Elsevier Editorial System(tm) for Food and Chemical Toxicology
Manuscript Draft

Manuscript Number: FCT-D-11-02062R1

Title: In vitro and in vivo activity of gallic acid and Toona sinensis leaf extracts against HL-60 human premyelocytic leukemia

Article Type: Full Length Article

Keywords: Toona sinensis; Gallic acid; HL-60 cells; Cell-cycle arrest; Xenografted nude mice

Corresponding Author: Mrs. Hsin-Ling Yang, PhD

Corresponding Author's Institution: China Medical University

First Author: Pei-Jane Huang, Ph.D.

Order of Authors: Pei-Jane Huang, Ph.D.; You-Cheng Hseu, Ph.D.; Meng-Shiou Lee, Ph.D.; K.J. Senthil Kumar, Ph.D.; Chi-Rei Wu, Ph.D.; Li-Sung Hsu, Ph.D.; Jiunn-Wang Liao, Ph.D.; I-Shiung Cheng, Ph.D.; Ya-Ting Kuo, Ph.D.; Shi-Ying Huang; Hsin-Ling Yang, PhD

Abstract: Toona sinensis is one of the most popular vegetarian cuisines in Taiwan and it has been shown to induce apoptosis in cultured human premyelocytic leukemia (HL-60) cells. In the present study, we examined the effects of Toona sinensis leaf extracts (TS extracts) on tumor regression using in vitro cell culture and an in vivo athymic nude mice model. We found that TS extracts (10-75 ug/mL) arrested HL-60 cells at the G1-S transition phase through the reductions of Cyclin D1, CDK4, Cyclin E, CDK2, and Cyclin A, and induction of CDK inhibitor p27KIP levels. Furthermore, VEGF expression and release was significantly inhibited by TS extracts. Notably, TS extracts treatment was effective in terms of delaying tumor incidence in the nude mice inoculated with HL-60 cells as well as reducing the tumor burden. Histological analysis confirmed that TS extracts significantly modulated tumor progression in xenograft tumor. Furthermore, a similar pattern of results were observed from gallic acid (5 and 10 ug/mL), a major compound in TS, caused G1 arrest through regulations of cell-cycle regulatory proteins. Our data suggest that Toona sinensis exerts antiproliferative effects on HL-60 cells in vitro and in vivo due mainly to the presence of gallic acid.

Response to Reviewers: Dear Editor-in-Chief,
Enclosed please find the revised version of our manuscript, entitled “In vitro and in vivo activity of gallic acid and Toona sinensis leaf extracts against HL-60 human premyelocytic leukemia” (Ms. ID. FCT-D-11-02062R1) by Pei-Jane Huang, You-Cheng Hseu, Meng-Shiou Lee, Chi-Rei Wu, K.J. Senthil Kumar, Ssu-Ching Chen, Li-Sung Hsu, Jiunn-Wang Liao, I-Shiung Cheng, Ya-Ting Kuo, Chih-Wei Chou, Hsin-Ling Yang which has been recently reviewed by your editorial board. An itemized list of changes made and our response to the reviewer’s comments is also enclosed.
Again we thank the valuable comments from the referees, the main suggestions and comments are list and response as following. Kindly referred the yellow highlights in the manuscript are modified sentence or word.

Reviewer #1:
Major Comments:
Comment: 1

In the section of Material and Methods, preparation of TS extracts: 50 g of crude extracts contain 130±26 mg total phenol compounds. The authors isolated 8 compounds (gallic acid, methyl gallate, ethyl gallate, kaempferol, ...) by using HPLC. The concentration of these 8 different compounds should be written in the text. It is also unclear why the authors used only gallic acid for in vitro experiments. The state of the art is that all compounds should be tested in vitro and the most effective one should be used for the main experiments.
Respond:
Thanks for pointing this out. As we mentioned in the text, eight compounds (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 TS extracts. Of these compounds, kaempferol, quercetin, rutinand methyl gallate has demonstrated cytotoxic effect against human leukemic cells (Wang et al., 1999; Chen et al., 2009; Ren et al., 2010; Bourogaa et al., 2011), while methyl gallate and ethyl gallate showed minimal cytotoxic effect to cancer cells when compared to gallic acid (Chia et al., 2005). Although, the anticancer activity of gallic acid a major compound of TS have been demonstrated in various cancer types such as human prostate, oral and leukemic cancer (Yang et al., 2006; Chen et al., 2009; Chia et al., 2010). However, its molecular mechanism underlying the cytotoxic effect towards human premyelocytic leukemia has poorly understood. Therefore, the present study was designed to address the anti-cancer potential of gallic acid a major compound of TS.
References:
Wang et al., 1999.European Journal of Cancer, 35(10):1517-1525.
Chen et al., 2009. Toxicology in Vitro. 23:603–609
Ren et al., 2010. Zhongguo Shi Yan Xue Ye XueZaZhi. 18(3): 629-33.
Bourogaa et al., 2011. Leukemia Research. 35:1093-1101
Chia et al., 2010. Molecules. 15(11):8377-89.
Chen et al., 2009. Cancer Lett. 286(2):161-71.
Yang et al., 2006. Food ChemToxicol. 44(12):1978-88.
Chia et al., 2005. http://www.fda.gov/ohrms/dockets/dockets/95s0316/95s-0316-rpt0294-04-Chia-vol230.pdf

Comment: 2
The authors used 10-75 μg/mL TS extracts and 5-10 μg/mL gallic acid in HL60 in vitro experiments. Is there any relation between the concentrations of TS extract and gallic acid concentrations? If there is no relation between the TS extracts and gallic acid concentrations which were used in in vitro experiments the whole experiments were waste of time and energy.
Respond:
Thanks for your query. There was no relation between the concentrations of TS and GA. However, the concentrations used in this study were previously established (Yang et al., 2006). The cytotoxic effect of gallic acid varying from cell to cell for example the IC50 value of TS against ovarian (SKOV3), cervical (Hela), endometrical (RL95-2), liver (HepJ5) and prostate cancer (DUI45) were 28, >100, >100, 30, and 17.5 μg/mL. In a similar way the IC50 value of GA against prostate cancer cells including DUI45, LNCaP, and PC-3 were 15.6, 20.7, and 16.1 μg/mL (Chen et al., 2009). Therefore, there was no relation between TS and GA concentrations; however the inhibitory potential of these drugs independently exhibited significant cytotoxic effects against leukemic cells.
References:
Chen et al., 2009. Cancer Lett. 286(2):161-71.
Yang et al., 2006. Food Chem Toxicol. 44(12):1978-88.

Comment: 3
In the same way the concentrations in the in vivo experiments in mice should be explained if there is any link between these 2 concentrations (7.5 and 10 mg/kg) and human consumption of TS or why authors choose these concentrations?
Respond:

Thanks for your query. There was no any link between these two concentrations. However, the concentration 7.5 mg/kg body weight of TS in PBS was a dose equal to ten-fold of the IC50 value of HL-60 cells (73 μg/mL) (Yang et al., 2006).
References:
Yang et al., 2006. Food Chem Toxicol. 44(12):1978-88.

Comment: 4
Toona sinensis (xiangchun) is used in two different ways, as it mentioned in the Yollow Emperors Book of Internal Medicine as TCM. In China and Taiwan the young leaves of T. senensis were used as vegetable. According to the preparation (decoction for pharmaceutical application, and whole cooked vegetable) the concentration of active compounds are different and this should be discussed as the authors mentioned that this vegetable is one of the most popular vegetarian cuisines in Taiwan.
Respond:
Thank you for your query. Yes we agreed that the composition of active compounds may vary in different preparations. Currently, there was no report which deals the bioavailability and pharmacokinetics of major compounds in T. sinensis. Whereas, the bioavailability of gallic acid was studied in human subjects (Shahrzd et al., 2000). Briefly, commercially available gallic acid supplement (Acidumgallicum tablets that contain 10% of gallic acid and 90% of glucose) and black tea brew which containing 93% of free forms gallic acid was performed to determine the pharmacokinetics and relative bioavailability of gallic acid in health human volunteers. After administration of acidumgallicum or black tea (each containing 0.3 mmoL of gallic aicd) to human subjects. The serum and urine concentrations of gallic acid and their metabolites were determined. A maximum serum concentration of 2.08 μM was observed in plasma, whereas 39.6% of the GA dose was extracted in urine as a gallic acid or gallic acid metabolites (Shahrzad et al., 2001). This study also concluded that the relative bioavailability of gallic acid from tea compared with that from the tablets was 1.06 ± 0.26, showing that gallic acid is as available from drinking tea as it is from swallowing tablets of gallic acid. According to this report, we believe that the half cooked leaves may contain higher amount of active constituents then that of herbal preparations.
Reference:
Shahrzd S, Aoyagi K, Winter A, Koyama A, Bitsch I. 2000. Pharmacokinetics of Gallic Acid and Its Relative Bioavailability from Tea in Healthy Humans. J. Nutr. 131: 1207-10.

Reviewer: 2
Opinion:
In general, I found the manuscript to be well written and logically presented and should be published with minor revision. Although there has been a substantial amount of research published in this area (antiproliferative activity of T. sinensis extracts and gallic acid), the work on the cell-cycle arrest of the HL-60 cell line and the tumor implant results adds new knowledge to the area.
I would suggest a few small changes to the manuscript:

Comment: 1
The title should be changed to reflect the work more accurately: e.g. In Vitro and In Vivo Activity of Gallic Acid and Toona sinensis Leaf Extract against HL-60 Leukemia Cells.
Respond
As per your suggestion, we changed the title of our manuscript.

Comment: 2
The author's make a case for TS extracts/gallic acid as potential chemopreventatives. I would suggest that they could improve their introduction by noting or briefly referring to any pharmacokinetic or uptake studies that may have been published. For compounds to be useful, a therapeutic concentration

must be obtained in vivo. I was able to find one reference in this area which they may want to include - Shahrzd S, Aoyagi K, Winter A, Koyama A, Bitsch I. 2000. Pharmacokinetics of Gallic Acid and Its Relative Bioavailability from Tea in Healthy Humans. J. Nutr. 131: 1207-10.
Respond
Thanks for your suggestion. As per your suggestion, in the revised version we have included the information regarding pharmacokinetics of GA.
Page 2-3, Line 18-21.

Comment: 3
Line 29-37, p.2 (Abstract) - could be simplified to: "...were observed with gallic acid (5 and 10 ug/mL), a major component of TS. Our data suggests that Toonasinensis exerts antiproliferative effects on HL-60 cells in vitro and in vivo due mainly to the presence of gallic acid.
Respond:
Thank you for pointing this out. In the revised version, we modified the sentence with your suggestion.
Page 2, Line 14-15.

Comment: 4
In the Discussion the author's conclude that ". these results imply that gallic acid as one of the major active constituents (is) responsible for the observed antiproliferative activity." Since this has already been stated in other previous work albeit with different cell lines (e.g. Chen et al 2009) it would be more accurate to say that ".these results corroborate (or confirm) other studies which have implicated gallic acid is the main constituent responsible for the antiproliferative activity (Chen et al, 2009, ...
Respond:
Thank you for pointing this out. In the revised version, we modified the sentence with your suggestion.
Page 21, Line 14-16.

Comment: 5
There are a few awkward phrases or grammar which I expect will be caught in the proofs - e.g. p.11 line 49-50 no need for "Bear this in mind" p. 12 line 12-13". cascade is still in debate" p12 line 20-22 it is not clear what the authors are attempting to say.
Respond:
Thank you for pointing this out. In the revised version, we corrected the above mentioned phrases.
Page 12, Line 6.
Page 12, Line 16-17.
Page 13, Line 3.

As per your suggestion, we also carefully double checked the typos errors in our manuscript. Once again thank you for your kind considerations and help. We are looking forward to hear from you soon.

Sincerely,
Dr. Hsin-Ling Yang
Professor
Institute of Nutrition
China Medical University, Taichung, Taiwan
Email: hlyang@mail.cmu.edu.tw

Reply to the comments by Referees

March 23, 2012

(**Ms. ID. FCT-D-11-02062R1**)

Dear Editor-in-Chief,

Enclosed please find the revised version of our manuscript, entitled **"*In vitro* and *in vivo* activity of gallic acid and *Toona sinensis* leaf extracts against HL-60 human premyelocytic leukemia" (Ms. ID. FCT-D-11-02062R1)** by Pei-Jane Huang, You-Cheng Hseu, Meng-Shiou Lee, Chi-Rei Wu, K.J. Senthil Kumar, Ssu-Ching Chen, Li-Sung Hsu, Jiunn-Wang Liao, I-Shiung Cheng, Ya-Ting Kuo, Chih-Wei Chou, Hsin-Ling Yang which has been recently reviewed by your editorial board. An itemized list of changes made and our response to the reviewer's comments is also enclosed.

Again we thank the valuable comments from the referees, the main suggestions and comments are list and response as following. Kindly referred the yellow highlights in the manuscript are modified sentence or word.

Reviewer #1:

Major Comments:

Comment: 1

In the section of Material and Methods, preparation of TS extracts: 50 g of crude extracts contain 130±26 mg total phenol compounds. The authors isolated 8 compounds (gallic acid, methyl gallate, ethyl gallate, kaempferol, ...) by using HPLC. The concentration of these 8 different compounds should be written in the text. It is also unclear why the authors used only gallic acid for in vitro experiments. The state of the art is that all compounds should be tested in vitro and the most effective one should be used for the main experiments.

Respond:

Thanks for pointing this out. As we mentioned in the text, eight compounds (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 TS extracts. Of these compounds, kaempferol, quercetin, rutinand methyl gallate has demonstrated cytotoxic effect against human leukemic cells (Wang et al., 1999; Chen et al., 2009; Ren et al., 2010; Bourogaa et al., 2011), while methyl gallate and ethyl gallate showed minimal cytotoxic effect to cancer cells when compared to gallic acid (Chia et al., 2005). Although, the anticancer activity of gallic acid a major compound of TS have been demonstrated in various cancer types such as human prostate, oral and leukemic cancer (Yang et al., 2006; Chen et al., 2009; Chia et al., 2010). However, its molecular mechanism underlying the cytotoxic effect towards human premyelocytic leukemia has poorly understood. Therefore, the present study was designed to address the anti-cancer potential of gallic acid a major compound of TS.

References:

Wang et al., 1999.European Journal of Cancer, 35(10):1517-1525.

Chen et al., 2009. Toxicology in Vitro. 23:603–609

Ren et al., 2010. Zhongguo Shi Yan Xue Ye XueZaZhi. 18(3): 629-33.

Bourogaa et al., 2011. Leukemia Research. 35:1093-1101

Chia et al., 2010. Molecules. 15(11):8377-89.

Chen et al., 2009. Cancer Lett. 286(2):161-71.

Yang et al., 2006. Food ChemToxicol. 44(12):1978-88.

Chia et al., 2005. http://www.fda.gov/ohrms/dockets/dockets/95s0316/95s-0316-rpt0294-04-Chia-vol230.pdf

Comment: 2

The authors used 10-75 µg/mL TS extracts and 5-10 µg/mL gallic acid in HL60 in vitro experiments. Is there any relation between the concentrations of TS extract and gallic acid concentrations? If there is no relation between the TS extracts and gallic acid concentrations which were used in in vitro experiments the whole experiments were waste of time and energy.

Respond:

Thanks for your query. There was no relation between the concentrations of TS and GA. However, the concentrations used in this study were previously established (Yang et al., 2006).

The cytotoxic effect of gallic acid varying from cell to cell for example the IC_{50} value of TS against ovarian (SKOV3), cervical (Hela), endometrical (RL95-2), liver (HepJ5) and prostate cancer (DUI45) were 28, >100, >100, 30, and 17.5 µg/mL. In a similar way the IC_{50} value of GA against prostate cancer cells including DUI45, LNCaP, and PC-3 were 15.6, 20.7, and 16.1 µg/mL (Chen et al., 2009). Therefore, there was no relation between TS and GA concentrations; however the inhibitory potential of these drugs independently exhibited significant cytotoxic effects against leukemic cells.

References:

Chen et al., 2009. Cancer Lett. 286(2):161-71.

Yang et al., 2006. Food Chem Toxicol. 44(12):1978-88.

Comment: 3

In the same way the concentrations in the in vivo experiments in mice should be explained if there is any link between these 2 concentrations (7.5 and 10 mg/kg) and human consumption of TS or why authors choose these concentrations?

Respond:

Thanks for your query. There was no any link between these two concentrations. However, the concentration 7.5 mg/kg body weight of TS in PBS was a dose equal to ten-fold of the IC_{50} value of HL-60 cells (73 µg/mL) (Yang et al., 2006).

References:

Yang et al., 2006. Food Chem Toxicol. 44(12):1978-88.

Comment: 4

Toona sinensis (xiangchun) is used in two different ways, as it mentioned in the Yollow Emperors Book of Internal Medicine as TCM. In China and Taiwan the young leaves of T. senensis were used as vegetable. According to the preparation (decoction for pharmaceutical application, and whole cooked vegetable) the concentration of active compounds are different and this should be discussed as the authors mentioned that this vegetable is one of the most popular vegetarian cuisines in Taiwan.

Respond:

Thank you for your query. Yes we agreed that the composition of active compounds may vary in

different preparations. Currently, there was no report which deals the bioavailability and pharmacokinetics of major compounds in *T. sinensis*. Whereas, the bioavailability of gallic acid was studied in human subjects (Shahrzd et al., 2000). Briefly, commercially available gallic acid supplement (Acidumgallicum tablets that contain 10% of gallic acid and 90% of glucose) and black tea brew which containing 93% of free forms gallic acid was performed to determine the pharmacokinetics and relative bioavailability of gallic acid in health human volunteers. After administration of acidumgallicum or black tea (each containing 0.3 mmoL of gallic aicd) to human subjects. The serum and urine concentrations of gallic acid and their metabolites were determined. A maximum serum concentration of 2.08 µM was observed in plasma, whereas 39.6% of the GA dose was extracted in urine as a gallic acid or gallic acid metabolites (Shahrzad et al., 2001). This study also concluded that the relative bioavailability of gallic acid from tea compared with that from the tablets was 1.06 ± 0.26, showing that gallic acid is as available from drinking tea as it is from swallowing tablets of gallic acid. According to this report, we believe that the half cooked leaves may contain higher amount of active constituents then that of herbal preparations.

Reference:

Shahrzd S, Aoyagi K, Winter A, Koyama A, Bitsch I. 2000. Pharmacokinetics of Gallic Acid and Its Relative Bioavailability from Tea in Healthy Humans. J. Nutr. 131: 1207-10.

Reviewer: 2

Opinion:

In general, I found the manuscript to be well written and logically presented and should be published with minor revision. Although there has been a substantial amount of research published in this area (antiproliferative activity of *T. sinensis* extracts and gallic acid), the work on the cell-cycle arrest of the HL-60 cell line and the tumor implant results adds new knowledge to the area.

I would suggest a few small changes to the manuscript:

Comment: 1

The title should be changed to reflect the work more accurately: e.g. In Vitro and In Vivo Activity of Gallic Acid and Toona sinensis Leaf Extract against HL-60 Leukemia Cells.

Respond

As per your suggestion, we changed the title of our manuscript.

Comment: 2

The author's make a case for TS extracts/gallic acid as potential chemopreventatives. I would suggest that they could improve their introduction by noting or briefly referring to any pharmacokinetic or uptake studies that may have been published. For compounds to be useful, a therapeutic concentration must be obtained in vivo. I was able to find one reference in this area which they may want to include - Shahrzd S, Aoyagi K, Winter A, Koyama A, Bitsch I. 2000. Pharmacokinetics of Gallic Acid and Its Relative Bioavailability from Tea in Healthy Humans. J. Nutr. 131: 1207-10.

Respond

Thanks for your suggestion. As per your suggestion, in the revised version we have included the information regarding pharmacokinetics of GA.

Page 2-3, Line 18-21.

Comment: 3

Line 29-37, p.2 (Abstract) - could be simplified to: "...were observed with gallic acid (5 and 10 ug/mL), a major component of TS. Our data suggests that Toonasinensis exerts antiproliferative

effects on HL-60 cells in vitro and in vivo due mainly to the presence of gallic acid.

Respond:

Thank you for pointing this out. In the revised version, we modified the sentence with your suggestion.

Page 2, Line 14-15.

Comment: 4

In the Discussion the author's conclude that ". these results imply that gallic acid as one of the major active constituents (is) responsible for the observed antiproliferative activity." Since this has already been stated in other previous work albeit with different cell lines (e.g. Chen et al 2009) it would be more accurate to say that ".these results corroborate (or confirm) other studies which have implicated gallic acid is the main constituent responsible for the antiproliferative activity (Chen et al, 2009, ...

Respond:

Thank you for pointing this out. In the revised version, we modified the sentence with your suggestion.

Page 21, Line 14-16.

Comment: 5

There are a few awkward phrases or grammar which I expect will be caught in the proofs - e.g. p.11 line 49-50 no need for "Bear this in mind" p. 12 line 12-13". cascade is still in debate" p12 line 20-22 it is not clear what the authors are attempting to say.

Respond:

Thank you for pointing this out. In the revised version, we corrected the above mentioned phrases.

Page 12, Line 6.

Page 12, Line 16-17.

Page 13, Line 3.

As per your suggestion, we also carefully double checked the typos errors in our manuscript. Once again thank you for your kind considerations and help. We are looking forward to hear from you soon.

Sincerely,

Dr. Hsin-Ling Yang
Professor
Institute of Nutrition
China Medical University, Taichung, Taiwan
Email: hlyang@mail.cmu.edu.tw

中 國 醫 藥 大 學

CHINA MEDICAL UNIVERSITY

91 Hsueh Shih Road

TAICHUNG, TAIWAN, R.O.C

Food and Chemical Toxicology *Dec 04, 2011*

Dear editor:

Enclosed is of "**Aqueous leaf extracts of *Toona sinensis* inhibit proliferation of human premyelocytic leukemia HL-60 cells *in vitro* and *in vivo***" for considered to be published in the **"Food and Chemical Toxicology".**

Toona sinensis (A. Juss.) M. Roem popularly known as Chinese mahogany or Chinese toon, a member of Meliaceae family, and is a type of deciduous perennial tree widely distributed in Asia. In Chinese and Taiwanese culture it is a very popular vegetarian cuisine. It has long been used as a traditional Chinese medicine for a wide variety of conditions. The edible leaves used as an oriental medicine for treating rheumatoid arthritis, cervicitis, urethritis, tympanitis, gastric ulcers, enteritis, dysentery, itchiness, and cancer. While the underlying pharmacological mechanisms of this new drug are still a matter of debate, previous scientific literatures have been reported that *T. sinensis* possessed variety of biological activities including anti-cancer, anti-angiogenesis, anti-inflammation, anti-diabetes, and antioxidant effects, as well as inhibiting Leydig cell steroidogenesis and improving the dynamic activity of human sperm quality.

Leukemia is one of the most threatening diseases today. Given that most adult leukemia patients are not candidates for transplantation, and that a more rational therapy is not adequately defined, they are typically treated with regimens that are based on (or at least include) chemotherapy. In our previous study, we demonstrated that the aqueous leaf extracts of *T. sinensis* (TS extracts) and gallic acid (3,4,5-trihydroxybenoic acid), a purified natural phenolic component, exhibited apoptosis against human premyelocytic leukemia (HL-60) cells. However, the effect of TS extracts against tumour cell-cycle regulation was poorly understood. Therefore, the present study aimed to investigate the anticancer effect of TS extracts and gallic acid in terms of tumor regression using *in vitro* cell culture model

(HL-60 cells) or *in vivo* athymic nude mice model of leukemia cancer. Furthermore, to establish the mechanism(s) underlying the *T. sinensis* anticancer properties, the levels of cell cycle control and the related molecules were assayed.

ABSTRACT: "*Toona sinensis* is one of the most popular vegetarian cuisines in Taiwan and it has been shown to induce apoptosis in cultured human premyelocytic leukemia (HL-60) cells. In the present study, we examined the effects of *Toona sinensis* leaf extracts (TS extracts) on tumor regression using *in vitro* cell culture and an *in vivo* athymic nude mice model. We found that TS extracts (10-75 μg/mL) arrested HL-60 cells at the G_1-S transition phase through the reductions of Cyclin D1, CDK4, Cyclin E, CDK2, and Cyclin A, and induction of CDK inhibitor $p27^{KIP}$ levels. Furthermore, VEGF expression and release was significantly inhibited by TS extracts. Notably, TS extracts treatment was effective in terms of delaying tumor incidence in the nude mice inoculated with HL-60 cells as well as reducing the tumor burden. Histological analysis confirmed that TS extracts significantly modulated tumor progression in xenograft tumor. Furthermore, a similar pattern of results were observed from gallic acid (5 and 10 μg/mL), a major compound in TS, caused G_1 arrest through regulations of cell-cycle regulatory proteins. Our data suggest that *Toona sinensis* exerts antiproliferative effects on HL-60 cells *in vitro* and *in vivo* due mainly to the presence of gallic acid. "

The study was performed according to the international, national and institutional rules regarding animal experiments, clinical studies and biodiversity rights and includes a clear explanation of the pharmaceutical importance of the study. The manuscript and its contents have not been published previously and are not under consideration for publication in another journal. We believe the paper may be of particular interest to your readers. Correspondence regarding the paper should be directed to me at the following address:

Prof. Hsin-Ling Yang
Institute of Nutrition,
China Medical University,
Huseh-Shih Road 91,
Taichung 40402, Taiwan.
Email: hlyang@mail.cmu.edu.tw
Tel: 886-4-22053366 ext 7503
Fax: 886-4-22078083

Thank you for your kind consideration and help. We look forward to hearing from you soon.

Sincerely yours,

Hsin-Ling Yang

***Conflict of Interest Statement**

Food and Chemical Toxicology

Conflict of Interest Policy

Supplement:
Article Title: *In vitro* and *in vivo* activity of gallic acid and *Toona sinensis* leaf extracts against HL-60 human premyelocytic leukemia

Author name: Pei-Jane Huang, You-Cheng Hseu, Meng-Shiou Lee, K.J. Senthil Kumar, Chi-Rei Wu, Li-Sung Hsu, Jiunn-Wang Liao, I-Shiung Cheng, Ya-Ting Kuo, Shi-Ying Huang, Hsin-Ling Yang

Declarations

Food and Chemical Toxicology requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

NONE

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

NONE

Signature (a scanned signature is acceptable, but each author must sign)	**Print name**
Pei-Jane Huang	Pei-Jane Huang
You-cheng Hseu	You-Cheng Hseu
Meng-Shiou Lee	Meng-Shiou Lee
[illegible]	K.J. Senthil Kumar
Chi-Rei Wu	Chi-Rei Wu
Li-Sung Hsu	Li-Sung Hsu
Jiunn-Wang Liao	Jiunn-Wang Liao
I-Shiung Cheng	I-Shiung Cheng
Ya-Ting Kuo	Ya-Ting Kuo
Shi-Ying Huang	Shi-Ying Huang
Hsin-Ling Yang	Hsin-Ling Yang

Research Article

In vitro and *in vivo* activity of gallic acid and *Toona sinensis* leaf extracts against HL-60 human premyelocytic leukemia

Pei-Jane Huang[a,1], You-Cheng Hseu[b,1], Meng-Shiou Lee[c], K.J. Senthil Kumar[b], Chi-Rei Wu[c], Li-Sung Hsu[d], Jiunn-Wang Liao[e], I-Shiung Cheng[f], Ya-Ting Kuo[h], Shi-Ying Huang[g,*], Hsin-Ling Yang[h,*]

[a]*Department of Health and Nutrition Biotechnology, Asia University, Taichung 41354, Taiwan*

[b]*Department of Cosmeceutics, College of Pharmacy, China Medical University, Taichung 40402, Taiwan*

[c]*School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, College of Pharmacy, China Medical University, Taichung 40402, Taiwan*

[d]*Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung 40402, Taiwan*

[e]*Graduate Institute of Veterinary Pathology, National Chung Hsing University, Taichung 40402, Taiwan*

[f]*Department of Physical Education, National Taichung University of Education, Taichung 40402, Taiwan*

[g]*Department of pediatrics, Armed Force Taoyuan General Hospital, Taoyuan 32551, Taiwan*

[h]*Institute of Nutrition, China Medical University, Taichung 40402, Taiwan*

*Corresponding authors. Tel.: +886 4 22053366 x 7503; fax: +886-4-22062891.

E-mail addresses: hlyang@mail.cmu.edu.tw (H-L, Yang) ; Huangsy56@yahoo.com.tw (S-Y, Huang)

[1] Both authors contributed equally.

Highlights

1. *Toona sinensis* leaf extracts (TS extracts) arrested HL-60 cell growth at G_1-S transition phase.
2. TS extracts induced cell-cycle arrest was mediated by ROS generation in HL-60 cells.
3. TS extracts inhibited VEGF expression and release in HL-60 cells.
4. TS extracts delayed tumor progression in HL-60 xenograft nude mice.

Research Article

In vitro and *in vivo* activity of gallic acid and *Toona sinensis* leaf extracts against HL-60 human premyelocytic leukemia

Pei-Jane Huang[a,1], You-Cheng Hseu[b,1], Meng-Shiou Lee[c], K.J. Senthil Kumar[b], Chi-Rei Wu[c], Li-Sung Hsu[d], Jiunn-Wang Liao[e], I-Shiung Cheng[f], Ya-Ting Kuo[h], Shi-Ying Huang[g,*], Hsin-Ling Yang[h,*]

[a]*Department of Health and Nutrition Biotechnology, Asia University, Taichung 41354, Taiwan*

[b]*Department of Cosmeceutics, College of Pharmacy, China Medical University, Taichung 40402, Taiwan*

[c]*School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, College of Pharmacy, China Medical University, Taichung 40402, Taiwan*

[d]*Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung 40402, Taiwan*

[e]*Graduate Institute of Veterinary Pathology, National Chung Hsing University, Taichung 40402, Taiwan*

[f]*Department of Physical Education, National Taichung University of Education, Taichung 40402, Taiwan*

[g]*Department of pediatrics, Armed Force Taoyuan General Hospital, Taoyuan 32551, Taiwan*

[h]*Institute of Nutrition, China Medical University, Taichung 40402, Taiwan*

*Corresponding authors. Tel.: +886 4 22053366 x 7503; fax: +886-4-22062891.

E-mail addresses: hlyang@mail.cmu.edu.tw (H-L, Yang); Huangsy56@yahoo.com.tw (S-Y, Huang)

[1] Both authors contributed equally.

ABSTRACT

Toona sinensis is one of the most popular vegetarian cuisines in Taiwan and it has been shown to induce apoptosis in cultured human premyelocytic leukemia (HL-60) cells. In the present study, we examined the effects of *Toona sinensis* leaf extracts (TS extracts) on tumor regression using *in vitro* cell culture and an *in vivo* athymic nude mice model. We found that TS extracts (10-75 μg/mL) arrested HL-60 cells at the G_1-S transition phase through the reductions of Cyclin D1, CDK4, Cyclin E, CDK2, and Cyclin A, and induction of CDK inhibitor $p27^{KIP}$ levels. Furthermore, VEGF expression and release was significantly inhibited by TS extracts. Notably, TS extracts treatment was effective in terms of delaying tumor incidence in the nude mice inoculated with HL-60 cells as well as reducing the tumor burden. Histological analysis confirmed that TS extracts significantly modulated tumor progression in xenograft tumor. Furthermore, a similar pattern of results were observed from gallic acid (5 and 10 μg/mL), a major compound in TS, caused G_1 arrest through regulations of cell-cycle regulatory proteins. Our data suggest that *Toona sinensis* exerts antiproliferative effects on HL-60 cells *in vitro* and *in vivo* due mainly to the presence of gallic acid.

Keywords:

Toona sinensis

Gallic acid

HL-60 cells

Cell-cycle arrest

Xenografted nude mice

1. Introduction

Toona sinensis (A. Juss.) M. Roem popularly known as Chinese mahogany or Chinese toon, a member of Meliaceae family, and is a type of deciduous perennial tree widely distributed in Asia. In Chinese and Taiwanese culture it is one of the popular vegetarian cuisine. It has long been used as a traditional Chinese medicine for a wide variety of conditions. The edible leaves used as an oriental medicine for treating rheumatoid arthritis, cervicitis, urethritis, tympanitis, gastric ulcers, enteritis, dysentery, itchiness, and cancer (Edmonds and Staniforth, 1998; Hseu et al., 2011a). While the underlying pharmacological mechanisms of this new drug are still a matter of debate, previous scientific literatures have been reported that *T. sinensis* possessed variety of biological activities including anti-cancer (Chang et al., 2002; Chang et al., 2006; Yang et al., 2006a; Chen et al., 2009; Wang et al., 2010; Yang et al., 2010a; Yang et al., 2010b; Chia et al., 2010), anti-angiogenesis (Hseu et al., 2011a) anti-inflammation (Bak et al., 2009), anti-diabetes (Hsu et al., 2003; Yang et al., 2003), and antioxidant (Cho et al., 2003; Hseu et al., 2008a; Jiang et al., 2009) effects, as well as inhibiting Leydig cell steroidogenesis and improving the dynamic activity of human sperm quality (Poon et al., 2005). Moreover, the safety levels and nontoxic characteristics of aqueous extracts of *T. sinensis* were evaluated using acute and sub-acute toxicity studies in mice (Liao et al., 2006).

Gallic acid (GA), a major phenolic compound that rich in TSL has a wide spectrum of biological and pharmacological effects. Various animal models or human studies proved that GA is extremely safe even at using high doses. Also, a few studies addressing the bioavailability of GA in human revealed that this compound is extremely well observed when compared to other polyphenols (Manach et al., 2005). When GA was given orally at a dose of 0.3 mmoL in Assam black tea (contained >93% of free form GA) to human, a maximum serum concentration of 2.08 μM was observed in plasma, whereas 39.6% of the

Formatted: Not Highlight

GA dose were extracted in urine as a GA or GA metabolites (Shahrzad et al., 2001). The pharmacological safety and efficacy of GA makes it a potential compound for treatment or prevention of a wide variety of human diseases.

Chemoprevention, which refers to the administration of natural or synthetic agents to prevent initiation and promotion events associated with carcinogenesis, is being increasingly appreciated as an effective approach for the management of neoplasia (Ahmad et al., 2001). Many studies have shown a clear link between abnormal cell-cycle regulation and apoptosis with cancer, as much as the cell-cycle inhibitors and apoptosis-inducing agents are being appreciated as armaments for the management of cancer (Stewart et al., 2003; Schmitt, 2003; Hsu et al., 2003). Eukaryotic cell-cycle progression involves a sequential activation of cyclin-dependent kinases (CDKs) whose activation is dependent upon their association with cyclins (Youn et al., 2008). Progression through the mammalian mitotic cycle is controlled by multiple holoenzymes comprising a catalytic CDK and a cyclin regulatory subunit (Takahashi et al., 1999; Hseu et al., 2008b). These cyclin-CDK complexes are activated at specific intervals during the cell-cycle but can also be induced and regulated by exogenous factors. Cell-cycle progression is also regulated by the relative balance between the cellular concentrations of cyclins/CDKs and CDKs inhibitors, including $p27^{KIP}$ (Hseu et al., 2008b; Kim et al., 2006). The cyclin-CDK complexes are subjected to inhibition *via* binding with CDK inhibitors (Kim et al., 2006). Recently, the relationship between cell-cycle arrest and cancer has been emphasized, with increasing evidence suggesting that the related processes of neoplastic transformation, progression and metastasis involve alteration of the normal cell-cycle regulation. Thus, anticancer (chemopreventive) agents may alter regulation of the cell-cycle machinery, resulting in an arrest of cells in different phases of the cell-cycle and, thereby, reducing growth and proliferation of cancerous cells, which may be useful in cancer therapy.

Leukemia is one of the most threatening diseases today. Given that most adult leukemia patients are not candidates for transplantation, and that a more rational therapy is not adequately defined, they are typically treated with regimens that are based on (or at least include) chemotherapy (Yang et al., 2006a). In our previous study, we demonstrated that aqueous leaf extracts of *T. sinensis* (TS extracts, 10-75 μg/mL) and gallic acid (3,4,5-trihydroxybenoic acid, 5-10 μg/mL), a purified natural phenolic component, exhibited apoptosis against human premyelocytic leukemia (HL-60) cells (Yang et al., 2006a). Notably, the significant inhibitory effects of tumor cell proliferation were observed only in leukemia HL-60 cells, whereas not in erythrocytes and human lymphocytes (Yang et al., 2006a). However, the effect of TS extracts against tumour cell-cycle regulation was poorly understood. Therefore, the present study aimed to investigate the anticancer effect of TS extracts and gallic acid in terms of tumor regression using *in vitro* cell culture model (HL-60 cells) or *in vivo* athymic nude mice model of leukemia cancer.

2. Materials and Methods

2.1. Chemicals

RPMI-1640 medium (Gibco BRL, Grand Island, NY), antibody against cyclin E, CDK2, cyclin B1, CDC2, caspase-8, Fas, FasL, VEGF, and β-actin (Santa Cruz Biotechnology Inc., Heidelberg, Germany) and antibody against cyclin D1, CDK4, cyclin A, $p27^{KIP}$, p15, caspase-9, and Bid (Cell Signaling Technology Inc., Danvers, MA) were obtained from their respective suppliers. All other chemicals were of the reagent or HPLC grade supplied either by Merck (Darmstadt, Germany) or Sigma (St. Louis, MO).

2.2. Preparation of TS extracts

Leaves of *T. sinensis* were sourced from Fooyin University, Kaohsiung, Taiwan. A

voucher specimen was characterized by Prof. Horng-Liang Lay, Graduate Institute of Biotechnology, National Pingtung University of Science and Technology, Pingtung County, Taiwan, and deposited at Fooyin University, Kaohsiung, Taiwan. The aqueous leaf extracts of *T. sinensis* (TS extracts) were prepared by adding 1000 mL of water to 1000 g of fresh *T. sinensis* leaves and boiled until 100 mL remained, as previously described (Chang et al., 2002; Hseu et al., 2008a). The crude extracts were centrifuged at 3000 × *g* for 12 min and the supernatant was 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 further analysis of its anticancer properties. The yield of TS extracts was 6%. The total phenolic content of the TS extracts was estimated to be 130 ± 26 mg gallic acid (pyrocatechol) equivalents/g of plant extracts as described previously (Yang et al., 2006a).

2.3. Isolation of gallic acid from *TS extracts*

TS extracts were dissolved in a mobile phase consisting of methanol-water (50:50, v/v) before high performance liquid chromatography (HPLC) analysis and separation. Chromatographic separation was achieved with a mobile phase consisting of methanol-water (50:50, v/v) in the first 15 min, gradually increasing the methanol to 100% in the next 10 min. A flow rate of 4.0 mL/min at room temperature was used. Eight compounds (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 TS extracts. The identification of the compounds was fully characterized by comparison of their spectral data (IR, NMR, and mass) with the analogous information reported in the literature (Yang et al., 2006a; Hsu et al., 2003). Gallic acid, a natural phenolic component purified from TS extracts was subjected in this study at a yield of 6% (Yang et al., 2006a).

2.4. Cell culture

Human acute promyeloblastic leukemia (HL-60) cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). 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 were counted by hemocytometer.

2.5. Flow cytometry analysis

Cellular DNA content was determined by flow cytometric analysis of propidium iodide (PI)-labeled cells as described previously (Hseu et al., 2007). In brief, HL-60 cells (2×10^5 cells/mL) were cultured in 6 cm culture dishes. After treatment with TS extracts or gallic acid, cells were harvested, washed and suspended in PBS and fixed in ice-cold 70% ethanol at -20 °C for overnight. After incubation, cells were re-suspended in PBS containing 1% Triton X-100, 0.5 mg/mL RNase, and 4 μg/mL PI at 37 °C for 30 min. A FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a single argon-ion laser (488 nm) was used for flow cytometric analysis. Forward and right-angle light scatter, which are correlated with the size of the cell and the cytoplasmic complexity, respectively, were used to establish size gates and exclude cellular debris from the analysis. DNA content of 1×10^4 cells per analysis was monitored using the FACSCalibur system. The cell-cycle was determined and analyzed using ModFit software (Verity Software House, Topsham, ME). Apoptotic nuclei were identified as a subploid DNA peak, and were distinguished from cell debris on the basis of forward light scatter and PI fluorescence.

2.6. Protein isolation and immunoblot analysis

HL-60 cells (2×10^6 cells/ 10 cm dish) were washed once in cold PBS, and suspended in 100 μL lysis buffer (10 mM Tris-HCl [pH 8], 0.32 M sucrose, 1% Triton X-100, 5 mM EDTA, 2 mM DTT, and 1 mM phenylmethyl sulfony flouride). The suspension was vortex and kept on ice for 20 min and then centrifuged at 15000 × *g* for 20 min at 4 °C. Total protein content was determined using Bio-Rad protein assay reagent, with bovine serum albumin (BSA) as the standard; protein extracts were reconstituted in sample buffer (0.062 M Tris-HCl, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol), and the mixture was boiled at 94 °C for 5 min. Equal amounts (50 μg) of the denatured proteins were loaded into each lane, separated by 10-15% SDS polyacrylamide gel, followed by transfer of the proteins to PVDF membranes overnight. Membranes were blocked with 0.1% Tween-20 in Tris-buffered saline containing 5% non-fat dry milk for 20 min at room temperature, and the membranes were reacted with primary antibodies for 2 h. They were then incubated with a horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody for 2 h before being developed by SuperSignal ULTRA chemiluminescence substrate (Pierce Biotechnology, Rockford, IL). For densitometry analysis band intensities were quantified by commercially available software AlpaEaseFc 4.0 (Genetic Technologies, Inc., Miami, FL).

2.7. Determination of VEGF release

To determine the effects of TS extracts on VEGF levels, HL-60 cells grown to 85% confluence were treated with 0-75 μg/mL of TS extracts for 6 h. Then, the medium was aspirated from the flasks and centrifuged at 500 × *g* for 10 min to remove cells from the medium. The level of VEGF released into the culture medium was estimated using commercially available VEGF ELISA kit (Chemicon International Inc., Temecula, CA).

2.8. Animal experiments

Eight weeks old male or female athymic nude mice (BALB/*c-nu* were purchased from GlycoNex Inc., (Taipei, Taiwan) and were maintained in cage housing separately in a specifically designed pathogen-free isolation facility with a 12 h light and 12 h dark cycle; the mice were provided rodent chow (Oriental Yeast Co, Tokyo, Japan) and water *ad libitum*. All experiments were conducted in accordance with the guidelines of the China Medical University Animal Ethics Research Board.

2.9. Tumor cell inoculation

HL-60 cells were grown in RPMI-1640 medium supplemented with 10% FBS, 2 mM glutamine, 1% penicillin-streptomycin-neomycin in a humidified incubator (5% CO_2 in air at 37°C). Experiments were carried out using cells less than 15 passages. HL-60 cells (1×10^6 cells in 200 μL matrix gel) were injected subcutaneously on the right hind flank of nude mice as described previously (Hseu et al., 2008b). Tumor volume, as determined by caliper measurements of tumor length, width and depth, were calculated using the formula: length × width^2 × 1/2 every 3 days (Collins et al., 2003). The two study groups received intraperitoneal injections of TS extracts (0.2 mL/mouse) dissolved in PBS buffer at 7.5 mg/kg and 10 mg/kg every 2 days, while the control group received vehicle only. After 21 days of treatment, the mice were sacrificed. The tumors were removed and weighed before fixing in 4% paraformaldehyde, sectioning and staining with hematoxylin-eosin for light microscopic analysis. Part of the tumor tissue was immediately frozen and the rest was fixed in 10% neutral-buffered formalin and embedded in paraffin. To monitor drug toxicity, the body weight of each animal was measured every 3 days. In addition, a pathologist examined the mouse organs, including liver, lungs and kidneys.

2.10. Statistical analysis

The results of the *in vitro* and *in vivo* experiments are presented as mean and standard deviation (mean ± SD) or standard error (mean ± SE), respectively. All study data were analyzed using analysis of variance (ANOVA), followed by Dunnett's test for pair-wise comparison. Statistical significance was defined as $p < 0.05$ for all tests.

3. Results

This study has investigated the anticancer effect of aqueous leaf extracts of *T. sinensis* (0-75 μg/mL) and gallic acid (0-10 μg/mL) *in vitro* using HL-60 premyelocytic leukemia cell line or *in vivo* nude mice xenograft model. The crude TS extracts were prepared from fresh *T. sinensis* leaves, yielding 6% based on the initial weight of *T. sinensis* leaves and the total yield of gallic acid from the TS extracts was 6% (Yang et al., 2006a).

3.1. TS extracts induce G_1 cell-cycle arrest in HL-60 cells

Flow cytometric analysis was used to obtain the profile of DNA content of the HL-60 cells treated with TS extracts to measure the fluorescence of PI-DNA complex. HL-60 cells with lower DNA staining relative to diploid analogs were considered apoptosis. A remarkable accumulation of subploid cells, the so-called sub-G_1 peak, was noted in those treated with TS extracts (75 μg/mL) for 0-18 h compared with the untreated group (Fig. 1). Furthermore, the stage at which growth inhibition was induced by TS extracts in the HL-60 cell-cycle progression was determined, from cellular distribution in the different phases of post treatment. Fig. 1 showed that exposure of cells to the TS extracts resulted in a time-dependent progressive and sustained accumulation of cells in the G_1 phase. Furthermore, in response to TS extracts the percentage of cells in the G_1 phase was gradually increased

whereas, those in the S and G_2/M phases was significantly decreased (Fig. 1). We have previously reported that TS extracts dose- and time-dependently inhibits the growth of HL-60 cells (Yang et al., 2006a). Consistent with our previous report, the current findings also suggest that TS extracts promote cell growth inhibition by inducing G_1 transition phase arrest in HL-60 cells.

3.2. TS extracts down-regulate Cyclin D1, CDK4, Cyclin E, CDK2, and Cyclin A expression and up-regulates P27^{KIP} expression

To examine the molecular mechanism(s) that may underlying changes in cell-cycle patterns, the effects of the TS extracts on various cyclins and cyclin-dependent kinases (CDKs) involved in cell-cycle control of the HL-60 cells were investigated. Our investigative approach was to treat the HL-60 cells with TS extracts (0-75 μg/mL) for 0-6 h. Dose and time-dependent reduction in cyclin D1, CDK4, cyclin E, CDK2, and cyclin A expression were observed after treatment with TS extracts (Fig. 2). Moreover, the experimental treatment did not appear to alter the amount of detectable cyclin B1 and CDC2 protein expression in HL-60 cells (Fig. 2). We also examined the effect of TS extracts on CDKs inhibitors including p27^{KIP} and p15. As shown in Fig. 2, treatment of HL-60 cells with TS extracts (0-75 μg/mL) for 0-6 h induced marked up-regulation of p27^{KIP} protein. However, we found there was no change in the detectable amount of p15 protein in HL-60 cells (Fig. 2). Taken together, TS extracts potentially arrest G_1-S transition phase as evidenced by down-regulation of cyclins and CDKs and enhanced CDKs inhibitors.

3.3. Activation of Fas-associated apoptotic pathway by TS extracts

To assess whether TS extracts (0-75 μg/mL for 0-6 h) promoted apoptosis *via* a death

receptor-associated pathway, the Fas and Fas ligand (FasL) protein levels in HL-60 cells were determined by Western blotting. Results showed that TS extracts appreciably stimulate the expression of Fas and FasL in a dose- and time-independent manner (Fig. 3). It is well understood that induction of Fas and FasL cleaves caspase-8 from procaspase-8, and the activated caspase-8 further stimulates caspase-3 *via* mitochondrial-dependent or –independent cascade (Nagata, 1997). Therefore, we verified whether TS extracts augment caspase-8 cleavage in HL-60 cells. Western blot results showed that TS extracts dose-and time-dependently induced cleavage of caspase-8 from the procaspase-8 (Fig. 3). In mitochondrial pathway of apoptosis, caspase-8 proteolytically activates a pro-apoptotic protein Bid, which targets mitochondrial membrane permiabilization and represents the mail link between extrinsic and intrinsic apoptotic pathways (Eskes et al., 2000). Our results also showed that down-regulation of Bid induced by TS extracts occurred in a dose- and time-independent manner (Fig. 3). In addition, we observed TS extracts activates caspase-9, which was concomitant with our previous report that TS extracts induced apoptosis through the release of cytochrome *c* (Yang et al., 2006a). However, the signaling mechanism is poorly understood. This data provided strong evidence that TS extracts-induced release of cyctochrome *c* further promotes apoptosome-mediated cleavage of caspace-9 from procaspase-9. With reference to our previous report, we assured that TS extracts-induced aberrant release of cytochrome *c* further amplified the cleavage of caspase-9 in HL-60 cells (Fig. 3).

3.4. Effect of catalase on TS extracts-induced cell-cycle arrest and apoptosis in HL-60 cells.

Our previous study demonstrated that catalase (H_2O_2 scavenger) significantly decreased *T. sinensis*-induced cytotoxicity, DNA fragmentation, and ROS generation in HL-60 cells

(Yang et al., 2006a). Further to confirm this issue, in the present study we examined the antioxidant catalase could effect TS extracts-induced cell-cycle arrest (cyclin D1, CDK4, cyclin E, CDK2, cyclin A, and $p27^{KIP}$) and apoptosis (Fas/FasL, caspase-8, Bid, and caspase-9) in HL-60 cells. Cells were simultaneously treated with TS extracts (75 μg/mL for 6 h) and catalase (10 U/mL) for indicated time period (Fig. 2 and 3). We found that catalase treatment significantly reduced TS extracts-induced G_1 arrest in HL-60 cells as evidenced by up-regulation of cell-cycle regulatory proteins including cyclin D1, CDK4, cyclin E, CDK2, cyclin A, and inhibits $p27^{KIP}$. Furthermore, catalase treatment markedly down-regulates death signaling cascades and pro-apoptotic proteins Fas, FasL, caspase-8, Bid, and caspase-9 in HL-60 cells (Fig. 2 and 3). These results also provided a positive mechanism that TS extracts-induced HL-60 cell-cycle arrest (G_1) and apoptosis was associated with the production of intracellular ROS, especially H_2O_2.

3.5. TS extracts induce down-regulation of VEGF in HL-60 cells

A number of studies have shown that VEGF is one the most important angiogenic factor closely associated with neovascularization in human tumors. Western blotting and ELISA assay were used to analyze the effects of TS extracts on the expression and release of angiogenic-related protein VEGF in HL-60 cells. As shown in Fig. 4A, treatment of HL-60 cells with TS extracts dose-dependently inhibits the expression of VEGF. In addition, control cells (without treatment) released detectable levels of VEGF into the serum-free culture media at approximately 27 pg/10^5 cells (Fig. 4B). A concomitant with protein level, TS extracts significantly inhibits VEGF release into culture media in a dose-dependent manner (Fig. 4B).

3.6. Effect of TS extracts on tumor growth in HL-60 xenograft nude mice.

Nude mice were used to evaluate the *in vivo* effect of TS extracts on tumor growth. HL-60 cells were xenograft into nude mice as described in materials and methods. All the animals appeared healthy with no loss of body weight noted during treatment with TS extracts (Fig. 5A). In addition, no signs of toxicity were observed (data not shown) in any of the nude mice. The time course for HL-60 xenograft growth with TS extracts (7.5 and 10.0 mg/kg) or without treatment (control) is shown in Fig. 5B. Evaluation of tumor volume showed significant growth inhibition associated with TS extracts treatment (Fig. 5B). At the end of 21 days, the HL-60 xenograft tumor of each mouse was excised from each sacrificed animal and weighed. Tumor weight in the TS extracts-treated (7.5 and 10.0 mg/kg) mice was inhibited as compared with the control group (Fig. 6A and 6B). In addition, abundant mitosis in nuclei was observed in xenograft tumor section, indicating the proliferating activity, with well differentiation of tumor cells were also noticed (Fig. 7A). While decreased mitotic figures shrunken tumor cells were noted in the 7.5 mg/kg TS extracts treated animals (Fig. 7B), and tumor cells became smaller and shrunken, indicating the regression of tumor cells, in the 10 mg/kg TS extracts treated animals (Fig. 7C). These *in vivo* data also strongly suggest that TS extracts exerted antitumor activity in HL-60 leukemia xenograft nude mice could be due to the modulation of cell-cycle regulation and/or induction of apoptosis.

3.7. Gallic acid causes G_1 arrest and regulates cell-cycle regulatory proteins in HL-60 cells.

Previously we reported that treatment of the HL-60 cells with gallic acid (5-10 μg/mL), purified from TS extracts, resulted in sequences of events marked by apoptosis in the HL-60 cells was accompanied by loss of cell viability, ROS generation, internucleosomal DNA fragmentation, cytochrome *c* release, activation of caspase-3, degradation of poly(ADP-

ribose) polymerase (PARP), and dysregulation of Bax/Bcl-2 (Yang et al., 2006a). Our present study also showed that TS extracts appreciably inhibits tumor progression through cell-cycle arrest at G_1 phase. Therefore, further we intended to investigate the effect of gallic acid (5-10 μg/mL), on cell-cycle control in HL-60 cells. The profile of the DNA content in gallic acid-treated HL-60 cells (5 μg/mL for 6-18 h) was obtained using flow cytometric analysis. Fig. 8A showed that gallic acid exposure resulted in progressive and sustained accumulation of cells in G_1 phase. Furthermore, the percentage of G_1 phase cells increased, while those in the S and G_2/M phase decreased after treatment with gallic acid (Fig. 8A). Notably, there was a remarkable accumulation of sub-G_1 peak in gallic acid-treated HL-60 cells (5 μg/ml for 6-18 h) compared with the untreated group (Fig. 8A). Our findings suggest that gallic acid also promotes cell growth inhibition by inducing G_1 phase arrest in human leukemia cells.

In order to examine the molecular mechanism(s) and underlying changes in cell-cycle patterns caused by gallic acid treatment, we investigated the effects of various cyclins and CDKs involved in cell-cycle regulation in HL-60 cells. Cells were treated with gallic acid (5-10 μg/mL) for 0-6 h. Dose-dependent reductions of cyclin D1, CDK4, cyclin E, CDK2, and cyclin A, and induction of $p27^{KIP}$ expression were observed (Fig. 8B). Notably, gallic acid treatment significantly inhibits the expression of cyclin D1, cyclin E, CDK2 and 4, which are critically required for G_1-S transition phase. Therefore, we believed that the gallic acid-induced G_1 cell-cycle arrest is mediated by the inhibition of cyclin D1, cyclin E, and CDK2 and 4. However, the experimental treatment did not appear to alter the amount of detectable cyclin B1, CDC2, and p15 protein levels, which was concomitant with TS extracts treatment (Fig. 8B).

4. Discussion

Differential regulation of the cell-cycle, and subsequent events leading to apoptotic cell death, account for the anticancer effect of some potential phytochemicals (Sporn and Suh, 2002). Several studies have demonstrated anticancer potential for extracts from a number of herbal medicines or mixtures *in vitro* or *in vivo*. Herbal medicine is one of the ancient forms of health care known to humankind and it has been used in most cultures throughout history. Typically, herbal medicines emphasize the use of whole extracts from a plant mix or from complex formulations (Sporn and Nuh, 2002). Our previous study has demonstrated that TS extracts induce apoptotic cell death in cultured human premyelocytic leukemia HL-60 cells (Yang et al., 2006a). The present investigation also a parallel study showing the effect of TS extracts an *in vivo* human tumor xenograft in nude mice as well as *in vitro* cell culture models involving HL-60 cells. Summary of our data suggests that TS extracts treatment could be effective in suppressing the proliferation of HL-60 cells as shown by growth inhibition, cell-cycle arrest, and apoptotic induction *in vivo* and *in vitro*. Investigation has shown the nontoxic characteristics of *T. sinensis* [oral administration of *T. sinensis* (1000 mg/kg/day) for 28 days in rats], which increases its potential for application in food and drug products (Liao et al., 2007). Furthermore, *in vivo* toxicity was also examined superficially from body weight changes and histological studies of vital organs (data not shown). There appeared to be no sign of significant toxicity at TS extracts exposures up to the concentration of 10 mg/kg. This likely indicates that there are no side effects at these doses. Future studies should test whether there is an optimal/effective dose for TS extracts exposure.

Disturbance of the cancer cell-cycle is one of the therapeutic targets in the development of new anticancer drugs. The results of cell-cycle analysis in the present study showed that TS extracts/gallic acid treatment had a profound effect on cell-cycle control, with the premyelocytic leukemia cells accumulating in G_1 phase. Progression through the first gap

phase (G_1) requires both cyclin D-dependent CDK4/CDK6 and CDK2/cyclin E holoenzymes (Takahashi et al., 1999; Youn et al., 2008). The CDK catalytic subunits CDK 4 and CDK 2, and their regulatory subunits, cyclin D1 and cyclin E, are believed to be a crucial event in the regulation of S-phase entry, which appears to define the restriction point in the late G_1 phase. Cyclin D expression is frequently deregulated in human neoplasias, and agents that can down-regulate cyclin D expression may be helpful in both their prevention and treatment (Sausville et al., 2000). Further, it has been found that cyclin E, which is one of the key cell-cycle regulators, is over-expressed in primary carcinoma tissue (Wang et al., 1994). Cyclin A is particularly interesting among the clyclin family because it can activate two different types of CDKs and function in both S-phase and mitosis. Cyclin A associated protein kinase activity is critically required for G_1 to S-phase transition and further entry into M-phase (Johnson and Walker, 1999). The results imply that the expression of cyclin D1, CDK4, cyclin E, Cyclin A, and CDK2 are down-regulated by TS extracts, which corroborates the G_1 block in HL-60 cells. It has been shown that impairment of a growth stimulation-signaling pathway induces the expression of CDK inhibitor, which binds to and subsequently inhibits cyclin-CDK activity (Sandal et al., 2002). Our results suggest that inducing $p27^{KIP}$ expression *via* treatment with TS extracts/gallic acid may account for a large part of the reduction in CDK activity and, subsequently, block cell-cycle progression. Our study has also demonstrated that there were no significant differences in the expression of cyclin B1 and CDC2 after treatment with the TS extracts and gallic acid. The evidence suggests that the complex formed by the association of cyclin B1 and CDC2 plays a major role at entry into mitosis (Kuo et al., 2006). These results suggest that the observed inhibition of proliferation in HL-60 cells associated with the *T. sinensis* treatment could be the result of cell-cycle arrest during the G_1 phase.

Investigations have shown that apoptosis is controlled by both mitochondrial and membrane death receptor pathways. The extrinsic pathway is initiated by the binding of transmembrane death receptors, including Fas, FasL, TNFR1, and TRAIL receptors with cognate extracellular ligands (Reed, 2000). Ligand receptors recruit adaptor proteins such as TRADD and FADD which interact with and trigger the activation of caspase-8. Activated caspase-8 further cleaves or activates downstream effector caspases, such as caspase-3 (Reed, 2009). The present study indicates that TS extracts-induced apoptosis is associated with up-regulation of Fas and FasL, caspase-8 activation, and down-regulation of Bid in HL-60 cells. Our previous investigation has been demonstrated that treatment of HL-60 cells with TS extracts can induce apoptosis *via* a mitochondrial pathway that is associated with loss of cell viability, internucleosomal DNA fragmentation, cytochrome *c* translocation, caspase-3 activation, poly ADP-ribose polymerase (PARP) degradation, and Bcl-2 and Bax dysregulation (Yang et al., 2006a). However, the activation of caspase-9 by TS extracts was still in debate. Caspase-9 is a crucial factor for activation of caspase-3, which cleave several cellular targets including poly ADP ribose polymerase (Reed, 2009). The current data filled the gape that TS extracts markedly activates caspace-9 from procaspase-9 followed by caspase-3 activation. Analysis of our data suggests that TS extracts-induced apoptosis is controlled by both a mitochondrial and a membrane DR pathway.

Our previous report demonstrated that catalase (H_2O_2 scavenger) significantly decreases TS extracts-induced cytotoxicity, DNA fragmentation, and ROS production in HL-60 cells (Yang et al., 2006a). The present investigation further confirmed that catalase significantly reduced TS extracts-induced cell-cycle arrest (cyclin D1, CDK4, cyclin E, CDK2, cyclin A, pRb, and $p27^{KIP}$) and apoptosis (Fas, FasL, caspase-8, Bid, and caspase-9) in HL-60 cells. Analysis of our data suggesting TS extracts-induced HL-60 cell-cycle arrest and apoptosis

could be due to the intracellular ROS generation, especially H_2O_2. Other workers have shown that gallic acid-induced intracellular ROS, especially H_2O_2, play an important role in eliciting an early signal of apoptosis (Sakaguchi et al., 1998; Inoue et al., 2000), and that catalase significantly reduces gallic acid-induced apoptotic cell death (Yang et al., 2006a; Isuzugawa et al., 2001). In addition, recent studies appear to support the notion that TS extracts may possess protective antioxidant properties (Cho et al., 2003; Hsieh et al., 2004; Hseu et al., 2008a). Several researchers have shown that antioxidants produce genetic changes that cause apoptosis in cancer cells by mechanisms other than antioxidant effect (Yang et al., 2006b). Thus, TS extracts might serve as a mediator for the reactive oxygen-scavenging system and potentially act as both a pro-oxidant and an antioxidant, depending on the redox state of the biological environment. However, the detailed mechanisms of the chemotherapeutic action of *T. sinensis* are unknown, and further investigations are needed.

Angiogenesis is tightly regulated by an intricate balance between stimulators and inhibitors. Among them, VEGF, a soluble angiogenic factor produced by many tumors as well as normal cell lines, plays a key role in regulating normal and pathologic angiogenesis (Tonini et al., 2003; Hseu et al., 2011b). A previous report clearly evidenced that increased angiogenesis in bone marrow region from patients with acute myeloid leukemia (Hussong et al., 2000). These observations also suggest that the increased anagiogeneis is critically mediated by VEGF expression, which play crucial role for the further onset of tumor progression. Therefore, the therapeutic strategies have been developed for acute myeloid leukemia also targets anti-angiogenic processes, with promising results, because of the critical dependence of tumor growth and metastasis on angiogenesis. It is noteworthy that TS extracts significantly down-regulates both VEGF expression and release in HL-60 cells. A similar pattern of results were found in our previous study that TS extracts potentially inhibits VEGF-induced angiogenesis in

vascular endothelial cells (Hseu et al., 2011a). Taken together, the inhibitory effect of TS extracts on VEGF activity or angiogenesis in lekemia or endothelial cells are strong evidence for development of anti-cancer/anti-angiogenic drug from this vital source.

Furthermore, tumor inhibition was observed after treatment with TS extracts in the nude mice xenograft model in this study. Both incidence and mean tumor volume and weight were significantly reduced by TS extracts. Experiments using animals and circulating blasts from leukemia patients have yielded evidence that apoptosis also occurs in response to chemotherapy *in vivo*. Human acute-leukemia cell lines (HL-60 cells) have proven particularly informative in study of chemotherapy-associated apoptotic proteolytic events (Hseu et al., 2004). Moreover, in this study the *in vivo* toxicity of TS extracts was also examined superficially from body weight change and histological study of vital organs (data not shown), with no apparent signs of significant negative effects at exposures of 7.5 and 10 mg/kg. Analysis of our data suggests that TS extracts exert anti-proliferative action and growth inhibition on HL-60 cells *in vitro* or *in vivo*.

Natural products, including plants, provide rich resources for anticancer drug discovery. 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, was isolated from the leaves of *T. sinensis*; identity of the compounds was determined by HPLC and based on the analogous information reported in the literature (Yang et al., 2006a; Hsu et al., 2003). The total phenolic content of the TS extract was estimated to be 130 ± 26 mg of gallic acid equivalent/g of plant extracts (Yang et al., 2006a). The yield of gallic acid, the natural phenolic component purified from TS extracts, was about 6%. Although it remains unclear which of the components of *T. sinensis* are active compounds, gallic acid has received increased attention recently because of some interesting new findings regarding its biological activities (Chen et al.,

2009). Gallic acid is widely distributed in various plants and fruits, such as gallnuts, sumac, oak bark, green tea, apple peels, grapes, strawberries, pineapples, bananas, lemons, and in red and white wine (You et al., 2010). Even though the therapeutic utility of gallic acid in this regard is unknown, its common occurrence in fruits and food as well as its small molecular weight (170 Da) might be an advantage in terms of safety and dosing design. Studies have demonstrated that gallic acid selectively induces cancer cell death by apoptosis; however, gallic acid shows no cytotoxicity against normal cells (Yang et al., 2006a). Other workers 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 (Agarwal et al., 2007). Raina et al (2008) revealed that gallic acid treatment remarkably decreased human prostate cancer cell xenografted tumor incidence in mice. Therefore, gallic acid may be a useful phytochemical for cancer chemoprevention (Surh, 2003). These results corroborate other studies which have implicated that gallic acid is the main constituent responsible for the antiproliferative activity (Chen et al., 2009). Moreover, in future we have planned to investigate antitumor effect of other bioactive compounds isolated from the aqueous leaf extracts of *T. sinensis*.

The results obtained *in vitro* and *in vivo* in this study imply that *T. sinensis* could act as a chemopreventive agent with respect to inhibition of the growth of human leukemia HL-60 cells through the induction of cell-cycle arrest and apoptosis. We also believed that the antitumor activity of *T. sinensis* may be the abundance of gallic acid. These data provide an important step that might help model the effects of *T. sinensis* for potential future studies with animal models and human patients, and thereby facilitate the development of nutraceutical products using this agent.

Conflict of interest statement

The authors have no conflict of interest to declare.

Acknowledgments

This work was supported by grants NSC-97-2320-B-039-042, NSC-99-2320-B-039-035-MY3, CMU95-037, and CMU95-333 from the National Science Council and China Medical University of Taiwan.

References

Agarwal, C., Veluri, R., Kaur, M., Chou, S.C., Thompson, J.A., Agarwal, R., 2007. Fractionation of high molecular weight tannins in grape seed extract and identification of procyanidin B2-3,3'-di-O-gallate as a major active constituent causing growth inhibition and apoptotic death of DU145 human prostate carcinoma cells. Carcinogenesis. 28, 1478-1484.

Ahmad, N., Adham, V.M., Afaq, F., Feyes, D.K., Mukhtar, H., 2001. Resveratrol causes WAF-1/p21-imediated G1-phase arrest of cell cycle and induction of apoptosis in human epridermoid carcinoma A431 cells. Clin Cancer Res. 7, 1466-1473.

Bak, M.J., Jeong, J.H., Kang, H.S., Jin, K.S., Ok, S., Jeong, W.S., 2009. *Cedrela sinensis* leaves suppress oxidative stress and expression of iNOS and COX-2 via MAPK signaling pathways in RAW264.7 cells. J Food Sci Nutr. 14, 269-276.

Chang, H.C., Hung, W.C., Huang, M.S., Hsu, H.K., 2002. Extract from the leaves of *Toona sinensis* Roemor exerts potent antiproliferative effect on human lung cancer cells. Am J Chin Med. 30, 307-314.

Chang, H.L., Hsu, H.K., Su, J.H., Wang, P.H., Chung, Y.F., Chia, Y.C., Tsai, L.Y., Wu, Y.C., Yuan, S.S.F., 2006. The fractioned *Toona sinensis* leaf extract induced apoptosis of human ovarian cancer cells and inhibits tumor growth in a murine xenograft model.

Gynecol Oncol. 102, 309-314.

Chen, H.M., Wu, Y.C., Chia, Y.C., Chang, F.R., Hsu, H.K., Hsieh, Y.C., Chen, C.C., Yuan, S.S., 2009. Gallic acid, a major compound of *Toona sinensis* leaf extracts, contains a ROS-mediated anti-cancer activity in human prostate cancer cells. Cancer Lett. 286, 161-171.

Chia, Y.C., Rajbanshi, R., Calhoun, C., Chiu, R.H., 2010. Anti-neoplastic effect of gallic acid, a major component of *Toona sinensis* leaf extract, on oral squmous carcinoma cells. Molecules. 15, 8377-8389.

Cho, E.J., Yokozawa, T., Rhyu, D.Y., Kim, H.Y., Shibahara, N., Park, J.C., 2003. The inhibitory effects of 12 medicinal plants and their component compounds on lipid peroxidation. Am J Chin Med. 31, 907-917.

Collins, A., Yuan, L., Kiefer, T.L., Cheng, Q., Lai, L., Hill, S.M., 2003. Overexpression of the MT1 melatonin receptor in MCF-7 human breast cancer cells inhibits mammary tumor formation in nude mice. Cancer Lett. 189, 49-57.

Edmonds, J.M., Staniforth, M., 1998. *Toona sinensis*: Meliaceae. Curtis's Bot Mag. 15, 186-193.

Eskes, R., Desagher, S., Antonsson, B., Martinou, J.C., 2000. Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. Mol Cell Biol. 20, 929-935.

Hseu, Y.C., Chang, W.H., Chen, C.S., Liao, J.W., Huang, C.J., Lu, F.J., Chia, Y.C., Hsu, H.K., Wu, J.J., Yang, H.L., 2008a. Antioxidant activities of *Toona Sinensis* leaves extracts using different antioxidant models. Food Chem Toxicol. 46, 106-114.

Hseu, Y.C., Chen, S.C., Chen, H.C., Liao, J.W., Yang, H.L., 2008b. *Antrodia camphorata* inhibits proliferation of human breast cancer cells *in vitro* and *in vivo.* Food Chem Toxicol. 46, 2680-2688.

Hseu, Y.C., Chen, S.C., Lin, W.H., Huang, D.Z., Lin, M.K., Kuo, Y.H., Wang, M.T., Cho, H.J., Wang, L., Yang, H.L., 2011a. *Toona sinensis* (leaf extracts) inhibit vascular endothelial growth factor (VEGF)-induced angiogenesis in vascular endothelial cells. J Ethnopharmacol. 134, 111-121.

Hseu, Y.C., Chen, S.C., Tsai, P.C., Chen, C.S., Lu, F.J., Yang, H.L., 2007. Inhibition of cyclooxygenase-2 and induction of apoptosis in estrogen-nonresponsive breast cancer cells by *Antrodia camphorata*. Food Chem Toxicol. 45, 1107-1115.

Hseu, Y.C., Wu, C.R., Chang, H.W., Kumar, K.J.S., Lin, M.K., Chen, C.S., Cho, H.J., Huang, C.Y., Huang, C.Y., Lee H.Z., Hsieh, W.T., Chung, J.G., Wang, H.M., Yang, H.L., 2011b. Inhibitory effects of *Physalis angulata* on tumor metastasis and angiogenesis. J Ethnopharmacol. 135, 762-771.

Hseu, Y.C., Yang, H.L., Lai, Y.C., Lin, J.G., Chen, G.W., Chang, Y.H., 2004. Induction of apoptosis by *Antrodia camphorata* in human premyelocytic leukemia HL-60 cells. Nutr Cancer. 48, 189-197.

Hsieh, T.J., Liu, T.Z., Chia, Y.C., Chern, C.L., Lu, F.J., Chuang, M.C., Mau, S.Y., Chen, S.H., Syu, Y.H., Chen, C.H., 2004. Protective effect of methyl gallate from *Toona sinensis* (Meliaceae) against hydrogen peroxide-induced oxidative stress and DNA damage in MDCK cells. Food Chem Toxicol. 42, 843-850.

Hsu, H.K., Yang, Y.C., Hwang. J.H., Hong, S.J., 2003. Effects of *Toona sinensis* leaf extract on lipolysis in differentiated 3T3-L1 adipocytes. Kaohsiung J Med Sci. 19. 3855-3890.

Hussong, J.W., Rodgers, G.M., Shami, P.J., 2000. Evidence increased aniogenesis in patients with acute myeloid leukemia. Blood. 95, 309-313.

Inoue, M., Sakaguchi, N., Isuzugawa, K., Tani, H., Ogihara, Y., 2000. Role of reactive oxygen species in gallic acid-induced apoptosis. Biol Pharm Bull. 23, 1153-1157.

Isuzugawa, K., Inoue, M., Ogihara, Y., 2001. Catalase contents in cells determine sensitivity to the apoptosis inducer gallic acid. Biol Pharm Bull. 24, 1022-1026.

Jiang, S.H., Wang, C.L., Chen, Z.Q., Chen, M.H., Wang, Y.R., Liu, C.J., Zhou, Q.L., Li, Z.J., 2009. Antioxidant properties of the extract and subtractions from old leaves of *Toona sinensis* Roem (Meliaceae). J Food Biochem. 33, 425-441.

Johnson, D. G., Walker, C.L., 1999. Cyclins and cell cycle checkpoints. Ann Rev Pharmacol Toxicol. 39, 295-312.

Kim, J.J., Kim, H.P., Jung, C.H., Hong, M.H., Hong, M.C., Bae, H.S., Lee, S.D., Park, S.Y., Park, J.H., Ko, S.G., 2006. Inhibition of cell cycle progression *via* $p27^{kip1}$ upregulation and apoptosis induction by an ethanol extract of *Rhus verniciflua* stokes in AGS gastric cancer cells. Int J Mol Med. 18, 201-208.

Kuo, P.L., Hsu, Y.L., Cho, C.Y., 2006. Plumbagin induced G2-M arrest and autophagy by inhibiting the AKT/mammalian target rapamycin pathway in breast cancer cells. Mol Cancer Ther. 5, 3209-3221.

Liao, J.W., Chung, Y.C., Yeh, J.Y., Lin, Y.C., Lin, Y.G., Wu, S.M., Chan, Y.C., 2007. Safety evaluation of water extracts of *Toona sinensis* Roemor leaf. Food Chem Toxicol. 45, 1393-1399.

Liao, J.W., Hsu, C.K., Wang, M.F., Hsu, W.M., Chan, Y.C., 2006. Beneficial effect of *Toona sinensis* Roemor on improving cognitive performance and brain degeneration in senescence-accelerated mice. Br J Nutr. 96, 400-407.

Manach, C., Williamson, G., Morand, C., Scalbert, A., Remesy, C., 2005. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am J Clin Nutr. 81, 230S-242S.

Nagata, S., 1997. Apoptosis by death factor. Cell. 88, 355-365.

Poon, S.L., Leu, S.F., Hsu, H.K., Liu, M.Y., Huang, B.M., 2005. Regulatory mechanism of

Toona sinensis on mouse leydig cell steroidogenesis. Life Sci. 76, 1473-1487.

Raina, K., Rajamanickam, S., Deep, G., Singh, M., Agarwal, R., Agarwal, C., 2008. Chemopreventive effects of oral gallic acid feeding on tumor growth and progression in TRAMP mice. Mol Cancer Ther. 7, 1258-1267.

Reed, J.C., 2000. Mechanisms of apoptosis. Am J Pathol. 157, 1415-1430.

Sakaguchi, N., Inoue, M., Ogihara, Y., 1998. Reactive oxygen species and intracellular Ca^{2+}, common signals for apoptosis induced by gallic acid. Biochem Pharmacol. 55, 1973-1981.

Sandal, T., Stapnes, C., Kleivdal, H., Hedin, L., Doskeland, S.O., 2002. A novel, extra neuronal role for cyclin-dependent protein kinase 5 (CDK5): modulation of cAMP-induced apoptosis in rat leukemia cells. J Biol Chem. 277, 20783-20793.

Sausville, E.A., Johnson, J., Alley, M., Zaharevitz, D., Senderowicz, A.M., 2000. Inhibition of CDKs as a therapeutic modality. Ann N Y Acad Sci. 910, 207-221.

Schmitt, C.A., 2003. Senescence, apoptosis and therapy--cutting the lifelines of cancer. Nat Rev Cancer. 3, 286-295.

Shahrzad, S., Aoyagi, K., Winter, A., Koyama, A., Bitsch, I., 2001. Pharmacokinetics of gallic acid and its related bioavailability from tea in healthy humans. J. Nutr. 131, 1207-1210.

Sporn, M.B., Suh, N., 2002. Chemoprevention: an essential approach to controlling cancer. Nat Rev Cancer. 2, 537-543.

Stewart, Z.A., Westfall, M.D., Pietenpol, J.A., 2003. Cell-cycle dysregulation and anticancer therapy. Trends Pharmacol Sci. 24, 139-145.

Surh, Y.J., 2003. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer. 3, 768-780.

Takahashi, A., Taniguchi, T., Ishikawa, Y., Yokoyama, M., 1999. Tranilast inhibits vascular

smooth muscle cell growth and intimal hyperplasia by induction of $p21^{wafi/cip1/sdi1}$ and p53. Circ Res. 84, 543-550.

Tonini, T., Rossi, F., Claudia, P.P., 2003. Molecular basis of angiogenesis and cancer. Oncogene. 22, 6549-6556.

Wang, C.Y., Lin, K.H., Yang, C.J., Tsai, J.R., Huang, J.Y., Wang, P.H., Hsu, H.K., Huang, M.S., 2010. *Toona sinensis* extracts induced cell cycle arrest and apoptosis in the human lung large cell carcinoma. Kaohsiung J Med Sci. 26, 68-75.

Wang, T.C., Cardiff, R.D., Zukerberg, L., Lees, E., Arnold, A., Schmidt, E.V., 1994. Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. Nature. 369, 669-671.

Yang, C.J., Huang, Y.J., Wang, C.S., Wang, P.H., Huang, J.Y., Wang, T.H., Hsu, H.K., Huang, H.W., Kumar, S.P., Huang, M.S., Weng, C.F., 2010b. Antiproliferative and antitumorigenic activity of *Toona sinensis* leaf extracts in lung adenocarcinoma. J Med Food. 13, 54-61.

Yang, C.J., Huang, Y.J., Wang, C.Y., Wang, P.H., Hsu, H.K., Tsai, M.J., Chen, Y.C., Kumar, V.B., Huang, M.S., Weng, C.F., 2010a. Antiproliferative effect of *Toona sinensis* leaf extract on non-small-cell lung cancer. Tran Res. 155, 305-314.

Yang, H.L., Chang, W.H., Chia, Y.C., Huang, C.J., Lu, F.J., Hsu, H.K., Hseu, Y.C., 2006a. *Toona sinensis* extracts induces apoptosis *via* reactive oxygen species in human premyelocytic leukemia cells. Food Chem Toxicol. 44, 1978-1988.

Yang, H.L., Chen, C.S., Chang, W.H., Lu, F.J., Lai, Y.C., Chen, C.C., Hseu, T.H., Kuo, C.T., Hseu, Y.C., 2006b. Growth inhibition and induction of apoptosis in MCF-7 breast cancer cells by *Antrodia camphorata*. Cancer Lett. 321, 215-227.

Yang, Y.C., Hus, H.K., Hwang, J.H., Hong, S.J., 2003. Enhancement of glucose uptake in 3T3-L1 adipocytes by *Toona sinensis* leaf extract. Kaohsiung J Med Sci. 9, 327-

333.

You, B.R., Moon, H.J., Han, Y.H., Park, W.H., 2010. Gallic acid inhibits the growth of HeLa cervical cancer cells *via* apoptosis and/or necrosis. Food Chem Toxicol. 48, 1334-1340.

Youn, M.J., Kim, J.K., Park, S.Y., Kim, Y., Kim, S.J., Lee, J.S., Chai, K.Y., Kim, H.J., Cui, M.X., So, H.S., Kim, K.Y., Park, R., 2008. Chaga mushroom (*Inonotus obliquus*) induces G_0/G_1 arrest and apoptosis in human hepatoma HepG2 cells. World J Gastroenterol. 14, 511-517.

Figure legends

Fig. 1. Fffects of TS extracts on cell-cycle distribution in HL-60 cells. Cells were treated with 75 μg/mL of TS extracts for 0-18 h, stained with PI, and analyzed by flow cytometry for sub-G_1 and cell-cycle. Distribution (as percentage) of cells in the different phases of the cell-cycle (sub-G_1, G_1, S, and G_2/M) after treatment is shown. Apoptotic nuclei were identified as a subploid DNA peak, and were distinguished from cell debris on the basis of forward light scatter and PI fluorescence. Representative flow cytometry patterns are also presented. Results are presented as mean±SD (n=3). *: indicates significant difference in comparison to control group (p<0.05).

Fig. 2. Western blot analysis of cyclin D1, CDK4, cyclin E, CDK2, cyclin A, $p27^{KIP}$, cyclin B, CDC2, and p15 protein levels in HL-60 cells after exposure to TS extracts. Cells were treated with 75 μg/mL of TS extracts for 2, 4, and 6 h, and with 0, 10, 25, 50, and 75 μg/mL of TS extracts for 6 h plus catalase (10 U/mL) as indicated. Protein (50 μg) from each sample was resolved on 10-12% SDS-PAGE, and western blot performed. β-actin was used as a control. Relative changes in protein bands were measured using densitometric analysis. Typical result from three independent experiments is shown.

Fig. 3. Western blot analysis of Fas, FasL, caspase-8, Bid, and caspase-9 protein levels in HL-60 cells after exposure to TS extracts. Cells were treated with 75 μg/mL of TS extracts for 2, 4 and 6 h, and with 0, 10, 25, 50, and 75 μg/mL of TS extracts for 6 h plus catalase (10 U/mL) as indicated. Protein (50 μg) from each sample was resolved on 12% SDS-PAGE, and western blot performed. β-actin was used as a control. Relative changes in protein bands were measured using densitometric analysis. Typical result from three

independent experiments is shown.

Fig. 4. TS extracts mediated down-regulation of VEGF. (A) Western blot analysis of the protein levels of VEGF in HL-60 cells after exposure to TS extracts (0-75 μg/mL) for 6 h. Proteins (50 μg) from each sample were resolved on 8-15% SDS-PAGE. β-actin was used as a loading control. Relative changes in protein bands were measured using densitometric analysis with the control being 100% as shown just below the gel data. Typical results from three independent experiments are shown. (B) ELISA assay of VEGF release in HL-60 cells after exposure to TS extracts (0-75 μg/mL) for 6 h. Concentration of VEGF released into the medium was determined by ELISA. Results are presented as mean ± SD of three assays. *Significant difference in comparison to the control group (p<0.05).

Fig. 5. Time-course effect of TS extracts on the growth of HL-60 xenograft was evaluated from measurements of body weight (A) and tumor volume (B) every 3 days. HL-60 cells were implanted subcutaneously into the flanks of nude mice on day 0, the animals were then treated with TS extracts (7.5 and 10.0 mg/kg/every two days) or without (as a control) as described in Materials and Methods. Results are presented as mean±SE (n=6). *: indicates significant difference in comparison to control group (p<0.05).

Fig. 6. *In vivo* inhibition of HL-60 xenograft tumors by TS extracts. Nude mice were treated with TS extracts (7.5 and 10.0 mg/kg/every two days) or without (control). On the 21th day after tumor implantation, the animals were sacrificed and the tumors removed (A) and weighed (B). Results are presented as mean±SE (n=6). *: indicates significant difference in comparison to control group (p<0.05).

Fig. 7. Histopathological findings of tumor cells in HL-60 xenograft tumors. The control HL-60 xenograft tumors and the HL-60 xenograft tumors after TS extract (7.5 and 10.0 mg/kg/every two days) treatments were sectioned, stained with hematoxylin and eosin, and examined with light microscopy (400×). Arrows indicate the mitotic nuclei in tumor cells (control), and TS extracts-induced (7.5 and 10.0 mg/kg) shrunken tumor cells. Typical results from three independent experiments are shown.

Fig. 8. (A**)** Effects of gallic acid on cell-cycle distribution in HL-60 cells. Cells were treated with 5 μg/mL of gallic acid for 0-18 h, stained with PI, and analyzed by flow cytometry for sub-G_1 and cell-cycle. Distribution (as percentage) of cells in the different phases of the cell-cycle (sub-G_1, G_1, S, and G_2/M) after treatment is shown. Representative flow cytometry patterns are also presented. Results are presented as mean±SD (n=3). *: indicates significant difference in comparison to control group (p<0.05). (B) Western blot analysis of cyclin D1, CDK4, cyclin E, CDK2, cyclin A, $p27^{KIP}$, cyclin B, CDC2, and p15 protein levels in HL-60 cells after exposure to TS extracts. Cells were treated with 5 μg/mL of gallic acid for 2, 4, and 6 h, and with 0, 5, and 10 μg/mL of gallic acid for 6 h. Protein (50 μg) from each sample was resolved on 12-15% SDS-PAGE, and western blot performed. β-actin was used as a control. Relative changes in protein bands were measured using densitometric analysis. Typical results from three independent experiments are shown.

Research Article

In vitro and *in vivo* activity of gallic acid and *Toona sinensis* leaf extracts against HL-60 human premyelocytic leukemia ~~Aqueous leaf extracts of *Toona sinensis* inhibit proliferation of human premyelocytic leukemia HL-60 cells *in vitro* and *in vivo*~~

Formatted: Font: Italic

Formatted: Font: Italic

Pei-Jane Huang[a,1], You-Cheng Hseu[b,1], Meng-Shiou Lee[c], K.J. Senthil Kumar[b], Chi-Rei Wu[c], Li-Sung Hsu[d], Jiunn-Wang Liao[e], I-Shiung Cheng[f], Ya-Ting Kuo[h], Shi-Ying Huang[g,*], Hsin-Ling Yang[h,*]

[a]*Department of Health and Nutrition Biotechnology, Asia University, Taichung 41354, Taiwan*

[b]*Department of Cosmeceutics, College of Pharmacy, China Medical University, Taichung 40402, Taiwan*

[c]*School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, College of Pharmacy, China Medical University, Taichung 40402, Taiwan*

[d]*Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung 40402, Taiwan*

[e]*Graduate Institute of Veterinary Pathology, National Chung Hsing University, Taichung 40402, Taiwan*

[f]*Department of Physical Education, National Taichung University of Education, Taichung 40402, Taiwan*

[g]*Department of pediatrics, Armed Force Taoyuan General Hospital, Taoyuan 32551, Taiwan*

[h]*Institute of Nutrition, China Medical University, Taichung 40402, Taiwan*

*Corresponding authors. Tel.: +886 4 22053366 x 7503; fax: +886-4-22062891.

E-mail addresses: hlyang@mail.cmu.edu.tw (H-L, Yang); Huangsy56@yahoo.com.tw (S-Y, Huang)

[1] Both authors contributed equally.

ABSTRACT

Toona sinensis is one of the most popular vegetarian cuisines in Taiwan and it has been shown to induce apoptosis in cultured human premyelocytic leukemia (HL-60) cells. In the present study, we examined the effects of *Toona sinensis* leaf extracts (TS extracts) on tumor regression using *in vitro* cell culture and an *in vivo* athymic nude mice model. We found that TS extracts (10-75 μg/mL) arrested HL-60 cells at the G_1-S transition phase through the reductions of Cyclin D1, CDK4, Cyclin E, CDK2, and Cyclin A, and induction of CDK inhibitor $p27^{KIP}$ levels. Furthermore, VEGF expression and release was significantly inhibited by TS extracts. Notably, TS extracts treatment was effective in terms of delaying tumor incidence in the nude mice inoculated with HL-60 cells as well as reducing the tumor burden. Histological analysis confirmed that TS extracts significantly modulated tumor progression in xenograft tumor. Furthermore, a similar pattern of results were observed from gallic acid (5 and 10 μg/mL), a major compound in TS, caused G_1 arrest through regulations of cell-cycle regulatory proteins. Our data suggest that *Toona sinensis* exerts antiproliferative effects on HL-60 cells *in vitro* and *in vivo* due mainly to the presence of gallic acid. ~~that may contribute critically to its cancer chemopreventive potential.~~

Keywords:

Toona sinensis

Gallic acid

HL-60 cells

Cell-cycle arrest

Xenografted nude mice

1. Introduction

Toona sinensis (A. Juss.) M. Roem popularly known as Chinese mahogany or Chinese toon, a member of Meliaceae family, and is a type of deciduous perennial tree widely distributed in Asia. In Chinese and Taiwanese culture it is one of the popular vegetarian cuisine. It has long been used as a traditional Chinese medicine for a wide variety of conditions. The edible leaves used as an oriental medicine for treating rheumatoid arthritis, cervicitis, urethritis, tympanitis, gastric ulcers, enteritis, dysentery, itchiness, and cancer (Edmonds and Staniforth, 1998; Hseu et al., 2011a). While the underlying pharmacological mechanisms of this new drug are still a matter of debate, previous scientific literatures have been reported that *T. sinensis* possessed variety of biological activities including anti-cancer (Chang et al., 2002; Chang et al., 2006; Yang et al., 2006a; Chen et al., 2009; Wang et al., 2010; Yang et al., 2010a; Yang et al., 2010b; Chia et al., 2010), anti-angiogenesis (Hseu et al., 2011a) anti-inflammation (Bak et al., 2009), anti-diabetes (Hsu et al., 2003; Yang et al., 2003), and antioxidant (Cho et al., 2003; Hseu et al., 2008a; Jiang et al., 2009) effects, as well as inhibiting Leydig cell steroidogenesis and improving the dynamic activity of human sperm quality (Poon et al., 2005). Moreover, the safety levels and nontoxic characteristics of aqueous extracts of *T. sinensis* were evaluated using acute and sub-acute toxicity studies in mice (Liao et al., 2006).

Gallic acid (GA), a major phenolic compound that rich in TSL has a wide spectrum of biological and pharmacological effects. Various animal models or human studies proved that GA is extremely safe even at using high doses. Also, a few studies addressing the bioavailability of GA in human revealed that this compound is extremely well observed when compared to other polyphenols (Manach et al., 2005). When GA was given orally at a dose of 0.3 mmoL in Assam black tea (contained >93% of free form GA) to human, a maximum serum concentration of 2.08 μM was observed in plasma, whereas 39.6% of the

Formatted: Not Highlight

GA dose were extracted in urine as a GA or GA metabolites (Shahrzad et al., 2001). The pharmacological safety and efficacy of GA makes it a potential compound for treatment or prevention of a wide variety of human diseases.

Chemoprevention, which refers to the administration of natural or synthetic agents to prevent initiation and promotion events associated with carcinogenesis, is being increasingly appreciated as an effective approach for the management of neoplasia (Ahmad et al., 2001). Many studies have shown a clear link between abnormal cell-cycle regulation and apoptosis with cancer, as much as the cell-cycle inhibitors and apoptosis-inducing agents are being appreciated as armaments for the management of cancer (Stewart et al., 2003; Schmitt, 2003; Hsu et al., 2003). Eukaryotic cell-cycle progression involves a sequential activation of cyclin-dependent kinases (CDKs) whose activation is dependent upon their association with cyclins (Youn et al., 2008). Progression through the mammalian mitotic cycle is controlled by multiple holoenzymes comprising a catalytic CDK and a cyclin regulatory subunit (Takahashi et al., 1999; Hseu et al., 2008b). These cyclin-CDK complexes are activated at specific intervals during the cell-cycle but can also be induced and regulated by exogenous factors. Cell-cycle progression is also regulated by the relative balance between the cellular concentrations of cyclins/CDKs and CDKs inhibitors, including $p27^{KIP}$ (Hseu et al., 2008b; Kim et al., 2006). The cyclin-CDK complexes are subjected to inhibition *via* binding with CDK inhibitors (Kim et al., 2006). Recently, the relationship between cell-cycle arrest and cancer has been emphasized, with increasing evidence suggesting that the related processes of neoplastic transformation, progression and metastasis involve alteration of the normal cell-cycle regulation. Thus, anticancer (chemopreventive) agents may alter regulation of the cell-cycle machinery, resulting in an arrest of cells in different phases of the cell-cycle and, thereby, reducing growth and proliferation of cancerous cells, which may be useful in cancer therapy.

Leukemia is one of the most threatening diseases today. Given that most adult leukemia patients are not candidates for transplantation, and that a more rational therapy is not adequately defined, they are typically treated with regimens that are based on (or at least include) chemotherapy (Yang et al., 2006a). In our previous study, we demonstrated that aqueous leaf extracts of *T. sinensis* (TS extracts, 10-75 μg/mL) and gallic acid (3,4,5-trihydroxybenoic acid, 5-10 μg/mL), a purified natural phenolic component, exhibited apoptosis against human premyelocytic leukemia (HL-60) cells (Yang et al., 2006a). Notably, the significant inhibitory effects of tumor cell proliferation were observed only in leukemia HL-60 cells, whereas not in erythrocytes and human lymphocytes (Yang et al., 2006a). However, the effect of TS extracts against tumour cell-cycle regulation was poorly understood. Therefore, the present study aimed to investigate the anticancer effect of TS extracts and gallic acid in terms of tumor regression using *in vitro* cell culture model (HL-60 cells) or *in vivo* athymic nude mice model of leukemia cancer.

2. Materials and Methods

2.1. Chemicals

RPMI-1640 medium (Gibco BRL, Grand Island, NY), antibody against cyclin E, CDK2, cyclin B1, CDC2, caspase-8, Fas, FasL, VEGF, and β-actin (Santa Cruz Biotechnology Inc., Heidelberg, Germany) and antibody against cyclin D1, CDK4, cyclin A, $p27^{KIP}$, p15, caspase-9, and Bid (Cell Signaling Technology Inc., Danvers, MA) were obtained from their respective suppliers. All other chemicals were of the reagent or HPLC grade supplied either by Merck (Darmstadt, Germany) or Sigma (St. Louis, MO).

2.2. Preparation of TS extracts

Leaves of *T. sinensis* were sourced from Fooyin University, Kaohsiung, Taiwan. A

voucher specimen was characterized by Prof. Horng-Liang Lay, Graduate Institute of Biotechnology, National Pingtung University of Science and Technology, Pingtung County, Taiwan, and deposited at Fooyin University, Kaohsiung, Taiwan. The aqueous leaf extracts of *T. sinensis* (TS extracts) were prepared by adding 1000 mL of water to 1000 g of fresh *T. sinensis* leaves and boiled until 100 mL remained, as previously described (Chang et al., 2002; Hseu et al., 2008a). The crude extracts were centrifuged at 3000 × *g* for 12 min and the supernatant was 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 further analysis of its anticancer properties. The yield of TS extracts was 6%. The total phenolic content of the TS extracts was estimated to be 130 ± 26 mg gallic acid (pyrocatechol) equivalents/g of plant extracts as described previously (Yang et al., 2006a).

2.3. Isolation of gallic acid from *TS extracts*

TS extracts were dissolved in a mobile phase consisting of methanol-water (50:50, v/v) before high performance liquid chromatography (HPLC) analysis and separation. Chromatographic separation was achieved with a mobile phase consisting of methanol-water (50:50, v/v) in the first 15 min, gradually increasing the methanol to 100% in the next 10 min. A flow rate of 4.0 mL/min at room temperature was used. Eight compounds (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 TS extracts. The identification of the compounds was fully characterized by comparison of their spectral data (IR, NMR, and mass) with the analogous information reported in the literature (Yang et al., 2006a; Hsu et al., 2003). Gallic acid, a natural phenolic component purified from TS extracts was subjected in this study at a yield of 6% (Yang et al., 2006a).

2.4. Cell culture

Human acute promyeloblastic leukemia (HL-60) cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). 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 were counted by hemocytometer.

2.5. Flow cytometry analysis

Cellular DNA content was determined by flow cytometric analysis of propidium iodide (PI)-labeled cells as described previously (Hseu et al., 2007). In brief, HL-60 cells (2×10^5 cells/mL) were cultured in 6 cm culture dishes. After treatment with TS extracts or gallic acid, cells were harvested, washed and suspended in PBS and fixed in ice-cold 70% ethanol at -20 °C for overnight. After incubation, cells were re-suspended in PBS containing 1% Triton X-100, 0.5 mg/mL RNase, and 4 μg/mL PI at 37 °C for 30 min. A FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a single argon-ion laser (488 nm) was used for flow cytometric analysis. Forward and right-angle light scatter, which are correlated with the size of the cell and the cytoplasmic complexity, respectively, were used to establish size gates and exclude cellular debris from the analysis. DNA content of 1×10^4 cells per analysis was monitored using the FACSCalibur system. The cell-cycle was determined and analyzed using ModFit software (Verity Software House, Topsham, ME). Apoptotic nuclei were identified as a subploid DNA peak, and were distinguished from cell debris on the basis of forward light scatter and PI fluorescence.

2.6. Protein isolation and immunoblot analysis

HL-60 cells (2×10^6 cells/ 10 cm dish) were washed once in cold PBS, and suspended in 100 μL lysis buffer (10 mM Tris-HCl [pH 8], 0.32 M sucrose, 1% Triton X-100, 5 mM EDTA, 2 mM DTT, and 1 mM phenylmethyl sulfony flouride). The suspension was vortex and kept on ice for 20 min and then centrifuged at 15000 × *g* for 20 min at 4 °C. Total protein content was determined using Bio-Rad protein assay reagent, with bovine serum albumin (BSA) as the standard; protein extracts were reconstituted in sample buffer (0.062 M Tris-HCl, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol), and the mixture was boiled at 94 °C for 5 min. Equal amounts (50 μg) of the denatured proteins were loaded into each lane, separated by 10-15% SDS polyacrylamide gel, followed by transfer of the proteins to PVDF membranes overnight. Membranes were blocked with 0.1% Tween-20 in Tris-buffered saline containing 5% non-fat dry milk for 20 min at room temperature, and the membranes were reacted with primary antibodies for 2 h. They were then incubated with a horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody for 2 h before being developed by SuperSignal ULTRA chemiluminescence substrate (Pierce Biotechnology, Rockford, IL). For densitometry analysis band intensities were quantified by commercially available software AlpaEaseFc 4.0 (Genetic Technologies, Inc., Miami, FL).

2.7. Determination of VEGF release

To determine the effects of TS extracts on VEGF levels, HL-60 cells grown to 85% confluence were treated with 0-75 μg/mL of TS extracts for 6 h. Then, the medium was aspirated from the flasks and centrifuged at 500 × *g* for 10 min to remove cells from the medium. The level of VEGF released into the culture medium was estimated using commercially available VEGF ELISA kit (Chemicon International Inc., Temecula, CA).

2.8. Animal experiments

Eight weeks old male or female athymic nude mice (BALB/*c-nu* were purchased from GlycoNex Inc., (Taipei, Taiwan) and were maintained in cage housing separately in a specifically designed pathogen-free isolation facility with a 12 h light and 12 h dark cycle; the mice were provided rodent chow (Oriental Yeast Co, Tokyo, Japan) and water *ad libitum*. All experiments were conducted in accordance with the guidelines of the China Medical University Animal Ethics Research Board.

2.9. Tumor cell inoculation

HL-60 cells were grown in RPMI-1640 medium supplemented with 10% FBS, 2 mM glutamine, 1% penicillin-streptomycin-neomycin in a humidified incubator (5% CO_2 in air at 37°C). Experiments were carried out using cells less than 15 passages. HL-60 cells (1×10^6 cells in 200 μL matrix gel) were injected subcutaneously on the right hind flank of nude mice as described previously (Hseu et al., 2008b). Tumor volume, as determined by caliper measurements of tumor length, width and depth, were calculated using the formula: length × width^2 × 1/2 every 3 days (Collins et al., 2003). The two study groups received intraperitoneal injections of TS extracts (0.2 mL/mouse) dissolved in PBS buffer at 7.5 mg/kg and 10 mg/kg every 2 days, while the control group received vehicle only. After 21 days of treatment, the mice were sacrificed. The tumors were removed and weighed before fixing in 4% paraformaldehyde, sectioning and staining with hematoxylin-eosin for light microscopic analysis. Part of the tumor tissue was immediately frozen and the rest was fixed in 10% neutral-buffered formalin and embedded in paraffin. To monitor drug toxicity, the body weight of each animal was measured every 3 days. In addition, a pathologist examined the mouse organs, including liver, lungs and kidneys.

2.10. Statistical analysis

The results of the *in vitro* and *in vivo* experiments are presented as mean and standard deviation (mean ± SD) or standard error (mean ± SE), respectively. All study data were analyzed using analysis of variance (ANOVA), followed by Dunnett's test for pair-wise comparison. Statistical significance was defined as $p < 0.05$ for all tests.

3. Results

This study has investigated the anticancer effect of aqueous leaf extracts of *T. sinensis* (0-75 μg/mL) and gallic acid (0-10 μg/mL) *in vitro* using HL-60 premyelocytic leukemia cell line or *in vivo* nude mice xenograft model. The crude TS extracts were prepared from fresh *T. sinensis* leaves, yielding 6% based on the initial weight of *T. sinensis* leaves and the total yield of gallic acid from the TS extracts was 6% (Yang et al., 2006a).

3.1. TS extracts induce G_1 cell-cycle arrest in HL-60 cells

Flow cytometric analysis was used to obtain the profile of DNA content of the HL-60 cells treated with TS extracts to measure the fluorescence of PI-DNA complex. HL-60 cells with lower DNA staining relative to diploid analogs were considered apoptosis. A remarkable accumulation of subploid cells, the so-called sub-G_1 peak, was noted in those treated with TS extracts (75 μg/mL) for 0-18 h compared with the untreated group (Fig. 1). Furthermore, the stage at which growth inhibition was induced by TS extracts in the HL-60 cell-cycle progression was determined, from cellular distribution in the different phases of post treatment. Fig. 1 showed that exposure of cells to the TS extracts resulted in a time-dependent progressive and sustained accumulation of cells in the G_1 phase. Furthermore, in response to TS extracts the percentage of cells in the G_1 phase was gradually increased

whereas, those in the S and G_2/M phases was significantly decreased (Fig. 1). We have previously reported that TS extracts dose- and time-dependently inhibits the growth of HL-60 cells (Yang et al., 2006a). Consistent with our previous report, the current findings also suggest that TS extracts promote cell growth inhibition by inducing G_1 transition phase arrest in HL-60 cells.

3.2. TS extracts down-regulate Cyclin D1, CDK4, Cyclin E, CDK2, and Cyclin A expression and up-regulates P27^{KIP} expression

To examine the molecular mechanism(s) that may underlying changes in cell-cycle patterns, the effects of the TS extracts on various cyclins and cyclin-dependent kinases (CDKs) involved in cell-cycle control of the HL-60 cells were investigated. Our investigative approach was to treat the HL-60 cells with TS extracts (0-75 μg/mL) for 0-6 h. Dose and time-dependent reduction in cyclin D1, CDK4, cyclin E, CDK2, and cyclin A expression were observed after treatment with TS extracts (Fig. 2). Moreover, the experimental treatment did not appear to alter the amount of detectable cyclin B1 and CDC2 protein expression in HL-60 cells (Fig. 2). We also examined the effect of TS extracts on CDKs inhibitors including p27^{KIP} and p15. As shown in Fig. 2, treatment of HL-60 cells with TS extracts (0-75 μg/mL) for 0-6 h induced marked up-regulation of p27^{KIP} protein. However, we found there was no change in the detectable amount of p15 protein in HL-60 cells (Fig. 2). Taken together, TS extracts potentially arrest G_1-S transition phase as evidenced by down-regulation of cyclins and CDKs and enhanced CDKs inhibitors.

3.3. Activation of Fas-associated apoptotic pathway by TS extracts

To assess whether TS extracts (0-75 μg/mL for 0-6 h) promoted apoptosis *via* a death

receptor-associated pathway, the Fas and Fas ligand (FasL) protein levels in HL-60 cells were determined by Western blotting. Results showed that TS extracts appreciably stimulate the expression of Fas and FasL in a dose- and time-independent manner (Fig. 3). It is well understood that induction of Fas and FasL cleaves caspase-8 from procaspase-8, and the activated caspase-8 further stimulates caspase-3 *via* mitochondrial-dependent or –independent cascade (Nagata, 1997). ~~Bear this in mind; further~~Therefore, we verified whether TS extracts augment caspase-8 cleavage in HL-60 cells. Western blot results showed that TS extracts dose-and time-dependently induced cleavage of caspase-8 from the procaspase-8 (Fig. 3). In mitochondrial pathway of apoptosis, caspase-8 proteolytically activates a pro-apoptotic protein Bid, which targets mitochondrial membrane permiabilization and represents the mail link between extrinsic and intrinsic apoptotic pathways (Eskes et al., 2000). Our results also showed that down-regulation of Bid induced by TS extracts occurred in a dose- and time-independent manner (Fig. 3). In addition, we observed TS extracts activates caspase-9, which was concomitant with our previous report that TS extracts induced apoptosis through the release of cytochrome *c* (Yang et al., 2006a). However, the signaling ~~cascade still in debate~~mechanism is poorly understood. This data provided strong evidence that TS extracts-induced release of cyctochrome *c* further promotes apoptosome-mediated cleavage of caspace-9 from procaspase-9. With reference to our previous report, we assured that TS extracts-induced aberrant release of cytochrome *c* further amplified the cleavage of caspase-9 in HL-60 cells (Fig. 3).

3.4. Effect of catalase on TS extracts-induced cell-cycle arrest and apoptosis in HL-60 cells.

Our previous study demonstrated that catalase (H_2O_2 scavenger) significantly decreased

T. sinensis-induced cytotoxicity, DNA fragmentation, and ROS generation in HL-60 cells (Yang et al., 2006a). Further to confirm this issue, in the present study we examined the ~~effects of~~ antioxidant catalase ~~on~~could effect TS extracts-induced cell-cycle arrest (cyclin D1, CDK4, cyclin E, CDK2, cyclin A, and $p27^{KIP}$) and apoptosis (Fas/FasL, caspase-8, Bid, and caspase-9) in HL-60 cells. Cells were simultaneously treated with TS extracts (75 μg/mL for 6 h) and catalase (10 U/mL) for indicated time period (Fig. 2 and 3). We found that catalase treatment significantly reduced TS extracts-induced G_1 arrest in HL-60 cells as evidenced by up-regulation of cell-cycle regulatory proteins including cyclin D1, CDK4, cyclin E, CDK2, cyclin A, and inhibits $p27^{KIP}$. Furthermore, catalase treatment markedly down-regulates death signaling cascades and pro-apoptotic proteins Fas, FasL, caspase-8, Bid, and caspase-9 in HL-60 cells (Fig. 2 and 3). These results also provided a positive mechanism that TS extracts-induced HL-60 cell-cycle arrest (G_1) and apoptosis was associated with the production of intracellular ROS, especially H_2O_2.

3.5. TS extracts induce down-regulation of VEGF in HL-60 cells

A number of studies have shown that VEGF is one the most important angiogenic factor closely associated with neovascularization in human tumors. Western blotting and ELISA assay were used to analyze the effects of TS extracts on the expression and release of angiogenic-related protein VEGF in HL-60 cells. As shown in Fig. 4A, treatment of HL-60 cells with TS extracts dose-dependently inhibits the expression of VEGF. In addition, control cells (without treatment) released detectable levels of VEGF into the serum-free culture media at approximately 27 pg/10^5 cells (Fig. 4B). A concomitant with protein level, TS extracts significantly inhibits VEGF release into culture media in a dose-dependent manner (Fig. 4B).

3.6. Effect of TS extracts on tumor growth in HL-60 xenograft nude mice.

Nude mice were used to evaluate the *in vivo* effect of TS extracts on tumor growth. HL-60 cells were xenograft into nude mice as described in materials and methods. All the animals appeared healthy with no loss of body weight noted during treatment with TS extracts (Fig. 5A). In addition, no signs of toxicity were observed (data not shown) in any of the nude mice. The time course for HL-60 xenograft growth with TS extracts (7.5 and 10.0 mg/kg) or without treatment (control) is shown in Fig. 5B. Evaluation of tumor volume showed significant growth inhibition associated with TS extracts treatment (Fig. 5B). At the end of 21 days, the HL-60 xenograft tumor of each mouse was excised from each sacrificed animal and weighed. Tumor weight in the TS extracts-treated (7.5 and 10.0 mg/kg) mice was inhibited as compared with the control group (Fig. 6A and 6B). In addition, abundant mitosis in nuclei was observed in xenograft tumor section, indicating the proliferating activity, with well differentiation of tumor cells were also noticed (Fig. 7A). While decreased mitotic figures shrunken tumor cells were noted in the 7.5 mg/kg TS extracts treated animals (Fig. 7B), and tumor cells became smaller and shrunken, indicating the regression of tumor cells, in the 10 mg/kg TS extracts treated animals (Fig. 7C). These *in vivo* data also strongly suggest that TS extracts exerted antitumor activity in HL-60 leukemia xenograft nude mice could be due to the modulation of cell-cycle regulation and/or induction of apoptosis.

3.7. Gallic acid causes G_1 arrest and regulates cell-cycle regulatory proteins in HL-60 cells.

Previously we reported that treatment of the HL-60 cells with gallic acid (5-10 μg/mL), purified from TS extracts, resulted in sequences of events marked by apoptosis in the HL-60 cells was accompanied by loss of cell viability, ROS generation, internucleosomal DNA

fragmentation, cytochrome *c* release, activation of caspase-3, degradation of poly(ADP-ribose) polymerase (PARP), and dysregulation of Bax/Bcl-2 (Yang et al., 2006a). Our present study also showed that TS extracts appreciably inhibits tumor progression through cell-cycle arrest at G_1 phase. Therefore, further we intended to investigate the effect of gallic acid (5-10 μg/mL), on cell-cycle control in HL-60 cells. The profile of the DNA content in gallic acid-treated HL-60 cells (5 μg/mL for 6-18 h) was obtained using flow cytometric analysis. Fig. 8A showed that gallic acid exposure resulted in progressive and sustained accumulation of cells in G_1 phase. Furthermore, the percentage of G_1 phase cells increased, while those in the S and G_2/M phase decreased after treatment with gallic acid (Fig. 8A). Notably, there was a remarkable accumulation of sub-G_1 peak in gallic acid-treated HL-60 cells (5 μg/ml for 6-18 h) compared with the untreated group (Fig. 8A). Our findings suggest that gallic acid also promotes cell growth inhibition by inducing G_1 phase arrest in human leukemia cells.

In order to examine the molecular mechanism(s) and underlying changes in cell-cycle patterns caused by gallic acid treatment, we investigated the effects of various cyclins and CDKs involved in cell-cycle regulation in HL-60 cells. Cells were treated with gallic acid (5-10 μg/mL) for 0-6 h. Dose-dependent reductions of cyclin D1, CDK4, cyclin E, CDK2, and cyclin A, and induction of $p27^{KIP}$ expression were observed (Fig. 8B). Notably, gallic acid treatment significantly inhibits the expression of cyclin D1, cyclin E, CDK2 and 4, which are critically required for G_1-S transition phase. Therefore, we believed that the gallic acid-induced G_1 cell-cycle arrest is mediated by the inhibition of cyclin D1, cyclin E, and CDK2 and 4. However, the experimental treatment did not appear to alter the amount of detectable cyclin B1, CDC2, and p15 protein levels, which was concomitant with TS extracts treatment (Fig. 8B).

4. Discussion

Differential regulation of the cell-cycle, and subsequent events leading to apoptotic cell death, account for the anticancer effect of some potential phytochemicals (Sporn and Suh, 2002). Several studies have demonstrated anticancer potential for extracts from a number of herbal medicines or mixtures *in vitro* or *in vivo*. Herbal medicine is one of the ancient forms of health care known to humankind and it has been used in most cultures throughout history. Typically, herbal medicines emphasize the use of whole extracts from a plant mix or from complex formulations (Sporn and Nuh, 2002). Our previous study has demonstrated that TS extracts induce apoptotic cell death in cultured human premyelocytic leukemia HL-60 cells (Yang et al., 2006a). The present investigation also a parallel study showing the effect of TS extracts an *in vivo* human tumor xenograft in nude mice as well as *in vitro* cell culture models involving HL-60 cells. Summary of our data suggests that TS extracts treatment could be effective in suppressing the proliferation of HL-60 cells as shown by growth inhibition, cell-cycle arrest, and apoptotic induction *in vivo* and *in vitro*. Investigation has shown the nontoxic characteristics of *T. sinensis* [oral administration of *T. sinensis* (1000 mg/kg/day) for 28 days in rats], which increases its potential for application in food and drug products (Liao et al., 2007). Furthermore, *in vivo* toxicity was also examined superficially from body weight changes and histological studies of vital organs (data not shown). There appeared to be no sign of significant toxicity at TS extracts exposures up to the concentration of 10 mg/kg. This likely indicates that there are no side effects at these doses. Future studies should test whether there is an optimal/effective dose for TS extracts exposure.

Disturbance of the cancer cell-cycle is one of the therapeutic targets in the development of new anticancer drugs. The results of cell-cycle analysis in the present study showed that TS extracts/gallic acid treatment had a profound effect on cell-cycle control, with the

premyelocytic leukemia cells accumulating in G_1 phase. Progression through the first gap phase (G_1) requires both cyclin D-dependent CDK4/CDK6 and CDK2/cyclin E holoenzymes (Takahashi et al., 1999; Youn et al., 2008). The CDK catalytic subunits CDK 4 and CDK 2, and their regulatory subunits, cyclin D1 and cyclin E, are believed to be a crucial event in the regulation of S-phase entry, which appears to define the restriction point in the late G_1 phase. Cyclin D expression is frequently deregulated in human neoplasias, and agents that can down-regulate cyclin D expression may be helpful in both their prevention and treatment (Sausville et al., 2000). Further, it has been found that cyclin E, which is one of the key cell-cycle regulators, is over-expressed in primary carcinoma tissue (Wang et al., 1994). Cyclin A is particularly interesting among the clyclin family because it can activate two different types of CDKs and function in both S-phase and mitosis. Cyclin A associated protein kinase activity is critically required for G_1 to S-phase transition and further entry into M-phase (Johnson and Walker, 1999). The results imply that the expression of cyclin D1, CDK4, cyclin E, Cyclin A, and CDK2 are down-regulated by TS extracts, which corroborates the G_1 block in HL-60 cells. It has been shown that impairment of a growth stimulation-signaling pathway induces the expression of CDK inhibitor, which binds to and subsequently inhibits cyclin-CDK activity (Sandal et al., 2002). Our results suggest that inducing $p27^{KIP}$ expression *via* treatment with TS extracts/gallic acid may account for a large part of the reduction in CDK activity and, subsequently, block cell-cycle progression. Our study has also demonstrated that there were no significant differences in the expression of cyclin B1 and CDC2 after treatment with the TS extracts and gallic acid. The evidence suggests that the complex formed by the association of cyclin B1 and CDC2 plays a major role at entry into mitosis (Kuo et al., 2006). These results suggest that the observed inhibition of proliferation in HL-60 cells associated with the *T. sinensis* treatment could be the result of cell-cycle arrest during the

G_1 phase.

Investigations have shown that apoptosis is controlled by both mitochondrial and membrane death receptor pathways. The extrinsic pathway is initiated by the binding of transmembrane death receptors, including Fas, FasL, TNFR1, and TRAIL receptors with cognate extracellular ligands (Reed, 2000). Ligand receptors recruit adaptor proteins such as TRADD and FADD which interact with and trigger the activation of caspase-8. Activated caspase-8 further cleaves or activates downstream effector caspases, such as caspase-3 (Reed, 2009). The present study indicates that TS extracts-induced apoptosis is associated with up-regulation of Fas and FasL, caspase-8 activation, and down-regulation of Bid in HL-60 cells. Our previous investigation has been demonstrated that treatment of HL-60 cells with TS extracts can induce apoptosis *via* a mitochondrial pathway that is associated with loss of cell viability, internucleosomal DNA fragmentation, cytochrome *c* translocation, caspase-3 activation, poly ADP-ribose polymerase (PARP) degradation, and Bcl-2 and Bax dysregulation (Yang et al., 2006a). However, the activation of caspase-9 by TS extracts was still in debate. Caspase-9 is a crucial factor for activation of caspase-3, which cleave several cellular targets including poly ADP ribose polymerase (Reed, 2009). The current data filled the gape that TS extracts markedly activates caspace-9 from procaspase-9 followed by caspase-3 activation. Analysis of our data suggests that TS extracts-induced apoptosis is controlled by both a mitochondrial and a membrane DR pathway.

Our previous report demonstrated that catalase (H_2O_2 scavenger) significantly decreases TS extracts-induced cytotoxicity, DNA fragmentation, and ROS production in HL-60 cells (Yang et al., 2006a). The present investigation further confirmed that catalase significantly reduced TS extracts-induced cell-cycle arrest (cyclin D1, CDK4, cyclin E, CDK2, cyclin A, pRb, and $p27^{KIP}$) and apoptosis (Fas, FasL, caspase-8, Bid, and caspase-9) in HL-60 cells.

Analysis of our data suggesting TS extracts-induced HL-60 cell-cycle arrest and apoptosis could be due to the intracellular ROS generation, especially H_2O_2. Other workers have shown that gallic acid-induced intracellular ROS, especially H_2O_2, play an important role in eliciting an early signal of apoptosis (Sakaguchi et al., 1998; Inoue et al., 2000), and that catalase significantly reduces gallic acid-induced apoptotic cell death (Yang et al., 2006a; Isuzugawa et al., 2001). In addition, recent studies appear to support the notion that TS extracts may possess protective antioxidant properties (Cho et al., 2003; Hsieh et al., 2004; Hseu et al., 2008a). Several researchers have shown that antioxidants produce genetic changes that cause apoptosis in cancer cells by mechanisms other than antioxidant effect (Yang et al., 2006b). Thus, TS extracts might serve as a mediator for the reactive oxygen-scavenging system and potentially act as both a pro-oxidant and an antioxidant, depending on the redox state of the biological environment. However, the detailed mechanisms of the chemotherapeutic action of *T. sinensis* are unknown, and further investigations are needed.

Angiogenesis is tightly regulated by an intricate balance between stimulators and inhibitors. Among them, VEGF, a soluble angiogenic factor produced by many tumors as well as normal cell lines, plays a key role in regulating normal and pathologic angiogenesis (Tonini et al., 2003; Hseu et al., 2011b). A previous report clearly evidenced that increased angiogenesis in bone marrow region from patients with acute myeloid leukemia (Hussong et al., 2000). These observations also suggest that the increased anagiogeneis is critically mediated by VEGF expression, which play crucial role for the further onset of tumor progression. Therefore, the therapeutic strategies have been developed for acute myeloid leukemia also targets anti-angiogenic processes, with promising results, because of the critical dependence of tumor growth and metastasis on angiogenesis. It is noteworthy that TS extracts significantly down-regulates both VEGF expression and release in HL-60 cells. A similar pattern of results were found in our

previous study that TS extracts potentially inhibits VEGF-induced angiogenesis in vascular endothelial cells (Hseu et al., 2011a). Taken together, the inhibitory effect of TS extracts on VEGF activity or angiogenesis in lekemia or endothelial cells are strong evidence for development of anti-cancer/anti-angiogenic drug from this vital source.

Furthermore, tumor inhibition was observed after treatment with TS extracts in the nude mice xenograft model in this study. Both incidence and mean tumor volume and weight were significantly reduced by TS extracts. Experiments using animals and circulating blasts from leukemia patients have yielded evidence that apoptosis also occurs in response to chemotherapy *in vivo*. Human acute-leukemia cell lines (HL-60 cells) have proven particularly informative in study of chemotherapy-associated apoptotic proteolytic events (Hseu et al., 2004). Moreover, in this study the *in vivo* toxicity of TS extracts was also examined superficially from body weight change and histological study of vital organs (data not shown), with no apparent signs of significant negative effects at exposures of 7.5 and 10 mg/kg. Analysis of our data suggests that TS extracts exert anti-proliferative action and growth inhibition on HL-60 cells *in vitro* or *in vivo*.

Natural products, including plants, provide rich resources for anticancer drug discovery. 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, was isolated from the leaves of *T. sinensis*; identity of the compounds was determined by HPLC and based on the analogous information reported in the literature (Yang et al., 2006a; Hsu et al., 2003). The total phenolic content of the TS extract was estimated to be 130 ± 26 mg of gallic acid equivalent/g of plant extracts (Yang et al., 2006a). The yield of gallic acid, the natural phenolic component purified from TS extracts, was about 6%. Although it remains unclear which of the components of *T. sinensis* are active compounds, gallic acid has received increased attention recently

because of some interesting new findings regarding its biological activities (Chen et al., 2009). Gallic acid is widely distributed in various plants and fruits, such as gallnuts, sumac, oak bark, green tea, apple peels, grapes, strawberries, pineapples, bananas, lemons, and in red and white wine (You et al., 2010). Even though the therapeutic utility of gallic acid in this regard is unknown, its common occurrence in fruits and food as well as its small molecular weight (170 Da) might be an advantage in terms of safety and dosing design. Studies have demonstrated that gallic acid selectively induces cancer cell death by apoptosis; however, gallic acid shows no cytotoxicity against normal cells (Yang et al., 2006a). Other workers 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 (Agarwal et al., 2007). Raina et al (2008) revealed that gallic acid treatment remarkably decreased human prostate cancer cell xenografted tumor incidence in mice. Therefore, gallic acid may be a useful phytochemical for cancer chemoprevention (Surh, 2003). These results corroborate other studies which have implicated that gallic acid is the main constituent responsible for the antiproliferative activity (Chen et al., 2009). ~~These results imply that gallic acid as one of the major active constituents responsible for the antitumor activities of *T. sinensis* leaf extracts.~~ Moreover, in future we have planned to investigate antitumor effect of other bioactive compounds isolated from the aqueous leaf extracts of *T. sinensis*.

Formatted: Not Highlight

Formatted: Not Highlight

The results obtained *in vitro* and *in vivo* in this study imply that *T. sinensis* could act as a chemopreventive agent with respect to inhibition of the growth of human leukemia HL-60 cells through the induction of cell-cycle arrest and apoptosis. We also believed that the antitumor activity of *T. sinensis* may be the abundance of gallic acid. These data provide an important step that might help model the effects of *T. sinensis* for potential future studies with animal models and human patients, and thereby facilitate the development of

nutraceutical products using this agent.

Conflict of interest statement

The authors have no conflict of interest to declare.

Acknowledgments

This work was supported by grants NSC-97-2320-B-039-042, NSC-99-2320-B-039-035-MY3, CMU95-037, and CMU95-333 from the National Science Council and China Medical University of Taiwan.

References

Agarwal, C., Veluri, R., Kaur, M., Chou, S.C., Thompson, J.A., Agarwal, R., 2007. Fractionation of high molecular weight tannins in grape seed extract and identification of procyanidin B2-3,3'-di-O-gallate as a major active constituent causing growth inhibition and apoptotic death of DU145 human prostate carcinoma cells. Carcinogenesis. 28, 1478-1484.

Ahmad, N., Adham, V.M., Afaq, F., Feyes, D.K., Mukhtar, H., 2001. Resveratrol causes WAF-1/p21-imediated G1-phase arrest of cell cycle and induction of apoptosis in human epridermoid carcinoma A431 cells. Clin Cancer Res. 7, 1466-1473.

Bak, M.J., Jeong, J.H., Kang, H.S., Jin, K.S., Ok, S., Jeong, W.S., 2009. *Cedrela sinensis* leaves suppress oxidative stress and expression of iNOS and COX-2 via MAPK signaling pathways in RAW264.7 cells. J Food Sci Nutr. 14, 269-276.

Chang, H.C., Hung, W.C., Huang, M.S., Hsu, H.K., 2002. Extract from the leaves of *Toona sinensis* Roemor exerts potent antiproliferative effect on human lung cancer cells. Am J Chin Med. 30, 307-314.

Chang, H.L., Hsu, H.K., Su, J.H., Wang, P.H., Chung, Y.F., Chia, Y.C., Tsai, L.Y., Wu, Y.C., Yuan, S.S.F., 2006. The fractioned *Toona sinensis* leaf extract induced apoptosis of human ovarian cancer cells and inhibits tumor growth in a murine xenograft model. Gynecol Oncol. 102, 309-314.

Chen, H.M., Wu, Y.C., Chia, Y.C., Chang, F.R., Hsu, H.K., Hsieh, Y.C., Chen, C.C., Yuan, S.S., 2009. Gallic acid, a major compound of *Toona sinensis* leaf extracts, contains a ROS-mediated anti-cancer activity in human prostate cancer cells. Cancer Lett. 286, 161-171.

Chia, Y.C., Rajbanshi, R., Calhoun, C., Chiu, R.H., 2010. Anti-neoplastic effect of gallic acid, a major component of *Toona sinensis* leaf extract, on oral squmous carcinoma cells. Molecules. 15, 8377-8389.

Cho, E.J., Yokozawa, T., Rhyu, D.Y., Kim, H.Y., Shibahara, N., Park, J.C., 2003. The inhibitory effects of 12 medicinal plants and their component compounds on lipid peroxidation. Am J Chin Med. 31, 907-917.

Collins, A., Yuan, L., Kiefer, T.L., Cheng, Q., Lai, L., Hill, S.M., 2003. Overexpression of the MT1 melatonin receptor in MCF-7 human breast cancer cells inhibits mammary tumor formation in nude mice. Cancer Lett. 189, 49-57.

Edmonds, J.M., Staniforth, M., 1998. *Toona sinensis*: Meliaceae. Curtis's Bot Mag. 15, 186-193.

Eskes, R., Desagher, S., Antonsson, B., Martinou, J.C., 2000. Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. Mol Cell Biol. 20, 929-935.

Hseu, Y.C., Chang, W.H., Chen, C.S., Liao, J.W., Huang, C.J., Lu, F.J., Chia, Y.C., Hsu, H.K., Wu, J.J., Yang, H.L., 2008a. Antioxidant activities of *Toona Sinensis* leaves extracts using different antioxidant models. Food Chem Toxicol. 46, 106-114.

Hseu, Y.C., Chen, S.C., Chen, H.C., Liao, J.W., Yang, H.L., 2008b. *Antrodia camphorata* inhibits proliferation of human breast cancer cells *in vitro* and *in vivo*. Food Chem Toxicol. 46, 2680-2688.

Hseu, Y.C., Chen, S.C., Lin, W.H., Huang, D.Z., Lin, M.K., Kuo, Y.H., Wang, M.T., Cho, H.J., Wang, L., Yang, H.L., 2011a. *Toona sinensis* (leaf extracts) inhibit vascular endothelial growth factor (VEGF)-induced angiogenesis in vascular endothelial cells. J Ethnopharmacol. 134, 111-121.

Hseu, Y.C., Chen, S.C., Tsai, P.C., Chen, C.S., Lu, F.J., Yang, H.L., 2007. Inhibition of cyclooxygenase-2 and induction of apoptosis in estrogen-nonresponsive breast cancer cells by *Antrodia camphorata*. Food Chem Toxicol. 45, 1107-1115.

Hseu, Y.C., Wu, C.R., Chang, H.W., Kumar, K.J.S., Lin, M.K., Chen, C.S., Cho, H.J., Huang, C.Y., Huang, C.Y., Lee H.Z., Hsieh, W.T., Chung, J.G., Wang, H.M., Yang, H.L., 2011b. Inhibitory effects of *Physalis angulata* on tumor metastasis and angiogenesis. J Ethnopharmacol. 135, 762-771.

Hseu, Y.C., Yang, H.L., Lai, Y.C., Lin, J.G., Chen, G.W., Chang, Y.H., 2004. Induction of apoptosis by *Antrodia camphorata* in human premyelocytic leukemia HL-60 cells. Nutr Cancer. 48, 189-197.

Hsieh, T.J., Liu, T.Z., Chia, Y.C., Chern, C.L., Lu, F.J., Chuang, M.C., Mau, S.Y., Chen, S.H., Syu, Y.H., Chen, C.H., 2004. Protective effect of methyl gallate from *Toona sinensis* (Meliaceae) against hydrogen peroxide-induced oxidative stress and DNA damage in MDCK cells. Food Chem Toxicol. 42, 843-850.

Hsu, H.K., Yang, Y.C., Hwang. J.H., Hong, S.J., 2003. Effects of *Toona sinensis* leaf extract on lipolysis in differentiated 3T3-L1 adipocytes. Kaohsiung J Med Sci. 19. 3855-3890.

Hussong, J.W., Rodgers, G.M., Shami, P.J., 2000. Evidence increased aniogenesis in

patients with acute myeloid leukemia. Blood. 95, 309-313.

Inoue, M., Sakaguchi, N., Isuzugawa, K., Tani, H., Ogihara, Y., 2000. Role of reactive oxygen species in gallic acid-induced apoptosis. Biol Pharm Bull. 23, 1153-1157.

Isuzugawa, K., Inoue, M., Ogihara, Y., 2001. Catalase contents in cells determine sensitivity to the apoptosis inducer gallic acid. Biol Pharm Bull. 24, 1022-1026.

Jiang, S.H., Wang, C.L., Chen, Z.Q., Chen, M.H., Wang, Y.R., Liu, C.J., Zhou, Q.L., Li, Z.J., 2009. Antioxidant properties of the extract and subtractions from old leaves of *Toona sinensis* Roem (Meliaceae). J Food Biochem. 33, 425-441.

Johnson, D. G., Walker, C.L., 1999. Cyclins and cell cycle checkpoints. Ann Rev Pharmacol Toxicol. 39, 295-312.

Kim, J.J., Kim, H.P., Jung, C.H., Hong, M.H., Hong, M.C., Bae, H.S., Lee, S.D., Park, S.Y., Park, J.H., Ko, S.G., 2006. Inhibition of cell cycle progression *via* $p27^{kip1}$ upregulation and apoptosis induction by an ethanol extract of *Rhus verniciflua* stokes in AGS gastric cancer cells. Int J Mol Med. 18, 201-208.

Kuo, P.L., Hsu, Y.L., Cho, C.Y., 2006. Plumbagin induced G2-M arrest and autophagy by inhibiting the AKT/mammalian target rapamycin pathway in breast cancer cells. Mol Cancer Ther. 5, 3209-3221.

Liao, J.W., Chung, Y.C., Yeh, J.Y., Lin, Y.C., Lin, Y.G., Wu, S.M., Chan, Y.C., 2007. Safety evaluation of water extracts of *Toona sinensis* Roemor leaf. Food Chem Toxicol. 45, 1393-1399.

Liao, J.W., Hsu, C.K., Wang, M.F., Hsu, W.M., Chan, Y.C., 2006. Beneficial effect of *Toona sinensis* Roemor on improving cognitive performance and brain degeneration in senescence-accelerated mice. Br J Nutr. 96, 400-407.

Manach, C., Williamson, G., Morand, C., Scalbert, A., Remesy, C., 2005. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am J Clin Nutr. 81, 230S-242S.

Nagata, S., 1997. Apoptosis by death factor. Cell. 88, 355-365.

Poon, S.L., Leu, S.F., Hsu, H.K., Liu, M.Y., Huang, B.M., 2005. Regulatory mechanism of *Toona sinensis* on mouse leydig cell steroidogenesis. Life Sci. 76, 1473-1487.

Raina, K., Rajamanickam, S., Deep, G., Singh, M., Agarwal, R., Agarwal, C., 2008. Chemopreventive effects of oral gallic acid feeding on tumor growth and progression in TRAMP mice. Mol Cancer Ther. 7, 1258-1267.

Reed, J.C., 2000. Mechanisms of apoptosis. Am J Pathol. 157, 1415-1430.

Sakaguchi, N., Inoue, M., Ogihara, Y., 1998. Reactive oxygen species and intracellular Ca^{2+}, common signals for apoptosis induced by gallic acid. Biochem Pharmacol. 55, 1973-1981.

Sandal, T., Stapnes, C., Kleivdal, H., Hedin, L., Doskeland, S.O., 2002. A novel, extra neuronal role for cyclin-dependent protein kinase 5 (CDK5): modulation of cAMP-induced apoptosis in rat leukemia cells. J Biol Chem. 277, 20783-20793.

Sausville, E.A., Johnson, J., Alley, M., Zaharevitz, D., Senderowicz, A.M., 2000. Inhibition of CDKs as a therapeutic modality. Ann N Y Acad Sci. 910, 207-221.

Schmitt, C.A., 2003. Senescence, apoptosis and therapy--cutting the lifelines of cancer. Nat Rev Cancer. 3, 286-295.

Shahrzad, S., Aoyagi, K., Winter, A., Koyama, A., Bitsch, I., 2001. Pharmacokinetics of gallic acid and its related bioavailability from tea in healthy humans. J. Nutr. 131, 1207-1210.

Sporn, M.B., Suh, N., 2002. Chemoprevention: an essential approach to controlling cancer. Nat Rev Cancer. 2, 537-543.

Formatted: Not Highlight

Stewart, Z.A., Westfall, M.D., Pietenpol, J.A., 2003. Cell-cycle dysregulation and anticancer therapy. Trends Pharmacol Sci. 24, 139-145.

Surh, Y.J., 2003. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer. 3, 768-780.

Takahashi, A., Taniguchi, T., Ishikawa, Y., Yokoyama, M., 1999. Tranilast inhibits vascular smooth muscle cell growth and intimal hyperplasia by induction of $p21^{wafi/cip1/sdi1}$ and p53. Circ Res. 84, 543-550.

Tonini, T., Rossi, F., Claudia, P.P., 2003. Molecular basis of angiogenesis and cancer. Oncogene. 22, 6549-6556.

Wang, C.Y., Lin, K.H., Yang, C.J., Tsai, J.R., Huang, J.Y., Wang, P.H., Hsu, H.K., Huang, M.S., 2010. *Toona sinensis* extracts induced cell cycle arrest and apoptosis in the human lung large cell carcinoma. Kaohsiung J Med Sci. 26, 68-75.

Wang, T.C., Cardiff, R.D., Zukerberg, L., Lees, E., Arnold, A., Schmidt, E.V., 1994. Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. Nature. 369, 669-671.

Yang, C.J., Huang, Y.J., Wang, C.S., Wang, P.H., Huang, J.Y., Wang, T.H., Hsu, H.K., Huang, H.W., Kumar, S.P., Huang, M.S., Weng, C.F., 2010b. Antiproliferative and antitumorigenic activity of *Toona sinensis* leaf extracts in lung adenocarcinoma. J Med Food. 13, 54-61.

Yang, C.J., Huang, Y.J., Wang, C.Y., Wang, P.H., Hsu, H.K., Tsai, M.J., Chen, Y.C., Kumar, V.B., Huang, M.S., Weng, C.F., 2010a. Antiproliferative effect of *Toona sinensis* leaf extract on non-small-cell lung cancer. Tran Res. 155, 305-314.

Yang, H.L., Chang, W.H., Chia, Y.C., Huang, C.J., Lu, F.J., Hsu, H.K., Hseu, Y.C., 2006a. *Toona sinensis* extracts induces apoptosis *via* reactive oxygen species in human premyelocytic leukemia cells. Food Chem Toxicol. 44, 1978-1988.

Yang, H.L., Chen, C.S., Chang, W.H., Lu, F.J., Lai, Y.C., Chen, C.C., Hseu, T.H., Kuo, C.T., Hseu, Y.C., 2006b. Growth inhibition and induction of apoptosis in MCF-7 breast cancer cells by *Antrodia camphorata*. Cancer Lett. 321, 215-227.

Yang, Y.C., Hus, H.K., Hwang, J.H., Hong, S.J., 2003. Enhancement of glucose uptake in 3T3-L1 adipocytes by *Toona sinensis* leaf extract. Kaohsiung J Med Sci. 9, 327-333.

You, B.R., Moon, H.J., Han, Y.H., Park, W.H., 2010. Gallic acid inhibits the growth of HeLa cervical cancer cells *via* apoptosis and/or necrosis. Food Chem Toxicol. 48, 1334-1340.

Youn, M.J., Kim, J.K., Park, S.Y., Kim, Y., Kim, S.J., Lee, J.S., Chai, K.Y., Kim, H.J., Cui, M.X., So, H.S., Kim, K.Y., Park, R., 2008. Chaga mushroom (*Inonotus obliquus*) induces G_0/G_1 arrest and apoptosis in human hepatoma HepG2 cells. World J Gastroenterol. 14, 511-517.

Figure legends

Fig. 1. Fffects of TS extracts on cell-cycle distribution in HL-60 cells. Cells were treated with 75 μg/mL of TS extracts for 0-18 h, stained with PI, and analyzed by flow cytometry for sub-G_1 and cell-cycle. Distribution (as percentage) of cells in the different phases of the cell-cycle (sub-G_1, G_1, S, and G_2/M) after treatment is shown. Apoptotic nuclei were identified as a subploid DNA peak, and were distinguished from cell debris on the basis of forward light scatter and PI fluorescence. Representative flow cytometry patterns are also presented. Results are presented as mean±SD (n=3). *: indicates significant difference in comparison to control group ($p<0.05$).

Fig. 2. Western blot analysis of cyclin D1, CDK4, cyclin E, CDK2, cyclin A, $p27^{KIP}$, cyclin B, CDC2, and p15 protein levels in HL-60 cells after exposure to TS extracts. Cells were treated with 75 μg/mL of TS extracts for 2, 4, and 6 h, and with 0, 10, 25, 50, and 75 μg/mL of TS extracts for 6 h plus catalase (10 U/mL) as indicated. Protein (50 μg) from each sample was resolved on 10-12% SDS-PAGE, and western blot performed. β-actin was used as a control. Relative changes in protein bands were measured using densitometric analysis. Typical result from three independent experiments is shown.

Fig. 3. Western blot analysis of Fas, FasL, caspase-8, Bid, and caspase-9 protein levels in HL-60 cells after exposure to TS extracts. Cells were treated with 75 μg/mL of TS extracts for 2, 4 and 6 h, and with 0, 10, 25, 50, and 75 μg/mL of TS extracts for 6 h plus catalase (10 U/mL) as indicated. Protein (50 μg) from each sample was resolved on 12% SDS-PAGE, and western blot performed. β-actin was used as a control. Relative changes in protein bands were measured using densitometric analysis. Typical result from three

independent experiments is shown.

Fig. 4. TS extracts mediated down-regulation of VEGF. (A) Western blot analysis of the protein levels of VEGF in HL-60 cells after exposure to TS extracts (0-75 μg/mL) for 6 h. Proteins (50 μg) from each sample were resolved on 8-15% SDS-PAGE. β-actin was used as a loading control. Relative changes in protein bands were measured using densitometric analysis with the control being 100% as shown just below the gel data. Typical results from three independent experiments are shown. (B) ELISA assay of VEGF release in HL-60 cells after exposure to TS extracts (0-75 μg/mL) for 6 h. Concentration of VEGF released into the medium was determined by ELISA. Results are presented as mean ± SD of three assays. *Significant difference in comparison to the control group (p<0.05).

Fig. 5. Time-course effect of TS extracts on the growth of HL-60 xenograft was evaluated from measurements of body weight (A) and tumor volume (B) every 3 days. HL-60 cells were implanted subcutaneously into the flanks of nude mice on day 0, the animals were then treated with TS extracts (7.5 and 10.0 mg/kg/every two days) or without (as a control) as described in Materials and Methods. Results are presented as mean±SE (n=6). *: indicates significant difference in comparison to control group (p<0.05).

Fig. 6. *In vivo* inhibition of HL-60 xenograft tumors by TS extracts. Nude mice were treated with TS extracts (7.5 and 10.0 mg/kg/every two days) or without (control). On the 21th day after tumor implantation, the animals were sacrificed and the tumors removed (A) and weighed (B). Results are presented as mean±SE (n=6). *: indicates significant difference in comparison to control group (p<0.05).

Fig. 7. Histopathological findings of tumor cells in HL-60 xenograft tumors. The control HL-60 xenograft tumors and the HL-60 xenograft tumors after TS extract (7.5 and 10.0 mg/kg/every two days) treatments were sectioned, stained with hematoxylin and eosin, and examined with light microscopy (400×). Arrows indicate the mitotic nuclei in tumor cells (control), and TS extracts-induced (7.5 and 10.0 mg/kg) shrunken tumor cells. Typical results from three independent experiments are shown.

Fig. 8. (A**)** Effects of gallic acid on cell-cycle distribution in HL-60 cells. Cells were treated with 5 μg/mL of gallic acid for 0-18 h, stained with PI, and analyzed by flow cytometry for sub-G_1 and cell-cycle. Distribution (as percentage) of cells in the different phases of the cell-cycle (sub-G_1, G_1, S, and G_2/M) after treatment is shown. Representative flow cytometry patterns are also presented. Results are presented as mean±SD (n=3). *: indicates significant difference in comparison to control group (p<0.05). (B) Western blot analysis of cyclin D1, CDK4, cyclin E, CDK2, cyclin A, $p27^{KIP}$, cyclin B, CDC2, and p15 protein levels in HL-60 cells after exposure to TS extracts. Cells were treated with 5 μg/mL of gallic acid for 2, 4, and 6 h, and with 0, 5, and 10 μg/mL of gallic acid for 6 h. Protein (50 μg) from each sample was resolved on 12-15% SDS-PAGE, and western blot performed. β-actin was used as a control. Relative changes in protein bands were measured using densitometric analysis. Typical results from three independent experiments are shown.

Figure 1

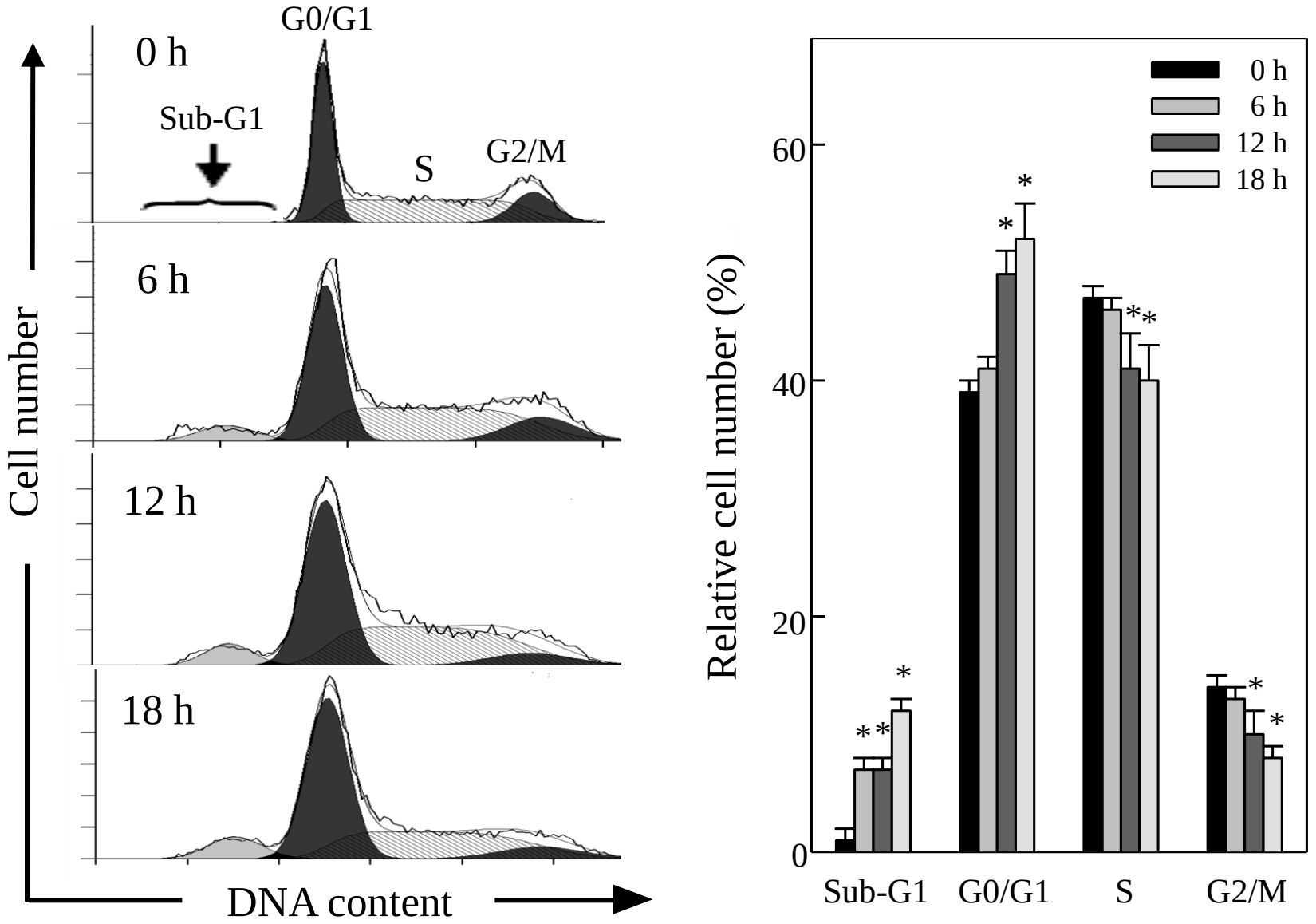

Fig. 1.

Figure 2

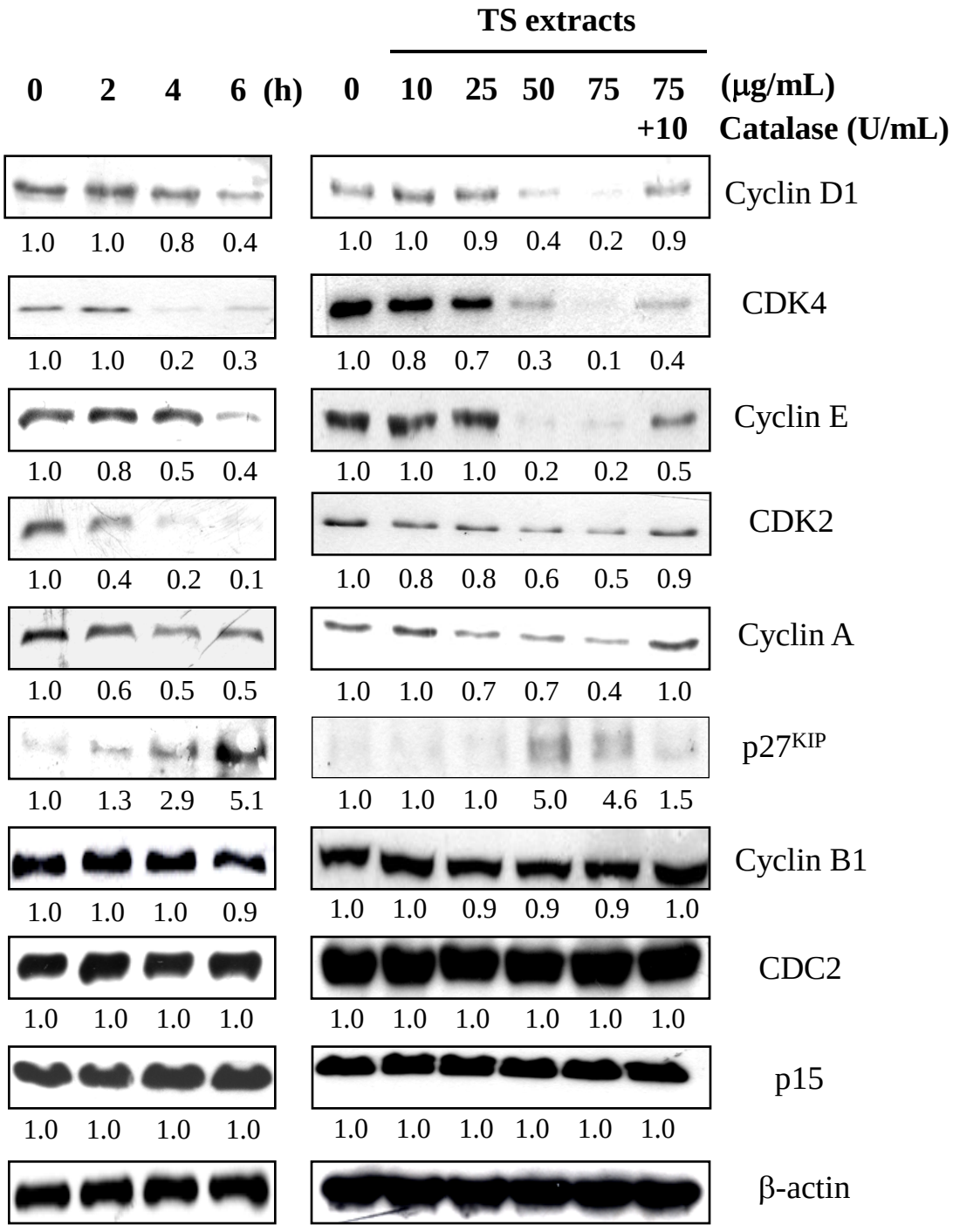

Fig. 2

Figure 3

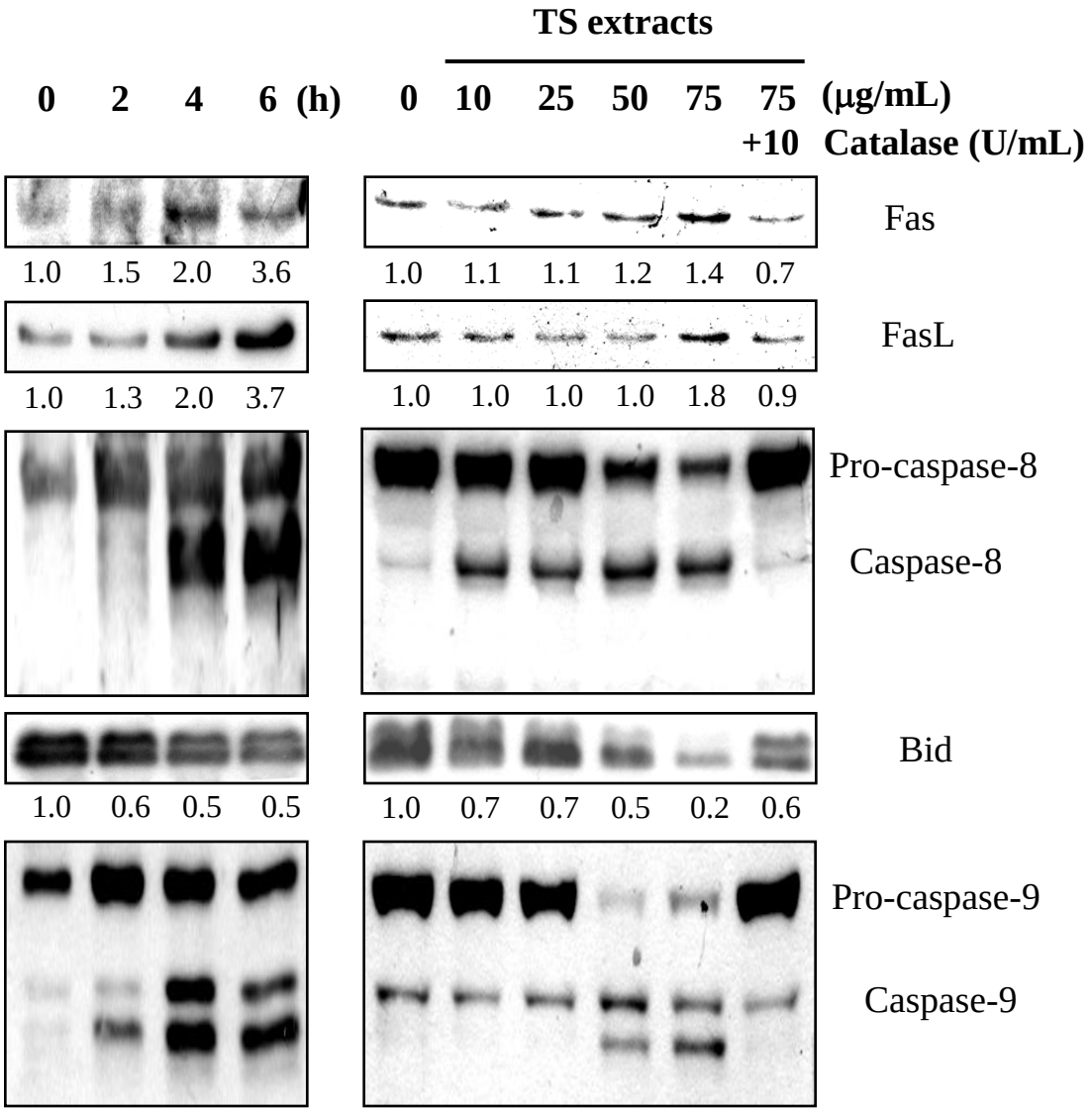

Fig. 3.

Figure 4

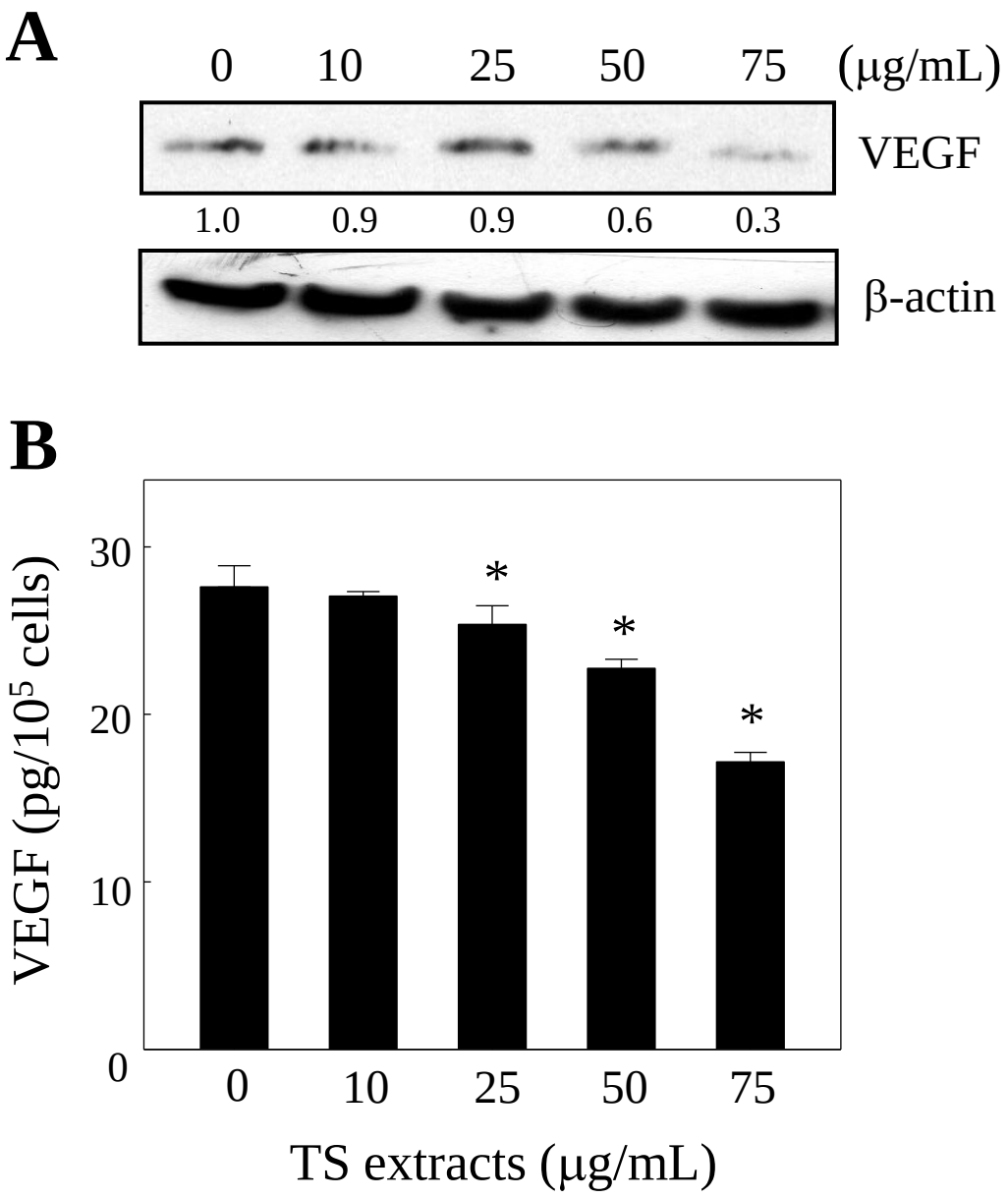

Fig. 4.

Figure 5

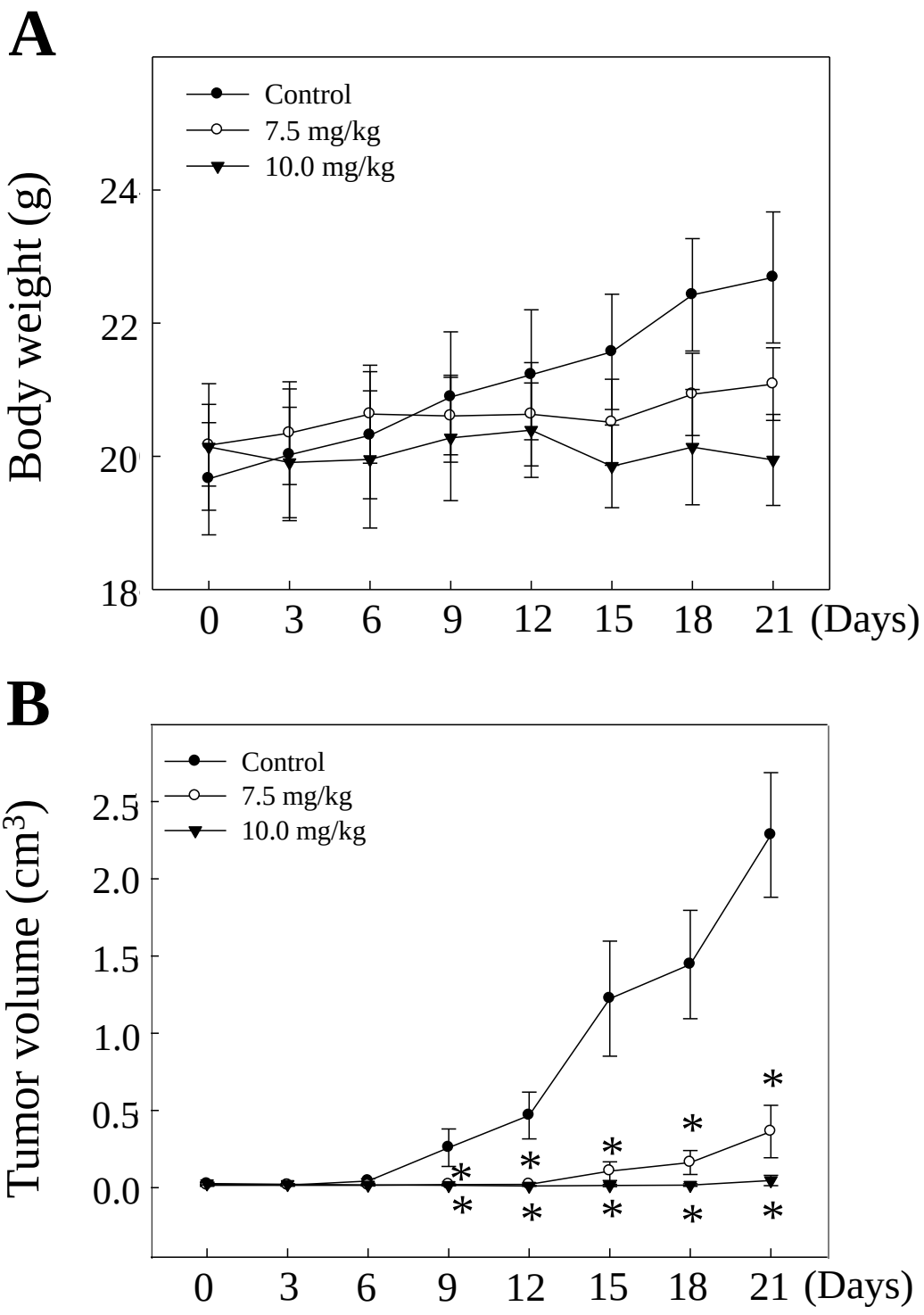

Fig. 5.

Figure 6

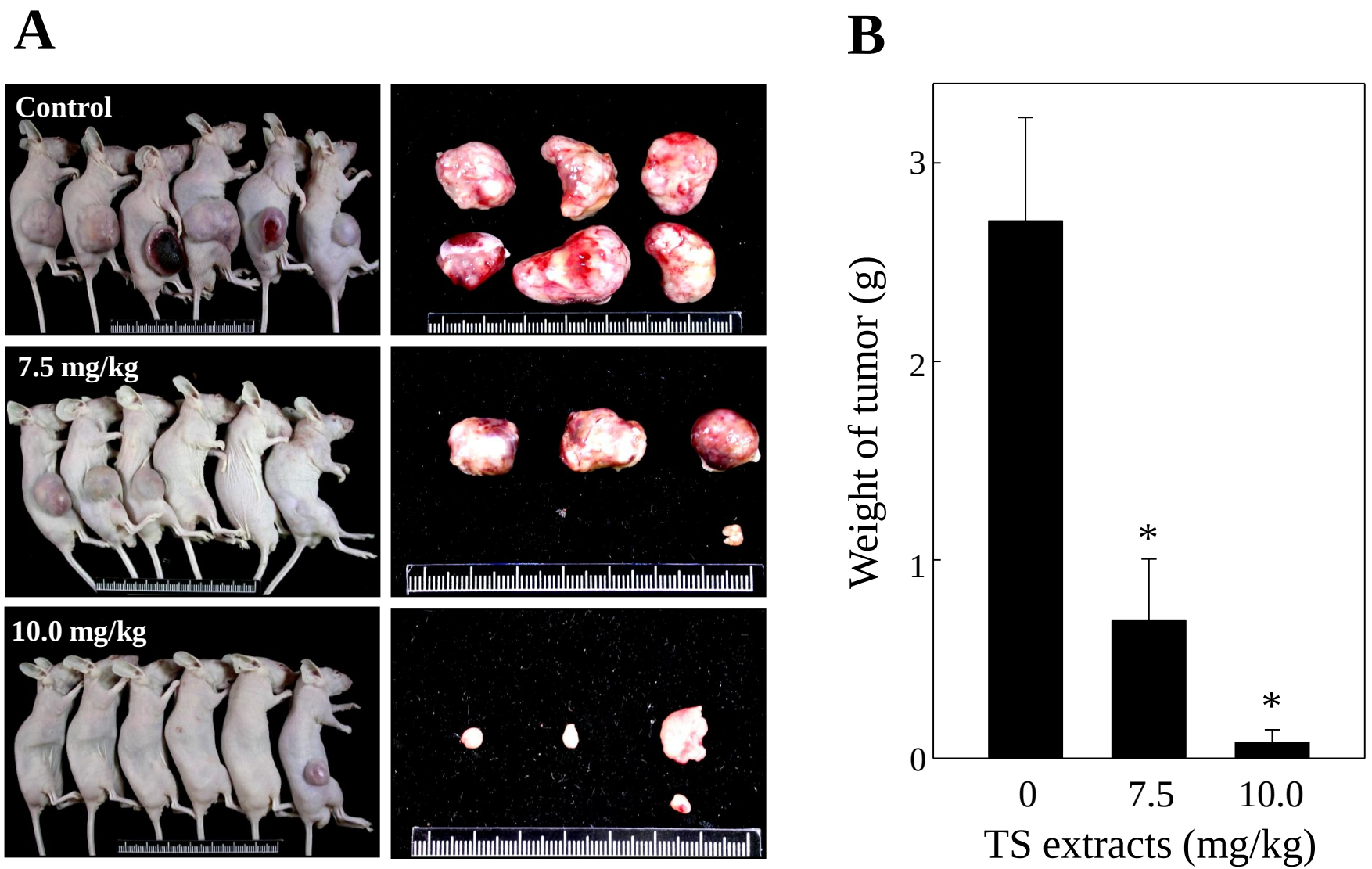

Fig. 6.

Figure 7

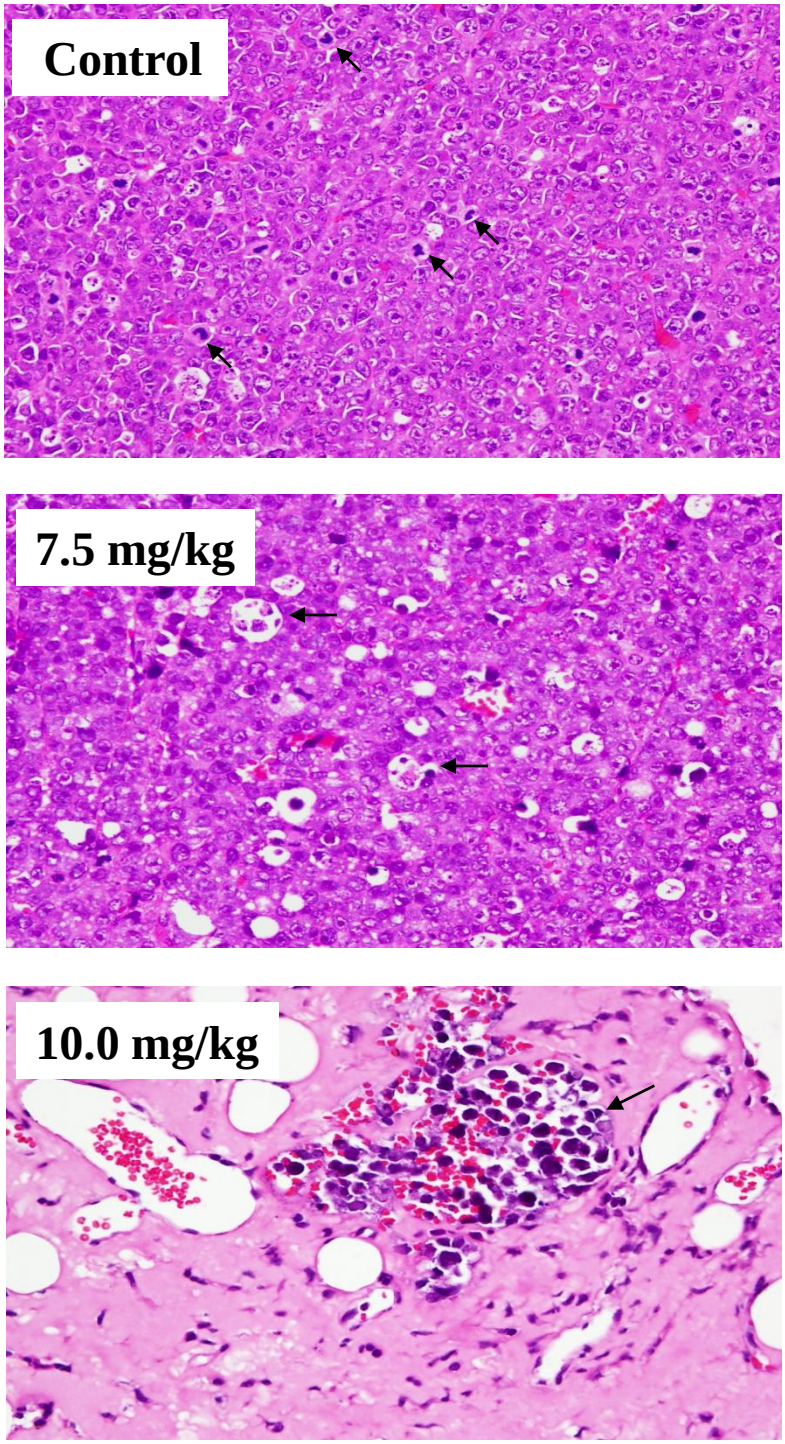

Fig. 7.

Figure 8

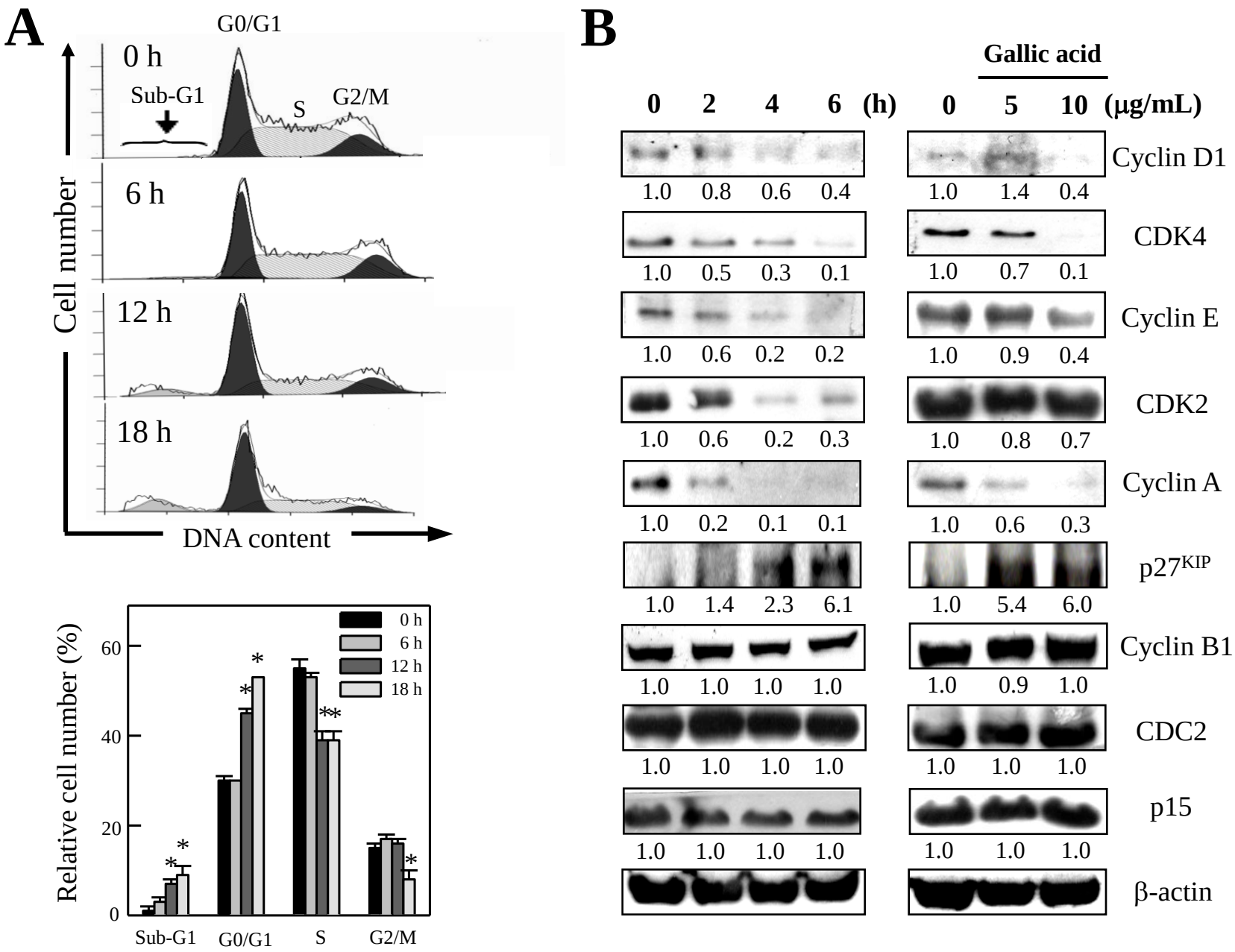

Fig. 8

*Abstract

ABSTRACT

Toona sinensis is one of the most popular vegetarian cuisines in Taiwan and it has been shown to induce apoptosis in cultured human premyelocytic leukemia (HL-60) cells. In the present study, we examined the effects of *Toona sinensis* leaf extracts (TS extracts) on tumor regression using *in vitro* cell culture and an *in vivo* athymic nude mice model. We found that TS extracts (10-75 μg/mL) arrested HL-60 cells at the G_1-S transition phase through the reductions of Cyclin D1, CDK4, Cyclin E, CDK2, and Cyclin A, and induction of CDK inhibitor $p27^{KIP}$ levels. Furthermore, VEGF expression and release was significantly inhibited by TS extracts. Notably, TS extracts treatment was effective in terms of delaying tumor incidence in the nude mice inoculated with HL-60 cells as well as reducing the tumor burden. Histological analysis confirmed that TS extracts significantly modulated tumor progression in xenograft tumor. Furthermore, a similar pattern of results were observed from gallic acid (5 and 10 μg/mL), a major compound in TS, caused G_1 arrest through regulations of cell-cycle regulatory proteins. Our data suggest that *Toona sinensis* exerts antiproliferative effects on HL-60 cells *in vitro* and *in vivo* due mainly to the presence of gallic acid.