Integrative Cancer Therapies
2017, Vol. 16(3) 308–318

DOI: 10.1177/1534735416642863
journals.sagepub.com/home/ict
SAGE

Toona sinensis Inhibits Murine Leukemia WEHI-3 Cells and Promotes Immune Response In Vivo

Hsin-Ling Yang, PhD[1], Varadharajan Thiyagarajan, PhD[1], Jiunn-Wang Liao, PhD[2], Yu-Lin Chu, MS[1], Chia-Ting Chang, MS[1], Pei-Jane Huang, PhD[3], Chih-Jung Hsu, PhD[4], and You-Cheng Hseu, PhD[1,3]

Abstract

Toona sinensis (TS) is one of the most popular vegetarian dishes in Taiwan. It has been shown to exhibit antioxidant, antiangiogenic, antiatherosclerotic, and anticancer properties. In this study, we demonstrated the ability of aqueous leaf extracts from TS to promote immune responses in BALB/c mice and to exhibit anti-leukemia activity in murine WEHI-3 cells. BALB/c mice were injected intravenously with WEHI-3 cells and then treated orally with TS (50 mg/kg). In vivo study showed that TS treatment reduced liver and spleen enlargement in WEHI-3 bearing mice compared with the untreated group. Furthermore, TS also decreased white blood cells (WBC), indicating inhibition of differentiation of the precursor of macrophages in WEHI-3 bearing mice. Treatment of WEHI-3 cells with TS (0-75 µg/mL for 24 hours) significantly reduced cell viability. Furthermore, TS treatment–induced late apoptosis was confirmed by Annexin-V/PI staining. Western blot analyses revealed that treatment of WEHI-3 cells with TS statistically increased the protein expression level of cytochrome *c* in the cytoplasm and activates caspase-3. Notably, TS treatment caused a dramatic reduction in Bcl-2 and increase in Bax protein levels. TS may disturb the Bcl-2 and Bax protein ratio and induce apoptosis. This reports confirms the antitumor activity of this nutritious vegetable potentially against leukemia.

Keywords

Toona sinensis, WEHI-3 cells, immune response, BALB/c mice, apoptosis

Submitted Date: 16 November 2015; Revised Date: 1 March 2016; Acceptance Date: 3 March 2016

Introduction

Leukemia is a group of cancers that affects the lymphoid system, blood, and bone marrow as well as the differentiation of normal hematopoietic cells. Leukemia cells are unable to undergo terminal differentiation, growth arrest, and apoptosis in response to appropriate environmental stimuli.[1] Based on the cell lineage transformation and clinical features, 4 main types of leukemia have been determined, namely acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), and chronic myeloid leukemia (CML).[2]

Acute myeloid leukemia arises within the bone marrow from a malignant hematopoietic progenitor cell. It has an annual overall incidence of 3.8 cases per 100 000 in the United States and Europe.[3] AML is primarily a disease of the elderly, with an incidence of 15 cases per 100 000 for those older than 60 years.[3] Unfortunately, the 5-year overall survival in patients older than 75 years is less than 10%.[4] Several factors have been implicated in the causation of AML, including antecedent hematologic disorders, familial syndromes, environmental exposures, and drug exposures. Though a number of experimental drugs have been designed for the therapy of AML, most have failed in clinical trials. Except for gemtuzumab ozogamicin that has been recently withdrawn from the market, no new agent has yet been

[1]China Medical University, Taichung, Taiwan
[2]National Chung Hsing University, Taichung, Taiwan
[3]Asia University, Taichung, Taiwan
[4]China Medical University Hospital, Taichung, Taiwan

Corresponding Author:
You-Cheng Hseu, Department of Cosmeceutics, College of Pharmaceutical and Food Sciences, China Medical University, 91 Hsueh-Shih Road, Taichung 404, Taiwan.
Email: ychseu@mail.cmu.edu.tw

approved for AML in the past 40 years.[5] Thus, the discovery of more effective therapeutic agents is needed for AML.

Numerous herbs used in traditional Chinese medicine may be of great value. *Toona sinensis* Roem (Meliaceae) widely distributed in Asia has been used as a nutritious food for a long time. The edible leaves were used as an oriental medicine with no significant side effects for treatment of enteritis, dysentery, and dermatitis.[6] The pharmacological and biological activities of extract of *Toona sinensis* (TS) have been reported, including anticancer,[7,8] antioxidant,[9] antiatherosclerotic/inflammatory,[10] antidiabetic,[11] antivirus,[12,13] as well as inhibiting Leydig cell steroidogenesis and SARS (severe acute respiratory syndrome) coronavirus replication.[14] The phytochemical analyses of TS have isolated of triterpenes and phenolic compounds.[6] Fifteen compounds of TS were identified, including methyl gallate, gallic acid, kaempferol, quercitin, quercitrin, ritin, catechin, epicatechin, oleic acid, palmitic acid, linoleic acid, and linolenic acid [15]. It has been demonstrated that the phenolic acid, gallic acid, a major component of TS leaf extracts possesses antioxidant and anticancer activities.[16,17] The biologically active compound in TS extracts may be similar to natural compounds such as phenolic compounds, flavonoids, alkaloids, polysaccharides, and glycoproteins, which are known to induce cell death in cancer cells.[18,19] The safety levels and nontoxic characteristics of TS were evaluated using acute and subacute toxicity studies in mice and rats, and no lethal effects were found at concentrations as high as 1 g/kg body weight.[20,21] Although various bioactivity studies of TS have been carried out, there is no report addressing the effects of TS on the regulation of immune responses and antileukemia activity in murine leukemia WEHI-3 cells. In these studies, we show the effects of TS on induction of apoptosis of WEHI-3 cells and the promotion of immune responses, and its antileukemia activity in WEHI-3 leukemia BALB/c mice in vivo.

Materials and Methods

Chemicals

RPMI-1640 medium was obtained from Gibco BRL (Grand Island, NY, USA), anti-mouse Bax, anti-mouse β-actin, anti-rabbit Bcl-2, anti-rabbit caspase-3, and anti-rabbit cytochrome *c* antibodies were purchased from Santa Cruz Biotechnology, Inc (Heidelberg, Germany). All other chemicals were of the highest grade commercially available and supplied either by Merck (Darmstadt, Germany) or Sigma (St Louis, MO, USA).

Cell Culture

WEHI-3 cells, a murine myelomonocytic leukemic cell line, were obtained from the American Type Culture Collection (Rockville, MD, USA). These cells were grown in RPMI-1640 supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 1% penicillin-streptomycin-neomycin in a 5% CO_2 humidified incubator at 37°C. Cultures were harvested and cell numbers enumerated by hemocytometer analysis of cell suspensions.

Toona sinensis *Preparation and Extraction*

The leaves of *Toona sinensis* (TS) were sourced from Fooyin University, Kaohsiung, Taiwan. A voucher specimen was characterized by Dr Horng-Liang Lay, Graduate Institute of Biotechnology, National Pingtung University of Science and Technology, Pingtung County, Taiwan.[22] The aqueous extracts of TS were prepared by adding 1000 mL of water to 1000 g of the leaves of fresh TS and boiling until 100 mL remained, as previously described.[23] The crude extracts were centrifuged at 3000 rpm for 12 minutes and the supernatant used for this study. The crude extracts (50 g) were concentrated in a vacuum and freeze dried to form powder, with the stock subsequently stored at −20°C for subsequent analysis of its anticancer properties. The yield of TS extracts was 5%.[8]

MTT Assay

The effect of TS on WEHI-3 cell viability was monitored using the MTT colorimetric assay. In brief, 2×10^5 cells/well were plated in 12-well plates, and they were pretreated with or without various concentrations of TS (10-75 μg/mL) for 24 hours. After treatment, the cells were incubated with 400 μL of 0.5 mg/mL MTT in phosphate-buffered saline (PBS) for 2 hours. Culture supernatants were removed and resuspended in 400 μL of isopropanol to dissolve the MTT formazan, and the absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader. The effect of TS on cell viability was assessed as the percent of viable cells compared to the vehicle-treated control group, which was arbitrarily assigned to represent 100% viability. This assay was performed in triplicate for each concentration.

Determination of Apoptotic Cells by Annexin V/PI Staining

Double staining for Annexin V-FITC and PI (propidium iodide) was performed to estimate the apoptotic rate of WEHI-3 leukemia cells. Briefly, cells were incubated with TS (75 μg/mL) for 0 to 24 hours. Cells were trypsinized, washed twice with PBS, and centrifuged at 800 rpm for 5 minutes. Then, cells (2×10^5cells/10-cm dish) were suspended in binding buffer (500 μL) and double-stained with Annexin V-FITC and PI for 15 minutes at room temperature. Then the result green (FITC) and red (PI) fluorescence of each sample was quantitatively analyzed by FACS Caliber flow cytometer (Becton Dickinson, San Jose, CA, USA) and Cell Quest software. The obtained results were interpreted as follows: (Q3) cells negative for both PI and

Annexin V-FITC staining were considered normal live cells. (Q4) PI-negative, Annexin V-FITC-positive stained cells were considered in early apoptosis. (Q2) PI positive, Annexin V-FITC-positive stained cells were considered in late apoptosis. (Q1) PI positive, Annexin V-FITC-negative stained cells were considered in necrosis.

Protein Isolation and Western Blot Analysis

WEHI-3 cells (2×10^6 cells) were seeded in a 10-cm dish. Once the cells reached 80% confluence, they were incubated with or without the indicated concentrations of TS for 24 hours. The treated cells were washed once in cold PBS and suspended in 100 μL of lysis buffer (10 mM Tris-HCl [pH 8], 0.32 M sucrose, 1% Triton X-100, 5 mM ethylenediamine tetra-acetic acid (EDTA), 2 mM DTT, and 1 mM phenyl methyl sulfonyl fluoride). The suspension was vortexed, kept on ice for 20 minutes, and then centrifuged at 15000 × *g* for 20 minutes at 4°C. Total protein content was quantified using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA), with bovine serum albumin (BSA) as the standard. The protein extracts were reconstituted in sample buffer (0.062 M Tris-HCl, 2% sodium dodecyl sulfate (SDS), 10% glycerol, and 5% β-mercaptoethanol), and the mixture was boiled at 94°C for 5 minutes. Equal amounts (50 μg) of the denatured proteins were loaded into each lane, separated by electrophoresis on 8% to 15% SDS polyacrylamide gels, and the proteins were transferred to PVDF (polyvinylidine fluoride) membranes overnight. The membranes were blocked with 0.1% Tween-20 in Tris-buffered saline containing 5% nonfat dry milk for 20 minutes at room temperature, and then incubated with the indicated primary antibody for 2 hours. The membranes were then incubated with a horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse antibody for 2 hours and developed using a chemiluminescence substrate (Millipore, Billerica, MA, USA). For densitometric analysis, band intensities were quantified using commercially available software (AlpaEaseFc 4.0; Genetic Technologies, Inc, Miami, FL, USA).

Animal Experiments

A total of 18 male BALB/c mice, 8 weeks of age were purchased from GlycoNex Inc in Taiwan and were maintained in cage housing in a specifically designed pathogen-free isolation facility with a 12/12-h light/dark cycle and fed rodent chow and water ad libitum. All experiments were conducted in accordance with the guidelines of the China Medical University, Animal Ethics Research Board.

Tumor Cell Inoculation

BALB/c mice were injected with WEHI-3 cells (3×10^6/100 μL) via the tail vein. Experiments were carried out using cells less than 15 passages. BALB/c mice were divided into 3 groups (n = 6). Group I was control, group II was injected with WEHI-3 cells, and group III was injected with WEHI-3 cells and then treated with TS extracts (0.2 mL/mouse) dissolved in PBS at 50 mg/kg every 2 days, while the control group received daily injections of vehicle only. After 21 days of treatment, the animals were then weighed and sacrificed. Blood was collected from all animals at the end of the experiments for hematological analysis.

Hematological Analysis

Blood samples (1 mL) were collected from animals of each group at the end of the experiments, and were treated immediately with ammonium chloride buffer for lysing of the red blood cells followed by centrifugation for 15 minutes at 1500 rpm at 4°C for the isolation of peripheral blood mononuclear cells and analyzed by flow cytometry.

Liver and Spleen Samples

All the animals (control and experimental groups) were weighed before whole blood was drawn. The tissues of liver and spleen were isolated and weighed for each animal.[24]

Histopathology

Tissue sample (spleen) of each group was fixed in 4% formaldehyde and embedded in paraffin. Five-millimeter sections were stained with hematoxylin and eosin as described previously.[24]

Statistical Analysis

The results of in vitro and in vivo experiments are presented as mean and standard deviation (mean ± SD) or mean and standard error (mean ± SE), respectively. All study data were analyzed using analysis of variance (ANOVA), followed by Dunnett's test for pairwise comparison. Statistical significance was defined as $P < .05$ for all tests.

Results

The Antiproliferative Effect of TS in Leukemia WEHI-3 Cells

To evaluate the cell viability of human leukemia cancer cells, WEHI-3 cells were treated with various concentrations (10, 25, 50, and 75 μg/mL) of TS for 24 hours and cells survival were evaluated by MTT assay. Treatment of TS with WEHI-3 cells resulted in a dose-dependent inhibition of WEHI-3 cell proliferation with the IC_{50} value of 21.9 ± 0.6 μg/mL (Figure 1A). This result suggests that TS was cytotoxic to murine WEHI-3 leukemia cells.

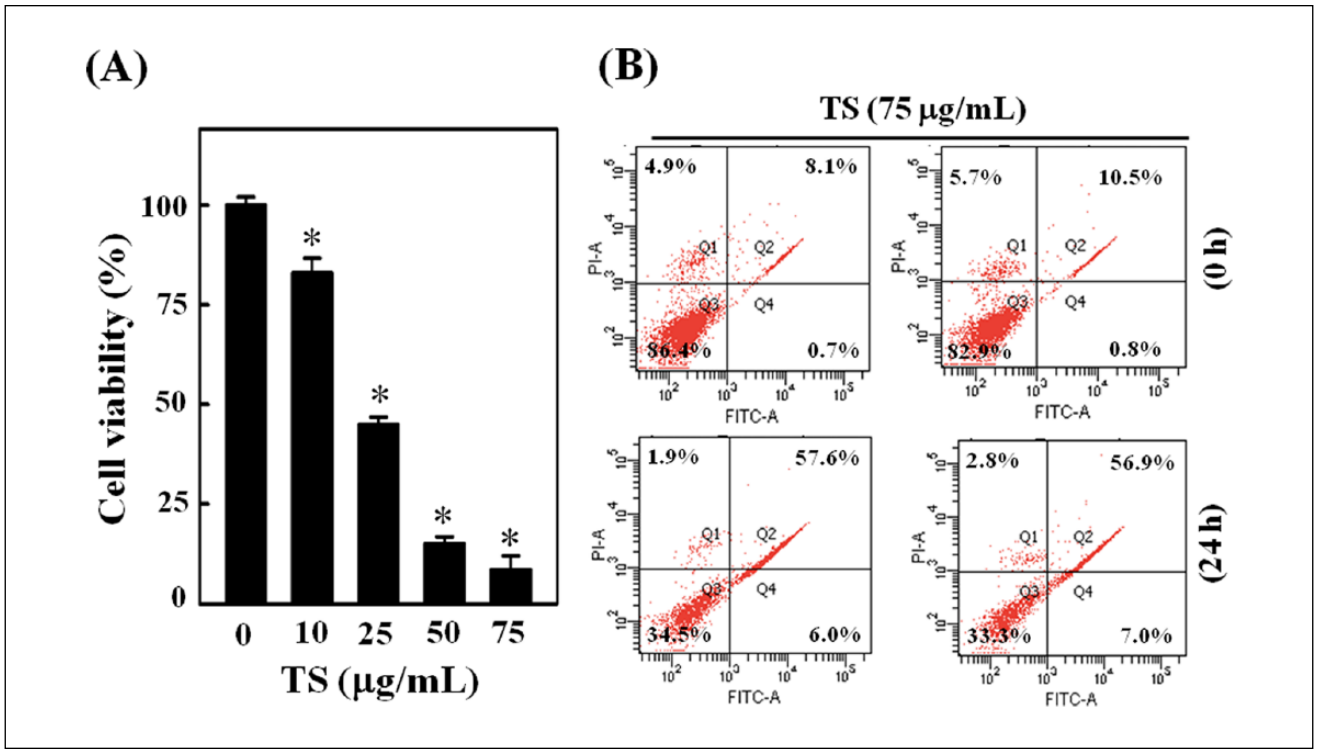

Figure 1. The effects of *Toona sinensis* (TS) on cell viability and apoptosis in murine myelocytic leukemia (WEHI-3) cells. (A) Anti-proliferative activity of TS on endothelial WEHI-3 cells. Cells were treated with increasing concentrations of TS (10-75 μg/mL) for 24 hours. Cell viability was determined before and after treatment with TS using MTT assay. The culture supernatant was removed and resuspended with isopropanol to dissolve the MTT formazan, and the absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader. The results are the mean ± SD of 3 assays. *Indicates a significant difference from the control group ($P < .05$). (B) TS induces apoptosis in WEHI-3 cells. Cells were treated with TS (75 μg/mL) for 24 hours, and apoptosis was measured by Annexin V/PI staining and flow cytometry. Data shown are representative of 2 independent experiments results.

TS Induces Apoptosis of WEHI-3 Leukemia Cells

Next, we determined the induction of apoptosis in WEHI-3 cells using Annexin-v/PI staining. The results showed that TS treatment resulted in a significant increase in late apoptotic cells (Q2), whereas no significant change in the necrotic cells was seen (Q1) (Figure 1B). These results demonstrate that TS exerts its cytotoxic effect on WEHI-3 leukemia cells via the induction of apoptotic cell death.

TS Upregulates Mitochondria-Mediated Apoptotic Cascades

To further delineate the manner in which TS induces apoptosis in WEHI-3 cells, mitochondria-dependent apoptotic protein markers were examined using Western blot analyses. Initially, the levels of cytochrome *c* in the cytosolic fraction were examined. Result indicates that TS treatment (10-75 μg/mL) for 24 hours significantly as well as dose-dependently increased the protein expression levels of cytochrome *c* in the cytoplasm (Figure 2A). Because cytochrome *c* is reportedly involved in the activation of the downstream caspases, which trigger apoptosis,[25] we investigated the effects of WEHI-3 cells on the downstream effector cascades of cytochrome *c*, including caspase-3. Western blot analyses revealed that treatment of WEHI-3 cells with TS significantly reduced the levels of procaspase-3 in a dose-dependent manner (Figure 2A). Taken together, these data suggest that TS-induced apoptosis in WEHI-3 cells was accompanied by cytochrome *c* release and caspase-3 activation.

TS Dysregulates the Bcl-2 and Bax Protein Ratio

Bcl-2 family proteins have been shown to play an important regulatory role in (mitochondria-mediated) apoptosis, either as activators (Bax, Bad, and Bok) or as inhibitors (Bcl-2, Bcl-xL, and Bcl-w).[26] To assess whether TS (0-75 μg/mL) promoted cell death was due to induction of apoptosis in WEHI-3 cells, we investigated apoptosis related protein by Western blotting. As shown in Figure 2, the anti-apoptosis protein Bcl-2 was decreased and pro-apoptosis protein Bax

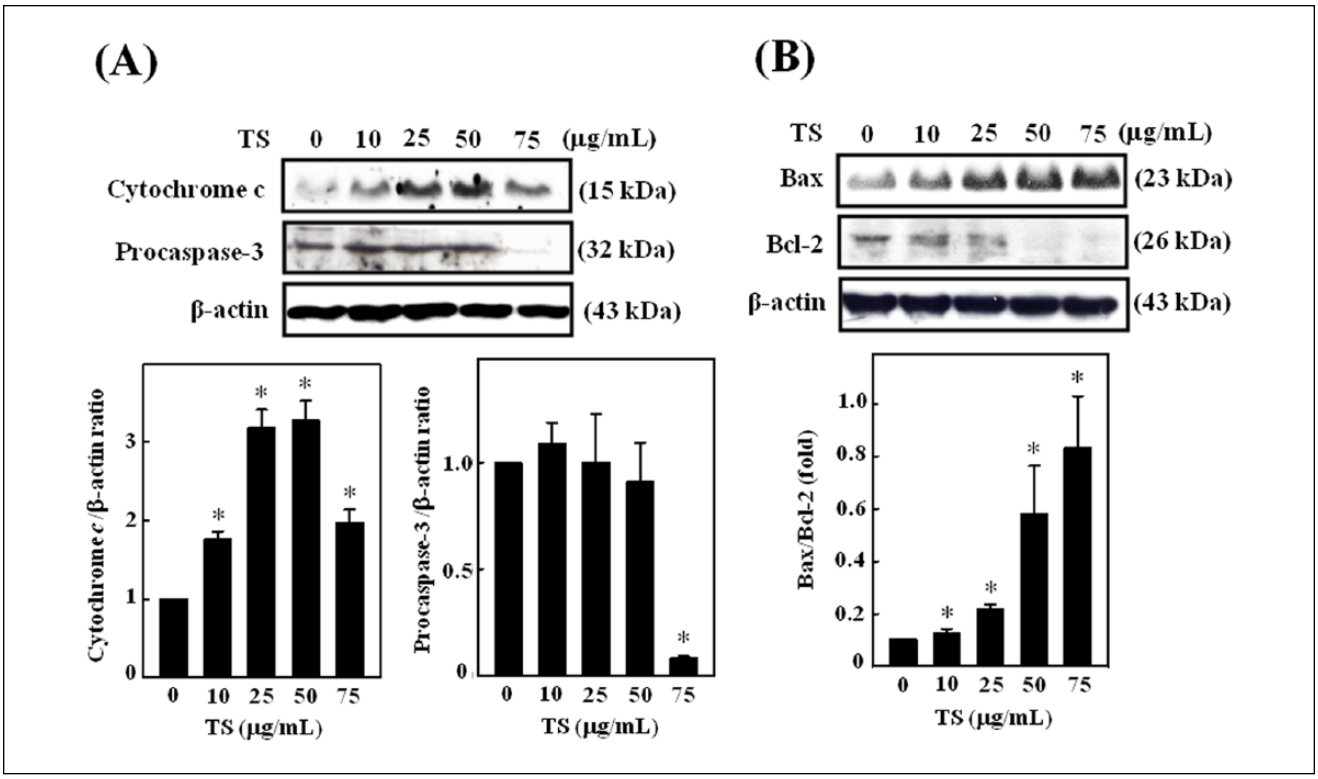

Figure 2. The effects of *Toona sinensis* (TS) on apoptotic proteins in WEHI-3 cells. The cells were treated with increasing concentrations of TS (10-75 μg/mL) for 24 h, and the protein levels of (A, B) Cytochrome *c*, procaspase-3, Bax, and Bcl-2 were analyzed. β-actin was used as a control. Relative changes in the Bcl-2 and Bax protein bands were measured by commercially available quantitative software (AlphaEase, Genetic Technology Inc, Miami, FL, USA), with the control representing 1.0-fold. The results are the mean ± SD of 3 assays. *Indicates a significant difference from the control group ($P < .05$).

was increase by TS treatment (10-75 μg/mL) for 24 hours. Furthermore, TS treatment increased Bax/Bcl-2 ratio in WEHI-3 cells (Figure 2B). These results indicate that TS may disturb the Bcl-2 and Bax protein ratio and induce apoptosis.

Injection of WEHI-3 cells induces leukemia in BALB/c mice

BALB/c mice were injected with WEHI-3 cells (3×10^6/100 μL) via the tail vein and treated with TS (50 mg/kg) by gavaged tube for 3 weeks. Animals were sacrificed for examination of liver and spleen tissues; they were individually photographed and weighed. Dissected animals are shown in Figure 3. After 19 days of treatment, the WEHI-3 injected group (all mice) exhibited hind leg paralysis, body curl, changes in hair brightness, and decrease in mouse activity, whereas the TS treated group had decreased paralysis and body curl, and improved hair brightness (Figure 3).

Effect of TS on spleen and liver

The weights of spleens and livers are presented in Figure 4 and 6, respectively. WEHI-3 injected groups significantly increased the weight of spleen and liver as compared with control groups (Figures 4 and 6). This is due to metastasis of WEHI-3 cells toward liver and spleen, which increased their sizes significantly. Whereas, treatment with TS (50 mg/kg) groups statistically decreased the weight of spleen and liver in tumor-bearing mice (Figures 4 and 6). Histopathology results suggested that TS-treated spleen tissues display increased megakaryocytes and decreased numbers of neoplastic cells. Neoplastic cells contained large irregular nuclei accompanied by clumped chromatin and prominent nucleoli, and abundant clear and light eosinophilic cytoplasm. Often mitotic figures were also noted; further there was a marked expansion in the red pulp, but little change in white pulp were seen in spleen tissues (Figure 5).

Hematology in WEHI-3-Injected Mice

The results of hematological analysis after TS treatment are shown in Table 1. The WEHI-3 injected group showed a significant increase in white blood cell (WBC) count as compared with control group, whereas treatment in the TS-treated group dramatically decreased the WBC count compared with the WEHI-3 group alone. Furthermore, the

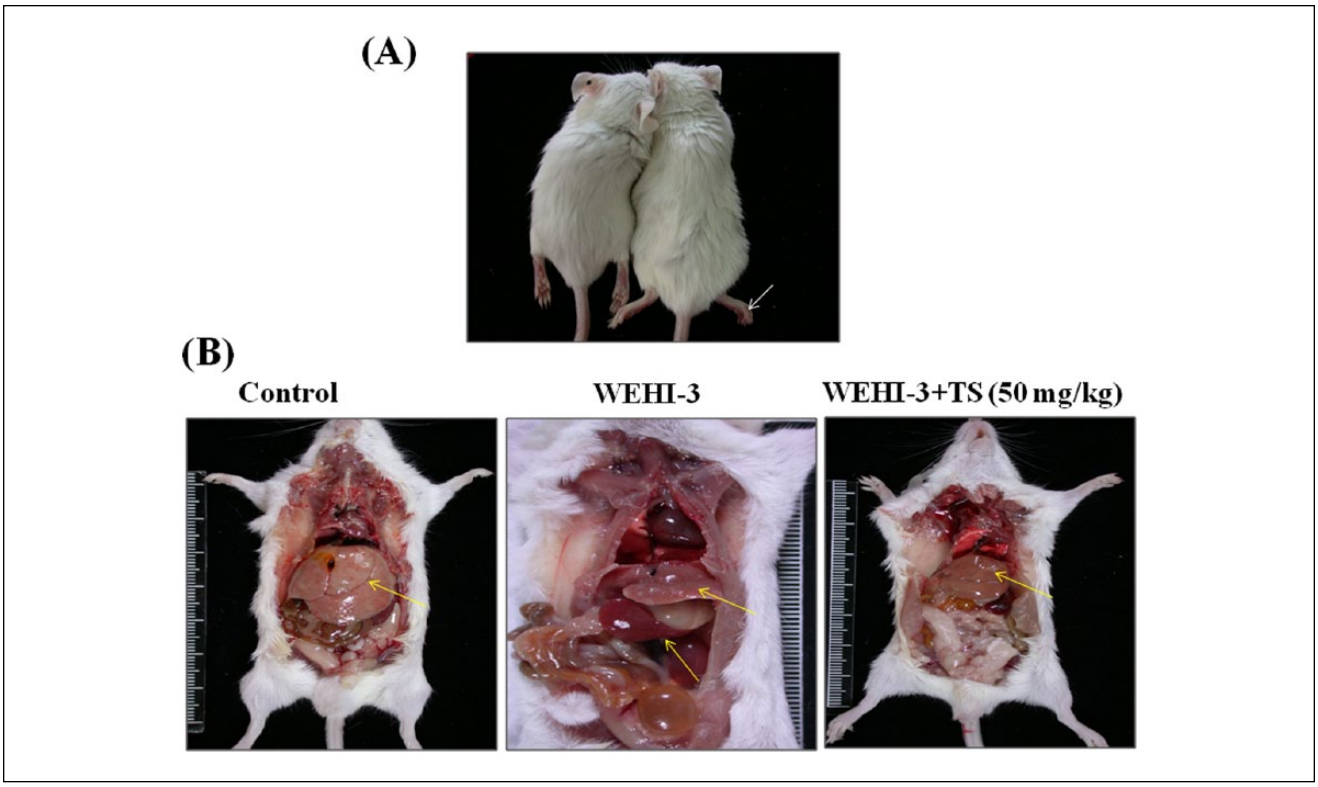

Figure 3. Representative pictures from dissected BALB/c mice after injection with WEHI-3 cells and/or treated with *Toona sinensis* (TS) for 3 weeks. BALB/c mice were injected with WEHI-3 cells in PBS for 3 weeks and co-treated with TS (50 mg/kg). (A) Mice, and (B) dissected section of control, WEHI-3, and WEHI-3 + TS (50 mg/kg) treated mice. White arrow indicates mice leg with paralysis. Yellow arrows indicate liver and spleen.

TS-treated group increased the concentration of red blood cells (RBC), hemoglobin (Hb), hematocrit (Hct), and mean corpuscular volume (MCV) levels compare to WEHI-3 group alone. In contrast, TS treatment reduced the concentration of mean corpuscular hemoglobin (MCH), and MCH concentration (MCHC) levels. Our results indicated that TS has antileukemia capacity and promoted the immune response in WEHI-3 bearing mice in vivo.

Leukocyte Differentiation

To determine the effect of TS on leukocyte differentiation, lymphocyte, neutrophil, monocyte, eosinophil, basophil, and nucleated RBC were analyzed. As shown in Table 2, the TS-treated group had significantly decreased expression of lymphocytes as compared with WEHI-3 alone group. In contrast, eosinophils were shown to be statistically decreased as compared with control and WEHI-3 injected mice. The observed changes in eosinophils could be due to excessive production of steroids (natural chemicals produced by the body and released into the blood). Furthermore, segmented neutrophils were increased in both the WEHI-3 injected and TS-treated groups as compared with control. The WEHI-3 injected group had increased nucleated RBC count, whereas the TS-treated group had decreased expression of RBC level (Table 2).

Discussion

It is well known that many natural compounds from food and plants have chemopreventive and chemotherapeutic efficacy in human cancer.[27,28] A number of natural products isolated from Chinese herbs have been found to induce apoptosis, suppress angiogenesis, inhibit proliferation, retard metastasis and enhance chemotherapy, exhibiting anti-cancer potential both *in vitro* and *in vivo*.[29,30] The different components in a given herb may have synergistic activities to offer greater therapeutic or preventive activity in combination.[31] In our previous study, we demonstrated that the aqueous extracts of TS (10-75 μg/mL) and gallic acid (5-10 μg/mL) promoted apoptosis against human premyelocytic leukemia (HL-60) cells.[8] To verify the possible anticancer effects of TS as a first step toward the development of a novel putative anticancer agent, we further investigated for its capacity to inhibit murine leukemia WEHI-3 cells growth and associated with immune responses in

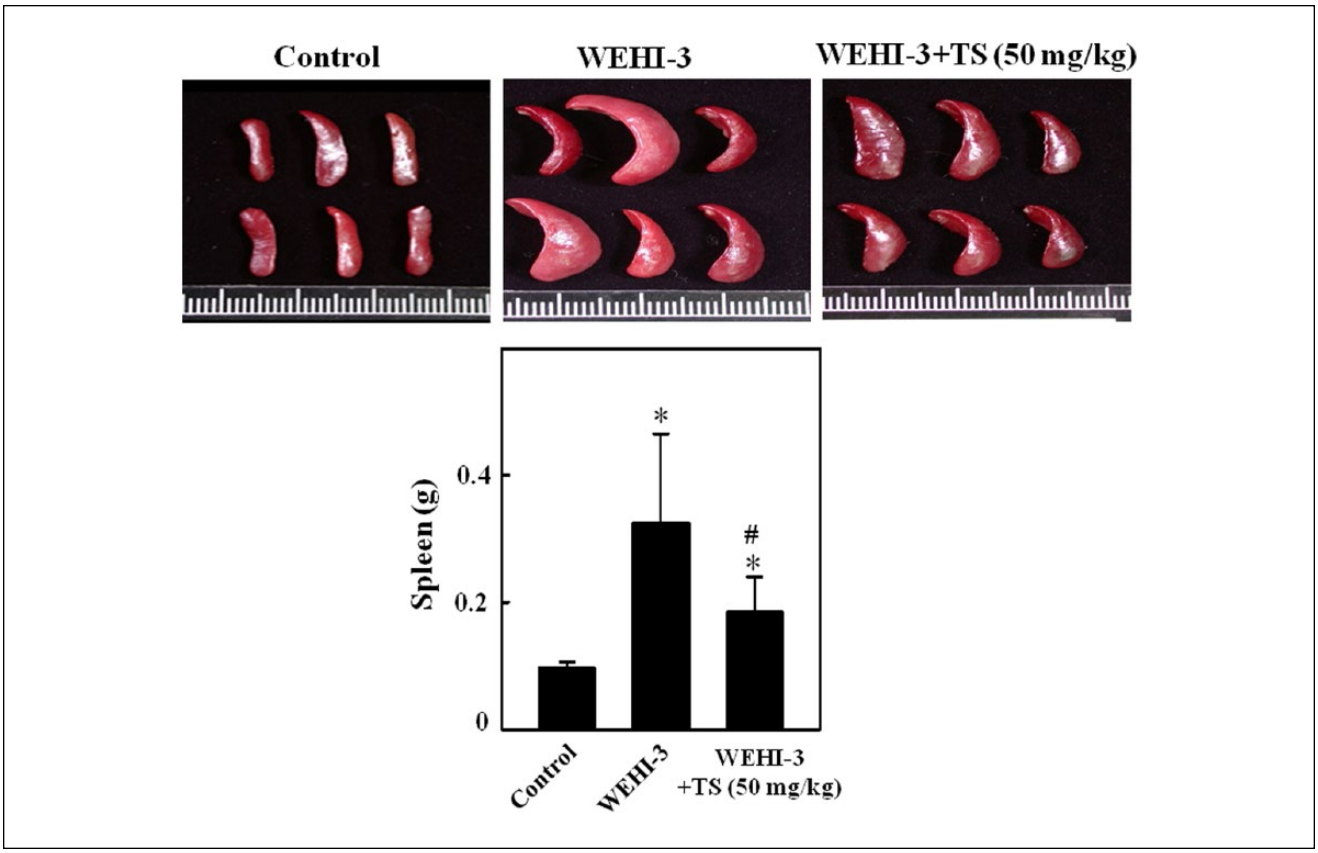

Figure 4. The effects of *Toona sinensis* (TS) on the morphology and weight of spleen in WEHI-3 bearing mice. BALB/c mice were intravenous injected with WEHI-3 cells and treated with TS by oral administration for 3 weeks, and animals were sacrificed for examinations of gross (A) and spleen weights (B). The results shown are the mean ± SD of 6 assays. *Indicates significant difference in comparison with control group ($P < .05$). #$P < .05$ compared with WEHI-injected groups.

WEHI-3 cells injected via tail vein of mice model. Leukemic animals have been used to explore for anticancer agents because of the low cost that promotes a short time to develop antileukemic agents in vivo. Our results indicated that TS significantly inhibited the WEHI-3 cells and induced apoptosis through the mitochondrial death pathway in vitro and immune response in vivo.

The mitochondrial death pathway is controlled by members of the Bcl-2 protein class and proapoptotic Bax proteins.[32] The proto-oncogene Bcl-2 antagonizes apoptosis in many cancers. On the contrary, overexpression of Bax accelerates apoptotic death.[33] Loss of mitochondrial membrane potential induces cytochrome *c* release from mitochondria to cytoplasm, which leads to the activation of caspase-9 and downstream cleavage of caspase-3.[34] Caspase-9 and caspase-3 are the main effector and executor caspases involved in apoptosis and play a pivotal role in the disintegration of the cells undergoing apoptosis.[35] Our finding showed that TS-induced apoptosis was associated with the downregulation of Bcl-2 and upregulation of Bax in murine WEHI-3 cells, and the changes of these proteins disrupted mitochondria membrane permeability and released cytochrome *c* into cytosol, leading to apoptosis via caspases activation (Figure 2).

The immune system is the vital defense against cancer growth, tumor, and infectious diseases. The adjustment of immune responses to alleviate such diseases has been of great interest for several years.[36] Recently, several medicinal plants and their products have been used to modulate immune response.[37] Furthermore, immunomodulation induced by medicinal plants can provide potential alternatives to conventional chemotherapies for various diseases, especially when the host defense mechanism needs to be activated under the conditions of impaired immune response.[38] Leukemia is the type of cancer in which bone marrow cells that produce white blood cells (leukocytes, the immune system cells) undergo neoplasia. The transformed cells unconditionally produce abnormal amounts of WBC, mainly in an immature form. The neoplastic cells crowd out the other cells in the marrow which leads to decreases in production of red cells, platelets, and other lines of WBCs. The destructive aspects of the disease are

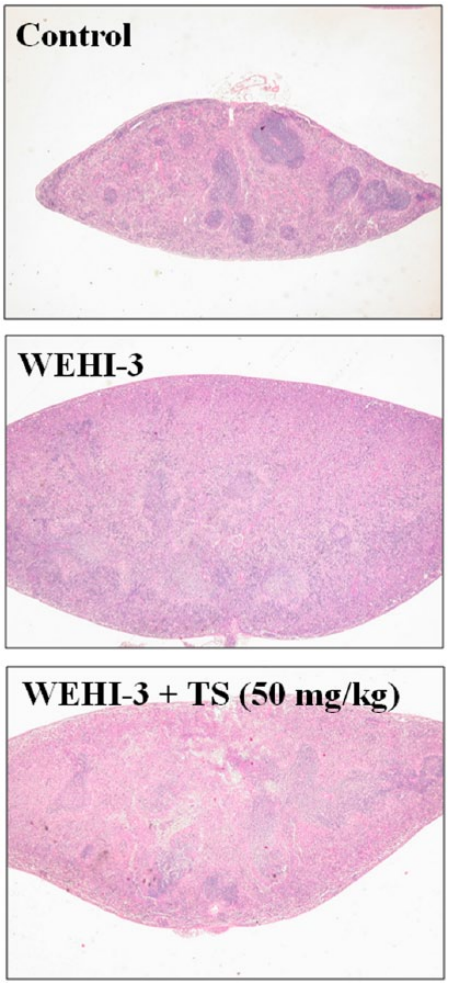

Figure 5. The effects of *Toona sinensis* (TS) on the histopathology of spleen. Spleens from each animal of each group were histopathologically excised as described in Materials and Methods secion. Control and TS treatment were examined using light microscopy (20× magnification).

often exhibit by the lack of these other cell types, which leads to formation of hemorrhage and anemia. Importantly, the internal organs (liver and spleen) bulge up with increased white cells. Furthermore, leukemic cells have markedly reduced immune functions, leading to excess incidence of infections.[39]

The in vivo model of mice injected with WEHI-3 has been well established.[40] Murine monomyelocytic WEHI-3 leukemia cells were derived from the BALB/c mouse[41] and used as an ideal system for the study of potential anticancer agents.[42] The effects of TS in vivo on WEHI-3 tumor cells in BALB/c mice were also examined. We selected the 50 mg/kg TS for in vivo studies based on our pretest (data not shown). The results indicated that TS significantly decreased the weights of liver and spleen in the examined animals (Figures 4 and 6). A notable reason for use of this model is the elevation of peripheral monocytes and granulocytes with immature morphology, causing enlarged and infiltrated spleens compared with the normal group. Also, numbers of white blood cells were increased in the WEHI-3 injection group and decreased in TS-treated group. Our experiments indicated that spleen and liver size decreased, which then decreased the white blood cells in the TS-treated group. The recruitment of leukocytes toward stimuli is of great significance for the production of effective immune response. Recently, studies showed that leukocyte values were raised by increasing malignancy in cancer cells.[43] Our previous literature suggests that TS exhibits anti-inflammatory potential in vitro and in vivo by regulating the immune system process and the physiological response to inflammatory stimuli. The anti-inflammatory effect of TS involves suppression of LPS-induced production of TNF-α and IL-1β in the serum and organs of mice.[44] In the present study, the TS-treated group had reduced levels of leukocytes, showing that TS promoted immune responses in BALB/c mice and exhibited antileukemia activity toward murine WEHI-3 cells.

Natural products, including plants, provide rich resources for anticancer drug discovery. As the different components in a given herb may buffer toxic effects or offer synergistic activities, extracts or mixtures of these herbs may offer greater therapeutic or preventive activity in combination.[45] In our previous study, a number of compounds, including gallic acid, methyl gallate, ethyl gallate, kaempferol, kaempferol-3-O-β-D-glucoside, quercetin, quercitrin, quercetin-3-*O*-β-D-glucoside, and rutin, were isolated from the leaves of TS, as verified by HPLC, with the analogous information reported in the literature.[10] The total phenolic content of TS was estimated to be 130 ± 26 mg gallic acid, equivalents/g of plant extracts. The yield of gallic acid, the natural phenolic component purified from TS, was approximately 10%.[8] Although it remains unclear which of the components of TS are active compounds, polyphenols have received increasing attention recently because of some interesting new findings regarding their biological activities. Of these compounds, it has been demonstrated that gallic acid possesses antioxidant and anticancer activities.[16] Gallic acid and its structurally related compounds are some of the main phenolic components of both black and green tea, and red wine. Studies have found that gallic acid induces apoptosis in cancer cells (such as HL-60RG, Hela, dRLh-84, PLC/PRF/5 and KB cells) with higher sensitivity than normal analogs (such as rat primary cultured hepatocytes, macrophages, endothelial cells and fibroblasts).[46] Some studies have shown that gallic acid causes inactivating phosphorylation of CDC25A/CDC25C- CDC2, leading to cell cycle arrest, and apoptosis induction in human prostate carcinoma DU 145 cells.[47] In the current work, TS

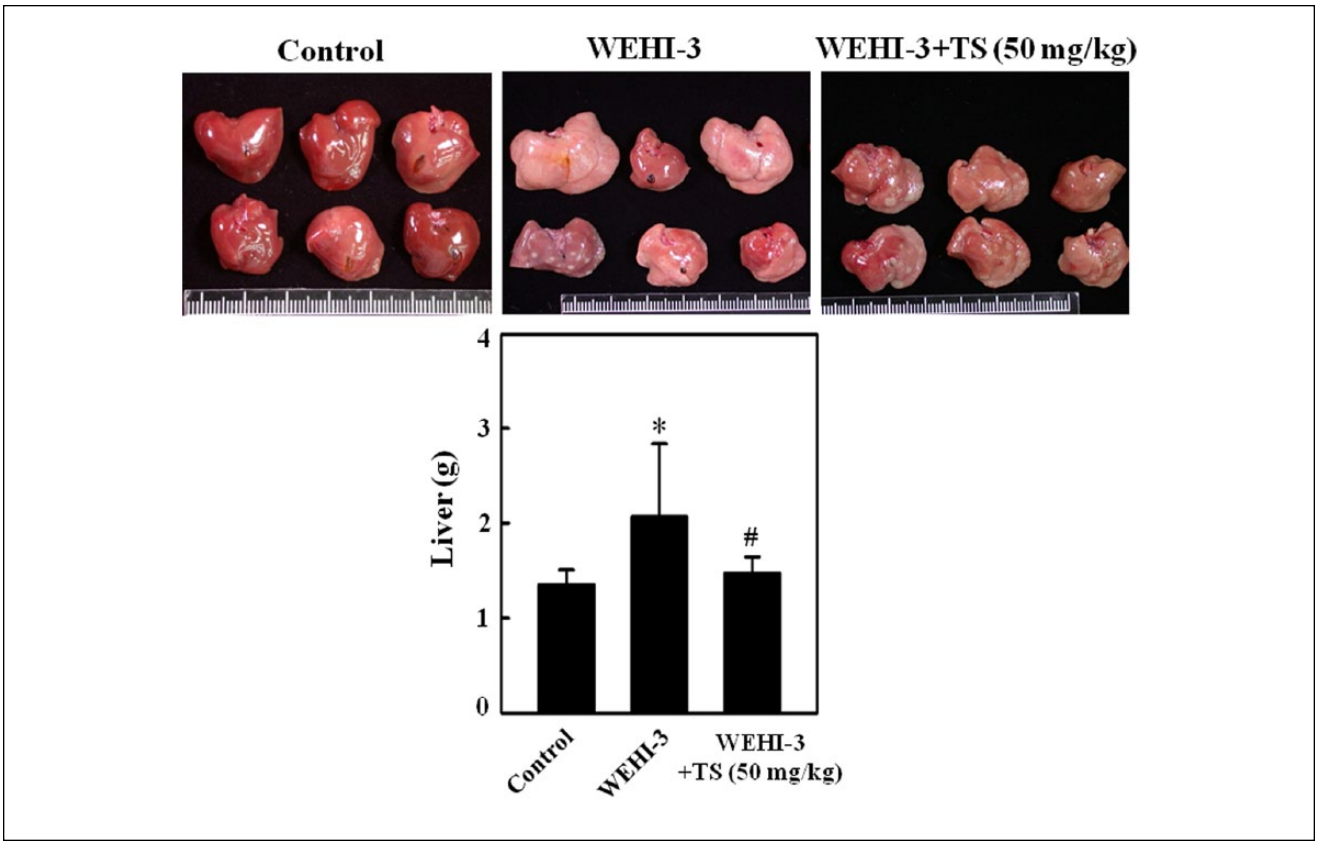

Figure 6. The effects of *Toona sinensis* (TS) on the morphology and weight of liver in WEHI-3 bearing mice. BALB/c mice were intravenously injected with WEHI-3 cells and treated with TS by oral administration for 3 weeks, and animals were sacrificed for examinations of gross (A) and liver weights (B). The results shown are the mean ± SD of 6 assays. *Indicates significant difference in comparison with control group ($P < .05$). $^{\#}P < .05$ compared with WEHI-injected groups.

Table 1. Hematology of *Toona sinensis* (TS) Treatment in WEHI-Injected Male Mice (n = 6).

Groups	WBC ($\times 10^3/\mu L$)	RBC ($\times 10^3/\mu L$)	Hb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)
Control	4.0 ± 0.9	9.1 ± 1.0	15.4 ± 1.8	49.5 ± 7.0	54.1 ± 1.9	16.8 ± 0.5	31.2 ± 1.5
WEHI-3	30.6 ± 27.1*	7.7 ± 1.3	13.5 ± 2.3	40.6 ± 7.1*	53.3 ± 3.8	17.7 ± 1.4	33.5 ± 4.2
WEHI-3+TS	12.2 ± 1.9*#	8.2 ± 0.9	13.9 ± 1.4	46.0 ± 4.8	56.0 ± 1.4	16.9 ± 0.4	30.2 ± 0.6

Abbreviations: WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, MCH concentration.
*Significant at $P < .05$ compared with control groups.
Significant at $P < .05$ compared with WEHI-injected groups.

Table 2. Leukocyte Differentiation ($10^3/mm^3$) of *Toona sinensis* (TS) Treatment in WEHI-Injected Male Mice (n = 6).

Groups	Lymphocytes	Segmented Neutrophils	Monocytes	Eosinophils	Basophils	Nucleated Red Blood Cells
Control	44.3 ±16.9	17.2 ± 6.8	0.0 ± 0.0	4.0 ± 1.9	0.0 ± 0.0	0.0 ± 0.0
WEHI-3	74.5 ± 12.8*	36.5 ± 11.6*	5.0 ± 4.1*	3.5 ± 5.0	0.2 ± 0.4	1.7 ± 2.9
WEHI-3+TS	43.0 ± 15.3#	37.3 ± 13.1*	5.0 ± 4.7*	1.2 ± 1.0*#	0.0 ± 0.0	0.2 ± 0.4

*Significant at $P < .05$ compared with control groups.
#Significant at $P < .05$ compared with WEHI-injected groups.

exhibited antileukemic potential that might be due to the presence of flavonoids and polyphenolics (gallic acids) in it. However, further isolation, structural characterization of active constituents is necessary to extrapolate the mechanism of action.

Conclusion

Taken together, our results confirm the potential of *Toona sinensis* as an agent of chemotherapeutic in murine myelocytic leukemia WEHI-3 cells in vitro and in vivo. These findings also sustain our contention that *T. sinensis* may possess anticancer properties potentially valuable for application in food and drug products. However, further in vivo studies using animal models are necessary to elaborate and exploit this nascent promise.

Authors' Note

Authors Hsin-Ling Yang and Varadharajan Thiyagarajan contributed equally to this study.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the grants MOST-104-2320-B-039-040-MY3, MOST-103-2320-B-039-038-MY3, NSC-103-2622-B-039-001-CC2, CMU102-ASIA-17, and CMU 102-ASIA-22 from the Ministry of Science and Technology, Asia University, and China Medical University, Taiwan.

References

1. Robak T, Wierzbowska A. Current and emerging therapies for acute myeloid leukemia. *Clin Ther*. 2009;31(pt 2): 2349-2370.
2. Morceau F, Chateauvieux S, Orsini M, Trecul A, Dicato M, Diederich M. Natural compounds and pharmaceuticals reprogram leukemia cell differentiation pathways. *Biotechnol Adv*. 2015;33(6 pt 1):785-797.
3. Dores GM, Devesa SS, Curtis RE, Linet MS, Morton LM. Acute leukemia incidence and patient survival among children and adults in the United States, 2001-2007. *Blood*. 2012;119:34-43.
4. Thein MS, Ershler WB, Jemal A, Yates JW, Baer MR. Outcome of older patients with acute myeloid leukemia: an analysis of SEER data over 3 decades. *Cancer*. 2013;119:2720-2727.
5. Nachliely M, Sharony E, Kutner A, Danilenko M. Novel analogs of 1,25-dihydroxyvitamin D combined with a plant polyphenol as highly efficient inducers of differentiation in human acute myeloid leukemia cells. *J Steroid Biochem Mol Biol*. 2016;164:59-65.
6. Edmonds JM SM. *Toona sinensis*: Meliaceae. *Curtis's Botanical Mag*. 1998;15:186-193.
7. Chang HC, Hung WC, Huang MS, Hsu HK. Extract from the leaves of *Toona sinensis* roemor exerts potent antiproliferative effect on human lung cancer cells. *Am J Chin Med*. 2002;30:307-314.
8. Yang HL, Chang WH, Chia YC, et al. *Toona sinensis* extracts induces apoptosis via reactive oxygen species in human premyelocytic leukemia cells. *Food Chem Toxicol*. 2006;44:1978-1988.
9. Yang H, Gu Q, Gao T, et al. Flavonols and derivatives of gallic acid from young leaves of *Toona sinensis* (A. Juss.) Roemer and evaluation of their anti-oxidant capacity by chemical methods. *Pharmacogn Mag*. 2014;10:185-190.
10. Yang HL, Huang PJ, Liu YR, et al. *Toona sinensis* inhibits LPS-induced inflammation and migration in vascular smooth muscle cells via suppression of reactive oxygen species and NF-κB signaling pathway. *Oxid Med Cell Longev*. 2014;2014:901315.
11. Hsieh TJ, Tsai YH, Liao MC, et al. Anti-diabetic properties of non-polar *Toona sinensis* Roem extract prepared by supercritical-CO_2 fluid. *Food Chem Toxicol*. 2012;50:779-789.
12. You HL, Chen CJ, Eng HL, Liao PL, Huang ST. The effectiveness and mechanism of *Toona sinensis* extract inhibit attachment of pandemic Influenza A (H1N1) virus. *Evid Based Complement Alternat Med*. 2013;2013:479718.
13. Chen CJ, Michaelis M, Hsu HK, et al. *Toona sinensis* Roem tender leaf extract inhibits SARS coronavirus replication. *J Ethnopharmacol*. 2008;120:108-111.
14. Poon SL, Leu SF, Hsu HK, Liu MY, Huang BM. Regulatory mechanism of *Toona sinensis* on mouse leydig cell steroidogenesis. *Life Sci*. 2005;76:1473-1487.
15. Hsieh TJ, Liu TZ, Chia YC, et al. Protective effect of methyl gallate from *Toona sinensis* (Meliaceae) against hydrogen peroxide-induced oxidative stress and DNA damage in MDCK cells. *Food Chem Toxicol*. 2004;42:843-850.
16. Chen HM, Wu YC, Chia YC, et al. Gallic acid, a major component of *Toona sinensis* leaf extracts, contains a ROS-mediated anti-cancer activity in human prostate cancer cells. *Cancer Lett*. 2009;286:161-171.
17. Chia YC, Rajbanshi R, Calhoun C, Chiu RH. Anti-neoplastic effects of gallic acid, a major component of *Toona sinensis* leaf extract, on oral squamous carcinoma cells. *Molecules*. 2010;15:8377-8389.
18. Wang CY, Lin KH, Yang CJ, et al. *Toona sinensis* extracts induced cell cycle arrest and apoptosis in the human lung large cell carcinoma. *Kaohsiung J Med Sci*. 2010;26:68-75.
19. Yang CJ, Huang YJ, Wang CY, et al. Antiproliferative effect of *Toona sinensis* leaf extract on non-small-cell lung cancer. *Transl Res*. 2010;155:305-314.
20. Liao JW, Hsu CK, Wang MF, Hsu WM, Chan YC. Beneficial effect of *Toona sinensis* Roemor on improving cognitive performance and brain degeneration in senescence-accelerated mice. *Br J Nutr*. 2006;96:400-407.
21. Liao JW, Yeh JY, Lin YC, Wei MM, Chung YC. Mutagenicity and safety evaluation of water extract of fermented *Toona sinensis* Roemor leaves. *J Food Sci*. 2009;74:T7-T13.
22. Hsu HK, Yang YC, Hwang JH, Hong SJ. Effects of *Toona sinensis* leaf extract on lipolysis in differentiated 3T3-L1 adipocytes. *Kaohsiung J Med Sci*. 2003;19:385-390.

23. Hseu YC, Chang WH, Chen CS, et al. Antioxidant activities of *Toona sinensis* leaves extracts using different antioxidant models. *Food Chem Toxicol*. 2008;46:105-114.
24. Yang JS, Kok LF, Lin YH, et al. Diallyl disulfide inhibits WEHI-3 leukemia cells in vivo. *Anticancer Res*. 2006;26: 219-225.
25. Earnshaw WC, Martins LM, Kaufmann SH. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem*. 1999;68:383-424.
26. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev*. 1999;13:1899-1911.
27. Eggler AL, Gay KA, Mesecar AD. Molecular mechanisms of natural products in chemoprevention: induction of cytoprotective enzymes by Nrf2. *Mol Nutr Food Res*. 2008;52(suppl 1):S84-S94.
28. Thiyagarajan V, Lin SH, Chia YC, Weng CF. A novel inhibitor, 16-hydroxy-cleroda-3,13-dien-16,15-olide, blocks the autophosphorylation site of focal adhesion kinase (Y397) by molecular docking. *Biochim Biophys Acta*. 2013;1830:4091-4101.
29. Tan W, Lu J, Huang M, et al. Anti-cancer natural products isolated from Chinese medicinal herbs. *Chin Med*. 2011;6:27.
30. Thiyagarajan V, Tsai MJ, Weng CF. Antroquinonol targets FAK-signaling pathway suppressed cell migration, invasion, and tumor growth of C6 Glioma. *PLoS One*. 2015;10:e0141285.
31. Vickers A. Botanical medicines for the treatment of cancer: rationale, overview of current data, and methodological considerations for phase I and II trials. *Cancer Invest*. 2002;20:1069-1079.
32. Czabotar PE, Lessene G, Strasser A, Adams JM. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nat Rev Mol Cell Biol*. 2014;15:49-63.
33. Lotem J, Sachs L. Regulation by bcl-2, c-myc, and p53 of susceptibility to induction of apoptosis by heat shock and cancer chemotherapy compounds in differentiation-competent and -defective myeloid leukemic cells. *Cell Growth Differ*. 1993;4:41-47.
34. Indran IR, Tufo G, Pervaiz S, Brenner C. Recent advances in apoptosis, mitochondria and drug resistance in cancer cells. *Biochim Biophys Acta*. 2011;1807:735-745.
35. Tripathi M, Singh BK, Mishra C, Raisuddin S, Kakkar P. Involvement of mitochondria mediated pathways in hepatoprotection conferred by *Fumaria parviflora* Lam. extract against nimesulide induced apoptosis in *vitro*. *Toxicol In Vitro*. 2010;24:495-508.
36. Abood WN, Fahmi I, Abdulla MA, Ismail S. Immunomodulatory effect of an isolated fraction from *Tinospora crispa* on intracellular expression of INF-γ, IL-6 and IL-8. *BMC Complement Altern Med*. 2014;14:205.
37. Bhat BA, Dhar KL, Puri SC, et al. Isolation, characterization and biological evaluation of datura lactones as potential immunomodulators. *Bioorg Med Chem*. 2005;13:6672-6677.
38. Ahmad W, Jantan I, Kumolosasi E, Bukhari SN. Immunostimulatory effects of the standardized extract of *Tinospora crispa* on innate immune responses in Wistar Kyoto rats. *Drug Des Devel Ther*. 2015;9:2961-2973.
39. Cotran RS, Kumar V, Robbins SL, Schoen FJ. Inflammation and repair: In: Cotran RS, Kumar V, Robbins SL, eds. *Robbins Pathologic Basis of Disease*. 5th ed. Philadelphia, PA: W. B. Saunders; 1994:51-93.
40. He Q, Na X. The effects and mechanisms of a novel 2-aminosteroid on murine WEHI-3B leukemia cells in vitro and in vivo. *Leuk Res*. 2001;25:455-461.
41. Zips D, Thames HD, Baumann M. New anticancer agents: in vitro and in vivo evaluation. *In Vivo*. 2005;19:1-7.
42. Li J, Sartorelli AC. Synergistic induction of the differentiation of WEHI-3B D+ myelomonocytic leukemia cells by retinoic acid and granulocyte colony-stimulating factor. *Leuk Res*. 1992;16:571-576.
43. Coussens LM, Werb Z. Inflammation and cancer. *Nature*. 2002;420:860-867.
44. Hsiang CY, Hseu YC, Chang YC, Kumar KJ, Ho TY, Yang HL. *Toona sinensis* and its major bioactive compound gallic acid inhibit LPS-induced inflammation in nuclear factor-κB transgenic mice as evaluated by in vivo bioluminescence imaging. *Food Chem*. 2013;136:426-434.
45. Ou TT, Wang CJ, Hung GU, Wu CH, Lee HJ. Aqueous extract of Shi-Liu-Wei-Liu-Qi-Yin induces G2/M phase arrest and apoptosis in human bladder carcinoma cells via Fas and mitochondrial pathway. *Evid Based Complement Alternat Med*. 2011;2011:nep016.
46. Sakaguchi N, Inoue M, Ogihara Y. Reactive oxygen species and intracellular Ca^{2+}, common signals for apoptosis induced by gallic acid. *Biochem Pharmacol*. 1998;55:1973-1981.
47. Agarwal C, Tyagi A, Agarwal R. Gallic acid causes inactivating phosphorylation of cdc25A/cdc25C-cdc2 via ATM-Chk2 activation, leading to cell cycle arrest, and induces apoptosis in human prostate carcinoma DU145 cells. *Mol Cancer Ther*. 2006;5:3294-3302.