# CHAPTER IV

# RESULTS

## 1. Effects of the ethanol extract from *D. reticulata* on nitric oxide production in LPS stimulated-macrophages

Nitric oxide (NO) is one of the key inflammatory mediators generated when macrophages are activated by several inflammatory stimuli as well as lipopolysaccharide (LPS). Inhibitory effect of the ethanol extract from *D. reticulata* on LPS stimulated-J774A.1 cells was first investigated by determining its effect on nitric oxide production. J774A.1 cells were treated with 6.25- 100 µg/ml of the extract for 24 h and then stimulated with 100 ng/ml LPS for the 24 h. The amount of nitric oxide in the supernatant of the treated cells was determined as nitrite concentration by using Griess reagents and nitrite standard curve.

The ethanol extract inhibit NO production in LPS-activated J774A.1 cells in a concentration dependent manner. The NO production of the activated cells was inhibited by 5.1%, 10.9%, 24.9%, 46.1% and 74.8% when the cells were pretreated with the extract at the concentrations 6.25, 12.5, 25, 50 and 100 µg/ml, respectively (Fig.10). The concentration required for 50% inhibition ($IC_{50}$ value) of ethanol extract was determined for selecting the concentrations of the extract used in the subsequent studies. The $IC_{50}$ value of the extract was 62.5 µg/ml (Fig.11). The effect of the ethanol extract on J774A.1 viability was also determined. The extract inhibited the NO production in LPS-activated J774A.1 cells without any effect on the cell viability (Fig.12).

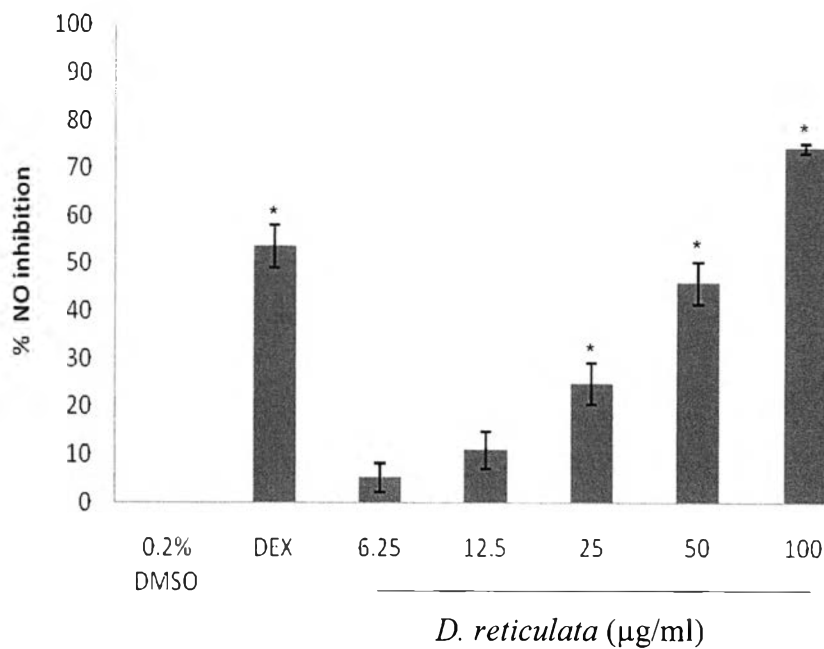


Figure 10: The inhibitory effect of *D. reticulata* ethanol extract on NO production in LPS stimulated-J774A.1 cells. The cells were treated with 6.25-100 µg/ml extract for 24 hours and then stimulated with 100 ng/ml LPS. Ten µM dexamethasone (DEX) was used as the positive control. The amount of NO produced in the supernatant of the treated cells was determined by Griess reagents. The percentage of NO inhibition was calculated by comparing with LPS-stimulated control. The data are expressed as the mean ± S.E. from four independent experiments (n=4).

*$p<0.05$ indicates significant difference from the 0.2% DMSO-treated LPS-activated cells.

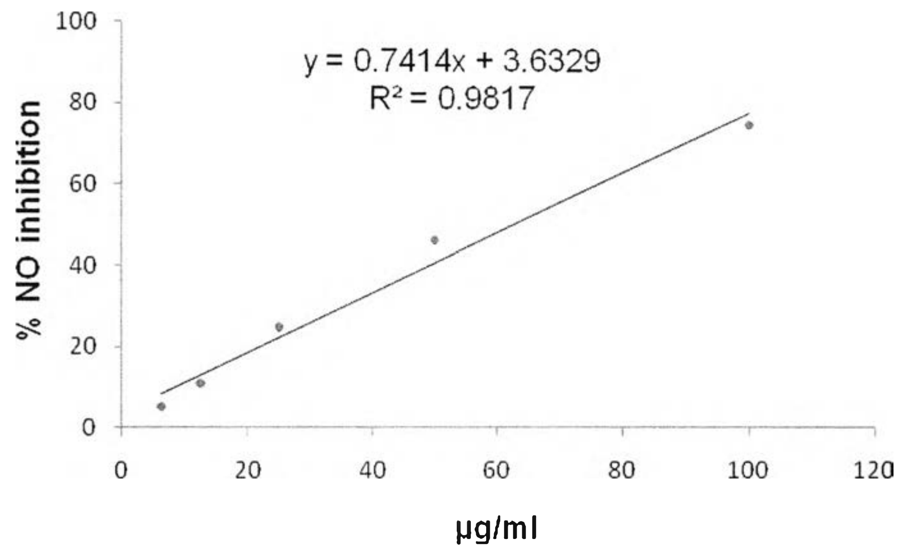


**Figure 11**: Determination of the $IC_{50}$ value of the ethanol extract from *D. reticulata*.

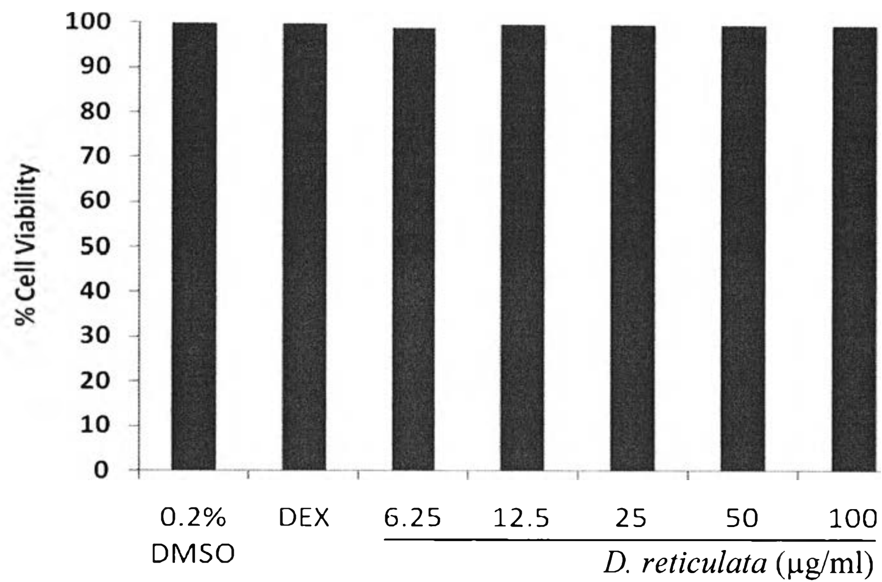


**Figure 12**: The effect of the ethanol extract on J774A.1 cell viability. The cells were treated with the ethanol extract for 24 h and activated with100 ng/ml LPS for 24 h. The viability of the treated cells was determined by resazurin assay. Data are expressed as the mean ± S.E. from four independent experiments (n=4).

## 2.Effect of the ethanol extract on phagocytic activity of LPS stimulated-macrophages

LPS activates the phagocytic activity of macrophages. The effect of the ethanol extract on phagocytic activity of J774A.1 cells was determined by zymosan-nitroble tetrazolium (NBT) assay. The cells were treated with 25, 50 and 100 µg/ml the ethanol extract for 24 h and then activated with 100 ng/ml for the next 24 h. The treated cells were incubated with 800 µg/ml of zymosan and 600 µg/ml of NBT for 1h. The supernatant was removed. The cells were washed and then lyses for detecting formazan product within the cells. The ethanol extract inhibited phagocytic activity of LPS-activated J774A.1 cells in a concentration dependent manner (Fig. 13). It inhibited the 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.

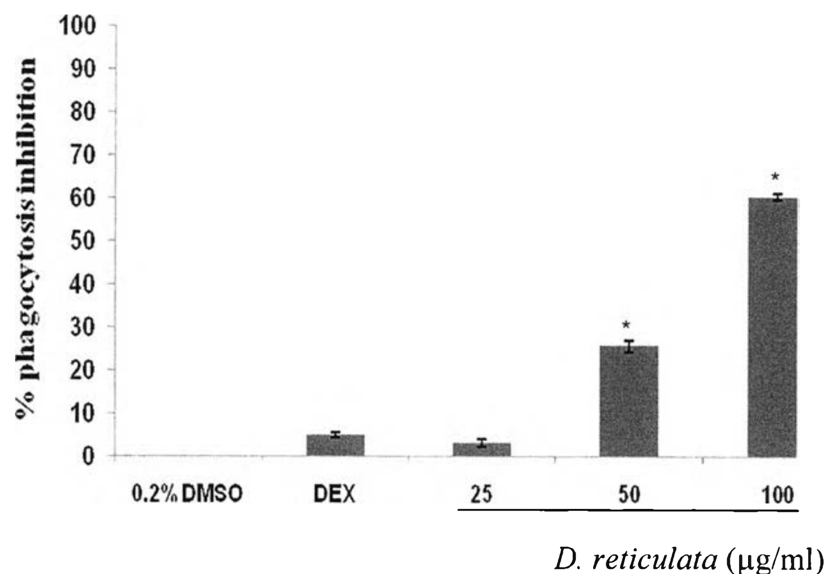


Figure 13: The effect of the ethanol extract on phagocytic activity of LPS stimulated-J774A.1 cells. The cells were treated with 25, 50 and 100 µg/ml extract for 24 hours. Ten µM dexamethasone (DEX) was used as the positive control. Phagocytic activity of the treated cells was determined by the production of formazan product of NBT in zymosan-NBT assay. The percentage of phagocytosis inhibition was determined by comparing with in the LPS-stimulated control. Data are expressed as the mean ± S.E. from three independent experiments (n=3).

### 3.The effect of the ethanol extract on the mRNA expressions of pro-inflammatory mediators in LPS stimulated-macrophages

LPS-stimulated macrophages synthesize and secrete several inflammatory mediators to generate innate immune response against microorganism and induce inflammatory process. These mediators include pro-inflammatory cytokines (TNF-$\alpha$, IL-1, IL-6 and IL-8), NO, reactive oxygen species, prostaglandins (PGs). The effects of the ethanol extract on the mRNA expression of pro-inflammatory cytokines (TNF-$\alpha$, IL-1, IL-6), iNOS which involves in NO production and COX-2 which plays role in PG production were investigated.

#### -The effect of the ethanol extract on the mRNA expression of pro-inflammatory cytokines

J774A.1 cells were treated with 25, 50 and 100 μg/ml ethanol extract for 24 h, then activated with 100 ng/ml LPS for 4 h. Total RNA was isolated from the treated cells and reverse transcribed to cDNA. The cDNA was amplified with specific primers of TNF-$\alpha$, IL-1$\beta$, and IL-6 gene by PCR. The PCR products were identified on 1.5% agarose gel electrophoresis and semi-quantiatated by gel documentation.

The ethanol extract significantly suppressed the mRNA expression of TNF-$\alpha$, IL-1$\beta$, and IL-6 in a concentration dependent manner (Fig. 14, 15, and 16). It suppressed mRNA TNF-$\alpha$ expression to 86.8%, 61.6 and 39.2% of the LPS-stimulated control at concentration of 25, 50 and 100 μg/ml, respectively (Fig. 14). It suppressed mRNA IL-1$\beta$ expression to 85.7%, 77.7% and 53% of the LPS-stimulated control at concentration of 25, 50 and 100 μg/ml, respectively (Fig. 15). 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 (Fig. 16).

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(a)

| | | | | | | |
|---|---|---|---|---|---|---|
| LPS (100 ng/ml) | - | + | + | + | + | + |
| 0.2 % DMSO | + | + | - | - | - | - |
| DEX (10 μM) | - | - | + | - | - | - |
| *D. reticulata* (μg/ml) | - | - | - | 25 | 50 | 100 |

(b)

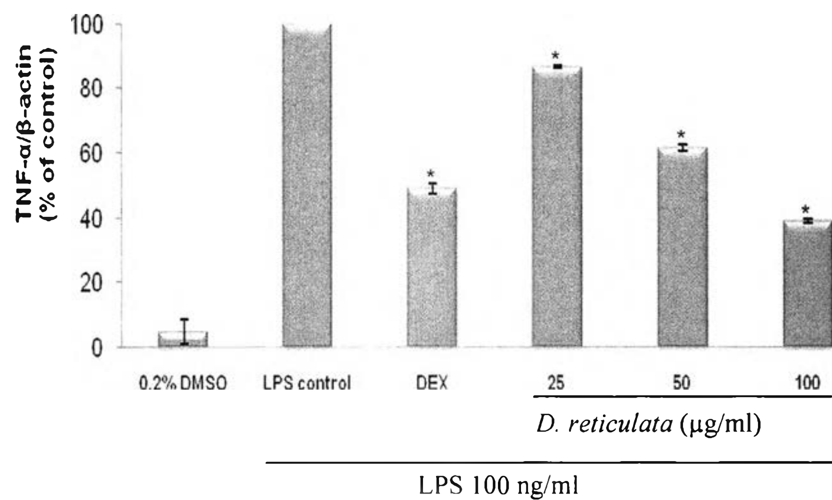



**Figure 14**: Effect of *D. reticulata* ethanol extract on the mRNA expression of TNF-α in LPS stimulated-J774A.1 cells. J774A.1 cells were treated with 25, 50 and 100 μg/ml for 24 h and then stimulated with 100 ng/ml LPS for 4 h. Total RNA was isolated from the treated cells and reverse transcribed to cDNA. The cDNA was amplified by PCR using TNF- α-specific primer. The PCR products were run in 1.5% agarose gel electrophoresis (a) and determine the quantities by using gel documentation and comparing with β-actin PCR product (b). Data are expressed as the mean ± S.E., *$p<0.05$ indicates significant difference from LPS-stimulated control.

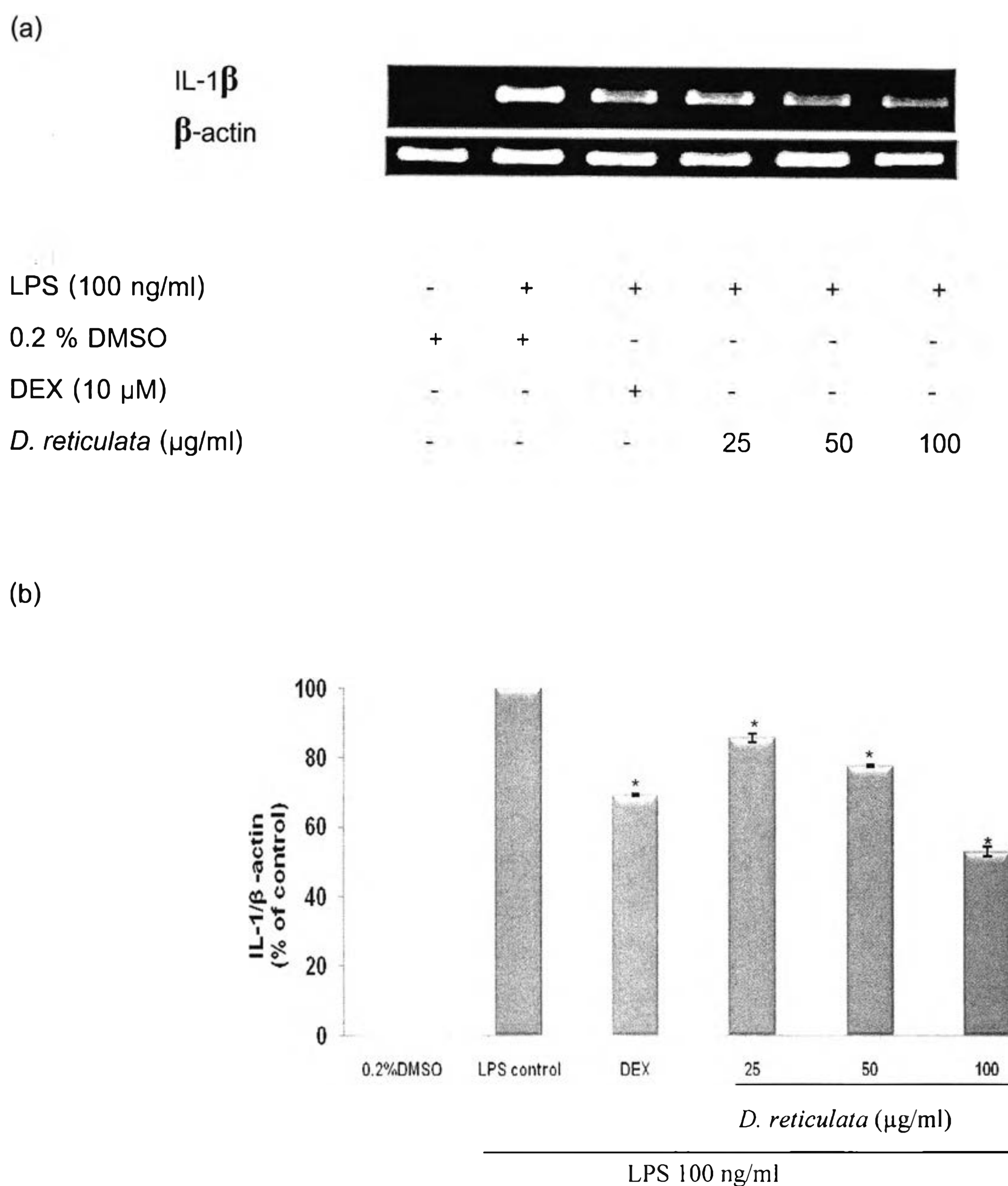
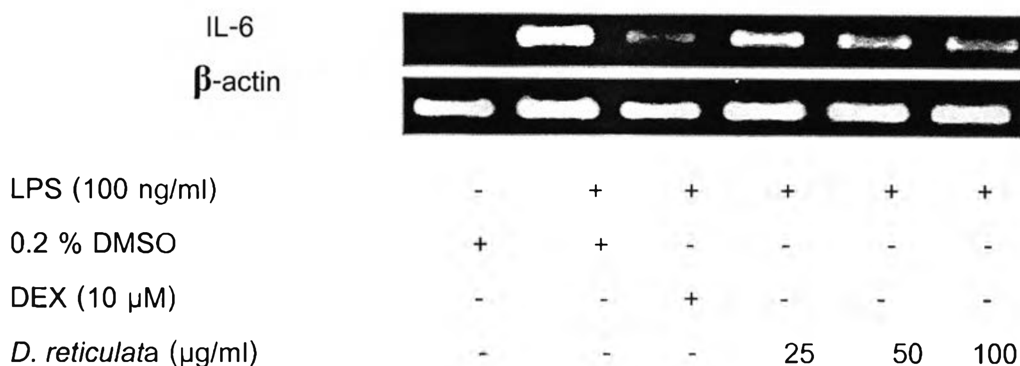



**Figure 15**: Effect of *D. reticulata* ethanol extract on the mRNA expression of IL-1β in LPS stimulated-J774A.1 cells. J774A.1 cells were treated with 25, 50 and 100 µg/ml for 24 h and then stimulated with 100 ng/ml LPS for 4 h. Total RNA was isolated from the treated cells and reverse transcribed to cDNA. The cDNA was amplified by PCR using IL-1β-specific primer. The PCR products were run in 1.5% agarose gel electrophoresis (a) and determine the quantities by using gel documentation and comparing with β-actin PCR product (b). Data are expressed as the mean ± S.E., *$p<0.05$ indicates significant difference from LPS-stimulated control.

(a)




(b)

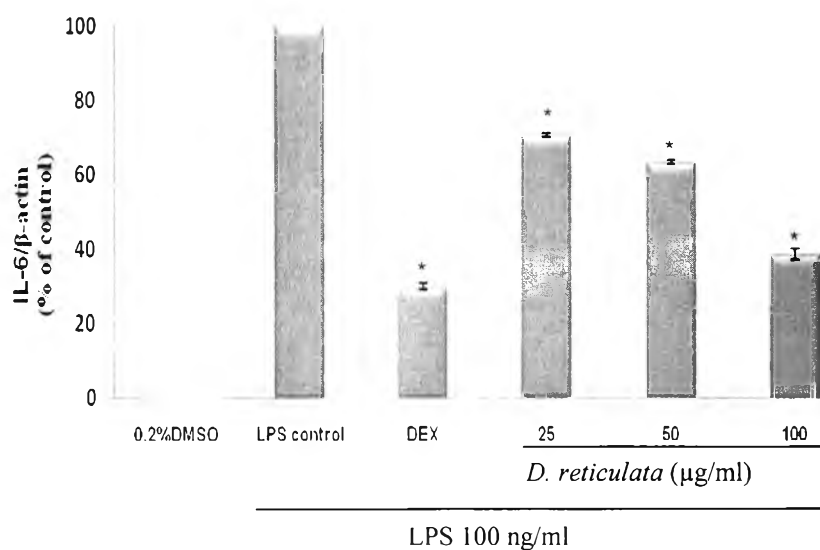



Figure 16: Effect of *D. reticulata* ethanol extract on the mRNA expression of IL-6 in LPS stimulated-J774A.1 cells. J774A.1 cells were treated with 25, 50 and 100 μg/ml for 24 h and then stimulated with 100 ng/ml LPS for 4 h. Total RNA was isolated from the treated cells and reverse transcribed to cDNA. The cDNA was amplified by PCR using IL-6-specific primer. The PCR products were run in 1.5% agarose gel electrophoresis (a) and determine the quantities by using gel documentation and comparing with β-actin PCR product (b). Data are expressed as the mean ± S.E., *$p<0.05$ indicates significant difference from LPS-stimulated control.

**-The effect of the ethanol extract on the mRNA expression of iNOS**

This study also investigated the effect of the ethanol extract on the mRNA expression of iNOS which is the inducible enzyme essential for NO production in activated macrophages. J774A.1 cells were treated with 25, 50 and 100 µg/ml ethanol extract for 24 h, then activated with 100 ng/ml LPS for 24 h. Total RNA was isolated from the treated cells and reverse transcribed to cDNA. The cDNA was amplified with iNOS-specific primers by PCR. The PCR products were identified on 1.5% agarose gel electrophoresis and determined their quantities by gel documentation. 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, respectively (Fig. 17).

(a)

iNOS

β-actin

| | | | | | | |
|---|---|---|---|---|---|---|
| LPS (100 ng/ml) | - | + | + | + | + | + |
| 0.2 % DMSO | + | + | - | - | - | - |
| DEX (10 μM) | - | - | + | - | - | - |
| *D. reticulata* (μg/ml) | - | - | - | 25 | 50 | 100 |

(b)

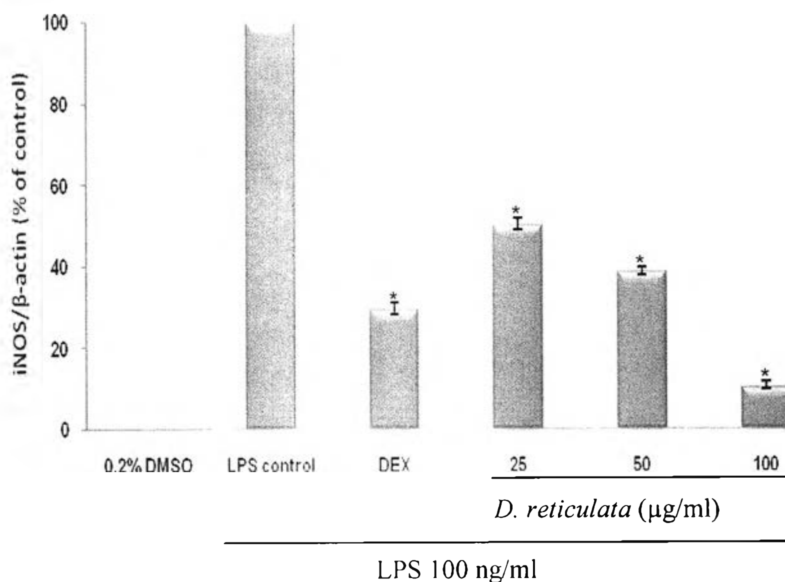



Figure 17: Effect of *D. reticulata* ethanol extract on the mRNA expression of iNOS in LPS stimulated-J774A.1 cells. J774A.1 cells were treated with 25, 50 and 100 μg/ml for 24 h and then stimulated with 100 ng/ml LPS for 24 h. Total RNA was isolated from the treated cells and reverse transcribed to cDNA. The cDNA was amplified by PCR using iNOS-specific primer. The PCR products were run in 1.5% agarose gel electrophoresis (a) and determine the quantities by using gel documentation and comparing with β-actin PCR product (b). Data are expressed as the mean ± S.E., *$p<0.05$ indicates significant difference from LPS-stimulated control.

**-The effect of the ethanol extract on the mRNA expression of COX-2**

This study also evaluated the effect of the ethanol extract on the mRNA expression of COX-2 which plays essential role in generating prostaglandins (PGs) of activated macrophages. PGs are key inflammatory mediators in inflammatory process. J774A.1 cells were treated with 25, 50 and 100 µg/ml ethanol extract for 24 h, then activated with 100 ng/ml LPS for 24 h. Total RNA was isolated from the treated cells and reverse transcribed to cDNA. The cDNA was amplified with COX-2-specific primers by PCR. The PCR products were identified on 1.5% agarose gel electrophoresis and determined their quantities by gel documentation. 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 (Fig. 18).

(a)

(b)

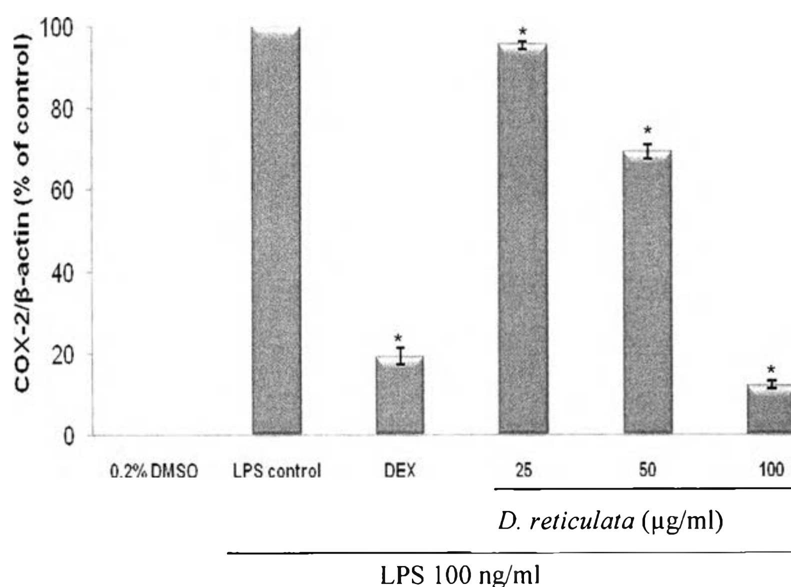



**Figure 18**: Effect of *D. reticulata* ethanol extract on the mRNA expression of COX-2 in LPS stimulated-J774A.1 cells. cells were treated with 25, 50 and 100 μg/ml for 24 h and then stimulated with 100 ng/ml LPS for 24 h. Total RNA was isolated from the treated cells and reverse transcribed to cDNA. The cDNA was amplified by PCR using COX-2-specific primer. The PCR products were run in 1.5% agarose gel electrophoresis (a) and determine the quantities by using gel documentation and comparing with β-actin PCR product (b). Data are expressed as the mean ± S.E., *$p<0.05$ indicates significant difference from LPS-stimulated control.