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Original article

Inhibitory effect of *Derris reticulata* ethanol extract on LPS-induced macrophage activation

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Background: *Derris reticulata* Craib is a Thai medicinal plant in the Leguminosae family. It has flavonoids as major active compounds similar to other plants in genus *Derris*. Several flavonoids have been identified to have anti-inflammatory activities. The anti-inflammatory mechanisms and potency of flavonoids from this medicinal plant is not known.
Objective: We investigated the effect of ethanolic extract from stem of *D. reticulata* on LPS-induced macrophage activation.
Methods: J774A.1 cells were treated with 6.25-100 g/ml the extract for 24 hours and then activated with 100 ng/ml lipopolysaccharide (LPS) for 24 hours. The extract inhibited nitric oxide production in LPS-activated J774A.1 cells in concentration-dependent manner with IC_{50} 62.5 μg/ml. The effect of this extract on phagocytosis activity of LPS-activated J774A.1 cells was also investigated.
Results: The extract at concentrations of 50 and 100 μg/ml significantly inhibited zymosan phagocytosis of LPS-activated cells in a concentration dependent manner. It decreased the mRNA expression of the inducible nitric oxide synthase (iNOS) which plays a role in NO production and the cyclo-oxygenase 2 (COX-2) which is responsible for prostaglandins (PGs) production in LPS-activated J774A.1 cells. This extract also inhibited the mRNA expression of pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 in the LPS-activated J774A.1 cells.
Conclusion: The results in this study reveal for the first time that the ethanolic extract of *D. reticulata* stem has potential anti-inflammatory activity. It inhibited production of several known inflammatory mediators in LPS-activated macrophages. These findings may be useful to study this plant further as a source of anti-inflammatory activity.

Keywords: Activated macrophage, anti-inflammation, *Derris reticulata*, phagocytosis, pro-inflammatory cytokines, pro-inflammatory mediators

Macrophages are important immune cells that function as regulators of immune responses in various tissues of the body including lympho-haematopietic organs, skin, gut, other portals of entry, and the nervous system [1, 2].

Many stimuli are able to activate macrophages to generate immune responses. Examples of these are phagocytosis of microbes or foreign antigen particles, components of bacterial cell walls such as lipopolysaccharide (LPS), cytokines from activated TH1 cells and mediators of the inflammatory response [3]. Activated macrophages are far more active than resting cells in eliminating microbes. They exhibit higher phagocytic activity and greater capacity to kill ingested microbes, increase production and secretion of inflammatory mediators, increase the ability to activate T cells, and secrete various cytotoxic proteins required for eliminating viral infected cells, tumor cells, and intracellular bacteria.

Activated macrophages release a wide range of mediators such as several pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-8, and IL-12), reactive oxygen species, nitric oxide, and prostaglandins. These mediators are involved in the inflammatory response that is a part of the innate immune defense. However, chronic infection or chronic inflammation can cause chronic release or overproduction of these mediators,

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which are harmful to host tissues and lead to tissue damage and chronic inflammatory diseases such as rheumatoid arthritis [4]. Many clinically used anti-inflammatory agents target and inhibit pro-inflammatory cytokine functions [2, 5-7].

It has been known for a long time that drugs derived from medicinal plants can contribute in drug discovery. Plants are rich sources of several clinically used drugs. Many compounds from plants have been reported to exhibit anti-inflammatory activities. Some of them are used in modern as well as alternative medicine. *Derris reticulata* Craib is a plant in Leguminosae family, which has flavonoids as its major active compounds similar to other plants in genus *Derris* [8, 9]. It has been used as expectorant. Flavonoids from several medicinal plants have been demonstrated to have a variety of biological activities including antibacterial, antiviral, antioxidant, antiulcerogenic, cytotoxic, antineoplastic, mutagenic, antihepatotoxic, antihypertensive, hypolipidemic, antiplatelet, and anti-inflammatory activities [10].

This study aimed to investigate potential anti-inflammatory activity of the ethanol extract from the stem of *D. reticulata* by investigating the effect of the ethanol extract on phagocytic activity and the production of inflammatory mediators in activated macrophages. This study intended to investigate the effect of the ethanolic extract from the stem of *Derris reticulata* on phagocytic activity, nitric oxide production and the RNA expression of several proteins involved in inflammation, especially in LPS-activated macrophages.

Materials and methods

Plant extract

Air-dried and grounded stems of *D. reticulata* was extracted with dichloromethane and then with absolute ethanol. The ethanol extract was dissolved in dimethylsulfoxide (DMSO). The constant final concentration of DMSO in this study was 0.2%.

Cells

Murine macrophages J774A.1 were obtained from ATCC. The cells were subcultured three times weekly and maintained in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin and incubated at 37°C in 5%CO_2/ 95% air.

Chemicals

The following reagents were used in this study, nitroblue tetrazolium (NBT) (Sigma, USA), lipopolysaccharide (LPS) (Sigma, USA), rezasurin (Sigma, USA), zymosan A from *Saccharomyces cervisiae* (Sigma, USA), DEPC (Molekula, UK), dimethylsulfloxide (DMSO) (Sigma, USA), ImProm-IITM Reverse Transcription system (Promega, USA), nitric oxide assay kit (Promega, USA), Taq polymerase (Invitrogen, UK), trypan blue dye (Sigma, USA), TRiZol reagent (Invitrogen, UK), dulbecco's modified eagle's medium (DMEM) (Gibco, USA), fetal bovine serum (Gibco, USA), RPMI 1640 medium (sigma, USA), sodium bicarbonate (Baker, USA).

Determination of NO production

1×10^5 cell/well J774A.1 cells in 96-well plates were treated with the ethanol extract at 6.25, 12.5, 25, 50 and 100 μg/ml for 24 hours. The treated cells were stimulated with 100 ng/ml LPS for 24 hours. The amount of NO released into the supernatant was determined in nitrite form by using Griess reagent [11]. The reaction mixture was detected with a microplate reader at 540 nm. The amount of nitrite was calculated from a sodium nitrite standard curve. The viability of the treated cells was determined by staining with resazurin for 2 to 4 hours at 37°C in 5% CO_2/ 95% air. The reduction of the product of resorufin in viable cells was detected by a microplate reader at 570 and 600 nm. The percentage of cell viability was calculated by comparing with LPS-activated condition.

Determination of phagocytosis activity

1×10^5 cell/well J774A.1 cells in 96-well plates were treated with 25, 50, and 100 μg/ml ethanol extract for 24 hours and then activated with 100 ng/ml LPS for 24 hours. The treated cells were carefully washed twice with DMEM and then incubated with 800 μg/ml of zymosan and 600 μg/ml of NBT for 1h. The cells were washed 3 times with methanol, air-dried, and lyses in 120 μl of 2M KOH and 140 μl of DMSO. The oxidized NBT product in blue color was detected at 570 nm. The percentage of phagocytosis inhibition was determined by comparing to LPS-activated condition [12].

Determination of mRNA expression of cytokines, iNOS and COX-2

1×10^5 cell/well J774A.1 cells in 96-well plates were treated with 25, 50, and 100 μg/ml ethanol extract for 24 hours. Ten μM dexamethasone was used as positive control. The treated cells were then activated with

100 ng/ml LPS for 24 hours. Total RNA was isolated from the treated cells using Trizol reagents and then reversed to cDNA using reverse transcription system kit. The cDNA was used as the template to amplified mRNA of cytokines, iNOS, and COX-2 with specific primers for iNOS and COX-2 genes. The PCR products were run on 1.5% agarose gel electrophoresis and their densities were measured by gel documentation.

Statistical analysis

Data were expressed as mean±standard error (mean S.E.). One-way ANOVA followed by Turkey's post hoc test was used for comparison between control and treatment groups. All statistical analysis was performed according to the statistic program, SPSS version 17. *P*-value <0.05 was considered statistically significant difference.

Results

Effect of the ethanol extract on NO production

D. reticulata ethanol extract decreased NO production in LPS-stimulated J774A.1 cells in a concentration-dependent manner with IC_{50} 62.5 μg/ml (**Figure 1**). The extract at the concentration of 6.25, 12.5, 25, 50, and 100 μg/ml inhibited NO production by 5.1%, 10.9%, 24.9%, 46.1%, and 74.8%, respectively. It did not affect cell viability of the treated macrophages (data not shown).

Effect of the ethanol extract on phagocytosis

The ethanol extract inhibited phagocytic activity of LPS-activated J774A.1 cells in a concentration-dependent manner (**Figure 2**). It inhibited zymosan-NBT phagocytosis of J774A.1 cells by 3.1%, 25.7%, and 60.8% when the cells were treated with the extract at 25, 50, and 100 μg/ml, respectively.

Effect of the ethanol extract on the mRNA expression of pro-inflammatory cytokines, iNOS and COX-2

The ethanol extract significantly suppressed the mRNA expression of TNF-α, IL-1β, IL-6, iNOS, and COX-2 in a concentration-dependent manner (**Figure 3**). The extract suppressed mRNA TNF-α expression to 86.8%, 61.6%, and 39.2% of the LPS-stimulated control at concentration of 25, 50, and 100 μg/ml, respectively (**Figure 3A**). The extract suppressed mRNA IL-1β expression to 85.7%, 77.7%, and 53% of the LPS-stimulated control at the concentration of 25, 50, and 100 μg/ml, respectively (**Figure 3B**). It also suppressed mRNA IL-6 expression to 70.8%, 63.5%, and 38.6% of the LPS-stimulated control at concentration of 25, 50, and 100 μg/ml, respectively (**Figure 3C**). The extract significantly suppressed LPS-induced mRNA of iNOS expression in a concentration-dependent manner to 50.5%, 38.7%, and 10.2% of the LPS-stimulated control at the concentrations of 25, 50, and 100 μg/ml,

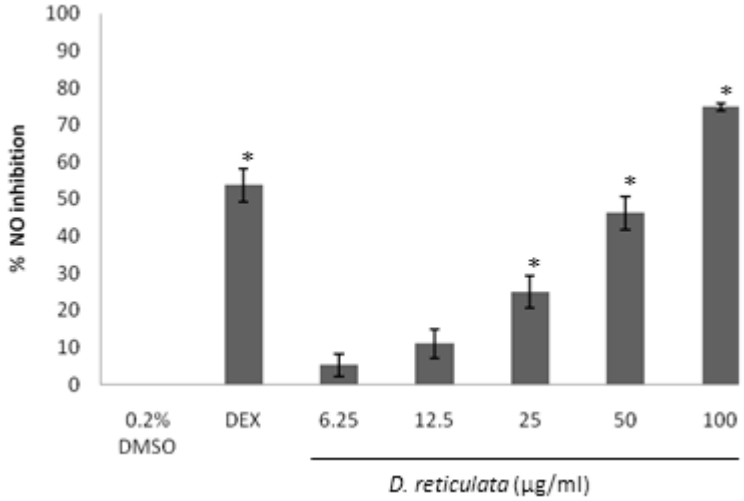


Figure 1. Inhibitory effect of *D. reticulata* ethanol extract on NO production in LPS stimulated-J774A.1 cells. The cells were treated with 6.25-100 μg/ml extract and then stimulated with 100 ng/ml LPS. Five μg/ml dexamethasone (DEX) was used as positive control. The NO production was determined by Griess reagent. The percentage of NO inhibition compared to LPS-activated condition is presented as mean S.E.M. of four independent experiments (n = 4), **p* <0.05 compared to untreated control (LPS-activated cells).

respectively (**Figure 3D**). The extract significantly suppressed LPS-induced mRNA of COX-2 expression in a concentration-dependent manner to 95.4%, 69.1%, and 12.2% of the LPS-stimulated control at the concentrations of 25, 50, and 100 μg/ml, respectively (**Figure 3E**).

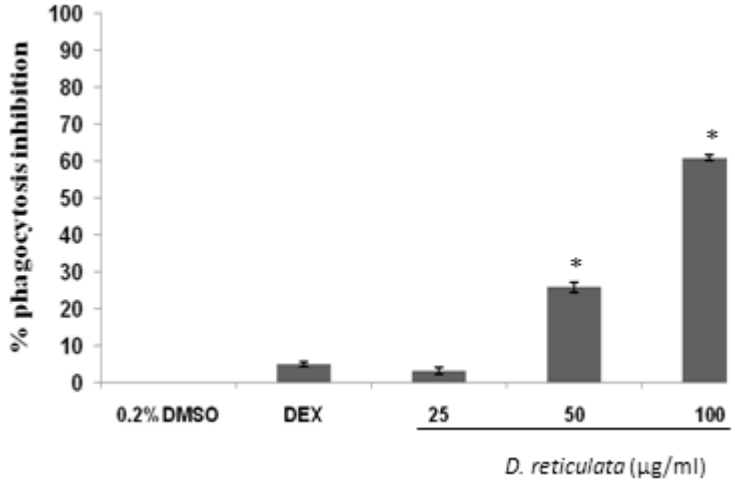
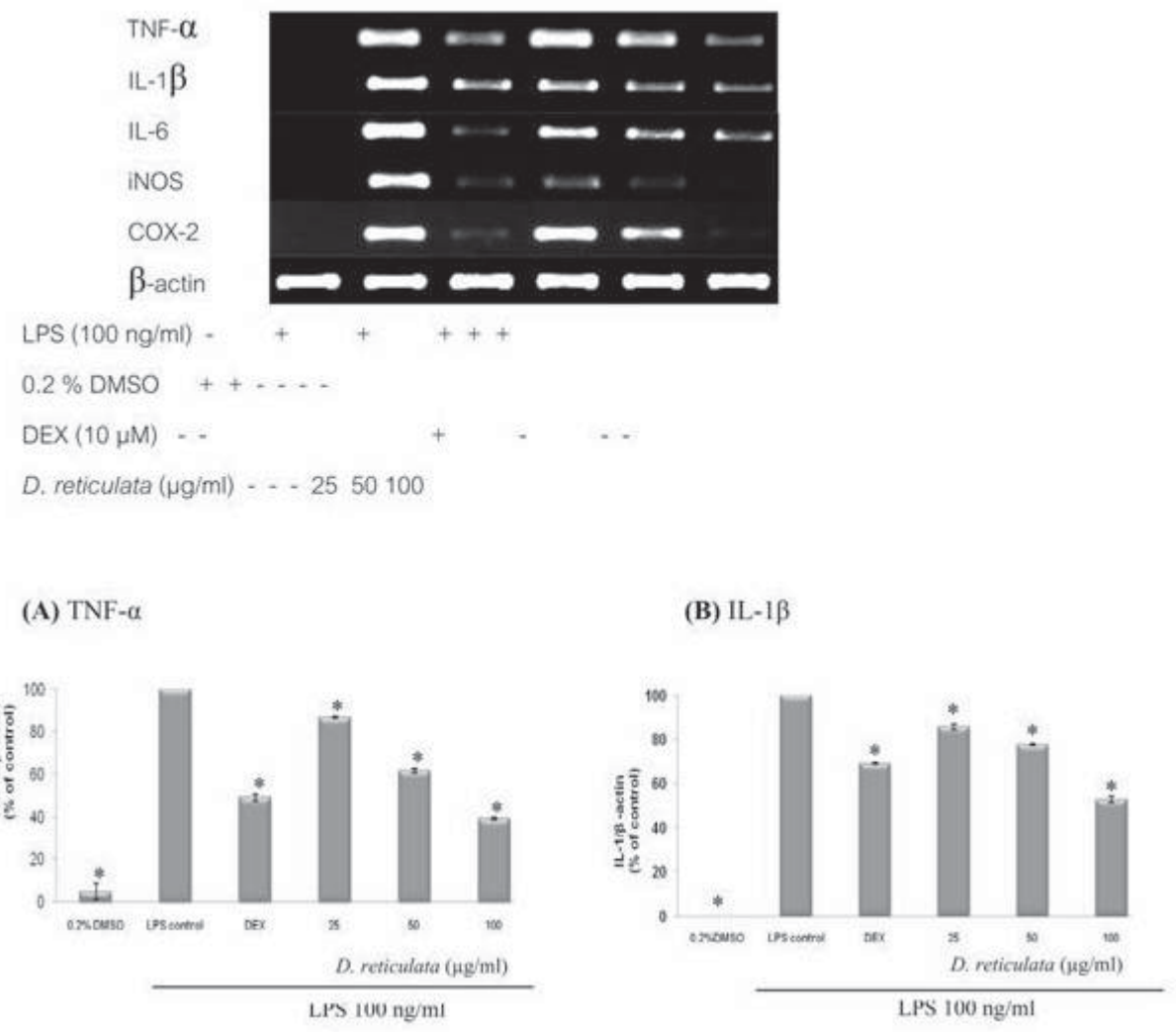


Figure 2. Effect of *D. reticulata* ethanol extract on phagocytosis in LPS stimulated-J774A.1 cells. The cells were treated at a dose of 25 to 100 μg/ml extract. Phagocytosis of treated cells was determined by zymosan-NBT assay. Data are presented as mean±SEM of three independent experiments (n = 3). *p <0.05 compared to LPS-activated condition (0.2% DMSO).



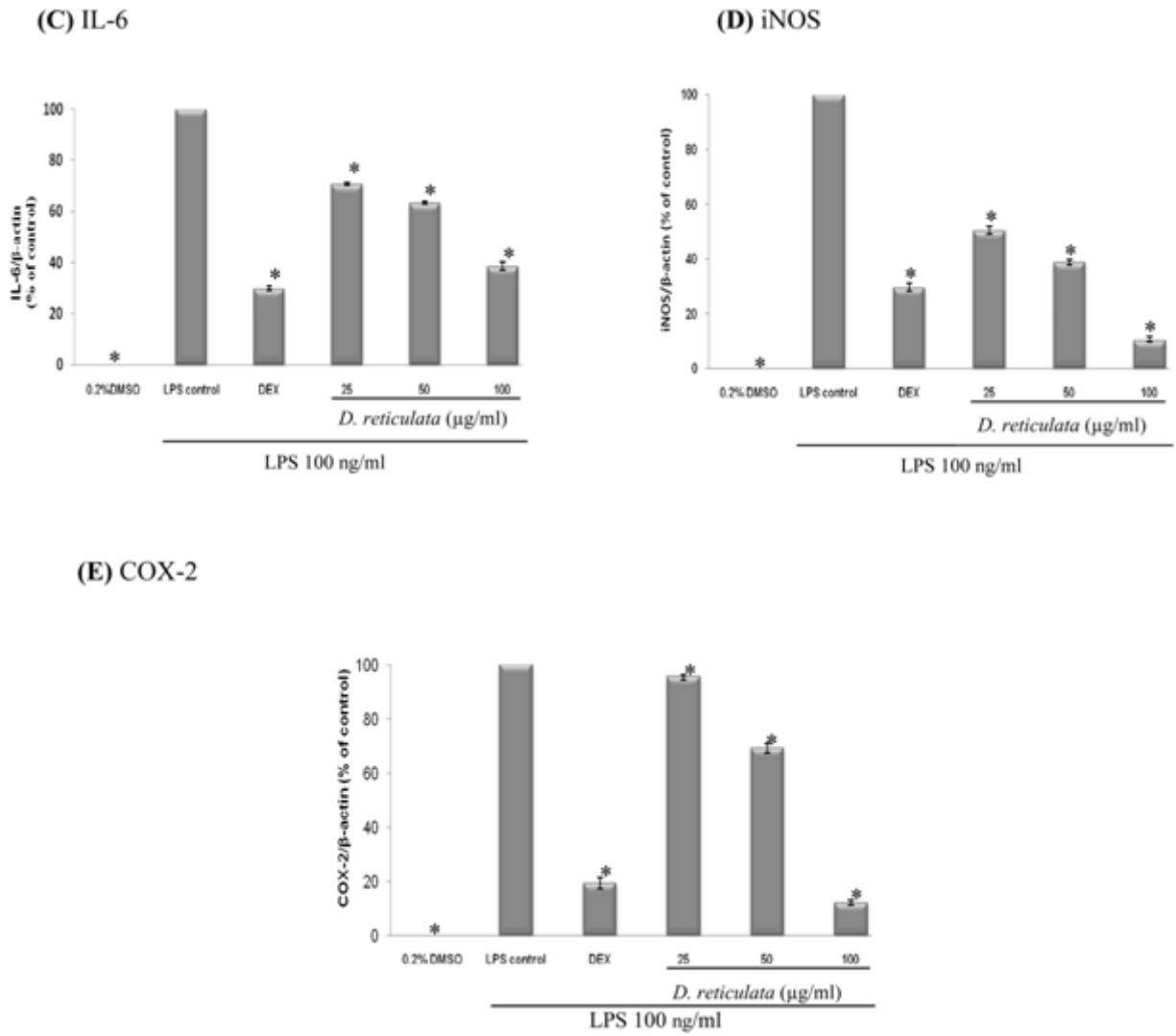


Figure 3. Effect of *D. reticulata* ethanol extract on mRNA expression of TNF-α (**A**), IL-1β (**B**), IL-6 (**C**), iNOS (**D**) and COX-2 (**E**) in LPS stimulated-J774A.1 cells. Data are presented as mean±SEM of three independent experiments (n = 3). **p* <0.05 compared to LPS-activated condition (0.2% DMSO).

Discussion

Many natural products are clinically used but many remain unexplored. Several groups of compounds have been explored for their potential anti-inflammatory agents especially flavonoids [13, 14]. Flavonoids have been reported to exhibit a variety of biological effects, both *in vitro* and *in vivo*, as antibacterial, antiviral, anti-ulcerogenic, cytotoxic, antineoplastic, anti-mutagenic, antihepatotoxic, antihypertensive, hypolipidemic, antiplatelet, antioxidant, and anti-inflammatory activities [10]. *Derris reticulata* Craib is a plant in the Leguminosae family, which has been used as expectorant. This plant contains flavonoids as its major active compounds similar to other plants in the genus *Derris*. Lupinifolin has been reported as the major constituent from the stem of *D. reticulate* [8]. Dereticulatin and 2', 3'-epoxylupinifolin have also been identified from the stems of this plant [15]. Very few pharmacological activities of these flavonoids have been evidenced. Lupinifolin demonstrated inhibitory effect, without cytotoxicity, on Epstein–Barr virus (EBV), early antigen activation induced by 12-O-tetradecanoylphorbol-13-acetate in Raji cells *in vitro* and exhibited chemopreventive effect on mouse skin tumor promotion *in vivo* [16]. Potential anti-inflammatory activity of the ethanolic extract from the stem of *D. reticulata* was investigated in this

study by evaluating the inhibitory effect of the extract on LPS-induced macrophage activation. All concentrations of the extract used in this study had no cytotoxic effect on J774A.1 macrophage cells, which were used in the study.

Activated macrophages play important roles during an inflammatory process. They produce several pro-inflammatory cytokines and several inflammatory mediators [4, 17-21]. The ethanolic extract of *D. reticulata* significantly decreased the mRNA expression of pro-inflammatory cytokines, TNF-α, IL-1, and IL-6 in LPS-activated J774A.1 macrophages. These inhibitory effects are concentration-dependent. It is well known that TNF-α acts as a potent endogenous pyrogen and plays an important role in the inflammatory process, cachexia, and septic shock. It triggers a cascade of cytokines responsible for attracting macrophages to the site of inflammation, activates the production of several inducible enzymes and proteins, and increases vascular permeability during the inflammatory process [22-24]. It is possible that the inhibitory effect of the ethanolic extract on IL-1β and IL-6 gene expression will come from the effect of the extract on TNF-α gene expression. The extract also inhibited the mRNA expression of iNOS and COX-2 enzymes in LPS-activated J774A.1 cells in a concentration dependent fashion. Both iNOS and COX-2 are inducible enzymes which are responsible for the production of a large amount of NO and PGE_2, respectively, in activated macrophages [25-29]. NO and PGE_2 are important inflammatory mediators in the inflammatory process. The inhibitory effect of the extract on the iNOS expression in activated J774A.1 cells was correlated with the reduction of NO production by the extract. The extract decreased the NO production in LPS-activated J774A.1 cells in a concentration-dependent manner, with the IC50 value at 62.5 μg/ml. These results demonstrate that the extract suppresses the NO production by directly or indirectly down regulating iNOS expression. The inhibitory effects of the extract on both iNOS and COX-2 expression may be the consequence from the decrease in the pro-inflammatory cytokine production.

It is known that LPS increases phagocytosis activity of LPS-activated macrophages. These activated cells phagocytose and got rid of recognized pathogens, foreign particles, or apoptotic cells by oxygen-dependent and oxygen-independent mechanisms. By oxygen-dependent mechanism, macrophages can generate several free radicals such as hydrogen peroxide, super oxide anion and NO for intracellular destroying pathogens. These substances may be released into the extracellular space and may injure host tissues leading to closely correlated with the pathophysiology of a variety of diseases and inflammation [19, 30, 31]. The inhibitory effect of the extract on activated macrophages was also confirmed by evaluating its effect on phagocytosis in LPS-activated J774A.1. The extract significantly inhibited zymosan phagocytosis of activated J774A.1 cells in a concentration dependent manner.

In conclusion, the results in this study demonstrate that the ethanol extract from the stems of *D. reticulata* suppresses LPS-activated macrophage from expression of several proteins which are pro-inflammatory cytokines, TNF-α, IL-1 and IL-6, and enzymes iNOS and COX-2 which are responsible for NO and PGE_2 production. This extract did not have any effect on cell viability. Therefore, *D. reticulata* may be a source of natural products that are candidates for developing novel anti-inflammatory agents in the future.

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