c release, Bcl-2 inhibition, expression of Bax, Fas, and FasL, as well as the cleavage of Bid in HL-60 cells. Moreover, this combined treatment overshadowed monotherapy in its ability to inhibit uPAR, MMP-9, MMP-2, COX-2 expression, and PGE_2 secretions. Our study strongly implies that this combined treatment offers more beneficial effects to suppress and treat leukemia due to apoptosis-mediated cell inhibition. Further *in vivo* studies related to the combined treatment could establish its future potential.

Keywords

Toona sinensis, *Antrodia camphorate*, leukemia, HL-60 cells, apoptosis, MMP, COX-2

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Introduction

Acute myeloid leukemia (AML) affects the differentiation of normal hematopoietic cells, lymphoid system, blood, and bone marrow. Leukemia cells cannot receive terminal differentiation, growth arrest, and apoptosis, which leads to cancer.[1] AML mainly affects the elderly and has an incidence rate of 15 cases per 1 00 000 in the United States and in Europe.[2] Antecedent hematologic disorders, environmental, drug exposures, familial syndromes, and other idiopathic factors are involved with AML. Increasing evidence indicates that there is a relationship among altered apoptotic pathways for neoplastic transformations, progression, and metastasis.[3] Apoptosis is defined as a mechanism for programmed cell death that is characterized as the condensation of chromatin, membrane blebbing, cell size reduction, chromosomal DNA cleavage, and caspase activation. Furthermore, it is triggered by caspase-8 and caspase-9 apoptosis mechanisms.[4] Inducing apoptosis in cancer cells offers several factors toward an effective anticancer therapy with fewer side-effects.[5]

Worldwide, ~80% of people are currently dependent on traditional medicine as their primary health care with majority of therapies using various herbal extracts.[6] In view of

this, use of natural substances could help control and treat various cancers as well as any associated mechanisms. The medical community is increasingly seeing these treatments as effective.[7]

Toona sinensis (A. Juss.) M.J. Roem., a member of the Meliaceae family, is broadly distributed across South-East Asia. In Taiwanese and Chinese cuisine, the leaves and young shoots of *T sinensis* are consumed as an edible vegetable. Liao et al assessed the nontoxic, acute, and subacute toxicities of *T sinensis* and reported it as safe.[8] In folk medicine, *T sinensis* is often used for the treatment of enteritis, dysentery, gastric ulcers, itchiness, diabetes, and cardiovascular diseases.[9,10] Accumulating evidence also indicates that leaf extract from *T sinensis* has lipolytic effects[11] and anticancer mechanisms for lung carcinoma (H661),[9] prostate cancer (DU145),[12] and oral squamous carcinoma (UM1, UM2, and SCC-4) cells. It also shows an inhibitory effect on the replication of the SARS coronavirus[13] as well as Leydig cell steroidogenesis.[14] Sun et al established an efficient and reliable HPLC-DAD (high-performance liquid chromatography diode-array detector) method for the characterization of phytochemical compounds from the *T sinensis* leaf extracts and reported that rutinoside, quercetin-3-O-β-D-glucoside, quercetin-3-O-α-L-rhamnoside, and kaempferol-3-O-α-L-rhamnoside were the 4 reported major flavonol glycoside compounds from these leaf extracts.[15]

Antrodia camphorata is a parasite that inhabits fungi on *Cinnamomum kanehirae* (Bull camphor tree) Hayata (Lauraceae). In Taiwan, *A camphorata* is better known as *Niu-Chang-Chih, Chang-Chih, Niu-Chang-Ku*, or *Chang-Ku. A camphorata* was used by aboriginal Taiwanese to treat various illnesses such as liver-related disease, intoxication, diarrhea, abdominal pain, hypertension, itchy skin, and other tumorigenic diseases. Recently, various *in vitro* and *in vivo* studies indicated a potential for anti-inflammatory/immunomodulatory, antiviral, and neuroprotective properties from its crude extracts.[6] *A camphorata* exerts effective hepatoprotective and other antioxidant characteristics for chronic chemical-induced hepatoxicity *in vivo*.[16] In 2006, Yang et al reported that the fermented culture broth of *A camphorata* exhibited an antiproliferative effect in breast cancer cells (MCF-7) by the induction of apoptosis. They also suggested that *A camphorata* metabolizes the culture medium and produces polysaccharides, crude triterpenoids, and total polyphenols during the fermentation process, which are considered to be the most effective fraction of *A camphorata* that possibly act as chemopreventive agents with regard to inhibition of the growth of cancer cells through the induction of apoptosis.[17]

Previous studies on *T sinensis* and *A camphorata* indicated that *T sinensis*[18] or *A camphorata*[19] induced apoptosis in human leukemia (HL-60) cells. However, a combined treatment has yet to be tested. To the best of our knowledge, our study is the first to focus exclusively on the combined treatment of *T sinensis* and *A camphorata* on HL-60 cells. Additionally, we tested whether this combination exhibited any anticancer activity in HL-60 cells through the apoptotic pathway. Furthermore, the synergistic effect was evaluated. Moreover, molecular mechanisms related to this effect were demonstrated.

Methods

Reagents and Antibodies

RPMI 1640, glutamine, fetal bovine serum (FBS), and penicillin-streptomycin were from GIBCO Laboratories (GIBCO BRL). We procured PARP and rabbit polyclonal antibody from Upstate Biotechnology. Bid was obtained from Cell Signaling Technology Inc. Rabbit polyclonal antibodies against Bcl-2, Bax, FasL, MMP-2, MMP-9, uPAR, caspase-3, cytochrome c, Fas, and β-actin were obtained from Santa Cruz Biotechnology Inc. All the remaining secondary antibodies were obtained from Santa Cruz Biotechnology. Propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and DiOC6 were obtained from Sigma-Aldrich. The chemiluminescence kit was from Pierce Company. All remaining reagents were of HLPC grade and bought either from Sigma Chemicals Co (MO, USA) or Merck & Co (NJ, USA).

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Extraction From Toona sinensis

Toona sinensis leaves were procured from Fooyin University, Kaohsiung, Taiwan. Dr Horng-Liang Lay (from the Graduate Institute of Biotechnology at National Pingtung University, Taiwan) characterized the leaf extract and a sample was deposited (FY-001) at China Medical University (CMU), Taichung, Taiwan. We used aqueous extracts of the *T sinensis* from its leaves, a procedure that was previously reported.[18] The supernatant was secured using the centrifugation of crude extracts of *T sinensis*. The yield (extract) from the *T sinensis* leaves was 10%.

Antrodia camphorata *Fermented Broth Preparation From Submerged Culture*

Antrodia camphorata was collected from Nantou County, Taiwan. All *A camphorata* specimens used in this study were saved in the CMU repository and named "CMU-AC010." Dr Shy-Yuan Hwang from the Endemic Species Research Institute in Nantou, Taiwan, characterized the fermented broth prepared from the *A camphorata*. The sample was plated on potato dextrose agar and incubated for 15 to 20 days at 30 °C. The procedure followed for the preparation of *A camphorata* fermented culture broth was the same as explained before.[20] The yield of the dry matter was determined to be 18.4 g/L. All powdered samples were rendered in Dulbecco's modified Eagle's medium containing 1% FBS (pH 7.4) and were saved at −20 °C. Approximately 2 to 4 batches of fermented *A camphorata* culture were involved in our experiments.

Culturing of HL-60 Cells

The human acute promyelocytic leukemia (HL-60) cell line was procured from Bioresource Collection and Research Center (BCRC), Hsinchu, Taiwan. They were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 1% streptomycin-neomycin-penicillin, and 2 mM glutamine in a humidified incubator supplemented with 5% CO_2 at 37 °C. The human umbilical vein endothelial cells (HUVECs) were cultured and maintained as given in our previous study without modification.[21]

Quantification of Viable Cells

We seeded as follows: 2×10^5 cells per well in a 12-well plate. These cells were resolved to increasing concentrations of *T sinensis* (6.25-25 μg/mL) and/or *A camphorata* (10-40 μg/mL) for 24 hours. After incubation, all viable cells were quantified using the trypan blue exclusion method. Drug treatment impact on cell morphology was visualized with a phase-contrast microscope (200× magnification).

Measurement of Toona sinensis *and* Antrodia camphorata *Combination Index Values*

The combination index (CI) for *T sinensis* and *A camphorata* was evaluated with the Chou-Talalay method.[22,23] We used Biosoft CalcuSyn software (Biosoft, Cambridge, UK) to tally the CI index. This index determined if the combined effect of both compounds showed additivity, synergy, or antagonism mechanisms. The formula used to tally the CI value is as follows:

$$\mathrm{CI} = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} + \frac{(D)_1(D)_2}{(D_x)_1(D_x)_2}$$

where $(D_x)_1$ and $(D_x)_2$ are the concentrations of the tested substances 1 and 2 used in the single treatment that was required to decrease the cell number by x%. $(D)_1$ and $(D)_2$ are the concentrations of the tested substance 1 in combination with the concentration of the tested substance 2 that together decreased the cell number by x%. We considered 1 and 2 as *T sinensis* and *A camphorata*, respectively. The CI value quantitatively defines synergism ($\mathrm{CI} < 1$), additive effect ($\mathrm{CI} = 1$), and antagonism ($\mathrm{CI} > 1$).

Flow Cytometry

We plated 2×10^5 HL-60 cells in a 60 mm dish. The cells were administered *T sinensis* (25 μg/mL) and/or *A camphorata* (40 μg/mL) treatment for 24 hours. Posttreatment, we harvested and fixed cells using 70% ethanol and saved them at −20 °C overnight. Later, we resuspended the cells in PBS that contained 1% Triton X-100, 0.5 mg/mL of RNase, and 4 μg/mL of PI for 30 minutes at 37 °C. We performed flow cytometry analysis with a FACS Calibur flow cytometer (488 nm; Becton Dickinson, CA, USA).

Western Blot

Cold PBS-washed HL-60 cells (5.0×10^5 cells/mL) were exposed to the lysis buffer (10 mM Tris-HCL [pH 8], 5 mM EDTA, 2 mM DTT, 1 mM phenylmethylsulphonyl fluoride, 1% Triton X-100, and 0.32 M sucrose) was added to the cold PBS-washed HL-60 cells (5.0×10^5 cells/mL) and incubated on ice for 20 minutes. Subsequently, we clarified total protein content from the cell suspension. Protein sample concentrations were determined using the Bio-Rad protein assay method.

We used equal concentrations of the denatured proteins and then separated them by using the SDS-PAGE gel method (10%); then, we transferred them on to PVDF membranes. We used and performed a Western blot method from the literature.[24] Protein bands from membranes were visualized and then these images were captured with a

Table 1. The Synergistic Effects of *Toona sinensis* and *Antrodia camphorata* on HL-60 Cells[a].

Treatment	μg/mL	Cell number (%)	Predicted value[b]	Combination index[c]
Toona sinensis	6.25	92.1 ± 3.1	–	–
	12.5	88.0 ± 4.3	–	–
	25	68.3 ± 5.7	–	–
Antrodia camphorata	10	91.9 ± 2.5	–	–
	20	86.2 ± 3.7	–	–
	40	75.5 ± 4.4	–	–
T sinensis + *A camphorata*	6.25 + 10	78.7 ± 3.1	84.6	0.55
	6.25 + 20	73.2 ± 2.5	79.4	0.60
	6.25 + 40	24.7 ± 2.2	69.5	0.12
T sinensis + *A camphorata*	12.5 + 10	72.7 ± 2.8	80.9	0.69
	12.5 + 20	64.1 ± 3.1	75.9	0.59
	12.5 + 40	16.8 ± 2.8	66.4	0.09
T sinensis + *A camphorata*	25 + 10	62.0 ± 3.1	62.8	0.76
	25 + 20	55.4 ± 3.1	58.9	0.66
	25 + 40	5.9 ± 1.6	51.6	0.04

[a]HL-60 cells were treated with increasing concentrations of either *Toona sinensis* (6.25-25 μg/mL) and/or in combination with *Antrodia camphorata* (10-40 μg/mL) for 24 hours. After the incubation period, the effect of individual or co-treatments of both *T sinensis* and *A camphorata* on HL-60 cell survival as well as proliferation were determined according to the Chou and Talalay method. Low combination index value signifies higher synergistic effect.
[b]Predicted value: (%A × %B)/100.
[c]Combination index according to Chou and Talalay[22]; values <1 indicates synergism.

Super Signal ULTRA chemiluminescence substrate (Pierce Biotechnology). We performed densitometric analysis using AlphaEase software (Genetic Technology Inc). We expressed protein data as the fold-over of control values for which the control value was given as one.

Measurement of Mitochondrial Membrane Potential

We incubated HL-60 cells (2×10^5 cells/dish) with *T sinensis* (25 μg/mL) and/or *A camphorata* (40 μg/mL) in a 60 mm dish for 24 hours. Posttreatment, we measured harvested cells and used a procedure that allowed mitochondrial membrane potential to be measured in accordance with a previous study.[25]

Quantification of PGE_2 Production

We seeded and grew HL-60 cells in a 12-well plate. Then, we incubated them with *T sinensis* (25 μg/mL) and/or *A camphorata* (40 μg/mL) for 24 hours. After treatment, 100 μL of conditioned medium was amassed and then we determined the PGE_2 concentration via the ELISA (Cayman Enzyme Immunoassay Kit) method.

Statistical Analysis

We used mean ± standard deviation for all expressed data used in this study. Then we used analysis of variance with Dunnett's test for pairwise comparison of the control group and the test groups. We assigned statistical significance as $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$ when compared with control cells, and $^{\#}P < .05$, $^{\#\#}P < .01$, and $^{\#\#\#}P < .001$ when compared with *T sinensis* or *A camphorata* cells only.

Results

Toona sinensis *and* Antrodia camphorata *Co-Treatment Exhibited Low CI Values in HL-60 Cells*

First, we looked at the effect of individual concentrations of *T sinensis* (6.25-25 μg/mL) or *A camphorata* (10-40 μg/mL) on HL-60 cell survival and proliferation. Table 1 shows that after 24 hours of treatment, *T sinensis* showed 68.3 ± 5.7% cell viability at 25 μg/mL. Whereas at 40 μg/mL concentration, *A camphorata* showed 75.5 ± 4.4% cell viability. Later, we tested the effects of various combinations using different concentrations of *T sinensis* and *A camphorata* on HL-60 cells; and we measured CI values. Our data showed that combined treatments (25 + 40 μg/mL) exhibited a lower CI value of 0.04 to signify that the synergistic growth inhibition effect in HL-60 cells was apparent. When compared with the individual treatments, the combined treatment has a more beneficial effects toward inhibiting HL-60 cell numbers.

Toona sinensis *and* Antrodia camphorata *Synergistically Inhibited HL-60 Cell Growth*

From the above observations, the synergistic effect of the combined treatment on HL-60 growth inhibition was further

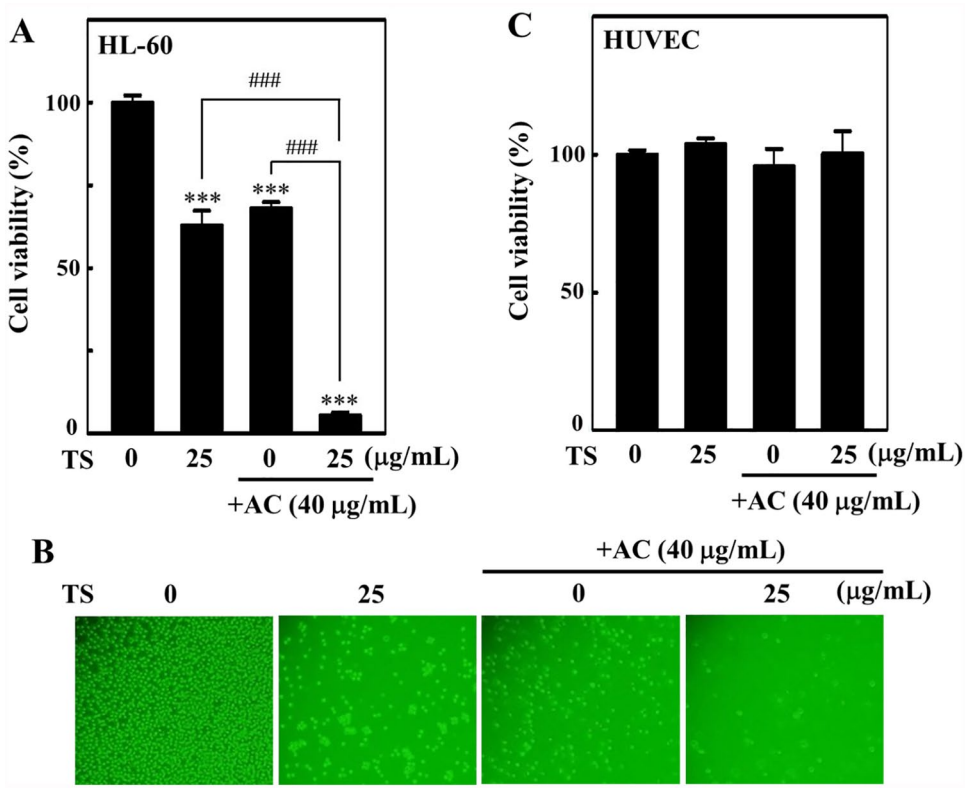

Figure 1. Effect of *Toona sinensis* and *Antrodia camphorata* co-treatment on HL-60 cell viability. (A, B) HL-60 cells were treated with 25 μg/mL *T sinensis* or 40 μg/mL *A camphorata* or in combination for 24 hours. Morphological changes were observed under the phase-contrast microscope (200× magnification). Using the trypan blue exclusion assay, HL-60 cell viability was determined. (C) HUVECs (human umbilical vein endothelial cells) were treated with 25 μg/mL *T sinensis* or 40 μg/mL *A camphorata* or combination of both for 24 hours and then the cell viability was measured. Values were expressed as mean ± standard deviation (n = 3). Statistical significance was assigned as $^{***}P < .001$ compared with untreated control cells and $^{\#\#\#}P < .001$ compared with *T sinensis* or *A camphorata* alone treated cells.

demonstrated. In comparison with untreated control cells, *T sinensis* (25 μg/mL) or *A camphorata* (40 μg/mL) alone treatments also significantly suppressed HL-60 cell viability by nearly 35% and 30%, respectively. Conversely, the combined treatment diminished the HL-60 cell viability by nearly 90%. These data signify the synergistic inhibitory effect of the combined treatment on HL-60 cells (Figure 1A). Furthermore, our phase-contrast microscope data also indicated that when compared with individual treatments, the combined treatment was responsible for significant inhibition of HL-60 cells (Figure 1B). We also tested for cytotoxic effects caused by the combined treatment on HUVECs. Interestingly, our cell viability data showed that neither *T sinensis* and *A camphorata* alone or the combined treatment showed significant cytotoxic effects in HUVECs (Figure 1C). These data indicated that anti-cell proliferative effects exhibited by individual extracts or in combination are associated with cancer cells only and had no significant deleterious effect on normal cells. Therefore, the combined treatment is safe for normal cells.

The Combined Treatment Induced the Accumulation of Sub-G_1 Cells and Apoptosis in HL-60 Cells

The effects of the combined treatment on the accumulation of sub-G_1 cells and apoptosis were examined with flow cytometry analysis to better quantify PI-DNA complex in HL-60 cells. Figure 2 shows that when compared with the *T sinensis* or *A camphorata* alone treatments, the combined treatment substantially increased the sub-G1 accumulation of HL-60 cells (Figure 2A). Also, this combinational treatment has significantly upregulated the apoptotic cells by about 45% compared with the control cells (Figure 2B). These data indicate that *T sinensis* and *A*

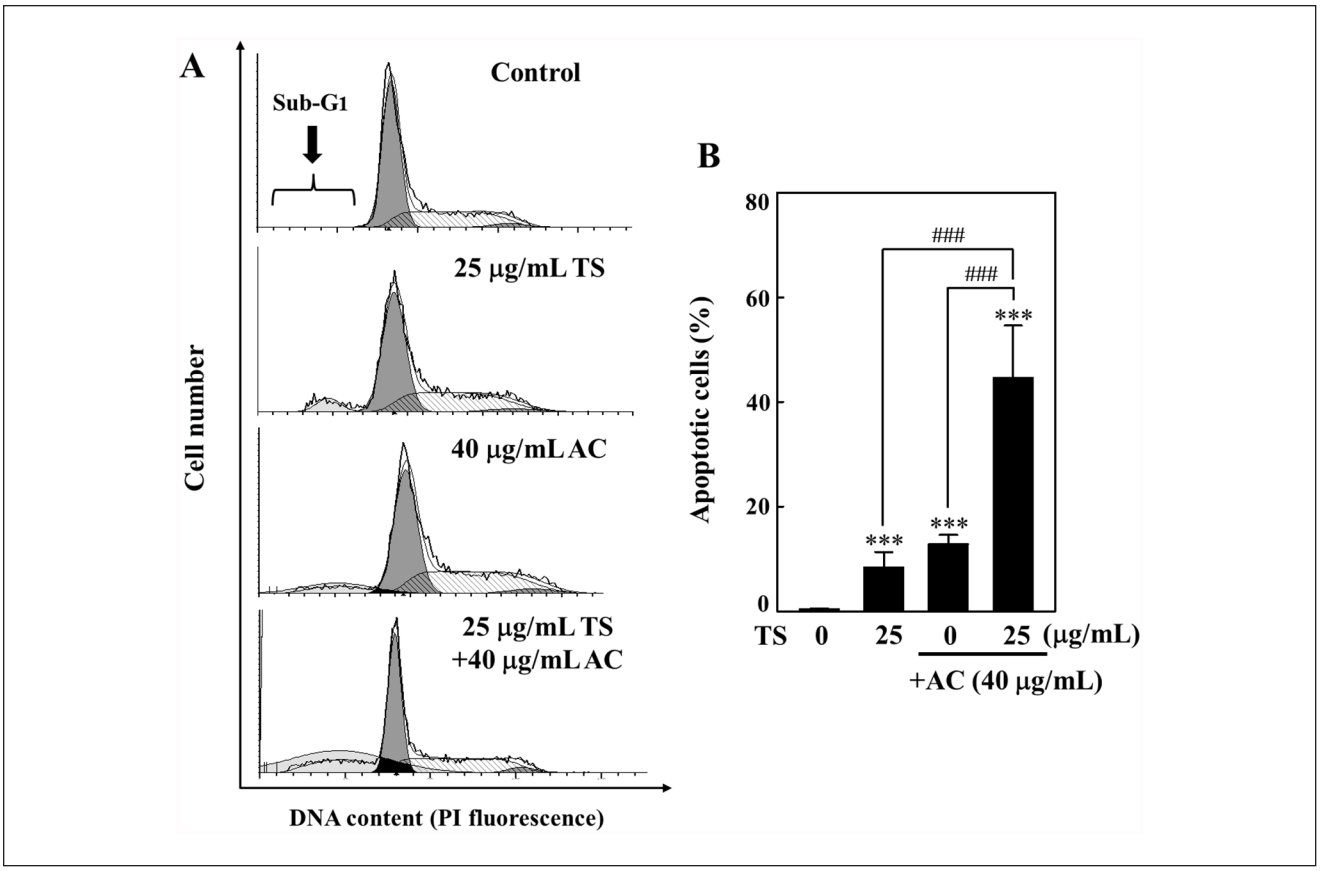

Figure 2. Effect of *Toona sinensis* and/or *Antrodia camphorata* treatment on sub-G_1 cell cycle of HL-60 cells. (A) HL-60 cells were treated with 25 μg/mL *T sinensis* or 40 μg/mL *A camphorata* or in combination for 24 hours and then analyzed by flow cytometry. (B) Percentage of sub-G_1 cell distribution after *T sinensis, A camphorata*, treatments were presented. All values are expressed as mean ± standard deviation (n = 3). Statistical significance was assigned as $^{***}P < .001$ compared with untreated control cells and $^{\#\#\#}P < .001$ compared with *T sinensis* or *A camphorata* alone treated cells.

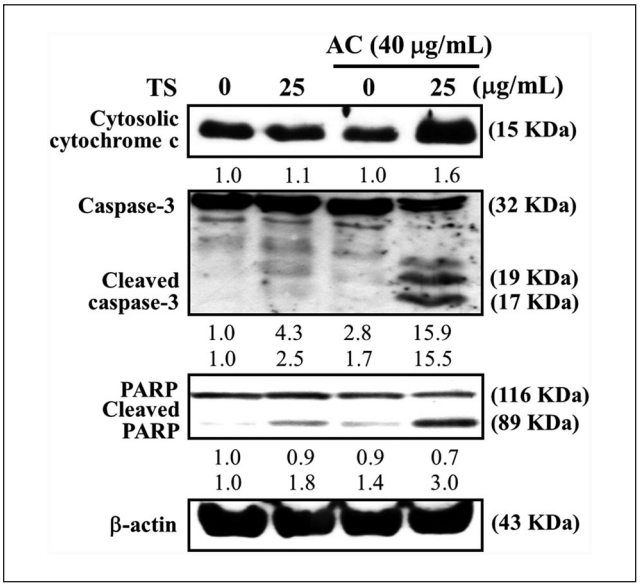

Figure 3. *(continude)*

Figure 3. *Toona sinensis* and/or *Antrodia camphorata* treatment induced the release of cytochrome c in HL-60 cells. HL-60 cells were treated with 25 μg/mL *T sinensis* or 40 μg/mL *A camphorata* or in combination for 24 hours. The expression of cytosolic cytochrome *c*, caspase-3, and PARP proteins were measured by Western blot method using β-actin as an internal control. Densitometric analysis was performed using the AlphaEase (Genetic Technology Inc) with the value of control assigned as 1.

camphorata exhibit protective effects in HL-60 cells via apoptotic mechanisms.

The Combined Treatment Induced the Release of Cytosolic Cytochrome c in HL-60 Cells

Furthermore, we have measured the expression patterns for various proteins associated with the combined treatment, which mediated apoptosis mechanisms in HL-60 cells. Our Western blot data showed that in comparison to untreated controls and *T sinensis* or *A camphorata* alone

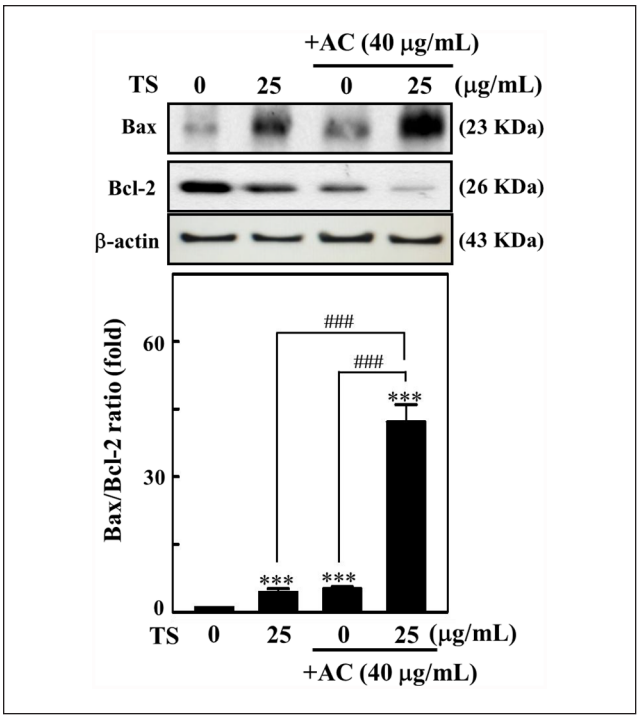

Figure 4. Effect of *Toona sinensis* and/or *Antrodia camphorata* treatment on Bax/Bcl-2 ratio in HL-60 cells. HL-60 cells were treated with 25 μg/mL *T sinensis* or 40 μg/mL *A camphorata* or in combination for 24 hours. The expression of Bax, Bcl-2 proteins were measured by Western blot using β-actin as an internal control. Densitometric analysis was performed using the AlphaEase (Genetic Technology Inc) with the value of control assigned to be 1.

treated cells, the combined treatment has increased the expression of cytosolic cytochrome c, cleaved caspase-3 (19, 17 KDa), and cleaved PARP (85 KDa) proteins. These results indicated that the combined treatment effectively induces apoptosis-mediated cell death in HL-60 cells. This indicates that a caspase-dependent mitochondrial mechanism is involved (Figure 3). This observation is in support of our previous 2 studies conducted on HL-60 cells that were tested with either *T sinensis* or *A camphorata*.[18,19]

The Combined Treatment Upregulated the Bax/Bcl-2 Ratio in HL-60 Cells

The Bcl-2 family of proteins plays a pivotal role as inhibitors or as activators in mitochondria-mediated apoptosis. These proteins are as follows: Bcl-xl, Bcl-w, and Bcl-2; and Bax, Bad, and Bok.[26] We determined the effect of the combined treatment on the expression patterns of Bax and Bcl-2 proteins in HL-60 cells. Figure 4 shows that when compared with control cells, the combined treatment has substantially increased pro-apoptotic Bax protein expression and decreased the expression of anti-apoptotic Bcl-2 protein levels in HL-60 cells. Also, the Bax/Bcl-2 ratio also indicated a significant increase (~45-fold) in the Bax value compared with the Bcl-2 expression. These data further showed that the combined treatment induces HL-60 cells to undergo apoptosis via the Bax pathway (Figure 4).

The Combined Treatment Downregulated the Mitochondrial Membrane Potential in HL-60 Cells

We used the flow-cytometry method to analyze whether *T sinensis* and *A camphorata* both cause dysfunction in mitochondrial membrane potentials ($\Delta\psi m$) in HL-60. The $DiOC_6$-stained cells showed that, when compared with the individual treatments, the combined treatment significantly reduced the mitochondrial membrane potential in HL-60 cells. Compared with the control cells, this effect seems significant. These data indicated that the combined treatment induced apoptosis and was mediated by mitochondrial dysfunction mechanisms (Figure 5).

The Combined Treatment Induced Apoptosis-Mediated Tumor Invasion in HL-60 Cells

CD95 (Fas/APO-1) and related ligand CD95L (FasL) have historically been viewed as a death receptor/death-ligand system via mediation of apoptosis induction in various cancer cells.[27] In this study, we measured the effect of *T sinensis* and *A camphorata* on the expression patterns of Fas and FasL proteins that lead to the death receptor–associated apoptosis pathway. Our Western blot data further showed that, when compared with the individual treatments, the combined treatment upregulated the expressions of Fas and FasL levels in HL-60 cells and simultaneously downregulated the Bid protein expression. Our data confirmed that the combined treatment has synergistically promoted apoptosis by the mitochondrial and death receptor pathways (Figure 6A).

MMP overexpression is linked to various cancer metastases.[28] MMP-9, urokinase plasminogen activator and its receptor (uPA, uPAR) system are linked to tumor migration and metastasis.[29] Therefore, we tested the effect of individual or combined treatment on the expression patterns of these proteins. Our Western blot data further indicated that the combined treatment significantly downregulated the expression of MMP-2, MMP-9, and uPAR proteins to induce anti-invasive and anti-migratory effects (Figure 6B). All these data suggested that the combined treatment has significant anti-tumor activity via the induction of apoptosis mechanisms in HL-60 cells.

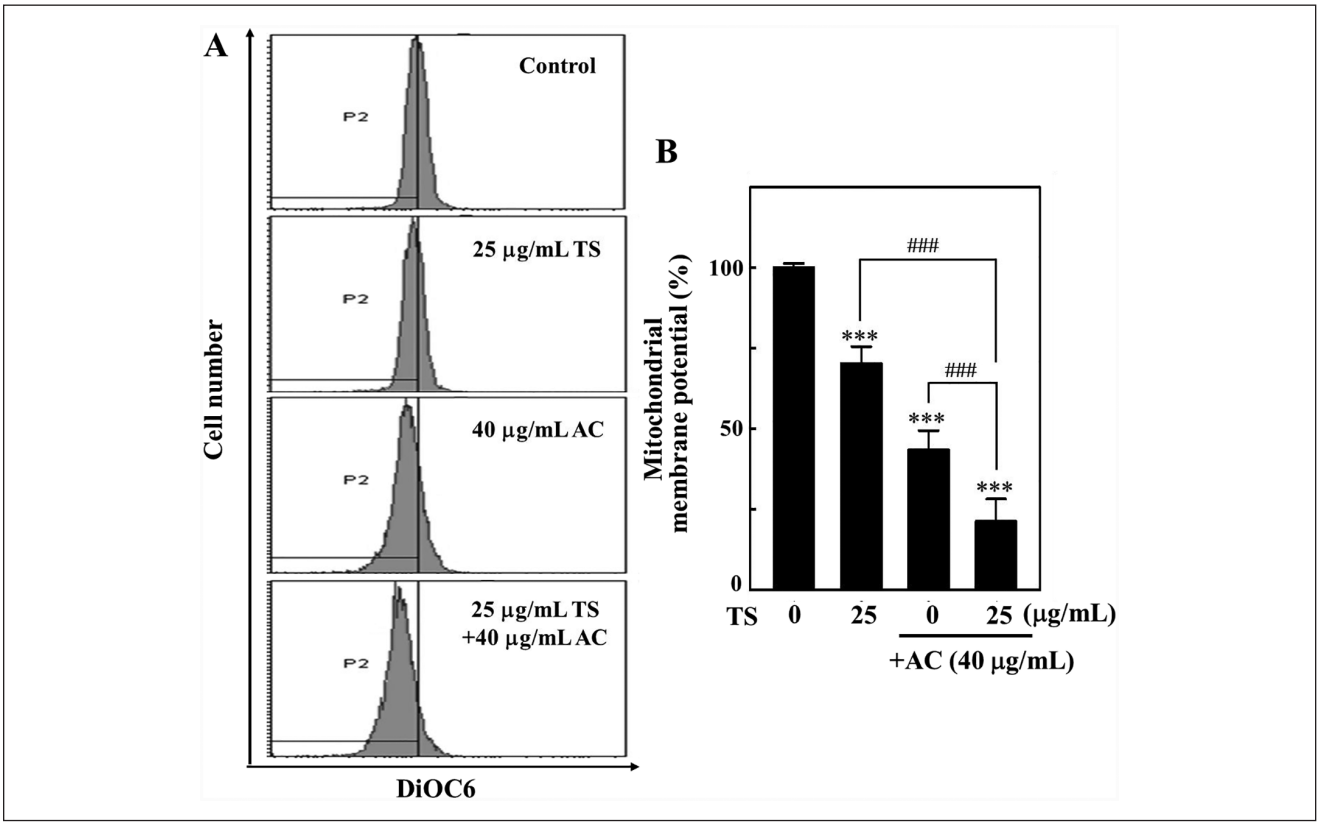

Figure 5. Effect of *Toona sinensis* and/or *Antrodia camphorata* treatment on HL-60 cell mitochondria membrane potential. (A, B) HL-60 cells were treated with 25 μg/mL *T sinensis* and/or 40 μg/mL *A camphorata* for 24 hours, followed by measurement of mitochondrial membrane potential by flow cytometry. The percentage of mitochondrial membrane potential was indicated by $DiOC_6$ fluorescence. All the values are expressed in mean ± standard deviation (n = 3). Statistical significance was assigned as $^{***}P < .001$ compared with untreated control cells and $^{###}P < .001$ compared with *T sinensis* or *A camphorata* alone treated cells.

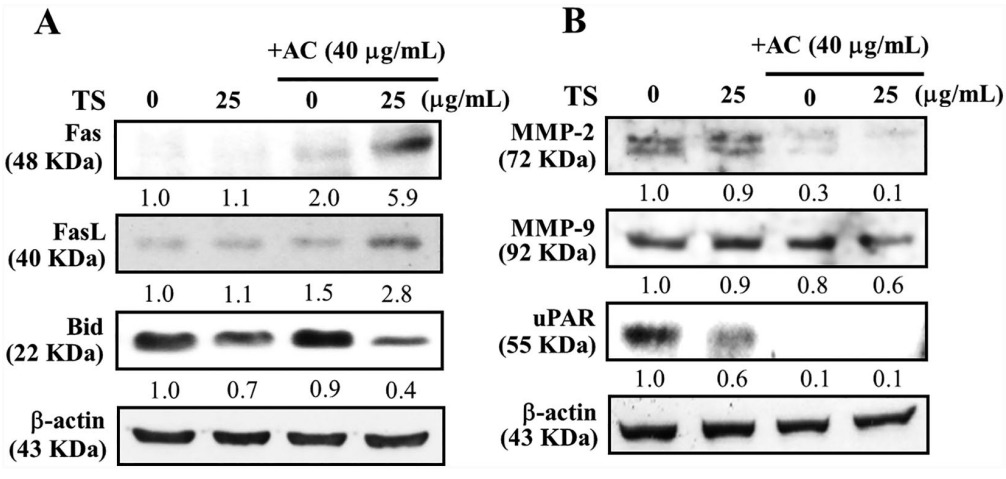

Figure 6. Effects of *Toona sinensis* and/or *Antrodia camphorata* treatment on the expression of various tumorigenic proteins in HL-60 cells. HL-60 cells were treated with 25 μg/mL *T sinensis* or 40 μg/mL *A camphorata* or in combination for 24 hours. Western blot method was used to measure (A) Fas, FasL, and Bid proteins; and (B) MMP-2, MMP-9, and uPAR proteins. All values were expressed as mean ± standard deviation (n = 3). β-actin was used as an internal control. Densitometric analysis was performed using the AlphaEase (Genetic Technology Inc) with the value of control assigned to be 1.

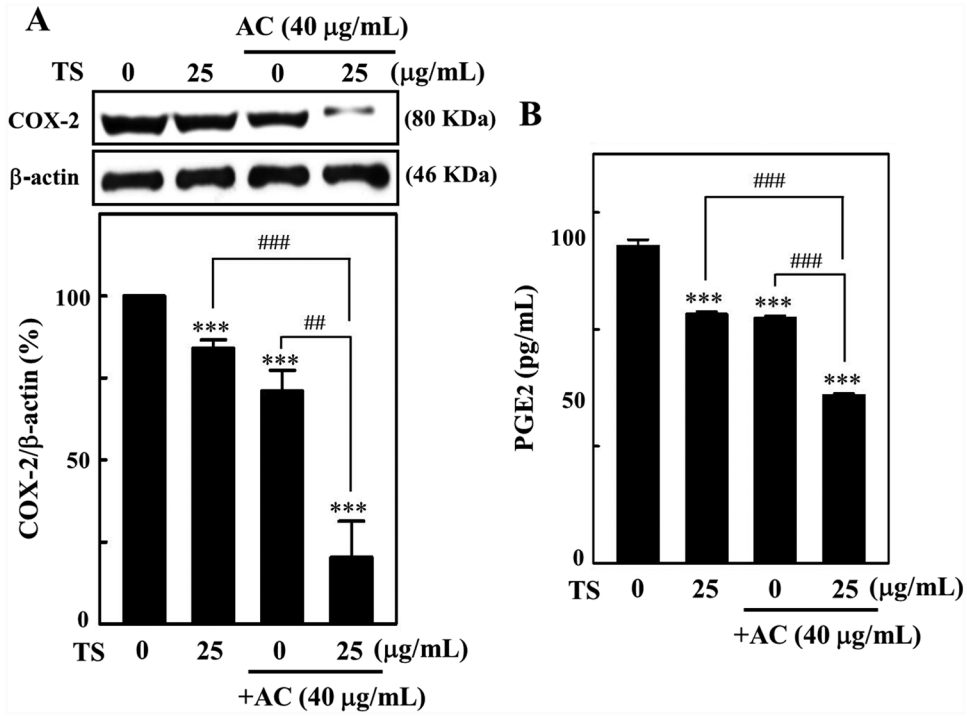

Figure 7. *Toona sinensis* and *Antrodia camphorata* co-treatment has downregulated the expression of COX-2 and PGE_2 production in HL-60 cells. HL-60 cells were treated with *T sinensis* (25 μg/mL), *A camphorata* (40 μg/mL), and *T sinensis* + *A camphorata* for 24 hours. (A) Western blot method was used to measure the expression of COX-2 protein. AlphaEase (Genetic Technology Inc) was used for the densitometric analysis. Control value was assigned as one. (B) Using the ELISA kit method, PGE_2 concentration in the culture media was also determined. All values were expressed as mean ± standard deviation (n = 3). Statistical significance was assigned as $^{***}P < .001$ compared with untreated control cells and $^{\#\#}P < .05$; $^{\#\#\#}P < .001$ compared with *T sinensis* or *A camphorata* alone treated cells.

Toona sinensis *and* Antrodia camphorata *Suppressed the Expression of COX-2 Enzyme Leading to Decreased Production of PGE_2 in HL-60 Cells*

Cyclooxygenase (COX-2) is an enzyme not generally expressed in resting cells; however, it can be induced in response to cytokines, various growth factors, and inflammatory stimuli. More recently, hematological malignancies are shown to constitutively express COX-2 when compared with normal cells. This indicates that COX-2 may act as an oncogene and its expression plays an important role in the pathogenesis of cancers of both nonhematological and hematological origin. Tumors that highly express COX-2 produce high levels of prostaglandins, including prostaglandin E_2 (PGE_2).[30] Our Western blot data obtained from *T sinensis* and *A camphorata* exposed HL-60 cells showed significant downregulation in the COX-2 enzyme expression as well as suppressed the production of PGE_2 (Figure 7).

Discussion

Folk and traditional medicine is an indispensable knowledge source of medicinal herbs and related curative properties. They also provide clues for further scientific research and continue to confirm their significance to medical science. In cancer treatment, chemotherapy is vital at nearly every phase. The idea of combined treatments enhances the effectiveness of various agents by prompting a synergistic therapy and lessens the side effects often associated with single-agent therapies.[31] In our study, we used a combined or individual treatments for leukemia and we also looked how synergistic activity against HL-60 cells occurred. From earlier studies, we noted that *A camphorata* induced apoptosis in HL-60 cells and blocked tumor growth in athymic nude mice,[32] while *T sinensis* demonstrated anti-cancer activity against the same through the induction of apoptosis.[18]

Table 1 shows that we first tested and measured the effects of various concentrations of *T sinensis* and *A camphorata* on HL-60 cells and their CI values. From the percentage of cell

number and CI values, we demonstrated that the combined treatments of 25 µg/mL of *T sinensis* and 40 µg/mL of *A camphorata* exhibited a synergistic effect in HL-60 cells. From this, we used the same concentrations of *T sinensis* and *A camphorata* to study any other effects on these cells.

Later, we tested the cytotoxic effect of the combined treatment on HL-60 cell proliferation. Our MTT data showed that, when compared with the individual treatments, the combined treatment significantly suppressed the HL-60 cell proliferation (Figure 1A and B). Interestingly, similar treatment in HUVEC cells did not show any cytotoxic effects (Figure 1C). These data clearly showed that the combined treatment has a potent effect against cancer cells only. This anti–cell proliferative effect of the combined treatment was further evidenced from the flow cytometry data that showed the accumulation of HL-60 cell population in the sub-G_1 phase and an increased percentage of cells that had undergone apoptosis. This was indicative that the combined treatment synergistically exhibited apoptotic mechanisms in HL-60 cells (Figure 2).

Apoptosis consists of caspase-dependent pathways through mitochondria (intrinsic) or death receptor (extrinsic) pathways.[33] Apoptosis initiation is caused by mitochondrial dysfunction along with potential losses of mitochondrial membranes and cytochrome c released to the cytosol from mitochondria. We looked for caspase-3 with the treatment of *T sinensis* and *A camphorata* individually and the combined treatment because cytochrome c participates in the activation of downstream caspases that trigger apoptosis. After cytochrome c is released, it initially activates caspase-9 and it binds with Apaf-1 to activate caspases-3. After the activated caspase-3 enters the nucleus, it cleaves PARP, a 115 KDa fragment, into an 89 KDa inactive fragment, which leads to apoptosis.[34] From this, we further tested the molecular parameters of the expression of various proteins that were associated with the *T sinensis*- and *A camphorata* -mediated apoptosis in HL-60 cells. Figure 3 shows that our Western blot data obtained from the expressions of cytosolic cytochrome c, cleaved caspase-3 (19, 17 KDa), and cleaved PARP (85 KDa) proteins showed that the combined treatment significantly upregulated the expression of these proteins to demonstrate that it is effectively inducing the caspase-dependent mitochondrial apoptotic mechanism in HL-60 cells. Our results are consistent with previous reports.[18,32]

The Bcl-2 family proteins play a vital regulatory role in the mitochondrial-related intrinsic apoptosis pathway, while Bax critically causes apoptotic cell death. It is noteworthy that Bcl-2 and Bcl-xL cause limited resistance to normal cancer treatments. From this, the balance between Bax and Bcl-2 protein levels is important for apoptosis to occur.[35] The literature shows that Bax promotes mitochondrial membrane permeability and is accompanied by the release of cytochrome c, which eventually causes apoptosis.[35] The combined treatment notably increased Bax expression and decreased Bcl-2 expression when compared with individual agents, which demonstrates it induced apoptosis in HL-60 cells by altering Bax/Bcl-2 ratio by 45-fold (Figure 4).

Ly et al reported that mitochondrial dysfunction participated in causing apoptosis, which suggests it is central to the apoptotic pathway.[36] We also tested if the combined treatment induced dysregulation in the mitochondrial membrane potential ($\Delta\psi m$) in HL-60 cells. Our flow cytometry data indicated that, when compared with the individual treatments, the combined treatment significantly reduced the mitochondrial membrane potential in HL-60 cells. Hence, the apoptosis process caused by the combined treatment was mediated via the dysfunction of mitochondrial membrane potential ($\Delta\psi m$; Figure 5).

The activation of the extrinsic pathway/death receptor is depicted by increased expression of Fas and FasL, which sequentially propagate cell death. The death receptors TRAIL and Fas activate the extrinsic pathway. Fas-FasL mediated signaling is well known because of its death-inducing function. When FasL is bound to the Fas receptor, they recruit the Death-Inducing Signaling Complex (DISC) that consists of Fas-Associated Death Domain (FADD) adaptor protein and inactive caspase-8. The link between the intrinsic and extrinsic apoptotic pathways is represented by caspase-8. Following the activation of caspase-8 in the DISC complex, it is released from FADD, which in turn subsequently initiates a cascade of events leading to apoptosis.[37] In certain conditions, a mitochondrial amplification loop is essential for the activated caspase-8, which mediates the partition of Bid into tBid. tBid becomes translocated into the mitochondria, which causes the eventual release of cytochrome c and causes apoptosis. This represents a link between intrinsic and extrinsic apoptotic pathways.[38] Peter et al reported that Fas and FasL have long been considered in the death ligand system, which has protective characteristics against cancer cells. This system supposedly caused apoptosis in cancer cells and exhibited a protective effect.[27]

MMPs and uPA help primary tumors to metastasize and includes the following processes: basement membrane degradation, cell migration via extracellular matrix, extravasation at distant organ sites, and new tumor growth.[28,39] Their inhibition often leads to the prevention of metastasis. Increased expressions of MMPs levels in leukemia patients correlate with lower survival rates and poorer responses to chemotherapy.[40] MMP-2 and MMP-9 are overexpressed in various cancer cells and serve in the invasion and metastasis of tumors.[41] Research shows that several chemopreventive or chemotherapeutic agents inhibit MMP-9 and suppress invasion and metastases in cancer cells.[42] Our Western blot data showed that, when compared with the individual treatments, the combined treatment has significantly induced the expression of Fas and FasL proteins and simultaneously downregulated the Bid expression (Figure 6A). However,

the combined treatment significantly downregulated the MMP proteins (MMP-2, MMP-9) and uPAR expression as well (Figure 6B). These data indicated that the combined treatment has significant antitumor activity in HL-60 cells via the induction of the apoptosis pathway.

Recent research has suggested that COX-2 inhibition and PGE_2 suppression elicit chemopreventive and chemotherapeutic effects that are combined with the cause of apoptosis in cancer cells.[43] COX-2 overexpression is linked apoptosis resistance in various cell types including leukemia cells, which disrupts organ homeostasis.[44] Research shows that intrinsic and extrinsic apoptotic pathways were activated by COX-2 mediated chemosensitization.[45] Numerous anticancer agents such as mitomycin C[46] and paclitaxel[47] induced COX-2 expression, which results in inflammation. *T sinensis* and *A camphorata* treatment showed a modest effect, whereas the combination treatment effectively inhibited the COX-2 and PGE_2 expression and elicited apoptosis in HL-60 cells (Figure 7A and B). We assumed that the combined treatment induced apoptosis, which might be due to the inhibition of COX-2 signaling.

NF-κB has been known to regulate the expression of many genes in modulating cellular proliferation, inflammatory responses, and apoptosis. Han et al investigated the effects of L-ascorbic acid on eukaryotic transcription factor NF-κB and COX-2 expressions in HL-60 cells. They reported that L-ascorbic acid downregulated the expression of COX-2, which has a NF-κB binding site on its promoter, through repressing NF-κB DNA binding activity. This demonstrates that the presence of NF-κB upstream to COX-2 has a specific role in HL-60 cells.[48] Besides this, in 2009, Kurihara et al reported that selective COX-2 inhibition suppresses the invasive activity of human oral squamous cell carcinoma cells via downregulation of an MMP-2 activating mechanism involving TIMP-2 and production of the MMP-2 protein by an interaction between cancer cells and stromal fibroblasts.[49] From this, it seems reasonable to suggest that NF-κB is playing a crucial role in the suppression of COX-2 and MMP-2. Future research needs to be conducted to study the detailed mechanisms by which the combined treatment modulates COX-2 expression.

Increasing evidence indicates that traditional medicines have exhibited chemotherapeutic or chemoprophylaxis effects against various cancers. *T sinensis* is rich in phytochemicals, such as steroids, polyphenols, alkaloids, tannins, lignans, and flavonoids.[50] Recently, many active compounds have been identified from *T sinensis* such as kaempferol, ethyl gallate, quercetin, gallic acid, kaempferol-3-O-β-D-glucoside, rutin, palmitic acid, catechin, and methyl gallate.[51] Among them, gallic acid causes mitochondrial apoptosis in various cancers.[52] Furthermore, quercetin and kaempferol causes apoptosis by interfering with the cell cycle.[53]

Antrodia camphorata contains the following bioactive components: steroids, benzenoids, triterpenoids, polysaccharides, and succinic/maleic acid derivatives, which indicate and exhibit several biological properties.[6,54] The literature reports yields of triterpenoids, polysaccharides, and total polyphenols as 47 mg/g, 23 mg/g, and 67 mg/g, respectively, in fermented *A camphorata* broth.[55] Furthermore, antroquinonol was extracted from mycelium broth and indicated cytotoxicity against breast cancer cells.[56] Three estrogen-type triterpenes and 5 lanostanes, extracted from *A camphorata* fruit have yielded potent cytotoxicity in vitro against various cancer cells.[57]

Future Perspectives

Although several compounds have been found in the extracts of *T sinensis*[15] and *A camphorata*,[17] the primary objective of this study was to demonstrate the synergistic contribution of the anticancer potential of *T sinensis* and *A camphorata* against HL-60 cells through an apoptotic pathway. Our results strongly suggested that the combined treatment of *T sinensis* and *A camphorata* has offered more beneficial effects on the suppression and treatment of leukemia. However, further investigations in the directions of measurement of MMP activity (zymography assay), the Fas/FasL-activated caspase-8 levels (extrinsic pathway of apoptosis pathway) in the individual as well as in combined groups, and *in vivo* studies could provide complete information about this synergistic mechanism. These aspects are in consideration for our future studies.

Conclusions

Recently, research has suggested that bioactive compounds isolated from various traditional herbs can augment chemotherapy because they have caused apoptosis and shown anticancer potential *in vivo* and *in vitro*.[51,58] Our study has established that the combined treatment demonstrated synergistic effects via apoptotic cell death and modulated both the COX-2 and MMP proteins. *T sinensis* and *A camphorata* have mechanisms related to the mitochondrial signaling pathways, which cause both intrinsic and extrinsic apoptotic pathways. Our findings strongly imply that the combined treatment could be an effective method in the suppression and treatment of leukemia.

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Author Contributions

YCH and HLY: Designed and supervised the study. YTK, YVG, KYL, LSH, PJH, HCL: Performed the experiments, collected and interpreted the data. YCH and HLY: Contributed reagents, materials, and analysis tools. YVG and YCH: drafted and reviewed the original manuscript. All authors read and approved the intellectual content and final version of the manuscript.

Declaration of Conflicting Interests

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