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Anti-inflammatory activities of tormentic acid from suspension cells of *Eriobotrya Japonica ex vivo* and *in vivo*

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Abstract

Anti-inflammatory effects of tormentic acid (**TA**) were investigated *ex vivo* and *in vivo*. **TA** decreased the paw edema at the 4th and 5th hour after λ-carrageenin (Carr) administration, and increased the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) in the liver tissue. **TA** also significantly attenuated the thiobarbituric acid reactive substances (TBARS) level in the edematous paw at the 5th hour after Carr injection. **TA** decreased the nitric oxide (NO) levels on the serum level and diminished the serum tumor necrosis factor (TNF-α) at the 5th hour after Carr injection. Western blotting revealed that the **TA** decreased Carr-induced inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expressions. As the results, the anti-inflammatory mechanisms of **TA** might be correlated to the decrease in the level of TBARS, iNOS, and COX-2 in the edema paw via increasing the activities of CAT, SOD, and GPx in the liver.

KEY WORDS: tormentic acid; anti-inflammation; TBARS; NO; TNF-*α*.

1. Introduction

Eriobotrya japonica Lindl is a rosaceous evergreen tree distributed in southeastern China and Taiwan. Astringent leaves of loquat have been used as nutrition supplements for chronic bronchitis, coughs, phlegm, high fever and gastroenteric disorders. Five main bioactive constituents of the leaf were characterized spectroscopically as tormentic acid (**TA**), maslinic acid, corosolic acid, oleanolic acid and ursolic acid. **TA** was also found in strawberry fruit (Hirai et al., 2000), the leaves of *Perilla frutescens* (Banno et al., 2004) and olive (Saimaru, Orihara, Tansakul, Kang, Shibuya & Ebizuka 2007). **TA** has been reported to exhibit anticancer, anti-inflammatory, and anti-atherogenic properties, and reduced vascular smooth muscle cell proliferation (Fogo, Antonioli, Calixto & Campos, 2009). TA also has a hypoglycemic effect in normoglycemic and glucose-induced hyperglycemic rats by increasing secretion of insulin (Ivorra, Paya & Villar 1988). However, little information is available on the anti-inflammatory effects of **TA**.

Inflammation leads to up-regulation of a series of enzymes in affected areas. Inducible nitric oxide synthase (iNOS) catalyzes the formation of nitric oxide (NO) from L-arginine (Lin & Tang, 2008). High concentration of NO are found to play important roles in inflammation and carcinogenesis. iNOS can be induced by bacterial endotoxic lipopolysaccahride (LPS), interferon-γ (IFN-γ), and a variety of pro-inflammatory cytokines (Wang, Zhou & Lin, 2011).

Inflammatory effect induced by λ-carrageenin (Carr) could be associated with free radicals, prostaglandin (PGI_2 and PGD_2) and NO which will be released when administrating with Carr. Edema effect reaches peak at the 3rd h and malondialdehyde (MDA), the major TBARS component was due to free radical attack plasma membrane

(Janero, 1990). Therefore, we examined the anti-inflammatory effects of **TA** on LPS-induced in RAW264.7 cells and Carr-induced edematous paw in mice. And we detected the levels of iNOS and COX-2 in either RAW264.7 cell or edematous paw. The activities of CAT, SOD and GPx in the liver at the 5^{th} h after Carr injection were also investigated to understand the relationship between the anti-inflammatory mechanism of **TA** and antioxidant enzymes.

2. Materials and methods

2.1. Chemicals

LPS, Carr and indomethacin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TNF-α was purchased from Biosource International Inc. (Camarillo, CA, USA). Anti-iNOS, anti-COX-2, and anti-β-actin antibodies (Santa Cruz, CA, USA) were obtained as indicated.

2.2. Isolation of TA callus induction and suspension cultures

Sterilized seeds of *E. japonica* (Department of Bio-industry and Agribusiness Administration, Taichung, Taiwan) were placed on the Murashige–Skoog (MS) basal medium containing 3% (w/v) sucrose and 0.3% (w/v) gelrite. One month later, the leaves were excised (2-3 mm) from seedings and placed on MS medium supplemented with 2.5 mg 6-benzyladenine and 1 mg 1-naphthalenacetic acid for callus induction. All medium were adjusted to pH 5.8 before autoclaving for 20 min at 121℃ and callus were subculture every 20 days at 25 ± 2 ℃ in dark.

2.3. Extraction and isolation of tormentic acid from suspension cells of *E. japonica*

The suspension cells were extracted with ethanol. The filtrate was concentrated under reduced pressure to yield a brown ethanol extract which was sonicated with 50% methanol, then kept at 4℃ for 24 h to remove aqueous soluble substance. The insoluble substance sonicated with 85% methanol at room temperature to remove insoluble substance. The filtrate was concentrated under reduced pressure to afford the white powder fraction. The white powder fraction (0.5g) was chromatographic on a reverse silica gel column (LiChroprep RP-18, E. Merck, 40-63 μm) using MeOH/H_2O gradient and then further purified by preparative high performance liquid chromatography (YMC, J'Sphere series ODS-H80, 10 x 250 mm, 85 % MeOH (v/v), 3 mL /min) to yield **TA**. ^1H spectra showed the identical data as reported (Taniguchi et al., 2002).

2.4. **Animals**

Male ICR mice (6-8 weeks) were obtained from the BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). The animals were kept in plexiglass cages at a constant temperature of 22 ± 1℃, and relative humidity of 55 ± 5 % with 12 h dark-light cycle for at least 2 weeks before the experiment. Animals were given food and water *ad libitum*. All experimental procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.5. **Cell culture**

A murine macrophage cell line RAW264.7 was purchased from Food Industry

Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in plastic dishes containing Dulbecco's Modified Eagle Medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma) in a CO_2 incubator (5% CO_2) at 37℃.

2.6. **Cell viability**

Cells (2×10^5) were cultured in 96-well plate containing DMEM supplemented with 10% FBS for 1 day. Then cells were cultured with **TA** in the presence of 100 ng/mL LPS for 24 h. Then , the cells were washed twice with DPBS and incubated with 100 μL of 0.5 mg/mL MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) for 2 h at 37℃ for cell viability. The medium was then discarded and 100 μL dimethyl sulphoxide (DMSO) was added. After 30-min incubation, absorbance at 570 nm was read using a microplate reader.

2.7. **Measurement of Nitric oxide/Nitrite**

Nitrite, a stable end product of NO, was then measured using the Griess reaction (Chang et al., 2009). 100 μL aliquots of sample mixed with 100 μL of Griess reagent (0.1% N-(1-naphthyl) ethylenediamide dihydrochloride, 1% sulfanilamide in 5% phosphoric acid), followed by spectrophotometric measurement at 550 nm. Nitrite concentrations in the supernatants were determined by comparison with a sodium nitrite standard curve.

2.8. **Carr-induced Edema**

Carr-induced hind paw edema model was used to determine anti-inflammatory activity (Chang et al., 2009). Animals were i.p. treated with **TA**, Indo or normal saline, 30 min prior to injection of 1% Carr (50 μL) in the plantar side of right hind paws of the mice. The paw volume was measured immediately after Carr injection at 1-5 h intervals using a plethysmometer (model 7159, Ugo Basile, Varese, Italy). The degree of swelling induced was evaluated by the ratio a/b, where a and b was the volume of the right hind paw after and before Carr treatment. Indo was used as a positive control. After 5 h, the animals were sacrificed and the Carr-induced edema paw were dissected and stored at -80 ℃. Also, blood were withdrawn and kept at -80 ℃. The protein concentration of the sample was determined by the Bradford dye-binding assay.

2.9. **Lipid Peroxidation assay**

Determination of lipid peroxidation from Carr-induced edematous paw by the TBARS was used an index (Chang et al., 2009). The amount of TBARS formed during the incubation was assessed by adding 1.5% thiobarbituric acid and then heating at 95℃ for 45 min. After cooling, the samples were centrifuged, and the absorbance of TBARS in the supernatant was measured at 532 nm. The levels of lipid peroxidation are expressed in terms of TBARS nmol /mg protein.

2.10. **Measurement of Serum TNF-α levels**

Serum levels of TNF-α were determined using a commercially available ELISA kit according to the manufacturer's instruction. TNF-α was determined from a standard curve. The concentrations were expressed as pg/mL.

2.11. **Antioxidant Enzyme Activity Measurements**

Total SOD activity was determined by the inhibition of cytochrome *c* reduction (Flohe and Otting, 1984). The reduction of cytochrome *c* was mediated by superoxide anions generated by the xanthine/xanthine oxidase system and monitored at 550 nm. One unit of SOD was defined as the amount of enzyme required to inhibit the rate of cytochrome *c* reduction by 50%. Total CAT activity was based on that of Aebi (Aebi, 1984). In brief, the reduction of 10 mM H_2O_2 in 20 mM of phosphate buffer (pH 7.0) was monitored by measuring the absorbance at 240 nm. The activity was calculated using a molar absorption coefficient, and the enzyme activity was defined as nanomoles of dissipating hydrogen peroxide per milligram protein per minute. Total GPx activity in cytosol was determined according to Paglia and Valentine's method (Paglia and Valentine, 1967). The enzyme solution was added to a mixture containing hydrogen peroxide and glutathione in 0.1 mM Tris buffer (pH 7.2) and the absorbance at 340 nm was measured. Activity was evaluated from a calibration curve, and the enzyme activity was defined as nanomoles of NADPH oxidized per milligram protein per minute.

2.12. **Western blot Analysis**

Total protein was extracted with a RIPA solution (radioimmuno-precipitation assay buffer) at -20℃ overnight. We used BSA (bovine serum albumin) as a protein standard

to calculate equal total cellular protein amounts. Protein samples (30 μg) were resolved by denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using standard methods, and then were transferred to PVDF membranes by electroblotting and blocking with 1% BSA. The membranes were probed with the primary antibodies (iNOS, COX-2, and β-actin) at 4℃ overnight, washed three times with PBST, and incubated for 1 h at 37 ℃ with horseradish peroxidase conjugated secondary antibodies. The membranes were washed three times and the immunoreactive proteins were detected by enhanced chemiluminescence (ECL) using hyperfilm and ECL reagent (Amersham International plc., Buckinghamshire, U.K.). The results of Western blot analysis were quantified by measuring the relative intensity compared to the control using Kodak Molecular Imaging Software and represented in the relative intensities.

2.13. **Histological Examination**

For histological examination, paws were dissected 5 h following the interplanetary injection of Carr. The tissue slices were fixed (1.85% formaldehyde, 1% acetic acid) for 1 week at room temperature, dehydrated by graded ethanol and embedded in Paraffin. Sections (5 μm) were deparaffinized with xylene and stained with hematoxylin and eosin (H&E) stain. All samples were observed and photographed with BH-2 Olympus microscopy. Every 3-5 tissue slices were randomly chosen from Carr, Indo and **TA**-treated (2.5 mg/kg) groups. Histological examination of these tissue slices revealed an excessive inflammatory response with massive infiltration of neutrophils [ploymorphonuclear leukocytes (PMNs)] by microscopy. The numbers of neutrophils were counted in each scope (400 x) and thereafter obtain their average count from 5

scopes of every tissue slice.

2.14. **Statistical Analysis**

Data are expressed as mean ± standard error of the mean (SEM). Statistical evaluation was carried out by one-way analysis of variance (ANOVA followed by Scheffe's multiple range tests). Statistical significance is expressed as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3. Results

3.1. **Cell Viability**

The effect of **TA** on RAW264.7 cell viability was determined by a MTT assay. Lower concentration of **TA** showed no effects in cell viability in the presence of 100 ng/mL LPS incubation for 24 h, however, 5 μg/mL **TA** significantly inhibited the cell viability.

3.2. **Effect of TA on LPS-induced NO Production in Macrophages**

When RAW264.7 macrophages treated with different concentrations of **TA** and LPS for 24 h, **TA** significantly inhibited nitrite production (Fig. 1C) and showed in a concentration-dependent manner.

3.3. **Inhibition of LPS-induced iNOS and COX-2 Protein by TA**

Results showed that incubation with **TA** and LPS for 24 h inhibited iNOS (53.2%) and COX-2 (47.6%) protein expression in mouse macrophage RAW264.7 cells in a dose-dependent manner (Fig. 2A) (Fig. 2B).

3.4. **Effects of TA on Carr-induced Mice Paw Edema**

TA (2.5 mg/kg) significantly inhibited ($p < 0.05$ or $p < 0.01$) the development of paw edema induced by Carr after 4 and 5 h of treatment (Fig. 3A). Indo (10 mg/kg) significantly decreased the Carr induced paw edema after 4 and 5 h of treatment ($p < 0.001$).

3.5. **Effects of TA on the TBARS, NO, and TNF-α levels**

The TBARS, NO, and TNF-α level increased significantly at the 5th h after Carr injection ($p < 0.001$), however, decreased TBARS level in the edematous paw (Fig. 3B) and NO level in serum (Fig. 3C) significantly by treatment with 2.5 mg/kg **TA** ($p < 0.001$) as well as 10 mg/kg Indo. And, **TA** (1.25 or 2.5 mg/kg) decreased the TNF-α level in serum at the 5th h after Carr injection ($p < 0.01$ or $p < 0.001$), as well as 10 mg/kg Indo (Fig. 3D).

3.6. **Effects of TA on activities of Antioxidant Enzymes**

At the 5th h after the intrapaw injection of Carr, liver tissues were analyzed for biochemical parameters such as CAT, SOD, and GPx activities (Table 1). CAT, SOD and

GPx activities in liver tissue were decreased significantly by Carr administration, however significantly increased after treated with 2.5 mg/kg **TA** and 10 mg/kg Indo (P<0.01) (Table 1).

3.7. **Effects of TA on Carr-induced iNOS and COX-2 protein expressions in Mice Edema Paw.**

Results showed that injection of **TA** (2.5 mg/kg) inhibited iNOS (55.8%) and COX-2 (68.8%) proteins expression in Carr-induced mouse paw edema at the 5^{th} h (Fig. 4A, and 4B). However, 10 mg/kg Indo showed an average down-regulation of iNOS (72.4%) and COX-2 (75.7%), respectively (Fig. 4B).

3.8. **Histological Examination.**

Paw biopsies of animals treated with **TA** (2.5 mg/kg) showed a reduction in Carr-induced inflammatory response. Inflammatory cells were reduced and confined to near the vascular areas. Intercellular spaces did not show any cellular infiltrations. Collagen fibers were regular in shape and showed a reduction of intercellular spaces. Moreover, the hypoderm connective tissue was not damaged (Fig. 5). Neutrophils increased with Carr treatment (P < 0.01). Indo and **TA** (2.5 mg/kg) could significantly decrease the neutrophils numbers compared to the Carr-treated group (P < 0.001 or P < 0.01) (Fig. 5E).

Discussion

Presently, more than 80 different triterpenoid structures have been identified in plants,

and an exponential increase in the number of reports regarding bioactive triterpenoids reflects their growing importance as sources of food and preventive medicines. In fact, some are increasingly being used for food or medicinal purposes for a variety of diseases in many countries as antioxidative (Somova, Nadar, Rammanan & Shode, 2003), anti-inflammatory (Mix, Mengshol, Benbow, Vincenti, Sporn & Brinckerhoff, 2001), hepatoprotective effects (Liu, Hartley & Liu, 1998), anti-tumour (Zou et al., 2004) and anti-diabetes effects (Chen et al., 2006). The formation of antifungal compounds (euscaphic acid, **TA,** and myrianthic acid) was observed in unripe strawberry which had been wounded and inoculated with conidia of *C. musae*. (Hirai et al., 2000). In the present study, we demonstrated anti-inflammatory activities of **TA** in *ex vivo* and *in vivo* systems. Dual inhibitory activities against iNOS as shown in *in vitro* assays appear to confer on **TA** a potent *in vivo* efficacy in mouse, Carr-induced, paw edema, comparable with a potent and well known COX inhibitor, Indo, suggesting its potential therapeutic usage as a novel topical anti-inflammatory source of healthy food.

Excessive production of NO plays a critical role in the aggravation of circulatory shock and chronic inflammatory diseases, such as septic shock, inflammatory hepatic dysfunctions, inflammatory lung disease and colitis (Huang & Ho, 2010). As many of these conditions exhibit rapid onset and development, often resulting in the failure of conventional anti-inflammatory therapies and extremely high mortality rates, a simultaneous suppression of NO production pathways, as shown by **TA**, may satisfy the control of the rapid progression of the inflammatory process.

The Carr test is highly sensitive to non-steroidal anti-inflammatory drugs, and has long been accepted as a useful phlogistic tool for investigating new drug therapies

(Spector and Willoughb, 1963). The degree of swelling of the Carr-injected paws was maximal 3 h after injection. Statistical analysis revealed that **TA** and Indo significantly inhibited the development of edema 4 h after treatment. They both showed anti-inflammatory effects in Carr-induced mice edematous paw.

This local acute inflammation model induces a biphasic edema consisting of an early phase (up to 2 h) followed by a more sustained late phase (2–6 h). The early phase of Carr edema is related to the production of inflammation mediators such as histamine, bradykinin, leukotrienes and cyclo-oxygenase products in the inflamed tissue. The late phase is related to neutrophil infiltration and the production of ROS. In a number of pathophysiological conditions associated with inflammation or oxidant stress, these ROS have been proposed to mediate cell damage via a number of independent mechanisms including the initiation of lipid peroxidation, the inactivation of a variety of antioxidant enzymes. In the studies of the mechanism on the inflammation, L-arginine–NO pathway has been proposed to play an important role in the Carr-induced inflammatory response (Cuzzocrea, Zingarelli, Calapai, Nava & Caputi, 1997). Our present results also confirm that Carr-induced paw edema model results in the production of NO. The expression of the inducible isoform of NO synthase has been proposed as an important mediator of inflammation (Lin & Tang, 2008). In our study, the level of NO was decreased significantly by treatment with **TA.** We suggest the anti-inflammatory mechanism of **TA** may be through the L-arginine–NO pathway because **TA** significantly inhibits the NO production.

TNF-α is a major mediator in inflammatory responses, inducing innate immune responses by activating T cells and macrophages, and stimulating secretion of other

inflammatory cytokines (Liao, Guo & Lin, 2011). Also, TNF-α is a mediator of Carr-induced inflammatory incapacitation, and is able to induce the further release of kinins and leukotrienes, which is suggested to have an important role in the maintenance of long-lasting nociceptive response (Dawson, Sedgwick, Edwards & Lees, 1991). In this study, we found that **TA** decreased the TNF-α level in serum after Carr injection.

The Carr-induced inflammatory response has been linked to neutrophils infiltration and the production of neutrophils-derived free radicals, such as hydrogen peroxide, superoxide and hydroxyl radicals, as well as the release of other neutrophils-derived mediators (Dawson et al., 1991). Some researches demonstrate that inflammatory effect induced by Carr is associated with free radical. Free radical, prostaglandin and NO will be released when administrating with Carr for 1-6 h. The edema effect was raised to the maximum at 3 h. TBARS production is due to free radical attack plasma membrane. Thus, inflammatory effect would result in the accumulation of TBARS. Glutathione (GSH) is a known oxyradical scavenger. Enhances the level of GSH conducive toward favor reduces TBARS production. Endogenous GSH plays an important role against Carr-induced local inflammation (Chaturvedi, 2008). In this study, there is significantly increased in CAT, SOD, and GPx activities with **TA** treatment. Furthermore, there are significantly decreases in TBARS level with **TA** treatment. We assume the suppression of TBARS production is probably due to the increases of CAT, SOD, and GPx activities.

In conclusion, these results suggested that **TA** possessed anti-inflammatory effects. The anti-inflammatory mechanism of **TA** may be related to iNOS and it is associated with the increase in the activities of antioxidant enzymes (CAT, SOD, and GPx). **TA** may be used as a pharmacological agent in the prevention or treatment of disease in which free

341 radical formation is a pathogenic factor.

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