**Anti-Inflammatory And Anti-Oxidant Activities Of Glochidion Daltonii Branch Extract**

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**Abstract**

Background: Glochidion daltonii (MÜll. Arg.) Kurz, Euphorbiaceae family, is a native medicinal plant in tropical regions of Asia. In Thailand, it has traditionally been used for treating pain and mouth inflammation. Objective: This study aims to investigate the anti-inflammatory and anti-oxidant effects of G. daltonii ethanolic extract (GDE) both in vitro and in vivo. Methods: The anti-inflammatory mechanism was examined in E.coli LPS-stimulated RAW264.7 cells by using semi-quantitative-reverse transcription-polymerase chain reaction and the potential anti-inflammatory effect of GDE was evaluated in Sprague-Dawley rat using carrageenan-induced rat paw edema method. In addition, DPPH assay was used for anti-oxidant evaluation. Results: The results showed that GDE at 0.063-0.250 mg/mL concentrations inhibited the expressions of tumor necrosis factor-α and interleukin-1β. Moreover, an intraperitoneal administration of GDE significantly reduced paw edema in rats. Furthermore, GDE showed high anti-oxidant property with IC₅₀ values at 6.35 ± 0.28 µg/mL. Conclusions: The results support the traditionally use of GDE for treating inflammation.

**Keywords:** glochidion daltonii, anti-inflammation, anti-oxidation

**INTRODUCTION**

Inflammation is a host defense mechanism to eliminate pathogens and to initiate healing process, but the uncontrolled or over production of inflammatory products can lead to injury of host cells, chronic inflammation and also chronic diseases (Mantovani et al., 2008). In inflammatory process, several reports demonstrated the participation of various biomarkers such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6 and cyclooxygenase (COX)-2. The TNF-α, a pro-inflammatory cytokine, is a key cytokine that plays role as a master regulator of inflammatory cytokine production involved in inflammation (Balkwill, 2009). In addition, TNF-α, IL-1 and IL-6 also play roles as endogenous pyrogens by stimulating the release of prostaglandins (Kagiwada et al., 2004). Besides, The inflammation that response to pathogen invasion, there are several free radicals occur in this process such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Aggarwal, 2004). These free radicals have high impact on physiological and pathological status by inducing oxidative stress mediated inflammatory response in several tissues (Kolls, 2006). Therefore, the substance that shows high anti-oxidative activity has high possibility to be anti-inflammatory agent.

Nowadays, various chemicals of plant show anti-oxidant and anti-inflammatory activities (Chi et al., 2001; Sripanidkulchai et al., 2009). In this study, we selected Glochidion daltonii extract (GDE) because it has traditionally been used for treating pain and mouth inflammation and our previous study revealed that GDE contained several phenolic compounds which may relate to anti-oxidant and anti-inflammatory activities. Therefore, in the present study, we investigated the anti-oxidant and anti-inflammatory activities of GDE using in vitro and in vivo studies.

**MATERIALS AND METHODS**

**Reagents**

In this study, the chemicals used were Molecular Biology Agarose (Bio-Rad, Spain), 1kb DNA ladder (Promega, USA), Blue/Orange 6X Loading dye (Promega, USA), Primer (Promilgo LLC, Boulder, co, USA), Tris base, Glacial acetic acid, EDTA (Ajax/Australiap), Omiscript RT Kit (QIAGEN), TopTaq MasterMix kit (QIAGEN) and Novel Juice (GeneDirex), RNA extraction kit (GE Healthcare, UK) was used to extract total RNA from the cells. DMEM media (Invitrogen, USA), FBS (Invitrogen, USA), Penicillin-streptomycin (Invitrogen, USA), Carrageenan (Fluka, Switzerland), dicrofenoacidiethy-lamoniumsult (Votarens Emulgel, Thailand), Escherichia coli lipopolysaccharides (LPS) (Sigma, USA), MTT (Invitrogen), DPPH (Sigma, USA) also were used in this study.

**Preparation of GDE**

The branch of G. daltonii was macerated in ethanol and then concentrated using a rotary evaporator and freeze-dried.

**Determination of DPPH radical scavenging activity**

The radical scavenging activity was determined by DPPH method (Shimada et al., 1992). The negative (methanol) and positive (vitamin C or vitamin E) controls were parallel run.

**Determination of phenolic content**

The total phenolic content was determined by the Folin-Ciocalteu method (Singleton et al., 1999).
Sample (5 mg) was dissolved with methanol and up to 1 mL, and then the sample solution was mixed with 0.25 mL of the 1N Folin-Ciocalteau reagent and 1.25 mL of 20% sodium carbonate. After mixing and standing for 40 minutes at the room temperature, the optical density was measured at 725 nm. The total phenolic contents were expressed as mg tannic acid equivalent (TAE)/g dry basis.

Cell culture
The murine macrophage cell line, RAW264.7 cells, was purchased from PromoCell, Germany. The cells were cultured in DMEM media supplemented with 10% heat-inactivated calf serum (HyClone, USA) and 1% penicillin (100 U/mL)-streptomycin (100 μg/mL) and incubated at 37°C in a humidified atmosphere with 5% CO2.

Animals
Male Sprague-Dawley rats, 7–8 weeks old, weighing 280–320 g was obtained from the National Animal Center, Mahidol University. The rats were housed separately 3–5 animals per hanging cage and maintained in air-conditioned room with a12-h light/dark cycle. A commercial pellet food and tap water were given ad libitum to animals. The experimental protocol was approved by the Animal Ethics Committee of Khon Kaen University (recordnumberAEKKU10/2556 and reference number 0514.1.12.2/8).

Cytotoxicity test
Macrophage RAW264.7 cells were treated with various concentrations of extract and then incubated at 37°C in the humidified atmosphere with 5% CO2 for 24h. Cell viability was analyzed by using MTT assay (Mosmann, 1983) and the absorbance measured at 570 nm. The results were calculated for % inhibition and expressed as %0 inhibitory concentration.

Determination of inflammatory-related gene expression
The cells were overnight cultured in 12-well plate and treated with various concentrations of extract and positive control. After incubation at 37°C in the humidified atmosphere with 5% CO2 for 22 hr, the LPS was added then further incubated for 24 h. Total RNA was extracted from the treated cells by using a GE Healthcare extraction kit. The first-strand cDNA was synthesized from total RNA (40ng) with Omniscript reverse transcriptase kit. The primers were used for amplifying the respective fragments. Polymerase chain reaction (PCR) was performed by incubation of each cDNA sample with the primers, Taq polymerase, and deoxynucleotide mix. Amplification was completed for 30 cycles and the conditions for PCR amplification followed previous reports (Sugawara et al., 2003; Won et al., 2006). The PCR products were then analyzed on 1.5% agarose gel, visualized by Novel Joice staining and RT-PCR product densities measured by Gel Documentation and System Analysis machine. The inflammatory-related gene expressions were calculated for the relative mRNA expression level compared with β-actin.

Carrageenan-induced rat paw edema assay
The rats were anesthetized throughout the experiment by intraperitoneal injection with 100 μL thiopental sodium (50 mg/Kg body weight). The animals were randomly divided into five groups of five animals or ten paws each. The animal paws were injected with 0.15 mL of 0.1% carrageenan into the sub plantar region just below the lateral malleolus of both left and right paws (Sripanikulchaisri et al., 2009) to induce the paw edema. Immediately after carrageenan injection, the animal paws were spread with five treatment conditions including Group1: distilled water as a negative control, Group 2: diclofenac as a positive control, Group 3–5: GDE treated group with various concentration. The edema was evaluated by using a plethysmometer (UgoBasile, model1740) before and after treatment. The swelling of paw was measured immediately and then hourly up to 6 hr.

Statistic analysis
All in vitro experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD). For in vivo study, the data were compared between treated groups and vehicle group and the results were expressed as mean ± S.D. One-Way ANOVA and multiple comparisons were used to analyze the significant difference by using SPSS version 19.0 software.

RESULTS AND DISCUSSION

Anti-oxidant activity

DPPH, a stable free radical with a characteristic absorption at 515 nm, was used to study the radical scavenging effects of GDE. When antioxidants donate protons to this radical, the decrease in absorption is taken as a measure of the extent of radical scavenging. The IC50 value for GDE was 6.35 ± 0.28 μg/mL which was very close to Vit.C and Vit.E as shown in Table 1.

| Table 1. Phenolic content, antioxidative activity by DPPH determination of GDE |
|-----------------------------|------------------|-------------------|-------------------|
| Test sample | Yield (%) | Total phenolic content | DPPH (IC50 (μg/mL)) |
| GDE | 1.86 ± 0.79 | 449.03 ± 32.63 | 6.35 ± 0.28 (0.983) |
| Vit.C | - | - | 3.21 ± 0.10 (0.998) |
| Vit.E | - | - | 2.99 ± 0.09 (0.994) |

Values are expressed as mean ± SD (n=3)
Toxicity effect of GDE on RAW 264.7 cells

The effect of GDE on the viability of RAW264.7 cells was determined using MTT assay. The cells were treated for 24 hr with various concentrations of GDE at 0.03-0.5 mg/mL. Figure 1 showed toxic effects on RAW264.7 cells as IC_{50} value (51 mg/mL). Based on these results, we evaluated the effect of GDE on anti-inflammation at doses lower than its IC_{50} value.

![Figure 1](image1.png)

**Figure 1.** Effect of GDE on RAW264.7 cells viability. After 24 hr of incubation with various concentrations of GDE, the cell viability was measured with MTT. Each value is a mean ± SD compared to control from three individual experiments.

Effect of GDE on the pro-inflammatory gene expression

The expression of the pro-inflammatory gene were not changed when treatment with GDE alone but were up-regulated after treatment with LPS compared with control. The gene expression of TNF-α was significantly suppressed by GDE in a higher level than that of indomethacin on TNF-α gene expression (Figure 2A). Similarly, GDE also exhibited significant suppressive effect on the expression of IL-1β (Figure 2B). However, GDE had no effect on the expression of COX-1 (Figure 2C). These result suggests that GDE has a selectively to suppress TNF-α and IL-1β.

![Figure 2](image2.png)

**Figure 2.** Inhibitory effect of GDE on mRNA expression of pro-inflammatory mediators. Relative mRNA expression of TNF-α (A), IL-1β (B), COX-1 (C) compared with β-actin mRNA expression and the amplified bands of TNF-α, IL-1β and COX-1 (D). * Significant difference from LPS treatment alone (p < 0.05)

GDE treatment suppresses an acute inflammatory in rats

In in vivo study, GDE showed suppressive effect on acute inflammation of carragenan-induced rat paw edema. The intraperitoneal of GDE significantly decreased the edema rate after carragenan injection for 120 min and significantly decreased the swelling of rat paws with dose and time-dependent manners (Figure 3). These results indicated that the GDE treatment exhibited anti-inflammatory effect against acute inflammation.
The results indicated that the GDE possessed high antioxidant activity by DPPH in comparison to the reference Vit.C and Vit.E. Anti-oxidation of this plant extract may related to its phenolics content due to high radical scavenging activity or anti-oxidant ability generally had high phenolics content with good correlation (Liu et al., 2008). To expand the usage of GDE for anti-inflammation, \textit{in vitro} and \textit{in vivo} studies were performed. \textit{In vitro} RT-PCR results demonstrated the significant dose-dependent, and selective suppressed the expression of TNF-α and IL-1β while it had no effect on the expression of COX-1. \textit{In vivo} study in carrageenan-induced paw edema which is a classical method assessing the acute inflammatory responses in antigenic challenges and irritants (Morris, 2003). Inflammatory induction by carrageenan is acute and non-immune response. It has been found that the injection of carrageenan into rat paw elevated the release of TNF-α, IL-6, IL-1, NO and COX-2 gene expression (Guay et al., 2004; Loram et al., 2007). From this method, GDE also revealed anti-inflammatory effect. This extract significantly suppressed the edema in a dose-dependent manner as similarly observed in the diclofenac treated group. These results support the suitable of using GDE for acute inflammatory treatment.

CONCLUSION

In conclusion, our study provides the first scientific support the utilization of \textit{Glochidion daltonii}. Its ethanolic extract exhibits \textit{in vitro} anti-oxidative and anti-inflammatory effects by modulating free radical generation and inflammatory related-gene expression. Moreover, the \textit{in vivo} study supports its anti-inflammatory effect of this plant as a folk medicine.

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