EFFECTS OF *PLEUROTUS SAJOR-CAJU* EXTRACTS ON PROLIFERATON, LIPOGENESIS AND LIPOLYSIS IN 3T3-LI CELL LINES.

5.1 Introduction

Adipose tissue is a huge energy storage system in the body, which plays an important role in the maintenance of the energy metabolic balance of the organism. At a cellular level, obesity is caused by increasing the amount and volume of adipocytes, induced by unceasing differentiation of adipocytes. Abnormal differentiation of adipocytes will affect glucolipid metabolism, induce obesity and insulin resistance and then influence the development of metabolic diseases (Zhou et al., 2007). Therefore, numerous studies in the world have recently focused on the regulation of the adipocyte differentiation and its relationship with mechanism of obesity and insulin resistance. Because proliferation and differentiation are two stages during the growth of cells, it is significant to study the effects of *P. sajor-caju* extracts on proliferation of preadipocytes and their differentiation into adipocytes for prevention and cure of those metabolic diseases (Hajer et al., 2008).

Adipocyte differentiation, known as adipogenesis or lipogenesis, is the process of fat cell development, accompanied by coordinated changes in cell morphology, hormone sensitivity, and gene expression (Pilch & Bergenhem, 2006). The regulation of adipogenesis has been studied extensively using various cell and animal models. In this study, the mouse preadipocyte 3T3-L1 cell line, which was originally generated from the Swiss albino mouse fibroblast cell line by Green and Kehinde (1974), was selected because this cell line is one of the most useful cell lines for investigating the characteristics of adipocytes and their secreted adipocytokines. In addition, these preadipocytes can easily be induced to differentiate into adipocytes (Mimura et al.,

2009) and is the most studied preadipocyte cell line. When introduced to appropriate differentiation inducers, including insulin, dexamethasone (a glucocorticoid) and 3-isobutyl-1-methylxanthine (a cylic AMP (cAMP) phosphodiesterase inhibitor that increases intracellular cAMP), 3T3-L1 preadipocytes differentiate into adipocytes (Guo & Liao, 2000).

The aims of this study were to assess the effects of *P. sajor-caju* extracts on lipogenic or lipolytic activities in 3T3-L1 adipocytes as well as to elucidate the possible pathway that could contribute to these activities. In addition, the effects of the *P. sajor-caju* extracts on oxidative stress level in 3T3-L1 cells during adipogenesis and lipolysis were also evaluated.

5.2 Materials and Methods

5.2.1 Materials

Applied Biosystem, United States of America

- High capacity cDNA reverse transcription kit
- MicroAmp™ optical 8-cap strip
- MicroAmp™ fast 8-tube strip
- RNAqueous® Micro kit
- TaqMan® gene expression assays
- TaqMan® gene expression master mix

American Type Culture Collection, United Sates of America

- Mouse 3T3-L1 cell line (preadipocytes)

Hirschmann Laborgerate (Germany)

- Hemocytometer

Flow Lab, Australia

- Phosphate buffer saline (PBS) tablet
- Amphotericin B (Fungizone®)

Fisher Scientific, United Kingdom,

- Isopropanol

ICN, United States of America

- Dexamethasone
- 1-methyl-3-isobutylxanthine, IBMX

Nunc, United States of America

- 75 cm^2 tissue culture flasks
- 25 cm^2 tissue culture flasks
- 96 well tissue culture plates
- 12 well tissue culture plates
- 6 well tissue culture plates

Sigma-Aldrich, United States of America

- Chloroamine-T
- Dulbecco's Modified Eagles's Medium, DMEM
- 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide, MTT

- Fetal bovine serum , FBS
- Insulin
- 1-Methyl-2-phenylindole, MPI
- Penicillin-streptomycin
- Oil red O dye, ORO
- 1,1,3,3-Tetraethoxypropane, $C_{11}H_{24}O_4$
- Trypsin-EDTA
- Trypan blue dye

Zen-Bio, United States of America

- Lipolysis kit

5.2.2 Methodology

5.2.2.1 Cell culture

a) Mouse preadipocyte culture

Cells (3T3-L1) obtained (from ATCC) were washed with phosphate buffered saline (PBS) and were cultured in 75 cm^2 culture flasks containing growth media (GM). These cells were then incubated in 5.0 % CO_2 incubator at 37 °C with 95 % humidity. The culture media was changed every 2-3 days until the preadipocytes reached confluent state.

b) Differentiation of mouse preadipocytes

Confluent preadipocytes were induced to differentiate in 75 cm^2 culture flasks, 25 cm^2 culture flasks, 6 well, 12 well and 96-well tissue culture plates according to a well established method (Madsen et al., 2003) but with some modifications. Table 5.1 shows the composition of growth media (GM), part-1 differentiation media (DM1), part-2 differentiation media (DM2) and freezing media.

The 3T3-L1 preadipocytes were initially differentiated in the 75 cm^2 culture flasks. Confluent preadipocytes were differentiated with 15 ml part-1 differentiation media, for two days at 37 °C and 5.0 % CO_2. On day four, part-1 differentiation media (DM1) was removed and the cells were replenished with 15 ml of part-2 differentiation media (DM2) until they were fully differentiated. After full differentiation, the cells were replenished with 15 ml of growth media. For differentiation in 25 cm^2 culture flasks, 5 ml instead of 15 ml of media was used for each similar step described previously. Meanwhile, for the differentiation process in the tissue culture plates, cells were seeded at a density of 3×10^4 cells/ml in each well. The differentiation induction was carried out as stated above. These fully differentiated adipocytes were used for subsequent studies. Cells differentiated in 6 well tissue culture plates were used for gene expression studies meanwhile cells differentiated in 12 and 96 well tissue culture plates were used for lipogenesis and lipolysis or MTT assay respectively.

c) Cell number estimation with trypan blue

The number of preadipocytes cultured in 25 cm^2 tissue culture flask was estimated using the improved haemocytometer grid system with trypan blue stain. This method is based on the principle that dead cells will take up trypan blue dye and stain blue, whereas viable cells are not stained. Trypan blue dye (10 µl) was added to 10 µl of

cell suspension (1:1 dilution). The mixture was loaded onto a haemocytometer and the number of viable cells was counted. The number of viable cells was determined using the following formula:

$$\text{Cell concentration (cells / ml)} = \text{mean cell count} \times \text{dilution factor} \times 10^4$$

$$\text{(cells/ml)} = \text{Mean cell count} \times 2 \times 10^4$$

Table 5.1: Composition of growth media, part-1 differentiation media and part-2 differentiation media

Growth Media (10%)	▪ **FBS** ▪ **2 mM Penicilin Streptomycin** ▪ **2 mM Fungizone** ▪ **DMEM media**
Part-1 Differentiation Media (DM1)	▪ FBS ▪ 2 mM Penicilin Streptomycin ▪ 2 mM Fungizone ▪ 1 µM Dexamethasone ▪ 0.5 mM IBMX ▪ Insulin (10 µg/ml) ▪ DMEM media
Part-2 Differentiation Media (DM2)	▪ FBS ▪ 2 mM Penicilin Streptomycin ▪ 2 mM Fungizone ▪ Insulin (10 µg/ml) ▪ DMEM media

5.2.2.2 Extracts

AE, BE, EE, EAE and GE were obtained as stated in Chapter Three. These extracts/compounds were assessed for antioxidant properties using the antioxidant assays described below.

5.2.2.3 Colorimetric MTT (tetrazolium) assay

Preparation of reagents

Five milligram of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was dissolved in one ml of PBS. Then the solution was filter-sterilized with 0.2 μm pore size filter.

Procedure

Confluent preadipocytes were trypsinized with 3 ml of trypsin-EDTA for 5 min to detach the adherent cells from the flask. Fibronectin helps the cells to attach to the flask. Trypsin will solubilise the fibronectin on the cell surface. However, trypsin can kill the cells in prolonged incubation. After trypsinization, the cells were transferred into a centrifuge tube and centrifuged at 196 × g for 5 minutes. The supernatant was discarded and the pellet was resuspended in one ml of cell culture media. The number of cells was determined using trypan blue method before the seeding process. Preadipocyte suspension (90 μL) was pipetted into each well of the sterile 96-well tissue culture plate. The culture was incubated in humidified air jacketed incubator for 24 hours until the preadipocytes were attached to the base of the tissue culture plate. A stock solution of 10 mg/ml was prepared for each *P. sajor-caju* extract. The stock solutions were filter-sterilized using 0.2 μm pore size filters. The filter-sterilized stock solution was then diluted appropriately with ultra pure water. The freshly prepared extracts (10 μl) were incorporated into the 96 well tissue culture plate containing preadipocytes after 24 hours of seeding process. The final concentrations of the various extracts were 0.01 μg /ml, 0.1 μg /ml, 1 μg /ml, 10 μg/ml, 100 μg/ml and 1000 μg/ml and in the control well, the extract was substituted with ultra pure water. Each concentration of the various extracts was tested in triplicates. The culture was then incubated for 24 and 48 hours to ensure

the maximum uptake of extracts introduced by the cells (Mosmann, 1983). Then, cytotoxic effect of the various *P. sajor-caju* extracts on preadipocytes was accessed using the MTT assay.

Ten µl of filter-sterilized MTT was added into each well after 24 and 48 hours of preadipocyte incubation with various *P. sajor-caju* extracts. The culture was incubated with MTT dye for 4 hours in the humidified air jacketed incubator containing 5 % CO_2 at 37 °C. Then, the medium and MTT solutions in each well was carefully removed using needle syringe. Hundred µl of DMSO was added into each well to dissolve the formazan crystals. The tissue culture plate was left at room temperature for 5 minutes to make sure all formazan crystals were dissolved. A purple colored substance will be formed after the crystals were dissolved. The absorbance was read at 560 nm against 700 nm using a microplate reader. The percentage of cell viability was calculated as stipulated below:

$$\text{Cell viability (\%)} = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{control}}}{\text{Abs}_{\text{blank}}} \times 100$$

5.2.2.4 Lipogenesis assay

Preparation of reagents

The Oil Red O stock solution was prepared by mixing 0.5 g of Oil Red O dye with 100 ml of isopropanol. Then, 18 ml of this stock solution was mixed with 12 ml of distilled water to prepare the Oil Red O working solution. The 60 % Oil Red O working solution was then filtered with a filter paper before using for the staining process.

Procedure

Lipogenesis assay was performed by replenishing the part-2 differentiating media with growth media and *P. sajor-caju* extracts. Insulin was used as a positive control meanwhile ultra pure water was used as negative control. After treating with

various concentrations of *P. sajor-caju* extracts for 48 hours, the cells were washed with PBS. The cells were fixed with formalin and rinsed with 60% isopropanol. It was then stained with Oil Red O working solution in the dark. Differentiation efficiency (lipid stained in the fully differentiated adipocytes) of the cells was assessed microscopically using an Olympus CK-40 inverted microscope. The cells were washed with PBS and subsequently dissolved in isopropanol. The absorbance was read at 510 nm using a microplate reader. Figure 5.1 shows mature differentiated adipocytes and mature adipocytes stained with Oil Red O dye. The percentages of lipogenic activity were calculated as stipulated below:

$$\text{Lipogenic activity (\%)} = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{control}}}{\text{Abs}_{\text{control}}} \times 100$$

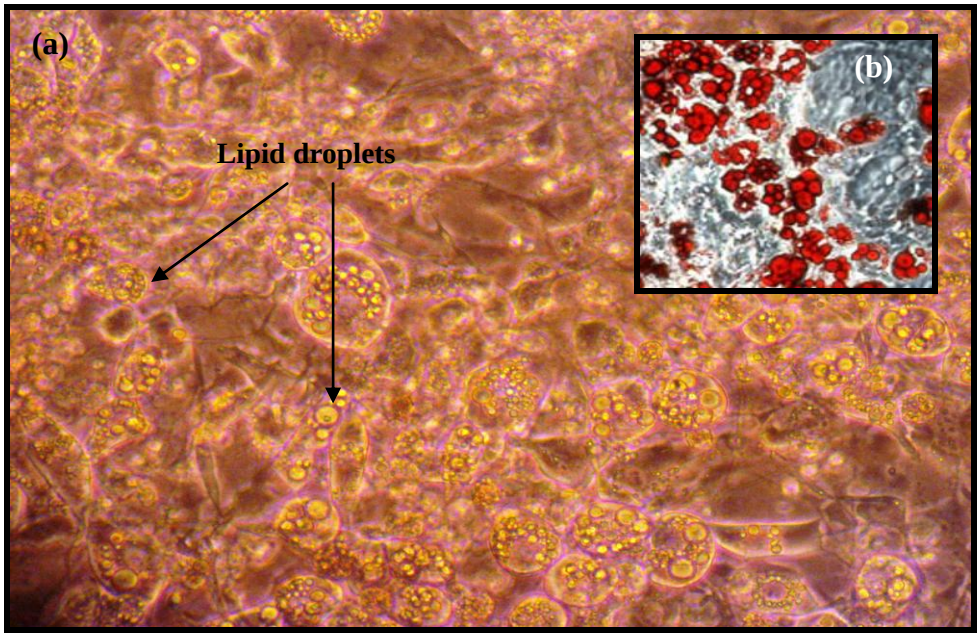

Figure 5.1: The series of pictures shows (a) mature adipocytes and (b) mature adipocytes stained with Oil Red O dye.

5.2.2.5 Lipolysis assay

Assay Principle

The assessment of lipolytic activity is investigated through a coupled reaction to measure non-esterified fatty acids and glycerol released by adipocytes. The glycerol and free fatty acids can be quantified by a series of chemical modifications to yield an equal amount of hydrogen peroxide (H_20_2) to glycerol or fatty acids which is measurable once modified into chromatic compounds. The concentration of glycerol or fatty acids detected is directly proportional to the increase in optical density.

Procedure

Lipolysis plays a central role in the regulation of energy balance. Lipolysis is a process in which triglycerides are hydrolysed into glycerol and free fatty acids. This process releases fatty acids into the bloodstream. Fully differentiated adipocytes are treated with various concentrations of *P. sajor-caju* extracts. Isoproterenol was used as positive control meanwhile ultra pure water was used as negative control. After 4 hours of treatment, lipolysis assay was performed according to the protocol of the assay kit. The absorbance reading was read at 550 nm using a microplate reader. Two parameters were measured, namely glycerol and free fatty acids (FFA). A standard curve of glycerol and fatty acid was plotted (Appendix B; pg 241). The fold of induction was calculated as stipulate below:

$$\text{Fold Induction} = \frac{\mu\text{M glycerol/ or FFA}_{\text{sample}}}{\mu\text{M glycerol/ or FFA}_{\text{control}}}$$

5.2.2.6 Protein carbonyl content assay (AOPP)

Preparation of Reagents

One volume of acetic acid (50%**)** was mixed with 1 volume of distilled water while potassium iodide (0.9628 g - 1.16 M) was dissolved in 5 ml of distilled water.

Procedure

Advanced oxidation protein products (AOPP) are uremic toxins created during oxidative stress through the reaction of chlorinated oxidants such as chloramines and hypochlorous acids. The level of AOPP present was determined using the method described by Witko-Sarsat et al., (1998). Fully differentiated adipocytes were treated with various concentrations of *P. sajor-caju* extracts. After 48 hrs of treatment, the media from each well was collected and was used to quantify the AOPP levels. The reagent mixture was prepared by adding 7.5 ml of 50% acetic acid with 2 ml of 1.16 M potassium iodide. PBS (16.2 ml) was added to the reagent mixture to make a final volume of 100 ml. The sample (18 µl) and 200 µl of the reagent mixture were added to 96 well tissue culture plates. Absorbance was read at 340 nm using a microplate reader. Chloramine-T solution of known concentrations (0,100, 200, 300, 400 and 500 µM) was used as standards for comparison (Appendix B; pg 242). The amount of damaged protein (AOPP) present was expressed in µmol chloramine-T/g. The concentration of AOPP was determined based on the standard curve.

5.2.2.7 Lipid peroxidation assay

Preparation of reagents

1-Methyl-2-phenylindole (MPI) (0.0213 g - 10.3 mM) was dissolved in 10 ml of acetonitrile until it was completely dissolved and hydrochloric acid (HCl) (37 % - 5M) was prepared by two × dilution from the stock.

Procedure

The assay was carried out based on the method described by Esterbauer & Cheeseman, (1990) with modifications. Fully differentiated adipocytes were treated with various concentrations of *P. sajor-caju* extracts. After 48 hours of treatment, the media from each extract was collected and was used to quantify lipid hydroperoxide levels. The media (150 µl), 375 µl of MPI in acetonitrile and 225 µl of HCl were mixed in a microcentrufuge tube. The mixture was incubated in a water bath at 45^{o}C for 40 minutes. Then, centrifuged at 19570 × g for 5 min. Absorbance was read at 586 nm using a microplate reader. 1,1,3,3-Tetraethoxypropane (TEP) solution of known concentration (2.5, 5, 10, 15 and 20 µM) was used as standard for comparison (Appendix B; pg 242). The amount of lipid hydroperoxides present was expressed in µmol TEP/g. The concentration of lipid hydroperoxides was determined based on the standard curve.

5.2.2.8 Assessment of gene expressions using real-time reverse transcription polymerase chain reaction (Real Time RT-PCR).

Preadipocytes were seeded into 6 well tissue culture plates containing 3 ml of growth media. Preadipocytes were differentiated to adipocytes using the method described in section 5.2.2.1. The fully differentiated adipocytes were treated with GE (50 µg/ml final concentration). Insulin (10 µg/ml final concentration) was used as positive control meanwhile ultra pure water was used as negative control. After 48 hours of incubation, the total RNA was isolated from the adipocytes using Ambion-RNAqueous Micro® kit. The isolation was done according to the product instructions. Briefly, the cells were lysed with lysis solution that distrupts cell membranes but capable of protecting the RNA from endogenous RNases. Subsequently, the cells were

homogenized by draining them through a small-bore needle syringe several times. The homogenate was then mixed with ethanol and centrifuged through a microfilter cartridge supplied with a silica-based membrane that selectively binds RNA. The impurities were effectively removed by specific washing step and total RNA in elution solution was eluted through an elution cartridge. Homogenates were kept ice-cold to prevent RNase activity. Purified RNA was used immediately for reverse transcription.

The purity of recovered total RNA was then estimated by diluting the sample 300× with ultra pure water and the ratio of absorbance reading A_{260}/A_{280} was calculated. An A_{260}/A_{280} of 1.8- 20 shows that RNA is reasonably clean of proteins and other UV chromophores that could either interfere with downstream applications or negatively affect the stability of stored RNA. The concentration of the RNA was calculated by the following formula:

Total RNA (µg) = A260 × 40 µg/ml × dilution factor × volume (ml)

Purified RNA was next used to synthesize complementary DNA (cDNA). High Capacity cDNA Reverse Transcription Kit which contains all reagents needed for reverse transcription (RT) of total RNA to single-stranded cDNA was used. Generally, 10 µl of RNA sample was mixed with 10 µl of High Capacity cDNA Reverse Transcriptase which contained all the reagents (RT buffer, dNTP mix, random primers, Multiscribe reverse ™ transcriptase enzyme and nuclease free water). The mixture was mixed thoroughly and was briefly centrifuged to spin down the contents and eliminate air bubbles. The mixture was loaded into a thermal cycler (Eppendorf, USA) and PCR was carried out according to optimized thermal cycling conditions as shown in Table 5.2. The cDNA product was used immediately for downstream analysis or stored under -80^{o}C. The integrity of DNA was estimated using NanoDrop2000 (Thermo Scientific, USA).

Table 5.2: Optimal reverse transcription (RT) conditions for cDNA synthesis

Step	**1**	**2**	**3**	**4**
Temperature (^{o}C)	25	37	85	4
Time	10 min	120 min	5 sec	Hold

5.2.2.9 Real – Time RT-PCR

Reverse transcription PCR based assays are currently the most common method used for confirming gene expression levels from different samples. Previously, DNA amplification and detection of the product of amplification used ethidium bromide (Higuchi et al., 1993) however today, with advent of fluorogenic DNA binding chemicals (SyberDyes®) and fluorogenic probes (TaqMan®), high sensitivity and rapid real time quantification of fluorescence amplicon are made possible. Besides that, this also eliminates the tedious need for post-PCR processing. Unlike SyberDyes® which intercalate with any double stranded DNA, TaqMan® probes only bind to the specific complementary sequence within the target gene.

In a real-time PCR reaction TaqMan® probes are used. The probe is designed with a reporter dye and a quencher moiety at the respective 5’ and 3’ ends. Due to the proximity of the reporter and quencher, the fluorescence of the reporter is quenched. The probes are designed to anneal to target sequence within the forward and reverse PCR primers. During the extension cycle, the Taq polymerase with intrinsic 5’ to 3’ nuclease activity cleaves the reporter dye from the probe. Once released, the reporter emits the fluorescence signal. The generated signal is proportional to the amount of amplicon in each cycle of amplification, therefore the relative amount of template can

be determined (Wang et al., 2006). Figure 5.2 shows the TaqMan® gene expression assay chemistry.

Reaction setup for all TaqMan® Gene Expression assay was performed according to the reaction setup instructions generated by the StepOne software (Ver 2.0, Applied Biosystems). Briefly, reaction which consisted of the TaqMan® Gene Expression Master Mix and assay mix was prepared separately, as each assay mix contained corresponding primers and probe for each gene targeted. All reagents were kept on ice, once thawed. Each reaction was assayed in triplicate. The reaction mix was mixed with either sterile ultra pure water for no template control reactions (NTC) or isolated cDNA. Subsequently the mixture was transferred into fluorescence compatible MicroAmp™ Fast Reaction Tube Strips and capped with MicroAmp™ Optical Cap Strips. The strips were centrifuged briefly and loaded into the real time PCR thermal cycler (StepOnePlus™ Real Time PCR System). Figure 5.3 shows the StepOnePlus™ Real Time PCR System setup in the laboratory.

Table 5.3 shows the list of the genes and corresponding accession numbers investigated in this study. Endogenous control (also known as housekeeping genes) used in this study is eukaryotic 18S rRNA with FAM/MGB probe. All TaqMan® probes used in this investigation were labeled with FAM™ reporter dye at the end 5' end and a MGB quencher at the 3' end.

Table 5.3: Genes investigated

No	Gene name and abbreviation	Assay ID	Accession number
1	Leptin (Lep)	Mm 00434759_m1	NM_008493.3
2	Adiponection (Adipoq)	Mm 00456425_m1	NM_009605.4
3	Lipoprotein lipase (LPL)	Mm 00434770_m1	NM_008509.2
4	Hormone sensitive lipase (HSL/Lipe)	Mm 00495359_m1	NM_001039507
5	Glucose transporter 4 (Glut-4)	Mm 00436615_m1	NM_009204.2
6	Adipose triglycerides lipase (ATGL/Pnpla2)	Mm 00503040_m1	NM_025802

General abbreviation of genes selected for this study and corresponding assay ID and accession number was obtained from Applied Biosystems website and NCBI database. Assay ID refers to the Applied Biosystems Gene Expression Assays inventoried kits with proprietary primer and TaqMan® probe mix. Assay ID with 'Mm' is referred to as '*Mus musculus'*. All Gene Expression Assay kits indicated are FAM/MGB probed.

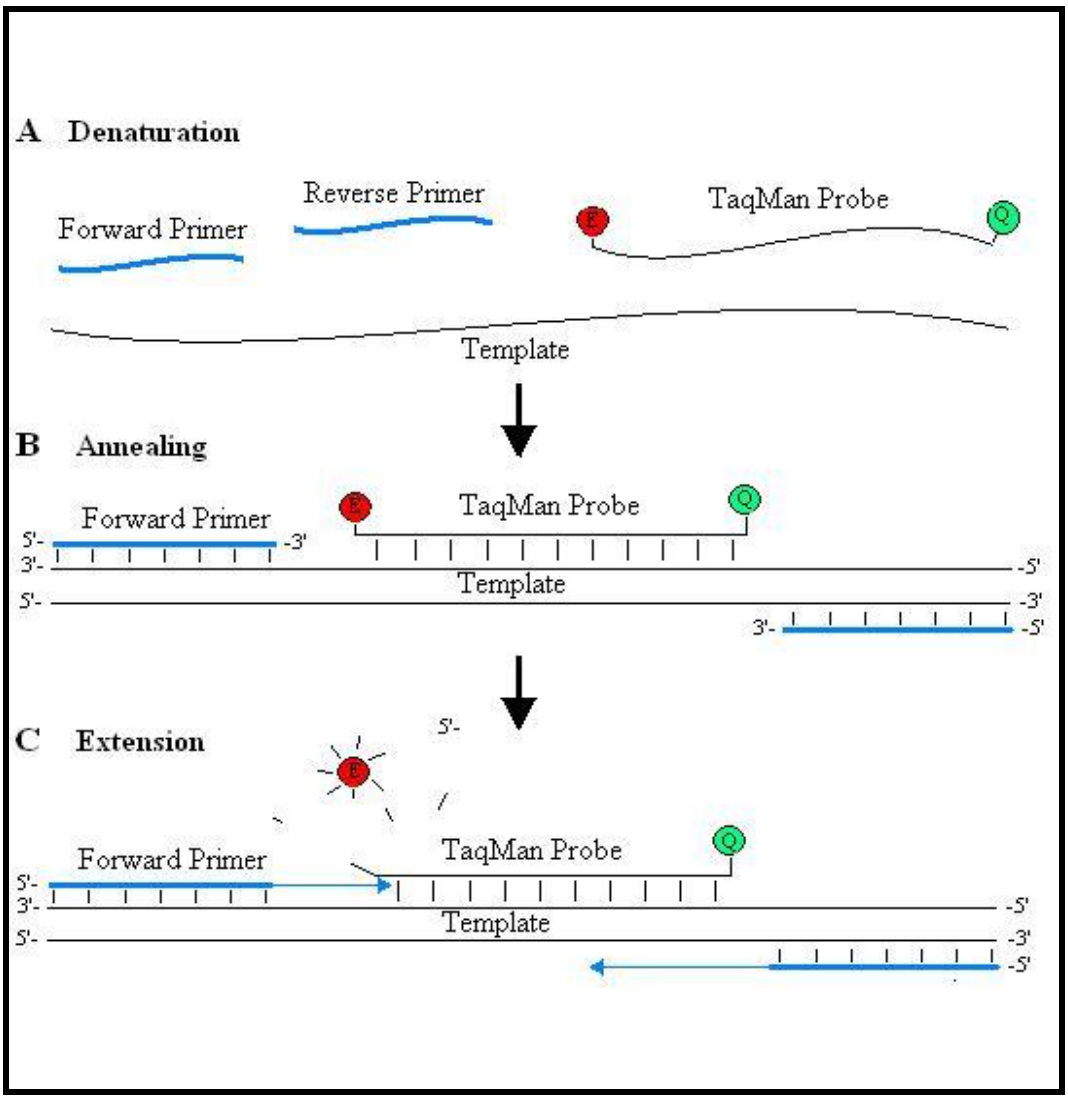

Figure 5.2: TaqMan® gene expression assay chemistry.

TaqMan® probes comprise of a short single stranded oligonucleotide usually conjugated at the 5' end to a reporter dye and a quencher dye at the 3' end. The TaqMan® probe is positioned downstream of both primer sites. During annealing/extension the probe and primers hybridize to the target sequence. During extension cycle the probe is dislodged and the 5' reporter dye cleaved by the 5'-3' exonuclease activity of Taq polymerase. The separation of the reporter and quencher dyes which leads to an increase in fluorescence which is directly proportional to the amount of TaqMan® probe cleaved during each cycle. The TaqMan® assay system requires discriminate hybridization of both primers and probe to the template, thus eliminating non specific amplification and contributions from primer dimmer formation (www.appliedbiosystems.com)

Figure 5.3: Real- Time RT PCR (Applied Biosystem), with StepOne® 2.0 software.

5.2.2.10 Calculations

In this study, the quantification approach used was comparative C_T method, also known as $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). C_T value refers to the number of amplification cycles required for a significant increase in the reporter's fluorescence and it is referred as threshold cycle. The passive reference dye, 6-carboxy-X-rhodamine (ROX™) is used in all real time PCR reactions to normalize fluorescent fluctuations and to compensate well-to-well variations including volume and concentrations differences. It was incorporated in the assay master mix.

The relative expression of the investigated genes was normalized with the endogenous control (18S rRNA). C_T values are means of triplicate measurements. The calculations and formulas involved were as follows.

$$C_{T\ Target} - C_{T\ Endogenous\ control} = \Delta C_T$$

$$\Delta C_{T\ Sample} - \Delta CT_{\ Calibrator} = \Delta\Delta C_T$$

$$\text{Relative fold change} = 2^{-\Delta\Delta Ct}$$

Target = gene of interest
Endogenous control = a gene that is present at a stable amount in total RNA despite experimental conditions (18S rRNA)
Sample = treated sample
Calibrator = untreated sample or control

In this study, relative fold change lower than 1 was expressed as negative numbers which indicates downregulation (e.g. when $\Delta\Delta C_T$ value is 2.00, the relative fold change value of 0.25 is expressed as -4.0).

5.2.2.11 Statistical Analysis

Data are shown as mean ± SD. One-way analysis of variance was used to determine the significant differences between groups. Duncan's multiple range tests (DMRT) was used to determine the significant differences between groups. STATGRAPHICS Plus software (version 3.0, Statistical Graphics Corp., Princeton, NJ, USA) was used for all statistical analysis. Statistical significance was accepted at $p < 0.05$. All figures were drawn using GraphPad Prism 5 (GraphPad Software Inc., California, USA)

5.3 Results and Discussion

5.3.1 Effects of *P. sajor-caju* extracts on proliferation of 3T3-L1 cells

In this study, the preadipocytes were treated with various concentrations of *P. sajor-caju* extracts. MTT assay was performed after 24 and 48 hr of incubation in the presence or absence of the extracts. All extracts tested showed a dose-dependent increase in proliferation of preadipocytes. The percentages of proliferation in descending order after 24 hour treatment were GE > AE > BE > EE > EAE (Figure 5.4 [a]). GE and AE exhibited highest the percentage of proliferation at 95±3.3 % and 93.89±5.5 % respectively at a concentration of 1000 µg/ml meanwhile, EAE significantly exhibited the lowest percentage of proliferation at 38.94± 1.1 %. Same pattern of proliferation was observed after 48 hours of treatment with *P. sajor-caju* extracts (Figure 5.4[b]). This suggests that, all extracts tested in this study showed no cytotoxicity to preadipocytes.

EAE exhibited very low percentage of preadipocyte proliferation and this could be due to the presence of palmitate compounds (see section 3.3.3). It has been reported that these compound can inhibit proliferation of preadipocytes in mouse 3T3-L1 and rat preadipocytes by modulating intracellular signal that induces endoplasmic reticulum stress (Guo et al, 2007). However in this study, EAE contributed to preadipocytes proliferation as the concentration increased because studies revealed that co-treatment with other unsaturated fatty acids may abolish the effect of palmitate (Wang et al., 2009). Thus in EAE, the presence of unsaturated fatty acids such as methyl linoleate, ethyl linoleate, oleic acid, ethyl oleate, ethyl linolenate and linoleic acid probably counter-acted with methyl palmitate and ethyl palmitate that were comprised in EAE.

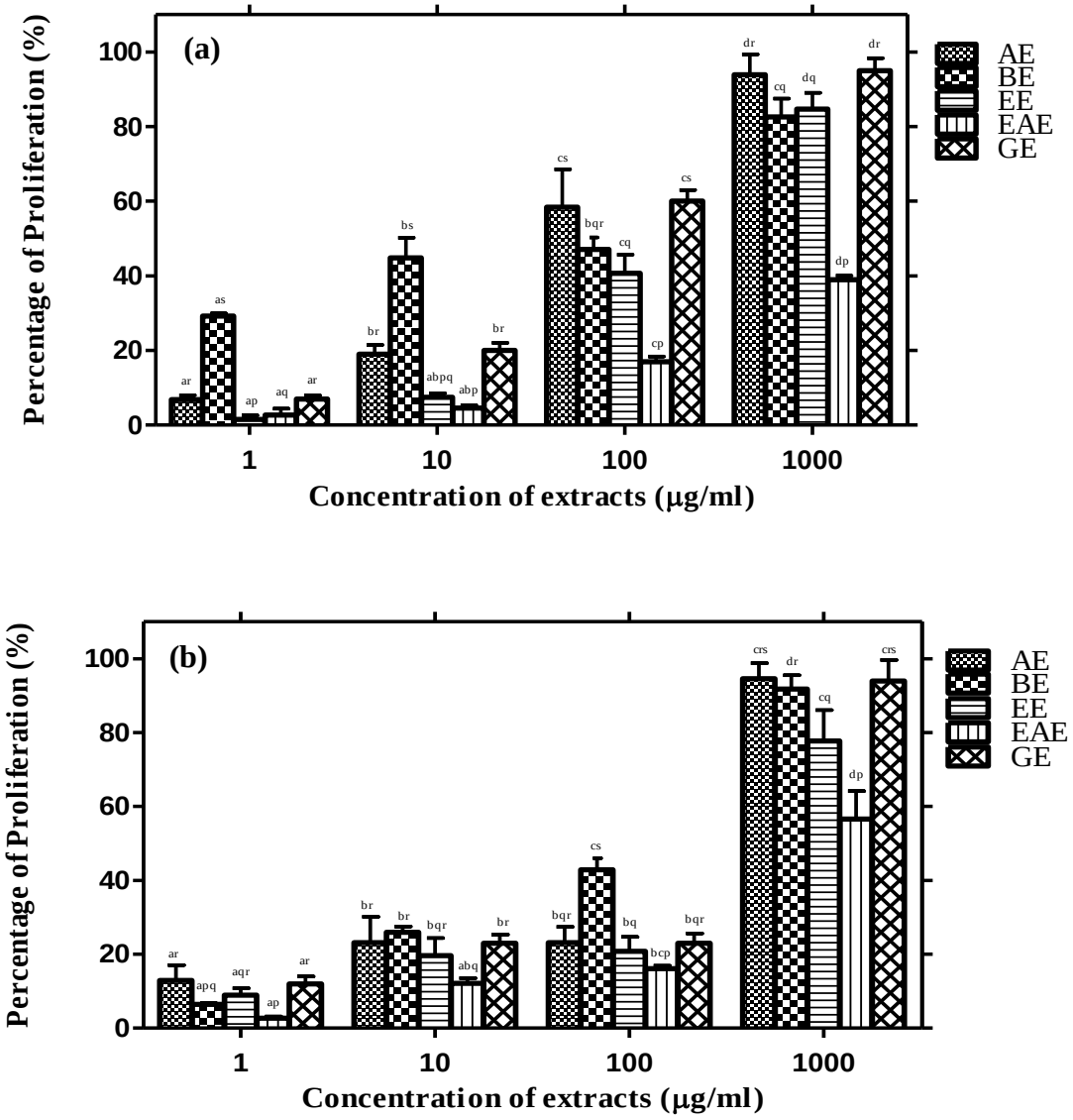

Figure 5.4: The percentages of proliferation of preadipocytes cells after treatment with *P. sajor-caju* extracts (a) after 24 hours and (b) after 48 hours treatment.

Values expressed are means ± S.D of triplicate measurements. For same extracts with different concentrations, means in the different bar with different letters (a-d) were significantly different ($p<0.05$).For different extracts with same concentrations, means in the different bar with different letters (p-r) were significantly different ($p<0.05$). AE is aqueous extract; BE is butanol extract; EE is ethanol extract; EAE is ethyl acetate extract; GE is polysaccharide extract.

5.3.2 Effects of *P. sajor-caju* extracts on lipogenesis of 3T3-L1 cells

Preadipocyte cell line (3T3-L1) is a useful model for investigating lipogenesis/ adipogenesis process. In this study, mature adipocytes were treated with various concentrations of *P. sajor-caju* extracts (0.01-1000 µg/ml). After 48 hours of incubation, the adipocytes were subjected to Oil Red O assay to determine the lipid content in each treatment. Figure 5.5 shows the lipogenic activity of *P. sajor-caju* extracts on adipocytes after staining with Oil Red O. At lower concentrations (0.01-1 µg/ml), all *P. sajor-caju* extracts increased the lipogenic activity, however, at higher concentrations (1-1000 µg/ml); all *P. sajor-caju* extracts decreased the lipogenic activity. Meanwhile, insulin promoted the lipogenic activity and this effect was dose-dependent. The lipogenic activity of *P. sajor-caju* extracts in descending order at 1000 µg/ml was insulin > BE > EE > GE > EAE > AE.

At lower concentrations (0.01 and 0.1 µg/ml), *P. sajor-caju* extracts showed insulin-like properties where the extracts stimulated lipogenic activity. AE and GE induced lipogenesis was significantly higher than insulin and the activity was comparable to 1000 µg/ml of insulin (74.98 %). Besides that, all the *P. sajor-caju* extracts tested showed better lipogenic activity than insulin, however as the concentrations increased (1-1000 µg/ml) insulin exhibited significantly higher lipogenic activity than the *P. sajor-caju* extracts.

BE showed higher lipogenic activity than AE, EE, EAE and GE at 1000 µg/ml and this could be attributed to the presence of nicotinamide in this extract (Table 3.7). It is reported that nicotinamide can stimulate the proliferation and differentiation of adipocytes by increasing the expression of FoxO1 protein and adipocyte specific genes (Bai et al., 2008; Hsu & Yen, 2007). Similarly, the saturated (palmitic acid) or monounsaturated (methyl palmitate, ethyl palmitate, methyl stearate, ethyl stearate,

ethyl oleate and oleic acid) fatty acids in EAE also may stimulate lipogenesis (Clarke et al., 1997).

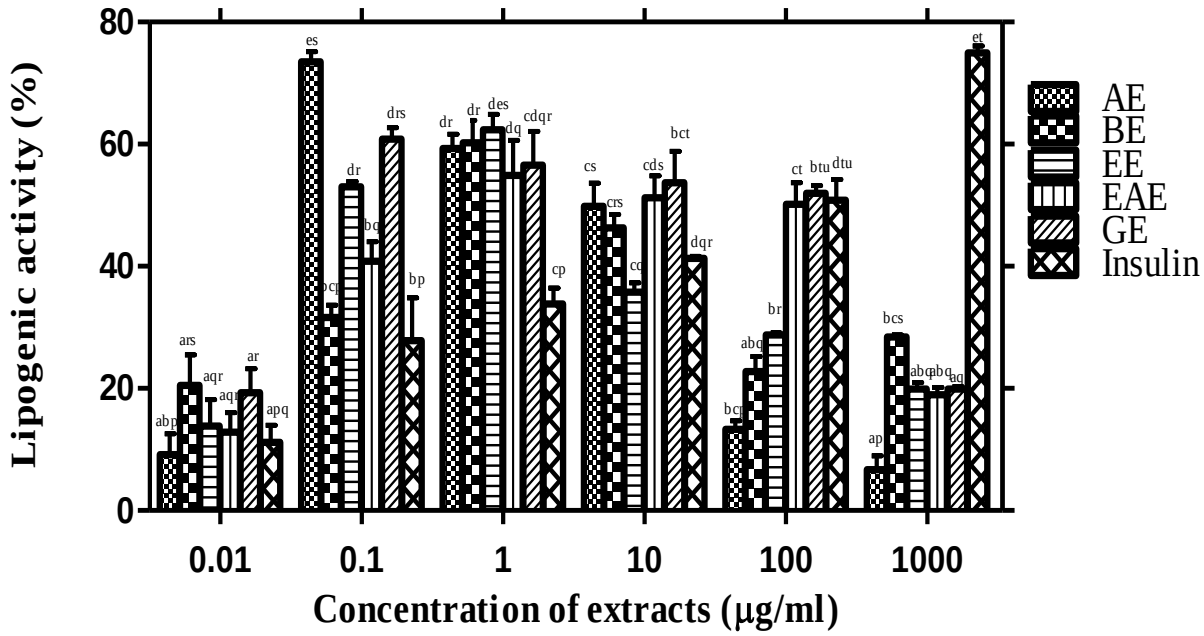

Figure 5.5: Lipogenic effect of *P. sajor-caju* extracts on adipocytes measured using Oil Red O quantification.

Values expressed are means ± S.D of triplicate measurements. For same extracts with different concentrations, means in the different bar with different letters (a-d) were significantly different ($p<0.05$).For different extracts with same concentrations, means in the different bar with different letters (p-t) were significantly different ($p<0.05$). AE is aqueous extract; BE is butanol extract; EE is ethanol extract; EAE is ethyl acetate extract; GE is polysaccharide extract.

5.3.3 Effects of *P. sajor-caju* extracts on lipolysis of 3T3-L1 cells

Lipolysis in adipocytes occurs at the surface of cytosolic lipid droplets, which have recently gained much attention as dynamic organelles integral to lipid metabolism

(Lass et al., 2011). In this study, all *P. sajor-caju* extracts stimulated lipolytic activity and this effect was dose-dependent. The lipolytic activity was highest at 1000 µg/ml however, at the lower concentrations, no significant lipolytic activity were observed between the extracts. The lipolytic activity in descending order was BE > EE > GE > AE > Isoproterenol > EAE as shown in Figure 5.6. BE and EE elicited high lipolytic activity because the presence of 4-hydroxybenzaldehyde may contribute to the lipolytic activity in these extracts (Dallas et al., 2008). Meanwhile EAE exhibited the lowest lipolytic activity because it is reported that only oleate compounds was able to enhance the lipolytic activity in 3T3-L1 cells (Fong, 1990) and in EAE, only 6% from the total fatty acids identified is oleate which was oleic acid and ethyl oleate (Table 3.3).

Isoproterenol was used as positive control to induce lipolysis. Isoproterenol is a non-specific β-adrenergic agonist and the lipolytic pathway for isoproterenol involves β-adregenic receptors (Robidoux et al., 2004). On the other hand, insulin inhibits lipolysis by the activation of low cAMP phosphodiesterase resulting in reduction of cAMP content in fat cells (Szkudelski et al., 2005). All extracts of *P. sajor-caju* exhibited same lipolytic effect as isoproterenol however, further investigations are required to elucidate the lipolytic mechanism of *P. sajor-caju* extracts.

Genistein, daidzein, coumestrol and zearalenone, are naturally occurring phytoestrogens, which have also demonstrated a similar lipolytic activity as *P. sajor-caju* extracts (Szkudelski et al., 2005; Szkudelski et al., 2002). Several plants have also been shown to stimulate lipolytic activity such as *Toona senensis* leaves (Hsu et al., 2003), citrus fruits (Saito et al, 2007; Dallas et al., 2008; Miyata et al., 2011), *Nelumbo nucifera* leaves (Ohkoshi et al., 2007) and *Curcuma longa* (El-Moselhy et al., 2011).

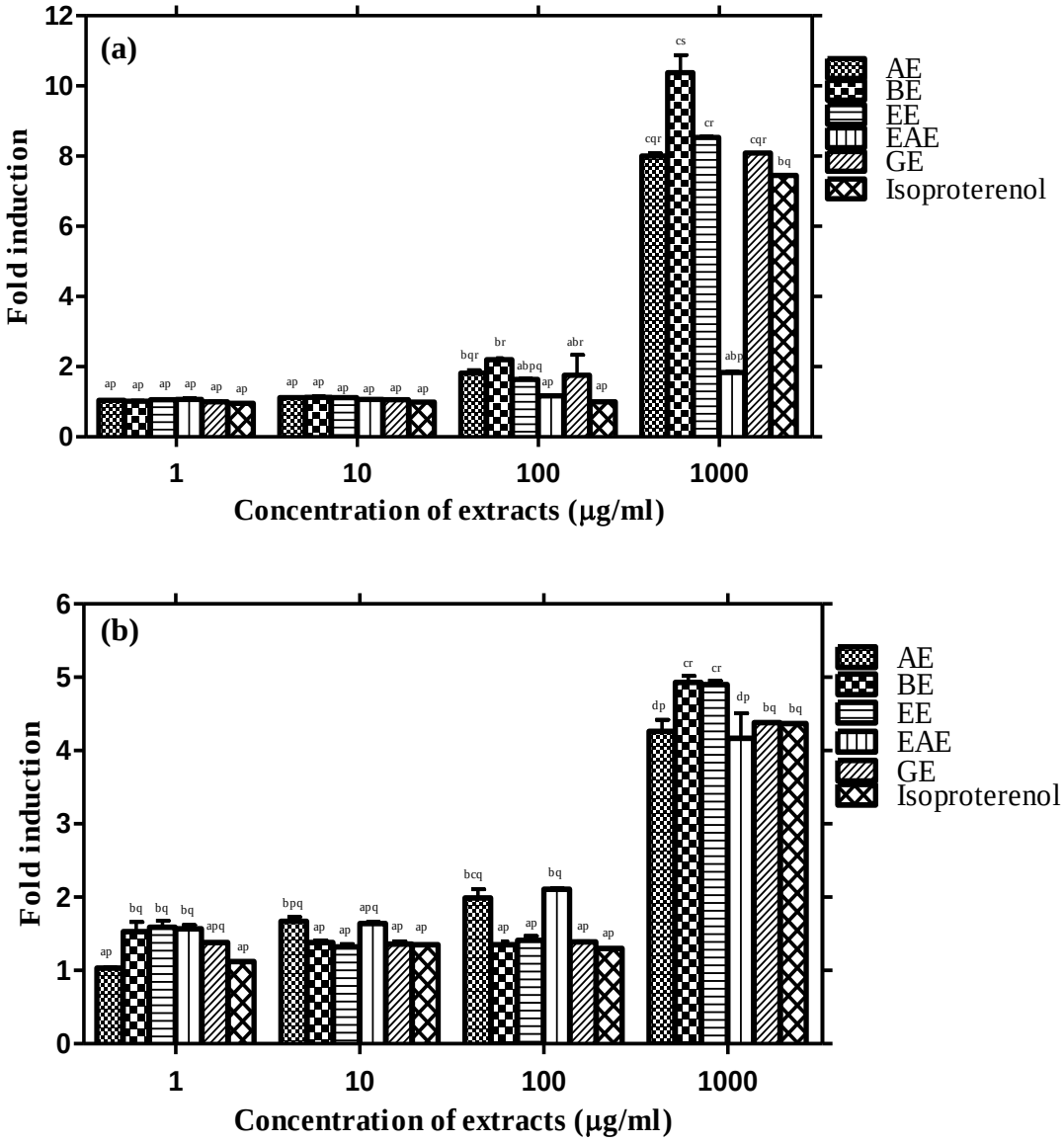

Figure 5.6: Lipolytic effect of *P. sajor-caju* extracts on 3T3-L1 adipocytes (a) quantification of glycerol (b) quantification of free fatty acids.

Values expressed are means ± S.D of triplicate measurements. For same extracts with different concentrations, means in the different bar with different letters (a-d) were significantly different ($p<0.05$). For different extracts with same concentrations, means in the different bar with different letters (p-t) were significantly different ($p<0.05$). AE is aqueous extract; BE is butanol extract; EE is ethanol extract; EAE is ethyl acetate extract; GE is polysaccharide extract.

5.3.4 Effects of *P. sajor-caju* extracts on oxidative stress indices – lipid hydroperoxides and protein carbonyl content (AOPP).

Oxidative stress has been identified as a key factor in numerous pathologies such as diabetes, inflammation and atherosclerosis, which are favored by obesity. For decades, adipocytes have been considered as low reactive cells. Indeed, it was thought that they were able only to increase or decrease their size according to the overall metabolism, resulting in either lipogenic or lipolytic conditions. More recently, the increased prevalence of obesity has raised an important research effort on adipose tissue (Talior et al., 2005). The present results bring evidence that adipose cells are susceptible to oxidative stress. In response to oxidative stress, cells may produce hydroperoxides and 4-HNE which have been identified as the main oxidative stress marker in adipocytes (Soares et al., 2005).

In this study, lipid hydroperoxides and protein carbonyl content was assessed to measure the oxidative stress level in adipocytes cultures. Table 5.4 shows the oxidative stress measurement in media after treating the 3T3-L1 cells with various *P. sajor-caju* extracts. The inhibitory potency of extracts against lipid hydroperoxide in descending order was GE > AE > EE > BE > EAE whilst, the inhibitory potency against protein damage in descending order was GE > EE > AE > EAE > BE. Adipocytes that are exposed to fatty acids caused down-regulation of the FoxO1 protein levels and the reduction of this protein are correlated with an increase in the production of reactive oxygen species (ROS) in mitochondria and pro-inflammatory adipokines (Subauste & Burant, 2007). Besides that, cells may produce hydroperoxides from polyunsaturated fatty acids such as linoleic acid (Soares et al., 2005). This supports the findings of high lipid hydroperoxide level in EAE treated cells, which mainly comprised of fatty acids.

Recent data have stressed that the role of glucose auto-oxidation and increased non-enzymatic glycation processes are major sources of oxidative stress in addition to impaired antioxidant defense mechanisms (Rudich et al., 1998). Prolonged oxidative stress can cause many alterations in the adipocytes. Sakurai et al., (2010) have reported that, oxidative stress in adipocytes can cause insulin resistance by activating the inflammatory- related adipokines such as TNF-α and MCP-1. Furthermore, oxidative stress also impairs the insulin-stimulated translocation of GLUT-4 to the plasma membrane (Rudich et al., 1999) and decreases plasma concentration of adiponectin which leads to fast transcription of NF-κB nuclear translocation in 3T3-L1 adipocyte (Soares et al., 2005). Lipoic acid (Rudich et al., 1999), resveratrol (Subauste & Burant, 2007) and polyphenols from grapes (Sakurai et al., 2010) are few compounds that have been reported to decrease oxidative stress level in 3T3-L1 adipocytes

Table 5.4: Oxidative indices measurement in media after treating with *P. sajor-caju* extracts in 3T3-L1 adipocytes.

Extracts	Lipid Hydroperoxides (µmol/g)	Protein Carbonyl Content (AOPP) (µmol/g)
AE	3.54 ± 0.41^{b}	12.28 ± 0.58^{b}
BE	7.77 ± 0.41^{c}	14.87 ± 0.66^{bc}
EE	6.98 ± 0.39^{c}	12.1 ± 0.6^{b}
EAE	14.1 ± 0.51^{d}	13.82 ± 0.14^{b}
GE	1.29 ± 0.45^{a}	10.45 ± 0.5^{a}
Untreated cells	18.64 ± 3.85^{e}	27.54 ± 0.5^{d}

Values expressed are means ± S.D of triplicate measurements. For different extracts with same concentrations, means in the different row with different letters (a-e) were significantly different ($p<0.05$). AE is aqueous extract; BE is butanol extract; EE is ethanol extract; EAE is ethyl acetate extract; GE is polysaccharide extract.

5.3.5 Effects of *P. sajor- caju* extracts on gene expressions

In this part of study, GE was selected to investigate the gene expression studies because:

1. GE exhibited anti-lipid peroxidation effect and may have potential as natural preservative (see section 4.3.3).
2. GE showed no cytotoxic effects on 3T3-L1 preadipocytes (see section 5.3.1)
3. GE stimulated lipogenesis at lower concentrations (0.1- 1µg/ml) in 3T3-L1 adipocytes and the activity was higher than insulin (positive control) (see section 5.3.2).
4. GE stimulated lipolysis and this effect was dose-dependent in 3T3-L1 adipocytes and the activity was similar to isoproterenol (positive control) (see section 5.3.3).
5. GE also attenuated oxidative stress that occured in adipocytes by reducing the hydroperoxide levels that may occur during glucose oxidase or non-enzymatic glycation (see section 5.3.4).

To determine if this extract had insulin like/anti-obesity properties, the relative expression of six selected genes, representing selective key intermediates in the insulin and isoproterenol pathways, were examined in adipocytes treated with GE and insulin as control. Figure 5.7 shows the regulation of the genes in 3T3-L1 adipocytes treated with 50 µg/ml of GE.

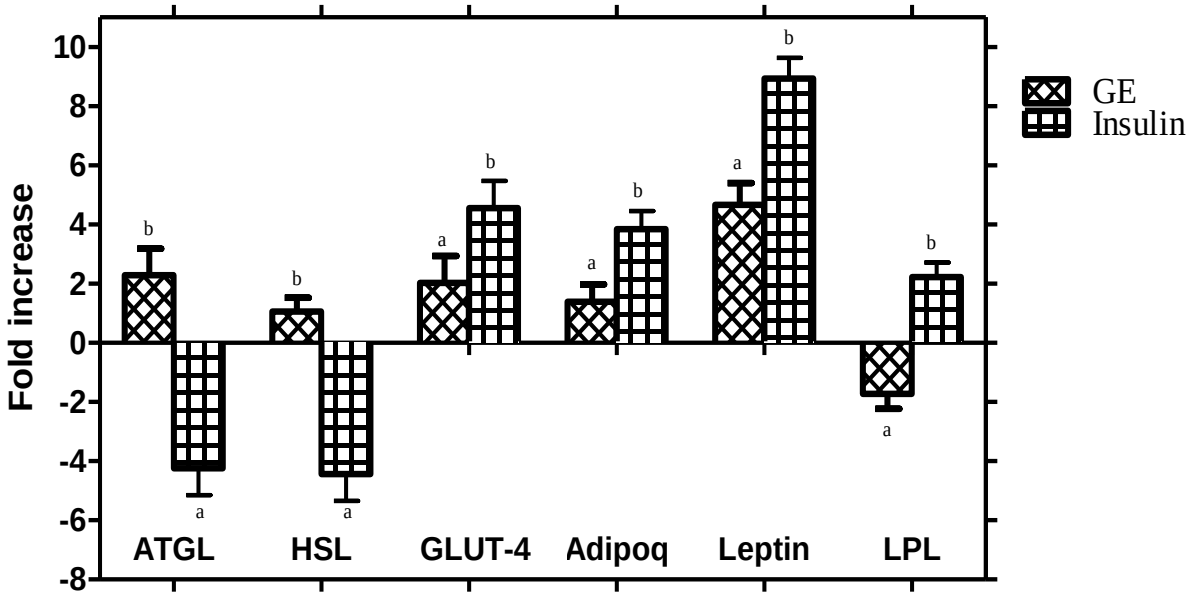

Figure 5.7: Effects of GE and insulin on gene expression in 3T3-L1 adipocytes.

Adipocytes were treated with GE or insulin for 48 hours. Total RNA was and cDNA were synthesized. RT-PCR was performed and the relative expression of the genes was calculated using the $\Delta\Delta$ C_T method. They were normalized with 18S eukaryotic rRNA. Results are expressed as –fold variation over carrier control group. Fold variations less than 0 were expressed as negative numbers (e.g., a –fold variation of 0.50 is expressed as -2.00). Values expressed are means ± S.D of triplicate measurements. Statistical significance was calculated based on the mean Δ C_T values by DMRT. For same gene with different treatment groups, means in the different bar with different letters (a-b) were significantly different ($p<0.05$). HSL is high sensitive lipase; ATGL is adipose triglyceride lipase; Adipoq is adiponectin; LPL is lipoprotein lipase; Lep is leptin; GLUT-4 is glucose transporter 4 and GE is polysaccharide extract.

Obesity is associated with increased triacylglycerol (TAG) storage in adipose tissue and insulin resistance. During times of energy shortage, TAG stored in lipid droplets is hydrolyzed to fatty acids and glycerol via lipolysis. Fatty acids released from adipose tissue can enter the circulation and be taken up by other organs for β-oxidation and subsequent ATP generation (Ahmadian et al., 2010). The mobilization of stored TAG is mediated by hormone-sensitive lipase (HSL) and the more recently discovered adipose triglyceride lipase (ATGL) (Jocken et al., 2008).

Jocken & Blaak, (2008), have reported that β-adrenoceptors coupled with stimulatory G (Gs) proteins activate adenylate cyclase (AC) so that the production of cyclic adenosine monophosphate (cAMP) increases and this in turn activates the protein kinases complex which are the protein kinase A (PKA) and AMP protein kinase (AMPK) leading to the phosphorylation of HSL and perilipin, a protein on the surface of lipid droplets in adipocytes. Phosphorylation of HSL triggers the translocation of HSL to the lipid droplet. It is known that, HSL is phosphorylated on several serine residues such as Ser-659 and Ser-660 which is the major PKA phosphorylation sites meanwhile AMPK phosphorylates Ser-565, another serine residue in the regulatory domain of HSL (Su et al., 2003).

Early experiments in HSL-deficient adipose tissue showed that the non-HSL lipolytic activity can also be activated by β-adrenergic stimulation. This suggested the existence of another ‘‘hormone-sensitive’’ enzyme besides HSL. Recently, the lipolytic pathway has been revisited by the identification of a new lipase: adipose triglyceride lipase (ATGL). In contrast to HSL, this lipase exhibits high substrate specificity for the hydrolysis of TAG. Adipose triglyceride lipase is regulated differently than HSL. An important regulatory mechanism for ATGL involves the activation of the enzyme upon interaction with CGI-58 (comparative gene identification 58). *In-vitro* experiments and

studies in living cells using bimolecular fluorescence complementation revealed that CGI-58-mediated activation of ATGL requires direct protein– protein interaction (Lass et al., 2011). The reversible binding of CGI-58 to perilipin could potentially represent an indirect PKA-dependent mechanism controlling ATGL activity. It is reported that CGI-58 is located at the surface of lipid droplets and is mostly bound to perilipin protein. In the activated state, perilipin is phosphorylated by cAMP-dependent PKA and this causes the dissociation of CGI-58 from perilipin, which is now available for the activation of ATGL activity (Granneman et al., 2005). Together, ATGL and HSL are responsible for more than 95% of the TAG hydrolase activity in adipocytes.

The present study demonstrated that, GE up-regulated the expression of HSL and ATGL genes by 2.99-fold and 2.28-fold respectively meanwhile insulin down-regulated the expression of these genes by 4.22-fold and 4.2-fold respectively. GE, is a polysaccharide thus, it cannot be hydrolysed by human enzymes (Manzi & Pizzoferrato, 2000) or absorb directly into the cells. It is possible that GE may bind with the β-adrenergic ligand to stimulate lipolysis in the adipocytes by increasing the expression of HSL and ATGL genes. Hence the activation of these genes leads to mobilization of TAG to form glycerol and fatty acids where HSL mainly breaksdown TAG to form diacylglycerol (DAG) whilst ATGL breaksdown DAG to form monoacylglycerol (MAG) and monoacylglycerol lipase (MGL) breaksdown MAG to form free fatty acids and glycerol. The gene regulation of GE is also in accordance with isoproterenol (a known β-adnergenic agent) (Yin et al., 2003; Robidoux et al., 2006). Figure 5.8 shows an overview of lipolytic pathway and possible involvement of GE.

In contrast, insulin binds to its insulin receptor substrates (IRS) in adipocytes and initiates a signaling event that, via phosphorylation and activation of phosphodiesterase 3B (PDE3B) protein decreases cAMP and ultimately inhibits

lipolysis. The importance of PDE3B is in suppressing adipocyte lipolysis and this correlates to the down-regulation of HSL and ATGL genes by insulin reported in this study (Liu et al., 2008; Dallas et al., 2008).

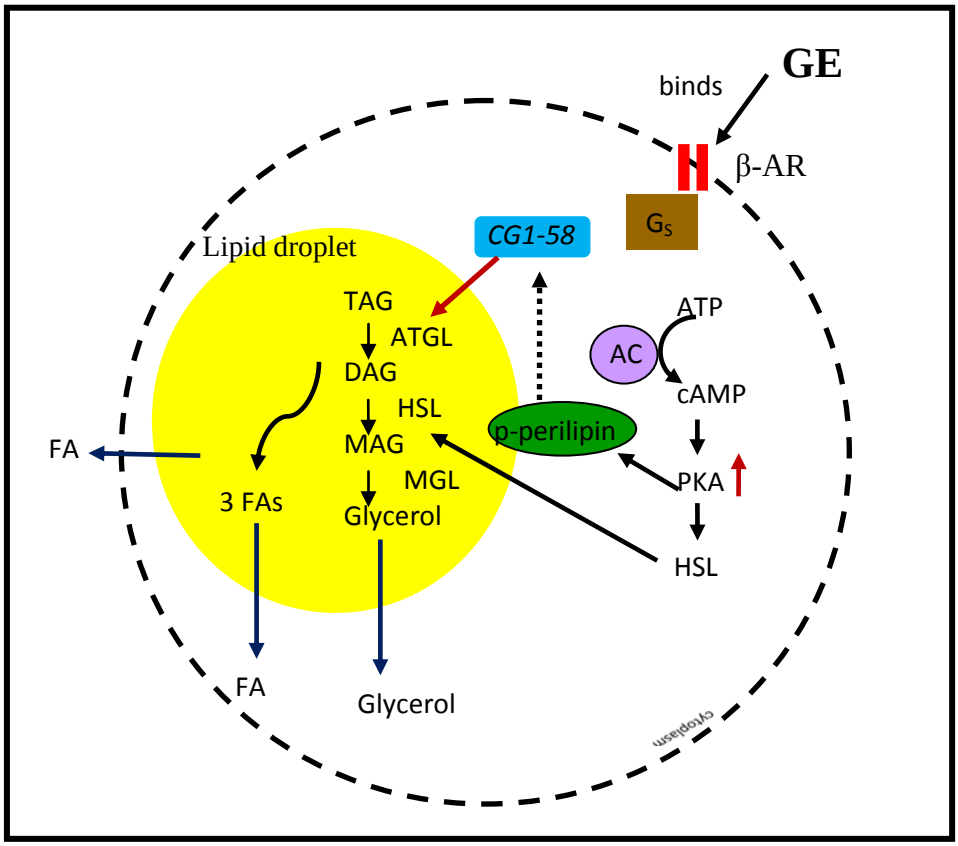

Figure 5.8: Overview of lipolytic pathway and possible involvement of GE

It is possible that, GE, by binding to Gs-coupled β-adrenergic receptors (β-AR), activates adenylate cyclase (AC) to increase cAMP and activate protein kinase A (PKA). PKA phosphorylates HSL and perilipin protein, resulting in translocation of HSL from the cytosol to the lipid droplet meanwhile perilipin protein releases the CGI-58 protein which then attaches to ATGL protein to increase the expression of ATGL gene in lipid droplets. TAG is triacylglycerol; DAG is diacylglycerol; MAG is monoacylglycerol; FA is fatty acids; GE is polysaccharide extract.

It is reported that, in adipocytes, β-adrenoceptor activation result in the simultaneous stimulation of both PKA and AMPK (Yin et al., 2003) and are likely to work in synergy to regulate key enzymes involved in lipid and glucose metabolism (Fu et al., 2007) by generating cellular events such as glucose uptake and lipid oxidation to produce energy, while turning off energy-consuming processes such as glucose and lipid production to restore energy balance (Chaves et al., 2011; Guo & Liao, 2000).

Increasing lipolysis in adipocytes would lead to chronically high levels of circulating fatty acids (Figure 5.8) that are correlated with adverse metabolic effects such as insulin resistance thus insulin, plays a key regulatory role in stimulating the transport of blood glucose into peripheral tissues through GLUT-4 transporter, which is mainly expressed in adipose tissues. In adipocytes, insulin stimulates the translocation and redistribution of GLUT-4 transporter from specific intracellular compartments to the plasma membrane where it facilitates glucose uptake (Huang et al., 2010). Adipocytes treated with GE showed elevated expression of GLUT-4 gene by 2.03-fold and adipocytes treated with insulin also showed elevated expression of GLUT-4 gene by 4.56-fold, which is two times more than GE. The GLUT-4 gene can be activated by two different signaling pathways which are phosphatidylinositol 3'-kinase (PI3K) which is activated by insulin (Liu et al., 2008) or AMPK. Exaggeration of glucose (R_a) commonly occurs in diabetes mellitus, GE may suppress exaggerated rates of glucose (R_a), and this in turn, lowers blood glucose level (hypoglycemic) by facilitating the glucose uptake and GLUT-4 translocation. (Hays et al., 2008; Huang et al., 2010) thus may prevents insulin resistance. Metformin (Tan et al., 2008) and glucosamine (Kong et al., 2009) are examples of compounds that are known to act via AMPK signalling pathway in 3T3-L1 adipocytes.

Besides the above described genes, adipocytes- derived cytokines (adipokines) namely; adiponectin and leptin were also investigated in this study. Adiponectin is a protein hormone circulating in the blood and administration of adiponectin is reported to decreases plasma glucose, free fatty acids, and triglycerides, and increases muscle fatty acid oxidation (Fruebis et al., 2001). The expression of adiponectin is in accordance with body fat mass thus insulin showed elevated level of adiponectin gene by 3.84-fold. This corresponds to results reported by Liu et al., (2008) and the up-regulation of adiponection in GE was 1.39-fold. Studies have demonstrated that, β-adrenoceptor agonists up-regulates the expression of adiponectin receptor 2, adenylyl cyclases and PKA. However the accompanying activation of AMPK may inhibit this regulation (Fu et al., 2007; Huypens et al., 2005). Thus it is unclear on how GE can directly influence adiponectin production and release in adipocytes. In this part of study, the function of adiponectin receptor 1 and 2 were not evaluated hence further investigations are needed to elucidate the role of adiponectin in glucose and lipid homeostasis.

Meanwhile leptin is involved in the regulation of body weight via its central actions on food intake and energy expenditure. Circulating leptin concentrations decrease, independently of body adiposity, and the decreases of leptin are proportional to changes of plasma insulin and glucose (Aliaga et al., 2003). Insulin and GE showed elevated levels of leptin by 8.93-fold and 4.66 respectively. Several *in vitro* studies have shown that insulin potently stimulates leptin secretion in adipocytes via PI3K independent signalling pathway (Hardie et al., 1996; Zeigerer et al., 2008). Well documented discoveries have also raised the possibility that leptin pathways act in concert with insulin to control glucose and lipids metabolism (Zou & Shao, 2008). In this part of study, leptin is suggested to improve insulin sensitivity by controlling the cellular concentrations of malonyl-CoA, via the inhibition of acetyl-CoA carboxylase

(the enzyme involved in malonyl-CoA transformation) which results in a decline of lipogenesis (Puente et al., 2008).

During the past decade, a great deal of interest has focused on the effect of LPL on lipid metabolism and atherogenesis, and its activity is tightly regulated at various levels including transcriptional, translational and post-transcriptional levels (Yokota et al., 2009). It plays an important role in lipid metabolism by hydrolyzing core TAG from circulating chylomicrons and very low-density lipoproteins (VLDLs) (Donahoo et al., 2011). Besides that, LPL has been shown to be one of the early genes induced during the differentiation of preadipocytes to adipocytes (Couturier et al., 1998). Adipocytes treated with GE down-regulated the expression of LPL by 1.73-fold whilst adipocytes treated with insulin, stimulated the expression of LPL by 2.2-fold. These results are in agreement with the suggestion that inhibition of LPL activity is induced due to increased cellular cAMP. Furthermore, adipocytes treated with epinephrine (Couturier et al., 1998) and isoproterenol (Antras et al., 1991) also elicited a down-regulation of LPL.

Phosphorylated AMPK plays a major role as a metabolic master. Based on the expression of the relevant genes studied, it points towards dual action of β-adrenergic receptors and AMPK signals, thus further investigations are required to validate the possible involvement of GE in AMPK signals.

5.4 Conclusion

From this part of study the following conclusions can be arrived at:

1) All *P. sajor-caju* extracts did not exhibit any cytotoxic effects on 3T3-L1 preadipocytes. AE, BE And GE stimulated proliferation of preadipocytes in a dose-dependent pattern after 24 and 48 hours treatment respectively.
2) All *P. sajor-caju* extracts stimulated lipogenesis however the effect was not dose-dependent. At lower concentrations (0.1-1 µg/ml), all *P. sajor-caju* extracts stimulated lipogenesis and was higher than insulin, but as the concentration of *P. sajor-caju* extracts increased the lipogenesis activity decreased.
3) All *P. sajor-caju* extracts stimulated lipolysis and this effect was dose-dependent. The activity was similar to isoproterenol (positive control). Among the extracts tested, AE and GE showed the highest lipolytic activity. The activation of HSL and ATGL genes in GE treated adipocytes may enhance the lipolysis observed in this study.
4) Adipocytes are highly sensitive to oxidative stress and this can alter the production of adipokines. GE was able to attenuate the oxidative stress that occurred in 3T3-L1 adipocytes. GE treated cultures displayed the lowest lipid and protein damage levels.
5) Furthermore, GLUT-4 and adiponectin genes were also up-regulated in adipocytes treated with GE. Thus this makes GE a good hypoglycemic agent and insulin sensitizer without increasing adiposity.
6) LPL is an adipogenesis initiator enzyme and leptin is important in regulation of body weight. Adipocytes treated with GE elicited down-regulation of LPL but showed elevated levels of leptin (adipokine).

7) The present study, strongly suggests that the lipolytic effects of GE were stimulated by activation of AMPK protein through expression of the down-stream genes. However to validate the involvement of AMPK in this part of study, more studies are required to be carried out.

At present, because of dissatisfaction with high costs and potentially hazardous side-effects, the potential of natural products for treating obesity and diabetes are under exploration, and GE may be an excellent alternative strategy for developing future effective and safe therapeutic agent. In order to elucidate the efficacy of GE on altering body fat and improving the glucose tolerance, Chapter Six investigates the potential of GE as anti-obesity and anti-diabetic agent in mice fed a high fat diet.