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Vol. 7(23), pp. 1546-1553, 22 June, 2013
DOI 10.5897/AJPP12.1327
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Analgesic and anti-inflammatory activities of the ethanol extract of *Taxillus sutchuenensis* in mice

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Accepted 27 May, 2013

In this study, the analgesic activities and the mechanism of anti-inflammatory activities of 50% ethanol extract from TS (ETS) *in vivo* were investigated. Investigations were performed in mice with 2 analgesic models: acetic acid-induced writhing response and formalin-induced paw licking. The anti-inflammatory effect was tested by λ-carrageenan (Carr)-induced mice paw edema. These analgesic results indicated that ETS at a dose of 0.5 and 1.0 g/kg reduced the acetic acid-induced writhing responses and the licking time in the late phase of the formalin test. Moreover, Carr-induced paw edema was significantly reduced when ETS (0.5 and 1.0 g/kg) was administered 1 to 5 h after Carr injection. ETS reduced the level of malondialdehyde (MDA) in the edema paw by increasing the activities of antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GRx), and catalase (CAT), in the liver and reducing tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 activities in the edema paw. This study demonstrates the analgesic and anti-inflammatory effects of ETS, thus verifying its popular use in traditional medicine.

Key words: *Taxillus sutchuenensis;* analgesia, anti-inflammation, mice, malondialdehyde (MDA).

INTRODUCTION

Inflammation is recognized as a vital process in response to the parthenogenesis of various diseases, such as cardiovascular disease, cancer, atherosclerosis, arthritis, diabetes mellitus, obesity, neurodegenerative disease, heart disease, and many other life-threatening and debilitating diseases. Inflammation is the activation of the immune system caused by infection, toxins, physical injury, or chemical irritation, and is a complex process characterized by the contribution of several mediators, such as nitric oxide (NO), prostaglandins (PGs), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), prostanoids, and leukotrienes (Moncada et al., 1991; Chiu et al., 2012). Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most commonly prescribed drugs for the treatment of inflammatory diseases (Sheeba and Asha, 2009). NSAIDs possess analgesic and anti-inflammation activity, because of the mechanism of inhibiting cyclooxygenases (COXs) for a decrease in PG production, which consequently reduces pain and inflammation. However, the NSAIDs, used clinically, are often of limited application, because of the occurrence of adverse digestive effects, most notably gastrointestinal hemorrhage, ulceration, and perforation (Wallace, 2001). Thus, developing a novel anti-inflammatory drug is crucial.

Taxillus sutchuenensis (TS) (Lecomte) Danser, which is

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called *Sang Ji Sheng* in Taiwan, is one species of the largest mistletoe family, Loranthaceae, which contains 75 genera and approximately 1000 species (Wilson and Calvin, 2006). This parasitical plant is capable of haustorial connections to various hosts, such as Aceraceae, Anacardiaceae, Euphorbiaceae, Fabaceae, Moraceae, Ruraceae, and Theaceae, and is widely distributed on land surfaces of the former supercontinent (Wilson and Calvin, 2006; Qui and Gilbert, 2003). For decades, the whole plant (stems and leaves) of TShas been used as herbal medicine, and has been used for the treatment of many human and animal ailments that include rheumatoid arthralgia, hypertension, obesity, cancer, and gastrointestinal tracts and wounds (Wang et al., 2008). In a previous study, the findings showed that TS possesses antioxidant, anti-inflammatory, and antiproliferative activities *in vitro* (Liu et al., 2012). However, research regarding information on the analgesic and anti-inflammatory activities of TS *in vivo* is scant.

In this study, the analgesic and anti-inflammatory activities of ETS *in vivo* were systematically investigated. Its analgesic activity was evaluated using the acetic acid-induced writhing response and the formalin test. The anti-inflammatory activity of ETS was determined using the Carr-induced paw edema model, which is a useful model for assessing inflammation. The activities of antioxidant enzymes including GRx, SOD, GPx, and CAT in the liver were subsequently determined.

MATERIALS AND METHODS

Plant and crude extract preparation

Plant materials were collected from Nantou County in Taiwan. They were identified and authenticated by Dr. Chao-Lin Kuo, head of the School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources (CPCR), College of Pharmacy, China Medical University. The voucher specimen (Number: CMU-CPCR-UL-096031) was deposited at CPCR. Coarse powder of TS (5 kg) was extracted 3 times at 5:5 (aqueous/ethanol). The extract was evaporated under reduced pressure by using a rota-vapor, and then stored under light protection. A yield equivalent to 15.517% of the original weight was obtained. The extract was stored in a refrigerator before use.

Chemicals

Formalin was purchased from Nihon Shiyaku Industry, Ltd. Acetic acid, λ-carrageenan (Carr), indomethacin (Indo), and other chemicals were purchased from Sigma-Aldrich, Inc. (St Louis, MO, USA). The SOD, GPx, GRx, CAT, and MDA activity assay kits were purchased from Randox Laboratory Ltd.

Experimental animals

Male Imprinting Control Region (ICR) mice (18 to 22 g) were obtained from BioLASCO Taiwan Co., Ltd. They were housed in standard cages at a constant temperature of 22 ± 1°C, with a relative humidity of 55 ± 5% and a 12 h light-dark cycle (8:00 AM to 8:00 PM) for a minimum of 1 week before the experiment. They were fed food and water *ad libitum*. The animals used in this study were housed and cared in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Committee on Animal Research, China Medical University. The placebo groups were administered orally with 0.1 ml/10 g body weight saline. All tests were conducted under the guidelines of the International Association for the Study of Pain (Zimmermann, 1983). The required minimum number of animals and duration of observations were used to obtain consistent data.

Acute toxicity studies

For the acute toxicity study, 10 male ICR mice (22 to 25 g) were randomly distributed. They were administered orally with doses (5000 mg/kg body weight) of TS. The animals were observed continuously for the first 4 h, and then the number of survivors was noted after 14 days of dosing.

Acetic acid-induced writhing response

The writhing test was performed as described by Fontenele et al. (1996). The writhes were induced by intraperitoneal (i.p.) injection of 1.0% acetic acid solution (v/v, 0.1 ml/10 g body weight). Positive-control animals were pretreated with indomethacin (10 mg/kg, i.p.) 25 min before acetic acid injection. Three doses (250, 500, and 1000 mg/kg) of ETS were administered orally to each group of mice 55 min before the chemical stimulus. Five minutes after i.p. injection of acetic acid, the total numbers of writhing and stretching during 10 min were recorded.

Formalin test

The formalin test that was administered was based on the method of a previous study (Liu et al., 2007). Nociception was induced by an i.p. injection of 20 µl of 5% formalin in saline to the right hind paw of the mice. ETS (0.25, 0.5, and 1.0 g/kg, p.o.) was administered 60 min before the formalin injection. Indo (10 mg/kg, i.p.) was administered 30 min before nociception was induced. The control group received the same volume of saline by oral administration. The time spent with responses of licking and biting of the injected paw was taken as a response for the indicator of pain. Responses were measured for 5 min after formalin injection (early phase) and 20 to 30 min after formalin injection (late phase).

Carr-induced mice paw edema

The anti-inflammatory activity of ETS was determined by the Carr-induced edema in the hind paws of the mice. Male ICR mice fasted for 24 h before the experiment, with free access to water. Twenty microliters of a 1% Carr suspension in saline was injected into the plantar side of the right hind paws of the mice (6 per each group) (Winter et al., 1962; Amabeoku and Kabatend, 2012). Animals were peritoneally-treated with the ETS (0.25, 0.5, and 1.0 g/kg), Indo, or normal control, 60 min prior to the injection of Carr. The paw volume was measured immediately by using a plethysmometer at 1, 2, 3, 4 and 5 h after Carr administration. The degree of swelling was evaluated by the delta volume (a-b), where a and b are the volumes of the right hind paw after and before Carr treatment, respectively. In the second experiment, the whole right hind paw tissue and liver tissue were taken at the third hour. The right hind paw tissue was rinsed in ice-cold normal saline, and immediately placed in 4 times their volume of cold normal saline, and homogenized at 4°C. The homogenate was then centrifuged at 12 000 rpm for 5 min. The supernatant was obtained and stored at -80°C in a refrigerator for MDA assays. The whole liver tissue was

immediately placed in cold normal saline and homogenized. The homogenate was then centrifuged at 12 000 rpm for 5 min. The supernatant was obtained and stored at -80°C in a refrigerator for the antioxidant enzyme (SOD, GPx, GRx, and CAT) activity assays.

Measurement of TNF-α, IL-1β, IL-6 by ELISA

Serum levels of TNF-*α*, IL-1*β*, and IL-6 were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Biosource International Inc., Camarillo, CA, USA), according to the manufacturer's instruction. The absorbance at 450 and 540 nm was measured on a microplate reader (VersaMax), and the concentrations of cytokines were determined as a difference in absorbance between 450 and 540 nm.

MDA assay

MDA was evaluated using the thiobarbituric acid-reacting substance (TRARS) method (Nakhai et al., 2007). First, the paw tissues were homogenized in buffered saline (1:4); afterward, 400 µl of 1,1,3,3-tetraethoxypropan trichloroacetic acid (28% w/v) was added to 200 µl of this mixture and centrifuged at 3000 g for 30 min. Thereafter, 300 µl of the supernatant was added to 150 µl of 2-thiobarbituric acid (1% w/v). The mixture was incubated for 45 min in a boiling water bath, and then 450 µl n-butanol was added; the solution was centrifuged and cooled, and absorption of the supernatant was recorded in 532 nm by the microplate reader (VersaMax, Massachusetts, USA). Tetramethoxypropane was used as the standard. MDA levels were expressed as nanomoles per milligram of protein. Protein concentration was measured using the Lowry method (Lowry et al., 1951). Bovine serum albumin was used as the standard.

Antioxidant enzymes activity measurements

Liver tissue homogenates were collected for the estimations of SOD (Misra and Fridovich, 1972), GPx (Flohe Gunzler, 1984), GRx (Carlberg and Mannervik, 1985), and CAT enzymes (Aebi, 1984) to detect the antioxidant activities of TS. The SOD enzyme activity was determined according to the method by Misra and Fridovich. In total, 100 µl of the tissue extract was added to 880 µl (pH 10.2, 0.1 mM EDTA) of a carbonate buffer. Twenty microliters of 30 mM epinephrine (in 0.05% acetic acid) was added to the mixture at 480 nm for 4 min on a Hitachi U2000 Spectrophotometer. The enzyme activity was expressed as the amount of enzymes that inhibit the oxidation of epinephrine by 50%, which is equal to one unit. The total CAT activity was based on that by Aebi (1984). In brief, the reduction of 10 mM H_2O_2 in 20 mM of the 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 nmoles of dissipating hydrogen peroxide per milligram of protein per minute. The GPx enzyme activity was determined according to the method by Flohe and Gunzler (1984). The reaction mixture was composed of 500 µl phosphate buffer, 100 µl 0.01 M GSH (reduced form), 100 µl 1.5 mM NADPH, and 100 µl GRx (0.24 units). In total, 100 µl of the tissue extract was added to the reaction mixture and incubated at 37°C for 10 min. Thereafter, 50 µl of 12 mM t-butyl hydroperoxide was added to 450 µl of the tissue reaction mixture, and was measured at 340 nm for 180 s. The molar extinction coefficient of 6.22×10^{-3} was used to determine the enzyme activity. One unit of activity is equal to the millimolar of NADPH oxidized per minute per milligram of protein. The GRx enzyme activity was determined following the method by Carlberg and Mannervik at 37°C (Carlberg and Mannervik, 1985). Fifty microliters of NADPH (2 mM) in 10 mM Tris buffer (pH 7.0) was added in the cuvette containing 50 µl of GSSG (20 mM) in the phosphate buffer. In total, 100 µl of tissue extract was added to the NADPH-GSSG-buffered solution and measured at 340 nm for 3 min. The molar extinction coefficient of 6.22×10^{-3} was used to determine the GRx enzyme activity. One unit of activity is equal to the millimolar of NADPH oxidized per minute per milligram of protein.

Statistical analysis

All the data were expressed as the mean ± standard error of mean (SEM). Statistical evaluation was conducted 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 ***$P < 0.001$.

RESULTS

Toxicity study

The acute toxicity of ETS was evaluated in the mice at doses of up to 5000 mg/kg body weight p.o. administered for 14 days. ETS did not cause behavioral changes, and no death was observed. The oral LD_{50} value of ETS was greater than 5000 mg/kg of body weight in the mice, and was considered to be a practically non-toxic substance.

Acetic acid-induced writhing response

The writhing method used in this study was similar to that described in a previous study (Chao et al., 2009). The writhes were induced by i.p. injection of 1% acetic acid (10 ml/kg of body weight). ETS–treated groups received ETS (0.25, 0.5, and 1.0 g/kg, p.o.), whereas the positive control group received Indo (10 mg/kg, i.p.). The findings showed that ETS (0.5 and 1.0 g/kg) and Indo (10 mg/kg) demonstrated inhibition in this model ($P < 0.01$ to 0.001) (Figure 1). The inhibitory effect of acetic acid-induced writhing by ETS was similar to that produced by a positive-control Indo.

Formalin test

The antinociceptive activity of ETS was determined using the formalin test, as shown in Figure 2 (Dubuisson and Dennis, 1977). The results demonstrated a dose-dependent relationship of ETS in both phases of formalin-induced pain. In the early phase, no significant inhibition was generated with the doses of ETS (0.25, 0.5, and 1.0 g/kg) and Indo (10 mg/kg), compared with the control group. In the late phase, doses of 0.5 and 1.0 g/kg significantly reduced nociception. The Indo treatment also produced significant inhibition in the late phase.

Carr-induced mice paw edema

Carr-induced mice paw edema was used to determine the

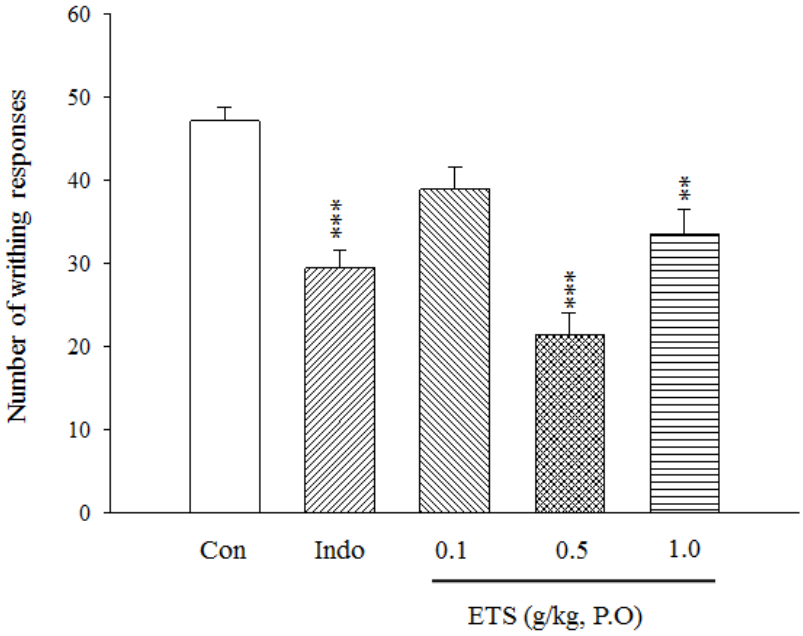

Figure 1. Analgesic effect of ETS and Indo on the acetic acid-induced writhing response in mice. Each value is represented as the mean ± SEM. **P < 0.01, ***P < 0.001, compared with the control (Con) group (one-way ANOVA followed by Scheffe's multiple-range test).

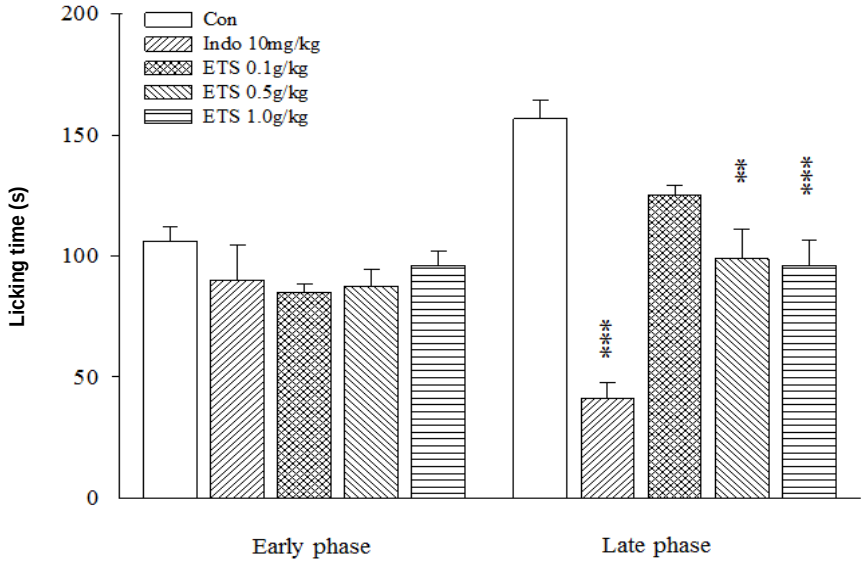

Figure 2. Analgesic effect of ETS and Indo on (a) early phase and (b) late phase in the formalin test on the mice. Each value is represented as the mean ± SEM. *P < 0.05, ***P < 0.001, compared with the control (Con) group (one-way ANOVA followed by Scheffe's multiple-range test).

anti-inflammatory activity of ETS (Figure 3). Carr-induced paw edema was dramatically reduced in a dose-dependent manner by administrate ETS at 1 to 5 h after Carr injection. A significant anti-inflammatory effect of Indo was also observed after the Carr injection.

The activities of anti-oxidant enzymes

After the third hour following the intrapaw injection of Carr, liver tissues were analyzed for biochemical parameters, such as those of GPx, GRx, SOD, and CAT activities

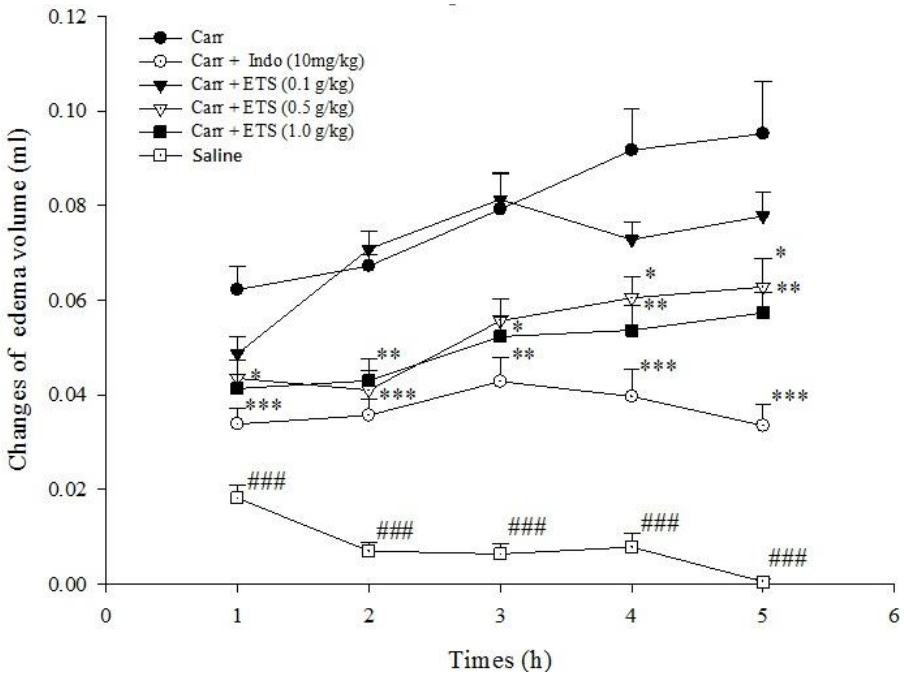

Figure 3. Effects of ETS and Indo on hind-paw edema induced by Carr in mice. Each value is represented as the mean ± SE (n = 6). *Significant difference from the Carr group (*P < 0.05, **P < 0.01, ***P < 0.001).

(Table 1). Carr reduced the activities of GPx, GRx, SOD, and CAT in Carr-induced paw edema by 61.3, 40.2, 42.3, and 53.9%, respectively, compared to the control group. In the range of 0.5 to 1.0 g/kg, ETS significantly increased the activities of GPx, GRx, SOD, and CAT, compared to that observed in the Carr group, as well as the Indo group.

MDA Level

In the control group, the MDA level in the edema paw significantly induced by Carr increased. However, MDA levels were lowered significantly after treatment with ETS at 0.5 and 1.0 g/kg. Indo, in the positive-control group, reduced the MDA level as compared to that observed in the Carr group.

Serum TNF-α, IL-1β, and IL-6 level

The TNF-α, IL-1β, and IL-6 levels increased significantly in serum at the fifth hour after Carr injection ($P < 0.001$). However, ETS (0.5 or 1.0 g/kg) reduced the TNF-α, IL-1β and IL-6 levels in serum ($P < 0.01$ or $P < 0.001$), as well as 10 mg/kg Indo (Table 2).

DISCUSSION

In this study, we evaluated the putative analgesic and anti-inflammatory activities of ETS to clarify the pain and inflammation-relieving effects. Two analgesic animal models were used to identify the possible peripheral and central effects of the test substances. In the acetic acid-induced writhing response, the visceral pain model, the analgesic mechanism of abdominal writhing, was induced by acetic acid, which involves the release of arachidonic acid (AA) through COX and PG biosynthesis (Koo et al., 2006). ETS at the oral dose of 0.5 to 1.0 g/kg significantly reduced the writhing response of acetic acid-induced mice. The effect may be due to the inhibition of the synthesis of AA or its metabolites. The formalin test, which is believed to be relatively more specific in identifying pain in central and/or peripheral components, was also used in this study. The formalin test consists of 2 biphasic phases: an early phase (lasting the first 5 min) corresponding to acute neurogenic pain, and a late phase (lasting from 15 to 30 min) corresponding to inflammatory pain responses (Huang et al., 2011). ETS was administered orally (0.5 and 1.0 g/kg) to the mice, which produced dose-related inhibition late-phase pain, but did not inhibit neurogenic (early phase) pain caused by intraplantar injection of formalin, suggesting that the antinociceptive effect of ETS could be due to its peripheral action (Figure 2).

The Carr-induced mice paw edema has been well established as a valid model for evaluating NSAIDs, and has been accepted as a useful phlogistic tool for investigating the antiedematous effect of natural products (Di Rosa et al., 1971; Garcia Leme et al., 1973). Carr-

Table 1. Effects of ETS and Indo on changes in GPx, GR, SOD, and CAT activities were studied in Carr-induced paw edema in mice.

Group	U/mg protein			
	GPx	GR	SOD	CAT
Control	40.07 ± 2.69###	2.87 ± 0.31###	0.087 ± 0.005###	8.39 ± 1.52###
Carr	23.11 ± 2.49	1.11 ± 0.11	0.052 ± 0.003	3.87 ± 0.44
Carr + Indo (10 mg/kg)	38.79 ± 3.51***	1.96 ± 0.24***	0.084 ± 0.008 ***	5.86 ± 0.43**
Carr + ETS (0.25 g/kg)	24.84 ± 2.00	1.23 ± 0.16	0.059 ± 0.005	3.82 ± 0.95
Carr + ETS (0.5 g/kg)	30.23 ± 1.81*	1.52 ± 0.16*	0.061 ± 0.007*	6.44 ± 0.54**
Carr + ETS (1 g/kg)	37.44 ± 4.04***	2.10 ± 0.29***	0.076 ± 0.006**	6.48 ± 0.93***

Table 2. Effects of ETS and indomethacin (Indo) on changes in TNF-α, IL-1, and IL-6.

Group	Protein (ng/mg)		
	TNF-α	IL-1	IL-6
Control	ND*	ND	ND
Carr	232.9 ± 13.8	216.9 ± 50.0	54.0 ± 13.1
Carr + Indo (10 mg/kg)	100.3 ± 9.6***	76.6 ± 17.2***	21.6 ± 5.1***
Carr + ETS (0.1 g/kg)	234.2 ± 15.2	196.7 ± 30.4	50.3 ± 5.8
Carr + ETS (0.5 g/kg)	162.8 ± 14.1**	141.1 ± 15.5*	28.0 ± 4.5 **
Carr + ETS (1 g/kg)	124.0 ± 13.0***	109.3 ± 17.4**	21.0 ± 5.2 ***

*ND: Not detectable

induced inflammatory responses fall into the category of acute inflammation, which involves the synthesis or release of several inflammatory mediators including bradykinin, prostaglandins (PGs), nitric oxide, and cytokines, which further cause pain and fever (Vinegar et al., 1969). The development of edema in the hind paw following Carr injection has been characterized as a biphasic event to generate a series of inflammatory reactions (Vinegar et al., 1969). The initial phase of edema (0 to 1 h) contributes to the release of several mediators, such as histamine, 5-hydroxytryptamine (5-HT), and bradykinin (Di Rosa et al., 1971). Conversely, the second phase, in which swelling accelerates (1 to 6 h), is correlated with an elevated production of TNF-α, NO, and PGs (Posadas et al., 2004). In this study, it was demonstrated that ETS and indomethacin, a potent COX inhibitor, significantly inhibited the development of edema at the second phase after treatment. It suggested that the anti-edema effects of ETS are due to its inhibition of TNF-α or cytokine synthesis in the blood cells (Figure 3 and Table 2), as described for the anti-inflammatory mechanism of indomethacin in inhibiting the inflammatory process, induced by carrageenans (Di Rosa et al., 1971).

The inflammatory response to Carr has been linked to a rapid, mainly polymorphonuclear leucocyte (PMN) infiltration and the production of PMN-derived free radicals, as well as the release of other PMN-derived mediators (Dawson et al., 1991). The free radicals, NO and PG, are released when administering Carr for 1 to 6 h, and the degree of paw edema was raised to the maximum level in the third hour. MDA is one of the most frequently used biomarkers for evaluating the level of oxidative stress in many systems (Nielsen et al., 1997). This compound is a reactive aldehyde and reacts with deoxyadenosine and deoxyguanosine in DNA, forming DNA adducts (Marnett, 1999; Farmer and Davoine, 2007). The excess accumulation of MDA results in a series of mutagenesis and carcinogenesis, as well as inflammatory reactions. Increasing the level of GSH, a known oxyradical scavenger, toward reduces MDA production. Endogenous GSH plays a critical role against Carr-induced local inflammation. With some pathophysiological conditions associated with inflammation or oxidative stress, these reactive oxygen species (ROS) have been proposed to mediate cell damage through various independent mechanisms, such as the initiation of lipid peroxidation, the depletion of glutathione, and the inactivation of various antioxidant enzymes. Because of the importance of the oxidative status in edema formation, the anti-inflammatory effect exhibited by the drug in this model might be related to its antioxidant properties. A comparison of the antioxidant enzymes of extract-treated mice with those of control mice showed that the activities of GRx, GPx, SOD, and CAT were significantly enhanced (Table 1). Moreover, significant decreases in the MDA level occurred with ETS treatment (Figure 4). The results suggest that ETS may act as a natural antioxidant to protect cells against the

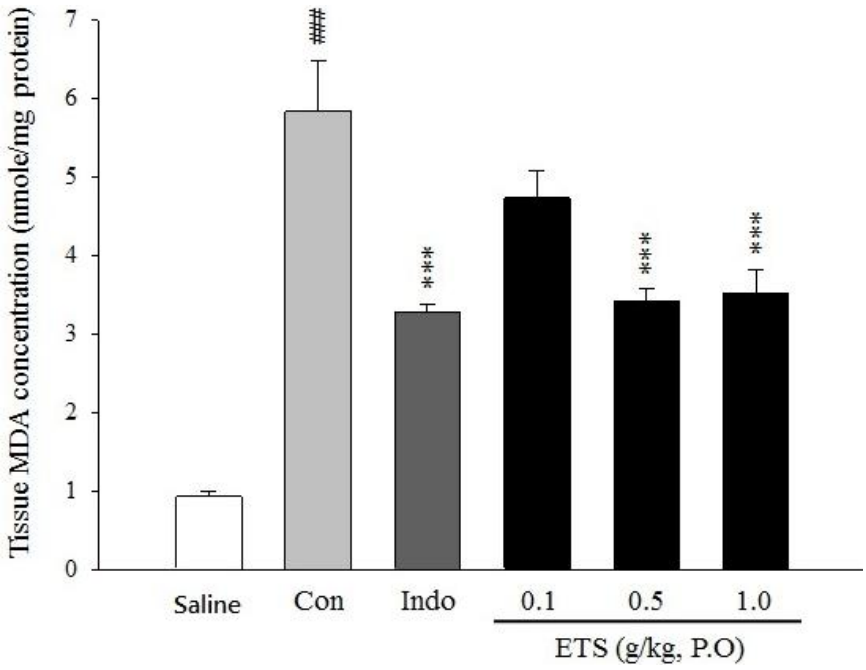

Figure 4. MDA accumulation of ETS and Indo at the third hour after Carr injection in the mice edema paw. Each value is represented as the mean ± SEM. ***P < .001, compared with the Carr group (one-way ANOVA followed by Scheffe's multiple-range test).

threat of reactive free radicals and inflammatory damage.

The pro-inflammatory cytokines, principally TNF-α and IL-1, are small and nonstructural proteins induced at sites of local inflammation, and they initiate the cascade of inflammatory mediators by targeting the endothelium (Dinarello, 2000). The TNF-α and IL-1 cytokines are involved in neutrophil migration in λ-carrageenan-induced inflammation. These mediators are able to recruit leukocytes, such as neutrophils, as reported in several recent experimental models (Salvemini et al., 1996; Loram et al., 2007). The TNF-α cytokine also induces a number of physiological and pathological effects, including septic shock, cytotoxicity, and inflammation (Salvemini et al., 2003). It is capable of inducing the further release of PGE2, iNOS, and COX-2, which is suggested to have a critical role in the maintenance of the long-lasting nociceptive response (Subbaramaiah and Dannenberg, 2003). In our previous study, we demonstrated that ETS reduced LPS-induced NO production and the expression of iNOS and COX-2 in RAW264.7 cells (Liu et al., 2012). In this study, it was found that ETS significantly reduced the TNF-α, IL-1β, and IL-6 levels in serum after Carr injection by treatment with 0.5 and 1.0 g/kg.

In conclusion, it was demonstrated that ETS exhibited anti-inflammatory activity against Carr-induced paw edema and analgesic activity against nociceptive responses triggered in mice by i.p. acetic acid or intraplantar formalin injections. The anti-inflammatory mechanisms of ETS are considered to be closely related to the increase in the activities of antioxidant enzymes (GPx, PGx, SOD, and CAT). Furthermore, ETS had an analgesic effect in both nociceptive models. The anti-inflammatory and analgesic effects of ETS are correlated and share common molecular pathways, including the inhibition of pro-inflammatory cytokine production. Therefore, TS may act as a pharmacological agent in the prevention or treatment of diseases in which free radical formation is a pathogenic factor.

ACKNOWLEDGEMENTS

This study is supported in part by the National Science Council, Taiwan (NSC 101-2320-B-039-032-MY2), the Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH100-TD-B-111-004), and the Committee on Chinese Medicine and Pharmacy, Department of Health, Executive Yuan (CCMP102-RD-014, CCMP102-RD-019).

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