EFFECT OF HERBAL PRODUCT ON THE BODY WEIGHT REDUCTION IN HIGH FAT DIET-INDUCED OBESE SPRAGUE DAWLEY RATS

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ABSTRACT

The aim of this study was to determine the effect of herbal product (HP) on the body weight reduction and lipid profile improvement in high fat diet (HFD)-induced obese Sprague Dawley (SD) rats. The ethyl acetate fraction (EAF) showed the highest total phenolic content (TPC) and total flavonoid content (TFC) at 30.48 ± 0.62 mg GAE/g and 156.52 ± 3.13 mg QE/g, respectively. It also showed the highest antioxidant capabilities to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) at IC_{50} = 0.03 ± 0.00 mg/mL, nitric oxide (NO) at IC_{50} = 1.00 ± 0.05 mg/mL and superoxide at IC_{50} = 0.16 ± 0.01 mg/mL and reduce ferric ions at 109.27 ± 3.35 μmol Fe^{2+}/g. However, the EAF showed low capability in chelating metal (< 50%). The oral acute toxicity test of the HP extract at a single dose of 5 g/kg body weight of rat showed no mortality and no toxic effect on SD rats. The body weight of HFD rats after two weeks treatment with water extract (WE), simvastatin and EAF was significantly reduced by 4.79%, 6.18% and 6.64%, respectively ($p < 0.05$). The body mass index (BMI) of the HFD rats after two weeks treatment with WE, simvastatin and EAF was also significantly reduced ($p < 0.05$). The treatment of HFD rats with EAF also led to significant reductions on triglyceride (TG), total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C), with a positive elevation of high density lipoprotein cholesterol (HDL-C) compared to WE and simvastatin. In conclusion, the results showed that the EAF had better potential for body weight reduction and lipid profile improvement in HFD rats than WE and simvastatin.

Keywords: high fat diet; total phenolic and flavonoid contents; antioxidant capabilities; body weight reduction; lipid profile improvement

KESAN PRODUK HERBA KE ATAS PENGURANGAN BERAT BADAN TIKUS SPRAGUE DAWLEY YANG DIBERI DIET TINGGI LEMAK

ABSTRAK

Tujuan kajian ini adalah untuk menentukan kesan produk herba (HP) ke atas pengurangan berat badan dan penambahbaikan profil lipid di dalam tikus-tikus Sprague Dawley (SD) yang obes disebabkan diet tinggi lemak (HFD). Fraksi etil asetat (EAF) memperlihatkan kandungan yang paling tinggi bagi jumlah fenol (TPC) dan jumlah flavonoid (TFC) dengan 30.48 ± 0.62 mg GAE/g dan 156.52 ± 3.13 mg QE/g, masing-masing. Ia juga memperlihatkan keupayaan yang paling tinggi untuk skaveng 2,2-difenil-1-pikril hidrazil (DPPH) dengan IC_{50}= 0.03 ± 0.00 mg/mL, nitrik oksida (NO) dengan IC_{50}= 1.00 ± 0.05 mg/mL dan superoksida dengan IC_{50}= 0.16 ± 0.01 mg/mL serta mengurangkan ion-ion ferik pada 109.27 ± 3.35 μmol Fe^{2+}/g. Walau bagaimanapun, ia memperlihatkan keupayaan yang rendah dalam pengkelatan logam (< 50%). Ujian ketoksikan akut oral HP dengan satu dos 5 g/kg berat badan tikus menunjukkan tiada kematian dan kesan toksik ke atas tikus-tikus SD. Berat badan tikus-tikus HFD selepas dua minggu rawatan dengan ekstrak air (WE), simvastatin dan EAF nyata sekali berkurang sebanyak 4.79%, 6.18% dan 6.64% masing-masing ($p < 0.05$). Indeks jisim tubuh (BMI) tikus-tikus HFD selepas rawatan dengan WE, simvastatin dan EAF juga nyata sekali berkurang ($p < 0.05$). Rawatan tikus-tikus HFD dengan EAF juga membawa kepada pengurangan berkesan pada trigliserida (TG), jumlah kolesterol (TC) dan lipoprotein ketumpatan rendah (LDL-C), dengan kenaikan lipoprotein ketumpatan tinggi (HDL-C) yang positif dibandingkan dengan WE dan simvastatin. Sebagai kesimpulan, keputusan-keputusan ini menunjukkan bahawa EAF mempunyai potensi yang lebih baik bagi pengurangan berat badan dan penambahbaikan profil lipid di dalam tikus-tikus SD yang obes disebabkan diet tinggi lemak (HFD) berbanding WE dan simvastatin.

Kata kunci: diet tinggi lemak; jumlah kandungan fenol dan flavonoid; keupayaan antioksida; pengurangan berat badan; penambahbaikan profil lipid

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LIST OF SYMBOLS AND ABBREVIATIONS

ºC	:	Degree Celsius
<	:	Less than
µ	:	Micro
≥	:	More than and equal to
%	:	Percent
±	:	Plus minus
ABA	:	Abscisic acid
AGEs	:	Advanced glycosylation end products
AgRP	:	Agouti-related protein
AMPK	:	Adenosine monophosphate-activated protein kinase
ATP	:	Adenosine triphosphate
BAT	:	Brown adipose tissue
BF	:	Butanol fraction
BMI	:	Body mass index
b.w.	:	Body weight
cAMP	:	Cyclic AMP
CAT	:	Catalase
C/EBPβ	:	CCAAT/enhancer binding protein beta
CF	:	Chloroform fraction
cm^2	:	Centimeter square
COMT	:	Catechol-o-methyl-transferase
CRP	:	C-reactive protein
CVD	:	Cardiovascular diseases
DCMF	:	Dichloromethane fraction

DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
EAF	:	Ethyl acetate fraction
ECG	:	Epicatechin gallate
EDTA-Na_2	:	Ethylenediaminetetraacetic acid disodium salt dihydrate
EGC	:	Epigallocatechin
EGCG	:	Epigallocatechin gallate
ESCP	:	Endocrine Society Clinical Practice
FDA	:	Food and Drug Administration
$FeCl_2$	:	Ferrous chloride
$FeCl_3$	:	Ferric chloride
FRAP	:	Ferric reducing antioxidant power
g	:	Gram
GPx	:	Glutathione peroxidase
HDL-C	:	High density lipoprotein cholesterol
HF	:	Hexane fraction
HFC	:	High fat diet control
HFD	:	High fat diet
HP	:	Herbal product
IC_{50}	:	Half maximal inhibitory concentration
IL-6	:	Interleukin-6
iNOS	:	Inducible nitric oxide synthase
kcal	:	Kilocalories
kg	:	Kilogram
L	:	Liter

LC/Q-TOF/MS	:	Liquid Chromatography/Quadrupole-Time-of-Flight/Mass Spectrometry
LD_{50}	:	Lethal dose
LDL-C	:	Low density lipoprotein cholesterol
LPL	:	Lipoprotein lipase
m^2	:	Meter square
MAF	:	Methanol aqueous fraction
MCP	:	Monocyte chemoattractant protein
MDA	:	Malondialdehyde
ME	:	Methanol extract
mg	:	Milligram
mL	:	Milliliter
MSH	:	Melanocyte-stimulating hormones
NADH	:	Nicotinamide adenine dinucleotide
NADPH	:	Nicotinamide adenine dinucleotide phosphate
NBT	:	Nitroblue tetrazolium
NC	:	Normal diet control
ND	:	Normal diet
NEFAs	:	Non-esterified fatty acids
nm	:	Nanometer
NO	:	Nitric oxide
NPY	:	Neuropeptide Y
PBS	:	Phosphate-buffered saline
PL	:	Pancreatic lipase
PMS	:	Phenazine methosulphate
p.o.	:	Perorally

POMC	:	Pro-opiomelanocortin
PPARγ	:	Peroxisome proliferator-activated receptor γ
ppm	:	parts-per-million, 10^{-6}
ROS	:	Reactive oxygen species
rpm	:	Revolutions per minute
SD	:	Sprague Dawley
SOD	:	Superoxide dismutase
TBARS	:	Thiobarbituric acid reactive substances
TC	:	Total cholesterol
TFC	:	Total flavonoid content
TG	:	Triglyceride
TLC	:	Thin Layer Chromatography
TNF-α	:	Tumor necrosis factor-α
TPC	:	Total phenolic content
TPTZ	:	2,4,6-Tris(2-pyridyl)-s-triazine
UCP	:	Uncoupling protein
UCP1	:	Uncoupling protein 1
VLDL	:	Very low density lipoprotein
WE	:	Water extract
w/v	:	Weight per volume

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background

Obesity incidence is increasing at an alarming rate and it becomes one of the serious health issues around the world. It has risen to the top of world health concern disease because it is the major contributors to the global burden of chronic diseases and other associated complications such as cancers, cardiovascular diseases (mainly heart disease and stroke), dyslipidemia, hypertension, osteoarthritis and type 2 diabetes (Derdemezis *et al.*, 2011; Singla *et al.*, 2010; Sun *et al.*, 2016). It also increases the rate of mortality and morbidity (Leonhardt *et al.*, 1999), increases the expenses for medication and impairs the quality of life (Haslam & James, 2005).

People are considered overweight when their BMI is from 25 kg/m^2 to 29.99 kg/m^2, while people are considered obese when their BMI are equal or exceed 30 kg/m^2 (World Health Organization (WHO), 2016a). According to WHO (2016b), more than 1.9 billion adults (18 years old and older) were overweight in the world and over 600 million people were obese among these adults in the year 2014. The worldwide prevalence of obesity is occurring not only in the adult population, but it is also occurring among adolescents and children. There were 41 million children under the age of 5 in the world who were overweight or obese in 2014 (WHO, 2016b).

Based on the Economist Intelligence Unit's "Tackling Obesity in Asean" report, which covered Indonesia, Malaysia, Philippines, Singapore, Thailand and Vietnam, it depicted that the prevalence of obesity in Malaysia was the highest in Southeast Asia (Rashid, 2017). The report also showed that the prevalence of obesity in Malaysia was 13.3%, while the prevalence of overweight in Malaysia was 38.5% (Rashid, 2017).

The increase prevalence of obesity around the world and in Malaysia raises the concern and awareness of government and citizens. Hence, strategies for obesity treatment such as dietary control, regular exercise, lifestyle changes and prescription of weight loss medication should be carried out. According to the "Pharmacological Management of Obesity: An Endocrine Society Clinical Practice (ESCP) Guideline", the most ideal treatment for obesity is to maintain a balanced diet and healthy lifestyle with regular exercise (Sun *et al.*, 2016). However, it is a challenge to maintain long term success with lifestyle modification and it is dependent on the compliance of obese patients (Sun *et al.*, 2016). Therefore, anti-obesity drug can be used to assist them during obesity treatment.

In the USA, there are several Food and Drug Administration (FDA)-approved weight loss synthetic drugs such as orlistat, Belviq, Contrave and Qsymia (Sun *et al.*, 2016). However, the synthetic drugs cause side effects such as liver injury, heart attack and stroke (Sun *et al.*, 2016). Due to the side effects of synthetic drugs, natural plant product made from herbs is more preferable to be used for obesity treatment since herbs are more economical, less or non-toxic, less prone to side effects and easily available (Chandrasekaran *et al.*, 2012; Park *et al.*, 2011).

Nowadays, combination of different herbs is used for obesity treatment because it provides a synergistic effect which increases their bioavailability and action on the multiple molecular targets (Mohamed *et al.*, 2014; Rayalam *et al.*, 2008). Besides, herbs contain large amount of phenolic compounds and flavonoids, which possess anti-obesity, hypolipidemic and antioxidant properties. Thus, a mixture of herbs is highly recommended to be used for obesity treatment.

The herbal product (HP) that used in this study is a mixture of citrus, green tea, Guarana, grape seed, lotus leaves, psyllium husk and *Spirulina*. All these herbs itself

showed good effects on body weight reduction and lipid profile improvement. They have been used alone as traditional remedies, but the mixture of these herbs has not been subjected into a comprehensive investigation in animal models. Therefore, the aim of this study was to determine the effect of HP on body weight reduction and lipid profile improvement in high fat diet (HFD)-induced obese Sprague Dawley (SD) rats.

1.2 Objectives

1. To characterize the compounds in the herbal extracts using Thin Layer Chromatography (TLC).
2. To identify the compounds present in the herbal extracts using Liquid Chromatography/Quadrupole-Time-of-Flight/Mass Spectrometry (LC/Q-TOF/MS).
3. To determine the total phenolic content (TPC) and total flavonoid content (TFC) of the herbal extracts using Folin-Ciocalteu method and aluminum chloride colorimetric method.
4. To evaluate antioxidant activities of the herbal extracts using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, ferric reducing antioxidant power (FRAP) assay, metal chelating assay, nitric oxide (NO) radical scavenging assay and superoxide radical scavenging assay.
5. To determine the effect of water extract (WE) and ethyl acetate fraction (EAF) of HP on the body weight reduction in high fat diet (HFD)-induced obese rats.
6. To determine the serum lipid profile levels of triglyceride (TG), total cholesterol (TC), low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) of the HFD-induced obese rats after treatment.

CHAPTER 2: LITERATURE REVIEW

2.1 Definition of obesity

Obesity is a word derived from the Latin *obesitas* which means fat or plump (Mohamed *et al.*, 2014). It is one of the metabolic diseases in which a person has excessive fat accumulation and it causes adverse effects on health, increases morbidity and mortality (Leonhardt *et al.*, 1999), increases expenses for medication and reduces the quality of life (Haslam & James, 2005).

Obesity is also a low-grade inflammatory chronic disease (Kadir *et al.*, 2015). It is featured by overproduction of pro-inflammatory adipocytokines and proteins such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS) and C-reactive protein (CRP), that can lead to oxidative stress (Charrière *et al.*, 2003). It is also featured by macrophage accumulation in white adipose tissue. Both of these inflammatory conditions are caused by the massive adipocytes production and enlargement of adipocytes (Jo *et al.*, 2009).

Besides, obesity is a chronic state of oxidative stress (Vincent & Taylor, 2006). Based on the research conducted by Furukawa *et al.* (2004), results showed that fat accumulation activated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase pathway and impaired antioxidant defense system, ultimately led to the generation of reactive oxygen species (ROS). The results also showed that high amount of ROS decreased mRNA expressions and activities of antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) (Furukawa *et al.,* 2004).

2.2 Obesity verification

2.2.1 BMI

Body weight itself cannot be used as an indicator of obesity since weight gain might be due to fat or muscle mass (Mohamed *et al.*, 2014). BMI is the most practical indicator of obesity and it is frequently used as a measurement of obesity to classify underweight, obese and over obese. BMI is defined as body weight in kilograms (kg) divided by the square of height in meters (m^2) of a person (Mohamed *et al.*, 2014). BMI classification is shown in Table 2.1.

Table 2.1: BMI classification (WHO, 2016a).

Classification	BMI (kg/m^2)
Underweight	<18.50
Normal weight	18.50-24.99
Overweight (Pre-obese)	25.00-29.99
Class I obesity	30.00-34.99
Class II obesity	35.00-39.99
Class III obesity	≥ 40

2.3 Causes of obesity

Prevalence of obesity is due to the interaction between behavioral, environmental and genetic factors, and it is associated with social and economic conditions of a person as well as their lifestyle (Ordovas & Shen, 2008). According to a review paper published by Keith *et al.* (2006), high prevalence of obesity was due to insufficient sleep, endocrine disruption, reduction of smoking rate as smoking reduces appetite, increased medication use, assortative mating, epigenetic factors that passed from one generation to the next generation and natural selection for higher BMI.

Obesity is mostly caused by imbalanced diet and sedentary lifestyle, but there are other rare causes like genetic factors and side effects of medication consumptions, such as atypical antipsychotic medications that cause elevation of weight gain as well as medical and psychiatric illnesses (Bleich *et al.*, 2008; Lau *et al.*, 2007).

2.3.1 Imbalanced diet

Obesity is caused by a long term effect of positive energy balance. Body weight is gained through a positive energy balance with high energy intake or low energy expenditure or both (Caballero, 2007). The energy balance is calculated using formula (1) below:

Energy Balance = Energy Intake - Energy Expenditure (1)

High energy intake is caused by imbalanced diet. When energy intake is higher than energy expenditure, gaining of weight will occur. Excess energy or calorie intake might be due to the high consumption of easily available fast food which contain high amount of fat and carbohydrate and due to the high consumption of snack food which is energy dense (Caballero, 2007; Schmidt *et al.*, 2005).

2.3.2 Sedentary lifestyle

People who do not maintain a regular exercise habit have higher chance to be obese (González-Castejón & Rodriguez-Casado, 2011). In this fast-paced world, most people rely on labor saving technology and mechanized transportation and these lead to low energy expenditure (Mohamed *et al.*, 2014). Hence, they are more prone to be obese.

2.3.3 Genetic factors

Obesity can be caused by polymorphisms in various genes that control appetite and metabolism of a person. Based on a study, people who had homozygous fat mass and obesity associated gene (FTO) risk alleles had 1.67 times higher chance to be obese and had weight with 3-4 kg more as compared to people who did not have the risk allele (Frayling *et al.*, 2007). Besides, obesity can also be caused by single-gene mutation in the gene that encoded dopamine receptor D4, melano-cortin-4 receptor, leptin or peroxisome proliferator-activated receptor γ2 (Mohamed *et al.*, 2014).

2.3.4 Illnesses and medical treatment

A person who has Cohen syndrome, Prader-Willi (causes food carving without limit), hypothyroidism, growth hormone deficiency or eating disorders has higher risk to be obese (Haslam & James, 2005; Rosen *et al.*, 1993). Besides, a person who has mental illness like bipolar disorder, depression or schizophrenia has a higher chance of being obese (McElroy, 2009). For example, people who are depressed like to eat desserts or keep on eating to release stress, finally it causes obesity. On the other hand, some side effects of medication used for mental illness treatment such as prescription of atypical antipsychotics and antidepressants can lead to weight gain (McElroy, 2009).

2.3.5 Social and economic factors

Social and economic status of a person is another factor that can cause obesity. In this fast-paced world, the population in a high social class has a higher risk to get obese. However, women in a high social class had a lesser chance to get obese (Sobal & Stunkard, 1989). Other studies showed that smokers had a lesser chance to be obese since nicotine suppressed appetite and increased energy expenditure (Chiolero *et al.*, 2008; Hofstetter *et al.*, 1986). On the other hand, Webbink *et al.* (2010) found that educated people were less likely to get obese and this was due to the supply of information on healthy lifestyle and obesity prevention by the organizer of educational programs in their education places.

2.4 Adverse effects of obesity on health

Obesity not only affects our health, but it also leads to mortality in severe cases. Osteoarthritis and back pain can be caused by obesity due to the extra weight placed on the skeleton of an obese person. There are other severe diseases that caused by obesity, such as Type 2 diabetes, hypertension, dyslipidemia, cancer, cardiovascular diseases (CVD) and metabolic syndrome.

2.4.1 Type 2 diabetes

Obesity is always associated with Type 2 diabetes. Type 2 diabetes is known as noninsulin-dependent diabetes mellitus, which is the most common primary form of diabetes and impaired glucose intolerance (Mohamed *et al.*, 2014). It is caused by insulin resistance and pancreatic β-cell dysfunction (Kasuga, 2006). Type 2 diabetes develops when body cells resist in responding to the signal that sent by insulin to uptake glucose in the bloodstream or when insufficient insulin secretion due to pancreatic β-cell dysfunction or both, which finally lead to abnormally high blood sugar level (Røder *et al.*, 1998).

In obese people, the secretion levels of non-esterified fatty acids (NEFAs), pro-inflammatory cytokines, glycerol and hormones from adipose tissue are increased due to the increased of adipose tissue mass (Karpe *et al.*, 2011). The elevated release of NEFAs causes insulin resistance and pancreatic β-cell dysfunction, ultimately lead to Type 2 diabetes (Al-Goblan *et al.*, 2014; Jelic *et al.*, 2007). According to Zhou and Grill (1994), continuous exposure of pancreatic β-cells to NEFAs caused adverse effects on β-cells function such as decreased insulin secretion and reduced efficiency of pro-insulin conversion to insulin within the β-cells. The low amount of insulin caused less uptake of glucose in the bloodstream and finally led to Type 2 diabetes. Another study showed that high levels of NEFAs competed with glucose for substrate oxidation, led to glucose uptake reduction. The elevation of glucose level caused overstimulation of pancreatic cells for insulin secretion and caused insulin resistance (Roden *et al.*, 1996; Stumvoll *et al.*, 2008). Finally, this caused Type 2 diabetes.

2.4.2 Hypertension

Epidemiological studies proposed that obese people had about 65-75% risk to get hypertension (Wofford & Hall, 2004). This associated pathological condition is

correlated to leptin secretion by adipose tissue (Hajer *et al.*, 2008). Many obese people have high level of leptin and they are in chronic hyperleptinemia with leptin resistance. However, a study showed that obese mouse was leptin resistant to satiety action on eating, but not in sympathetic nervous system (Mark *et al.*, 1999). Thus, sympathetic nervous system of obese people is activated even they are leptin resistant. This condition causes blood vessel constriction and high blood pressure, finally lead to hypertension.

2.4.3 Dyslipidemia

Obese people have a higher chance to get dyslipidemia. Dyslipidemia is characterized by hypertriglyceridemia with high amount of fasting and postprandial TG, elevated level of NEFAs, high level of LDL and low level of HDL (Klop *et al.*, 2013). These lipid abnormalities are due to impaired lipolysis, which is caused by low mRNA expression of lipase in adipose tissue, low lipoprotein lipase (LPL) activity in skeletal muscle and high competition between very low density lipoprotein (VLDL) and chylomicrons for lipolysis (Clemente-Postigo *et al.*, 2011; Klop *et al.*, 2012).

2.4.4 Cancer

Based on the classification from International Agency for Research on Cancer, obese people have high risk of getting breast, colon, kidney, ovary, uterus or prostate cancer (WHO, 2002). Many obese people are associated with high rates of tumourigenesis. A study proved that obese people had high levels of inflammatory tumor growth factors and this could be due to the high prevalence of neoplasia in obese people (Cottam *et al.*, 2010). Moreover, obese people have high secretion of adipocytokines by adipocytes, infiltrating macrophages and stromal cells due to the inflammation caused by adipocyte enlargement. Studies showed that elevated level of adipocytokines such as leptin and hepatic growth factor (except adiponectin) promoted cell invasion, increased tumor

growth, up-regulated cell proliferation and inhibited apoptosis which could cause cancer (Vona-Davis & Rose, 2007). Besides, excess estrogen that produced by adipose tissue may lead to the growth of targeted tumor cells. A study showed that estrogen was needed for the growth of breast cancer cells to invasive and metastatic phenotypes (Vona-Davis & Rose, 2007).

2.4.5 CVD

Obesity is always linked to CVD with the increased risk of heart failure, stroke, chest pain and abnormal heart rhythm (Artham *et al.*, 2009). Obese people have an increased electrical alterations and this causes frequent ventricular dysrhythmias even though they have an absence of heart dysfunction (Messerli *et al.*, 1987). Furthermore, studies also proved that obese people had high levels of small dense LDL particles which consisted of atherogenic properties, and these contributed to cardiovascular risk (Klop *et al.*, 2013; Packard, 2003).

2.4.6 Metabolic syndrome

Obesity is the risk factor for metabolic syndrome. This syndrome is featured by the co-occurrence of multiple metabolic disorders such as overall and central obesity, insulin resistance, hypertension, hyperglycemia and impaired glucose tolerance, and the presence of low HDL-C and elevated TG levels as well as with pro-thrombotic and pro-inflammatory states (González-Castejón & Rodriguez-Casado, 2011; National Institutes of Health (NIH), 2002; Visioli, 2011).

2.5 Potential mechanisms of obesity

2.5.1 Irregular food intake and energy metabolism with elevation of body weight

The brain has a significant role in regulating food intake and energy metabolism by sending signals to inform the central controllers through a feedback system (Ravussin & Bouchard, 2000). The brain activates a system to control its own energy by modulating

feeding behaviors via two mechanisms either homeostatic need to eat or hedonic to eat even we are not hungry (Volkow *et al.*, 2008; Volkow *et al.*, 2011). The hypothalamus is the brain region, which mainly regulates homeostatic food intake based on the sensing of neuropeptides (ghrelin, leptin, neuropeptide Y and insulin) and nutrients (amino acids, fatty acids and glucose) (Blouet & Schwartz, 2010).

Regulation of food intake and energy metabolism is controlled by neural and hormonal signals. For example, appetite-suppressing neuronal group produces pro-opiomelanocortin (POMC) in order to secrete melanocyte-stimulating hormones (MSH) that helps to reduce food intake and increase energy utilization (Bertolini *et al.*, 2009). Another example is an appetite-stimulating neuronal group produces neuropeptide Y (NPY) and agouti-related protein (AgRP) to stimulate food intake and reduce energy expenditure (Castañeda *et al.*, 2005). The interaction between appetite suppressing neurons and appetite stimulating neurons is vital in the regulation of food intake and energy metabolism.

Ghrelin is a hormone produced by gut and it is involved in regulating food intake by sending signal to the brain and activates NPY/AgRP appetite-stimulating neurons to stimulate food intake in response to negative energy balance (González-Castejón & Rodriguez-Casado, 2011). Ghrelin stimulates food intake and reduces energy utilization when stomach is empty and inhibits food intake when the stomach is stretched (Mohamed *et al.*, 2014). A study showed that ghrelin secretion level was suppressed less effectively by meals in obese people as compared to lean people (Cummings *et al.*, 2001; Cummings *et al.*, 2002). Nutrients obtained from the diet also affect the secretion level of ghrelin. Ingested lipids were shown to suppress ghrelin secretion less effectively than carbohydrates and proteins and this promoted body weight gain (Foster-Schubert *et al.*, 2004; Monteleone *et al.*, 2003; Overduin *et al.*, 2005). Moreover,

consumption of fructose-sweetened beverages suppresses ghrelin secretion and stimulates leptin less effectively than people who consume isocaloric glucose-sweetened beverages (Teff *et al.*, 2004). Therefore, the increased of ghrelin and decreased of leptin secretion stimulate food intake, ultimately lead to body weight gain.

Studies showed that obese people had an elevated level of ghrelin. Over secretion of ghrelin increases mitochondrial activity in NPY/AgRP cells, which help to transfer adenosine triphosphate (ATP) for cell metabolism. The increased of mitochondrial activity increases the generation of free radicals, which derived from mitochondria respiration and these free radicals can react with other molecules and trigger cell damage (González-Castejón & Rodriguez-Casado, 2011). The mitochondria uncoupling proteins (UCPs) which generally activated by a mechanism signaled by NPY/AgRP cells to protect the cell against free radicals are affected due to the formation of the high amount of free radicals. Therefore, UCPs should be up-regulated in order to prevent oxidative stress caused by free radicals (González-Castejón & Rodriguez-Casado, 2011).

Meanwhile, satiety is important in the maintenance of energy balance to prevent weight gain and obesity. Leptin is a satiety hormone primarily produced by white adipose tissue and it regulates food intake by sending homeostatic signals to the brain based on the amount of energy storage (Flier, 1997). It stimulates food intake and inhibits energy expenditure when low fat storage, whereas it inhibits food intake when high fat storage (Mohamed *et al.*, 2014). Leptin is produced at a level proportional to body fat content and enters into the central nervous system in proportional to its plasma level (Kim *et al.*, 2005). Activation of leptin receptors inhibits the effects of NPY, which is produced by appetite-stimulating neurons, but it stimulates POMC which is produced by appetite-suppressing neurons (González-Castejón & Rodriguez-Casado,

2011). Thus, energy expenditure, metabolic rate, thermogenic capacity and oxidation of dietary fat are increased, whereas food intake is decreased (Billington *et al.*, 1994; Novin *et al.*, 1976). Deficiency in leptin leads to intense hyperphagia and morbid obesity (Montague *et al.*, 1997). On the other hand, most of the obese people have high level of leptin, but they are leptin resistant (Hamann & Matthaei, 1996). Due to leptin resistance, obese people cannot suppress their food intake and finally lead to overeating, thus they easily gain weight (Flier, 2004).

2.5.2 Oxidative stress in obesity

Oxidative stress is highly correlated with most of the inflammatory and metabolic disease states such as obesity (Noeman *et al.*, 2011). It is due to the cumulative damage that caused by pro-oxidants where antioxidants are not sufficient to neutralize them. High concentrations of nitrogen species, ROS and other free radicals are involved in the etiology of obesity and it is indicated as the signs of oxidative stress (Parke, 1999).

Free radicals are highly reactive and unstable molecules with unpaired electrons which bind quickly to nearby molecules, while ROS are oxygen containing molecules which may or may not have unpaired electrons and they are also highly reactive (Vincent & Taylor, 2006). Examples of ROS are superoxide anion, NO and hydrogen peroxide (Vincent & Taylor, 2006). These free radicals and ROS are harmful as they are able to damage cellular components such as deoxyribonucleic acid (DNA), lipids and proteins in our body (Temple, 2000). However, defense systems in our body are able to protect us from the free radicals by the contribution of endogenous enzymes (CAT and SOD), endogenous factors (glutathione, urate and coenzyme Q) and nutritional factors (antioxidant nutrients and phytochemicals) (Temple, 2000).

According to Vincent and Taylor (2006), there are some possible sources of oxidative stress in obesity, such as hyperglycemia, increased metabolic ROS

production, insufficient antioxidant defenses due to increased rates of free radical formation, lipids and lipid oxidizability and chronic inflammation.

2.5.2.1 Hyperglycemia

Obesity may be associated with hyperglycemia and insulin resistance due to Type 2 diabetes (Vincent & Taylor, 2006). Hyperglycemia leads to the generation of advanced glycation end products (AGEs). AGEs are produced from lipids, protein and nucleic acids as a result of exposure to high amounts of sugars (Vincent & Taylor, 2006). AGEs bind to specific cell surface receptors and cause further production of ROS. Besides, AGEs also activate intracellular nuclear factor-$_{\kappa}$B in order to activate protein kinase C, sorbitol and transcriptions of vascular cell adhesion molecule-1 and intracellular adhesion molecule-1, which then lead to the production of ROS (Rodriguez-Manas *et al.*, 2003; Vincent & Taylor, 2006). The formation of ROS from the reactions of AGEs causes oxidative stress, which contributes to cell damage and accelerated monocyte homing to endothelium (Aronson & Rayfield, 2002; Evans *et al.*, 2002; Vincent & Taylor, 2006).

On the other hand, elevation of intracellular glucose level stimulates the polyol pathway in which aldose reductase acts as catalyst to convert glucose to sorbitol. The excessive accumulation of sorbitol causes cell damage and activates stress genes (Evans *et al.*, 2002). At the same time, hyperglycemia also activates NADPH oxidase activity, leads to the formation of superoxide which is highly reactive in the endothelium (Zhang *et al.*, 1999). Furthermore, elevation of glucose uptake from the diet into adipocytes also increases the formation of ROS since glucose can oxidize itself to form free radicals (Talior *et al.*, 2003). Therefore, overstimulation of polyol pathway and glucose auto-oxidation can cause oxidative stress in obesity.

2.5.2.2 Increased metabolic ROS production

Increased muscle activity activates metabolic pathways that form free radicals such as increased electron transport chain activity and conversion of hypoxanthine to urate (Vincent & Taylor, 2006). During exercise, active skeletal muscle requires high amount of oxygen for respiration. Electrons leak out from electron transport chain and reduce the oxygen when there is an occurrence of rapid electron transfer with increased respiration (Vincent & Taylor, 2006). Oxygen is partially reduced and forms superoxide, leads to the generation of hydrogen peroxide which is highly reactive and finally causes oxidative stress (Ji, 1995; Ji, 1996).

Besides, studies showed that cellular respiration rates and oxygen depletion in muscle cells of obese people during exercise were higher than non-obese people and it was because of the increased energy utilization due to the additional load of carrying excessive body weight (Salvadori *et al.*, 1999; Vincent *et al.*, 2004). Another study showed that obese patients were also less mechanically efficient during exercise and the inefficiency led to the increased of energy utilization (Vincent & Taylor, 2006). The elevated energy utilization in obese patients increases mitochondrial respiration and causes increased of lipid hydroperoxide production, then leads to oxidative stress in obese people (Vincent *et al.*, 2005).

In addition, obese patients have high levels of resting and post-exercise hypoxanthine and uric acid as compared to non-obese people (Saiki *et al.*, 2001). A study showed that an increased amount of hypoxanthine during exercise was observed in obese patients (Saiki *et al.*, 2001). When hypoxanthine is converted to urate, superoxide is formed (Vincent & Taylor, 2006). The formation of superoxide leads to the slight increase of oxidative stress in obese patients.

2.5.2.3 Insufficient antioxidant defenses

Sufficient dietary, enzymatic and non-enzymatic antioxidant defenses are important to maintain antioxidant-prooxidant balance (Vincent & Taylor, 2006). The combination of insufficient dietary, blood and enzymatic antioxidants and increased production of ROS cause prooxidant-antioxidant imbalance (Vincent & Taylor, 2006). It leads to lipid and protein oxidation and causes oxidative stress.

Antioxidant defenses of an obese patient can be disrupted due to high consumption of HFD food and low intake of antioxidants and phytochemicals rich foods (Vincent & Taylor, 2006). A study showed that phytochemical intake of obese people was inversely correlated to their BMI (Vincent & Taylor, 2006), lipid peroxidation and degree of adiposity (Reitman *et al.*, 2002; Wallström *et al.*, 2001). Another study also showed that the serum levels of dietary antioxidants (β-carotene and vitamin E) and antioxidant minerals (copper, selenium, and zinc) were found to be lower in obese people than non-obese people (Ozata *et al.*, 2002).

Moreover, a study was conducted to determine antioxidant protection within circulating lipids and the result showed that obese girls had low α-tocopherol/LDL and β-carotene/LDL as compared to non-obese girls. The study also showed that the obese girls had a high peroxidizability index (lipid peroxidation per amount LDL). Both results depicted that high amount of LDL obtained by obese people reduced the level of antioxidant and increased the rate of lipid peroxidation (Kljno *et al.*, 1998). Based on the result obtained from the study, researchers found out that there were insufficient amount of antioxidants within the large LDL pool since the available antioxidants were used up rapidly to fight against the free radicals produced in obese people, then led to lower defense from free radicals and caused oxidative stress (Kljno *et al.*, 1998).

In addition, activity of antioxidant enzymes is insufficient in obese people. This was proved by a study that showed low SOD and GPx activities in HFD-induced obese rats (Beltowski *et al.*, 2000). Another study proposed that antioxidant enzymes in the human body were able to combat against the free radicals produced in the early stage of obesity. However, chronic obesity slowly decreased the source of antioxidant enzymes (Olusi, 2002; Vincent *et al.*, 2001). Furthermore, researchers also found out that low antioxidant enzyme activities of GPx and SOD were coupled with high level of cholesterol, LDL and VLDL in obese patients as compared to non-obese people (Ozata *et al.*, 2002). The activity of antioxidant enzymes is also reduced by the elevated degree of adiposity since a study showed that erythrocyte CuZn-SOD activity was lower in very obese and GPx activity was lower in obese than in non-obese people (Vincent & Taylor, 2006). All these studies proved that the activities of antioxidant enzymes were reduced when antioxidants were used up to fight against the free radicals produced in obese people.

2.5.2.4 Lipids and lipid oxidizability

Obesity is featured by excessive intracellular TG, increased dietary fat intake and fat storage and dyslipidemia. Lipids are substrate for oxidation and the oxidation of lipids may increase the risk of atherosclerosis, endothelial dysfunction and thrombosis (Lyon *et al.*, 2003; Rodriguez-Porcel *et al.*, 2002). Lipids may also cause free radical generation and increase accumulation of oxidative by-products (Furukawa *et al.*, 2004).

Oxidative stress in obesity can be caused by metabolic impact of intracellular TG. Excessive TG causes accumulation of cytosolic long-chain acyl-CoA esters and elevation of superoxide production in mitochondrial electron transport chain. The accumulation of cytosolic long-chain acyl-CoA esters may interfere with the function of mitochondrial adenine nucleotide translators by disabling them to import adenine

disphosphate (ADP) into the mitochondrial matrix (Bakker *et al.*, 2000). Electrons are then accumulated in the electron transport chain and reacted with oxygen, finally leads to the formation of superoxide which causes oxidative stress (Bakker *et al.*, 2000).

Moreover, increased fat intake, such as conjugated fatty acids can cause oxidative stress (Vincent & Taylor, 2006). Conjugated fatty acids are polyunsaturated fatty acids where at least one pair of its double bonds is separated by a single bond. Conjugated fatty acids also contain two or more conjugated double bonds and they are prone to free radical attack because of the conjugated nature of double bonds adjacent to a methylene group (C-H), which weaken the methylene bond and make them vulnerable to oxidation (Almeida & Rato, 2017). The rate of lipid oxidation by ROS increases with the number of double bonds present in fatty acid and the increased amount of lipid molecules (Olusi, 2002; Vincent *et al.*, 2001). For instance, the food that is rich in trans-fat, such as deep fried foods, snack food and foods cooked with lard can cause the formation of oxidized LDL-C. The oxidized LDL-C causes oxidative stress in obese people and leads to obesity-related metabolic syndromes such as atherosclerosis and coronary heart disease since the oxidized LDL-C is atherogenic than native LDL (Kritchevsky & Kritchevsky, 1999).

On the other hand, the increased level of NEFAs in the blood, which caused by excess HFD intake can lead to oxidative stress in obesity (Stojiljkovic *et al.*, 2002). The blood glucose level is increased and nitroxide radicals are generated in smooth vascular and endothelial cells by NEFAs through protein kinase C mechanism (Inoguchi *et al.*, 2000). The high blood glucose level and generation of nitroxide radicals cause oxidative stress.

Furthermore, oxidative stress in obesity is caused based on the pattern of adiposity such as gynoid (peripheral fat distribution) and android (visceral fat deposition) fat

patterns of an obese person (Davì *et al.*, 2002). According to a study conducted by Davì *et al.* (2002), android fat pattern contributed high oxidative stress as compared to gynoid fat pattern. Besides, abdominal or visceral adiposity is associated with high level of NEFAs. High level of NEFAs induces oxidative respiratory burst in white blood cells and leads to increased ROS production (Inoguchi *et al.*, 2000).

In addition, studies proved that fat storage in obese patients can cause oxidative stress. A study showed that the increased rate of LDL oxidation and low plasma level of vitamin E were positively correlated with adiposity in obese patients (Myara *et al.*, 2003). Another study also showed that fat storage activated NADPH oxidase activity and decreased SOD, GPx and CAT activities in white adipose tissue due to an excessive ROS formation induced by fat, ultimately led to oxidative stress (Furukawa *et al.*, 2004).

Moreover, obese patients are always associated with dyslipidemia. They have high levels of TG and LDL and low level of HDL (Dobrian *et al.*, 2000). A study showed that hypercholesteremia is associated with high oxidizability of LDL molecules (Vincent & Taylor, 2006). Besides, the lag phase of lipid oxidation is shorter in LDL particles, thus rapid lipid peroxidation of LDL molecules occurs (Ozata *et al.*, 2002; Van Gaal *et al.*, 1998). Based on a study of the measurement of oxidizability of LDL and VLDL in obese patients by Van Gaal *et al.* (1998), high level of oxidized LDL was detected in obese patients and it was positively correlated to high level of malondialdehyde (MDA). MDA is an oxidative by-product and it is the biomarker of oxidative stress (Olusi, 2002). Therefore, this study proved that excessive lipids caused oxidative stress in obese people.

2.5.2.5 Chronic low-grade inflammation

Obesity is known as chronic inflammation (Saito *et al.*, 2003). The inflammation is featured by increased inflammatory cytokines and CRP productions, elevated white blood cell counts and increased white blood cell activity.

Adipocytes are known as fat cells or lipocytes and they are the main constituent of adipose tissues (Weisberg *et al.*, 2003; Xu *et al.*, 2003). The roles of adipose tissue are to store energy in the form of fat, to dissipate energy through thermogenesis and to insulate body as well as to support and protect the internal organs (Sun *et al.*, 2011). Adipose tissue also helps to secrete cytokines (pro-inflammatory cytokine) and adipokines (adiponectin and resistin) as well as to regulate intermediary metabolism (Ouchi *et al.*, 2011). In addition, adipose tissue is the target of hormones such as estrogens, glucocorticoids and growth hormone (Ouchi *et al.*, 2011).

There are two mechanisms involved in the growth of adipose tissue in obese people, which are through the development of adipocytes enlargement (hypertrophy) and through the increasing number of adipocytes (hyperplasia) (Arner *et al.*, 2010). Hypertrophy is predominated and developed dependent on diet, while hyperplasia is developed dependent on genetic factors (Jo *et al.*, 2009; Surmi & Hasty, 2008). Both mechanisms contribute to adipose tissue expansion and enlargement (Surmi & Hasty, 2008). Extremely adipocytes enlargement causes adipocytes swollen through ectopic fat deposition or adipose tissue inflammation, then the ability of adipose tissue is affected and leads to apoptosis (González-Castejón & Rodriguez-Casado, 2011).

Fat is not only stored in adipose tissue. However, excessive dietary fat can be stored in non-adipose tissue such as liver, skeletal muscle, heart and pancreas. Excessive accumulation of fat in non-adipose tissue is called steatosis or ectopic fat deposition. It causes cell dysfunction, followed by cell death or apoptosis, which is known as

lipotoxicity (Schaffer, 2003; Unger, 2003). This condition also causes oxidative stress in obesity.

On the other hand, excessive fat accumulation in adipose tissue triggers a low-grade inflammation and causes apoptosis with the secretion of pro-inflammatory cytokines such as IL-6 and TNF-α, and chemokines such as monocyte chemoattractant protein-1 (MCP-1), MCP-2 and MCP-4 (González-Castejón & Rodriguez-Casado, 2011; Surmi & Hasty, 2008). Excessive fat accumulation also leads to the production of CRP, which is secreted by the liver in response to inflammation (Kopp *et al.*, 2003).

As obesity develops, increased adipocyte volume and fat pad size raise the expression of inflammatory molecules such as TNF-α and leptin secretion (Vincent & Taylor, 2006). The elevated inflammatory molecules increase the expression of atherogenic adhesion molecules and trigger the attachment and migration of monocytes into vessel walls, then lead to the development of monocytes to macrophages (Lyon *et al.*, 2003). The elevated inflammatory molecules also attract more macrophages into fat tissue, lead to elevated ROS production and cause oxidative stress (Wellen & Hotamisligil, 2003). Besides, TNF-α inhibits insulin signal transduction and down-regulates expression of insulin receptor in adipocytes. It causes abnormal high level of glucose and hyperglycemia, which then leads to pancreatic β-cell dysfunction and apoptosis, followed by oxidative stress (Hotamisligil *et al.*, 1994; Weyer *et al.*, 2002).

CRP level is used to indicate vascular inflammation and predict LDL level. Obese people have higher levels of CRP secretion with increased vascular inflammation and high LDL level (Ridker *et al.*, 2000). On the other hand, obese people have low level of adiponectin with decreased insulin sensitivity and increased vascular inflammation (Lyon *et al.*, 2003; Yang *et al.*, 2001). Therefore, the occurrence of vascular

inflammation can be indicated by high levels of CRP and low level of adiponectin secretions.

Moreover, white blood cell counts and white blood cell activity of obese people are higher than normal people. They have high levels of monocyte and neutrophil sub-fractions (Kullo *et al.*, 2002). The white blood cells increase ROS formation and cause oxidative stress. Monocytes generate hydrogen peroxide, hypochlorous acid and myeloperoxidase, and when monocytes develop into macrophages, they secrete interleukins and TNF-α (Vincent & Taylor, 2006). Meanwhile, neutrophils produce superoxide through NADPH oxidase with hydrogen peroxide as intermediate. Monocytes and neutrophils convert hydrogen peroxide to hypochlorous acid via myeloperoxidase (Vincent & Taylor, 2006). Chloramines (oxidants), reactive aldehydes and tyrosine peroxide are generated from myeloperoxidase-hydrogen peroxide system and cause oxidative stress (Kullo *et al.*, 2002).

2.6 Treatment of obesity

It is a must to find a way to treat obesity or prevent obesity. Balanced diet and exercise should be conducted regularly. However, the compliance of a patient is the most important factor for long term success. Persistence and determination of obese patients are needed to control their weight and prevent obesity. There are several methods to treat obesity, such as maintenance of a balanced diet, regular exercise, weight loss program, medication and consumption of natural product.

2.6.1 Diet control

Energy is obtained in calories form and it is obtained from the nutrients (carbohydrate or sugars, proteins and fats) in food and drinks that we consumed. It is utilized for essential body functions such as breathing, food digestion, regulation of

body temperature and carrying out physical activities as well as for fat storage (NIH, 2017).

Excess calorie intake can cause obesity, thus it is important to control our diet. The daily calories needed for a person with moderate physical activity vary with age and gender (Mohamed *et al.*, 2014). The daily range of calories for a male is 2550-3200 kilocalories (kcal), while 1800-2300 kcal for female (Mohamed *et al.*, 2014). A well-balanced diet which is rich in fiber, low in fats and with multiple vitamins are needed for body to function well (Marlett *et al.*, 2002). Therefore, knowledge on nutrition is important in obesity treatment and diet control.

2.6.2 Regular exercise

Diet control alone is less effective in weight management (Mohamed *et al.*, 2014). Diet control should be accompanied with regular exercise to maintain a healthy lifestyle for long term success in obesity treatment (Mohamed *et al.*, 2014). Weight gain occurs when energy intake is higher than energy expenditure. Therefore, the equilibrium between food intake and energy expenditure should be maintained to prevent weight gain or obesity.

2.6.3 Weight loss program

Weight loss program is another choice for obesity treatment. This program has become increasingly popular for targeted weight loss recently. There are several types of weight loss programs such as a usual care group, person center-based program and telephone-based weight loss counseling program. The usual care group provides weight loss counseling sessions and monthly contacts to obese patients, but they do not receive prepackaged meals (Mohamed *et al.*, 2014). However, the person center-based and telephone-based weight loss counseling programs provide obese patients with

prepackaged food, a planned food menu and a timetable for conducting exercises regularly (Rock *et al.*, 2010).

2.6.4 Medication

There are some common anti-obesity drugs such as fluoxetine, orlistat, phentermine and sibutramine (Mohamed *et al.*, 2014). Based on the ESCP Guideline, pharmacological obesity treatment should be given only for obese patients who have BMI $\geq$ 30 kg/m^2 or BMI $\geq$ 27 kg/m^2 with comorbidity (Apovian *et al.*, 2015). Anti-obesity medication is always needed for long term prescription since body weight may regain after stop consumption (Mohamed *et al.*, 2014). Except the long-term compliance with the medication, there are other factors of concern, such as high cost of medication and lack of insurance coverage on the medical consumption and adverse effects as a result of these anti-obesity drugs. The adverse effects caused by these synthetic drugs are liver injury, heart attack and stroke (Sun *et al.*, 2016).

2.6.5 Natural products

As compared to synthetic drugs, natural plant products are less or non-toxic, less prone to side effects, easily available and more economical (Chandrasekaran *et al.*, 2012; Park *et al.*, 2011). Studies also showed that natural products possessed antioxidant, anti-obesity and hypolipidemic properties and they could be used to treat obesity. Therefore, crude extracts and isolated pure compounds from natural plant products are preferable to be used as an alternative to treat obesity. For example, a study showed that natural products such as tea saponin, *Platycodi radix* saponin, chitosan and chondroitin sulfate were useful in body weight reduction and lipid profile improvement for obesity treatment (Han *et al.*, 2005).

2.7 Bioactive phytochemicals in natural product

Phytochemicals are natural bioactive compounds which can be found in plant and the compounds may have beneficial effects to our body (Temple, 2000). Phytochemicals are grouped into different classes such as phenolic compounds, alkaloids and terpenoids.

2.7.1 Phenolic compounds

Phenolic compounds are the most abundant and widely distributed class of bioactive compounds in the plant kingdom (Bravo, 1998). They can be distributed into different groups such as simple phenolic acids, stilbenes, curcuminoids, chalcones, lignans, flavonoids, and isoflavones based on their structure with different number of phenol rings and the type and number of structural elements binding (González-Castejón & Rodriguez-Casado, 2011). Studies showed that phenolic compounds exhibited strong antioxidant activities to protect us from oxidative stress (González-Castejón & Rodriguez-Casado, 2011). Besides, other studies also showed that phenolic compounds had good effects on lipid metabolism and inhibiting enzymes such as pancreatic lipase (PL) and glycerophosphate dehydrogenase in order to reduce body weight, fat, plasma NEFAs levels and hepatic lipid accumulation (Park *et al.*, 2013; Yoshikawa *et al.*, 2002).

2.7.1.1 Simple phenolic acids

Simple phenolic acids are non-flavonoid phenolic compounds conjugated with other natural chemicals such as alcohols, flavonoids, glucosides, hydroxyfatty acids and sterols (González-Castejón & Rodriguez-Casado, 2011). These compounds are hydroxy derivatives of aromatic carboxylic acids, which are from benzoic acid or cinnamic acid group (Neo *et al.*, 2010). For instance, ellagic, *ρ*-hydroxybenzoic and gallic acids are the

derivatives of benzoic acid, while *ρ*-coumaric, caffeic and ferulic acids are the derivatives of cinnamic acid (Neo *et al.*, 2010).

Ferulic acid is one of the examples of phenolic acids and it can be found in rice bran oil, cereals and orange (Nicholson *et al.*, 2008). It possesses a high potential of *in vitro* antioxidant activities and has hypolipidemic properties that lower the risk of obesity (Son *et al.*, 2010; Srinivasan *et al.*, 2007). It is also able to reduce cholesterol level, protect liver from injury and inhibit tumor growth (Srinivasan *et al.*, 2007; Wilson *et al.*, 2007).

2.7.1.2 Stilbenes

Stilbenes are rarely found in plant and they occur as monomers, oligomers and as conjugate to sugars (González-Castejón & Rodriguez-Casado, 2011). They are considered as phytoestrogens (Shanle & Xu, 2010). Moreover, they are produced through the phenylpropanoid pathway in response to environmental stress, infections, disease and ultraviolet exposure (González-Castejón & Rodriguez-Casado, 2011; Roupe *et al.*, 2006).

One of the examples of stilbenes is resveratrol. It can be found in apples, blueberries and red grapes in very low amount (Vitrac *et al.*, 2002). It has a high antioxidant capacity and it is able to reduce LDL-C level in order to prevent atherosclerosis (Rahman *et al.*, 2006; Ramprasath & Jones, 2010). Furthermore, it also helps to decrease adipogenesis and reduce the viability of mature pre-adipocytes by down-regulating adipocyte transcription factors and adipocyte specific genes like peroxisome proliferator-activated receptors γ (PPARγ) and CCAAT-enhancer-binding proteins α (Baile *et al.*, 2011; Rayalam *et al.*, 2008). Moreover, it also helps to reduce lipid accumulation *in vitro* by increasing lipolysis, inducing apoptosis and reducing lipogenesis (Rayalam *et al.*, 2008; Szkudelska *et al.*, 2009). Besides, it helps to increase

insulin sensitivity in adipocytes (Kang *et al.*, 2010) and inhibit differentiation and proliferation of pre-adipocytes that trigger fat mobilization (Fischer-Posovszky *et al.*, 2010).

2.7.1.3 Curcuminoids

Curcuminoids consist of two linked molecules of ferulic acid (González-Castejón & Rodriguez-Casado, 2011). The most abundant curcuminoids are curcumin, demethoxycucumin and bisdemethoxycurcumin. They can be found in turmeric and ginger. Curcuminoids have some properties such as anticancer, antioxidant and anti-inflammatory activities. Studies showed that curcuminoids were able to inhibit the accumulation of lipid in rats (Pongchaidecha *et al.*, 2009).

Among the curcuminoids, curcumin is the best studied phytochemical since it has a lot of benefits. It helps to regulate gene expression in order to increase energy expenditure, decrease lipid accumulation and reduce intracellular lipids (Alappat & Awad, 2010; Ejaz *et al.*, 2009; Tang & Chen, 2010). It also helps to inhibit angiogenesis to prevent the growth of adipose tissue and reduce body fat and body weight (Ejaz *et al.*, 2009). Moreover, it helps to prevent obesity-associated inflammation, insulin resistance, hyperglycemia, hyperlipidemia and hypercholesterolemia (Aggarwal, 2010). It is also able to inhibit LDL oxidation that causes inflammation to liver by regulating leptin, which inhibits hunger and reduces food intake (Naidu & Thippeswamy, 2002). Furthermore, it helps to reduce leptin resistance, enhance adiponectin level and decrease inflammatory signals caused by obesity (Aggarwal, 2010).

2.7.1.4 Chalcones

Chalcones contain open-chain flavonoids and the two aromatic rings are joined by a three-carbon α, β-unsaturated carbonyl system (Nowakowska, 2007). They are antioxidant, anti-inflammatory, anti-proliferative and anti-cancer agents (Nowakowska,

2007). Dihydrochalcone phlorizin is one of the examples of chalcones and it is used to treat obesity. It can be found mostly in the leaves of Sweet Tea. It helps to inhibit glucose absorption in the small intestine (Ehrenkranz *et al.*, 2005). Naringenin is another example of chalcones. It helps to inhibit the production of inflammatory mediators (Hirai *et al.*, 2007; Yu *et al.*, 2009) and suppress macrophage infiltration to the enlarged adipocytes in obese people (Hirai *et al.*, 2010).

2.7.1.5 Lignans

Lignans are a group of phytoestrogen with two phenyl-propane units and they are mostly found in flaxseed and grain (González-Castejón & Rodriguez-Casado, 2011). Lignans can be categorized into plant lignans and mammalian lignans. Plant lignans (matairesinol and secoisolariciresinol) in human foods are converted to mammalian lignans (enterodiol and enterolactone) by intestinal microflora (Adlercreutz, 2007). The mammalian lignans are involved in antioxidant activities and estrogen-like activities, thus the risk of chronic diseases like hormone-related obesity can be reduced (Prasad, 2000). Plant lignans matairesinol helps to lower the risk of coronary heart disease, CVD and cancer (Milder *et al.*, 2006). On the other hand, plant lignan secoisolariciresinol diglucoside which is isolated from flaxseed has antihypertensive effects (Prasad, 2004). According to Prasad (1999), secoisolariciresinol diglucoside was also able to reduce the level of serum TC and prevented atherosclerosis in high-cholesterol diet fed rabbits.

2.7.1.6 Flavonoids

Flavonoids constitute a big family of 6000 different phytochemicals, found in fruits and vegetables (González-Castejón & Rodriguez-Casado, 2011). They are the most common group of phenolic compounds. They help to protect plants from external influences, thus it can be found in the highest concentration in outer leaves or shells. Flavonoids contain two aromatic carbon rings, benzopyran (A and C rings) and benzene

(B ring) (González-Castejón & Rodriguez-Casado, 2011). They can be categorized into different subgroups such as flavonols, flavanols, anthocyanidins, flavones, flavanones and flavanonols.

(a) ***Flavonols***

Flavonols are commonly found in low amount in fruits and vegetables compared to other phytochemicals. They can be found in onions, curly kale, broccoli and blueberries. They are anti-oxidant, anti-inflammatory and anti-proliferative agents as well as anti-hypertensive agent (Perez-Vizcaino & Duarte, 2010). One of the examples of flavonols is quercetin. It shows high anti-lipase activity as compared to luteolin (Zheng *et al.*, 2010). It is able to decrease *in vitro* adipogenesis by activating adenosine monophosphate-activated protein kinase (AMPK) signal pathway in preadipocytes and to down-regulate the expression of adipogenesis-related factors (Ahn *et al.*, 2008). It also helps to induce apoptosis in preadipocytes and mature adipocytes (Ahn *et al.*, 2008; Hsu & Yen, 2006).

(b) ***Flavanols***

Flavanols exist as monomers (catechins) and polymers (proanthocyanidins) (González-Castejón & Rodriguez-Casado, 2011). Catechins are found in green tea, chocolate, red wine and apricots. They help to reduce body weight and waist circumference through gastric lipase inhibition and through increased of thermogenesis (Boschmann & Thielecke, 2007; Chantre & Lairon, 2002). Furthermore, green tea catechin like epigallocatechin gallate (EGCG) has been studied by researchers and the results showed that it was able to inhibit adipocyte differentiation and proliferation, lipogenesis and fat absorption, to reduce fat mass, body weight, TG level, NEFAs, cholesterol and glucose as well as to increase β-oxidation and thermogenesis (Richard *et al.*, 2009; Wolfram *et al.*, 2006). Moreover, it was able to reduce intracellular lipid

accumulation and inhibit glucose and fatty acid transport (Kim *et al.*, 2010). It had anti-obesity effect and it inhibited lipid absorption in the intestine and inflammation (Bose *et al.*, 2008; Hill *et al.*, 2007; Wolfram *et al.*, 2006). On the other hand, caffeine and EGCG in green tea produce a synergistic effect by increasing energy expenditure and fat oxidation and by reducing total and abdominal fat (Nagao *et al.*, 2005).

Proanthocyanidins are condensed tannins and mostly found in apples, cocoa, grape seed, grape skin and green and black teas (Guyot *et al.*, 1998; Souquet *et al.*, 1996). These compounds provide astringent character to the fruit and bitterness to chocolate (González-Castejón & Rodriguez-Casado, 2011). Proanthocyanidins from grape seed possess antioxidant properties with cardio-protective effect (Shao *et al.*, 2009). Besides, they are able to induce lipolysis by increasing cyclic AMP (cAMP) production in adipocytes (Pinent *et al.*, 2005).

(c) ***Anthocyanins***

Anthocyanins are antioxidant pigments which exist in different forms (González-Castejón & Rodriguez-Casado, 2011). They are unstable in the aglycone form (anthocyanidins), but they are resistant to light, pH and oxidized situation when they are in plants (González-Castejón & Rodriguez-Casado, 2011). They are stable when they are conjugated with other flavonoids (co-pigmentation) and their degradation is prevented by glycosylation and esterification with organic acids (citric and malic acids) and phenolic acids (González-Castejón & Rodriguez-Casado, 2011). Anthocyanins can be found in red wine and purple grapes (Clifford, 2000). Example of anthocyanins is cyanidin. Its content is proportional to the color intensity of the fruits (González-Castejón & Rodriguez-Casado, 2011). Cyanidin is an anti-obesity agent and it exhibits anti-inflammatory effect on obese adipose tissues (Tsuda, 2008).

(d) ***Flavones***

Flavones are less common than flavonols and they possess glycosides of luteolin and apigenin (González-Castejón & Rodriguez-Casado, 2011). They can be found in parsley and celery. Large amount of flavones like tangeretin, nobiletin, and sinensetin can also be found from the skin of citrus fruit (Shahidi & Naczk, 1995). One of the examples of flavones is luteolin. It exhibits antioxidant, anti-lipase and anti-inflammatory effects (Middleton *et al.*, 2000; Zheng *et al.*, 2010). For example, it suppresses inflammation induced by macrophages due to adipocytes enlargement (Ando *et al.*, 2009). It also helps to enhance insulin sensitivity in obese people (Zheng *et al.*, 2010).

(e) ***Flavanones***

Flavanones can be found in tomatoes and mint, but they are found with large amounts in citrus fruit (González-Castejón & Rodriguez-Casado, 2011). The main aglycones are eriodictyol in lemons, hesperetin in oranges and naringenin in grapefruit (González-Castejón & Rodriguez-Casado, 2011). Naringenin is an antioxidant agent as it inhibits lipid peroxidation and it also acts as an agent that lower cholesterol by suppressing cholesteryl ester synthesis (Borradaile *et al.*, 1999). Moreover, it helps to inhibit inflammation caused by adipocytes enlargement (Hirai *et al.*, 2007), to reduce preadipocyte proliferation (Hsu & Yen, 2006) and to prevent body weight gain (González-Castejón & Rodriguez-Casado, 2011).

(f) ***Flavanonols***

Flavanonols have different oxidation state from anthocyanins and they possess 3-hydroxy-2,3-dihydro-2-phenylchromen-4-one backbone (González-Castejón & Rodriguez-Casado, 2011). Some examples of flavanonols are taxifolin and aromadedrin. Taxifolin is involved in *in vitro* anti-inflammatory activities (Makena *et al.*, 2009). It also helps to inhibit cholesterol production by suppressing HMG-CoA reductase activity

and to prevent the production and secretion of triacylglycerol and phospholipids as well as to reduce the secretion of apoB into LDL particles (Casaschi *et al.*, 2004).

2.7.1.7 Isoflavones

Isoflavones are diphenolic compounds and they have same chemical structures with estradiol. They are usually known as estrogen-like molecules or non-steroidal estrogens (González-Castejón & Rodriguez-Casado, 2011). They trigger genomic and non-genomic estrogen signalling pathways and interact with the metabolism of steroid hormones (Pilsakova *et al.*, 2010).

Soy is rich in isoflavones and the isoflavones are made up of genistein, daidzein and glycitein (Manach *et al.*, 2004). Consumption of soy is able to increase HDL-C and decrease TC, LDL-C and TG levels as well as protect us from getting obese and CVD (Pilsakova *et al.*, 2010). Daidzein and glycitein in soy help to inhibit adipocyte differentiation and help to suppress the expression of adipogenic markers, PPARγ and Glut-4, whereas genistein in soy helps to decrease preadipocyte proliferation (Kim *et al.*, 2010). A study showed that the combination of genistein with resveratrol and quercetin helped to reduce adipogenesis (Rayalam *et al.*, 2008). Another study showed that the combination of genistein with vitamin D prevented adipogenesis and promoted apoptosis in mature preadipocytes (Rayalam *et al.*, 2008). Last but not least, a study showed that the consumption of soy protein isolates and genistein was able to reduce body weight, abdominal fat pad, adipose tissue weight, hepatic lipid droplets and TG level (Simmen *et al.*, 2010). Hence, all these studies proved that the combination of phytochemicals could be the potential remedy for obesity treatment.

2.7.2 Alkaloids

Alkaloids are one of a large class of organic and basic substances found in plants which mostly contain basic nitrogen atoms and they also possess compounds with

neutral and even weak acidic properties (Babbar, 2015). Some of the examples of alkaloids are capsaicin, ephedrine, caffeine and nicotine. A study showed that capsaicin helped to suppress the inflammatory response that induced by obesity through decreasing the secretion levels of TNFα, IL-6, and MCP-1 and through increasing the adiponectin level in adipose tissue and liver (Kang *et al.*, 2007; Kang *et al.*, 2010). The study also showed that capsaicin was able to decrease food consumption and increase energy utilization and lipid oxidation (Kang *et al.*, 2010).

On the other hand, ephedrine helps to enhance the noradrenaline level in order to suppress appetite and increase energy expenditure (Astrup *et al.*, 1995; Kaufman *et al.*, 1998). Caffeine is also one of the examples of alkaloids and it is used to increase lipolysis and thermogenic effect (Astrup *et al.*, 1992; Astrup *et al.*, 1995), while nicotine helps to reduce food intake and to increase fat oxidation and energy utilization (Jensen *et al.*, 1995; Tucci, 2010).

2.7.3 Terpenoids

Terpenoids are one of the largest and diverse classes of natural compounds. They are chemically modified terpenes where methyl groups are removed or oxygen atoms are added (González-Castejón & Rodriguez-Casado, 2011). Terpenes which consist of hydrocarbons with the general formula of C10H16 are modified by adding oxygen-containing compounds such as alcohols, aldehydes and ketones in order to form terpenoids (Park & Kim, 2011). Isoprene unit (CH_2=C(CH_3)-CH=CH_2) is the structure of all terpenoids (González-Castejón & Rodriguez-Casado, 2011). Therefore, terpenoids are classified based on the number of isoprene units used. They are classified into monoterpenoids, sesquiterpenoids, diterpenoids, sesterpenoids, triterpenoids, carotenoids (tetraterpenoids) and polyisoprenoids.

Monoterpenes and sesquiterpenes are the chief constituents of essential oils in plants. Sesquiterpenes have the function as an anti-inflammatory agent and this is due to the presence of one or more sesquiterpenes lactones (Zhang & Demain, 2005). One of the examples of natural sesquiterpenes is an abscisic acid (ABA), which is able to treat obesity-related inflammation (González-Castejón & Rodriguez-Casado, 2011). Studies showed that ABA was able to reduce fasting blood glucose level, inhibit glucose intolerance, improve obesity-associated inflammation and suppress the expression of PPARγ and responsive genes in adipose tissue of high fat diet mice (Guri *et al.*, 2007).

On the other hand, carotenoids are lipophilic pigments tetraterpenoids. They have a long chain of conjugated double bonds in the center of molecule, thus they are susceptible to oxidation and cis-trans isomerization. Therefore, this provides them chemical reactivity, light-absorbing properties and shape (Britton *et al.*, 2004). Carotenoids are categorized into hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls). They are responsible for red, orange and yellow color of food. Carotenes such as α-carotene, β-carotene and lycopene are the precursors for vitamin A and they play vital roles in antioxidant activities, cellular differentiation and immune system (Fraser & Bramley, 2004). Studies showed that carotenoids were able to prevent the development of inflammation-associated diseases such as obesity (González-Castejón & Rodriguez-Casado, 2011; Krinsky *et al.*, 2004).

Triterpenoids form a big group of natural compounds which are derived from C_{30} precursors (Everlyne *et al.*, 2016). Steroids and sterols are the examples of triterpenoid. Squalene is the immediate biological precursor of all triterpenoids (Everlyne *et al.*, 2016). Plant steroids (phytosterols) are natural compounds that have the same structure with mammalian cell-derived cholesterol (Gupta *et al.*, 2011). Examples of phytosterols are sterols and stanols. These compounds can be found in unrefined vegetable oils, nuts

and legumes and they exist in esterified and free alcohol forms (Berger *et al.*, 2004). Brassicasterol, campesterol, diosgenin, sitosterol and stigmasterol are the phytosterols that have anti-obesity effect (González-Castejón & Rodriguez-Casado, 2011). High consumption of these compounds can reduce TC and LDL levels since the compounds have the ability to compete with cholesterol for micelle formation in the intestinal lumen in order to prevent cholesterol absorption (González-Castejón & Rodriguez-Casado, 2011; Izar *et al.*, 2011).

One of the examples of phytosterols is protodioscin which is a furostanol saponin. It is the active compound of the rhizomes of *Dioscorea gracillima* (González-Castejón & Rodriguez-Casado, 2011). It has anti-hyperlipidemic effect. A study showed that the supplementation of protodioscin in hyperlipidemic rats significantly lowered the blood levels of TG, TC and LDL as well as increased HDL (González-Castejón & Rodriguez-Casado, 2011; Mohamed *et al.*, 2014). Another example of phytosterols is diosgenin. Diosgenin is a steroidal saponin which can be found in different type of plants such as fenugreek and the roots of yam (González-Castejón & Rodriguez-Casado, 2011). It helps to inhibit the elevation of TG and suppress the expression of lipogenic genes in HepG2 cells. It is also used to treat hypercholesterolemia (González-Castejón & Rodriguez-Casado, 2011).

2.8 Anti-obesity mechanisms of natural product

Phytochemical compounds in natural product are bioactive non-nutrient compounds and they help to reduce the risk of obesity (Liu, 2003). A significant amount of bioactive compounds in natural product may provide multifunctional anti-obesity effects beyond the essential nutrients in order to treat obesity. The anti-obesity effects of bioactive compounds from natural product are employed in several pathways through reducing lipid absorption and food intake, increasing energy expenditure, inhibiting

differentiation and proliferation of pre-adipocytes, inducing lipolysis and preventing lipogenesis (Mohamed *et al.*, 2014).

2.8.1 Lipase inhibitor

Ingested fats that are absorbed in intestine are important for energy supply, thermal regulation, energy storage and membrane components as well as transmission/reservoir agents for essential fatty acids and fat-soluble vitamins (Buchholz & Melzig, 2015; Carey & Hernell, 1992; Tucci *et al.*, 2010). Fats cannot be absorbed in intestine before they are hydrolyzed. Hence, PL plays an important role in hydrolyzing triacylglycerols to monoacylglycerols and NEFAs before absorption (Mohamed *et al.*, 2014). PL is the main lipid-digesting enzyme which removes fatty acids from α and α' position of dietary TG in order to yield β-monoglyceride and long-chain saturated and polyunsaturated fatty acids (Zheng *et al.*, 2010). Therefore, inhibition of PL is a targeted approach for obesity treatment.

Some phytochemicals are discovered as anti-obesity agent or lipase inhibitor such as phenolic compounds from the leaves of *Nelumbo nucifera*. Besides, phytochemicals such as saponins, phenolic compounds, flavonoids and caffeine as well as carbohydrates (chitosan) have lipase inhibitory effects. For example, phenolic compounds obtained from tea leaves such as epicatechin gallate (ECG), epigallocatechin (EGC) and EGCG have strong lipase inhibitory effect (Thielecke & Boschmann, 2009). Moreover, metabolites from microorganisms such as lipstatin from *Streptomyces toxytricini* and panclicins from *Streptomyces* sp. also possess lipase inhibitory effect (Mutoh *et al.*, 1994).

2.8.2 Appetite suppressant

Body weight gain can be prevented by controlling appetite and satiety via the interaction between neurological and hormonal signals (Sun *et al.*, 2016). An elevation

of noradrenaline secreted by the adrenal gland is released into the bloodstream and activate sympathetic nervous system activity, then leads to increased satiety, energy utilization, hunger suppression and fat oxidation (Morton *et al.*, 2014). On the other hand, neural signal peptides such as dopamine, histamine and serotonin and their associated receptors are responsible in satiety regulation. Therefore, these receptors can be the targets for anti-obesity agent to treat obesity through food intake reduction by increasing satiety (Chantre & Lairon, 2002).

Some bioactive compounds are discovered as the appetite suppressants to control the hunger center in the brain in order to suppress appetite and reduce food intake (Mohamed *et al.*, 2014). For instance, the total extract of *Hypericum perforatum* is used as appetite suppressant and anti-obesity agent to suppress appetite and reduce food intake since this extract helps to increase serotonin in synaptosomes in order to inhibit serotonin uptake by synaptosomal (Husain *et al.*, 2011).

2.8.3 Energy expenditure stimulant

Obesity is caused by a positive energy balance with increased energy intake or decreased energy utilization or both occurred simultaneously. In order to prevent weight gain, we have to control our food intake and conduct exercise regularly (Sun *et al.*, 2016). Phytochemical compounds from natural product can help to control or reduce body weight. It also helps to promote energy expenditure by boosting metabolic rate through increasing thermogenesis and helps to burn calories and extra body fat (Kazemipoor *et al.*, 2012). For example, phytochemical compounds such as caffeine and capsaicin as well as natural compounds from green tea and its extract were proven as energy expenditure stimulants for obesity treatment (Rayalam *et al.*, 2008).

Brown adipose tissue (BAT) plays a critical role in maintaining energy balance via non-shivering thermogenesis by dissipating the excess energy to heat with the assistance

of uncoupling protein 1 (UCP1). UCP1 is responsible for oxidative phosphorylation, and it oxidizes nutrients from food by using enzymes in order to release energy in the form of ATP. Therefore, up-regulation of UCP1 gene expression can help to increase energy utilization for body weight reduction (Cannon & Nedergaard, 2004). According to Yoon *et al.* (2008), the expression of UCP in BAT and liver was activated by an ethanolic extract of *Solanum tuberosum* and the fat weight of HFD rats was significantly reduced. Thus, the ethanolic extract of *S. tuberosum* could be used as an energy expenditure stimulant in obesity treatment.

2.8.4 Adipocyte differentiation inhibitor

Adipocytes are the cells that form adipose tissues and they play an important role to maintain lipid homeostasis and energy balance (Kim *et al.*, 2006). White adipose tissue stores TG when there is energy excess, while it releases NEFAs to provide energy when there is energy deprivation (Kwan *et al.*, 2015). However, excess accumulation of adipose tissue causes obesity. Hence, adipogenesis must be inhibited in obesity treatment.

According to Kang *et al.* (2013), down-regulation of the expression of CCAAT/enhancer binding protein beta (C/EBPβ) and PPARγ inhibited adipocytes differentiation and prevented adipogenesis. Based on Xiao *et al.* (2010), adipocytes differentiation was inhibited by *Sibiraea angustata* extracts through down-regulating the expression of C/EBPβ and PPARγ. Besides, lipid accumulation in adipose tissue could also be decreased by *S. angustata* extracts. Other studies showed that phytochemical compounds such as resveratrol, quercetin, genistein, EGCG and capsaicin had an apoptotic effect on mature pre-adipocytes and an ability to prevent adipogenesis (Yang *et al.*, 2006).

2.8.5 Lipid metabolism regulator

Lipid metabolism is the crucial mechanism that has to be controlled for maintaining energy homeostasis. Lipolysis stimulation contributes to TG hydrolysis, thus it decreases fat storage in our body (Sun *et al.*, 2016). Therefore, lipolysis should be promoted to prevent obesity. Based on a study carried out by Bordicchia *et al.* (2014), the activation of β-adrenergic receptor promoted lipolysis in white adipose tissue and non-shivering thermogenesis in BAT. For instance, flavonoids from *N. nucifera* leaves are the examples of the natural product involved in β-adrenergic receptor activation (Ohkoshi *et al.*, 2007). Another study showed that activation of AMPK promoted fat oxidation and glucose uptake in skeletal muscle in order to trigger lipid metabolism (Kola *et al.*, 2008; O'Neill *et al.*, 2013). For example, flavonols like quercetin from natural product activates AMPK pathway, then leads to activation of lipoxygenase and finally causes lipolysis (Ahn *et al.*, 2008).

2.9 Antioxidant mechanisms of natural product

Natural product contains bioactive compounds such as phenolic compounds, especially flavonoids. The compounds have antioxidant properties due to their redox properties and chemical structure (Baba & Malik, 2015). They were known as effective hydrogen donors and good antioxidants. Studies showed that the scavenging ability of phenolic compounds was due to their hydroxyl groups (El-Tantawy, 2015). Antioxidants are able to treat oxidative stress in obesity induced by free radicals. Obese patients have elevated level of TC, LDL-C and TG and low level of HDL-C. The elevation of LDL-C in the bloodstream is easily attacked by free radicals in the body and forms oxidized LDL. The oxidized LDL will then deposit in blood vessel and causes atherosclerosis. Antioxidants can prevent the formation of atherosclerosis by donating hydrogen or electron to the free radicals in order to reduce susceptibility of LDL from oxidation.

According to Colla *et al.* (2008), the therapeutic properties of *Spirulina* in obesity treatment and lipid profile improvement were due to the antioxidant abilities of polyunsaturated fatty acids (γ-linolenic acid), phycocyanin and phenolic compounds in *Spirulina*. Besides, other studies also depicted that the antioxidant capacity of phenolic compounds was correlated with lipid profile improvement (Belguith-Hadriche *et al.*, 2010). For example, a study proved that LDL-C level of the rats was reduced after brown algae extract supplementation and the researchers suggested that LDL-C reduction might be due to the ability of phenolic compounds in the extract to form phenoxyl radicals in the presence of peroxidases in order to inhibit lipid peroxidation and prevent free radical accumulation (Ahmed *et al.*, 2016). The study also showed that the treatment of rats with the brown algae extract had great improvement in HDL-C level and the researchers suggested that the HDL improvement might be due to the ability of phenolic compounds in the extract in accelerating the decomposition of free radicals (Ahmed *et al.*, 2016). HDL improvement can help to inhibit oxidative stress in obesity since HDL is known as a free radical scavenger and it prevents peroxidation of β-lipoproteins. In addition, studies showed that the increased HDL-C and the reduced LDL-C levels were due to the flavonoids intake since flavonoids was able to remove cholesterol from peripheral tissue to the liver for catabolism and excretion (McCrindle *et al.*, 2003).

2.10 Herbal product for obesity treatment

In order to search for more efficient and effective obesity treatment, combination of multiple natural products such as a mixture of herbs can be used to treat obesity because it may provide synergistic effect by increasing their bioavailability and action on the multiple molecular targets (Liu, 2003; Rayalam *et al.*, 2008). A study proved that the combined natural products not only had multifunctional anti-obesity effects with combined actions of lipase inhibitory, appetite reduction, increased energy utilization,

decreased adipocyte differentiation and lipolysis stimulation, but it also had hypolipidemic and antioxidant effects (Thielecke & Boschmann, 2009).

The HP used in this study for obesity treatment is a mixture of herbs which has not been subjected into a comprehensive investigation in animal models and has not been commercialized in the market. Therefore, an investigation is carried out to determine its effect on body weight reduction and lipid profile improvement in HFD rats. The main ingredients of the HP consist of citrus, green tea, Guarana, grape seed, lotus leaves, psyllium husk and *Spirulina*.

2.10.1 Citrus

Citrus is native to tropical Asia, but it is also found in all tropical and subtropical countries (Suryawanshi, 2011). Citrus contains five adrenergic amines such as N-methyltyramine, hordenine, octopamine, synephrine and tyramine (Preuss *et al.*, 2002). One of the amines, synephrine is a type of alkaloids which increases energy expenditure, increases metabolism and suppresses appetite in order to reduce body weight (Haaz *et al.*, 2006). It is also recognized as a fat burner. Besides, it is well-known as a replacement of the banned ephedra stimulant on body weight reduction without ephedra side effects (Suryawanshi, 2011).

Consumption of citrus helps in weight loss and increases thermogenesis (Shekelle *et al.*, 2003) with minimal effect on blood pressure and heart rate (Penzak *et al.*, 2001). A study was conducted by the Department of Physiology, Medicine and Pathology at Georgetown University and they found out that the consumption of citrus was able to reduce body weight and able to promote energy expenditure by increasing metabolic rate which increase thermogenesis (Suryawanshi, 2011). Another study conducted by Colker *et al.* showed a significant body weight reduction and body fat loss after consuming citrus (Suryawanshi, 2011).

Citrus also consists of vitamins, minerals, flavonoids, phenolic compounds and terpenoids (Yi *et al.*, 2017). Citrus flavonoids are the most important compounds and more than 60 types of flavonoids are discovered in citrus (Lv *et al.*, 2015). Flavonoids exert their antioxidant activity by scavenging free radicals, preventing damage effect of ROS, chelating metal ions, inhibiting lipid peroxidation, inhibiting the activity of oxidant enzymes and enhancing the activity of antioxidant enzymes (Zou *et al.*, 2016).

Besides, high amount of citrus flavonoids is positively correlated with antioxidant capacity (Islam *et al.*, 2015). They inhibit oxidant enzymes, which play important roles in the redox reaction of biological systems and they are the main promoters of cellular ROS (Souza *et al.*, 2016). They also trigger the activity of antioxidant enzymes such as SOD and CAT in order to enhance antioxidant defenses (Ferlazzo *et al.*, 2016). For example, consumption of naringin which is a type of flavonoids from citrus is able to increase the activity of antioxidant enzymes, enhance hepatic SOD and CAT activities and decrease hepatic mitochondrial hydrogen peroxide content (Jeon *et al.*, 2002).

Furthermore, citrus flavonoids are also able to inhibit lipid peroxidation since these compounds can reduce ROS and eliminate them from biological system in order to prevent accumulation of ROS that causes oxidative stress (Paul *et al.*, 2015; Yi *et al.*, 2017). For example, citrus flavonoids such as naringin, naringenin, hesperidin, hesperetin and tangeretin are able to inhibit the production of ROS (Manna *et al.*, 2016).

Moreover, citrus is rich in vitamin C and flavonoid such as hesperidin. Hesperidin is able to revive vitamin C after it is quenched by free radicals. Based on a human clinical trial, the result showed that the consumption of citrus was able to increase HDL-C level and reduce LDL-C level and this was due to the presence of hesperidin in citrus (Suryawanshi, 2011). In addition, fiber in citrus, known as pectin was able to lower cholesterol and stabilize blood sugar levels (Suryawanshi, 2011).

2.10.2 Green tea

Green tea contains catechin polyphenols and caffeine. These bioactive compounds help to stimulate thermogenesis and fat oxidation (Dulloo *et al.*, 2000). According to Dulloo *et al.* (2000), their study showed that the consumption of 100 mg of caffeine from green tea was able to increase the metabolite rate over 150 minutes in 3-4%. Their study also showed that the consumption of green tea was able to increase energy expenditure. Therefore, these proved that body weight could be reduced through the intake of green tea.

Green tea contains EGCG and this compound is a type of flavanols, which inhibits the action of catechol-o-methyl-transferase (COMT) that degrades noradrenaline. When the amount of noradrenaline increases, a signal will be given to fat cells to break down fat. The fat will then be released into the bloodstream and used as energy by muscle cells. Therefore, it helps to reduce body weight. On the other hand, caffeine in green tea inhibits transcellular phosphodiesterases that break down intracellular cAMP and this causes high level of noradrenaline secretion, leading to increased thermogenesis and satiety (Dulloo *et al.*, 1999).

Several studies proved that the consumption of green tea in Caucasians, Chinese, Japanese and Thais helped to reduce their body weight by increasing energy expenditure and fat oxidation (Auvichayapat *et al.*, 2008). Furthermore, *in vitro* studies suggested that catechins especially EGCG in green tea helped to inhibit adipocyte differentiation and proliferation (Hung *et al.*, 2005), reduce fat absorption (Raederstorff *et al.*, 2003) and suppress COMT (Dulloo *et al.*, 2000). Meanwhile, *in vivo* animal studies showed that catechins especially EGCG in green tea were able to reduce fat mass (Klaus *et al.*, 2005), decrease TG level (Yang *et al.*, 2001), reduce NEFAs and TC level (Ashida *et*

al., 2004) as well as increase β-oxidation with improved exercise capacity (Murase *et al.*, 2006).

The effects of major green tea polyphenol, EGCG with a dose of 3.2 g/kg diet on HFD-induced obese mice (60% energy as fat) for 16 weeks were studied. According to Bose *et al.* (2008), the body weight, body fat and visceral fat weight of the mice were decreased with the increased of fecal lipids as compared to HFD control mice after EGCG treatment. The EGCG treatment also decreased plasma cholesterol, liver weight and liver TG in HFD mice. On the other hand, they also carried out another experiment by giving short term EGCG treatment to 3-month-old HFD-induced obese mice with a dose of 3.2 g/kg diet for 4 weeks and the result showed that the mesenteric fat weight of the mice was reduced as compared to HFD control mice. Their study showed that short term and long term EGCG treatments were able to prevent the development of obesity and other obesity complication signs. They also concluded that EGCG might reduce fat absorption, reduce inflammation, reduce fat formation and promote fat oxidation.

2.10.3 Guarana

Guarana is native to Amazonas State and it is able to boost energy. Guarana can be found in energetic beverages (Smith & Atroch, 2010). Besides, it is also the ingredient of dietary supplements as a weight loss-promoting adjuvant (Lima *et al.*, 2005). The Guarana's seed contains two times higher amount of caffeine (2-4.5%) compared to coffee beans (1-2%). It also contains methylxanthines, catechins, saponins, epicatechins and proanthocyanidols (Ângelo *et al.*, 2008). Several studies proved that Guarana is able to reduce body weight, increase energy expenditure and regulate lipid metabolism (Boozer *et al.*, 2001; Lima *et al.*, 2005). Guarana is also able to delay gastric emptying (Pittler *et al.*, 2005).

Based on a study conducted by Suleiman *et al.* (2016), TG level of overweight people who consumed Guarana was reduced 20% as compared to basal values. The reduction of the TG level might be due to the high concentration of caffeine, methylxanthines and catechins in Guarana (Bittencourt *et al.*, 2013). Another study showed that catechins in Guarana were able to prevent fat absorption in the intestine and inhibit the action of glycerol-3-phosphate dehydrogenase that catalyze β-nicotinamide adenine dinucleotide (NADH)-dependent reduction of dihydroxyacetone phosphate (DHAP) to produce glycerol-3-phosphate, which act as one of the vital precursors of triacylglycerol (Bérubé-Parent *et al.*, 2005; Kao *et al.*, 2010).

Moreover, a study on Guarana supplementation with a dose of 50 mg/kg b.w./day for 30 days in hypercholesterolemic rats was conducted and the results revealed that Guarana was able to lower TC, LDL-C and inflammatory process (Krewer *et al.*, 2014). Furthermore, the effect of decaffeinated Guarana and whole Guarana extracts on lipid metabolism in trained and sedentary rats was examined and it showed that the fat content and body weight of treated rats were significantly reduced after the rats were treated with whole Guarana extract (Lima *et al.*, 2005). The study depicted that the changes of lipid metabolism in treated rats were associated with the methylxanthine content of Guarana such as caffeine and theophylline. Theophylline is an adenosine receptor antagonist and it is able to increase lipolysis, which causes a body weight reduction.

Furthermore, *in vitro* and *in vivo* studies were performed to test the potential effects of Guarana on LDL oxidation. According to Portella *et al.* (2013), the *in vivo* study showed that healthy elderly people who ingested Guarana exhibited low LDL oxidation compared to the control subjects and this proved that the consumption of Guarana was able to provide antioxidant protection to LDL. This result was supported by their *in*

vitro data which showed that Guarana had high antioxidant activity mainly at the concentrations of 1 and 5 μg/mL and was able to improve lag phase of LDL in order to prevent LDL oxidation. They also suggested that long term exposure of LDL particles to Guarana polyphenols might make them less susceptible to oxidative stress.

2.10.4 Grape seed

Grape is one of the world's major fruit crops. Several grape products such as fruits, juice, raisins and wine are available in the market. Grape is a plant rich in phenolic compounds such as the flavonoids. Flavonoids such as (+)-catechins, (-)-epicatechin and procyanidin polymers can be found in grape seeds (Xia *et al.*, 2010). The flavonoids in grape seed are known to have strong antioxidant and anti-inflammatory effects *in vitro* and *in vivo* (Li *et al.*, 2001). A study showed that grape seed had the highest antioxidant capability, followed by its skin and its flesh (Pastrana-Bonilla *et al.*, 2003).

Procyanidins are the main phenolic compounds in grape seed and these compounds can be used to treat obesity. Preliminary study showed that procyanidins from grape seed were able to prevent low-grade inflammation *in vivo* by increasing anti-inflammatory molecules, reducing pro-inflammatory molecules and regulating adipose tissue cytokine imbalance in HFD-induced rats (Terra *et al.*, 2009). Grape-seed procyanidin extract (30 mg/kg/day) was also used to treat the rats fed with 60% kcal fat diet for 19 weeks and the result showed that the extract was able to decrease body weight and systemic inflammatory markers such as TNF-α and CRP (Terra *et al.*, 2011).

According to Moreno *et al.* (2003), the ethanol extract of grape seed which is rich in proanthocyanidins (condensed tannins) and other bioactive compounds was able to inhibit enzymatic activities of PL and LPL *in vitro* in order to prevent fat absorption and inhibit fat accumulation in adipose tissue. Besides, the extract was also able to prevent the activity of hormone-sensitive lipase in order to reduce the release of free fatty acids

from adipocytes into blood circulation by reducing lipolytic activity of cultured 3T3-L1 adipocytes (Moreno *et al.*, 2003). These results showed that ethanol extract of grape seed contained bioactive compounds which were able to inhibit lipases and the extract was effective in obesity treatment.

On the other hand, a study was conducted to determine the effects of phenolic Chardonnay grape seeds extract on obesity and oxidative stress in HFD-induced hamsters for 12 weeks. According to Décordé *et al.* (2009), the extract prevented the increased of abdominal fat, plasma glucose level, TG level, insulin level and insulin resistance value in obese hamsters as compared to the control group. The extract was able to reduce the levels of insulin and leptin by 16.5% and 45%, respectively. Besides, the extract was also able to reduce superoxide production and NADPH oxidase activity by 74% and 30%, respectively (Décordé *et al.*, 2009). Hence, the study proved that a phenolic extract of grape seed was able to treat obesity with the association of reducing oxidative stress.

2.10.5 Lotus leaves

N. nucifera (Gaertn.) is an aquatic herb and it belongs to the family Nymphaeaceae. It has some common names such as a Chinese water lily, sacred lotus and Indian lotus, but it is generally known as lotus (Moro *et al.*, 2013). Lotus leaf is traditionally used as folk medicine to treat obesity in China (Ono *et al.*, 2006). The lotus leaves are bitter and sweet. They contain flavonoids and alkaloids (Shoji *et al.*, 1987). Various bioactive compounds are identified from lotus leaves such as anonaine, nuciferine, pronuciferine, roemerine, N-nornuciferine, liriodenine, armepavine quercetin, leuco-anthocyanidin and leucodelphinidin (Mehta *et al.*, 2013).

Study depicted that the methanol extract of lotus leaves contained the highest TPC and TFC as compared to its seeds, stamens, embryos and rhizomes (Jung *et al.*, 2008).

According to Wu *et al.* (2003), *in vitro* assay showed that the methanol extract of lotus leaves had good abilities in scavenging free radicals, chelating metal and reducing metal. Their study also showed that the methanol extract of lotus leaves at concentrations of 0.1-0.3 mg/ml was able to protect Caco-2 cells from hydrogen peroxide. The ability of the extract in preventing cell damage caused by oxidative stress was due to the antioxidant properties of the extract (Wu *et al.*, 2003). Besides, their study also showed that the methanol extract of lotus leaves exhibited dose-dependent antioxidant activities against hemoglobin-induced linoleic acid peroxidation and Fenton reaction-mediated plasmid DNA oxidation (Wu *et al.*, 2003).

Lotus leaf extract has anti-obesity and anti-hyperlipidemia effects on rodents (Ono *et al.*, 2006). A study showed that lotus leaves were able to inhibit the activities of α-amylase and lipase, up-regulate lipid metabolism and increase expression of uncoupling protein-3 mRNA in C2C12 (mouse myoblast cell line) myotubes (Ono *et al.*, 2006). Another study showed that lotus leaf extract was able to reduce TC, phospholipids and TG levels (Onishi *et al.*, 1984). In addition, lotus leaf extract is able to trigger lipolysis and decrease adipogenesis in human pre-adipocytes (Siegner *et al.*, 2010). For example, a study showed that 50% ethanol extract of lotus leaves was able to stimulate lipolysis in white adipose tissue of mice and the bioactive compounds that contributed to the lipolytic activity were astragalin, catechin, hyperoside, isoquercitrin and quercetin-3-O-α-arabinopyranosyl-(1→2)-β-galactopyranoside (Ohkoshi *et al.*, 2007).

Furthermore, another study showed that lotus leaf flavonoids had a high percentage of *in vitro* porcine pancreatic lipase and α-amylase inhibitory activities with the IC_{50} values of 0.38 ± 0.022 and 2.20 ± 0.18 mg/mL, respectively (Liu *et al.*, 2013). Besides, the TC, TG, LDL-C and MDA levels of HFD Wistar rats were reduced and the level of HDL-C of HFD Wistar rats was increased after supplementation with lotus leaf

flavonoids (Liu *et al.*, 2013). The lipid accumulation in the liver of the HFD Wistar rats was also reduced after supplementation with lotus leaf flavonoids (Liu *et al.*, 2013).

2.10.6 Psyllium husk

Psyllium is a viscous and water-soluble fiber, obtained from the husks of the seeds of *Plantago ovate* (Slavin & Green, 2007). Psyllium can be transited throughout the gut without extensive fermentation compared to other viscous fibers (Fischer *et al.*, 2004). It is able to reduce blood lipids (Slavin & Green, 2007). A randomized, crossover, double-blind trial was conducted on 12 healthy people by giving them 10.8 g of psyllium husk and placebo, respectively. The result showed that psyllium husk was able to delay gastric emptying from the third hour after a meal, and increase satiety and reduce hunger at the sixth hour after the meal (Bergmann *et al.*, 1992).

Besides, a triple-blind crossover study was conducted by Turnbull and Thomas (1995) to compare the result of the subjects who received psyllium treatment with 200 mL of water, placebo with 200 mL of water, and 200 mL of water. A meal was then given at lunch time after 3 hours of treatment. The result showed that psyllium treatment provided significantly more satiety at 1 hour post-meal compared to the other two groups. On the day of the meal, the total fat intake was also significantly lower in grams per day and as a percentage of energy after psyllium treatment as compared to the subjects that received water only.

In addition, a double-blind study was conducted by giving a dose of 7.4 g of psyllium to normal volunteers. After a meal, the hungry feeling and energy intake of the psyllium treatment group were significantly reduced compared to placebo (Rigaud *et al.*, 1998). Moreover, a 24-hour appetite study was conducted by comparing the effect of psyllium husk with wheat bran fiber as breakfast. The result showed that the hungry feeling and

voluntary energy intake of the subjects were decreased after consumption of psyllium husk (Delargy *et al.*, 1997).

Another study was carried out to compare the metabolic effects of three types of fibers such as psyllium, sugar cane fiber and cellulose on carbohydrate metabolism, body weight and ghrelin gene expression in HFD mice. According to Wang *et al.* (2007), the body weight gains of mice treated with psyllium and sugar cane fiber were significantly lower than the mice treated with cellulose. There was no significant difference in body weight between mice treated with psyllium and sugar cane fiber. Besides, the fasting plasma concentration of leptin and ghrelin mRNA level in mice treated with psyllium and sugar cane fiber was significantly lower than mice treated with HFD and cellulose (Wang *et al.*, 2007). These suggested that psyllium supplementation was able to lower the body weight, fasting plasma concentration of leptin and ghrelin mRNA level of HFD mice.

Hypercholesterolemia is one of the risk factors for obesity. A study was conducted on men and women with hypercholesterolemia by giving 5.1 g of psyllium or cellulose placebo twice per day for 26 weeks. The result showed that the serum level of TC and LDL-C were 4.7% and 6.7% lower in the psyllium treatment group than the placebo group after 26 week treatment (Anderson *et al.*, 2000). Therefore, this study proved that psyllium husk was also able to reduce TC and LDL-C levels.

2.10.7 *Spirulina*

Spirulina is a microscopic filamentous blue-green alga or cyanobacterium. It is found in a very high pH alkaline lake (sometime reach pH 11). The pH prevents the growth of other algae (Belay *et al.*, 1993). *Spirulina* is traditionally used as food and it is safe for consumption. It contains 60-70% protein by weight, vitamins (pro-vitamin A), essential amino acids, minerals (iron) and essential fatty acids (γ-linolenic acid). *Spirulina* could

also be used to treat hypercholesterolemia and obesity. According to Colla *et al.* (2008), the therapeutic properties of *Spirulina* in obesity treatment and lipid profile improvement were due to the antioxidant abilities of polyunsaturated fatty acids (γ-linolenic acid), phycocyanin and phenolic compounds in *Spirulina*.

Based on a double-blind-crossover study versus placebo group, researchers found out that the consumption of 2.8 g of *Spirulina* at 3 times per day over 4 weeks led to significant body weight reduction in obese patients (Becker *et al.*, 1986). Besides, a study was conducted on 30 obese patients and the result showed that the consumption of *Spirulina* was able to lower the serum levels of TC and LDL-C as well as increase HDL-C (Nakaya *et al.*, 1988). Another study showed that the consumption of *Spirulina* for 4 weeks was able to lower the level of TG in fructose-induced hyperlipidemic rats with significant increase in LPL activity (Iwata *et al.*, 1990). Furthermore, a study showed that water extract of *Spirulina* was able to suppress PL activity that hydrolyzes dietary fat. Without hydrolysis, the dietary fat could not be absorbed in the intestine. Thus, body weight gain was prevented (Miranda *et al.*, 1998).

Moreover, *Spirulina platensis* contains phycobiliproteins such as phycocyanin and allophycocyanin (Boussiba & Richmond, 1979; Brejc *et al.*, 1995). Both of these compounds are accessory pigment to chlorophyll. A preliminary study showed that protean extract of *S. platensis* exerted a strong free radical scavenging effect and inhibited lipid peroxidation (Estrada *et al.*, 2001). Another study was conducted and researchers found out that phycocyanin was the main compound that contributed to antioxidant activity and the increase of phycocyanin content was correlated to the increased antioxidant activity (Estrada *et al.*, 2001). Furthermore, a study showed that chromophore phycocyanobilin obtained from *Spirulina* was a potent inhibitor of NADPH oxidase, thus consumption of *Spirulina* could inhibit NADPH oxidase activity

that activated by fat accumulation in obese patients (McCarty, 2007). In addition, *Spirulina* contains high amount of phytopigments like chlorophyll, carotenoids and phycocyanin which help to increase antioxidant enzyme activities such as SOD, CAT and GPx (Hirahashi *et al.*, 2002).

According to Miranda *et al.* (1998), *Spirulina maxima* possess phenolic acids, tocopherols and β-carotene. These compounds exert antioxidant properties. Study on the *in vitro* and *in vivo* antioxidant capability of methanolic extract of *S. maxima* has been implemented by measuring the reduction of thiobarbituric acid reactive substances (TBARS) in brain homogenate. From the result of *in vitro* antioxidant activity, lipid peroxidation of brain homogenate was inhibited by almost 95% with 0.5 mg of methanolic extract of *S. maxima* and the IC_{50} for lipid peroxidation inhibition was 0.18 mg (Miranda *et al.*, 1998). From the result of *in vivo* antioxidant activity, lipid peroxidation of brain homogenate was inhibited by 97% and 71% for experimental groups, while it was inhibited by 74% and 54% for control groups after 2 and 7 weeks of treatment with daily dose of 5 mg of *S. maxima* methanolic extract, even though there was no significant difference in TBARS formation from liver homogenates between the experimental and the control groups (Miranda *et al.*, 1998).

CHAPTER 3: METHODOLOGY

3.1 Materials and standard drug

HP was provided by GlucosCare (M) Sdn. Bhd. Each capsule of the HP contained 42 mg of each citrus, grape seed, green tea, Guarana, lotus leaves and *Spirulina*, and 48 mg of psyllium husk. Simvastatin (Pharmaniaga Berhad, Malaysia) for treatment study was purchased from a pharmacist.

3.2 Chemicals and reagents

DPPH, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), ferrozine, nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), sodium nitroferricyanide, curcumin, gallic acid, vanillin, basic bismuth nitrate, potassium iodide, sodium acetate, potassium hydrogen phosphate, potassium dihydrogen phosphate, Tris-base and Griess reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC grade of formic acid, water and acetonitrile, dimethyl sulfoxide (DMSO), Tween 80 and Folin-Ciocalteu reagent were purchased from Merck (Darmstadt, Germany).

Analytical grade of methanol, hexane, chloroform, dichloromethane, ethyl acetate, n-butanol, sulfuric acid (H_2SO_4), glacial acetic acid, absolute ethanol, and hydrochloride acid (HCl) were purchased from Classic Chemicals Sdn. Bhd., Malaysia. Chemicals such as sodium carbonate, sodium nitrate, aluminum chloride, quercetin, sodium hydroxide, ascorbic acid, ferric chloride ($FeCl_3$), ferrous sulfate, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-Na_2), ferrous chloride ($FeCl_2$) and nicotinamide adenine dinucleotide (NADH) were also purchased from Classic Chemicals Sdn. Bhd., Malaysia.

3.3 Preparation of herbal powder

The capsule of the HP was discarded, while the herbal powder was kept in an airtight container under cool and dry place before it was used.

3.4 Preparation of crude 10% methanol extract (10% ME) and crude water extract (WE)

The obtained herbal powder (50 g) was extracted with 10% methanol (300 mL) in a beaker. The mixture was stirred for 30 minutes by using magnetic stirrer and then left at room temperature overnight. After that, the mixture was centrifuged at 3500 × g for 15 minutes and the supernatant was collected. The pellet from the mixture was extracted again with 10% methanol (300 mL) and the following steps as above were repeated. The extraction process was repeated thrice. The same extraction steps were carried out in the preparation of crude WE.

The three batches of supernatants from 10% methanol extraction (crude 10% ME) were filtered by using Whatman filter paper No. 3 and then pooled together for fractionation as described in Section 3.5 before running TLC. Another three batches of supernatants from water extraction (crude WE) were filtered by using Whatman filter paper No. 3, then pooled together and sent for freeze drying under normal procedure after running TLC as described in Section 3.6.1.

3.5 Fractionation

The filtered 10% ME was fractionated sequentially with hexane (250 mL), chloroform (250 mL), dichloromethane (250 mL), ethyl acetate (250 mL) and n-butanol (250 mL), in order to obtain soluble fraction of hexane (HF), chloroform (CF), dichloromethane (DCMF), ethyl acetate (EAF) and n-butanol (BF), respectively. The final residue layer was obtained as a methanol aqueous fraction (MAF). The whole fractionation process above was repeated thrice and each type of obtained soluble

fraction was filtered by using Whatman filter paper No. 3 and pooled together in a Schott bottle. All the six different types of fractions (HF, CF, DCMF, EAF, BF and MAF) were then used to run TLC as described in Section 3.6.1. After TLC analysis, HF, CF, DCMF, EAF, BF and MAF were concentrated at 40 °C by using a rotary evaporator (Rotavapor R-200, Buchi) and then air-dried in fume hood, while WE was sent to freeze dry. The dried HF, CF, DCMF, EAF, BF and MAF and the freeze-dried WE were then stored at 0 ºC and ready to be used for further analysis.

3.6 Preliminary screening of phytochemical compounds

3.6.1 TLC

HF, CF, DCMF, EAF, BF, MAF and WE were subjected to TLC analysis. TLC analysis was carried out as described by Bahrani *et al.* (2014). Each test sample was spotted on the sample line of aluminium-backed silica gel TLC plate (Merck, Germany) using a tapered capillary tube. The TLC plates were then transferred to a covered jar which filled with a solvent such as chloroform and 10% methanol in chloroform, respectively. When the solvent reached solvent line, the TLC plate was immediately taken out from the covered jar. The TLC plate was then dried in a fume hood and colored bands were identified under visible light and UV light (254 nm). After that, each TLC plate was sprayed with the prepared visualizing reagents such as Dragendorff, Vanillin-H_2SO_4 and Folin-Ciocalteu reagents (Appendix A), respectively. The TLC plates sprayed with Dragendorff and Folin-Ciocalteu reagents were dried in a fume hood and the colored bands were marked, whereas the plates sprayed with Vanillin-H_2SO_4 reagent were heated for 3 minutes at 110 °C and the colored bands were marked (Pyka, 2014). Analysis of phytochemical compounds was done by calculating the retention factor (R_f) value of each band using the formula (2) below:

$$R_f = \frac{\text{Distance Travelled of the Compound}}{\text{Distance Travelled of the Solvent}} \quad (2)$$

3.6.2 LC/Q-TOF/MS

The freeze-dried WE was dissolved in HPLC grade of water and sent for LC/Q-TOF/MS analysis using the facility available at the Infra Analysis Laboratory, University of Malaya. The instrument used was Liquid Chromatography coupled with Agilent 6550 Q-TOF mass spectrometer. A 10 μL aliquot of WE (10 ppm) was injected and analyzed on C18 column. The separation was conducted at 30°C (column temperature) using a gradient elution method with 0.1% formic acid in water (solvent A) and 100% acetonitrile (solvent B). The solvent gradient in volumetric ratios was set as follows: 0-1 min at 99% A; 1-12 min from 99% A to 0% A; 12-12.5 min from 0% A to 99% A; 12.5-13.5 min at 99% A. The flow rate was 0.3 mL/min.

3.7 *In vitro* studies

3.7.1 Preparation of test samples

The dried HF, CF, DCMF, EAF and BF were dissolved in methanol for DPPH radical scavenging assay. However, the dried HF, CF, DCMF, EAF and BF were dissolved in DMSO for TPC, TFC and all the conducted antioxidant assays as described under Section 3.7.4.

The HF, CF, DCMF, EAF and BF dissolved in DMSO were diluted using distilled water for TPC, TFC, FRAP and metal chelating assays. On the other hand, the HF, CF, DCMF, EAF and BF dissolved in DMSO were diluted using prepared phosphate-buffered saline (PBS) (Appendix A) for NO scavenging assay and diluted using prepared Tris-HCl buffer (Appendix A) for superoxide scavenging assay. The final DMSO concentration did not exceed 0.1% (Chou *et al.*, 2011).

The dried MAF and the freeze-dried WE were dissolved in distilled water for TPC, TFC and all the conducted antioxidant assays, except NO and superoxide scavenging assays. The dried MAF and freeze-dried WE were dissolved in PBS for NO scavenging

assay, while the dried MAF and freeze-dried WE were dissolved in Tris-HCl buffer for superoxide scavenging assay.

3.7.2 Determination of TPC

The TPC of the seven test samples was determined using the Folin-Ciocalteu method described by Ablat *et al.* (2014) with slight modification. It was carried out in the dark. Gallic acid (5 mg) was prepared by dissolving it in 1 mL of methanol, and it was further diluted with distilled water to concentrations of 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 mg/mL. A volume of 20 µL of gallic acid or blank or test sample (1 mg/mL) was pipetted into the well of a 96-well microtiter plate, followed by the addition of 100 µL Folin-Ciocalteu reagent (diluted 10 fold with distilled water). The mixture was mixed thoroughly using a microplate reader (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek). The mixture was then incubated for 5 minutes at room temperature. After that, 75 µL of sodium carbonate solution (0.075 g/mL) was added into each well and the mixture was mixed and incubated for 2 hours at room temperature. The absorbance was measured at 740 nm with the microplate reader. Gallic acid was used as a standard to plot calibration curve, while test sample was replaced with distilled water as blank. The TPC of each test sample was estimated from calibration curve, and the result was expressed as milligram gallic acid equivalent per gram of dried sample (mg GAE/g). The TPC of the test samples was calculated using the formula (3) below (Iqbal *et al.*, 2012):

$$\text{TPC} = \frac{\text{C} \times \text{V}}{\text{M}} \quad (3)$$

where, C is the concentration of gallic acid obtained from the calibration curve (mg/mL); V is the volume of the test sample (mL) and M is the dried weight of the test sample (g).

3.7.3 Determination of TFC

The TFC of the seven test samples was determined using the aluminum chloride colorimetric method described by Ablat *et al.* (2014) with slight modification. It was carried out in the dark. Quercetin (5 mg) was prepared in 1 mL of methanol, and it was further diluted to concentrations of 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 mg/mL using distilled water. A volume of 50 µL of quercetin or blank or test sample (1 mg/mL) was added into the well of a 96-well microtiter plate, followed by the addition of 70 µL of distilled water and 15 µL of 5% sodium nitrate solution. The mixture was mixed thoroughly using a microplate reader (Tecan Sunrise, Austria) and incubated for 5 minutes at room temperature. Then, 15 µL of 10% aluminum chloride solution was added into the mixture and mixed. After 6 minutes of incubation, another 100 µL of 1M sodium hydroxide solution was added and mixed well. The absorbance was then measured at 510 nm with the microplate reader. Quercetin was used as a standard to plot calibration curve, while test sample was replaced with distilled water as blank. The TFC of each test sample was estimated from calibration curve, and the result was expressed as milligram quercetin equivalent per gram of dried sample (mg QE/g). The TFC of the test samples was calculated by using the formula (4) below (Abebe *et al.*, 2017):

$$\text{TFC} = \frac{\text{C} \times \text{V}}{\text{M}} \quad (4)$$

where, C is the concentration of quercetin obtained from the calibration curve (mg/mL); V is the volume of the test sample (mL) and M is the dried weight of the test sample (g).

3.7.4 Determination of antioxidant activities

3.7.4.1 DPPH radical scavenging assay

The DPPH radical scavenging activity of the seven test samples (0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 mg/mL) was determined according to the method described by Ablat *et al.* (2014). It was carried out in the dark. 40 µL of test sample or ascorbic acid (0.05, 0.1,

0.2, 0.4, 0.8 and 1.6 mg/mL) or negative control was added into the well of a 96-well microtiter plate, followed by the addition of 200 µL of 50 µM DPPH in methanol. The mixture was immediately mixed using a microplate reader (Tecan Sunrise, Austria) and incubated for 15 minutes at room temperature. The absorbance was then measured at 517 nm with the microplate reader. Ascorbic acid in methanol was used as positive control, while test sample was replaced with methanol as negative control. The percentage (%) of DPPH radical inhibition of the seven samples was calculated according to equation (5) below (Abebe *et al.*, 2017):

$$\text{DPPH Radical Scavenging Activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100\% \quad (5)$$

where, A_0 is the absorbance of negative control and A_1 is the absorbance of test sample or positive control.

The graph of the % of DPPH radical inhibition against the concentration of test samples and positive control was plotted. The concentration of samples required to scavenge 50% of DPPH radical (IC_{50}) was determined and the value was compared with IC_{50} of ascorbic acid.

3.7.4.2 FRAP assay

The FRAP activity of the seven test samples was determined using the method described by Ablat *et al.* (2014). It was carried out in the dark. Ferrous sulfate was dissolved in distilled water and further diluted into different concentrations (0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 mg/mL). 20 µL of ferrous sulfate or blank or test sample (1 mg/mL) was pipetted into the well of 96-well microtiter plate, followed by the addition of 200 µL of freshly prepared FRAP reagent which consisted of 2.5 mL of 10 mM TPTZ in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3$ and 25 mL of prepared 0.3 M acetate buffer (pH 3.6) (Appendix A). The mixture was mixed thoroughly using a microplate reader (Tecan Sunrise, Austria). After 8 minutes of incubation, the absorbance was measured at 595

nm with the mircoplate reader. Ferrous sulfate solution was used as a standard to plot calibration curve, while test sample was replaced with distilled water as blank. The FRAP value of each test sample was estimated from the calibration curve and it was expressed as micromole Fe^{2+} per gram of dried sample (μmol Fe^{2+}/g). The FRAP value of each test sample was calculated using the formula (6) below (Abebe *et al.*, 2017):

$$\text{FRAP} = \frac{\text{C} \times \text{V}}{\text{M}} \quad (6)$$

where, C is the concentration of quercetin obtained from the calibration curve (mg/mL); V is the volume of the test sample (mL) and M is the dried weight of the test sample (g).

3.7.4.3 Metal chelating assay

The ferrous ion chelating activity of the seven test samples (0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 mg/mL) was determined according to the method described by Ablat *et al.* (2014). It was carried out in the dark. EDTA-Na_2 was prepared in distilled water and further diluted into different concentrations (0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 mg/mL). 100 μL of test sample or EDTA-Na_2 or negative control was pipetted into the well of a 96-well microtiter plate, followed by the addition of 120 μL of distilled water and 10 μL of 2mM $FeCl_2$. Then, 20 μL of 5 mM ferrozine was added into the mixture and mixed using a microplate reader (Tecan Sunrise, Austria). After 20 minutes of incubation at room temperature, the absorbance was measured at 562 nm with the microplate reader. EDTA-Na_2 was used as positive control, while test sample was replaced with distilled water as a negative control. On the other hand, blank was prepared by adding 20 μL of distilled water instead of ferrozine. The % of the formation of Fe^{2+}-ferrozine complex was calculated according to the following equation (7) below:

$$\%\ \text{of ferrous ion chelating activity} = \frac{\text{A}_0 - \text{A}_1}{\text{A}_0} \times 100\% \quad (7)$$

where, A_0 is the absorbance of negative control and A_1 is the absorbance of test sample or positive control.

The graph of the % of ferrous ion chelating activity against the concentration of test samples and positive control was plotted. The concentration of each test sample required to chelate 50% of Fe^{2+} ion (IC_{50}) was determined and the value was compared with IC_{50} of EDTA-Na_2.

3.7.4.4 NO radical scavenging assay

NO radical scavenging activity of the seven test samples (0.2, 0.4, 0.6, 0.8 and 1 mg/mL) was determined using the method described by Ablat *et al.* (2014) with slight modification. It was carried out in the dark. 50 µL of test sample or curcumin (0.2, 0.4, 0.6, 0.8 and 1 mg/mL) or negative control was pipetted into the well of a 96-well microtiter plate, followed by the addition of 50 µL of sodium nitroferricyanide (10 mM) in 20 mM PBS (pH 7.4). The mixture was mixed using a microplate reader (Tecan Sunrise, Austria) and incubated at room temperature for 150 minutes, followed by the addition of 125 µL of Griess reagent. After 10 minutes of incubation, the absorbance was measured at 546 nm with the microplate reader. Curcumin in methanol was used as positive control, while test sample was replaced with PBS as negative control. Blank was prepared by adding all the reaction mixture without Griess reagent. The % of NO radical inhibition was calculated according to the equation (8) below:

$$\% \text{ of NO radical scavenging activity} = \frac{A_0 - A_1}{A_0} \times 100\% \quad (8)$$

where, A_0 is the absorbance of negative control and A_1 is the absorbance of test sample or positive control.

The graph of the % of NO radical scavenging activity against the concentration of test samples and positive control was plotted. The concentration of each sample required to scavenge 50% of NO (IC_{50}) was determined and the value was compared with IC_{50} of curcumin.

3.7.4.5 Superoxide radical scavenging assay

Superoxide radical scavenging activity of the seven test samples (0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 mg/mL) was measured using the NBT reduction method described by Balogun and Ashafa (2016). It was carried out in the dark. Gallic acid was dissolved in Tris-HCl buffer and then further diluted into different concentrations (0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 mg/mL) using Tris-HCl buffer. 50 µL of each 0.3 mM NBT, 0.936 mM NADH, 16 mM Tris-HCl buffer (pH 8) and 0.12 mM PMS was pipetted into the well of a 96-well microtiter plate, followed by the addition of 100 µl of test sample or gallic acid or negative control. The mixture was mixed thoroughly using a microplate reader (Tecan Sunrise, Austria). After 5 min of incubation at room temperature, the absorbance was then measured at 560 nm with the microplate reader. Gallic acid was used as positive control, while test sample was replaced with Tris-HCl buffer as negative control. The % of superoxide radical inhibition was calculated according to the equation (9) below:

$$\% \text{ of superoxide radical scavenging activity} = \frac{A_0 - A_1}{A_0} \times 100\% \quad (9)$$

where, A_0 is the absorbance of negative control and A_1 is the absorbance of test sample or positive control.

The graph of the % of superoxide radical scavenging activity against the concentration of test samples and positive control was plotted. The concentration of each sample required to scavenge 50% of superoxide (IC_{50}) was determined and the value was compared with IC_{50} of gallic acid.

3.8 Animal studies

SD rats at the age of 8 weeks with the body weight around 200-250 g were purchased and kept in cages at ambient temperature with 12 hours/12 hours light/dark cycle. They were fed with ND pellet 702P (Gold Coin) and tap water *ad libitum*. The rats were

maintained in animal house until they acclimatized to the laboratory conditions for five days (Organisation for Economic Co-operation Development (OECD), 2002). After acclimatization, the rats were divided into different groups for acute toxicity test and for obesity induction and treatment studies. Before giving treatment, the rats were weighed. The volume of the sample given was 10 ml/kg b.w. of the rat (Erhirhie *et al.*, 2014). The experimental procedures under an ethic number of S/16012017/10102016-01/R were implemented according to the guidelines from "Guide for the Care and Use of Laboratory Animals (Eighth Edition)" and the procedures were approved by the Institutional Animal Care and Use Committee, University of Malaya (UM IACUC).

3.8.1 Oral acute toxicity test

The oral acute toxicity test was conducted according to the "OECD guidelines 423" (OECD, 2002). After acclimatization, male and female SD rats were divided into two groups as shown in Table 3.1 with 3 rats in each group (n = 3). The rats in both of the groups were fed with ND pellet and tap water *ad libitum*. The rats were fasted overnight (16 hours) and weighed before oral administration.

Table 3.1: Groupings for oral acute toxicity test.

Group (s)	Treatment
Male rats	WE (5g/kg b.w.; p.o)
Female rats	WE (5g/kg b.w.; p.o)

WE: water extract in distilled water; b.w.: body weight; p.o: perorally

The acute toxicity test was performed by administrating a single dose of WE (5 g/kg b.w. of rat) via oral gavage to the rats in both of the groups on the first day of treatment. Each rat was observed after oral administration during the first 30 minutes, periodically during the first 24 hours with special attention spent for the first 4 hours, and daily afterwards for a total of 14 days (OECD, 2002). Observations on food and water consumption, feces and urine, shivering, gasping, response to touch and changes on

skin, fur, eyes and mucous membranes as well as lethality and length of recovery of the rats were recorded. The body weight of the rats was also recorded once every 2 days.

3.8.2 Obesity induction

After acclimatization, the male SD rats were separated into ND group and HFD group. Obesity induction was conducted for 8 weeks before giving treatment. During obesity induction, the rats in the ND group were fed with ND pellet *ad libitum*, whereas the rats in the HFD group were fed with handmade HFD pellet *ad libitum*.

The HFD pellet was made based on the composition provided by Kadir *et al.* (2015) with modification. It was prepared by mixing 52% of powdered ND pellet 702P (Gold Coin), 16% of Tapioca Starch, 24% of maize oil (Mazola), 4% of ghee (Crispo) and 4% from 50% (w/v) palm sugar (Windmill) solution. All the ingredients for making the HFD pellet were mixed and then baked overnight in oven at 62ºC. The HFD pellet contained 482.0 kcal/100 g with 51.6% of carbohydrate, 13.1% of protein and 35.3% of fat as shown in Table 3.2. Meanwhile, ND pellet contained 306.2 kcal/100 g with 48.8 % of carbohydrate, 21 % of protein and 3 % of fat as shown in Table 3.2.

Table 3.2: Composition of HFD and ND pellets.

HFD Pellet		ND Pellet	
Nutrient	**%/100 g**	**Nutrient**	**%/100 g**
Carbohydrate	51.6	Carbohydrate	48.8
Protein	13.1	Protein	21
Fat	35.3	Fat	3
Ingredients	**g/100 g**	Calcium	0.8
Normal diet pellet	52	Phosphorus	0.4
Tapioca Starch	16	Fiber	5
Maize oil	24	Moisture	13
Ghee	4	Ash	8
Palm sugar solution	4		
Total Energy (kcal/100 g)	482.0	Total Energy (kcal/100 g)	306.2

The body weight and body length of the rats in ND and HFD groups were measured weekly. After 8 weeks of obesity induction, the HFD rats whose body weight were 20% higher than the average body weight of rats in the ND group (Sun *et al.*, 2012) and whose BMI ranged above 0.68 ± 0.05 g/cm^2 (Novelli *et al.*, 2007) were verified as obese rats.

3.8.3 Treatment with WE

The ND rats and verified obese rats were separated into five groups as shown in Table 3.3 with six rats per group (n = 6).

Table 3.3: Groupings for WE treatment.

Group (s)	Treatment
1 (NC)	ND (tap water)
2 (HFC)	HFD (tap water)
3 (ND + WE)	ND + WE (250 mg/kg b.w., p.o.)
4 (HFD + WE)	HFD + WE (250 mg/kg b.w., p.o.)
5 (HFD + Simvastatin)	HFD + Simvastatin (3 mg/kg b.w., p.o.)

NC: normal diet control; HFC: high fat diet control; ND: normal diet; HFD: high fat diet; WE: water extract in distilled water; b.w.: body weight; p.o.: perorally

Group 1 (NC) represented ND control group in which the rats were provided with ND pellet, group 2 (HFC) represented HFD control group in which the rats were provided with HFD pellet, group 3 (ND + WE) represented treatment group in which the rats were administrated with WE (250 mg/kg b.w.) once daily via oral gavage along with ND pellet, group 4 (HFD + WE) represented treatment group in which the rats were administrated with WE (250 mg/kg b.w.) once daily via oral gavage along with HFD pellet and group 5 (HFD + Simvastatin) represented standard group in which the rats were administrated with simvastatin (3 mg/kg b.w.) once daily via oral gavage along with HFD pellet (Bais *et al.*, 2014). All the rats were fed with pellet and tap water *ad libitum*. WE and simvastatin were prepared by dissolving it in distilled water.

The treatment study was implemented for 2 weeks. Observations on food and water consumption, feces and urine, shivering, gasping, response to touch and changes on skin, fur, eyes and mucous membranes as well as lethality and length of recovery of the rats were recorded daily.

3.8.4 Treatment with EAF

The ND rats and verified obese rats were separated into four groups as shown in Table 3.4 with six rats per group (n = 6).

Table 3.4: Groupings for EAF treatment.

Group (s)	Treatment
1 (ND + 2% Tween 80)	ND + 2% Tween 80
2 (HFD + 2% Tween 80)	HFD + 2% Tween 80
3 (ND + EAF)	ND + EAF (250 mg/kg b.w., p.o.)
4 (HFD + EAF)	HFD + EAF (250 mg/kg b.w., p.o.)

ND: normal diet; HFD: high fat diet; EAF: ethyl acetate fraction in 2% Tween 80; b.w.: body weight; p.o.: perorally

Group 1 (ND + 2% Tween 80) represented ND vehicle control group in which the rats were administrated with 2% Tween 80 in distilled water once daily via oral gavage along with ND pellet, group 2 (HFD + 2% Tween 80) represented HFD vehicle control group in which the rats were administrated with 2% Tween 80 in distilled water once daily via oral gavage along with HFD pellet, group 3 (ND + EAF) represented treatment group in which the rats were administrated with EAF (250 mg/kg b.w.) once daily via oral gavage along with ND pellet and group 4 (HFD + EAF) represented treatment group in which the rats were administrated with EAF (250 mg/kg b.w.) once daily via oral gavage along with HFD pellet. All the rats were fed with pellet and tap water *ad libitum*. EAF was prepared by dissolving it in 2% Tween 80 (Badole *et al.*, 2015).

The treatment study was implemented for 2 weeks. Observations on food and water consumption, feces and urine, shivering, gasping, response to touch and changes on skin, fur, eyes and mucous membranes as well as lethality and length of recovery of the rats were recorded daily.

3.9 Analyses during and after treatment studies

3.9.1 Food intake, body weight, body length and BMI

The amount of pellet given (g) was recorded and subtracted with the amount of leftover pellet (g) that weighed daily in order to calculate the daily food intake of each rat within 24 hours. On the other hand, the body weight of each rat was weighed and recorded on the first day before treatment, and once every 3 days during treatment phase. The rats were fasted overnight (16 hours) before weighing. Body length (cm) was also measured from nasal to anus of each rat on the first day of treatment and on the day of scarification in order to measure BMI. The BMI for normal adult rat ranged between 0.45 ± 0.02 and 0.68 ± 0.05 g/cm^2, while the BMI for obese rats ranged above 0.68 ± 0.05 g/cm^2 (Novelli *et al.*, 2007). BMI of each rat was calculated using the formula (10) below:

$$\text{BMI} = \frac{\text{Body weight (g)}}{\text{Body Length}^2\ (\text{cm}^2)} \qquad (10)$$

3.9.2 Preparation of blood and serum samples

On the 15th day of the experiment, the rats were sacrificed using cervical dislocation method. Blood was collected immediately from the rats, which were fasted for 16 hours before scarification by carotid bleeding. The collected blood was allowed to coagulate for 30 minutes at room temperature. It was then centrifuged at 1450 × g for 10 minutes in order to obtain blood serum. After centrifugation, the blood serum was aspirated out from the serum separating tube and transferred into 1.5 mL micro-centrifuge tubes. All

the blood serum samples were stored at -80 ºC before they were sent for lipid profile analysis which was described under Section 3.9.3.

3.9.3 Biochemical test for lipid profile

The blood serum samples were sent to the Division of Laboratory Medicine, University of Malaya Medical Centre for analysis on the lipid profile levels of TG, TC, LDL-C and HDL-C of the rats.

3.10 Statistical analysis

Statistical analysis was carried out using SPSS 20 for Windows. Data analysis for *in vitro* studies was performed in triplicate and the results were expressed in mean ± standard deviation (SD), except for animal studies which were performed in sextuplicate. Tests were carried out at 5% significance level, $\alpha = 0.05$ for data analysis. One-way ANOVA followed by Fisher's Least Significant Difference (LSD) test and Duncan's multiple range test were carried out to test for significant differences of samples between groups, while one-way ANOVA followed by Dunnett's multiple comparison test were carried out to test for significant differences of samples between control and experimental groups. In addition, paired-sample t-test was carried out to test for significant differences between initial and final values. The correlations between antioxidant capacities, TPC and TFC were analyzed using Pearson's correlation test. On the other hand, the IC_{50} values were calculated using Microsoft Excel.

CHAPTER 4: RESULTS

4.1 Extractions

Crude WE and crude 10% ME were obtained from the herbal powder extractions with distilled water and 10% methanol, respectively. A clear dark brown solution was collected after the filtration of crude WE. The filtered WE gave a flask of light brown crystal (36.01 g) with the yield of 27.98% after freeze drying. On the other hand, a clear dark green solution was collected after the filtration of crude 10% ME. The filtered 10% ME was pooled together and subjected for fractionation.

4.2 Fractionation

The filtered 10% ME was fractionated sequentially with hexane, chloroform, dichloromethane, ethyl acetate and butanol. Two immiscible layers (organic layer and aqueous layer) were formed in each time of fractionation. During hexane fractionation, an organic layer which contained dissolved compounds in hexane was formed above the aqueous layer. Same condition was observed during fractionation with ethyl acetate and butanol. However, an organic layer which contained dissolved compounds in chloroform was formed below the aqueous layer during chloroform fractionation. Same condition was observed during dichloromethane fractionation. On the other hand, foam was formed between the two immiscible layers during fractionation with chloroform and dichloromethane (Figure 4.1).

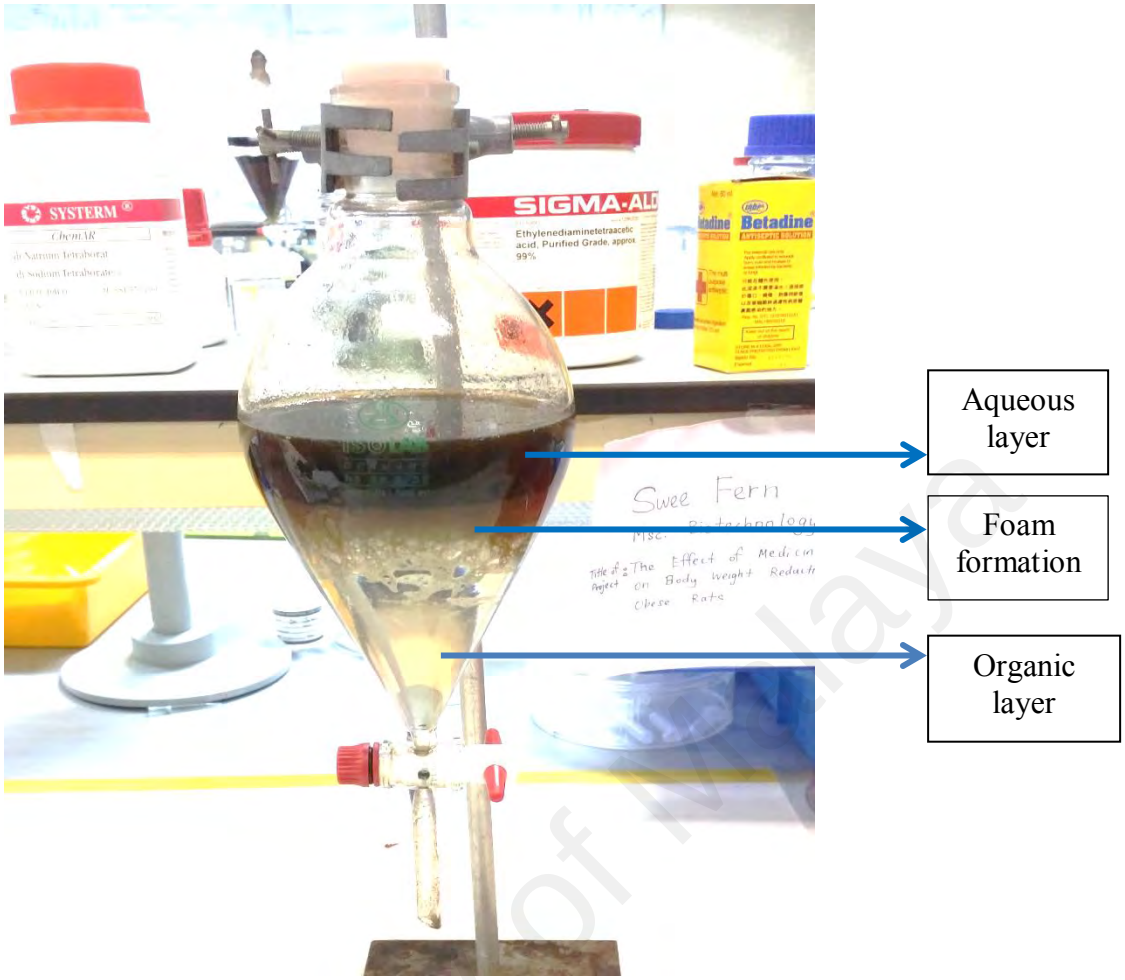

Figure 4.1: Foam was formed between two immiscible layers during chloroform fractionation.

Figure 4.2: The soluble fractions of hexane, chloroform, ethyl acetate, butanol and dichloromethane were obtained.

Six different soluble fractions such as HF (colorless solution), CF (light yellow solution), DCMF (very light yellow solution), EAF (golden yellow solution), BF (brownish yellow solution) and MAF (dark brown solution) were obtained from the fractionations of filtered 10% ME (Figure 4.2). The yield and color of each dried fraction and freeze-dried crude WE were recorded in Table 4.1.

Table 4.1: The yield and color of dried fractions and crude WE.

Fraction (s) /Crude Extract	Yield (g)	Observation
HF	0.02	Colorless oil
CF	0.26	Light yellow fibrous powder
DCMF	0.03	Yellowish green fibrous powder
EAF	0.14	Yellowish brown sticky semisolid
BF	0.45	Brownish orange sticky semisolid
MAF	28.06	Dark brown crystal
WE	36.01	Light brown crystal

HF: hexane fraction; CF: chloroform fraction; DCMF: dichloromethane fraction; EAF: ethyl acetate fraction; BF: butanol fraction; MAF: methanol aqueous fraction; WE: water extract.

4.3 Preliminary screening of phytochemical compounds

4.3.1 TLC

HF, CF, DCMF, EAF, BF, MAF and WE were subjected to TLC analysis. The results of TLC analysis for each fraction and crude WE are shown in Table 4.2 to Table 4.15 (Appendix B to Appendix E).

Table 4.2: TLC analysis of HF in chloroform solvent.

Compound	R_f value	Visible light	UV light (254 nm)	Visualizing Reagents			Presence of compound
				Dragendorff	Vanillin-H_2SO_4	Folin-Ciocalteu	
1	0.15	ND	ND	ND	Dark blue (+)	ND	Terpenoid
2	0.18	ND	ND	ND	Purple (+)	ND	Terpenoid
3	0.25	ND	ND	ND	Purple (+)	ND	Terpenoid
4	0.35	ND	ND	ND	Purple (+)	ND	Terpenoid
5	0.52	ND	ND	ND	Dark blue (+++)	ND	Terpenoid
6	0.58	ND	ND	ND	Purple (+++)	ND	Terpenoid
7	0.81	ND	ND	ND	Blue (+)	ND	Terpenoid
8	0.93	ND	ND	ND	Dark blue (++)	ND	Terpenoid

Indication of intensity of band's color:

+++ = Strong ++ = medium + = weak ND = No band was observed

Table 4.3: TLC analysis of HF in methanol: chloroform (1: 9) solvent.

Compound	R_f value	Visible light	UV light (254 nm)	Visualizing Reagents			Presence of compound
				Dragendorff	Vanillin-H_2SO_4	Folin-Ciocalteu	
1	0.36	ND	ND	Orange (++)	ND	ND	Alkaloid
2	0.56	ND	ND	Orange (+)	ND	ND	Alkaloid
3	0.68	ND	ND	ND	Dark blue (+)	ND	Terpenoid
4	0.81	ND	ND	ND	Dark blue (+++)	ND	Terpenoid
5	0.85	ND	ND	ND	Purple (+++)	ND	Terpenoid
6	0.94	ND	Red (++)	ND	Dark blue (++)	ND	Terpenoid
7	0.98	ND	Red (++)	ND	ND	ND	Natural fluorescence compounds

Indication of intensity of band's color:

+++ = Strong ++ = medium + = weak ND = No band was observed

Table 4.4: TLC analysis of CF in chloroform solvent.

Compound	R_f value	Visible light	UV light (254 nm)	Visualizing Reagents			Presence of compound
				Dragendorff	Vanillin-H_2SO_4	Folin-Ciocalteu	
1	0.02	ND	Light red (++)	ND	ND	ND	Natural fluorescence compounds
2	0.04	ND	Light red (++)	ND	Dark purple (++)	ND	Terpenoid
3	0.06	ND	Purple (++)	ND	ND	ND	Natural fluorescence compounds
4	0.09	ND	Purple (++)	ND	Dark purple (++)	ND	Terpenoid
5	0.11	ND	Green (++)	ND	ND	ND	Natural fluorescence compounds
6	0.14	ND	Dark blue (++)	ND	ND	ND	Natural fluorescence compounds
7	0.15	ND	Light green (+)	ND	Dark purple (+)	ND	Terpenoid
8	0.20	ND	Blue (++)	ND	ND	ND	Natural fluorescence compounds
9	0.21	ND	Light green (+)	ND	ND	ND	Natural fluorescence compounds
10	0.26	ND	Light blue (+++)	ND	ND	ND	Natural fluorescence compounds
11	0.28	ND	Red (+)	ND	ND	ND	Natural fluorescence compounds
12	0.40	ND	Light blue (+)	ND	Dark purple (++)	ND	Terpenoid
13	0.45	ND	Blue (++)	ND	ND	ND	Natural fluorescence compounds
14	0.48	Brownish green (+)	Light blue (+++)	ND	ND	ND	Plant pigment, natural fluorescence compounds

Table 4.4, continued

Compound	R_f value	Visible light	UV light (254 nm)	Visualizing Reagents			Presence of compound
				Dragendorff	Vanillin-H_2SO_4	Folin-Ciocalteu	
15	0.55	ND	Fluorescent blue (+++)	ND	ND	ND	Natural fluorescence compounds
16	0.62	ND	Fluorescent red (+++)	ND	ND	ND	Natural fluorescence compounds
17	0.66	ND	ND	ND	Dark purple (+)	ND	Terpenoid
18	0.85	ND	Fluorescent blue (+++)	ND	Orange (+)	ND	Phenol (phloroglucinol, catechin or epicatechin)
19	0.92	ND	Fluorescent red (+++)	ND	Blue (+)	ND	Terpenoid
20	0.94	Brownish green (+)	ND	ND	ND	ND	Plant pigment
21	0.97	ND	ND	ND	Purple (++)	ND	Terpenoid

Indication of intensity of band's color:

+++ = Strong ++ = medium + = weak ND = No band was observed

Table 4.5: TLC analysis of CF in methanol: chloroform (1: 9) solvent.

Compound	R_f value	Visible light	UV light (254 nm)	Visualizing Reagents			Presence of compound
				Dragendorff	Vanillin-H_2SO_4	Folin-Ciocalteu	
1	0.25	ND	ND	Orange (+)	ND	ND	Alkaloid
2	0.28	ND	Purple (+)	ND	ND	ND	Natural fluorescence compounds
3	0.35	ND	ND	ND	Dark purple (+)	ND	Terpenoid
4	0.42	ND	Light green (+++)	ND	Purple (++)	ND	Terpenoid
5	0.54	ND	Red (++)	ND	ND	ND	Natural fluorescence compounds
6	0.62	ND	Light green (+++)	ND	ND	ND	Natural fluorescence compounds
7	0.70	ND	Blue (++)	ND	Purple (+)	ND	Terpenoid
8	0.74	ND	Fluorescent red (+)	ND	Red (+)	ND	Phenol
9	0.85	ND	Light green (+)	ND	Blue (+)	ND	Terpenoid
10	0.89	ND	Fluorescent blue (++)	ND	ND	ND	Natural fluorescence compounds
11	0.95	Brownish green (+)	Bright blue (+++)	ND	ND	ND	Plant pigment, natural fluorescence compounds
12	0.98	Brownish green (++)	Bright red (+++)	ND	Dark purple (+++)	ND	Plant pigment, terpenoid

Indication of intensity of band's color:

+++ = Strong ++ = medium + = weak ND = No band was observed

Table 4.6: TLC analysis of DCMF in chloroform solvent.

Compound	R_f value	Visible light	UV light (254 nm)	Visualizing Reagents			Presence of compound
				Dragendorff	Vanillin-H_2SO_4	Folin-Ciocalteu	
1	0.68	ND	Fluorescent red (+)	ND	ND	ND	Natural fluorescence compounds
2	0.88	ND	Light red (+)	ND	ND	ND	Natural fluorescence compounds

Indication of intensity of band's color:
+++ = Strong ++ = medium + = weak ND = No band was observed

Table 4.7: TLC analysis of DCMF in methanol: chloroform (1: 9) solvent.

Compound	R_f value	Visible light	UV light (254 nm)	Visualizing Reagents			Presence of compound
				Dragendorff	Vanillin-H_2SO_4	Folin-Ciocalteu	
1	0.25	ND	Blue (+)	Orange (+)	ND	ND	Alkaloid
2	0.89	ND	ND	Orange (+++)	ND	ND	Alkaloid
3	0.92	ND	ND	ND	Dark purple (+)	ND	Terpenoid
4	0.97	ND	Red (+)	ND	Dark purple (++)	ND	Terpenoid

Indication of intensity of band's color:
+++ = Strong ++ = medium + = weak ND = No band was observed

Table 4.8: TLC analysis of EAF in chloroform solvent.

Compound	R_f value	Visible light	UV light (254nm)	Visualizing Reagents			Presence of compound
				Dragendorff	Vanillin-H_2SO_4	Folin-Ciocalteu	
1	0.03	ND	Light yellow (++)	ND	ND	ND	Natural fluorescence compounds
2	0.06	ND	Red (++)	ND	ND	Dark purple (++)	Phenol
3	0.12	ND	Fluorescent red (+)	ND	ND	ND	Natural fluorescence compounds
4	0.17	ND	Fluorescent red (+)	ND	ND	ND	Natural fluorescence compounds
5	0.23	ND	Fluorescent red (+)	ND	Purple (+)	ND	Terpenoid
6	0.29	ND	Fluorescent red (++)	ND	ND	ND	Natural fluorescence compounds
7	0.32	ND	Fluorescent red (++)	ND	ND	ND	Natural fluorescence compounds
8	0.89	Green (+)	Fluorescent red (+++)	ND	ND	ND	Plant pigment, natural fluorescence compounds
9	0.92	ND	Red (+)	ND	ND	ND	Natural fluorescence compounds

Table 4.8, continued

Compound	R_f value	Visible light	UV light (254nm)	Visualizing Reagents			Presence of compound
				Dragendorff	Vanillin-H_2SO_4	Folin-Ciocalteu	
10	0.94	ND	Red (+)	ND	ND	ND	Natural fluorescence compounds
11	0.95	Green (+)	Fluorescent red (+++)	ND	ND	ND	Plant pigment, natural fluorescence compounds
12	0.97	Green (+)	Fluorescent red (+++)	ND	ND	ND	Plant pigment, natural fluorescence compounds
13	0.98	ND	ND	ND	Dark purple (++)	ND	Terpenoid

Indication of intensity of band's color:

+++ = Strong ++ = medium + = weak ND = No band was observed

Table 4.9: TLC analysis of EAF in methanol: chloroform (1: 9) solvent.

Compound	R_f value	Visible light	UV light (254 nm)	Visualizing Reagents			Presence of compound
				Dragendorff	Vanillin-H_2SO_4	Folin-Ciocalteu	
1	0.06	Brown (++)	Reddish brown (+++)	ND	Dark red (+++)	ND	Plant pigment, phenol
2	0.08	ND	Reddish brown (+++)	ND	Dark red (++++)	Dark blue (++)	Phenol
3	0.11	ND	Light yellow (+++)	ND	ND	ND	Natural fluorescence compounds
4	0.12	Yellowish brown (++)	Fluorescent light green (+++)	ND	ND	ND	Plant pigment, natural fluorescence compounds
5	0.14	Light brown	Purple (++)	ND	ND	ND	Plant pigment, natural fluorescence compounds
6	0.15	ND	Light yellow (+)	ND	ND	ND	Natural fluorescence compounds
7	0.18	ND	Light yellow (+)	ND	Red (+++)	Dark blue (++)	Phenol
8	0.23	ND	Light blue (++)	ND	ND	ND	Natural fluorescence compounds
9	0.32	Yellowish brown (++)	Fluorescent light green (+++)	ND	ND	ND	Plant pigment, natural fluorescence compounds
10	0.36	ND	Flueorescent red (+++)	ND	ND	ND	Natural fluorescence compounds
11	0.39	ND	Fluorescent light blue (+++)	ND	ND	ND	Natural fluorescence compounds
12	0.42	ND	Light blue (+)	ND	ND	ND	Natural fluorescence compounds
13	0.45	ND	Light blue (+)	ND	Dark purple (+)	ND	Terpenoid
14	0.46	ND	Fluorescent light green (++)	ND	ND	ND	Natural fluorescence compounds

Table 4.9, continued

Compound	R_f value	Visible light	UV light (254 nm)	Visualizing Reagents			Presence of compound
				Dragendorff	Vanillin-H_2SO_4	Folin-Ciocalteu	
15	0.49	ND	Light green (+)	ND	ND	ND	Natural fluorescence compounds
16	0.50	ND	Red (++)	ND	ND	ND	Natural fluorescence compounds
17	0.54	ND	Light purple (+)	ND	ND	ND	Natural fluorescence compounds
18	0.55	ND	Light blue (+)	ND	ND	ND	Natural fluorescence compounds
19	0.57	Greenish brown (++)	Fluorescent red (+++)	ND	ND	ND	Plant pigment, natural fluorescence compounds
20	0.60	ND	Light purple (+)	ND	ND	ND	Natural fluorescence compounds
21	0.65	ND	Fluorescent red (++)	ND	ND	ND	Natural fluorescence compounds
22	0.68	ND	Fluorescent red (+++)	ND	ND	ND	Natural fluorescence compounds
23	0.91	Green	Fluorescent red (+++)	ND	Blue (++)	ND	Plant pigment, terpenoid
24	0.99	ND	Fluorescent red (+++)	ND	ND	ND	Natural fluorescence compounds

Indication of intensity of band's color:

+++ = Strong ++ = medium + = weak ND = No band was observed

Table 4.10: TLC analysis of BF in chloroform solvent.

Compound	R_f value	Visible light	UV light (254 nm)	Visualizing Reagents			Presence of compound
				Dragendorff	Vanillin-H_2SO_4	Folin-Ciocalteu	
1	0.05	ND	ND	ND	Red (+)	Dark blue (++)	Phenol
2	0.63	ND	Light red (+)	ND	ND	ND	Natural fluorescence compounds

Indication of intensity of band's color:
+++ = Strong ++ = medium + = weak ND = No band was observed

Table 4.11: TLC analysis of BF in methanol: chloroform (1: 9) solvent.

Compound	R_f value	Visible light	UV light (254nm)	Visualizing Reagents			Presence of compound
				Dragendorff	Vanillin-H_2SO_4	Folin-Ciocalteu	
1	0.07	ND	ND	ND	Red (+)	Dark blue (++)	Phenol
2	0.13	ND	Light blue (+)	ND	ND	ND	Natural fluorescence compounds
3	0.31	ND	Light blue (+)	Orange (++)	ND	ND	Alkaloid
4	0.94	ND	Light blue (+)	Orange (+++)	ND	ND	Alkaloid

Indication of intensity of band's color:
+++ = Strong ++ = medium + = weak ND = No band was observed

Table 4.12: TLC analysis of MAF in chloroform solvent.

Compound	R_f value	Visible light	UV light (254nm)	Visualizing Reagents			Presence of compound
				Dragendorff	Vanillin-H_2SO_4	Folin-Ciocalteu	
ND	ND	ND	ND	ND	ND	ND	ND

Indication of intensity of band's color:
+++ = Strong ++ = medium + = weak ND = No band was observed

Table 4.13: TLC analysis of MAF in methanol: chloroform (1: 9) solvent.

Compound	R_f value	Visible light	UV light (254nm)	Visualizing Reagents			Presence of compound
				Dragendorff	Vanillin-H_2SO_4	Folin-Ciocalteu	
1	0.05	ND	ND	ND	ND	Dark purple (+)	Phenol
2	0.12	ND	Light blue (+)	ND	ND	ND	Natural fluorescence compounds
3	0.31	ND	ND	Orange (+)	ND	ND	Alkaloid
4	0.89	ND	Light blue (++)	ND	Dark blue (++)	ND	Terpenoid
5	0.97	ND	Light blue (+)	ND	Dark blue (++)	ND	Terpenoid

Indication of intensity of band's color:
+++ = Strong ++ = medium + = weak ND = No band was observed

Table 4.14: TLC analysis of WE in chloroform solvent.

Compound	Rf value	Visible light	UV light (254nm)	Visualizing Reagents			Presence of compound
				Dragendorff	Vanillin-H_2SO_4	Folin-Ciocalteu	
1	0.03	ND	ND	ND	ND	Dark blue (++)	Phenol
2	0.05	ND	ND	ND	Red (+)	ND	Phenol

Indication of intensity of band's color:
+++ = Strong ++ = medium + = weak ND = No band was observed

Table 4.15: TLC analysis of WE in methanol: chloroform (1: 9) solvent.

Compound	R_f value	Visible light	UV light (254nm)	Visualizing Reagents			Presence of compound
				Dragendorff	Vanillin-H_2SO_4	Folin-Ciocalteu	
1	0.09	ND	ND	ND	Red (++)	ND	Phenol
2	0.15	ND	ND	ND	ND	Dark blue (+)	Phenol
3	0.42	ND	ND	Orange (+)	ND	ND	Alkaloid
4	0.97	ND	Light blue (++)	ND	Blue (++)	ND	Terpenoid

Indication of intensity of band's color:
+++ = Strong ++ = medium + = weak ND = No band was observed

4.3.2 LC/Q-TOF/MS

Several compounds were detected in WE of the HP by using an LC/Q-TOF/MS in negative-mode electrospray-mass spectrometry. The result is shown in Table 4.16. Amino acids, benzoic acid, flavonoids, phenolic compounds, polysaccharides and sugars were detected in the WE.

Table 4.16: Detection of compounds in WE by LC/Q-TOF/MS.

	Compound Name	Retention Time (RT)	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula	DB Diff (ppm)	Hits (DB)
1	Nigerose (Sakebiose)	3.429	342.1163	C12 H22 O11	C12 H22 O11	-0.26	C12 H22 O11	-0.26	10
2	Levan	3.598	504.1682	C18 H32 O16	C18 H32 O16	1.57	C18 H32 O16	1.57	10
3	Nigerose (Sakebiose)	3.616	342.1154	C12 H22 O11	C12 H22 O11	2.46	C12 H22 O11	2.46	10
4	Stachyose	3.622	666.2203	C24 H42 O21	C24 H42 O21	2.28	C24 H42 O21	2.28	8
5	Maltopentaose	3.628	828.2724	C30 H52 O26	C30 H52 O26		C30 H52 O26	2.74	4
6	Maltohexaose	3.63	990.3257	C36 H62 O31	C36 H62 O31		C36 H62 O31	1.83	3
7	Celloheptaose	3.631	1152.3787	C42 H72 O36	C42 H72 O36		C42 H72 O36	1.41	2
8	Gallocatechin 3-O-gallate	8.26	458.0838	C22 H18 O11	C22 H18 O11	2.43	C22 H18 O11	2.43	5
9	(E)-2-Butenyl-4-methyl-threonine	8.48	187.1205	C9 H17 N O3	C9 H17 N O3	1.99	C9 H17 N O3	1.99	10
10	3,4-Dimethylbenzoic acid	11.022	150.0677	C9 H10 O2	C9 H10 O2	2.62	C9 H10 O2	2.63	10
11	His His Ser	12.82	379.1599	C15 H21 N7 O5	C15 H21 N7 O5	1.46	C15 H21 N7 O5	1.46	10
12	6-Gingerol	12.82	294.1835	C17 H26 O4	C17 H26 O4	-1.2	C17 H26 O4	-1.21	4

4.4 *In vitro* studies

4.4.1 Determination of TPC

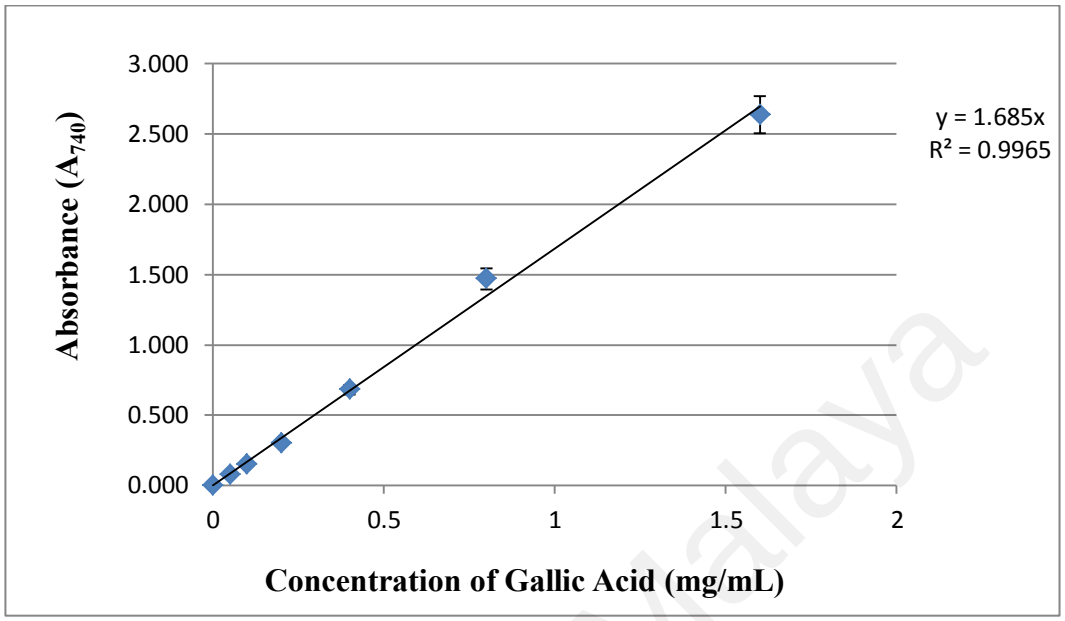

Figure 4.3: Calibration curve for the determination of total phenolic content. Each point represents mean ± SD (n = 3).

In TPC assay, the pattern of the color changes of gallic acid was from colorless to dark blue with the increased concentration of gallic acid. The calibration curve for the determination of TPC is shown in Figure 4.3 and it was plotted according to the absorbance readings of gallic acid with the concentrations of 0 to 1.6 mg/mL (Appendix F). Linear equation (y = 1.685 x) was obtained from the calibration curve.

Table 4.17: Total phenolic content of HF, CF, DCMF, EAF, BF, MAF and WE.

Fraction (s) /Crude Extract	TPC (mg/GAE g of dry sample)
HF	0.97 ± 0.03^{a}
CF	1.01 ± 0.02^{a}
DCMF	1.31 ± 0.01^{a}
EAF	30.48 ± 0.62^{b}
BF	15.98 ± 0.57^{c}
MAF	1.24 ± 0.06^{a}
WE	2.81 ± 0.05^{d}

Values represent mean ± SD (n = 3). The values with different case letters (a, b, c, d) differ significantly at $p < 0.05$ (one-way ANOVA, Fisher's LSD post hoc test, followed by Duncan's multiple range test). TPC: total phenolic content; HF: hexane fraction; CF: chloroform fraction; DCMF: dichloromethane fraction; EAF: ethyl acetate fraction; BF: butanol fraction; MAF: methanol aqueous fraction; WE: water extract.

The TPC of the fractions and the extract is shown in Table 4.17 (Appendix G) and it was in the following order: EAF (30.48 ± 0.62 mg/GAE g) > BF (15.98 ± 0.57 mg/GAE g) > WE (2.81 ± 0.05 mg/GAE g) > DCMF (1.31 ± 0.01 mg/GAE g) > MAF (1.24 ± 0.06 mg/GAE g) > CF (1.01 ± 0.02 mg/GAE g) > HF (0.97 ± 0.03 mg/GAE g).

Duncan's test was carried out in order to determine homogenous group. Four homogenous subset groups were defined (Appendix H). Based on the result of the test, it showed that the highest mean of TPC obtained by EAF was significantly different ($p < 0.05$) from the mean of TPC obtained by all the other fractions (HF, CF, DCMF, BF and MAF) and crude extract (WE). The result also depicted that the mean of TPC between HF, CF, DCMF and MAF were statistically not significant ($p > 0.05$). These were supported by the result obtained from Fisher's LSD post hoc test (Appendix H).

4.4.2 Determination of TFC

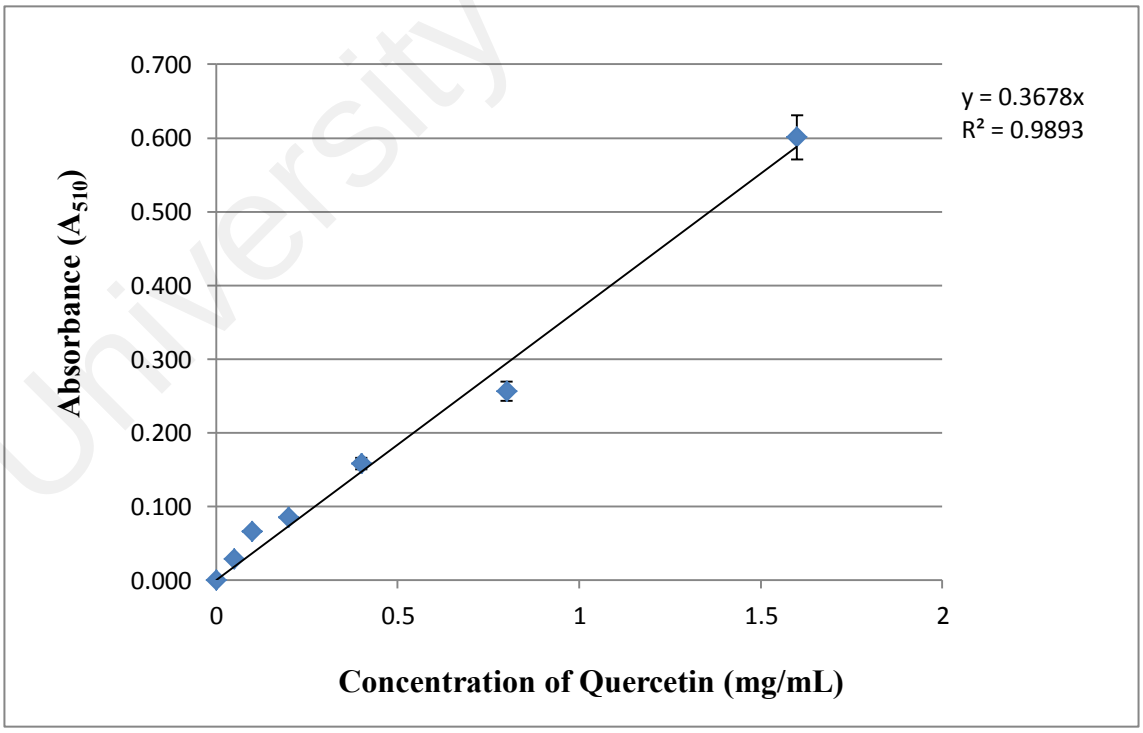

Figure 4.4: Calibration curve for the determination of total flavonoid content. Each point represents mean ± SD (n = 3).

In TFC assay, the pattern of the color changes of quercetin standard set was from colorless to yellowish brown with the increased concentration of quercetin. The calibration curve for the determination of TFC is shown in Figure 4.4 and it was plotted according to the absorbance readings of quercetin with the concentrations of 0, 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 mg/mL (Appendix I). Linear equation (y = 0.3678 x) was obtained from the calibration curve.

Table 4.18: Total flavonoid content of HF, CF, DCMF, EAF, BF, MAF and WE.

Fraction (s) /Crude Extract	TFC (mg/QE g of dry sample)
HF	6.34 ± 0.28^{a}
CF	6.84 ± 0.44^{a}
DCMF	8.07 ± 0.16^{a}
EAF	156.52 ± 3.13^{b}
BF	107.58 ± 5.51^{c}
MAF	9.02 ± 0.21^{a}
WE	14.32 ± 0.16^{d}

Values represent mean ± SD (n = 3). The values with different case letters (a, b, c, d) differ significantly at $p < 0.05$ (one-way ANOVA, Fisher's LSD post hoc test, followed by Duncan's multiple range test). TFC: total flavonoid content; HF: hexane fraction; CF: chloroform fraction; DCMF: dichloromethane fraction; EAF: ethyl acetate fraction; BF: butanol fraction; MAF: methanol aqueous fraction; WE: water extract.

The TFC of each fraction and extract is shown in Table 4.18 (Appendix J). Based on Table 4.18, the TFC of the fractions and the extract was in the following order: EAF (156.52 ± 3.13 mg/QE g) > BF (107.58 ± 5.51 mg/QE g) > WE (14.32 ± 0.16 mg/QE g) > MAF (9.02 ± 0.21 mg/QE g) > DCMF (8.07 ± 0.16 mg/QE g) > CF (6.84 ± 0.44 mg/QE g) > HF (6.34 ± 0.28 mg/QE g).

Duncan's test was used to determine homogenous group. Four homogenous subset groups were defined (Appendix K). Based on the result of the test, it showed that the highest mean of TFC obtained by EAF was significantly different ($p < 0.05$) from the mean of TFC obtained by all the fractions (HF, CF, DCMF, BF and MAF) and extract

(WE). The result also depicted that the mean of TFC between HF, CF, DCMF and MAF were statistically not significant ($p > 0.05$). These were supported by the result obtained from Fisher's LSD post hoc test (Appendix K).

4.4.3 Determination of antioxidant activities

4.4.3.1 DPPH radical scavenging assay

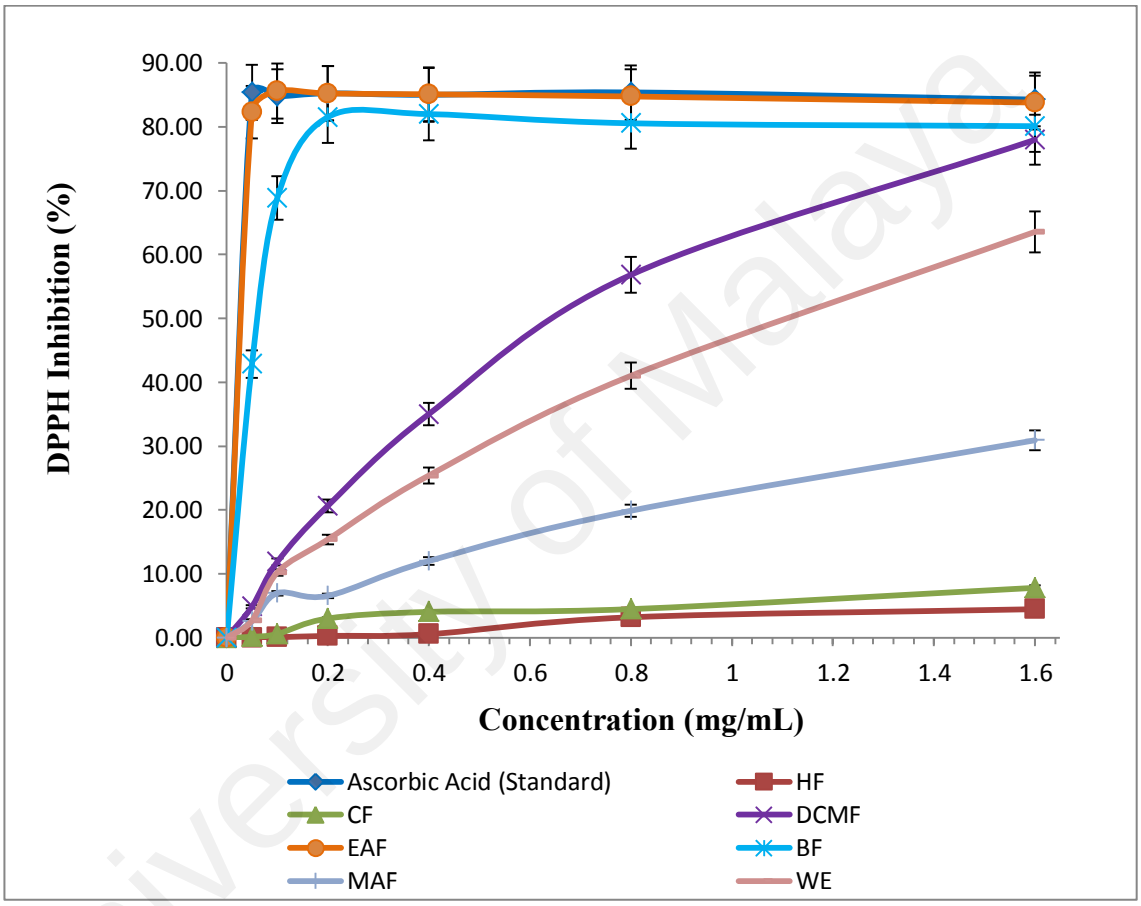

Figure 4.5: Percentage of DPPH radical scavenging activity of ascorbic acid, HF, CF, DCMF, EAF, BF, MAF and WE against concentration. Each point represents mean ± SD (n = 3). HF: hexane fraction; CF: chloroform fraction; DCMF: dichloromethane fraction; EAF: ethyl acetate fraction; BF: butanol fraction; MAF: methanol aqueous fraction; WE: water extract.

In DPPH assay, the pattern of the color changes of ascorbic acid standard set was from purple to light yellow with the increased concentration of ascorbic acid after the addition of DPPH solution. Ascorbic acid showed 84.28% of DPPH inhibition at the concentration of 1.6 mg/mL, with IC_{50} value of 0.03 ± 0.00 mg/mL. Based on Figure

4.5, it showed that EAF exhibited the highest percentage of DPPH inhibition (83.81%), followed by BF (80.11%), DCMF (77.98%), WE (63.56%), MAF (30.92%), CF (7.81%) and HF (4.46%) at the concentration of 1.6 mg/mL (Appendix L). Figure 4.5 also showed that the potential of EAF and BF in scavenging DPPH radicals was comparable to ascorbic acid which acted as standard in this assay.

Table 4.19: IC_{50} values of ascorbic acid, HF, CF, DCMF, EAF, BF, MAF and WE for DPPH radical scavenging activity.

Fraction (s) / Crude Extract	DPPH Inhibition (IC_{50} mg/mL)
HF	ND
CF	ND
DCMF	$0.63 \pm 0.40^{a, b}$
EAF	0.03 ± 0.00^{a}
BF	0.06 ± 0.01^{a}
MAF	ND
WE	1.11 ± 0.64^{b}
Ascorbic acid (Standard)	0.03 ± 0.00^{a}

Values are shown in mean ± SD (n = 3). The values with different case letters (a, b) differ significantly at $p < 0.05$ (one-way ANOVA, Fisher's LSD post hoc test, Duncan's multiple range test). HF: hexane fraction; CF: chloroform fraction; DCMF: dichloromethane fraction; EAF: ethyl acetate fraction; BF: butanol fraction; MAF: methanol aqueous fraction; WE: water extract; ND: not detected.

The IC_{50} value of ascorbic acid, HF, CF, DCMF, EAF, BF, MAF and WE for DPPH radical scavenging activity was determined and the result is shown in Table 4.19. From Table 4.19, the IC_{50} value of the fractions and the extract for DPPH scavenging activity was in the following order: EAF (0.03 ± 0.00 mg/mL) < BF (0.06 ± 0.01 mg/mL) < DCMF (0.63 ± 0.40 mg/mL) < WE (1.11 ± 0.64 mg/mL). However, the IC_{50} values of HF, CF and MAF were not detected.

Duncan's test was used to determine homogenous group. Two homogenous subset groups were defined (Appendix M). The results of the test were supported by the result obtained from Fisher's LSD post hoc test (Appendix M). Based on the result of

Duncan's test, it showed that the lowest IC_{50} obtained by EAF was significantly different ($p < 0.05$) from IC_{50} obtained by WE. The result also showed that the IC_{50} between EAF, BF, DCMF and ascorbic acid were statistically not significant ($p > 0.05$).

Moreover, the relationship between DPPH radical scavenging activity, TPC and TFC of the herbal extracts were analyzed using Pearson correlation method (Appendix N). The result revealed that there was a significant inverse and strong relationship between DPPH radical scavenging activity and TPC of the herbal extracts with a correlation coefficient of -0.678, which was significant at 0.05 level. Besides, the result also revealed that there was a significant inverse and strong relationship between DPPH radical scavenging activity and TFC of the herbal extracts with a correlation coefficient of -0.724, which was significant at 0.01 level.

4.4.3.2 FRAP assay

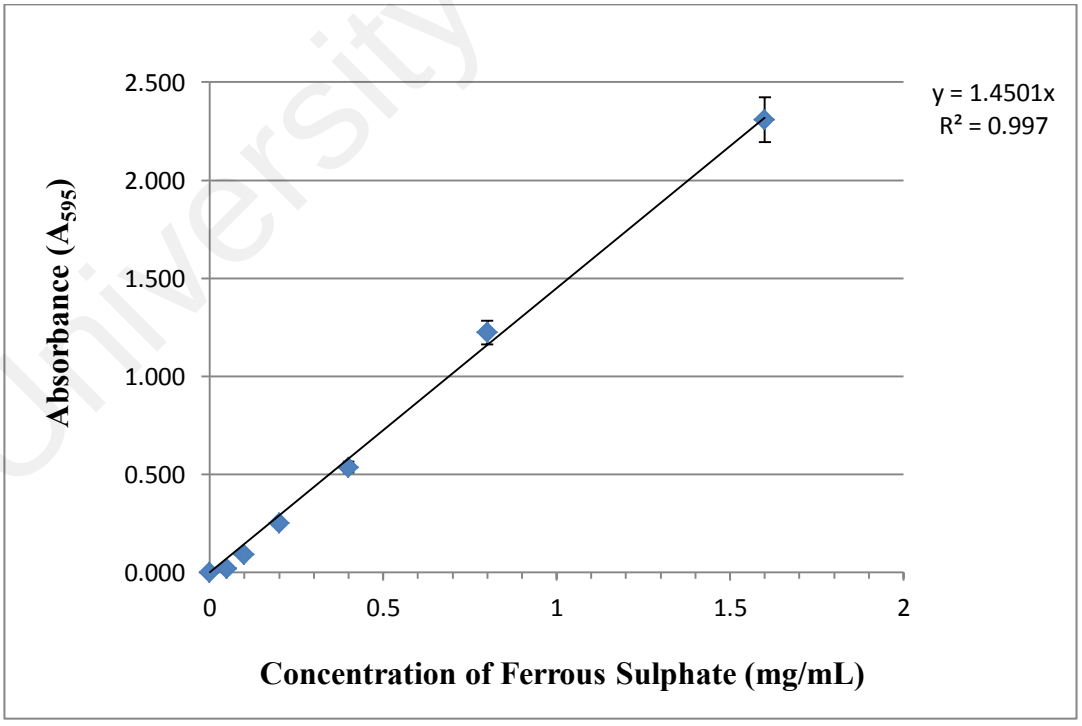

Figure 4.6: Calibration curve for the determination of ferric reducing antioxidant power. Each point represents mean ± SD (n = 3).

In FRAP assay, the pattern of the color changes of ferrous sulfate standard set was from colorless to violet blue with the increased concentration of ferrous sulfate. The calibration curve for the determination of FRAP is shown in Figure 4.6 and it was plotted based on the absorbance readings of ferrous sulfate with the concentrations of 0, 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 mg/mL (Appendix O). Linear equation (y = 1.4501 x) was obtained from the calibration curve.

Table 4.20: Ferric reducing antioxidant power of HF, CF, DCMF, EAF, BF, MAF and WE.

Fraction (s) /Extract	FRAP (μmol Fe^{2+}/g)
HF	7.45 ± 0.05^{a}
CF	$9.16 \pm 1.08^{a, b}$
DCMF	11.00 ± 0.47^{b}
EAF	109.27 ± 3.35^{c}
BF	106.67 ± 2.37^{c}
MAF	15.08 ± 0.69^{d}
WE	25.53 ± 0.12^{e}

Values represent mean ± SD (n = 3). The values with different case letters (a, b, c, d, e) are significantly different at $p < 0.05$ (one-way ANOVA, Fisher's LSD post hoc test, Duncan's multiple range test). FRAP: ferric reducing antioxidant power; HF: hexane fraction; CF: chloroform fraction; DCMF: dichloromethane fraction; EAF: ethyl acetate fraction; BF: butanol fraction; MAF: methanol aqueous fraction; WE: water extract.

The FRAP value of each fraction and extract is shown in Table 4.20 (Appendix P). The FRAP value of the fractions and the extract was in the following order: EAF (109.27 ± 3.35 μmol Fe^{2+}/g) > BF (106.67 ± 2.37 μmol Fe^{2+}/g) > WE (25.53 ± 0.12 μmol Fe^{2+}/g) > MAF (15.08 ± 0.69 μmol Fe^{2+}/g) > DCMF (11.00 ± 0.47 μmol Fe^{2+}/g) > CF (9.16 ± 1.08 μmol Fe^{2+}/g) > HF (7.45 ± 0.05 μmol Fe^{2+}/g).

Duncan's test was used to determine homogenous group. Five homogenous subset groups were defined (Appendix Q). Based on the result of the test, it showed that the highest mean of FRAP value obtained by EAF was significantly different ($p < 0.05$) from the mean of FRAP value obtained by HF, CF, DCMF, MAF and WE, except BF.

The result also depicted that the mean of FRAP value between EAF and BF were statistically not significant ($p > 0.05$). These were supported by the result from Fisher's LSD post hoc test (Appendix Q).

Furthermore, the relationship between FRAP, TPC and TFC of the herbal extracts was analyzed using Pearson correlation method (Appendix R). The result revealed that there was a significant strong and positive relationship between FRAP and TPC, and also between FRAP and TFC of the herbal extracts with correlation coefficients of 0.933 and 0.972, respectively which were significant at 0.01 level.

4.4.3.3 Metal chelating assay

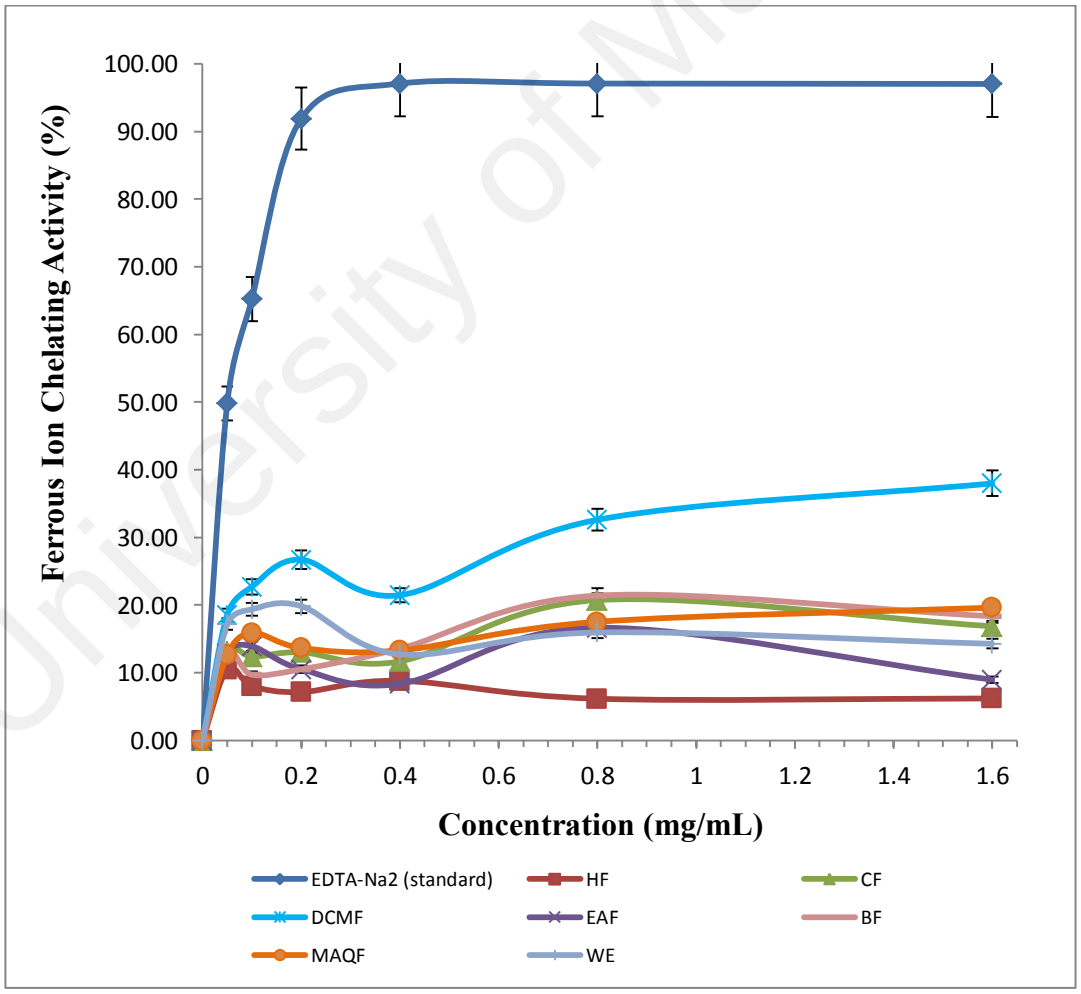

Figure 4.7: Ferrous ion chelating activity of EDTA-Na_2, HF, CF, DCMF, EAF, BF, MAF and WE by the effect of concentration. Each point represents mean ± SD (n = 3). HF: hexane fraction; CF: chloroform fraction; DCMF: dichloromethane fraction; EAF: ethyl acetate fraction; BF: butanol fraction; MAF: methanol aqueous fraction; WE: water extract.

Table 4.21: Ferrous ion chelating activity of EDTA-Na_2, HF, CF, DCMF, EAF, BF, MAF and WE.

Fraction (s) /Extract	Ferrous Ion Chelating Activity (IC_{50} mg/mL)
HF	ND
CF	ND
DCMF	ND
EAF	ND
BF	ND
MAF	ND
WE	ND
EDTA-Na_2	0.05 ± 0.00

Values represent mean ± SD (n = 3). HF: hexane fraction; CF: chloroform fraction; DCMF: dichloromethane fraction; EAF: ethyl acetate fraction; BF: butanol fraction; MAF: methanol aqueous fraction; WE: water extract; ND: not detected.

In this assay, the pattern of the color changes of EDTA-Na_2 standard set was from purple to colorless with the increased concentration of EDTA-Na_2 after the addition of ferrozine. EDTA-Na_2 was used as standard in this assay and it showed 97.01% of ferrous ion chelating activity at the concentration of 1.6 mg/mL, with the IC_{50} value of 0.05 ± 0.00 mg/mL. Based on Figure 4.7, the percentage of ferrous ion chelating activity of all the fractions and extract was lower than 50% (Appendix S). Therefore, the IC_{50} value of all the fractions and the extract was not detected (Table 4.21). The percentage of ferrous ion chelating activity of HF, CF, DCMF, EAF, BF, MAF and WE reached 6.19%, 16.85%, 37.97%, 8.96%, 18.34%, 19.61% and 14.27%, respectively at the concentration of 1.6 mg/mL. Besides, DCMF exhibited the highest percentage of ferrous ion chelating activity (37.97%) among all the herbal extracts.

4.4.3.4 NO radical scavenging assay

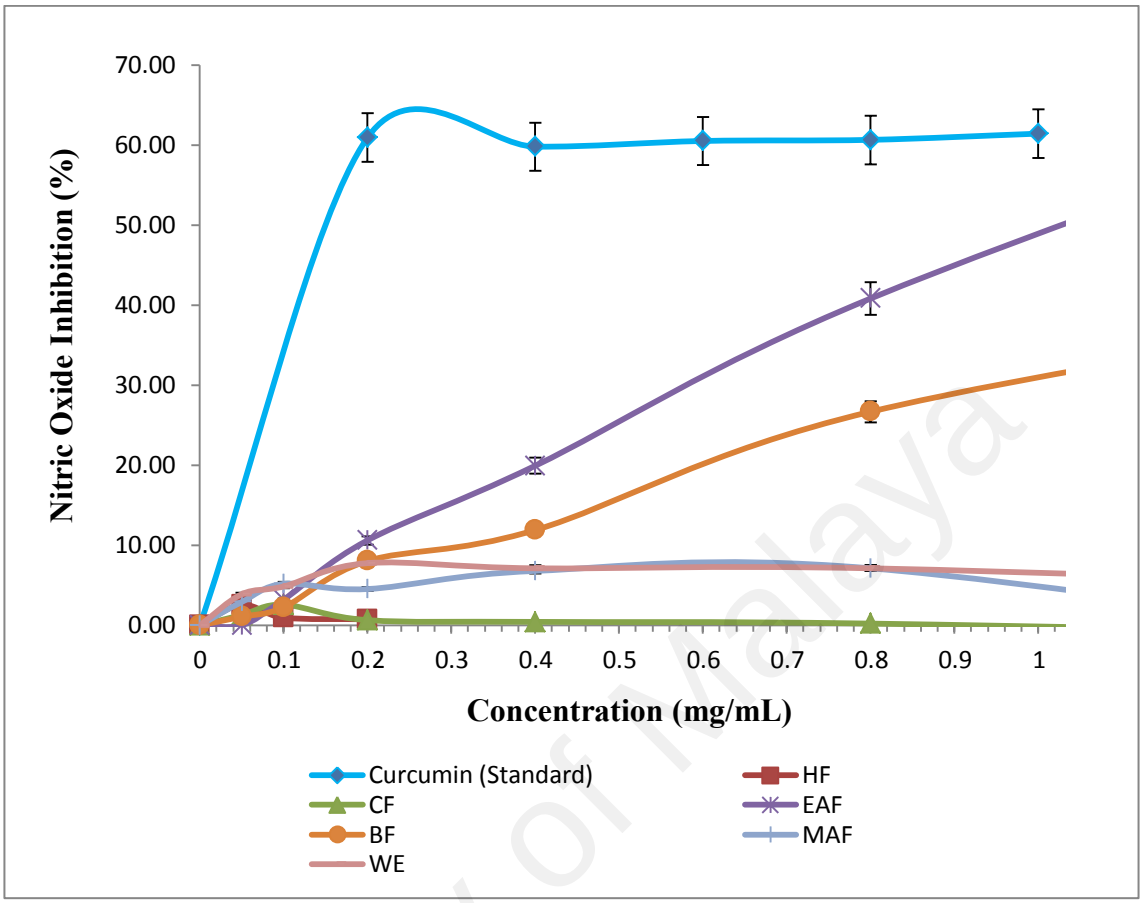

Figure 4.8: Nitric oxide radical scavenging activity of curcumin, HF, CF, EAF, BF, MAF and WE by the effect of concentration. Each point represents mean ± SD (n = 3). HF: hexane fraction; CF: chloroform fraction; EAF: ethyl acetate fraction; BF: butanol fraction; MAF: methanol aqueous fraction; WE: water extract.

In this assay, the pattern of the color changes of curcumin standard set was from light pink to light yellow with the increased concentration of curcumin. Curcumin was used as standard in this assay and it showed 61.44% of NO inhibition at the concentration of 1 mg/mL (Appendix T), with IC_{50} value of 0.16 ± 0.01 mg/mL (Table 4.22).

Based on Figure 4.8, the percentage of NO scavenging activity of all the fractions and the extract was lower than 50%, except EAF (Appendix T). Therefore, the IC_{50} value of all the fractions and the extract was not detected, except EAF. EAF not only obtained the highest TPC and TFC, it also exhibited the highest percentage of NO inhibition (Table 4.17, Table 4.18 and Figure 4.8). EAF exhibited the highest

percentage of NO inhibition among the fractions and extract, which was 71.70% at the concentration of 1.6 mg/mL (Appendix T), with IC_{50} value of 1.00 ± 0.05 mg/mL (Table 4.22). On the other hand, DCMF did not have any capability in scavenging NO.

Table 4.22: IC_{50} values of HF, CF, DCMF, EAF, BF, MAF and WE for nitric oxide scavenging activity.

Fraction (s) /Extract	Nitric Oxide Inhibition (IC_{50} mg/mL)
HF	ND
CF	ND
DCMF	ND
EAF	1.00 ± 0.05^{a}
BF	ND
MAF	ND
WE	ND
Curcumin (Standard)	0.16 ± 0.01^{b}

Values represent mean ± SD (n = 3). The values with different case letters (a, b) are significantly different at $p < 0.05$ (one-way ANOVA). HF: hexane fraction; CF: chloroform fraction; DCMF: dichloromethane fraction; EAF: ethyl acetate fraction; BF: butanol fraction; MAF: methanol aqueous fraction; WE: water extract; ND: not detected.

Based on the result of one-way ANOVA between groups, it showed that the IC_{50} value of the EAF for NO scavenging activity was significantly different ($p < 0.05$) from the IC_{50} value of curcumin (Appendix U).

4.4.3.5 Superoxide radical scavenging assay

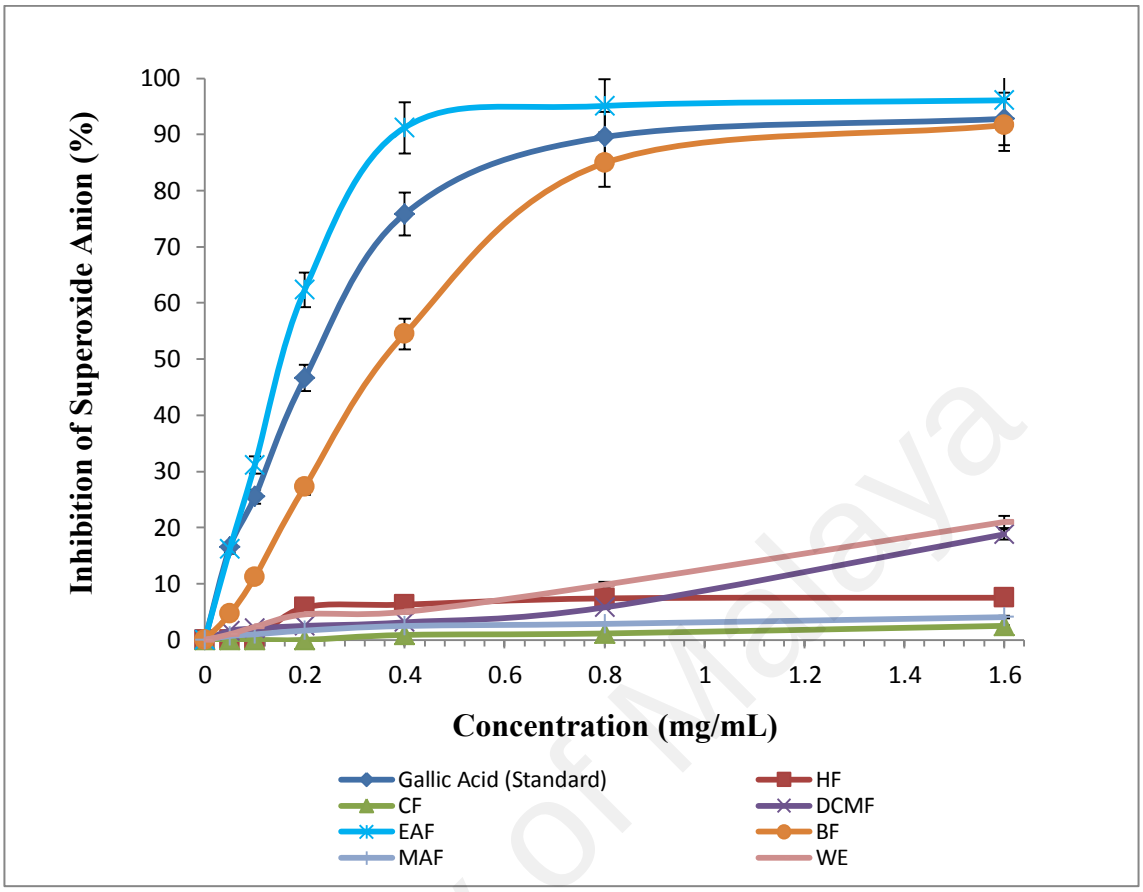

Figure 4.9: Superoxide radical scavenging activity of gallic acid, HF, CF, DCMF, EAF, BF, MAF and WE by the effect of concentration. Values are shown in mean ± SD (n = 3). HF: hexane fraction; CF: chloroform fraction; DCMF: dichloromethane fraction; EAF: ethyl acetate fraction; BF: butanol fraction; MAF: methanol aqueous fraction; WE: water extract.

In this assay, the pattern of the color changes of gallic acid standard set was from dark purple to yellow with the increased concentration of gallic acid. Gallic acid was used as standard in this assay and it exhibited 92.80% of superoxide radical inhibition at the concentration of 1.60 mg/mL, with IC_{50} value of 0.21 ± 0.01 mg/mL. Based on Figure 4.9, it showed that EAF exhibited the highest percentage of superoxide radical inhibition (96.09%), followed by BF (91.68%), WE (21.00%), DCMF (18.00%), HF (7.51%), MAF (4.05%) and CF (2.50%) at the concentration of 1.6 mg/mL (Appendix V).

Table 4.23: IC_{50} values of gallic acid, HF, CF, DCMF, EAF, BF, MAF and WE for superoxide radical scavenging activity.

Fraction (s) /Extract	Superoxide Inhibition (IC_{50} mg/mL)
HF	ND
CF	ND
DCMF	ND
EAF	0.16 ± 0.01^{a}
BF	0.37 ± 0.02^{b}
MAF	ND
WE	ND
Gallic acid (Standard)	0.21 ± 0.01^{c}

Values represent mean ± SD (n = 3). The values with different case letters (a, b, c) are significantly different at $p < 0.05$ (one-way ANOVA, Fisher's LSD post hoc test, Duncan's multiple range test). HF: hexane fraction; CF: chloroform fraction; DCMF: dichloromethane fraction; EAF: ethyl acetate fraction; BF: butanol fraction; MAF: methanol aqueous fraction; WE: water extract; ND: not detected.

The IC_{50} value of gallic acid, HF, CF, DCMF, EAF, BF, MAF and WE for superoxide radical scavenging activity is shown in Table 4.23. The IC_{50} value of the EAF for superoxide radical scavenging activity was 0.16 ± 0.01 mg/mL; whereas the IC_{50} value of BF for superoxide radical scavenging activity was 0.37 ± 0.02 mg/mL. The EAF exhibited the highest capability in scavenging superoxide radical, followed by gallic acid and then BF ($p < 0.05$). However, the IC_{50} values of HF, CF, DCMF, MAF and WE were not detected.

Duncan's test was used to determine homogenous group. Three homogenous subset groups were defined (Appendix W). Based on the result of the test, it showed that the lowest IC_{50} value obtained by EAF was significantly different ($p < 0.05$) from the IC_{50} value obtained by BF and gallic acid. This was supported by the result obtained from Fisher's LSD post hoc test (Appendix W).

In addition, the relationship between superoxide radical scavenging activity, TPC and TFC of the herbal extracts was analyzed using Pearson correlation method (Appendix X). The result revealed that there was a significant inverse and strong

relationship between superoxide radical scavenging activity and TPC, and also btween superoxide radical scavenging activity and TFC of the herbal extracts with correlation coefficients of -0.994 and -0.966, respectively which was significant at 0.01 level.

4.5 Animal studies

4.5.1 Oral acute toxicity test

No mortality was observed during the oral acute toxicity test in both groups of rats. Male and female rats in each group showed normal consumption of food and water, normal feces, no shivering, no gasping, active and sensitive to touch. The body weight of rats in both groups was recorded and the result is shown in Figure 4.10.

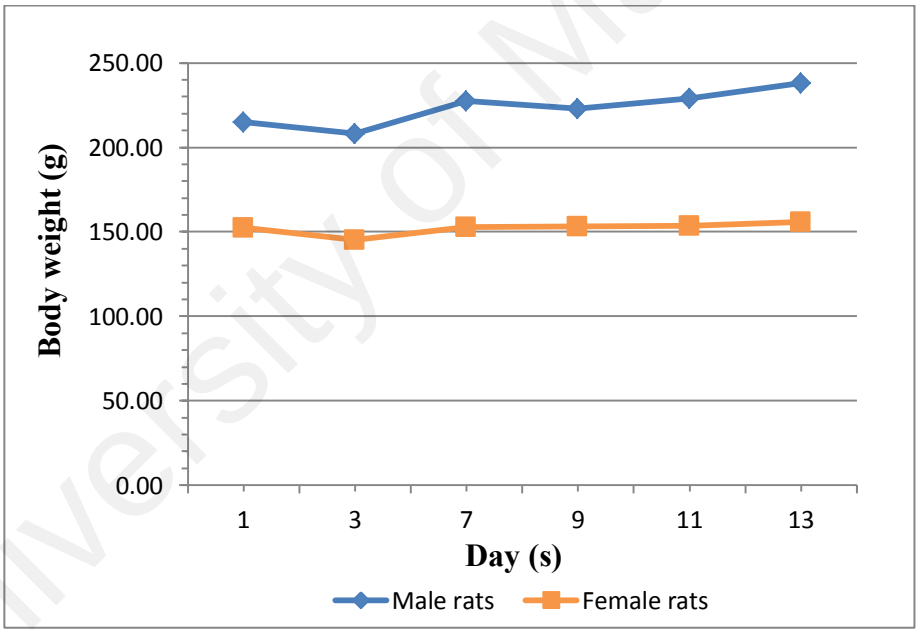

Figure 4.10: Body weight of rats during the 14-day oral acute toxicity test. Each point represents mean ± SD (n = 3).

The mean difference between the initial and final body weight of male and female rats for the oral acute toxicity test were analyzed using paired-sample t-test (Appendix Y and Appendix Z). The mean of the final body weight of male rats (238 ± 27.84 g) was higher than the mean of initial body weight of male rats (215 ± 28 g). The test depicted that there was a significant change between the mean of initial and final body weight of

male rats ($p < 0.05$). Meanwhile, the mean of the final body weight of female rats (156 ± 17.44 g) was higher than the mean of initial body weight of female rats (152.33 ±14.74 g). However, the test depicted that there was no significant change between the mean of initial and final body weight of female rats ($p > 0.05$).

4.5.2 Analyses before and after WE treatment

4.5.2.1 Body weight and BMI

All rats were healthy throughout the experiment and they consumed the provided food during the whole experiment period. The body weight and BMI of rats in each group before and after WE treatment are shown in Table 4.24 (Appendix AA to Appendix CC).

Table 4.24: The mean of initial (after obesity induction) and final (after WE treatment) body weight and BMI of rats.

Group (s)	Body weight (g)		BMI (g/cm^2)	
	Initial	Final	Initial	Final
NC	334.33 ± 12.04^{a}	363.50 ± 13.77$^{*, a}$	0.65 ± 0.03^{a}	0.65 ± 0.04^{a}
HFC	404.50 ± 5.01^{b}	467.83 ± 5.60$^{*, b}$	0.70 ± 0.00^{b}	0.77 ± 0.02$^{*, b}$
ND + WE	334.33 ± 13.19^{a}	349.50 ± 12.66$^{*, a}$	0.63 ± 0.03^{a}	0.63 ± 0.03^{a}
HFD + WE	403.83 ± 2.14^{b}	384.50 ± 7.99$^{*, a, b}$	0.72 ± 0.02^{b}	0.64 ± 0.03$^{*, a}$
HFD + Simvastatin	404.33 ± 2.50^{b}	379.33 ± 3.56$^{*, a, b}$	0.71 ± 0.02^{b}	0.63 ± 0.01$^{*, a}$

Values represent mean ± SD (n = 6). The final value in the same row with * are significantly different at $p < 0.05$ compared to the initial value (paired-sample t-test). The values in the same column with different case letters (a, b) are significantly different at ^{a} $p < 0.05$ versus HFC; ^{b} $p < 0.05$ versus NC (one-way ANOVA, Fisher's LSD post hoc test, followed by Dunnett's multiple comparison test). NC: normal diet control; HFC: high fat diet control; ND + WE: normal diet with water extract; HFD + WE: high fat diet with water extract; HFD + Simvastatin: high fat diet with simvastatin; BMI: body mass index.

After 8 weeks of obesity induction, all the HFD-induced obese rats had significantly 20% higher body weight and higher BMI (more than 0.68 ± 0.05 g/cm^2) than the ND rats. All the rats were successfully distributed into five groups as shown in Table 4.24. Based on the observation, the mobility of HFD rats were slower than ND rats. Post hoc

test was conducted and the result (Appendix BB) showed that there were significant body weight and BMI differences observed between ND rats in NC and ND + WE groups and HFD-induced obese rats in HFC, HFD + WE and HFD + Simvastatin groups after obesity induction ($p < 0.05$). The result also showed that there were no significant body weight and BMI differences observed between ND + WE and NC after obesity induction ($p > 0.05$). Similarly, there were no significant body weight and BMI differences observed between HFC and HFD + WE, and also HFC and HFD + Simvastatin after obesity induction ($p > 0.05$).

Paired-sample t-test was conducted and the result (Appendix CC) showed that there were significant body weight reductions observed in HFD + WE and HFD + Simvastatin after two weeks of treatment ($p < 0.05$). The mean of body weight of rats in HFD + Simvastatin was significantly reduced from 404.33 ± 2.50 g to 379.33 ± 3.56 g by 25 g which was 6.18% after treatment, while the mean of body weight of rats in HFD + WE was significantly reduced from 403.83 ± 2.14 g to 384.50 ± 7.99 g by 19.33 g which was 4.79% after treatment. Dunnett's multiple comparison test was also conducted and the result (Appendix BB) showed that the final body weight of both obese treated groups (HFD + WE and HFD + Simvastatin) was significantly different from the final body weight of HFC and NC. In addition, the result of paired-sample t-test revealed that there were significant BMI reductions observed in HFD + WE and HFD + Simvastatin after treatment ($p < 0.05$). The mean of BMI of rats in HFD + Simvastatin was significantly reduced from 0.71 ± 0.02 g/cm^2 to 0.63 ± 0.01 g/cm^2 by 0.08 g/cm^2 after treatment, whereas the mean of BMI of rats in HFD + WE was also significantly reduced from 0.72 ± 0.02 g/cm^2 to 0.64 ± 0.03 g/cm^2 by 0.08 g/cm^2 after treatment. The result of Dunnett's multiple comparison test revealed that the final BMI of both obese treated groups (HFD + WE and HFD + Simvastatin) was significantly different from the final BMI of HFC ($p < 0.05$). Post hoc test showed that there were no

significant final body weight and final BMI differences observed between HFD + WE and HFD + Simvastatin ($p > 0.05$) (Appendix BB).

On the other hand, the result (Appendix CC) of paired-sample t-test revealed that there were significant body weight increments observed in ND + WE and NC after two weeks ($p < 0.05$). The mean of body weight of rats in ND + WE was significantly increased from 334.33 ± 13.19 g to 349.50 ± 12.66 g by only 15.17 g after treatment, while the mean of body weight of rats in NC was significantly increased from 334.33 ± 12.04 g to 363.50 ± 13.77 g by 29.17 g after two weeks. Moreover, there were no significant change between initial BMI and final BMI of rats in ND + WE and NC after two weeks ($p > 0.05$). The BMI of rats in ND + WE (0.63 ± 0.03 g/cm^2) was maintained after treatment, while the mean of BMI of rats in NC was also maintained from 0.65 ± 0.03 to 0.65 ± 0.04 g/cm^2 (Table 4.24). Post hoc test showed that the final body weight of ND + WE were significantly different from the final body weight of NC ($p < 0.05$). Meanwhile, there was no significant final BMI difference observed between ND + WE and NC ($p > 0.05$).

4.5.2.2 Food intake

The food intake of rats in each group was recorded daily during treatment (Appendix AA) and the result is shown in Figure 4.11 (Appendix DD).

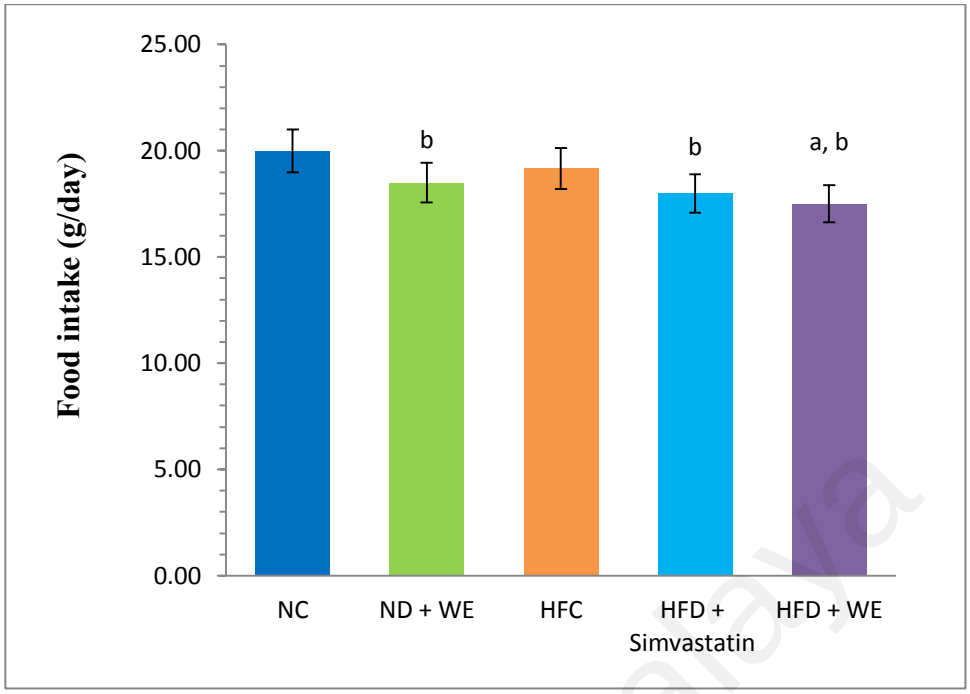

Figure 4.11: Food intake of the rats during WE treatment. Values represent mean ± SD (n = 6). The value with different case letters (a, b) is significantly different at [a] $p < 0.05$ versus HFC; [b] $p < 0.05$ versus NC (one-way ANOVA, Fisher's LSD post hoc test, followed by Dunnett's multiple comparison test). NC: normal diet control; ND + WE: normal diet with water extract; HFC: high fat diet control; HFD + WE: high fat diet with water extract; HFD + Simvastatin: high fat diet with simvastatin.

The result of post hoc test revealed that there was no significant food intake difference observed during treatment between NC and HFC ($p > 0.05$). However, there was a significant food intake reduction observed in HFD + WE compared to NC and HFC ($p < 0.05$) (Appendix DD). There was also a significant food intake reduction observed in ND + WE compared to NC ($p < 0.05$) (Appendix DD). Meanwhile, there was no significant food intake reduction observed in HFD + Simvastatin compared to HFC ($p > 0.05$).

4.5.2.3 Biochemical test for lipid profile

The lipid profile levels of TG, TC, HDL and LDL of the rats after WE treatment is shown in Figure 4.12a to Figure 4.12d (Appendix EE and Appendix FF). There were significant TG, TC, HDL and LDL differences observed between NC and HFC ($p < 0.05$).

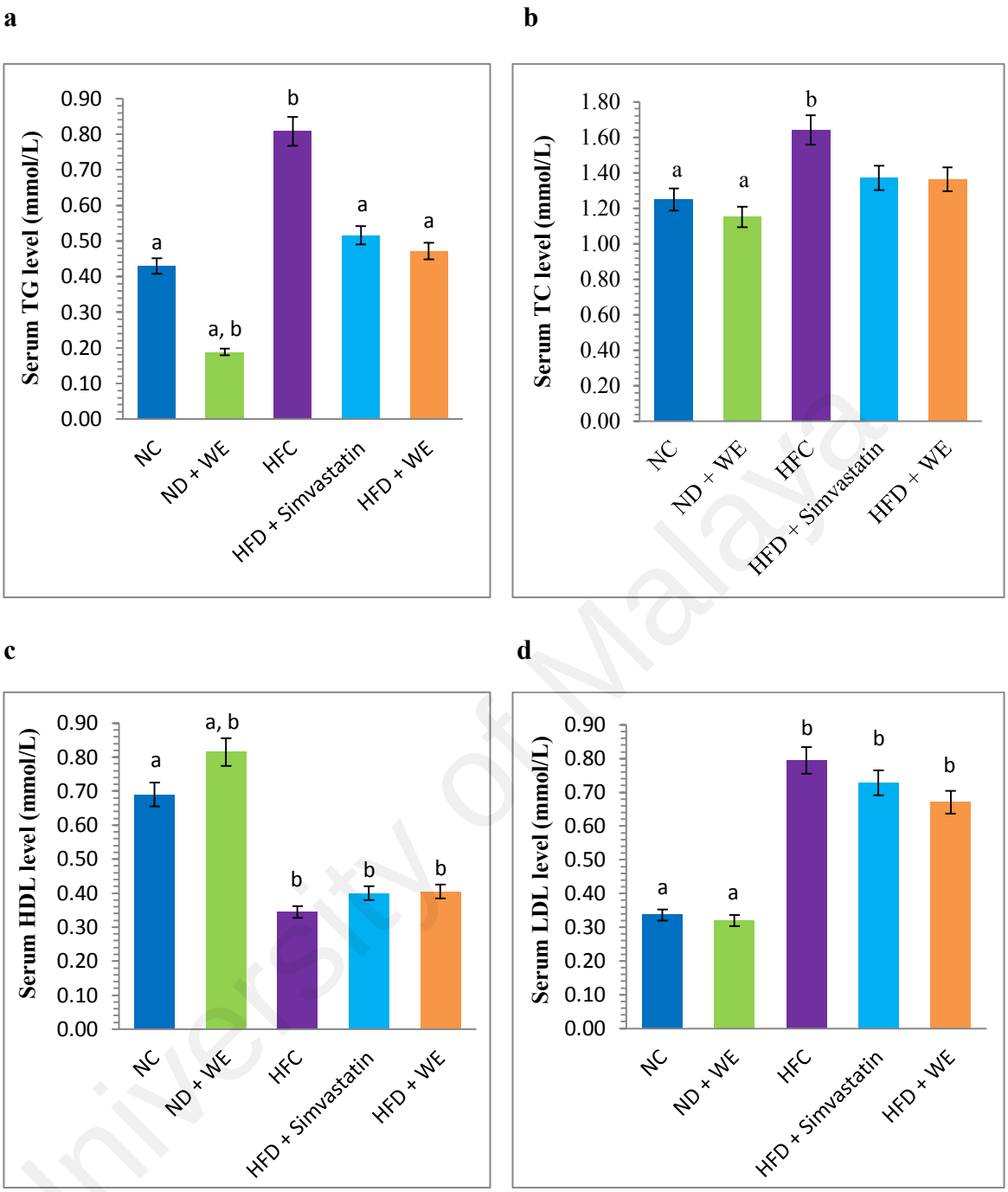

Figure 4.12: Lipid profile of rats in each group after WE treatment. **a:** serum triglyceride level **b:** serum total cholesterol level **c:** serum high-density lipoprotein cholesterol level **d:** serum low-density lipoprotein cholesterol level. Values represent mean ± SD (n = 6). The values with different case letters (a, b) are significantly different at [a] $p < 0.05$ versus HFC; [b] $p < 0.05$ versus NC (one-way ANOVA, Fisher's LSD post hoc test, followed by Dunnett's multiple comparison test). NC: normal diet control; ND + WE: normal diet with water extract; HFC: high fat diet control; HFD + WE: high fat diet with water extract; HFD + Simvastatin: high fat diet with simvastatin; TG: triglyceride; TC: total cholesterol; HDL: high-density lipoprotein; LDL: low-density lipoprotein.

The lipid profile levels of TG of the rats after WE treatment is shown in Figure 4.12a. Dunnett's multiple comparison test revealed that there were significant TG reductions detected after two weeks of treatment in HFD + WE and HFD + Simvastatin compared to HFC ($p < 0.05$). Rats in HFD + WE showed the lowest TG level (0.47 ± 0.19 mmol/L) compared to HFC (0.81 ± 0.04 mmol/L), followed by HFD + Simvastatin (0.52 ± 0.04 mmol/L). However, post hoc test revealed that there were no significant TG differences observed between HFD + WE and HFD + Simvastatin ($p > 0.05$) (Appendix FF). The TG level of HFD + WE and HFD + Simvastatin were not significantly different from NC ($p > 0.05$). Besides, Dunnett's multiple comparison test also showed that there was a significant TG reduction detected after treatment in ND + WE compared to NC and HFC ($p < 0.05$).

The lipid profile levels of TC of the rats after WE treatment is shown in Figure 4.12b. Rats in HFD + WE showed the lowest TC level (1.36 ± 0.19 mmol/L) compared to HFC (1.64 ± 0.20 mmol/L), followed by HFD + Simvastatin (1.37 ± 0.18 mmol/L). As compared to HFC, there was a positive TC reduction detected after treatment in HFD + WE and HFD + Simvastatin. However, Dunnett's multiple comparison test showed that there was no significant reduction detected in both treated HFD groups compared to NC and HFC ($p > 0.05$). Post hoc test also revealed that there was no significant TC difference observed between HFD + WE and HFD + Simvastatin ($p > 0.05$) (Appendix FF). On the other hand, there was also no significant TC reduction observed in ND + WE compared to NC even there was a positive reduction of the TC level detected in ND + WE ($p > 0.05$).

The lipid profile levels of HDL of the rats after WE treatment is shown in Figure 4.12c. There were positive HDL increments in HFD + WE and HFD + Simvastatin after treatment compared to HFC. However, Dunnett's multiple comparison test showed that

there were no significant HDL increments observed in HFD + WE and HFD + Simvastatin after treatment compared to HFC ($p > 0.05$). HFD + WE showed the highest HDL level (0.41 ± 0.06 mmol/L) as compared to HFC (0.35 ± 0.08 mmol/L), followed by HFD + Simvastatin (0.40 ± 0.06 mmol/L). Post hoc test revealed that there were also no significant HDL differences observed between HFD + WE and HFD + Simvastatin ($p > 0.05$) (Appendix FF). Meanwhile, there was a significant HDL increment detected in ND + WE compared to NC and HFC ($p < 0.05$).

The lipid profile levels of LDL of the rats after WE treatment is shown in Figure 4.12d. The rats in HFD + WE showed the lowest LDL level (0.67 ± 0.21 mmol/L) as compared to HFC (0.80 ± 0.25 mmol/L), followed by HFD + Simvastatin (0.73 ± 0.14 mmol/L). There were positive LDL reductions in HFD + WE and HFD + Simvastatin compared to HFC, but there were no significant LDL reduction observed in HFD + WE and HFD + Simvastatin compared to HFC ($p > 0.05$). Post hoc test revealed that there were also no significant LDL differences observed between HFD + WE and HFD + Simvastatin ($p > 0.05$) (Appendix FF). In addition, there was a positive reduction of LDL level detected in ND + WE after treatment compared to NC, but there were no significant LDL reduction observed in ND + WE compared to NC ($p > 0.05$).

4.5.3 Analyses before and after EAF treatment

4.5.3.1 Body weight and BMI

All rats were healthy throughout the experiment and they consumed the provided food during the whole experiment period. The body weight and BMI of rats in each group before and after treatment are shown in Table 4.25 (Appendix GG to Appendix II).

Table 4.25: The mean of initial (after obesity induction) and final (after EAF treatment) body weight and BMI of rats.

Group (s)	Body weight (g)		BMI (g/cm^2)	
	Initial	Final	Initial	Final
ND + 2% Tween 80	334.00 ± 2.37^{a}	363.33 ± 3.77$^{*, a}$	0.65 ± 0.03^{a}	0.65 ± 0.02^{a}
HFD + 2% Tween 80	405.00 ± 1.67^{b}	465.00 ± 4.00$^{*, b}$	0.70 ± 0.01^{b}	0.76 ± 0.02$^{*, b}$
ND + EAF	334.50 ± 2.43^{a}	335.50 ± 2.26$^{a, b}$	0.63 ± 0.02^{a}	0.61 ± 0.03$^{*, a, b}$
HFD + EAF	404.17 ± 2.99^{b}	377.33 ± 4.50$^{*, a, b}$	0.72 ± 0.05^{b}	0.64 ± 0.04$^{*, a}$

Values represent mean ± SD (n = 6). The final value in the same row with * are significantly different at $p < 0.05$ compared to the initial value (paired-sample t-test). The values in the same column with different case letters (a, b) are significantly different at ^{a} $p < 0.05$ versus HFD + 2% Tween 80; ^{b} $p < 0.05$ versus ND + 2% Tween 80 (one-way ANOVA, Fisher's LSD post hoc test, followed by Dunnett's multiple comparison test). ND + 2% Tween 80: normal diet with 2% Tween 80; HFD + 2% Tween 80: high fat diet with 2% Tween 80; ND + EAF: normal diet with ethyl acetate fraction; HFD + EAF: high fat diet with ethyl acetate fraction; BMI: body mass index.

After 8 weeks of obesity induction, all the HFD-induced obese rats had significantly 20% higher body weight and higher BMI (more than 0.68 ± 0.05 g/cm^2) than the ND rats. All the rats were successfully distributed into four groups as shown in Table 4.25. Based on the observation, the mobility of HFD rats was slower than the ND rats. Post hoc test was conducted and the result (Appendix HH) showed that there were significant body weight and BMI differences observed between ND rats in ND + 2% Tween 80 and ND + EAF groups and HFD-induced obese rats in HFD + 2% Tween 80 and HFD + EAF groups after obesity induction ($p < 0.05$). The result also showed that there were no significant body weight and BMI differences between ND + 2% Tween 80 and ND + EAF after obesity induction. Similarly, there were no significant body weight and BMI differences between HFD + 2% Tween 80 and HFD + EAF after obesity induction.

On the other hand, the body weight of HFD vehicle control rats in HFD + 2% Tween 80 was significantly increased from 405.00 ± 1.67 g to 465.00 ± 4.00 g by 60 g after two weeks, whereas the body weight of HFD rats treated with EAF was significantly

reduced from 404.17 ± 2.99 g to 377.33 ± 4.50 g by 26.84 g which was 6.64% after treatment ($p < 0.05$) (Appendix II). Dunnett's multiple comparison test was conducted and the result (Appendix HH) showed that the final body weight of HFD + EAF was significantly different from the final body weight of HFD + 2% Tween 80 and ND + 2% Tween 80 ($p < 0.05$). On the other hand, the BMI of HFD vehicle control rats in HFD + 2% Tween 80 was significantly increased from 0.70 ± 0.01 g/cm^2 to 0.76 ± 0.02 g/cm^2 by 0.06 g/cm^2 after two weeks, whereas the BMI of HFD rats treated with EAF was significantly decreased from 0.72 ± 0.05 g/cm^2 to 0.64 ± 0.04 g/cm^2 by 0.08 g/cm^2 after treatment. Dunnett's multiple comparison test showed that the final BMI of HFD + EAF was significantly different from the final BMI of HFD + 2% Tween 80.

Furthermore, the body weight of ND vehicle control rats in ND + 2% Tween 80 was significantly increased from 334.00 ± 2.37 g to 363.33 ± 3.78 g by 29 g after 2 weeks, but the body weight of ND rats treated with EAF was increased not significantly from 334.50 ± 2.43 g to 335.50 ± 2.26 g by only 1 g. Dunnett's multiple comparison test showed that the final body weight of ND + EAF was significantly different from the final body weight of ND + 2% Tween 80 and HFD + 2% Tween 80 ($p < 0.05$). Meanwhile, the BMI of rats in ND + EAF was significantly decreased from 0.63 ± 0.02 g/cm^2 to 0.61 ± 0.03 g/cm^2 by 0.02 g/cm^2 after treatment, whereas the BMI of ND vehicle control rats (0.65 g/cm^2) was maintained. The final BMI of ND + EAF was significantly different from ND + 2% Tween 80 and HFD + 2% Tween 80 ($p < 0.05$).

4.5.3.2 Food intake

The food intake of rats in each group was recorded daily during EAF treatment (Appendix GG) and the result is shown in Figure 4.13 (Appendix JJ).

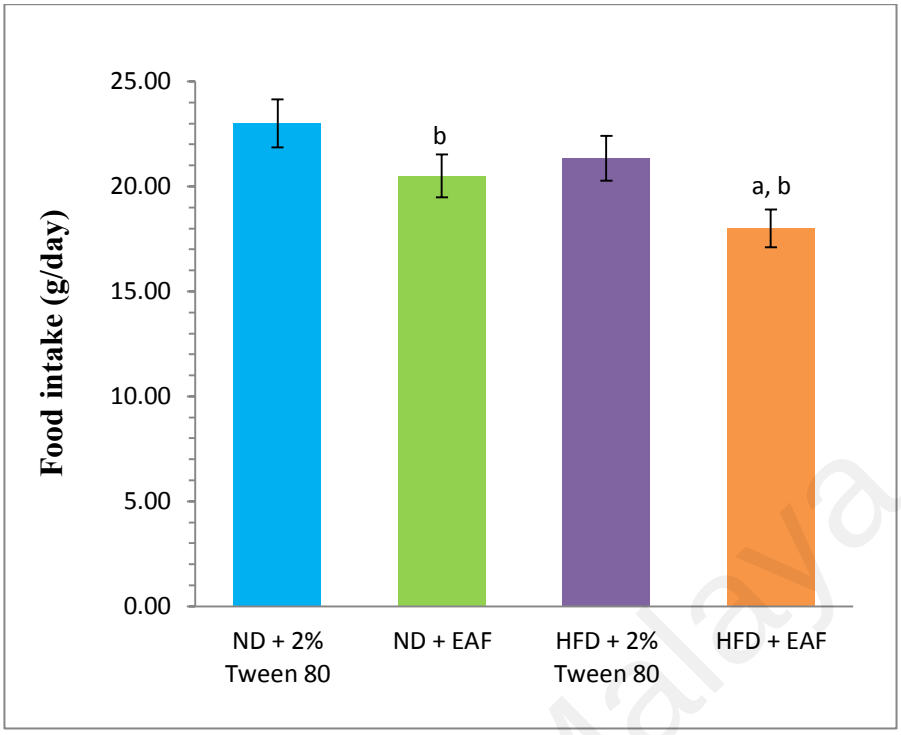

Figure 4.13: Food intake of rats during EAF treatment. Values represent mean ± SD (n = 6). The values with different case letters (a, b) are significantly different at [a] $p < 0.05$ versus HFD + 2% Tween 80; [b] $p < 0.05$ versus ND + 2% Tween 80 (one-way ANOVA, Fisher's LSD post hoc test, followed by Dunnett's multiple comparison test).

There was no significant food intake difference observed between ND + 2% Tween 80 and HFD + 2% Tween 80 ($p > 0.05$). On the other hand, there was a significant food intake difference found in ND + EAF compared to ND + 2% Tween 80 ($p < 0.05$). Besides, there was also a significant food intake difference observed in HFD + EAF as compared to ND + 2% Tween 80 and HFD + 2% Tween 80 ($p < 0.05$).

4.5.3.3 Biochemical test for lipid profile

The lipid profile levels of TG, TC, HDL and LDL of the rats after EAF treatment are shown in Figure 4.14a to Figure 4.14d (Appendix KK and Appendix LL). There were significant TG, TC, HDL and LDL differences observed between ND + 2% Tween 80 and HFD + 2% Tween 80 ($p < 0.05$).

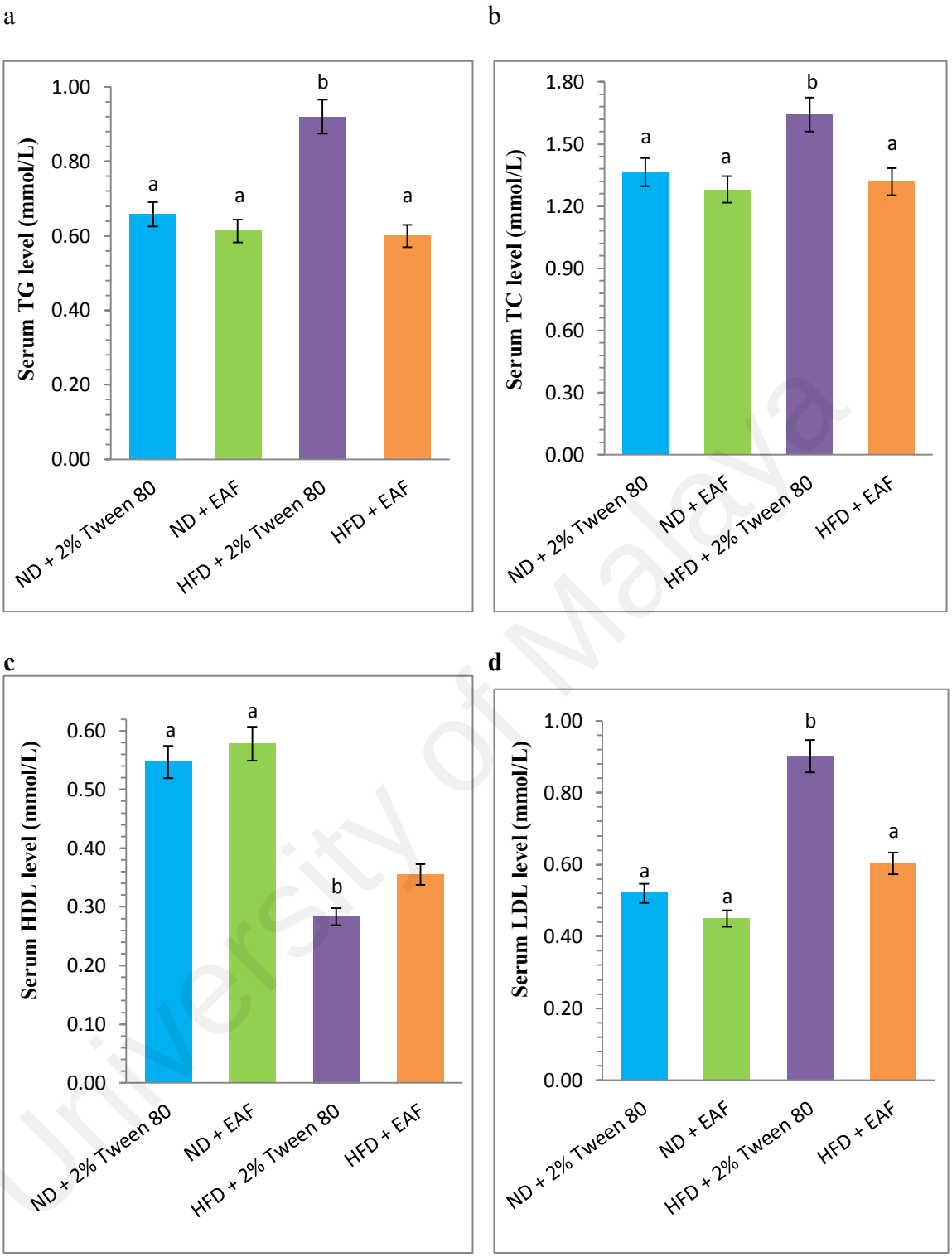

Figure 4.14: Lipid profile of rats in each group after EAF treatment. **a** serum triglyceride level **b** serum total cholesterol level **c** serum high-density lipoprotein cholesterol level **d** serum low-density lipoprotein cholesterol level. Values represent mean ± S (n = 6). The values with different case letters (a, b) are significantly different at [a] $p < 0.05$ versus HFD + 2% Tween 80; [b] $p < 0.05$ versus ND + 2% Tween 80 (one-way ANOVA, Fisher's LSD post hoc test, followed by Dunnett's multiple comparison test). ND + 2% Tween 80: normal diet with 2% Tween 80; HFD + 2% Tween 80: high fat diet with 2% Tween 80; ND + EAF: normal diet with ethyl acetate fraction; HFD + EAF: high fat diet with ethyl acetate fraction; TG: triglyceride; TC: total cholesterol; HDL: high-density lipoprotein; LDL: low-density lipoprotein.

There were significant TG, TC and LDL reductions found in HFD + EAF compared to HFD + 2% Tween 80 ($p < 0.05$). Moreover, there was a positive increment of HDL found in HFD + EAF compared to HFD + 2% Tween 80. However, there was no significant increment of HDL level observed in HFD + EAF compared to ND + 2% Tween 80 and HFD + 2% Tween 80 ($p > 0.05$).

On the other hand, there were positive TG, TC, and LDL reduction and HDL elevation observed in ND + EAF compared to ND + 2% Tween 80. However, there was no significant TG, TC and LDL reductions and no significant increment of HDL found in ND + EAF compared to ND + 2% Tween 80 ($p > 0.05$).

CHAPTER 5: DISCUSSION

5.1 Extraction

Phytochemicals extraction could be performed through solid-liquid extraction and liquid-liquid extraction. Both types of extractions were used in this study. Solid-liquid extraction is one of the extraction methods by extracting phytochemical compounds from solid material into a solvent. In this study, crude WE and crude 10% ME were obtained from solid-liquid extraction by extracting phytochemical compounds from the herbal powder into distilled water and 10% methanol, respectively.

According to Wang *et al.* (2009), phenolic compounds were best soluble in polar organic solvent than water. A lot of studies showed that most of the phenolic compounds were extracted by organic solvent that had high polarity and the amount of the extracted phenolic compounds was increased with the increased polarity of organic solvent (Ablat *et al.*, 2014). Therefore, 10% methanol was used to extract compounds from herbal powder and other organic solvents were used for fractionation in this study in order to compare the amount of extracted phenolic compounds with WE.

5.2 Fractionation

Liquid-liquid extraction is also one of the extraction methods by extracting phytochemical compounds from a solvent to another solvent. In this study, liquid-liquid extraction was applied through fractionation. The phytochemical compounds in filtered 10% ME crude extract were fractionated according to their polarity (from non-polar to high polarity) by using non-polar organic solvent to high polar organic solvent (hexane, chloroform, dichloromethane, ethyl acetate and butanol). Generally, a polar solvent dissolves polar compounds, while non-polar solvent dissolves non-polar compounds.

Two immiscible layers (organic layer and aqueous layer) were formed in each fractionation. During chloroform fractionation, the compounds that dissolved in chloroform were formed as an organic layer below the aqueous layer. Same condition was observed during dichloromethane fractionation. This condition was observed since the density of chloroform (1.4799 g mL^{-1}) and dichloromethane (1.25 g mL^{-1}) were higher than the density of aqueous layer which was nearly 1 g mL^{-1} (Yazdi *et al.*, 2008). However, the compounds that dissolved in hexane were formed as an organic layer above the aqueous layer. Same condition was observed during fractionation with ethyl acetate and butanol. This was because the density of organic solvents such as hexane, ethyl acetate and butanol were not as dense as the aqueous layer. Therefore, the organic layer appeared above or below the aqueous layer was dependent on the density of the organic solvent.

Furthermore, foam was observed during fractionation with chloroform and dichloromethane. The formation of foam might be due to the presence of saponins and this observation was proved by the saponins test conducted by Mir *et al.* (2016). According to the review by Aboutalebi and Monfared (2016), saponins are high molecular weight glycosides. They possess polycyclic aglycones part with one or more sugar side chains and the aglycone part is known as sapogenin, either steroid or triterpene (Aboutalebi & Monfared, 2016). Saponins can be classified into steroid saponin or triterpenoid saponin based on their aglycone part. The foaming ability of saponins is caused by the hydrophobic sapogenin and a hydrophilic sugar part of saponin (Aboutalebi & Monfared, 2016).

From Table 4.1, MAF showed the highest yield compared to the other fractions obtained from the crude 10% ME. The yield and color of each obtained dried fraction varied based on the solvent used during extraction and fractionation. This was because

different solvents dissolved different types of compounds and the characteristics and chemical structures of the compounds affected their solubility in the solvent (Bekele *et al.*, 2016). On the other hand, HF showed the lowest yield among the fractions obtained from crude 10% ME. The low yield of HF indicated that there was a minute amount of lipids in crude 10% ME since hexane was used to extract lipids (Ablat *et al.*, 2014).

5.3 Preliminary screening of phytochemical compounds

5.3.1 TLC

TLC is one of the easiest phytochemical screening methods used to separate and identify phytochemical compounds due to its fast, simplicity, cheap, high sensitivity and good reproducibility (Santana & Meireles, 2016). It is a chromatographic technique which the stationary phase is aluminum-backed TLC plate with silica gel (very polar adsorbent) and the mobile phase is a solvent or mixture of solvents. The principle of this method is based on capillary action which draws the solvent up the TLC plate.

In this experiment, the TLC plates were dried in a fume hood since the solvents (chloroform and 10% methanol in chloroform) used to run this TLC analysis were toxic and easily evaporated. The R_f value of each phytochemical compound was calculated based on the travelling distance of the compounds. Nonpolar compounds travelled further from the sample line compared to polar compounds (Santana & Meireles, 2016). Polar compounds travelled slowly because they strongly adsorbed on the stationary phase, which was a polar adsorbent (Columbia University, 2007).

Solvent system which was chosen in running TLC analysis was important for a good separation of compounds. A very high polar solvent system used in TLC analysis could cause all the compounds running to the top of a TLC plate. However, a very non-polar solvent used in TLC analysis could stop the movement of compounds and cause improper compounds separation (Columbia University, 2007). In this study, a good

separation of compounds was observed in each herbal extract when a mixture of non-polar solvent with a polar solvent (10% methanol in chloroform) was used as a solvent system to run the TLC analysis compared to chloroform. For example, no compound was detected in TLC analysis of MAF when chloroform was used as a solvent system. However, alkaloid, natural fluorescence compounds or compounds that contained benzene rings or conjugated systems, terpenoid and phenolic compounds were detected in TLC analysis of MAF when 10% methanol in chloroform was used as a solvent system.

Colored compounds such as plant pigments could be observed under visible light (Rhodium, 2017). Brownish green bands were observed under visible light in TLC analysis of CF and this indicated that the presence of plant pigments in CF. On the other hand, non-colored compounds could be observed as bright zones on a dark background under UV light (Columbia University, 2007). UV light is a non-destructive visualization technique used to observe compounds that absorbed UV light (Columbia University, 2007). Compounds that contained benzene rings or conjugated systems could absorb UV light, thus it could be observed under UV light (Columbia University, 2007). Natural fluorescence compounds such as aflatoxins, polycyclic aromatic hydrocarbons, riboflavin and quinine could also be observed under UV light at 254 nm (Rhodium, 2017). Colored and fluorescent bands were observed under UV light in this study (Appendix B). This indicated that the presence of natural fluorescence compounds or compounds that contained benzene rings or conjugated systems in the herbal extract.

On the other hand, the compounds that could not be observed under visible light and UV light could be visualized by spraying the TLC plate with visualizing reagents such as Dragendorff, Vanillin-H_2SO_4 and Folin-Ciocalteu reagents in order to form colored bands. Dragendorff reagent was used to detect alkaloid and other nitrogen-based

compounds with the formation of orange bands (Wall, 2000). After spraying with Dragendorff, orange bands were observed on the TLC plates which were spotted with HF, CF, DCMF (Appendix C), BF, MAF and WE. This indicated that the presence of alkaloids in HF, CF, DCMF, BF, MAF and WE. The presence of alkaloid in the herbal extracts might be due to the synephrine, which is a type of alkaloids from citrus (Haaz *et al.*, 2006). Synephrine could help to increase energy expenditure, increase metabolism and suppress appetite in order to reduce body weight (Haaz *et al.*, 2006). It is a fat burner and well-known as a replacement of the banned ephedra stimulant on body weight reduction, without ephedra side effects (Suryawanshi, 2011). Besides, the presence of alkaloid in the herbal extracts might be due to the caffeine, which is a type of alkaloids from green tea (Dulloo *et al.*, 2000) and Guarana (Ângelo *et al.*, 2008). Caffeine from green tea could help to increase lipolysis and thermogenesis (Astrup *et al.*, 1992; Astrup *et al.*, 1995). The Guarana's seed contains two times higher amount of caffeine (2-4.5%) compared to coffee bean (1-2%) (Ângelo *et al.*, 2008). A study conducted by Suleiman *et al.* (2016) showed that the TG level of overweight people who consumed Guarana was reduced 20% compared to basal values. The reduction of the TG level was due to the high concentration of caffeine in Guarana (Bittencourt *et al.*, 2013). Another study showed that the fat content and body weight of treated rats were significantly reduced after the rats were treated with whole Guarana extract compared to decaffeinated Guarana (Lima *et al.*, 2005). In addition, the presence of alkaloid in the herbal extracts might be due to the alkaloids from lotus leaves (Shoji *et al.*, 1987). An investigation showed that some of the alkaloids in lotus leaves could significantly inhibit pancreatic lipase and inhibit adipocyte differentiation (Ahn *et al.*, 2013). All these alkaloids might be the contributors of body weight reduction and lipid profile improvement in WE treatment of this study.

Meanwhile, vanillin-sulfuric acid reagent was used to detect terpenoids such as essential oil and steroids with the formation of purple or blue bands (Kristanti & Tunjung, 2015; Nithya & Muthumary, 2011; Wall, 2000). After spraying with vanillin-sulfuric acid reagent, purple, blue and dark blue bands were observed on the TLC plates which were spotted with HF, CF, DCMF, EAF (Appendix D), MAF and WE. This indicated that the presence of terpenoids in HF, CF, DCMF, EAF, MAF and WE. The presence of terpenoid in the herbal extracts might be due to the D-limonene, which is a type of terpenoids from citrus (Yi *et al.*, 2017). D-limonene helped to reduce the size of white and brown adipocytes, lower the serum level of TG and LDL-C and increase the serum level of HDL-C in obese mice (Jing *et al.*, 2013). Furthermore, the presence of terpenoid in the herbal extracts might be due to the β-carotene, which is a type of terpenoids from *Spirulina* (Colla *et al.*, 2008). β-carotene was known to exhibit antioxidant properties (Miranda *et al*., 1998). Hence, these terpenoids might be the contributors of body weight reduction and lipid profile improvement in WE and EAF treatments of this study.

Vanillin-sulfuric acid reagent was also used to detect phenolic compounds with the formation of red or other colored bands. According to a study conducted by Sharma *et al.* (1998), vanillin-sulfuric acid reagent could be used to detect phenolic compounds such as gallic acid, methyl gallate, pyrogallol, phloroglunicol, catechol, resorcinol, hydroquinone, catechin, epicatechin, ferulic acid and *p*-coumaric acid. In this study, orange band was observed after spraying vanillin-sulfuric acid reagent on the TLC plate that spotted with CF which used chloroform as a solvent system. The formation of orange band might be due to the presence of phloroglucinol, catechin and epicatechin in CF and this observation was supported by Sharma *et al.* (1998). Moreover, red bands were observed on TLC plates that spotted with CF, BF, EAF (Appendix D) and WE after spraying with vanillin-sulfuric acid reagent. This indicated that the presence of

phenolic compounds in the CF, BF, EAF and WE. The presence of phenolic compounds in WE might be due to the gallocatechin 3-O-gallate from green tea and this was identified by LC/Q-TOF/MS in this study.

Folin-Ciocalteu reagent was also used to detect phenolic compounds with the formation of blue or purple band (Jindal & Mohamad, 2012; Wall, 2000). In this study, dark blue and dark purple bands were observed on TLC plates that spotted with EAF (Appendix E), BF, MAF and WE after spraying with Folin-Ciocalteu reagent. This indicated that the presence of phenolic compounds in the EAF, BF, MAF and WE. The phenolic compounds in the herbal extracts might be extracted from citrus (Yi *et al.*, 2017), green tea (Dulloo *et al.*, 1999), Guarana (Ângelo *et al.*, 2008), grape seed (Xia *et al.*, 2010), lotus leaves (Ohkoshi *et al.*, 2007), psyllium husk (Patel *et al*, 2016) and *Spirulina* (Colla *et al.*, 2008). The phenolic compounds in the herbal extracts might be due to the hesperidin, which is a type of flavonoids from citrus. A human clinical trial showed that the consumption of citrus was able to increase HDL-C and reduce LDL-C and this was due to the presence of hesperidin in citrus (Suryawanshi, 2011). Besides, a study proved that a phenolic extract of grape seed was able to treat obesity with the association of reducing oxidative stress (Décordé *et al.*, 2009). Furthermore, a study showed that the lotus leaf flavonoids exhibited high percentage of *in vitro* porcine pancreatic lipase and α-amylase inhibitory activities, improved lipid profile of the HFD rats and reduced lipid accumulation in the liver of HFD rats (Liu *et al.*, 2013). Thus, these phenolic compounds might be the contributors of body weight reduction and lipid profile improvement in WE and EAF treatments of this study.

5.3.2 LC/Q-TOF/MS

Amino acids, benzoic acid, flavonoids, phenolic compounds, polysaccharides and sugars were detected in the WE. All these compounds might be the contributors of body

weight reduction and lipid profile improvement in the WE treatment of this study. Gallocatechin 3-O-gallate was detected in WE. It is a type of flavonoids and its presence in WE might be due to the green tea in HP. It is a well-known and major antioxidant from green tea (Ikeda *et al.*, 2003). Ikeda *et al.*, 2003 reported that gallocatechin 3-O-gallate is a type of catechin, which could lower dietary cholesterol absorption and inhibit obesity. Besides, levan was detected in WE. Levan is a dietary fiber and a homopolysaccharide, which consists of a type of monosaccharide repeating unit where sugar monomers are bound to form linear chains (Öner, 2013). It is water soluble, strongly adhesive and film-forming biopolymer, thus it acts as emulsifier, stabilizer, encapsulating agent and thickener (Öner, 2013). Kazemipoor *et al.* (2012) reported that due to these properties, levan could inhibit the PL activity by preventing the absorption of lipid in the intestine. Hence, the non-absorbed fat could be excreted through feces. Sun *et al.* (2016) also reported that levan could suppress appetite and provide satiety. In addition, 6-gingerol was also detected in WE. It is a type of phenolic compounds. Tzeng *et al.* (2014) reported that it could help to reduce adipogenesis by down-regulating the PPARγ activity in order to prevent adipocyte differentiation. Therefore, all these compounds might be the contributors of body weight reduction and lipid profile improvement in the WE treatment.

5.4 *In vitro* studies

5.4.1 Determination of TPC and TFC

Phenolic compounds are the most important secondary metabolites found in plants. These compounds play an important role as antioxidants due to their redox properties (Jing *et al.*, 2015). In this study, the TPC of herbal extracts was determined using the Folin-Ciocalteu method and this method was based on the reaction of oxidation-reduction. Folin-Ciocalteu reagent used in this assay contained phosphomolybdic/phosphotungstic acid complexes and the addition of sodium

carbonate into the mixture in each microtiter plate well was to adjust the pH of the mixture into alkaline condition (Bioquochem, 2017). When phenolic compounds donated electrons to phosphomolybdic/phosphotungstic acid complexes in alkaline medium, blue color complexes would form (Lallianrawna *et al.*, 2013). In this study, the color of the mixture in some microtiter plate wells was changed from colorless to dark blue. This indicated that the presence of electron donating phenolic compounds in the herbal extracts (Lallianrawna *et al.*, 2013). The color intensity was proportional to the concentration of the electron donating phenolic compounds in the herbal extracts.

Flavonoids are also the most important and widely distributed group of phenolic compounds in plants. These compounds also play an important role as antioxidants (Jing *et al.*, 2015). The TFC of herbal extracts was determined using aluminum chloride colorimetric method in the presence of sodium nitrite in alkaline medium. The addition of sodium hydroxide solution into the mixture in each microtiter plate well was to adjust the pH of the mixture into alkaline condition (Pękal & Pyrzynska, 2014). The basic principle of using aluminum chloride colorimetric method for the determination of TFC was based on the complexation of aluminum ion with C-4 keto and either C-3 or C-5 hydroxyl, or with ortho hydroxyl groups in the A or B ring of flavonoids (Pękal & Pyrzynska, 2014). In this study, the color of the mixture in some microtiter plate wells was changed from colorless to yellowish brown and this indicated that there was a formation of aluminum-flavonoid complexes in the herbal extracts (Pękal & Pyrzynska, 2014). The color intensity was proportional to the concentration of the aluminum-flavonoid complexes in the herbal extracts.

Table 4.17 and Table 4.18 showed the TPC and TFC of the herbal extracts. The contents varied due to different solvents used in the extraction. EAF had higher TPC and TFC than HF and WE. This indicated that ethyl acetate, a semi-polar solvent was

more effective in extracting phenolic compounds and flavonoids than low or high polar solvent. The same result was also obtained from a study conducted by Jing *et al.* (2015).

Based on the theory, the TPC of each extract should be higher than its TFC since flavonoid is one of the groups of phenolic compounds. However, the result in this study showed that the TFC of each fraction and crude extract was higher than its TPC. This might be due to the gallic acid, a standard used for the determination of TPC in this study was not the predominant phenolic compound in the HP (Tharmadurai, 2016). Except gallic acid, catechin could be used as a standard for the determination of TPC and this was supported by Tharmadurai (2016). Besides, catechin should be used as a standard for the determination of TFC instead of quercetin since catechin might be the predominant flavonoid in the HP. This was because green tea, Guarana, grape seed and lotus leaves in the HP consisted of catechin and it was proved by several studies (Ângelo *et al.*, 2008; Dulloo *et al.*, 2000; Ohkoshi *et al.*, 2007; Xia *et al.*, 2010). When same standard (catechin) was used to express the TPC and TFC of the herbal extracts, both of the contents could be easily and fairly compared (Tharmadurai, 2016).

5.4.2 Determination of antioxidant activities

5.4.2.1 DPPH radical scavenging assay

DPPH assay is a relatively fast, simple and economical assay compared to the other antioxidant assays (Alam *et al.*, 2013). It is a commonly used *in vitro* antioxidant assay, which is conducted based on the reduction of DPPH radical to a non-radical form of DPPH in the presence of hydrogen donating antioxidant (Afshar *et al.*, 2012). DPPH is a stable free radical that possessed a delocalized electron over the molecule in order to prevent dimerization. The delocalized electron contributes to the formation of dark purple color (Alam *et al.*, 2013). The hydrogen donation ability of antioxidants in the herbal extracts was detected by measuring the color changes at 517 nm. In this assay,

the color of the mixture was changed from dark purple to light yellow, which was observed as a decrease in absorbance. This indicated that the presence of hydrogen donating antioxidants in the herbal extracts, which was able to reduce DPPH radical to a non-radical form of DPPH. The same observation was also obtained by Irshad *et al.* (2012).

From Figure 4.5, it showed that the percentage of DPPH scavenging activity of the herbal extracts was increased with the increased concentration of the extracts. EAF and BF exhibited the highest DPPH scavenging ability with the lowest IC_{50} values of 0.03 ± 0.00 mg/mL and 0.06 ± 0.01 mg/mL, respectively, which were comparable to ascorbic acid. EAF had the same IC_{50} value as the ascorbic acid. IC_{50} value was defined as the concentration of antioxidant needed to reduce the initial radical by 50%. The lower the IC_{50} value, the more potent the antioxidant was in DPPH scavenging activity (Irshad *et al.*, 2012). Therefore, these results indicated that EAF and BF contained the most potent antioxidants in scavenging DPPH radicals among the other herbal extracts. This was supported by a study conducted by Irshad *et al.* (2012) since they found out that the methanolic extracts of pulp and seed of *Cassia fistula* were the strongest DPPH radical scavengers due to the lowest IC_{50} value obtained by the extracts.

5.4.2.2 FRAP assay

FRAP assay was conducted to determine the ferric reducing power of the herbal extracts. This assay is conducted based on the reduction of colorless ferric complex (Fe^{3+} tripyridyltriazine) to violet blue colored ferrous complex (Fe^{2+} tripyridyltriazine) in the presence of electron donating antioxidants at low pH (Irshad *et al.*, 2012). In this assay, the color intensity was proportional to the ferric reducing power. The same observation was also obtained by Irshad *et al.* (2012).

From Table 4.20, EAF and BF showed the highest ferric reducing power, while HF and CF showed the lowest ferric reducing power. These postulated that the EAF and BF contained the most potent antioxidants, which were able to donate electrons and react with free radicals in order to convert them into a more stable product and inhibit the free radical chain reactions. This result was supported by a study conducted by Irshad *et al.* (2012). Besides, the EAF with the highest TPC and TFC exhibited the highest FRAP value, whereas HF and CF with the lowest TPC and TFC exhibited the lowest FRAP value. This suggested that phenolic compounds and flavonoids might be the contributors in reducing ferric ions and the same result was obtained by Jing *et al.* (2015).

5.4.2.3 Metal chelating assay

This assay was used to measure the formation of ferrous-ferrozine complex based on the chelating capacity of ferrous ion with ferrozine (Ablat *et al.*, 2014). The complexation was detected by the color changes of mixture from purple to colorless, which was observed as a decrease in absorbance. EDTA-Na_2 which was a standard in this assay exhibited the highest percentage of ferrous ion chelating activity (97.01%) at the concentration of 1.6 mg/mL as compared to the herbal extracts. However, the percentage of ferrous ion chelating activity of all the herbal extracts in this study was lower than 50%. Since a good result was obtained by EDTA-Na_2 in this assay, thus this confirmed that there was no careless mistake when conducting this assay.

In this study, the result depicted that the metal chelating capability of the antioxidants in the herbal extracts was far lower than EDTA-Na_2. This result was supported by a study conducted by Andjelkovic *et al.* (2006). In addition, researchers reported that the metal chelating ability was a minor role for some antioxidants compared to the other antioxidant properties (Jindal & Mohamad, 2012).

5.4.2.4 NO radical scavenging assay

NO is a very unstable, diatomic molecule that possesses an unpaired electron and it is produced by endothelial cells, macrophages and neurons (Parul *et al.*, 2013). NO helps to mediate memory, neurotransmission, synaptic plasticity and vasodilation. Besides, it also controls platelet function, antimicrobial and antitumor activity (Jagetia *et al.*, 2004). Overproduction of NO which is caused by obesity can lead to cell damage, DNA fragmentation and neuronal cell death (Parul *et al.*, 2013). In this assay, NO was generated spontaneously when sodium nitroferricyanide mixed with PBS (pH 7.4). The NO could react with oxygen and decompose to form nitrite ions. The amount of nitrite ions was determined by Griess reagent (Jagetia *et al.*, 2004). Griess reagent contained sulphanilamide, N-1-napthylethylenediamine dihydrochloride and phosphoric acid. Light pink color was observed when diazotization occurred between nitrite ions and sulphanilamide, followed by coupling with N-1-napthylethylenediamine dihydrochloride under acidic (phosphoric acid) condition (Jagetia *et al.*, 2004). In this study, the color of the mixture was changed from light pink to light yellow, which was observed as a decrease in absorbance. This indicated that the presence of antioxidants in the herbal extracts were able to compete with oxygen in order to prevent decomposition of NO and formation of nitrite ions. This result was supported by Jagetia *et al.* (2004).

From Figure 4.8, it showed that the percentage of NO scavenging activity of the herbal extracts was increased with the increased concentration of the extracts. The percentage of NO scavenging activity of all the herbal extracts was lower than 50%, except EAF. EAF exhibited the highest percentage of NO scavenging activity with the IC_{50} value of 1.00 ± 0.05 mg/mL, but still weaker than curcumin. Hence, these results showed that the curcumin was excellent in scavenging NO compared to all the herbal extracts. However, EAF contained the most potent antioxidants in scavenging NO radicals among the herbal extracts. The same result was also obtained by Ablat *et al.*

(2014) as they found out that the EAF of *Brucea javanica* seed exhibited the highest NO scavenging activity among all the obtained extracts (HF, CF and WE), but still weaker than curcumin.

5.4.2.5 Superoxide scavenging assay

Superoxide anion is a weak oxidant, but it can produce highly reactive hydroxyl radicals and singlet oxygen, which can cause oxidative stress (Meyer & Isaksen, 1995). NBT was used as a probe in this assay. Superoxide radicals were generated from dissolved oxygen by PMS-NADH coupling reaction in the assay. The superoxide radicals were able to reduce NBT into purple formazan which could be measured at 560 nm (Iqbal *et al.*, 2012). In this assay, the color of the mixture was changed from dark purple to yellow, which was observed as a decrease in absorbance. The decrease in absorbance showed that the presence of antioxidants in the herbal extracts, which were able to inhibit the reduction of NBT by scavenging superoxide radicals. The same observation was also obtained by Iqbal *et al.* (2012).

Based on Figure 4.9, it showed that the percentage of superoxide scavenging activity of the herbal extracts was increased with the increased concentration of the extracts. EAF exhibited the highest superoxide scavenging ability with IC_{50} values of 0.16 ± 0.01 mg/mL, which was lower than gallic acid (0.21 ± 0.01 mg/mL). Therefore, these results showed that EAF contained the most potent antioxidants in scavenging superoxide radicals compared to the herbal extracts and gallic acid. The same result was also obtained by Jing *et al.* (2015) as they found out that the BF of *Rhododendron anthopogonoides* exhibited the highest superoxide scavenging ability with IC_{50} values of 270.67 ± 0.58 μg/ml, which was lower than standard and the other extracts.

5.4.3 Correlation between TPC, TFC and antioxidant activity

The antioxidant activities of the herbal extracts were the indication of the presence of phenolic compounds and flavonoids with varying antioxidant properties (Jing *et al.*, 2015). This might be due to the redox properties of the antioxidants, which allow them to act as hydrogen donors, reducing agents, singlet oxygen quenchers and transition metal ion chelators (Rice-Evans *et al.*, 1997). In this study, the correlation between TPC, TFC and antioxidant activity of the herbal extracts was determined using Pearson's correlation test. A significant correlation was found between TPC, TFC of the extracts with DPPH scavenging activity, ferric reducing power and superoxide scavenging activity. Besides, EAF not only obtained the highest TPC and TFC, but it also exhibited the highest percentage of NO inhibition. These indicated that the phenolic compounds and flavonoids in the herbal extracts were the contributors in scavenging DPPH radicals, reducing ferric ions to ferrous ions, scavenging NO radicals and scavenging superoxide radicals. These results were supported by the studies conducted by Ablat *et al.* (2014) and Jing *et al.* (2015) as they also found out that the extract with higher TPC and TFC showed the higher antioxidant capability.

The phenolic compounds and flavonoids that contributed to the antioxidant activities in this study might be obtained from citrus (Yi *et al.*, 2017), green tea (Dulloo *et al.*, 1999), Guarana (Ângelo *et al.*, 2008), grape seed (Xia *et al.*, 2010), lotus leaves (Ohkoshi *et al.*, 2007), psyllium husk (Patel *et al*, 2016) and *Spirulina* (Colla *et al.*, 2008) in the HP. Citrus flavonoids are the most important compounds since they could exert antioxidant activities by scavenging free radicals, preventing damage effect of ROS, chelating metal ions, inhibiting lipid peroxidation, inhibiting the activity of oxidant enzymes and enhancing the activity of antioxidant enzymes (Zou *et al.*, 2016). Besides, Portella *et al.* (2013) proved that Guarana polyphenols could provide antioxidant protection to LDL. Moreover, flavonoids such as (+)-catechins, (-)-

epicatechin and procyanidin polymers could be found in grape seeds (Xia *et al.*, 2010). The flavonoids in grape seed are known to have strong antioxidant and anti-inflammatory effects *in vitro* (Li *et al.*, 2000) and *in vivo* (Chang *et al.*, 2007). Colla *et al.* (2008) also reported that the phenolic compounds in *Spirulina* had antioxidant abilities and they were contributed in obesity treatment and lipid profile improvement.

5.5 Animal studies

5.5.1 Oral acute toxicity test

The oral acute toxicity test of the HP extract showed no mortality and no toxic effect with a dose of 5 g/kg b.w. of rat after 14 days of observation in both groups of the male and female rats. Thus, the LD_{50} value was expected to exceed 5 g/kg b.w. of rat (OECD, 2002). Based on Figure 4.10, the body weight of rats in both groups was decreased from day 1 to day 3. This might be due to the effect of WE on body weight reduction after administration of a single dose of WE to the rats on the first day of the test. However, the body weight of rats in both groups was increased gradually from day 3 until the last day of the test. The elevation of body weight showed that the WE had no toxic effect on the rats in both groups.

The paired t-test revealed that there was a significant change between initial and final body weight of male rats ($p < 0.05$), while there was no significant change between initial and final body weight of female rats ($p > 0.05$). This indicated that male SD rats were sensitive and more suitable for obesity induction and for treatment studies. Besides, this also showed that normal male SD rats could be induced into obese rats faster than female SD rats.

5.5.2 Development of high fat diet-induced obese rats

HFD-induced obese rat is always used to conduct experiments related to obesity since the induced obese rat has the same characteristics and usual pathway of obesity as

humans and the body weight of rats increases when they are fed with HFD pellet (Kadir *et al.*, 2015). Fat is the most energy dense nutrient and the addition of this nutrient in the food can increase calories and energy. High calorie intake increases body weight and energy intake, thus leads to obesity. Kadir *et al.* (2015) showed that a diet which contained more than 30% of total energy as fat could cause obesity. Therefore, HFD pellet with 35.3% of total energy as fat was prepared in this study in order to induce normal rats into obese rats.

Woods *et al.* proposed that the body weight of HFD-induced obese rats were higher than ND rats (Kadir *et al.*, 2015). In this study, the degree of obesity was evaluated by comparing the body weight of HFD-induced obese rats with ND rats. However, body weight was not enough for obesity verification since body weight gain might be due to increase of fat or muscle mass (Mohamed *et al.*, 2014). Hence, BMI was used together with body weight gain to verify the obese status of rats. Novelli *et al.* (2007) proposed that the range of BMI for ND adult rat was between 0.45 ± 0.02 and 0.68 ± 0.05 g/cm^2, while the BMI for HFD rats were higher than 0.68 ± 0.05 g/cm^2.

There were significant body weight and BMI differences observed between ND groups and all the HFD induced obese groups after obesity induction at week 8 ($p < 0.05$) (Table 4.24 and Table 4.25). Since all the HFD induced obese rats had significantly 20% higher body weight and higher BMI (more than 0.68 ± 0.05 g/cm^2) than the ND rats, thus the rats were verified as obese rats and then proceeded with treatment studies.

5.5.3 Analyses before and after WE and EAF treatment

Citrus, grape seed, green tea, Guarana, lotus leaves, *Spirulina* and psyllium husk itself have the potential to reduce body weight and improve the lipid profile. However, the effect of the mixture of these herbs on body weight reduction and lipid profile

improvement in animal model has not been investigated. Therefore, the mixture of the herbs from a prepared HP was used to test for its effectiveness on body weight reduction and lipid profile improvement in HFD-induced SD rats.

The dose of HP for humans was 2500 mg per day (M. Jamaludin, personal communication, January 19, 2016). According to Huang *et al.* (2016), the rat dose could be converted from human equivalent dose based on the body surface area and formula from US FDA by assuming a human weight of 60 kg. The rat dose was obtained as 2500 mg/day/60 kg = 41.67 x 6 = 250 mg/kg. The conversion coefficient, 6 from the calculation represented the differences of body surface area between human and rat. Thus, the rat dose used for treatment in this study was set at 250 mg/kg b.w. of rat.

A lot of studies showed that body weight reduction and lipid profile improvement were due to the presence of phenolic compounds and flavonoids (Bordicchia *et al.*, 2014; Colla *et al.*, 2008; McCrindle *et al.*, 2003; Suryawanshi, 2011; Thielecke & Boschmann, 2009; Yang *et al.*, 2001). The herbs used in this study were rich in phenolic compounds and flavonoids, which had anti-obesity, antioxidant and hypolipidemic properties. For example, body weight was reduced after the consumption of flavonoids from *N. nucifera* leaves since the flavonoids was able to activate β-adrenergic receptor in order to promote lipolysis in white adipose tissue and non-shivering thermogenesis in BAT (Bordicchia *et al.*, 2014; Ohkoshi *et al.*, 2007). Besides, a study showed that EGCG, which is a flavonoid in green tea was able to reduce TG (Yang *et al.*, 2001), TC (Ashida *et al.*, 2004), body weight and body fat (Thielecke & Boschmann, 2009). Human clinical trial also showed that the consumption of citrus increased HDL level and reduced LDL level since citrus is rich in vitamin C and flavonoids such as hesperidin (Suryawanshi, 2011). On the other hand, various studies showed that lipid profile improvement was correlated with the antioxidant activity of phenolic compounds

(Belguith-Hadriche *et al.*, 2010; Hassan *et al.*, 2011). A study also showed that the therapeutic properties of *Spirulina* on obesity treatment and lipid profile improvement were due to the antioxidant abilities of polyunsaturated fatty acids (γ-linolenic acid), phycocyanin and phenolic compounds in *Spirulina* (Colla *et al.*, 2008). In this study, EAF possessed the highest TPC and TFC among HF, CF, DCMF, BF and MAF. The EAF also exhibited the highest antioxidant capability in scavenging DPPH, NO and superoxide radicals as well as reducing ferric ion into ferrous ion among HF, CF, DCMF, BF and MAF. Therefore, EAF was chosen to test for its effectiveness on body weight reduction and lipid profile improvement in HFD-induced SD rats and its effectiveness was compared with WE.

Ethyl acetate extract/fraction of other plants was also chosen by other researchers for animal studies due to its high TPC, TFC and antioxidant capacity. Belguith-Hadriche *et al.* (2010) reported that the ethyl acetate extract of fenugreek seed had a significant lipid lowering and antioxidative effects on high-cholesterol-fed rats and these effects were correlated with its TPC, TFC and antioxidant activities. Besides, Ablat *et al.* (2014) reported that the EAF of *B. javanica* seed had a hypoglycemic effect on non-diabetic rats and the effect were correlated with its high TPC, TFC, antioxidant activities and glycogen phosphorylase α inhibition.

5.5.3.1 Body weight and BMI

The body weight of HFD control rats in HFC and HFD + 2% Tween 80 was increased approximately 2-fold than the ND control rats in NC and ND + 2% Tween 80. This showed that the high fat diet pellet in this study could significantly increase the body weight of rats and this might be due to the high caloric intake and high energy density of the pellet. This was supported by Kadir *et al.* (2015) since they also found out that the consumption of high fat diet led to the significant high caloric intake compared

to the normal diet and this was due to the addition of fat to the food which could increase calories and energy density. However, there was no significant change between initial and final BMI of ND control rats in NC and ND + 2% Tween 80, while the BMI of HFD control rats in HFC and HFD + 2% Tween 80 was significantly increased after two weeks. This indicated that the body weight gain in ND control rats was not due to the body fat gain, but it might be due to muscle mass gain. This was supported by Mohamed *et al.* (2014) as they reported that body weight alone cannot be used as an indicator of obesity since weight gain might be due to fat or muscle mass.

After two weeks of treatment, the body weight and BMI of HFD rats treated with WE, EAF and simvastatin were significantly reduced compared to HFD control rats ($p < 0.05$). The result showed that WE (250 mg/kg), simvastatin and EAF (250 mg/kg) was able to reduce body weight of HFD rats by 4.79%, 6.18% and 6.64% after two weeks of treatment. This indicated that EAF had the highest ability in reducing body weight of HFD rats after two weeks of treatment, followed by simvastatin and then WE. Besides, the significant BMI reduction indicated that WE, EAF and simvastatin were able to reduce body fat of HFD rats significantly.

On the other hand, the body weight of ND rats was controlled by increasing only 4.54% after WE treatment compared to ND control rats in NC, where their body weight was significantly increased by 8.72%. There was no significant change between initial and final BMI of NC and ND + WE after two weeks treatment ($p > 0.05$). These indicated that WE was able to control body weight of ND rats and maintain the BMI of ND rats after treatment. Besides, the body weight of ND rats was controlled by increasing only 0.30% after EAF treatment compared to ND control rats in ND + 2% Tween 80, where their body weight was significantly increased by 8.78%. The BMI of ND rats was significantly decreased after EAF treatment ($p < 0.05$), while there was no

significant change between initial and final BMI of ND + 2% Tween 80 ($p > 0.05$). These results indicated that the EAF was able to control body weight of ND rats better than WE, and the EAF was able to reduce BMI of ND rats but WE was not able to reduce BMI of ND rats.

All these results concluded that EAF had better potential in body weight and BMI reductions in HFD and ND rats compared to WE. The effectiveness of body weight and BMI reductions by EAF might be due to its highest TPC and TFC. It was reported that phenolic compounds, especially flavonoids had the potential in body weight and body fat reductions due to their anti-obesity, antioxidant and hypolipidemic effects (El-Tantawy, 2015). The compounds could be obtained from green tea, Guarana, grape seed and lotus leaves in the HP. Researchers reported that flavonoids obtained from green tea such as ECG, EGC and EGCG had a strong lipase inhibitory effect and they could reduce body weight and body fat (Thielecke & Boschmann, 2009). Catechins in Guarana were able to prevent fat absorption in the intestine (Bérubé-Parent *et al.*, 2005; Kao *et al.*, 2010). Besides, phenolic extract of grape seed was able to treat obesity (Décordé *et al.*, 2009). Liu *et al.* (2013) also reported that the lotus leaf flavonoids had a high percentage of *in vitro* pancreatic lipase and α-amylase inhibitory activities. Therefore, the highest abilities of body weight and body fat reductions obtained by EAF might be contributed by the highest amount of phenolic compounds and flavonoids in the EAF and the compounds might have anti-obesity and hypolipidemic properties.

5.5.3.2 Food intake

There were no significant food intake differences observed between NC and HFC as well as between ND + 2% Tween 80 and HFD + 2% Tween 80 ($p > 0.05$). This indicated that the amount of food intake was not affected by the amount of calories in the HFD and ND pellets. The same result was obtained by Kadir *et al.* (2015). However,

there were significant food intake reductions found in all treated HFD groups compared to their own control group (HFC or HFD + 2% Tween 80), except HFD rats treated with simvastatin. There were also significant food intake reductions found in all treated ND groups compared to their own control group (NC and ND + 2% Tween 80) ($p < 0.05$). These indicated that the compounds in EAF and WE were able to suppress appetite and provide satiety, in order to decrease food intake and ultimately reduce or control the body weight of the rats. The compounds could be obtained from citrus, Guarana, green tea and psyllium husk in the HP. Researchers reported that caffeine obtained from green tea (Dulloo *et al.*, 1999) and Guarana (Pittler *et al.*, 2005) could increase satiety and delay gastric emptying. In addition, Haaz *et al.* (2006) reported that synephrine, which is a type of alkaloids obtained from citrus could suppress appetite in order to reduce body weight. Based on the mini review by Moon *et al.* (2007), they mentioned that EGCG, which was a polyphenolic flavonoid from green tea had the ability to suppress appetite. Besides, Mohamed *et al.* (2014) reported that psyllium fiber was able to absorb water in the stomach in order to create a feeling of fullness and decrease appetite. All these compounds might be the contributors in suppressing appetite and providing satiety.

The result also showed that the EAF was able to reduce food intake of rats more efficiently than WE and simvastatin among all the treated groups. The food intake of HFD and ND rats treated with EAF was significantly reduced by 15.61% and 10.87%, respectively, compared to their control group ($p < 0.05$). However, the food intake of HFD and ND rats treated with WE was significantly reduced by 8.71% and 7.50%, respectively, compared to their control group ($p < 0.05$). The lowest % of food intake reduction was obtained by HFD rats treated with simvastatin among the treated groups, which was 6.10%. These depicted that the food intake of rats was affected by EAF more than WE and simvastatin. This might be due to the highest TPC and TFC obtained by

EAF in this study and the phenolic compounds, especially flavonoids in the EAF might act as anti-obesity agents which could suppress appetite, delay emptying and decrease food intake of the rats. The phenolic compounds in EAF could be obtained from citrus and green tea in the HP. For example, EGCG, which was a polyphenolic flavonoid from green tea had the ability to suppress appetite (Moon *et al.*, 2007). Besides, flavonoids such as naringenin, hesperetin and polymethoxylated flavones from citrus could also suppress appetite (Kim & Park, 2011).

5.5.3.3 Lipid profile

TG is the main component in vegetable oil and animal fats. It is the energy sources and transporters of dietary fat. TG is frequently carried in VLDL and chylomicrons. VLDL is made by the liver and has cholesterol, while chylomicrons can be obtained from dietary fat. However, cholesterol is a sterol and lipid which presents in cell membranes and is delivered into the bloodstream. It is used to form cell membranes and hormones and it plays a role in cell signaling processes. On the other hand, TC is the measure of the cholesterol components such as LDL, HDL and VLDL. HDL-C is a good cholesterol, which removes cholesterol from cells and atheroma and confers protection against cardiac complications and obesity. Meanwhile, LDL-C carries 60% to 70% of TC in serum and it has atherogenic properties. Thus, high levels of LDL-C can lead to cardiovascular morbidity and mortality (Sun *et al.*, 2012).

There were significant increments of TG, TC and LDL-C level as well as significant reduction of HDL level in HFD control rats compared to ND control rats ($p < 0.05$). This was due to HFD supplementation. The same result was obtained by Bais *et al.* (2014) and Kadir *et al.* (2015). The elevation of LDL-C in HFD control rats might be due to high content of cholesterol and saturated fatty acids in the HFD pellet and it caused down-regulation of LDL receptors, which responsible to mediate endocytosis of

LDL and maintain LDL-C level (Zulet *et al.*, 1999). According to Kadir *et al.* (2015), the rate of TG production was increased by LDL through transferring fat from the liver to adipose tissue. Hypertriglyceridemia might occur due to the high consumption of HFD and it was caused by elevated liver VLDL-TG secretion into the bloodstream. Besides, down-regulation of LDL receptors occurred with saturated TG compared to unsaturated TG. All these showed that dietary intake of HFD could change cellular membrane lipids and affect metabolic properties such as receptor-mediated uptake of lipoproteins (Kadir *et al.*, 2015; Oliveros *et al.*, 2015). In addition, hypercholesteremia might also occur due to the high consumption of HFD and this was associated with enhanced oxidizability of LDL molecules. High level of oxidized LDL caused oxidative stress in obese patient (Olusi, 2002). Therefore, lipid profile of obese patient should be improved.

There were significant reductions of TG, TC and LDL-C detected in HFD + EAF compared to HFD control rats ($p < 0.05$). EAF had a better effect on TC and LDL-C reduction in HFD rats than WE and simvastatin. This might be due to the highest TPC and TFC and the best antioxidant capabilities obtained by EAF compared to WE. The compounds in EAF, which might reduce TG, TC and LDL-C could be extracted from green tea, Guarana, grape seed, lotus leaves, psyllium husk and *Spirulina* in the HP. EGCG obtained from green tea could reduce TG (Richard *et al.*, 2009; Wolfram *et al.*, 2006) and TC (Ashida *et al.*, 2004). Catechins in Guarana could reduce TG (Bittencourt *et al.*, 2013), TC and LDL-C (Krewer *et al.*, 2014). Besides, phenolic Chardonnay grape seed extract could reduce TG in obese hamsters (Décordé *et al.*, 2009). Lotus leaf flavonoids could also reduce TG, TC and LDL-C (Liu *et al.*, 2013). Moreover, consumption of *Spirulina* was able to lower the level of TG (Iwata *et al.*, 1990), TC and LDL-C (Nakaya *et al.*, 1988). Psyllium was also able to reduce TC and LDL-C levels (Anderson *et al.*, 2000). Furthermore, LDL-C reduction might be due to the presence of

flavonoids in EAF since flavonoids were able to reduce LDL-C by removing cholesterol from peripheral tissue to the liver for catabolism and excretion (El-Tantawy, 2015). Flavonoids are potent hypolipidemic since they were able to increase fecal sterol by reducing the absorption of dietary cholesterol in the intestine, increase lipid metabolism and modulate enzymes involved in lipid metabolism (El-Tantawy, 2015). Besides, the LDL-C reduction might be due to the ability of antioxidants in EAF to inhibit lipid peroxidation and prevent free radical accumulation in the presence of peroxidases (Ahmed *et al.*, 2016).

There were positive elevation of HDL-C in HFD + WE, HFD + EAF and HFD + Simvastatin after treatment. EAF exhibited the highest elevation level of HDL-C (28.57%), followed by WE (17.14%) and simvastatin (14.29%). EAF had a better effect in increasing HDL-C than WE and simvastatin. This might be due to the EAF had the highest TPC and TFC and the best antioxidant capabilities compared to WE. It has been proved that the increased of HDL-C might be due to the flavonoids intake since flavonoids was able to remove cholesterol from peripheral tissue to the liver for catabolism and excretion (McCrindle *et al.*, 2003). In addition, the increased of HDL-C might be due to the ability of phenolic compounds in the extract in accelerating the decomposition of free radicals that caused by oxidative stress in obesity (Ahmed *et al.*, 2016).

CHAPTER 6: CONCLUSION

The TLC results showed the presence of alkaloids, phenolic compounds and terpenoids in WE. The LC/Q-TOF/MS analysis showed the presence of amino acids, benzoic acid, flavonoids, phenolic compounds, polysaccharide and sugars in WE. The TLC results also showed the presence of phenolic compounds and terpenoids in EAF. The EAF exhibited the highest TPC and TFC. It also exhibited the highest antioxidant capabilities in scavenging DPPH, NO and superoxide radicals and reducing ferric ions. Its highest antioxidant capabilities were due to its TPC and TFC. However, it exhibited low capability in chelating metal (< 50%). The EAF was chosen to orally treat HFD-induced obese SD rats for two weeks and its effect on body weight reduction and lipid profile improvement was compared with WE and simvastatin. The body weight of HFD rats treated with WE (250 mg/kg b.w.), simvastatin and EAF (250 mg/kg b.w.) was significantly reduced by 4.79%, 6.18% and 6.64%, respectively ($p < 0.05$). The BMI of HFD rats treated with WE, simvastatin and EAF were also significantly reduced ($p < 0.05$). The food intake of rats treated with WE and EAF was also significantly reduced ($p < 0.05$). Besides, the EAF could significantly lower TG, TC and LDL-C levels, as well as positively increase HDL-C level of HFD rats compared to WE and simvastatin. All these results showed that the EAF had better potential for body weight reduction and lipid profile improvement in HFD rats compared to WE and simvastatin.

Further research is recommended to more clearly examine the safety and effect of EAF supplementation on the liver and kidney of the rats. Besides, further research on the effect of the higher dose of EAF on body weight reduction and lipid profile improvement can be conducted in order to compare its effectiveness with the effect of EAF at a daily dose of 250 mg/kg b.w. of rats that conducted in this study.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Conference and presentation:

Chai, S. F. & Mohamad, J. (2016, December). *Effect of herbal product on the body weight reduction in high fat diet-induced obese Sprague Dawley rats*. Poster presented at the Biological Sciences Graduate Congress (BSGC), University of Malaya, Kuala Lumpur, Malaysia.