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# Studies on the Anti-Inflammatory Effects of *Clerodendron trichotomum* Thunberg Leaves

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*Clerodendron trichotomum* Thunberg Leaves (CTL) have been used for centuries in Chinese folk medicine for their anti-inflammatory properties. We have studied the anti-inflammatory effects of CTL extracts in rats, mice and in Raw 264.7 cells. 1 mg/kg solutions of the 30% and 60% methanol extracts of CTL were used and a 1 mg/kg of indomethacin was used as a positive anti-inflammatory standard; these were then administrated to rats. Carrageenan was injected subcutaneously to induce hind paw edema in rats. The result of carrageenan-induced rat paw oedema showed that a 1 mg/kg of the 30%, and 60% methanol fraction of CTL and 1 mg/kg of indomethacin inhibited the hind paw edema by 19.5%, 23.0%, and 20.5% respectively. The effect of CTL on inflammation in mice by a capillary permeability assay was examined by detecting Evans blue leakage from capillaries after the intraperitoneal injection of acetic acid, a potent inflammatory stimulus. The 60% methanol fraction of CTL inhibited Evans blue dye leakage by 47.0%, which was 10% higher than that of the inhibition of 1 mg/kg of indomethacin. Also, the 60% methanol fraction of CTL suppressed the prostaglandin $E_2$ ($PGE_2$) generation in RAW 264.7 macrophage cells after treatment with lipopolysaccharide (LPS) by as much as the inhibition of 1 mg/kg of indomethacin and this led to the synthesis of $PGE_2$ by COX-2 induction. The inhibition of the carrageenan-induced rat paw oedema, vascular permeability and the $PGE_2$ generation demonstrates that the 60% methanol fraction of CTL contains a potent anti-inflammatory activity.

**Key words:** Anti-inflammatory effect, Capillary permeability, $PGE_2$

## INTRODUCTION

*Clerodendron trichotomum* Thunberg (Verbenaceae) is a native plant commonly found in China, Korea, and Japan. The stems and leaves of this plant have been used in folk remedies for the treatment of inflammation for centuries (Lee, 1993; Kim, 1996). Inflammation is an important part of the body's defense reaction for the repair of damaged tissue. It is divided into acute and chronic inflammation, the latter of which is a repeated acute inflammatory reaction or is developed from an acute inflammation. Acute inflammation occurs in the vessels supplying blood to the damaged area and it is controlled by hemodynamic and permeability changes, leukocytes and phagocytosis. These processes are mediated by chemokines, which are the vasoactive mediators released from leukocytes or by products from the plasma enzyme systems. These mediators cause an increase in vascular permeability, redness and pain. They also cause an increase in levels of vasoactive amines and leukotriene in the vessel walls, the tissue that's damage by oxygen-derived free radicals and the macrophages that engulf or kill bacteria and damaged tissue fragments; in addition, old neutrophils are activated and modulate the inflammatory response. Systemic symptoms of inflammation are fever, weakness, a loss of appetite, chills, and arthralgia. Local symptoms of inflammation are redness, swelling, heat, pain, and loss of function. Studies of the anti-inflammatory reaction are based on local symptoms. In our experiments, we investigated the anti-inflammatory effects of the 30% and 60% methanol extracts of *Clerodendron trichotomum* Thunberg Leaves (CTL) versus indomethacin, which is used as a positive anti-inflammatory standard. Indomethacin is a nonsteroid also known as heterocyclic acid, suppresses prostaglandin synthesis by inhibiting cyclooxygenase-2

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(COX-2) (Han *et al.*, 2001). Exudates that accumulate in damaged areas cause the expression of inflammatory mediators (Yesilada *et al.*, 2002). For our *in vivo* experiments, we injected carrageenan, an inflammatory inducer into the right hind paw of a test group treated with the methanol extracts of CTL and into members of a control group and measured the swelling to compare oedema inhibition. Also, Evans blue release into the abdominal cavity was used to detect any inhibition of capillary permeability after the injection of 0.6% acetic acid. In an *in vitro* experiment, we compared $PGE_2$ production in RAW 264.7 macrophages induced by LPS (lipopolysaccharide). (Pilbram *et al.*, 1993). $PGE_2$ is related to COX-2, which is an isoform of COX. COX-1 is expressed in all tissues and cells and is present at the luminal side of the ER (Endoplasmic reticulum) (DeWitt *et al.*, 1988; Merlie *et al.*, 1988; Funk *et al.*, 1991; Otto *et al.*, 1994) whereas COX-2 is present at the perinuclear envelope as well as the ER (Morita *et al.*, 1995).

## MATERIALS AND METHODS

### Plant material

CTL plants collected from Naejangsan (Mt.) in July 2001 were identified by Professor Wan-Kyun Whang (Chung-Ang University). Voucher specimens were deposited in the Pharmacal Resources Botany Laboratory (CTL-360). CTL plants were verified botanically and dried in the dark for these experiments.

### Extract of the methanol-soluble fraction

The preparation of the methanol soluble fraction from CTL is shown in Fig. 1. 600 g of dried CTL was milled, extracted with methanol/water (70:30 v/v) and then concentrated to a 58 g extracts under a vacuum. Extracts were then resuspended in 500 mL of hot distilled water and next fractionated with ether yielding 2.4 g of ether extracts and 44.5 g of water extracts. The water extracts were subjected to chromatographic separation with Amberlite XAD-2 and elution was performed using a methanol/water gradient, which yielded a 30% and a 60% methanol fraction.

### *In vivo* tests

#### Animals

Male Sprague-Dawley rats (190-250 g) and Swiss albino mice (25-30 g) were purchased from an animal breeding company (Daehan Biolink, Seoul, Korea). The animals were left for 2 days to acclimatize and were maintained on a standard pellet diet and water. Food was withdrawn on the day before the experiment, but the animals were allowed a free access to water.

#### Preparation of test samples for bioassay

Test samples, indomethacin, and Evans blue (after suspending in water and physiological saline of 0.9% NaCL) were given orally to test animals. Oral doses of extract were made at 1 mg/kg. The animal control group received the same experimental handling as animals of the test group, except that the drug treatment was replaced with appropriate volumes of the dosing medium.

#### Carrageenan-induced oedema

To determine effects on acute inflammation, the carrageenan-induced paw oedema model described by Kasahara *et al.* (1985) was used, with some modifications. Sixty minutes after the oral administration of either the test sample or indomethacin, each rat was injected with a freshly prepared (1 μg/100 μL) suspension of carrageenan (Sigma, St. Louis, Missouri, USA) in physiological saline (0.9% NaCl) into the sub plantar tissue of the right hind paw. Paw oedema was measured in every 60 minutes over the next 5 h after the injections. Differences in footpad thickness were measured using a

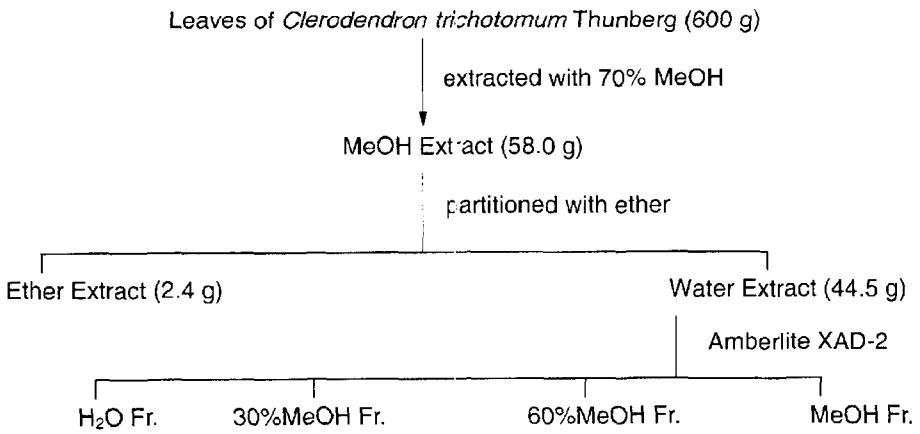


**Fig. 1.** Extraction and fractionation of *Clerodendron trichotomum* Thunberg Leaves

digital gauge caliper. Mean values of the treated groups were compared with those of the control group and the results were then analyzed.

**Acetic acid induced increase in capillary permeability**

The effect of the test samples on the vascular permeability induced by acetic acid in mice was determined according to the Whittle method with some modifications (Yesilada *et al.*, 1988). Each test sample (1 mg/kg body weight) was administered orally to 5 mice. Thirty minutes after this administration, each mouse was injected intravenously into the tail vein with 0.1 mL of 4% Evans blue dye solution in saline solution. Ten minutes after this injection of dye solution, 0.5 mL of 0.6% (v/v) acetic acid was injected intraperitoneally. Twenty minutes later, the mice were killed by neck dislocation, and the viscera were irrigated with distilled water. This was then collected and centrifuged at 2,000 rpm for 10 min. The supernatants were increased in volume to 10 mL with distilled water and 0.1 mL of 0.1 N NaOH solution was added to clear any turbidity. The adsorption of the washing solution was measured at 590 nm. In the control animals, a mixture of distilled water and saline was given orally, and then the animals were treated in the manner described above.

### *In vitro* test

**Cell culture**

The murine macrophage/monocyte cell line RAW 264.7 macrophages (Korean Cell Line Bank, Seoul, Korea) were maintained at 37°C and 5% $CO_2$ in DMEM containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 μg/mL penicillin and 100 μg/mL streptomycin. Cells were plated at a density of $1.0\times10^6$ cells/mL.

**LPS-induced $PGE_2$**

$PGE_2$ production was measured in the culture medium in order to determine COX-2 activity (Han *et al.*, 2001). For the $PGE_2$ induction assay, RAW 264.7 macrophages were plated into 24-well plates at a density of $5.0\times10^5$ cells/well in 1 mL of DMEM and then treated with 500 μM of acetylsalicylic acid (Asprin) to inactivate COX-1. After 2 h incubation, the cells were washed 3 times with fresh DMEM (5% heat inactivated fetal bovine serum, 2 mM glutamine, 100 μg/mL penicillin and 100 μg/mL streptomycin) and the culture media were replaced with fresh DMEM containing LPS (1 mg/mL) and test samples (1 mg/mL) or LPS (1 μg/mL) and indomethacin samples (1 mg/mL). After 16 h culture, the culture supernatants were either used immediately or stored at -70°C until required for $PGE_2$ determination. $PGE_2$ concentration was measured using a commercial competitive enzyme immunoassay kit (Cayman Chem. Co., Ann Arbor, MI, USA) according to the manufacturer's protocol.

**Statistical analysis of data**

Data obtained from animal experiments were expressed as mean standard error (SEM). Statistical differences between the treatments and the control were tested using the two tailed Student's *t*-test. $P<0.01$, or $P0.05$ was considered to be significant.

## RESULTS AND DISCUSSION

**Carrageenan-induced oedema**

The anti-inflammatory effect of the methanol fraction of CTL on the carrageenan-induced hind paw oedema model is shown in Table I. Since oedema gradually developed over a 2-4 h and was stable after 4-5 h, we compared the anti-inflammatory effects over this period. The anti-inflammatory effect was most pronounced during the 5$^{th}$ h of the inflammatory response and the 30% and 60% methanol extracts of CTL (1 mg/kg) inhibited oedema by 19.5% and 23.0%, respectively compared with the control group. The 1 mg/kg of the standard anti-inflammatory drug, indomethacin inhibited oedema by 20.5%. In particular, the anti-inflammatory effect of the 60% methanol extract of CTL was 2.5% more than that of indomethacin. These results indicate that a 60% methanol extract of CTL has potent anti-inflammatory activity. It has been reported that carrageenan is toxic to macrophages and reduces the phagocytosis of alveolar macrophage and circulating mononuclear cells (Tobacman, 2001). These results show

**Table I.** Effect of the methanol extracts from CTL on carrageenan induced paw edema in rats

| Group | Dose (mg/kg) | N [a] | Swelling thickness (mm) (Inhibition%)[b] | | | | |
|---|---|---|---|---|---|---|---|
| | | | 1 h | 2 h | 3 h | 4 h | 5 h |
| Control [c] | | 5 | 1.6±0.4 | 2.7±0.3 | 3.6±0.4 | 3.8±0.3 | 3.89±0.3 |
| Indomethacin | 1 | 5 | 1.6±0.4 | 2.2±0.4 | 2.9±0.4 | 3.1±0.4 | 3.18±0.4 (20.5)* |
| 30% Extract | 1 | 5 | 1.6±0.7 | 2.2±0.6 | 2.6±0.7 | 2.9±0.7 | 3.07±0.6 (19.5)* |
| 60% Extract | 1 | 5 | 1.2±0.3 | 2.2±0.5 | 2.4±0.4 | 2.6±0.4 | 2.74±0.3 (23.0)* |

a) N means the number of animals in each group.
b) Aberage value of three experiments
c) Treatment with 0.9% saline solution (0.5 mL)
*$P<0.05$, **$P<0.001$

**Table II.** Effects of the active extracts on increased vascular *permeability induced by acetic acid in mice*

| Group | Dose (mg/kg) | N[a] | Evans blue Conc. (μg/mL)±SEM [b] | Inhibition(%)[b] |
|---|---|---|---|---|
| Control [c] | | 5 | 17.0±2.2 | 0 |
| Indomethacin | 1 | 5 | 10.7±1.5 | 37.0* |
| 30% Extract | 1 | 5 | 12.6±2.1 | 23.7* |
| 60% Extract | 1 | 5 | 9.1±0.8 | 47.0** |

a) N means the number of animals in each group.
b) Average value of three experiments
c) Treatment with 0.9% saline solution (0.5 mL)
*$P<0.05$, **$P<0.01$

that the methanol extracts of CTL inhibit the oedema induced by carrageenan by reviving the damaged macrophages.

### Acetic acid-induced increase in capillary permeability

The antis-inflammatory effect of CTL extracts by capillary permeability assay was determined by detecting Evans blue leakage from the capillaries after the intraperitoneal injection of acetic acid, a potent inflammatory stimulus. As shown in Table II, the concentration of Evans blue in the group injected with the 30% methanol extract of CTL (1 mg/mL) was 12.65 μg/mL, which was 23.7% lower than the control group. In a test group injected with a 60% methanol extract of CTL, the concentration of Evans blue was 9.1 μg/mL and dye leakage was reduced by 47.0% versus the control group. On the other hand, indomethacin inhibited the capillary permeability by 37.0%. Therefore, the 30% and 60% methanol extracts of CTL exhibited potent anti-inflammatory activities by blocking capillary expansion. Moreover, the inhibition of capillary permeability in a group injected with a 60% methanol extract of CTL was 10% more than that observed in a group injected with the anti-inflammatory drug indomethacin. This result suggests that the 60% methanol extract of CTL has a significant anti-inflammatory effect. Capillary expansion induces a blood pressure increase and causes pain and redness. Therefore, we believe that the methanol extracts of CTL are potentially of great benefit for the management of pain or arthritis by suppressing vascular permeability. The results obtained show that methanol extracts of CTL have potent anti-inflammatory activity, which is entirely consistent with the ability of extracts to inhibit rat a paw oedema that's induced by carrageenan.

### LPS-induced $PGE_2$

$PGE_2$ was measured in the supernatant of cultured RAW 264.7 macrophages after LPS induction. As shown in Fig. 2, the cells treated with the 30% and the 60%

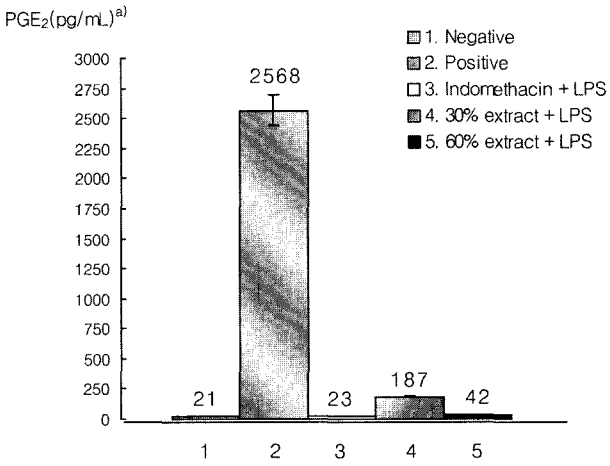


**Fig. 2.** $PGE_2$ concentration in RAW 264.7 macrophages treated with LPS. $PGE_2$ in the supernatant of cultured RAW 264.7 macrophages after LPS induction was measured. Negative indicates only distilled water. Positive represents only LPS (1 μg/mL). a) Average value of three experiments.

methanol extracts of CTL (1 mg/mL) produced 187 pg/mL and 42 pg/mL of $PGE_2$ respectively. Cells treated with the potent anti-inflammatory agent, indomethacin produced 23 pg/mL, and cells treated with the 60% methanol extract showed a 61 fold reduction in $PGE_2$ synthesis versus the control and a 1.8 fold increase in $PGE_2$ synthesis versus cells treated with indomethacin. In particular, the inhibition of $PGE_2$ production by the 60% methanol extract was similar to that caused by indomethacin. These results demonstrate that the methanol extracts of CTL has an anti-inflammatory effect. Since the nonsteroidal anti-inflammatory drug indomethacin causes side effects by inducing COX-1 (Meade *et al.*, 1993), cells were pretreated with acetylsalicylic acid to irreversibly inactivate COX-1 by acetylation (Lecomte *et al.*, 1994; Vane, 1994). The anti-inflammatory mechanism of indomethacin reduces $PGE_2$ production by inhibiting prostaglandin synthetase. Therefore, indomethacin inhibits $PGE_2$ production in RAW 264.7 cells more so than the methanol extracts of CTL. Nitrogen Oxide production stimulated by LPS, Fas and TNF-α may also be inhibited by the methanol extracts of CTL, which could be used for the treatment of asthma and malaria (Leme *et al.*, 2002), but these are issues for further study.

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