CHAPTER 2

Material and Methods

2.1 Chemicals

2.2 Research Design

In this research (figure 2.1), aqueous and ethanolic extracts of *A. burmannicus* were prepared. Phytochemical contents in the extracts including total phenolic and flavonoids were determined. Next, the anti-oxidant ability of the extracts was determined by 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays. Lipopolysaccharide-activated RAW 264.7 macrophages were used to investigate the anti-inflammatory effects of the extracts. Cytotoxicity assay was performed to define non-toxic concentration of the extracts and lipopolysaccharide (LPS) on the cells. RAW 264.7 macrophages were treated with aqueous or ethanolic extract (0-200 µg/mL) for 2 hour and then the cells were induced by LPS, a potent inducer of inflammation, and further incubated for 24 hour. After that, cultured medium was subjected to determine nitric oxide production by griess's reagent, and to quantity TNF-α and IL-6 protein level by ELISA. Moreover, the treated-cells were extracted for (i) mRNA in order to determine mRNA expression of IL-1β, IL-6 and TNF-α by RT-PCR. (ii) Protein in order to investigate the amount of iNOS and COX-2. To investigate whether the extracts could inhibit inflammation-induced insulin resistance, TNF-α-stimulated 3T3-L1 adipocytes were treated with the extracts and the level of glucose taken by the cells was examined.

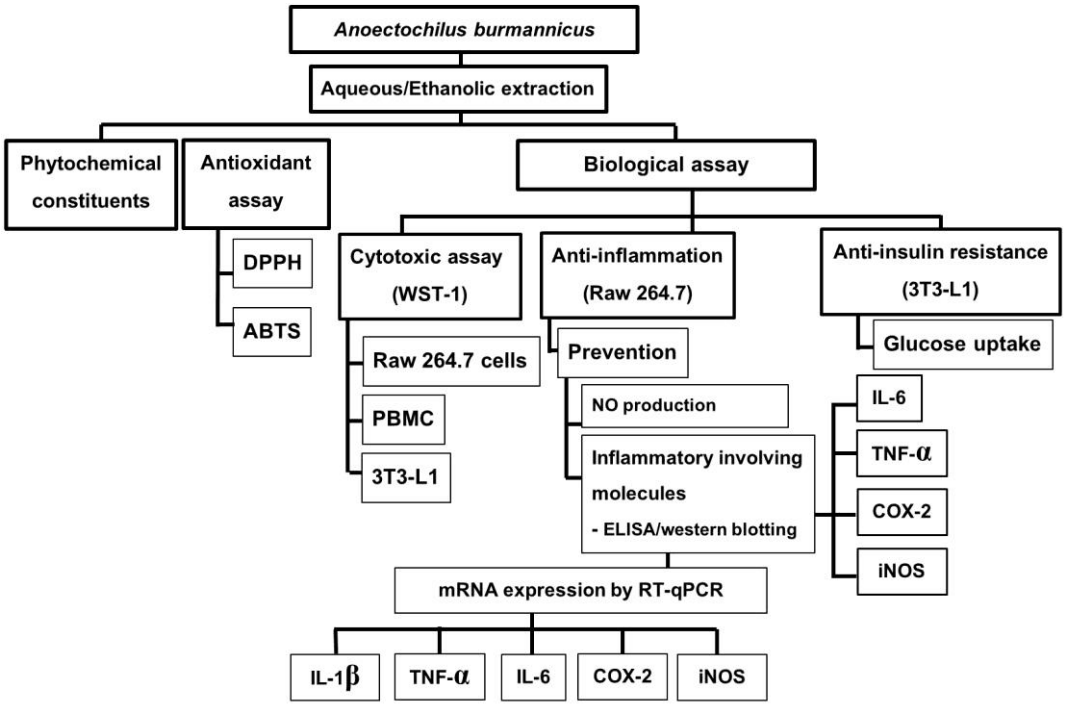


Figure 2.1 Scope of Experimental Design.

2.3 Plant extraction

2.3.1 Plant material

A. burmannicus (AB) was regeneration by tissue cultured at Pong kai Agriculture and Agricultural Cooperatives, that support by Queen Sirikit Botanic Garden located in Amphoe Mae Rim, Chiangmai, Thailand. The dried and powdered whole plant was prepared and then extracted either with water or 80% v/v ethanol.

2.3.2 Plant aqueous extraction

To obtain the aqueous extract of *A. burmannicus* (ABAE), the dried whole plant of AB was soaked in diH_2O at the ratio of 1:200 (v/w), then the mixture was autoclaved at 121 °C for 30 minutes, following the method of Kim GN, et al. (2011) with slightly modifications (180). Then , to obtain crude powder, the filtrate was subsequently freezed and dried. The extract sample was kept at -20°C until used.

2.3.3 Plant ethanolic extraction

Dried whole plant of AB was soaked for overnight with 80% ethanol at the ratio of 1:100 w/v with occasional stirring. The ethanolic fraction was filtered and was

evaporated using a rotary evaporator at temperature 50 °C. Next, the extract fraction was freeze-dried by lyophilization. The crude ethanolic extract of *A. burmannicus* (ABEE) was kept at -20°C until used.

2.4 Phytochemical Screening

2.4.1 Determination of Total Phenolic Content

Total soluble phenolic level was determined using Folin–Ciocalteu reagent assay with slightly modifications by Subedi L, et al. (2014) (181). Briefly, 0-200 µg/mL of the extracts was prepared in 400 uL in DMSO. A volume of 300 µL of 10% equivalent Folin–Ciocalteu reagent was added and the mixture was incubated for 3 min in dark, room temperature. Then 300 µL of 7.5% equivalent Na_2CO_3 was added. Then the mixture was incubated for 30 min in the dark, after which absorbance readings at 765 nm was measured using a spectrophotometer. Acidified ethanol was used as the blank. The content of phenolics was expressed as gallic acid equivalents (GAE) in mg/g of sample, using a standard curve of 0–20 µg of gallic acid/mL. Data was recorded as mean ± SD for three replicates.

2.4.2 Determination of Total Flavonoid Content

Total flavonoids content was determined using aluminum chloride colorimetric method (181). Two hundred and fifty µl of each plant extract (0-200 µg/mL) was mixed with 125 µL of 10% w/v aluminum and 125 µL sodium nitrate and 1 mL of 1 M sodium hydroxide. The reaction was kept at room temperature for 15 min in the dark. The absorbance of the reaction mixture was measured at 510 nm with a spectrophotometer. The standard curve was prepared using quercetin solutions in DMSO at concentrations 2.5 to 20 µg/mL. All values were expressed as milligram of catechin equivalents per 1 gram dry weight. Data was recorded as mean ± SD for three replicates.

2.4.3 Determination of phenolic and flavonol constituents

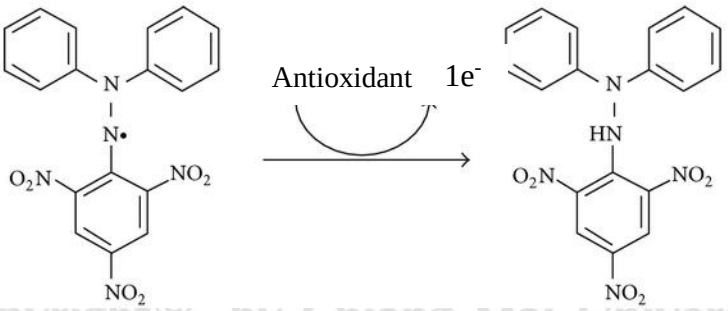
To determine the constituent of phenolic and flavonol in ABAE and ABEE, High-Performance Liquid Chromatography (HPLC) of ABE was performed according to the

method described by Yizhong Cai, et al (2003) with some modification (182). Briefly, 25 mg of ABE extracts was dissolved in 1 mL of HPLC-grade water. An aliquot of the ABE solution was subjected to a C18 column (250 mm×4.6 mm, 5 µm) and eluted with gradient elution of 0.1% (v/v) trifluoroaetic acid (TFA) at flow rate 1ml/min and monitored by the absorbance at 280 and 325 nm. Peak area and retention time of each sample of the present HPLC method were determined to compare with standard curve of various concentration of standard gallic acid, protocatecheuic acid, catechin, chlorogenic acid, vanillic acid, hydroxybenzoic acid, coumaric acid and ferrulic acid.

2.5 Antioxidant Activity Screening

2.5.1 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) assay

2, 2-diphenyl-1-picrylhydrazyl radical or DPPH is a well-known radical and a trap ("scavenger") for other radicals. DPPH is used as an indicator of the radical nature of the reaction. The principle of DPPH assay is depicted on figure 2.2. The DPPH radical has a deep violet color in solution, and it becomes colorless or pale yellow when neutralized (183). This reaction was monitoring and the number of initial radicals can be counted from the change in the optical absorption at 520 nm of the DPPH.



DPPH radical DPPH

Figure 2.2 Principle of DPPH Assay (184).

The reaction for scavenging DPPH radical was prepared in 96-well microplate at room temperature following the method of Chi-Yang Li, et al. (2014) with some modification. Twenty µl of the extracts dissolved in DMSO was added into 180 µL of DPPH radical (2 mmol/L in ethanol) and incubated in dark for 15 min. After that an absorbance of DPPH was then measured at 540 nm in a photodiode array spectrophotometer. Trolox (0-20 µg/mL) was used as a standards, and the mixture of

180 µL of DPPH with 20 µL of DMSO was used as a vehicle control. The antioxidant activity was expressed as % inhibition (quenching) of DPPH absorbance.

2.5.2 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay

ABTS radical cation (ABTS•+) was produced by the reaction of 7 mM final concentration ABTS with 2.45 mM potassium persulfate (final concentration) and then the mixture was standed in the dark at room temperature for 12–16 h before following the method of. Jatinder Kumar, et al. (2014) with some modification (185, 186). Then, the ABTS•+ solution, which are 2,29-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, and potassium persulfate (di-potassium peroxdisulfate), was diluted with deionized water to an absorbance of 0.70 (±0.02) at 734 nm, at 30°C. After added 10 µL of extract or Trolox standards (final concentration 0–20 µg/ml) in ethanol into 1.0 mL of diluted ABTS•+ solution, the absorbance reading was taken at 30°C exactly 1 min after initial mixing and up to 6 min. All determination was carried out at least three times, and in triplicate. The percent inhibition of absorbance at 734 nm was calculated and plotted as a function of concentration of antioxidants and that of Trolox for the standard reference data. Trolox equivalent antioxidant capacity (TEAC) values and calculated as mean value ± standard deviation (SD) (n = 3).

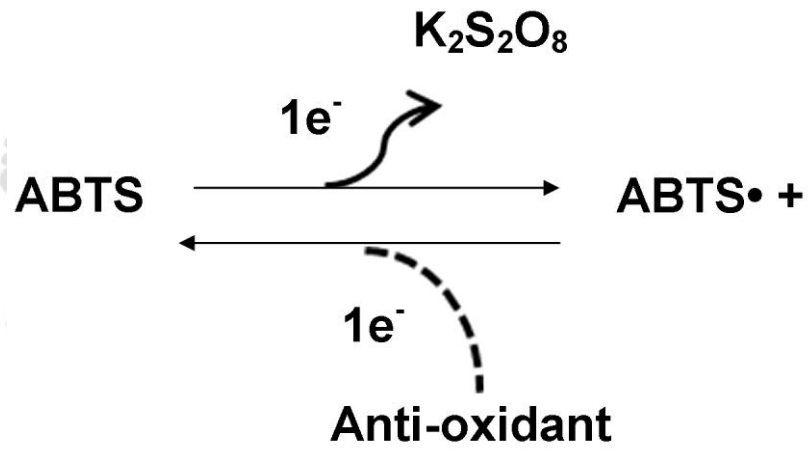


Figure 2.3 Antioxidants inhibit the oxidation of ABTS by electron transfer radical scavenging (187).

2.6 Cell culture

RAW 264.7 macrophage cell line was obtained from CLS-Cell Lines Service, Germany. The cells were cultured as suspension in ultra-low attachment culture dish in DMEM with L-glutamine supplemented with 10% FBS and 1% penicillin/streptomycin solution under 5% CO_2 at 37°C. The cells were harvested and subjected to experiments when the cell confluent reached 80%.

3T3-L1 preadipocyte cells were purchased from ATCC (American Type Culture Collection). The cells were cultured in DMEM with L-glutamine supplemented with 10% bovine serum and 1% penicillin/streptomycin solution and maintained at 37 °C in a 5% CO2 humidified atmosphere (CO_2 incubator, Heal Force). After reach 80% confluence on day 2, 3T3-L1 preadipocyte were differentiated by inducing with 0.5 mM 3-isobutyl-1-metylxanthine (IBMX), 0.5 µg/ml dexamethasone, 5 µg/mL insulin and 10% fetal ovine serum for 72 hours, after re-suspended with the same medium without IBMX and dexamethasone. For completed differentiation the cells was incubated with DMEM containing 10%FBS for 7-14 days.

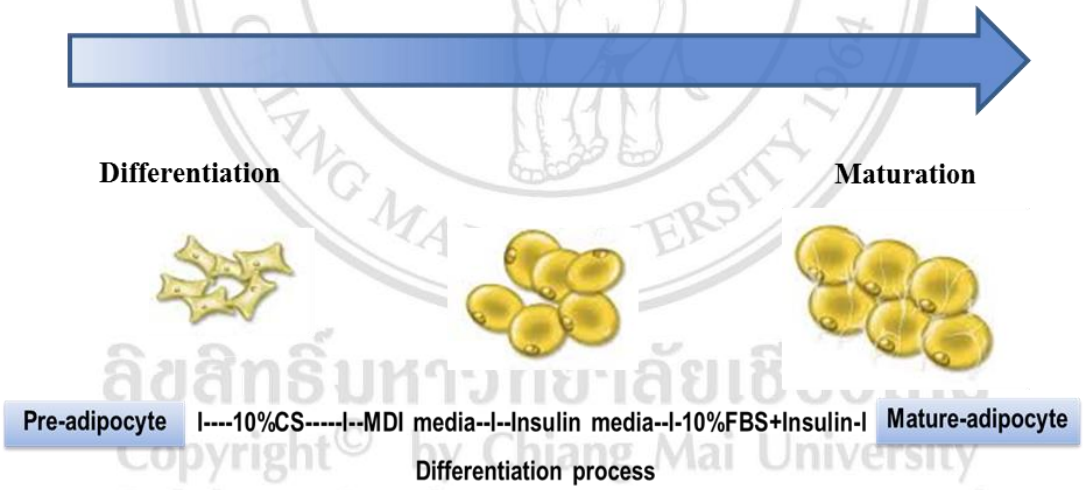


Figure 2.4 3T3-L1 adipocyte differentiation processes.

Human peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-hypaque, according to the manufacturer's instructions. The mononuclear cells were carefully collected and washed twice with ice-cold PBS buffer pH 7.4. After completely isolated, PBMC was counted and suspended in RPMI medium.

2.7 Cytotoxicity of the extracts in RAW 264.7 macrophage, PBMCs and 3T3-L1 adipocyte cells

(2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) or WST-1 was a reagent to measure cell proliferation. The assay utilizes the enzymatic cleavage of tetrazolium salt to complex formazan by cellular mechanism that occurs primarily at the surface (188). This mechanism is dependent on the glycolytic NAD(P)H production of viable cells, which is mitochondrial dehydrogenases enzyme (Figure 2.5). The formazan dye are formed directly correlates to the number of metabolically active cells in the culture (189). In brief, WST-1 assay would be added followed by incubation at 37°C for 2 hours. The absorbance (Optical density; OD) at 450 nm was measured using a microplate reader. Percentage of cellular viability was calculated using the following equation.

$$\% \text{ Cellular viability} = \frac{(\text{OD of treatment group} - \text{OD of blank})}{(\text{OD of control group} - \text{OD of blank})} \times 100$$

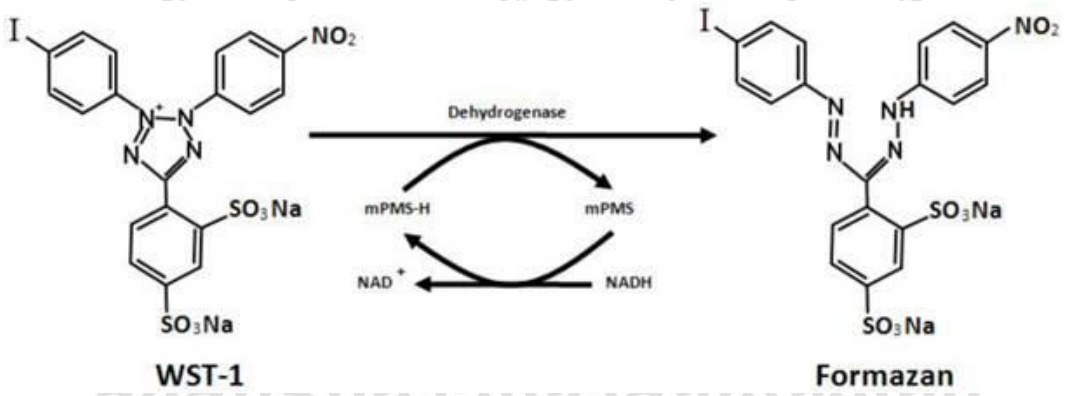


Figure 2.5 Principle of WST-1 assay (190).

Macrophage (RAW 264.7) and 3T3-L1 adipocyte cells were seeded at 1×10^4cells/well and 5×10^3 cells/well, respectively, in 96-well culture plates. Then, the cells were treated with various doses (5-200 µg/mL) of ABAE and ABEE for 24 hours. Cytotoxicity of the extracts was also determined by WST-1 assay.

Non-cytotoxic concentration of the extracts on the macrophages and adipocytes were used in further experiments.

Human peripheral blood mononuclear cells (PBMCs) were used to determine cytotoxicity of the extracts on human normal cells. PBMCs were seeded at 8×10^4cells/well in 96-well culture plates. Then, the cells were treated with various doses (5-200 µg/mL) of the ABAE and ABEE for 24 hours. Cytotoxicity was determined using WST-1 assay.

2.8 Anti-inflammation effect of the extracts from *Anoectochilus burmannicus*

2.8.1 Inhibition of NO production from macrophage (RAW 264.7) stimulated with lipopolysaccharide

The assay principle is based on the reaction of nitrite ions with 1% sulfanylamide in acidic medium and the diazo compound, which is N-(1-naphthyl)ethylenediamine (NED) obtained further reacts with diamine yielding in an azo color (Figure 2.6) (191). One hundred µL of cell culture supernatant was removed and combined with 100 µL of griess reagent in 96 well plate. The nitrite ion concentration was determined by measuring the absorbance of the azo color at 540 nm by using microplate reader. The amount of nitric oxide was determined in comparison with a sodium nitrite standard curve.

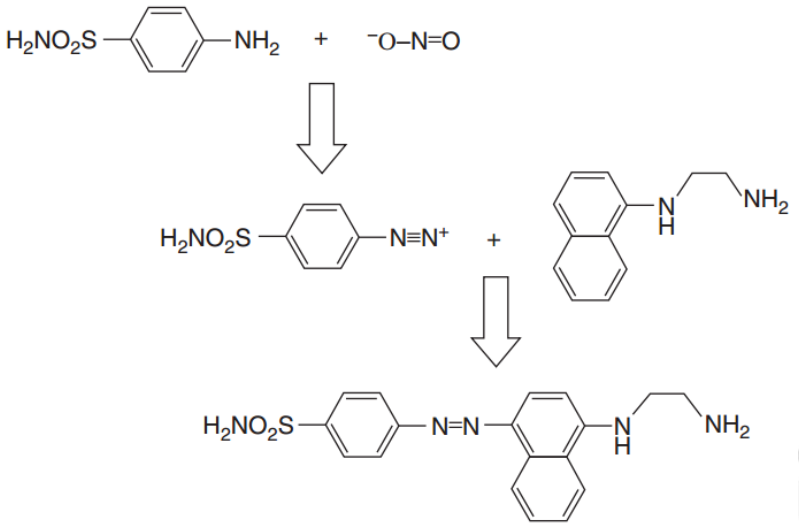


Figure 2.6 Griess reaction principle (191).

Macrophage cells (RAW 264.7) were seeded at 1×10^4cells/well in 96-well culture plates. Next, the cells were pre-treated with non-toxic concentrations (50, 100 and 200 µg/mL) of the aqueous or ethanolic extracts for 2 hour. Then, the cells were further treated with the same concentration of the ABAE and ABEE in the presence or absence of LPS for 24 hour. Later, the supernatants were collected into less colorimetric 96-well plate. Griess reagent was added and incubated at room temperature for 15 minutes in the dark. The absorbance was determined at 540 nm using a microplate reader. Percentage

of cellular viability was calculated using the following equation. Results were expressed as mean ± SD, n=3

$$\% \text{ Cell viability} = \frac{\text{OD of treatment group - OD of blank}}{\text{OD of control group - OD of blank}} \times 100$$

2.8.2 Inhibition of TNF-α, IL-1β, IL-6, iNOS and COX-2 expression in macrophage RAW 264.7 stimulated with lipopolysaccharide (LPS)

The RAW 264.7 cells were seeded at 5×10^5cells in a 35 mm^2 dish and cultured overnight to allow cells become nearly confluent. Next, the cells were pretreated with varying doses (50, 100 and 200 µg/mL) of aqueous or ethanolic extracts for 2 hour. Then, LPS (1 µg/mL) was added and further incubated for 24 hour. The cells and culture supernatant from the experiment were used to quantify TNF-α, IL-1β, IL-6, iNOS and COX-2 mRNA expression by RT-PCR. Moreover, the cells were collected and determined and protein level of TNF-α, IL-6 by ELISA, and protein expression of iNOS and COX-2 by western blot analysis.

2.8.2.1 Gene expression analysis by quantitative RT-PCR (qRT-PCR)

Reverse transcription-polymerase chain reaction (RT-PCR) is commonly used in molecular biology to detect RNA expression via the creation of complementary DNA (cDNA) transcripts from RNA (192). PCR method was used DNA polymerase to synthesize new strand of cDNA to the offered template strand. The ability of DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group by the help of a primer that can add the first nucleotide to synthesize cDNA from template strand. The primer will ammeal to the mRNA and extended by an RNA-dependent DNA polymerase to transcribe a cDNA copies (Figure 2.7). The researcher was used this method for increasing a specific region of template sequence. Billions copies of PCR product was accumulated at the end of the PCR reaction.

Quantitative RT-PCR (qRT-PCR) is based on using the amplication of cDNA to hybridize a particular target gene by specific forward and reverse oligonucleotide primer. qRT-PCR can also be applied to the detection and quantification of DNA in samples. qRT-PCR was using a thermal cycler with the

capacity to illuminate each sample with a beam of light of at least one specified wavelength and detect the fluorescence emitted by the excited fluorophore. Fluorescent reporter probes detect only the DNA containing the sequence complementary to the probe; therefore, use of the reporter probe significantly increases specificity, and enables performing the technique even in the presence of other doublestrand DNA (dsDNA). SYBR Green was bind to any amplified dsDNA PCR products.

2.8.2.1.1 RNA Extraction

Total RNA was extracted with TriZol reagent (Invitrogen, USA) according to the manufacturer's instruction. In brief, cell culture supernatant was removed then cells monolayer was lysed with 1 mL of TriZol reagent and passed several times through a pipette. The homogenate solution was transferred to the fresh tube and incubated for 10 min at room temperature. After, added 200 µL of chloroform into the homogenized sample, the tube was shaken for 15 sec and incubated at room temperature for 3 min. The homogenized solution was separated in to three phases, including a lower red of phenol-chroroform phase, a middle-phase, and a colorless upper aqueous phase by centrifugation at 12,000×g for 15 min at 4°C. RNA remained in the upper aqueous phase was transferred into a fresh tube and mixed with 500 µL of isopropanol at -20°C for overnight. After that, the RNA sample was centrifuged at 12,000×g for 10 min at 4°C to obtained RNA pellet by removing the supernatant. One mL of 75% ethanol was added to the RNA pellet and then centrifuged at 12,000×g for 5 min at 4°C. The RNA pellet was left for air-dried and re-dissolved with diethylpyrocarbonate (DEPC)-treated water. The purity of total RNA was quality and quantity by the ratio of OD260/OD280 value 1.8-2.0.

2.8.2.1.2 cDNA synthesis by reverse transcription

One µg of total RNA was used as a template strand to synthesize cDNA with oligo (deoxythymidine) primers and *Superscript II* reverse transcriptase (Invitrogen) (Figure 2.7). In brief, the reaction contained 1µg of total RNA of each sample, 0.5 µg of oligo-$(dT)_{18}$, 1mM dNTPs, 200 units of reverse transcriptase and 20 units of ribonuclease inhibitor was added with DEPC-treated water for adjusted volume into 20 µl. The reaction was incubated at 70°C for 5 min, 4°C for 1 min, and 42°C for 60 min. At the end of these processes, the cDNA was strored at -20°C until used.

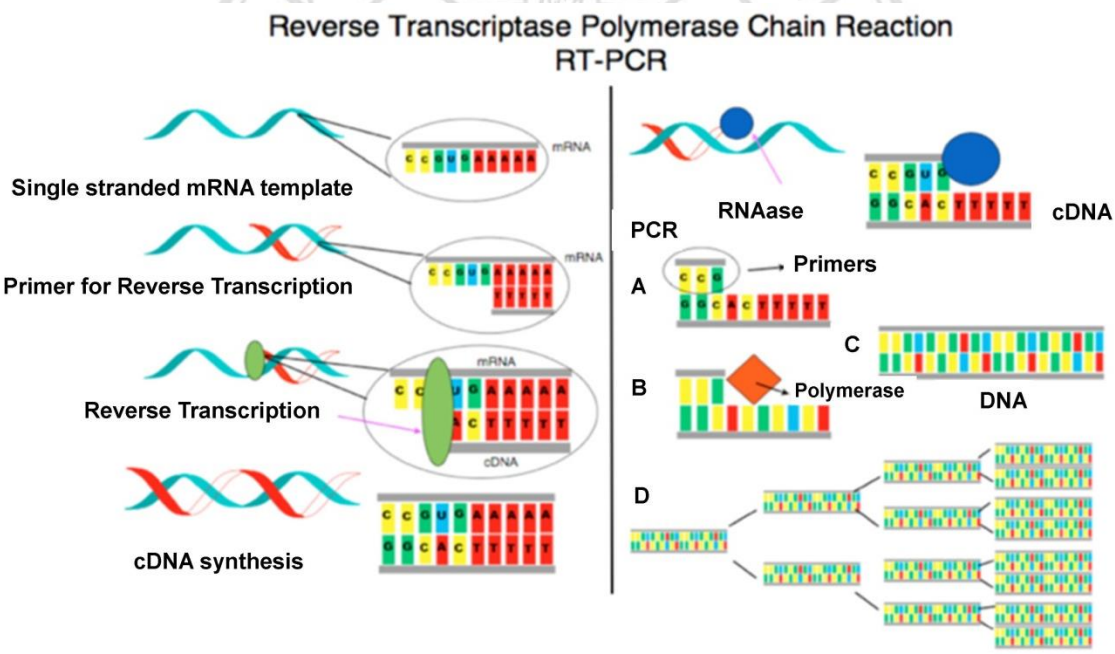


Figure 2.7 Processes of Reverse Transcriptase Polymerase Chain Reactions (RT-PCR) (193).

2.8.2.1.3 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR is modern methodologies for studying gene expression from a tiny amount of starting material. The monitor of qRT-PCR product uses fluorescent technologies that PCR product concentration is correlated with fluorescence intensity (194, 195). SYBG (Figure 2.8) was used in this study. The quantity can be measured as an absolute number of copies or a relative amount when normalized by the expression of GAPDH control.

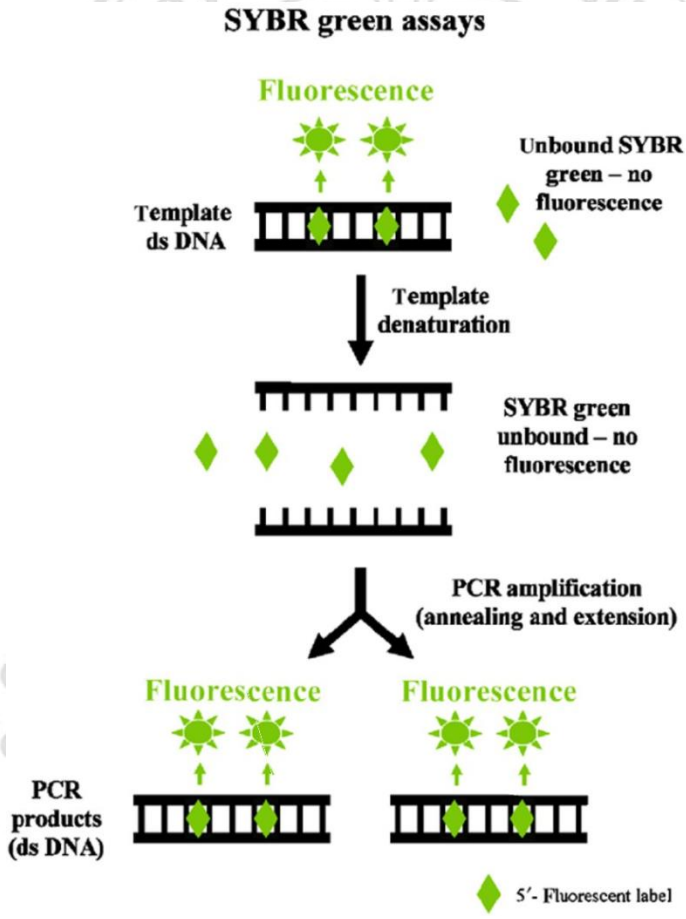


Figure 2.8 Real time PCR chemistry by SYBR Green detection (196).

In this study, the proinflammatory molecules (TNF-α, IL-1β and IL-6) and inflammation enzymes (iNOS and COX-2) gene expressions were quantified using THUNDERBIRD™ SYBR® qPCR Mix (Toyobo) and performed using ABI 7500 Real-time PCR system (Applied Biosystems, USA). In brief, the cDNA target was prepared as described above in section 2.8.3.1 and 2.8.3.2. Target cDNA was amplified by reverse transcription using the specific primer for the quantitative RT-PCR. The primer sequences in this study are listed in the following Table 2.1.

Table 2.1 Oligonucleotide sequences for real-time RT-PCR.

Oligonucleotide Name	Sequence 5´-3´
Murine TNF-α	
Forward/Sense	5´- CTCCAGGCGGTGCCTATGT
Reverse/Antisense	5´- GAAGAGCGTGGTGGCCC
Murine IL-6	
Forward/Sense	5´- CCAGAAACCGCTATGAAGTTCC
Reverse/Antisense	5´- TCACCAGCATCAGTCCCAG
Murine IL-1β	
Forward/Sense	5´- AAGGGCTGCTTCCCAACCTTTGAC
Reverse/Antisense	5´- ATACTGCCTGCCTGAAGCTCTTGT
Murine COX-2	
Forward/Sense	5´- CCGAGGTGTATGTATGAG
Reverse/Antisense	5´- TGGGTAAGTATGTAGTGC
Murine iNOS	
Forward/Sense	5´- CTTTGGTGCTGTATTTCC
Reverse/Antisense	5´- TGTGACCTCAGATAATGC
Murine GAPDH	
Forward/Sense	5´- TGGCAAAGTGGAGATTGTTGCC
Reverse/Antisense	5´- AAGATGGTGATGGGCTTCCCG

2.8.2.2 Enzyme-Linked Immuno-Sorbent Assay (ELISA) assay

Enzyme-linked Immunosorbent Assays (ELISA) is biological assay technique. ELISA is used for the detection and quantification of proteins typically secreted or released from cells. ELISA can provide a useful measurement of antigen or antibody concentration. The ELISA can be used to detect the presence of antigens that are recognized by an antibody or it can be used to test for antibodies that recognize an antigen (197). There are five types of ELISA are indirect ELISA protocol, direct ELISA protocol, sandwich ELISA protocol, competitive ELISA protocol and ELISPOT protocol (198). However, the main ELISA principle and lots of procedures are the same based on the binding structure between the antibody and antigen. One of the most common types of ELISA are “direct ELISA” and "sandwich ELISA".

The basic principle of ELISA is based on immobilizing a target-specific capture antibody or antigen onto a high protein binding capacity ELISA plate enables capture of target protein. The captured protein is then detected by a protein-specific biotinylated antibody. The target protein is quantified using a colorimetric reaction based on activity of avidin-horseradish peroxidase (bound to the biotinylated detection antibody) on a specific substrate (e.g., ABTS, SuperAqua Blue or TMB). The optical density of the end-product is measured using a spectrophotometer.

A general ELISA is a four-step procedure. In this study was using a sandwich ELISA, which uses two separate antibodies: the first to recognize and bind the target analyze, the second to detect the bound target. Typically step of sandwich ELISA as showed in Figure 2.9, In Step 1, one of the antibodies is applied to the well of a microliter plate: this is known as the capture antibody. The capture antibody binds to the plate via passive absorption and this step is often performed at 4 °C overnight. A blocking solution (typically milk protein (casein), bovine serum albumin or fish gelatin) is applied. These proteins adhere to any vacant sites on the plastic surface of the well that are not occupied by capture antibodies thereby minimizing the effect of nonspecific binding by other reagents to the plate surface during subsequent incubation steps. Excess blocking agent is removed and the plate is rinsed before addition of the test sample (wash steps are

incorporated between all incubation steps to minimize the background signal due to non-specific binding). If the test sample contains the target analyze, this is bound by the capture antibody that is anchored to the plate (Step 2). After the incubation step with the test sample, the plate is washed prior to the addition of the enzyme-linked detection antibody (Step 3). The detection antibody is conjugated with an enzyme which is commonly either horseradish peroxidise, alkaline phosphatase or β-D-galactosidase. These enzymes are proteins that catalyze the hydrolysis of a chromogenic substrate, such as 3,3',5,5'-tetramethylbenzidine or 2,6- dichlorophenolindophenol, which undergoes a colorimetric change that is measurable using a spectrophotometric plate reader at specified wavelengths (Step 4). With the catalysis of the traditional chromogenic substrates the reaction is terminated by the addition of a stop solution prior to measuring the absorbance of each of the wells of the microliter plate.

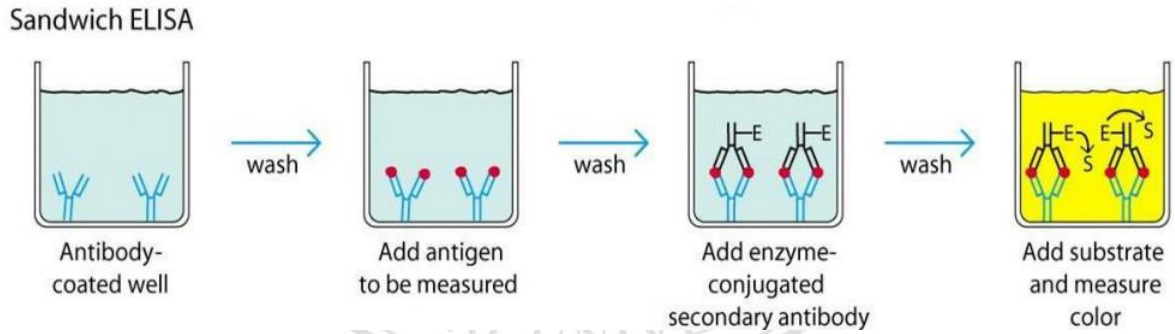


Figure 2.9 Typically sandwich ELISA (199).

Determination of protein level of pro-inflammatory cytokines, TNF-α and IL-6 in culture supernatant of the treated-cells was determined using a sandwich Enzyme Link Immuno-Sorbent Assay (BioLegend's ELISA MAX^{TM} Deluxe Set, CA).

Briefly, 100 µl of specific monoclonal capture antibody was added into each well on 96 well plates for 16-18 hours at 4°C. Then, the plate was washed 3 times with 0.05% PBS-Tween washing buffer and 200 µl of blocking solution was added in each well and incubated for 1 hour at room temperature for blocking non-specific binding sites. After that, the plate was washed 3 times with washing buffer and then the cytokine standard (7.8-500 pg/mL) or the sample was added to each well and incubated at room temperature for 2 hours. The specific cytokine in standard or sample would be allowed to bind with the capture antibody. Unbound

material was washed away by washing buffer. The capture cytokine proteins were detected by further incubating with 100 µl of biotin-conjugated anti-cytokine antibodies (detection antibody) for 1 hour at room temperature and followed by 100 µl of enzyme-labeled avidin-horseradish peroxidase in each well and incubated for 30 min at room temperature. Then, 100 µl of TMB chromogenic substrate was added and incubated for 30 min at room temperature to generate blue colored product. The concentration of cytokine present in the sample was belonging to the level of colored product. The final step, 100 µl of stop solution was added to each well to generate yellow colored instead blue color. The colored product can be measured by spectrophotometer at 450 optical density (OD). The cytokine level of samples was calculated from each cytokine standard curve.

2.8.2.3 Western Blot Analysis

Western blotting, also known as immunoblotting, is a well-established and widely used to detect and analyze specific proteins in a complex mixture extracted from cells (200). The method is based on specificity of polyclonal or monoclonal antibodies to protein immobilized on a membrane and detecting the bound antibody. For detection the target protein can visualized as a band on a blotting membrane and X-ray film (201).

It is an analytical method wherein the sample or denature protein would be separated by the size of polypeptide with electrophoresed on a sodium dodecyl sulphate-polypeptide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose membrane. The transferred protein could be detected using specific primary antibody, which specific for the protein of interest. After incubation with secondary antibody labeled with peroxidase enzyme which binds to the primary antibody's Fc domain, chemiluminescent substrate would be used to detect the expression of specific proteins (202).

2.8.2.3.1 Total Protein Extraction

Cell pellet of treated-RAW 264.7 macrophage was collected by scratching the cells with cold PBS on ice and centrifuged at 10,000 rpm for

1 min. Then, the cells were lysed with RIPA buffer (GE Healthcare, USA) that contains protease inhibitor cocktail tablet (Roche diagnostics, Germany), on ice for 30 min. Cell debris was eliminated by centrifugation at 12,000 rpm for 10 min at 4°C. The lysate solution was collected to determined protein concentration.

2.8.2.3.2 Determination of total protein concentration

The concentration of total protein in each sample was measured by Bio-Rad Protein Assay (Bio-Rad, USA), which is a protein determination method that involves the binding of Coomassie Brilliant Blue G-250 dye to protein. The color of dye depends on the binding of solution on amino acids or peptide, which is the acid solution status. At a pH of the solution less than 0 the dye has a red color (cationic) and it can absorbed maximum wavelength at 470 nm. At a pH of around 1 the dye is green (neutral) and absorbed maximum at 620 nm while above pH 2 the dye is bright blue (anionic) and absorbed a maximum at 595 nm (203, 204). The blue colored protein dye complex was measured at 595 nm using a microplate reader (Bio-TEK, USA). The protein concentration could be qualified by calculation using a standard protein dilution curve.

The protein concentration of each sample was determined by Bio-Rad Protein assay reagent as follows: 190 µl of diluted (1:5) Coomassie Brilliant Blue G-250 dye was mixed with 10 µl of Bio-Rad standard protein assay II (62.5-500 µg/ml) or unknown protein samples in each well of 96-well plate. Then, the absorbance was measured at 595 nm using a microplate reader. The absorbance values of all individual standard and unknown samples replicates were subtracted by that blank.

2.8.2.3.3 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis [SDS-PAGE]

The samples are separated according to molecular weight using SDS-PAGE (gel electrophoresis). By the way, protein have been denatured by

loading buffer containing SDS detergent, which is strong reducing agents to remove secondary and tertiary structure of protein such as disulfide bonds [S-S] to sulhydryl groups [SH and SH] (205). In addition, the protein is becomes negatively charged that it can move through the gel to the positive electrode in an electric field. Smaller proteins are migrating faster though the mesh of gel and separate according to their size (206).

The separating gel that contained 10% acrylamide, which is poured between two glasses and topped by a short stacking gel was used for fractionation of protein. Small volumes of protein (20 µL) of each samples were mixed in 4X loading buffer (1.25M Tris-HCl pH6.8, glycerol, 10% SDS, 0.2M DTT and 0.02%bromophenol blue) and boiled at 95°C for 10 min before loading into the gel. Each sample was added to each individual well, and then, the gel was connected to a power supply at 90 volts and allowed to run for 1 hour and 30 min in a buffer tank (25mM Tris-HCl, 193 mM glycine, 10%SDS) to separate the proteins or until the tracking dye reached the bottom of the gel. Protein standard marker was used to estimate size of separated protein.

2.8.2.3.4 Protein transfer (Blotting)

Following gel electrophoresis, the separated protein mixtures need to transfer into a solid support such as nitrocellulose or polyvinylidene difluoride (PVDF), which is a chemically inert substance. The separated protein mixtures can move to the membrane from gel by the driving of electric field, which consist between the gel surface and the positive electrode. The filter paper was used for protect and help the gel and blotting membrane are close contact between their surfaces. Mostly, the method of electrophoretic transfer can be dividing into wet and semi-dry methods (207, 208). In a wet transfer, the cassette of gel/blotting paper/filter paper sandwich is immersed in a buffer tank and subjected to an electrical field. Semi-dry transfer, the stack of gel/blotting paper/filter paper sandwich is assembled on large electrode plates which generate the electric field, while the buffer is restricted to the stack of wet filter paper (201, 207).

In this study was using wet transfer, which is usually considered to be more safe the gel from drying out, and is often preferred for larger proteins. The procedure following by prewet the nitrocellulose membrane and sponge in transfer buffer. For negatively charged proteins, build the stack on the half of the cassette that will face the anode (+). Place the blotting cassette in a tray filled to transfer buffer until it cover the haft of the cassette then place sponge on the submerged part of the cassette. Place two wetted blotting papers on to the sponge the cover with the nitrocellulose membrane on top of the blotting papers. Place the separated protein gel on top of the membrane, which cut away the stacking gel and cut one corner from the separating gel for enable you to correctly orientate the gel if it "flips over" during equilibration. Cover the separating gel with blotting papers and sponge on the gel, respectively. After, pressing the stack to remove air bubbles. Place the cassette in the transfer tank on ice with stirring the buffer by magnet to circulate the buffer during transfer. Connect the transfer tank to the power supply and transfer with 100 volts for 1 hour and 30 min. (Figure 2.10)

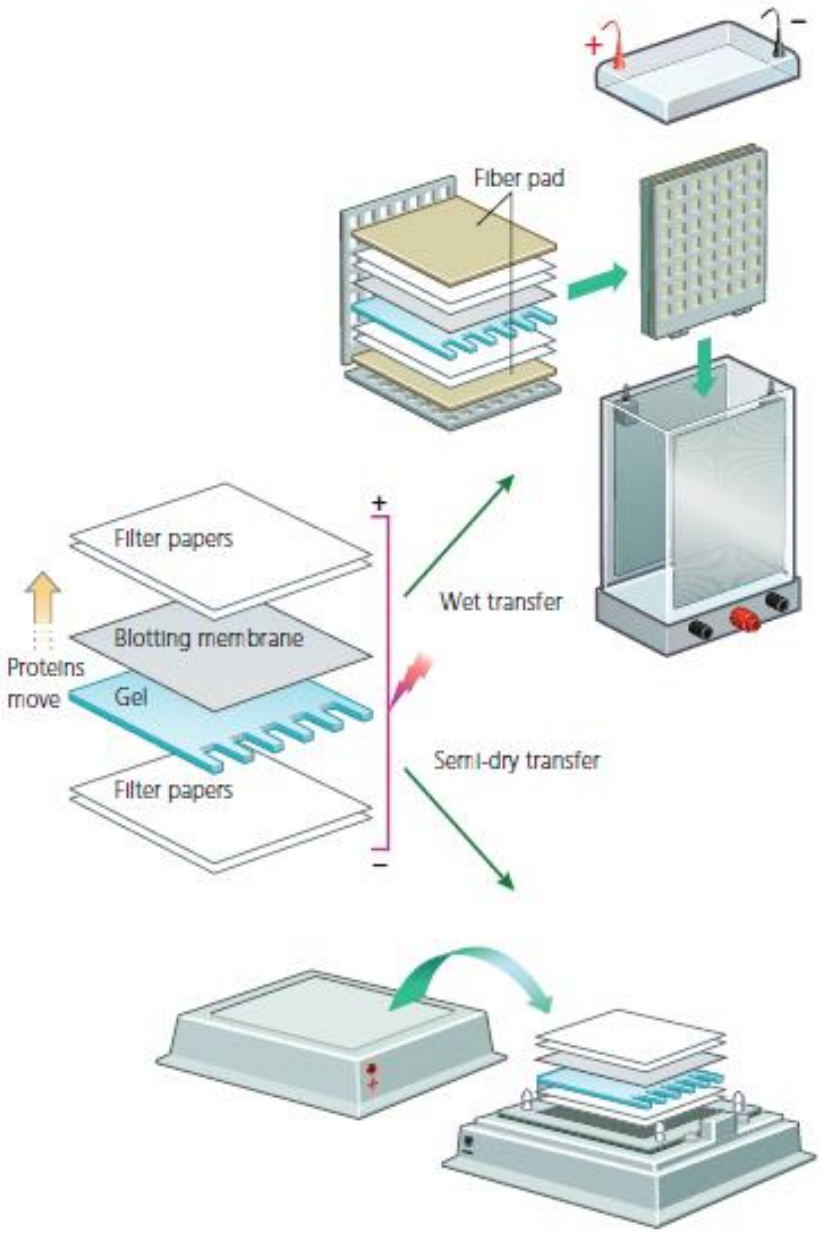


Figure 2.10 Western blot transferred method (207).

2.8.2.3.5 Antibody probing

After blotting, nitrocellulose membrane was collected and cut the membrane by sizes of iNOS (molecular weight, 125 kDa), COX-2 (molecular weight 74 kDa) and β-actin (molecular weight, 42 kDa) into the box and then incubated with 3% BSA in Tris-buffed saline solution (TBS) for 2 hours at room temperature.

After blocking and washing 5 times with TBS-T for 5 min, the blot was incubated in a dilute solution of primary antibodies 1 : 5000 µL of anti-COX-2 antibody (Cell signaling, USA) or 1 : 5000 µL of anti-iNOS antibody (Merck, USA) or 1: 10,000 µL of anti-actin (Sigma, USA) in TBS containing 3% BSA for 2 hours at room temperature. Then, the blot was washed 5 times for 5 min with TBS containing 0.15% Tween 20 (TBST) to remove excess antibodies.

Primary antibody was detected by using a secondary antibody, which has been covalently joined to detected label molecule (biotin, an enzyme, or a fluorescent dye). In this study was used goat anti-mouse or anti-rabbit IgG linked to peroxidase (Bio-Rad, USA) at a 1:8000 dilution in 3% BSA in TBS for 2 hour at room temperature and washed with TBS-T buffer for 5 times. In this time, excess antibody has been washed away and target protein was tagged with labeled antibody, the label was used to identify the target protein on the blot.

2.8.2.3.6 Detection

For detection the target protein are based on chemiluminescence, chemifluorescence, fluorescence, chromogenic or radioisotopic detection. Chemiluminescence and chemifluorescence are enzymatic method that used an enzyme conjugated to a secondary antibody and it requires a reagent that reacts with the enzyme and then emits light (209, 210).

Chemiluminescence is commonly used for detect the target protein, which based on antibodies conjugated to horseradish peroxidase (HRP) that

catalyze the oxidation of luminol which is oxidized by HRP in the presence of H_2O_2 and an enhancer to produce light emission. HRP can tag to antibodies or streptavidin and can be used with different chemiluminescent reagents. Moreover, HRP is obtained higher detection sensitivity, stronger light intensity and long lasting signals (210).

After, incubated with secondary antibody, the blotting membrane was incubated with ECL (enhanced chemiluminescence) detection for 5 min. The emitted light is detected by exposing the western blot to Kodak X-ray film.

2.9 Anti-inflammation-induced insulin resistance by aqueous and ethanolic extracts from *Anoectochilus burmannicus*

Pre-adipocytes (3T3-L1) were seeded 5×10^5 cells/well in 96 well plate and allowed to differentiate into matured adipocytes as described in section. After that, mature adipocytes were induced insulin resistance by 50 ng/mL of TNF-α for 24 hours. Then, the cells were washed twice with PBS. Various concentrations (50, 100, 200 μg/mL) of the extracts were added and further incubated for 24 hours.

2.9.1 Glucose uptake assay

Glucose is a ubiquitous energy source in most organisms (211). Glucose metabolism, a process which converts glucose into energy in the form of adenosine triphosphate (ATP), is a primary source of energy and biomaterials for the maintenance of cell homeostasis. In humans, glucose is absorbed by the intestines and then into the blood. Extra glucose is stored in the muscles and liver as glycogen which hydrolyzed to glucose and released into the blood when needed (212). Blood glucose levels must be maintained within homeostatic levels to ensure optimal supply of glucose for normal cell function and survival. Glucose uptake in cells is achieved by the action of glucose transporters, which facilitate glucose movement down a concentration gradient. The rate of glucose uptake in cells is dynamic and tightly regulated by hormones and/or growth factors including insulin (213). In obesity-induced insulin resistance (type 2 diabetes)

involve in inflammation found high concentration of glucose in blood circulation instead of being absorbed by cells leading to type 2 diabetes (214). Type 2 diabetes mellitus arises from insulin resistance that is inability of store glucose and out of control glucose output in adipose tissue, liver and muscle (214). Thus, fluorescent-tagged blucose bioprobes is usually used to monitor glucose utilization into the cells (215, 216).

A fluorescent derivative of glucose, 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG), which is a glucose analogue that is follows the metabolic pathway of glycolysis, similarly to D-glucose and fluorodeoxyglucose (FDG). Microscopy can be performed to examine 2- NBDG uptake in living cells, and the intracellular concentration of transported 2-NBDG can be measured with fluorescence spectroscopy (215, 217). 2-NBDG molecule featured the same fluorescent group as 6-NBDG, which implying a low affinity for the transporters. 6-NBDG had a fluorescent group on the C-6 position of glucose, but 2-NBDG had it on the C-2 position rather than the C-6 position. 2-NBDG is transported into the cells via glucose transporters (GLUT), and is phosphorylated at the C-6 position by hexokinases I–II (HK). The phosphorylated fluorescent metabolite 2-NBDG-6-phosphate remains in the cell until further decomposition occurs into a non-fluorescent form (218). 2-NBDG was then rapidly degraded to non-fluorescent products by the glycolytic pathway, which is the enzyme glucose 6-phosphatase (219). The cellular breakdown of 2-NBDG is a useful property, because 2-NBDG signal should indicate both the cellular glucose uptake rate and metabolic activity (217), although it should be noted that 2-NBDG may also accumulate as glycogen. Indeed, the usefulness and simplicity of 2-NBDG as a tracer for eukaryote cell viability. 2-NBDG can be maximally excited at 485 nm and emits at 535 nm. Mostly, 2-NBDG has been used to screen and identify of new regulators of glucose uptake.

After the treatment, the cells were washed twice with cold-PBS and low glucose DMEM medium was added and incubated for 3 hours at 37°C. Then, the cells were washed with cold-PBS and 100 µl of glucose-free DMEM containing 80 µM of fluorescent glucose analog 2-NBD-glucose (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) (Figure 2.11) and 100 nM of insulin

was added and incubated for 1 hour at 37°C. The cells were washed again with cold-PBS for 2 times. And then 100 µl of 90% DMSO/10% PBS was added and incubated for 10 min at 37°C, The cells were transferred to 96-well black plate. The intensity of fluorescence was measured by a fluorescence microplate reader at λex=485 nm and λem=535 nm.

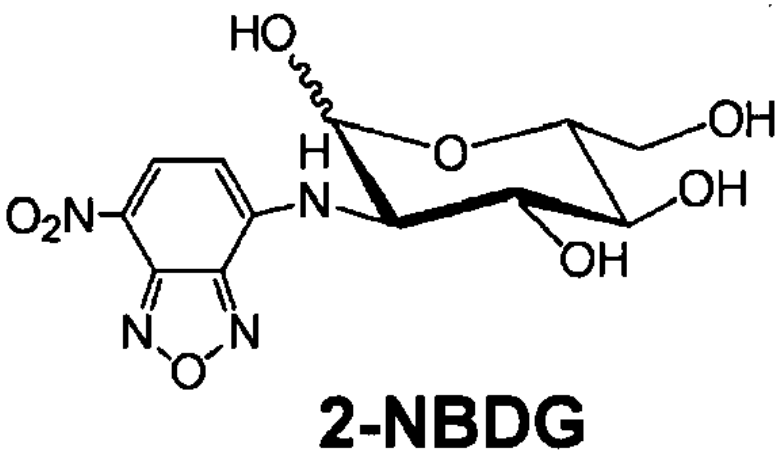


Figure 2.11 Structure of 2-NBDG (220).

(2-NBDG. In: DOCUMENTS USP, editor. 2010.)

2.10 Statistical Analysis

All value was given as mean ± standard derivation (X ± SD) from triplicate samples of three independent experiments. Overall differences among the treatment groups were determined using one-way analysis of variance (ANOVA) by Prism 5.0 software. P values < 0.05 are regarded as significant.