EFFECTS OF POLYSACCHARIDE (GE) ON HIGH-FAT DIET INDUCED METABOLIC CHANGES AND OXIDATIVE STRESS IN C57BL/6J (ob/ob) OBESE MICE.

6.1 INTRODUCTION

Studies on animal models have clearly demonstrated two distinct animal models of obesity; the first type is genetic obesity, as seen in rodent strains such as the Zucker fatty (fa/fa) rat and the leptin-deficient obese (lep<sup>ob</sup>/lep<sup>ob</sup>) mouse, which become obese under various experimental conditions (Phillips et al., 1996). The second type of obesity reflects more closely to the human condition where the animals develop central adiposity, hyperinsulinemia, hyperglycemia and hypertension which results from a combination of genetic and environmental factors (Schreyer et al., 1998; Moon et al., 2009) such as long term high-fat intake has been most intensively investigated because of its contribution to the development of both obesity and diabetes in humans and rodents (Kim et al., 2004; Crevel et al., 1992).

In this part of study, the C57BL/6J (ob/ob) mouse strain was selected to investigate the high fat diet induced metabolic changes such as hyperlipidemia, hyperglycemia and hyperinsulinemia. Besides that, the potential effects of GE on oxidative stress indices and antioxidant enzyme was investigated and it was compared with an oral antidiabetic agent (metformin) which is also known to reduce weight (Glueck et al., 2008; Despres, 2003).
6.2 Material and Methods

6.2.1 Materials

Applied Biosystems, United State of America
- RNAlater® solution
- TaqMan® gene expression assays

BD Vacuitaner®, United States of America
- 13 × 7 mm× 2.5 ml BD SST™ glass serum tube with gold BD Hemogard™
  closure

Bioassay Systems, United State of America
- QuantiChrom® Uric acid kit

Calbiochem®, Germany
- Catalase assay kit (CAT)
- Glutathione peroxides assay kit (GPx)
- Reduced glutathione assay kit (GR)
- Superoxide dismutase assay kit (SOD)

Cayman Chemicals, United State of America
- 8-hydroxy-2-deoxy-Guanosine EIA kit
Merck Sante, France

- Metformin

Roche Diagnostic, Germany

- ACCU-CHEK Advantage II Glucometer
- ACCU-CHEK Advantage II Test strips

Sigma-Aldrich, United State of America

- Glucose, C₆H₁₂O₆

SPI-BIO Bertin pharma, France

- Rat Insulin Enzyme Immunoassay Kit

TestDiet®, United State of America

- 45 % of high-fat diet
- 60 % of high fat diet

6.2.2 Methodology

6.2.2.1 Animals and Experimental Design

This study was conducted in conformity with the policies and procedures of the Animal Care and Use Committee of Faculty of Medicine, University of Malaya. (Approval number: ISB/14/07/2010/GK [R]). Female C57BL/6J (ob/ob) mice (7-week old) were purchased from BioLasco Laboratory, Taiwan. The animals were maintained in stainless steel wire–mesh cages in a room kept at 21°C with a standard conditions of
12-hr light/dark cycle (light period: 8:00–20:00 hr) with free access to food and water, which were provided fresh every day. After one week for acclimatisation, the mice were randomly assigned (based on weight) into 7 groups. Six mice were assigned in each group. The groups were divided as stated below:

- Group 1: ND (Normal diet only)
- Group 2: ND240 (Normal diet + 240 mg/kg of body weight GE)
- Group 3: HFD (High-fat diet only)
- Group 4: HFD60 (High-fat diet + 60 mg/kg of body weight of GE)
- Group 5: HFD120 (High-fat diet + 120 mg/kg of body weight of GE)
- Group 6: HFD240 (High-fat diet + 240 mg/kg of body weight of GE)
- Group 7: HFDMET (High-fat diet + 2 mg/kg of body weight of metformin [antidiabetic drug])

The composition of normal diet was 5% of total energy as fat whilst the high-fat diet was 45 and 60% of fats. GE was administered thrice a week, via epi-gastric route using a feeding needle (size 20) to groups ND240, HFD60, HFD120 and HFD240 for 15 weeks. Metformin was administered to HFDMET as positive control meanwhile for groups ND and HFD, distilled water (vehicle) was administrated the same way as GE was administrated to the animals until sacrificed. Water was provided ad libitum. After 7 weeks of feeding with 45 % of fat, the animal diet was substituted with 60% of fat for groups HFD, HFD60, HFD120, HFD240 and HFDMET whilst for groups ND and ND240 the diet was not altered throughout the experiment. Body weight and food consumption were monitored daily. During the experimental period, urine was collected from each group weekly (every Monday mornings at 10:00 hr) for assessment of oxidative damage.
6.2.2.2 Oral Glucose Tolerance Test (OGTT)

After 15 weeks of experiment, the mice were fasted for 12 hr before the oral glucose test was carried out. Blood samples were obtained from a cut in the tail vein of the mice and blood glucose levels were determined using ACCU-CHEK® glucometer and ACCU-CHEK® Advantage test strips. Firstly, the fasting blood glucose levels were measured. Then, GE was administrated to the mice via epic-gastric route using a feeding needle and the blood glucose levels were measured again. Glucose (2 g/kg of body weight) was also administrated to the mice via epic-gastric route. Finally, the blood glucose levels were measured every 30 min for 2 hr (30, 60, 90 and 120 min). Figure 6.1 shows the instruments used in OGT test.

![Instruments used in OGT test](image)

Figure 6.1: Instruments used to study oral glucose tolerance test (OGTT). (a) ACCU-CHEK® Advantage glucometer with test strips; (b) feeding needle (size20)
6.2.2.3 Sample collection and analytical methods

After 16 weeks of experiment, the mice were anesthetized with ether after withholding food for 12 hr. Mice was sacrificed by aortic exsanguinations. Blood samples were collected in a SST- treated blood collection tubes. Serum samples were prepared by centrifugation of blood at 2400 × g for 15 min and stored at -80°C until further analysis was carried out. Immediately after blood collection, the liver and kidney was perfused in-situ with ice-cold saline. The weight of the livers and kidneys from each mouse from each group were recorded. Eight ml of ice-cold phosphate buffer saline (PBS) was added to one gram of liver or kidney. The samples were then homogenized using a homogenizer and one millilitre aliquots of the supernatant were stored in microcentrifuge tubes at -80°C for further analysis. Adipose tissues were removed and stored in RNAlater® solution and kept in 4°C overnight before storing the samples in -80°C for RNA extraction and further analysis. Figure 6.2 shows the work flow of the animal experiment.
Figure 6.2: The experimental design and analytical methods for the in vivo study. CAT is catalase; SOD is superoxide dismutase; GPx is glutathione peroxidase, GR is reduced glutathione; TBARS is thiobarbituric acid reactive species.
6.2.2.4 **Insulin Sensitivity Test**

This Enzyme Immuno Assay (EIA) is based on the competition between unlabelled rat insulin and acetylcholinesterase (AChE) linked to rat insulin (tracer) for limited specific Guinea-Pig anti-rat insulin antiserum sites. Briefly, the complex Guinea-Pig antiserum-rat insulin complex binds to the Goat anti-Guinea-Pig antibody that is attached to the well. The plate was incubated for 16 hours for the immunological reaction to occur. The plate is then washed and Ellman’s Reagent (enzymatic substrate for AChE and chromogen) was added into the wells. The AChE tracer acts on the Ellman’s Reagents to form a yellow compound. The absorbance was read at 410 nm. The intensity of the colour, which is determined by spectrophotometry, is proportional to the amount of tracer bound to the well and is inversely proportional to the amount of free rat insulin present in the well.

6.2.2.5 **DNA Damage (8-hydroxy-2-deoxy-Guanosine) Test**

8-hydroxy-2-deoxy-Guanosine (8-OHdG) is produced by the oxidative damage of DNA. Briefly, the principle of this assay is based on the competition between 8-OH-dG and a 8-OH-dG acetylcholinesterase (AChE) conjugate for a limited amount of 8-OH-dG monoclonal antibody. Since, the concentration of the 8-OH-dG tracer is held constant while the concentration of 8-OH-dG varies, the amount of 8-OH-dG tracer that is able to bind to the 8-OH-dG monoclonal antibody will be inversely proportional to the concentration 8-OH-dG in the well. This antibody-8-OH-dG complex binds to goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate was washed to remove any unbound reagents and then Ellman’s Reagent was added to the well. The product of this enzymatic reaction has a distinct yellow colour and absorbs strongly at 412 nm.
6.2.2.6 Antioxidant enzymes

The aliquots of the tissue supernatant were used to measure the enzymic antioxidant activities namely, superoxide dismutase (SOD-1.15.1.1), glutathione peroxidase (GPx-1.11.1.9) and catalase (CAT-1.11.1.6) and non-enzymic antioxidant namely reduced glutathione (GR). Commercially available kits were used for SOD, CAT, GPx and GR assays. The SOD activity was measured by using a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase at 450 nm. Total GPx activity was quantified spectrophotometrically by measuring the decrease in reduced nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm using cumene hydroperoxide as a substrate. The GR assay is based on the reduction of oxidized glutathione by NADPH and catalase activity in the liver and kidney tissues was measured with H₂O₂ as a substrate. The formaldehyde produced was measured spectrophotometrically with purpald chromogen at 540 nm. The protein content of hepatic and renal homogenates was determined by the method of Bradford (1976) using the Bio-Rad Protein Assay (Barcelona, Spain), with bovine serum albumin as a standard (Appendix B; pg 243).

Enzyme activities were expressed in units per milligram of protein. One unit of SOD activity was defined as the amount of enzyme that exhibited 50% dismutation of the superoxide radical. One unit of CAT activity was defined as the amount of enzyme that caused the formation of 1.0 nmol formaldehyde per min. The unit for GPx and GR activity were expressed as nanomoles of NADPH per min using an extinction coefficient of 0.00373 µM⁻¹
6.2.2.7 Gene Expression

The gene expression investigation was carried out based on the methods described in Chapter 5. Table 6.1 shows the genes investigated in this part of study.

Table 6.1: Genes investigated

<table>
<thead>
<tr>
<th>No</th>
<th>Gene name and abbreviation</th>
<th>Assay ID</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Retinol binding protein 4 (RBP-4)</td>
<td>Mm 00803266_m1</td>
<td>XM_993476</td>
</tr>
<tr>
<td>2</td>
<td>Nuclear factor-κB (NF-κB)</td>
<td>Mm 00482418_m1</td>
<td>NM_003998</td>
</tr>
<tr>
<td>3</td>
<td>Sterol regulatory binding protein (SREBP-1c)</td>
<td>Mm 00550338_m1</td>
<td>NM_011480.3</td>
</tr>
<tr>
<td>4</td>
<td>Tumor necrosis factor-α (TNF-α)</td>
<td>Mm 00443258_m1</td>
<td>NM_013693.2</td>
</tr>
<tr>
<td>5</td>
<td>Peroxisome proliferator activated-receptor-γ (PPAR-γ)</td>
<td>Mm 01184322_m1</td>
<td>NM_011146</td>
</tr>
<tr>
<td>6</td>
<td>Serum amyloid A 2 (SAA-2)</td>
<td>Mm 00656927_g1</td>
<td>NM_009117.3</td>
</tr>
<tr>
<td>7</td>
<td>Interleukin 6 (IL-6)</td>
<td>Mm 00446190_m1</td>
<td>NM_031168.1</td>
</tr>
<tr>
<td>8</td>
<td>Monocyte chemoattractant protein-1 (MCP-1)</td>
<td>Mm 00437433_m1</td>
<td>NM_011331.2</td>
</tr>
<tr>
<td>9</td>
<td>C-reactive proteins (CRP)</td>
<td>Mm 00432680_g1</td>
<td>NM_007768.4</td>
</tr>
</tbody>
</table>

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.

6.2.2.8 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).

6.3 Results and Discussion

6.3.1 Effects of GE on the changes in body weight

In the previous chapter, GE was shown to stimulate lipolysis in 3T3-L1 adipocytes. Thus, in this study, the potential effect of GE in preventing weight gain in C57BL/6J \((ob/ob)\) mice on high-fat diet induced obesity was investigated. The experiment was conducted for 15 weeks and GE, metformin or distilled water (control) were fed via epi-gastric route thrice a week. The test compounds were not administered daily to avoid physical stress. To evaluate the lipid profile measurement, the blood collected from each mouse from each group was centrifuged to obtain the serum layer. The serum was pooled together in order to have sufficient serum samples for further analysis. The pooled serum samples were sent to the Clinical Diagnostic Laboratory Unit, University Malaya Medical Centre for the serum lipid analysis. Figure 6.3 (a-b) shows the effects of GE and metformin on body weight changes and lipid profile of C57BL/6J mice fed on a high-fat diet or normal diet.

The body weight in ND group gradually increased during the 15 week period. In contrast, the body weight of mice in HFD group showed rapid increase of body weight. The descending order for the percentages of weight gain in each groups were HFD > HFD60 > HFD120 > HFDMET > ND > HFD240 > ND240. The mice in HFD group
gained 49.1% of body weight by the end of the experiment. The weight gain of HFD60, HFD120 and HFD240 groups were 32.16%, 29.63% and 22.2% respectively. For the normal diet groups, the weight gain of ND and ND240 groups were 27.34% and 20.68% respectively. Metformin was used as a positive control in this study, because metformin is a known anti-diabetic drug with weight reduction properties and HFDMET group displayed a weight gain of 26.01%.

In addition, mice in HFD60, HFD120 and HFD240 groups had 27.55%, 36.69% and 39.76% lower body weight respectively compared to HFD group. Meanwhile, ND240 group elicited a 15.70% lower body weight compared to ND group. HFD MET group showed 31.90% lower body weight compared to HFD group. The potential weight lowering effect of GE treated groups were comparable to HFD MET group. The weight lowering potential of each treatment group in descending order was HFD240 > HFD120 > HFD MET > HFD240 > ND240. Figure 6.4 shows the size of mice in each group in the end of the 15 weeks experiment.

The serum lipid analysis includes the measurement for triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c). In the present study, the serum lipid levels were increased in HFD group compared to the ND group. In the HFD control group, the TG level was increased 1.3-fold, TC increased by 1.4-fold, HDL-c increased by 1.3-fold and LDL-c increased by 2.7-fold compared to those in ND group thus the mice in HFD were hyperlipidemia. Meanwhile, mice in HFD60, HFD120 and HFD240 treated groups showed considerably reduced levels of TG, TC, HDL-c and LDL-c and this effect was dose-dependent. Similarly, in HFD MET group, the TG, TC, HDL-c and LDL-c levels were also decreased compared to HFD group. GE treated groups remarkably improved
high-fat diet induced hyperlipidemia, and the lipid levels and was comparable to HFD MET group.

The atherogenic index and cardiac risk factor were calculated based on the measurement obtained from the lipid analysis. The atherogenic index (AIP) was defined by TC minus HDL-c divided by HDL-c whilst the cardiac risk factor was calculated as TC divided by HDL-c (Jeong et al., 2010). From the present study, the AIP risk predictor indices for HFD group increased compared to those in ND and GE or metformin treated groups. The reductions in the atherogenic indexes in GE treated groups, nevertheless, indicate a decreased risk of vascular disease since high atherogenic index has been positively correlated with cardiovascular risk (Keefe & Bell, 2007). In accordance to the high AIP risk factor, the cardiac risk factor was also elevated in HFD group compared to those in ND and GE or metformin treated groups.

![Graph showing weight changes over weeks for different groups](caption in following page)
Figure 6.3: Effects of GE and metformin on (a) body weight changes and (b) lipid profile of C57BL/6J mice fed on a high-fat diet or normal diet.

The concentrations of GE were 60, 120 and 240 mg/kg/day. Metformin (2 mg/kg/day) was used as positive control. Values expressed are means ± S.D of (n=6 per group) measurements. TG is triglycerides; TC is total cholesterol; HDL-c is high density lipoprotein cholesterol; LDL-c is low density lipoprotein cholesterol.
Figure 6.4: The size of mouse from each group after 15 weeks experiment. (a) ND; (b) ND240; (c) HFD; (d) HFD60; (e) HFD120; (f) HFD240 and (g) HFDMET. Pictures were taken using Canon Digital Ixus 8015 model (actual size -3264x2448).
Numerous naturally-occurring compounds have been proposed as treatments for lowering the body weight including fish oil and green tea extract, where the catechins such as EGC and EGCG have received tremendous attention (Kasaoka et al., 1999). The anti-obesity activity of green tea extract was reported to have resulted from the combined actions of appetite reduction, greater lipolytic activity and energy expenditure, and less lipogenic activity and adipocyte differentiation (Hsu & Yen, 2007).

Previous studies have shown that soluble fibers, such as high methoxylated apple pectin (HMAP), soluble cocoa fiber (SCF) and β-glucan, attenuated some of the main clinical alterations that characterize the metabolic syndrome (Sanchez et al., 2011). Beta-glucan from Aureobasidum sp. reduced the body weight, blood glucose and blood triglyceride as well as cholesterol concentrations in db/db mice. The beta-1,3-glycosidic bond is known to act as an antioxidant which is also associated with prevention of metabolic disorders such as obesity and diabetes (Kang et al., 2008). Pramanik et al., (2007), have reported that the β-glucan from the polysaccharide in P. sajor-caju consists of a beta-1,3-glycosidic bond. Thus it is possible to speculate that, the beta-1,3-glycosidic linkages present in GE could prevent weight gain in high-fat induce obese C57BL/6J (ob/ob) mice. However, further investigations are required to prove that similar structure is present in the polysaccharide (GE) used in this study.

It is also well documented that elevated levels of TC, TG and LDL-c (hyperlipidemia) are associated with complications of diabetes mellitus (Aas et al., 2009) and atherosclerosis (Kim et al., 2008b) and these serum cholesterol concentrations can be lowered by ingestion of water-soluble fibers such as pectin and oat bran (Fukushima et al., 2001). In the present study, lower TC and TG concentrations were observed in mice fed with GE, indicating that consumption of GE had beneficial
effect in suppressing TC and TG levels. About 30% of blood cholesterol is carried in the form of HDL-c and it can remove cholesterol from antheroma within arteries and transport it back to the liver for excretion or re-utilization. Thus high level of HDL-c in GE treated groups, may protect the body against cardiovascular disease (Kwiterovich, 2000). On the other hand, GE even at the lowest dose used, reduced approximately 60% of LDL-c concentration compared to HFD control group. Most studies have found LDL-c to be the most dangerous among the serum lipids and the oxidation of LDL leads to its increased penetration of arterial walls (Kim et al., 2008b). LDL-c transport cholesterol to the arteries where they can be retained in arterial proteoglycans that contributes to the formation of artherosclerotic plague lesions. LDL-c poses a risk of cardiovascular disease when it invades endothelium and become oxidized since the oxidized form is more easily retained by the proteoglycans. Thus, increased level of LDL-c is associated with atherosclerosis, heart attack, stroke and peripheral vascular disease (Crowwell & Otvos, 2004). GE may aid in the prevention or reduction of cardiovascular risk factors.

Similar serum cholesterol lowering activity was observed in Maitake and Enokitake mushroom fibers and attempts to elucidate the mechanism involved in the lipid-lowering effect of mushroom have been reported (Fukushima et al., 2001). It has been suggested that dietary mushroom fibers (i.e. polysaccharides) might bind to bile acids to reduce their entry into enterohepatic circulation, which then leads to an increase in gut bile acid secretion. As a result, the liver responds by increasing hepatic conversion of cholesterol into bile acids, thus reducing its circulating levels (Fukushima et al., 2001). In summary, GE had hypolipidemic effects on high-fat induced obese mice, where GE lowered the TG, TC and LDL-c levels that may have decreased the body weight, atherogenic index and cardiac risk factor.
6.3.2 Effects of GE on the glucose tolerance

Overweight and obesity are risk factors for dyslipidemia, insulin resistance (Gokalp et al., 2008), type 2 diabetes and cardiovascular diseases (Pasimeni et al., 2006). Diabetes may lead to microvascular (blindness, renal failure and neuropathy) and macrovascular (stroke and myocardial infarction) complications (Yumuk et al., 2005). Glucose tolerance test was performed on the mice to investigate the glucose tolerance levels in mice on high fat diet. The mice were fasted 12 hours before the oral glucose tolerance test was carried out. Figure 6.5 shows the oral glucose tolerance test results of obese mice on high-fat or normal diets. According to Lee et al., (2006) and the protocol from the kit, mice with fasting blood glucose of 7.8 mmol/L (200 mg/dL) and above was considered hyperglycemic meanwhile mice with fasting blood glucose of below 3.9 mmol/L (70 mg/dL) were categorised as hypoglycemic.

In this study, mice in HFD and HFD60 groups were considered to be moderately hyperglycemic but the blood glucose level of mice in HFD60 group was lower compared to HFD group. Mice in the other groups remained euglycemic (normal). Glucose challenge dramatically increased the blood glucose level in HFD group compared to those in ND group, while GE treated groups prevented the blood glucose levels from rising. In HFD group, the hyperglycemia was exacerbated after glucose administration and the blood glucose level in descending order was HFD > HFD60 > HFDMET > HFD120 > ND > HFD240 > ND240.

The blood glucose was further measured every 30 minutes for 2 hours (30, 60, 90, 120 minutes). At the end of 120 minutes, the blood glucose levels for each group in descending order was HFD > HFD60 > ND > HFDMET > ND240 > HFD120 > HFD240. The blood glucose level in all the groups decreased in a time dependent pattern. After 120 minutes of glucose administration, the blood glucose level in HFD
group did not return to the fasting glucose level, indicating glucose intolerance. However for GE and metformin treated groups, the blood glucose levels were below the fasting glucose level indicating the glucose tolerance was improved when GE or metformin were administrated to the mice. In summary, GE treated groups (HFD60, HFD120 and HFD240) showed the lowest blood glucose levels and good glucose tolerance after 120 minutes of glucose administration.

Figure 6.5: Effects of GE on oral glucose tolerance (OGT) of C57BL/6J mice fed on a high-fat diet or normal diet.

GE concentrations were 60, 120, 240 mg/kg/day. Metformin (MET) was used as positive control. Blood glucose was measured 30 minutes before glucose administration (-30 min) and every 30 minutes after glucose administration (0, 30, 60, 90, 120 min). Values expressed are means ± S.D of triplicate measurements (n=6 per group).
6.3.3 Effects of GE on blood glucose, serum insulin levels and insulin resistance index

The systemic inflammation has been recognized as a key link between obesity and insulin resistance (Vroegrijk et al., 2011). Insulin resistance is characterized by increased expression of pro-inflammatory cytokines, macrophage infiltration into white adipose tissue and an impaired response to insulin in the main insulin target tissues. On the molecular level, obesity-induced insulin resistance involves lipid overload, cytokine and ER stress- mediated activation of variety of serine/threonine kinases which phosphorylate the main insulin receptor substrates IRS-1 and IRS-2 on multiple sites, thereby inhibiting proper signal propagation (Scheja et al., 2011).

The fasting blood glucose level was measured before the mice were sacrificed on the 16th week using the ACCU-CHEK® Advantage glucometer with ACCU-CHEK® Advantage test strips and the blood was drawn from the tail vein meanwhile the serum insulin was measured using the kit described in section 6.1.2.4. The index of insulin resistance was estimated by the homeostasis model assessment (HOMA) and was calculated using relationships between the blood glucose and insulin levels according to the following formula (Matthews et al., 1985):

\[
\text{HOMA-IR} = \frac{\text{Insulin (µUI/L)} \times \text{Blood glucose (mmol/L)}}{22.5}
\]

Figure 6.6 (a-c) shows the effect of GE and metformin on blood glucose levels, serum insulin concentration and HOMA-IR value in high-fat diet induced obese mice and mice on normal diet. The fasting blood glucose in descending order was HFD > HFD60 > HFDMEN > HFD > ND > HFD120 > HFD240 > ND240. A modest but a significant hyperglycemia developed in HFD group because severe hyperglycemia and hyperinsulinemia in C57BL/6J mice is only known to develop after 24 weeks of feeding.
with high-fat diet (Nagata et al., 2008). GE administrated mice (HFD120 and HFD240), however showed a significant decrease in the fasting blood glucose levels compared to the mice in HFD group, nevertheless there were no significant differences observed in the normal diet groups (ND and ND240). The serum insulin level was significantly elevated in HFD group and was 1.9 -fold higher than ND group which indicates hyperinsulinemia. However, GE treated groups and metformin treated group showed a significant decrease in serum insulin concentration compared to HFD group.

Homeostatic model assessment values for insulin resistance (HOMA-IR) were calculated using the described formula. The HOMA-IR indices for HFD group showed an increase of 150% compared to ND group. In spite of feeding high-fat diet, the insulin resistance indices of HFD60, HFD120 and HFD240 groups were significantly reduced by 44 %, 62.4 % and 60.8 % respectively whilst in HFDMET group, the insulin resistance indices was significantly reduced by 56 % when compared to HFD group. Amelioration of insulin resistance in HFD240 treated group was comparable to the metformin treated group. This result suggests that GE was able to lower the blood glucose level partially due to the improvement of insulin resistance.

Reactive oxygen species (ROS) production is one of many factors that have been suggested to play a role in the development of insulin resistance. It remains unclear, however, whether increased ROS production causes insulin resistance in-vivo (Ando & Fujita, 2009). However, Nagata et al. (2008) have demonstrated that the up-regulation of genes responsible for ROS production occurs in both the liver and adipose tissue before the onset of insulin resistance and obesity in mice fed on a high fat-diet. Besides that, Roberts & Sindhu, (2009) have also reported systemic oxidative stress is indeed associated with insulin resistance. Accumulating evidence thus points to the concept that oxidative stress may contribute to insulin resistance.
Figure 6.6: Effects of GE on (a) fasting blood glucose concentrations, (b) serum insulin levels and (c) HOMA-IR value of C57BL/6J mice fed on a high-fat diet or normal diet.

GE concentrations were 60, 120, 240 mg/kg/day. Metformin (MET) is used as positive control. Values expressed are means ± S.D of triplicate measurements (n=6 per group). Means with different letters in different bar (a-e) are significantly different (p<0.05).
6.3.4 Effects of GE on the oxidative stress in urine samples (protein carbonyl, lipid hydroperoxides, uric acid and DNA damage levels)

Generally, when the mice’s immune system is triggered by disorders, a massive production of ROS or oxidative burst is activated by macrophages that are coupled with the inflammatory system (Rosen et al., 1995). Macrophages or phagocyte activation cause release of reactive species which leads to damaged biological molecules include oxidized lipids (i.e. lipid hydroperoxides, \( F_2 \)-isoprostanes, oxidized low-density lipoproteins and 4-hydroxynone) (Rio et al., 2005), oxidized proteins (i.e. nitrated tyrosines and protein carbonyls), oxidized carbohydrates (glycated products), oxidized nucleic acid bases (8-oxo-2-deoxyguanosine) and enzymatic markers of oxidative stress, such as myeloperoxidase, an endogenous generator of oxidants (Ohshima & Bartsch, 1994; Roberts & Sindhu, 2009). Finally, these damaged metabolites may cause the development of insulin resistance (Wu et al., 2004).

In this part of study, urine was collected to estimate the levels of oxidative indices. Oxidation products can be found in the urine and is considered to reflect local and systemic oxidative stress (Kirschbaum, 2001). Although short-lived, ROS leave a detectable trace of modified oxidative products, and an array of these damaged end products can be detected using a variety of assays. The urine samples were collected from each mouse in each group on every Monday (11:00 hr) for 15 weeks. The urine samples from each group were pooled together in order to have sufficient samples to measure the protein carbonyl, lipid hydroperoxides, uric acid and DNA damage levels. Figure 6.7 (a-d) shows the protein carbonyl, lipid hydroperoxide, uric acid and DNA damage levels for each group during the 15 weeks of experiment respectively.
Figure 6.7: Effects of GE and metformin on (a) protein carbonyl and (b) lipid hydroperoxides levels in urine samples of C57BL/6J mice fed on a high-fat diet or normal diet.

GE concentrations were 60, 120, 240 mg/kg/day. Metformin (MET) is used as positive control. Values expressed are means ± S.D of triplicate measurements (n=6 per group). Means with different letters in different bar (a-e) are significantly different (p<0.05).
Figure 6.7 (continued): Effects of GE and metformin on (c) uric acid and (d) DNA damage levels (8-OHdG) in the urine samples of C57BL/6J mice fed on a high-fat diet or normal diet.

GE concentrations were 60, 120, 240 mg/kg/day. Metformin (MET) is used as positive control. Values expressed are means ± S.D of triplicate measurements (n=6 per group). Means with different letters in different bar (a-e) are significantly different (p<0.05).
The protein carbonyl, lipid hydroperoxides, uric acid and DNA damage levels in ND group gradually increased every week, however, these oxidative stress indices were significantly elevated in HFD throughout the 15 weeks of experiment compared to ND. The protein carbonyl level in HFD group increased 29.6% by the end of the experiment. Similar patterns were observed in the lipid hydroperoxides and uric acid levels. The percentage of increase in lipid hydroperoxides level was 30.40% and in uric acid was 44.71% by the end of the 15th week in untreated HFD group. This could be due to the development of hyperglycemia and insulin resistance in the HFD group mice (Figure 6.6). It is reported that insulin resistance or diabetes may attribute to the rise of oxidative stress in animals (Galli et al. 2005). Elevated glucose level has been shown to cause increased production ROS, non-enzymatic glycation of proteins, and glucose auto-oxidation which collectively lead to oxidative stress (Cvetkovic et al., 2009).

The protein carbonyl levels in GE treated groups were increased but the percentage of increase was lower compared to the percentage of increase in HFD group. HFDMET also had decreased protein carbonyl level compared to HFD group. Similarly, the lipid hydroperoxides and uric acid levels in GE treated groups (HFD60, HFD120 and HFD240) were significantly reduced during the 15 weeks experiment and this effect was dose-dependent. GE attenuated the progression of hyperglycemia and weight gain (Figure 6.3 and 6.6) and this possibly contributed to the reduced oxidative stress in the mice. Matsuura et al. (1998) have reported that weight reductions have shown to be associated with significant decrease in uric acid in patients and it is mainly attributed to an impaired renal clearance of uric acid. This finding supports the observation of decreased uric acid concentrations in GE treated mice. The DNA damage level was elevated in HFD group, however, there was no significant differences observed between all the groups tested (p>0.05).
In this part of study, lipid hydroperoxides, protein carbonyl, uric acid and 8-hydroxy-2-deoxyguanosine (8-OHdG) were selected as oxidative markers because among the many biological targets of oxidative stress, lipids and proteins are the most involved class of biomolecules with fundamental roles such as biological catalysts, gene regulators and as structural components of cells. Uric acid is also selected as one of the oxidative markers because clinical and epidemiological researches have recently suggested that uric acid is an important predictive marker of the metabolic syndrome while 8-OHdG is one of the major forms of oxidative DNA damage and a useful marker of cellular oxidative stress (Kasai, 1997). Besides that, studies have shown that, elevated levels of malondialdehyde (MDA) (Agarwal & Chase, 2002), protein carbonyl (Leeuwenbergh et al., 1999), uric acid (Nagata et al., 2009) and 8-OHdG (Wu et al., 2004) in animals are associated with several conditions, including hypertension, cardiovascular diseases, renal diseases and diabetes (Yoshino et al., 2009).

6.3.5 Effects of GE on enzymatic and non-enzymatic antioxidant levels in liver, kidney and serum

The antioxidant capacity of dietary components has been linked to the prevention of diabetes mellitus (Koutelidakis et al., 2009; Xie et al., 2009). Dietary components such as fruits, vegetables, spices, herbs and mushrooms have been studied for their antioxidant properties \textit{in-vitro} extensively (Kanagasabapathy, et al., 2011; Palacios et al., 2011). However, the demonstration of the antioxidant properties of these components \textit{in-vivo} has remained elusive but it is gaining importance nowadays. Previously, antioxidant capacity has been mainly approached by measuring the increase of antioxidant capacity in serum or plasma after an oral intake of a food infusion. Nevertheless, numerous studies have also suggested that oxidative processes occurring
in various tissues and organs in the human body may be crucial in the onset of metabolic disease (Boer et al., 2005; Wood et al., 2006). Little research has been conducted with the objective to measure the increase of antioxidant capacity in tissues and organs as an index of antioxidant effect of diet *in-vivo*. It is reasonable to hypothesize that an increase in the antioxidant capacity of organs and tissues may occur as a result of consumption of food rich in antioxidant compounds. It is reported that, after absorption, the antioxidant compounds are transferred through the blood circulation to various organs. For example, it has been recently demonstrated that, catechin ingested through the diet may be detected in the kidney of the rats (Boer et al., 2005). Oxidative stress is known to increase antioxidant enzyme expression, although this response appears to be tissue dependent (Castrillejo et al., 2011). For this reason, we studied the non-enzymic and enzymic antioxidants and lipid peroxidation levels in the liver and kidney because they are the key organs in the mammalian oxidative metabolism as shown in Table 6.2.

Briefly, the natural antioxidant system consists of a series of antioxidant enzymes and numerous endogenous and dietary antioxidant compounds that react with and inactivate reactive oxygen species (ROS). The primary antioxidant enzymes include, but are not limited to, superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidase (GPx). Meanwhile, the non-enzymatic antioxidants include vitamin C, vitamin E, β-carotene, reduced glutathione (GR) and numerous phytochemicals. Cells must maintain their levels of antioxidants, often defined as their antioxidant potential, through dietary intake and/or de novo synthesis (Roberts & Sindhu, 2009; Rains & Jain, 2011).

In this study, HFD group displayed reduced levels of SOD, CAT and GPx in the kidney, liver and serum compared to the ND or GE or metformin treated groups.
Table 6.2: Effects of GE on antioxidant enzyme system, GR content and TBARS levels in the kidney, liver and serum of C5BL/6J (ob/ob) mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Antioxidant activity (nmol/min/mg protein)</th>
<th></th>
<th>TBARS (mmol/L)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GPx</td>
<td>CAT</td>
<td>SOD (U/mg protein)</td>
<td>GR</td>
</tr>
<tr>
<td>ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>71.08±6.3cd</td>
<td>103.93±4.5d</td>
<td>0.034±0.0d</td>
<td>14.13±4.2a</td>
</tr>
<tr>
<td>Liver</td>
<td>83.95±10.8b</td>
<td>29.96±3.9c</td>
<td>0.017±0.0b</td>
<td>103.42±8.1d</td>
</tr>
<tr>
<td>Serum*</td>
<td>162.72±4.0b</td>
<td>78.49±1.4d</td>
<td>0.079±0.00a</td>
<td>12.73±2.4ab</td>
</tr>
<tr>
<td>ND240</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>82.72±6.9d</td>
<td>112.96±4.6e</td>
<td>0.037±0.0d</td>
<td>20.48±4.6e</td>
</tr>
<tr>
<td>Liver</td>
<td>90.42±11.7b</td>
<td>32.57±6.7d</td>
<td>0.032±0.00d</td>
<td>68.59±8.8b</td>
</tr>
<tr>
<td>Serum*</td>
<td>178.57±5.0c</td>
<td>106.23±1.4e</td>
<td>0.0795±0.00a</td>
<td>8.49±1.7a</td>
</tr>
<tr>
<td>HFD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>32.31±3.2a</td>
<td>52.64±1.2a</td>
<td>0.017±0.0a</td>
<td>22.53±2.1d</td>
</tr>
<tr>
<td>Liver</td>
<td>45.22±5.5a</td>
<td>15.18±8.7a</td>
<td>0.01±0.0a</td>
<td>96.19±4.1c</td>
</tr>
<tr>
<td>Serum*</td>
<td>158.76±4.7a</td>
<td>53.51±8.9a</td>
<td>0.0775±0.00a</td>
<td>25.47±1.7b</td>
</tr>
<tr>
<td>HFD60</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>32.46±4.9a</td>
<td>79.72±1.3b</td>
<td>0.023±0.06c</td>
<td>15.1±3.3b</td>
</tr>
<tr>
<td>Liver</td>
<td>68.20±8.3b</td>
<td>22.98±5.7b</td>
<td>0.019±0.00b</td>
<td>62.13±6.1ab</td>
</tr>
<tr>
<td>Serum*</td>
<td>151.97±4.0a</td>
<td>59.22±3.1b</td>
<td>0.0785±0.0a</td>
<td>18.04±1.5ab</td>
</tr>
<tr>
<td>HFD120</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>36.16±5.1ab</td>
<td>86.40±11.6bc</td>
<td>0.027±0.00c</td>
<td>17.23±3.5ab</td>
</tr>
<tr>
<td>Liver</td>
<td>74.86±8.7b</td>
<td>24.91±9.7bc</td>
<td>0.023±0.00c</td>
<td>57.58±6.1a</td>
</tr>
<tr>
<td>Serum*</td>
<td>178.28±4.2bc</td>
<td>79.11±9.9d</td>
<td>0.079±0.00a</td>
<td>21.22±2.0ab</td>
</tr>
<tr>
<td>HFD240</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>44.58±5.3b</td>
<td>84.29±1.5bc</td>
<td>0.022±0.0ab</td>
<td>16.37±3.4ab</td>
</tr>
<tr>
<td>Liver</td>
<td>61.39±8.9b</td>
<td>24.30±1.1bc</td>
<td>0.026±0.00c</td>
<td>52.71±6.7a</td>
</tr>
<tr>
<td>Serum*</td>
<td>176.87±7.2bc</td>
<td>79.35±6.0d</td>
<td>0.0801±0.00a</td>
<td>19.1±1.0ab</td>
</tr>
<tr>
<td>HFD300</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>66.83±5.5c</td>
<td>89.49±2.7c</td>
<td>0.020±0.0ab</td>
<td>32.44±3.6c</td>
</tr>
<tr>
<td>Liver</td>
<td>119.06±9.3c</td>
<td>75.80±8.5bc</td>
<td>0.019±0.00b</td>
<td>132.20±7.0f</td>
</tr>
<tr>
<td>Serum*</td>
<td>177.44±7.9c</td>
<td>73.12±2.5c</td>
<td>0.079±0.00a</td>
<td>10.61±2.7ab</td>
</tr>
</tbody>
</table>

*a unit of measurement is in ng/min/ml.

GE concentrations were 60, 120, 240 mg/kg/day. Metformin (MET) is used as positive control. Values expressed are means ± S.D of triplicate measurements. Means with different letters in different bar (a-e) are significantly different between treatment groups (p<0.05). GPx is glutathione peroxidase; GR is reduced glutathione; CAT is catalase; SOD is superoxide dismutase and TBARS is lipid peroxidation.
This corresponds to the high levels of oxidative stress markers (protein carbonyl, lipid hydroperoxides and uric acid levels) detected in the urine of the mice of HFD group (see chapter 6.3.4). However, the serum SOD activity in GE treated groups, did not exhibit any significant difference with HFD group. SOD activity has been reported to be higher in the plasma than serum thus, this could contribute to the low SOD activity that was observed in this study (Coudray et al., 2009). Similar pattern was observed in HFD-MET, where the GPx, SOD and CAT levels were significantly higher than in HFD group. The increased antioxidant activity in GE and metformin treated groups may protect the body against oxidative damage (protein carbonyl, lipid hydroperoxides and uric acid levels).

GR is a major non-enzymatic antioxidant molecule that is involved in the second line of defence against free radical damage in the body (Hong & Lee, 2009). HFD group showed the highest GR activity whilst GE treated groups showed significantly lower GR activity in liver, kidney and serum. Furukawa et al. (2004) determined the activities of the anti-oxidative proteins in the white adipose, liver, and muscle tissues from the mouse model of metabolic syndrome; and they showed that the levels of these enzymes differed depending on which tissue they were measured in. Besides that, this is also probably because of the uniqueness of the kidney with respect to GR homeostasis. Renal proximal tubular cells can take up intact GR from the extracellular space to maintain the levels of GR necessary for renal function. The low GR levels found in the liver and serum may be counteracted and controlled in the kidney because of this peculiarity (Castrillejo et al., 2011).

TBARS assay was carried out to measure the lipid damage level in liver, kidney and serum. HFD group showed the highest TBARS concentration whilst GE treated groups had significantly lower TBARS concentrations in the liver, kidney and serum.
The decreased lipid damage levels observed in the GE treated groups may correspond with the high enzymic antioxidant activity. In chapter 4, GE was shown to inhibit in vitro lipid peroxidation and this study confirms that GE was also able to inhibit in vivo lipid peroxidation.

The primary lines of defense preventing biological macromolecules from ROS attack are the antioxidant enzymes. SODs are a ubiquitous family of enzymes that catalyze the conversion of superoxide anion into H$_2$O$_2$. In the subsequent step of the detoxifying cascade, H$_2$O$_2$ previously produced is converted to water and molecular oxygen by CAT or GPx, which uses GR as the hydrogen donor. Increased levels of ROS in cells and tissues may act as a signal to enhance the activity and expression of antioxidant enzymes (Roberts & Sindhu, 2009). In this part of study, GE treated groups were able to significantly increase the antioxidant enzymes and reduce the membrane lipid damage. The increase in SOD activity observed in GE treated groups maybe a consequence of high intracellular superoxide anion (O$_2$•-) levels produced due to the high-fat diet consumption. SOD then converts the O$_2$•- into hydrogen peroxide. The GPx and CAT enzymes eliminate the excess H$_2$O$_2$ and other hydroperoxide products of secondary ROS reactions hence, the GPx and CAT activity were also elevated in this study. However, in spite of the increase in GPx and CAT activity, there was a decrease in GR activity thus further investigations is required to study the effects of GE in activating GR proteins. Similar results were observed in HFDMET group, suggesting that GE can exert the same or better activity than metformin in preventing oxidative stress.

Sanchez et al. (2011) have also reported that, feeding Zucker fatty acids rats with β-glucan reduced the MDA levels in the plasma and significantly increased the antioxidant activity. In fact, it was observed that a diet enriched in whole-grain and
refined wheat flours produced an increase of liver glutathione in rats, and consequently improved the redox status in these animals (Fardet et al., 2007). Moreover, water-soluble corn bran hemicellulose suppressed the development of liver injury in rats (Daizo et al., 2005), and it was suggested that the observed finding was partly due to the increase in antioxidant concentration in this tissue. Thus, this study could also suggest that the soluble polysaccharide (GE) that was used may improve the redox state and attenuate the oxidative injury.

6.3.6 Effects of GE on the expressions of adipokines and inflammatory markers in adipose tissue.

Adipose tissue is actively involved in sensing the nutritional and metabolic status of the organism through several signalling pathways and regulates energy metabolism by secreting molecules in response to these cues (Kim et al., 2004; Zou & Shao, 2008). Thus, adipose tissue is a complex and active secretory organ that both sends and receives signals that modulate energy expenditure, appetite, insulin sensitivity, endocrine function, inflammation and immunity (Shoelson et al., 2007).

In this study, consumption of high–fat diet leads to obesity and the mice fed a high-fat diet weighed more than mice on normal diet, they developed substantially more adipose tissue than the normal diet mice and developed hyperlipidemia typically associated with obesity (Harwood, 2012). Besides that, a high-fat intake and increased circulation of free fatty acids led to insulin resistance, and ultimately to diabetes mellitus in this genetically prone mice. This observation was also similar with the findings reported by Park et al. (2005).

Thus, in this part of the study, attempt to increase the understanding of how GE possibly regulates the pathways responsible for preventing obesity, hyperglycemia,
insulin resistance and also modulating the associated low-grade inflammation was evaluated. Table 6.3 shows the expression of adipocytes derived markers (adipokines) meanwhile Table 6.4 shows the expression of markers associated with anti-inflammatory signalling pathway.

Table 6.3: Effects of GE on the expression of molecular markers in adipose tissue

<table>
<thead>
<tr>
<th>Genes investigated</th>
<th>ND240</th>
<th>HFD60</th>
<th>HFD120</th>
<th>HFD240</th>
<th>HFDMET</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPAR-γ</td>
<td>-1.69±0.19</td>
<td>-2.08±0.69ab</td>
<td>-1.69±0.48ab</td>
<td>-1.02±0.36b</td>
<td>-1.07±0.16b</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>-1.27±0.65</td>
<td>-1.01±0.16b</td>
<td>-3.10±0.44a</td>
<td>-2.25±0.30ab</td>
<td>-2.30±1.13ab</td>
</tr>
<tr>
<td>LPL</td>
<td>-1.05±0.09</td>
<td>-1.93±0.18ab</td>
<td>-1.12±0.42b</td>
<td>-1.17±0.47b</td>
<td>-2.22±0.99a</td>
</tr>
<tr>
<td>HSL</td>
<td>1.98±0.07</td>
<td>2.99±0.17a</td>
<td>6.73±0.42c</td>
<td>6.54±0.32c</td>
<td>4.63±1.16b</td>
</tr>
<tr>
<td>ATGL</td>
<td>1.34±0.34</td>
<td>1.78±0.67a</td>
<td>6.05±0.42c</td>
<td>5.69±0.34c</td>
<td>3.84±0.98b</td>
</tr>
<tr>
<td>GLUT-4</td>
<td>1.49±0.24</td>
<td>1.78±0.32b</td>
<td>2.41±0.42c</td>
<td>2.05±0.32c</td>
<td>1.13±0.13a</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>1.56±0.15</td>
<td>-3.81±0.37a</td>
<td>1.13±0.62c</td>
<td>1.66±0.30c</td>
<td>-1.68±0.24b</td>
</tr>
<tr>
<td>RBP-4</td>
<td>-1.10±0.13</td>
<td>-1.20±0.16a</td>
<td>-1.30±0.44a</td>
<td>-1.63±0.33a</td>
<td>-1.50±0.17a</td>
</tr>
</tbody>
</table>

Total RNA was extracted from the adipose tissue from each group and cDNA were synthesized. RT-PCR was performed and the relative expression of the genes was calculated using the ΔΔ C_T method. They were normalized with 18S eukaryotic rRNA. Results are expressed as –fold variation over carrier control groups (ND with ND240 and HFD with HFD60, HFD120, HFD240 and HFDMET). 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-c) were significantly different (p<0.05).
The molecular markers that were studied were PPAR-γ, SREBP-1c, HSL, ATGL, adiponectin, LPL, GLUT-4 and RBP-4 (Table 6.1). These markers play an important role in modulating the lipid and glucose metabolism in adipose tissue. PPAR-γ and SREBP-1c genes are the key adipose transcription factors that play important roles in adipogenesis and insulin sensitivity (Kim & Park, 2008). These genes act cooperatively and sequentially to trigger terminal adipocyte differentiation. The PPAR-γ is expressed selectively in the adipose tissues and it promotes the differentiation and proliferation of the adipocytes from the fibroblasts, thereby causing an increase in fat mass (Brun et al., 1997) whilst SREBP-1c controls the production of endogenous ligands for PPAR-γ as a mechanism for coordinating the actions of these adipogenic factors (Kim & Park, 2008) and has been implicated as being a key regulator for fatty acid and triglyceride synthesis (Hasani & Joost, 2005). GE treated groups had lower expression of PPAR-γ and SREBP-1c compared to HFD group and the down-regulation of these genes may have contributed to lower weight gain observed in the GE treated groups. Fatty acid binding protein (FABP) 4, fatty acid synthase (FAS) and LPL are some of the significant proteins involved in lipid metabolism. LPL is the key enzyme that regulates the disposal of lipid in the body and its role is to hydrolyse triglyceride circulating in the lipoprotein particles in order to uptake fatty acids into the cells (Fielding & Frayn, 1998). The expression of LPL was significantly down-regulated in GE treated groups compared to HFD group because PPAR-γ protein binds to the promoter regions of adipocyte-expressed LPL gene (Lee et al., 2010). Since GE induced reduction of PPAR-γ gene, thus this could attribute to the reduced expression of LPL.

Furthermore, down – regulation of these genes also may indicate that, GE did not bind to the insulin receptor substrate (IRS) that in turn could activate the
phosphodiesterase 3B (PDE3B) pathway which is the main signalling pathway for insulin (Cho et al., 2011).

HSL and ATGL genes are reported to play an important role in the mobilization of stored TAG (Jocken et al., 2007). The detailed mechanism for HSL and ATGL has been described in Chapter Five. The GE treated groups, significantly up-regulated the expression of HSL and ATGL genes and this effect was dose-dependent. This indicates that, in GE treated groups, the differentiation process was decreased but the lipolysis process was stimulated in adipose tissue. This also corresponds to the lipolytic effect observed in GE treated 3T3-L1 adipocytes (Chapter 5).

Adiponectin has attracted considerable interest in the area of obesity and diabetes over the last few years (Bonnard et al., 2008). After 15 weeks of feeding high-fat diet, HFD group mice had reduced glucose tolerance indicating hyperglycaemia and also altered in-vivo insulin responsiveness and this could be due to the reduced expression of adiponectin meanwhile, GE treated groups (HFD120 and HFD240) had increased adiponectin expression and this may explain the better glucose tolerance observed in these groups. Adiponectin is considered to be an anti-diabetic and anti-atherogenic hormone, but conflicting results on its levels in obese patients and animal models have been shown by previous studies (Kern et al., 2001). Some of them demonstrated declined circulating levels of adiponectin in high-fat diet fed rats (Lee et al., 2009) and mice (Peng et al., 2009). In contrast, some studies did not find changes in adiponectin in rats (Yang et al., 2006) and different strains of mice fed a high-fat diet (Barnea et al., 2006; Townsend et al., 2008). A possible explanation for such difference is that the expression of adiponectin, could be altered by multiple factors including both genetic and environmental factors, and could be modified by even resting and fasting state (Zhang et al., 2010). Meanwhile, GLUT-4 gene, the insulin-responsive glucose
transporter, plays a major role for glucose transport and maintenance of insulin sensitivity *in-vivo* in adipose tissue and the insulin resistance (IR) is a result of impaired signalling pathway from insulin receptor to GLUT-4 vesicle in adipose tissues (Miura et al., 2003). In this study, the mice in GE treated groups, showed better glucose tolerance than the mice on HFD group, and the improvement in glucose tolerance maybe reflected in an increase of the GLUT-4 expression in these groups. Expression of adiponectin along with GLUT-4 genes could lead to sensitization of insulin and improvement of hyperglycemia/glucose tolerance in the GE treated groups.

In contrary, RBP-4 now emerges as a new adipokine, linking glucose uptake with insulin sensitivity. RBP-4 is reported as a factor that is derived from adipose tissue that can cause insulin resistance (Graham et al., 2006). Eventough the activation mechanism of RBP-4 is still unclear but reports have shown that increased expression of RBP-4 was found in adipose tissue of mice with adipocyte-specific ablation of GLUT-4 (Wang et al., 2007) and increased plasma RBP-4 levels were observed in obese children were correlated not only with indices of obesity and IR but also with inflammatory factors (Balagopal et al., 2007).This correlates with the findings in this study that, GE treated groups, decreased the expression of RPB-4 compared to HFD group. In HFDMET group, the regulation of these molecular markers was similar to the GE treated groups. The regulation of these genes in HFDMET group also supports the improvement in glucose tolerance and insulin resistance.

Phosphorylated AMPK plays a major role as a metabolic master. Based on the expression of the relevant genes studied, it can be proposed that the action of GE points towards dual action of β-adrenergic receptors, which then activates the AMPK signalling pathway. Thus further investigations are required to validate the possible involvement of GE in AMPK signals.
Table 6.4: Effects of GE on the expression of inflammatory markers in adipose tissue.

<table>
<thead>
<tr>
<th>Genes investigated</th>
<th>ND240</th>
<th>HFD60</th>
<th>HFD120</th>
<th>HFD240</th>
<th>HFDMET</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>-1.17±0.67</td>
<td>-1.18±0.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-1.11±0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-3.27±0.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-1.35±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CRP</td>
<td>-2.10±0.60</td>
<td>-3.10±0.78&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>-4.00±0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-3.40±0.87&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>-2.30±0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCP-1</td>
<td>-1.63±0.45</td>
<td>1.09±0.14&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>-2.33±0.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-1.33±0.69&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>-1.20±1.10&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>SAA-2</td>
<td>-1.55±0.66</td>
<td>-5.53±0.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-5.39±0.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-5.67±0.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-3.82±0.77&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NF-κB</td>
<td>-1.11±0.37</td>
<td>-2.30±0.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-2.90±0.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-2.30±0.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-2.45±1.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>TNF-α</td>
<td>-3.46±0.09</td>
<td>1.16±0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-4.50±0.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-4.11±0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-1.79±1.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
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</table>

Total RNA was extracted from the adipose tissue from each group and cDNA were synthesized. RT-PCR was performed and the relative expression of the genes was calculated using the ΔΔC<sub>T</sub> method. They were normalized with 18S eukaryotic rRNA. Results are expressed as –fold variation over carrier control groups (ND with ND240 and HFD with HFD60, HFD120, HFD240 and HFDMET). 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 ΔC<sub>T</sub> values by DMRT. For same gene with different treatment groups, means in the different bar with different letters (a-c) were significantly different (p<0.05).
The regulations of inflammatory markers (IL-6, CRP, MCP-1, SAA-2, NF-κB and TNF-α) were also evaluated in the adipose tissue. The links between obesity and inflammation have existed for decades (Ferrante, 2007) and more recent epidemiological evidence have confirmed these findings by showing increase of additional acute phase reactants in obese subjects, including TNF-α, IL-6 and CRP (Dandona et al., 2004). Glucose and fat intake have both been shown to induce inflammation, potentially through increase of oxidative stress (Mohanty et al., 2002; Mohanty et al., 2004). In this part of the study, mice fed with high-fat diet have been shown to have increased oxidative stress status in the urine, liver, kidney and serum where the TBARS levels were elevated whilst the antioxidant enzyme levels were reduced.

Briefly, NF-κB controls the regulation of genes encoding proteins involved in immune and inflammatory responses (i.e., cytokines, chemokines, growth factors immune receptors, cellular ligands, and adhesion molecules) (Kuhad & Chopra, 2009). Obesity is associated with a gradual expansion of the adipose cells and leads to a reduction in the production of anti-inflammatory adipokines (like adiponectin) while pro-inflammatory cytokines (like IL-6, CRP and SAA-2) are markedly increased. This, in turn, leads to the infiltration of monocytes and macrophages into the adipose tissue facilitated by MCP-1 (Smith et al., 2007; Ferrante, 2007; Terra et al., 2011). The inflamed state leads to the release of cytokines into adipose tissues that are not normally secreted by the adipose cells. A key cytokine that is released by macrophages, but not in differentiated adipose cells, is TNF-α which is the key regulator of inflammation and was strongly correlated to the level of insulin resistance in obese animal models (Kern et al., 2003). However, Li et al (2002), have reported that TNF-α is also released by adipose tissue. Studies have demonstrated that, TNF-α along with IL-6 not only inhibits
adiponectin (anti-inflammatory adipokines) expression and impair insulin sensitivity of other tissues (Fasshauer et al., 2002) but also interferes with adipocyte metabolism at numerous sites including transcriptional regulation, glucose and fatty acid metabolism (Kim et al., 2004). The pro-inflammatory cytokine IL-6 was among the first to be implicated as a predictor of insulin resistance. Finally, these pro-inflammatory cytokines (IL-6 and TNF-α) mediate distant inflammatory effects, including activation of CRP and SAA-2 (Koh et al., 2005). The SAA proteins are derived from distinct genes; human express SAA-1, SAA-2, SAA-3 and SAA-4. In this study, only SAA-2 protein was studied because SAA-2 expression increases dramatically during acute inflammatory responses and recent studies have suggested adipocytes may be a major contributor of SAA-2 (Shoelson et al., 2007). Meanwhile, CRP is an acute phase reactant. Circulating concentrations of SAA-2 and CRP are also increased in individuals with impaired glucose tolerance (Muller et al., 2002) and are considered to be a marker for insulin resistance (Pannacculli et al., 2001).

HFD group had higher expression of the pro-inflammatory markers compared to the GE treated groups and metformin treated group. It has been reported that, ROS and endoplasmic reticulum (ER) stress are increased by adiposity and consumption of high-fat diet that may activate NF-κB signalling cascade in the adipose tissue (Shoelson et al., 2007; Cho et al., 2011). When NF-κB signaling cascade is activated, increased MCP-1 results in a dramatic elevation of macrophage infiltration, promoting the downstream secretion of pro-inflammatory markers namely, TNF-α and IL-6 and finally this increases the expression of CRP and SAA-2 in HFD group. The expression of these pro-inflammatory markers in HFD, are known to initiate and amplify insulin resistance in the adipose tissue as observed in this study (Tsigos et al., 1997). Figure 6.8 shows the
overview of inflammatory markers of adipose tissue and altered secretion of adipokines in HFD group.

However, the NF-κB activity in GE treated groups, was significantly down-regulated compared to HFD group because there was no size expansion in the adipose tissue and this in turn did not increase the expression of MCP-1, where macrophages were not released into the cells (Hajer et al., 2008) and thus, the expression for TNF-α and IL-6 also decreased in GE treated groups compared to HFD group. Similarly, the expressions in SAA-2 and CRP were also down-regulated in GE treated groups compared to HFD group. Besides that, the local enhancement in adiponectin expression produced by GE might also be partly responsible for the reduced inflammatory-cytokine expression levels in these groups (Kern et al., 2001). Furthermore, significant reduced weight gain observed in mice of HFD60, HFD120 and HFD240 groups had decreased expression of TNF-α and this could partly reverse the insulin resistance (Bruun et al., 2002). Some authors have recently reported similar results in adipose tissue treated with various natural occurring substances such as grape seed procyanidins (Terra et al., 2011), lotus (Liao & Lin, 2011), carvacrol (Cho et al., 2011) and tocotrienol (Kuhad & Chopra, 2009).

The pro-inflammatory markers were significantly decreased in HFDMET group compared to HFD group. The NF-κB signalling cascade which includes the expression of MCP-1, TNF-α, IL-6, CRP and SAA-2 were down-regulated in the adipose tissue and the expression was similar to GE treated groups. It has been reported that, treating patients with metformin, decreased the CRP concentration in the serum (Akbar et al., 2003). GE may be a potential candidate for preventing weight gain, glucose lowering effect, insulin sensitization and attenuating anti-inflammatory response in adipose tissue.
Inflammation and altered secretion of inflammatory markers in HFD group.

Inflammation in adipose tissue is probably initiated by increased size of the adipocytes. NF-κB is able to regulate the secretion of other pro-inflammatory by stimulating adipocytes to produce MCP-1 and then the recruitment of macrophages into adipose tissue. Subsequently, TNF-α creates a hierarchy of cytokines within adipose tissue and changed the excretion profile of adipose tissue (Bakker et al., 2009). GE treated groups did not alter the size of adipocytes thus the expression of NF-κB and the down-stream markers are inhibited but not the expression of adiponectin.
6.4 Conclusion

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

1) GE significantly reduced the weight gain in high-fat diet induced obese C57BL/6J (ob/ob) mice. The mice in GE treated groups (HFD60, HFD120 and HFD240) remained healthy and lean while the mice in HFD control group were obese. GE was able to protect against hyperlipidemia in mice.

2) GE treated groups were able to reduce the blood glucose level significantly compared to HFD group. GE was able to improve the glucose tolerance where the blood glucose level normalises to the fasting blood glucose level.

3) GE treated groups were able to protect against hyperglycemia and hyperinsulinemia in mice by up-regulating the expression of adiponectin and GLUT-4 genes. These genes plays important role in glucose homeostasis and insulin sensitization hence, GE treated groups were not insulin-resistance.

4) GE treated groups decreased the concentrations of oxidative damaged products (protein carbonyl content, lipid hydroperoxides and uric acid) compared to HFD group thus GE may protect the mice against oxidative stress.

5) GE treated groups significantly increased the antioxidant activities (SOD, CAT and GPx) in the kidney, liver and serum compared to HFD group.

6) GE down-regulated the expression of PPAR-γ, SREBP-1c and LPL while up-regulating the expression of HSL and ATGL. This means, GE decreased lipogenesis by inhibiting the key adipose transcription factors and stimulated lipolysis via HSL and ATGL expressions.

7) The inflammatory associated genes (TNF-α, IL-6, MCP-1 and SAA-2) were down-regulated in the GE treated groups. The inhibition NF-κB cascade also
leads to lower glucose levels and attenuation of insulin resistance in GE treated groups.

In summary, the different dosages of GE (HFD60, HFD120 and HFD240) have potential in mitigating anti-obesity and obesity related disorders mainly hyperlipidemia hyperglycemia and hyperinsulinemia in addition to the anti-inflammatory activity in C57BL/6J mice fed a high-fat diet. The properties displayed by GE were comparable to HFDMET group thus, making GE a safer and cost-effective anti-obesity and anti-diabetic therapeutic agent.