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Antioxidant, Analgesic, and Anti-inflammatory activities of the ethanolic extracts of *Taxillus liquidambaricola*

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1. **Introduction**

Inflammation is recognized as a biological process in response to tissue injury. At the injury site, an increase in blood vessel wall permeability followed by migration of immune cells can lead edema formation during inflammation. However, excessive inflammation contributes to many acute and chronic human diseases (Rao et al., 2007). Inflammatory response is characterized by the abundant productions of nitric oxide (NO) and prostaglandin E2 (PGE_2), and of cytokines, such as tumor necrosis factor α (TNF-α), and thus, these pro-inflammatory mediators are important anti-inflammatory targets (Sheeba, and Asha 2009). Lipopolysaccharide (LPS) is an endotoxin and a constituent of the outer membrane of gram-negative bacteria. LPS stimulates innate immunity, by regulating the productions of inflammatory mediators, like, NO, TNF-α, Interleukin-6, prostanoids, and leukotrienes (Liu, et al., 2007). And in the animal the inflammation model of a carrageenan (Carr) induced edema is usually used to assess the contribution of natural products in resisting the biochemical changes associated with acute inflammation. Carr can induce acute inflammation beginning with infiltration of phagocytes, the production of free radicals as well as the release of inflammatory mediators (Salvemini et al., 1996).

Intracellular antioxidant mechanisms against these inflammatory stresses involve antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT) and

glutathione peroxidase (GPx) in tissues. Recently, it has been shown that faulty cellular antioxidant systems cause organisms to develop a series of inflammatory and cancer diseases (Valko et al., 2006). However, it appears that the various roles of enzymatic antioxidants help to protect organisms from excessive generation of oxidative stress in the inflammatory process, which has triggered studies focusing on the role of natural products in suppressing the production of oxidation by increasing enzymatic antioxidants in tissues (Huang et al., 2011).

Taxillus liquidambaricola (Hayata) Hosok, a parasitic plant that attacks the plant, which is called “Sang Ji Sheng” in Taiwan, and the whole plant (stems and leaves) has been traditionally used for the treatment of rheumatoid arthralgia, threatened abortion and hypertension and also been applied as an anti-obesity herbal medicine (Wang et al., 2008). Although *Taxillus liquidambaricola* has showed some physiological effects, there are no studies focusing on its inhibitory effects on the antioxidant, analgesic activities, and the mechanism of anti-inflammatory activities of the ethanolic extracts of *Taxillus liquidambaricola* (ETL) in cell and animal models. Consequently, the objective of the present study is to determine the therapeutical effects of ETL against antioxidant, analgesic, and anti-inflammatory activities.

2. **Materials and methods**

2.1. Materials

Lipopolysaccharide (LPS, *Escherichia coli* O127:B8), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), *N*-(1-naphthyl) ethylenediamine dihydrochloride, sulfanilamide, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), thiobarbituric acid (TBA), 3-[4,5-dimethyl-thiazol- 2-yl]-2,5-diphenyl tetrazolium bromide (MTT), λ-Carrageenan (Carr), indomethacin (Indo), quercetin and other chemical reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). Plant materials were collected from Taichung country in Taiwan. They were identified and authenticated by Dr. Yuan-Shiun Chang, Professor, School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, College of Pharmacy, China Medical University.

2.2. Preparation of the extracts of plant materials

Dried sample of ETL (100 g) was macerated with 1L ethanol for 24 h at room temperature. Filtration and collection of the extract was done three times. The filtrates were collected, concentrated with a vacuum evaporator until the volume was below 10 mL and then freeze-dried. The yield obtained was 6.2 % (w/w).

2.3. Fingerprint chromatogram of ETL extracts by HPLC

The chromatographic system consisted of a Qaternary Gradient Pump SFD 2100, a SFD 5200 autosampler, a Merck LiChrospher 100 RP-18e column (5 μm, 4.0 I.D.×250 mm) and a S-3210 photodiode-array detector (PDA) (Schambeck SFD GmbH, Bad Honnef, Germany). Peak area was calculated by using a Schambeck HPLC-GPC-Software in the computer integrator. The samples were analyzed by HPLC on a Lichrospher 100 RP-18e column and they were detected at 360 nm with methanol / 0.5% phosphate solution (50: 50, *v/v*) as the mobile phase at a flow rate of 1.0 mL/min.

To the first sample (unhydrolyzed), 0.1 g of ETL was dissolved in 1 mL of LC-grade methanol and filtered through ultra membrane filter (pore size 0.45 μm; Millipore) before HPLC analysis. The second sample (hydrolyzed), 0.1 g of ETL, was hydrolysis for 60 min in the presence of 8 mL 2% H_2SO_4 at 100℃ heated in water bath, efficiently released quercetin from quercetin glycosides, and partitioned five times with 20 mL ethyl acetate. The ethyl acetate portions were combined, filtered and then concentrated by a rotary evaporator and the residues dissolved in 1 mL of LC-grade methanol and filtered through ultra membrane filter before HPLC analysis. The analysis processes included separation, hydrolysis and structure elucidation of

quercetin and quercetin glycosides. The separation of quercetin was carried out by solvent partition and high performance liquid chromatography (HPLC). For the identification of quercetin, photodiode-array detection was used.

2.4. In vitro antioxidant activities of crude extracts

2.4.1. Determination of antioxidant activity by DPPH radical scavenging ability

The effects of crude extracts and positive controls (BHT) on DPPH radicals were estimated according to the method of Huang et al., (2006). Aliquot (20 µL) of crude extracts at various concentrations were each mixed with 100 mM Tris-HCl buffer (80 µL, pH 7.4) and then with 100 µL of DPPH in ethanol to a final concentration of 250 µM. The mixture was shaken vigorously and left to stand at room temperature for 20 min in the dark. The absorbance of the reaction solution was measured spectrophotometrically at 517 nm. The percentages of DPPH decolorization of the samples were calculated according to the equation: % decolorization = [1- (ABS_{sample} /$ABS_{control}$)] ×100. EC_{50} value was the effective concentration at which DPPH radicals were scavenged by 50% and was obtained by interpolation from linear regression analysis.

2.4.2. Determination of antioxidant activity by $ABTS^{\cdot+}$ scavenging ability

The $ABTS^{\cdot+}$ scavenging ability was determined according to the method of Huang et al., (2006). Aqueous solution of ABTS (7 mM) was oxidized with potassium peroxodisulfate (2.45 mM) for 16 hrs in the dark at room temperature. The $ABTS^{\cdot+}$ solution was diluted with 95% ethanol to an absorbance of 0.75 ± 0.05 at 734 nm (Beckman UV-Vis spectrophotometer, Model DU640B). An aliquot (20 µL) of each sample (125 µg/mL) was mixed with 180 µL $ABTS^{\cdot+}$ solution and the absorbance was read at 734 nm after 1 min. Trolox was used as a reference standard.

2.5. Determination of total polyphenol content

The total polyphenol contents of crude extracts were determined according to the method of Huang et al (2008). 20 µL of each extract (125 µg/mL) was added to 200 µL distilled water and 40 µL of Folin-Ciocalteu reagent. The mixture was allowed to stand at room temperature for 5 min and then 40 µL of 20 % sodium carbonate was added to the mixture. The resulting blue complex was then measured at 680 nm. Catechin was used as a standard for the calibration curve. The polyphenol content was calibrated using the linear equation based on the calibration curve. The total polyphenol content was expressed as mg catechin equivalence (CE)/g dry weight.

2.3. Cell culture

A murine macrophage cell line RAW 264.7 (BCRC No. 60001) was purchased from the Bioresources Collection and Research Center (BCRC) of the 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, USA) in a CO_2 incubator (5% CO_2 in air) at 37℃ and subcultured every 3 days at a dilution of 1:5 using 0.05% trypsin–0.02% EDTA in Ca^{2+}-, Mg^{2+}- free phosphate-buffered saline (DPBS).

2.3.1. Cell viability.

Cells (2 x 10^5) were cultured in 96-well plates containing DMEM supplemented with 10% FBS for 1 day to become nearly confluent. Then cells were cultured with samples in the presence of 100 ng/mL LPS for 24 hrs. After that, the cells were washed twice with DPBS and incubated with 100 μL of 0.5 mg/mL MTT for 2 hrs at 37℃ testing for cell viability. The medium was then discarded and 100 μL dimethyl sulfoxide (DMSO) was added. After 30-min incubation, absorbance at 570 nm was read using a microplate reader.

2.3.2. Measurement of Nitric oxide/Nitrite

Nitrite levels in the cultured media and serum, which reflect intracellular NO synthase activity, were determined by Griess reaction (Huang et al., 2007). The cells

were incubated with samples in the presence of LPS (100 ng/mL) at 37℃ for 24 hrs. Then, cells were dispensed into 96-well plates, and 100 μL of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride and 5% phosphoric acid) and incubated at room temperature for 10 min. Using sodium nitrite to generate a standard curve, the concentration of nitrite was measured form absorbance at 540 nm.

2.4. Animals

This study was conducted in conformity with the policies and procedure details in the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 86–23 1985) and was approved by the ethics committee of the Institutional Animal Care and Use Committee (IACUC) of China Medical University, Taichung, Taiwan. ICR strain male mice (6−8 weeks old) were obtained from BioLASCO Taiwan Co., Ltd., Taipei, Taiwan. The animals were housed in an environmentally controlled room (temperature 22 ± 1 ℃; relative humidity 55 ± 5%; 12 h dark–light cycle). They were given food and water *ad libitum*.

After a 2-week adaptation period, male ICR mice (18-25 g) were randomly assigned to five groups (n=6) of the animals in acetic acid-induced writhing (1%, 0.l mL/10 g i.p.) and formalin-induced licking (5%, 20 μL/per mice i.p.) experiments.

These include a pathological model group (received acetic acid or formalin), a positive control (acetic acid or formalin + Indo), and ETL administered groups (acetic acid or formalin+ ETL: 0.25, 0.5, and 1.0 g/Kg). In the Carr-induced edema experiment, there were randomly assigned to six groups (n=6) of the animals in the study. The control group receives normal saline (i.p.). The other five groups include a Carr-treated, a positive control (Carr + Indo) and ETL administered groups (Carr + ETL: 0.25, 0.5, and 1.0 g/Kg).

2.4.1. Acetic acid-induced writhing response

After a 2-week adaptation period, male ICR mice (18 to 25 g) were randomly assigned to six groups (n = 8) including a normal control, an Indo positive control and four ETL-treated groups. Control group received 1% acetic acid (10 mL/Kg body weight) and the positive control group received Indo (10 mg/Kg, i.p.) 25 min before intraperitoneal injection of 1% acetic acid (10 mL/Kg body weight). ETL-treated groups received ETL (0.25, 0.5, and 1.0 g/Kg, p.o.) 55 min before intraperitoneal injection of 1% acetic acid (10 mL/Kg body weight). Five minutes after the i.p. injection of acetic acid, the number of writhing during the following 10 minutes was recorded (Huang et al., 2010).

2.4.2. Formalin test

The antinociceptive activity of the drugs was determined using the formalin test (Dubuisson and Dennis, 1977). Control group received 5% formalin. Twenty micro-liter of 5% formalin was injected into the dorsal surface of the right hind-paw 60 min after administration of ETL (0.25, 0.5, and 1.0 g/Kg, p.o.) and 30 min after administration of Indo (10 mg/Kg, i.p.). The mice were observed for 30 min after the injection of formalin, and the amount of time spent licking the injected hind paw was recorded. The first 5 min post formalin injection is referred to as the early phase and the period between 15 min and 40 min as the late phase. The total time took licking or biting the injured paw (pain behavior) was measured with a stop watch. The activity was recorded in 5 min intervals.

2.4.3. Determination of carrageenan (Carr) induced edema

Carr-induced hind paw edema model was used for determination of anti-inflammatory activity (Winter et al., 1962). After a 2-week adaptation period, male ICR mice (18 to 25 g) were randomly assigned to five groups (n = 6) including Carr, positive Indo control and three ETL-treated groups. Carr group received 1% Carr (50 µL). ETL at doses of 0.25, 0.5, and 1.0 g/Kg were orally administered 2 hrs before the injection with 1% Carr (50 µL) in the plantar side of right hind paws of the

mice. And Indo (10 mg/Kg) was intraperitoneally administered 90 min before the injection with 1% Carr (50 μL) in the plantar side of right hind paws of the mice. Paw volume was measured immediately after Carr injection at 1, 2, 3, 4, and 5 h intervals using a plethysmometer (model 7159, Ugo Basile, Varese, Italy). The degree of swelling induced was evaluated by *a* minus *b*, where *a* was the volume of the right hind paw after Carr treatment and *b* was the volume of the right hind paw before Carr treatment. Indo was used as a positive control.

In the later experiment, the right hind paw tissue was taken at the 5^{th} h. The right hind paw tissue was rinsed in ice-cold normal saline, and immediately placed in cold normal saline four times their volume and homogenized at 4 ℃. Then the homogenate was centrifuged at 12,000×*g* for 5 min. The supernatant was obtained and stored at −20 ℃ refrigerator for MDA and the antioxidant enzymes (CAT, SOD, and GPx) activities assays.

2.4.4. Determination of tissue lipid peroxidation

MDA was evaluated by the thiobarbituric acid reacting substances (TRARS) method (Ohishi et al., 1985). Briefly, MDA reacted with thiobarbituric acid in the acidic high temperature and formed a red-complex TBARS. The absorbance of TBARS was determined at 532 nm.

2.4.6. Measurement of tumor necrosis factor (TNF-α) in serum

Serum levels of TNF-α were determined using a commercially available ELISA kit (Biosource International, Inc., Camarillo, CA) according to the instructions of the manufacturer. TNF-α was determined from a standard curve.

2.4.7. Determination of antioxidant enzyme activity in paw tissue

The following biochemical parameters were analyzed to check the protective activity of ETL by the methods given below. 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 estimation was based on the previously reported (Armstrong & Browne, 1994). In brief, the reduction of 10 mM H_2O_2 in 20 mM of phosphate buffer (pH 7) was monitored by measuring the absorbance at 240 nm. The activity was calculated by 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 as previously reported (Flohe & Gunzler, 1984). 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. The protein concentration of the tissue was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, CA).

2.4.8. Histological examination

For histological examination, biopsies of paws were taken 5^{th} hrs following the interplanetary injection of Carr. The tissue slices were fixed in (1.85% formaldehyde, 1% acetic acid) for 1 week at room temperature, dehydrated by graded ethanol and embedded in Paraffin (Sherwood Medical). Sections (thickness 5 μm) were deparaffinized with xylene and stained with H & E stain. All samples were observed and photographed with BH2 Olympus microscopy. Every 3~5 tissue slices were randomly chosen from Carr, Indo and ETL treated (1.0 g/Kg) groups. The numbers of neutrophils were counted in each scope (400 x) and thereafter obtain their average count from 5 scopes of every tissue slice.

2.4.9. Protein Lysate Preparation and Western blot Analysis of iNOS and COX-2

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.

2.5. Statistical analysis

Data are expressed as mean ± S.E. Statistical evaluation was carried out by one-way analysis of variance (ANOVA followed by Scheffe's multiple range test).

Statistical significance is expressed as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3. Results

3.1 Fingerprint Analysis by HPLC

To establish the fingerprint chromatogram for the quality control of ETL, quercetin was used as markers. Quercetin rarely occurs in the free state but usually present as O-glycosides and are linked to sugars like glucose, galactose or rhamnose. Determination of quercetin presented as glycosides and aglycone forms in ETL for quality assessment. To accurately determinate the quercetin and quercetin glycosides, the glycosyl groups on the quercetin glycosides should be removed by acid hydrolysis and converted to quercetin (aglycone) before HPLC. An optimized HPLC-DAD technique was employed. According to the plot of peak-area ratio (y) vs. concentration (x, μg/mL), the regression equations and correlation coefficient (r) was $y = 0.094x + 0.033$ (r^2=0.9992). Fig. 1 shows HPLC fingerprint chromatograms of quercetin (Fig. 1A), ETL (Fig. 1B), and ETL after acid hydrolysis (Fig. 1C). Quercetin component has been identified as quercetin by the retention time (29.8 min) and UV absorbance of purified standard. The extract without hydrolysis contained only traces of free quercetin (10.5 μg/g dry weight). The content of quercetin after hydrolysis in ETL was consistently high (126.6 μg/g dry weight) and comparable with the content before hydrolysis. The aglycone form of quercetin accounts for only about 8% of the total quercetin content. A significant amount of quercetin was found in hydrolyzed ETL while only low content of free quercetin was detected in original

samples, suggesting that quercetin exists in combined forms.

3.1. The contents of phytochemicals extracted and the antioxidant activities of ETL.

Plants containing polyphenols have been reported to possess strong antioxidant activities (Hung et al., 2006). The results showed that ETL had the highest phenolic contents of 352.31 ± 2.68 μg CE/mg, respectively (Table 1). Total flavonoid content was expressed as mg of rutin equivalent per gram of dry weight. As shown in Table 1, the total flavonoid content of ETL was 38.48± 1.38 μg RE/mg.

Table 1 also shows ABTS and DPPH scavenging activities of ETL. TEAC value of ETL was 1063.53 ± 6.34 μg/mg. And ETL exhibited the strongest antioxidant activities in scavenging DPPH radicals, with EC_{50} values of 88.72 ± 3.57 μg/mL, respectively. We also evaluated the reference compound of quercetin exhibited the strongest antioxidant activities in ABTS and DPPH scavenging radicals in the ETL.

3.2. Effect of the ETL on LPS-induced NO Production in Macrophages

In a cellular model of inflammation, the NO inhibitory activity of ETL was determined by using the LPS activated macrophages to produce NO radicals that were measured as nitrites in the culture medium by the Griess reaction. As shown in Table 1,

ETL reduced the NO production of activated macrophages with an IC_{50} value of 386.38 ± 2.54 μg/mL, respectively. This suggests ETL could be a potential inhibitor of NO related inflammation pathway. In addition, no cell toxicity was observed with ETL (0, 250, 500, and 1000 μg/mL), as measured by the MTT cell viability test. And the reference compounds of quercetin (0, 5, 10, and 20 μM) in the ETL also showed the NO inhibitory activity induced by LPS in RAW264.7 macrophages with an IC_{50} value of 16.42 ± 0.21 μg/mL, respectively (Fig. 2 and Table 1).

3.3. Inhibition of LPS-induced iNOS and COX-2 Protein by ETL and Quercetin.

The results showed that incubation with ETL and quercetin in the presence of LPS for 24 hrs inhibited iNOS and COX-2 protein expression in mouse macrophage RAW264.7 cells in a dose-dependent manner (Fig. 3A). The intensity of protein bands were analyzed and showed an average of 73.8% and 76.2% down-regulation of iNOS and COX-2 proteins, respectively, after treatment with ETL at 1000 μg/mL compared with the LPS-alone (Fig. 3B). And the protein expression showed an average of 78.8%, and 19.2% down-regulation of iNOS and COX-2 protein after treatment with quercetin at 20 μM (Fig. 3A). The down-regulation of iNOS and COX-2 activity of ETL (1000 μg/mL) was better than quercetin (20 μM).

3.3. Acetic acid-induced writhing response

The cumulative amount of abdominal stretching correlated with the level of acetic acid-induced pain (Fig. 4A). ETL treatment (0.25, 0.5, and 1.0 g/Kg) significantly inhibited the number of writhing in comparison with the pathological model group. The inhibition rates of the number of writhing compared with the pathological model group are 22.84%, 40.08%, and 57.78% respectively. The inhibiting effect of acetic acid-induced writhing by ETL (1.0 g/kg) was similar to that produced by a positive control Indo (10 mg/kg) ($P < 0.001$).

3.2.2. Formalin test

ETL significantly inhibited formalin-induced pain in the late phase; however, there was no inhibition in the early phase (Fig. 1B). ETL treatment (0.25, 0.5, and 1.0 g/Kg) significantly inhibited the formalin-induced pain (late phase) in comparison with the pathological model group. The inhibition rates of formalin-induced licking compared with the pathological model group are 30.53%, 43.78%, and 52.55%, respectively. This inhibiting effect of formalin-induced licking time by ETL (1.0 g/kg; $P < 0.001$) was better than a positive control Indo (10 mg/kg) ($P < 0.001$).

3.2.3. λ-Carrageenan (Carr)-induced edema

Fig. 5A shows the effect of ETL on Carr induced paw edema in mice. Indo is an

anti-inflammatory drug used to reduce acute inflammatory response such as swelling. According to Fig. 5A, Indo (10 mg/Kg) reduced the edema volumes about 53.3% in comparison to the Carr group during the 5th h of Carr treatment. Further, in the range of 0.25–1.0 g/Kg, ETL showed a concentration dependent inhibition of edema development. For ETL at the concentration of 1.0 g/Kg, the levels of edema volume were decreased to 49.5% of that observed in the Carr group after 5th h treatment. These data imply that ETL can exhibit an inhibitor of edema in acute inflammatory processes.

3.2.4. Effects of ETL on MDA, NO, and TNF-α levels

Lipid oxidation serves as a marker of cellular damage and has been recognized as a marker of inflammatory damage. As shown in Fig. 5B, Carr increased the level of lipid oxidation by 3.57 folds in comparison with the control group. Meanwhile, Indo decreased the level of lipid oxidation to 51.2% of that observed in the Carr group. In fact, in the range of 0.25-1.0 g/Kg, ETL inhibited the level of lipid oxidation down to 0.27-54.4% of that observed in the Carr group. These data imply that ETL can protect against tissue lipid oxidation in Carr induced inflammatory processes.

Many studies demonstrated that Carr-induced inflammatory processes increased NO, iNOS expression, and elevated TNF-α production. The level of nitrite is a

regular index for intracellular NO and iNOS production *in vivo*. As shown in Fig. 5C, Carr increased the level of nitrite by 9.2 folds in comparison to the control group in serum. Meanwhile, Indo decreased the level of serum nitrite to 56.7% of that observed in the Carr group. In fact, in the range of 0.25-1 g/Kg, ETL reduced the level of nitrite to 29.5-61.5% of that observed in the Carr group. And Carr increased the level of TNF-α in the serum by 5.6 folds in comparison to the control group (Fig. 5D). Indo decreased the level of serum TNF-α to 58.5% of that observed in the Carr group. ETL also inhibited the production of TNF-α to 15.3-61.9% of that observed in the Carr group. These data imply that ETL acts as an inhibitor of Carr induced tissue inflammation by decreasing NO and TNF-α production *in vivo*.

3.2.5. Effects of ETL on the activities of antioxidant enzymes in Carr-induced paw edema

Under healthy conditions, free radicals are prevented by enzymes directly interacting with ROS. Table 2 shows the activities of CAT, SOD, and GPx in Carr-induced paw edema of treated mice. Carr decreased the activities of CAT, SOD, and GPx in Carr-induced paw edema by 41.1%, 56.4%, and 50.3% respectively, in comparison to the control group (p<0.001). In the range of 0.25-1.0 g/Kg, ETL increased the activities of CAT to 116.3%-156.1%, SOD to 133.8%-202.1%, and GPx

to 133.5%-187.4% respectively, compared to that observed in the Carr group. Indo also exhibited increase effects in the activities of CAT (147.8%), SOD (194.1%), and GPx (174.8%) in comparison to the Carr group. These data imply that the anti-inflammatory effects of ETL *in vivo* might be attributed to its elevation in the antioxidant enzymes activities of Carr-induced mice.

3.2.6. Effects of ETL on Carr-induced iNOS and COX-2 protein expressions in Mice Paw Edema

The results showed that administered of ETL (1.0 g/Kg) on Carr-induced for 5th h inhibited iNOS and COX-2 proteins expression in mouse paw edema (Fig. 6A). The intensity of protein bands was analyzed and showed an average of 57.6% and 72.4% down-regulation of iNOS and COX-2 protein ($p < 0.001$) respectively, after treatment with ETL at 1.0 g/Kg compared with the Carr-induced alone (Fig. 6B). In addition, the protein expression showed an average of 47.4% and 49.1% down-regulation of iNOS and COX-2 protein after treatment with Indo at 10 mg/Kg compared with the Carr-induced alone.

3.2.7. Histological examination.

Further, paw biopsies of animals treated with ETL showed a reduction in

inflammatory response Carr-induced. Actually inflammatory cells were reduced in number and were 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. 7A). Neutrophils were notified increased with Carr treatment ($p < 0.001$). Indo and ETL (1.0 g/Kg) could significantly decrease the neutrophils numbers as compared to the Carr-treated group ($p < 0.001$) (Fig. 7B).

4. Discussion

Free radicals could play an important role in the degenerative or pathological processes of various serious diseases, such as aging, cancer, coronary heart disease, Alzheimer's disease, neurodegenerative disorders, atherosclerosis, cataracts, and inflammation (Hung et al., 2006). The use of traditional medicine is widespread and plants still present a large source of natural antioxidants that might serve as leads for the development of novel drugs. The higher radical scavenging activity of ETL seems to be closely correlated with its polyphenolic constituents though active components could play important roles in its antioxidative effect. Consequently, it is possible that the total phenolic constituents may contribute to anti-inflammatory activity of ETL. In this paper, we demonstrated that ETL inhibited radical scavenging and NO production. And the reference compound of quercetin in the ETL also with the antioxidant and anti-inflammatory activities (Table 1).

Triterpenoid, flavonoids, and phenolic acids possessed analgesic and anti-inflammatory effects on animal models and the pharmacological effects (Arslan et al., 2010). Studies have also demonstrated that flavonoids such as rutin, quercetin, luteolin produced significant antinociceptive and anti-inflammatory activities (Deliorman et al., 2007). Hence, it was suggested that the antioxidant, analgesic, and anti-inflammatory activities of ETL may be related to its phenolic content.

Tωo analgesic testing methods were employed with the objective of identifying possible peripheral and central effects of the test substances. The acetic writhing test is used to study the peripheral analgesic effects of drugs (Koster et al., 1959). Related studies have demonstrated that acetic acid indirectly induces the release of endogenous mediators of pain that stimulate the nociceptive neurons, which are sensitive to nonsteroidal anti-inflammatory drugs (Arslan et al., 2010). When compared antinociceptive activities, ETL was relatively potent in acetic acid writhing test indicating peripheral antinociception. In contrast, ETL (1.0 g/Kg) exhibited an action in similar magnitude with Indo, a reference drug for peripheral antinociception (Fig. 4A). Formalin-induced paw pain produced a distinct biphasic nociception, a first phase (lasting the first 5 min) corresponding to acute neurogenic pain, and a second phase (lasting from 15 to 30 min) corresponding to inflammatory pain responses (Huang et al., 2011). Therefore, the test can be used to clarify the possible mechanism of an antinociceptive effect of a proposed analgesic. The inhibitory effect of ETL on the nociceptive response in the late phase of the formalin test suggested that the anti-nociceptive effect of ETL could be due to its peripheral action (Fig. 4B).

Carr-induced inflammation has been well established as a valid model to study free radical generation in paw tissue after inflammatory states. The cellular and molecular mechanism of the Carr-induced inflammation is well characterized, and

these models of inflammation are standard models of screening for anti-inflammatory activity of various experimental compounds (Kumar and Kuttan, 2009). It appears that the early phase of the Carr edema is related to the production of histamine, leukotrienes and possibly cyclooxygenase products, while the delayed phase of the Carr-induced inflammatory response has been linked to neutrophil infiltration and the production of neutrophil-derived free radicals, such as hydrogen peroxide, superoxide and OH radicals, as well as to the release of other neutrophil-derived mediators. The degree of paws swelling was maximal at 3^{th} hrs after injection of Carr. However, a reduction in paw swelling size is a good index in determining the protective action of anti-inflammatory agents. According to Fig. 5A, ETL (1.0 g/Kg) inhibited the development of edema at 5^{th} hrs after treatment. And quercetin also administered before Carr clearly blocked Carr-induced inflammation in the rats (Morikawa et al., 2003).

In the process of inflammation, a burst of NO is synthesized from L-arginine by iNOS in activated macrophages. In fact, the overproduction of NO could induce cell damage as well as inflammation. Our data imply that the inhibitory effects of ETL on NO production could contribute to the decrease of oxidative stress and inflammation development in tissues. It has been proposed that free radicals play an important role in the Carr-induced acute inflammatory response (Salvemini et al., 1996). NO

produced by activated macrophages also plays an important mediator in the cytotoxic/cytostatic mechanism of non-specific immunity. Therefore, ETL decreased NO production *in vitro* (Table 1) and *in vivo* (Fig. 2B), which could further lead to reduce the edema response in inflammation.

During inflammatory processes, large amounts of the proinflammatory mediators, NO and PGE_2, are generated by inducible iNOS and COX-2, respectively. INOS, is generally not present in resting cells, but is induced by various stimuli, which include bacterial LPS, TNF-α, IL-1β and interferon-γ (Salvemini et al., 2003). However, COX-2 is induced by pro-inflammatory stimuli, including LPS and cytokines in cells *in vitro* and in inflamed sites *in vivo.* In this study, there is a significant decrease in iNOS and COX-2 activities with ETL treatment (Fig. 3A and 6A). We assume the suppression of NO production is probably due to the decreases of iNOS and COX-2 activities. Moreover, ETL act as herbal antioxidants and its antioxidative action may partly be responsible for the inhibition of NO production. Therefore, the inhibitory effect of ETL on NO production could be contributed to its total polyphenols inhibition to iNOS protein expression. Quercetin also exerts its anti-inflammatory property by suppressing NO production and iNOS through the inhibition of extracellular signal-regulated protein kinase (ERK) and p38 mitogen-activated protein kinase (MAPK), and nuclear factor kappa-B (NF-κB)/IκB signal transduction

pathways (Cho, et al 2003).

Lipid oxidation not only serves as a marker of cellular damage *in vivo* but also has been recognized to be the inducer of inflammatory processes. Some researches demonstrate that inflammatory effect induced by Carr is associated with free radicals. Free radicals, prostaglandin and NO will be released when administrating with Carr for 1-6 hrs (Huang et al., 2011). MDA production is due to free radical attack plasma membrane. Thus, inflammatory effect would result in the accumulation of MDA. In this study, ETL not only exhibited radicals scavenging capacity and could decrease Carr induced lipid damage *in vivo* (Fig. 5B).

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 inactivation of a variety of antioxidant enzymes. Giving the importance of the oxidative status in the formation of edema, the anti-inflammatory effect exhibited by drug in this model might be related to its antioxidant properties (Bignotto et al., 2009). The role of CAT is to decompose H_2O_2. Increased SOD activity can protect cells against threat of reactive free radicals. GPx is regarded as a crucial enzyme which catalyses the reduction of hydroperoxide. As shown in Table 2, there was a significant increase in CAT, SOD, and GPx activities with ETL treatment. This data implied the anti-inflammatory effect of ETL could be

due at least in part to elevate intracellular antioxidant enzyme activities and decrease inflammatory stress in tissue. However, we found that ETL decreased radical production and lipid oxidation *in vitro* as well as *in vivo*. These data suggest that ETL could serve as a natural antioxidant to protect cells against inflammatory damage.

In conclusion, our data suggest that ETL shows anti-inflammatory effects *in vitro* and *in vivo*. The anti-inflammatory effects of ETL may be related to iNOS and COX-2 reduction and reduce excess TNF-α generation in physiological systems. The antioxidant effects of ETL can be due to increase in the activities of antioxidant enzymes and its effects on radicals scavenging. Therefore, we suggest that ETL contain herbal antioxidants and exhibit analgesic, anti-inflammatory activity *in vivo*.

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References

Armstrong, D., Browne, R., 1994. The analysis of free radicals, lipid peroxides, antioxidant enzymes and compounds related to oxidative stress as applied to the clinical chemistry laboratory. Advances in Experimental Medicine and Biology 366, 43-58.

Arslan, R., Bektas, N., Ozturk, Y., 2010. Antinociceptive activity of methanol extract of fruits of Capparis ovata in mice. Journal of Ethnopharmacology,131, 28-32.

Bignotto, L., Rocha, J., Sepodes, B., Eduardo-Figueira, M., Pinto, R., Chaud, M., de Carvalho, J., Moreno, H., Mota-Filipe, H. Anti-inflammatory effect of lycopene on carrageenan-induced paw oedema and hepatic ischaemia-reperfusion in the rat**.** British Journal of Nutrition 102, 126-33.

Cho, S.Y., Park, S.J., Kwon, M. J., Jeong, T.S., Bok, S.H., Choi, W.Y., Jeong, W.I., Ryu, S.Y., Do, S.H. Lee, C.S., Song, J. C., Jeong, K.S., 2003. Quercetin suppresses proinflammatory cytokines production through MAP kinases and NF-κB pathway in lipopolysaccharide-stimulated macrophage. Molecular and Cellular Biochemistry 243, 153–160.

Deliorman, O.D., Hartevioğlu, A., Küpeli, E., Yesilada, E., 2007. *In vivo* anti-inflammatory and antinociceptive activity of the crude extract and fractions from *Rosa canina* L. fruits. Journal of Ethnopharmacology, 112, 394-400.

Dubuisson, D., Dennis, S.G., 1977. The formalin test: A quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain 4, 161-174.

Flohe, L., Gunzler, W.A., 1984. Assays of glutathione peroxidase. Methods in Enzymology 105, 114-121.

Flohe, L., Otting, F., 1984. Superoxide dismutase assays. Methods in Enzymology 105, 93-104.

Huang, M.H., Huang, S.S., Wang, B.S., Wu, C.H., Sheu, M.J., Hou, W.C., Lin, S.S., Huang, G.J., 2011. Antioxidant and anti-inflammatory properties of *Cardiospermum halicacabum* and its reference compounds *ex vivo* and *in vivo.* Journal of Ethnopharmacology 133, 743-750.

Huang, D.J., Chen, H.J., Hou, W.C., Lin, Y.H., 2006. Sweet potato (*Ipomoea batatas* [L.] Lam 'Tainong 57') storage roots mucilage with antioxidant activities *in vitro*. Food Chemistry 98, 774-781.

Huang, S.S., Huang, G.J., Ho, Y.L., Lin, Y.H., Hung, H.J. Chang, T.N., Chan, M.J., Chen, J.J., Chang, Y.S., 2008. Antioxidant and antiproliferative activities of the four *Hydrocotyle* species from Taiwan. Botanical studies 49, 311-322.

Huang, G.J., Sheu, M.J., Chen, H.J., Chang, Y.S., Lin, Y.H., 2007. Inhibition of Reactive Nitrogen Species *in Vitro* and ex *Vivo* by Trypsin Inhibitor from sweet potato 'Tainong 57' storage roots. Journal of Agricultural and Food Chemistry 55, 6000-6006**.**

Huang, G.J., Huang, S.S., Lin, S.S., Shao, Y.Y., Chen, C.C., Hou, W.C. Kuo, Y.H., 2010. Analgesic Effects and the Mechanisms of Anti-inflammation of Ergostatrien-3 beta-ol from *Antrodia camphorata* Submerged Whole Broth in Mice. Journal of Agricultural and Food Chemistry 58, 7445-7452.

Hung, T.M., Na, M.K., Thuong, P.T., Su, N.D., Sok, D.E., Song, K.S., Seong, Y.H., Bae, K.H., 2006. Antioxidant activity of caffeoyl quinic acid derivatives from the roots of Dipsacus asper Wall. Journal of Ethnopharmacology 108, 188-192.

Huang, S.S., Chiu, C. S., Chen, H. J., Lin, S. S., Hsieh, I.C., Hou, W.C., Huang, G.J., 2011. Antinociceptive activities and the mechanisms of anti-inflammation of asiatic acid in mice. *Evidence Based Complementary and Alternative Medicine* doi:10.1155/2011/895857.

Koster R, Anderson M, De BEJ. 1959. Acetic acid for analgesic screening. Federation Proceeding 18, 412-416.

Kumar, P.P., Kuttan, G., 2009. Vernonia cinerea L. scavenges free radicals and

regulates nitric oxide and proinflammatory cytokines profile in carrageenan induced paw edema model. Immunopharmacology and Immunotoxicology 31, 94-102.

Liu, D.Z., Liang, H.J., Chen, C.H., Su, C.H., Lee, T.H., Huang, C.T., Hou, W.C., Lin, S.Y., Zhong, W.B., Lin, P.J., Hung, L.F., Liang, Y.C., 2007. Comparative anti-inflammatory characterization of wild fruiting body, liquid-state fermentation, and solid-state culture of *Taiwanofungus camphoratus* in microglia and the mechanism of its action. Journal of Ethnopharmacology 113, 45–53.

Morikawa, K., Nonaka, M., Narahara, M., Torii, I., Kawaguchi, K., Yoshikawa, T., Kumazawa, Y., Morikawa, S., 2003. Inhibitory effect of quercetin on carrageenan-induced inflammation in rats. Life Sciences 74, 709–721.

Ohishi, N., Ohkawa, H., Miike, A., Tatano, T., Yagi, K., 1985. A new assay method for lipid peroxides using a methylene blue derivative. Biochemistry International 10, 205-211.

Rao, Y.K., Fang, S.H., Tzeng, Y.M., 2007. Evaluation of the anti-inflammatory and anti-proliferation tumoral cells activities of *Antrodia camphorata*, *Cordyceps sinensis*, and *Cinnamomum osmophloeum* bark extracts. Journal of Ethnopharmacology 114, 78–85.

Salvemini, D., Wang, Z.Q., Bourdon, D.M., Stern, M.K., Currie, M.G. and Manning, P.T., 1996. Evidence of peroxynitrite involvement in the carrageenan-induced rat paw edema. European Journal of Pharmacology 303, 217-220.

Salvemini, D., Ischiropoulos, H., Cuzzocrea, S., 2003. Roles of nitric oxide and superoxide in inflammation. Methods in Molecular Biology 225, 291–303.

Sheeba, M.S., Asha, V.V., 2009. *Cardiospermum halicacabum* ethanol extract inhibits LPS induced COX-2, TNF-alpha and iNOS expression, which is mediated by NF-kappa B regulation, in RAW264.7 cells. Journal of Ethnopharmacology 124, 39-44.

Valko, M., Rhodes, C.J., Moncol, J., Izakovic, M. and Mazur, M., 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chemico-Biological Interactions 160, 1-40.

Wang, Y., Deng, M., Zhang, S.Y., Zhou, Z.K., Tian, W.X., 2008. Parasitic loranthus from Loranthaceae rather than Viscaceae potently inhibits fatty acid synthase and reduces body weight in mice. Journal of Ethnopharmacology 118, 473–478.

Winter, C.A., Risley, E.A., Nuss, G.W., 1962. Carrageenin-induced edema in hind paw of the rat as an assay for antiiflammatory drugs. Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and

Medicine (New York, N.Y.) 111, 544-547.