



## CHAPTER V

### DISCUSSION CONCLUSION AND SUGGESTION

#### Discussion and conclusion

Macrophage and monocyte are activated in response to component of pathogenic bacteria invading the host and a cascade intracellular response or tissue injury are initiated. Immune cells are stimulated by adhesion molecule activation signal in order to enhance the migration capacity to inflamed tissue. Lipopolysaccharide is one of the inflammatory stimuli which activated immune cells to up-regulate the inflammatory state. Nitric oxide, IL-1 $\beta$ , IL-6, TNF- $\alpha$  and other pro-inflammatory cytokines are produced by immune cells during the inflammatory process. They possess a variety of biological activities in response to the immunopathology of acute and chronic inflammatory diseases for examples osteoarthritis, rheumatoid arthritis, Crohn's disease etc.

The hexane, ethyl acetate, butanol and water extracts (G1-G8) from leaves and branches of *G.parva*, a Thai herbal plant were investigated ( G1-hexane extract from branches, G2-ethyl acetate extract from branches, G3-butanol extract from branches, G4-water extract from branches, G5-hexane extract from leaves, G6-ethyl acetate extract from leaves, G7-butanol extract from leaves, G8-water extract from leaves). Since their effects have not been investigated for the immunopathological view point in response to anti-inflammatory process. *G.parva* in difference fractions of solvent extract demonstrated a magnitude responses in cytokines secretory effect of the LPS-stimulated macrophage J774A.1 cells used in this study. Among the fractions that produced nitric oxide inhibition, (G1, G2, G5 and G6). G6 demonstrated the most potent effect ( $IC_{50} = 11.12 \mu\text{g/ml}$ ) while cytotoxic effect occurred at dose of  $50 \mu\text{g/ml}$ . G5, G2 and G1 were less potent than G6, their  $IC_{50}$  for NO inhibition were 11.76, 16.70 and  $44.96 \mu\text{g/ml}$  respectively. G3, G4, G7 and G8 exhibited minimal NO production in macrophage J 774A.1 stimulated with LPS, so they got no further investigation in this study. NO generation is stimulated during nitric oxide synthase ( NOS ) catalyse the

conversion of L-arginine to citrulline. The excess production of NO especially in macrophage can bring about inflammation, cytotoxicity, carcinogenicity and autoimmune diseases. (Liu RH, 1995, Nguyen *et al* 1992) Thus suppression of NO production is important for anti-inflammatory action. The extraction of *G. parva*, G1, G2, G5 and G6 inhibited NO generation in dose dependence manner ( Figure 10a, 11a, 12a, 13a ) which are related to their capability on iNOS mRNA expression. ( Figure 18, 19, 20, 21 )

In this present study, fraction from branches and leaves of *G. parva* extracted by different polarity of solvents vary from hexane, ethylacetate (G1, G2, G5, G6) demonstrated inhibitory effect on the expression of the pro-inflammatory cytokines, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in difference magnitude. Their inhibitory activity didn't correlate with their activity on NO production, for example., the hexane extract of *G. parva* branches (G1) at near IC<sub>50</sub> concentration of NO production ( 50  $\mu$ g/ml) exhibited 54.3% inhibition of TNF- $\alpha$  expression by the LPS-treated macrophage while the hexane extract from the leaves of *G. parva* (G5) at the near its IC<sub>50</sub> for NO production (12.5  $\mu$ g/ml) demonstrated much more potent inhibition of the expression of TNF- $\alpha$  (95% inhibition). The inhibition effect on TNF- $\alpha$ , IL-1 $\beta$  and IL-6 might be associated with the reduction in pain and inflammation. In addition, all *G. parva* extract used in this study also significantly inhibited the expression of COX-2 and iNOS mRNA in LPS-treated macrophage for 24 h except for G5 and G6 at 6-25  $\mu$ g/ml. The maximum inhibition effect of COX-2 expression was found in G6 at high concentration (25 $\mu$ g/ml) (93%, Appendix B-30). Both NO and prostaglandin (PGs) are known to be important mediators involve in acute and chronic inflammation. They are produced by nitric oxide synthase (NOS) and cyclooxygenase enzyme (COX) activation of their corresponding precursor, L-arginine and arachidonic acid (AA) respectively. Increase generation of NO is known to activate COX enzyme which in turn converts arachidonic acid to prostaglandins leading to pain and inflammation. In consideration of each *G. parva* extract, it was found that G6 which was the leaves extracts with ethyl acetate at the concentration of 25  $\mu$ g/ml predominantly inhibited the expression of TNF- $\alpha$  for about 98.6% ( Appendix B-30) and its inhibitory

effect was greater than dexamethasone 10 $\mu$ M which caused only about 21% inhibition. The same results also obtained when the hexane extract from the leaves (G5) was tested for its inhibitory effect on TNF- $\alpha$  generation in the LPS-stimulated macrophages (Appendix B-29). Dexamethasone, a steroidal anti-inflammatory drugs, showed 88.6% inhibition of IL-1 $\beta$  mRNA expression. Only 69.8% inhibition of IL-1 $\beta$  was demonstrated in G2, the branch extract with ethyl acetate and it was found at the concentration of 20  $\mu$ g/ml. Surprisingly, the ethyl acetate extract from branches (G2) and hexane extract from leaves (G5) failed to demonstrate the inhibition effect on IL-6 expression (Fig. 15 and Fig.16) As mentioned before, acridone alkaloids and mixture of  $\beta$  - sitosterol/stigmasterol are among the major compounds found in hexane extract of *G. parva*. The results obtained from this study were in accordance with the previous study which demonstrated that  $\beta$  -sitosterol possesses inhibitory activity of COX-2, IL-6 and TNF- $\alpha$  in macrophages treated with LPS (Ding *et al.*, 2009). The down regulation of the expression of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 mRNA expression may be useful for improvement of inflammatory disorder. However, other constituents of the *G. parva* extract e.g. acridone alkaloids (N-methylataphilline and 5-hydroxy-N methylseverifoline) might participate in immune regulation of these inflammatory cells. Difference constituents in each *G. parva* extract might possess difference role in modulation of inflammatory cytokines expression. Although the molecular mechanism associated with the inhibition of LPS-induced macrophage J774A.1 expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, COX-2 and iNOS by the fractions of *G. parva* extracts in this study were not investigated, it is well established that several common pathways are known to linked the transcription factor, nuclear factor (NF)- $\kappa$ B, because it controls the expression of pro-inflammatory genes such as adhesion molecule and cytotoxic molecular generating enzyme including iNOS and COX-2 (Harmut *et al* 2004., Hong *et al* 2002.) The association of NF- $\kappa$ B and inhibitory effect of *G.parva* extract are needed to be elucidated. At present, investigators are focussing on the development of potent inhibitor of NF- $\kappa$ B for a novel anti-inflammatory drugs.

In summary , production of NO and the expression of iNOS and COX-2 mRNA as well as TNF- $\alpha$  , IL-1 $\beta$  and IL-6 by LPS-stimulated macrophage J774A.1 were measured in response to *G.parva* extracts (G1, G2, G5, G6). All different fractions of the extract were found to significantly inhibit COX-2 and iNOS gene expression in different magnitude and subsequent decrease production of prostaglandins and NO. Furthermore TNF- $\alpha$  , IL-1 $\beta$  and IL-6 were decrease in their mRNA expression due to the inhibitory effect of *G.prava* extract. The implication of these compounds for their anti-inflammatory effects were suggested.

### **Suggestion**

Four fractions of *G.parva* extracts (G1, G2, G5 and G6) are screened on their activity toward pro-inflammatory cytokines generations on the LPS-stimulated macrophage J774A.1. Further studies needed to clarify the mechanism of action of their inhibiting effect on these pro-inflammatory cytokines production and enzyme generation. The in vivo anti-inflammatory potential of these extracts should be confirmed.