

Association of Activation of Induced COX-2, iNOS and Cytokines with NF-kappa B Depression by Taiwan Wild Grape Ethanolic Extract in Mice

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Abstract

Taiwan wild grape (*Vitis thunbergii* var. *taiwaniana*, VTT) is an important traditional herbal medicine used to treat muscle injuries and acute and chronic pain of the ligaments. Information on its bioactivity and the underlying mechanisms, which have not been elucidated thus far, is needed to demonstrate its value for pharmacological and clinical use. This study presents evidence to clarify the antinociceptive and anti-inflammatory activities of an ethanolic extract of VTT stem (VTT_{EtOH}) and the possible molecular mechanisms involved in such bioactivities. In the mice, VTT_{EtOH} significantly reduced the acetic acid-induced writhing response ($P < 0.01$), formalin-induced licking time ($P < 0.01$), and edema paw volume at 4 and 5 h after λ -carrageenan (Carr) injection. VTT_{EtOH} obviously decreased the levels of tumor necrosis factor (TNF)- α ($P < 0.01$), interleukin (IL)-1 β ($P < 0.05$), IL-6 ($P < 0.001$), nuclear factor (NF)- κ B. ($P < 0.001$), inducible nitric oxide synthase (iNOS) ($P < 0.001$), cyclooxygenase (COX)-2 ($P < 0.001$) and nitric oxide (NO) ($P < 0.001$) in edema-paw tissue. The molecular mechanisms underlying these effects might involve significant inhibition of the activity of COX-2 through suppression of NF- κ B. and iNOS expressions and reduction of the levels of various inflammatory mediators, including TNF- α , IL-1 β , IL-6, and NO. Our findings provided pharmacological and histopathological evidences that VTT_{EtOH} alleviates inflammatory pain-related diseases.

Key Words: anti-inflammation, cyclooxygenase-2, inducible nitric oxide synthase, nuclear factor-kappa B, *Vitis thunbergii* var. *taiwaniana*, wild grape

Introduction

Drug resistance and tolerance can markedly decrease the clinical efficacy of medicines. Thus, the use of botanicals to alleviate diseases is becoming increasingly popular. Plants of the genus *Vitis* are common edibles with health effects and are found worldwide. Many studies have reported their anti-oxidant, anti-inflammatory, analgesic, anti-pyretic (3, 14, 17, 35) and anti-dementia (21) effects, among others. However, the molecular mechanisms underlying their anti-acute inflammatory effects remain to be determined.

Taiwan wild grape (*Vitis thunbergii* Sieb. & Zucc. var. *taiwaniana* Lu, VTT) is an important folk herbal medicine in Taiwan, and alcoholic extracts of the VTT stem are used to treat muscle injuries and acute and chronic pain of the ligaments. As a potential dietary supplement, VTT was reported to prevent joint deterioration and inhibit inflammation. Its two major active ingredients, quercetin and resveratrol (15, 19), have been shown to exhibit inhibitory activities against neurodegenerative diseases and knee damage associated with arthritis (35). However, the molecular mechanisms underlying the analgesic and anti-inflammatory effects of ethanolic extracts from VTT stem (VTT_{EiOH}) have not been fully defined. Therefore, developing VTT_{EiOH} as a phytomedicine for clinical use was limited.

Pain is a biological mechanism that can stimulate cells to produce and release a series of intracellular signaling substances (23). In animal models, acetic acid indirectly triggers the release of endogenous nociceptive mediators, such as serotonin and prostaglandins (PGs), and proinflammatory cytokines, such as tumor necrosis factor (TNF)- α and interleukin (IL)-1 β , to cause a painful sensation (24). The formalin-induced licking response is a frequently used animal model to study analgesic and anti-inflammatory effects, because the response is highly specific for identifying the central and peripheral components of pain. Therefore, the formalin test can effectively assess the effects of a drug against inflammatory and non-inflammatory pain (28, 37). Inflammation is one of the most important defense mechanisms against invading pathogens or damaged cells (9). Pain is the main clinical manifestation of inflammation. Acute inflammatory reactions can repair and rebuild damaged tissues. However, inflammation eventually progresses to a chronic state if not treated rapidly and effectively (7). Excessive and long-term chronic inflammation can cause various diseases and establish a breeding ground for carcinogenesis (32). Thus, understanding the mechanisms of acute inflammation and preventing its progression to chronic inflammation are crucial.

Lambda-carrageenan (Carr)-induced paw edema has been described as a highly reproducible model of acute inflammation. Paw edema is widely used to evaluate the anti-inflammatory activity of different test compounds and was the basis for the discovery of various nonsteroidal anti-inflammatory drugs (NSAIDs) and selective cyclooxygenase (COX)-2 inhibitors (2). Pro-inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6, are produced in large quantities by activated macrophages and monocytes, which stimulate cellular responses by increasing PGs or reactive oxygen species (ROS) (1). TNF is one of the most effective physiological inducers of the transcription factor nuclear factor (NF)- κ B. It should be noted that TNF and NF- κ B mutually influence each other, and the induction of some TNF-responsive genes of immunological relevance is mediated, at least in part, through the activation of NF- κ B (30). NF- κ B also plays an essential role in immune and inflammatory responses through the regulation of inducible nitric oxide synthase (iNOS) and COX-2 (13). Thus, inhibition of PG and nitric oxide (NO) production by blocking TNF- α and NF- κ B could alleviate inflammation-related diseases, and inhibition of NF- κ B expression is another strategy for the development of anti-inflammatory agents (4, 16).

The purpose of this study was to establish reference information on VTT_{EiOH} for the treatment of inflammatory pain-related diseases. In this study, two mouse models, the acetic acid-induced writhing model and the formalin-induced paw licking model, were used to investigate the antinociceptive effects of VTT_{EiOH} (33, 34). Subsequently, the Carr-induced mouse paw edema model was used to study VTT_{EiOH} anti-inflammatory effects (20). To elucidate the possible molecular mechanisms underlying these effects, the activities of TNF- α , IL-1 β , IL-6, COX-2 and NO, and the expression of NF- κ B and iNOS in paw tissues were analyzed. In addition, histopathological examinations were conducted to verify the inflammatory changes in mouse plantar. For quality control of VTT_{EiOH}, quercetin and resveratrol were used as reference chemical standards. Fingerprint chromatography and quantitative analysis were performed using high-performance liquid chromatography (HPLC) to analyze the quality and components of VTT.

Materials and Methods

Wild Grape Plant Collection

Fresh stems from wild grape plants were collected from the countryside of the Yunlin county in Taiwan. The plants were identified through plant taxonomy and histologic examination by Dr. Yu-

Chin Lin who is the laboratory moderator of New Phytomedicine R&D Laboratory of Da-Yeh University (Changhua county, Taiwan). Plant classification and identification were based on information on the flora of Taiwan. Histologic characteristics, including the morphology and distribution of vascular bundles, catheters and fibers were investigated by microscopy. A voucher specimen (Number: TWU-Plant-VTT-001) and a plant specimen were deposited at a botanical laboratory (Department of Biotechnology, College of Health Sciences, TransWorld University, Yunlin county, Taiwan).

Extraction of Plant Materials and Preparation of Test Samples

Fresh stem tissues were washed, sliced and dried in an oven at 50–60°C until the moisture content was less than 5%. Ethanol was added, and the stem tissues were heated at 35°C for 60 min twice. The filtrate was then concentrated and dried with a rotary evaporator (Laborota 20 compact; Heidolph Instruments GmbH & Co., Germany) at 40°C and 120–180 mbar. The yield was 4.10%. The VTT_{EtOH} was then dissolved in distilled water for pharmacological testing.

Experimental Animals

Male ICR mice (22–25 g) were obtained from BioLASCO Taiwan Co., Ltd., Taipei, Taiwan. The mice were housed in a regulated environment at a temperature of 22 ± 2°C and 50 ± 10% humidity with free access to standard food pellets and tap water under a 12-h light-dark cycle. Before the experiments began, the mice were acclimated for 1 week. Then, the mice were randomly divided into five groups contained control group, positive control group, and three test-sample groups. There were eight mice in each group. The control group was the experimental animal models by treating with acetic acid, formalin, or 1% Carr in mice. The positive control group was treated with 20 mg/kg Indomethacin (Indo) and the test-sample groups were administered with differential doses of VTT_{EtOH} (100, 200, and 400 mg/kg) in the experimental animal models. The drugs and test samples were administered to the mice. The analgesic and anti-inflammatory activity assays were performed according to standard protocols. The experimental protocol (ORDR-AS-2012-P002) was approved by the Institutional Animal Care and Use Committee (IACUC) of Transworld University, and all care and experimentation of animals were carried out in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Chemicals and Drugs

Resveratrol, quercetin, Carr, Indo and Griess reagent were purchased from Sigma-Aldrich Chemical Co. (Missouri, USA). Formalin was purchased from Nihon Shiyaku Industry Ltd. (Taipei, Taiwan). Murine IL-1 β , IL-6 and TNF- α enzyme-linked immunosorbent assay (ELISA) development kits were purchased from Bio-Legend, Inc. (California, USA). The NO ELISA kit was purchased from Cayman Chemical Company (Michigan, USA). The COX-2 ELISA kit was purchased from R&D Systems, Inc. (Minnesota, USA). The rabbit NF- κ B and iNOS polyclonal antibodies were purchased from Novus (Colorado, USA).

Chromatographic Analysis

The resveratrol and quercetin contents of VTT_{EtOH} were qualitatively analyzed and quantified by high performance liquid chromatography (HPLC) according to the modified method of Gambuti (12). In the qualitative analysis, the retention time and maximum absorption of the sample were compared to those of the reference standards. The HPLC system consisted of a Shimadzu LC-10ATvp liquid chromatograph (Kyoto, Japan) equipped with a DGU-14A degasser, an FCV-10ALvp low-pressure gradient flow control valve, an SIL-10A SHIMADZU auto injector, an SPD-M10Avp diode array detector, and an SCL-10Avp system controller. Peak areas were calculated using Shimadzu Class-LC10 software (Version 6.12 sp5). A GL Sciences Inertsil ODS-3V column (5 μ m, 4.6 \times 150 mm) was used.

The mobile phase was a mixture of methanol (MeOH) with 2.5% acetic acid (A) and distilled water with 2.5% acetic acid (B). The sample was injected in a volume of 10 μ L (n = 3), and the flow rate was 0.8 mL/min. The gradient profile was run at 1.0 mL/min for 60 min.

The reference standards were resveratrol (ASB-00018090-100, Lot[#] 00018090-719, purity 99.4%; Sigma-Aldrich) and quercetin (ASB-00017030-100, Lot[#] 00017030-566, purity 93.4%; Sigma-Aldrich). The resveratrol gradient program was as follows: 0–25 min, 25% B, 25–55 min, 90% B, 55–60 min, 25% B. The scan wavelength for both quercetin and resveratrol was 338 nm. Peaks were detected with an SPD-M10AVP detector (Shimadzu). The above conditions were used for both the HPLC assay and HPLC fingerprinting of VTT_{EtOH}.

Acute Toxicity Testing

An acute toxicity test was performed according to the method of Liao *et al.* (18). Experimental animals,

10 mice in each group, were administered VTT_{EtOH} orally at 5 g/kg, and were given free access to standard food pellets and tap water. The mice were regularly observed for any mortality or behavioral changes for 14 days.

Analgesic Activity: Acetic Acid-Induced Writhing Response

The acetic acid-induced writhing response test was carried out according to a previously reported method (33). Mice received an intraperitoneal (i.p.) injection of 1.0% acetic acid (v/v, 0.1 ml/10 g), and writhing responses were then observed. VTT_{EtOH} (100, 200, or 400 mg/kg) or Indo (20 mg/kg) was administered orally prior to the acetic acid injection.

Analgesic Activity: Formalin-Induced Paw Licking

The formalin-induced licking response in mice has been established as a valid and reliable model for nociceptive pain (34). Formalin (20 μ l of a 5% solution) was administered to the animals in the right subplantar region, and two distinct periods of intense licking were observed. VTT_{EtOH} (100, 200, or 400 mg/kg, p.o.) and Indo (20 mg/kg, p.o.) were administered 60 min before formalin injection.

Analgesic Activity: Anti-Inflammatory Activity

Carr (50 ml of a 1% solution) was injected into the plantar aponeurosis of the right hind foot. Inflammation was quantitated in ml with a plethysmometer (MK-101CMP, Muromachi, Tokyo, Japan), which recorded small differences in water levels caused by volume displacement. The percentage of edema was then calculated by comparing the average volume of the back paws of each animal after Carr injection (V_t) with the average volume of the back paws of each animal before treatment (V_0). The percent inhibition was obtained for each group by using the following equation: $[(V_t - V_0) \text{ control} - (V_t - V_0) \text{ treated}] (20)$. All animals were sacrificed by cervical dislocation upon completion of the experiment.

Histopathological Examination

Four hours after Carr injection, the paw was excised and used to prepare tissue specimens. After formalin fixation, decalcification, paraffin embedding and Hematoxylin and eosin (H&E) staining, histopathological changes were examined with a BX60 microscope (Olympus, Melville, NY, USA) and a camera (Macrofire 599831). The results were analyzed at the Animal Disease Diagnostic Center, Chung

Hsing University, Taichung, Taiwan.

TNF- α , IL-1 β and IL-6 Assays

TNF- α was measured by ELISA at 450 nm in an ELISA plate reader (5). The captured TNF- α antibody was pre-seeded in the wells of a 96-well plate overnight. On the following day, a second set of biotinylated antibodies was incubated with the test articles or standard antigens in the plate, and streptavidin was added. The method used to assess TNF- α was adapted to assess IL-1 β and IL-6.

Western Blotting for NF- κ B and iNOS

Freshly separated paw samples were homogenized in a lysis buffer. The protein concentration of the whole tissue homogenate, cytosolic and microsomal fractions was assessed with the western blotting method (9). Proteins from paw homogenates (100 μ g) or purified microsomes (50 μ g) were loaded into each well of an 8–12% polyacrylamide gel and separated by electrophoresis. The separated proteins were transferred to nitrocellulose membranes, and the membrane was blocked overnight with a buffer solution and then incubated with appropriate primary antibodies for 2 h using 1:1,000 dilution of rabbit anti-NF- κ B (1:100 dilution, 2 h at 25°C; Novus, Littleton, CO, USA) or rabbit anti-iNOS antibodies (1:100 dilution, 2 h at 25°C, Santa Cruz, Dallas, TX, USA). The membranes were washed three times with Tris-buffered saline containing Tween 20 (TBST) for 15 min, and then incubated with the secondary antibody, alkaline phosphatase-conjugated anti-rabbit IgG (1:1,000 dilution) for 1 h. The Enhanced Chemiluminescence Western Blotting Detection Kit (Amersham, Pittsburgh, PA, USA) was used to visualize the antibody-bound proteins. For quantification, signal intensity was evaluated with Image Gauge version 3.0 (Fuji Film; Kanagawa, Japan) by using the Fuji film LAS-4000 system.

NO Assay

The NO assay was performed according to the method of Moshage *et al.* (26). Briefly, NO⁻³ was converted to NO⁻² by nitrate reductase, and the NO⁻² was subsequently reacted with sulfanilic acid to produce diazonium ions, which coupled with N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative (purplish red). The change in color was recorded at 540 nm.

COX-2 Assay

COX-2 was determined by measuring the per-

Table 1. Analgesic activity of an VTT_{EiOH} in mice

Groups/Dose	No. of writhing responses	No. of licking responses	
		Early phase	Late phase
Control	31.57 ± 1.41	77.00 ± 5.73	165.90 ± 9.93
Indo 20 mg/kg	11.33 ± 1.80***	74.80 ± 8.28	88.88 ± 15.09**
VTT _{EiOH} 100 mg/kg	23.00 ± 1.37*	65.20 ± 4.26	93.00 ± 13.49**
VTT _{EiOH} 200 mg/kg	21.00 ± 2.70*	60.10 ± 3.69	9.50 ± 10.41**
VTT _{EiOH} 400 mg/kg	20.83 ± 1.66**	71.80 ± 8.05	91.77 ± 13.71**

Values are the mean ± standard error of the mean (SEM). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 compared to the control group.

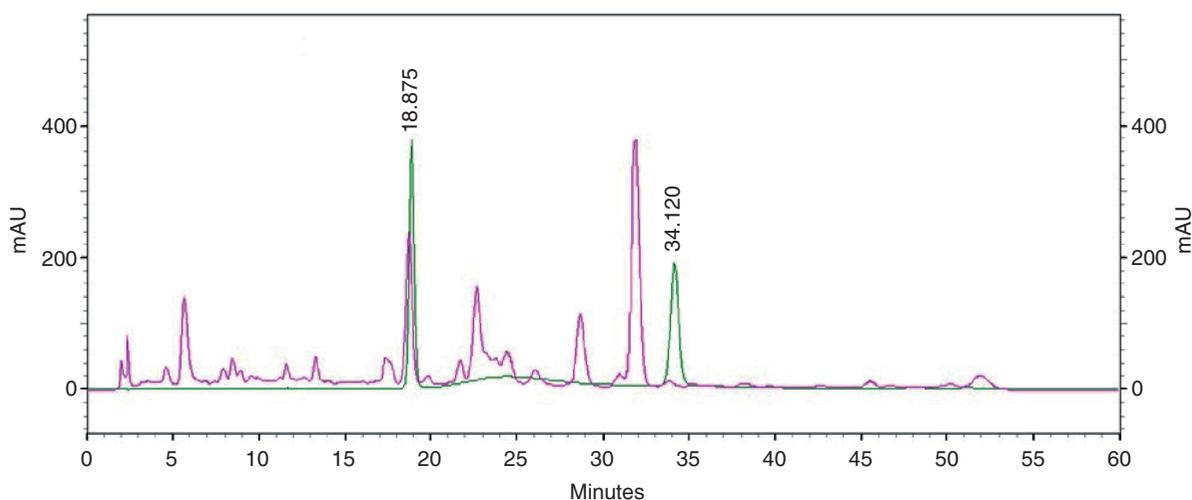


Fig. 1. Fingerprints of VTT_{EiOH} determined by HPLC. The red line is the fingerprint of VTT_{EiOH}, the green line (left peak, 18.87 min) is resveratrol, and the green line (right peak, 34.12 min) is quercetin.

oxidase activity of prostaglandin endoperoxide H2 synthase (PGHS) (27). The peroxidase activity of PGHS was assessed following the oxidation of N, N, N', N'-tetramethyl-p-phenylenediamine (TMPD) at 37°C using arachidonate as the substrate. The color changes were recorded at 590 nm.

Statistical Analysis

All data obtained in this study are reported as the mean and standard error and were analyzed using one-way analysis of variance (ANOVA) followed by Scheffe's test. *P* values less than 0.05 were considered significant.

Results

Resveratrol and quercetin are two known active ingredients in grape plant. In this study, the quantitative analysis methods of two reference standards,

resveratrol and quercetin, were built up individually by HPLC. Afterwards, the fingerprints of VTT_{EiOH} and the contents of resveratrol and quercetin in VTT_{EiOH} were measured. The fingerprint spectrum is shown in Figure 1. The red line is the fingerprint of VTT_{EiOH}, green line (left peak, 18.87 min) is that of resveratrol, and green line (right peak, 34.12 min) is that of quercetin. The maximum absorbance values were observed at 309 and 369 nm, and the relative amounts of resveratrol and quercetin were 1.6147 and 0.2461 µg/mg, respectively.

The acute toxicity of VTT_{EiOH} was assessed in mice at a dose of 5 g/kg. No behavioral change or mortality was observed after 14 days of oral administration.

The acetic acid-induced abdominal writhing model was used to test the effect of VTT_{EiOH} on visceral pain (Table 1). Indo was used as a positive control. The number of writhes in the Carr group was 31.57 ± 1.41. VTT_{EiOH} and Indo had significant effects on the number of abdominal constrictions and

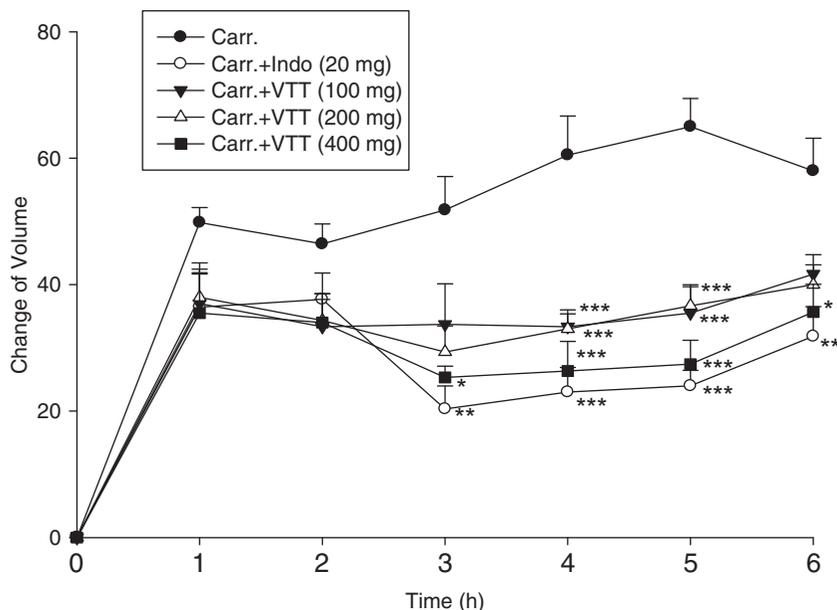


Fig. 2. Effects of VTT_{EIOH} and Indo on hind paw edema induced by Carr in mice. Values are the mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to the Carr group.

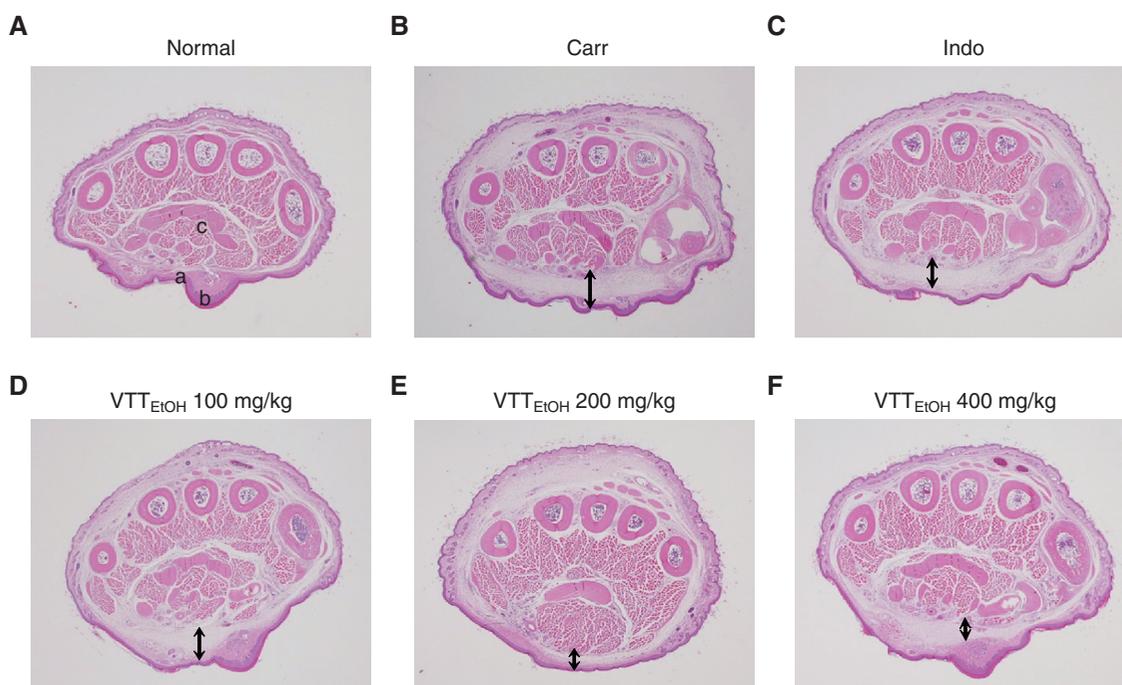


Fig. 3. Histopathological findings in footpad tissue sections obtained 4 h after injection with 1% Carr. (a: epidermis, b: connective tissue, c: muscle fibers). The connective tissue swelling was shown in the H&E stain data (20X). The gap distance is greater in Carr and Indo groups.

writhes. Pretreatment with 100 mg/kg (23.00 ± 1.37 , $P < 0.05$), 200 mg/kg (21.00 ± 2.70 , $P < 0.05$) or 400 mg/kg VTT_{EIOH} (20.83 ± 1.66 , $P < 0.01$), or 20 mg/kg Indo (11.33 ± 1.80 , $P < 0.001$) significantly reduced the writhing response. The acetic acid-induced writhing response in mice was significantly decreased by pretreatment in a dose-dependent manner.

The formalin-induced licking response has been reported to be biphasic. Centrally acting drugs can inhibit both phases, whereas peripherally acting drugs, such as NSAIDs, can inhibit only the second phase (28, 37). In the early phase of this study, neither VTT_{EIOH} nor Indo exhibited any antinociceptive activity compared to that in the control (Table 1).

Table 2. Effects of VTT_{EtOH} and Indo on TNF- α , IL-1 β , and IL-6 levels in Carr-induced paw edema

Groups	Tissue TNF- α (pg/mg protein)	Tissue IL-1 β (pg/mg protein)	Tissue IL-6 (pg/mg protein)
Carr	9431.85 \pm 1603.83	32185.93 \pm 5052.24	49108.55 \pm 5149.53
Carr +Indo 20 mg/kg	2807.75 \pm 436.82 ***	11083.33 \pm 1706.45 ***	8611.20 \pm 2080.48 ***
Carr +VTT _{EtOH} 100 mg/kg	4867.02 \pm 562.33 *	13213.13 \pm 1856.06 **	21417.71 \pm 1166.86 ***
Carr +VTT _{EtOH} 200 mg/kg	3101.52 \pm 416.40 ***	17048.28 \pm 1499.55 *	18045.32 \pm 1290.70 ***
Carr +VTT _{EtOH} 400 mg/kg	3963.87 \pm 533.64 **	17233.54 \pm 2518.45 *	21904.57 \pm 1641.03 ***

Values are the mean \pm SEM. * P < 0.05, ** P < 0.01, *** P < 0.001 compared to the Carr group.

In the later phase, the responses were significantly decreased by pretreatment with 100, 200 or 400 mg/kg VTT_{EtOH} (93 \pm 13.49, 89.50 \pm 10.41, and 91.77 \pm 13.71, respectively, P < 0.01), or 20 mg/kg Indo (88.88 \pm 15.09, P < 0.01). The licking responses after administration of VTT_{EtOH} at all three doses were similar to the response after administration of 20 mg/kg Indo (P < 0.01). However, the effects of VTT_{EtOH} on the later phase of the formalin-induced pain response appeared to not be dose-dependent.

The Carr-induced mouse paw edema model was used to evaluate the anti-inflammatory activity of VTT_{EtOH} (Fig. 2). At 3 h, only VTT_{EtOH} at 400 mg/kg (P < 0.05) and Indo (20 mg/kg, P < 0.01) significantly reduced paw edema. At 4 and 5 h, VTT_{EtOH} at 100, 200 and 400 mg/kg (P < 0.001) and Indo at 20 mg/kg (P < 0.001) significantly reduced paw edema volume. At 6 h, only 400 mg/kg VTT_{EtOH} (P < 0.05) exhibited nearly the same anti-inflammatory effect as that of 20 mg/kg Indo (P < 0.01). Therefore, at 4 and 5 h, VTT_{EtOH} at 100, 200, and 400 mg/kg showed almost the same anti-inflammatory effect as that of Indo (20 mg/kg).

The histopathological findings showed that VTT_{EtOH} slightly reduced the inflammatory response of paw edema and neutrophil infiltration (Fig. 3). For the histopathological examination, paw biopsies were harvested 4 h after Carr injection. Both VTT_{EtOH} and Indo significantly decreased Carr-induced paw edema. The paws of normal mice showed no inflammation, tissue destruction or swelling phenomenon (Fig. 3A). In contrast, the Carr-injected mice displayed enlarged cavities in the paw tissue (Fig. 3B). As for the positive control and the experimental groups, the edematous condition was obviously abated by treatment with Indo (20 mg/kg) and VTT_{EtOH} (100, 200, and 400 mg/kg), as shown in Figures 3C to 3F.

The experiment was also used to explore whether the anti-acute inflammatory effects of VTT_{EtOH} were associated with inhibition of the major cytokines or proteins involved in the inflammatory response. The effects of VTT_{EtOH} on TNF- α , IL-1 β and IL-6 levels are shown in Table 2. Compared with the results for the control group, a significant reduction in the TNF- α levels was observed in mice treated with 20 mg/kg Indo (2807.75 \pm 436.82, P < 0.001) and 100, 200 or 400 mg/kg VTT_{EtOH} (4867.02 \pm 562.33, P < 0.05; 3101.52 \pm 416.40, P < 0.001; and 3906.87 \pm 533.64, P < 0.01, respectively). Although the effects of the three VTT_{EtOH} doses did not show a standard dose-dependent response, VTT significantly inhibited the TNF- α levels. Pretreatment with 200 mg/kg VTT_{EtOH} showed a potent effect, similar to that of Indo. Compared to the control group, treatment with 100, 200 or 400 mg/kg VTT_{EtOH} (P < 0.01, P < 0.05 and P < 0.05, respectively), or 20 mg/kg Indo (P < 0.001) significantly reduced IL-1 β levels. Although the inhibitory effect of VTT_{EtOH} on IL-1 was slightly attenuated with increasing dose, the effect was still within the effective range.

A significant reduction in IL-6 was also observed following treatment with 100, 200 or 400 mg/kg VTT_{EtOH} (P < 0.001), or 20 mg/kg Indo (P < 0.001). The reduction observed following administration of VTT_{EtOH} at the three tested doses was similar to that of 20 mg/kg Indo.

VTT_{EtOH} attenuated NF- κ B protein expression in mouse paws injected with Carr (Figs. 4A & 4B). At 4 h, 100, 200 or 400 mg/kg VTT_{EtOH} showed inhibitory effects on NF- κ B protein expression to 80.72%, 84.74%, and 79.37%, respectively, of the untreated control; 20 mg/kg Indo also downregulated NF- κ B expression to 86.96% (P < 0.001).

VTT_{EtOH} and Indo also inhibited the expression

Table 3. Effects of VTT_{EtOH} and Indo on NO and COX-2 protein levels in Carr-induced paw edema

Groups	Tissue NO (nM protein)	Tissue COX-2 (pg/mg protein)
Carr	1086.95 ± 33.38	2.65 ± 0.17
Carr +Indo 20 mg/kg	501.65 ± 43.48 ^{***}	1.42 ± 0.08 ^{***}
Carr +VTT _{EtOH} 100 mg/kg	584.05 ± 50.98 ^{***}	1.55 ± 0.08 ^{***}
Carr +VTT _{EtOH} 200 mg/kg	682.94 ± 30.72 ^{***}	1.58 ± 0.06 ^{***}
Carr +VTT _{EtOH} 400 mg/kg	691.47 ± 33.75 ^{***}	1.64 ± 0.09 ^{***}

Values are the mean ± SEM. ^{***}*P* < 0.001 compared to the Carr group.

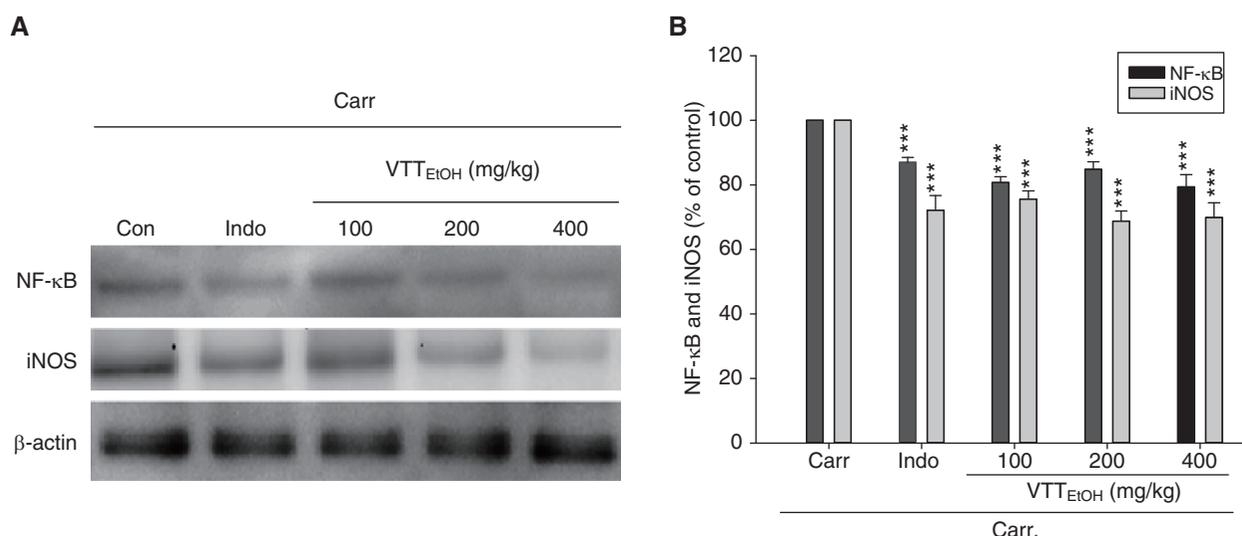


Fig. 4. Effects of VTT_{EtOH} and Indo on NF-κB and iNOS expression in the Carr-induced paw edema model at 4 h. Tissue suspensions were prepared and subjected to western blotting using antibodies specific for NF-κB and iNOS. Beta-actin was used as an internal control. (A) A representative western blot from three separate experiments is shown. (B) Relative NF-κB and iNOS protein levels were calculated with reference to the levels of the Carr-injected mice. Data are the mean ± SEM of three experiments performed in triplicates. ^{***}*P* < 0.001 compared to the Carr-alone group.

of iNOS in mouse paws with Carr-induced edema (Figs. 4A & 4B). The results showed that administration of VTT_{EtOH} at 100, 200 and 400 mg/kg inhibited iNOS protein expression to 75.61%, 68.78% and 69.87%, respectively, of the control level (*P* < 0.001) associated with Carr-induced mouse paw edema at 4 h. Indo at 20 mg/kg also significantly inhibited iNOS protein expression (72.14%, *P* < 0.001).

iNOS stimulates NO formation in a Carr-induced paw edema model in mice. The nitrite level was continuously monitored in the Carr group of mice (Table 3). Pretreatment with 100, 200 or 400 mg/kg VTT_{EtOH} (584.05 ± 50.98, 682.94 ± 30.72, and 691.47 ± 33.75, respectively, *P* < 0.001), or 20 mg/kg Indo (501.65 ± 43.48, *P* < 0.001), significantly inhibited the increase in NO levels associated with mouse paw edema when compared to the levels in the mice in the Carr group (1086.95 ± 33/38 nM).

The COX-2 level markedly increased (2.65 ± 0.17 pg/mg) in Carr-induced paw edema (Table 3). After treatment with 20 mg/kg Indo (1.42 ± 0.08, *P* < 0.001) or VTT_{EtOH} at 100, 200 or 400 mg/kg (1.55 ± 0.08, 1.58 ± 0.07, or 1.64 ± 0.09, respectively; *P* < 0.001), a significant decrease of COX-2 levels was observed (Table 3). Although reduced levels of NO and COX-2 were observed in mice administered the three doses of VTT_{EtOH}, the levels were within the error range.

Discussion

In relation to its folk application as an analgesic for muscle and ligament injuries, VTT_{EtOH} was tested using experimental animal models to confirm its efficacy and determine its pharmacological activities and to determine the underlying mechanisms.

Though many Scholars reported that resveratrol existed in VTT, resveratrol content was seldom to be investigated. Tsai *et al.* (2014) reported that resveratrol content (mg/g) in water extract from root and stems of VTT was 1.1 mg/g (35). Additionally, quercetin remains to be identified and be quantified in order to prove its importance in VTT. In this study, the resveratrol and quercetin contents in VTT_{EiOH} were qualitatively and quantitatively analyzed in the same time. This method is fast and accurate. The resveratrol content (1.6147 $\mu\text{g}/\text{mg}$) in VTT_{EiOH} shows the better extraction method. The quercetin content (0.2461 $\mu\text{g}/\text{mg}$) in VTT_{EiOH} provides the important reference to quality control.

In animals, acetic acid indirectly triggers the release of nociceptive endogenous mediators, such as serotonin and PGs, and proinflammatory cytokines, such as TNF- α and IL-1 β , to cause a painful sensation (24). It is also an established model of visceral pain that involves processing *via* COX to release arachidonic acid, which causes increased swelling and pain (10). This pain can be inhibited by NSAIDs or other central nerve inhibitors. In this study, VTT_{EiOH} at 100-400 mg/kg quickly relieved abdominal pain in a similar dose-dependent manner as it decreased the writhing response. Thus, we inferred that the antinociceptive effect of VTT_{EiOH} may be related to COX-2 production and a subsequent effect on arachidonic acid metabolite synthesis. As VTT_{EiOH} is further separated and purified, it may be possible to obtain a similar effect at a lower dose.

The formalin test can effectively assess the effects of a drug on inflammatory and non-inflammatory pain. In the formalin experiment, neither VTT_{EiOH} nor Indo exhibited any antinociceptive activity compared to that reported for the control in the early phase (Table 1). However, in the later phase, the responses were significantly decreased by VTT_{EiOH} pretreatment or Indo. However, the effect of VTT_{EiOH} on reducing the licking response in the later phase was not dose-dependent, and the dose-time relationship curve should be further explored.

The inflammatory reactions during the late phase of the formalin-induced model and in the Carr-induced mouse paw edema model are similar. The Carr-induced inflammation model is a widely employed model of inflammation used for screening anti-inflammatory drugs, and most studies used paw edema as the dependent measure of inflammation (39, 41). This *in vivo* model also has two phases (42). Histamine, bradykinin, and 5-hydroxytryptamine are released in the first phase of edema (0–1 h), and then TNF- α , IL-1 β , COX-2, and PGs are produced and the degree of swelling increases in the second phase (1–6 h). Usually, edema in the hind paw peaked at 3 h post injection (2). Our experimental results showed

that only VTT_{EiOH} at 400 mg/kg and Indo (20 mg/kg) significantly reduced paw edema at 3 and 6 h. In contrast, at 4 and 5 h, VTT_{EiOH} and Indo at the tested concentrations significantly reduced paw edema volume (Fig. 2). VTT_{EiOH} exhibited its effects at 3 h, which plateaued at 4–5 h, and declined after 6 h. At 4–5 h, the most effective period, the minimum effective dose was 100 mg/kg.

Microscopic examination of paw sections from Carr-injected rats revealed dense inflammatory infiltrates, mainly lymphocytes and plasma cells, in the interstitial and prevascular spaces and between muscle bundles (11). This is evidenced by the decrease of paw edema induced by Carr and the presence of several inflammatory mediators known to participate in the Carr response and myeloperoxidase activity, as well as the amelioration of inflammatory histopathological changes (8). As shown in Figure 3, Carr-induced mouse paw edema decreased after VTT_{EiOH} treatment. The intercellular spaces in the connective tissues of the paws also obviously decreased. Therefore, histopathological examination provided a secondary evidence supporting the anti-inflammatory effect of VTT_{EiOH}. Vazquez *et al.* (38) demonstrated that Carr-induced paw edema has systemic implications, characterized by increased levels of acute phase proteins and lung inflammatory responses, including lung edema, fibrin deposition and leukocyte infiltration (38).

As described above, VTT_{EiOH} has anti-inflammatory effects. The results of this study indicated that the antinociceptive effect of VTT_{EiOH} may be similar to that of peripherally acting drugs and may be due to its anti-inflammatory effects.

Recent studies have shown that Carr induces the release of specific pro-inflammatory cytokines, namely TNF- α , IL-1 β , and IL-6. These cytokines play a critical role in edema formation, mechanical allodynia, neutrophil migration and pain hypersensitivity. However, few studies have addressed the role of these key cytokines in Carr-induced inflammation (2). In this work, a significant reduction in TNF- α levels was observed following treatment with VTT_{EiOH} (Table 2). Although the reductions induced by VTT_{EiOH} were not dose-dependent, VTT_{EiOH} did significantly inhibit TNF- α . The reduction induced by VTT_{EiOH} was slightly attenuated with increasing dose; however, it was still within the effective range at the highest tested dose. The three doses of VTT_{EiOH} also significantly decreased IL-6 levels, similar to Indo.

TNF- α is a potent proinflammatory cytokine produced by activated macrophages that promotes IL-1 β production, which contributes to upregulation of β -nerve growth factor (NGF) and hypersensitivity to inflammatory pain (2, 25,

36). Additionally, some researchers have reported that TNF- α and IL-1 β are involved in neutrophil migration, and the mRNA expression levels of IL-1 β , IL-6 and TNF- α were elevated in rat paw muscle tissue at 3 h after Carr injection. However, IL-6 induces the production of the pro-inflammatory cytokines TNF- α and IL-1 β , indirectly augmenting iNOS and COX-2 protein expression and activity, and ultimately the degree of peroxynitrite formation and tissue injury (29). The results of this study showed that different doses of VTT_{EIOH} indeed reduced the activity of TNF- α , IL-1 β and IL-6, although the efficacies were different. However, the effect of VTT_{EIOH} on IL-6 in the Carr-induced model peaked within the “plateau period” at 4–5 h.

After treatment with VTT_{EIOH}, the levels of TNF- α , IL-1 β and IL-6 decreased, and the protein expression of NF- κ B and iNOS was inhibited (Fig. 4). At 4 h, VTT_{EIOH} showed significant inhibitory effects on NF- κ B and iNOS protein expression that were similar to the effects of Indo. NF- κ B, a crucial transcription factor, can regulate the expression of COX-2 and iNOS (1, 22). In turn, COX-2 can increase PG levels to promote inflammatory reactions (31), and iNOS produces large amounts of NO in response to inflammatory and nociceptive events (29).

Pretreatment with VTT_{EIOH} or Indo significantly inhibited the increase in NO and COX-2 levels in the mouse paw edema model. Although inhibitory effects of the three different doses of VTT_{EIOH} on NO and COX-2 were observed, they were still within the error range. During the plateau period (4–5 h after Carr-injection), VTT_{EIOH} showed the maximum anti-inflammatory effect, and maximum effects were also observed on NF- κ B, iNOS, NO, and COX-2 levels. COX-2 activity was the highest in the late phase of Carr-induced paw edema, which increased the PG levels (31). Reduction of iNOS and COX-2 expression to suppress NO and PG levels may be beneficial for treating inflammatory diseases (6).

Accordingly, the antinociceptive effect of VTT_{EIOH} may be similar to that of peripherally acting drugs, and may be due to its anti-inflammatory effect. The molecular mechanisms underlying the anti-inflammatory effect of VTT_{EIOH} may involve inhibition of the activity of cytokines, such as TNF- α . TNF is one of the most effective physiological inducers of NF- κ B, and TNF and NF- κ B influence each other (30). In addition, VTT_{EIOH} inhibited Carr-induced mouse paw edema at 4 h as well as NF- κ B and iNOS protein expressions. It also indirectly affected the activities of NO and COX-2. iNOS is regulated by TNF- α and NF- κ B (1, 29). Consequently, another putative mechanism for the anti-inflammatory effects of VTT_{EIOH} may involve inhibition of NF- κ B.

NF- κ B may play a key role in the anti-inflammatory effects or anti-inflammatory analgesic abilities of VTT_{EIOH}. This mechanism is similar to that reported by Killeen *et al.* (16). Killeen *et al.* also presented that the regulation and control of NF- κ B activation is a potential therapeutic strategy for the prevention and treatment of NF- κ B-related human inflammatory diseases (16).

In addition to its analgesic and anti-inflammatory effects, Indo has many undesirable side effects. In contrast, in the current study, even a high dose of VTT_{EIOH} (5 g/kg) did not cause any mortality or behavioral changes in the mice, which indicated that acute toxicity was undetectable in this test sample. The active ingredients and their chronic toxicity and safety to clarify the commercial development value of VTT_{EIOH} should be further evaluated.

Conflict of Interest

The authors declared no conflicts of interest.

Acknowledgments

The authors extend special thanks to Dr. Fu-Du Chen, Dr. Yi-Hung Chiang, Dr. Wen-Te Chang, Chia-Fang Hsu, Dr. Shidoaseeing (Chiu-Chuan Chien) and Chi-Rei Wu for their contributions to the present study.

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