CHAPTER 4

Discussion and conclusion

Inflammation is a biological reaction, which is response to disrupted tissue homeostasis. The primary functions of inflammation are rapid degradation or elimination of the underlying source of the disturbance, removal of damaged tissue, and restoration of tissue homeostasis (221). However, sometimes inflammation can cause further inflammation; and it can develop into long-term repair and recovery leading to complications in therapies. Chronic inflammation can cause pathological diseases such as cancer (10), rheumatoid arthritis (14), metabolic syndromes (6) and diabetes mellitus (43). Mostly, the important mediators involved in inflammation are inflammatory cytokines. Therefore, inflammation is recognized as an overwhelming concern to the health status of population and the underlying basis of significant pathological diseases.

Several studies have reported that one of the underlying mechanisms of chronic diseases involves the inflammation. For example, obesity could induce inflammation, which can develop to insulin resistance leading to type II DM (6). The link between obesity and insulin resistance is demonstrated by the expression of the pro-inflammatory cytokines such as IL1-β, IL-6, and especially TNF-α in adipose tissue of obese mice (222). During obesity, adipose tissue become hypertrophy and hyperplasia, in order to respond rapidly to alterations in nutrient deprivation and excess, thereby responding its major role in whole-body energy homeostasis (223). Moreover, accumulations of fat in other tissues such as muscle, liver, hearth and pancreas could induce lipotoxicity leading to inflammation in these tissues. These situations could induce oxidative stress leading to dysfunctionand macrophage accumulation in the tissue, that contribute to inflammation and insulin resistance. During inflammation, adipocyte and macrophage secrete a large number of biomolecules, hormones and cytokines such as free fatty acid, TNF-α, IL-6, leptin, adiponectin and resistin, that could induce the inflammatory response and block the insulin signalingOtherwise, other key molecules that modulate inflammation are PGE2 and NO which produced by the

help of COX-2 and iNOS, respectively (224, 225). These molecules therefore could be targets of the pharmaceutical in the treatment of inflammation-involved diseases, including insulin resistance. Inhibition of inflammation mediator production would be a strategy to reduce chronic inflammation that could prevent people from several chronic diseases including typeII DM and cancer. Recently, the researchers therefore have tried to investigate the novel knowledge to treat or relieve the disorders derived from inflammation by phytochemicals from natural products that have no or less side effect.

This research aimed to investigate biological effects, including, anti-oxidant, anti-inflammation and anti-insulin resistance of *Anoectochilus burmannicus* (AB). Hot water and 80% v/v ethanol extractions were used in the present study to prepare AB extracts (ABE). Herb plants as folk medicine are widely used in Asian people, including Chinese, Vietnamese and Thai, which in form of decoction, which is prepared by boiling mixed herbs with water for approximately 1-4 hours or medicinal liquor, which the herbs are soaked in liquors at least for 3-5 days. Thus, extraction protocol in this study would like to mimic the instruction for use of the plant sample in daily life. Content of phenolic compounds that found in several plant extracts is correlated with free radical scavenger activity. In this study, ABAE which contained higher level of total phenolic and phenolic derivatives (vanillic acid, coumaric acid, ferulic acid) than ABEE, exerted greater anti-oxidant capacity compare to ABEE. The amounts of phenolic detected in ABAE and ABEE probably involving in their anti- oxidant property. However, previous study reported that *Anoectochilus formosanus* and *Anoectochilus roxburghii* contained other active components such as kinsenoside, triterpene derivative and glycosidic constituents (175, 177, 179, 226, 227). Therefore, anti-oxidants included in AB might be not only the phenolic compounds. Bioassay-guided fractionation therefore should be further performed to identify active compound(s) included in AB. Moreover, preparation the plant extract by imitation the brew coffee or tea by infusion the plant with hot water for 5 min at 94-95°C and examine its biological activities would be further performed for developed *A. burmannicus* aqueous extract to consume as hot tea.

During obesity, obese adipocyte can induce macrophage infiltration in adipose tissue and generate higher among of free radicals. The free radical can prolong the

chronic inflammation (43). The results from DPPH and ABTS assays suggested that ABE exerted anti-oxidant property which might provide protective effect on free radicals related inflammation. ABAE and ABEE were next tested for their inhibitory activities on inflammation in LPS-induced RAW 264.7 macrophages and on inflammation-induced insulin resistance in TNF-α-treated 3T3-L1 adipocytes. Nitric oxide (NO), an important pro-inflammatory mediator, is generated from L–arginine, by the catalysis of inducible nitric oxide synthase (iNOS). Overexpression of NO significantly regulates inflammation, which is relevant to the pathogenesis. Besides, NO could be used as a marker for the diagnosis and monitoring of response to anti-inflammatory therapy. In this study, NO production was used to investigate the anti-inflammatory activity of ABAE and ABEE in LPS-stimulated RAW 264.7 macrophages. The results showed that ABE significantly inhibited LPS-induced NO production and iNOS mRNA and protein expresions compared to LPS-induced macrophages. Therefore, ABAE and ABEE may attenuate iNOS expression at the transcription and translation levels leading to the reduction of NO production. Several studies showed phenolic compounds including gallic acid, vanillic acid, coumaric acid and ferulic acid directly inhibited the expression of iNOS gene and protein. Phenolic compounds included in ABAE and ABEE probably play a role in the inhibition of LPS-induced iNOS and NO levels.

The effect of AB on level of IL-1β, IL-6 and TNF-α, important inflammatory cytokines, secreted by macrophages that contributed the inflammation process was next examined. The result showed that ABAE could suppress the expression of IL-6 protein and mRNA levels, IL-1β mRNA levels, but not TNF-α. Several studies reported that many kind of plant extracts can suppress production of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α). Recently, in the study of Feng D et al (2010) found that lycopene and methanolic extract from edible mushrooms inhibited LPS-induced production of NO and IL-6 and decreased mRNA expression of NO, iNOS, IL-1β and IL-6, but they did not alter TNF-α level (228). The authors examined which the signal transduction pathways was modulated by lycopene and suggested that lycopene significantly inhibited the effects of LPS by suppressing a key inflammatory pathway related to mitogen-activated protein kinase (MAPK) ,ERK1/2 and p38 MAPK, and NF-κB, but not JNK. This supports the hypothesis that the expression of IL-1β and IL-6 is

predominantly affected by ERK1/2 whereas JNK is the mainly transduction pathway of TNF-α, after LPS stimulation. Therefore, it might be suggested that ABAE and ABEE may inhibit LPS-induce the activation of MAPK and NF-kB pathways leading to the decreased of IL-1β and IL-6. The molecular mechanism of AB inhibited the cytokine production in LPS-induced macrophages should be further determined.

Prostaglandin E2 (PGE2) is a lipid metabolite derived from arachidonic acid by the COX-2, an inducible enzyme that response to biological effects associated with inflammation (36). Inflammatory stimuli such as hormones, growth factors and LPS can induce the expression of COX-2, which is important during inflammation and in proliferative diseases. Effect of AB on LPS-stimulated expression of COX-2 was next elucidated. The result showed that ABEE could inhibite LPS-induced COX-2 expression in the macrophages whereas ABAE did not alter COX-2 expression. These results suggested that ABEE could inhibit the expression of COX-2 that might be further attenuated the PGE2 production leading to reduce the severity of inflammation dieseases. Hae-Young Kim et al. (2014) showed even chloroform layer of *Actinidia arguta* stems extract did not alter the COX-2 level, but it strongly reduced NO production, pro-inflammatory cytokines in both protein and mRNA expressions and inhibited an activation of NF-κB and MAPKs (229). Besides, Jenny P Castro el al. (2014) reported that the reduction of NO production could contribute to the anti-inflammatory effect in mice treated with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (230). Therefore, the reduction of NO production and IL-1β level, but not COX-2 expression by ABAE might be sufficient to diminish inflammation.

In addition, it has been reported that obesity could induce chronic inflammation which correlated with insulin resistance and diabetes. Obesity causes adipost tissue dysfunction that attenuated lipid metabolism leading to increasing free fatty acid level in serum. These free fatty acid could stimulate inflammatory signaling cascades in immune cells, especially macrophage cells. Then, overproduction of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) would drive immune cell infiltration, cytokine secretion and disrupts the insulin signaling cascade. Impairment of the insulin signaling cascade would lead to a reduction of glucose uptake in adipocytes and muscle (231). Current study reported TNF-α is an important inflammation cytokine that play a critical role in insulin

resistance in adipocytes (232). To determine whether ABE could abolish inflammation-induced insulin resistance, TNF-α was used to stimulate inflammation in murine 3T3-L1 adipocytes. We found that ABAE and ABEE only at 50μg/mL could sinificantly inhibit TNF-α-induced the insulin resistance in adipocyte as represent by the increasing of glucose uptake. Although ABAE and ABEE did not alter TNF-α production in the LPS-treated macrophages, but it could inhibit TNF-α-induced insulin resistance in adipocytes, which might be due to the inhibition the TNF-α signaling. The molecular mechanisms of ABE inhibit TNF-α-induced insulin resistance must be further determined. These results suggested that ABE did not only showed anti-inflammation, but also exhibit inhibitory effect on inflammation-induced insulin resistance. Previous reports showed kinsennoside and polysaccharose from *A. roxburghii* showed significant in anti-hyperglycemic activity in streptozotocin diabetic rats by prevented weight loss in diabetic animals, and ameliorated β-cells damage caused by oxidative stress and NO. In the study of Jian-Gang Zhang (2015) showed polysaccharose isolated from *A. roxburghii* exerted anti-oxidant effect on diabetic mice leading to an improvement of glucose and lipid metabolism, increase of immune protection and reduction of oxidative stress, which might be a possible reason for its anti-diabetic effect (233). Therefore, the active compound(s) included in ABE that provide anti-inflammation and anti-insulin resistance shoud be further identify.

It was shown that the effective doses of ABE used in all experiment in this study did not toxic to murine macrophages and adipocytes. Besides, the safety of ABE was determined in normal human PBMCs and found that ABAE and ABEE at the concentration up to 200 μg/ml had no effect on the cell viability. Thus, ABAE and ABEE might be considered to be safe for further development and reseach in animal and clinical models.

In summary, this study demonstrated apparently for the first time, anti-oxidant, anti-inflammation and anti-insulin resistance of aqueous and ethanolic extracts of *A. burmannicus* extracts (ABAE and ABEE). The effects of ABAE and ABEE on inhibition of NO production and expression of pro-inflammatory cytokines including, IL-6, and IL-1β in activated macrophages might be related to its anti-insulin resistance effect in TNF-α-induced adipocyte. In addition, the effects of ABAE and ABEE on anti-

inflammation might be protect other chronic dieseases related inflammation. Our study suggests scientific information to develop and use the *A. burmannicus* as fork medicine, food ingredient or food supplement for prevention of inflammation-related chronic diseases. Further studies should be carried out to insight into the accuracy mechanisms of these actions and the effective components included in ABE have to be investigated.

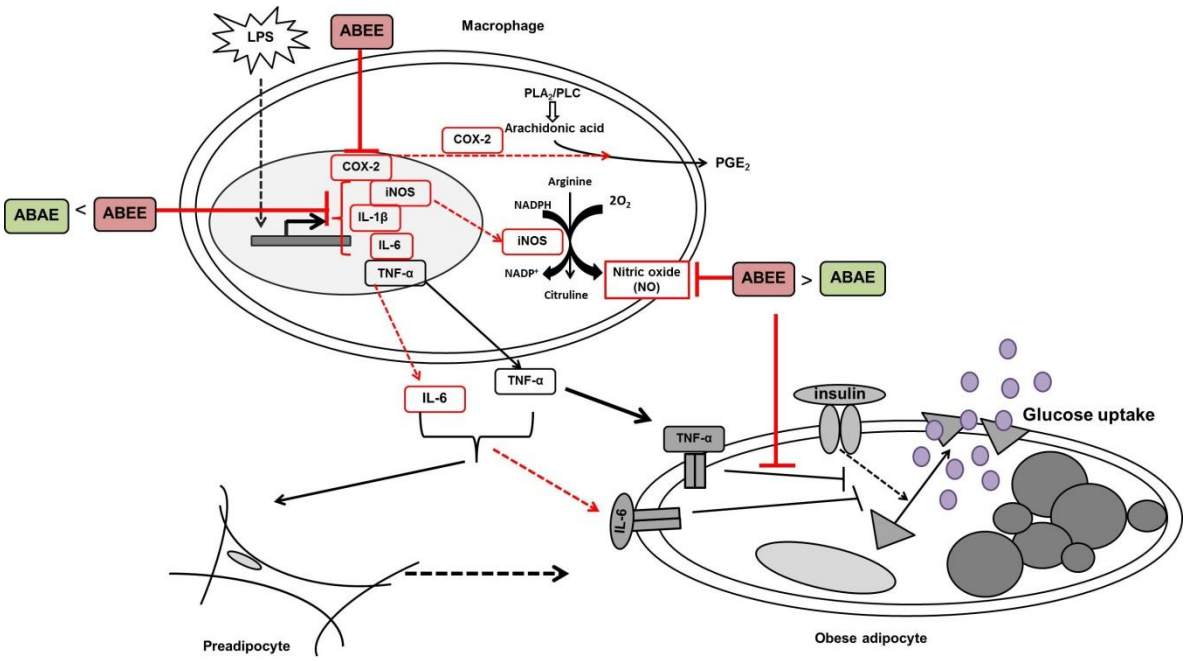

Figure 4.1 Model of ABE actions