Antioxidant activities of aqueous leaf extracts of *Toona sinensis* on free radical-induced endothelial cell damage

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ARTICLE INFO

ABSTRACT

Ethnopharmacological relavence: In Taiwan, *Toona sinensis* (*Toona sinensis*) is well known as a traditional Chinese medicine, while the underlying pharmacological mechanisms of this drug are still a matter of debate.

Materials and methods: The purpose of this study was to evaluate the protective effects of non-cytotoxic concentrations of aqueous leaf extracts of *Toona sinensis* (TS extracts; 50–100 µg/mL) and gallic acid (5 µg/mL), a major component of these extracts, against AAPH-induced oxidative cell damage in human umbilical vein endothelial cells (ECs).

Results: Exposure of ECs to AAPH (15 mM) decreased cell viability from 100% to 43%. However, ECs were pre-incubated with TS extracts prior to AAPH induction resulted in increased resistance to oxidative stress and cell viability in a dose-dependent manner. An increase in ECs-derived PGI_2 and IL-1β in response to AAPH exposure was positively correlated with cytotoxicity and negatively with TS extracts concentrations. In addition, gallic acid also suppressed PGI_2 and IL-1β production in AAPH-induced ECs. Notably, TS extracts/gallic acid treatment significantly inhibited ROS generation, MDA formation, SOD/catalase activity, and Bax/Bcl-2 dysregulation in AAPH-stimulated ECs. Pretreatment of ECs with TS extracts/gallic acid also suppressed AAPH-induced cell surface expression and secretion of VCAM-1, ICAM-1 and E-selectin, which was associated with abridged adhesion of U937 leukocytes to ECs. Moreover, TS extracts/gallic acid treatment significantly inhibited the AAPH-mediated up regulation of PAI-1 and down regulation of t-PA in ECs, which may decrease fibrinolytic activity.

Conclusions: Therefore, *Toona sinensis* may possess antioxidant properties that protect endothelial cells from oxidative stress. Our results also support the traditional use of *Toona sinensis* in the treatment of free radical-related diseases and atherosclerosis.

1. Introduction

Toona sinensis Roem. (Meliaceae; *Toona sinensis*) is a type of arbor that is widely distributed in Asia. It has long been used as a traditional Chinese medicine for a wide variety of conditions in Chinese society and is very popular in vegetarian cuisine in Taiwan. The edible leaves have been used as an oriental medicine for treating rheumatoid arthritis, cervicitis, urethritis, tympanitis, gastric ulcers, enteritis, dysentery, itchiness, and cancer (Xien, 1996). Notably, peoples from Sanxia region in China, *T. sinesis* were used for traditional medicine preparation for curing cerebrovascular and cardiovascular diseases (Li and Chen, 2009). While the underlying pharmacological mechanisms of this drug are still a matter of debate, various biological activities of *Toona sinensis* leaf extracts have been reported, including anti-cancer (Chang et al., 2002, 2006; Yang et al., 2006), anti-diabetes (Hsu et al., 2003), and antioxidant (Hseu et al., 2008b) effects, as well as inhibiting Leydig cell steroidogenesis (Poon et al., 2005) and suppressing brain degeneration in senescence-accelerated mice (Liao et al., 2006). Moreover, the safety levels and nontoxic characteristics of aqueous extracts of *Toona sinensis* were evaluated using a bacterial reverse

mutation assay (Ames test), and both acute and subacute toxicity studies were conducted in mice (Liao et al., 2006).

Atherosclerosis is a complicated inflammatory process that can lead to vascular endothelial dysfunction. The early pathogenesis of atherosclerosis appears to be activation of the endothelium in response to a variety of relevant stimuli including oxidized lipids, cytokines, or turbulent flow (Kim et al., 2007). The vascular endothelium plays a critical role in the preservation of normal vessel wall structure and function. Vascular endothelial cells (ECs) control vascular permeability, vessel tone, coagulation, fibrinolysis and inflammatory responses. Changes in EC membrane function induced by reactive oxygen species (ROS) appear to play a key role in the pathogenesis of atherosclerosis (Li and Shah, 2004). Prostacyclin (PGI_2) is produced by ECs and possesses anti-aggregatory and -vasodilatory properties and decreasing polymorphonuclear adhesion in ECs (Hseu et al., 2008a). It has been reported that, in response to oxidative stress, ECs can produce and release eicosanoids like PGI_2 (Cohen and Tong, 2010). Production of PGI_2 by ECs is directly proportional to the severity of oxidative stress and cell damage, which is important for the maintenance of vascular homoeostasis (Egan and FitzGerald, 2006). Interleukin (IL)-1 has also been implicated in the pathogenesis of atherosclerosis (Kleemann et al., 2008). EC produce IL-1 in response to oxidative stress, which stimulates EC release of chemotactic factors and increases the expression of cell surface adhesion molecules important for neutrophil, monocyte and lymphocyte adhesion. IL-1 production also increased in parallel with oxidative stress-induced cell damage (Kleemann et al., 2008).

Localized accumulation of monocytes/macrophages and T lymphocytes in the arterial intima appears to play a key role in early atherogenesis, as well as in plaque rupture in advanced atherosclerotic lesions (Liuzzo et al., 2005). Molecular mechanisms mediating the recruitment of monocytes/macrophages and T lymphocytes into atherosclerotic lesions may depend on multiple and complex processes; however, adhesion of monocytes and T lymphocytes to the vascular endothelium is among the earliest and essential processes during atherogenesis, as well as during inflammatory responses (Liuzzo et al., 2005). This process appears to be mediated by endothelial–leukocyte adhesion molecules expressed on the surface of the vascular endothelium covering atherosclerotic and inflammatory lesions (Blankenberg et al., 2003). These adhesion molecules include vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin, and their expression can be transcriptionally induced by ROS or inflammatory cytokines (Zapolska-Downar and Naruszewicz, 2009).

Free-radical-mediated cell injury may be critically important in various pathological phenomena (Piao et al., 2004). Therefore, antioxidants that prevent damage caused by free radicals are worth of additional investigation. The goal of this investigation was to explore the antioxidant potential of the aqueous leaf extracts of *Toona sinensis* (TS extracts) and gallic acid (3,4,5-trihydroxybenoic acid), with natural purified phenolic component of TS extracts, in terms of protecting human umbilical vein endothelial cells from oxidative damage after exposure to free radical initiator AAPH. The AAPH-induced DNA damage was assayed by comet assay. ELISA was performed to quantify PGI_2, IL-1β, MDA and SOD/catalase activity in AAPH-challanged ECs. Bax, Bcl-2, ICAM-1, VCAM-1, E-selectin, PAI-1 and t-PA was determined by Western blotting analysis using appropriate antibodies. ROS and U937 adhesion was monitored using fluorescence based assay systems. In the present study, 2,2′-azobis-2-amidinopropane dihydrochloride (AAPH), a water-soluble free radical generator, was used to stimulate oxidative stress *in vitro*, and the peroxyl radicals were generated by thermal decomposition of an azo compound in oxygen, eventually causing the oxidation of lipid, protein and DNA in biomolecules (Mayo et al., 2003).

2. Materials and methods

2.1. Chemicals

Fetal bovine serum (FBS), M-199 medium, glutamine, and penicillin–streptomycin–neomycin (PSN) were obtained from GIBCO Laboratories (Grand Island, NY, USA). AAPH [2,2′-azo-bis(2-amidinopropane) hydrochloride] was obtained from Wake pure chemical (Osaka, Japan). PGI_2 immunoassay was obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). IL-1β, VCAM, ICAM, and E-selectin immunoassay was obtained from R&D Systems (Minneapolis, MN, USA). SOD activity kit was obtained from Randox Laboratories Ltd. (Crumlin, UK). Catalase activity kit was obtained from Calbiochem (La Jolla, CA, USA). t-PA and PAI-1 immunoassay was obtained from American diagostica (Stamford, CT, USA). Anti-β-actin, anti-Bax, anti-Bcl-2, anti-ICAM, anti-VCAM, anti-E-selectin, anti-tPA and anti-PAI-1 antibody were obtained from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). All other chemicals were of the highest grade commercially available and supplied either by Merck (Darmstadt, Germany) or Sigma.

2.2. Toona sinensis preparation and extraction

The aqueous extracts of *Toona sinensis* were prepared by adding 1000 mL water to 1000 g fresh *Toona sinensis* leaves and boiling until it was reduced to 100 mL, as previously described (Hsu et al., 2003). The crude extracts were centrifuged at $860 \times g$ 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; the stock (2 mg/mL in double distilled water) was subsequently stored at $-20\,^{\circ}$C until analyzed for antioxidant properties. The crude TS extracts separated from fresh *Toona sinensis* leaves had a yield of 5%, which was based on the initial weight of the crude extracts (Yang et al., 2006).

2.3. Isolation of gallic acid from TS extracts

The 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. Eight compounds (gallic acid, methyl gallate, ethyl gallate, kaempferol, kaempferol-3-O- β-ᴅ-glucoside, quercetin, quercitrin, quercetin-3-O-β-ᴅ-glucoside and rutin) were isolated from the TS extracts, as previously described (Yang et al., 2006). The identity 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., 2006; Hsu et al., 2003). Gallic acid, a major component of leaf extracts of *Toona sinensis* was collected for use in this study at a yield of 6% (Yang et al., 2006; Chia et al., 2010). The stock (5 mg/mL in DMSO) was subsequently stored at $-20\,^{\circ}$C until analyzed for antioxidant properties.

2.4. Cell culture

Human umbilical vein endothelial cells (HUVEC) were prepared from human umbilical veins essentially as described (Hseu et al., 2008a). In brief, the umbilical cord was infused with 0.02% collagenase solution containing the ECs and flushed from the cord by perfusion with cord buffer and then centrifuged. The resulting cell suspension was divided equally between several 10-cm petri dishes, and grown to confluence in M-199 medium and supplemented with 20% FBS and PNS at $37\,^{\circ}$C in 5% CO_2. Upon confluence, the primary culture cells were detached with trypsin–EDTA and subcultured in tissue culture wells at $37\,^{\circ}$C. All experiments were carried out using cells at least 4 days after a single passage only. The cells were removed from each well with 0.4% trypsin and counted

using a hemocytometer. Cell viability (4×10^5 cells/12 wells) were checked after treatment using trypan blue exclusion and examined using phase contrast microscopy.

For all experiments, ECs were incubated with the indicated concentration of TS extracts (0, 50, 75 or 100 μg/mL) or with 5 μg/mL gallic acid for 1 h. The supernatant was removed following supplementation, the ECs were washed with cord buffer and the culture media was replaced with fresh media containing 15 mM AAPH (in PBS, pH 7.4). This reaction mixture was shaken gently while being incubated for 14 h at 37 °C; for the ROS assay, cells were incubated for 2 h. In order to induce free radical chain oxidation in ECs, aqueous peroxyl radicals were generated by thermal decomposition of AAPH (an azo compound) in oxygen (Mayo et al., 2003). The advantages of this method included the fact that AAPH decomposes thermally to generate radicals without biotransformations or enzymes and the rate of radical generation could be easily controlled by adjusting the concentration of the initiator (Mayo et al., 2003).

2.5. Comet assay (single-cell gel electrophoresis assay)

The assay was essentially the same as that described by Sing et al. (1988). The ECs (8×10^5 cells/6 wells) were suspended in 1% low-melting-point agarose in PBS (pH 7.4) and pipetted onto superfrosted glass microscope slides precoated with a layer of 1% normal-melting-point agarose (warmed at 37 °C prior to use). The agarose was allowed to set at 4 °C for 10 min before immersion of the slides in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris [pH 10], 1% Triton X-100) at 4 °C for 1 h in order to remove cellular proteins. Slides were then placed in single rows in a 30 cm wide horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM EDTA (pH 13.4) at 4 °C for 40 min to allow separation of the two DNA strands (alkaline unwinding). Electrophoresis was performed in the unwinding solution at 30 V (1 V/cm) and 300 mA for 30 min. The slides were then washed three times for 5 min each with 0.4 M Tris (pH 7.5) at 4 °C before staining with PI (3 μg/mL). The PI-stained nucleoids were examined under a fluoroescence microscope using a 510–550 nm excitation filter at 200× magnification. The damage was not homogeneous and visual scoring of the cellular DNA on each slide was based on characterization of 100 randomly selected nucleus/nucleoids. DNA damage in the ECs, as DNA strand breaks including double and single-strand variants at alkali-labile sites, was analyzed in an alkaline condition (pH 13.4). Tail Moment: DNA migration from the nucleus of each cell was measured with a computer program using the comet moment parameters as follows: comet moment = $\Sigma 0 \rightarrow n$ [(amount of DNA at distance X) × (distance X)]/total DNA. The above-described procedures are referred to as the standard comet assay. Observation and analysis of the results were always performed by the same experienced person. The analysis was blinded, with the observer having no knowledge of slide identity.

2.6. Enzyme-linked immunosorbent assay for PGI$_2$ and IL-1β metabolites

After the ECs (4×10^5 cells/well) in 12 well plate were incubated with or without TS extracts/gallic acid and AAPH, 500 μL aliquots of supernatant were collected and quantified the PGI$_2$ concentration in culture media by measuring the level of the stable metabolite of PGI$_2$, 6-keto-PGF1α, using an enzyme-linked immunosorbent assay (ELISA) kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Meanwhile, concentrations of IL-1β secreted into the media were quantified using a commercially available ELISA kit (R&D Systems).

2.7. Measurement of ROS generation

Production of intracellular ROS was detected by fluorescence microscopy and flow cytometry using 2′,7′-dihydrofluorescein-diacetate (DCFH-DA). ECs (2×10^5 cells/24 wells) were cultured in M-199 medium, with renewal of the culture medium when the cells reached 80% confluence. Samples were then incubated with 10 μM DCFH-DA in culture medium at 37 °C for 30 min. During loading, the acetate groups on DCFH-DA were removed by intracellular esterase, trapping the probe inside the ECs. After loading, cells were washed with warm PBS. Production of intracellular ROS can be measured by changes in fluorescence due to intracellular production of DCF caused by oxidation of DCFH. Intracellular ROS, as indicated by dichlorofluorescein (DCF) fluorescence, was measured with a fluorescence microscope (Olympus 1× 71).

2.8. Assay for formation of thiobarbituric acid reactive substances (TBARS)

To estimate the generation of malondialdehyde (MDA) equivalents during EC oxidation, the TBARS assay was performed (Nagai et al., 2011). After treatment, ECs (4×10^5 cells/well in 12 well) were mixed with 1.5 mL of 0.67% thiobarbituric (TBA) and 1.5 mL of 20% trichloroacetic acid and heated at 100 °C for 30 min, then the temperature of the reaction product was maintained at 25 °C for 30 min, followed by the incubation samples were centrifuged at $860 \times g$ for 15 min at 4 °C. The reaction product was assayed fluorometrically using an F2000 optical spectrofluorometer (Hitachi Ltd., Tokyo, Japan), with excitation at 515 nm and emission at 553 nm. Freshly diluted tetra-ethoxypropane, which produces MDA, was used as a standard. Results are expressed as pmol of MDA/10^5 cells.

2.9. Measurement of SOD activity in ECs

This procedure is a variation of the classical NBT method (Spitz and Oberley, 1989). ECs were seeded on a 12-well plate with 4×10^5 cells/well. ECs suspension (100 μL) was centrifuged at $860 \times g$ for 2 min, the supernatant was discarded and 35 μL ice water was added for cell disruption. SOD kit (Randox laboratories) was used to measure SOD activity, with xanthine and xanthine oxidase employed to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. SOD activity is measured by the degree of reaction inhibition. The absorbance of samples was measured at 505 nm against a reagent blank. The calculation was performed according to the manufacturer's instructions; SOD activity is expressed as unit/mg protein.

2.10. Catalase activity

To evaluate catalase activity, 4×10^5 cells/well were seeded on a 12-well plate and washed twice with PBS, homogenized and immunoprecipitated with an anti-catalase antibody (Iwai et al., 2003). Catalase activity was measured using a Catalase Assay Kit (Calbiochem) following the manufacturer's protocol. Briefly, catalase protein samples were incubated in the presence of a known concentration of H$_2$O$_2$. After 1 min incubation, the reaction was quenched with sodium azide. Catalase activity was determined by a spectrophotometric assay (520 nm) that measures the rate of dismutation of H$_2$O$_2$.

2.11. U937 adhesion assay

U937 cells were labeled with 10 μg/mL BCECF-AM for 30 min at 37 °C, washed and resuspended in serum-free media. ECs (2×10^5 cells/wells) were cultured in 24 well plate and incubated

with reagents prior to being co-cultured with 1×10^5 cells/mL BCECF-AM-labeled U937 cells for 30 min at 37 °C. Non-adhering U937 cells were removed by gentle aspiration, and wells were washed with PBS. Cells were lysed using 0.1% Triton X-100 in 0.1 M Tris–HCl, pH 7.4, to evaluate U937 adhesion to ECs. Fluorescence was measured using a microplate fluorescence reader with excitation at 510 nm and emission at 531 nm.

2.12. Western blot analysis

ECs (4×10^6 cells/100-mm dish) were detached and 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 sulfonyflouride). The suspension was put on ice for 20 min and then centrifuged at 16,000 × g for 20 min at 4 °C. Total protein content was determined using Bio-Rad protein assay reagent, with bovine serum albumin 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 for 5 min. Equal amounts (20 μg) of the denatured proteins were loaded into each lane, separated on 8% SDS polyacrylamide gel, followed by transfer of the proteins to PVDF membranes in 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 using the SuperSignal ULTRA chemiluminescence substrate from Pierce Biotechnology (Rockford, IL, USA). Band intensities were quantified by densitometry with absorbance of the mixture at 540 nm determined using an ELISA plate reader.

2.13. Assessment of VCAM-1, ICAM-1, E-selectin, PAI-1, and t-PA secretion

ECs were seeded on a 6-well plate using 8×10^5 cells/well, and the cells were pretreated with TS extracts or gallic acid, followed by the addition of AAPH. Cell culture supernatant was collected for evaluation of ICAM-1, VCAM-1 and E-selectin using commercially available kits (R&D systems). Additionally, the level of t-PA and of PAI-1 was determined in the supernatant of ECs cultures using commercially available ELISA kits (American diagostica) followed by the manufacturer's instructions.

2.14. Statistics

Data is presented as mean ± S.D. of at least three independent experiments ($n = 3$). One way ANOVA followed by Dunnett's test were performed to determine statistical differences between groups. Statistical significance was defined as $p < 0.05$ for all tests.

3. Results

In this study, a human umbilical vein endothelial cells (ECs) culture system was used to evaluate the antioxidant effects of the aqueous leaf extracts of *Toona sinensis* (TS extracts) and gallic acid, the natural phenolic component purified from TS extracts, in response to oxidative stress induced by the free-radical generator, AAPH. AAPH was used to stimulate *in vitro* conditions of oxidative stress and the peroxyl radicals were generated by thermal decomposition of an azo compound in oxygen (Mayo et al., 2003). In the presence of oxygen, these radicals can attack membrane polyunsaturated fatty acids and initiate lipid peroxidation chain reactions. As previously reported, the concentration of TS extracts and gal-

lic acid used in these studies was not cytotoxic to ECs (Hseu et al., 2011). Therefore, for all subsequent experiments, we employed the non-cytotoxic concentration of TS extracts (i.e., ≤100 μg/mL) and of gallic acid (5 μg/mL), and focused on the effect of TS extracts or gallic acid on AAPH-induced ECs damage.

3.1. Effect of TS extracts and gallic acid on AAPH-induced cytotoxicity in ECs

In this study, ECs exposed to 15 mM AAPH in M-199 medium for 14 h displayed decreased cell viability as assessed by trypan blue exclusion (Fig. 1A). ECs treated with 50–100 μg/mL TS extracts and 5 μg/mL gallic acid significantly restored cell viability when compared with AAPH alone treatment (Fig. 1A) ($p < 0.05$). Although morphological changes (cell shrinkage) detected by phase-contrast microscopy were evident in the AAPH-exposed cell damage. However, the extent of these changes and cytotoxicity attributable to AAPH exposure were reduced with increasing TS extracts/gallic acid pre-treatment (Fig. 1A). These results clearly indicate that the exposure of ECs to TS extracts/gallic acid confers a significant protective effect against AAPH-induced oxidative damage.

3.2. Effects of TS extracts and gallic acid on DNA damage

To determine whether TS extracts or gallic acid modulates AAPH-induced DNA damage in ECs was examined. The formation of DNA strand breaks (DNA damage) in the cellular DNA was evaluated using single-cell gel electrophoresis (comet assay). As shown in Fig. 1B, treatment with AAPH significantly induced ECs DNA damage after 14 h treatment; however, addition of 50–100 μg/mL TS extracts or 5 μg/mL gallic acid significantly ($p < 0.05$) decreased AAPH-induced DNA damage in ECs.

3.3. Effects of TS extracts and gallic acid on AAPH-induced PGI$_2$ and IL-1β production in ECs

It has been reported that the production of PGI$_2$ and IL-1β is directly proportional to the extent of AAPH-induced oxidative stress and cell damage (Mayo et al., 2003). Bear this in mind; further we monitored AAPH-induced PGI$_2$ and IL-1β production in cultured ECs. Results obtained from this study showed that ECs incubated with AAPH alone (15 mM) dramatically increased PGI$_2$ and IL-1β production compared with control cells (Fig. 2A and B). AAPH-induced PGI$_2$ and IL-1β production was significantly ($p < 0.05$) inhibited by TS extracts and gallic acid in a dose-dependent manner with an IC$_{50}$ value of 73 and 56 μg/mL of TS extracts, respectively (Fig. 2A and B). Furthermore, cells were pre-incubated with 100 μg/mL of TS extracts and 5 μg/mL gallic acid alone, the PGI$_2$ or IL-1β levels were maintained at a background level similar to the untreated samples (data not shown). These findings provided evidence that AAPH-mediated oxidative stress and resulting injury in ECs was significantly diminished by TS extracts/gallic acid treatment. Our data demonstrating that increased PGI$_2$ and IL-1β production by ECs in response to AAPH exposure was positively and negatively correlated with cell damage and TS extracts/gallic acid concentration, respectively.

3.4. Effects of TS extracts and gallic acid on AAPH-induced ROS production and MDA formation in ECs

ROS can cause deleterious oxidative damage to cellular components and a loss of ECs function (Li and Shah, 2004). To investigate the participation of oxidative stress in AAPH-treated ECs, intracellular ROS generation was monitored. Fluorescence microscopic analyses using DCFH-DA as a fluorescence probe demonstrated
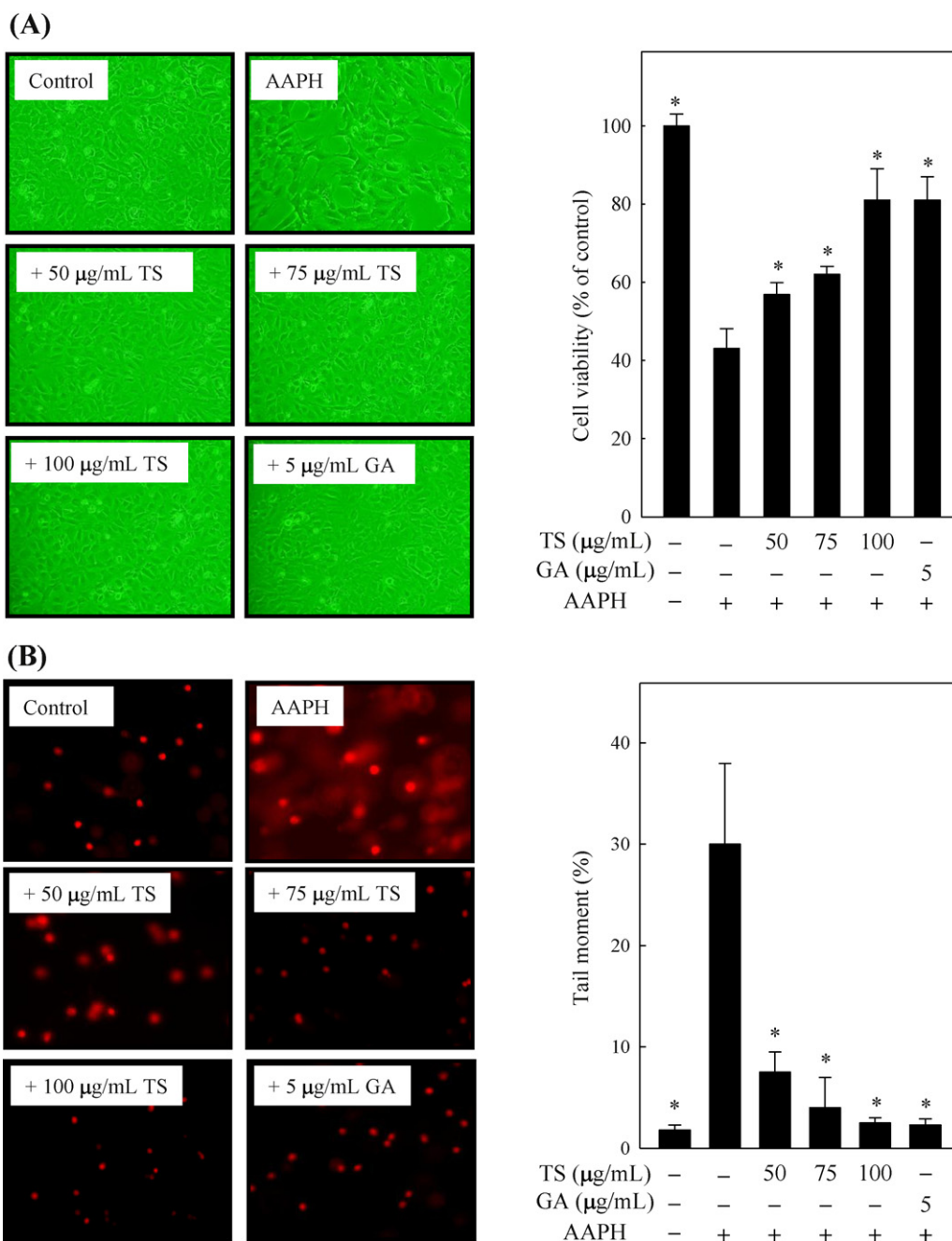
Fig. 1. TS extracts and gallic acid inhibited AAPH-induced cytotoxicity (A) and DNA damage (B) in ECs. Cells were treated with TS extracts (50–100 μg/mL) and/or gallic acid (5 μg/mL) in the absence or presence of AAPH (15 mM) for 14 h. (A) Viable cell number in cultures was monitored by counting cell suspensions using a hemocytometer (200× magnification). (B) DNA damage was assessed using an alkaline comet assay as reported in Section 2. Tail Moment of each cell was measured with a computer program using the comet moment parameters. Results are the mean ± SD of three assays; * indicates significant differences ($p < 0.05$) relative to AAPH groups. TS: TS extracts; GA: gallic acid.

that incubation of ECs with 15 mM AAPH caused a significant increase in fluorescence 2 h after treatment. However, the AAPH-induced increase of ROS generation was significantly ($p < 0.05$) inhibited by exposure to TS extracts (50–100 μg/mL) and/or gallic acid (5 μg/mL) in a concentration-dependent manner (Fig. 3A).

Oxidative stress is associated with the peroxidation of cellular lipids, which can be determined by measuring MDA. As shown in Fig. 3B, MDA level in the culture media was significantly increased with response to AAPH-exposure; when cells were incubated with TS extracts (50–100 μg/mL) and gallic acid (5 μg/mL), the elevated MDA formation was significantly decreased onto basal level ($p < 0.05$). These findings provide positive evidence that both TS extracts and gallic acid treatment significantly inhibited AAPH-

induced oxidative stress in ECs by the reduction of intracellular ROS accumulation and MDA elevation.

3.5. Effects of TS extracts and gallic acid on SOD and catalase activity in AAPH-induced ECs

Biological systems protect themselves against the damaging effects of activated ROS by several means, including free radical scavengers, chain reaction terminators and enzymes such as SOD and catalase (Gutteridge and Halliwell, 2006). Therefore, the effects of TS extracts and gallic acid on AAPH-induced SOD depletion were evaluated in ECs. As shown in Fig. 4A, AAPH exposure reduced the SOD activity in ECs and this reduction was significantly ($p < 0.05$)
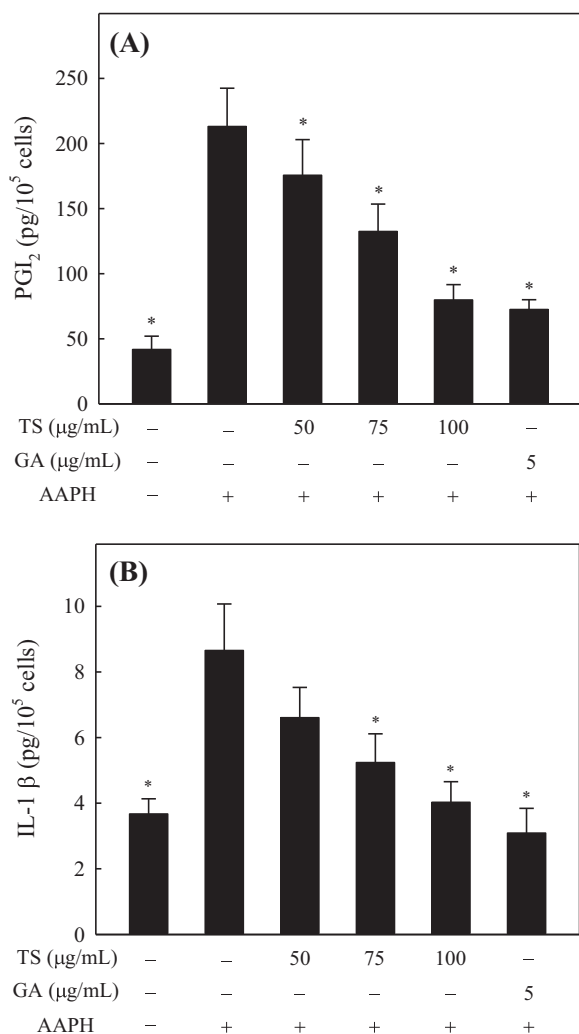
Fig. 2. Inhibitory effects of TS extracts and gallic acid on PGI$_2$ (A) and IL-1β (B) production in ECs exposed to AAPH. Cells were harvested after incubation with TS extracts (50–100 μg/mL) and gallic acid (5 μg/mL) in the absence or presence of AAPH (15 mM) for 14 h. PGI$_2$ and IL-1β concentrations were measured in the culture media using a commercial ELISA kit. Results are the mean ± SD of three assays; * indicates significant differences ($p < 0.05$) relative to AAPH groups.

augmented by the treatment of TS extracts or gallic acid in a dose-dependent manner with an IC$_{50}$ value of 25 μg/mL. Catalase is a key component of the antioxidant defense system and inhibition of this protective mechanism results in enhanced sensitivity to free radical induced cellular damage (Sampathkumar et al., 2005). As shown in Fig. 4B, treatment with AAPH significantly decreased catalase activity in ECs with an IC$_{50}$ value of 35 μg/mL. However, pre-treatment with TS extracts (75 or 50–100 μg/mL) and gallic acid (5 μg/mL) increased the catalase activity in AAPH-induced ECs ($p < 0.05$). The present study reveals that there is an increase of SOD and catalase activity suggesting that TS extracts/gallic acid have a protective effect in response to ROS. Therefore, administration of TS extracts/gallic acid increases the SOD and catalase activity in AAPH-induced EC damage, thus preventing the accumulation of excessive free radicals and protecting ECs from AAPH.

3.6. Effects of TS extracts and gallic acid on AAPH-induced Bax and Bcl-2 protein expression

It has been shown that the Bcl-2 family of proteins has an important regulatory role in apoptosis, both in activation (Bax) and inhibition (Bcl-2) of apoptosis; the Bax/Bcl-2 protein ratio is rec-

ognized as a key factor in the regulation of the apoptotic process (Yang et al., 2006). Therefore, we studied the effects of TS extracts (75 μg/mL) and gallic acid (5 μg/mL) on Bcl-2 and Bax protein levels in AAPH-treated ECs. Cells incubated with AAPH for 14 h caused an increase in Bax protein and a decrease in Bcl-2 protein, which was significantly ($p < 0.05$) reversed by TS extracts and gallic acid (Fig. 5). Analysis of our data indicates that TS extracts or gallic acid may prevent the dysregulation of Bax/Bcl-2, and thereby lead to up-regulate anti-apoptotic signals in ECs.

3.7. Effects of TS extracts and gallic acid on AAPH-activated U937 adhesion

To explore the effects of TS extracts and gallic acid on endothelial cell–leukocyte interactions, we examined adhesion of U937 cells, a monocyte cell line, to AAPH-activated ECs under static conditions. Unstimulated confluent ECs exhibited minimal binding to U937; however, U937 adhesion was substantially increased when ECs were incubated with AAPH alone (Fig. 6A and B). Concurrent incubation of ECs confluent with TS extracts (50–100 μg/mL) and gallic acid (5 μg/mL) dose-dependently inhibited U937 adhesion to AAPH-activated ECs ($p < 0.05$) (Fig. 6 A and B).

3.8. Effects of TS extracts and gallic acid on AAPH-induced adhesion molecule expression/secretion in ECs

To examine the effect of TS extract and gallic acid exposure on adhesion molecule expression/secretion, ECs were treated with the indicated concentration of TS extracts (50, 75 and 100 μg/mL) or gallic acid (5 μg/mL) in serum-free medium. ECs were exposed to 15 mM AAPH for 14 h, and then evaluated ICAM-1, VCAM and E-selectin expression and secretion by Western blotting and ELISA analysis, respectively. Results revealed that AAPH-induced increases in the expression and secretion of ICAM-1, VCAM-1 and E-selectin were reduced by TS extracts (100 μg/mL) and gallic acid (5 μg/mL) pre-treatment in a statistically significant ($p < 0.05$) manner (Fig. 7A and B).

3.9. Effects of TS extracts and gallic acid on PAI-1 and t-PA expression/secretion from ECs

Since the balance between PAI-1 and t-PA determines fibrinolytic activity (Matsumoto and Horie, 2011). Therefore, we also investigated the effects of indicated concentration of TS extracts (50, 75 and 100 μg/mL) or gallic acid (5 μg/mL) on the production of PAI-1 and t-PA from ECs. ECs were exposed to 15 mM AAPH for 14 h, and then evaluated PAI-1 and t-PA expression and secretion by Western blotting and ELISA, respectively. Results showed that incubation of ECs with AAPH led to increase PAI-1 level (Fig. 8A). Notably, AAPH also promoted t-PA depletion in ECs (Fig. 8B). TS extract and gallic acid treatment significantly inhibited AAPH-induced increase in PAI-1 and reduction in t-PA (Fig. 8A and B). Therefore, TS extracts/gallic acid treatment significantly inhibited AAPH-induced disruption of the balance between PAI-1 and t-PA, which may decrease EC coagulation and fibrinolysis.

4. Discussion

There is evidence that an injury to arterial endothelial cells reflects one of the key events in the initiation and evolution of atherosclerosis (Kim et al., 2007). It has been shown that antioxidants protect cells against free radical-induced oxidative damage in several *in vitro* models including ECs (Hseu et al., 2008a). In our previous study, we concluded that TS extracts and gallic acid, a major component of TS extracts, possesses effective antioxidant activity, which includes scavenging of free and superoxide anion
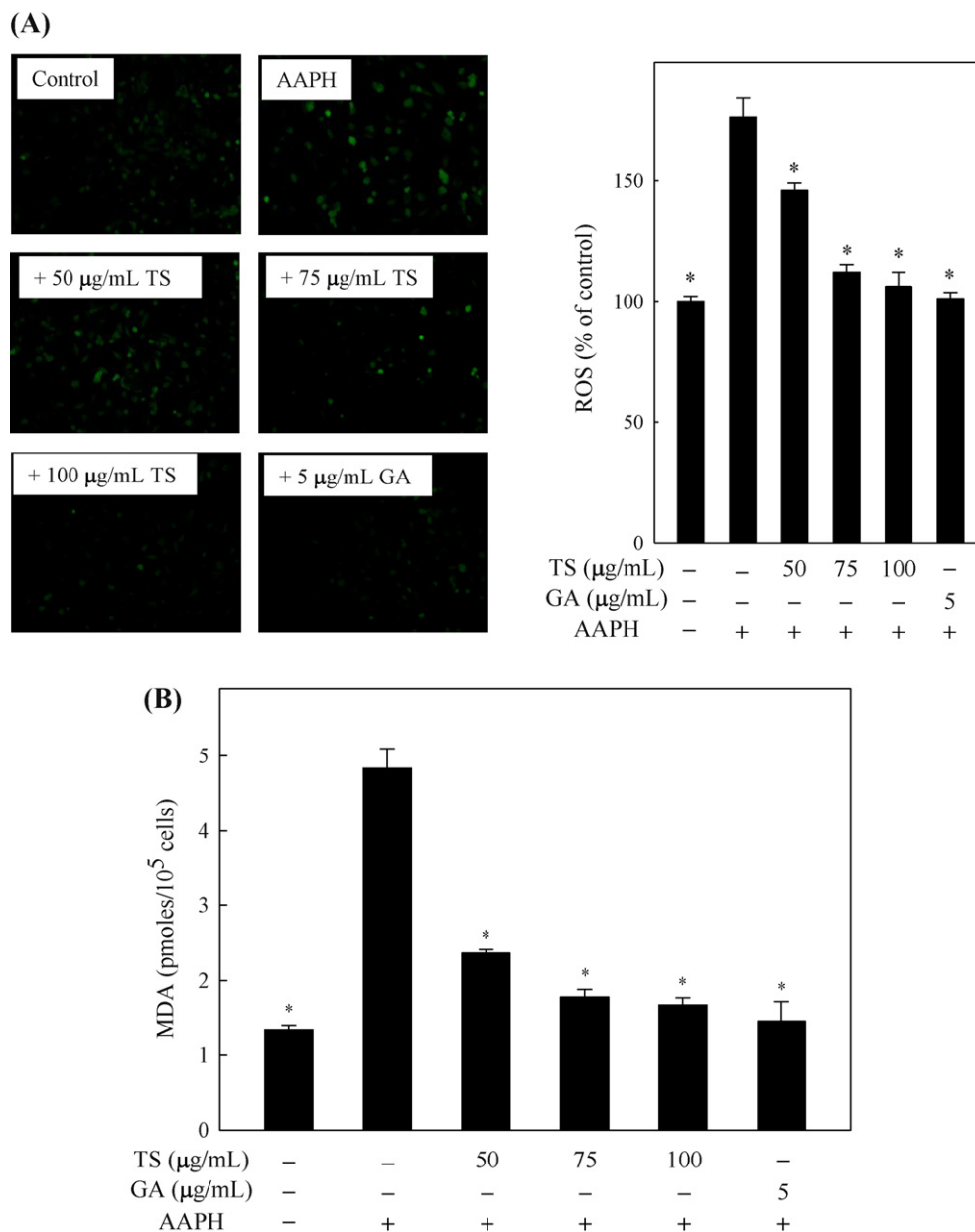
Fig. 3. TS extracts and gallic acid attenuated AAPH-induced ROS generation (A) and MDA formation (B) in ECs. (A) Cells were treated with TS extracts (50–100 μg/mL) and gallic acid (5 μg/mL) in the absence or presence of AAPH (15 mM) for 2 h. The intracellular ROS level, was indicated by DCF fluorescence, was measured by fluorescence microscopy (200× magnification). (B) MDA production was measured by the TBARS assay as described in Section 2. Results are the mean ± SD of three assays; * indicates significant differences ($p < 0.05$) relative to AAPH groups.

radicals, and greater reducing power and metal chelating activity (Hseu et al., 2008b). Further, supplementation with TS extracts and gallic acid appears to reduce $CuSO_4$, AAPH- and SNP (sodium nitroprusside)-induced oxidative modification of LDL and AAPH-induced erythrocyte hemolysis (Hseu et al., 2008b). In the present work, we demonstrate that both TS extracts and gallic acid are able to inhibit the AAPH-induced oxidative damage in human umbilical vein endothelial cells. The free radicals generated from AAPH react with oxygen molecules rapidly to yield peroxyl radicals. The lipid peroxyl radicals attack other lipid molecules to form lipid hydroperoxide and new lipid radicals. This reaction takes place repeatedly resulting in attacks on various biological molecules and the production of physiochemical alterations and cellular damage (Yokozawa et al., 2000). It has been reported that AAPH intoxication results death of vascular endothelial cells in various organs and caused atherosclerosis, ischemia-reoxygeneration injury and

inflammatory diseases (Yokozawa et al., 2000). Therefore, AAPH-intoxication experiment may be a promising assay system for the quantitative study of cellular response to oxidative stress and biological activities of antioxidants (Yokozawa et al., 2000). Based on these findings, it seems reasonable to suggest that TS extracts and gallic acid protects ECs from free radical-induced oxidative damage, further reducing the risk of free radical-related diseases and atherosclerosis.

ROS can trigger signal transduction pathways in ECs that lead to an increase in the expression of cytokines, such as PGI_2 and IL-1β, which are known to play important roles in atherosclerotic pathogenesis (Mayo et al., 2003). Prostacyclin (PGI_2) is produced by ECs and possesses anti-aggregatory and vasodilatory properties, decreasing polymorphonuclear adhesion to ECs *in vitro* (Hseu et al., 2008a). Under physiological conditions, the secretion of PGI_2 and IL-1β are extremely low in endothelial cells. However, in
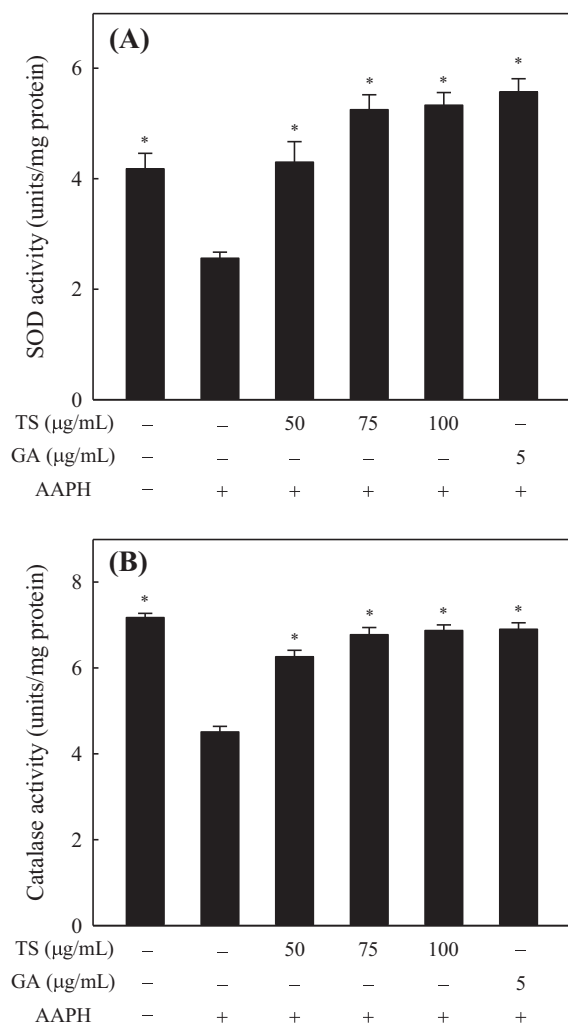
Fig. 4. Inhibitory effects of TS extracts and gallic acid on AAPH-induced SOD (A) and catalase (B) activity in ECs. Cells were harvested after incubation with TS extracts (50–100 μg/mL) and gallic acid (5 μg/mL) in the absence or presence of AAPH (15 mM) for 14 h. SOD or catalase activity was determined using a spectrophotometric assay (520 nm or 505 nm) as reported in materials and methods. Results are the mean ± SD of three assays; '*' indicates significant differences ($p < 0.05$) relative to AAPH groups.

Fig. 5. Effects of TS extracts and gallic acid on AAPH-induced Bcl-2 and Bax expression in ECs. Cells were harvested after incubation with TS extracts (75 μg/mL) and gallic acid (5 μg/mL) in the absence or presence of AAPH (15 mM) for 14 h. Protein (20 μg) from each sample was resolved on a 8% SDS-PAGE, and western blot was performed. A typical result from three independent experiments is shown. Relative changes in protein bands were measured using densitometric analyses with the control being 100% as shown just below the gel data. Results are the mean ± SD of three assays; * indicates significant differences ($p < 0.05$) relative to AAPH groups.

response to ROS/oxidative stress or injury, PGI$_2$ and IL-1β production were dramatically increases in ECs, thereby increasing adhesion of circulating monocytes and lymphocytes to the activated ECs at the site of injury (Renier et al., 2003). Thus, IL-1β secretion by vascular cells appears to be intimately involved in monocyte recruitment and adhesion, concomitant with the production of other cytokines and growth factors, which are crucial events in the initiation and progression of the atheromatous plaque (Singh et al., 2002). Therefore, when the cells were enriched with antioxidants, oxidative stress and resultant injury were significantly diminished. In congruence with these lines, our results also showed that AAPH-induced PGI$_2$ and IL-1β production in ECs were significantly inhibited by TS extracts/gallic acid in a dose-dependent manner (Fig. 2). These inhibitory effects of TS extracts/gallic acid on PGI$_2$ and IL-1β production may contribute to the effect of *Toona sinensis* at reducing the risk of free radical-related diseases and atherosclerosis.

ROS-induced endothelial dysfunction plays an important role in the initiation and development of vascular disease (Hseu et al., 2008a). Mount of scientific literatures indicate that antioxidant administration improves endothelial function (Siow and Mann,
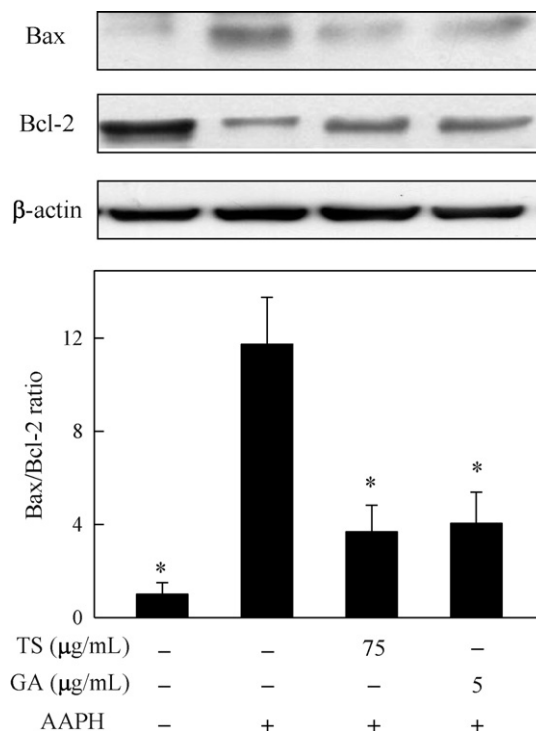
2010; Farbstein et al., 2010). ROS can cause deleterious oxidative damage to cellular components and a loss of ECs function (Li and Shah, 2004). Notably, ROS produce DNA lesions such as base modifications, single-strand breaks, double strand breaks, and the cross-linking of bases in living cells (Valko et al., 2004). DNA damage in the cells can be repaired, although the repair may be error-prone with low fidelity leading to mutations, genomic instability, and cell death through apoptotic mechanisms (Shen, 2011). Therefore, the presented data provides convincing evidence that TS extracts and gallic acid can provide protection from AAPH-induced intracellular ROS and DNA damage in ECs, which may play an important role in the prevention of endothelial dysfunction and atherosclerosis *in vitro*.

Lipid peroxidation in biological systems has long been thought of as a toxicological phenomenon that results in pathological consequences (Yokozawa et al., 2000). It has been well documented that AAPH enhanced lipid peroxidation in cellular systems (Yokozawa et al., 2000). Consistence with these views, we measured the lipid peroxidant, MDA, to determine the antioxidant function of TS extracts and gallic acid against AAPH-induced cellular damage in ECs. Results also reveled that, pretreatment with TS extracts and gallic acid decreased AAPH-induced MDA formation in ECs. Thus TS extracts/gallic acid preventing the accumulation of excessive free radicals and protecting the ECs from AAPH-derived cellular injury.

SOD and catalase is a key component of the antioxidant defense system. Inhibition of this protective mechanism results in enhanced sensitivity to free radical-induced cellular damage (Gutteridge and Halliwell, 2006). The SOD converts superoxide radicals (O_2^-) into H_2O_2 plus O_2, thus participating with other antioxidant enzymes in the enzymatic defense against oxygen
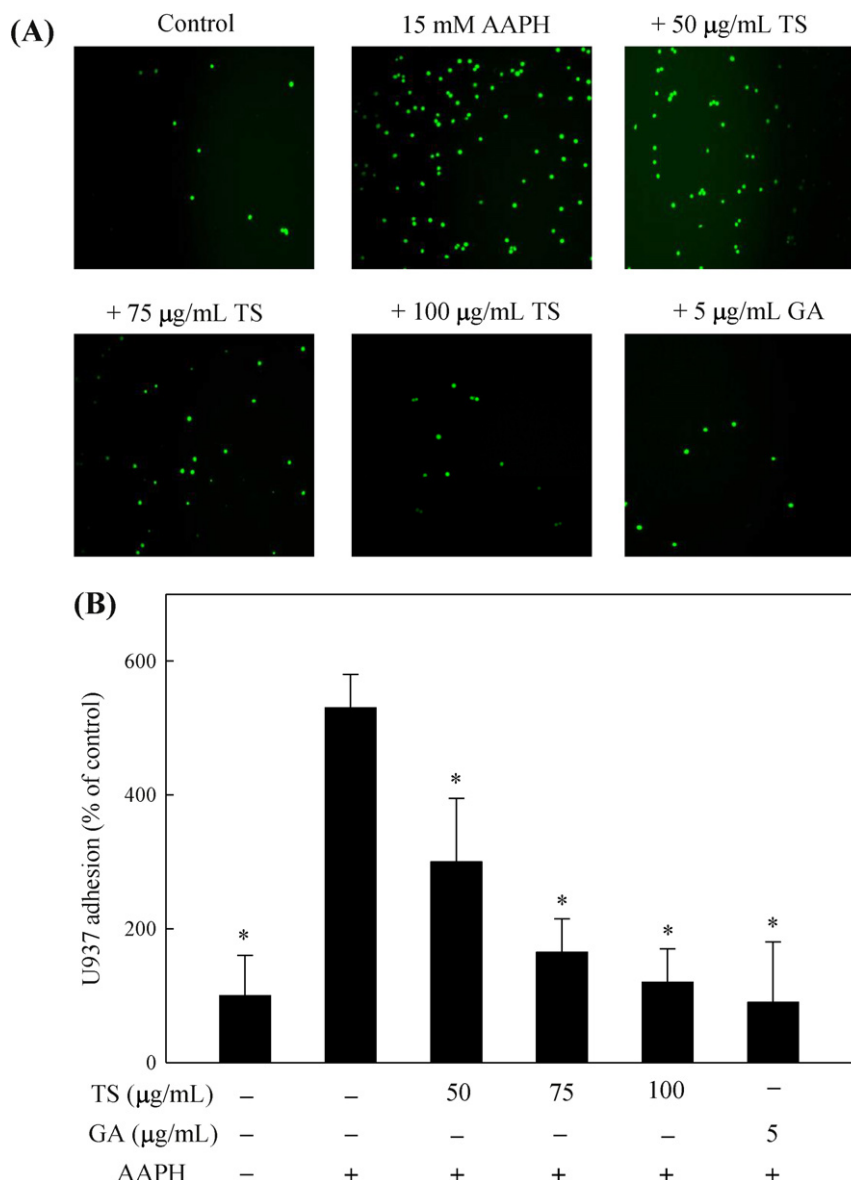
Fig. 6. TS extracts and gallic acid inhibits AAPH-induced U937 cell adhesion to ECs. ECs were harvested after incubation with TS extracts (50–100 μg/mL) and gallic acid (5 μg/mL) in the absence or presence of AAPH (15 mM) for 14 h. (A) After ECs were washed, BCECF-AM-labeled U937 cells were added to each well and cultures were incubated for an additional hour at 37 °C. (B) The level of U937 cells bound to ECs (shown as a percentage of the control), indicated by BCECF-AM fluorescence, was measured by fluorescence microscopy (200× magnification). Results are the mean ± SD of three assays; * indicates significant differences ($p < 0.05$) relative to AAPH groups.

toxicity. Further, the reduction in the activity of catalase may, therefore, result in a number of deleterious effects due to the accumulation of O_2^- and H_2O_2 (Sampathkumar et al., 2005). Our present data demonstrates that the AAPH-treatment dramatically depleted endogenous SOD/catalase levels in ECs. Pretreatment with TS extracts/gallic acid significantly ($p < 0.05$) augmented AAPH-depleted SOD/catalase levels in ECs. Thus, the potential elevation of SOD/catalase activity by *Toona sinensis* is accordance with the inhibition of AAPH-induced oxidative damage, thus, providing effective protection from oxidative modification of ECs.

Growing evidence demonstrates that the Bcl-2 family plays an important regulatory role in apoptosis, either as activators (Bax) or inhibitors (Bcl-2) (Yang et al., 2006). Endothelial apoptosis is a physiological process that contributes to vessel homeostasis by eliminating damaged cells from the vessel walls (Harrod et al., 2006). If increased, endothelial apoptosis may lead to disturbed endothelial function, which may promote atherogenesis and thrombosis (Stoneman and Bennett, 2004). Many of the agents that induce apoptosis are oxidants or stimulators of cellular oxidative metabolism, whereas antioxidants have been shown to prevent apoptosis in some systems (Qin et al., 2001). Recent studies reveled that Bcl-2 protein is also responsible for cellular defense against oxidative stress. In addition, activation of Bcl-2 can inhibit lipid peroxidation. Therefore it has been supposed that Bcl-2 act as free radical scavenger (Murakoshi et al., 2003). These evidences indicate that the anti-apoptotic Bcl-2 protein associated with mitochondrial membrane integrity by preventing cytochrome *c* release, caspase activation, Bax redistribution, and apoptosis (Er et al., 2006). In this study, we also observed that AAPH-treatment suppressed Bcl-2 and increased Bax protein levels in ECs. However, AAPH-induced Bax/Bcl-2 dysregulation are significantly reversed by TS extracts/gallic acid pretreatment in ECs. The results concluded that TS extracts/gallic acid reduces apoptosis in ECs, possibly via it ability to up regulate Bcl-2 and down regulate Bax expression. Therefore, *Toona sinensis* may have valuable antioxidant properties.
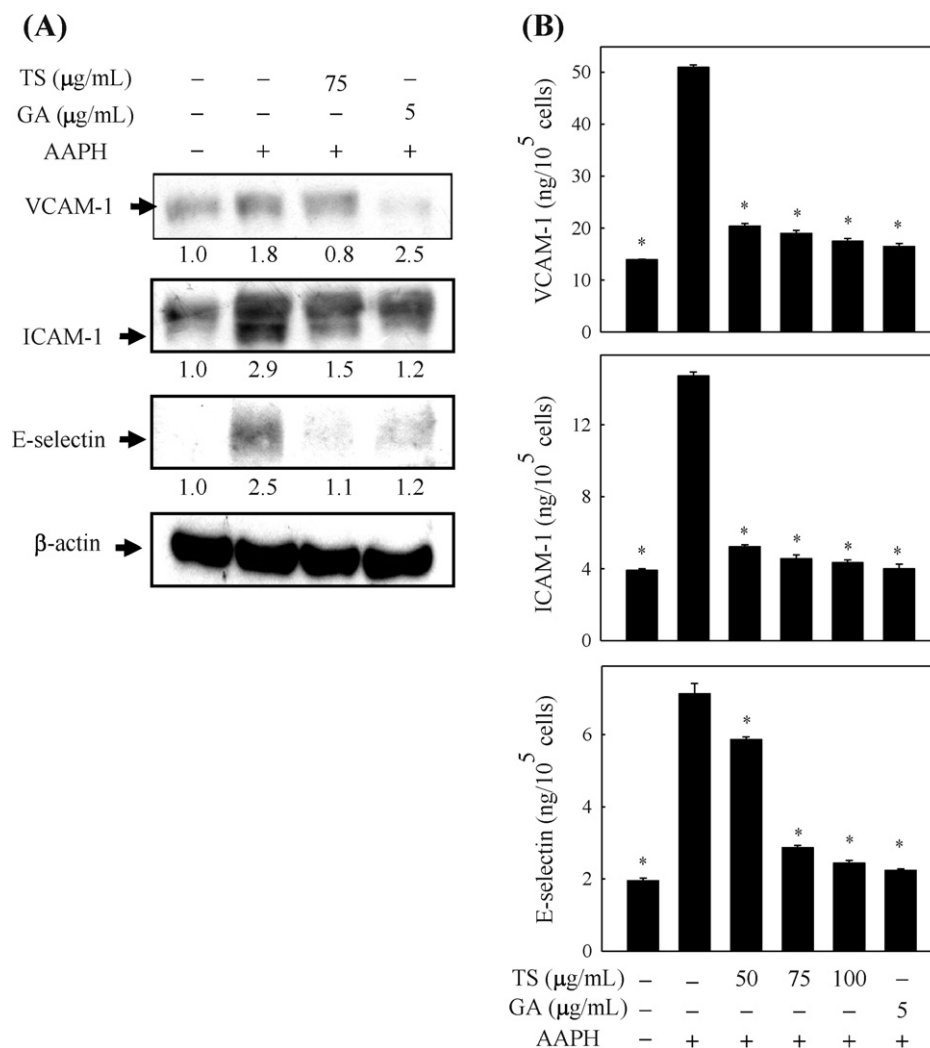
Fig. 7. TS extracts and gallic acid suppressed AAPH-induced VCAM-1, ICAM-1, and E-selectin expression (A) and secretion (B) in ECs. Cells were harvested after incubation with the indicated concentration of TS extracts and gallic acid in the absence or presence of AAPH (15 mM) for 14 h. (A) Immuno blotting was performed against anti-VCAM-1, ICAM-1 and E-selectin. Relative changes in protein bands were measured using densitometric analysis with the control being 100% as shown just below the gel data. (B) The supernatants were collected for analyses of VCAM-1, ICAM-1 and E-selectin concentration using ELISA. Results are the mean $\pm$ SD of three assays; * indicates significant differences ($p < 0.05$) relative to AAPH groups.

Leukocyte adhesion to arterial endothelial cells is thought to be an important step in the development of atherosclerosis (Matsumoto et al., 2002). It is well known that adhesion molecules are strong predictors of atherosclerotic lesion development and further onset of cardiovascular events (Blankenberg et al., 2003). These include both secreted molecules such as the chemokine family of chemoattractant cytokines and surface-expressed cell adhesion molecules of the selectin and immunoglobulin families (Kim et al., 2007). Our present data demonstrates that TS extracts and gallic acid dose-dependently decreased the AAPH-induced adhesion of U937 cells to the endothelial cells. Furthermore, TS extracts and gallic acid significantly diminished both expression and secretion levels of ICAM-1, VCAM-1, and E-selectin in AAPH-challenged ECs. Thus, TS extracts and gallic acid can down regulate VCAM-1, ICAM-1, and E-selectin associated with reduced adhesion of leukocytes, which are playing major role in the prevention of atherosclerosis and inflammatory responses.

Vascular ECs also produce many modulatory proteins, such as PAI-1 and t-PA, which regulate coagulation and fibrinolysis (Cheng et al., 2008). PAI-1, a 50 kDa glycoprotein secreted by various cells including endothelial cells, hepatocytes, platelets, and smooth muscle cells (Binder et al., 2002). Increased local expression of PAI-1 is observed in restenosis and in atherosclerotic plaques (Binder et al., 2002). *In vitro*, PAI-1 plays a critical role in the regulation of fibrinolysis and serving as a primary inhibitor of t-PA, which is responsible for the intravascular plasminogen activation (Cheng et al., 2008). Endogenous t-PA release from the endothelium regulates the dissolution of intravascular thrombus, and is a critical determinant of cardiovascular outcome (Cheng et al., 2008). The balance between PAI-1 and t-PA is known to control the development of thrombosis. In the present study, we observed that AAPH treatment modulates balance between PAI-1 and t-PA in ECs to become procoagulative and hypofibrinolytic. Interestingly, TS extracts/gallic acid treatment significantly inhibited the AAPH-induced up-regulation of PAI-1 and down-regulation of t-PA expression in ECs, which may reduce ECs coagulation and fibrinolysis.

Natural products, including plants, provide rich resources for food and 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, were isolated from the
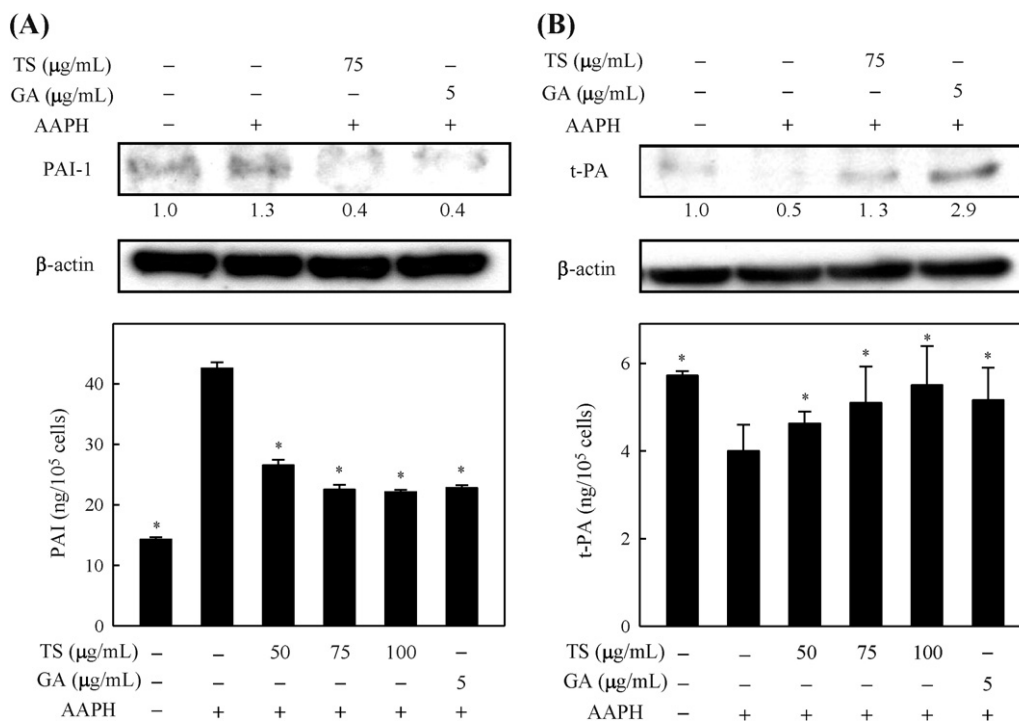
Fig. 8. Inhibitory effects of TS extracts and gallic acid on AAPH-induced PAI-1 (A) and t-PA (B) expression and secretion in ECs. Cells were harvested after incubation with the indicated concentration of TS extracts and gallic acid in the absence or presence of AAPH for 14 h. Immuno blotting was performed against anti-VCAM-1, ICAM-1 and E-selectin. Relative changes in protein bands were measured using densitometric analysis with the control being 100% as shown just below the gel data. The supernatants were collected, and the content of PAI-1 and t-PA were analyzed by ELISA. Results are the mean ± SD of three assays; * indicates significant differences ($p < 0.05$) relative to AAPH groups.

leaves of *Toona sinensis*; identity of the compounds was determined by HPLC and based on the analogous information reported in the literature (Hsu et al., 2003; Yang et al., 2006). The total phenolic content of TS extracts was estimated to be 130 mg ± 26 mg gallic acid (pyrocatechol) equivalents/g of plant extracts (Yang et al., 2006). The yield of gallic acid, the natural phenolic component purified from TS extracts, was about 6% (Yang et al., 2006). Although it remains unclear which of the components of *Toona sinensis* are active compounds, in recent years much attention has been focused for polyphenols regarding their biological activates, especially the antioxidant activity of gallic acid (Ow and Stupans, 2003). This study also agreement with previous reports showing antioxidant properties of gallic acid (Hsieh et al., 2004; Hseu et al., 2008b). 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. 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. These results imply that gallic acid is one of the active compounds responsible for the inhibition of free radical-induced ECs damages *in vitro*. Further bioassay-directed fractionations leading to the identification and purification of the compounds responsible for the antioxidant effects are warranted.

In conclusion, the present study revealed that *Toona sinensis* supplementation reduced AAPH-induced oxidative modification in ECs. Based on these findings, it seems reasonable to suggest that the antioxidant properties of *Toona sinensis* protect vascular endothelial cells from oxidative stress. Our results also contribute towards the validation of the traditional use of *Toona sinensis* in the treatment of cardiovascular diseases. However, further *in vivo* experiments are critically warranted to confirm these promising results.

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