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 IC_{50} 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 (Proligo LLC, Boulder, co, USA), Tris base, Glacial acetic acid, EDTA (Ajax/Australia), 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), diclofenacdiethy-lammoniumsalt (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).

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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 37ºC in a humidified atmosphere with 5% CO_2.

Animals

Male Sprauge-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 37ºC in the humidified atmosphere with 5% CO_2 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 50% 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 37ºC in the humidified atmosphere with 5% CO_2 for 22 hr, the LPS was added then further incubated for 2 hr. 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 (Sripanikulchai *et al.*, 2009) to induce the paw edema. Immediately after carageenan 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, model7140) 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 IC_{50} 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 (r^2), 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)

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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.

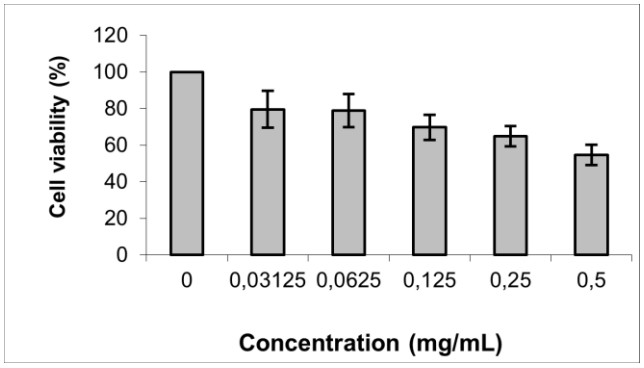

Figure 1. Effect of GDE on RAW264.7 cells viability. After 24 hr of incubation with various concentrations of GDE, the cell viability was measured with MTT. Each value is a mean ± SD compared to control from three individual experiments.

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.

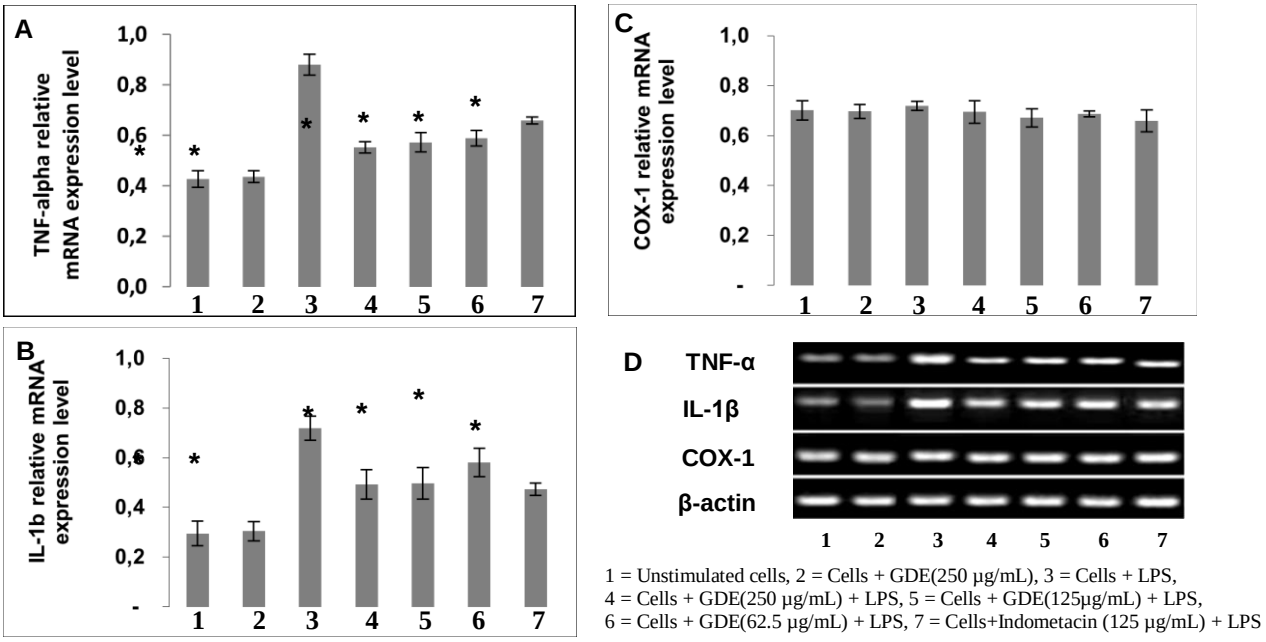

Figure 2. Inhibitory effect of GDE on mRNA expression of pro-inflammatory mediators, Relative mRNA expression of TNF-α (A), IL-1β (B), COX-1 (C) compared with β-actin mRNA expression and the amplified bands of TNF-α, IL-1β and COX-1 (D). * Significant difference from LPS treatment alone ($p < 0.05$)

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.

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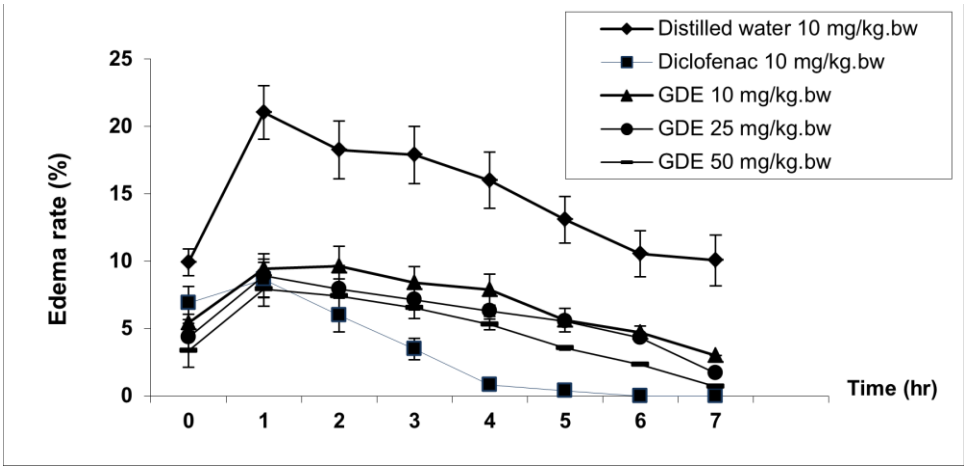

Figure 3. Suppressive effect of GDE on carrageenan-induced rat paw edema (n=10), the results expressed as mean ± SD

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, *in vitro* and *in vivo* studies were performed. *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. *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 *Glochidion daltonii*. Its ethanolic extract exhibits *in vitro* anti-oxidative and anti-inflammatory effects by modulating free radical generation and inflammatory related-gene expression. Moreover, the *in vivo* study supports its anti-inflammatory effect of this plant as a folk medicine.

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REFERENCES

Aggarwal, B. B. (2004). Nuclear factor-kappa B: the enemy within. *Cancer Cell;* 6; 203–208.

Balkwill, F. (2009). Tumor necrosis factor and cancer. *Nature Reviews Cancer; 9*; 361–371.

Chi, Y. S., Cheon, B. S. & Kim, H. P. (2001). Effect ofwogonin, a plant flavone from *Scutellaia radix*, on the suppression of cyclooxygenase-2 and the induction of inducible nitric oxide synthase in lipopolyssacharide-treatedRAW264.7 cells. *Biochemical Pharmacology; 61*; 1195–1203.

Guay, J., Bateman, K., Gordon, R., Mancini, J. & Riendeau, D. (2004). Carrageenan-induced paw edema in rat elicits a predominant prostaglandinE2 (PGE2) response in the central nervous system associated with the induction of micro somal PGE2 synthase-1. *Journal of Biological Chemistry; 279*; 24866–24872.

Kagiwada, K., Chida, D., Sakatani, T., Asano, M., Nambu, A., Kakuta, S. & Iwakura, Y. (2004). Interleukin (IL)-6, but not IL-1, induction in the brain downstream of cyclooxygenase-2 is essential for the induction of febrile response against peripheral IL-1α. *Endocrinology; 145*; 5044–5048.

Kolls, J. K. (2006). Oxidative stress in sepsis: a redox redux. *Journal of Clinical Investigation; 116*; 860-863.

Liu, X., Zhao, M., Wang, J., Yang, B. & Jiang, Y. (2008). Antioxidant activity of methanolic extract of emblica fruit (*Phyllanthus emblica* L.) from six regions in China. *Journal of Food Composition and Analysis; 21;* 219-228.

Loram, L. C., Fuller, A., Fick, L. G., Cartmell, T., Poole, S. & Mitchell, D. (2007). Cytokine profiles during carrageenan-induced

inflammatory hyperalgesia in rat muscle and hind paw. *Journal of Pain; 8;* 127–136.

Mantovani, A., Allavena, P., Sica, A. & Balkwill, F. (2008). Cancer-relatedinflammation. *Nature; 454;* 436–444.

Morris, C. J. (2003). Carrageenan-induced paw edema in the rat and mouse. Methods in Molecular Biology; 225; 115–121.

Mosmann T. (1983). Rapid colorimetric assay for cellular growth and survival application to proliferation and cytotoxicity assay. *Journal of Immunological Methods; 65*; 55-63.

Shimada, K., Fujikawa, K., Yahara, K. & Nakamura, T. (1992). Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *Journal of Agricultural and Food Chemistry; 40*; 945–948.

Singleton, V. L., Orthofer, R. & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteau reagent. *Methods in Enzymology; 299*; 152-178.

Sripanidkulchai, B., Junlatat, J., Wara-aswapati & N., Hormdee, D. (2009). Anti-inflammatiory effect of *Streblus asper* leaf extract in rats and its modulation on inflammation-associated genes expression in RAW264.7 macrophage cells. *Journal of Ethnopharmacology; 124*; 566-570.

Sugawara, I., Yamada, H. & Mizuno, S. (2003). Relative importance of STAT4 in murine tuberculosis. *Journal of Medical Microbiology; 52*; 29–34.

Won, J. H., Im, H. T., Kim, Y. H., Yun, K. J., Park, H. J., Choi, J. W. & Lee, K. T. (2006). Anti-inflammatory effect of buddlejasaponinIV through the inhibition of iNOS and COX-2 expression in RAW264.7 macrophages via the NF-kappaB inactivation. *British Journal of Pharmacology; 148*; 216–225.

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