CHAPTER 3

Results

3.1 *Anoectochilus burmannicus* extract yield

One hundred gram of dried whole plant of *Anoectochilus burmannicus* (AB) was extracted by hot water or 80% ethanol to get crude aqueous extract (ABAE) and ethanolic extract (ABEE), respectively. The yield of AABE and ABEE was about 16.79% and 17.37%, respectively as shown in Table 3.1.

Table 3.1 Yield of *Anoectochilus burmannicus* extract (ABE)

***Anoectochilus burmannicus* extract (ABE)**	**% (w/w)**
Anoectochilus burmannicus aqueous extract (ABAE)	16.79
Anoectochilus burmannicus ethanolic extract (ABEE)	17.37

3.2 Phytochemical analysis

3.2.1 Total phenolic and flavonoid contents

Phenolics and flavonoids are typically known as phytochemical antioxidants. The total phenolic content in the fractions was quantitatively measured by Folin assay as described in Section 2.4. The total phenolic content was expressed in mg of gallic acid equivalent. Total phenolic contents of the extract was shown in Table 3.2. The amount of total phenolic content in ABAE (19.09 ±1.18 mg GAE/g extract) was higher than in ABEE (6.82 ±0.62 mg GAE/g extract). While total flavonoid content in the extracts was not detectable.

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Table 3.2 Content of total phenolics and flavonoids in ABE

***Anoectochilus burmannicus* extract (ABE)**	**Phenolic content* (g GAE/g extract)**	**Flavonoid content* (g CE/g extract)**
ABAE	19.09 ± 1.18	n.d.
ABEE	6.82 ± 0.62	n.d.

*****Results were expressed as mean±SD, n=3

* n.d. = not detectable

3.2.2 Phenolic and flavonoid derivatives analysis by high-performance liquid chromatography (HPLC)

Phenolic acids are composed of two classes: hydroxybenzoic acid and hydroxycinnamic acid derivatives. Examples of hydroxybenzoic acid derivatives are gallic acid, protocatechuic acid, vanillic acid and that of hydroxycinnamic acid derivatives are chlorogenic acid, coumaric acid and ferulic acid. The chromatographic gradient system was described in Section 2.4. As shown in Table 3.3., HPLC fingerprint data showed that ABAE and ABEE contained chlorogenic acid, vanillic acid, coumaric acid and ferulic acid, when compared the fingerprint of the samples with standard compounds. The Retention Time (RT) of gallic acid, protocatechuic acid, catechin, chlorogenic acid, vanillic acid, hydroxybenzoic acid, coumaric acid and ferulic acid was 7.43,11.25, 13.03, 14.80, 16.23, 16.66, 19.78 and 20.43, respectively. The results showed that ABAE contained chlorogenic acid (0.612±0.02 mg/g extract), vanillic acid (1.647±0.013 mg/g extract), coumaric acid (0.058±0.001 mg/g extract) and ferulic acid (0.169±0.008 mg/g extract) whereas ABEE contained chlorogenic acid (0.75±0.009 mg/g extract), vanillic acid (0.810±0.01 mg/g extract), coumaric acid (0.003±0.003 mg/g extract) and ferulic acid (0.035±0.005 mg/g extract) as shown in Table 3.3. Almost of phenolic derivatives determined in this study was higher in ABAE than

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ABEE, excluding chlorogenic acid. The HPLC chromatograms were presented in Appendix D.

Table 3.3 HPLC analysis of phenolic and flavonoid derivatives in ABE

ABE	Chlorogenic acid (mg/g extract)	Vanillic acid (mg/g extract)	Coumaric acid (mg/g extract)	Ferulic acid (mg/g extract)
ABAE	0.612±0.029	1.647±0.013	0.058±0.001	0.169±0.008
ABEE	0.750±0.009	0.810±0.010	0.003±0.003	0.035±0.005

*Results were expressed as mean ± SD, n=3

3.3 The free radical scavenging activity of ABE

Numerous methods are used to evaluate anti-oxidant activities of matural compounds in foods or biological systems with varying results. Two free radicals that are commonly used to assess antioxidant activity *in vitro* are DPPH and ABTS method ref. ABE were evaluated for their abilities to neutralize the stable free radicals. The DPPH and ABTS free radical scavenging activities of the ABAE and ABEE were compared and shown in Figure 3.1 and Figure 3.2, respectively. The scavenging activity of ABAE was higher than ABEE. As shown in Table 3.4 and 3.5, the SC_{50} of ABAE and ABEE was 4,551 ± 357 µg/mL and 7,506 ± 832 µg/mL, respectively, analyzed by DPPH assay. While the SC_{50} of ABAE and ABEE was 421 ± 30 µg/mL and 834 ± 58 µg/mL, respectively, examinedby ABTS assay.The results indicated that the antioxidant capacities in ABE might due to the compounds that have high polarity included in ABE.

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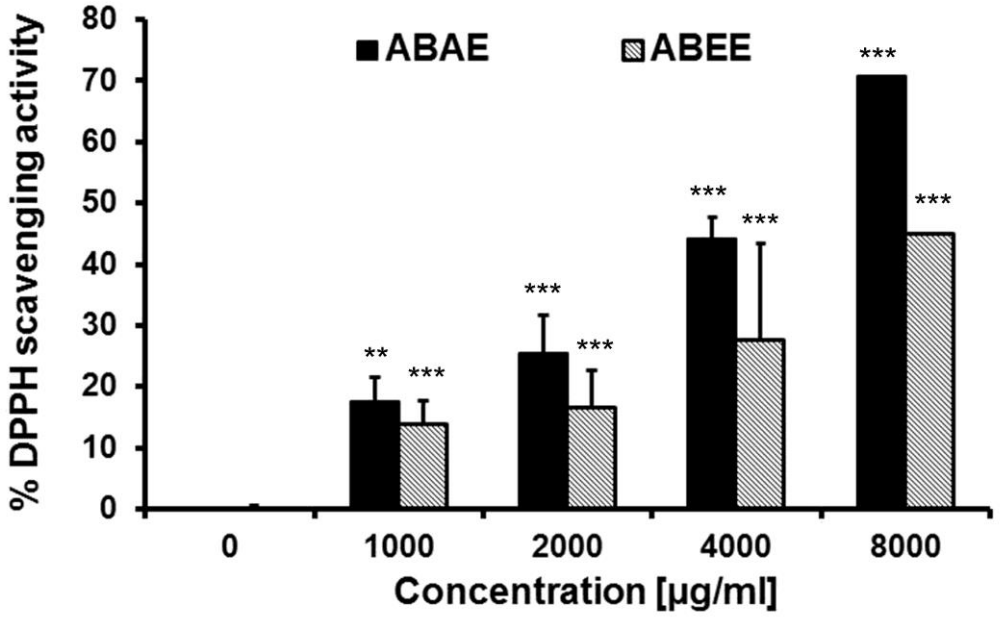

Figure 3.1 The percentage of DPPH radical scavenging activity by ABE. The result represented mean value of triplicate independent experiment. ****p < 0.001, versus* control treated without the extracts.

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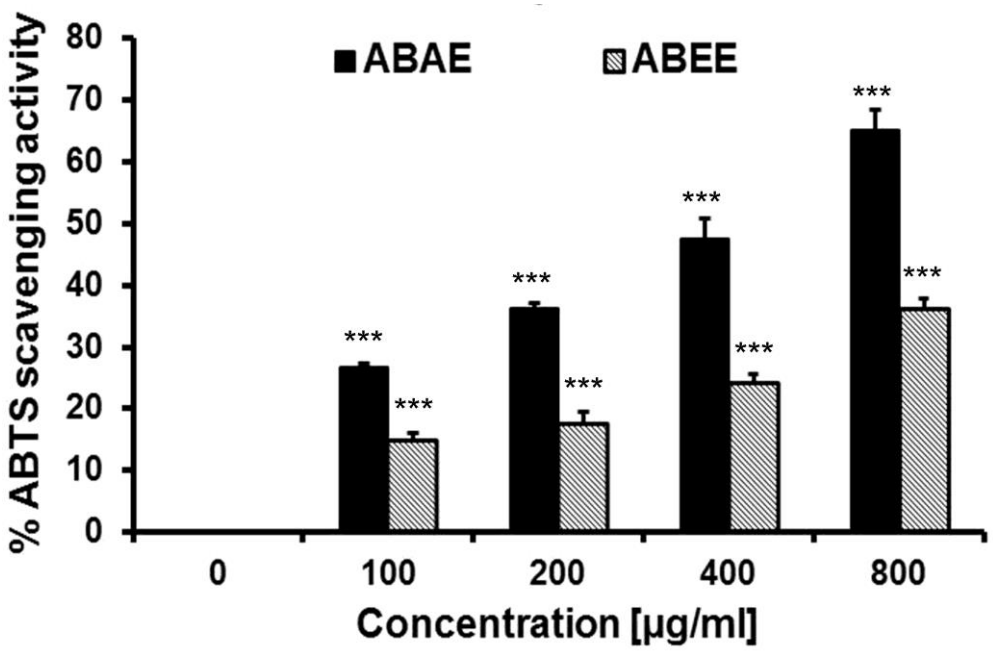

Figure 3.2 The percentage of ABTS acavenging activity of ABE. The result represented mean value of triplicate independent experiment. ****p < 0.001, versus* control treated without the extracts.

Table 3.4 The SC_{50} of ABE in DPPH assay

ABE	**SC_{50} (µg/mL)**
ABAE	4,551 ± 357
ABEE	7,506 ± 832

SC_{50}= the concentration of the extracts that scavenge 50% of DPPH free radical

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Table 3.5 The SC_{50} of ABE in ABTS assay

ABE	**SC_{50} (µg/mL)**
ABAE	421 ± 30
ABEE	834 ± 58

SC_{50}= the concentration of the extracts that scavenge 50% of ABTS free radical

3.4 The effect of ABE on growth of RAW 264.7 macrophage

To examine whether ABE affect the viability of RAW 264.7 macrophage, the cells were treated with varies concentrations of ABAE or ABEE for 24 hours and subjected to WST-1 assay as described in section 2.7. The extracts at the concentration up to 200 µg/mL did not show toxicity on the cells, as shown in Figure 3.3.

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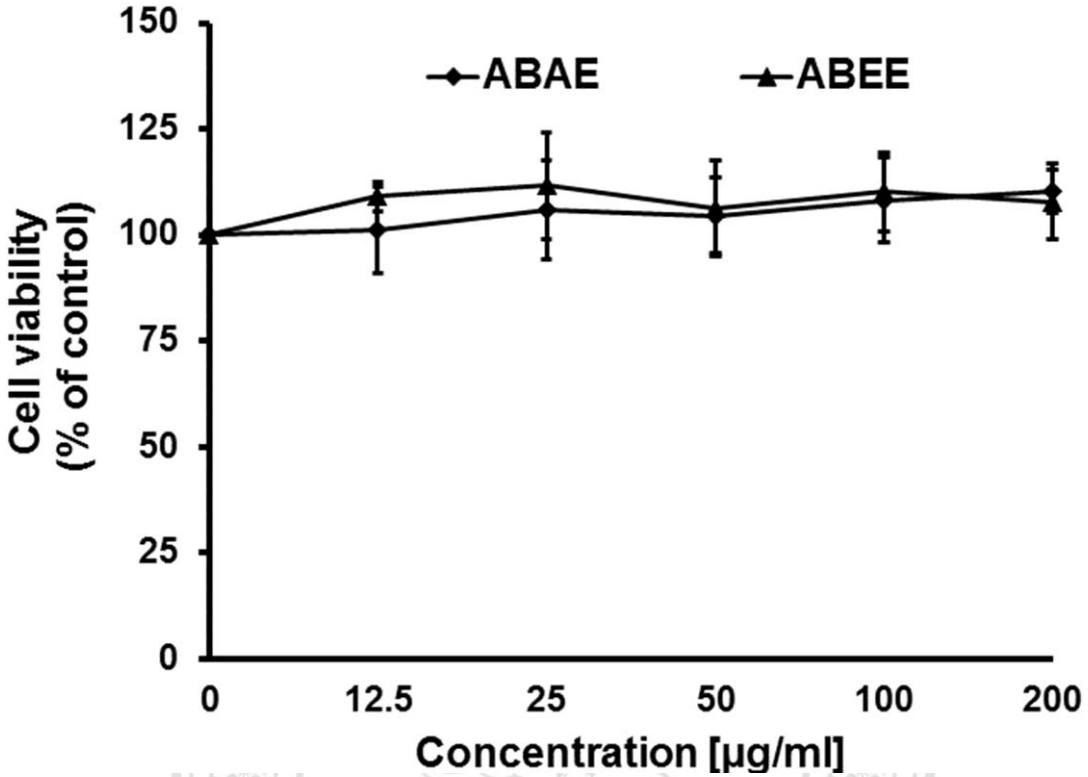

Figure 3.3 Effect of ABE on cell viability of RAW 264.7 macrophages. The results were expressed as the percentage of cell viability compared to the vehicle control (mean ± SD of three independent experiments).

3.5 *In vitro* anti-inflammatory activity of the ABE on NO production

Nitric oxide (NO) is a signaling molecule that plays a key role in the pathogenesis of inflammation ref. To examine whether the ABE exhibit anti-inflammatory activity, NO production was determined in culture medium of RAW264.7 cells which stimulated by lipopolysaccharide (LPS). As shown in Figure 3.4, ABAE and ABEE showed efficiency to inhibit the NO production in a dose dependent manner. ABEE strongly inhibited NO production up to 40% ($p<0.001$) which more effective than ABAE that inhibited the production by 20% ($p<0.001$) in LPS-stimulated RAW 264.7 macrophages.

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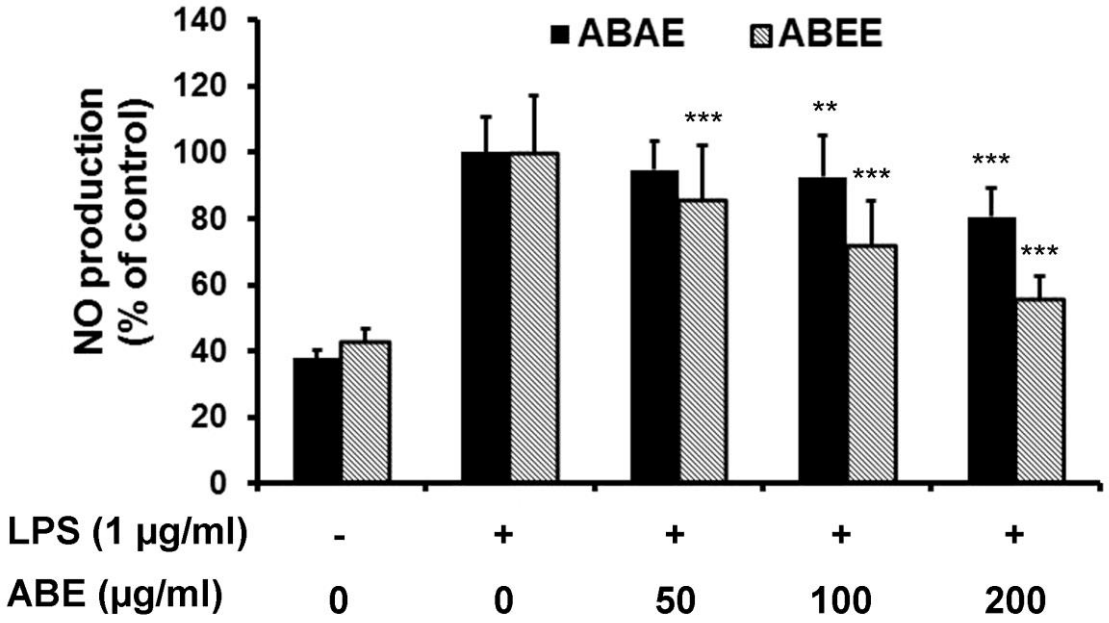

Figure 3.4 Effect of the ABE on LPS-induced NO production in RAW 264.7 macrophages. The cells were pretreated with ABAE or ABEE (0-200 µg/mL) for 2 hours and then 1 µg/mL of LPS was added and further incubated for 24 hours. The condition medium was colloected for determination of nitrite content using Griess reagent assay. Percentage of NO production was relative to LPS-treated group. The result was expressed as the percentage of NO production relative to the LPS-treated control (mean ± SD of three independent experiments) $**p<0.01$ and $***p < 0.001$, *versus* LPS-treated control (without the extracts).

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3.6 Effect of ABE extracts on inflammatory cytokines protein expression in LPS-treated RAW 264.7 macrophage by ELISA

Over production of pro-inflammatory cytokines could induce inflammation in pathogenic conditions. The inhibitory effect of ABAE and ABEE on IL-6 protein expression induced by LPS in RAW 264.7 macrophage was shown in Figure 3.5A. LPS treatment significantly increased IL-6 protein expression, whereas ABAE slighly decreased LPS-stimulated IL-6 protein level in RAW 264.7 macrophages. ABEE at high concentration (200 µg/ml) significantly inhibited LPS-induced IL-6 protein level by >40% ($p<0.001$) in RAW 264.7 macrophages when compared with inflammation control.

The level of TNF-α protein was shown in Figure 3.5B. LPS could induce TNF-α protein level by 50% when compared with non treatment group. ABAE amd ABEE did not affect on TNF-α protein expression in LPS-stimulated RAW 264.7 macrophages.

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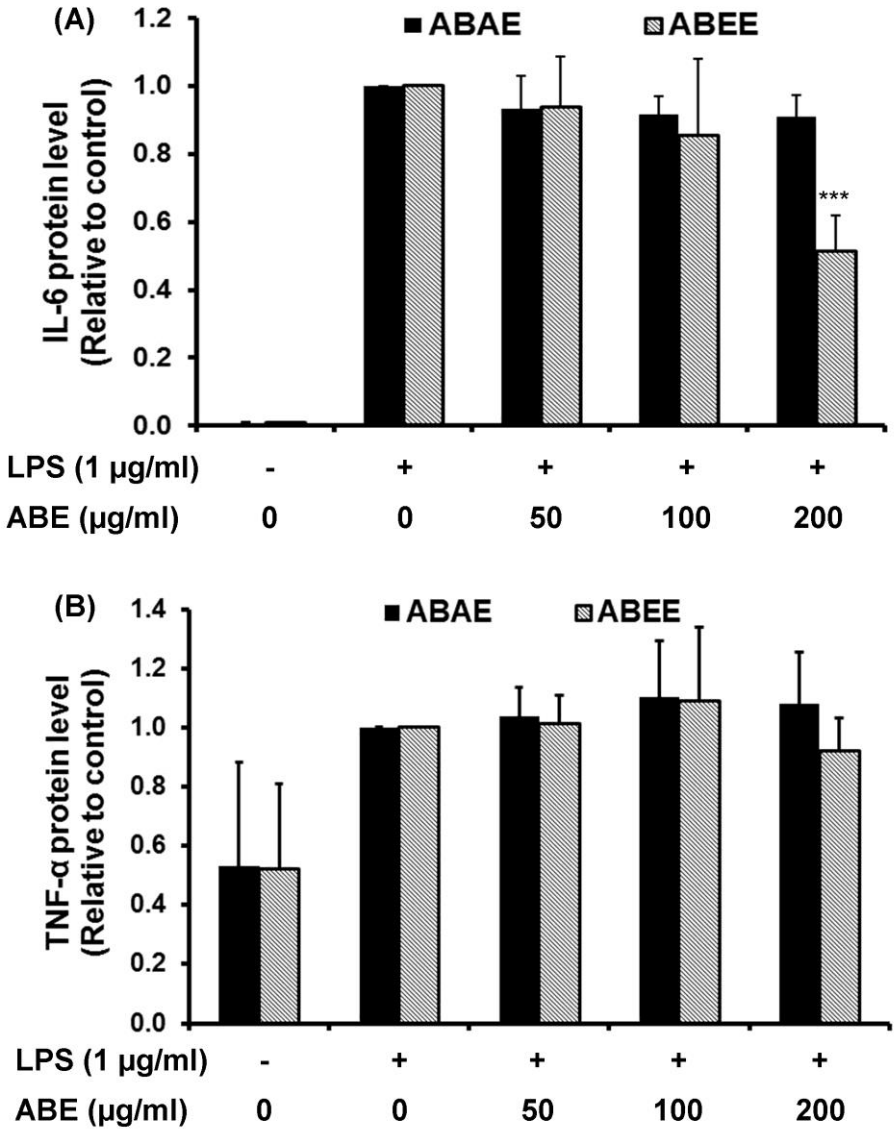

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Figure 3.5 Effect of ABE on IL-6 (A) and TNF-α (B) protein expressions in LPS-treated RAW 264.7 macrophages determined by ELISA. RAW 264.7 cells were treated with various concentrations of each ABE for 2 hours and 1 µg/ml of LPS was added and further incubated for 24 hours. After the treatment, cell supernatant was collected and then subjected to ELISA to detect IL-6 and TNF-α protein levels. The results were expressed as the relative of protein expression compared to the inflammation group (LPS-treated cells without ABE). The data representd as mean ± SD of three indepensent experiment, ****p < 0.001* was significantly different from the LPS-treated group.

3.7 Effect of ABE on protein expression of proinflammatory enzymes in LPS-induced RAW 264.7 macrophage by western blotting

Inducible NO synthase (iNOS) could convert arginine into citrulline, which produces NO in the inflammation process, while COX-2, which is selectively induced by pro-inflammatory cytokines at the site of inflammation. Previous results showed that ABE could decreased LPS-induced NO and IL-6 levels. Researcher next ivestigated whether ABE inhibit LPS-stimulated the expression of inflammatory enzymes, including COX-2 and iNOS by western blot analysis.

As shown in Figure 3.6, 1 µg/mL of LPS could induce the expression of COX-2 in RAW 264.7 macrophages up to 90% when compared to untreated group. ABEE significantly inhibited LPS-induced COX-2 protein expression in RAW 264.7 macrophages by 65% ($p<0.001$) when compared to LPS-treated group.

The level of iNOS protein expression was shown in Figure 3.7. The expression of iNOS protein expression was strongly induced up to >95% in LPS-treated cells when compared to non-inflammation control. ABAE at high dose (200 µg/ml) significantly inhibited LPS-stimulated iNOS protein expression up to 60% ($p<0.001$) in the cells when compared to LPS-treated group. ABEE at lower concentration (50 µg/ml) could significantly decreased LPS-induced iNOS protein expression and the decreased was up to 90% at 200 µg/ml when compared to LPS-treated group.

The results suggested that ABAE and ABEE might inhibit LPS-induced NO production via the down-regulation of iNOS expression. ABEE had higher efficiency than ABAE to diminish COX2 and iNOS protein level in the LPS-treated cells.

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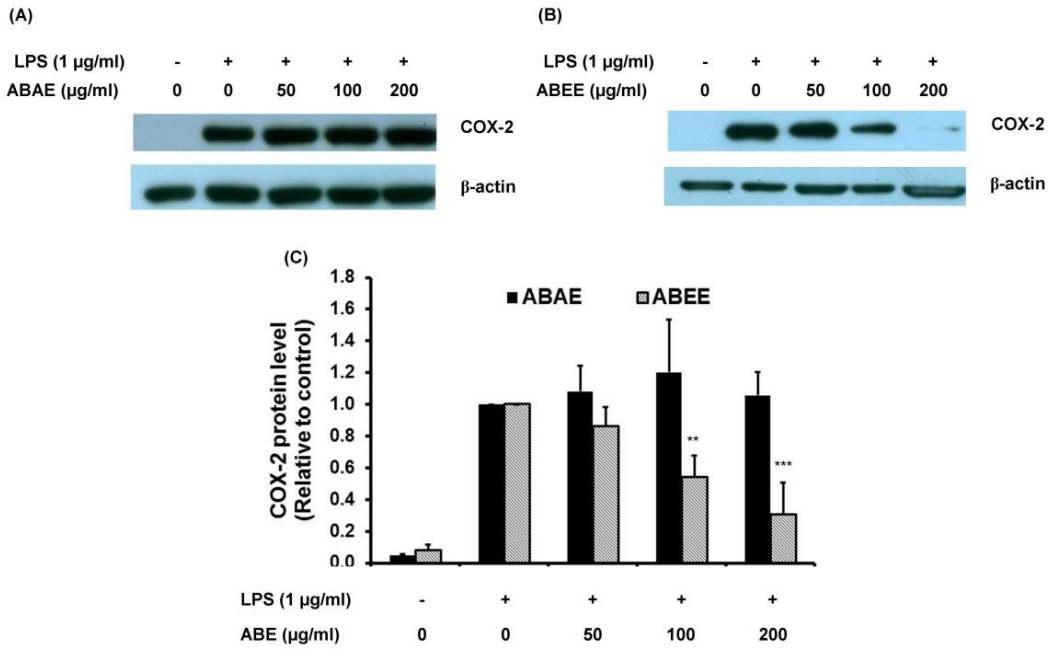

Figure 3.6 Effect of ABE on COX-2 protein expression in LPS-treated RAW 264.7 macrophages using western blotting. RAW 264.7 cells were treated with various concentrations of ABAE or ABEE for 2 hours and 1 µg/mL of LPS was added and further incubated for 24 hours. After the treatment, cell pellet was collected and lysed for protein extraction. Pretein smples were subjected to western blotting for detection of COX-2 expression. The results were expressed as the relative of protein expression compared to the inflammation group. The data representd as mean ± SD of three indepensent experiment, $**p<0.01$, $***p < 0.001$ was significantly different from the LPS-treated group.

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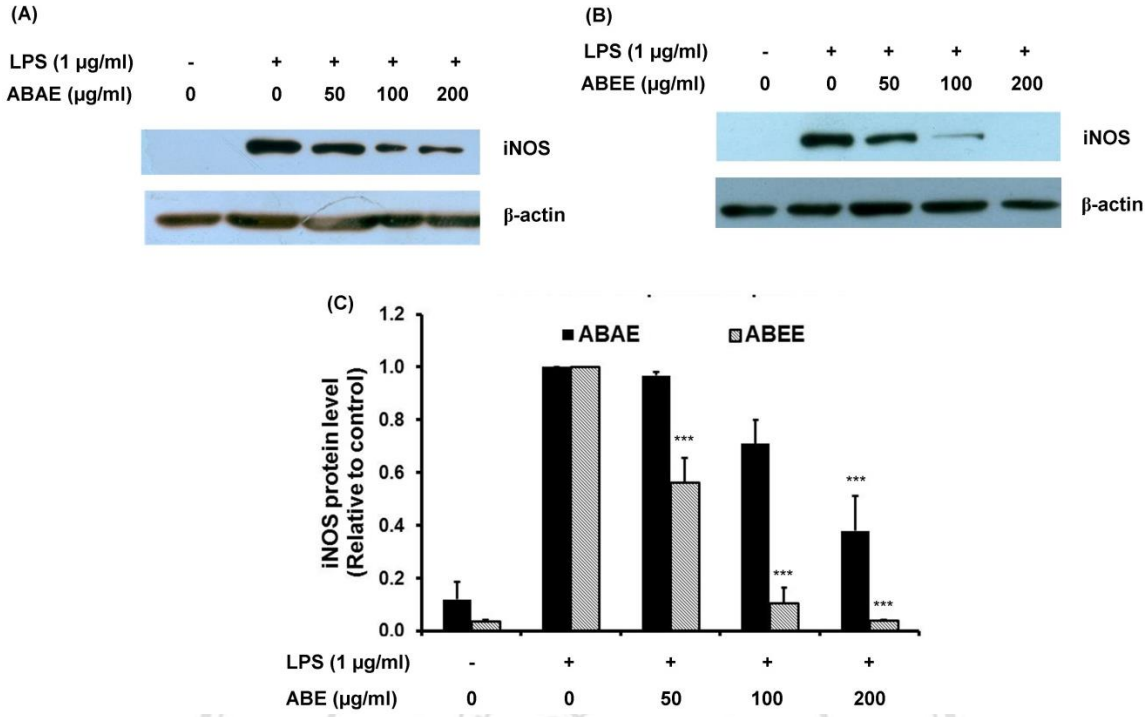

Figure 3.7 Effect of ABE on LPS-stimulated iNOS protein expression in RAW 264.7 macrophages by western blotting. RAW 264.7 cells were treated with various concentrations of ABAE or ABEE for 2 hours and 1 μg/mL of LPS was added and further incubated for 24 hours. After the treatment, cell pellet was collected and lysed for protein extraction. Pretein smples were subjected to western blotting for detection of iNOS expression. The results were expressed as the relative of protein expression compared to the inflammation group. The data representd as mean ± SD of three indepensent experiment, ***$p < 0.001$ was significantly different from the LPS-treated group.

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3.8 Effect of ABE on LPS-stimulated gene expression of inflammatory-associated molecules in RAW 264.7 macrophages by RT-PCR

It was found that ABE could inhibit LPS-induced protein expression of inflammatory-associated molecules, including IL-6, COX-2 and iNOS. Investigation of the effect of ABE on LPS-induced gene expression of the molecules was performed by Real-Time quantitative PCR. Moreover, the effect of ABE on IL-1β and TNF-α mRNA expression was quantified in the LPS-treated cells. As shown in Figure 3.8-3.111, the mRNA expression of IL-1β, IL-6, TNF-α, COX-2 and iNOS was induced by LPS in RAW 264.7 macrophages when compared with untreated group.

Figure 3.8 showed that ABAE and ABEE could inhibit IL-1β mRNA expression in LPS-stimulated RAW 264.7 macrophage in a dose dependent manner. ABAE at high dose (200 µg/mL), whereas ABEE at only 100 µg/mL significantly inhibited IL-1β mRNA expression in LPS-treated macrophages.

The inhibitory effect of ABEts on IL-6 mRNA expression induced by LPS in RAW 264.7 macrophage was shown in Figure 3.9. ABAE (200 µg/mL) and ABEE (100 and 200 µg/mL)significantly inhibited LPS-induced IL-6 mRNA expression up to 30% and 80%, respectively, in RAW 264.7 macrophages. Figure 3.10 showed ABAE and ABEE did not alter LPS-induced the mRNA expression of TNF-α. This results confirmed the previous data of TNF-α protein expression was not decreased by ABE in the LPS-treated cells.

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In Figure 3.11A, ABAE showed slightly inhibition of COX-2 mRNA expression in LPS-stimulated RAW 264.7 macrophages by 20%. While, ABEE (200 µg/mL) significantly inhibit COX-2 mRNA expression by >40% in LPS-stimulated RAW 264.7 macrophages.

Figure 3.11B showed the inhibitory effect of ABE on iNOS mRNA expression induced by LPS in RAW 264.7 macrophages. It was found that ABAE sligtly inhibited iNOS mRNA expression by 30% in LPS-stimulated RAW 264.7 macrophages. Whereas, ABEE at 100 and 200 µg/mL significantly diminished LPS-stimulated iNOS mRNA expression by 25 and 30, respectively, in the cells.

These results suggested that ABE might trigger LPS-induced IL-6, COX-2 and iNOS expression at transciption level.

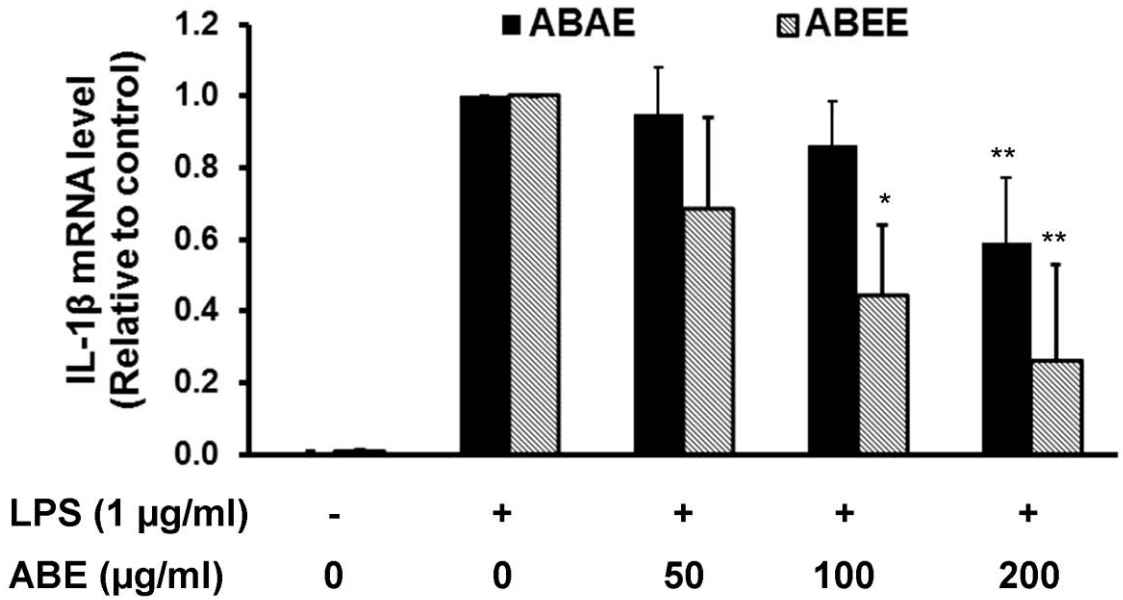

Figure 3.8 Effect of ABE on IL-1β mRNA expressions in LPS-treated RAW 264.7 macrophages determined by Real-Time PCR. RAW 264.7 cells were treated with various concentrations of ABAE or ABEE for 2 hours and 1 µg/mL of LPS was added and further incubated for 24 hours. After the treatment, cell pellet was collected and RNA was extracted with TriZol and then Real-Time PCR was performed using SYBG green as DNA fluorescent dye to detect IL-1β mRNA expressions. The results were expressed as the relative of gene expression compared to the inflamed cell that treated with 1 µg/mL of LPS. The data representd as mean ± SD of three independent experiment, *$p<0.05$ **$p<0.01$, and ***$p < 0.001$ were significantly different from the LPS-treated group.

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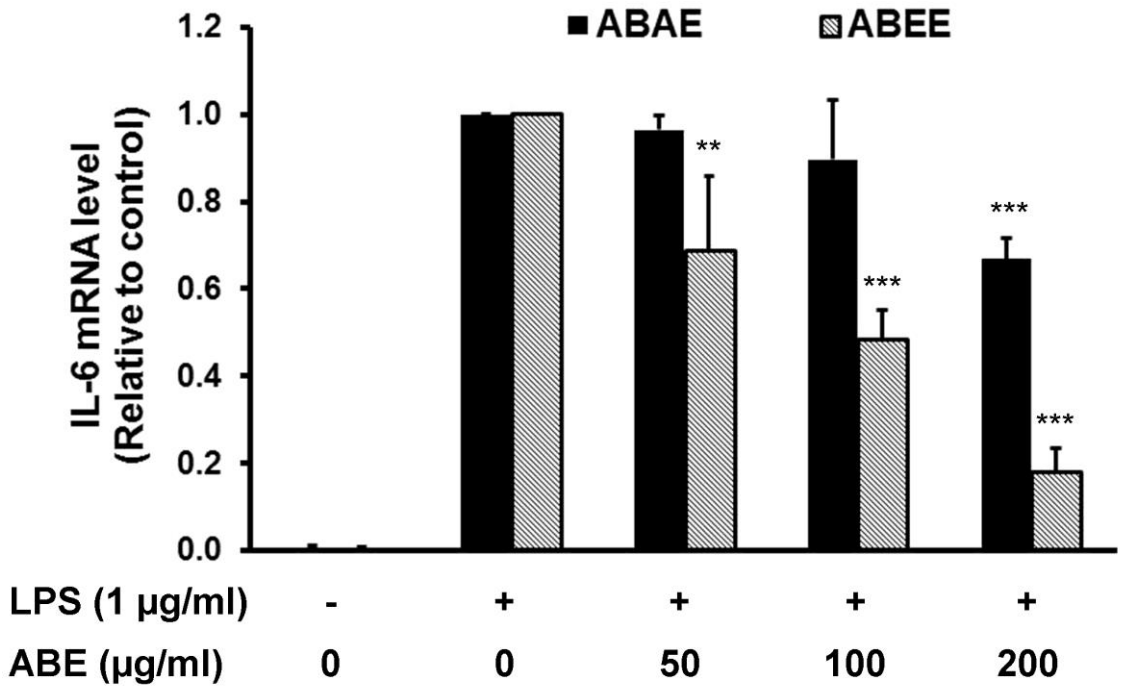

Figure 3.9 Effect of ABE on IL-6 mRNA expressions in LPS-treated RAW 264.7 macrophages determined by Real-Time PCR. RAW 264.7 cells were treated with various concentrations of ABAE or ABEE for 2 hours and 1 µg/mL of LPS was added and further incubated for 24 hours. After the treatment, cell pellet was collected and RNA was extracted with TriZol and then Real-Time PCR was performed using SYBG green as DNA fluorescent dye to detect IL-6 mRNA expressions. The results were expressed as the relative of gene expression compared to the inflamed cell that treated with 1 µg/mL of LPS. The data representd as mean ± SD of three independent experiment, $*p<0.05$ $**p<0.01$, and $***p < 0.001$ were significantly different from the LPS-treated group.

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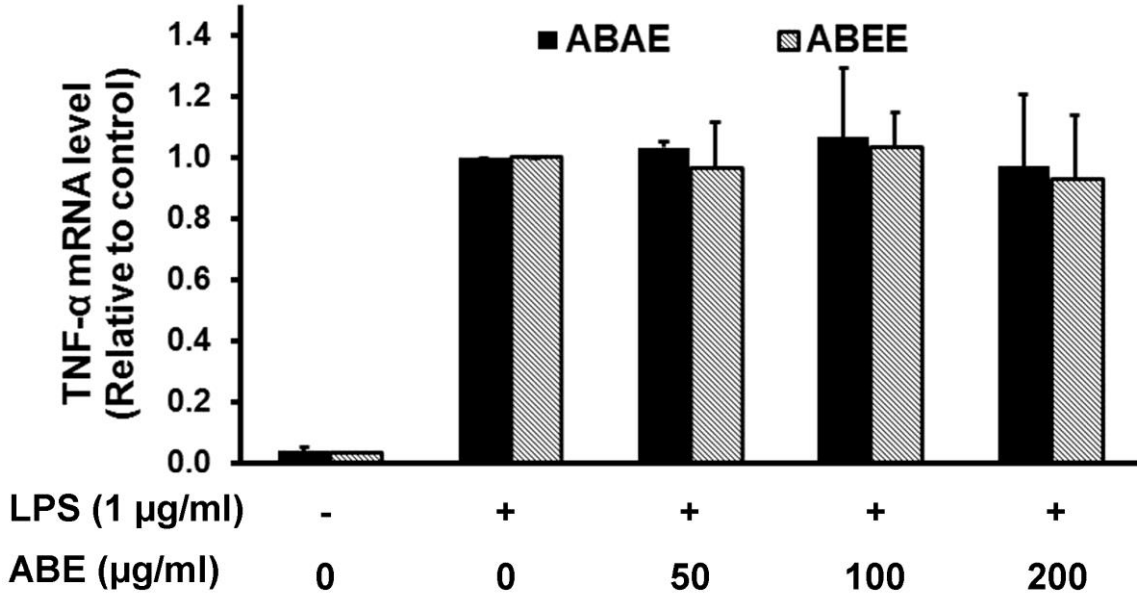

Figure 3.10 Effect of ABE on TNF-α mRNA expressions in LPS-treated RAW 264.7 macrophages determined by Real-Time PCR. RAW 264.7 cells were treated with various concentrations of ABAE or ABEE for 2 hours and 1 μg/mL of LPS was added and further incubated for 24 hours. After the treatment, cell pellet was collected and RNA was extracted with TriZol and then Real-Time PCR was performed using SYBG green as DNA fluorescent dye to detect TNF-α mRNA expressions. The results were expressed as the relative of gene expression compared to the inflamed cell that treated with 1 μg/mL of LPS. The data representd as mean ± SD of three independent experiment, *$p<0.05$ **$p<0.01$, and ***$p < 0.001$ were significantly different from the LPS-treated group.

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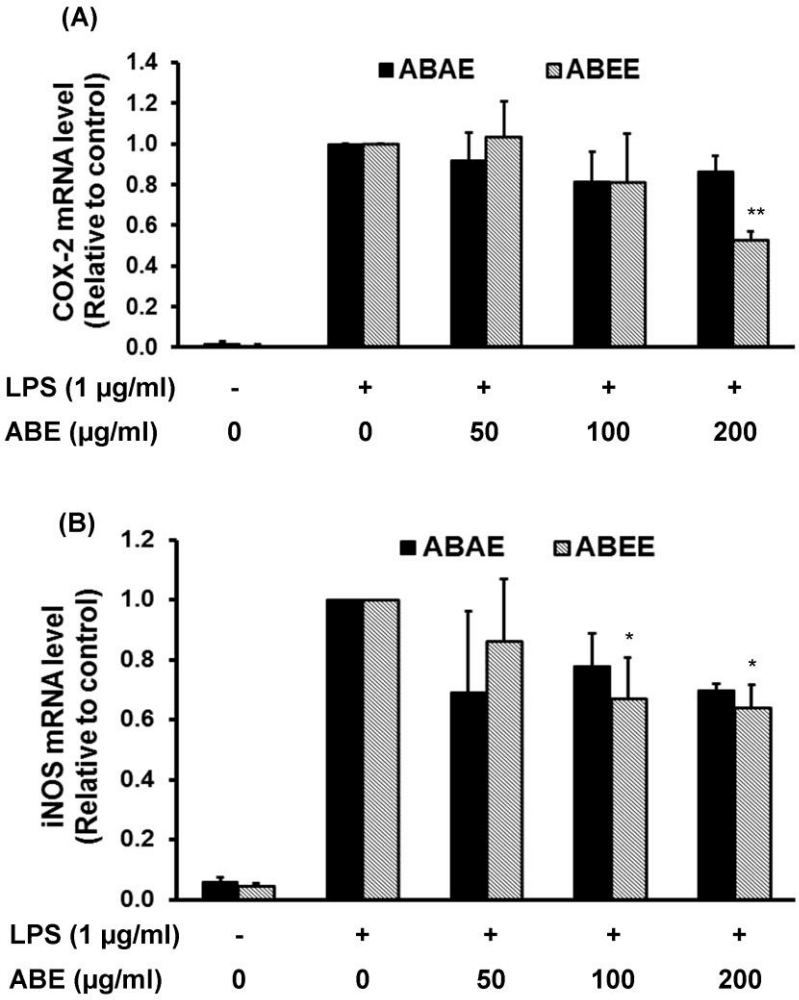

Figure 3.11 Effect of ABE on COX-2 (A) and iNOS (B) mRNA expressions in LPS-treated RAW 264.7 macrophages determined by Real-Time PCR. RAW 264.7 cells were treated with various concentrations of ABAE or ABEE for 2 hours and 1 µg/mL of LPS was added and further incubated for 24 hours. After the treatment, cell pellet was collected and RNA was extracted with TriZol and then Real-Time PCR was performed using SYBG green as DNA fluorescent dye to detect COX-2 and iNOS mRNA expressions. The results were expressed as the relative of gene expression compared to the inflamed cell that treated with 1 µg/mL of LPS. The data representd as mean ± SD of three indepensent experiment, *$p<0.05$, **$p<0.01$ and were significantly different from the LPS group.

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3.9 Cytotoxicity of the ABE on 3T3-L1 adipocytes

The cells were treated with various concentrations of ABAE or ABEE extracts for 24 hours and the cell viability was determined using WST-1 assay. The percentage of cell viability was calculated relative to untreated control. The results demonstrated that ABAE and ABEE at the concentration up to 200 µg/mL did not toxic to the cells as show in Figure 3.12.

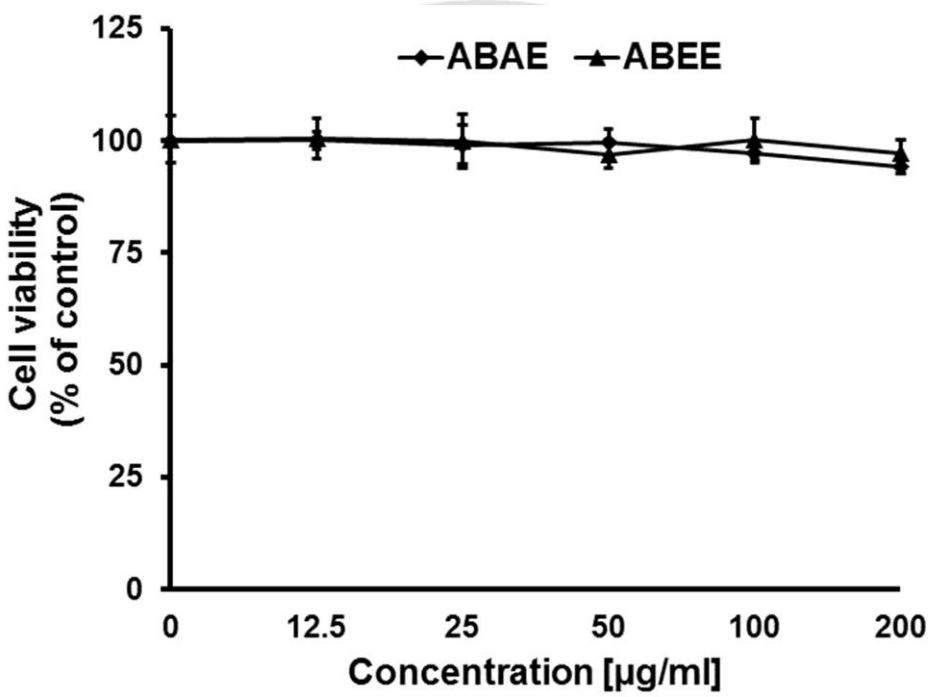

Figure 3.12 Effect of ABE on cell viability of 3T3-L1 cell lines. Cells were treated with each *Anoectochilus burmannicus* extracts (0-200 µg/mL) for 24 hours and the cell viability was determined using WST-1 assay without the extracts, the percentage of cell viability value is 100%. The result was expressed as the percentage of cell viability compared to the control (mean ± SD of three independent experiments).

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3.10 Effect of ABE on anti-insulin resistance in 3T3L-1 adipocytes

Obesity could induce chronic inflammation condition leading to insulin resistance which diminish glucose uptake and stimulate lipolysis in adipocytes ref. Inflamed macrophages are found to be associated with this process by the secretion of pro-inflammatory cytokines, especially TNF-α that has been reported to induce insulin resistance condition ref. Besides, the adipocyte itself is integral to the development of obesity-induced inflammation. Activated pro-inflammatory macrophages and stressed adipocytes repeatedly secrete IL-6 and TNF-α, other cytokines and adipokines to the circulation ref. To investigate the inhibition of inflammation-induced insulin resistance by ABE, obese adipocytes (3T3-L1 matured adipocyte) were treated with TNF-α (50 ng/mL) for 24 hours to induce insulin resistance and then treated with various concentrations of ABE for 24 hours. Then, determination of glucose uptake was performed using fluorescent D-glucose analog (2-NBDG) in the present of insulin.

As shown in Figure 3.13, treatment with nontoxic doses (50 and 200 μg/mL) of ABAE or ABEE significantly improved the insulin response of the TNF-α-treated adipocyte as the insulin stimulation of glucose uptake was induced by >50% when compared to the control cells that induced with TNF-α alone. Therefore, these results evidently demonstrated the inhibitory effect of ABE on the inflammation-induced insulin resistance in 3T3-L1 adipocytes.

The previous results showed anti-inflammation effects of ABAE and ABEE which may result in the prevention of inflammation-related chronic diseases. The results from this study could confirm the effective of ABAE and ABEE to inhibit inflammation-stimulated insulin resistance that is a cause of diabetes type 2.

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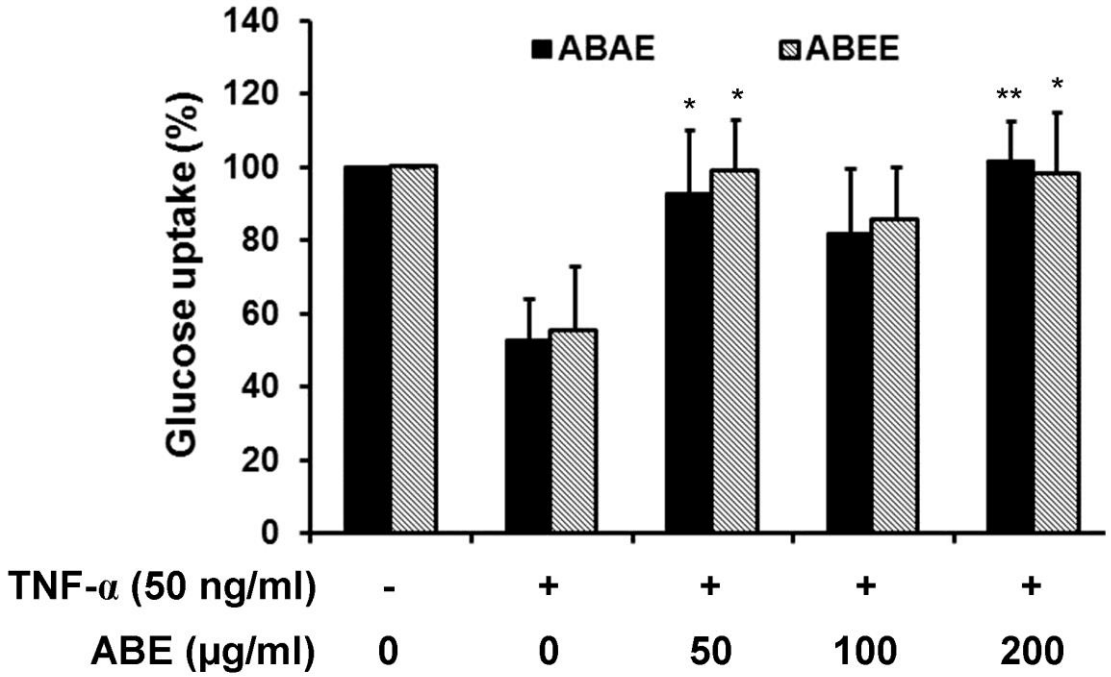

Figure 3.13 The effect of ABE on TNF-α-induce insulin resistance by impaired glucose uptake in 3T3-L1 adipocytes. 3T3- L1 adipocytes were incubated with 50 ng/mL of TNF-α for 24 hours and then treated with ABE (50-200 μg/mL) for 24 hours. The cells were collected and subjected to the assay of insulin-stimulated glucose uptake. *p< 0.05, **$p<0.01$ compared to the inflammation control (TNF-α-treated cells).

3.11 Cytotoxicity of the ABE on PBMCs

The present study found that ABE showed no toxicity on murine macrophages and murine adipocytes. Cytotoxicity of ABE on normal human cells was determined to proof whether the ABAE and ABEE are safe. Human PBMCs were isolated and treated with various concentrations of ABAE or ABEE for 24 hours and the cell viability was assessed by WST-1 assay. As show in Figure 3.14, treatment the cells with ABAE or ABEE at the concentration up to 200 μg/mL, which was an effective concentration, did not alter the cell viability. The result suggested the possibility for futher animal and clinical studies to develop and use the ABE as chemoprevention for inflammation-related diseases.

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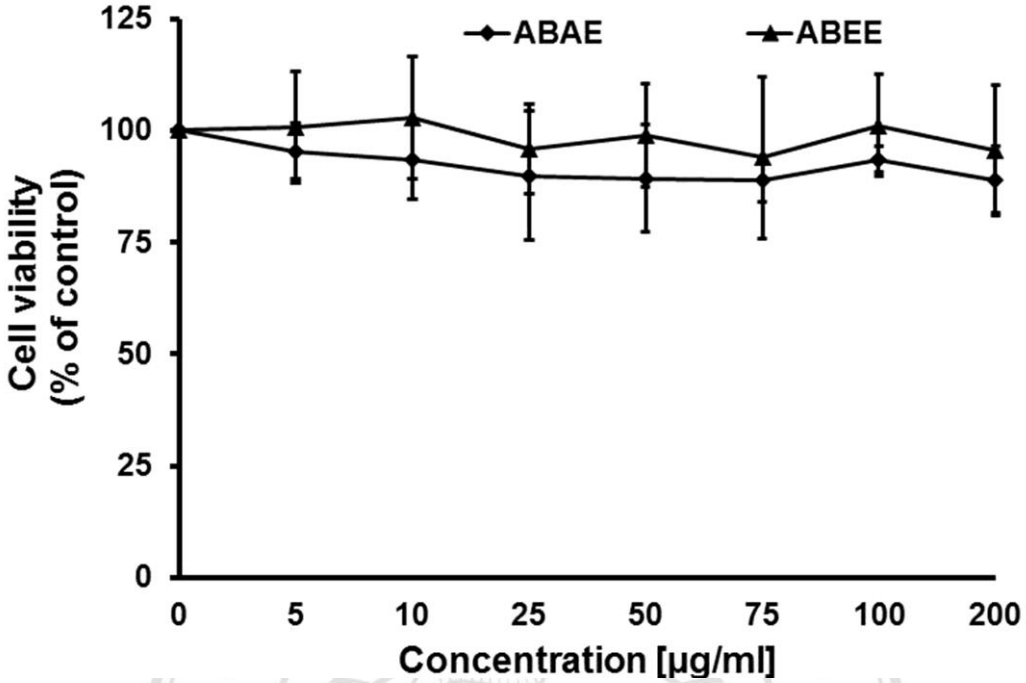

Figure 3.14 Effect of ABE on cell viability of PBMCs. The cells were treated with ABE for 24 hours, and then examined the cell number by WST-1 assay. The result was expressed as the percentage of cell viability compared to the control (mean ± SD of three independent experiments).

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